© 2021. Published by The Company of Biologists Ltd | Journal of Cell Science (2021) 134, jcs258324. doi:10.1242/jcs.258324

RETRACTION

Retraction: Regulation of neural markers nestin and GFAP expression by cultivated bone marrow stromal cells

S. Wislet-Gendebien, P. Leprince, G. Moonen and B. Rogister

The authors are retracting *J. Cell Sci.* (2003) **116**, 3295-3302 (doi:10.1242/jcs.00639).

Allegations of image manipulation were made for Fig.1A and Fig. 2E,F on PubPeer. As the data were obtained for these figures 20 years ago, the authors no longer have all of the original images and blots. A cropped blot was found for data shown in Fig. 2E, but the resolution was poor and no definitive conclusion on any band duplication could be made.

The authors have no explanation for how these issues could have arisen and say that although these issues do not modify the scientific message, they are retracting the paper. They apologise to readers for any inconvenience.

Journal of Cell Science

Regulation of neural markers nestin and GFAP expression by cultivated bone marrow stromal cells

S. Wislet-Gendebien[1,*], P. Leprince[1], G. Moonen[1] and B. Rogister[1,2]

[1]Center for Cellular and Molecular Neurobiology, University of Liège, 17 Place Delcour, B-4020 Liège, Belgium
[2]Department of Neurology, University of Liège, C.H.U. (B35) Sart Tilman, B-4000 Liège, Belgium
*Author for correspondence (e-mail: s.wislet@ulg.ac.be)

Accepted 28 April 2003
Journal of Cell Science 116, 3295-3302 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00639

Summary

Bone marrow stromal cells can differentiate into many types of mesenchymal cells, i.e. osteocyte, chondrocyte and adipocyte, but can also differentiate into non-mesenchymal cells, i.e. neural cells under appropriate in vivo experimental conditions (Kopen et al., 1999; Brazelton et al., 2000; Mezey et al., 2000). This neural phenotypic plasticity allows us to consider the utilization of mesenchymal stem cells as cellular material in regenerative medicine. In this study, we demonstrate that cultured adult rat stromal cells can express nestin, an intermediate filament protein predominantly expressed by neural stem cells. Two factors contribute to the regulation of nestin expression by rat stromal cells: serum in the culture medium inhibits nestin expression and a threshold number of passages must be reached below which nestin expression does not occur. Only nestin-positive rat stromal cells are able to form spheres when they are placed in the culture conditions used for neural stem cells. Likewise, only nestin-positive stromal cells are able to differentiate into GFAP (glial fibrillary acidic protein)-positive cells when they are co-cultivated with neural stem cells. We thus demonstrated that adult rat stromal cells in culture express nestin in absence of serum after passaging the cells at least ten times, and we suggest that nestin expression by these cells might be a prerequisite for the acquisition of the capacity to progress towards the neural lineage.

Key words: Nestin, Bone marrow stromal cells, GFAP, Neural stem cells, Glial differentiation

Introduction

Stem cells have the capacity to self-replicate or to produce progeny of one or several specific differentiated cell types. They are not committed to a particular type of tissue and can therefore generate cells belonging to different cell types. Two types of stem cells have been identified: embryonic stem cells (ES) cells, found in the inner cell mass of the early embryo, and tissue-specific, including adult stem cells. Adult or somatic stem cells have been located in bone marrow (Bianco et al., 1999), blood (Domen et al., 1999), cornea and retina (Wu et al., 2001), brain (Davis et al., 1994), skeletal muscles (Seale et al., 2000), dental pulp (Gronthos et al., 2000), liver (Sell, 1990) and skin (Gandarillas and Watt, 1997). Much information concerning the characteristics of adult stem cells is derived from studies of hematopoietic stem cells, because they are easily isolated, transplant and delivered in the treatment of several hematologic diseases and cancers.

Stem cells located in continuously renewing tissues such as skin, gut, bone marrow, are able to regenerate or repair these tissues throughout life. However, in the non-regenerating adult central nervous system (CNS), neural stem cells (NSC) have a poor capacity to generate new neurons or oligodendrocytes to replace cells lost after injury or degeneration. In nervous system disorders in which specific neuronal cell loss occur (e.g. Parkinson's disease) transplantation of neural cells allows for the replacement of lost cells and recovery of some degree of function (Freed et al., 2001).

Embryonic stem cells are totipotent and are thus able to differentiate in any kind of cell type present during development and in adulthood. At present, there is no evidence that adult stem cells are totipotent, but some may have the capacity to differentiate into phenotypes that belong to tissues other than the one from which they originated, a property usually referred to as plasticity or transdifferentiation. A recently reported example of such plasticity is the finding that, after intravascular delivery of genetically labelled adult mouse bone marrow into lethally irradiated adult hosts, donor cells expressing neuronal markers were found in the host CNS (Brazelton et al., 2000). In vitro, a tiny fraction (2-5%) of bone marrow stromal cells cultured in the presence of EGF or BDNF express nestin, glial fibrillary acidic protein (GFAP) and neuron-specific nuclear protein (NeuN) (Sanchez-Ramos et al., 2000). The addition of dibutiryl cyclic AMP has been reported to induce the differentiation of human mesenchymal stem cells (MSCs) into early progenitors of neural cells (Deng et al., 2001).

During the development of the CNS, proliferating neuroepithelial cells express nestin (neural stem cells protein), an intermediate filament protein (Lendahl et al., 1990), which is also expressed by NSC in adult mammals and then used to identify adult neural progenitors in culture (Dahlstrand, et al., 1995). Although nestin is not a specific marker of neural stem cells because it is also transiently expressed in muscle progenitors and in some epithelial derivatives (Mokry and Nemecek, 1998), the analysis of neurospheres obtained from ES cells (which are known to be nestin-negative in vivo)

demonstrates that all the cells within those spheres express nestin, suggesting that nestin expression is correlated to or is coincident with the initiation of sphere formation (Tropepe et al., 2001).

The use of MSCs in auto-graft protocols in neurological diseases necessitates the identification of the molecular events that are important for the induction of neural differentiation of MSCs. We found that the presence of serum in the culture medium represses nestin expression by rat stromal cells (rSCs). Moreover, only nestin-positive rSCs are able to form aggregates in suspension when they are transferred to NSCs culture conditions. But, when those spheres or aggregates were placed in culture conditions known to favour neural differentiation of NSCs, only modifications of cell shape were observed. In contrast, when nestin-positive rSCs were grown in co-culture with mouse neural stem cells (mNSCs), heterogenous spheres formed which, when plated on polyornithine-coated surfaces, released 40% rSCs that differentiated into GFAP-positive cells. Nestin-negative rSCs cells, when grown in the same condition, gave rise to less than 5% GFAP-positive rSCs. Nestin expression by rSCs should thus be regarded as a first step in their progression to the neural lineage.

Materials and Methods

Preparation and culture of rat stromal cells

Adult rat bone marrow was obtained from femurs and tib[illegible] aspiration and was resuspended into 5 ml of DEM (Invitr[illegible]n, Merelbeke, Belgium) (Azizi et al., 1998). Between 100 and 200×[illegible] marrow cells were plated on 175-cm tissue culture flask in DEM/10[illegible] foetal bovine serum (Invitrogen). After 24 hours, the non-adherent cells were removed by replacing the medium. When t[illegible] became confluent, they were resuspended with 0.25% T[illegible]sin and [illegible] mM EDTA and then sub-cultured. To initiate nes[illegible] expres[illegible]n, the cultures were washed with PBS and grown i[illegible]DEM/F1[illegible] (Invitrogen). After 3 days, the cells were fixe[illegible]SC[illegible] were then processed for immunocytochemistry or we[illegible]n blot[illegible]s described below.

Preparation and culture of mNSC

Green C57BL/6 mice embryos (Jackson Immunor[illegible]rch Laboratory, West Grove, PA, USA) were us[illegible] as a source of mN[illegible]n the green mouse, GFP expression is u[illegible]er the β-actin promoter activity and NSC can therefore be iden[illegible]ed by th[illegible] green fluorescence (Okabe et al., 1997). The day of [illegible]ception [illegible]s determined by the presence of a vaginal plug (embr[illegible]c day [illegible]. E15 stri[illegible] were isolated and triturated in DEM/F12 (Invit[illegible] with a ste[illegible] Pasteur pipette. The cell suspension was filtered with[illegible]0 μm-[illegible]e filter and viable cells were estimated by[illegible] pa[illegible]ue exclus[illegible]. [illegible]e cells were plated (1×10^6 cells/75-cm tis[illegible] cultur[illegible]flask) in D[illegible]M/F12 supplemented with epidermal gr[illegible]th factor (EGF, 20 ng/ml, Sigma), N2 and B27 (Neurobasal[illegible]edium, I[illegible]gen), which are two multicomponent cell culture supple[illegible]ents [illegible]oid of any growth factor. When the size of neurosphere reach[illegible]pproximately 50 cells, they were dissociated to a single cell suspens[illegible]by trituration and replated in fresh culture medium.

Immunological characterization of rSCs

rSCs have been characterized by immunocytochemical labelling for CD45 (Pharmingen, The Netherlands; 1:200), CD11b (Pharmingen; 1:200), Thy1.1 (Chemicon, Wevelgem, Belgium; 1:200) and P75 NGF-R (Chemicon; 1:200) (Goodell et al., 1997). Fluorescence-activated cell sorting of first passage rSCs was performed with anti-CD45 and anti-CD11b antibodies. Briefly, the cells were suspended with 10 mM ice-cold EDTA (Fluka, Bornem, Belgium) and 500,000 cells were washed in 3 ml of PBS co[illegible]% foetal calf serum. After centrifugation, the pellet was s[illegible]ended in 2[illegible]l of the primary antibodies solutions, for 1 hour at [illegible]m temperature. They were then washed 3 times in PBS containin[illegible]% foetal calf seru[illegible]nd incubated with anti-mouse IgG (1:500) sec[illegible]ry antibody c[illegible]pled to FITC (Jackson Immunoresearch L[illegible]oratory) [illegible] hour at r[illegible]m temperature and in the dark. Before an[illegible]sis using FA[illegible]the [illegible]s were fixed by a 15-minute incubation[illegible]n 1% [illegible]araformal[illegible]de solution. The analysis was perfor[illegible]d with [illegible] FACSort instrument (Becton Dickinson) and the r[illegible]ts were [illegible]alyzed usi[illegible] the Cellquest program (Becton Dickinson).

Functional characterization of r[illegible]

The adipos[illegible]ic [illegible]erentiation of rSCs was induced by treatment with 1-methyl[illegible]isobutyr[illegible]hine (0.5 mM, Sigma, Belgium), dexamethasone (1 μM, [illegible]ma), bovine insulin (0.01 mg/ml) and indomethacin (0.2 mM, S[illegible]a). rSCs were placed in the above adi[illegible]cyte induction medium for 24 days. The differentiation was [illegible]luated by accumulation of lipid vacuoles and staining with Oil Red [illegible] (Sigma) follow[illegible] fixation with 4% paraformaldehyde. To induce [illegible]teocyte differenti[illegible]on, the rSCs were incubated in DEM containing [illegible]amethasone (0.[illegible]M, Sigma), ascorbate (0.05 mM, Sigma) and β-gly[illegible]phosphate[illegible]0 mM, Sigma) for 12 days. A significant increase in alka[illegible]phatase activity was measured with the alkaline phosphatase colorimetric test, following the manufacturer's [illegible]tions (Sigma). Chondrogenic differentiation was induced with D[illegible]M me[illegible]um containing dexamethasone (0.1 μM, Sigma), sodium [illegible]yruvate (1 mM, Janssen Chemica), ascorbic-2-phosphate acid (0.15 mM, Sigma), proline (0.35 mM, Sigma), bovine insulin (0.25 μg/ml), [illegible]selenic acid (6.25 μg/ml, Sigma) and linoleic acid (5.35 μg/ml, [illegible]gma). Chondrocytes were obtained when rSCs were grown as a [illegible]ellet in the induction medium for 20 days. The cell aggregates were fixed with 4% paraformaldehyde, paraffin-embedded, cut at 5 μM sections and stained with toluidine blue.

Induction of sphere formation by rSCs

After being induced to express nestin, rSCs were trypsinized and suspended in DEM/F12 containing N2 and B27 supplements for 24 hours. During this time, the cells aggregated. These aggregates were plated onto polyornithine-coated dishes for 5 days in the same medium and were then processed for immunocytochemistry as described below.

Co-culture rSCs and mNSC

Nestin-positive rSCs were trypsinized and were co-incubated with GFP-positive mNSC (1×10^6 mNSC and 1×10^4 rSCs) for 48 hours in DEM/F12 containing EGF, N2 and B27 supplements. During this time, the cells aggregated, forming heterogenous spheres. For a good observation of the presence of rSCs into the heterogenous spheres, the rSCs were colored in red with the DiD Vybrant™ cell-labelling solutions (Molecular Probes) following the manufacturer's instructions. These spheres were plated on polyornithine-coated dishes for 5 days, in DEM/F12 containing N2 and B27 supplements and were processed for immunocytochemistry as described below. Nestin-negative rSCs were trypsinized and directly replated with GFP-positive mNSC onto polyornithine-coated dishes for 5 days following the same culture condition as described for the nestin-positive cells. The cells were then processed for immunocytochemistry.

Immunocytochemistry

The cultures were fixed with 4% (v/v) paraformaldehyde for 15 minutes at room temperature and washed 3 times in TBS buffer. They were then permeabilized in 1% Triton X-100 (v/v) for 15 minutes and washed 3 times in TBS buffer. Non-specific binding was blocked by a 1-hour treatment in TBST (TBS buffer with 0.1% Tween) containing fat-free milk powder (30 mg/ml). The cells were then incubated for 1 hour at room temperature with either anti-p75/NGF-R, or anti-Thy1.1, or anti-nestin (Rat401, Pharmingen; mouse IgG, dilution 1:1500), or anti-GFAP (Dako; mouse IgG, dilution 1:500), or anti-M2 (Developmental Studies Hybridoma Bank; rat IgG, dilution 1:500), or anti-GLAST (Shibata et al., 1997) (rabbit IgG, 1:4000) primary antibodies (diluted in blocking buffer). After 3 washes, cells were incubated in FITC- or Cy5-conjugated anti-mouse IgG (Jackson Immunoresearch; 1:500) or rhodamine-conjugated anti-rat IgG (Jackson Immunoresearch, 1:500) for 1 hour at room temperature and in the dark. The nuclei were stained with ethidium homodimer (0.2 μM, Sigma). The preparations were then mounted in Fluoprep™ (bioMérieux, Marcy L'Etoile, France) and observed using a Bio-Rad MRC1024 laser scanning confocal microscope.

Western blot

Total protein extracts were obtained from confluent cells that had been cultured in the different media. The cells were harvested by scraping the dish in 500 μl lysis buffer (0.6 M KCl, 5 mM EGTA, 5 mM EDTA, 1% Triton X-100 and 1 mM PMSF in PBS). Extracts were then fractioned into pelletable (insoluble) and non-pelletable (soluble) proteins by centrifugation at 30,000 ***g*** for 15 minutes at 4°C. The pellet was resuspended in loading buffer (glycerol 10% v/v; T[illegible]05 M, pH 6.8; SDS 2%, bromophenol blue and 2.5% [illegible] β-mercaptoethanol) and the suspension was centrifuged at 30,000 [illegible] for 15 minutes at 4°C. The supernatant was used for protein concentration measurement using the RC DC Protein Assay (Bio-Rad). The same protein quantities in each lane were separated by electrophoresis using the Phastgel 4-15% SDS gradient (Amersham Pharmacia Biotech) and transferred to a PVDF membrane. The membranes were saturated with 3% gelatin (BioRad), incubated for 1 hour with a [illegible] antibody against rat nestin (Pharmingen, 1:[illegible]00) or β-actin, as control for protein loading (Sigma, 1:5000) at room temperature and then washed several times with PBS-0.1% Tween. The membrane was then incubated in biotinylated goat anti-mouse antibody (Boehringher Mannheim; 1:5000) for 1 hour at room temperature. After several washes in PBS-0.1% Tween, the membrane was incubated with peroxidase-coupled streptavidin (1:100,000, Sigma) at 37°C for 1 hour. Radial glial cells were used as a positive control for nestin expression.

DNA ploidy

DNA content per cell was determined by FACS analysis after staining the cells with propidium iodide. After 5 days of co-culture, rSCs and mNSCs were trypsinised and fixed to 70% ethanol, at 4°C during 16 hours. The cells were stained with propidium iodide (400 μg/ml, Sigma) just before FACS analysis.

Results

Immunological and functional characterization of rSCs

Rat stromal cells were isolated from the femoral and tibial bones of adult rats and propagated in culture. rSCs have been reported to be CD45- and CD11b-negative, but Thy1.1- and P75 NGF-R-positive (Goodell et al., 1997). Fluorescent cell sorting at passage 1 demonstrated that the cells were negative for CD11b (Fig. 1A) and CD45 (Fig. 1B) – two cell surface markers associated with lymphohematopoietic cells. In contrast, immunofluorescent labeling demonstrated that 100% of the rSCs were positive for Thy1.1 (Fig. 1C) and p75 NGF receptor (Fig. 1D). Classically, SCs can differentiate either into adipocytes, or chondrocytes and osteocytes (Pereira et al., 1995; Kuznetsov et al., 2001; Bruckop, 1999; Majumdar et al., 1998; Pittenger et al., 1999). When our putative rSCs fraction was placed in adipogenic, osteogenic and chondrogenic induction media, adipocytes, osteocytes and chondrocytes, respectively, were obtained (Fig. 1E-G).

Differentiation of rSCs into Nestin-positive cells

To induce neural differentiation of rSCs in long-term cultures, we placed them in a classical NSC culture medium (DEM/F12 supplemented with N2 and B27). After 72 hours most rSCs expressed nestin as revealed by immunocytochemistry (Fig. 2A). When rSCs are placed in DEM/F12 (Fig. 2B), DEM/F12+N2 (Fig. 2C) and DEM/F12+B27 (Fig. 2D), the same result is obtained, suggesting that the removal of serum from the culture medium was actually responsible for the induction of nestin expression by rSCs. These results were confirmed by Western blotting (Fig. 2F). In all of these experiments, no alteration in the morphology of nestin-positive SCs was observed. We demonstrated that the absence of serum was necessary but not sufficient for the induction of nestin expression. Indeed, the number of passages of rSCs in vitro also regulates their ability to express nestin when grown in serum-free condition (Fig. 3A-E): a minimum of ten passages is needed before rSCs are able to express nestin when placed in serum-free culture conditions. However, the capacity of these cells to differentiate into adipocytes or osteocytes did not change as a function of the number of passages (Fig. 3F,H).

Induction of sphere formation by rSCs

Recently, Tropepe et al. (Tropepe et al., 2001) suggested that nestin expression by ES cells is correlated with the capacity to form neurospheres and that ES cells have to go through a nestin-expression stage before differentiating into neural cells. When nestin-positive and nestin-negative rSCs were trypsinised and replaced in an NSC growth medium (DEM/F12, N2 and B27) in non-adherent conditions, we observed that passage 15 nestin-positive rSCs aggregated in suspension (Fig. 4A). In contrast, passage 4 nestin-negative rSCs which had been cultivated in serum-free conditions for 48 hours prior to being transferred to NSCs growth medium, remained in suspension and did not form spheres or aggregates (Fig. 4B). Passage 15 rSCs that did not express nestin (because they had been cultivated in serum-containing medium) adhered spontaneously to the dish (Fig. 4C). We then plated the nestin-positive rSCs aggregates on a polyornithine-coated surface for 5 days as is done for neurospheres in order to stimulate cell differentiation. The cell morphology changed from the flat and elongated shape of MSCs (Fig. 4D) to more rounded morphology (Fig. 4E), but immunocytological labelling with antibodies recognizing astrocytic (anti-GFAP, anti-GLAST), oligodendroglial (anti-O4, anti-A2B5) and neuronal markers (anti-NeuN, anti-NSE, anti-Tuj1, anti-MAP2B) were all negative (data not shown). Moreover, after 5 days in these

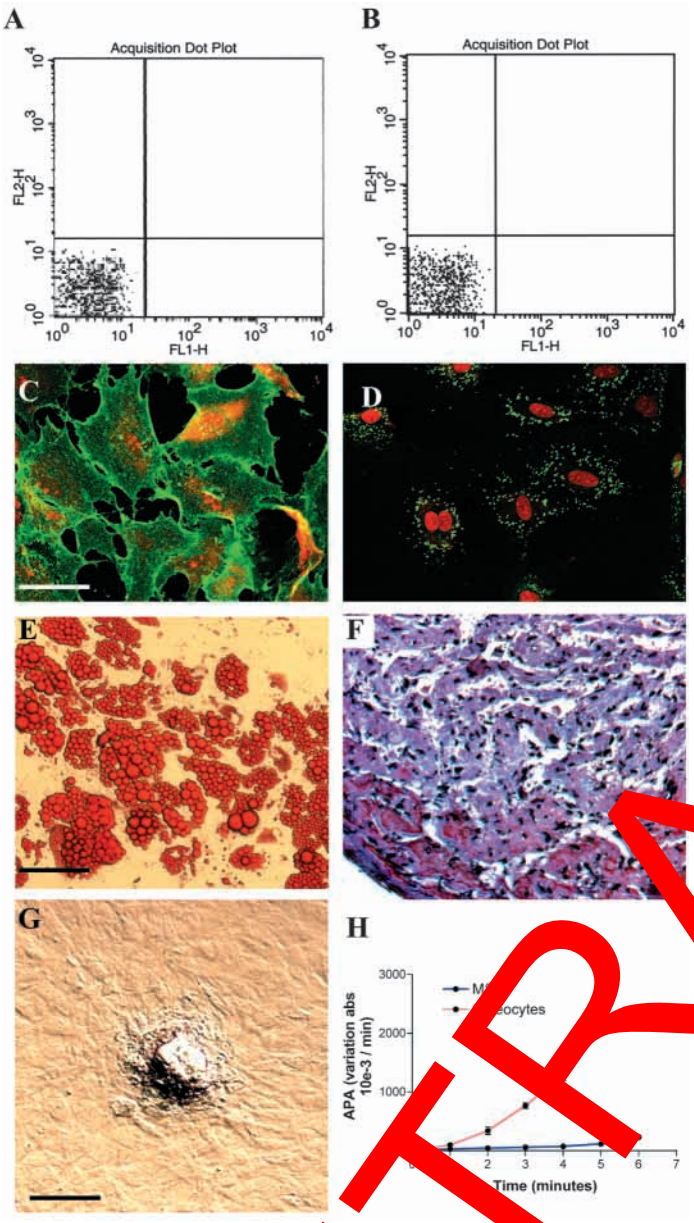

Fig. 1. Immunological and functional characterization of undifferentiated rSCs. FACS demonstrates that the cells are negative for CD11b (A) and CD45 (B), two lymphohematopoietic surface markers. Immunofluorescent labelling shows that rSCs are positive for Thy1.1 (C) and p75 NGF receptor (D). Adipocyte induction was revealed by accumulation of lipid vacuoles that were stained with Oil Red O (E). Chondrocytes were obtained by culturing rSCs as a pellet for 20 days in chondrogenic induction medium. Sections of the paraffin-embedded cells were stained with toluidine blue. The formation of cartilaginous structures is characterized by differentiated chondrocytes in lacunae surrounded by extracellular matrix (F). When rSCs were placed in osteocyte induction medium, cells formed nodules (G) with multi-layered regions, and a significant increase in alkaline phosphatase was observed (H). Enzyme activity of the cells was measured in triplicate cultures with the alkaline phosphatase colorimetric test. In this test, the variation of the absorbency was measured as a function of time at 405 nm. Scale bars: (C-E) 40 μm; (F,G) 150 μm.

conditions, nestin expression decreased from 80% to 15% (Fig. 4F).

Co-culture of rSCs and mNSCs

As nestin expression by rSCs appears insufficient to stimulate their differentiation into neural cells, we have co-cultivated nestin-positive rSCs with mNSCs because the neural differentiation of MSC has been observed mainly in vivo when cells had been grafted in newborn rat brain. When we co-cultivated nestin-positive red-labelled rSCs for 5 days with NSCs obtained from E15 'green mouse' striata (Okabe et al., 1997), we observed that nestin-positive rSCs formed heterogenous spheres (Fig. 5M) with the NSC. These heterogenous spheres were then transferred on polyornithine-coated dishes for 5 days allowing cellular differentiation. Immunolabeling revealed the differentiation of 40±2.39% nestin-positive rSCs into GFAP-expressing cells (n=3, representing 2000 cells) (Fig. 5A-C). A similar percentage of GLAST-positive rSCs was also observed in these conditions (Fig. 5G-I). However, no nestin-positive rSCs expressed neuronal (anti-NeuN, anti-NSE, anti-Tuj1, anti-MAP2B) or oligodendroglial markers (anti-O4, anti-A2B5) (data not shown). Moreover, passage 4 nestin-negative rSCs were unable to form heterogenous spheres, and when plated directly with mNSC onto a polyornithine-coated surface, only 4% of them expressed GFAP (Fig. 5D-F). Three justifications could be formulated to explain the GFAP expression by rSC: 1) a decrease of GFP expression in mNSCs, 2) a rSC-mNSC fusion event, and 3) phenotypic plasticity of the rSCs. To exclude a GFP downregulation, we cultivated GFP-positive mNSC alone in the same conditions, and a GFAP immunoreactivity was observed within GFP-positive cells (Fig. 5J-L). To exclude a cell fusion process, we first used antibody M2, which specifically recognizes mouse-specific astrocytes (Lagenaur and Schachner, 1981) to demonstrate that the GFP-negative astrocytes that developed under these conditions were of rat origin: no GFP-negative, GFAP-positive cells were recognized by the M2 antibody, but the GFAP- and GFP-positive cells were (Fig. 5N,O). Furthermore, we analyzed the ploidy of the co-cultivated cells in order to determine if rSCs adopt the astroglial phenotype of the recipient cells by a hypothetical cell fusion event. We compared DNA ploidy of the rSCs maintained in co-culture with mNSC for 5 days (Fig. 5P) with the DNA ploidy of rSCs and mNSC cultivated separately for the same period, and we found the same DNA content in the three cultures, thus excluding a cell fusion event.

Discussion

Cellular therapies are promising approaches in the treatment of several neurological diseases such as Parkinson's disease (Isacson et al., 2001) or Huntington's disease (Dunnett, 2000), but also for spinal cord injury (Hall, 2001), and, in association with gene therapy, the treatment of glioma (Armstrong et al., 2000; Isacson and Sladek, Jr, 1999). One main problem concerns the origin and nature of the cells to be used for such procedures. The ideal cell should exhibit several key properties, including: (1) a high level of proliferation in vitro, allowing the production of a large number of cells from a minimal amount of donor material,

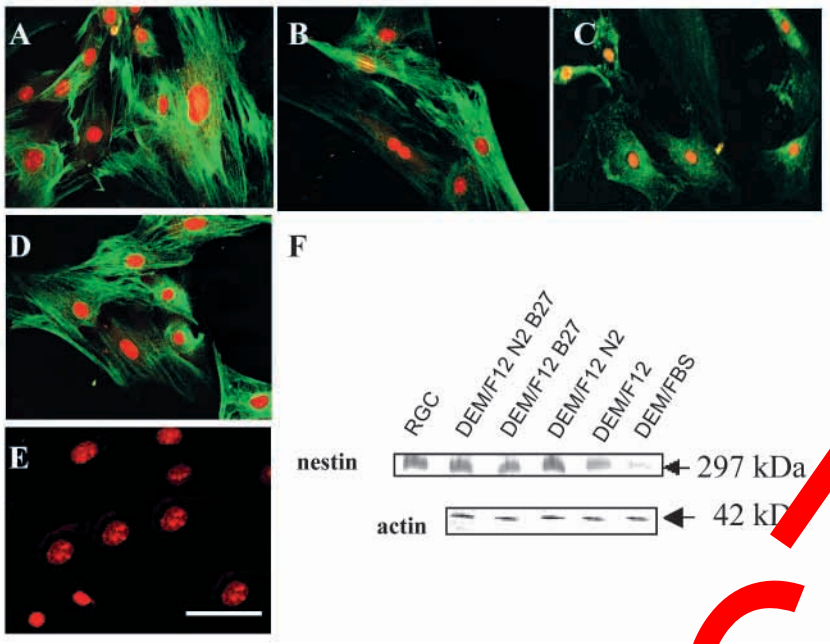

Fig. 2. Regulation of nestin expression by rSCs by the serum. rSCs were cultivated in different culture medium: DEM/F12+N2+B27 (A), DEM/F12 (B), DEM/F12+N2 (C) or DEM/F12+B27 (D) and DEM/10% FBS (E). C[illegible]ere labeled with anti-nestin antibody (gr[illegible]) and cel[illegible]lei were counterstained wi[illegible]ethidium home[illegible]ner (red). Scale bar: (A-E) 40 µ[illegible] Nestin expression present in cells grown in an[illegible]m-free media, [illegible]d this result was confirmed by we[illegible]n blotting [illegible]. Radial glial cells (RG[illegible]) were used a[illegible]estin-[illegible]sitive control and β-actin [illegible]s used as protein-l[illegible]g control.

(2) a good control of this proliferative activity in vivo, and (3) a phenotypic plasticity allowing the differentiation into appropriate neuronal or glial phenotype. The cells used for transplantation into patients with neurological disorde[illegible] hitherto been derived from the foetal human CNS. For example, the cells grafted in Parkinson's disease patients [illegible]ve been obtained from foetal donors. They are isolated as po[illegible] mitotic neurons from foetal mesencephalic tis[illegible] in whic[illegible] dopaminergic neurons are normally found. T[illegible]s app[illegible]ach has several pitfalls: a limited number of cells i[illegible]vailable[illegible] grafted patients need to be immunosuppressed[illegible]he mate[illegible] easily available and there are a numbe[illegible]f ethic[illegible] problems (Freed et al., 2001).

Several recent reports suggest t[illegible] bone ma[illegible]w stromal cells could be a non-embryonic [illegible]non-foetal sourc[illegible]f stem cells suitable for cell replacemen[illegible]st[illegible]gies in the treatment of CNS disorders (Kopen et al.[illegible]999; A[illegible]ama et al., 2002a; Akiyama et al., 2002b; Brazelton et [illegible] 2000). An understanding of the [illegible]olecular regulation of such a 'mesenchymal-neural' t[illegible]sition may be very important when considering the use of [illegible]Cs in th[illegible]reatment of CNS disorders.

In our study, rSC[illegible]ave be[illegible]isolated [illegible]om adult rat bone marrow using a differe[illegible]l ad[illegible]esion pro[illegible]dure and selected by their capacity of rapid pro[illegible]ation. W[illegible]demonstrate that these stromal cells [illegible]to exp[illegible]tin. This expression is dependent on[illegible]wo fac[illegible]s. The first [illegible]e is the absence of serum-derived co[illegible]ponents [illegible]the culture medium. Indeed, in our study usi[illegible] weste[illegible]lot analysis and cell counting, higher levels of [illegible]tin expression were found in serum-free conditions, alt[illegible]h RT-PCR and western blot demonstrate a faint signal for ne[illegible] even in the presence of serum. The second factor is the nu[illegible]ber of cell passages. A minimum of ten passages is required for nestin expression by 75% of rSCs. However, the capacity of rMSC to differentiate into adipocytes, osteocytes or chondrocytes does not change as a function of the number of passages. This requirement of 10 passages (which correspond to 25 doubling populations) in vitro was reproc[illegible]ble in 4 i[illegible]ependent experiments. [illegible]ecently, [illegible]ng et [illegible]. (Jiang et al., 2002), demonstrated [illegible]t [illegible]e cells within murine bone marrow MSC cul[illegible]res can differentiate not only [illegible] the mesenchymal lineage cells but also into endo[illegible]ium, ectoderm and endoderm. These rare cells, [illegible]hich have been named as multipotent adult progenitor cells (or MAPCs), can be expanded for more than 100 population d[illegible]blings, bringing about an enrichment in M[illegible]PCs in MSCs culture. The increase in the [illegible]rcentage of n[illegible]in-positive MSCs as a function of the number o[illegible]assages [illegible]ld possibly be explained by the presence of these [illegible]s in our cultures and their increase in number with additional passages.

[illegible]how that only rSCs from nestin-positive cultures are [illegible]ble to form clusters or aggregates in the non-adherent conditions used to cultivate NSCs. Nestin expression by rSCs and their ability to grow in suspension in such defined culture conditions bring them nearer to the NSCs phenotype. However, when nestin-positive rSCs spheres were plated onto an adherent surface, no glial and/or neuronal differentiation was observed. It seems that the complete neural differentiation of MSCs observed in vivo, may require the involvement of several induction signals which have not been reproduced in vitro. It is for this reason that rSCs and NSCs were co-cultivated. It was hoped that such an experiment would reproduce in vitro some of the complex molecular interactions that are required to induce a full neural differentiation of mesenchymal cells in vivo.

Under these co-culture conditions, nestin-positive rSCs were able to express GFAP, the astroglia-specific intermediate filament protein, but also GLAST, another marker of astroglia (Shibata et al., 1997). When nestin-negative rSCs were co-cultivated with NSCs under identical conditions, a low percentage of GFAP-positive cells of mesenchymal origin was observed, suggesting that nestin expression is a prerequisite for rSCs differentiation into a GFAP- or a GLAST-positive cell type. This observation is in agreement with the notion that an ordered succession of stimuli is needed to promote such a differentiation.

Given the fact that it has been reported that 0.2-1 of ES cells per 10^5 bone marrow cells can fuse with an other cell-type and thus mimic a differentiation and/or plasticity (Terada and al., 2002), we have excluded this possibility by two methods: first, we could not find any mouse-specific antigenic labeling on GFAP-positive cells of rSCs origin, and we exclude a tetraploidy in our co-culture by FACS analysis. So, GFAP-

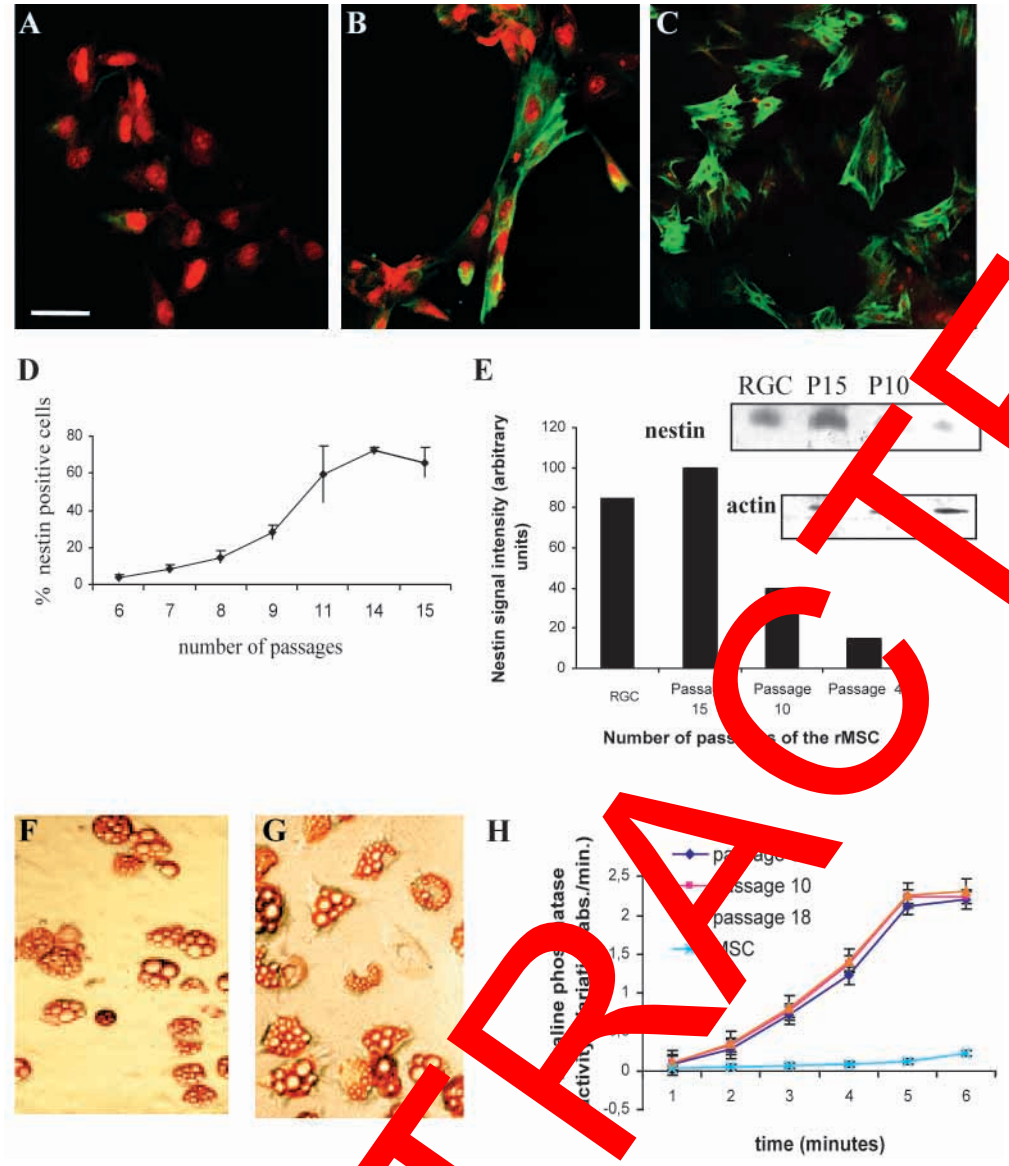

Fig. 3. Regulation of nestin expression in rSCs, by the number of passages. rSCs express nestin weakly between passage 0 and passage 6 (A, rSCs passage 4). The expression increases between passage 6 and passage 10 (B, rSCs passage 10). Beyond passage 11, ~75% of rSCs express nestin in serum-free medium (C, rSCs passage 15). Cultures were labeled with nestin antibody (green labelling) and cell nuclei were counterstained with ethidium homodimer (red labelling). (D) The evolution of nestin expression as function of the number of passages of rSCs. This graphic was established from results of immunocytochemical analysis on passage 4, 6, 7, 8, 9, 11, 14 and 15. In this experiment, results are expressed as percentages of nestin-positive cells in each passage in serum-free condition (n=4, representing a minimum of 1500 counted cells in each passage). These results were confirmed by western blotting on passage 4 (P4), 10 (P10) and 15 (P15) (E). Radial glial cells (RGCs) were used as nestin-positive control and actin was used as protein-loading control. No alteration of the capacity of differentiation: (F) P10 adipocyte, (G) P18 adipocyte, (H) osteocytes can be observed even in the long-term cultures. Scale bar: (A-C,F,G) 40 μm.

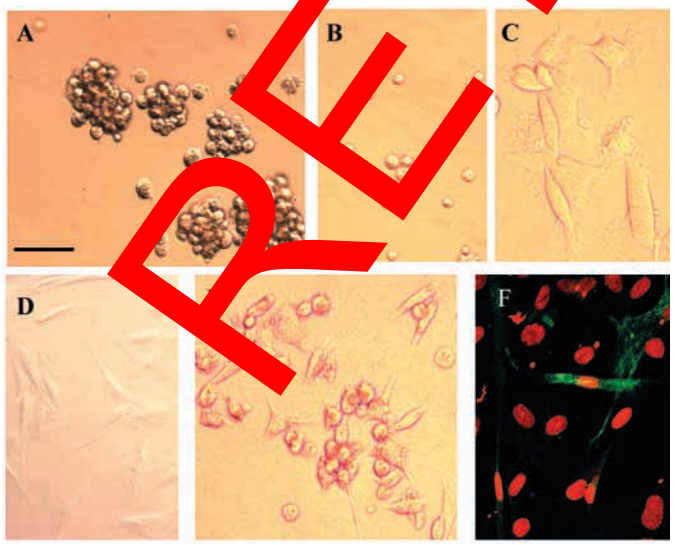

Fig. 4. Simultaneous nestin expression and aggregates formation. After 72 hours incubation in serum-free medium, the cells were trypsinised and resuspended in DEM/F12 with N2 and B27 for 24 hours. During that time, passage 15 cells aggregated to form clusters or aggregates resembling neurospheres (A), whereas passage 4 rSCs remained in suspension (B). Nestin-negative passage 15 cells immediately adhere on the culture dish (C). The spheres obtained in condition A were then placed on polyornithine for 5 days in DEM/F12, N2 and B27 (E) where they exhibited a different morphology than that observed in DEM/20% FBS medium (D). Nestin immunocytochemistry (green) reveals that only 15% of the cells still express nestin under these conditions (F). Scale bar: (A-F) 40 μm.

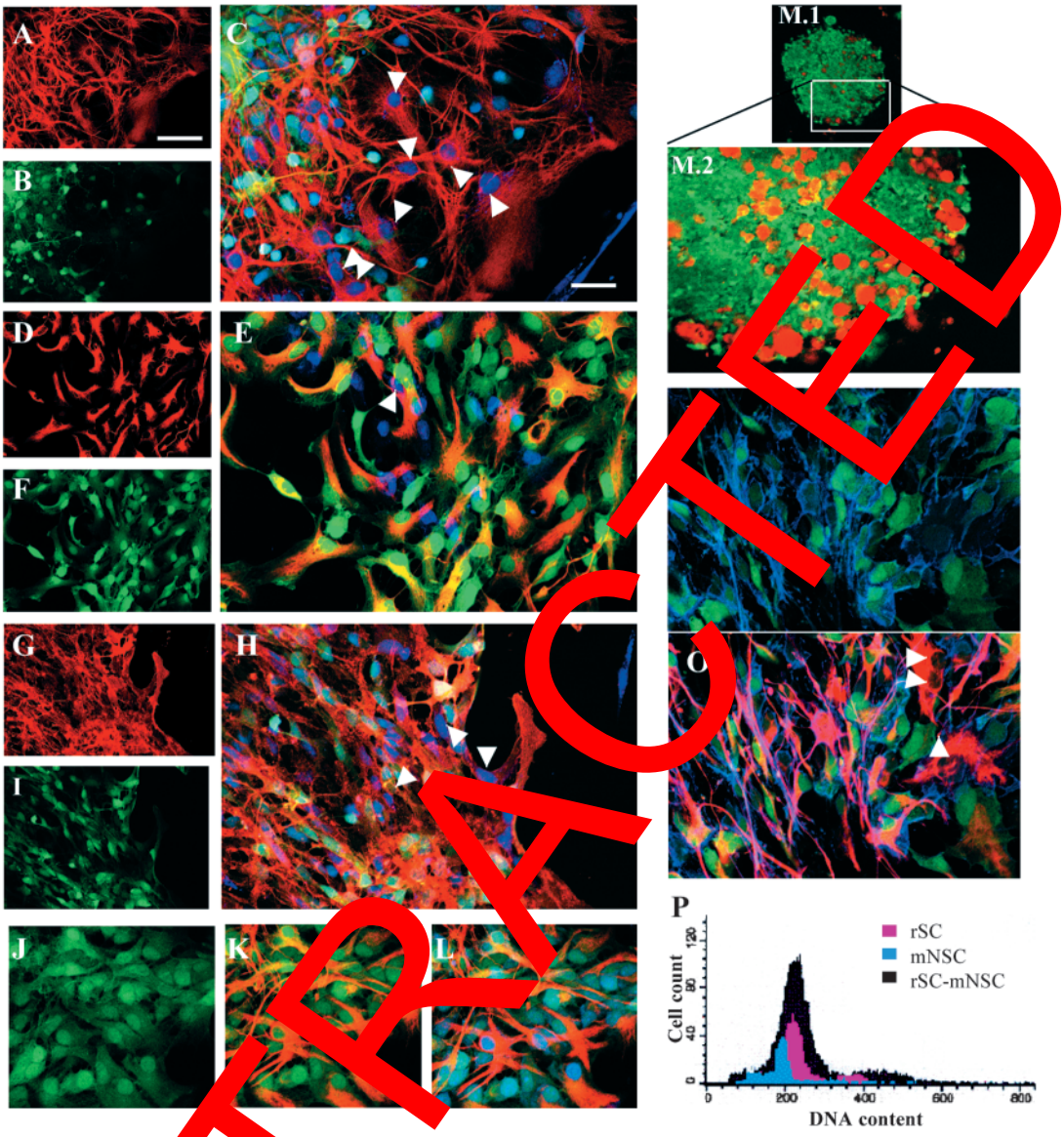

Fig. 5. Effect of co-culture on ne[illegible]n. Heterogenous spheres of nestin-positive rSCs stained with DiD Vybrant™ cell-labelling solution (red) (Molecular Probes) and GFP-positi[illegible]SCs (M.1,M.2) are placed on polyornithine-coated dishes for 5 days. Some rSCs have differentiated into GFAP-pos[illegible]e cells (indicated by a[illegible]owheads). GFAP (red) is expressed by a large fraction of cells (A), of which some also contained green fluorescent [illegible]otein (B), and thus originate from mNSC. Triple labelling (including nuclei stained by EtD1 in blue) allow the identification of non-gree[illegible]SCs that [illegible]press GFAP (C). Co-culture of passage 4 nestin-negative rSCs with GFP-positive mNSC demonstrated that only a small percen[illegible]ge of GF[illegible]-positive [illegible]ells were derived from MSCs (D-F). Astroglial differentiation of passage 15 rSCs were confirmed with Glast m[illegible] (red) (G-I). Pur[illegible]FP-positive mNSC were used as a control to demonstrate that all GFAP (red)-positive cells from mNSC remain GFP-p[illegible] (green) (J-L). A double-labelling with M2 (blue) and GFAP (red) antibodies allows the confirmation of the mesenchymal origin of some G[illegible] positi[illegible] cells (O). rSC-derived GFAP-positive cells are not recognized by the M2 antibody. The GFP-positive astrocy[illegible] (gr[illegible]astrocytes) differentiate from mNSC are recognized by the M2 antibody (N). rSCs, mNSC and co-cultured rSCs and mNSC w[illegible] stained [illegible]ith propidium iodide and subjected to FACS analysis (P). Arrowheads in C,E,H,O indicate the mesenchymal-derived cells that ex[illegible]ess neural [illegible]arkers. Scale bars: (A,B,D,F,G,I,M.1) 150 μm; (C,E,H,J,K-M.2) 40 μm; (N,O) 60 μm.

expression by rSC[illegible]e independent of cell-fusion events or GFP downregulation i[illegible]mNSC, but well a neural phenotypic plasticity of rSCs.

In conclusion, nestin expression by rSCs should be regarded as a first step in their progression to the neural lineage. A better knowledge of the regulation of their differentiation into astrocytes and the definition of appropriate culture conditions to obtain their differentiation into neurons and/or oligodendrocytes is still needed before considering MSCs as an appropriate cellular material to be used for cell replacement therapies in CNS disorders.

This work was supported by the grant of the Fonds National de la Recherche Scientifique (FNRS) of Belgium, by the Fondation

Medical Reine Elisabeth (FMRE), by the Fonds Charcot, and by the Belgian League against Multiple Sclerosis. S.W. is a research fellow of the Télévie (FNRS). B.R. and P.L. are senior research associate and research associate of the FNRS, respectively. We thank Dr André Gothot (Department of Haematology, University of Liège, C.H.U. Sart Tilman, Belgium) and Bernard Coomans (Center for Cellular and Molecular Neurobiology, University of Liège, Belgium) for help with the FACS analysis, Dr Gary Brook (Department of Neurology, RWTH, Aachen, Germany) for his comments during the preparation of the manuscript, and Dr Tanaka (Department of Neurological Diseases, Kodaira, Japan) for the gift of anti-GLAST antibody.

References

Akiyama, Y., Radtke, C., Honmou, O. and Kocsis, J. D. (2002a). Remyelination of the spinal cord following intravenous delivery of bone marrow cells. *Glia* **39**, 229-236.

Akiyama, Y., Radtke, C. and Kocsis, J. D. (2002b). Remyelination of the rat spinal cord by transplantation of identified bone marrow stromal cells. *J. Neurosci.* **22**, 6623-6630.

Armstrong, R. J., Watts, C., Svendsen, C. N., Dunnett, S. B. and Rosser, A. E. (2000). Survival, neuronal differentiation, and fiber outgrowth of propagated human neural precursor grafts in an animal model of Huntington's disease. *Cell Transplant.* **9**, 55-64.

Azizi, S. A., Stokes, D., Augelli, B. J., DiGirolamo, C. and Prockop, D. J. (1998). Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats – similarities to astrocyte grafts. *Proc. Natl. Acad. Sci. USA* **95**, 3908-3913.

Bianco, P., Riminucci, M., Kuznetsov, S. and Robey, P. G. (1999). Multipotential cells in the bone marrow stroma: regulation in the context of organ physiology. *Crit. Rev. Eukaryotic Gene Expr.* **9**, 159-173.

Brazelton, T. R., Rossi, F. M., Keshet, G. I. and Blau, H. M. (2000). From marrow to brain: expression of neuronal phenotypes in adult mice. *Science* **290**, 1672-1674.

Dahlstrand, J., Lardelli, M. and Lendahl, U. (1995). Nestin mRNA expression correlates with the central nervous system progenitor cell state in many, but not all, regions of developing central nervous system. *Brain Res. Dev. Brain Res.* **84**, 109-129.

Davis, A. and Temple, S. (1994). A self-renewing multipotential stem cell in embryonic rat cerebral cortex. *Nature* **372**, 263-266.

Deng, W., Fischer, O. and Prockop, D. J. (2001). In vitro differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase intracellular cyclic AMP. *Biochem. Biophys. Res. Commun.* **282**, 148-152.

Domen, J. and Weissman, I. L. (1999). Self-renewal, differentiation or death: regulation and manipulation of hematopoietic stem cell fate. *Mol. Med. Today* **5**, 201-208.

Dunnett, S. B. (2000). Functional analysis of fronto-striatal reconstruction by striatal grafts. *Novartis Found. Symp.* **231**, 21-41.

Freed, C. R., Greene, P. E., Breeze, R. E., Tsai, W. Y., DuMouchel, W., Kao, R., Dillon, S., Winfield, J., Culver, S. and Trojanowski, J. Q. et al. (2001). Transplantation of embryonic dopamine neurons for severe Parkinson's disease. *New Engl. J. Med.* **344**, 710-719.

Gandarillas, A. and Watt, F. M. (1997). c-Myc promotes differentiation of human epidermal stem cells. *Genes Dev.* **11**, 2869-2882.

Goodell, M. A., Rosenzweig, M., Kim, H., Marks, D. F., DeMaria, M., Paradis, G., Grupp, S. A., Sieff, C. A., Mulligan, R. C. and Johnson, R. P. (1997). Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nat. Med.* **3**, 1337-1345.

Gronthos, S., Mankani, M., Brahim, J., Robey, P. G. and Shi, S. (2000). Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc. Natl. Acad. Sci. USA* **97**, 13625-13630.

Hall, E. D. (2001). Pharmacological treatment of acute spinal cord injury: how do we build on past successes? *J. Spinal Cord. Med.* **24**, 142-146.

Isacson, O. and Sladek, J. R., Jr (1999). Cellular and molecular treatments of neurological diseases. *Exp. Neurol.* **159**, 1-3.

Isacson, O., van Horne, C., Schumacher, J. M. and Brownell, A. L. (2001). Improved surgical cell therapy in Parkinson's disease. Physiological basis and new transplantation methodology. *Adv. Neurol.* **86**, 447-454.

Jiang, Y., Jahagirdar, B. N., Reinhardt, R. L., Schwartz, R. E., Keene, C. D., Ortiz-Gonzalez, X. R., Reyes, M., Lenvik, T., Lund, T. and Blackstad, M. et al. (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418**, 41-49.

Kopen, G. C., Prockop, D. J. and Phinney, D. G. (1999). Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal murine brains. *Proc. Natl. Acad. Sci. USA* **96**, 10711-10716.

Kuznetsov, S. A., Mankani, M. H., Gronthos, S., Satomura, K., Bianco, P. and Robey, P. G. (2001). Circulating skeletal stem cells. *J. Cell Biol.* **153**, 1133-1140.

Lagenaur, C. and Schachner, M. (1981). Monoclonal antibody (M2) to glial and neuronal cell surfaces. *J. Supramol. Struct. Cell Biochem.* **15**, 335-346.

Lendahl, U., Zimmerman, L. B. and McKay, R. D. (1990). CNS stem cells express a new class of intermediate filament protein. *Cell* **60**, 585-595.

Majumdar, M. K., Thiede, M. A., Mosca, J. D., Moorman, M. A. and Gerson, S. L. (1998). Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. *J. Cell. Physiol.* **176**, 57-66.

Mezey, E., Chandross, K. J., Harta, G., Maki, R. A. and McKercher, S. R. (2000). Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. *Science* **290**, 1779-1782.

Mokry, J. and Nemecek, S. (1998). Immunohistochemical detection of intermediate filament nestin. *Acta Medica* (Hradec Kralove) **41**, 73-80.

Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T. and Nishimune, Y. (1997). 'Green mice' as a source of ubiquitous green cells. *FEBS Lett.* **407**, 313-319.

Pereira, R. F., Halford, K. W., O'Hara, M. D., Leeper, D. B., Sokolov, B. P., Pollard, M. D., Bagasra, O. and Prockop, D. J. (1995). Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. *Proc. Natl. Acad. Sci. USA* **92**, 4857-4861.

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S. and Marshak, D. R. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143-147.

Prockop, D. J. (1997). Marrow stromal cells as stem cells for non hematopoietic tissues. *Science* **276**, 71-74.

Sanchez-Ramos, J., Song, S., Cardozo-Pelaez, F., Hazzi, C., Stedeford, T., Willing, A., Freeman, T. B., Saporta, S., Janssen, W. and Patel, N. et al. (2000). Adult bone marrow stromal cells differentiate into neural cells in vitro. *Exp. Neurol.* **164**, 247-256.

Seale, P. and Rudnicki, M. A. (2000). A new look at the origin, function, and 'stem-cell' status of muscle satellite cells. *Dev. Biol.* **218**, 115-124.

Sell, S. (1990). Is there a liver stem cell? *Cancer Res.* **50**, 3811-3815.

Shibata, T., Yamada, K., Watanabe, M., Ikenaba, K., Wada, K., Tanaka, K. and Inoue, Y. (1997). Glutamate transporter GLAST is expressed in the radial glia-astrocyte lineage of developing mouse spinal cord. *J. Neurosci.* **17**, 9212-9219.

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D. M., Nakano, Y, Meyer, E., Morel, L., Petersen, B. and Scott, E. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature* **416**, 542-545.

Tropepe, V., Hitoshi, S., Sirard, C., Mak, T. W., Rossant, J. and van der Kooy, D. (2001). Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. *Neuron* **30**, 65-78.

Wu, D. M., Schneiderman, T., Burgett, J., Gokhale, P., Barthel, L. and Raymond, P. A. (2001). Cones regenerate from retinal stem cells sequestered in the inner nuclear layer of adult goldfish retina. *Invest. Ophthalmol. Vis. Sci.* **42**, 2115-2124.