Phenotypic and Functional Characteristics of Mesenchymal Stem Cells Differentiated Along a Schwann Cell Lineage

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ABSTRACT
We have investigated the phenotypic and bioassay characteristics of bone marrow mesenchymal stromal cells (MSCs) differentiated along a Schwann cell lineage using glial growth factor. Expression of the Schwann cell markers S100, P75, and GFAP was determined by immunocytochemical staining and Western blotting. The levels of the stem cell markers Stro-1 and alkaline phosphatase and the neural progenitor marker nestin were also examined throughout the differentiation process. The phenotypic properties of cells differentiated at different passages were also compared. In addition to a phenotypic characterization, the functional ability of differentiated MSCs has been investigated employing a co-culture bioassay with dissociated primary sensory neurons. Following differentiation, MSCs underwent morphological changes similar to those of cultured Schwann cells and stained positively for all three Schwann cell markers. Quantitative Western blot analysis showed that the levels of S100 and P75 protein were significantly elevated upon differentiation. Differentiated MSCs were also found to enhance neurite outgrowth in co-culture with sensory neurons to a level equivalent or superior to that produced by Schwann cells. These findings support the assertion that MSCs can be differentiated into cells that are Schwann cell-like in terms of both phenotype and function.

INTRODUCTION

It is recognized that Schwann cells (SC) are essential for peripheral nerve development and regeneration (Bunge et al., 1994; Jessen and mirsky, 1999). Addition of cultured SC into nerve conduits enhanced axonal regeneration across nerve gaps (Guenard et al., 1992; Mosahebi et al., 2001; Rodriguez et al., 2000). However, the culture of sufficient numbers of autologous SC is time consuming, limiting the usefulness of this technique as delay compromises axonal regeneration (Sulaiman and Gordan, 2000). Use of allogeneic cells elicits an intense immune response (Guenard et al., 1992), which clinically would require immune suppression, difficult to justify within the context of peripheral nerve repair.

An alternative to SC transplantation is stem cell differentiation to achieve the same functional properties. Clinical application of embryonic stem cells raises ethical dilemmas (Gilbert, 2004), while adult stem cells circumvent many of these issues. Increasing evidence of adult stem cell plasticity (Bjornson et al., 1999; Bonilla et al., 2002; Orlic et al., 2001) prompted investigation of readily accessible tissue sources for the regeneration potential to support axon regeneration in the CNS (Amoh et al., 2005; Safford et al., 2004; Sigurjonsdottir et al., 2005).

Clinically, mesenchymal stromal cells (MSCs) derived from bone marrow (marrow stromal cell, MSCs) are easily accessible, and their plasticity was demonstrated both in vitro and in vivo (Ferrari et al., 1998; Jiang et al., 2002; Petersen et al., 1999; Pittenger et al., 1999; Schwartz et al., 2002). In particular, MSCs showed potential to regenerate neurons in CNS (Azizi et al., 1998; Brazleton et al., 2000; Kopen et al., 1999; Mezey et al., 2000; Woodbury et al., 2000) and glial tissue in spinal cord (Akiyama et al., 2002; Sasaki et al., 2001). MSCs were also differentiated in vitro to express markers of SCs and enhanced peripheral nerve regeneration (Dezawa et al., 2001; Tohill et al., 2004).

The ease of isolation and availability of MSCs, their differentiation into Schwann-like cells, and ability to promote axonal regeneration are appealing for tissue engineering. Despite encouraging early reports, the true phenotype and function of these cells remains to be fully established. While in vivo transplantation suggests that differentiated MSCs confer benefits on axonal regeneration, it remains unclear whether this is a direct effect due to SC phenotype differentiation, or an indirect effect from MSCs interaction with surrounding regenerating environment.

Our aim was to assess the phenotypic and bioassay characteristics of MSCs differentiated along SC lineage.
using glial growth factor (GGF), which also stimulates cell proliferation (Mahanthappa et al., 1996). Immunocytochemistry and Western blotting were used to identify the SC markers S100, P75, and GFAP. Expression of nestin, a neural stem cell precursor marker, Stro-1, a nonhaematopoietic bone marrow progenitor cell marker, and alkaline phosphatase, a marker of undifferentiated state, were also quantified throughout the differentiation process. Cells at different passages were compared to identify possible changes in differentiation potential with age. Importantly, we assessed the functional potential of differentiated MSCs employing a co-culture with dissociated primary sensory neurons.

MATERIALS AND METHODS

Schwann Cell Harvest and Culture

Schwann cells (SC) were harvested from the sciatic nerves of rats according to a previously developed technique (Mosahebi et al., 2001). Briefly, the sciatic nerves of 20 Lewis neonatal rats (1–2 day old) were exposed, removed, and placed in chilled Dulbecco’s Modified Eagle’s Medium containing 1% penicillin-Streptomycin (DMEM, Gibco, UK). Nerves were digested with 37.5 U/mL collagenase Type 1 (Worthington, USA) and 0.03% trypsin-EDTA (Gibco) for 15 min at 37°C. The medium was aspirated and the enzyme digestion cycle repeated a further three times. Fetal bovine serum (FBS) (10% in DMEM) was added to neutralize the enzymes. Nerves were mechanically triturated, then passed through a 70 μm Falcon filter to remove residual debris. The resulting cell suspension was centrifuged at 600g for 5 min, the supernatant gently aspirated from the cell bolus and fresh cell growth medium added. Cells were then plated onto poly-D-Lysine (PDL) coated 25 cm² flasks and incubated in 5% CO₂ at 37°C overnight.

Twenty four hours after plating, the medium was removed and replaced with fresh cell growth medium containing 100 μM cytosine-β-D-arabinoside (Sigma-Aldrich, UK). This antimitotic agent effectively reduces the numbers of the more rapidly proliferating fibroblasts relative to the SC population. After a further 48 h, the medium was replaced with cell growth medium supplemented with 4.1 μg/mL Forskolin (Sigma-Aldrich) and 63 ng/mL GGF (Acorda Therapeutics, USA).

Further fibroblast elimination was carried out by resuspending the SCs in mouse anti-rat Thy 1.1 (1:500, Serotec, UK), for 10 min at 37°C. Rabbit complement was then added to the cells for a further 30 min, followed by centrifugation at 600g for 5 min. The supernatant was aspirated and the cell bolus resuspended in SC growth medium. Cells were then plated on a PDL-coated flask and incubated in 5% CO₂ at 37°C.

Bone Marrow Cell Harvest and Culture

The proximal and distal ends of adult Lewis rat long bones were removed to reveal the marrow cavity. Mesenchymal cell growth medium (MEM-Alpha; Gibco; supplemented with 10% FBS and 1% penicillin-streptomycin) was injected through each marrow cavity using a 21G needle. The resulting cell suspension was triturated, filtered through a 70 μm Falcon filter and centrifuged for 5 min at 600g. The supernatant was aspirated, the cell bolus resuspended in mesenchymal cell growth medium, and the cells plated in 75 cm² tissue culture flasks and incubated in 5%CO₂ at 37°C. Haematopoietic cells were eliminated by washing daily with DMEM until all nonadherent cells were removed. The cells were allowed to grow to confluence.

To confirm the presence of multi-potent MSCs, cultures were treated for three weeks with either osteogenic induction medium (100 μg/mL ascorbate, 0.1 μM dexamethasone, 10 mM β-glycerophosphate in DMEM) or adipogenic induction medium (1 μM dexamethasome, 0.5 mM isobutyl-methylxanthine, 100 μM indomethacin, 10 μg/mL insulin in MEM) and then stained with Alizarin Red and Oil Red O to confirm differentiation as described previously (Pittinger et al., 1999).

Mesenchymal Stem Cell Differentiation with GGF

At confluence, MSCs were passaged, split, and replated. Undifferentiated MSCs (uMSCs) were maintained up to passage 8. For differentiation, cells of either passage 2 or passage 5 were used. Medium from subconfluent cells was removed and replaced with medium supplemented with 1 μM β-mercaptoethanol (Sigma-Aldrich) for 24 h. Cells were then washed and fresh medium supplemented with all-trans-retinoic acid (35 ng/mL) was added.

After 72 h, cells were washed and medium replaced with MSCs differentiation medium, i.e. mesenchymal cell growth medium supplemented with platelet derived growth factor-AA 5 ng/mL, basic fibroblast growth factor 10 ng/mL (both Peptotech EC, UK), Forskolin 5.7 μg/mL (Sigma-Aldrich), and GGF 126 ng/mL (Acorda Therapeutics). Cells were incubated for two weeks in these conditions with fresh medium added approximately every 72 h. (Dezawa et al., 2001; Tohill et al., 2004).

Immunostaining of Cultured Cells

Differentiated MSCs (dMSCs) cultured on chamber slides (Lab-Tek) were fixed in 4% (w/v) paraformaldehyde at 4°C for 20 min. Cell nuclei were labelled with Hoechst (Bisbenzimide H 33342, 10 μg/mL in PBS, Fluka, Switzerland) for 15 min at room temperature. Cells were then incubated overnight at 4°C with primary antibodies to S100 (rabbit polyclonal; 1:200; Dako, Denmark), glial fibrillary acidic protein (GFAP, mouse monoclonal; 1:200; Chemicon, USA), P75 neurotrophin receptor (mouse monoclonal; 1:333; Alomone, Israel), Nestin (mouse monoclonal; 1:500; Chemicon), and Stro-1.
(mouse monoclonal; 1:50, R&D Systems, UK). The following day, slides were incubated for 2 h with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (horse anti-mouse or goat anti-rabbit; 1:100; Vector Labs., USA). After washing with PBS slides were mounted with Vectashield (Vector Labs). Separate chamber slides were labelled for alkaline phosphatase using the Vector Red Alkaline Phosphatase Substrate Kit 1 (Vector Labs).

Slides were examined under a fluorescence microscope (Olympus BX60) at ×20 magnification. For each marker, 10 random fields per slide were photographed and the number of cells staining positively expressed as a percentage of the total number of cells as indicated by Hoechst nuclear staining. All staining was repeated in 2–4 independent experiments. Cultures of SC and uMSCs were similarly stained as positive and negative controls accordingly to the antibodies used.

**Western Blotting of Cell Lysates**

Lysates were prepared from cells cultured to confluence. Cells were treated with buffer containing 50 mM HEPES, 150 mM NaCl, 10% glycerol, 0.1% (v/v) Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, and protease inhibitors (Sigma-Aldrich) for supernatant only lysates, or with 100 mM PIPES, 5 mM MgCl₂, 20% (v/v) glycerol, 0.5% (v/v) Triton X-100, 5 mM EGTA, and protease inhibitors for whole cell lysates. Supernatant only lysates were incubated for 15 min on ice, then centrifuged at 13,000g for 5 min, and the supernatant retained for analysis.

Proteins were denatured by heating to 95°C in Laemmli buffer for 5 min, and then separated on 15% (for S100) or 10% (for P75) acrylamide gels by SDS-PAGE (200 V), and transferred to nitrocellulose membranes. For S100, membranes were blocked for 2 h (5% (w/v) nonfat dry milk in TBS Tween, 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween), and then incubated overnight at 4°C with anti-S100 (mouse monoclonal; 1:1000; Affiniti, UK). For P75, membranes were blocked overnight in 5% casein (Sigma-Aldrich), 5% BSA (Fluka) in TBS Tween, then incubated with anti-P75 (rabbit polyclonal; 1:500; Promega, USA) for 24 h. Membranes were incubated for 1 h with HRP-conjugated secondary antibodies [horse anti-mouse 1:1000 for S100 (Sigma-Aldrich); goat anti-rabbit 1:5000 for P75 (Cell Signalling, USA)]. Membranes were washed and treated with ECL chemiluminescent substrate (Amersham, UK) for 1 min and developed by exposure to Kodak X-OMAT light-sensitive film.

The linear range of the film was determined by running initial gels with lysates of varying protein concentrations. Subsequent blots at protein concentrations determined from initial experiments were reprobed for Actin C-2 (mouse monoclonal; 1:200; Santa-Cruz, USA) as a loading control. For each experiment 4–8 lysates from different cell cultures were used. Blots were analysed using the Scion Image program for Windows.

**Dorsal Root Ganglion Neurons Functional Bioassay**

Dorsal root ganglia (DRG) were removed from adult male Lewis rats, cleaned of connective tissue and chemically dissociated in 0.125% collagenase Type IV (Worthington) in Ham's F12 (Gibco) for 1.5 h. DRG were then transferred to 0.25% trypsin (Worthington) for 30 min in 5% CO₂ at 37°C. Trypsin was then inactivated using 30% FBS (Gibco). Ganglia were washed in Ham's F12, before mechanical dissociation by gentle trituration using a glass pipette. Dissociated neurons were passed through 70 µm mesh (Cadiusch, UK) to remove nondissociated cells and myelin debris. The cell suspension was then centrifuged at 9g for 5 min. Cells were resuspended in Ham's F12 and centrifuged through 15% bovine serum albumin at 100g for 10 min, to remove non-neuronal cells. Neurons were resuspended in modified Bottenstein and Sato's medium (BS containing: 0.1 mg/mL transferrin, 20 nM progesterone, 100 µM putrescine, 30 nM sodium selenite, and 1 mg/mL BSA, 0.01 mM cytosine arabinoside, and 10 µM insulin) in Ham's F12. Sensory neurons were seeded onto coverslips precoated with 2 µg/mL laminin-1 (Sigma-Aldrich). Each coverslip was placed in a 6 well plate and incubated overnight in BS in 5%CO₂ at 37°C.

Twenty four hours prior to DRG harvest, dMSCs, control cultures of SCs, and uMSCs were seeded onto 1.0 µm pore size cell culture inserts (Becton Dickinson, USA) at a concentration of 150,000 cells/insert. Inserts were initially placed in 6-well plates containing culture medium only and incubated overnight in 5% CO₂ at 37°C. Forty-eight hours later the inserts were checked for cell adherence and placed in the wells containing DRG neurons. The inserts were placed in a way that allowed the molecules secreted by the cells to permeate through the pores of the membrane and reach the DRG neurons underneath. However, there was no direct contact between SC or MSC and DRG neurons. Additional wells, with filter inserts containing BS medium but no cells, were incubated under the same conditions as controls. After 24 h of incubation as co-culture, neuron seeded coverslips were washed, fixed in 4% (w/v) ice-cold paraformaldehyde for 15 min and incubated with the pan-neuronal marker, anti-β tubulin III (mouse monoclonal; 1:100); Sigma-Aldrich) overnight. The following day, incubation with FITC-conjugated horse anti-mouse (1:100; Vector Labs) was carried out for 1 h, the coverslips washed in PBS and mounted on slides with Vectashield for fluorescence.

Slides were examined under a fluorescence microscope (Olympus BX60) at ×10 magnification. For each condition, 30 random fields were photographed for quantification. Neurite-outgrowth was then assessed using 3 independent parameters: percentage of process-bearing neurons (the proportion of cells bearing neurites at least twice the length of their soma), length of longest neurite (measured by tracing the length), and total neurite density (as assessed by counting the number of intersection points of neurites crossing a series of 33 µm concentric
circles radiating from the cell soma, using a macro developed using SigmaScan Pro 5 software; SPSS). Three independent co-culture experiments were carried out for each cell type.

Statistical Analysis

Data are expressed as mean ± SD. Statistical analysis for Western blotting densitometry and sensory neuron co-cultures was conducted using Prism software by one way ANOVA followed by Bonferroni’s Multiple Comparison Test.

RESULTS

Morphological Assessment of Differentiated MSCs

uMSCs appeared as a monolayer of large, fibroblast-like flattened cells (Fig. 1A) which when treated with appropriate induction media where able to adopt an osteogenic (Fig. 1B left panel) or adipogenic (Fig. 1B right panel) phenotype. uMSCs cells were treated with GGFs and assessed morphologically at regular intervals. Evidence of morphological changes including an elongated shape and bipolar morphology were already visible in many cells after seven days in differentiation medium (Fig. 1C) and in the majority of cells by day 14 (Fig. 1D). At confluence the dMSCs displayed a whorling pattern (Fig. 1E) characteristic of SC (Fig. 1F). Similar changes were evident in cells differentiated at passages 2 and 5 (data not shown).

Phenotypic Expression of Schwann Cell Markers

MSCs differentiated at 2 different passages (T2 and T5) were assessed for expression of the SC markers S100, GFAP, and P75 to determine evidence of phenotypic progression along a SC lineage, and to determine the effect of passage on the ability to differentiate. Control SC cultures were immunoreactive (IR) for all three markers (data not shown). uMSCs cultures were negative for GFAP, P75 (Figs. 2A,B), and S100 (Fig. 3A) at passage 2 and 5. However if uMSCs were cultured up to passage 8, there was occasional, faint expression of S100 (Fig. 3B). Immunostaining for GFAP (Figs. 2C,E) and P75 (Figs. 2D,F) was observed in all dMSCs after 14 days whether differentiated from passage 2 or 5. Immunostaining for S100 was detected in approximately half of the dMSCs at both passage 2 (49%) and passage 5 (55%) differentiation (Figs. 3C,D).
Densitometric analysis of Western blots of dMSCs lysates probed with S100 and P75 antibodies supported the results found on immunocytochemical staining (see Fig. 4). Bands at 10.5 and 75 kDa were detected in dMSCs lysates exposed to S100 and P75 antibodies respectively. S100 expression in dMSCs lysates showed a 6-fold increase compared with uMSCs (**P < 0.01). Similarly P75 expression by dMSCs increased 4-fold in comparison to uMSCs, (***P < 0.001). The level of expression of both markers in dMSCs was not significantly different to that observed in SC (P > 0.05 dMSCs vs. SC).

**Phenotypic Expression of Stem Cell Markers**

Stro-1 staining was detected in 10% of uMSCs (Fig. 5A). Following differentiation, no Stro-1 positive cells were observed in dMSCs (Fig. 5B). As expected, control SC cultures were also uniformly negative for Stro-1 (Fig. 5C).

The majority of uMSCs stained positively for alkaline phosphatase at passages 2 (93%) and 5 (89%) (Figs. 6A,B). However, by passage 8 this expression had decreased to 37% (Fig. 6C). Following 14 days culture in differentiation medium, expression of alkaline phosphatase declined in cells differentiated at both passage 2 and 5% to 36% and 21%, respectively (Figs. 6D,E). Control SC cultures were uniformly negative for alkaline phosphatase (Fig. 6F).

Positive nestin expression was seen in a small proportion (3%) of uMSCs at passage 2 (Fig. 7A). This rose transiently to 32% in cells cultured to passage 5 and by passage 8 nestin expression had fallen to 16% (Figs. 7B,C). In MSCs cultured for 14 days in differentiation medium, on average 10% expressed nestin (8% in cells differentiated at passage 2 and 13% in cells differentiated at passage 5) (Fig. 7D).

**DRG Functional Assay**

The functional capacity of cultured SC, uMSCs, and dMSCs to promote neurite outgrowth was determined by quantification of their effect on co-cultured adult sensory neurons. Three separate parameters of neurite outgrowth were quantified: percentage of process-bearing neurons, length of longest neurite, and neurite density.

Following 24 h culture in BS media alone, few adult sensory neurons (4.8 ± 1.8)% extend neurites, which are typically short and few in number (Fig. 8A). DRG co-cultured with uMSCs showed some neurite outgrowth (Fig. 8B), which was not as abundant as seen with co-cultures of dMSCs and SC. Notably, dMSCs appear to stimulate outgrowth of long, sparsely branched neurites (Fig. 8C), while SC produce a highly-branched, arboreal outgrowth (Fig. 8D).

There is an increase in the percentage of neurons extending neurites (Fig. 9A), and the length and complexity of the neurite arborisation (Fig. 9B,C) following co-culture with all cells types tested. The percentage of neurons extending neurites was significantly increased.
by the presence of uMSCs [(38.4 ± 5.7)%,

\( P < 0.01 \)],
dMSCs (63.2 ± 12.4)%,

\( P < 0.001 \), and SC [(46.6 ± 5.6)%,

\( P < 0.001 \) compared with media alone (4.8 ± 1.8)%.

In addition, the percentage of neurite-bearing cells induced by the dMSCs was significantly greater than that observed with the uMSCs (\( P < 0.05 \)).

The length of the longest neurite developing from each cell increased significantly with the addition of both SCs (\( P < 0.01 \) or dMSCs (\( P < 0.001 \) but not the uMSCs \( P > 0.05 \), (Fig. 9B). As with the percentage sprouting, the largest increase was seen in the dMSCs group, with a slight improved effect to that of cultured SC.

Neurite density was also significantly increased by the addition of both SC and dMSCs (\( P < 0.01 \) and \( P < 0.05 \) respectively) (Fig. 9C). By this method of analysis the largest increase was seen with SC co-culture. This may indicate a difference in the pattern of neurite outgrowth stimulated by the different cell types, with SC producing a denser, more highly arborised pattern of neurite outgrowth than the dMSCs (Figs. 8C,D).

**DISCUSSION**

These results provide further evidence that differentiated MSCs have similar morphological and phenotypic characteristics as SC and, more importantly, the two cell types have comparable functional properties. Morphologic changes are clearly evident within 7 days of culture in GGF containing medium, with some cells exhibiting classic bipolar morphology within 4–5 days. By two weeks these changes are uniform and, if grown to confluence, the differentiated cells show a whorling pattern not seen when they are cultured without GGF.

Morphological changes alone may be stimulated simply by the addition of \( \beta \)-mercaptoethanol, which can induce neurite-like processes formation in neuronal and MSCs cultures (Ishii et al., 1993; Woodbury et al., 2000). However, immunostaining for S100, GFAP, and P75, all known SC markers (Jessen and Mirsky, 1999; Morrisey et al., 1991) indicates that differentiated cells have undergone a phenotypic change. Although positive immunoreactivity may be an artefact of cellular shrinkage (Lu et al., 2004), S100 and P75 detection by Western blotting strongly suggests that this is not the case, as
both protein levels increase significantly following differentiation, with equivalent expression to that seen in SC cultures.

The influence of cell passage on dMSCs ability to express SC markers has not been reported before. In vitro MSCs differentiation towards neuronal phenotype was shown using cells expanded beyond passage 20 (Woodbury et al., 2000), and MSCs expansion for at least 25 population doublings (equivalent to passage 10) is a prerequisite for neuronal differentiation (Wislet-Gendebien et al., 2003). Previous in vitro studies of MSCs differentiation to SC lineage used cells cultured to passage 5 (Dezawa et al., 2001; Tohill et al., 2004). In this study, a comparison of cells differentiated to either passage 2 or passage 5 showed no difference of expression for any SC markers, suggesting that cell age, at least at this relatively early stage, does not affect their differentiation ability.

Consistently, uMSCs cultures at passages 2 and 5 were uniformly negative for all SC markers, indicating absence of glial cells contamination. Interestingly, there was occasional weak S100 expression in uMSCs expanded to passage 8, possibly due to the innate potential of a small cell proportion to differentiate spontaneously. Rare multipotent adult progenitor cell (MAPC) were identified in mouse, rat, and human bone marrow, which can be expanded for over 120 population doublings and differentiate in vitro and in vivo into all three germ layer cells (Jiang et al., 2002). MSCs isolated by their adherence to plastic (Owen and Friedenstein, 1988) contain a heterogeneous mix of stromal cells, with a small proportion likely to be true multipotent progenitors or stem cells with a broader developmental plasticity. It might be possible that, with increasing passage, this cell population is selectively enhanced as a proportion of the total MSCs. Without the restraint of in vivo mechanisms controlling cell differentiation and proliferation, in vitro culture may permit spontaneous differentiation and maturation within this subpopulation of cells.

Notably, there was progressive decline of alkaline phosphatase expression, an undifferentiation marker in uMSCs, which should only decline with differentiation (Chambers et al., 2003; Horiuchi et al., 2003; Shamblott et al., 1998). This decline in uMSCs was noticed from passage 2 (93%) to passage 8 (37%), with levels similar to those seen in dMSCs (21–36%). This result is not unexpected, as spontaneous differentiation of MSCs in vitro has been described previously for cells of mesodermal lineage. In our study this would indicate that there might have been some spontaneous differentiation in vitro, possibly explaining the occasional appearance of S100 expression in older cultured cells.

To assess the true proportion of progenitor cells within this heterogeneous culture we used Stro-1, a stromal cell marker (Simmons and Torok-Storb, 1991; Stewart et al., 2003) that recognizes non-haematopoietic bone marrow progenitor cells with stable undifferentiated phenotype. These cells proliferate extensively while retaining the potential to differentiate even when cultured long term (Gronthos et al., 1999; Tuli et al., 2003). Our results showed 10% Stro-1 positive cells, declining to zero with differentiation. This would indicate that Stro-1 positive cells undergo glial differentiation, but it is unclear whether in cohort with or at the expense of Stro-1 negative cells.
Nestin is expressed by neural stem cells (Dahlstrand et al., 1992) and identifies adult neuronal and glial progenitor cells in culture (Dahlstrand et al., 1995; Mignone et al., 2004). Nestin expression is an essential prerequisite for MSCs to progress toward a neural lineage (Wislet-Gendebien et al., 2003). It is unknown whether this holds true for MSCs differentiated to glial lineage, but we found nestin expression in a small proportion (3%) of passage 2 uMSCs. This rose transiently to 32% in passage 5 cells, falling to 16% by passage 8, consistent with previous reports of 18% nestin expression in long-term MSCs cultures (Mezey et al., 2000). Nestin expression in precursor cells is known to be transient (Woodbury et al., 2000), declining rapidly as differentiation occurs, consistently with the lower levels of nestin expression (10%) seen in differentiated cells. However, the significance of marked expression elevation at passage 5 is harder to explain. Results of positive nestin staining need to be considered with caution. Nestin expression is indicative that MSCs can develop into neural precursors (Mezey et al., 2000), but there are also reports of nestin expression in progenitor cells of diverse tissue types (About et al., 2000; Ha et al., 2003; Kachinsky et al., 1995; Mokry and Nemecek, 1998; Niki et al., 1999; Sjersen and Lendahl, 1993; Zulewski et al., 2001). It might be that this marker is indicative of a general precursor cell rather than one specifically committed to a neural lineage.

Immunostaining and Western blotting results are strongly indicative that GGF induces a phenotypic change in dMSCs along SC lineage. However, phenotypic and morphological results do not always equate with cell function. Hence, we tested the functional potential of dMSCs to elicit neurite outgrowth from isolated primary sensory neurons. By separating stem cells and neurons with microfilters, the neurite outgrowth is likely to result from growth factors or other molecules secreted from the overlying cells, excluding indirect effects possibly encountered in vivo. Our results indicate that dMSCs promote neurite outgrowth to similar levels as achieved by SC, consistent with previous in vivo results of enhanced nerve regeneration (Dezawa et al., 2001; Tohill et al., 2004).

DRG neurons in culture display distinct neurite growth, with early short arborisation, and a later elongating growth pattern (Smith and Skene, 1997). These discrete patterns are influenced by different growth factors (Cafferty et al., 2001; Gavazzi et al., 1999) and reflect progressive recovery phases, with the later elongating phase being crucial for successful nerve regeneration (Smith and Skene, 1997). Different quantification techniques may reflect these different patterns, the percentage of process-bearing neurons being analogous to arboreal growth, and the longest neurite length analogous to elongating growth (Gavazzi et al., 1999). The measure of neurite density is likely to be influenced by both arboreal and elongating growth patterns. It may be that the small results variations seen here between quantification techniques reflect differences in growth type or progression from one phase to the next stimulated by SC and dMSCs.

dMSCs produce a neurite extension superior to that seen with uMSCs, and the moderate neurite growth caused by the latter would suggest that stem cell can confer some benefit even before differentiation. Alternatively this may be further evidence of stem cell potential to develop spontaneously along glial lineage with appropriate cellular or extracellular stimuli. Similar finding were noted previously in vivo (Cuevas et al., 2002; Tohill et al., 2004), and this occurrence in vitro would suggests that the regenerating neurons secreting GGF (Mahanthappa et al., 1996), rather than the regenerating microenvironment, may promote differentiation. Also, it is not excluded that other factors in the culture might...
participate in this possible differentiation, and this aspect is currently under investigation.

In conclusion, our results show that MSCs from bone marrow can differentiate into cells expressing both morphologic and phenotypic progression along a Schwann cell lineage. Furthermore, differentiated MSCs enhance neurite outgrowth in co-culture bioassay, indicating the functional potential of differentiated stem cells.

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