In vitro Differentiation of Human Embryonic Stem Cells into Precursors of the Central Nervous System

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CORINNE ISABEL BENZING

aus Mainz

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1. Referent: Prof. Dr. Oliver Brüstle
2. Referent: Prof. Dr. Michael Hoch

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Abbreviations

°C	Degree celsius
A	Anterior
A.0	Adherent stage 0
A.1	Adherent stage 1
A.2	Adherent stage 2
AA	Ascorbic acid
BLBP	Brain lipid binding protein
BMP	Bone morphogenetic protein
bp	Basepair
cDNA	Complementary DNA
ChAt	Choline Acetyltransferase
CMV	Cytomegalovirus
CNPase	2',3'-cyclic nucleotide 3'-phosphodiesterase
CNS	Central nervous system
CP	Cortical plate
D, d	Day
DAPI	4’,6-diamidino-2-phenylindole
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
dNTPs	Desoxynucleosid-triphosphate mix
DTT	Dithiotreitol
E.	Embryonic day
EB	Embryoid body
EB/SR	Embryoid body medium containing Serum Replacement
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
eGFP	Enhanced green fluorescent protein

EMA	Epithelial membrane antigen
ES cells	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FGF	Fibroblast growth factor
Fig.	Figure
g	Gram
GC	Granular cell layer
GDF	Growth differentiation factor
GFAP	Glial fibrillary acidic protein
GLAST	Astrocyte-specific glutamate transporter
h	Hour
H&E	Hematoxylin and Eosin
HRP	Horse raddish peroxidase
Hz	Hertz
ICM	Inner cell mass
IGF	Insulin-like growth factor
ITSFn	Medium containing insulin, transferrin, sodium-selenite and fibronectine
Kan	Kanamycin
KO/SR	Medium containing KO-DMEM and Serum Replacement
l	Liter
LIF	Leukemia inhibitory factor
M	Molar
MAP	Microtubule-associated protein
MEF	Mouse embryonic fibroblasts
mg	Milligram
min	Minute
mM	Millimolar
mV	Millivolt
MZ	Mantle zone
N3FL	Neural differentiation medium for murine ES cells

NAA	Neural differentiation medium containing ascorbic acid
Neo	Neomycin
ng	Nanogram
NGS	Normal goat serum
NIH	National Institute of Health
nM	Nanomolar
NRS	Normal rat serum
P	Posterior
P	Promoter
P.	Postnatal day
P.0	Precursor cell population 0
P.1	Precursor cell population 1
P.5	Precursor cell population 5
pA	Picoampere
PBS	Phosphate-buffered saline
PC	Pyramidal cell layer
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PO	Poly-ornithine
PSA-NCAM	Polysialylated neural cell adhesion molecule
RA	Retinoic acid
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Reverse transcriptase
rt	Room temperature
SDIA	Stromal cell-derived inducing activity
sec	Second
SMA	Smooth muscle actin
SR	Serum Replacement
SSEA	Stage-specific embryonic antigen
SV40	Simian virus 40

SVZ	Subventricular zone
TEA	Triethanolamine
TGF	Transforming growth factor
TH	Tyrosine hydroxylase
Tra	Tumor-related antigen
UV	Ultraviolet
VZ	Ventricular zone
µm	Micrometer
µM	Micromolar

1 Introduction

A stem cell is a unique cell type with the ability of substantial self-renewal and the potential to differentiate into various cell types. Somatic stem cells differ from their pluripotent counterpart mainly in two aspects. They are tissue-specific and have only limited self-renewing capacities as well as a restricted differentiation potential. In contrast, pluripotent stem cells can self-renew in unlimited numbers and give rise to all three germ layers. This includes embryonic stem cells (ES cells) and embryonic germ cells. Due to these features, ES cells have become a promising source for future therapeutic approaches and can serve as a model system for developmental processes. One key prerequisite for both applications are defined conditions for the differentiation of ES cells into specific somatic cell types.

In particular, the generation of cells of the central nervous system (CNS) is of broad interest. The human CNS is characterized by unsurpassed complexity and a very limited capacity for regeneration. For that reason, defined conditions for the generation of human ES cell-derived neural precursors may build the foundation for a variety of applications.

First of all, enriched ES cell-derived neural precursor cells are promising candidates for future transplantation strategies, due to their potential to differentiate into all three neural lineages i.e. neurons, astrocytes and oligodendrocytes. Experiments in rodents have already demonstrated that neural precursors are able to migrate and differentiate after transplantation into the host tissue (Kim *et al.*, 2002; Scheffler *et al.*, 2003; Wernig *et al.*, 2004).

Another challenging application for human ES cell-based neural differentiation protocols is basic research. Human ES cells provide the opportunity to study developmental events that, due to ethical reasons, cannot be studied in the living human. Thus, defined conditions for human ES cell differentiation have to be established. In the case of neural differentiation, this may help to uncover early processes underlying the development of the human CNS.

1.1 Early developmental processes in the vertebrate CNS

Fundamental knowledge about early developmental processes in vertebrates has come from studies on the clawed frog *Xenopus laevis*. The early *Xenopus* embryo is divided into three germ layers: ectoderm, mesoderm and endoderm. During gastrulation, the dorsal mesoderm involutes between endoderm and ectoderm (Fig. 1.1). The area where invagination starts is called the dorsal or blastopore lip. The ventral part of the ectoderm gives rise to epidermis, the dorsal part generates the neuroectoderm. Generation of the neuroectoderm depends on inductive signals from the underlying mesoderm. With the onset of neurulation, neuroectoderm folds into the neural plate and subsequently into the neural tube. This primary structure consists of neural stem cells and gives rise to the central nervous system including brain and spinal cord.

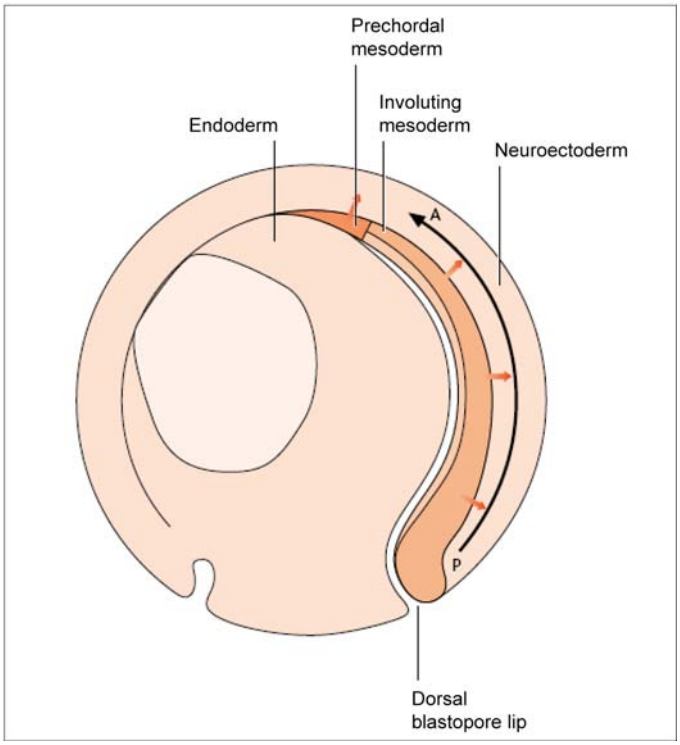

Fig. 1.1: Early gastrula stage *Xenopus* embryo

Sagittal section. During early gastrulation, mesoderm invaginates between endoderm and ectoderm, beginning at the blastopore lip. The involuting mesoderm induces the dorsal ectoderm to acquire a neuroectodermal fate (red arrows). The prechordal mesoderm induces the neural plate and gives later rise to the chordamesoderm. P=posterior, A=anterior. Adapted from Itasaki (2002).

1.1.1 Neural induction

In the 1920's, Spemann and Mangold performed ground breaking experiments on *Xenopus laevis* embryos through investigating the onset of neural differentiation, the so-called neural induction (1924). They were able to demonstrate that the dorsal blastopore lip of a gastrula stage embryo induces a second axis when transplanted to the ventral side of a host embryo. As the generated neuroectoderm is derived from the host tissue, they concluded that the generation of neural tissue is induced by factors secreted from this region – the so-called 'organizer' (reviewed by Harland and Gerhart, 1997). They considered the onset of neural differentiation as a result of early interaction processes between mesodermal and ectodermal tissue during gastrulation.

Inspired by these findings, many groups focused on the identification of neural-inducing factors. Hemmati-Brivanlou and Melton (1997) found blocking of the transforming growth factor-ß (TGF-ß)-receptor-mediated bone morphogenetic protein (BMP)-signaling pathway to be sufficient to directly induce neural tissue without the presence of mesoderm. The inhibition of the BMP-pathway, normally leading to epithelial differentiation, results in neural differentiation of ectodermal cells.

Interestingly, findings from *Xenopus* embryos showed that neuroectodermal tissue could also be generated without the existence of an organizer or additional neural-inducing factors. If the presumptive ectoderm is explanted and cultivated with cells of the dorsal blastopore lip, neural tissue will develop. Without cocultivation only epidermis will form. On the other hand, repeated dissociation of the explanted ectodermal tissue without cocultivation results in the generation of cells expressing neural markers (Godsave and Slack, 1989; Grunz and Tacke, 1989; Sato and Sargent, 1989).

Together these results led to the 'default model' of neural induction (Fig. 1.2, Munoz-Sanjuan and Brivanlou, 2002). The model postulates neural differentiation as a result of non-activation of specific signal transduction pathways. Thus, cells of the embryonic ectoderm would differentiate into neuroectoderm if signals from their cellular environment were completely absent. Conversely, this theory implicates that epithelia, as the other possible fate of ectoderm, has to be actively induced. Earlier experiments even demonstrated, that all embryonic tissues seem to have a neural fate in the absence of inhibiting signals (Eggen and Hemmati-Brivanlou, 2001).

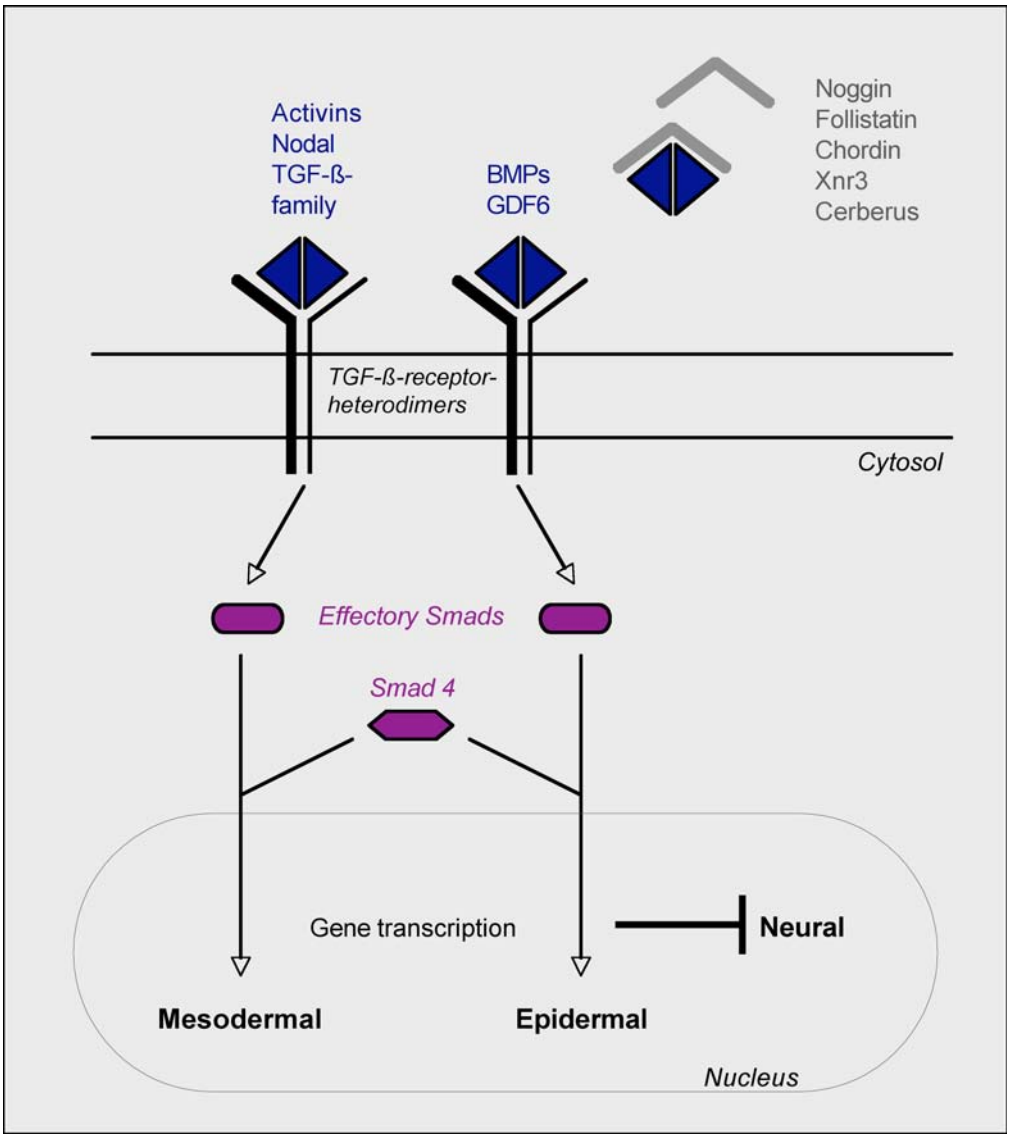

Fig. 1.2: The default model of neural induction

The acquisition of a neural fate as postulated by the default model (simplified scheme). In *Xenopus*, activation of the BMP-pathway leads to mesodermal respectively epithelial fate. After binding of a ligand to the TGF-ß-receptor, intracellular effectory Smad-proteins bind to Smad4 and translocate into the nucleus. They associate with various transcription factors and induce mesodermal or epithelial gene transcription. In the absence of BMP signaling, cells acquire a neural identity. From an extracellular route, neural differentiation can either be promoted by BMP-inhibitors, or by a strong dilution of the BMP ligands. Various additional factors modulate the BMP-pathway in the cytosol and in the nucleus.
Adapted from Munoz-Sanjuan and Brivanlou (2002).

Studies on dissociated cells of the animal blastocyst pole identified specific factors, which induce epithelial fate and, by doing so, inhibit neural differentiation. They all belong to the TGF-ß-family: BMP2, 3, 4, 7, and growth differentiation factor 6 (GDF6).

In analogy activins, nodal, nodal-related Vg1, TGF-ß1, -2, and -3 actively induce mesoderm.

In *Xenopus* embryos, secreted BMPs initiate a TGF-ß-receptor-mediated signal transduction pathway leading to activation of intracellular factors including the family of Smad-proteins. This family can be classified into 3 groups: signal pathway-specific effectory, regulatory and inhibitory Smads (Munoz-Sanjuan and Brivanlou, 2002). Signal transduction finally leads to the association of single effectory Smads to Smad4 and subsequent translocation into the nucleus. Here they act as transcription factors and induce the expression of mesodermal or epithelial genes.

In accordance with the default model of neural induction, several factors at different levels of the signal transduction cascade could be identified, which lead to a neuroectodermal fate by inhibiting the induction of epithelia. Secreted factors like noggin, chordin, follistatin, Xnr3 and cerberus bind to members of the TGF-ß-family and inhibit their association with the TGF-ß-receptor (Smith and Harland, 1992; Lamb *et al.*, 1993; Hemmati-Brivanlou *et al.*, 1994; Sasai *et al.*, 1995; Yamashita *et al.*, 1995; Bouwmeester *et al.*, 1996; Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996; Hansen *et al.*, 1997; Piccolo *et al.*, 1999). Follistatin and cerberus are able to inhibit both epithelial and mesodermal differentiation (Hemmati-Brivanlou *et al.*, 1994; Piccolo *et al.*, 1999). Neural differentiation can be induced not only at the extracellular, but also at the intracellular level. For example, activation of further signal transduction steps can be impeded if inhibitory Smad-proteins compete with regulatory Smads (Nakao *et al.*, 1997; Hata *et al.*, 1998). The BMP-pathway can also be inhibited by transcription-repressor-complexes present in the nucleus (Nomura *et al.*, 1999; Wang *et al.*, 2000b).

In general, the induction of neural tissue can be direct or indirect. Indirect induction is characterized by the induction of differentiation by an intermediate cell type like dorsal mesoderm. In contrast, direct induction refers to inducing factors which directly – in absence of other cell types – lead to the generation of neural tissue (Eggen and Hemmati-Brivanlou, 2001).

1.1.2 Properties of neural stem cells

Fetal neural stem cells

Fetal neural stem cells lead to both cell types of the nervous system, neurons and glia cells. Neurons are able to transmit information in form of electric signals. Their excitable membranes allow them to generate and propagate action potentials. In general, they consist of a cell body containing the nucleus, and two types of cell processes, the axon and the dendrites. A neuron receives electric input through its dendrites and forwards the information along its axon. Glia cells are the most abundant cells in the nervous system. Important glia cell types are oligodendrocytes and astrocytes, and the Schwann-cells of the peripheral nervous system (PNS). Oligodendrocytes generate myelin sheets around the axons of neurons in the CNS. Schwann-cells have a similar function in the PNS. Astrocytes perform several tasks, e.g. participation in the blood-brain barrier and support of neurons.

The process of neurulation begins shortly after neural induction. During neurulation, the neuroectoderm folds into the neural tube. This early neuroectodermal structure is composed of a germinal neuroepithelium that is one cell layer thick (Gilbert, 2000). It consists of rapidly dividing stem cells, which are continuous from the luminal surface of the neural tube to the outside, the pial surface (Fig. 1.3 a). During the cell cycle, these cells undergo interkinetic nuclear migration. This means that the nucleus is at the pial side of the neural tube during S-phase, and at the luminal side during M-phase (Gilbert, 2000). These first neuroepithelial stem cells are morphologically homogeneous and multipotent, meaning that they can give rise to several neural cell types (Sauer, 1935; Huttner and Brand, 1997). This includes neurons, glia cells and radial glia cells, the latter will be described below (Price *et al.*, 1987; Luskin *et al.*, 1988; Price and Thurlow, 1988).

As a feature of all somatic stem cells, neuroepithelial stem cells can divide clonally. Studies in mouse demonstrated that their survival and proliferation *in vitro* is strongly FGF-2 (fibroblast growth factor 2)-dependent (Kilpatrick and Bartlett, 1995; Qian *et al.*, 1997).

Extensive proliferation within the germinal neuroepithelium during early developmental stages leads to the generation of two different cell populations forming the ventricular

zone (VZ): Radial glia cells through symmetric, neuroblasts through asymmetric cell division (Fig. 1.3 b). Later, radial glia cells can divide asymmetrically to generate a neuron and a radial glia cell. Radial glia cells elongate, but keep in contact with both the pial and the luminal side of the neural tube. They serve as guiding tracts for the neuroblasts, which migrate along the radial glia to the pial side, where they settle as postmitotic neurons. This process leads to the generation of the cortical plate and the marginal zone, outer layers consisting of postmitotic neurons (Fig. 1.3 c).

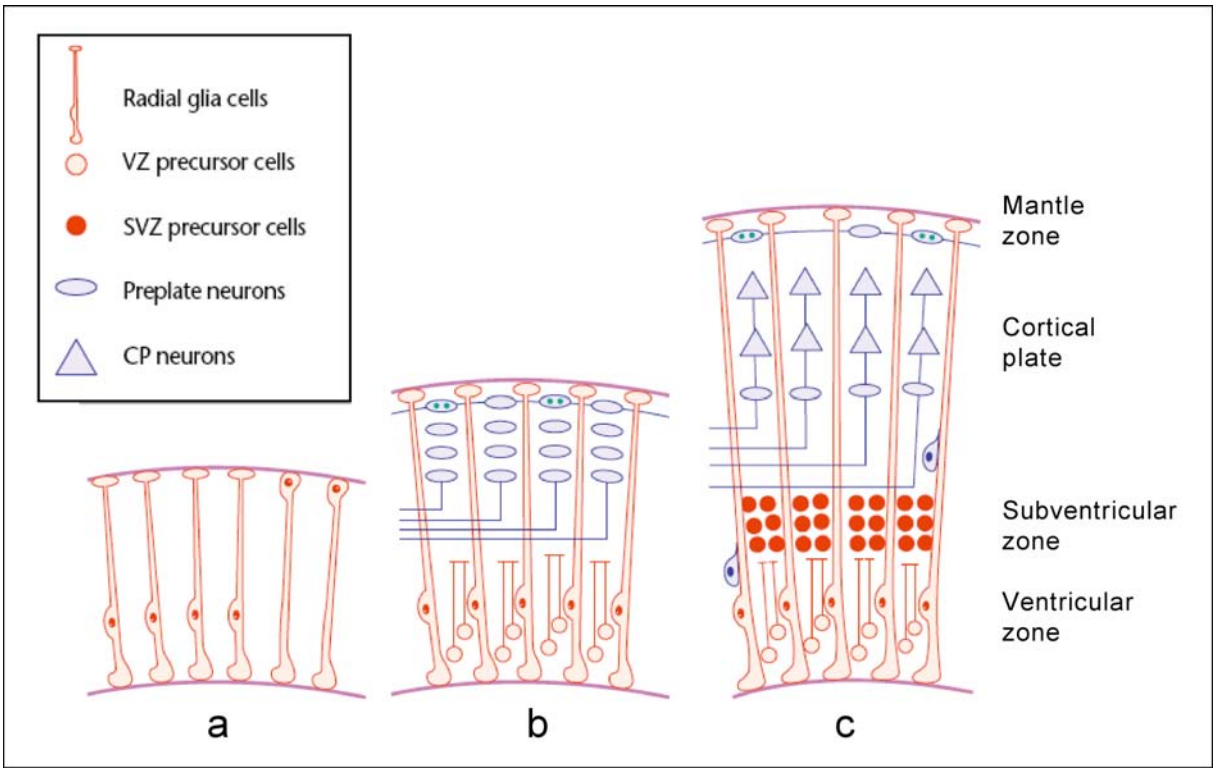

Fig. 1.3: Early development of the mammalian isocortex

The cortex is depicted with the pial (basal) side up. Dividing precursor cells are depicted in red; postmitotic neurons are in blue.
(**a**) Before neurogenesis, all cells appear similar. They span the entire thickness of the cerebral wall and proliferate. During the cell cycle the nuclei move between the ventricular and pial surface (interkinetic nuclear migration).
(**b**) After the onset of neurogenesis, the first postmitotic neurons (preplate neurons) settle underneath the pial surface. The precursor cells that remain attached to both surfaces are the radial glia cells. The area of the former neuroepithelium is now called the ventricular zone (VZ).
(**c**) At midneurogenesis a secondary proliferative zone is formed, the subventricular zone (SVZ). After further thickening of the cerebral wall, neurons migrate along radial glia fibres and settle above the SVZ, building the mantle zone (MZ) and below the newly forming cortical plate (CP).
Adapted from Götz (2001).

Neuroepithelial stem cells give rise to another population of neural stem cells, generating the subventricular zone (SVZ). *In vitro*, the proliferation of SVZ precursors depends on FGF and EGF (Reynolds et al., 1992; Gritti et al., 1999). *In vivo*, two distinct neural stem cell populations were postulated: an early, depending on FGF, followed by an epithelial growth factor (EGF)-depending population (Tropepe *et al.*, 1999; Martens *et al.*, 2000).

Due to their characteristic morphology and their appearance in nearly all regions of the CNS, radial glia cells were described early (reviewed by Bentivoglio and Mazzarello, 1999). Their function as leading tracks misled from the fact, that radial glia cells are neural precursor cells themselves (Malatesta *et al.*, 2000; Hartfuss *et al.*, 2001; Miyata *et al.*, 2001; Noctor *et al.*, 2001; 2002). In the meantime it was postulated that even the majority of proliferating cells of the VZ are radial glia cells (Noctor *et al.*, 2002). They undergo interkinetic nuclear migration (Alvarez-Buylla *et al.*, 1998) and can divide asymmetrically (Kamei *et al.*, 1998). Further studies revealed, that radial glia cells can generate both neurons and glia (Malatesta *et al.*, 2000; Hartfuss *et al.*, 2001; Miyata *et al.*, 2001; Noctor *et al.*, 2001; Tamamaki *et al.*, 2001). Radial glia cells exhibit a specific elongated morphology and express a characteristic set of markers, which distinguishes them from other neural precursor cells. RC2 is a highly specific marker only expressed in radial glia cells. Furthermore, radial glia cells express BLBP and GLAST, but they share these features with astrocytes (Edwards *et al.*, 1990; Feng *et al.*, 1994; Kurtz *et al.*, 1994; Shibata *et al.*, 1997; Hartfuss *et al.*, 2001).

Adult neural stem cells

Over a long time period, the vertebrate brain was thought to be incapable of adult neurogenesis. Conclusive evidence for the generation of new neurons in adult vertebrates came from observations in the adult avian brain (Goldman and Nottebohm, 1983; Burd and Nottebohm, 1985). In the adult mammalian brain, neurogenesis seems to concentrate on mainly two regions. The dentate gyrus of the hippocampus was demonstrated to be capable of generating new neural cells (Altman and Das, 1965; Kuhn *et al.*, 1996). Furthermore, new neurons can be generated in the SVZ of rodents (Altman, 1969; Doetsch *et al.*, 1999). In addition, many groups have been able to isolate multipotent neural progenitor cells, which can generate neurons from other regions of the adult rodent brain and spinal cord (Reynolds and Weiss, 1992; Morshead *et al.*,

1994; Gage *et al.*, 1995; Gritti *et al.*, 1996; Johe *et al.*, 1996; Weiss *et al.*, 1996) and from the adult human brain (Kirschenbaum *et al.*, 1994; Palmer *et al.*, 2001).

In the meantime, astrocytes were identified as potential stem cells in the adult brain. Although they posses attributes of fully differentiated cells, SVZ astrocytes were shown to produce progeny that can differentiate into neurons and glia (Doetsch *et al.*, 1999). When cultivated under defined conditions *in vitro*, they do behave as stem cells (Laywell *et al.*, 2000). Malatesta and coworkers (2000) were able to demonstrate that both radial glia cells and astrocytes give rise to neurons in the neonatal brain. Adult neurogenesis is now accepted as a common feature of vertebrate brains, also in the human (Alvarez-Buylla and Lois, 1995; Eriksson *et al.*, 1998).

1.2 Embryonic stem cells

Embryonic stem cells can be obtained from the pre-implantation embryo, i.e. the blastocyst consisting of the trophectoderm and the inner cell mass (ICM). For this purpose, the ICM is isolated and plated onto fetal fibroblasts (Fig. 1.4). The emanating ES cell colonies can then be further cultivated. For the first time in 1981, two research groups succeeded in isolating murine ES cells using this strategy (Evans and Kaufman, 1981; Martin, 1981).

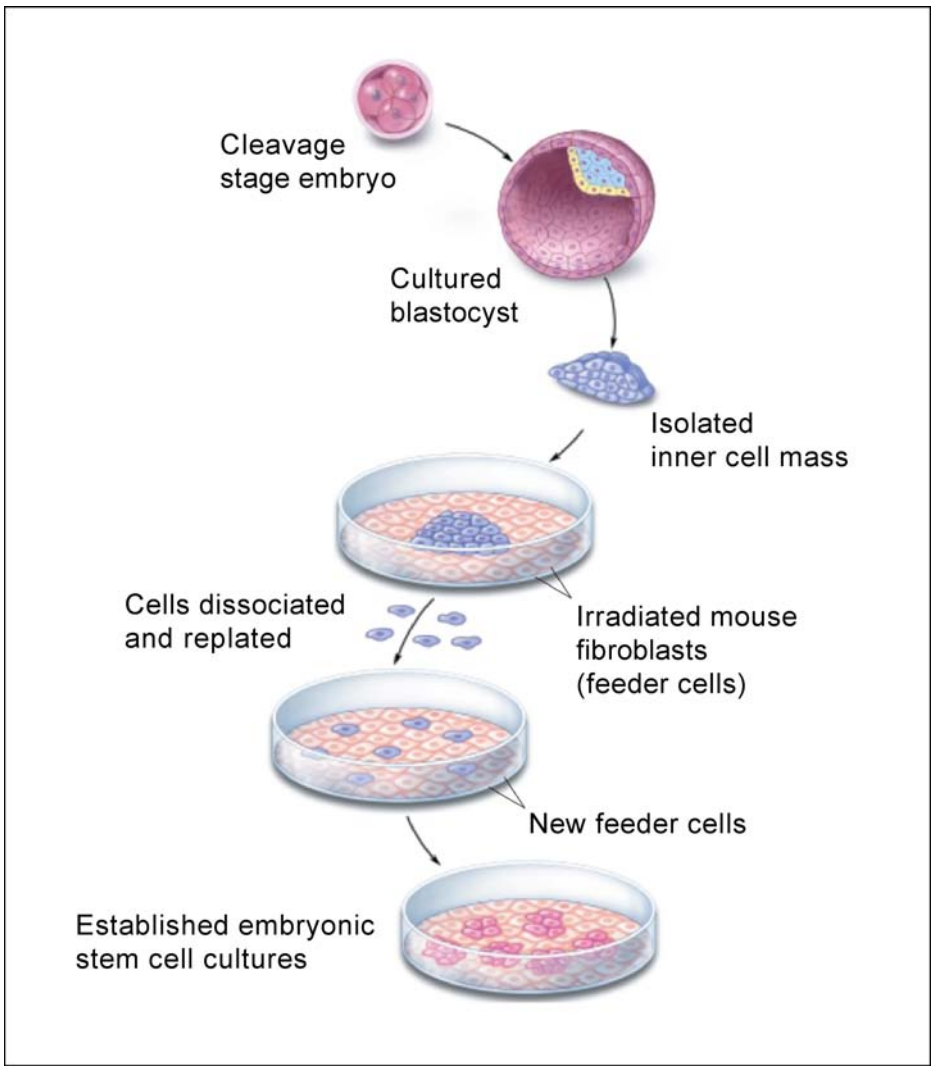

Fig. 1.4: Generation of embryonic stem cell cultures

The inner cell mass of a pre-implantation embryo is isolated and cultivated on fetal fibroblasts. Cell lines can be established by dissociation and propagation of the obtained colonies. Adapted from NIH (2001).

By now, ES cells have been generated from various other species including humans (Thomson *et al.*, 1998). More recently, ES cells have also been isolated from later stage blastocysts and from the morula stage (Stojkovic *et al.*, 2004; Strelchenko *et al.*, 2004).
Another promising technique for the generation of ES cells is therapeutic cloning. For this purpose, the nucleus of a somatic cell is transferred into an enucleated oocyte (Wilmut *et al.*, 1997). By isolating the ICM of the generated blastocyst, ES cells with the same nuclear genome as the cell donor can be obtained. Transferred to human, this technology could offer numerous advantages for cell replacement strategies and basic research. On the one hand, patient-specific ES cell lines can be generated to prevent rejection of transplanted cells. On the other hand, human ES cell lines with the genotype of specific human diseases can be generated. This offers the opportunity to study molecular mechanisms of disease processes *in vitro*.

1.2.1 Properties of murine and human ES cells

Blastocyst-derived human and murine embryonic stem cells have the ability to generate all somatic cell types and to self-renew in unlimited number. However, mouse and human ES cells differ in many aspects. ES cell colonies and human ES cells themselves, are bigger and more flattened, whereas murine ES cell colonies are small and have a defined border. Murine ES cells grow faster with a doubling time of 12 h compared to human ES cells, which need 35 h (Amit *et al.*, 2000). Furthermore, in contrast to murine ES cells, human ES cells are able to differentiate into extra-embryonic tissue, i.e. trophoblast-like cells (Thomson *et al.*, 1998; Odorico *et al.*, 2001; Xu *et al.*, 2002).
ES cells of both species differ also in their pattern of gene expression (Ginis *et al.*, 2004; Rao, 2004). Pluripotent ES cells always express a characteristic set of markers, which are down-regulated upon differentiation. In particular, human ES cells express the cell surface markers Tra-1-60 (Tumor-related antigen) and Tra-1-81 (Thomson *et al.*, 1998; Draper *et al.*, 2002; Henderson *et al.*, 2002). These markers are human-specific and are not found in other species. Both human and murine ES cells express surface-antigens from the group of SSEA-markers (stage-specific embryonic antigen). Pluripotent human ES cells show expression of SSEA-3 and SSEA-4, whereas murine ES cells express SSEA-1 (Krupnick *et al.*, 1994; Thomson *et al.*, 1998; Draper *et al.*,

2002; Henderson *et al.*, 2002). Other genes, associated with the pluripotent state of ES cells include *Oct3/4*, *nanog*, and *Rex-1* (Ben-Shushan *et al.*, 1998; Nichols *et al.*, 1998; Lebkowski *et al.*, 2001; Chambers *et al.*, 2003; Mitsui *et al.*, 2003; Rao, 2004).

ES cells are usually cultivated on a layer of murine or human fetal fibroblasts (Evans and Kaufman, 1981; Martin, 1981; Thomson *et al.*, 1998; Reubinoff *et al.*, 2000). These cells are also called feeder cells, because they secret specific factors thereby keeping ES cells in an undifferentiated state. In murine ES cells, leukemia inhibitory factor (LIF) was identified as a factor with pluripotency-promoting effect, even in absence of feeder cells (Williams *et al.*, 1988). LIF inhibits differentiation along the LIF/Stat3-pathway in murine ES cells, but it does not have this effect in human ES cell cultures (Evans and Kaufman, 1981; Martin, 1981; Thomson *et al.*, 1998; Raz *et al.*, 1999; Reubinoff *et al.*, 2000; Metcalf, 2003). In general, human ES cells have to be cultivated on feeder cells or in feeder-conditioned medium on an appropriate coating (Xu *et al.*, 2001). Due to the dependency of human ES cells on fetal fibroblasts, many groups have focused on conditions for feeder-free cultivation (Amit *et al.*, 2000; Sato *et al.*, 2004; Li *et al.*, 2005b; Xu *et al.*, 2005a; Xu *et al.*, 2005b). FGF-2 seems to be an indispensable factor for the maintenance of pluripotency in human ES cells. Addition of FGF-2 to a medium containing serum-replacement is commonly used for the propagation of human ES cells on feeder cells (Amit *et al.*, 2000). Furthermore, inhibition of the BMP-pathway seems to be a possibility for maintaining human ES cell pluripotency. Recently, Xu *et al*. demonstrated that the BMP-inhibitor Noggin in combination with FGF-2 keeps human ES cells in an undifferentiated state under feeder-free conditions (2005b).

1.2.2 Strategies for the generation of ES cell-derived enriched somatic cell populations

The most challenging aim of ES cell technology is the generation of enriched somatic cell types. This is in particular important for future transplantation strategies, which require pure cell populations, as resident pluripotent ES cells may cause the formation of teratomas after transplantation (Thomson *et al.*, 1998; Amit *et al.*, 2000; Reubinoff *et al.*, 2000). In general, two different strategies exist for the generation of highly enriched somatic cell populations: Directed differentiation and lineage-selection (Fig. 1.5).

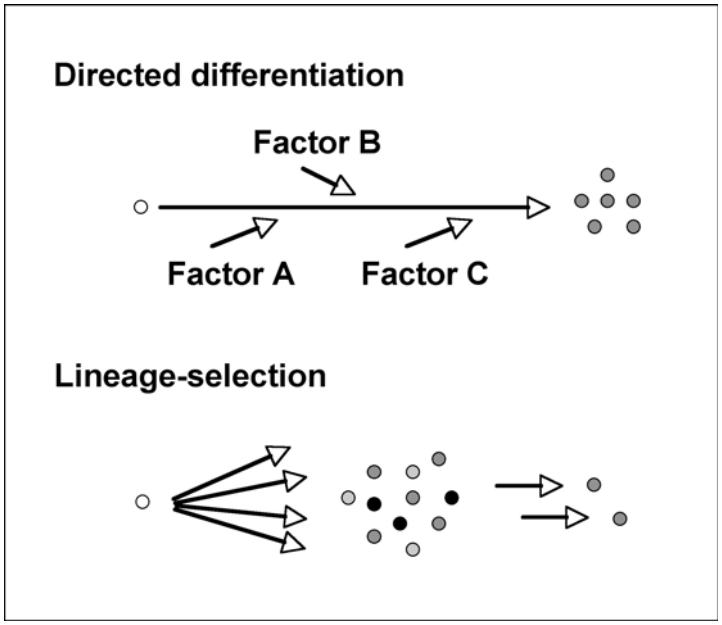

Fig. 1.5: Strategies for the generation of ES cell-derived enriched somatic cell types

Directed differentiation is based on the sequential treatment with specific growth factors in defined media. All cells are guided towards the desired phenotype. For application of lineage-selection, all cells are differentiated spontaneously into several phenotypes. The desired phenotype can then be isolated by cell type-specific selectable markers. Both strategies can be combined.
Adapted from Wernig *et al.* (2003).

Directed differentiation *in vitro* is based on the application of specific media compositions and extrinsic factors in a defined manner and sequence (Schuldiner *et al.*, 2000). The aim of this strategy is to induce the entire cell population to differentiate into the desired cell type. Differentiation is commonly induced by aggregation of ES cells

into embryoid bodies (EBs), which are multicellular aggregates that comprise all three germ layers (Desbaillets *et al.*, 2000; Itskovitz-Eldor *et al.*, 2000; Bhattacharya *et al.*, 2005). Specific cultivation conditions can then promote the selective enrichment of the desired cell type. In earlier studies, retinoic acid (RA) was frequently used to induce neural differentiation (Bain *et al.*, 1995; Fraichard *et al.*, 1995; Strübing *et al.*, 1995; Li *et al.*, 1998; Bibel *et al.*, 2004). Other strategies are based on cocultivating ES cells with a somatic cell line. In several studies, the somatic cell line PA6 was used for the induction of neural differentiation. These cells appear to exert a yet not defined 'stromal cell-derived inducing activity' (SDIA, Kawasaki *et al.*, 2000).

In contrast, lineage-selection is based on the selection of a desired phenotype from a pool of heterogeneously differentiated cell types. Two different techniques can be used to isolate ES cell-derived specific cell types. One possibility is to select cells on the basis of expression of a specific surface antigen by using immunological methods like immunopanning, magnetic- or fluorescence-activated cell sorting (FACS; Roy *et al.*, 1999; Malatesta *et al.*, 2000; Roy *et al.*, 2000; Uchida *et al.*, 2000; Wang *et al.*, 2000a; Carpenter *et al.*, 2001; Cassidy and Frisen, 2001; Kawaguchi *et al.*, 2001; Keyoung *et al.*, 2001; Rietze *et al.*, 2001; Schmandt *et al.*, 2005).

Another method is to genetically modify ES cells with a selectable marker only expressed in the desired cell population. For selection, an antibiotics resistance gene under the control of a cell-type specific promoter is commonly used. When a mixed cell population is treated with the specific antibiotic, only those cells expressing the marker are resistant and survive (Klug *et al.*, 1996; Pasumarthi and Field, 2002; Glaser *et al.*, 2005). Cells can also be selected by transfection with a fluorescent transgene under the control of a specific promoter, for example the enhanced green fluorescent protein (eGFP). Subsequent FACS-sorting leads to an enriched cell population expressing the desired cell type-specific marker. This method was already successfully used for the selection of neural precursors and neurons derived from ES cells and primary tissue (Wang *et al.*, 1998; Roy *et al.*, 1999; Roy *et al.*, 2000; Wang *et al.*, 2000a; Rietze *et al.*, 2001; Wernig *et al.*, 2002).

1.2.3 *In vitro* differentiation of murine ES cells into neural phenotypes

Studies on murine ES cells have demonstrated that directed differentiation can be used to generate multipotent neural and glial precursors at high purities (Okabe *et al.*, 1996; Brüstle *et al.*, 1997b; Brüstle *et al.*, 1999). Based on the differentiation into EBs, Okabe and coworkers (1996) generated a protocol for the directed differentiation of murine ES cells into neural precursors (Fig. 1.6). For this purpose, EBs were plated after 4 days of cultivation and further propagated in ITSFn medium containing insulin, transferrin, sodium-selenite and fibronectin. This medium selectively promotes the survival of neural precursor cells. They can be further cultivated in a defined neural medium supplemented with FGF-2, which strongly promotes proliferation of neural precursor cells (Sensenbrenner, 1993; Brickman *et al.*, 1995). At this stage, neural precursor cells express the intermediate filament nestin. After growth factor withdrawal, neural precursors differentiate into cells of all three neural lineages: neurons, astrocytes and oligodendrocytes. After transplantation into the ventricular system of the developing rat brain, they integrate into numerous regions of the host brain and differentiate into neurons and glia (Brüstle *et al.*, 1997b).

Based on the protocols from the group of Okabe (1996), Brüstle and coworkers (1999) established a new protocol for the generation of highly enriched glial precursor cells (Fig. 1.6). For this aim, neural precursor cells were cultivated in FGF-2. Subsequently, these so-called N3FL cells were replated in a defined medium containing FGF-2 and epidermal growth factor (EGF), leading to the cell population N3EFL. After reaching subconfluency, cells were again harvested and replated in FGF-2 and platelet-derived growth factor (PDGF), these cells are now called N2FP cells. Growth factor combination of FGF-2 and PDGF is known to promote the proliferation of glial precursor cells (Bögler *et al.*, 1990). Murine ES cell-derived glial precursor cells can be easily identified by the expression of the cell surface antigen A2B5. After growth factor withdrawal, the majority of the generated glial precursors differentiate into astrocytes and oligodendrocytes. Upon transplantation into the ventricular system of myelin-deficient rats, the engrafted cells migrate into several regions of the host CNS and have the capacity to myelinate host axons (Brüstle *et al.*, 1999).

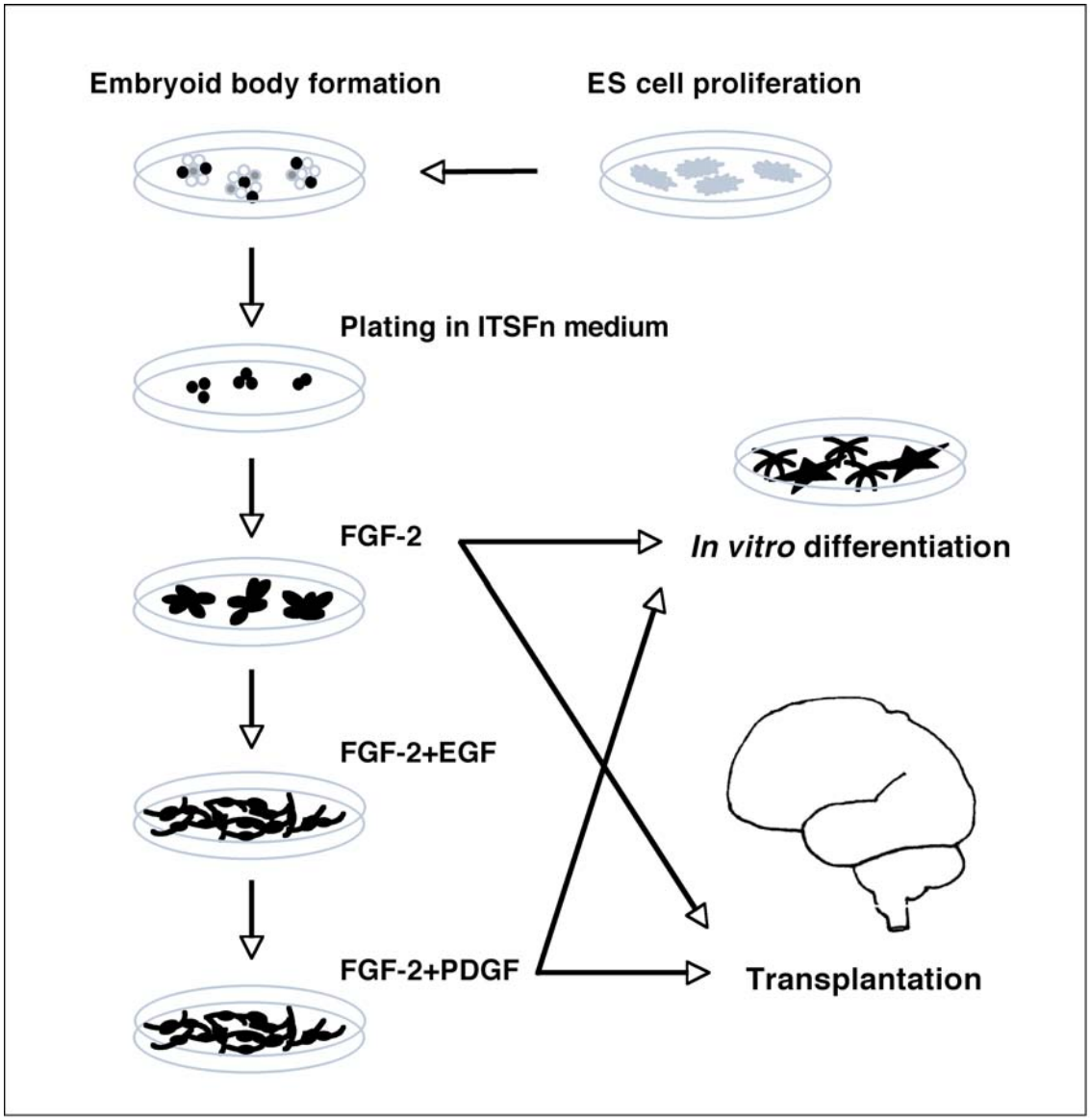

Fig. 1.6: Protocol for the directed differentiation of murine ES cells into neural and glial precursors according to Okabe *et al*. (1996) and Brüstle *et al*. (1999).

After initial proliferation in LIF-containing medium, murine ES cells were aggregated to EBs. After plating, the cells were further cultivated in serum-free ITSFn medium. After 7 days, the cells were triturated to a single cell suspension and subsequently plated on poly-ornithine (PO)-coated dishes in defined neural medium containing FGF-2, leading to neural precursor cells. Following growth factor withdrawal in this stage, the cells differentiate into both neurons and glia. After further cultivation of precursors in FGF-2 and EGF followed by cultivation in FGF-2 and PDGF, highly enriched glial precursors can be obtained. The majority of the precursors give rise to astrocytes and oligodendrocytes after growth factor withdrawal.

In combination with lineage selection strategies or further growth factor exposure, purified neural precursor cells allow for the enrichment of specific neural subtypes such as dopaminergic neurons, mid- and hindbrain neurons and oligodendrocytes (Lee *et al.*,

2000; Rolletschek *et al.*, 2001; Barberi *et al.*, 2003; Glaser *et al.*, 2005; Schmandt *et al.*, 2005). Transplantation studies in animals have already demonstrated the potential of ES cell-derived neural subtypes to functionally integrate into host tissue (Kim *et al.*, 2002; Scheffler *et al.*, 2003; Wernig *et al.*, 2004).

Other approaches for neural differentiation based on coculture conditions with stromal cell lines demonstrated that induction of neuroectodermal differentiation does not necessarily require an EB stage (Kawasaki *et al.*, 2000; Barberi *et al.*, 2003). It has also been shown that murine ES cells can be converted into neural lineages without the presence of other tissues or morphogens like retinoic acid, simply by cultivating them in multicellular aggregates using low-density conditions in defined media (Tropepe *et al.*, 2001). Additionally, studies on murine ES cells have already proven the principle that conversion into an neuroectodermal precursor cell type can be performed by adherent monoculture (Ying *et al.*, 2003). In this case, murine ES cells can be adherently converted without co-cultivation steps or the formation of EBs.

1.2.4 Differentiation potential of human ES cells

Similar to murine ES cells, human ES cells have the ability to spontaneously differentiate into cells of all three germ layers (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000). Aggregation of human ES cells leads to the generation of EBs, which give rise to derivatives of all 3 germ layers (Itskovitz-Eldor *et al.*, 2000). Schuldiner and coworkers (2000) were able to demonstrate that exposure to single growth factors preferentially induces the differentiation of EBs into cells of a specific germ layer. Over the last years several clinically relevant somatic cell types such as cardiomyocytes, insulin-producing cells, endothelial cells, and hematopoietic cells were generated (Assady *et al.*, 2001; Kaufman *et al.*, 2001 2002; Kehat *et al.*, 2001; Kehat *et al.*, 2002).

In contrast to murine ES cell culture systems, the derivation of highly purified neural precursors from human ES cells is still at an early stage. The protocols communicated so far for the generation of human ES cell-derived neural lineages mostly rely on mechanical or enzymatic isolation of neural cell clusters from differentiated human ES cells or plated EBs (Reubinoff *et al.*, 2001; Zhang *et al.*, 2001; Conti *et al.*, 2005). Some depend on lineage-selection strategies (Carpenter *et al.*, 2001) or on coculture conditions (Buytaert-Hoefen *et al.*, 2004; Perrier *et al.*, 2004; Zeng *et al.*, 2004; Park *et*

al., 2005). Others directly aggregate single human ES cell colonies in neural differentiation media (Schulz *et al.*, 2004). Like murine ES cells, human ES cell-derived neural precursors were already shown to incorporate into the host tissue after transplantation into an animal model (Reubinoff *et al.*, 2001; Zhang *et al.*, 2001; Tabar *et al.*, 2005).

Several groups have already communicated the generation of human ES cell-derived enriched neural subtypes such as peripheral neurons (Li *et al.*, 2005a; Pomp *et al.*, 2005), midbrain and hindbrain neurons (Lee *et al.*, 2000), dopaminergic neurons (Buytaert-Hoefen *et al.*, 2004; Perrier *et al.*, 2004; Zeng *et al.*, 2004; Park *et al.*, 2005), and oligodendrocytes (Nistor *et al.*, 2005).

1.3 Aim of this study

The major goal of this project was to establish new protocols for the generation of enriched neural precursor cells from human ES cells. The long-term aim of this study is the generation of human ES cell-derived neural precursor cells for future transplantation approaches and basic research. For both applications, defined protocols leading to highly enriched neural precursor cells are key prerequisites. For therapeutic approaches, neural precursors have promising capacities due to their ability to differentiate into cells of all three neural lineages such as neurons, astrocytes and oligodendrocytes. For basic research, neural differentiation protocols performed under defined conditions offer various applications for the recapitulation of early developmental processes in the CNS.

To this end, strategies for the generation of enriched neural precursors were explored. The first part of the study addresses the question whether EB-based differentiation protocols such as those established for murine ES cells could be developed for human ES cells, and whether cells generated by such an approach can differentiate upon transplantation into host CNS tissue.

A second question to be addressed was whether neural induction could also be brought about without an intermediate embryoid body-stage. For this purpose, a new protocol for the adherent conversion of human ES cells into the neuroectodermal lineage had to be established. Precursor cells generated according to both protocols had to be characterized to reveal their neural identity. Furthermore, their capacity to differentiate into glia cells and neurons had to be demonstrated.

2 Materials

Cell culture plastic ware, including dishes, pipettes, centrifugation tubes, cell strainer, cell scraper and incubation tubes were obtained from BioRad Laboratories (München), Corning Coster (New York, USA), Eppendorf (Hamburg), Falcon/Becton Dickinson (Heidelberg), Greiner (Nürtingen), Millipore (Billerica, USA) and Nunc (Wiesbaden). Glass materials were obtained from Schott (Mainz).

2.1 Technical equipment

Appliance	Name	Supplier
Balances	LA310S	Sartorius (Göttingen)
	BL610	Sartorius (Göttingen)
Centrifuges	Megafuge 1.OR	Heraeus Instruments (Hanau)
	Centrifuge 5415 C	Eppendorf (Hamburg)
Cryostat	HM 560	Microm Laborgeräte GmbH (Walldorf)
Digital camera	C 5050 Zoom	Olympus (Hamburg)
Electroporator	Gene-Pulser® II	BioRad Laboratories GmbH (München)
Freezing container	Nalgene^{TM}Cryo 1°C	Nalge Nunc (New York, USA)
Gel chamber	Agagel Midi Agagel Midi-Wide	Biometra (Göttingen)
Gel documentation	Chemidoc	BioRad (München)
Hemocytometer	Neubauer	Roth (Karlsruhe)
Imaging Software	Adobe Photoshop 7.0	Adobe (München)
	OpenLab 4.0	Improvision (Tübingen)
Incubator	Heracell	Heraeus Instruments (Hanau)
Microscope slides for immunohistochemistry	Superfrost plus	Menzel-Gläser (Braunschweig)

Microscopes	Axiovert 25	Zeiss (Jena)
	Axiovert 40 CFL	Zeiss (Jena)
	Axiovert 200M	Zeiss (Jena)
	Axioskop 2	Zeiss (Jena)
	LSM-510	Zeiss (Jena)
	SMZ 1500	Nikon GmbH (Düsseldorf)
pH-meter	CG840	Schott (Mainz)
Photometer	SmartSpec^{TM}3000	Biorad (München)
Polyester membrane	Transwell-Clear	Corning (Bodenheim)
Power supply	Standard power pack P2.5	Biometra (Göttingen)
Shaker	Bühler Schüttler WS 10	Johanna Otto GmbH (Hechingen)
Sterile hood	Herasafe (vertical)	Heraeus Instruments (Hanau)
	Heraguard (horizontal)	Heraeus Instruments (Hanau)
Thermocycler	T3	Biometra (Göttingen)
Vibroslicer	VSLM1	Campden Instruments (Sileby, GB)
Water bath	1008	GFL (Burgwedel)

2.2 Chemicals and reagents

Product	**Supplier**
Apo-Transferrin	Chemicon (Temecula, USA)
Ascorbic acid	Sigma (Deisenhofen)
Blue Alkaline Phosphatase Substrat Kit	Vector Lab. (Burlingame, USA)
ß-Mercaptoethanol	Invitrogen (Karlsruhe)
Collagenase type IV	Invitrogen (Karlsruhe)
DMEM, high glucose	Invitrogen (Karlsruhe)
DMEM-F12	Invitrogen (Karlsruhe)
DMSO	Sigma (Deisenhofen)
DNA ladder 100 bp plus	Fermentas (St. Leon-Rot)
DNA loading buffer (6x)	Fermentas (St. Leon-Rot)
dNTPs	Peqlab (Erlangen)
DTT	Roche (Basel)
EDTA	Sigma (Deisenhofen)

Ethanol	Merck (Darmstadt)
Ethidiumbromide	Sigma (Deisenhofen)
Fetal calf serum	Invitrogen (Karlsruhe)
Fetal bovine serum (defined)	Hyclone (Logan, USA)
Freezing medium (serum-free)	Sigma (Deisenhofen)
FGF-2 for human ES cell culture	Invitrogen (Karlsruhe)
FGF-2 for neural differentiation	R&D Systems (Wiesbaden)
G418	PAA Lab. (Cölbe)
G5-supplement (100x)	Invitrogen (Karlsruhe)
Gelatine type A	Sigma (Deisenhofen)
Glacial acetic acid	Sigma (Deisenhofen)
Glycerol	Sigma (Deisenhofen)
Hanks' Balanced Salt Solution	Sigma (Deisenhofen)
Human fibronectinee	ICN Biomedicals (Eschwege)
Human Laminin	Sigma (Deisenhofen)
Insulin	Sigma (Deisenhofen)
Isopropanol	Sigma (Deisenhofen)
Ketanest	Parke Davis GmbH (Karlsruhe)
Knockout-DMEM	Invitrogen (Karlsruhe)
L-glutamine	Invitrogen (Karlsruhe)
Matrigel (not growth factor reduced)	BD Bioscience (Heidelberg)
$MgCl_2$	Invitrogen (Karlsruhe)
Mowiol 4-88	Merck (Darmstadt)
N2-supplement (100x)	Invitrogen (Karlsruhe)
$NaHCO_3$	Sigma (Deisenhofen)
NGS	Sigma (Deisenhofen)
Non-essentiell aminoacids	Invitrogen (Karlsruhe)
NRS	Sigma (Deisenhofen)
Oligo (dT)-Primer	Roche (Mannheim)
Paraformaldehyde	Sigma (Deisenhofen)
PBS (cell culture)	Invitrogen (Karlsruhe)
PBS (immunochemistry)	Biochrom (Berlin)
PCR-Buffer RXN (10x)	Invitrogen (Karlsruhe)
PDGF-AA	R&D Systems (Wiesbaden)
PeqGold Universal Agarose	PeqLab (Erlangen)
Poly-L-Ornithine	Sigma (Deisenhofen)
Progesterone	Sigma (Deisenhofen)

Putrescine	Sigma (Deisenhofen)
Reverse Transcriptase	Roche (Mannheim)
Rompun	Bayer (Leverkusen)
RNAse inhibitor	Roche (Mannheim)
RNeasy Mini Kit	Qiagen (Hilden)
RT-buffer	Roche (Mannheim)
Serum Replacement	Invitrogen (Karlsruhe)
Sodiumazide	Merck (Darmstadt)
Sodiumchloride	Sigma (Deisenhofen)
Sodiumpyruvate	Invitrogen (Karlsruhe)
Sodiumselenite	Sigma (Deisenhofen)
Taq DNA polymerase	Invitrogen (Karlsruhe)
Tris	Sigma (Deisenhofen)
Triton-X-100	Sigma (Deisenhofen)
Trypsin-EDTA (10x)	Invitrogen (Karlsruhe)
Trypsin-inhibitor	Invitrogen (Karlsruhe)
TSA biotin Tyramide Reagent Pack	Perkin-Elmer (Wellesley, USA)

2.3 Cell lines and animal stocks

Human ES cell line H9.2	Haifa, Israel (Amit *et al.*, 2000)
Human ES cell line H9.2eGFPneo	Henrike Siemen, Bonn
Human ES cell line H9	Haifa, Israel (Thomson *et al.*, 1998)
Human ES cell line I3	Haifa, Israel
Human ES cell line I6	Haifa, Israel
CD-1 mice	Charles River, Sulzfeld
Sprague-Dawley rats	Charles River, Sulzfeld
Wistar rats P9	Charles River, Sulzfeld

2.4 Plasmid

The human ES cell line H9.2eGFPneo was transfected with the plasmid **pEGFP-C1**, encoding the reporter gene eGFP under the control of the CMV-promotor (BD Biosciences, Clontech; Heidelberg). The cell line H9.2eGFPneo was established and kindly provided by Henrike Siemen.

Length: 4.7 kb

Resistance genes: Neo^r, Kan^r

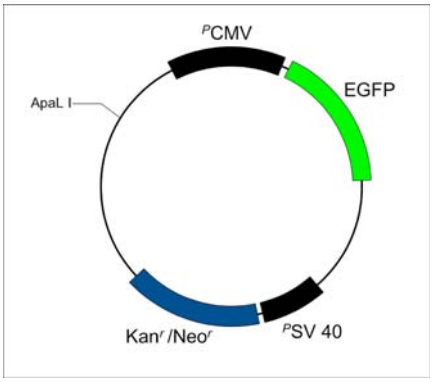

Fig. 2.1: Plasmid pEGFP-C1

P=Promotor, CMV=Cytomegalus-virus, SV 40=Simian Virus 40, Kan=Kanamycin, Neo=Neomycin.
(Kindly provided by H. Siemen)

2.5 Cell culture reagents

All cell culture reagents except of growth factors were sterile filtrated through a Millipore filtration unit (Millipore; Billerica, USA) before application.

2.5.1 Cell culture media

Cultivation medium for human ES cells (KO/SR medium)

Knockout-DMEM	80%
Serum Replacement	20%
Non-essential amino acids	1%
L-glutamine	1 mM
ß-mercaptoethanol	0.1 mM
FGF-2	4 ng/ml

Freezing medium for human ES cells

Knockout-DMEM	70%
DMSO	10%
Defined bovine serum	20%

Cultivation medium for EBs (EB/SR medium)

Knockout-DMEM	80%
Serum Replacement	20%
Non-essential amino acids	1%
L-glutamine	1 mM
Nucleoside stock solution	10%

ITSFn medium

Insulin	5 µg/ml
Apo-Transferrin	50 µg/ml
Sodium-selenite	30 nM
Human fibronectin	2.5 µg/ml
in DMEM-F12	

NAA medium

Insulin	25 µg/ml
Apo-transferrin	50 µg/ml
Sodium-selenite	30 nM
Progesterone	20 nM
Putrescine	10 µM
Ascorbic acid	200 µM
in DMEM/F12	

G5 medium

G5-supplement	1:100
in DMEM/F12	

N2 medium

N2-supplement	1:100
in DMEM/F12	

2.5.2 Cell dissociation reagents

Accutase II

Applied as provided

Collagenase type IV

Collagenase type IV	1 mg/ml
in Knockout-DMEM	

Trypsin-EDTA

10x Trypsin-EDTA	1:10
in PBS	

2.5.3 Growth factors

FGF-2 (stock solution)

Human recombinant FGF-2	10 µg/µl
BSA	0.1%
in PBS	

PDGF (stock solution)

Human recombinant PDGF	10 µg/ml
HCl	4 mM
BSA	0.1%

2.5.4 Coatings

Gelatine type A

Gelatine type A	0.1%
in $_{dd}H_2O$	

⇒ Incubation: 20 min, 37°C

Matrigel

Matrigel	1:30
in Knockout-DMEM	

⇒ Incubation: over night, 4°C

Poly-ornithine (PO)

Poly-ornithine	1.5 mg/ml
in $_{dd}H_2O$	

⇒ Incubation: 2 h

Laminin

Human laminin	1 µg/ml
in PBS	

⇒ For PO/laminin-coating: incubation with PO for 2 h and laminin for 2 h.

Fibronectin

Human fibronectin	1 µg/µl
in $_{dd}H_2O$	

2.5.5 Reagents for molecular biology

Tris-EDTA (pH 8.0)

Tris	10 mM
EDTA	1 mM
in $_{dd}H_2O$	

1x TAE (pH 7.8)

Tris-HCl	40 mM
Sodium-acetate	5 mM
EDTA	1 mM

in $_{dd}H_20$

2.5.6 Reagents for immunocyto- and immunohistochemistry

Fixation reagent

Paraformaldehyde	4%

in PBS

Buffers for immunohistochemistry on hippocampal slices

1)	NGS	10%
	in PBS	
2)	NGS	5%
	in PBS	

Blocking buffer for immunocytochemistry

FCS	5%

in PBS

Blocking buffer for immunohistochemistry

NGS	10%
Triton-X-100	0.5%

in PBS

Incubation buffer I for immunohistochemistry

NGS	1%

in PBS

Incubation buffer II for immunohistochemistry

NGS	1%
NRS	3%
in PBS	

Peroxidase (0.3%)

H_2O_2 (30%)	1%
in 100 ml $_{dd}H_2O$	

Tyramide solution

Prepared according to manufacturers manual

Mowiol

Glycerol	6 g
Mowiol	2.49 g
in 6 ml $_{dd}H_2O$	
+ 0.2 M Tris-HCl (pH 8.5)	12 ml

Sodium azide

Sodium azide	1 mg/ml
in PBS	

Triton-X-100 (1%)

Triton-X-100	10 mg/ml
in PBS	

PBS

PBS	9.55 mg/ml
in $_{dd}H_2O$	

Hepes Buffer (pH 7.5 – 8.0)

Hepes	10 mM
in $_{dd}H_2O$	

$\underline{NaHCO_3 \text{ Buffer}}$

$NaHCO_3$ 10 mM

in $_{dd}H_2O$

2.6 Antibodies

Ms = mouse, Rb = rabbit, GP = guinea pig

Primary antibodies	**Isotype**	**Dilution**	**Provider**
A2B5	Ms IgM	1:300	Chemicon (Temecula, USA)
Alpha-fetoprotein	Rb	1:200	Dako Cytomation (Hamburg)
ß-III-tubulin	Ms IgG	1:1.000	BAbCo (Covance, USA)
CHAT (choline acetyltransferase)	Rb	1:500	Chemicon (Temecula, USA)
CNPase (2',3'-cyclic nucleotide 3'-phosphodiesterase)	Ms IgG	1:250	Sigma (Deisenhofen)
Cytokeratin	Ms IgG	1:500	Dako Cytomation (Hamburg)
Desmin	Ms IgG	1:500	Dako Cytomation (Hamburg)
GAD 67 (glutamic acid decarboxylase)	Rb	1:500	Chemicon (Temecula, USA)
GFAP (glial fibrillary acidic protein)	Ms IgG	1:100	ICN Biomedicals (Eschwege)
GLAST (astrocyte-specific glutamate transporter)	GP	1:500	Chemicon (Temecula, USA)
Human nuclei	Ms IgG	1:50	Chemicon (Temecula, USA)
Human nestin	Ms IgG	1:100	Chemicon (Temecula, USA)
MAP2ab (microtubule-associated protein)	Ms IgG	1:200	Chemicon (Temecula, USA)
Musashi	Rb	1:100	Chemicon (Temecula, USA)
O4	Ms IgM	1:100	Chemicon (Temecula, USA)
Oct4	Rb	1:400	Santa Cruz Biot. (Santa Cruz, USA)
Pax6	Rb	1:200	Acris antibodies (Hiddenhausen)
PSA-NCAM (polysialylated neural cell adhesion molecule)	Ms IgM	1:1.000	Chemicon (Temecula, USA)
Serotonin	Rb	1:500	Sigma (Deisenhofen)
SMA (Smooth muscle actin)	Ms IgG	1:25	Dako Cytomation (Hamburg)

Sox1	Rb	1:100	Sigma (Deisenhofen)
Tra-1-60 (tumor-related antigen)	Ms IgM	1:100	Chemicon (Temecula, USA)
Tra-1-81	Ms IgM	1:100	Chemicon (Temecula, USA)
Tyrosine hydroxylase clone TH-2	Ms IgG	1:5.000	Sigma (Deisenhofen)
vGlut1 (vesicular glutamate transporter)	GP	1:500	Chemicon (Temecula, USA)
EMA (endothelial membrane antigen)	Ms IgG	1:50	Dako Cytomation (Hamburg)

Secondary antibodies	**Dilution**	**Provider**
Fluorescein-Avidin	1:125	Vector Laboratories (Burlingame, USA)
Biotin anti-rabbit	1:200	Dako Cytomation (Hamburg)
Biotin anti-mouse IgG	1:200	Dako Cytomation (Hamburg)
CY3 goat anti-mouse IgG + IgM	1:250	Jackson Imm. Res. (West Grove, USA)
FITC goat anti-rabbit	1:200	Jackson Imm. Res. (West Grove, USA)

2.7 PCR-Primers

Gene product, product length (bp) **Primer sequence**	**Annealing temp.**	**$MgCl_2$**	**Cycles**
GAPDH, 197 bp *Fw*: 5'-CTG CTT TTA ACT CTG GTA AAG T-3' *Rv*: 5'-GCG CCA GCA TCG CCC CA-3'	60°C	4 mM	30
Mash1, 219 bp *Fw*: 5'-GTC GAG TAC ATC CGC CTG-3', *Rv*: 5'-AGA ACC AGT TGG TGA AGT CGA-3'	65°C	2 mM	30
Nanog, 901 bp *Fw*: 5-'GAT CGG GCC CGC CAC CAT GAG TGT GGA TCC AGC TTG-3' *Rv*: 5'-GAT CGA GCT CCA TCT TCA CAC GTC TTC AGG TTG-3'	60°C	1.5 mM	30
Oct4, 219 bp *Fw*: 5'-GAG AAC AAT GAG AAC CTT CAG GAG A-3' *Rv*: 5'-TTC TGG CGC CGG TTA CAG AAC CA-3'	60°C	2 mM	30
Pax6, 275 bp *Fw*: 5'-AAC AGA CAC AGC CCT CAC AAA CA-3' *Rv*: 5'-CGG GAA CTT GAA CTG GAA CTG AC-3'	66°C	1.5 mM	30

3 Methods

3.1 Cultivation of pluripotent human ES cells

Generally, cell culture was performed under sterile conditions in a sterile hood with sterile media, glass and plastics. Cells were cultivated in an incubator at 37°C, 4% CO_2 and saturated air humidity.

3.1.1 Generation, cultivation and mitotic inactivation of murine fetal fibroblasts

All working steps were performed according to the Standard Operating Procedures of LIFE & BRAIN GmbH, Bonn.

3.1.2 Cultivation of human ES cells

Human ES cells were cultivated on a layer of irradiated mouse embryonic fibroblasts (MEF) in 6-well cell culture dishes (6-well plate). Cells were grown in serum-free KO/SR medium. Medium was changed daily and human ES cells were passaged every 4 days. For passaging, medium was removed and the cells were incubated in 1 mg/ml Collagenase IV (0.5 ml/well) for one hour. Subsequently, cells were rinsed off and centrifuged in a 15 ml-centrifugation tube (800 rpm, 3 min, 4°C). The pellet was re-suspended using a 1 ml-pipette until only small aggregates remained. The cells were plated at a ratio of 1:4 on fresh MEF.
If the proportion of morphologically differentiated cells exceeded 10%, human ES cells were manually cleaned. All steps were performed in a horizontal sterile hood using a binocular. In the cleaning process, differentiated colonies were removed by scraping with a sterile 1 ml-syringe with needle (26 g 3/8, 0.45 x 10). Subsequently, differentiated colonies were removed together with the KO/SR medium. The remaining undifferentiated colonies were passaged as described.

3.1.3 Freezing and thawing of human ES cells

Prior to freezing of human ES cells, cells from 3 wells of a 6-well plate were treated with collagenase IV, rinsed off and centrifuged as described (3.1.2). Afterwards, the supernatant was discarded and the pellet was carefully re-suspended with a 5 ml-pipette in KO/SR medium (0.5 ml). Human ES cell freezing medium (0.5 ml) was added, the cell suspension was re-suspended 2 times (2 x), and transferred to a cryo-vial. The vial was frozen over night in an isopropanol-filled freezing container at -80°C. The following day, the frozen cells were transferred to liquid N_2.
Human ES cells were thawed by gently swirling the cryo-vial in a 37°C water bath until only a small clump of frozen cells remained. After melting, the cells were quickly placed into a KO/SR medium-filled 15 ml-centrifugation tube and centrifuged (800 rpm, 3 min, 4°C). The supernatant was discarded, and the pellet was carefully re-supended in 2 ml KO/SR medium with a 5 ml-pipette. Cells of one cryo-vial were placed onto fresh MEF in 1 well of a 6-well plate.

3.2 *In vitro* differentiation of human ES cells

In vitro differentiation of human ES cells into neural precursors was performed according to two different, newly established protocols. One strategy involves the induction of differentiation in an EB-stage, the other one depends on direct conversion of human ES cells in monolayer culture.

3.2.1 Embryoid body-induced neural differentiation

For the generation of EBs, human ES cells were detached by collagenase-treatment as described (3.1.2). The pellet was re-suspended only 5 x with a 5 ml-pipette to preserve the colonies. Aggregates were transferred into 6 cm-bacterial petri dishes to avoid adherence. Medium was changed every 2 days by transferring the EBs to a 50 ml-centrifugation tube. After sedimentation of the aggregates, the supernatant was replaced with fresh EB medium. The cells were cultivated as floating aggregates in serum-free EB medium for a total of 14 days. Subsequently, EBs were plated onto PO

(poly-ornithine)-coated cell culture dishes, and 48 h later transferred to ITSFn medium containing 20 ng/ml FGF-2. Medium was changed every second day. After cultivation for 7 days, outgrowing cells were triturated to a single cell suspension, yielding a neural precursor population designated as P.0. For this purpose, plated EBs were first washed 2 x with PBS and then treated with trypsin/EDTA for up to 10 min at 37°C. The enzymatic reaction was stopped by a trypsin-inhibitor at a ratio of 1:1. Afterwards, the dishes were rinsed with NAA medium, and the cell suspension was centrifuged in a 15 ml-centrifugation tube (1100 rpm, 5 min, 4°C). The supernatant was discarded and the pellet was re-suspended in NAA medium with a 2 ml-pipette. A cell strainer was used to sort out cell clumps. The cells were plated on PO-coated dishes in NAA medium containing 10 ng/ml FGF-2 and 10 ng/ml laminin at a density of 6 x 10^4 cells/cm^2. Medium was changed every second day and FGF-2 was added daily. After reaching confluency, neural precursor cells were passaged by trypsin/EDTA-treatment as described, yielding the subsequent precursor populations P.1, P.2, etc.

Human ES cell-derived neural precursors generated according to this protocol could be easily frozen and thawed. To freeze them, cells were harvested by trypsin/EDTA-treatment and transferred to serum-free freezing medium in cryo-vials. Cell concentrations in 1 ml/cryo-vial ranged between 2 and 4 x 10^6 cells. Cells were placed in a polystyrene box at -80°C and transferred to liquid N_2 on the following day. Neural precursors could be thawed in a water bath and rapidly transferred into a 15 ml-tube filled with NAA medium. After centrifugation (1100 rpm, 5 min, 4°C), the supernatant was discarded and the pellet was re-suspended in fresh NAA medium. Cells from 1 cryo-vial were plated onto a 10 cm-cell culture dish previously coated with PO.

For induction of neuronal differentiation, neural precursor cells P.1 were cultivated on PO-coated cell culture dishes in N2 medium under growth factor withdrawal for 1 week.

To further differentiate human ES cell-derived neural precursors to the glial lineage, P.1 cells were transferred and propagated in PO/laminin-coated cell culture dishes in G5 medium supplemented with 2 ng/ml PDGF. Cells were cultivated for 8 weeks with a medium change every other day.

3.2.2 Adherently induced neural differentiation

To prepare a substrate for cell plating, 6-well plates were coated with Matrigel. Matrigel was diluted at a ratio of 1:30 in cold KO-DMEM and the dishes were coated over night at 4°C.

In a first step, human ES cells were cultivated on Matrigel under adherent conditions. Human ES cells were washed 1 x in PBS, followed by an incubation with Accutase II for 20 min at 37°C. After Accutase II-treatment, human ES cells detached, while MEF and differentiated cells remained largely adherent. The cell culture dishes were rinsed in KO/SR and the detached human ES cells were centrifuged in a 15 ml-centrifugation tube (1100 rpm, 5 min, 4°C). The supernatant was discarded and the pellet was re-suspended in KO/SR medium. Afterwards, the cells were plated onto Matrigel-coated 6-well plates at a density of 1 x 10^6 cells/well, yielding adherent stage A.0 cells. Twenty-four hours later, the adherent cells were changed to NAA medium containing 10 ng/ml FGF-2. The cells were adherently cultivated for a total of 8 days. During this time, they were passaged twice at a ratio of 1:3 onto fresh Matrigel-coated dishes by Accutase II-treatment as described (yielding stage A.1 and A.2 cells. A schematic protocol is depicted in Fig. 4.10). Medium supplemented with FGF-2 had to be changed daily.

The generated colonies of adherent stage A.2 (see Fig. 4.10) were then treated with collagenase IV for 20 min and gently detached from the dish with a cell scraper in NAA medium. The aggregates were collected in a 15 ml-centrifugation tube and centrifuged (800 rpm, 3 min, 4°C). The supernatant was discarded and the pellet was re-suspended in fresh medium. To avoid any adherence, aggregates were further cultivated in suspension culture in 10 cm-bacterial petri dishes placed on a shaker. FGF-2 was added daily and medium was changed every other day. Medium change was performed similar to floating EBs (see 3.2.1), with only 2/3 of the NAA medium being replaced during each change. After 15 days, neurospheres were plated on tissue culture dishes in fresh medium. FGF-2 was added daily, and the medium was changed on the second day. Three days after plating, the outgrowing neural precursor cells were triturated to a single cell suspension. The cells were washed 1 x in PBS and incubated in Accutase II for 15 min at 37°C. Afterwards, the dishes were rinsed with NAA medium and the cells were collected in a 15 ml-centrifugation tube and centrifuged (1100 rpm, 5 min, 4°C), strained, and transferred to PO-coated dishes in NAA medium supplemented with 10

ng/ml FGF-2 (stage P.0). Subsequent passaging resulted in the neural precursor cell stages P.1, P.2, etc.
To induce neuronal differentiation, 15-day-old neurospheres were cultivated under FGF-2-withdrawal in DMEM/F12 supplemented with N2 (Invitrogen) for another 15 days and subsequently plated onto poly-l-lysine and laminin-coated cell culture dishes. The cells were immunocytochemically analyzed 12 days later. For glial differentiation, P.0 cells were propagated under growth factor withdrawal in N2 medium for 2 weeks, followed by cultivation in NAA medium for 3 weeks and again in N2 medium for 1 week on PO-coated dishes.

3.3 Transplantation

In order to prepare neural precursor cells for transplantation, they were harvested after treatment with trypsin/EDTA and centrifuged (1100 rpm, 5 min, 4°C). Subsequently, cells were triturated to a single cell suspension, quantified, again centrifuged under the same conditions, and re-suspended at the desired cell concentration in Hanks' buffer.

3.3.1 Intra-uterine transplantation

Intra-uterine transplantations were performed as described (Brüstle *et al.*, 1995; Brüstle *et al.*, 1997a). Timed pregnant embryonic day 14.5 (E14.5) Sprague-Dawley rats were anesthetized (10 mg/kg Rompun, 80 mg/kg Ketanest) and placed on a 37°C plate. The uterine horns were exposed and the telencephalic vesicles of the embryos were identified under transillumination (Fig. 3.1 B). 0.5 – 1.0 x 10^5 cells were injected into the telencephalic vesicle using a glass capillary. Injected embryos were placed back into the abdominal cavity for spontaneous delivery.
On postnatal days 7 and 14, live-born recipient animals were sacrificed under deep anesthesia, followed by transcardial fixation with 4% paraformaldehyde in PBS. The brains were removed and postfixed in 4% PFA in PBS over night. The next day, brains were transferred to a sucrose-solution (30%) for 2 days. Afterwards, cryostat sections (50 µm) were prepared and placed onto microscopic slides. The slices were stored at -80°C until examination (see 3.5).

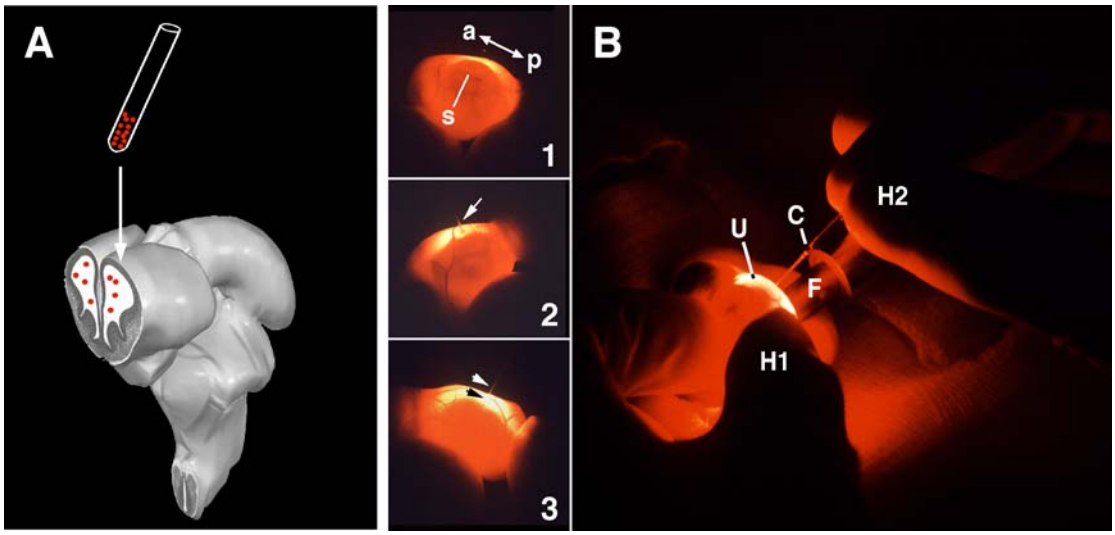

Fig. 3.1: Intrauterine transplantation approach

(A) Schematic representation. Donor cells are transplanted into the telencephalic vesicle of E16.5 embryonic rats. **(B)** Photograph of the transplantation process. Orientation of the cranial sutures before injection (1), and the path the injection capillary (arrows) from a dorsal (2) and a lateral (3) view. a=anterior, C=injection capillary, F=fiber optic light source, H=experimenter's hand, p=posterior, s=sagittal suture, U=uterus.
Brüstle (1997a).

3.3.2 Transplantation into the postnatal rat brain

Two-day-old (P.2) Sprague-Dawley rats were used for studying migration and differentiation of human ES cell-derived neural precursors in postnatal host CNS tissue. The rats were shortly anasthetized by hypothermia on ice for 4 min. Hypothermia was chosen to anesthetize P.2-rats, as anesthetics like ketamine or xylezine cannot be applicated to animals at this age without an pronounced increase in the mortality rate. The animals received 2 µl of a neural precursor cell suspension (1,5 x 10^5 cells/µl) in 2 deposits along the rostral-caudal axis of the right hemisphere by using a glass capillary. After transplantation, the rats were placed on a 37°C plate. Upon reaching their regular body temperature, they were placed back to the mother animal. 1, 2, 3, 4 and 8 weeks after transplantation, recipient rats were deeply anesthetized and perfused with 4% paraformaldehyde in PBS. The brains were removed and treated as described (see 3.5).

3.3.3 Rat hippocampal slice culture model

Using a vibroslicer, 400 µm horizontal sections were generated from the hippocampus of 9 – 10-day-old Wistar rats. The slices included the gyrus dentatus and the entorhinal and temporal cortex (Scheffler *et al.*, 2003). They were transferred onto a polyester membrane and cultivated at 35°C, 5% CO_2 and saturated air humidity in an initial culture medium containing 25% normal horse-serum, which was gradually replaced after 3 – 5 days by a chemically defined, serum-free culture medium based on DMEM/F12, N2-supplement and B27-supplement. Medium was changed every second day, and 5 – 7 days after explantation, a cell suspension of 1 – 25 x 10^4 neural precursor cells in a total volume of 20 µl was deposited onto the slices using an injection device.

For immunohistochemical analyses, cultures were fixed in 4% paraformaldehyde for 1 h and subsequently washed several times in PBS.

(The hippocampal slices were prepared and maintained by Barbara Steinfarz from our institute).

3.4 Immunocytochemistry

Immunocytochemical analyses of the cells were performed using primary antibodies and appropriate secondary antibodies labeled with CY3 or FITC (see 2.6). Nuclei were visualized by DAPI staining (1:10.000 in $NaHCO_3$, 4 min incubation).

Antibodies raised against O4, Tra-1-60 and Tra-1-81 were applied on living cells at 37°C in cultivation medium for 1 h, washed 2 x with appropriate culture medium and subsequently incubated with secondary antibodies for 1 h. The cells were washed 2 x for a total of 20 min in cultivation medium and were then directly fixed and stained with DAPI.

For all other markers, cells were fixed in 4% PFA for 10 min and washed in PBS. Cells were then blocked for 10 min in blocking solution containing FCS and incubated with primary antibodies diluted in blocking solution over night at 4°C (with 0.1 % Triton-X-100 for intracellular markers). Secondary antibodies were diluted in blocking solution and incubated for 2 h at room temperature (rt). The cells were washed in PBS, subsequently

stained with DAPI and again washed 2 x for a total of 20 min in PBS. The cells were then embedded in Mowiol and covered with a cover slide.
For immunocytochemical staining of human ES cell-derived neurospheres, spheres were fixed in 4% PFA and embedded in paraffin. Spheres were sliced with a microtome (4 µm) and subsequently examined histologically after hematoxylin and eosin (H&E) staining.
For quantification of the immunofluorescence stainings of stage P.1 neural precursors, a minimum of 500 cells was analyzed. For the quantification of neuronal and glial marker expression in differentiated cell populations, a minimum of 1000 cells per marker was scored in random fields (at 400x).
Expression of pluripotency markers in adherent colonies was quantified by counting 100 colonies in every experiment. Colonies were scored positive when ≥ one cell showed positive marker expression.

3.5 Immunohistochemistry

Different protocols had to be performed for immunohistochemical analyses of transplanted human ES cell-derived neural precursor cells, depending on the host tissue and the transplanted cell lines.

3.5.1 Staining of hippocampal slices

For immunohistochemical analyses, the sections were washed in PBS. Unspecific activity was blocked applying 10% normal goat serum for 30 min (rt). Primary antibodies were diluted in 5% normal goat serum over night (rt). On the following day, sections were washed in PBS and incubated with specific Cy3-conjugated secondary antibodies for 1 h. Following washing in PBS, the sections were mounted in Vectashield. For intracellular antigen-detection, 0.1%-0.3% Triton X-100 was added to the blocking- and antibody solutions.

3.5.2 Staining of transplanted human ES cells in host brain tissue

To identify human ES cell-derived transplanted cells in the rat brain tissue, cells first had to be labeled with a primary antibody raised against human nuclei. This antibody shows optimal results when applied in combination with tyramide signal amplification. The protocol consists of two steps:

Step 1: Rat brain slices on microscope slides were washed in PBS to dissolve the remaining tissue-tek. The slices were blocked in peroxidase (0.3%) for 15 min, and subsequently washed in PBS. This was followed by incubation with blocking buffer for 30 min. The slices were incubated with the primary antibody against human nuclei diluted in incubation buffer I with 0.5% Trition-X-100 over night in a wet chamber (rt). The following day, slices were washed in PBS and an appropriate secondary antibody coupled to biotin was applied in incubation buffer II with 0.5% Triton-X-100 for 4 h. Thereafter, the tissue was washed in PBS and streptavidin coupled to HRP (horse radish peroxidase) was applied for 1 hour. The slices were washed in PBS and tyramide solution (tyramide coupled to biotin) was added for 10 min. To visualize the labeled cells, slices were washed in PBS and incubated in fluorescein-coupled avidin in Hepes buffer (10 mM) for 2 h.

Step 2: To subsequently label the slices with another primary antibody, the slices were fixed again in 4% PFA for 15 min. After washing in PBS, they were incubated in blocking solution for 30 min. The primary antibody was applied in incubation buffer I with 0.1% Triton-X-100 over night in a wet chamber (rt). The following day, slices were washed in PBS, and an appropriate secondary antibody diluted in incubation buffer II with 0.1% Triton-X-100 coupled to CY3 was applied for 4 h. After washing in PBS, nuclei were visualized by DAPI-staining (1:10.000). Slices were washed as before, embedded in Mowiol, and covered with a glass cover slide.

Transplanted eGFP-expressing human ES cell-derived cells were not additionally labeled with a human-specific antibody. Staining with primary antibodies raised against the antigens of interest and appropriate secondary antibodies was performed as described above in step 2.

3.6 RT-PCR assays

Total RNA was extracted from human ES cells at different stages using the RNeasy Mini Kit according to manufacturers instructions (spinning protocol). The isolated RNA was quantified photometrically and 1 µg was used for the synthesis of cDNA. One µg RNA in $_{dd}H_2O$ and 1 µl oligo (dT) primer were diluted to a final volume of 10.5 µl in $_{dd}H_2O$ and incubated in a thermocycler for 10 min at 65°C. After that, 4 µl RT-buffer, 2 µl DTT, 0.5 µl RNAse inhibitor, 1 µl Reverse Transcriptase and 2 µl dNTPs were added and again incubated in a thermocycler at 37°C for 60 min, followed by incubation at 93°C for 5 min. Two µl of the generated cDNA was used in a PCR-reaction. Different primer sets were utilized, an overview of the sequences and reaction conditions can be found in chapter 2.7.
The composition of the components in the PCR-reaction were as follows:

2 µl cDNA
2 µl dNTPs
2 µl PCR-buffer
1 µl each forward und reverse primer
0.1 µl Taq polymerase
$MgCl_2$ (varies depending on primer, see 2.7)
$_{dd}H_2O$ (varies depending on the amount of $MgCl_2$)
Σ 20 µl

The PCR-reaction was performed in a thermocycler, with annealing temperatures varying depending on the primer. Negative and positive control templates were included in each PCR-reaction. PCR was performed in 0.5 ml-reaction tubes.

The individual steps were performed as follows:

94°C	4 min		
94°C	2 min	⎫	
X°C*	30 sec	⎬	30 cycles
72°C	1 min	⎭	
72°C	10 min		
4°C	∞		

For details see 2.7.

After the PCR-reaction was performed, 3.5 µl 6x loading buffer was added to each tube. The samples were electrophoretically separated on an agarose-gel (1.5% agarose in TAE-buffer, + 1 µl ethidiumbromide/10 ml buffer) at 100 V for approximately 20 min. The Agarose gels were exposed to UV-light in a gel documentation system to visualize DNA-bands.

4 Results

Prior to the establishment of two new strategies for the directed differentiation of human ES cells into neural precursor cells, preparatory work had to be performed.

4.1 Preparatory work

The first part of this work was to establish techniques for the handling of human ES cells in our laboratory. During a visit at the Technion Institute in Haifa, Israel, I gained insights into human ES cell culture techniques and transferred them to our Institute.
Within 6 months, basic procedures were established including cultivating, thawing, freezing, passaging and proliferation of human ES cells. For the long-term cultivation of undifferentiated human ES cells, optimal cell concentrations, cultivation periods, passaging procedures and cleaning processes (i.e. removal of differentiated cells) had to be established. This preparatory work resulted in operating protocols and media formulation protocols for the efficient cultivation of human ES cells. Four different human ES cell lines were cultivated and expanded: H9, H9.2, I3 and I6.
Two types of fibroblasts used as feeder layer were set up and compared: mouse fetal fibroblasts and human foreskin fibroblasts. Methods for freezing, thawing, cultivating and mitotic inactivation of feeder cells were optimized and adapted to the requirements of human ES cell culture.
Furthermore, several techniques for the analysis of pluripotent and differentiated human ES cells were established: Immunofluorescence markers, indicating pluripotency or differentiation into derivatives of the 3 germ layers, as well as neural differentiation markers were identified and optimized for utilization in human cells. This included also the adaptation of staining procedures, such as incubation times and temperatures, concentrations and buffer compositions.
In a set of pilot experiments, pluripotency markers were employed in FACS-sorting of undifferentiated human ES cells. To this aim, treatment of the cells prior to the sorting, sorting parameters and subsequent cultivation procedures were searched out.

Moreover, human-specific primers for RT-PCR indicating differentiation into the 3 germ layers or neural differentiation were established, as well as primer-specific PCR conditions. Different procedures for quantitative PCR were set up for the same primers.

4.2 Neural differentiation of human ES cells via EB formation

Since protocols for the differentiation of murine ES cells already exist (Okabe *et al.*, 1996; Brüstle *et al.*, 1999), this study aimed at establishing a similar strategy for neural differentiation of human ES cells. In proof-of-principle experiments, the enriched human ES cell-derived neural precursors were further analyzed after transplantation into host tissue.

In brief, the murine protocols are based on the induction of differentiation by aggregating ES cells to EBs. Subsequently, the EBs were plated and propagated in ITSFn medium to support proliferation and selection of neural precursor cells. Cells were then triturated to a single cell suspension and further cultivated in neural differentiation medium containing FGF-2. This strategy led to the enrichment of neural precursor cells from murine ES cells (for details, see 1.2.3 and the enclosed Fig. 1.6 of the murine differentiation protocol). The following results demonstrate that crucial differentiation steps had to be modified to translate the murine protocols for neural differentiation to human ES cells.

4.2.1 Pilot studies

First it was investigated, whether protocols for the neural differentiation of murine ES cells can be directly translated to human ES cells without any changes of cultivation conditions. This experiment revealed, that human ES cells under murine differentiation conditions did not differentiate into enriched neural precursors, but into a strongly heterogeneous cell population containing flattened epithelium-like cells and beating heart muscle cells of mesodermal origin.

To identify promising neural differentiation conditions for human ES cells, preparatory work had to follow. The time period of the individual differentiation steps, media constituents and coatings were varied and combined.

At this time point, neural differentiation was predominantly determined by morphological criteria. Early neural differentiation could be easily identified already within plated EBs or during the ITSFn-stage of the differentiation protocol. In these stages, where cells partially grew in multilayered cell communities, neural differentiation displayed a characteristic pattern, as primitive neural tube-like structures emerged, with the cells arranging in rosettes. Later on, after the cells were triturated to a single cell suspension, monolayer cultures of neural precursor cells still had a characteristic phenotype. The cells grew in rosette-like clusters, with their nuclei arranged radially and their processes building a star-shaped pattern.

4.2.2 Establishment of an EB-based protocol for the neural differentiation of human ES cells

Fig. 4.1 A schematically represents the crucial modifications of the murine protocols for neural differentiation, which made it possible to establish an EB-based protocol for human ES cells. The essential differentiation stages remained the same, such as aggregation to EBs, plating, cultivation in ITSFn medium, and subsequent trituration to a single cell suspension (Fig. 4.1 B-E). However, differentiation steps within individual stages had to be adapted to human ES cells.

At first, the differentiation conditions of the initial EB-stage had to be modified. The generation process itself had to be altered, as single human ES cells did not aggregate into EBs as murine ES cells. Human ES cell colonies (Fig. 4.1 B) had to be detached by collagenase IV-treatment instead of Trypsin-EDTA. This enabled the transfer of human ES cells as almost complete colonies to EB medium. Following this step, evenly shaped EBs could be obtained (Fig. 4.1 C). Compared to the murine protocol, the EB medium contained commercial Serum Replacement (SR) instead of fetal calf serum (FCS). EB medium containing FCS resulted in a more heterogeneous differentiation of plated EBs, in particular into epithelial phenotypes and tissue indicating beating heart muscle cells. In contrast, cultivation in SR yielded more neural tube-like structures within the plated EBs. The number of these neural structures could be even increased when the period of EB cultivation was extended to 14 days (murine protocol: 4 days).

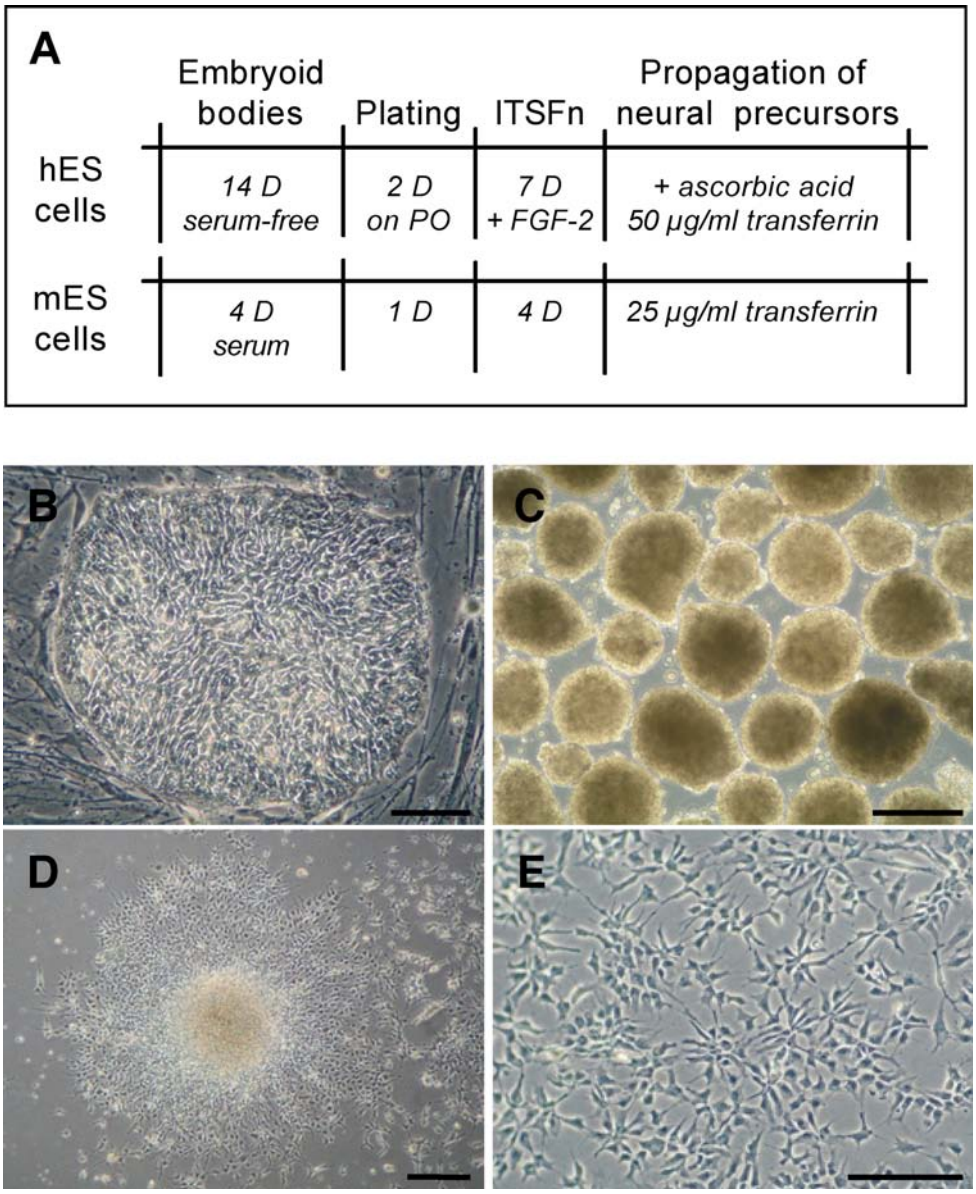

Fig. 4.1: Directed differentiation of human ES cells into neural precursor cells

(**A**) Schematic procedure for the generation of human ES cell (hES cell)-derived neural precursors (upper panel) in comparison to the existing protocol for murine ES cells (mES cells) (lower panel). Human ES cells grown on irradiated mouse fibroblasts (**B**) were aggregated to EBs and cultivated in serum-free suspension culture for 14 days (**C**). Subsequently, EBs were plated onto PO-coated dishes and after 2 days transferred to ITSFn medium supplemented with 20 ng/µl FGF-2 (**D**). Seven days later, outgrowing cells were triturated to a single cell suspension and transferred onto PO-coated dishes in NAA medium containing 10 ng/µl FGF-2 and 200 µM ascorbic acid. Passaging to stage P.1 led to highly enriched neural precursor cells (**E**). Scale bars: B 100 µm, C 250 µm, D 250 µm, E 200 µm.

Several coatings were tested to identify optimal plating conditions for human ES cell-derived EBs, as plating on uncoated dishes did lead to the appearance of neural tube-like structures, but not to a characteristic neural outgrowth with rosette-shaped structures. As murine neural precursors were cultivated on PO-coated dishes in later stages, it was tested if plating the EBs on PO may have a positive effect on neural differentiation. Indeed, it turned out that plating on PO-coated dishes led to more neural rosette-like morphologies in the outgrowth of plated EBs (Fig. 4.1 D). However, after 24 h not every single EB was plated. To avoid this problem, plating was extended to 2 days, leading to a higher number of plated EBs. Plated EBs were then transferred to ITSFn medium, with an extended cultivation time of 7 days compared to the murine protocol. Similar to murine ES cells, ITSFn medium promotes the elaboration of a neural phenotype, although elimination of non-neural cells is less efficient. In murine ES cells, massive cell death occurs in this stage, whereas in human ES cells such an effect could not be observed. This problem was challenged by simultaneously promoting neural precursor cell proliferation by the addition of 20 ng/ml FGF-2 to the ITSFn medium.

After 1 week in ITSFn medium, outgrowing neural precursor cells were triturated to a single cell suspension and further propagated in neural differentiation medium containing FGF-2 (10 ng/ml). In contrast to murine neural precursor medium N3, the human neural differentiation medium was supplemented with ascorbic acid (AA), which improved the survival of neural precursor cells. Doubling the amount of transferrin also had a positive effect, as it decreased cell death within the culture and led to higher plating efficiencies after passaging. Additional passaging on PO in NAA medium led to a homogenous precursor cell population referred to as P.1 (Fig. 4.1 E).

4.2.3 Characterization of human ES cell-derived neural precursor cells

The generated neural precursors in stages P.0 and P.1 could be easily frozen and thawed. Upon plating, stage P.1 precursors showed the typical morphology of primitive neural cells, as they arranged in rosette-like architectures (Fig. 4.1 E). To further characterize their phenotype, proliferating cells in stage P.1 were subjected to immunofluorescence analyses.

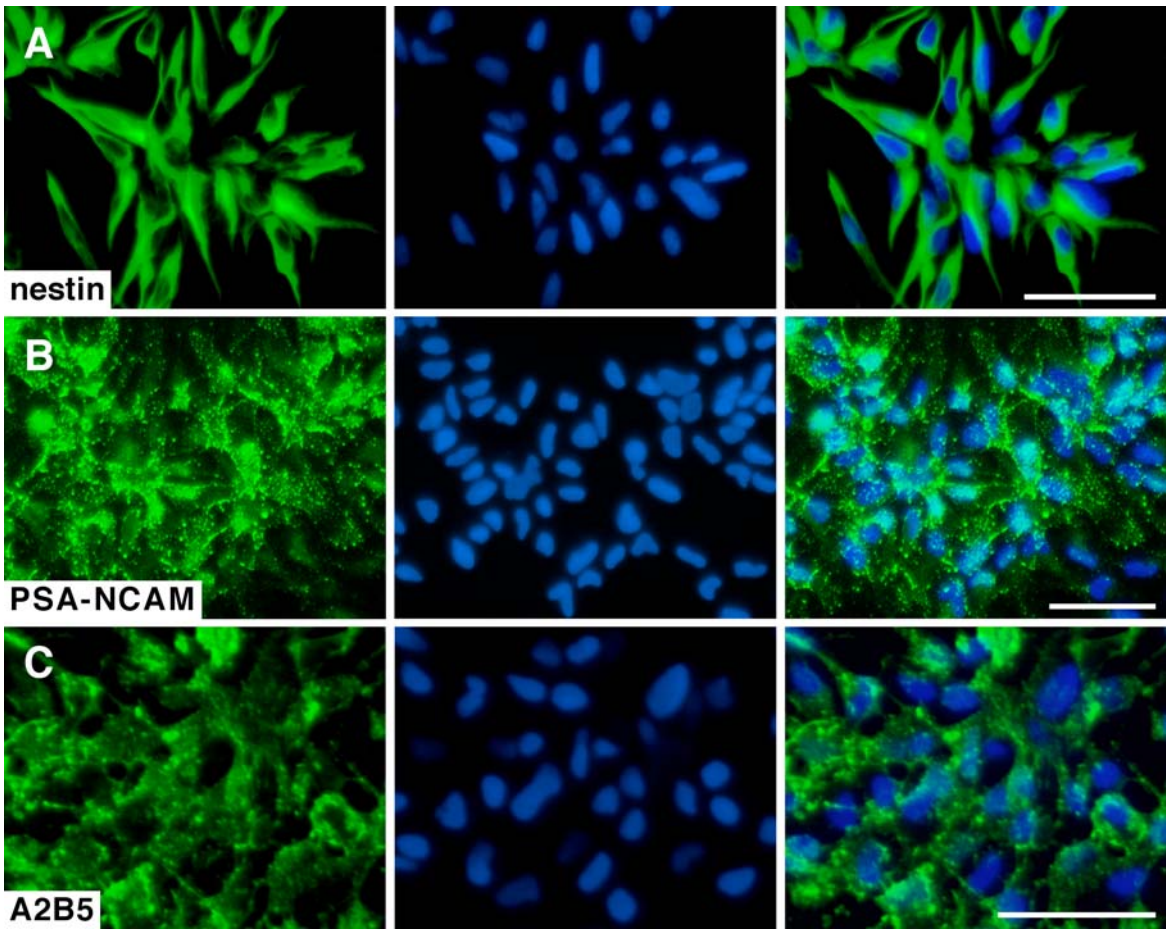

Fig. 4.2: Human ES cell-derived neural precursors express neural markers

Immunofluorescence analyses of proliferating neural precursors (stage P.1). Left column: Cells were stained with antibodies to nestin (**A**), PSA-NCAM (**B**) and A2B5 (**C**) and counterstained with DAPI (middle column). Right column: overlay. Scale bars: A – C 50 μm.

Neural precursor cells showed expression of nestin (98±0.6%) and the neural markers PSA-NCAM (65±3.1%) and A2B5 (63±6.9%) (Figs. 4.2 + 4.3). Only occasional cells had differentiated spontaneously into neurons expressing ß-III-tubulin (5±1.8%). To explore contamination of the obtained neural precursor population with non-neural cell types, immunofluorescence analyses were performed with markers specific for other lineages. Cells of endodermal, epithelial, and mesodermal origin expressing alpha-fetoprotein (AFP), epithelial membrane antigen (EMA) and smooth muscle actin (SMA) respectively were only detected in negligible amounts (< 1 of 10^6 cells).

We were able to passage human ES cell-derived neural precursor cells up to 3-4 times, in 1 experiment also up to passage 5 (P.5). During these propagation steps, an up-regulation of PSA-NCAM and A2B5 could be obtained, accompanied by a down-

regulation of nestin. Already in passage P.1, the number of cells expressing PSA-NCAM (65%) and A2B5 (63%) raised in contrast to passage P.0 (Fig. 4.3). In a representative experiment performed with P.5 cells, 78% expressed PSA-NCAM and 74% expressed A2B5, whereas only 85% expressed nestin. The number of spontaneously differentiated neurons expressing ß-III-tubulin slightly decreased in passage P.1 (5%).

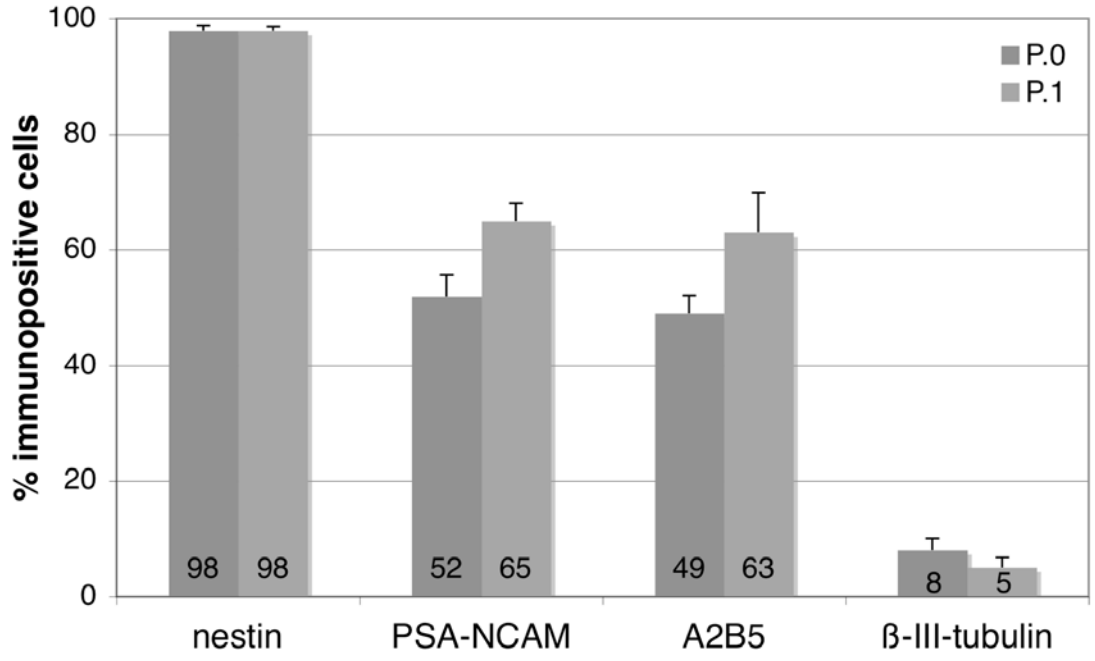

Fig. 4.3: Changes in the expression profile of human ES cell-derived neural precursors during cultivation

Fluorescence analyses revealed that during P.0 to P.1, expression of nestin remained constant in neural precursor cells, whereas PSA-NCAM and A2B5 is up-regulated. The number of cells spontaneously differentiating into neurons expressing ß-III-tubulin decreased slightly during prolonged cultivation.

4.2.4 *In vitro* differentiation potential of human ES cell-derived neural precursors

Differentiation of neural precursor cells into neuronal phenotypes was performed with highly confluent P.1 precursor cells in N2-supplemented media by growth factor withdrawal. One week after growth factor withdrawal, a huge population of the cells had adapted a neuronal morphology, accompanied by expression of the neuronal markers MAP2ab and ß-III-tubulin (42±15%, Fig. 4.4 A-B). The high variability might depend on the cell density, as highly confluent cells were used. In this case, a standardized and

similar cell density could not be ensured, as the amount of spontaneous cell death differed in the individual experiments, although similar starting densities were used.

The cells were stained immunocytochemically for dopaminergic, cholinergic, serotonergic, glutamatergic and GABAergic transmitter expression, whereas serotonergic transmitter types could not be detected. Other transmitter types were not analyzed. Staining for vGlut1, a transporter of glutamate, revealed no reliable results, as all cells were positive in several experiments. Thus, a background effect is more likely. Remarkably, 22±1.2% of all cells showed expression of tyrosine hydroxylase (TH), amounting to 54% of all ß-III-tubulin-expressing neurons (Fig 4.4 C).

In a single experiment, several clusters of CHAT expressing neurons could be obtained, a transmitter associated to cholinergic neurons. The CHAT-positive cells were not counted, as 3 positive experiments are necessary for representative numbers. In all 3 experiments, occasional cells showed the expression of GAD67, a marker of GABAergic differentiation. The numbers of GAD67-positive cells were very low, approximately under 1% of all cells.

To initiate glial differentiation, confluent P.1 cells were cultivated in G5 media supplemented with 0.2 µl/ml PDGF. After 8 weeks, 54%±8% of the cells expressed GFAP (Fig 4.4 D). Under these conditions, only the minority of the cells showed a neuronal phenotype. Differentiation into oligodendrocytes expressing O4 or CNPase was not detectable.

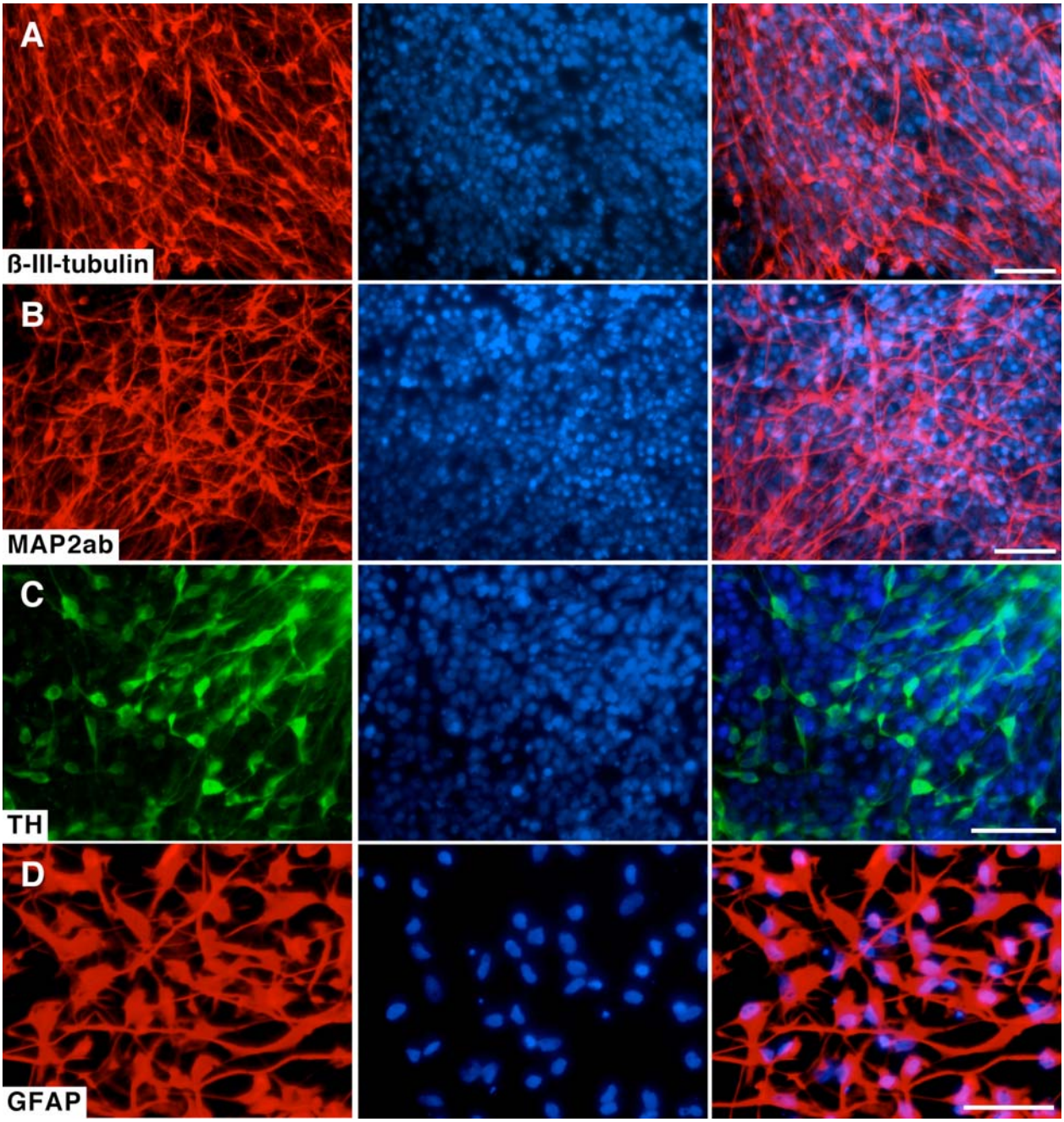

Fig. 4.4: Differentiation potential of human ES cell-derived neural precursors

Immunofluorescence analyses of human ES cell-derived neurons and glia. One week after growth factor withdrawal, neural precursors stage P.1 cells had differentiated into neurons expressing ß-III-tubulin (42±12%, **A**) and MAP2ab (**B**). The most frequently detected neurotransmitter-associated marker was TH (22±1.2%, **C**). After 8 weeks in a glial-promoting medium, the majority of the cells expressed GFAP (54±8%, **D**). Cells were counterstained with DAPI. Scale bars: A-D 50 µm.

4.2.5 *In vitro* differentiation of the stably eGFP-transfected human ES cell line H9.2eGFPneo

A stably transfected ES cell line ubiquitously expressing eGFP is a helpful tool for transplantation experiments in animal models, as the donor cells can be easily identified by virtue of fluorescence. For this reason, Henrike Siemen from our institute transfected the human ES cell line H9.2 by electroporation with a viral construct encoding for eGFP under control of the CMV-Promotor. The viral construct carried a neomycin resistance gene to select for successfully transfected cells. The cell line can be permanently maintained in the presence of G418.

To explore whether our established *in vitro* differentiation protocol can be applied to other human ES cell populations, the eGFP-expressing subclone H9.2VI was subjected to the neural differentiation paradigm. During the differentiation process, eGFP-expression was down-regulated (Fig. 4.5). Already at the EB-stage, single EBs showed only partial expression of green fluorescence. During the ITSFn-stage, preferentially cells within the remaining clusters of plated EBs and cells in the outer outgrowth area showed eGFP-expression. Fluorescence was further down-regulated in neural precursor stages P.0 and P.1, with only 2.4±0.6% of all cells visibly expressing eGFP in the latter stage.

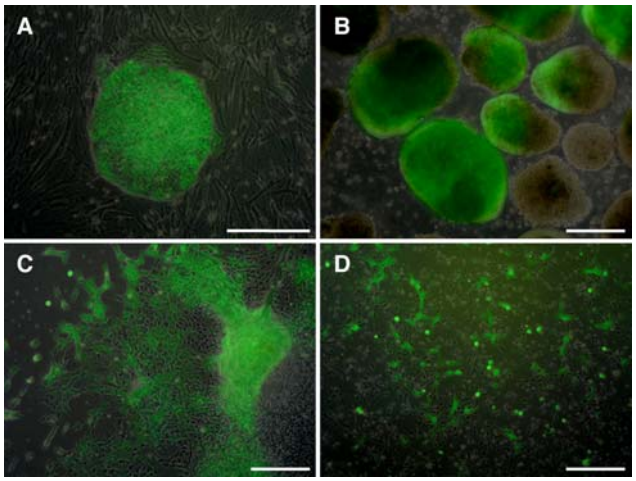

Fig. 4.5: Neural differentiation of the stably transfected cell line H9.2eGFPneo

Stably transfected human ES cells expressed eGFP throughout the individual colonies (**A**). Following aggregation to EBs, eGFP is expressed in a heterogeneous pattern (**B**). During the further differentiation stages ITSFn and neural precursor population P.0, a heterogeneous pattern of cells expressing eGFP and cells which have down-regulated the expression could be obtained (**C**+**D**). Scale bars: A 125 µm, B-D 250 µm.

4.2.6 Transplantation of human ES cell-derived neural precursors in a hippocampal slice culture model

A rat organotypic hippocampal slice culture model was used to assess the migration and integration potential of human ES cell-derived neural precursors after transplantation into host brain tissue. For this purpose, the human ES cell subclone H9.2VI, stably transfected with an eGFP expression construct was employed (see 4.1.5). Differentiation of human ES cells into neural precursors was performed according to the newly established protocol (see 4.1). Two days after passaging, neural precursors of the proliferative stage P.0 were deposited on a hippocampal slice.

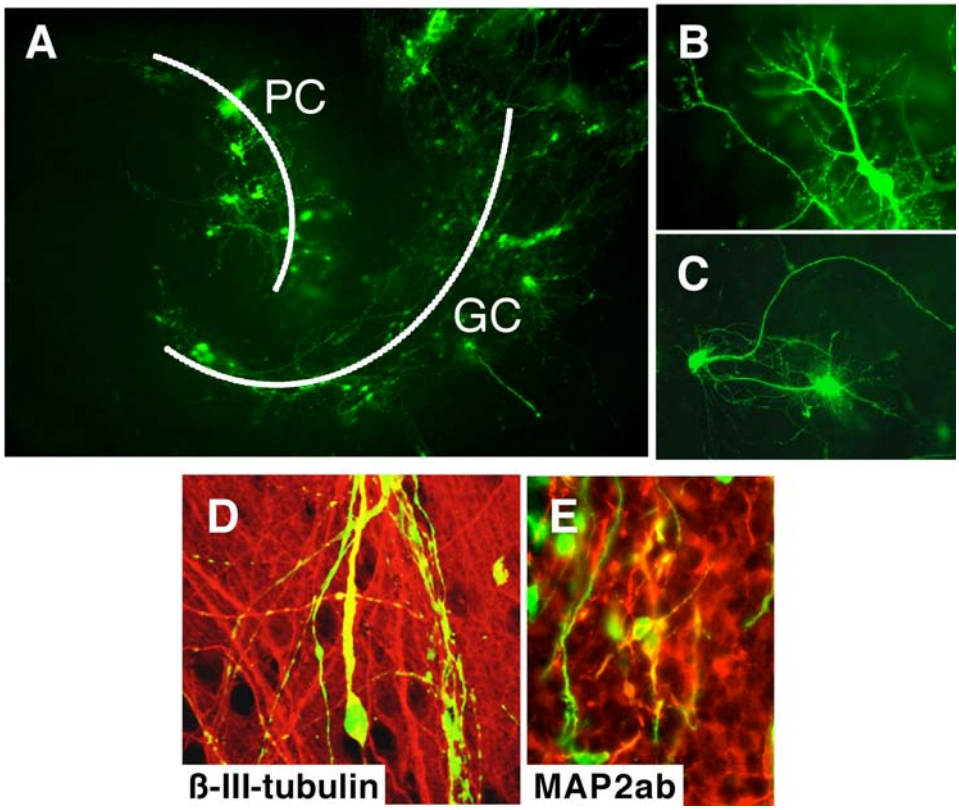

Fig. 4.6: Neuronal differentiation of engrafted human ES cell-derived neural precursor cells within a hippocampal slice culture

Nineteen days after transplantation, human ES cell-derived donor cells had engrafted in the hippocampal slice. Remarkably, they clustered within the pyramidal (PC) and granule cell layers (GC) (**A**). Incorporated donor-derived neurons showed long extensions (**B**, **C**) and expressed ß-III-tubulin (red, **D**) and MAP2ab (red, **E**). Donor cells were detectable by virtue of their native eGFP expression.

Three weeks after transplantation, the grafted cells had invaded the slice up to a depth of 150 µm. In particular in the area of the pyramidal and the granule cell layer, the cells had specifically clustered along these structures (Fig. 4.6 A). In the entire slice, engrafted cells had differentiated into neurons displaying long axon-like extensions and dendritic processes. The human ES cell-derived cells expressed the neuronal markers ß-III-tubulin and MAP2ab (Fig. 4.6 D-E).
Although eGFP-expression is down-regulated in later differentiation stages *in vitro*, these experiments revealed a strong expression after transplantion into host tissue.

4.2.7 Functional characterization of ES-cell derived progeny following incorporation into hippocampal slice cultures

In a next step, it was investigated whether transplanted human ES-cell derived neural precursor cells have the ability to functionally integrate into CNS host tissue. To that end, cells from neural precursor stage P.0 were transplanted onto 5 – 7-day-old hippocampal slices on day 2 after passaging or after 5 days of growth factor withdrawal. Subsequently, the engrafted cells were analyzed electrophysiologically. Electrophysiological studies were carried out by Christiane Rüschenschmidt and Heinz Beck from the Department of Epileptology, Bonn. Whole-cell voltage- and current-clamp recordings were carried out 15 to 27 days after transplantation from a total of 90 transplanted cells (for details of the methodology see Benninger *et al.*, 2003; Wernig *et al.*, 2004).
Cells could be clearly identified due to the backflow of eGFP into the recording pipette after attaining the whole-cell configuration (Fig. 4.7 A). To test for the capability of the cells to generate action potentials, current-clamp experiments were carried out in all cells. Only a small fraction (5 cells) of the recorded eGFP-positive cells displayed regenerative spikes upon current injection with an amplitude ranging from 30 to 75 mV that could be classified as immature action potentials (Fig. 4.7 B). However, none of these cells were capable of generating repetitive discharges. Moreover, action potential morphology was immature (regarding amplitude and half width) compared to host neurons recorded within the same culture preparation. Fig. 4.7 B shows traces of an eGFP-positive cell that was capable of generating action potentials and received spontaneous synaptic input at a frequency of ~3 Hz (Fig. 4.7 C). Although these

findings indicate that human ES-cell derived neurons can, in principle, develop excitable membrane properties and receive synaptic contacts, it is important to note that the large majority of cells did not develop into functional neurons.

Most of the eGFP-positive cells displayed very small spikes upon prolonged current injection (250 ms), with amplitudes ranging from 2.5 to 20 mV. These were suppressed by blocking Na^+ channels with 0.5 µM tetrodotoxin in 3 cells, whereas 50 µM Ni^{2+}, a blocker of T-type Ca^{2+} channels, did not have an effect. Thus, such spikelets seem to be mediated predominantly by voltage-gated Na^+ channels. The remainder of eGFP-positive cells showed passive membrane properties upon current injection.

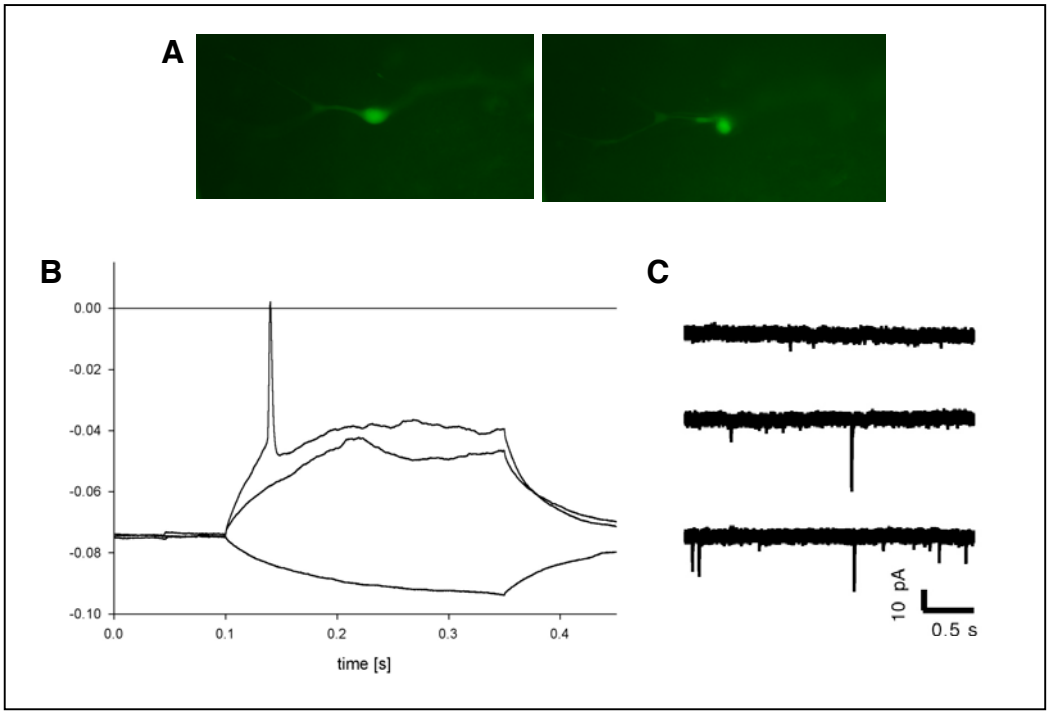

Fig. 4.7: Functional analysis of eGFP-labeled human ES-cell derived neural precursors after incorporation into hippocampal slice cultures

Example of a recorded cell: Cell before the recording (left) and after rupturing of the cell membrane with eGFP backflow into the pipette (right) (**A**). Current-clamp recording: an immature action potential is elicited by depolarizing current injection. (**B**) Spontaneous synaptic input in the same cell recorded in voltage-clamp mode at a holding potential of -80 mV (**C**). (Mesurements were perfomed by Christiane Rüschenschmidt and Heinz Beck, Department of Epileptology, Bonn).

In voltage-clamp mode, the cells were analyzed for their ability to receive synaptic input. The current patterns mirrored the variability seen in current-clamp experiments.

Fig. 4.7 C shows a cell, which displays excitatory postsynaptic currents. Cells that were capable of generating action potentials displayed the highest density of voltage-gated Na^+ currents. A subset of the remaining cells also showed Na^+ currents (36% of cells). Most cells expressed a variety of K^+ currents, such as fast (A-type, 26% of cells) and slowly (delayed rectifier, 90% of cells) inactivating currents. Moreover, many cells exhibited inward rectifier K^+ currents as a reaction to hyperpolarisation. The slow K^+ currents were sensitive to 10 mM TEA-chloride, as expected (n=3). A small fraction of GFP-positive cells corresponding to those with passive membrane properties appeared to lack voltage-dependent currents, exhibiting instead very large leak conductances reminiscent of mature astroglial cells.

4.2.8 Transplantation into the pre- and postnatal rat brain

With the aim to investigate the migration, integration and survival potential of human ES cell-derived neural precursors *in vivo*, we performed intrauterine transplantations into the ventricular system of embryonic rats. For this purpose, human ES cell-dereived neural precursor cells generated according to the new EB-based protocol were injected into the ventricles of E16.5 rats. Two weeks after transplantation, animals were fixed and the brains were explanted for immunhistochemical analysis. The donor cells had formed clusters at the ventricular wall and integrated as single cells. Cells were found in a variety of host brain regions including cortex, hippocampus, bulbus olfactorius, striatum, septum, thalamus, hypothalamus, tectum, corpus callosum, subventricular zone, brain stem and cerebellum (Fig. 4.8 A). Human ES cell-derived neural precursors had differentiated and displayed a neuronal phenotype with axon-like extensions. The majority of the incorporated cells expressed the neural markers ß-III-tubulin and MAP2ab (Fig. 4.8 B - C). Expression of specific transmitter phenotypes (GABAergic, dopaminergic, cholinergic, serotonergic and glutametergic) could not be detected. However, after two weeks, the transplanted neurons might still be in a very immature stage. Glial cells could not be found in both transplantation approaches.

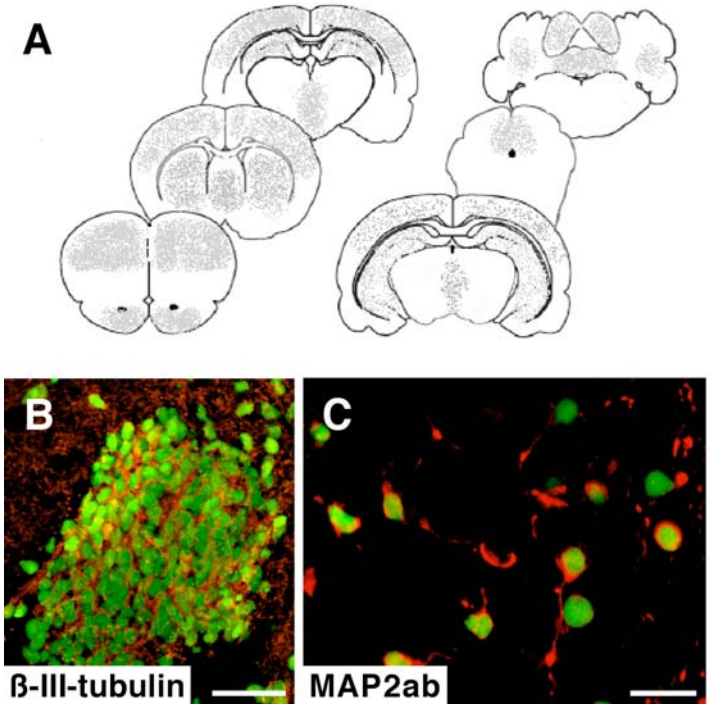

Fig. 4.8: Integration and neuronal differentiation of transplanted human ES cell-derived neural precursors after transplantation into the developing rat brain

Four weeks after transplantation, human ES cell-derived neural precursor cells were found in numerous host brain regions. (**A**) Grey dots symbolize regions harboring integrated donor-derived cells. (**B**) Donor cell cluster showed prominent expression of ß-III-tubulin (red; septal region). (**C**) MAP2ab-positive human ES cell-derived neurons (red) incorporated into the host cortex. Donor cells were identified with an antibody to human nuclei (green). Scale bars: B 40 µm, C 20 µm.

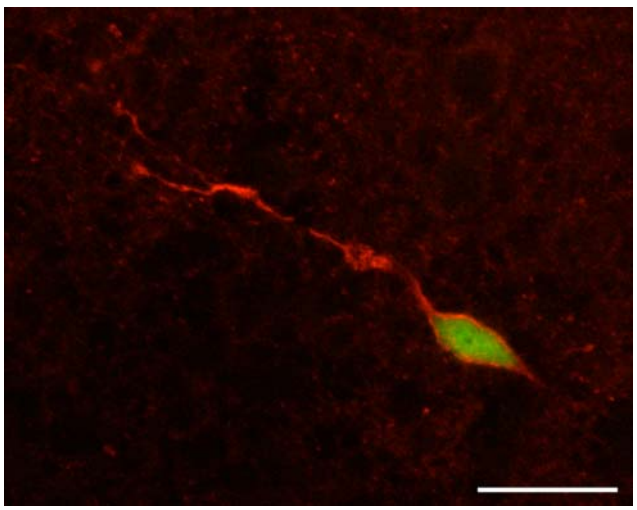

Fig. 4.9: Neuronal differentiation after transplantation into P2-rats

Immunofluorescence analysis of a human ES cell-derived neuron two weeks after transplantation into P2-rats. The majority of the transplanted cells differentiated into neurons expressing ß-III-tubulin (red) and migrated deep into the host tissue. Donor-derived cells were identified with an antibody to human nuclei. Scale bar: 20 µm.

4.3 Differentiation of human ES cells into neural precursors in a two-step protocol

A major aim of this study was to establish a new protocol for the generation of human ES cell-derived neural precursor cells, which bypasses the formation of embryoid bodies and permits the direct conversion of human ES cells into neurogenic precursors. The new strategy includes two main differentiation steps. In a first step, human ES cells propagated as adherent cultures on extracellular matrix proteins are induced to differentiate into the neural lineage in differentiation media containing FGF-2. In a second step, the adherent cells are proliferated to form detaching neurospheres. Upon plating, these neurospheres give rise to a homogenous population of neural precursors capable of generating neurons, astrocytes and oligodendrocytes.

4.3.1 Strategy for the direct neural conversion of human ES cells

The newly established *in vitro* differentiation protocol comprises several sequential steps including induction of differentiation of human ES cells under adherent culture conditions, followed by cultivation as neurospheres. A schematic protocol of the key steps is depicted in Fig. 4.10.

In a first step, human ES cells were treated with the cell-dissociation reagent Accutase II. This step permits efficient enzymatic detachment of the human ES cell colonies, whereas feeder cells and differentiated flat cells remain largely attached. Detached human ES cells were triturated to a single cell suspension and transferred onto Matrigel-coated dishes (stage A.0). As human ES cells did not plate in neural differentiation medium, KO/SR medium had to be used in this step. After 24 h, the cells were transferred to neural differentiation medium containing FGF-2, and subsequently passaged twice in the same medium on Matrigel (stages A.1 and A.2). In the adherent stages A.0 – A.2, the cells changed their morphology towards more densely packed colonies (Fig. 4.10 B+C). After a total cultivation time of 8 days in monolayer culture, colonies from stage A.2 were detached by collagenase IV-treatment and subsequent scraping.

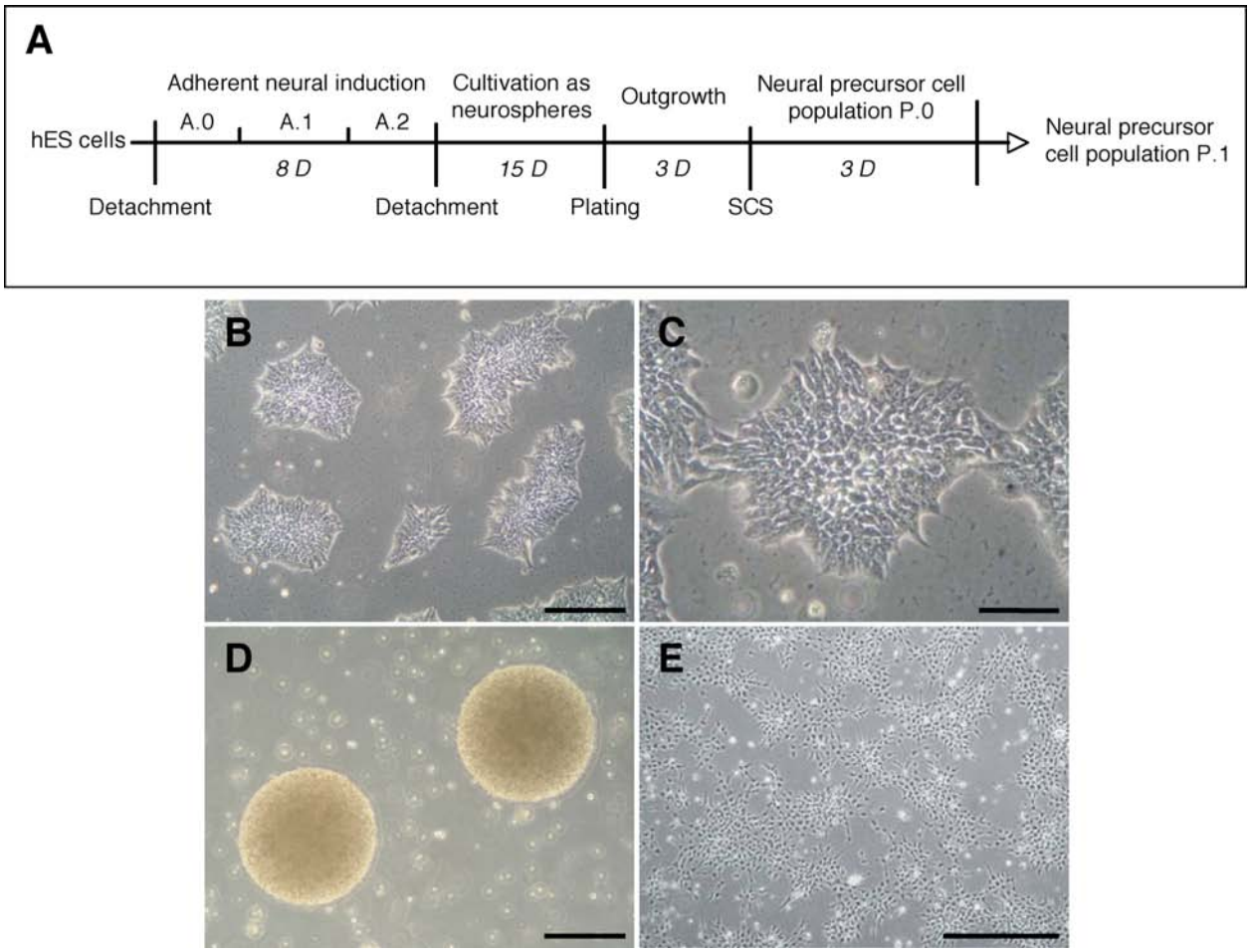

Fig. 4.10: Directed differentiation of human ES cells into neural precursor cells

(**A**) Schematic representation for the generation of human ES cells (hES cell)-derived neural precursors. Pluripotent human ES cells were detached by Accutase II-treatment and plated onto Matrigel-coated dishes in serum-free KO/SR medium (stage A.0). The following day, cells were transferred to NAA medium containing 10 ng/µl FGF-2. After 3 days, cells were transferred onto fresh Matrigel-coated dishes (stage A.1). This procedure was repeated once (stage A.2) with the cells being propagated as adherent culture for a total of 8 days. At this time point, the cells grew as well-defined radially oriented colonies (**B**+**C**). These colonies were detached and further cultivated for 15 days in suspension culture as neurospheres (**D**). After this period, neurospheres were plated onto tissue culture dishes. After 3 days, outgrowing neural precursor cells were triturated to a single cell suspension (SCS), replated (population P.0.), and subsequently passaged (population P.1, **E**). Scale bars: B 250 µm, C 100 µm, D 250 µm, E 500 µm.

The aggregates were further cultivated in suspension culture as spheres in neural differentiation medium (Fig. 4.10 D). To avoid any adherence, the spheres were cultivated in petri dishes placed on a shaker. Under these conditions, spheres developed within 1 day. After 15 days, the neurospheres were plated on cell culture dishes. Within 3 days, massive outgrowth of nestin-positive neural precursor cells was

observed. The cells were triturated to a single cell suspension leading to neural precursor stage P.0. After 3 days, the cells were further passaged to neural precursor stage P.1. This strategy yielded a homogenous neural precursor cell population (Fig. 4.10 E).

4.3.2 Human ES cells differentiate into the neuroectodermal lineage in monolayer culture

Under monolayer conditions in stages A.0 – A.2, human ES cells changed their morphology towards more densely packed 'puffy' colonies (Fig. 4.11 B+C). During these stages, immunofluorescence analyses showed a decreasing expression of the pluripotency markers TRA-1-60 and TRA-1-81 (Fig. 4.11 A-C).

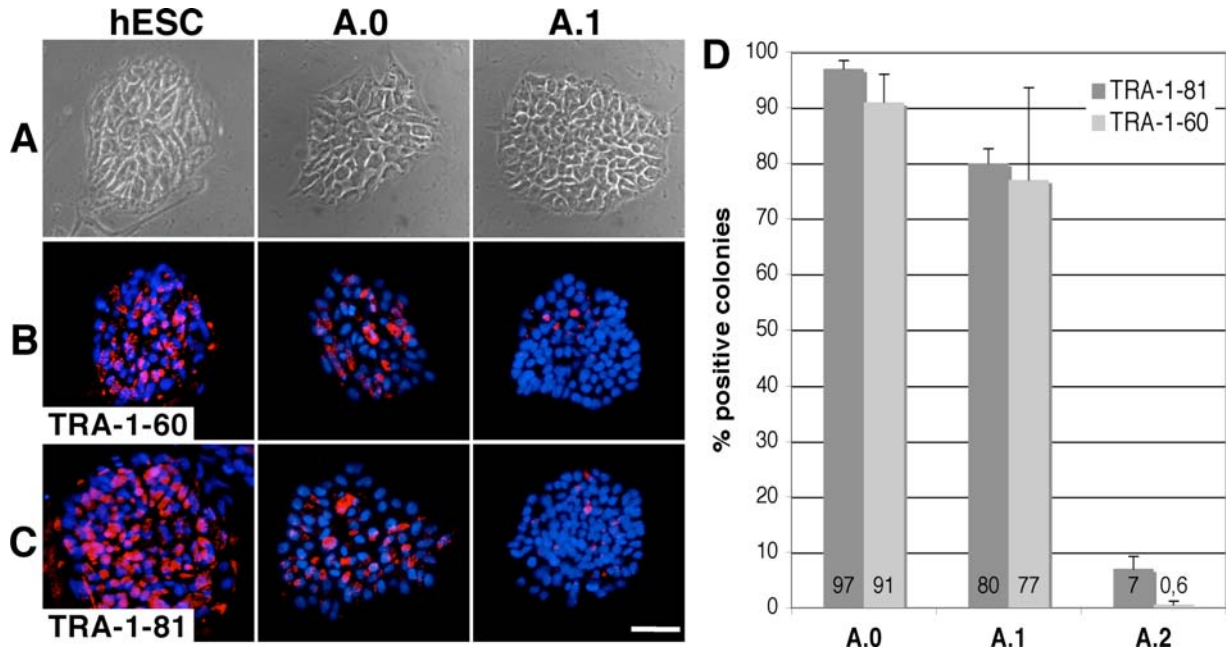

Fig. 4.11: Down-regulation of pluripotency markers during adherent differentiation

Phase contrast (**A**) and immunofluorescence analysis of TRA-1-60 (**B**) and TRA-1-81 expression (**C**). Both markers were down-regulated during the adherent differentiation stages A.0 and A.1. In stage A.2, only occasional cells had retained residual expression of the pluripotency markers (not shown). Scale bar: 50 µm. (**D**) The diagram shows the percentage of positive colonies expressing TRA-1-60 and TRA-1-81. Every colony that showed at least 1 positive cell was counted as positive. In stages A.0, expression of both markers exceeded 90%. In stage A.1, still up to 80% of the colonies expressed pluripotency markers, followed by a pronounced down-regulation in stage A.2.

Pluripotent human ES cells cultivated on feeder cells showed prominent expression of the pluripotency markers. During cultivation under adherent conditions in stages A.0 and A.1, pluripotency markers were strongly down-regulated. In stage A.2, hardly any expression was detectable by immunofluorescence analyses (data not shown).

To study the down-regulation of pluripotency-associated markers in detail, colonies expressing TRA-1-60 and TRA-1-80 were counted. Whereas at stage A.0 more than 90% of the colonies were immunoreactive for both markers, these values dropped to 0.6% TRA-1-60-positive and 7% TRA-1-81-positive colonies at stage A.2, respectively (Fig. 4.11 D).

RT-PCR analyses were performed to further investigate the declining pluripotent character of the colonies and the onset of differentiation.

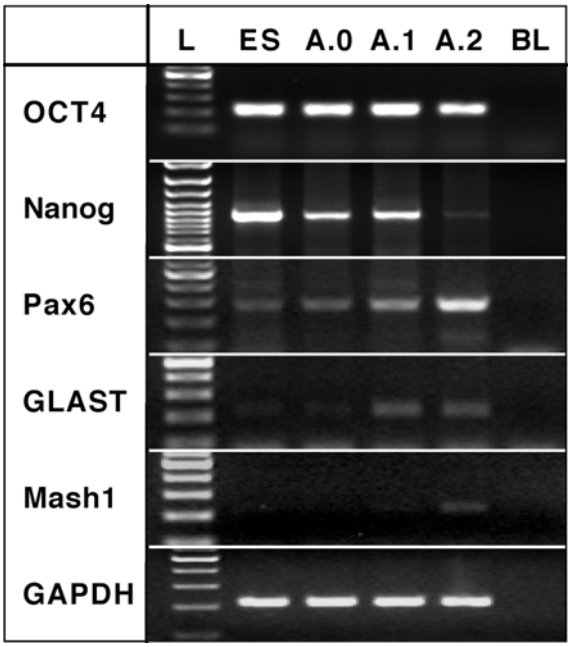

Fig. 4.12: RT-PCR-analyses of pluripotency-associated and early neural markers during adherent differentiation

RT-PCR analyses were performed with pluripotent human ES cells and cells of the adherent stages A.0 – A.2 (L: DNA-ladder (thick band: 500 bp), BL: blank). During adherent differentiation, cells showed a down-regulation of the pluripotency markers Oct4 and nanog and a strong up-regulation of Pax6. The neural marker Mash1 was up-regulated in A.2, and the radial glia marker GLAST was up-regulated in stages A.1 and A.2.

The analyses revealed changes in the expression profile during stages A.0 – A.2, which are compatible with the onset of neural differentiation (Fig. 4.12). The cells showed a down-regulation of the pluripotency markers Oct4 and nanog, with almost no expression

of nanog and a decreased expression of Oct4 in stage A.2. This was accompanied by an up-regulation of the pro-neural markers Pax6 and Mash1. In addition, GLAST, which is expressed in radial glia cells, was found slightly up-regulated.

4.3.3 Characterization of the human ES cell-derived neurospheres

Colonies from stage A.2 were detached and further cultivated as neurospheres in NAA medium. Under these conditions, spheres developed within 1 day. In contrast, no sphere formation was observed in medium used for the growth of EBs, suggesting that not EBs but neurospheres had developed.

Neural differentiation was supported by histological analysis of 5-day-old spheres, which exhibited rosette formations characteristic of primitive neural tissue (Fig. 4.13 A). On day 10, these rosette formations had disappeared in the majority of the spheres. At this time point, the cells showed a more homogeneous distribution. Focally, the nuclei formed cellular ribbons with rhythmic nuclear palisading (Fig. 4.13 B). Fifteen days after generation, the cells in the majority of neurospheres were less densely packed and the spheres displayed an evenly rounded shape (Fig. 4.13 C).

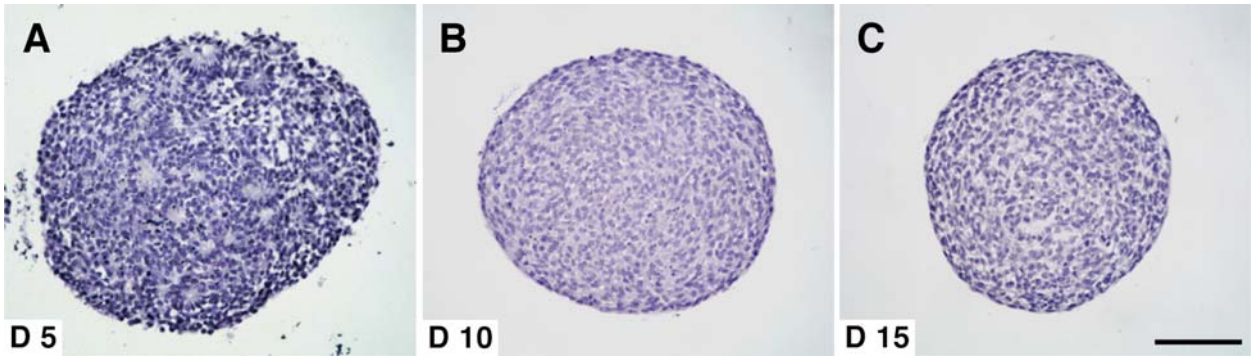

Fig. 4.13: Histological analyses of human ES cell-derived neurospheres

During neurosphere development, the cells exhibited characteristic changes in histoarchitecture (H&E-staining). At day 5 (**A**), the spheres exhibited rosette architectures with virtual centers. At later stages (day 10, **B**; day 15, **C**), the cells showed a more homogeneous distribution with focal cell ribbons and rhythmic nuclear palisading. Scale bar: 100 µm.

Immunofluorescence analyses were used to further characterize the differentiation of the spheres in slices of 5-, 10- and 15-day-old spheres. The spheres showed no expression of alpha-fetoprotein (endoderm), cytokeratin (epithelium) and desmin

(mesoderm), suggesting, that the cells had not entered into multi-germlayer differentiation. However, we noticed occasional areas within the neurospheres containing smooth muscle actin-positive cells.

To confirm the neural identity of the spheres, immunofluorescence analyses on 5-day-old neurospheres were performed with antibodies to nestin, and the neural marker A2B5 (Fig. 4.14). Nestin-expressing cells were distributed equally across the spheres, forming neural rosette-like structures (Fig. 4.14 A). A2B5 showed a patchy expression within the single spheres (Fig. 4.14 B). Occasional neural precursors had already differentiated spontaneously into neurons expressing ß-III-tubulin (data not shown). Immunofluorescence analyses with the neural marker PSA-NCAM revealed a homogenous but weak expression throughout the spheres (data not shown).

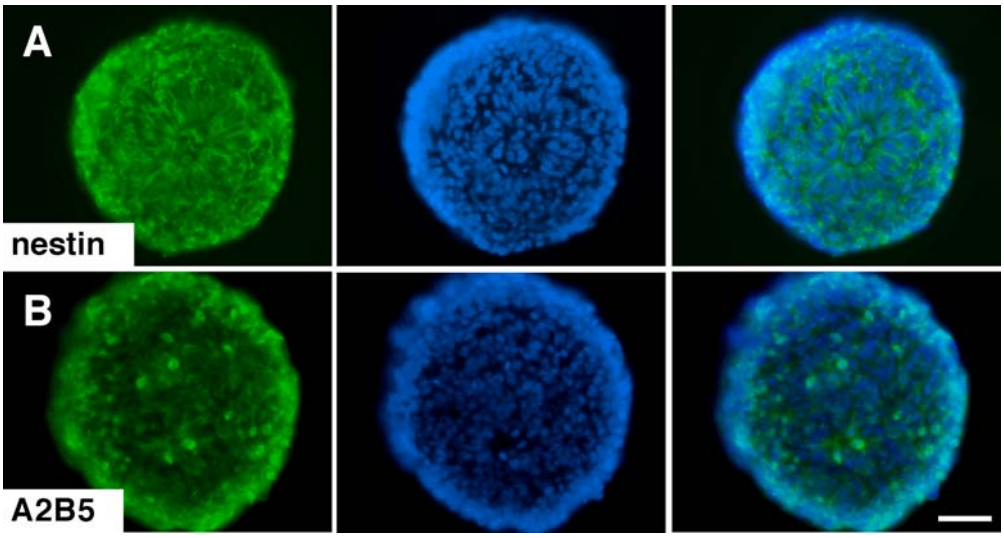

Fig. 4.14: Expression of neural markers in 5-day-old neurospheres

Immunofluorescence analyses of 5-day-old human ES cell-derived neurospheres to nestin (**A**) and A2B5 (**B**). Nuclei were visualized by DAPI-staining. Scale bar: 50 μm.

4.3.4 Neurospheres give rise to enriched neural precursor cells

After 15 days, neurospheres were plated on uncoated tissue culture dishes. Within 3 days, a massive outgrowth of nestin-positive neural precursor cells was observed. The cells were triturated to a single cell suspension (population P.0) and passaged again 3 days later (population P.1).

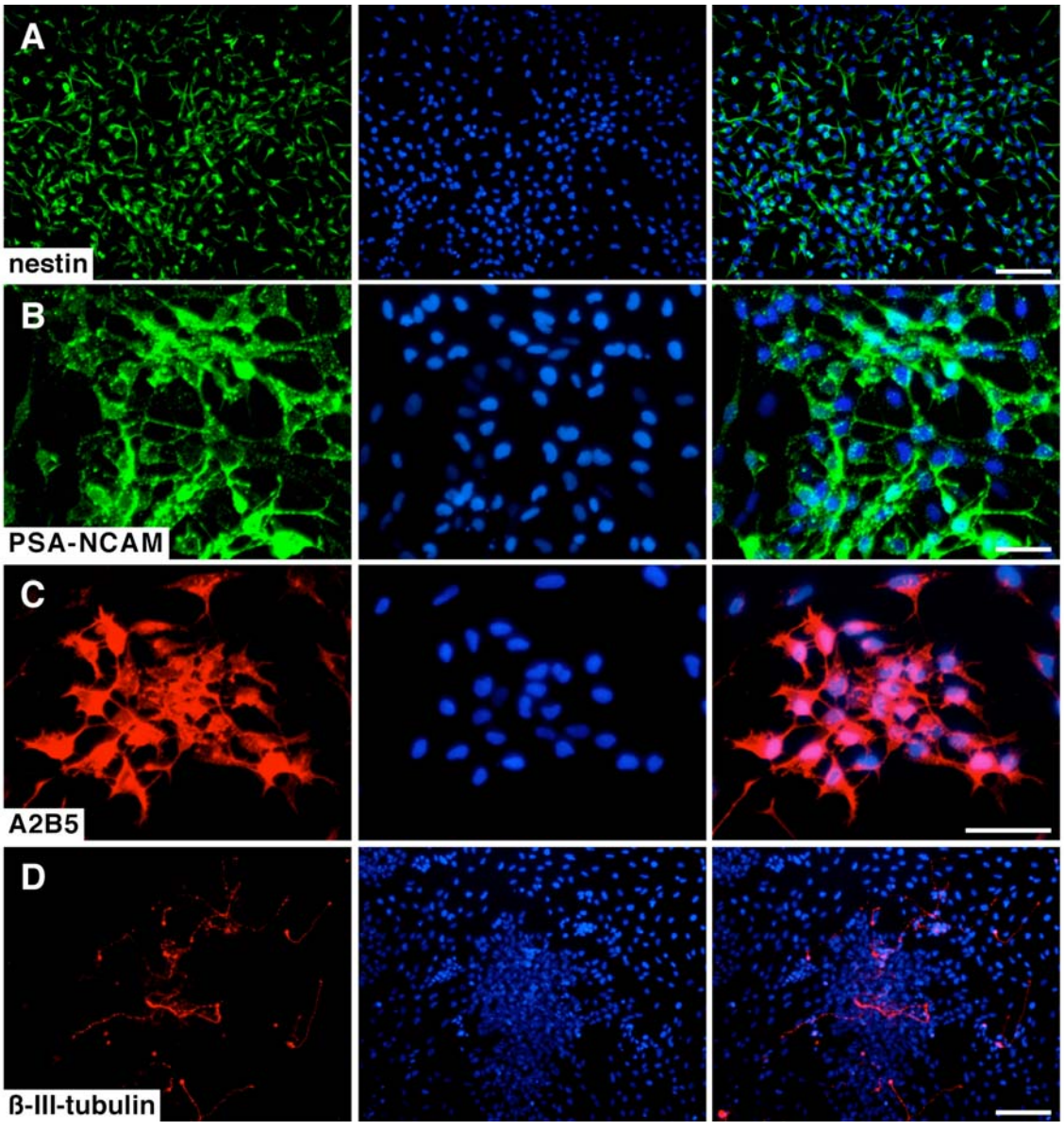

Fig. 4.15: Immunofluorescence analysis of neurosphere-derived neural precursor cells

Neural precursors derived from plated neurospheres (population P.1) express nestin (**A**), PSA-NCAM (**B**) and A2B5 (**C**). In the presence of FGF-2, only a small fraction of the cells undergo spontaneous differentiation into ß-III-tubulin-positive neurons (**D**). Scale bars: A, D 100µm, B, C 50 µm.

P.1 cells showed typical neural rosette formation and expressed nestin (96±3.5%) and the neural markers PSA-NCAM (37±40.7%) and A2B5 (96±1.7%; Fig. 4.15 A-C). Few cells spontaneously differentiated into neurons expressing ß-III-tubulin (4±3%, Fig. 4.15 D). Furthermore, immunofluorescence analyses were performed to detect contamination with other lineages. Although no cells positive for markers of other lineages were detected in the neurospheres, cells of endodermal (AFP), epithelial (EMA), and mesodermal (SMA) origin were detected in negligible amounts in the neurosphere-derived monolayer cultures (< 1 of 10^6cells). These cells might have arisen from resident pluripotent cells in the neurospheres.

4.3.5 Differentiation potential of human ES cells, directly converted into the neural lineage

To study the differentiation potential of neurospheres, the spheres were propagated under growth factor withdrawal for 15 days in N2 medium. Massive axonal and dendritic outgrowth from neurons within the plated neurospheres occurred within few days after plating, indicating neuronal differentiation. Neuronal differentiation increased during further cultivation. Fluorescence analysis revealed accompanying expression of the neuronal marker ß-III-tubulin (Fig. 4.16 A).

To induce glial differentiation, P.0 cells were propagated under growth factor withdrawal for a total of 6 weeks. They were first propagated in N2 medium for 2 weeks, followed by a 3-week-cultivation in NAA medium and another week in N2 medium. These conditions yielded extensive differentiation into astrocytes expressing GFAP and displaying typical multipolar extensions (Fig. 4.16 B). Furthermore, occasional 04-positive oligodendrocytes were noticed (Fig. 4.16 C+D). This cell type was located in clusters, with the cell bodies of single cells branching out into fine extensions. Together, these findings demonstrate that cells generated according to the newly established protocol have the potential to give rise to all three neural lineages.

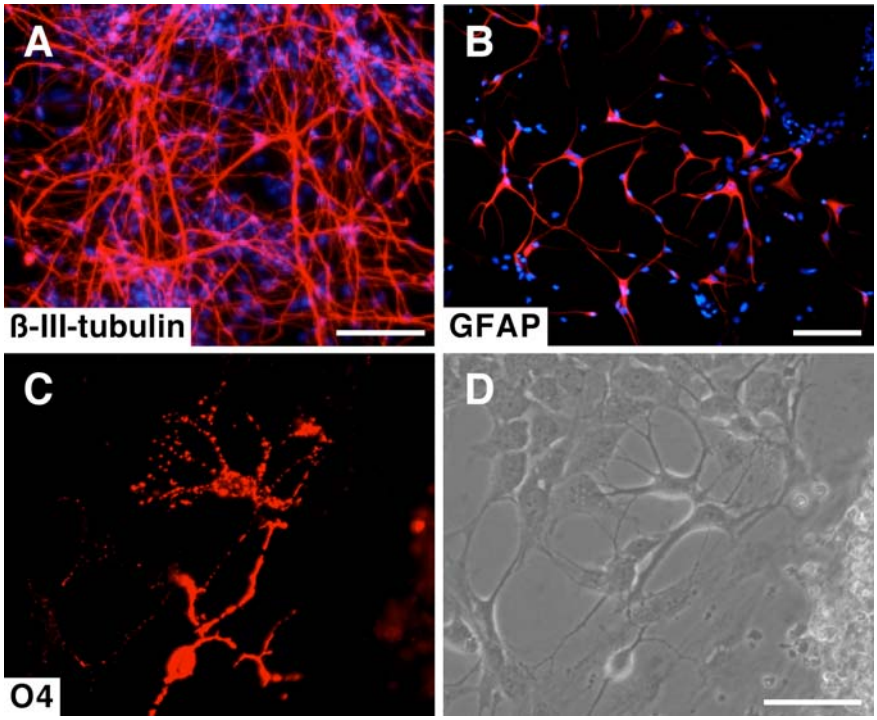

Fig. 4.16: Pan-neural differentiation of neurosphere-derived precursors

Following growth factor withdrawal in NAA and N2 media, immunofluorescence analyses revealed differentiation into ß-III-tubulin-positive neurons (**A**), GFAP-positive astrocytes (**B**) and oligodendrocytes expressing O4 (**C**, **D**; phase contrast).
Nuclei are counterstained with DAPI. Scale bars: A-B 100 µm, C-D 50 µm.

5 Discussion

5.1 A newly established EB-based differentiation protocol permits the generation of enriched human ES cell-derived neural precursors

During the last couple of years, a variety of protocols based on EB formation were established for murine ES cells to generate multipotent neural and glial precursors at high purities (Bain *et al.*, 1995; Fraichard *et al.*, 1995; Strübing *et al.*, 1995; Okabe *et al.*, 1996; Li *et al.*, 1998; Brüstle *et al.*, 1999; Bibel *et al.*, 2004). The first part of this study focused on the question whether such protocols can be established for human ES cells. To this aim, protocols for the directed differentiation of murine ES cells into neural precursors were modified and translated to human ES cells (Okabe *et al.*, 1996; Brüstle *et al.*, 1999).

The newly established protocol enables a directed differentiation into human ES cell-derived neural precursors. The majority of protocols already existing for human ES cells are mainly based on the mechanical isolation and further propagation of primitive neural tube-like structures from plated EBs or differentiated ES cells. (Reubinoff *et al.*, 2001; Zhang *et al.*, 2001; Conti *et al.*, 2005). Others depend on lineage selection strategies to specifically isolate neural cell types from a heterogeneously differentiated cell pool (Carpenter *et al.*, 2001). Such intermediate isolation steps are not required in this newly established protocol. Likewise it is not required to induce differentiation into the neural lineage by cocultivation with somatic cell lines (e.g. PA6) or the usage of stromal-cell conditioned medium (Park *et al.*, 2005; Shin *et al.*, 2005).

5.1.1 Human and murine ES cells do not react similar to neural differentiation conditions

Findings from this study demonstrate that differentiation protocols for murine ES cells based on an EB-differentiation step, can - in principle – be established for human ES cells too. However, it is necessary to modify crucial steps in the differentiation protocol.

This is due to the different properties of murine and human ES cells (see 1.2.1). First of all, human ES cells have a far longer doubling time than murine ES cells in their pluripotent stage. In their differentiated state, they also show longer differentiation periods. For example, differentiation of human ES cell-derived neural precursors into oligodendrocytes after growth factor withdrawal may take up to 6 weeks, in contrast to 4 days for murine neural precursors cultivated in FGF-2 and EGF (see 1.2.3). This is probably due to the longer and more complex human embryonic development. Furthermore, although many molecular pathways are conserved between mouse and human, crucial pathways for the maintenance of pluripotency or differentiation processes differ strongly or do not exist in one of the species. An obvious example is the Lif/Stat3 pathway, which leads to the maintenance of pluripotency in murine ES cells, but not in human ES cells. Therefore it is not surprising, that protocols for neural ES cell differentiation cannot be transferred 1:1 from one species to the other. The unique properties of human ES cells have to be taken into account by extending single differentiation steps or by varying growth factors and morphogens to induce neural differentiation.

The most obvious difference in the neural differentiation protocol is the different reaction of human and murine cells in ITSFn medium. In murine ES cells, a strong selective effect induces massive cell death of non-neural cells, resulting in a relatively homogenous neural population after 4 days of cultivation (Okabe *et al.*, 1996; Brüstle *et al.*, 1999). A promoting effect of ITSFn towards a neural phenotype was previously shown in the neuronal differentiation of embryonic carcinoma cell lines (Rizzino and Crowley, 1980).

In contrast, nearly no visible cell death occurred in human ES cell-derived populations, and non-neural cells with flat epithelial morphology could still be observed. However, a promoting effect of ITSFn medium on neural precursor cells remained in human ES cells, although it is less strong than in murine ES cells. This effect seems not to be caused primarily by elimination of other lineages, but by support of the endogenous neural differentiation bias.

As a sufficient selective effect on neural precursors was not achievable under these conditions, subsequent experiments focused on modifications aiming at a stronger proliferation of neural precursors to expand the already existing population. For this reason, FGF-2 was added to the ITSFn medium. This growth factor was previously employed to promote proliferation of mechanically isolated human ES cell-derived

neural precursors in ITSFn medium (Zhang *et al.*, 2001). Members of the FGF family possess a broad mitogenic potential. FGF signaling is mediated by binding to and activation of a family of receptor tyrosine kinases (Lee *et al.*, 1989; Givol and Yayon, 1992; Jaye *et al.*, 1992; Eswarakumar *et al.*, 2005). Binding to these receptors leads to phosphorylation of several down-stream proteins, resulting in the activation of the Ras/Map kinase signaling pathway (Kouhara *et al.*, 1997). In addition, FGFs can also bind to heparan sulfate proteoglycans (HSPG). These low-affinity receptors do not mediate a biological signal but function as mediators for FGF-binding to and activation of their signaling receptors (Yayon *et al.*, 1991; Ornitz *et al.*, 1992; Spivak-Kroizman *et al.*, 1994; Lin *et al.*, 1999; Eswarakumar *et al.*, 2005). FGF-2 itself is implicated in several biological processes as for example tumor growth, tissue repair and angiogenesis (Givol and Yayon, 1992; Grose and Dickson, 2005; Presta *et al.*, 2005). Of specific interest for this study is the influence of FGF-2 on nervous system development. In the developing brain, it has effects on proliferation, differentiation, migration and cell survival of neural cell types (Sensenbrenner, 1993; Brickman *et al.*, 1995; Gremo and Presta, 2000; Abe and Saito, 2001). Early studies already demonstrated a strong mitogenic effect of FGF-2 on neural stem and precursor cells (Gensburger *et al.*, 1987; Cattaneo and McKay, 1990; Kilpatrick and Bartlett, 1993; Ray *et al.*, 1993; Ghosh and Greenberg, 1995; Vicario-Abejón *et al.*, 1995). Indeed, after addition of FGF-2 to the ITSFn medium, a visible increase in the number of typical neural morphologies emerging from the plated EBs, as neural rosette-like structures and the cells arranged in a star-shaped pattern, could be observed.

Another crucial factor, which strongly influences ES cell differentiation, is the supplementation of FCS and SR, respectively. In the protocols for neural differentiation of murine ES cells, EBs were cultivated in medium containing FCS. Repeating these conditions with human ES cells yielded a heterogenous cell population. The outgrowth of the plated EBs contained cells resembling flattened epithelium or primitive endoderm, as well as beating heart muscle cells. In contrast, when human EBs were cultivated in SR, neural tube-like structures frequently appeared after plating of the EBs. These findings suggest that FCS inhibits early neural differentiation. Similar observations were made by Gerecht-Nir (Rambam Medical Center, Haifa, Israel; personal communication). However, FCS has also been used for the maturation of terminally differentiated murine ES cell-derived neurons (Okabe *et al.*, 1996; Li *et al.*, 1998).

The initiating pilot studies depicted the cultivation time of the EBs as another key factor for the enrichment of neural precursors from human ES cells. In other protocols for the generation of human ES cell-derived neural cell types, EBs were only cultivated for up to 10 days before plating (Zhang *et al.*, 2001). In contrast, our experiments showed that a cultivation time of more than 10 days led to higher numbers of neural rosette-like structures in the outgrowth of plated EBs. On the other hand, a cultivation time of more than 20 days was too long, as neuronal morphologies with long axon-like extensions frequently appeared within the plated EBs. Finally, a cultivation time of 14 days provided an optimal balance between high numbers of neural precursors and low rates of spontaneous differentiation. This notion is confirmed by a recent study on human EBs demonstrating that the early neural marker Sox1 is strongly up-regulated in 14-day-old EBs (Bhattacharya *et al.*, 2005).

Following trituration to a single cell suspension, neural precursors were cultivated on PO-coated dishes, where they formed characteristic morphologies as neural precursors arranged in neural rosette-like structures with compact cell bodies. On other coatings, such as fibronectin-, laminin- or gelatine, these properties were not that prominent and the cells were often flat and evenly distributed on the dish. Future studies might address the question, what kind of interaction processes between the neural precursors and specific coatings are underlying these morphological changes.

Although the conditions were already optimized, neural precursor populations still had to be stabilized. During monolayer cultivation and in particular after passaging, cell death could be observed, resulting in poor plating efficiencies. Furthermore, this cell death impaired proliferation, as neural precursors need to be cultivated in a sufficient density. In the murine ES cell differentiation protocol, cells are cultivated in N3FL medium, which is supplemented with transferrin, progesterone, putrescine, sodium-selenite, laminin, and FGF-2 (Okabe *et al.*, 1996; Brüstle *et al.*, 1999). After doubling the amount of transferrin, an improved survival effect could be obtained. Transferrin is the key protein for iron transport and uptake in the organism, an element which is essential for many homeostatic functions including enzymatic activities. Furthermore, transferrin seems to have other functions. This might at least partially contribute to its neurotrophic properties, but the underlying mechanism is not fully elucidated yet (Gomme *et al.*, 2005). Okabe *et al*. already demonstrated for ITSFn medium that the component transferrin has a general survival effect on murine ES cell-derived neural precursors (1996). Earlier experiments already demonstrated a strong proliferative

effect of transferrin in various cell types (Trowbridge and Omary, 1981; Mendelsohn *et al.*, 1983; Kan and Yamane, 1984; Neckers, 1984; Barakat-Walter *et al.*, 1991; Bruinink *et al.*, 1996).

In addition, ascorbic acid (AA) was added to the medium, which has already been shown to have a positive effect on cell survival due to its antioxidantic and antitoxic properties (Studer *et al.*, 2000; Gaetke and Chow, 2003; Duarte and Lunec, 2005). Together, these modifications yielded enriched human ES cell-derived neural precursor cells in high quantities, comparable to the murine protocol. Human ES cell-derived precursors differentiated into neurons and astrocytes after growth factor withdrawal. These properties demonstrate that the generated cell population consists of multipotent neural precursors that have the potential to differentiate into the neuronal and the glial lineage.

The high numbers (22%) of TH-positive neurons observed after growth factor withdrawal may be caused by the treatment with AA prior to induction of differentiation. For murine ES cells, Lee *et al*. demonstrated that treatment of neural precursor cells with sonic hedgehog (SHH) and FGF-8 followed by AA-treatment led to a more than 2-fold increase in TH-positive cell yield (2000). Even if differentiating neural precursor cells were treated with AA alone, the number of TH-positive cells significantly increased compared to untreated cells. In cultured proliferating midbrain neuroblasts, even a 5- to 7-fold enhancement of TH-positive neurons could be obtained, when AA was added to a medium containing SHH and FGF-8 (Volpicelli *et al.*, 2004). Other studies on the same cell type revealed an increase of neurons in general and of TH-positive neurons in particular after incubation with AA (Yu *et al.*, 2004). Similar observations were made with other cell types. Studies from Seitz and coworkers revealed that human neuroblastoma cells treated with AA for 5 days had a 3-fold increase of tyrosine hydroxylase gene expression (Seitz *et al.*, 1998). Generally, AA was demonstrated to promote the generation of neurons from ES cells (Shin *et al.*, 2004). In human ES cells, too, AA was already applied to enhance differentiation into TH-positive neurons (Perrier *et al.*, 2004; Park *et al.*, 2005; Yan *et al.*, 2005). Advantageously, the new EB-protocol requires no additional treatment with SHH and FGF-8 or co-cultivation with stromal cell lines to generate high numbers of TH-positve cells (Buytaert-Hoefen *et al.*, 2004; Perrier *et al.*, 2004; Zeng *et al.*, 2004; Park *et al.*, 2005; Yan *et al.*, 2005).

In contrast to murine neural precursors, human ES cell-derived precursors were not able to generate oligodendrocytes under the established conditions (Okabe *et al.*, 1996;

Brüstle *et al.*, 1999). However, they gave rise to both the neuronal and glial lineage, as they generated neurons and astrocytes. This observation may be due to a more restricted neural precursor cell type or the fact that medium conditions have to be further optimized for the generation of oligodendrocytes. Another possibility is to extend the differentiation period.

Murine ES cell-derived neural precursors, which were first cultivated in FGF-2 and EGF, followed by a treatment with EGF and PDGF, led to enriched glial precursors (Brüstle *et al.*, 1999). In human ES cells, this growth factor combination did not lead to a comparable enrichment of this cell type. Under such a treatment, human ES cell-derived neural precursors proliferated very slowly, and the survival rate after passaging was quite low. Experiments will have to follow to identify optimal conditions for the generation of enriched human ES cell-derived glial precursors.

5.1.2 Neural-specific markers confirm the identity of human ES cell-derived neural precursors

In general, neural precursor cells are multipotent, meaning that they can differentiate into neurons and glial cells. Neural precursor cells also express characteristic antigens.

The intermediate filament nestin was used to identify ES cell-derived neural precursor cells in earlier studies (Okabe *et al.*, 1996). In the meantime, nestin expression has also been found in precursor cells of the skeletal muscle, testes and teeth (Sejersen and Lendahl, 1993; Kachinsky *et al.*, 1995; Terling *et al.*, 1995; Frojdman *et al.*, 1997). It was also detected in mature cells such as adrenal cortex cells of the kidney, blood vessel cells, interstitial cells and astrocytes (Lin *et al.*, 1995; Tsujimura *et al.*, 2001; Bertelli *et al.*, 2002; Klein *et al.*, 2003). This broad expression pattern demonstrates that this marker is not sufficient for the identification of neural precursor cells. However, it is crucial to demonstrate nestin expression when characterizing neural precursor cells as it is expressed in neuroepithelial cells of the early neural tube and in radial glia cells (Hockfield and McKay, 1985; Lendahl *et al.*, 1990), as well as in cultured neural stem cells (McKay, 1997). Two surface markers are more reliable and commonly used to identify neural precursors: A2B5 and NCAM with its polysialylated isoform PSA-NCAM. PSA-NCAM is expressed in neural precursor cells of the developing CNS (Hekmat *et al.*, 1990; Kleene and Schachner, 2004). However, it is not restricted to neural precursor

cells, but is expressed again in later stages of the neuroectodermal lineage (Seki and Arai, 1993; Minana *et al.*, 1998; Cremer *et al.*, 2000; Bruses and Rutishauser, 2001; Glaser *et al.*, 2005). In human ES cell-derived neurons, PSA-NCAM was found to be expressed after 1 week of growth factor withdrawal (not shown). This finding further supports the view that terminal maturation of human ES-cell derived neurons takes more than 1 week, as PSA-NCAM is down-regulated at later stages of neuronal differentiation. Although PSA-NCAM is used as a specific marker for the neural lineage, it has also been shown to be ectopically expressed in cells of other tissues (Husmann *et al.*, 1989; Gerety and Watanabe, 1997; Muller-Rover *et al.*, 1998).

A2B5 was demonstrated to be expressed by glial precursor cells in mouse and human (Raff *et al.*, 1983; Gard and Pfeiffer, 1990; Fok-Seang and Miller, 1994; Scolding *et al.*, 1999; Dietrich *et al.*, 2002). In human ES cells, it was previously used as a pan-neural marker, since it was expressed by precursor cells developing into astrocytes and neurons (Carpenter *et al.*, 2001). In murine ES cells on the contrary, it is used as a glial precursor marker (Raff *et al.*, 1983; Brüstle *et al.*, 1999) .

PSA-NCAM, A2B5 and nestin were expressed by human ES cell-derived neural precursor cells generated according to the new protocol and thus confirm their neural identity. Other studies obtained a similar spectrum of marker expression in human ES cell-derived neural precursor cells (Carpenter *et al.*, 2001; Reubinoff *et al.*, 2001; Zhang *et al.*, 2001; Gerrard *et al.*, 2005; Tabar *et al.*, 2005).

Another frequently utilized marker for the identification of neural precursor cells is the RNA-binding protein musashi. It has been detected in mammalian neural stem and progenitor cells, as well as in astroglial progenitors and astrocytes (Sakakibara *et al.*, 1996; Sakakibara and Okano, 1997; Kaneko *et al.*, 2000; Keyoung *et al.*, 2001). Apart from the CNS, musashi is also expressed in distinct cells of the small intestine (Kayahara *et al.*, 2003; Potten *et al.*, 2003). In this study, human ES cell-derived neural precursors could not be stained specifically with this marker, probably due to a problem with the antibody.

Two transcription factors involved in early neural differentiation are also used to identify neural precursor cells: Pax6 and members of the Sox gene family encoding for Sry-related transcription factors. Sox1, 2, and 3 are expressed in the developing neuroepithelium of vertebrate embryos and have been implicated in the specification and maintenance of the neuroectodermal lineage (Pevny *et al.*, 1998; Bylund *et al.*, 2003; Graham *et al.*, 2003; Kan *et al.*, 2004; Zhao *et al.*, 2004). Sox1 is a useful tool for

neural stem cell identification, as it is the earliest neuroectodermal marker expressed during neural plate and neural tube formation (Pevny *et al.*, 1998). However, recent studies revealed the expression of Sox genes in pluripotent human ES cells (Okumura-Nakanishi *et al.*, 2005; Rodda *et al.*, 2005 2005; Western *et al.*, 2005). Therefore, other neural-specific markers have to be applied to confirm the neural identity of human ES cell-derived progeny.

Pax6 is a homeobox-transcription factor, whose first expression can be detected as the neural tube begins to fold. During further development of the nervous system, Pax6 is expressed in forebrain, midbrain, hindbrain and the spinal cord (Walther and Gruss, 1991 1997; Mansouri *et al.*, 1994; Grindley *et al.*, 1995). It was also found to be expressed in radial glia cells and ES cell-derived neural precursors (Callaerts *et al.*, 1997; Sun *et al.*, 1998; Schwarz *et al.*, 1999; Englund *et al.*, 2005; Ikeda *et al.*, 2005).

Immunofluorescence analyses with commercially available antibodies to Sox1 and Pax6 showed specific expression in murine ES cell-derived neural precursor cells. However, no expression could be detected in neural precursors derived from human ES cells according to the newly established protocol. In accordance with the results generated in this work, other authors, too, did not detect an expression of Sox1 (Carpenter *et al.*, 2001; Reubinoff *et al.*, 2001) or Pax6 (Zhang *et al.*, 2001) in human ES cell-derived neural precursors. This is probably due to different cultivation conditions and time periods yielding distinct populations of neural stem and precursor cells. Li and coworkers reported sequential stages of generated neural progenitors. They demonstrated that an early population of Sox1-negative and Pax6-positive cells within neural rosette-like structures is followed by a population positive for both markers (Li *et al.*, 2005a). This neural precursor cell type appears within further differentiated neural tube-like structures with an inner lumen.

In the developing CNS, distinct precursor cell populations expressing different sets of markers are also generated. For example, Pax6 is expressed by radial glia cells, but not by proliferating precursors of the VZ and the SVZ (Gotz *et al.*, 1998; Hartfuss *et al.*, 2001; Tarabykin *et al.*, 2001; Malatesta *et al.*, 2003; Englund *et al.*, 2005). In contrast, members of the Sox-family were detected in proliferating neural progenitors throughout embryogenesis except in radial glia cells (Pevny *et al.*, 1998; Wu *et al.*, 1999). However, since differentiation of ES cells is not precisely recapitulating *in vivo* developmental processes, it is hard to discuss observations from the *in vitro* system in the context of developmental data. The generated cells *in vitro* are somehow artificial, thus the

expression or the absence of a specific markers don't have to be similar to *in vivo* expression patterns.

5.1.3 Human ES cell-derived precursors differentiate into immature neurons after transplantation onto a hippocampal slice

To analyze whether human ES cell-derived neural precursors generated according to the EB-based protocol were able to functionally integrate into the CNS tissue, cells were transplanted onto rat hippocampal slice cultures. Although immunofluorescence analyses revealed differentiation into neurons, only a minority of the cells showed weak regenerative spikes upon current injection that could be classified as immature action potentials. The cells did not reserve synaptic input.

The cultivation period on hippocampal slice cultures (up to 27 days) might be to short for human ES cells to differentiate into functionally active neurons. Other authors obtained comparable results. For example, neurons derived from human neural progenitor cells and human ES cells were not able to fire spontaneous action potentials, although they expressed sodium and potassium channels (Piper *et al.*, 2000; Carpenter *et al.*, 2001). Progenitors derived from the human brain required up to 70 days before they developed ligand-gated currents (Chalmers-Redman *et al.*, 1997).

In future experiments, the differentiation period for human ES cell-derived neural precursors has to be extended. Consequently, hippocampal slice cultures may not be the suitable tool for such an application, as they can be cultivated for a maximum of 4 – 5 weeks. A more suitable approach might be the transplantation of human ES cell-derived precursors into the rat brain, and electrophysiological examination after a defined time period in acute brain slices. Recently, such experiments were performed with human ES cells by Muotri *et al*. (2005). Human ES cells were induced to differentiate by cocultivation with PA6 cells and subsequently transplanted intra-uterine. Acute slices from 18-month-old rats, which were transplanted according to this strategy, revealed human ES cell-derived neurons, which were capable of generating mature action potentials.

5.1.4 Human ES cell-derived neural precursors have the capacity to migrate and differentiate *in vivo*

After transplantation of neural precursor cells generated according to the EB-based protocol into the pre- and postnatal rat brain, the cells showed the potential to migrate within the adjacent CNS tissue and to differentiate into neurons expressing ß-III-tubulin and MAP2ab. The transplanted cells migrated into the host brain regions in a similar pattern as already demonstrated in previous studies with mouse and human fetal stem cells (Brüstle *et al.*, 1995; Brustle *et al.*, 1998). Longer survival of the engrafted cells might be required to detect glial cell types. The absence of astrocytes and oligodendrocytes might be due to the long differentiation periods human ES cells require to generate these cell types. Other groups found glial cell types 11 weeks after engraftment of human ES cell-derived neural precursors (Tabar *et al.*, 2005).

Transplantation into P2 rats is an elegant method, as the cranial bone of P2 rats is not completely hardened at this time point and can be easily injected with a glass capillary. The transplantation takes not longer than 30 sec for every animal and the mortality rate can be minimized to zero. Furthermore, the mother animal is not affected at all compared to intrauterine transplantations.

Both transplantation approaches were first proof-of-concept experiments, conducted to demonstrate the integration and differentiation potential of neural precursors generated according to the EB-based protocol in CNS tissue. Two different approaches (pre- and postnatal) were chosen to investigate whether the transplanted cells show similar properties in the brain tissue of embryonic and newborn rats. Further experiments will have to follow with higher quantities of transplanted animals to evaluate the full potential of these cells concerning differentiation, integration and functional maturation.

5.2 Human ES cells can be adherently converted into the neuroectodermal lineage

In the second part of this work, a protocol for the direct conversion of human ES cells into neural precursor cells was established. In a first step, human ES cells propagated as adherent cultures on extracellular matrix proteins were induced to differentiate into the neural lineage in differentiation medium containing FGF-2. In a second step, the adherent cells were proliferated to form detaching neurospheres. The results of this study show that human ES cell colonies propagated in the presence of FGF-2 can be directly converted into neurogenic precursors without intermediate cultivation steps. Converted neural cells readily gave rise to neurospheres, which, upon plating, generated an outgrowth of neural precursors expressing nestin, PSA-NCAM and A2B5. Following growth factor withdrawal, neurosphere-derived precursors were able to differentiate into all 3 neural lineages. In contrast to other methods aiming at the generation of neural precursors, this protocol avoids the generation of EBs and coculture with stromal cells (Reubinoff *et al.*, 2001; Zhang *et al.*, 2001; Park *et al.*, 2005). EBs are notoriously heterogeneous and typically contain derivatives of several germ layers (Itskovitz-Eldor *et al.*, 2000). Bypassing the EB stage should thus reduce contamination with non-neural cells and their potential influences on neural differentiation. Avoiding coculture steps with other cell types such as stromal cells should also prevent ill-characterized paracrine effects between different cell populations and reduce the risk of contamination associated with the use of primary cells and cell lines (Martin, 2005).

Importantly, this new protocol permits the directed differentiation into neural cell types, without the necessity of mechanical isolation of neural rosette-like structures or lineage-selection approaches (Carpenter *et al.*, 2001; Reubinoff *et al.*, 2001; Zhang *et al.*, 2001; Conti *et al.*, 2005).

5.2.1 Neural conversion of human ES cell colonies in the presence of FGF-2

Data from previous studies suggest that mouse ES cells plated at low density can be directly recruited into a neural fate (Tropepe *et al.*, 2001; Ying *et al.*, 2003). This

phenomenon appears to depend critically on FGF signaling. Whereas Tropepe *et al*. have used FGF-2 in their conversion paradigm, Ying *et al*. have found that blockade of the FGF signaling pathway disrupts the neural conversion process. On the other hand, studies have shown that FGF-2 promotes the self-renewal of human ES cells both on fibroblasts (Amit *et al.*, 2000) and Matrigel (Xu *et al.*, 2005b). In the new protocol, the presence of FGF-2 appears to support neural differentiation. These differences might be due to lower concentrations of FGF-2 and the absence of SR in the neural differentiation medium. Furthermore, several other factors may influence the neural differentiation of human ES cells in the newly established differentiation protocol. This is particularly true for the constituents of Matrigel, including epidermal growth factor (EGF), insulin-like growth factor (IGF) and platelet-derived growth factor (PDGF), i.e. growth factors which are known to modulate the proliferation and differentiation of neural precursors (Reddy and Pleasure, 1992; Reynolds *et al.*, 1992; Arsenijevic *et al.*, 2001; Erlandsson *et al.*, 2001; Anderson *et al.*, 2002). In addition, Matrigel contains the already mentionend factor heparan sulphate proteoglycan, an important cofactor of FGF-2 (Lin *et al.*, 1999; Perrimon and Bernfield, 2000).

Previously available methods for the generation of human ES cell-derived neural precursors have frequently relied on the mechanical isolation of neural rosette-like structures from mixed cultures (Reubinoff *et al.*, 2001; Zhang *et al.*, 2001; Conti *et al.*, 2005). In contrast, in the new protocol, plated human ES cell colonies themselves gradually adopt a neural fate. This is supported by the down-regulation of pluripotency-associated markers (nanog and Oct4) and the concomitant induction of neural antigens (Pax6, Mash1, GLAST). Following detachment from the cell culture plates, these colonies continue to grow and form neurospheres. Disruption of the colonies and trituration to single cells strongly decreased the efficiency of neurosphere formation. This could indicate that the maintenance of cell-cell contacts within a single colony favors neurosphere formation. The observation is also compatible with the notion that only a small subset of neurosphere cells represent *bona fide* stem cells (Reynolds and Weiss, 1996; Svendsen and Caldwell, 2000; Tropepe *et al.*, 2001). However, in contrast to other studies (Conti *et al.*, 2005), this study was not primarily aimed at the production of human ES cell-derived neural *stem* cells but at the efficient enrichment of neural *precursors*. It can be hypothesized that the pronounced propensity of neural cells for sphere formation further contributes to this purification effect of the established protocol. Indeed, the spheres showed no expression of desmin, alpha-fetoprotein or keratin, i.e.,

markers indicative of multi-germ layer differentiation. Within the spheres scattered SMA-positive cells could be rarely noticed, a cell population which may also arise from derivatives of the neural crest (Le Douarin, 1982; Phillips *et al.*, 1987; Beall and Rosenquist, 1990).

During proliferation of the spheres, only occasional spontaneous differentiation into ß-III-tubulin-expressing neurons could be observed (data not shown). No differentiation into astrocytes was noticed within the 15 days of neurosphere proliferation. After plating, the neurospheres gave rise to an outgrowth of highly enriched bipolar cells expressing nestin, A2B5 and PSA-NCAM, i.e., antigens typically present in human ES cell-derived neural precursors (Carpenter *et al.*, 2001). As expected, further growth factor withdrawal induced differentiation of these precursors into neurons and – at later time points – astrocytes and oligodendrocytes.

Interestingly, the human ES cell-derived neurospheres displayed pronounced changes in histoarchitecture. Within the first week they were composed of typical rosette-like clusters. In the second week, this characteristic histomorphology vanished and gave rise to a neural tissue-like architecture with a more homogenous distribution of the cells. Focally, they formed rhythmic architectures with their nuclei being arranged in cellular ribbons. Considering that both rosette formation and nuclear palisading are features frequently observed in primitive neuroepithelial tumors, it is tempting to speculate that human ES cell-derived neurospheres may provide access to developmental stages relevant for brain tumor induction.

5.2.2 Direct conversion of human ES cells recapitulates early induction processes

BMP signaling is generally regarded as an inhibitor of neural differentiation (Munoz-Sanjuan and Brivanlou, 2002). To account for this, available protocols for the neural conversion of ES cells involve steps which reduce auto- and paracrine BMP signaling within the cultures, either by dilution in low density cultures (Tropepe *et al.*, 2001; Ying *et al.*, 2003) or the use of BMP inhibitors (Gerrard *et al.*, 2005). In contrast, the protocol prescribed here operates at high cell densities (up to 2 x 10^5 cells/cm^2 at the end of stage A.2) and without BMP inhibitors, which could point to pro-neural inductive effects between the evolving neural colonies. Indeed, we found that the formation of neural

colonies was less efficient at lower cell densities. The presence of direct pro-neural inductive effects within our high density cultures is further supported by the fact that our protocol does not depend on additional neural-promoting substances such as the B27-supplement used in other studies (Ying *et al.*, 2003).

According to the default model, BMP inhibition is required for neural induction (see 1.1.1). Several authors have questioned this interpretation, claiming that other pathways might be involved in neural induction (Streit and Stern, 1999). Two other candidates are Wnts and FGFs, which are thought to act concomitantly with or separately from BMP inhibition. This discussion will focus on FGF signaling because FGF-2 is the only growth factor applied in the adherent protocol.

Evidence for a concerted action of BMP signaling and FGFs came from experiments demonstrating that the neuralizing activity of chordin and noggin requires an intact FGF-signal transduction pathway (Launay *et al.*, 1996; Sasai *et al.*, 1996). Furthermore, *in vitro* experiments on explanted chick epiblasts indicate that early FGF signaling in prospective neural epiblast cells attenuates BMP signaling by repressing BMP expression (Wilson *et al.*, 2000; Wilson *et al.*, 2001).

These studies also point to a second mechanism, which is BMP-independent but is regulated by FGF signaling, although the underlying processes of such a signaling pathway are not yet elucidated. They demonstrated, that neural markers were not expressed and the epidermal fate was restored, when FGF signaling was blocked *in vitro* by a FGF receptor inhibitor (Wilson *et al.*, 2000; Wilson *et al.*, 2001). In earlier studies, FGF-2 was already postulated as a direct neural inducer. Following application of FGF-soaked beads to primitive streak chick embryos *in vitro*, an ectopic induction of neural plates independent of the endogenous neural tube was observed (Alvarez *et al.*, 1998). In chick, FGF signaling also seems to control the timing of the neural transcription factor Pax6 (Bertrand *et al.*, 2000).

Also in *Xenopus* it has been demonstrated that FGF signaling can directly induce neural differentiation (Kengaku and Okamoto, 1993; Lamb and Harland, 1995; Launay *et al.*, 1996). Findings from the work of Lamb and Harland (1995) showed that exposure to FGF-2 induced cells from mid- and late-gastrula ectoderm to express the neural marker NCAM. As the existence of mesodermal tissue could not be detected, the neural inducing activity of FGF-2 was classified as direct (Lamb and Harland, 1995).

In general, FGF signaling might actively induce neural tissue in early developmental stages (Wilson *et al.*, 2000; Linker and Stern, 2004). As ES cells represent an early

developmental cell type, FGF signaling might have the potential to actively induce neural differentiation in this system. In this context, a direct inductive effect of FGF-2 on neural differentiation in the adherent protocol seems to be probable. Furthermore, FGF-2 signaling seems to be sufficient to effectively induce neural differentiation in human ES cells. Further experiments might help to confirm this theory. For example, the generation of cells which are non-responsive to FGF, by inducible expression of a dominant-negative FGF-receptor.

Later in embryonic development, FGF has also an effect on regionalization. Further findings from Xenopus revealed that FGF signaling is probably only needed for posterior neural tissue formation (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995; Storey *et al.*, 1998). To test if this hypothesis could be confirmed in the adherent protocol, further experiments have to follow aiming at the identification of regionalization patterns in all stages of the protocol by analyzing region-specific transcription factors.

5.3 Perspectives

The newly established protocols provide tools for the purification of human ES cell-derived neural precursors. Highly enriched neural precursors are promising candidates for future transplantation strategies, basic research, and model systems for screening approaches.

First of all, cell-based repair within the CNS depends on purified human ES cell-derived neural precursors. Purification of the neural cell population avoids teratoma formation upon transplantation. In particular, neural precursor cells generated according to the EB-based protocol presented here are promising candidates for transplantation approaches, as they already demonstrated their capacity to migrate and differentiate into neurons in host tissue. For the adherent protocol, similar properties have to be demonstrated in future studies.

For basic research, both protocols provide a starting point for further differentiation into specific neural subtypes. Neural precursor populations derived from the newly established protocols proved their capacity to differentiate into neurons and glia. Optimization of differentiation strategies may provide highly enriched populations of specific neuronal and glial subtypes.

Furthermore, the EB-based protocol could serve as a tool for further studies aiming at elucidating differences and similarities in the neural differentiation behavior of human and murine ES cells.

The adherent differentiation protocol could serve as a culture system for studying neural differentiation under highly defined conditions. The established monolayer conditions for adherent conversion provide the ability to recapitulate neural induction processes *in vitro.* Of broad interest may be to elucidate processes of early neural development, in particular the molecular signals underlying the transition from an ectodermal to a neuroectodermal fate. Furthermore, primitive neural rosette-like structures within the neurospheres offer ideal conditions to study both the formation of primitive neural architectures as well as community effects within multicellular aggregates. Considering that both rosette formation and nuclear palisading are features frequently observed in primitive neuroepithelial tumors, it is tempting to speculate that human ES cell-derived neurospheres may provide access to developmental stages relevant for the induction of these malignancies. Additionally, homogenous populations of neural precursors could

be used as an *in vitro* system to study the function of neural genes and proteins – in their native or genetically modified form.

Both protocols are also of commercial relevance. The highly enriched neural precursor cells could serve as model systems for pharmaceutical or toxicological screening approaches and for compound development in pharmaceutical industries. Such a system will be an advantage compared to already existing murine-based methods, as species-specific effects could be studied in human cells.

6 Abstract

Human embryonic stem cells with the property of pluripotency and unlimited self-renewal are a promising tool for basic research and future transplantation strategies. Following the establishment of basic techniques of human ES cell cultivation, 2 different strategies for the generation of human embryonic stem cell (human ES cell)-derived neural precursor cells were established.

Protocols for the neural differentiation of murine embryonic stem cells (murine ES cells) already exist (Okabe *et al.*, 1996; Brüstle *et al.*, 1999). In these strategies, differentiation is induced by aggregation of ES cells to embryoid bodies (EBs). The first part of this project focused on the question whether such a protocol for neural differentiation could be established for human ES cells too. Indeed, modification of several key steps, such as differentiation periods, media formulations and specific coatings permitted to generate an EB-based protocol for human ES cells. This new strategy led to the generation of highly enriched neural precursor cells expressing nestin and the neural markers PSA-NCAM and A2B5. After growth factor withdrawal, the generated neural precursors differentiated into neurons and glia cells, indicating their potential to generate cells of the neuronal and the glial lineage.

In proof-of-principle experiments, it was investigated whether human ES cell-derived neural precursor cells have the potential to integrate into host tissue upon transplantation. For this aim, eGFP-expressing neural precursors were transplanted onto hippocampal slice cultures. Immunohistological analyses revealed that the transplanted cells were able to migrate into the slice and differentiate into neurons.

To investigate whether the transplanted cells have the capacity to functionally mature within the host tissue, the cells were analyzed electrophysiologically at the Department of Epileptology (University of Bonn Medical Center). The experiments revealed that only a minority of cells were able to induce action potentials after 3 weeks of engraftment in hippocampal slices.

In an additional set of experiments, the differentiation and migration properties of human ES cell-derived neural precursors upon *in vivo* transplantation were analyzed. For this purpose, precursor cells were transplanted into the developing brain of pre- and postnatal rats. In both cases, the cells formed clusters, from where single cells migrated

into the host tissues. The transplanted cells differentiated into neurons, as confirmed by the expression of neuron-specific markers.

So far, protocols for the neural differentiation mostly depend on EB-formation, coculture with stromal cells, lineage-selection strategies or mechanical isolation of neural rosette-like structures from differentiated human ES cell cultures. For that reason, the second part of this thesis addresses the question, whether such intermediate steps could be avoided and human ES cells could be directly converted into neurogenic precursors.

The newly established direct conversion paradigm consists of an adherent cultivation step, followed by cultivation as neurospheres. In the first step, human ES cells propagated as adherent cultures on extracellular matrix proteins were induced to differentiate into the neural lineage in differentiation media containing fibroblast growth factor-2 (FGF-2). In the second step, the adherent cells were proliferated to form detaching neurospheres. Upon plating, these neurospheres gave rise to a homogenous population of neural precursors capable of generating neurons, astrocytes and oligodendrocytes. In addition to the practical advantage, also a mechanistic knowledge could be gained with the adherently converted human ES cells: The findings suggest that FGF-2 exposure alone suffices to promote neural conversion of adherently growing human ES cell cultures. The results of this study should provide a basis for the efficient generation of neural cell types for analytic and biomedical applications.

7 Zusammenfassung

Humane embryonale Stammzellen (humane ES Zellen) sind durch ihre Eigenschaft der Pluripotenz und der uneingeschränkten Vermehrbarkeit viel versprechende Kandidaten sowohl für die Grundlagenforschung als auch für zukünftige Transplantationsstrategien.
Nachdem im Rahmen dieser Dissertation grundlegende Techniken zur Kultivierung humaner ES Zellen etabliert wurden, konnten 2 verschiedene Verfahren entwickelt werden, die zum Ziel hatten, neurale Vorläuferzellen aus humanen embryonalen Stammzellen (humane ES Zellen) zu gewinnen.
Es existieren bereits neurale Differenzierungsstrategien für murine embryonale Stammzellen (murine ES Zellen), bei denen die Differenzierung der ES Zellen in Embryoidkörperchen, den so genannten Embryoid bodies (EB) induziert wird.
In einem ersten Projektteil dieser Dissertation wurde daher untersucht, ob ein solches Verfahren auch für humane embryonale Stammzellen etabliert werden kann. Tatsächlich machte die Modifikation entscheidender Schritte, wie der Differenzierungszeiträume, Medienzusammensetzungen und spezifischer Beschichtungen möglich, ein EB-basiertes Protokoll für humane ES Zellen zu etablieren.
Das auf diese Weise optimierte Verfahren führte zu einer hoch aufgereinigten Population neuraler Vorläuferzellen aus humanen ES Zellen, die Nestin und die neuralen Marker PSA-NCAM und A2B5 exprimieren. Diese Zellen besitzen die Fähigkeit, nach Wachstumsfaktorentzug in Neurone und Gliazellen auszudifferenzieren.
In Pilot-Experimenten wurde anschließend untersucht, ob die humanen ES Zell-abgeleiteten neuralen Vorläuferzellen die Fähigkeit besitzen, nach Transplantation in das Empfängergewebe zu integrieren. Zu diesem Zweck wurden eGFP-exprimierende neurale Vorläufer auf hippocampale Schnittkulturen transplantiert. Immunhistologische Untersuchungen ergaben, dass die neuralen Vorläufer in das Gewebe migrierten und Marker reifer Neurone exprimierten. Um zu analysieren, ob sich die Zellen auch funktionell integrierten, wurden die Schnittkulturen am Institut für Epileptologie (Unikliniken Bonn) elektrophysiologisch untersucht. Es zeigte sich während der begrenzten Kultivierungsdauer von 3 Wochen auf den Schnittkulturen, dass nur eine Minderheit der Zellen die Fähigkeit besaß, Aktionspotentiale zu induzieren

Zusätzlich wurde untersucht, wie sich die humanen ES Zell-abgeleiteten Vorläufer nach *in vivo* Transplantation verhalten. Hierfür wurden die Zellen prä- und postnatal in das sich entwickelnde Gehirn der Ratte transplantiert. Die neuralen Vorläufer integrierten und migrierten in beiden Fällen in das Empfängergewebe, differenzierten in Neurone aus und exprimierten Neuron-spezifische Marker.

Die meisten Protokolle zur neuralen Differenzierung humaner ES Zellen beruhen bislang auf EB-Bildung, Kokultur mit Stroma-Zellen, Lineage-selection-Strategien oder der mechanischen Isolierung neuraler Strukturen aus differenzierten humanen ES Zell-Kulturen.

Aus diesem Grund sollte in dem zweiten Teil der Arbeit die Frage beantwortet werden, ob solche Zwischenschritte vermieden und humane ES Zellen direkt in neurale Vorläufer differenziert werden können.

Dieses neue Differenzierungsprotokoll besteht aus einer adhärenten Konversion und der Kultivierung als Neurosphären. Im ersten Schritt werden humane ES Zellen zunächst adhärent auf extrazellulären Matrixproteinen in FGF-2-haltigem Differenzierungsmedium kultiviert und so die neurale Differenzierung induziert. Anschließend werden die entstanden Kolonien als Neurosphären kultiviert. Nachdem diese plattiert sind, entsteht eine hoch aufgereinigte Population neuraler Vorläuferzellen, die das Potential besitzen, in Neurone, Astrocyten und Oligodendrocyten zu differenzieren.

Zusätzlich zu dem praktischen Vorteil, den dieses Protokoll liefert, können daraus auch mechanistische Erkenntnisse gewonnen werden: Die Ergebnisse zeigen, dass die Zugabe von FGF-2 ausreicht, um humane ES Zellen unter adhärenten Bedingungen neural zu konvertieren. Aus diesem Grund stellt dieses Protokoll eine Basis dar, um effizient neurale Zelltypen für analytische und biomedizinische Applikationen zu erzeugen.

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