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SPECIFICATION AND POTENCY OF HUMAN NEURAL STEM CELLS FOR CLINICAL TRANSPLANTATION

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To my family with love

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Specification and Potency of Human Neural Stem Cells for Clinical Transplantation

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ABSTRACT

Neural stem cells hold promise for future treatment of spinal cord injury. Various aspects regarding cell fate specification, manufacturing and monitoring, with implications for clinical applications of these cells, are discussed herein. Neural stem cells can be obtained from a number of sources, including fetal tissue and pluripotent embryonic stem cells. Transplantation of cells derived from immature sources is associated with tumor risk, which needs to be thoroughly investigated. We have shown that assessment of pluripotency should preferably be performed in the intended target compartment, as commonly used teratoma tests failed to detect pluripotent cells remaining after neural induction, cells that after transplantation gave rise to tumors in the central nervous system of model animals. We also found that among non-pluripotent neural stem/progenitor cells (NPCs) from human fetal tissue, occasional cells expressed mRNA for genes associated with the pluripotent phenotype. The phenotype of these NPCs showed no overt differences from the others, but live-cell imaging showed that all NPCs constantly changed their morphology. Surprisingly, mRNA for pluripotency genes was not restricted to a certain subpopulation of cells. Rather, transcripts of these genes transiently appeared in most cells, but during short periods. In a similar way, we found that expression of markers such as PSA-NCAM and A2B5, associated with more differentiated phenotypes, entailed a propensity for differentiation, but not fate restriction. Isolated cell populations with either high or low immunoreactivity for CD133, CD15, CD24, CD29, PSA-NCAM or A2B5 both reconstructed the parental distribution of immunoreactivity after about two weeks in culture. Transcriptome analysis and *in vitro* studies confirmed that the reversible expression of markers was a reflection of reversible phenotypic identity. This finding requires that phenotype interconversion is added to the hierarchical model of neural fate determination *in vitro*. Furthermore, we have developed and evaluated a device for automatized mechanical dissociation of cell aggregates in culture, in compliance with regulatory guidelines for production of cells for transplantation, and shown its usefulness in long-term NPC cultures.

LIST OF PUBLICATIONS

I. Sundberg M, **Andersson PH**, Åkesson E, Odeberg J, Holmberg L, Inzunza J, Falci S, Öhman J, Suuronen R, Skottman H, Lehtimäki K, Hovatta O, Narkilahti S, Sundström E. Markers of pluripotency and differentiation in human neural precursor cells derived from embryonic stem cells and CNS tissue. Cell Transplant. 2011;20(2):177-91.

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III. **Vincent PH**, Odeberg J, Åkesson E, Samuelsson E-B, Holmberg L, Falci S, Seiger Å, Sundström E. Phenotype interconversion in human neural stem and progenitor cells. Manuscript.

IV. Wallman L, Åkesson E, Ceric D, **Andersson PH**, Day K, Hovatta O, Falci S, Laurell T, Sundström E. Biogrid – a microfluidic device for large-scale enzyme-free dissociation of stem cell aggregates. Lab Chip. 2011 Oct7;11(19):3241-8.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BBB	Basso, Beattie, and Bresnahan
BCIs	Brain computer interfaces
BD	Becton Dickinson
bFGF	Basic fibroblast growth factor
BLBP	Brain lipid binding protein
BMP	Bone morphogenetic protein
cDNA	Complementary DNA
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CSPG	Chondroitin sulfate proteoglycan
CTV	Cell trace violet
DCX	Doublecortin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix molecules
EGF	Endothelial growth factor
FACS	Fluorescence-activated cell sorting
Fbr	(Subcortical) forebrain
FBS	Fetal bovine serum
FCS	Forward scatter
FDA	Food and drug administration
FES	Functional electrical stimulation
FMO	Fluorescence minus one
GAPDH	Glyceraldehyde dehydrogenase
gDNA	Genomic DNA
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GMP	Good manufacturing practices
FT	FACS flow through control

GO	Gene ontology
hESC-NPCs	Human embryonic stem cell-derived neural precursor cells
hESCs	Human embryonic stem cells
hfbrNPCs	Human fetal forebrain-derived neural precursor cells
HFFs	Human foreskin fibroblasts
hNPCs	Human fetal-derived neural precursor cells
hiPSCs	Human induced pluripotent stem cells
hNSCs	Human neural stem cells
hscNPCs	Human fetal spinal cord-derived neural precursor cells
IR	Immunoreactive
LDH	Lactate dehydrogenase
LIF	Leukemia inhibitory factor
MAP2	Microtubule-associated protein 2
MBP	Myelin basic protein
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem/stromal cells
NBM	Neurobasal medium
NCAM	Neural cell adhesion molecule
NDM	Neural differentiation medium
NeuN	Neuronal nuclear antigen
NS	Neurosphere medium
NSE	Neuron specific enolase
NSG	Neurosphere growth medium
NSM	See NSG
OPCs	Oligodendrocyte progenitor cells
PBS	Phosphate-buffered saline
PEST	Penicillin/streptomycin
PFA	Paraformaldehyde (formaldehyde solution)
PO	Polyornithin
PSA-NCAM	Polysialylated neural cell adhesion molecule
PSI	Pounds per square inch

qPCR	Quantitative polymerase chain reaction
RA	Retinoic acid
RAGT	Robot-assisted gait training
RFP	Red fluorescent protein
RT-PCR	Reverse transcriptase polymerase chain reaction
sc	Spinal cord
SCI	Spinal cord injury
SCID	Severe combined immunodeficiency
SHH	Sonic hedgehog
SR	Serum replacement
SSC	Side scatter
TF	Transcription factor
VPA	Valproic acid

1 INTRODUCTION

1.1 CLINICAL BACKGROUND

1.1.1 Spinal Cord Injury (SCI)

Traumatic injury to the spinal cord (SCI) causes loss of sensory and motor function as well as disruption of autonomic functions. The most common causes for SCI are traffic accidents and falling accidents, and often, young individuals are affected. Assessments of quality-of-life parameters for afflicted individuals indicate that medical problems such as neurogenic pain, pressure sores, spasticity, bladder dysfunction, bowel dysfunction and sexual dysfunction affect their well-being, often to a larger degree than the extent of their motor impairment, which is the disability that most people associate with SCI (Westgren and Levi, 1998). In addition, these patients face increased risk of cardiovascular complications and shorter life expectancy (Middleton et al., 2012). The reported global incidence of SCI - between 8 and 246 cases per million inhabitants per year - varies between regions. The variation is attributable to both socioeconomic and cultural factors as well as to differences in definition and survey methodology. Global reported prevalence varies from about 100 to 1,000 per million inhabitants. Prevalence is rising mainly due to increased survival of SCI patients (Furlan et al., 2013; Singh et al., 2014).

1.1.2 Pathophysiology of SCI

The progress of traumatic SCI is usually divided into three phases, acute, sub-acute/secondary and chronic. In the acute phase, encompassing the immediate injury and the following days, mechanical damage immediately ruptures neural and vascular tissue, inducing cell necrosis and ischemia as well as neurogenic chock, which lasts for about 24 hours and represents a general failure of the neuronal network. The secondary phase overlaps the acute and includes excitotoxic insult induced by glutamate released by ruptured cells. Apoptosis, or programmed cell death, occurs as a result from excess glutamate signaling. Lipid peroxidation and release of free radicals further damage the tissue. Astrocyte activation and gliosis are accompanied by inflammatory immune responses by invading neutrophils and lymphocytes. In addition, factors inhibiting neurite growth are expressed at the lesion site, obstructing regenerative sprouting of axons. Finally, in the chronic phase, scar tissue is formed, and alterations in receptor and channel protein expression and behavior create a new environment, further exacerbating demyelination and apoptosis (Hulsebosch, 2002).

Spontaneous recovery occurs in the period after traumatic spinal cord injury as the initial spinal chock abates over weeks or months (Ditunno et al., 2004) and some of the consequences of injury are compensated for by neuronal plasticity. Although the neurological symptoms may still improve a year after injury, there is in most cases very limited spontaneous recovery long term, and, historically, no treatments and certainly no cures have been available. In ancient Egypt, records were kept on spinal cord injured

patients, and somewhat laconically describing them as having “an ailment not to be treated”. The limited ability of the human spinal cord to heal itself after injury can most certainly be ascribed to the fact that there is limited favorable evolutionary pressure for such a capacity in a severely injured spinal cord. Rather, the regenerative compensatory plasticity that does exist, albeit restricted, has been shown to form aberrant neuronal circuits, leading to progressive neuronal dysfunction (Beauparlant et al., 2013). With the advent of modern medicine and improved hygiene, lethality following SCI has dropped dramatically and the hope for effective treatment and even cure is rising steadily.

1.1.3 Therapies for SCI

1.1.3.1 Rescue Strategies and Pharmacological Treatment

Immediate intervention following injury is potentially important to restrict the secondary deleterious effects that include ischemia and inflammation at system level, and glutamate excitotoxicity and free radical-induced cell death at the molecular level. Strategies that routinely have been employed clinically include decompression surgery and high-dose infusion of corticosteroids, primarily methylprednisolone, which robustly ameliorates injury symptoms in rodent SCI models, and initially was claimed to lead to significant clinical improvement of neurological outcome (Bracken et al., 1990). However, clinical use of methylprednisolone (A-Methapred, Solu-Medrol) has in recent years been advised against due to questionable beneficial effects in prospective blinded randomized trials, and adverse deleterious effects such as gastro-intestinal bleeding, wound infections and hyperglycemia. Although the scientific data does not support the use of high-dose methylprednisolone after SCI, there is no absolute consensus among clinicians (Bracken, 2012; Cheung et al., 2015; Evaniew et al., 2016).

Other strategies aiming for neuroprotection include cooling of the spinal cord, administration of tetracyclins and glutamate antagonists and hyperbaric oxygen treatment (Asamoto et al., 2000; Casha et al., 2012; Hansebout and Hansebout, 2014; Xu et al., 2004).

Strategies to promote the regeneration of injured tissue after SCI strive to alter the microenvironment, which is generally non-permissive to growth. Cells in the intact and injured spinal cord express a variety of attractant and repulsive molecules such as netrins, integrins and matrix molecules including chondroitin sulfate proteoglycans (CSPGs). Several clinical strategies to inhibit CSPGs have been developed (Kim et al., 2017). The most famous and well-studied neurite growth inhibitor is probably Nogo, a protein expressed in the central nervous system (CNS) known to inhibit axon regeneration. Blocking Nogo activity at its receptor or by sequestering it has been shown to protect and rescue neurons in animal injury models. However, it is likely that such an approach will have to be combined with other therapeutic strategies to maximize functional recovery after SCI (Schweigreiter and Bandtlow, 2006).

1.1.3.2 Cell Transplantation

Several possible ways by which transplanted cells can contribute to the improvement of motor function after SCI have been conceived. The beneficial effects in SCI have been suggested to include neuroprotection, enhanced regenerative growth, immunomodulation, cell replacement and functional support. In most animal studies the time window of successful transplantation has been limited, and the biggest effect is seen when transplantation is performed in the acute or sub-acute stage of the injury. Several studies have showed that the mechanism behind the treatment effect is that the graft provides support for neurons-at-risk, possibly through the release of trophic factors. It has been claimed that acute transplantation is less effective than sub-acute because the inflammatory situation acutely after injury decreases graft survival, but results from our research group contradicted this statement (Emgard et al., 2014). With a delay of more than 1–2 weeks the time window for protective effects in rodents closes, but a window of opportunity to enhance regeneration remains for some time, until the subsequent formation of the glial scar impedes the effect of the transplant. The relation between post-injury time of transplantation and injury progression of rodent models eventually needs to be translated into the human setting. Different cell types are believed to exert different effects, and a few clinical trials have been initiated following different tracks, using fetal cells, oligodendrocyte progenitors, autologous Schwann cells, mesenchymal stromal cells or microglia (Assinck et al., 2017).

Human fetal neural progenitor cells (hNPCs) from abortion material, similar to cells used in this thesis, have been used in the Pathway Study, conducted by the company StemCells Inc. Part of the study was randomized and single-blinded, designed to evaluate the efficacy of allogeneic transplantation of fetal brain-derived hNPCs to 12 patients with thoracic, sub-acute SCI. The results of interim analysis revealed differences in motor strength that favored the treatment group, but the magnitude of the effect was lower than the company had hoped for, which led to termination of the study (see (Anderson et al., 2017a)), (clinicaltrials.gov). Preclinical data showed that mice and rats transplanted with the cells could recover sensory as well as motor function after SCI. Mechanisms thought to be responsible for functional recovery included neuroprotection, remyelination and synaptic integration (Tsukamoto et al., 2013). However, a recent evaluation in rodent models of the specific cell lines used in the Pathway Study found no evidence of efficacy, raising questions about the validation of cells intended for clinical trials (Anderson et al., 2017a).

The first study to use cells derived from human embryonic stem cells (hESCs) for treatment of SCI was conducted by the US-based company Geron Inc. Oligodendrocyte progenitors (OPCs) derived from hESCs were transplanted to five SCI patients, aiming primarily for re-myelinisation of host neurons. The study found no adverse effects, but was discontinued for financial reasons in 2011. The company Asterias Biotherapeutics used higher doses of the same cells in a phase 1 clinical trial that was completed in 2014 without finding adverse effects. They are currently recruiting patients to a study investigating even higher doses. The hESC-derived OPC transplants have been shown to attenuate lesion pathogenesis and improve recovery of forelimb function in rat SCI

models (Sharp et al., 2010). Interestingly, for a short period Geron Inc. published in their web site information that the OPCs released several factors that could be protective, and that antibodies to TGFβ2 blocked the treatment effects in a study of the OPCs in SCI rats. This information was removed after a few weeks.

The rationale behind cell transplantation of Schwann cells for SCI is to support regenerative axonal growth by myelinating sprouting host axons, to remyelinate axons that were demyelinated after the injury, as well as release of neurotrophic factors and extra-cellular matrix molecules. An Iranian study of 33 patients transplanted with autologous Schwann cells with a 2-year follow-up found no adverse effects and signs of improved sensory function (Saberi et al., 2011). The Miami Project to Cure Paralysis has also successfully completed a phase 1 study using autologous Schwann cells (Anderson et al., 2017b).

Mesenchymal stem/stromal cells (MSCs) are comparatively simple to culture and can be harvested from various tissues in patients and used for autologous transplantation without immunosuppression. The effects of MSCs are mainly attributed to their anti-inflammatory effects, but some studies suggested they could differentiate into neurons (Jiang et al., 2002). The latter hypothesis is however questioned by many researchers (Franco Lambert et al., 2009). Several safety studies in SCI patients have been conducted using MSCs, none of which have reported any concerns. However, functional effects, presumably resulting from neuroprotection, have been absent or very small (Dasari et al., 2014).

All cell transplantation strategies require cells cultured in accordance with regulations, good manufacturing practices (GMP), stipulated by regulatory agencies. The basic principles of GMP include good hygiene, controlled and well-defined conditions, thorough documentation and traceability of compounds. Production of human cells for transplantation under GMP specifically demands minimal contribution of xenogeneic compounds in the culturing procedures, to avoid transfer of pathogens or other genetic material. This has expedited the production of feeder-free conditions and defined media devoid of animal products for culturing of cells intended for transplantation, for instance. Splitting of cell cultures still relies on proteolytic enzymes that catalyze separation of cell aggregates by cutting cell adhesion molecules. We have developed and evaluated a mechanical device, called the Biogrid, described in paper IV, that dissociates cell aggregates without relying on the use of enzymes.

1.1.3.3 Other Treatment Strategies

The CNS is partly “immune privileged”, meaning that introduction of antigens from infection or trauma fail to elicit a proper inflammatory immune response. The blood-brain barrier was widely believed to constitute an impenetrable wall separating the CNS from the systemic immune system until Michal Schwartz and co-workers discovered in the late 90ies that immune cells are pivotal for CNS neuroprotection and repair, although their spontaneous recruitment to the CNS is insufficient (Moalem et al.,

1999). Treatment strategies involving modulation of immune response, including transplantation of activated macrophages, to resolve deleterious inflammatory responses following CNS injury are currently being developed, but the first clinical study, ProCord, conducted by the company ProNeuron, was closed prematurely due to a combination of funding problems and lack of obvious beneficial effects (Jones et al., 2010).

Recent rapid development in areas such as micro-robotics, biosensors and material research has made ideas about human/machine interactions increasingly realistic. Robot-assisted gait training (RAGT) has been used in rehabilitation since the late 90ies. Brain computer interfaces (BCIs) are devices that measure brain activity and translate it into control signals used for communication, environment control or upper extremity neuroprostheses in which Functional Electrical Stimulation (FES) uses surface electrodes to create purposeful contractions for improved motor function (Rupp, 2014). Importantly, the development of mechanical devices such as exoskeletons focuses on motor performance, and although increased mobility is linked to better cardiac function, bowel function and quality of life, many dysfunctions troubling SCI patients, such as urinary bladder problems, pain and spasticity, are not addressed. For complete restoration of spinal cord function, replacement of lost neuronal signaling is most likely necessary. In the future, combinations of beneficial therapies will likely be used, possibly using gene therapy or other ways to manipulate the microenvironment (Baptiste and Fehlings, 2006; Muheremu et al., 2016; Peng et al., 2015; Sahni and Kessler, 2010; Zhao and Fawcett, 2013).

The recent shut-down of three high-profile clinical trials (ProNeuron, StemCells Inc. and Geron) demonstrates the tremendous financial pressure that lies on companies performing stem cell-based clinical trials in SCI. As a matter of fact, StemCells Inc. has recently merged with the Israeli company Microbot Medical, which specializes in micro-robotic technologies. The failures also show there is a mismatch between the requirements of stem cell researchers and the expectations of investors (Smalley, 2016). And that stem cell science is hard. Really hard.

1.2 CELLULAR DEVELOPMENT *IN VITRO* AND *IN VIVO*, POTENCY

1.2.1 The Epigenetic Landscape

“The epigenetic landscape” is a visualization of cellular differentiation that Conrad Waddington introduced in 1957, and consists of a mountain with ridges and valleys along its slopes (see figure 1). Waddington envisioned a completely undifferentiated cell – a totipotent cell – as a marble starting its journey of differentiation from the top of the mountain along the valleys all the way down to rest at the lowest points at the foot of the mountain while acquiring more and more features of a mature, differentiated cell as it loses potential energy (=potency). At certain points along the way, decisions are made as to which slope the cell should follow to which final fate. In his publication “The Strategy of the Genes”, Waddington also included another, less famous, image of the underside of the landscape, were genes are attached via strings that exert tension on

the landscape above. The tension varies depending on gene expression, which in turn depends on genetic and epigenetic traits as well as environmental cues, altering the topography of the landscape and changing the likelihoods of different outcomes in cellular differentiation. The first image became popular about a decade ago when Shinya Yamanaka and co-workers introduced the concept of induced pluripotent stem cells (iPSCs). By adding a defined set of transcription factors, Yamanaka managed to revert somatic cells back to pluripotency, which is often illustrated with an image of cells traveling up Waddington's mountain, against the fictitious gravitational field. A more suitable homage to Waddington would be using the second image including the Yamanaka factors pulling the mountain from below at the top, inversing it, creating a local minimum were the top used to be (or actually close to the top: iPSCs are pluripotent, not totipotent). Nevertheless, the work of Yamanaka, and John Gurdon before him, on the reversibility of differentiation earned them the Nobel Prize in physiology or medicine in 2012, and initiated a whole new field of research with tremendous potential for both experimental biology and clinical applications. It also overturned the somewhat prevailing dogma that differentiation is a one-way street.

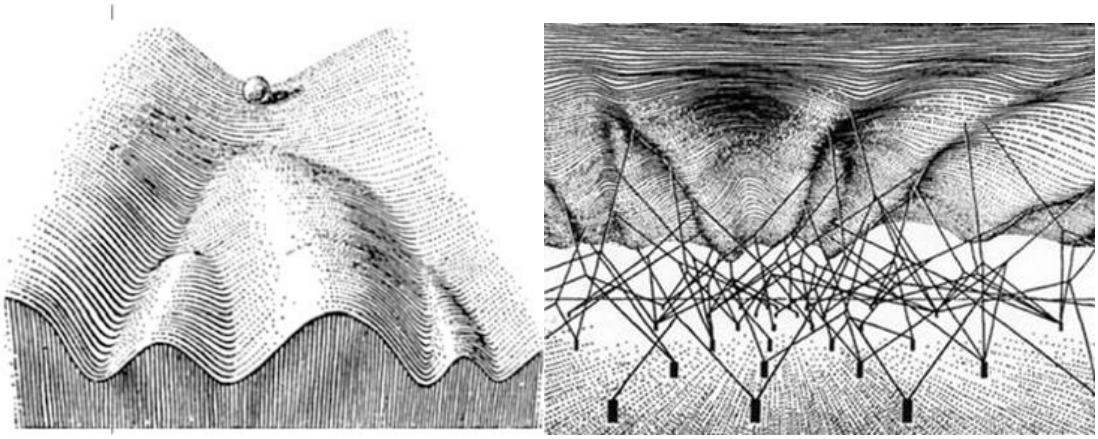


Figure 1. Waddington's epigenetic landscape. Left: The marble at the top represents a totipotent cell beginning its journey towards differentiation. Right: Gene expression alters the topography of the landscape. Images from "The Strategy of the Genes", Waddington, 1957 (Waddington, 1957).

1.2.2 *In Vivo*

In the mammalian embryo, cell development is tightly controlled and progresses in an orderly sequence. This is accomplished by cross talk between neighboring cells (local induction) or distant signaling eventually leading to differential gene expression in cells destined for different fates. The ability of organisms to stop cells from travelling against the gravitational field of Waddington's mountain is crucial to keep structural integrity and avoid neoplasm formation. Much of mammalian cell machinery is devoted to keeping cells in check, making sure that the only way to go is down the mountain, or into controlled cell death – apoptosis, and in that regard, to some extent, differentiation *is* a one-way street. There are four major ways for maintaining differentiation once it has been initiated (Gilbert, 2006):

1. Transcription factors can act on the enhancer of its own gene, leading to independence of the signal that originally induced it.

2. Chromatin modification keeps the correct genes accessible and blocks access to inappropriate genes.
3. Self-regulatory autocrine signaling maintains phenotype.
4. Laterally enforced regulation: neighboring cells enforce the differentiation of each other.

Much of the principles governing *in vivo* development can be directly applied to *in vitro* studies because of the robustness of the developmental machinery, but as the three-dimensional structure is lost, things certainly change. The process of specification *in vivo*, where cells acquire more mature features and eventually become post-mitotic (in the case of neurons), includes asymmetric cell division, where one daughter cell becomes more mature and the other one remains an un-specified progenitor cell. A symmetric cell division gives rise to two progenitor cells. In a relatively homogeneous cell culture *in vitro*, spatial organization of cells is disrupted, and the local cues, such as cell adherence molecules and morphogen gradients are absent or disturbed, which leads to less predictable mitotic results. The *in vivo* situation can, however, be partially mimicked by artificial morphogen gradients to achieve asymmetric cell division *in vitro* (Habib et al., 2013).

1.2.3 Levels of Cell Potency: Totipotency, Pluripotency, Multipotency, Restricted Potency and Differentiated Progeny

In mammals, the fertilized oocyte and the blastomeres of the first division(s) are totipotent, which means that they can give rise to embryonic as well as extra-embryonic tissue. But, according to the commonly used definition stating that the term "stem cell" denotes a cell that has the capacity to self-replicate, totipotent mammalian cells are not stem cells *in vivo.* Rather, they rapidly give rise to pluripotent stem cells and placental cells. Correspondingly, their developmental potential has not been captured *in vitro*. Pluripotency is commonly defined as a cellular state from which progeny of all three germ layers (ectoderm, mesoderm and endoderm) can be obtained. Although pluripotency, like totipotency, is a transient state *in vivo*, pluripotent cells can be derived from different stages of early embryonic development and maintained indefinitely in an artificially induced self-renewal state *in vitro* by supplementing exogenous cues (Nichols and Smith, 2012). In 2009, Nichols and Smith proposed two pluripotent phases: naïve and primed (Nichols and Smith, 2009). The naïve state is defined by the unrestricted developmental potential to give rise to all somatic lineages and the germline, exists only transiently in the preimplantation epiblast and is characterized, among a few other traits, by two active X chromosomes in female cells. In the primed state, one X chromosome is inactivated. The term primed alludes to the developmental pluripotent state that resembles the post-implantation embryonic configuration rather than the even less specified pre-implantation state. Cultured embryonic stem cells from rodents present the features of naïve stem cells, but conventional generation of embryonic stem cells from human blastocysts yields primed cells, representing a slightly more specified developmental stage, requiring *in vitro* manipulation with kinase inhibitors to reset them to the naïve state. Recently however,

the same group of researchers that defined the two different phases succeeded in deriving naïve cells from the inner cell mass of a human embryo (Guo et al., 2016).

1.2.3.1 Pluripotency Network

Some markers are differentially expressed in human naïve and primed pluripotent stem cells, but both cell types share expression of the transcription factors at the core of the pluripotency network, NANOG, OCT-4 and SOX2. These transcription factors (TFs) constitute a regulatory level of the transcriptional profile of the cell. In mammalian pluripotent cells, the core gene regulatory network actively maintains cells in a pluripotent state by repressing differentiation (Kalmar et al., 2009; Silva and Smith, 2008). As well as acting on their own promoters, OCT-4 and SOX2 form heterodimers and bind to the proximal *NANOG* promoter, thereby creating cross-regulatory and auto-regulatory transcriptional loops that maintain pluripotency through both activation and repression (Boyer et al., 2005; Pan et al., 2006; Rodda et al., 2005; Wang et al., 2006), reviewed in (Johnson et al., 2008). It has been shown that the core TFs do not act as pan-repressors of differentiation, but each factor controls specific fates. For instance, high levels of OCT-4 entail mesodermal differentiation and low levels ectoderm, in close interaction with the BMP4 signaling pathway. SOX2 represses mesendoderm differentiation whereas NANOG specifically represses ectoderm (Wang et al., 2012). Mouse ES cells expressing parts of the transcription factor network contribute poorly to chimaera formation compared to cells expressing all parts of the network (Toyooka et al., 2008), although studies of gene expression in hESCs at single cell level has revealed significant heterogeneity in the pluripotent stem cell compartment, showing that there is a gradient and a hierarchy of expression of pluripotency genes. Not all pluripotency-associated gene transcripts are found even in the population most likely to give rise to hESC colonies under conditions that promote hESC renewal. Rather than representing a binary choice, pluripotency and specification in hESCs exist along a continuum that is associated with an apparent probabilistic element of fate determination and interconversion between states (Enver et al., 2009; Hough et al., 2009).

1.2.3.2 NANOG

NANOG is the first known transcription factor to appear after compaction in eutherian mammals, and its expression is down-regulated after implantation, precisely corresponding to the pluripotent phenotype *in vivo* (Chambers et al., 2003). When overexpressed in ES cells, NANOG is sufficient to sustain the pluripotency state in the absence of exogenously added leukemia inhibitory factor (LIF), a feature that no other TFs have been shown to possess (Chambers et al., 2003; Mitsui et al., 2003). NANOG levels undergo slow, random fluctuations, giving rise to heterogeneous cell populations without loosing pluripotency, but low NANOG expression entails higher propensity to differentiate (Chambers et al., 2007; Kalmar et al., 2009). However, in reprogramming of iPSCs, appearance of NANOG does not mark the fully reprogrammed state, as NANOG+ cells often do not give rise to human iPS cell colonies (Chan et al., 2009).

1.2.3.3 OCT-4

While the function of OCT-4 in non-pluripotent cells has not been widely studied (although its presence in somatic cells has been questioned (Lengner et al., 2008)), its function in maintaining the pluripotent state of cells has been studied extensively for more than 25 years (Scholer et al., 1990; Nichols et al., 1998; Radzisheuskaya and Silva, 2014), and it is considered a master regulator for initiation and maintenance of pluripotency during embryonic development (reviewed in (Shi and Jin, 2010)). Pluripotency is maintained mainly by buffering the transcriptional activity of OCT-4, which also appears to be the main determinant to exit pluripotency (Munoz Descalzo et al., 2013)

1.2.3.4 REX1

The zinc finger transcription factor reduced expression 1 (REX1) displays similar, but not identical, expression pattern as OCT-4 in pluripotent cells *in vivo* (REX1 expression is restricted to the ICM and is down-regulated in the epiblast and the primitive ectoderm), and is mostly absent from differentiated cells (Toyooka et al., 2008; Mitsui et al., 2003). REX1 is tightly and directly regulated by NANOG and SOX2, which bind to *REX1* promoter elements and cooperatively enhance transcription.

1.2.3.5 Other Pluripotency-associated Markers

Within the pluripotency continuum of hESCs *in vitro*, the growth factor GDF3 and the NODAL receptor TDGF-1 are the only proteins found selectively expressed by hESCs residing in the state suggested to be at the top of the pluripotency hierarchy (Hough et al., 2009). Some other secreted factors, such as CRIPTO and LEFTY, and receptors, such as CD133 and CD326, have also been positively associated with the pluripotent state (Abeyta et al., 2004; Boyer et al., 2005; Sundberg et al., 2009). The stage-specific glycolipid embryonic antigens SSEA-3, SSEA-4 are expressed by hESCs, but their function is unclear, and it has been shown that they are dispensable in maintaining the pluripotency in hESCs (Brimble et al., 2007). The glycan SSEA-5 and the keratan sulfate antigens Tra-1-60 and Tra1-81 are also expressed by hESCs with some degree of specificity (Schopperle and DeWolf, 2007; Tang et al., 2011). STELLA (DPPA3) is necessary for successful conversion of iPSCs (Xu et al., 2015), and signs of differential expression between naïve and primed pluripotent cells have been shown for STELLA, as well as for DPPA5, KLF17 and TFCP2L1 (Qian et al., 2016; Weinberger et al., 2016; Ye et al., 2013). The DNA methyltransferase DNMT3B is expressed in pluripotent cells and down-regulated upon differentiation together with LIN28 and HESX1, although HESX1 is also expressed in several other tissues (Richards et al., 2004; Watanabe et al., 2002). The Activin A signaling pathway has been shown to be indispensible for maintaining pluripotency (Vallier et al., 2009).

1.2.4 Neural Stem, Progenitor and Restricted Precursor Cells

Generally, the term stem cell is used for a cell with a seemingly unlimited capacity for self-renewal, whereas a progenitor cell gives rise to terminally differentiated progeny

after a finite number of mitoses, but there are no universally accepted definitions and researchers within different fields of biology use the terms differently (Seaberg and van der Kooy, 2003). Neural progenitor cells (NPCs) are multipotent, and can give rise to neurons, astrocytes and oligodendrocytes as well as self-replicate. In 1992, Reynolds and Weiss proved for the first time the existence of NPCs in adult mammals by demonstrating that adult mouse striatal cells could be propagated *in vitro*. They also, more or less by chance, developed the neurosphere culture assay, in which neural progenitors proliferate as free-floating spheres (see figure 2) without growth substrate (Reynolds et al., 1992; Reynolds and Weiss, 1992). Since then, the neurosphere culture assay has been used to culture and evaluate NPCs, sometimes with the aim to find a subpopulation that would constitute the true Neural Stem Cells (NSCs). NSCs are multipotent, slow dividing and self-replicate while giving rise to more committed progenitors. They are thought to constitute a small proportion of cells in neurosphere culture and have been suggested to make up the sphere-forming population *in vitro* (Capela and Temple, 2002; Cummings et al., 2005; Reynolds and Rietze, 2005; Tamaki et al., 2002; Uchida et al., 2000). In 2005, Reynolds and Rietze calculated the NSC frequency to be 0.16 % (Reynolds and Rietze, 2005). The use of the neurosphere assay to determine the proportion of NSCs has, however, been questioned since neurospheres are highly motile and are prone to fuse and divide (Singec et al., 2006).

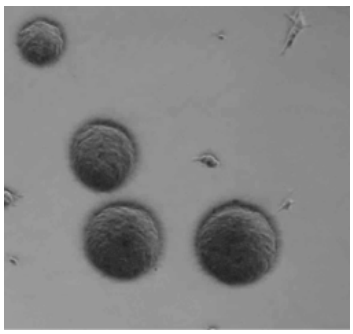


Figure 2. Neurospheres derived from human fetal spinal cord in culture.

A number of markers have been proposed to label murine and/or human NSCs *in vitro*, including Prominin1 (CD133), LeX (CD15), Notch1, Syndecan-1 (CD138), β-1-Integrin (CD29), CXCR4 (CD184) and FGFR4 (reviewed in (Ramasamy et al., 2013)). Conversely, a number of markers have been proposed to label progenitor cells more specified towards a certain lineage: glial progenitors express the ganglioside epitope A2B5 and the glycoprotein CD44 (Dietrich et al., 2002; Liu et al., 2004), and neuronal progenitors express the poly-sialylated neural cell adhesion molecule (PSA-NCAM) and the glycoprotein CD24 (Han et al., 2002; Lepore and Fischer, 2005; Mayer-Proschel et al., 1997). Unspecified NPCs almost uniformly express the intermediate filament protein nestin (Lendahl et al., 1990). Ravin and co-workers showed in 2008 that fate specification of rat neural progenitors to bi-potent (able to generate astrocytes and oligodendroglia) or uni-potent (generating either neurons or astrocytes or oligodendroglia) precursors *in vitro* occurs early, while cells are dividing in the presence of mitogens, implying that restricted precursor cells exist *in vitro*. Surprisingly, the specification was lost at passage, showing that progenitor fate restriction is reversible under certain circumstances, which certainly dilutes the meaning of the word “restricted” (Ravin et al., 2008). Nevertheless, the terms neural restricted precursors (NRPs) and glial restricted precursors (GRPs) have been widely used for cells expressing PSA-NCAM and A2B5, respectively (Cao et al., 2005; Cao et al., 2002; Davies et al., 2006; Haas and Fischer, 2013; Han et al., 2002; Han et al., 2004; Lepore and Fischer, 2005; Lepore et al., 2004; Mayer-Proschel et al., 1997; Rao

and Mayer-Proschel, 1997; Sandrock et al., 2010). Many studies on specification of NSCs via NPCs and NRPs/GRPs to differentiated, mature cells have been conducted on rodent cells *in vitro*, and the translation to injury models has not been entirely straightforward. For instance, the injured spinal cord is far less permissive to neuronal maturation of transplanted neuronal progenitors than the uninjured cord (Cao et al., 2002), and mouse neuronal precursors generate glial progeny after ectopic transplantation (Seidenfaden et al., 2006). The translation further to human cells has presented additional challenges vis-à-vis cell specification and marker specificity (Sim et al., 2009). For instance, A2B5 has been reported to co-localize with neuron-specific enolase (NSE) in human fetal brain cells (Satoh and Kim, 1995).

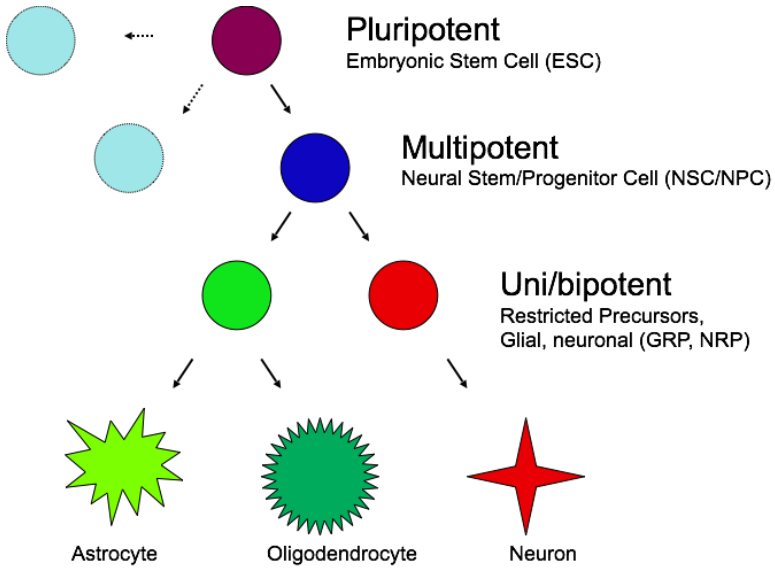


Figure 4. Schematic representations of the hierarchy of potency of cells along the neuroectodermal lineage, as the hypothesis under which the studies in the present thesis were initiated. A pluripotent cell (magenta) gives rise to a multipotent neural progenitor (blue), which gives rise to restricted progeny, glial (green) and neuronal (red), which in turn generates terminally differentiated cells.

1.2.5 Differentiated Cells

The primary purpose and final goal of neural cell maturation is of course to generate functionally mature progeny that are properly incorporated in the tissue. Immature neurons are characterized by expression of the cytoskeletal proteins β-tubulin III, doublecortin (DCX) and microtubule-associated protein 2 (MAP2), while further maturation induces expression of neuronal nuclei antigen (NeuN), NSE and synaptic markers such as PSD-95, synaptophysin and synaptotagmin. In human spinal cord development, neuronal precursors leave the proliferative zone lining the central canal and undergo changes in transcription factor (TF) expression profile under the influence of dorso-ventral molecular gradients of Sonic hedgehog (SHH) and bone morphogenetic proteins (BMPs), secreted from the notochord and roof plate, respectively. Subtype specification is thus carefully governed by expression of mutually exclusive pairs of TFs depending on perceived concentrations of SHH and BMP (Jessell, 2000). Other secreted molecules, such as fibroblast growth factor (FGF) and retinoic acid (RA), induce simultaneous anterior-posterior identity specification via expression of homeobox (HOX) genes (Duester, 2008). Astroglial maturation in the human spinal cord occurs later, beginning at 6 weeks after conception, followed by oligodendrocyte maturation (Marklund et al., 2014). Astrocytes are characterized by glial fibrillary acidic protein (GFAP) immunoreactivity. Oligodendrocyte progenitors

express OLIG1, OLIG2, chondroitin sulfate proteoglycan (NG2) and platelet-derived growth factor α, while mature oligodendrocytes express myelin basic protein (MBP) and galactolipids (GALC, O1, O4) (Lu et al., 2001). The specificity of glial markers is generally low in humans. Cellular identity is confirmed based on functionality and morphology.

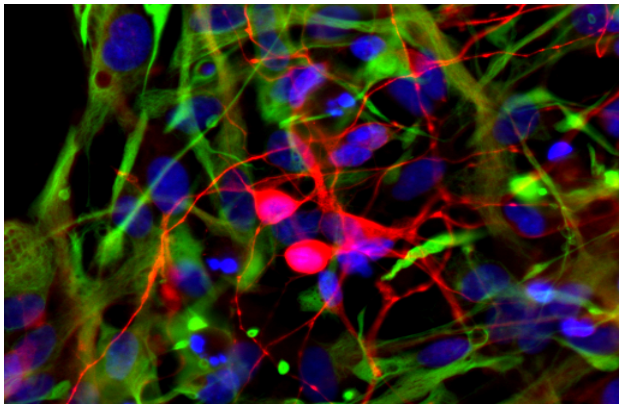


Figure 3. Differentiated progeny of hscNPCs: GFAP-immunoreactive cells (green, presumably astrocytes), β-tubulin III-immunoreactive cells (red, presumably neurons). Cell nuclei are counterstained with Hoechst (blue).

1.2.6 Sources of Neural Progenitor Cells

1.2.6.1 Fetal Tissue

The primary source of cells used in this thesis was human fetal CNS tissue from gestational week 5-11, specifically spinal cord and sub-cortical forebrain. The reason for excluding cortical cells is that the human cortex develops rapidly during 5-9 weeks after conception, from a thin cell layer at week 5 to making up the major fraction of the CNS at week 9, which makes it difficult to compare fetuses of varying ages using cortical material. Fetal tissue is only obtained after initial neuroectodermal specification has occurred, and the cells, although proliferating *in vitro*, are maintained within the neuroectodermal lineage. After about 9.5 weeks of gestation, the ability of human fetal spinal cord to generate viable stem/progenitor cultures is severely hampered (Akesson et al., 2007).

1.2.6.2 Adult Human Tissue

The regenerative capacity of the adult CNS in humans is very limited. Although various compartments of the adult CNS contain cells that posses proliferative ability, there seems to be very little production of neurons that actually incorporate into functioning neural networks after birth, certainly not in the cortex. Neurogenesis in the human hippocampus contributes to a turnover rate of 0.004 % daily in the dentate gyrus, but it is still unclear whether this exchange is of any significance for either normal brain function or disease progress (Bergmann et al., 2015). Nevertheless, cells from the subventricular zone of the lateral ventricle, the hippocampal granular zone, the filum terminale and a few other places of adult human CNS can be cultured *in vitro* and generate neurons (Nam et al., 2015). It has been suggested that adult stem cells is a preferred source of cells for clinical application, because of the possibility to do autologous transplantation – extract cells from the patient's stem cell niche and transplant them back to the patient, with or without *in vitro* culturing.

1.2.6.3 Embryonic Stem Cells

Neuroectodermal fate seems to be the default pathway of differentiating ESCs, although at protracted differentiation pace and with low yield (Kamiya et al., 2011). Media supporting neural cell growth can be used to enrich for neural progeny (Nat et al., 2007). More efficient differentiation can be accomplished by synergistic dual inhibition of the SMAD pathway with the BMP inhibitor Noggin and the molecule SB431542 (Chambers et al., 2009). It has been shown that ESC-derived NPCs retain self-renewal and neuronal differentiation potential to larger degree than fetal-derived NPCs, which lose neurogenic potential during extended culture and eventually undergo senescence (Anderson et al., 2007; Oikari et al., 2016; Wright et al., 2006).

1.2.6.4 Induced Pluripotent Stem Cells

Since they were first generated in 2006, iPSCs have held phenomenal promise for clinical use. The first clinical trial using iPSCs is currently ongoing, using autologous fibroblasts transformed into retinal pigment epithelial cells for transplantation to patients with neovascular age-related macular degeneration (Mandai et al., 2017). Similar neural induction methods as for hESCs can be used. No iPSCs were used in the present thesis.

1.2.7 Growth Substrates

Extracellular matrix molecules (ECM) provide structural and biochemical support to surrounding cells *in vivo,* and *in vitro* culture conditions designed to mimic the *in vivo* situation use the appropriate formulation of ECM when possible. All 2-dimensional *in vitro* studies containing live cells in this thesis were performed on growth substrates chosen to provide a situation similar to the *in vivo* setting while maximizing the survival of cells. To make the ECM stick to the plastic or glass surface, polyaminoacids are routinely used. Cells were also cultured as neurospheres (see section 3.2.2.1) without exogenously added growth substrates.

1.2.8 Fluctuating Gene Expression

A recurring theme throughout this thesis is transcriptional fluctuation generating phenotypic flexibility. Although on many levels tightly controlled, transcription of large parts of the genome is pulsating in transcriptional bursts with irregular intervals, but with surprisingly constant length and height of the pulses (Chubb et al., 2006) and with gene-specific kinetics (Suter et al., 2011). Stochastic gene expression has been linked to benefits of phenotypic variability (Acar et al., 2008; Kussell and Leibler, 2005; Losick and Desplan, 2008). Speculation can be made that there are evolutionary benefits associated with limited random transcription if it, for instance, improves the ability to rapidly adapt to changing environmental conditions. Genes essential for cellular basic functions tend to have little noise in their expression patterns, while genes associated with stress responses are noisier (Blake et al., 2006). A little flexibility is good. A completely static system remains static.

2 AIMS OF THESIS

The overall aim of the present thesis was to advance human neural stem cells towards spinal cord injury transplantation.

The specific aims were as follows:

- To compare fetal NPCs with hESC-derived NPCs over time in culture and with regard to safety and outcome after *in vivo* model injury transplantation, and to investigate features of fetal NPCs related to pluripotency
- To study specification of NPCs
- To develop and evaluate a method to dissociate cell aggregates suitable for GMP production of cells

3 MATERIALS AND METHODS

3.1 ETHICAL CONSIDERATIONS

All studies were conducted in accordance with the principles of the Declaration of Helsinki and with proper ethical permits from the Regional Ethics Vetting Board in Stockholm, Sweden, the National Board of Health and Welfare ("Socialstyrelsen") and, were applicable, the Southern Stockholm Animal Experiment Ethics Board, Sweden.

3.2 HUMAN FETAL TISSUE

3.2.1 Obtaining Fetal Tissue

Human fetal tissue was obtained from elective routine abortions (5.5-10.5 weeks after conception) in collaboration with the gynecology clinic at Karolinska University Hospital, Huddinge. Written, informed consent was given by the donors prior to donation of the abortion material. The donors were ensured that there would be no consequences coupled to their decision on participation in the research program apart from that a blood sample was required should they agree to participate. The blood samples were subjected to virology and serology screening. Experienced medical staff carried out the abortion procedures using gentle low-pressure aspiration, and the abortion material was age determined, categorized and dissected. Personal data were entered into a bio bank database and the tissue samples were assigned non-traceable serial numbers.

3.2.2 Culturing Conditions for Fetal Cells

3.2.2.1 Propagation

In most experiments in the present thesis, cells from fetal spinal cord (sc) and subcortical forebrain (fbr) were used after careful removal of meninges and blood vessels. The tissues were separated and homogenized in neurosphere (NS) medium (1x DMEM/F12, (Life Technologies), 1x N-2 supplement (R&D Systems), 5 mM HEPES (Invitrogen), 0.6 % w/v glucose, and 2 µg/ml heparin (Sigma)) with a glass-Teflon homogenizer. Live and dead cells were counted in a Bürker chamber after addition of trypan blue, a dye that enters and labels cells with compromised membrane integrity, and therefore presumed dead, to a small aliquot of single cells using a phase contrast microscope. In a typical experiment, 1-2 million live cells and 2-4 million dead cells could be obtained from a spinal cord of gestational week 7 using this method, while a 7-week sub-cortical forebrain typically generated 2-4 million live cells and 6-8 million dead cells.

The single-cell suspensions were transferred to neurosphere growth (NSG) medium (NS medium supplemented with the mitogens endothelial growth factor (EGF, 20 ng/ml), basic fibroblast growth factor (bFGF, 20 ng/ml) and ciliary neurotrophic factor

(CNTF, 10 ng/ml), all recombinant, from R&D Systems)) at a concentration of 100-200 cells/µl and incubated at 37° C in a humidified atmosphere with 5 % CO_2 in culture flasks or dishes. 20 % fresh medium was added twice a week. After a few days, neurospheres – spherical or almost spherical, free-floating cell aggregates – would form (see figure 2).

When passaged, the neurospheres were enzymatically and mechanically dissociated using TrypLE Express (Invitrogen) and gentle trituration, and single cells were re-seeded in fresh medium every 7-21 days depending on growth rate and sphere size.

Passage of neurospheres with the Biogrid device described in paper IV was accomplished by aspirating neurospheres directly from the culture flasks through a plastic tube attached to the Biogrid, into a plastic 10 ml syringe and then ejected through the Biogrid again into fresh medium in new culture flasks. The 120-µM spacing between the sharp edges of the Biogrid allows small cell aggregates and single cells to pass, but cuts larger spheres.

Adherent cultures were achieved by dissociating neurospheres and seeding the cell suspensions on polyornithin/laminin-111-treated surfaces at a concentration of 15,000 – 30,000 cells/cm^2, and culturing in NSG medium. The polyornithin (PO, Sigma) was diluted 1:6 in PBS, applied to the growth surface for 1 h, removed and allowed to dry for 10 min and subsequently rinsed 3x with autoclaved dH_2O. 1 µg of laminin-111 (Sigma) per cm^2 was then added to the PO-treated surface in NSG medium and incubated at 37° C in a humidified atmosphere with 5 % CO_2 for 24 h before use. The adherent cells were passaged by a method similar to that described for the neurospheres, using TrypLE Express-mediated dissociation from the surface and gentle mechanical trituration. Sphere cultures could be turned into adherent cultures and vice versa simply by adjusting the surface coating and replating.

Spheres from both sc and fbr could readily be cryo-preserved in NS-medium supplemented with 30 % human serum albumin, 7.5 % dimethyl sulphoxide (DMSO) and 20 ng/ml EGF and put in the vapor phase of liquid nitrogen. Throughout all the studies, we have kept records of the number of passages and freeze-thaw cycles of all biological samples – henceforth referred to as cases – used, thus allowing correlation analysis of data to those factors. Experiments in the present thesis were performed using fetal cells passaged no more than 15 times and no less than twice, except when freshly acquired tissue was investigated specifically.

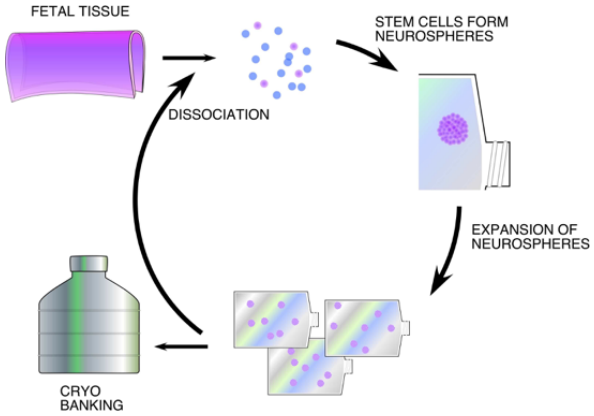


Figure 5. Routine procedures to obtain and culture fetal NPCs.

Samples of fetal cultures were checked for karyotype abnormalities, and although most of them were found to be karyotypically normal, one case presented with the X-monosomal karyotype 45,X, known as Turner syndrome. Since 3 % of all pregnancies start with 45,X embryos and 99 % of all Turner syndrome pregnancies result in spontaneous abortion during the first trimester, fetal samples collected during the first trimester will contain a larger proportion of Turner syndrome samples than the human population. The same can be argued for other karyotype aberrations (Urbach and Benvenisty, 2009). However, no evidence was found that large chromosomal alterations had been introduced in fetal cultures *in vitro* as a result of culture adaptation. The Turner case was excluded from studies in this thesis.

3.2.2.2 Generation of Differentiated Neurons, Astrocytes and Oligodendrocytes

To acquire progeny of differentiated phenotypes from multipotent progenitor cells, a number of media formulations were used. It is important to remember that there are no unbiased differentiation conditions. Simply removing mitogens from the culture medium induces various types of cell death and senescence rather than differentiation.

3.2.2.2.1 Astrocyte-permissive Medium

NS medium supplemented with fetal bovine serum (FBS) promotes glial growth and maturation. 10 % FBS was routinely used and cells were plated on poly-D lysine-coated glass slides in 24-well plates at a concentration of $1.5x10^5$ cells/cm^2 for 12 days.

3.2.2.2.2 Neurobasal Medium (NBM)

NBM is a proprietary cell culture medium designed to increase the survival of embryonic hippocampal rat neurons and is now widely used for many types of neural cell cultures. B27 supplement was added 1:100 and cells were plated on poly-D lysine- and fibronectin-coated glass slides in 24-well plates at a concentration of $3x10^5$ cells/cm^2 for 12 days.

3.2.2.2.3 NS-medium with Serum Replacement (SR) and Retinoic Acid (RA)

In paper III, NS medium supplemented with 5 % SR and 10 µM RA (NS-SR-RA), which promotes neuronal differentiation, was used. Cells were plated on poly-D lysine- and fibronectin-coated glass slides in 24-well plates at a concentration of $3x10^5$ cells/cm^2 for 12 days.

3.3 HUMAN EMBRYONIC STEM CELLS (hESCs)

3.3.1 Obtaining hESCs

hESCs were derived from surplus embryos donated by couples undergoing infertility treatment at the fertility unit of the Karolinska University Hospital. Supernumerary embryos of high quality are cryopreserved for a period of five years, after which the patients get to decide whether the embryos should be discarded or be donated for research. Embryos of low quality can be donated or discarded immediately after generation and evaluation.

3.3.2 Culturing Conditions for hESCs

3.3.2.1 hESCs on Feeders

Human foreskin fibroblasts (HFFs) were used as feeder cells, providing auxiliary substances including attachment substrates, nutrients and other factors needed for hESC growth in culture. HFFs were cultured on Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10 % FBS and 25 U/ml penicillin/streptomycin (PEST, Cambrex Bio Science). HFFs were irradiated (40 Gy) to stop their proliferation, and the medium was gradually changed into hESC medium, consisting of KnockOut Dulbecco's Modified Eagle's Basal Medium (kDMEM) supplemented with 20 % Knockout Serum Replacement (kSR), 2 mM GlutaMax, 25 U/ml PEST, 1 % minimal essential medium non-essential amino acids, 0.5 mM β-mercaptoethanol and 8 ng/ml human bFGF, and hESCs, freshly passaged either by mechanical cutting or dissociation with TrypLE and gentle trituration, were put on the feeders. At passage with TrypLE, the feeder cells create gelatinous clumps that can be removed, and the hESCs can be transferred as single cells in solution. Contaminating feeder cells die after a few days after passage. All cultures were maintained in a humidified atmosphere at 37° C and 5 % CO_2.

3.3.2.2 hESCs on Chemical Substrates

Culturing hESCs on chemical substrates is believed to be of importance for future clinical translation, avoiding possible transfer of pathogens from feeder cells or feeder cell contamination. The support normally given by feeder cells is replaced with nutrients added to the media. In this thesis, Matrigel and Laminin-511 were used as substrates and the medium mTeSR (STEMCELL Technologies) was used with TrypLE-mediated passaging.

3.3.2.3 hESCs as Spheres

In paper IV, suspension cultures of hESCs were established as described by Steiner and collaborators (Steiner et al., 2010). Cell aggregates were mechanically removed with a scalpel from colonies of undifferentiated hESCs grown on feeders. The aggregates were placed in 10 cm^2 ultra low attachment plates in 2 ml of the suspension medium containing NBM with 14 % kSR, 2 mM glutamine, 50 U/ml PEST, 1 % minimal essential medium non-essential amino acids and 4 ng/ml bFGF.

The medium was replaced every second day by tilting the plate and removing 50–80 % of the medium and adding a fresh medium. Cell aggregates were initially passaged every 7 or 8 days using a 1 ml pipette to break up the aggregates into smaller clusters. Dissociation performed using the Biogrid device described in paper IV was done by replacing most of the cell culture medium and aspirating the spheres through the Biogrid at an approximate flow rate of 1 ml/s and immediately ejecting the cut spheres back into the well.

3.3.3 *In Vitro* Differentiation of hESCs

3.3.3.1 hESCs Induced to Neural Progenitor Cells – hESC-NPCs

To induce neural specification, hESC colonies grown on laminin, Matrigel or feeders were mechanically dissected into two different media in suspension culture: neural differentiation medium (NDM) and neural stem cell medium (in paper I called NSM, throughout the rest of this thesis called NSG). NDM contained DMEM/F-12 and NBM (1:1) supplemented with 1xB27, 1xN2, 2 mM GlutaMax (Gibco Invitrogen), 25 U/ml PEST, and 20 ng/ml bFGF. The neurospheres obtained were mechanically split by cutting with a scalpel once a week and cultured up to 20 weeks.

3.3.3.2 Terminal Differentiation of hESC-NPCs

Differentiated neural progeny of hESC-NPCs were generated using the same protocols for differentiation as described for fetal cells under section 3.2.2.2.

3.4 TERATOMA TEST

In paper I, to study teratoma formation, 10–12 intact neurospheres (in total ≈100,000 cells) derived from hESCs and human fetal CNS were transplanted into the right testis of severe combined immunodeficiency (SCID) mice (n = 10), as previously described (Hovatta et al., 2003; Inzunza et al., 2005). Equivalent subcutaneous transplantations of 10–12 neurospheres (≈100,000 cells) in the left groin were also performed in the same animals. Undifferentiated hESCs of the same cell lines (100,000 cells/injection) were used as positive controls. After the cell injections, the development of tumors in the testes and subcutis in transplanted animals was followed by manual palpation during 12 weeks. Animals were sacrificed by a lethal dose of intravenous barbiturates before transcardiac perfusion with 4 % PFA in 0.1 M PBS. Testes were dissected out and a 1 cm^2 piece of the skin and superficial layer of underlying skeletal muscle at the location of the subcutaneous transplantation was cut out, the tissue was post-fixed for 4 h in PFA, and then transferred to 10 % sucrose for a minimum of 24 h. Sections (10 µm) were cut on a cryostat (Micron) and stained with hematoxylin-eosin (Sigma) for histological analysis. Immunohistochemistry was performed as described in section 3.6.1.

3.5 REVERSE TRANSCRIPTASE – POLYMERASE CHAIN REACTION (RT-PCR) AND QUANTITATIVE PCR (qPCR)

3.5.1 *RNA Extraction and cDNA Synthesis*

In paper I, RNA was extracted from hESC-NPCs and fetal NPCs using the RNeasy® Micro Kit (Qiagen). RNA was quantified using a NanoDrop spectrophotometer. 50 ng of RNA was used for cDNA synthesis using oligo-dT primers at 50° C in a total reaction volume of 25 µl. In paper II, new primers were designed using Vector NTI and Primer3 software to span exon-exon boundaries and were checked for sequence homologies using NCBI BLAST. RNA was extracted using the RNeasy Mini Kit (Qiagen). Cells were pre-homogenized by passage through a 20-gauge needle in lysis buffer 10 times. 100 ng of RNA was used for cDNA synthesis using target-specific reverse primers for PCR and random hexamers for qPCR. The reverse transcription reactions using target-specific primers were performed at 55° C (*NANOG, OCT-4, DNMT3B, CRIPTO*), 50° C (*GDF3, GAPDH*) or 51° C (*REX1*) in 25 µl, and at room temperature with random hexamers.

3.5.2 *PCR*

In paper I, each PCR reaction contained 1 µl of cDNA, corresponding to 2 ng of RNA, and the PCR program included: denaturation in 95° C for 3 min followed by 35 cycles of 95° C for 30 s, 55° C for 30 s, 72° C for 1 min, and final extension for 5 min at 72° C. Primers designed to amplify the transcripts of the following genes specific for the following cell types of interest were used a) pluripotent cells: *NANOG*, *OCT-4*, *DNMT3B*, *Activin A receptor*; b) mesodermal cell lineages: *Brachyury*, endodermal cell lineage: *alpha-fetoprotein* (*AFP*); c) NPCs: *SOX2*, *NESTIN*, *PAX-6*, *MUSASHI*, *MASH1*, *neural cell adhesion molecule* (*NCAM*); d) radial glial cells: *brain lipid binding protein* (*BLBP*); e) neuronal cells: *Doublecortin* (*DCX*), *MAP2*, β-*tubulin III*; f) astrocytes: *glial fibrillary acidic protein* (*GFAP*); g) oligodendrocytes: *OLIG1*, *OLIG2*. The housekeeping gene *GAPDH* was used as reference gene. The expression of the genes was analyzed at 2, 4, 6, 8, and 12 weeks of culture. In paper II, The PCR reaction was performed with Stratagene Paq 5000 DNA polymerase. The products were run on agarose gels, and single bands were obtained, cut out, purified using the QIAquick gel extraction kit (Qiagen) and sequenced to confirm the identity of the original mRNA. Negative controls included ‘no template’ and ‘no reverse transcriptase’ samples.

3.5.3 *qPCR*

Quantitative RT-PCR (qPCR) was performed with FastStart Universal SYBR Green Master Mix (ROX, Roche) for the genes *OCT-4*, *DNMT3B*, *GDF3*, *REX1* and *GAPDH,* and with a TaqMan assay for *NANOG* (Applied Biosystems). Primers for SYBR Green chemistry were designed specifically for qPCR and ordered from Thermo Electron GmbH (Germany), and primers and probes for *NANOG* were custom designed by Applied Biosystems. Primers were designed to span exon–exon

boundaries, and primer sequence specificity was confirmed with NCBI BLAST to eliminate the risk of genomic contamination. Primers were analyzed with PCR; amplified products were run on agarose gels, cut out, and sequenced. qPCR was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems) with the following profile: 1 cycle of 95°C for 10 min, 40 cycles of alternating 95°C for 15 s, and 60°C for 30 s followed by a melting curve analysis for specificity control. The quantification was performed using the Pfaffl (ΔΔCt) method as described in (Bookout et al., 2006) using pooled undifferentiated hESCs HS999 and HS980 as a reference sample and *GAPDH* as reference gene. Software-generated thresholds and Ct values for each gene were used (7500 Fast System version 2.0.3). The amplification efficiencies were obtained by running a dilution series of hESC mRNA and used to calculate relative fold induction with the formula

$$\text{fold induction} = \frac{\text{Eamp}_t^{\Delta Ct_{t(\text{control}-\text{sample})}}}{\text{Eamp}_r^{\Delta Ct_{r(\text{control}-\text{sample})}}}$$

where

$Eamp_t$ = Amplification efficiency for the target gene = PCR efficiency for the target gene + 1
$Eamp_r$ = Amplification efficiency for the reference gene = PCR efficiency for the control gene + 1
$\Delta Ct_{t\ (control\text{-}sample)}$ = Cycle number at which the signal from the target gene in the control sample reaches the threshold minus the cycle number at which the signal from the target gene in the sample reaches the threshold
$\Delta Ct_{r\ (control\text{-}sample)}$ = Cycle number at which the signal from the reference gene in the control sample reaches the threshold minus the cycle number at which the signal from the reference gene in the sample reaches the threshold. The data analysis was performed with Microsoft Excel. See papers I and II for lists of primers.

3.6 IMMUNOCHEMISTRY

All methods in the following sections (immunohistochemistry, immunocytochemistry, FACS and flow cytometry) rely on antibodies produced by immunized animals. Antibodies specifically bind the desired target epitopes with varying affinities, and, importantly, also display cross-reactivity to similar epitopes on other proteins as well as unspecific binding of other parts of the antibody than the antigen-binding site. For all techniques using antibodies in surplus, the signal from the specific binding asymptotically reaches a maximum value as all epitopes are occupied. The signal from the unspecific binding increases quasi-linearly with increasing concentration. Titration of appropriate antibody concentration is performed to maximize the proportion of specific, desired, binding to unspecific, undesired binding (see figure 6). Different methods to decrease unspecific binding can also be implemented. Problems regarding antibody-generated signal noise inherent to individual techniques are further discussed in the sections below.

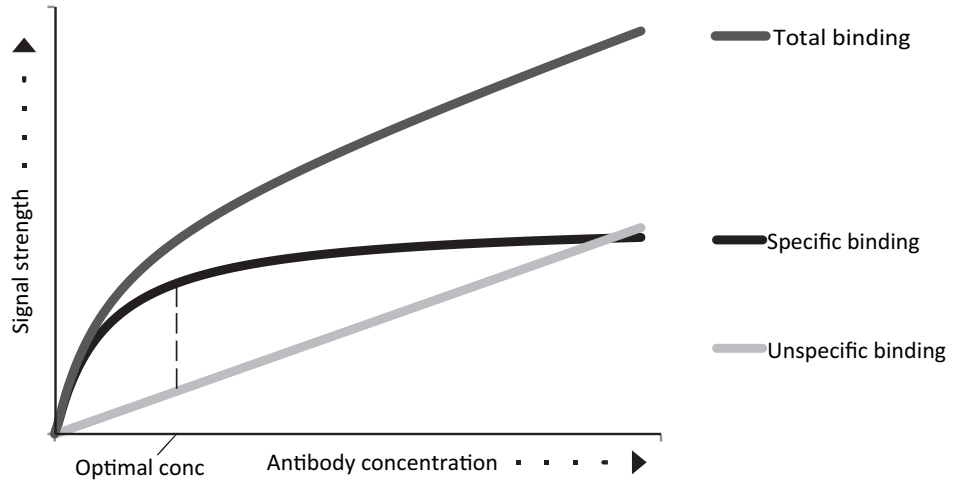


Figure 6. Signal output and specificity of immunochemistry. For all methods using antibodies, the specific binding reaches a maximum as epitopes get occupied (black line). The unspecific binding (background) increases quasi-linearly (grey line). The obtained signal is shown in dark grey. Optimal antibody concentration and staining conditions must be determined individually and should maximize discrimination of specific from unspecific binding.

3.6.1 Immunohistochemistry

In a typical immunohistochemical analysis, the tissue was fixed in 4 % formaldehyde solution (PFA), treated with 30% sucrose over night at 4° C, mounted in TissueTek mounting medium (Sakura Finetek), and frozen on dry ice. In paper I, neurospheres were sectioned in a cryostat at a thickness of 5 µm. Tissue sections were blocked with 1.5 % goat/donkey serum in PBS at 30 min in room temperature (RT), and primary antibodies were diluted in 0.3 % Triton X-100 in PBS and incubated overnight at 4° C. After rinsing in PBS the sections were incubated with secondary antibody in 0.3% Triton X-100 PBS for 1 h at RT. To detect neurons, anti-β-tubulin type III (1:800, Sigma) and anti-MAP2 (1:50, Chemicon) antibodies were used; to detect immature cells, anti-BLBP (1:500, Chemicon) and anti-nestin (1:200, Chemicon) antibodies were used; to detect astrocytes, anti-GFAP (1: 500, DAKO) antibody was used. Secondary antibodies used were Cy3-conjugated AffiniPure goat anti-mouse IgG (1:2400, Jackson Immuno Research Laboratories), Alexa Fluor 488-conjugated goat anti-mouse IgG, and Alexa Fluor 488-conjugated goat anti-rabbit IgG (1: 1200, both from Molecular Probes). For nuclear counterstaining, Hoechst 33342 (1: 200, Sigma) was used. For negative control, samples were incubated with secondary antibody only. At least five sections/sample were analyzed. For paper I, analysis was performed on hESC-NPCs cultured in NSM and NDM, and human fetal NPCs (hfNPCs) cultured in NSM *in vitro* for 2, 4, 8, 12, and 20 weeks. Samples were evaluated semi-quantitatively using a fluorescence microscope (Zeiss, Axiophot). Human cells in the teratoma tests in paper I were detected using human nuclear marker HuNu (1:250, Chemicon) and differentiated hESC-NPCs using anti-β-tubulin III (1:800, Sigma) antibody.

Human fetal spinal cord (paper III) was fixed in 4 % PFA, cryo-preserved in 30 % sucrose, mounted and frozen as described for neurospheres above. For paper I, rat

spinal cords were perfusion fixed with 400 ml of 4 % PFA in phosphate buffer, pH 7.4, followed by excision, postfixation in 4 % PFA for 90 min, rinse and storage in 30 % sucrose. 10 µm sections were cut on a cryostat.

3.6.2 Immunocytochemistry

Adherent cell cultures were washed with PBS, fixed in 4 % PFA for 10 min, washed 3x5 min with PBS, blocked in 1.5 % serum from the animal the secondary antibody was produced in in 0.3 % Triton-X in PBS for 30 min. Primary antibodies were diluted in block and samples were incubated over night at 4° C and washed with 0.1 % triton in PBS 3x5 min the next day. Incubation with the appropriate secondary antibodies in block for 1 h was followed by 3x5 min wash in 0.1 % triton in PBS. A DAPI:PBS 1:50,000 solution was added for 1 min and removed before the cells were mounted in PVA mounting media (DABCO) and analyzed using a Zeiss fluorescence microscope with OpenLab software. The primary antibodies used were NANOG AF1997 Goat (R&D Systems), OCT-4 C-20 Goat (Santa Cruz Biotech), REX-1 AP2051a Rabbit (Abgent). Secondary antibodies: Rabbit anti-goat Alexa488 (Invitrogen Molecular Probes), Rabbit anti-goat Alexa488 (Invitrogen Molecular Probes) and goat anti-rabbit Cy3 (Jackson Immuno Research).

3.7 FLOW CYTOMETRY AND FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

Flow cytometry relies on specific labeling of cells by e.g. antibodies coupled to fluorescent proteins or reporter gene expression with fluorescent proteins. The technique allows analysis of single particles – in this case cells – in solution. A narrow stream of the solution is created with the help of a carrier (sheath) fluid so that cells come in single file. The stream is directed through a laser beam orthogonal to the stream. Detectors appropriately placed outside the flow chamber analyze the refracted and fluorescent light. The different parameters detected are Forward Scatter (FSC), roughly reflecting cell size; Side Scatter (SSC), representing cell granularity and fluorescent light intensity.

In papers II and III, FACS was used to sort out subpopulations of live cells from heterogeneous populations. The method allows simultaneous analysis of single cells by flow cytometry and sorting of cells. The stream of sheath fluid carrying cells in single file passes through a rapidly oscillating nozzle head, creating droplets. Flow speed is adjusted to maximize the probability that each droplet contains one cell. Depending on set parameters, droplets are given a small electrical charge, allowing sorting by an electrostatic field acting on the droplets (see figure 7). PBS was routinely used as sheath fluid. 20 PSI (pounds per square inch ≈ 1.4 bar) sheath pressure and a 100 µm nozzle was found to be optimal for cell survival and FACS efficiency.

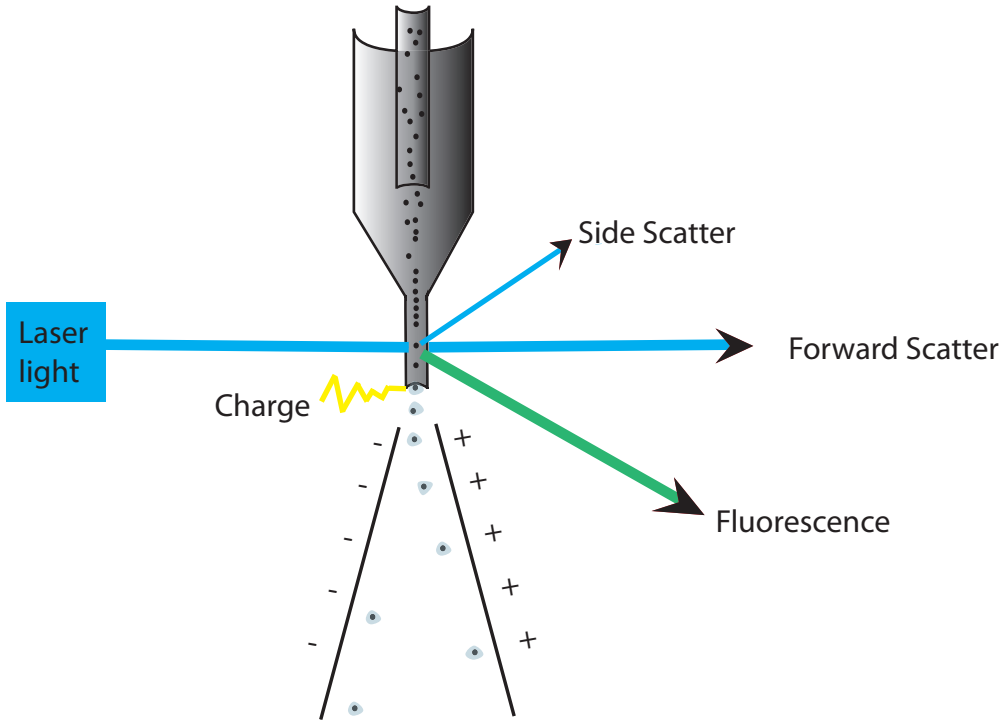


Figure 7. FACS basics.

Spectral overlap of fluorescent species requires careful consideration when designing flow cytometry experiments. Primarily, fluorescent molecules with minimal spectral overlap were combined when possible (e.g. allophycocyanin – APC, and phycoerythrin – PE), and proper compensation between fluorescent channels was implemented. Compensation is the process of correcting the spillover from the primary signal in each of the other detectors, rendering the signal identical to background in all inappropriate detectors. This was accomplished by using unstained sample controls for setting background, single stained sample controls and FMO (fluorescence-minus-one) controls, each containing all fluorescent species included in the experiment except one, for which the compensation of that particular channel was adjusted so that the signal was identical to background.

Dissociation of cell aggregates before FACS comes with an inherent conflict between efficiency of separation and cell viability. Thorough dissociation inevitably yields a proportion of dead cells, most of which were distinguished from live cells by their compromised membrane integrity and inability to exclude the DNA-binding fluorescent agent Sytox® Blue (Molecular Probes). In some experiments, LIVE/DEAD Cell Viability Stain (Molecular Probes), which binds protein rather than DNA, and can therefore distinguish dead cells from live cells even after permeabilization and fixation, was used for exclusion of dead cells. Remaining doublets or larger aggregates were excluded by analyzing their FSC-H (forward scatter height) versus FSC-A (forward scatter area) signal ratio. A properly calibrated cytometer yields almost the same value for a spherical object in FSC-H and FSC-A, meaning that non-spherical objects, such as

aggregates of two or more cells, will deviate from the diagonal in an FSC-H vs. FSC-A signal plot.

FACS was performed on a Becton Dickinson (BD) FACSDiva and on a BD FACSAria Fusion. Flow cytometry analysis was performed on a BD LSRFortessa, a BD Accuri C6 and on a BD FACSCalibur. Bead calibration was performed before every FACS experiment and regularly on analysis machines. Immediate post-sort analysis was performed after FACS experiments to determine the purity of the sorted populations. In paper II, single cell sort mode was used for reporter-transduced cells, and purity mode for CD133/CD24 sorting. In paper III, purity mode was used for all FACS experiments except the CTV experiment (see section 3.12), in which yield mode was used.

Target antigen	Method	Dilution	Source	Used in paper
A2B5	FC	1:50	R&D Systems	I, IV
A2B5-APC, PE	FC	3:100	Miltenyi	I, IV
BLBP	ICC	1:500	Chemicon	I
CD133-PE	FC	3:100	Miltenyi	I, III, IV
CD15-PC5	FC	3:100	Beckman Coulter	I, IV
CD24-PerCP-Cy5.5	FC	1:50	BD	III, IV
CD326	FC	1:100	Chemicon	I
CD44-PE	FC	1:50	BD Pharmingen	I
CD45-FITC	FC	1:100	BD	III
CD56-PC5	FC	1:50	BD Pharmingen	I
GFAP	ICC, IHC	1:500	Dako	I-IV
DCX	ICC	1:500	Abcam	IV
HuNu	IHC	1:250	Chemicon	I
Ki67	ICC	1:100	DAKO	II
MAP2	ICC	1:50	Chemicon	I
NANOG	ICC	1:100	R&D Systems	III
Nestin	ICC, IHC	1:200	Chemicon	I
Nestin	ICC	1:400	Millipore	IV
OCT-4	FC, ICC	1:200	Santa Cruz	III
PSA-NCAM-APC, PE	FC	3:100	Miltenyi	I, IV
PSA-NCAM	FC, ICC	1:400	Milipore	I, IV
REX1	IHC	1:200	Abgent	III
SSEA-4	FC	1:100	Millipore	I
β-tubulin III	ICC, IHC	1:800	Sigma	I-IV
β-tubulin III	ICC	1:1200	Biosite	IV

Table 1. List of antibodies used in the present thesis.

3.8 BATCH SPHERE COUNTING BY PHOTO ANALYSIS

For large-scale, unbiased image analysis of sphere sizes and sphere number, phase contrast images were obtained using an Olympus IMT-2 inverted microscope with an Olympus CMOS camera in 9 pre-determined positions in each circular 10 cm^2 well. A

schedule of well positions was created by recording the number of turns of the stage control knobs needed to cover as much of each well as possible, while avoiding including the edges of the wells that otherwise could distort the image, and so that including any part of the well in more than one photo was avoided. Standardization was achieved by placing the culture plate edge in parallel with the edge of the microscope stage, aligning the leftmost edge of the well in the eyepiece crosshair and turning the stage control knobs according to the pre-determined schedule. Focus was adjusted manually.

The resulting images required pre-processing before analysis to distinguish background and debris from spheres, and to generate binary (black-and-white) images. All images from one well were put in a separate folder and, typically, four wells of each condition were analyzed. This allowed for estimation of the technical variance and evaluation of the method. Three and eight biological replicates were used in paper II and paper III, respectively.

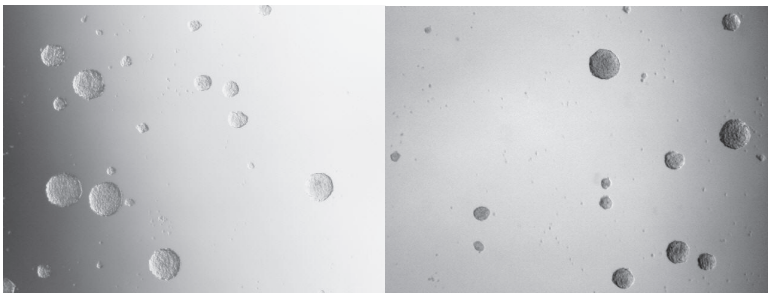
The following ImageJ macro was used to count spheres and to analyze individual sphere sizes in all images in a folder chosen by the user:

```
macro "Batch Measure" {
   dir = getDirectory("Choose a Directory ");
   list = getFileList(dir);
   setBatchMode(true);
   for (i=0; i<list.length; i++) {
      path = dir+list[i];
      showProgress(i, list.length);
      if (!endsWith(path,"/")) open(path);
      if (nImages>=1) {run("Find Edges");
                                          run("Enhance Contrast", "saturated=0.5");
                                          run("Subtract Background...", "rolling=50");
                                          run("Make Binary");
                                          run("Outline");
                                          run("Fill Holes");
                                          run("Find Edges");
                                          run("Analyze Particles...", "size=200-Infinity
circularity=0.60-1.00 show=Outlines display exclude summarize record");
         close();
      }
   }
}
```

The data output given by this ImageJ macro is the number of circular (with a circularity=$4\pi(\text{area}/\text{perimeter}^2)> 0.6$) objects larger than 200 pixels in each picture and their individual two-dimensional size in pixels, enabling the quantification of spheres as well as individual and total sphere volume (assuming that all neurospheres are perfect spherical objects), which can be considered to be directly proportional to the number of

cells (assuming that all spheres have equal and homogenous cell densities). Data compilation, calculations and statistical analysis were performed using Microsoft Excel.

a)



b)

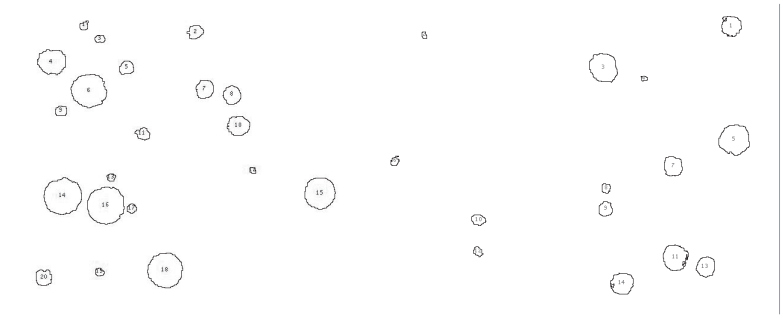


Figure 9. a) Phase-contrast images of spheres. b) Images processed in ImageJ using the batch sphere counting macro described in section 3.8.

3.9 TRANSCRIPTOME ANALYSIS

In order to compare the transcriptomes of cells immunoreactive for different markers associated with glial or neuronal phenotype commitment at sorting and after the re-setting of the immunoreactivity profile three passages later, cells were FACS-sorted based on A2B5 and PSA-NCAM immunoreactivity as described above. 50,000 cells of each of the four populations flow-through (FT), double negative (-/-), A2B5+/PSA-NCAM- (A+/P-) and A2B5-/PSA-NCAM+ (A-/P+) from four different hscNPC cases (biological replicates) were sorted directly into 300 µl of RNAlater (LifeTechnonogies) and frozen at -80°C before RNA extraction. 50,000 cells per population were also sorted directly into NSG medium, cultured adherently for one passage and subsequently as spheres for two more passages before RNA extraction, generating a total of 32 samples (four populations from four biological replicates measured at two time points). RNAlater was removed by addition of 300 µl PBS and centrifugation at 16,000 x g. RNA extraction of sorted cells was done with the RNeasy Micro Kit (QIAGEN) according to the manufacturer's instructions. RNA extraction of cultured cells was done with TRIzol/chloroform separation after which RNA in the hydrophilic phase was transferred to PureLink RNA Mini Kit (Ambion) columns and eluted according to the manufacturer's instructions. RNA quality was determined on a BioAnalyzer 2100 with the Agilent RNA 6000 Pico Kit (Agilent Technologies). RNA quantity was determined using the Qubit® RNA HS Assay Kit (ThermoFischer Scientific).

The RNA samples were further processed using the Illumina® TotalPrep™ RNA Amplification kit according to the manufacturer´s instructions. Briefly, 80 ng total RNA was subjected to reverse transcription using oligo(dT)-primers to synthesize first strand cDNA, followed by a second strand synthesis to generate double-stranded (ds)DNA, which was then purified on column. *In vitro* transcription of cDNA generated multiple copies of biotinylated antisense cRNA, which was purified on column, quality-checked as described above and quantified using a NanoDrop™ spectrophotometer. Biotinylated cRNA was hybridized to the HumanHT-12 v4 Expression BeadChip Kit (Illumina) according to the manufacturer's instructions. Preliminary quality control was performed using the Illumina BeadStudio software.

Data were processed in R using the Limma package from Bioconductor. Probes with signal intensity higher than 5 on a $\log_2$ scale compared to background in at least three samples were considered expressed and retained for downstream analysis, keeping 32,456 out of 47,323 probes. Differentially expressed genes were obtained by blocking the donor factor, yielding a paired samples analysis, comparing the transcriptome of sorted cells immediately after sorting to that of the same, sorted culture three passages later.

3.10 LENTIVIRAL TRANSDUCTION OF REPORTERS

To investigate whether transcripts of pluripotency-associated mRNA were evenly or unevenly distributed among NPCs, reporter lentiviral constructs for Nanog and Oct-4 expressing GFP and Rex1 expressing RFP under specific promoters were used in paper II. In paper II and III, control viruses with a constitutively active promoter (EF1α) expressing GFP were used. All viruses were from Allele Biotechnology Inc. Viruses were transduced into newly passaged adherent cell cultures. The positively charged poly-cation hexadimethrine bromide (Polybrene) was added to facilitate virus envelope binding to the cell membrane at a concentration of 2 μg/ml. A multiplicity of infection (MOI) virus ratio >3 was employed, which typically yielded >95 % transduction efficiency in control experiments with control viruses without a discernible effect on cell survival or proliferation. The viruses and Polybrene were removed by extensive rinsing with NSG medium after 24 h and subsequent passaging.

3.11 LIVE-CELL IMAGING

In paper II, live-cell imaging of reporter-transduced NPCs in a humidified atmosphere at 37° C with 5 % CO_2 was done by acquiring time-lapse movies using a Zeiss Cell Observer system with a 10x objective, rendering photos with a 150 x 120 μm surface area (=1,388 x 1,040 pixels x 1.1 μm resolution/10x magnification) containing between approximately 10 and 100 cells each. Locations were chosen non-randomly where live, fluorescent cells were present. Phase contrast images and fluorescence images were obtained. Thorough optimization was performed, since live-cell imaging requires compromises between what is good for the cell cultures and

what generates high quality images. High excitation intensity, frequent image acquisition and long exposure time effectively kills cells (Schroeder, 2011). The image intervals were typically 7.5 min, and the cultures were monitored for 24 h. The image analysis was performed with ImageJ using the LSM Reader plugin and with Adobe Illustrator CC.

3.12 ANALYSIS OF CELL EXPANSION

In paper III, to determine if cell division is necessary for interconversion to occur, or whether clonal expansion or culture adaptation is responsible for the re-setting of proportions of cells expressing different antigens, single cells were labeled with CellTrace Violet (CTV), anti-CD133 and anti-CD15 antibodies and subsequently sorted with FACS and then cultured in NSG medium to allow the antigen profiles of sorted hscNPCs to reset. Dead cells were excluded in the FACS analysis on the basis of LIVE/DEAD fluorescence. CTV content decreases as cells proliferate, diluting the fluorescent signal for each cell division. This allows for analysis of accumulated proliferation over a longer period of time. At first and third passage after sorting (10 days and 33 days after sorting, respectively), cells were analyzed with flow cytometry for expression of the same markers used for sorting correlated to CTV content, with the purpose of determining if cells that had interconverted also had undergone a higher number of mitoses.

3.13 BIOGRID FABRICATION

Silicon microgrids were fabricated at Lund University by anisotropic wet etching with conditions and dimensions listed in paper IV.

3.14 ANALYSIS OF PRESSURE EXPOSURE TO SPHERES USING BIOGRID

In paper IV, pressure changes occurring during normal Biogrid passage were measured using a differential pressure sensor connected via a three-way connection to the Biogrid setup. To study neurosphere pressure tolerance, the three-way connection was switched to connect the sensor to the syringe, closing the outlet tubing, after which pressure transients were applied by aspiration or compression.

3.15 VIABILITY ASSAY

In paper IV, cell viability was evaluated with the LDH assay, in which the activity of the enzyme lactate dehydrogenase (LDH) is measured. LDH leaks from apoptotic or necrotic cells. By analyzing its activity in the medium, the level of cell death can be estimated. The method uses a coupled enzymatic reaction, where a tetrazolium salt is converted into a red formazan product by the enzyme diaphorase using the NADH generated from NAD+ by LDH when converting lactate to pyruvate (see fig. 10). The formazan content was analyzed with a microplate photometer at 492 nm absorbance using absorbance at 650 nm as reference. Confounding factors include medium

additives with LDH activity and/or absorbance at 492 nm.

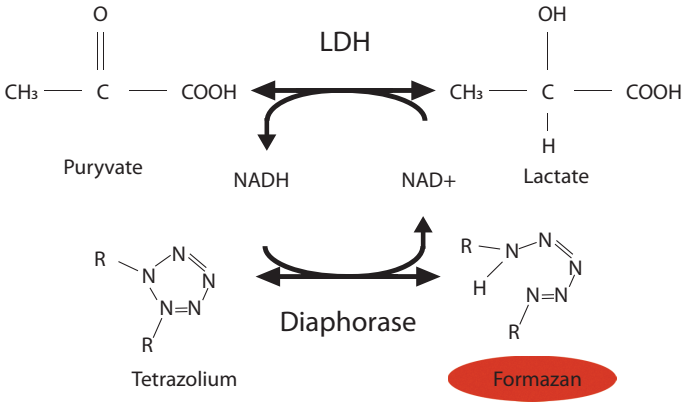


Figure 10. Principle of cell viability analysis with LDH. The activity of LDH in the medium is measured via a coupled enzymatic reaction generating a red formazan product, which is measured spectrophotometrically.

3.16 SCI *IN VIVO* MODEL

A total of 24 adult immunodeficient female rats (170–200 g, HsdHan:RNU-rnu) were included in paper I. After a laminectomy of thoracic vertebra 9, a spinal cord contusion injury was produced using the IH Spinal Cord Impactor set at a force of 150 kilodynes, with no dwell time. The lesioned spinal cords were covered with one layer of Lyoplant meningeal substitute prior to suture of the wound. Eight days later the animals were re-anaesthetized and 10–12 hESC-NPC neurospheres (cultured for 8 weeks to allow neural induction) selected by size to contain a total of approximately 100,000 cells were transplanted to the lesion area. Three groups of injured rats (6 animals/group) were included and transplanted with: a) hESC-NPCs cultured in NDM (HS360), b) hESC-NPCs cultured in NSG (HS360), c) hESC-NPCs cultured in NDM (HS181). One group of rats with a laminectomy but without contusion injury (6 animals/ group = sham rats) was injected with hESC-NPCs cultured in NDM (HS360). Animals were followed up to 12 weeks after injury and testing of hind limb motor function was performed one day before injury and 1, 3, 6, 10, and 12 weeks after injury. Hind limb function was assessed using the Basso, Beattie, and Bresnahan (BBB) motor performance scale, with 21 as the score for normal hind limb function, and 0 for complete paralysis. An experienced investigator blinded to the experimental design performed functional testing. After the motor assessment at 12 weeks, the rats were sacrificed, and perfusion-fixed with 400 ml of 4 % PFA in phosphate buffer, pH 7.4. The dissected spinal cords were post-fixed in 4 % PFA for 90 min and thereafter rinsed and kept in 30 % sucrose until they were cut in 10 µm sections and processed for histological analysis.

3.17 STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS Statistics. In paper II, Student's t-test with Holm-Bonferroni's correction for multiple comparisons was used, assuming random sampling from normally distributed data sets with equal variance. The statistical analysis of qPCR data was done using the C_t values (in $\log_2$ scale). In paper

III, to compare phenotype outcome after FACS and differentiation or propagation, two-way repeated measures analysis of variance (ANOVA) followed by Dunnett's post hoc test was used. Clonal expansion calculations were performed using Wolframalpha.com. The likelihood of a density of genes associated with certain gene ontology (GO) terms occurring by chance was calculated using Wolframalpha.com using the cumulative binomial distribution formula $L(k;n,p) = \sum_k^n \binom{n}{k} p^k (1-p)^{n-k}$, where p was set to be equal to the estimate 10 %, based on the frequency of the GO terms of interest annotated to 150 randomly chosen genes.

4 RESULTS AND DISCUSSION

4.1 SIGNIFICANCE OF PLURIPOTENCY MARKER EXPRESSION IN NPCS

Surprisingly, we found in paper I and II that hNPCs as well as human fetal tissue contain mRNA transcripts of genes strongly associated with the pluripotent phenotype. *NANOG*, *OCT-4*, *SOX2*, *REX1*, *CRIPTO*, *DNMT3B* and *GDF3* mRNA was found in multiple cases. SOX2 is ubiquitously expressed by NPCs, hence included in our panel when comparing hESC-NPCs with fetal NPCs in paper I, but excluded from paper II. However, we could not confirm protein expression using antibodies that were able to demonstrate these proteins in hESCs. A possible explanation – and our initial hypothesis – for the existence of pluripotency-associated markers at the mRNA level in non-pluripotent hscNPCs is that the markers distinguish a subpopulation of cells in the heterogeneous hscNPC cultures forming the true stem cell population, the NSCs, should the expression be of significance to the phenotype of the cells, and limited to that population. As described in paper II, we have found no evidence for this, and although the phenotype of the NPCs expressing pluripotency-associated markers can be studied more extensively, for example by transcriptome analysis, the available data do not suggest that they have a specific phenotype. The question if these cells are or could become tumorigenic after transplantation should be thoroughly investigated before they are applied clinically, but among the large number of studies on human fetal CNS transplants to animal models, we are not aware of any reports of tumor formation after transplantation of human NPCs either to SCID mouse testis or to the injured spinal cord. Expression in an individual cell of one or a couple of transcription factors associated with a complex and tightly regulated phenotype such as pluripotency is most likely not enough to elicit expression of the complete proteome necessary for maintaining the phenotype (Toyooka et al., 2008).

One single report of aberrant growth after clinical transplantation of fetal cells has surfaced (Amariglio et al., 2009). Beginning in 2001, a boy with the disease ataxia telangiectasia repeatedly received transplants of fetal cells, the origin of which is not entirely clear but seems to have been fetal tissue dissociated and transplanted immediately after abortion. Five years later, a tumor diagnosed as glioneural neoplasm was removed from his spinal cord. The tumor was found to consist of cells from at least two donors, but no host cells. The boy also presented with a slow-growing abnormal mass in the brain, which was not removed at the time. The spinal cord tumor cells appeared well-differentiated, also grew slowly and were multiclonal, which suggests that both tumors were relatively benign. Telangiectasia is associated with an increased cancer risk and it is possible that out-of-control donor cells could have escaped the boy's immune system, which was weakened by the disease (Miyagi et al., 1995). The case may be drastically un-representative of likely future transplantation settings, both regarding graft origin and diagnosis, but nevertheless, the case provides the first proof that transplantation of fetal neural cells carries a risk of tumor formation.

Our results in paper II demonstrate that expression of pluripotency-associated TFs occurs in all or most fetal NPCs, but is concentrated to a small sub-population at any given moment. No particular phenotype could be ascribed to cells in that sub-population; sphere formation characteristics as well as proliferation and cell death rates were similar to non-expressing cells. Is anomalous expression of pluripotency-associated TFs the result of promoter-activated transcription or are they products of random transcriptional bursts, or both? We used lentiviral reporters driven by promoters for NANOG, REX1 and OCT-4, which have previously been shown to correctly mimic endogenous expression of TFs (Rodda et al., 2005). Importantly though, reporter genes using fluorescent proteins do not reproduce translation of the endogenous gene. Rather, GFP reporter expression and fluorescence intensity is directly proportional to GFP mRNA in eukaryotic cells (Soboleski et al., 2005). We confirmed, using qPCR after FACS selection of reporter-positive cells, that expression was two orders of magnitude higher in reporter-positive cells. Lentiviruses are incorporated semi-randomly into the genome (Schambach et al., 2013), implying that the position of integration can affect the probability of transcription, which is not evenly distributed in the mammalian genome (Chakalova and Fraser, 2010). The precise genomic positions of lentiviral incorporation can be determined, but with substantial effort. This was not done in our studies. Promoters are also known to leak, described as having one state with highly efficient transcription and one inactive state with very lowly efficient transcription (Huang et al., 2015). One conclusion that can be drawn in this particular case is that reporter-expression correctly mimics the behavior of transcription for a gene, but does not necessarily reflect concentration of the endogenous transcript. In other words, both random and specific transcription is reflected. Leaky promoters could be biologically relevant to sense the environment and activate transcription programs, while a completely static system remains static. The multitude of TFs required to elicit pluripotency may reflect that pluripotency is a "protected cell state", well guarded by the cell. As pluripotent cells differentiate, chromatin rearrangements and other features block transcription of pluripotency-associated mRNA, and the likelihood of the correct combination of mRNA species being transcribed by stochastic events is in that case low enough to avoid unwanted de-differentiation.

There are reports on results contradictory to ours. Ilieva and Dufva reported in 2013 that *OCT-4*-and *SOX2*-mRNA positive cells were organized in the center of neurospheres derived from human fetal embryonic mesencephalon (Ilieva and Dufva, 2013), suggesting that expression of pluripotency-associated mRNA species is accompanied with stem cell features in those cells.

Either way, in future clinical applications, close monitoring of transplanted cells is required. For instance, positron emission tomography can be used non-invasively to monitor transplanted cells (Haralampieva et al., 2016). If aberrant growth is seen, various steps can be taken to eliminate the threat. For that purpose, systems to

selectively induce apoptosis of transplanted cells have been conceived, using “suicide genes” (Di Stasi et al., 2011).

4.2 CORRELATION BETWEEN mRNA AND PROTEIN

The phenotypic identity of a cell is arguably best described by its proteome, i.e. the whole protein content at any given moment, but the transcriptome, i.e. the RNA content, is much easier to analyze. The protein concentration generally co-varies with the mRNA concentration in a cell, but the relationship is not absolute; about 40 % of the variation in the concentration of a typical protein species can be explained by knowing the concentration of the corresponding mRNA (Vogel and Marcotte, 2012). The poor correlation between mRNA and protein quantity is due to a number of characteristics that differ between mRNA and protein processing, and the fact that there seems to be no correlation between the processing of an mRNA species and the processing of its protein product. For instance: mean mRNA half-life is 5 hours in mammalian cells, and the range is narrow. Mean protein half-life is 46 hours with large variation. Furthermore, mRNA is produced at a rather slow rate, about 2 mRNA molecules per cell per hour, while hundreds of protein molecules can be produced per mRNA per hour, resulting in an estimated average mRNA:protein ratio of 1:2,800 (Schwanhausser et al., 2011).

In paper I and II, we demonstrated that mRNA, but not protein, of pluripotency-associated species is present in non-pluripotent cells. How can the presence of mRNA but absence of detectable concentrations of protein be explained? There is a multitude of mechanisms regulating translation from mRNA to protein, such as microRNA and repressor proteins binding to mRNAs, inhibiting translation or mediating degradation of transcripts. Mechanisms modulating initiation of translation depend on secondary structure of mRNA, ribosome recruitment proteins and phosphorylation status of the species involved. In the specific case of pluripotency, there is a requirement of co-expression of core transcription factors to elicit the phenotype (Wang et al., 2006). Likely, activation of translation is dependent on presence of multiple mRNA species; a feature that would ensure that developmental direction is maintained towards differentiation once cells down-regulate the pluripotent machinery and leave the pluripotent state (see section 1.2.2). When analyzed with virally transduced fluorescent reporters under the expression of promoters for *OCT-4*, *NANOG* and *REX1*, no co-transcription of *REX1/NANOG* or *OCT-4/NANOG* was detected in the fetal NPCs studied in paper II.

Piao et al. reported in 2006 that about 0.5 % of hscNPCs were immunoreactive for the markers Tra-1-60, Tra-1-81 and SSEA4 when analyzed with flow cytometry. During the work on paper I and II, using more rigid flow cytometry settings and other isotype control antibodies, we were unable to confirm those findings. Furthermore, no immunoreactivity to CD386 or OCT-4 could be demonstrated. Immunocytochemistry

experiments with antibodies against REX1 and NANOG also failed to detect protein in hscNPCs (see figure 11).

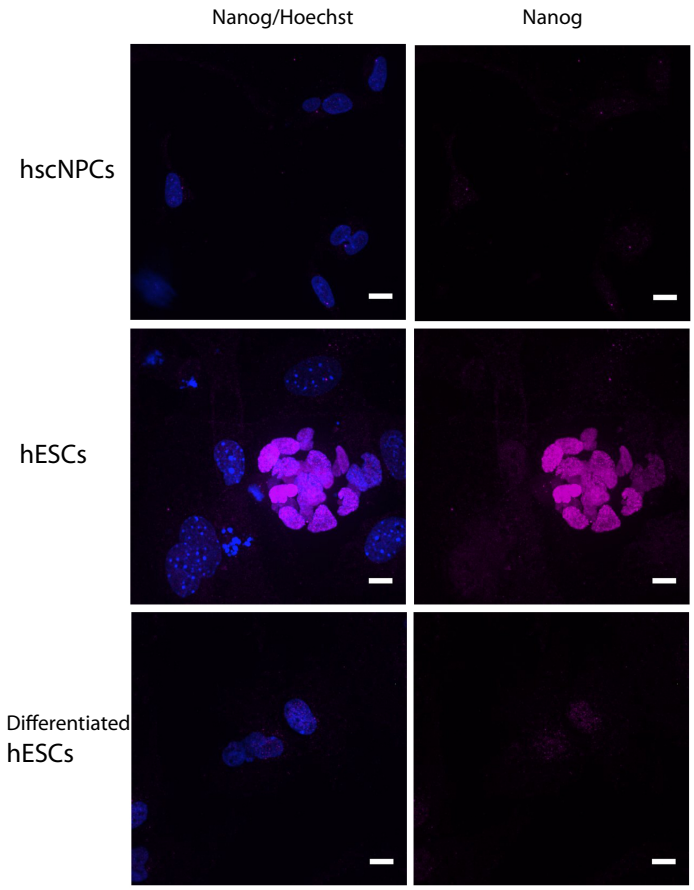


Figure 11. Immunocytochemical analysis of NANOG in fetal hscNPCs, naïve hESCs and spontaneously differentiated hESCs (12d). hscNPCs do not stain for the NANOG antibody, whereas naïve hESCs are strongly stained, and spontaneous differentiation of hESCs for 12 days generates weakly stained cells. 5,724 fetal cells were imaged, none of which showed detectable NANOG-immunoreactivity. Nuclear staining (Hoechst, blue) and NANOG (magenta). Scale bar 10 μM. Image credit: John Schell.

A more trivial explanation to the lack of protein, that of course should be addressed, is experimental failure. We cannot with absolute certainty claim that proteins of pluripotency-associated genes are absent from fetal NPCs, due to the possibility of failed immunochemistry experiments. The detection of immunoreactivity on hESCs in control experiments makes inability to detect protein less likely, but we have no information on lower detection limit.

Two main types of experimental failure are further addressed below: Failure to exclude false positive RT-PCR results (representing a type I error); and failure to detect protein, although present (representing a type II error).

4.2.1 Validation of RT-PCR Data

RT-PCR is a sensitive method and proper negative controls are needed to minimize the risk of false positive results, which is ever present. We constructed primers to selectively bind mRNA by choosing primer positions traversing exon-exon boundaries, and to distinguish between genomic DNA and cDNA based on product length by placing primers in different exons, and evaluated primer specificity *in silico* as well as experimentally. Temperature-optimized RT-PCR experiments yielded single products with the expected size, evaluated by gel electrophoresis, and the products were

sequenced using the Sanger method, which confirmed that the products originated from mRNA. We can therefore conclude with a high level of certainty that mRNA was present. It should be noted, though, that detection of pluripotency-associated mRNA can be affected by the presence of mRNA transcripts of pseudogenes, particularly OCT-4 (Liedtke et al., 2007; Poursani et al., 2016).

4.2.2 Validation of Protein Data

Cyclic methods analyzing mRNA content are more sensitive than immunochemical methods to detect protein. Chances are that proteins are present but below detection level. The use of proper immunochemical controls confirms the specificity and sensitivity of the antibody, but the absolute absence of protein cannot be proved. Although the principle of linearity of protein sequence is similar to that of DNA and RNA, the central dogma of molecular biology states that no flow of information can go from protein to RNA or DNA, which probably prevents the possibility to develop cyclic methods for protein analysis. The alternatives to antibody-based techniques available today for unbiased detection of low levels of proteins rely on separation chromatography and mass spectrometry (Lesur and Domon, 2015).

4.3 PLURIPOTENCY AND NEURAL PROGENITORS

Arguably, the most important finding in paper I is that standard teratoma tests can fail to detect pluripotent cells, that give rise to teratomas in other transplantation settings. This was the obvious but slightly surprising conclusion of the fact that we repeatedly found tumor growth in the spinal cords of rats transplanted with NPCs derived from hESCs, in spite of the negative result from so called teratoma tests. In paper I and II, we also reported that the expression of pluripotency-associated mRNA species, either measured qualitatively or quantitatively, is not predictive of the potency state of human cells.

A number of tests for validating pluripotency have been developed. The reasons for evaluating pluripotency of cells vary depending on the scientific question at hand. Many pluripotency tests are designed to confirm that the cells of interest (iPSCs, ESCs) are in fact pluripotent, and the tests' validity therefore depend on their specificity. Tests to ascertain that no pluripotent cells remain prior to clinical transplantation require high sensitivity to be able to pick up all cells that could give rise to unwanted progeny *in vivo*.

The methods for evaluating pluripotency include production of germ line competent chimeric organisms, tetraploid complementation, teratoma formation, embryoid body formation, and *in vitro* assays such as analysis of pluripotent-specific protein or mRNA marker expression, analysis of epigenetic state and morphological analyses (De Los Angeles et al., 2015; Smith et al., 2009). The tests mentioned above are listed in order of diminishing stringency regarding the ability to identify truly pluripotent cells. All methods have their advantages and drawbacks further discussed below (Smith et al., 2009; Buta et al., 2013).

4.3.1 Tetraploid Complementation

The most compelling evidence that a cell is pluripotent is if it can generate an entire healthy, fertile animal. When applying an electrical current to an embryo at the 2-cell stage, the cells fuse and a single tetraploid cell is formed, containing four copies of each chromosome instead of the normal two. That cell cannot give rise to a viable individual. However, it can give rise to the trophoblast, which later develops into the extra-embryonic tissue, the placenta and the umbilical cord. By combining the tetraploid cell with pluripotent cell candidates and implanting into a surrogate uterus, a viable embryo can develop. The extra-embryonic tissue will develop exclusively from the tetraploid cells; the fetus will develop from the pluripotent cells. Although arguably being the most stringent pluripotency test, tetraploid complementation is ethically and practically not feasible for human cells.

4.3.2 Chimaera with Germline Transmission

In chimaera formation, presumed pluripotent cells are injected into an embryo, which generates a chimeric organism, consisting of a mixture cells from the original embryo as well as from the injected cells. The chimaera is then allowed to mature and give offspring. If successful germline transmission of the injected cells is accomplished, i.e. if some offspring of the chimaera carries genetic material only from the injected cells, the cells were pluripotent. Lately, interspecies chimaeras have been created, raising hope that entire human organs intended for transplantation could be grown from autologous stem cells in pigs, for example (Wu et al., 2016). For the purpose of the evaluation of pluripotency, creation of a chimeric organism will however remain restricted to non-human species due to the low contribution of foreign species stem cells in an interspecies chimaera and hence low sensitivity of chimaera formation as a test of pluripotency.

4.3.3 Teratoma Formation Analysis

Teratomas are spatially restricted, most often benign, tumors consisting of tissue or organ components not normally found in the organ in which they arise. The name teratoma stems from the Greek word ‘teras’ – monster, and the suffix –oma, denoting a tumor or neoplasm, because its constituents can resemble normal tissue, and have been known to include teeth, hair and even more complex organs. When evaluating cells for pluripotency by teratoma formation transplantation assay, the cells are usually injected into well-defined sites of immunocompromised (SCID) mice. Most commonly, cells are put under the skin (sub-cutaneously), directly in the muscle tissue (intra-muscularly), under the kidney capsule or in the testis capsule. The major reasons for choosing the testis capsule are that the testis is easily accessible, both for transplantation and for evaluation of the tumor by palpation, and that the testis capsule provides a compartment in which the transplanted cells most often will stay and differentiate (Gertow et al., 2007). The resulting teratoma is cut out and processed for histochemistry. Naturally occurring teratomas can consist of cells deriving from one,

two or all three germ layers, endoderm, mesoderm and ectoderm. A teratoma from a homogeneous solution or body of pluripotent cells should consist of cells from all three germ layers, and to confirm this, antibodies labeling mesoderm-, endoderm- and ectoderm-specific markers are employed together with antibodies specifically labeling cells of the species from which the cells under investigation originated. Alternatively, the injected cells have been pre-labeled with stably expressed reporter genes. Haematoxylin-eosin staining is employed to facilitate microscope evaluation with normal, transmitted, light. The haematoxylin is positively charged and binds to negatively charged substances, primarily DNA and RNA, thereby staining the nuclei blue; eosin, on the other hand, is negatively charged, and binds to positively charged amino acid residues such as arginine and lysine, thereby staining proteins red. The local protein concentration determines the intensity of the staining and the color depth, which means that protein-rich structures, like muscle fibers, turn dark red, while structures with lower protein content, like mitochondria, turn palely pink. Structures high in fat, such as membranes and myelin, neither bind haematoxylin nor eosin.

Teratoma formation assays are laborious, time consuming, ethically disputable, expensive and, as we and others have shown, subject to type II errors (Sundberg et al., 2011; Zhang et al., 2008; Dressel et al., 2008; Hentze et al., 2009b) but nevertheless standard procedure in the scientific community for the evaluation of pluripotency *in vivo*. Several groups have used dilution strategies to evaluate the detection limit of pluripotent cells in teratoma formation assays. Non-pluripotent cells grafts have been spiked with pluripotent cells in low numbers and the rate and frequency of teratoma formation has been evaluated in various compartments in different animal species and strains. The most important factors increasing the sensitivity of the assays were low immunocompetence of the receiving animal and species similarity between donor and recipient. Other factors influencing the outcome were target compartment, graft embedding in Matrigel® and origin of the pluripotent graft. But, importantly, as few as two pluripotent cells could generate a teratoma after transplantation in the most sensitive model, highlighting the importance of rigorous methods to make sure pluripotent cells are excluded from clinical grafts (FDA, 2008; Hentze et al., 2009a; Lawrenz et al., 2004). The teratoma tests in paper I, were hESC-NPCs either in single-cell suspension or as neurospheres were injected subcutaneously and into the testes of mice with severe combined immunodeficiency (SCID), did not give rise to any identifiable teratomas. However, cells from the same cultures produced rapidly growing teratomas in the lesioned spinal cords of immunodeficient rats. Species differences as well as differences between target compartments – immunological or other – likely explain the different outcomes. The fact that the spinal cord was injured, a situation associated with long-term inflammation, may have increased the propensity for local tumor development. We therefore suggest that safety testing of cell therapy should always be performed in the intended target regions.

4.3.4 Embryoid Body Formation and Directed Differentiation

Correspondingly to *in vivo* teratoma formation, embryoid body (EB) formation can be used to retrospectively identify pluripotent cells *in vitro*. But, in contrast to *in vivo* teratoma formation, EB formation is, due to low sensitivity, primarily not an assay for analysis of small contribution of remaining pluripotent cells. EBs, irregular lumps consisting of cells from all three germ layers, form when pluripotent cells are differentiated under certain conditions *in vitro*. The process recapitulates many aspects of cell differentiation in early development (Kurosawa, 2007). Other models of spontaneous or directed *in vitro* differentiation can also be used to specifically determine the potential of cells to form different progeny such as neural (Chambers et al., 2009) and cardiac (Hoebaus et al., 2013) stem/progenitor cells.

4.3.5 Protein Expression Analysis

As mentioned in the background (section 1.2.3), a number of proteins have been associated with the pluripotent state, but claims that any single protein (most notably *NANOG*) is both selective and specific for pluripotent cells, have been refuted (Chan et al., 2009; Kalmar et al., 2009). The work by Sundberg et al. preceding paper I identified the transmembrane glycoprotein CD326 (EpCam) as differentially expressed between hESCs and their neural progeny (Sundberg et al., 2009). It is not, however, specific to pluripotent cells, but expressed in a variety of human epithelial tissues. Thomson et al. described the first hESC cultures in 1998 and established that they expressed cell surface markers previously known to characterize primate ESCs, including TRA-1-60, TRA-1-81, SSEA3, SSEA4 and alkaline phosphatase (Thomson et al., 1998). Although non-pluripotent cell types express the cell surface markers listed by Thomson et al. in various combinations, the complete cell surface marker profile listed in that seminal paper has been a model for hESC research ever since, and, to my knowledge, no non-pluripotent cell has been shown to express the whole profile. This may, of course, be for technical reasons. Importantly though, there have been reports of human pluripotent cells lacking expression of markers on that list, leading to the conclusion that any comprehensive list of markers is not selective for hESCs (Brimble et al., 2007). Likely, co-detection of many or all of the factors involved in maintaining the pluripotent state is necessary to absolutely unambiguously claim that a cell is pluripotent, but more research is needed before any protein data-based claim that cells are *not* pluripotent is possible. Multiplex proteomic assays using mass spectrometry constitute a promising means to that end (Baud et al., 2017). Alongside antibody-based techniques to detect and quantify proteins, spectrometry-based techniques are rapidly evolving.

In paper I, small populations (1-5 %) of the hESC-NPCs were immunoreactive for the pluripotency-associated proteins SSEA-4, OCT-4 and CD386, while fetal NPCs were not, indicating that protein data is more predictive of teratogenic potential than mRNA data.

4.3.6 mRNA Expression Analysis

Differentiation of pluripotent cells is routinely monitored over time by analyzing changes in the mRNA pool. In neural progenitor populations derived from pluripotent cells *in vitro* described in paper I, *OCT-4*, *NANOG* and *DNMT3B* mRNA concentrations declined to undetectable levels already within 2 weeks of neural induction, while the same mRNA species remained at detectable levels in fetal cells, that did not create teratomas is any compartment tested. Although not statistically confirmed, a slow down-regulation of *DNMT3B* mRNA could be discerned in fetal cells over 12 weeks, but *OCT-4* and *NANOG* mRNA levels were oscillating without following any salient pattern over the 5 time points investigated during 12 weeks. In paper II, using retroviral reporters, we found that expression of *NANOG*, *OCT-4* and *REX1* mRNA was most prominent in distinct, small subsets of hscNPCs at any given time point, and that those populations were reestablished after depletion sorting, indicating that fluctuating expression is inherent to all or most hscNPCs *in vitro*. Multiplex, single cell qPCR and whole transcriptome analysis of single cells with other cyclic methods have become more accessible, and data from such experiments will most certainly add important information in the future.

4.3.7 Analysis of Epigenetic State

The epigenetic states of pluripotent cells, as well as the epigenetic changes associated with differentiation have been studied (Zhao et al., 2007), and theoretically, it should be possible to determine the potency of a cell solely based on its genetic code and epigenetic configuration, but the biochemical processes involved in transcription and translation are far too complicated for any kind of reliable prediction to be possible in the foreseeable future.

4.3.8 Morphology Analysis

Distinguishing between a pluripotent and a differentiated cell based on morphology alone is not possible, but certain distinct morphological traits characterize pluripotent stem cells: Regardless of whether pluripotent stem cells are cultured on matrix substrate or feeder cells, they usually form flat and compact colonies with sharp edges. Individual pluripotent stem cells are typically round and have a large nucleus compared to the soma. Significant experience is required to be able to validate the quality of stem cell cultures based on morphology (Holm, 2012).

4.4 ANALYSIS OF SPHERE FORMATION

Culturing cells as neurospheres entails some difficulties regarding for instance monitoring the morphology of living cells or estimating the cell number in a culture without dissociating the spheres. The neurosphere culture method has been used for more than 20 years and for many types of cells, including cancer cells, mesenchymal stromal cells, pancreatic cells etc., to propagate cells without substrate. It is also used as a method of analysis measuring the cells' ability to form presumably clonal spheres,

which would indicate ‘stem-ness’. The notion that all spheres originate from a single sphere-forming cell has however been refuted (Pastrana et al., 2011; Singec et al., 2006). We have also seen that sphere fusion is a common event and have substantial (or at least circumstantial) evidence that cell-cell contact may be necessary for proliferation of hscNPCs in a non-adherent setting. When individual hscNPCs are put in one 96-well each with conditioned medium, no spheres are formed.

If the sphere-forming cells were the true stem cells, it would indicate that only a certain subset of cells have the intrinsic ability to form spheres. In an attempt to identify that putative subset we FACS sorted cells with specific traits in different wells and analyzed their sphere-forming ability with image analysis. Neurospheres are somewhat transparent and best viewed with phase contrast microscopy, a technique which converts phase shifts in light passing through cells, that have different refractive index than the growth medium, to brightness changes in the image. In order to make an automated, un-biased analysis of a large number of images, we created a script that could be run in the freeware ImageJ. There are several commercially and freely available software and hardware solutions that all have emerged since we initiated this project: Celígo 3D Tumor Spheroid Analysis from Nexelcom; VisionGauge from Visionx; Cell Profiler and ImageJ macros. For an overview, see (Choudhry, 2016).

A more thorough analysis of sphere forming is feasible using time-lapse video monitoring of single cells in the process of sphere forming, possibly in gelatinous medium to reduce the motility of cells. Freely floating hNPCs *in vitro* exhibit not only Brownian motion, but also motion probably associated with elongation of processes and contact inhibition/attraction. 3D Matrigel matrices have previously been used for the purpose of monitoring proliferating and/or differentiating cell aggregates *in vitro*, but in this case, the growth factor constituents of regular Matrigel, as well as the laminins (still present in growth factor-reduced Matrigel) would most likely affect sphere formation and proliferation.

4.5 CHOOSING TYPE OF CELL FOR SCI TRANSPLANTATION

Identifying the optimal cell mixture for SCI transplantation necessarily involves the identification of the mechanism(s) primarily responsible for functional recovery. We have previously shown that transplantation of human NPCs in the form of neurospheres improves functional outcome in several rat SCI models, and is associated with a survival of donor cells, which surpasses that of transplanted dissociated cells. The mechanisms by which the transplanted cells exert their effect are probably several but rescue of injured host neurons is an important factor. We have also shown that SCI rats transplanted with cells derived from human fetal spinal cord improve to a higher degree than rats transplanted with forebrain-derived human fetal cells (Emgard et al., 2014). Speculation can be made that this is the result of regional-specific cell characteristics – such as cell surface antigen expression or factors excreted – that remains during cell culture (Piao et al., 2006). Others have shown functional recovery after transplantation of fetal forebrain-derived spheres as well as spinal cord-derived spheres (Watanabe et

al., 2004). The initial rationale for paper III was to use cell surface molecules to select for certain phenotypes (see section 1.2.4), which would make it possible to transplant purified populations of cells and elucidate which mechanisms are responsible for functional recovery. Isolation, *in vitro* proliferation and subsequent transplantation of lineage restricted NPCs would aid in determining the cell type, and thereby the mechanisms, responsible for the functional improvement of transplanted SCI rats. Also, since transplantation acutely or possibly subacutely seems preferred (Emgard et al., 2014), cells intended for a prospective clinical application of neurospheres will most likely be cultured after sorting and banked in sufficient number for fast access upon request. Cells can be isolated by immunolabeling and subsequent cell sorting by fluorescence-activated cell sorting (FACS), immunopanning or magnetic-activated cell sorting (MACS). We decided to use FACS, which has the advantage of simultaneous analysis and sorting of the cells, although the yield can be substantially lower, and there is a risk of exposing the cells to shear forces and pressure transients in the FACS apparatus. As described in paper IV, pressure changes in the Biogrid device far surpassing those induced by the FACS have no obvious effect on cell survival or phenotype outcome. Furthermore, confusing preliminary data generated with immunopanning led to a decision to avoid that technique. In hindsight, the confusing data may have represented the main finding of paper III, that NPCs interconvert *in vitro*, but we had no idea at the time.

4.6 PHENOTYPE INTERCONVERSION

Surprisingly, culture homogeneity after FACS sorting for the cell surface antigens CD15, CD133, CD24, A2B5 or PSA-NCAM could not be maintained over time in culture with regard to 1) expression of the antigen for which the cells were sorted, 2) the transcriptome and 3) phenotype outcome in various *in vitro* assays presented in paper III. We believe that the observations reflect consequences of phenotype interconversion. Phenotype interconversion has previously been reported to occur in non-neural cells, e.g. hematopoietic progenitors (Chang et al., 2008), breast cancer cell lines (Gupta et al., 2011) and primary muscle cells (Stockholm et al., 2010). Chang et al. showed in 2008 that phenotype cell-to-cell variability within a stem cell culture is a manifestation of transcriptome-wide expression noise, which controls lineage choice but does not generate irreversibly committed precursors. The slow relaxation (requiring more than 12 cell doublings) of FACS sorted progenitors with distinct transcriptional profiles to the parental distribution of the marker for which the cells were sorted suggested the existence of multiple stable cell states – attractor states – along the way (Chang et al., 2008). Similarly, Gupta et al. described in 2011 the dynamics of interconversion of breast cancer cells between differentiated states and a stem cell state using a Markov model, with probabilities of interconversion between all states (Gupta et al., 2011). We have not demonstrated direct conversion between states far out on either side of the neuronal-glial fate spectrum (i.e. A2B5-/PSA-NCAM+ neuronal precursors and A2B5+/PSA-NCAM- glial precursors), but rather propose that interconversion of NPCs goes via an un-biased cell state. It has previously been shown that the original cell fate of mouse neural precursor cells is lost in neurosphere cultures

(Machon et al., 2005) and that rat oligodendrocyte precursors can revert back to a multipotent stage in the presence of bFGF (Kondo and Raff, 2000), which may, in the light of our findings, be effects of phenotype interconversion. In contrast to the finding by Ravin and co-workers that uni-potent cells in rat cerebral cultures are reset to a tri-potent stage at passage (Ravin et al., 2008), fetal hNPCs in our system lost their antigen profile and presumed uni- or bi-potency also without passaging, which may reflect differences between species or culture conditions. We evaluated the possible contribution to the observed results of two confounding factors: low sorting purity and clonal expansion. Increasing the stringency of the FACS sorting by only collecting a small proportion of NPCs with the highest immunoreactivity, to compare them with NPCs with no immunoreactivity did not change the outcome. Likewise, mathematical models of culture behavior into which small populations with deviating proliferative features were used including parameters based on experimental observations (see figure 14). These models produced outcomes that did not at all resemble experimental observations. Furthermore, no NPC cultures investigated have presented with detectable changes in karyotype. Clonal expansion *in vitro* is associated with genetic alterations and chromosomal aberrations of rapidly growing cells.

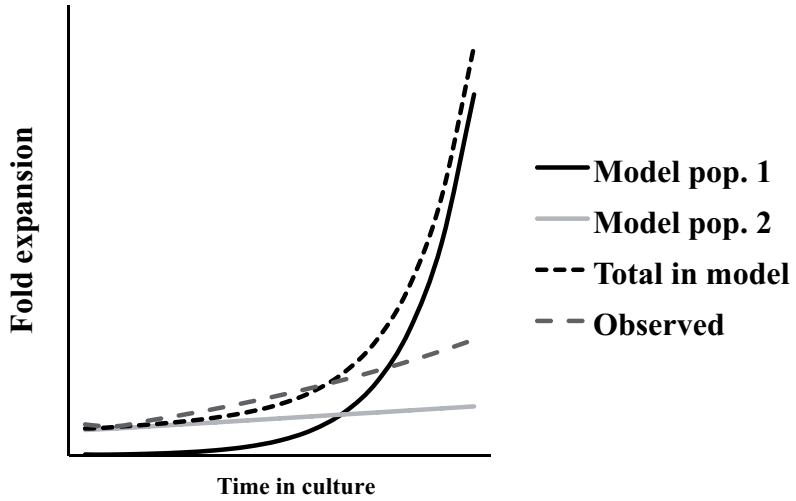


Figure 13. Graph illustrating the results of mathematical modelling of clonal expansion using the Deasy equation (Deasy et al., 2003), which are inconsistent with experimental observations. Model population 1 represents a 1 % rapidly growing contaminating subpopulation. Population 2 represents the 99 % cells in the population with one 10^{th} the proliferation rate of pop. 1. The resulting fold expansion in the model is illustrated with the dotted black line. A segmented grey line represents the *in vitro* proliferation of hscNPCs observed experimentally.

To further examine the possible involvement of clonal expansion by a small subpopulation, we sorted CTV-stained cells based on both CD15- and CD133-immunoreactivity, cultured them in NSG medium, and analyzed CTV fluorescence intensity and CD15- and CD133-immunoreactivity 10 and 33 days later. Initially, CD15+/CD133+ cells proliferated faster than CD15-/CD133- cells, but the effect of the sorting waned after about one week. As expected, CTV stain intensity measured with flow cytometry was higher in the population that was CD133-/CD15- at sorting than in their CD133+/CD15+ counterparts at 10 and 33 days after sorting, confirming that CD133-/CD15- cells initially proliferated slower than CD15+/CD133+ cells. However, the proportions of CD15- and CD133-immunoreactive cells had been reset, partially

with respect to CD15 at 10 days, completely with respect to CD133 at both time points and CD15 at 33 days, and CTV fluorescence intensity was not correlated to CD133 or CD15 *immunoreactivity* at 10 or 33 days after sorting, indicating that interconversion took place in all populations to similar extent. Furthermore, there was no difference in the rate of CTV signal decline between the subpopulations between 10 and 33 days, presumably after interconversion had occurred, indicating that there was no subpopulation solely responsible for reconstituting the population via clonal expansion.

It is also important to note that clonal expansion of a small subpopulation – although not consistent with any of our observations – would not contradict the concept of interconversion, since the proportions of cell expressing markers for which the cells had been sorted approached the same value as FACS flow-through control cells asymptotically, indicating a population attractor state for each marker in our culture system (see paper III, figure 1). Rapidly dividing cells falsely detected as negative in the FACS that would only give rise to clones of positive cells in a negative population would take over the culture and all cells would be positive, which was never the case. The reversed scenario would consist of cells falsely registered as positive giving rise to negative progeny in a positive population, rendering it negative, which also never occurred. Also, for NPCs sorted with respect to PSA-NCAM, A2B5 and CD24 immunoreactivity, no differences in growth rate between positive and negative populations could be seen at any time point. As demonstrated in paper III, fig. 7b, fresh tissue from human CNS contains about 2-20 % CD133-immunoreactive cells, which rapidly acquire CD133 expression *in vitro*, initially from selective growth of CD133+ cells, and later also from interconversion (paper III, fig. 1, left panel).

4.6.1 Phenotype Accompanies Re-setting of Surface Marker Profile

CD15+ hscNPCs did not form a higher number of spheres than CD15- cells, but displayed a higher proliferation rate, which resulted in larger spheres (paper III, fig. 5). This was analyzed by un-biased, semi-automated image analysis as described in section 3.8. The difference in proliferation rate was linked to CD15-immunoreactivity, which changed over time in culture. Similar results have been reported for the proposed stem cell marker β-1-integrin, the loss of which is accompanied by lower proliferation rate and higher mortality, but not loss of sphere forming capacity (Leone et al., 2005). CD15 expression in mouse cortical progenitor cells has been shown not to change with the cell cycle, indicating that it reflects proliferative potential rather than cell cycle stage (Capela and Temple, 2006). It has also been reported that serially sorted CD15+,- mouse NPCs do not form neurospheres (Kelly et al., 2009). hscNPCs in our system were able to form spheres after serial sorting (CD15-,- or CD15+,-), both with and without passaging in between. This may indicate a higher propensity of hscNPCs to interconvert.

A2B5-/PSA-NCAM+ cells generated a higher proportion neuronal progeny than A2B5+/PSA-NCAM- cells, but were not committed to the neuronal fate. As PSA-NCAM immunoreactivity was reset within weeks of continued culturing with mitogens

after cell sorting, differentiation fate bias was also lost. FACS sorting *per se* actually increased the ratio of GFAP:β-tubulin III-IR cells after subsequent differentiation, probably because neuronal progenitors are more sensitive to the FACS conditions, which include rapid pressure changes and comparatively long periods of both mitogen and cell-cell contact depravation. Other sorting methods, such as MACS or immuno-panning, may be gentler for neuronal progenitors (discussed in section 4.5). PSA-NCAM-IR neuronal progenitors have longer, thinner processes and smaller cell bodies than PSA-NCAM-negative hscNPCs, and co-localize with MAP2 and β-tubulin III immunoreactivity to some extent (see figure 14). When cells are dissociated, having long, thin processes may be disadvantageous for maintenance of membrane integrity and cell survival, which could explain the lower yield of neurons directly after sorting as well as the accumulation of glial progenitors over time in fetal NPC cultures (see section 1.2.6.3). We have not detected any difference in growth rate or Ki-67 immunoreactivity related to PSA-NCAM immunoreactivity. Co-localization of Ki-67 and PSA-NCAM staining in adherent hscNPCs is shown in figure 13. Ki-67 immunoreactivity also largely co-localized with MAP2 immunoreactivity in hscNPC adherent cultures, but not with MAP2 immunoreactivity in NBM cultures after 12 days' differentiation.

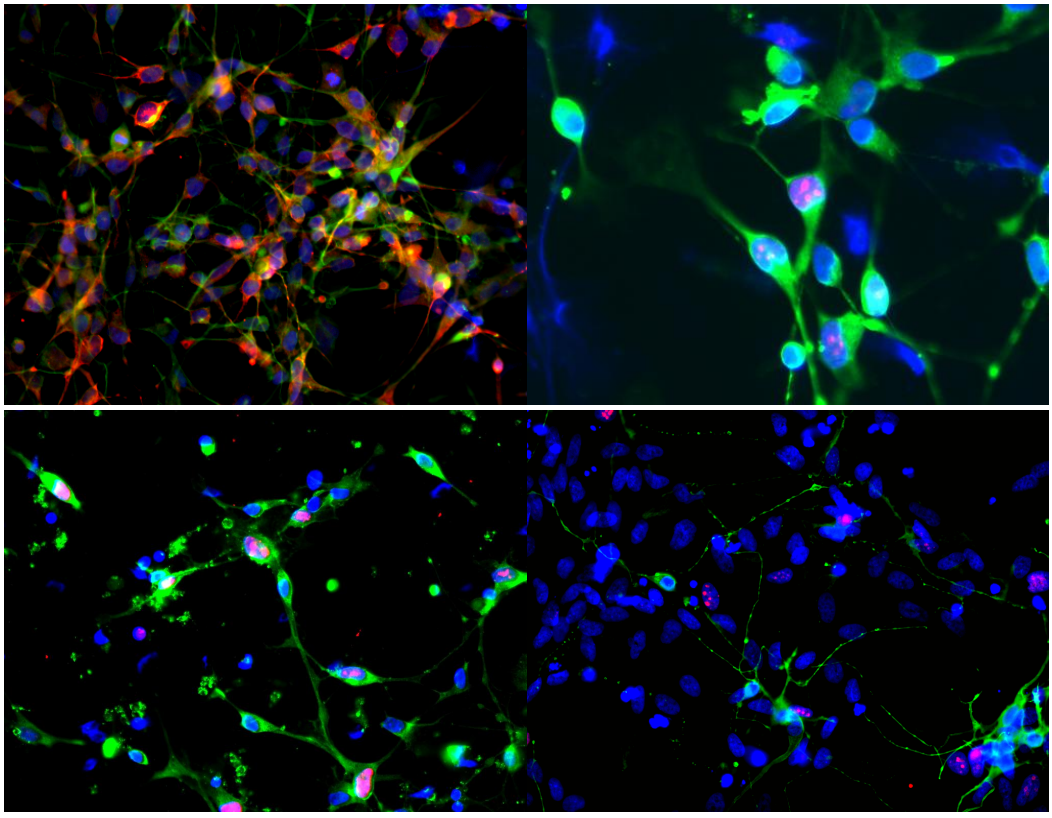


Figure 14. Upper left: MAP2- (red) and PSA-NCAM-IR (green) adherent hscNPCs. Upper right: MAP2- (green) and Ki-67-IR (red) adherent hscNPCs. Lower left: PSA-NCAM- (green) and Ki-67-IR (red) adherent hscNPCs. Lower right: PSA-NCAM- (green) and Ki-67-immunoreactivity (red) does not co-localize after 12d differentiation in NBM. Similar results were obtained using MAP2/Ki-67 staining of cells differentiated 12d in NBM (data not shown).

Most hscNPCs differentiate into GFAP-immunoreactive cells after transplantation *in vivo* or serum-induced *in vitro* differentiation (Emgard et al., 2014) irrespective of

sorting. In our studies, the differences in proportion of GFAP-IR progeny between A2B5+ and A2B5- cells did not reach statistical significance (data not shown). Evaluation of their morphology revealed that not all GFAP-IR cells had typical astrocytic morphology, but the difference in proportions of GFAP-IR cells with typical astrocytic morphology did also not reach statistical significance (data not shown). Although the proportion of β-tubulin III IR progeny from A2B5+ cells after differentiation in NBM was significantly lower than FT control cells (data not shown), transcriptome analysis revealed that there was no significant transcriptome profile associated with A2B5 immunoreactivity, indicating that A2B5 sorting is of little, if any, consequence for hscNPCs, especially if culturing afterwards is required.

CD133 has been used to prospectively sort out neural stem cells from adult mouse subependymal zone (Beckervordersandforth et al., 2010), and it has been shown that human embryonic forebrain CD133+/SSEA-4+ and CD133+/CD15+ subfractions are enriched in neurosphere-initiating cells (Barraud et al., 2007). CD133 immunoreactivity correlates with cell DNA content and thus to cell cycle stage and has been shown to interconvert in human embryonic stem cells and various cancer cell lines (Jaksch et al., 2008). As described in paper III, while CD133- cells from freshly dissected human fetal spinal cord and subcortical forebrain did not form spheres, CD133- cells from already established hscNPCs cultures did, but much slower than CD133+ or FT control cells. There is a possibility that the CD133- population of hscNPCs was contaminated by CD133+ cells that have been enzymatically stripped of their surface expression of the CD133 molecule by the dissociation enzyme TrypLE Express, and therefore falsely registered by the FACS as CD133-. Freshly dissected human fetal spinal cord and subcortical forebrain, on the other hand, was routinely dissociated mechanically, without enzymes.

4.6.2 Phenotype Interconversion Is Likely a Cell Intrinsic Feature

In paper III, we tried two different approaches to investigate whether cell-cell interactions govern interconversion kinetics. First, we treated cells with the γ-secretase inhibitor L-685,458 without detecting any effect on interconversion. Second, we used GFP-expressing cells to be able to follow the behavior of PSA-NCAM+ cells in a mixed culture with PSA-NCAM- cells, and, similarly, A2B5+ cells in a mixed culture with A2B5- cells. The results indicated that there was no that mechanism sensing the proportions of cells expressing PSA-NCAM or A2B5. Also, PSA-NCAM IR cells are randomly distributed in adherent and neurosphere hscNPCs, contradictory to the hypotheses that either mitotic bias or lateral inhibition is responsible for resetting population frequencies. However, we have shown in paper II that adherent hscNPCs are highly motile and probably move around too fast for any conclusion to be drawn from a still image regarding population control mechanisms. We have not investigated the kinetics with which individual cells transform from, for example, a PSA-NCAM+ state to a PSA-NCAM- state. The fact that PSA-NCAM-IR cells are disproportionally slim and neuron-like combined with the relatively short time course of dramatic morphology changes of adherent hNPCs described in paper II (supplementary video 1)

indicate that individual cell state transition kinetics may be of importance for understanding interconversion. On population level, we have shown that full interconversion occurs within 3 passages. Individual cells may flip between states much more rapidly, with certain probabilities to exist in any given state, and slower cellular events may primarily affect the probabilities, but not the cell state *per se*. Such events could probably be investigated using reporters and time-lapse imaging, as has been done for NOTCH1 (Shimojo et al., 2008) and NANOG (Kalmar et al., 2009). We nevertheless conclude that relaxation to a population attractor state is driven by mechanisms inherent to individual hNPCs.

4.7 TRANSCRIPTOME ANALYSIS OF PHENOTYPE INTERCONVERSION

In paper III, to determine if resetting of the cell phenotype occurs in parallel to surface antigen profile, transcriptional analysis was performed on acutely sorted hscNPCs as well as hscNPCs sorted and then cultured for 3 passages. The transcriptional profiles of cells sorted based on immunoreactivity for A2B5 and PSA-NCAM were surprisingly homogenous within biological replicates. Un-biased, automated clustering revealed larger differences between biological replicates than between sorted populations. Nevertheless, the differences in the transcriptome between sorted populations were missing three passages later, indicating that interconversion takes place on the transcriptome level as well as on the cell surface.

Transcriptome analysis revealed 19 genes significantly upregulated in the A2B5-/PSA-NCAM+ population, and an analysis of GO terms associated to those 19 genes suggested that the cells were biased towards a neuronal fate, which was confirmed by *in vitro* experimental data (see sections 3.2.2.2.3 and 4.6.1). Three passages later, when the A2B5-/PSA-NCAM+ population had interconverted with regard to cell surface profile as well as preferential differentiation bias, there was no memory of the sorting among the previously significantly up-regulated 19 genes as evaluated by ranking score (paper III, fig. 8 and table 3). Whether transcriptional events or cell surface expression mechanisms govern interconversion kinetics is a chicken-or-egg-type of question that cannot be answered by our present data, but we conclude that alterations in cell surface expression of A2B5 and PSA-NCAM is accompanied by transcriptome profile alterations.

4.8 EPIGENETICS AND STOCHASTIC EXPRESSION

In paper II, we showed that the proportion of cells expressing pluripotency-associated TFs decreased after differentiation. We have also seen that after 14 days of *in vitro* differentiation in NS-FBS, hsc-derived cells detached and died when NS-FBS medium was exchanged for NSG medium containing mitogens (data not shown) and could therefore no longer be investigated for interconversion, and may have irreversibly lost their NPC features. For each final cell fate, there seems to be a point of no return where cell fate decision is made irreversible (see figure 15). Epigenetic regulation of transcription and chromatin status probably affects the likelihood of stochastic expression for genes that are silenced as cells differentiate (Chalancon et al., 2012;

Trott et al., 2012). To validate this hypothesis with regard to interconversion, we treated hscNPCs with valproic acid (VPA), a histone deacetylase inhibitor, whose actions lead to higher histone acetylation levels and thereby higher accessibility for transcription. 1 mM VPA treatment of sorted cells did not significantly affect the kinetics of interconversion (data not shown).

Just as epigenetics influence the probability of stochastic events, gene expression noise can direct epigenetic changes. Studies on the evolutionary advantages of using epigenetics to exploit stochastic expression revealed a trade-off between adaptation time and robustness of the acquired phenotype, favoring epigenetic changes in a rapidly changing environment, and genetic adaptation in slowly changing environments (Gomez-Schiavon, 2016).

Although the *in vitro* conditions for propagating NPCs are well defined, one could argue that the environment is less rigid and contains fewer determining cues than the *in vivo* situation, which could further induce the mechanisms governing stochastic expression. The lack of proper developmental control *in vitro* can be illustrated by the fact that hscNPCs express combinations of transcription factors not found *in vivo* (unpublished data, Alekseenko et al.). Austin Smith has formulated a similar remark regarding pluripotency *in vitro* and *in vivo*:

> "Observations of fluctuating gene expression and phenotypic heterogeneity in vitro have fostered a conception of pluripotency as an intrinsically metastable and precarious state. However, in the embryo (…) the properties of pluripotent cells change in an orderly sequence." (Smith, 2017)

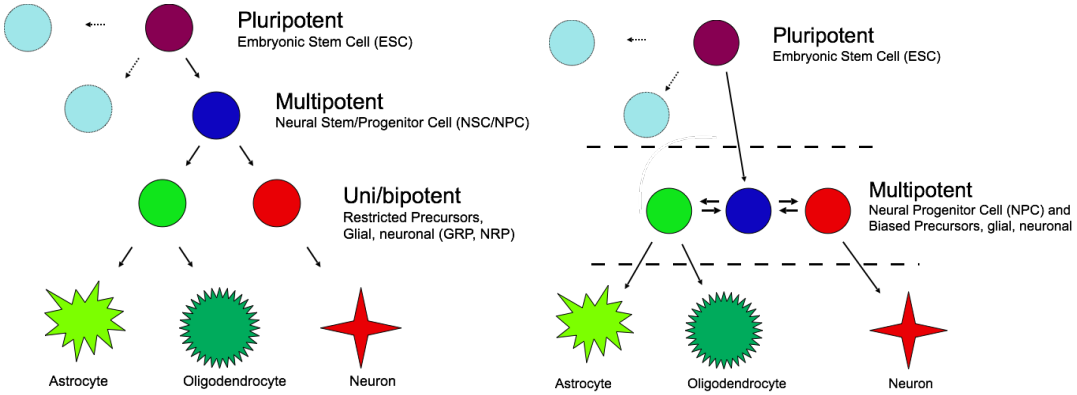


Figure 15. Schematic representations of the hierarchy of potency of cells along the neuroectodermal lineage *in vitro*. On the left: Hypothesis under which the studies in the present thesis were initiated. A pluripotent cell (plum) gives rise to a multipotent neural progenitor (blue), which gives rise to restricted progeny, glial (green) and neuronal (red), which in turn generates terminally differentiated cells. On the right: Model of hierarchical relationships that better fits experimental *in vitro* observations. Neural progenitor cells exist in different states, associated with varying probabilities of differentiation, but with maintained multipotency. Flipping between states – phenotype interconversion – involves alterations of the transcriptome. Dotted lines signify points of no return, which only allow passage from above.

4.9 ROAD TO CLINIC/PRODUCTION OF CELLS UNDER GMP

Culturing and transplanting spheres has several advantages compared to single cells. First, there is no need for addition of exogenous ECMs to culture plates. Cells in neurospheres create their own microenvironment containing appropriate cell adhesion molecules and growth substrates, promoting growth and survival. Second, from published transplantation studies it can be concluded that cell survival after xenotransplantation of neurospheres surpasses that of single cells (see discussion in Emgård et al., 2009). The possible drawbacks of using neurospheres are at first glance threefold, two of which have to do with standardization rather than outcome. First, precise quantification of cell number is difficult, if not impossible. We have determined the equation describing the relation between sphere diameter and the number of hNPCs in individual spheres, based on counting the number of cells in a large number of spheres of different diameters. Second, using neurospheres adds a level of complexity to the transplantation situation. Which sphere size is optimal for transplantation? Choosing sphere size for transplantation depends on the delivery method and the status of the cord. Our preclinical data suggest that acute or possibly sub-acute transplantation is preferred (Emgard et al., 2014), which would in many occasions allow access to the cord during acute decompressing or stabilizing surgery. Open surgery at a later time point for the sole purpose of cell transplantation entails additional risks of infection, bleeding and other complications that are less likely to be accepted by the patients as well as the ethical review board. Secondary surgery was also a reason for significant dropout in the Pathway study conducted by StemCells Inc. In our setting of experimental SCI model transplantation, a Hamilton syringe with a pulled glass capillary is used for administration of spheres in open surgery, setting an upper limit to the sphere size at about 300 μM. Irrespective of the transplantation method and knowing that pulled glass capillaries will never be used for injections into patients, it is beneficial to be able to see the tissue to be transplanted to increase the chances that it ends up were it's supposed to go, especially when transplanting to fluid-filled compartments such as posttraumatic syringomyelia cysts, which we currently are studying (but not as a part of this thesis). A 300-μm sphere is clearly visible to the naked eye.

Sphere size is also related to the third drawback of a clinical use of neurospheres. There have been reports of increased cell death and differentiation in the middle of neurospheres derived from mouse brain cells and human glioma cells as sphere size increases, maybe because of limited diffusion of mitogens or other factors into the spheres (Azari et al., 2011; Rahman et al., 2015; Walker and Kempermann, 2014). We have not been able to confirm those observation using fetal NPCs, as apoptotic markers and markers for differentiated cells were not unevenly distributed in sectioned spheres (data not shown), and increased cell death was not seen in the center of hscNPC spheres as large as 1 mm across. Contradictory observations regarding the heterogenic distribution of proliferating cells in neurospheres highlight the need for thorough investigation of each neurosphere model system: rapidly proliferating progenitors have been reported to constitute the outer rim as terminally differentiated cells accumulated

in the middle of neurospheres derived from mouse dentate gyrus (Babu et al., 2011; Reynolds and Rietze, 2005), while neurospheres from human fetal mesencephalon displayed the opposite, as differentiation occurred primarily at the rim and undifferentiated cells remained in the center (Ilieva and Dufva, 2013).

We have preliminary data indicating that cells grown as spheres behave slightly differently compared to adherent cells, especially with regard to dorso-ventral identity and nestin expression. Adherent NPCs – which are routinely cultured on laminin-111 – presented a more ventral identity than NPCs in neurospheres, as shown by the expression of positional transcription factors. Adherent NPCs also contained a higher proportion cells immunoreactive for the marker nestin, implying that adherent cultures are more homogeneously immature than neurospheres (unpublished data, Alekseenko et al.), which has also been suggested by others (Conti and Cattaneo, 2005). Nevertheless, the features of the two culture systems seem reversible, as spheres of hscNPCs and hfbrNPCs can be made adherent and vice versa (see section 3.2.2.1).

Thus, depending on the actual situation, there may be strong reasons to choose neurospheres for clinical transplantations. In these applications, culture conditions under GMP will likely require mechanical instead of enzymatic dissociation, to eliminate the risk of contaminating the transplanted tissue with proteolytic enzymes. In paper IV we describe the Biogrid device that reliably and with high reproducibility allows dissociation of neurospheres and hESC aggregates in solution in automated systems. It is reasonable to believe that cell aggregates of other origins, such as hepatocytes, MSCs or even plant cells could be dissociated as well. We showed that the pressure transients at Biogrid passage do not induce cell death of hNPCs, and that the proliferation achieved using Biogrid – ≈100-fold during 10-13 weeks – is similar to enzymatically passaged hNPC cultures and sufficient for efficient *in vitro* expansion of hNPC cultures. Proliferation after enzymatic dissociation was more variable. The proportions of differentiated progeny within neurospheres were not affected by passage method.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

Clinical transplantation of progenitor cells to the human CNS requires in-depth investigation of tumorigenic potential of grafted cells, not only by standard SCID mouse teratoma tests, but also by long-term follow up of transplantation to the intended target compartments of other model animals. Furthermore, we have corroborated the findings that mRNA data is less predictive of pluripotency than protein data.

We have shown that phenotype interconversion is a common feature of neural stem/progenitor cells derived from human fetuses *in vitro*, a feature that also affects pluripotency gene expression. Whether this is a phenomenon attributable to the artificial *in vitro* milieu and of limited interest to the *in vivo* situation should be more thoroughly investigated. Specified progenitors certainly exist *in vivo*, but our results show that it is not possible to propagate them with retained specificity *in vitro*. Applications such as clinical transplantation of stem cells will most likely depend on *in vitro* expansion of cells to secure the accessibility of material, and phenotype interconversion should be taken into account, especially when culturing selected subpopulations of heterogeneous cultures. To selectively culture specified progenitors of hscNPCs may be possible by using other culturing methods than the ones used in this thesis, including other media formulations and growth conditions. In NSG medium, cell surface expression, or even transcriptional profile, of a cell population or individual cell only provides a snapshot of the population's or individual cell's propensity to differentiate. Therefore, the hierarchical model of phenotype specification for these cells must be questioned. In the absence of truly determining cues for entering terminal differentiation, hscNPCs seem to remain within the unspecified realm, exploring it.

Finally, production of cells for clinical transplantation will require new, standardized culturing techniques devoid of animal-derived content and factors such as proteolytic enzymes that may have unforeseen effects on the cells to be transplanted or the recipient. The Biogrid device can be used in automated systems for dissociation of cell aggregates intended for therapeutic use, enabling large-scale culturing of hNPCs as well as other cells.

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