Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells

Andreas Hermann[1,*], Regina Gastl[1,*], Stefan Liebau[1], M. Oana Popa[2], Jörg Fiedler[3], Bernhard O. Boehm[4], Martina Maisel[1], Holger Lerche[1,2], Johannes Schwarz[5,6], Rolf Brenner[3] and Alexander Storch[1,‡]

[1]Department of Neurology, [2]Department of Applied Physiology, [3]Division for Biochemistry of Joint and Connective Tissue Diseases, Department of Orthopaedics and [4]Division of Endocrinology, Department of Internal Medicine, University of Ulm, Helmholtzstr. 8/1, 89081 Ulm, Germany
[5]Department of Neurology, University of Leipzig, Liebigstr. 22a, 04103 Leipzig, Germany
[6]Division of Biology, California Institute of Technology, 1200 East California Boulevard, Pasadena, CA 91125, USA
*Authors contributed equally
‡Author for correspondence (e-mail: alexander.storch@neuro.med.tu-dresden.de)

Accepted 5 May 2004
Journal of Cell Science 117, 4411-4422 Published by The Company of Biologists 2004
doi:10.1242/jcs.01307

Summary

Clonogenic neural stem cells (NSCs) are self-renewing cells that maintain the capacity to differentiate into brain-specific cell types, and may also replace or repair diseased brain tissue. NSCs can be directly isolated from fetal or adult nervous tissue, or derived from embryonic stem cells. Here, we describe the efficient conversion of human adult bone marrow stromal cells (hMSC) into a neural stem cell-like population (hmNSC, for human marrow-derived NSC-like cells). These cells grow in neurosphere-like structures, express high levels of early neuroectodermal markers, such as the proneural genes *NeuroD1*, *Neurog2*, *MSl1* as well as otx1 and nestin, but lose the characteristics of mesodermal stromal cells. In the presence of selected growth factors, hmNSCs can be differentiated into the three main neural phenotypes: astroglia, oligodendroglia and neurons. Clonal analysis demonstrates that individual hmNSCs are multipotent and retain the capacity to generate both glia and neurons. Our cell culture system provides a powerful tool for investigating the molecular mechanisms of neural differentiation in adult human NSCs. hmNSCs may therefore ultimately help to treat acute and chronic neurodegenerative diseases.

Key words: Mesodermal stromal cells, Neural stem cells, Transdifferentiation, Neural differentiation, Human stem cells

Introduction

Tissue-specific stem cells were thought to be lineage restricted and therefore only able to differentiate into cell types of the tissue of origin. However, several recent studies suggest that this type of stem cell might be able to break the barriers of germ layer commitment and differentiate in vitro and/or in vivo into cells of different tissues. For instance, transplanted bone marrow cells contribute to endothelium (Asahara et al., 1997; Jackson et al., 2001; Lin et al., 2000; Orlic et al., 2003; Orlic et al., 2001; Rafii et al., 1994) and skeletal muscle myoblasts (Ferrari et al., 1998; Gussoni et al., 1999) and acquire properties of hepatic and biliary duct cells (Lagasse et al., 2000; Petersen et al., 1999; Theise et al., 2000), lung, gut, and skin epithelia (Krause et al., 2001) as well as neuroectodermal cells (Brazelton et al., 2000; Mezey et al., 2000; Mezey et al., 2003). Buzanska and colleagues reported recently that human umbilical cord blood-derived cells attain neuronal and glial features in vitro (Buzanska et al., 2002). Furthermore, neural stem cells (NSCs) may repopulate the hematopoietic system (Bjornson et al., 1999; Shih et al., 2002; Shih et al., 2001), and muscle cells may differentiate into hematopoietic cells (Jackson et al., 1999).

Mesodermal stromal cells (MSCs) derived from bone marrow cell suspensions by their selective attachment to tissue culture plastic can be expanded efficiently (Friedenstein et al., 1976; Reyes et al., 2001; Sekiya et al., 2002). MSCs are capable of differentiating into many mesodermal tissues, including bone, cartilage, fat, and muscle (Friedenstein et al., 1976; Orlic et al., 2001; Prockop, 1997; Reyes et al., 2001; Sekiya et al., 2002). In addition, these cells were reported to differentiate in vitro and in vivo into cells expressing neuronal and glial markers (Sanchez-Ramos et al., 2000; Woodbury et al., 2000; Zhao et al., 2002). Jiang and co-workers recently demonstrated a rare multipotent adult progenitor cell (MAPC) within MSC cultures from rodent bone marrow (Jiang et al., 2002a; Jiang et al., 2002b). This cell type differentiates not only into mesenchymal lineage cells but also into endothelium and endoderm. Mouse MAPCs injected in the blastocyst contribute to most if not all somatic cell lineages including brain (Jiang et al., 2002a). Furthermore, mouse MAPCs can also be induced to differentiate in vitro using a co-culture system with astrocytes into cells with biochemical, anatomical, and electrophysiological characteristics of neuronal cells (Jiang et al., 2003). However, in vitro conversion of human MSCs into clonogenic undifferentiated hNSCs, which can be proliferated and subsequently differentiated into all major cell lineages of the brain, such as neurons, astroglia and oligodendroglia has not been reported. This immature hNSC population would be more suitable for neuroregenerative strategies using transplantation than fully differentiated neural cells because terminal differentiated neuronal cells are well known to survive detachment and subsequent transplantation

procedures poorly (Bjorklund and Lindvall, 2000; Carvey et al., 2001; Kim et al., 2002b; Pluchino et al., 2003). We show here that comparable to embryonic stem (ES) cells, adult human MSCs can be converted into a clonogenic neural stem cell-like population (hmNSC, for human marrow-derived NSC-like cells) growing in neurosphere-like structures, which can be differentiated in vitro into cells with morphological and functional characteristics of neuronal, astroglial and oligodendroglial cells.

Materials and Methods

Cell culture

Adult human bone marrow was harvested from routine surgical procedures (pelvic osteotomies; 4 samples; age 18-35 years) following informed consent and in accordance with the terms of the ethics committee of the University of Ulm. hMSC were isolated and cultured as described earlier (Fickert et al., 2003; Fiedler et al., 2002). Cells were passaged once a week. The hMSC phenotype was confirmed by FACS analysis with CD9, CD90, CD105, and CD166 (positive), as well as CD14, CD34, and CD45 (negative), and by the potential to differentiate in osteoblasts, chondrocytes and adipocytes. After passage 2-10 (≈10-50 population doublings) conversion of hMSC into neurosphere-like structures was initiated. Specifically, cells were dissociated with 0.05% trypsin/0.04% EDTA and plated on low-attachment plastic tissue culture flasks (Nalge Nunc International, Rochester, NY, USA) at a concentration of 1-2×10^5 cells/cm^2 in P4-8F medium (AthenaES, Baltimore, MD) supplemented with 20 ng/ml of both epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2; both from Sigma, St Louis, MO) at 5% CO_2, 92% N_2 and 3% O_2. After 10-20 days, sphere formation could be observed. These neurosphere-like structures were expanded for an additional 2-10 weeks (2-4 passages; ≈5-30 population doublings) before glial or neuronal differentiation was started. The medium was changed once a week and growth factors were added twice a week. Induction of terminal neural differentiation was initiated by plating the cells on poly-L-lysine coated glass cover slips at a concentration of 1.5-2.0×10^5 cells/cm^2 in Neurobasal® medium (Gibco, Tulsa, OK) supplemented with 0.5 μM all-trans-retinoic acid (Sigma), 1% FCS, 5% horse serum, 1% N2 supplement and 1% penicillin/streptomycin (all from Gibco). 10 ng/ml rh-PDGF-BB was then added for glial induction (R&D Systems, Minneapolis, MN) or 10 ng/ml rh-BDNF for neuronal induction (Promega, Madison, WI). Cells were differentiated for 10-14 days.

Clonal analysis of hmNSCs

Clonal analysis was performed according to Uchida and co-workers (Uchida et al., 2000). In brief, hmNSCs were serially diluted into hmNSC expansion medium in 96-well plates. Only single cells were expanded by supplementing the medium to 50% with filtered medium conditioned for 48 hours on hmNSC and containing growth factors. Cells were expanded for 3-6 weeks and further processed as described above for hmNSC using the neuronal induction protocol.

Osteogenic differentiation of hmNSCs

In order to differentiate hMSCs and hmNSCs into osteoblasts, we used standard protocols described earlier (Fiedler et al., 2002; Reyes et al., 2001). We used both cell types from passage 6-10. Briefly, osteogenic differentiation was induced by plating the cells at 2×10^4 cells/cm^2 in DMEM medium containing 10% FCS, 1% glutamine and 1% penicillin/streptomycin supplemented with 0.1 μM dexamethasone, 50 mg/ml ascorbic acid, and 2.16 mg/ml β-glycerophosphate (all from Sigma, St Louis, MO). Cells were cultured for 14 days and medium was changed twice a week.

Flow cytometry

hMSC and hmNSC were treated with trypsin-EDTA (Gibco) and washed with PBS. Dead cells were excluded from analysis by forward-scatter gating. Samples were analyzed using FACSCalibur flow cytometer and Cellquest software (both from Becton Dickinson, Franklin Lakes, NJ). A minimum of 12,000 events was acquired for each sample.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde in PBS. Immunocytochemistry was carried out using standard protocols. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Antibodies and dilutions were as follows: TH monoclonal, 1:1000; β-tubulin III monoclonal, 1:1000; fibronectin monoclonal, 1:400 (all from Sigma); MAP2ab monoclonal, 1:300 and GFAP monoclonal, 1:1000 (both Pharmingen, San Diego, CA); GalC monoclonal, 1:750; GFAP polyclonal, 1:1000; nestin polyclonal, 1:500 (all from Chemicon International, Temecula, USA). Fluorescence labeled secondary antibodies (Jackson, West Grove, PA) were then applied. For visualizing osteoblasts, the cultures were analyzed by enzymatic testing for alkaline phosphatase (AP) activity according to standard protocols using an AP staining kit (Sigma) (Fiedler et al., 2002).

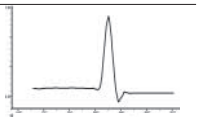
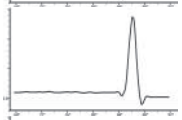
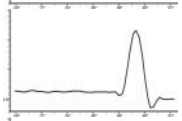
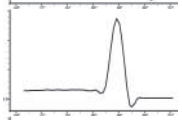
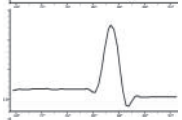
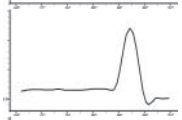
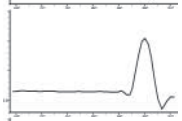
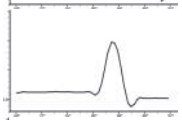
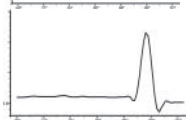
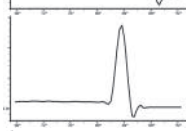
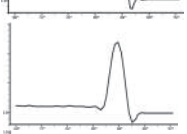
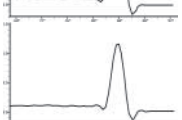
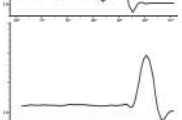
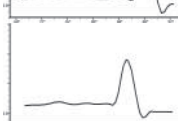
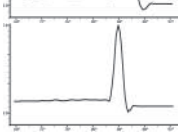
RNA extraction, RT-PCR and quantitative real-time RT-PCR analysis

Total cellular RNA was extracted from hMSCs, hmNSCs and terminally differentiated hmNSCs (neuronal induction medium) using RNAeasy total RNA purification kit followed by treatment with RNase-free DNase (Qiagen, Hilden, Germany). RT-PCR reactions were performed essentially as described previously (Fiedler et al., 2002). Primer sequences (forward, reverse) and lengths of the amplified products were as follows: AP (5′-ACC TCG TTG ACA CCT GGA AG-3′, 5′-CCA CCA TCT CGG AGA GTG AC-3′, 189); c-fos (5′-AGC TCT GTG GCC ATG GGC CCC-3′, 5′-AGA CAG ACC AAC TAG AAG ATG A-3′, 457); GAPDH (5′-CGG AGT CAA CGG ATT TGG TCG TAT-3′, 5′-AGC CTT CTC CAT GGT TGG TGA AGA C-3′, 188); RUNX2 (5′-TAC CAG ACC GAG ACC AAC AGA G-3′, 5′-CAC CAC CGG GTC ACG TCG C-3′, 239); SOX9 (5′-CTA CGA CTG GAC GCT GGT GC- 3′, 5′-CGA TGT CCA CGT CGC GGA AG-3′, 234). Quantitative real-time one step RT-PCR was carried out using the LightCycler® System (Roche, Mannheim, Germany), and amplification was monitored and analyzed by measuring the binding of the fluorescent dye SYBR Green I to double-stranded DNA. 1 μl total RNA was reverse transcribed and subsequently amplified using QuantiTect SYBR Green RT-PCR Master mix (Qiagen) and 0.5 μM each of the sense and antisense primers. Tenfold dilutions of total RNA were used as external standards. Standards and samples were simultaneously amplified. After amplification, melting curves of the RT-PCR products were acquired to demonstrate product specificity. Results are expressed relative to the housekeeping gene *HMBS* (hydroxymethylbilane synthase). Primer sequences, lengths of the amplified products and melting analyses are summarized in Table 1.

Electrophysiology

Cells were investigated for membrane currents between days 10 and 20 after differentiation using the standard whole-cell patch-clamp technique with an EPC-7 amplifier (List Electronics, Heidelberg, Germany) and pClamp data acquisition (Axon Instruments) as

Table 1. Primers used for quantitative real-time RT-PCR

Gene (Protein)	Sequence (forward; reverse)	Product length (bp)	Melting point analysis*
FN1 (Fibronectin)	5′-GAG ATC AGT GGG ATA AGC AGC A-3′ 5′-CCT CTT CAT GAC GCT TGT GGA-3′	150	
GFAP (GFAP)	5′-GAG GCG GCC AGT TAT CAG GA-3′ 5′-GTT CTC CTC GCC CTC TAG CA-3′	168	
HMBS (Hydroxymethylbilane synthase)	5′-TCG GGG AAA CCT CAA CAC C-3′ 5′-CCT GGC CCA CAG CAT ACA T-3′	155	
MBP (Myelin basic protein)	5′-CGG CAA GAA CTG CTC ACT ATG-3′ 5′-GTG CGA GGC GTC ACA ATG–3′	106	
MSI1 (Musashi 1)	5′-GCC CAA GAT GGT GAC TCG-3′ 5′-ATG GCG TCG TCC ACC TTC-3′	114	
NeuroD1 (Neurogenic differentiation 1)	5′-CGC TGG AGC CCT TCT TTG-3′ 5′-GCG GAC GGT TCG TGT TTG-3′	118	
Neurog2 (Neurogenin 2)	5′-CGC ATC AAG AAG ACC CGT AG-3′ 5′-GTG AGT GCC CAG ATG TAG TTG TG-3′	173	
NES (Nestin)	5′-TGG CTC AGA GGA AGA GTC TGA-3′ 5′-TCC CCC ATT TAC ATG CTG TGA-3′	148	
NTRK1 (Neurotrophic tyrosine kinase, receptor, type 1)	5′-CTA CAG CAC CGA CTA TTA CCG–3′ 5′-CGA TTG CCT CCG TGT TG–3′	128	
OCT-4 (Octomer-binding transcription factor 4)	5′-GTA TTC AGC CAA ACG ACC ATC-3′ 5′-CTG GTT CGC TTT CTC TTT CG-3′	176	
OTX1 (Orthodenticle homolog 1)	5′-CAC TAA CTG GCG TGT TTC TGC-3′ 5′-AGG CGT GGA GCA AAA TCG-3′	121	
SNCA (α-Synuclein)	5′-AGG ACT TTC AAA GGC CAA GG-3′ 5′-TCC TCC AAC ATT TGT CAC TTG C-3′	187	
SOX1 (Sex determining region Y-box 1)	5′-GCC CAG GAG AAC CCC AAG-3′ 5′-CGT CTT GGT CTT GCG GC-3′	177	
TH (Tyrosine hydroxylase)	5′-AGC TCC TGA GCT TGT CCT TG-3′ 5′-TGT CCA CGC TGT ACT GGT TC-3′	142	
TUBB4/III (β-Tubulin III)	5′-AGT GAT GAG CAT GGC ATC GA-3′ 5′-AGG CAG TCG CAG TTT TCA CA-3′	317	

*x-axis represents temperature (from 68-92°C in 4°C steps), *y*-axis indicates fluorescence [d(F1)dT].

described previously (Labarca et al., 2001; Storch et al., 2003). We added 10% FCS 3-5 days prior to patch-clamp experiments for better sealing. Extracellular solution contained 100 mM NaCl, 54 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM D-Glucose. The pipette solution was 130 mM KCl, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES, 3 mM Na-ATP. Using these solutions, borosilicate pipettes had resistances of 3-4 MΩ. Data were analyzed using pClamp 8.0, Microsoft Excel 97 and Origin 5.0 software.

Reverse-phase HPLC with electrochemical detection

For determination of dopamine production and release, media were supplemented with 100 μM tetrahydrobiopterin and 200 μM ascorbate 3 days prior to medium harvest. Dopamine levels were determined in medium and extracellular buffer stabilized with EGTA/glutathione solution as reported previously (Storch et al., 2001), and stored at –80°C until analysis. Aluminum absorption and HPLC analysis of dopamine have been described (Storch et al., 2001).

Cell counting and statistics

For quantification of the percentage of cells producing a given marker, in any given experiments the number of positive cells of the whole well surface was determined relative to the total number of DAPI-labeled nuclei. In a typical experiment, a total of 500-1000 cells were counted per marker. Statistical comparisons were made by Dunnett's *t*-test. If data were not normally distributed, a non-parametric test (Mann-Whitney U-test) was used for comparisons of results. All data are expressed as mean±s.e.m.

Results

Characterization of undifferentiated hMSCs

Bright staining was seen with antibodies against the mesodermal marker fibronectin in all cells (Fig. 1A), but only 4±3% (n=3) of cells were slightly positive for nestin (Fig. 1A). No staining was observed against markers of mature neuroectodermal cell types, such as MAP2ab, TH, GFAP and GalC (data not shown). Flow cytometry revealed that hMSC were $CD9^{+/low}$, $CD15^{-}$, $CD34^{-}$, $CD45^{-}$, $CD90^{+}$, $CD166^{+}$, $CD133^{-}$ (Fig. 2). In quantitative RT-PCR, hMSCs expressed high levels of FN1, SOX1 and NTRK1, significant levels of OCT-4, but very low levels of Neurog2, NES, GFAP and TUBB4/III. hMSCs did not significantly express MSl1, OTX1, NeuroD1, MBP, SNCA and TH (Fig. 3; for complete names of genes and encoded proteins, refer to Table 1). hMSCs proliferated for at least 10 weeks without changing their morphology and phenotype (doubling time: 1.5 days; ≈50 population doublings).

Culture conditions for conversion of hMSCs into neuroprogenitor-like cells

To convert adult hMSCs into cells with characteristics of NSCs, we detached hMSCs after 2-10 passages (≈10-50 population doublings) and cultured them in uncoated flasks in serum-free P4-8F medium supplemented with EGF and FGF-

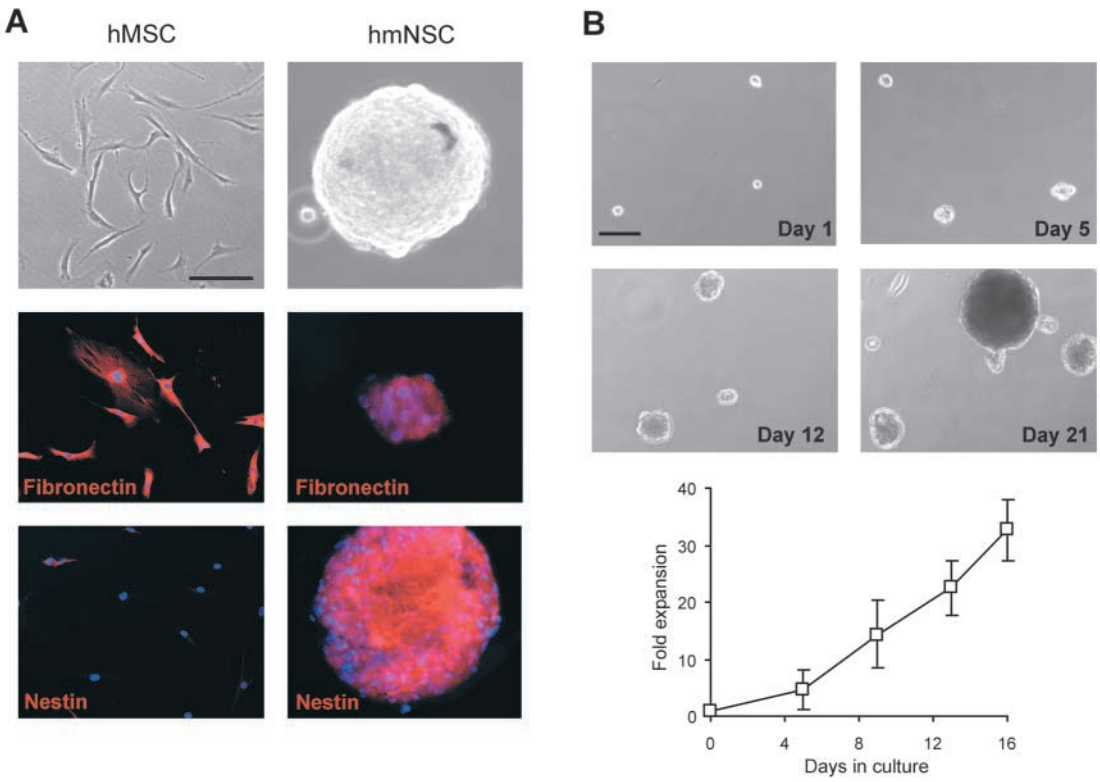


Fig. 1. Characteristics of adult human mesodermal stromal cells (hMSC) and human marrow-derived neural stem cell-like cells (hmNSC) during expansion. (A) Morphology, fibronectin and nestin expression of hMSC (left panels) and hmNSC (right panels). All hMSCs express high levels of fibronectin, but only 4±3% of cells express very low levels of the NSC marker nestin, whereas some hmNSCs express low levels of fibronectin, but nearly all cells express high levels of nestin. Nuclei are counterstained with DAPI (blue). Bar, 100 μm. (B) Representative sequence of phase-contrast photomicrographs of hmNSCs 1, 5, 12 and 21 days after conversion from hMSCs. A growth curve of hmNSCs revealed by enumerating the cells at each time point under a hemocytometer is also shown (n=5; calculated doubling time 2.6 days). Bar, 50 μm.

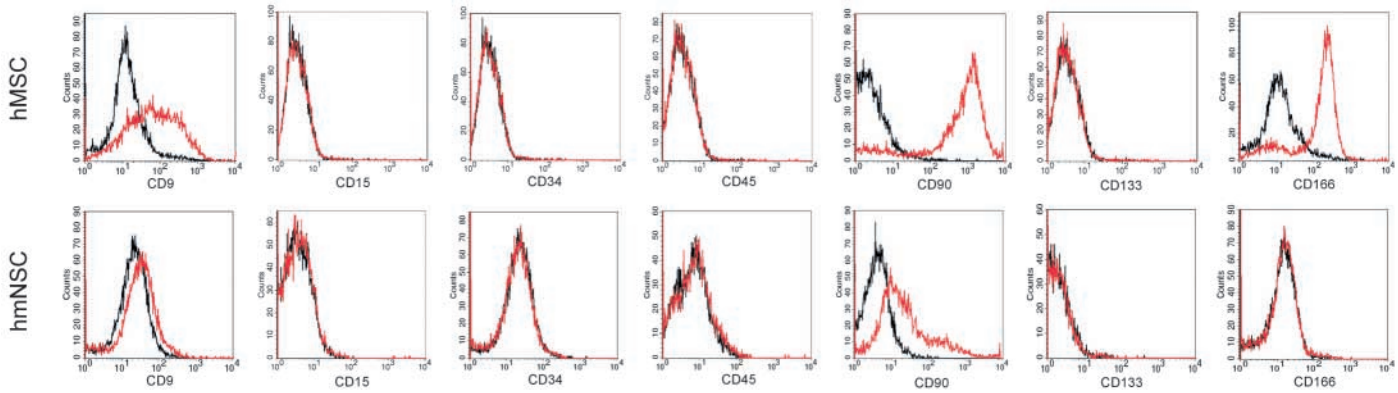


Fig. 2. Flow cytometry of hMSCs (upper panels) and hmNSCs (lower panels) cultured for 10-50 and 5-30 population doublings, respectively. Cells were labeled with fluorescence-coupled antibodies against CD9, CD15, CD34, CD45, CD90, CD133, CD166 or immunoglobulin isotype control antibodies. Cells were analyzed using a FACSCalibur flow cytometer. Black line, control immunoglobulin; red line, specific antibody.

2. The cells did not adhere to the surface of tissue culture flasks. One third (37±17%, *n*=5) of hMSCs died after 3 days, but after 10-14 days, the remaining cells formed small spheres of floating cells (Fig. 1A,B). hmNSCs proliferated with an estimated doubling time of 2.6 days in vitro for at least up to 10 weeks (≈5-30 population doublings; Fig. 1B) without changing morphology or phenotype. Immunocytochemistry showed that 87±2% of hmNSCs expressed high levels of nestin, but only a few cells expressed low levels of fibronectin (Fig. 1A). No staining was seen using antibodies against MAP2ab, GFAP and GalC. The phenotype of these hmNSCs was CD15, CD34, CD45, CD133 and CD166 negative; hmNSCs expressed low levels of CD9, but higher levels of CD90 (Fig. 2). Notably, CD133 expression was only detected on a small hmNSC subset (<1%), consistent with previous reports on neural progenitors (Uchida et al., 2000; Vogel et al., 2003). Quantitative analysis of *FN1* mRNA showed similar levels in hmNSCs compared to those in hMSCs, but significantly decreased levels of *SOX1*, *OCT-4* and *NTRK1* (Fig. 3). On the other hand, quantitative RT-PCR of hmNSC demonstrated acquisition of neuroectodermal transcripts. In hmNSC, mRNA encoding for otx1, neuroD1, neurogenin2, musashi1 and nestin could be detected at levels between 4.5- and 77.1-fold those seen in hMSCs (Fig. 3B). Similar to hMSCs and as expected for NSCs, the mRNA levels for marker genes of mature neural cell types, such as *GFAP*, *MBP*, *TUBB4/III*, *SNCA* and *TH*, were undetectable or very low (Fig. 3B).

Neuronal differentiation of hmNSCs

For terminal differentiation in vitro, we used a neuronal and a glial induction protocol, by plating the cells onto poly-L-lysine and adding cytokines and growth factors known to induce differentiation of NSCs into mature neuronal or glial cells. Differentiation into neuronal cells required plating of hmNSCs in medium without EGF and FGF-2, but with brain-derived neurotrophic factor (BDNF) and retinoic acid (neuronal induction medium). Ten to fourteen days after plating, quantitative mRNA analysis revealed that the expression of *FN1* as well as the proneural genes *SOX1*, *OTX1*, *NeuroD1* and *Neurog2* was down-regulated during terminal neuronal differentiation of hmNSCs (Fig. 3B). In contrast, marker mRNA levels of mature neural cell types (*GFAP*, *MBP*, *TUBB4/III*, *SNCA* and *TH*) were significantly increased (Fig. 3B). No changes were seen in the expression level of *OCT-4* and *NTRK1*. Consistently, we obtained 42±6% of hmNSCs with early neuronal characteristics (β-tubulin III expression) and 6±2% expressing the marker molecule for mature neurons (MAP2ab) but only 13±4% of cells with GFAP expression and astroglial morphology (Fig. 4A,B). MAP2ab and GFAP were never found in the same cell. Interestingly, in these cultures 11±7% of cells expressed the catecholaminergic marker TH (*n*=3), which is the rate-limiting enzyme for dopamine synthesis. To test whether these TH-positive cells displayed the ability to synthesize dopamine and release it in response to membrane depolarization, media of hMSC and hmNSCs were harvested before and after terminal differentiation and assayed by HPLC. Dopamine was not detectable in the media of hMSCs and undifferentiated hmNSCs with or without KCl depolarization (data not shown). In contrast, when media of in vitro differentiated hmNSCs (day 14) were assayed, total dopamine concentrations were 108±83 pg/ml (Fig. 5A,B). Following these results dopamine release following membrane depolarisation was measured. After treatment of differentiated hmNSCs with 56 mM KCl, the released dopamine in the media was 709±269 pg/ml (Fig. 5A,B). Differentiation of hMSCs using the neuronal induction protocol produced neither mature MAP2ab$^+$ neurons nor TH$^+$/dopamine producing cells (*n*=5), and did not lead to an up-regulation of *TUBB4/III* or *GFAP* gene expression (data not shown). Patch-clamp experiments to demonstrate neuronal characteristics in these cells were not successful as addition of 10% FCS was necessary to obtain sufficiently high seal resistances, but this led to an extensive overgrowth of glial cells.

Glial differentiation of hmNSCs

Using the glial induction medium containing platelet-derived growth factor (PDGF-BB) and retinoic acid for 10-14 days, 45±4% of hmNSCs acquired morphologic and phenotypic characteristics of astrocytes (GFAP$^+$), 27±5% acquired

characteristics of oligodendrocytes [galactocerebrosidase (GalC)$^+$], whereas only a few cells exhibited the early neuronal marker class III β-tubulin (Fig. 4B). There were no relevant morphological differences between cells differentiated with the neuronal and the glial induction protocol, respectively. No mature MAP2ab$^+$ neurons could be detected. Electrophysiological analysis of hmNSCs, that were differentiated using the glial induction protocol, revealed that about 40% of cells (19 out of 47 recorded cells) showed a sustained outward current of a few hundred pA up to 6 nA.

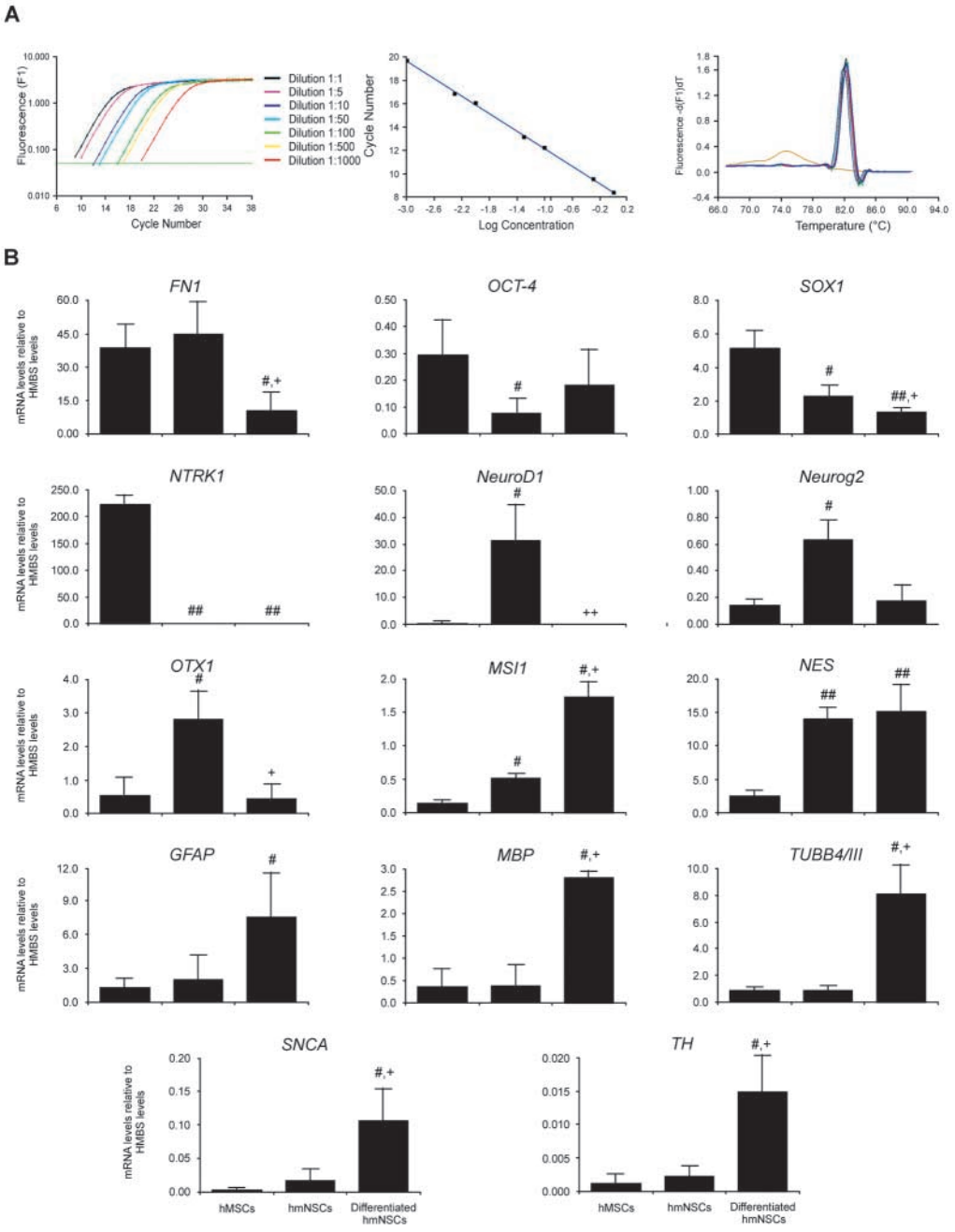


Fig. 3. Quantitative transcription profile of hMSC, hmNSCs and terminal differentiated hmNSCs into glial and neuronal cell types. (A) Representative real-time RT-PCR analysis using the LightCycler® technique. Plot of the fluorescence versus the cycle number obtained from SYBR Green detection of serially diluted *FN1* mRNA (encoding fibronectin) (left). The horizontal line represents the position of the threshold. The standard curve obtained by plotting cycle number of crossing points versus dilution factor is shown (center), in addition to melting curve analysis showing the specificity of the amplified PCR product (right). (B) Quantitative real-time RT-PCR analyses of mesodermal genes (*FN1*), proneural genes (*SOX1, OTX1, NeuroD1, Neurog2, MSl1*), NSC marker genes (*NES*), glial genes (*GFAP, MBP*) and neuronal genes (*TUBB4/III, SNCA, NTRK1, TH*) as well as *OCT-4* as a marker for pluripotency in hMSC, hmNSCs and differentiated hmNSCs, respectively, by the neuronal induction protocol for 14 days. Expression levels are expressed relative to the housekeeping gene *HMBS* (hydroxymethylbilane synthase). For primers, complete names of genes and melting curve analyses demonstrating the specificity of amplified PCR products see Table 1. #, $P<0.05$; ##, $P<0.01$ when compared to mRNA levels in hMSCs; +, $P<0.05$; ++, $P<0.01$ when compared to mRNA levels in hmNSCs.

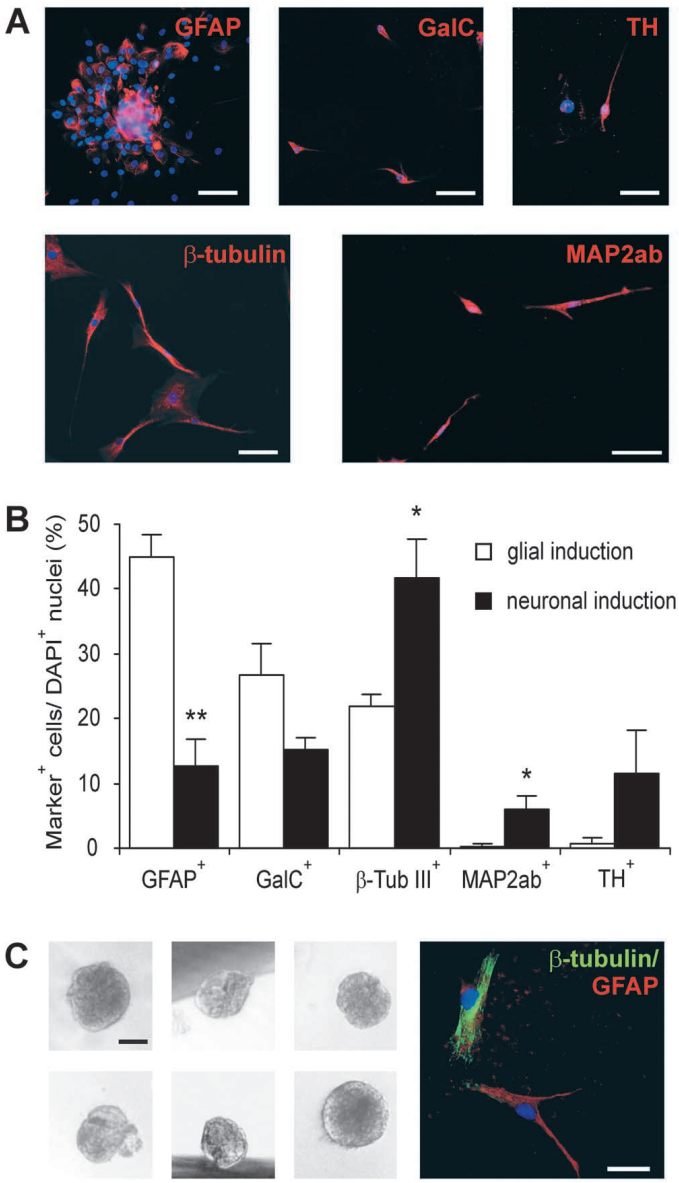


Fig. 4. In vitro differentiation of marrow-derived neurosphere-like structures into astroglial, oligodendroglial and neuronal cell types. hmNSCs were differentiated after 5-30 population doublings using the glial induction or the neuronal induction protocol on poly-L-lysine coated coverslips for 14 days. (A) hmNSCs differentiated using the neuronal induction medium were stained for markers for astrocytes (GFAP), oligodendrocytes (GalC), neurons (β-tubulin-III, MAP2ab), or catecholaminergic cells (TH). Nuclei are counterstained with DAPI (blue). Bars, 50 μm. (B) Quantification of 14-day cultures of hmNSCs differentiated with the glial and the neuronal induction protocols. Data shown are mean±s.e.m. from at least three independent hMSC preparations. *, $P<0.05$; **, $P<0.01$ when compared to the percentage of positive cells in the glial induction protocol. (C) Clonal expansion from single hmNSCs. Six representative photomicrographs of neurosphere-like structures derived from single hmNSCs (left). Bar, 50 μm. Differentiation capacity of clonally derived neurosphere-like structures (right panel). Progeny of single cell-derived neurosphere-like structures can be differentiated into neurons (β-tubulin III$^+$, green) and astrocytes (GFAP$^+$, red). Nuclei are counterstained with DAPI (blue). Bar, 30 μm.

These currents showed a voltage-dependence and kinetics characteristic for delayed rectifier K^+ channels (Fig. 5C). In a few cells, we could identify small inward currents with voltage dependence and kinetics typical for voltage-activated Na^+ channels (Fig. 5D).

hmNSCs are clonogenic multipotent progenitor cells

To determine whether individual hmNSCs could generate both neurons and glia, juvenile sphere-like structures were dissociated and individual cells were isolated by limiting dilution into ultra low attachment multi-well plates; single cells were cultured in hmNSC expansion medium for 3 to 6 weeks. After this period 11% of the single cells proliferated to generate neurosphere-like structures (Fig. 4C). Clonal cells were then differentiated for 10 days. Double immunostaining revealed that differentiation capacities were very variable between cell clones with 40±39% (range: 0 to 100%) of individual cells acquiring morphologic and phenotypic characteristics of astrocytes (GFAP$^+$) and 26±27% (0-60%) characteristics of neurons (β-tubulin III$^+$) (Fig. 4C); 28% of the clones showed only one of the investigated cell types.

hmNSC lose their capacity to differentiate into mesodermal lineages

To determine whether hmNSCs were still able to differentiate into mesenchymal cell types in vitro, we compared the ability of hMSCs and hmNSCs to differentiate into osteoblasts using standard protocols and high density cultures (Fiedler et al., 2002; Reyes et al., 2001). After 14 days, 57±5% of hMSCs acquired morphologic and phenotypic characteristics of osteoblasts, whereas only 17±5% of hmNSCs expressed alkaline phosphatase (AP)$^+$ without displaying typical morphology of osteoblasts (Fig. 6A,B; $n=3$; $P<0.01$, t-test). Consistently, expression levels of AP and runt-related transcription factor 2 (*RUNX2*) as osteogenic markers, *SOX9* as a mesodermal (chrondogenic/osteogenic) marker (Healy et al., 1999) and the transcription factor c-fos were very low or absent in osteogenic differentiated hmNSCs (Fig. 6C).

Discussion

The central finding of this study is that cells with major characteristics of NSCs can be efficiently generated from human MSCs obtained from adult bone marrow, in a similar way as NSCs are derived from ES cells (Bain et al., 1995; Lee et al., 2000). These bone marrow-derived NSCs were cultured in neurosphere-like structures using a technique similar to those used for isolation and propagation of NSCs (Carvey et al., 2001; Cattaneo and McKay, 1990; Johansson et al., 1999; Reynolds and Weiss, 1992; Storch et al., 2001; Storch and Schwarz, 2002; Uchida et al., 2000). The phenotype of these cells is similar to that of human neural progenitor cells derived from fetal forebrain (Uchida et al., 2000; Vogel et al., 2003), but different from that of hMSC (Fickert et al., 2003; Fiedler et al., 2002; Vogel et al., 2003). Our hmNSCs are clonogenic, self-renewing cells that maintain the capacity to differentiate into mature functional brain-specific cell types, while losing mesodermal characteristics. The differentiation capacity of hmNSCs is similar to that of hNSCs derived from fetal or adult

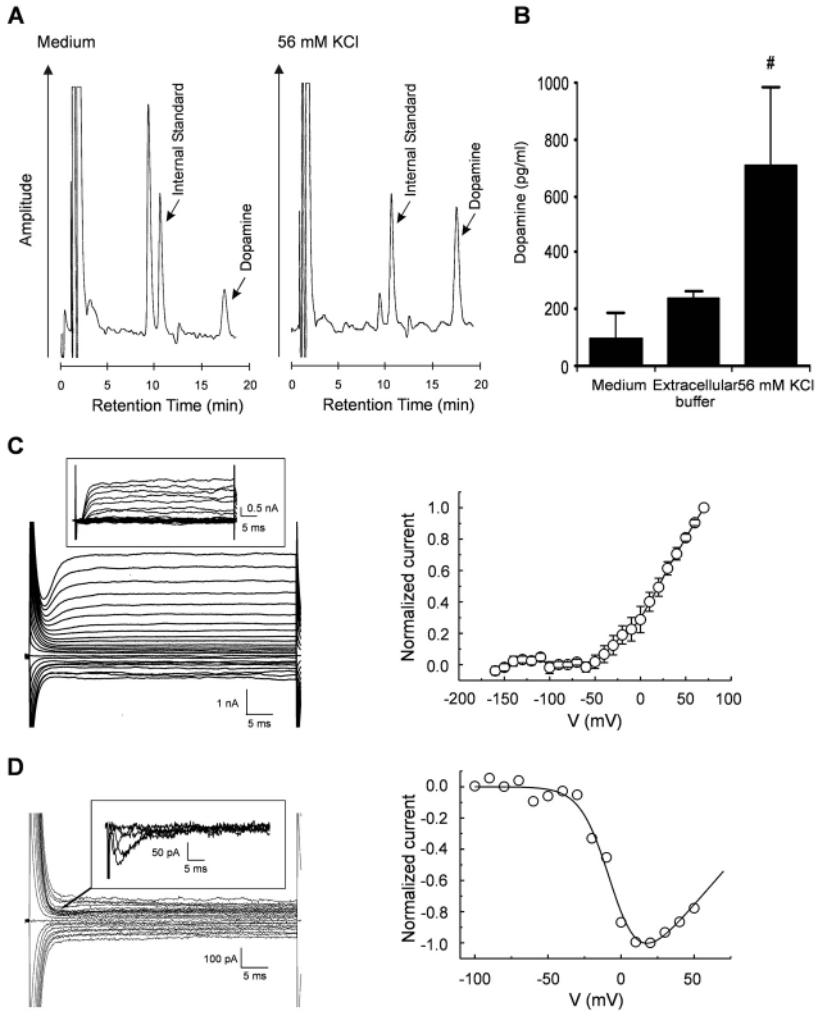


Fig. 5. Functional properties of terminal differentiated hmNSC. (A-B) Dopamine production and release was measured in hmNSCs differentiated using the neuronal induction protocol for 14 days. (A) Representative chromatograms of HPLC-ECD determination of dopamine in medium conditioned for 3 days (left) and extracellular buffer with 56 mM KCl conditioned for 45 minutes (right). (B) Quantification of dopamine in medium conditioned for 3 days (left), in extracellular buffer conditioned for 45 minutes (center), and in extracellular buffer with 56 mM KCl conditioned for 45 minutes (right). # , $P<0.05$ when compared to extracellular buffer dopamine levels (paired *t*-test). (C,D) Electrophysiological recordings on hmNSCs differentiated using the glial induction protocol. For voltage-clamp measurements, cells were held at –80 mV and hyperpolarized or depolarized in 10 mV steps between –160 and +70 mV. (C) Example of a sustained outward current shown without and with (inset) leak subtraction using a –P/4 protocol (left). Current-voltage relationship of the normalized outward currents recorded with leak subtraction (n=7, right panel). (D) Example of an inward current without and with leak subtraction (inset: only currents for depolarizing steps to –40, –20, 0, 20 and 60 mV are shown) (left). Peak current-voltage relationship for the same cell with leak subtraction (right panel). The line represents a fit to the following equation: $I(V)/I_{max}= g(V–V_{rev})/1+\exp[(V–V_{0.5})/k_V]$, where I (I_{max}) is the (maximum) membrane current; g, the maximum conductance; V, the applied voltage; V_{rev}, the reversal potential for Na^+; $V_{0.5}$, the potential of half-maximal activation and k_V, a slope factor.

brain (Fricker et al., 1999; Johansson et al., 1999; Storch et al., 2001; Uchida et al., 2000; Westerlund et al., 2003).

Oct-4, a member of the POU family of transcription factors, is characteristically expressed in embryonic cells and necessary for their pluripotency (Pesce and Scholer, 2000). Recently, Pochampally and co-workers reported the expression of the *OCT-4* gene in hMSCs with higher expression in cells cultured in serum-free medium (Pochampally et al., 2004). We also demonstrated Oct-4 expression in our initial hMSC population suggesting that these cultures contain pluripotent progenitors, which might be related to pluripotent MAPCs (Jiang et al., 2003; Jiang et al., 2002a). Consistent with the more restricted (neuroectodermal) differentiation capacity of hmNSCs the expression level of Oct-4 was reduced after the conversion of hMSCs into hmNSCs. A large body of knowledge exists on lineage determination in NSCs, because unlike many types of stem cells, NSCs undergo self-renewal and multilineage differentiation in culture. SOXB1 transcription factors, including the three closely related genes *SOX1*, *SOX2* and *SOX3*, universally mark neural progenitor and stem cells throughout the vertebrate CNS (Bylund et al., 2003; Pevny et al., 1998; Uwanogho et al., 1995). Recent data further suggest that Sox1-3 signaling is both necessary and sufficient to maintain pan-neural properties of undifferentiated NSCs and therefore generation of neuronal cells from NSCs depends on the inhibition of Sox1-3 expression (Bylund et al., 2003; Graham et al., 2003). Consistently, in undifferentiated hmNSCs we detected significant *SOX1* gene expression with decreasing mRNA levels during terminal differentiation of hmNSCs. The highest level of *SOX1* gene expression was found in hMSCs, suggesting a multipotency of this progenitor cell type including a neuroectodermal potential. These data are in line with results by Woodbury and co-workers showing expression of some early and late neuroectodermal genes in hMSCs (Woodbury et al., 2002).

Proneural genes, which encode transcription factors of the basic helix-loop-helix (bHLH) class such as neuroD1 and neurogenin2, are both necessary and sufficient to initiate the development of neural lineages and to promote the generation of progenitors that are committed to neural differentiation (Bertrand et al., 2002; Bylund et al., 2003; Graham et al., 2003). Proneural genes are mostly expressed in neuroprogenitor cells including NSCs in early neuroectodermal tissues (Franklin et al., 2001; Nieto et al., 2001; Simmons et al., 2001). The exact mechanisms of action of proneural genes are only partially understood, but one major effect is the inhibition of Sox1-3 expression (Bylund et al., 2003; Graham et al., 2003). In hMSCs with high Sox1

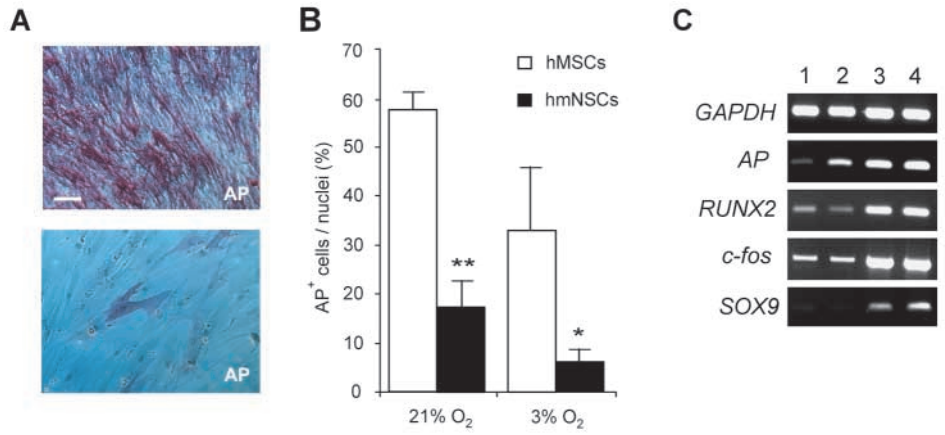


Fig. 6. Osteogenic differentiation ability of both hMSCs and hmNSCs. Both cell types were differentiated after 6-10 passages using the osteoblast differentiation protocol for 10 days. (A) Differentiated hMSCs (upper panel) and hmNSCs (lower panel) were stained for the osteogenic marker alkaline phosphatase (AP). Bar, 50 μm. (B) Quantification of 10-day cultures of hMSCs and hmNSCs differentiated into osteoblasts under normal atmospheric oxygen levels routinely used for osteoblast cultures (21%) and reduced atmospheric oxygen levels used in our neural culture system (3%). Data shown are mean±s.e.m. from at least three independent cell preparations. *, $P<0.05$; **, $P<0.01$ when compared to the percentage of AP^+ cells in hMSCs. (C) Expression of osteogenic and mesodermal marker genes as well as the transcription factor c-fos in hMSCs and hmNSCs after osteogenic differentiation under 21% (lanes 1, 4) and 3% (lanes 2, 3) atmospheric oxygen levels. Semiquantitative RT-PCR analysis of *AP*, runt-related transcription factor 2 (*RUNX2*), *SOX9* and the transcription factor *c-fos*, as well as *GAPDH* (housekeeping gene) was performed in hmNSC (lanes 1 and 2) and hMSC (lanes 3 and 4).

expression, the levels of proneural genes are absent or very low, but conversion into hmNSC with lower levels of Sox1 leads to significant up-regulation of the expression of all investigated proneural genes. Terminal differentiation of hmNSCs resulted in down-regulation of proneural genes leading to very low or absent expression levels.

The *Otx* homeobox gene *OTX1* is widely and exclusively expressed at early stages of neuroectoderm differentiation (Acampora et al., 2001; Simeone, 1998). In contrast to hMSCs, hmNSCs express significant levels of *OTX1*, which nearly disappear after terminal differentiation of these cells into neuronal/glial cells. The RNA-binding protein musashi1 is involved in post-transcriptional gene regulation in neuroprogenitors and/or NSCs and is used as a marker for those cell types (Kaneko et al., 2000). Consistently, musashi1 is not expressed in hMSCs, but up-regulated during the conversion of hMSCs into hmNSCs. NSCs can be further defined by the presence of the intermediate filament protein nestin (Cattaneo and McKay, 1990; Dahlstrand et al., 1995; Lendahl et al., 1990), which is also found in young neurons, reactive glial cells and ependymal cells (Johansson et al., 1999). Taken together, after the conversion, hmNSCs acquire a specific expression pattern of early neuroectodermal and/or NSC marker genes, such as SOXB1 transcription factors, proneural genes and nestin. However, it is unclear from the present experiments whether reprogramming of a committed mesenchymal stem cell (transdifferentiation) or proliferation and differentiation of a more pluripotent stem cell already harbored in the hMSC suspension is responsible for the cell type conversion in our system (Jiang et al., 2002a; Sanchez-Ramos, 2002).

Further evidence for the stem cell-like nature of hmNSCs within the adult marrow-derived neurosphere-like aggregates is the demonstration of clonogenicity of individual cells in the limiting dilution assay. In the presence of growth factors/cytokines known to induce glial/neuronal differentiation in brain-derived NSCs, hmNSCs differentiate in vitro into all major cell types of the CNS, including mature neurons expressing MAP2ab. Unfortunately, we have not yet characterized these neurons electrophysiologically. Electrophysiological characterization of neurons derived from human progenitor cells have only been reported in two studies (Carpenter et al., 2001; Westerlund et al., 2003). However, Jiang and co-workers showed functional characteristics of neurons (generation of action potentials, expression of sodium channels) in neurons derived from MAPCs by using co-cultures with primary astrocytes (Jiang et al., 2003). These differentiated mouse multipotent adult progenitor cells also show a morphological phenotype with expression of TH, dopamine and dopa-decarboxylase, like dopaminergic neurons in vitro (Jiang et al., 2003; Jiang et al., 2002a). Jin and colleagues showed by immunocytochemical techniques that murine bone marrow cells can be induced to synthesize the neurotransmitter GABA using similar differentiating factors as we used in the present study (Jin et al., 2003). However, no depolarization-dependent release of GABA was shown. Our differentiated hmNSCs also express TH at both the mRNA and protein levels, but also produce and release dopamine similar to functional dopaminergic neurons (Lee et al., 2000; Storch et al., 2001). Using the glial induction protocol and FCS to differentiate hmNSCs, we were able to demonstrate electrophysiological properties typical for developing and adult glial cells (Bordey and Sontheimer, 2000; Gritti et al., 2000; Kressin et al., 1995). Consistent with the neuroectodermal nature of hmNSCs, their mesodermal/osteogenic differentiation potential is nearly absent. In summary the differentiation capacity of hmNSCs seems similar to that of NSCs derived from fetal and adult brain of various species (Carvey et al., 2001; Cattaneo and McKay, 1990; Kilpatrick and Bartlett, 1993; Reynolds and Weiss, 1992; Song et al.,

2002; Uchida et al., 2000). Furthermore, we showed for the first time, functional properties of neuronal/glial cells generated from human MSCs.

In a typical experiment, for every 1×10^6 hMSCs, we routinely obtained 1.6×10^8 marrow-derived NSCs, 0.1×10^8 neurons, 0.7×10^8 astroglial and 0.4×10^8 oligodendroglial cells. Considering all parameters of proliferation, cell death and selective differentiation throughout the multiple conversion and differentiation steps, at least 7×10^7 hmNSCs, or 0.5×10^7 neurons, 3×10^7 astroglial and 2×10^7 oligodendroglial cells, respectively, could numerically be produced from one hMSC harvested from the bone marrow sample. These cell quantities are sufficient for most transplantation protocols as well as for extensive characterizations of adult NSCs by molecular biology or protein biochemistry, such as gene array analysis or proteomics. Thus, hmNSCs could facilitate understanding the molecular mechanisms of neural differentiation in adult human NSCs. In contrast to most previous protocols for the conversion of MSCs into neuroectodermal cells generating mature neuronal and/or glial cells attached to the culture surface (Hofstetter et al., 2002; Jiang et al., 2003; Jiang et al., 2002a; Kim et al., 2002a; Sanchez-Ramos et al., 2000; Woodbury et al., 2000), we demonstrate here the possibility to generate undifferentiated NSCs or premature neural cells from human origin. Those are commonly used and more suitable for neurotransplantation compared to fully differentiated neural cells (Bjorklund and Lindvall, 2000; Carvey et al., 2001; Kim et al., 2002b; Pluchino et al., 2003), as differentiated neuronal cells are well known to poorly survive detachment and subsequent transplantation procedures. Although studies in animal models of neurodegenerative diseases are needed to assess the function and safety of hmNSC-derived neural cells in vivo further, our data show that hmNSC differentiate into mature neural cells showing functional properties, such as dopamine production and potassium-dependent release after neuronal differentiation as well as expression of outward-rectifying potassium channels and sodium channels in glial cells. However, future experiments are warranted to define further functional properties of terminal differentiated hmNSCs into neuronal and glial cells by using co-culture methods with astroglial cells (Jiang et al., 2003; Song et al., 2002) and longer periods of differentiation time to achieve more mature neurons and glial cells (Westerlund et al., 2003). Our method provides the means to study autologous approaches in neurotransplantation using adult human NSCs derived from bone marrow, an accessible tissue in every individual.

We would like to thank Thomas Lenk and Giovanni Ravalli for their excellent technical assistance and Hans-Jörg Habisch for fruitful discussions. This work was supported in part by the Interdisziplinäres Zentrum für klinische Forschung (IZKF) Ulm (Project D6) to A.S., the BMBF (Polish-German Cooperation in Neuroscience Program) to A.S., and the Landesstiftung Baden-Württemberg (Förderprogramm 'Adulte Stammzellen' to A.S. und R.B.). A.H. was supported by an IZKF fellowship as member of the graduate college GRK460, Ulm.

References

Acampora, D., Gulisano, M., Broccoli, V. and Simeone, A. (2001). Otx genes in brain morphogenesis. *Prog. Neurobiol.* **64**, 69-95.

Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G. and Isner, J. M. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. *Science* **275**, 964-967.

Bain, G., Kitchens, D., Yao, M., Huettner, J. E. and Gottlieb, D. I. (1995). Embryonic stem cells express neuronal properties in vitro. *Dev. Biol.* **168**, 342-357.

Bertrand, N., Castro, D. S. and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**, 517-530.

Bjorklund, A. and Lindvall, O. (2000). Cell replacement therapies for central nervous system disorders. *Nat. Neurosci.* **3**, 537-544.

Bjornson, C. R., Rietze, R. L., Reynolds, B. A., Magli, M. C. and Vescovi, A. L. (1999). Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. *Science* **283**, 534-537.

Bordey, A. and Sontheimer, H. (2000). Ion channel expression by astrocytes in situ: comparison of different CNS regions. *Glia* **30**, 27-38.

Brazelton, T. R., Rossi, F. M., Keshet, G. I. and Blau, H. M. (2000). From marrow to brain: expression of neuronal phenotypes in adult mice. *Science* **290**, 1775-1779.

Buzanska, L., Machaj, E. K., Zablocka, B., Pojda, Z. and Domanska-Janik, K. (2002). Human cord blood-derived cells attain neuronal and glial features in vitro. *J. Cell Sci.* **115**, 2131-2138.

Bylund, M., Andersson, E., Novitch, B. G. and Muhr, J. (2003). Vertebrate neurogenesis is counteracted by Sox1-3 activity. *Nat. Neurosci.* **6**, 1162-1168.

Carpenter, M. K., Inokuma, M. S., Denham, J., Mujtaba, T., Chiu, C. P. and Rao, M. S. (2001). Enrichment of neurons and neural precursors from human embryonic stem cells. *Exp. Neurol.* **172**, 383-397.

Carvey, P. M., Ling, Z. D., Sortwell, C. E., Pitzer, M. R., McGuire, S. O., Storch, A. and Collier, T. J. (2001). A clonal line of mesencephalic progenitor cells converted to dopamine neurons by hematopoietic cytokines: a source of cells for transplantation in Parkinson's disease. *Exp. Neurol.* **171**, 98-108.

Cattaneo, E. and McKay, R. (1990). Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. *Nature* **347**, 762-765.

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

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

Fickert, S., Fiedler, J. and Brenner, R. E. (2003). Identification, quantification and isolation of mesenchymal progenitor cells from osteoarthritic synovium by fluorescence automated cell sorting. *Osteoarthritis Cartilage* **11**, 790-800.

Fiedler, J., Roderer, G., Gunther, K. P. and Brenner, R. E. (2002). BMP-2, BMP-4, and PDGF-bb stimulate chemotactic migration of primary human mesenchymal progenitor cells. *J. Cell. Biochem.* **87**, 305-312.

Franklin, A., Kao, A., Tapscott, S. and Unis, A. (2001). NeuroD homologue expression during cortical development in the human brain. *J. Child Neurol.* **16**, 849-853.

Fricker, R. A., Carpenter, M. K., Winkler, C., Greco, C., Gates, M. A. and 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.

Friedenstein, A. J., Gorskaja, J. F. and Kulagina, N. N. (1976). Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp. Hematol.* **4**, 267-274.

Graham, V., Khudyakov, J., Ellis, P. and Pevny, L. (2003). SOX2 functions to maintain neural progenitor identity. *Neuron* **39**, 749-765.

Gritti, A., Rosati, B., Lecchi, M., Vescovi, A. L. and Wanke, E. (2000). Excitable properties in astrocytes derived from human embryonic CNS stem cells. *Eur. J. Neurosci.* **12**, 3549-3559.

Gussoni, E., Soneoka, Y., Strickland, C. D., Buzney, E. A., Khan, M. K., Flint, A. F., Kunkel, L. M. and Mulligan, R. C. (1999). Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* **401**, 390-394.

Healy, C., Uwanogho, D. and Sharpe, P. T. (1999). Regulation and role of Sox9 in cartilage formation. *Dev. Dyn.* **215**, 69-78.

Hofstetter, C. P., Schwarz, E. J., Hess, D., Widenfalk, J., El Manira, A., Prockop, D. J. and Olson, L. (2002). Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. *Proc. Natl. Acad. Sci. USA* **99**, 2199-2204.

Jackson, K. A., Mi, T. and Goodell, M. A. (1999). Hematopoietic potential

of stem cells isolated from murine skeletal muscle. *Proc. Natl. Acad. Sci. USA* **96**, 14482-14486.

Jackson, K. A., Majka, S. M., Wang, H., Pocius, J., Hartley, C. J., Majesky, M. W., Entman, M. L., Michael, L. H., Hirschi, K. K. and Goodell, M. A. (2001). Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J. Clin. Invest.* **107**, 1395-1402.

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

Jiang, Y., Vaessen, B., Lenvik, T., Blackstad, M., Reyes, M. and Verfaillie, C. M. (2002b). Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. *Exp. Hematol.* **30**, 896-904.

Jiang, Y., Henderson, D., Blackstad, M., Chen, A., Miller, R. F. and Verfaillie, C. M. (2003). Neuroectodermal differentiation from mouse multipotent adult progenitor cells. *Proc. Natl. Acad. Sci. USA* **100**, 11854-11860.

Jin, K., Mao, X. O., Batteur, S., Sun, Y. and Greenberg, D. A. (2003). Induction of neuronal markers in bone marrow cells: differential effects of growth factors and patterns of intracellular expression. *Exp. Neurol.* **184**, 78-89.

Johansson, C. B., Momma, S., Clarke, D. L., Risling, M., Lendahl, U. and Frisen, J. (1999). Identification of a neural stem cell in the adult mammalian central nervous system. *Cell* **96**, 25-34.

Kaneko, Y., Sakakibara, S., Imai, T., Suzuki, A., Nakamura, Y., Sawamoto, K., Ogawa, Y., Toyama, Y., Miyata, T. and Okano, H. (2000). Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. *Dev. Neurosci.* **22**, 139-153.

Kilpatrick, T. J. and Bartlett, P. F. (1993). Cloning and growth of multipotential neural precursors: requirements for proliferation and differentiation. *Neuron* **10**, 255-265.

Kim, B. J., Seo, J. H., Bubien, J. K. and Oh, Y. S. (2002a). Differentiation of adult bone marrow stem cells into neuroprogenitor cells in vitro. *Neuroreport* **13**, 1185-1188.

Kim, J. H., Auerbach, J. M., Rodriguez-Gomez, J. A., Velasco, I., Gavin, D., Lumelsky, N., Lee, S. H., Nguyen, J., Sanchez-Pernaute, R., Bankiewicz, K. et al. (2002b). Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* **418**, 50-56.

Krause, D. S., Theise, N. D., Collector, M. I., Henegariu, O., Hwang, S., Gardner, R., Neutzel, S. and Sharkis, S. J. (2001). Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* **105**, 369-377.

Kressin, K., Kuprijanova, E., Jabs, R., Seifert, G. and Steinhauser, C. (1995). Developmental regulation of Na+ and K+ conductances in glial cells of mouse hippocampal brain slices. *Glia* **15**, 173-187.

Labarca, C., Schwarz, J., Deshpande, P., Schwarz, S., Nowak, M. W., Fonck, C., Nashmi, R., Kofuji, P., Dang, H., Shi, W. et al. (2001). Point mutant mice with hypersensitive alpha 4 nicotinic receptors show dopaminergic deficits and increased anxiety. *Proc. Natl. Acad. Sci. USA* **98**, 2786-2791.

Lagasse, E., Connors, H., Al-Dhalimy, M., Reitsma, M., Dohse, M., Osborne, L., Wang, X., Finegold, M., Weissman, I. L. and Grompe, M. (2000). Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. *Nat. Med.* **6**, 1229-1234.

Lee, S. H., Lumelsky, N., Studer, L., Auerbach, J. M. and McKay, R. D. (2000). Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nat. Biotechnol.* **18**, 675-679.

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

Lin, Y., Weisdorf, D. J., Solovey, A. and Hebbel, R. P. (2000). Origins of circulating endothelial cells and endothelial outgrowth from blood. *J. Clin. Invest.* **105**, 71-77.

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

Mezey, E., Key, S., Vogelsang, G., Szalayova, I., Lange, G. D. and Crain, B. (2003). Transplanted bone marrow generates new neurons in human brains. *Proc. Natl. Acad. Sci. USA* **100**, 1364-1369.

Nieto, M., Schuurmans, C., Britz, O. and Guillemot, F. (2001). Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. *Neuron* **29**, 401-413.

Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S. M., Li, B., Pickel, J., McKay, R., Nadal-Ginard, B., Bodine, D. M. et al. (2001). Bone marrow cells regenerate infarcted myocardium. *Nature* **410**, 701-705.

Orlic, D., Kajstura, J., Chimenti, S., Bodine, D. M., Leri, A. and Anversa, P. (2003). Bone marrow stem cells regenerate infarcted myocardium. *Pediatr. Transplant.* **7**, 86-88.

Pesce, M. and Scholer, H. R. (2000). Oct-4: control of totipotency and germline determination. *Mol. Reprod. Dev.* **55**, 452-457.

Petersen, B. E., Bowen, W. C., Patrene, K. D., Mars, W. M., Sullivan, A. K., Murase, N., Boggs, S. S., Greenberger, J. S. and Goff, J. P. (1999). Bone marrow as a potential source of hepatic oval cells. *Science* **284**, 1168-1170.

Pevny, L. H., Sockanathan, S., Placzek, M. and Lovell-Badge, R. (1998). A role for SOX1 in neural determination. *Development* **125**, 1967-1978.

Pluchino, S., Quattrini, A., Brambilla, E., Gritti, A., Salani, G., Dina, G., Galli, R., del Carro, U., Amadio, S., Bergami, A. et al. (2003). Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. *Nature* **422**, 688-694.

Pochampally, R. R., Smith, J. R., Ylostalo, J. and Prockop, D. J. (2004). Serum deprivation of human marrow stromal cells (hMSCs) selects for a subpopulation of early progenitor cells with enhanced expression of OCT-4 and other embryonic genes. *Blood* **103**, 1647-1652.

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

Rafii, S., Shapiro, F., Rimarachin, J., Nachman, R. L., Ferris, B., Weksler, B., Moore, M. A. and Asch, A. S. (1994). Isolation and characterization of human bone marrow microvascular endothelial cells: hematopoietic progenitor cell adhesion. *Blood* **84**, 10-19.

Reyes, M., Lund, T., Lenvik, T., Aguiar, D., Koodie, L. and Verfaillie, C. M. (2001). Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. *Blood* **98**, 2615-2625.

Reynolds, B. A. and Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255**, 1707-1710.

Sanchez-Ramos, J. R. (2002). Neural cells derived from adult bone marrow and umbilical cord blood. *J. Neurosci. Res.* **69**, 880-893.

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

Sekiya, I., Larson, B. L., Smith, J. R., Pochampally, R., Cui, J. G. and Prockop, D. J. (2002). Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. *Stem Cells* **20**, 530-541.

Shih, C. C., Weng, Y., Mamelak, A., LeBon, T., Hu, M. C. and Forman, S. J. (2001). Identification of a candidate human neurohematopoietic stem-cell population. *Blood* **98**, 2412-2422.

Shih, C. C., Mamelak, A., LeBon, T. and Forman, S. J. (2002). Hematopoietic potential of neural stem cells. *Nat. Med.* **8**, 535-536.

Simeone, A. (1998). Otx1 and Otx2 in the development and evolution of the mammalian brain. *EMBO J.* **17**, 6790-6798.

Simmons, A. D., Horton, S., Abney, A. L. and Johnson, J. E. (2001). Neurogenin2 expression in ventral and dorsal spinal neural tube progenitor cells is regulated by distinct enhancers. *Dev. Biol.* **229**, 327-339.

Song, H., Stevens, C. F. and Gage, F. H. (2002). Astroglia induce neurogenesis from adult neural stem cells. *Nature* **417**, 39-44.

Storch, A. and Schwarz, J. (2002). Neural stem cells and neurodegeneration. *Curr. Opin. Investig. Drugs* **3**, 774-781.

Storch, A., Paul, G., Csete, M., Boehm, B. O., Carvey, P. M., Kupsch, A. and Schwarz, J. (2001). Long-term proliferation and dopaminergic differentiation of human mesencephalic neural precursor cells. *Exp. Neurol.* **170**, 317-325.

Storch, A., Lester, H. A., Boehm, B. O. and Schwarz, J. (2003). Functional characterization of dopaminergic neurons derived from rodent mesencephalic progenitor cells. *J. Chem. Neuroanat.* **26**, 133-142.

Theise, N. D., Badve, S., Saxena, R., Henegariu, O., Sell, S., Crawford, J. M. and Krause, D. S. (2000). Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. *Hepatology* **31**, 235-240.

Uchida, N., Buck, D. W., He, D., Reitsma, M. J., Masek, M., Phan, T. V., Tsukamoto, A. S., Gage, F. H. and Weissman, I. L. (2000). Direct isolation of human central nervous system stem cells. *Proc. Natl. Acad. Sci. USA* **97**, 14720-14725.

Uwanogho, D., Rex, M., Cartwright, E. J., Pearl, G., Healy, C., Scotting, P. J. and Sharpe, P. T. (1995). Embryonic expression of the chicken Sox2,

Sox3 and Sox11 genes suggests an interactive role in neuronal development. *Mech. Dev.* **49**, 23-36.

Vogel, W., Grunebach, F., Messam, C. A., Kanz, L., Brugger, W. and Buhring, H. J. (2003). Heterogeneity among human bone marrow-derived mesenchymal stem cells and neural progenitor cells. *Haematologica* **88**, 126-133.

Westerlund, U., Moe, M. C., Varghese, M., Berg-Johnsen, J., Ohlsson, M., Langmoen, I. A. and Svensson, M. (2003). Stem cells from the adult human brain develop into functional neurons in culture. *Exp. Cell Res.* **289**, 378-383.

Woodbury, D., Schwarz, E. J., Prockop, D. J. and Black, I. B. (2000). Adult rat and human bone marrow stromal cells differentiate into neurons. *J. Neurosci. Res.* **61**, 364-370.

Woodbury, D., Reynolds, K. and Black, I. B. (2002). Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. *J. Neurosci. Res.* **69**, 908-917.

Zhao, L. R., Duan, W. M., Reyes, M., Keene, C. D., Verfaillie, C. M. and Low, W. C. (2002). Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. *Exp. Neurol.* **174**, 11-20.