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In vitro neurogenesis of adult neural stem cells from bone marrow and brain

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List of abbreviations

AP	action potential
BrdU	5-bromo-2-deoxyuridine
CD	cluster of differentiation
CFU-F	colony forming unit fibroblasts
CM	conditioned medium
CNS	central nervous system
DNA	desoxyribonucleinacid
EGF	epidermal growth factor
ES cells	embryonic stem cells
FACS	fluorescence activated cell sorting (also flow cytometry)
FN	fibronectin
GABA	gamma aminobutyric acid
GalC	Galactosylceramidase
GFAP	Glial fibrillary acidic protein
hmNSCs	human marrow-derived neural stem cell-like cells (also hMSC-NSC)
hMSCs	human marrow stromal cells
hNPCs	adult human hippocampal NPCs
hNSCs	human neural stem cells
HPLC	high performance liquid chromatography
KCL	potassium chloride
MAP2	microtubule associated protein
MAPCs	multipotent adult progenitor cells
MBP	myelin basic protein
MEFs	mouse embryonic fibroblasts
MIAMI cells	marrow-isolated adult multilineage inducible cells
mRNA	messenger ribonucleinacid
MSCs	marrow stromal cells
NCAM	neural cell adhesion molecule
NGFR	nerve growth factor receptor
NMDA	n-Methyl-D-Aspartat
NPCs	neural progenitor cells
NSCs	neural stem cells
NSE	neuron specific enolase
PCR	polymerase chain reaction
PD	Parkinson's disease
PDGF-RB	platelet derived growth factor receptor b
PI-FACS	propidium iodide FACS
PSA-NCAM	polysialic acid NCAM
RMP	resting membrane potential
RNA	ribonucleinacid
RT-PCR	reverse transcriptase polymerase chain reaction
Shh	sonic hedgehoc
SVZ	subventricular zone
TH	tyrosine hydroxylase
tNSCs	tegmental neural stem cells
TTX	tetrodotoxin
Tuj1	neuron specific class III Beta-tubulin

1. Introduction

1.1. Neural stem cells: Origin and definition

The discovery of neural stem cells (NSCs) in the developing and adult brain that can generate neural tissue has raised new possibilities for repairing the nervous system. Despite the debate on how to classify these germinal cells, there is consensus that NSCs are a subtype of progenitor cells that are capable of extended self-renewal and that they have the ability to generate all major cell types of nervous tissue, such as neurons and glial cells (reviewed in [28,60]). Thus, NSCs are usually identified operationally by their behaviour after isolation. During expansion, they normally grow in floating, multicellular aggregates, so called 'neurospheres' [28,60]. Markers that define NSCs have recently been developed [28,75,86,89]. The first marker defining NSCs was the intermediate filament protein nestin, but some neural precursor cells are nestin-negative [51] and, on the other hand, nestin is expressed by other cell types, including non-neuronal cells [77] as well. Recently, Uchida and co-workers performed an extensive analysis of surface markers on clonogenic human neurosphere cultures and defined a subset of human NSCs as phenotypically CD133-positive, but negative for CD34 and CD45 [86]. Vogel and colleagues characterised commercially available neural progenitor cells as CD15, CD56, CD90, CD164, nerve growth factor receptor, 57D2 and W4A5 positive, whereas they were negative for CD45, CD105 (endoglin), CD109, CD140b (PDGF-RB) and W8B2 [89]. Recently, the expression of specific neural transcription factors, such as Sox-1, Musashi-1, Otx-1, Otx-2, Neurod1 and neurogenin-2 was reported in neural stem or progenitor cells, demonstrating their neuroectodermal progeny [8,15].

Early studies reported the isolation of stem-like cells from embryonic mammalian CNS [18,42,57,68,69,82] and the peripheral nervous system [79]. Since then, stem cells have been isolated from many regions of the embryonic CNS, indicating their ubiquity. Recently, the first isolation of NSCs from adult brain was demonstrated [57,69,79]. Adult NSCs have now been found in the two principal neurogenic regions, the hippocampus and the subventricular zone (SVZ) of the lateral ventricles, and in some non-neurogenic regions, such as the spinal cord [28,57,59,60,66,69], adult *substantia nigra* [55] and mesencephalon [94]. However, other research groups were not able to fully replicate some of these reports [27,29,45,47]. The mentioned regions, however, are of special interest as they reveal spontaneous neurogenesis throughout the entire lifetime, suggesting to play a functional role in physiological cell replacement in aging, learning and cognition, as well as proposing a therapeutic potential in neurological disease [1,14,85].

1.2. Neural stem cells in culture

NSCs can be either directly extracted from fetal or adult nervous tissue and proliferated in culture [18,28,42,56,60,81,88], or embryonic stem (ES) cells can be extracted, proliferated and differentiated into neural precursor cells (Fig. 1). Furthermore, there is evidence for multipotent adult stem cells from other tissues, which seems to have the potential to transdifferentiate into neuroectodermal lineages [36,74,83]; (Fig 1). ES cells are totipotent cells isolated from the inner cell mass of the preimplantation blastocyst, which give rise to all cells in the organism. ES cells can be differentiated into neural stem or precursor cells and subsequently neurons [40,41,53]. However, their proliferative potential seems to be responsible for the high risk of tumor formation after transplantation of ES cells [6,12]. Similarly, multipotent stem cells are also able to regenerate, but are believed to have a more restricted potential than ES cells, and are often defined by the organ from which they are derived. NSCs have been categorized as multipotent stem cells derived from the nervous system with the capacity to regenerate and to give rise to cells belonging to all three major cell lineages of the nervous system; neurons, oligodendrocytes, and astrocytes [2,7,18,28,42,60,81,88]. Furthermore, NSCs seem to lack the risk of tumor formation, most likely because of their more restricted proliferation potential [34].

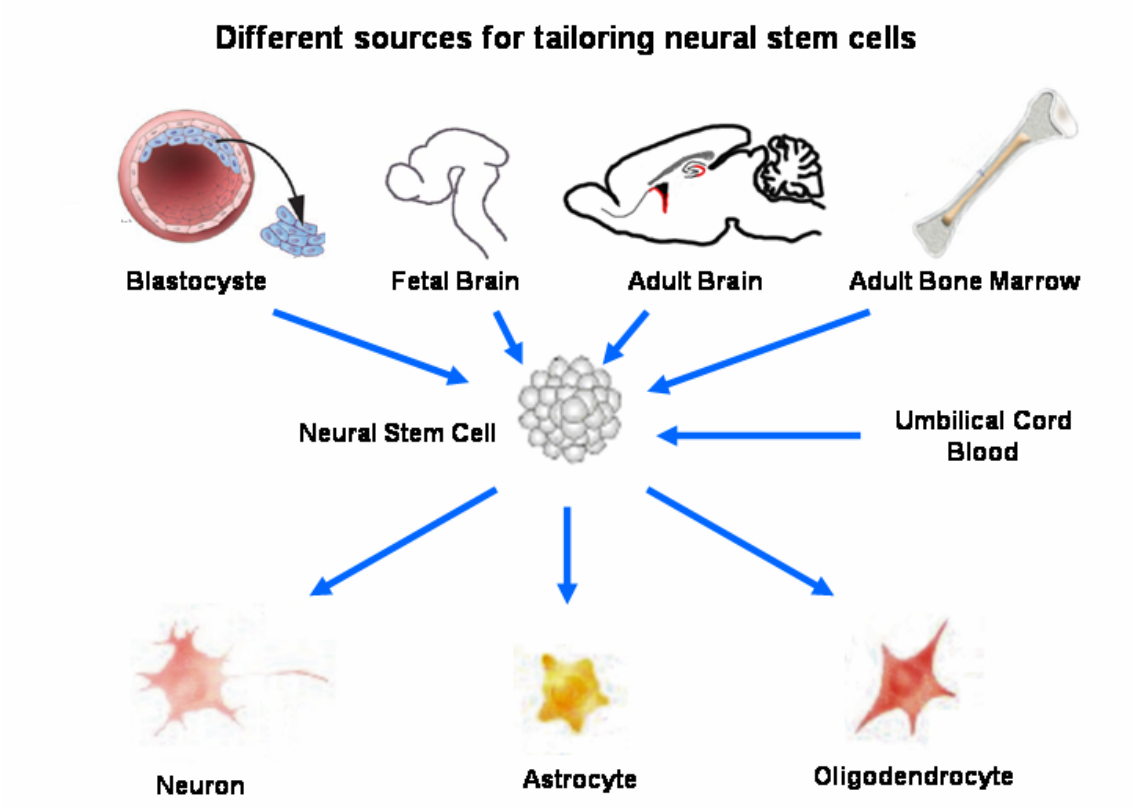

Figure 1: Schematic overview of various sources of neural stem cells (NSCs) with the capacity to differentiate into all major cell types of the central nervous system, namely astroglia, oligodendroglia and neurons. Most protocols produce NSCs growing in multicellular spheroid aggregates called “neurospheres”.

1.2.1. Brain-derived adult neural stem cells

Multipotent NSCs with the potential to generate mature cells of all neural lineages, have been consistently demonstrated within the hippocampus and SVZ of the lateral ventricles [4,23,32,33,38,69,70]. These cells grow *in vitro* as neurospheres in the presence of epidermal growth factor (EGF) [31,69]. Previous work has reported that SVZ-NSCs correspond to a rare population of relatively quiescent cells. However, not ciliated ependymal cells correspond to the NSCs [38], but SVZ astrocytes act as NSCs in both the normal and regenerating adult brain [21,22]. In line with these latter reports, a model for adult neurogenesis was developed, showing SVZ astrocytes as the *in vivo* stem cells, so called Type B cells, which give raise to transit amplifying cells, so-called Type C cells, which can further become restricted to neuroblasts, so-called Type A cells [21]. In recent years, neurogenesis was reported to occur in other regions of the adult brain under normal conditions, such as the neocortex [30], *amygdala* [9] and *substantia nigra* [94]. In contrast to SVZ-NSCs, the proliferation of these NSCs depends on both, EGF and fibroblast growth factor 2 (FGF-2) [23,91]. Furthermore, a few recent studies demonstrated the isolation of neural progenitor cells (NPCs) from different regions of the adult human brain including cortex, *amygdala*, hippocampus and SVZ [7,39,44,50,61,63,72,92], but only three previous studies investigated human adult NPCs from the hippocampus region *in vitro* [39,50,71]. These progenitor cells are reported to be multipotent cells differentiating into both glial and neuronal cells with some functional properties of neurons, such as the expression of sodium and potassium channels [71]. In contrast to extensive analyses of the phenotype and differentiation capacity of adult non-human mammalian hippocampal NPCs, little is known about their human counterparts, most likely due to the lack of tissue availability.

1.2.2. Adult multipotent stem cells

Alternatively, stem cells derived from blood, bone marrow, or skin may be converted into neural cell types. Adult stem cells were believed to be lineage restricted, which means that they only can differentiate into cells of their tissue origin [35,74]. However, there are several reports that these cells can break barriers of germ layer commitment and differentiate *in vitro* and *in vivo* into cells expressing neuronal and glial markers [35,67,83]. Bone marrow stromal cells (MSCs) are a heterogeneous population of cells providing ultrastructural and biochemical scaffolds within the hematopoietic microenvironment [10,89]. These cells are derived from bone marrow cell suspensions either by their selective attachment to tissue culture plastic or by different depletion methods [84] and can be expanded efficiently. These cell cultures contain some multipotent, mesenchymal stem cells that can differentiate into osteogenic, chondrogenic, adipogenic, myogenic, and fibroblastic lineages [26,67,76]. First reports in 2000 showed that mesodermal cells

from rat and human were able to convert into cells with neuronal and glial markers, but no functional test were reported [73,93]. Jiang and co-workers recently demonstrated a rare multipotent adult progenitor cell (MAPC) within MSC cultures from rodent bone marrow [36,37]. This cell type differentiates not only into mesenchymal lineage cells but also endothelium and endoderm. 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 [35].

1.3. Aim of this study

The scope of my thesis has been to identify possible alternative cell sources for neural stem cells which fulfill important requirements for the use of these cells in regenerative treatment strategies: (i) Generation of high yields of cells out of a small starting population without loosing their differentiation potential, (ii) on-demand availability of cells without major logistical and ethical problems and (iii) the possibility to standardize the cell source in a future clinical setting. Isolation and characterization of human NPCs from adult human hippocampus derived from epileptic surgery procedures (selective hippocampectomy or medio-temporal lobectomy) served as positive control.

A second approach has been the identification and *in vitro* characterization of neural stem cells in the adult midbrain as a cell source for possible endogenous regeneration in the brain region where the neurodegenerative process in Parkinson's disease is located. Since the reported data on *in vivo* dopaminergic neurogenesis in the adult brain are conflicting, the aim of the second part of this study was to investigate dopaminergic neurogenesis from adult neural stem cells *in vitro* to provide data on adult dopaminergic differentiation of neural stem cells as one major prerequisite for endogenous regenerative approaches in Parkinson's disease by recruiting endogenous stem cells in the adult brain.

2. Results and discussion

2.1. Epigenetic conversion of human adult bone marrow stromal cells into neural stem cells

This section refers to the following publications:

Hermann A, Gastl R, Liebau S, Popa OM, Fiedler J, Boehm BO, Maisel M, Lerche H, Schwarz J, Brenner R, Storch A: Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells. Journal of Cell Science, Vol. 117, No. 19, 2004, pp. 4411-4422.

Hermann A, Liebau S, Gastl R, Fickert S, Habisch HJ, Fiedler J, Schwarz J, Brenner R, Storch A: Comparative analysis of neuroectodermal differentiation capacity of human bone marrow stromal cells using various conversion protocols. Journal of Neuroscience Research, Vol. 83, No. 8, 2006, pp. 1502-14.

Hermann A, Maisel M and Storch A: Epigenetic conversion of human adult bone mesodermal stromal cells into neural stem cells. Expert Opinion in Biological Therapy, Vol. 6, No. 7, 2006, pp. 653-670.

Human adult bone marrow-derived mesodermal stromal cells (hMSCs) are capable to differentiate into multiple mesodermal tissues, including bone and cartilage [26,67,76]. Several recent studies suggested that multipotent adult stem cells might be able to break barriers of germ layer commitment and differentiate *in vitro* and/or *in vivo* into cells of different tissues [36,67,83]. The conversion of MSCs into neuroectodermal cells generating neuronal and/or glial cells attached to the culture surface [73,93] as well as the *in vitro* differentiation of mice MSCs into cells with biochemical, anatomical, and electrophysiological characteristics of neuronal cells using a co-culture system with astrocytes [35] has been reported previously. However, *in vitro* conversion of *human* MSCs into clonogenic undifferentiated hNSCs, which proliferate and subsequently differentiate 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 known to survive detachment and subsequent transplantation procedures poorly [11,43].

In the present study, we were able to develop a unique multistep protocol for the generation of NSC-like cells (hmNSCs) from human adult bone marrow stromal cells (hMSCs) using an epigenetic conversion approach (the resulting cell are called hmNSCs for human marrow-derived neural stem cell-like cells). Furthermore, to compare the resulting phenotype with original adult NSCs from brain tissue, we established a long-term culture of human NPCs derived from adult hippocampus by epileptic surgery (hNPCs, for adult human hippocampal NPCs), providing the ideal positive control by having both adult and human NSCs (please also refer to 2.2.). The hmNSCs grew in neurosphere-like structures, showed the typical exponential growth curve of slowly dividing cells with an estimated doubling time of 2.6 days and expressed high levels of early neuroectodermal markers, such as the proneural genes *NEUROD1*, *NEUROG2*, *MSl1* as well as *OTX1* and nestin (for genes and encoded proteins, please refer to 6.1.). Phenotypically, there

were no major differences detectable between the hmNSCs and the brain-derived hippocampal NPCs. FACS analysis revealed that similar to hNPCs, hmNSCs were $CD34^-$, $CD45^-$ and $CD133^-$. Interestingly, when compared to the initial hMSCs population, hmNSCs lose the marker expression of mesodermal stromal cells and became $CD9^-$ and $CD166^-$.

NSC are operationally characterized by their potential to proliferate as well as the capacity to generate both neurons and glia [34]. To prove the neuroectodermal potential of the converted hMSCs, we developed two different protocols, a neuronal and glial induction protocol, respectively. Investigation of marker gene expression on the protein level by immunocytochemistry revealed that both protocols significantly reduced the expression of the mesodermal marker protein fibronectin when compared to hMSCs. Furthermore, the glial induction protocol induced an increase of cells expressing glial and oligodendroglial markers, while the neuronal protocol generated a mature neuronal phenotype, demonstrated by the expression of MAP2ab (6±2% $MAP2ab^+$ cells). Quantitative mRNA analyses revealed that the expression of the mesodermal marker gene *FN1* was down-regulated during terminal neuronal differentiation of hmNSCs whereas marker mRNA levels of mature neural cell types (*GFAP, MBP, TUBB4/III, SNCA* and *TH*) were significantly increased. To determine whether individual hmNSCs could generate both neurons and glia and thereby proving their multipotency, we performed clonal analysis as described by Uchida and co-workers using the limiting dilution technique [86]. Double immunostaining revealed that individual hmNSCs can generate cells acquiring morphologic and phenotypic characteristics of astrocytes ($GFAP^+$) and neurons (β-tubulin III^+). Furthermore, to investigate the remaining mesodermal potential of hmNSCs *in vitro*, we compared the ability of hMSCs and hmNSCs to differentiate into osteoblasts using standard protocols and high density cultures [25]. By immunocytochemistry and RT-PCR, we showed that hmNSCs lose their mesodermal differentiation potential compared to the initial hMSC population underlining the successful conversion towards a neuroectodermal phenotype.

Using the glial induction protocol and fetal calf serum to differentiate hmNSCs, we were able to demonstrate electrophysiological properties typical for developing and mature glial cells [13,48]. Due to technical reasons, we were not yet able to functionally characterize the resulting neurons using the patch-clamp technique. On the one hand, electrophysiological characterization of neurons derived from human progenitor cells seem to be very delicate concerning technical aspects and therefore have hitherto only been reported in two studies [16,92]. On the other hand, Jiang and co-workers only succeeded to show electrophysiological properties typical for neurons of their mice MAPCs by using a co-culture system [35]. In line with these facts, our hmNSCs continued to express nestin after terminal differentiation while acquiring several more mature neuroectodermal markers. To test whether the 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. In contrast, when media of *in vitro* differentiated hmNSCs were assayed, total dopamine production was detectable with higher concentrations following membrane depolarisation of differentiated hmNSCs with 56 mM KCl. Direct differentiation of hMSCs using the neuronal induction protocol produced neither mature MAP2ab$^+$ neurons nor TH$^+$/dopamine producing cells, and did not lead to an up-regulation of *TUBB4/III* or *GFAP* gene expression.

Taken together, we demonstrated that similar to embryonic stem (ES) cells, adult human MSCs can be converted into a clonogenic neural stem cell-like cell population (hmNSCs), which can be differentiated *in vitro* into cells with morphological and functional characteristics of neuronal, astroglial and oligodendroglial cells. However, there is still debate whether this results from cell fusion, aberrant marker gene expression or real neuroectodermal differentiation. Indeed, some controversy persists regarding the *in vivo* differentiation potential of MSCs into neurons and glia [5,17,90]. In addition, several *in vitro* studies re-evaluating the differentiation potential of MSCs towards a neural phenotype could not reproduce some of the transdifferentiation protocols [58,62]. Furthermore, neuroectodermal gene expression was already seen in MSCs before any differentiation [19,84]. Taken together, a critical examination of the differentiation process including a comparative analysis of the initial cell population (MSCs) and the converted neural cell types seems to be indispensable to determine the validity of neuroectodermal differentiation capacity of bone marrow-derived MSCs. Therefore, we extended our work on neuroectodermal conversion of adult hMSCs in a second study by re-evaluating our previous findings.

We consequently compared various established *in vitro* protocols for the neuroectodermal differentiation of human MSCs using epigenetic stimulation by analyzing the expression pattern of a large set of both early and late neuroectodermal maker genes in the undifferentiated MSC population as well as the converted neural cell types using quantitative real-time PCR and immunocytochemistry. We found that neuroectodermal genes commonly used to characterize neural cell types including neuroectodermally converted MSCs were already expressed by undifferentiated human MSCs, such as neural stem cells markers (*NES*, *MSI1*) or early neurogenic markers (*OTX1*, *SOX1* and *SOX10*, *NEUROG2*, *NEUROD1*). Interestingly, also some glial (*GFAP*, *MBP*) as well as immature neuronal markers (*TUBB4/III*) were expressed by hMSCs on both, mRNA and protein level. However, hMSCs did not significantly express *SNCA* or MAP2ab as mature neuronal markers or *TH*, a marker for catecholaminergic cells.

Regarding the neuroectodermal differentiation potential of hMSCs, all protocols using a direct differentiation of hMSCs into a neural phenotype failed to induce significant changes in morphology and/or expression of markers of early and mature glial as well as neuronal cell types. In contrast, only the multi-step protocol (as described in the first study) with conversion of hMSCs into hmNSCs and subsequent terminal differentiation into mature glia and neurons generated relevant morphological changes as well as a significant increase of expression levels of marker genes for early and late neural cell types, such as *NES*, *NEUROG2*, *MBP* and MAP2ab, accompanied by a loss of their mesenchymal properties. Interestingly, neither step one nor step two of our multistep protocol alone were able to induce these changes. Noteworthy, investigation of early neurogenic markers showed an upregulation only during the conversion from hMSCs to the neural stem cell-like state of hmNSCs, followed by a downregulation during terminal differentiation, as one would expect. However, the maintenance of the high expression level of nestin and the lack of action potential generation suggests a still immature neuronal phenotype (also refer to [35]). This provides impetus for a conversion, in means of change of one undifferentiatied cell type into another undifferentiatied cell type more likely than a transdifferentiation, defined as the conversion of one differentiated cell type to another differentiated cell type. Whether this conversion is more likely due to a reprogramming of the gene expression profile of a committed cell into that of a more pluripotent cell or the proliferation and differentiation of a pluripotent progenitor/stem cell already harboured in the initial tissue remains to be shown in future experiments [36,74]. Furthermore, future studies are warranted to define further functional properties of terminally differentiated hmNSCs into neuronal and glial cells by using co-culture methods with astroglial cells [35,78] and longer periods of differentiation time to achieve more mature neurons and glial cells [92]. In parallel, transplantation studies are needed to clarify both the differentiation and migration behaviour of hmNSCs as well as their regenerative potential in different disease models of acute and chronic neurodegeneration, such as stroke and Parkinson's disease.

2.2. Isolation, expansion and *in vitro* characterization of adult human hippocampal progenitor cells

This section refers to the following publication:

Hermann A, Maisel M, Liebau S, Gerlach M, Kleger A, Schwarz J, Kim KS, Antoniadis G, Lerche H, Storch A: Mesodermal cell types induce neurogenesis from adult human hippocampal progenitor cells. Journal of Neurochemistry, Vol. 98, No. 2, pp. 629-640.

A few recent studies demonstrated isolation of NPCs from different regions of the adult human brain including cortex, amygdala, hippocampus and SVZ [7,39,44,50,61,63,92], but only three

previous studies investigated human adult NPCs from the hippocampus region *in vitro* [39,50,71]. However, due to limited tissue availability, the knowledge of their phenotype and differentiation behaviour is restricted and further studies are necessary to improve our knowledge on this cell type. For this purpose we established a long-term culture of human neural progenitor cells derived from adult hippocampus by epileptic surgery (hNPCs, for adult human hippocampal NPCs). Furthermore, this cell type would be the perfect positive control of our conversion studies of marrow stromal cells as mentioned above. In this study we extensively characterized the phenotype and differentiation capacity of hNPCs including co-culture methods on various feeder cells such as mouse cortical astrocytes, mouse embryonic fibroblasts (MEFs) and PA6 stromal cells. Isolated hNPCs were cultured at clonal density by transferring the cells to serum-free media supplemented with FGF-2 and EGF in 3% atmospheric oxygen. These hNPCs showed neurosphere formation, expressed high levels of early neuroectodermal markers, such as the proneural genes *NEUROD1* and OLIG2, the NSC markers nestin and Musashi1, the proliferation marker Ki-67 and significant activity of telomerase. The phenotype was $CD15^{low/-}$, $CD34^{-}$, $CD45^{-}$ and $CD133^{-}$ as shown by FACS analysis.

After removal of mitogens and plating the hNPCs on poly-L-lysine, they spontaneously differentiated into a neuronal ($MAP2ab^{+}$), astroglial ($GFAP^{+}$), and oligodendroglial ($GALC^{+}$) phenotype. Differentiated hNPCs showed functional properties of neurons, such as sodium channels, action potentials and production of the neurotransmitters glutamate and GABA. To further characterize their *in vitro* differentiation potential we did co-culture experiments of hNPCs with astrocytes, MEFs and PA6 cells similar to that shown in section 2.3. Interestingly, co-culture on all feeder cells increased neurogenesis of hNPCs, while only MEFs and PA6 cells also led to a morphological neurogenic maturation seen by elongated neurites. Remarkably, co-culture on astrocytes increased the differentiation of hNPCs towards astroglial lineages, which is controversial to data on mice subventricular zone NSCs [78], but could be explained by different cell sources for astrocyte cultures in the two studies. However, future studies are warranted to show detailed neuron subtype specification as well as to further define functional neuronal properties including action potentials accompanied by synaptic neurotransmitter release in fully differentiated hNPCs [35,78,92].

2.3. Functional neuronal and dopaminergic differentiation of multipotent neural stem cells from the adult tegmentum

This section refers to the following publications:

Hermann A, Maisel M, Wegner F, Liebau S, Kim DW, Gerlach M, Schwarz J, Kim KS, Storch A: Multipotent Neural Stem Cells from the Adult Tegmentum with Dopaminergic Potential Develop Essential Properties of Functional Neurons. Stem Cells, Vol. 24, No. 4, pp. 949-64.

Hermann A, Gerlach M, Schwarz J, Storch A: Neurorestoration in Parkinson's disease by cell replacement and endogenous regeneration. Expert Opinion on Biological Therapy, Vol. 4, No. 2, 2004, pp. 131-143.

Storch A, **Hermann A**, Schwarz J: Stammzell-basierte regenerative Therapie des Morbus Parkinson, in: Die Parkinson-Krankheit und atypische Parkinson-Syndrome (Oertel WH, ed.), 2005, Edition Roche, Grenzach-Wyhlen, pp. 49-58.

Neurogenesis in the adult brain occurs within the two principle neurogenic regions, the hippocampus and the SVZ of the lateral ventricles [3,30,49,54,57]. The occurrence of adult neurogenesis in non-neurogenic regions including the midbrain remains controversial, but isolation of neural stem cells (NSCs) from several parts of the adult brain including the *substantia nigra* has been reported [9,30,94]. However, other research groups were not able to replicate some of these reports [45,46], and some have been challenged on methodological grounds [27,29,45,46,65]. Lie and co-workers (2002) described the isolation of multipotent NSCs from the adult *substantia nigra* with neurogenic differentiation capacity *in vitro* and after transplantation into the adult hippocampus [55]. However, the authors neither reported on detailed characterization of these progenitor cells *in vitro* nor provided data on neuronal subtype specification or functional properties after differentiation. Moreover, similar to all other adult CNS stem cells, these midbrain-derived NSCs did not differentiate into dopaminergic nerve cells [55]. Thus it remains unclear whether adult NSCs do have the capacity to produce functional dopaminergic neurons, the cell type lost in Parkinson's disease, which would be feasible for regenerative approaches by recruiting endogenous stem or progenitor cells.

To investigate the dopaminergic potential of adult NSCs, we established the isolation, expansion and *in vitro* characterization of adult mouse tegmental (midbrain and hindbrain) neural stem cells (tNSCs) and their differentiation into functional nerve cells including dopaminergic neurons. For this purpose, we chose defined serum-free *in vitro* conditions in this study, in order to omit possible unknown interactions which could inhibit a possible neurogenic/dopaminergenic potential of tNSCs. After isolation, these tNSCs showed neurosphere formation, expressed high levels of early neuroectodermal markers, such as the proneural genes *Neurod1*, *Neurog2* and *Olig2*, the NSC markers nestin and Musashi1 and the proliferation markers Ki-67 and BrdU. In cell cycle analysis using PI-FACS technology the cells displayed a pattern typical for slowly dividing cells. Consistently, they expressed significant levels of telomerase. FACS analysis

revealed that the phenotype of tNSCs was $CD24^{low/-}$, $CD34^{-}$, $CD45^{-}$, $prominin1^{-}$ and $SSEA1^{-}$. Markers for mature neural cell types, such as *Mbp*, *Plp*, *Ntrk1*, MAP2ab and TH, were undetectable or very low. This phenotype is similar to that of NSCs derived from adult mouse SVZ or hippocampus [21,33,70]. tNSCs could be passaged and cultured as secondary and tertiary neurospheres without changing morphology and phenotype for 3 to 12 weeks (≈ 5 to 10 passages).

In the presence of selected growth factors, tNSCs differentiated into neurons, oligodendroglia and astrocytes with an approximate ratio of 1:1.5:14, respectively. Looking for neuronal subtype specification, tNSCs could be differentiated into cells expressing the markers for cholinergic, GABAergic or glutamatergic neurons with similar amounts of the distinct neuronal subpopulations with respect to the various differentiation protocols. Interestingly, no serotonergic cells could be observed with any of the differentiation protocols tested. Electrophysiological analyses revealed functional properties of mature nerve cells, such as TTX-sensitive sodium channels, action potentials as well as GABA-, glutamate and NMDA-induced currents, with the latter ones known to be exclusively expressed by neuronal cells [87]. Clonal analysis demonstrated that individual tNSCs retain the capacity to generate both glia and neurons. Herewith we showed for the first time that adult NSCs from the tegmental area are multipotent neural stem cells *in vitro* which can be differentiated into fully functional neurons. However, the major remaining question was whether these NSCs residing in the region of interest in Parkinson's disease are able to differentiate into dopaminergic neurons.

Therefore we developed a multiple-step differentiation protocol using co-culture conditions with different feeder cell types. There were no relevant differences in the differentiation capacity of tNSCs into astroglia and neurons with respect to the various differentiation conditions. In contrast, only using PA6 stromal cells as feeder cells, we were able to induce dopaminergic neurogenesis in a small percentage of cells (2.4 ± 1.4 % of all neurons), showing typical morphological features of dopaminergic neurons with small irregularly shaped soma and bipolar or multipolar branching processes with varicosities. Consistently, using quantitative RT-PCR, we demonstrated the expression of various dopaminergic marker genes in tNSCs differentiated on PA6 cells, such as tyrosine hydroxylase, dopa-decaboxylase and dopamine transporter, as well as dopamine production using HPLC.

Taken together, we demonstrate not only the existence of adult multipotent NSCs from the tegmental area with functional neurogenic potential, but also the proof-of-principle of dopaminergic differentiation of stem cells in the region of interest in Parkinson's disease, a prerequisite for future endogenous cell replacement strategies in Parkinson's disease.

3. Summary

Parkinson's disease (PD) is characterized by a continuous and selective loss of dopaminergic neurons in the *substantia nigra pars compacta* with a subsequent reduction of the neurotransmitter dopamine [24,52]. Thus, the prospect of replacing the missing or damaged dopaminergic cells is very attractive. Possible regenerative therapies include transplanting developing neural tissue or neural stem cells into the degenerated host brain, and inducing proliferation of endogenous stem cells by pharmacological manipulations. Neural stem cells with the capacity to self-renew and produce the major cell types of the brain, exist in the developing and adult central nervous system. These cells can be generated and expanded *in vitro* while retaining the potential to differentiate into nervous tissue. However, major problems are control of cell growth and differentiation of these cells as well as ethical aspects concerning the use of human fetal tissue [34,80].

Producing specific cell types from a patient´s own stem cells would provide immunological advantages and could avoid these ethical concerns. Here we demonstrate the identification of a possible autologous cell source for neural stem cells by providing evidence that hMSCs can be differentiated *in vitro* into mature and functional neuroectodermal cells by a multi-step protocol. Importantly, the acquired phenotype is very similar to brain-derived human adult neural progenitor cells. This technique therefore fulfills important requirements for the use of these cells in regenerative treatment strategies: (i) Generation of high yields of cells out of a small starting population without loosing their differentiation potential, (ii) on-demand availability of cells without major logistical and ethical problems and (iii) the possibility to standardize the cell source in a future clinical setting. Neuroectodermally converted hMSCs may therefore ultimately help to treat acute and chronic neurodegenerative diseases. However, there is a hypothetical risk that the patients own cells might carry genetic information which increases their vulnerability to the pathogenetic process underlying PD. Nevertheless, conditions for generating dopaminergic neurons from human adult stem cells need to be optimized before these cells can be considered for transplantation into PD. In addition, adult stem or progenitor cells seem to lack the proliferative capacity of fetal NSCs or ES cells [34,80].

Regeneration and neurogenesis in the adult mammalian brain is a long recognized phenomenon [57,60,64,69,70]. It has been shown that cells in the adult subependymal layer divide and migrate several millimeters to the olfactory bulb, where they differentiate into neurons [57]. These data demonstrate that adult neural precursors are at least in part associated with neuronal reconstitution, but targeted recruitment of endogenous cells in other brain regions remains to be shown. In a second series of experiments we were able to show the existence of NSCs in the adult *tegmentum* as well as their extensive characterization *in vitro* under defined serum-free conditions.

Furthermore, we could show functional dopaminergic differentiation of these tNSCs. However, factors which govern this differentiation process remain to be identified. Interestingly, there are data suggesting that endogenous neural precursor cells are amenable to external modulation: Intraventricular treatment of adult mice with EGF increased proliferation and migration of subependymal cells [20]. Therefore, another regenerative approach could be to replace neuronal densities and circuitries by stimulation of endogenous stem cells. Conclusively, in the presented studies, we provide the proof-of-principle for dopaminergic neurogenesis in the region of interest in Parkinson's disease, a prerequisite for future endogenous cell replacement strategies in Parkinson's disease.

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6. Appendices

6.1. Genes and encoded proteins

Gene	Protein	Function	Marker protein
SNCA	alpha-Synuclein	Scleroprotein	Marker for mature neurons
β-TUBB4/III	β-Tubulin class III	Scleroprotein	Marker for young neurons
FN1	Fibronectin 1	Scleroprotein	Mesodermal marker
FZD1	Frizzled homolog 1	Receptor	Wnt signaling, neuronal development
GALC	Galactosylceramidase	Enzyme	Oligodendrocyte marker
GFAP	Glial fibrillary acidic protein	Scleroprotein	Astrocyte marker
HMBS	Hydroxymethyl-bilane-Synthase	Enzyme	Housekeeping gene
MBP	Myelin basic protein	Scleroprotein	Oligodenrocyte marker
MSI1	Musashi homolog 1 (Drosophila)	Post-transcriptionfactor	NSC marker
NEUROD1	Neurogenic differentiation 1	Transcriptionfactor	Early neurogenic marker protein
NEUROG2	Neurogenin 2	Transcriptionfactor	Early neurogenic marker protein
NOTCH1	Notch homolog 1, translocation-associated	Receptor	
NES	Nestin	Intermediate filament	NSC maker
NTRK1	Neurotrophic tyrosine kinase, receptor, type 1	Receptor	
OLIG2	Oligodendrocyte transcription factor 2	Transcriptionfactor	*In vitro* NSC marker
OTX1	Orthodenticle homolog 1 (Drosophila)	Transcriptionfactor	Early neurogenic marker protein
OTX2	Orthodenticle homolog 2 (Drosophila)	Transcriptionfactor	Early neurogenic marker protein
PTCH	Patched homolog (Drosophila)	Integral-membran protein	Shh signaling
SOX1	SRY (sex determining region Y)-box 1	Transcriptionfactor	Early neurogenic marker protein
SOX2	SRY (sex determining region Y)-box 2	Transcriptionfactor	Early neurogenic marker protein
SOX10	SRY (sex determining region Y)-box 10	Transcriptionfactor	Early neurogenic marker protein
TH	Tyrosine hydroxylase	Enzyme	Marker for dopaminergic neurons

6.2. Curriculum vitae

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10/2002-10/03	Speaker of the Graduate College 460, Ulm, Germany

Prices and Fellowships:

2005	„Carl-Gustav-Carus Förderpreis"
2005	„Förderpreis junger Neurowissenschaftler" of the Felgenhauer foundation
2005	Travel Grant of the German Parkinson Society to the 4th German Parkinson Congress
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2003	Travel Grant of the German Parkinson Society to the 3rd German Parkinson Congress
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5-6/2005	Spring School in Regenerative Medicine: "Differentiation of neuronal progenitor cells" and practical course on "Electrophysiology and Proteomics of developing neuronal cells", Rostock, Germany

8/2002 European Summer School of Channelopathies in Ulm, Germany

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March 2005 Oral presentation at the 3rd German Pakinson Congress on "Isolation, Expansion und *in-vitro*-Charakterisierung adulter mesenzephaler neuraler Stammzellen", Frankfurt, Germany, *Akt Neurologie* (2005) 32, S15, 56.

October 2004 Oral presentation at the 34th Annual Meeting of the Society for Neuroscience in San Diego, California; USA: "Isolation, long-term expansion and *in vitro* characterization of adult human neural progenitor cells" *Program No. 825.1, Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience, 2004. Online.*

November 2003 Oral presentation at the 33rd Annual Meeting of the Society for Neuroscience in New Orleans, Louisiana; USA: "Conversion of human adult bone marrow stromal cells into neural stem cells*" Program No. 880.6. 2003 Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience, 2003. Online.*

March 2003 Oral presentation at the 3rd German Pakinson Congress on " Conversion of human adult mesenchymal progenitor cells into neural stem-like cells", Dresden, Germany, *J. Neural. Transm.* (2003) **110:** VII

Publications:

Hermann A, Maisel M, Liebau S, Gerlach M, Kleger A, Schwarz J, Kim KS, Antoniadis G, Lerche H, Storch A: Mesodermal cell types induce neurogenesis from adult human hippocampal progenitor cells. Journal of Neurochemistry, accepted for publication March 2006.

Hermann A, Liebau S, Gastl R, Fickert S, Habisch HJ, Fiedler J, Schwarz J, Brenner R, Storch A: Comparative analysis of neuroectodermal differentiation capacity of human bone marrow stromal cells using various conversion protocols. Journal of Neuroscience Research, accepted in January 2006, DOI: 10.1002/jnr.20840.

Hermann A, Maisel M, Wegner F, Liebau S, Kim DW, Gerlach M, Schwarz J, Kim KS, Storch A: Multipotent Neural Stem Cells from the Adult Tegmentum with Dopaminergic Potential Develop Essential Properties of Functional Neurons. *Stem Cells Express*, 2005; doi:10.1634/stemcells.2005-0192.

Storch A, **Hermann A**, Schwarz J, 2005, Stammzell-basierte regenerative Therapie des Morbus Parkinson, *in: Die Parkinson-Krankheit und atypische Parkinson-Syndrome* (Oertel WH, ed.), Edition Roche, Grenzach-Wyhlen, pp. 49-58.

Hermann A, Gastl R, Liebau S, Popa OM, Fiedler J, Boehm BO, Maisel M, Lerche H, Schwarz J, Brenner R, Storch A: Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells. *Journal of Cell Science*, Vol. 117, No. 19, 2004, pp.4411-4422.

Hermann A, Gerlach M, Schwarz J, Storch A: Neurorestoration in Parkinson's disease by cell replacement and endogenous regeneration. *Expert Opinion on Biological Therapy*, Vol. 4, No. 2, 2004 pp.131-143.

List of referred publications

Hermann A, Gastl R, Liebau S, Popa OM, Fiedler J, Boehm BO, Maisel M, Lerche H, Schwarz J, Brenner R, Storch A: Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells. Journal of Cell Science, Vol. 117, No. 19, 2004, pp. 4411-4422.

Hermann A, Liebau S, Gastl R, Fickert S, Habisch HJ, Fiedler J, Schwarz J, Brenner R, Storch A: Comparative analysis of neuroectodermal differentiation capacity of human bone marrow stromal cells using various conversion protocols. Journal of Neuroscience Research, Vol. 83, No. 8, 2006, pp. 1502-14.

Hermann A, Maisel M and Storch A: Epigenetic conversion of human adult bone mesodermal stromal cells into neural stem cells. Expert Opinion in Biological Therapy, Vol. 6, No. 7, 2006, pp. 653-670.

Hermann A, Maisel M, Liebau S, Gerlach M, Kleger A, Schwarz J, Kim KS, Antoniadis G, Lerche H, Storch A: Mesodermal cell types induce neurogenesis from adult human hippocampal progenitor cells. Journal of Neurochemistry, Vol. 98, No. 2, pp. 629-640.

Hermann A, Maisel M, Wegner F, Liebau S, Kim DW, Gerlach M, Schwarz J, Kim KS, Storch A: Multipotent Neural Stem Cells from the Adult Tegmentum with Dopaminergic Potential Develop Essential Properties of Functional Neurons. Stem Cells, Vol. 24, No. 4, pp. 949-64.

Hermann A, Gerlach M, Schwarz J, Storch A: Neurorestoration in Parkinson's disease by cell replacement and endogenous regeneration. Expert Opinion on Biological Therapy, Vol. 4, No. 2, 2004, pp. 131-143.

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

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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).

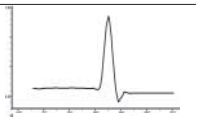
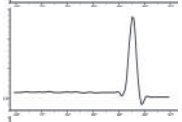
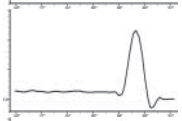
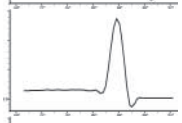
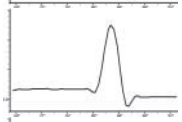
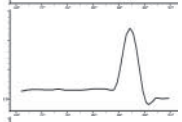
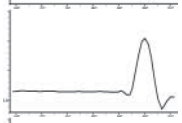
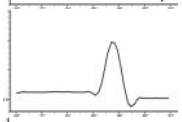
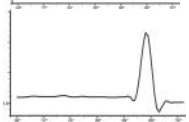
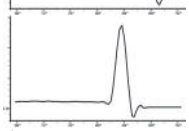
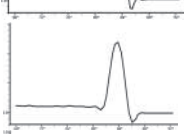
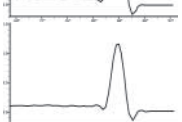
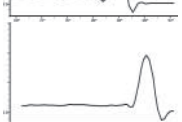
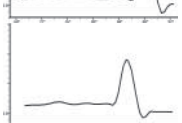
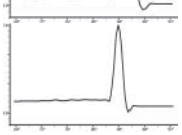
RNA extraction, RT-PCR and quantitative real-time RT-PCR analysis

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-

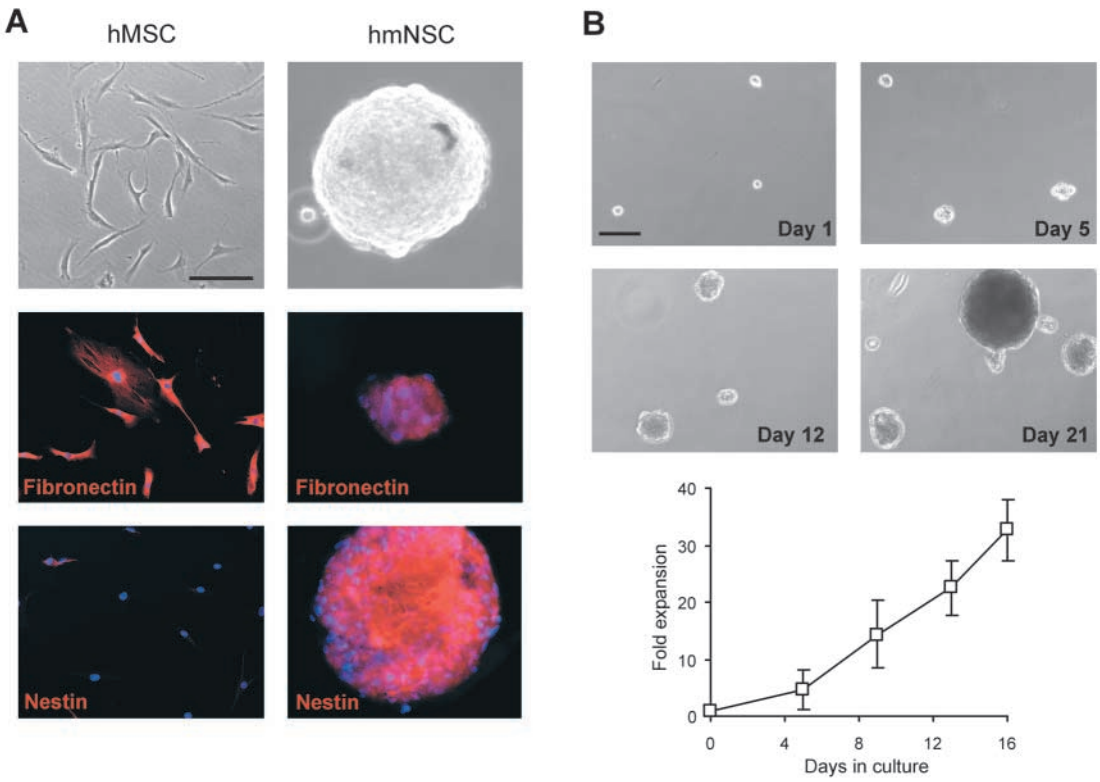


Fig. 1. Characteristics of adult human mesodermal stromal cells (hMSC) and human marrow-derived neural stem cell-like cells (hmNSC) during expansion. (A) Morphology, fibronectin and nestin expression of hMSC (left panels) and hmNSC (right panels). All hMSCs express high levels of fibronectin, but only 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.

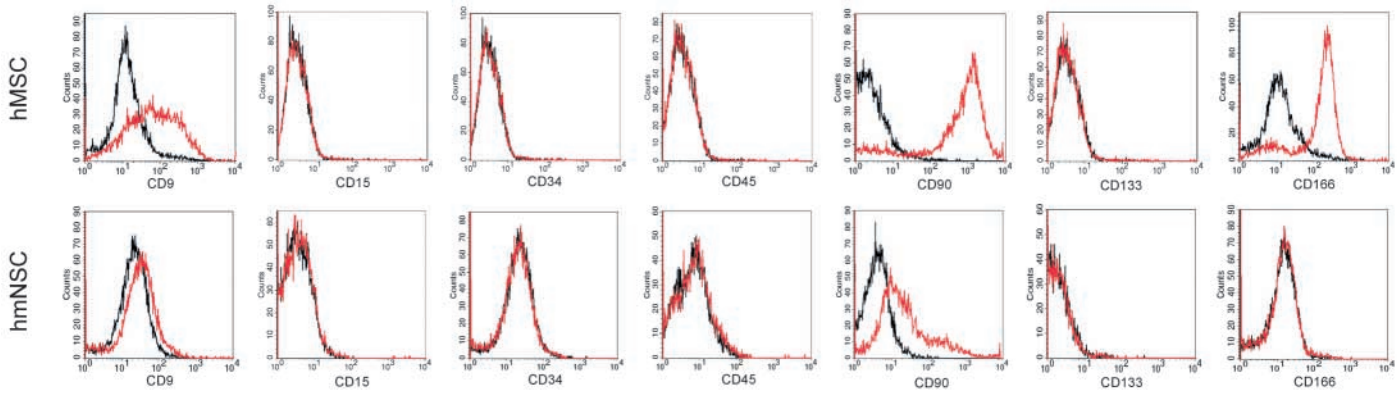


Fig. 2. Flow cytometry of hMSCs (upper panels) and hmNSCs (lower panels) cultured for 10-50 and 5-30 population doublings, respectively. Cells were labeled with fluorescence-coupled antibodies against CD9, CD15, CD34, CD45, CD90, CD133, CD166 or immunoglobulin isotype control antibodies. Cells were analyzed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

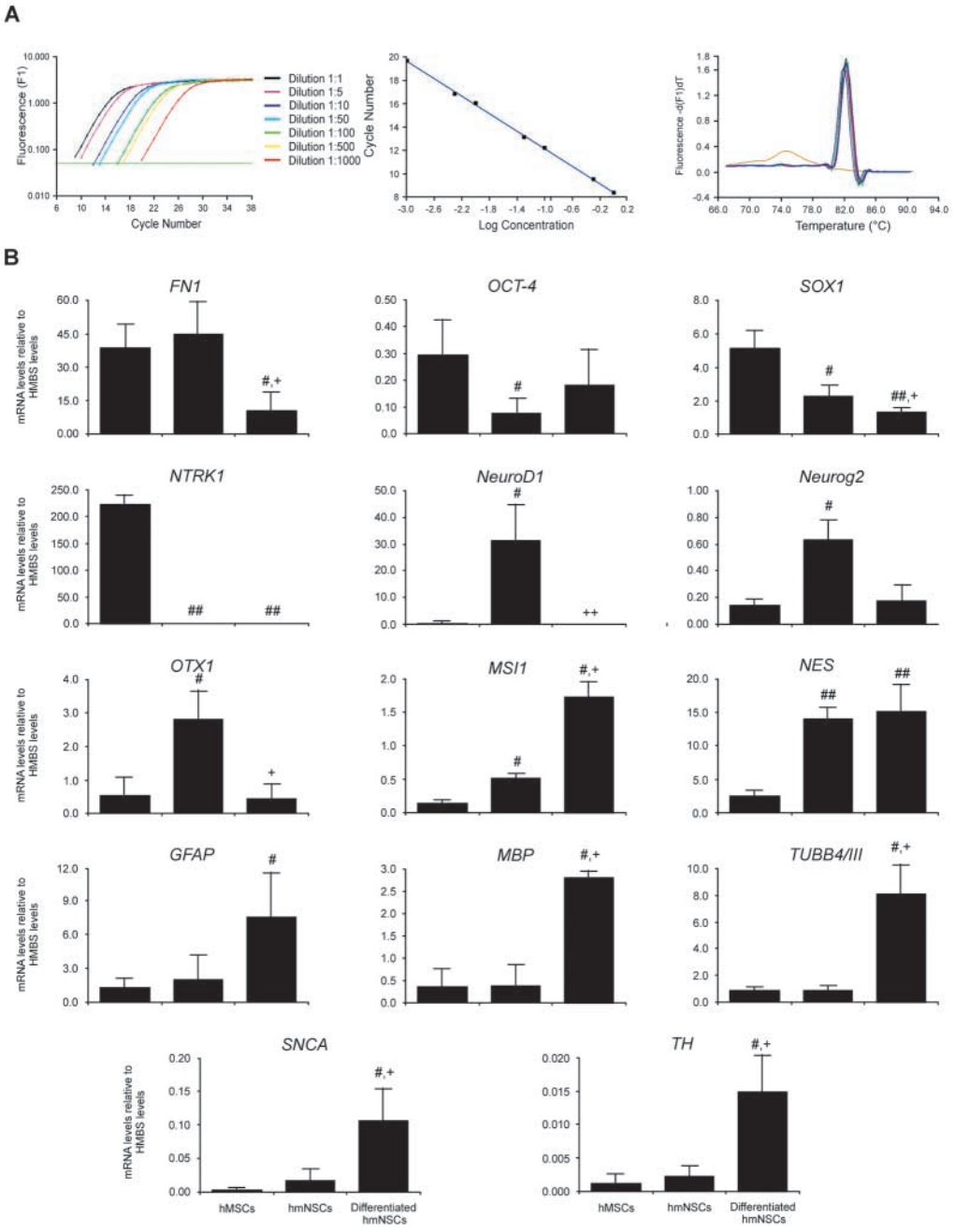


Fig. 3. Quantitative transcription profile of hMSC, hmNSCs and terminal differentiated hmNSCs into glial and neuronal cell types. (A) Representative real-time RT-PCR analysis using the LightCycler® technique. Plot of the fluorescence versus the cycle number obtained from SYBR Green detection of serially 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.

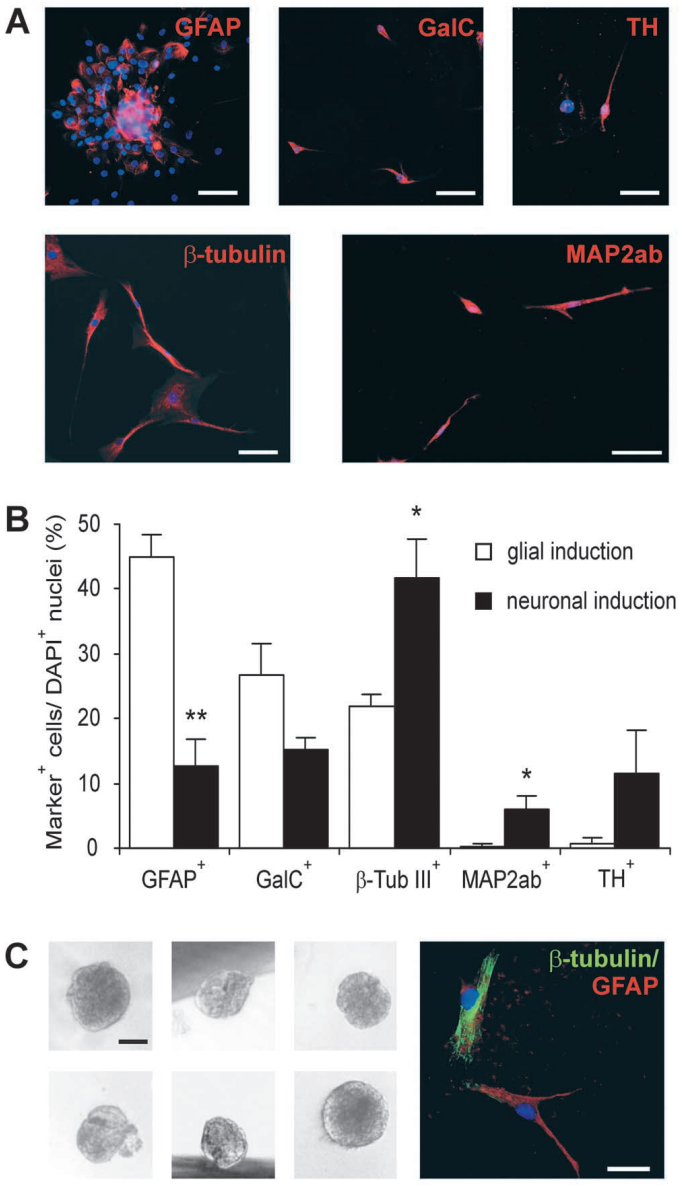


Fig. 4. In vitro differentiation of marrow-derived neurosphere-like structures into astroglial, oligodendroglial and neuronal cell types. hmNSCs were differentiated after 5-30 population doublings using the glial induction or the neuronal induction protocol on poly-L-lysine coated coverslips for 14 days. (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

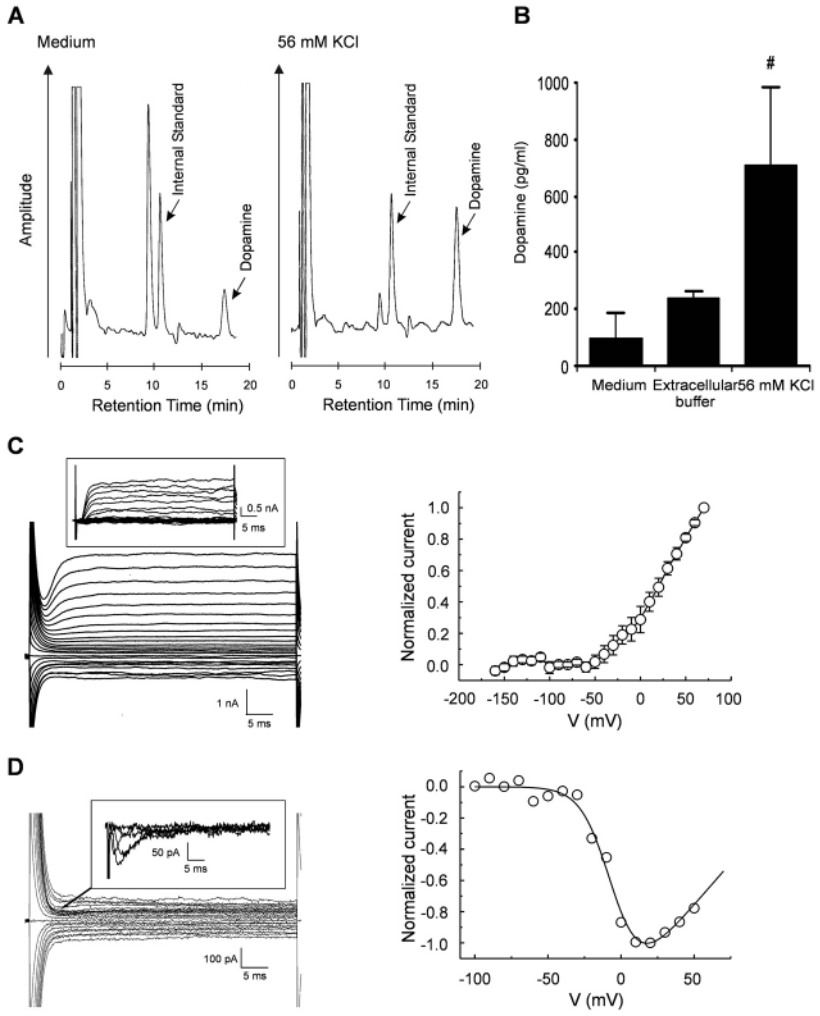


Fig. 5. Functional properties of terminal differentiated hmNSC. (A-B) Dopamine production and release was measured in hmNSCs differentiated using the neuronal induction protocol for 14 days. (A) Representative chromatograms of HPLC-ECD determination of dopamine in medium conditioned for 3 days (left) 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

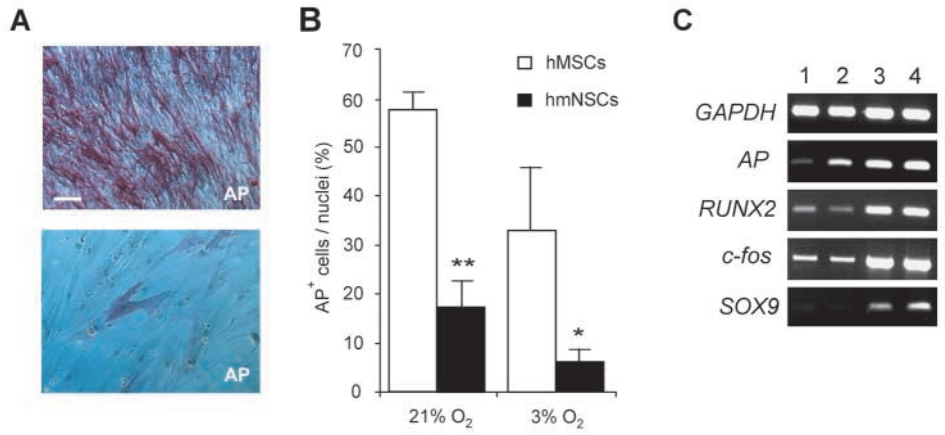


Fig. 6. Osteogenic differentiation ability of both hMSCs and hmNSCs. Both cell types were differentiated after 6-10 passages using the osteoblast differentiation protocol for 10 days. (A) Differentiated hMSCs (upper panel) and hmNSCs (lower panel) were stained for the osteogenic marker alkaline phosphatase (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.

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Comparative Analysis of Neuroectodermal Differentiation Capacity of Human Bone Marrow Stromal Cells Using Various Conversion Protocols

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Human adult bone marrow-derived mesodermal stromal cells (hMSCs) are able to differentiate into multiple mesodermal tissues, including bone and cartilage. There is evidence that these cells are able to break germ layer commitment and differentiate into cells expressing neuroectodermal properties. There is still debate about whether this results from cell fusion, aberrant marker gene expression or real neuroectodermal differentiation. Here we extend our work on neuroectodermal conversion of adult hMSCs in vitro by evaluating various epigenetic conversion protocols using quantitative RT-PCR and immunocytochemistry. Undifferentiated hMSCs expressed high levels of fibronectin as well as several neuroectodermal genes commonly used to characterize neural cell types, such as nestin, β-tubulin III, and GFAP, suggesting that hMSCs retain the ability to differentiate into neuroectodermal cell types. Protocols using a direct differentiation of hMSCs into a neural phenotype failed to induce significant changes in morphology and/or expression of markers of early and mature glial/neuronal cells types. In contrast, a multistep protocol with conversion of hMSCs into a neural stem cell-like population and subsequent terminal differentiation in mature glia and neurons generated relevant morphological changes as well as significant increase of expression levels of marker genes for early and late neural cell types, such as nestin, neurogenin2, MBP, and MAP2ab, accompanied by a loss of their mesenchymal properties. Our data provide an impetus for differentiating hMSCs in vitro into mature neuroectodermal cells. Neuroectodermally converted hMSCs may therefore ultimately help in treating acute and chronic neurodegenerative diseases. Analysis of marker gene expression for characterization of neural cells derived from MSCs has to take into account that several early and late neuroectodermal genes are already expressed in undifferentiated MSCs. © 2006 Wiley-Liss, Inc.

Key words: mesodermal stromal cells; neural differentiation; transdifferentiation; human stem cells

Bone marrow stromal cells are a heterogeneous population of cells providing ultrastructural and biochemical scaffolds within the hematopoietic microenvironment (Bianco et al., 2001; Vogel et al., 2003). These cells are derived from bone marrow cell suspensions either by their selective attachment to tissue culture plastic or by different depletion methods (Tondreau et al., 2004) and can be expanded efficiently (Friedenstein et al., 1976; Reyes et al., 2001; Sekiya et al., 2002). These cell cultures contain some multipotent, mesenchymal stem cells (or mesodermal stromal cells; MSCs) that can differentiate into osteogenic, chondrogenic, adipogenic, myogenic, and fibroblastic lineages (Friedenstein et al., 1976; Reyes et al., 2001; Sekiya et al., 2002). Even though adult stem cells are lineage restricted, which means that they can differentiate only into cells of their tissue origin, there is a growing body of evidence

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that these stem cells can break the barriers of germ layer commitment. For example, transplanted bone marrow cells contribute to endothelium (Rafii et al., 1994; Asahara et al., 1997; Lin et al., 2000; Jackson et al., 2001; Orlic et al., 2001, 2003) and skeletal muscle myoblasts (Ferrari et al., 1998; Gussoni et al., 1999) and acquire properties of hepatic and biliary duct cells (Petersen et al., 1999; Lagasse et al., 2000; Theise et al., 2000), lung, gut, and skin epithelia (Krause et al., 2001). Jiang and coworkers (2002b, 2003) recently demonstrated a rare multipotent adult progenitor cell (MAPC) within MSC cultures from rodent bone marrow. This cell type differentiates not only into mesenchymal lineages but also into endothelial and endodermal lineages. Mouse MAPCs injected into the blastocyst contribute to most if not all somatic cell lineages, including brain cell types (Jiang et al., 2002b). D'Ippolito and colleagues (2004) recently presented a method to purify human MSCs from dead specimens similar to the methods used by Jiang et al., the so called MIAMI cells (for marrow-isolated adult multilineage inducible cells).

Recent studies have supported the fact that MSCs are additionally able to express properties of neuroectodermal cells in vitro (Sanchez-Ramos et al., 2000; Woodbury et al., 2000, 2002; Black and Woodbury, 2001; Kohyama et al., 2001; Deng et al., 2001; Jiang et al., 2002b, 2003; Kabos et al., 2002; D'Ippolito et al., 2004; Tondreau et al., 2004) and in vivo after transplantation into the brain and spinal cord (Eglitis et al., 1999; Kopen et al., 1999; Brazelton et al., 2000; Chopp et al., 2000; Mezey et al., 2000, 2003; Nakano et al., 2001; Hofstetter et al., 2002). However, most of these studies reported only morphological data and/or marker expression and no functional properties of the neuroectodermally converted MSCs. Recently, Jiang and coworkers (2003) reported that mouse MAPCs can be induced to differentiate in vitro into cells with biochemical, anatomical, and electrophysiological characteristics of neuronal cells by using a coculture system with astrocytes. Dezawa and colleagues (2004) showed the induction of a mature neuronal phenotype in MSCs by transfection with the Notch intracellular domain, with high amounts of dopaminergic nerve cells and some cells firing action potentials. However, all functional data in this article were obtained in rodent MSCs. We recently reported a protocol for in vitro conversion of human MSCs into a neural stem cell-like cell type that can be subsequently differentiated into functional neuroectodermal cells without genetically modifying these cells (Hermann et al., 2004).

Despite these studies, some controversy persists regarding the in vivo differentiation potential of MSCs into neurons and glia (Wagers et al., 2002; Castro et al., 2002; Alvarez-Dolado et al., 2003; Ono et al., 2003; Weimann et al., 2003). In addition, several in vitro studies reevaluating the differentiation potential of MSCs towards a neural phenotype could not reproduce some of the transdifferentiation protocols (Lu et al., 2004; Neuhuber et al., 2004). Furthermore, neuroectodermal gene expression was already seen in MSCs before any differentiation (Corti et al., 2003; Tondreau et al., 2004). Together, a critical examination of the differentiation process, including a comparative analysis of the initial cell population (MSCs) and the converted neural cell types, is necessary to determine the validity of neuroectodermal differentiation capacity of bone marrow-derived MSCs.

In this report, we consequently compared various established in vitro protocols for the neuroectodermal differentiation of human MSCs via epigenetic stimulation by analyzing the expression pattern of a large set of both early and late neuroectodermal maker genes in the undifferentiated MSC population as well as the converted neural cell types by using quantitative real-time PCR and immunocytochemistry. We found that neuroectodermal genes commonly used to characterize neurectodermal cell types were already expressed in undifferentiated human MSCs. All protocols tested with a direct differentiation of MSCs into a neural phenotype did not induce significant changes in morphology and/or expression of markers of early and mature glial as well as neuronal cell types. However, the two-step conversion-differentiation protocol introduced by Hermann et al. (2004) was able to induce the combination of significant changes of the expression pattern of marker genes of early and late neural cell types accompanied by a loss of their mesenchymal properties.

MATERIALS AND METHODS

Cell Culture

Adult human bone marrow samples were harvested from routine surgical procedures (pelvic osteotomies; four samples; ages 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 (Fiedler et al., 2002; Fickert et al., 2003). Briefly, the hMSCs were cultured in a basal medium consisting of DMEM with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (all from Gibco, Tulsa, OK) at 37°C, 5% CO_2 in 95% humidity. Cells were passaged once per week. The hMSC phenotype was proved by FACS analysis with CD9, CD90, CD105, and CD166 (positive) as well as CD14, CD34, and CD45 (negative) and by the potential to differentiate into osteoblasts, chondrocytes, and adipocytes. hMSCs were expanded up to passage 10 (approximately 50 population doublings) and used for differentiation according to the protocols described below.

Neuroectodermal Conversion Protocols

We used six protocols for the in vitro conversion of hMSCs into neural cell types in order to analyze comparatively the neuroectodermal potential of hMSCs derived from adult human bone marrow (Fig. 1).

Protocol 1 (Sanchez-Ramos et al., 2000). Neurobasal medium (Gibco) was supplemented with 0.5 μmol $liter^{-1}$ all-trans-retinoic acid (Sigma) and 1% FCS, 5% horse serum, 1% N2 supplement, and 1% penicillin/streptomycin (all from Gibco) as well as 10 ng ml^{-1} rh-BDNF (neuronal induction; Promega, Madison, WI). Cells were differentiated for 10–14 days.

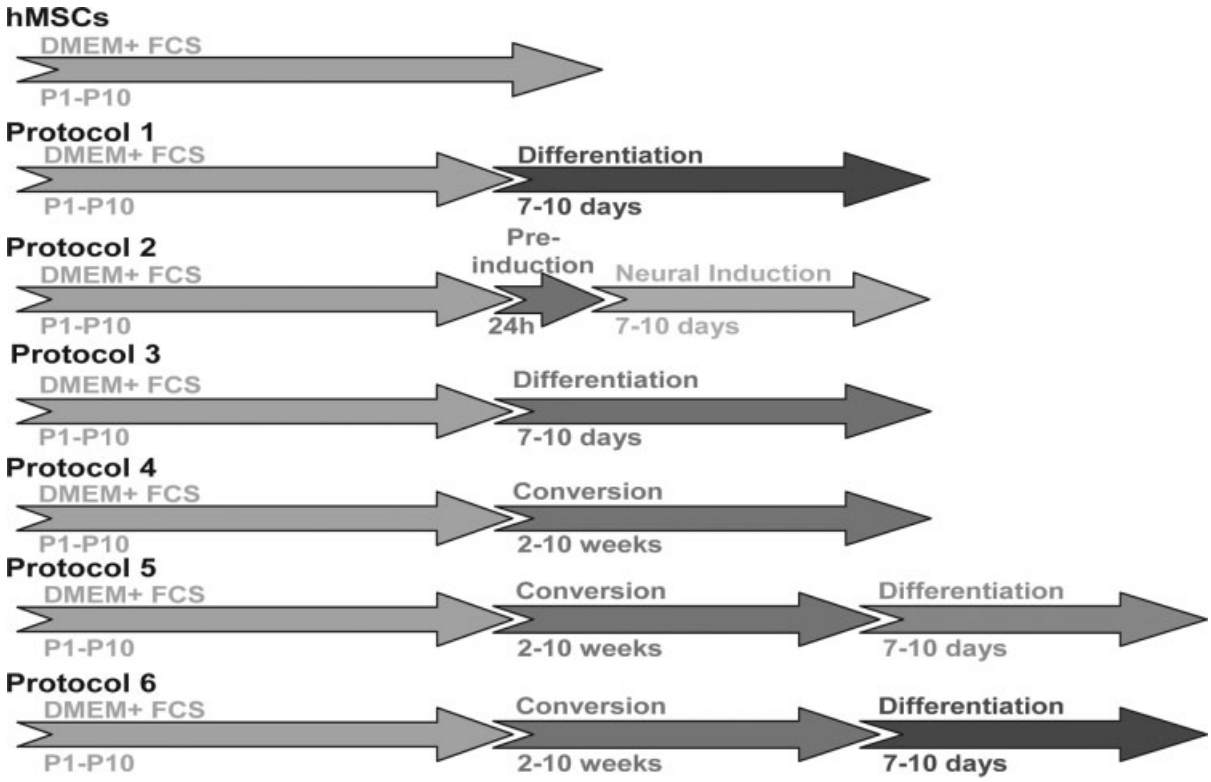

Fig. 1. Diagrams of protocols for neuroectodermal differentiation of human mesodermal stromal cells (MSCs; see Materials and Methods for details).

Protocol 2 (Woodbury et al., 2000). Protocol 2 was adapted from the methods described by Woodbury et al. (2000) for MSCs derived from rat bone marrow. Subconfluent cultures of hMSCs were maintained in DMEM/10% FBS. Twenty-four hours prior to neuronal induction, the medium was replaced with preinduction medium consisting of DMEM, 20% FBS, and 1 mM β-mercaptoethanol (BME). To initiate neuronal differentiation, the preinduction medium was removed, and the cells were washed with PBS and transferred to neuronal induction medium composed of DMEM, 2% dimethylsulfoxide (DMSO), and 200 mM butylated hydroxyanisole (BHA).

Protocol 3 (modified from Hermann et al., 2004). Induction of neuroectodermal differentiation was initiated by plating the cells on poly-L-lysine-coated glass coverslips at a concentration of 1.5–2.0 × 10^5 cells cm^{-2} in P4-8F medium (AthenaES, Baltimore, MD) without epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2) and differentiated for 10–14 days. The medium was changed once per week.

Protocols 4–6 (Hermann et al., 2004). In contrast to the previous protocols, these protocols comprised two conversion-differentiation steps (Fig. 1): First, the hMSCs were converted into neurosphere-like structures (hmNSCs, for human mesodermal-derived neural stem-like cells) by dissociation by 0.05% trypsin/0.04% EDTA and plating on tissue culture low-attachment plastic flasks (Nalge Nunc International, Rochester, NY) at a concentration of 1.0–2.0 × 10^5 cells cm^{-2} in P4-8F medium (AthenaES) supplemented with 20 ng ml^{-1} of both EGF and FGF-2 (both from Sigma, St. Louis, MO) at 5% CO_2, 92% N_2, and 3% O_2 (protocol 4). After 10–20 days, sphere formation could be observed. These neurosphere-like structures were then expanded for an additional 2–10 weeks (2–4 passages; 5–30 population doublings). The medium was changed once per week, and growth factors were added twice per week. The second step included the induction of terminal glial (protocol 5) or neuronal (protocol 6) differentiation. Thus, the hmNSCs were plated on poly-L-lysine-coated glass coverslips at a concentration of 1.5–2.0 × 10^5 cells cm^{-2} in Neurobasal medium (Gibco) supplemented with 0.5 μmol $liter^{-1}$ all-trans-retinoic acid (Sigma), and 1% FCS, 5% horse serum, 1% N2 supplement, and 1% penicillin/streptomycin (all from Gibco). For glial differentiation, the medium was supplemented with 10 ng ml^{-1} rh-PDGF-BB (R&D Systems, Minneapolis, MN), for neuronal differentiation with 10 ng ml^{-1} rh-BDNF (Promega, Madison, WI). Cells were differentiated for 10–14 days.

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 follow: TH monoclonal 1:1,000, β-tubulin III monoclonal 1:1,000, fibronectin monoclonal 1:400 (all from Sigma), MAP2ab

TABLE I. Primers for Quantitative Real-Time RT-PCR

Gene (protein)	Sequence (forward; reverse)	Product length (bp)
CALB1 (calbindin 1, 28 kDa)	5′-AGC TCC TGA GCT TGT CCT TG-3′	151
	5′-TGT CCA CGC TGT ACT GGT TC-3′	
FN1 (fibronectin)	5′-GAG ATC AGT GGG ATA AGC AGC A-3′	150
	5′-CCT CTT CAT GAC GCT TGT GGA-3′	
FZD1 (frizzled homologue 1)	5′-GGA TTG GCA TTT GGT CAG TG-3′	166
	5′-CTT GTC ATT ACA CAC CAC TCG G-3′	
GFAP (GFAP)	5′-GAG GCG GCC AGT TAT CAG GA-3′	168
	5′-GTT CTC CTC GCC CTC TAG CA-3′	
HMBS (hydroxymethylbilane synthase)	5′-TCG GGG AAA CCT CAA CAC C-3′	155
	5′-CCT GGC CCA CAG CAT ACA T-3′	
MBP (myelin basic protein)	5′-CGG CAA GAA CTG CTC ACT ATG-3′	106
	5′-GTG CGA GGC GTC ACA ATG-3′	
MSI1 (musashi 1)	5′-GCC CAA GAT GGT GAC TCG-3′	114
	5′-ATG GCG TCG TCC ACC TTC-3′	
NEUROD1 (neurogenic differentiation 1)	5′-CGC TGG AGC CCT TCT TTG-3′	118
	5′-GCG GAC GGT TCG TGT TTG-3′	
NGN2 (neurogenin 2)	5′-CGC ATC AAG AAG ACC CGT AG-3′	173
	5′-GTG AGT GCC CAG ATG TAG TTG TG-3′	
NES (nestin)	5′-TGG CTC AGA GGA AGA GTC TGA-3′	148
	5′-TCC CCC ATT TAC ATG CTG TGA-3′	
NOTCH1 (Notch homolog 1 translocation associated)	5′-GCG ACA ACG CCT ACC TCT-3′	133
	5′-GCA CAC TCG TAG CCA TCG-3′	
NTRK1 (neurotrophic tyrosine kinase, receptor, type 1)	5′-CTA CAG CAC CGA CTA TTA CCG-3′	128
	5′-CGA TTG CCT CCG TGT TG-3′	
OCT-4 (octomer-binding transcription factor 4)	5′-GTA TTC AGC CAA ACG ACC ATC-3′	176
	5′-CTG GTT CGC TTT CTC TTT CG-3′	
OTX1 (orthodenticle homolog 1)	5′-CAC TAA CTG GCG TGT TTC TGC-3′	121
	5′-AGG CGT GGA GCA AAA TCG-3′	
OTX2 (orthodenticle homolog 2)	5′-CAC TTC GGG TAT GGA CTT GC-3′	153
	5′-CGG GTC TTG GCA AAC AGT G-3′	
PTCH (patched homolog)	5′-CTG GCA GGA GGA GTT GAT TG-3′	171
	5′-GCC GCT TTG TCC TCG TTC-3′	
SNCA (α-synuclein)	5′-AGG ACT TTC AAA GGC CAA GG-3′	187
	5′-TCC TCC AAC ATT TGT CAC TTG C-3′	
SOX1 (sex determining region Y-box 1)	5′-GCC CAG GAG AAC CCC AAG-3′	177
	5′-CGT CTT GGT CTT GCG GC-3′	
SOX2 (sex determining region Y-box 2)	5′-CAG GAG AAC CCC AAG ATG C-3′	112
	5′-GCA GCC GCT TAG CCT CG-3′	
SOX10 (sex determining region Y-box 10)	5′-GCA AGG CAG ACC CGA AGC-3′	140
	5′-GTC CAA CTC AGC CAC ATC AAA G-3′	
TH (tyrosine hydroxylase)	5′-AGC TCC TGA GCT TGT CCT TG-3′	142
	5′-TGT CCA CGC TGT ACT GGT TC-3′	
TUBB4/III (β-tubulin III)	5′-AGT GAT GAG CAT GGC ATC GA-3′	317
	5′-AGG CAG TCG CAG TTT TCA CA-3′	

monoclonal 1:300 and GFAP monoclonal 1:1,000 (both Pharmingen, San Diego, CA), GalC monoclonal 1:750, GFAP polyclonal 1:1,000, nestin polyclonal 1:500 (all from Chemicon, Temecula, CA), and fluorescence-labeled secondary antibodies (Jackson, West Grove, PA).

RNA Extraction and Quantitative Real-Time RT-PCR Analysis

Total cellular RNA was extracted from hMSCs, hmNSCs (protocol 4) and neuroectodermally differentiated hMSCs or hmNSCs (protocol 1–3, 5, 6) by using RNAeasy total RNA purification kit, followed by treatment with RNase-free DNase (Qiagen, Hilden, Germany). Quantitative real-time one-step RT-PCR was carried out by using the LightCycler System (Roche, Mannheim, Germany), and amplification was monitored and analyzed by measuring the binding of the fluorescence dye Sybr green I to double-stranded DNA. One microliter of total RNA was reversely transcribed and subsequently amplified by using QuantiTect Sybr green RT-PCR Master mix (Qiagen) and 0.5 μmol liter^{-1} of both sense and antisense primers. After amplification, melting curves of the RT-PCR products were acquired, demonstrating product specificity. Results are expressed relative to the housekeeping gene *HMBS* (hydroxymethylbilane synthase). Primer sequences and lengths of the amplified products are summarized in Table I.

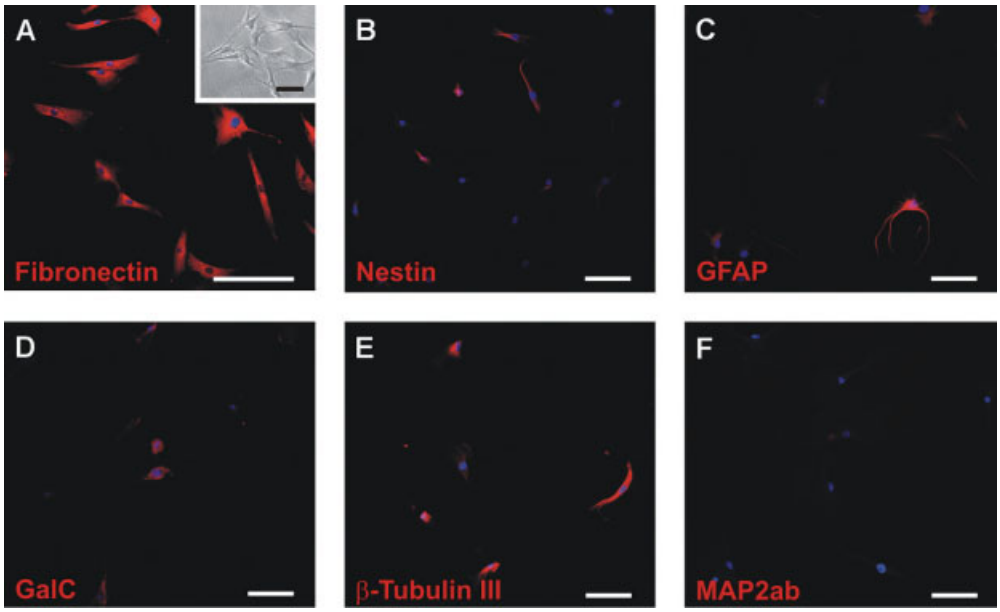

Fig. 2. Morphology and marker expression in adult human mesodermal stromal cells (hMSC) during expansion. hMSCs were expanded for up to 10 passages and stained for the mesodermal marker fibronectin (**A**), neural stem cells marker nestin (**B**), astroglial marker GFAP (**C**), oligodendroglial marker GalC (**D**), and neuronal markers β-tubulin class III (**E**) and MAP2ab (**F**). Nuclei were counterstained with DAPI (blue). Panels show representative immunofluoresence images; *inset* displays phase-contrast microphotography of hMSCs. Note that hMSCs express all markers tested (in particular, fibronectin) except for the mature nerve cell marker MAP2ab (for semiquantitative results of immunostainings refer to Table II). Scale bars = 100 μm.

Cell Counting and Statistical Analysis

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, 500–1,000 cells in total were counted per marker. Statistical comparisons were made by Dunnett's *t*-test. If data were not normally distributed, a nonparametric test (Mann-Whitney U-test) was used for comparisons of results. All data are expressed as mean ± SEM.

RESULTS

Neuroectodermal Marker Expression in Human MSCs

Although the neuroectodermal conversion of adult MSCs was investigated in several studies (Sanchez-Ramos et al., 2000; Woodbury et al., 2000; Hermann et al., 2004; Bossolasco et al., 2005), knowledge of the neuroectodermal properties and marker expression in hMSCs is limited. We investigated marker molecules for neurons (β-tubulin III, MAP2ab), astrocytes (GFAP), and oligodendrocytes (GalC) as well as neural progenitor cells (nestin) by immunostaining. As displayed in Figure 2, all neuroectodermal markers were already expressed by hMSCs, except for the mature nerve cell marker MAP2ab, with 44% ± 13% of cells being β-tubulin III$^+$, 29% ± 3% GFAP$^+$, and 19% ± 6% GalC$^+$ (n = 3). Only 4% ± 3% of cells were slightly positive for nestin, whereas bright staining was seen with antibodies against the mesodermal marker fibronectin in over 70% of the cells (n = 3; Fig. 2, Table II). We extended our gene expression analysis by using quantitative real-time RT-PCR. hMSCs expressed high levels of *FN1*, encoding the mesodermal marker gene fibronectin, FZD1 (human frizzeled homolog 1), and *NTRK1* (neurotrophin receptor kinase 1; Fig. 3). Interestingly, *OCT-4*, a marker for multipotency normally expressed by embryonic stem cells, was expressed in low levels in human MSCs. With regard to the expression of neuroectodermal genes, hMSCs expressed low, but detectable, levels of stem cells markers [*NES* (nestin), *MSI1* (Musashi1)] or early neurogenic markers (*OTX1*, *SOX1*, *SOX2*, *SOX10*, *NGN2* (neurogenin2), and *NEUROD1* (neuroD1); Fig. 3]. Interestingly, also some glial (*GFAP*, *MBP*) as well as immature neuronal markers [*TUBB4/III* (β-tubulin class III)] are expressed by hMSCs. However, hMSCs did not significantly express *OTX2*, *SNCA* (α-synuclein) as a mature neuronal marker or TH, a marker for catecholaminergic cells (Fig. 3; for complete names of genes and encoded proteins, refer to Table I). hMSCs proliferated for at least 10 weeks barely changing their morphology and phenotype (doubling time 1.5 days; 50 population doublings).

Gene Expression Pattern in hmNSCs

We next compared the gene expression pattern of hMSCs differentiated into immature neuroectodermal

TABLE II. Comparative Analysis of Various Protocols for Neuroectodermal Differentiation of hMSCs*

Differentiation conditions	β-Tubulin III$^+$ neurons	MAP2ab$^+$ neurons	GFAP$^+$ astrocytes	GalC$^+$ oligoden drocytes	FN$^+$ cells
Human MSCs	+++	−	++	++	++++
Protocol 1	+++	−	++	n.d.	++++
Protocol 2	+++	−	++	n.d.	++
Protocol 3	++++	−	+++	n.d.	++++
Protocol 4	n.d.	−	n.d.	n.d.	++
Protocol 5[a]	++	−	+++	++	++
Protocol 6[a]	+++	++	++	++	++

*Immunostainings were scored as − if no cells were positive for the respective marker, + if up to 5% of cells were positive, ++ if more than 5% and less than 40% of cells were positive, +++ if more than 40% and less than 70% of cells were positive, and ++++ if more than 70% of cells were positive; n.d., not determined.
[a]Data from Hermann et al. (2004).

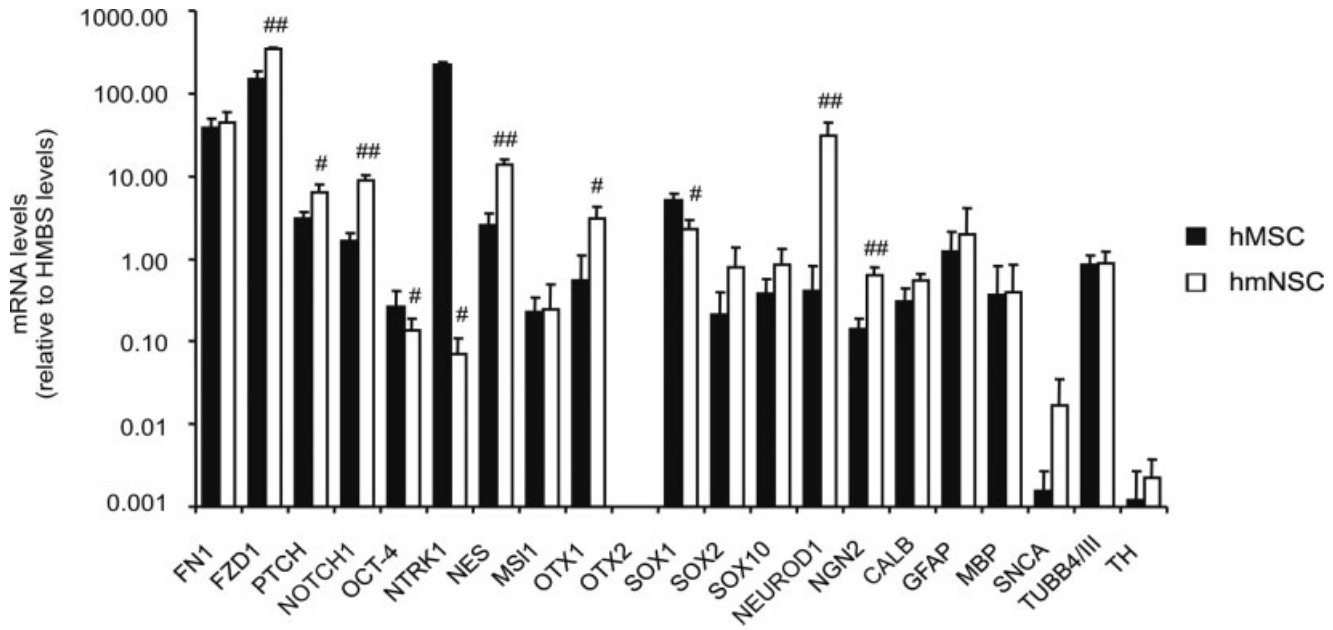

Fig. 3. Quantitative transcription profile of undifferentiated hMSCs and hmNSCs (protocol 4). Real-time RT-PCR analyses of mesodermal genes (*FN1*), genes expressed by multiple tissues (*FZD1, PTCH, NOTCH1*), proneural genes (*SOX1, SOX2, SOX10, OTX1,OTX2, NEUROD1, NGN2, CALBB*), NSC marker genes (*NES, MSl1*), glial genes (*GFAP, MBP*), and neuronal genes (*TUBB4/III, SNCA, TH*) as well as *OCT-4* as a marker for pluripotency. Expression levels are expressed relative to the housekeeping gene *HMBS* (hydroxymethylbilane synthase). For primers and complete names of genes of amplified PCR products see Table I. Data shown are mean values ± SEM from at least three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with levels measured in hMSCs

neural stem cell-like cells (hmNSCs) in protocol 4 with that described for hMSCs. Therefore, we extended our quantitative real-time RT-PCR analysis in hMSCs as well as in hmNSCs (Hermann et al., 2004). hmNSCs expressed high levels of *FN1*, *FZD1*, and *NOTCH1* (Notch homolog 1 translocation associated) as well as the neuroectodermal gene *NES* (encoding nestin) and the proneural genes *NEUROD1* and *PTCH* (patched homolog; Fig. 3). We additionally found detectable mRNA levels of *OCT-4*; the proneural genes *MSI1*, *NGN2*, and *SOX1/10*; the glial genes *GFAP* and *MBP* (myelobasic protein); and the neuronal marker gene *TUBB4/III*. hmNSCs did not significantly express *OTX2*, *SOX2*, *SNCA*, or *TH* (Fig. 3). In comparison with the initial cell population (hMSCs), we found a significant up-regulation of *FZD1*, *PTCH*, and *NOTCH1* as well as the neuroectodermal genes *NES*, *OTX1*, *NEUROD1*, and *NGN2* (Fig. 3). mRNA levels of *OCT-4*, *NTRK1*, and *SOX1* were significantly reduced in hmNSCs compared with hMSCs (Fig. 3). Together, the conversion of hMSCs into an NSC-like cell population (hmNSCs) by protocol 4 resulted in an up-regulation of various proneural and early neuroectodermal genes.

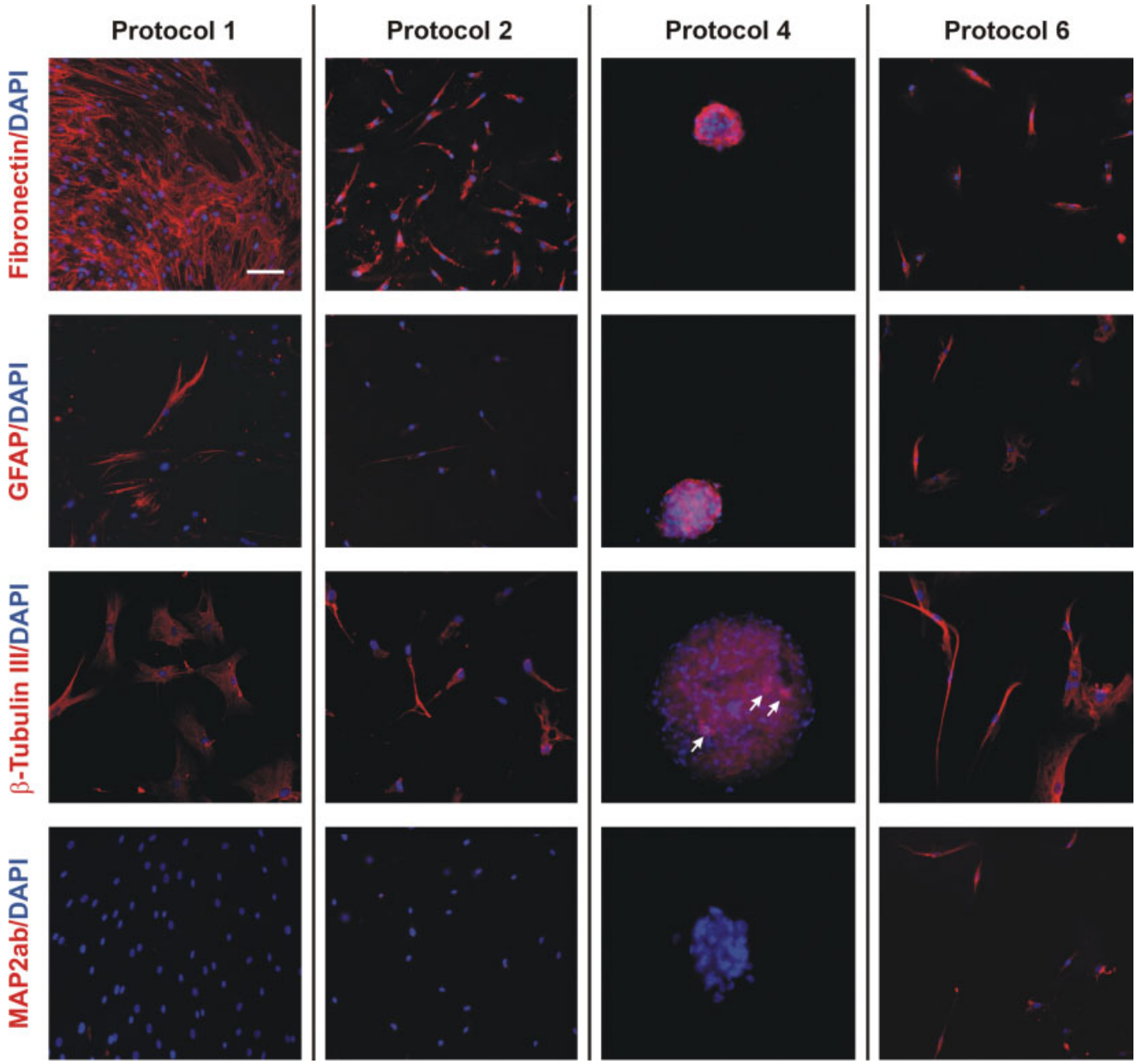

Fig. 4. Morphology and marker expression of adult human mesodermal stromal cells (hMSC) after differentiation according to protocol 1, 2, 4, or 6. hMSCs or hmNSCs were differentiated according to the protocol and stained for fibronection, GFAP, β-tubulin III, or MAP2ab. Nuclei are counterstained with DAPI (blue). Note that only hmNSCs differentiated according to protocol 6 expressed the mature nerve cell marker MAP2ab. For semiquantitative results of immunostainings refer to Table II. Scale bar = 100 μm.

Immunocytochemical Analyses of Neuroectodermally Differentiated hMSCs

To prove the neuroectodermal potential of hMSCs, we compared various recently published protocols for neural induction of human MSCs (Sanchez-Ramos et al., 2000; Woodbury et al., 2000; Hermann et al., 2004; Fig. 1). First we investigated marker gene expression on the protein level by immunocytochemistry. We looked for markers of mesodermal cell types (fibronectin), astroglia (GFAP), oligodendroglia (GalC), and immature and mature neurons (β-tubulin III and MAP2ab, respectively) in hMSCs neuroectodermally differentiated by using protocols 1–3, 5, and 6 (Fig. 4, Table II). With protocol 1 according to Sanchez-Ramos and colleagues (2000), 12% ± 12% of cells acquired morphological and phenotypical characteristics of astrocytes ($GFAP^+$) and 62% ± 25% acquired characteristics of immature neurons (β-tubulin III^+), whereas none of the cells was positive for the mature neuronal marker MAP2ab (n = 3; Fig. 4, Table II).

Furthermore, 89% ± 1% of the cells still showed bright staining against the mesodermal marker fibronectin (Fig. 4). Together, protocol 1 did not produce significant changes in neural marker expression and morphology compared with the initial cell population of hMSCs. We next tested the neural induction protocol 2 developed by Woodbury and coworkers (2000), including a 1-day preincubation step with high serum levels, followed by a serum-free inducing medium. After 7–10 days of differentiation via protocol 2, we obtained under 15% glial cells ($GFAP^+$) as well as 60–70% β-tubulin III-expressing cells, whereas no $MAP2ab^+$ cell could be observed (Fig. 4, Table II); 30–40% of the differentiated hMSCs were still positive for fibronectin (Fig. 4, Table II). Similarly to the case with protocol 1, the neural induction failed to show significant changes from the starting population (hMSCs), except for a marked reduction of fibronectin expression. Protocol 3, using serum-free conditions to induce neural differentiation (Hermann et al., 2004), did not lead to relevant changes in neural marker expression compared with undifferentiated hMSCs (Table II).

The two-step protocols for differentiation into glial (protocol 5) and neuronal (protocol 6) cell types significantly reduced the expression of mesodermal marker protein fibronectin compared with hMSCs, but the expression of the immature neuronal marker β-tubulin III was unchanged (Fig. 4, Table II). Furthermore, protocol 5 induced an increase of cells expressing glial and oligodendroglial markers, whereas only protocol 6 generated mature neuronal phenotype demonstrated by the expression of MAP2ab (6% ± 2% $MAP2ab^+$ cells; n = 3; Fig. 4, Table II).

Gene Expression Pattern of Neuroectodermal Marker Genes in Differentiated hMSCs

We next extended our work previously published (Hermann et al., 2004) and systematically compared the gene expression pattern using quantitative real-time RT-PCR (refer to Fig. 5) in cells derived from hMSCs by the classical direct neuroectodermal differentiation protocols 1–3 (Sanchez-Ramos et al., 2000; Woodbury et al., 2000) and in cells derived from hmNSCs by the second differentiation step (protocols 5 and 6; Hermann et al., 2004). First, fibronectin mRNA was reduced only after differentiation using protocol 6 but was unchanged by protocol 1 compared with hMSCs (Fig. 5), which is in line with immunofluorescence data (Table II). Interestingly, with the glial induction protocol (protocol 5), *FN1* was even up-regulated. Next we compared the expression of the neural stem cell marker genes *NES* and *MSI1* as well as the proneural genes *SOX1*, *OTX1*, *NEUROD1*, and *NGN2* with the six protocols. Whereas there was a 5.6-fold increase in nestin expression through the conversion and differentiation protocol (protocol 6), differentiation by protocol 1 induced an 8,000-fold down-regulation of nestin expression. Furthermore, there were no significant changes in *NES* expression induced by protocols 2 and 3, but we detected a down-regulation of *NES* by protocol 5. Whereas all other early neuroectodermal marker genes were up-regulated during the conversion from hMSCs into hmNSCs by between 4.5- and 77.1-fold, they were unchanged (*OTX1*, *NEUROD1*), decreased (*NGN2*), or even undetectable (*MSI1*) in cells that had undergone differentiation according to protocols 1–3 (Fig. 5). With protocol 6 for terminal differentiation of hmNSCs, the early neuroectodermal marker gene *MSI1* was up-regulated, but *NES* was unchanged, and *NEUROD1*, *NGN2*, and *OTX1* were down-regulated during the terminal differentiation process (Fig. 5). Similarly, *NEUROD1*, *NGN2*, and *OTX1* were down-regulated during terminal differentiation with protocol 5, whereas *MSI1* remained unchanged (Fig. 5).

We next investigated mRNA levels of marker genes for more mature neuroectodermal cell lineages, such as *TUBB4/III* and *SNCA* for neurons, *GFAP* for astroglial cells, and *MBP* (myelin basic protein) for oligodendroglia, in neuroectodermally differentiated hMSCs (Fig. 5). With protocol 1–5 for differentiation of hMSCs or hmNSCs, there were no significant changes in mRNA levels of *TUBB4/III* in differentiated cells compared with their undifferentiated counterparts. In contrast, only protocol 6 induced a 8.9-fold increase of *TUBB4/III* expression in terminally differentiated hmNSCs compared with the initial hMSC population. We analyzed, as a second marker gene for more mature neurons, expression of the *SNCA* gene (α-synuclein), which is expressed only in neuronal cells. Although there were no significant changes in the expression pattern after differentiation using protocol 1–5, we could show a 70-fold up-regulation using our multistep protocol (6; refer to Fig. 5). Interestingly, TH, the rate-limiting enzyme for dopamine synthesis, was also uniquely up-regulated by protocol 6 (Fig. 5). The expression of the astroglial marker gene *GFAP* was down-regulated on the mRNA levels under the detection limit by protocol 1–3 but was up-regulated by about 6-fold in protocol 6 (Fig. 5). *MBP*, a marker gene for oligodendroglial cells (Barbarese et al., 1988), was already expressed in undifferentiated hMSCs but was highly down-regulated (133-fold) or remained unchanged by differentiating the cells with protocol 1–3 (Fig. 5). Protocol 6 led to a 6.7-fold increase of *MBP* mRNA levels compared with that measured in hMSCs (Fig. 5). Interestingly, the glial marker expression remained unchanged or even decreased using the ''glial'' induction protocol 5. Together, the direct differentiation of hMSCs into neural cells types by protocol 1–3 did not lead to a relevant up-regulation of early or late neuroectodermal marker genes, but the second step of the two-step conversion-differentiation protocol (protocol 6) resulted in a down-regulation of most early neural genes combined with an up-regulation of all marker genes for mature neuronal cell types tested (Fig. 5).

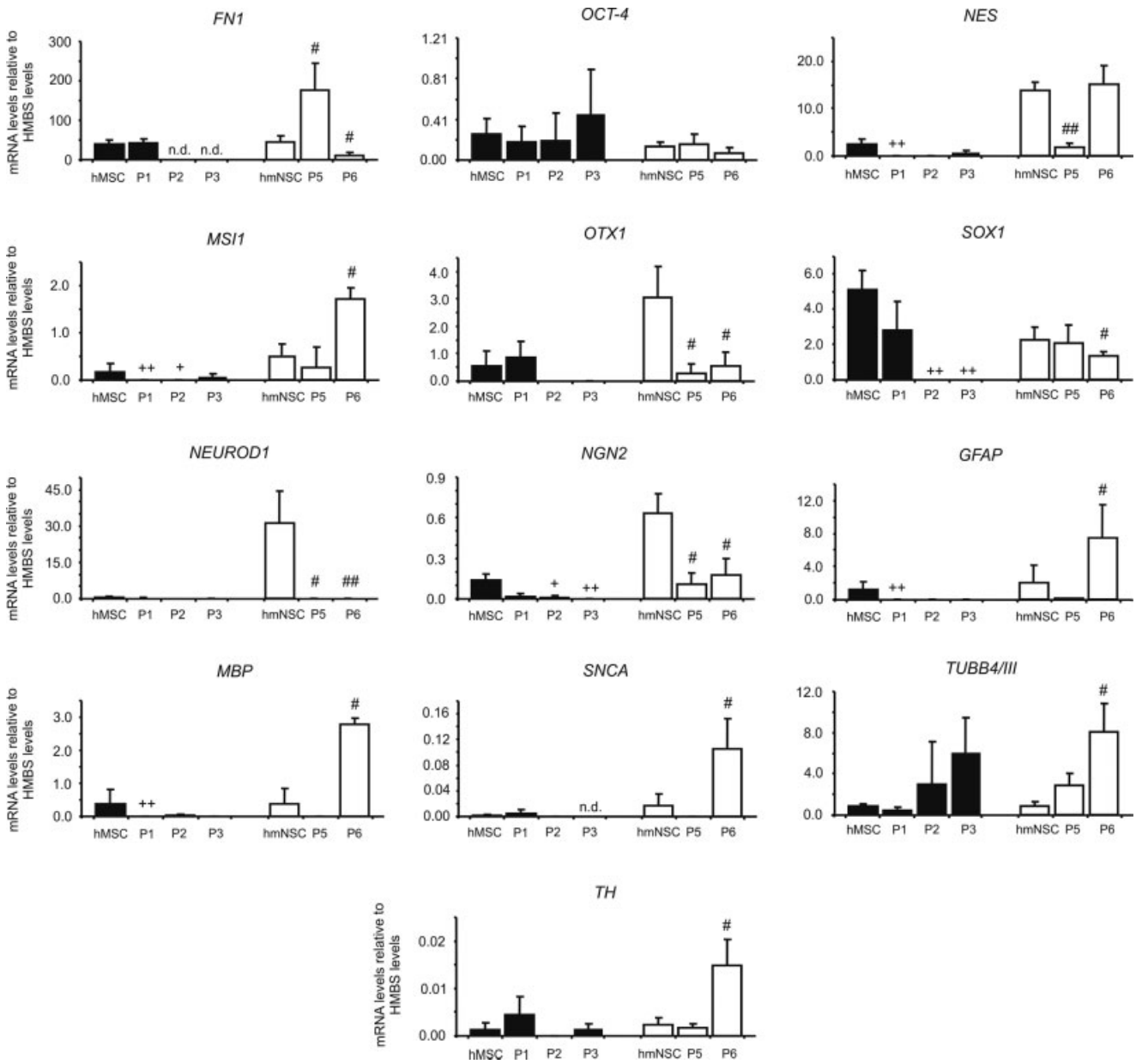

Fig. 5. Quantitative transcription profile of neuroectodermally differentiated hMSCs (protocols 1–3; solid bars) as well as terminal differentiated hmNSCs into glial and neuronal cell types (protocols 5 and 6; open bars) compared with their undifferentiated counterparts (hMSCs and hmNSCs, respectively). Real-time RT-PCR analyses of mesodermal genes (*FN1*), proneural genes (*SOX1, OTX1, NEUROD1, NGN2*), NSC marker genes (*NES, MSI1*), glial genes (*GFAP, MBP*), and neuronal genes (*TUBB4/III, SNCA, TH*) as well as *OCT-4* as a marker for pluripotency. Expression levels are expressed relative to the housekeeping gene *HMBS* (hydroxymethylbilane synthase). For primers and complete names of genes of amplified PCR products see Table I. Data shown are mean values ± SEM from at least three independent experiments. $^{+}P < 0.05$, $^{++}P < 0.01$ compared with levels measured in hMSCs. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with levels measured in hmNSCs. n.d., not determined.

DISCUSSION

The central findings of the present study are 1) that undifferentiated hMSCs obtained from human adult bone marrow express major markers commonly used to characterize early and mature neuroectodermal cells and 2) that conversion protocols using epigenetic stimulation for differentiation of hMSCs directly into mature neuroectodermal cells did not induce significant changes of morphology or neural marker expression. 3) In comparison, neuroectodermal differentiation of hMSCs by using a two-step protocol with initial conversion of hMSCs into immature neural stem cell-like cells (hmNSCs) and

subsequent specification into glial and neuronal cells seems to be more efficient and leads to significant morphological changes and a marker gene expression pattern typical for neural cell types.

Several studies have focused on multipotency of adult MSCs, showing that undifferentiated MSCs express marker genes from all three embryonic germ layers and the germline (Tremain et al., 2001; Woodbury et al., 2002; Jiang et al., 2002a; Seshi et al., 2003), including neuroectodermal genes (Woodbury et al., 2002; Corti et al., 2003; Tondreau et al., 2004; Bossolasco et al., 2005; Lu and Tuszynski, 2005). However, these studies have reported expression of only single or very few genes in mature glial or neuronal cells. Here we focus on the expression pattern of both early and late neuroectodermal marker genes expressed in hMSCs on mRNA and/or protein level. In our cell system, undifferentiated hMSCs already expressed low levels of early neuroectodermal markers such as NSC marker proteins (nestin and musashi1), several SOXB1 transcription factors, and proneural genes (neurogenin2 and neuroD1). Various glial genes and β-tubulin class III, a marker already expressed in immature neurons (Lee et al., 1990; Iacopetti et al., 1999; Rakic, 2002), were also found in hMSCs. On the other hand, no marker exclusively expressed by mature neurons, such as MAP2ab or α-synuclein, was observed, and additionally hMSCs lacked expression of neuronal functions such as sodium channels or action potentials (Kohyama et al., 2001; Hofstetter et al., 2002; Hung et al., 2002; Padovan et al., 2003; Hermann et al., 2004; Lu and Tuszynski, 2005). The pluripotency of hMSCs is further supported by the expression of Oct-4 (Pesce and Scholer, 2000; Hermann et al., 2004; Pochampally et al., 2004), which is characteristically expressed in embryonic cells and necessary for their pluripotency (Pesce and Scholer, 2000; Pochampally et al., 2004). Together, undifferentiated hMSCs express at low levels a pattern of neuroectodermal genes supporting their neural differentiation capacity. The expression of these genes demands a quantitative expression analysis of a complete set of early and late neuroectodermal marker genes to demonstrate conclusively neuroectodermal conversion of hMSCs.

There have been many studies over the last years reporting the conversion or transdifferentiation of hMSCs into neuroectodermal cells in vitro (Sanchez-Ramos et al., 2000; Woodbury et al., 2000; Jiang et al., 2003; Dezawa et al., 2004; Hermann et al., 2004; Bossolasco et al., 2005; Lu and Tuszynski, 2005). However, most of these studies lack data on neuroectodermal marker expression of the initial hMSC population and on quantitative analysis of changes of neuroectodermal and/or mesodermal properties of hMSCs during the conversion process (Sanchez-Ramos et al., 2000; Woodbury et al., 2000; Jiang et al., 2002a; Lee et al., 2003; Munoz-Elias et al., 2004; Neuhuber et al., 2004; D'Ippolito et al., 2004; Bossolasco et al., 2005; Lu and Tuszynski, 2005). To contribute to this discussion, we evaluated the differentiation process of hMSCs and compared various conversion protocols used to induce hMSCs towards a neuroectodermal phenotype by epigenetic stimulation. Because the presence of a subset of neuronal markers cannot be taken to prove commitment to a neuronal fate (Lu et al., 2004; Neuhuber et al., 2004), we analyzed a large set of both early and late neuroectodermal marker genes by using quantitative real-time PCR and/or immunocytochemistry. Undifferentiated hMSCs already express several phenotypic properties of neural cells, so all data were quantitatively compared with data obtained from undifferentiated hMSCs.

Consistently with previous reports (Sanchez-Ramos et al., 2000; Woodbury et al., 2000; D'Ippolito et al., 2004; Bossolasco et al., 2005), we could detect several neuronal markers after differentiation of hMSCs by direct conversion protocols [protocol 1 (Sanchez-Ramos et al., 2000), protocol 2 (Woodbury et al., 2000), and protocol 3 (modified from Hermann et al., 2004)]. However, in comparison with undifferentiated hMSCs, there were no significant differences in any of the markers tested (Table II, Fig. 5). Furthermore, no expression of the mature neuronal marker MAP2ab was observed, and there was no significant reduction in the expression levels of fibronectin and *OCT-4*. Together, these data pointed most likely towards aberrant induced gene expression rather than towards a sequence of gene expression as is required for neurogenesis (Neuhuber et al., 2004). Indeed, Neuhuber and colleagues (2004) showed that changes in morphology upon addition of the chemical induction medium are caused by rapid disruption of the cytoskeleton. Thus the decrease in fibronectin staining by protocol 2 seems to be most likely derived from these cytoskeleton changes, as recently described by two independent groups (Lu et al., 2004; Neuhuber et al., 2004). In contrast, two-step transdifferentiation with initial conversion into immature NSC-like cells and subsequent terminal neural differentiation led to significant changes in morphology and marker expression pattern by both conversion steps. As shown previously (Lee et al., 2003; Hermann et al., 2004; Bossolasco et al., 2005), the conversion of hMSCs into NSC-like cells under serum-free condition, but supplementation with EGF and FGF-2 revealed a neurosphere-like morphology. Here we additionally demonstrated a dramatic change of the expression pattern compared with that of hMSCs, with reduction of the mesodermal marker fibronectin as well as increase of early neuroectodermal genes, such as NSC markers, proneural genes, and other markers expressed by neural progenitor or stem cells, such as OTX1, PTCH, or NOTCH1 (Fig. 3).

Terminal neural differentiation of hmNSCs into mature neural cell populations (protocol 5 for glial and protocol 6 for neuronal specification) revealed a maturation of the cells, with an increase of cells with a glial/oligodendroglial phenotype ($GFAP^+/GalC^+$) by protocol 5 and an increase of neuronal cells, with up to 7% of cells showing a mature neuronal phenotype expressing MAP2ab produced by protocol 6. There is to our

knowledge no report showing MAP2ab$^+$ cells on the protein level derived from hMSCs. Interestingly, in contrast to the protein level, re-evaluating protocol 5 on mRNA level revealed no changes in GFAP or MBP expression compared with hmNSCs, most likely because of different time courses of gene expression on the mRNA and protein level. The systematic investigation of neuroglial differentiation capacity of hMSCs by Bossolasco and colleagues (2005) also described neurosphere-like structures derived from hMSCs but did not report on subsequent differentiation of these cellular aggregates (Bossolasco et al., 2005). For directly differentiated hMSCs, this research group was able to show low levels of MAP2ab at the mRNA level by conventional semiquantitative RT-PCR, but no MAP2ab$^+$ cells (Bossolasco et al., 2005). Noteworthy, concerning the two-step conversion protocol, was that neither step 1 (protocol 4) nor step 2 (identical to protocol 1) alone was able to induce a mature neuroectodermal phenotype, but the sequential two-step approach seems to be important. However, all protocols tested in the present study have to be further optimized to improve maturation and purity of glial and/or neuronal cells derived from human MSCs.

Our systematic analysis of early and late neural genes via quantitative real-time PCR during the conversion-differentiation procedure further supports that the changes in gene expression are not due to aberrant gene expression but seem to be a sequence related to a neuroectodermal conversion. We therefore analyzed two common NSC markers, namely, the posttranscriptional regulator musashi1 (Kaneko et al., 2000) and the intermediate filament protein nestin (Cattaneo and McKay, 1990; Lendahl et al., 1990; Dahlstrand et al., 1995; Johansson et al., 1999), the SOXB1 transcription factor *SOX1* marking neural progenitor cells (Uwanogho et al., 1995; Pevny et al., 1998; Bylund et al., 2003), the neural Otx homeobox gene *OTX1*, as well as several proneural transcription factors of the basic helix-loop-helix (bHLH) family (Simeone, 1998; Acampora et al., 2001; Franklin et al., 2001; Nieto et al., 2001; Simmons et al., 2001). Overall, the conversion of hMSCs into hmNSC (protocol 4) leads to significant up-regulation of the expression of these early neuroectodermal genes (Fig. 3), whereas terminal differentiation of hmNSCs (protocols 5 and 6) resulted in down-regulation of most of these genes (Fig. 5). The remaining high expression levels of some of these early genes (for example, nestin and musashi1) after terminal neural differentiation most likely indicate still a premature state of neuronal development. Consistently, Jiang and colleagues showed the acquisition of a neuronal phenotype using a similar protocol to differentiate mouse MAPCs with a fully mature neuronal phenotype only after coculturing the cells with primary glial cells (Jiang et al., 2003). In contrast to the two-step conversion protocols, direct neuroectodermal differentiation of hMSCs (protocols 1–3) did not lead to significant changes in the neural gene expression pattern compared with undifferentiated hMSCs (Fig. 5). Together, the transient expression of early neurogenic/NSC marker genes exclusively within this conversion protocol further suggests the importance and necessity of the two-step technique, including a NSC stage. However, according to Neuhuber and colleagues (2004), future studies should investigate the whole transcriptome with the gene array technique during the neuroectodermal conversion process of hMSCs to confirm further the neuroectodermal differentiation capacity of these multipotent adult stem cells.

The present study relies only on morphological changes and neural marker gene expression on the mRNA and protein level to define the events during neuroectodermal conversion of hMSCs. Some previous studies attempted to perform electrophysiological experiments on putative neurons derived from MSCs without showing voltage-gated potassium/sodium channels or action potentials (Kohyama et al., 2001; Hofstetter et al., 2002; Hung et al., 2002; Padovan et al., 2003). In our preliminary analysis of neural cells derived by the two-step conversion protocol, we were able to show that hmNSCs differentiate into mature glial cells showing functional expression of outward-rectifying potassium channels and sodium channels in glial cells (Hermann et al., 2004). However, future experiments are warranted to define the functional neuronal properties further, including action potentials accompanied by synaptic neurotransmitter release in fully differentiated hmNSCs via coculture methods with astroglial cells and/or longer differentiation periods (Song et al., 2002; Jiang et al., 2003; Westerlund et al., 2003; Neuhuber et al., 2004). The data presented here provide further evidence for a neuroectodermal differentiation capacity of human MSCs, but a sequential differentiation of MSCs into NSC-like cell type and subsequently into mature neural cells seems to be necessary for neuroglial specification of human adult bone marrow stromal cells.

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Expert Opinion

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Epigenetic conversion of human adult bone mesodermal stromal cells into neuroectodermal cell types for replacement therapy of neurodegenerative disorders

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Tissue-specific stem cells, such as bone marrow-derived mesodermal stromal cells (MSCs), are thought to be lineage restricted and, therefore, could only be differentiated into cell types of the tissue of origin. Several recent studies, however, suggest that these types of stem cells might be able to break barriers of germ layer commitment and differentiate *in vitro* and/or *in vivo* into cells of different tissues, such as neuroectodermal cell types. Recently, protocols for high-yield generation of undifferentiated neural stem cell (NSC)-like cells from MSCs of primate and human origin were reported. Undifferentiated NSCs are commonly used and are more suitable for neurotransplantation compared with fully differentiated neural cells, as differentiated neural cells are well known to poorly survive detachment and subsequent transplantation procedures. These human MSC-derived NSC-like cells (MSC-NSCs) grow in neurosphere-like structures and express high levels of early neuroectodermal markers, but lose characteristics of MSCs. In the presence of selected growth factors, human MSC-NSCs can be differentiated into the three main neural phenotypes: astroglia, oligodendroglia and neurons. Compared with direct differentiation of human MSCs into mature neural cells, the conversion step seems to be essential to generate mature functional neuroectodermal cells. This review describes the techniques for the conversion of human MSCs into NSCs and summarises the data on epigenetic conversion of human MSCs into immature neuroectodermal cells. These cells provide a powerful tool for investigating the molecular mechanisms of neural differentiation, and might serve as an autologous cell source to treat acute and chronic neurodegenerative diseases.

Keywords: bone marrow cells, mesodermal stromal cells, neural stem cells, neurodegenerative disease transplantation

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1. Introduction

The ongoing obsolescence of our society raises new aspects in modern medicine. Specifically, neurologists are confronted with increasing prevalences of neurodegenerative diseases. Not only have Parkinson's or Alzheimer's disease become more frequent with increasing lifespan, but stroke and traumatic brain injury are also responsible for a decline in neuronal function. Furthermore, in chronic inflammatory CNS diseases, such as multiple sclerosis, a loss of several types of neuroectodermal cells occurs. There is, therefore, a growing need for therapeutic

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approaches to restore neural cell loss or reconstitute physiological function. Cell transplantation is one of the strategies with potential for treatment of such neurological disorders, and many kinds of cells, including embryonic stem (ES) cells and neural stem cells (NSCs), have been considered as candidates for transplantation therapy (for review see [1-5]). ES cells and NSCs seem to be very feasible for neurorestorative strategies compared with primary tissue, as these cell systems fulfil many important requirements for the use of these cells in regenerative treatment strategies [1,6,7], such as the generation of high yields of cells from a small starting population without losing their differentiation potential, on-demand availability of cells without major logistical problems, and the possibility to standardise the cell source in a clinical setting.

Stem cells are unspecialised cells with the ability to self-renew indefinitely and, under appropriate conditions, they can give rise to a definite range of mature cell types. Compared with totipotent ES cells, neural stem or progenitor cells have been categorised as multipotent tissue-specific stem cells, producing all kinds of brain-specific cell types, such as neurons, glia and oligodendroglia, and may also replace or repair diseased brain tissue. Clonogenic NSCs can be directly isolated from fetal or adult nervous tissue, or derived from ES cells [4,5,8-12]. In the adult brain, NSCs have been consistently demonstrated within the hippocampus and subventricular zone (SVZ) of the lateral ventricles [13-15]. These cells grow *in vitro* as spherical floating clusters (neurospheres) in the presence of epidermal growth factor (EGF) and/or fibroblast growth factor (FGF)-2 (for review see [1]).

Alternatively, multipotent adult stem cells are of special interest with regards to autologous transplantation approaches, probably, without immunological rejection. An important source of multipotent adult stem cells is bone marrow stromal cells ([MSCs]; also known as mesodermal stromal cells or mesenchymal stem cells). In contrast to human ES cells or human brain-derived NSCs, human MSCs (hMSCs) are easy to isolate and can be expanded over a long period of time without serious ethical and technical problems. Bone marrow-derived MSCs can differentiate into several types of mesenchymal cells, including osteocytes, chondrocytes and adipocytes [16-18]. In addition, it has been reported that under appropriate experimental conditions MSCs differentiate into non-mesenchymal cell populations, such as glial and neuronal cells [19-23]. These observations have raised interest in the possible use of MSCs in cell therapy strategies for various neurological disorders. The data on neuroectodermal transdifferentiation of hMSCs are reviewed here. The authors focus on protocols using epigenetic approaches to change the phenotype without alterations of sequences of bases in genomic DNA (**Table 1**). Furthermore, the characteristics of generated neural cell types, as well as their potential in regenerative approaches in acute and chronic neurodegenerative diseases, are discussed.

2. Transgerminal conversion of tissue-specific stem cells

During development, a stepwise commitment of cell fate is occurring, starting from the totipotent ES cell, followed by germ layer commitment (pluripotent stem cell with the potential to differentiate into all cell types of the respective germ layer) and further lineage restriction (multipotent stem cell), ending up in the terminal differentiated cell stage, which is the functional cell stage in most tissues. This developmental process is called differentiation or specification. It is noteworthy that there is no conclusive evidence for the presence of a pluripotent germ layer-committed stem cell from the literature. On the other hand, there are processes called metaplasia with conversions between developmentally unlinked cell stages or cell types. Naturally occurring metaplasias are normally associated with excessive growth, for example, arising from abnormal response to hormonal stimulation or wound healing. However, not all metaplasias represent a transformation to normal tissues, and many of the final states are atypical or dysplastic [24]. From the literature, there is no uniform nomenclature to describe the different possible cell type conversions through the developmental stages. In order to facilitate the description of these conversion processes, the authors would suggest the following nomenclature modified from [24] (**Figure 1**): metaplasia is the conversion of one cell type into another, including dedifferentiation, transdifferentiation and also conversion between undifferentiated stem cells of different tissues or germ layers; dedifferentiation is the process of the conversion from one developmental cell stage into a more undifferentiated cell stage; transdifferentiation is defined as the conversion of one differentiated cell type to another differentiated cell type within one germ layer, with or without an intervening cell division. From the literature, there is no conclusive evidence of transdifferentiation between differentiated cells from different germ layers. Conversion between germ layer-committed or pluripotent stem cells is called transgerminal conversion (for example, the conversion of MSCs from the mesoderm into NSC-like cells of neuroectodermal origin [25,26]), whereas translineage conversion represents the conversion between lineage-restricted multipotent stem cells within one germ layer (for example the conversion of skin-derived progenitors into neuroectodermal stem cells [27]). These conversion processes include potential dedifferentiation–redifferentiation steps.

This review summarises the data on the conversion of mesoderm-derived hMSCs into immature and mature neuroectodermal cell types, which represents a transgerminal conversion process according to the classification defined above. However, it is unclear from most of the presented studies whether reprogramming of a committed MSC (transgerminal conversion), the dedifferentiation/redifferentiation cycle, or proliferation and differentiation of a more pluripotent stem cell already harboured in the hMSC suspension

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Table 1. Summary of neuroectodermal differentiation studies in hMSCs.

Cell source	Induction media	Neuroectodermal markers	Comments	Ref.
One-step differentiation protocols				
hMSCs depleted of CD34$^+$	DMEM, HS, FBS, RA, BDNF; on polyethylimine-coated substrate or murine fetal midbrain cells	Nestin, β-tubulin III, NeuN, GFAP	Morphology and marker expression, no comparison to undifferentiated hMSCs	[21]
Plastic adherent hMSCs	24-h preinduction with DMEM/20% FBS and 1 mM β-mercaptoethanol (BME) followed by DMEM/2% DMSO/200 μM BHA	Nestin, NSE, NF-M, TrkA (no GFAP$^+$ cells)	No function, downregulation of all neuroectodermal markers; cytoskeleton disruption?	[23,34]
hMSCs depleted of CD45$^+$/glycophorin A$^+$ cells; passaged for 20 – 70 doublings	Low glucose DMEM + FBS + EGF + PDGF; FGF-2 for 3 weeks; fibronectin-coated substrate	β-Tubulin III, NSE, glutamate, GFAP, Gal-C	No further characterisation	[64]
hMSCs	α-MEM + FBS; passaged when 70 – 90% confluent, isobutylmethylxanthine/dibutyryl cAMP	NSE, vimentin	Only western blot analysis after 6 days; no expression of MAP2, GFAP, MBP, Tau, NF-M	[19]
hMSCs and CD133$^+$ subpopulation under culture conditions appropriate for neural stem cells	Various differentiation conditions, with the best being DMEM/F12; neurotrophin 3 or BDNF on fibronectin.	GFAP, Nestin, NF-M/-H, MAP2; β-tubulin III for quantification	CD133$^+$ MSCs, but not CD34$^+$/CD133$^-$ cells with neuroectodermal potential, differentiated CD133$^+$ cells more resembled neuron-like cells; no Na$^+$ channels, no GalC$^+$ cells	[65]
hMSCs	Neurobasal medium, 5 mM cAMP, 5 mM IBMX, 25 ng/ml NGF, 2.5 mg/ml insulin	Nestin, β-tubulin III, GFAP, TH, MAP2, SH2 and SH3 (as mesodermal markers)	Neuroectodermal genes expressed in undifferentiated MSCs, increase of TH, GFAP, MAP2, decrease of β-tubulin III; SH2 and SH3 unchanged	[30]
hMSCs	M199 Media, serum free conditions, tricyclodecane-9-yl-xanthogenate (D609)	NSE, but no GFAP	Only protein expression after 6 h investigated, no comparison to initial MSC population	[90]
hMSCs	FGF-1, protein kinase C activator and reagents increasing cellular cAMP	β-Tubulin III, NF, NSE, GFAP protein and mRNAs, higher levels than in undifferentiated MSCs; the mesenchymal marker FN decreased following differentiation.	All changes within 24 h, cell death after 72 h or 5 days	[89]
Whole bone marrow, low-density mononuclear cells, hMSCs, and CD90$^+$, CD133$^+$, CD45$^-$/Gly$^-$, NGFR^{p75+} or NCAM$^+$ subppopulations	Various epigenetic conditions: serum-free conditions, growth factors and supplements; conditioned media from neuronal or glial cultures or coculture systems	GAP43, NSE, β-tubulin III, MAP2, GFAP, Nestin on RNA level, no MAP2 staining	hMSCs and the CD90$^+$ subpopulation already express neuroectodermal marker genes prior to differentiation	[61]

α-MEM: Minimum Essential Medium α; A2B5: Immature glial lineage marker; β-tubulin III: Neuron-specific class III beta tubulin; BDNF: Brain-derived neurotrophic factor; BHA; t-Butylhydroxyanisol; BME: β-Mercaptoethanol; CD: Cluster of differentiation; D609: Tricyclodecane-9-yl-xanthogenate; dbcAMP: Dibutyryl-cyclo-amino-monophosphate; DMEM: Dulbecco's modified Eagle's medium; DMSO: Dimethylsulfoxide; EGF: Epidermal growth factor; F12: Ham F12 medium; FBS: Fetal bovine serum; FGF: Fibroblast growth factor; FN: Fibronectin; GABA: Gamma-aminobutyric-acid; GalC: Galactocerebrosidase C (marker for oligodendrocytes); GAP43: Growth-associated protein 43; GFAP: Glial fibrillary acidic protein; hMSC: Human MSC; HNK-1: HNK-1 carbohydrate epitope; HS: Horse serum; IBMX: Isobutylmethylxanthine (phosphodiesterase inhibitor); LIF: Leukaemia inhibitory factor; M199: M199 medium; MAP2: Microtubulin-associated protein 2 (marker for mature neurons); MBP: Myelin basic protein (marker for oligodendrocytes); MSC: Mesodermal stromal cells; NCAM: Neural cell adhesion molecule; NeuN: Neuronal marker; NF: Neurofilament; NF-H: NF heavy chain; NF-M: NF middle chain; NGF: Nerve growth factor; NGF-R: NGF receptor (p75); NGFR^{p75}: NGF-R (p75); NGHRI: http://www.nhgri.nih.gov; NSE: Neuron-specific enolase; pan-NF: NF-H and NF-M; PDGF: Platelet-derived growth factor; RA: Retinoic acid; SH2 and SH3: Mesodermal markers; SHH: Sonic hedgehog; S100β: S100 calcium-binding protein-β; Tau: Mictotubule-associated protein tau; TH: Tyrosine hydroxylase; TrkA: Tyrosine receptor kinase A.

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Table 1. Summary of neuroectodermal differentiation studies in hMSCs (continued).

Cell source	Induction media	Neuroectodermal markers	Comments	Ref.
hMSCs	Sodium ferulate in serum-free conditions after 24h preinduction using 10%FBS + FGF-2	Nestin, NSE, GFAP	Morphology and immunocytochemistry of 3 markers only	[91]
hMSCs (plastic adherent; immunomagnetic bead selected)	Serum- and feeder cell-free condition + FGF-2, EGF and PDGF	NSE, β-tubulin III, NF-H, GABA, TH, Serotonin	Stable neuroectodermal gene expression over weeks, no functional data or mesodermal characterisation after conversion	[88]
hMSCs from osseus debris	24 h preinduction with DMEM/20% FBS and 1 mM BME followed by DMEM/2% DMSO/200 μM BHA	NSE, β-tubulin III, NF-H and HNK-1, a subpopulation expressed CD15 and synaptophysin	Only morphological data without implications of neuroectodermal function; no comparison to undifferentiated MSCs	[92]
hMSCs	DMSO + BHA; serum free; 5% HS, 1% FBS, RA or BDNF	Nestin, GFAP, β-tubulin III, MAP2, FN; early end late neuroectodermal genes	No significant changes of neuroectodermal gene expression compared to undifferentiated MSCs	[62]
Multiple-step differentiation protocols				
hMSCs	EGF+ FGF-2 Forskolin + DMSO or hippocampal astrocyte conditioned media	Nestin, Musashi-1, β-tubulin III, pan-NF, FN, vimentin	Morphological data by immunocytochemistry only; necessity of two-step assay	[97]
Whole bone marrow	15% serum, LIF, FGF-2, SHH and FGF-8 Serum-free, FGF-2, SHH, FGF-8 Serum-free, FGF-2 Removal of FGF-2 or 10% serum	Nestin, A2B5, GFAP, S100β, β-tubulin III; NGHRI gene chip analysis	First neurosphere assay for MSCs, whole bone marrow was used; neither neuroectodermal function nor mesodermal properties tested	[26]
hMSCs from dead specimens	FGF-2 BME or NT3 NT3/NGF/BDNF	Nestin β-tubulin III, NGF-R NF; NeuN	Only morphological data published	[86]
hMSCs	Serum-free, EGF, FGF-2 HS, FBS, RA, BDNF	Increase of Nestin, Musashi-1, early neurogenic genes Increase of β-tubulin III, MAP2, GFAP, GalC reduction of FN; loss of mesodermal differentiation potential, neuroectodermal functions	First report showing detailed analysis of a two-step protocol, including functional data	[25,62]
hMSCs and subpopulations (see above)	Serum-free conditions and EGF	Neurospheres	Too few neurospheres for further investigations	[61]

α-MEM: Minimum Essential Medium α; A2B5: Immature glial lineage marker; β-tubulin III: Neuron-specific class III beta tubulin; BDNF: Brain-derived neurotrophic factor; BHA; t-Butylhydroxyanisol; BME: β-Mercaptoethanol; CD: Cluster of differentiation; D609: Tricyclodecane-9-yl-xanthogenate; dbcAMP: Dibutyryl-cyclo-amino-monophosphate; DMEM: Dulbecco's modified Eagle's medium; DMSO: Dimethylsulfoxide; EGF: Epidermal growth factor; F12: Ham F12 medium; FBS: Fetal bovine serum; FGF: Fibroblast growth factor; FN: Fibronectin; GABA: Gamma-aminobutyric-acid; GalC: Galactocerebrosidase C (marker for oligodendrocytes); GAP43: Growth-associated protein 43; GFAP: Glial fibrillary acidic protein; hMSC: Human MSC; HNK-1: HNK-1 carbohydrate epitope; HS: Horse serum; IBMX: Isobutylmethylxanthine (phosphodiesterase inhibitor); LIF: Leukaemia inhibitory factor; M199: M199 medium; MAP2: Microtubulin-associated protein 2 (marker for mature neurons); MBP: Myelin basic protein (marker for oligodendrocytes); MSC: Mesodermal stromal cells; NCAM: Neural cell adhesion molecule; NeuN: Neuronal marker; NF: Neurofilament; NF-H: NF heavy chain; NF-M: NF middle chain; NGF: Nerve growth factor; NGF-R: NGF receptor (p75); $NGFR^{p75}$: NGF-R (p75); NGHRI: http://www.nhgri.nih.gov; NSE: Neuron-specific enolase; pan-NF: NF-H and NF-M; PDGF: Platelet-derived growth factor; RA: Retinoic acid; SH2 and SH3: Mesodermal markers; SHH: Sonic hedgehog; S100β: S100 calcium-binding protein-β; Tau: Mictotubule-associated protein tau; TH: Tyrosine hydroxylase; TrkA: Tyrosine receptor kinase A.

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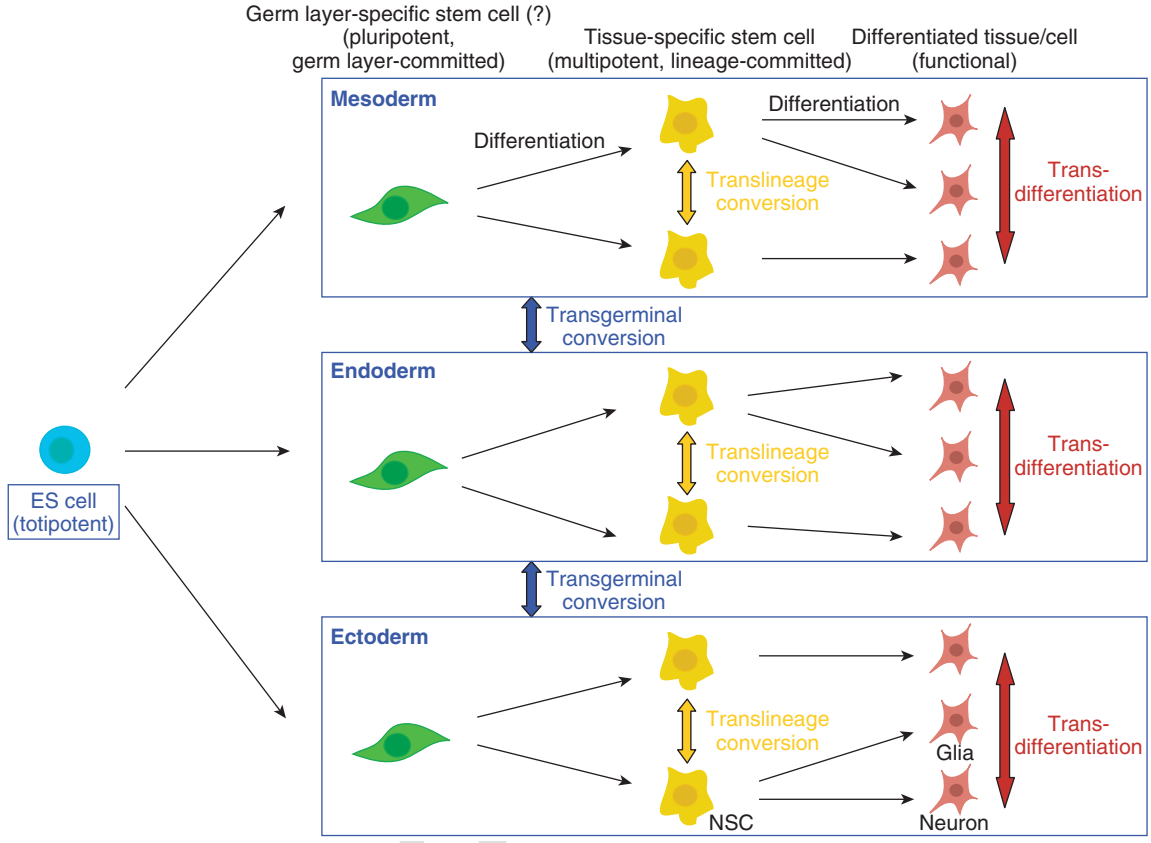

Figure 1. During development, a stepwise commitment of stem cells is occurring, starting from totipotent ES cells, followed by germ layer commitment (pluripotent stem cell) and further lineage restriction (multipotent stem cell), and ending up in the terminal differentiated stage. Conversions between germ layer-committed cells are called transgerminal conversions, whereas translineage conversion represents conversions between lineage-restricted multipotent stem cells within one germ layer. Finally, transdifferentiation means the conversion of one fully differentiated cell type to another fully differentiated type within one germ layer (see text for details). As there is no conclusive report on the existence of a pluripotent germ layer-committed stem cell, the authors marked this cell type with a (?).
ES: Embryonic stem; NSC: Neural stem cell.

(refer to section 3.1) is responsible for the neuroectodermal cell type conversion.

3. Mesodermal stromal cells: origin and definition

Bone marrow-derived MSCs are a heterogeneous population of cells providing ultrastructural and biochemical scaffolds within the haematopoietic microenvironment [28,29]. These cells are derived from bone marrow cell suspensions, either by their selective attachment to tissue culture plastic or by different depletion methods [30], and can be expanded efficiently. After isolation, MSCs can be cultivated *in vitro* and contain progenitors capable of generating bone, cartilage, fat and other connective tissues [16-18]. These non-haematopoietic precursors found in bone marrow stromal cells are also known as colony-forming unit fibroblasts or MSCs [16]. Adult stem cells were believed to be lineage restricted, which means that they can only differentiate into the cells of their tissue origin [31,32]; however, there is a growing body of evidence that stem cells derived from blood or bone marrow can break barriers of germ-layer commitment and differentiate *in vitro* [19,21-23,31,33-36] and *in vivo* after transplantation into the brain and spinal cord [37-44] into cells expressing neuronal and glial markers [17,32,33]. Many reports on multipotency of MSCs followed; for example, transplanted bone marrow cells contribute to endothelium [45-50] and skeletal muscle myoblasts [51,52], and acquire properties of hepatic and biliary duct cells [53-55], lung, gut and skin epithelia [56].

In the authors' studies, the hMSC phenotype was proven by FACS analysis with positivity for CD9, CD90, CD105 (endoglin) and CD166, as well as negativity for CD14, CD34 and CD45, and by the potential to differentiate into osteoblasts, chondrocytes and adipocytes [25,57,58]. In a systematic investigation of surface marker expression of

hMSCs, Vogel and colleagues showed by FACS analysis that CD10$^+$, CD13$^+$, CD61$^+$, CD90$^+$, CD105$^+$ (endoglin), CD45$^-$, CD34$^-$ and CD133$^-$. MSCs also expressed CD109, CD140b (PDGF-RB), CD164 and CD172a (SIRPa) [28]. Interestingly, they also described a heterogeneity within this hMSCs, as demonstrated by the preferential expression of nestin and W8B2 antigen, an antibody of unknown specificity raised against the retinoblastoma cell line WERI-RB-1, on distinct MSC subpopulations. Morphologically, these populations comprised small single cells and larger cells with polygonal appearance [59,60].

Concerning neuroectodermal marker expression, Deng and colleagues found in 2001 that hMSCs expressed several markers characteristic of neural cells, such as MAP1B, neuron-specific enolase (NSE), TuJ-1 and vimentin, before any differentiation [19]. The expression of neuroectodermal transcripts by undifferentiated hMSCs was first investigated systematically by Tondreau and colleagues in 2004 [30]. In undifferentiated hMSCs, expression of Nestin, β-tubulin-III, glial fibrillary acidic protein (GFAP) and tyrosine hydroxylase (TH) could be detected. They concluded that the constitutive expression of Nestin or β-tubulin-III by hMSCs shows that these cells are 'multidifferentiated' cells [30]. Bossolasco and co-workers showed that hMSCs and the CD90$^+$ subpopulation thereof already express neuroectodermal marker genes, such as β-tubulin-III, NSE, GAP43 or GFAP. Interestingly, CD90$^+$ cells were negative for Nestin, neurofilament (NF)-M, MAP2 and GFAP, and became positive for these markers, at least on the RNA level, after neuroectodermal differentiation. None of the other subpopulations of MSCs analysed (CD133$^+$, CD45$^-$/Gly$^-$, NGFR^{p75+} or neural cell adhesion molecule [NCAM]$^+$ cells) were positive for the neural markers [61]. The authors consequently analysed the expression pattern of a large set of both early and late neuroectodermal maker genes in undifferentiated hMSC populations described above [57]. All neuroectodermal markers tested on immunocytochemistry (β-Tubulin III, GFAP, GalC, Nestin) were already expressed by hMSCs except the mature nerve cell marker MAP2ab, but only 4% of cells were slightly positive for Nestin. Using quantitative real-time polymerase chain reaction (RT-PCR), hMSCs expressed high levels of *FZD1* (human frizzled homologue 1) and *NTRK1* (neurotrophin receptor kinase 1), low levels of *NES* (Nestin), *MSI1* (Musashi-1) and early neurogenic markers (*OTX1*, *SOX1* and *SOX10*, *NGN2* [Neurogenin-2], *NEUROD1* [NeuroD1]). Furthermore, some glial (*GFAP*, *MBP*) as well as immature neuronal markers (*TUBB4/III* [β-tubulin-III]) are expressed by hMSCs [25,57].

Another type of adult multipotent stem cell was reported by Toma and co-workers, demonstrating a stem cell in the rodent skin that proliferates and differentiates in culture to produce neurons, glia, smooth muscle cells and adipocytes [32]. Similar precursors that produce neuron-specific proteins following differentiation can be iolated from the adult human scalp [32]. Belicchi has been devoted to purified human skin-derived stem cells that are capable of neural differentiation, based on the presence or absence of the CD133 cell surface marker [62]. The enriched skin-derived CD133$^+$ cells expressed the CD34 and Thy-1 antigens. These cells, cultured in a growth medium containing EGF and FGF-2, proliferated in spheres and differentiated *in vitro* into neurons, astrocytes and, rarely, oligodendrocytes. Single cells from sphere cultures initiated from human purified CD133$^+$ cells were replanted as single cells and were able to generate new spheres, demonstrating the self-renewing ability of these stem cell populations.

3.1 Potential 'neuroectodermal' progenitor cells within hMSC populations

Three interesting studies focused on identifying a putative 'neuroectodermal' progenitor cell within the hMSC population capable of transdifferentiation into neuroglial cells [17,61,63,64]. Bossolasco and colleagues used the following cell preparations [61]: whole bone marrow, low-density mononuclear cells, hMSCs, and subpopulations sorted for CD90$^+$, CD133$^+$, CD45$^-$/Gly$^-$, nerve growth factor receptor (NGFR)$^{p75+}$ or NCAM$^+$ cells. Many epigenetic differentiation conditions were tested, for example, serum-free conditions, as well as various growth factors and supplements. Furthermore, cellular interaction influences on cell fate were evaluated using conditioned media derived from neuronal or glial cell cultures, or using coculture systems. In directly differentiated hMSCs, this research group was able to show low levels of MAP2ab on the mRNA level by conventional semiquantitative RT-PCR, but no MAP2ab$^+$ cells [61]. Furthermore, only hMSCs and CD90$^+$ MSCs showed neuronal/astroglial morphology under diverse differentiation conditions. The NCAM$^+$ as well as NGF^{p75-} subpopulations acquired a neuronal-like morphology, but were not further investigated [61]. Together, hMSCs seem to already contain a subpopulation of cells capable of neuroglial differentiation, for example, the CD90$^+$ subpopulation. Unfortunately, most of the data were obtained by semiquantitative RT-PCR and only confirmed for GFAP on the protein level. However, these data are consistent with the authors' studies that also used CD90$^+$ hMSCs (> 90% purity of CD90$^+$ cells) for neuroectodermal conversion of hMSCs [25,57,65]. Padovan and co-workers compared the unsorted cell population with CD133$^+$ stem-like cells from hMSCs cultures [64]. CD133$^+$ mesenchymal cells, but not CD34$^+$/CD133$^-$ cells, generally showed a higher expression of neuronal markers than unsorted marrow stromal cells and, at least morphologically, differentiated CD133$^+$ cells more resembled neuron-like cells. However, this subpopulation also did not show neuronal functions, such as Na$^+$ currents or action potentials [64].

Reyes and Verfaille reported on a population of human multipotent adult progenitor cells (MAPCs) within the MSC population [17,63] similar to the mouse MAPCs reported earlier by the same research group [20]. They showed evidence that MAPCs can be induced to differentiate into cell types of the neuroectodermal lineage, including β-tubulin-III-,

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neurofilament-, NSE- and glutamate-positive neurons, GFAP-positive astrocytes, and myelin basic protein (MBP)- and galactocerebroside (GalC)-positive oligodendrocytes, which was proven by immunohistochemistry and western blot analysis [17,63]. It is as yet unclear whether human MAPC cultures contain pluripotent stem cells without germ layer commitment, or whether these cultures contain 'neuroectodermal' stem cells committed to neuroectodermal differentiation.

4. Neural stem cells: origin and definition

NSCs are consensually classified as a subtype of progenitor cells that are capable of extended self-renewal and have the ability to generate all major brain-specific cell types, such as neurons, glia and oligodendroglia [4,5]. Due to the lack of a specific pattern of marker expression, NSCs are still identified operationally by their growing and differentiation behaviour after isolation. During expansion, they normally grow in floating, multicellular aggregates, the so-called 'neurospheres' [4,5]. NSCs can be generated from ES cells [6,66,67] or directly extracted from fetal or adult nervous tissue and proliferated in culture [4,5,8-12]. Similar to tissue-specific stem or progenitor cells, NSCs have been categorised as multipotent stem cells derived from the nervous system with the capacity to regenerate and to give rise to cells belonging to all three major cell lineages of the nervous system: neurons, oligodendrocytes and astrocytes [4,5,8,9,11,12,68,69,70,71]. This definition of NSCs includes the generation of the cells from nervous system tissue (with the exception of ES cell-derived NSCs) and, therefore, raises the problem of defining NSC or NSC-like populations derived from other tissues or multipotent stem cells by conversion or transdifferentiation.

During the last couple of years, molecular markers that define NSCs have been developed [4,28,72,73]. The first marker was the intermediate filament protein Nestin, but some neural precursor cells are Nestin-negative [74] and, on the other hand, Nestin is expressed by other cell types, including non-neuronal cells [75]. Uchida and co-workers performed an extensive analysis of surface markers on clonogenic fetal human neurosphere cultures and defined a subset of human NSCs as phenotypically CD133-positive, but negative for CD34 and CD45 [73]. Vogel and colleagues characterised commercially available fetal neural progenitor cells as positive for CD15, CD56, CD90, CD164, NGFR, 57D2 and W4A5, but negative for CD45, CD105, CD109, CD140b (PBGF-RB) and W8B2 [28]. Recently, the expression of specific neural transcription factors, such as Sox-1, Musashi-1, Otx-1, Otx-2, NeuroD1 and Neurogenin-2, was reported in neural stem or progenitor cells, demonstrating their neuroectodermal progeny [76,77]. Only a very few studies characterised adult human NSCs with respect to their growing behaviour, morphology and marker expression pattern [70,78-84]. These cells are $CD15^{low/-}$, $CD34^{-}$, $CD45^{-}$, $CD133^{-}$ and express various NSC markers, such as Nestin and Musashi-1. Together with the operational definition of NSCs, these markers, or, even better, this pattern of NSC-marker expression, help to identify NSC or NSC-like populations generated from non-neural tissue or (adult) multipotent stem cells.

5. One-step neuroectodermal conversion of human mesodermal stromal cells

Several studies provide evidence that MSCs are able to express properties of neuroectodermal cells *in vitro* [18,19,21,22,23,30,34,85] and *in vivo* after transplantation into the brain and spinal cord [37-40,42-44]. Here the focus is on the *in vitro* generation of neuroectodermal cells out of hMSCs or similar cell populations. The first reports appeared in 2000 by Sanchez-Ramos and co-workers and Woodbury and colleagues [21,23]. Both groups developed two independent differentiation protocols that revealed neuroectodermal protein expression after neuronal induction. Since these early articles, several studies have reported direct 'one-step' neural differentiation of hMSCs using three completely different approaches (**Figure 2**):

- incubation of hMSCs with a combination of growth factors, cytokines, glial feeder cell layers and/or conditioned media known to induce neuroectodermal differentiation in ES cells or NSCs [21,57,64,86,87]
- increase of intracellular cAMP levels reported to induce neuronal differentiation in fetal and adult brain-derived NSCs [19,88]
- chemical-defined media with various compounds, including antioxidants such as β-mercaptoethanol (BME), butylated hydroxyanisole (BHA), sodium ferulate (SF), dimethylsulfoxide (DMSO), as well as tricyclodecane-9-yl-xanthogenate (D609) [22,23,57,89,90,91]

5.1 Growth factors, cytokines and conditioned media for neuroectodermal conversion

Sanchez-Ramos and colleagues demonstrated that hMSC can be differentiated into neural cells in the presence of EGF or brain-derived neurotrophic factor (BDNF), expressing the protein and mRNA for Nestin, GFAP and neuron-specific nuclear protein (NeuN). Re-evaluating this protocol, however, fibronectin mRNA and protein levels were unchanged in differentiated hMSCs compared with undifferentiated hMSCs [57]. Early neuroectodermal marker genes were unchanged (*OTX1*, *NEUROD1*), decreased (*NES*, *NGN2*) or even undetectable (*MSI1*) in cells that had undergone differentiation according to the Sanchez-Ramos protocol [57]. The investigation of late neuroectodermal genes, including *TUBB4/III*, *MAP2ab* and *SNCA* for neurons, *GFAP* for astroglial cells and *MBP* for oligodendroglia, revealed no relevant upregulation of these marker genes on the mRNA and/or protein level [57]. In coculture experiments with rat fetal mesencephalic or striatal feeder cell layers, Sanchez-Ramos and colleagues showed that a small proportion of hMSC-derived cells differentiated into cells

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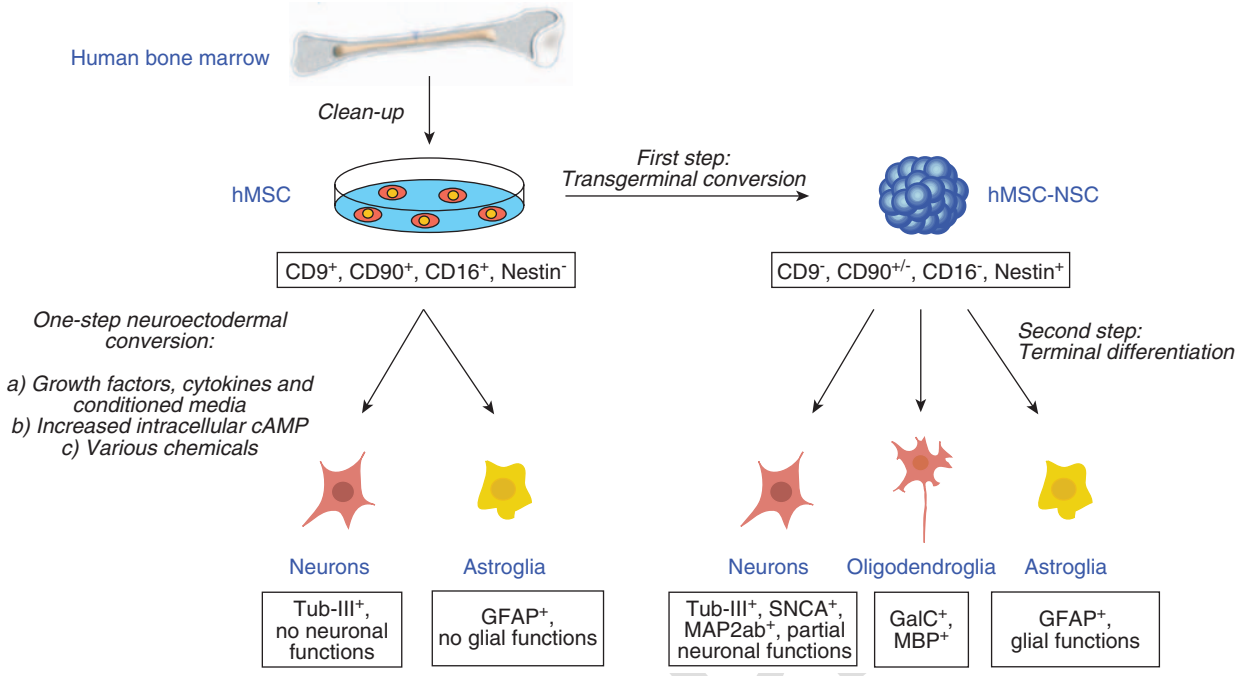

Figure 2. Scheme of available protocols for epigenetic conversion of hMSCs into neuroectodermal cell populations. Concerning the one-step neuroectodermal conversion, there are three principle approaches using (a) growth factors, conditioned media and/or neural feeder cell layers, (b) increased intracellular cAMP levels, or (c) various chemicals (antioxidants and others) to induce a neuroectodemal phenotype. Note that the two-step procedures for neuroectodermal conversion include (step one) transgerminal conversion of hMSCs into an immature NSC-like cell type (hMSC-NSC) and, subsequently, (step two) terminal differentiation into functional glial and neuronal cells. Data in boxes display the marker expression and/or functional data of the respective cell type (see text for details).

GalC: Galactocerebrosidase C; GFAP: Glial fibrillary acidic protein; hMSC: Human mesodermal stem cell; hMSC-NSC: hMSC-derived NSC-like cell; MAP2: Microtubulin-associated protein 2; MBP: Myelin basic protein; NSC: Neural stem cell; SNCA: Synuclein alpha; Tub-III: β-Tubulin III.

expressing NeuN and GFAP [21]. Bossolasco and co-workers also showed that several culture conditions using neural induction factors and/or astroglia feeder layers can induce changes of the morphology and marker expression towards a neuroglial phenotype [61]. The effects were pronounced in the CD90+ subpopulation of MSCs. However, neither functional analysis of the achieved neuroectodermal cells nor their remaining mesodermal properties have been evaluated in these studies.

Padovan and colleagues examined the expression of neuronal and glial markers by hMSCs under diverse culture conditions appropriate for neural stem cells, such as combinations of growth factors or cytokines, as well as different extracellular matrix proteins [64]. Furthermore, they compared the unsorted cell population with CD133+ stem-like cells using immunofluorescence, western blot and functional patch-clamp analysis. Following differentiation, hMSCs expressed Nestin, NF-M/-H, MAP2, β-tubulin III and GFAP, but did not become GalC-positive. Overall, the expression of the early neuronal marker β-tubulin III was most pronounced in the presence of DMEM/F12 and neurotrophin-3 or BDNF when hMSCs were cultured onto fibronectin. However, electrophysiological investigations could not show fast Na^+ currents or functional neurotransmitter receptors in differentiated MSCs. Interestingly, CD133+ mesenchymal cells, but not CD34+/CD133- cells, generally showed a higher expression of neuronal markers than unsorted marrow stromal cells, and, at least morphologically, differentiated CD133+ cells more resembled neuron-like cells. However, this subpopulation also did not show Na^+ currents [64]. Tao and co-workers showed that a serum- and feeder cell-free condition and supplementation of EGF, FGF-2 and platelet-derived growth factor (PDGF) induced neuronal morphology in hMSCs [87]. In addition to the markers mentioned above, these cells expressed neurotransmitters or associated proteins, such as gamma-aminobutyric acid (GABA), TH or serotonin. Interestingly, these changes were maintained for up to 3 months. However, neither functional data nor the remaining mesodermal properties after transgerminal conversion have been shown.

5.2 Increased intracellular cAMP for neuroectodermal conversion

Deng and colleagues investigated the effects of intracellular cAMP levels on neuroectodermal differentiation of hMSCs

using dibutyryl-cAMP (dbcAMP) and the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) [19]. Increasing the intracellular cAMP level is known to induce neuronal differentiation in fetal and adult brain-derived NSCs. By comparing the expression levels of neuroectodermal genes before and after incubation with dbcAMP and IBMX for 6 days, they showed that the expression levels of both NSE and vimentin were increased during the neuroectodermal differentiation process. They stated that the increase of NSE and vimentin expression coincided with the appearance of neuron-like cells in the cultures [19]. However, there are no data correlating the morphological changes with immunocytochemistry staining against NSE or vimentin. Thus, it is not possible to correlate NSE/vimentin expression and neuron-like morphology of the cells. Furthermore, the expression levels of MAP1B or TuJ-1 remained unchanged through the differentiation process, and they could not detect any expression of NF-M, MAP2, tau, S-100β, GFAP and MBP in either untreated or IBMX/dbcAMP-treated hMSCs. Suon and co-workers investigated the differentiation potential of hMSCs by various differentiation cocktails [88], particularly those including reagents that increase cellular cAMP levels, similar to Deng and co-workers [19]. The study focused on changes in gene expression within the first 48 h of the conversion process. Their best differentiation cocktail, consisting of IBMX, forskolin and a protein kinase C activator, produces a rapid (1 – 4 h) and transient (24 – 48 h) transformation of nearly all hMSCs into neuron-like cells displaying a complex network of processes [88]. In addition, differentiated hMSCs expressed increased levels of neuron- and glial-specific proteins and mRNAs compared with undifferentiated hMSCs. In contrast, the mesenchymal marker fibronectin, which is highly expressed in the undifferentiated state, was reduced following differentiation. However, analysing the time course of differentiation, primary hMSCs remained transformed for up to 48 h before spontaneously returning morphologically to the undifferentiated state. Moreover, the continuous reexposure of cultures every 24 or 48 h to the differentiation cocktail could not sustain their neuron-like appearance and often resulted in cell death of hMSCs. Interestingly, this phenomenon could be delayed by incubation of differentiated cells with glial-conditioned media (glial-CM) for up to 5 days, but the cells died after that time period if they grew in glial-CM [88]. It is noteworthy that, in contrast to mouse MAPCs, hMSCs did not differentiate into neuronal-appearing cells using a sequential incubation protocol described by Jiang and co-workers for mouse MAPCs (unpublished observations; [31,88]).

5.3 Various chemicals for neuroectodermal conversion

Woodbury and co-workers developed a protocol for the neuroectodermal conversion of rat MSCs using a preinduction with high serum and BME for 24 h and subsequent incubation with a chemical induction medium including DMSO and BHA [22,23]. This protocol showed a rapid acquirement of neuroectodermal marker gene expression and morphological changes, even within hours after neural induction was initiated [23]. However, only a few markers have been tested and all analysis was based on immunocytochemistry or semiquantitative RT-PCR [23]. In their follow-up study published in 2002, Woodbury *et al.* compared gene expression during this conversion/transdifferentiation process [22]. Interestingly, almost all of the investigated genes were downregulated during the 'neuronal' induction process, accompanied by a retraction of the cell body. The so-called redifferentiation by withdrawing the neuronal induction medium yielded in a recovery of the original cell shape [22]. The authors adapted the Woodbury protocol to MSCs from human origin and quantitatively measured the expression of several early and late neuroectodermal marker genes [57]. There were no relevant changes of the expression of the neural stem cell marker genes *NES* and *MSI1*, as well as the proneural genes *SOX1*, *OTX1*, *NEUROD1* and *NGN2*. In addition, there was no significant upregulation of mRNA levels of late neural genes, including *TUBB4/III, SNCA*, *GFAP* and *MBP* [57]. Consistently, the authors did not find any MAP2ab$^+$ mature neurons after the differentiation of hMSCs using the Woodbury protocol [57]. In a very recent study, Wenisch and colleagues investigated the sequence of ultrastructural changes of bone-derived stem cells during neuronal induction using a protocol similar to the Woodbury protocol, and compared these data with immunocytochemical and electrophysiological properties of the cells [91]. The cells showed neuronal morphologies and expressed NSE, β-tubulin III, NF-H and HNK-1, whereas only a subpopulation expressed CD15 and synaptophysin. However, electrical signalling could not be detected, neither spontaneously nor after electrical stimulation. Using transmission electron microscopy, the authors claimed that induced hMSCs revealed cellular features of neuritogenesis and synaptogenesis, and suggested that MSCs have features similar to those observed in immature neurons [91]. However, there is no report on the further survival of these cells in the mentioned studies, as this protocol was shown to produce poor cell survival [87]. Together, these data pointed most likely towards aberrantly induced gene expression and cytoskeleton disruption rather than towards a sequence of gene expression as required for neuroectodermal differentiation [92]. Indeed, Neuhuber and colleagues showed that changes in morphology following addition of the chemical induction medium introduced by Woodbury and co-workers are caused by the rapid disruption of the cytoskeleton [92].

Wang and co-workers investigated the effect of the tricyclodecane-9-yl-xanthogenate (D609) on the neural differentiation of hMSCs [89]. D609 exhibits a variety of biological functions, including antiviral, anti-inflammatory, antiapoptotic and antitumour activity. As they stated, exposure of hMSCs to D609 leads to a neuronal-like morphology after 3 h. Six hours later, > 80% of hMSCs displayed intensive NSE, but no GFAP staining, and

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remained positive until 72 h after differentiation. However, no comparison with the starting population was shown, and this report only showed morphological data as well as protein expression by immunocytochemistry [89]. Another study from the same research group explored the effects of SF on the differentiation of hMSCs into neural cells *in vitro* [90]. SF is an antioxidant that exhibits a variety of biological functions, including clearing free radicals, antilipid oxidation and antiplatelet aggregation. They found that hMSCs could be induced to cells with neural morphology when cultured with SF and when expressing neural proteins such as Nestin, NSE and GFAP. In time course experiments, ~ 30% of the hMSC-derived cells expressed Nestin when cultured with SF for 3 h, but no expression was detected after 24 h. In contrast, the percentages of positive cells for NSE or GFAP were ~ 67 and 39%, respectively, at 6 h, and these cells survived > 7 days, suggesting that SF can induce hMSCs to differentiate into neural-like cells *in vitro* [90]. However, only morphology and immunocytochemistry for three markers have been shown.

Most studies mentioned above that used a chemical-defined medium for the induction of a neural phenotype in hMSCs showed a rapid change in the morphology and the acquirement of neuroectodermal transcripts within hours after initiation of the transdifferentiation process. Furthermore, these changes could often be reversed either by the removal of inductors or even spontaneously. Thus, it is questioned whether these effects result from real neuroectodermal conversion/transdifferentiation or probably from aberrant gene expression and cytoskeleton disruption. Lu and co-workers, therefore, explored the potential of simple chemical methods to transdifferentiate cell types other than MSCs, including primary rat fibroblasts, primary human keratinocytes, HEK-293 cells, rat PC-12 cells, and as positive control rat bone MSCs [93]. Surprisingly, apart from keratinocytes, all cell types adopted at least partial 'neuron-like' cell morphology, showing tiny extensions resembling neurites following stimulation with BME or DMSO/BHA. In time-lapse microscopy they showed evidence that the chemical exposure of rat MSCs did not result in new neurite growth, but cellular shrinkage with retraction of the majority of existing cell extensions, leaving only a few, tiny neurite-like processes [93]. Rat MSCs were exposed to various stressors and cellular shrinkage, and adoption of pseudoneuronal morphology was displayed in all cases. In parallel to cellular shrinkage, an increase in immunolabelling for neuronal markers NSE and NeuN was apparent in the cell soma, which could not be confirmed by RT-PCR. Furthermore, blockade of protein synthesis with cycloheximide did not prevent cells from adopting 'neuron-like' morphology after chemical induction. Morphological changes and increases of marker expression by immunolabelling following 'chemical induction' of rat MSCs are, therefore, probably the result of cellular toxicity, cell shrinkage and changes in the cytoskeleton, and do not represent regulated steps in a complicated cellular differentiation process [93].

Another weakness of most studies in the one-step conversion of hMSCs is the missing quantitative comparison of the results with the data on the initial cell populations (undifferentiated MSCs). As hMSCs express several early and late neuroectodermal markers routinely used to characterise NSCs or mature neural cell populations [30,57,94], a critical examination of the differentiation process, including a comparative analysis of the initial cell population (hMSCs) and the converted neural cell types, is necessary to determine the validity of the neuroectodermal differentiation capacity of bone marrow-derived hMSCs [57,92,93,95]. Furthermore, the irreversible transgerminal conversion should be proven by demonstrating the decreased mesodermal differentiation capacity of converted neuroectodermal cell populations [25].

Together, there is no report conclusively demonstrating the differentiation of hMSCs into mature functional neuronal cells using a one-step differentiation protocol. However, protocols using specific differentiation conditions with growth factors/cytokines known to influence neurogenic specification of ES cells or NSCs, such as a combination of EGF, BDNF, PDGF and/or cAMP, are able to induce a neuron-like morphology, but no functional properties of neuroectodermal nerve cells have been reported as yet. Most studies lack quantitative comparison of the neurally differentiated cells with the initial MSC population, as well as the analysis of their mesodermal differentiation capacity to exclude transient or incomplete transdifferentiation.

6. Two-step neuroectodermal conversion of human mesodermal stromal cells

A novel approach for the neuroectodermal specification of hMSCs is the two-step technology (**Figure 2**), with initial conversion of hMSCs into immature NSC-like cells, and subsequent terminal differentiation into mature neuronal and glial cell types [6,25,57,61]. These two steps are independent from each other and are needed to carefully characterise the intermediate cell population to demonstrate their similarity to brain-derived NSCs. This cell type is named bone marrow-derived NSC-like cell (BM-NSC). In this case, whole bone marrow cell suspensions were used as the initial cell population, or hMSC-derived NSC-like cells (hMSC-NSCs) when hMSCs were used for conversion.

6.1 First step: conversion into NSC-like cells

The *in vitro* conversion of bone marrow cells or hMSCs into clonogenic undifferentiated hNSC-like cells, which can be proliferated and subsequently differentiated into all major cell lineages of the brain, such as neurons, astroglial and oligodendroglial cells, has been reported recently [25,26]. This immature BM-NSC or hMSC-NSC population is more suitable for neuroregenerative strategies using transplantation procedures compared with fully differentiated neural cells, as

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terminal differentiated neuronal cells are well known to poorly survive detachment and subsequent transplantation procedures.

Joannides and colleagues showed that sequential treatment of hMSCs with the mitogens EGF and FGF-2 in serum-free media for 7 days, followed by exposure to postnatal hippocampal astrocyte-conditioned medium (HACM) for another 7 days, significantly promotes the generation of neuroectodermal cells (shown by neurofilament/β-tubulin expression) [96]. Interestingly, during step one, NSC markers appeared (Nestin and Musashi-1), and after 7 days the first NF-positive cells could be detected having a round cell shape with only short processes. Removal of mitogens and further culturing in serum-free conditions partly reversed this effect, and cells acquired mesodermal properties. Addition of HACM to the serum-free media after step one led to neuroectodermal maturation, but replacing HACM with DMSO also revealed a neuroectodermal phenotype in some of the cells. Thus, the requirement of two stages, notably 'commitment' and 'differentiation', seems to be of considerable interest [96]. However, the mechanisms underlying this process remain unknown. Furthermore, the presented data only focused on morphological data by immunocytochemistry. The systematic investigation of neuroglial differentiation capacity of hMSCs by Bossolasco and colleagues also described neurosphere-like structures derived from hMSCs, but did not report on subsequent differentiation of these cellular aggregates [61].

D'Ippolito and colleagues recently presented that the neural induction of MIAMI cells also required a sequential conversion–induction process. This process has been divided into three steps: specification (incubation with FGF-2), followed by commitment (exposure to BME or NT-3) and differentiation (incubation with NT-3/NGF/BDNF). Each stage was characterised by the stage-specific expression of specific markers: Nestin was expressed in the first cell stage, followed by induction of β-tubulin-III and NGFR in ~ 50% of the cells (stage two), and then expression of NF-M and NeuN in ~ 40% of plated cells (stage three). However, only morphological analysis has been performed and no further characterisation has been carried out or been published, for example, concerning their mesodermal potential after neuroectodermal conversion or functional data [85].

In 2003, Lee and colleagues showed the *in vitro* isolation and expansion of cells capable of forming neurosphere-like aggregates from human adult bone marrow [26], and called them marrow-derived neural-competent cells (BM-NSCs). Assuming the similarity to embryonic cells, as well as given that neural fate is one of the principle default differentiation pathways of uncommitted multipotent cells *in vitro* [20,97], they developed a multiple-step protocol similar to that used in ES cell culture using serum-containing media, supplemented with leukaemia inhibitory factor, FGF-2, sonic hedgehog (SHH) and FGF-8. Clusters of cells were first seen after 10 days and transferred to serum-free conditions supplemented with FGF-2, SHH and FGF-8. After another 10 – 20 days, Nestin$^+$ neurospheres were appearing, which could be propagated in serum-free media containing FGF-2. Cells within these passaged spheroids could be further differentiated into astrocytes and neurons [26]. *In vivo*, these cells also differentiated into neuronal subtypes and oligodendrocytes. Furthermore, gene expression analysis using the gene array technique showed the acquirement of an expression profile similar to that of human fetal brain-derived NSCs, indicating a successful conversion process [26]. However, two points must be taken into account. First, the authors used whole bone marrow cell suspensions after depletion of red blood cells. As shown by many groups, whole bone marrow contains a heterogeneous set of cells, containing mainly stromal and haematopoietic stem cells. In the mentioned study, it is therefore impossible to distinguish whether the neural-like cells were derived from haematopoietic stem cells or from hMSCs. Second, neither functional data nor the mesodermal properties after conversion were investigated.

According to the protocol established by Hermann and colleagues (2004) [25], hMSCs are converted into cells with characteristics of immature neuroectodermal cells bearing all major characteristics of NSCs by culturing hMSCs in uncoated flasks in serum-free medium supplemented with the mitogens EGF and FGF-2 [25]. It is important to note that only cells that do not adhere to the surface of tissue culture flasks should be kept in culture. In the authors' experience, usually a third of the hMSCs die after 3 days, but after 7 – 14 days, the remaining cells form small spheres of floating cells. These hMSC-NSCs proliferated with an estimated doubling time of 2.6 days *in vitro* for at least 10 weeks (~ 5 – 30 population doublings), neither changing morphology nor phenotype [25]. The extensive marker expression analyses revealed that 90% of hMSC-NSCs express high levels of Nestin, but only a few cells still express low levels of the mesodermal cell marker fibronectin. Quantitative RT-PCR of hMSC-NSCs demonstrated acquisition of neuroectodermal transcripts, including mRNA encoding Otx1, NeuroD1, Neurogenin-2, Musashi-1 and Nestin, with levels between 5- and 78-fold of those seen in the initial cell population of hMSCs [25,57]. 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. It is noteworthy that hMSCs from young donors proliferate faster and seem to have a more multipotent potential, including neuroectodermal differentiation potential (unpublished observations). The mesodermal differentiation capacity of hMSC-NSCs was dramatically decreased during the conversion step [25].

6.2 Second step: terminal neuroectodermal differentiation

The second step of most two- or multistep protocols for neuroectodermal differentiation of hMSCs comprises the terminal differentiation of hMSC-NSCs by plating the cells

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on specific surfaces, withdrawal of mitogens and incubation with various differentiation factors. Johannides and colleagues showed the production of β-tubulin/NF double-labelled cells from hMSCs after terminal differentiation. However, only immunocytochemistry data were shown without a second method confirming the marker expression. Furthermore, neither the initial population was characterised nor has functional data of neuroectodermal cells been presented [96]. Similar to that, D'Ippolito and co-workers showed evidence for neuroglial transdifferentiation of hMSCs without further characterisation of the obtained cell types [85]. One study failed to further characterise the produced neurospheres due to paucity of converted cells [61]. It, therefore, remains unknown whether these cells develop in a stepwise conversion from a mesodermal to a neuroectodermal progeny, or whether these changes are mediated by aberrant gene expression.

Lee and colleagues convincingly showed the acquirement of neuroectodermal phenotypes even in a clonal setting, thus showing the multipotency of their human BM-NSCs (hBM-NSCs) [26]. Interestingly, terminal differentiated hBM-NSCs had a similar gene expression profile compared to terminal differentiated fetal NSCs, as shown by gene chip analysis. Furthermore, *in vivo* migration and differentiation analysis showed the acquirement of neuroectodermal transcripts after transplantation [26]. As this study used whole bone marrow cell suspension as the primary cell source, it remains unclear whether the obtained neural cell types were derived from MSCs or haematopoietic stem cells.

In the authors' two-step conversion–differentiation protocol, the authors demonstrated the appearance of mature neuroectodermal cell types after terminal differentiation [25]. Mature neuroectodermal transcripts, such as MAP2ab, GFAP or GalC, were upregulated on mRNA and protein levels during terminal differentiation. These changes of marker expression correspond to a morphological change towards a glial and neuronal morphology. It is noteworthy that investigation of early neurogenic markers showed a downregulation during this terminal differentiation. Using a HPLC-based method, the authors detected the production and depolarisation-dependent release of the neurotransmitter dopamine in terminally differentiated hMSC-NSCs. Electrophysiology revealed the expression of small Na^+ currents and K^+ channels, a pattern usually seen in astroglial cells. However, the authors failed to show action potentials as a major obligate property of neurons [25]. Together, the authors were able to detect properties of astroglial cells as well as of an immature neuronal phenotype. In line with that, Nestin expression still remained high after terminal differentiation [25]. Similarly to the authors' observations, Jiang and colleagues showed for mouse MAPCs that fully mature neurons could only be achieved after coculturing these cells with astrocytes [31]. Thus, further investigations are warranted to improve the maturity of obtained neuroectodermal cell types in the authors' two-step protocol using human MSCs. Furthermore, *in vivo* data demonstrating the behaviour of hMSC-NSCs after transplantation are needed to further characterise the obtained cell type.

7. Comparison of hMSC-NSCs with brain-derived adult hNSCs

As described in section 4 in detail, there is a lack of a single NSC marker, and NSCs are, therefore, defined by a specific pattern of marker gene expression and operationally by their growing and differentiation behaviour in culture [1,3]. As these properties are species-specific and are, in addition, dependent on the developmental stage of the tissue of origin, hMSC-derived NSC-like cells should be compared with NSCs directly isolated from the adult human brain in order to define their neuroectodermal specification. There are a few recent studies demonstrating isolation and characterisation of NSCs from different regions of the adult human brain, including cortex, amygdala, hippocampus and SVZ [70,78-84]). Unfortunately, there is no comparative study of hMSC-NSCs and human brain-derived NSCs available. However, the authors used similar culture conditions for the expansion of both hMSC-NSCs and hippocampus-derived hNSCs, and presented a detailed analysis of the expression of surface markers, early neuroectodermal and NSC markers in both cell types [25,57,84]. In general, the growing behaviour with neurosphere formation and a doubling time of 1 – 2 weeks, the surface marker expression, as well as the expression pattern of early and late neuroectodermal marker genes, are similar in hMSC-NSCs and adult human hippocampal NSCs (**Table 2**).

Morphological and expression analysis of late neuroectodermal marker genes by immunocytochemistry and quantitative RT-PCR revealed that the differentiation capacity of hMSC-NSCs is similar to that found in adult human hippocampal NSCs (**Table 2**; [25,57,84]). Electrophysiological measurements showed functional properties of glial cells in differentiated hMSC-NSCs [25], but, in contrast to brain-derived adult NSCs, there is no single report on the functional properties characteristic for neurons in cells derived from hMSCs [81,83,84,98]. Together, the two-step protocols for the conversion of hMSCs into neuroectodermal cell types demonstrated that:

- the resulted NSC-like cells display all major characteristics of NSCs
- the terminal differentiated cell populations showed morphological characteristics of the three main neural phenotypes
- the functional maturation of the resulted neuroglial cell types is incomplete

8. Conclusions

Here the authors discuss the neuroectodermal conversion of hMSCs by epigenetic stimulation of the cells, but without the genetic engineering of the cells as reported by Dezawa and co-workers [99,100]. There have been many studies in recent

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Table 2. Comparison of marrow-derived NSC-like cells and hippocampal NSCs.

Properties	Marrow-derived NSC-like cells	Adult hippocampal NSCs
Morphology	Neurosphere-like structures	Neurospheres
Doubling time	2.6 days	~ 7 – 14 days
Surface markers	$CD9^{low}$, $CD15^{-}$, $CD34^{-}$, $CD45^{-}$, $CD90^{+}$, $CD133^{-}$, $CD166^{-}$	$CD15^{low/-}$, $CD34^{-}$, $CD45^{-}$, $CD133^{-}$
NSC markers	$Nestin^{+}$, Musashi-1^{+}	$Nestin^{+}$, Musashi-1^{+}
Early neuroectodermal markers	$OTX1^{+}$, $NeuroD1^{+}$, $Neurogenin2^{+}$	$OTX1/2^{+}$, $Olig2^{+}$, $NeuroD1^{+}$, $Neurogenin2^{+}$
Late neuroectodermal markers	$MAP2ab^{-}$, β-tubulin III^{-}, $GFAP^{-}$, MBP^{-}, $GalC^{-}$	$MAP2ab^{-}$, $GFAP^{-/low}$, $GalC^{-}$
Other markers	$Fibronectin^{+}$	$Fibronectin^{-}$
Differentiation capacity	$MAP2ab^{+}$ or β-tubulin III^{+} neurons (no functions), $GFAP^{+}$ and functional astroglia, $GalC^{+}$ oligodendroglia	$MAP2ab^{+}$ or β-tubulin III^{+} functional neurons, $GFAP^{+}$ and functional astroglia, $GalC^{+}$ oligodendroglia

Marrow-derived NSC-like cells and adult human hippocampal NSCs were cultured under similar conditions and analysed by microscopy (morphology, differentiation capacity), FACS (surface markers), quantitative RT-PCR and/or immunocytochemistry (NSC markers, early and late neuroectodermal markers, other markers, differentiation capacity), as well as functional assays (differentiation capacity). Data are from [25,62,85].

β-tubulin III: Neuron-specific class III beta tubulin; GalC: Galactocerebrosidase C (marker for oligodendrocytes); CD: Cluster of differentiation; GFAP: Glial fibrillary acidic protein; MAP2ab,: Microtubulin-associated protein 2ab; MBP: Myelin basic protein (marker for oligodendrocytes); NeuroD1: Neurogenic differentiation 1; NSC: Neural stem cell; Olig2: Oligodendrocyte lineage transcription factor 2; OTX1: Orthodenticle *Drosophila* homologue of 1; OTX2: Orthodenticle *Drosophila* homologue of 2; RT-PCR: Real-time polymerase chain reaction.

years reporting the transdifferentiation of hMSCs into neuroectodermal cells *in vitro* [19,21,22,23,31,33,34]. Several *in vitro* studies re-evaluating the differentiation potential of MSCs towards a neural phenotype could not reproduce some of the transdifferentiation protocols [92,93]. In contrast, it is reported that cells with major characteristics of the neuroectoderm can be generated from hMSCs obtained from adult bone marrow [25,26,57], similar to the way neural cells are derived from ES cells [6,7]. However, for a complete and stable change of the mesodermal cell into the mature neuroectodermal phenotype, it is necessary to demonstrate morphology and marker expression of neuroectodermal cell types, functional properties of glial cells and neurons, and loss of mesodermal properties, for example, mesodermal differentiation capacity. Most reports present morphological and marker gene expression studies without functional data on the resulting neuroglial cells. Moreover, all but one study lack data on mesodermal properties of neuroectodermally converted hMSCs [25].

As mentioned by Neuhuber and colleagues, the presence of a subset of neuronal markers cannot be taken to prove commitment to a neuronal fate. To clearly identify a differentiated MSC as a neuron, it should display a complete set of neuronal markers [92,93]. The authors provided data on a set of early and late neuroectodermal marker gene expression during all conversion–differentiation steps, showing that hMSC-derived NSC-like cells express a similar marker pattern compared to brain-derived NSCs [25,57,84]. Furthermore, terminal differentiation of hMSC-NSCs resulted in the downregulation of NSC-marker genes and early neuroectodermal genes, but an upregulation of marker genes of mature neuroglial cell types [25,57,84]. Lee and co-workers performed a detailed comparative study on the transcription profile of hMSC-NSCs using the gene array technique [26]. In their multistep protocol, they found a dramatic change of the transcriptome of their initial whole bone marrow cell suspension towards a transcription profile similar to that determined in fetal brain-derived NSCs. Together, the morphological and marker expression data demonstrate a significant shift from the mesodermal phenotype of hMSCs towards a neuroglial phenotype with astroglial, oligodendroglial and neuronal properties, in particular in the case of a multistep conversion–differentiation approach. On the other hand, the differentiation techniques using a chemical induction medium seem to induce a pseudoneuronal morphology, and the changes of the expression profile are probably a result of aberrant gene expression [57,92,93,95].

With respect to functional properties of the generated neural cell types, mature and functional neuroectodermal cells were only seen when using the multiple-step protocol introduced by Hermann and colleagues [25]. The authors were able to show electrophysiological properties of astroglial cells (astroglia type 2), as well as neurotransmitter (dopamine) production and depolarisation-dependent release, in terminally differentiated hMSC-NSCs [25]. Interestingly, neither the conversion step nor the terminal differentiation step of the two-step conversion–differentiation protocol alone were able to induce a mature and functional neuroectodermal phenotype, underlining the importance of the multiple-step protocol [25,57]. However, there is no study demonstrating functional properties obligate for neurons, such as the expression of sodium channels and action potentials in cells from hMSCs.

To demonstrate the stable phenotype change of hMSCs into a neural cell population, the authors investigated the mesodermal differentiation capacity and the marker

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expression for mesodermal cell types in hMSC-NSCs [25,57]. They showed a dramatic decrease of mesodermal (osteogenic) differentiation capacity of hMSC-NSCs compared with MSCs, as well as a significant reduction of mesodermal marker expression during the neuroectodermal differentiation process. These data are supported by gene array analysis of hMSC-NSCs in comparison to blood cells [26].

9. Expert opinion

The major advantage of the hMSC system discussed here is the possibility of an autologous approach in stem cell therapy without the need of therapeutic cloning. Furthermore, the presented protocols exclusively use epigenetic factors for neuroectodermal conversion without genetically engineering the cells, which facilitates their potential use in humans by reducing the risk for tumourigenesis, as well as for unwanted gene transfer to the host organism. hMSCs are an easily accessible and on-demand available cell source in every individual. Using routine bone marrow punction to isolate these cells for further *in vitro* proliferation and subsequent sorting or priming towards the lineage of interest would revolutionise stem cell therapy. The first transplantation studies using neuroectodermally converted immature cells derived from human bone marrow showed that these cells survive and migrate into the diseased brain area [26]. However, there is a hypothetical risk that the patient's own cells might carry genetic information that increases their vulnerability to the pathogenetic process underlying the patient's disease. Many questions remain to be answered before we can translate these results to human therapy:

- Which disorders are most feasible to cell-based therapeutic approaches, such as transplantation, gene delivery or cytokine production by transplanted hMSCs? Parkinson's disease? Alzheimer's disease? Tumours? Stroke?
- What is the best way to administer hMSCs: intracerebral, intraventricular or intravenous transplantation?
- What is the best cell stage for transplantation: hMSC, hMSC-NSCs or terminal differentiated hMSC-NSCs? (refer to **Figure 2**)
- Concerning neurotransplantation, are the beneficial effects of transplantation of new cells into the brain more likely to come from secretion of neurotrophic factors rather than integration of new cells into the existing circuitries? [104,105]
- Concerning gene delivery via hMSCs, what are the side effects, especially after long-term survival of animals?
- What is the immune response following (autologous) intracerebral implantation of hMSC-derived cells?
- Is there a hypothetical risk that the patient's own cells carry genetic information that increases their vulnerability to the pathogenetic process underlying the different diseases?
- Is there a risk for tumour formation after intracerebral transplantation of these adult stem cells?

Producing specific cell types from the patient's own stem cells would provide many advantages, for example, the availability of cells, ethical issues, as well as the lack of need for immunosupression. Nevertheless, conditions for generating neurons from human adult stem cells need to be optimised before these cells can be considered for transplantation procedures in humans. The cell culture system of human marrow-derived NSCs provides a powerful tool for investigating the molecular mechanisms of neural differentiation in adult hNSCs. hMSC-NSCs may, therefore, ultimately help to treat acute and chronic neurodegenerative diseases.

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Mesodermal cell types induce neurogenesis from adult human hippocampal progenitor cells

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Abstract

Neurogenesis in the adult human brain occurs within two principle neurogenic regions, the hippocampus and the subventricular zone (SVZ) of the lateral ventricles. Recent reports demonstrated the isolation of human neuroprogenitor cells (NPCs) from these regions, but due to limited tissue availability the knowledge of their phenotype and differentiation behavior is restricted. Here we characterize the phenotype and differentiation capacity of human adult hippocampal NPCs (hNPCs), derived from patients who underwent epilepsy surgery, on various feeder cells including fetal mixed cortical cultures, mouse embryonic fibroblasts (MEFs) and PA6 stromal cells. Isolated hNPCs were cultured in clonal density by transferring the cells to serum-free media supplemented with FGF-2 and EGF in 3% atmospheric oxygen. These hNPCs showed neurosphere formation, expressed high levels of early neuroectodermal markers, such as the proneural genes *NeuroD1 and Olig2*, the NSC markers Nestin and Musashi1, the proliferation marker Ki67 and significant activity of telomerase. The phenotype was $CD15^{low/-}$, $CD34^-$, $CD45^-$ and $CD133^-$. After removal of mitogens and plating them on poly D-lysine, they spontaneously differentiated into a neuronal ($MAP2ab^+$), astroglial ($GFAP^+$), and oligodendroglial ($GalC^+$) phenotype. Differentiated hNPCs showed functional properties of neurons, such as sodium channels, action potentials and production of the neurotransmitters glutamate and GABA. Co-culture of hNPCs with fetal cortical cultures, MEFs and PA6 cells increased neurogenesis of hNPCs *in vitro*, while only MEFs and PA6 cells also led to a morphological and functional neurogenic maturation. Together we provide a first detailed characterization of the phenotype and differentiation potential of human adult hNPCs *in vitro*. Our findings reinforce the emerging view that the differentiation capacity of adult hNPCs is critically influenced by non-neuronal mesodermal feeder cells.

Keywords: human adult neural progenitor cells, neuronal differentiation, neural stem cells, PA6 stromal cells.

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Abbreviations used: hNPCs, hippocampal neural progenitor cells; MEFs, mouse embryonic fibroblasts, NPCs, neural progenitor cells; PDL, poly-D-lysine.

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Neural precursor cells (NPCs) have been the focus of increased attention over recent years because of their potential in cell replacement and gene therapy in the adult brain of patients with neurological diseases, for example stroke and Parkinson's disease. Recent *in vitro* studies indicated that multipotent, self-renewing NPCs with the capacity to differentiate into glial and neuronal cell types can be isolated from several mammalian adult brain regions namely the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Reynolds and Weiss 1992; Johansson *et al*. 1999b; Rietze *et al*. 2001; Doetsch 2003; Hack *et al*. 2004) and in lower amounts from non-neurogenic areas, such as neocortex and the spinal cord (Magavi *et al*. 2000; Shihabuddin *et al*. 2000; Wachs *et al*. 2003). Indeed, a few recent studies demonstrated isolation of NPCs from different regions of the adult human brain including cortex, amygdala, hippocampus and SVZ (Kirschenbaum *et al*. 1994; Johansson *et al*. 1999a; Kukekov *et al*. 1999; Arsenijevic *et al*. 2001; Nunes *et al*. 2003; Westerlund *et al*. 2003; Moe *et al*. 2005), but only three previous studies investigated human adult NPCs from the hippocampus region *in vitro* (Johansson *et al*. 1999a; Kukekov *et al*. 1999; Roy *et al*. 2000). However, there is no detailed analysis yet of the expression of early neuroectodermal or neural stem cell markers in proliferating adult human hippocampal NPCs (hNPCs). These progenitor cells are reported to be multipotent cells differentiating into both glial and neuronal cells with some functional properties of neurons, such as the expression of sodium and potassium channels (Roy *et al*. 2000). In contrast to extensive analyses of the differentiation capacity of adult non-human mammalian hNPCs in the absence and presence of various feeder layer cell types, such as astrocytes and fibroblasts (Song *et al*. 2002), there is no systematic evaluation of the influence of feeder cell layers on the differentiation behavior of adult NPCs from human origin.

There is a growing body of evidence that cell-to-cell contacts are important for neuroectodermal specification of fetal and adult NPCs (Kawasaki *et al*. 2000; Storch *et al*. 2001; Song *et al*. 2002). Various cell types derived from neuroectodermal tissue including cortical, striatal and mesencephalic astrocytes support not only the neuronal differentiation, but also the neuronal subtype specification of fetal and adult mammalian brain-derived NPCs (Ling *et al*. 1998; Storch *et al*. 2001; Song *et al*. 2002; Nakayama *et al*. 2004; Hermann *et al*. 2005). Additionally, several mesodermal-derived cell types such as fibroblasts and PA6 stromal cells are reported to significantly alter the differentiation capacity of various embryonic, fetal and adult neuroprogenitor cell types (Kawasaki *et al*. 2000; Perrier *et al*. 2004; Hermann *et al*. 2005; Kitajima *et al*. 2005). Adult and embryonic fibroblasts promote the differentiation of various NPC types including embryonic stem (ES) cell-derived and adult NPCs into all neural cell types including astroglial and neuronal cells. PA6 cells drive the differentiation of embryonal stem (ES) cell-derived NPCs and adult mesencephalic NPCs into nerve cells with a subset of cells showing a functional dopaminergic phenotype. In most studies the conditioned media were not as effective as the corresponding cocultured feeder cell layer (Kawasaki *et al*. 2000; Song *et al*. 2002) suggesting that direct cell-to-cell contacts promote the neuroectodermal differentiation of NPCs.

Although there are a few reports on isolation and short-term propagation of adult hippocampal NPCs from human origin (Johansson *et al*. 1999a; Kukekov *et al*. 1999; Roy *et al*. 2000), the phenotype of long-term expanded adult human hippocamal NPCs as well as their neuroectodermal differentiation capacity is largely unknown. Here we describe the isolation, long-term expansion and differentiation potential of multipotent adult hippocampal NPCs derived from epileptic-surgery procedures. We further analyzed the expression of early neuroectodermal and NPC marker genes as well as the telomerase activity during the proliferation and their differentiation capacity on various coculture systems including mouse cortical astrocytes, mouse embryonic fibroblasts (MEFs) and PA6 stromal cells.

Materials and methods

Cell culture

Adult human hippocampal tissue was obtained from routine epilepsy surgery procedures (selective amygdale-hippocampectomy or anterior temporal lobectomy; 14 samples; age 18–55 years) following informed consent of the patients. All procedures were in accordance with the Helsinki convention and approved by the Ethical Committee of the University of Ulm. The tissue was taken from the removed hippocampi and stored in ice-cold Hank's balanced salt solution (HBSS) supplemented with 11 mM glucose and 1% penicillin/streptomycin for transporting the tissue from the operating theatre. All patients underwent high-resolution magnetic resonance imaging excluding tumors and were screened for the presence of infectious disease. In all cases, the hippocampus and the neuropathological examination did not reveal evidences for tumor formation. For expansion of neurospheres consisting of hNPCs, tissue samples were cut into small pieces with a scalpel, incubated in 0,1% trypsin (Sigma, St Louis, MO, USA) for 30 min at RT, incubated in DNase (40 mg/mL; Sigma) for 10 min at RT and homogenized to a quasi single cell suspension by gentle triturating (Storch *et al*. 2001). The cells were added to 25 cm^2 flasks ($2 \times 3\ 10^6$ viable cells per flask) in Knock-Out DMEM (Gibco BRL, Life Technologies; Tulsa, OK, USA), supplemented with 10% serum replacement (Gibco); 0.5 mM glutamine; 1% penicillin/streptomycin and 20 ng/mL of both EGF and FGF-2 (both from Sigma) at 5% CO_2; 92% N_2 and 3% O_2 using an incubator equipped with an O_2-sensitive electrode system (Haereus, Germany). After 10–20 days neurosphere formation was observed and these spheres were expanded for additional 5–8 weeks (in total 7–12 weeks, 5–12 passages) before differentiation was initiated. The medium was changed once a week, while the growth factors were added twice a week. For BrdU labeling, cells were incubated for 30 h with 10 μM BrdU.

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Differentiation conditions

Induction of neural differentiation was initiated by plating the cells on poly-D-lysine (PDL) coated glass cover-slips in Knock-out-DMEM, 10% serum replacement, 0.5 mM glutamine, 1% penicillin/streptomycin, 10 ng/mL rh-BDNF (Promega, Madison, WI, USA), 100 μM di-butyryl-(db) cAMP and 0.5 μM retinoic acid (both from Sigma). Cells were differentiated for 14 days. For coculture analyses, various feeder cells (mouse embryonic fibroblasts [MEFs]), PA6 stromal cells as well as primary mixed cortical cultures (containing astrocytes and neurons) from E17 mouse embryos were cultured on gelatine-coated cover-slips as described earlier (Kawasaki *et al*. 2000; Storch *et al*. 2001). In coculture experiments, hNPCs were pre-stimulated with expansion media supplemented with 500 ng/mL Shh and 100 ng/mL FGF-8 for 48 h prior to seeding the cells. When coculture was started, confluent feeder layers were washed twice with PBS. The hNPCs were seeded at a concentration of $1.5–2.0 \times 10^5$ cells cm^{-2} in D-MEM high glucose/F_{12} (50 : 50) containing 5% fetal calf serum, 5% horse serum; 1% Penicillin/Streptomycin. For conditioned media experiments confluent PA6 cell layers were washed three times with the medium described above for coculture experiments and incubated further with the same medium. The media, conditioned by exposure to PA6 cells for 72 h, was then removed, centrifuged at 1800 × g for 15 min, and the supernatant was used immediately or stored at −20°C until for further use. For conditioned media experiments, hNPCs were seeded on PDL at a concentration of $1.5–2.0 \times 10^5$ cells cm^{-2} similar to coculture experiments, but the media were supplemented with PA6 conditioned medium (50%). Medium change was performed on day 4 and every other day following that.

Flow cytometry

Human hNPCs were treated with Accumax® (Gibco) and washed with PBS. Dead cells were excluded from analysis by forward scatter gating. Samples were processed using a FACSCalibur flow cytometer and analyses were performed with the Cellquest software (both from Becton Dickinson, Franklin Lakes NJ, USA). Antibodies were used as follows: CD15-FITC 1 : 10, CD34-FITC 1 : 10, CD45-PE 1 : 10, CD133-PE 1 : 10 (all from Miltenyi Biotech, Bergisch Gladbach, Germany). A minimum of 10 000–12 000 events were acquired for each sample.

Immuncytochemistry

Cell cultures were fixed in 4% paraformaldehyde in PBS or with 4% paraformaldehyde/PBS followed by ice-cold acidic ethanol and 2 N HCl for BrdU staining. Immunocytochemistry was carried out using standard protocols. Cell nuclei were counter stained with 4,6-diamidino-2-phenylindole (DAPI). The following primary antibodies were used: Mouse anti-human nuclei 1 : 200; mouse anti-Nestin 1 : 500, rabbit anti-GFAP 1 : 1000, mouse anti-GalC 1 : 750, rabbit anti-Neurogenin2 1 : 500, rabbit anti-Musashi1 1 : 500, rabbit anti-NeuroD1 1 : 500 (all from Chemicon International, Temecula, USA); rabbit anti-Tuj1 1: 2000, mouse anti-Tuj1 1 : 500 (both from Covance, Richmond, CA); rabbit anti-Ki67 1 : 500 (Novocastra, Newcastle, UK), rabbit anti-Olig2 1: 2000 (kindly provided by Dr H. Takebayashi) and secondary antibodies conjugated to Alexa 488, 568 or 647 1 : 500 (all from Invitrogen-Molecular Probes, Carlsbad, California), and rat anti-BrdU 1 : 40 with fluorescence labeled secondary antibody (both from Abcam, Cambridge, UK). Images were captured using a fluorescence microscope (Axiovert 135, Zeiss, Oberkochen, Germany) or a Leica TCS/NT confocal microscope equipped with krypton, krypton/argon and helium lasers.

Telomerase activity

A highly sensitive *in vitro* assay known as the quantitative real-time telomeric repeat amplification protocol (Emrich *et al*. 2002; Saldanha *et al*. 2003) has been used for detecting telomerase activity (OTD kit; Allied Biotech, Ijamsville, MD). The telomerase activity in the cell or tissue extract is determined through its ability to synthesize telomeric repeats onto an oligonucleotide substrate *in vitro*. Telomerase from the cell extract or tissue adds telomeric repeats onto a substrate oligonucleotide and the resultant extended product are subsequently amplified by PCR. The PCR products are then visualized using DNA fluorochromes SYBR Green as described above. Mouse ES cells (D3 ES cell line) were used as positive control and cultured as described previously (Chung *et al*. 2002), whereas adult mouse cortical tissue was used as negative control (Milosevic *et al*. 2005).

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

Total cellular RNA was extracted from hNPCs during expansion using RNAeasy total RNA purification kit followed by treatment with RNase-free DNase (Qiagen, Hilden, Germany). 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 fluorescence dye SYBR Green I to double-stranded DNA. 1 μL (50 ng) of total RNA was reverse transcribed and subsequently amplified using QuantiTect SYBR Green RT-PCR Master mix (Qiagen) and 0.5 μmol l^{-1} of both 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. The results are expressed relative to the housekeeping gene *HMBS* (hydroxymethylbilane synthase). Primer sequences, lengths of the amplified products and melting point analyses are summarized in Table 1.

Electrophysiology

Cells were investigated for membrane currents 3–10 days after initiation of differentiation on PDL using the standard whole cell patch clamp technique with an EPC-7 amplifier (List Electronics, Heidelberg, Germany) and pClamp data acquisition (Axon Instruments, Union City, CA) essentially as described previously (Storch *et al*. 2003; Hermann *et al*. 2004). For better sealing we added 5% FCS 5 h prior to the patch clamp experiments. The extracellular solution contained (in mmol l^{-1}): 142 NaCl, 8.1 KCl, 1$CaCl_2$, 6 $MgCl_2$, 10 HEPES, 10 D-Glucose, pH value 7.4. The pipette solution contained (in mmol L^{-1}): 153KCl, 1 MgCl2, 5 EGTA, 10 HEPES, pH value 7.3. Using these solutions, borosilicate pipettes had resistances of 6–10 MΩ. Seal resistances in the whole cell mode were between 0.5 and 1 GΩ. Data were analysed using pClamp 8.0, Microsoft Excel 97 and Origin 5.0 software. Resting membrane potentials (RMP) were determined in the current clamp mode immediately after establishing the whole cell configuration. Action potentials (APs) were elicited by applying increasing depolarizing current pulses (300 ms, 10 pA current steps).

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Table 1 Primers for quantitative real-time RT-PCR

Gene (Protein)	*Sequence* (*forward; reverse)*	Accession Number
FZD1	5′-GGA TTG GCA TTT GGT CAG TG-3′	NM003505
(frizzled homologue 1)	5′-CTT GTC ATT ACA CAC CAC TCG G-3′	
HMBS	5′-TCG GGG AAA CCT CAA CAC C-3′	NM000190
(hydroxymethylbilane synthase)	5′-CCT GGC CCA CAG CAT ACA T-3′	
MSI1	5′- GCC CAA GAT GGT GAC TCG-3′	NM002442
(musashi 1)	5′-ATG GCG TCG TCC ACC TTC-3′	
NeuroD1	5′-CGC TGG AGC CCT TCT TTG-3′	NM002500
(neurogenic differentiation 1)	5′-GCG GAC GGT TCG TGT TTG-3′	
Neurog2	5′-CGC ATC AAG AAG ACC CGT AG-3′	NM024019.2
(neurogenin 2)	5′-GTG AGT GCC CAG ATG TAG TTG TG-3′	
NES	5′-TGG CTC AGA GGA AGA GTC TGA-3′	NM006617.1
(nestin)	5′-TCC CCC ATT TAC ATG CTG TGA-3′	
NOTCH1	5′-GCG ACA ACG CCT ACC TCT-3′	NM017617
(Notch homolog 1 translocation associated)	5′-GCA CAC TCG TAG CCA TCG-3′	
NTRK1	5′-CTA CAG CAC CGA CTA TTA CCG – 3′	NM001012331.1
(neurotrophic tyrosine kinase, receptor, type 1)	5′-CGA TTG CCT CCG TGT TG – 3′	
OTX1	5′-CAC TAA CTG GCG TGT TTC TGC-3′	NM014562.2
(orthodenticle homolog 1)	5′-AGG CGT GGA GCA AAA TCG′-3′	
OTX2	5′-CAC TTC GGG TAT GGA CTT GC-3′	NM021728.2
(orthodenticle homolog 2)	5′-CGG GTC TTG GCA AAC AGT G-3′	
P75 (NTR)	5′-CTTTTGGGGTATCCATAGCAGT-3′	NM002507
(nerve growth factor receptor)	5′-CCACGGGACCCTTCATTC-3′	
SOX1	5′-GCC CAG GAG AAC CCC AAG-3′	NM005986
(sex determining region Y-box 1)	5′-CGT CTT GGT CTT GCG GC-3′	
SOX2	5′-CAG GAG AAC CCC AAG ATG C-3′	NM003106.2
(sex determining region Y-box 2)	5′-GCA GCC GCT TAG CCT CG-3′	
SOX10	5′-GCA AGG CAG ACC CGA AGC-3′	NM006941.3
(sex determining region Y-box 10)	5′-GTC CAA CTC AGC CAC ATC AAA G-3′	

Determination of GABA, glutamate, dopamine and serotonin production

For determination of dopamine production, media were supplemented with 100 μmol l^{-1} tetrahydrobiopterin and 200 μmol l^{-1} ascorbate 2 days prior to medium harvest. Dopamine levels were determined in medium stabilized with EGTA/glutathione solution as reported previously (Storch *et al*. 2001), and stored at − 80°C until analysis. Dopamine was assayed by reverse-phase HPLC with an electrochemical detector as previously described (Gerlach *et al*. 1996). GABA, glutamate and serotonin were assayed by HPLC with fluorescence detection (Gerlach *et al*. 1996), employing precolumn derivatisation with ortho-phtaldialdehyde and an automatic HPLC system (Kontron Instruments, Neufahrn, Germany). The excitation and emission wave-length of the fluorescence detector were set at 330 and 450 nm, respectively.

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 100–500 cells were counted per marker. In coculture experiments, only cells positive for the antihuman nuclei antibody were determined in confocal microscopic images to avoid the counting of overlaying cells. Human nuclei/$GFAP^+$ and human nuclei/$Tuj1^+$ cells, respectively, were counted in sister cultures. 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. Data presented are pooled from experiments performed on cells obtained from tissue of all 14 patients. All data are presented as mean ± SEM.

Results

Isolation and long-term expansion of neurosphere-forming cells from the adult human hippocampus

After 10–20 days *in vitro*, the cells formed small spheres with all typical morphological properties of neurospheres (Fig. 1a). FACS analysis revealed that the phenotype of these neurosphere-forming NPCs was $CD15/Lex1^{low/-}$, $CD34^-$, $CD45^-$, $CD133^-$ (Fig. 1b). Extensive analyses of the expression of early and late neuroectodermal marker genes using quantitative real-time PCR and immunocytochemistry (Figs 1a,c, for complete names of genes and encoded proteins refer to Table 1) revealed that hNPCs expressed high levels the early neuroectodermal or CNS precursor cell markers, such as Nestin and Musashi1 (Cattaneo and McKay 1990;

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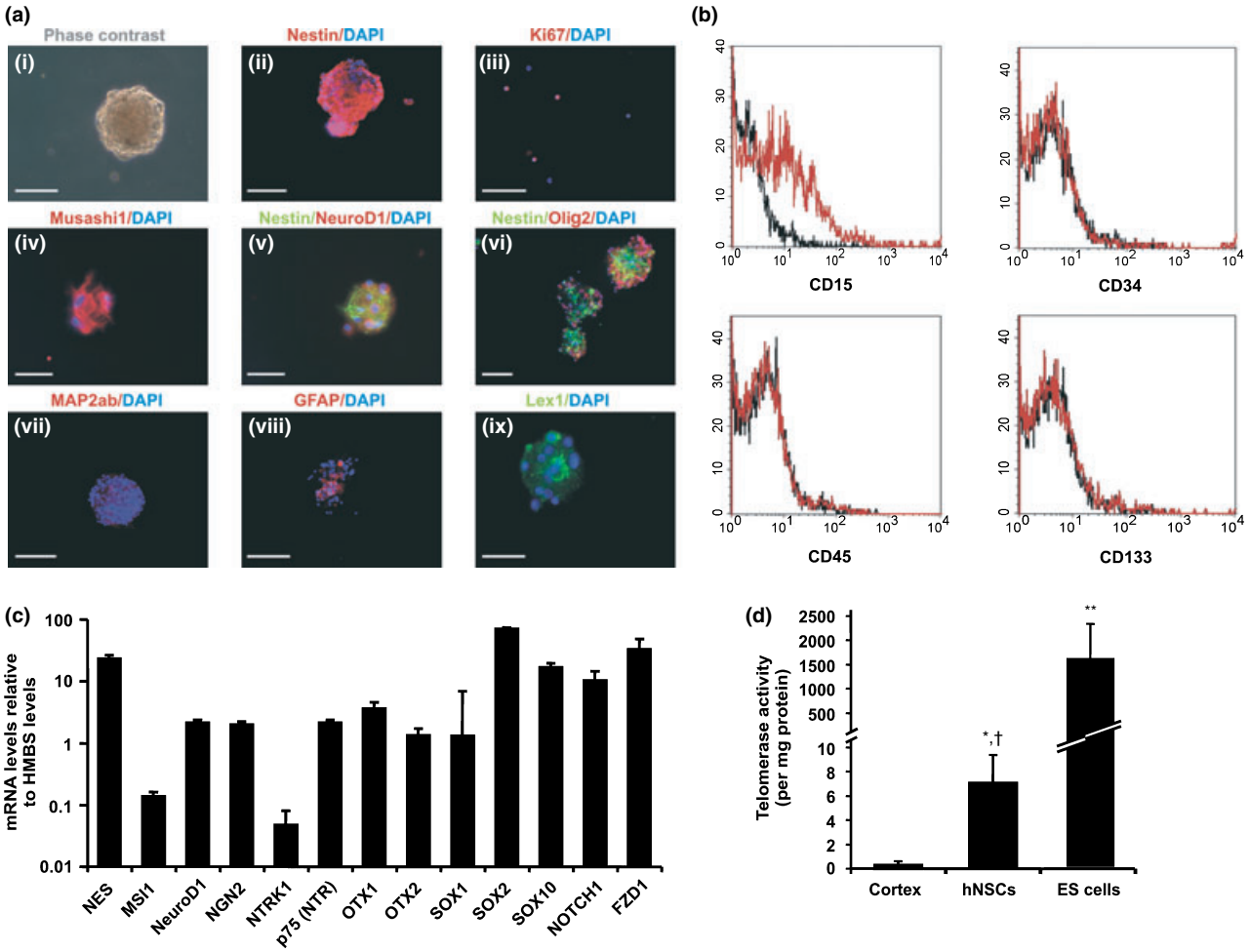

Fig. 1 Characteristics of adult human hippocampal NPCs during *in vitro* expansion. (a) Morphology and marker expression of adult hNPCs during expansion in the presence of EGF and FGF-2. Spheres (phase contrast) were cultured for 2–3 h to allow attachment and then stained for Nestin, Ki-67, Musashi1, Nestin and NeuroD1 or Olig2, MAP2ab, GFAP and Lex1/CD15. Nuclei were counterstained with DAPI. Scale bars, 100 μm (all except Musashi1 and NeuroD1 stainings, respectively) or 50 μm (Musashi1 and NeuroD1 stainings). (b) Flow cytometry of adult hNPCs cultured for 4–12 weeks (5–10 passages). Cells were labeled with fluorescence-coupled antibodies against CD15, CD34, CD45, CD133 or immunoglobulin isotype control antibodies. Cells were analyzed using a FACSCalibur flow cytometer. Black line, control immunoglobulin; red line, specific antibody. (c) Quantitative transcription profile of hNPCs. Results of real-time RT-PCR analysis of the NSC markers Nestin and Musashi1 (*NES, MSI1*), proneural genes (*NeuroD1, NGN2*), genes early expressed in the development (*Frizzled 1* (*FZD1*)), *Notch1, OTX1, OTX2, SOX1, SOX2* and *SOX10*) and tyrosin-receptor kinases like $p75^{NGF.}$ and *NTRK1* are displayed. Expression levels are expressed relative to the housekeeping gene HMBS. For primers complete names of the genes and accession numbers see Table 1. Results are mean values ± s.e.m. from at least three independent experiments. (d) Telomerase activity in human NPCs after 3 to 8 weeks as well as adult cortical tissue and mouse ES cells during expansion measured by the quantitative real-time telometric repeat amplification protocol. Telomerase activity was normalized to protein content. Results are mean values ± s.e.m. from three independent experiments. * indicates $P < 0.05$, ** represents $P < 0.01$ when compared to adult cortical tissue; + indicates $P < 0.05$ when compared to ES cells.

Reynolds *et al*. 1992; Kaneko *et al*. 2000) on both the mRNA and protein level. Furthermore, adult hNPCs showed detectable expression levels of the proneural genes NeuroD1 and Olig2 on the mRNA and/or the protein level (Figs 1a,c). Consistent with the FACS analysis data, only a few cells were weakly positive for CD15 protein (Fig. 1a). Quantitative real-time PCR additionally showed the expression of several early neuroectodermal marker genes, such as *NTRK1*, *OTX1/2* and *SOX1,2,10* as well as surface receptors important for neural development [*NOTCH1, NTRK1, FZD1, p75*(*NTR*); Fig. 1(c)]. This phenotype is similar to that of NSCs derived from adult mouse SVZ or hippocampus (Rietze *et al*. 2001; Doetsch *et al*. 2002; Hack *et al*. 2004). hNPCs could be passaged and cultured as secondary and tertiary neurospheres without changing morphology and phenotype for up to 12 weeks (≈ 8–10 passages).

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To confirm the proliferation potential of adult hNPCs, we used the proliferation marker Ki67 as well as BrdU incorporation to identify DNA-synthesizing cells (Fig. 1a). Ki67 is detected in the nucleus of proliferating cells in all active phases of the cell cycle from the late G1 phase through the M-phase but is absent in non-proliferating and early G1-phase cells and cells undergoing DNA repair (Gerdes *et al*. 1983; Gerdes *et al*. 1984; Key *et al*. 1994). As expected, total Ki-67-staining (representing late G1- through M-phase) was higher at each time point (67 ± 8% of cells were $Ki67^+$ $n = 3$) than total BrdU staining (11 ± 1% $BrdU^+$ cells; $n = 3$), which only marks cells within the S-phase of the cell cycle. After 4–7 weeks, an average number of 7 ± 6% of hNPCs were found as spontaneously apoptotic (chromatin condensation and margination, apoptotic bodies in some cells) by DAPI staining. These cells were negative for Ki67 (data not shown) as well as for most other markers tested such as Nestin and Olig2 (Fig. 1a).

Telomerase is inactive in most somatic cells, but present in various stem cell populations (Meyerson *et al*. 1997; Ostenfeld *et al*. 2000; Mattson and Klapper 2001). Quantification of telomerase activity in hNPCs in comparison to adult human differentiated tissue using the telomeric repeat amplification protocol (Emrich and Karl 2002; Saldanha *et al*. 2003) showed significant telomerase activity in hNPCs compared to human cortical differentiated tissue as negative control without changes over the three months expansion period (Fig. 1d). Together, investigations of the proliferation characteristics revealed the typical pattern of slowly dividing cells with a small amount of spontaneous apoptosis within the hNPC neurospheres.

Adult human hippocampal NPCs differentiate into functional neuronal cell types

Differentiation of adult hNPCs was initiated by removal of mitogens, plating the cells onto poly D-lysine and addition of BDNF, db-cAMP and retinoic acid. After 14 days, 36 ± 8% acquired morphologic and phenotypic characteristics of astrocytes ($GFAP^+$), 31 ± 13% that of oligodendrocytes ($GalC^+$), 29 ± 7% that of young neurons ($Tuj1^+$) and 11 ± 8% that of mature neurons ($MAP2ab^+$ $n = 7$; Figs 2a,b). As we did not find $MAP2ab^+$ cells during expansion of hNPCs within the neurospheres (Fig. 1a), all $MAP2ab^+$ neurons must be derived from progenitor cells not expressing this marker for mature neurons. GFAP and Tuj1 were never found in the same cell (Fig. 2a). Very few cells expressed markers for dopaminergic (tyrosin hydroxylase) or serotoninergic (5-hydroxytyramine, 5-HT) cells (data not shown). Pre-stimulation of hNPCs with expansion media supplemented with Shh (500 ng/mL) and FGF-8 (100 ng/mL) for 48 h prior to seeding the cells did not lead to significant changes in their differentiation behavior (compare Fig. 2b with 3b).

To analyze the functional properties of these differentiated hNPCs, we performed both whole-cell voltage-clamping to record voltage-gated sodium and potassium currents and current-clamping to search for the capacity to generate action potentials after differentiation for 3–7 days. The resting potential of the cells, determined in the current clamp mode immediately after establishing the whole cell conformation, was −41 ± 12 mV ($n = 8$). The majority of differentiated adult hNPCs (5 out of 8 recorded cells) expressed a sustained outward current ranging from a few hundred pA to up to 4 nA (Fig. 2c). These currents showed a voltage dependence and kinetics characteristic for delayed rectifier K^+ channels (Fig. 2d). In a few cells, we could identify inward currents with voltage dependence and kinetics typical for voltage-activated Na^+ channels and were able to record action potentials (Fig. 2c–e). Neurotransmitter (glutamate, GABA, dopamine and serotonin) production was studied on hNPCs differentiated for 14 days using a HPLC-based method. We found significant production of glutamate and GABA by differentiated hNPCs (Fig. 2f–g), but no generation of dopamine and serotonin (data not shown).

PA6 stromal cells and mouse embryonic fibroblasts induce neurogenesis from adult human hippocampal NPCs

We next tested the neuronal differentiation potential of adult hNPCs by plating the progenitor cells on feeder layers of several cell types known to promote neuronal differentiation of ES cells and/or NPCs including fetal mixed cortical cultures, mouse embryonic fibroblasts (MEFs) and PA6 stromal cells (Kawasaki *et al*. 2000; Perrier *et al*. 2004; Hermann *et al*. 2005; Kitajima *et al*. 2005). The human origin of immunostained cells was confirmed by costaining the cultures with a human-specific antibody and investigating the cells using a confocal microscope. As shown in Fig. 3a and b, coculture of hNPCs with primary cortical cells, MEFs or PA6 stromal cells for 14 days resulted in a significant increase of $Tuj1^+$ neurons, while only astrocytes and MEFs produced more $GFAP^+$ glial cells compared to control conditions (PDL-coated culture surface). The potential of different feeder cells to induce neurogenesis from adult hNPCs could be ordered as follows: PA6 cells > MEFs > cortical feeder cells > PDL, whereas the ranking order for the promotion of an astroglial phenotype was: Cortical feeder cells > MEFs > PA6 cells = PDL. Consistently, morphological analysis of the $Tuj1^+$ neurons derived from adult hNPCs by measuring the length of neurites revealed that coculturing the hNPCs with MEFs or PA6 cells promoted neurite outgrowth of differentiating hNPCs compared to cultures on PDL or mixed cortical cultures (Fig. 3c). Media conditioned by PA6 stromal cells for 72 h did not show any relevant effects on glial or neuronal differentiation of hNPCs (no significant changes of $GFAP^+$ or $Tuj1^+$ cell counts compared to hNPCs differentiated in normal media; Figs 3a,b). Furthermore, hNPCs on MEFs or PA6 cells produced significant higher amounts of the neurotransmitters

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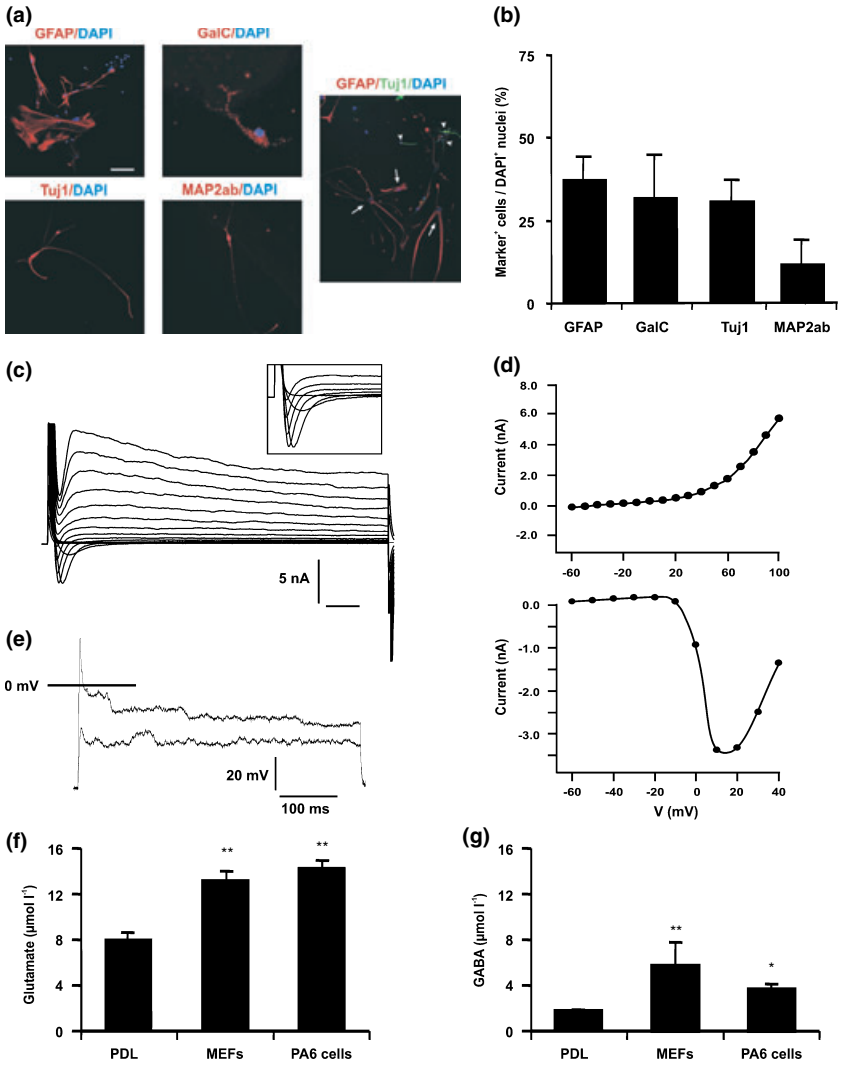

Fig. 2 *In vitro* differentiation capacity of adult human hippocampal NPCs. (a) hNPCs differentiated by plating onto poly-D-lysine (PDL), mitogen withdrawal and incubation with BDNF (10 ng/mL), db-cAMP (100 μM) and retinoic acid (0.5 μM) for 14 days. The cells were stained against markers for astrocytes (GFAP), oligodendrocytes (GalC), and/or neurons (Tuj1; MAP2ab), respectively. In the right panel, arrows mark $GFAP^+$ astrocytes and arrowheads mark $Tuj1^+$ neurons. Nuclei are counterstained with DAPI (blue). Scale bar, 30 μm. (b) Quantification of differentiation capacity of 14-day cultures of hNPCs shown in (a). Data shown are mean values ± SEM from three to seven independent experiments. (c–e) Electrophysiological recordings on hNPCs differentiated without feeder layers as described under (a). For voltage-clamp measurements, cells were held at − 70 mV and hyper- or depolarized in 10 mV steps to + 100 mV for 50 ms. (c) Example of a fast inward current and a sustained outward current (inset: only currents for depolarizing steps to − 60, − 50, 0, 20 and 60 mV are shown). (d) Current-voltage relationships of the sustained outward currents (upper diagram) and the inward currents (lower diagram) of the cell displayed in (c). (e) Representative voltage traces of current-clamp recordings of differentiated hNPCs in response to depolarizing current pulses of increasing amplitude. The resting membrane potential was ∼ − 65 mV and the action potential peaked at ∼ + 30 mV. (f–g) Neurotransmitter production of differentiated hNPCs. Glutamate (f) and GABA (g) production were measured in hNPCs differentiated on poly-D-lysine (PDL), MEFs or PA6 cells for 14 days (see Material and methods) using a HPLC-based assay. Quantification of the neurotransmitters was performed in medium conditioned for 2 days. Feeder cell layer did not produce detectable amounts of glutamate or GABA. *$p < 0.05$; ** $p < 0.01$ when compared to cells differentiated on PDL.

glutamate and GABA compared to cells cultured on PDL or mixed cortical cultures (Fig. 2f–g). We did not find any dopamine or serotonin production under all culture conditions.

Discussion

Here we describe the isolation, long-term *in vitro* expansion and the differentiation potential of multipotent adult human

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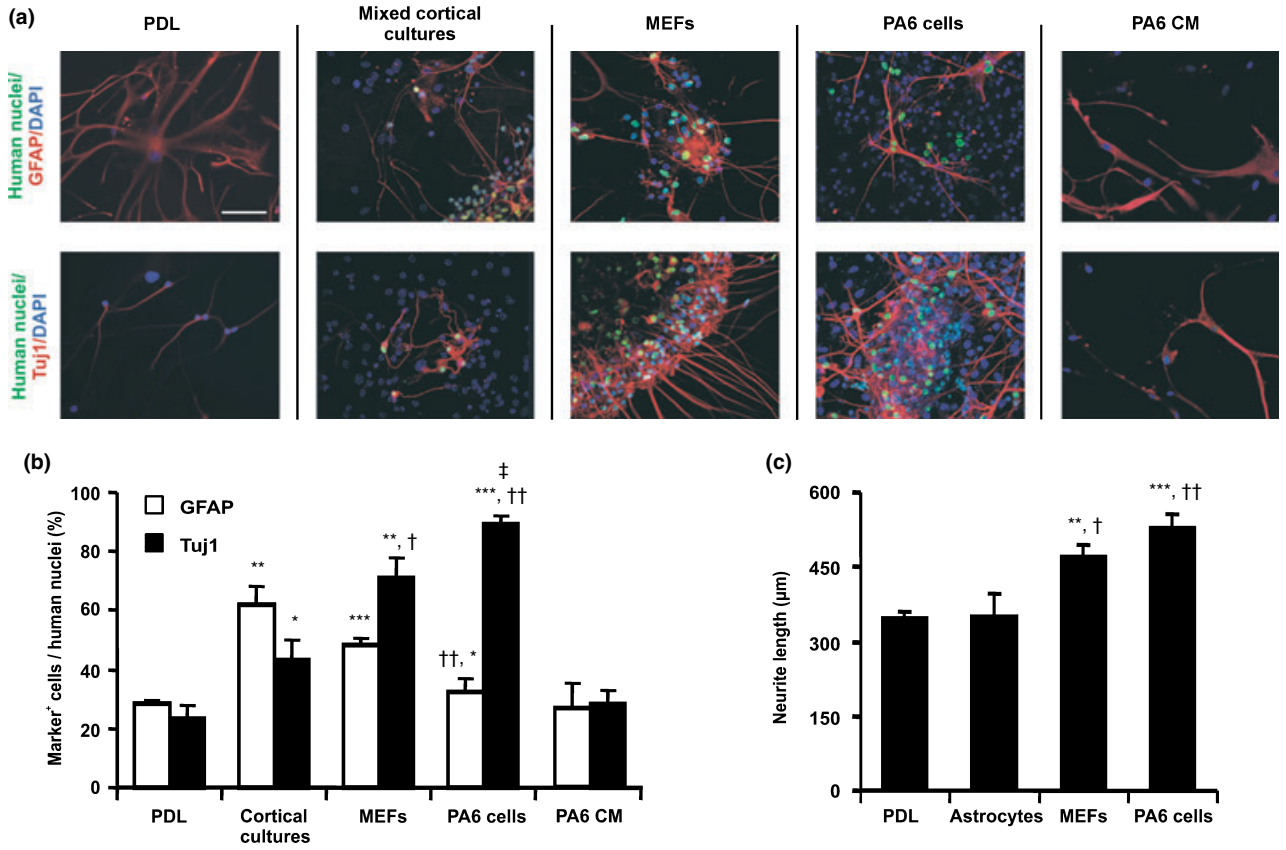

Fig. 3 Differentiation capacity of adult hippocampal hNPCs on various coculture systems including mouse fetal mixed cortical cultures, mouse embryonic fibroblasts (MEFs), and PA6 stromal cells, as well as by PA6 cell conditioned media (CM). Differentiation was initiated after an expansion phase of 3–10 weeks as described in the Material and methods. (a) Confocal microphotographs of triple immunostainings of hNPCs for markers of astroglia (GFAP) or neurons (Tuj1) as well as human cells (human nuclei). Nuclei were counterstained with DAPI (blue). Scale bar, 75 µm. (b) Quantification of GFAP$^+$ or Tuj1$^+$ derived from hNPCs (counterstained with human nuclei antibody) by differentiation on PDL, mixed cortical feeder cell layers, MEFs, PA6 cells, or on PDL with PA6 cell conditioned media (50%). (**c**) Length of the neurites of Tuj1$^+$/human nuclei$^+$ neurons derived from hNPCs under coculture conditions. Results are mean values ± SEM from at least three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ in comparison to PDL and PA6 CM, respectively; $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ in comparison to cortical cocultures; $\ddagger p < 0.05$ in comparison to MEFs.

hippocampal NPCs derived from epilepsy surgery. Noteworthy, we used diseased hippocampal tissue to isolate hNPCs that most likely contains mesangial sclerosis reported to include an increased number of neural progenitor cells *in vivo* (Blumcke *et al*. 2001; Crespel *et al*. 2005). These brain-derived NPCs were expanded in neurosphere cultures similar to fetal human NPCs (Svendsen *et al*. 1998; Svendsen *et al*. 1999; Caldwell *et al*. 2001; Storch *et al*. 2001) and expressed several markers for early neuroectodermal cells or NPCs as well as significant telomerase activity. The adult NPCs differentiated into all major cell types of the brain, namely astroglia, oligodendroglia and neurons. Functional analyses of the differentiated NPCs revealed obligate functional properties of neurons including the expression of voltage-gated sodium and potassium channels, the generation of action potentials, and the production of neurotransmitters such as glutamate and GABA. The central finding of our study is that non-neuroectodermal cells derived from the mesoderm (mouse embryonic fibroblasts [MEFs] and PA6 stromal cells) promote neurogenesis and maturation of neurons from adult human hippocampal NPCs.

Since there are only a few reports on isolation and short-term propagation of adult hippocampal NPCs from human origin (Johansson *et al*. 1999a; Kukekov *et al*. 1999; Roy *et al*. 2000), detailed data on their morphological properties and marker expression during expansion as well as their differentiation capacity are missing. NPCs are often defined *in vitro* by the presence of the fetal and adult CNS stem cells marker Nestin (Cattaneo and McKay 1990; Lendahl *et al*. 1990; Dahlstrand *et al*. 1995), and the recently recognized NPC marker Musashi1 (Kaneko *et al*. 2000). Consistently, Nestin and Musashi1 are highly expressed in adult human hippocampal NPCs on both mRNA and protein levels. Another family of genes expressed in early neuroectodermal

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cells are the proneural genes encoding transcription factors of the basic helix-loop-helix (bHLH) class such as NeuroD1, Neurogenin2 and Olig2 (Franklin *et al*. 2001; Nieto *et al*. 2001; Simmons *et al*. 2001; Hack *et al*. 2004). Adult hippocampal hNPCs expressed these proneural genes, which are necessary and sufficient to promote the generation (proliferation and self-renewal) of progenitors that are committed to neural differentiation (Bertrand *et al*. 2002; Bylund *et al*. 2003; Graham *et al*. 2003). The early neurogenic markers *OTX1/2*, several SOXB1 transcription factors (*SOX1,2,10*) and surface receptors important for neural development (*NOTCH1, NTRK1, FZD1, p75*(*NTR*)) were also found on the mRNA level in adult hippocampal NPCs. FACS analysis revealed that the hNPCs are $CD15^{low/-}$, $CD34^-$, $CD45^-$ and $CD133^-$. This phenotype is similar to that found in fetal human and adult mouse NPCs (Capela and Temple 2002; Vogel *et al*. 2003). The stem cell marker CD133 (or prominin-1) is expressed on several primitive cells such as hematopoietic stem or progenitor cells, embryonic neuroepithelial stem cells and endothelial stem cells (Weigmann *et al*. 1997; Uchida *et al*. 2000; Bhatia 2001), but there is no systematic report on CD133 expression in adult NPCs. However, in neuroepithelial stem cells, CD133 is mainly localized at the apical cell membrane facing the ventricular wall (Weigmann *et al*. 1997; Corbeil *et al*. 1999; Corbeil *et al*. 2000). Consistently, the adult hNPCs isolated from the hippocampus region without ventricular contacts were $CD133^-$. Together, human adult hippocampal NPCs expressed a typical pattern of early neuroectodermal and/or NPC marker genes, including major factors for proliferation/self-renewal and neurogenic differentiation. Due to a very slow growing behavior of the adult hNPCs (approx. 8–14 days doubling time), we failed to perform single cell (clonal) analysis of their differentiation capacity. However, we did not found $MAP2ab^+$ cells during expansion of hNPCs within the neurospheres demonstrating that all $MAP2ab^+$ neurons after differentiation must be *de novo* generated from MAP2ab-negative progenitor cells. Finally, showing proliferation by Ki67 and BrdU immunostaining as well as the expression of telomerase (Mattson and Klapper 2001), these adult NPCs fulfill the major characteristics of NPCs during *in vitro* expansion.

Differentiation of adult hNPCs was initiated by removal of mitogens and plating the cells onto poly-lysine and incubation for 14 days with BDNF, db-cAMP and retinoic acid with or without pre-stimulation with Shh and FGF-8 for 48 h. This mixture of differentiation factors was reported to induce neurogenic effects in adult hippocampal and tegmental NPCs from mouse without affecting the feeder cell layers (Hermann *et al*. 2005). In the present study, the pre-incubation of adult hNPCs with Shh and FGF-8 did not induce significant changes of the neuro-astroglia differentiation behavior of the adult hNPCs. Overall, hNPCs differentiated into all major cell types of the CNS, namely mature ($MAP2ab^+$) neurons with long neurites ($\approx$ 350 μm after 14 days of differentiation), oligodendrocytes and astrocytes. The majority of these cells expressed sustained outward currents with the typical properties of delayed rectifier potassium channels, but no detectable sodium channels. A few cells showed properties of young neurons, such as expression of sodium channels and generation of immature action potentials with a long duration and no after-hyperpolarization. This *in vitro* differentiation capacity is similar to that previously described for adult human SVZ and hippocampal NPCs (Johansson *et al*. 1999a; Kukekov *et al*. 1999; Roy *et al*. 2000; Westerlund *et al*. 2003; Moe *et al*. 2005). Roy and coworkers described potassium and sodium channels, but no action potentials in neuronal cells derived from adult hippocampal hNPCs expanded for only a short period of time ($\approx$ 2 weeks) (Roy *et al*. 2000). In contrast, Westerlund *et al*. 2003 and Moe *et al*. 2005 reported detailed electrophysiological analysis of adult SVZ NPCs showing not only sodium channels and action potentials in differentiated NPCs, but also functional neuronal networks (synaptic connections) from single differentiated hNPCs (Moe *et al*. 2005). However, these cells were derived from the ventricular wall and not from hippocampal hNPCs and it seems to be important to distinguish well between NPCs from different brain regions as shown for mice counterparts (Liu *et al*. 1999; Doetsch 2003; Kempermann *et al*. 2004). In addition to these previous results, we were able to demonstrate that differentiated hNPCs produce various neurotransmitters, such as glutamate and GABA.

There is a growing body of evidence that cell-to-cell contacts and/or soluble trophic factors produced by feeder cells are important for neuroectodermal specification of fetal and adult NPCs (Kawasaki *et al*. 2000; Storch *et al*. 2001; Song *et al*. 2002). Various cell types derived from neuroectodermal tissue including cortical, striatal and mesencephalic astrocytes support not only neuronal differentiation, but also neuronal subtype specification of fetal and adult brain-derived NPCs (Kawasaki *et al*. 2000; Perrier *et al*. 2004; Hermann *et al*. 2005; Kitajima *et al*. 2005). Additionally, several mesoderm-derived cell types, such as fibroblasts and PA6 stromal cells, are reported to significantly alter the differentiation capacity of ES cells as well as various fetal and adult NPC types (Kawasaki *et al*. 2000; Perrier *et al*. 2004; Hermann *et al*. 2005; Kitajima *et al*. 2005). In contrast to the data from Song and coworkers showing that adult astrocytes derived from neurogenic regions (hippocampus) promote neurogenesis from adult mouse hNPCs (Song *et al*. 2002), mixed cortical cultures from E17 mouse embryos containing mainly astroglial did not alter neurogenesis from adult human hNPCs in the present study. This discrepancy most likely resulted from differences of the developmental stage and the brain region from which the primary cultures were isolated. In contrast, both mesoderm-derived cell types (MEFs and PA6 cells) induced neurogenesis of adult human

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hNPCs with over 80% Tuj1$^+$ neurons in cocultures of hNPCs together with PA6 cells. Moreover, MEFs and PA6 cells promote morphological and functional maturation of adult hNPC-derived neurons. These neurons showed longer neurites with more branches and produced higher amounts of neurotransmitters. On the other hand, trophic support provided by PA6 conditioned media did not lead to significant changes of glial and neuronal differentiation of hNPCs. This is in agreement with studies on mouse and human ES cell-derived NPCs showing that fibroblasts and PA6 cells promote neurogenesis from these NPCs with coculturing being much more effective than PA6 conditioned media (Kawasaki *et al*. 2000; Zeng *et al*. 2004). Interestingly, PA6 feeder cells did not influence the glial potential of hNPCs, whereas MEFs and particularly mixed cortical cultures increased the number of astrocytes differentiated from hNPCs. Together, our results demonstrate a neurogenesis-inducing activity of mesoderm-derived cell types not only in ES cells as reported previously (Kawasaki *et al*. 2000; Zeng *et al*. 2004), but also in adult human hNPCs. The mechanisms or factors of this neurogenic activity are largely unknown, but most likely include cell-to-cell contacts and/or cell (membrane)-bound factors, such as the Wnt ligands (Kawasaki *et al*. 2000; Storch *et al*. 2001; Song *et al*. 2002; Schmidt and Patel 2005). However, due to the different neuronal-glia induction potential of the investigated feeder cell types, it seems to be most likely a combination of different trophic factors and cell surface interactions which influence the neuro-glia fate determination. We used rodent feeder cell layers to analyse the neural differentiation capacity of adult hNPCs and therefore it is unclear whether human feeder might have different trophic effects.

In summary, we provide a detailed characterization of long-term expanded human adult hippocampal neuroprogenitor cells derived from tissue obtained from epilepsy surgery procedures. The described technique for long-term expansion of these adult human NPCs allows the generation of high yields of cells for further characterizations by molecular biology or protein biochemistry, such as gene array analysis or proteomics. This study provides therefore a framework for standardized comparative analysis of brain-derived adult NPCs with adult NPCs derived from other human cell sources, such as bone marrow stromal or skin cells (Toma *et al*. 2001; Hermann *et al*. 2004). However, future studies are warranted to further define the functional (electrophysiological) properties including synaptic neurotransmitter release of differentiated adult human hNPCs and compare these data with data obtained on primary human astrocytes and neurons. Furthermore, we demonstrated that mesoderm-derived cell types, such as MEFs and PA6 stromal cells, not only induce neurogenesis from adult human NPCs, but also promote morphological and functional maturation of neurons derived from these progenitor cells. The analysis of the underlying molecular mechanisms in the future might help to better understand neurogenesis and neuronal maturation in the adult human brain.

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Multipotent Neural Stem Cells from the Adult Tegmentum with Dopaminergic Potential Develop Essential Properties of Functional Neurons

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Key Words. Adult neurogenesis • Neural stem cells • Dopaminergic differentiation • Parkinson's disease • Electrophysiology Neuroregeneration

ABSTRACT

Neurogenesis in the adult brain occurs within the two principal neurogenic regions: the hippocampus and the subventricular zone of the lateral ventricles. The occurrence of adult neurogenesis in non-neurogenic regions, including the midbrain, remains controversial, but isolation of neural stem cells (NSCs) from several parts of the adult brain, including the substantia nigra, has been reported. Nevertheless, it is unclear whether adult NSCs do have the capacity to produce functional dopaminergic neurons, the cell type lost in Parkinson's disease. Here, we describe the isolation, expansion, and in vitro characterization of adult mouse tegmental NSCs (tNSCs) and their differentiation into functional nerve cells, including dopaminergic neurons. These tNSCs showed neurosphere formation and expressed high levels of early neuroectodermal markers, such as the proneural genes *NeuroD1*, *Neurog2*, and *Olig2*, the NSC markers Nestin and Musashi1, and the proliferation markers Ki67 and BrdU (5-bromo-2-deoxyuridine). The cells showed typical propidium iodide–fluorescence-activated cell sorting analysis of slowly dividing cells. In the presence of selected growth factors, tNSCs differentiated into astroglia, oligodendroglia, and neurons expressing markers for cholinergic, GABAergic, and glutamatergic cells. Electrophysiological analyses revealed functional properties of mature nerve cells, such as tetrodotoxin-sensitive sodium channels, action potentials, as well as currents induced by GABA (γ-aminobutyric acid), glutamate, and NMDA (*N*-methyl-d-aspartate). Clonal analysis demonstrated that individual NSCs retain the capacity to generate both glia and neurons. After a multistep differentiation protocol using co-culture conditions with PA6 stromal cells, a small number of cells acquired morphological and functional properties of dopaminergic neurons in culture. Here, we demonstrate the existence of adult tNSCs with functional neurogenic and dopaminergic potential, a prerequisite for future endogenous cell replacement strategies in Parkinson's disease. STEM CELLS *2006;24:949–964*

INTRODUCTION

Neurogenesis in the adult mammalian brain is a generally accepted phenomenon in two discrete neurogenic regions: the hippocampus and the subventricular zone (SVZ) of the lateral ventricles [1–5]. In recent years, neurogenesis was reported to occur in other regions of the adult brain under normal conditions, such as the neocortex [2], amygdala [6], and substantia nigra [7]. Moreover, various brain insults have been shown to induce the production of new neurons in the striatum [8], cortex [9, 10], and substantia nigra [7]. However, other research groups were not able to replicate some of these reports [11, 12], and some have been challenged on methodological grounds [11–15].

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For example, a recent study by Frielingsdorf and colleagues [13] could not reproduce the data reported by Zhao and coworkers [7] showing adult dopaminergic neurogenesis in the substantia nigra, including re-establishment of the nigro-striatal dopaminergic connections. They did not find newly generated dopaminergic neurons by the 5-bromo-2-deoxyuridine (BrdU) incorporation assay in normal or 6-hydroxydopamine–lesioned animals. They further argued that in vivo labeling of new neurons in the substantia nigra with DiI might be related to retrograde labeling of existing nigral neurons [13]. These data are in line with earlier studies showing that adult substantia nigra progenitor cells give rise to glial cells only [16]. However, the in vivo differentiation pattern may not reflect the entire lineage potential of resident progenitor cells, because regional environmental factors appear to restrict in situ differentiation to distinct neural lineages. For example, in vitro analysis and transplantation studies have suggested that progenitor cells from the adult substantia nigra, similar to progenitors from the adult spinal cord, have the potential to give rise to cells of all neural lineages [16–18]. In addition, isolated cortical progenitor cells have the potential to give rise to glia and neurons in vitro [19].

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. Multipotent NSCs with the potential to generate mature cells of all neural lineages, including astroglia, oligodendroglia, and neurons, have been consistently demonstrated within the hippocampus and the SVZ of lateral ventricles [20–26]. These cells grow in vitro as spherical floating clusters (neurospheres) in the presence of epidermal growth factor (EGF) [21, 27]. Previous work has reported that SVZ NSCs correspond to a rare population of relatively quiescent cells. More recently, it has been suggested that not the ciliated ependymal cells correspond to the NSCs [24] but that SVZ astrocytes act as NSCs in both the normal and regenerating adult brain [28]. Recent studies demonstrated the presence of multipotent central nervous system (CNS) stem cells in non-neurogenic regions of the adult brain, including the spinal cord and the ventricular neuraxis [17, 18]. In contrast to SVZ NSCs, the proliferation of these NSCs depends on both EGF and fibroblast growth factor-2 (FGF-2) [18, 26]. Lie and coworkers described the isolation of multipotent NSCs from the adult substantia nigra with neurogenic differentiation capacity in vitro and after transplantation into the adult hippocampus [16]. However, the authors neither reported on detailed characterization of these progenitor cells in vitro nor provided data on neuronal subtype specification or functional properties after differentiation. Moreover, similar to all other adult CNS stem cells, these midbrain-derived NSCs did not differentiate into dopaminergic nerve cells [16].

Here, we show the presence of multipotent clonogenic NSCs in the adult mouse tegmentum (tegmental NSCs [tNSCs]) with all major properties of CNS stem cells such as neurosphere formation, expression of Nestin and other NSC markers, as well as significant telomerase activity. They show the capacity to differentiate in vitro into all neural cell lineages, including astroglia, oligodendroglia, and neurons. Moreover, the resulting nerve cells displayed morphological and functional properties of mature neuronal subpopulations, such as GABAergic and dopaminergic neurons.

Materials and Methods

Sphere Culture

Adult mice (6–10-week-old male C57BL/6 mice) were killed by cervical dislocation, and their brains were removed and placed into ice-cold Hanks' balanced saline solution supplemented with 1% penicillin/streptomycin and 1% glucose (all from Gibco, Tulsa, OK, http://www.invitrogen.com). The tegmental tissue (midbrain and hindbrain), including the subependymal zone/SVZ of the aqueduct and fourth ventricle, was then aseptically prepared using a dissection microscope. The meninges were carefully removed from the tissue after preparation of the tegmental area. For comparative studies with NSCs from adult's principal neurogenic regions, we isolated the hippocampus from the same mice as described previously [19]. For expansion of neurospheres, tissue samples from both regions were incubated in 0.1% trypsin (Sigma, St. Louis, http://www.sigmaaldrich.com) for 10 minutes at room temperature, incubated in DNase (40 mg/ml; Sigma) for 10 minutes at 37°C, and homogenized to a quasi–single-cell suspension by gentle triturating [29]. The cells were added to 25-cm^2 flasks (2–3 $\times$ 10^6 viable cells per flask) in serum-free Neurobasal (NBa) medium containing 1% glutamate, 2% B27 supplement, and 1% penicillin/streptomycin (all from Gibco) supplemented with 20 ng ml^{-1} of both mitogens FGF-2 and EGF (both from Sigma). After 10–20 days, sphere formation was observed. These neurospheres were expanded for an additional 3–12 weeks (in total, five to 10 passages) before terminal differentiation was initiated. The medium was changed once a week, and growth factors were added twice a week. For BrdU labeling, cells were incubated for 30 hours with 10 μM BrdU.

Differentiation Conditions

Induction of neural differentiation (protocol 1) was initiated by plating the cells on poly-L-lysine (PLL)-coated glass coverslips at a concentration of 1.5–2.0 $\times$ 10^5 cells cm^{-2} in NBa medium containing 1% glutamate, 2% B27 supplement, and 1% penicillin/streptomycin supplemented with 0.5 μmol l^{-1} all-trans-retinoic acid (Sigma), 10 ng ml^{-1} brain-derived neurotrophic factor (BDNF; Promega, Madison, WI, http://www.promega.com), and 100 μM dibuteryl(db)-cAMP (Sigma). Cells were differentiated for 14 days. For analyzing the differentiation capacity of tNSCs, several other combinations of growth factors/cytokines were used (Table 1). The following substances were used: 10% fetal bovine serum (Biochrom AG, Berlin, http://www.biochrom.de), 100 pg/ml interleukin (IL)-1b, 1 ng/ml IL-11, 10 ng/ml glial cell line–derived neurotrophic factor, 1 ng/ml leukemia inhibitory factor (all from Sigma), 10 ng/ml FGF-4, 10 ng/ml FGF-8, and 10 ng/ml sonic hedgehog (Shh; all from R&D Systems, Minneapolis, http://www.rndsystems.com). For co-culture analyses, various feeder cells (mouse embryonic fibroblasts [MEFs]), PA6 stromal cells, as well as cortical and striatal astrocytes from embryonic day 14.5 [E14.5] rat embryos) were cultured on gelatine-coated cover slides as described earlier [29, 30]. Before plating the tNSCs on the feeder layers, the cells were incubated with expansion media supplemented with 500 ng/ml Shh and 100 ng/ml FGF-8 for 48 hours ("pre-stimulation"). When co-culture was started, confluent feeder cells where washed twice with phosphate-buffered saline (PBS) and tNSCs were seeded at a concentration of

Table 1. Differentiation capacity of adult tegmental neural stem cells

Differentiation conditions (growth factors/cytokines/co-cultures)	MAP2ab$^+$ or Tuj1$^+$ neurons	GFAP$^+$ astrocytes	GalC$^+$ oligodendrocytes	TH$^+$ neurons
BDNF on PLL	+	+++	+	−
dbcAMP on PLL	+	+++	++	−
Retinoic acid on PLL	+	++++	−	−
BDNF, retinoic acid on PLL	+	++	+	−
BDNF, dbcAMP, retinoic acid on PLL (protocol 1)	+	++	+	−
FBS on PLL	+	+++	+	−
IL-1b on PLL	+	++	+	−
IL-1b, IL-11, GDNF, LIF on PLL	++	++++	++	−
FGF-4, Shh on PLL	+	++++	+	−
FGF-8, Shh on PLL	++	++++	+	−
dbcAMP, FGF-8, Shh on PLL	+	++	+	−
Pre-stimulation (FGF-8) on PLL	+	n.d.	n.d.	−
Pre-stimulation (FGF-8) + CM on PLL	++	n.d.	n.d.	−
Pre-stimulation (Shh) on PLL	++	n.d.	n.d.	−
Pre-stimulation (Shh) + CM on PLL	++	n.d.	n.d.	−
Pre-stimulation (FGF-8 + Shh) on PLL (protocol 2)	+	+++	n.d.	−
Pre-stimulation (FGF-8 + Shh) + CM on PLL	+	+++	n.d.	−
Pre-stimulation (FGF-8 + Shh) on MEFs (protocol 3)	++	+++	+	−
Pre-stimulation (FGF-8 + Shh) on cortical astrocytes	+	n.d.	n.d.	−
Pre-stimulation (FGF-8 + Shh) on striatal astrocytes	+	n.d.	n.d.	−
Pre-stimulation (FGF-8 + Shh) on PA6 cells (protocol 4)	++	+++	+	+

Immunostainings were scored as − if no cells were positive for the respective marker, + if up to 5% of cells were positive, ++ if 5%–25% of cells were positive, +++ if 25%–75% of cells were positive, and ++++ if more than 75% of cells were positive.
Abbreviations: BDNF, brain-derived neurotrophic factor; CM, conditioned medium on PA6 cells; dbcAMP, dibuteryl-cAMP; FBS, fetal bovine serum; FGF, fibroblast growth factor; GalC, galactocerebrosidase C; GDNF, glial cell line–derived neurotrophic factor; GFAP, glial fibrillary acidic proetin; IL, interleukin; LIF, leukemia inhibitory factor; MAP2ab, monoclonal antimicrotubule-associated protein 2ab; MEF, mouse embryonic fibroblast; n.d., not determined; PLL, poly-L-lysine; Shh, sonic hedgehog; TH, tyrosine hydroxylase.

1.5–2.0 $\times$ 10^5 cells cm^{-2} in Glasgow's modified Eagle's medium (Gibco) containing 1% glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 0.1 mM 2-mercaptoethanol ("differentiation medium" [DM]) supplemented with 10% knockout-serum replacement (Gibco). Medium change was performed on day 4 and every other day after that. After 7 days, the medium was changed to "differentiation medium" supplemented with 1% N2 (DM-N2 medium; Gibco).

Clonal Analysis

Clonal analysis was performed according to Uchida and coworkers [31]. In brief, tNSCs were serially diluted into expansion medium in 96-multiwell plates. On the next day, single cell–containing wells (assessed microscopically) were expanded by supplementing the medium to 50% with conditioned medium containing growth factors. Cells were expanded for 3–6 weeks and then differentiated using feeder cell–free differentiation protocol 1.

Flow Cytometry

tNSCs were treated with Accumax (Gibco) and washed with PBS. Dead cells were excluded from analysis by forward-scatter gating. Samples were processed using a FACSCalibur flow cytometer, and analyses were performed with the Cellquest software (both from Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). Antibodies were used as follows: CD34–fluorescein isothiocyanate (FITC) 1:10, CD45-phycoerythrin (PE) 1:10, CD24-FITC 1:200 (all from Chemicon, Temecula, CA, http://www.chemicon.com), rat anti-prominin1 monoclonal antibody 13A4 1:300 (BD Biosciences, San Diego, http://www.bdbiosciences.com), and mSSEA1 1:5 (kindly provided by Dr. Beltinger, University of Ulm) followed by fluorescence-labeled secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com). Propidium iodide (PI)–fluorescence-activated cell sorting (FACS) was performed as previously described [32]. In brief, the cells were dissociated by trypsin (Gibco) and washed with PBS. The cells were fixed with 80% ice-cold ethanol and incubated at −20°C for 30 minutes. The cells were washed with PBS and PI-stained (0.5 mg/ml PI in 0.038 M sodium citrate buffer [pH 7.0]) for 30 minutes in the dark (37°C). Dead cells were excluded from analysis by forward-scatter gating. A minimum of 10,000–12,000 events were acquired for each sample.

Immunocytochemistry

Cell cultures were fixed in 4% paraformaldehyde in PBS or with 4% paraformaldehyde/PBS followed by ice-cold acidic ethanol and HCl for BrdU staining. Immunocytochemistry was carried out using standard protocols. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). The following primary antibodies were used: mouse anti-Tuj1 1:500 or rabbit anti-Tuj1 1:2,000 (Covance, Richmond, CA, http://www.covance.com), sheep or rabbit anti–tyrosine hydroxylase (TH) 1:200 (Pel-Freez, Rogers, AR, http://www.pel-freez.com), rabbit anti-serotonin (5-HT) 1:2,500 (DiaSorin, Stillwater, MN, http://www.diasorin.com), rabbit anti-cholineacetyltransferase 1:500, mouse anti-galactocerebrosidase C (GalC) 1:750, rabbit anti–glial fibrillary acidic protein (GFAP) polyclonal 1:1,000, mouse anti-Nestin monoclonal 1:500, anti-CD-24-FITC 1:200, rabbit anti-neurogenin2 1:500, rabbit anti-neuroD1 1:500, rabbit anti-Musashi1 1:500 (all from Chemicon), rabbit anti-Ki-67 1:500 (Novocastra Ltd., Newcastle upon Tyne, U.K., http://www.novacastra.co.uk), rabbit anti–γ-aminobutyric acid (GABA) 1:1,000, rabbit anti-glutamate 1:10,000 (both from Sigma), mouse anti-GFAP monoclonal 1:1,000, mouse anti–monoclonal antimicrotubule-associated protein 2ab (MAP2ab) 1:300 (all from BD Pharmingen, San Diego, http://www.bdbiosciences.com/pharmingen), rat anti–prominin1-FITC monoclonal antibody 13A4 1:800 (BD Biosciences), rat anti–PSA-NCAM (polysialylated neural cell adhesion molecule) 1:250 (BD Pharmingen), rabbit anti-olig2 1:1,000 (kindly provided by Dr. Takebayashi; [33]) and fluorescence-labeled secondary antibodies (Jackson ImmunoResearch Laboratories), and rat anti-BrdU 1:40 with fluorescence-labeled secondary antibody (both from Abcam, Cambridge, U.K., http://www.abcam.com). Images were captured using a fluorescence microscope (Axiovert 135; Zeiss, Oberkochen, Germany, http://www.zeiss.com) or a Leica TCS/NT confocal microscope (Leica, Heerbrugg, Switzerland, http://www.leica.com) equipped with krypton, krypton/argon, and helium lasers.

RNA Extraction and Quantitative Real-Time RT-PCR Analysis

Total cellular RNA was extracted from tNSCs and 14-day differentiated tNSCs (differentiation protocol 1) using RNAeasy total RNA purification kit followed by treatment with RNase-free DNase (Qiagen, Hilden, Germany, http://www.qiagen.com). Quantitative real-time one-step reverse transcription–polymerase chain reaction (RT-PCR) was carried out using the LightCycler System (Roche, Mannheim, Germany, http://www.roche.com), and amplification was monitored and analyzed by measuring the binding of the fluorescence dye SYBR Green I to double-stranded DNA. One microliter (50 ng) of total RNA was reverse-transcribed and subsequently amplified using QuantiTect SYBR Green RT-PCR Master mix (Qiagen) and 0.5 μmol l^{-1} of both 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. The results are expressed relative to the housekeeping gene *HMBS* (hydroxymethylbilane synthase). Primer sequences, lengths of the amplified products, and melting point analyses are summarized in Table 2.

Telomerase Activity

A highly sensitive in vitro assay known as the quantitative real-time telomeric repeat amplification protocol [34, 35] has been used for detecting telomerase activity (OTD kit; Allied Biotech, Inc., Ijamsville, MD, http://www.alliedbiotechinc.com). The telomerase activity in the cell or tissue extract is determined through its ability to synthesize telomeric repeats onto an oligonucleotide substrate in vitro. Telomerase from cell or tissue extracts adds telomeric repeats onto a substrate oligonucleotide, and the resulting extended products are subsequently amplified by the PCR. The PCR products are then visualized using the DNA fluorochrome SYBR Green as described above. Mouse embryonic stem cells (ESCs) (D3 ESC line) were used as positive control and cultured as described previously [36], whereas adult mouse cortical tissue was used as negative control [37].

Electrophysiology

Cells were investigated 7–20 days after differentiation using standard whole-cell patch-clamp technique. Data were recorded using an EPC-7 or EPC-9 amplifier (HEKA Electronics Inc., Lambrecht/Pfalz, Germany, http://www.heka.com) and pClamp data acquisition software (Axon Instruments, Union City, CA, http://www.moleculardevices.com) essentially as described previously [38, 39]. Extracellular solution contained (in mmol l^{-1}) 142 NaCl, 8.1 KCl, 1 $CaCl_2$, 6 $MgCl_2$, 10 HEPES, and 10 D-glucose (pH 7.4; 320 mOsm). Pipette solution contained (in mmol l^{-1}) 153 KCl, 1 $MgCl_2$, 5 EGTA, and 10 HEPES (pH 7.3; 305 mOsm). Using these solutions, borosilicate pipettes had resistances of 6–10 MΩ. Seal resistances in the whole-cell mode were between 0.1 and 1 GΩ. For recordings of GABA-induced inward currents, the solutions were balanced giving an equilibrium potential of 0 mV for chloride ions. For recordings of glutamate-induced inward currents, the external bath solution contained (in $mmol^{-1}$) 162 NaCl, 1.2 $CaCl_2$, 2.4 KCl, 0.01 glycine, 11 glucose, 10 HEPES (pH 7.3; 320 mOsm). Picrotoxin (100 μM; Sigma) and strychnine (2 μM; Sigma) were added to inhibit ligand-gated chloride channels. The internal solution contained (in $mmol^{-1}$) 95 CsCl, 6 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, and 11 EGTA (pH 7.2; 300 mOsm). Drugs were applied rapidly via gravity with an SF-77B perfusion fast-step system (Warner Instruments LLC, Hamden, CT, http://www.warnerinstruments.com). Data were analyzed using pClamp 8.0, Microsoft Excel 97 (Microsoft, Redmond, WA, http://www.microsoft.com), and Origin 5.0 software. Resting membrane potentials (RMP) were determined immediately after gaining whole-cell access. Action potentials (APs) were elicited by applying increasing depolarizing current pulses (10-pA current steps). The afterhyperpolarization (AHP) amplitude was measured from peak to beginning of plateau reached during the current injection, AP duration was measured at half amplitude, and time to peak AHP from spike onset.

Determination of Dopamine, GABA, and Serotonin Production and Release

For determination of dopamine production and release, media were supplemented with 100 μmol l^{-1} tetrahydrobiopterin and 200 μmol l^{-1} ascorbate 2 days prior to medium harvest. Dopamine levels were determined in medium, and extracellular buffer was stabilized with EGTA/glutathione solution as reported previously [29] and stored at −80°C until analysis.

Table 2. Primers for quantitative real-time reverse transcription–polymerase chain reaction

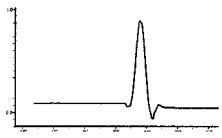
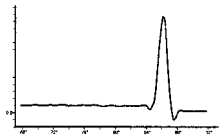
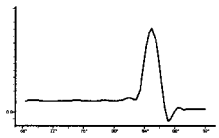
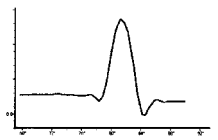
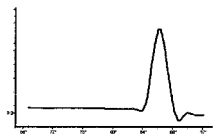
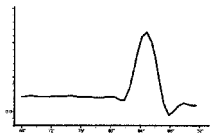
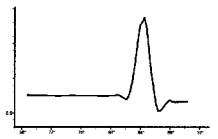
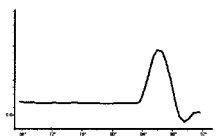
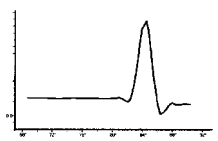
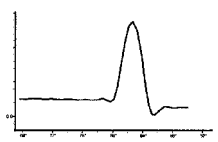
Gene (protein)	Sequence (forward; reverse)	Product length (bp)	Melting point analysis[a]
DDC (Dopa-decarboxylase)	5′-GTG GGT GAA GAG GAC TGA-3′ 5′-GCA GCC CCT TGA CTC CGT A-3′	182	
DAT (Dopamine transporter)	5′-ATG CTG CTC ACT CTG GGT ATC-3′ 5′-CAG GAA AGT AGC CAG GAC AAT-3′	135	
GFAP (Glial fibrillary acidic protein)	5′-GAT CTA TGA GGA GGA AGT TCG-3′ 5′-TCT GCA AAC TTA GAC CGA TAC C-3′	183	
HMBS (Hydroxymethylbilane synthase)	5′-TGT ATG CTG TGG GTC AGG GAG-3′ 5′-CTC CTT CCA GGT GCC TCA GA-3′	144	
MBP (Myelin basic protein)	5′-CAC GGG CAT CCT TGA CTC-3′ 5′-GCC GTG CTG CGA CTT C-3′	130	
MSI1 (Musashi 1)	5′-GCA GAC CAC GCA GGA AG-3′ 5′-CGC CAG CAC TTT ATC CAC-3′	151	
NeuroD1 (Neurogenic differentiation 1)	5′-CCG CCA CAC GCC TAC A-3′ 5′-CAA ACT CGG CGG ATG G-3′	148	
Neurog2 (Neurogenin 2)	5′-CGG CGT CAT CCT CCA AC-3′ 5′-CGG GTA GAG GAC GAG AGA GG-3′	179	
NES (Nestin)	5′-TGG AAC AGA GAT TGG AAG GC-3′ 5′-TCT TGA AGG TGT GCC AGT TGC-3′	153	
NF (Neurofilament-160kd)	5′-CAT CAG CAA GTC GGT AAA GG-3′ 5′-CTG CGG GCT ACG GTC A-3′	133	

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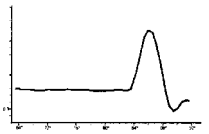
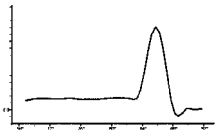
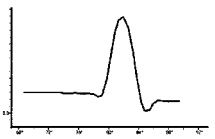
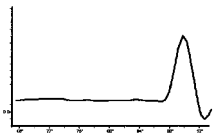
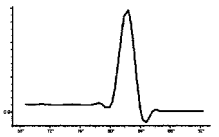
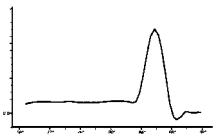
Table 2. (Continued)

Gene (protein)	Sequence (forward; reverse)	Product length (bp)	Melting point analysis[a]
NTRK1 (Neurotrophic tyrosine kinase, receptor, type 1)	5′-GAG TTG AGA AGC CTA ACC ATC-3′ 5′-GCA TTG GAG GAC AGA TTC AGG-3′	104	
NR4A2 (Nuclear receptor Nurr1)	5′-TGT CAG CAC TAC GGT GTT CG-3′ 5′-AGG GTA AAC GAC CTC TCC G-3′	2,263	
Olig2 (Oligodendrocyte transcription factor 2; Olig2)	5′-GAC CGA GCC AAC ACC AGC-3′ 5′-GGG ACG ATG GGC GAC TAG-3′	166	
Pax6 (Paired box protein PAX6)	5′-GCA CCA AAG GGT CAT CGC-3′ 5′-TGG GGG GTG GAT GGA AG-3′	193	
PLP (Myelin proteolipid protein)	5′-CAC CTA TGC CCT GAC TGT TG-3′ 5′-GTG GAA GGT CAT TTG GAA CAT A-3′	176	
TH (Tyrosine hydroxylase)	5′-AGC TCC TGC ACT CCC TGT C-3′ 5′-CGA GAG GCA TAG TTC CTG AGC-3′	175	

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

Dopamine was assayed by reverse-phase high-performance liquid chromatography (HPLC) with an electrochemical detector as previously described [40]. GABA and serotonin were assayed by HPLC with fluorescence detection [40], employing precolumn derivatisation with ortho-phthaldialdehyde and an automatic HPLC system (Kontron Instruments, Neufahrn, Germany). The excitation and emission wave lengths of the fluorescence detector were set at 330 and 450 nm, respectively.

Cell Counting and Statistics

For quantification of the percentage of cells producing a given marker, in any given experiment 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 500–1,000 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 ± SEM.

RESULTS

Isolation and In Vitro Characterization of Neurosphere-Forming Cells from the Adult Tegmentum

To isolate tNSCs, the tegmentum (midbrain and hindbrain) was selectively dissected from adult C57BL/6 mice using a dissection microscope. Cells were isolated and cultured in uncoated flasks in serum-free NBa medium supplemented with EGF and FGF-2. After 10–20 days in vitro, the cells formed small spheres with all typical morphological properties of neurospheres (Fig. 1A). FACS analysis revealed that the phenotype of the tNSCs was $CD24^{low/-}$, $CD34^-$, $CD45^-$, $Prominin1^-$, and $SSEA1^-$ (Fig. 1B). Furthermore, we used quantitative real-time RT-PCR and immunocytochemistry on unfixed or fixed neurospheres to investigate the expression pattern of several early and late neuroectodermal genes (Fig. 2A, 2C; for complete names of genes and encoded proteins, refer to Table 2). tNSCs expressed high levels of early neuroectodermal markers such as Nestin, an

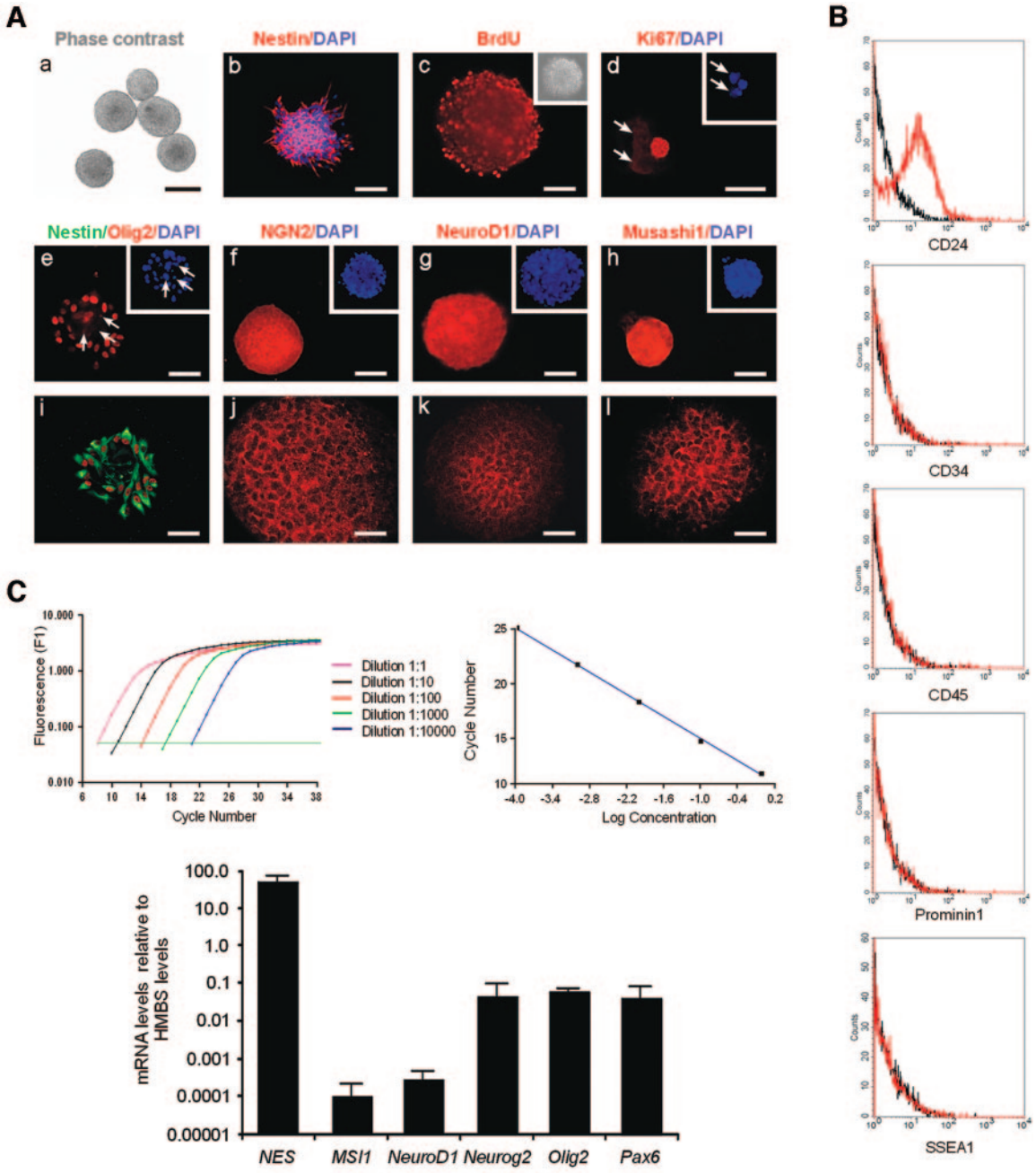


Figure 1. Characteristics of adult tNSCs during in vitro expansion. **(A):** Morphology and marker expression of adult tNSCs during expansion in the presence of EGF and FGF-2. Spheres were cultured for 2–3 hours to allow attachment and then stained for Nestin (b, i), Ki67 (d), Olig2 (e, i), Neurogenin2 (f, j), NeuroD1 (g, k), and/or Musashi1 (h, l). For BrdU staining (c), the spheres were cultured in the presence of 10 μM BrdU for 30 hours before plating. Nuclei were counterstained with DAPI. Confocal images (i–l) confirm the presence of the respective marker protein in nearly all cells within the neurosphere. Arrows mark Ki67$^-$/DAPI$^+$ cells (d) and apoptotic cells not expressing Nestin/Olig2 (e), respectively. Scale bars = 15 μm (d), 30 μm (e, i, j–l), 50 μm (b, c, f–h), and 200 μm (a). **(B):** Flow cytometry of tNSCs cultured for 4–12 weeks (five to 10 passages). Cells were labeled with fluorescence-coupled antibodies against CD24, CD34, CD45, prominin1, and SSEA1 or immunoglobulin isotype control antibodies. Cells were analyzed using a FACSCalibur flow cytometer. Black line, control immunoglobulin; red line, specific antibody. **(C):** Quantitative transcription profile of tNSCs. Top panel: 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 *NES* mRNA (encoding for nestin). The crossing line represents the position of the threshold. Diagram on right shows the standard curve obtained by plotting cycle number of crossing points versus dilution factor. Bottom panel: Quantitative real-time RT-PCR analyses of NSC markers (*NES, MSl1*), proneural genes (*NeuroD1, Neurog2, Olig2*), and the neuronal transcription factor *Pax6* in tNSCs during expansion. Expression levels are expressed relative to the housekeeping gene *HMBS*. Data shown are mean values ± SEM from at least three independent experiments. For primers, complete names of genes, and melting curve analyses demonstrating the specificity of amplified PCR products, see Table 2. Abbreviations: BrdU, 5-bromo-2-deoxyuridine; DAPI, 4,6-diamidino-2-phenylindole; EGF, epidermal growth factor; FGF, fibroblast growth factor; HMBS, hydroxymethylbilane synthase; PCR, polymerase chain reaction; RT-PCR, reverse transcription–polymerase chain reaction; tNSC, tegmental neural stem cell.

intermediate filament protein present in CNS stem cells (Fig. 1A, 1C) [21, 41], and the CNS stem cell marker Musashi1 [42], as well as the proneural genes *Olig2*, *Neurogenin2*, and *NeuroD1* on both the mRNA and protein levels (Fig. 1A, 1C). Proneural genes, which encode transcription factors of the basic helix-loop-helix class, are important factors for NSC self-renewal, multipotency, and commitment to neural lineages of NSCs [43–48]. Confocal microscopy of the spheres revealed that nearly all (>95%) living cells without nuclear condensation (DAPI stain) expressed Nestin (Fig. 1A) with some cells (<5%) being double-positive for Nestin and GFAP (data not shown). Co-labeling with anti-olig2 antibody further demonstrated co-expression of both early neuroectodermal markers in nearly all living cells (Fig. 1A). Furthermore, the proneural genes *Neurogenin2* and *NeuroD1*, as well as the NSC marker *Musashi1*, were expressed in nearly all cells within the neurospheres as shown by confocal microscopy (Fig. 1A). The neurogenic fate-determining transcription factor Pax6 mRNA was also expressed in these neurospheres (Fig. 1C). Only very few cells were weakly positive for CD24 or PSA-NCAM protein (data not shown). Markers for mature neural cell types, such as *MBP*, *PLP*, *NTRK1, MAP2*, and *TH*, were undetectable or very low at the mRNA level; at the protein level, MAP2ab, GalC, and TH were absent (data not shown). This phenotype is similar to that of NSCs derived from adult mouse SVZ or hippocampus [20, 22, 23]. tNSCs could be passaged and cultured as secondary and tertiary neurospheres without changing morphology and phenotype for 3–12 weeks (~5–10 passages).

Cell Cycle Distribution, Proliferation Potential, and Apoptosis of tNSCs

Cell cycle analysis using PI-FACS demonstrated that, besides apoptosis (see below), there was a large fraction of tNSCs distributed in G_1, S, and G_2/M phases (see Fig. 2A, 2B for details of cell cycle distribution). In addition, we used the proliferation marker Ki67 as well as BrdU incorporation to identify DNA-synthesizing cells (Figs. 1A and 2B). Ki67 was detected in the nucleus of proliferating cells in all active phases of the cell cycle from the late G_1-phase through the M-phase but is absent in non-proliferating and early G_1-phase cells and in cells undergoing DNA repair [49–51]. As expected, total Ki67 staining (representing late G_1 through M phase) was higher than total BrdU staining at each time point. When the percentage of G_1-phase cells was subtracted from total Ki67 staining, the resulting percentage was very similar to that of BrdU-stained cells (Fig. 2B).

To evaluate cell death by apoptosis during cultivation of tNSCs, we used PI-FACS analysis and DAPI staining. After 4–12 weeks, an average number of 4% ± 1% of cells were found by PI-FACS analyses to be apoptotic. Histograms representing FACS analysis and DAPI staining of apoptotic events in tNSCs are shown in Figure 2B. Spontaneously apoptotic cells exhibited chromatin condensation and margination, sometimes followed by appearance of apoptotic bodies, as revealed by DAPI staining, and were negative for Ki67 (data not shown) as well as for most other markers tested such as Nestin and Olig2 (Fig. 1A).

Telomerase is inactive in most somatic cells but present in various stem cell populations [52–54]. Quantification of telomerase activity in tNSCs using the telomeric repeat amplification protocol [34, 35] showed stable telomerase activity in tNSCs over the 3-month expansion period with significantly higher activity compared to that measured in mouse cortical tissue (negative control) but lower activity compared to that in mouse ESCs (Fig. 2C). Together, investigations of the proliferation characteristics revealed the typical pattern of slowly dividing cells with a small amount of spontaneous apoptosis within the tNSC neurospheres, similar to fetal mouse mesencephalic NSCs [55].

Differentiation Capacity of Adult tNSCs

Differentiation of tNSCs was initiated after 3–12 weeks by removal of mitogens, plating the cells onto poly-L-lysine, and addition of BDNF, db-cAMP, and retinoic acid (protocol 1, Fig. 1A). After 14 days, the majority of cells (71% ± 27%) acquired morphologic and phenotypic characteristics of astrocytes ($GFAP^+$), 6% ± 5% acquired those of oligodendrocytes ($GalC^+$), and 5% ± 2% those of mature neurons ($MAP2ab^+$; $n = 8$; Fig. 3B). GFAP, GalC, and Tuj1/MAP2ab were never found in the same cell (Figs. 1D and 4A). Consistently, quantitative RT-PCR of tNSCs after differentiation using protocol 1 for 14 days confirmed the differentiation of tNSCs into mature neuroectodermal cells (Fig. 3E). The NSC marker gene *NES*

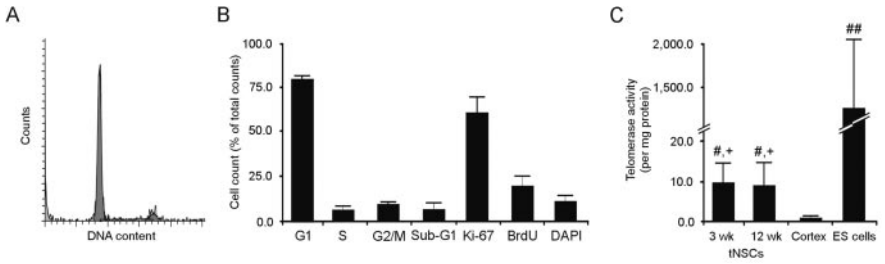


Figure 2. Cell cycle distribution, proliferation potential, and apoptosis of adult tNSCs. **(A):** PI-FACS analysis of tNSCs showing the cell cycle distribution typical for slowly dividing cells. **(B):** Quantitative data of PI-FACS analyses, Ki67 immunostaining, BrdU staining, and nuclei with chromatin clumping and fragmentations (apoptotic nuclei) in DAPI stainings. (See Fig. 2A for Ki67, BrdU, and DAPI stainings of neurospheres.) Results are the mean ± SEM from three to six independent experiments. **(C):** Telomerase activity in tNSCs after 3 and 12 weeks, respectively, as well as adult cortical tissue and mouse ESCs during expansion measured by the quantitative real-time telomeric repeat amplification protocol. Telomerase activity was normalized to protein content. Results are mean values ± SEM from three independent experiments. #$p < .05$, ##$p < .001$ when compared with adult cortical tissue; +$p < .001$ when compared with ESCs. Abbreviations: BrdU, 5-bromo-2-deoxyuridine; DAPI, 4,6-diamidino-2-phenylindole; ESC, embryonic stem cell; PI-FACS, propidium iodide–fluorescence-activated cell sorting; tNSC, tegmental neural stem cell.

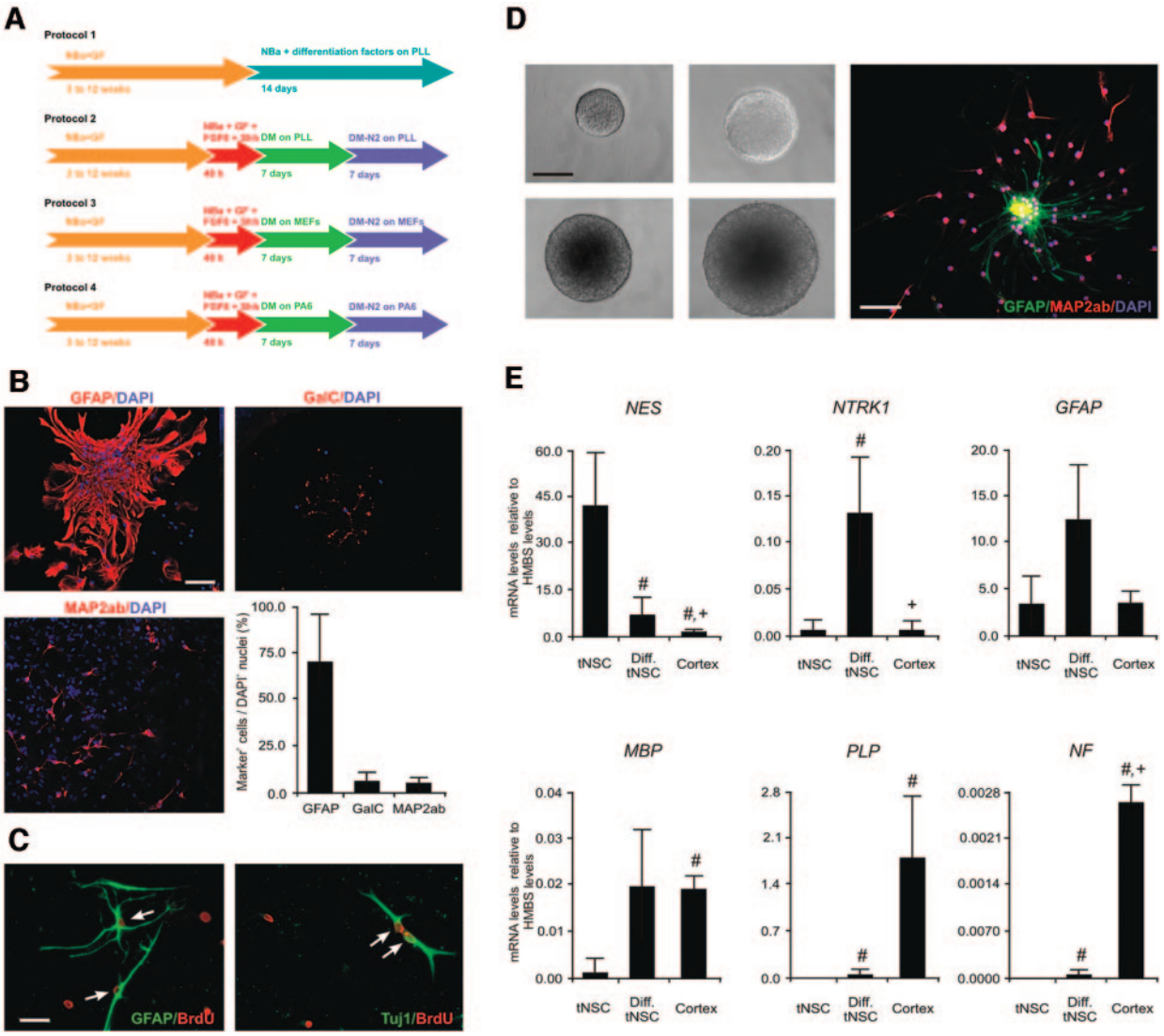


Figure 3. In vitro differentiation capacity of tNSCs. **(A):** Schematic diagrams of protocols for differentiation of tNSCs (see Materials and Methods for details). **(B):** tNSCs differentiated using protocol 1 were stained against markers for astrocytes (GFAP), oligodendrocytes (GalC), or neurons (MAP2ab). Nuclei are counterstained with DAPI (blue). Scale bar = 30 μm. Quantification of differentiation capacity of 14-day cultures of tNSCs differentiated using protocol 1. Data shown are mean values ± SEM from eight independent experiments. **(C):** Immunocytochemical analysis of differentiated tNSCs expanded for only 7 days. Prior to differentiation, tNSCs were incubated with 10 μM BrdU for 30 hours and then differentiated using protocol 1 and double-stained against GFAP or Tuj1 (green) and BrdU (red). Arrows indicate double-labeled cells. Scale bar = 15 μm. **(D):** Clonal analysis of single tNSCs. Left panel: Secondary neurospheres can be derived from a single tNSC from expanded neurospheres. Scale bar = 200 μm. Right panel: Differentiation capacity of clonally derived neurosphere cells. Progeny of single cell–derived neurospheres can be differentiated into neurons (MAP2ab) and astrocytes (GFAP). Nuclei were counterstained with DAPI (blue). Scale bar = 30 μm. **(E):** Quantitative transcription profile of tNSCs, differentiated tNSCs using protocol 1, and primary mouse cortical tissue. Results of quantitative real-time RT-PCR analyses of the NSC marker gene Nestin (*NES*), the neural gene *NTRK1,* glial genes (*GFAP, MBP, PLP*), and the neuronal gene *NF* are displayed. Expression levels are expressed relative to the housekeeping gene *HMBS*. For primers, complete names of genes, and melting curve analyses demonstrating the specificity of amplified PCR products as listed in Table 2. Results are mean values ± SEM from at least three independent experiments. $^{\#}p < .05$ when compared with tNSCs; $^{+}p < .05$ when compared with differentiated tNSCs. Abbreviations: BrdU, 5-bromo-2-deoxyuridine; DAPI, 4,6-diamidino-2-phenylindole; DM, differentiation medium; DM-N2, differentiation medium supplemented with N2; FGF, fibroblast growth factor; GalC, galactocerebrosidase C; GF, growth factor; GFAP, glial fibrillary acidic protein; MAP2ab, microtubule-associated protein 2ab; MEF, mouse embryonic fibroblast; NBa, neurobasal medium; NF, neurofilament; NSC, neural stem cell; PLL, poly-L-lysin; RT-PCR, reverse transcription–polymerase chain reaction; Shh, sonic hedgehog; tNSC, tegmental neural stem cell.

(encoding Nestin) was downregulated sixfold, whereas mRNA levels of *NTRK1*, *GFAP*, *MBP*, *PLP*, and *NF* increased six- to 430-fold during the differentiation process (Fig. 3E). Most mRNA levels of markers for mature neural cell types in differentiated tNSCs were lower compared with those in adult mouse cortex (Fig. 3E). No dopaminergic or serotoninergic cells could be detected using protocol 1. Replacement of BDNF/db-cAMP/retinoic acid by various combinations of growth factors/cytokines known to induce the dopaminergic phenotype in several murine stem cell types [38] led to changes in the differentiation

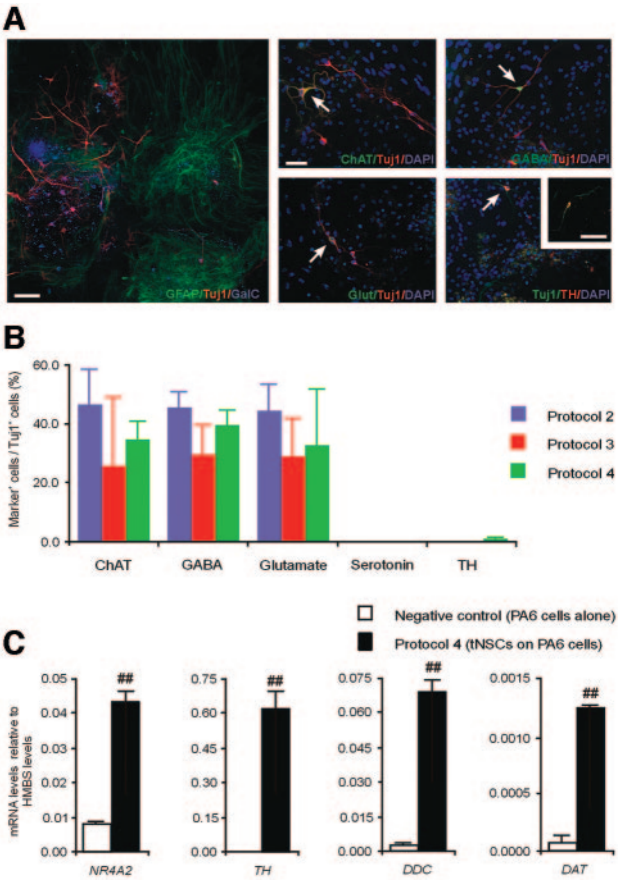


Figure 4. Neuronal subtype differentiation capacity of tNSCs in vitro established by using differentiation protocols 2–4 after an expansion phase of 3–12 weeks. **(A):** Triple immunostaining of tNSCs differentiated using protocol 4 for markers of astroglia (GFAP), neurons (Tuj1), and oligodendroglia (GalC), as well as cholinergic (ChAT), GABAergic (GABA), glutamatergic (glutamate), and dopaminergic (TH) neurons. Nuclei were counterstained with DAPI (blue). Arrows mark double-stained neurons. The inset shows high-magnification confocal image of a $Tuj1^+/TH^+$ neuron. Scale bars = 30 μm (left panel) or 15 μm. **(B):** Quantitative data of triple immunostainings of tNSCs differentiated with protocols 2–4, respectively. Results are mean values ± SEM from at least three independent experiments. **(C):** Quantitative real-time RT-PCR analyses of the dopaminergic marker genes nuclear receptor Nurr1 (*NR4A2*), TH (*TH*), dopa-decarboxylase (*DDC*), and dopamine transporter (*DAT*) on tNSCs differentiated using protocol 4 (closed bars) and negative control (open bars). Expression levels are expressed relative to the housekeeping gene *HMBS*. For primers, complete names of genes, and melting curve analyses, see Table 2. Results are mean values ± SEM from at least three independent experiments. $^{\#\#}p < .01$ when compared with negative control (PA6 cells alone). Abbreviations: ChAT, cholineacetyltransferase; DAPI, 4,6-diamidino-2-phenylindole; DAT, dopamine transporter; DDC, dopa-decarboxylase; GABA, γ-aminobutyric acid; GalC, galactocerebrosidase C; GFAP, glial fibrillary acidic protein; HMBS, hydroxymethylbilane synthase; RT-PCR, reverse transcription–polymerase chain reaction; TH, tyrosine hydroxylase; tNSC, tegmental neural stem cell.

capacity into astroglial, oligodendroglial, and neuronal cells, but no TH^+ neurons were observed (Table 1). For analyzing the differentiation capacity of acutely isolated tNSCs before neurosphere formation (7 days after preparation), we used the BrdU incorporation assay. The tNSCs were incubated during the expansion phase with BrdU (10 μM) for 30 hours. Then, differentiation was initiated using protocol 1. We were able to detect $BrdU^+/Tuj1^+$ neurons and $BrdU^+/GFAP^+$ glial cells (Fig. 3C), demonstrating that these neurons and glial cells were de novo–generated from proliferating progenitor cells.

Next, we tested various multistep protocols with pre-stimulation using FGF-8 and/or Shh for 48 hours in expansion medium before plating the cells on PLL or feeder layers of several cell types, including astroglia, MEFs, and PA6 stromal cells (protocols 2–4; Fig. 3A). In fact, protocol 4 is similar to the protocol for dopaminergic differentiation of mouse ESCs reported by Kawasaki and coworkers [30]. There were no relevant differences in the differentiation capacity of tNSCs into astroglia and neurons with respect to the various differentiation conditions (Table 1). In contrast, only protocol 4 was able to induce dopaminergic neurogenesis in a small percentage of cells (2.4% ± 1.4% of $Tuj1^+$ cells; $n = 3$), showing typical morphological features of $Tuj1^+/TH^+$ dopaminergic neurons with small irregularly shaped soma and bipolar or multipolar branching processes with varicosities (Fig. 4A, 4B). Using quantitative RT-PCR, we consistently demonstrated expression of various dopaminergic marker genes, including *NR4A2* (encoding the nuclear receptor Nurr1), *TH* (TH), *DDC* (dopa-decarboxylase), and *DAT* (dopamine transporter), in tNSCs differentiated using protocol 4 (Fig. 4C). No dopaminergic neurogenesis could be observed in cultures without PA6 cells (including protocols using PA6 conditioned media), and there were no differences in the amounts of glutamatergic, GABAergic, cholinergic, and serotoninergic neurons in cultures with PA6 cells compared with PA6 cell–free cultures (Fig. 4B; Table 1). Remarkably, the morphology of the different nerve cell subtypes was significantly different with large soma and multiple neurites in cholinergic cells and relatively small soma with two to three neurites in GABAergic and glutamatergic neurons as described for primary cultures (Fig. 4A). In contrast, protocol 4 also induced glutamatergic, GABAergic, and cholinergic neurons out of corresponding adult mouse hippocampal NSCs but was unable to produce TH^+ dopaminergic cells out of hippocampal NSCs (data not shown).

Clonal Analysis of Adult tNSCs

To determine whether individual tNSCs could generate both neurons and glia, neurospheres were dissociated and individual cells were isolated by the single-cell culture technique in ultra low–attachment multiwell-plates. Single cells were cultured in expansion medium for 3–6 weeks. One third of the single cells proliferated to generate secondary neurospheres (Fig. 3D). Clonal cells were then differentiated for 14 days using differentiation protocol 1. Double immunostaining revealed that each of these clonally derived neurospheres differentiated into neurons and astrocytes (Fig. 3D). In addition to the BrdU incorporation experiments (Fig. 3C), these results demonstrate that neurons and glia are de novo–generated from proliferating tNSCs.

Functional Properties of Differentiated Adult tNSCs

To analyze the functional properties of differentiated tNSCs, we performed whole-cell voltage-clamp recordings to measure voltage-gated sodium and potassium currents and current-clamp recordings to measure the capacity to generate action potentials

after differentiation for 10–20 days using protocol 1. The RMP of differentiated tNSCs was −46 ± 4 mV (n = 34), the input resistance was 536 ± 45 MΩ (n = 48), and the cell membrane capacity was 14.7 ± 1.3 pF (n = 48). Of differentiated tNSCs, 83 out of 95 recorded cells (87.4%) expressed a sustained outward current of a few hundred pA up to 5 nA (Fig. 5A). These currents showed a voltage dependence and kinetics characteristic for A-type currents as well as tetraethylammonium-sensitive delayed rectifier potassium channels (Fig. 5A, 5B). In 67 out of 95 cells (70.5%), we could identify inward currents of up to 2 nA with voltage dependence and kinetics typical for voltage-activated sodium channels (Fig. 5C, 5D). These currents were tetrodotoxin (TTX)-sensitive (Fig. 5C). Current-clamp recordings revealed that differentiated tNSCs generated TTX-sensitive APs with a duration of 9.6 ± 0.2 ms (n = 8) and a short AHP (Fig. 5E). The length of AHPs was 22.7 ± 0.8 ms, and the maximal amplitude 9.8 ± 0.4 mV (n = 8).

To further characterize the functional properties of differentiated tNSCs, we measured the response of tNSCs differentiated for 7 days using protocol 1 to various concentrations of GABA (10–1,000 μM), glutamate (1–300 μM), or *N*-methyl-D-aspartate (NMDA; 100 μM). GABA elicited inward currents in a dose-dependent manner with a half-maximal effective concentration (EC_{50} value) of 42 μM (Fig. 6A, 6B). These currents could be antagonized by co-application of bicuculline and enhanced by co-application of diazepam (Fig. 6C). The characteristics of GABA-induced currents in our cells are typical for $GABA_A$ receptors. Furthermore, glutamate induced fast inward currents in a dose-dependent manner with an EC_{50} value of 8 μM (Fig. 6D, 6E). Nine out of 10 cells expressing glutamate-induced currents also showed functional NMDA receptors with amplitudes of approximately 27% of the mean glutamate amplitude (Fig. 6F). Because there is no reliable evidence for functional glutamate receptors of the NMDA subtype in glial cells [56], these data further demonstrate functional properties of mature nerve cells.

Neurotransmitter (dopamine and GABA) production and release were studied on tNSCs differentiated using protocol 4.

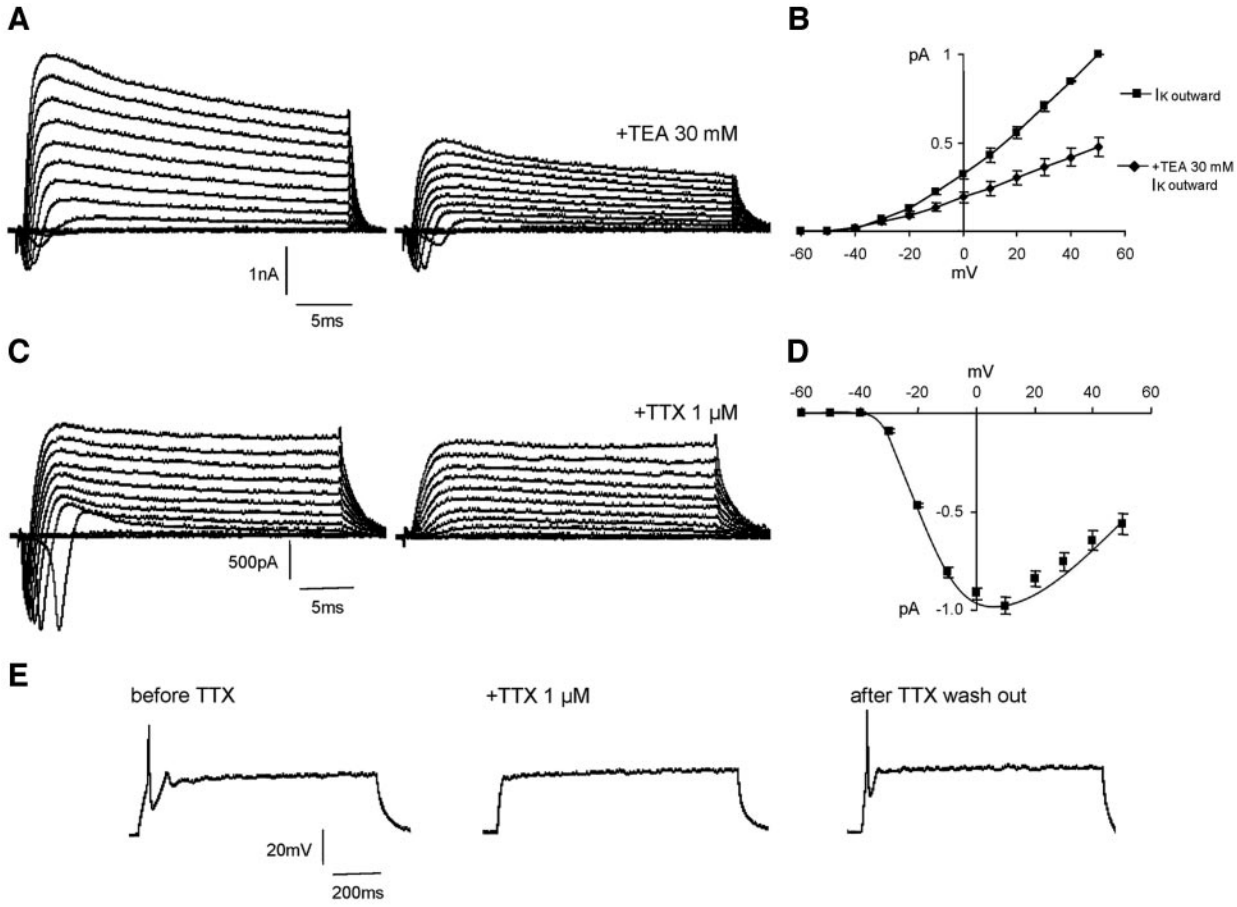


Figure 5. Electrophysiological properties of adult tegmental NSCs differentiated using protocol 1 for 10 days. For voltage-clamp measurements **(A–D)**, cells were held at −70 mV and depolarized to +50 mV with increasing amplitudes in steps of 10 mV. **(A):** Representative traces from a differentiated tNSC displaying inward and sustained outward currents. Potassium outward currents were markedly reduced by application of TEA (30 mM). **(B):** Current-voltage (I-V) relationships of the outward currents in steady state at the end of the voltage steps in the absence (■) and presence of 30 mM TEA (♦). Data are mean values ± SEM from three to 11 independent experiments. **(C–D):** Fast inward currents could be completely blocked by application of TTX (1 μM) and displayed the characteristic I-V relationship of voltage-gated sodium channels (n = 7). **(E):** Current-clamp recordings of the same cell recorded in **(C)** in response to depolarizing current pulses of increasing amplitude. The resting membrane potential was approximately −65 mV, and TTX-sensitive action potentials peaked at approximately +40 mV. Abbreviations: TEA, tetraethylammonium; tNSC, tegmental neural stem cell; TTX, tetrodotoxin.

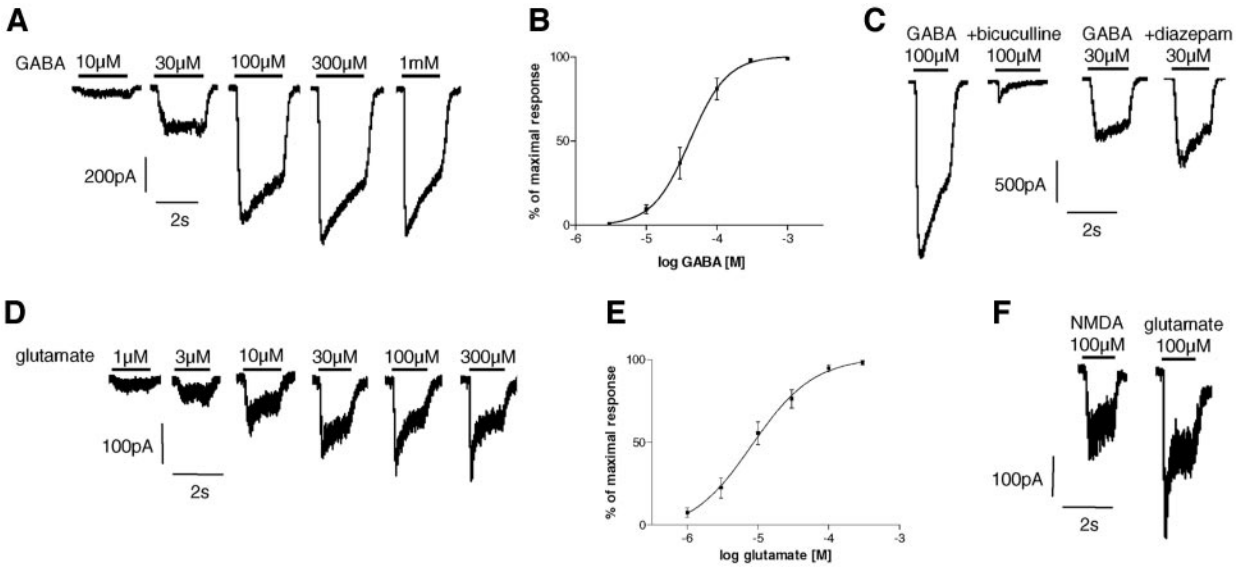


Figure 6. Electrophysiological properties of ligand-gated ion channels in adult tegmental neural stem cell (tNSCs) after in vitro differentiation for 7 days using differentiation protocol 1. The holding potential was −70 mV in whole-cell voltage-clamp mode, and substances were applied with a rapid perfusion system every 30 seconds. **(A):** Representative recordings of GABA-induced inward currents after applications of increasing concentrations of GABA (10–1,000 μM). **(B):** GABA dose-response curve with an half-maximal effective concentration (EC_{50}) value = 41.5 ± 1.1 μM. Data are mean values ± SEM from five independent experiments. **(C):** GABA-induced currents could be antagonized by co-application of bicuculline and enhanced by co-application of diazepam. **(D):** Representative recordings of glutamate-induced inward currents after applications of increasing concentrations of glutamate (1–300 μM). **(E):** Glutamate dose-response curve with an EC_{50} value = 8.2 ± 1.8 μM. Data are mean values ± SEM from eight independent experiments. **(F):** NMDA-induced currents could be elicited in nine out of 10 cells expressing glutamate-induced currents (mean NMDA amplitude was 27% of mean glutamate amplitude). Abbreviations: GABA, γ-aminobutyric acid; NMDA, *N*-methyl-D-aspartate.

HPLC electrochemical detector analysis showed significant amounts of dopamine in media conditioned by differentiated tNSCs for 2 days (38.3 ± 10.7 pg/ml; n = 4; Fig. 7A, 7B), but only small amounts of dopamine were found in extracellular buffer or after stimulation of the cells with 56 mM KCl for 45 minutes. (Fig. 7B), consistent with the amounts of TH^+ cells and the expression of various dopaminergic marker genes, including the key enzymes of dopamine synthesis (TH and dopa-decarboxylase; Fig. 4). Under the same conditions, we detected GABA production and potassium-dependent release of GABA by differentiated tNSCs (Fig. 7B). There was no dopamine production by undifferentiated tNSCs or tNSCs differentiated using protocol 1. Consistent with the immunocytochemical data, we did not find the neurotransmitter serotonin in any sample.

DISCUSSION

Here, we provide evidence for the existence of multipotent NSCs in the adult mouse tegmentum (midbrain and hindbrain region) with the capacity to differentiate in vitro into functional

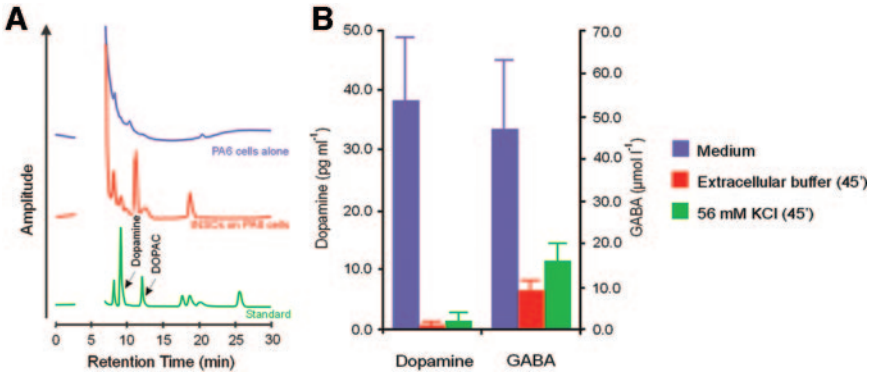


Figure 7. Neurotransmitter (dopamine, GABA) production and release of tNSCs differentiated on PA6 cells using protocol 4. **(A):** Representative chromatograms of HPLC-ECD determination of dopamine in medium conditioned for 2 days by tNSCs on PA6 cells (protocol 4, red) and PA6 cells alone (blue). Standard is presented in green. **(B):** Quantification of dopamine and GABA production in medium conditioned for 2 days (blue bars), in extracellular buffer conditioned for 45 minutes (red bars), and in extracellular buffer + 56 mM KCl conditioned for 45 minutes (green bars). Abbreviations: DOPAC, dihydroxyphenyl acetic acid; GABA, γ-aminobutyric acid; HPLC-ECD, high-performance liquid chromatography–electrochemical detector; KCl, potassium chloride; tNSC, tegmental neural stem cell.

nerve cell types, including dopaminergic neurons. We isolated and cultured NSCs from the adult tegmentum (tNSCs) of C57BL/6 mice using the neurosphere culture technique similar to those used for isolation and propagation of fetal mesencephalic NSCs [29, 38, 57, 58]. Their phenotype was similar to that of NSCs derived from adult mouse hippocampus, mouse SVZ, or human fetal brain samples [20, 31, 59]. The tNSCs were clonogenic, self-renewing cells that maintain the capacity to differentiate into mature functional nerve cells, including dopaminergic neurons (Figs. 3–7). Their potential to proliferate was slower compared with embryonic/fetal NSCs, whereas their differentiation capacity was similar to that of NSCs derived from fetal rodent or human mesencephalon [29, 38, 55, 57, 58, 60].

NSCs are often defined in vitro by the presence of the CNS stem cells marker Nestin [41, 61, 62], a class IV intermediate filament protein [61, 63, 64] and a marker for precursor cells in the adult brain [24, 61, 65–67]. Nestin is highly expressed in tNSCs during in vitro expansion. Furthermore, the RNA-binding protein Musashi1 is involved in post-transcriptional gene regulation in neuroprogenitors and/or NSCs and is used as a marker of those cell types [42]. Consistently, Musashi1 is expressed in tNSCs both at the mRNA and protein levels (Fig. 1). 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. Proneural genes, encoding transcription factors of the basic helix-loop-helix class, such as *NeuroD1*, *Neurogenin2*, and *Olig2*, 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 [43–45]. Proneural genes are therefore expressed mostly in neuroprogenitor cells, including NSCs in early neuroectodermal tissues [23, 46–48]. Consistently, tNSCs expressed mRNA and protein of NeuroD1, Neurogenin2, as well as Olig2 (Fig. 1). For example, *NeuroD1* and *Neurogenin2* are considered neuronal differentiation genes and promote exit from the cell cycle and inhibit gliogenesis [68–70]. In addition, Olig2 is a pivotal factor for neurogenesis and oligodendroglial specification of adult NSCs in vitro [23, 71]. Although Olig2 showed no co-localization with adult CNS stem cell markers in vivo, it becomes highly upregulated under expansion conditions with high levels of EGF and FGF-2 (Fig. 1 and [23]). Olig2 is required for the proliferation and self-renewal of neurosphere cells, as shown by interference analysis [23]. The transcription factor Pax6 is the major neurogenic determinant for radial glial cells and adult NSCs [23, 72–74]. Indeed, neurogenic differentiation in neurospheres fully depends on the expression of the transcription factor Pax6, independent of the region of origin [23]. Analysis of Pax6 expression in tNSCs demonstrated Pax6 expression during the expansion phase, further confirming their neurogenic potential (Fig. 2C). Together, tNSCs expressed a typical pattern of early neuroectodermal and/or NSC marker genes during the expansion phase in vitro, including major factors for proliferation and self-renewal as well as neurogenic differentiation, such as proneural genes and Pax6.

Further evidence for the stem cell–like nature of tNSCs within the neurospheres is the demonstration of clonogenicity of individual cells in the single-cell culture assay showing the potential of clonally derived neurospheres to differentiate into both neurons and astrocytes. In addition, these results demonstrate that neurons and glia are de novo–generated from proliferating cells (Fig. 3C, 3D). Finally, showing proliferation by PI-FACS analysis, BrdU labeling and Ki67 immunostaining as well as the expression of telomerase [54], these tNSCs fulfill the major characteristics of multipotent NSCs. After removal of mitogens, plating the cells onto PLL, and incubation with BDNF, db-cAMP, and retinoic acid for 14 days, tNSCs differentiate into all major cell types of the CNS, namely mature ($MAP2ab^+$) neurons, oligodendrocytes, and astrocytes with an approximate ratio of 1:1.5:14, respectively. Replacement of the mentioned growth factors with various combinations of growth factors and/or cytokines did not lead to major changes of this differentiation capacity (Table 1). In the presence of selected growth factors (see Materials and Methods for details), tNSCs differentiated into cells expressing the markers for cholinergic, GABAergic, or glutamatergic neurons with similar amounts of the distinct neuronal subpopulations with respect to the various differentiation protocols. Thus, differentiation of tNSCs into these neuronal subtypes did not depend on pre-stimulation with FGF-8 and Shh or co-culture with feeder cells such as MEFs, astrocytes, or PA6 cells (Fig. 4B). Interestingly, no serotoninergic cells could be observed with any differentiation protocol tested.

We further demonstrated that a high percentage of differentiated adult tNSCs acquired functional properties of nerve cells, such as generation of TTX-sensitive sodium channels and action potentials similar to differentiated fetal mouse mesencephalic NSCs [38] or adult NSCs derived from mouse SVZ [75]. A subset of these cells expressed currents with the characteristics of functional $GABA_A$ receptors as well as ionotropic glutamate receptors, including the NMDA-subtype known to be expressed exclusively by neuronal cells [56]. Together, this in vitro differentiation capacity of tNSCs is similar to that of NSCs derived from fetal brain as well as the principal neurogenic regions (hippocampus and SVZ of the lateral ventricles) of the adult brain of various species [21, 31, 41, 76–78]. Zhao and colleagues demonstrated neurogenesis in the adult substantia nigra of C57BL/6 mice in vivo [7]. Two other studies did not find newly born neurons in the adult substantia nigra of normal or lesioned rodents [13, 16]. Moreover, Lie and colleagues found the production of glial cells, but not of neuronal cells, from adult substantia nigra progenitor cells in vivo [16]. In contrast, the in vitro differentiation capacity of these substantia nigra NSCs seems to include neuronal cell lineages [16]. However, this study reported only differentiation into immature neurons ($NeuN^+$ or $Tuj1^+$ cells) and did not show any functionality or neuron subtype specification [16].

The analysis of neuronal subtype differentiation of tNSCs revealed that without pre-stimulating the cells with FGF-8 and Shh and subsequent co-culture with PA6 stromal cells, no TH^+ dopaminergic neurons could be achieved. However, after a multistep differentiation protocol using pre-stimulation and subsequent co-culture of tNSCs with PA6 cells, a small subset of cells not only acquired typical morphological features of dopaminergic cells but also produced the neurotransmitter dopamine, demonstrating the differentiation of tNSCs into functional dopaminergic neurons (Figs. 4, 7). No dopaminergic neurogenesis was detected using other cell types for feeder layer cultures, such as cortical or striatal astrocytes known to induce neuro-

genesis in adult NSCs [78] or MEFs, normally used for proliferation of ESCs. These data suggest a crucial role of PA6 cells (or the so-called stromal cell–derived inducing activity [30]) on dopaminergic neurogenesis not only for ESCs but also for adult tNSCs. However, in contrast to ESCs, tNSCs seem to need an additional priming step to become dopaminergic, such as the pre-incubation procedure with FGF-8 and Shh used in the present study.

Zhao and colleagues demonstrated dopaminergic neurogenesis in the adult substantia nigra of C57BL/6 mice in vivo [7]. A subsequent study was not able to reproduce these results or demonstrate newly generated dopaminergic neurons by the BrdU incorporation assay in normal or 6-hydroxydopamine–lesioned animals [13]. In contrast to the latter results established in vivo, we were able to show dopaminergic specification of isolated adult tNSCs in vitro. This fact is in contrast to previous results on adult NSCs not reporting dopaminergic specification of adult NSCs in vitro independent of the region of origin of the NSCs [7, 13, 16, 78]. The reasons for these discrepancies are likely the following: (a) The in vivo differentiation capacity may not reflect the entire lineage potential of resident progenitor cells, because regional environmental factors appear to restrict in situ differentiation to distinct neural lineages [16–18]. We established a multistep differentiation protocol including coculturing the tNSCs with PA6 stromal cells known to have potent effects on dopaminergic differentiation of ESCs in vitro [30] to provide an environment sufficient for dopaminergic specification in vitro of isolated and long-term expanded adult tNSCs. However, the amounts of dopaminergic neurons generated from adult tNSCs are very low (≈2.5% of all neurons or ≈0.25% of all cells, which is five- to 10-fold less than differentiated fetal mesencephalic NSCs derived from rodents or humans) [29, 38, 79]. The knowledge of the specific factors produced by PA6 stromal cells, which induce dopaminergic specification, will help to further optimize the efficiency of dopaminergic specification of adult NSCs. High yields of dopaminergic cells would be essential for characterization of the intracellular mechanisms of adult dopaminergic neurogenesis as well as the NSC-derived dopaminergic neurons. (b) We used, for the first time, NSCs from the whole adult tegmentum and not just from the substantia nigra or the principal neurogenic regions of the adult brain [7, 16, 78]. Future studies are warranted to further define the exact region within the adult tegmentum from which "pre-dopaminergic" NSCs can be generated.

SUMMARY

We demonstrated the presence of multipotent clonogenic NSCs in the tegmentum of adult mice with the differentiation capacity similar to embryonic/fetal mesencephalic NSCs, including specification into functional nerve cells such as dopaminergic and GABAergic neurons. Previous reports suggest that these cells were derived from the SVZ of the aqueduct and/or fourth ventricle, a structure that is nearby the substantia nigra pars compacta (A9 region) where the degenerating dopaminergic neurons in Parkinson's disease (PD) are located. Recent studies failed to conclusively demonstrate dopaminergic neurogenesis in the adult mammalian midbrain in vivo [7, 13, 16, 80], but our results suggest that NSCs residing in the adult midbrain/hindbrain area can give rise to new functional (dopaminergic) neurons when exposed to appropriate environmental signals. The presented developmental potential of adult tNSCs is one prerequisite for potential future endogenous cell replacement strategies in PD. However, future studies are warranted to clarify the mechanisms for controlling proliferation and dopaminergic differentiation of these adult tNSCs. The presented novel cell culture system provides a powerful tool for investigating these molecular mechanisms of adult neurogenesis and dopaminergic differentiation.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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Expert Opinion

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Cell- & Tissue-based Therapy

Neurorestoration in Parkinson's disease by cell replacement and endogenous regeneration

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Parkinson's disease (PD) is characterised by a continuous and selective loss of dopaminergic neurons in the *substantia nigra pars compacta* with a subsequent reduction of the neurotransmitter dopamine. Thus, the prospect of replacing the missing or damaged dopaminergic cells is very attractive. Possible regenerative therapies include transplanting developing neural tissue or neural stem cells into the degenerated host brain and inducing proliferation of endogenous stem cells by pharmacological manipulations. Neural stem cells, with the capacity to self renew and produce the major cell types of the brain, exist in the developing and adult CNS. These cells can be generated and expanded *in vitro* while retaining the potential to differentiate into nervous tissue. However, one major problem is the control of growth and differentiation of these cells. This review discusses new data on stem cell technology in cell replacement strategies in PD as well as endogenous dopaminergic regeneration.

Keywords: dopaminergic neurons, neural stem cells, neurorestoration, Parkinson's disease, therapy, transplantation

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1. Introduction

Parkinson's disease (PD) is characterised by the continuous degeneration of dopaminergic neurons in the A9 region of the *substantia nigra pars compacta* with a relative sparing of ventral tegmental area (A10 region) neurons [1,2]. This ongoing loss of nigral dopaminergic presynaptic neurons mainly leads to a reduction of striatal dopamine content and subsequently to the occurrence of motor symptoms such as rigidity, tremor and bradykinesia [1,2]. This cell loss may be due to genetic abnormalities of the diseased cell or may be due to the absence of enzymes, neurotransmitters, tropic factors or the presence of toxic substances or physical injuries (for review see [2,3]). At present, there is no cure for the nigrostriatal dopaminergic deficit associated with PD. Intervention strategies for the treatment of PD have used both surgical interventions (pallidotomy, fetal cell transplantation) and pharmacological therapies (levodopa, deprenyl, dopamine agonists) [1,4,5]. This antiparkinsonian drug therapy mainly substitutes the nigrostriatal dopamine deficit and subsequently improves motor symptoms in PD. However, in advanced stages of PD, response to dopaminergic substitution becomes increasingly less satisfactory and treatment-refractory signs appear, for example, balance disorders, freezing, dementia or affective changes [6]. More recently, deep brain electrical stimulation was developed. However, these treatments have several limitations (reviewed in [2]). Consequently, there is a pressing need for innovative approaches such as micropumps, viruses, genetically modified cell systems, small molecules [7], transplantation of primary mesencephalic tissue or stem cell therapy (Figure 1).

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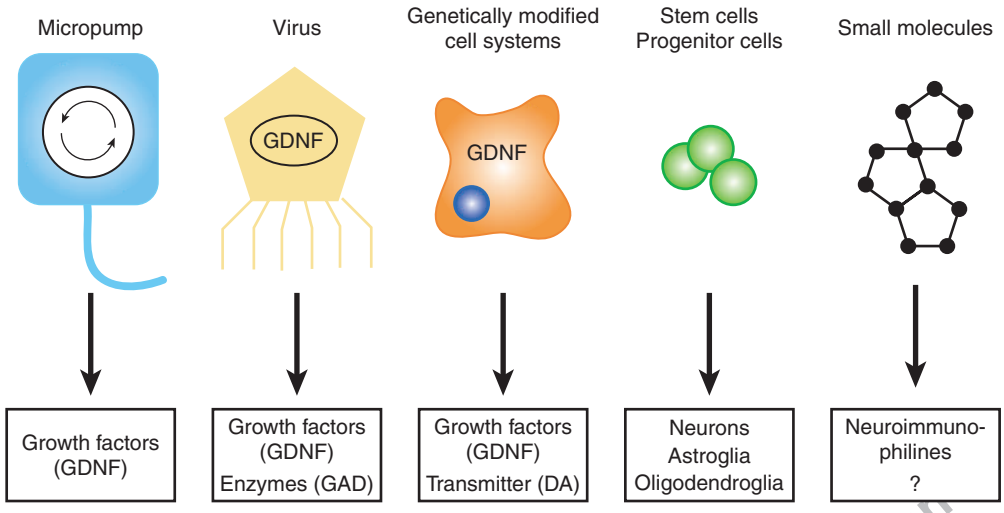

Figure 1. As classical treatments of PD have several limitations, there is a pressing need for innovative approaches such as micropumps, viruses or genetically modified cell systems, with the goal of delivering the lacking enzymes, transmitters or growth factors to stimulate endogenous regeneration. Transplantation of primary mesencephalic tissue or stem cell therapy, on the other hand, should directly replace the lost nerve cells and consequently provide an adequate amount of dopamine release. Small molecules seem to have influence on neurogenesis and, therefore, could be used to stimulate endogenous restitution. See text for details.
DA: Dopamine; GAD: Glutamate decarboxylase; GDNF: Glial cell line-derived neurotropic factor; PD: Parkinson's disease.

Transplantation is one of the most effective strategies for restoring these lost cell or tissue functions. The loss of a specific type of dopaminergic cells in PD makes the prospect of replacing the missing or damaged cells very attractive. Indeed, recent clinical transplantation trials with primary human embryonic dopaminergic neurons provide the proof-of-principle for the cell replacement strategy in PD [8-10], but also demonstrate several limitations for general clinical application: although functional neuroimaging and neuropathological investigations showed the integration of transplanted dopaminergic neurons in the host striatum, only subpopulations of PD patients displayed significant clinical benefits. Moreover, 7 – 56% of patients suffered from dyskinesias after a 12 h drug-free period [9-11]. Furthermore, there are major logistical problems with the clinical use of human cells or tissue. These problems are amplified when tissue comes from a small region of the developing brain, such as the *substantia nigra*. Thus, transplantation of primary dopaminergic cells in PD presents numerous logistical, ethical and scientific concerns [12,13]. The development of techniques to isolate, expand and differentiate neural precursor cells would overcome most of these problems. This paper reviews new data on stem cell technology in cell replacement strategies in PD, as well as new results on endogenous dopaminergic regeneration.

2. Neural stem cells: origin and definition

The discovery of neural stem cells (NSCs) in the developing and adult brain that can generate neural tissue has raised new possibilities for repairing the nervous system in PD. Despite the debate on how to classify these germinal cells, there is consensus that NSCs are a subtype of progenitor cells that are capable of extended self-renewal and have the ability to generate all major cell types of nervous tissue, such as neurons and glial cells (reviewed in [14,15]). NSCs are usually identified operationally by their behaviour after isolation. During expansion, they normally grow in floating, multicellular, so-called 'neurospheres' [14,15]. However, markers that define NSCs have recently been developed [14,16-18]. The first marker defining NSCs was the intermediate filament protein nestin, but some neural precursor cells are nestin-negative [19] and, on the other hand, nestin is expressed by other cell types, including non-neuronal cells [20]. Recently, Uchida and co-workers performed an extensive analysis of surface markers on clonogenic human neurosphere cultures and defined human NSCs as phenotypically CD133-positive, but negative for CD34 and CD45 [17]. Vogel and colleagues characterised commercially available neural progenitor cells as CD15, CD56, CD90, CD164, nerve growth factor receptor, 57D2 and W4A5 positive, whereas they were negative for CD45, CD105 (endoglin), CD 109, CD 140b(PBGF-RB) and W8B2 [18]. Recently, the expression of specific neural transcription factors, such as Sox-1, Musashi-1, Otx-1, Otx-2, NeuroD1 and neurogenin-2 was reported in neural stem or progenitor cells, demonstrating their neuroectodermal progeny [21,22]. Furthermore, there are reports on surface markers of committed precursors, such as high polysialic acid neural cell adhesion molecule for neuronal-restricted precursors [23] or A2B5 for glial precursors [24].

Early studies reported the isolation of stem-like cells from embryonic mammalian CNS [25-28] and the peripheral nervous system [29]. Since then, stem cells have been isolated from many regions of the embryonic CNS, indicating their ubiquity. Recently, the first isolation of NSCs from adult brain was demonstrated [30,31]. However, adult NSCs have now been

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found in the two principal neurogenic regions, the hippocampus and the subventricular zone (SVZ), and in some non-neurogenic regions, such as the spinal cord [14,15,23,24,30-33] and adult *substantia nigra* [34], as well as mesencephalon [35]. However, the amount of neurogenesis outside the two principal neurogenic regions in the adult brain should not be overestimated compared with the embryonic CNS.

3. Neural stem cells in culture

3.1 Isolation and expansion of neural stem cells

NSCs can either be directly extracted from fetal or adult nervous tissue and proliferated in culture [14,15,25,26,36-41], or embryonic stem (ES) cells can be extracted, proliferated and differentiated into neural precursor cells [42-46]. ES cells are pluripotent cells isolated from the inner cell mass of the preimplantation blastocyst, which give rise to all cells in the organism. There is evidence that ES cells can also produce germ cells and, therefore, these cells are now classified as totipotent [47]. Similarly, multipotent stem cells are also able to regenerate, but are believed to have a more restricted potential than ES cells and are often defined by the organ from which they are derived. NSCs have been categorised as multipotent stem cells derived from the nervous system with the capacity to regenerate and give rise to cells belonging to all three major cell lineages of the nervous system; neurons, oligodendrocytes and astrocytes [14,15,25,26,36-41].

The finding of Gensburger and co-workers in 1987 that fibroblast growth factor (FGF)-2 could induce proliferation of neural precursors in embryonic hippocampal cultures initiated a new field of cell culture experiments studying proliferation of neural precursors *in vitro* [48]. Later, epidermal growth factor (EGF) was found to stimulate the growth of striatal precursors, retaining their ability to differentiate into all major types of CNS cell types [27]. Since these early studies, most protocols for the *in vitro* proliferation of embryonic or fetal NSCs have used both mitogens (FGF-2 and EGF) alone or in combination in serum-free media (for review, see [40]). Subsequently, various other epigenetic approaches have been employed for *in vitro* propagation of NSCs, including erythropoietin [49] and reduced atmospheric oxygen [50,51], as well as less defined additives such as conditioned media and chicken extracts (for review, see [52]). However, at present, it is not completely clear how these proliferation factors influence differentiation of NSCs into distinct subsets of neurons or glial cells [53]. On the other hand, there are some interesting candidate factors that have not been investigated for their potency to stimulate proliferation of NSCs *in vitro*: for example, neuropeptide Y is reported to function as a neuroproliferative factor in adult mammals [54]. In addition, when added to the culture medium, leukaemia inhibitory factor (LIF) allows continual cell growth in human NSC cultures [55]. LIF acts through the gp130 signal transducing subunit. It is also required for the continual growth of mouse, but not human, ES cells and appears to maintain these cultures in a proliferative state by preventing differentiation [56]. Furthermore, telomerase activity is reported to have a pivotal role in the development and apoptosis of neural tissue (for review, see [57]). Another strategy to enhance proliferation and to increase culture time is genetic manipulation by transduction of immortalising genes into neural progenitors (for review, see [58,59]).

Adult NSCs have been found in the principal neurogenic regions (hippocampus and the SVZ) and in non-neurogenic regions in many species, ranging from chickadees to humans [15,30-32,60-62]. By isolation of NSCs from adult mouse brain and direct examination of cell properties, Rietze and co-workers recently showed that a predominant, nestin-positive functional type of stem cell exists in the periventricular region of adult brain, with the ability to generate both neuronal and non-neuronal cell types [60]. Lai and colleagues identified sonic hedgehog (SHH) as a regulator of adult hippocampal NSCs showing a dose-dependent proliferative response [63]. Another factor, named noggin, was shown to increase neurogenesis of SVZ stem cells while decreasing gliaogenesis by inhibiting the bone morphogenic protein (BMP) signalling pathway [64]. Interestingly, noggin is expressed by ependymal cells adjacent to the SVZ, whereas BMP is expressed by NSCs themselves [65].

Most of the reports mentioned above used rodent material for their studies, but what about human tissues? Several studies show that neural stem or precursor cells can be isolated from embryonic/fetal human CNS [40,41,50,58,59,66-70] and human ES cells can be differentiated into neuronal-like cells by nerve growth factor and retinoic acid [46]. Isolation and propagation of adult human neural precursor cells were recently reported [37,71-73]. Isolated hippocampal NSCs derived from surgical specimens of patients undergoing selective hippocampectomy could also be transfected and sorted using fluorescent proteins expressed under control of tissue-specific promoters [73]. Cells expressing nestin and/or α-tubulin were highly committed to neuronal differentiation. However, the proliferative potential of such cells is not clear. Besides deriving NSC cultures from 'fresh' surgical specimens, it is also possible to isolate and expand NSCs derived from post mortem tissue [72]. As many as 10^{12} cells could be expanded from a single specimen.

3.2 Neuronal differentiation of neural stem cells

NSCs derived from embryonic central nervous tissue were noted to generate oligodendrocytes, neurons and astrocytes in an approximate ratio of 1:5:25, respectively, when allowed to spontaneously differentiate after removal of growth factors or mitogens in serum-free media [74,75], although this differentiation programme can be influenced by exposure to different factors, such as growth factors or cytokines. However, neurons generated from expanded populations of NSCs are to a large degree GABAergic. In the CNS, the salient pattering in anterior–posterior and dorsal–ventral axes occurs early, concomitantly with neural induction. Consequently, it was postulated

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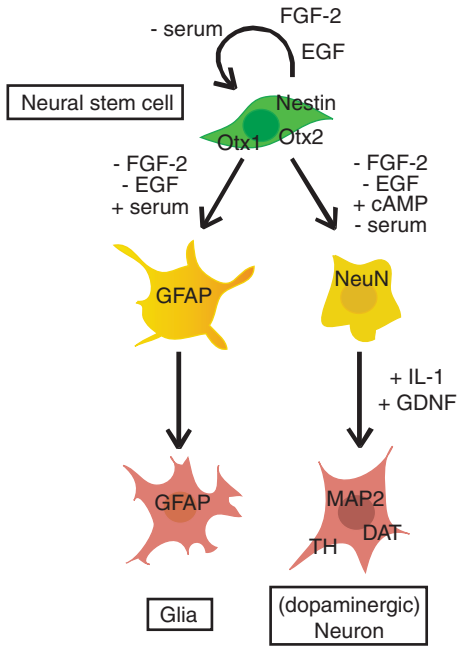

Figure 2. Schematic representation of tentative model for differentiation of mesencephalic neural stem cells into dopaminergic neurons. This diagram summarises the events and molecules during expansion as well as early and late stages of differentiation of mesencephalic-derived neural precursor cells. Removal of the mitogens FGF-2/EGF and plating the cells onto PLL induces the differentiation of neural precursors into both glial and neuronal cells. Addition of serum biased the differentiation into glial cell lines, but addition of substances increasing the intracellular level of cAMP biased differentiation into neuronal phenotypes. Subsequently, exposure to the cytokine/growth factor-mixture led to generation of the dopaminergic phenotype, expressing several dopaminergic marker molecules, such as TH and DAT. See text for details.

DAT: Dopamine transporter; EGF: Epidermal growth factor; FGF: Fibroblast growth factor; GDNF: Glial cell line-derived neurotropic factor; GFAP: Glial fibrillary acidic protein; MAP: Microtubule-associated protein; PLL: Poly-L-lysine; TH: Tyrosine hydroxylase.

that stem cells and restricted precursors exhibit regionalisation. Indeed, neurospheres generated from different CNS regions from insects and vertebrates express region-appropriate markers and, in addition, stem cells isolated from different neural regions generate region-appropriate progeny. Spinal cord stem cells generate spinal cord progeny [23], and stem cells from the forebrain generate more GABA-containing neurons than dorsal stem cell cultures under identical conditions [39]. Furthermore, only NSCs isolated from the midbrain SVZ generate functional dopaminergic neurons [16,38,39,50].

As NSCs are suggested as a new source of tissue for regenerative therapy in PD [76], most studies on both directly isolated and ES cell-derived NSCs have been focused on the induction of a dopaminergic phenotype. The first studies showed that mesencephalic-derived dopaminergic precursors from rodent embryonic brain could be isolated and expanded *in vitro* using FGF-2. However, these cells retained their capability to generate dopaminergic neurons only for a few divisions, not allowing long-term expansion of these cells [77,78]. Ling and co-workers demonstrated that IL-1 is able to induce a mature dopaminergic phenotype in long-term expanded rodent mesencephalic precursors, with significantly increased effects from the addition of other factors, such as IL-11, LIF, glial cell line-derived neurotropic factor (GDNF) and membrane fragments (see Figure 2 for a schematic overview of dopaminergic differentiation of mesencephalic NSCs) [38]. These dopaminergic cells show all major morphological features as well as functional properties of mature dopaminergic cells, such as dopamine production/release and expression of sodium and hyperpolarisation-induced cation (I_h) channels [79]. However, in contrast to precursor cells derived from human forebrain samples, which are easily expanded using EGF/FGF-2 in 21% O_2, precursors derived from human mesencephalon do not show any proliferation under these conditions or do not differentiate into dopaminergic neurons *in vitro* [14,40,50,55,68,69].

The authors have established an improved culture method for long-term proliferation of human mesencephalic precursors *in vitro* using 'physiological' O_2 levels of 3% known to stimulate mesencephalic precursors from rat [50,51,80]. Using these conditions, the authors successfully expanded EGF/FGF-2-responding cells from human midbrain tissue < 10 weeks post-fertilisation for > 1 year. These cells formed spheres which enlarged over time and showed immunoreactivity for the CNS precursor cell marker nestin, but not for the dopaminergic marker tyrosine hydroxylase (TH) or the glial marker glial-fibrillary acidic protein. Following incubation in differentiation media containing IL-1b, IL-11, LIF and GDNF, 1 – 5% of the precursor cells converted into cells exhibiting morphological and functional properties characteristic of dopamine neurons in culture, including dopamine production and release (Figure 2) [50,79].

During the last few years, many aspects of genetic and epigenetic control of induction and phenotypic maturation of dopaminergic neurons have been characterised [81-83]. For example, transcription factors, such Nurr1 and Ptx3, have been discovered to play an important role in this process. Furthermore, Nurr1 is reported to be upregulated by the differentiation factor IL-1 in mesencephalic precursor cells from rat [84]. Consistently, overexpression of Nurr1 is able to induce midbrain dopaminergic phenotype after stimulation of NSCs with astrocyte-conditioned medium [85,86]. On the other hand, these mechanisms and factors are potential targets for drug development to influence dopaminergic neuron development or regeneration *in vivo*.

Another approach to generate dopaminergic neurons includes differentiation of ES cells into neural stem or precursor cells and subsequently into dopaminergic neurons [43-45]. These protocols used FGF-8, SHH and ascorbic acid or stromal cell-derived inducing activity to increase the yield of TH-positive neurons [43-45]. Interestingly, FGF-8 and SHH, known to be involved in dopaminergic differentiation during development in mammals [83,87], are ineffective in generating

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dopaminergic neurons from NSCs [40]. However, these techniques are established for mouse and primate ES cells and there is no report on human ES cells [88].

As yet, there is no report on dopaminergic differentiation of adult NSCs from rodent or human origin. There is evidence for progenitor cells in the adult *substantia nigra pars compacta* in rodent systems, but these precursor cells failed to differentiate into dopaminergic cells [34,35].

Alternatively, stem cells derived from blood, bone marrow or skin may be converted into dopaminergic neurons. Adult stem cells were believed to be lineage restricted, which means that they can only differentiate into the cells of their tissue origin [89,90]. However, there are several reports that these cells can break barriers of germ layer commitment and differentiate *in vitro* and *in vivo* into cells expressing neuronal and glial markers [91-93]. The first reports in 2000 showed that mesodermal cells from rat and human are able to convert into cells with neuronal and glial markers, but no functional tests were reported [94,95]. Recently, Jiang and co-workers showed the differentiation of mesenchymal stem cells (MSCs) not only into mesodermal cells, but also into cells with visceral mesoderm, neuroectoderm and endoderm characteristics *in vitro* and *in vivo* [89,91]. Thirty per cent of the cells expressed markers of dopaminergic neurons, such as dopadecarboxylase and TH [89]. Toma and co-workers found a stem cell in the rodent skin which proliferates and differentiates in culture to produce neurons, glia, smooth muscle cells and adipocytes [92]. Similar precursors that produce neuron-specific proteins following differentiation can be isolated from adult human scalp [92]. Future experiments are warranted to determine whether reprogramming of a committed stem cell (transdifferentiation) or proliferation and differentiation of a more pluripotent stem cell took place in these experiments.

Producing specific cell types from a patient's own stem cells would provide immunological advantages. On the other hand, there is a hypothetical risk that the patient's own cells might carry genetic information which increases their vulnerability to the pathogenetic process underlying PD. Nevertheless, conditions for generating dopaminergic neurons from human adult stem cells need to be optimised before these cells can be considered for transplantation in PD. In addition, adult stem or progenitor cells seem to lack the proliferative capacity of fetal NSCs or ES cells.

4. Cell replacement in PD: transplantation of neural stem cells

PD seems to be a major target for restorative treatment, as only a relatively small number of neurons need to be replaced in a specific target area (striatum) [1,2]. On the other hand, neural transplantation in PD is based on a well-defined biological mechanism: recovery of function by restoration of dopaminergic transmission in the striatum. Moreover, clinical trials using primary dopaminergic neurons provide the proof-of-principle [8-10]. However, transplantation studies in PD or animal models of PD using ES cells or NSCs have three different goals:

- transplantation of such cells are an excellent tool to characterise stem and progenitor cell behaviour *in vivo*
- transplantation studies are performed to explore the regenerative potential of these cells in PD (Table 1)
- NSCs may also be used as vectors for stable transfer of genes with therapeutic relevance to the CNS (reviewed in [52,58])

Many laboratories and companies hope to use NSCs or ES cells to provide tissue for restoration of neuronal deficits via transplantation (see Figure 3 for schematic overview [96]). Stem cells are a promising source for transplantation for large numbers of patients. The *in vitro* storage, expansion and characterisation of neural progenitor cells may help to alleviate the ethical and logistic problems associated with the 'gold standard' of neural transplantation, the use of fresh embryonic tissue. Furthermore, allogenic transplantation into the host brain does not cause severe immune responses [9,10,97]. Studies focusing on the potential of NSCs or ES cells to restore lost cell or tissue function in PD are summarised in Table 1. However, there is no doubt that a lack of acceptance of the grafted cells in the adult brain will result in no (clinical) benefit, even though the production of high amounts of cells would be possible. Finally, it has to be clarified whether the beneficial effects of transplantation of NSCs or their derivatives into the diseased brain are more likely to be from secretion of neurotropic factors, from integration of these cells into the existing neuronal circuitries or from both [98].

4.1 Transplantation of fetal tissue: proof-of-principle for cell replacement in PD

Open and double-blinded clinical trials have demonstrated that transplantation of dopaminergic neurons taken from human fetuses into PD patients shows the survival of the tissue up to 12 years following transplantation and a remarkable ability to replace endogenous degenerating dopaminergic neurons and to ameliorate disease symptoms [9-11,99-104]. So far, > 300 patients worldwide have been transplanted with embryonic ventral mesencephalic neurons. However, in two double-blinded clinical trials, only subpopulations of PD patients (< 60 years of age; < 49 points in the Unified Parkinson's disease Rating Scale [UPDRS] part III) displayed significant improvement of their motor function [9-11]. Furthermore, several studies in animal models of PD and early transplantation studies in PD patients showed poor graft survival in the adult striatum [104-106], which can be improved by addition of several growth factors or antioxidants [105,106]. However, two recent controlled clinical trials reported robust graft survival with normal-appearing reinnervation of the striatum in autopsy cases and normalisation of F-Dopa uptake in the striatum of transplanted PD patients using positron emission tomography technology [9,10]. Taken together, tissue availability, ethical concerns over the use of tissue from aborted

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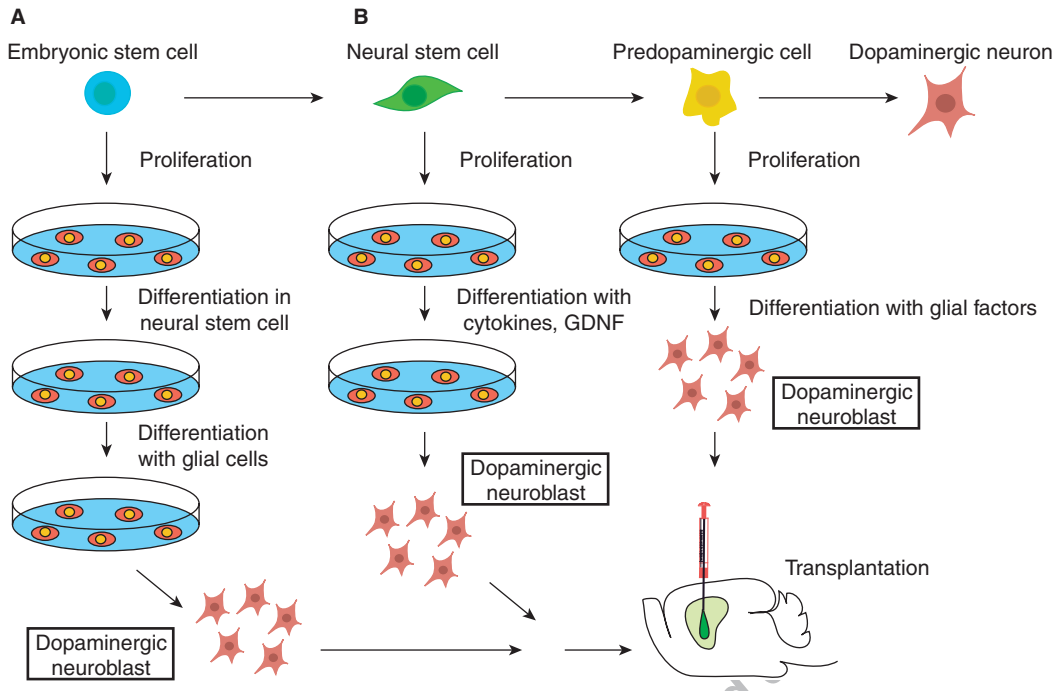

Figure 3. Schematic presentation of different approaches for transplantation strategies in PD. A) ES cells, with a programme including several steps of proliferation and subsequent differentiation in dopaminergic neuroblasts followed by the transplantation procedure. **B**) NSCs, which can be proliferated and differentiated in a similar pattern compared to the ES cells, using cytokines or co-culture techniques. To get a higher yield of dopaminergic neurons in the transplant, it is recommended to select the cells of interest by using surface markers or genetic engineering, with the consequence of having an even more restricted cell. See text for details.
ES: Embryonic stem; GDNF: Glial cell line-derived neurotropic factor; NSC: Neural stem cells; PD: Parkinson's disease.

human fetuses, the need to perform microbiological testing on a tissue that can only be stored for short periods of time, along with the reliability of dopamine neuron content of this tissue, present numerous logistical, ethical and scientific concerns [12,13]. Moreover, 7 – 56% of treated patients developed dyskinesias after short-term removal of dopaminergic drug therapy [9-11]. There is evidence that these side effects might be related to the implantation of dopaminergic neurons not only from the A9 region of the *substantia nigra*, but also from the ventral tegmental area (A10 region). For example, transplanted A9 dopaminergic (AHD2+ and dopamine-D2 receptor+) neurons selectively reinnervate the appropriate motor regions of the affected putamen and correlate better with improved behavioural function in animal models than other dopaminergic neurons [107]. Furthermore, non-A9 dopaminergic neurons have a lower capacity to regulate synaptic dopamine release than A9 dopaminergic cells, which might lead to unstable dopamine levels in the striatum, provoking dyskinesia and other motor side effects [1,4-6].

4.2 Embryonic stem cells

As mentioned earlier, one approach to generate dopaminergic neurons includes differentiation of ES cells into neural stem or precursor cells and subsequently into dopaminergic neurons [43-45]. In contrast to other cell types, they seem to be a reliable source for dopaminergic neurons because of their unlimited proliferative potential. Björklund and co-workers recently reported that transplanting low doses of undifferentiated mouse ES cells into rat striatum results in a spontaneous differentiation of ES cells into mature dopaminergic neurons [108]. This procedure leads to sustained behavioural reconstitution in an animal model of PD. Recently, two research groups showed that a highly enriched population of midbrain NSCs can be derived from mouse ES cells by overexpression of Nurr1 [109,110]. The dopamine neurons generated from these stem cells show morphological, functional and electrophysiological properties expected of neurons from the midbrain after transplantation into the striatum of parkinsonian rats [109]. Full behavioural recovery of transplanted parkinsonian rats was observed, therefore providing evidence that the beneficial effects are coming at least in part from functional integration of grafted cells [109]. However, these techniques are established for mouse and primate ES cells and there is no report on human ES cells [88]. Although these results encourage the use of ES cells in cell replacement therapy for PD, there is the problem of controlling cell growth and differentiation. In most studies using ES cells for transplantation into the brain, tumour or teratoma formation is reported. In addition, one patient with advanced PD died 23 months following intrastriatal transplantation of embryonic mesencephalic dopaminergic neurons [102]. No surviving TH-immunoreactive neurons were found at autopsy. Instead,

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Table 1. Summary of transplantation studies assessing the neuroregenerative potential of stem cells in PD.

Donor cells	Host site (animal model)	Effects	References
ES cells			
Mouse ES cells (undifferentiated)	Adult rat striatum (6-OHDA model)	Normalisation of dopaminergic presynaptic markers; behavioural restoration	[108]
Mouse ES cell-derived neural precursors	Adult mouse striatum (MPTP model)	Integration of TH-positive cells into host striatum	[45]
Mouse ES cells overexpressing Nurr1-derived neural precursors	Adult rat striatum (6-OHDA-model)	Integration of functional dopaminergic neurons into host striatum; behavioural restoration	[109,110]
Embryonic or fetal neural cells			
Human hippocampal precursors	Adult rat striatum (6-OHDA model)	Survival (10% of transplanted cells after 4.5 months); integration and migration	[41]
Rat mesencephalic precursor cells (differentiated into dopaminergic neurons)	Adult rat striatum (6-OHDA model)	Integration of TH-positive cells; normalisation of striatal dopamine content; behavioural (functional) restoration	[16,78,84]
Rat mesencephalic precursor cells (differentiated into dopaminergic neurons)	Adult rat striatum (6-OHDA model)	Development of dopaminergic neurons is pronounced in dopamine-depleted striatum compared with intact striatum	[113]
Human cortical precursors	Adult rat striatum (6-OHDA model)	Solid graft in host striatum 2 – 20 weeks after transplantation; maturation into dopaminergic neurons and restoration of function in a small number of animals	[69]
Human mesencephalic precursors (differentiated into dopaminergic neurons)	Adult rat striatum (6-OHDA model)	Integration of TH-positive cells; partial behavioural (functional) restoration; poor cell survival after long-term proliferation of neural precursor cells	[67,68]
Adult stem cells			
Human retinal dopaminergic stem cells	Adult striatum of PD patients	Survival, dopamine release seen by PET-analysis; long-term improvement of motor function in the UPDRS-Score; no incompatibility reaction	[122]
Marrow stromal cells from mice	Mouse striatum (MPTP model)	Survival of labelled marrow cells in host striatum; some TH-positive cells; some functional benefit	[118]

6-OHDA: 6-Hydroxydopamine; ES: Embronic stem; MPTP: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD: Parkinson's disease; PET: Positron emmission tomography; TH: Tyrosine hydroxylase; UPDRS: Unified Parkinson's disease Rating Scale.

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pieces of bone, hair, cartilage and squamous epithelium were evident at multiple sites in the ventricles. Death was probably caused by obstruction of the fourth ventricle with secondary brainstem compression due to growth from the cell suspension implant [102]. The risk of tumour formation is a problem, particularly of ES and immortalised cell sources.

4.3 Embryonic/fetal neural stem cells

The possibility of propagating and differentiating NSCs in culture makes this cell type an attractive cell source for reconstructive transplantation strategies in PD. Numerous *in vivo* transplantation studies addressing the fate and potential of neural stem or precursor cells have been performed. These studies indicate the existence of both pluripotent and unipotent NSCs and suggest that interactions between implanted cells and the host tissue play a role in the determination of glial and neural cell fate. Furthermore, different treatment of NSCs *in vitro* can significantly alter their behaviour after implantation at different host sites. Interestingly, NSCs display extensive tropism for pathology in adult brain [111], supporting the hypothesis that NSCs play an important role in neuroregeneration in the adult brain. For more details, see [28,112].

Several studies demonstrated that implantation of precursor-derived dopaminergic neurons from rodents leads to histological, biochemical and functional recovery in animal models of PD [16,67,68,78,84,113], but only very few studies used precursors after long-term expansion [84]. The authors recently demonstrated long-term expansion of human predopaminergic mesencephalic NSCs [50]. After transplantation into a rat parkinsonian model, these cells produced histological and functional recovery (Schwarz, unpublished observations).

Therefore, the authors' long-term culture system fulfils all important requirements for the use of these cells in regenerative treatment strategies:

- generation of high yields of cells from a small starting population without losing their differentiation potential
- on-demand availability of cells without major logistical problems
- the possibility to standardise the cell source in a clinical setting

Another very interesting area is the use of NSCs for stable transfer of foreign genes or application of growth factors in the CNS. These cells could be transfected not only with various viral vectors [114,115], but also with standard transfection techniques, such as lipofection or calcium-phosphate precipitation, reducing the risk of viral infection of the host. Furthermore, NSCs display extensive migratory capacity and the ability to integrate throughout the brain after implantation into germinal zones or after intravenous administration, with an extensive tropism to pathology within the brain [111,116]. Finally, NSCs migrate even more readily in the aged brain, even without overt lesioning, than in the young adult intact brain [98]. Thus, NSCs might be used to restore enzyme or cellular deficiencies in a global fashion in the aged brain or preferentially in diseased areas of the brain.

In contrast to ES and immortalised cell sources, tumourigenicity seems to be a minor problem with fetal or adult stem cells. Although these cells are less flexible with regards to multiplication and differentiation, there is increasing evidence that it is more beneficial to use cells that are already committed to becoming a particular cell type (neuronal versus glial cells). As Han and colleagues showed in the adult spinal cord [117], transplantation of cells already committed to become neurons integrate as neurons even in a non-neurogenic CNS environment, which prevents the transition of multipotential NSCs to the neuronal commitment stage by directing uncommitted precursors towards glial differentiation. Furthermore, long-term survival of dopaminergic neurons generated from stem cells must also be demonstrated in experimental models of PD. Finally, it should be demonstrated in experimental animal models of PD that stem cells intended for use in human transplantation studies have an effect similar to that seen for fetal mesencephalic dopamine neurons.

4.4 Adult stem cells

Although there are several reports about the plasticity of adult NSCs in transplantation studies [65], or even the existence of NSCs in the adult *substantia nigra pars compacta* [34,35], there is no report on transplantation of adult NSCs in animal models of PD. Looking at alternative adult cell sources, Li and colleagues give impetus for the therapeutic potential of MSCs for the treatment of PD [118]. MSCs prelabelled with 5-bromo-2'-deoxyuridine (BrdU) were grafted into the striatum of parkinsonian (MPTP-treated) mice. The grafted mice exhibited a significant improvement in the rotarod test at 35 days post-transplant compared to non-grafted controls. Immunohistochemistry revealed BrdU reactive cells in the striatum of the grafted animals at least 4 weeks after transplantation. Scattered BrdU-reactive cells expressed TH-immunoreactivity [90,118].

5. Endogenous stem cells: regeneration without transplantation?

Regeneration and neurogenesis in the adult mammalian brain is a long recognised phenomenon [15,30-32,60-62]. It has been shown that cells in the adult subependymal layer divide and migrate several millimetres to the olfactory bulb, where they differentiate into neurons [30,119]. These data demonstrate that adult neural precursors are at least in part associated with neuronal reconstitution, but targeted recruitment of endogenous cells in other brain regions remain to be shown. However, there are data suggesting that endogenous neural precursor cells are amenable to external modulation: intraventricular treatment of adult mice with EGF increased proliferation and migration of subependymal cells [120]. Therefore, another regenerative approach could be to replace neuronal densities and circuitry by stimulation of endogenous stem cells. Such cells may be derived from persisting stem cells in the brain or from tissue infiltrating the brain, which are likely to originate from bone marrow. Recently, some cells in the adult brain have been reported to retain their proliferative potential and their ability to differentiate into glial and neuronal cells [72,73]. Most reports showed progenitors in the two major neurogenic regions, the hippocampus and the SVZ [14,15,23,24,30,31], but there are several recent reports which also show proliferating precursors in non-neurogenic regions, such as the spinal cord and the cortex [32,33,36,37] or the adult *substatia nigra* [34]. Zhao and colleagues showed evidence for a continuous turnover of dopaminergic cells in the adult *substantia nigra pars compacta* and, in addition, these newly produced neurons seem to establish connections to the striatum [35]. These recent reports are interesting, as they provide the proof-of-principle for regeneration of dopaminergic neurons by *in vivo* neurogenesis. However, these findings must not be overestimated, as the provided amount of newly generated neurons is almost negligible compared with the two major regions of neurogenesis. Another crucial point is the role of the graft in evoking regeneration and neuroprotective mechanisms in the host. Ourednik and colleagues recently found intrinsic ability of NSCs to induce rescue and regeneration of impaired dopaminergic neurons in the aged host [98]. Stimulation with molecules that govern proliferation and dopaminergic differentiation would, therefore, dramatically change the therapeutic spectrum of PD.

6. Conclusion

The data summarised here suggest that:

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- controlled production of dopaminergic cells in sufficient amounts is technically possible
- adult brain tissue shows, at least in part, a regenerative potential that is comparable to early stages of development

Comparing the different sources of NSCs, ES cells might have the greatest potential, concerning either their proliferative or differentiation capacities. However, these same features also cause immense problems, such as uncontrolled proliferation and differentiation with subsequent tumour formation. Therefore, ES cells are not feasible for neuroregenerative strategies in humans until these problems are reliably resolved. There is no report of tumour formation using fetal or adult NSCs, surely in part due to their limited proliferation and differentiation potentials. However, several studies showed long-term proliferation and subsequent differentiation of fetal NSCs in functional dopaminergic neurons, therefore providing an on-demand source for transplantation strategies [50,79,84]. On the other hand, there is increasing evidence that it is more beneficial to use cells that are already committed to becoming a particular type of cell (neuronal versus glial) or are derived from the same brain region they are being transplanted into [117]. Finally, the proliferation and differentiation capabilities of adult NSCs as well as multipotent adult stem cells, such as mesenchymal stem cells, are poorly understood, and further studies are warranted to improve their *in vitro* expansion and functional dopaminergic differentiation.

7. Expert opinion

Developing NSC technology represents a prime research interest with profound economic potential, not only concerning the use of stem cells as a source of dopaminergic neurons for transplantation in PD, but also for studying the development and degeneration of human dopaminergic neurons. This approach might identify new targets for novel pharmacological substances for neuroprotective and neurorestorative treatments of PD. However, in the CNS, the complexity of the tissue as well as the limited knowledge of the mechanisms of neuronal integration have restricted therapeutic efforts to reconstitute local, well-characterised neurotransmitter deficits, such as restoring the nigrostriatal dopaminergic system in PD [2,99,100]. In future studies, the following issues for regenerative strategies in PD need to be addressed:

- Will it be feasible to generate large quantities of cells that will reliably differentiate into specific human neurons with properties of the highly specialised dopaminergic neurons of the A9 region of the *substantia nigra pars compacta*?
- Is there any chance that transplanted NSCs may give rise to tumours?
- Which is the best way to administer the stem cells: intracerebral, intraventricular or intravenous transplantation?
- Which is the best cell stage for transplantation: stem cell, committed precursor or premature neuron?
- Are the beneficial effects of transplantation of new cells into the brain more likely come from secretion of neurotropic factors than integration of new cells into the existing circuitries [98]?
- What is the immune response following intracerebral implantation?
- What is the potential of alternative cell sources, such as retinal stem cells or mesenchymal stem cells?

The paramount goal of stem cell research, in the authors' view, is to regenerate neuronal densities and circuitry by stimulation of endogenous stem cells. However, their *in vivo* and *in vitro* behaviour is still poorly understood. In the authors' opinion, the cell sources being closest to use in neuroregenerative therapies at present are fetal or adult human NSCs, providing long-term expansion and subsequent differentiation in functional dopaminergic neurons without showing any tumour formation. Future therapeutic approaches using stem cell therapy may also use a combination of NSCs, neurotropic factors, genetic manipulations and transplantation techniques [98,121]. Alternatively, progenitors may be used to introduce tropic support or neuroactive substances into specific brain regions.

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STAMMZELLBASIERTE REGENERATIVE THERAPIE DES MORBUS PARKINSON

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Einführung

Der Morbus Parkinson oder das idiopathische Parkinson-Syndrom (IPS) ist eine chronisch-progressive neurodegenerative Bewegungsstörung, die durch einen kontinuierlichen und selektiven Verlust von dopaminergen Nervenzellen in der *Substantia nigra pars compacta* mit einer nachfolgenden Reduktion des Neurotransmitters Dopamin, insbesondere im Striatum, gekennzeichnet ist. (1, 2) Dieser fortschreitende Verlust der nigralen dopaminergen präsynaptischen Neuronen führt zu den Symptomen wie Rigor, Tremor und Bradykinese. Die Selektivität des Zelluntergangs beim Morbus Parkinson, die im Wesentlichen auf die dopaminergen Neuronen in der Substantia nigra pars compacta (Area A9) beschränkt ist, macht den Ersatz dieser Nervenzellen außerordentlich attraktiv. In ersten klinischen Studien zur dopaminergen Zellersatztherapie bei Patienten mit IPS wurde dopaminproduzierendes autologes Gewebe des Nebennierenmarks verwendet. Nach der Implantation dieses Gewebes konnten allerdings keine anhaltenden klinischen Effekte beobachtet werden. Nachfolgende Studien mit allogenem fetalem oder embryonalem mesenzephalem Gewebe konnten zunächst in zahlreichen Tierversuchen eine vollständige Kompensation experimenteller Läsionen der Substantia nigra zeigen. Aus diesem Grund wurden bereits Mitte der 80er Jahre des 20. Jahrhunderts intrastriatale Transplantationen von humanem dopaminergem Mittelhirngewebe bei IPS-Patienten in offenen klinischen Anwendungen durchgeführt. Während initial nur das Gewebe eines Spenders verwendet wurde, zeigte sich später der Vorteil der Implantation von Gewebe von mindestens 4 Mittelhirnen pro Gehirnhälfte. Die erheblichen ethischen und klinischen Probleme bei der Gewinnung des Gewebes verhinderten bisher jedoch den großzügigen Einsatz dieser Therapie. Insgesamt sind jedoch inzwischen mehr als 400 Patienten mit embryonalem humanem Mittelhirngewebe behandelt worden. Das Anwachsen der Transplantate konnte durch Aufnahme von radioaktivmarkiertem L-Dopa (Fluorodopa) und der Positronen-Emissions-Tomographie (PET) nachgewiesen werden. In diesen offenen Studien wurden überwiegend lang anhaltende Effekte auf alle Parkinson-Symptome und eine deutliche Reduktion der dopaminergen Medikation beim fortgeschrittenen IPS berichtet. (1) Zwei aktuelle doppelblinde plazebokontrollierte Transplantationsstudien belegen das „*proof-of-principle*" dieser Zellersatzstrategie beim IPS, trotzdem ist diese Technik zum generellen Einsatz nicht geeignet (3, 4): Zwar

werden durch die funktionelle Bildgebung und neuropathologische Untersuchungen die initialen offenen Studien bestätigt und eine Integration der dopaminergen Nervenzellen des Transplantats im Striatum des Patienten demonstriert. (3, 4) In den kontrollierten Studien konnten jedoch nur bei Subpopulationen von Patienten (Alter < 60 Jahre in der ersten Studie; UPDRS Part III ≤ 49 in der zweiten Studie) signifikante Verbesserungen der klinischen Parameter beobachtet werden. (3, 4) Zusätzlich sind bei 7-56 % der Patienten unwillkürliche Dyskinesien im definierten Off-Zustand berichtet worden, deren Ursachen bisher nicht vollständig verstanden sind. (3-5) Insgesamt ergeben sich bei der klinischen Verwendung von humanen Zellen oder Geweben erhebliche wissenschaftliche, logistische und ethische Probleme, die durch die Verwendung von embryonalem fetalem Gewebe noch potenziert werden. Die Entwicklung von Techniken zur Expansion von Vorläufer- oder Stammzellen könnte eine Lösung dieser Probleme bedeuten. Die vorliegende Übersicht diskutiert die neuen Erkenntnisse zur Stammzelltechnologie mit besonderer Berücksichtigung der Zellersatztherapie bei Morbus Parkinson und insbesondere auch der Verwendung von adulten Stammzellpopulationen.

Grundsätzlich sind zwei prinzipielle Strategien in der Entwicklung von regenerativen Therapien beim Morbus Parkinson mittels Stammzelltechnologie denkbar (6):

1. Der Ersatz der verloren gegangenen dopaminergen Zellpopulation durch Transplantation der entsprechenden Zellen
2. Die Rekrutierung endogener neuraler Stamm- oder Progenitorzellen innerhalb des adulten erkrankten Gehirnareals, beim Morbus Parkinson der Substantia nigra pars compacta

Für die stammzellbasierte Neurotransplantation beim Morbus Parkinson stehen folgende Zelltypen zur Verfügung (Abb. 1): Embryonale Stammzellen (ES-Zellen), fetale und adulte gewebsspezifische neurale Stammzellen (NSZ) aus dem zentralen Nervensystem sowie konvertierte und/oder differenzierte humane adulte Knochenmarks-Stammzellen (MSZ). (6) Von adulten MSZ oder multipotenten adulten Progenitorzellen (MAPZ) wurde bisher angenommen, dass sie auf ihren Zelltyp bzw. ihre Keimblattzelllinie determiniert sind; in der letzten Zeit wurden jedoch eine Vielzahl von Studien publiziert, die belegen, dass diese Zellen ihre Keimblattdifferenzierung überwinden können und in Zellen mit neuronalen und glialen Eigenschaften differenzieren können. (7, 8, 13-16) Im Folgenden werden die einzelnen Zelltypen und ihr regeneratives Potential beim IPS diskutiert.

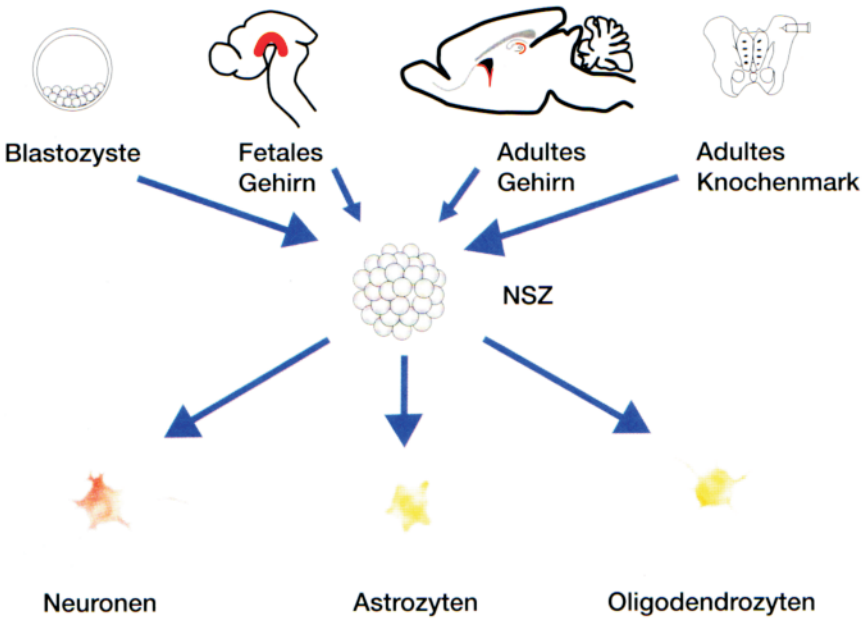

Abb. 1: Schematische Übersicht über die verschiedenen Quellen neuraler Stammzellen (NSZ) mit der Kapazität in alle wesentlichen Zelltypen des zentralen Nervensystems zu differenzieren, also in Astroglia, Oligodendroglia und Neuronen. Die meisten Protokolle generieren NSZ, die in Neurophären wachsen. Im fetalen Gehirn ist das ventrale Mittelhirn als Region zur Gewinnung der prädopaminergen NSZ rot hervorgehoben, im adulten Gehirn sind die neurogenen Regionen (Hippocampus und SVZ) rot markiert.

Embryonale Stammzellen

ES-Zellen weisen wesentliche Charakteristika einer optimalen Zellquelle für Zellersatztherapien auf. (17, 18) ES-Zellen sind selbst erneuernde totipotente Stammzellen, die aus der inneren Masse der Blastozyste gewonnen werden können. (19) Diese ES-Zellen können in neurale Stamm- oder Vorläuferzellen und nachfolgend in funktionelle dopaminerge Neuronen differenziert werden. (11, 20, 21) Zwei unabhängige Arbeitsgruppen konnten eine hoch reine Population von Mittelhirnstammzellen und dopaminergen Neuronen durch die Überexpression des dopaminergen Differenzierungsfaktors Nurr1 generieren. (10, 12) Die aus diesen ES-Zellen gewonnenen dopaminergen Neuronen zeigen alle morphologischen, funktionellen und elektrophysiologischen Eigenschaften dopaminerger Zellen aus dem Mittelhirn. (11, 20, 21) Sie integrieren sich funktionell in das Striatum von 6-Hydroxydopamin-Ratten und führen zu einer funktionellen und histologischen Rekonstitution in diesem Parkinson-Tiermodell. (10) Diese Techniken sind insbesondere für ES-Zellen aus der Maus und nichthumanen

Primaten etabliert. (21) Bei humanen ES-Zellen konnte die Differenzierung in dopaminerge Nervenzellen ebenfalls demonstriert werden, in den Parkinson-Tiermodellen zeigt sich jedoch bisher ein Verlust des dopaminergen Phänotyps nach deren Transplantation in das Striatum. Insgesamt sind die Ergebnisse mit den ES-Zellen in der Zelltherapie des IPS sehr viel versprechend, jedoch besteht ein erhebliches Problem in der Kontrolle des Zellwachstums und der Differenzierung. In vielen Studien zur intrazerebralen Transplantation von ES-Zellen zeigte sich nach Transplantation ein Hirntumor bzw. Teratom an der Implantationsstelle.

Embryonale und fetale neurale Stammzellen

Im Unterschied zu ES-Zellen zeigen embryonale oder fetale gewebsspezifische neurale Stammzellen (NSZ) ein stärker eingeschränktes Differenzierungspotential. (18,22-28) Sie können im Wesentlichen in Zelltypen des zentralen Nervensystems, also Neuronen, Oligodendrozyten und Astrozyten differenzieren. Mitte der 80er Jahre des letzten Jahrhunderts konnten Gensburger und Mitarbeiter zeigen, dass durch die Inkubation mit *fibroblast growth factor-2* (FGF-2) eine neurale Vorläuferzellpopulation aus dem embryonalen Hippocampus expandiert werden kann. (29) Später zeigte sich ein erheblicher Expansionseffekt durch *epidermal growth factor* (EGF) auf die NSZ. (30) Unter geeigneten Bedingungen differenzieren diese NSZ *in-vitro* und *in-vivo* in funktionelle Neuronen, in der Mehrzahl GABAerge Neuronen, Oligodendrozyten mit der Fähigkeit zur Myelinbildung und Astrozyten. (18, 22-28) Die Induktion des dopaminergen Phänotyps oder noch besser des mesenzephalen (A9) dopaminergen Phänotyps ist eine wesentliche Voraussetzung für eine erfolgreiche Zellersatztherapie beim IPS. Aufgrund der sehr frühen rostrokaudalen und dorsoventralen Differenzierung des Säugetiergehirns, zeigen NSZ und andere determinierte ZNS-Vorläuferzellen eine erhebliche Spezialisierung bzw. Regionalisierung. Dementsprechend konnten bisher nur NSZ aus dem Mittelhirn in funktionelle dopaminerge Neuronen differenziert werden. (25, 26, 31, 32) Diese Mittelhirnvorläuferzellen aus dem fetalen und embryonalen Gehirn von Maus, Ratte und Mensch können isoliert und über lange Zeit *in-vitro* expandiert werden, so dass große Zellmengen aus geringem Ausgangsmaterial generiert werden können. (25, 31) Ling und Mitarbeiter demonstrierten, dass Interleukin-1 (IL-1) einen dopaminergen Phänotyp in langzeit-expandierten NSZ aus der Ratte induzieren kann. (25) Diese Zellen zeigen alle morphologischen und funktionellen Eigenschaften von ausdifferenzierten dopaminergen Nervenzellen, so produzieren sie den Neurotransmitter Dopamin und schütten diesen kaliumabhängig aus. (31) Weiterhin exprimieren sie Natriumkanäle und hyperpolarisationsinduzierte Kationenkanäle (Ih-Kanäle). Ähnliche Ergebnisse konnten wir mit mesenzephalen NSZ aus humanem Material nach einer Expansion von bis zu 12 Monaten erhalten. (31) Eine Vielzahl von Studien demonstriert, dass die Implantation

von dopaminergen Neuronen aus mesenzephalen NSZ aus Ratte und Maus zu einer histologischen, biochemischen und funktionellen Rekonstitution in Tiermodellen des IPS führt. (18, 35-39) Nur sehr wenige dieser Studien verwendeten jedoch Vorläuferzellen nach einer Langzeitexpansion, die für eine klinische Anwendbarkeit aus logistischen und wissenschaftlichen Gründen unerlässlich ist. Wir konnten die erfolgreiche Transplantation von dopaminergen Neuronen aus langzeitexpandierten humanen mesenzephalen NSZ in einem Parkinson-Modell bei der Ratte demonstrieren (40): So konnten wir sowohl histologische als auch funktionelle Verbesserungen in diesem Modell zeigen. Dabei war die funktionelle Rekonstitution von der Anzahl der überlebenden TH-positiven dopaminergen Nervenzellen im Wirtsstriatum abhängig. Bisher konnten weder ein Tumorwachstum noch eine immunologische Abstoßungsreaktion der NSZ beobachtet werden. Aus diesem Grund erfüllen die humanen fetalen mesenzephalen NSZ wesentliche Voraussetzungen für die Verwendung dieser Zellen in der regenerativen Therapie des IPS:

1) Die Generierung einer großen Zellmenge aus einer kleinen Startpopulation ist ohne Verlust des Differenzierungspotentiales möglich.
2) Diese Zellen sind ohne wesentliche logistische Probleme zu jeder Zeit erhältlich.
3) Dieser Zelltyp ist unter standardisierten Bedingungen auch in einer klinischen Studie verwendbar (mögliche Qualitätskontrolle; Herstellung unter GMP).

Wesentliche offene Fragen bei der Verwendung der NSZ bestehen im Langzeitüberleben der dopaminergen Nervenzellen im Wirtsstriatum, der funktionellen Integration im Langzeitverlauf, der immunologischen Reaktion auf das Überleben und die Funktion der Zellen und die Entstehung von Dyskinesien nach Transplantation.

Adulte neurale Stammzellen aus dem zentralen Nervensystem

Multipotente adulte gewebsspezifische NSZ mit dem Potential zur Differenzierung in alle neuralen Zelllinien einschließlich astroglialer, oligodendroglialer Zellen und Neuronen konnten konsistent im Hippocampus und der subventrikulären Zone (SVZ) der lateralen Ventrikel nachgewiesen werden. (41-43) Diese Zellen wachsen in so genannten „Neurosphären" in Anwesenheit von EGF und/oder FGF 2 (44-48). Bisher konnte jedoch nicht schlüssig gezeigt werden, dass adulte NSZ in dopaminerge Neuronen differenzierbar sind. (49, 50) Dementsprechend existiert meines Wissens kein Bericht über die Transplantation von adulten NSZ in Tiermodelle des Morbus Parkinson.

Adulte multipotente Stammzellen

Knochenmarksstromazellen (MSZ) oder mesenchymale Stammzellen von Erwachsenen können durch ihre selektiven Adhäsionseigenschaften zu Plastikoberflächen isoliert und sehr effektiv expandiert werden. MSZ besitzen die Kapazität in multiple mesodermale Gewebe einschließlich Knochen, Knorpel, Fett und Muskel zu differenzieren. (51) In einigen Untersuchungen konnte gezeigt werden, dass diese Zellen ihre Keimblattdeterminierung überwinden und ebenso in Zellen differenzieren können, die neuronale und gliale Marker exprimieren. (13-15) Funktionelle Eigenschaften neuraler Zellpopulationen konnten jedoch nicht demonstriert werden. Jiang und Mitarbeiter konnten zeigen, dass eine seltene multipotente adulte Progenitorzelle (MAPC) innerhalb der Knochenmarkszellsuspension aus der Maus vorkommt. (7) Dieser Zelltyp kann nicht nur in mesodermale Zellreihen, sondern ebenso in endotheliale und endodermale Zelltypen ausdifferenzieren. (7, 8) In einem Kokultursystem mit Astrozyten können diese Zellen in neuronale Zellen mit allen wesentlichen biochemischen, morphologischen und elektrophysiologischen Charakteristika von Nervenzellen differenzieren. (8) Wir selbst konnten eine Technologie entwickeln, mit der adulte humane MSZ in eine klonogene NSZ-ähnliche Zellpopulation konvertiert werden können. Diese wachsen in Neurosphären, können *in-vitro* in Zellen mit morphologischen und funktionellen Charakteristika von Nervenzellen, astroglialen und oligodendroglialen Zellen differenziert werden. Diese NSZ aus den humanen MSZ exprimieren die typischen NSZ-Markerproteine Nestin und Musashi1, sowie verschiedene proneurale Gene, wie Neuro D, Neurogenin 2 und Otx2. (9) Kürzlich konnten Dezawa und Mitarbeiter zeigen, dass durch die Expression der intrazellulären Notch-Domäne eine Konvertierung von humanen MSZ in funktionelle Nervenzellen erfolgen kann. (52) Unter Stimulation dieser Zellen mit *glial cell line-derived neurotrophic factor* (GDNF) sind dort funktionelle dopaminerge Nervenzellen beschrieben. (52) Nach Transplantation dieser Zellen in ein Parkinson-Tiermodell konnte eine funktionelle und histologische Rekonstitution gezeigt werden. Diese Ergebnisse sind jedoch bisher nicht reproduziert worden.

Zusammenfassung und Schlussfolgerungen

Die hier diskutierten Daten zeigen, dass die kontrollierte Produktion von dopaminergen Zellen in großer Menge aus verschiedenen Stammzellpopulationen für die Zellersatztherapie (Neurotransplantation) beim IPS technisch möglich ist. ES-Zellen haben wahrscheinlich das größte Potenzial in der Zellersatztherapie des Morbus Parkinson, zum einen durch ihre ausgesprochen hohe proliferative Potenz, zum anderen durch ihre Kapazität in funktionelle dopaminerge Neuronen zu differenzieren. Die wesentliche Aufgabe wird sein, die Entwicklung von Tumoren/Teratomen an der

Implantationsstelle bei ES-Zelltransplantationen vollständig zu verhindern. Im Gegensatz dazu sind bisher keine Tumorformationen in Tieren nach Transplantation von adulten oder fetalen gewebsspezifischen NSZ beschrieben. Auf der anderen Seite ist jedoch das Poliferationspotential dieser Zellen eingeschränkt. Trotzdem ist eine hohe Zahl von funktionellen dopaminergen Nervenzellen aus neuralen Stammzellen verfügbar. Das regenerative Potential von adulten neuralen Stammzellen oder neuralen Stammzellen aus Knochenmarksstammzellen beim IPS kann derzeit noch nicht abgeschätzt werden. Besonders attraktiv sind diese Zellen jedoch aufgrund der Möglichkeit einer autologen Transplantationsstrategie. Kürzlich konnten mehrere Studien erste Hinweise auf neurale Stamm- oder Progenitorzellen in der adulten Substantia nigra zeigen (50, 53), so dass die zielgerichtete Rekrutierung dieser Zellen für eine *in-vivo*-Regeneration beim Morbus Parkinson möglich erscheint. Zukünftige Studien müssen die exakten molekularen Mechanismen für die Proliferation und dopaminergen Differenzierung dieser adulten prädopaminergen Stamm- bzw. Vorläuferzellen dargestellt werden, um dadurch potentielle „*drug targets*" für die endogene Regeneration beim Morbus Parkinson zu definieren.

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Eidesstattliche Erklärung

Ich versichere hiermit, dass ich die Arbeit selbständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt sowie die wörtlich oder inhaltlich übernommenen Stellen als solche kenntlich gemacht habe.

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