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Neuronal Differentiation of Stem Cells Isolated From Adult Muscle

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Lineage uncommitted pluripotent stem cells reside in the connective tissue of skeletal muscle. The present study was carried out with pluripotent stem cells (PPSCs) isolated from 6-month old rat muscle. Before differentiation, these cells were vimentin+, CD90+, CD45−, and varied in their expression of CD34. The PPSCs were expanded as non-adherent aggregates under similar conditions to those used to generate neurospheres from embryonic or neural stem cells. The PPSC-derived neurospheres were positive for nestin, an early marker present in neuronal precursors, and expressed the two alternative mRNA forms of the neuroectodermal marker Pax-6, as well as mRNA for Oct-4, a gene related to the pluripotentiality of stem cells. To confirm their neural potential, PPSC-derived neurospheres were plated on coated coverslips under varying conditions: Neurobasal medium with N2 or B27, and either NT3 or BDNF. After 4–6 days the cells expressed neuronal (Tuj1+, NF68), astrocytic (GFAP) and oligodendrocytic (MOSP+, MBP+) markers, both by immunocytochemistry and RT-PCR. In addition, PPSCs were cultured as monolayers under adherent conditions, exposed to growth factors and defined differentiating conditions for 5 hr, and subsequently kept for 2 days in a maturation medium. At this point they gave rise to a mixed population of early neural progenitors (Nestin+ or NG2+), immature and mature neurons (Tuj1+ and NF145+) and myelin producing oligodendrocytes (CNPase + and MOSP+). Our study shows that PPSCs present in adult muscle can overcome germ lineage restrictions and express the molecular characteristics of brain cells. Therefore, PPSCs isolated from adult muscle could provide a novel source for autologous cell replacement in neurodegenerative and demyelinating diseases.

Key words: neuron; differentiation; neurospheres; oligodendroglia, myelin

Stem cells were thought previously to be life time-committed to specific lineages depending on their germ layer (Faust and Magnuson, 1993). Recent studies, however, have demonstrated lineage interconversion within the same or even different germ layers (Ferrari et al., 1998; Gussoni et al., 1999; Galli et al., 2000; Toma et al., 2001). Thus, stem cells derived from adult tissues may retain plasticity in their commitment, and their differentiation may be influenced by environment rather than by lineage (for review see Clarke and Frisen, 2001). In some cases, however, apparent lineage interconversion may be the result of the continuous presence of a small number of cells from a different lineage in adult tissue (Jackson et al., 1999; McKinney-Freeman et al., 2002).

Previous studies have shown that stem cells capable of differentiating into several mesodermal phenotypes reside within adult muscle tissue (Pate et al., 1993; Lucas et al., 1995; Cornelison and Wold, 1997; Katagiri et al., 1997; Gussoni et al., 1999; Jackson et al., 1999; Bosch et al., 2000; Lee et al., 2000; Young et al., 2001a;b). By clonal analysis we have shown that this population contains progenitors stem cells (lineage committed) and pluripotent stem cells (lineage uncommitted) capable of differentiating into muscle, osteogenic, adipogenic and chondrogenic cells (Young et al., 1999; Young et al., 2001a;b). These lineage uncommitted cells are able of extended self-renewal and of generating different lineage committed progenitor cells from a single cell clone as expected from stem cells. It is unclear, however, whether this population of pluripotent stem cells (PPSCs) can be induced to primarily express a neural phenotype.

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If stem cells isolated from adult muscle could differentiate into neurons and glia, they could provide a unique source of cells for neural repair. Clinical evidence indicates that transplantation of fetal brain tissue might be a viable therapy for some neurodegenerative diseases (Bjorklund and Lindvall, 2000); however, restricted availability of fetal human tissue, ethical hurdles and the need for toxic immunosuppressant drugs seriously limit this approach. Autologous transplantation avoids the use of immunosuppressants and the risk of infection from the graft.

Recent studies have shown that exogenous bone marrow stem cells can differentiate into neurons in vitro (Sanchez-Ramos et al., 2000; Woodbury et al., 2000; Deng et al., 2001) and when transplanted into brain (Azizi et al., 1998; Kopen et al., 1999; Brazelton et al., 2000; Mezey et al., 2000). The use of bone marrow cells, however, has its own limitations and an alternate source of stem cells that could be easily and safely harvested from the patient would be highly desirable. The goal of the present study was to determine whether pluripotent stem cells isolated from adult muscle could be directed to express a neural phenotype in vitro as a prelude to future transplantation studies.

MATERIALS AND METHODS

Cell Isolation

PPSCs were isolated from the gastrocnemius and flexor digitorum of 6-month-old Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) as described previously (Lucas et al., 1995). PPSCs cultured originally in OptiMem (GibcoBRL, Grand Island, NY) with 15% fetal bovine serum (FBS) (Omega Scientific, Tarzana, CA) were released with Trypsin-EDTA buffer, and then plated in gelatin coated flasks with a "Basic Medium" consisting of OptiMem, 0.01 mM β-mercaptoethanol (Sigma, St. Louis, MO), 0.028 M sodium bicarbonate (Sigma), 100 U/ml penicillin G, 100 U/ml streptomycin and 250 ng/ml amphotericin B (GibcoBRL), supplemented with 10% horse serum (HS) (Gemini Bioproducts, Woodland, CA; Basic Medium/HS). Cells used in this study were passaged 13 times and cryopreserved three times.

Neurosphere Formation:

Cryopreserved PPSCs were thawed and counted using the Trypan blue method. Cells were plated as a suspension on nontreated 6-well plates at a concentration of 100,000 cells/ml in a medium consisting of Neurobasal A (GibcoBRL), 100 U/ml penicillin G, 100 U/ml streptomycin, L-glutamine (2 mM; Nb medium), supplemented with B27 (GibcoBRL), basic fibroblast growth factor (bFGF; 40 ng/ml; Alomone Lab.) and epidermal growth factor (EGF; 20 ng/ml; GibcoBRL) modified from that used by Svendsen et al. (1998). Cells were maintained in this medium for 7–15 days and the growth factors replaced every 3–4 days. After 7–15 days when *neurospheres* (cluster of floating cells) were present, the cells in suspension were passaged (after trituration with a polished Pasteur pipette), and replated in a nontreated T-25 flask in Nb medium supplemented with B27, EGF and bFGF (20 ng/ml). This expansion protocol was repeated every 7–10 days as described previously (Reynolds et al., 1992). When desired, whole neurospheres were cryopreserved in fresh medium with 7.5% of dimethylsulfoxide (DMSO) as described previously (Gritti et al., 2001).

Neurosphere Differentiation

The neurospheres were collected, centrifuged and passaged after trituration, then plated on poly-ornithine/laminin (PO/L; Sigma) coated coverslips. Two different media were used: Nb medium supplemented with laminin (1 μg/ml; Sigma) and B27 (Nb-B27; GibcoBRL) or laminin and N2 (Nb-N2; GibcoBRL). Cells were plated in these two media supplemented with EGF and bFGF at 20 ng/ml for 1–2 days. Subsequently, these growth factors were substituted by one of following growth factors: NT3 (20 ng/ml; Alomone Lab) or BDNF (20 ng/ml; R&D). Cells were incubated in one of these media for 4–6 days and then processed for immunocytochemistry or RT-PCR.

Neuroectodermal Differentiation

Cryopreserved cells were thawed and plated on 1% gelatin coated plastic dishes or PO/L coated coverslips and maintained in Basic Medium/HS for at least two days. When cells reached 70% confluency, they were transferred to a Basic Medium/HS or Basic Medium/FBS containing bFGF (10 ng/ml) for 24 hr. After this preincubation, the cells were washed with Dulbecco's phosphate buffered saline (PBS; GibcoBRL) and transferred to a differentiation medium modified from that used by Woodbury et al. (2000). This medium consisted of the Basic Medium without serum, containing 2% DMSO, 200 μM butylated hydroxyanisole, 25 mM KCl, 2 mM valproic acid, 10 μM forskolin, 1 μM hydrocortisone, and 5 μg/ml of insulin (Sigma). The cells were maintained in this "Differentiation Medium" for 5 hr and then transferred to a "Maturation Medium" consisting of Neurobasal A, N2 supplement, 100 U/ml penicillin G, 100 U/ml streptomycin (GibcoBRL), L-glutamine (2 mM) and supplemented with laminin (1 μg/ml), bFGF and EGF (both 20 ng/ml) and kept in the incubator for 2 days. They were then washed with 0.1 M PBS and processed for immunocytochemistry or RT-PCR analysis.

RNA Extraction and Reverse Transcription-PCR Analysis

Total RNA was isolated from cells in culture using Trizol Reagent (GibcoBRL) followed by treatment with RQ1 DNase (Promega). For reverse transcription (RT), 250 ng of RNA was used in a final volume of 10 μl containing 250 pmol of random hexamers (Promega), 1× buffer (GibcoBRL), 25mM of dithiothreitol (GibcoBRL), 10 mM of each dNTP, 100 U of Superscript II reverse transcriptase (GibcoBRL). The reaction was carried out at 42°C for 45 min, then at 92°C for 5 min.

PCR amplification was carried out using 10 μl of the RT reaction in a final volume of 50 μl containing 1× PCR buffer (GibcoBRL), 0.2 mM of each dNTP, 1.5mM $MgCl^2$, 1.5 U of Taq DNA polymerase (GibcoBRL), 1 μM of primer forward (F) and 1 μM of primer reverse (R). The PCR cycling profile was as follows: 94°Cfor 3 min, then 30–36 cycles of 94°C for 45 sec, 56–65°C (Tm) for 30 sec, 72°C for 1 min, and a final extension at 72°C for 10 min (Mastercycler gradient thermocycler, Eppendorf). Products were separated on 2% agarose gels and visualized by ethidium bromide staining. Primer sequences (F: forward; R: reverse), length of amplified products and an-

nealing temperatures (T_m) were as follows: β-actin (F: 5′-AGGCATCCTGACCCTGAAGTAC-3′; R: 5′-TCTTCATGAGGTAGTCTGTCAG-3′; 376 bp, 56°C, 30 cycles), Oct-4 (Vassilieva et al., 2000; 312 bp, 61°C, 36 cycles), Pax 6 (Jones et al., 1998; 337 bp or 295 bp, 56°C, 35 cycles), Nestin (Scherer and Gallo, 1998; 431 bp, 56°C, 30 cycles), β tubulin III (Law et al., 1999; 240 bp, 56°C, 30 cycles), 68 kDa neurofilament protein (NF68; Wakabayashi et al., 1999; 327 bp, 60°C, 31 cycles), Glial fibrillary acidic protein (GFAP; Matsuura et al., 2001; 141 bp, 61°C, 30 cycles), MyoD (Kraus and Pette, 1997; 489 bp, 65°C, 30 cycles), Myogenin (Kraus and Pette, 1997; 328 bp, 65°C, 30 cycles), Myelin basic protein (MBP; Richter-Landsberg et al., 2000; 313 bp, 56°C, 32 cycles).

Immunocytochemistry

Each antigen was examined in at least three independent experiments. After each treatment the cells were fixed for 20 min in cold (4°C) para-formaldehyde (PFA 4%) in 0.1 M phosphate buffer. The cells were washed with PBS and treated with a quenching solution of 3.6 mg/ml glucose, 0.13 mg/ml sodium azide and 0.1 mg/ml glucose oxidase (Sigma) in PBS for 1 hr at 37°C to decrease internal peroxidase activity. Cells were then washed and incubated for 2 hours at room temperature (RT) in a blocking solution (BSA 1%, 0.25 Triton X-100, and 5% normal serum). The cells were then covered with a solution containing Triton X-100 (0.25%), 2.5% serum, and the primary antibody, and incubated at RT overnight. The following day, the cells were washed in PBS and incubated for 1 hr at RT with one of the following secondary antibodies: goat anti-mouse IgM 1:200 (Vector, Burlingame, CA) or an IgG antibody supplied in the Vector ABC Elite kit, 1:200 in 0.25% Triton X-100 and 1% serum. The cells were washed in PBS and then incubated for 45 min in the AB solution of the ABC Elite kit (Vector). After washes, the antigen/antibody complexes were visualized with 0.33 mg/ml diaminobenzidine as chromagen and 0.06% H_2O_2. Cells on coverslips were dehydrated, defatted in xylene, and mounted with Eukit (Calibrated Instruments, Hawthorne, NY).

For fluorescence, the secondary antibodies were Cy3- or FITC-coupled anti-rabbit and anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA). Cells were incubated in the secondary antibodies for 2 hr at RT in 0.25% Triton and 3% normal serum. The cells were washed in PBS and the coverslips were mounted on slides with Vectashield containing DAPI (Vector) for counterstaining.

Sources and concentrations of the primary antibodies were as follows: mouse anti-Nestin, monoclonal (1:1,000) and mouse anti-myogenin, monoclonal (1:100) (Hybridoma Bank, Iowa). Mouse anti-smooth muscle actin, monoclonal (1:300; Sigma). Rabbit anti-β-tubulin type III (Tuj1), polyclonal (1:1,500; Babco). Mouse anti-tau protein, monoclonal (1:250), mouse anti-NeuN, monoclonal (1:200), rabbit anti-Neurofilament 145 kDa, polyclonal (1:600), rabbit anti-NG2, polyclonal (1:600), mouse anti-myelin oligodendrocytes specific protein, monoclonal (1:600) and rabbit anti-GFAP, polyclonal (1:1,200; Chemicon, Temecula, CA). Mouse anti-CD45, monoclonal (1:100), mouse anti-CD90, monoclonal (1:100; Serotec). Mouse anti-CD34, monoclonal (1:40; Res. Diagnostic Inc.).

Each experiment included wells without secondary antibodies, as controls. Furthermore, control mouse or rabbit immunoglobulin G or M (IgG or IgM) were used at the same concentration as the primary antibodies to control for nonspecific staining.

Cells on coverslips were visualized with an Axioscope Zeiss microscope (Germany) and photographed with a Spot camera (Diagnostic Instruments Inc., USA). Cells in wells were visualized with an inverted microscope (Leica DMIL) under 20× and 40× magnification, and photographed with a Kodak Digital Still Camera DKC-CM30. For quantification, six non-overlapping fields of each well were viewed with a 20× objective and captured. The number of positive cells and total cell number were counted in each field (50–80 cells per field), the numbers added for each well, and the percentage of positive cells calculated. Means and SD were calculated from three separate experiments.

For single color fluorescence: specimens were imaged with an Axioscope Zeiss microscope (Germany) equipped with a mercury short arc lamp (Osram, Germany). Filter for DAPI (360 nm excitation; 460 nm emission, Chroma), Cy3 (545 nm excitation; 610 nm emission, Chroma) and FITC (450–90 nm excitation; 535 nm emission, Chroma) were used and cells were photographed with a Spot camera (Diagnostic Instruments Inc.). For confocal microscopy: specimens were imaged on a Leica TCS-SP MP Confocal and Multiphoton Microscope (Heidelberg, Germany) equipped with an argon laser for 488 nm blue excitation (for FITC) and a krypton laser for 568 nm yellow excitation (for TRITC or Texas Red). DAPI images were collected using a picosecond two-photon laser system (Millenia X-Tsunami, Spectra-Physics) tuned to 765 nm. Images were collected using Leica Confocal Software and processed to make maximum projection through-focus images and double label overlay images.

RESULTS

Nontreated PPSCs Are Negative for Neuronal and Glial Markers

Virtually all undifferentiated PPSCs isolated in our experimental conditions (Lucas et al., 1995; Young et al., 2001a) were positive for CD90 as shown by immunocytochemistry (Fig. 1A). In contrast, all cells were negative for the hematopoietic marker CD45 (Fig. 1B; Trowbridge and Thomas, 1994). When stained for CD34 the population seemed more heterogeneous: a small subset of positive cells of variable intensity was observed but other cells showed no staining (Fig. 1C).

Undifferentiated PPSCs were intensely positive for vimentin, which is a marker for early muscle cells and early glial progenitors (Fig. 1D; Cochard and Paulin, 1984); however, they were negative for myogenin and smooth muscle actin (data not shown).

PPSCs were also tested for nestin, an early marker for brain cells that is present in neural progenitors (Lendahl et al., 1990) and in muscle precursors cells (Zimmerman et al., 1994). Only one or two cells in the well were occasionally positive for this marker (data not shown). A few undifferentiated PPSCs showed weak staining for NG2, an early oligodendrocytic marker but no staining was observed with any of the antibodies for neural or glial markers (data not shown). Expression of β-tubulin III, GFAP,

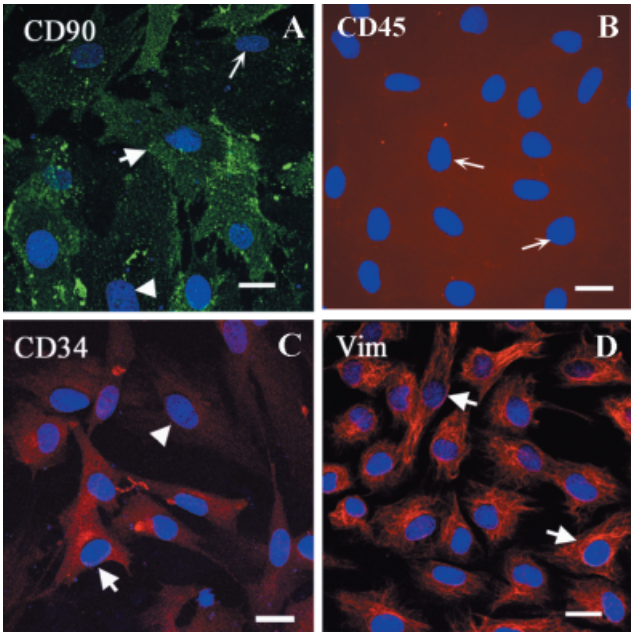

Fig. 1. Antigenic properties of undifferentiated PPSCs. **A:** CD90 positive cells represent the majority of the population. Both intensely (large arrow) and moderately stained cells (arrowheads) were observed, and negative cells were very rare (top small arrow). **B:** PPSCs showed no staining when immunocytochemistry for CD45 was carried out (arrows). **C:** Immunolabeling for CD34: moderate (arrows) and low intensity (arrowhead) immunostaining was found. **D:** Immunolabeling for vimentin: the entire cell population was intensely immunoreactive for this cytoskeleton protein. Nuclei in blue were stained with DAPI. Scale bar = 20 μm.

NF145 and NF68 was not detected by either immunocytochemistry or RT-PCR at this stage (data not shown).

Morphological Changes of PPSCs Cultured in Adherent and Non-Adherent Conditions

When grown in basic medium with serum, undifferentiated PPSCs retained a flat, polygonal morphology (Fig. 2A). To test the ability of PPSCs to give rise to neural progenitors, cells that are known to generate neurospheres, PPSCs were cultured in a serum-free medium under non-adherent conditions. In these conditions some of the cells attached to the dish and grow in monolayers, whereas others remained floating and continued to divide, forming cluster of cells after 7–15 days (Fig. 2B,C). These cells resembled those described previously as neurospheres (Reynolds et al., 1992). The clusters seemed bright under the phase contrast microscope and showed cilia at the perimeters (Fig. 2B,C). These clusters were successfully passaged and expanded; after each passage a decreasing percentage of cells attached to the surface of the wells, and after 4–5 passages all cells formed neurospheres. When the neurospheres were dissociated and plated in PO/L coverslips, a variable number of bipolar, tripolar and large flat cells were observed (Fig. 2D).

An alternate protocol was used to determine whether PPSCs could give rise to neurons and glia under adherent conditions as previously shown in other non-neural stem cells. After 5 hours in serum-free differentiation medium under adherent conditions, 90–98% of the PPSCs showed a contraction of the cell body and the emergence of processes (Fig. 2E). After 2 days in the maturation medium a variable number of cells died, whereas the remaining cells belonged to two different populations according to the presence or absence of processes. The cells with processes showed small round cell bodies and usually two or three processes similar to the ones observed after 5 hr; some of the cells had multipolar processes (Fig. 2F). As many as 30% of the cells were polygonal with a large nucleus and no processes at this time, whereas 15% of cells had a fibroblast-like morphology (data not shown).

Neural Genes Expression in Neurospheres Derived From PPSCs

To ascertain whether the neurospheres derived from PPSCs contained cells of neuroectodermal lineage, they were dissociated by trituration, plated on PO/L-coated coverslips and maintained in Nb-B27 or Nb-N2 medium with EGF-bFGF for 1–2 days to improve the survival of neural progenitors, as described previously (Gritti et al., 2001). Thereafter, cells were transferred to a medium with NT3 or BDNF. Cells were processed for immunocytochemistry and RT-PCR after 4–5 days in these media. After reverse transcription, the expression of several genes was determined by PCR under non-saturating conditions. The housekeeping gene β-actin served as an internal control of RT-PCR.

The POU-domain transcription factor Oct-4 is a gene related to embryonic pluripotent cells (Pesce and Scholer, 2000). Low levels of Oct-4 expression were observed in undifferentiated PPSCs (Fig. 3A). During neurospheres formation, Oct-4 mRNA expression was highly increased after two days, but decreased slightly after 10 days (Fig. 3A).

Undifferentiated PPSCs expressed Pax6, a neuroectodermal marker (Mansouri et al., 1996). Primers used for the detection of Pax6 expression were located in regions spanning the differentially spliced exon 5a (Jones et al., 1998). Expression of both forms (Pax6+5a and Pax6-5a) was detected in undifferentiated PPSCs (Fig. 3A). Although this RT-PCR was not a strictly quantitative analysis, the level of the Pax6-5a form was much higher than Pax6+5a in undifferentiated PPSCs. PPSCs and cells within the neurospheres expressed the two alternative transcripts Pax6+5a (337 bp PCR product) and Pax6−5a (295 bp PCR product). In contrast to undifferentiated PPSCs, (Figs. 3A, 5A), the relative abundance of the two Pax6 transcripts seemed to be the same in neurospheres (Fig. 3A).

Neurospheres were also tested for expression of the mRNA encoding the neuroectodermal marker nestin, which is absent in PPSCs (Figs. 3A, 5A). Nestin mRNA in neurospheres increased with time in culture. When neurospheres were plated, β-tubulin III and GFAP mRNAs

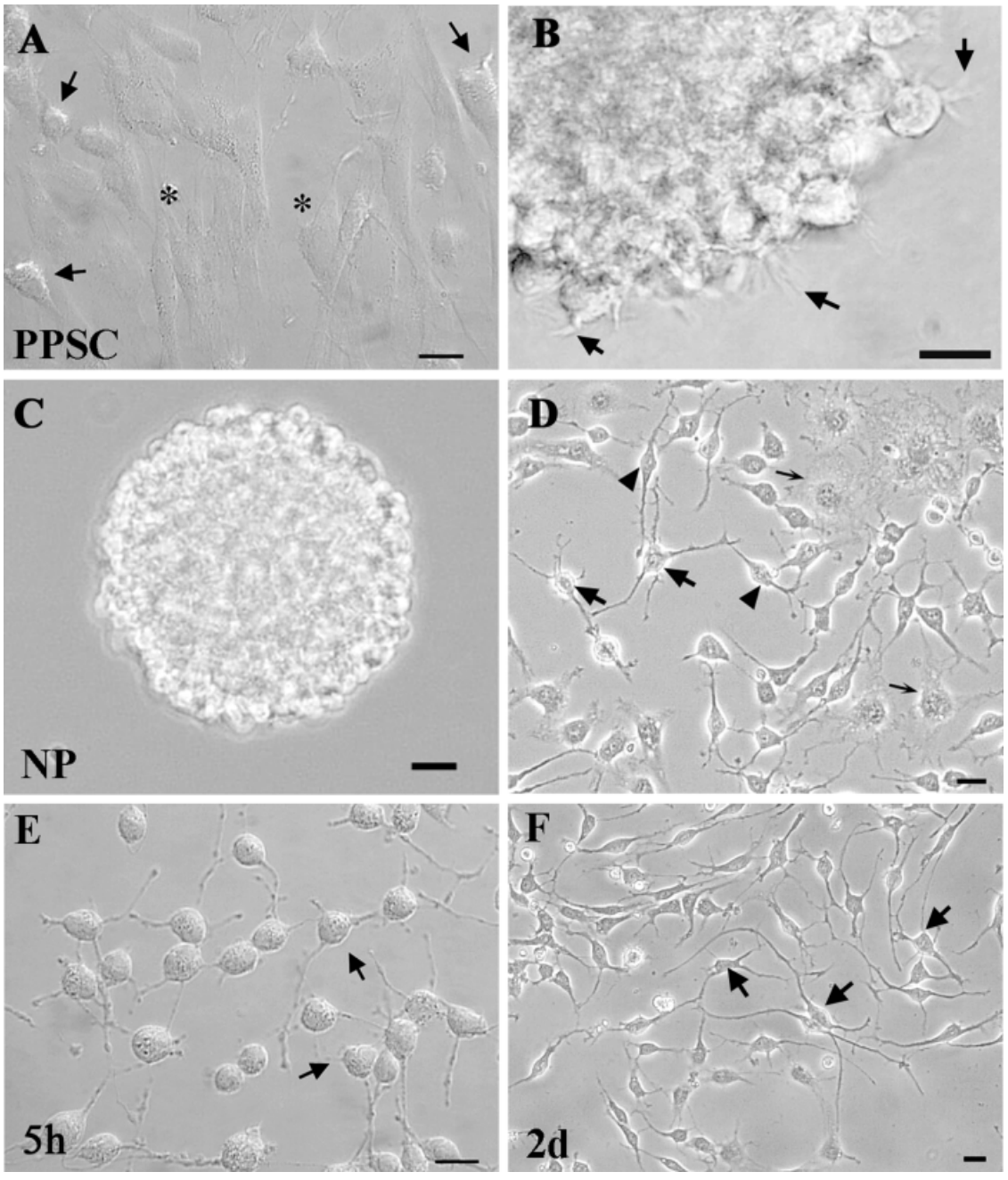

Fig. 2. Pre and postdifferentiation morphology of PPSCs grown in monolayer or as neurospheres. **A:** Undifferentiated PPSCs: polygonal flat cells (*) predominated, with a few cells with round or triangular cell body (arrows). **B:** Detail of a PPSCs-derived-neurosphere; note the cilia extension at the perimeter of the cluster (arrows). **C:** PPSC-derived neurospheres observed after 10 days in the neurospheres medium. **D:** Morphology of cells isolated from neurospheres and plated on coverslips after 3 days. Bipolar (arrowheads), tripolar (large arrows) and large flat cells (small arrows) were observed. **E:** Morphology of PPSCs grown as a monolayer after 5 hr in the differentiation medium: most cells had round small cell bodies and processes (arrows). **F:** Representative field after 2 days in the maturation medium showing a majority of round cells with processes (arrows). Scale bar = 20 μm.

were detected in all the conditions tested (Fig. 3B). NF68 mRNA expression was detected in all conditions, except Nb-B27 medium supplemented with NT3 (Fig. 3B). MBP expression was detectable only in the neurospheres plated with Nb-N2 medium with NT3 or BDNF (Fig. 3B). These data suggests that the Nb-N2 medium is more favorable than Nb-B27 for oligodendroglial differentiation.

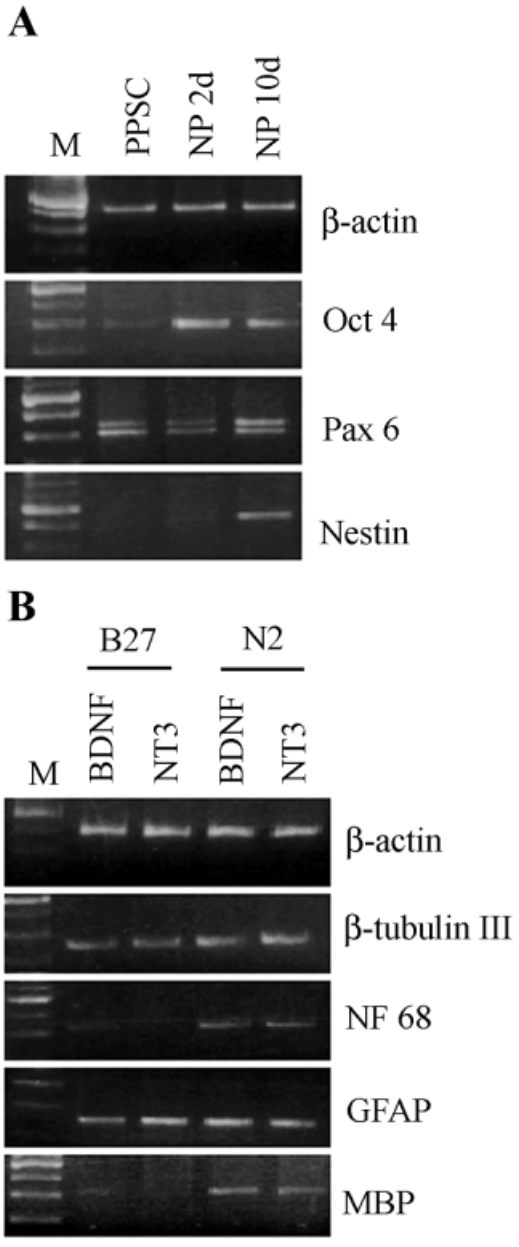

Fig. 3. Gene expression in PPSCs and PPSCs-derived-neurospheres. **A:** RT-PCR analysis of Oct-4, Pax 6 and nestin mRNA expression in PPSCs and in neurospheres after 2 days (NP 2d) and 10 days (NP 10d). **B:** Analysis of β-tubulin III, NF68, GFAP and MBP mRNA expression after neurospheres differentiation. Cells from neurospheres were plated in two different media (Nb-B27 or Nb-N2) supplemented with BDNF or NT3. Total RNA was extracted after 4 days in these differentiation media. M: 100 bp DNA ladder. The expression of the β-actin gene was used as a control.

Expression of Neuronal and Glial Markers in PPSC-Derived Neurospheres

Immunocytochemistry was carried out to further confirm the neural differentiation of the PPSC-derived neurospheres. As was shown previously by RT-PCR, neurospheres were positive for nestin. Nestin was still detected a few days after plating in cells isolated from the neurospheres (Fig. 4A) but its expression decreased with time in these conditions. A similar pattern was observed for vimentin staining, which was very intense in the neurospheres but decreased with time in a subset of cells when the neurospheres were cultured on coverslips (data not shown).

Cells plated in Nb-B27 media gave rise to neurons (Tuj1+; Fig. 4C) and astroglial cells (GFAP+) when exposed to either BDNF or NT3. In these conditions numerous cells were positive for NG2, a proteoglycan present in the membranes of progenitors as well as differentiated oligodendrocytes (Dawson et al., 2000) and in newly generated cells in adult hippocampus (van Praag et al., 2002; Fig 4B); however, few oligodendrocytes (MOSP+) were observed. Conversely, when cells were plated in Nb-N2 medium, numerous oligodendrocytes (MOSP+; Fig. 4E) were found in addition to neurons (Tuj1+; Fig. 4D) and astroglia (GFAP+; Fig. 4F). Cells plated in Nb-B27 showed better survival than those in Nb-N2. In addition, cells exposed to BDNF, whether in Nb-N2 or in Nb-B27, showed better survival than those in NT3. Tuj1 + cells cultured in NT3 displayed the most mature neuronal morphology.

Differentiation of the PPSCs Cultured Under Adherent Conditions Into Neurons and Glia

RT-PCR analysis was used to address the differentiation of the cells under adherent conditions. Total RNA was extracted from undifferentiated PPSCs, after 5 hr in the differentiation medium, and after 2 days in the maturation medium.

As mentioned above, expression of both forms of Pax6 (Pax6+5a and Pax6−5a) was detected in undifferentiated PPSCs; however, only Pax6−5a transcript was detected after neural differentiation and maturation (Fig. 5A). Undifferentiated PPSCs showed no expression of the neuroectodermal marker nestin. They expressed nestin mRNA, however, after 5 hr of differentiation and 2 days of maturation (Fig. 5A).

The neuroectodermal differentiation of the cells was confirmed by the expression of mRNA encoding the early neural markers β-tubulin III and NF68. The levels of expression of β-tubulin III and NF-68 were the same after 5 hr in the differentiation medium and after 2 days in the maturation medium (Fig. 5B). GFAP mRNA was only detected after 5 hr in the differentiation medium (Fig. 5B). In contrast, MBP, a marker of mature oligodendrocytes (Zhang, 2001), was only expressed after 2 days of maturation (Fig. 5B). The muscle specific mRNAs myogenin and Myo-D were not detected by RT-PCR in PPSCs at any time points of the monolayer protocol (data not shown).

PPSCs Cultured in Monolayers Differentiated Into Neurons and Oligodendroglia

To verify the ability of PPSCs to give rise to neurons and oligodendrocytes under adherent conditions, immu-

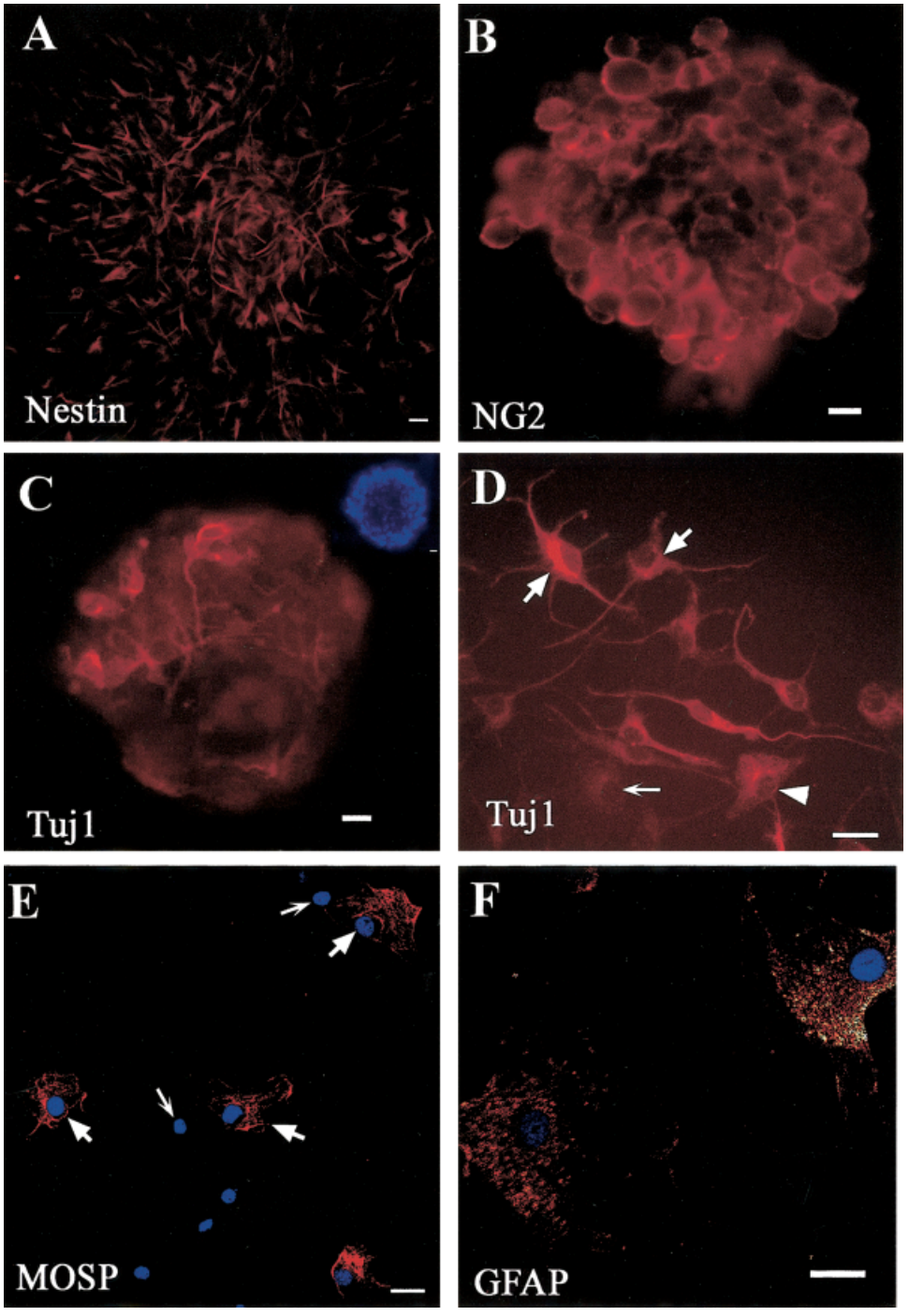

Fig. 4. Immunocytochemical staining of PPSC-derived neurospheres. Cells in the neurospheres stained intensely for **A:** Nestin, **B:** NG2, and **C:** Tuj1. Insert in C shows cell nuclei counterstained with DAPI. **D:** When plated, the neurospheres gave rise to round cells with numerous processes. These cells were positive for β-tubulin III (large arrows), and were surrounded by negative cells (bottom small arrow). In addition, some flat cells stained positively for β-tubulin III (arrowhead). **E:** Cells immunostained for myelin oligodendrocyte specific protein (MOSP) in red (large arrows); cells were counterstained with DAPI; small arrows point to nuclei of negative cells. **F:** Large flat cells were positive for GFAP immunostaining. Scale bar = 20 μm for A,B,D–F; 10 μm for C.

nocytochemistry was carried out after initial differentiation (5 hr) and further maturation (2 days). To show that the potential of the PPSCs was not serum-dependent we carried out some of our experiments with two different serums, HS and FBS, in parallel. The results showed similar neural potential of the cells regardless the serum used (data not shown). Therefore, all the data reported were obtained with cells expanded in HS.

Confirming the neuroectodermal potential of the cells, after 5 hr of differentiation ≈40–50% of the cells exhibiting round cell bodies and processes showed a high level of immunostaining for nestin (data not shown). In addition, approximately 60% of the cells showed immunostaining of strong or medium intensity for NG2 (data not shown). At this time point a subset of cells co-expressed neuronal (NF145 and Tau) and glial markers

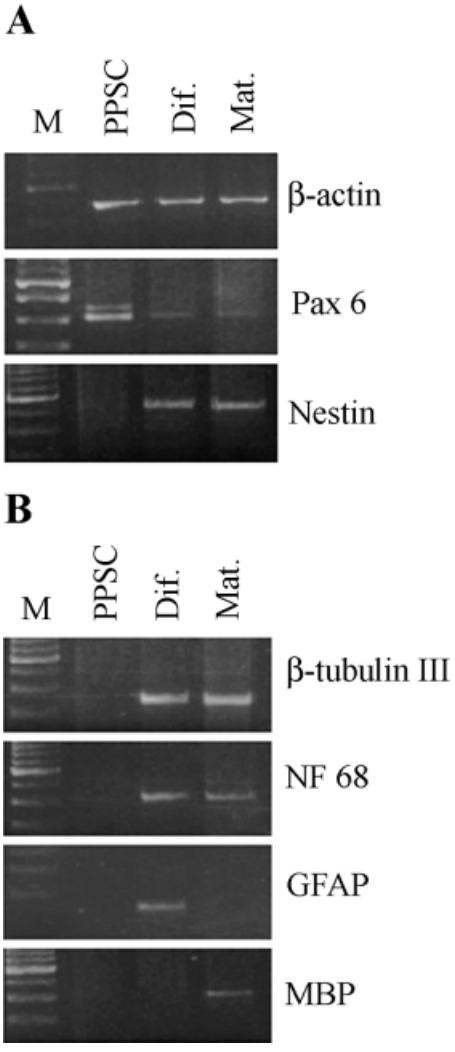

Fig. 5. RT-PCR analysis of the expression of several genes in PPSCs plated as monolayer. Total RNA was extracted from undifferentiated PPSCs, after the 5 hr differentiation step (Dif.) and after 2 days in a maturation medium (Mat.). **A:** Analysis of the expression of the genes Pax 6 and nestin. **B:** Analysis of the expression of the genes β-tubulin III, NF68, GFAP and MBP. M: 100 bp DNA ladder. Expression of the β-actin gene was used as an internal control.

(GFAP) (data not shown). The percentage of cells that stained for NF145 was 71.2 ± 3.8 and for GFAP, 68.4 ± 11.1% (the results are the average of three experiments ± SD). In contrast to the detection of β-tubulin III mRNA at 5 hr, immunostaining was absent at that time. This could have been due to a delay in the detection of the protein, probably attributable to post-translational modifications (Laferriere and Brown, 1996), or to a greater sensitivity of the RT-PCR method vs. immunocytochemistry. MOSP immunostaining was not detected after 5 hr of differentiation.

After the cells were incubated in the maturation medium for 2 days under adherent conditions, clusters of cells still expressed nestin. These cells were usually round with processes while others were flat (Fig. 6A). Also, a variable number of cells showed intense staining for vimentin (Fig. 6B).

A variable number of cells (20–60%) became positive for β-tubulin III (Tuj1) at this time (Fig. 7A–D). Generally these cells were round with processes, although some flat cells also showed positive staining for this antibody. Typically Tuj1+ cells appeared in clusters. NF145 was found only in a very small number of cells after 2 days (Fig. 7E–G). Tau expression was not detected after 2 days in culture. Only very rare cells were positive for NeuN indicating that the neurons originating from the PPSCs are not postmitotic at this stage (data not shown).

No staining for GFAP was observed after 2 days in the maturation medium. At this point, NG2 staining was heterogeneous, with positive cells of variable intensity and cells that did not express the protein (Fig. 6A,B). A high percentage of cells were positive for CNPase. These cells were round and multipolar, with typical oligodendroglial morphology (Fig. 6C,D). In addition, approximately 10–20% of the cells expressed the myelin oligodendrocyte specific protein (MOSP) (Fig. 6E and F). These MOSP+ cells were isolated and often located at the edge of the wells. They mostly included round cells with short or no processes, and cells surrounded by a large sheet of myelin, as observed typically in cultures of oligodendrocytes.

MOSP+ cells were clearly different from the Tuj1+ cells, both in their morphology and their distribution. Although cells expressing Tuj1, NF145 or MOSP were present in each experiment, their number was variable. Therefore, quantitative assessments were not carried out.

DISCUSSION

Previous studies have shown that stem cells capable of differentiating into several mesodermal phenotypes reside in adult muscle tissue (Pate et al., 1993; Lucas et al., 1995; Katagiri et al., 1997; Cornelison and Wold, 1997; Gussoni et al., 1999; Jackson et al., 1999; Bosch et al., 2000; Lee et al., 2000; Young et al., 2001a;b). We show that these cells, isolated from adult rat muscle, can be directed toward the neuroectodermal lineage in vitro and express phenotypic markers of neurons, astroglial and oligodendroglial cells. To our knowledge, this is the first report of differentiation of stem cells isolated from muscle into a neural fate. We suggest that these cells represent a novel source for neuroglial replacement in vivo.

In our study, PPSCs were directed to a non-mesenchymal lineage, as shown by the expression of mRNAs and proteins characteristic of neurons and glia, by using two different protocols: floating cultures and monolayer.

PPSCs Give Rise to Neurospheres That Differentiate Into Neurons and Glia

To explore the neural potential of PPSCs, we tested the ability of the cells to give rise to neurospheres when grown floating in a typical serum-free medium. Neurospheres are cluster of cells formed from single neural stem

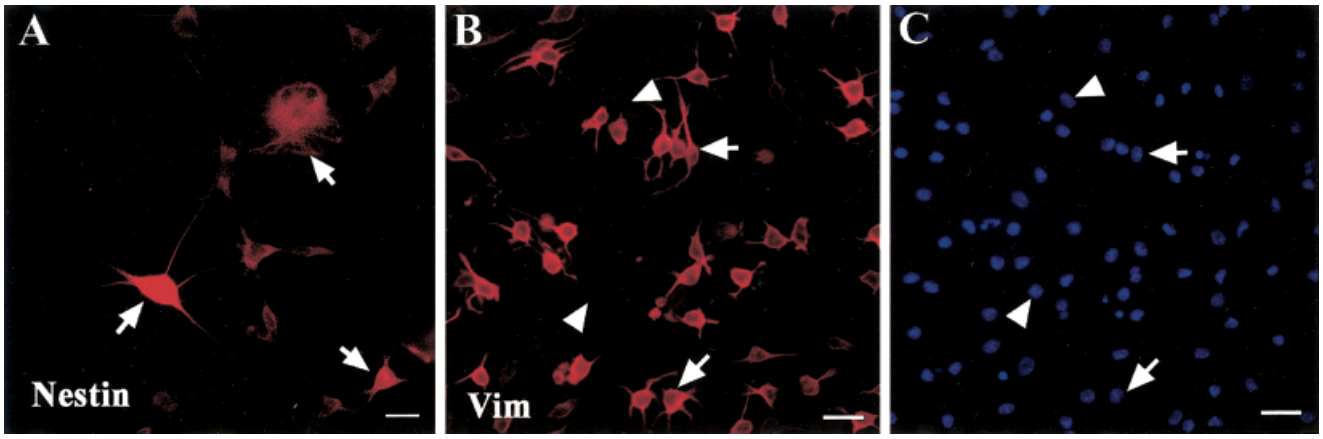

Fig. 6. Immunocytochemical staining of PPSCs after 2 days in maturation medium. **A:** Cells of diverse morphology were immunostained for nestin: large flat cells (top right arrow), large round multipolar cells (left arrow) and small cells with processes (bottom right arrow). **B:** In red, positive cells with round bodies and processes (arrows) immunostained for vimentin, intermingled with negative cells (arrowheads). **C:** Field shown in B counterstained in blue with DAPI. Scale bar = 20 μm.

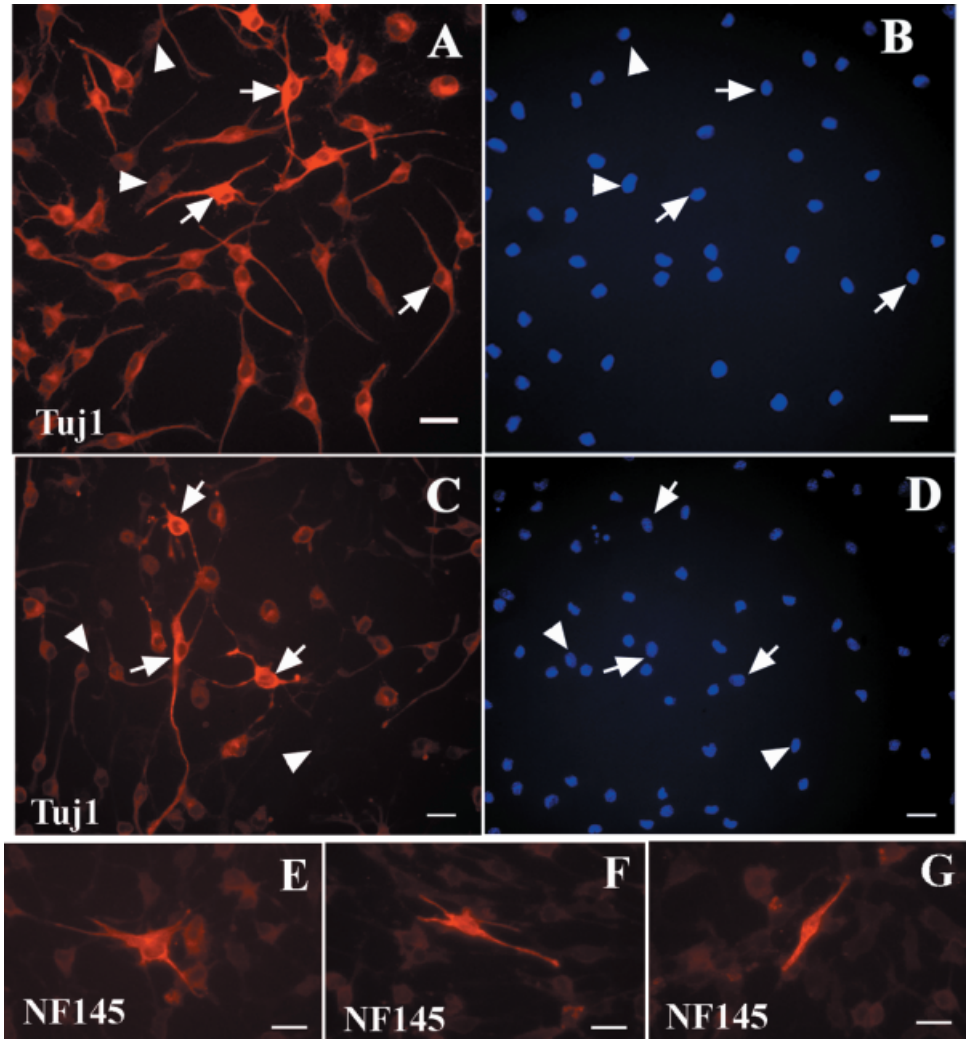

Fig. 7. Expression of neuronal markers in PPSCs after 2 days in maturation medium. **A** and **C:** Cells with a neuronal-like morphology were positive for β-tubulin III (Tuj1; arrows), and were surrounded by negative cells whose cell nuclei are shown in (**B**,**D**), counterstained in blue with DAPI (arrowheads). **E–G:** Scattered cells show intense staining for NF145. Scale bar = 20 μm.

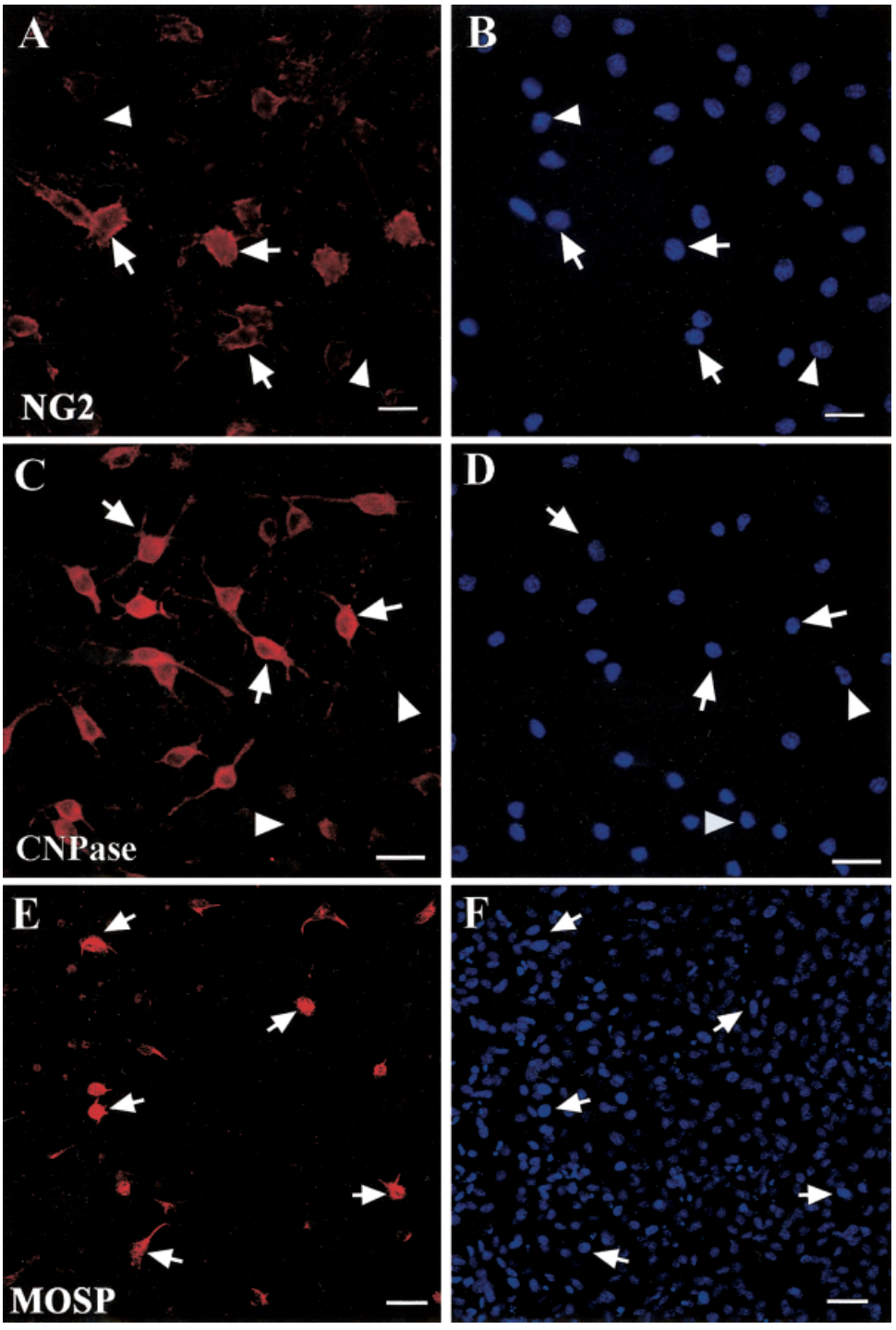

Fig. 8. Expression of oligodendroglial markers in PPSCs after 2 days in maturation medium. **A:** In red, membrane of cells immunostained for NG2 (arrows). Cell nuclei were counterstained in blue with DAPI shown in **B**. Arrowheads in (A,B) point to negative cells for NG2. **C:** Typical multipolar cells positive for CNPase (arrows) surrounded by negative cells (arrowheads). **D:** Counterstained in blue with DAPI. **E:** Cells immunostained for MOSP in red were usually round with very short processes (arrows). They were scattered among negative cells (arrowheads). **F:** Field shown in E counterstained in blue with DAPI. Scale bar = 20 μm for A–D; 40 μm for E,F.

cells obtained from either adult or fetal CNS or from embryonic stem cells (Gritti et al., 2001). The neurospheres contain progenitor cells capable of either self-renewal or differentiation into neurons and glia (Reynolds et al., 1992). Clusters of cells were observed after 7–10 days and were passaged, expanded and cryopreserved. These clusters presented cilia extensions surrounding the perimeter as is observed commonly on neurospheres from embryonic or neural stem cell origin. When plated in different conditions, these cells differentiated into neurons, astroglia and oligodendrocytes. Time course of marker expression seemed similar to that seen previously in other neurospheres (Reynolds and Weiss, 1992). After plating, early markers of neurospheres, such as nestin and NG2,

were highly expressed. The expression of these early markers decreased during differentiation and neuronal, oligodendroglial and astroglial markers appeared in the same order as described previously (Gritti et al., 2001). The percentages of the different cell types differed, however, depending on the growth factor and the supplement used. In particular, PPSC-derived neurospheres plated in N2 supplement gave rise to a relatively high number of oligodendrocytes, which are usually sparse in CNS neurospheres. This suggests that PPSCs have a particular potential to differentiate into myelin producing cells.

PPSCs Differentiate Into Neuron and Glia in Monolayer Cultures

Two distinct time points were examined in monolayer cultures. After 5 hr of differentiation, the PPSCs expressed nestin, an intermediate filament used as a marker for neural progenitors in the mammalian CNS. At this time point, a variable proportion of cells expressed NG2, a protein present in oligodendrocyte progenitors (Dawson et al., 2000) and in newly generated cells in adult hippocampus (van Praag et al., 2002). The expression of these two early neural markers indicates the induction of the neuroectodermal lineage in the culture.

In addition, a transient co-expression of glial and neuronal markers was observed after 5 hr in the differentiation medium. A similar stage has been observed previously in vitro in hippocampal stem cells after treatment with bFGF in a serum free medium (Gage et al., 1995), in rat embryonic cells from striatum at early stages of differentiation (Rosser et al., 1997), and in immortalized embryonic mesencephalic mouse cells (Colucci-D'Amato et al., 1999). Similarly, postnatal forebrain neural progenitors can display mixed glial and neuronal properties in vitro, when they co-express NSE, NF145 and GFAP (Feldman et al., 1996). Thus, co-expression of glial and neuronal markers is a pattern that reflects multipotentiality at early stages of differentiation in culture.

After 5 hr in the differentiation medium PPSCs were at an early stage in the neuroectodermal lineage and did not yet commit to a specific fate. In contrast, after 2 days in the maturation medium immunostaining for nestin and NG2 decreased and the co-expression of glial and neuronal markers disappeared. Moreover, GFAP staining was no longer present, whereas the neuronal markers Tuj1 and NF145 were strongly expressed in a variable number of cells. In addition, some cells expressed CNPase and MOSP, suggesting that a larger number of the early progenitors that are present at 5 hr are committed toward the neuronal and oligodendroglial than the astroglial phenotypes in these conditions. Interestingly, conditions that are roughly similar to our monolayer protocol can induce the differentiation of bone marrow cells into cells expressing neuronal markers (Woodbury et al., 2000). Although the potential of the PPSCs was qualitatively reliable in every experiment, quantitatively the number of neurons and oligodendroglia was not always consistent. This suggests some heterogeneity in the initially plated PPSCs population, which might affect the final outcome of the experiment.

PPSCs Express Genes Characteristic of Neural and Embryonic Stem Cells

Further confirming their neural potential, the mRNA encoding the neuroectodermal marker Pax6, a gene expressed in neural stem cells and in neurospheres (Reubinoff et al., 2001), was detected in PPSCs. The homeobox gene Pax6 encodes a transcriptional factor expressed from early developmental stages in embryo to adulthood in the CNS and the eye (Callaerts et al., 1997). This gene is involved in the development of several areas of the CNS (Walther and Gruss, 1991), and is also related to dopaminergic fate specification (Vitalis et al., 2000; Mastick and Andrews, 2001). The RT-PCR product distinguished two alternative forms Pax6+5a and Pax6−5a that encode proteins with different DNA recognition capacities and probably distinct roles in CNS development (Epstein et al., 1994; Jaworski et al., 1997). We observed the expression of both transcripts in PPSCs; however, the relative abundance of the two Pax6 transcripts changed during the two protocols. Further studies will be necessary to characterize the role of these two transcripts and their function in stem cell differentiation.

Oct-4 gene is involved directly in the self-renewal capacity and totipotency of mammalian embryonic stem cells (for a review see Pesce and Scholer, 2001). Undifferentiated PPSCs expressed low levels of Oct-4; however, Oct-4 expression increased rapidly when PPSCs were transferred to the neurospheres medium. This could be the result of a selection process by the culture conditions: during the formation of neurospheres, a subset of cells remained attached to the flask surface and only a percentage of the whole population floated, divided and formed very small neurospheres (multipotent cells with neural potential). When the neurospheres were kept for 10 days in culture, Oct-4 expression decreased slightly probably indicating an increase in committed cells within the neurospheres.

PPSCs Isolated From Adult Muscle May Provide a Novel Source of Stem Cells for Neural Replacement

The present results confirm the ability of PPSCs to give rise not only to different types of mesodermal cells (Young et al., 1999; Young et al., 2001a), but also to cells of a different germ origin: neuroectodermal. This is in accordance with studies published during the last few years showing the ability of stem cells to dedifferentiate, redifferentiate or transdifferentiate (for a review see Clarke and Frisen, 2001). Non-neural stem cells have been shown previously to give rise to neurons in vitro. Several groups have reported the differentiation of bone marrow stem cells into neurons and glia in monolayer cultures, although none, to our knowledge, has generated tri-potent neurospheres from bone marrow cells. Interestingly, only one group has reported obtaining neurospheres from non-

neuronal adult tissue, specifically from skin stem cells (Toma et al., 2001).

Our initial cell population is a mixture of progenitors (committed cells) and pluripotent stem cells (uncommitted) as we have shown previously by clonal analysis (Young et al., 2001a). The clonal analysis revealed cells with unlimited self-renewal ability and the potential to give rise to various cell types of different lineages, a characteristic of stem cells. Successive episodes of freezing and thawing act as a selection process for the PPSCs. A selection process including extended passaging has also been used for marrow stem cells, which are rare, as are our cells, in the initially harvested population (Reyes et al., 2001; Reyes and Verfaillie, 2001). The experiments reported here were carried out with cells passaged 13 times and cryopreserved 3 times, which corresponds to an optimal enrichment in PPSC; however, we have been able to obtain similar neurospheres from cells passaged 12–16 times.

Undifferentiated PPSCs were negative for early and late markers of muscle, like myogenin, myoD and smooth muscle actin, which suggests that they are not myoblast or cells committed toward the myogenic phenotype. Immunocytochemistry revealed that similar to the mesenchymal stem cells from bone marrow (Pittenger et al., 1999), PPSCs are positive for CD90. The presence in muscle of stem cells with the potential to repopulate all major peripheral blood lineages has been reported (Jackson et al., 1999). These cells were found to be CD45+, however, which suggests a hematopoietic origin (McKinney-Freeman et al., 2002). Conversely, PPSCs isolated by the thaw-freeze protocol from both rat (this study) and human muscle (Young et al., 1999, 2001b) were negative for the hematopoietic marker CD45, which suggests that PPSCs represent a different cell population, and argues against a hematopoietic origin for the PPSCs. The same was true for the population of stem cells isolated from muscle by Gussoni et al. (1990), who also were able to reconstitute the hematopoietic compartment in vivo and for another group that explored osteogenic potential of muscle derived stem cells (Lee et al., 2000). In addition, PPSCs were heterogeneous for CD34 expression and able to grow in suspension, in contrast to the bone marrow cells that are known to be CD34− and particularly adhesive to plastic (Pittenger et al., 1999; Colter et al., 2001; Toma et al., 2001). Stem cells isolated from skin expressed nestin but not vimentin; conversely PPSCs expressed high levels of vimentin and almost never expressed nestin when nondifferentiated (Toma et al., 2001). Therefore, the PPSCs have distinct characteristics from all other types of pluripotent stem cells shown previously to differentiate into neurons and glia in vitro.

Although the expression of markers depends on culture conditions, no previous cell population matched our cells characteristic or potential. In addition, despite the fact that the exact origin of the PPSCs remains unknown, this population can be harvested from a very accessible tissue, making these cells an excellent source of stem cells for autologous transplantation. Our data suggest that they represent a distinct population of pluripotent cells and a new source of neurons and glia from an accessible adult tissue. These pluripotent cells isolated from adult rat muscle expressed in vitro the same markers as newly generated cells in vivo (NG2, Tuj1, and GFAP) that give rise to functional neurons in the adult hippocampus (van Praag et al., 2002).

The experimental conditions used in this study led to immature neurons and glia. Although the ability of PPSCs to maintain these differentiated phenotypes in vitro for longer periods remains untested, the early differentiation demonstrated here indicates that they can reach a stage useful for transplantation in vivo. A recent study has shown that a similar mixed population of multipotent neural progenitors and lineage restricted cells, from embryonic human forebrain derived neurospheres, can be a useful source of cells for neural replacement. Indeed, these cells were able of integration and site specific differentiation in adult brain (Fricker et al., 1999).

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REFERENCES

Azizi SA, Stokes D, Augelli BJ, DiGirolamo C, Prockop DJ. 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.

Bjorklund A, Lindvall O. 2000. Cell replacement therapies for central nervous system disorders. Nat Neurosci 3:537–544.

Bosch P, Musgrave DS, Lee JY, Cummins J, Shuler T, Ghivizzani TC, Evans T, Robbins TD, Huard J. 2000. Osteoprogenitor cells within skeletal muscle. J Orthop Res 18:933–944.

Brazelton TR, Rossi FM, Keshet GI, Blau HM. 2000. From marrow to brain: expression of neuronal phenotypes in adult mice. Science 290: 1775–1779.

Callaerts P, Halder G, Gehring WJ. 1997. PAX-6 in development and evolution. Annu Rev Neurosci 20:483–532.

Clarke D, Frisen J. 2001. Differentiation potential of adult stem cells. Curr Opin Genet Dev 11:575–580.

Cochard P, Paulin D. 1984. Initial expression of neurofilaments and vimentin in the central and peripheral nervous system of the mouse embryo in vivo. J Neurosci 4:2080–2094.

Colter DC, Sekiya I, Prockop DJ. 2001. Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proc Natl Acad Sci USA 98:7841–7845.

Colucci-D'Amato GL, Tino A, Pernas-Alonso R, French-Mullen JM, di Porzio U. 1999. Neuronal and glial properties coexist in a novel mouse CNS immortalized cell line. Exp Cell Res 252:383–391.

Cornelison DD, Wold BJ. 1997. Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. Dev Biol 191:270–283.

Dawson MR, Levine JM, Reynolds R. 2000. NG2-expressing cells in the central nervous system: Are they oligodendroglial progenitors? J Neurosci Res 61:471–479.

Deng W, Obrocka M, Fischer I, Prockop DJ. 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.

Epstein J, Cai J, Glaser T, Jepeal L, Maas R. 1994. Identification of a Pax paired domain recognition sequence and evidence for DNA-dependent conformational changes. J Biol Chem 269:8355–8361.

Faust C, Magnuson T. 1993. Genetic control of gastrulation in the mouse. Curr Opin Genet Dev 3:491–498.

Feldman DH, Thinschmidt JS, Peel AL, Papke RL, Reier PJ. 1996. Differentiation of ionic currents in CNS progenitor cells: dependence upon substrate attachment and epidermal growth factor. Exp Neurol 140:206–217.

Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F. 1998. Muscle regeneration by bone marrow-derived myogenic progenitors. Science 279:1528–1530.

Fricker RA, Carpenter MK, Winkler C, Greco C, Gates MA, Bjorklund A. 1999. Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain. J Neurosci 19:5990–6005.

Gage FH, Coates PW, Palmer TD, Kuhn HG, Fisher LJ, Suhonen JO, Peterson DA, Suhr ST, Ray J. 1995. Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. Proc Natl Acad Sci USA 92:11879–11883.

Galli R, Borello U, Gritti A, Minasi MG, Bjornson C, Coletta M, Mora M, De Angelis MG, Fiocco R, Cossu G, Vescovi AL. 2000. Skeletal myogenic potential of human and mouse neural stem cells. Nat Neurosci 3:986–991.

Gritti A, Galli R, Vescovi AL. 2001. Cultures of stem cells of the central nervous system. In: Fedoroff S, Richardson A, editors. Protocols for neural cell culture. Totowa, NJ: Humana Press. p. 173–198.

Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF, Kunkel LM, Mulligan RC. 1999. Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature 401:390–394.

Jackson KA, Mi T, Goodell MA. 1999. Hematopoietic potential of stem cells isolated from murine skeletal muscle. Proc Natl Acad Sci USA 96:14482–14486.

Jaworski C, Sperbeck S, Graham C, Wistow G. 1997. Alternative splicing of Pax6 in bovine eye and evolutionary conservation of intron sequences. Biochem Biophys Res Commun 240:196–202.

Jones SE, Jomary C, Grist J, Thomas MR, Neal MJ. 1998. Expression of Pax-6 mRNA in the retinal degeneration (rd) mouse. Biochem Biophys Res Commun 252:236–240.

Katagiri T, Akiyama S, Namiki M, Komaki M, Yamaguchi A, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T. 1997. Bone morphogenetic protein-2 inhibits terminal differentiation of myogenic cells by suppressing the transcriptional activity of MyoD and myogenin. Exp Cell Res 230:342–351.

Kopen GC, Prockop DJ, Phinney DG. 1999. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc Natl Acad Sci USA 96:10711–10716.

Kraus B, Pette D. 1997. Quantification of MyoD, myogenin, MRF4 and Id-1 by reverse- transcriptase polymerase chain reaction in rat muscles–effects of hypothyroidism and chronic low-frequency stimulation. Eur J Biochem 247:98–106.

Laferriere NB, Brown DL. 1996. Expression and posttranslational modification of class III beta-tubulin during neuronal differentiation of P19 embryonal carcinoma cells. Cell Motil Cytoskeleton 35:188–199.

Law AK, Pencea V, Buck CR, Luskin MB. 1999. Neurogenesis and neuronal migration in the neonatal rat forebrain anterior subventricular zone do not require GFAP-positive astrocytes. Dev Biol 216:622–634.

Lee JY, Qu-Petersen Z, Cao B, Kimura S, Jankowski R, Cummins J, Usas A, Gates C, Robbins P, Wernig A, Huard J. 2000. Clonal isolation of muscle-derived cells capable of enhancing muscle regeneration and bone healing. J Cell Biol 150:1085–1100.

Lendahl U, Zimmerman LB, McKay RD. 1990. CNS stem cells express a new class of intermediate filament protein. Cell 60:585–595.

Lucas PA, Calcutt AF, Southerland SS, Warejcka D, Young HE. 1995. A population of cells resident within embryonic and newborn rat skeletal muscle is capable of differentiating onto multiple mesodermal phenotypes. Wound Repair Regen 3:457–468.

Mansouri A, Hallonet M, Gruss P. 1996. Pax genes and their roles in cell differentiation and development. Curr Opin Cell Biol 8:851–857.

Mastick GS, Andrews GL. 2001. Pax6 regulates the identity of embryonic diencephalic neurons. Mol Cell Neurosci 17:190–207.

Matsuura N, Lie DC, Hoshimaru M, Asahi M, Hojo M, Ishizaki R, Hashimoto N, Noji S, Ohuchi H, Yoshioka H, Gage FH. 2001. Sonic hedgehog facilitates dopamine differentiation in the presence of a mesencephalic glial cell line. J Neurosci 21:4326–4335.

McKinney-Freeman SL, Jackson KA, Camargo FD, Ferrari G, Mavilio F, Goodell MA. 2002. Muscle-derived hematopoietic stem cells are hematopoietic in origin. Proc Natl Acad Sci USA 99:1341–1346.

Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR. 2000. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. Science 290:1779–1782.

Pate DW, Southerland SS, Grande DA, Young HE, Lucas PA. 1993. Isolation and differentiation of mesenchymal stem cell from rabbit muscle. Surgical Forum XLIV , 587–589.

Pesce M, Scholer HR. 2000. Oct-4: control of totipotency and germline determination. Mol Reprod Dev 55:452–457.

Pesce M, Scholer HR. 2001. Oct-4: gatekeeper in the beginnings of mammalian development. Stem Cells 19:271–278.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.

Reubinoff BE, Itsykson P, Turetsky T, Pera MF, Reinhartz E, Itzik A, Ben Hur T. 2001. Neural progenitors from human embryonic stem cells. Nat Biotechnol 19:1134–1140.

Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, Verfaillie CM. 2001. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood 98:2615–2625.

Reyes M, Verfaillie CM. 2001. Characterization of multipotent adult progenitor cells, a subpopulation of mesenchymal stem cells. Ann N Y Acad Sci 938:231–233.

Reynolds BA, Tetzlaff W, Weiss S. 1992. A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J Neurosci 12:4565–4574.

Reynolds BA, Weiss S. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707–1710.

Richter-Landsberg C, Gorath M, Trojanowski JQ, Lee VM. 2000. Alpha-synuclein is developmentally expressed in cultured rat brain oligodendrocytes. J Neurosci Res 62:9–14.

Rosser AE, Tyers P, ter Borg M, Dunnett SB, Svendsen CN. 1997. Co-expression of MAP-2 and GFAP in cells developing from rat EGF responsive precursor cells. Brain Res Dev Brain Res 98:291–295.

Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR. 2000. Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol 164:247–256.

Scherer SE, Gallo V. 1998. Expression and regulation of kainate and AMPA receptors in the rat neural tube. J Neurosci Res 52:356–368.

Svendsen CN, ter Borg MG, Armstrong RJ, Rosser AE, Chandran S, Ostenfeld T, Caldwell MA. 1998. A new method for the rapid and long term growth of human neural precursor cells. J Neurosci Methods 85: 141–152.

Toma JG, Akhavan M, Fernandes KJ, Barnabe-Heider F, Sadikot A, Kaplan DR, Miller FD. 2001. Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nat Cell Biol 3:778–784.

Trowbridge IS, Thomas ML. 1994. CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. Annu Rev Immunol 12:85–116.

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH. 2002. Functional neurogenesis in the adult hippocampus. Nature 415: 1030–1034.

Vassilieva S, Guan K, Pich U, Wobus AM. 2000. Establishment of SSEA-1- and Oct-4-expressing rat embryonic stem-like cell lines and effects of cytokines of the IL-6 family on clonal growth. Exp Cell Res 258:361–373.

Vitalis T, Cases O, Engelkamp D, Verney C, Price DJ. 2000. Defect of tyrosine hydroxylase-immunoreactive neurons in the brains of mice lacking the transcription factor Pax6. J Neurosci 20:6501–6516.

Wakabayashi T, Messing A, Brenner M. 1999. Nonlethal detection of transgene expression in the CNS of founder mice. Biotechniques 26:302–307.

Walther C, Gruss P. 1991. Pax-6, a murine paired box gene, is expressed in the developing CNS. Development 113:1435–1449.

Woodbury D, Schwarz EJ, Prockop DJ, Black IB. 2000. Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 61:364–370.

Young HE, Duplaa C, Young TM, Floyd JA, Reeves ML, Davis KH, Mancini GJ, Eaton ME, Hill JD, Thomas K, Austin T, Edwards C, Cuzzourt J, Parikh A, Groom J, Hudson J, Black AC, Jr. 2001a. Clonogenic analysis reveals reserve stem cells in postnatal mammals: I. Pluripotent mesenchymal stem cells. Anat Rec 263:350–360.

Young HE, Steele TA, Bray RA, Detmer K, Blake LW, Lucas PW, Black AC. 1999. Human pluripotent and progenitor cells display cell surface cluster differentiation markers CD10, CD13, CD56, and MHC class-I. Proc Soc Exp Biol Med 221:63–71.

Young HE, Steele TA, Bray RA, Hudson J, Floyd JA, Hawkins K, Thomas K, Austin T, Edwards C, Cuzzourt J, Duenzl M, Lucas PA, Black AC Jr. 2001b. Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. Anat Rec 264:51–62.

Zhang SC. 2001. Defining glial cells during CNS development. Nat Rev Neurosci 2:840–843.

Zimmerman L, Parr B, Lendahl U, Cunningham M, McKay R, Gavin B, Mann J, Vassileva G, McMahon A. 1994. Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors. Neuron 12:11–24.