Differentiation assays of bone marrow-derived Multipotent Adult Progenitor Cell (MAPC)-like cells towards neural cells cannot depend on morphology and a limited set of neural markers

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Abstract

There are accumulating studies that report a neurogenic potential of bone marrow-derived cells both in vitro as well as in vivo. Most claims of neural “transdifferentiation” have based their conclusions on morphology and neural gene expression. Recently, doubts have been raised about the validity of both outcome parameters since non-neural cells can extend neurites and show aberrant neural gene expression as a response to stress inducing factors. In this study, we compared bone marrow-derived Multipotent Adult Progenitor Cell (MAPC)-like cells and neural stem cells (NSC) in their morphology and neural gene expression profile after neural differentiation using three differentiation protocols. We evaluated the expression of five neuroglial antigens [neurofilament 200 (NF200); beta III tubulin (β3 tub); tau; Glial Fibrillary Acidic Protein (GFAP); Myelin Basic Protein (MBP) and RIP antigen] using real-time PCR (RT-PCR) and immunocytochemistry (ICC). MAPC-like cells adopted a neural-like morphology in one protocol but a fibroblast-like morphology in the two other protocols. RT-PCR and ICC show that MAPC-like cells already express the neural antigens beta III tubulin and NF200 at baseline, but no upregulation of these genes after exposure to three distinct differentiation protocols was seen. In contrast, NSC adopt neural and glial morphologies with a clear increase in expression of all neuroglial genes in all differentiation protocols used. In conclusion, our data demonstrate that neural-like morphology and expression of a limited set of neural marker genes by MAPC-like cells after differentiation are not absolute proof of neural transdifferentiation because MAPC-like cells only partially meet the criteria which are fulfilled by NSC after neural differentiation.

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Introduction

An adult stem cell source that is easy accessible and can be differentiated towards different neural cell types could be the ultimate cell source for cell therapy of neurodegenerative diseases. It is therefore not surprising that initial reports of a possible neurogenic potential of bone marrow-derived cells led to an explosion of reports which claimed that bone marrow-derived cells can adopt a neural identity (Eglitis and Mezey, 1997; Brazelton et al., 2000; Mezey et al., 2000; Priller et al., 2001; Nakano et al., 2001; Zhao et al., 2002; Mezey et al., 2003; Weimann et al., 2003a; Munoz-Elias et al., 2004). However, more recent studies have clearly shown that such in vivo differentiation may be partial, extremely rare (Wagers et al., 2002) or may be artifactual (Alvarez-Dolado et al., 2003). In some studies, donor derived cells are found in the brain after transplantation but these do not express neural antigens (Castro et al., 2002; Massengale et al., 2005). In other studies, a fraction of the transplanted bone marrow cells do express neural antigens but these cells have a morphology distinct from adult
neurons (Brazelton et al., 2000; Mezey et al., 2000; Zhao et al., 2002). Studies in which differentiation from blood stem cells to neurons was quantified, measured an extremely low efficiency of differentiation towards neural cells (Wagers et al., 2002). Original reports that bone marrow cells could differentiate towards Purkinje neurons were challenged by others, reporting that these cells were the result of fusion of donor cells with endogenous Purkinje neurons (Alvarez-Dolado et al., 2003; Weimann et al., 2003b).

Next to these in vivo studies, several in vitro studies have shown that stem cells could be isolated out of bone marrow and that these stem cells were capable of transdifferentiation towards neural-like cells (Woodbury et al., 2000; Sanchez-Ramos et al., 2000; Kohyama et al., 2001; Deng et al., 2001; Kabos et al., 2002; Jiang et al., 2003, 2002; Kim et al., 2002; Munoz-Elias et al., 2003; Rismanchi et al., 2003; Levy et al., 2003; Locatelli et al., 2003; Padovan et al., 2003; Dezawa et al., 2004; Tondreau et al., 2004; Magaki et al., 2005; Bossolasco et al., 2005). In some of these studies, bone marrow-derived cells were even capable of differentiation towards cells of the three different germinal layers (endoderm, mesoderm and ectoderm). These cells were called Multipotent Adult Progenitor Cells (MAPC) (Reyes et al., 2001; Schwartz et al., 2002; Jiang et al., 2002) or Marrow-Isolated Adult Multilineage Inducible (MIAMI) cells (D’Ippolito et al., 2004).

Most differentiation studies base their conclusions on morphological data, namely the formation of neurite-like extensions, and immunohistochemical data, namely the expression of neural antigens. However, recent evidence suggests that a neural-like morphology or neural antigen expression may not be as specific as previously thought. For example, Woodbury et al. recently developed a differentiation protocol where bone marrow stromal cells rapidly (within several hours) display neural morphology and show an enhanced expression of neural antigens. However, recent evidence suggests that these cells were the result of fusion of donor cells with endogenous neural antigens as an indication for neural differentiation should also be questioned for several reasons. First, some neural antigens are already expressed at baseline in native, non-neural bone marrow-derived cells (Woodbury et al., 2002; Goolsby et al., 2003; Tondreau et al., 2004; Ratajczak et al., 2004; Bertani et al., 2005). Second, the exposure of mesenchymal stem cells to stressors also causes an enhancement of the expression of neural markers Neuronal Nuclei (NeuN) and Neuron-Specific Enolase (NSE) (Lu et al., 2004), but also Neurofilament 200 (NF200) and tau (Bertani et al., 2005). Finally, when different studies using the same type of stem cells and the same differentiation protocol are compared for antigen expression before and after differentiation, several inconsistencies can be found for different markers (Woodbury et al., 2000; Lu et al., 2004; Neuhuber et al., 2004; Bertani et al., 2005).

To better evaluate neural differentiation of bone marrow-derived cells, we compared neural differentiation of bone marrow-derived MAPC-like cells and brain derived neural stem cells (NSC) using various differentiation protocols. We evaluated the expression of five different neural antigens (NF200, beta III tubulin, tau, Glial Fibrillary Acidic Protein, Myelin Basic Protein) at baseline and after differentiation. The expression profile of the neuroectodermal genes was measured using immunocytochemistry (ICC) and real-time PCR (RT-PCR). It was found that, although bone marrow-derived cells can obtain some characteristics of neural cells, only neural stem cells gave rise to cells which met all criteria screened for.

Materials and methods

Culture of MAPC-like stem cells

All animal procedures and experiments were approved by the local Ethical Committee of the University of Ghent. Isolation of bone marrow-derived stem cells was performed using the same procedure as described for MAPC (Jiang et al., 2002). In brief, the femurs of adult Sprague–Dawley rats (n = 2, body weight 250 g, Harlan) were flushed, and bone marrow mononucleated cells (BMMNC) were collected by Ficoll-Hypaque separation. BMMNCs were initially plated in expansion medium on fibronectin coated plates at a density of $6 \times 10^5$ cells/cm².

MAPC expansion medium consisted of the following: 57.5% low glucose DMEM (Cambrex, Verviers, Belgium), 39% MCDB-201 (Sigma, Bornem, Belgium), 1% insulin–transferrin selenium (ITS, Sigma), 0.5% linoleic acid–bovine serum albumin (LA-BSA, Sigma), 100 μM ascorbic acid 2-phosphate (Sigma), 100 U/ml penicillin (Cambrex), 100 U/ml streptomycin (Cambrex), 10 ng/ml PDGF-BB (R&D), 10 ng/ml EGF (Sigma), 2% Fetal Calf Serum (FCS, Serum Supreme, Cambrex) and 1000 units/ml mLIF (Chemicon, Temecula, CA, USA). Half of the medium was changed every 3 days. After 3 weeks, the hematopoietic fraction (CD45+ and red blood cells) was depleted using paramagnetic beads. Cells were separated with 2 sequential MACS LD columns (Miltenyi Biotech, Utrecht, The Netherlands). After depletion, cells were plated in fibronectin coated flat-bottomed 96-well plates at a density of 1 cell per well to generate single cell clones. Clones were expanded at densities between 0.5 and 1.5 $\times 10^5$/cm².

Five single cell MAPC-like clones were withdrawn on the basis of their spindle-like morphology, their expansion capacity...
(>40 population doublings) and their phenotypic expression profile (MHC I, MHC II, CD44<sup>dim</sup>) using flow cytometry analysis. Using real-time polymerase chain reaction (RT-PCR), relative expression of Oct4 was compared between the five MAPC-like cell clones and the clone with the highest expression was chosen to be used for further analysis in this study.

**Culture of neural stem cells**

Neural stem cells were isolated according to a published protocol (Gobbel et al., 2003). In brief, the brain of female Sprague–Dawley rats (<i>n</i> = 2, body weight 250 g, Harlan) was removed and placed in artificial cerebrospinal fluid (124 mM NaCl, 5 mM KCl, 3.2 mM MgCl<sub>2</sub>, 105 mM NaHCO<sub>3</sub>, 10 mM glucose and 2 mM CaCl<sub>2</sub>). The subventricular zone was removed bilaterally by microscopic dissection. To dissociate the tissue, it was placed into a digestion medium containing 0.5 mM Na<sub>2</sub>EDTA (Sigma), 1.0 mM cysteine (Sigma), 0.9 mg/ml papain (Worthington, Lakewood, NJ) and 1 mg/ml DNase (Sigma), dissolved in Earle’s balanced salt solution and this for 45 min. Digestion was stopped by adding ovomucoid (Sigma). After a mechanical dissociation, the cells were plated in a 96-well flat-bottomed plate at a density of 2000 cells per well in a chemically defined medium. The NSC growth medium consisted of NS-A medium (Euroclone, Milan, Italy) with an additional 2 mM L-glutamine (Cambrex), penicillin 3 mM D-glucose (Sigma), 1.2 mM sodium bicarbonate (Sigma), 0.46 mM HEPES (Sigma), 2% B27 (Gibco BRL, Rockville, MD), 1% N2 supplement (Gibco BRL), 100 U/ml penicillin (Cambrex), 100 U/ml streptomycin (Cambrex), 20 ng/ml of human recombinant epidermal growth factor (EGF; Sigma) and 20 ng/ml of human recombinant basic fibroblast growth factor (bFGF; R&D, Minneapolis, MN, USA). Cells were grown at 37°C in 5% CO<sub>2</sub> and 95% air with saturated humidity. They were passaged once cell clusters were formed, approximately 2 weeks after initial isolation and every 1–2 weeks thereafter. At the fourth passage, the cells were plated in a flat-bottomed 96-well plate at a density of 1 cell per well. A single cell clone was chosen to be used in this study on the basis of its capacity to form neurospheres and the survival and morphology after in vitro differentiation.

**Karyotyping**

MAPC-like cells and NSC, subcultured at a 1:4 dilution, 24 h before harvesting, were collected and subjected to a 1.5-hour colcemid incubation followed by lysis with hypotonic KCl and fixation in acid/alcohol. Metaphases were analyzed after G banding (Dewald et al., 1985).

**Differentiation of MAPC-like cells into mesenchymal lineages**

**Adipogenesis**

To induce MAPC-like cells to differentiate into adipocytes, cells were plated into six-well plates at a density of 20 × 10<sup>3</sup>/cm<sup>2</sup> and incubated in adipogenic induction medium consisting of DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin, 1 μM dexamethasone, 0.2 mM indomethacin, 10 μg/ml insulin, 0.5 mM methylisobutyl-xanthine (all from Sigma). Medium was replaced every 3–4 days during 3 weeks. Differentiation was appreciated by morphological examination, and Oil Red O staining was performed to detect lipid accumulation (Pittenger et al., 1999).

**Osteogenesis**

To induce MAPC-like cells to differentiate into osteoblasts, cells were plated into six-well plates at a density of 3 × 10<sup>5</sup>/cm<sup>2</sup> and incubated in osteogenic induction medium consisting of DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin, 0.1 μM dexamethasone, 50 μM ascorbic acid 2-phosphate and 10 mM beta-glycerophosphate (Sigma). Medium was replaced every 3–4 days during 3 weeks. Von Kossa’s staining was performed in order to demonstrate calcium deposition in differentiated cultures (Pittenger et al., 1999).

**Neural differentiation of MAPC-like cells and NSC**

All neural differentiations were done with the cells plated on matrigel in DMEM/B27 medium (Gibco BRL) with an additional 2 mM l-glutamine (Cambrex), penicillin 3 mM D-glucose (Sigma), 1.2 mM sodium bicarbonate (Sigma), 0.46 mM HEPES (Sigma), 2% B27 (Gibco BRL, Rockville, MD), 1% N2 supplement (Gibco BRL), 100 U/ml penicillin (Cambrex), 100 U/ml streptomycin (Cambrex), 20 ng/ml of human recombinant basic fibroblast growth factor (bFGF; Sigma) and 20 ng/ml of human recombinant epidermal growth factor (EGF; Sigma) and 20 ng/ml of human recombinant basic fibroblast growth factor (bFGF; Sigma) and 20 ng/ml of human recombinant epidermal growth factor (EGF; Sigma) and 20 ng/ml of human recombinant epidermal growth factor (EGF; Sigma) and 20 ng/ml of human recombinant epidermal growth factor (EGF; Sigma) and 20 ng/ml of human recombinant epidermal growth factor (EGF; Sigma). Neurons were differentiated parallel by three methods based on previously published protocols: the Jiang method (Jiang et al., 2003), the Kohyama method (Kohyama et al., 2001) and the Kabos method (Kabos et al., 2002). In brief, for MAPC-like cells, the Jiang method consisted of a predifferentiation phase in which the cells are exposed to bFGF (100 ng/ml, R&D) for 7 days. After this neural induction phase, MAPC-like cells were exposed to Sonic Hedgehog (100 ng/ml, R&D) and FGF-8 (10 ng/ml, R&D) for another 7 days. In the case of NSC differentiation, the cells were directly plated for 7 days in the presence of SHH (100 ng/ml) and FGF-8 (10 ng/ml) without a predifferentiation phase. In the Kohyama protocol, MSC cells were incubated for 3 days with 10 μmol/ml 5-azacytidine, 10% FCS, Nerve Growth Factor (NGF, R&D, 50 ng/ml), Brain Derived Factor (BDNF, R&D, 50 ng/ml) and Neurotrophin-3 (NT-3, R&D, 50 ng/ml) for the induction of neural differentiation. After the induction phase, cells were incubated with serum-free medium containing NGF, NT-3 and BDNF (all 50 ng/ml) for 7 days. NSC were not subjected to the induction phase and were directly plated for 7 days in the presence of the cytokines NGF, NT-3 and BDNF (all 50 ng/ml). In the Kabos method, cells were incubated for 7 days with 20 ng/ml bFGF and 20 ng/ml EGF in order to induce differentiation. In the following phase, cells were exposed to 1 μM retinoic acid (RA, Sigma) and 1 mM dibutyryl cyclic AMP
RNA was extracted from the cells using a kit (Aurum™ total RNA kit, Bio-Rad, Hercules, CA, USA). cDNA was prepared using a first strand synthesis kit (iScript™ cDNA Synthesis Kit, Bio-Rad). Using the standard curve method, the relative amount of expression of Oct4 and neuroglial genes was determined by normalizing using hypoxanthine phosphoribosyl transferase (HPRT) as a housekeeping gene (Peinnequin et al., 2004). For HPRT, the oligonucleotides used were GCG TCT CTT TTG ATT TGC A (sense primer) and TCA CAA GGG AAG TGA CAA TCT ACC T (antisense primer), for Oct4 CTG TAA CCG GCG CCA GAA (sense primer) and TGC ATG GGA GAG CCC AGA (antisense primer), for beta III tubulin (β3-tub) GGA GCT GTT CAA GCG CAT CT (sense primer) and CCC GTG TAC CAG TGG AGG AA (antisense primer), for NF200 GCTG TCC TTC TTG AGC TTC TTT G (sense primer) and TTG TTG TCG GGC TAT GAA GAA A (antisense primer), for tau AAG CCA GTG GAC CTG AGC AA (sense primer) and TTC GAC TGG ACT CTA TCC TTG AAG T (antisense primer), for Glial Fibrillary Acidic Protein (GFAP) GAG AGA TTC GCA CTC AGT AC (sense primer) and TGG ACC GAT ACC ACT CTG TT (antisense primer), for Myelin Basic Protein (MBP) ATT CCG AGG AGA GTG TGG GTT T (sense primer) and GAA GTT TCG TCC CTG CGT TTC (antisense primer). All primers were designed with Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) and tested for specificity using NCBI’s BLAST software. All primers were manufactured and HPLC purified by Operon Biotechnologies (Cologne, Germany). PCR reagents were obtained from Eurogentec (Seraing, Belgium) as SYBR Green I mastermixes and used according to the manufacturer’s instructions. Reactions were run on ABI Prism 7000 Sequence Detection System (Applied Biosystems). The cycling conditions were as follows: 10 min at 95°C, 40 cycles at 95°C for 15 s and 60°C for 60 s. After PCR amplification, a melting curve was generated for every PCR product to check the specificity of the PCR reaction (absence of primer–dimers or other non-specific amplification products). To quantify gene expression, normalized mRNA expression levels of Oct4 in the five MAPC-like cells clones were compared with levels in rat testis. Normalized mRNA expression levels of neuralglial genes in MAPC-like cells and NSC before (respectively M0 and N0) and after differentiation according to the Jiang (M1, N1), the Kohyama (M2, N2) and the Kabos (M3, N3) protocol were compared with levels of expression in an NSC clone that was differentiated following a standard protocol ($N_{\text{standard}}$). According to this protocol, cells are plated on poly-lysine in medium devoid of growth factors but with the addition of 3% Fetal Bovine Serum (Reynolds and Weiss, 1996; Gobbel et al., 2003). Therefore, the formula to quantify expression of the genes of interest ($Genes_int$) is: Relative expression ($Genes_int$) in $N_{x}$ or $M_{x}$/Relative expression (HPRT) in $N_{0}$ or $M_{0}$/[Relative expression (Genes_int) in $N_{\text{standard}}$/Relative expression (HPRT) in $N_{\text{standard}}$].

**Immuncytochemistry for CNS antigens**

Undifferentiated and differentiated NSC and MAPC-like cells were stained for the same markers from which the gene expression was analyzed using RT-PCR, except for oligodendrocytes, which were stained with RIP antibody because this resulted in much better stainings compared to immuncytochemistry for MBP. Cells were fixed with 4% paraformaldehyde for 10 min and subjected to immuncytochemistry. MAPC-like cells and differentiated NSC are adherent and can be processed easily. In order to prevent differentiation induced by plating undifferentiated NSC on coated surface, cytocentrifuged preparations (Cytospin 2; Cytospin, Shandon, UK) were made and fixed. The PFA-fixed preparations were quenched with 50 mM NH$_4$Cl for 10 min, permeabilized with 0.2–0.5% Triton X-100 (TX100) in phosphate-buffered saline (PBS) for 5 min and blocked using 0.4% fish skin gelatin/PBS for 30 min followed by incubation first with primary antibodies (ms, mouse monoclonal; rb, rabbit polyclonal): ms anti-nestin 1:50 (Chemicon, MAB353), ms anti-β3 tub 1:100 (Sigma SDL-SD10), ms anti-NF200 1:100 (Chemicon, MAB5262), rb anti-tau 1:5000 (Dako, A 0024), rb anti-GFAP, 1:100 (Dako, Z0334), ms anti-oligodendrocytes 1:5000 (Chemicon, RIP, MAB1580) and then with secondary antibodies Alexa Fluor 488 goat anti-mouse IgG (H+L) (Molecular Probes) 1:1000 and goat anti-rabbit Texas Red IgG (H+L) (Vector Laboratories, Burlingame, CA) 1:1000 diluted in 0.4% fish skin gelatin/phosphate-buffered saline for 2 h each. To test the specificity of the secondary antibodies, the primary antibody step was omitted. Cross reactivity of the primary antibodies with human antigens allowed testing the antigen specificity by staining HUVEC cells as a negative control and rat brain as a positive control. The coverslips were mounted in Mowiol supplemented with 4,6-diamidino-2-phenylindole (DAPI). Immunoreactive (IR) cells were visualized using a Bio-Rad Radiance 2100 confocal laser scanning microscope.

**Results**

**Isolation of MAPC-like cells**

We successfully isolated cells from the bone marrow of adult rats using the protocol described by Jiang et al. (2002). After 3 weeks, bulk cultures were depleted of CD45$^+$ and red blood cells were subsequently plated in MAPC expansion medium at a density of one cell per well. Five clones were selected on the basis of a similar morphology (Fig. 1), flow cytometric data (MHC I and MHC II neg, CD44$^{+}$dim) and growth characteristics (data not shown) as true MAPC cells. All these five clones
were subjected to the neural differentiation protocol described in the study by Jiang et al. (2002). Since none of the clones differentiated towards cells positive for tau, GFAP or RIP using this protocol, we will designate these cells as MAPC-like cells. After these initial differentiation attempts, we used RT-PCR analysis to determine the levels of Oct4 expression in these five cell clones (Table 1). The clone with the highest expression, clone 3.5b1, was chosen to be used for further analysis.

Cytogenetic analysis after 40 population doublings showed that all clones had a normal karyotype (Fig. 2).

**Isolation and phenotypic characterization of NSC cells**

Neural stem cells were isolated from the subventricular zone of the brain by plating dissociated, subventricular cells in a special defined neural stem cell expansion medium (Gobbel et al., 2003). Two weeks after initial isolation, large spherical clusters of cells, or neurospheres, which grew loosely attached to the substrate, were present in the culture. These neurospheres were passaged mechanically when the diameter of the neurospheres ranged from 100 to 150 μm. The dissociated cells were plated again, and secondary neurospheres appeared in culture. Neurospheres were passaged every 7 to 10 days after plating. At the fourth passage, the cells were plated at a density of 1 cell per well in a 96-well plate. Approximately, 4% of the cells formed neurospheres which could be passaged continuously (>10 passages). Using the culture and isolation technique especially designed for adult rat neural stem cells, we were able to keep single cell clones of NSC in culture for over 1 year (Gobbel et al., 2003). Formation of neurospheres and expansion capacity were used as criteria for the selection of a clone which was used in this study. Cytogenetic analysis of the cells after 40 population doublings showed that the cells also had a normal karyotype (data not shown).

**Mesenchymal differentiation of MAPC-like cells**

When inducing the adipogenic differentiation, the cells adopted a round morphology and accumulated large, cytoplasmic vacuoles in which accumulation of lipid was detected with Oil Red O staining (Fig. 3a), while in non-induced cells, no staining was detected (Fig. 3b). The osteogenic differentiation was demonstrated by calcium deposition in cell culture by positive Von Kossa’s stain in large portion of cells (Fig. 3c) while non-induced cells were all negative (Fig. 3d).

**Neural differentiation of MAPC-like cells and NSC**

MAPC-like cells and NSC were differentiated according to three previously described protocols: the Jiang method (Jiang et al., 2003), the Kohyama method (Kohyama et al., 2001) and the Kabos method (Kabos et al., 2002). In contrast to NSC, MAPC-like cells were first predifferentiated by incubation with neural induction factors: bFGF (Jiang method); 5-azacytidine/NGF/NT-3/BDNF (Kohyama method) and bFGF/EGF (Kabos method). In all three methods, MAPC-like cells formed neurite-like extensions and in case of the Jiang and Kabos method the cells rounded up and appeared as very bright cells by phase contrast microscopy (Fig. 4A). Quantification however showed that the proportion of cells that immunostained positive for nestin, a marker for neuroprogenitor cells, was lower after predifferentiation (about 15%) than at baseline conditions (about 30%, Fig. 4B). Predifferentiated MAPC-like cells and NSC were then differentiated by culturing them for 7 days in the presence of other differentiation factors (Jiang method: SHH, FG-8; Kohyama method: NGF, BDNF, NT-3; Kabos method: RA, cAMP). MAPC-like cells differentiated with the Jiang method displayed a bipolar morphology and rounded cell bodies, which is typical for young neurons. MAPC-like cells differentiated with the Kohyama or Kabos method also developed long extensions but adopted a more fibroblast-like morphology (Fig. 5). NSC displayed a large number of neurite-like processes in all three conditions. Cells with a bipolar morphology and cells with a polarized morphology were present. In addition, a significant fraction of cells with glial-like morphology could be detected between the differentiated cells.

<p>| Table 1 |</p>
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<th>Levels of Oct4 mRNA expression in five MAPC-like cell clones relative to expression in rat testes</th>
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RNA was extracted from five MAPC-like cell clones. cDNA was prepared using a first strand synthesis kit (iScript™ cDNA Synthesis Kit, Bio-Rad). RT-PCR reactions were run on ABI Prism 7000 Sequence Detection System. The cycling conditions were as follows: 10 min at 95°C, 40 cycles at 95°C for 15 s and 60°C for 60 s. After PCR amplification, a melting curve was generated for every PCR product to check the specificity of the PCR reaction. Using the standard curve method, the relative amount of expression of Oct4 was determined by normalization using HPRT as a housekeeping gene. To quantify gene expression, normalized mRNA expression levels of Oct4 in MAPC-like cell clones were compared with levels of expression in rat testes.
Quantitative real-time PCR for neuroglial mRNAs

Normalized mRNA expression levels of neuroglial genes in MAPC-like cells and NSC before (respectively M0 and N0) and after differentiation according to the Jiang (M1, N1), the Kohyama (M2, N2) and the Kabos (M3, N3) protocol were quantified. The results demonstrate that neural markers β3-tub and NF200 are expressed in NSC before (N0) and after N1, N2 and N3 differentiation. Expression is however less than in the NSC clone, differentiated using the standard protocol. In addition, the increase of β3-tub and NF200 expression after differentiation is limited: β3-tub expression is 1.5 to 2 times higher after differentiation, NF200 expression is 7 to 8 times higher after differentiation. RT-PCR analysis showed that β3-tub and NF200 were also expressed in MAPC-like cells. β3-tub expression was even higher in MAPC-like cells before (M0) than after differentiation (M1, M2, M3). In the case of NF200, expression in M0 was lower than M1 and M2 but higher than M3 expression.

We subsequently analyzed expression of differentiation markers for neurons (tau), astrocytes (GFAP) and oligodendrocytes (MBP). Expression of tau, GFAP and MBP could not
be demonstrated in MAPC-like cells before or after differentiation. In contrast, all three markers showed a baseline expression in NSC before differentiation. After differentiation, increase in expression was noted: a tenfold increase for tau, a 100-fold increase for MBP and a 1000-fold increase for GFAP. So, for all three markers, there is a clear augmentation in expression due to differentiation (Table 2).

Immunocytochemical staining of neuroglial antigens

Immunocytochemistry of NSC and MAPC-like cells yielded the following results. The presence of β3-tub and NF200 could not be demonstrated in non-differentiated NSC; but a fraction of differentiated NSC stained positive for these markers. β3-tub and NF200 could also be demonstrated in MAPC-like cells and this before and after differentiation (Fig. 6A). Immunocytochemistry for tau, RIP and GFAP showed that MAPC-like cells did not stain positive for these markers before or after differentiation in contrast to differentiated NSC. Undifferentiated NSC did only stain positive for RIP but not for tau or GFAP (Fig. 6B). One can conclude from both techniques that β3-tub and NF200 are expressed in MAPC-like cells both in undifferentiated as in differentiated state; whereas tau, GFAP and MBP are not expressed in MAPC-like cells neither in undifferentiated nor in differentiated state. Both RT-PCR and ICC demonstrated that differentiated NSC were positive for all markers investigated and this in all three differentiation paradigms. RT-PCR could detect expression of β3-tub, NF200, tau and GFAP genes in undifferentiated neural stem cells while ICC could not detect the presence of these antigens.

Discussion

Most studies that demonstrated neural differentiation of bone marrow-derived cells based their conclusion on the adoption of a neural-like morphology and the expression of neural genes. Recent evidence, however, show an that a non-neural cell can adopt a neural morphology and show an aberrant expression of neural antigens as a response to different stressors rather than being the result of neural transdifferentiation (Lu et al., 2004; Neuhuber et al., 2004; Bertani et al., 2005). To further investigate the significance of neural morphology and neuroglial antigen expression as proof of neural differentiation, bone marrow-derived MAPC-like cells were compared in their morphology and neuroglial gene expression profile with positive control NSC before and after neural differentiation by three different protocols.

In this study, we were able to isolate stem cells out of the mesenchymal cell fraction of rat bone marrow with an expansion capacity and a spindle-like morphology characteristic for MAPC, a rare subpopulation of MSC, described by Verfaillie et al. (Jiang et al., 2002). MAPC cells have been shown to differentiate to cells of the three germinal layers (endoderm, ectoderm and mesoderm). We were able to differentiate the MAPC-like cells towards mesodermal cell fates, such as osteoblasts and adipocytes, using protocols described for mesenchymal stem cells. To date, no other group has published that pluripotent cells could be generated using the method described by Jiang in 2002 suggesting that the critical parameters necessary for terminal transdifferentiation are unknown. Pluripotency is a required property of MAPC but
since we were also unable to reproducibly obtain differentiation to neuronal cells, we designate the cells MAPC-like. Because we were especially interested in the neural transdifferentiation capacity of bone marrow-derived MAPC-like cells, we compared the neural transdifferentiation capacity of a MAPC-like cell clone with that of tissue-specific NSC isolated from the subventricular zone of adult rats. Several studies have shown that NSC can differentiate towards electrically active neurons, astrocytes and oligodendrocytes (Whittemore et al., 1999; Liu et al., 1999; Toda et al., 2000; Song et al., 2002).

We selected a MAPC-like cell clone on the basis of its Oct4 expression which was highest compared to four other clones analyzed. Oct4 is also expressed in precursors of gametes or primordial germ cells (PGCs), in undifferentiated embryonic stem (ES), embryonic germ (EG) and embryonic carcinoma (EC) cell lines. Its exact role is not yet elucidated, but expression of Oct4 in ES is important in maintaining self-renewal and a pluripotent state (Niwa et al., 2000; Cavaleri and Schöler, 2006). This seems also to be the case in MAPC since clones with high Oct4 expression have a higher pluripotency than clones with low Oct4 expression (Verfaillie, personal communication).

NSC could be successfully isolated, cultured and differentiated using a standard protocol (Gobbel et al., 2003). The NSC were subjected to three differentiation protocols which were specifically designed for neuronal differentiation of bone marrow-derived stem cells. The protocols, however, were slightly modified taking into account the different nature of the cells. The most important modification was the omission of the neural pre-induction phase in which bone marrow stem cells are forced to differentiate towards the neural stem or progenitor cell phase. In the Jiang and Kabos methods, the factors used in this pre-induction phase are growth factors used to culture neural stem cells. Therefore, in the case of NSC differentiation, this step could be omitted without any influence on the outcome of differentiation. In the neural induction phase of the Kohyama
method, 5-azacytidine is used. This demethylating agent is believed to have enhancing effects on transdifferentiation of cells and has been used for transdifferentiation of bone marrow-derived cells to cardiomyocytes (Makino et al., 1999) and hepatocytes (Yamazaki et al., 2003). In our study, this factor was omitted since no transdifferentiation is needed in case of NSC differentiation. MAPC-like cells were differentiated using the protocols as originally described by the authors. The Jiang method has been optimized for neural differentiation of MAPC-like cells. The Kohyama method is designed for differentiating mesenchymal stem cells, and the Kabos method uses unfractionated bone marrow as starting material.

Another neural differentiation method is a protocol described by Woodbury et al. (2000). Initially, MAPC-like cells and NSC were compared in their potential for neural differentiation using this method, but this protocol caused massive cell death of NSC (data not shown). This is in line with recent evidence showing that the adoption of neural morphology and the enhancement of neural gene expression in cells that are differentiated using the Woodbury protocol are likely to be stress artefacts (Lu et al., 2004; Neuhuber et al., 2004; Bertani et al., 2005). Therefore, this differentiation protocol was omitted from this study.

In all three protocols, NSC differentiated towards cells with appropriate neural and glial morphology and positive for all neural or glial markers investigated with ICC and RT-PCR analysis. Semi-quantitative analysis showed that there was a clear augmentation in expression of three markers (tau, GFAP and MBP) already at baseline, but there was no clear upregulation of these genes after exposure to all differentiation protocols. In the case of tau, GFAP and MBP, cells did not express these markers before or after differentiation at levels detectable with RT-PCR or ICC.

In contrast to our study, the Minneapolis group did report the differentiation of bone marrow-derived MAPC towards cells positive for different neural, astrocyte and oligodendrocyte markers using the protocol we used in our study (Jiang et al., 2002). This group succeeded in differentiating murine MAPC-like cells towards functional neurons using a complex co-culture differentiation protocol (Jiang et al., 2003). Our MAPC-like cells also had multipotent capacity since we were able to differentiate them towards different mesodermal cell fates. However, we did not succeed in differentiating rat MAPC-like cells towards neuroectodermal phenotypes on a persuasive basis, although several attempts were made to reproduce the neural differentiation results of the Minneapolis group. By comparing the outcome of the neural differentiation of the MAPC-like cells with that of a positive control, adult NSC, we demonstrate that the protocols used in this study can induce neural differentiation but only in cells that have this potential.

In summary, our results show that bone marrow-derived MAPC-like cells express "neural markers" nestin, β3-tub and NF200 and can be induced to extend neurite-like extensions. However, these factors cannot be seen as proof for a neuropotency since the differentiation protocols tested have
no clear inducing or enhancing effects on the expression of neuroglial genes in MAPC-like cells while, for \(\beta\)3-tub and NF200, they do in the positive control NSC. Recent studies have demonstrated that the formation of neurite-like structures is not always the result of the development of axons or dendrites but can also be induced by exposing cells to several stressors (Lu et al., 2004; Neuhuber et al., 2004; Bertani et al., 2005). In all protocols used in this study to differentiate MAPC-like cells, the FCS used to culture MAPC-like cells is withdrawn from the medium. Several studies have demonstrated that removal of

![Fig. 6. Comparative analysis of antigen reactivity of MAPC-like cells and NSC at the end of differentiation using 3 different protocols. Immunocytochemistry analysis of MAPC-like cells and NSC before (respectively M0 and N0) and after differentiation according to the Jiang protocol (M1, N1); the Kohyama method (M2, N2) and the Kabos method (M3, N3). A: M0 cells stained slightly positive for \(\beta\)3-tub and NF200. N0 cells did not stain for NF200 and \(\beta\)3-tub. Between the differentiated NSC and MAPC-like cells, there were cells that stained positive for NF200 and \(\beta\)3-tub. B: no tau-, RIP- or GFAP-positive cells could be demonstrated either before or after differentiation. Undifferentiated NSC were negative for tau and GFAP but some stained positive for the RIP marker. After all, three differentiations, tau-, GFAP- and RIP-positive cells, were present among the differentiated cells.](image)
serum induces oxidative stress and DNA fragmentation. So as an alternative explanation, the formation of neurite-like extensions by MAPC-like cells in our study could be the result of stress induced by serum withdrawal. There are also several indications why the expression of \( \beta \)-tub and NF200 is no proof for a true neural phenotype. First, the present study demonstrates that differentiated MAPC-like cells do not express other panneural or glial markers such as tau and GFAP or MBP, while these are expressed in differentiated NSC. Second, \( \beta \)-tub and NF200 but also other presumed neural-specific markers are not exclusively expressed in neural cells but also in hematopoietic progenitor cells (Goolsby et al., 2003) and undifferentiated bone marrow stromal cells (Tondreau et al., 2004; Bertani et al., 2005). Third, a recent study showed that it is possible to induce expression of neural-specific antigens and neurotransmitters by exposing bone marrow cells to different mixes of growth factors. A closer evaluation, however, showed that the intracellular distribution of the antigens was different from that seen in adult neurons, implicating that these cells probably cannot function as true neurons (Jin et al., 2003).

Fig. 6 (continued).
The main conclusion of our study is that the expression of neural antigens by MAPC-like cells in this study probably is a phenomenon of aberrant expression rather than true transdifferentiation. We conclude this based on the fact that subjection of multipotent MAPC-like cells, to neural differentiation protocols, which were adopted from other transdifferentiation studies and proven to work for NSC, did not induce or up-regulate expression of the analyzed neural and glial antigens. Our study does not rule out that MAPC or mesenchymal stem cells may have the potential to differentiate towards neural cells. The data, however, show very clearly that validation of transdifferentiation of cells towards neural cell types, using only morphology and a limited set of neural antigen expression as outcome parameters, is not sufficient and that one has to be very rigorous in the interpretation of these neural transdifferentiation experiments. Furthermore, we show that the critical steps in neural differentiation of MAPC are not yet fully understood and undefined parameters may profoundly influence the results obtained in different laboratories.

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References


