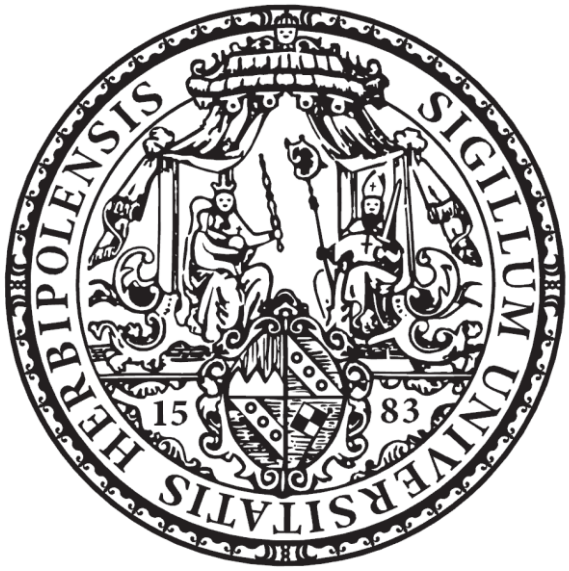

Neurogenesis from parthenogenetic human embryonic stem cells

Neurogenese von parthenogenetischen humanen embryonalen Stammzellen

Doctoral thesis for a doctoral degree
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1. SUMMARY

Imprinted genes play important roles in brain development. As the neural developmental capabilities of human parthenogenetic embryonic stem cells (hpESCs) with only a maternal genome were not assessed in great detail, hence here the potential of hpESCs to differentiate into various neural subtypes was determined. In addition DNA methylation and expression of imprinted genes upon neural differentiation was also investigated.

The results demonstrated that hpESC-derived neural stem cells (hpNSCs) showed expression of NSC markers *Sox1*, *Nestin*, *Pax6,* and *Musashi1* (*MS1*), the silencing of pluripotency genes (*Oct4*, *Nanog*) and the absence of activation of neural crest (*Snai2, FoxD3)* and mesodermal *(Acta1)* markers. Moreover, confocal images of hpNSC cultures exhibited ubiquitous expression of NSC markers *Nestin, Sox1, Sox2* and *Vimentin*. Differentiating hpNSCs for 28 days generated neural subtypes with neural cell type-specific morphology and expression of neuronal and glial markers, including *Tuj1*, *NeuN*, *Map2*, *GFAP*, *O4*, *Tau*, *Synapsin1* and *GABA*. hpNSCs also responded to region-specific differentiation signals and differentiated into regional phenotypes such as midbrain dopaminergic- and motoneuron-type cells. hpESC-derived neurons showed typical neuronal Na^+/K^+ currents in voltage clamp mode, elicited multiple action potentials with a maximum frequency of 30 Hz. Cell depicted a typical neuron-like current pattern that responded to selective pharmacological blockers of sodium (tetrodotoxin) and potassium (tetraethylammonium) channels. Furthermore, in hpESCs and hpNSCs the majority of CpGs of the differentially methylated regions (DMRs) KvDMR1 were methylated whereas DMR1 (*H19/Igf2* locus) showed partial or complete absence of CpG methylation, which is consistent with a parthenogenetic (PG) origin. Upon differentiation parent-of-origin-specific gene expression was maintained in hpESCs and hpNSCs as demonstrated by imprinted gene expression analyses.

Together this shows that despite the lack of a paternal genome, hpNSCs are proficient in differentiating into glial- and neuron-type cells, which exhibit electrical activity similar to newly formed neurons. Moreover, maternal-specific gene expression and imprinting-specific DNA-methylation are largely maintained upon neural differentiation. hpESCs are a means to generate histocompatible and disease

allele-free ESCs. Additionally, hpESCs are a unique model to study the influence of imprinting on neurogenesis.

2. ZUSAMMENFASSUNG

Imprinted Gene spielen eine wichtige Rolle bei der Gehirnentwicklung. Da das neurale Entwicklungspotenzial von hpESCs bisher noch nicht ausführlich untersucht wurde, war das Ziel dieser Arbeit das Differenzierungspotenzial von hpESCs zu verschiedenen neuralen Subtypen zu untersuchen. Außerdem wurden die DNA-Methylierung und Expression *imprinted* Gene in hpESCs während der neuralen Differenzierung analysiert.

Die Ergebnisse zeigten, dass von hpESCs abgeleitete neurale Stammzellen (hpNSCs) die NSC-Marker *Sox1, Nestin, Pax6* und *Musashi1* (*MS1*) exprimierten, Pluripotenzmarker-Gene (*Oct4*, *Nanog*) abschalteten und keine Aktivierung von Markern der Neuralleistenzellen (*Snai2, FoxD3*) sowie dem mesodermalen Marker *Acta1* stattfand. Immunfärbungen zeigten weiterhin, dass aus hpESCs abgeleitete Stammzellen die NSC-Marker Nestin, Sox1, Sox2 und Vimentin auf Proteinebene exprimierten. Durch gerichtete neurale Differenzierung für 28 Tage konnten aus hpESCs neurale Subtypen abgeleitet werden, die eine neurale Zelltyp-spezifische Morphologie aufweisen und positiv für neuronale und gliale Marker wie *Tuj1, NeuN, Map2, GFAP, O4, Tau, Synapsin1* und *GABA* sind. Um aus hpNSCs dopaminerge und Motoneuronen abzuleiten, wurden während der Differenzierung Morphogene und trophische Faktoren zugegeben. Elektrophysiologische Analysen konnten zeigen, dass die *in vitro* differenzierten Neuronen, die von hpESCs abgeleitet wurden, für Neurone typische Na^+/K^+ Ströme sowie Aktionspotentiale (30 Hz) vorweisen ausbilden und auf ausgewählte pharmakologische Natrium- (Tetrodotoxin) und Kalium- (Tetraethylammonium) Kanal-Blocker reagierten.

Desweiteren war der Großteil der CpGs von differentiell methylierten Regionen (DMRs) KvDMR1 in hpESCs und hpNSCs methyliert, während DMR1 (*H19/Igf2* Locus) eine partiell oder komplett abwesende CpG-Methylierung zeigte, was dem parthenogenetischen Ursprung entspricht. Während der Differenzierung wurde die elternabhängige (*parent-of-origin)* spezifische Genexpression in hpESCs und hpNSCs aufrechterhalten, wie mit Genexpressionsanalysen *imprinted* Gene gezeigt werden konnte.

In der Summe zeigen die hier dargestellten Ergebnisse, dass hpESCs, die kein paternales Genom besitzen, keine Beeinträchtigung im neuralen

Differenzierungspotential zeigten und zu Gliazellen und Neurone differenziert werden konnten. Elektrophysiologische Analysen zeigten ferner, dass von hpESCs abgeleitete Neurone funktionell sind. Zudem wird die Expression maternal-spezifischer Gene und die *Imprinting*-spezifische DNA-Methylierung während der Differenzierung größtenteils aufrechterhalten. In der Summe stellen hpESCs ein einzigartiges Modell dar, um den Einfluss des *Imprintings* auf die Neurogenese zu untersuchen.

3. ABBREVIATIONS

AG	androgenetic
ASCs	adult stem cells
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
bFGF2	basic fibroblast growth factor 2
cDNA	complementary deoxyribonucleic acid
cAMP	cyclic adenosine monophosphate
6-DMAP	6-dimethylaminopurine
DAPI	4,6-diamidino-2-phenylindole
DEPC	diethylpyrocabonate
DMSO	dimethyl sulfoxide
DMEM	dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EB	embryoid bodies
EDTA	ethylenediaminetetraacetic acid
ESC	embryonic stem cell
EGF	epidermal growth factor
et al.	*et alii* (masculine plural) or *et aliae* (feminine plural), and others
FCS	fetal calf serum
FGF8b	fibroblast growth factor 8b
GG	gynogenetic
GDNF	glial cell-derived neurotrophic factor
hESCs	human embryonic stem cells
hNSCs	human neural stem cells

hpESCs	human parthenogenetic embryonic stem cells
hpNSCs	human parthenogenetic neural stem cells
ICM	inner cell mass (of blastocyst)
iPS cells	induced pluripotent stem cells
IVF	*in vitro* fertilisation
KSR	knockout serum replacement
max	maximum
min	minimum
N	biparental (normally fertilised)
NEAA	non-essential amino acids
NSCs	neural stem cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PG	parthenogenetic
RCF	relative centrifugal force
RNA	ribonucleic acid
rpm	revolutions per minute
RT- PCR	real time polymerase chain reaction
SDS	sodium dodecyl sulfate
SHH	sonic hedgehog homolog
UBS	umbilical cord blood serum

4. INTRODUCTION

4.1 *Stem cells: features and functions*

The single celled embryo, the zygote, is formed when the oocyte is fertilized by a sperm. In vertebrates the zygote and early blastomere are considered to be totipotent as characterised by the ability to develop into a complete embryo including the placenta (Mitalipov *et al.,* 2009). Every individual totipotent cell is a self-contained entity which can develop into a complete organism. The cells of later developmental stages lose the totipotent property. With the progression of embryonic development beyond the 8-cell stage or later (varies in different species), blastomeres lose their totipotent characteristics (Fig. 1) (Hemmat *et al.,* 2010).

The cells derived from the inner cell mass (ICM) of the blastocyst stage embryo, are limited in their differentiation capability and thus are pluripotent in nature (Fig. 1). Embryonic stem cells (ESCs) are isolated from the ICM of preimplantation embryos (Evans *et al.,* 1981; Martin 1981). Surprisingly, *in vitro* ESCs maintain embryo-seeding and germline-transmission capacity together with self-renewal and pluripotent potential. Other hallmarks of ESCs are the expression of telomerase that prevents shortening of DNA during repetitive replication cycles and the ability to sustain a stable karyotype after numerous divisions. ESCs are the prototype of pluripotent stem cells.

Notably, a transient totipotent cell population was identified within mouse ES and induced pluripotent stem (iPS) cell cultures which possessed the capability to differentiate into embryonic and extra-embryonic tissues (Macfarlan *et al.,* 2012). These ESCs showed high expression levels of transcripts that are usually expressed in the totipotent two-cell embryos (Macfarlan *et al.,* 2012). This may point towards a transient state ESCs pass through, during which they can form all the tissue types of an organism, including placental cells (Surani *et al.,* 2012).

In many adult tissues stem cells are the only permanently present cell types, as differentiated cells have a limited life span and therefore have to be continuously replaced (Young *et al.,* 2004; Hemmat *et al.,* 2010). These stem cells are known as somatic/adult stem cells (ASCs). ASCs are multi- or unipotent as they can give rise to various specialized cell types of one organ system (Poulsom *et al.,* 2002). Therefore, ASCs are lineage-restricted. ASCs divide symmetrically or asymmetrically

(Forbes *et al.,* 2002; Tsai *et al.,* 2002) and mostly exist in a quiescent state; hence they divide infrequently (Li *et al.,* 2010).

Due to their unique developmental potential mentioned above stem cells are crucial for embryonic development and adult tissue homeostasis. Stem cells are also valuable tools for basic and applied research areas such as developmental biology, drug discovery, development of diagnostics, stem-cell-based therapies, modelling and understanding disease origin and progression.

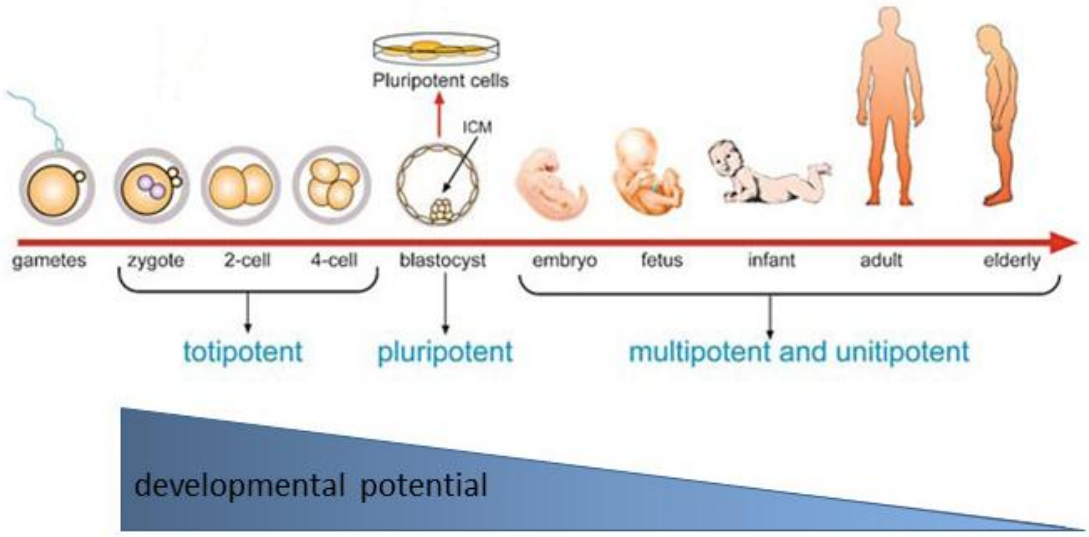


Fig. 1 Developmental potential of stem cells.
Embryonic development starts with the formation of the zygote. The zygote and each blastomere till the 4 cell stage are considered to be totipotent (Mitalipov *et al.,* 2009). As development proceeds, the developmental potential decreases resulting in pluripotent ICM cells of the blastocyst from which the ESCs are derived. The developmental ability further declines subsequently resulting in multipotent, unipotent and differentiated cells (adapted from Mitalipov *et al.,* 2009).

4.1.1 *Human embryonic stem cells*

Human embryonic stem cells (hESCs) are derived from the ICM of human blastocysts (Thomson *et al.,* 1998). Similar to mouse ESCs, hESCs can be cultured and expanded *in vitro.* hESCs possess analogous properties to ICM cells such as self-renewal and pluripotency, the hallmarks of ESCs. To identify the *in vivo* differentiation ability of hESC lines, teratoma formation in mice is considered to be a stringent method. hESCs when injected into immunodeficient mouse develop teratomas containing derivatives from the three germ layers. Using hESCs in the

teratoma assay demonstrate that hESCs maintained their three germ layer differentiation potential when cultured *in vitro* (Thomson *et al.,* 1998).

Under differentiation conditions, hESCs can generate any of the 200 cell types present in the adult body (Yu *et al.,* 2008). *In vitro* expansion of hESCs as a pluripotent population requires culturing on fibroblast feeder cells and supplementation with fibroblast growth factor 2 (FGF-2).

FGF signalling is critical for hESC self-renewal and is required to maintain hESCs in an undifferentiated state (Eiselleova *et al.,* 2009; Zoumaro-Djayoon *et al.,* 2011). Along with FGF signalling, TGF/Activin/Nodal signalling is essential for hESCs (Beattie *et al.,* 2005; James *et al.,* 2005; Vallier *et al.,* 2005). FGF-2 indirectly acts on fibroblast feeder cells to modulate transforming growth factor b1 (TGFβ1) and activin A signalling, which are growth factors that promote self-renewal along with FGF-2 (Greber *et al.,* 2007). FGF-2 was shown to activate the mitogen-activated protein kinase (MAPK) pathway (Li *et al.,* 2007). Moreover, FGF-2 induces the production of TGFβ and insulin-like growth factor-II (IGF-II) (Bendall *et al.,* 2007). Bendall *et al.,* 2007 also observed that FGF-2 regulates the hESC niche including hESC-derived fibroblast-like cells which support self-renewal of hESCs. Furthermore, FGF-2 signalling regulates NANOG promoter activity which supports hESCs proliferation in an undifferentiated state (Xu *et al.,* 2008). On the contrary, leukemia inhibitory factor (LIF) is required for long-term maintenance of mouse ESCs (Evans *et al.,* 1981; Martin 1981). Thus, it seems that human and mouse ESCs differ. Further proteomic, transcriptomic, epigenetic and cell culture analyses indicated that hESCs are similar to mouse epiblast-derived stem cells (Brons *et al.,* 2007; Tesar *et al.,* 2007).

Similar to the transient pluripotent cells found in an embryo, hESCs express stage-specific markers, embryonic antigens, alkaline phosphatase and telomerase activities, together with genes responsible for maintaining the molecular pluripotency network. A unique core transcriptional regulatory circuit is responsible for the regulation of the pluripotency network in hESCs which comprises of the transcription factors Oct4, Sox2 and Nanog (Chambers *et al.,* 2009; Kashyap *et al.,* 2009; Ng *et al.,* 2011). Polycomb repressive complexes (PRC1 and PRC2) and stem cell-related microRNA are involved with Oct4, Sox2 and Nanog in a complex regulatory mechanism controlling the unique features of ESCs (Kashyap *et al.,* 2009; Ng *et al.,* 2011).

The properties mentioned above make hESCs a promising source for future regenerative applications. By now some hESC-based therapies have reached clinical trials (Goldring *et al.,* 2011). So far results of only some of these clinical trials are published. Schwartz *et al.,* 2012 reported the safe engraftment of hESC-derived retinal pigment epithelium cells into patients suffering from macular degeneration with no signs of hyperproliferation, tumorigenicity, ectopic tissue formation or graft rejection (Schwartz *et al.,* 2012). However, the road ahead for the application of hESCs in regenerative medicine remains long and tortuous. One of the major concerns for the clinical application of hESCs is that transplantation of hESC-derived cells evokes an immune response from the host body (Muller *et al.,* 2009; Tang *et al.,* 2011). This might lead to graft rejection (Muller *et al.,* 2009; Tang *et al.,* 2011). Hence there is the need to develop approaches for the derivation of histocompatible pluripotent stem cells.

4.1.2 Nuclear reprogramming

Development from a pluripotent stem cell to a differentiated cell was for a long time considered as a one-way road. But surprisingly, differentiated cells can be reverted into pluripotent cells using specific conditions. This process is known as nuclear reprogramming. The genetic and epigenetic changes needed for reversing the developmental process can be achieved by two principal approaches: somatic cell nuclear transfer (SCNT) (Wilmut *et al.,* 1997) and induced reprogramming using ectopic gene expression (Takahashi *et al.,* 2006).

SCNT requires removal of the nucleus from an unfertilized oocyte or a recently fertilized zygote and replacement by the nucleus of a somatic cell (Campbell *et al.,* 2007). This technology is successful in a variety of mammals such as mice (Wakayama *et al.,* 1998), cattle (Cibelli *et al.,* 1998; Kato *et al.,* 1998), pigs (Polejaeva *et al.,* 2000), goats (Baguisi *et al.,* 1999), rabbits (Chesne *et al.,* 2002), cats (Shin *et al.,* 2002), mules (Woods *et al.,* 2003), horses (Galli *et al.,* 2003), rats (Zhou *et al.,* 2003), and dogs (Lee *et al.,* 2005). So far it seems that SCNT is not successfully applicable with human cells. The human SCNT embryos were not able to cleave properly; as a result the embryos were arrested at early cleavage stages and were unable to develop into blastocysts (Stojkovic *et al.,* 2005; Cibelli 2007). In 2004 Hwang *et al.* claimed that they successfully produced SCNT-derived hESCs.

However this report was later retracted because of fabrication of data and the SCNT-derived hESCs were later found to be PG in origin (Kim *et al.,* 2007). Recently Noggle *et al.,* 2011 reported that adding the nucleus of a differentiated adult cell into a haploid oocyte allowed the growth of the oocyte till the blastocyst stage which, lead to the derivation of triploid pluripotent stem cells. However, critical limitations are required to overcome for the derivation of diploid hESCs by SCNT (Daley *et al.,* 2011; de Souza 2011).

Murine iPS cells were first derived by reprogramming somatic cells by ectopic expression of four transcription factors: Oct4, Sox2, Myc and Klf4 (Takahashi *et al.,* 2006). Human iPS cells were generated by two independent research groups using a set of different transcription factors: Oct4, Sox2, Myc and Klf4 (Takahashi *et al.,* 2007) or Oct4, Sox2, Nanog and Lin28 (Yu *et al.,* 2007). These human iPS cells express ESC-specific markers, differentiate into multiple lineages *in vitro*, and form teratomas containing cell types of all three germ layers. iPS cells are a potential source of histocompatible patient-specific pluripotent stem cells for future regenerative applications. Clinical trials of human iPS cells in regenerative medicine are at an early stage (Lengner 2010; Vitale *et al.,* 2011). However, many obstacles have to be crossed before iPS cells can be considered to be used as therapeutics. Several analyses indicate that iPS cells are similar to ESCs including differentiation capacity, gene expression and chromatin modification profiles (Puri *et al.,* 2012). But still there are considerable number of reports that point out differences between ESCs and iPS cells. For example, various reports showed differences between hESCs and iPS cells in gene expression (Chin *et al.,* 2009), DNA methylation (Doi *et al.,* 2009) and differentiation potential (Hu *et al.,* 2010). Also, iPS cells maintain epigenetic memories of somatic cells after reprogramming (Barrero *et al.,* 2011; Ohi *et al.,* 2011; Panopoulos *et al.,* 2011). Nevertheless, iPS cells not only provide a tool for researchers to understand the mechanistic basis of diseases, but also offer the possibility of *in vitro* drug testing and deciphering the molecular processes of reprogramming and differentiation (Kiskinis *et al.,* 2010).

4.1.3 *Germline stem cells*

Earlier studies suggested that primordial germ cells derived from midgestation embryos had similar pluripotent characteristics as ESCs (Donovan *et al.,* 2003). Studies in mice (Kanatsu-Shinohara *et al.,* 2004; Guan *et al.,* 2006; Moraveji *et al.,*

2012) and humans (Conrad *et al.,* 2008) showed that pluripotent germline stem cells (GSCs) can be derived from neonatal and adult testis. GSCs in adult testis are suggested to be derived from spermatogonial stem cells (Guan *et al.,* 2006; Conrad *et al.,* 2008). Spermatogonial stem cells can be enriched by culturing them with specific cytokines and extracellular matrix proteins (for example laminin) followed by fluorescence-activated cell sorting with antibodies specific for cell-surface pluripotency markers. Culturing sorted cells in the presence of LIF enables the derivation of pluripotent GSC lines (Guan *et al.,* 2006; Conrad *et al.,* 2008). The generation of GSCs from spermatogonial stem cells probably happens due to the initiation of a reprogramming process caused by the culture conditions (Conrad *et al.,* 2008; Kimura *et al.,* 2011). Similar to ESCs and iPS cells, GSCs can differentiate into tissues of all three germ layers in embryoid bodies and teratoma analyses (Kee *et al.,* 2010). However, further validation of GSCs is needed to evaluate their potential for clinical uses, particularly given that they carry two copies of paternal genome and therefore have only paternal imprint.

4.2 *Pluripotent uniparental stem cells: generation and developmental potential*

Pluripotent uniparental stem cells are diploid and carry copies of either paternal or maternal genomes. Cells, which inherited genetic material only from the maternal side, are called gynogenetic (GG) or PG cells. Conversely, androgenetic (AG) cells carry two copies of the paternal genome (Barton *et al.,* 1984; McGrath *et al.,* 1984).

4.2.1 *Androgenetic ESCs*

AG ESCs are generated from AG blastocyst. AG embryo generation by pronuclear transfer involves the reciprocal exchange of maternal with paternal pronuclei from two zygotes, resulting in embryos with two paternal genomes from different zygotes (McGrath *et al.,* 1983). Mammalian AG embryos cease development at an early embryonic stage (Barton *et al.,* 1984; McGrath *et al.,* 1984). Despite the inability of full development, AG embryos reach the blastocyst stage from which ESCs can be produced (Mann *et al.,* 1990; Eckardt *et al.,* 2007). AG ESCs have been derived from a variety of mammals such as mice (McGrath *et al.,* 1984; Surani *et al.,* 1984; Eckardt *et al.,* 2007) and bovine (Lagutina *et al.,* 2004). However, until now there are no reports on the derivation of human AG ESCs.

4.2.2 *Parthenogenetic ESCs*

Pluripotent uniparental stem cells that contain the maternal genome only can be GG and PG derived. Murine GG embryos are generated by exchanging paternal pronuclei with maternal pronuclei from two zygotes. Conversely, parthenogenetic human embryonic stem cells (hpESCs) are obtained from oocytes that undergo artificial *in vitro* parthenogenesis (Kim *et al.,* 2007; Lin *et al.,* 2007; Mai *et al.,* 2007; Revazova *et al.,* 2007). The development of offspring from an oocyte without male genetic contribution is known as parthenogenesis. The oocyte is activated by exposure to ionomycin that mimics the calcium waves produced by sperm entry during normal fertilization (Nakagawa *et al.,* 2001; Paffoni *et al.,* 2008). The increase in intracellular calcium ionophore simulates the biochemical events caused by sperm penetration that triggers the oocyte into further development (Nakagawa *et al.,* 2001; Paffoni *et al.,* 2008). The extrusion of the polar body at meiotic divisions is prevented by 6-dimethylaminopurine (6-DMAP) leading to the derivation of a blastocyst with two copies of the maternal genome (Brevini *et al.,* 2008; Paffoni *et al.,* 2008). Parthenogenesis occurs naturally in some invertebrate species and in vertebrates like lizards, birds and fish (Lampert *et al.,* 2008; Neaves *et al.,* 2011). Even though mammals are not capable of reproducing by parthenogenesis, PG ESCs can be derived from PG blastocyst originating from artificial activation of oocytes. However in mammals, PG embryos do not develop to adulthood majorly due to the absence of a paternal genome (Kono *et al.,* 2004; Solter 2006). PG ESCs have been isolated from several species such as mouse (Kaufman *et al.,* 1983; Surani *et al.,* 1986; Kim *et al.,* 2007; Eckardt *et al.,* 2011), rabbit (Fang *et al.,* 2006; Wang *et al.,* 2007), pig (Brevini *et al.,* 2010), sheep (Nandedkar *et al.,* 2009), bovine (Pashaiasl *et al.,* 2010), buffalo (Sritanaudomchai *et al.,* 2007) and monkey (Marshall *et al.,* 1998; Cibelli *et al.,* 2002; Vrana *et al.,* 2003; Dighe *et al.,* 2008; Wei *et al.,* 2011). In addition, the derivation of pluripotent human ESCs from PG blastocysts were described (Kim *et al.,* 2007; Lin *et al.,* 2007; Mai *et al.,* 2007; Revazova *et al.,* 2007).

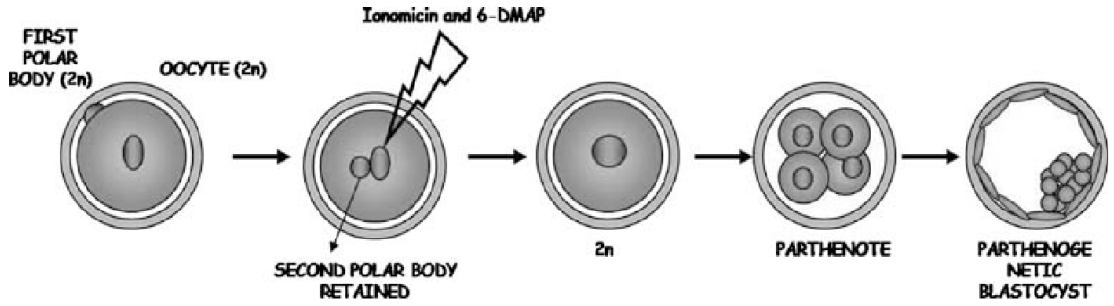


Fig. 2 Schematic representation of in vitro parthenogenetic activation of human oocytes. Human oocytes can be stimulated to divide by ionomycin and 6-DMAP exposure. hpESCs can be derived from the ICM of PG blastocysts (adapted from Brevini *et al.,* 2008).

4.2.3 *Developmental capabilities of uniparental cells*

In general mouse uniparental embryos do not develop past the late somite stages. AG embryos barely grow past early developmental stages, with an impeded growth of the embryo proper and a relatively well developed trophoblast (Barton *et al.,* 1984). On the other hand, PG embryos rarely develop to late somite stages. PG embryos are largely deprived of extraembryonic tissues (Kaufman *et al.,* 1977; Surani *et al.,* 1983). To better analyse the developmental potential of uniparental murine ESCs, chimeras were produced either by aggregating preimplantation stage embryos of biparental (N) with AG or PG cleavage stage blastomeres or by injecting AG or PG ICM cells or ESCs into biparental blastocysts (Nagy *et al.,* 1989; Fundele *et al.,* 1990; Barton *et al.,* 1991). The analysis of chimeras revealed that uniparental ICM cells and ESCs contributed to all tissues of mice, including germline cells (Nagy *et al.,* 1989; Fundele *et al.,* 1990; Barton *et al.,* 1991). However, AG and PG favoured specific differentiation pathways (Nagy *et al.,* 1989; Fundele *et al.,* 1990; Barton *et al.,* 1991). While AG cells were present in mesoderm-derived tissues, PG cells were mostly found in brain and occasionally in tissues derived from mesoderm (Fundele *et al.,* 1989; Nagy *et al.,* 1989; Paldi *et al.,* 1989; Fundele *et al.,* 1990; Barton *et al.,* 1991; Mann *et al.,* 1991). AG chimerism caused severe skeletal defects and often leads to lethality in chimeras (Mann *et al.,* 1990). In contrast, PG chimeras were not lethal. However, cells derived from PG chimeras showed low proliferation rate, shorter cell cycle and increased senescence (Hernandez *et al.,* 2003).

Further analyses of AG and biparental ICM cell chimeras demonstrated that in AG/N chimeras the brain size was smaller and the body was larger. AG cells contribute extensively to hypothalamus and less to the cortex and striatum (Allen *et al.,* 1995;

Keverne *et al.,* 1996). On the contrary, PG and N ICM cell derived chimeric fetal brains were larger in size but the body of the chimeras was smaller. PG cells were found to contribute more to striatum and cortex, and less to the hypothalamus (Allen *et al.,* 1995; Keverne *et al.,* 1996). This biased developmental potential of AG and PG cells might be in part because of the lack or overexpression of imprinted genes, as these genes are preferentially expressed from only one parental allele. Moreover, this also points out towards contrasting impacts of imprinted genes in neural development (Allen *et al.,* 1995; Keverne *et al.,* 1996; Keverne 2001).

Upon *in vitro* differentiation mouse uniparental ESCs are capable to produce derivatives of the three germ layers (Eckardt *et al.,* 2008). Murine AG or PG ESCs can differentiate into engrafting hematopoietic stem cells and into neural stem cells (NSCs) (Eckardt *et al.,* 2007; Lengerke *et al.,* 2007; Dinger *et al.,* 2008; Teramura *et al.,* 2009). Uniparental mouse NSCs were further shown to differentiate into neural subtypes including dopaminergic neurons (Dinger *et al.,* 2008; Teramura *et al.,* 2009; Choi *et al.,* 2010). Moreover, it was shown that the genetic correction strategy is applicable with PG ESCs for diseases associated with dominant alleles. This is possible by deriving PG ESCs from oocytes of a patient containing an allele that is free of the disease (Eckardt *et al.,* 2011).

4.2.4 *hpESCs: features and prospective*

Cibelli *et al.,* 2002 and Vrana *et al.,* 2003 described the derivation of PG ESCs from non-human primates. The differentiation into the three germ layers and long-term engraftment of dopaminergic neurons in rats demonstrated that PG cells can be derived from primates which can further form functional neural cell types (Sanchez-Pernaute *et al.,* 2005). In 2007, four groups independently published reports on the derivation of hpESCs after artificial activation of unfertilized oocytes (Kim *et al.,* 2007; Lin *et al.,* 2007; Mai *et al.,* 2007; Revazova *et al.,* 2007).

The PG activation of an oocyte can be achieved using two different procedures (Rougier *et al.,* 2001). In the first protocol, chemical activation of metaphase-2 oocytes is performed before inhibition of the second polar body extrusion, which results in oocytes with a haploid genome (Lin *et al.,* 2003; Lin *et al.,* 2007; Revazova *et al.,* 2008). The oocyte becomes diploid by spontaneous duplication of the genome by a phenomenon known as diploidization (Ito *et al.,* 1991; Kim *et al.,* 2007). Diploidy is maintained after the sister chromatids are segregated, which subsequently leads

to the formation of homozygous parthenotes (Fig. 3b). However, crossing over takes place during the prophase of the first meiotic division and the heterozygosity depends on the level of crossing over (Rougier *et al.,* 2001). In the second method, the first polar body extrusion is inhibited, which results in a tetraploid oocyte (Kubiak *et al.,* 1991; Kim *et al.,* 2007). Diploidy is restored by exclusion of the second polar body at the end of oocyte maturation (Fig. 3C). Homologous chromosomes are not segregated when the first polar body extrusion is inhibited. The segregation of sister chromatids occurs after the exclusion of the second polar body. This leads to the derivation of a parthenote with genetic make-up identical to the oocyte (Kubiak *et al.,* 1991; Kim *et al.,* 2007; Brevini *et al.,* 2008). Together this indicates that the genetic constitution of PG embryos depends on the method employed for its activation (Fig. 3) (Kim *et al.,* 2007; Brevini *et al.,* 2008).

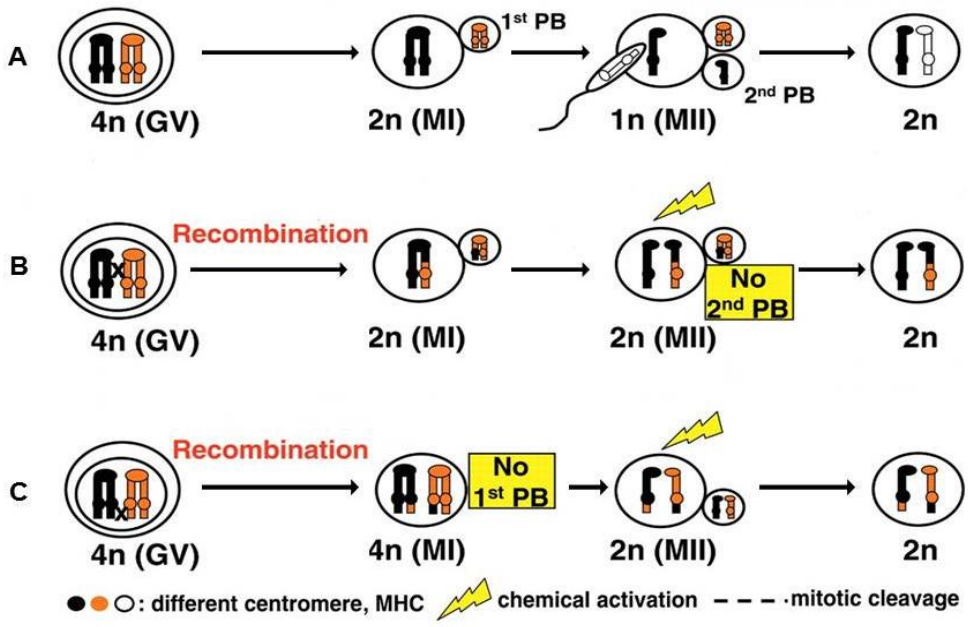


Fig. 3 Schematic representation of chromosomal changes occurring during normal or artificially induced oocyte maturation. **(A)** Genetic composition of *in vitro* fertilized embryos. **(B)** Homozygous parthenotes are formed by extrusion of the second polar body. Diploid status is reached by spontaneous duplication of the haploid genome (diploidization). **(C) The** first polar body is inhibited so extrusion of homologous chromosomes does not take place when activation is carried out. Diploidy is reached after the extrusion of the second polar body (adapted from Kim *et al.,* 2007).

One of the major concerns before the application of hESCs in clinical studies is the immune response after transplantation into genetically unrelated recipients (Dawson *et al.,* 2003; Ginis *et al.,* 2003). The activation of the host's immune system which leads to graft rejection is triggered by alloantigens or antigenic proteins present on the graft such as blood group antigens (ABO) and the major histocompatibility complex (MHC) proteins (known as human leukocyte antigens (HLA) in humans) (Tang *et al.,* 2011). Matching donor and host HLA types is necessary to avoid a cytotoxic T-cell response in the host after transplantation (Drukker 2004; Utermohlen *et al.,* 2009). But due to extensive polymorphism in HLA proteins the chance to find a donor-host HLA match is low (Mitalipov *et al.,* 2009; Tang *et al.,* 2011). Therefore, homozygosity in parthenotes represents an alternative strategy for deriving histocompatible pluripotent cells which reduces the immunogenicity of ESC-derived progeny. Pluripotent HLA homozygous hpESC lines have been derived from human oocytes (Kim *et al.,* 2007; Lin *et al.,* 2007; Revazova *et al.,* 2008). Generation of hpESC lines that are homozygous for all three sets of HLA (A, B and DR) will increase the number of recipients a graft can match. These hpESC lines can be used for establishing HLA histocompatible ESC banks (Nakajima *et al.,* 2007). Therefore, hpESCs are suggested to have a potential application in cell replacement therapies. Moreover, homozygous hpESCs were proposed to be advantageous for selecting cell lines with drug response, disease or cancer genes (Eckardt *et al.,* 2011). Additionally, *in vitro* hpESCs were shown to generate various cell lineages for example, mesenchymal stem cells, hepatocytes, pancreatic endocrine cells, retinal pigmented epithelial and neural progenitor cells (Harness *et al.,* 2011; Chen *et al.,* 2012; Isaev *et al.,* 2012; Turovets *et al.,* 2012). On the other hand, homozygosity can cause amplification of an undesirable genetic component due to the loss of heterozygosity (Tang *et al.,* 2011).

4.3 *Neural stem cells: properties and derivation from various sources*

Neural stem cells (NSCs) represent a self-renewable and multipotent adult stem cell type existing in the embryonic and adult mammalian central and peripheral nervous system (Kokovay *et al.,* 2008; Goritz *et al.,* 2012). In the developing brain various types of neuronal and glial cells originate from NSCs *via* the formation of intermediate precursors. Although less frequent, NSCs have regenerative

capabilities in the postnatal and adult brain where they generate neurons and glia (Ma *et al.,* 2009; Goritz *et al.,* 2012). Neurogenesis in vertebrate central nervous system starts with the induction of neuroectoderm and leads to the formation of a neural plate. Then the neural tube is formed with the invagination of the neural plate. These structures are made up by neuroepithelial progenitor cells (Gotz *et al.,* 2005). During the maturation of the neural tube NSCs are present in the ventricular layer, closest to the lumen (Gotz *et al.,* 2005; Goritz *et al.,* 2012).

In the adult mammalian brain, NSCs are present in two distinct regions, the dentate gyrus (DG) of the hippocampus (Eriksson *et al.,* 1998) and the subventricular zone (SVZ) of the lateral ventricular wall (Johansson *et al.,* 1999). NSCs exist in two developmentally different niches that maintain the self-renewal and regulate fate-committed asymmetrical division of NSCs (Miller *et al.,* 2009; Fuentealba *et al.,* 2012).

NSCs can be isolated from the human embryonic and adult brains and cultured *in vitro* in defined media (Temple 2001). NSCs show self-renewal and multipotent characteristics in culture. Additionally, NSCs can be generated following *in vitro* differentiation of pluripotent hESCs similar to NSCs isolated from the human embryonic and adult brains.

In vitro neural differentiation of pluripotent stem cells mimics the stages observed in normal embryonic brain development (Nishikawa *et al.,* 2007). By using various differentiation protocols numerous groups reported the generation of a range of distinct neural precursor populations that are similar to the precursors present during various stages of a developing brain. For instance, NSCs generated by Tropepe *et al.,* 2001; Elkabetz *et al.,* 2008; Chambers *et al.,* 2009 show the molecular characteristics of neuroepithelial progenitors while NSCs derived by Koch *et al.,* 2009 resembles cells from the neural tube. Moreover, NSCs described by Bibel *et al*. 2007 and Conti *et al*. 2005 were similar to fetal brain progenitor cells and adult brain NSCs respectively.

There are three common approaches to promote neural induction of pluripotent stem cells (Dhara *et al.,* 2008; Denham *et al.,* 2009; Conti *et al.,* 2010). The first is culturing cells in suspension to form aggregates of differentiated cells, called embryoid bodies, which in defined medium generate NSCs. The second procedure is to co-culture pluripotent stem cells on a feeder layer of stromal cells, such as PA6 or MS5 cell lines which induce neural differentiation as the factors required are

secreted by the feeder cells. Until now, the only approach that has been shown to induce neural differentiation in a defined manner is by directly inhibiting the BMP and/or SMAD signaling pathways. Using these neural differentiation approaches, various reports have shown that PG ESCs from different mammalian species including human are capable of differentiating into various neuroectodermal cell types, such as retinal pigment epithelium and NSCs (Harness *et al.,* 2011; Isaev *et al.,* 2012).

4.4 *Imprinting: mechanism and influence*

The majority of the mammalian genes are expressed from both the parentally inherited copies of the chromosomes. However, for some genes only one copy is expressed, depending on the parent of origin. These genes are termed imprinted genes, and the phenomenon is referred to as genomic imprinting (Surani 1994; Solter 1998). Imprinting is a temporal- and spatial-specific phenomenon. Imprinted genes are regulated by epigenetic mechanisms (Ferguson-Smith 2011). The term ‘epigenetic’ refers to heritable changes in gene expression that are not accompanied by alteration of the DNA sequence (Wolffe *et al.,* 1999). Epigenetic marks include changes in DNA methylation, covalent modifications of histone proteins causing conformational changes of the chromatin, and noncoding RNA (Smith *et al.,* 2007). Various combinations of epigenetic modifications are necessary for imprinted gene regulation. In mammalian cells, DNA methylation of CpG dinucleotides is associated with a transcriptionally repressed state (Bird 2002). CpG dinucleotides are often found as CG-rich sequences called CpG-islands. CpG-islands are mostly differentially methylated at the promoters of imprinted genes, where the repressed allele is methylated and the active allele is unmethylated. These regions are known as differentially methylated regions (DMRs) (Pfeifer 2000; Ferguson-Smith *et al.,* 2001). Up till now, nearly 145 imprinted genes have been described in mice and approximately 80 in humans (catalogue of imprinting effects: http://igc.otago.ac.nz and the medical research council harwell genomic imprinting homepage: http://www.mousebook.org). Although isolated single imprinted genes have been identified in the human genome, a peculiar feature of imprinted genes is that they are frequently clustered in the genome (Spahn *et al.,* 2003; Verona *et al.,* 2003). Almost 80% of the imprinted genes are clustered in various 1-5Kb sized chromosomal domains (Bartolomei *et al.,* 2011; Ferguson-Smith 2011). Genes in clusters are

regulated by an element called imprinting control region (ICR), which is composed of DMRs (Pfeifer 2000; Spahn *et al.,* 2003). ICRs regulate imprinted gene expression and epigenetic marks at many genes in a cluster, even those which are located several mega bases away (Verona *et al.,* 2003; Lewis *et al.,* 2006).

Another characteristic of the clusters is that they mostly express noncoding RNAs (ncRNAs), which are expressed from the maternal allele (O'Neill 2005). The mechanism of regulation of imprinted clusters over long distances is currently explained by two models (Pauler *et al.,* 2006; Wan *et al.,* 2008). The first model involves ncRNA-mediated silencing of protein-coding genes in the same cluster (Peters *et al.,* 2008). The maternal allele lacks ICR methylation therefore ncRNAs are expressed, which repress the genes present in the cluster; while genes are expressed from the paternal allele as the ncRNAs is repressed by the methylation of ICR. This model is demonstrated in the case of *Kcnq1* and *Igf2r* ICRs (Pauler *et al.,* 2006; Peters *et al.,* 2008; Mohammad *et al.,* 2009). Another model involves the insulation of genes on one side of the ICR by CTCF (CCCTC binding insulator protein)-mediated blocking of enhancer elements as exemplified in the case of the *H19*/*Igf2* locus (Thorvaldsen *et al.,* 1998; Reik *et al.,* 2000). There are several imprinted genes that demonstrate tissue- or cell- type specific imprinting (Hudson *et al.,* 2011; Fedoriw *et al.,* 2012). Placenta and brain are major sites for region-specific imprinting (Hudson *et al.,* 2011; Fedoriw *et al.,* 2012). For example, *Igf2* is maternally silenced, except in the choroid plexus and leptomeninges of the brain where it is biallelically expressed (DeChiara *et al.,* 1991). Another tissue type-specific imprinted gene, delta-like homologue 1 (*Dlk1*), shows absence of imprinting in the postnatal neurogenic niche whereas it is maternally silenced in all the other tissue types (Ferron *et al.,* 2011). Ubiquitin protein ligase E3A (*Ube3a*) also shows tissue type-specific imprinting: it is paternally imprinted only in the human brain (Rougeulle *et al.,* 1997; Vu *et al.,* 1997), where it displays neuron-specific imprinting (Yamasaki *et al.,* 2003).

4.4.1 *Role of imprinting in the brain*

In a developing embryo imprinted gene are involved in growth and development, mainly in neurodevelopment (Keverne 2001; Wilkinson *et al.,* 2007). Studies on chimeras obtained from AG or PG ESCs mixed with N embryos revealed that AG and PG cells localised to distinct regions of the brain. This provided evidence of

distinct roles of imprinted genes in the development of particular cell lineages in the brain (Keverne *et al.,* 1996). Brain-specific imprinted genes interact with molecules of different signalling pathways that control the survival, patterning and differentiation of neural cells (Wilkinson *et al.,* 2007). Imprinted genes also affect adult behavior for example the imprinted Grb10 gene regulates social behavior (Curley 2011; Garfield *et al.,* 2011). Aberrations in imprinted gene function in human brain also lead to imprinting-related neurological disorders (Wilkins *et al.,* 2011). Imprinting-related growth disorder Beckwith–Wiedemann is caused by abnormalities of the imprinted genes *Igf2 and Kcnq1ot11, H19, Cdkn1c* and *Kcnq1* located on human chromosome 11p15 (Weksberg *et al.,* 2003; Abu-Amero *et al.,* 2008). Prader–Willi and Angelman syndromes are also imprinting-related behavioural and neurodevelopmental disorders. These syndromes are caused by loss of function of imprinted genes Ube3a, Snrpn and Necdin situated on human chromosome 15q11-13 (Horsthemke *et al.,* 2006).

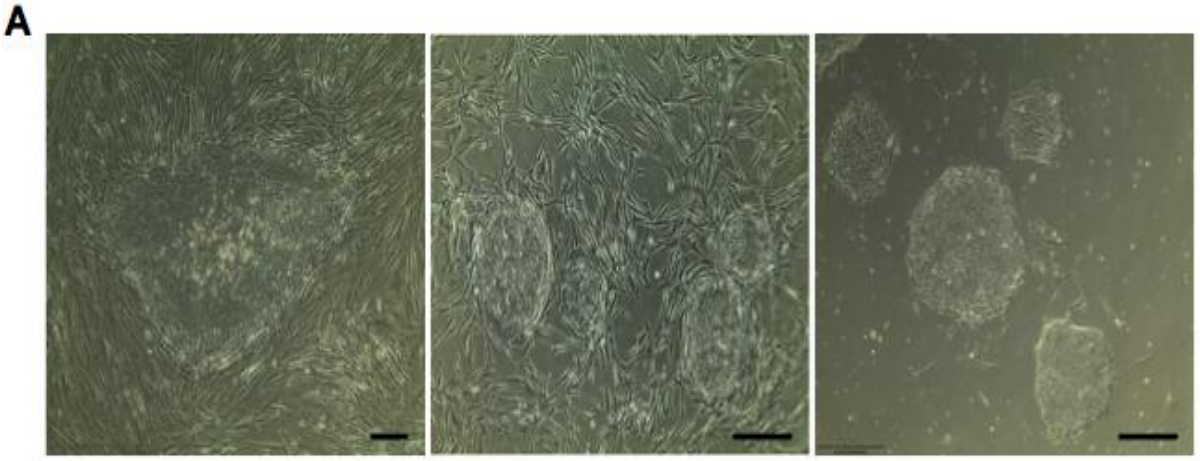
4.5 *Aim and strategy*

The preferential localization of murine PG and AG ICM cells to distinct brain regions in chimeras (Keverne *et al.,* 1996) Indicates distinct roles of the maternal and paternal genomes on brain development and function (Keverne 2001; Wilkinson *et al.,* 2007; Curley 2011). Together with reports of centrosome amplification and chromosomal instability in PG cell lines derived from mammals including humans (Brevini *et al.,* 2012), this could indicate a restricted neurogenic competency of hpESCs. The differentiation potential of hpESCs, particularly their potential for neurogenesis and differentiation into functional neuronal subtypes, is currently not analysed in detail. Therefore here I investigated the *in vitro* differentiation capability of hpESCs into NSCs and further into neural subtypes including midbrain TH-positive neurons and motoneurons. In parallel I examined the functional aspects and the imprinting status of hpESCs and hpNSCs.

5. RESULTS

5.1 *Optimization of hpESCs culture conditions*

hpESCs (cell lines: LLC6P, LLC9P) were grown on mitomycin C-inactivated human foreskin fibroblast (HFF) or feeder free on matrigel (Fig. 4A). hpESCs were reported to have an improved propagation upon addition of umbilical cord blood serum (UBS) to the culture medium (Revazova *et al.,* 2007). Furthermore, it was reported that hypoxic culturing allows long-term propagation of hESCs without spontaneous differentiation (Prasad *et al.,* 2009; Lim *et al.,* 2011). To optimise culture conditions, hpESCs were propagated under 4% or 20 % oxygen and in different serum conditions. The following conditions were tested: 10% knockout serum replacement (KSR) plus 10% UBS, 10% KSR plus 5% UBS and 20% KSR, respectively. hpESCs propagated under different serum and oxygen concentrations showed minor variations in cell numbers (Fig. 4B). hpESCs grown under 4% oxygen and 20% KSR or under 20% oxygen and 20% KSR stained positive for alkaline phosphatase (AP) activity (Fig. 4C). In addition, hpESCs homogenously expressed pluripotency marker *Oct4* and *Sox2* and showed absence of expression of the differentiation marker *SSEA1* (stage specific embryonic antigen 1) (Lin *et al.,* 2007; Mai *et al.,* 2007; Revazova *et al.,* 2007). All together this showed that hpESCs could be propagated in KSR under normal oxygen concentrations with no major change in cell numbers, or AP activity cells propagated with KSR and under 20% oxygen expressed pluripotency markers and lacked expression of differentiation associated marker.



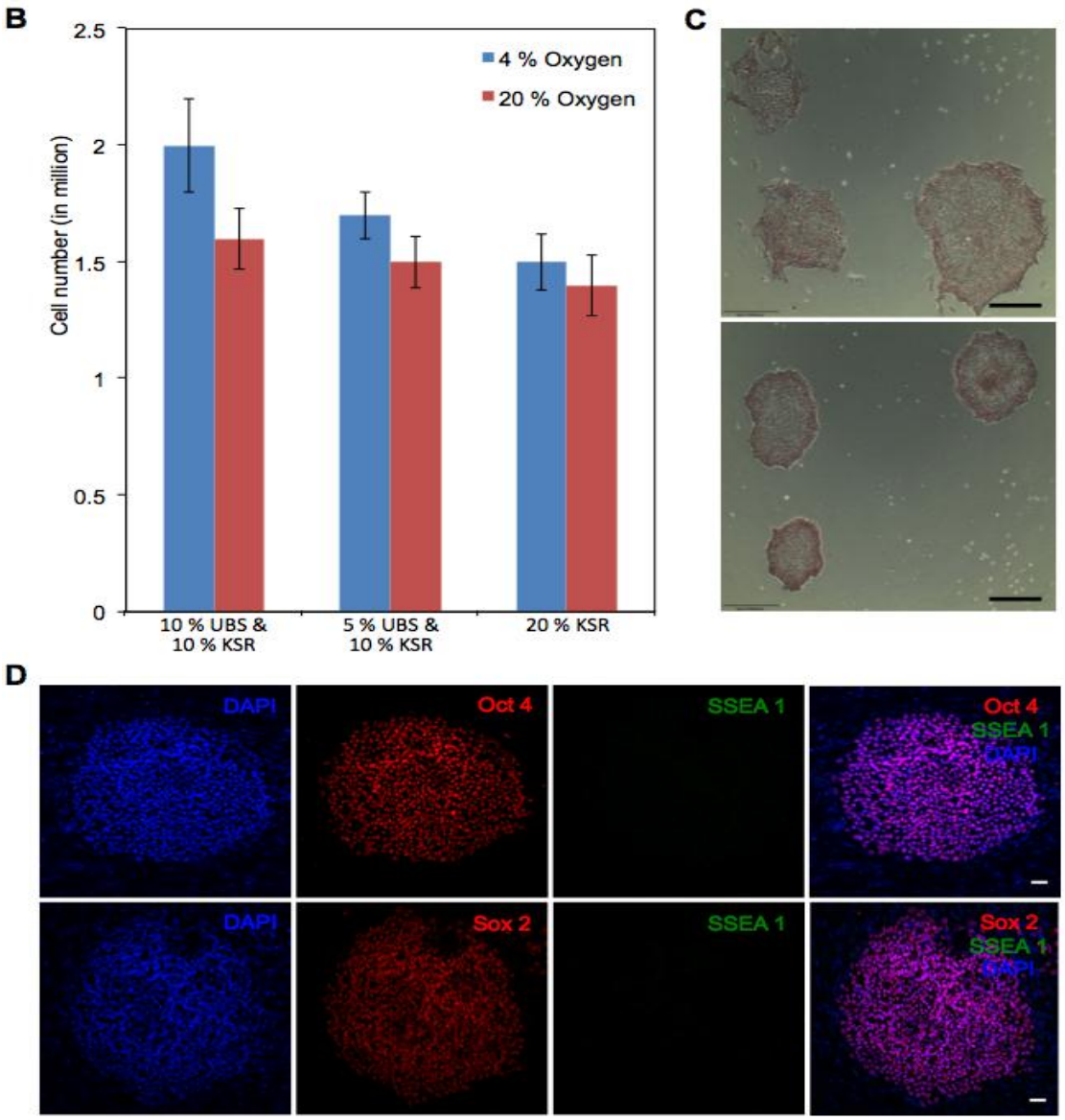


Fig. 4 Optimization of hpESCs culture conditions. **(A)** Shown are phase-contrast images of hpESC cell lines LLC6P (left), LLC9P (middle). Cells grown on mitomycin C-inactivated HFF or matrigel (right, cell lines LLC9P). **(B)** Graph represents cell counts of hpESCs which were propagated under 4% or 20 % oxygen conditions as well as on 10% KSR plus 10% UBS, 10% KSR plus 5% UBS or 20% KSR. **(C)** Images of alkaline phosphatase staining of hpESCs grown under 4% oxygen and 20% KSR (top image) or 20% oxygen and 20% KSR (lower image) are shown. **(D)** Confocal images of hpESCs (LLC9P) co-immunostained with *Oct4*- and *SSEA1*-specific antibodies. Nuclei were co-stained with DAPI. Scale bars: 50 µm; n=3.

5.2 *hpESC-derived teratoma in NOD-SCID mice*

To assess the ability of hpESCs (LLC9P) to form derivatives of the three germ layers, teratomas were generated by injection of hpESCs into a cryolesioned brain of immunodeficient NOD-SCID mice. Transplantations were performed one week after

lesion induction to avoid excessive loss of the transplanted cells due to acute post-injury inflammation (Siren *et al.,* 2006). Two recipients, each were transplanted per dose with $2x10^4$, $5x10^4$ or $10x10^4$ cells. Transplantation of undifferentiated hpESCs resulted in the formation of teratomas three months after injection in 2 out of 6 transplanted brains. Animals transplanted with $5x10^4$ or $10x10^4$ cells developed teratomas. Hematoxylin and eosin staining of transplanted brain sections demonstrated the presence of organized structures, including smooth muscle, cartilage, and blood representing mesodermal germ layer and ciliated epithelium, respiratory epithelium, as well as gastrointestinal lining cells of endodermal origin (Fig. 5A). Donor origin of teratomas was verified by positive immunostaining of teratoma with human nuclei-specific antibody (HNu) (Fig. 5B). This data showed that hpESCs are capable of forming teratomas that comprises cells of various cell types.

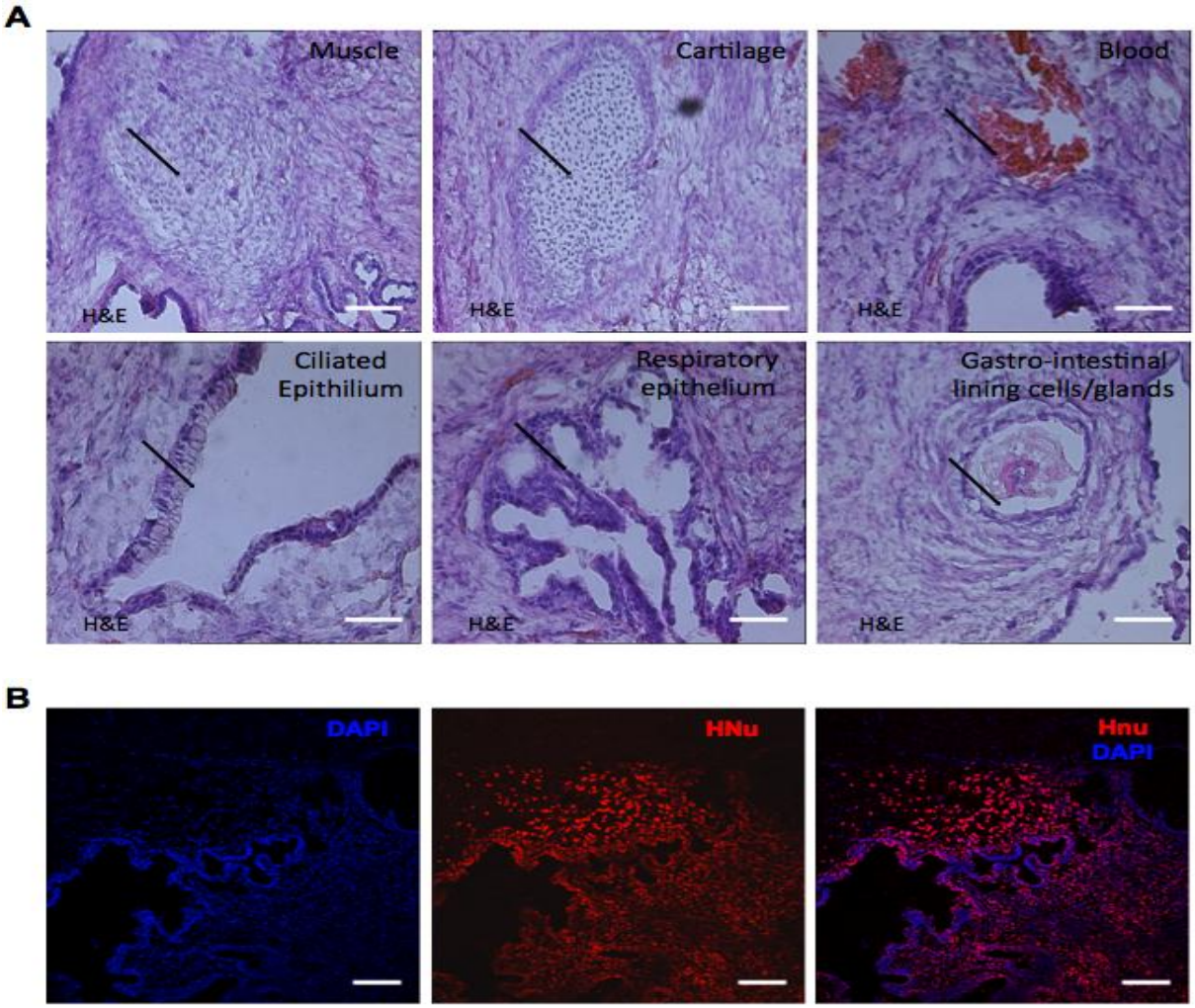


Fig. 5 hpESC-derived teratoma. **(A)** Depicted are bright field images of haematoxylin and eosin stainings of teratomas derived from transplantations of

hpESCs into brain-injured NOD-SCID mice. On the basis of morphology we observed muscle (upper left), cartilage (upper middle), blood (upper right), ciliated epithelium (lower left), respiratory epithelium (lower middle) and gastro-intestinal lining cells (arrows indicate the cell types). Scale bars: 100 µm **(B)** Representative confocal images of hpESC-derived teratomas immunostained for human nuclei-specific antibody (HNu) and DAPI. Scale bars: 100 µm; n=2.

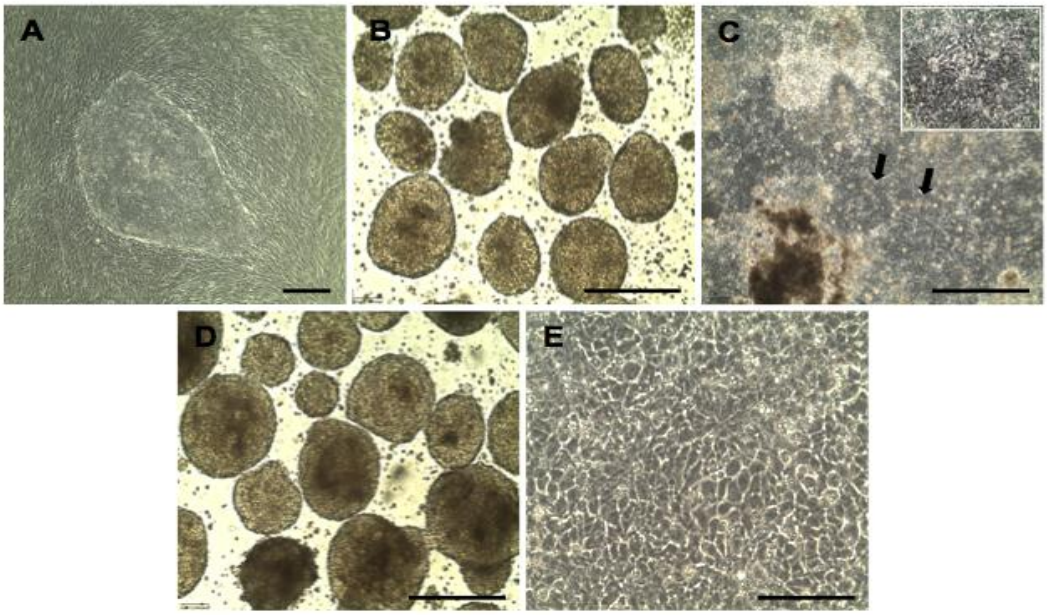
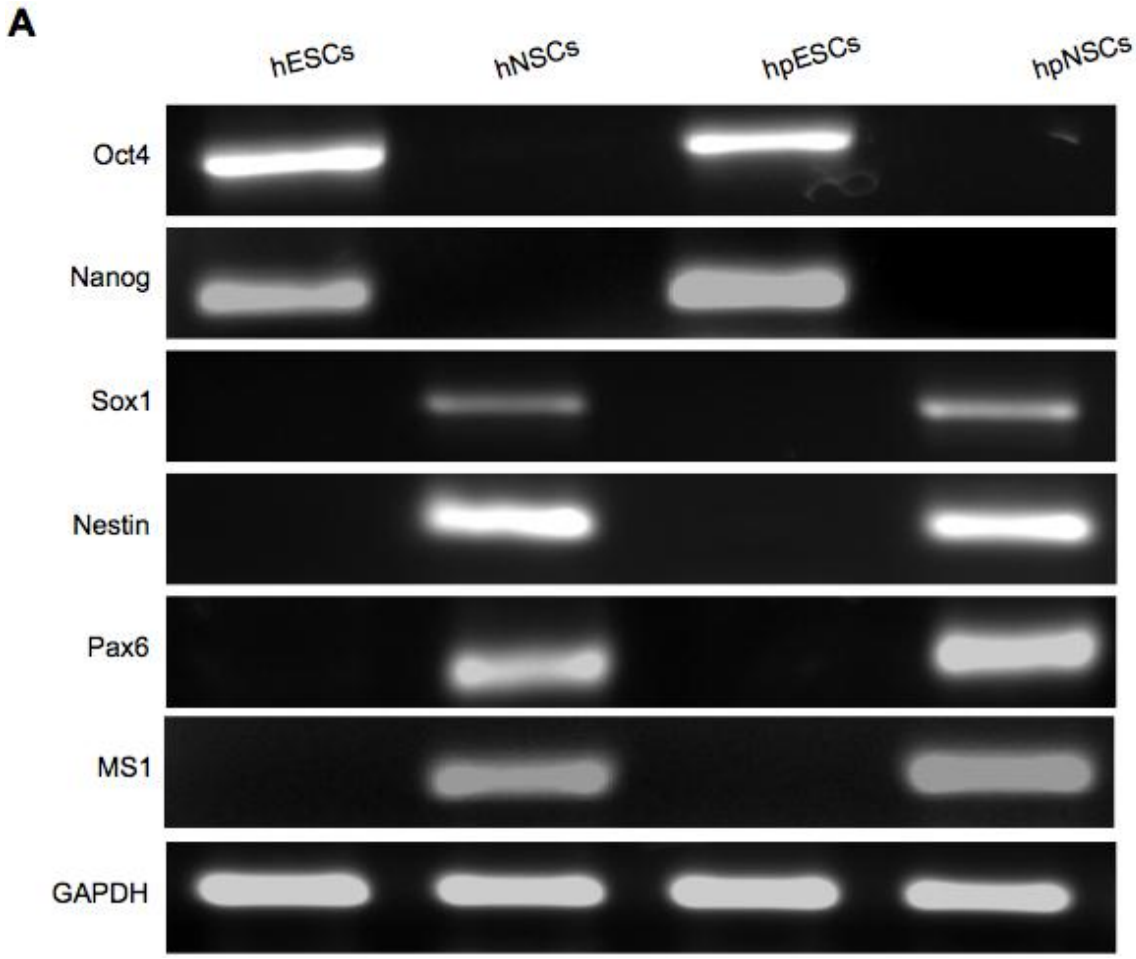


Fig. 6 hpNSCs generated from hpESCs (cell line LLC9P). Images illustrate critical stages of *in vitro* neural differentiation of hpESCs towards hpNSCs. Starting from hpESCs grown on HFF **(A)**, to hpESC-derived floating embryoid bodies **(B),** followed by attached embryoid bodies (**C**, arrows indicate a rosette-like pattern) then to floating neurospheres **(D),** and finally to hpNSCs **(E)**. Scale bars: left panel: 0.5 mm; other panels: 0.25 mm; n=3.

5.3 *Neural differentiation of hpESCs*

To evaluate the *in vitro* neural differentiation potential, hpESCs (LLC9P and LLC6P) were neuralized using a multi-step protocol that induces the differentiation of hESCs into NSCs (Koch *et al.,* 2009). Upon differentiation of hpESCs neural rosettes appeared after attaching 4-day-old embryoid bodies on polyornithine/laminin-coated dishes (Fig. 6A&B). 10 to 12 days later rosettes formed 3-dimensional structures (Fig. 6C). These structures were detached from the culture dishes and grown as floating neurospheres for 1 to 2 days (Fig. 6D). Next, neurospheres were dissociated to form single cells and plated as a monolayer under defined culture conditions on polyornithine/laminin-coated dishes (Fig. 6E). These conditions yielded NSC-like cells with homogeneous morphology. The NSC identity was confirmed by gene expression analysis, that revealed up-regulation of NSC markers *Sox1*, *Nestin*,

Pax6, and *Musashi1*, loss of expression of the pluripotency markers *Oct4* or *Nanog* (Fig. 7A) and absence of activation of the markers for non-neural lineages like neural crest (*Snai2, FoxD3)* and mesoderm *(Acta1)* (Fig. 7B). Further hpESC-derived neural stem cells (hpNSCs) were stained positive for NSC markers *Nestin, Sox1, Sox2* and *Vimentin* (Fig. 8). The NSC marker expression in hpNSCs was ubiquitous and not limited to subsets of cells. Similar results were obtained with hpESC cell line LLC6P (Fig. 9). Upon differentiation, two 10 cm^2 culture dishes of LLC9P hpESCs yielded a mean of 29 (±3.5) million hpNSCs whereas, LLC6P hpESCs generated 11.8 (±1.7) million cells. Together these results showed that hpESCs can differentiate into hpNSCs that expressed NSC-specific markers and lacked gene expression of pluripotency and non-neural lineage markers.



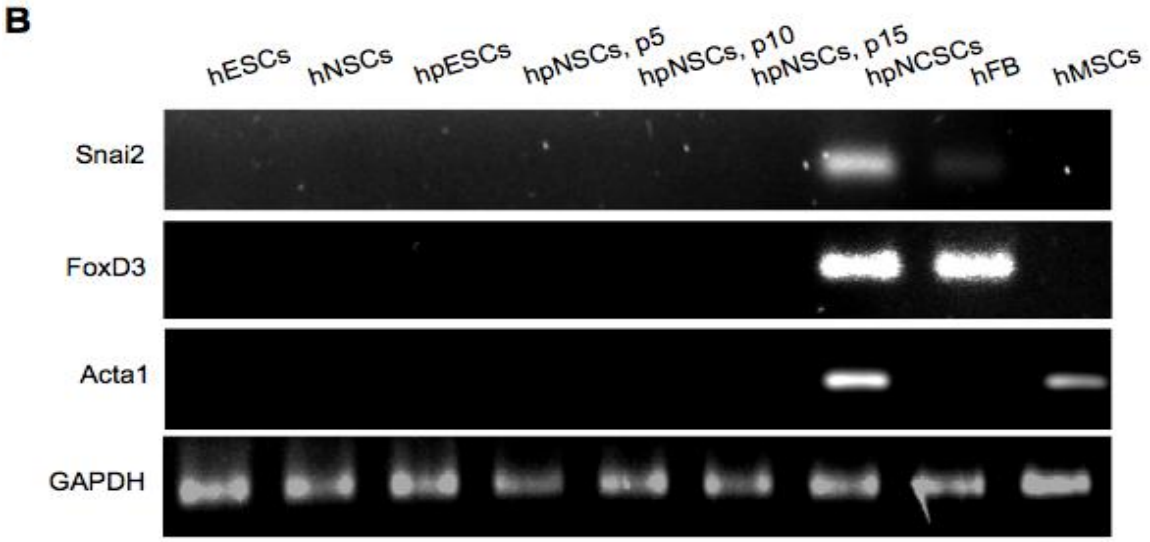
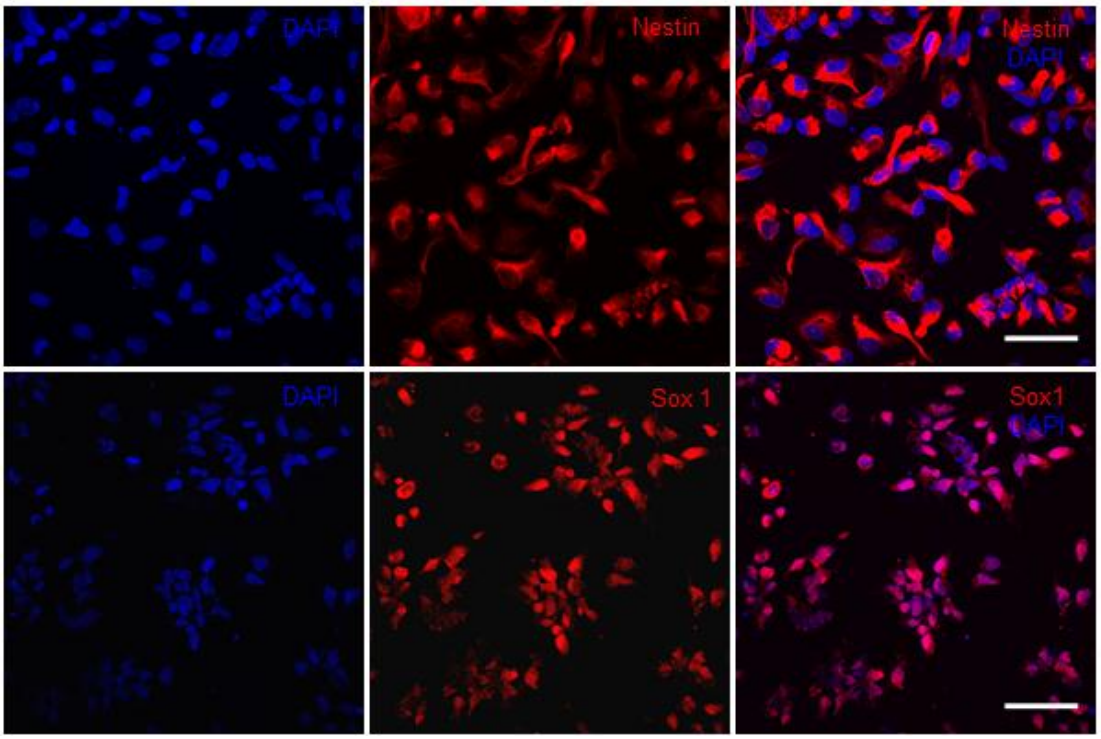


Fig. 7 Expression analyses of pluripotency, NSC and neural crest cell markers (cell line LLC9P). **(A)** Representative semi-quantitative RT-PCR analyses for the expression of pluripotency markers *Oct4, Nanog* and neural stem cell markers *Sox1*, *Nestin*, *Pax6* and *Mushashi1* (*MS1*) in hESCs, hNSCs, hpESCs, and hpNSCs are shown. GAPDH is the house-keeping control. **(B)** Shown are expression analyses of neural crest cell markers *Snai2* and *FoxD3* and mesodermal marker *Acta1* in hESCs, hNSCs, hpESCs, and hpNSCs at passages 5, 10 and 15 by RT-PCR. Human PG neural crest stem cells (hpNCSCs), human fetal brain (hFB) and human adipose tissue-derived mesenchymal stromal cells (hMSCs) are shown as controls.



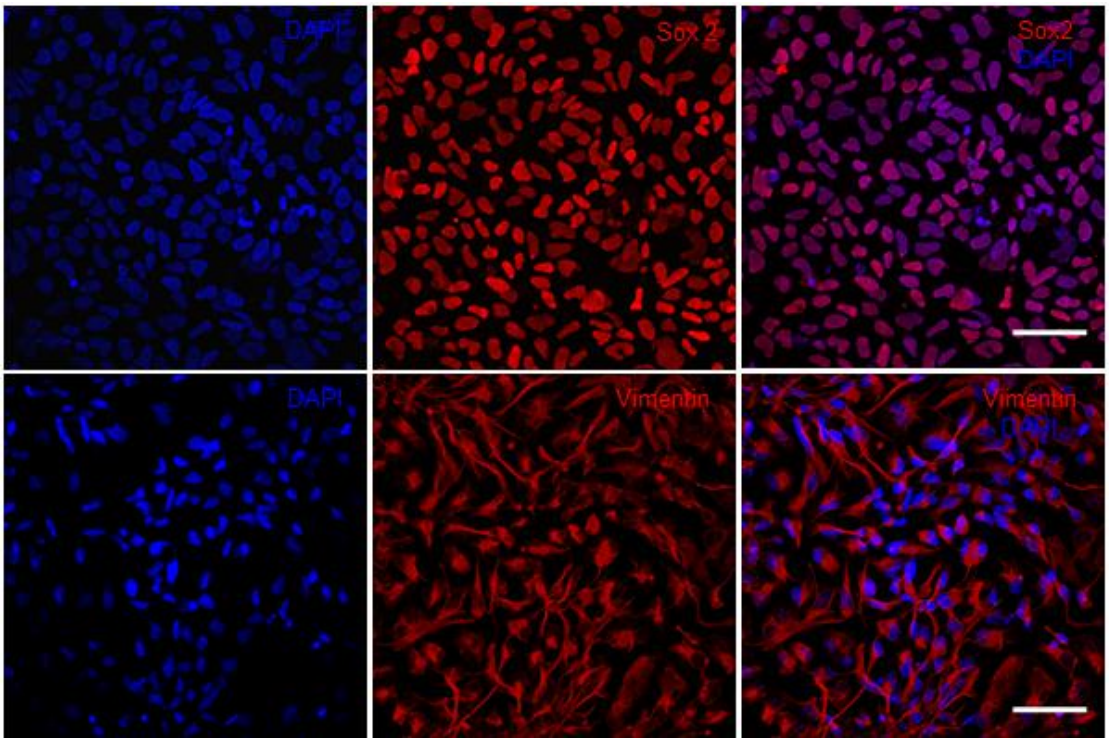
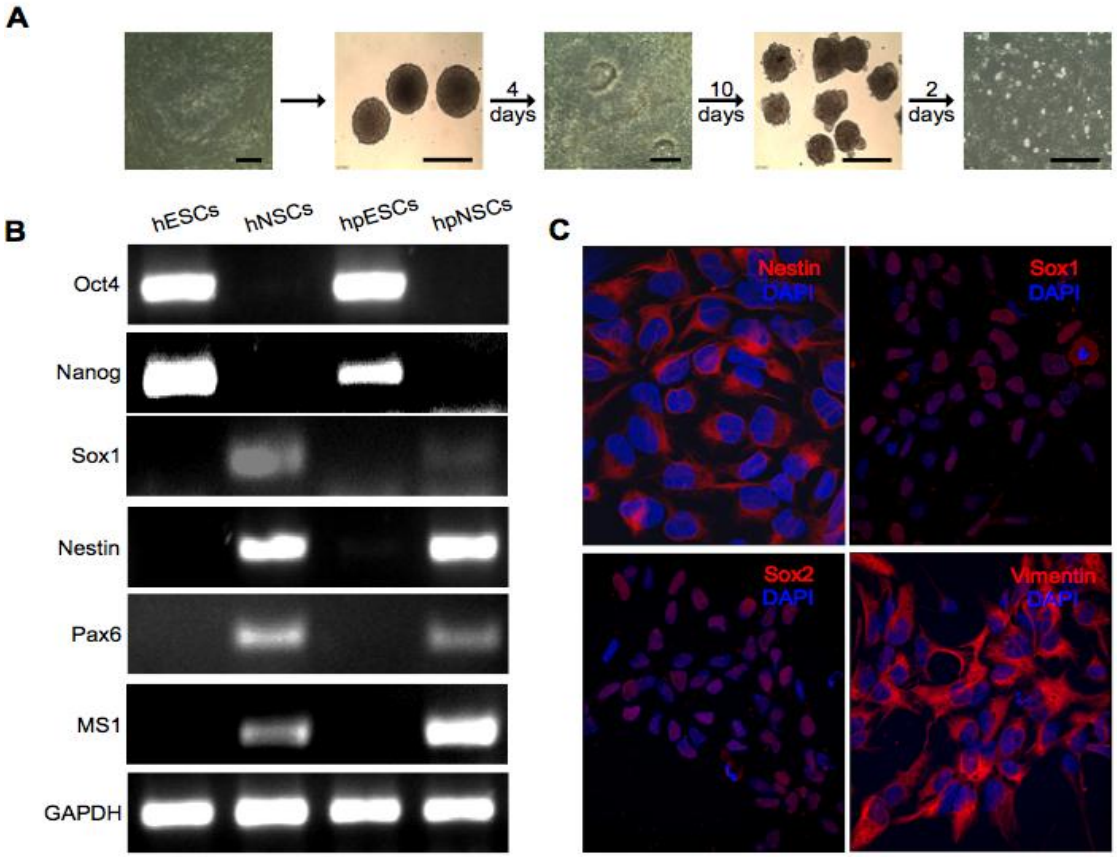


Fig. 8 Immunophenotypic characterization of hpNSCs (cell line LLC9P). Shown are representative confocal images of hpNSCs immunostained for neural stem cell markers *Nestin*, *Sox1*, *Sox2*, and *Vimentin*. Cells were co-stained with DAPI. Scale bars: 50 µm; n=3.



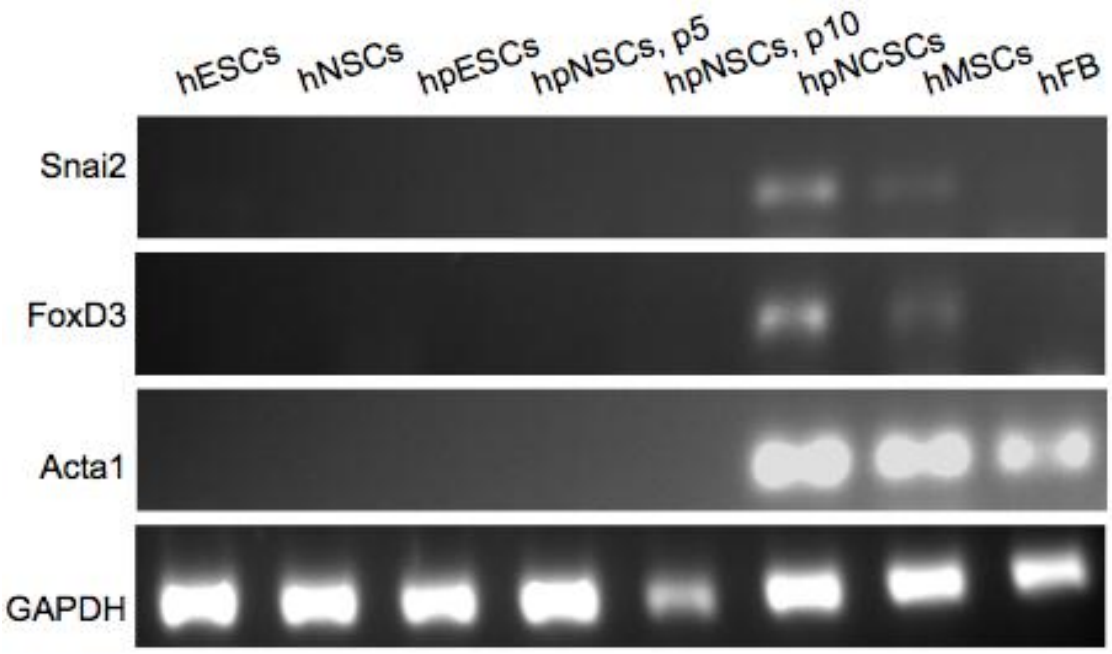


Fig. 9 hpESC-derived hpNSCs from LLC6P cell line. **(A)** Images depict stages of *in vitro* neural differentiation of hpESC towards hpNSCs. Scale bars, left panel: 0.5 mm; other panels: 0.25 mm. **(B)** Shown are representative RT-PCR analyses for the expression of *Oct4*, *Nanog*, S*ox1*, *Nestin*, *Pax6* and *MS1* in hESCs, hNSCs, hpESCs and hpNSCs. Also represented are expression analyses of neural crest cell markers *Snai2* and *FoxD3* and the mesodermal marker *Acta1* in hESCs, hNSCs, hpESCs, and hpNSCs (passages 5, 10). hpNCSCs, hFB and hMSCs are shown as controls. **(C)** Images exhibit immunostaining of hpNSC cultures with antibodies specific for *Nestin*, *Sox1*, *Sox2*, and *Vimentin*. Images were taken by confocal microscopy. Scale bars: 50 µm; n=3.

5.4 *Expression of mitotic checkpoint and extracellular matrix genes in hpESCs*

A recent report demonstrated abnormal levels of gene expression of molecules related to spindle formation in hpESCs compared to hESCs (Brevini *et al*., 2009). Particularly *MAD1*, *MAX* and *SIN3* showed higher levels of gene expression, indicating deregulation in the MAD1-dependent pathway. Further, low transcription levels of *CENP-E*, *TTK* and *Aurora A kinase*, may point towards aberration at different spindle check points (Brevini *et al*., 2009). Together, this may indicate that alteration of proliferation mechanisms occurs in hpESCs, probably related to the uniparental origin (Brevini *et al*., 2009). Therefore, the expression levels of specific mitotic spindle check-point proteins was assessed, in PG (LLC6P and LLC9P) compared to conventional ESCs (I3 and H9). As shown in (Fig 10A) variation in gene expression level was detected. This dissimilarity was apparent not only between

hpESCs and hESCs cells but also between individual ESC lines independent of PG or N origin.

In addition, differences in differentiation potential between hpESCs and hESCs were reported. This might be due to reduced levels of extracellular matrix (ECM) transcripts in hpESCs (Harness *et al*., 2011). A comparison of expression levels of the ECM transcripts in hpESCs and hESCs showed that, LLC6P hpESCs had lower ECM gene expression compared to LLC9P cells and hESCs (Fig. 10B).

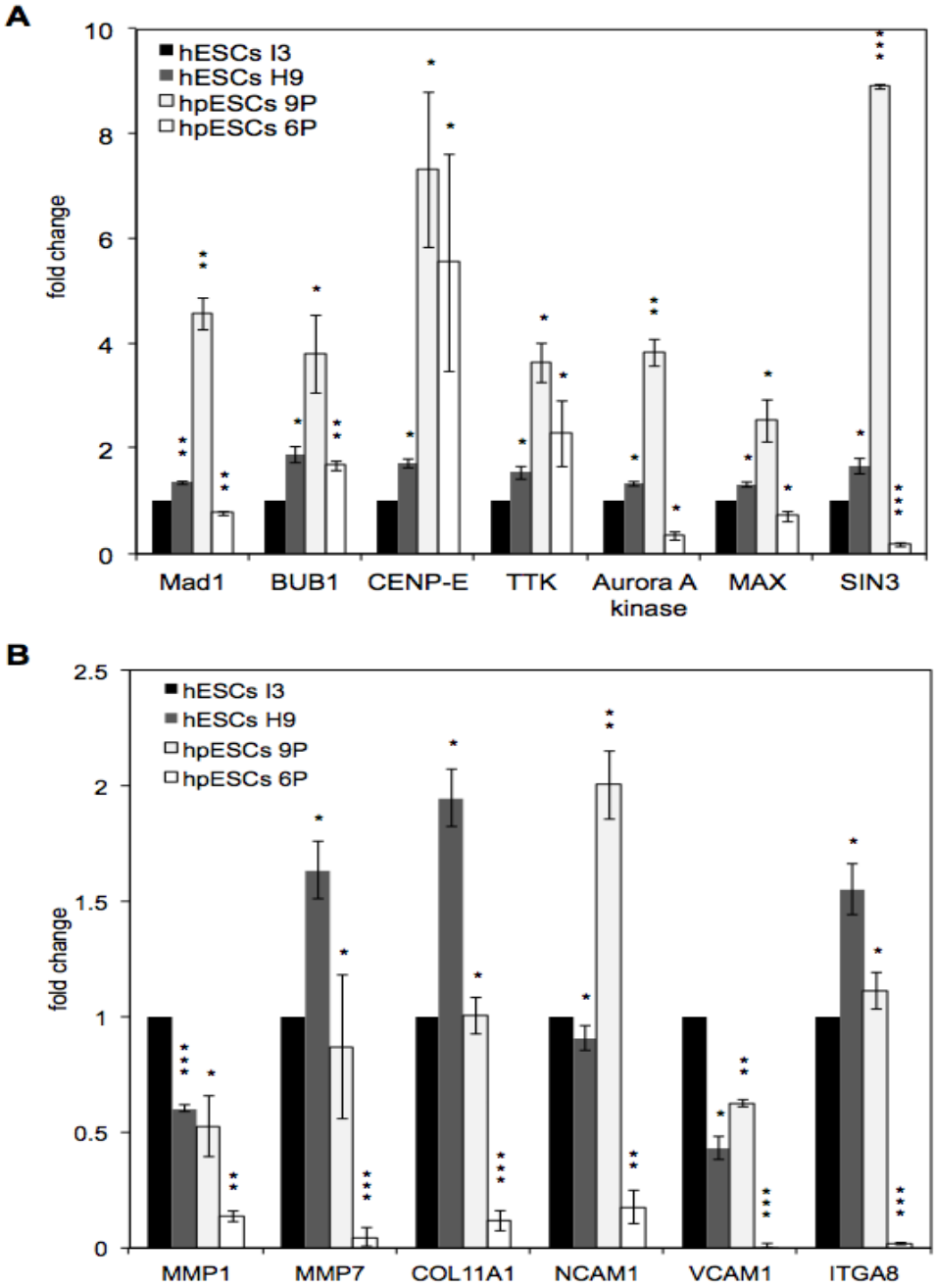


Fig. 10 Analyses of mitotic checkpoint and extracellular matrix gene expression by quantitative RT-PCR. **(A)** RNA expression of spindle formation- and chromosome segregation-related genes were analyzed in hpESCs compared to

hESCs (I3 and H9) ESCs, respectively. The genes analyzed were *MAD1*, *BUB1*, *CENPE*, *TTK kinase*, *aurora A kinase*, *MAX* and *SIN3*. **(B)** Representative expression analyses of extracellular matrix molecules in hpESCs compared to hESCs. The genes analyzed were *MMP1*, *MMP7*, *COL11A1*, *NCAM1*, *VCAM1* and *ITGA8*. Expression levels of hESCs were set to 1. Fold change was calculated by the $2^{-\Delta\Delta Ct}$ method. The housekeeping gene *GAPDH* was used as a reference. n=3, * p<0.05, ** p<0.01, *** p<0.001 by Student's *t*-test.

5.5 *Terminal differentiation of hpESCs-derived hpNSCs*

To study the in vitro differentiation potential of hpNSCs into neural sub-types, hpNSCs were differentiated by growth factor (FGF-2 and EGF) withdrawal (Fig 11A). 28 days differentiated hpNSC-derived cells expressed transcripts of *Tuj-1* (class III beta-tubulin, neurons), *GFAP* (glial fibrillary acidic protein, astrocytes), *S100B* (S100 calcium binding protein B, astrocytes), *Olig2* (oligodendrocyte transcription factor 2, oligodendrocyte), (Fig. 11B). Expression levels were similar to RNA isolated from a human fetal brain isolate, while these transcripts were not detected in undifferentiated hpESCs. hpNSC-derived cells immunostained positive for neural sub-types markers *NeuN* (neuronal nuclei), neuron-specific *Tuj-1*, *MAP2* (microtubule associated protein 2), astrocyte marker *GFAP* and oligodendrocyte marker (O4) (Fig. 11C). hpNSC-derived neurons also stained positive for expression of presynaptic vesicle protein Synapsin-1, axonal marker Tau and MAP2. Furthermore, *Tuj1*/DAPI positive cells co-expressed the neurotransmitter GABA (γ-aminobutyric acid) (Fig. 11C). Overall, hpNSCs favor neuronal differentiation (61 ± 1.6% of cells), specifically GABAnergic neurons (95 ± 1.3%), whereas glial cells were less frequently detectable (17 ± 0.3% of cells). On the other hand, oligodendrocytes were only detectable after 6 weeks of differentiation (2 ± 0.3% of cells) (Fig. 11D). Similar cell percentages of neuronal and astroglial differentiation were observed for the hpESC line LLC6P, with the exception that O4-positive cells were not detected (Fig. 11D). Together this shows that hpESC-derived hpNSCs can differentiate *in vitro* into cells with immunophenotypic features of neurons, astrocytes and oligodendrocytes. hpNSCs favored neuronal differentiation particularly GABAnergic neurons. hpNSC-derived neurons also express presynaptic vesicle protein Synapsin-1 which indicates that these neurons may be capable of forming a synapse.

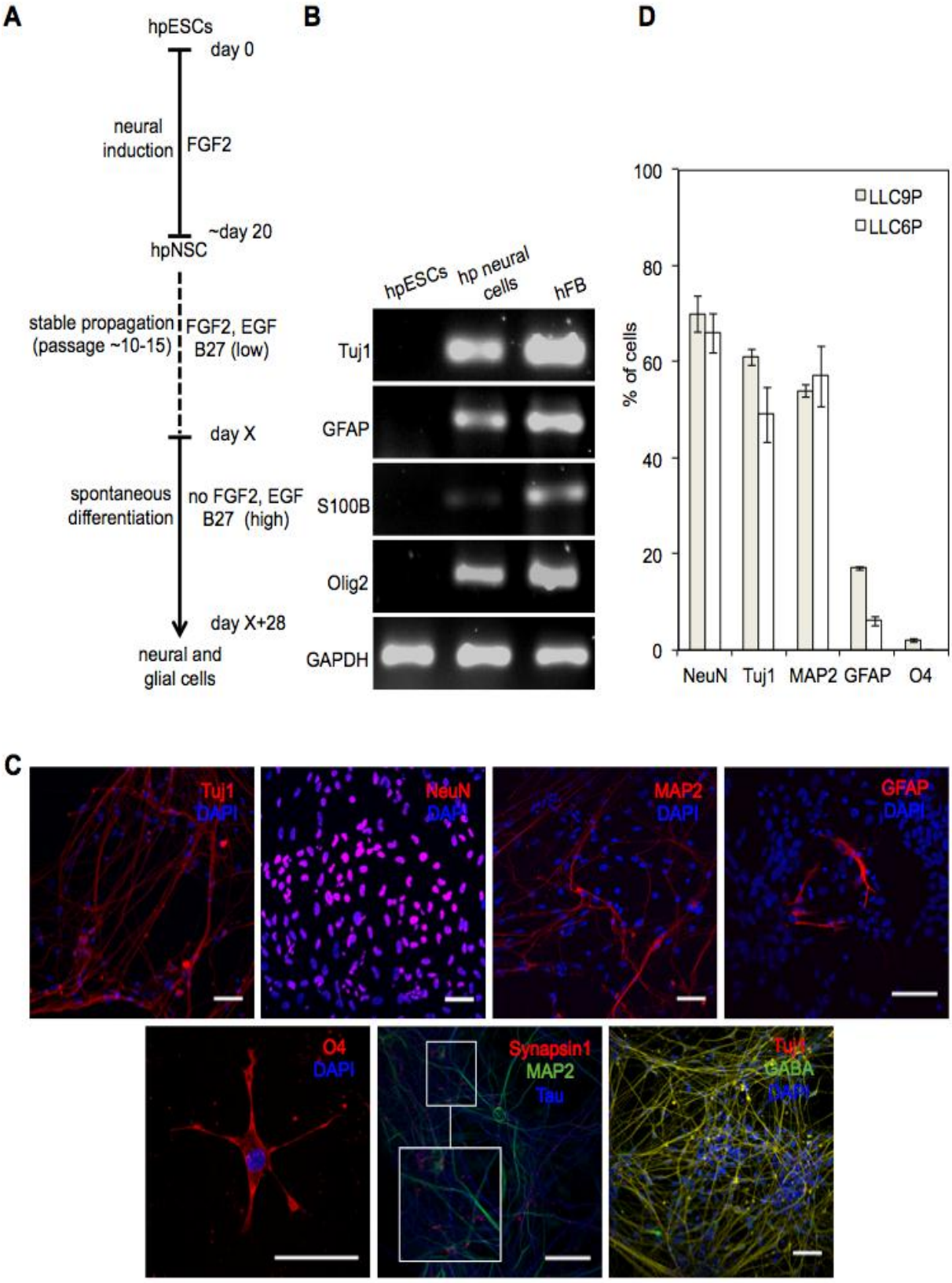


Fig. 11 Terminal differentiation of hpNSCs into neural and glial cell types (cell line LLC9P). **(A)** Schematic representation of *in vitro* neural differentiation of hpNSCs towards neural subtypes (scheme adapted from Koch et al., 2007). **(B)** Shown are semi-quantitative RT-PCR analyses of neural and glial markers *Tuj1*,

GFAP, *S100B*, *Olig2* in hpESCs, hpESC-derived neuronal and glial cells (hp neural cells) and hFB. GAPDH was used as house keeping control. **(C)** Images show immunostaining of hp neural cells with neural subtype-specific antibodies: *Tuj1*, *NeuN*, *Map2*, *GFAP*, *O4*, *Synapsin1/Map2/Tau* (insert shows higher magnification) and *Tuj1*/*GABA*. Cells were counterstained with DAPI. Images were taken by a confocal microscope. **(D)** Neural subtypes: *NeuN*, *Tuj1*, *Map2*, *GFAP* and *O4* percentages are given for cell lines LLC9P and LLC6P. Neuronal or glial marker- and DAPI-positive cells were counted to determine percentages. ImageJ software was used for counting. Scale bars: 50 µm; n≥ 3.

5.6 *Region-specific differentiation of hpNSCs*

Induction of regional phenotypes such as dopaminergic neurons and motoneurons was utilized to assess the responsiveness of hpNSCs towards instructive regionalization cues.

For induction of dopaminergic neurons hpNSCs were cultured in the presence of sonic hedgehog (SHH) and FGF8b for 8 days as described (Perrier *et al*. 2004). Then cells were cultured for three weeks in the presence of BDNF and GDNF (Fig. 12A). Midbrain-specific transcripts *Nurr1* (nuclear receptor related 1 protein), *En1* (engrailed homeobox 1) and *Pax2* (paired box gene 2) were expressed in human fetal brain isolate and hpNSC-derived cells (after 28 days of differentiation) and were absent in undifferentiated hpESCs (Fig. 12B). Immunocytochemical staining revealed expression of midbrain markers *En1* and *Pitx3* (paired-like homeodomain 3), which are transcription factors required for differentiation and survival of midbrain dopaminergic neurons and for *TH* (dopamine biogenesis) (Fig. 12B). Three independent differentiations generated 79.8 ± 3.2% *En1,* 10.8 ± 0.6% *Pitx3* and 13.3 ± 1.6% *TH* positive cells (Fig. 12B).

To explore the potential induction of motoneurons from hpNSCs (Li *et al*. 2005), cells were exposed to 1 µM retinoic acid and SHH alternatively with or without FGF2 and EGF for 6 days each. Then cells were cultured with BDNF and GDNF for 14 days (Fig. 13A). After 28 days of differentiation, transcripts of the motoneuron markers *HoxA1* and *HoxA2* were detectable in differentiated cultures but not in undifferentiated hpESCs (Fig. 13B). Correspondingly, immunostaining revealed nuclear expression of *Isl1* (ISL LIM homeobox1, marker for motoneuron progenitors), *Nkx2.2* (NK2 homeobox 2, ventral brain marker), *HB9* (motor neuron and pancreas homeobox 1, motoneuron marker) and *MAP2* (neuronal marker) (Fig. 13B). In aggregate hpNSCs generated 80.1 ± 3% *Isl1,* 70.9 ± 2.6% *Nkx2.2* and 79.1 ± 2.9%

HB9 positive cells (Fig. 13B). In summary, the data indicated that hpNSCs are responsive to instructive regionalization cues and hpNSCs can differentiate into cells that express dopaminergic and motoneuron markers. Neuronal cells that express dopaminergic or motoneuron markers were also observed upon differentiation of hpESC line LLC6P. Altogether, this set of data showed that hpNSCs have the capability to be modulated towards different regional phenotypes such as dopaminergic neurons and motoneurons.

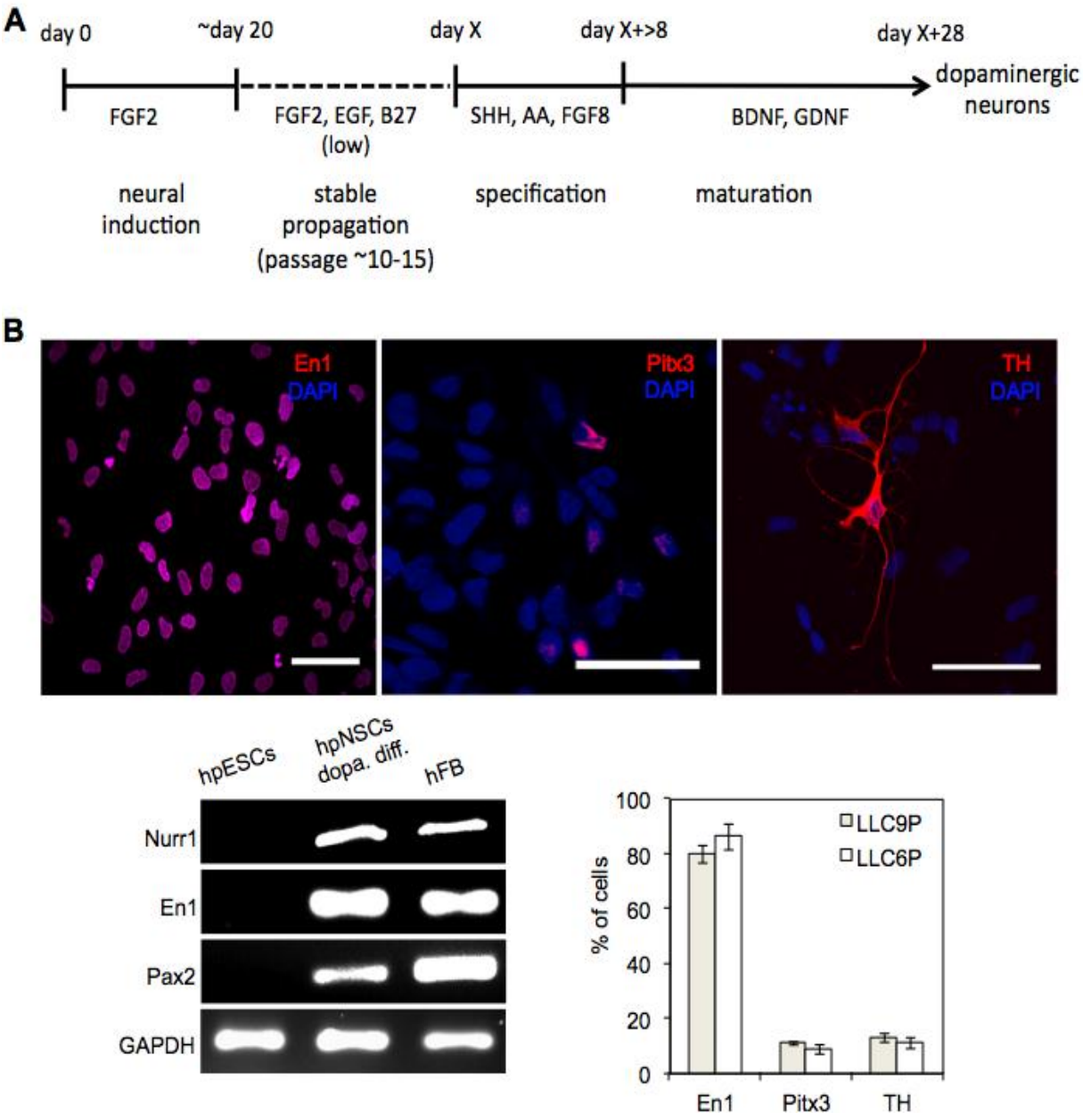


Fig. 12 Differentiation of hpNSCs towards dopaminergic neurons (A) Schematic representation of *in vitro* neural differentiation of hpESCs (LLC9P) towards

dopaminergic neurons and into motoneuron fates (scheme adapted from Koch et al., 2007). **(B)** Representative analyses for the expression of midbrain transcripts *Nurr1, En1* and *Pax2* by RT-PCR. Images exhibit immunostaining with antibodies specific for *En1, Pitx3* and *TH*. Cells were co-stained with DAPI. Percentages of DAPI and *En1, Pitx3* or *TH* positive cells are indicated (LLC9P and LLC6P). Scale bars: 50 µm; n=3

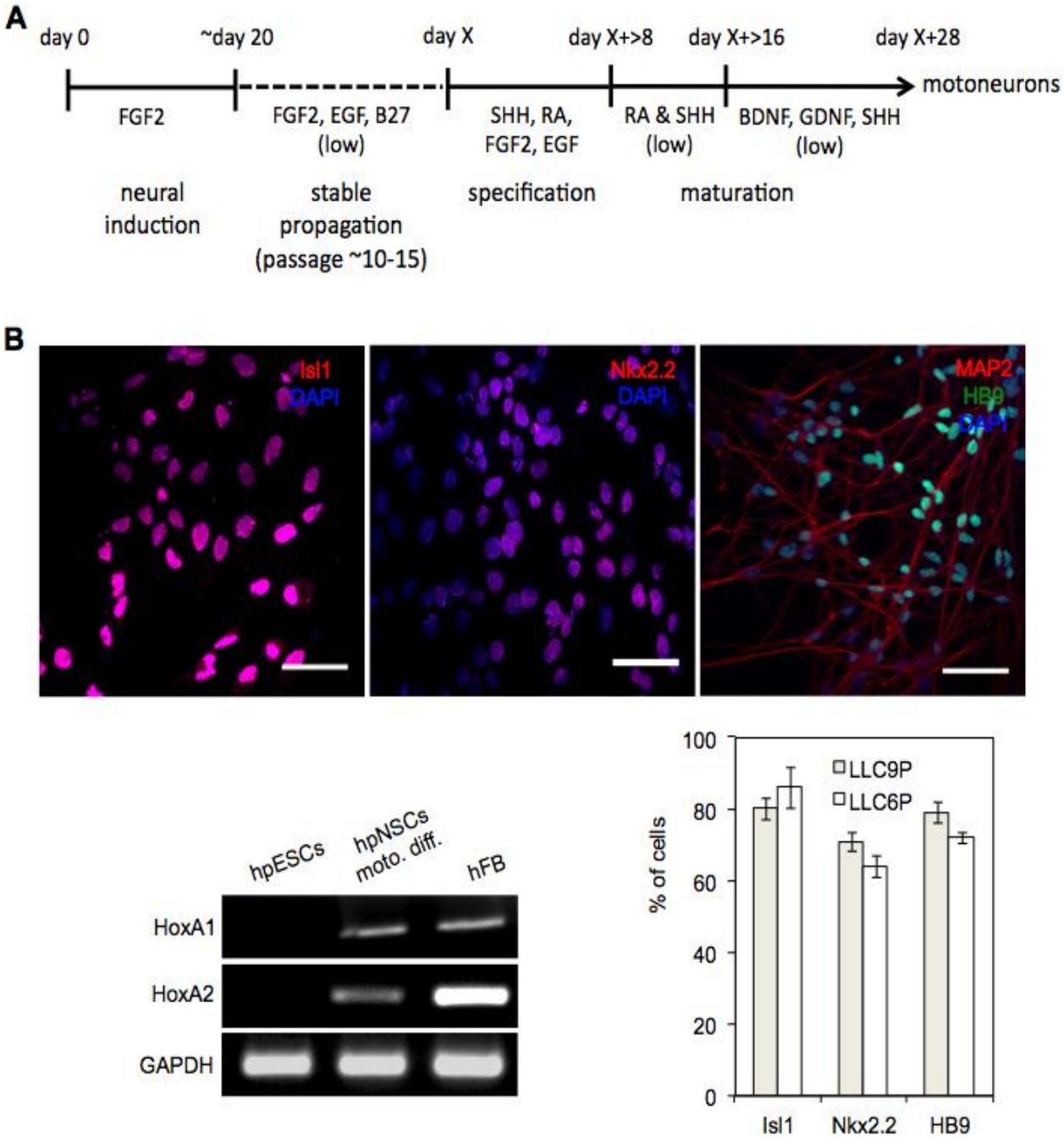


Fig. 13 Motoneurons generated from hpNSCs (LLC9P).
(A) Schematic representation of *in vitro* neural differentiation of hpESCs (LLC9P) towards motoneurons (scheme adapted from Koch et al., 2007). **(B)** Analyzed are the expression of *HoxA1* and *HoxA2* by RT-PCR. Corresponding images of

immunostainings for the expression of motoneuron markers: *Isl1*, *Nkx2.2* and *HB9*. Nuclei are counterstained with DAPI. Given are the percentage cell counts of *Isl1*, *Nkx2.2* and *HB9*- and DAPI-positive cells for LLC9P and LLC6P. Scale bars: 50 µm; n=3.

5.7 *Electrophysiological analyses of hpESCs-derived neurons*

Next it was investigated whether hpNSCs-derived neurons (PG neurons) can functionally mature *in vitro*. Patch-clamping was performed on the PG neurons to assess their electrophysiological properties. As shown in Fig. 14A typical neuronal Na^+/K^+ currents in voltage clamp mode (VC stimulation pattern: -80 mV to +55 mV, step size 15 mV, stimulation time 20 ms) were exhibited by PG neurons. Multiple action potentials with a maximum frequency of 30 Hz were derived with depolarizing step current injections over a 500 ms time period (Fig. 14B). PG neurons exhibited a typical neuron-like current pattern when maximum in- and outward currents were plotted against the corresponding stimulation voltage (Fig. 14C). Further, PG neurons were responsive to pharmacological channel blockers of sodium (tetrodotoxin) and potassium (tetraethylammonium) (Fig. 14D). These results show that electrophysiological properties of PG neurons at 28 days of differentiation were comparable to those reported in literature for human *in vitro* induced neuronal cells (Table 1).

Table 1. Electrophysiological characteristics of PG neurons

	mean	**SD**	**N**
resting membrane potential (mV)	- 32.50	± 7.27	14
membrane capacitance (pF)	9.31	± 3.88	14
series resistance (MΩ)	7.83	± 2.91	14
number of induced action potentials	12 out of 12 recordings		

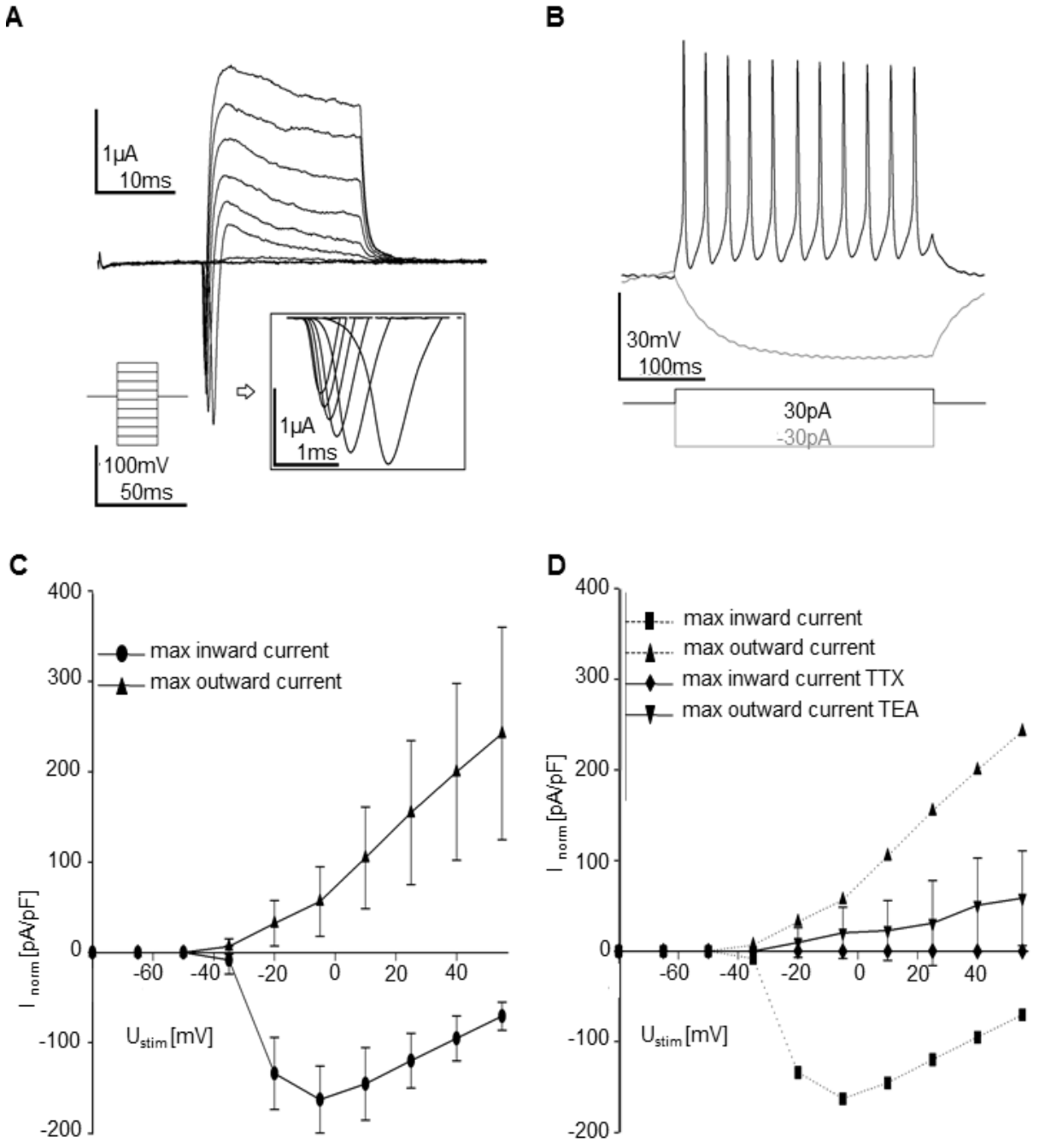


Fig. 14 Electrophysiology of PG neurons. Electrophysiological studies (patch-clamping) were performed on PG neurons (LLC9P) differentiated for 28 days. **(A)** Shown are representative current traces of PG neurons in whole cell configuration in response towards step depolarization (insert: sodium stimulation). Stimulus in VC mode was provided by stepwise increment of membrane potential (-80mV to +55mV, step size 15mV) **(B)** Membrane potential traces in response to step depolarization by current injection in current clamp mode; depolarization - black line, hyperpolarization - grey line. **(C)** Current (I) / voltage (V) curves from VC-stimulation. The maximum and the minimum measured currents were used to plot the graph of stimulation potential [mV] (current normalized to cell size [pA/pF]). **(D)** The graph shows I/V

curves of VC-stimulation before and after treatment of PG neurons with the Na^+ channel blockers tetrodotoxin (TTX) or K^+ channel blocker tetraethylamonium (TEA). The highest and the lowest measured currents were plotted against each other to produce the stimulation potential [mV] graph (current was normalized to cell size [pA/pF]). n=3.

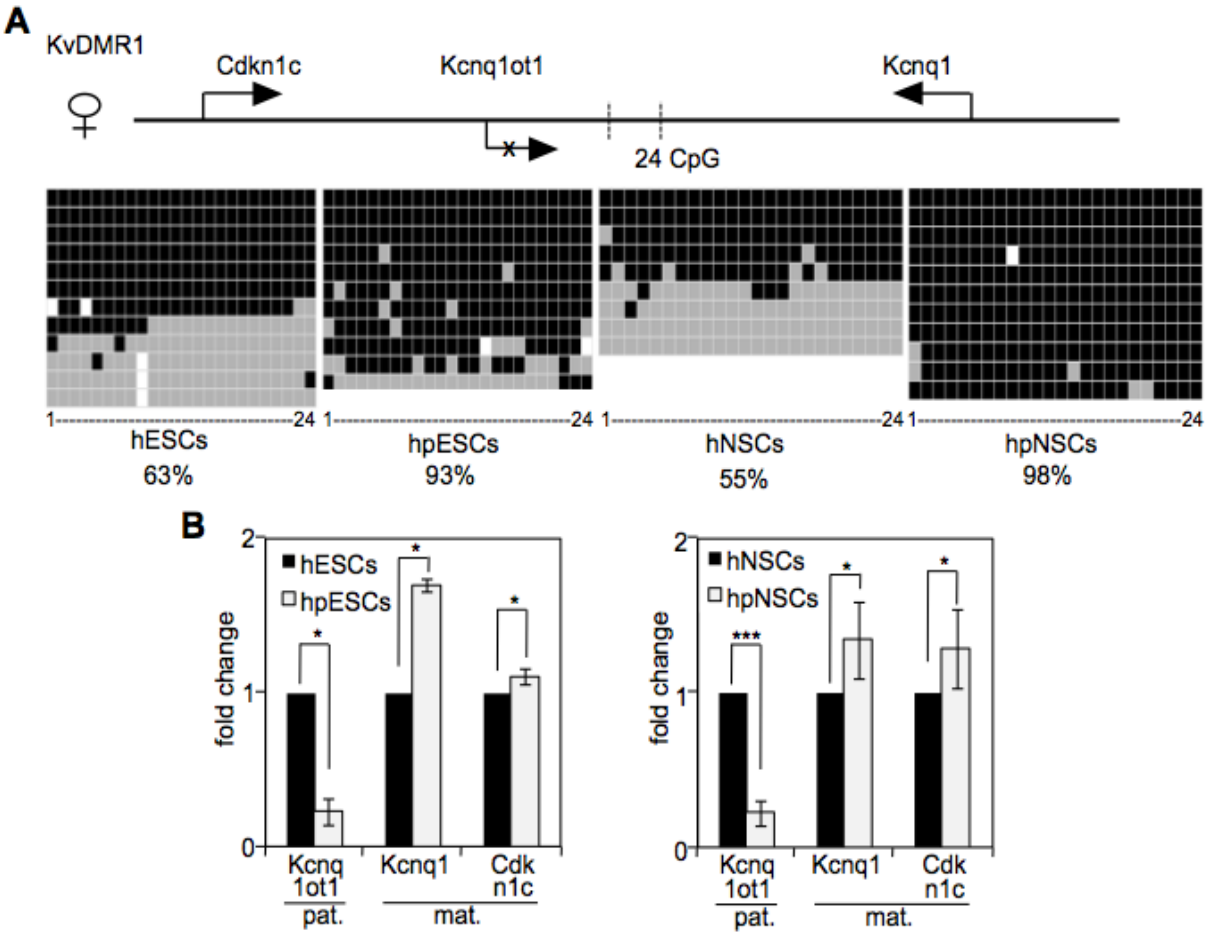
5.8 *Analysis of differentially methylated regions of imprinted brain genes by bisulfate sequencing*

Alterations of genomic imprinting in human embryos can contribute to the development of disorders linked to maternally or paternally expressed genes (Gabriel et al., 1998). hpESCs require a detailed imprinting analysis because of the possible influence on differentiation and the functionality of differentiated progeny. Expression of imprinted genes is achieved by parent-of-origin-specific epigenetic modifications mainly by DNA methylation of ICR (Ferguson-Smith, 2011). Therefore to assess the methylation status of ICR during neural differentiation CpG islands of two differentially methylated regions (DMR) were analyzed in hpESCs and hpNSCs (PG cells) in comparison to hESCs and hNSCs (N cells). 24 CpGs of KvDMR1 were analyzed by bisulfate sequencing. KvDMR1 controls the imprinting of one paternally expressed long-noncoding RNA, *Kcnq1ot1*, and eight maternally expressed protein-coding genes, including *Cdkn1c* (cyclin-dependent kinase inhibitor 1c), *Kcnq1* (potassium voltage-gated channel, KQT-like subfamily, member 1) (Verona *et al*., 2003). The *Kcnq1ot1* transcription start site (TSS) is located within the KvDMR1. When methylated on the maternal allele, *Kcnq1ot1* is silent (Mancini-DiNardo *et al*., 2003). On the paternal allele, the KvDMR is unmethylated and *Kcnq1ot1* is transcribed which represses the expression of eight maternally imprinted genes (Fitzpatrick *et al*., 2002). As shown in Fig. 15A, 50% methylation at the CpGs of KvDMR1 in N cells indicating biparental origin was observed. As anticipated from PG cells, CpGs of KvDMR1 in hpESCs and hpNSCs were hypermethylated. Quantitative RT-PCR analysis revealed lack of *Kcnq1ot1* expression and higher expression of *Kcnq1* but not *Cdkn1c* in PG compared to N cells (Fig 15B & 16B).

H19 is solely expressed from the maternal allele (Zhang *et al.,* 1992) while *Igf2* is expressed only from the paternal allele (Giannoukakis *et al.,* 1993). The two genes share an enhancer region that is located downstream of *H19* (Leighton *et al*., 1995) The ICR of *H19* is a boundary element, controlled by DNA methylation (Bell *et al*., 2000, Hark *et al*., 2000, Szabo *et al*., 2000). The CTCF protein (transcriptional

repressor) binds to the unmethylated maternal ICR, which prevents the promoters located in the *Igf2* gene from interacting with the enhancers resulting in the transcriptional silencing of *Igf2* (Bell *et al*., 2000, Hark *et al*., 2000, Szabo et al., 2000, Kanduri *et al.,* 2000). The methylated paternal ICR prevents the binding of CTCF, which allows the enhancers to contact the promoters of the paternal *Igf2* and the gene is transcribed (Hark *et al*., 2000, Schoenherr *et al*., 2002). Bisulfite sequencing showed that most of the H19 DMR1 CpGs were methylated in N cells while PG cells showed partial or complete absence of methylation of H19 DMR1 CpGs (Fig 15C). Gene expression analysis revealed absence of *Igf2* expression and over-expression of *H19* in PG compared to N cells (Fig. 15D & 16B).

Together these results show that imprinted genes in hpESCs and hpNSCs upon differentiation maintained imprinting-specific DNA-methylation and maternal-specific gene expression.



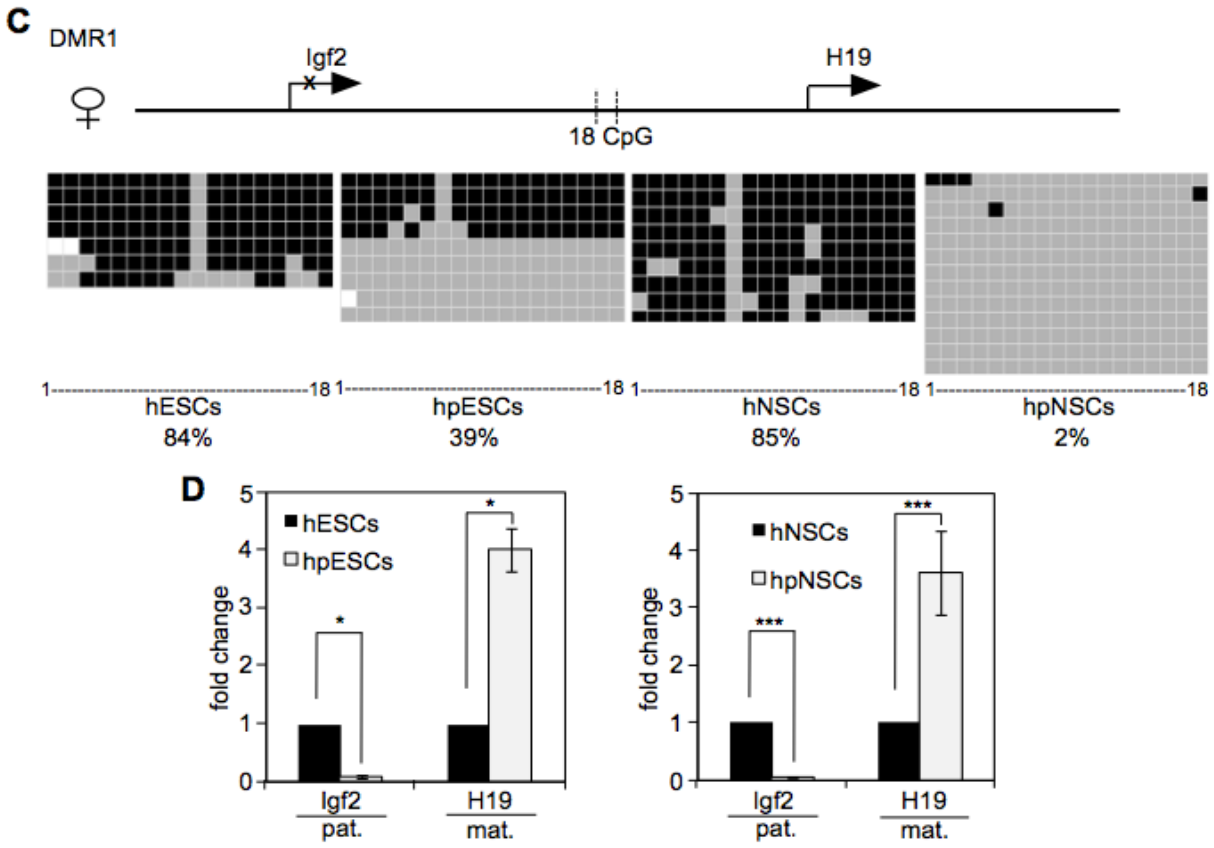


Fig. 15 Analysis of the methylation status of differentially methylated regions (DMRs) and expression analysis of imprinted genes. Bisulfite sequencing and imprinted gene expression analyses in hESCs, hNSCs, (I3) and in hpESCs, hpNSCs (LLC9P), respectively are shown. **(A)** Shown are the location of KvDMR1 and the transcriptional start sites of *Cdkn1c*, *Kcnq1ot1* and *Kcnq1* as line diagram (maternal allele). Data exhibits bisulfite sequencing results of KvDMR1 (position: 66531-66801) in N and PG cells. Black boxes: methylated CpGs; grey boxes: unmethylated CpGs; white boxes: not analyzed. Percentages of CpG methylation are indicated. **(B)** Representation of RT-PCR analyses of imprinted genes *Kcnq1ot1*, *Kcnq1* and *Cdkn1c* in PG and N cells. The relative expression represents the fold change of gene expression in PG compared to N cells, respectively. Fold change was calculated by the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as a reference gene. Expression levels of N cells were set to 1. n=3. **(C)** The line diagram depicts the location of DMR1 (position: 66531-66801) and the transcriptional start sites of *Igf2* and *H19* (maternal allele). Also included are bisulfite sequencing analyses of DMR1 in PG and N cells. **(D)** Shown are *Igf2*- and *H19*-specific gene expression analyses on N and PG cells. n=3, * p<0.05, ** p<0.01, *** p<0.001.

5.9 *Analysis of imprinted brain genes by quantitative real-time PCR*

The expression level of imprinted brain genes was analyzed by quantitative RT-PCR. As shown in Fig. 16 the paternally expressed *Snrpn* and *Nnat* genes were not detected or were poorly expressed while the maternal expressed *Gtl2*, *Dlx5* and *Kcnk9* genes exhibited higher levels of expression in both hpESCs and hpNSCs

compared to hESCs and hNSCs, respectively. However, *Igf2r* expression was elevated only in hpESCs but not in hpNSCs. These results indicate that the analyzed imprinted genes maintain their parent-of origin-specific gene expression bias during neural differentiation in hpESC lines LLC6P and LLC9P.

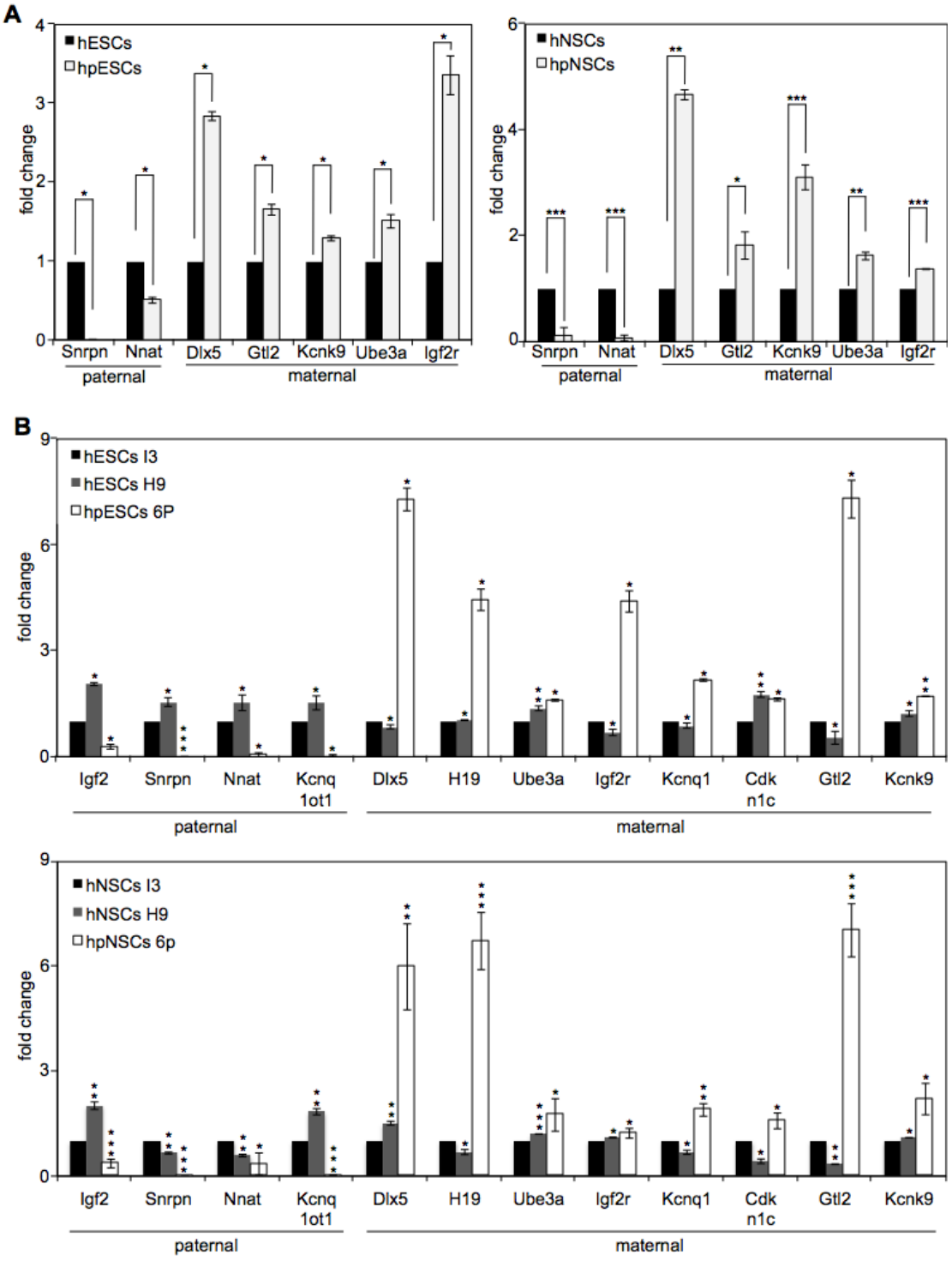


Fig. 16 Analyses of imprinted gene expression in hpESCs and hpNSCs (LLC9P). (A) Relative expression levels of imprinted genes in hpESCs compared to

hESCs (I3) are given. Fold change was calculated by the $2^{-\Delta\Delta Ct}$ method. Expression levels of hESCs were set to 1. In brain *Snrpn* and *Nnat* are preferentially paternally expressed, and *Gtl2*, *Dlx5*, *Ube3a* and *Igf2r* are preferentially maternally expressed. **(B)** Shown are expression levels of imprinted genes: *Snrpn*, *Nnat*, *Gtl2*, *Dlx5*, *Ube3a* and *Igf2r* in hpNSCs compared to hNSCs. Fold change was calculated by the $2^{-\Delta\Delta Ct}$ method. Expression levels of hNSCs were set to 1. n=3, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

6. DISCUSSION

The aim of this study was to define the capability of hpESCs to differentiate towards neural cell lineages *in vitro*. In summary, the results demonstrated that despite the lack of paternal genome, hpESCs generated proliferating NSCs that were capable to differentiate into neural and glial cell types. The hpESC-derived neurons were physiologically functional. Furthermore after neural differentiation PG cells maintained allele-specific expression of imprinted genes.

6.1 *Maintenance of hpESCs cultures*

ESCs, which are derived from the ICM of blastocyst stage embryos, have the ability to self-renew. The other defining characteristic of ESCs is pluripotency—that is the ability to differentiate into any cell type of the adult body (Thomson *et al.,* 1998). Earlier reports suggested that hpESCs propagation was improved by the addition of human UBS to the culture medium (Revazova *et al.,* 2007). However, results gathered in this thesis indicated that human UBS supplementation had no effect on hpESCs propagation. hpESCs were able to self-renew in medium containing synthetic KSR medium. Similar to previous reports hpESCs cultured with KSR maintained the expression of pluripotency markers (Lin *et al.,* 2007; Mai *et al.,* 2007; Revazova *et al.,* 2007). Furthermore, KSR-cultured hpESCs were able to generate teratomas comprising of three germ layers when transplanted into immunodeficient mice (Lin *et al.,* 2007; Mai *et al.,* 2007; Revazova *et al.,* 2007). This verifies that hpESCs are pluripotent and have the potential to differentiate into the various germ layers *in vivo*.

6.2 *Neural differentiation of hpESCs*

The results shown here also confirmed that hpESCs can differentiate into hpNSCs. Similar to previous reports on conventional ESC-derived NSCs, expression of pluripotent cell-specific transcription factors was absent in hpNSCs (Conti *et al.,* 2005; Koch *et al.,* 2009). hpNSCs exhibited appropriate expression of NSC-specific genes and the absence of neural crest (*Snai2, FoxD3)* and mesodermal marker (*Acta1*). A comparative study on neural differentiation potential of PG and N ESCs indicated that hpESCs yielded low quantities and impaired maturation of neural cells mainly due to reduced cell-cell interaction in hpESCs (Harness *et al*., 2011). The

protocol used by Harness *et al*., 2011 for neural differentiation included a neurosphere formation step, which depends greatly on cell-cell interactions. They concluded that the differences in cell-cell interaction were possibly due to variation in gene expression profile of ECM molecules in PG compared to N ESCs. This was discussed to contribute to decreased neurosphere formation capability of hpESCs. Although one of the hpESC line (LLC6P) analyzed in this thesis was also studied by Harness *et al*., 2011, the results obtained here are contradictory. A possible reason could be that in this thesis an alternative differentiation procedure was followed, which was optimized for producing standardized NSCs with reduced spontaneous differentiation and minimized restriction of lineage-specific differentiation (Koch *et al.,* 2009). The different culture systems can be an explanation for the contrasting results. In addition, I noticed that hpNSCs yields varied between the two hpESC lines (LLC6P and LLC9P). Harness *et al*., 2011 observed that hpESCs showed low expression levels of the early neuroblast marker *NCAM1* compared to hESCs. On the contrary, I observed high yields of hpNSCs from LLC9P cell line, which could be explained by the profound expression of NCAM1 in these cells (Knoth *et al.,* 2010). Hence the neurogenic capability of hpESC lines was paralleled by the dissimilarity in *NCAM1* gene expression that indicated cell-to-cell heterogeneity as reported earlier between independent hESC lines (Allegrucci *et al.,* 2007; Osafune *et al.,* 2008; Pal *et al.,* 2009; Tavakoli *et al.,* 2009; Lappalainen *et al.,* 2010). I also observed variations in gene expression levels of molecules related to spindle formation and mitotic checkpoint genes in PG in comparison to N ESCs as well as between the two hpESC lines. These variations were likely due to differences between the cell lines (Allegrucci *et al.,* 2007; Osafune *et al.,* 2008; Pal *et al.,* 2009; Tavakoli *et al.,* 2009; Lappalainen *et al.,* 2010). Another reason might be the genetic imbalance observed in uniparental ESCs (Brevini *et al.,* 2009).

6.3 *hpNSC-derived neural subtypes*

The present study indicated that hpNSCs were capable of generating mature neurons and glia. The stem cell status of hpNSCs was proven by the stable neuro- and gliogenic differentiation potential through various passages. hpNSCs responded to cues which directed them to differentiate towards ventral midbrain dopaminergic and ventral spinal cord motoneurons. This showed that hpNSCs were receptive to growth factors responsible for directing differentiation of NSCs into regional

phenotypes such as midbrain TH- neurons and HB9-positive neurons (Perrier *et al.,* 2004; Li *et al.,* 2005). I further showed that upon exposure to optimized differentiation conditions PG neurons generated action potentials and exhibited electrophysiological membrane properties similar to newly formed or hESC-derived neurons (Conti *et al.,* 2005; Koch *et al.,* 2009). Studying the PG neurons by immunocytochemical analyses revealed high frequencies of GABAergic neurons. Preferential differentiation into GABAergic neurons is a well-known phenomenon in NSCs, regardless of whether they are derived from cortex, striatum or from hESCs, cultured in monolayer or in aggregation (Conti *et al.,* 2005; Zhang 2006; Koch *et al.,* 2009). The reason of this bias is not known. A possible explanation is that during NSCs expansion the presence of growth factors can lead to quick loss of the proficiency of NSCs to generate site-specific neuronal subtypes (Conti *et al.,* 2010). Nonetheless, monolayer culture results in the disruption of the three-dimensional tissue structure and modification of the extracellular environment that probably alters the factors responsible for the generation of neuronal subtypes.

Altogether this shows that EGF- and FGF2-dependent monolayered hpNSCs retain multipotentiality and neurogenic efficiency after *in vitro* expansion and show competence to efficiently generate electrophysiologically active PG neurons when exposed to differentiation conditions. The unperturbed neural differentiation potential of hpESCs is consistent with earlier reports of successful murine AG ESC-derived neurogenesis (Mann *et al.,* 1991; Dinger *et al.,* 2008; Teramura *et al.,* 2009). The analyses indicate that uniparental ESCs are less restricted in their neural developmental potential than predicted from *in vivo* studies (Mann *et al.,* 1990; Keverne *et al.,* 1996). However, sophisticated animal transplant models are needed to assess the broader neural differentiation potential of hpESCs.

6.4 *Imprinting in hpESCs and hpNSCs*

Genomic imprinting is based on an epigenetic mechanism of gene regulation which results in parent-of-origin–dependent monoallelic gene expression (Bartolomei *et al.,* 2011). A genome-wide analysis of imprinted genes in embryonic and adult mouse brains demonstrated that nearly 1300 loci show parent-of-origin-specific expression (Gregg *et al.,* 2010). However, independent repetitions of this genome-wide analysis suggested that a large number of false-positive imprinted genes were reported mainly due to discrepancies in the analyses and experimental methodology

(DeVeale *et al.,* 2012). Nevertheless, this high-throughput transcriptome sequencing estimated the number of imprinted brain genes, which was comparable to the known number of imprinted genes in the brain (DeVeale *et al.,* 2012; Kelsey *et al.,* 2012). Moreover, previous reports using similar global screen approaches reported only a few novel imprinted genes (Babak *et al.,* 2008; Wang *et al.,* 2008).

Imprinted genes expressed in the brain are associated with a variety of neural functions including feeding, behavior and biochemical pathways governing metabolism and cell adhesion (Wilkinson *et al.,* 2007; Curley 2011). The *in vitro* neural differentiation of hESCs is astonishing, taking into consideration the large number of imprinted genes expressed in the brain and the contribution of imprinted genes in brain development (Keverne 2001; Wilkinson *et al.,* 2007; Curley 2011). We observed in PG cells that CpG methylation in the KvDMR was consistent with PG origin. The expression level of paternally expressed *Kcnq1ot1* was reduced and maternally expressed *Kcnq1* was up regulated in PG cells compared to N cells. However, maternally expressed *Cdkn1c* transcripts were only upregulated in one PG cell line (LLC6P) in comparison to N cells. The reason could be cell-to-cell heterogeneity as reported earlier between different hESC lines (Allegrucci *et al.,* 2007; Osafune *et al.,* 2008; Pal *et al.,* 2009; Tavakoli *et al.,* 2009; Lappalainen *et al.,* 2010). Nevertheless the exact reason for the down regulation of *Cdkn1c* remains unclear.

Analyses of DMR1 of the *H19/Igf2* locus in PG cells showed complete lack of methylation, which was in line with the PG origin. On the contrary, in N cells hypermethylation of CpGs in DMR1 of *H19/Igf2* locus was observed. The transcript level of Igf2 was low and *H19* gene expression was up regulated in PG cells, which were in line with PG origin. This indicates that various mechanisms apart from CpG methylation are regulating the allele-specific expression of *H19*/*Igf2* (Reik *et al.,* 2000).

One of the regulatory mechanisms controlling the allele-specific expression of *H19*/*Igf2* involves CTCF, a transcriptional repressor which controls reciprocal allelic silencing of the *Igf2* and *H19* genes by binding to the *H19* imprinting control region (Hark *et al.,* 2000; Schoenherr *et al.,* 2003). Another regulatory mechanism might involve a transcript that is antisense to *Igf2* (*Igf2-as*). *Igf2-as* is maternally imprinted and it is transcribed in a reverse direction within the *Igf2* gene (Okutsu *et al.,* 2000). It is unclear if *Igf2-as* is translated into a protein, although it encodes a putative

polypeptide (Okutsu *et al.,* 2000). It is speculated that putative *Igf2-as* peptide can regulate parent-of-origin-specific gene expression of *Igf2* (Vu *et al.,* 2003).

Comparative analyses of various hESC lines indicated a substantial degree of epigenetic stability, despite differences in genetic background, derivation and expansion conditions (Rugg-Gunn *et al.,* 2005; Kim *et al.,* 2007). However, certain genomic loci were epigenetically more stable than others (Rugg-Gunn *et al.,* 2005; Kim *et al.,* 2007; Rugg-Gunn *et al.,* 2007). For example, *Kcnq1ot1* locus was shown to be stable while the *H19/Ifg2* locus was unstable based on methylation patterns. The report further suggested that late passage hESCs are prone to hypermethylation in the DMR1 region of the *H19/Ifg2* cluster. In my study the hESCs used were also of higher passages, therefore I also observed that the *Kcnqot1* locus was epigenetically stable in PG and N cells while the *H19/Ifg2* locus was not. The observed alterations of the methylation patterns in *H19/Ifg2* DMR1 may have occurred during establishment and *in vitro* expansion of ESCs that can add heterogeneity to cells (Rugg-Gunn *et al.,* 2005; Kim *et al.,* 2007; Rugg-Gunn *et al.,* 2007). Despite the irregularity of DMR methylation the gene expression of *H19* and *Igf2* in hpESCs and hpNSCs was consistent with a PG origin. Furthermore, other paternal (*Snrpn* and *Nnat)* and maternal (*Dlx5, Gtl2*, *Ube3a* and *Kcnk9)* imprinted genes maintained their parent-of-origin-specific gene expression pattern. However, *Igf2r* expression was elevated only in hpESCs but not in hpNSCs: the molecular basis was unclear.

I observed that imprinted genes in hpESCs and hpNSCs upon differentiation maintained maternal-specific expression and imprinting-specific DNA-methylation. This suggests that either there is a less stringent role for imprinted gene expression during neural *in vitro* differentiation, or the balanced expression of imprinted genes is not required for differentiation to the stages analyzed. However *in vivo* animal transplant models are needed to assess the impact of imprinted genes on neural differentiation potential of hpESCs.

6.5 *Pluripotent stem cells*

Various types of human pluripotent stem cells derived from different sources and by different methodologies have been studied. They include hESCs (Thomson *et al.,* 1998), iPS cells (Takahashi *et al.,* 2007; Yu *et al.,* 2007), hpESCs (Kim *et al.,* 2007; Lin *et al.,* 2007; Mai *et al.,* 2007; Revazova *et al.,* 2007) and testis-derived pluripotent stem cells (Conrad *et al.,* 2008) (Fig. 17). Although, mammalian SCNT-

derived ESCs can be generated in multiple species, the derivation of hESCs through SCNT has failed so far (Cibelli 2007). However, Noggle *et al.,* 2011 generated triploid pluripotent stem cells from human oocytes by injecting the diploid nucleus of an adult cell into an human oocyte with SCNT. But still a lot of obstacles that need to be overcome before obtaining diploid SCNT-derived ESCs (Daley *et al.,* 2011; de Souza 2011).

Transplantation studies involving hESC-derived NSCs into mice and rats indicated that the stem cell–based approach can induce considerable improvements in animal models with neurodegenerative disorders (Goldring *et al.,* 2011). However, the clinical application of any type of stem cells to treat neurodegenerative diseases requires more detailed investigation, considering the complexity of the human brain structure and function and the alterations in the brain related to neurodegenerative diseases. Thus, in-depth transplantation studies are needed to understand the mechanisms regulating the proliferation, migration, differentiation, survival and function of ESC-derived donor cells.

Another hurdle before clinical application of hESC-derived cells is graft rejection triggered by MHC antigens that induce T-cell responses finally leading to graft destruction (Kadereit *et al.,* 2011; Tang *et al.,* 2011). Taylor *et al.,* 2005 predicted that 150 HLA heterozygous cell lines would provide a full match of MHC for less than 20% of recipients. Therefore a large number of cell lines would be needed to cover the complete population, keeping in mind the diversity of HLA haplotypes specifically in different ethnic groups.

Patient-specific iPS cells are generated by exogenous expression of combinations of transcription factors in somatic cells (Takahashi *et al.,* 2007; Yu *et al.,* 2007). iPS cells can provide the opportunity for specific individuals to undergo cell replacement therapy given that autologous transplantation of genetically identical cells does not lead to immune rejection (Yamanaka 2007). It has been estimated that a stem cell bank containing approximately 150 iPS cell lines with homozygous HLA haplotypes can provide histocompatible cells for vast majority of the population with minimum requirement of immunosuppression (Taylor *et al.,* 2011; Taylor *et al.,* 2012). Importantly, iPS cells can be utilized for future gene therapy strategies for known brain-specific genetic defects (Lengner 2010; Vitale *et al.,* 2011; Marchetto *et al.,* 2012). Despite the previous enthusiasm for the application of iPS cells in regenerative medicine, differences between iPS cells and ESCs have been reported

(Chin *et al.,* 2009; Doi *et al.,* 2009; Hu *et al.,* 2010). These differences prevent the use of common methodologies for iPS cells and hESCs. Moreover, reprogramming to iPS cells leaves traces of somatic cell identity, known as epigenetic memory (Barrero *et al.,* 2011; Ohi *et al.,* 2011; Panopoulos *et al.,* 2011).

hpESCs are an alternative method to generate patient-specific pluripotent stem cells as they are an exact match to the oocyte donor's genome (both nuclear and mitochondrial). hpESCs can be obtained without destroying a viable embryo and their derivation raises less ethical, socio-political and legal concerns in comparison to conventional hESCs. Furthermore, HLA homozygous hpESC lines can partially match at the MHC locus to a larger population of transplant recipients compared to conventional hESCs (Revazova *et al.,* 2008). Furthermore, Nakajima *et al.,* 2007 described that HLA homozygous hpESC lines established from only 55 oocytes will provide matched donor cells for 80% of the patients. The number of hpESCs lines proposed by Nakajima *et al.,* 2007 is considerably lesser than the number of hESCs (Taylor *et al.,* 2005) and iPS cells (Taylor *et al.,* 2011; Taylor *et al.,* 2012) lines suggested for a pluripotent stem cell bank. Further detailed analyses of imprinted genes in hpESCs and hpESC-derived progenitor cells are needed to better address concerns about safety and differentiation efficiency of these cells *in vivo* before they can be considered as an alternative source for regenerative cell therapies. hpESCs provide a unique opportunity to study the role of maternal or paternal genome in organ development and to gain insights into a variety of imprinting-related disorders.

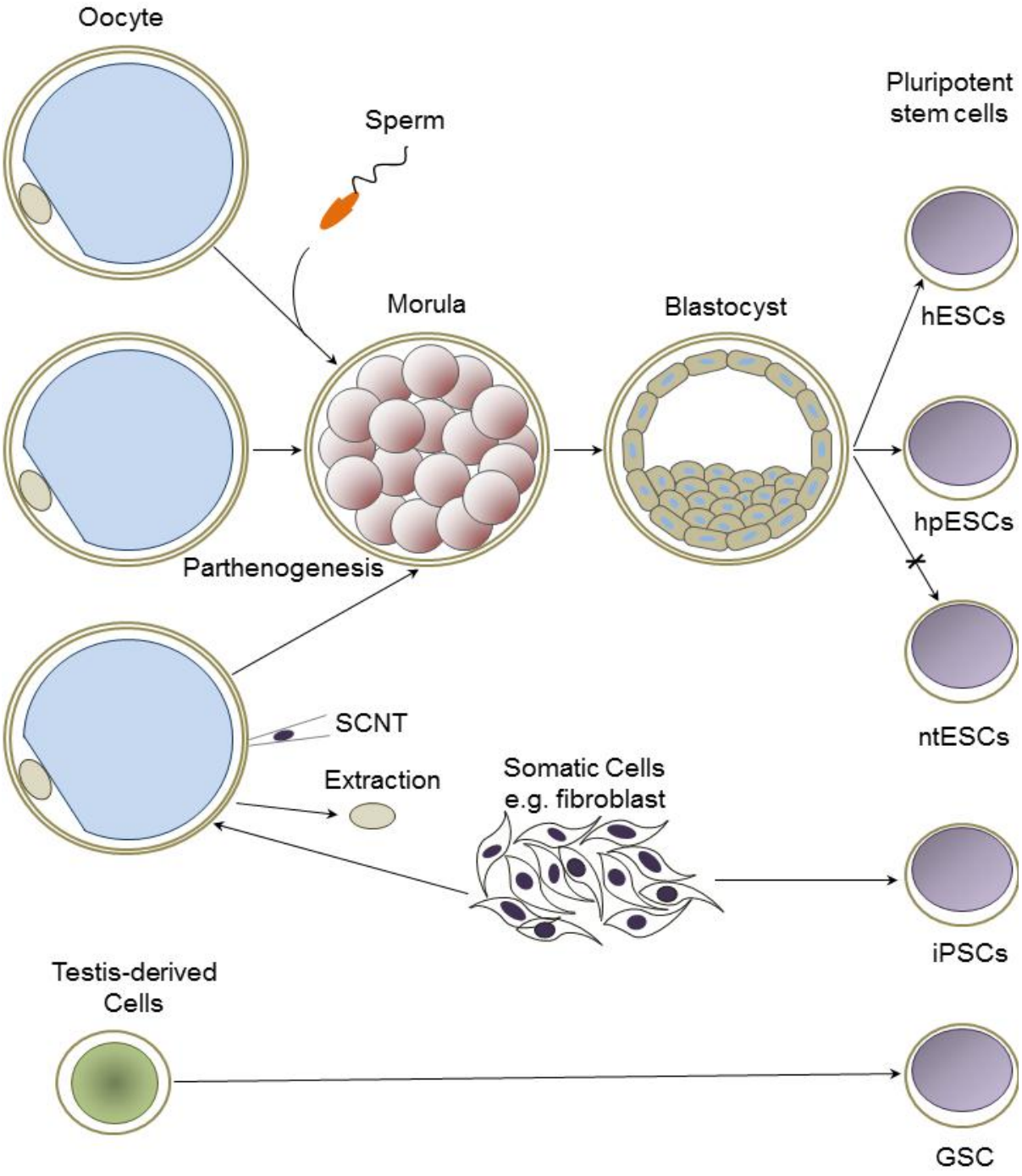


***Fig. 17: Origin of various types of pluripotent stem cells*.**
hESCs are isolated from the ICM of the blastocysts. hpESCs are derived from direct oocyte activation. ntESCs (nuclear-transfer embryonic stem cells) are formed by transferring the nucleus of a somatic cell into an enucleated oocyte (cross signifies that the strategy is not successful in humans). iPS cells can be obtained by transfecting the somatic cells with transcription factors such as *Klf4*, *c-Myc*, *Oct4* and *Sox2* which genetic reprogram the cells. Germline stem cells (GSC) are derived from adult testis by culture under specific conditions (adapted and modified from Muller *et al.,* 2009).

6.6 *Conclusion*

The results presented here demonstrate that despite the lack of paternal genome, hpESCs generate proliferating NSCs that are capable of differentiation into functional neuron-like cells and maintain allele-specific expression of imprinted genes. Additional studies are needed to further assess the *in vivo* functionality of hpESC-derived neural cells. hpESCs can have important implications for studying the role of maternal and paternal genomes on neural development and to better understand the alterations in the structure and function of the brain due to imprinting-associated brain diseases.

7. MATERIAL AND METHODS

7.1 Material

7.1.1 *Cell Lines*

Cell Line	Cell Type	Source
phESC-3 (LLC6P)	Human parthenogenetic embryonic stem cells	Revazova *et al*., 2007 International Stem Cell Corporation, California
phESC-6 (LLC9P)	Human parthenogenetic embryonic stem cells	Revazova *et al*., 2007 International Stem Cell Corporation, California
HFF-1	Human foreskin fibroblast	ATCC-LGC Standards, Wesel, Germany

7.1.2 *Antibodies*

7.1.2.1 *Primary Antibodies*

Specificity	Clone	Isotype	Supplier
GABA	polyclonal	Mouse IgG	Sigma-Aldrich, Schnelldorf, Germany
GFAP	GA5	Mouse IgG	Novocastra, Wetzlar, Germany
Glutamate			Sigma-Aldrich
HB9	polyclonal	Goat IgG	Santa cruz biotechnology, Heidelberg, Germany
Human Nuclei	3E1.3	Mouse IgG	Milipore , Billerica, MA, USA
MAP2	AP-20	Mouse IgG	Sigma-Aldrich
Nestin	2C1.3A11	Mouse IgG	Abcam, Cambridge, UK
NeuN	A60	Mouse IgG	Chemicon, Schwalbach, Germany
Oct4	polyclonal	goat IgG	Santa cruz biotechnology
O4	O4	Mouse IgM	R&D Systems, Minneapolis, USA
Sox1	polyclonal	Rabbit IgG	Milipore , Billerica, MA, USA
Sox2	245610	Mouse IgG	Abcam
SSEA1	145-2C11	Hamster IgG	BD Biosciences, Heidelberg, Germany
Synapsin-1	polyclonal	Rabbit IgG	Synaptic Systems, Göttingen,

			Germany)
TH	polyclonal	Rabbit IgG	Sigma-Aldrich
Tubulin-ß-III	Tuj 1	Mouse IgG	R&D Systems
Tau	polyclonal	Mouse	Synaptic Systems
Vimentin	polyclonal	Mouse IgG	Abcam

7.1.2.2 *Secondary Antibodies*

Specificity	Host	Conjugate	Supplier
Goat IgG	Donkey	Cy3	Santa cruz biotechnology
Goat IgG	Donkey	FITC	Santa cruz biotechnology
Mouse IgG	Goat	Cy3	Chemicon
Mouse IgG	Goat	Cy5	Chemicon
Rabbit IgG	Goat	Cy3	Chemicon
Rabbit IgG	Goat	Cy5	Chemicon
Rabbit IgG	Goat	Daylight488	Jackson Immunoresearch laboratory, Hamburg, Germany

7.1.3 Cell Culture-related materials

7.1.3.1 *Media and Supplements*	*Supplier*
Amino acids (non-essential)	PAA Laboratories, Cölbe, Germany
B27 supplement	Gibco Invitrogen, Karlsruhe, Germany
Dispase	BD Biosciences
DMEM/ F12	PAA Laboratories
FCS cell-culture grade	Biochrom, Berlin, Germany
Knockout DMEM high glucose	Gibco Invitrogen
Knockout Serum replacement	Gibco Invitrogen
HEPES buffer (100 mM)	PAA Laboratories
L-glutamine (200 mM)	PAA Laboratories
ß-Mercaptoethanol	Sigma-Aldrich
N2 supplement	Gibco Invitrogen
Neurobasal Medium	Gibco Invitrogen
Non-Essential Amino Acids	PAA Laboratories

PBS without Mg^{2+}/Ca^{2+}	PAA Laboratories
Trypsin/EDTA	PAA Laboratories
Matrigel	BD Biosciences

7.1.3.2 *Growth Factors*	*Supplier*
Fibronectin	Sigma-Aldrich
Human BNDF	R&D Systems
Human EGF, recombinant	PeproTech, Hamburg, Germany
Human GNDF	R&D Systems
Human FGF-2, recombinant	R&D Systems
Human FGF-2, recombinant	PeproTech
Human FGF-8, recombinant	PeproTech
Human SHH	PeproTech
Human transferrin	Sigma-Aldrich

7.1.3.3 *Medium for hpESCs culture*

Supplements	Volume (500 mL)	Final concentration
Knockout-DMEM	380 mL	-
Knockout serum replacement	100 mL	20 %
Amino acids (non-essential)	5 mL	1x
L-Glutamine (200mM)	5 mL	2mM
Penicillin (10KU/mL) Streptomycin (10mg/ml)	5 mL	10 mL/L
ß-Mercaptoethanol (14.3M) (3.5 ß-Merc. in 10 mL Hepes)	5 mL (Sterile filtered)	0.1 mM

7.1.3.4 *Medium for hpESC-derived embryoid bodies culture*

Supplements	Volume (500 mL)	Final concentration
Knockout-DMEM	430 mL	-
Knockout serum replacement	50 mL	10 %
Amino acids (non-essential)	5 mL	1x
L-Glutamine (200mM)	5 mL	2mM
Penicillin (10KU/mL)	5 mL	10 mL/L

Streptomycin (10mg/ml)		

7.1.4 *Chemicals*

Chemicals	Supplier
Acetic acid	PAA Laboratories
Agarose	Sigma-Aldrich
Alkaline Phosphatase Kit	Applichem, Darmstadt, Germany
BSA	Sigma-Aldrich
Chloroform	Applichem
DAPI	Sigma-Aldrich
DNAse I	Ambion, Austin, USA
DNAse 10X buffer	Ambion
DNeasy Tissue Kit	Ambion
DEPC	Sigma-Aldrich
DMSO	Applichem
EZ DNA Methylation-Gold kit	Zymo Research, Irvine, CA, USA
EDTA	Applichem
Ethanol	Applichem
Ethidium bromide	Merck, Darmstadt, Germany
Fluorescence mounting medium	Dako, Hamburg, Germany
Glucose	Applichem
Glycerol	Applichem
Goat serum	Sigma-Aldrich
Isopropanol	Applichem
Laminin	Sigma-Aldrich
Methanol	Applichem
Mitomycin C	Sigma-Aldrich
Paraformaldehyde	Applichem
peq Gold RNA pure	Peqlabs
Phenol	Applichem
Poly-L-ornithine	Sigma-Aldrich
QPCR SYBR green mix	ABgene, Hamburg, Germany

Sodium citrate	Applichem
Sodium hydroxide	Applichem
Retanoic acid	Sigma-Aldrich
Tetraethylammonium chloride	Sigma-Aldrich
Tetrodotoxin	Sigma-Aldrich
TissueTek O.C.T.	Sakura Finetek, Heppenheim, Germany
Tris	Applichem
TritonX-100	Sigma-Aldrich

7.1.5 *Consumables*

Consumables	Supplier
Bacteriological Petri dishes	Greiner Bio One, Essen, Germany
Cell culture flasks	Sarstedt, Nümbrecht, Germany Nunc , Wiesbaden, Germany
Centrifuge tubes (15 mL & 50 mL)	Greiner Bio One
Cover slip	Hartenstein Laborbedarf, Würzburg, Germany
Disposable gloves	Kimberly-Clark, Koblenz, Germany
Multi-well cell culture plates	Nunc
Parafilm M	Hartenstein Laborbedarf
Scalpels	Ratiomed, Schossholte-stuckenbrock, Germany
Sterile filter	Schleicher & Schuell, Dassel, Germany
Syringes	B. Braun, Melsungen, Germany
Tissue culture plates	Greiner Bio One, BD Biosciences

7.1.6 *Instruments*

Instruments	Supplier
Cell freezing container	Nalgene, Hereford, UK Cell culture
EPC 10 double patch clamp amplifier	HEKA, Lambrecht, Germany
Centrifuges	Eppendorf, Wesseling-Berzdorf and Thermo Scientific, Dreieich, Germany

Confocal microscope	Leica SP5, Wetzlar, Germany
Digital weighting balance	Sartorius, Göttingen, Germany
Gene Rotor Real Time PCR	Corbett Life Sciences (LTF Labortechnologie), Germany
Incubator	Thermo Scientific
Inverted microscope	Carl Zeiss, Jena, Germany
Microscope	EVOS, AMG, USA
Neubauer chamber	Marienfeld,Lauda-Königshofen,Germany
Sterile bench	Heraeus, Hanau, Germany
Vortexer	Scientific Industries, New York, USA
Water bath	GFL Gesellschaft für Labortechnik, Burgwedel, Germany

7.1.7 *Buffers and Solutions*

Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3 in H_2O.

MPS buffer: 80 mM PIPES, 1 mM $MgCl_2$, 5 mM EGTA, 0.5 % Triton X-100; pH 6.8 in H_2O.

10 mM Citrate buffer pH6: 9 mL 0.1 M citric acid in H_2O + 41 mL 0.1 M Trisodium citrate dehydrate in H_2O + 450 mL H_2O.

7.2 Methods

7.2.1 *Human foreskin fibroblast cell culture (HFF)*

HFF were cultured in HFF medium containing DMEM high glucose supplemented with 15 % cell culture-grade FCS, Penicillin (100 U/mL)/ Streptomycin (100 U/mL) and L-glutamine (2 mM). The fibroblasts were cultured at 37°C, 5 % CO_2. Medium was changed twice a week. Cells were passaged at 1:4 -1:5 split ratio. For passage of HFFs, medium was removed, cells were washed once with PBS and 5 ml of Trypsin/EDTA was added. After 5 minutes at 37°C trypsinisation was stopped by adding 5 mL of HFF medium. Cells were centrifuged at 700 rpm, resuspended in HFF medium and plated on tissue culture plates. To generate mitotically inactive feeder cells for culturing hpESCs, confluent HFF cultures plates were incubated at 37°C, 5 % CO_2 with 10 µg/mL mitomycin C in HFF medium for 2.5 hours. Culture dishes were then washed twice with PBS and fresh HFF medium was added.

7.2.2 *hpESC culture*

hpESCs (cell lines LLC6P (previously referred to as phESC-3) and LLC9P, phESC-6) were derived and described by the International Stem Cell Corporation (Revazova *et al.* 2007). hpESCs culture was performed as previously described with slight modifications (Revazova *et al.* 2007). hpESCs were maintained on mitomycin C inactivated HFFs at 5% CO_2 in medium containing Knockout-DMEM, 20% Knockout Serum replacement, 1% non-essential amino acids, 2 mM L-glutamine, 0.1 mM ß-mercaptoethanol and 4 ng/mL FGF2. Cultures were passaged at a 1:3–1:4 split ratio every 5–7 days. Medium was changed every day. For passage of hpESCs, medium was removed cells were incubated with dispase. After 8-10 minutes, the reaction was stopped by centrifugation and removing the supernatant. Cells were washed once more with medium then resuspended in fresh medium and replated on 10 cm^2 culture dishes with mitomycin C inactivated HFFs. For AP staining the colonies were fixed and the activity of their AP was visualized by applying the Alkaline Phosphatase Kit according to manufacturer (see Material).

7.2.3 *Freezing of HFFs and hpESCs*

HFFs were spun down at 700 rpm for 5 minutes at 4°C and resuspend in 90% cell culture-grade FCS and 10% DMSO. Cells were frozen at densities ranging from 1 x 10^6 / mL (min) to 5 x 10^6 / mL (max), approximately 1.5 mL per vial. Freezing took place in a freezing box with Isopropanol at -80°C for 3 days; afterwards vials were transferred in liquid nitrogen storage. hpESCs were frozen in 40% cell culture-grade FCS, 40% Knockout serum replacement and 20% DMSO.

7.2.4 *Thawing of hpESCs and HFFs from liquid nitrogen storage*

Vials with frozen cells were thawed rapidly in a water bath at 37°C, cell suspension were diluted with 5 mL appropriate cell culture medium, spun down at 700 RPM for 5 minutes at 4°C, supernatant was removed and the cells were resuspended in appropriate cell culture medium, seeded and cultured.

7.2.5 *Neural in vitro differentiation*

Neural differentiation was performed as previously described (Koch *et al.* 2009) with slight modifications. Briefly, 4-day-old embryoid bodies were transferred to

polyornithine/laminin-coated tissue culture dishes and propagated in N2 medium containing DMEM/F12, N2 supplement (1:100) with 10 ng/mL FGF2. Within 10-12 days, embryoid body outgrowths develop into neural tube-like structures. First signs of neural differentiation are small rosettes showing a columnar shape. These small rosettes become further organized and increase in size forming neural tube like structures with a central lumen and 3-dimensional growth. These structures were mechanically isolated with a needle. These clusters where further propagated as free-floating neurospheres in N2 medium containing 10 ng/mL FGF2 for 1 to 3 days. These neurospheres were distributed into single cells by incubating the spheres with trypsin/EDTA for 10 minutes. Neural stem cells were plated on polyornithine/laminin-coated tissue culture dishes. Media was changed to neural stem cell medium (NSCM) containing DMEM/F12, N2 supplement (1:100), 1.6 g/L glucose, 10 ng/mL FGF2, 10 ng/mL EGF, and 1 µL/mL B27 supplement. High cell densities were essential during initial plating and media was changed daily. Passaging was performed at very high cell density and cells were split at a 1:2-1:3 ratio using trypsin/EDTA. Trypsin was inhibited by trypsin-inhibitor and cells were centrifuged at 300X*g* for 5 minutes at 4°C. Terminal differentiation of neural stem cells was performed in differentiation media containing DMEM/12 (N2 supplement; 1:50) and Neurobasal (B27 supplement; 1:50) mixed at 1:1 ratio. cAMP (300 ng/mL) was added to the media for 28 days. For induction of dopaminergic neurons (Perrier *et al*. 2004), neural stem cells were cultured in N2 medium with addition of 200 ng/mL SHH, 100 ng/mL FGF8b, and 160 µM ascorbic acid for 8 days. Differentiation was performed for 20 days in differentiation media with addition of BDNF (20 ng/mL), 10 ng/mL GDNF, 160 µM ascorbic acid, and 0.5 mM dibutyryl-cAMP. For induction of motoneurons (Li *et al*. 2005), 1 µM retinoic acid was added to NSCM for 6 days in the presence of additional B27 supplement (1:50) and adding 1 µg/mL SHH from day 5. From day 7, media was changed to NSCM (without FGF2 and EGF) but with B27 (1:50), 1 µg/mL SHH and 0.01 µM retinoic acid for another 6 days. SHH was reduced to 50 ng/mL for another 14 days, and cells were differentiated in the presence of 20 ng/mL BDNF and 20 ng/mL GDNF in differentiation media.

7.2.6 *Immunostaining of cultured cells*

Cover slips with differentiated cells were washed with PBS once. After removal of PBS, cells were permeabilized with 1 mL MSP buffer each well for 30 seconds.

Afterwards, cells were fixed with 1 mL Methanol for 3 minutes at –20°C and washed three times with 1 mL PBS + 0.1 % Triton X-100. For blocking unspecific binding, cells were treated with 0.1 % Triton X-100, 5 % goat serum in PBS. Then cells were incubated for 30 minutes with 250 µL of different primary antibodies. Another protocol followed for cells grown on coverslips was that the cells were fixed in 4 % formaldehyde, permeabilized in 0.1 % Triton-X and 0.2 % gelatin. Slides were stained with the following primary antibodies: mouse anti-Nestin (1:500), rabbit anti-Sox1 (1:500), mouse anti-Sox2 (1:500), mouse anti-vimentin (1:500), mouse anti-NeuN (1:1000), mouse anti-Tuj1 (1:1000), mouse anti-MAP2 (1:1000), mouse anti-GFAP (1:500), mouse anti-O4 (1:500), goat anti-Oct4 (1:200), mouse anti-SSEA1 (1:500), rabbit anti-Synapsin1 (1:1000), mouse anti-Tau (1:500), rabbit anti-TH (1:500), goat anti-HB9 (1:500). After washing three times with 1 mL 0.1 % Triton X-100 in PBS for 5 minutes, 250 µL of secondary antibodies: Anti-mouse Cy3, Cy5, and DyLight 488, anti-rabbit Cy3 and Cy5 and anti-goat Cy3 and FITC were used. The cells were incubated for 1hour in the dark at room temperature. Lastly, the cells were washed three times with 0.1 % Triton X-100 in PBS and after that, nuclei were stained with 1 mL DAPI-solution (5 mg/mL DAPI stock solution was diluted 1:500 in PBS with 0.1 % Triton X-100, yielding a DAPI concentration of 10 µg/mL) for 5 minutes. Then cells were washed with PBS and cover slips were put upside down on a drop of Fluorescent Mounting Medium on slides. Fluorescent imaging was done using a SP5 Confocal Microscope.

7.2.7 *Brain sample preparation and* Immunohistochemistry *of cryosections*

Brains of NOD-SCID (NOD.Cg-prkdscid Il2rgtm1Wjl/Sz) mice with teratomas were fixed in 4 % (w/v) paraformaldehyde in PBS for a day, then for two days cryoprotected in 16 % (w/v) glucose in PBS. Afterwards the brains were embedded in TissueTek O.C.T and stored at – 80 °C. 10 µm thick sagittal cryosections were cut with a microtome at – 20 °C. Sections with teratomas were stained with hematoxylin-eosin for histological analysis. Histological analysis was performed on hematoxylin-eosin stained sections.

hpESC-derived teratomas were assessed by immunohistochemical staining by human nuclei-specific antibody (HNu). Cryosections were thawed for 30 minutes at room temperature and then boiled in a microwave with 10mM sodium citrate buffer pH 6. The slides were cooled down to room temperature for 30 minutes.

Cryosections were washed once with water and three times with PBS. Followed by 2 hour incubation with blocking solution containing PBS, 5% Goat Serum and 0,1% Triton-X, slides. Then the slides were incubated with 1:500 HNu antibody in blocking solution over night at 4°C in a moist chamber. To avoid evaporation slides were covered with parafilm. The day after, slides were rinsed three times in PBS and incubated for 1 hour with the blocking solution containing 1:200 Cy3-labelled goat anti-mouse secondary antibody. The slides were rinsed 3 times in PBS and incubated with DAPI for 5 minutes then again washed once with PBS. Lastly the cryosections were fixed in mowiol, an anti-bleaching reagent.

7.2.8 *RNA isolation*

Total RNAs were isolated from feeder-free cultures of hESCs or from hpESCs. Feeder cells were depleted by repeated passages on Matrigel-coated plates. Total RNA was isolated from hESCs (I3 and H9 cell lines), from hESC-derived neural stem cells (hNSCs) and from hpESCs and hpNSCs by using peqGOLD RNAPure^{TM}. Passage numbers of hpESCs that were used to generate hpNSCs were identical. As controls, cDNA from human parthenogenetic neural crest stem cells (hpNCSCs) (isolated from attached EBs stage of differentiation), total RNA, human fetal brain (hFB), 18 weeks, female (Stratagene, Santa Clara, CA, USA) and human adipose tissue-derived mesenchymal stromal cells (hMSCs) were used. 1 million cells were washed once in PBS and pellet by centrifuging for 10 minutes at 5000 g (3500rpm). The cell pellet was resuspended in 700 µL of RNA peq gold, mix by gentle pipetting, and then incubated for 3 minutes. 100 µL of chloroform was added and mixed by gentle vortexing. Next, samples were centrifuged for 10 minutes at 20000 g (14000rpm). The supernatants were transferred to fresh tubes and equal volumes of isopropanol were added to the aqueous phase and mixed gently by inverting. The nucleic acid was left to precipitate at -20°C for 20 minutes. Following precipitation samples were centrifuged for 10 minutes at 20000 g (14000 rpm). Following centrifugation, the supernatant was discarded and the pellet was resuspended in 1 mL of 70% ethanol. Samples were centrifuged for 10 minutes at 20000 g (14000 rpm). The supernatant was discarded, the pellet was again resuspended with 1mL of ethanol for a second wash and centrifuged for 10 minutes at 20000xg (14000 rpm). Finally, the supernatant was decanted and the pellet was air dried and resuspended

in 20 µL DEPC-treated water. The extracted amounts of RNA in the samples were photometrically quantified using an Eppendorf BioPhotometer.

7.2.9 *DNAse treatment*

To remove any contaminating genomic DNA from total RNA, RNA samples were treated with RNAse-free recombinant DNAse. 1 µg of RNA was mixed with 1 µL of 10X DNAse buffer, DEPC treated water was added to 12 µL and 1 µL (2U) of DNAse I was added. Samples were incubated for 30 minute at 37°C. DNAse was inactivated by adding 1 µL of 25mM EDTA and 10 minutes incubation at 65°C. Half of the reaction mix containing 0.5 µg RNA was used for cDNA synthesis.

7.2.10 *First strand synthesis*

Reverse transcription reaction to synthesize first cDNA strand from DNAse treated total RNA included 0.5 µg of RNA in 6.5 µL DEPC-water, 1µL (200 ng) of oligo dT (16-mer) and 5 µL of DEPC treated water, the reaction-mix was heated at 65°C for 5 minutes to resolve all secondary RNA structures. Then 1 µL of dNTPs (10mM), 4 µL of 5X first strand synthesis buffer, 2 µL of 100mM DTT and 0.5 µL of M-MLV reverse transcriptase were added, yielding final 20 µL reaction-mix per sample. Samples were incubated for 1 hour at 37°C and cDNAs were subsequently stored at -20°C.

7.2.11 *Analysis of gene expression using semi-quantitative RT-PCR*

PCR reactions were run by using Taq Polymerase. PCR conditions used were 35 to 40 cycles of denaturation at 94 °C for 1 minute, annealing temperatures at 58 °C to 62 °C for 1 minute according to the primers, and elongation at 72 °C for 1 minute. GAPDH was used a house-keeping gene to compare the expression. Omission of transcriptase during RT or cDNA sample during PCR served as negative controls. All reactions were performed on a T3 thermocycler. Primers (Eurofins MWG Operon, Ebersberg, Germany) used were: Primers (Eurofins MWG Operon, Ebersberg, Germany) used were: *Acta1* forward (f): 5'-CAG GGC CCG AGC CGA GAG TAG-3', reverse (r): 5'-ATA CCG ACC ATG ACG CCC TGG TG-3', Tm: 60°C; *En1* f: 5'-GAC TCG CAG CAG CCT CTC-3', r: 5'-GCC TGG AAC TCC GCC TTG-3', 55.3°C; *FoxD3* f: 5'-CTG GAA GAG AAG GAC AGC GAC GCA-3', r: 5'-GCT GTT CTT GGG CTT GCT CGG G-3', 60°C; *Gapdh* f: 5'-ACG ACC CCT TCA TTG ACC TCA ACT-3', r: 5'- ATA TTT CTC GTG GTT CAC ACC CAT-3', 60°C; *GFAP* f: 5'-GGC ACG TGC

GGG AGG CGG CC-3', r: 5'-TCT CAT CAC ATC CTT GTG C-3', 59°C; *HoxA1* f: 5'-GGG TGT CCT ACT CCC ACT CA-3', r: 5'-GGA CCA TGG GAG ATG AGA GA-3', 62.4°C; *HoxA2* f: 5'-TTC AGC AAA ATG CCC TCT CT-3', r: 5'-TAG GCC AGC TCC ACA GTT CT-3', 60.5°C; *Musashi1* (*MS1*) f: 5'-GTC CTG TCG CCC ACC ATC TC-3', r: 5'-CCC TCC CAA CGC CAC TGA C-3', 60°C; *Nanog* f: 5'-GCT TGC CTT GCT TTG AAG CA-3', r: 5'-TTC TTG ACT GGG ACC TTG TC-3', 57°C; *Nestin* f: 5'-AGA GGG GAA TTC CTG GAG-3', r: 5'-CTG AGG ACC AGG ACT CTC TA-3', 58°C; *Nurr1* f: 5'-TTC TCC TTT AAG CAA TCG CCC-3', r: 5'-AAG CCT TTG CAG CCC TCA CAG-3', 60°C; *Oct4* f: 5'-CGA CCA TCT GCC GCT TTG AG-3', r: 5'-CCC CCT GTC CCC CAT TCC TA-3', 62°C; *Olig2* f: 5'-CAG AAG CGC TGA TGG TCA TA-3', r: 5'-TCG GCA GTT TTG GGT TAT TC-3', 60°C; *Pax2* f: 5'-CAG GCA TCA GAG CAC AT C-3', r: 5'-GTC ACG ACC AGT CAC AAC-3', 55.7°C; *Pax6* f: 5'-AAT AAC CTG CCT ATG CAA CCC-3', r: 5'-AAC TTG AAC TGG AAC TGA CAC AC-3', 59°C; *Snai2* f: 5'-ATA CCA CAA CCA GAG ATC CTC A-3', r: 5'-GAC TCA CTC GCC CCA AAG ATG-3', 60°C; *Sox1* f: 5'-TAC AGC ATG TCC TAC TCG CAG-3', r: 5'-CTC TGG ACC AAA CTG TGG CG-3', 61°C; *S100B* f: 5'- AAA GAG CAG GAG GTT GTG G A-3', r: 5'- AGG AAA GGT TTG GCT GCT TT-3', 60°C; *Tuj1* f: 5'- CAA CAG CAC GGC CAT CCA GG-3', r: 5'-CTT GGG GCC CTG GGC CTC CGA-3', 60°C. Expression analyses of mitotic checkpoint and extracellular matrix genes by RT-PCR were performed using QuantiTect Primer Assays (Qiagen, Düsseldorf, Germany).

7.2.12 Analysis of imprinted brain gene expression using quantitative RT-PCR

RT-PCR reactions were performed and quantified using a Rotor-Gene™ 3000 and ABsolute™ QPCR SYBR® Green Mix. PCR conditions used were 40 cycles of denaturation at 94 °C for 40 seconds, annealing temperatures at 60 °C for 40 seconds and elongation at 72 °C for 1 minute. The relative gene expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method. The ΔCt-values indicate a difference of Ct-values between reference gene and target gene. The housekeeping gene GAPDH was used as the reference gene. The expression level of target genes in hESCs and hNSCs was set to 1 in order to determine differences of the target gene expression in hpESCs and hpNSCs, respectively. The primer sequences (Eurofins MWG Operon, Ebersberg, Germany) used were: *Cdkn1c* f: 5'-TGA AGG ACC AGC CTC TCT CG-3', r: 5'-TTC TCC TGC GCA GTT CTC TTG-3'; *Dlx5* f: 5'-CCA ACC AGC

CAG AGA AAG AA-3', r: 5'-GCA AGG CGA GGT ACT GAG TC-3'; GAPDH f: 5'-GGA GTC AAC GGA TTT GGT CG-3', r: 5'-TCC TGG AAG ATG GTG ATG GG-3': *Gtl2* f: 5'-ATC AGC CAA GCT TCT TGG AA-3', r: 5'-AGC TTC CAT CCG CAG TTC T-3'; *H19* f: 5'-CGG ACA CAA AAC CCT CTA GCT TGG AAA-3', r: 5'-GCG TAA TGG AAT GCT TGA AGG CTG CTC-3'; *Igf2* f: 5'-CTT GGA CTT TGA GTC AAA TTG G-3', r: 5'-CCT CCT TTG GTC TTA CTG GG-3'; *Igf2r* f: 5'-CCA TTC AGA CAA CGA CGG ATA C-3', r: 5'-ACG TTA TAT CCT TGC GAA CTG TTT AG-3'; *Kcnk9* f: 5'-CTA CTT TGC GAT CAC GGT CA-3', r: 5'-GTA GCG CAC GAA GGT GTT C-3'; *Kcnq1* f: 5'-TGT CCA CCA TCG AGC AGT ATG-3', r: 5'-CCG TCC CGA AGA ACA CCA C-3'; *Kcnq1ot1* f: 5'-CCA CCT TCT CCA TCT GCT CA-3', r: 5'-AAT CCA GTG GGG AAA AGG TC-3'; *Nnat* f: 5'-AAT CAA AAC ACC GCA CCA G-3', r: 5'-ATC AGT GAG GGG CAA GGG GGG TTC-3'; *Snrpn* f: 5'-TGG CAC CTT TAA GGC TTT TG-3', r: 5'-CCG CTT TTC TTC ACG CTC T-3'; Ube3a f; 5'-AGC CGG AAT CTA GAT TTC CA-3', r: 5'-TGT CTG TGC CCG TTG TAA ACT-3'.

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10. AFFIDAVIT

I hereby declare that my thesis entitled

Neurogenesis from parthenogenetic human embryonic stem cells

is the result of my own work. I did not receive any help or support from commercial consultants.

All sources and / or materials used are listed or specifed in the thesis.

Furthermore, I verify that this entire thesis or any part of its contents has not been submitted or is not under consideration for another examination process, neither in identical nor in similar form.

Place, Date …… Signature ……..

Hereby it is confirmed that Ruhel Ahmad has worked on his own on following parts of his doctoral thesis:
hpESC expansion and differentiation, hpNSC expansion and differentiation, analysis of gene expression, immunofluorescence, and analysis of imprinted gene expression.
Prof. Dr. Albrecht Müller
Place, Date Würzburg 28.09.2012 *Signature*

Fig. 14 and Table 1: Ruhel Ahmad performed hpNSCs culture, differentiation of hpNSCs towards neurons. The following co-authors performed and analyzed the electrophysiological properties of PG neurons.
Wanja Wolber
Place, Date Würzburg 28.9.12 *Signature* ...
Prof. Dr. Anna-Leena Sirén
Place, Date Würzburg, 28.09.12 *Signature* .,

Fig. 15: For bisulfite sequencing analysis Ruhel Ahmad performed hpESC culture, neural differentiation and DNA isolation from hpESC and hpESC-derived hpNSCs. The following co-authors performed the bisulfite sequencing analysis on genomic DNA of hpESCs, hESCs, hESC-derived NSCs and hpESC-derived NSCs.
Dr. K. John McLaughlin
Place, Date Columbus OH, 25/09/12 *Signature*
Dr. Sigrid Eckardt
Place, Date Columbus OH, 25/09/12 *Signature*

Fig. 7, 9, 10,15, 16: The following collaborators provided hESCs and hESC-derived NSCs (cell lines I3 & H9) to be used as control samples. Ruhel Ahmad isolated DNA and RNA.
Dr. Philipp Koch
Place, Date Bonn., 26/09/12 *Signature*
Prof. Dr. Oliver Brüstle
Place, Date Bonn., 26/09/12 *Signature* .

12. PUBLICATIONS

1. Choi SW, Eckardt S, **Ahmad R**, Wolber W, McLaughlin KJ, Sirén AL and Müller AM. *Two paternal genomes are compatible with dopaminergic in vitro and in vivo differentiation*. Int. J. Dev. Biol ; 54: 1755 - 1762 (2010).

2. **Ahmad R**, Wolber W, Koch P, Eckardt S, Geis C, Heckmann M, Semechkin R, McLaughlin KJ, Brüstle O, Sirén AL, Müller A M. *Functional neuronal cells generated by human parthenogenetic stem cells*. PLoS One. 2012;7(8):e42800; 2012.

3. Wolber W, Choi SW, **Ahmad R**, Eckardt S, McLaughlin, KJ, Geis C, Heckmann M, Sirén A-L, Müller AM: *Phenotype and stability of neural differentiation of androgenetic murine ES cell-derived neural progenitor cells*. In revision.

Poster Presentation

1. Ahmad R, Wolber W, Koch P, Eckardt S, Geis C, Heckmann M, Semechkin R, McLaughlin KJ, Brüstle O, Sirén AL, Müller A M. *Parthenogenetic human embryonic stem cells form functional neurons.* 6th International Symposium organized by the students of the Graduate School of Life Sciences of the University of Würzburg, 13-14 October, 2011, Würzburg, Germany.

2. Ahmad R, Wolber W, Koch P, Eckardt S, Geis C, Heckmann M, Semechkin R, McLaughlin KJ, Brüstle O, Sirén AL, Müller A M. *Parthenogenetic human embryonic stem cells form functional neurons.* 2nd Inter-academic Symposium of the National Academy of Sciences, Leopoldina and the Israel Academy of Sciences and Humanities. From Molecules to Circuits in Neuropsychiatric Disease 18-21 September, 2011, Würzburg, Germany. (poster number: p5)

3. Ahmad R, Wolber W, Koch P, Eckardt S, Geis C, Heckmann M, Semechkin R, McLaughlin KJ, Brüstle O, Sirén AL, Müller A M. *Parthenogenetic human embryonic stem cells form functional neurons.* Annual Conference of the German Genetics Society 14-16 September 2011, Würzburg, Germany. (poster number: p76)

4. Ahmad R, Wolber W, Koch P, Eckardt S, Geis C, Heckmann M, Semechkin R, McLaughlin KJ, Brüstle O, Sirén AL, Müller A M. *Parthenogenetic human ES cells form functional neurons***.** 9th annual meeting of International Society for Stem Cell Research (ISSCR) 15-18 June, 2011, Toronto, Canada (poster number: 3347)

5. Ahmad R, Wolber W, Koch P, Eckardt S, Geis C, Heckmann M, Semechkin R, McLaughlin KJ, Brüstle O, Sirén AL, Müller A M. *Pluripotent parthenogenetic human*

stem cells form functional neurons. 1st International PhD symposium of the Berlin-Brandenburg School for Regenerative Therapies, 1-3 December 2010, Berlin, Germany (poster number: I.1)

6. Ahmad R, Wolber W, Koch P, Eckardt S, Geis C, Heckmann M, Semechkin R, McLaughlin KJ, Brüstle O, Sirén AL, Müller A M. *Pluripotent parthenogenetic human stem cells form functional neurons.* Awarded 2nd best poster prize, 5th International Symposium organized by the students of the Graduate School of Life Sciences of the University of Würzburg, 13-14 October, 2010, Würzburg, Germany.

Place, Date …… *Signature* ……