Derivation, propagation and differentiation of human stem and progenitor cells

Mathilda Zetterström Axell

Centre for Brain Repair and Rehabilitation,
Department of Clinical Neuroscience and Rehabilitation
Institute of Neuroscience and Physiology
at Sahlgrenska Academy
University of Gothenburg
2009

..

Mathilda Zetterström Axell
Tryck: Intellecta infolog
Göteborg 2009
ISBN 978-91-628-7841-2
Cover illustration: Devil-shaped human embryonic stem cell colony, Cellartis AB, Dundee, Scotland

Derivation, propagation and differentiation of human stem and progenitor cells

Mathilda Zetterström Axell

Centre for Brain Repair and Rehabilitation, Institute of Neuroscience and Physiology at Sahlgrenska Academy, University of Gothenburg, 2009

Abstract

Neuronal loss is a common feature of many neurological disorders, including stroke, Parkinson's disease, Alzheimer's disease and traumatic brain injury. Human embryonic stem cells (hESCs) and hESC-derived neural progenitors (NPs) may provide a number of new ways for studying and treating diseases and injuries in the brain. Studying the proliferation and differentiation characteristics of hESCs and NPs is important for three main reasons: 1, they represent an almost unlimited source of cells for neuron replacement therapies after neurodegeneration in the brain; 2, they are a good source of normal human cells for studying functional genomics, proteomics or for drug screening; and 3, they allow us to study early human brain development.

The general aims of this thesis were four-fold: 1, to develop efficient and simple methods for the large scale propagation of hESCs and hESC-derived NPs; 2, to optimise NP differentiation into mature neurons and glia; 3, to find suitable materials to promote migration and differentiation of stem and progenitor cells, and; 4, to uncover critical differentiation factors expressed in common between neuroblasts in the rostral migratory stream (RMS; the only long distance cell migration system in the human brain) and that of hESC-derived NPs.

To address these aims, we used a range of techniques including cell culture, morphometric analysis, immunocytochemistry, immunohistochemistry and RT-PCR.

Here we report the development of an improved method for the transfer and culture of undifferentiated hESCs in the absence of a cell feeder-layer, which is more cost effective and reduces the contact with murine feeder cells that render the hESCs

unusable for future transplantation into humans. We have also developed a simple method for producing NPs from hESCs, suitable for large scale expansion and long term propagation of NPs. The production of large quantities of NPs allows us to readily compare the properties of NPs in culture to those in the human brain. Studying the differentiation of hESCs on permissive substrates has also been a focus and is of importance because of the relevance to the developing and adult human brain, where a complex extracellular matrix exists as scaffolding for neuronal development. We found electrospun fibrous scaffolds suitable for propagation and differentiation of hESCs, deriving predominantly tyrosine hydroxylase positive neurons indicating a dopaminergic fate. Finally, we studied the adult human brain for the presence of progenitor cells with migratory characteristics. We used a combination of serial sectioning, immunostaining and RT-PCR of human post-mortem brain material. This was the first study to reveal the presence of a human RMS by which neuroblasts migrate long distances from the subventricular zone to the olfactory bulb where they differentiate into mature neurons. Further, we discovered a number of differentiation factors expressed (Pax6, NCAM, DCX, βIII-tubulin) in common between the human RMS neuroblasts and hESC-derived NPs. Taken together, this thesis reveals improved ways to propagate and differentiate hESCs in culture, and has uncovered common differentiation factors present in both human neuroblasts and NPs. These studies further our understanding of human brain development, allow large scale production of NPs for further study, and may one day be useful for treating central nervous system disorders.

Key Words

Human embryonic stem cells, neural progenitor cells, stem cells, differentiation, propagation, migration, cell culturing, rostral migratory stream, electrospun scaffolds

Populärvetenskaplig sammanfattning på svenska

Förlust av nervceller är en gemensam nämnare för många neurologiska sjukdomar som stroke, Parkinsons sjukdom, Alzheimers sjukdom och traumatisk hjärnskada. Den vuxna hjärnans kapacitet att reparera sig själv är begränsad varför mycket forskning fokuserar på att kunna ersätta och reparera skadad hjärnvävnad. Humana embryonala stamceller (hESC; omogna, självreplikerande, kan bilda alla celltyper i den vuxna kroppen) och neurala progenitorceller (NPC; självreplikerande, förstadium till mogna hjärnceller) deriverade från hESC kan ge oss nya sätt att studera och behandla skador på hjärnan efter sjukdom eller trauma. Detta genom att förse oss med en nästan oändlig källa av celler för att studera geners och proteiners funktion, för läkemedelutveckling, för att studera tidig utveckling av den mänskliga hjärnan och för utveckling av transplantationsterapier.

Det är känt sedan tidigare att progenitorceller förflyttar sig en lång sträcka via en specifik bana/”motorväg” (rostral migratory stream; RMS) i den vuxna hjärnan hos gnagare. Vi har här för första gången identifierat migrerande progenitorceller (neuroblaster) i den vuxna mänskliga hjärnan och visar även för första gången att RMS även finns hos människan. Dessa neuroblaster visade sig uttrycka flera markörer (Pax6, NCAM, DCX och βIII-tubulin) gemensamt med NPC deriverade från hESCs, enligt en ny enkel, effektiv och billig metod som vi utvecklat här. Stora mängder NPC behövs bl.a. för att kunna jämföra egenskaper hos NPC i odling med de i hjärnan. Traditionellt sett odlas hESC på att stödlager av så kallade feederceller (bindvävsceller från mus eller människa). Dessa feederceller utsöndrar näring och bidrar dessutom med andra idag ej kända faktorer, vilka bidrar till att behålla hESC i ett stabilt omoget stadium. Vi har här även utvecklat ett förbättrat och stabilt protokoll för överföring av hESC till ett feederfritt odlingsunderlag och vidare expandering. Feederfri odling minskar risken för kontamination av skadliga molekyler från feedercellerna till hESC, vilket måste elimineras för

att kunna använda cellerna för transplantationsterapier. För att ta ytterligare steg mot transplantationsterapier och för att lättare kunna styra mognaden av hESC till specifika nervcellstyper har vi har tagit fram ett biokompatibelt 3-dimentionellt material som är lätt att odla hESC på/i vilket främjar bildandet av dopaminerga nervceller. Det är denna celltyp som dör vid Parkinsons sjukdom.

Sammanfattningsvis så har våra studier bidragit till att förbättra och utveckla mer effektiva metoder för att odla hESC och få dem att bilda nervceller. Vi har även hittat faktorer involverade i mognadsprocessen gemensamma hos neuroblaster i den vuxna mänskliga hjärnan och hos NPC deriverade från hESC enligt vår metod. Våra studier främjar förståelsen för hjärnans utveckling och visar att vi kan producera stora mängder NPC för vidare studier, något som i framtiden kan vara mycket viktigt för att behandla skador på centrala nervsystemet (CNS).

Papers included in the thesis

I. Eva Sjögren-Jansson, **Mathilda Zetterström**, Karina Moya, Jenny Lindqvist, Raimund Strehl, and Peter S. Eriksson. "Large-Scale Propagation of Four Undifferentiated Human Embryonic Stem Cell Lines in a Feeder-Free Culture System". *Developmental Dynamics, 233:1304–1314, 2005.*

II. **Mathilda Zetterström Axell**, Suzana Zlateva, Maurice A. Curtis. "A method for rapid derivation and propagation of neural progenitors from human embryonic stem cells". *In manuscript.*

III. Björn Carlberg*, **Mathilda Zetterström Axell*,** Ulf Nannmark, Johan Liu, H. Georg Kuhn. "Electrospun polyurethane scaffolds for proliferation and neuronal differentiation of human embryonic stem cells".*** equal contribution**. *Biomed. Mater. 4 (2009) 045004.*

IV. Maurice A. Curtis, Monica Kam, Ulf Nannmark, Michelle F. Anderson, **Mathilda Zetterström Axell**, Carsten Wikkelso, Stig Holtås, Willeke M. C. van Roon-Mom, Thomas Björk-Eriksson, Claes Nordborg, Jonas Frisén, Michael Dragunow, Richard L. M. Faull, Peter S. Eriksson. "Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension". *Science. 2007 Mar 2;315(5816):1243-9. Epub 2007 Feb 15.*

Additional papers not included in the thesis;

Camilla Karlsson, Katarina Emanuelsson, Fredrik Wessberg, Kristina Kajic, **Mathilda Zetterström Axell**, Peter S. Eriksson, Anders Lindahl, Johan Hyllner, Raimund Strehl. “Human embryonic stem cell-derived mesenchymal progenitors-Potential in regenerative medicine”. Stem Cell Res. 2009 May 19.

Abbreviations

ALP - alkaline phosphatase

AS - Akademiska Sjukhuset

ASCs - adult stem cells

BMPs - bone morphogenetic proteins

BrdU - bromodeoxyuridine

CN - caudate nucleus

CNS - central nervous system

CSF - cerebrospinal fluid

DAB - 3,3 diaminobenzidine

DAPI - 4´-6´Diamidino-2-phenylindole

DCX - doublecortin

DG - dentate gyrus

D-MEM - Dulbecco's modified eagle medium

DMEM/F12 - DMEM/nutrient mixture F-12

DMF - *n,n*-dimethylformamide

DMSO - dimethyl sulfoxide

EBs - embryoid bodies

ECM - extracellular matrix

EGF - epidermal growth factor

ELISA - enzyme-linked immunosorbent assay

En1 - engrailed 1

ESCs - embryonic stem cells

FBS - fetal bovine serum

FGFs - fibroblast growth factors

FISH - fluorescence *in situ* hybridization

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

Gbx - gastrulation brain homebox

GFAP - glial fibrillary astrocytic protein

HBSS - Hank's Balanced Salt Solution

hEF - human embryonic fibroblasts

hESCs - human embryonic stem cells

ICM - inner cell mass

iPSCs - induced pluripotent stem cells

LV - lateral ventricle

mEF - mouse embryonic fibroblasts

MRI - magnetic resonance imaging

NCAM - neural cell adhesion molecule

NEAA - non essential amino acids

NPs - neural progenitors

NSCs - neural stem cells

OB - olfactory bulb

Oct-4 - POU Transcription Factor-4

Olig2 - oligodendrocyte lineage transcription factor 2

OT - olfactory tract

Otx - orthodentical homologue

Pax - paired box

PBS - phosphate buffered saline
PCNA - proliferating cell nuclear antigen
PD - Parkinson's disease
PEST - penicillin-streptomycin
PFA - paraformaldehyde
PH3 - phosphorylated histone H3
PSA - polysialic acid
RA - retinoic acid
RMS - rostral migratory stream
RT-PCR - reverse transcriptase-polymerase chain reaction
SA - Sahlgrenska University hospital
SCID - severe combined immunodeficient
SD - standard deviation
SEM - scanning electron microscopy
SGZ - subgranular zone
Shh - sonic hedgehog
Sox - sex determining region of Y-chromosome
SR - serum replacement
SSEA - stage specific embryonic antigens
SVZ - subventricular zone
TEM - transmission electron microscopy
TFs - transcription factors
TGFβ - transforming growth factor beta
THF - tetrahydrofuran
TRA - tumour rejection antigen
TUNEL - terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling
VOE - ventriculo-olfactory extension
VONS - ventriculo-olfactory neurogenic system
VZ - ventricular zone

Table of contents

Abstract 3

Populärvetenskaplig sammanfattning på svenska 5

Papers included in the thesis 7

Abbreviations 8

Background 15

Stem cells from concept to thought 15

What is a stem cell? 15

Stem cells at different levels of maturation 16

Stem cells in the embryo 17

Stem cells in the developing brain 18

Differentiation and migration of neural progenitor cells 18

Neural inducing/directing signals 19

Neural inducing molecules 20

Stem cells in the adult brain 21

Neurogenesis and gliogenesis 21

The rostral migratory stream (RMS) 22

Migration and differentiation inducing molecules 22

The function of the olfactory system 23

Differentiation from embryo to adult brain 24

Differentiation 24

Neural stem cells 24

Neuroectodermal cell type markers 24

Generation of and *in vitro* culturing methods for human embryonic stem cells 27

Derivation of a human embryonic stem cell line 27

Characterization of an hESC line 30

Feeder-free culture of hESCs 31
Neural progenitors from hESCs 32

Potential benefits of embryonic stem cell research 32
Problems to be overcome for the success of cell-based therapies 33
Stem cell therapy in neurological disorders 34

Tissue engineering 35
Scaffolds for hESC propagation and differentiation 35

Aim of these studies 37

Experimental procedures 38

Ethical approval 38

Human embryonic stem cell (hESC) lines (paper I, II, III) 38

Preparation of conditioned VitroHES-medium (paper I and II) 38

Transfer of hESCs to Matrigel (paper I) 39

Viability study on hESC clusters dissociated mechanically vs. enzymatically (paper I) 40

The hESC cluster sizes after dissociation (paper I) 40

Passage of Matrigel propagated hESCs (paper I, II and III) 40

Derivation and propagation of neural progenitor cells (paper II) 41

In vitro differentiation of hESC-derived neural progenitor cells (paper II) 42

Electrospun fiber for co-culture and differentiation of hESCs (paper III) 42

Human tissue collection (paper IV) 43

Characterization of undifferentiated hESCs, NPs, mature derivates, and RMS neuroblasts (paper I, II, III, IV) 44
Immunocytochemistry (paper I, II, III) 44
Immunohistochemistry (paper IV) 44
Alkaline phosphatase (ALP) expression (paper I, II) 47
Telomerase activity (paper I) 47
Karyotyping and fluorescence in situ hybridization (FISH) (paper I) 48
Teratomas (paper I) 48
Reverse transcriptase-polymerase chain reaction (RT-PCR) (paper I, II, IV) 49
TUNEL staining (paper IV) 52
Cryopreservation of Matrigel cultured hESCs and hESC-derived NPs (paper I and II) 52
Statistics (paper II, III) 53
Electrospun polymer fiber generation (paper III) 53
Surface morphology and structural properties of electrospun polymer scaffolds (paper III) 54
Scanning electron microscopy (SEM), (paper III) 55
Transmission electron microscopy (TEM), (paper IV) 55
Magnetic resonance imaging (MRI) of human brains (paper IV) 56
Results and discussion *57*
Paper I 57
Mechanical dissociation is more efficient than enzymatic dissociation when transferring hESC cultures to feeder-free conditions 58
The cluster size after dissociation is important for transfer and for passage 59
The conditioned medium was optimal from mEFs in passage 2, day 1-3 60
The percentage of mitotic cells was similar in feeder and feeder-free hESC cultures 60

Successful cryopreservation by slow rate freezing and rapid thawing of feeder-free hESC cultures 60
Our hESCs maintained pluripotency and other hESC characteristics after transfer to feeder-free conditions 61

Paper II 62

Matrigel propagated hESCs for NP generation 62
Gelatine and laminin substrates function equally well for cell attachment and NP derivation 63
Rosette formations in passage 1 63
FGF2 is required for the derivation and maintenance of NPs 64
Cell density affects cell fate 64
Neuroectodermal markers are expressed by our NP cultures 65
PAX6 and Sox1 gene expression in the NP cultures 66
Sox3 gene expression in the NP cultures and its mature derivates 66
Gradually declining Oct-4 expression required for NP derivation 67
GFAP is expressed by undifferentiated NPs and its derivates 67
Mature neurons and glial cells are derived from the NP populations 67
Some mesodermal markers are found in the NP cultures 68

Paper III 68

Human ESCs attach and proliferate on electrospun fibrous scaffolds 68
A neuronal cell fate was induced in cells grown on electrospun scaffolds 69
The 3-dimentional scaffolds affect hESC cell fate determination 70
The interaction between hESCs and the scaffolds were shown by SEM micrographs 71

Paper IV 71

The anatomical location of the RMS in the human brain 71
Cell death is not the fate of most of the RMS progenitor cells 72
Progenitors in the RMS have migratory proteins and a migratory morphology 72
Ultrastructural studies verify that the human SVZ contains cells with migratory-like morphology 73

Ultrastructural studies reveal progenitors at all levels of the RMS that have migratory morphology 74
Directed migration of progenitors in human VONS 74
Progenitor cells become neurons in the OB 75
The human RMS is organized around a tubular extension of the lateral ventricle that reaches the OB 75
Pax6, Olig 2, and DCX gene expression is consistent with differentiation along the VONS 76

Conclusions and Significance ***77***

Conclusions from paper I **77**

Conclusion from paper II **77**

Conclusions from Paper III **78**

Conclusions from paper IV **78**

Responses to given aims ***79***

Acknowledgements ***80***

References ***82***

Background

Stem cells from concept to thought

What is a stem cell?

All stem cells, regardless of their source, have three important characteristics that distinguish them from other types of cells in the body; 1, they are capable of dividing and self-renewal for long periods; 2, they are unspecialized cells; and 3, and they give rise to all specialized cell types. Under certain physiologic or experimental conditions, they can be induced to become tissue- or organ-specific cells with special functions such as the beating cells of the heart muscle or dopamine producing neurons of the brain [1].

Most work on stem cells is done with either embryonic stem cells (ESCs) or adult stem cells (ASCs) from rodent or primate species. ESCs are primitive (undifferentiated) cells derived from a preimplantation embryo with the capacity to self-replicate indefinitely. They have the potential to become any cell type of the adult body, thus they are said to be pluripotent. The ASCs (or somatic) on the other hand are undifferentiated cells found in a differentiated tissue that typically generate the cell types of the tissue in which they reside. They can self-renew, with limitations, and they can differentiate to form cell types of tissues other than the type in which they reside [1]. Only very recently (in 2006) a new type of stem cell was derived. Researchers made a breakthrough by identifying conditions that would allow some specialized adult cells to be "reprogrammed" genetically to assume a stem cell-like state. These new stem cells are called induced pluripotent stem cells (iPSC; [2]).

Stem cells at different levels of maturation

The ultimate stem cell is the fertilized egg, the zygote, which has the ultimate potential, since it can generate a fetus, and is thus said to be totipotent (figure 1). As the totipotent cells divide it generates pluripotent stem cells that have the unique ability to self-renew indefinitely and can generate all cell types of the adult body [3]. Although, they do not have the capacity to generate a fetus, since the pluripotent cells lack the ability to generate the placenta and other tissue necessary for development in the uterus. As the pluripotent cells differentiate, their capacity for self-renewal becomes limited and they gain the potential to differentiate. As time goes on they become multipotent like neural stem cells (NSCs) that can only generate the cell types of the tissue in which they reside, that is neurons and glial cells. Finally, a unipotent stem cell is a specialised cell in adult organisms capable of differentiating along only one lineage. The adult stem cells in many differentiated, undamaged tissues are typically unipotent and give rise to just one cell type under normal conditions (figure 1), [1].

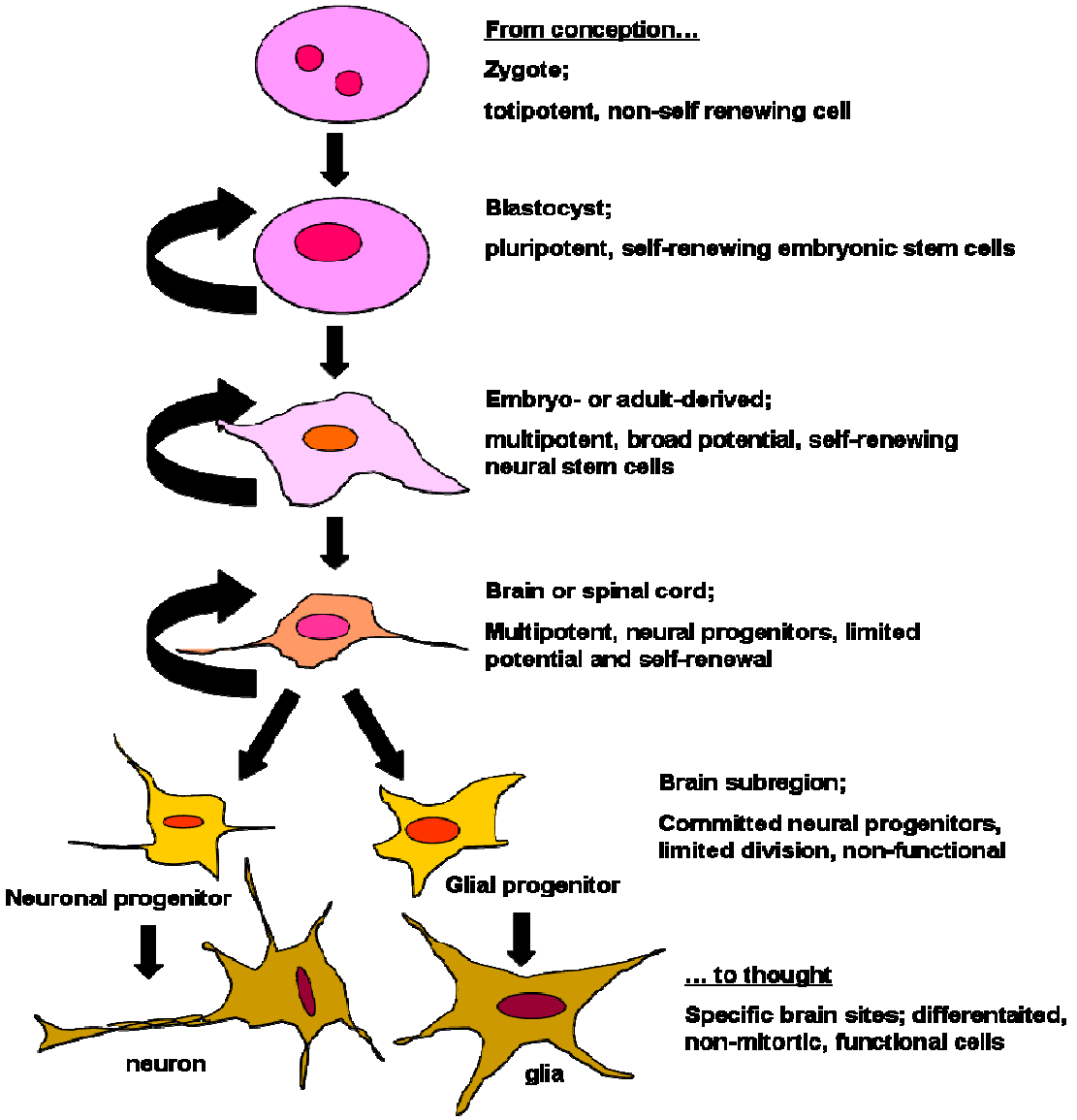

Figure 1; Stem cells from conception to thought. A schematic figure of stem cells with neural capacity at different levels of maturation.

Stem cells in the embryo

The fertilised egg undergoes multiple divisions to generate a blastocyst. The blastocyst is primarily composed of three structures; 1, the outer trophoblast, which is the layer of cells that surrounds the blastocoel; 2, a hollow cavity inside the blastocyst; 3, the inner cell mass (ICM), which is a group of cells at one end of the blastocoel that develop into the embryo proper [1]. From the ICM regionalised cellular differentiation takes place to form three major embryonic germ layers; endoderm, mesoderm, and ectoderm. These cells go

on to form all the tissue types of the body in a strictly temporal and spatial order [4]. The ectoderm (external layer) gives rise to neural cells and skin; the mesoderm (middle layer) gives rise to muscle and blood cells; the endoderm (internal layer) gives rise to the internal organs [1, 3], (figure 2).

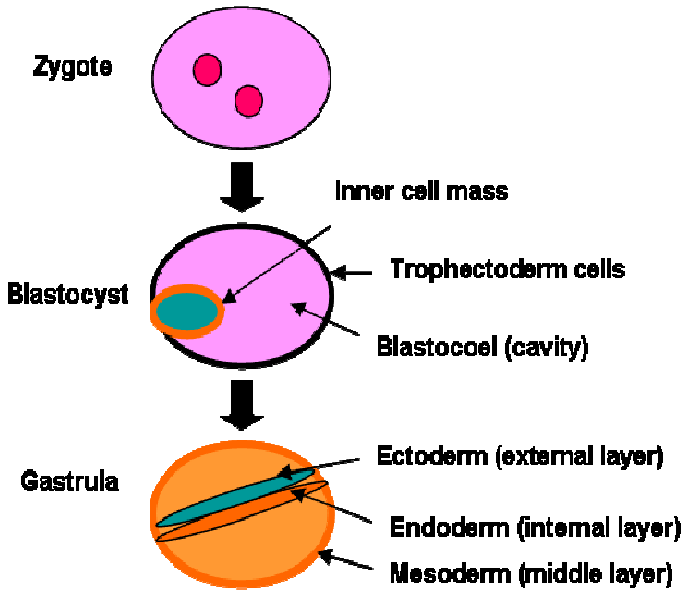

Figure 2; A schematic figure of the stem cell development in a blastocyst, from the fertilized egg (zygote) to the gastrulation stage. The embryonic germ layers (mesoderm, endoderm and ectoderm) are the source of all cell types in the adult body.

Stem cells in the developing brain

Differentiation and migration of neural progenitor cells

In the embryo, precursor cells are located in the ventricular zone (VZ), in the inner most cell layer surrounding the lumen of the neural tube. These precursors undergo a number of cell divisions and the postmitotic differentiated cells then migrate away from the ependymal zone towards the marginal zone. Extracellular factors direct the cells either to become neurons or glial cells and the migration of neurons in several regions occurs by neurons migrating along specialized type of glial cells called radial glia [5]. The generation of new functional neurons is a complex process that is tightly controlled by extrinsic signals and that is characterized by stage-specific gene expression programs and cell biological processes. Although, the

transcription factors (TFs) regulating such stage-specific developmental steps in adult neurogenesis are largely unknown [6].

Neural inducing/directing signals

Neural induction represents the earliest step in the determination of ectodermal cell fates [7]. The neuroectoderm is first seen as a sheet of cells, the neural plate, which differentiates to finally generate the three major cell types in the nervous system; neurons, astrocytes and oligodendrocytes. Neuralization of the pluripotent embryonic stem cells is coupled to inductive signals, highly conserved among different species, driving this pathway [7]. These signals include bone morphogenetic proteins (BMPs; downregulation), fibroblast growth factors (FGFs), Wnt-proteins, Sonic hedgehog (Shh), retinoic acid (RA) and other TFs like paired box (Pax), engrailed (En1), gastrulation brain homebox (Gbx) and orthodentical homologue (Otx). The inductive signals are secreted either by the cells themselves or by neighboring tissues in unique spatial and temporal order during the embryonic development [5, 8]. These signals (Wnts, FGFs and RA) direct the ectoderm germ layer to transform into the neural plate, then further into the neural tube, but also to maintain the neural fate and subdivision of the neural tube into spinal cord, fore- mid- and hindbrain [5, 8]. The "organizer" region/node both induce and organize these neuralizing signals by secreting factors like noggin, chordin and follistatin (inducing neural signals or inhibiting mesodermal signals), activin, members of the BMPs (BMP4 and BMP7) and FGF3 [7]. The FGF3 signal has an active role in the neural induction through inactivation of the BMPs. BMPs are multi-functional growth factors that belong to the transforming growth factor beta (TGFβ) superfamily [9]. BMP signaling plays critical roles in heart, neural and cartilage development. The activity of BMPs is regulated extracellularly by several families of secreted, negatively-acting factors like noggin, chordin and follistatin. These BMP

antagonists participate in the control of a diverse range of embryonic processes, such as establishment of the dorsal-ventral axis, neural induction, and formation of joints in the developing skeletal system. The ongoing process of neurogenesis in the adult brain also requires inhibition of BMP ligand activity [10]. BMP inhibition is a conserved feature across all species and stand as as the hallmark of neural induction. This inhibition may be achieved through distinct mechanisms in different species, at the level of transcriptional regulation of BMP messages, by the clearance of secreted BMP proteins by multiple inhibitors and, possibly, by other mechanisms such as translational control that are necessary to ensure a complete elimination of BMP signals [7].

Neural inducing molecules

The combination of FGF, TGFβ, activin, Wnts, antagonist of BMP signalling and other growth factors have been reported to sustain human embryonic stem cells (hESCs) in an undifferentiated state [11-18]. The morphogen FGF2 has the ability to effect in both neuralizing embryos and in keeping the hESCs in an undifferentiated state [19-21]. The TFs FGF8, Wnts and Shh are required for generation of midbrain dopamine neurons from hESCs [22], while a combination of the factors RA and Shh have a central role in directing ESCs into more mature neurons [23]. Many Sox (sex determining region of Y-chromosome) transcription factors play important roles in regulating cell differentiation. The numerous members of this family are organized in several subgroups according to structural identities found within the proteins [24]. Further, neural cell adhesion molecule (NCAM) is a member of the Ig superfamily of adhesion molecules [25] and its expression of non-polysialylated NCAM enables interactions that induce differentiation and neurite outgrowth while reducing proliferation and motility. The underlying mechanism leading to differentiation consists of enabling

heterophilic NCAM signals at homotypic cell-cell contacts that otherwise are prevented by polysialyation [26, 27].

Stem cells in the adult brain

Neurogenesis and gliogenesis

During adult neurogenesis, NSCs generate functional neurons through a coordinated series of steps, including cell fate specification, migration, axonal and dendritic growth, and synaptic integration into the brain [28]. In the adult brain neurogenesis occurs in the subventricular zone (SVZ) of the lateral ventricle and in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG), while in newborns the neurogenesis is mainly focused in the VZ. In the adult SVZ, the glial fibrillary astrocytic protein (GFAP)+, S-100 calcium-binding protein beta subunit (S100B)-, Nestin+, Sox2+ quiescent population of radial cells called the B cells, give rise to Mash1+ transient amplifying progentiors (the C cells; [29, 30], which in turn give rise to polysialic acid (PSA)-NCAM+ neuroblasts (A-cells) that migrate towards the olfactory bulb (OB) through what is known as the rostral migratory stream (RMS), in all mammals including humans [30, 31]. Within the OB these new neurons differentiate into two types of interneurons; granular neurons and periglomerular neurons [28]. On the other hand, in the SGZ, GFAP+, Sox2+, Nestin+ radial glia-like cells, believed to be quiescent NSCs [32], give rise to transient amplifying progenitors which in turn give rise to doublecortin (DCX)+ neuroblasts that give rise to local granule cells presumably glutamatergic excitatory neurons [28]. In contrast, the gliogenesis occurs throughout the whole central nervous system (CNS) [5].

The rostral migratory stream (RMS)

The RMS (figure 3) is the main pathway by which newly born SVZ cells reach the OB in rodents, rabbits and primates. However, the RMS in the adult human brain has been elusive. In the rodent brain the RMS contains progenitor cells that migrate from the SVZ, adjacent to the lateral ventricle, out to the OB. The RMS takes a course rostral to the striatum and then the cells migrate forward in the olfactory tract (OT) to the OB. The human forebrain follows the basic structural organization of the mammalian brain, but is extensively developed compared to the rodent. The human OB, and hence the olfactory interneuron replacement system, is comparatively smaller than in rodents and is anatomically organized differently and therefore the RMS has remained elusive in the human brain [31, 33].

Migration and differentiation inducing molecules

Many factors affect cell migration and differentiation in the adult human brain. Four important factors that regulate differentiation and migration of neural progenitors along the human ventriculo-olfactory neurogenic system (VONS; figure 3) are Pax6, oligodendrocyte lineage transcription factor 2 (Olig2), DCX, and PSA-NCAM. The human VONS include the SVZ, the RMS, the OT, and the OB [31]. Pax6 induces differentiation, important for the fate specification of progenitor cells into periglomerular neurons in the OB [34]; Olig2 inhibits olfactory neuron differentiation; DCX promotes cell migration; and PSA-NCAM is expressed by migrating cells and promotes migration (see Rutishauser 2007/8 for review) [35, 36]. Both proteins PSA-NCAM and DCX are important for cell migration in rodent RMS [37-39].

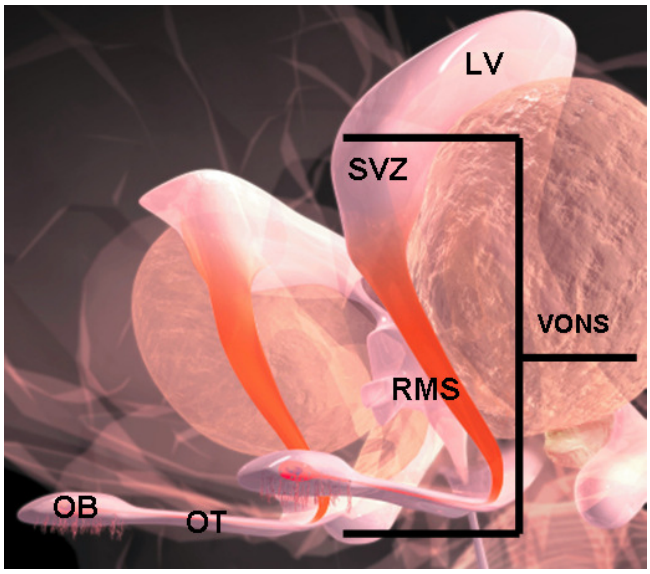

Figure 3; Schematic figure showing the pathway of the migrating neural progenitors (NPs) of the human rostral migratory stream (RMS). The NPs migrate from the subventricular zone (SVZ) adjacent to the lateral ventricle (LV), overlaying the caudate nucleus (CN), through the olfactory tract (OT) and finally reach the olfactory bulb (OB). The human ventriculo-olfactory neurogenic system (VONS) contains the SVZ, the RMS, the OT, and the OB. Illustration modified from Curtis el al. 2007.

The function of the olfactory system

The olfactory system is an important survival system to detect signs of danger such as smoke or contaminated food. The addition of new neurons in the human OB in adulthood may contribute to plasticity in this system. In rodents, the turnover of neurons in the adult OB is regulated by experience and modulates the circuitry in response to external stimuli. Some indications on the functional role of adult OB neurogenesis in humans may be gained from pathological conditions. Progenitor proliferation in the SVZ and neuroblast migration is reduced both in animal models and in patients with Parkinson's disease (PD) [40, 41]. Reduced OB neurogenesis in rodents results in impaired odor discrimination [42], a common and early sign of Parkinson's disease in humans. The presence, but also the function, of adult neurogenesis may be conserved from lower mammals to humans.

Differentiation from embryo to adult brain

Differentiation

Differentiation is the process by which unspecialized stem cells give rise to specialized cells (figure 2). During this process the cell passes through several stages, and becomes more specialized at each step. The internal signals for differentiation are controlled by a cell's genes, carrying coded instructions for all cellular structures and functions. The external signals on the other hand, come from chemicals secreted by other cells, physical contact with neighboring cells, and certain molecules in the microenvironment. Many of the triggers for these inside and outside signals for differentiation process is not yet fully understood and many questions about stem cell differentiation remains. To address these questions may give us new ways to control stem cell differentiation in vitro, thereby growing cells or tissues that can be used for specific purposes such as cell-based therapies or drug screening [1].

Neural stem cells

NSCs are primary progenitors that give rise to neurons and glia in the embryonic, neonatal and adult brain. NSCs divide asymmetrically and often amplify the number of progeny they generate via symmetrically dividing to form intermediate progenitors [30]. NSCs in the brain are considered to be restricted in terms of cell fate and will only give rise to three major cell types of the CNS: neurons and two categories of non-neuronal cells, astrocytes and oligodendrocytes. The NSCs are thus said to be multipotent (figure 1), [1].

Neuroectodermal cell type markers

Most markers used to identify neural cells are not limited to neural cells but may also be expressed by other non-neural cell types. To define a specific cell type therefore requires a large battery of markers (show presence or

absence of expression) combined with morphological and functional indicators. Through the differentiation process hESCs alter their gene expression profile and hence their protein production. The TF Oct4 (Octamer Transcription Factor-3) is expressed by pluripotent cells of the ICM (the ESCs) and by the primitive ectoderm [43-45]. Oct4 gradually decreases with the onset of neural progenitor (NP) markers like Sox2, Pax6, Nestin, musashi1, and NCAM. A rapid downregulation of the POU Transcription Factor-4 (Oct-4) in the differentiating hESCs results in a extraembryonic cell fate rather than a neural differentiation [11, 46], suggesting that transiently sustained levels of Oct4 expression may be required for *in vitro* differentiation of hESCs into neural lineages. Rapid down regulation of Oct4 expression in hESCs might promote the formation of primitive endoderm [12, 46-49]. Neuroectodermal cells within the neural tube are characterized by expression of several markers including Sox1, Sox2, nestin [50-52], musashi-1 [53] and NCAM [54, 55]. Sox1, Sox2 and Sox3 have equivalent functions. The TF Sox2 belongs to the highly conserved Sox gene family [56]. The Sox2 protein is essential for neural induction of the ectoderm, expressed in neuroepithelial stem cells during embryonic development [57], and retains neural progenitor identity thus counteracts neuronal differentiation [58]. Sox2 has also been detected in some differentiated neurons and is expressed in the adult brain, by subtypes of postmitotic neurons. [50-55]. Nestin, a neural intermediate filament protein, is expressed during early developmental stages and during regenerative processes in muscle and neuronal cells. It is a primary marker for the identification of neuroepithelial/neural progenitor cells [59, 60], but is also widely expressed in the developing embryo e.g. in endocrine progenitor cells, vascular endothelial cells [61], testis [62] and skeletal muscle [63]. Musashi-1 is an RNA-binding protein that is highly expressed in neural progenitor cells, including neural stem cells. Musashi-1 is gradually down-regulated during the course of neural differentiation and

localized in the cytoplasm in embryonic neural progenitor cells [53, 64, 65]. Pax6, a member of the paired box gene family, is expressed in developing and adult brain [66], and is one of the key factors for CNS patterning [67]. It has a dual role in controlling both the degree of adult neurogenesis and periglomerular neuron fate, and was shown to be crutial for generation of neuronal progenitors as well as for direction of neurons towards the glomerular layer and acquisition of a dopaminergic phenotype [34]. Olig2 promotes adult oligodendrogliogenesis, opposes the neurogenic role of Pax6, and specifies the transient amplifying precursor state of neural progenitors in the adult brain [34]. DCX, a microtubule-associated protein expressed in migrating neuroblasts, promotes cell migration [68]. DCX is a widely used marker for newly generated neurons in mammals, and has a critical function in the movement of newly generated neurons in the adult brain. DCX is required for nuclear translocation and maintenance of bipolar morphology during migration in the adult forebrain [38]. DCX is necessary for embryonic radial migration and migration of adult SVZ cells [39]. Another important marker for neuroblasts is PSA and it is attached exclusively to NCAM. PSA on NCAM is developmentally regulated thus playing a prominent role in different forms of neural plasticity spanning from embryonic to adult nervous system including axonal growth, cell migration, synaptic plasticity, neuronal-glial plasticity, embryonic and adult neurogenesis [36]. PSA-NCAM is a molecule abundant in the developing nervous system, although absent during the early phases of neurogenesis [69]. During CNS development PSA-NCAM is considered a marker of immature NPs [70]. PSA-NCAM is associated with most parts of the olfactory system, including the RMS [71]. βIII-tubulin (TuJ1) is an early neuronal marker [72].

Generation of and *in vitro* culturing methods for human embryonic stem cells

Human ESCs are derived from embryos generated through *in vitro* fertilization procedures and donated for research after informed consent by the donor pair. They are *not* derived from eggs fertilized in a woman's body. The hESCs *in vitro* cannot give rise to a complete organism, because they do not have the three dimensional environment that is essential for embryonic development *in vivo*, and they lack the trophectoderm and other tissue that support fetal development [1].

Derivation of a human embryonic stem cell line

To generate a hESC line *in vitro* several steps are taken;

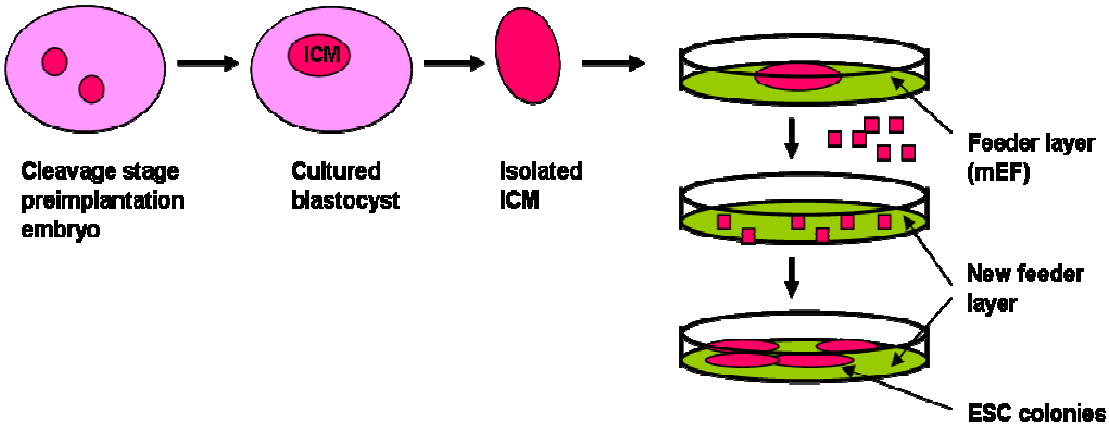

Figure 4; Schematic figure describing the process of deriving a human embryonic stem cell line (hESC) from a preimplantaion embryo at the blastocyst stage, by isolating the inner cell mass (ICM). The ICM is transferred to a culture dish coated with mouse embryonic feeder (mEFs) cells. Outgrowths of the ICM is dissociated into small pieces and transferred to a new culture dish coated with new mEFs. The small cell pieces will attach and divide to form hESC colonies, thus a hESC line is formed.

1. Culturing of the preimplantation embryo to one of the earliest stages of embryonic development called the blastocyst (figure 4).

2. The hESC line is established by isolation of the ICM from the 4-5 day old blastocyst (figure 4). Isolation of the ICM can be done by spontaneous hatching or by isolation of the ICM by enzymatic treatment to remove the zona pellucida [73].

3. The ICM is then plated on to a tissue culture dish precoated with mEF or human embryonic fibroblasts (hEF) in defined hESC medium (nutrition mix including serum; [20, 74]), (figure 4). The feeder cells in the bottom of the culture dish provide the ICM cells a sticky surface to which they can attach. Also, the feeder cells release nutrients into the culture medium. The ICM cells divide and spread over the surface of the dish. The feeder layer cells are treated (by irradiation or enzymatic mitomycine C treatment) so that they can not divide.

4. After 9-15 days of culturing the ICM-derived outgrowths are dissociated into small pieces by enzymatic (dispase or collagenase IV) or mechanical treatment (figure 5A) and replated on fresh mEF or hEF layers in new hESC medium.

5. Individual colonies with undifferentiated morphology are then selected and mechanically dissociated into small pieces (figure 5A) and replated under the same conditions, thus generating a hESC line (figure 4).

6. Through the process of re-plating or subculturing, the cells can be repeated many times over and for many months/years. Each cycle of subculturing the cells is referred to as a passage that results in the expansion of the cell cultures. When growing hESCs on feeder cell

layers, the colonies are mechanically cut every five days and the pieces are then transferred to new feeder layers in fresh hESC culturing media. When growing without feeder layers (figure 5B) the hESCs are treated with an enzyme every 5-8 days to detach the colonies from the culture surface. To grow the hESCs without mEF cells is a significant scientific advantage since the risk of viruses or other macromolecules being transmitted to the human cells is eliminated [1].

7. Once the cell line is established, the original cells yield millions of ESCs. Human ESCs that have proliferated in cell culture for six months or more without differentiating, are pluripotent, and appear genetically normal are referred to as an hESC line. At any stage in the process, batches of cells can be frozen and shipped to other laboratories for further culture and experimentation [1].

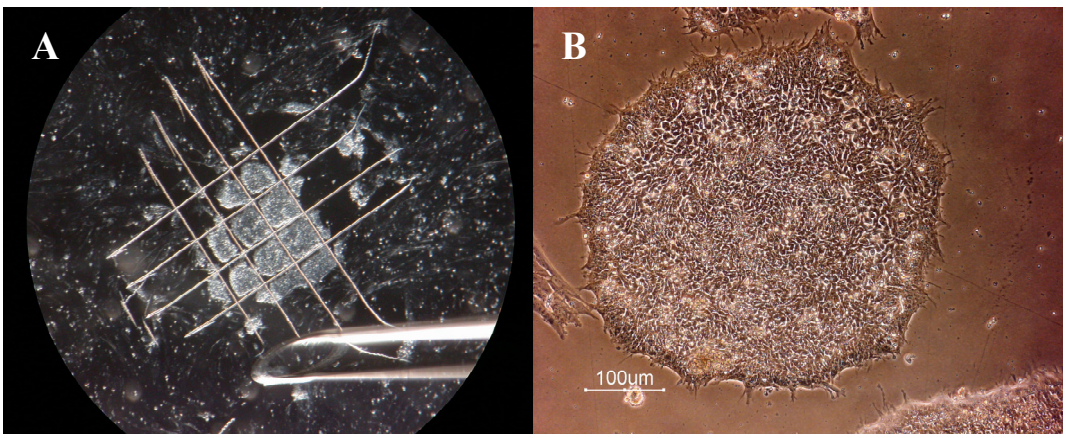

Figure 5; Undifferentiated human embryonic stem cell (hESC) colonies. A. Manual passage by cutting the hESC colony propagated on a layer of mitomycine C treated mouse embryonic feeder (mEFs) cells. B. Feeder-free culture of hESCs on Matrigel without the use of an mEF layer.

Characterization of an hESC line

To date there is not an agreed upon standard battery of tests that measure the cells' fundamental properties, however, several different kinds of tests can be used to say that you have a true ESC line [1, 3, 75] and these are;

1. Identification of specific cell surface markers associated with undifferentiated hESC, cell surface antigens; SSEA-3 and SSEA-4 (stage specific embryonic antigens), the lack of SSEA-1 which is up regulated in differentiated cells, TRA-1-60 and TRA-1-81 (tumour rejection antigen 1).
2. The expression of alkaline phosphatase (ALP; enzyme).
3. Morphological appearance; growing in tight monolayer colonies with spherical cells devoid of processes.
4. Proof of pluripotency, generation of progeny from all three embryonic germ layers (mesoderm, endoderm and ectoderm). This can be done *in vitro* by spontaneous differentiation (embryoid bodies; EBs) or by manipulating the cells to differentiate into specific cell types of all three germ layers, and *in vivo* by generation of teratomas (injecting hESCs into severe combined immunodeficent beige mice; SCID mice).
5. Growing and subculturing the hESC line for many months to ensure that the cells are capable of long-term growth and self-renewal [1].
6. Retain pluripotency for at least twelve months, while retaining a normal karyotype. The chromosomes are examinined under a microscope to assess whether the chromosomes are damaged or if the number of chromosomes has changed.
7. Active telomerase (high levels) and long telomeres. High levels of telomerase activity [3, 20, 74], hTERT (the catalytic component of

telomerase) is expressed at high levels in undifferentiated hESCs and downregulated upon differentiation [76].

8. Determine the presence of TFs that are typically produced by undifferentiated cells. TF help turn genes on and off at the right time, which is an important part of the processes of cell differentiation and embryonic development. Expression of Oct-4 and Nanog, two of the most important TFs, that function to keep the ESCs in a proliferating and non-differentiating state [1].
9. Determining whether the cells can be re-grown, or subcultured, after a cycle of freeze, thawing, and re-plating [1].

Feeder-free culture of hESCs

Human and mouse ESCs are traditionally derived and cultured on mEF layers [3] but can be propagated on hEFs [77-79] or in a feeder-free environment [15, 74, 80-83]. Future replacement therapies using hESC-derived cells or tissues will require that the cells and tissues are produced without contact with any animal source (xeno-free). Contamination of hESCs grown on animal feeder layers has been shown [84]. The standard methods for derivation of hESC lines requires the use of either hEFs or mEFs for co-culturing [3, 77, 85-87]. Even though today there are feeder-free [14] and xeno-free [88] derived hESC lines, most hESC lines used today where initially derived on mEFs and hence are not allowed in human therapies. Furthermore, the use of hESCs for replacement therapies, functional genomics, and drug screening also relies on the availability of routine large-scale culturing protocols for undifferentiated hESCs [83, 89]. The use of enzymes for dissociation when expanding the cultures makes passaging much less laborious, thus enabling large scale production of these cells.

Neural progenitors from hESCs

The most commonly used protocols for the generation of NPs from hESC involve multicellular aggregates called EBs, long term culturing, co-cultures and/or genetic manipulations [59, 60, 90-98]. These methods are often practically inconvenient and also involve poorly defined medium conditions that can lead to varying culture conditions. More recent publications describe the derivation of monolayer cultures of NPs from hESC [46, 99-102]. Even though the latter protocols are simpler than previously published methods they still contain multiple steps, the use of conditioned medium, extended derivation times or the addition of many growth factors. Moreover, little is reported on long term maintenance, large scale production, and/or cost efficient generation of stable hESC-derived NP populations in adherent monolayer cultures. Large cell quantities of NPs will be required for future replacement therapies, toxicology testing and drug screening in the pharmaceutical industry.

Potential benefits of embryonic stem cell research

The potential applications of hESCs to human disease are many, as shown by real advances made recently with human diseases in animal models. Studies transplanting cells derived from human and monkey ESCs into animal models have shown correction or partial correction of PD [103, 104] and hESC- derived oligodendrocytes have improved spinal cord injuries in rats [105, 106]. Furthermore, in late January 2009, the California-based company Geron received FDA clearance to begin the first human clinical trial of cells derived from hESCs in patients with acute spinal cord injury [107]. Perhaps the most important potential application of hESCs is the generation of cells and tissues that could be used for cell-based therapies. Today, donated organs and tissues are often used to replace ailing or destroyed tissue, but the need for transplantable tissues and organs far outweighs the available supply. Stem

cells, directed to differentiate into specific cell types, offer the possibility of a renewable source of replacement cells and tissues to treat diseases [1] and disorders throughout the body. Potential cell therapies with differentiated hESCs include retinal neurons for retinitis pigmentosa, dopaminergic neurons for PD, and motoneurons and oligodendrocytes for spinal cord injury [108]. For nervous system disorders, a possible restoration of cellular and functional loss is the goal. Many other diseases could benefit from hESC research, for instance autoimmune diseases including diabetes, rheumatism, multiple sclerosis, and lupus; also chronic heart failure (after stroke); end-state kidney disease; cancer; muscular dystrophy; fibrosis and hepatitis; and burns. Other benefits from hESC research will be to better understand the complex events occurring during human development, like finding out which genes regulate cell differentiation. Furthermore, to understand the kind of errors that cause abnormal cell differentiation and division causing cancer and birth defects, chromosomal defects and determination of the proteins stem cells express during differentiation. In addition, the methods for developing new drugs and security tests, like screening for toxins, in the pharmaceutical industry could undergo dramatic changes thanks to the development of hESC based methods [1].

Problems to be overcome for the success of cell-based therapies

Regenerative medicine in human subjects using transplanted stem cells, or their progeny, faces serious technical hurdles like; 1, transplantation rejection and the monitoring of this, 2, efficient guidance of hESCs down the correct pathway of differentiation using growth factors, and 3, and ensuring that cells of such great proliferative potential do not develop into cancers [109].

Stem cell therapy in neurological disorders

Neuronal loss is a common feature of many neurological disorders, including stroke, PD, Alzheimer's disease as well as traumatic brain injury. These neurological disorders are all highly debilitating diseases that usually require long-term hospitalization and/or rehabilitation at an enormous cost to the patient as well as to society. Therefore, there is an urgent need to develop effective treatments for these patients.

Recent studies have presented results that support the idea that hESCs or hESC-derivates used as donor cells transplantation may provide a future method for repairing damaged brain structures as well as enhancing functional recovery of the brain after stroke [110-113] or PD [95, 103, 114-116]. The potential of hESCs to differentiate into neural lineages have been demonstrated both *in vivo* and *in vitro* [85, 92, 95, 99, 117-121]. Large amounts of purified hESC-derived NPs are needed for the creation of an unlimited source of donor cells for neural transplantation and gene therapy, for creating standardised transplantation experiments (undifferentiated hESCs and precursors of other lineages may form tumours and foreign tissues upon transplantation). Transplantation therapies using stem cells may provide potential treatment for the restoration of cellular and functional loss in the nervous system after traumatic brain injury or other neurological disorders. Stem cells as donor elements will require the availability of a renewable source of transplantable cells. For this purpose hESCs are the ultimate source since they can be propagated indefinitely in culture, remaining undifferentiated and maintaining potential to differentiate into all the cell types of the adult body. Ideally, they will have optimal survival capacity and can be differentiated into appropriate region-specific cell types according to the requirements of the injured host tissue. Although, appropriate functional integration of grafted cells into the host nervous system and prevention of

tumor formation are concerns associated with transplantation studies that needs to be addressed before commencing with therapies in humans.

Tissue engineering

Tissue engineering is one of the major components of regenerative medicine and follows the principles of cell transplantation, materials science and engineering towards the development of biological substitutes that can restore and maintain normal function in diseased and injured tissues [122]. The most urgent problem in transplantation medicine is the shortage or lack of suitable donor organs and tissue. Human ESCs could be utilized as a cellular source to replace damaged tissue by cell transplantation or implantation of cellular scaffolds [123].

Scaffolds for hESC propagation and differentiation

Polymer fibers produced via electrospinning, widely studied both theoretically and experimentally, have been proposed for a number of applications due to their unique properties, like high surface-to-volume ratio, related to their small physical dimensions [124-127]. Generally for biomedical applications electrospun biocompatible or biodegradable polymer fibers are intended to act as three-dimensional scaffolds mimicking natural *in vivo* extracellular matrices and supporting cell proliferation [128-130]. This physical support provided by the three-dimensional porous polymer networks make them exceptionally promising within tissue engineering. Numerous studies have been performed with different applications in mind, for instance cardiovascular, cartilage, osseous and nervous tissue implants [20, 131-139]. The adult CNS has a limited capacity to recover from damage after trauma or disease, and has therefore been subjected to intense research efforts concerning tissue engineering and implants [20, 131-133, 136, 137, 140-145]. Electrospun porous scaffolds, mimicking the natural three-dimensional

environment of the *in vivo* extracellular matrix (ECM) and providing physical support, have been identified as promising candidates for CNS tissue engineering. Studies have been published where electrospun fibers are used as a scaffold to support undifferentiated cells with the intent of differentiating them into nervous tissue [136, 137]. The nanofibrous scaffold can support differentiation and promote cell adhesion, indicating that nanofibrous scaffolds may play an important role in nervous tissue engineering [131, 133].

Aim of these studies

The overall aim of this thesis was to improve and derive methods for the propagation of hESCs and hESC-derived NPs and to study the derivation, propagation and differentiation of human stem and progenitor cells.

Specific aims

I) To develop an improved and more robust protocol for the transfer of hESCs to feeder-free conditions (paper I).

II) To develop a culture method that facilitates long-term propagation and large-scale production of undifferentiated hES cells in a feeder-free environment (paper I).

III) To develop an efficient protocol for the rapid generation of an expandable population of fast growing hESC-derived NP cells with the capacity to generate mature neurons and glial cells in vitro (paper II).

IV) To find suitable materials to promote migration and differentiation of stem and progenitor cells (paper III).

V) To find critical differentiation factors and markers expressed in common between human RMS neuroblasts and hESC-derived NPs (paper IV).

Experimental procedures

Ethical approval

Ethical approval was given for studies of stem cell function and survival in the adult human brain by the regional etikprövningsnämnden in Gothenburg (Dnr 448-06). Ethical approval was also given by the research committee at Uppsala University for the research project concerning the culture of hESCs (Dnr 00-536).

Human embryonic stem cell (hESC) lines (paper I, II, III)

Initially, the hESC lines were established and maintained on a monolayer of Mitomycin C (Sigma Aldrich, Sweden) treated mEF cells (Thomson et al., 1998), and cultured in standard hESC medium (Xu et al., 2001; Amit et al., 2000), currently manufactured as VitroHES™ medium by Vitrolife AB (Kungsbacka, Sweden), and characterized according to standard criteria [3, 146]. The hESC lines SA002 [146], SA121 [146], SA167 [83], AS034 [146], and AS038 [146] were established from blastocysts collected from Sahlgrenska University hospital (SA) and Akademiska Sjukhuset (AS), respectively.

Preparation of conditioned VitroHES-medium (paper I and II)

To prepare mEF cells for conditioning of VitroHES-medium or hESC medium, mEF cells (Mitomycin C treated) where seeded in a culture flask and cultured to a confluent monolayer for 24 hours in Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 1% penicillin-streptomycin (PEST), 10% fetal bovine serum (FBS) and 2 mM GLUTAMAX-I supplement (200 mM). The mEFs where then wash with phosphate buffered saline (PBS) and the culture medium replaced with VitroHES or hESC medium (0.28 ml/cm^2) for a 24 hour conditioning period. Conditioned

medium (k-VitroHES or k-hESC medium) was collected every day for up to three times from the same mEF culture, sterile filtered, and used either fresh or after freezing (-20ºC) and supplemented with 4 ng/ml of FGF2 prior to use.

Transfer of hESCs to Matrigel (paper I)

Two different techniques (mechanical or enzymatic dissociation) were evaluated for the transfer of the hESCs onto rehydrated Matrigel plates. First the hESC colonies were mechanically cut into small square pieces carefully detached and transferred to Hanks' Balanced Salt Solution (HBSS) solution. For the enzymatic dissociation collagenase IV was used for a 30 min incubation during which repeated mechanical dissociations was preformed and the process monitored in a microscope. For the mechanical dissociation, the clusters where only mechanically dissociated with a pipette, and the process completed at an aggregate cluster size of 400-600 cells/cluster. The cell suspension generated was then pelleted, washed, resuspended in k-VitroHES medium, and transferred to rehydrated Matrigel plates at a cluster density of 10 to 15 clusters/well (6-well plate). Each experiment was repeated four times in 4 wells/dissociation technique, with the same amount of cells seeded each time. The number of cells initially used for the two different dissociation protocols were identical. After two and six days the colony size and number was calculated. The colony area was calculated by measuring the X and Y diameter of all hESC colonies with undifferentiated morphology, allowing for an approximation of cell growth. These colonies consist of a monolayer of homogenously sized cells making this approximation possible. Colony area to cell number correlation was calculated and an approximately linear correlation between colony size and cell number was found in undifferentiated Matrigel cultures. The area/cell relationship averaged at 82 μm^2 with individual cell areas ranging from 30 to 134 μm^2.

Viability study on hESC clusters dissociated mechanically vs. enzymatically (paper I)

The viability test was performed by using calcein/ethidium homodimer (calcein/EthD) kit and a comparison was preformed of the hESCs dissociated mechanically vs. enzymatically in the transfer step. The dissociated cells were resuspended in 100 μL calcein/EthD solution respectively and incubated for 10 minutes in room temperature. Each cell suspension was placed on a glass slide and a cover glass placed on top. The dead and live cells were counted manually in a microscope (Nikon Eclipse TE2000-U).

The hESC cluster sizes after dissociation (paper I)

Colonies were dissociated mechanically and enzymatically from equally sized undifferentiated hESC colonies. Suspensions of cell clusters were then incubated with Nile-red staining solution (1μM in PBS) for 10 minutes and photo documented using a fluorescence microscope (Nikon Eclipse TE2000-U). All clusters were counted and measured using ImageJ image analysis software.

Passage of Matrigel propagated hESCs (paper I, II and III)

The cell cultures were observed visually by using an inverted microscope. When ready for passage, the medium was aspirated and a collagenase IV solution was added to each well and incubated for 15 to 20 minutes. To facilitate cell detachment from the surface careful mechanical dissociation was performed followed by another 15 minutes of incubation. The cells were then washed, resuspended in k-VitroHES medium (paper I) or mEF conditioned hESC medium (paper II and III) and seeded at a split ratio of 1:3 to 1:6 onto Matrigel. The hESC cultures were passaged every 5 to 6 days and the medium was changed three times a week.

Derivation and propagation of neural progenitor cells (paper II)

At 0 days undifferentiated hESC colonies propagated on Matrigel were enzymatically dissociated (collagenase IV; 200 U/mL, 30 minutes, 37 ^{o}C) generating a cell suspension containing small cell aggregates and single cells. The cells were washed in warm basal media, pelleted and resuspended in warm culturing media. The different culturing media evaluated for NP derivation and propagation included standard hESC medium, mEF conditioned hESC medium [83], Dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM/F12) or neurobasal A medium with various supplements in different combinations (table 1; paper II). We observed that the standard hESC medium supplemented with FGF2 was the best media for the induction and propagation of the NPs from hESCs, hence this is the media used in the following experiments. The cell suspension was then plated on to different adhesive substrates (laminin, gelatine, polyornithine/laminin, polyornithine-coated Primaria treated plastic plates, Primaria plastic plates, Nunclon treated culture plates or Matrigel plates). Laminin and gelatine were the substrates used for NP derivation and propagation in the following experiments. The seeding cell density was 150-200x10^3cells/cm^2 for the NP generation. Half of the culturing medium was changed three times a week. Cells were cultured under these conditions for 8 days during which time neural rosettes [147, 148] were formed. On day 8 the cultures where treated briefly with collagenase IV to detach the rosettes (flattened cells at the edge of rosette colonies did not detach), and then mechanically dissociated to a single cell suspension by gently triturating the cell solution with a Pasteur pipette. The cells were washed and reseeded under the same conditions as before. In passage 2 adherent monolayer NP populations were generated and these cultures where expanded 1:2 or 1:3, every 4 to 7 days or when ~80% confluent. Passage for expansion of the NP cultures hereafter was preformed using collagenase IV to generate a single cell suspension that was then re-

seeded as high density cultures (50-100x10^3 cells/cm^2; lower cell densities generated differentiated cultures) to maintain a proliferating NP population.

In vitro differentiation of hESC-derived neural progenitor cells (paper II)

The hESC derived NPs where differentiated into mature neurons and glial cells as adherent monolayer cultures on laminin in hESC medium supplemented with TGF-β1 (10 ng/mL) for 7 days, or supplemented with Shh (500 ng/mL) and FGF8 (100 ng/mL) for 9 to 16 days, or supplemented with Shh 500 ng/mL, 40 ng/mL FGF2, and 1% N2, for 14 days. Alternatively, the NPs where differentiated on laminin in DMEM/F12 medium (supplemented with 1% PEST, 1% L-glutamine and 1% N2 supplement, 1% non essential amino acids (NEAA), 0.2% β-mercaptoethanol, and 20 ng/mL of FGF2) for 16 days. The NPs where also differentiated as free floating neurospheres, by seeding the cell suspension onto a non-adherent substrate, in hESC medium supplemented with Shh (500 ng/mL) and FGF8 (100 ng/mL), generating spheres, that where then plated onto an adherent (laminin) surface for 6 days.

Electrospun fiber for co-culture and differentiation of hESCs (paper III)

Undifferentiated feeder-free Matrigel hESC cultures where dissociated enzymatically by collagenase IV treatment, washed, and seeded onto Nunclon treated cell culture plates, together with 1 cm^2 sized pieces of electrospun fiber scaffolds. The culturing medium consisted of standard hESC medium supplemented with 1 % N2 and 10 ng/mL of FGF2. After 5 to 7 days of co-culture with the fibers the medium was changed to a neural differentiation medium consisting of neurobasal A basal medium supplemented with 1 % B27 and 1 % N2, omitting the growth factor FGF2. Additionally, for long term differentiation culture (18-47 days) 20 ng/mL of

epidermal growth factor (EGF) and FGF2 was added to the differentiation medium. The differentiation medium was changed three times a week and the co-cultures were allowed to propagate and then differentiate for up to 47 days.

Human tissue collection (paper IV)

The human brain tissue was obtained from the Neurological Foundation of New Zealand Human Brain Bank at the University of Auckland or from Sahlgrenska University Hospital, Sweden. These latter patients had been diagnosed with squamous cell carcinomas at the base of the tongue, in the larynx or in the pharynx and had received 5-bromo-2´-deoxyuridine (BrdU; 250 mg) dissolved in saline and given as an intravenous infusion (2.5 mg/ml, 100 ml). The BrdU was given to patients to assess the proliferative activity of the tumor cells [149, 150]. The full consent of all families was obtained prior to autopsy and the respective University Human Subjects Ethics committees approved these studies. In all cases pathological examination excluded any neuropathology. The brains were removed at autopsy and in some cases the hemispheres were separated and one hemisphere was dissected and frozen fresh and the other half was fixed or the whole brain was fixed. The brains were fixed by perfusion through the cerebral arteries, first with PBS with 1% sodium nitrite and followed by 15% formalin in 0.1 M phosphate buffer, pH 7.4. After perfusion, approximately 5 cm x 5 cm blocks of the brain containing the midline cortical surface medially, middle frontal gyrus laterally, thalamus caudally, corpus callosum rostrally, cingulate gyrus dorsally and the OT and trigone ventrally was dissected and postfixed in the same fixative for 24 h. The blocks were cryoprotected in 20% sucrose and then 30% sucrose until equilibrated. The blocks were serially sectioned in a sagittal or coronal plane on a freezing microtome (50-µm sections) and stored

serially in PBS and 0.1% azide. Hemispheres that were unfixed were dissected, frozen and stored at -80°C until required for further processing.

Characterization of undifferentiated hESCs, NPs, mature derivates, and RMS neuroblasts (paper I, II, III, IV)

Immunocytochemistry (paper I, II, III)

Immunocytochemistry is a technique used to assess the presence of a specific protein or antigen in cells by use of a specific antibody that binds the antigen and thus allow the visualization and examination under a microscope. In paper I, II, III, and IV this technique was utilized to determine the cell types present using cell type specific markers. Cell cultures and fiber co-cultures were washed in PBS, fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature and then washed again three times in PBS. The primary antibodies and cells were incubated over night at 4°C before being visualized using appropriate secondary antibodies. Cultures were also incubated with a cell nuclei stain 4´-6´Diamidino-2-phenylindole (DAPI), at a final concentration of 0.5 ug/mL for 5 minutes at room temperature, to visualize all the cell nuclei. The stained cultures were rinsed and mounted using DAKO fluorescent mounting medium or ProLong Gold and visualized with an inverted fluorescent microscope. See table 1 for primary and secondary antibodies and for dilutions. Control staining included omission of either primary or secondary antibodies and revealed neither non-specific staining nor antibody cross-reactivity.

Immunohistochemistry (paper IV)

Immunohistochemistry is a technique for localization of proteins of interest using antibodies raised against specific proteins of interest. In paper IV sagittal and coronal sections for staining with proliferating cell nuclear

antigen (PCNA) were first incubated overnight in a citric acid solution containing citric acid (Na3 salt) and Na2HPO4 (pH 4.5) before undergoing a standard antigen retrieval protocol [151]. All sections were incubated in a 50% methanol solution with 1% H_2O_2 to block endogenous peroxidases in the tissue. To detect proliferating cells, three PCNA antibodies were used (table 1), each giving identical results. In three cases detailed quantitative studies were undertaken on the number of PCNA-positive cells in the RMS. Images from serial sagittal sections (every 3rd section) were photographed at x20 magnification. Images were analyzed for PCNA-positive cells using the FindSpots algorithm (which detects objects based upon their intensity and size) in the Metamorph (v.6.2.6, Molecular Devices) image analysis program. To detect neuronally committed neuroblasts we used PSA-NCAM antibody and DCX (table 1). For fluorescent triple-labeling for BrdU and other antigens tissue underwent a standard hydrochloric acid pretreatment to enable BrdU presentation, prior to incubation in BrdU antibodies, Neuronal nuclei antibodies and GFAP (table 1). For visualization with the light microscope, species specific biotinylated secondary antibodies (dilution 1:500) were added serially, followed by Extravidin (dilution 1:1,000; Sigma) and finally 3, 3 diaminobenzidine was used to visualize the staining. The sections were mounted onto glass slides, dehydrated in graded alcohol, cleared in xylene and coverslipped. Primary antibodies where detected by addition of species specific secondary antibodies (table 1). Between each of the steps above, the sections were washed for 3 x 10 minutes in PBS and 0.1% triton X-100 for 3,3 diaminobenzidine (DAB) labeled sections and tris-buffered saline for BrdU labeled sections. The fluorescent-labeled sections were mounted and coverslipped with Citifluor and imaged by using a confocal laser scanning microscope. Each fluorescent label was imaged serially to eliminate detection of bleed-through and other artificial fluorescence. The confocal images were captured in a Z-series with an interslice gap of 1-μm. Bright-field images

were taken with a digital camera on a light microscope and the images were captured in Photoshop. Macro-photographs were captured on a free standing digital camera. Illustrations were compiled in Illustrator.

Table 1: Primary and secondary antibodies used in this study.

Antibody	Source	Immunogen	Dilution	Company
Primary abs for immunocytochemistry (paper I, II, III):				
Oct-3/4	mouse	Human Oct-3/4	1:100-1:200	Santa Cruz Biotech
SSEA-1	mouse	stage specific embryonic antigen-1	1:200	DSHB
SSEA-3	Rat	stage specific embryonic antigen-1	1:200	DSHB
SSEA-4	mouse	stage specific embryonic antigen-4	1:200	DSHB
Tra-1-60	mouse	High molecular weight glycoprotein	1:200	Santa Cruz/SDS
Tra-1-81	mouse	High molecular weight glycoprotein	1:200	Santa Cruz/SDS
Nestin	mouse	Intermediate filament protein	1:200-1:500	BD Pharmingen
Sox2	goat	the SRY-related HMG-box	1:200	Santa Cruz Biotech
Sox2	mouse	the SRY-related HMG-box	1:1000	Chemicon
NCAM	rabbit	Neural cell adhesion molecule	1:500	Chemicon
Pax6	mouse	Human recombinant Pax6	1:200	Chemicon
Musashi-1	rabbit	Neural RNA binding protein.	1:200-1:1000	Chemicon
Internexin	rabbit	Alpha-internexin	1:750	Chemicon
A2B5	mouose	Neuron cell surface antigen	1:250	Chemicon
β_{III}-tubulin	mouse	Human III β-tubulin isotype III	1:200	Sigma-Aldrich
β_{III}-tubulin	rabbit	Neuronal class III β-tubulin	1:1000	Biosite
MAP2ab	mouse	Bovine microtubule associated protein	1:100-1:200	Sigma-Aldrich
NF200	mouse	Neurofilamant 200	1:200	Sigma
TH	mouse	Tyrosine hydroxylase	1:2000	Sigma-Aldrich
TH	rabbit	Tyrosine hydroxylase	1:250	Chemicon
GFAP	rabbit	Cow glial fibrillary acidic protein	1:250-500	DAKO
GFAP			1:250	Chemicon
APC	mouse	A recombinant ammino terminal fragment of APC	1:20-100	Calbiochem
GalC	rabbit	Galactoserebroside	1:75-1:180	Sigma-Adrich
GalC	mouse	Galactoserebroside	1:200	Chemicon
Ct-1	rabbit	Human cardiotrophin-1	1:200	Chemicon
AFP	mouse	Alfafetoprotein	1:500	Sigma
PH3	rabbit	Phospho-Histone H3	1:100-1:150	KeLab
Primary abs for immunohistochemistry (paper IV);				
PCNA	rabbit	Proliferating cell nuclear antigen FL261	1:750	Santa Cruz
PCNA	mouse	Proliferating cell nuclear antigen PC10	1:500	Santa Cruz
PCNA	mouse	Proliferating cell nuclear antigen	1:500	Chemicon
PSA-NCAM	mouse	Polysialic acid neural cell adhesion molecule	1:1,000	Gift G Rougon
DCX			1:500	
BrdU	rat	Accurate	1:500	
Neuronal nuclei	mouse		1:20	

GFAP	guinea pig		1:250	
Secondary abs for immunocytochemistry (paper I, II, III):				
Alexa 488	goat	Mouse IgG	1:2000	Molecular Probes
Alexa 488	donkey	Rabbit IgG	1:2000	Jackson Laboratories
Alexa 594	goat	Rabbit IgG	1:2000	Molecular Probes
Alexa 555	donkey	Mouse IgG	1:2000	Molecular Probes
FITC	sheep	Rabbit IgG	1:800	Chemicon
FITC	donkey	Mouse IgG	1:800	Termo
FITC	donkey	Rat IgG	1:800	Jackson Laboratories
FITC	goat	Mouse IgM	1:300	Jackson Laboratories
Texas Red	donkey	Mouse IgG	1:800	Jackson Laboratories
Cy3	goat	Rat IgM	1:300	Jackson Laboratories
Secondary abs for immunohistochemistry (paper IV);				
Texas Red		Mouse IgM		
FITC	donkey	Rat	1:250	
Cy3	donkey	Mouse	1:250	
Cy5	donkey	guinea pig	1:250	
Alexa 594	donkey	Mouse	1:200	
Alexa 647	donkey	Rabbit	1:200	

Alkaline phosphatase (ALP) expression (paper I, II)

Expression of ALP in undifferentiated hESCs and NPs was analyzed following fixation of cells with citrate-acetone-formaldehyde fixative solution using a Sigma diagnostics kit.

Telomerase activity (paper I)

Enzyme-linked immunosorbent assay (ELISA), an immunoassay utilizing an antibody labeled with an enzyme marker such as horseradish peroxidase. While either the enzyme or the antibody is bound to an immunosorbent substrate, they both retain their biologic activity; the change in enzyme activity as a result of the enzyme-antibody-antigen reaction is proportional to the concentration of the antigen and can be measured spectrophotometrically or with the naked eye. Many variations of the method have been developed. In paper I we used a PCR-based ELISA, to analyse the telomerase activity in Matrigel and mEF cultured hESCs that were first harvested, lysed.

Karyotyping and fluorescence in situ hybridization (FISH) (paper I)

Karyotyping is the mapping of the full chromosome set of the nucleus of a cell. The chromosome characteristics of an individual or a cell line are usually presented as a systematized array of metaphase chromosomes from a photomicrograph of a single cell nucleus arranged in pairs in descending order of size and according to the position of the centromere. In paper I the hESCs designated for karyotyping were incubated for 1 to 3 hours in colcemid, dissociated, fixated, mounted on glass slides and the chromosomes visualized by using a modified Wright's staining.

In situ hybridization is a technique that localizes specific nucleic acid sequences within intact chromosomes, eukaryotic cells, or bacterial cells through the use of specific nucleic acid-labeled probes. Fluorescence *in situ* hybridization (FISH) is a type of *in situ* hybridization in which target sequences are stained with fluorescent dye so their location and size can be determined using fluorescence microscopy. This staining is sufficiently distinct that the hybridization signal can be seen both in metaphase spreads and in interphase nuclei. For the FISH analysis in paper I, a commercially available kit (MultiVysion™ PB Multicolour Probe Panel) containing probes for chromosome 12, 13, 17q, 18, 21 and the sex chromosomes (X and Y) was used. Slides were analyzed using an inverted microscope equipped with appropriate filters and software (CytoVision, Applied Imaging).

Teratomas (paper I)

For the teratoma formation experiment, immunodeficient SCID mice (C.B-17/lcrCrl-scidBR) were used. Matrigel propagated hESC colonies were enzymatically detached from the surface by using collagenase IV, mechanically dissociated into small cell aggregates and approximately 50 000 to 100 000 cells/organ were injected under the kidney capsule. Control animals were treated with Cryo-PBS injections or with primary brain cells

from a littermate. The animals were sacrificed eight weeks after injection and the tumors were immediately fixed in 4% PFA and paraffin embedded. For histological analysis the teratoma were sectioned to 8 μm and stained with Alcian Blue/Van Giesson.

Reverse transcriptase-polymerase chain reaction (RT-PCR) (paper I, II, IV)

PCR is an *in vitro* method for producing large amounts of specific DNA or RNA fragments of defined length and sequence from small amounts of short oligonucleotide flanking sequences (primers). The essential steps include thermal denaturation of the double-stranded target molecules, annealing of the primers to their complementary sequences, and extension of the annealed primers by enzymatic synthesis with DNA polymerase. The reaction is efficient, specific, and very sensitive. Reverse transcriptase-polymerase chain reaction (RT-PCR) is a variation of the PCR technique in which cDNA is made from RNA via reverse transcription. The resultant cDNA is then amplified using standard PCR protocols. In paper I, II and IV RT-PCR was used to detect the gene expression of cell type specific genes to determine the cell types present. Total RNA was extracted from the cell cultures (paper II) using RNeasy Plus Mini kit. In paper IV total RNA was extracted from the AOC, OT, OB and from putamen, caudate nucleus and hippocampus (as controls; paper IV). Reverse transcription of 1 μg of total RNA to cDNA was performed by using AMV First Strand cDNA Synthesis Kit (paper I) or M-MLV reverse transcriptase (paper II, IV). The PCR was carried out using 5 uL of cDNA with Platinum Taq DNA Polymerase (paper I) or TaqBead Hot Start Polymerase (paper II, IV) in a total volume of 50 uL in the presence of 50 pmol of each primer, 1.5-2 mM MgCl2. The primer pair sequences, number of cycles, annealing temperatures, and product size (bp) are summarized in table 2 for each gene analyzed. The PCR products were size

fractioned by gel electrophoresis using a 1-2% agarose gel electrophoresis and visualized after ethidium bromide staining or SYBR Safe™ DNA gel stain using a Fuji LAS-3000 imaging system. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal positive control (paper II and IV), and β-actin in paper I. For all mRNA samples a minus RT-PCR step was performed using the mRNA as template in the PCR reaction and the GAPDH primers to detect any contaminating DNA in the RNA samples. Human genomic DNA was used as a positive control for PCR reactions (paper II). Human liver was used as a positive control and water as negative control for the PCR reaction (paper I).

Comments: In paper IV the Olig2 PCR assay failed in the AOC due to unknown technical reasons. However, we were able to detect the Olig2 protein within the AOC using immunohistochemistry.

Table 2: Primer sequences, PCR cycles, annealing temperatures and product length for the RT-PCR reactions

Gene	Sequence (forward; reverse)	Cycles	Annealing (°C)	Prod. Length (bp)
Oct-4 paper II	5'-CGTGAAGCTGGAGAAGGAGAAGCTG-3' 5'-CAAGGGCCGCAGCTTACACATGTTC-3'	30	55	247
Oct-4 paper I	5′-GGCGTTCTCTTTGGAAAGGTGTTC-3′ 5′-CTCGAACCACATCCTTCTCT-3′,	2+2+2 +2+35	66+64+62+ 60+58	312
Sox2 paper II	5'-CGG AAA ACC AAG ACG CTC A-3' 5'-GCC GTT CAT GTA GGT CTG CG-3'	35	55	318
Nestin paper II	5'-CAG CTG GCG CAC CTC AAG ATG-3' 5'-AGG GAA GTT GGG CTC AGG ACT GG-3'	30	55	208
Sox1 paper II	5′-CTCACTTTCCTCCGCGTTGCTTCC -3′ 5′-TGCCCTGGTCTTTGTCCTTCATCC -3′	35	58	848
Sox3 paper II	5'-GAGGGCTGAAAGTTTTGCTG-3′ 5'-CCCAGCCTACAAAGGTGAAA-3′	35	60	131
Musashi-1 paper II	5'-GAGACTGACGCGCCCCAGCC-3' 5'-CGCCTGGTCCATGAAAGTGACG-3'	36	60	212
NCAM paper II	5′-AGGAGACAGAAACGAAGCCA-3′ 5′-GGTGTTGGAAATGCTCTGGT-3′	35	60	160
Pax6 paper II, IV	5'-CAG CTC GGT GGT GTC TTT GTC A-3' 5'-CTG CGC CCA TCT GTT GCT TTT C-3'	3+30	68+65	433
DCX paper II, IV	5′-AATCCCAACTGGTCTGTCAAC -3′ 5′-GTTTCCCTTCATGACTCGGCA -3′	30	57	405
HuD paper II	5′-CTGCTCTCCCAGCTCTA-3′ 5′-AGGCTTGTCATTCCATC-3′	45	60	148
Olig2 paper II, IV	5′-GCTGTGGAAACAGTTTGGGT-3′ 5′-AAGGGTGTTACACGGCAGAC-3′	30	57	291
GFAP paper II	5'-TCATCGCTCAGGAGGTCCTT-3' 5'-CTGTTGCCAGAGATGGAGGTT-3'	30	65	383
MAP2 paper II	5'-GCACTTCAAGGGAAGCTGAT-3' 5'-ATCAAATGGTCCACTAGGCG-3'	30	58	412
TH paper II	5'-ATCACCTGGTCACCAAGTTC-3' 5'-GTGGTGTAGACCTCCTTCCA-3'	30	60	180
Desmin paper II	5'-CAG GGA CAT CCG GGC TCA GTA T-3' 5'-AGC TTC CGG TAG GTG GCA ATC T-3'	2+2+2 +2+30	58+56+54+ 52+50	402
AFP paper II	5'-ACT CCA GCA TCG ATC CCA CTT T-3' 5'-TTC CCC ATC CTG CAG ACA ATC C-3'	2+2+2 +2+30	58+56+54+ 52+50	453
Brachiury paper II	5'-CAA TGC CAG CCC ACC TAC CA-3' 5'-GGG CCA ACT GCA TCA TCT CCA-3'	2+2+2 +2+35	65+63+61+ 59+57	562
HNF3-α paper II	5′-GAG TTT ACA GGC TTG TGG CA-3′ 5′-GAG GGC AAT TCC TGA GGA TT-3′	30	55	408
GAPDH paper II, IV	5'-ACC ACA GTC CAT GCC ATC AC-3' 5'-TCC ACC ACC CTG TTG CTG TA-3'	30	54	452
β-actin paper I	5'-TGGCACCACACCTTCTACAATGAGC-3' 5'-GCACAGCTTCTCCTTAATGTCACGC-3'	2+2+2 +2+35	66+64+62+ 60+58	400
-RT step paper II, IV	GAPDH primers and PCR program using the mRNA as template	30	54	452

TUNEL staining (paper IV)

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) is an *in situ* method for detecting areas of DNA which are nicked during apoptosis. Terminal deoxynucleotidyl transferase is used to add labeled dUTP, in a template-independent manner, to the 3 prime OH ends of either single- or double-stranded DNA. The TUNEL assay labels apoptosis on a single-cell level, making it more sensitive than agarose gel electrophoresis for analysis of DNA fragmentation. In this study TUNEL staining was preformed to detect DNA fragmentation to examine if PCNA expressed in the RMS cells was due to repair or apoptosis instead of proliferation.

Cryopreservation of Matrigel cultured hESCs and hESC-derived NPs (paper I and II)

The hESCs colonies or NP cultures were dissociated with collagenase IV before freezing. After washing and centrifugation, the cells were transferred to a freezing medium, consisting of k-VitroHES medium or hESC medium supplemented with 10% dimethyl sulfoxide (DMSO), 4 ng/ml FGF2, and serum replacement (SR) to a final concentration of 30%, with a cell density 1 million cells per ml freeze medium. The final cell suspension was added to Nunc CryoTubes and rapidly transferred to a Nalgene freezing container for storages in -80°C over night or at least for 4 hours before moved to long-term storage in a Liquid Nitrogen tank. To thaw the cells, k-VitroHES medium or hESC medium was prepared and preheated to 37°C before thawing the cells by rapidly transferring the cryotubes to a 37°C water bath until all of the cell suspension was thawed. The cell suspension was then transferred to the preheated k-VitroHES medium or hESC medium, for 5 minutes equilibration, before centrifugation. The cell pellet was washed once, resuspended in the

culturing medium and seeded into culturing plates at the same cell density as before freezing.

Statistics (paper II, III)

Cell counting data was generated from three repeated experiments, counting at least 10 randomly selected visual fields (40x magnification) per antibody and experiment. The number of positively stained cells were counted and expressed as the percentage of positive cells out of the total number of cells. The total number of cells was quantified by counting the total number of DAPI stained cell nuclei in all fields of view. The results are presented as mean ± standard deviation (SD), (paper II and III). In paper III data were analyzed using Student's t-test. * $p < 0.05$ was considered statistically significant.

Electrospun polymer fiber generation (paper III)

Biocompatible aromatic polyether based polyurethane resin (Desmopan 9370A, Bayer MaterialScience AG) was selected due to its flexible elastomeric properties, making it a suitable candidate material for electrospun cell carrier or tissue engineering applications. Polyurethane resin was dissolved in a solvent composed of 60 wt % tetrahydrofuran (THF, Scharlau) and 40 wt % *n,n*-dimethylformamide (DMF, Scharlau), obtaining a solution with 11 wt % polymer concentration. The homogenized polyurethane solution was transferred to a 20 ml plastic syringe (Omnifix, Braun). Fibrous scaffolds were manufactured with a vertical electrospinning setup. The syringe, loaded with polymer solution, was fitted with a 0.85 mm inner diameter cannula (1.2 mm outer diameter) (Sterican, Braun) connected to the positive potential of a high voltage power supply (Gamma High Voltage Research). A grounded collector was placed at a distance of 22 cm from the cannula tip. With the described setup (figure 6), scaffolds were manufactured

on aluminum foil substrates. Experiments were carried out with the cannula tip at a potential of 18 kV.

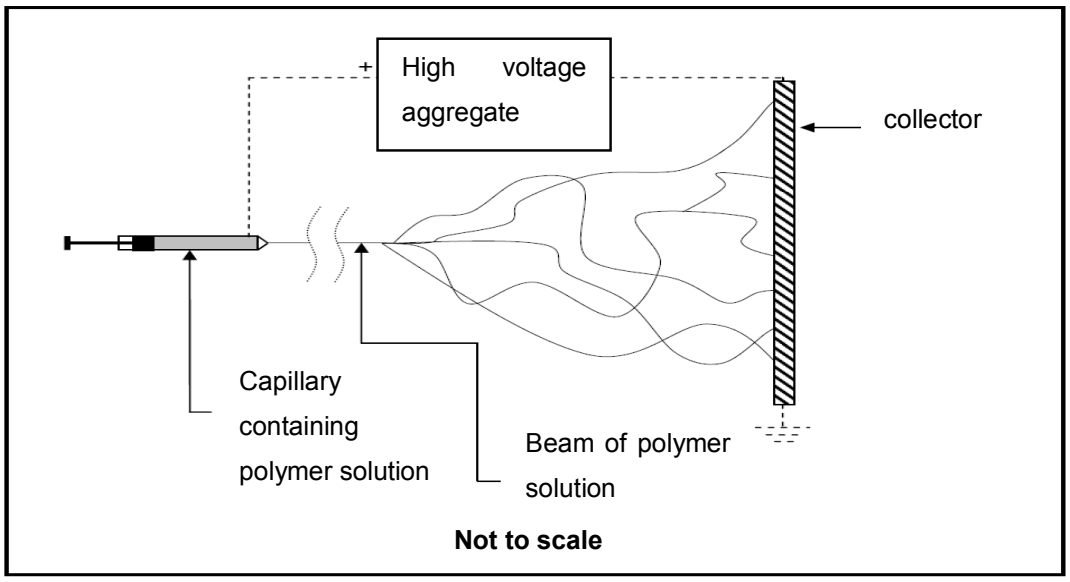

Figure 6; A schematic illustration of the electrospinning process, developed at SMIT Center & BioNano Systems Laboratory, Department of Microtechnology and Nanoscience at Chalmers University of Technology. Illustration by Björn Carlberg.

Surface morphology and structural properties of electrospun polymer scaffolds (paper III)

The surface morphology and structural properties of the electrospun fiber scaffolds were inspected using scanning electron microscopy (SEM) (JSM-6301F, JEOL). Prior to electron microscopy samples were sputter coated with 20 nm gold to avoid charging phenomena. The fiber diameter distribution was determined by image analysis of SEM-micrographs (8 random locations with 20 samples at each location under 4,000x magnification). Pore diameter distribution, porosity and total pore volume of the electrospun polyurethane scaffolds were determined utilizing mercury intrusion porosimetry (AutoPore III 9410, Micromeritics).

Scanning electron microscopy (SEM), (paper III)

SEM is a microscopy technique in which the object is examined directly by an electron beam scanning the specimen point-by-point. The image is constructed by detecting the products of specimen interactions that are projected above the plane of the sample, such as backscattered electrons. To prepare the samples for SEM analysis, the fibers released from the aluminum substrate were fixed at different time points of hESC co-culture and differentiation, using EM-fixative (2 % PF, 2.5 % GA 0.05M Na-kak, pH 7.2, Na-acid) and treated for SEM analysis.

Transmission electron microscopy (TEM), (paper IV)

Transmission electron microscopy (TEM) is an electron microscope technique in which the electrons or their reaction products that pass down through the specimen are imaged below the plane of the specimen while other electrons are absorbed in the specimen. To prepare the specimen for TEM a post-fixation procedure was performed after initial post-mortem fixation of the specimens. Specimens were cut into smaller blocks and immersion fixed in a modified Karnowsky solution (2% paraformaldehyde, 2.5% glutaraldyde and 0.05M Na-azide in 0.05M Na-cacodylate) overnight and post fixed in 1% OsO4 and 1% potassium ferrocyanide in 0.1 M Na-cacodylate for 2 hrs. Specimens were treated with 0.5% uranylacetate en bloc, dehydrated and embedded in Agar 100 resin. A first series of 0.5µm sections were cut, stained with a Richardson's stain and evaluated in a light microscope. This evaluation made it possible to trim the blocks in order to make ultrathin (50-60-nm) sections of selected regions for TEM. Electron microscope sections were examined after contrasting by lead citrate and uranylacetate in a LEO912AB TEM equipped with a CCD camera.

Magnetic resonance imaging (MRI) of human brains (paper IV)

Magnetic resonance imaging (MRI) is a non-invasive method of demonstrating internal anatomy based on the principle that atomic nuclei in a strong magnetic field align and radiofrequency energy can be used to force them out of alignment. As the protons return to alignment radio waves are generated at different frequencies for different tissue types that can be reconstructed into computerized images. In order to further visualise the ventricular extension, we performed MRI using high resolution heavily T2 weighted spin-echo sequences developed to accentuate fluid for the purpose of tracking cerebrospinal fluid (CSF) leakage in patients with hydrocephalus. A previous study in humans suggested a nasal route of CSF drainage in man [152]. It is also a well established clinical phenomenon that patients with acute hydrocephalus often develop leakage of CSF from the nose (liquorhea). Therefore we wanted to investigate if the ventriculo-olfactory extension could be visualized better with MRI in hydrocephalic patients. Hydrocephalus did not dilate the ventriculo-olfactory extension; we saw similar results in normotensive patients. The evaluation of the morphology of the OB was a retrospective analysis and was not part of, or influenced by, the clinical evaluation. None of the patients had findings on MRI indicating ongoing CSF leakage. The patients were examined in a 1.5 Tesla MR scanner with a special T2 weighted sequence designed for high resolution and maximum contrast between soft tissue and fluid with the following parameters: 3D TSE (TSE factor 53, DRIVE), TR/TE 1500/130, FOV 130 mm, Reconstructed resolution 0.25 x 0.25 (in plane) x 0.5 (slice thickness), acquisition time 10 min 9 sec. Images were obtained in the coronal plan with axial and sagittal reconstructions. All images were examined and interpreted by a neuroradiologist (S.H.).

Results and discussion

Paper I

Future replacement therapies using hESC derived cells or tissues will require xeno-free culture conditions. The use of hESCs will require the availability of routine large-scale culturing protocols for undifferentiated hESCs. In this study, we have developed an efficient technique for the transfer of hESCs from feeder to feeder-free culture based on mechanical dissociation. On the other hand, enzymatic dissociation was used for passage of the cultures. The transfer of cells using the mechanical dissociation technique is more efficient in terms of generating larger amounts of cells. The current method is a more-efficient method that leads to higher expansion efficiency combined with markedly improved purity of undifferentiated cells by virtually eliminating the presence of differentiated hESCs from the cultures compared with previously published methods [15, 74]. The pure undifferentiated cultures generated using the present method are more useful for further applications such as genetic analysis (DNA array), and differentiation experiments, where uncontrolled spontaneous differentiation could interfere.

Four different hESC lines SA 002, AS 038, SA 121 [146], and SA 167 were used in all experiments. The cell lines were propagated on Matrigel for up to 35 passages, and the morphological appearance and other hESC characteristics remained unaltered even after a cycle of freeze/thawing. All cultures consisted of well-defined colonies of hESCs without morphological signs of differentiation. According to the current protocol, the hESCs can be successfully frozen and thawed using conventional cryopreservation techniques. Furthermore, this method provides a culturing system that more closely resembles conditions used for routine propagation of various cell lines.

Mechanical dissociation is more efficient than enzymatic dissociation when transferring hESC cultures to feeder-free conditions

Enzyme treatment in the transfer step may negatively affect the cell survival, propagation, and/or differentiation. Therefore, we compared enzymatic treatment with mechanical dissociation when transferring the hESC cultures to Matrigel. In the present study, mechanical dissociation was found to be superior to the enzyme treatment regarding success rate, initial adhesion, colony size, and propagation of undifferentiated hESC cultures. The colony sizes (total colony area), after using the mechanical dissociation for transfer, was significantly larger on day 2 ($p < 0.001$) and 6 ($p < 0.036$) after plating, compared with when using the enzymatic dissociation protocol. The colonies were dense with sharp edges and a homogenous, compact morphological appearance, characteristic for undifferentiated hESCs. These features were maintained throughout the entire propagation period on Matrigel. When transferring the hESCs to Matrigel, the colonies were mechanically cut from the feeder, using only the centre part of each colony eliminating the risk of contaminating the cultures with feeder cells, a possible problem in protocols where enzymatic dissociation is utilised for the transfer [74]. Furthermore, the use of enzymes, at the very delicate step of transferring the feeder cultured hESCs to a feeder-free surface, may cause inactivation of important surface molecules involved in cell adhesion and growth. The major components in Matrigel are extracellular matrix proteins, such as collagen type IV and laminin. Activation of the cell surface integrins upon binding to extracellular matrix proteins is a crucial step for the regulation of cell adhesion, survival and proliferation. For example, integrin alpha 1 has a unique role among the collagen receptors in regulating both *in vivo* and *in vitro* cell proliferation in collagenous matrices [153]. Laminin-specific receptors, possibly formed by integrin $\alpha 6$ and $\beta 1$, which are highly expressed by hESCs [74], may also play a major role in the adhesion of hESC to the

matrix surface. Thus, one possibility is that some of the important surface receptors for attachment or survival might be negatively affected by the rough initial collagenase IV treatment before the cells have adapted to the new surface.

The cluster size after dissociation is important for transfer and for passage

In the present study, the cell cluster size also proved to be of importance for adhesion, survival, propagation, and maintenance of undifferentiated hESC cultures on Matrigel. Large aggregates in the cell suspension tended to form EB-like structures containing differentiated cells, and too small aggregates or single cell suspensions did not support adhesion and survival of the cells. For serial passages on Matrigel, the cluster size was also important but not as crucial as it was for the transfer step. The average cluster size after enzymatic treatment (used for passage/expansion) was generally smaller than after mechanical dissociation, and more single cells were found in the enzyme-treated cell suspensions. The enzymatic treatment produced an average of 1,850 counts with a median size of 34μm^2 (ranging from 8 to 535 μm^2), whereas the mechanical treatment resulted in an average of 160 counts with a size median of 243 μm^2 (ranging from 10 to 11,780 μm^2). The larger cluster size generated by mechanical treatment proved to be favourable at the delicate step of transferring the hESCs to the feeder-free conditions. However, for passage/expansion on Matrigel, the enzyme dissociation technique was superior. All our attempts to use mechanical dissociation for passaging have failed. Mechanical passaging from Matrigel required the use of considerable mechanical force and resulted in individual floating cells and differentiated EB-like structures but yielded no analyzable undifferentiated colonies. Our conclusion, therefore, is that mechanical dissociation for passaging is inapplicable.

The conditioned medium was optimal from mEFs in passage 2, day 1-3

The quality of the conditioned medium was an important factor for hESC maintenance on Matrigel. In earlier, unpublished data, it was noted that mEF cells in passage 2 and no older than 3 days gave the optimal conditions for coculturing with hESCs. An explanation for this finding may be that, after day 3, the ability of the feeder cells to produce or release the undefined factors needed for hESC survival, proliferation, and maintenance gradually declined. Based on these observations, it was decided to only use the mEF cells in passage 2 for a maximum of 3 days, for conditioning the VitroHES^{TM} medium.

The percentage of mitotic cells was similar in feeder and feeder-free hESC cultures

We calculated the mitotic index to compare the growth rate between our feeder-free cultures and conventional mEF cultures by quantifying the number of cells in mitosis as defined by immunoreactivity for phosphorylated histone H3 (PH3). The mitotic index (percentage of cells in mitosis) was similar in cultures grown under feeder-free (3.50% ± 0.655) conditions and to feeder (4.19% ± 0.939) conditions. Furthermore, the doubling time for our feeder-free cultures was roughly the same (approximately 35 hr) as previously reported for feeder-free [74, 154] and mEF [20] propagated hESCs.

Successful cryopreservation by slow rate freezing and rapid thawing of feeder-free hESC cultures

Slow-rate freezing and rapid thawing methods are most commonly used for cryopreservation of cell lines [155] and are effective for freezing of mESCs [156]. However, this method has not been reported as successful for freezing

hESC lines. We, however, used this standardized cryopreservation technique for our feeder-free hESCs and proved it to be efficient; the survival rate was high and no morphological or cell specific marker differences could be seen after a cycle of freezing and thawing. This technique has advantages over the more common but complicated vitrification methods used for freezing of hESCs [85, 87, 157, 158] in that the risk of contamination is lowered, and it is less laborious. In vitrification techniques, colonies are cut and frozen in large pieces compared with the present technique in which a mixture of single cells and small aggregates were frozen. When freezing large aggregates using the present technique, the cells did not survive after thawing (unpublished data). These observations suggested that the size of the aggregates was very important for survival after thawing, depending on the freezing technique used.

Our hESCs maintained pluripotency and other hESC characteristics after transfer to feeder-free conditions

Pluripotency and maintenance of the hESC lines under feeder-free conditions was demonstrated and compared with previous results for mEF cultures of the respective cell lines ([146]; table 3, paper I). The characterization was performed by examining the morphology, expression of undifferentiated markers, telomerase activity, karyotype, and differentiation *in vivo*. All the cell lines used in the present study, cultured under feeder-free conditions, expressed the ESC TF Oct-4, high levels of ALP reactivity, high levels of telomerase activity, normal stable karyotype and the expression of ECS specific cell surface markers SSEA-3, SSEA-4, Tra 1-60, Tra 1-81. Teratoma formation proved the capacity of the feeder-free propagated hESCs to differentiate in to all three embryonic germ layers (proof of pluripoteency). All hESC lines showed similar results in characterization before [146] and after the transfer to feeder-free cultures (figure 4, 5, 6, 7, 8; paper I), thus,

proving the maintenance of pluripotency and other hESC features even when propagated in a feeder-free environment (on Matrigel). Although, it has been shown that three independent hESC lines gained chromosomes 12 and 17q after propagation in feeder-free conditions [102, 159], and this chromosomal gain was suggested to provide a selective advantage for the propagation of undifferentiated hESC. We, therefore, performed FISH analyses on all our cell lines cultured on Matrigel for chromosomes 12 and 17q without detecting any abnormalities (table 3, paper I).

Paper II

In this study we found gelatine and laminin substrates, together with standard hESC medium supplemented with FGF2, to efficiently generate proliferating NPs in only 8 days (figure 1, paper II). These NPs, derived from undifferentiated feeder-free hESCs cultures on Matrigel, have the potential to generate mature neurons and glia. The advantage of this simple and novel method is that it makes the NP generation less laborious and more cost efficient than previously published protocols [46, 59, 60, 90-102].

Matrigel propagated hESCs for NP generation

In our initial experiments, we found that mEF cultured hESCs did not survive well on other culture substrates. Using Matrigel propagated hESCs though; we could derive proliferating cultures on other substrates. We believe that this was because Matrigel propagated hESCs were better equipped to handle the harsh conditions used for NP derivation, without feeder cells, resulting in a much higher cell survival of proliferating cells.

Gelatine and laminin substrates function equally well for cell attachment and NP derivation

It has been shown that Matrigel propagated, undifferentiated hESCs do not adhere well to gelatine substrates [46], whereas in our study gelatine was used to efficiently generate NPs. We therefore wanted to know if this difference in attachment properties could be due to differences in cell lines or in handling technique. To this aim, we used two well characterized hESC lines, SA002 and AS034 [146], for this evaluation and each cell line had the ability to generate NP populations both on gelatine and laminin substrates in standard hESC medium [20, 74]. Laminin as a substrate promotes neural differentiation in hESCs [98], but not in combination with standard hESC medium. Here we demonstrate that gelatine as a substrate functions equally well as laminin to generate NP cultures in combination with hESC medium and FGF2; this has not previously been shown. This combination with short term, adherent cultures and gelatine substrate using only FGF2 as growth supplement has never before been reported for the generation of NPs from hESCs.

Rosette formations in passage 1

On gelatine or laminin substrates rosette formations were generated in passage 1 (figure 1C, paper II). After the first passage on these substrates, the NPs grew in a homogenous adherent monolayer with elongated cells displaying a bi- or tri-polar morphology (figure 1D and E, paper II) and maintained the same morphology for the sequential passage. ALP, expressed by undifferentiated hESCs [3] and human mesenchymal stem cells (hMSCs) [160], was not found in our NP cultures (figure 1F and G, paper II), indicating that the undifferentiated hESCs had undergone a complete shift from pluripotent hESCs to NPs.

FGF2 is required for the derivation and maintenance of NPs

In this study, we found that two hESC lines on two different substrates gave rise to the same type of NPs with virtually the same gene expression profile, as shown by RT-PCR (figure 2; paper II) and immunocytochemistry (figure 3; paper II). The growth factor FGF2 seems to play a role in the derivation, proliferation and maintenance of the progenitor state, as previously reported [102]. Without the FGF2 addition we observed increased differentiation into various cell types, hence no generation of a homogenous NP culture.

Cell density affects cell fate

Furthermore, cell density affected the cell fate in our cultures, as previously reported [161]. In this respect, we observed that a high seeding cell density was required for rosette formation when deriving the NPs. Low seeding cell density ($<150x10^3$ cells/cm^2) resulted in fibroblast-like cell types instead. This observation was further supported by a study where the authors used a similar method for the derivation of skeletal myoblasts and found high cell densities to yield neural cell fates, whereas low cell densities gave rise to mesenchymal precursors [162]. Furthermore, cell density in the local environment affects cell-fate determination [161]. Furthermore, we observed that the derived NP cultures maintained a progenitor state when using a continuously high cell density (50-100 $x10^3$ cells/cm^2) when for passaging/expanding the cultures. We observed that the NPs more readily differentiated into mature non proliferating cell types when seeding at a low density (<50 $x10^3$ cells/cm^2). Also, seeding at a high cell density ($>200x10^3$ cells/cm^2) when expanding, caused the cultures to form 3-dimensional colonies instead of monolayer NP cultures.

Neuroectodermal markers are expressed by our NP cultures

The undifferentiated neuroectoderm, or NPs, within the neural tube are characterized by expression of several markers including Sox1, Sox2, nestin [50-52], musashi-1 [53] and NCAM [54, 55]; consistent with the marker expression of our NP cultures (figure 2 and 3; paper II). We used a combination of RT-PCR and immunocytochemical analysis to ensure that not only the RNA was translated but also the proteins where being expressed. The immunocytochemical results reviled that our NPs expressed the progenitor marker nestin (97.8% ± 0.5 of total cells), the neuroectodermal markers Sox2 (17.2% ± 11.7 of total cells), Pax6 (6.3% ± 1.0 of total cells), NCAM (96% ± 2.9 of total cells), internexin and A2B5 (glial progenitors), as well as occasional cells positive for markers of more mature cell types like the astrocyte marker GFAP, GalC (oligodendrocytes) and βIII -tubulin (neurons). Furthermore, the progenitor populations displayed no immunoreactivity for markers of pluripotent hESCs (SSEA-1, 3, and -4, Tra-1-81), nor markers of other germ layers; mesoderm (Ct-1) or endoderm (AFP). To confirm the immunocytochemical results RT-PCR was performed reveling that on both gelatine and laminin substrates, NPs derived from the hESCs displayed a gene expression for the progenitor marker nestin, the neuroectodermal markers Sox1, Sox2, Sox3, NCAM, Musashi-1, the dorsal specification/neuroectodermal marker Pax6, and occasional DCX expression (figure 2A and B, paper II). Thus, these NPs express many markers (Pax6, NCAM, DCX, Sox2 and βIII-tubulin) in common with that of the migrating neuroblasts of the adult human brain [31]. These gene and protein expression results confirm the derivation of a homogenous population of expandable NPs using our rapid and simple protocol.

PAX6 and Sox1 gene expression in the NP cultures

Simple monolayer protocols for the generation of NPs from mESCs have previously been described [44], although there are differences between mESCs and hESCs that prevent the direct transfer of protocols [163]. Furthermore, and in contrast to our novel method, no evidence of large scale production or long-term culture was given in that study [44]. Human ESCs express Pax6 before Sox1 in neural differentiation [23], which is the opposite of previous observations in mESCs. In mESCs Sox1 is instead the earliest neuroectodermal marker during neural plate and tube formation [164]. During neuroectodermal differentiation of hESCs, early neuroectodermal cells express Pax6 but not Sox1 (rosettes) and late neuroectodermal cells express both Pax6 and Sox1 (neural tube-like structures) [23]. The Pax6/Sox1 expressing cells are more mature (corresponding to neuroectodermal cells in the neural plate/tube that are regionally specified) than the Pax6 expressing cells (early neuroectodermal cells) [23]. These examples highlight the importance of studying hESCs and NP cells derived from human material rather than assuming the mESC data translate to hESC lines. Our NP cultures expressed both Sox1 and Pax6 indicating that we have mature NPs that are regionally specified.

Sox3 gene expression in the NP cultures and its mature derivates

Sox3 has previously been described in neural stem cell maintenance where Sox3 immunoreactivity in hESCs appears upon differentiation to NPs and then decrease as cells differentiate further into neurons [165]. Although, Sox3 can also persist in specific post-mitotic neuronal populations [165]. We found that our method also yielded Sox3 expressing NPs, lending further support for the identity of the NP populations derived using our protocol. Furthermore, Sox3 gene expression was also found in our differentiated cultures.

Gradually declining Oct-4 expression required for NP derivation

Pluripotent cells of the ICM and primitive ectoderm express the pluripotent marker Oct-4 [43, 45]. The NPs derived according to our simple and rapid protocol express the pluripotent marker Oct-4 from passage 1 to at least passage 11 (later passages were not analyzed for Oct-4 expression), with a declining expression level as passage number increased. The maintenance of Oct-4 expression in our NP populations is supported by results from other studies [46, 166] where the loss of pluripotency and formation of definitive ectoderm (the progenitor of both surface ectoderm and neuroectoderm) is marked by down regulation, but not a complete loss, of Oct-4 expression [166]. Also, Oct-4 is temporally retained before down regulation when hESCs are induced to become NP-type cells and the cells that lost Oct-4 expression rapidly did not turn into neural cells but rather to flattened extrembryonic cells [46].

GFAP is expressed by undifferentiated NPs and its derivates

In the present study the proliferating NP populations expressed the astrocytic marker GFAP, although GFAP is expressed by NSCs of the adult brain [29, 30, 32]. Further, post-mortem human cortical neural progenitor cells express GFAP among other markers (DCX, EGF-R, nestin, nucleostemin and Sox2) under proliferating conditions [167]. In addition, our NP populations also differentiated into mature GFAP expressing astrocytes and approximately 15% of the differentiated NPs were immunopositive for GFAP.

Mature neurons and glial cells are derived from the NP populations

The NPs derived on both laminin and gelatine substrates in hESC medium generated both mature neurons and glial cells upon differentiation *in vitro* resulting in 82.2% ± 8.6 βIII-tubulin (neurons) positive cells, 15.5% ± 2,6 GFAP (astrocytes) positive cells, and 10.4% ± 4.6 GalC (oligodendrocytes)

positive cells (figure 4A-C, paper II). Further, MAP2ab (figure 4D, paper II) and TH positive cell types (figure 4E, paper II) could also be seen. These immunocytochemical results where also confirmed by RT-PCR analysis of differentiated NPs, reveling positive expression for GFAP, MAP2, TH and a negative gene expression for mesodermal and endodermal markers (figure 2A and 4H, paper II). Furthermore, we found that the NPs maintained their progenitor characteristics and were able to differentiate into mature neurons/glia even after freezing and thawing.

Some mesodermal markers are found in the NP cultures

The NP cultures were negative for AFP (endoderm), HNF3-α (endoderm), Brachiury (mesoderm) and MyoD (mesoderm) gene expression; although, they expressed Sma and desmin, genes typically associated with the mesodermal lineage. However, coexpression of neural and mesodemal markers occurs in mesenchymal stem cells by the differentiation of these cells to neural cell types [168-170], and also NSC can differentiate into endothelial lineages (from mesoderm), [171]. Furthermore, Sma and GFAP positive cells can be derived from the same progenitors and GFAP positive cortical stem cells turned into Sma positive smooth muscle cells when plated at a low density [161]. This could potentially explain the Sma expression that occurred in our cultures, even if only weak gene expression was detected.

Paper III

Human ESCs attach and proliferate on electrospun fibrous scaffolds

The hESCs where able to attach to and proliferate on the electrospun fibrous scaffolds (figure 4a; paper III) as shown by the data derived from counting of DAPI stained cell nuclei. A statistically significant increase in cell number from an average of 9.7 ± 8.1 cells per visual field counted on day 5 to almost

10 times the number of cells (91.2 ± 64.1; $p < 0.001$) on day 18 (figure 4b and figure 5; paper III) was seen. The cell number remained more constant after day 18 (figure 4c-d; paper III) and even decreased slightly (figure 5; paper III) at day 32 (77 ± 51.7; $p < 0.001$) and 47 (72.9 ± 45; $p < 0.001$), indicating that the cells are differentiating (terminally differentiated/mature cells do not divide) rather than propagating at later time points in co-culture as a result of the differentiating conditions.

A neuronal cell fate was induced in cells grown on electrospun scaffolds

The immunocytochemistry results indicate that the hESCs cultured on/in the fiber structure could be induced to differentiate towards a neuronal cell fate (figure 4e-l; paper III). After 18 to 47 days of co-culture and differentiation positive stainings (positive labeling to indicate differentiation had taken place) were found for the mature neuronal markers MAP2ab (figure 4g-I; paper III), βIII-tubulin (80-100%; figure 4e, f; paper III) and for the dopaminergic marker tyrosine hydroxylase (80-100%; TH; figure 4k; paper III). However, little or no positive staining was observed for the astrocyte marker GFAP (figure 4e, f, l; paper III), indicating that mostly neuronal cells were derived in these culture conditions. On the other hand, reference cultures under the same differentiation conditions, but omitting the fibers, showed a large proportion of GFAP (95-100%; figure 4m-p; paper III) positive cell types and only occasional MAP2ab, TH, and GalC positive cells. These control cultures displayed a negative immunoreactivity for the hESC marker Oct-4 and the progenitor marker Sox2. Similarly, the differentiated fiber/hESC co-cultures displayed negative immunoreactivity for Oct-4, Sox2 and nestin, indicating the absence of undifferentiated hESCs and proliferating progenitors in the cultures. Additionally, only a few cells were positive for the oligodendrocyte marker GalC, further confirming the conclusion that

mature neurons being the primary cell type derived in the co-cultures (data not shown). Although we can not totally exclude the generation of mature cell types of other germ layers since we did not include and markers for mesoderm and endoderm in the immunocytologial analyses made. However, we could clearly see that virtually all cells where reactive to MAP2ab, TH, βIII-tubulin, GalC or GFAP.

The 3-dimentional scaffolds affect hESC cell fate determination

Evidently, the results indicate that the three dimensional culture structure provided by the electrospun scaffolds directs hESC differentiation towards a neuronal cell fate. Cell fate is determined by intrinsic programs and external physical cues, for instance cell-cell contact [161]. Indeed, the importance of external physical cues on cell differentiation and proliferation induced by the geometrical and topological structures provided by three dimensional scaffolds mimicking the natural extracellular environment has received attention and been confirmed in the literature [138, 144, 145, 172]. Proliferation and differentiation of mESCs (E14.5) on electrospun polyamide surfaces has been studied, demonstrating that physical cues originating from the topology and structure of these surfaces affect cell signaling pathways promoting cell proliferation [138]. Similarly, proliferation and differentiation of hESCs on highly porous PLLA/PLGA three dimensional scaffolds promotes cell differentiation, connecting these results to the physical and structural properties of the scaffolds via reference cultures [144, 145]. Furthermore, the structural morphology of the fibrous scaffolds physically influence stem cell differentiation, in this case towards a neuronal fate [145], consistent with reports in literature.

The interaction between hESCs and the scaffolds were shown by SEM micrographs

SEM micrographs were acquired to analyze the interaction between cells and scaffolds. The hESC-derived neuronal cells displayed neurite outgrowths thet interacted with the scaffold (figure 6a; paper III), elongated neural cell bodies with neurite outgrowths connecting to and spreading over the nanofibrous network (figure 6b; paper III), elongated neural cell bodies established connection through outgrowths (figure 6c; paper III), and the cells exhibit excellent mechanical attachment to individual fibers of the scaffold (figure 6d; paper III). These results reinforce the potential of utilizing electrospun polyurethane scaffolds for neural tissue engineering in adult human CNS repair and rehabilitation and also as cell carriers for enhanced *in vitro* culturing of stem cells.

Paper IV

In this study, we provide a characterization of the human VONS containing the SVZ, the RMS, the OT, and the OB. We demonstrate that the human RMS is organized around a lateral ventricular extension reaching the OB.

The anatomical location of the RMS in the human brain

Analysis of PCNA-stained sagittal human forebrain sections (counterstained with a Nissl stain) showed the presence of an RMS-like pathway. The human RMS takes a caudal path en route from the SVZ to the olfactory cortex (figure 1; paper IV). The dorsal RMS is a broad band of proliferating cells, but ventrally there are fewer cells in the RMS (figure 2; paper IV). This work demonstrates a remarkable similarity between human and rodent olfactory systems. However, because of the pronounced enlargement of the frontal cortex in the human forebrain, most of the rostral caudate nucleus (CN), SVZ, and frontal cortex are located at levels rostral to the olfactory tubercle. The

RMS in the human brain must, therefore, take a caudal path before entering the olfactory tract. In fact, forward rotation of the rodent forebrain by about 75° shows the RMS to be situated in an orientation comparable to that of the human; the larger frontal cortex and relatively smaller OB in the human accounts for this geometric difference between the human and the rodent RMS. Because the human RMS is relatively small and takes a caudal path before entering the OT (figure 1C; paper IV), rigorous serial sagittal sectioning of whole forebrains was required to see the long axis of it (figure 1A vs figure 2C; paper IV). Previous attempts to find a human RMS [173] were probably unsuccessful because of the use of coronal sections in these studies. Our group has the access to large numbers of normal, well-preserved, perfused, whole human brains that was necessary for this extensive analysis of the VONS.

Cell death is not the fate of most of the RMS progenitor cells

To examine whether PCNA was being expressed in RMS cells because of DNA repair or apoptosis and not proliferation [151], we performed TUNEL to detect DNA fragmentation. No TUNEL was present in the descending limb of the RMS despite the presence of many TUNEL-positive cells in the adjacent CN.

Progenitors in the RMS have migratory proteins and a migratory morphology

Immunostaining for PSA-NCAM, which is expressed by migrating cells in the rodent RMS [35, 36], was investigated in the SVZ near the cleft, the descending and rostral limbs of the RMS, the OT core and periphery, and the OB. In each region examined, PSA-NCAM–positive cells were present. Neuronal precursors in the human RMS express the migratory protein PSA-NCAM. PSA-NCAM is expressed by precursors in the SVZ, suggesting the

presence of migratory cells at the start of the RMS (figure 3a; paper IV). ßIII-tubulin, an immature neuronal marker, was examined in the SVZ, the descending limb, and the OT. Immature neurons that express both ßIII-tubulin and PSA-NCAM are present in the RMS, and some mature in the olfactory bulb and express NeuN. Fiber staining was observed in the SVZ beneath the gap region and close to the SVZ, as well as in the descending limb of the RMS (figure 4a-c; paper IV). We performed double labeling and laser scanning confocal microscopy for PSA-NCAM and ßIII-tubulin, which demonstrated that in the SVZ PSA-NCAM and ßIII-tubulin are colocalized on the same bipolar cells (figure 4d-f; paper IV). Double-labeled PSA-NCAM– and ßIII-tubulin–positive cells were also seen in the OT (figure 4g-j; paper IV), as well as in the OB.

Ultrastructural studies verify that the human SVZ contains cells with migratory-like morphology

In the adult mouse brain, where neuronal migration from the SVZ is common, ultrastructural and immunolabeling studies demonstrated that migrating neuronal precursors in the SVZ express PSA-NCAM (Doetsch classification, type A cells) [29, 174]. We sought to determine whether the human SVZ had similar migratory, type A cells. Electron microscopy (EM) revealed different types of cells that could be identified on the basis of their nuclear morphology and overall shape (figure 4K; paper IV). Type A cells had an elongated cell body and a smooth contour. Their nuclei contained lax chromatin with one or two small nucleoli. They were located between the myelin layer and the ependymal layer. Positioned at the interface of the SVZ and myelin layer were the type B1 cells. These cells contained irregular nuclei with frequent invaginations and clumped chromatin.

Ultrastructural studies reveal progenitors at all levels of the RMS that have migratory morphology

Next, we wanted to determine the cell types present in the human VONS compared with the rodent brain, focusing on the OB, the olfactory tract, and the AOC with light microscopy (LM) and TEM. Collectively, progenitor cells in the RMS share some ultrastructural features with cells undergoing chain migration in the rodent RMS [174]. Furthermore, immuno-TEM revealed that the cells with a migratory-like morphology were also positive for PSA-NCAM.

Directed migration of progenitors in human VONS

Progenitor cells in the human VONS express PSA-NCAM and DCX, two proteins important for cell migration in the rodent RMS [37-39]. These cells have many ultrastructural characteristics that have previously been used as reliable indicators of neuroblast migration in the rodent RMS [175, 176]. The close association of the progenitor cells to one another suggests that the progenitors may migrate in chains, as in rodents; alternatively, the progenitors may use the extracellular matrix surrounding the ventriculo-olfactory extension (VOE) [176]. On the basis of the morphological characteristics of cells within the human VONS, the expression of migratory proteins, the orientation of the leading processes in the direction of migration, and the emergence of new neurons in the OB, we conclude that the cells within the human VONS probably undergo directed migration toward the OB. Studies in higher mammals have established that insults to the brain can induce neurogenesis via the recruitment of progenitor cells from the RMS [177]. Our study provides a foundation for this possibility in the adult human brain.

Progenitor cells become neurons in the OB

We used human OB from patients that had been administered BrdU for the purpose of tracing the development of laryngeal and pharyngeal carcinomas. BrdU is a thymidine analog and labels cells in the S-phase of the cell cycle [178, 179]. We double-labeled the OB with neuronal nuclei (NeuN), a mature neuronal marker, and an antibody against BrdU. Laser-scanning confocal Z-series analysis revealed the colocalization of BrdU and NeuN in the same cells in the periglomerular layer (figure 4L; paper IV), indicating that adult-born neurons exist in the OB.

The human RMS is organized around a tubular extension of the lateral ventricle that reaches the OB

The VONS contains an extension of the lateral ventricle, the VOE, which appears to be fluid-filled and is connected via a patent duct to the OB in the ventral forebrain. Thus, it appears that the human brain contains a continuous SVZ organized around the VOE that connects the lateral and the olfactory ventricles. The organization of the RMS around the VOE could support the notion that the migration of neuroblasts may be influenced by CSF circulation, as recently suggested [175]. In conclusion, in the adult human brain proliferating cells migrate from the SVZ adjecent to the lateral ventricle (LV) overlaying the CN to the OT in the base of the forebrain to the OB. We performed gross dissection of the OB, this revealed that the rostral OB region contains a hollow ventricle (figure 5a, b; paper IV). Dissections of the human OB reveal a hollow OB. The double arrow indicates the width of the olfactory ventricle. By using MRI scans, we observed a central high signal consistent with fluid in the center of the OB. Surrounding the central core there was low-signal (black) OB tissue that was in the shape of the OB overlying the cribriform plate (figure 5f, h; paper IV). The visualized (with

MRI) olfactory ventricle measured about 7 mm in length (figure 5f, g; paper IV), with a transverse diameter of about 1.5 mm (figure 5h; paper IV).

Pax6, Olig 2, and DCX gene expression is consistent with differentiation along the VONS

Next, we investigated the differential gene expression pattern in different parts of the olfactory system. We performed RT-PCR on fresh human brain homogenates from the AOC, the OT, and the OB. We chose to examine mRNA for three factors that regulate differentiation of olfactory neurons: Pax6, which induces differentiation; Olig2, which inhibits differentiation; and DCX, which promotes cell migration (figure 6M and N; paper IV). GAPDH was used as an internal control [34, 180, 181]. As expected, Pax6 was highly expressed in the OB and weakly expressed in the AOC and the OT (figure 6M; paper IV). This is consistent with previous studies showing the importance of Pax6 for the fate specification of progenitor cells into periglomerular neurons in the OB [34]. In contrast, expression of Olig2, a TF thought to inhibit olfactory neuron differentiation, was much higher in the OT compared with in the OB (figure 6M; paper IV). RT-PCR for DCX revealed extensive expression in each region of the VONS examined, although the expression was higher in the OB than in the OT and the AOC (figure 6M; paper IV). In the rodent brain, DCX is expressed throughout the olfactory system by migrating progenitors from SVZ to the OB [182]. Therefore, it is not surprising that we see DCX mRNA evenly abundant in all regions of the VONS in the human brain. In conclusion, Pax6 expression is increased during progenitor differentiation in the adult human brain and Olig2 expression decreases as the neural progenitor cells mature. DCX is evenly abundant in all regions of the VONS in the human brain (figure 6N; paper IV). To confirm PCR results immunostaining of various parts of the VONS with antibodies against Pax6, Olig2, and DCX was performed.

Conclusions and Significance

Conclusions from paper I

We have derived a robust and versatile method for the transfer of hESCs to feeder-free cultures, where propagation of the hESCs in an undifferentiated state can be carried out without laborious manual cutting and transfer of colonies. This method is fully comparable to feeder culturing with regard to differentiation and growth rates as well as for maintaining all the normal hESC features. This improved method facilitates propagation without laborious manual cutting and transfer of colonies, fully comparable to feeder culturing (differentiation, growth rates, maintaining all the normal hESC features). This feeder-free protocol facilitates large-scale production of hESCs and makes hESC culturing less dependant on extensive prior experiences. This culturing system can be effectively used for optimization experiments of feeder-free hESC cultures in the future, regarding for example, medium development, comparative studies of the effect of different substrates, and also facilitate a standardized production of hESCs for various experiments such as animal studies, where large amounts of cells are required.

Conclusion from paper II

We have derived a simple and effective method for the generation of proliferating NP populations. The method is a rapid protocol generating proliferating adherent monolayer NP cultures on gelatine or laminin substrate in standard hESC medium supplemented only with FGF2 in 8 days. The NPs derived according to this novel method express many markers of early neuroectoderm and progenitors, and can easily be differentiated into mature neurons and glial cells. The NPs can be frozen slowly and rapidly thawed for prolonged time storage, and still retain their essential characteristics after

thawing. These cultures could serve as a renewable source of hESC-derived NPs, suitable for large-scale expansion. They may be a valuable source of cells for various studies of the human neural development, for experimental work like toxicology testing and drug screening, and as a potential source of cells for the restoration of cellular and functional loss in the nervous system.

Conclusions from Paper III

In this study electrospun fibrous polyurethane scaffolds have been proven feasible as a substrate for hESC cultivation and neural differentiation. Furthermore, cells displayed favorable interaction with the substrate, spreading outgrowths, establishing connections to adjacent cells and attaching to individual fibers. Immunocytochemistry results show that fibers can support neuronal differentiation in hESC cultures, predominantly dopaminergic, TH positive neurons. Results indicate that physical cues induced by the fibrous scaffolds direct stem cells towards a neuronal fate. Hence, these scaffolds exhibit potential as cell carriers in neural tissue engineering repair and rehabilitation of the adult human CNS.

Conclusions from paper IV

In this paper we demonstrate the presence of a human RMS and illustrate the neuroblasts in it. The RMS is unexpectedly organized around a lateral ventricular extension reaching the OB. Further, the RMS ensheathing the lateral olfactory ventricular extension, as seen by MRI, cell-specific markers, and electron microscopy, contains progenitor cells with migratory characteristics and cells that incorporate BrdU and become mature neurons in the OB.

Responses to given aims

I) We have derived a efficient method for the transfer of undifferentiated hESCs to a feeder-free surface, Matrigel, based on mechanical dissociation (paper I).

II) Furthermore, this feeder-free method faciclitats non laborious production of large amounts of undifferentiated hESCs and also maintain the hESCs in a stable undifferentiated state for long term cultures (paper I).

III) We have developed a rapid, simple and cost efficient method for the derivation and propagation of proliferating hESC-derived NP cultures, with the capacity to generate mature neurons and glial cells *in vitro* upon differentiation (paper II).

IV) We have developed a biocompatible electrospun fiber that promotes propagation and neuronal differentiation of feeder-free hESC cultures (paper III).

V) We found that many of the markers expressed in the RMS by migrating neuroblasts also were expressed by the NPs derived from feeder-free hESC cultures according to our new method described in paper II, including Pax6, NCAM, DCX, Sox2 and βIII -tubulin (paper IV).

Acknowledgements

Many thanks to;

My first supervisor, the late prof. Peter S. Eriksson, for giving me this opportunity, for believing in me and for inspiration.

My new supervisor Maurice Curtis, for being an excellent supervisor, for letting me take part in his fantastic RMS project, for all his help and support.

Eva Kilmare, former Sjögren-Jansson, who took care of me in the lab and thought me everything I know about cell culturing.

Johan Hyllner, who recruited me for my first position after university studies and who introduced me to Peter. I'm very grateful!

Michelle Anderson who took really good care of me when I started my PhD studies and who also has been my co-supervisor almost all the way. I've really missed you at work!

Friends and colleagues at CBR; Helene Andersson, Ina Nodin, Jonas Faijerson, Ann-Marie Alborn, Jenny Nyberg, Åsa Persson, Charlotta Lindwall, Linda Paulson, Karin Gustavsson, Cecilia Bull, Birgit Linder, Nina Hellström, Jenny Zhang, Maire Kalm, Michael Nilsson, Niklas Karlsson, Georg Kuhn, Christi Kuhn, Axel Jansson, Suzana Zlateva, Karin Hultman, Sheila Engdahl, Klas Blomgren, Åsa Widestrand, Lizhen Li, Rita Grandér, Henrik Landgren, Olle Lindberg, Ulrika Wilhelmsson, Milos Pekny and Marcela Pekna.

Everyone at Cellartis AB; Angelica, Raimund, Jenny L, Karina M, Katarina Em, Katarina A, Jenny G, Marie, Gunilla, Karin, Ulrika F, Gabriella, Karolina, Josefina, Catharina El, Boo, Petter, Peter, Mikael, Fredrik W, Ulrika K, Nico, Mats.

Everyone at “Brosklab”; Narimin Bigdeli, Camilla B, Kristina, Tommi T, Camilla K, Katrin, Anders Lindahl.

Collaborators at Chalmers University of Technology, Department of Microtechnology and Nanoscience; Rebuan Lim Yee Bun, Björn Carlberg and Johan Liu.

Gunnel Nordström, Anki Nyberg, Ulla Ohlsson, Carina Mallard, Patrik and Oskar for help with practical things, administration and IT.

Everyone at the 4th floor for nice lunch conversations.
Ulf Nannmark for collaborations and SEM analysis.

Tack till Familj och Vänner! Det finns inget viktigare!
Tack Inger, Lasse och Maria; min härliga svärfamilj!
Tack Mamma, Pappa, Rasmus, och Ellen med Familjer; den bästa och tokigaste familj jag kan önska mig!
Tack min underbara Calle och våra fina ungar Wille och Maja; utan er vore allt värdelöst! Älskar er!

References

1. NIH. *Stem Cell Basics*. [In Stem Cell Information [World Wide Web site].] 2009 Tuesday, April 28, 2009 [cited 2009 2009-05-26]; Available at <http://stemcells.nih.gov/info/basics/defaultpage>].
2. Woltjen, K., et al., *piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells.* Nature, 2009. **458**(7239): p. 766-70.
3. Thomson, J.A., et al., *Embryonic stem cell lines derived from human blastocysts.* Science, 1998. **282**(5391): p. 1145-7.
4. Zhang, S.C., et al., *Human embryonic stem cells for brain repair?* Philos Trans R Soc Lond B Biol Sci, 2008. **363**(1489): p. 87-99.
5. Gilbert, S., *Developmental biology 8th edition*. 8 ed. 2006: Sinauer Associates, Inc.
6. Haslinger, A., et al., *Expression of Sox11 in adult neurogenic niches suggests a stage-specific role in adult neurogenesis.* Eur J Neurosci, 2009.
7. Munoz-Sanjuan, I. and A.H. Brivanlou, *Neural induction, the default model and embryonic stem cells.* Nat Rev Neurosci, 2002. **3**(4): p. 271-80.
8. Moore, K.L., Persand, T.V.N., *The developing human clinically oriented embryology, 8th edition*. 8 ed. 2008: WB Saunders Company.
9. Chen, D., M. Zhao, and G.R. Mundy, *Bone morphogenetic proteins.* Growth Factors, 2004. **22**(4): p. 233-41.
10. Groppe, J., et al., *Structural basis of BMP signaling inhibition by Noggin, a novel twelve-membered cystine knot protein.* J Bone Joint Surg Am, 2003. **85-A Suppl 3**: p. 52-8.
11. Xu, R.H., et al., *Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells.* Nat Methods, 2005. **2**(3): p. 185-90.
12. Wang, L., et al., *Human embryonic stem cells maintained in the absence of mouse embryonic fibroblasts or conditioned media are capable of hematopoietic development.* Blood, 2005. **105**(12): p. 4598-603.
13. Xu, C., et al., *Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium.* Stem Cells, 2005. **23**(3): p. 315-23.
14. Klimanskaya, I., et al., *Human embryonic stem cells derived without feeder cells.* Lancet, 2005. **365**(9471): p. 1636-41.
15. Amit, M., et al., *Feeder layer- and serum-free culture of human embryonic stem cells.* Biol Reprod, 2004. **70**(3): p. 837-45.
16. Sato, N., et al., *Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a*

pharmacological GSK-3-specific inhibitor. Nat Med, 2004. **10**(1): p. 55-63.
17. Kim, S.J., et al., *Contribution of the PI3K/Akt/PKB signal pathway to maintenance of self-renewal in human embryonic stem cells.* FEBS Lett, 2005. **579**(2): p. 534-40.
18. Beattie, G.M., et al., *Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers.* Stem Cells, 2005. **23**(4): p. 489-95.
19. Storey, K.G., et al., *Early posterior neural tissue is induced by FGF in the chick embryo.* Development, 1998. **125**(3): p. 473-84.
20. Amit, M., et al., *Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture.* Dev Biol, 2000. **227**(2): p. 271-8.
21. Lamb, T.M. and R.M. Harland, *Fibroblast growth factor is a direct neural inducer, which combined with noggin generates anterior-posterior neural pattern.* Development, 1995. **121**(11): p. 3627-36.
22. Barberi, T., et al., *Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice.* Nat Biotechnol, 2003. **21**(10): p. 1200-7.
23. Li, X.J., et al., *Specification of motoneurons from human embryonic stem cells.* Nat Biotechnol, 2005. **23**(2): p. 215-21.
24. NCBI. ***SOX Transcription Factors***. [web page] 2009 [cited 2009-06-12]; http://www.ncbi.nlm.nih.gov/sites/entrez?Db=mesh&Cmd=ShowDetailView&TermToSearch=68055747&ordinalpos=1&itool=EntrezSystem2.PEntrez.Mesh.Mesh_ResultsPanel.Mesh_RVDocSum].
25. Durbec, P. and H. Cremer, *Revisiting the function of PSA-NCAM in the nervous system.* Mol Neurobiol, 2001. **24**(1-3): p. 53-64.
26. Seidenfaden, R., et al., *Polysialic acid directs tumor cell growth by controlling heterophilic neural cell adhesion molecule interactions.* Mol Cell Biol, 2003. **23**(16): p. 5908-18.
27. Seidenfaden, R., A. Krauter, and H. Hildebrandt, *The neural cell adhesion molecule NCAM regulates neuritogenesis by multiple mechanisms of interaction.* Neurochem Int, 2006. **49**(1): p. 1-11.
28. Gage, F.H., Kempermann, G., Song, H., *Adult neurogenesis*. 2008, Cold spring Harbor, New York: Cold spring harbor laboratory press.
29. Doetsch, F., J.M. Garcia-Verdugo, and A. Alvarez-Buylla, *Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain.* J Neurosci, 1997. **17**(13): p. 5046-61.
30. Merkle, F.T. and A. Alvarez-Buylla, *Neural stem cells in mammalian development.* Curr Opin Cell Biol, 2006. **18**(6): p. 704-9.

31. Curtis, M.A., et al., *Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension.* Science, 2007. **315**(5816): p. 1243-9.
32. Seri, B., et al., *Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus.* J Comp Neurol, 2004. **478**(4): p. 359-78.
33. Kam, M., et al., *The cellular composition and morphological organization of the rostral migratory stream in the adult human brain.* J Chem Neuroanat, 2009. **37**(3): p. 196-205.
34. Hack, M.A., et al., *Neuronal fate determinants of adult olfactory bulb neurogenesis.* Nat Neurosci, 2005. **8**(7): p. 865-72.
35. Bedard, A., et al., *The rostral migratory stream in adult squirrel monkeys: contribution of new neurons to the olfactory tubercle and involvement of the antiapoptotic protein Bcl-2.* Eur J Neurosci, 2002. **16**(10): p. 1917-24.
36. Bonfanti, L., *PSA-NCAM in mammalian structural plasticity and neurogenesis.* Prog Neurobiol, 2006. **80**(3): p. 129-64.
37. Chazal, G., et al., *Consequences of neural cell adhesion molecule deficiency on cell migration in the rostral migratory stream of the mouse.* J Neurosci, 2000. **20**(4): p. 1446-57.
38. Koizumi, H., et al., *Doublecortin maintains bipolar shape and nuclear translocation during migration in the adult forebrain.* Nat Neurosci, 2006. **9**(6): p. 779-86.
39. Ocbina, P.J., et al., *Doublecortin is necessary for the migration of adult subventricular zone cells from neurospheres.* Mol Cell Neurosci, 2006. **33**(2): p. 126-35.
40. Hoglinger, G.U., et al., *Dopamine depletion impairs precursor cell proliferation in Parkinson disease.* Nat Neurosci, 2004. **7**(7): p. 726-35.
41. Freundlieb, N., et al., *Dopaminergic substantia nigra neurons project topographically organized to the subventricular zone and stimulate precursor cell proliferation in aged primates.* J Neurosci, 2006. **26**(8): p. 2321-5.
42. Lledo, P.M., M. Alonso, and M.S. Grubb, *Adult neurogenesis and functional plasticity in neuronal circuits.* Nat Rev Neurosci, 2006. **7**(3): p. 179-93.
43. Scholer, H.R., et al., *New type of POU domain in germ line-specific protein Oct-4.* Nature, 1990. **344**(6265): p. 435-9.
44. Ying, Q.L., et al., *Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture.* Nat Biotechnol, 2003. **21**(2): p. 183-6.

45. Rosner, M.H., et al., *A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo.* Nature, 1990. **345**(6277): p. 686-92.
46. Gerrard, L., L. Rodgers, and W. Cui, *Differentiation of human embryonic stem cells to neural lineages in adherent culture by blocking bone morphogenetic protein signaling.* Stem Cells, 2005. **23**(9): p. 1234-41.
47. Niwa, H., J. Miyazaki, and A.G. Smith, *Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells.* Nat Genet, 2000. **24**(4): p. 372-6.
48. Hay, D.C., et al., *Oct-4 knockdown induces similar patterns of endoderm and trophoblast differentiation markers in human and mouse embryonic stem cells.* Stem Cells, 2004. **22**(2): p. 225-35.
49. Wang, G., et al., *Noggin and bFGF cooperate to maintain the pluripotency of human embryonic stem cells in the absence of feeder layers.* Biochem Biophys Res Commun, 2005. **330**(3): p. 934-42.
50. Lendahl, U., L.B. Zimmerman, and R.D. McKay, *CNS stem cells express a new class of intermediate filament protein.* Cell, 1990. **60**(4): p. 585-95.
51. Pevny, L.H., et al., *A role for SOX1 in neural determination.* Development, 1998. **125**(10): p. 1967-78.
52. Wood, H.B. and V. Episkopou, *Comparative expression of the mouse Sox1, Sox2 and Sox3 genes from pre-gastrulation to early somite stages.* Mech Dev, 1999. **86**(1-2): p. 197-201.
53. Kaneko, Y., et al., *Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells.* Dev Neurosci, 2000. **22**(1-2): p. 139-53.
54. Rutishauser, U., *NCAM and its polysialic acid moiety: a mechanism for pull/push regulation of cell interactions during development?* Dev Suppl, 1992: p. 99-104.
55. Ronn, L.C., B.P. Hartz, and E. Bock, *The neural cell adhesion molecule (NCAM) in development and plasticity of the nervous system.* Exp Gerontol, 1998. **33**(7-8): p. 853-64.
56. Wegner, M. and C.C. Stolt, *From stem cells to neurons and glia: a Soxist's view of neural development.* Trends Neurosci, 2005. **28**(11): p. 583-8.
57. Zappone, M.V., et al., *Sox2 regulatory sequences direct expression of a (beta)-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells.* Development, 2000. **127**(11): p. 2367-82.
58. Bylund, M., et al., *Vertebrate neurogenesis is counteracted by Sox1-3 activity.* Nat Neurosci, 2003. **6**(11): p. 1162-8.

59. Zhang, S.C., et al., *In vitro differentiation of transplantable neural precursors from human embryonic stem cells.* Nat Biotechnol, 2001. **19**(12): p. 1129-33.
60. Reubinoff, B.E., et al., *Neural progenitors from human embryonic stem cells.* Nat Biotechnol, 2001. **19**(12): p. 1134-40.
61. Lardon, J., I. Rooman, and L. Bouwens, *Nestin expression in pancreatic stellate cells and angiogenic endothelial cells.* Histochem Cell Biol, 2002. **117**(6): p. 535-40.
62. Frojdman, K., et al., *The intermediate filament protein nestin occurs transiently in differentiating testis of rat and mouse.* Differentiation, 1997. **61**(4): p. 243-9.
63. Sejersen, T. and U. Lendahl, *Transient expression of the intermediate filament nestin during skeletal muscle development.* J Cell Sci, 1993. **106 (Pt 4)**: p. 1291-300.
64. Sakakibara, S., et al., *Mouse-Musashi-1, a neural RNA-binding protein highly enriched in the mammalian CNS stem cell.* Dev Biol, 1996. **176**(2): p. 230-42.
65. Imai, T., et al., *The neural RNA-binding protein Musashi1 translationally regulates mammalian numb gene expression by interacting with its mRNA.* Mol Cell Biol, 2001. **21**(12): p. 3888-900.
66. Stoykova, A. and P. Gruss, *Roles of Pax-genes in developing and adult brain as suggested by expression patterns.* J Neurosci, 1994. **14**(3 Pt 2): p. 1395-412.
67. Goetz, M., *How are neurons specified: master or positional control?* Trends Neurosci, 1998. **21**(4): p. 135-6.
68. Liu, Y.W., et al., *Doublecortin expression in the normal and epileptic adult human brain.* Eur J Neurosci, 2008. **28**(11): p. 2254-65.
69. Seki, T. and Y. Arai, *Expression of highly polysialylated NCAM in the neocortex and piriform cortex of the developing and the adult rat.* Anat Embryol (Berl), 1991. **184**(4): p. 395-401.
70. Nguyen, L., et al., *Untangling the functional potential of PSA-NCAM-expressing cells in CNS development and brain repair strategies.* Curr Med Chem, 2003. **10**(20): p. 2185-96.
71. Bonfanti, L. and D.T. Theodosis, *Expression of polysialylated neural cell adhesion molecule by proliferating cells in the subependymal layer of the adult rat, in its rostral extension and in the olfactory bulb.* Neuroscience, 1994. **62**(1): p. 291-305.
72. Doetsch, F. and A. Alvarez-Buylla, *Network of tangential pathways for neuronal migration in adult mammalian brain.* Proc Natl Acad Sci U S A, 1996. **93**(25): p. 14895-900.
73. Solter, D. and B.B. Knowles, *Immunosurgery of mouse blastocyst.* Proc Natl Acad Sci U S A, 1975. **72**(12): p. 5099-102.

74. Xu, C., et al., *Feeder-free growth of undifferentiated human embryonic stem cells.* Nat Biotechnol, 2001. **19**(10): p. 971-4.
75. Carpenter, M.K., E. Rosler, and M.S. Rao, *Characterization and differentiation of human embryonic stem cells.* Cloning Stem Cells, 2003. **5**(1): p. 79-88.
76. Lebkowski, J.S., et al., *Human embryonic stem cells: culture, differentiation, and genetic modification for regenerative medicine applications.* Cancer J, 2001. **7 Suppl 2**: p. S83-93.
77. Amit, M., et al., *Human feeder layers for human embryonic stem cells.* Biol Reprod, 2003. **68**(6): p. 2150-6.
78. Choo, A.B., et al., *Expansion of pluripotent human embryonic stem cells on human feeders.* Biotechnol Bioeng, 2004. **88**(3): p. 321-31.
79. Lee, J.B., et al., *Available human feeder cells for the maintenance of human embryonic stem cells.* Reproduction, 2004. **128**(6): p. 727-35.
80. Amit, M. and J. Itskovitz-Eldor, *Feeder-free culture of human embryonic stem cells.* Methods Enzymol, 2006. **420**: p. 37-49.
81. Amit, M. and J. Itskovitz-Eldor, *Maintenance of human embryonic stem cells in animal serum- and feeder layer-free culture conditions.* Methods Mol Biol, 2006. **331**: p. 105-13.
82. Cheon, S.H., et al., *Defined feeder-free culture system of human embryonic stem cells.* Biol Reprod, 2006. **74**(3): p. 611.
83. Sjogren-Jansson, E., et al., *Large-scale propagation of four undifferentiated human embryonic stem cell lines in a feeder-free culture system.* Dev Dyn, 2005. **233**(4): p. 1304-14.
84. Martin, M.J., et al., *Human embryonic stem cells express an immunogenic nonhuman sialic acid.* Nat Med, 2005. **11**(2): p. 228-32.
85. Reubinoff, B.E., et al., *Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro.* Nat Biotechnol, 2000. **18**(4): p. 399-404.
86. Richards, M., et al., *Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells.* Stem Cells, 2003. **21**(5): p. 546-56.
87. Hovatta, O., et al., *A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells.* Hum Reprod, 2003. **18**(7): p. 1404-9.
88. Ellerstrom, C., et al., *Derivation of a xeno-free human embryonic stem cell line.* Stem Cells, 2006. **24**(10): p. 2170-6.
89. Kim, M.S., et al., *Microfabricated embryonic stem cell divider for large-scale propagation of human embryonic stem cells.* Lab Chip, 2007. **7**(4): p. 513-5.

90. Peh, G.S., et al., *CD133 expression by neural progenitors derived from human embryonic stem cells and its use for their prospective isolation.* Stem Cells Dev, 2009. **18**(2): p. 269-82.
91. Carpenter, M.K., et al., *Enrichment of neurons and neural precursors from human embryonic stem cells.* Exp Neurol, 2001. **172**(2): p. 383-97.
92. Schuldiner, M., et al., *Induced neuronal differentiation of human embryonic stem cells.* Brain Res, 2001. **913**(2): p. 201-5.
93. Schulz, T.C., et al., *Directed neuronal differentiation of human embryonic stem cells.* BMC Neurosci, 2003. **4**: p. 27.
94. Davidson, K.C., et al., *Wnt3a regulates survival, expansion, and maintenance of neural progenitors derived from human embryonic stem cells.* Mol Cell Neurosci, 2007. **36**(3): p. 408-15.
95. Hong, S., et al., *Neural precursors derived from human embryonic stem cells maintain long-term proliferation without losing the potential to differentiate into all three neural lineages, including dopaminergic neurons.* J Neurochem, 2008. **104**(2): p. 316-24.
96. Jiang, X., et al., *Isolation and characterization of neural crest stem cells derived from in vitro differentiated human embryonic stem cells.* Stem Cells Dev, 2008.
97. Koch, P., et al., *A rosette-type, self-renewing human ES cell-derived neural stem cell with potential for in vitro instruction and synaptic integration.* Proc Natl Acad Sci U S A, 2009.
98. Ma, W., et al., *Cell-extracellular matrix interactions regulate neural differentiation of human embryonic stem cells.* BMC Dev Biol, 2008. **8**: p. 90.
99. Erceg, S., et al., *Differentiation of human embryonic stem cells to regional specific neural precursors in chemically defined medium conditions.* PLoS ONE, 2008. **3**(5): p. e2122.
100. Shin, S., S. Dalton, and S.L. Stice, *Human motor neuron differentiation from human embryonic stem cells.* Stem Cells Dev, 2005. **14**(3): p. 266-9.
101. Yao, S., et al., *Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions.* Proc Natl Acad Sci U S A, 2006. **103**(18): p. 6907-12.
102. Benzing, C., et al., *Neural conversion of human embryonic stem cell colonies in the presence of fibroblast growth factor-2.* Neuroreport, 2006. **17**(16): p. 1675-81.
103. Ben-Hur, T., et al., *Transplantation of human embryonic stem cell-derived neural progenitors improves behavioral deficit in Parkinsonian rats.* Stem Cells, 2004. **22**(7): p. 1246-55.

104. Takagi, Y., et al., *Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model.* J Clin Invest, 2005. **115**(1): p. 102-9.
105. Keirstead, H.S., et al., *Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury.* J Neurosci, 2005. **25**(19): p. 4694-705.
106. Faulkner, J. and H.S. Keirstead, *Human embryonic stem cell-derived oligodendrocyte progenitors for the treatment of spinal cord injury.* Transpl Immunol, 2005. **15**(2): p. 131-42.
107. GeronCorporation. *Geron Receives FDA Clearance to Begin World's First Human Clinical Trial of Embryonic Stem Cell-Based Therapy*. [News Release] 2009 January 23, 2009 [cited 2009 2009-05-26]; press release; http://www.geron.com/media/pressview.aspx?id=1148].
108. Erceg, S., M. Ronaghi, and M. Stojkovic, *Human embryonic stem cell differentiation toward regional specific neural precursors.* Stem Cells, 2008.
109. Penington, D.G. and G.F. Mitchell, *Human embryonic stem cells leap the barrier.* Med J Aust, 2007. **187**(3): p. 139-40.
110. Wei, L., et al., *Transplantation of embryonic stem cells overexpressing Bcl-2 promotes functional recovery after transient cerebral ischemia.* Neurobiol Dis, 2005. **19**(1-2): p. 183-93.
111. Tabar, V., et al., *Migration and differentiation of neural precursors derived from human embryonic stem cells in the rat brain.* Nat Biotechnol, 2005. **23**(5): p. 601-6.
112. Hicks, A.U., et al., *Transplantation of human embryonic stem cell-derived neural precursor cells and enriched environment after cortical stroke in rats: cell survival and functional recovery.* Eur J Neurosci, 2009.
113. Zhang, P., et al., *Transplanted human embryonic neural stem cells survive, migrate, differentiate and increase endogenous nestin expression in adult rat cortical peri-infarction zone.* Neuropathology, 2009.
114. Arenas, E., *Stem cells in the treatment of Parkinson's disease.* Brain Res Bull, 2002. **57**(6): p. 795-808.
115. Brederlau, A., et al., *Transplantation of human embryonic stem cell-derived cells to a rat model of Parkinson's disease: effect of in vitro differentiation on graft survival and teratoma formation.* Stem Cells, 2006. **24**(6): p. 1433-40.
116. Cho, M.S., D.Y. Hwang, and D.W. Kim, *Efficient derivation of functional dopaminergic neurons from human embryonic stem cells on a large scale.* Nat Protoc, 2008. **3**(12): p. 1888-94.

117. Carpenter, M., et al., *Derivation and characterization of neuronal precursors and dopaminergic neurons from human embryonic stem cells in vitro.* Methods Mol Biol, 2006. **331**: p. 153-67.
118. Dottori, M. and M.F. Pera, *Neural differentiation of human embryonic stem cells.* Methods Mol Biol, 2008. **438**: p. 19-30.
119. Itsykson, P., et al., *Derivation of neural precursors from human embryonic stem cells in the presence of noggin.* Mol Cell Neurosci, 2005. **30**(1): p. 24-36.
120. Itskovitz-Eldor, J., et al., *Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers.* Mol Med, 2000. **6**(2): p. 88-95.
121. Schuldiner, M., et al., *Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells.* Proc Natl Acad Sci U S A, 2000. **97**(21): p. 11307-12.
122. Atala, A., *Recent developments in tissue engineering and regenerative medicine.* Curr Opin Pediatr, 2006. **18**(2): p. 167-71.
123. Ameen, C., et al., *Human embryonic stem cells: current technologies and emerging industrial applications.* Crit Rev Oncol Hematol, 2008. **65**(1): p. 54-80.
124. Hohman M M, S.M., Rutledge G and Brenner M P *Electrospinning and electrically forced jets. I. Stability theory Physics of Fluids* 2001. **13**: p. 2201-20.
125. Hohman M M, S.M., Rutledge G and Brenner M P *Electrospinning and electrically forced jets. II. Applications Physics of Fluids* 2001. **13**: p. 2221-36.
126. Theron S A, Z.E.a.Y.A.L., *Experimental investigation of the governing parameters in the electrospinning of polymer solutions Polymer* 2004. **45**: p. 2017-30.
127. Thompson C J, C.G.G., Yarin A L and Reneker D H *Effects of parameters on nanofiber diameter determined from electrospinning model Polymer* 2007. **48**: p. 6913-22.
128. Lutolf, M.P. and J.A. Hubbell, *Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering.* Nat Biotechnol, 2005. **23**(1): p. 47-55.
129. Venugopal, J., et al., *Interaction of cells and nanofiber scaffolds in tissue engineering.* J Biomed Mater Res B Appl Biomater, 2008. **84**(1): p. 34-48.
130. Sell, S.A., et al., *Cross-linking methods of electrospun fibrinogen scaffolds for tissue engineering applications.* Biomed Mater, 2008. **3**(4): p. 45001.
131. Bini, T.B., et al., *Peripheral nerve regeneration by microbraided poly(L-lactide-co-glycolide) biodegradable polymer fibers.* J Biomed Mater Res A, 2004. **68**(2): p. 286-95.

132. Ahmed, I., et al., *Three-dimensional nanofibrillar surfaces covalently modified with tenascin-C-derived peptides enhance neuronal growth in vitro.* J Biomed Mater Res A, 2006. **76**(4): p. 851-60.
133. Bini, T.B., et al., *Development of fibrous biodegradable polymer conduits for guided nerve regeneration.* J Mater Sci Mater Med, 2005. **16**(4): p. 367-75.
134. Sell, S.A., et al., *Electrospun polydioxanone-elastin blends: potential for bioresorbable vascular grafts.* Biomed Mater, 2006. **1**(2): p. 72-80.
135. Sui, G., et al., *Poly-L-lactic acid/hydroxyapatite hybrid membrane for bone tissue regeneration.* J Biomed Mater Res A, 2007. **82**(2): p. 445-54.
136. Yang, F., et al., *Electrospinning of nano/micro scale poly(L-lactic acid) aligned fibers and their potential in neural tissue engineering.* Biomaterials, 2005. **26**(15): p. 2603-10.
137. Yang, F., et al., *Characterization of neural stem cells on electrospun poly(L-lactic acid) nanofibrous scaffold.* J Biomater Sci Polym Ed, 2004. **15**(12): p. 1483-97.
138. Nur, E.K.A., et al., *Three-dimensional nanofibrillar surfaces promote self-renewal in mouse embryonic stem cells.* Stem Cells, 2006. **24**(2): p. 426-33.
139. Sun, T., et al., *Development of a 3D cell culture system for investigating cell interactions with electrospun fibers.* Biotechnol Bioeng, 2007. **97**(5): p. 1318-28.
140. Nisbet, D.R., et al., *Interaction of embryonic cortical neurons on nanofibrous scaffolds for neural tissue engineering.* J Neural Eng, 2007. **4**(2): p. 35-41.
141. Zietlow, R., et al., *Human stem cells for CNS repair.* Cell Tissue Res, 2008. **331**(1): p. 301-22.
142. Carone, T.W. and J.M. Hasenwinkel, *Mechanical and morphological characterization of homogeneous and bilayered poly(2-hydroxyethyl methacrylate) scaffolds for use in CNS nerve regeneration.* J Biomed Mater Res B Appl Biomater, 2006. **78**(2): p. 274-82.
143. Hou, S., et al., *The repair of brain lesion by implantation of hyaluronic acid hydrogels modified with laminin.* J Neurosci Methods, 2005. **148**(1): p. 60-70.
144. Levenberg, S., et al., *Neurotrophin-induced differentiation of human embryonic stem cells on three-dimensional polymeric scaffolds.* Tissue Eng, 2005. **11**(3-4): p. 506-12.
145. Levenberg, S., et al., *Differentiation of human embryonic stem cells on three-dimensional polymer scaffolds.* Proc Natl Acad Sci U S A, 2003. **100**(22): p. 12741-6.

146. Heins, N., et al., *Derivation, characterization, and differentiation of human embryonic stem cells.* Stem Cells, 2004. **22**(3): p. 367-76.
147. Zhou, J.M., J.X. Chu, and X.J. Chen, *An improved protocol that induces human embryonic stem cells to differentiate into neural cells in vitro.* Cell Biol Int, 2008. **32**(1): p. 80-5.
148. Wilson, P.G. and S.S. Stice, *Development and differentiation of neural rosettes derived from human embryonic stem cells.* Stem Cell Rev, 2006. **2**(1): p. 67-77.
149. Bjork-Eriksson, T., et al., *The immunohistochemical expression of DNA-PKCS and Ku (p70/p80) in head and neck cancers: relationships with radiosensitivity.* Int J Radiat Oncol Biol Phys, 1999. **45**(4): p. 1005-10.
150. Bjork-Eriksson, T., et al., *The lack of correlation between proliferation (Ki-67, PCNA, LI, Tpot), p53 expression and radiosensitivity for head and neck cancers.* Br J Cancer, 1999. **80**(9): p. 1400-4.
151. Tomasevic, G., F. Kamme, and T. Wieloch, *Changes in proliferating cell nuclear antigen, a protein involved in DNA repair, in vulnerable hippocampal neurons following global cerebral ischemia.* Brain Res Mol Brain Res, 1998. **60**(2): p. 168-76.
152. Lowhagen, P., B.B. Johansson, and C. Nordborg, *The nasal route of cerebrospinal fluid drainage in man. A light-microscope study.* Neuropathol Appl Neurobiol, 1994. **20**(6): p. 543-50.
153. Pozzi, A., et al., *Integrin alpha1beta1 mediates a unique collagen-dependent proliferation pathway in vivo.* J Cell Biol, 1998. **142**(2): p. 587-94.
154. Rosler, E.S., et al., *Long-term culture of human embryonic stem cells in feeder-free conditions.* Dev Dyn, 2004. **229**(2): p. 259-74.
155. Freshney, R.I., *A manual of basic technique*
Culture of animal cells, ed. F. RI. 1994, New York: Wiley-Liss Inc. . p 255-265.
156. Robertson, E.J., *Embryo-derived stem cell lines*. *Teratocarcinomas and embryonic stem cells: a practical approach*, ed. R. EJ. 1987, Oxford: UK: IRL Press. p 71-112.
157. Reubinoff, B.E., et al., *Effective cryopreservation of human embryonic stem cells by the open pulled straw vitrification method.* Hum Reprod, 2001. **16**(10): p. 2187-94.
158. Richards, M., et al., *Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells.* Nat Biotechnol, 2002. **20**(9): p. 933-6.
159. Draper, J.S., et al., *Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells.* Nat Biotechnol, 2004. **22**(1): p. 53-4.

160. Pittenger, M.F., et al., *Multilineage potential of adult human mesenchymal stem cells.* Science, 1999. **284**(5411): p. 143-7.
161. Tsai, R.Y. and R.D. McKay, *Cell contact regulates fate choice by cortical stem cells.* J Neurosci, 2000. **20**(10): p. 3725-35.
162. Barberi, T., et al., *Derivation of engraftable skeletal myoblasts from human embryonic stem cells.* Nat Med, 2007. **13**(5): p. 642-8.
163. Li, Y., et al., *Expansion of human embryonic stem cells in defined serum-free medium devoid of animal-derived products.* Biotechnol Bioeng, 2005. **91**(6): p. 688-98.
164. Wichterle, H., et al., *Directed differentiation of embryonic stem cells into motor neurons.* Cell, 2002. **110**(3): p. 385-97.
165. Wang, T.W., et al., *Sox3 expression identifies neural progenitors in persistent neonatal and adult mouse forebrain germinative zones.* J Comp Neurol, 2006. **497**(1): p. 88-100.
166. Rathjen, J., et al., *Directed differentiation of pluripotent cells to neural lineages: homogeneous formation and differentiation of a neurectoderm population.* Development, 2002. **129**(11): p. 2649-61.
167. Schwartz, P.H., et al., *Isolation and characterization of neural progenitor cells from post-mortem human cortex.* J Neurosci Res, 2003. **74**(6): p. 838-51.
168. Alessandri, G., et al., *Isolation and culture of human muscle-derived stem cells able to differentiate into myogenic and neurogenic cell lineages.* Lancet, 2004. **364**(9448): p. 1872-83.
169. Kania, G., et al., *Somatic stem cell marker prominin-1/CD133 is expressed in embryonic stem cell-derived progenitors.* Stem Cells, 2005. **23**(6): p. 791-804.
170. Minguell, J.J., et al., *Nonstimulated human uncommitted mesenchymal stem cells express cell markers of mesenchymal and neural lineages.* Stem Cells Dev, 2005. **14**(4): p. 408-14.
171. Wurmser, A.E., et al., *Cell fusion-independent differentiation of neural stem cells to the endothelial lineage.* Nature, 2004. **430**(6997): p. 350-6.
172. Berthiaume, F., et al., *Effect of extracellular matrix topology on cell structure, function, and physiological responsiveness: hepatocytes cultured in a sandwich configuration.* Faseb J, 1996. **10**(13): p. 1471-84.
173. Sanai, N., et al., *Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration.* Nature, 2004. **427**(6976): p. 740-4.
174. Lois, C., J.M. Garcia-Verdugo, and A. Alvarez-Buylla, *Chain migration of neuronal precursors.* Science, 1996. **271**(5251): p. 978-81.

175. Sawamoto, K., et al., *New neurons follow the flow of cerebrospinal fluid in the adult brain.* Science, 2006. **311**(5761): p. 629-32.
176. Schaar, B.T. and S.K. McConnell, *Cytoskeletal coordination during neuronal migration.* Proc Natl Acad Sci U S A, 2005. **102**(38): p. 13652-7.
177. Arvidsson, A., et al., *Neuronal replacement from endogenous precursors in the adult brain after stroke.* Nat Med, 2002. **8**(9): p. 963-70.
178. Dolbeare, F., *Bromodeoxyuridine: a diagnostic tool in biology and medicine, Part III. Proliferation in normal, injured and diseased tissue, growth factors, differentiation, DNA replication sites and in situ hybridization.* Histochem J, 1996. **28**(8): p. 531-75.
179. Dolbeare, F., *Bromodeoxyuridine: a diagnostic tool in biology and medicine, Part I: Historical perspectives, histochemical methods and cell kinetics.* Histochem J, 1995. **27**(5): p. 339-69.
180. Kohwi, M., et al., *Pax6 is required for making specific subpopulations of granule and periglomerular neurons in the olfactory bulb.* J Neurosci, 2005. **25**(30): p. 6997-7003.
181. Lee, A., et al., *Markers of cellular proliferation are expressed in cortical tubers.* Ann Neurol, 2003. **53**(5): p. 668-73.
182. Brown, J.P., et al., *Transient expression of doublecortin during adult neurogenesis.* J Comp Neurol, 2003. **467**(1): p. 1-10.