Regionalisation of human ES cell derived neural precursors

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Abbreviations

°C	Degree **C**elsius
A	**A**mpere
ANR	**A**nterior **n**eural **r**idge
AzaC	5′-**Aza**-2′-deoxy **c**ytidine
BLBP	**B**rain **l**ipid **b**inding **p**rotein
BMP	**B**one **m**orphogenetic **p**rotein
bp	**B**ase**p**air
BrdU	5′-**Br**omo-2′-**D**eoxy**u**ridine
cDNA	**C**opy **D**eoxyribo**n**ucleic **A**cid
CNP	2′,3′-**c**yclic **n**ucleotide 3′-**p**hosphodiesterase
CNS	**C**entral **n**ervous **s**ystem
CP	**C**rossing **p**oint
Cy3	**Cy**anine **3**
Cy5	**Cy**anine **5**
D, d	**D**ay
DAPI	4′6-**Dia**midino-2′-**p**heny**l**indol-dihydrochloride
DEPC	**Die**thyl**p**yro**c**arbonate
Dlx1	**D**istal-**l**ess homeo bo**x 1**
Dlx2	**D**istal-**l**ess homeo bo**x 2**
DMEM	**D**ulbecco's **m**odified **e**agle **m**edium
DMSO	**Dim**ethyl**s**ulf**o**xide
DNA	**D**eoxyribo**n**ucleic **A**cid
dNTP	**D**esoxy**n**ucleosid-**tri**p**h**osphate mix
E10	Day **10** of **e**mbryonic mouse development
E2F1	**E2F** transcription factor **1**
EB	**E**mbryoid **b**ody
EDTA	**E**thylene**d**iamine**t**etraacetic **a**cid
EGF	**E**pidermal **g**rowth **f**actor
EGFP	**E**nhanced **g**reen **f**luorescent **p**rotein
Emx1	**Em**pty spiracles homolog **1**
Emx2	**Em**pty spiracles homolog **2**
En1	**En**grailed
ES cell	**E**mbryonic **S**tem **C**ell
EtBr	**Et**hidium **br**omide
FACS	**F**luorescence-**a**ctivated **c**ell **s**orting
FCS	**F**etal **C**alf **S**erum
FGF-2	**F**ibroblast **g**rowth **f**actor **2**
FISH	**F**luorescence **i**n **s**itu **h**ybridisation

FITC	**F**luorescein-**i**so**t**hio**c**yanate
FoxG1	**Fo**rkhead bo**x** 1
G	**G**ram
GABA	**G**amma-**a**mino **b**utyric **a**cid
Gad67	**G**lut**a**mate **d**ecarboxylase
GAPDH	**G**lycer**a**ldehyd-3-**p**hosphat-**d**e**h**ydrogenase
Gbx2	**G**astrulation **b**rain homeo bo**x 2**
GFAP	**G**lial **f**ibrillary **a**cidic **p**rotein
h	**H**our
hES	**H**uman **e**mbryonic **s**tem cell
hESNSCs	Long-term expandable **hES** cell derived **n**eural **s**tem **c**ells
HoxA2	**Ho**meobo**x** A2
HoxB4	**Ho**meobo**x** B4
ICM	**I**nner **c**ell **m**ass
ITSFn	**I**nsulin, **t**ransferrin, **s**odium selenite & **f**ibronectin medium
kb	**K**ilo-**b**ases
Knockout-DMEM	Knockout- **D**ulbecco's **M**odified **E**agle **M**edium
Krox20	Early growth response
l	**L**iter
L27	Ribosomal protein **L27**
LGE	**L**ateral **g**anglionic **e**minence
LIF	**L**eukemia **i**nhibitory **f**actor
Mash1	**A**chaete-**s**cute **h**omolog **1**
MEF	**M**urine **e**mbryonic **f**ibroblast
mES	**M**urine **e**mbryonic **s**tem cell
min	**M**inute
mRNA	**M**essenger **r**ibo**n**ucleic **a**cid
ms	**M**ou**s**e
mV	**M**illi**v**olt
ng	**N**ano**g**ram
Nkx2.2	**NK2** homeobo**x 2**
NSC	**N**eural **s**tem **c**ell
Otx2	**Ot**hodenticle homolog **2**
P0	**P**ostnatal day **0** in mouse development
Pax2	**Pa**ired bo**x** gene **2**
Pax3	**Pa**ired bo**x** gene **3**
Pax5	**Pa**ired bo**x** gene **5**
Pax6	**Pa**ired bo**x** gene **6**
Pax7	**Pa**ired bo**x** gene **7**
PBS	**P**hosphate-**b**uffered **s**aline

PCR	**P**olymerase **c**hain **r**eaction
PFA	**P**ara**f**orm**a**ldehyde
PNS	**P**eripheral **n**ervous **s**ystem
PO	**P**oly-**o**rnithine
Rb	**R**a**b**bit
Rpm	**R**ounds **p**er **m**inute
RT	**R**everse **t**ranscription
SVZ	**S**ub**v**entricular **z**one
SD	**S**tandard **d**eviation
TH	**T**yrosine **h**ydroxylase
Tubb3	**Tub**ulin, **b**eta **3**
vGlut1	**V**esicular **glu**tamate **t**ransporter 1
vGlut2	**V**esicular **glu**tamate **t**ransporter 2
VPA	**V**al**p**roic **a**cid
μ	Micro

1 Introduction

Embryonic stem cells, this term evokes mixed feelings whenever mentioned. Often the related research is condemned for ethical reasons and fear that it will encourage reproductive cloning. Human embryonic stem (hES) cells consequently represent an ethical and social challenge, a fact reflected in the distinctive laws and regulations governing their use worldwide and the ongoing debate surrounding these legislations. Still, ten years after the first derivation of hES cell lines, it is clear that the availability of these cells, from which all major somatic cell lineages can be generated, has profoundly altered our approaches in human biology and medicine. A major part of the research is focused on the use of hES cell derivatives in treating brain diseases and spinal cord injuries. Yet, these diseases also represent the greatest challenges for cell replacement strategies given the complexity of the adult central nervous system. However, due to the progressive aging of our society and the subsequent increase in neurodegenerative diseases stem cell-based therapies of neural repair are of immense importance.

1.1 Stem cells

Unlike most cells in the human body stem cells are undifferentiated cells capable of self-renewal and differentiation into specialized tissue types under certain physiologic or experimental conditions. Hence they enable tissues homeostasis as well as repair in response to injury (Blau et al. 2001). The term “progenitor cell”, on the other hand, refers to any dividing cell with the capacity to differentiate, including putative stem cells whose self-renewal has not yet been demonstrated (Smith 2006). Here the term “precursor” is used interchangeably with the term “progenitor”.

Different types of stem cells are found at distinct sites in the body often throughout the lifetime of the organism. The division of stem cells can be either symmetric – yielding two identical daughter cells – or asymmetric, resulting in one daughter cell with the properties of a stem cell and one more differentiated cell. In case of symmetric stem cell division both daughter cells can be stem cells or both can be more differentiated cells; in this instance the self-renewal capability is lost (Molofsky et al. 2004).

Regarding the second stem cell characteristic, a stem cell is termed totipotent if it can give rise to all cell types of an organism including the extra-embryonic tissues. A

pluripotent stem cell, on the other, hand is only capable of generating cells of the three germ layers i.e. all somatic cell types (Fig. 1).

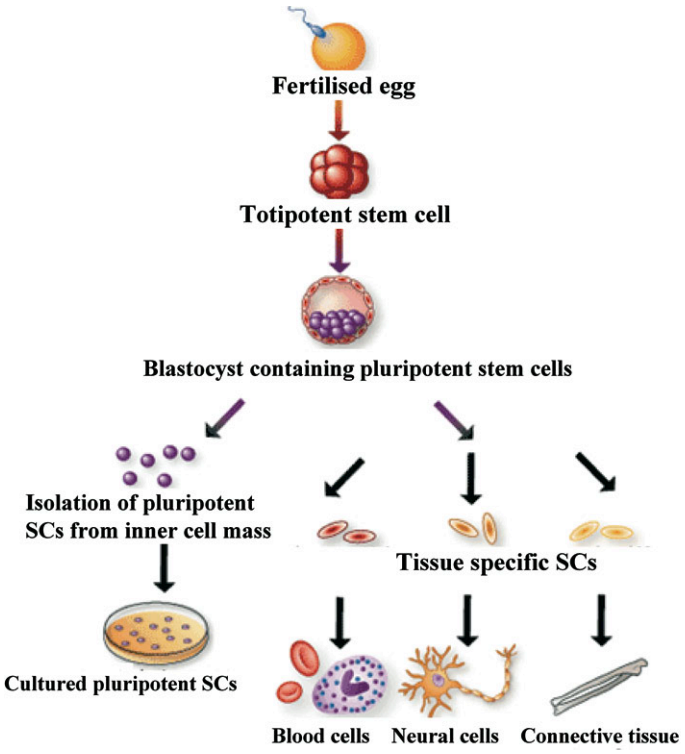

Fig. 1. Stem Cell Hierarchy
Stem cells (SC) differ in their differentiation potential. While totipotent cells can generate a complete organism, pluripotent cells give rise to all somatic cell lineages and multipotent stem cells reside in different organs (http://www.scq.ubc.ca/ 2008).

The differentiation potential of multipotent cells – somatic stem cells present in many tissues – is limited even further as they usually only differentiate into cells of their own germ layer (Table 1). Moreover their self-renewal capacity is restricted. Nevertheless, these cells ensure an adequate supply of differentiated cells in many tissues, such as the gut and skin, and they have been in clinical use for many years for instance in bone marrow transplantations (Jaenisch and Young 2008).

Table 1: Overview – Developmental options of stem cells (Jaenisch and Young 2008)

Term	Potency
Totipotent	Ability to form all lineages of an organism; in mammals only the zygote and the first cleavage blastomeres are totipotent
Pluripotent	Ability to form all lineages of a body. Example: embryonic stem cells
Multipotent	Ability of (adult) stem cells to form multiple cell types of one lineage. Example: hematopoietic stem cells
Unipotent	Cells form one cell type. Example: spermatogonial stem cells

Interestingly, the notion that somatic (i.e. multipotent) stem cells do not differentiate into cells of other germ layers has been challenged in recent years (Brazelton et al. 2000; Mezey et al. 2000). However, since at least some of the reported transdifferentiation events following transplantation of somatic stem cells were evidently due to cell fusion with host cells, it is not yet clear to what extent somatic stem cells are capable of transdifferentiation without prior de-differentiation (as seen in iPS cells, see 1.1.1.3) (Terada et al. 2002; Ying et al. 2002).

1.1.1 Embryonic stem cells

According to the above definition embryonic stem cells are pluripotent since they are capable of forming cells from all three germ layers and continuously self-renew. They were first isolated from mice in 1981 (Evans and Kaufman 1981; Martin 1981) and at present have been generated from several species (Graves and Moreadith 1993; Wheeler 1994; Thomson et al. 1995; Pain et al. 1996; Li et al. 2006). The first human embryonic stem cells – isolated 10 years ago by James A. Thomson – marked the start of the current excitement about (human) embryonic stem cell research and the first step towards pharmaceutical and clinical applications in humans (Thomson et al. 1998).

1.1.1.1 Derivation and characteristics of human embryonic stem cells

Conventionally, embryonic stem cells are isolated at day 5 of human embryonic development. At this time point the blastocyst consists of the inner cell mass and an outer layer of trophoblast cells. While the former will go on to generate the embryo proper the latter will give rise to the extraembryonic tissues including the placenta (NIH 2007). To generate ES cells, the inner cell mass is isolated via immunosurgery and subsequently its outgrowths are passaged on a feeding layer of irradiated mouse embryonic fibroblasts to establish an ES cell line (Solter 1975; Thomson et al. 1998) (Fig. 2).

Under standard culture conditions hES cells are grown in medium containing serum replacement (Amit and Itskovitz-Eldor 2002) and fibroblast growth factor 2 (FGF-2). Alternatively hES cells are grown on Matrigel in fibroblast-conditioned medium.

In vitro the pluripotency of hES cells is associated with the expression of several markers including Oct3/4, Nanog, Telomerase and Rex1 as well as alkaline phosphatase activity and it is demonstrated via embryoid body formation (NIH 2007). If cultured as a suspension hES cells will spontaneously aggregate to structures termed embryoid bodies (EBs), that mirror the first steps of inner cell mass

differentiation *in vivo*. In these EBs, hES cells will differentiate into cells from all three germ layers namely ectoderm, endoderm and mesoderm (Murray and Edgar 2004).

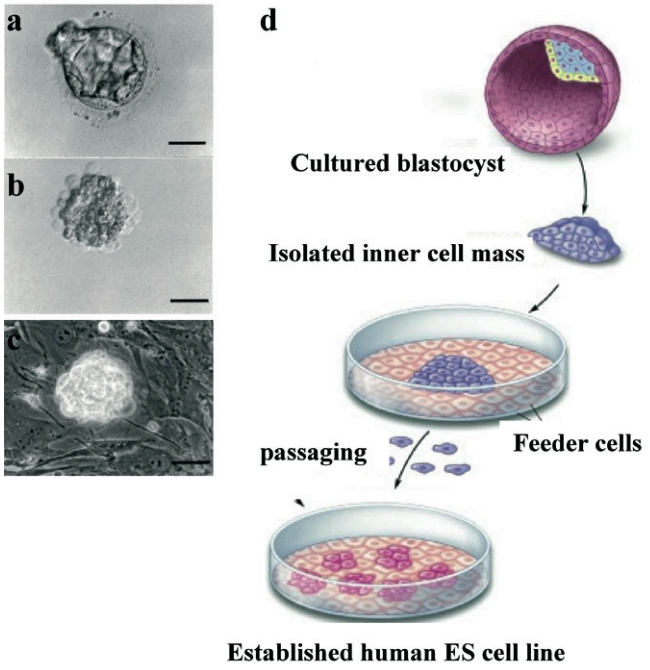

Fig. 2. Derivation of human ES cell line.
(a) Donated human embryo produced by *in vitro* fertilization at the blastocyst stage. **(b)** Human blastocyst after zona pellucida removal by immunosurgery. **(c)** Inner cell mass immediately after immunosurgery on mitotically inactivated mouse embryonic fibroblast feeder layer (Amit and Itskovitz-Eldor 2002) (Scale bar = 50 μm). **(d)** Schematic overview of hES cell generation: after the inner cell mass has been isolated via immunosurgery, it is cultivated on mouse embryonic feeder cells. If the inner cell mass cells can be subsequently dissociated and replated, a new ES cell line has been established (NIH 2007).

In vivo pluripotency is proven via transplantation of hES cells to host organisms, which results in formation of tumours called teratomas consisting of cells from all three germ layers (Evans and Kaufman 1981). The ultimate proof of pluripotency, however, is tetraploid aggregation. In this procedure the blastomeres of a two-cell embryo are fused thereby generating a one cell tetraploid embryo. When this embryo is combined with ES cells the generated offspring is solely derived from the ES cells (Nagy et al. 1993; Duncan 2005). For obvious ethical reasons this ultimate evidence cannot be provided in humans.

First clues concerning the quality of the hES cell culture can be gained from observing the morphology of the cells. Whereas undifferentiated hES grow in flat, relatively compact colonies with a clear edge (Fig. 3), the first signs of differentiation are the loss of the sharp outer boundary as well as structures arising in the middle of the colony. It is important to keep in mind that the growth and molecular characteristics of hES cells are the product of tissue culture selection. Thus the hES

cells differ epigentically and biologically from their cells of origin (Jaenisch and Young 2008).

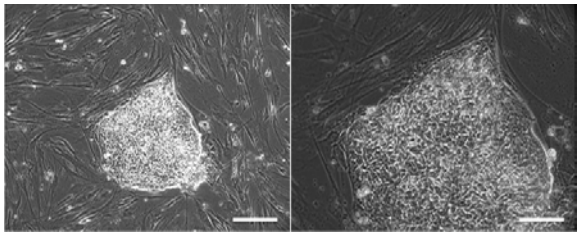

Fig. 3. hES cell colony on mouse embryonic fibroblasts
Human ES cells (H9.2 P50) grow as flat, compact colonies (Scale bar: 280μm & 70μm).

1.1.1.2 Developments in hES derivation

When using the classical derivation method as described above, resulting hES cells can be contaminated with animal substances through cultivation on mouse fibroblast feeder cells and the employment of matrix components of animal origin (Jaenisch and Young 2008). This contamination may lead to expression of non-human sialic acid that would be immunogenic on cells used for human transplantation (Martin et al. 2005). Therefore hES cells cultured in the presence of animal substances would not be approved for clinical use and hence new approaches have been developed to avoid this exposure and to increase hES cell derivation efficiency. For instance, the inner cell mass has recently been isolated via micromanipulation instead of immunosurgery and was subsequently cultured on human feeder cells or in completely defined medium in the absence of animal cells and derivatives (Inzunza et al. 2005; Kim et al. 2005; Ludwig et al. 2006). In addition it has been tried to address the ethical issue of destroying an embryo while deriving hES cells via creation of mES and hES cells from single cell biopsies at the eight-cell stage, which leave the embryo intact (Chung et al. 2006; Klimanskaya et al. 2006) (Fig. 4).

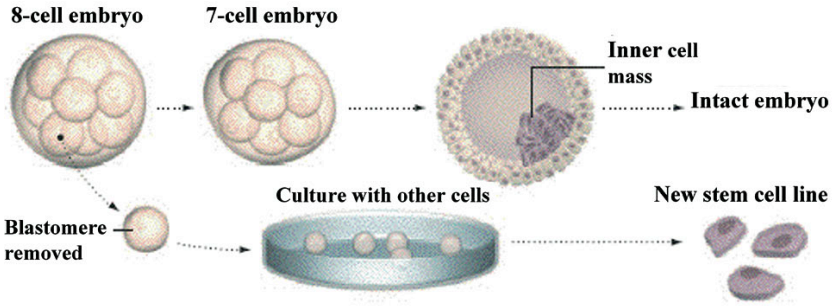

Fig. 4. Derivation of ES cell line from single blastomere
A single blastomere is removed from an 8-cell stage embryo as routinely done in pre-implantation diagnostics. The blastomere is then cultured with other cells to derive a new ES cell line, while the embryo continues to develop into a blastocyst (Chung et al. 2006; Klimanskaya et al. 2006; Wade and Granberg 2006).

However, to date no live offspring have been reported after this procedure was applied.

1.1.1.3 New developments in pluripotent cell derivation

An immense amount of research is currently focused with increasing success at generating cells with the main characteristics of embryonic stem cells – unlimited self-renewal and pluripotent differentiation potential. In the mouse and other mammals it has been demonstrated some time ago that somatic cells can be reprogrammed through somatic cell nuclear transfer (SCNT). In SCNT the nucleus from a donor somatic cell is introduced into an enucleated oocyte. Via activation using chemicals or electric shock cell division is stimulated up to the blastocyst stage. Subsequently an ES cell line can be derived, or if the blastocyst is transplanted to a surrogate mother it can give rise to a clone as demonstrated with the sheep Dolly (Wilmut et al. 1997; Hipp and Atala 2004). The first primate ES cells derived via SCNT were reported last year (Byrne et al. 2007) and blastocysts have been obtained after SCNT of human cells. Yet, from these no hES cell lines were established (French et al. 2008). Still, these studies proved that terminal differentiation does not restrict the potential of the nucleus (Jaenisch and Young 2008). This notion is supported further by the fact that cells with the characteristics of ES cells can be created through culture induced reprogramming and from testis (Guan et al. 2006; Jaenisch and Young 2008).

The most striking approach, however, was the establishment of induced pluripotent cells – iPS cells. After demonstrating that adult somatic cells could be transformed into pluripotent cells via fusion with ES cells, the search was out to identify these pluripotency-conferring factors (Tada et al. 2001). The breakthrough came 2006 in the study by Takahashi and Yamanka. It showed that via retrovirally transducing just four factors namely Oct3/4, Sox2, c-Myc and Klf4 and subsequent selection of cells that expressed the marker of pluripotency *Fbx15,* iPS cells could be derived. This was first demonstrated in fetal and then in adult mouse fibroblasts. However, with these "first generation" iPS cells no chimeras could be generated (Takahashi and Yamanaka 2006). Therefore, adult fibroblasts expressing drug selectable markers under the control of Nanog or Oct4 – two of the best studied and most essential genes associated with pluripotency – were chosen instead of Fbx15 expression to selected for truly reprogrammed cells (Maherali et al. 2007; Okita et al. 2007; Rossant 2007; Wernig et al. 2007). Using the previously described strategy in combination with Nanog or Oct4, three groups derived stable iPS lines. These are almost identical

to ES cells in their transcriptional imprinting and chromatin modification and passed the "gold standard" test for pluripotenty by extensively contributing to all adult cell types including the germ line (Wernig et al. 2007) (Fig. 5).

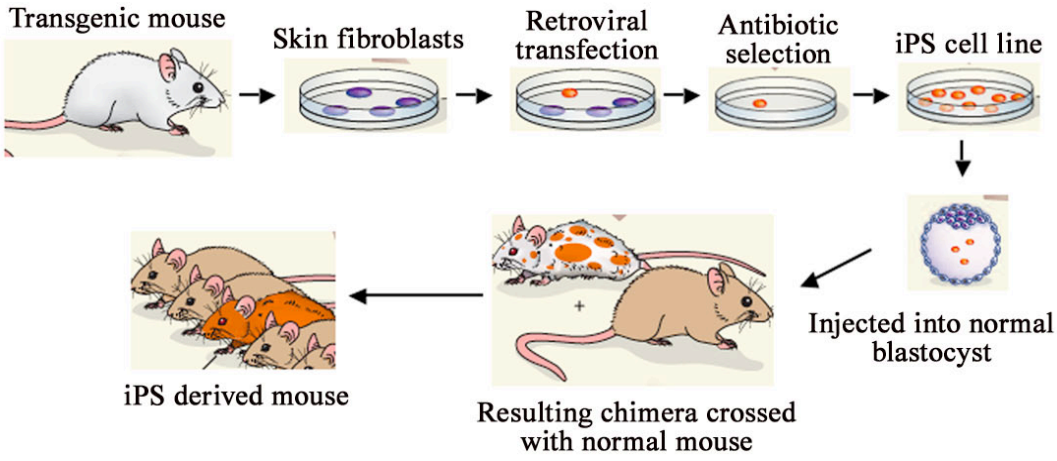

Fig. 5. Generation of induced pluripotent stem cells (Takahashi and Yamanaka 2006)
Fibroblasts were isolated from mice carrying a drug-selectable marker linked to the expression of *Oct/4* (Wernig et al. 2007) or *Nanog* (Maherali et al. 2007; Okita et al. 2007) and retrovirally transfected with the genes for Oct3/4, Sox2 Klf4 and cMyc. In the next step, transfected cells were subjected to antibiotic selection thereby selecting for those cells expressing *Oct4* or *Nanog*. These rare cells were expanded to stable iPS cell lines. When iPS cells were injected into blastocysts of normal mice they contributed to all cell types of the body including the germ line. Thus, when chimaeric animals were crossed with normal mice this resulted in mice carrying the genetic content of an iPS cell (Okita et al. 2007; Rossant 2007).

The question of whether the same reprogramming was possible with human cells did not remain open for long. In the same year two groups presented evidence that human somatic cells can be reprogrammed into iPS cells. Yamanka's group successfully applied the advanced technique, which they had employed in mice, but this time omitting the selection transgene (Takahashi et al. 2007; Yu et al. 2007). These iPS cells were subjected to various assays comparing them with hES cells including morphological studies, surface-marker expression, epigenetic status, formation of embryoid bodies *in vitro*, directed differentiation to neural cells and cardiomyocytes as well as teratoma formation *in vivo*. Importantly genomic DNA analysis together with analysis of short tandem repeats demonstrated the genetic origin of independent human iPS clones from their parental fibroblast populations (Takahashi et al. 2007). Thomson and his team, on the other hand, screened for genes highly enriched in hES cells relative to myeloid precursors. This approach resulted in a list of 14 genes including Oct4 and Sox2 but not Klf-4 or cMyc. They went on to show that out of these 14 genes Oct4, Sox2, Nanog and Lin28 were sufficient to reprogram human somatic cells to iPS cells, displaying the main characteristics of ES cells including differentiation into the three germ layers (Yu et al. 2007).

The intriguing possibility provided by induced pluripotent stem cells is to use non-controversial adult sources to derive iPS cells and then employ these instead of the ethically controversial human embryonic stem cells for treatment of degenerative diseases and traumatic injuries. Particularly promising is the potential for derivation of patient specific iPS cells to provide tissue-matched cells for transplantation medicine – thereby eliminating the concern of immune rejection (Rossant 2007).
In summary, this reprogramming system offers exciting and powerful approaches especially since it is feasible to use nonintegrating adenoviruses for delivering the inducing transcription factors (Stadtfeld et al. 2008). Nevertheless hES cells will not be obsolete for the foreseeable future as it has to be ensured that iPS cells do not differ in clinically important ways from hES cells (Yu et al. 2007).

1.1.1.4 *In vitro* differentiation of hES cells

In order to realize their therapeutic potential ES cells have to be differentiated into pure somatic cell populations, eliminating all hES cells in the process as these may cause the formation of teratomas after transplantation (Thomson et al. 1998; Reubinoff et al. 2000; Amit and Itskovitz-Eldor 2002). The widely used differentiation strategies of lineage-selection, over-expression of instructive factors, directed differentiation and co-culture are illustrated below (Fig. 6).
The lineage selection approach is based on selecting the desired phenotype from a pool of heterogeneously differentiating hES cells. From this starting population relevant cells can be segregated, exploiting the distinctive expression of surface antigens on different cell types e.g. via immunopanning or fluorescence-activated cell sorting (FACSorting). In immunopanning the antibody against the desired surface antigen is anchored to a surface, whereas FACSorting isolates the desired cells as part of an antigen-antibody complex (Li et al. 1998; Roy et al. 2000; Wang et al. 2000; Keyoung et al. 2001; Schmandt et al. 2005). Alternatively, hES cells are genetically modified with a selection marker under the control of a promoter only expressed in the desired cell type. Drug-resistance genes as well as fluorescence-conferring genes such as EGFP are commonly employed as selectable markers. In the second step the cell population can be enriched for the desired cell type via application of the drug thereby killing all non-expressing cells or via isolation using FACSorting (Klug et al. 1996; Li et al. 1998; Pasumarthi and Field 2002; Glaser et al. 2005).

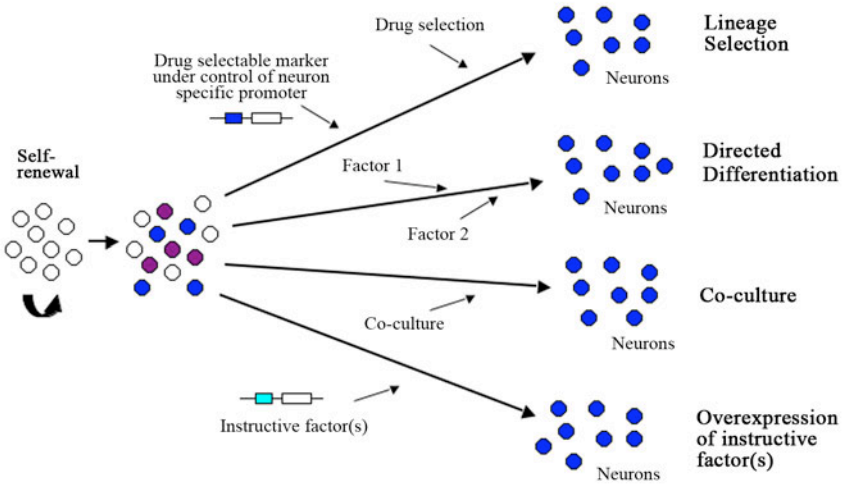

Fig. 6. *In vitro* differentiation strategies for ES cells
Enriched somatic cell populations can be generated from ES cells via co-culture, directed differentiation, over-expression of instructive factors and lineage selection (Terstegge 2006).

A second possibility to substantially enrich for neural subtypes is activation of key determinants i.e. over-expression of instructive factors. Examples of this strategy are the overexpression of Nurr1 or Lmx1 in mES cells that resulted in enhanced differentiation into midbrain dopaminergic neurons and oligodendrocytes (Chung et al. 2002; Andersson et al. 2006; Scheffler et al. 2006).

The aim of directed differentiation *in vitro*, on the other hand, is to induce the entire cell population to differentiate into the desired cell type. Initial differentiation is often accomplished via formation of embryoid bodies (EBs) (Bhattacharya et al. 2005), which are then cultured in specific media and with the addition of extrinsic factors in a pre-determined sequence (Bain et al. 1995; Brustle et al. 1999; Bibel et al. 2004).

In addition co-culture can be used to regulate the differentiation of ES cells. This approach could also be classified as a direct differentiation method, because it is often used in combination with that method (Table 2). However, as the co-culture method is of central relevance in this work, it was depicted separately in Fig. 6. It has been demonstrated that astrocytic co-culture accelerates onset of synaptic activity (Johnson et al. 2007) and stromal cell-derived inducing activity promotes differentiation of neural cells from mouse embryonic stem cells (Kawasaki et al. 2000). Additionally other co-culture settings have proven to be effective i.e. non-cell autonomous signals were capable of exerting an influence on cell differentiation. For instance, telomerase-immortalized fetal midbrain astrocytes enriched hES populations for dopaminergic neurons (Roy et al. 2006, 2007).

Using these different strategies clinically relevant cell types such as cardiomyocytes, neurons, glia and insulin-producing cells have already been derived from hES cells (Assady et al. 2001; Zhang et al. 2001; Kehat et al. 2004; Hoffman and Carpenter 2005). Additionally ES cell derivatives have been used successfully in animal models to repair heart damage (Cai et al. 2007), to reverse functional deficits in Parkinsonian rats (Yang et al. 2008) and to reduce blood glucose levels in hyperglycaemic mice (Shim et al. 2007). However, most of these cell populations lack the purity and homogeneity necessary for human cell replacement therapies. Other challenges include concerns about tumour formation, immune rejection, heart arrhythmia and seizure activity in the central nervous system after transplantation. In addition more research is needed to determine the right graft size and the correct model for preclinical testing (Murry and Keller 2008).

Even though the application of hES cells in the clinic is still some way off, the characteristics of hES cells make them vital for research and development in the pharmaceutical industry. For example, uniformly differentiated hES can serve as model with minimal deviation of original characteristics for toxicity testing (Klimanskaya et al. 2008). Finally a wide range of hES cell lines harbouring genetic disorders such as Duchenne muscular dystrophy, fragile-X syndrome, Huntington's disease and others have been generated and are available to study these diseases *in vitro* (Verlinsky et al. 2005).

1.1.2 Neural stem cells

1.1.2.1 Endogenous neural stem cells

By now the hypothesis that one variety of neural stem cell gives rise to neurons, astrocytes and oligodendrocytes has been firmly established by several studies proving that primary stem cells isolated from the different sites of the embryonic brain (Fig. 7) differentiate into these three cell types (Davis and Temple 1994; McKay 1997).

Central nervous system (CNS) stem cells undergo repeated asymmetric cell divisions and over time alter their responses to growth factors (Fig. 8) thus producing diverse neural and glial subtypes (Allen 2008). During embryonic development early neural progenitors proliferate in response to growth factors such as FGF and are highly neurogenic (Fig. 8). Late foetal neural stem cells, on the other hand, proliferate mainly in response to EGF and predominantly generate glial cells (Fig. 8). Currently the mechanisms regulating this temporal responsiveness to patterning cues are still

poorly understood, but it is clear that integration of both intrinsic and extrinsic signals is involved (Edlund and Jessell 1999; Allen 2008). Especially cell autonomous mechanisms e.g. epigenetic determinants seem to play an essential role in establishing the required competence for an appropriate response to patterning and differentiation cues (Kobayashi et al. 2002; Allen 2008).

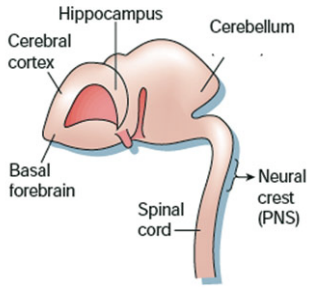

Fig. 7. Locations of neural stem cells
The principal regions of embryonic nervous system from which neural stem cells have been isolated (Temple 2001).

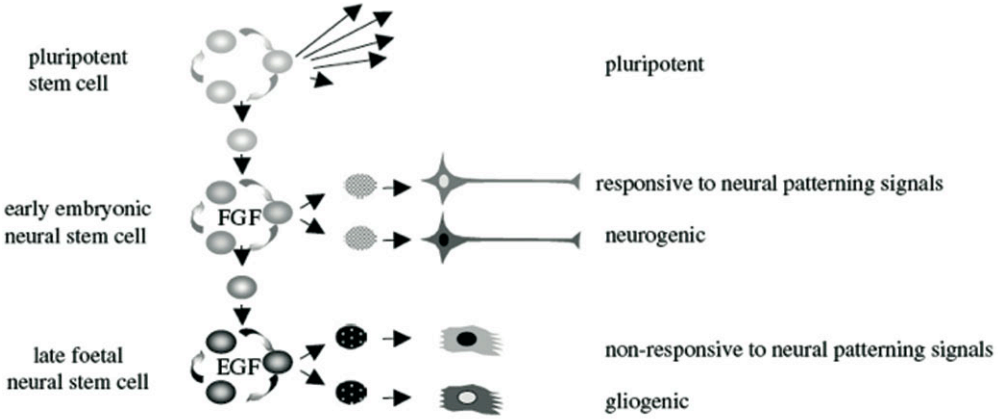

Fig. 8. Developmental potential of neural stem cells is temporally regulated
First neural stem cells divide symmetrically but then switch to asymmetric division passing through a neurogenic phase (responsive to FGF) followed by a gliogenic phase (influenced by EGF)(Temple 2001; Allen 2008).

Multipotent neural precursors cells have also been isolated from the adult rodent brain (Reynolds and Weiss 1992; Gage et al. 1998) as well as from fetal and adult human brain (Zhao et al. 2008). In the adult brain neurogenesis is restricted under normal conditions to the subgranular zone (SGZ) of the dendate gyrus in the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Kuhn et al. 1997; Eriksson et al. 1998). Neurons born in the adult SVZ migrate over great distances along the rostral migratory stream to the olfactory bulb, whereas neurons born in the adult SGZ migrate to the granule cell layer of the dentate gyrus. Both types of newborn neurons integrate into existing circuits and receive functional input

(Zhao et al. 2008). Whether or not neurogenesis also occurs in other areas of the adult mammalian brain remains controversial, but isolation of proliferative precursors from adult neocortox as well as adult temporal cortex has been reported (Palmer et al. 1999; Walton et al. 2006; Gould 2007).

In order to proliferate multipotent CNS cells – especially those derived from adult organisms – *in vitro* they are often grown as floating cell clusters termed neurospheres (Reynolds and Weiss 1992; Vaccarino et al. 2001). Neurospheres predominantly consist of committed progenitors intermingled with differentiated astrocytes and neurons, which seem to provide a niche for the maintenance of a few stem cells (Garcion et al. 2004). Typically proliferation is promoted through the addition of growth factors such as fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF) (Fig. 8) (Vaccarino et al. 2001). Upon withdrawal of these factors from the cultures, the multipotent cells spontaneously differentiate into glial cells and neurons, although the efficiency of neuronal differentiation tends to decline with increasing passage number (Cattaneo and McKay 1990; Vaccarino et al. 2001). Further disadvantages of this culture system include that stem cells maintained within neurospheres are not directly accessible and have not yet been purified (Suslov et al. 2002).

1.1.2.2 ES cell derived neural cells

In principle there are two strategies of using ES cell derived neural cells in cell replacement therapies. In the first approach the desired fully differentiated cells are generated *in vitro* and subsequently transplanted. In the second approach the ES cell derived neural stem cells are transplanted, relying on the host environment to provide appropriate signals to direct differentiation into the desired neural fate.

Utilizing the first strategy – generating differentiated cells *in vitro* – it was demonstrated in 1996 that neural cells could be generated from ES cells via EB formation and subsequent exposure to RA (Bain et al. 1996; Okabe et al. 1996). Since then a variety of neural cell types with specific transmitter profiles have been derived from hES cells via directed differentiation using growth factor combinations as summarized in Table 2.

Table 2 Examples for neural subtype specification from human embryonic stem cells
(Trounson 2006)

Primary inducer	Tissue Type	Reference
Noggin	Neuroectoderm	(Pera et al. 2004)
SDIA + GFs	Midbrain neural cells	(Perrier et al. 2004)
FGF2, FGF8, Shh	Midbrain TH neurons	(Perrier et al. 2004; Yan et al. 2005)
FGF2	TH neurons	(Schulz et al. 2003; Schulz et al. 2004)
SDIA + BMP4, Shh	Neural crest	(Trounson 2004)
bFGF, EGF, RA	Oligodendrocytes	(Nistor et al. 2005)
RA, FGF, SHH, BDNF, GDNF, IGF1	Motoneurons	(Li et al. 2005)

GFs, growth factors; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; GDNF, glial-derived neurotrophic factor; SCF, stem cell factor; GSCF, granulocyte stem cell factor; EB, embryoid body; SDIA, stromal cell derived inducing activity

Employing immature neural progenitors for transplantation appears to be a more promising option than using fully differentiated neural cells, since the precursors should integrate more readily into the highly complex CNS. Hence, in addition to differentiated neural cell populations, neural precursor cells have been generated from ES cells. These neural precursors are capable of proliferation and generation of neurons, astrocytes and oligodendrocytes (Conti et al. 2005; Glaser et al. 2007; Koch et al. 2009). A protocol used for this purpose begins with embryoid body formation in defined medium. In the next step the EBs are plated on adhesive substrates in minimal serum free medium – termed ITSFn – which does not support the survival of non-neuroectodermal cells including undifferentiated ES cells. Finally plating on laminin substrates in the presence of FGF2 promotes proliferation of tri-potential neural stem cells (NSCs) (Okabe et al. 1996; Brustle et al. 1999; Zhang et al. 2001; Conti et al. 2005). The long-term expandable human embryonic stem cell derived neural stem cells (hESNSCs) (Fig. 9) employed here were generated using a protocol based on this procedure (Koch et al. 2009).

In addition to the above described technique NSC differentiation from mouse ES cells can be achieved directly without EB formation through culture at low and very low densities under feeder-free conditions (Tropepe et al. 2001; Glaser et al. 2007). This direct differentiation protocol has been adapted to create NSCs from hES cells (Benzing et al. 2006).

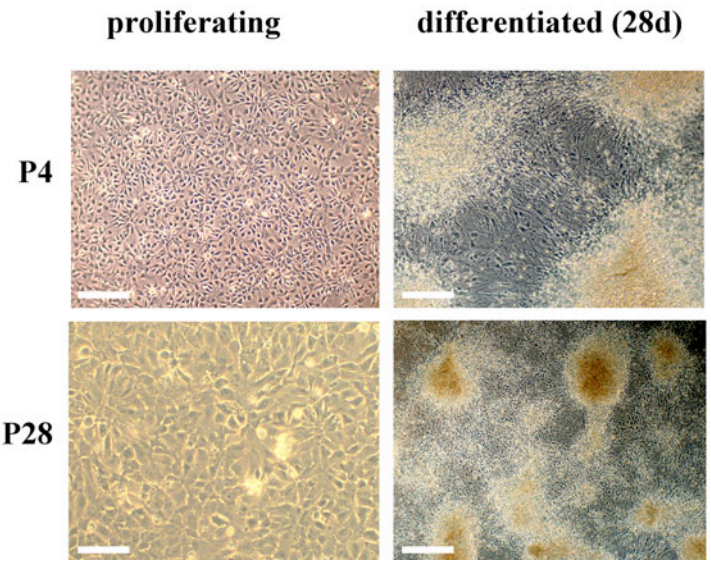

Fig. 9. hESNSCs in their proliferative and differentiated state
Homogenous long-term expandable human embryonic stem cell derived neural stem cells (hESNSCs) used here maintain their characteristic morphology and robust neurogenic potential over the passages *in vitro* (scale bar top: 90μm, bottom: 50 &120μm).

In summary, once the complex inductive interactions regulating ES cell neurogenesis are uncovered, the addition of appropriate factors at the correct stage might allow optimization of NSC production as well as the derivation of desired neural cell types from undifferentiated ES cells (Cai and Grabel 2007).

1.2 Development and specification of the nervous system

The vertebrate central nervous system initially arises from the dorsal region of the embryonic ectoderm. This process of neural plate induction requires active repression of bone morphogenetic protein (BMP) signalling from the non-neural ectoderm by diffusible factors such as noggin, chordin and follistatin (Smith and Harland 1992; Lamb et al. 1993; Hemmati-Brivanlou et al. 1994; Stern 2001; Liu and Niswander 2005). Following induction, the neural plate rolls up and forms the neural tube, which consists of neural stem cells and in turn gives rise to the central nervous system (CNS) including the brain and spinal cord (Lumsden and Krumlauf 1996). Grafting experiments in chick embryos gave insights into mechanisms of subsequent CNS development. For instance duplication of a brain region was accomplished via application of FGF8 (Crossley et al. 1996) suggesting that a single factor is sufficient to bias the differentiation cascade and establish major regional features of the CNS (McKay 1997).

1.2.1 Regionalisation and patterning

Initially the neural plate is anterior in character and posterior regions form during anteriorposterior patterning (Wilson and Maden 2005). The initiation of patterning along the anteroposterior axis is marked by the appearance of several vesicles at the anterior end of the neural tube namely the prosencephalon (or forebrain), the mesencephalon (or midbrain) and the rhombencephalon (or hindbrain) (Lumsden and Krumlauf 1996). Meanwhile the underlying meso-endodermal tissue as well as the non-neural ectoderm participate in patterning along the dorsoventral axis (Wurst and Bally-Cuif 2001). This dorso-ventral patterning has been elucidated in detail. It leads to the generation of cellular and regional diversity due to interplay of extrinsic mechanisms and expression of regulatory genes in a graded and restricted fashion. The patterning depends on the ratio between ventralizing factors of the sonic hedgehog (Shh) family, emanating from the notochord and floor plate (Beadle and Tatum 1941; Mohler 1988; Tabata et al. 1992) as well as dorsalizing factors of the bone morphogenetic protein (BMP) family produced by the roof plate (Sulston et al. 1983). In the next step the ventricular zone is progressively subdivided into zones expressing distinctive homeodomain transcription factors, from which different subclasses of neuron as well as glia later emerge (Anderson 2001) (Fig. 10).

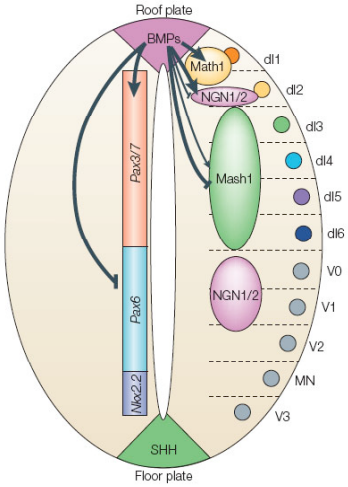

Fig. 10. Schematic illustration of dorso-ventral patterning
The dorsoventral axis is initially specified by opposing diffusion gradients of Sonic hedgehog (Shh) and bone morphogenetic proteins (BMPs) secreted by signalling centres at the ventral (floorplate) and dorsal (roofplate) midline, respectively (Liu and Niswander 2005).

In contrast patterning events along the anteroposterior axis are less well understood. Local signalling centres acting as secondary organizers account at least in part for the induction and maintenance of refined patterning in the rhombencephalon and possibly in the prosencephalon. Two such signalling centres have been identified so far: the anterior neural ridge (ANR), positioned at the junction between prosencephalon and anterior ectoderm, and the isthmic organizer (IsO), which marks

the boundary between midbrain and hindbrain (Shimamura and Rubenstein 1997; Houart et al. 1998). The IsO, which is found in all vertebrate species, is necessary and sufficient for the development of mesencephalic and metencephalic structures (Wurst and Bally-Cuif 2001). When grafted to ectopic neural locations such as the diencephalic prosomere 1 the IsO induced mesencephalic and metencephalic structures in clear rostrocaudal polarity i.e. the posterior side of the induced mesencephalon and the rostral side of the induced metencephalon were in contact with the graft (Marin and Puelles 1994; Wurst and Bally-Cuif 2001).

Removal of the ANR, on the other hand, results in a failure to express FoxG1 (previously Bf1). This transcription factor, which selectively marks future cortical progenitors before the telencephalon is morphologically distinguishable is required for normal cortical morphogenesis (Tao and Lai 1992; Xuan et al. 1995; Shimamura and Rubenstein 1997).

Furthermore as discussed above with regard to BMPs and Shh in dorsoventral patterning, single factors, which play a role in anteriorposterior patterning, have been identified. One example is retinoic acid (RA). As a patterning factor RA in conjunction with FGFs and WNTs is involved in the organization of the posterior hindbrain and spinal cord (Liu et al. 2001). In the absence of RA signalling these structures are not developed (Maden 2007). Overall patterning events result in the establishment of a rough three-dimensional grid in the embryonic neural axis, conferring a positional identity on precursor cells in the CNS (Kiecker and Lumsden 2005). This identity is reflected by the expression of distinctive regional transcription factors. Some of these transcription factors were used as candidate markers representative of the brain region where they are found *in vivo* (Table 3 and Table 4).

Table 3 Region specific expression of candidate transcription factors

Brain Region	**Forebrain**	**Midbrain**	**Hindbrain**
Candidate transcription factors	FoxG1, Emx1, Emx2, Otx2, Dlx1, Dlx2	En1, Pax2, Pax5	Gbx2, HoxA2, HoxB4, Krox20

In this context, it should be noted that some of the candidate markers change their expression with stage of development and upon differentiation (Pasini and Wilkinson 2002; Mo and Zecevic 2008).

1.2.2 Plasticity and regional gene expression in neural progenitors

1.2.2.1 Endogenous neural progenitors

Transplantations of P0 neural progenitors into the adult rat brain resulted in inefficient heterotopic neuronal integration thereby limiting the possibilities of this approach (Zigova et al. 1998; Turner and Shetty 2003). The limitation was not seen if cells from areas exhibiting postnatal neurogenesis (i.e. the hippocampus, olfactory bulb and cerebellum) were used or if precursor cells were transplanted into these areas, suggesting that local guidance cues might be maintained in these regions (Renfranz et al. 1991; Vicario-Abejon et al. 1995; Suhonen et al. 1996). Moreover grafting neural cells across the uterine wall into the embryonic mammalian brain revealed a remarkable plasticity of transplanted neuroepithelial precursor cells (Brustle et al. 1995; Campbell et al. 1995; McKay 2004). For instance striatal precursors injected into the embryonic mouse brain were incorporated into cortex and various other host brain regions, suggesting that they were not restricted to a striatal fate (Fishell 1995). Furthermore, transplanted precursor cells from ventral and dorsal mouse telencephalon were shown to participate in the generation of telencephalic, diencephalic, and mesencephalic brain regions, generating widespread CNS chimerism (Brustle et al. 1995). Additional grafting experiments showed that immortalized hippocampal cells transplanted to the developing cerebellum differentiated into typical cerebellar neurons (Renfranz et al. 1991). Moreover primary cerebellar cells grafted into the hippocampus of neonatal rats and wild type mice acquired morphological and immunohistochemical features of hippocampal granule neurons (Vicario-Abejon et al. 1995). Therefore, these studies suggest that precursor cells originating in a distinct part of the neuroepithelium are not restricted to a local fate but are capable of migration and differentiation upon heterotopic transplantation thereby responding to signals of their new environment.

In this context it is important to note that growth factor mediated expansion of neural precursors *in vitro* has been reported to compromise positional identity information. Specifically a candidate transcription factor expression analysis of neurospheres and stem progenitor cells expanded in the presence of EGF and FGF-2 revealed profound changes in expression patterns of region specific transcription factors compared with primary precursors *in vivo* (Santa-Olalla et al. 2003; Hack et al. 2004). In accordance with these results Dr. Jakupoglu of our group found a blurring of the region specific transcriptional code after cultivation of primary neural cells in EGF and FGF (Jakupoglu, C., unpublished data). Furthermore neural subtypes that

occur only in specific regions such as dopaminergic neurons are difficult to produce *in vitro* even if the neurospheres originated from the region generating dopamine neurons *in vivo* (Hitoshi et al. 2002; Parmar et al. 2002; Ciccolini et al. 2003; Machon et al. 2005; Yan et al. 2005). However, since not all reports confirmed the observation that propagation in EGF and FGF alters the original region specific code (Hitoshi et al. 2002) an explanation for the different results could be the effect of cell density. As long as cells still interact with their normal neighbours they might still receive positional information that is only partially overridden by the potent growth factor signalling of EGF and FGF. This phenomenon, termed "community effect" may account for the maintenance of some regional differences in high-density neurosphere cultures (Ostenfeld et al. 2002; Parmar et al. 2002; Hack et al. 2004). Nevertheless, these outcomes underline the concept that regionalisation and neurogenesis are inseparably linked and the loss of regionalization impairs the generation of distinct phenotypes (Goetz 1998; Hack et al. 2004). The notion that maintenance of positional identity requires ongoing interaction with environmental cues was further supported by a study from Jensen and co-workers suggesting that impaired regionalization may be restored by co-culture with non-expanded primary cells. It was demonstrated that expanded neural precursors derived from the lateral ganglionic eminence (LGE) regain their potential to differentiate into striatal projection neurons upon co-culture with primary LGE cells (Jensen et al. 2004). The phenomenon seemed dependent on cell-to-cell contact and could not be mimicked by conditioned media (Eriksson and Wictorin 2003). This suggests that during development neural precursors can be regionally specified by their environment and that through mimicking such an environment one can achieve the same differentiation behaviour *in vitro*.

1.2.2.2 ES cell-derived neural progenitors

Several studies have demonstrated the potential of hES and mES cell derived neural precursors to incorporate into the CNS, where they differentiate into neurons and glia morphologically indistinguishable from neighbouring host cells (Brustle et al. 1997; McDonald et al. 1999; Reubinoff et al. 2001; Zhang et al. 2001).

When investigated in more detail, it was revealed that at single cell level grafted mES cells derived neurons exhibited morphological and functional integration into a large variety of regions of the developing rodent brain. Yet, many of the incorporated neurons failed to express appropriate region specific genes. Hence it was concluded

that lack of an appropriate regional code does not prelude morphological and synaptic integration of ES cell derived neurons (Wernig et al. 2004).
In vitro the responsiveness and potential of both mouse and human ES cell derived neural progenitors for directed differentiation seems temporally regulated (Li et al. 2005; Bouhon et al. 2006). The mechanisms that limit temporal responsiveness to patterning cues are unknown. However, since extrinsic cues conferred by the spatial organization and interaction of cells *in vivo* are absent *in vitro,* it has been suggested that temporal responsiveness is a mainly intrinsic property (Allen 2008).
So if the herein used hES cell-derived neural progenitors are as pliable as early mouse derived neural progenitors it might be possible to guide their region specific gene expression using a co-culture approach. Through combining this approach of regional pre-specification with the controlled generation of neuronal, astrocytic and oligodendroglial subpopulations, regionally defined donor cells may be created for future regenerative therapies.

1.3 Epigenetic regulation of developmental potential

The term epigenetics refers to a collection of mechanisms that together define the phenotype of a cell without affecting its genotype (Sasaki and Matsui 2008). It is clear that epigenetic changes via coordinating fate-determining gene expression influence the developmental potential of both embryonic and neural stem cells thus regulating neural lineage progression.
Gene expression is not only dependent on the presence of appropriate transcription factors interacting with enhancer as well as promoter elements in a cell-type or tissue-specific manner but is also based on the availability of binding sites regulated by higher orders of chromatin structure (Allen 2008). These specific patterns and their alterations are somatically heritable and the propagation of epigenetic status to the next cell generation is important for establishing cell type specific gene expression and maintaining cell identity (Avots et al. 2002; Jaenisch and Bird 2003). It has been known for some time that in transcriptionally active chromatin domains the histones are hyperacetylated and the DNA is demethylated (or showing very little methylation). In contrast, in inactive chromatin regions the DNA is methylated and the histones are hypoacetylated (Cedar 1988). Apart from these two modifications histone methylation, phosphorylation, ubiquination and sumoylation as well as polycomb-trithorax proteins affect the epigenetic status (Jenuwein and Allis 2001).

Histone acetylation occurs reversibly on lysine e-$NH3^+$ groups of core histones, effecting a change in electrostatic charge and a subsequent loosening of the chromatin. The level of histone acetylation depends on the opposing activities of histone acetyltransferases (HATs) and deacetylases (HDACs) (Eberharter and Becker 2002). For the latter enzyme family several specific inhibitors are known for instance trichostatin A (TSA) (Yoshida et al. 1990) and valproic acid (2-propylpentanoic acid, VPA). VPA is a well-tolerated anticonvulsant inhibiting both class I and II HDACs resulting in hyperacetylation of histone H3 and H4 (Kramer et al. 2003). In addition to its HDACs inhibiting properties, VPA has been reported to modulate GABA levels in the brain and to activate ERK pathway mediated neurotropic actions (Biggs et al. 1994; Hao et al. 2004).

AzaC, on the other hand, is often used as a DNA methylation inhibitor. When incorporated into DNA, it covalently binds to and irreversibly blocks the ubiquitously expressed maintenance methyltransferase Dnmt1, allowing passive demethylation to take place as cells divide (Pietrobono et al. 2002). Treatment with AzaC in combination with TSA resulted in a synergist reactivation of silenced genes in tumour cells (Cameron et al 1999). This result might suggest that epigenetic modifications can influence the developmental potential of a cell.

1.4 Aim

Future application of human embryonic stem cell derived neural precursors will depend critically on the ability of patterning the cells in a predictable manner. This capacity may then result in generation of regionally defined cell types capable of functionally integrating into their new environment. So far only few regionally specified cell populations relevant to transplantation strategies of neurodegenerative disorders have been obtained *in vitro*, e.g. dopaminergic neurons and motoneurons (Perrier et al. 2004; Li et al. 2005). Therefore the purpose of this work was to systematically investigate whether primary cells from different regions of the developing rodent brain – which themselves are known to be regionally specified (Zappone et al. 2000; Hitoshi et al. 2002) – can influence the regional identity of human ES cell-derived neural stem cells *in vitro*.

As a basis for all experiments the first aim was to investigate the ground state of regional gene expression of the hES cell derived neural stem cells established in our group. As readout of this and the following investigations human specific

quantitative RT-PCR expression analysis of region specific transcription factors was used.

To subsequently investigate the main objective – whether regionalisation cues from primary cells can be utilized to prime the regional identity of the hESNSCs – paradigmatic co-culture settings were chosen. In these, hESNSCs were exposed to forebrain, midbrain, hindbrain/cerebellum and spinal cord of embryonic and P0 mice as well as hippocampal and cerebellar slices of 9-day-old rats. The influence of both – cell-mediated and diffusible – cues was assessed.

Since it is known that the developmental potential of ES cells can be in part ascribed to their dynamic epigenetic state, a further question to be addressed was whether DNA demethylation and histone hyperacetylation alter the response of hESNSCs to primary cell derived cues.

2 Materials and Methods

2.1 General

All work involving human embryonic stem cells was approved by the Robert-Koch-Institute and was carried out in accordance with the German Stem Cell law.
Materials and solutions for the cell culture were appropriately sterilized (autoclaving, heat-sterilisation, sterile filtration) prior to use or bought sterile from the manufacturer. A complete list of the used equipment, materials, chemicals, antibodies and primers can be found in the appendix.

2.2 Cell types

The human embryonic stem cell lines were provided by Joseph Itskovitz-Eldor, Haifa, Israel. The human embryonic stem cell derived neural precursors of the H9.2 line used in this work were established by myself whereas the I3 hES-derived neural precursors – including a EGFP over-expressing line – were kindly provided by Dr. Koch.
For the co-cultures primary cells derived from embryonic and neonatal brains of C57B6 mice were used. For the slice cultures, brains of 9-day old Wistar rats were employed.

2.3 Human embryonic stem cells

To preserve the undifferentiated state of hES cells, they are cultured on mitotically inactivated mouse fibroblasts under standard conditions.

2.3.1 Culture of murine embryonic fibroblasts

The serum-free medium used for cultivation of hES cells is not suitable for fibroblasts. Under serum-free conditions they develop a spindle-like appearance and detach from the tissue culture dish after approximately one week. Therefore, the fibroblasts are thawed in medium supplemented with serum and are only cultured under serum-free conditions when they serve as feeder cells for hES cells.
Vials with cryopreserved murine embryonic fibroblasts were thawed in a water bath at 37°C, transferred in 5ml MEF Medium, centrifuged at 1200 rpm for 5 minutes, and plated on one 15cm dish per vial. The cells were cultured at 37°C and 5% CO_2. When

they had reached confluence, cells were washed twice in PBS, incubated with trypsin/EDTA for 5 minutes at 37°C and washed of with FCS containing MEF-Medium. The cell suspension was spun down at 1200 rpm for 5min and re-plated onto 15cm dishes in a 1:3 ratio. In this way the cells were cultured for up to 4 passages.

2.3.2 Mitotic inactivation of murine embryonic fibroblasts via γ-irradiation

A prerequisite for the culture of hES cells on fibroblasts is the mitotic inactivation of the feeder cells since fibroblasts proliferate faster than hES cells and would otherwise outgrow the stem cells.

For γ-irradiation confluent 15 cm dishes were trypsinized as described above (2.3.1). The cell pellets of 24-26 dishes were pooled in 35ml MEF Medium and transferred to a T175 culture flask. The γ-irradiation was carried out in the therapeutic radiology unit at the University of Bonn medical centre either on a linear accelerator (MEVATRON MD2, Siemens) using 6MV photons at maximal dosage (17mm) or in a tissue equivalent solid phantom (RW3, PTW Freiburg) at a focus-tissue–distance of 100 cm. After irradiating the fibroblasts with 15 Gy, they were plated onto gelatine coated 15cm cell culture dishes. After 24h the irradiated fibroblasts were frozen at a concentration of $2.4x10^6$ cells per vial.

2.3.3 Thawing of mitotically inactivated murine embryonic fibroblasts

Vials with cryopreserved γ-irradiated murine embryonic fibroblasts were thawed in a water bath at 37°C, transferred in 5ml MEF-Medium, centrifuged at 1200 rpm for 5 minutes and plated onto two gelatine-coated six-well tissue culture plates per vial. This results in $2x10^5$ fibroblasts per well of a six-well tissue culture plate. They were cultured at 37°C and 5% CO_2. No less than eight hours and no longer than three days after plating, hES cells were seeded on the mitotically inactivated fibroblasts.

2.3.4 Thawing of human embryonic stem cells

Both freezing and thawing of hES cells is an inefficient process, because survival rates are low and spontaneous differentiation rates are high. Therefore, human ES cells are kept as permanent cultures and thawed as seldom as possible.

The hES cells were thawed quickly in a water bath at 37°C, transferred to a Falcon tube and 5ml hES cell medium were added slowly and cautiously. While the hES cells were centrifuged at 800 rpm for 3 minutes the medium of one well of a six-well

plate containing irradiated fibroblasts was changed from MEF medium to hES cell medium. The hES cell pellet was re-suspended cautiously in 1ml hES cell medium. Subsequently the cells were seeded homogenously onto the previously prepared fibroblast feeder layer and incubated at 37°C/ 5% CO_2.

2.3.5 Passaging of human ES cells

Human ES cells are usually passaged as small aggregates, since this approach increases the plating efficiency and reduces spontaneous differentiation as well as karyotypic abnormalities. Thus, the application of trypsin, which dissolves hES cell aggregates, is not suitable. Instead a combination of collagenase IV treatment and mechanical methods is employed.

HES cell culture medium was changed every day and the cells were passaged every three to six days. In order to do so, the medium was aspirated and 1mg/ml collagenase IV (0.5ml/well) was administered for 45 minutes. Afterwards the collagenase was diluted with 1ml hES medium per well and the hES cell colonies were rinsed of the tissue culture plate. Following centrifugation at 800 rpm for 3 minutes 1ml hES cell medium was added to the cell pellet which was then re-suspended using a 1000μl Eppendorf pipette until only small aggregates remained visible. Finally, the cells were seeded homogenously in ratios between 1:1 and 1:6 in hES cell medium onto the previously prepared fibroblast feeder layer (see 2.3.3) and incubated at 37°C/ 5% CO_2.

2.3.6 Freezing of human ES cells

Human ES cells were detached from the tissue culture plates by collagenase IV treatment and centrifuged as described in 2.3.5. Depending on the density of the culture one to three wells were frozen per cryovial. The pellet was cautiously re-suspended in 0.5ml hES medium per cryovial employing a 5ml plastic pipette in order to preserve the cell aggregates (colonies). Subsequently the cell suspension was aliquoted into cryovials and 0.5ml hES freezing medium were added drop wise to each vial. The cells were slowly frozen down at 1°C per minute to -80°C in a freezing container and transferred to liquid nitrogen on the next day.

2.3.7 Induction of multi-lineage differentiation from hES *in vitro*

When grown as aggregates in suspension culture under favourable conditions ES cells form structures termed embryoid bodies (EBs), which consist of cells from all three germ layers.

This is generally considered as indication for the maintenance of the hES cell state. Moreover it is the first step in deriving human embryonic stem cell derived neural precursors.

Human ES cells were detached from the feeder layer and spun down as described above (2.3.5). In the next step the pellet was re-suspended very cautiously in EB medium supplemented with FCS in order to preserve the hES cells as aggregates. The aggregates where then cultured in suspension in bacterial grade Petri dishes. The medium was changed every other day through transferring the EBs into a Flacon tube. After the cells had settled at the bottom the medium was aspirated and replaced with fresh EB medium.

2.4 HES cell derived neural precursors

The hESNSCs established in the course of this project were derived from human embryonic stem cells of the H9.2 line in passage 48-52.

2.4.1 Derivation and culture

Human neural precursors were derived as described previously (Zhang et al. 2001; Koch et al. 2009). Briefly, ES cell colonies were detached from the feeder layer and EB formation was induced as described above using serum replacement (2.3.8). After 4 days the EBs were plated on polyornithine treated tissue culture dishes on which they attached. On the next day the medium was changed to ITSFn medium and thereafter medium was exchanged every other day. Within 10 to 14 days the embryoid bodies generated outgrowths in which neural rosettes like structures developed. These structures were mechanically isolated using a gauge needle under a dissection microscope at the horizontal hood. They were rinsed off with N2 medium and transferred to bacterial grade Petri dishes. Thereafter they were cultured as free-floating neurospheres in N2 medium at 37°C/ 5% CO_2 on a shaker at low setting. Medium was changed every other day. After 3 weeks the neurospheres were pooled in a 15ml Falcon tube and after addition of 1ml trypsin they were incubated for 5 min at 37 °C. In the next step 1ml trypsin inhibitor and 10% DNase were added before the neurospheres were triturated to single cells. The cells were centrifuged in 7ml N2 medium at 1000 rpm for 4 minutes, resuspended in 1ml N2 medium and plated onto 1 well of a polyornithine/laminin coated 12-well tissue culture dish. From then on cells were proliferated in N2 medium supplemented with 10 ng/ml FGF2, 10 ng/ml EGF and B27 1:1000. During the first seven days medium was changed daily.

2.4.2 Propagation

2.4.2.1 Preparation of polyornithine/laminin coated dishes

The cells were routinely passaged every three days. In order to do so tissue culture dishes were first coated with polyornithine for at least two hours. After this time the dishes were washed twice with PBS and subsequently coated in laminin 1μg/ml for at least 45 minutes. The treated dishes were used no longer than three days after coating.

2.4.2.2 Passaging of hESNSCs

Usually hESNSCs were split in a 1:2 or 1:3 ratio every three days. After aspirating medium from the cells, 0.5ml trypsin were added per well of a six-well tissue culture dish. After incubation for three minutes at room temperature or at 37°C, 0.5ml trypsin inhibitor were added to each well, the cells were rinsed off and collected in a Falcon tube. While the cells were centrifuged at 1000 rpm for 5 minutes, laminin was aspirated from polyornithine/laminin treated six-well tissue culture dishes and 1,5ml N2 medium supplemented with FGF, EGF and B27 were added per well. In the next step the cell pellet was resuspended in 0.5ml N2 medium per well and seeded on the prepared tissue culture dishes. On the next day EGF, FGF and B27 were added (see 2.4.1). On the second day after passaging the medium was changed completely.

2.4.3 Induction of differentiation from hESNSCs

2.4.3.1 Preparation of Matrigel coated dishes

Matrigel coated dished were routinely used for long-term differentiation of hESNSCs since the cells adhered better to this coating than to polyornithine/laminin.

Matrigel® was thawed over night at 4°C and diluted 1:30 in ice-cold Kockout-DMEM before coating tissue culture dishes for 2 hours at room temperature or overnight at 4°C, respectively. After this time the Matrigel-solution was aspirated and cells were seeded on the plates as described above (2.4.2.2).

2.4.3.2 Induction of differentiation

When cultured in the presence of FGF and EGF the hESNSCs proliferate, whereas upon withdrawal of these two factors they will cease proliferating and start to differentiate.

Matrigel was aspirated and cells were passaged as described above (2.4.2.2) in a ratio of 1:2 or 1:3 onto the Matrigel-pre-treated tissue culture dishes. On the next day N2 medium was exchanged with NGMC medium to induce differentiation. Thereafter NGMC medium was changed every other day.

2.4.4 Co-cultures and transplantation of hESNSCs

Non co-cultured hESNSCs of the same passage as co-cultured cells were used as control. They were differentiated for the duration of the co-cultures on Matrigel. In the case of the organotypic slice model, control cells were cultured on the insert without an orgonotypic slice or in the case of re-aggregation co-culture, re-aggregated without rodent cells.

2.4.4.1 Shared medium co-culture

Cells were trypsinized as described above (2.4.2.2) and 150.000 hESNSCs were plated in each well of a 12-well cell culture plate pre-coated with Matrigel in N2 medium. On the next day the medium was changed to NGMC and single cell suspensions of the cerebellum, mesencephalon and telencephalon were prepared from newborn (P0) C57/B6 mice following a previously described protocol (Polleux and Ghosh 2002). The whole dissection procedure was carried out on ice in R6 medium. Briefly, the pups were anesthetized using isolfuran. Following cervial dislocation and removal of the skull, the brain was dissected out. In the next step the telencephalon, mesencephalon and cerebellum were separated under a dissection microscope using forceps and cleaned of blood vessels and meninges. Each tissue type was placed in a separated 15ml Falcon tube, in which all tissues of the same type were subsequently pooled, i.e. the cerebella of all dissected pups were pooled in one Falcon tube. The tissue was then digested in papain for 20 minutes at 37°C. After this time the reaction was halted using trypsin inhibitor and the tissue was rinsed several times in R6 medium. Subsequently, a single cell preparation was derived via triturating the primary cells to a near single cell suspension in R6 medium supplemented with DNase (0.1%) using flamed Pasteur pipettes and passing them through a cell strainer. Afterwards the cells were centrifuged at 1000 rpm for 5min and the pellet was re-suspended in NGMC medium supplemented with 1μg/ml laminin. Finally 200.000 primary cells of each region were placed in separate cell culture inserts (Falcon, 0.4μm pore size) above the neural precursor cells (Fig. 11). During the whole culture period of 28 days the medium was changed every 3-4 days via carefully inserting a Pasteur pipette into the space between insert wall and the wall of the tissue culture well.

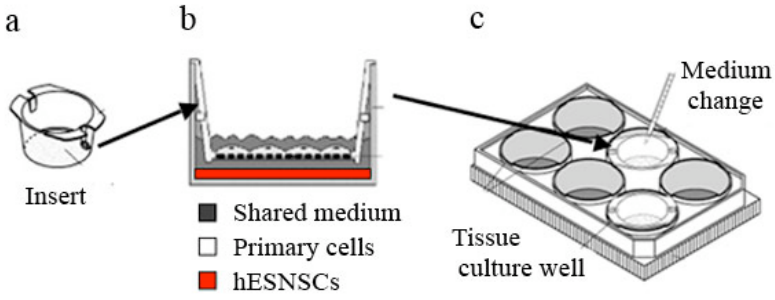

Fig. 11. Shared medium co-culture setup (modified from BD Falcon)
(a) An insert was used for culturing primary cells and hESNSCs in the same medium **(b)** in normal tissue culture dishes. **(c)** Medium was changed via carefully inserting a pipette into the space between insert and wall of the tissue culture well.

2.4.4.2 Physical contact co-culture

In the first experiments hESNSCs expressing EGFP under control of the phosphoglycerate kinase (PGK) promoter and a blasticidine drug resistance gene under control of the SV40 promoter derived from I3 human embryonic stem cells kindly provided by Dr. Koch were used.

In the case of physical contact co-culture with embryonic cells, timed-pregnant C57/B6 mice (the day when a vaginal plug was detected was designated as embryonic day 0) were anesthetized on the appropriate day using isolfuran. Following cervical dislocation, the embryos were removed form the uterus and placed in a Petri dish containing PBS. They were freed of the uterus using forceps and placed in pairs in 3 cm Petri dishes in R6 medium. Under a dissecting microscope the brains were exposed and the following regions, depending on the individual experiments, were placed into separated Falcon tubes in R6 medium on ice:

E15: anterior forebrain, midbrain hindbrain organizer region (MHO) (Fig. 12a).

E12.5: forebrain, midbrain or MHO, hindbrain, spinal cord (Fig. 12b)

The midbrain-hindbrain boundary was used as anatomical cut-off point to separate the midbrain from the hindbrain (Fig. 12).

All tissues of one type were pooled, i.e. the spinal cords of all embryos dissected at one time were pooled in one Falcon tube. In the next step primary cells were slowly and carefully resuspended to near single cell suspension in R6 medium in the presence of DNAse (0.1%) using flamed Pasteur pipettes. After counting of the cells and centrifugation at 1000 rpm for 4 minutes the cell pellet was resuspended in N2 medium. Cells were plated on polyornithine/laminin coated 12 well plates (each region on a separate plate) at densities of 100.000 cells/cm^2 (anterior telencephalon, spinal cord) or 200.000 cells/ cm^2 (midbrain, hindbrain). After 7h, when the primary

cells had begun to settle, the cell debris was washed off and 200.000 hESNSCs were added per well. On the following day the N2 medium was exchanged with NGMC medium. Thereafter medium was changed every other day.

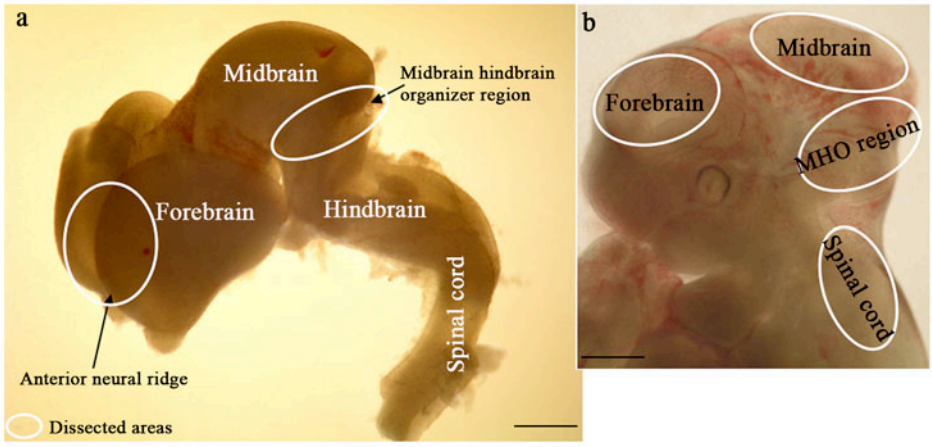

Fig. 12. Areas approximately prepared from a total E14/ E15 and E12.5 mouse brain
(a) Total E14 mouse brain, indicated are the regions approximately prepared for the different experiments (scale bar 600μm). **(b)** Head of an E12.5 embryo. Primary cells were prepared from the highlighted regions (scale bar 700μm).

In direct co-culture with cells of P0 animals, the primary cell preparation was similar to the procedure described in 2.4.4.1 above.

2.4.4.3 Re-aggregation co-culture

Primary cells were prepared from the midbrain and hindbrain of E12.5 C57/B6 embryos as described above (2.4.4.2, for approximate areas see Fig. 12b). After centrifugation all cells of one brain region e.g. the midbrain (Fig. 12) were mixed with hESNSCs at a ratio of 10:1 in 1ml NGMC medium.

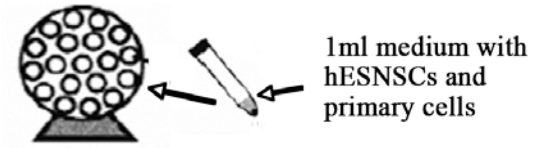

Fig. 13. Re-aggregation co-culture set up (modified from Studer et al 1998)
Cells were grown as free-floating aggregates at slow rotation.

Co-cultures were grown as free floating aggregates in 15ml Flacon tubes in a roller drum system (Studer et al. 1998) (Fig. 13) for 10 days, while the medium was changed every other day.

2.4.4.4 Transplantation of neural precursors onto organotypic slice cultures

Organotypic slice cultures of the hippocampus, and cerebellum were prepared by Ms Steinfarz from 9 day old Sprague Dawlesy rats as described (Scheffler et al. 2003). Briefly, slices (400μm) containing the dentate gyrus, entorhinal cortex, and adjacent

areas of the temporal cortex as well as the cerebellum were prepared and cultured in interphase conditions at 5% CO_2 and 35°C (Stoppini et al., 1991). Cultures were started in a horse serum-containing medium, which was gradually replaced until day 5 in culture by a serum-free, defined solution based on DMEM supplemented with N2 and B27. On day five of culture 200.000 hESNSCs were seeded on each slice. Medium was changed every other day throughout the culture period.

2.4.4.5 Transplantation into neonatal rats

HESNSCs were trypsinised and centrifuged as described above (2.4.2.2), resuspended at 100.000 cells/μl in Cytocoon buffer and placed on ice. CD1 rat pups (n=16, P1) were cryo-anesthetized in melting ice and 2 μl of the cell suspension were injected by Dr. Koch into the left hippocampus and the cerebellum using a glass micropipette (70μm). The pups were subsequently warmed on an electrical blanket and when they had regained consciousness returned to their cage. At P20 the animals were anesthetized, perfused with ice cold 4% paraformaldehyde at room temperature for 5-10 minutes at a low rate of approximately 10ml/min followed by perfusion with PBS at the same flow rate for 10min. The brain was excised; the left hippocampus as well as the cerebellum were dissected out and immersed in 4% paraformaldehyde.

2.4.5 DNA demethylation/ histone hyperacetylation experiments

2.4.5.1 VPA/AzaC treatment

Two hours after passaging the hESNSCs at a 1:1 ½ ratio, N2 medium supplemented with FGF, EGF and B27 medium (as described in 2.4.1) was changed and 1mM VPA and 250-500nM AzaC were added to the culture. Thereafter medium with VPA/AzaC was changed every day for 3 days (72h), while control cells were left untreated.

After this time any other treatment was started immediately without passaging the cells again, since the survival rate of trypsin application directly following VPA/AzaC treatment was very low.

2.4.5.2 Methylation status analysis

Commonly the methylation status of a DNA sequence is determined by incubation with sodium bisulfite resulting in conversion of unmethylated cytosine residues to uracil and leaving methylated cytosines unchanged, thereby giving rise to different sequences for methylated and unmethylated DNA.

For the methylation status analysis total DNA was extracted with the DNeasy Blood and Tissue kit followed by bisulfite treatment using the EpiTect Bisulfite Kit according to the manufacturer's instructions. Once the DNA was bisulfite treated methylation status of heavily methylated long interspersed repetitive elements (LINEs) was analysed as indicator for the whole genome. This was done via the COBRA assay published by Yang and co-workers. Briefly, 25µl PCR reactions were carried out under the conditions as specified in the appendix. The final PCR product was digested with HinF1 at 90 minutes at 37°C. This enzyme cut the sequence GANTC, which was only present at originally methylated sites since the bisulfite treatment converted all unmethylated cytosine residues to uracil. Therefore it was possible to distinguish between originally methylated and unmethylated sites using this strategy. Finally the digested PCR products were separated by electrophoresis on a 3% agarose gel and stained with ethidium bromide.

2.4.5.3 Western Blot

Western blot was used to assess the acetylation status of histone 4 in VPA treated cells. The employed antibody recognised acetylation on lysines 5, 8, 12 and 16 of histone 4.
Compositions of all buffers and solutions are listed in the appendix. During protein separation a prestained marker containing proteins of 250kDa, 150kDa, 100kDa, 75kDa, 50kDa 37kDa, 25kDa, 20kDa, 15kDa und 10kDa obtained from Biorad was used.
Equal amounts of protein were denatured at 95°C for 5 minutes in Laemmli sample buffer and separated on a 10% SDS PAGE gel, which was run for 1 hour at 100V. The proteins were transferred to a nitrocellulose membrane using the Biorad mini trans-blot system at 100V for one hour. Protein binding was confirmed via Ponceau staining before the membrane was blocked with 5% skimmed milk for 1 hour at room temperature. Thereafter the membrane was probed with anti-acetyl histone H4 rabbit antiserum (recognizing acetylation on K5, 8, 12 and 16), the primary antibody, diluted in antibody blocking solution at 4°C over night. On the next day the membrane was washed with PBS/Tween and the peroxidase-conjugated secondary antibody was applied for 1 hour at room temperature. Finally the bound target protein was visualized using ECL (Pierce) reagents following the manufacturer's instructions.

2.4.5.3 Karyotypic stability

To ensure that histone hyperacetylation and DNA demethylation do not have an adverse effect on karyotypic stability FISH analysis and karyotypic analysis as

described in 2.5.3 were carried out with the help of Ms Limbach. At least 20 metaphases were counted for each condition.

2.4.5.4 Regional expression profile and neurogenic potential after treatment

After 72 hours of VPA/AzaC administration as described in 2.4.5.1, total RNA was extracted from the cells and analysed for the expression of region-specific markers via quantitative PCR. Additionally VPA/AzaC treated cells were differentiated for 18 days in NGMC medium. After this time they were stained for Tubb3 and GABA and their region-specific gene expression was analysed again using quantitative PCR.

2.4.5.5 Co-culture with VPA/AzaC pre-treated cells

To test whether hESNSCS are more amenable to primary cell derived patterning cues after epigenetic alteration a direct co-culture with cells of E12.5 C57/B6 mice was carried out.
The primary cells were prepared from forebrain, midbrain and hindbrain as described in 2.4.4.2 and mixed in a 1:1 ratio with hESNSCs. The cells were cultured for 5 and 9 days, respectively on polyornithine/laminin coated 12-well dishes in NGMC medium.

2.4.5.6 Treatment with BMP4/ RA following VPA/AzaC administration

To test whether neural precursors are more susceptible to morphogens after DNA demethylation and histone hyperacetylation, cells were treated with 3μM RA for 7-10 days in NGMC medium or 10ng/ml BMP4 for 4 days in N2 medium. At the end of the culture periods cells were either fixed in paraformaldehyde for immunohistochemical analysis or total RNA was extracted for quantitative PCR analysis.

2.5 Analytical Methods

2.5.1 Determination of cell number

Cell viability was determined via trypan blue staining. This diazo dye is not absorbed into live cells, but can permeate the membrane of dead cells. Therefore cells with intact membranes are not coloured, whereas dead cells show a distinctive blue staining. Since live cells are excluded from staining, this method is also called dye exclusion method.
To determine total cell number, a cell suspension generated via trypsin treatment was diluted 1:2 with trypan blue and counted in a Neubauer haemocytometer.

2.5.2 Proliferation assay

*5-bromo-2-deoxyuridine (BrdU) is a synthetic analogue of thymidine and can be incorporated into the newly synthesised DNA of replicating cells during S phase of cell cycle. It is commonly used to detect proliferatin*g cells.

To quantify the number of cells in the S-phase of cell cycle, hESNSCs were pulsed with 10μg/ml BrdU on the first day after passaging for 3 hours. Afterwards they were fixed in 4% paraformaldehyde. Simultaneously, hESNSCs were also analysed for the expression of Ki67, the prototypic cell cycle related nuclear protein, expressed by proliferating cells in all phases of the active cell cycle. It is absent in resting (G0) cells (Gerdes et al. 1991).

2.5.3 RNA extraction and quantitative RT-PCR analysis

2.5.3.1 RNA extraction

All work involving RNA was carried out using RNase free tubes and filter tips. Moreover only diethylpyrocarbonate (DEPC) treated water was employed.

Medium was aspirated and cells were lysed in 100μl Trifast per cm^2 of a cell culture dish. Complete brain slices and brain sections, on the other hand, were transferred to Falcon (15ml) or Eppendorf (1ml) tubes containing 1-2ml Trifast and homogenized using the Bandelin Bioplus homogenizer. The lysate of both cells and tissue was either stored at -80°C or immediately processed. In both cases a 15-minute incubation at room temperature followed the lysing step. Then 200μl chloroform were added per ml Trifast, and mixed thoroughly with the lysate. After another 15 minutes of incubation the protein, DNA and RNA fractions were separated via centrifugation at 12000xg/4°C. In the next step the (uppermost) aqueous phase was transferred to a new Eppendorf tube and mixed with 1 volume 70% ethanol in DEPC-water. From this step onwards the RNeasy kit was employed following the supplier's instructions including DNase treatment. RNA was quantified by optical density measurement on the Nanodrop at 260 nm (OD_{260}), where 1 OD_{260} equals 40μg/ml RNA. The RNA was either stored at -80°C or immediately reverse transcribed.

Since cell numbers following the first FACS experiments were very low, RNA was isolated from sorted cells using the PicoPuro RNA Isolation Kit according to the manufacturer's instructions.

2.5.3.2 Reverse transcription

For reverse transcription oligo$(dT)_{15}$ primers were employed to ensure that only mRNA was used as template.

For each sample 0.8μg total RNA were transcribed into cDNA using the I-script cDNA synthesis kit according to the manufacturer's protocol. As negative control the reverse transcriptase was omitted at the cDNA synthesis step once for each sample. The cDNA was either stored at -20°C or a (q)PCR reaction was carried out immediately following the reverse transcription step.

2.5.3.3 Design of human-specific primers

Since the human cells were not separated from the rodent cells at the end of the direct, the re-aggregation and the slice co-cultures or the in vivo transplantation experiments, human specific primers were designed to distinguish between gene expression in human and rodent cells.

For different genes of interest human-specific primers and rodent primers were designed in parallel using the Beacon Designer 5 software (Premier Biosoft) allowing a species specificity genome blast prior to ordering. The species-specificity of the primer pairs was controlled by quantitative RT-PCR using total fetal and adult mouse and/or rat brain cDNA and human fetal or adult brain cDNA (Stratagene) as positive controls.

2.5.3.4 Quantitative RT-PCR

All quantitative RT-PCRs were performed in triplicates on the Biorad Icylcer in 25μl reactions. Per reaction 1μl cDNA, 30pmol of each primer, 200μM of each dNTP, 3mM $MgCl_2$, 1:2.000.000 SYBR I Green, 10μM Fluorescein and 0.5U Tag-polymerase were added. Primer sequences and PCR conditions are listed in 11.6. The specificity of the PCR products was confirmed via melt curve analysis and gel electrophoresis.

In order to compare the expression level of the different genes, all data was normalized against the housekeeping gene L27, a ribosomal protein that is expressed at a constant level at all times. Threshold cycle, ct, was measured as the cycle number at which the SYBR I Green emission increased above a background (threshold) level. Specific mRNA transcript levels were calculated using Q-Gene Core Module 1.2 software (Muller et al. 2002).

2.5.4 Karyotype analysis

During long-term cultivation hESNSCs might acquire chromosomal abnormalities. Especially gaining a chromosome 12 or 17 leads to a growth advantage for the cells (Draper et al. 2004).

2.5.4.1 G-Banding

One day after passaging when the cells had reached 70% confluence medium was changed and 0.2μg/ml Colcemid were added for six hours. At the end of this period cells were washed twice in PBS, detached from the tissue culture dish via trypsin/trypsin inhibitor treatment and spun down. The pellet was cautiously re-suspended in 1ml 0.075M Potassium chloride (KCL) solution before adding another 2ml 0.075M KCl-solution and incubating the cell suspension for 10 minutes at 37°C. Afterwards three drops of fixative were added and the sample was centrifuged for 10 minutes at 1500 rpm. The pellet was washed twice more with fixative and finally re-suspended in 1ml fixative. At this point the samples were either stored at -20°C or directly dropped onto degreased microscope slides, which hat been pre-cooled at minus 20°C. When the samples had been checked for correct chromosome spreading they were incubated over a 60°C water bath for 20 seconds followed by drying on a heating plate at 60°C. The samples were incubated at 60°C over night and G-banding was performed the next day by Ms Limbach. Briefly, the samples were incubated for 55 seconds in 2,5μl bactrotrypsin/ml PBS at 37C, rinsed twice in PBS, stained with Giemsa-solution for 20 minutes, rinsed, dried, swirled in xylol and mounted in corbit. The analysis was carried out at the Institut für Humangenetik of the Medical University Bonn at a Leitz Diaplan 68236 microscope using the CytoVision Ultra software.

2.5.4.2 Fluorescence in situ hybridisation (FISH)

Cells were treated as described in 2.5.3.1. After re-suspension in fixative they were dropped onto microscope slides and subsequently Fish analysis was performed by Ms Limbach using the Vysis cep12 (32-132012) and cep17 (32-130017) probes according to the manufacture's protocol.

2.5.5 Agarose gel electrophoresis

DNA and RNA are negatively charged, due to the phosphate moieties of the sugar-phosphate backbone, thus they will migrate towards the positive pole of an electric field. During gel electrophoresis DNA (and RNA) fragments are separated according to their molecular weight and size, the greater the size of the fragments in any one analysis the lower the agarose concentration.

For separation of DNA on an agarose gel a 1kb (kilo base) marker and/or a 100bp (base pair) marker from Peqlab were used. Generally gels consisting of 1% agarose and TAE were employed. In the case of detecting fragments following restriction

digest with HinF1 3% gels were used. In order to analyse the size of the DNA fragments 0,5µg/µl ethidium bromide (EtBr) were added to the gel directly prior to pouring. EtBr intercalates between adjacent DNA base pairs and was visualized on a transilluminator. Gels were run at 85mV and 200mA for 45 minutes.

2.5.6 Fluorescence – activated cell sorting

At the end of the first slice co-culture experiments hESNSCs were separated from the primary cells using fluorescence-activated cell sorting. However, considering that the purity of the re-isolated EGFP-positive cells did not exceed 90%, residual host cells represented a potential source for false-positive results in the planned RT-PCR studies. To bypass these problems the use of species-specific RT-PCR primers was initiated.

For re-isolation of transplanted cells lentivirally transduced hESNSCs expressing EGFP under control of the phosphoglycerate kinase (PGK) promoter and a blasticidine drug resistance gene under control of the SV40 promoter kindly provided by Dr. Koch were used. All hESNSCs co-cultured with one brain region (e.g. all cells co-cultured with hippocampal slices) were pooled together and digested in papain (20 units/ml) for 20 minutes. The digestion was stopped by adding trypsin inhibitor solution and the tissue was triturated to a single cell suspension using flamed Pasteur pipettes. The mixture was re-suspended at 1-2 million cells/ml in FACS buffer containing 0,1% DNAse and all subsequent steps were performed on ice. For staining of dead cells 0,5µg/ml propidium iodide were added and the cell suspension was passed through a cell sieve. The EGFP-expressing cells were collected via flow cytometry using a 70µm ceramic nozzle at a speed of 5000 – 8000 events per second at the Institute of Molecular Medicine and Experimental Immunology, University of Bonn on a fluorescence-activated cell sorter FACS DiVa with the kind help of Mrs. Meiners. Viable cells as indicated by propidium iodide staining were gated by their forward and side scatter characteristics and gates were set to sort EGFP positive cells. Probes were collected in 1.5ml Eppendorf tubes, containing 20 units/ml RNAsin and 20µl cell extraction buffer. For quality control analysis of the FACS machine a GFP negative cell fraction was always collected in parallel. Immediately after sorting the GFP positive cells were frozen on dry ice.

2.5.7 Immunocytochemistry

Primary and secondary antibodies as well as the used dilutions, producers of the antibodies and peculiarities of the staining are summarized in 11.5.

Analysis of immunocytochemical staining was usually carried out on a fluorescence microscope (Axioskop2, Zeiss) with the exception of the organotypic slice cultures. These were analysed on a confocal laser scanning microscope (Olympus Fluoview 1000).

2.5.7.1 Detection of surface markers in cells grown as monolayer

Cells were fixed in 4% paraformaldehyde for 20 min at room temperature. When detecting GABA 0,03% glutaraldehyde were added to the paraformaldehyde. After this time the cells were washed twice in PBS, blocked for 30 minutes in blocking solution (10% normal goat serum, NGS, in PBS) and incubated with the primary antibody in blocking solution overnight at 4°C. On the next day the primary antibody was aspirated, cells were washed twice in PBS and the corresponding Cy3 or FITC conjugated secondary antibody was applied for 1 hour at room temperature in order to visualize the antigen. Finally cell nuclei were stained with DAPI (1:10000 in $NaHCO_3$) and the samples were mounted in Vectashield.

2.5.7.2 Detection of nuclear markers in cells grown as monolayer

For the detection of nuclear antigens the cells were fixed for 15 minutes at room temperature in 4% paraformaldehyde and subsequently the cell membrane was permeabilized via application of 0.5% Triton-X100 in PBS for 15 minutes at room temperature. Thereafter the staining continued with the blocking step and was carried out as described above (2.5.7.1).

2.5.7.3 Immunohistochemistry of organotypic slices cultures

Slices were fixed in 4% paraformaldehyde overnight at 4°C. In the morning they were rinsed twice in PBS and then either stored in PBS + sodium azide (0.1%) at 4°C or immediately processed. Immunolabelling was performed on free-floating specimens in 12-well tissue culture plates, with one slice per well. After blocking in 10% fetal calf serum in 0.1% Triton in PBS for at least 6 hours, the primary antibodies were applied in blocking solution for 24 hours at room temperature. During this time slices were rotated on a shaker at low setting. Afterward the slices were washed in PBS + sodium azide for 48 hours before the secondary antibodies were applied in blocking solution for approximately 12 hours. Finally the slices were washed again in PBS + sodium azide over the course of one day and mounted in Vectashield.

2.5.7.4 Immunochistochemistry using tyramide amplification

Tyramide signal amplification is an enzyme based method utilizing the catalytic activity of horseradish peroxidase (HRP) to achieve greatly enhanced (100-fold) labelling of the target antigen.

When using the tyramide amplification system slices were fixed as described above (2.5.7.3) and then treated with 0.003% H_2O_2 for four hours to quench any endogenous peroxidase activity. Afterwards the specimens were blocked and stained with rabbit-anti-human FoxG1 and mouse anti-human nuclei. Subsequently secondary antibodies were applied as described above (2.5.6.3). The tyramide enhanced antigen was detected using a biotinylated anti-rabbit antibody. In the next step streptavidin coupled horseradish peroxidase was applied for 6 hours and finally the tyramide development was performed in 0.003% H_2O for 35 min.

2.5.7.5 Detection of BrdU

The detection of BrdU was accomplished following the method described in 2.5.6.1 except that after permeabilising the cell membrane also the DNA was denatured via incubation in 2N hydroxychloride (HCl) for 10 minutes. Subsequently the HCl was neutralized using 0.1 M Borate buffer and the procedure continued with the blocking step as described in 2.5.6.1.

2.5.8 Extraction of total protein

Medium was aspirated; cells were washed once in ice-cold PBS and PBS containing 1mM PMSF was applied. In the next step cells were scraped from the dish, centrifuged at 1800 rpm/4°C for 10 minutes, resuspended in 50μl lysing buffer per well of a 6-well plate (Nunc) and lysed for 1 hour on ice. The lysate was then spun down at 12000 rpm for 15 minutes and the supernatant was transferred to a 1.5 ml Eppendorf tube. The protein was quantified using the BCA assay kit and frozen at -20°C.

2.5.9 Statistical Analysis

Unless stated otherwise, errors are given as ± SD and data were analysed using students-t test with $p^* \leq 0.05$. The quantitative RT-PCR data of the co-cultures was analysed using two-way ANOVA (SPSS), following the kind instructions of Mr. Mende from the Institut für Medizinische Biometrie, Informatik und Epidemiologie (IMBIE) with $p^* \leq 0.05$ and $p^* \leq 0.01$.

3 Results

3.1 HESNSCs adopt a specific regional fate under standard culture conditions

As a basis for any meaningful analysis of the hESNSCs regionalization potential the ground state of region-specific gene expression was analysed. For the differential gene expression analysis several transcription factors were used as candidate markers, which are characteristic for specific brain regions (see Table 3). In addition also cell type specific markers were tested (Table 4), since some regional transcription factors are only expressed in specific cell types. For instance the telencephalic marker FoxG1 is expressed in neural precursors and neurons but not in mature glia. Hence if FoxG1 expression is investigated the expression of a neuronal marker has to be assessed in parallel in order to ascertain whether a change in FoxG1 expression level can be explained by a change in the ratio of neurons in the cell population.

In order to establish a regionalization profile, hESNSCs (H9.2, I3) of passages 3, 14 and 28 in their proliferative state, i.e. during expansion mediated by the growth factors EGF and FGF-2 were tested for region and cell type specific gene expression. Additionally, cells from these three passages that had been cultured for 28 days without growth factors, i.e. which were differentiated, were also assayed. Total human fetal brain RNA at 19 weeks of age obtained from Stratagene was used as positive control. While the morphology of the cells was not altered over the passages, it was evident from the quantitative RT-PCR data that the regional expression profile changed. The hESNSCs showed highest levels of the forebrain markers FoxG1 and Dlx1 in the first 9 passages (Fig. 14), but propagation in EGF and FGF resulted in a marked down-regulation of the forebrain markers already in passage 14. In cells of passage 28 the expression level of the forebrain candidate genes was almost undetectable (Fig. 15). This loss of forebrain identity was confirmed by immunocytochemical analysis of FoxG1 protein expression (Fig. 16). At the same time increased passaging resulted in an up-regulation of the hindbrain markers HoxB1 and Gbx2 from undetectable or very low levels to levels 5 fold higher in passage 28 compared to passage 3. This up-regulation of hindbrain gene expression was already observed in passage 14 and was especially obvious in passage 28 in both hESNSC cell lines (Fig. 14, Fig. 15).

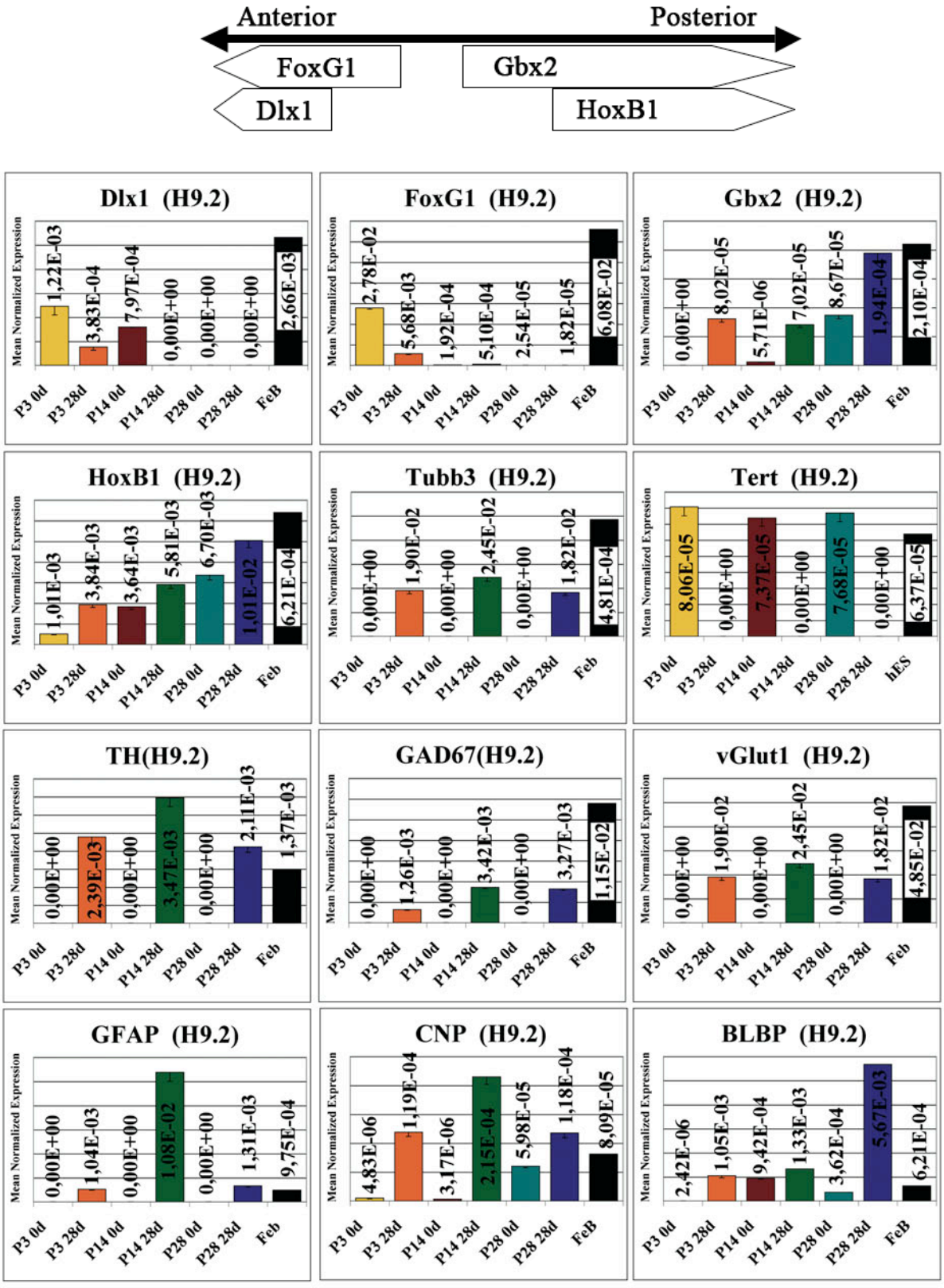

Fig. 14. Expression of region and cell type specific markers in proliferating and differentiated H9.2 derived hESNSCs

Dlx1: ventral forebrain, FoxG1: telencephalon, HoxB1/Gbx2: hindbrain (see schematic overview on top), Tubb3: early neurons, Tert.: telomerase, TH: tyrosine hydroxylase – rate limiting enzyme in dopamine synthesis, vGlut1: glutamatergic neurons, Gad67: inhibitory interneurons, GFAP: astrocytes, CNP: oligodendrocytes, BLBP: radial glia, (P: passage number of hESNSCs used, d: days of differentiation, FeB = total human fetal brain cDNA was used as control). All results were normalised to L27.

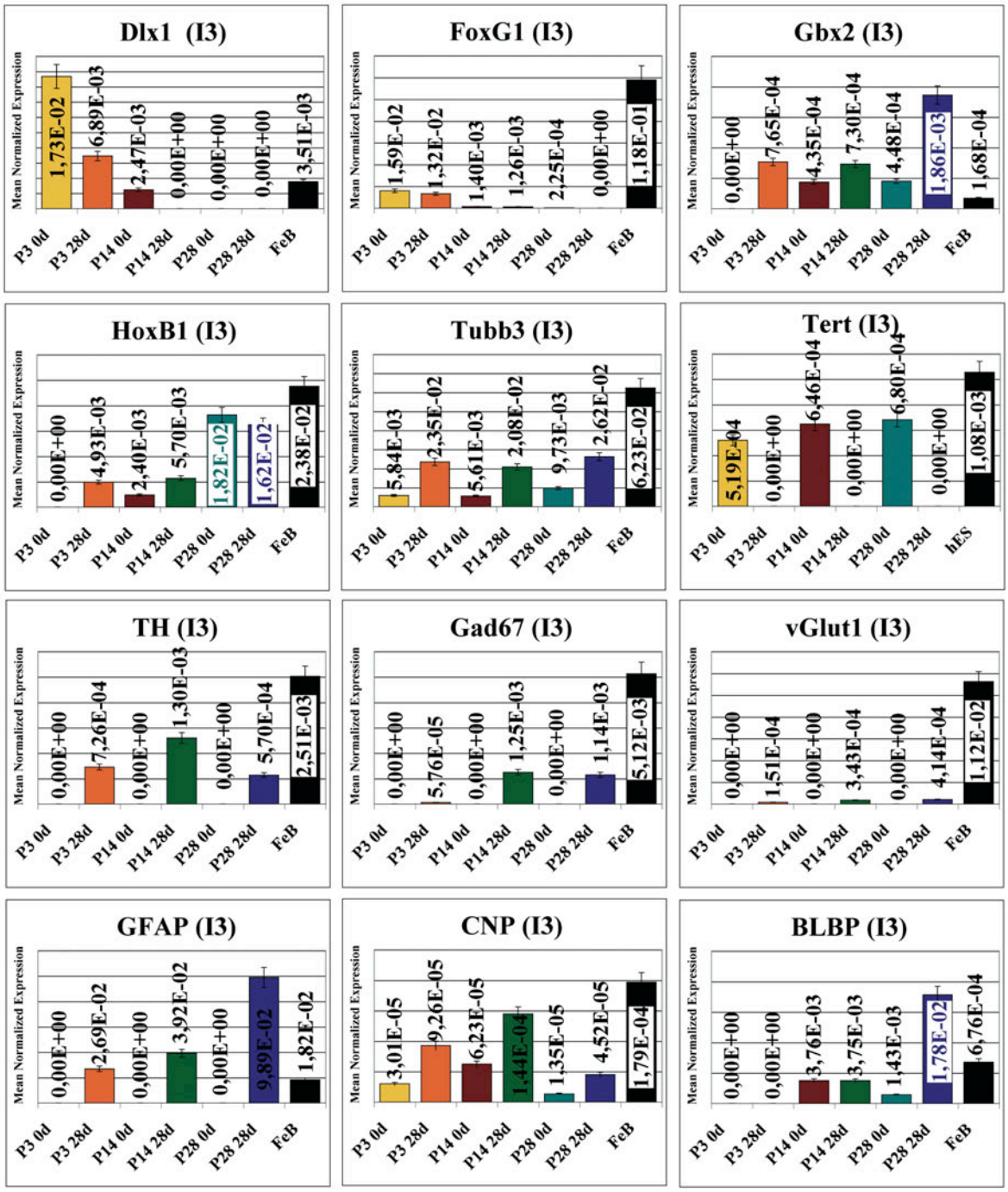

Fig. 15. Expression of region and cell type specific markers in proliferating and differentiated I3 derived hESNSCs
see Fig. 14 for details

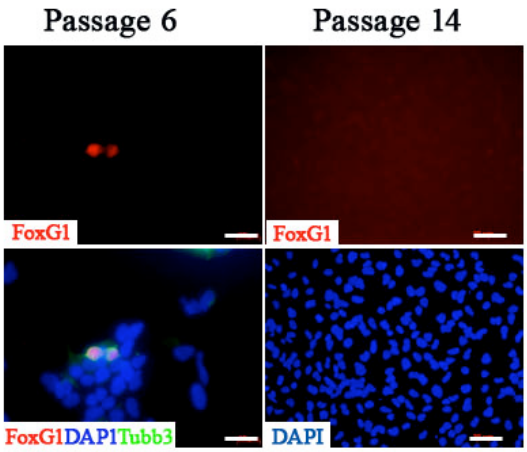

Fig. 16. Prolonged passaging reduced the number of FoxG1-positive cells
Neural precursors derived from H9.2 human embryonic stem cells showed FoxG1 staining in passage six. In passage 14, however, FoxG1 staining could not be detected anymore (scale bar left side 40μm, right side 80μm).

In addition to region specific markers cell type specific transcription factors were assessed. HESNSCs expressed markers characteristic for neuronal (Tubb3), astrocytic (GFAP) and oligodendrocytic (CNP) differentiation in a pattern reflecting proliferation or differentiation, e.g. proliferating cells showed less Tubb3 expression than differentiated cells of the same passage. The neurogenic potential – i.e. the Tubb3 expression level – remained constant over the passages (Fig. 14, Fig. 15). As expected Telomerase expression decreased upon differentiation. Moreover differentiating neural precursors expressed vGlut1, one of the three known glutamate transporters at excitatory synapses, tyrosine hydroxylase, the rate limiting enzyme in dopamine synthesis and glutamic acid decarboxylase (GAD), the main enzyme in Gamma amino butyric acid (GABA) synthesis, which was used as candidate gene for inhibitory interneurons (Watling 1998).

It has been shown previously that hESNSCs differentiate first into mainly GABAergic neurons, then into astrocytes and finally oligodendrocytes started to appear in the cultures (Koch et al. 2009). In accordance with these results it was found on protein level that hESNSCs in passage 28 which had been differentiated for 18 days expressed Tubb3 and GABA, the primary inhibitory neurotransmitter of the brain. At this time point half of the hESNSCs were Tubb3 positive and of these 53± 2,9% expressed GABA (Fig. 17).

Interestingly, expression of the radial glia marker BLBP, which was undetectable in passage 3, rose over the passages and during differentiation.

Taken together these results indicate that during *in vitro* culture stably proliferating hESNSCs loose anterior telencephalic identities as indicated by the loss of FoxG1 and seemed regionally biased towards posterior fates compatible with a hindbrain location as indicated by the increased expression levels of HoxB1 and Gbx2. This

shift might be caused by FGF, which is known to alter developmental competence and posteriorize neuroepithelial cells (Gabay et al. 2003).

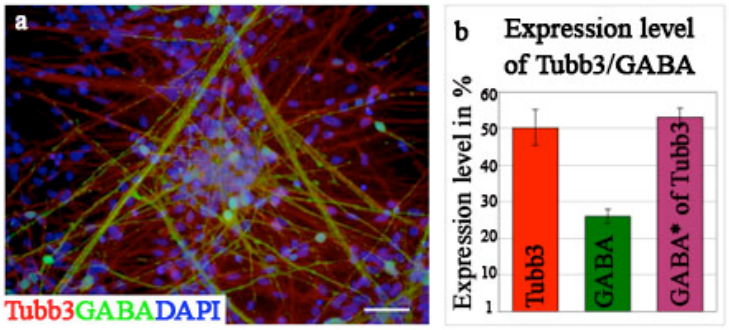

Fig. 17. Differentiating hESNSCs expressed Tubb3 and GABA
After 18 days of differentiation more than 50% of the hESNSCs **(b)** had developed into Tubb3 **(a)** positive neurons of which 53± 2,9% were GABAergic (scale bar 50μm).

3.1.1 Regional gene expression can be influenced via retinoic acid application

After establishing the regional ground state of the neural precursor cells it was investigated whether it is possible to change their regional fate using retinoic acid. This morphogen was chosen since it plays a crucial role in the specification of the vertebrate hindbrain and induces Hox gene expression (Sirbu et al. 2005). Thus neural precursors were treated with RA (5μM) for 14 days. After this time staining for HoxB4, which is rarely expressed at the protein level under normal differentiation conditions, revealed that 6,4± 0,49% of the RA treated cells and 0,23± 0,11% of the control cells expressed the marker. Thus significantly more RA treated cells expressed HoxB4 compared to non–treated control cells (Fig. 18). This indicates that it is possible to change the regional identity of the hESNSCs using the potent morphogen retinoic acid.

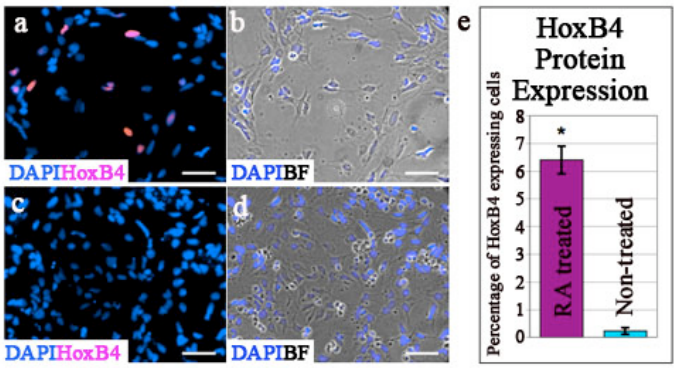

Fig. 18. Induction of HoxB4 protein expression following RA treatment
6,4± 0,49% of the neural precursors, which have been treated with RA (5μM) **(a,b,e),** showed HoxB4 expression, whereas control cells **(c,d,e)** only occasionally expressed the marker (scale bar 70μm, p* ≤ 0.05).

3.2 HESNSCs are not easily amenable to regional re-specification by tissue derived cues

In addition to posteriorization by RA, parallel studies revealed that the application of FGF8 in combination with Shh induced a ventralisation of the hESNSCs (Koch et al. 2009). These observations suggest that hESNSCs are amenable to regional specification by environmental cues. This finding is in accordance with the results of several studies suggesting that neural precursor cells originating in a distinct part of the neuroepithelium are not restricted to a local fate but are capable of migration and differentiation upon heterotopic transplantation, thereby responding to non-cell-autonomous signals of their new environment, if this new environment provides appropriate cues (Renfranz et al. 1991; Brustle et al. 1995; Vicario-Abejon et al. 1995; Suhonen et al. 1996; Brustle et al. 1997; Olsson et al. 1998). Therefore it was investigated to what extent environmental cues from primary cells and tissues of the young rodent brain were capable of influencing the regional transcription factor profile of hESNSCs via co-culture.

To this end paradigmatic co-culture conditions were selected using primary cells of different stages of CNS maturation as regional cues are differentially expressed during development of the CNS and many are down-regulated as the nervous system matures (Zappone et al. 2000). Primary cells from different brain regions are known to express distinct regional transcription factor profiles both *in vivo* and *in vitro* (Ostenfeld et al. 2002; Parmar et al. 2002; Kim et al. 2006). Changes in regional gene expression profile of the hESNSCs were monitored using quantitative RT-PCR employing human specific candidate transcription factors. It was assumed that the transcription factor expression may be up– or down-regulated depending on the primary cell types the neural precursors were co-cultured with. For instance co-culture with cells of hindbrain origin should result in less expression of the forebrain marker FoxG1 than co-culture with cells from the forebrain.

3.2.1 Shared medium co-culture does not interfere with the intrinsic regional expression profile of the hESNSCs

First a shared medium co-culture setting was chosen to investigate the effect of primary cell derived diffusible factors on the hESNSCs.

In order to do so, 150.000 long term passaged hESNSCs (passage > 28) were plated per well of a 6-well tissue culture plate. On the next day primary cells from the forebrain, midbrain and hindbrain of newborn (P0) mice were prepared and 200.000

primary cells of each region were placed in separate cell culture inserts above the neural precursor cells. Through this arrangement the two cell types shared a medium throughout the four weeks of co-culture, but were not in direct contact.

The candidate transcription factor analysis revealed that shared medium culture as used in this setting was not sufficient to induce a significant difference in expression of regional markers in the hESNSCs. Furthermore no significant change in Tubb3 or GFAP expression was observed (Fig. 19).

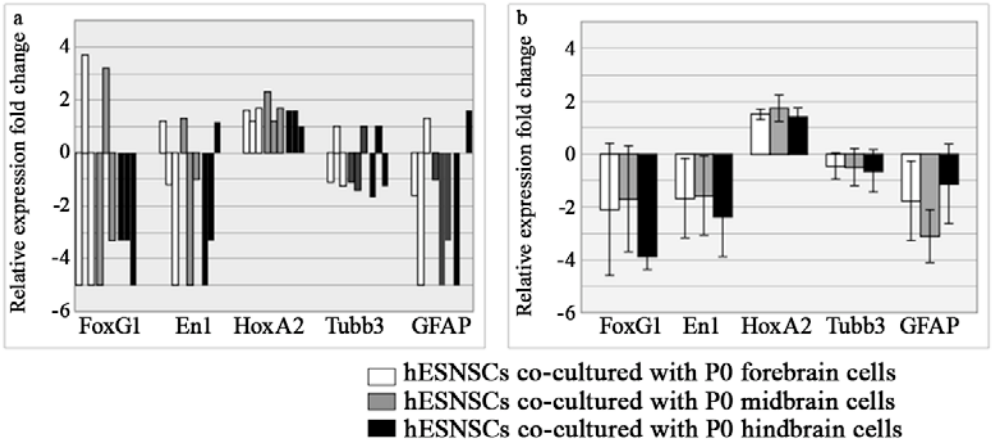

Fig. 19. Expression of region and cell type specific candidate genes in hESNSCs following shared medium co-culture with postnatal day 0 mouse cells
hESNSCs were co-cultured for four weeks in the same medium as P0 mouse primary cells, but were not in direct contact. **(a)** Independent experiments were performed in triplicates and summarized as averages **(b).** There was no significant induction, due to high variability in expression levels of the marker genes corresponding to each of the different brain regions FoxG1 (forebrain), En1 (midbrain), HoxA2 (hindbrain) or cell types Tubb3 (young neurons), GFAP (astrocytes). The gene expression levels were analysed using two way ANOVA (SPSS). All results were normalized to L27. Data are shown as fold expression relative to control treatment, i.e. hESNSCs that were not co-cultured but differentiated for the duration of the experiment.

3.2.2 HESNSCs are not amenable to regionalisation cues from direct co-cultures with P0 mouse cells

One possibility why the shared medium co-culture did not induce region-specific gene expression could be that specification of regional phenotype is dependent on direct cell-cell contact. Such a finding would be in accordance with the fact that co-culture settings, which have been shown to be effective e.g. stromal cell-derived inducing activity (Kawasaki et al. 2000) rely on direct cell-cell contact. Furthermore it is known that cell-cell and cell-matrix contacts such as collagens, proteglycans and glyoproteins influence the local microenvironment that elicits the intracellular signalling events important in regionalisation (Faissner and Steindler 1995; Olsson et al. 1998; Czyz and Wobus 2001). In order to include and use these short range signals a co-culture was set up to investigate whether it is necessary for the rodent primary

cells to be in direct contact with the hESNSCs in order to exert a regional influence on them.

Primary cells were prepared from the forebrain, midbrain and hindbrain of P0 mice, 200.000 primary cells of one region e.g. of the forebrain were pooled with 150.000 long term passaged hESNSCs (passage > 28) in a Falcon tube and the cells of the two species were plated together into the well of a 12-well tissue culture plate.

3.2.2.1 RNA analysis of directly co-cultured cells using species specific primers

At the end of the co-culture period of 28 days, region and cell type specific gene expression was analysed using species-specific primers specially designed for this purpose (Fig. 20, Table 4). This ensured that no false positive results were generated via the analysis of gene expression of co-cultured primary rodent cells.

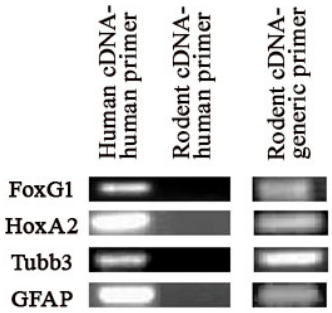

Fig. 20. Design of human-specific primers for qRT-PCR
Shown are examples of human specific primers used to analyse gene expression in the hESNSCs following direct co-culture. Lane 1) RT-PCR product with human-specific primers and total human fetal brain cDNA; lane 2) same conditions as in lane 1, but with a rodent brain cDNA of the appropriate age; lane 3) RT-PCR product with species-unspecific primers and with a rodent brain cDNA of the appropriate age.

Table 4 List of established and validated human specific primers

Primer	**Expression Domain/ Cell type** (MGI database)	**Approximately expressed in mouse brain**
BLBP	(Radial) glial cells	E11 – adult
Darpp32	Striatal projection neurons	E15 – adult
DCX	Early neurons	E11 – E17
Dlx1	Ventral diencephalon	E8 – P12
Dlx2	Ventral diencephalon	E9 – P7
E2F1	Ubiquitous	E9 - adult
Emx1	Dorsal diencephalon	E9,5 – P0
Emx2	Dorsal diencephalon	E8 – P0
En1	Midbrain	E8 – adult
FoxG1	Dorsal telencephalon	E8,5 – adult
Gad67	Inhibitory interneurons	E10,5 – adult
Gbx2	Hindbrain	E7,5 – P0

List of established and validated human specific primers continued

Primer	**Expression Domain/ Cell type** (MGI database)	**Approximately expressed in mouse brain**
GFAP	Astrocytes	E10 – adult
HB9	Motoneurons	E10,5 – P0
HoxA2	Anterior hindbrain	E8 – Postnatal week 6-8
HoxB4	Hindbrain	E8 – P0
Krox20	Rhombomeres 3,5; neural crest	E8 – P0
L27	Ribosomal protein	E1,5 – adult
Mash1	Ventral telencephalon/Cerebellum	E10 – P7
Nestin	Neural precursors	E8,5 – adult
Nkx2.2	Ventral neural tube	E8 – P7
Otx2	Forebrain	E7,5 – P0
Pax2	Midbrain	E7,5 – adult
Pax3	Dorsal neural tube	E7,5 – adult
Pax5	Midbrain	E8 – P0
Pax6	Proliferating neurons/ midbrain	E6,5 – adult
Pax7	Dorsal neural tube	E10 – P0
Sox10	Neural crest	E8,5 – adult
Tubb3	Developing neurons	E9,5 – adult
vGlut1	Glutamatergic neurons	E18,5 – adult
vGlut2	Glutamatergic neurons	E18, 5 – adult

3.2.2.2 Gene expression analysis following direct co-culture with P0 mouse cells

The direct co-culture did not induce a significant region-specific induction of gene expression (Fig. 21). There was no significant reduction or induction of FoxG1 or En1 expression. Additionally the expression levels of Tubb3 and GFAP varied from co-culture to co-culture with no reproducible trend detectable. However, hESNSCs co-cultured with P0 midbrain cells expressed repeatedly (Fig. 21a) and significantly (Fig. 21b) less HoxA2 than cells co-cultured with other brain regions or non co-cultured control cells. Moreover the same cells also expressed repeatedly less En1, but this reduction was not significant compared to expression levels of hESNSCs co-cultured with hindbrain cells. Nevertheless, this finding differed from the expectation since midbrain cells, which themselves express En1 did not induce but down-regulated En1 expression in co-cultured hESNSCs.

In contrast to the finding, that direct co-culture had little influence on the regional candidate marker gene expression in hESNSCs, a study by Roy and co-workers showed that direct co-culture had an effect on hES cell derived cells. Specifically it was demonstrated that telomerase-immortalized human fetal midbrain astrocytes substantially potentated dopaminergic neurogenesis (Roy et al. 2006). One possible explanation for this discrepancy is the fact the cells used by Roy and colleagues were co-cultured considerably earlier after their derivation from hES cells than the hESNSCs. This is an important consideration since the growth factor FGF, which is used in to proliferate the hESNSCs under standard culture conditions may alter developmental competence of neuroepithelial cells (Gabay et al. 2003).

Therefore it was investigated in the next step whether short term passaged hESNSCs (passage 6) were more responsive to co-culture derived cues of an 28 day direct co-culture. These hESNSCs were exposed to FGF and EGF for a considerably shorter period i.e. at least 22 passages less than the long-term passaged hESNSCs used previously.

However, as seen before the expression level of the representative transcription factors was not significantly up– or down-regulated (Fig. 21). On average the co-cultured hESNSCs showed a down-regulation of all representative candidate markers.

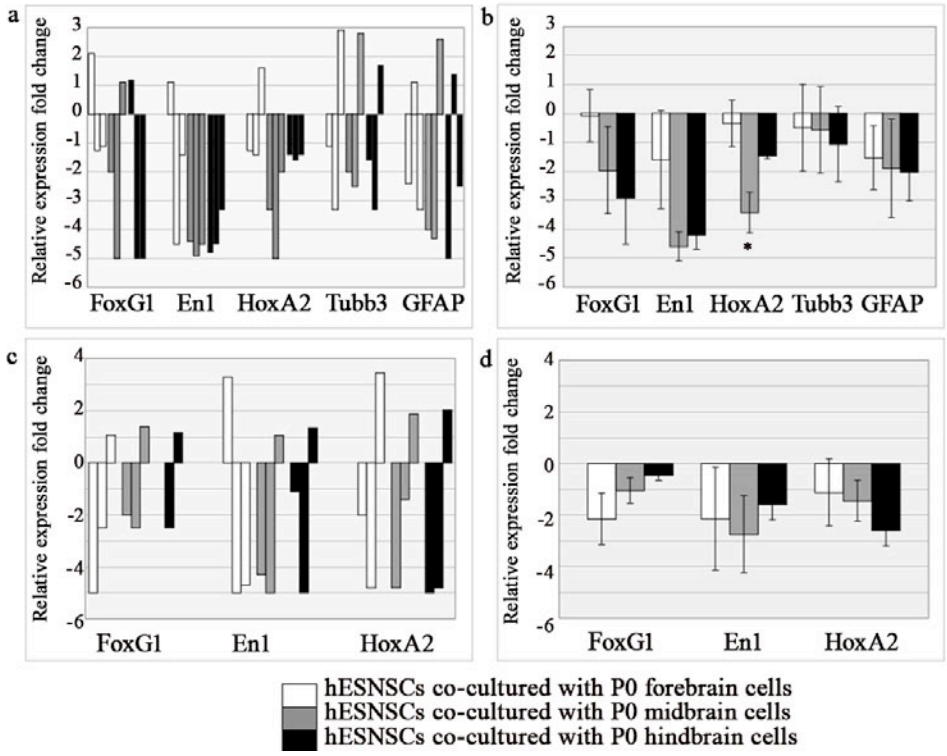

Fig. 21. Expression of representative regional markers in hESNSCs following direct co-culture with postnatal day 0 mouse cells
200.000 P0 mouse cells of the forebrain, midbrain or hindbrain, were pooled with 150.000 hESNSCs and subsequently cultured in a direct co-culture for 4 weeks. In the first set of experiments long term passaged hESNSCs (passage > 28) were used **(a,b)**, whereas in the second set of experiments hESNSCs of passage 6 were employed **(c,d)**. Each set of experiments was performed in triplicates **(a,c)** and summarized as averages **(b,d)**. There was no significant induction in hESNSCs of the marker genes FoxG1 (forebrain), En1 (midbrain), HoxA2 (hindbrain), Tubb3 (young neurons), GFAP (astrocytes). There was, however, a significant reduction in HoxA2 expression in hESNSCs co-cultured with cells from the midbrain **(b)**. The co-cultures were analysed using two way ANOVA (SPSS) with $p^* \leq 0.05$. All results were normalized to human specific L27. Data are shown as fold expression relative to control cells i.e. hESNSCs that were not co-cultured but differentiated for the duration of the experiment.

3.2.3 Effect of organotypic slice co-culture on regional gene expression of hESNSCs

In parallel with the direct co-cultures an organotypic slice co-culture was set up to elucidate whether it is necessary for the primary cells to retain their three-dimensional structure/position to induce regionalisation of co-cultured neural precursors. This model has the advantage that the cell-cell and cell-matrix contacts as well as the intracellular signalling events elicited through these contacts, which are known to be important in regionalisation (Olsson et al. 1998), more closely resemble those *in vivo,* i.e. the primary cells retain their position within the tissue, which is important for their regional identity.

The challenge of the experimental setup in the organotypic slice co-culture was the re-isolation of the co-cultured hESNSCs in a way enabling analysis of gene expression via quantitative RT-PCR.

3.2.3.1 Re-isolation of hESNSCs after slice co-culture transplantation via FACS

Organotypic brain slices were prepared from the hippocampus and cerebellum of 9-day-old Sprague Dawley rats (Fig. 22). Five days after derivation 200.000 EGFP positive hESNSCs (> passage 28) were seeded on each slice. In this setting hESNSCs typically migrate into the slice tissue where they differentiate (Scheffler et al. 2003).

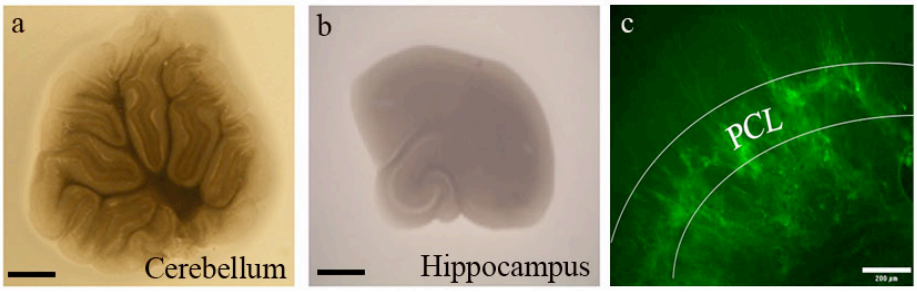

Fig. 22. Culture on organotypic slices
Organotypic slices of the cerebellum **(a)** and hippocampus **(b)** were prepared from 9-day-old Sprague Dawley rats (scale bar size= 2,5mm) and 200.000 hESNSCs were deposited on each slice 5 days after derivation. **(c)** Example of hESNSCs on a hippocampal slice, that was fixed after 21 days of co-culture and stained for GFP to detect the transplanted hESNSCs, which expressed EGFP (PCL: pyramidal cell layer, scale bar = 200μm) (pictures taken by: bright field Ms Steinfarz, fluorescence Dr. Jakupoglu).

After 14-21 days FACSorting was used to re-isolate the hESNSCs. To this end all engrafted slices of one region were pooled and processed together to a single cell suspension. For staining of dead cells 0.5μg/ml propidium iodide were added and the cell suspension was passed through a cell sieve. Then the EGFP-expressing cells were collected via fluorescence activated cell sorting (FACS). Under optimized conditions cell viabilities between 55-95% and sorting efficiencies ranging from 80-90% (overview in Table 5) were reached.

Table 5 Summary of FACSortings following organotypic slice co-culture experiments

	Experiment I	**Experiment II**	**Experiment III**
Culture period	14d	18d	21d
Number of grafted slices	Hippo.: 12 Cereb.: 12	Hippo.: 18 Cereb.: 17	Hippo.: 23 Cereb.: 28
Total number of transplanted hESNSCs	Hippo.: 1,2 x10^6 Cereb.: 1,6 x10^6	Hippo.: 1,6 x10^6 Cereb.: 2 x10^6	Hippo.: 2,3 x10^6 Cereb.: 2,8 x10^6
GFP positive events (automatically counted)	Hippo.: 66 000 Cereb.: 80 000	Hippo.: 190 000 Cereb.: 130 000	Hippo.: 450 000 Cereb.: 225 000
Percentage of viable cells as measured by propidium iodide exclusion	55-75%	58-95%	75%
Sort efficiency (purity after sort re-analysis)	**80-85%**	**80-90%**	**85-90%**
Percentage of GFP $^+$ cells during FACSorting	**5%**	**1-2%**	**6-11%**

However, only 1-11% of the transplanted cell could be re-isolated. Therefore the numbers obtained here were not sufficient for extensive subsequent quantitative RT-PCR analysis. Furthermore, as the purity of the re-isolated EGFP positive cells did not exceed 90% residual rodent cells were likely to still represent a potential source for false-positive results in the planned qRT-PCR analysis.
To bypass these problems, in subsequent experiments total RNA was isolated from 2-3 engrafted slices of the same brain region and species specific primers (3.2.2.1) were employed for gene expression analysis.

3.2.3.2 Transplantation onto slice co-cultures does not regionally specify the hESNSCs

At the end of the slice co-culture period gene expression in transplanted human cells was compared to gene expression level of control cells – hESNSCs, that were not transplanted, but differentiated on a tissue culture insert for the duration of the organotypic slice co-culture. It was found that the anterior marker FoxG1 was increased 12,5 fold in hESNSCs transplanted onto hippocampal slices compared to neural precursors cultured on cerebellar slices (Fig. 23). This increase in FoxG1 expression was investigated in more detail, revealing that the telencephalic transcription factor was reproducibly and significantly induced between day 6 and day 11 of co-culture, with a further significant increase in expression level until day 15 (Fig. 23d). However, none of the other investigated regional transcription factors showed a differential expression pattern in cells transplanted onto hippocampal or cerebellar slices. Especially the other employed forebrain marker Otx2 – even though it was slightly up-regulated on average – did not mimic the significant induction seen in FoxG1 gene expression (Fig. 23a).
En1 and HoxA2 were both on average down-regulated in transplanted cells compared to control cells (Fig. 23a). However, this trend was neither significant nor region specific, since hESNSCs transplanted onto both hippocampal and cerebellar slices showed decreased En1 and HoxA2 expression compared to control cells.
Furthermore differentiation of the hESNSCs during the co-culture period had to be considered, since FoxG1 is expressed in differentiated neurons but not in differentiated astrocytes. Therefore an overall increase in the number of neurons generated from hESNSCs transplanted onto hippocampal slices compared to cerebellar slices would have led to an overall increase in FoxG1 expression without affecting the actual gene expression level. Thus Tubb3 expression was analysed to account for the possibility that more neurons might be generated by one of the slice

cultures. However, as Tubb3 expression was on average even higher in hESNSCs transplanted to cerebellar slices. Thus the significant up-regulation of FoxG1 on mRNA level in hESNSCs transplanted to hippocampal slices could not be explained by the generation of more neurons in these cultures (Fig. 23a). Hence, it was investigated whether the induction of FoxG1 mRNA was mimicked at protein level using tyramide-enhanced immunohistochemistry. As shown in Fig. 23b the pronounced change in FoxG1 expression observed in the qRT-PCR analysis was not adequately reflected at protein level.

Furthermore the expression of the transcription factor E2F1, which acts as transcriptional activator important for progression through G1/S phase transition was analysed (DeGregori et al. 1997). Under normal culture conditions E2F1 was highly expressed in proliferating neural precursors but down-regulated upon differentiation (Fig. 23c). The significant increase in FoxG1 expression in hESNSCs transplanted onto hippocampal slices was accompanied by an increase of E2F1 in the same cells (Fig. 23a).

As with the mixed monolayer co-culture in 3.2.2 an organotypic slice co-culture was set up also with short-term expanded hESNSCs (passage 3) to study, whether these neural precursors keep their forebrain identity when co-cultured on hippocampal slices. A different forebrain candidate gene (Dlx1) was used to bypass potential differential regulation of FoxG1 at mRNA and protein level.

However, no significant induction of a representative marker gene was observed after transplanting short-term expanded hESNSCs onto organotypic slices. On average hESNSCs transplanted to cerebellar slices expressed the forebrain candidate gene Dlx1 at higher levels than cells transplanted onto hippocampal slices (Fig. 23e).

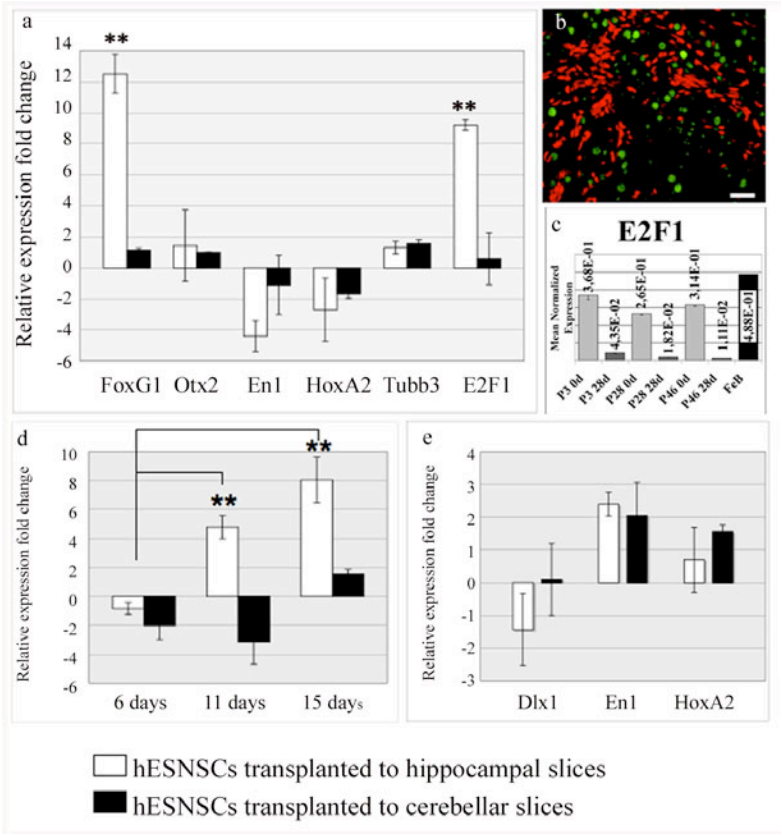

Fig. 23. Effect of transplanting hESNSCs onto organotypic brain slices on regional gene expression in the neural precursor cells
200.000 long-term expanded hESNSCs were seeded per hippocampal or cerebellar slice. At the end of the co-culture period the expression of candidate transcription factors was assessed using the regional markers FoxG1/Otx2/Dlx1 (forebrain), En1 (midbrain), HoxA2 (hindbrain), the cell type specific marker Tubb3 as well as the cell cycle associated transcription factor E2F1. **(a)** A reproducible and significant induction of FoxG1 and E2F1 expression was found in hESNSCs transplanted onto hippocampal slices but not in any other marker gene. The slice co-cultures were analysed using two way ANOVA (SPSS) with p**≤ 0.01. All results were normalized to the human specific housekeeping gene L27. Data are shown as fold expression relative to control cells, i.e. hESNSCs that were not transplanted but differentiated for the duration of the experiment. **(b)** After 21 days of culture on a hippocampal slice hESNSCs (human nuclei: red) show no FoxG1 (FoxG1: green) expression, whereas the endogenous cells are clearly immunoreactive (scale bar 50μm). **(c)** The transcription factor E2F1 was highly expressed in proliferating neural precursors and down-regulated upon differentiation (P: passage number of hESNSCs; d: days of differentiation). **(d)** FoxG1 expression increased significantly between day 6 and day 11 and day 15 of co-culture in hESNSCs transplanted to hippocampal slices. **(e)** The slice co-culture was repeated with hESNSCs of passage 3 using a different forebrain marker gene (Dlx1), but no significant induction of a marker gene was found in this setting.

3.2.4 Embryonic primary cells do not induce region specific gene expression in directly co-cultured human neural precursors

It is known that regional patterning is predominantly taking place during embryonic development, whereas in the neonatal brain mainly neurite outgrowth, myelination and apoptosis are ongoing (Zappone et al. 2000; Brumwell and Curran 2006). Therefore - i.e. to ensure the maximal presence of local patterning cues - an embryonic environment was chosen for the next co-culture.

In this setting rodent cells were derived from the anterior forebrain and parts of the mid- and hindbrain encompassing the midbrain-hindbrain organizer (MHO), a region suggested to act as organizer in midbrain/hindbrain development. After derivation primary cells were plated at densities of 100.000 cells/cm^2 (anterior forebrain) and 200.000 cells/cm^2 (MHO) respectively. After 7h, when the primary cells had begun to settle, the cell debris was washed off and 200.000 long term passaged hESNSCs (> passage 28) were added per well and co-cultured for 14-18 days.

The gene expression analysis of co-cultured hESNSCs showed that even though En1 and HoxA2 expression were repeatedly up-regulated in hESNSCs co-cultured with the MHO region (Fig. 24a) the overall relative fold change in expression was not significant (Fig. 24b). Moreover neither Tubb3 nor GFAP expression were significantly changed by the co-culture conditions.

A direct co-culture with E12.5 primary cells, the earliest cell type amenable to preparation, was investigated next. In this setting a slightly different set of candidate genes was employed in order to detect regionalising trends, which might have been missed using the previous set of marker genes. Otx2 was used instead of FoxG1, Pax2 was used in addition to En1 and Gbx2 was used in addition to HoxA2.

However, overall no significant induction or reduction of region-specific gene expression was observed in this co-culture setting (Fig. 24). The forebrain marker Otx2 and the midbrain marker Pax2 were up-regulated in all co-cultured cells, compared to non co-cultured control cells, while the other midbrain marker En1 was on average down-regulated in all co-cultured hESNSCs.

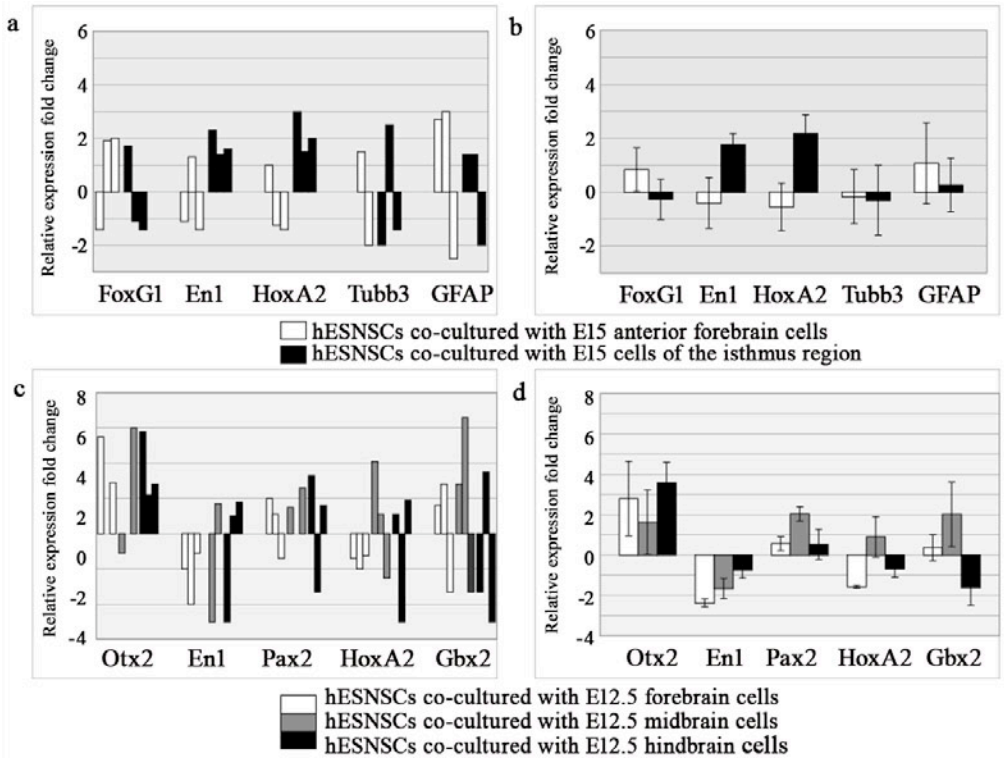

Fig. 24. Regional marker gene expression in hESNSCs following direct co-culture with embryonic primary cells
Primary cells were isolated from the E15 (**a,b)** or E12,5 **(c,d)** mouse brain and co-cultured in a direct setting with hESNSCs. Independent experiments are shown in **(a)** and **(c),** and summarized as averages in **(b)** and **(d).** There was no significant induction in hESNSCs of the marker genes corresponding to the brain regions FoxG1/Otx2 (forebrain), En1/Pax2 (midbrain), HoxA2/Gbx2- (hindbrain) or cell types Tubb3 (young neurons), GFAP (astrocytes). The co-cultures were analysed using two way ANOVA (SPSS). All results were normalized to the expression of the human specific L27 primer. Data are shown as fold expression relative to control cells, i.e. hESNSCs that were not co-cultured but differentiated for the duration of the experiment.

3.2.5 Effect of re-aggregation co-culture on regional gene expression in hESNSCs

Except for the slice co-culture, where the hESNSCs could invade the slice, the previous experiments employed co-culture settings, in which the two cell types were mainly cultured as a monolayer. Therefore it was investigated whether the primary cells need to surround the hESNSCs – not just grow in direct contact with them – in order to induce a change in regional gene expression. To this end a re-aggregation co-culture system, where single cell suspensions of freshly primary cells and hESNSCs were pooled in a 10:1 ratio in 15ml Falcon tubes and subsequently cultured in a roller drum system, was employed (Studer et al. 1998).

As the hESNSCs exhibited a hindbrain phenotype under standard culture conditions and since they did not show region specific adaptation when co-cultured with regions remote from this endogenous phenotype, primary cells from the forebrain were no longer used for the co-culture. Instead only E12.5 primary cells from the

midbrain and hindbrain were prepared for the co-culture using the midbrain-hindbrain boundary as anatomical cut-off point to separate the two regions. Consequently also the candidate transcription factors employed to assess changes in regional gene expression profile were changed. Here the midbrain markers Pax5 and En1 in combination with the anterior hindbrain marker HoxA2 were employed.
To visualize re-aggregation between mouse and human cells, EGFP expressing hESNSCs were used. The two cell types aggregated well with each other and typically formed one sphere-to-bean shaped structure per Falcon tube (Fig. 25).

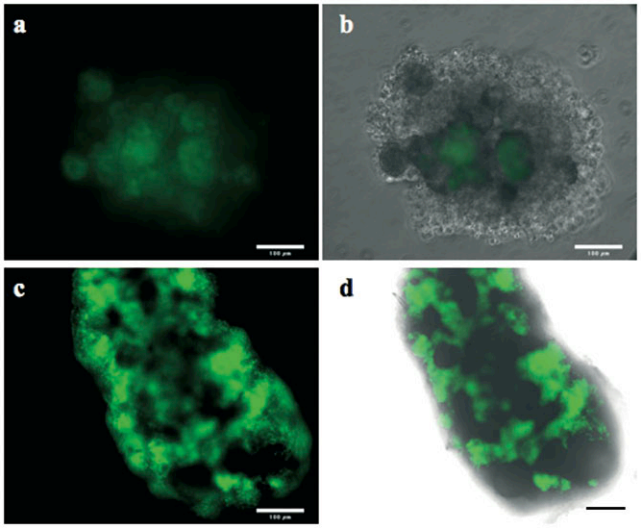

Fig. 25. Aggregates of primary cells and hESNSCs after 7 days of co-culture
Typically one aggregate developed per Falcon tube. EGFP expressing hESNSCs aggregated with E12.5 midbrain cells **(a,b)** and hindbrain cells **(c,d)** (scale bar 100μm).

After 10 days regional gene expression of the human neural precursors was assessed using human specific quantitative RT-PCR. Overall there was no significant up- or down-regulation of any regional candidate marker in hESNSCs in this co-culture model. The midbrain marker En1 was repeatably down-regulated in all re-aggregated hESNSCs, and the other midbrain marker Pax5 was – though highly variable - on average also down-regulated in hESNSCs re-aggregated with midbrain primary cells (Fig. 26).

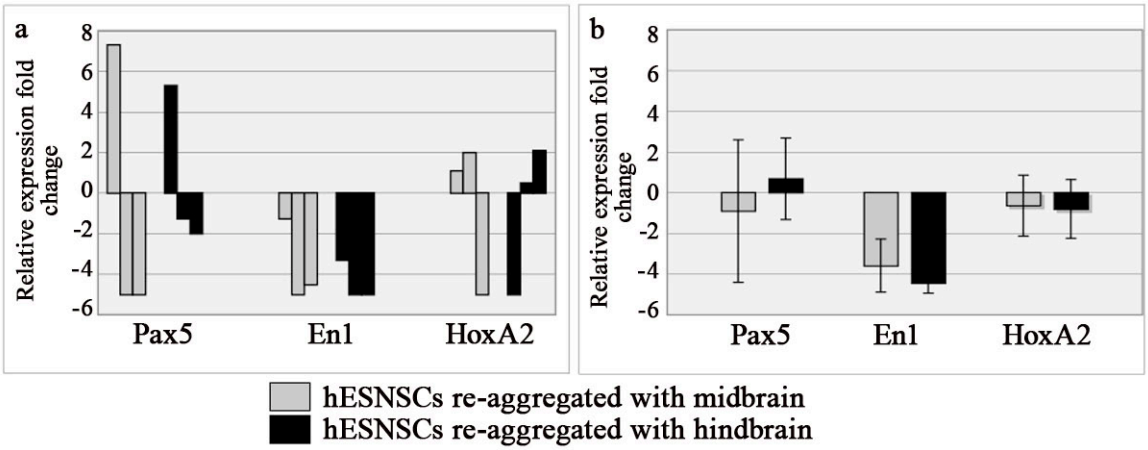

Fig. 26. Expression of regional candidate genes in hESNSCs following re-aggregation co-culture with mouse E12.5 cells
Neural precursors were pooled in a 1:10 ration with midbrain or hindbrain primary cells and cultured in a roller drum system to achieve aggregation. At the end of the co-culture experiments expression of the region specific markers was analysed using human specific quantitative RT-PCR, normalized to human specific L27. Independent experiments were performed in triplicates **(a)** and summarized as averages **(b)** Pax5 /En1 (midbrain), HoxA2 (hindbrain). The data were analysed using two way ANOVA (SPSS). Data are shown as fold expression relative to control cells, i.e. hESNSCs that were not co-cultured but aggregated for the duration of the experiment.

3.2.6 Transplantation into different regions of the neonatal rat brain does not have a significant influence on the intrinsic regional gene expression of hESNSCs

In a proof-of-principle study it was assessed whether the human specific primers could be used to analyse gene expression of hESNSCs transplanted *in vivo*. In order to do so 200.000 hESNSCs were transplanted unilaterally into the forebrain and cerebellum of P1 rat pups. After 20 days the animals were sacrificed and total RNA was isolated from the forebrain and the cerebellum. Subsequently human specific qRT-PCR was performed using this RNA.

The human specific primers designed in the course of this work were sensitive enough to detect gene expression of the transplanted hESNSCs. Although there was a trend showing that on average the forebrain markers FoxG1 and Otx2 were expressed highest in hESNSCs transplanted to the forebrain, there were no significant changes, since the expression levels varied substantially between individual samples (Fig. 27).

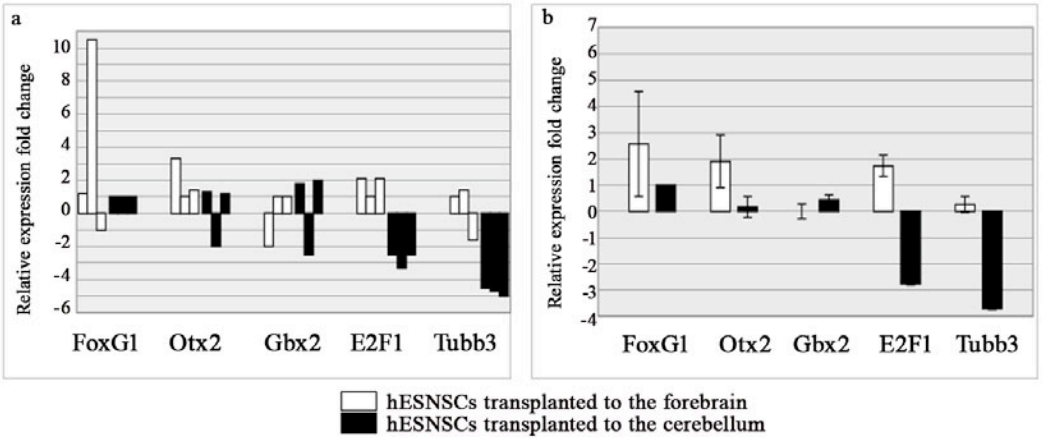

Fig. 27. Expression of candidate genes in neural precursors after *in vivo* transplantation to P1 rats
200.000 hESNSCs were transplanted to the forebrain and cerebellum of P1 rats. Gene expression of the human cells was analysed after 20 days. Data from the individual samples are shown in **(a)** and the average expression fold changes are shown in **(b).** Otx2 and FoxG1 expression were markedly but not reproducibly or significantly increased in hESNSCs transplanted to the hippocampus. There was no detectable FoxG1 expression in hESNSCs transplanted to the cerebellum. Gene expression was analysed using two way ANOVA (SPSS). All quantitative RT-PCR results were normalized to human specific L27. Data are shown as fold expression relative to control cells, i.e. hESNSCs that were not transplanted but differentiated for the duration of the experiment.

3.3 Effect of DNA methylation and histone hyperacetylation

One of several explanations for the finding that neither co-culturing with nor transplantation into different brain regions induced significant changes in region-specific gene expression might be that the hESNSCs are not amenable to the regionalisation signals of primary cells due to epigenetic mechanisms. Together with transcriptional regulators and micro RNAs, epigenetic DNA and chromatin alterations play pivotal roles in controlling the plasticity of the transcriptional profile of a cell (Chen and Daley 2008). Epigenetic states are somatically heritable and the propagation of the specific patterns to the next cell generation is important for maintaining cell identity (Avots et al. 2002). Histone acetylation, methylation and phosphorylation are among the best know modifications and involved in controlling the access of regulatory proteins to specific sequences in the genome (Jenuwein and Allis 2001). The level of histone acetylation, which occurs reversibly on lysine e-$NH3^+$ groups of core histones depends on opposing activities of histone acetyltransferases (HATs) and deacetylases (HDACs). For the latter enzyme family several specific inhibitors are known for instance trichostatin A (TSA; Yoshida et al. 1990) and valproic acid (2-propylpentanoic acid, VPA). VPA is a well-tolerated anticonvulsant inhibiting both class I and II HDACs resulting in hyperacetylation of histone H3 and H4 (Kramer et al. 2003). Acetylation of histones H3 and H4 is associated with active transcription (Jenuwein and Allis 2001).

A second epigenetic modification of the vertebrate genome is methylation of cytosine within CpG dinucleotides leading to a stable repression of transcription (Bird 2002). While in adult somatic cells DNA methylation patterns are stable, they are very dynamic during early development (Schmutte and Jones 1998). AzaC is often used as a DNA methylation inhibitor. When incorporated into DNA, it covalently binds to and irreversibly blocks the maintenance methyltransferase Dnmt1, allowing passive demethylation to take place as cells divide (Pietrobono et al. 2002).
It is known that the developmental potential of ES cells can be in part ascribed to their dynamic epigenetic state. Thus it was tested whether treating the hESNSCs with the HDAC inhibitor VPA and the nucleotide analogue AzaC alters their developmental competence.

3.3.1 Treatment with VPA and AzaC results in histone hyperacetylation and DNA demethylation

It has been reported that VPA at a concentration of 1mM inhibits HDAC in adult rat hippocampal neural progenitors (Hsieh et al. 2004). Therefore it was tested in the first step whether administration of the standard concentration of 1mM would result in hyperacetylation in the hESNSCs. This was assessed through detecting changes in histone acetylation using Western blot analysis with an antibody specific for acetylated histone 4. The outcome suggested that under normal culture conditions treatment with 1mM VPA for 3 days greatly enhanced acetylation on histone 4 in hESNSCs (Fig. 28a).
In the next step it was studied, whether the second agent – AzaC – caused DNA demethylation in the hESNSCs at concentrations of 250nM and 500nM (Schmittwolf et al. 2005). Neural precursors were cultured under standard culture conditions for 3 days with or without the addition of AzaC. For detecting changes in methylation pattern the method of Yang and co-workers was used. In this approach global DNA methylation was determined via employing bisulfite treatment of DNA, followed by non-specific PCR amplification of long interspersed nucleotide elements (LINEs), which are usually heavily methylated. Finally restriction enzyme digest of the PCR product was performed using HinF1. This endonuclease only cuts repetitive elements that were originally methylated because the cytosine residues of the enzyme recognition site in the unmethylated repetitive elements were converted into uracil during the bisulfite reaction. As expected HinF1 generated digestion products of 285bp, 247bp, 166bp and 128bp when using control DNA, that was not AzaC

treated (Yang et al. 2004). In Fig. 28b the lower bands represent methylated repetitive elements. The upper band represents unmethylated repetitive elements.

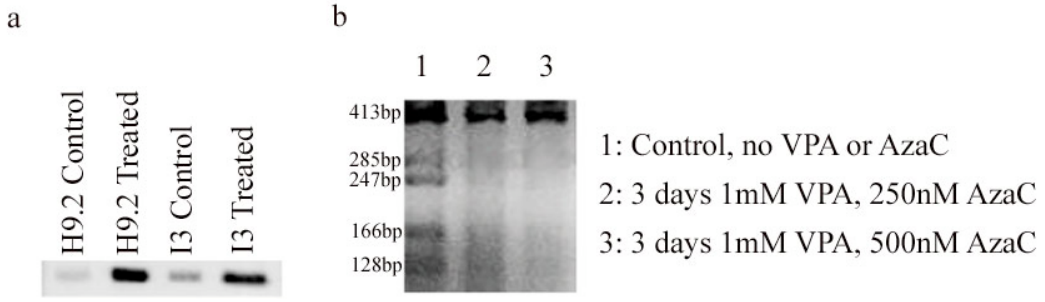

Fig. 28. VPA/AzaC treated hESNSCs were demethylated and hyperacetylated
(a) Cells had been treated with 1mM VPA for 72h. Histone acetylation status was investigated using an anti-acetyl-H4 antibody. **(b)** Genomic DNA was treated with sodium bisulfite and a non-specific PCR was performed, which amplified a pool of LINE-1 repetitive elements. The PCR product was digested with HinFI, which only cuts repetitive elements that were originally methylated. The digested PCR product was separated by agarose gel electrophoresis and stained with Ethidium bromide. The lower bands (285bp, 247bp, 166bp and 128bp) represent methylated repetitive elements, suggesting that the untreated cells were methylated (lane 1). The upper band (413bp) represents unmethylated repetitive elements, indicating that the treated cells were demethylated (lanes 2/3).

The untreated neural precursors were methylated (lanes 1) whereas the treated cells were demethylated (Fig. 28, lanes 2 and 3).

3.3.2 VPA/AzaC treatment does not affect karyotypic stability, proliferation rate or regional gene expression in hESNSCs

In the preliminary experiments of 3.3.1. 250nM AzaC were sufficient to induce demethylation. Since it has been suggested that AzaC may induce chromosomal abnormalities at high concentrations (3μM) the low dose of 300nM AzaC was chosen for subsequent experiments (Harrison et al. 1983). After 72h of 1mM VPA and 300nM AzaC administration the morphology of treated hESNSCs was undistinguishable from control cells (Fig. 29).

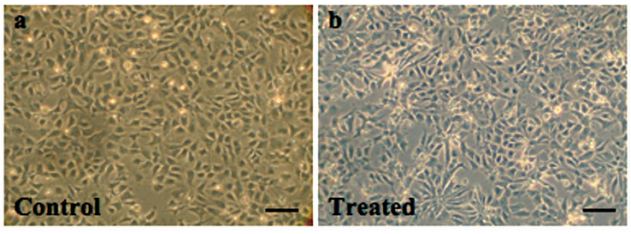

Fig. 29. Morphology after VPA/AzaC treatment (I3 P30)
After 72 h of VPA/AzaC administration treated cells **(b)** were indistinguishable from control cells **(a)**. Scale bar 30μm.

FISH analysis was carried out on chromosomes 12 and 17q, since hES cells are susceptible to anaploidy of these chromosomes leading to increased proliferation and hence a selective advantage (Draper et al. 2004). FISH analysis of the hotspots on chromosomes 12 and 17q as well as G-banding (at least 20 metaphases were counted

for each condition) demonstrated that 72h of VPA/AzaC administration did not cause an increase in chromosomal abnormalities compared to control cells (Fig. 30).

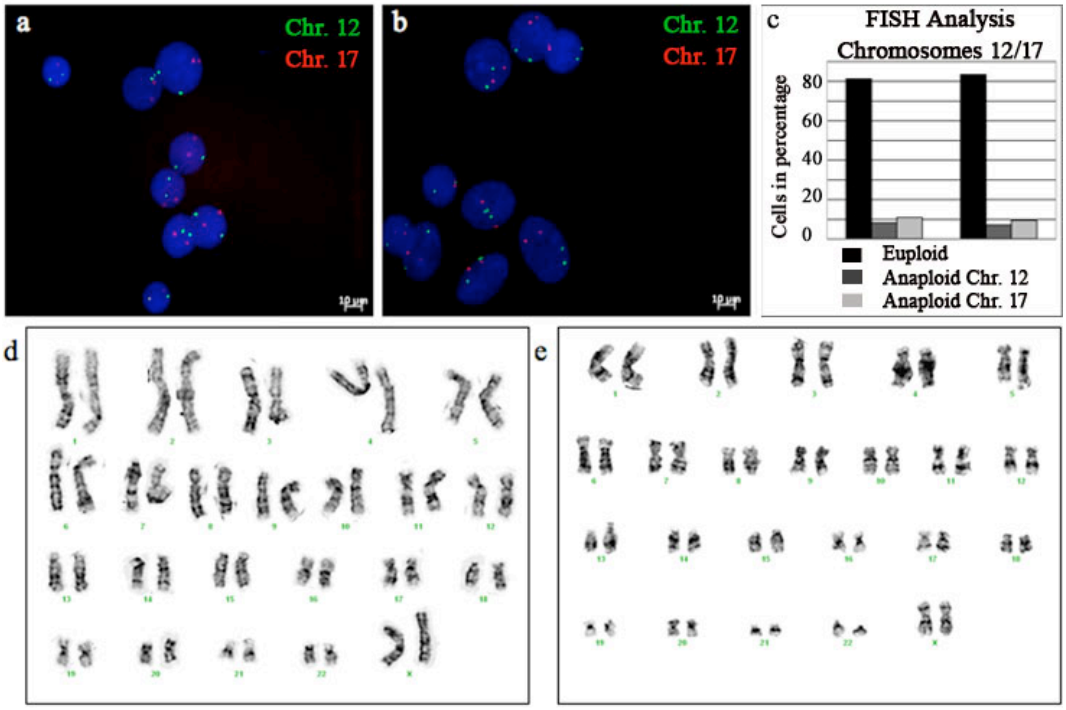

Fig. 30. Treatment with 1mM VPA and 300nM AzaC did not cause an increase in chromosomal abnormalities as measured by FISH analysis and G-banding
Control hESNSCs **(a,d)** were cultured under standard conditions for three days, while treated neural precursors **(b,e)** received a daily dose of 1mM VPA and 300nM AzaC. **(a,b,c)** The results of the FISH analysis for chromosomes 12 and 17q as well as **(d,e)** G-banding suggested that this treatment regime did not have adverse effects on chromosome numbers compared to control cells.

Additionally the treatment with VPA and AzaC did not change proliferation rate (as determined by BrdU incorporation and Ki67 staining, Fig. 31) and 72h of VPA (1mM) and AzaC (300nM) administration did not change the expression levels of regional and cell type specific marker genes (Fig. 32a).

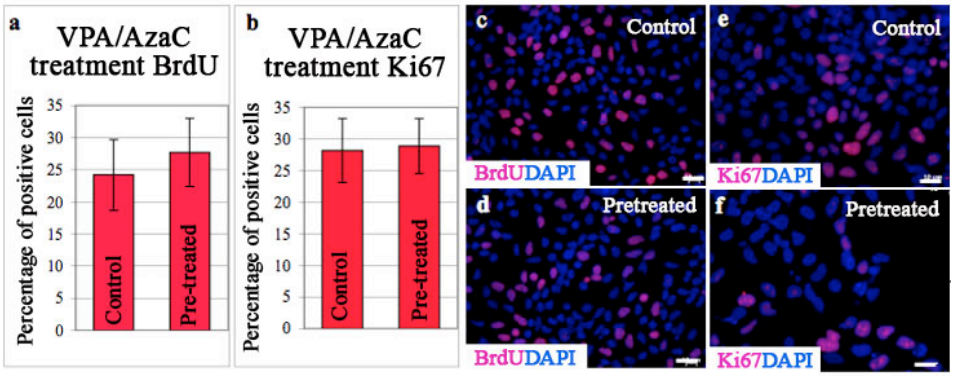

Fig. 31. Treatment with 1mM VPA and 300nM AzaC for 72h did not significantly affect the proliferation rate of hESNSCs.
Shown are the averages of three independent experiments, in which proliferation rate was analysed via BRDU incorporation **(a,c,d)** and Ki67 expression **(b,e,f),** respectively. Scale bar 15μm.

In order to investigate potential long-term effects of VPA/AzaC treatment, 1mM VPA and 300nM AzaC were administered to proliferating hESNSCs for 72h. Subsequently the neural precursors were differentiated for 18 days. At the end of this period gene expression levels of cell type specific markers (Tubb3, Gad67, vGlut1) as well as regional specific markers (Otx2, Pax3, HoxA2) were compared to expression levels in control cells, which were not treated with VPA or AzaC prior to differentiation. The findings of these analyses suggested that on mRNA level pre-treatment resulted in a slight but significant decrease of Tubb3 expression (Fig. 32b). The expression level of the other candidate genes was statistically unaltered after 18 days of differentiation (Fig. 32b). The decrease in Tubb3 expression on mRNA level was not confirmed on the protein level (Fig. 32 e,f). 50, 2 ±4,9% of the control cells and 48,3 ±4,3% of the pre-treated hESNSCs differentiated into Tubb3 positive neurons. Of these Tubb3 positive neurons 53,7 ±2,4% and 51,7 ±3,3% were GABA positive in control and pretreated cells, respectively (Fig. 32c-f).

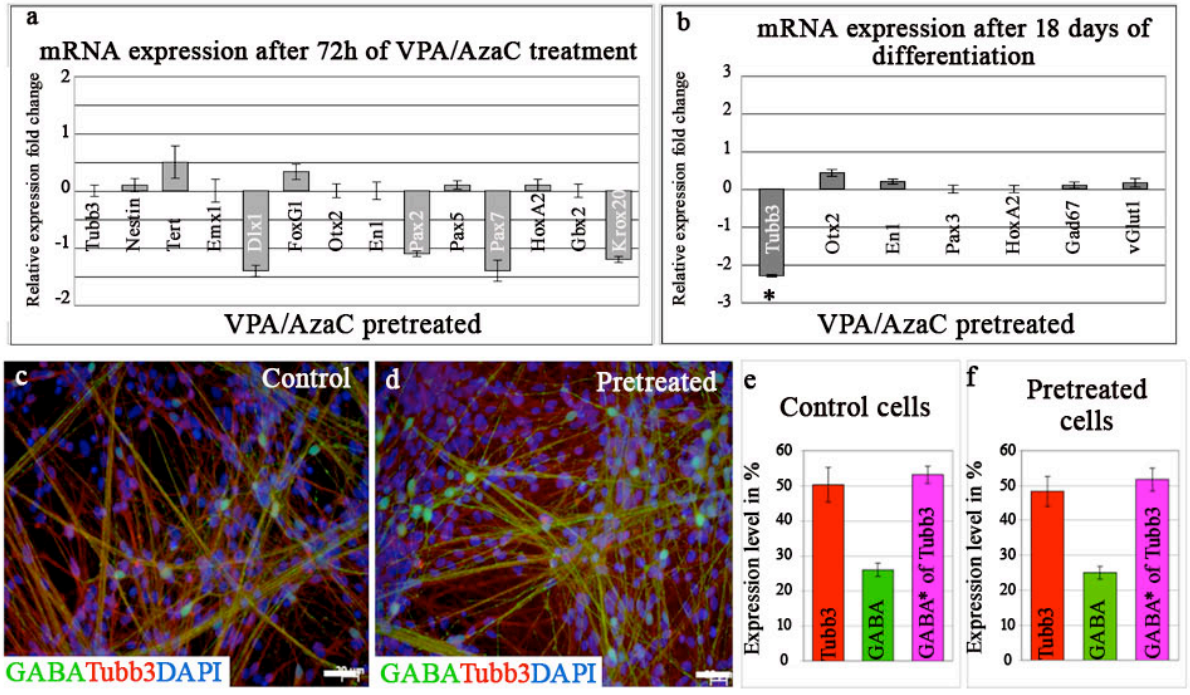

Fig. 32. Expression of region and cell type specific markers after 72h of VPA/AzaC treatment and subsequent differentiation of pre-treated hESNSCs for 18 days
(a) hESNSCs treated with 1mM VPA/300nM AzaC for 72h did not show a significant alteration in the expression of candidate genes compared to non-treated control cells. (b) Pretreated hESNSCs differentiated for 18 days showed a significant decrease in Tubb3 expression. No significant changes in regional (Otx2, Pax3, HoxA2) or other cell type specific (Gad67, vGlut1) gene expression were observed. (c,e) After differentiation for 18 days 50.2± 4,9% of the control cells expressed Tubb3 and of these cells 53,7± 2,4% were GABA positive. Of the pretreated cells 48± 4,3% had differentiated into Tubb3 positive neurons (d,f). Of these 51.7± 3.3% were GABA positive. All gene expression data were normalized to L27 and analyzed using students t-test, p*≤0.05. Data are shown as fold expression relative to control cells, i.e. hESNSCs, which were proliferating or differentiated under standard conditions but not treated with VPA or AzaC (Scale bar 30μm)

3.3.3 Pre-treatment with VPA and AzaC does not render the hESNSCs more susceptible to co-culture derived environmental cues

The previous results showed that there was no significant difference in regional gene expression and differentiation behaviour between VPA/AzaC pre-treated and non-pretreated control cells. To test whether demethylation and simultaneous hyperacetylation render the hESNSCs more susceptible to regionalisation cues of primary cells, co-cultures with hESNSCs, which had been pre-treated for 72h were set up. To this end long term passaged hESNSCs (> passage 28) were treated with 1mM VPA and 300nM AzaC for 72h. After this time freshly prepared primary cells from the forebrain, midbrain-hindbrain organizer region and spinal cord of E12.5 mice were added to the hESNSCs. The cells were not actively mixed, because the human neural precursors showed very limited tolerance to trypsin treatment, following VPA/AzaC administration. In this setting also a spinal cord preparation was included to analyse whether it might be possible to further posteriorize the hESNSCs.

As demonstrated in Fig. 33a/b after 5 days of direct co-culture no significant difference in gene expression levels was detectable in the co-cultured hESNSCs. Therefore the exposure time of the hESNSCs to the primary cells was increased to 9 days of co-culture. In this setting slightly different candidate genes were employed. Pax2 was used instead of En1 and Gbx2 was used instead of HoxA2. The rationale for choosing transcription factors, which are specific for slightly different regions in the midbrain and hindbrain, was that it might be possible to pick up regionalizing trends, which were overlooked using the previous set of marker genes. Due to limited amounts of RNA it was not possible to analyse all transcription factors simultaneously.

However, also with this set of candidate markers no significant induction of gene expression in VPA/AzaC pretreated cells was detected after 9 days of co-culture (Fig. 33c/d). Gene expression of both co-culture settings was also compared to gene expression of non-treated non-co-cultured cells (data not shown), but no differential induction of gene expression was observed.

These findings suggest that VPA/AzaC treatment did not substantially increase the responsiveness of hESNSCs to primary cell derived cues.

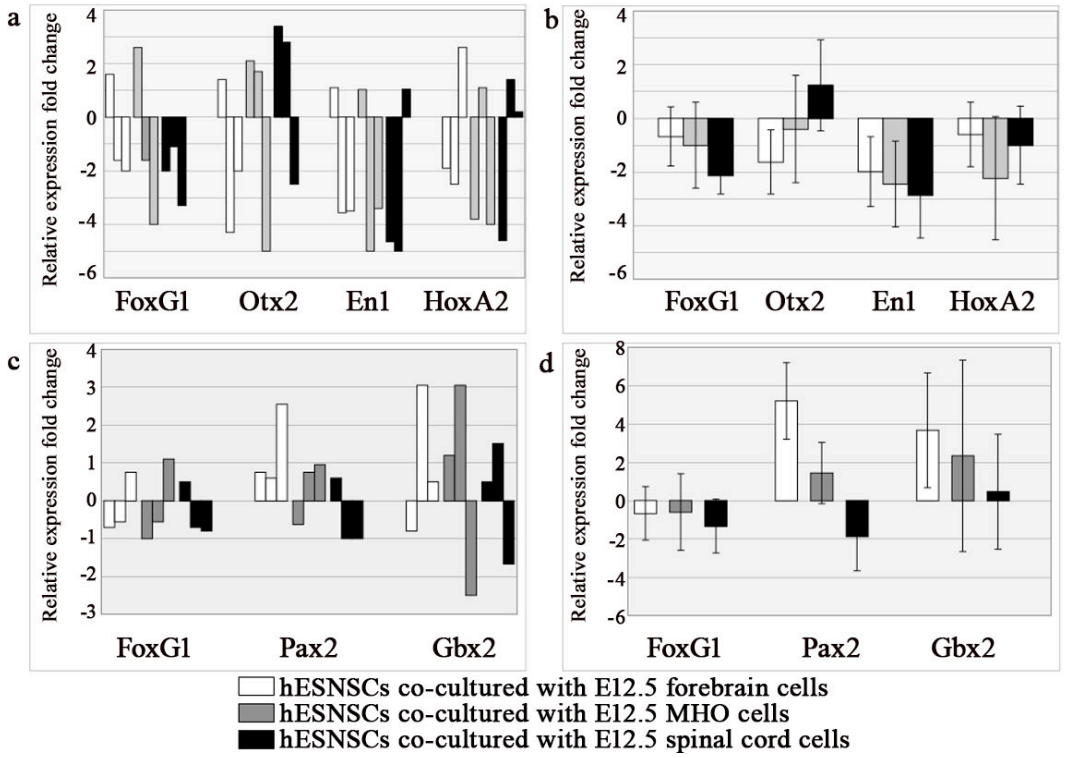

Fig. 33. Expression level of candidate markers genes in hESNSCs, which were pre-treated with VPA and AzaC and subsequently co-cultured with different regions of the embryonic mouse brain. hESNSCs were treated with 1mM VPA and 300nM AzaC for 72h. After this time freshly prepared primary cells from the forebrain, MHO region and spinal cord of E12.5 mice were added. The two cell types were co-cultured for 5 (a,b) or 9 days (c,d). There was no significant induction of the marker genes corresponding to each of the different brain regions FoxG1/Otx2 (forebrain), En1/Pax2 (midbrain), HoxA2/Gbx2 (hindbrain). The co-cultures were analysed using two way ANOVA (SPSS). All results were normalized to human specific L27. Data are shown as fold expression relative to control cells, i.e. hESNSCs, which were pre-treated but non co-cultured.

3.3.4 VPA/AzaC treatment enhances the response to morphogens

From previous experiments it was known that the neural precursors respond to high levels of single morphogens (3.3.1,(Koch et al. 2009). Therefore it was investigated whether demethylation and simultaneous hyperacetylation render the hESNSCs more susceptible to regionalisation cues of defined morphogens.

In order to do so cells, which had been treated with VPA and AzaC for 72h as described above, were cultured for an additional 7-10 days in the presence of 3μM retinoic acid. RA is involved in posteriorization and induction of Hox gene expression during normal embryogenesis (Maden et al. 1998). At the end of the culture period induction of hindbrain genes was assessed via immunohistochemical staining of HoxB4 (Fig. 34) as well as quantitative RT-PCR of HoxB4 and additional hindbrain genes (HoxB8, HoxD11). 30,7± 4% of the VPA/AzaC pre-treated cells expressed HoxB4 on protein level compared to 3,9± 1,7% of control cells. Thus the treated cells showed significantly increased HoxB4 protein expression after RA application suggesting that they were more posteriorized than control cells (Fig. 34

a,b,c). This observation was confirmed via quantitative PCR (Fig. 34d), which also detected a significant induction of the additional hindbrain genes HoxB8 (3,7 fold increase) and HoxD11 (6 fold increase) as well as a down regulation of the forebrain gene Otx2 (1,6 fold decrease).

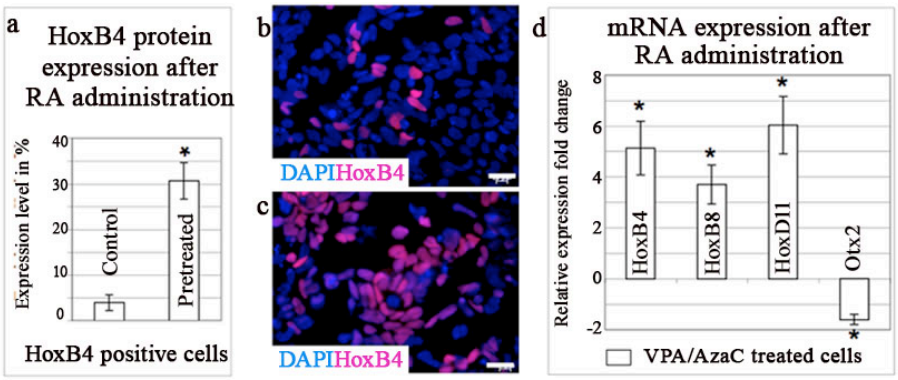

Fig. 34. Increased responsiveness of hESNSCs to RA following pre-treatment with VPA/AzaC
After treatment with 1mM VPA/300nM AzaC for 72h hESNSCs were subsequently cultured in the presence of 3µM RA for an additional 7-10 days. 3,9±1,7% of control cells (**a,b**) and 30,7±4 of pre-treated hESNSCs showed HoxB4 protein expression **(a,c)**. This significant increase was confirmed by quantitative PCR data **(d)** demonstrating a significant induction of further hindbrain genes as well as a down-regulation of Otx2. Data were analysed using student's t-test, with $p^* \leq 0.05$, Data are shown as fold expression relative to control cells, i.e. hESNSCs, which were not treated with VPA/AzaC but treated with RA (scale bar 10μm).

These results suggest that demethylation and hyperacetylation render neural precursors more responsive to potent morphogens. In order to further substantiate this notion, the effect of the increased responsiveness mediated by pre-treatment with VPA/AzaC had to be translated from the anterior-posterior axis to the dorso-ventral axis. For this purpose the morphogen BMP4 was chosen, which is involved in dorsalization of the spinal cord during development (Liu and Niswander 2005). The hypothesis was that if VPA/AzaC treatment increased the responsiveness of the neural precursors to morphogens this treatment scheme should result in a dorsalization, i.e. an increased Pax3 expression in the hESNSCs. As demonstrated in Fig. 35 BMP4 administration to VPA/AzaC treated neural precursors resulted in an significant induction of Pax3 expression on protein level from 0,8± 0,7% in control hESNSCs (Fig. 35a/b) to 5,5± 1,2% in pre-treated hESNSCs (Fig. 35a/c) as well as a significant increase of Pax3 on mRNA level (4,3 fold), thereby indicating a dorsalization of the hESNSCs. The up-regulation of the dorsal marker Pax3 on mRNA level was accompanied by a significant down-regulation of the ventral marker Nkx2.2 (2 fold decrease) (Fig. 35d).

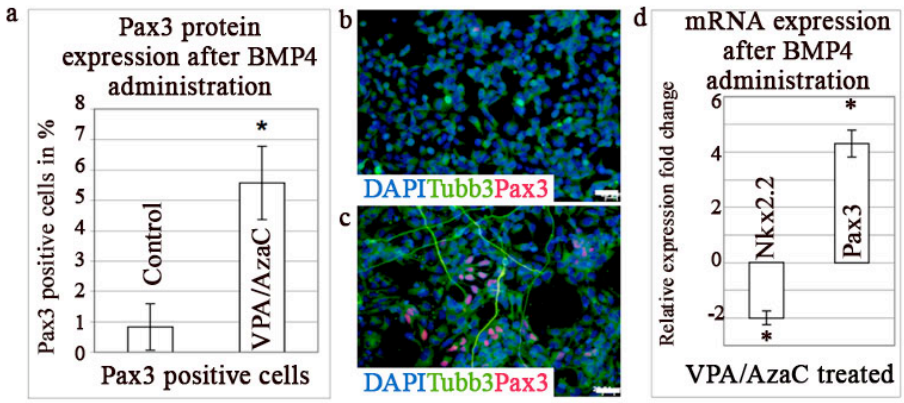

Fig. 35. Increased responsiveness of hESNSCs to BMP4 following pre-treatment with VPA/AzaC
Following administration of 1mM VPA/ 300nM AzaC for 72h neural precursors were subsequently cultured in the presence of 10ng/ml BMP4 for 4 days. The control cells **(a,b)** no application of VPA/AzaC, but administration of BMP4 showed only occasional expression of the dorsal marker Pax3. HESNSCs, which had been pretreated, however, showed a significant increase of Pax3 protein expression **(a,c).** This finding was confirmed by quantitative PCR data **(d)** demonstrating a significant induction of Pax3 as well as a down-regulation of the ventral marker Nkx2.2. Data were analysed using student's t-test, with p*≤0.05, Data are shown as fold expression relative to control cells, i.e. hESNSCs, which were not treated with VPA/AzaC but treated with BMP4 (scale bar 20μm).

These observations substantiate the notion that pre-treatment with VPA and AzaC, i.e. DNA demethylation and histone hyperacetylation, renders the hESNSCs more responsive to the effect of potent morphogens.

4 Discussion

All major somatic cell lineages can be generated from human embryonic stem cells. Several studies have demonstrated the capacity of embryonic stem cells to generate a variety of neural subtypes including motoneurons and dopaminergic neurons (Bain et al. 1995; Okabe et al. 1996; Li et al. 1998; Kawasaki et al. 2000; Tropepe et al. 2001; Yang et al. 2008).

However, until now grafted cells often show insufficient differentiation, a process very likely controlled by correct region-specific gene expression and indispensable for functional improvement (Kim et al. 2003). Therefore research is focused on the challenge of generating neural populations with a defined region-specific phenotype. Here multipotent, stable proliferating, long-term expandable human embryonic stem cell derived neural stem cells were chosen as a model system. Under standard culture conditions these neural precursors show a defined regional gene expression profile, which corresponds to a hindbrain identity. This defined regional phenotype provides an ideal starting point for detecting minimal changes of regional transcription factor expression. Therefore the hESNSCs were chosen for systematically studying whether primary cell derived cues are capable of influencing the regional phenotype of neural precursor cells.

4.1 Neural precursors maintain their neurogenic potential and posteriorize during prolonged expansion in the presence of growth factors

In contrast to other embryonic stem cell derived neural precursors the hESNSCs showed robust neuronal differentiation over the passages as demonstrated by Tubb3 expression in quantitative RT-PCR (Fig. 15) as well as immunocytochemistry (Fig. 17) (Elkabetz et al. 2008). They did not acquire a gliogenic bias upon prolonged passaging (Koch et al. 2009). This was an important observation as neural stem/progenitor cell expansion was until recently accompanied by a diminished potential to generate neurons over glial cells (Tropepe et al. 2001). In mES cell derived neural stem cells this bias was only resolved in 2005, when it was shown that they could be expanded for over 100 passages and still retain the capability to produce large proportions of neurons (Conti et al. 2005).

In order to further characterize the hESNSCs, it was investigated whether the regional gene expression changes. Both I3 and H9.2 derived hESNSCs showed a

distinct pattern of region-specific gene expression. In early passages the cells displayed high expression levels of the representative forebrain markers FoxG1 and Dlx1 (Fig. 14, Fig. 15). During prolonged proliferation using EGF and FGF these anterior markers were down-regulated. Simultaneously the expression of the posterior markers HoxB1 and Gbx2 increased (Fig. 14, Fig. 15). From at least passage 10 onwards the neural precursors displayed a defined hindbrain phenotype (Koch et al. 2009). This phenotype was maintained during prolonged *in vitro* culture.

The original acquisition of an anterior phenotype was reminiscent of earlier observations in both mouse and human ES cells. It was found by several groups that ES cells derived neural cells adopt anterior CNS characteristics by default (Wichterle et al. 2002; Li et al. 2005; Watanabe et al. 2005). The finding that the hESNSCs first expressed anterior markers is in accordance with the anterior neural default model postulated in classical studies of Xenopus CNS development. These studies detected that during development the neuroectoderm at the head region forms first and is then shifted towards posterior fates in response to secreted signals including RA, FGFs and Wnts (Nieuwkoop 1952; Sasai and De Robertis 1997). At present prolonged proliferation of NSC and neural progenitors *in vitro* – including our own protocol – relies on the presence of FGF. FGF family members possess a broad mitogenic potential. They signal via receptor tyrosine kinases resulting in the activation of the Ras/MAP kinase signalling pathway (Jaye et al. 1992; Kouhara et al. 1997; Eswarakumar et al. 2005). Specifically FGF2 is known to alter developmental competence and posteriorize neuroepithelial cells (Gabay et al. 2003; Hack et al. 2004; Zhang 2006). Possibly due to the almost universal employment of FGF for proliferation, no protocol for stably proliferating forebrain precursors has been published yet. Since in humans the forebrain is the largest part of the CNS, derivation of such a protocol is a major goal, as it would allow the generation of the rich array of neuronal subtypes that are present in the forebrain. One of currently most promising approaches to generate forebrain fates is via inhibiting endogenous Wnt signals, which are also implicated in anterior-posterior specification. For mouse ES cells Watanabe and co-workers have demonstrated that this strategy increased the level of neural progenitors displaying forebrain characteristics by 30% (Watanabe et al. 2005).

Recently, also a different type of hES cell derived neural stem cells has been reported. These cells, generated from the early rosette stage of neural stem cell development (conclusively termed R-NSCs), showed broad differentiation potential toward CNS

and PNS fates as well as *in vivo* engraftment. Just like the early rosette stage cells of which they are derived, R-NSCs exhibited robust FoxG1 expression. Elkabetz and co-workers hypothesized that this conservation of forebrain fate is due to supplementation with SHH and the Notch agonists DLL4 and Jagged-1. It was demonstrated that in the absence of SHH and Notch pathway activation the R-NSCs progressed to a more restricted – i.e. less patternable – NSC stage, probably comparable to the hESNSCs. Despite this Notch pathway activation, the R-NSCs cannot be maintained in long-term *in vitro* culture (Elkabetz et al. 2008).

Hence it seems as if neural stem cells expressing forebrain marker genes and displaying a broad differentiation potential can be proliferated via Notch pathway activation for a limited period of time (up to 4 passages) (Elkabetz et al. 2008), but in order to achieve long-term *in vitro* cultivation, protocols not relying on FGF still need to be developed.

4.2 Neural precursors remain amenable to patterning by retinoic acid

Treatment of mouse embryonic stem cells or embryonic carcinoma cells with RA has been the most commonly used approach for neural differentiation due to its simplicity and high efficiency (Jones-Villeneuve et al. 1982; Bain et al. 1996). In contrast, if neural fate is already determined RA instead acts mainly to posteriorize neural cells. This was observed first in Xenopus and then also in mouse embryos. If the embryos were treated with excess RA, anterior structures were lost, whereas the hindbrain and spinal cord seemed to expand. At the same time anterior genes such as Otx2, Emx1 and Dlx1 were repressed and posterior genes such as Krox20 and various Hox genes were up-regulated (Agarwal and Sato 1993; Avantaggiato et al. 1996; Maden 2002; Sirbu et al. 2005).

To test whether the hESNSCs are amenable to change in regional gene expression when treated with morphogens, retinoic acid was chosen. The treated cells showed significantly increased expression of the hindbrain gene HoxB4 (Fig. 18). These data suggest that neural precursors possess the potential for directed region specific differentiation *in vitro* when treated with RA.

Data from parallel studies performed by Koch et al. suggested that the hESNSCs were amenable to alterations in regional gene expression when treated with FGF8 and Shh ((Koch et al. 2009). This is a well-established combination of morphogens resulting in neural progenitors with mid/hindbrain characteristics and subsequent specification of dopaminergic neurons (Zhang 2006).

Other groups obtained comparable results. Bouhon and co-workers reported that they could progressively posteriorize their mouse embryonic stem cell derived neural progenitors. When treated with FGF2 and RA the cells down-regulated Pax6 and up-regulated midbrain and hindbrain markers including En1 and HoxB4 (Bouhon et al. 2006). Furthermore Pankratz and colleagues have recently reported a similar observation in human embryonic stem cells (Pankratz et al. 2007). Their primitive neuroepithelial cells initially exhibited an anterior identity as judged by widespread Otx2 expression. It was possible to transform this anterior phenotype via retinoic acid administration to a more posterior fate. Treatment with RA for 8 days resulted in most cells expressing the hindbrain marker HoxB4 and only a few cells maintaining Otx2 expression. In this context it should be noted that the cells used by Pankratz and co-workers were analysed four weeks after initial differentiation from hES cells. This was a time point considerably earlier than that at which our stable neural precursor population was assayed (Pankratz *et al.* 2007). Importantly, other findings indicated that mouse and human ES cell derived neural progenitors lost their responsiveness to single morphogens after *in vitro* cultivation (Bouhon et al. 2006; Elkabetz et al. 2008). For example in the above mentioned study Bouhon and co-workers observed that they could induce motoneuron formation from their mouse embryonic stem cell derived neural progenitors between day 4 and 8 but not anymore between day 16 and 20 of differentiation (Bouhon et al. 2006).
Thus the hESNSCs, in contrast to other ES cell derived neural precursors, appear to retain their responsiveness to single morphogens even after prolonged *in vitro* expansion.

4.3 Tissue derived cues are not sufficient to induce and consolidate regional re-specification in hESNSCs

In principle, it should be possible to generate specific subtypes of neurons and glia by adjusting the concentration of morphogens or combination of morphogens. This was successfully accomplished in the case of progenitors from different sub-regions of the spinal cord, which were induced to form motoneurons via application of RA or FGFs and GDF11 (Wichterle et al. 2002; Li et al. 2005). Yet, many morphogens are expressed at different locations and varying time-points during CNS development. Consequently additional factors such as timing, space and quantity of patterning cues or other molecular events are necessary to produce distinct neural subtypes (Zhang 2006). One possibility to address this complex interaction is to model the

specific regional environment of representative brain regions *in vitro* using acutely isolated primary cells. Employing this approach it was studied systematically to which extent cues from primary cells of different brain regions are capable of influencing region-specific gene expression of hESNSCs using paradigmatic co-culture settings.

This approach seemed feasible, as earlier studies suggested that mES derived neural precursors were capable of migration and differentiation upon transplantation into the embryonic rat brain displaying extensive axonal innervation of the host brain. These results indicate that *in vitro* derived neural progenitors are capable of responding to signals of their new environment (Brustle et al. 1997).

Moreover it has been demonstrated that cues from co-culture paradigms can induce region specific marker gene expression in neural stem cells. Specifically it was reported that co-culture with E14.5 mouse ventral forebrain tissue induced expression of an early onset ventral forebrain specific reporter gene in neural stem cells from the dorsal forebrain as well as from the midbrain/hindbrain. This was possible even though the stem cells from the different regions expressed distinct regional identities *in vitro* prior to co-culture (Hitoshi et al. 2002).

These different results suggest that tissue derived cues may influence both primary neural stem cells and ES cell derived neural progenitors.

Diffusible primary cell derived cues are not sufficient to induce changes in regional gene expression in hESNSCs

In the first instance a shared medium co-culture setting was chosen to investigate, whether diffusible factors released by primary cells were sufficient to induce regional specification of hESNSCs. Of special interest was the question whether long term passaged hESNSCs (> passage 28), which displayed a hindbrain phenotype prior to co-culture, could be reverted to a forebrain fate. Thus it was investigated whether expression of forebrain transcription factors could be induced (again).

The results (Fig. 19) suggested that the shared medium co-culture did not induce any significant and reproducible changes in the expression of representative transcription factors corresponding to the distinct brain regions, i.e. no effect of the co-culture on region specific gene expression was evident.

Some examples of co-culture settings that were applied successfully by other groups include telomerase-immortalized fetal midbrain astrocytes that enriched for human embryonic stem cell derived dopaminergic neurons (Roy et al. 2006, 2007). Moreover

astrocytic co-culture was shown to accelerate the onset of synaptic activity (Johnson *et al.* 2007) and stromal cell-derived inducing activity is known to promote differentiation of neural cells from mouse embryonic stem cells (Kawasaki *et al.* 2000). Even though these approaches are dependent on a range of different effects they all use direct cell-cell contact. Therefore a direct co-culture model was set up next.

Direct co-culture derived cues do not induce regional re-specification

Also the direct co-culture of hESNSCs with P0 mouse cells did not result in a significant induction of regional gene expression corresponding to the employed brain regions (Fig. 21). In contrast, hESNSCs co-cultured with the forebrain showed on average the highest expression of the hindbrain marker HoxA2 of all co-cultured cells.

One explanation for this finding could be that human embryonic stem cell derived neural cells, which – in contrast to our hESNSCs – have been shown to be amenable to co-culture mediated effects were tested considerably earlier after their derivation from hES cells than the cells used in this study. Johnson and colleagues started their co-culture using astrocytes at three weeks of hES cell differentiation, while Vazin et al. employed stromal co-culture directly from the start of their differentiation protocol (Johnson et al. 2007; Vazin et al. 2008). Thus the hESNSCs used in this study were proliferated substantially *in vitro,* whereas cells used by other groups in comparable experiments were not.

In our setting employing cells earlier after derivation from hES cells would be problematic. It would be difficult to judge how much of any occurring change in regional phenotype was due to long-term proliferation using FGF and how much to co-culture derived cues.

Despite these considerations a direct co-culture using short-term passaged hESNSCs (passage 3-6) was conducted. The reason were reports (study by Bouhon and colleagues see above) suggesting that with increasing passage number embryonic stem cell derived neural precursors loose their responsiveness to local inductive cues (Bouhon et al. 2006). In addition to the number of divisions the time of differentiation might be impacting on the ability of neural precursors to respond to patterning signals. For example Li et al. claimed that the capacity of human embryonic stem cell derived neural progenitors to generate region specific neurons is progressively restricted (Li et al. 2005). They observed that HB9, characteristically expressed in motoneurons, could be effectively induced via treatment with RA and Shh in early

neural precursors 10 days after differentiation from hES cells but not in late neural precursors 15 days after initial differentiation. Thus it seemed as if the cells had lost their responsiveness to the patterning cues in just 4-5 days. This notion corresponds to findings in primary neural stem cells, which were shown to respond differently to extrinsic cues at different stages of cortical development. It was observed that E12 mouse cortical precursors do not respond to CNTF/LIF administration. In contrast, in adult CNS stem cells CNTF/LIF treatment resulted in astrocytic differentiation (Molne et al. 2000).

However, the outcome of our experiment showed that the expression level of the candidate transcription factors was not significantly up- or down-regulated in hESNSCs of passage 6 in three independent experiments (Fig. 21). This suggests that the passage number of the hESNSCs was not the main reason for the lack of induction of region specific gene expression in the direct co-cultures.

To maximise the presence of patterning factors two direct co-culture models employing embryonic day 15 and 12.5 primary cells, one of the earliest cell types amenable to preparation, were studied. Yet, gene expression analysis following co-culture with both embryonic cell types revealed no region-specific induction of gene expression (Fig. 24).

Isolated induction of the regional transcription factor FoxG1 in slice co-cultures

To explore whether the primary cells need to maintain their three-dimensional position to induce regionalisation a slice co-culture model was investigated. This setting has the advantage that the cell-cell and cell matrix contacts such as collagens, proteoglycans and glycoproteins more closely resemble the *in vivo* situation (Faissner and Steindler 1995). An example for the potency of extra cellular matrix components is the suggestion by Cyzy and Wobus that interaction of embryonic stem cells with the extra cellular matrix via integrins determines the expression of signalling molecules BMP-4 and Wnt-1. This activation is important for mesodermal and neuroectodermal lineage induction (Czyz and Wobus 2001).

Differences between brain regions also seem to be reflected in the adhesive properties of the primary cells. Specifically, it was reported that regional differences in neuronal differentiation were reflected by differential expression of Ca^{2+} independent CAMs (Whitesides and LaMantia 1995). Thus in a slice co-culture, where hESNSCs are surrounded by local cues and gradients of patterning factors might arise, extra cellular matrix components might have an influence on co-cultured

hESNSCs. In contrast, in a monolayer co-culture, primary cells might release patterning cues, which then simply drift off into the medium. Another property of *in vivo* arealization is mirrored more closely in a three-dimensional setting than in a direct co-culture. This is the fact that neural progenitors encounter multiple competitive instructive signals from all sides at any one time (Anderson 2001).

The slice co-culture model significantly increased expression of the forebrain gene FoxG1 in hESNSCs transplanted onto the hippocampus (Fig. 23). Since the hippocampus itself expresses FoxG1, this might indicate a region specific marker gene induction. Moreover, it suggested a possible reversal of the hindbrain phenotype. Yet, none of the other regional transcription factors were differentially regulated. Particularly, the other employed forebrain marker Otx2 did not mirror the significant induction of FoxG1 in hESNSCs co-cultured on hippocampal slices. Furthermore the anterior hindbrain marker HoxA2 was not down-regulated in cells transplanted to hippocampal slices compared to those transplanted to cerebellar slices. This, however, would be expected if there was ongoing regional specification. Finally the FoxG1 induction was not reflected at protein level (Fig. 23).
The significant robust increase of FoxG1 expression at the RNA level argues against a random induction of the gene. Nevertheless, considering that none of the other transcription factors was significantly induced or reduced and that FoxG1 expression was not increased at protein level, it became unlikely that the induction of FoxG1 on mRNA level was due to a regionalizing effect of the slice co-culture.
Interestingly, it has been reported that FoxG1 up-regulation is associated with increased proliferation of neural precursors (Martynoga et al. 2005). Such an interpretation would be compatible with the observed significant induction of E2F1 on mRNA level in hESNSCs transplanted to hippocampal slices. E2F1 is a factor acting as transcriptional activator important for progression through G1/S phase transition (DeGregori et al. 1997). To further address this issue a detailed proliferation analysis including BrdU-pulse experiments would be required.

Re-aggregation co-culture does not suffice for induction of region-specific gene expression in hESNSCs

The three-dimensional slice co-culture, where the hESNSCs can invade the slice and acquire a three-dimensional position within the regionally specified primary cells, had yielded a significant induction of the marker gene FoxG1. Thus the influence of a re-aggregation co-culture, where the hESNSCs are also surrounded by primary cells,

on the regional gene expression of the hESNSCs was tested. The advantage of the re-aggregation co-culture, where primary cells and hESNSCs were cultured in a roller drum system (Studer et al. 1998), was that embryonic primary cells could be used thereby maximising the presence of patterning factors. Usually one sphere per Falcon tube developed. In this sphere hESNSCs were evenly distributed among the primary cells. Therefore the conditions for maximal interaction between primary cells and hESNSCs were provided. However, the long term expanded human neural precursors did not show region specific gene induction in this model. This outcome was surprising since the setting was in our opinion the most powerful one, because the co-cultured cells completely intermingled with each other and the MHO alone is necessary and sufficient for induction of mesencephalic and metencephalic structures (Marin and Puelles 1994).

hESNSCs remain insensitive to regionalisation cues of the intact neonatal brain

Finally *in vivo* transplantation was used to assess the power of the generated and validated human-specific primers established in the course of this project. To this end hESNSCs were transplanted into the forebrain and cerebellum of P1 rat pups. After 21 days regional marker gene expression was analysed via quantitative RT-PCR. While the gene expression of the hESNSCs could be analysed using the human specific primers, transplantation into the intact neonatal brain did not induce significant changes in region-specific gene expression (Fig. 27).

There are several explanations, which could account for the fact the primary cell derived cues were not sufficient to influence the regional gene expression of the hESNSCs.

First of all, the variability in the quantitative PCR results might have been too high to detect significant, reproducible changes in gene expression level. One reason for the high variability between individual experiments might be the primer design. Since the primers had to be human specific in order to distinguish between gene expression in primary cells and hESNSCs, only a restricted choice of primer sequences was available. Therefore primer selection was limited. For instance it was not always possible to generate a primer pair with melting temperatures within 1°C of each other. The variability in gene expression level between individual experiments could also be a result of subtle differences in the primary cell populations used for the co-cultures. Even though the derivation procedure was standardized such subtle differences cannot be completely ruled out.

Furthermore only rodent tissue was available for the co-cultures. This tissue might not have been able to exert the same influence on human cells given their longer maturation time and differing molecular signature as human tissue might have been able to do. An indication that human cells can influence human cells in a co-culture setting was published by Roy and co-workers. Their immortalized mesencephalic astrocytes of human origin were shown to drive development of dopaminergic neurons from hES cells (Roy et al. 2006). In the same study hES derived cells were treated with the patterning factors Shh and FGF8 and co-cultured simultaneously (Roy et al. 2006), whereas the hESNSCs were only co-cultured. Therefore it could be tried to supplement our co-cultures with defined morphogens.
Besides it has been hypothesized that cells might have to undergo their final division in their new environment to respond appropriately to local cues (Brustle et al. 1995). A potential avenue to further investigate this possibility would be to normalize the candidate gene expression in a co-culture experiment to those hESNSCs that divided again using a BrdU-pulse experiment. Furthermore it might be possible that long-term proliferation using FGF does not only compromise positional identity (Hack et al. 2004), but also compromises the ability to respond to tissue derived patterning cues. Even hESNSCs of passage 3, the youngest passage used for experiments in this study, might already have a restricted capacity for region specific differentiation. As discussed above Li et al. published that human ES cell derived neural progenitors respond effectively to directed posteriorization 10 days after derivation from hES cells. However, already 4-5 days later the neural progenitors had lost their responsiveness to RA and Shh (Li et al. 2005). The mechanisms that cause this limited temporal responsiveness to patterning cues are unknown. However, it has been suggested that temporal responsiveness is a mainly intrinsic property (Allen 2008), possibly related to epigenetic events.

4.4 DNA demethylation and histone hyperacetylation enhance the response of neural precursors to defined morphogens

The lack of response of the hESNSCs to primary cell derived regional stimuli after long-term *in vitro* propagation might also be a result of their non-permissive epigenetic organisation. As stated above alterations in histone acetylation and DNA methylation patterns are fundamental for establishing and maintaining cell type specific gene expression patterns (Jaenisch and Bird 2003). To change these

consolidated gene expression patterns the DNA in the hESNSCs was demethylated via AzaC treatment and histones were hyperacetylated using VPA administration.
VPA, a widely employed antiepileptic drug and antimanic mood stabilizer, has been reported to inhibit histone deacetylase, to modulate GABA levels in the brain, to activate the ERK pathway and to induce ERK pathway mediated neurotropic actions (Biggs et al. 1994; Bowden et al. 1994; Gottlicher et al. 2001; Phiel et al. 2001; Hao et al. 2004). In our setting administration of 1mM VPA caused HDAC inhibition and thus histone hyperacetylation. To account for any possible influence of VPA on GABA expression in the predominantly GABAergic neural precursors (Fig. 17) immunohistochemical analysis was performed. No effect on the ratio of detected GABA positive neurons could be found (Fig. 32).
Moreover, it was necessary to investigate whether VPA via modulation of the ERK-signalling pathway had any effect on neural differentiation or proliferation.
It was found that DNA demethylation and histone hyperacetylation did not cause significant alterations in proliferation rate or regional expression profile in the hESNSCs. Treatment with VPA and AzaC resulted in significantly less Tubb3 expression on mRNA level compared to non-treated control cells. This observation is in contrast to previous findings. For example inhibition of HDAC activity using VPA was claimed to result in neuronal differentiation of adult hippocampal neural progenitors. This effect was attributed to VPA inducing neurogenic transcription factors including the basic helix-loop-helix factor NeuroD, thereby promoting neuronal fates and inhibiting glial differentiation (Hsieh et al. 2004). Another study reported that HDAC inhibition significantly stimulated neuronal lineage progression in E14 mouse neural stem cell differentiation. In the employed minimal serum-free culture medium HDAC inhibition promoted the development of normal electrophysiological recordings and correct morphological maturation (Balasubramaniyan et al. 2006). The differing results might be ascribed to the fact that the hESNSCs were embryonic stem cell derived and not acutely isolated from rodents.
After administration of 1mM VPA and 300nM AzaC for 72h hESNSCs were subsequently treated with 3μM RA. This treatment scheme resulted in a significantly increased expression of the hindbrain gene HoxB4 on both protein and mRNA level (Fig. 34). Previous experiments had already shown that RA alone is sufficient to posteriorize the hESNSCs (3.1.1). Now, these experiments indicated that posteriorization via RA application following pre-treatment with VPA and AzaC is

approximately 9 times as potent as RA application alone. Simultaneously with HoxB4 also HoxB8 and HoxD11, two additional hindbrain genes, were significantly up-regulated and expression of the forebrain marker Otx2 was down-regulated. Furthermore, after pre-treatment with VPA and AzaC and subsequent administration of 10ng BMP4 the hESNSCs exhibited a significantly increased expression of the dorsal marker Pax3. At the same time levels of the ventral marker Nkx2.2 decreased. Taken together these results suggest that DNA demethylation and histone hyperacetylation enhance the effect of the defined morphogens RA (posteriorization) and BMP4 (dorsalization) on the hESNSCs.

Nevertheless, treatment with VPA and AzaC did not increase the responsiveness of the neural precursors to co-culture derived regionalisation cues. This is surprising since Schmittwolf and co-workers have reported that following DNA demethylation and histone hyperacetylation, neurospheres contributed to chimeric haematopoietic systems after transplantation into irradiated adult recipients (Schmittwolf et al. 2005). Hence these demethylated and hyperacetylated neurospheres showed a destabilisation of cell lineage commitment. This finding is also in agreement with the observation that histone deacetylase inhibitors and DNA demethylation increase the efficiency of reprogramming (Kishigami et al. 2006; Rybouchkin et al. 2006). Especially, it was claimed recently that VPA improves reprogramming efficiency by more than 100-fold. As a consequence it was possible to generate iPS cells without using the oncogene c-Myc (Huangfu et al. 2008).

A possible explanation for the apparent discrepancy regarding the potency of VPA treatment in our setting is the fact that epigenetic modifications do not alter the complete transcriptome. On the contrary, only 3% of all genes were influenced by DNA methylation and histone hyperacetylation in one report (Van Lint et al. 1996). In further experiments it could be elucidated whether treatment with VPA/AzaC during the co-culture period renders the hESNSCs more susceptible to primary cell derived cues. However, in such a setting the primary cells themselves could loose their regional identity upon exposure to VPA and AzaC.

Moreover the lack of responsiveness of the hESNSCs might be attributed to the co-culture conditions themselves: if they do not sufficiently mimic *in vivo* conditions (dilution of patterning cues etc. see 4.3) rendering the cells more responsive to regionalising cues will be in vain.

Nevertheless, this study suggests in accordance with others (Ruau et al. 2008) that by modifying the epigenetic state of precursor cells one can extend their developmental

capacity. In our case this might open the possibility to generate region specific neural precursors under controlled conditions that can be readily manipulated.

In summary the observations of this work suggest that hESNSCs retain a responsiveness to high levels of defined morphogens, which is enhanced by DNA demethylation and histone hyperacetylation, but are not easily amenable to regional specification by tissue derived environmental cues. These results support the notion that region specific gene expression is not easily overcome by tissue derived instructive factors *in vitro*.

5 Perspectives

The studies described here represent the first systematic investigation of the influence of tissue-derived cues on the regional gene expression of hESNSCs. The results suggest several approaches for further pursuing the objective of elucidating methods for the generation of neuronal subtypes expressing distinct regional transcription factor profiles similar to those observed in the CNS *in vivo.*

For instance, the finding that histone hyperacetylation and DNA methylation seem to lead to an increased responsiveness to defined morphogens could be used to increase the effectiveness of existing protocols employing morphogens. For instance it would be interesting to investigate whether the efficiency of procedures already leading to a specific neuronal population expressing a distinct neurotransmitter as well as regional transcription factor profile is enhanced if the cells are subjected to DNA demethylation and histone hyperacetylation. The generation of motoneurons using Shh and retinoic acid would be an excellent target for such an experiment (Li et al. 2005)

Further studies could also address whether the combination of epigenetic alteration and tissue derived environmental cues can be utilized to induce regional specification in human ES cell derived neural precursor cells *in vivo.* To this end VPA/AzaC pre-treated hESNSCs could be transplanted into neonatal rodents. After a sufficient period of time their regional gene expression could be analysed using quantitative RT-PCR as well as immunohistochemical analysis.

An interesting question would be to investigate in detail whether the presence of both VPA and AzaC is required to induce an increased responsiveness to defined morphogens.

Moreover elucidating exactly which transcription factors are affected directly by the treatment with VPA and AzaC might identify regulatory genes involved in patterning the CNS. This could be achieved using a CHIP-based method thereby moving from the candidate gene approach of this work to a more global gene analysis.

Overall the likelihood of pluripotent cell derived neural precursors to be used in regenerative medicine is increasing with the growing knowledge about their characteristics, their potential, and their limits. The finding that the susceptibility of hESNSCs to patterning by morphogens seems enhanced after DNA demethylation and histone hyperacetylation might lead to more protocols providing region specific neural cells for use in future cell replacement therapies.

However, it should be noted that the results of a number of previous studies implied that neural and even non-neural cells can be easily recruited into a region specific fate (Flax et al. 1998; Brazelton et al. 2000; Mezey et al. 2000; Munoz-Elias et al. 2004; Bae et al. 2007). Although care has to be taken to extrapolate our findings to other cell populations, the results of this study should serve as a note of caution, since the findings suggest that neural precursors are not easily recruited into a specific regional phenotype by tissue derived cues but need to be specified to a high extent, before envisaging future region-specific cell replacement strategies.

6 Abstract

The derivation of region-specific neural cells from human embryonic stem cells holds great promise for future cell replacement strategies of neurodegenerative diseases. However, the possibility to recreate the diversity of the mature CNS with its numerous types of neurons displaying distinct region-specific transcription factor expression profiles *in vitro* using defined morphogens seems limited. Therefore this study sought to investigate whether primary cells from different regions of the developing rodent brain – which themselves are known to be regionally specified – can influence the regional identity of human ES cell-derived neural stem cells (hESNSCs).

After establishing the regional gene expression profile of the hESNSCs under standard culture conditions *in vitro* the cells were exposed to paradigmatic co-culture settings. To that end cells from forebrain, midbrain, hindbrain/cerebellum and spinal cord of embryonic and P0 mice as well as hippocampal and cerebellar slices of 9-day-old rats were used. To analyse the gene expression of co-cultured human cells human-specific real time RT-PCR primers were designed and implemented. Surprisingly, neither co-culturing with nor transplantation onto or even *in vivo* transplantation into different brain regions induced significant changes in region-specific gene expression in the hESNSCs.

As it is known that the developmental potential of ES cells can be in part ascribed to their dynamic epigenetic state it was subsequently tested whether subjecting the hESNSCs to DNA demethylation and histone hyperacetylation would alter their developmental competence. Remarkably, even modifying the epigenetic state did not suffice to increase the responsiveness of hESNSCs to primary cell derived cues. However, administration of VPA and AzaC seemed capable of intensifying the effect of defined morphogens such as retinoic acid and BMP4 to posteriorize and dorsalize the hESNSCs, respectively.

These observations suggest that hESNSCs retain a responsiveness to high levels of single morphogens but are not easily amenable to regional specification by tissue derived environmental cues even after DNA demethylation and histone hyperacetylation.

7 Zusammenfassung

Die Herstellung von regional-spezifischen neuralen Vorläuferzellen aus embryonalen Stammzellen ist eine viel versprechende Möglichkeit zur Gewinnung von Zellen für Zellersatztherapien neurodegenerativer Erkrankungen. Allerdings ist es unwahrscheinlich, dass es *in vitro* gelingt das hochkomplexe Zentralnervensystem mit seinen vielen verschieden neuronalen Phänotypen, die unterschiedliche regional Transkriptionsfaktoren exprimieren, mit Hilfe von Morphogenen nachzubilden. Das Ziel dieser Arbeit war daher eine systematische Untersuchung der Frage, ob Primärzellen aus definierten Gehirnregionen, die selbst ein regionalspezifisches Genexpressionsmuster aufweisen, neurale Vorläufer (hESNSCs) so beeinflussen können, dass diese eine regional-spezifische Differenzierung und damit eine Adaptation an ihre Umgebung zeigen.

Nachdem das regionale Genexpressionsmuster der hESNSCs unter Standardkulturbedingungen ermittelt worden war, wurden paradigmatische Kokulturexperimente durchgeführt. Für die Kokulturen wurden Zellen aus dem Vorderhirn, dem Mittelhirn, dem Hinterhirn/Cerebellum und dem Rückenmark von embryonalen und P0 Mäusen sowie hippocampale und cerebellare Schnittkulturen von 9 Tage alten Ratten verwendet. Um die Genexpression von kokultivierten hESNSCs analysieren zu können, wurden humanspezifische RT-PCR Primer entworfen und implementiert.

Überraschenderweise konnte in keiner der untersuchten Ko- bzw. Schnittkulturen eine signifikante Veränderung der regionalen Genexpression humaner neuraler Vorläuferzellen festgestellt werden.

Da bekannt ist, dass das Entwicklungspotential von ES Zellen teilweise auf ihren dynamischen epigenetischen Zustand zurückzuführen ist, sollte im zweiten Teil der Arbeit geklärt werden, ob die Plastizität neuraler Vorläufer hinsichtlich ihres regionalen Phänotyps durch Histonhyperazetylierung und DNA-Demethylierung zunimmt. Bemerkenswerterweise schien auch eine Modifizierung dieser Prozesse nicht auszureichen, um die Empfänglichkeit der hESNSCs für die regionalspezifischen Einflüsse der Primärzellen zu steigern. Allerdings schien die Behandlung mit VPA und AzaC zu einer Verstärkung der Effekte der definierten Morphogene Retinsäure (Posteriorisierung) und BMP4 (Dorsalisierung) zu führen.

Daher wird davon ausgegangen, dass hESNSCs die Fähigkeit besitzen, auf hohe Dosen definierter Morphogene zu reagieren, aber selbst nach DNA-Demethylierung

und Histonhyperazetylierung nicht einfach durch regionale Stimuli aus ihrer Umgebung regionalisierbar sind.

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10 Declaration

Hiermit versichere ich, dass ich diese Dissertation selbständig und ausschließlich unter Zuhilfenahme der angegebenen Hilfsmittel angefertigt habe. Die Daten und Ideen, die aus anderen Quellen direkt oder indirekt übernommenen wurden, sind unter Angabe der Quelle gekennzeichnet.
Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form in einem anderen Prüfungsverfahren vorgelegt. Ich habe noch keinen früheren Promotionsversuch unternommen.

11 Appendix

11.1 Consumables

Consumables	Manufacturer
15cm cell culture dishes	TPP
1ml injection needles	BD Bioscience
8-stripes for quantitative RT-PCR	Biozym
Cell sieve	(40 μm) BD Bioscience
Cryovials	Nunc
Cuvettes	Eppendorf
Disposable Pipettes	(1ml, 2ml, 5ml, 10ml, 25ml) Falcon
Disposable Pipettetips	(10μl, 100μl, 200μl, 1000μl) ART
Falcon-tubes	Falcon, Heidelberg
Filter	(0,2μm) Nalgene
Gauge-needles	Microlance 3 BD Bioscience
Microscope slide	Menzel
Reaction vessels	Eppendorf
Stericup-filters	(0,22μm) Millipore
T175 flask	BD Falcon
Vitrics	Schott, Mainz
12 well tissue culture plates	BD Bioscience
6 well tissue culture plates	Nunc

11.2 Equipment

Appliance	Name/Manufacturer
Bühler shaker	**EB KS-15 Control, Johanna Otto GmbH**
Centrifuges	**Megafuge 1.OR, Heraeus Instruments** **Eppendorf Centrifuge 5415 C, Eppendorf** **RC5B Plus, Sorvall Heraeus Instruments**
Cleanbench (horizontal)	**HERAguard Thermo Electron**
Cleanbench (vertical)	**Holten Safe 2000 Thermo Electron**
Confocal laser scanning Microscpe	**Mikroskop Fluoview1000 Olympus**
Cryostat HM 560	**Microm Laborgeräte GmbH**
Digital Camera	**C 5050 Zoom Olympus Optical**
Electrophoresis	**Agagel Mini Biometra, Göttingen**
FACS DiVa	**Becton Dickinson**
Freezing unit	**Cryo 1°C Freezing Container Nalgene**
Gel documentation	**Geldoc2000 Bio-Rad, München**
Heated plate	**OTS40 Medite, Burgdorf**
Heating unit	**UNITEK HB-130/E**

Homogenizer	**Bandelin Plus**
Hybridisiation oven OV3	**Biometra**
Incubator	**Heracell, Heraeus Instruments**
Incubator	**HERAcell Thermo Electron, Waltham**
Laminar-Air-flow workbench	**Herasafe, Heraeus Instruments**
Microscopes	**Axiovert 25, Zeiss** **Axiovert 135, Zeiss** **Axioskop 2, Zeiss** **Leica** **LSM-510, Zeiss**
Neubauer cell counting camber	**Roth**
pH-electrode CG840	**Schott**
Photometer	**Biophotometer Eppendorf** **Nanodrop ND-1000, Fisher Scientific**
Pipettes	**10μl, 100μl, 200μl, 1000μl Eppendorf**
Pipetting aid	**Pipetboy Brand, Faust**
Quantitative PCR Cycler	**iCycler Bio-Rad**
Scales	**Precision Balance LA310S Sartorius**
Thermocycler	**T3 Biometra**
Thermomixer	**Compact Eppendorf**
Ultrasound bath	**Bandelin Sonorex**
Vacuumpump BVC21 + BVC01	**Vacuubrand GmbH und Co**
Vortex	**Genie 2 Scientific Industries**
Water bath	**1008, GFL**

11.3 Chemicals, solutions, enzymes

Substance	**Manufacturer**
1kb plus DNA ladder	Invitrogen
2-Mercaptoethanol	Invitrogen
30% acrylamide mix	Biorad
6x DNA loading buffer	Fermentas
Acetic acid	Merck
Agarose	PeqGold Universal PeqLab,
APS	Sigma
B27	Gibco
Bactotrypsin	BD Bioscience
Bromphenol blue	Sigma
BSA	Sigma
Chloroform	Sigma
Colcemid	Invitrogen
Cyclic AMP	Sigma

Cytocoon buffer	Evotec
DAPI	Sigma
DMEM	Invitrogen
DMEM/F12	Invitrogen
DMSO	Sigma
DNase	Sigma
dNTP	Peqlab
Dulbecco's Phosphate Buffered Saline	Invitrogen
EDTA	Roth
EGF	R&D Systems
Ethanol	Merck
Ethidiumbromide	Sigma
FCS	Invitrogen
FGF-2	Invitrogen
Fibronectin	MP Biomedicals
Ficole	Sigma
Fluorescein	Sigma
Gelatine Typ A	Sigma
Glucose	Sigma
Glutaraldehyde	Sigma
Glycin	Sigma
Gycerol	Sigma
HinF1	Invitrogen
Insulin	Sigma
Isopropanol	Sigma
Knockout-DMEM	Invitrogen
Knockout-Serum Replacement	Invitrogen
Kollagenase Typ IV	Invitrogen
L-Glutamine	Invitrogen
Ligase	Invitrogen
Lipofectamine 2000	Invitrogen
Magnesiumchloride	Invitrogen
Matrigel	BD Bioscience
Methanol	Carl Roth
Mowiol	Sigma
NaN_3	Sigma
Neurobasal medium	Gibco
Non-essential amino acids	Invitrogen, Metabion
Oligo-(dt)-Primer	Invitrogen
Papain	Sigma

Paraformaldehyde	Sigma
Potassiumchloride	Sigma,
RNAsin	Promega
SDS	Roth
Skimmed milk powder	Roth
Sodiumselenite	Sigma
Sodiumacetate	Sigma
Sodiumhydrogencarbonate	Sigma
Sodiumpyruvate	Invitrogen
Sucrose	Roth
SYBR Green I	Sigma
Taq-Polymerase	Invitrogen
TEMED	Sigma
Tissue Tek	Sakura
Transferrin	Sigma-Alrich
Trifast	Peqlab
Tris-HCl	ICN Biomedicals
Triton-X100	Sigma
Trypan-Blau	Invitrogen
Trypsin-EDTA (10x)	Invitrogen
Tween 20	Sigma
Vectashield	Vector laboratories
Xylene Cyanole	Biorad

11.4 Kits

BCA Assay	Pierce
DNase Kit	Qiagen
DNeasy Blood and Tissue Kit	Qiagen
EpiTect Bisulfite Kit	Qiagen
Iscipt Kit	Biorad
Peqlab Cycle Pure Kit	Peqlab
PicoPuro RNA Isolation Kit	Arcturus
RNeasy Kit	Qiagen
Super Signal West Femto Kit	Pierce

11.5 Antibodies

11.5.1 Primary Antibodies

Antigen	Description	Dilution	Type	Pre-treatment	Producer
BrdU	Uridin-Derivative which can incoporate into the DNA instead of thymidine	1:33	ms	HCl	Sigma
BrdU		1:200	rat	HCl	BD
GABA	Gamma-amino-butyric acid; inhibitory neurotransmitter of the CNS	1:300	rat	Triton –X 100	DSHB, Iowa City
FoxG1	Regional transcription factor expressed in the telecenphalon	1:5000	rb	Triton –X 100	Kind gift of Dr. Studer
Nkx2.2	Nkx2.2 is a member of the mammalian NK2 homeobox transcription factor family that is expressed in the ventral CNS and the pancreas	1:300	ms	Triton –X 100	DSHB, Iowa City
GFAP	Glial fibrillary acidic protein; intermediate filament in astrocytes	1:1000	rb		Dako Cytomation
GFAP		1:100	ms		ICN
GFP	Green fluorescent protein	1:3000	rb	Triton –X 100	Abcam
GFP		1:700	ms	Triton –X 100	Chemicon
HoxB4	Homeodomain protein expressed in the hindbrain and spinal cord	1:10	rat	Triton –X 100	DSHB, Iowa City
Nestin	Intermediate filament	1:100	ms	Triton –X 100	RD&Systems
Pax3	Paired box 3 gene, expressed during early to midgestation in the dorsal neural tube	1:250	ms	Triton –X 100	DSHB, Iowa City
S100β	Calcium-binding protein expressed by glial cells of the CNS and PNS	1:2000	ms		Sigma
Tuj1	Beta-III tubulin	1:1000	ms		Covance
Tuj1		1:1000	rb		Covance

11.5.2 Secondary Antibodies

Conjugate	Type	Dilution	Producer
Cy3	Goat anti-mouse IgM IgG	1:300	Jackson Immuno Research
Cy3	Goat anti-rabbit IgG	1:300	Jackson Immuno Research
FITC	Goat anti-rabbit IgG	1:200	Jackson Immuno Research
FITC	Goat anti-mouse IgM IgG	1:250	Jackson Immuno Research
Biotin	Anti-rabbit	1:200	Dako
Streptavidin coupled HRP	Anti-rabbit	1:500	Dako

11.6 Primers

If not stated otherwise quantitative RT-PCRs were run for 38 cycles and at 3mM MgCl.

11.6.1 Human specific quantitative RT-PCR primers

Primer	Sequence (5′-3′)	Annealing temp.	Product melting temp.	Product length
BLBP	CGACCTACTCCGCTAACC CTCCAGTGTCCCTCTTTCC	63	87	119
Darpp32	CTTTGGCTGATACCCAGAGAACC GAGTAGAATTGTGAAGGGCAAATCC	62	91	181
DCX	CCGCACTATCTACACCATC TCACAGAGGAGGCAGCAG	62	90	190
Dlx1	AGACAGCCCAAGCAGCAAGATAAAC AATGAGGACAGCGGACGGATGAG	65	89	198
Dlx2	AGATTTTCCCACTGATTTATTTG CAACAACAAAGACTTTAGGATAC	64	89	194
E2F1	GCAGAGCAGATGGTTATGGTG TATGGTGGCAGAGTCAGTGG	62	92	218
Emx1	AGTCCGAGCAGAAGAAGAAGG CAGCAGCAAGCAGCACTC	64	91	146
Emx2	TCCTCAACGGCTTCCACTC TCGGCGAACACCAAGTCC	63	91	78
En1	GGCTATCCTACTTATGGGCTCAG CTTCTCGTTCTTCTTCTTCTTCAG	64	93	160
FoxG1	CCCTCCCATTTCTGTACGTTT CTGGCGGCTCTTAGAGAT	65	86	203
Gad67	GATCTGCTTCCGGCTAAGAACGGTG AAGTGTTATTTGTTCCATGAGGACA	63	94	386
Gbx2	TGGAGAGCGATGTGGACTAC CCTGTCTTGGAATTGGCATTG	62	89	342
GFAP	ATCAACTCACCGCCAACAGCGCC CTCATACTGCGTGCGGATCTCTT	63	92	346
HB9	GTTACTCATCCATTGCCATC CCAGGTTGCTACAAGAGG	62	88	199
HoxA2	GAACTGCTTACACCAACAC TTCCTCGTCCTCCTCTAC	63	89	253
HoxB4	TTTCATCTTTAATCACGCCAGGTC GAAAGGAAATCCAGGCTGTCTTC	62	93	204
Krox20	GCTACCCAGAAGGCATAATC TGACAGGAACGCAGAAGG	62	90	250
L27	GGTGGTTGCTGCCGAAATG ATCTCTGAAGACATCCTTATTGACG	58-69	92	325

Human specific qRT-PCR primers continued

Primer	Sequence (5′-3′)	Annealing temp.	Product melting temp.	Product length
Mash1	GTCCTGTCGCCCACCATCTC CCCTCCCAACGCCACTGAC	63	92	251
Nestin	GGAGAAGGACCAAGAACTG ACCTCCTCTGTGGCATTC	63	91	152
Nkx2.2	ATGCCTCTCCTTCTGAAC TCAAGTGACGACATTAACG	62	90	129
Otx2	TGTGATCTCCTGTTATTGTATGC CTTCTCTTCTCTGACTCTCTTTG	60	85	195
Pax2	GCGACTCTCACAGCACAG CCACTCCACTCCTCCCATC	62	87	288
Pax3	ATGAAGCAAGAATGGAGGAATC CACAGGAAAGGGAAACAAGTC	62	88	222
Pax5	GCAGAATGTCATCCGAGGTATT ATGGCAGGTGTCCGAAGTG	62	91	179
Pax6	TACCAACGATAACATACCAAGC AGCCTCATCTGAATCTTCTCC	63	90	264
Pax7	GCGTGCTCAGAATCAAGTTC AGGTTCCGACTCCACATCC	62	88	154
Sox10	GAACAGGCTGGACAGAGGAGAAG AACAGGAGTCATAGGAGTGGTAAGG	64	89	182
Tubb3	CCGAAGCCAGCAGTGTCTAAACC GCAATAGATTTATTAAGTATCCC	63	92	237
vGlut1	ACCTCCATTCCACTCATCTC TTTGGGTATCCTTGAAACTGTC	62	89	179
vGlut2	AACCGCATCTACCCAAATACC TGAGAATGAGAATACAACAGAATAGC	62	89	199

11.6.2 Non human specific quantitative RT-PCR primers

Primer	Sequence (5′-3′)	Annealing temp.	Product melting temp.	Product length
CNP	TGGCGCCCCTCCTCATCATGAGG CATCACGGTACTTGTCCAC	61	90	241
GAPDH	ACGACCCCTTCATTGACCTAACT ATATTTCTCGTGGTTCACACCCA	55-69	92	325
HoxB1	GGGCTTGTCCGATGGCTACG GGTACTTGTTGAAATGGAACTCC	63	90	122
HoxB6	CGTGGATGCAGCGGATGAAT CAGCACCTTCACTCGGCCT	62	88	297
HoxC6 (Takahashi et al. 2004)	TCAAACGTGGACCTGAAAGTCA GGGAAAAGGGCCTGTAGACAA	62	89	126

Non human specific quantitative RT-PCR primers continued

Primer	Sequence (5′-3′)	Annealing temp.	Product melting temp.	Product length
HoxC8 (Takahashi et al. 2004)	CGCACCACGTTCAAGACTTCT TAAGCGAGCACGGGTTCT	62	90	81
HoxD11 (Hye et al. 2006)	GCCTCCAACTTCTACAGCGC TTGAGCATCCGAGAGAGTTG	62	88	289
Olig2	CAGAAGCGCTGATGGTCATA TCGGCAGTTTTGGGTTATTC	63	90	207
Telomerase	TGGCTGCGTGGTGAACTTG GCGGTTGAAGGTGAGACTGG	68	95	205
TH	CAGTTCTCGCAGGACATTG CGTCTGGTCTTGGTAGGG	63	89	243

11.6.3 Primers for COBRA assay

Primer	Sequence (5′-3′)	Reference
Line fw	TTGAGTTGTGGTGGGTTTTATTTAG	(Yang et al. 2004)
Line rev	TCATCTCACTAAAAAATACCAAACA	(Yang et al. 2004)

11.6.4 PCR conditions

Line PCR

Time (seconds)	Temperature (°C)	Repeats
30	95	
30	50	
30	72	35
600	72	

Quantitative RT-PCR

Time (min)	Temperature (°C)	Repeats
3.00	95.5	
0.20	94.5	
0.20	Various	
0.50	72.0	
1.00	95.0	
1.00	55.0	35
10	55.0	

PCR Mastermix (10ml)

Reagent	Volume (μl)
PCR buffer (10x)	2000
MgCl (50mM)	1200
Each dNTP (100nM)	40
SYBR Green (1: 10 0000)	15
Fluorescein (100μM)	2
ddH_2O	6623

11.7 Animals

Species	Type	Supplier	Age
Rat	Wistar	Charles-River	P9
Mouse	C57 B6	Jackson Laboratories	E10 – P3
Mouse	CD1	Jackson Laboratories	E15
Mouse	C57 B6 GFAP GFP	(Nolte et al. 2001)	P0

11.8 Solutions and buffers:

11.8.1 Cell culture

CM (Conditioned Medium)

50ml hES Cell Medium without FGF-2 per 10^6 mitotically inactivated fibroblast
Incubation overnight at 37°C, 5% CO_2
Store at -20°C
Add 4ng/ml FGF-2 prior to use

EB-Medium

FCS	20%
Non-essential amino acids	1%
L-Glutamine	1mM
Knockout DMEM	80%

hES cell medium

Knockout serum replacement	20%
Non-essential amino acids	1%
L-Glutamine	1mM
2-Mercapthoethanol	0,1mM
FGF-2	4ng/ml
In Knockout-DMEM	80%

ITSFn medium

Insulin	25µg/ml
Transferrin	100µg/ml
Sodium selenite	5ng/ml
Fibronectin	2,5µg/ml
FGF-2	20ng/ml
In DMEM/F12	

MEF Medium

FCS	10%
Sodiumpyruvate	1%
Non-essential amino acids	1%
in DMEM	

N2 Medium

N2 supplement	5ml
Glucose	0,1g/ml
Insulin	4ng/ml
DMEM-F12	500ml

Prior to use add 10ng/ml FGF,10ng/ml EGF and 1:1000 B27

NGMC medium

B27	1:50
Cyclic AMP	100ng/ml
N2 medium	50%
Neurobasal medium	50%

R6-Medium

Sodium sulfide	98mM
Potassium sulfide	30mM
Magnesium chloride	5.8mM
Calcium chloride	0.25 mM
Hepes	1mM
Glucose	20mM
Sodium hydroxide	0.125mM
Phenol Red	0.001%

Poly-ornithine (PO)

Poly-ornithine	1,5mg/ml
in ddH2O (Millipore, sterile)	
Coating of culture plates for at least 2 hours	

11.8.2 Western blot

Laemmli sample buffer (2x)

SDS	4%
Glycerol	20%
Bromphenol blue	0.004%
Tris-HCl	0.125 M

Prior to use add 20% 2-mercaptoethanol

SDS-PAGE running gel (10%)

dd H_2O	2 ml
30% acrylamide mix	1.7 ml
1.5 M Tris HCl	1.3 ml
10% SDS	0.05 ml
10% APS	0.05 ml
TEMED	0.002 ml

Stacking gel

dd H_2O	1.15 ml
30% acrylamide mix	0.33 ml
0.5 M Tris HCl	0.5 ml
10% SDS	0.02 ml
10% APS	0.02 ml
TEMED	0.002 ml

5x Running buffer

Tris	25 mM
Glycin	956 mM
SDS	0.5%
H_2O	add 1000ml

10x Transfer Buffer

Tris HCl	250 mM
Glycin	1.95 mM
H_2O	add 1000ml

Prior to use add 20% methanol

Antibody blocking solution

Tris-HCl pH7.5	10mM
NaCl	150mM
Tween 20	0.1%
Skimmed milk powder	5%
BSA	2%
NaN_3	10%
H_2O	Add 200ml

Aliquot and store at -20°C

PBS-Tween
250μl Tween in 500ml PBS

Blocking solution

Skimmed milk powder	5%
PBS-Tween	100ml

11.8.3 Other

Tris-Acetate-buffer 50x (TAE)

Tris-Base	242g
Acetic acid	570,1 ml (100%)

add 5.0 M EDTA to a total volume of 100ml and adjust the pH to 8,0

Agarose gel (1%)

Agarose	0,5g
TAE	1ml (50x)

Dissolve in 49ml ddH_20

Ethidiumbromide	2μl

add when cooled down and poor gel without creating bubbles into the gel chamber

Fixative for FISH analysis and G-banding

Methanol	3 parts
Glacial acetic acid	1 part

KCl (0.075M)

KCl	5,6g/l
H_2O	

FACS buffer

HBSS	1 part
Hepes (pH 7,4)	2,5mM
Glucose	30mM
Calcium chloride	1mM
Magnesium sulfide	1mM
Sodium bicarbonate	4mM
EDTA	1mM
Penicillin/streptomycine	1x

Add 500 ml destilled water

Loading buffer

EDTA	0,1M
Sucrose	60%
Bromphenol Blue	0,04%
Xylene Cyanole	0,04%
Ficole	2%
H_2O dd	

DMSO freezing medium 2x

FCS	5ml
DMSO	3,6ml

dissolve in 22,8 ml DMEM-ES, aliquot and store at –20°C

Sodium azide

Sodium azide	1 mg/ml

in PBS

11.9 Supplements

Fibronectin (Fn 1μg/μl)
Fibronectin 1mg
dissolve in 1ml ddH_2O, filter sterilize (0,2 μm), aliquot and store at –80°C

Insulin stock (5 mg/ml)
Insulin 1g
dissolve in 200ml 10 mM sodium hydroxide, aliquot and store at –80°C

Sodium selenite stock (500 μM)
Sodium selenite 86,5 mg
dissolve in 10 ml ddH2O and store at –20°C (100x)
dissolve 150μl stock solution in 15 ml ddH2O, aliquot and store at +4°C

Progesterone stock (20 μM)
Progesterone 32 mg
dissolve in 50 ml EtOH abs. and store at –80°C (100x)
mix 30μl of stock with 3 ml EtOH, aliquot and store at –80°C

Trypsin/EDTA 1x (0,125%)
10x Trypsin/EDTA 1ml
dissolve in 9ml PBS, aliquot and store at –20°C

Trypsin inhibitor (25 mg/ml)
Trypsin inhibitor 500mg
dissolve in 20ml PBS and store at –20°C (100x)

12 Curriculum Vitae

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