MARIA SUNDBERG

Differentiation and Purification of Human Pluripotent Stem Cell-derived Neuronal and Glial Cells

Graft designing for spinal cord injury repair

ACADEMIC DISSERTATION
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To my father

Abstract

Human embryonic stem cells (hESCs) are pluripotent cells that can be differentiated into all three germ layer cell types of the human body. The potential of these cells to efficiently proliferate and differentiate into neuronal and glial cells makes them a desirable cell source to be used for neural graft production for different neurological disorders, such as spinal cord injuries (SCI). Multipotent human neural stem/precursor cells (NSCs/NPCs) can also be derived from human fetal central nervous system (CNS) forebrain and spinal cord tissues. In the case of SCI, neural cell transplantation therapies could be one option for regeneration of the damaged tissue and restoring loss of locomotor function. However, currently existing differentiation protocols for hESC-derived neural cell production often result in heterogeneous populations, which contain undifferentiated or partly differentiated hESCs or NSCs/NPCs proliferating in an uncontrolled manner and are tumorigenic upon grafting. Also, although it has been shown that fetal CNS-derived NSCs/NPCs are effective for the treatment of SCI in animal models some studies have suggested that these cells may be tumorigenic after transplantation. Thus, it is important to evaluate the safety of these human neural cell grafts properly and in reliable animal models to avoid grafting of unsafe cells for the patients in the future. Related to this it has been shown that in SCI animal models more specialized cell populations, such as oligodendrocyte precursors (OPCs), are safer upon grafting and can have more beneficial effects in the regeneration of damaged tissue compared to multipotent NSC/NPC transplantations. However, the oligodendrocyte differentiation protocols for pluripotent stem cells are not so effective and contain undefined and animal-derived products, which are not to be recommended for clinical applications due to the safety risks of possible immunological reactions or pathogen cross-transfers.

In this thesis work the first aim was to characterize hESC surface proteins to find novel markers related to these cells that could be used for the purification of hESC-derived neural cell populations from tumorigenic pluripotent stem cells. In addition, the aim was to characterize the surface protein expression profiles of hESC-derived neural cell populations during the neuronal differentiation process and select appropriate markers for the sorting of pure neuronal populations with fluorescence activated cell sorter (FACS). The second aim was to compare the differences between hESC- and fetal CNS-derived NPCs to ascertain their specific characteristics of pluripotency and neural marker expression levels. In addition, different immunodeficient rodent host tissues were evaluated for reliability in the determination of hESC- and fetal CNS-derived NPCs tumorigenicity and safety. Furthermore, the hESC-derived NPCs were grafted into SCI-model rats to evaluate their effects for regeneration after injury. In the third project the aim was to develop a novel differentiation protocol for hESC-derived OPCs and oligodendrocytes, including optimization of purification step for disposing of pluripotent stem cells. Finally, in the fourth project the protocol for hESC-derived OPC production was optimized into xeno-free conditions aiming at clinical grade cell production.

According to the results from the first study a novel marker related to pluripotent stem cells, namely epithelial cell adhesion molecule EpCAM/CD326 was found. This marker was successfully used for the purification of hESC-derived neural cell populations from pluripotent stem cells with FACS. The results from the second study showed that in contrast to the fetal NPCs, the hESC-derived NPC populations

expressed pluripotency related markers at protein level, which indicated the presence of undifferentiated cells in the graft and made the cell population tumorigenic upon grafting. Also, the animal studies showed that there are remarkable differences between different tissues' permissiveness for tumor formations caused by undifferentiated hESCs. After grafting of hESC-derived NPCs in immunodeficient mice testicles and subcutaneous tissues, no tumors or teratomas were observed. By contrast, grafting of the same cells in immunodeficient rats' spinal cords resulted in tumor formations, and significant decline in the animals' locomotor function was detected. According to the third study, a novel differentiation and purification protocol for hESC-derived OPC production was developed using only human recombinant growth factors and extracellular matrix proteins to induce the differentiation. Also, for purification of produced hESC-derived OPCs a gentle sorting method for FACS with NG2-antibody was developed. In addition, the myelination capacity of hESC-derived OPCs was demonstrated in co-cultures with neurons. Finally, the results from the fourth study showed that it is possible to efficiently differentiate OPCs from pluripotent stem cells in totally xeno-free conditions, and the xeno-free medium also supported subculturing and differentiation of sorted $NG2^+$ OPCs.

These studies suggest that hESCs are a promising cell source for neuronal and oligodendroglial differentiation. These results also showed that it is very important to purify the differentiated populations of pluripotent stem cells prior grafting them to avoid tumor formations. Importantly, since remarkable differences were detected between different tissues' and animal species' propensity for tumor formations caused by hESCs, we concluded that there may be similar or even bigger differences in graft tumorigenicity between commonly used animal models and human patients. In conclusion, for the future treatment of SCI it will be important that pluripotent - or multipotent - stem cell-derived neural grafts are properly produced and characterized using reproducible and traceable manufacturing and characterization methods.

Tiivistelmä

Ihmisen alkion kantasolut ovat pluripotentteja soluja jotka voidaan erilaistaa kaikiksi kolmeksi eri alkiosolukerroksen solutyypiksi. Alkion kantasolujen tehokas kyky jakaantua ja erilaistua hermosoluiksi ja hermotukisoluiksi tekee niistä erinomaisen lähteen neuraalisten solusiirteiden tuotantoa varten. Näille solusiirteille on tarvetta erilaisten neurologisten sairauksien ja vaurioiden, kuten selkäydinvaurioiden, hoidossa. Multipotentteja ihmisen neuraalisia kantasoluja ja esiastesoluja voidaan myös eristää sikiön keskushermostosta; etuaivoista ja selkäytimestä. Tulevaisuudessa neuraalisten solujen siirto selkäydinvauriopotilaille voisi olla yksi vaihtoehtoinen hoitomuoto vaurioituneen kudoksen korjaamisessa ja liikuntakyvyn palauttamisessa potilaille. Tällä hetkellä olemassa olevat erilaistamismenetelmät neuraalisten solujen tuottamiseksi alkion kantasoluista usein kuitenkin johtavat heterogeenisten populaatioiden syntyyn, jotka sisältävät erilaistumattomia tai vain osittain erilaistuneita alkion kantasoluja tai kontrolloimattomasti jakaantuvia neuraalisia esiastesoluja. Nämä solut voivat aiheuttaa kasvainten muodostumista siirtojen yhteydessä. Lisäksi, vaikkakin on osoitettu että sikiön keskushermostosta eristetyt neuraaliset kantasolut ja niiden esiastesolut ovat toimivia selkäydinvaurion korjaamisessa eläinmalleissa, jotkut tutkimukset ovat esittäneet että nämä solut voivat aiheuttaa kasvaimia solusiirtojen yhteydessä. Tämän vuoksi on hyvin tärkeätä että neuraalisten solusiirteiden turvallisuus arvioidaan huolellisesti ja luotettavissa eläinmalleissa, jotta vältettäisiin vaaraa aiheuttavien solujen siirto potilaisiin tulevaisuudessa. Tähän liittyen on näytetty että erilaistuneemmat solupopulaatiot, kuten oligodendrosyyttien esiastesolut, ovat turvallisempia ja hyödyllisempiä vaurioituneen kudoksen korjaamisessa selkäytimessä verrattuna neuraalisiin kantasoluihin ja esiastesoluihin. Tällä hetkellä olemassa olevat menetelmät oligodendrosyyttien erilaistamiseksi pluripotenteista kantasoluista eivät kuitenkaan ole täysin tehokkaita ja sisältävät eläinperäisiä aineita, jotka eivät ole suositeltavia kliinisiä sovelluksia ajatellen, turvallisuusriskien kuten immunologisten reaktioiden tai patogeenikontaminaatioiden vuoksi.

Tämän väitöskirjan ensimmäisen osatyön tarkoituksena oli karakterisoida ihmisen alkion kantasolujen pintaproteiineja, jotta löydettäisiin uusia merkkiaineita, joita voitaisiin käyttää alkion kantasoluista erilaistettujen neuraalisten solupopulaatioiden puhdistamiseen kasvaimia aiheuttavista kantasoluista. Lisäksi, tavoitteena oli karakterisoida ihmisen alkion kantasoluista erilaistettujen neuraalisten solupopulaatioiden pintaproteiinien ilmentymisprofiilit erilaistuksen aikana ja valikoida sopivat merkkiaineet puhtaiden hermosolupopulaatioiden lajittelua varten virtaussytometrillä. Toisessa osatyössä tavoitteena oli verrata ihmisen alkion kantasoluista ja sikiön keskushermostosta-eristettyjen neuraalisten esiastesolujen välisiä eroja, selvittääksemme näiden solujen erityispiirteet monikykyisyyden ja neuraalisten pintaproteiinien ilmentymisen suhteen. Lisäksi arvioitiin erilaisten immunopuutteisten jyrsijöiden kudosten käytännöllisyyttä ihmisen alkion kantasolujen- ja sikiön keskushermostosta eristettyjen neuraalisten esiastesolujen turvallisuuden arvioinnissa. Selvittääksemme myös voitaisiinko näitä soluja käyttää tulevaisuudessa selkäydinvaurion korjaamisessa siirsimme näitä soluja selkäydinvauriomalliin rotille. Kolmannessa osatyössä oli tavoitteena kehittää uudenlainen erilaistamismenetelmä oligodendrosyyttien esiastesolujen ja

oligodendrosyyttien tuottamiseksi ihmisen alkion kantasoluista. Lopuksi, neljännessä osatyössä optimoitiin eläinperäisiä aineita sisältämätön menetelmä oligodendrosyyttien esiastesolujen erilaistamiseksi alkion kantasoluista, joka tähtää kliinisen tason solutuotantoon.

Tutkimustulosten perusteella ensimmäisessä osatyössä löydettiin uusi pintaproteiini pluripotenteille kantasoluille: epiteeli soluadheesio molekyyli/CD326. Tätä merkkiainetta käytettiin ihmisen alkion kantasoluista erilaistettujen neuraalisten solupopulaatioiden puhdistukseen erilaistumattomista kantasoluista virtaussytometrillä. Toisessa osatyössä näytettiin että toisin kuin sikiön keskushermostosta eristetyissä neuraalisissa esiastesoluissa ihmisen alkion kantasoluista erilaistetuissa neuraalisissa esiastesolupopulaatioissa ilmentyi pluripotenteille kantasoluille tyypillisiä proteiineita, joka viittaa siihen että nämä solupopulaatiot sisälsivät erilaistumattomia kantasoluja jotka voivat aiheuttaa kasvaimia solusiirtojen yhteydessä. Lisäksi tässä tutkimuksessa pystyttiin osoittamaan, että eri kudoksissa ihmisen alkion kantasolujen kyky muodostaa kasvaimia vaihteli huomattavasti. Ihmisen alkion kantasoluista erilaistettujen neuraalisten esiastesolujen siirto immunopuutteisten hiirten kiveksiin ja ihonalaiseen kudokseen ei aiheuttanut kasvainten muodostumista, toisin kuin immunopuutteisten rottien selkäytimeen siirretyt solut, jotka aiheuttivat kasvaimia ja johtivat huomattavaan liikuntakyvyn huononemiseen rotilla. Kolmannessa osatyössä kehitettiin uusi menetelmä ihmisen alkion kantasolujen erilaistamiseksi oligodendrosyyttien esiastesoluiksi. Tässä menetelmässä käytettiin vain ihmisen rekombinantti kasvutekijöitä ja soluvälitilan proteiineja erilaistuksen indusoimisessa. Lisäksi tässä työssä optimoitiin hellävarainen lajittelumenetelmä puhdistamaan erilaistetut oligodendrosyyttien esiastesolut virtaussytometrillä käyttäen NG2-vasta-ainetta. Tämä tutkimus osoitti myös, että erilaistuvat oligodendrosyyttien esiastesolut muodostivat myeleenituppea aksonien ympärille yhteisviljelmissä hermosolujen kanssa. Lopuksi, neljännessä osatyössä näytettiin, että käyttämällä eläinperäisiä aineita sisältämätöntä erilaistamismenetelmää pystyttiin erilaistamaan oligodendrosyyttien esiastesoluja ihmisen alkion kantasoluista, lisäksi eläinperäisiä materiaaleja sisältämätön kasvatusliuos ylläpiti lajiteltujen $NG2^+$ oligodendrosyyttien esiastesolujen jatkokasvatusta ja erilaistumista.

Nämä tutkimustulokset osoittivat, että ihmisen alkion kantasolut ovat hyvä solulähde hermosolujen ja oligodendrosyyttien erilaistamiselle. Kaikkein tärkeimpänä tuloksena pystyttiin osoittamaan, että on erittäin tärkeätä puhdistaa erilaistetut solupopulaatiot pluripotenteista kantasoluista ennen solujen siirtoa kohdekudoksiin, jotta kasvainten muodostumista voitaisiin välttää. Tutkimustulokset osoittivat myös, että alkion kantasolujen kyky muodostaa kasvaimia vaihteli huomattavasti eri kudosten ja eläinlajien välillä, jonka vuoksi päättelimme, että samankaltaisia tai jopa suurempia eroja siirteiden turvallisuudessa saattaa esiintyä yleisesti käytettyjen eläinmallien ja ihmisten välillä. Yhteenvetona voidaan todeta, että on erittäin tärkeätä että tulevaisuudessa selkäydinvaurion hoitoon käytettävät kantasoluista erilaistetut neuraaliset solusiirteet tuotetaan ja karakterisoidaan huolellisesti käyttäen toistettavia ja jäljitettäviä tuotanto- ja tutkimusmenetelmiä.

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Abbreviations

AA	Ascorbic acid
APC	Allophycocyanin
ASIA	American Spinal Injury Association
BDNF	Brain derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BLBP	Brain lipid binding protein
BMP	Bone morphogenetic protein
BrdU	5-bromo-2'-dehoxyuridine
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	complementary deoxyribonucleic acid
c-Myc	V-myc myelocytomatosis viral oncogene homolog
CNP	2',3'-cyclic nucleotide 3'-phosphohydrolase (CNPase)
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
Cy	Cyanine
DMEM/F12	Dulbecco's Modified Eagle Medium: nutrient mixture F-12
DNMT3b	DNA (cytosine-5-)-methyltransferase 3 beta
EB	Embryoid body
EpCAM	Epithelial cell adhesion molecule
ECM	Extra cellular matrix
EGF	Epidermal growth factor
ESC	Embryonic stem cell
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FDA	United States Food and Drug Administration
FP	Floor plate
FITC	Fluorescein iso thiocyanate isomer 1
GABA	Gamma-aminobutyric acid
GalC	Galactocerebroside
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDF3	Growth differentiation factor-3
GDNF	Glial cell derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GLAST	Glutamate transporter
GMP	Good manufacturing practice
GRM	Glial restriction medium
hESC	Human embryonic stem cell
HuNu	Human nuclei
HS	Human serum
HSC	Hematopoietic stem cell
ICM	Inner cell mass
IGF-1	Insulin like growth factor 1
i.p.	Intraperitoneal injection
iPS cell	Induced pluripotent stem cell
ISCI	International Stem Cell Initiative
ISSCR	International Society for Stem Cell Research

LIF	Leukemia inhibitor factor
MAG	Myelin associated glycoprotein
MAP-2	Microtubule associated protein 2
MBP	Myelin basic protein
MEA	Microelectrode array
MEF	Mouse embryonic fibroblast
MOG	Myelin Oligodendrocyte Glycoprotein
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
Myt1	Myelin transcription factor 1
Nanog	Nanog homeobox
NCAM	Neural cell adhesion molecule
NG2	Chondroitin Sulfate Proteoglycan
NDM	Neural differentiation medium
NSM	Neural stem cell medium
NT	Neurotrophin
Oct-4	Octamer-4, POU domain, class 5, transcription factor 1
OEG	Olfactory ensheathing glia
Olig1/2	Oligodendrocyte transcription factor 1/2
OPC	Oligodendrocyte precursor cell
Pax-6	Paired box gene 6
PBS	Phosphate buffered saline (d, Dulbecco's)
PDGF-AA	Platelet derived growth factor-aa
PDGFR	Platelet derived growth factor receptor
PE	Phycoerythrin
PFA	Paraformaldefyde
pM	Motor neuron progenitor
qRT-PCR	Quantitative real-time reverse transcriptase polymerase chain reaction
PLP	Proteolipid protein
RA	Retinoic acid
RP	Roofplate
RT-PCR	Reverse transcriptase polymerase chain reaction
s.c.	Subcutaneous injection
SCI	Spinal cord injury
SCID	Severe combined immunodeficiency
SHH	Sonic hedgehog
SOX	Sex determining region Y-box
SR	Serum replacement
SSEA	Stage-specific embryonic antigen
T3	3,3',5-triiodo-L-thyronine
TH	Thyroid hormone
XF	Xeno-free

Original publications

The present study is based on following articles, which are referred to in the text by their Roman numerals (**I-IV**):

I. **Sundberg M**., Jansson L., Ketolainen J., Pihlajamäki H., Skottman H., Suuronen R., Hovatta O., Narkilahti S. CD marker expression profiles of human embryonic stem cells and their neural derivatives determined using flow cytometric analysis reveals a novel CD marker for exclusion of pluripotent cells. Stem Cell Research (2009) 2: 113-124.

II. **Sundberg M.**, Andersson P-H., Åkesson E., Odeberg,J., Holmberg L., Inzunza J., Falci, S., Öhman J., 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 Transplantation (2010, *in press*).

III. **Sundberg M.**, Skottman H., Suuronen R., Narkilahti S. Production and isolation of $NG2^+$ oligodendrocyte precursors from human embryonic stem cells in defined serum-free medium. Stem Cell Research (2010) 2: 91-103.

IV. **Sundberg M.**, Skottman H., Shin S., Vemuri M., Suuronen R., Narkilahti S. A xeno-free culturing protocol for pluripotent stem cell-derived oligodendrocyte precursor cell production (*Submitted* 2010).

1. Introduction

Central nervous system (CNS) injuries, such as spinal cord injuries (SCI) are often severe; causing patients lifelong deficits due to varying degrees of paralysis. Today, there is no effective treatment for SCI. The recovery of CNS from trauma is restricted due to the limited potential of the CNS to regenerate lost or damaged neurons or glial cells, regenerate myelin producing oligodendrocytes to restore saltatory conduction and reform functional neural connections (Thuret et al., 2006). For this reason stem cell therapies have been considered to be one option for the treatment of SCI, which could be used for replacing the dead and injured cells and remyelinate damaged neurons and support hosts cells with delivering trophic factors (Coutts and Keirstead, 2008).

Stem cells provide tools for cellular replacement strategies due to their capacity to multiply *in vitro* as well as differentiate into desired cell populations. Human embryonic stem cells (hESC) especially are a usable source of stem cells since they are pluripotent cells which can be expanded in large amounts in cell culture in undifferentiated state and differentiated into all three germ layer cell types of human body; ectodermal, mesodermal, and endodermal cells (Thomson et al., 1998). By following established differentiation protocols these cells can be induced to differentiate for example into neuroectodermal cells; neural stem/ precursor cells (NSC/NPC) capable of differentiating into specialized neuronal and glial cells (Carpenter et al., 2001; Zhang et al., 2001). It has been shown in several animal models that these hESC-derived neural cells are a promising source for cellular transplants to be used in the treatment of various neurodegenerative injuries and diseases, reviewed by (Erceg et al., 2009). In addition, multipotent NSC/NPCs can also be derived from fetal CNS tissue with the ability for long term cell proliferation and differentiation capacity into neuronal and glial cell types both *in vitro* and *in vivo* (Carpenter et al., 1999; Sun et al., 2008; Vescovi et al., 1999b). These fetal CNS-derived NSC/NPCs can also improve the remyelination and enhance the locomotor recovery of SCI animals (Cummings et al., 2005; Hwang et al., 2009; Iwanami et al., 2005). However, since there are remarkable differences in tumorigenicity and level of neural commitment between hESC-derived neural cells and human fetal CNS-derived neural cells (Brederlau et al., 2006; Iwanami et al., 2005; Shin et al., 2007), more studies are needed to compare the features required of optimal neural graft for the treatment of SCI.

Furthermore, for the production of hESC-derived neural cell grafts safety issues need to be addressed, since undifferentiated pluripotent stem cells are capable of producing teratomas after grafting (Brederlau et al., 2006). For this reason it is very important to characterize the pluripotent stem cells and their neural derivates and determine specific markers that can be used for the purification of hESC-derived neural cell populations from pluripotent stem cells prior to grafting (Pruszak et al., 2009). Also, the use of proper animal models for evaluation of hESC-derived neural

cell grafts safety in terms of teratoma formation capacity needs to be addressed, since wide variation occurs between different organs and even between different animal strain permissiveness for teratoma formation caused by undifferentiated pluripotent stem cells (Hentze et al., 2009; Kishi et al., 2008).

For the treatment of SCI it has been shown that transplantation of stem cells that can induce functional recovery through remyelination of damaged neurons is conducive to recovery (Faulkner and Keirstead, 2005). Thus, several differentiation protocols have been developed to induce hESCs to differentiate into these myelinating cells of CNS; oligodendrocyte progenitor/precursor cells (OPCs) and oligodendrocytes (Hu et al., 2009a; Izrael et al., 2007; Kang et al., 2007; Nistor et al., 2005). In addition, these hESC-derived OPCs have been shown to enhance remyelination and restore locomotor function of SCI animals (Erceg et al., 2010a; Keirstead et al., 2005; Sharp et al., 2009). More importantly, it has been shown that differentiation of the pluripotent stem cells into more specialized neural cell types reduces the risk of teratoma formation (Cloutier et al., 2006; Erceg et al., 2010a). These results have made the hESC-derived OPCs an ideal cell population for graft development for future treatment of SCI patients, and the *Phase I* studies with these cells are ongoing in USA (Geron, www.geron.com, 22nd of October). Nevertheless, the production of hESC-derived OPCs and oligodendrocytes is far from the optimal (Hu et al., 2009a; Izrael et al., 2007; Nistor et al., 2005) and some of the protocols are not even reproducible in different laboratories.

To reduce the risks of stem cell-therapies, production of hESC-derived neural cell grafts for clinical use requires that the differentiation methods should be performed according to new EU directives, or United States Food and Drug Administration (FDA) guidelines, and according to good manufactoring practice (GMP) instructions, and countries individual regulations (Ahrlund-Richter et al., 2009; Skottman et al., 2007). Currently the existing differentiation methods for hESC-derived OPCs or oligodendrocytes include undefined xenogenic components (Hu et al., 2009a; Izrael et al., 2007; Nistor et al., 2005), which may increase graft rejection or pathogenic cross transfer between different species (Heiskanen et al., 2007; Martin et al., 2005). Although it is not mandatory for the differentiation and culturing conditions to be totally free of animal–derived products (xeno-free), the minimal use of animal derived components diminishes the risks of unidentified components' effect on grafted cells and diminishes the risk of rejection events in host tissue (Ahrlund-Richter et al., 2009; Unger et al., 2008). Thus, development of xeno-free differentiation protocols for hESC-derived OPC differentiation is important for the future safe cell graft production for treatment of SCI.

2. Review of the literature

2.1 Definition of stem cells

The most prominent features of stem cells are their ability for self-renewal and their ability to differentiate into different cell types. Stem cells are divided into several different classes according to their differentiation potential: totipotent stem cells, pluripotent stem cells, and multipotent stem cells, as presented in Figure 1. In addition, there are some other types of stem cells, which are oligopotent or unipotent stem cells e.g. lymphoid or myeloid stem cells, and muscle stem cells respectively. Totipotent stem cells are cells from fertilized egg or morula stage embryo, and these cells are able to build a whole organism, including the trophoectoderm (Mitalipov and Wolf, 2009). Pluripotent stem cells originate from the isolated inner cell mass of blastocyst stage embryo (Thomson et al., 1998) or are induced from the adult somatic cells by transfer of pluripotency inducing genes (Takahashi et al., 2007; Yu et al., 2007). Pluripotent stem cells are able to differentiate into all three germ layers of the human body including ectodermal, endodermal and mesodermal cell types. Multipotent stem cells are cells isolated from specific tissues, from fetuses or adults, and these cells are able to generate differentiated cells of the same tissue origin or closely related tissues (Mitalipov and Wolf, 2009).

2.2 Pluripotent stem cells

2.1.1 Human embryonic stem cells

The first stable human embryonic stem cell (hESC) lines were derived in 1998 by Thomson and colleagues (Thomson et al., 1998). hESC lines are derived from the isolated inner cell mass (ICM) of blastocyst stage embryos, which are plated on top of a supporting fibroblast cell layer to form a tight stem cell colony. These stem cells can also be cultured on top of different protein matrices which support cells' maintenance in undifferentiated stage (Mallon et al., 2006). hESCs have high telomerase activity level (Heins et al., 2004) and they have been shown to retain normal karyotype in prolonged culturing *in vitro* (Buzzard et al., 2004). Due to the stem cells' efficient self-renewal ability they can be further passaged and cultured in undifferentiating stage *in vitro* for prolonged periods of time (Reubinoff et al., 2000). Moreover, these cells can be differentiated *in vitro* into all three germ layer cells of the human body (Heins et al., 2004; Reubinoff et al., 2000). This differentiation potential has also been detected *in vivo* where transplantations of

undifferentiated hESCs have led to the formation of teratomas, a specific type of tumor, containing ectodermal, endodermal, and mesodermal cell types (Thomson et al., 1998). As such, these human cells are considered to be an excellent source for differentiation into different cell types, including various subtypes of neural cells. Nowadays hESCs are used for studying of human developmental processes as well as for further differentiation of specific cells for transplantation purposes.

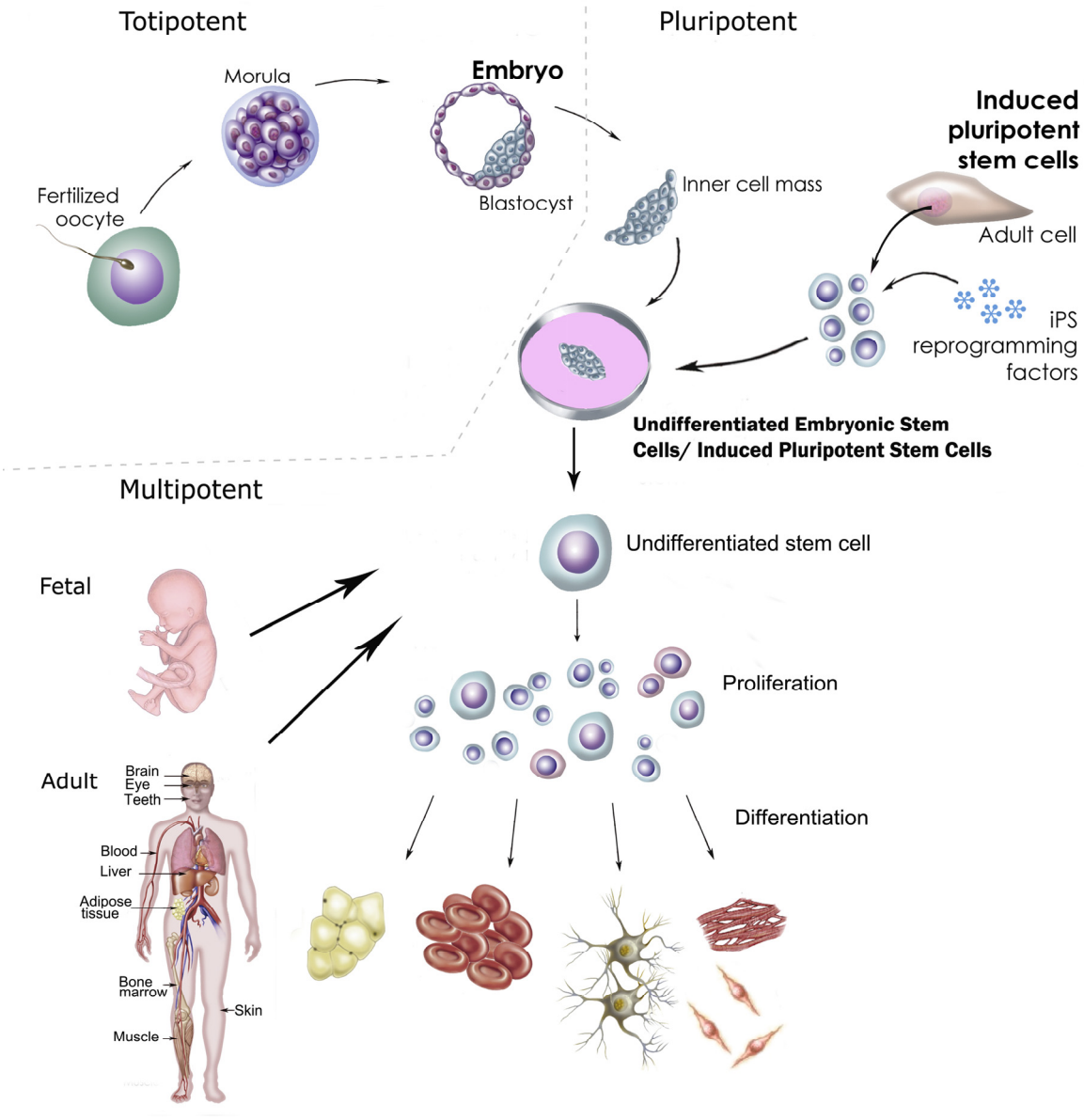

Figure 1. Sources of totipotent-, pluripotent-, and multipotent stem cells. Modified from a picture made by Bettina Mannerström. The original figure was made by Catherine Twomey for the National Academies, http://www.nationalacademiues.ord/stemcells.

2.1.2 Induced pluripotent stem cells

The first human induced pluripotent stem cell lines were first discovered by Yamanaka's research group (Takahashi et al., 2007). In this method somatic cells from adults were isolated and transduced with genes encoding transcription factors related to pluripotency *Oct4*, *Sox2*, *Klf4*, and *c-Myc*. The produced induced pluripotent stem cells (iPS cells) formed colonies on top of supporting fibroblast cell layers, and these colonies could be passaged *in vitro* for prolonged periods of time. Also, iPS cells resembled hESCs in their morphology, and gene and protein expressions, proliferation capacity, and telomerase activity (Takahashi et al., 2007). The pluripotency of iPS cells was confirmed by teratoma formation capacity and differentiation potential to produce cell types from ectodermal, endodermal, and mesodermal lineages (Takahashi and Yamanaka, 2006). However, since *c-Myc* is a powerful oncogene and increases the tumorigenicity of these cells the gene pattern to induce pluripotent stem cells has been modified to contain only genes for *Oct4*, *Sox2*, and *Klf4* (Nakagawa et al., 2008). Related to this, another research group published a protocol for iPS cell generation with four factors *Oct4*, *Sox2*, *Nanog*, and *Lin28* (Yu et al., 2007). These reprogramming technologies make it possible to isolate somatic cells from patients suffering from severe diseases and produce patient specific iPS cell lines, for example from patients suffering from Parkinson's disease (Soldner et al., 2009). The use of patient-specific iPS cells for differentiation allows to study disease processes *in vitro* and to test different drugs that can be used to prevent disease progression in patients, and in the future they may be utilized for graft production for clinical use.

2.2 Characterization of pluripotent stem cells

There are several genes and markers for the characterization of hESCs. Transcription factors such as Octamer-4 (Oct- 4) commonly called POU5F1 (POU class 5 homeobox 1) and Nanog, are expressed in undifferentiated hESCs and are related to pluripotency and tumor formation (Babaie et al., 2007; Chambers et al., 2003; Ding et al., 2008; Gopalan et al., 2009; Kehler et al., 2004; Looijenga et al., 2003; Mitsui et al., 2003; Rosner et al., 1990). These genes regulate the self-renewal of undifferentiated ESCs and it has been reported that knockdown of these genes promotes cell differentiation (Zaehres et al., 2005). Markers related to DNA methylation, such as DNA methyl transferase *DNMT3b*, are also associated with undifferentiated hESCs (Assou et al., 2009). It has been suggested that the higher expression levels of *DNMT3b* in hESCs compared to somatic cells implies a central role of this DNA methyltransferase in the control of the epigenome of these cells (Assou et al., 2009). In addition, pluripotent stem cells express transcription factor *Gdf3* (*Vgr1*) a member of the transforming growth factor beta (*TGFβ*) superfamily. It has been reported that this *Gdf3* expression is related to stem cells' ability to stay in undifferentiated stage and retain their differentiation capacity *in vitro* (Levine and Brivanlou, 2006). Other markers related to pluripotent stem cells are the stage-specific embryonic antigens -3 and -4 (SSEA-3, SSEA-4) and the tumor related antigens Tra-1-60 and Tra-1-81, which are expressed on the surface of human teratocarcinoma stem cells, human embryonic germ cells and hESCs (Andrews et

al., 1984; Badcock et al., 1999; Henderson et al., 2002; Kannagi et al., 1983; Koivisto et al., 2004; Lajer et al., 2002).

There are also Cluster of Differentiation (CD) markers related to pluripotent stem cells. CD-markers recognize adhesion molecules, receptors, and ligands, which are expressed on the surface of cells. This CD-molecule identification system was initially established for studying of leukocytes and different nomenclatures of antibodies used were gathered together by the Human Leukocyte Differentiation Antigens (HLDA) Workshop in 1984 (Bernard and Boumsell, 1984). After the establishment of first hESC-lines several of these CD-markers were associated with undifferentiated pluripotent hESCs; CD9, CD24, CD90, CD117, CD133, and CD135 (Assou et al., 2007; Bhattacharya et al., 2004; Carpenter et al., 2004). However, most of these CD-markers are not specific to pluripotent stem cells, and their expression can be found from several different subtypes of specialized cells, as shown in Table 1.

During the reprogramming of human fibroblasts into iPS cells it has been shown that the expression of TRA-1-60, *DNMT3b* and *REX1* can be used for the detection of iPS cells that are fully reprogrammed to pluripotent state, whereas alkaline phosphatase, SSEA-4, *GDF3*, *hTERT* and *NANOG* are not reliable markers for distinguishing partially reprogrammed cells from *bona fide* iPS cell lines (Chan et al., 2009b). Consistent with this, although there exist several different types of markers for the detection of cells' pluripotency, the specificity of these markers is not so straightforward. For example, the expressions of SSEA-3 and SSEA-4, are not critical for maintaining hESC pluripotency (Brimble et al., 2007). Previous meta-analysis by the International Stem Cell Initiative (ISCI) indicated that a significant proportion of hESC-lines do not express SSEA-3, and there is wide variation in the expressions of SSEA-3 and -4 between different hESC-lines (Adewumi et al., 2007). Furthermore, SSEA-4 is expressed by a subset of dorsal root ganglion cells (Holford et al., 1994) and in the early neuroepithelial cells in the developing forebrain (Barraud et al., 2007). In addition, expression of *Oct-4* has been detected in non-pluripotent NPCs isolated from adult rhesus macaque brain (Davis et al., 2006) and rat NSCs (Singh et al., 2009). *GDF3* expression has been detected in neural cells from the human hippocampus, cerebral cortex, and cerebellum (Hexige et al., 2005). Also, DNMTs have been shown to be present in NSCs during culturing, indicating that DNA methylation is not a process solely affecting the maintenance of pluripotent stem cells, but is also an active and dynamic process affecting renewal and maintenance of neural progenitor cells (Singh et al., 2009). Thus several of these markers related to pluripotent stem cells are also expressed in non-pluripotent cell types. For this reason, more studies for detection and screening of different molecules specific for pluripotent stem cells are needed.

Table 1. CD-marker expression in hESCs, NSC/NPCs, neural cells, mesenchymal stem cells (MSC), and hematopoietic stem cells (HSC) as reported in published literature. X human cells, ¤ animal cells.

Antigen	hESC	NSC/ NPC	Neural cells	MSC	HSC	References
CD4				x	x, ¤	(Di Ianni et al., 2008; Ivanisevic et al., 2010; Wineman et al., 1992)
CD9	x	x	x		¤	(Carpenter et al., 2004; Heinz et al., 2002; Klassen et al., 2001; Nakamura et al., 2001)
CD10				x		(Vogel et al., 2003)
CD13		x		x	x	(Portmann-Lanz et al., 2006; Taussig et al., 2005; Tocci and Forte, 2003; Vogel et al., 2003)
CD15		x			x	(Ivanisevic et al., 2010; Pruszak et al., 2009; Tong et al., 1993)
CD24	x	x	x			(Assou et al., 2007; Pruszak et al., 2009; Schwartz et al., 2003)
CD29	x	x	x	x		(Hall et al., 2006; Milner and Campbell, 2002; Portmann-Lanz et al., 2006; Schwartz et al., 2003; Tocci and Forte, 2003; Xu et al., 2001)
CD31				x	x	(Pranke et al., 2005; Tocci and Forte, 2003)
CD34		x		x	x	(D'Arena et al., 1998; Kaiser et al., 2007; Klassen et al., 2001; Pranke et al., 2005; Schwartz et al., 2003)
CD38			x		x	(Mizuguchi et al., 1995; Nilsson et al., 2002)
CD44		x	x	x	x	(Lian et al., 2007; Martins et al., 2009; Portmann-Lanz et al., 2006; Sackstein et al., 2008; Schwartz et al., 2003; Tocci and Forte, 2003; Vogel et al., 1992)
CD45		x	¤	x	x	(Dahlke et al., 2004; Martins et al., 2009; Nakahara et al., 2005; Schwartz et al., 2003; Tocci and Forte, 2003)
CD49b				x		(Katz et al., 2005; Tocci and Forte, 2003)
CD49d		¤		x	x	(Katz et al., 2005; Kil et al., 1998)
CD49f		¤	x			(Fortunel et al., 2003; Hall et al., 2006)
CD56		x	x	x	x	(Alvarnas et al., 2001; Lanier et al., 1989; Schwartz et al., 2003; Vogel et al., 2003)
CD59	x		x	x	x	(Storstein et al., 2004; Taylor and Johnson, 1996; Terstappen et al., 1992; Vedeler et al., 1994)
CD61				x	x	(Pranke et al., 2005; Vogel et al., 2003)
CD71				x		(Martins et al., 2009; Tocci and Forte, 2003)
CD90	x	x	x	x	x	(Draper et al., 2002; Hamann et al., 1980; Portmann-Lanz et al., 2006; Schwartz et al., 2003; Tocci and Forte, 2003; Vogel et al., 2003)
CD105				x	x,¤	(Chan et al., 2009a; Lian et al., 2007; Martins et al., 2009; Portmann-Lanz et al., 2006; Vogel et al., 2003)
CD106				x		(Nishihira et al. 2010)
CD117	x				x	(Carpenter et al., 2004; D'Arena et al., 1998; Pranke et al., 2005; Yin et al., 1997; Zambidis et al., 2005)
CD133	x	x	¤	x	x	(Bhatia, 2001; Carpenter et al., 2004; Katz et al., 2005; Martins et al., 2009; Schwartz et al., 2003; Tamaki et al., 2002; Uchida et al., 2000; Vogel et al., 2003; Yin et al., 1997; Zambidis et al., 2005)
CD135	x				¤	(Carpenter et al., 2004; Zeigler et al., 1994)
CD144					¤	(Kim et al., 2005)
CD146		x		x		(Astori et al., 2007; Pruszak et al., 2009)
CD166				x	¤	(Lian et al., 2007; Ohneda et al., 2001; Portmann-Lanz et al., 2006)
CD184		x	x	x	¤	(Mohle et al., 1998; Ni et al., 2004; Peng et al., 2007; Schwartz et al., 2003; Sordi et al., 2005; Wynn et al., 2004)
CD271		¤	x	x		(Anderson et al., 2004; Buhring et al., 2007; Casaccia-Bonnefil et al., 1999; Paratore et al., 2001; Pruszak et al., 2009)

2.3 Multipotent stem cells

2.3.1 Fetal stem cells

Fetal tissues represent a usable source for the isolation of specific types of stem cells which are multipotent and capable of differentiating into specialized cell types. Fetal stem cells can be isolated, for example, from CNS, blood, bone marrow, heart, liver, lung, spleen, and pancreas (Campagnoli et al., 2001; in 't Anker et al., 2003a; Mimeault et al., 2007; Vescovi et al., 1999b). Also, stem cells can be isolated from isolated amniotic membrane and fluid, which is an extra embryonic tissue (in 't Anker et al., 2003b).

Human NSCs can be isolated from the developing fetal brain, or from the spinal cord (Vescovi et al., 1999b). NSC lines have been derived from these regions either by inducing sphere formation in suspension cultures (Carpenter et al., 1999; Vescovi et al., 1999b) or in adherent monolayers (Sun et al., 2008). Single cells have also been isolated from dissociated tissue by sorting with different CD-markers and allowing these cells to form neurospheres (Uchida et al., 2000). The markers used for isolation: CD34, CD45, and CD133 (Uchida et al., 2000) and other CD-markers related to NSC/NPCs are listed in Table 1.

Fetal NSCs are able to proliferate efficiently and differentiate into neuronal, astrocytic, and oligodendrocyte cells (Carpenter et al., 1999; Vescovi et al., 1999b). Upon grafting these cells survive, migrate and differentiate appropriately (Cummings et al., 2005; Kelly et al., 2004; McBride et al., 2004; Vescovi et al., 1999a; Wu et al., 2002), also delivering neuroprotective factors (Behrstock et al., 2006; Ebert and Svendsen, 2005; Lee et al., 2007b; Suzuki et al., 2007). Therefore, at the present time fetal NSCs are studied intensively and tested in experimental transplantations for the treatment of SCI, where survival and proliferation of these cells has been detected (Akesson et al., 2007; Emgard et al., 2009), in addition, with the enhancement of locomotor recovery of injured animals (Cummings et al., 2005; Hwang et al., 2009; Iwanami et al., 2005; Salazar et al., 2010). Furthermore, human fetal neural cells have been grafted in animal models of intracerebral hemorrhage (Jeong et al., 2003; Lee et al., 2007b) and ischemic stroke (Chu et al., 2004; Darsalia et al., 2007), where the cells survived and improved the animals' recovery. In addition, clinical trials for the treatment of Parkinson's disease have been performed with fetal solid mesencephalic tissue or cell suspensions (Freed et al., 2001; Mendez et al., 2008). Previously these cells have also been considered to be safe upon grafting in the CNS, but recently one study showed that human fetal NSCs led to massive tumor formation for a patient suffering from ataxia telangiectasia (Amariglio et al., 2009). However, such tumor formations have newer been associated with fetal NSC/NPCs grafted in animals with SCI, intracerebral hemorrhage, or Huntington's disease (Cummings et al., 2005; Jeong et al., 2003; McBride et al., 2004; Salazar et al., 2010). As such, human fetal neural cells are considered to be a promising cell source for future transplantation therapies for several neurological disorders. Although, in addition to some safety risks, the poor availability of fetuses, the existence of ethical questions, and country-specific regulations may limit the usefulness of these cells in several countries.

2.3.2 Adult stem cells

From adult tissues like brain tissue, adipose tissue, bone marrow, placenta and cord blood it is possible to isolate several different types of multipotent stem cells (Mimeault et al., 2007; Pincus et al., 1998). For example; neural, mesenchymal or hematopoietic stem cells can be isolated from adults, and the various CD-markers associated with these cell types are described in Table 1.

Historically, it has been thought that the adult mammalian CNS is vulnerable to injuries and deficits due its poor ability to regenerate damaged neural tissue. This assumption was based on the evidence that neurogenesis completes shortly after birth. Nowadays, the plasticity of brain tissue is more evident and it is known that neurogenesis occurs in the adult nervous system (Eriksson et al., 1998; Gould et al., 1990; Gould et al., 1992; Gould and Tanapat, 1997; Gould et al., 1999; Kempermann et al., 1997; Kempermann et al., 2004; Kuhn et al., 1996; van Praag et al., 1999). Subsequently rodent NPCs have been isolated from two different neurogenic regions of the adult brain: I) the subventricular zone of the lateral ventricles and II) the subgranular zone of the dentate gyrus (Kempermann et al., 2006), as well as human NPCs (Eriksson et al., 1998; Pincus et al., 1998). However, the adult NPCs have less migration capacity than embryonic NPCs, they require extensive growth factor treatment for sustaining, and their capacity to proliferate and differentiate decreases after prolonged periods of culturing *in vitro* (Doetsch et al., 1999; Morshead et al., 1998; Wright et al., 2006).

Although tissue specific stem cells have a restricted ability to differentiate into any other tissue cell lineages, it has been reported that human adipose tissue derived mesenchymal stem cells (MSCs) can be differentiated into neural and glial cells in certain culturing conditions (Jang et al., 2010). In addition, it has been shown that human umbilical cord blood-derived mesenchymal stromal cells have the capacity to differentiate into cells with an oligodendrocyte phenotype (Luo et al., 2010), although the ability of these cells to myelinate axons has not been tested. Also, a conditioned medium from MSCs has been reported to induce oligodendrogenesis of adult neural progenitors (Rivera et al., 2008). According to earlier studies, MSCs have also been shown to enhance the recovery of animals with SCIs (Cizkova et al., 2006; Lee et al., 2007c; Yang et al., 2008), and they have been used in transplantation studies for several neurological disorders; stroke, multiple sclerosis, Huntington's disease, and Parkinson's disease (Kim and de Vellis, 2009). In spite of this, the exact mechanisms of MSCs function after transplantations are still unclear and animals' follow-up times after cell graftings have been quite short, leaving doubts about the actual benefits of cells for regeneration. Nevertheless there is a great potential for the MSCs to respond for several differentiation cues, act as inducer for neural cells to differentiate via secreting trophic factors, and also improve the outcomes of neurologically deficient animals.

In the adult olfactory epithelium there exists a population of basal stem cells, which are responsible for the continuing replacement of neurons and their supporting cells (Leung et al., 2007; Moran et al., 1982; Schwob, 2002). These cells can be isolated from the dissociated adult olfactory neuroepithelium from cadavers or patients undergoing endoscopic nasal sinus surgery (Barnett et al., 2000; Roisen et al., 2001; Winstead et al., 2005). It has been shown that these neurosphere forming cells have

the potential to differentiate into neurons or glial cells depending on environmental signals (Zhang et al., 2005). In addition, olfactory ensheathing glia (OEG) cells from olfactory bulb have been widely used in different acute and chronic models of rodent SCI (Bartolomei and Greer, 2000; Franklin and Barnett, 2000; Ramon-Cueto and Valverde, 1995). OEGs have been shown to promote regeneration of injured dorsal root axons into the spinal cords of adult rodents (Ramon-cueto 1994), as well as regeneration of descending and ascending tracts after severe injuries, like complete spinal transaction (Santos-Benito and Ramon-Cueto, 2003). More importantly, some studies have shown that OEGs can promote the recovery of sensimotor functions in injured animals after transplantation of acute or subacute stage of the injury (Santos-Benito and Ramon-Cueto, 2003). Thus, adult neural cells can be isolated and differentiated from several sources and in the future they have wide potential to be utilized in the treatment of neurological disorders.

2.4 Neural differentiation of pluripotent stem cells

2.4.1 Neural induction and Neuronal differentiation

There are several protocols to induce pluripotent stem cells to differentiate into neuroectodermal cell lineages including neuronal and glial cells. In the initial establishment of hESC-lines these cells' capacity to spontaneously differentiate into neural cells was detected when cells were continuously cultured on top of the same fibroblast cell layers for several weeks (Reubinoff et al., 2000). Also, neural differentiation of hESCs has been induced by derivation of embryoid bodies (EB) together with different kinds of substances; growth factors, their blockers, and different morphogens (Carpenter et al., 2001; Reubinoff et al., 2001). These studies described for the first time the differentiation potential of hESC-derived neural cells, via detection of specialized cell types of neuronal, astrocytic, and oligodendrocytic cells shown in Figure 2 (Carpenter et al., 2001; Reubinoff et al., 2001).

Recently introduced genetic programming technologies have also made it possible to convert mouse embryonic and postnatal fibroblasts into functional neurons *in vitro* using the transcription factors *Ascl1*, *Brn2*, and *Myt1l* (Vierbuchen et al., 2010). These induced neuronal cells expressed multiple neuron-specific markers, they formed functional synapses, and were able to generate action potentials (Vierbuchen et al., 2010). In the future, such technologies may enable the production of patient specific neurons and overcome the risks of tumorigenesis related to hESC- or iPS cell-derived neural cells use in regenerative medicine.

Currently most of the hESCs or iPS cells neural differentiation methods utilize either EB formation or stromal cell lines (MS5 and PA6), as well as combinations of suspension and adherent culturing (Barberi et al., 2003; Carpenter et al., 2001; Erceg et al., 2009; Hargus et al., 2010; Kawasaki et al., 2002; Nat et al., 2007;

Zhang et al., 2001). Formatted EBs are three dimensional cell clusters (Itskovitz-Eldor et al., 2000), and their spontaneous differentiation results in only a few percent of neural cells. Thus, to induce neural differentiation EBs require stimulation by different growth factors and medium supplements, such as retinoic acid (RA) or basic fibroblast growth factor (bFGF) (Carpenter et al., 2001; Zhang et al., 2001). To induce neural differentiation directly in suspension cultures hESC colonies can be dissected out from the fibroblast cell layers and allowed to form free floating cell aggregates that, in neural induction medium, form neural precursor containing neurospheres (Itsykson et al., 2005; Nat et al., 2007). These neural precursor cells can further be differentiated into specialized cell types, for example into motor neurons (Itsykson et al., 2005; Li et al., 2008). In adherent cultures the differentiating cells form neural tube-like structures containing neuroepithelial cells, called rosettes (Erceg et al., 2008; Gerrard et al., 2005). To induce neuronal specification of these cells, they can be cultured on top of a specific growth platform like Matrigel or cell dishes coated with collagen, fibronectin, laminin, Poly-D-Lysine (PDL), or vitronectin (Erceg et al., 2008; Ma et al., 2008). Additionally, it has been shown that laminin is a key extracellular matrix (ECM) molecule to enhance hESCs neural progenitor cells generation, expansion and differentiation into neurons (Ma et al., 2008). Furthermore, stromal cell lines (MS5 and PA6) have been used for neural induction of hESCs and iPS cells. These stromal cell lines are mouse pre-adipocytic mesenchymal cells, which were originally developed for the maintenance of purified hematopoietic stem cells (Itoh et al., 1989). Currently, co-cultures of hESCs or iPS cells with mouse stromal cell lines are routinely used for differentiation of dopaminergic (DA) neurons (Hargus et al., 2010; Park et al., 2005; Perrier et al., 2004; Vazin et al., 2008).

Regarding the factors affecting CNS development it has been shown that RA has an important role, for example, in posteriorizing CNS tissue (Durston et al., 1989; Li et al., 2005) and also in neural induction of ESC (Li et al., 2005; Zhang, 2006), where RA signaling causes a very strong level of caudalization (Irioka et al., 2005). In addition, it has been shown that bFGF induces neural specification of hESCs and blocking of bFGF signaling inhibits neural induction (LaVaute et al., 2009). bFGF has caudalizing activity in early neural induction (Kudoh et al., 2002) and in the presence of RA and Sonic hedgehog (Shh) it differentiates hESCs into motor neurons (Li et al., 2005). In addition, it has been shown that several other growth factors, inhibitors, and vitamins enhance the neuronal differentiation of hESCs, such as ascorbic acid (AA), brain derived growth factor (BDNF), FGF8, glial derived neurotrophic growth factor (GDNF), and noggin (Gerrard et al., 2005).

The early neural differentiation can be detected with expressions of Pax6 and Sox1, which are transcription factors affecting early neuroectodermal development (Li et al., 2005). In addition the expressions of nestin, musashi, A2B5 and neural cell adhesion molecule (NCAM) has been detected during early neural differentiation of hESCs (Gerrard et al., 2005; Nat et al., 2007; Reubinoff et al., 2001; Zhang et al., 2001). After further differentiation of neural precursors into specialized neuronal cell types, micro-tubule associated protein -2 (MAP-2)-, synaptophysin-, glutamic acid decarboxylase-, gamma-aminobutyric acid-, or tyrosine hydroxylase-positive neurons can be detected (Gerrard et al., 2005; Nat et al., 2007; Reubinoff et al., 2001; Zhang et al., 2001). However, the hESC-derived neuronal cell populations are usually not homogeneous after differentiation and may contain astrocytes

positive for glial fibrillary acidic protein (GFAP), or a few oligodendrocytes positive for O4, or even a few pluripotent stem cells expressing Oct-4 (Brederlau et al., 2006; Gerrard et al., 2005; Nat et al., 2007; Reubinoff et al., 2001; Zhang et al., 2001).

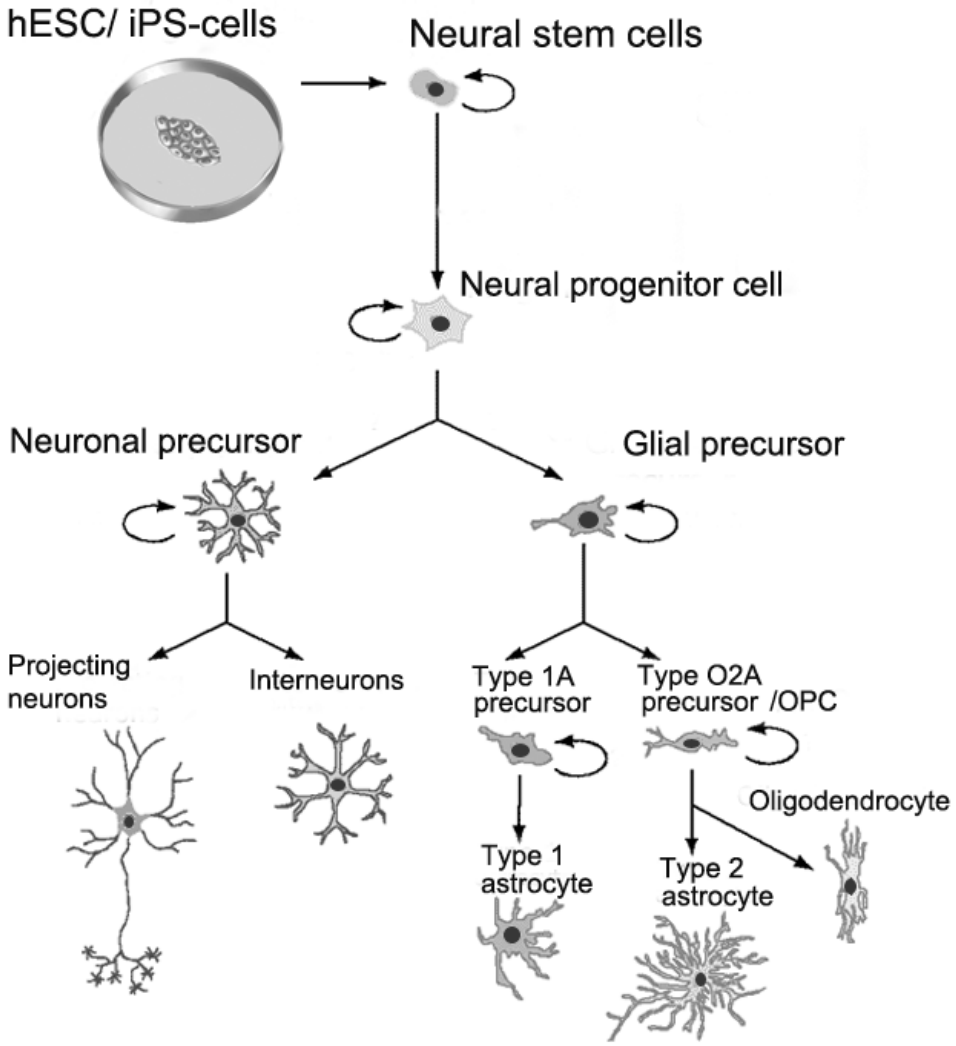

Figure 2. Neural differentiation of pluripotent stem cells results in the formation of neuronal and glial precursors and maturation of specialized neurons and glial cell types. Figure modified from a picture originally made by Bob Crimi (www. http://cmbi.bjmu.edu.cn/cmbidata/stem/specific/specific03.htm, 20th of October 2010).

Currently several protocols are available for the differentiation of specialized neural subtypes from pluripotent stem cells (Erceg et al., 2009). The ability of these cell populations to regenerate damaged CNS tissue has been studied intensively in animal models of SCI, ischemic brain injury, multiple sclerosis, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (Goldman and Windrem, 2006; Hargus et al., 2010; Lindvall and Kokaia, 2006). Thus, these pluripotent stem cell-derived neural cell populations are a great resource for studying human CNS development stages *in vitro* and their regenerative capacities *in vivo*, while also offering a great opportunity to expand the treatment options for several neurological disorders of the CNS in the future.

2.4.2 Oligodendrocyte differentiation

2.4.2.1 Transcription factors and growth factors involved in oligodendrocyte differentiation

Human OPCs arise from the ventral developing spinal cord near the floorplate (FP) and it has been reported that the human oligodendroglial differentiation process *in vitro* and *in vivo* follows the same pathways as rodent cells, although it takes a longer time (Hajihosseini et al., 1996; Zhang et al., 2000). Developmental studies with animals have shown that transcription factor Shh, derived from the FP and the notochord, is essential in activating several transcription factors involved in OPC development (Poncet et al., 1996), shown in Figure 3. For example, expressions of Olig1 and Olig2 are induced by Shh stimulation in dose dependent mechanism (Lu et al., 2002). Similarly, Shh also affects ventral spinal Olig2-expressing progenitors and OPCs developmental processes from hESC-derived neuroepithelia (Hu et al., 2009a). In addition, it has been shown that the transcription factors Olig2 and Olig1, specifically affect the motor neuron progenitor (pMN) domain in the neural tube (Fu et al., 2002; Lu et al., 2002), leading to the rise of the OPCs. These factors are also important for oligodendrocyte specification in the spinal cord (Lu et al., 2002). The secretion of bone morphogenetic proteins (BMPs), from the neural tube roofplate (RP, Figure 3), affects developmental process by inhibiting oligodendrocyte development in animals, (Cate et al., 2010; Gomes et al., 2003; Mekki-Dauriac et al., 2002), as well as in human cells (Izrael et al., 2007).

Sox10 is a transcription factor expressed throughout oligodendrocyte development according to animal studies (Stolt et al., 2002). Furthermore, Sox10 is important for neural crest and peripheral nervous system development (Pevny and Placzek, 2005), and is especially involved in OPC differentiation from hESCs (Izrael et al., 2007; Nistor et al., 2005). This transcription factor belongs to the SRY-related HMG-box family (SOX) which regulates embryonic neural development and cell fate decision (Pevny and Placzek, 2005). During OPC development the coexpression of Sox10 with Sox9 is important for transcriptional regulator of platelet-derived growth factor receptor α (PDGFRα) (Finzsch et al., 2008). OPCs also express Nkx2.2, which is an important transcription factor affecting the differentiation of precursor cells into mature oligodendrocytes (Qi et al., 2001), also stimulating the expression of genes involved in myelination (Qi et al., 2001). Furthermore, expression of Nkx6.2/GTX has been detected in mature oligodendrocytes and regulates expressions of myelin basic protein (*MBP*) and proteolipid protein (*PLP*) (Awatramani et al., 1997; Cai et al., 2010). The notch signaling pathway affects the fate decision of OPCs and oligodendrocytes, since it has been shown that notch ligands Jagged and Delta can inhibit oligodendrocyte differentiation (Wang et al., 1998a). Figure 3 summarizes the transcriptional network affecting oligodendrocyte development based on studies with animal cells.

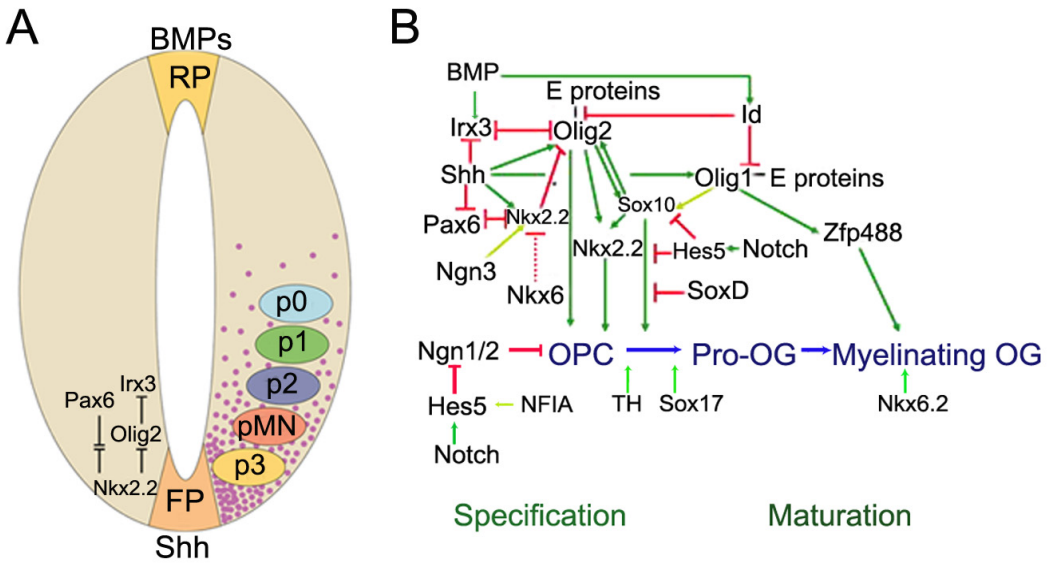

Figure 3. Patterning of the neural tube (A) and transcriptional network affecting oligodendrocytes development (B). Interneuron subtypes are derived from p0-p3 domains, whereas motor neurons arise from pMN, as well as oligodendrocytes at a later stage of development (A). Shh, BMP, and Notch signaling pathways are important for oligodendrocyte specification in pMN domain (red line inhibits; green lines promote the transcription factors, B). Figure modified from original pictures made by David Rowitch (Nature Reviews, 2004) and by Danette Nicolay (Glia, 2007). Reprinted with permission from Macmillan Publishers Ltd: Nature Reviews, (Rowitch, 2004), http://www.nature.com/nrn/index.html and John wiley and Sons: Glia, (Nicolay et al., 2007), www.interscience.wiley.com.

Several growth factors are involved in the differentiation process of OPCs to oligodendrocytes, as shown in Figure 4. One of these factors is the PDGF-AA, which acts via its receptors (PDGFRα). When OPCs start to differentiate the expression of this receptor is downregulated (Hart et al., 1989; Zhang et al., 2000). PDGF-AA signaling affects survival and proliferation of OPCs, especially in combination with bFGF (McKinnon et al., 1990). bFGF functions via four different tyrosine kinase receptors, and three of these are expressed in OPCs in varying levels, depending on the maturation stage of the cells (Bansal et al., 1996). Interestingly, it has been reported that when hESC-derived OPCs were stimulated with bFGF their differentiation was inhibited and cells were maintained in proliferating state (Hu et al., 2009a). Also, insulin and insulin-like growth factor (IGF-1) can regulate oligodendrocyte development through their receptors, which are present in OPCs (Baron-Van Evercooren et al., 1991). IGF-1 promotes OPC proliferation, increases the number of matured oligodendrocytes in rodent cell cultures (McMorris and Dubois-Dalcq, 1988), and affects the myelination capacity of oligodendrocytes (Carson et al., 1993). Moreover, ciliary neurotrophic factor (CNTF) and leukemia inhibitor factor (LIF) have both been shown to enhance the generation of oligodendrocytes in cultures of dividing rodent oligodendrocyte progenitors (Mayer et al., 1994) as well as in cultures of human oligodendroglial cells (Zhang et al., 2000). CNTF and LIF also promote oligodendrocyte maturation and survival together with other growth factors; such as bFGF and IGF-1 (Mayer et al., 1994). OPCs also express the ErbB family of tyrosine kinase receptors and neuregulin/glial growth factor (GGF) acts via these receptors by promoting the

survival of pro-oligodendrocytes and inhibiting the differentiation of oligodendrocytes (Canoll et al., 1996). It has been also detected that neurotrophins (NT) affects OPC survival and proliferation capacity (Barres et al., 1994b). Co-stimulation with NT-3 and PDGF-AA especially promotes the expansion of OPCs and has been shown to affect the timing of oligodendrocyte development (Barres et al., 1994b), similarly as stimulation with thyroid hormone (TH) (Barres et al., 1994a). Laminin receptor alpha6beta1 integrin is expressed on oligodendrocytes, and enhances the sensitivity of oligodendrocytes to the survival effect of other growth factors (Frost et al., 1999). Laminins also regulate CNS myelination by interacting with both integrin receptors and dystroglycan receptors which are both expressed on oligodendrocytes (Colognato et al., 2007).

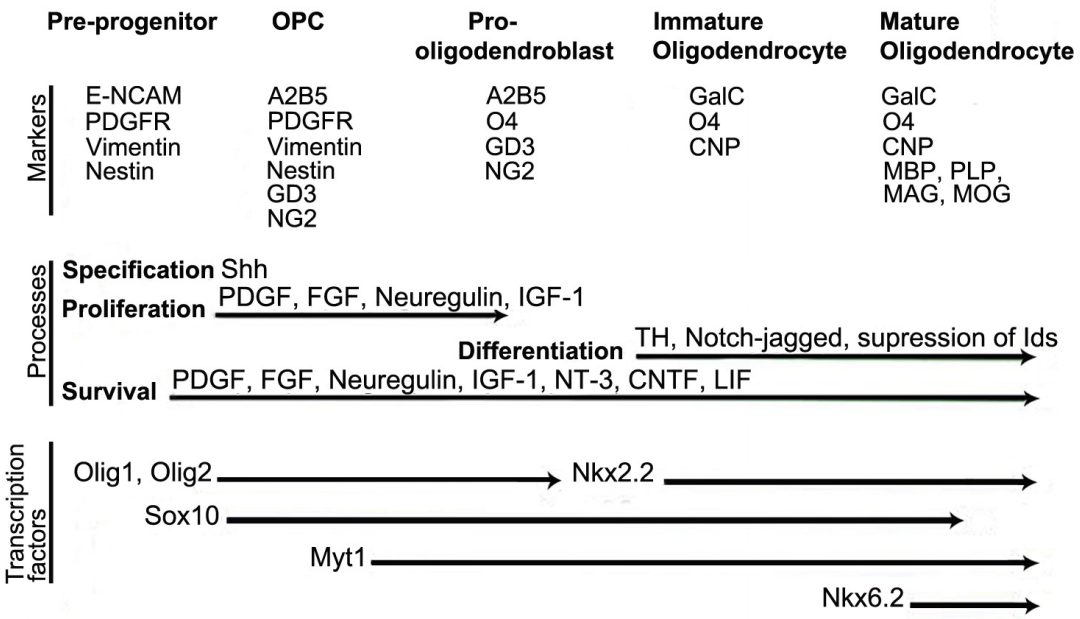

Figure 4. Schematic presentation of markers, growth factors, and transcription factors affecting on oligodendrocyte development and differentiation. Figure modified from a picture made by Judith Grinspan (Journal of Neuropathology and Experimental Neurology, 2002).

During the oligodendrocyte differentiation process, OPC populations can be identified with antibodies against: A2B5, GD3, NG2, and PDGFRα (Baumann and Pham-Dinh, 2001), shown in Figure 4. A2B5 is expressed in both neurons and glial cells, but usually it is used to monitor the maturation of oligodendrocyte progenitors, since it is downregulated during differentiation (Farrer and Quarles, 1999). GD3, ganglioside 3, is expressed in oligodendrocytes' progenitors (Hardy and Reynolds, 1991), like NG2, chondroitin sulfate proteoglycan, which is usually coexpressed together with PDGFRα (Nishiyama et al., 1996). When OPCs start to differentiate they begin to express a sulfated surface antigen known as pro-oligodendroblast antigen, which can be detected with O4-antibody (Bansal et al., 1992). When the final maturation occurs, the differentiated oligodendrocytes express galactocerebroside on the cell surfaces (Raff et al., 1978) and cell proliferation decreases. In mature oligodendrocytes monoclonal antibodies against RIP-antigen detect oligodendrocyte processes and myelin sheaths (Friedman et al., 1989).

Furthermore, the oligodendrocyte structural formation of myelin sheaths is dependent on the expression of MBP and PLP, which are expressed after the appearance of galactocerebrosides (Grinspan et al., 1998; Shine et al., 1992). Previously it was thought that PLP only offers structural support for myelin membrane, but after further investigation it has been shown that it also regulates the differentiation processes (Yang and Skoff, 1997). There are also many other markers related to the characterization of oligodendrocytes' myelination, like 2',3'-Cyclic nucleotide-3'-phosphohydrolase (CNP) (Baumann and Pham-Dinh, 2001), myelin-associated glycoprotein (MAG) (Filbin, 1996), and myelin/oligodendrocyte glycoprotein (MOG) (Solly et al., 1996), as presented in Figure 4.

2.4.2.2 Oligodendrocyte differentiation of human embryonic stem cells

Currently there are at least six different methods for OPC and oligodendrocyte differentiation from hESCs (Hu et al., 2009a; Hu et al., 2009b; Izrael et al., 2007; Kang et al., 2007; Nistor et al., 2005; Zhang et al., 2006b). The production of hESC-derived OPCs is complicated and requires several different growth factors and inducers to be accomplished, and most attempts to directly transfer mouse ES cell differentiation methods (Brustle et al., 1999; Glaser et al., 2005; Zhang et al., 2006a) to hESC cultures have been time consuming and required lot of optimization (Izrael et al., 2007).

The first method describing hESC-derived oligodendrocyte precursor production and differentiation was published in 2005 by Nistor and colleagues (Nistor et al., 2005). In this method neurospheres were formed in the presence of RA stimulation in glial restriction medium (GRM) containing epidermal growth factor (EGF). The authors described the formation of yellow spheres, which contained precursor cells highly positive for Pax6, A2B5, Olig1, Sox10, NG2, and PDGFRα. This protocol included a selection step where, after 28 days of differentiation, yellow spheres were plated down onto Matrigel-coated dishes to attach yellow spheres and other cells were shaken out. After 42 days of differentiation, cell aggregates were re-plated on poly-l-lysine and human laminin-coated cell dishes to differentiate into oligodendrocytes, which were positive for; GalC, RIP, and O4 (Nistor et al., 2005). Importantly, the authors showed *in vivo* that these cells could integrate into brain tissue and myelinate axons from *shiverer* mutant mouse brain (Nistor et al., 2005). Regardless of this, one obstacle with this protocol reliability is the fact that this method could not be replicated with the same efficiency in other laboratories using other hESC-lines. Recently, Erceg and colleagues (2010a) showed that according to this protocol differentiated hESC-derived OPC population was not as pure as previously described (Nistor et al., 2005). Instead, the differentiated population had multipotent differentiation capacity producing of neuronal and astrocytic cells in addition to oligodendrocytes after *in vitro* analysis and after grafting onto the injured spinal cords. (Erceg et al., 2010a). In addition, Izrael and colleagues modified this Nistor's method by adding noggin, BMP-antagonist, to the differentiation medium (Izrael et al., 2007). It is known that RA treatment induces expression of BMPs which inhibit the differentiation of cells to oligodendrocytes (Mehler et al., 2000), and the addition of noggin prevented the upregulation of BMP expressions in cells (Izrael et al., 2007). In this study the addition of noggin

increased the formation of mature oligodendrocytes with ramified cell branches and expression of MBP. Especially, after transplantation of noggin treated cells into MBP-deficient mouse brain the myelination of axons could be detected (Izrael et al., 2007).

In the most recent study Hu and colleagues presented the novel effects of different growth factors for hESC-derived oligodendrocyte differentiation (Hu et al., 2009a). In this method the hESC-derived OPCs were differentiated in the glial differentiation medium modified with the additions of RA, Shh, bFGF, T3, cAMP, EGF, noggin, PDGF-AA, IGF-1, and NT3 (Hu et al., 2009a). The addition of Shh activated expression of transcription factors *Olig2*, *Nkx2.2* and *Sox10*, which are important for OPC development. In addition, transition of pre-OPCs to OPCs was inhibited by bFGF stimulation, which repressed Shh-dependent co-expression of Olig2 and Nkx2.2 (Hu et al., 2009a). In total the differentiation process took 3 months, during which time Olig2 and Nkx2.2 co-expressing as well as NG2 and PDGFRα co-expressing cells and Sox10-positive cells appeared in cultures. Moreover, during additional 2-4 weeks differentiation in glial medium containing PDGF-AA, IGF-1 and NT3 O4-positive cells were detected and after prolonged 6-8 weeks' differentiation MBP-positive branched oligodendrocytes were detected (Hu et al., 2009a). In this study the functionality of these cells was shown in *shiverer* mouse brain, where electron microscopy analyses revealed the formation of multilayered myelin sheaths around axons 3 months after transplantation (Hu et al., 2009a). The authors underlined also the importance of using a longer differentiation protocol for hESCs compared to earlier studies (Izrael et al., 2007; Nistor et al., 2005), since it resembles the longer developmental processes in humans compared to rodents.

Another method published in 2007 by Kang and colleagues have described hESC-derived oligodendrocyte differentiation through EB formation step (Kang et al., 2007). Since the EB formation is mainly an uncontrolled event the starting material for OPC differentiation is not ideal compared to the method of Hu and colleagues, where the initial neural differentiation was induced in adherent cultures for 10 days in a neural induction medium (Hu et al., 2009a). According to Kang's method the EBs were formatted in the hESC medium without bFGF for 4 days and then they were plated down onto Matrigel-coated culture dishes for 5 days in ITS (insulin, transferrin, selenium) medium, and cultured thereafter in the presence of bFGF and N2 supplement for 5 days to form and amplify neural precursors (Kang et al., 2007). Furthermore, the formatted neural rosettes were isolated and replated onto Matrigel in the presence of EGF, PDGF-AA, and bFGF and cultured for 8 days. After this stage developed OPCs expressed markers such as PDGFR, A2B5, NG2 (Kang et al., 2007). For the final maturation of oligodendrocytes the authors described the withdrawal of all the growth factors from the medium and addition of 3,3'5-triiodo-l-thyroine (T3) to induce differentiation. In this final stage cells expressed markers such as O4, O1, and MBP, and the total differentiation process took 46 days (Kang et al., 2007). Moreover, in contrast to the other three methods (Hu et al., 2009a; Izrael et al., 2007; Nistor et al., 2005), in this study the myelination capacity of hESC-derived oligodendrocytes was confirmed *in vitro* in co-cultures with neurons isolated from the fetal rat brain, and no *in vivo* studies were performed (Kang et al., 2007).

Thus, there are major differences among existing protocols for oligodendrocyte differentiation from hESCs, and some of the protocols have not been reliably developed or are not reproducible in other laboratories. Taken together, although more optimization is needed for efficient OPC and oligodendrocyte production from hESCs, these existing methods offer a great opportunity for studies of the human oligodendroglial developmental processes and the signaling pathways affecting differentiation. In the future, these human OPCs may also offer an opportunity to expand treatment options for patients suffering demyelination disorders of the CNS.

2.5 Safety aspects concerning human embryonic stem cell-derived neural grafts for clinical use

2.5.1 Tumorigenicity of human embryonic stem cell-derived neural grafts

Upon grafting pluripotent stem cells have ability to form teratomas *in vivo* (Thomson et al., 1998). Thus, when considering the use of the pluripotent stem cells as a source for neural cell graft production, the propensity of these cells to form teratomas or other kind of tumors after differentiation should be thoroughly investigated.

It has been shown that when differentiating specific neural cell populations from pluripotent stem cells the cell grafts produced include cells capable of forming teratomas in brain tissue after grafting (Brederlau et al., 2006; Roy et al., 2006; Schulz et al., 2004; Sonntag et al., 2007). According to these studies the reason for this teratoma formation was the fact that all the cells did not receive or respond to the differentiation guides of cell culturing conditions (Brederlau et al., 2006; Roy et al., 2006; Schulz et al., 2004; Sonntag et al., 2007). One of the studies showed that low levels of Oct-4, SSEA-4 and Tra-1-60-positive cells were detected in some of the neural grafts which caused teratomas (Sonntag et al., 2007). Furthermore, it has been shown that neural differentiation of hESCs may result in the appearance of two morphologically distinct populations of cells that are either pluri- or multipotent-progenitors able to proliferate in an uncontrolled manner, thus increasing the occurrence of teratoma formation (Roy et al., 2006; Sonntag et al., 2007). In addition, the differentiation time crucially affects the propensity for teratoma formation (Brederlau et al., 2006). Rats that received hESC-derived neural cells after 16 days of neural differentiation developed teratomas, whereas rats that received cells differentiated for 20-23 days did not show teratoma formation after 13 weeks of follow-up (Brederlau et al., 2006). These results permit the conclusion that even though pluripotent hESCs are guided to differentiate into neuronal cells, the populations produced are often heterogeneous and contain unwanted cell types (Brederlau et al., 2006; Roy et al., 2006; Sonntag et al., 2007). Thus, the composition of hESC-derived neural grafts affects the outcome of transplantations and if the cell graft contains pluripotent cells the occurrence of teratoma formation increases (Sonntag et al., 2007). Whereas prolonged differentiation time can decrease the risk of teratoma formation (Brederlau et al., 2006) likewise an efficient

method for the specialization of cells (Cho et al., 2008; Erceg et al., 2010b; Geeta et al., 2008; Lee et al., 2007a).

When evaluating the safety of hESC-derived grafts it should be noted that there are differences between the permissiveness of the different target organs for teratoma formation caused by undifferentiated hESCs (Hentze et al., 2009). It has been shown that intramuscular injections of undifferentiated hESCs led to the most convenient and reproducible teratoma formation, and subcutaneously transplanted pluripotent cells did not form teratomas without co-injecting them in the presence of Matrigel in mice (Hentze et al., 2009). In line with this, a recent study showed that when the mouse ESC clumps were injected subcutaneously, the number of cells required for teratoma formation was greater than intramuscular injected cell numbers (Kishi et al., 2008). In addition, it has been shown that the number of hESCs required for teratoma formation correlates negatively with the time required for teratoma formation (Hentze et al., 2009). When 20 colonies of undifferentiated hESCs were injected into immunodeficient mice hind leg teratomas were detected after 5 weeks of follow-up, whereas two colonies of undifferentiated hESCs required 12 weeks for teratoma formation (Hentze et al., 2009). Thus, if a hESC-derived differentiated cell population contains only a few pluripotent cells, it is obvious that the time for them to form teratomas may be several months. Moreover, these results raise the question about the safety of produced hESC-derived neural cells grafts, since in most of the transplantation studies the follow-up times are quite short, showing only the first few weeks of animals' functional recovery (Keirstead et al., 2005), but omitting the prolonged follow-up times required for reliable evaluation of graft safety. Furthermore, it has been reported that, in addition to the number of ESCs required for teratoma formation, graft composition also affects the propensity for teratoma formation; more single ESCs are required for teratoma formation than clumps of ESCs (Kishi et al., 2008), stating that in the cell clumps the tumorigenic cells survive better and are able to proliferate effectively after transplantation. This study also showed that the mouse strains had crucial effects on the appearance of teratomas. When mouse ESC-derived progenitor cell grafts containing pluripotent stem cells were transplanted into the lower limb muscle of nonobese diabetic/ severe combined immunodeficiency (NOD/SCID) or knock-out NOD/SCIDγc^{null} mice, teratomas developed in all NOD/SCIDγc^{null} mice, but only in two out of five NOD/SCID mice (Kishi et al., 2008). The reason for this was the differences in the immunological responses of these two mouse strains, since NOD/SCIDγc^{null} mice lack NK as well as B and T cells, whereas NK cells are present in NOD/SCID mice affecting their lower susceptibility to hESC induced teratoma formation (Kishi et al., 2008). Thus, it is important to be aware of these immunological differences also when evaluatin the safety of hESC-derived cell populations in different immunodeficient animal models.

Taken together, it is very important to be aware of hESC-derived neural cell populations' risks for tumor formation if any pluripotent cells or NSCs/NPCs proliferating in an uncontrolled manner have remained in the graft. Moreover, proper animal models with prolonged follow-up times are needed for evaluating graft safety.

2.5.2 Purification of human neural subpopulations

Currently most of the differentiation methods developed for pluripotent stem cells and techniques for human neural cell culturing aim at the efficient and large scale production of highly pure human neural cells for transplantation purposes. However, often the differentiated neural cell populations are heterogeneous containing several impurities like undifferentiated or partly differentiated neural cells causing tumors after grafting (Brederlau et al., 2006; Roy et al., 2006; Schulz et al., 2004; Sonntag et al., 2007). For this reason, efficient purification steps like fluorescence activated cell sorting (FACS), magnetic activated cell sorting (MACS), genetic modifications, suicide genes, killing agents and antibiotic selection, have been developed for isolating specific cell types or eliminating of the harmful tumorigenic pluripotent stem cells from the cell population prior to grafting (Herzenberg et al., 1976; Herzenberg et al., 2002; Knoepfler, 2009; Miltenyi et al., 1990; Pruszak et al., 2009; Roy et al., 2000a; Roy et al., 2000b; Wang et al., 1998b). Nevertheless, the use of some of these methods for clinical use is still restricted for reasons of safety, because it is possible that genetic modifications increase the risks of cell tumorigenicity, killing agents may be toxic causing side effects for patients, and antibiotic selections specificity may not be high enough for the elimination of solely pluripotent stem cells (Knoepfler, 2009). Thus, utilization of FACS and MACS may be preferable in clinical use.

In recent decades in the hematology and immunology research field, flow cytometric analysis with surface CD-markers and sorting methods, e.g. FACS and MACS, have been routinely used for the separation of specific subpopulations of high purity, also for clinical-grade cell production (Baumgarth and Roederer, 2000; Horan et al., 1986; Lowdell and Theocharous, 1997; Tricot et al., 1998; Vose et al., 2001). In the neurological research field the usefulness of these markers and methods have also been noted. For example, CD133-positive selection has been used in the isolation of human CNS stem cells from fetal brain tissue using FACS (Tamaki et al., 2002; Uchida et al., 2000). These cells' ability to survive, differentiate and integrate into the tissue has been tested in the brains of immunodeficient newborn mice (Tamaki et al., 2002; Uchida et al., 2000), as well as in the spinal cords of immunodeficient mice (Salazar et al., 2010). In addition, it has been shown that CD-markers can be used to study the differentiation stages of hESC-derived neural cell populations and for the sorting of live neural cells for transplantation purposes (Pruszak et al., 2007; Pruszak et al., 2009).

The stem cell sorting strategies with FACS or MACS are based on the distinct surface molecule expressions of cells and use of specific markers for different subpopulations of cells (BD Biosciences www.bdbiosciences.com, Milteney www.miltenyibiotec.com). Commonly used CD-markers are expressed in several different types of cells and their lineage specificity is not so straightforward, see Table 1 for example. As such panel of different CD-markers and combinatorial sorting with several markers results in a more reliable way to produce specific safe cell grafts, especially in cases where undifferentiated pluripotent stem cells are used as the initial source for differentiation (Pruszak et al., 2009). It has been reported that the use of different combinations of positive and negative selection and different intensities for the expression of CD-markers enables isolation of different subpopulations from hESC-derived neural cells (Pruszak et al., 2009). According to

this study, NSC population was sorted with $CD15^{+}/CD24^{LO}/CD29^{HI}$ selection, neural crest cell populations were sorted with $CD15^{-}/CD24^{LO}/CD29^{HI}$ selection, and neuronal cell populations were isolated with $CD15^{-}/CD24^{HI}/CD29^{LO}$ selection (Pruszak et al., 2009). Upon grafting these sorted NSCs resulted in the formation of neuroepithelial rosette-like tumors, sorted neural crest cells did not show neuroepithelial rosettes, but tumors formed with highly proliferative cells and myosin-positive cells (Pruszak et al., 2009). In contrast to this, the sorted neuronally differentiated population did not form any tumors after grafting, and cells integrated into host brain tissue by extending neuronal processes in brain (Pruszak et al., 2009). These results suggest that with combinatorial labeling sorted hESC-derived neuronally differentiated cells can be used for transplantation therapies without risk of tumor formation.

One option for conducting sorting without surface molecule labeling is the use of gene-expression based sorting with fluorescence-tagged promoters (Wang et al., 1998b), which are activated when certain progenitor specific genes are activated during neural development (Chalfie et al., 1994). Although, it is doubtfull if these techniques will lead to clinical use due the safety risks of cells genetic manipulations, the purified populations can be further studied and characterized *in vitro* from the developmental point of view. For example, by using green fluorescent protein (GFP) sorting method, neuronal progenitors have been isolated from the hippocampus and ventricular zone of adult brain tissue (Roy et al., 2000a; Roy et al., 2000b). In these studies the human adult brain tissues were obtained from adult patients undergoing therapeutic lobectomy and after dissociation of the tissues cultured cells were transduced with either Promoter/Talpha1:hGFP or Enhancer/nestin:GFP plasmid DNA, and sorted with FACS according to neural promoter-driven GFP expression (Roy et al., 2000a; Roy et al., 2000b). In addition, neural stem cells have been sorted and enriched from dissociated human fetal brain cells by their expression of nestin and musashi and co-activation of GFP expression. Prior to sorting these fetal cells were infected with adenoviruses bearing the gene for GFP under the control of nestin or musashi1 promoters (Keyoung et al., 2001). Moreover, oligodendrocytes have been isolated from dissociated human adult brain tissues by their expression of 2'3'-cyclic nucleotide 3'-phosphodiesterase 2 (CNP2) tagged on GFP and sorting with FACS (Roy et al., 1999). Furthermore, several studies have described the gene transfection and promoter specific selection and sorting for ESC-derived neuronal cells (Wernig et al., 2002) for the production of purified ESC-derived dopamine neurons (Hedlund et al., 2008), or for ESC-derived motorneurons (Ozdinler and Macklis, 2006), as well as for hESC-derived Synapsin-GFP expressing neuronally differentiated cells (Pruszak et al., 2007).

There are thus several methods and markers available for the purification of neural cell populations derived from different origins; CNS tissue or pluripotent stem cells, as shown in Figure 5. Since stem cell based therapies are studied intensively for treatment options for neurological disorders, it will be very important to use these methods to achieve purified neural cell populations that are free from tumorigenic cells.

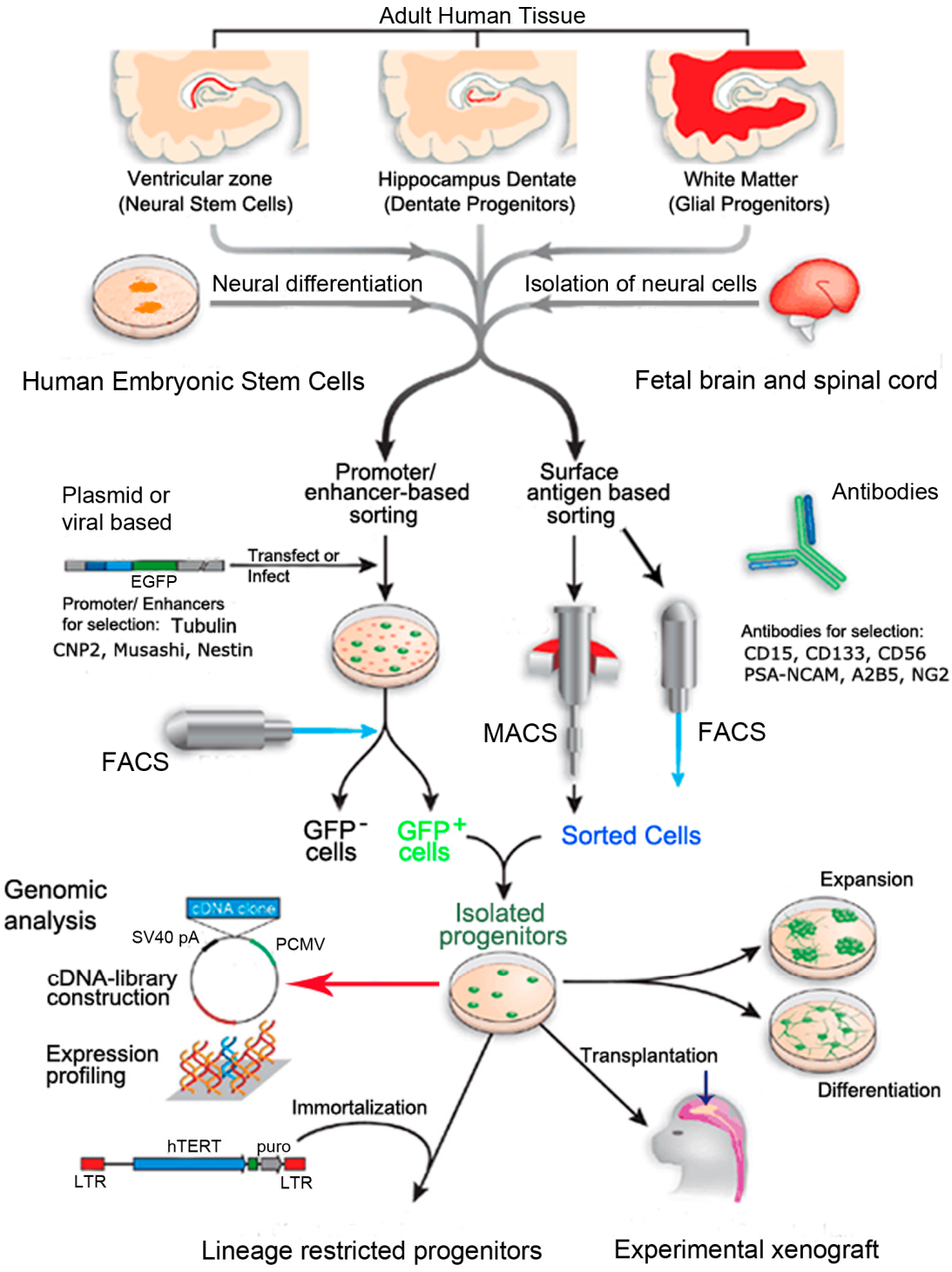

Figure 5. Purification methods for neural cell populations derived from hESCs, adult or fetal CNS tissues. Gene transfection based sorting methods and surface antibody labeling based sorting methods performed either with FACS or MACS are presented. Figure modified from a picture originally made by Katie Ris (Nature Biotechnology, 2005), reprinted with permission from Macmillan Publishers Ltd: Nature Biotechnology (Goldman, 2005), originally published in http://www.nature.com/naturebiotechnology.

2.5.3 Xeno-free culturing and neural differentiation methods for human embryonic stem cells

Different hESC lines were previously directly or indirectly exposed to animal substances during derivation or maintenance of cells *in vitro* (Skottman et al., 2006; Wang and Sun, 2005). Some of these hESC lines have been cultured on mice fibroblast feeder cells and hESC colonies passaged enzymatically (Adewumi et al., 2007). Mouse fibroblasts may affect the differentiation capacity and contaminate hESC cultures with animal substances, leading to changes in carbohydrate chains on the cell surfaces and increasing the rejection prospects of these cells (Heiskanen et al., 2007; Martin et al., 2005). Moreover, enzymatic passaging of hESCs can increase abnormal mutations in chromosomes (Thomson et al., 2008), and may cause differences between gene and protein expression levels on the cells (Adewumi et al., 2007). To overcome these problems, several research groups have used human foreskin fibroblasts for hESC derivation (Inzunza et al., 2005; Richards et al., 2002; Richards et al., 2003), culture medium supplemented with human serum or serum replacement to avoid use of animal serum, and mechanical cell passaging to avoid the use of enzymes (Ellerstrom et al., 2006; Inzunza et al., 2005; Rajala et al., 2007). Human fibroblasts, also known as 'feeder cells', support colony formation of hESCs and secretes several factors to the hESC cultures which affect cell maintenance in undifferentiated stage (Richards et al., 2002; Richards et al., 2003). However, currently developed feeder-free cultures for hESCs will make it easier to define the exact components and soluble factors affecting hESCs and help standardization of the culturing conditions (Akopian et al., 2010; Ludwig et al., 2006). To gain totally xeno-free hESC lines several research groups have also reported the removal of animal components from the ICM isolation procedure (Ellerstrom et al., 2006; Heins et al., 2004; Suss-Toby et al., 2004). There are also xeno-free culturing media for the maintenance and propagation of undifferentiated pluripotent stem cells (Akopian et al., 2010; Ludwig et al., 2006). In addition, a recently published method described new xeno-free medium for the maintenance and culturing of undifferentiated hESCs and multipotent stem cells which aims at clinical grade cell production (Rajala et al., 2010). These advances increase the safety of stem cell products and add to the potential of these cells to be used in transplantation therapies for human patients.

At the moment, most of the differentiation protocols for hESC-derived neural- or oligodendroglial cells still contain animal derived substances. For example, for the production of hESC-derived dopaminergic neurons, mouse stromal cell lines (MS5 and PA6) have been used to induce differentiation (Park et al., 2005; Sonntag et al., 2007). The use of these cells for clinical therapy is not advisable since stromal cell lines may increase the risk of pathogen cross-transfer and changes on cell surface molecules, as previously shown with hESCs and mouse feeders (Heiskanen et al., 2007; Martin et al., 2005). Regarding xenogenic cell culturing conditions, glial restriction medium (GRM) used for hESC-derived oligodendrocyte progenitor cell production for clinical grade cell grafting for human patients (Geron, www.geron.com) contains B27-supplement with animal derived substances (Nistor et al., 2005). It has been shown that several differences occurs between different manufacturers' B27-supplements (Chen et al., 2008), and most of these differences were due to variability in the quality of different sources of bovine serum albumin (BSA) included in the supplement (Chen et al., 2008). It has therefore been hard to

define the exact medium compositions, and this has increased the variability of culture conditions between different laboratories affecting the replicability of the differentiation methods (Chen et al., 2008). In addition, BSA may have different effects on human cells compared to human serum albumin. Furthermore, one of the commonly used coating matrices utilized for hESCs differentiation is the commercial xenogenic gel product Matrigel (BD Biosciences). Matrigel is originally derived from mouse sarcomagelatinous protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells, and the exact composition of the factors included in this product has not been identified. As an example, Matrigel is used for hESC-derived oligodendrocyte progenitor cell differentiation (Nistor et al., 2005) for clinical grade cell grafting (Geron, www.geron.com). Although, at the moment the regulations of FDA do not require the differentiation protocols to be totally xeno-free, the use of xenogenic undefined products for clinical grade cell production is not advisable due the existing risks of pathogen contaminations or risks of rejection events after grafting.

2.5.4 Criteria for optimal human embryonic stem cell-derived clinical grade cell grafts

For the future establishment of stem cell therapies it is good to be aware of the differences between properly designed cell therapies and illegal and ineffective 'stem cell therapies' offered by unauthorized and unprofessional people at great expense and with unsafe cell grafts. For this reason the International Society for Stem Cell Research (ISSCR) has published Guidelines for the Clinical Translation of Stem Cells (December 3, 2008) to control stem cell based medical innovations and to protect patients. In keeping with this, prior to entering into clinics investigators, research facilities, and clinics should be aware of the general regulations related to stem cell therapies, such as conduct of cell processing and manufacturing processes in a quality controlled environment, in addition to controlled preclinical studies, and clinical research, and followed by the FDA or the European Medicines Agency (EMEA) guidelines, or country specific regulations (ISSCR, 2008).

To ensure that the stem cell-based products are developed in the safest possible way, GMP practices are utilized for cell derivation and culturing processes (Ahrlund-Richter et al., 2009). The basic principle of GMP is to ensure that all the different work steps are performed under quality controlled conditions followed by established validated protocols, documented properly, and that the methods used are reliable, reproducible, and performed with defined and traceable raw materials (Ahrlund-Richter et al., 2009). The quality management also includes environmental monitoring, error management, validation of equipment, performation of auditions, and training of personnel. GMP production in case of hESCs covers meeting the donor eligibility rules and testing, processing, screening, storing, distribution, labeling, packaging, and recovery of hESCs (Carpenter et al., 2009). Table 2 summarizes the main criteria for producing of optimal transplantable OPC population from pluripotent stem cells. The aim of this quality control throughout stem cell processing is to ensure that unsafe products are not used in clinical trials or treatments. However, the first ongoing pluripotent stem cell based clinical trial with

hESC-derived oligodendroglial progenitors is conducted with hESCs derived in non-GMP conditions with xeno materials (Geron, www.geron.com). The results of this trial will in the future show how precisely these GMP regulations and xeno-free differentiation methods need be followed. In the meantime, several research groups have derived 'clinical-grade' hESC-lines, which are characterized and produced under GMP conditions to accomplish safety regulations and to produce best possible cell material for clinical transplantations (Crook et al., 2007).

Table 2. Criteria for production of optimal transplantable OPC population from pluripotent stem cells. (www.geron.com, Carpenter et al., 2009).

Manufacturing process	**Characterization of cells**	**Preclinical animal studies**

GMP production
• clean room facilities
• validated and controlled processes
• comprehensive documentation and records of processes
 	
Selection and conduction of OPC differentiation with stem cell line derived and characterized under GMP conditions
 	
Defined differentiation protocol
• repeatable method
• defined, traceable and quality checked medium ingredients and growth factors
• purification methods
• (xeno-free products)
 	
Cryopreservation
• sustain viability
• sustain stability
	Use of qualified characterization methods
	
	Determination of cells
	• viability
	• proliferation capacity
	• differentiation efficiency
	• gene- and protein-expression profiles
	  ◦ absence of pluripotency-, endo- and mesodermal markers
	  ◦ presence of OPC/ oligodendrocyte specific markers
	• safety
	  ◦ sterility
	  ◦ virus free
	  ◦ mycoplasma free
	  ◦ no chromosomal mutations
		Safety
		• absence of teratomas
		• absence of cysts
		• absence of toxic effects
		• absence of allodynia
		• absence of immunological rejection events
		
		Efficiency
		Determination of cells
		• survival
		• differentiation capacity
		• myelination capacity
		• ability to improve animals locomotor function
		• ability for permanent regeneration

2.6 Neural cell therapy for treatment of spinal cord injury

2.6.1 Spinal cord injury

SCIs are heterogeneous in nature, and their severity depends largely on the level of the spinal cord at which the injury occurred (http://www.asia-spinalinjury.org/, 8th of October). Cervical injury causes quadriplegia, thoracic injuries cause paraplegia, and lumbar injuries cause paraplegia. The incidences of SCI varies approximately between 10 and 71 per million per year around the world. In 2008 the incidence of SCI in Australia was 14 per million, the Canadian Paraplegic Association reports 35 per million, China 65 per million, the United States 40 per million (based on National Spinal Cord Injury database), in the United Kingdom 10-15 per million (Royal College of Physicians, 2008), in Germany 18 per million, and in Finland 20 per million per year (http: //www.kaypahoito.fi, 24th of November 2010). The average age of injured patients varies around 30-35 years, and most of the patients are male (> 80%). There are several causes for acute spinal cord injuries: motor vehicle accidents (44%), acts of violence (24%), falls (22%), sports (8%), and others (2%) (http://www.sci-recovery.org/, 24th of November 2010). Currently existing treatment options are administration of high doses of methylprednisolone, which is a steroid, or administration of tirilazad, which is a potent lipid preoxidation inhibitor (Bracken et al., 1997). The monosialoganglioside compound GM1 (also called Sygen) has also been tested on Phase *III* trial for treatment of SCI (Hall and Springer, 2004). Although clinical data have shown that the benefits from these drugs are modest for the patients, it has been suggested that methylprednisolone and tirilazad could be used for minimizing the secondary effects of SCI when administered within 8 hours of injury (Bracken et al., 1997), but the benefits of GM1 administrations are still controversial (Hall and Springer, 2004). Very often the treatment is mainly supportive, including surgical and medical treatment as well as physical therapy. The outcome of physical therapy is to help to preserve some range of motion. Curative treatments for SCI are currently not available.

The SCIs can be divided into four different types: 1) contusion injury, which leads to central hematomyelia also causing possible syringomyelia, 2) solid cord injury, 3) cord maceration, where spinal cord morphology is severely distorted, and 4) cord lacerations (Hulsebosch, 2002). After SCI there follow three different phases: the acute, secondary, and chronic phase (Tator, 1995; Tator, 1996; Tator, 1998).The acute phase includes 1) hemorrhage, ischemia, and hypoxia, 2) secretion of pro-inflammatory cytokines and glutamate cytotoxicity, 3) secretion of free radicals and nitric oxide, as well as activation of proteases (Okano, 2010). On the cellular level this leads to death of neural cells in the spinal cord, where neurons, oligodendrocytes, astrocytes, and their precursors die (Horky et al., 2006). After the acute phase, one of the secondary processes in spinal cord is the death of oligodendrocytes through apoptosis and loss of myelin sheaths around axons (Crowe et al., 1997). In line with this, activation of caspases has been detected at the lesion site of injury (Knoblach et al., 2005). Another significant event after SCI is the activation of inflammatory responses, such as activation of macrophages, microglia, neutrophils, and T-cells, which can have either destructive or reparative

effects on the spinal cord after injury (Jones et al., 2005). Furthermore, although the circumferential white matter is not usually affected by the injury, cavities and cysts which form interrupt ascending and descending axonal tracts. Also, the formation of glial scar after SCI disturbs the rearrangement of spared axons. This irreversible event involves reactive astrocytes, glial progenitors, microglia, macrophages (Jones et al., 2002; Jones et al., 2003), fibroblasts, and Schwann cells (Bruce et al., 2000; Guest et al., 2005). The scar which forms is impenetrable and contains several molecular inhibitors for axon growth (Fawcett, 2006; Silver and Miller, 2004). Sometimes in chronic responses the injury area expands progressively over several months or even years because of continuing cell apoptosis. These events lead to the loss of normal axonal function, cyst formation, and permanent hyperexcitability develops in cells that are unable to connect to each other properly, resulting in development of pain syndromes (Hulsebosch, 2002). Although, some spontaneous repair mechanisms do occur after SCI, including proliferation of cells that can differentiate into glial cells (Azari et al., 2005; Horky et al., 2006; Yamamoto et al., 2001; Yang et al., 2006), the detected recovery of function is usually modest and incomplete. Hence much research effort has been invested in developing stem cell based therapies for SCI.

2.6.2 Grafting of human neural precursors and oligodendrocyte precursors after spinal cord injury

The stem cell based therapies developed for SCI aims to 1) replace damaged or dead cells, 2) re-connect injured neurons, 3) secret neurotrophic factors to support survival and regeneration of remaining endogenous cells, 4) re-myelinate damaged axons, or 5) induce neovascularization (Coutts and Keirstead, 2008).

Transplantations of NSCs/NPCs derived from hESCs, fetal and adult tissues have been studied in rodents and non-human primates after SCI (Coutts and Keirstead, 2008; Cummings et al., 2005; Iwanami et al., 2005). It has been shown that human NPCs can differentiate after grafting and migrate in the injured spinal cords, diminish the size of the lesion, and also promote the remyelination and improve the functional recovery of animals with SCI (Coutts and Keirstead, 2008; Cummings et al., 2005; Iwanami et al., 2005; Salazar et al., 2010). For example, $CD133^{+}$ human fetal stem cells isolated from CNS can be grown as neurospheres and these cells survive, migrate, and express differentiation markers for neurons and oligodendrocytes after long-term engraftment in SCI immunodeficient mice (Cummings et al., 2005). According to this study, also locomotor recovery was detected in injured animals, in addition to the occurrence of remyelination in both in the SCI-model and in the myelin-deficient mice (Cummings et al., 2005). Related to this, it has been reported that $CD133^{+}/CD24^{LO}$ human fetal NSCs can survive, differentiate, and improve locomotor function after grafting onto an early chronic SCI microenvironment of immunodeficient mice (Salazar et al., 2010). In addition, after cell graftings no tumor formations were detected in spinal cords in 16 weeks of follow-up studies (Cummings et al., 2005; Salazar et al., 2010). Human fetal CNS isolated NSCs/NPCs have also been studied for the treatment of cervical contusion injury of adult marmosets (Iwanami et al., 2005). According to this study the cells survived 8 weeks after transplantation into the spinal cord, diminishing the cavity

site of the injury and differentiating into neurons, oligodendrocytes, and astrocytes. The transplanted cells also improved the locomotor recovery of the injured animals (Iwanami et al., 2005). Although these results are quite promising, the statistical significance of this study is not so strong, since the number of animals used in this study was only 10, of which 5 received the cell grafts and 5 were included in the control group (Iwanami et al., 2005). Recently, it has also been reported that transplantation of genetically modified Olig2-expressing immortalized human NSCs increased the volume of spared white matter and reduced the cavity volume after SCI (Hwang et al., 2009). This study showed that, although the genetic modifications increased NSCs proliferation capacity, the grafted cells were non-tumorigenic (Hwang et al., 2009), but this is debatable, since the animals' follow-up time after transplantation was only 7 weeks (Hwang et al., 2009). Nevertheless, the Olig2-NSC grafts significantly increased the thickness of myelin sheath around the axons in the spared white matter and animals with Olig2-NSC grafts showed improvement in the hind limbs locomotor function (Hwang et al., 2009).

Since demyelination is a prominent feature of SCI, several research groups have concluded that transplantation of myelinogenic cell populations to the injury site enhances structural repair and functional recovery following injury. For example, OPCs and glial precursor cells derived from hESCs survive, differentiate, and have been shown to improve the locomotor function of injured rats at the same time with remyelination of injured axons (Keirstead et al., 2005). Furthermore, using the same OPC differentiation protocol (Nistor et al., 2005) recent animal studies have proven that in addition to treatment of contusion thoracic injury (Keirstead et al., 2005), these cells can be used for the treatment of cervical injuries (Sharp et al., 2010), and complete spinal cord transections (Erceg et al., 2010a) and improve animals' locomotor functions. According to Erceg and colleagues' study the transplantation of hESC-derived OPCs together with hESC-derived motoneurons promoted partial recovery of the hind limb movement of the injured rats after complete spinal cord transection (Erceg et al., 2010a). However, the OPC population used in this study was not pure and contained multipotent progenitors able to differentiate into neurons, astrocytes, and oligodendrocytes (Erceg et al., 2010a). In any case, follow-up studies of animals have indicated that the transplantations of these hESC-derived OPCs are safe, and tumor formation, scarring, tissue pathogenesis, or behavioral declines have not been associated to these grafts (Cloutier et al., 2006; Erceg et al., 2010a).

2.6.3 Current situation of cell therapies for spinal cord injury

Several studies have described the outcomes of cell transplantations in clinical experimental trials for SCI patients (Sahni and Kessler, 2010), see Table 3 for examples. Previously, autologous olfactory ensheathing cells have been transplanted to SCI patients, resulting in non- or very modest improvement for the patients (Feron et al., 2005; Mackay-Sim et al., 2008). Related to this, a more recent study claimed that olfactory mucosal cells have some beneficial effect on improving the American Spinal Injury Association (ASIA) motor scores of patients after 12-45 months of follow-up (Lima et al., 2010). Also, autologous macrophages have been tested in *Phase I* trial showing that three of the eight patients studied recovered significantly in neurological motor and sensory functions (Knoller et al., 2005).

However, this study did not include control groups and the patient material was quite small for reliable statistical analysis (Knoller et al., 2005). In another small trial with two patients transplantation of bone marrow mesenchymal stem cells (MSC) co-cultured with the patient's autoimmune T (AT) cells and transdifferentiated into NSCs increased some sensory improvement in the patients (Moviglia et al., 2006). In addition, a study with schwann cell transplantations from the sural nerve have shown that the cells were not harmful to the patients, but their efficiency was not studied (Saberi et al., 2008). A recent study by Geffner and colleagues reported that autologous bone marrow stromal cell (BMSC) transplantations improved patients' bladder function and quality of their lives (Geffner et al., 2008). Also autologous intra-arterial bone marrow cell transplantations have induced partial improvements in the motor and/or sensory functions of acute SCI patients and one chronic SCI patient monitored for one year (Sykova et al., 2006). Although it remained unclear if recovery occured solely due the cell grafts (Sykova et al., 2006). In line with this, trials with bone marrow cell transplantations have shown that acute or subacute SCI patients improved their ASIA Impairment Scale (AIS) grade 30.4% after transplantation, but no improvement was detected in chronic SCI patients. Also, the safety aspects of these cells are still under investigation (Yoon et al., 2007). Although these trials show beneficial effects with bone marrow stem cell transplantations for SCI, the restricted ability of these cells to differentiate into functional neural cells (Alexanian, 2010), that could actually regenerate the damaged spinal cord tissue, is a big challenge for future regenerative medicine.

The future holds great promises for stem cell therapy aiming at treatment of SCI, since Geron started the first *Phase I* trial for acute thoracic level SCI patients with hESC-derived oligodendrocyte progenitor cells (www.geron.com, 24th of November 2010). Fetal NSCs have also been recruited for the *Phase I* trial for chronic SCI patients by Neuralstem Inc. (http://www.neuralstem.com/, 24th of November 2010) and StemCells Inc. has planned to start the first *Phase I* clinical trials with human CNS-stem cells for chronic SCI patients in 2011 (http://www.stemcellsinc.com/The-rapeutic-Programs/Spinal-Cord-Injury.htm, 24th of November 2010). Taken together, although issues like efficiency and safety are still under investigation with these cell grafts and more controlled clinical trials are needed, these studies suggest that hESC-derived oligodendroglial cells and NSCs have a wide therapeutic potential for SCI treatment.

Table 3. Cell transplantations and clinical trials for SCI patients

SCI	Injury Level	Grafted cells	Follow up time	Advantage	References
chronic, complete	thoracic	olfactory ensheathing cells	1-3 years	No advantage, no harm	(Feron et al., 2005; Mackay-Sim et al., 2008)
chronic, sensorimo-tor comple-te or motor complete	cervical, thoracic	olfactory mucosal cells	12-45 months	Some improvement in AIS grading, relatively safe, but AIS grades declined in 1 patient	(Lima et al., 2010)
acute, complete	cervical 5-thoracic 11	macrophages	1 year	Some patients had improvements in ASIA grading, safe	(Knoller et al., 2005)
chronic	cervical, thoracic	T cells and MSCs transdiffe-rentiated into NSC	3-6 months	Some sensory improvement	(Moviglia et al., 2006)
chronic	mid-thoracic	schwann cells	1 year	Safe, but not effective	(Saberi et al., 2008)
acute and chronic	cervical, thoracic	BMSCs	2 years	Improve bladder function and quality of life, safe	(Geffner et al., 2008)
subacute and chronic, complete	cervical, thoracic	Bone marrow cells	1 year	Partial improvements in motor and/or sensory functions, safe	(Sykova et al., 2006)
acute, subacute, chronic, complete	cervical, thoracic	bone marrow cells	~10 months	The AIS grade increased in 30.4% of the acute and subacute treated patients	(Yoon et al., 2007)
chronic, complete	cervical, thoracic	Human CNS stem cells		Clinical trial planned to be started in 2011	StemCells Inc. http://www.stemc ellsinc.com/Thera peutic-Programs/Spinal-Cord-Injury.htm
subacute or chronic, complete	cervical 5-lumbar 5	olfactory ensheathing cells		Clinical trial recruiting/ongoing	Wroclaw Medical University, http://www.am.w roc.pl/en/
chronic, complete	below cervical 5-spine,	Bone marrow derived stem cells		Clinical trial recruiting/ongoing	TCA Cellular Therapy http://www.tcacel lulartherapy.com
acute, complete	thoracic	hESC-derived OPCs		Clinical trial recruiting/ongoing	Geron, www.geron.com
chronic, complete	thoracic	Fetal NSCs		Clinical trial recruiting/ongoing	Neuralstem, Inc. http://www.neura lstem.com/

3. Aims of the present study

The aim of this thesis work was to determine the human neural cell characteristics through proliferation, differentiation, and teratoma formation studies both *in vitro* and *in vivo*. In addition, the aim was to develop an efficient and safe method for hESC-derived OPC differentiation and purification and optimize this method for totally xeno-free conditions. This thesis work consists 4 parts and specified aims of each study are outlined below.

I. To study and characterize specific surface proteins for hESCs and analyze the surface protein expression changes during hESCs neural differentiation, and develop a sorting method for enabling production of pure human neural cell populations free from pluripotent stem cells.

II. To specify the characteristics of NPCs derived from hESCs or human fetal CNS tissue in terms of pluripotency and differentiation marker expressions *in vitro*, tumor formation capacity *in vivo*, and to test the suitability of hESC-derived NPCs for SCI repair *in vivo*.

III. To develop a differentiation method for OPC production from hESCs by using minimal amounts of animal derived substances, and characterize the cells' gene, and protein expression profiles, viability and proliferation capacity during differentiation. Also to test the myelinization capacity of differentiating oligodendrocytes in co-cultures with neural cells *in vitro*, and purify the hESC-derived OPC population with sorting method.

IV. To develop and test xeno-free culturing conditions for hESC-derived oligodendrocyte differentiation aiming at clinical grade cell production and purification, and to analyze the cells' gene, and protein expression levels during differentiation.

4. Materials and Methods

4.1 Ethical approval

For the use of the hESC-lines derived in Karolinska Institute (Hovatta et al., 2003; Inzunza et al., 2005) in Studies **I-III**, ethical approval was admitted by the Regional Ethical Vetting Board, Stockholm, Sweden. The ethics committee of the Pirkanmaa Hospital District, Finland (Hovatta, R05051) provided a supportive statement regarding the conduct of research utilizing the hESC-lines derived at the Karolinska Institute. For the hESC-lines derived at Regea and used in studies **III-IV**, Regea received a supportive statement from the ethics committee of the Pirkanmaa Hospital District to derive and expand new hESC-lines from surplus embryos, which cannot be used in the fertility treatment of the donating couples (Skottman, R05116). For Study **II** the fetal CNS-tissue derived NPC culturing procedure was approved by the Regional Ethical Vetting Board, Stockholm, Sweden. The experimental procedure of SCI and cell grafting for immunodeficient rodents was approved by The Experimental Animal Ethics Committee of Southern Stockholm, Sweden (Study **II**).

4.2 Cell cultures

4.2.1 Human embryonic stem cells

hESC lines (HS181, HS360, HS362, HS346, HS401) used in Studies **I-III**, were derived from the inner cell mass of supernumerary blastocyst-stage embryos at the Karolinska Institute, Karolinska University Hospital Huddinge, Sweden (Hovatta et al., 2003; Inzunza et al., 2005). Two novel hESC lines (Regea 06/040 and Regea 08/023), were derived and cultured in Regea Institute for Regenerative Medicine (Rajala et al., 2010; Skottman, 2010), and these lines were used in Studies **III-IV**. All the hESC lines used were cultured similarly on top of mitotically inactivated human foreskin fibroblasts (CRL-2429, ATCC, Manassas, CA) in hESC-medium containing knockout Dulbecco's modified Eagle's medium (Gibco Invitrogen, Carlsbad, CA), 20% knockout-Serum replacement (Gibco Invitrogen), 2 mM GlutaMax (Gibco Invitrogen), 0.1 mM minimal essential medium non-essential amino acids (Cambrex Bio Science, Karlskoga, Sweden), 0.1 mM beta-mercaptoethanol (Gibco Invitrogen), 50 U/ml penicillin/streptomycin (Cambrex), and 8 ng/ml human basic fibroblast growth factor (bFGF, R&D Systems, Minneapolis, MN). hESC colonies were passaged by mechanical splitting at 5 to 7-day intervals and re-plated on fresh fibroblasts. All cultures were maintained in a

humidified atmosphere at 37 °C and 5% CO_2. Karyotype normality was confirmed between 25-30 passages and absences of mycoplasma infection were confirmed routinely.

4.2.2 Induced pluripotent stem cells

In Study **IV**, the iPS-cell lines used were StemPro and A116. The iPS-cell line StemPro passage 43 (Invitrogen), was made from human fetal dermal fibroblasts, with four retrovirus factors, *Oct4*, *Sox2*, *Klf4*, and *c-Myc*. The other iPS-cell line A116 passage 28 (made by Professor Otonkoski, University of Helsinki, Finland) was produced from adult fibroblasts with four retrovirus factors; *Oct-4*, *Sox-2*, *Klf-4*, *c-Myc*. Prior differentiation both iPS-cell lines were cultured on top of mitotically inactivated human foreskin fibroblasts in hESC-medium, as described in 4.2.1.

4.2.3 Neural induction of human stem cells

4.2.3.1 Neural precursor cell and neuronal differentiation of human embryonic stem cells

Neural differentiation of hESC-NPCs, in Study **II**, was performed in suspension cultures. Briefly, the hESC colonies were mechanically cut from the feeder layer with a surgical knife (blade 15) and these cut cell clusters were transferred into ultra low attachment 6-well plates (Costar, Corning Inc., Corning, NY) and allowed to form neural cell aggregates in neural induction media: neural stem cell medium (NSM) or neural differentiation medium (NDM). During further culturing these neural cells' aggregates formed neural spheres, which were mechanically split once a week and cultured for up to 20 weeks. NSM contained; Dulbecco's modified Eagle's medium/F-12 (DMEM:F12, 1:1; Life Technologies) supplemented with 0.6% glucose, 5 mM Hepes, heparin (2 µg/ml; Sigma, St. Louis, MO), 1% N2 supplement (Life Technologies), EGF (20 ng/ml; R&D Systems, Minneapolis, MN), bFGF (20 ng/ml; R&D systems), and CNTF (10 ng/ml; R&D Systems). NDM contained; DMEM:F12 and Neurobasal medium (1:1) supplemented with 1× B27, 1× N2, 2 mM GlutaMax (all from Gibco Invitrogen), 25 U/ml penicillin/streptomycin, and 20 ng/ml bFGF.

In Studies **I**, **III**, and **IV**, neuronal differentiation of hESCs was performed in suspension and adherent cultures using a protocol (Lappalainen et al., 2010). Briefly, in suspension cultures the cells formed neural spheres which were mechanically split once a week and cultured for 6 to 8 weeks in NDM. Thereafter, spheres were dissected and plated on laminin (10µg/ml, Sigma) coated well plates in NDM without bFGF. On the next day after plating, bFGF (4 ng/ml) and BDNF (5 ng/ml; Gibco Invitrogen) were added to the medium to support the growth and survival of maturing neuronal cells, and cultured for 1-2 weeks, after which cells were characterized (Study **I**) or co-cultured together with OPCs (Studies **III-IV**). All cultures were maintained in a humidified atmosphere at 37 °C and 5% CO2.

4.2.3.2 Fetal Stem Cells neural differentiation

Human fetal NPC cultures, used in Study **II**, were established from human first trimester derived CNS tissue (6–10 weeks of gestation). The establishment and passaging of human forebrain and spinal cord derived neurospheres have been described elsewhere (Odeberg et al., 2005; Akesson et al., 2006). The protocol used here was modified from the method of Carpenter and co-workers (Carpenter et al., 1999). Briefly, embryonic-fetal tissue was retrieved from clinical first trimester routine abortions with informed consent by women undergoing termination of pregnancy. Cells were cultured at a density of 100000–200000 cells/ml in NSM or in NDM, to form free-floating neurospheres. The cultures of free-floating neurospheres were passaged every 7–14 days by enzymatic dissociation with TrypLE Express (Invitrogen) and gentle trituration, and fresh medium was added twice a week. Cultures were maintained in a humidified atmosphere at 37 °C and 5% CO_2.

4.2.4 Oligodendrocyte precursor cell differentiation of human pluripotent stem cells

In Studies **III** and **IV**, the pluripotent stem cells were differentiated into OPCs in a suspension culture in ultra-low cell cluster 6-well plates (Costar). The OPC differentiation was performed using two different methods which both included 3 stages. *Stage 1. Neural induction of hESCs:* In the beginning, hECS/iPS-cell colonies were mechanically dissected out of feeders and directly transferred into neural induction medium to form neural spheres. *Stage 2. OPC production:* Cells were cultured in the presence of different growth factors that induced OPC development and proliferation in spheres. *Stage 3. OPC maturation:* OPCs were cultured in medium with reduced growth factor concentrations to induce the maturation of OPCs to oligodendrocytes. Throughout the experiment, the spheres were dissected mechanically once a week and the medium was changed 3 times per week. The spheres were plated down into adherent cultures prior immunocytochemical and flow cytometric analysis, and plates were coated with mixture of ECM-proteins: laminin 10 µg/ml (Sigma), collagen IV 10 µg/ml (Sigma), and nidogen1 1µg/ml (R&D Systems) in dPBS, over night 4 ºC. All cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2.

4.2.4.1 Method 1

In Study **III**, 4 different combinations of growth factors were tested in basic N2-medium in Stage 2, and 3 different combinations of growth factors were tested in Stage 3. Combinations and concentrations of different growth factors used in media are listed in Table 4.

4.2.4.2 Method 2

In Study **IV**, 6 different combinations of growth factors in basic medium NS or xeno-free medium (XF) were tested. Combinations of different growth factors used in media are listed in Table 5.

In Studies **III-IV**, the basal N2/NS-medium used throughout the experiments contained DMEM/F-12 supplemented with 1× N2 supplement and 2 mM GlutaMax (both from Gibco Invitrogen), 0.6% glucose, 5 mM Hepes, 2 µg/ml heparin (all from Sigma, St. Louis, MO), and 25 U/ml penicillin and streptomycin. In Study **IV** the XF-medium used contained: DMEM/F12, XF-NSC-supplement StemPro (Invitrogen, product under testing), 2 mM GlutaMax (Gibco Invitrogen), 25 U/ml penicillin and streptomycin.

Table 4. Different combinations of growth factors and proteins tested in OPC differentiation protocol for pluripotent stem cells, Study **III**.

Differentiation Stage/Time	**Combination of growth factors**
Stage 1 / 8w	N2 1 = EGF, bFGF, CNTF
Stage 2 / 3w	N2 2.1= EGF, bFGF, PDGF-AA, CNTF, laminin N2 2.2= EGF, bFGF, IGF-1, PDGF-AA, CNTF, laminin N2 2.3= EGF, bFGF, PDGF-AA, laminin N2 2.4= EGF, bFGF, IGF-1, PDGF-AA, laminin
Stage 3 / 2w	N2 3.1= T3, CNTF N2 3.2= AA, T3, CNTF N2 3.3= AA, T3

Growth factor concentrations in N2-medium were: EGF 20 ng/ml, bFGF 20 ng/ml (in Stage 1) and 10 ng/ml (in Stage 2), CNTF 10 ng/ml (all from R&D Systems), T3 40 ng/ml, AA 200 µM, IGF-1 100 ng/ml, laminin 1 µg/ml (all from Sigma), PDGF-AA 20 ng/ml (Peprotech Inc.).

Table 5. Different combinations of growth factors and proteins tested in OPC differentiation protocol for pluripotent stem cells, Study **IV**.

Differentiation Stage/Time	**Combination of growth factors**
Stage 1/ 4w	NS 1= EGF, bFGF, CNTF NS 2= B27, EGF, bFGF, additional supplements NS 3= B27, EGF, bFGF, CNTF, additional supplements XF 1= EGF, bFGF, CNTF XF 2= EGF, bFGF, additional supplements XF 3= EGF, bFGF, CNTF, additional supplements
Stage 2/ 3w	NS 2.1= EGF, bFGF, IGF-1, PDGF-AA, CNTF, laminin NS 2.2= B27, EGF, bFGF, IGF-1, PDGF-AA, laminin, additional supplements NS 2.3= B27, EGF, bFGF, IGF-1, PDGF-AA, CNTF, laminin, additional suppl. XF 2.1= EGF, bFGF, IGF-1, PDGF-AA, CNTF, laminin XF 2.2= EGF, bFGF, IGF-1, PDGF-AA, laminin, additional supplements XF 2.3= EGF, bFGF, IGF-1, PDGF-AA, CNTF, laminin, additional supplements
Stage 3/ 2-4w	NS 3.1= T3, AA, CNTF NS 3.2= B27, T3, AA, laminin, additional supplements NS 3.3= B27, T3, AA, CNTF, laminin, additional supplements XF 3.1= T3, AA, CNTF XF 3.2= T3, AA, laminin, additional supplements XF 3.3= T3, AA, CNTF, laminin, additional supplements

Growth factor concentrations in NS and XF- media were: EGF 20 ng/ml, bFGF 20 ng/ml (in Stage 1) and 10 ng/ml (in Stage 2), CNTF 10 ng/ml (all from R&D Systems), B27 1× (Gibco, Invitrogen), T3 40 ng/ml, AA 200 µM, IGF-1 100 ng/ml, laminin 1 µg/ml (all from Sigma), PDGF-AA 20 ng/ml (Peprotech Inc.). Additional supplements are not presented since the protocol is under publication.

4.3 Automated monitoring of human embryonic stem cell-derived neural cells

During neuronal and oligodendroglial differentiation of hESCs in Studies **I** and **III**, the growth and morphological characteristics of cells were monitored using a cell culture platform Cell-IQ (Chip-Man Technologies, Tampere, Finland) with phase-contrast microscope optics (10×) and a camera, as previously described (Narkilahti et al., 2007). This software allowed time-lapse imaging of 500 × 670-µm areas in the culture wells. Captured images were saved as JPEG files in separate folders and converted into movie format using Cell-IQ analysis software available from Chip-Man Technologies.

4.4 Gene expression analysis

4.4.1 RNA isolation and cDNA synthesis

In Studies **I-IV**, cells were lysed on RNA extraction lysis buffer and RNA was extracted using either the RNeasy® Micro Kit (Qiagen, Hilden, Germany) or NucleoSpin® RNA II kit (Machinery-Nagel GmbH & Co, Düren, Germany) according to manufacturer's instructions. The concentration and purity of extracted mRNAs were measured using Nanodrop (Thermo Fisher Scientific). For RT-PCR total of 50 ng of mRNA/sample was used for reverse transcription synthesis of single-strand complementary DNA (cDNA), for quantitative RT-PCR 200 ng of mRNA/sample was used. cDNA synthesis was performed with Sensiscript RT Kit (Qiagen) or with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

4.4.2 RT-PCR

In Studies **I-IV**, the reverse transcriptase polymerase chain reaction (RT-PCR) was performed with 1µl of cDNA, 0.25 µM forward and reverse primers (Biomers, Germany), 1× Taq buffer (−MgCl, +KCl) (Fermentas, Leon-Rot, Germany), 2.5 mM dNTP (Fermentas), 25 mM MgCl (Fermentas), dH_2O, and 0.6 Unit Taq DNA polymerase enzyme (Fermentas). The PCR reaction was run in PCR-machine (Eppendorf AG, Germany) and the program parameters were: cDNA denaturation at 95 °C for 3 min followed with 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min and a final extension for 5 min in 72 °C. The final products of PCR were run electrophoretically on 1-2% agarose gels (peqGOLD Universal Agarose, peQlab, Erlangen) containing containing 0.5 µg/ml ethidium bromide (Sigma) and gels were visualized with Molecular Imager Chemidoc XRS System (Bio-Rad Laboratories, Hercules, CA, USA) under UV light.

4.4.3 Quantitative RT-PCR

In Study **II**, quantitative RT-PCR (qRT-PCR) was performed with FastStart Universal SYBR Green Master Mix (ROX, Roche) for the genes *Oct-4, DNMT3b, Gdf3, GAPDH*, and with a TaqMan assay for *Nanog* (Applied Biosystems, Foster City, USA). Primers for SYBR Green chemistry were designed specifically for qPCR and ordered from Thermo Electron GmbH (Germany), primers were also designed to span exon-exon boundaries and primer sequence specificity was confirmed with NCBI BLAST to eliminate the risk of genomic contamination. In studies **III-IV** qRT-PCR was performed with TaqMan assays for *Olig2, Nkx2.2, Sox10, SHH* and *GAPDH* (Applied Biosystems). qRT-PCR was done using the 7500 Fast Real-Time PCR System (**II**) or 7300 Real-Time PCR System (**III-IV**, Applied Biosystems) with the following profile: 1 cycle of 95 ^{o}C for 10 min, 40 cycles of alternating 95 ^{o}C for 15 seconds and 60 ^{o}C for 30 seconds followed by a melting

curve analysis for specificity control. Genes' relative expression levels were measured using the ΔΔCt-method as described in (Livak and Schmittgen, 2001). Undifferentiated hESCs were used as a reference sample and *GAPDH* as reference gene. The Ct values were calculated by the software (7500 Fast System ver 1.3.1, or 7300 sequence detecting software).

4.5 Flow Cytometry

In Studies **I-IV**, protein expression profiles of hESC-derived neural cells and fetal NPCs were analyzed using flow cytometry. FACSAria (BD Biosciences, NJ, USA) with FACS Diva software (BD) was used in Studies **I, III,** and **IV**, FACsort flow cytometer (Beckton Dickinson) with the Cell Quest software was used in Study **II**. For sample preparation, cells were trypsinized either with 1× trypsin-EDTA (in the case of neuronal cells), or with 0.05× trypsin-EDTA (in the case of OPCs), or with Tryple Express (Invitrogen, in the case of NPCs) for 5 min at 37ºC and inactivated with 5% human serum (HS) in PBS, centrifuged at 1900 rpm for 4 min at 4ºC, and resuspended in 2% HS in PBS. Aliquots of 150 000 to 200 000 viable cells per sample were used for flow cytometric analysis. Cells were labeled with primary antibodies without fluorochrome conjugates for 30 min followed by incubation with secondary antibodies for 20 min. For labeling with fluorochrome conjugated CD-markers cells were incubated for 20 min in the dark. After labeling, cells were washed twice with 2% HS in PBS and centrifuged at 1900 rpm (Multifuge 1S-R Heraeus, Siehe Rotor, Kendro Laboratory Products, Germany) for 4 min at 4ºC. Cells were suspended in 2% HS in PBS and analyzed with flow cytometry. During flow cytometry analysis the cell population of interest was determined and dead cells excluded using forward and side scatter parameters (FSC and SSC). Background fluorescence was excluded using unlabeled cells or cells incubated with only secondary antibodies or isotype controls. For each sample run, 10 000-20 000 events were recorded and analyzed. CD-markers used for these analyses are listed in Table 6.

4.6 Sorting and re-culturing of neural subpopulations

4.6.1 Sorting of neuronal cells

FACSAria (BD Biosciences) was used for sorting in Studies **I**, **III**, and **IV**, for a schematic presentation of sorting see Figure 6. Stream was stabilized by turning sweat spot on, and Accudrop Beads (BD Biosciences) was used for stabilizing the drop delay. Sort setup was selected according to the nozzle size, which was 100-µm. For setting the sorting parameters sheath pressure was set at 20.00 PSI, sort precision mode for purity, flow rate 2, and voltage plates to 5.0 V. Live cells were gated using FSC and SSC parameters and positive cells were collected into 5-ml polystyrene tubes (BD Biosciences).

In Study **I** tubes contained NDM with reduced amount of bFGF (4 ng/ml) supplemented with BDNF (5 ng/ml) to support the growth and survival of maturing neural cells. Neuronal sub-populations were sorted with $CD56^+$, $CD133^+$, $CD166^+$, $CD271^+$, $CD184^+$, or $CD326^-$ and plated on laminin 10 µg/ml coated well plates and cultured for 1 week in adherent cultures. Twelve hours before fixation cells were monitored using automatic cell monitoring system (Cell IQ) and stained afterwards.

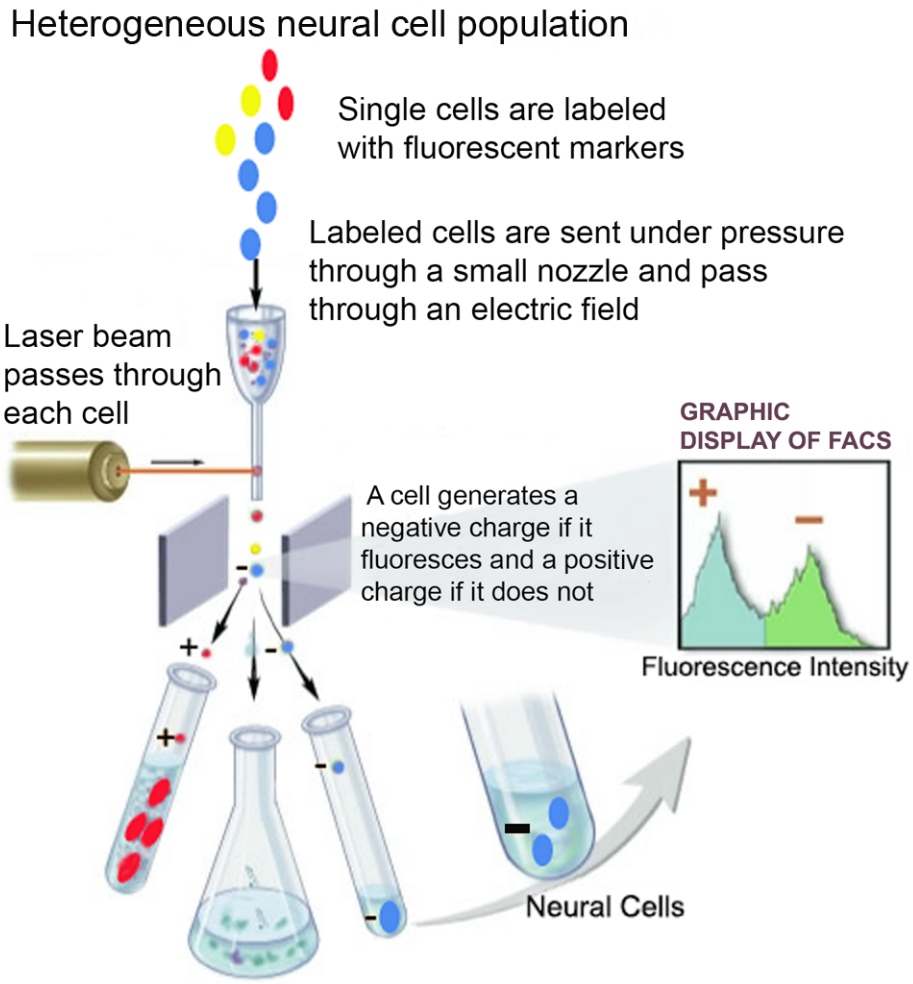

Figure 6. Fluorescence activated cell sorting for hESC-derived neural cells. Figure modified from a picture originally published in http://php.med.unsw.edu.au/cellbiology/index.php?title=2010_Lab_4#Fluorescence_Activated_Cell_Sorting_.28FACS.29.

Table 6. List of used CD-markers.

CD-marker	Fluorochrome	Name	Manufacturer
CD4	FITC	OKT4	Immunotools
CD9	FITC	MRP1, TM4SF	Immunotools
CD9	PE	MRP1, TM4SF	BD Bioscience
CD10	PE-Cy7	CALLA, neprilysin	BD Bioscience
CD13	PE	Aminopeptidase N	Immunotools
CD24	FITC	HSA	Immunotools
CD29	APC	β1 integrin	BD Bioscience
CD31	FITC	PECAM1	Immunotools
CD34	APC	Monomeric sialomucin-like glycoprotein	Immunotools
CD38	PE	Cyclic ADP ribose hydrolase	Immunotools
CD44	FITC	H-CAM	Immunotools
CD44	PE	H-CAM	BD Bioscience
CD45	FITC	Leucocyte common R	Miltenyi Biotech
CD49b	FITC	α2 integrin	Immunotools
CD49d	PE	α4 integrin	BD Bioscience
CD49f	APC	α6 integrin	BD Bioscience
CD56	PE	NCAM	Immunotools
CD59	FITC	Protectin	Immunotools
CD61	FITC	β3 integrin	Immunotools
CD71	PE	Transferrin receptor	Immunotools
CD90	APC	Thy-1	BD Bioscience
CD99R	FITC	MEM-131	Immunotools
CD105	PE	SH-2, endoglin	R&D Systems
CD106	PE-Cy5	VCAM1	BD Bioscience
CD117	APC	c-KIT, SCFR	Miltenyi Biotech
CD133	PE	AC133, prominin	Miltenyi Biotech
CD140a	PE	PDGFRα	BD Bioscience
CD144	PE	VE-cadherin	R&D Systems
CD146	PE	MEL-CAM	BD Bioscience
CD166	PE	ALCAM	BD Bioscience
CD184	PE-Cy5	CXCR4, SDF1	BD Bioscience
CD271	FITC	LNGFR	Miltenyi Biotech
CD326	APC	EpCAM	Miltenyi Biotech

4.6.1.1 Multielectrode array measurements

In Study **I**, for measuring the electrophysiological activities of sorted hESC-derived neural cells, the $CD56^+$ neural subpopulation (20 000 cells/ well) was plated on the 6-well Multielectrode arrays (MEAs, Multichannel Systems, Germany). MEAs were coated with polyethylene imine (PEI, 0.05% w/v) (Fluka/ Sigma-Aldrich) over night at 4ºC, after which laminin (20 µg/ml in dPBS) was added to dish and incubated over night at 4ºC. Cells were cultured in NDM with bFGF (4 ng/ml) and BDNF (5 ng/ml). The electrophysiological activities of formed neural network were measured with MEA-amplifier (MEA-1060-Inv-BC, Multi Channel Systems) and signals were sampled at 20 or 50 kHz using a data acquisition card controlled through the MC_Rack software (Multi Channel Systems). NeuroExplorer (Nex Technologies, Littleton, MA) was used to visualize the processed signalling data. A high-pass filter (2nd order Butterworth filter) with a bandpass cut-off frequency set to 200 Hz was used to remove baseline fluctuations. For the background noise less than 10 μV_{rms} was allowed. During recordings the MEA-dish temperature was maintained at 37 °C using a TC02 temperature controller (Multi Channel Systems). Imaging was performed with Olympus microscope camera (ALTRA 20, Olympus) connected to CellD software (version 2.6, build 1210, Olympus Soft Imaging Solutions GmbH, Munich, Germany).

4.6.2 Sorting of oligodendrocyte precursor cells

For the purification of hESC-derived OPC populations, in Studies **III-IV**, OPCs were sorted with $NG2^+$ using FACSAria (BD Biosciences), as described in 4.6.1. In Study **III**, the $NG2^+$ cells were plated in ultra-low-attachment 24-well culture plates 100 000 cells/well (Costar, Corning Inc.) in OPC-medium 2.2 supplemented ± 1× B27 for sphere formation. For control samples the cells that were not sorted were sub-cultured similarly to form spheres. Formed spheres were examined microscopically every second day and they were mechanically dissected once per week. After 6 weeks of sub-culturing $NG2^+$ spheres and unsorted spheres cultured either with or without B27 were dissected and re-plated on adherent cultures for 1 week for oligodendrocyte differentiation as described in 4.2.3. In Study **IV**, the $NG2^+$ cells were plated down in 48-well culture plates 60 000 cells/well (Nunc., Roskilde, Denmark), coated with ECM-mixture as described in 4.2.3., in OPC-medium XF2.

4.7 Co-cultures of oligodendrocyte precursor cells with neurons

4.7.1 GFP-neurons and oligodendrocyte precursor cells

In Study **III**, the myelination capacity of OPCs was studied in co-cultures with green fluorescent protein (GFP)-transfected hESC-derived neuronal cells,

differentiated as described above in Section 4.1.2.1. The neuronal cells were transduced with lentivirus LV-PGK-GFP containing the green fluorescent protein (GFP) marker gene, under the control of human phosphoglycerate kinase promoter (PGK) and the regulatory elements: self-inactivating modifications (SIN), central polypurine tract (cPPT), and woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). Virus titers were 1.9×10^6 transducing units (TU)/ml and 6×10^8 TU/ml, and cells were transfected with multiplicity of infection of 30 for 5 h at 37°C, (viruses kindly donated by Professor Ylä-Herttuala, University of Eastern Finland). After 1 week, the mechanically split hESC-derived OPC spheres were plated with GFP-trasfected neuronal cells and cultured in OPC medium 2.2 for 2 weeks.

4.8 Immunocytochemical characterization

In Studies **I**, **III**, **IV**, adherently cultured cells from 24-well plates, coated with laminin (10 µg/ml, in the case of neuronal cells) or a mixture of ECM-proteins (laminin 10 µg/ml, collagen IV 10 µg/ml, nidogen-1 1 µg/ml, in case of OPCs and oligodendrocytes) were characterized immunocytochemically. Prior stainings cells were fixed with 4% paraformaldehyde (PFA) for 10 min (in the case of OPCs and oligodendrocytes) and 20 min (in the case of neuronal cells) at room temperature (RT) and washed twice with PBS. For inhibition of unspecific antibody attachment well plates were blocked with 10% normal donkey serum (NDS) and 1% BSA in PBS (containing 0.1% Triton X-100 for detection of intracellular proteins) prior antibody incubations. Primary antibodies were diluted in 1% BSA and 1% NDS in PBS (containing 0.1% Triton X-100 for detection of intracellular proteins) and incubated with cells overnight at 4°C. Cells were washed with 1% BSA in PBS and secondary antibodies were diluted in 1% BSA in PBS and incubated with cells for 1 h at RT in the dark. Cells were then washed with PBS and with phosphate buffer without saline to remove traces of salts. For nuclear staining cells were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Inc. Burlingame, CA, USA). Cells were microscoped with Olympus IX51 phase-contrast microscope equipment with fluorescence optics and Olympus DP71 camera (Olympus, Finland). The primary and secondary antibodies and dilutions used are listed in Table 7 and Table 8.

4.8.1 Live staining

In Study **IV**, 10 days after sorting the $NG2^+$ cells, as well as oligodendrocytes differentiated for 11 weeks were live-stained with O4-antibody for 30 min at 37º C, washed once with dPBS, incubated for 30 min with secondary antibody (anti-mouse IgM Alexa568, Invitrogen), washed with dPBS and microscoped. After microscoping cells were fixed and mounted with Vectashield-mounting medium containing DAPI, as previously described in Section 4.8.

4.9 Immunohistochemical characterization

To study the neural differentiation in Study **II**, the neurospheres derived from hESCs and human fetal CNS tissue were collected in Tissue-Tek Cryomolds (Sakura Finetek, Zoeterwoude Netherlands). Cells were fixed with 4% PFA, treated with 30% sucrose overnight at 4 ºC, and mounted in Tissue-Tek mounting medium (Sakura Finetek) and frozen on dry ice. The neurospheres were cryostat-sectioned at a thickness of 5 µm. Tissue sections were blocked with 1.5% goat/donkey serum in PBS at 30 min in RT, and primary antibodies were diluted in 0.3% Triton X-100 in PBS and incubated overnight at 4 ºC. After rinsing in PBS the sections were incubated with secondary antibody in 0.3% Triton X-100-PBS for 1 hour at RT. For nuclear counter-staining Hoechst 33342 (1:200, Sigma) was used. For background staining control samples were incubated with secondary antibody only. The primary and secondary antibodies and dilutions used are listed in Table 7 and Table 8.

4.10 Cell proliferation

For the cell proliferation analysis in Study **III**, adherently cultured OPCs from stage 2 were incubated with 5'-bromo-2-deoxy-uridine (BrdU, 10 µM; Sigma) in 2.1, 2.2, 2.3, or 2.4 media for 12h. Thereafter, cells were fixed with 4% PFA for 20 min, washed three times with PBS, and treated with 2 M HCl at RT for 30 min. Cells were neutralized with 0.1 M sodium borate (pH 8.5) for 2 min and blocked in 10% NDS (Sigma), 1% BSA (Sigma) in 0.1% Triton X-100 in PBS for 1 h. Next, cells were processed for immunocytochemistry for mouse anti-BrdU (DAKO) and rabbit anti-NG2 (1:100, Chemicon) as described in Section 4.8. To study the proliferation capacity of the cells after sorting in Study **III** $NG2^+$ cells were plated on adherent cultures straightly after sorting and cultured in OPC-medium 2.2 supplemented ± 1× B27. After 1 week cells were fixed and stained with proliferation marker Ki67 (Chemicon) as described in Section 4.8. For the proliferation analysis, in Study **III**, the numbers of co-labeled BrdU and NG2-positive OPCs or Ki67-positive OPCs were counted from five to ten non-overlapping pictures taken with 20× camera objective.

Table 7. List of antibodies and dilutions used in immunocytochemical (ICC) and immunohistochemical (IHC) stainings.

Antibody	Origin	Ig	Dilution	Manufacturer
Pluripotent markers				
anti-EpCAM	mouse	IgG	ICC 1:200	Chemicon
anti-Nanog	goat	IgG	ICC 1:200	R&D Systems
anti-Oct-3/4	goat	IgG	ICC 1:100	R&D Systems
anti-SSEA-4	mouse	IgG	ICC 1:200	Millipore
anti-Tra-1-81	mouse	IgM	ICC 1:200	SantaCruz Biotechnology
Glial markers				
anti-BLBP	rabbit	IgG	ICC 1:800 IHC 1:500	Chemicon
anti-CD44	rat	IgG	ICC 1:100	Biolegend
anti-A2B5	mouse	IgM	ICC 1:400	R&D Systems
Neural precursor markers				
anti-PSA-NCAM	mouse	IgM	ICC 1:200	Chemicon
anti-Nestin	mouse	IgG	ICC 1:200 IHC 1:200	Chemicon
anti-Sox2	goat	IgG	ICC 1:200	SantaCruz Biotechnology
Oligodendrocyte markers				
anti-GalC	mouse	IgG	ICC 1:200	Chemicon
anti-MBP	rabbit	IgG	ICC 1:200	Chemicon
anti-NG2	rabbit	IgG	ICC 1:100	Chemicon
anti-O4	mouse	IgM	ICC 1:100	Chemicon
anti-Olig2	rabbit	IgG	ICC 1:400	Chemicon
anti-PDGFR-α	rabbit	IgG	ICC 1:500	SantaCruz Biotechnology
Astrocyte Marker				
anti-GFAP	sheep	IgG	ICC 1:800 IHC 1:500	R&D System Dako
Neuronal markers				
anti-β-tubulinIII	mouse	IgG	ICC/IHC 1:800	Sigma
anti-CXCR4	mouse	IgG	ICC 1:2000	R&D Systems
anti-MAP-2	rabbit	IgG	ICC 1:600 IHC 1:50	Chemicon
anti-NCAM	rabbit	IgG	ICC 1:800	Chemicon
Proliferation markers				
anti-BrdU	mouse	IgG	ICC 1:400	DAKO
anti-Ki67	rabbit	IgG	ICC 1:400	Chemicon
Human nuclear marker				
anti-HuNu	mouse	IgG	IHC 1:250	Chemicon

Table 8. List of secondary antibodies and dilutions used in immunocytochemical (ICC) and immunohistochemical (IHC) stainings.

Antibody	Origin	Ig	Dilution	Manufacturer
anti-goat Alexa Fluor 488	donkey	IgG	ICC 1:400	Invitrogen
anti-mouse Alexa Fluor 488	donkey	IgG	ICC 1:400	Invitrogen
anti-mouse Alexa Fluor 568	donkey	IgG	ICC 1:400	Invitrogen
anti-mouse Alexa Fluor 568	goat	IgM	ICC 1:400	Invitrogen
anti-mouse Alexa Fluor 488	goat	IgG	IHC 1:1200	Invitrogen
anti-mouse Cy3	goat	IgG	IHC 1:2400	Jackson ImmunoResearch
anti-rabbit Alexa Fluor 488	donkey	IgG	ICC 1:400	Invitrogen
anti-rabbit Alexa Fluor 568	goat	IgG	ICC 1:400	Invitrogen
anti-rabbit Alexa Fluor 488	goat	IgG	IHC 1:1200	Invitrogen
anti-rat Alexa Fluor 488	donkey	IgG	ICC 1:400	Invitrogen
anti-sheep Alexa Fluor 568	donkey	IgG	ICC 1:400	Invitrogen

4.11 Cell viability

For cell viability study in Study **III**, the OPC spheres from stage 2 were dissected into single cell suspensions with 0.05× Trypsin-EDTA (BioWhittaker, Fisher Scientific Inc., Leicestershire, UK) and plated on poly-D-ornithine (50 µg/ml in PBS) coated 24-well plates in 2.1, 2.2, 2.3, or 2.4 media. After 2 to 3 hours, calcein-AM and ethidium homodimer-1 were added into the medium and incubated with the cells for 30 min according to manufacturer's instructions (LIVE/DEAD® Viability/cytotoxicity Kit for mammalian cells, Molecular Probes, Invitrogen). Viable cells incorporated calcein-AM and were detected as green in 488 nm wavelength whereas dead cells incorporated ethidium homodimer-1 and were detected as red in 568 nm. Both live and dead cells were counted at least from 4 non-overlapping pictures taken with 20× camera objective and at least 400 cells (live and dead together) were counted from each group.

4.12 Scanning electron microscopy and transmission electron microscopy

For electron microscopy analyses, in Studies **III** and **IV**, OPCs (Stage 3) and neurons were co-cultured on 4-well Lab-Tek™ Chamber Slides (Nunc Corporation, Denmark) in Medium 3.2 supplemented with 1× B27 for 2 weeks (**III**) or in media NS1, NS2, NS3, XF4, XF5, and XF6 (**IV**). Cells were then fixed with 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at RT. Samples were washed with PBS, post-fixed with 1% osmium tetroxide, and dried with a critical point dryer to preserve the cellular structures. Scanning electron microscope (SEM) samples were sputtered with gold and observed with a JEOL JSM-6335F (Jeol Ltd, Tokyo, Japan). For transmission electron microscopy (TEM) blocks were sectioned, stained with

toludine blue, and mounted on copper grids, stained with uranyl acetate and lead citrate. TEM was performed using a JEOL JEM-100SX (Jeol Ltd) at 15000-25000× and 50 000× magnification. Sample preparation and imaging were performed in the Electron Microscopy Laboratory, University of Turku, Finland.

4.13 Teratoma formation studies

The experimental procedure of cell grafting for immunodeficient mice was approved by the Experimental Animal Ethics Committee of Southern Stockholm, Sweden (Studies **I-II**). To study teratoma formation in Studies **I** and **II**, undifferentiated hESCs, hESC-derived neural cells as single suspension, intact hESC-derived NPC neurospheres, and human fetal NPC neurospheres were transplanted into the right testis of severe combined immunodeficient (SCID) mice. In Study **I**, the hESCs and hESC-derived neural cell populations were transplanted into SCID mice testes as previously described (Hovatta et al., 2003). Animals were divided into three different groups according to different cell types transplanted: group 1) hESCs 200 000 cells/testis (n=3), group 2) hESC-derived neural cells 1 000 000 cells/testis (n=7), group 3) hESC-derived sorted CD326-negative neural cells 1 000 000 cells/testis (n=3). After cell transplantations the animals were monitored for 2 months for teratoma formation with manual palpation and detection of macroscopic tumors.

In Study **II**, 10-12 intact neurospheres (in total approximately 100 000 cells) derived from hESCs and human fetal CNS were transplanted into the SCID mice testicles (n= 10). Equivalent subcutaneous transplantations of 10-12 neurospheres (~100 000 cells) in the left groin were also performed on the same animals. Transplantation of undifferentiated hESC of the same cell lines were used as positive controls for teratoma formation capacity of pluripotent cells. After the cell injections, the development of tumors in the testes and subcutis in transplanted animals was followed by manual palpation for 12 weeks. Animals were sacrificed by a lethal dose of intravenous barbiturates before trans-cardiac perfusion with 4% PFA in 0.1 M PBS.

For histological analysis in Studies **I-II**, testes were dissected and in Study **II**, a 1cm^2 piece of the skin and superficial layer of underlying skeletal muscle at the location of the subcutaneous transplantation was cut out. All the tissues were post-fixed for 4 h, and then transferred to 10% sucrose for at least 24 h. 10 μm sections were cut on a cryostat (Micron) and stained with hematoxylin-eosin (Sigma) and human cells were detected using human nuclear marker HuNu (1:250, Chemicon) and neural cells were detected with β-tubulin III (1:800, Chemicon).

4.14 Transplantation of neural precursor cells into spinal cord injured animals

4.14.1 Spinal cord injury

The experimental procedure of SCI and cell grafting for immunodeficient rodents was approved by The Experimental Animal Ethics Committee of Southern Stockholm, Sweden (Study **II**). In Study **II**, the spinal cord compression lesion was performed in 24 adult immunodeficient female rats (HsdHan: RNU-rnu, Harlan, UK) weighing 170–200 g. Figure 7 presents the spinal cord impactor equipment (Precision instruments, Kentucky, US) and spinal cord prior and post compression injury. Prior to surgery the animals were injected with Atropin (0.05mg/kg i.p., NM Pharma AB, Stockholm, Sweden) 30 minutes prior to anesthesia with a mixture of Hypnorm (fentanyl citrate, 0.216 mg/kg, and fluanisome, 6.75 mg/kg, Janssen Pharmaceutical, Breese, Belgium) and Dormicum (midazolam, 3.375 mg/kg, Hoffman-La Roche, Basel, Switzerland). The rats's body temperature was monitored and kept at 38°C throughout the surgical procedure (CMA/150, Carnegie Medicine). Thoracic spinal cord was surgically exposed by a laminectomy at Th9 and treated with a few drops of Xylocain (lidocain hydrocloride 20mg/ml, AstraZeneca Sweden AB, Södertälje, Sweden) on the exposed spinal cord surface. A moderate and bilateral spinal cord contusion injury was achieved with an infinite horizon spinal cord impactor set at 150 kdyns force, (Precision Instruments, Kentucky) with a dwell time of 10 ms (Figure 8). The sham operated animals were not exposed to contusion injury (n= 6). Prior to suturing the wound the lesioned spinal cord was covered with one layer of meningeal substitute (Lyoplant, B/Braun Aesculap). Post-surgery buphrenorphine (Temgesic, 7 μg/kg, Reckitt & Colman) was administered intramuscularly twice a day for 4 days. The urinary bladders were emptied manually twice of day. Borgal (Trimetoprim sulfa, 15 mg/kg s.c., Intervet International B.V., Boxmeer, The Netherlands) was administered to the rats if signs of infections appeared.

4.14.2 Cell grafting

In Study **II**, eight days after SCI/sham operation the animals were re-anaesthetized and 10–12 neurospheres (cultured for 8 weeks to allow neural induction) containing in total approximately 100,000 cells were transplanted into the lesions. Four groups of rats were included in the study and 6 animals/group were grafted with: a) hESC-derived NPCs cultured in NDM (HS360) b) hESC-derived NPCs cultured in NSM (HS360), c) hESC-derived NPCs cultured in NDM (HS181), d) sham operated animals (a group of rats with a laminectomy but without contusion injury), were injected with hESC-derived NPCs cultured in NDM (HS360). After hind-limb 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 minutes and thereafter rinsed and kept in 30% sucrose until they were cut in 10 μm sections and processed for histological analysis and hematoxylin-eosin staining (Sigma).

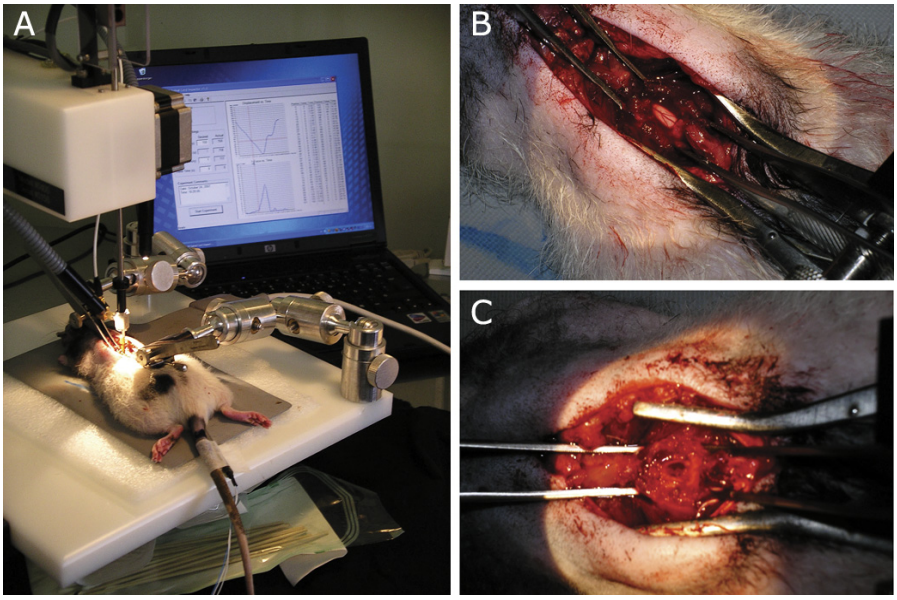

Figure 7. Schematic presentation of rat's spinal cord contusion-injury model. Rat under spinal cord impactor precision system (A), exposed spinal cord after removal of thoracic vertebra 9 (B), spinal cord after contusion injury (C).

4.14.3 Behavioral analysis of locomotor function

In Study **II**, hind-limb function was assessed using the the Basso, Beattie and Bresnahan (BBB) motor performance scale (Basso et al., 1995), with 21 as the score for normal hind-limb function, and 0 for complete paralysis. Animals were followed up for 12 weeks after injury and testing of hind-limb motor function was performed 1 day before injury and 1, 3, 6, 10 and 12 weeks after injury. The researcher performing the behavioral analysis was blinded for the experimental design.

4.15 Statistical analysis

In Studies **I** and **III**, statistical analysis was performed with SPSS program for Windows (v. 16.0, SPSS Inc. Chicago, IL). Nonparametric Kruskal-Wallis test followed by Mann-Whitney U test were used for all analyses. P values of less than 0.05 were considered statistically significant. In Study **II**, the BBB-locomotor ratings were analyzed statistically using nonparametric method based on median values and Kruskal-Wallis test for the nonparametric statistical program Statview 4.12 (Abacus Concepts Inc).

5. Results

5.1 Detection of a novel marker for pluripotent stem cells

In Study **I**, for characterization of seven different hESC-lines and their neural derivates CD-marker expression analysis was performed with 30 different CD-markers using flow cytometry. According to this analysis a novel marker related to pluripotent stem cells CD326/EpCAM was found, which was expressed in > 95% of all the studied hESC-lines co-labeled with Tra1-81. In addition, expression of CD326 was absent or low (< 10%) in hESC-derived neuronal cells differentiated for 46 days (Figure 8 and 9, Study **I**). Interestingly, the expressions of markers previously associated with undifferentiated hESCs, CD24 and CD90, were also detected from the hESC-derived neuronal cell populations at similar levels. Expression of CD133, marker previously associated with stem cells and neural stem cells, was > 60% in undifferentiated hESCs and its expression was downregulated during neuronal differentiation (Figure 9). hESC-derived neuronal cells also expressed CD56, CD117, CD184, and CD271 at higher levels (>40-80%) compared to undifferentiated hESCs (Study **I**).

For further characterization of the novel pluripotency marker CD326, its expression was co-localized on the surface of Nanog and Oct-4 positive hESCs (Figure 8, Study **I**). Most importantly, during neuronal differentiation of hESCs the expressions of *Nanog* and *Oct-4* were downregulated (Figure 8) simultaneously with the downregulation of CD326 expression, according to the conventional immunocytochemistry and flow cytometric analysis (Figures 8-9, Study **I**). We detected the neuronal differentiation of hESCs with RT-PCR analysis, which showed that the expressions of *Mash1*, *MAP-2,* and *CXCR4 (CD184)* were upregulated (Figure 8, Study **I**) concurrently with the upregulation of CD184 and CD56 at the protein level during 46 days of differentiation (Figure 9, Study **I**). The neuronal differentiation was confirmed with the expression of MAP-2 that was first detected in the neural rosette- like structures after 11 days of differentiation and in later timepoints, after 23-46 days of neuronal differentiation, in cells resembling more mature neurons (Figure 8).

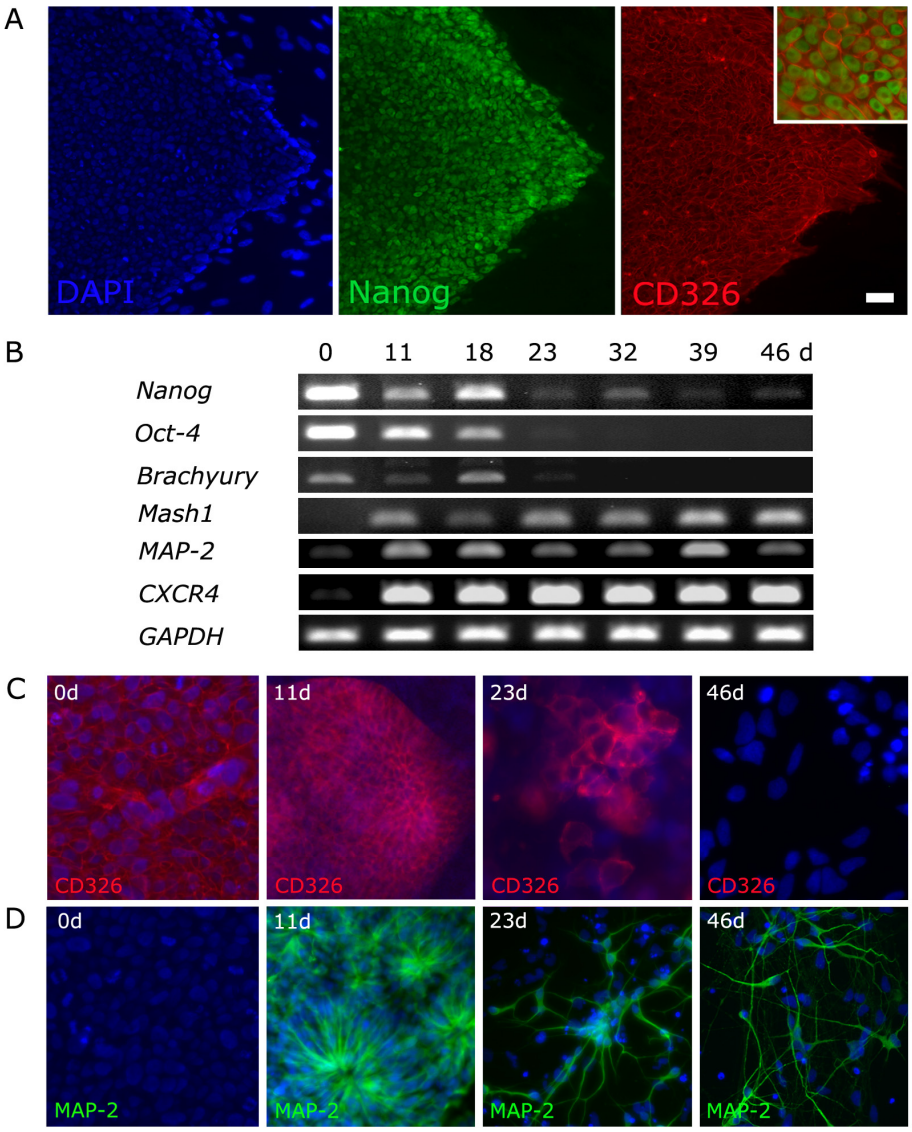

Figure 8. Expression of Nanog and CD326/EpCAM in undifferentiated hESCs, higher magnification shows merged figures of Nanog and CD326, DAPI was used for detection of cell nuclei, (scale bar 100 µm, panel A). RT-PCR analysis of *Nanog*, *Oct-4*, *Brachyury*, *Mash1*, *MAP-2*, *CXCR4*, and *GAPDH* expression during neuronal differentiation of hESCs (timepoints 0, 11, 23, and 46 days, B). Panels of CD326 (C) and MAP-2 (D) expressions during neuronal differentiation of hESCs (timepoints 0, 11, 23, and 46 days). (hESC-line HS360).

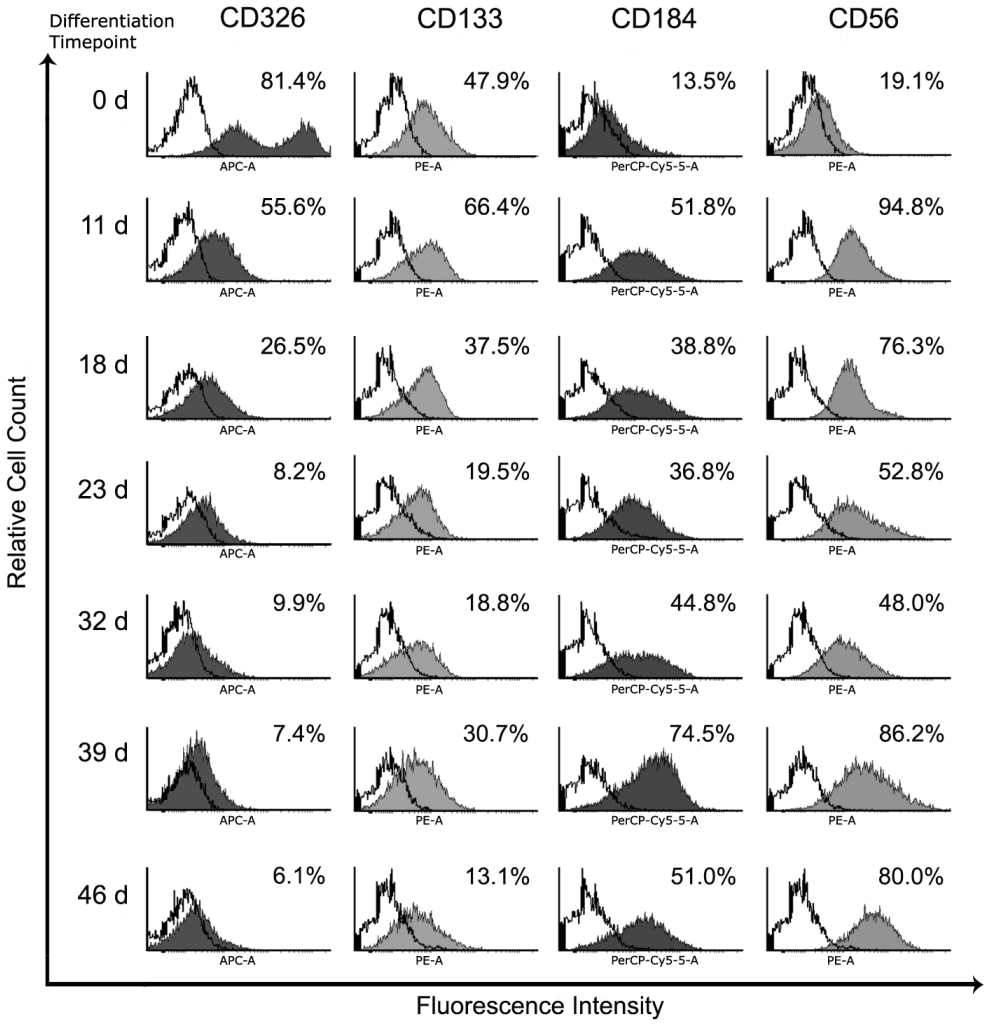

Figure 9. Flow cytometric analysis of CD326, CD133, CD184, and CD56 expression during neuronal differentiation of hESCs (timepoints 0, 11, 18, 23, 32, 36 and 46 days). (hESC-line HS360).

5.2 Sorting of functional CD56$^+$ human embryonic stem cell-derived neural cell populations

In Study **I**, for the production of pure neuronal cell populations for grafting purposes, the hESC-derived neuronal populations were sorted with CD56$^+$, CD117$^+$, CD133$^+$, CD166$^+$, CD184$^+$, CD271$^+$, and CD326$^-$ selection using FACS. The sorted neural subpopulations remained viable after sorting and resembled typical neuronal cells, also differentiating into MAP-2 positive neurons (Figure 10, Study **I**), and after CD56$^+$ and CD326$^-$ selection no tumor formation could be detected in mice testicles (Study **I**). Most importantly this study showed that the sorted CD56$^+$ neural cells formed electrophysiologically active neuronal networks when cultured on a MEA dish (Figure 10).

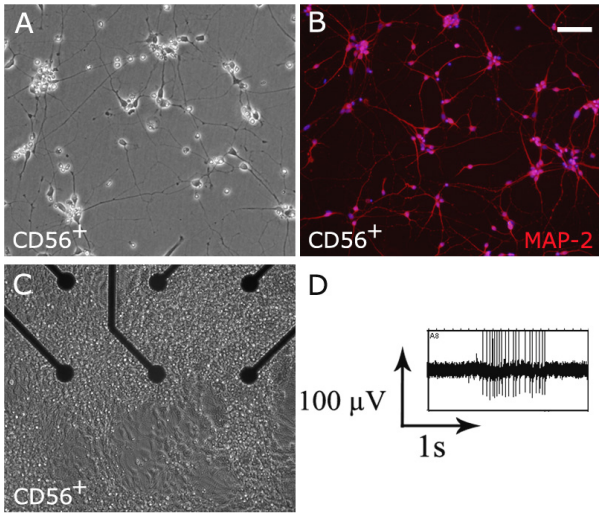

Figure 10. hESC-derived CD56$^+$ neuronal cells after 1 week of sorting (hESC-line Regea 08/023, A), cells were positively stained with MAP-2 antibody (scale bar 100 µm, B). On the MEA-dish the CD56$^+$ neurons formed neuronal network (C) and the electrophysiological activity of the network was detected (D). Unpublished results by Sundberg, Nurmi, Ylä-Outinen and Narkilahti.

5.3 Tumorigenicity of human embryonic stem cell-derived neural precursor cells

In Study **II**, the differences of hESC-derived NPCs and human fetal derived NPCs were analyzed in terms of pluripotency, differentiation, and propensity for tumor formation after grafting in different target tissues of immunodeficient rodents. According to the results of this study, higher expression levels of pluripotency related genes *Oct4*, *Nanog*, *DNMT3b* were detected in fetal NPCs compared to hESC-derived NPCs (Figure 11A, Study **II**). This difference was also confirmed with qRT-PCR analysis, which showed similar results (Study **II**). In contrast to this, no pluripotency related markers, Oct-4, CD326, and SSEA-4, were detected on fetal NPCs at protein level, whereas small amounts of these proteins were detected in hESC-derived NPCs (Figure 11B, Study **II**).

In Study **II** we compared the neural differentiation efficiency of hESC-derived NPCs and fetal-derived NPCs with semiquantitative RT-PCR analysis and expression of neural stem cell and neural differentiation related genes. According to this analysis the fetal-derived NPCs were already more neuronally committed from the beginning of the cell culture establishment (Figure 11A, Study **II**), whereas the neural differentiation with pluripotent hESCs required more time (Figure 11A, Study **II**). In addition, differences between hESC-derived NPCs cultured on different medium compositions, NSM versus NDM, were detected (Study **II**). hESC-derived NPCs cultured in NDM expressed higher levels of neural markers at gene and protein level. However, the effect of different media for neural differentiation of fetal-derived NPCs could not be detected (Study **II**). Interestingly, according to flow cytometric analysis the neural stem/precursor cell markers CD15,

CD133, NCAM, PSA-NCAM, and A2B5 were expressed at higher levels in hESC-derived NPCs after 8 weeks of culturing compared to fetal-derived NPCs (Study **II**).

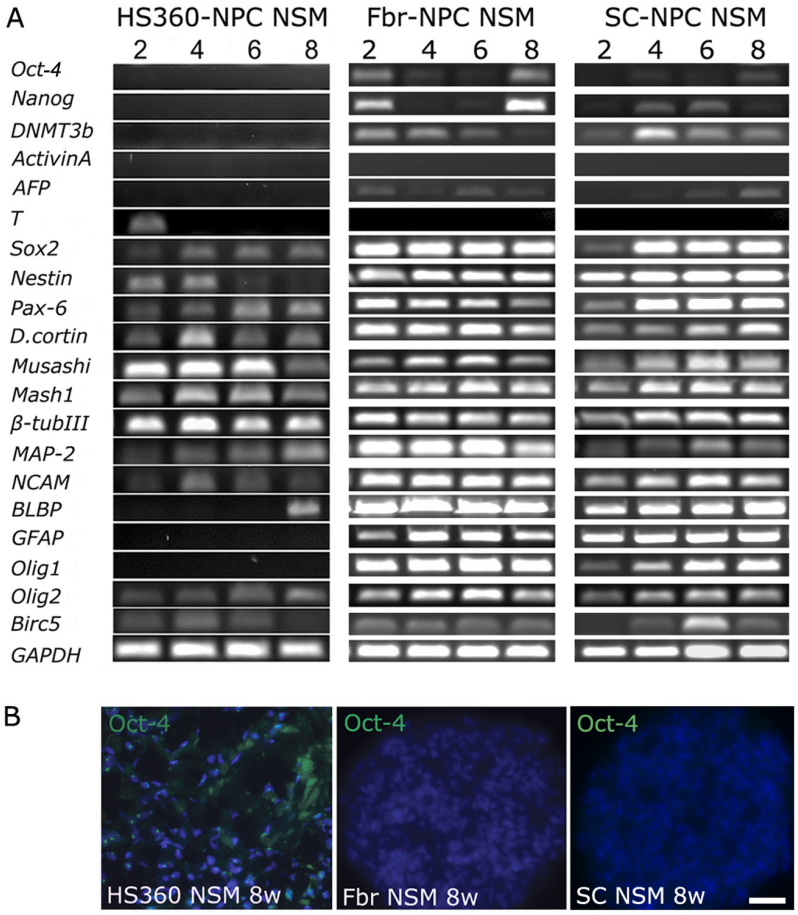

Figure 11. Gene expression analysis of hESC-derived NPCs (hESC-line HS360) and fetal-derived NPCs; forebrain (Fbr), spinal cord (SC) in NSM (timepoints 2, 4, 6, and 8 weeks, A). Oct-4 expression on hESC-derived NPCs and fetal-derived NPCs after 8 weeks of culturing in NSM (scale bar 100 µm, B).

In Study **II**, for the evaluation of the teratoma formation capacity of hESC-derived NPCs versus fetal-derived NPCs, these cells were grafted into immunodeficient mouse testicles and subcutaneous tissue. This analysis showed that the NPC grafts from both of the cell origins survived in the testicles and morphologically and fenotypically resembled neuronal cells, with no signs of teratoma formation (Figure 12A-B, Study **II**). Also, the subcutaneously grafted NPCs did not result in teratoma formations (Study **II**). After the hESC-derived NPC grafting onto the spinal cords of immunodeficient rats some of the animals with SCI improved their locomotor function during the first 6 weeks of follow-up. According to behavioral analysis these animals reached BBB-scoring 15 at 6-week time point (score 21 represents healthy animals, Study **II**). However, a significant decline in locomotor function and BBB-scorings was detected in all the animals after 10-12 weeks of hESC-derived NPC grafting, both in the SCI and sham operated animals (Study **II**). Moreover, teratoma formations were detected in the spinal cords after 12 weeks of cell grafting (Figure 12C, Study **II**).

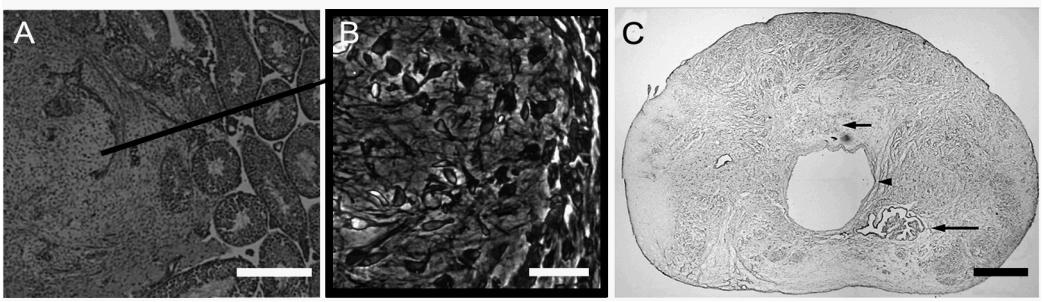

Figure 12. Histological sections of immunodeficient mouse testicle shows the hESC-derived NPC graft survival in the testicle (scale bar 500 μm, A). Higher magnification shows the morphology of the grafted neural cells (scale bar 100 μm, B). Teratoma formation was detected in injured spinal cord after 12 weeks of hESC-derived NPC grafting, different germ layer cell types are pointed out with black arrows (scale bar 500 μm, C).

5.4 Differentiation and purification of oligodendrocyte precursor cells from human embryonic stem cells

In Study **III**, a new differentiation protocol for hESC-derived OPC production was developed. The protocol was based on neural sphere formation in suspension culturing where the neural precursor cells differentiated into OPCs in response to different growth factors. Several combinations of growth factors for the induction of OPC differentiation were tested in protocol development process (see Table 3). This protocol consisted of three stages; the first stage was aimed at neural precursor differentiation and initial neural induction, the second stage was aimed at OPC derivation, and the final stage was aimed at inducing the differentiation of OPCs to oligodendrocytes (Study **III**).

According to the findings of Study **III**, during hESCs differentiation upregulation of important genes affecting oligodendrocyte development was detected. As shown in Figure 12 the hESC-derived OPCs expressed *NG2*, *Sox10*, *Olig2*, *PDGFR*, *OMG*, and *MBP,* after Stage 2. Also, according to studies of different growth factors' effects on OPC differentiation a novel effect of CNTF was detected; CNTF upregulated *Olig2* expression in OPCs after differentiation Stage 2 (Study **III**). The addition of IGF-1 for OPC cultures, together with CNTF, increased the number of proliferative BrdU/NG2-positive cells in the cell population significantly compared to OPCs cultured without it ($p < 0.05$, Study **III**).

Furthermore, according to flow cytometric analysis and immunocytochemistry in Study **III** the expressions of OPC specific markers NG2 and PDGFRα were > 80% in hESC-derived OPCs, whereas the expressions of astrocytic marker GFAP and neuronal marker MAP-2 were low (< 20%) in the differentiated cell populations (Figure 13B, Study **III**). When the maturing GalC-positive oligodendrocytes at differentiation Stage 3 (Figure 13C) were co-cultured with GFP-transfected neurons, the cells' attachment was detected (Figure 13D) likewise the formation of MBP-positive wraps around axons (Study **III**). This myelin layer formation around axons was further confirmed with transmission electron microscopy from co-cultures (Study **III**).

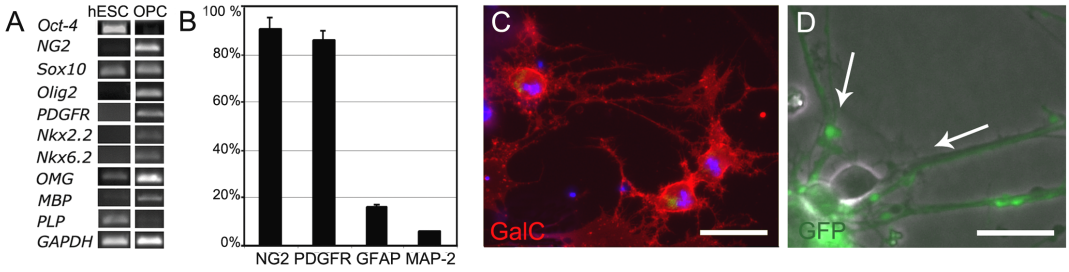

Figure 13. Gene expression analysis of hESCs and hESC-derived OPC population (A). Percentages of NG2, PDGFR, GFAP, and MAP-2 positive cells in hESC-derived OPC population (B). GalC-positive maturing hESC-derived oligodendrocytes (scale bar 20 µm, C). Co-culture of hESC-derived oligodendrocytes and GFP-transfected neuronal cells, white arrows show the connection of oligodendrocyte cell brances with axons (scale bar 20 µm, D). (hESC-line HS360).

For purification of the hESC-derived OPC population the cells were sorted with $NG2^+$ selection and allowed to aggregate into spheres (Figure 14A-B, Study **III**). During subculturing of the $NG2^+$ cells for 6 weeks the gene expression levels related to OPC differentiation remained stable (Study **III**). Compared to the unsorted cell population, the expressions of NG2 and O4 were higher in $NG2^+$ cells after 7 weeks of subculturing (Study **III**). In addition, the $NG2^+$ cells differentiated into GalC-positive oligodendrocytes (Figure 14C, Study **III**). Most importantly, only few pluripotent cells were detected (0.6% $CD326^+$) from the sorted $NG2^+$ OPC population (Figure 14D), and the cells were negative for Tra1-81 and Oct-4 (Study **III**).

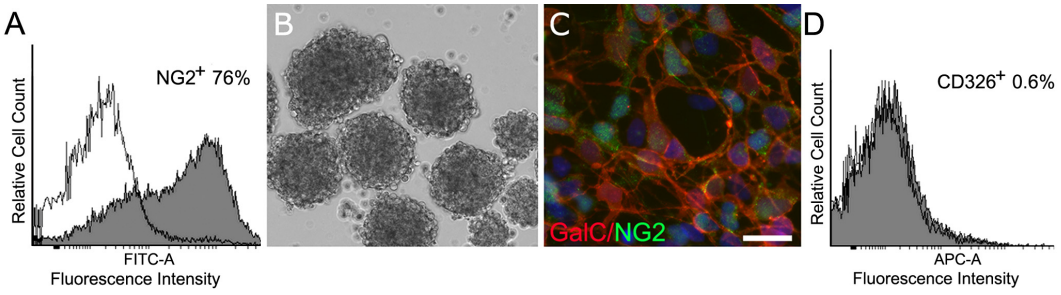

Figure 14. Histogram of NG2-positive cells prior sorting (A). $NG2^+$ cells formed spheres 8 days after sorting (B). After 7 weeks of subculturing $NG2^+$ cells were differentiated into GalC-positive oligodendrocytes (red) and few NG2-positive OPCs were detected (green, scale bar 25 µm, C) and the cells were 0.6% CD326-positive (D). (hESC-line Regea 08/023).

5.5 Development of xeno-free differentiation protocol for human embryonic stem cell-derived oligodendrocyte precursor cells

In Study **IV**, for the production of a safe cell graft for the future treatment of SCI patients, a novel xeno-free differentiation protocol for pluripotent stem cell-derived OPCs was optimized. According to flow cytometric analysis the expression of glial precursor marker A2B5 was 89.3% and PDGFR (CD140a) was 38.2% in hESC-derived glial precuror cell population after 5 weeks of differentiation in the xeno-free medium. During further differentiation the expressions of these markers were downregulated (Figure 15, Study **IV**). The expression of glial/astroglial precursor marker CD44 was constant during differentiation being 20-30% (Figure 15, Study **IV**). The expression of OPC specific marker NG2 upregulated from 27.1% (after 5 weeks of differentiation) to 61.7% (after 8 weeks of differentiation). During further differentiation of cells in the xeno-free medium the expression of NG2 was downregulated, being 27.2% after final stage of differentiation. At the same time the expression of maturing oligodendrocyte specific marker O4 upregulated from 7.7% (after 5 weeks of differentiation) to 85.3% (after 11 weeks of differentiation, Figure 15, Study **IV**).

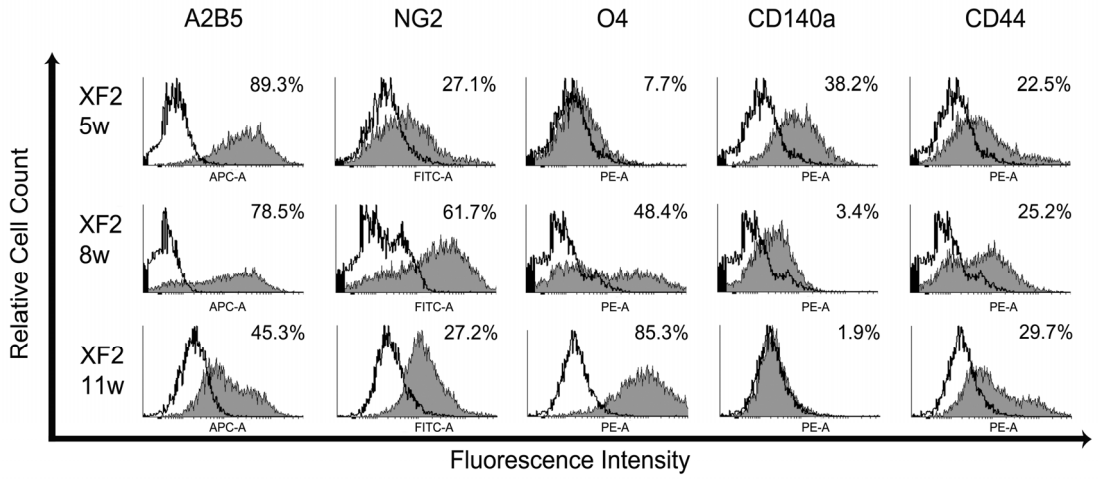

Figure 15. Flow cytometric analysis during hESC-derived OPC differentiation in xeno-free medium XF2. Expressions of A2B5, NG2, O4, CD140a, and CD44 were analyzed at 5 weeks, 8 weeks, and 11 weeks after differentiation (hESC-line Regea 08/023).

According to RT-PCR and qRT-PCR analysis the *Sox10, Olig2,* and *Nkx2.2* expressions upregulated during OPC differentiation in xeno-free medium (Figure 16A, Study **IV**). Also, expressions of *Nkx6.2* and *PLP* were detected in OPCs after 7 weeks of differentiation in xeno-free medium (Figure 16A, Study **IV**). Immunocytochemical analysis confirmed that in xeno-free medium differentiated hESC-derived oligodendrocytes expressed GalC and O4, and morphologically resembled mature oligodendrocytes (Figures 16B-C, Study **IV**). In addition, in the xeno-free conditions differentiated hESC-derived OPCs could be sorted with NG2-positive selection and further differentiated into O4-positive cells. These sorted cell populations were free from Tra-1-81 and Oct-4 –positive pluripotent stem cells (Study **IV**).

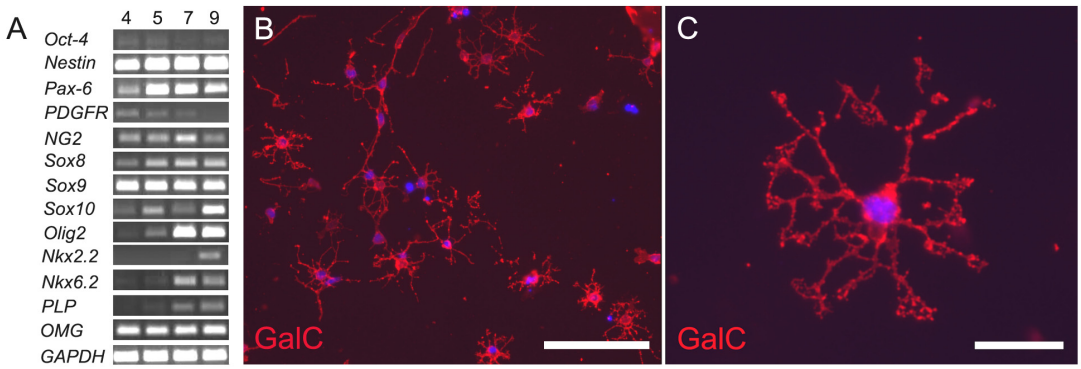

Figure 16. Gene expression analysis during hESC-derived OPC differentiation in xeno-free medium XF2 (timepoints: 4, 5, 7, and 9 weeks, hESC-line Regea 06/040, A). hESC-derived oligodendrocytes differentiated in xeno-free medium for 11 weeks were positive for GalC (B-C) and morphologically resembled mature oligodendrocytes with ramified cell branches (C). Scale bars 100 µm B; 25 µm C, (hESC-line Regea 08/023).

6. Discussion

Currently SCIs cause lifelong impairments for patients and curative treatments are not available. Stem cell therapies are one option for regeneration of damaged CNS tissue, and pluripotent hESCs are one of the cell sources considered to be ideal for this purpose due to the cells' efficient differentiation capacity (Coutts and Keirstead, 2008). However, pluripotent stem cell-derived neural grafts entail risks for tumor formation upon transplantation if the cell populations contain undifferentiated cells (Brederlau et al., 2006; Roy et al., 2006; Schulz et al., 2004; Sonntag et al., 2007). Related to this there are differentiation protocols for production of more specialized cell types like OPCs and oligodendrocyte from hESCs, but often these protocols result in heterogeneous populations and contain animal derived products which may be unsafe in clinical use (Erceg et al., 2010a; Kang et al., 2007; Nistor et al., 2005). Thus, this dissertation focuses on the characterization of undifferentiated hESCs and their neural derivates and finding of novel markers for purification of hESC-derived neural cell populations, and the development of novel methods for efficient and xeno-free OPC differentiation from hESCs. In the following chapters, the importance of our results is discussed and evaluated in comparison to those of other studies.

6.1 Characterization of human embryonic stem cells and their neural derivates

According to previous meta-analysis by the International Stem Cell Initiative (ISCI), it has been shown that different hESC-lines cultured around the world have similar expression levels of some of the commonly used pluripotent markers, whereas some markers are expressed quite distinct levels (Adewumi et al., 2007). That is why novel markers related to pluripotent stem cells are needed that would be expressed constantly in different cell lines and could be used for the validation of different hESC-lines and their pluripotency, as well as for detecting of pluripotent stem cells in iPS cell cultures. According to our results from the CD-marker expression analysis with seven different hESC-lines, derived from two independent laboratories, we were able to detect a novel marker related to pluripotent stem cells: CD326 (Study **I**). CD326 is a glycoprotein known as epithelial cell adhesion molecule (EpCAM) and it is related to cell adhesion (Litvinov et al., 1997). In hESC colonies, the cells are packed tightly to attach to each other and the expression of CD326 on the cell surfaces might explain these tight cell-to-cell contacts. Previously the expression of CD326 has been detected on hepatic progenitor cells (Schmelzer et al., 2006) and carcinoma cells (Munz et al., 2004). EpCAM affects carcinoma cells for the upregulation of the proto-oncogene c-myc and cyclin A/E, via which it has a direct impact on the cell cycle and proliferation capacity of the cells (Munz et

al., 2004). Thus, we suggest that it might have a similar regulatory role in a pluripotent stem cell proliferation capacity. According to our results in Study **I**, the expression of this marker was detected with combinatorial FACS analysis in Tra-1-81 colabeled hESCs and immocytochemically stained Oct-4 and Nanog-positive hESCs. Since we published our results related to this marker and its expression on pluripotent stem cells, several other research groups have confirmed our finding, by showing that EpCAM was localized in Oct-4 positive pluripotent cells and its expression was downregulated during differentiation (Lu et al., 2010; Ng et al., 2010). Also, it has been shown that in EpCAM-silenced cells endodermal and mesodermal genes were upregulated while cell proliferation was decreased >40% (Ng et al., 2010). Thus, the expression of EpCAM is essential for pluripotent stem cells to maintain their undifferentiated state as well as their proliferation capacity.

Neuronal specification of hESCs with several differentiation protocols is usually a complicated process and results in the formation of heterogeneous populations, containing immature neural cells and fully matured neurons (Brederlau et al., 2006; Roy et al., 2006; Sonntag et al., 2007). Thus surface protein expression profiling is important for the detection of different developmental stages of neural cell differentiation, as described here (Study **I**) and also by other research groups (Pruszak et al., 2009). Here, we performed the neural differentiation of hESCs using a simple culturing medium: DMEM/F12/ Neurobasal medium including N2-, and B27-neural supplements in combination with bFGF (Lappalainen et al., 2010). During neuronal differentiation of hESCs we detected the downregulation of pluripotency markers: *Oct-4, Nanog*, Tra-1-81 and CD326 (Study **I**). Similarly, previous studies with variable neural differentiation protocols have shown the downregulation of pluripotency genes and proteins during neural differentiation of hESCs (Gerrard et al., 2005; Nat et al., 2007; Pruszak et al., 2007). The formation of neural rosette-like structures is the first sign of neuroectodermal differentiation of hESCs (Reubinoff et al., 2001; Zhang et al., 2001), and we detected typical rosette structures after 11 days of neural induction (Study **I**). In addition, during 46 days of culturing we detected the neuronal differentiation of hESCs by the upregulation of *Mash1, MAP-2,* and *CXCR4 (CD184)* expression, and CD56 (NCAM) and CD184 (CXCR4) expression (Study **I**). These results are in line with earlier studies where CD56 has been used to monitor neural specification of hESCs (Pruszak et al., 2007) and this marker has been shown to be important in early neuroectodermal differentiation (Reubinoff et al., 2001). In studies of fetal CNS derived NPCs, the CD184 expression has been associated with the efficient migratin ability of neural cells (Ni et al., 2004). This suggests that CD184 expressing hESC-derived neural cell populations may have migration capacities similar to those of fetal NPCs. In Study **I** we detected CD184 expression in the MAP-2 positive neurites, confirming its expression in maturing human neuronal cells, as previously shown in human fetal cells (Peng et al., 2007). Taken together, these results confirmed that CD56 and CD184 can be used for the detection of different maturation stages during neuronal differentiation of hESCs.

6.2 Purification of human embryonic stem cell-derived neural and oligodendroglial cell populations

Although there are several differentiation protocols available for neural differentiation of hESCs, the resulting populations are often impure and may contain undifferentiated cells or partly differentiated proliferating stem cells (Brederlau et al., 2006; Kozubenko et al., 2009; Sonntag et al., 2007). This causes significant risks that the cell population will form tumors when engrafted onto the CNS tissue. Therefore we (Studies **I**, **III**, and **IV**) and other research groups used sorting methods like FACS to purify the hESC-derived heterogeneous neural cell populations (Chung et al., 2006; Guzman et al., 2008; Peh et al., 2009; Pruszak et al., 2007).

Most interestingly, a recent study by Pruszak and co-workers revealed that by using a certain CD-marker expression 'code' distinct subpopulations from neurally differentiated hESCs can be isolated (Pruszak et al., 2009). In their study different combinations of negative, positive or intensity based selection (high or low) were used for hESC-derived neural cells labeled with CD15, CD24, and CD29 (Pruszak et al., 2009). In contrast to Pruszak and co-workers study, we detected high expression levels of CD24 and CD29 both in undifferentiated hESCs and their neural derivates, so we could not use either of these surface markers for specific selection of neural subpopulations derived from hESCs (Study **I**). Despite this, we were able to purify the hESC-derived neural cell populations by sorting with other CD-markers: CD56, CD117, CD133, CD166, CD184, and CD271 (Study **I**). We showed that the neurites grew effectively after sorting, especially in $CD56^+$ sorted cells, and $CD56^+$ and $CD184^+$ neural cells matured effectively into viable neurons expressing MAP-2 (Study **I**). Most importantly, the neural cells sorted with $CD56^+$ formed electrophysiologically active neuronal networks when plated onto an MEA-dish, as previously shown with unsorted hESC-derived neural cells (Heikkilä et al., 2009). These results also concur with earlier studies using $CD56^+$ selection to isolate functional neuronal cell populations from hESCs or iPS cells for transplantation and regeneration of CNS deficit animals (Hargus et al., 2010; Pruszak et al., 2007). Moreover, these results can in the future be confirmed by electrophysiological modulation tests such as the addition of neurotransmitters, activators, and inhibitors for cells (Heikkilä et al., 2009), and recordings from single cells with a patch-clamp system would also demonstrate the existence of real action potential (Erceg et al., 2008). This is important for demonstrating that hESC-derived heterogeneous neural cell populations can be purified with FACS and that after sorting the cells are able to connect with each other and are electrophysiologically active, which is an important feature of neuronal cells when they are considered to be used for regenerative medicine.

Considering the safety issues of hESC-derived neural cell populations, we were able to show that by sorting with CD326-negative selection, we could eliminate the remaining pluripotent stem cells from the differentiated hESC-derived neural cell populations (Study **I**). This is an important finding since remaining pluripotent stem cells in the hESC-derived neural cell populations are tumorigenic upon transplantation, as we (in Study **II)**, and other research groups have shown (Brederlau et al., 2006; Kozubenko et al., 2009; Sonntag et al., 2007). In addition,

according to our results the hESC-derived neural cell populations negatively selected and sorted against CD326 remained viable after sorting, had neuronal morphology, and were non-tumorigenic in testicle transplantations (Study **I**). In the future, this negative selection procedure will allow us to purify hESC-derived neural cell populations prior to cell grafting. Since negatively selected cell populations do not contain any bound antibodies on the cell surfaces it may make them safer to use for transplantation therapies than positively selected cells containing bound antibodies on the cell surfaces. Thus, our finding of this CD326 marker novel purpose for the detection and elimination of pluripotent cells from hESC or iPS-derived cell populations is important for achieving pure differentiated cell populations free from tumorigenic pluripotent stem cells.

To approach the safety issues of the hESC-derived OPC populations produced in Studies **III-IV**, we decided to sort the OPCs with $NG2^+$ selection using FACS. As previously shown, NG2 is a chondroitin sulfate proteoglycan which is a surface marker detected in OPCs (Nishiyama et al., 2009; Wilson et al., 2006). A recent study reported that $NG2^+$ cells can give rise to GABAergic neurons, olfactory interneurons, or oligodendrocytes, depending on the region specific cues in the brain tissue in which the cells are transplanted (Aguirre and Gallo, 2004; Aguirre et al., 2004). According to our results in Study **III**, the sorted $NG2^+$ cells cultured in our OPC induction medium expressed genes and proteins specific for OPC development. Also, in Study **IV** we were able to show that OPCs differentiated in xeno-free medium could be sorted by $NG2^+$ selection and further differentiated into O4-positive oligodendrocytes. Most importantly, the $NG2^+$ cell populations were free from pluripotent cells, as they were negative for Tra1-81, Oct-4, and CD326 (Studies **III-IV**), known markers for pluripotency as described in Study **I**. According to our Studies **I** and **II**, this is crucial for safe non-tumorigenic cell population production. Furthermore, the sorted $NG2^+$ population matured into GalC-positive oligodendrocytes, and we detected only very few astrocytes but no neurons (Study **III**). In line with this, a recent study reported that rodent glial cell populations sorted with $NG2^+$ and subcultured in OPC specific medium differentiated into oligodendrocytes *in vitro*, but did not give rise to neurons (Horiuchi et al., 2010). Thus, we concluded that sorting with $NG2^+$ selection is a reliable way to perform hESC-derived OPC population purification (Studies **III-IV**).

Regarding methodology, the use of fluorescence-activated flow cytometry offers an opportunity to use variable CD-markers and other surface antibodies labeled with different fluorescent labels for multicolor analysis and subpopulation characterizations of heterogeneous cell populations (Baumgarth and Roederer, 2000). Especially for sorting of neural subpopulations use of FACSAria (BD) is quite a reliable and safe way to achieve viable, purified cell populations from hESCs, as we (Studies **I**, **III**, **IV**) and other research groups have shown (Hargus et al., 2010; Peh et al., 2009; Pruszak et al., 2007; Pruszak et al., 2009). Several research groups also use this method routinely for sorting of hESC-derived or iPS-derived neural cells for transplantation studies (Hargus et al., 2010; Pruszak et al., 2007; Pruszak et al., 2009). Nevertheless, the great number of cells needed for sorting with FACS and the loss of cells after sorting detracts the usability of this method. However, according to several decades of hematology research, several laboratories have used different flow cytometry equipment and sorting methods for hematopoietic cell analysis and sortings prior to grafting cells onto human patients

(Bornhauser et al., 2005; Lowdell and Theocharous, 1997). For the future consideration of GMP-requirements for stem cell graft productions there is also sorting equipment available that ensures the quality control and sterility of the cell sorting as well as better cell survival (BD, www.bdbiosciences.com, 10th of October 2010). MACS also holds great promise for the future, since it is a gentle separation method for distinct cell populations, based on magnetic bead labeling without massive cell loss (Milteney www.miltenyibiotec.com, 10th of October 2010). As such, the flow cytometry and sorting methods are the 'gold standard' methods, which are used widely and reliably for analyzing different cell populations and for the purification of cell populations.

6.3 Comparison of human embryonic stem cell- and fetal CNS tissue-derived neural precursor cells' pluripotency and differentiation capacity

The differences between NPCs of different origins have been studied intensively to determine the proper cell population to be used for transplantation purposes for CNS disorders (Guillaume and Zhang, 2008; Kim and de Vellis, 2009; Okano, 2010; Shin et al., 2007). The importance of these characterizations relies on the evaluation of the safety of pluripotent cell-derived neural cell populations and the detection of proper differentiation stage of neural cells derived from tissues or pluripotent stem cells according to their expression of specific neural markers, epigenetic regulators, and transcription factors (Okano, 2010). In our Study **II** we performed gene expression profiling for hESC-derived NPCs and human fetal NPCs during their neural culturing time *in vitro* in neural differentiation medium and in neural stem cell medium. According to our results, we detected down-regulation of the pluripotency related genes *Nanog*, *Oct-4*, *DNMT3b* and *Gdf3* in hESC-derived NPCs compared to undifferentiated hESCs. On the other hand, we detected mRNAs of *Nanog*, *Oct-4*, *DNMT3b,* and *Gdf3* at fetal NPCs expressed at even higher levels than in hESC-derived NPCs, according to quantitative PCR analysis. In line with these results, a recent study showed that rat NSC population contained a large number of cells immunoreactive to Oct-4 and DNMT1 (Singh et al., 2009). Earlier studies have also reported that non-pluripotent NPCs isolated from adult rhesus macaque brain expressed *Oct-4* (Davis et al., 2006), and *Gdf3* has been found in the human hippocampus, cerebral cortex, and cerebellum (Hexige et al., 2005). These results suggest that these commonly known pluripotency markers may have different effects on different developmental stages affecting cell proliferation capacity and DNA methylation events and are not solely associated with the pluripotency or tumorigenicity of cells.

According to the results of Study **II**, despite the low pluripotency marker mRNA levels in hESC-derived NPCs, we also detected low protein expression levels of pluripotency markers Oct-4, SSEA4, and CD326 in these cell populations. These hESC-derived NPC spheres, which contained only a few pluripotent cells, resulted in tumor formation after grafting into spinal cords of immunodeficient rats (Study **II**). In line with these results, a previously published study showed that neural differentiation of hESCs lead to the derivation of heterogeneous neural cell

populations, which expressed pluripotency markers at protein level and upon grafting these cells formed tumors in the CNS tissue (Kozubenko et al., 2009). Interestingly, in contrast to hESC-derived NPCs the human fetal NPCs did not express detectable levels of these pluripotency proteins (Study **II**), and no tumor formations have previously been detected in the spinal cords of rats grafted with these cells (Akesson et al., 2007; Erceg et al., 2009). Thus, it seems that even relatively low expression levels of pluripotency mRNAs results in protein translation in hESC-derived NPCs but not in fetal NPCs, and this difference is crucial for the tumorigenicity of the NPC populations derived from pluripotent stem cells.

According to Study **II,** compared to the neural differentiation capacity of the fetal NPCs the hESC-derived NPCs neural induction took a longer time. The expression of neural markers was detected after a longer period of culturing and medium compositions affected the neural gene and protein expression levels of hESC-derived NPCs. This suggests that pluripotent hESCs were more modifiable and altered by culture conditions and neural inducing growth factors compared to more neurally committed fetal NPCs that did not change their phenotype considerably in the two media studied (Study **II**). These results concur with an earlier meta-analysis, which showed that the fetal NPCs expressed neural differentiation genes more elevated levels than hESC-derived NPCs (Shin et al., 2007). It has also been previously shown that regional differences exist between fetal forebrain and spinal cord derived NPCs. Spinal cord derived NPCs express elevated levels of astroglial marker GFAP compared to much lower expression of it in forebrain derived NPCs (Piao et al., 2006), similarly as we detected in Study **II**. To conclude, it is evident that fetal NPCs are exposed to regional neural guiding prior isolation from the CNS tissues and that this explains their differences in neural commitment and low propensity for tumorigenicity compared to more modifiable and tumorigenic hESC-derived NPCs.

6.4 Safety studies of human embryonic stem cell- and fetal CNS tissue-derived neural precursor cells

In Study **II** prior to grafting the hESC-derived NPCs into spinal cords, we performed teratoma formation studies with these cells and also with fetal-derived NPCs in immunodeficient mice testicles and subcutaneous tissue. We selected these tissue origins, since previous teratoma formation studies with undifferentiated stem cells have been performed on them (Kishi et al., 2008; Reubinoff et al., 2000). However, we could not detect any teratomas or abnormal overgrowth of transplanted NPCs in the testicles or subcutaneous tissues, although the graft survival and neural differentiation in grafting sites could be detected (Study **II**). Previously differences have been shown between target organs with regard to the propensity of transplanted hESCs to develop teratomas (Hentze et al., 2009). In addition, it has been shown that the number of ESCs required for teratoma formation differed between different transplantation sites (Kishi et al., 2008). In line with this, we think that if more hESC-derived NPC spheres were transplanted into mouse testicles teratomas could have been detected. However, transplanted hESC-

derived NPC spheres, containing a total of 100,000 cells, consistently gave rise to teratomas in the rat spinal cords, even though similar amounts were non-tumorigenic in mice testicles and subcutaneous tissues. This remarkable difference in the permissiveness of teratoma formation between SCID mice testicles and subcutaneous tissue compared to immunodeficient rats' spinal cords have not so far been described elsewhere. We also believe that similar consequences are possible between the human spinal cord and the animal models commonly used to evaluate cell graft tumorigenesis. This leads to the risk that even though hESC-derived neural cells have been shown to be non-tumorigenic in animals (Erceg et al., 2010a; Keirstead et al., 2005) they could be tumorigenic in human spinal cords after increase of the dose of transplantable cells. Related to this, a recent study by Kishi and colleagues showed that even two different immunodeficient mouse strains had differences in teratoma formation propensity, due to the differences in immunological responses between mouse strains (Kishi et al., 2008). We concluded that this immunological difference between different strains (Kishi et al., 2008) might be more marked between different species and that it might be one explanation for the big difference in teratoma formation occurrences in our Study **II**. This also raises concerns about the reliability of using immunodeficient animals for the safety studies of human pluripotent stem cell-derived grafts.

In addition, it has been shown that the number of hESCs required for teratoma formation is related to the time required for teratoma formation (Hentze et al., 2009). In our Study **II**, to ensure that there was sufficient time for the teratomas to develop both in testicles and spinal cords, the transplanted animals were monitored for 12 weeks. Previously published follow-up times after cell grafting onto injured spinal cords have been quite short, describing the first few weeks of animals' improvement (Keirstead et al., 2005), but leaving out the long-term follow up times required for reliable evaluation of graft safety. Most importantly, we detected a similar kind of recovery in some SCI animals during the first 6 weeks of follow-up after hESC-derived NPC transplantations, as described earlier with hESC-derived oligodendroglial cells (Keirstead et al., 2005). However, a dramatic decrease in locomotor function was detected after 6 weeks, followed by continuous deterioration of locomotor function until 12 weeks time point. Thus, this prolonged follow-up time revealed the tumorigenicity of hESC-derived neural graft. For future studies, sufficient follow-up times will also be important when evaluating the safety of hESC-derived neural cells in different neurological deficient animal models.

Study **II** emphasizes the importance of using a reliable animal model for preclinical transplantation studies of hESC-derived neural grafts. We also propose that evaluation of the safety of cell therapies should be performed in the same location of the CNS as the actual therapeutic cell grafting and include extended observation times. In the future, to avoid tumor formations in human patients, clear safety requirements for hESC-derived neural grafts should be set out in the legislation and these regulations should be followed prior to granting permission to transfer these cells for clinical use.

6.5 Differentiation of oligodendrocyte precursor cells from human embryonic stem cells

According to our results on hESC-derived NPCs tumorigenicity after grafting onto spinal cord and the irrefutable evidence that remaining pluripotent stem cells and proliferating precursors were the cause of it (Study **II**), in Study **III** we wanted to develop a more specialized cell population from hESCs that would be free from pluripotent stem cells and that could be used for the treatment of SCI in the future. Since oligodendrocytes are myelin forming cells of the CNS, and since earlier studies have shown that hESC-derived OPCs can restore the locomotor function of SCI animals (Erceg et al., 2010a; Keirstead et al., 2005), we developed our own method for the differentiation and purification of OPCs from hESCs (Study **III**).

Many protocols have been developed for the differentiation of hESCs towards glial cell fate and oligodendrocytes (Hu et al., 2009a; Izrael et al., 2007; Kang et al., 2007; Nistor et al., 2005). These protocols include undefined growth conditions and contain animal-derived substances and therefore are not designed as such for clinical grade production. Our method differs from these previously published methods in several ways. First of all we used hESC-lines cultured on top of human foreskin fibroblasts, to avoid xenogenic contamination caused by mouse fibroblasts (Heiskanen et al., 2007; Martin et al., 2005). Secondly throughout the differentiation protocol we passaged the cell population mechanically, avoiding the use of enzymes that can lead to abnormal chromosomal mutations in the longer run, as described for hESCs (Thomson et al., 2008). Most importantly, our differentiation protocol was based on suspension culturing where OPCs were differentiated in spheres in the presence of human-recombinant growth factors, human mitogens, and human ECM proteins (Study **III**). Finally, to purify the differentiated OPC population we performed sorting with FACS and $NG2^{+}$ selection, and subcultured cells in sphere format. The culturing and differentiation of cells in sphere format allows the utilization of bioreactor cultivation in the future, where gas flows, medium changes, and cell passaging are performed in invariable conditions under computer monitoring (Gilbertson et al., 2006; Portner et al., 2005; Sen et al., 2004). All these considerations make the protocol easily convertible into GMP-level cell production for clinical purposes in the future.

To induce OPC differentiation from hESC-derived NPCs in Study **III**, we stimulated the cells with bFGF, EGF, PDGF-AA, CNTF, and IGF-1. We showed that CNTF induced *Olig2* expression in OPCs, which is a novel finding of CNTF effects on OPC differentiation. Furthermore, we detected that addition of IGF-1 for the OPC cultures increased the number of proliferating NG2-positive cells, and together with CNTF, increased the cell survival. These results are in line with earlier studies with rodent OPCs (Barres et al., 1992; Barres et al., 1993; Carson et al., 1993; Hart et al., 1989; Hsieh et al., 2004; McKinnon et al., 1990; McMorris and Dubois-Dalcq, 1988; Zaka et al., 2005), showing that the similar pathways affecting on rodent OPC development are present in hESC-derived OPCs, as previously predicted with human fetal cells (Zhang et al., 2000).

The expressions of *PDGF-R, Nkx2.2, Sox10, Olig1/2, MBP,* and *PLP* are prominent factors used for identifying hESC-derived OPCs (Izrael et al., 2007; Kang et al.,

2007). According to Study **III**, we detected the expression of these genes in developing OPC population, and in addition we showed that hESC-derived OPCs expressed *Ngn3, Sox9, Sox8, Gli1, Gli2, Nkx6.2,* and *oligodendrocyte-myelin glycoprotein*, which are crucial genes for oligodendrocyte development (Nicolay et al., 2007). Furthermore, we characterized the hESC-derived OPC population immunocytochemically and showed that hESC-derived OPCs were positive for NG2 and PDGFRα, two markers routinely used to identify OPCs (Levine et al., 2001; Nishiyama et al., 1996). Also, during further differentiation >80% of cells were GalC-positive. In line with a previous study which demonstrated that hESC-derived maturing oligodendrocytes were able to myelinate neurons *in vitro* (Kang et al., 2007), we showed with transmission electron microscopy that differentiated OPCs in co-cultures with neurons formed multilayer myelin sheaths around axons (Study **III**). Thus, these characterizations confirmed that our differentiation protocol for hESC-derived OPC production resulted in effective and functional OPC differentiation.

Considering the existence of several different differentiation protocols for human OPC production from hESCs (Hu et al., 2009a; Izrael et al., 2007; Kang et al., 2007; Nistor et al., 2005), it is important to be aware of the differences between different hESC-lines used in different laboratories, which also affects the outcome of differentiation protocols (Adewumi et al., 2007). This is because there are several differences in the derivation and maintenance of hESC-lines prior to differentiation and use of mouse/human feeders, animal substances, serum/knock-out serum replacement, and enzymatic/mechanical cell passaging (Adewumi et al., 2007). It has been shown that distinct hESC-lines differ quite a lot from their differentiation potential and adaptation capacity for changed culture conditions, as shown by differences between several hESCs-lines' neural differentiation capacity (Lappalainen et al., 2010). As such, our differentiation protocol may be efficient for OPC production using the hESC-lines derived similarly (Hovatta et al., 2003; Inzunza et al., 2005; Rajala et al., 2007) but its usefulness for other hESC-lines needs to be studied in the future, and may thus require further optimization of the protocol.

6.6 Xeno-free differentiation protocol for human embryonic stem cell-derived oligodendrocyte precursor cells

The use of stem cell-derived neural cell products for clinical cell therapies is one of the main objectives in the stem cell research field. However, several issues should be taken into account prior to transfer these cells to clinical use. These issues include selection of a reasonable and safe cell source, use of defined and repeatable differentiation protocols and graft quality control tests (Ahrlund-Richter et al., 2009). In our Study **IV**, we approached these matters aiming to achieve a totally xeno-free differentiation protocol for hESC-derived OPC production. Although this protocol described in Study **IV** was not performed at the GMP-level due to cost constraints; the methods and tests used were performed in such a way that they can

be easily transferred to GMP-level graft production and characterization.

According to Study **IV**, we were able to show that OPCs differentiated in xeno-free medium expressed higher levels of *Sox10*, *Olig2*, and *Nkx2.2* according to qRT-PCR analysis compared to control conditions. According to the flow cytometric analysis the hESC-derived glial precursor cell population expressed A2B5, NG2 and PDGFR after 5 weeks of differentiation in xeno-free medium. After 8 weeks of differentiation the expression of NG2 and O4 increased in hESC-derived OPC population, and after final stage of differentiation at 11 weeks > 80% of cells were O4-positive and expressed GalC (Study **IV**). These results concur with earlier studies describing hESC-derived OPC differentiation in xenogenic conditions (Hu et al., 2009a; Izrael et al., 2007; Kang et al., 2007; Nistor et al., 2005). Also, we were able to purify the hESC-derived OPC population in xeno-free conditions with $NG2^+$ selection (Study **IV**), which is important, since in the future this will enable us to eliminate harmful tumorigenic pluripotent stem cells prior to grafting. Taken together, in Study **IV** we developed a xeno-free differentiation method for hESC-derived OPC production that also supported the cells' differentiation and subculturing after the sorting process.

As we and other research groups have shown, some of the hESCs and hESC-derived neural differentiation procedures can be transferred into xeno-free conditions (Study **IV**) (Rajala et al., 2010; Swistowski et al., 2009) and in the future cell grafts can be produced under GMP conditions (Carpenter et al., 2009). Furthermore, commonly used medium supplements for neural differentiation, such as B27-supplement, are now available as a xeno-free product (Invitrogen, www.invitrogen.com, 15th of December 2010), and Matrigel can also be replaced with xeno-free ECM proteins for oligodendrocyte differentiation; laminin (Hu et al., 2009a), or vibronectin (Gil et al., 2009). All these advances make it possible to convert the existing differentiation protocols for xeno-free production, or for the future development of totally new ones with existing xeno-free hESC-lines, to avoid risks of animal derived components for human cells (Heiskanen et al., 2007; Martin et al., 2005). Although this entails a lot of work and optimization for the differentiation protocols, in the future it will enable us to have safer and more patient-friendly ways of producing neural grafts for human patients.

6.7 Future perspectives for cell transplantation therapies for spinal cord injury

In light of our research data and data from several other laboratories, it is evident that prior to transferring stem cells to clinical treatment of SCI many issues must be addressed. First of all, since SCI is complex event involving acute, secondary, and chronic processes at physical and cellular level, effective stem cell treatment should take into account all these events. In addition, selection of the optimal cell source for grafting purposes is crucial to ensure the success of the treatment. It is also important to take into account the proper time window after SCI when selecting

patients for stem cell therapies, to avoid harmful effects of tissue inflammation processes for the cell graft at the too early stage of injury or physical barriers of glial scar formation at a later stage of injury. To ensure patient safety clinical treatments should be well justified, and performed by professionals and described in detail (ISSCR, 2008). In addition, patients selected for cell graftings should be aware of the risks and possible adverse effects of the treatment. It is also very important to provide reliable information to the public and to the scientific community about clinical trials and treatments. This includes, for example, scientific publications including both positive and negative results, likewise information about the risks, harms and concerns involved in stem cell therapies.

Researchers have suggested that the optimal cell source for SCI transplantations could be NSCs or NPCs. These cells may either have interaction abilities, or abilities to release neurotrophic factors, thereby promoting reconstructions of own tissue by endogenous cells, protecting damaged host cells from cell death or toxic influences, or differentiating into specific cells to replace dead cells from injury areas, like neurons, astrocytes and oligodendrocytes (Coutts and Keirstead, 2008; Okano, 2010). In fact, fetal CNS tissue derived NSCs have been transplanted into SCI animal models, showing reconstruction abilities and improvement in non-human primates' locomotor recovery (Iwanami et al., 2005). Related to this, a company called Neuralstem, Inc. (http://www.neuralstem.com/, 24th of November 2010) has announced that it has filed an Investigational New Drug application with the FDA to begin a *Phase I* clinical trial for chronic SCI to test the safety of human fetal spinal cord-derived stem cell transplantations. Furthermore, StemCells Inc. will start *Phase I* trials in 2011 with human NSCs for chronic SCI, based on the encouraging results of regeneration in pre-clinical animal studies (Salazar et al., 2010). However, prior to transferring these cells for clinical use the exact mechanisms affecting the actions of these cells in the host tissue, and the therapeutic effects, needs to be thoroughly ascertained. The safety risks of these cells also need to be taken into account properly, since one recent transplantation experiment with fetal NSC graft led to the the development of a massive tumor formation in the brain for a patient suffering from ataxia telangiectasia (Amariglio et al., 2009). In addition, the ethical issues need to be taken into account when considering the wider utilization of fetal cells for transplantation purposes, since the tissues are not so easily available, the isolated cell number is usually limited, and some countries limit the use fetal cells for legislative and religious reasons.

To overcome the limitations of poor availability and restricted use of fetal cells, pluripotent stem cells offer for the future an almost unlimited cell supply, to be used for the differentiation of specialized neural cell types. Currently the first clinical trials with hESC-derived oligodendrocyte progenitors (GRNOPC) are ongoing for acute thoracic level spinal cord injury patients, and the first patients were treated with these cells in fall 2010. This first human trial with an hESC-derived product will concentrate on safety issues of grafts, which is the most important thing to be tested prior to increasing the dose of transplantable cells for studies on effectiveness and regeneration (www.geron.com, 24th of November 2010). Thus, at the moment the use of pluripotent stem cells as a source for neural graft production for treatment of SCI is a reality, and in the future we will see if these cells are the long waited treatment option for this otherwise lifelong neurological deficit.

Recently, wide interest in using of iPS cell-derived neural cells for studies on neural deficits, like Parkinson's disease have raised the question of the usefulness of these cells for regenerative purposes and for future transplantation therapies, for example, for SCI (Soldner et al., 2009; Tsuji et al., 2010). iPS cells offer several advantages; they are derived without use of human fertilized eggs or embryos, diminishing the ethical questions related to embryonic stem cell therapies. Furthermore, there is an almost unlimited cell supply to generate patient specific iPS cell lines from skin biopsies, which offers the opportunity to produce autologous cell grafts without fear of immunorejections (Salewski et al., 2010). A recent study by Tsuji and colleagues described the use of mouse iPS cell-derived neurospheres for the treatment of SCI animals (Tsuji et al., 2010). According to this study, the SCI animals grafted with iPS-derived neurospheres improved their locomotor function and some remyelination of axons was also detected (Tsuji et al., 2010). Although no tumors or abnormal cell proliferation were detected in spinal cords with grafts (Tsuji et al., 2010), the safety of these cells needs further investigation since follow-up of the animals was only 42 days. In keeping with this, the use of iPS cell-derived neural cells for the future treatment of SCI is one possibility, although it entails similar or even higher risks for teratoma formations than hESC-derived neural cells (Okano, 2010). There are also many more problems related to the use of iPS cells in regenerative medicine, among them cells' epigenetic memory (Kim et al., 2010), old mitochondria (Parker et al., 2009), and uncontrolled reprogramming technology (Mikkelsen et al., 2008; Okita et al., 2007). iPS cells are made traditionally by insertion of oncogenic genes and viral vectors, which can lead to incomplete reprogramming processes and makes iPS cells oncogenic (Mikkelsen et al., 2008; Okita et al., 2007). However, in the future the use of mutagenesis and virus-free iPS cells can help to overcome these obstacles and widen the use of these cells in clinical research (Kaji et al., 2009; Soldner et al., 2009; Woltjen et al., 2009; Yu et al., 2009; Zhou et al., 2009).

The usage of multipotent stem cells and autologous cells for grafting is one option to overcome several safety issues and immunorejections related to pluripotent stem cell-derived grafts. Clinical trials with autologous bone marrow stem cells and olfactory derived cells for patients with SCI, have shown that grafting of these cells is safe for patients and induces partial improvement in motor and/or sensory functions (Lima et al., 2010; Sykova et al., 2006), as well as improvement in bladder function (Geffner et al., 2008), and ASIA grading (Yoon et al., 2007), although wider patient groups are needed for reliable evaluation of the effectiveness and safety of these treatments.

Since SCI is a complex injury, for its effective treatment the future prospects might actually cover a combination of several cell transplantation strategies, with combinations of different cell types; autologous-, multipotent- or pluripotent stem cell-derived cells, together with possible gene therapy, material engineering, and a pharmacological approach (Blits and Bunge, 2006; Hains et al., 2001; Hendriks et al., 2004; Iwanami et al., 2005; Keirstead et al., 2005; Nesic et al., 2001; Sykova et al., 2006; Teng et al., 2002). In the future the whole manufacturing process for stem cell products can be performed under controlled conditions, where all the materials including culturing media, nutrient supplements, and growth factors are traceable, and quality controlled prior to use (Ahrlund-Richter et al., 2009). For the future large scale cell production the possibilities of using suspension culturing would

enable the transfer of these cells for bioreactor cultivation, where gas flows and medium changes are performed in invariable conditions under computer monitoring (Gilbertson et al., 2006; Portner et al., 2005; Sen et al., 2004). In addition, developed cryopreservation techniques will make it possible to establish cell graft banks for quality checked clinical products (Ahrlund-Richter et al., 2009; Kuleshova et al., 2009). Most importantly, prior to entering clinics with these stem cell-based grafts, transplantable cell populations must be characterized thoroughly in terms of; purity, sterility, identity, stability, proliferation and differentiation capacity, safety, and efficacy. Hopefully, after accomplishing all these requirements in the future it will be possible to offer reliable and efficient stem cell therapy for the treatment of SCI.

7. Conclusions

The aim of this study was to characterize hESC and fetal CNS tissue-derived neural cell properties and their safety for the treatment of SCI. First of all, surface protein expression profiles of hESCs and their neural derivates were characterized to find suitable markers for the purification of hESC-derived neural populations from pluripotent tumorigenic stem cells. Subsequently, hESCs and fetal CNS tissue-derived NPCs were compared with each other in terms of pluripotency and neural differentiation capacity *in vitro*. Also, in order to determine the properties important for future safe cell grafts for SCI treatment, hESC and fetal CNS tissue-derived NPCs tumor formation capacity was evaluated *in vivo* in immunodeficient animal tissues. Furthermore, a new protocol for OPC differentiation from hESCs was developed, and further optimized aiming at xeno-free differentiation and purification for future graft production for the treatment of SCI. Based on these studies, the following conclusions were drawn:

- According to wide surface marker expression screening with flow cytometry a novel marker related to pluripotent hESC was found: CD326, also known as Epithelial Cell Adhesion Molecule (EpCAM). CD326 co-localized in Tra-1-81, Oct-4, and Nanog positive cells, and these cells formed teratomas in SCID mouse testicles. The > 90% expression of CD326 was detected in seven different hESC-lines, derived in two independent laboratories (**I**).

- hESC-derived neural cell populations could be purified from pluripotent stem cells with a combination of specific CD-markers. This purification was performed with FACS, either using positive or negative selection against markers $CD326^-$, $CD56^+$, and $CD184^+$. After sorting neural cells were viable, proliferative and able to mature (**I**). In addition, we were able to show that homogeneous $CD56^+$ neural cell populations formed pure neuronal networks and the cells were electrophysiologically active.

- According to Study **II**, hESC-derived NPCs were more modifiable than fetal NPCs. Fetal NPCs were more committed to neural cell types than hESC-derived NPCs, since hESC-derived NPCs expressed pluripotent markers at the protein level, whereas these proteins were not expressed in fetal NPCs. This was the main difference that caused the increased tumorigenicity of hESC-derived NPCs compared to non-tumorigenic fetal NPCs after grafting onto immunodeficient rats (**II**).

- Safety studies with hESC-derived neural cell grafts, which contain only few pluripotent stem cells, should not be performed in testicles or subcutaneous

tissues. These conclusions were drawn when hESC-derived NPCs did not form any tumors in the testicles or subcutaneous tissue of immunodeficient mice, but robust tumor formation could be detected after transplantation into the intact or injured spinal cords of immunodeficient rats. Thus, when using pluripotent stem cells as a source for neural graft production, safety studies should always be performed in the same target region in CNS as the actual therapeutic grafting (**II**).

- In Study **III** we developed a novel method for the differentiation and purification of human OPCs from hESCs. These OPCs were differentiated in serum-free media with human-derived growth factors, mitogens, and human ECM proteins. These maturing OPCs were able to myelinate neurons *in vitro*. Importantly, purification of $NG2^+$ OPCs from hESCs and formation of $NG2^+$ spheres enabled amplification of the pure OPC population in the suspension culture. This sorted population remained stable over 7 weeks of culturing *in vitro* and further differentiated into GalC-positive oligodendrocytes. This purification step allowed the production of cell grafts that were free from pluripotent stem cells (**III**).

- In Study **IV** we developed xeno-free differentiation protocol for hESC-derived OPCs using commercial xeno-free medium supplement and combinations of various human growth factors. According to our results we were able to show that the xeno-free supplement could be used as efficiently as two other known medium supplements N2 and B27 for OPC differentiation (**IV**). In xeno-free medium the OPCs differentiated efficiently into O4- and GalC-positive cells. Also, this xeno-free medium supported the $NG2^+$ cell population subculturing and differentiation after sorting, enabling purification of OPC populations.

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Tampere, November 2010

Doing science is like dancing jazz
– There is always room for improvisation –

References

Adewumi O, Aflatoonian B, Ahrlund-Richter L, Amit M, Andrews PW, Beighton G, Bello PA, Benvenisty N, Berry LS, Bevan S, Blum B, Brooking J, Chen KG, Choo AB, Churchill GA, Corbel M, Damjanov I, Draper JS, Dvorak P, Emanuelsson K, Fleck RA, Ford A, Gertow K, Gertsenstein M, Gokhale PJ, Hamilton RS, Hampl A, Healy LE, Hovatta O, Hyllner J, Imreh MP, Itskovitz-Eldor J, Jackson J, Johnson JL, Jones M, Kee K, King BL, Knowles BB, Lako M, Lebrin F, Mallon BS, Manning D, Mayshar Y, McKay RD, Michalska AE, Mikkola M, Mileikovsky M, Minger SL, Moore HD, Mummery CL, Nagy A, Nakatsuji N, O'Brien CM, Oh SK, Olsson C, Otonkoski T, Park KY, Passier R, Patel H, Patel M, Pedersen R, Pera MF, Piekarczyk MS, Pera RA, Reubinoff BE, Robins AJ, Rossant J, Rugg-Gunn P, Schulz TC, Semb H, Sherrer ES, Siemen H, Stacey GN, Stojkovic M, Suemori H, Szatkiewicz J, Turetsky T, Tuuri T, van den Brink S, Vintersten K, Vuoristo S, Ward D, Weaver TA, Young LA, Zhang W (2007): Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. Nat Biotechnol 25: 803-816.

Aguirre A, Gallo V (2004): Postnatal neurogenesis and gliogenesis in the olfactory bulb from NG2-expressing progenitors of the subventricular zone. J Neurosci 24: 10530-10541.

Aguirre AA, Chittajallu R, Belachew S, Gallo V (2004): NG2-expressing cells in the subventricular zone are type C-like cells and contribute to interneuron generation in the postnatal hippocampus. J Cell Biol 165: 575-589.

Ahrlund-Richter L, De Luca M, Marshak DR, Munsie M, Veiga A, Rao M (2009): Isolation and production of cells suitable for human therapy: challenges ahead. Cell Stem Cell 4: 20-26.

Akesson E, Piao JH, Samuelsson EB, Holmberg L, Kjaeldgaard A, Falci S, Sundstrom E, Seiger A (2007): Long-term culture and neuronal survival after intraspinal transplantation of human spinal cord-derived neurospheres. Physiol Behav 92: 60-66.

Akopian V, Andrews PW, Beil S, Benvenisty N, Brehm J, Christie M, Ford A, Fox V, Gokhale PJ, Healy L, Holm F, Hovatta O, Knowles BB, Ludwig TE, McKay RD, Miyazaki T, Nakatsuji N, Oh SK, Pera MF, Rossant J, Stacey GN, Suemori H (2010): Comparison of defined culture systems for feeder cell free propagation of human embryonic stem cells. In Vitro Cell Dev Biol Anim 46: 247-258.

Alexanian AR (2010): An efficient method for generation of neural-like cells from adult human bone marrow-derived mesenchymal stem cells. Regen Med 5: 891-900.

Alvarnas JC, Linn YC, Hope EG, Negrin RS (2001): Expansion of cytotoxic CD3+ CD56+ cells from peripheral blood progenitor cells of patients undergoing autologous hematopoietic cell transplantation. Biol Blood Marrow Transplant 7: 216-222.

Amariglio N, Hirshberg A, Scheithauer BW, Cohen Y, Loewenthal R, Trakhtenbrot L, Paz N, Koren-Michowitz M, Waldman D, Leider-Trejo L, Toren A, Constantini S, Rechavi G (2009): Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient. PLoS Med 6: e1000029.

Anderson KN, Potter AC, Piccenna LG, Quah AK, Davies KE, Cheema SS (2004): Isolation and culture of motor neurons from the newborn mouse spinal cord. Brain Res Brain Res Protoc 12: 132-136.

Andrews PW, Damjanov I, Simon D, Banting GS, Carlin C, Dracopoli NC, Fogh J (1984): Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. Differentiation in vivo and in vitro. Lab Invest 50: 147-162.

Assou S, Le Carrour T, Tondeur S, Strom S, Gabelle A, Marty S, Nadal L, Pantesco V, Reme T, Hugnot JP, Gasca S, Hovatta O, Hamamah S, Klein B, De Vos J (2007): A meta-analysis of human embryonic stem cells transcriptome integrated into a web-based expression atlas. Stem Cells 25: 961-973.

Assou S, Cerecedo D, Tondeur S, Pantesco V, Hovatta O, Klein B, Hamamah S, De Vos J (2009): A gene expression signature shared by human mature oocytes and embryonic stem cells. BMC Genomics 10: 10.

Astori G, Vignati F, Bardelli S, Tubio M, Gola M, Albertini V, Bambi F, Scali G, Castelli D, Rasini V, Soldati G, Moccetti T (2007): "In vitro" and multicolor phenotypic characterization of cell subpopulations identified in fresh human adipose tissue stromal vascular fraction and in the derived mesenchymal stem cells. J Transl Med 5: 55.

Awatramani R, Scherer S, Grinspan J, Collarini E, Skoff R, O'Hagan D, Garbern J, Kamholz J (1997): Evidence that the homeodomain protein Gtx is involved in the regulation of oligodendrocyte myelination. J Neurosci 17: 6657-6668.

Azari MF, Profyris C, Zang DW, Petratos S, Cheema SS (2005): Induction of endogenous neural precursors in mouse models of spinal cord injury and disease. Eur J Neurol 12: 638-648.

Babaie Y, Herwig R, Greber B, Brink TC, Wruck W, Groth D, Lehrach H, Burdon T, Adjaye J (2007): Analysis of Oct4-dependent transcriptional networks regulating self-renewal and pluripotency in human embryonic stem cells. Stem Cells 25: 500-510.

Badcock G, Pigott C, Goepel J, Andrews PW (1999): The human embryonal carcinoma marker antigen TRA-1-60 is a sialylated keratan sulfate proteoglycan. Cancer Res 59: 4715-4719.

Bansal R, Stefansson K, Pfeiffer SE (1992): Proligodendroblast antigen (POA), a developmental antigen expressed by A007/O4-positive oligodendrocyte progenitors prior to the appearance of sulfatide and galactocerebroside. J Neurochem 58: 2221-2229.

Bansal R, Kumar M, Murray K, Morrison RS, Pfeiffer SE (1996): Regulation of FGF receptors in the oligodendrocyte lineage. Mol Cell Neurosci 7: 263-275.

Barberi T, Klivenyi P, Calingasan NY, Lee H, Kawamata H, Loonam K, Perrier AL, Bruses J, Rubio ME, Topf N, Tabar V, Harrison NL, Beal MF, Moore MA, Studer L (2003): Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice. Nat Biotechnol 21: 1200-1207.

Barnett SC, Alexander CL, Iwashita Y, Gilson JM, Crowther J, Clark L, Dunn LT, Papanastassiou V, Kennedy PG, Franklin RJ (2000): Identification of a human olfactory ensheathing cell that can effect transplant-mediated remyelination of demyelinated CNS axons. Brain 123 (Pt 8): 1581-1588.

Baron-Van Evercooren A, Olichon-Berthe C, Kowalski A, Visciano G, Van Obberghen E (1991): Expression of IGF-I and insulin receptor genes in the rat central nervous system: a developmental, regional, and cellular analysis. J Neurosci Res 28: 244-253.

Barraud P, Stott S, Mollgard K, Parmar M, Bjorklund A (2007): In vitro characterization of a human neural progenitor cell coexpressing SSEA4 and CD133. J Neurosci Res 85: 250-259.

Barres BA, Hart IK, Coles HS, Burne JF, Voyvodic JT, Richardson WD, Raff MC (1992): Cell death and control of cell survival in the oligodendrocyte lineage. Cell 70: 31-46.

Barres BA, Schmid R, Sendnter M, Raff MC (1993): Multiple extracellular signals are required for long-term oligodendrocyte survival. Development 118: 283-295.

Barres BA, Lazar MA, Raff MC (1994a): A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development. Development 120: 1097-1108.

Barres BA, Raff MC, Gaese F, Bartke I, Dechant G, Barde YA (1994b): A crucial role for neurotrophin-3 in oligodendrocyte development. Nature 367: 371-375.

Bartolomei JC, Greer CA (2000): Olfactory ensheathing cells: bridging the gap in spinal cord injury. Neurosurgery 47: 1057-1069.

Basso DM, Beattie MS, Bresnahan JC (1995): A sensitive and reliable locomotor rating scale for open field testing in rats. J Neurotrauma 12: 1-21.

Baumann N, Pham-Dinh D (2001): Biology of oligodendrocyte and myelin in the mammalian central nervous system. Physiol Rev 81: 871-927.

Baumgarth N, Roederer M (2000): A practical approach to multicolor flow cytometry for immunophenotyping. J Immunol Methods 243: 77-97.

Behrstock S, Ebert A, McHugh J, Vosberg S, Moore J, Schneider B, Capowski E, Hei D, Kordower J, Aebischer P, Svendsen CN (2006): Human neural progenitors deliver glial cell line-derived neurotrophic factor to parkinsonian rodents and aged primates. Gene Ther 13: 379-388.

Bernard A, Boumsell L (1984): The clusters of differentiation (CD) defined by the First International Workshop on Human Leucocyte Differentiation Antigens. Hum Immunol 11: 1-10.

Bhatia M (2001): AC133 expression in human stem cells. Leukemia 15: 1685-1688.

Bhattacharya B, Miura T, Brandenberger R, Mejido J, Luo Y, Yang AX, Joshi BH, Ginis I, Thies RS, Amit M, Lyons I, Condie BG, Itskovitz-Eldor J, Rao MS, Puri RK (2004): Gene expression in human embryonic stem cell lines: unique molecular signature. Blood 103: 2956-2964.

Blits B, Bunge MB (2006): Direct gene therapy for repair of the spinal cord. J Neurotrauma 23: 508-520.

Bornhauser M, Eger L, Oelschlaegel U, Auffermann-Gretzinger S, Kiani A, Schetelig J, Illmer T, Schaich M, Corbeil D, Thiede C, Ehninger G (2005): Rapid reconstitution of dendritic cells after allogeneic transplantation of CD133+ selected hematopoietic stem cells. Leukemia 19: 161-165.

Bracken MB, Shepard MJ, Holford TR, Leo-Summers L, Aldrich EF, Fazl M, Fehlings M, Herr DL, Hitchon PW, Marshall LF, Nockels RP, Pascale V, Perot PL, Jr., Piepmeier J, Sonntag VK, Wagner F, Wilberger JE, Winn HR, Young W (1997): Administration of methylprednisolone for 24 or 48 hours or tirilazad mesylate for 48 hours in the treatment of acute spinal cord injury. Results of the Third National Acute Spinal Cord Injury Randomized Controlled Trial. National Acute Spinal Cord Injury Study. Jama 277: 1597-1604.

Brederlau A, Correia AS, Anisimov SV, Elmi M, Paul G, Roybon L, Morizane A, Bergquist F, Riebe I, Nannmark U, Carta M, Hanse E, Takahashi J, Sasai Y, Funa K, Brundin P, Eriksson PS, Li JY (2006): Transplantation of human embryonic stem cell-derived cells to a rat model of Parkinson's disease: effect of in vitro differentiation on graft survival and teratoma formation. Stem Cells 24: 1433-1440.

Brimble SN, Sherrer ES, Uhl EW, Wang E, Kelly S, Merrill AH, Jr., Robins AJ, Schulz TC (2007): The cell surface glycosphingolipids SSEA-3 and SSEA-4 are not essential for human ESC pluripotency. Stem Cells 25: 54-62.

Bruce JH, Norenberg MD, Kraydieh S, Puckett W, Marcillo A, Dietrich D (2000): Schwannosis: role of gliosis and proteoglycan in human spinal cord injury. J Neurotrauma 17: 781-788.

Brustle O, Jones KN, Learish RD, Karram K, Choudhary K, Wiestler OD, Duncan ID, McKay RD (1999): Embryonic stem cell-derived glial precursors: a source of myelinating transplants. Science 285: 754-756.

Buhring HJ, Battula VL, Treml S, Schewe B, Kanz L, Vogel W (2007): Novel markers for the prospective isolation of human MSC. Ann N Y Acad Sci 1106: 262-271.

Buzzard JJ, Gough NM, Crook JM, Colman A (2004): Karyotype of human ES cells during extended culture. Nat Biotechnol 22: 381-382; author reply 382.

Cai J, Zhu Q, Zheng K, Li H, Qi Y, Cao Q, Qiu M (2010): Co-localization of Nkx6.2 and Nkx2.2 homeodomain proteins in differentiated myelinating oligodendrocytes. Glia 58: 458-468.

Campagnoli C, Roberts IA, Kumar S, Bennett PR, Bellantuono I, Fisk NM (2001): Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood 98: 2396-2402.

Canoll PD, Musacchio JM, Hardy R, Reynolds R, Marchionni MA, Salzer JL (1996): GGF/neuregulin is a neuronal signal that promotes the proliferation and survival and inhibits the differentiation of oligodendrocyte progenitors. Neuron 17: 229-243.

Carpenter MK, Cui X, Hu ZY, Jackson J, Sherman S, Seiger A, Wahlberg LU (1999): In vitro expansion of a multipotent population of human neural progenitor cells. Exp Neurol 158: 265-278.

Carpenter MK, Inokuma MS, Denham J, Mujtaba T, Chiu CP, Rao MS (2001): Enrichment of neurons and neural precursors from human embryonic stem cells. Exp Neurol 172: 383-397.

Carpenter MK, Rosler ES, Fisk GJ, Brandenberger R, Ares X, Miura T, Lucero M, Rao MS (2004): Properties of four human embryonic stem cell lines maintained in a feeder-free culture system. Dev Dyn 229: 243-258.

Carpenter MK, Frey-Vasconcells J, Rao MS (2009): Developing safe therapies from human pluripotent stem cells. Nat Biotechnol 27: 606-613.

Carson MJ, Behringer RR, Brinster RL, McMorris FA (1993): Insulin-like growth factor I increases brain growth and central nervous system myelination in transgenic mice. Neuron 10: 729-740.

Casaccia-Bonnefil P, Gu C, Khursigara G, Chao MV (1999): p75 neurotrophin receptor as a modulator of survival and death decisions. Microsc Res Tech 45: 217-224.

Cate HS, Sabo JK, Merlo D, Kemper D, Aumann TD, Robinson J, Merson TD, Emery B, Perreau VM, Kilpatrick TJ (2010): Modulation of bone morphogenic protein signalling alters numbers of astrocytes and oligodendroglia in the

subventricular zone during cuprizone-induced demyelination. J Neurochem 115: 11-22.

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994): Green fluorescent protein as a marker for gene expression. Science 263: 802-805.

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A (2003): Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113: 643-655.

Chan CK, Chen CC, Luppen CA, Kim JB, DeBoer AT, Wei K, Helms JA, Kuo CJ, Kraft DL, Weissman IL (2009a): Endochondral ossification is required for haematopoietic stem-cell niche formation. Nature 457: 490-494.

Chan EM, Ratanasirintrawoot S, Park IH, Manos PD, Loh YH, Huo H, Miller JD, Hartung O, Rho J, Ince TA, Daley GQ, Schlaeger TM (2009b): Live cell imaging distinguishes bona fide human iPS cells from partially reprogrammed cells. Nat Biotechnol 27: 1033-1037.

Chen Y, Stevens B, Chang J, Milbrandt J, Barres BA, Hell JW (2008): NS21: re-defined and modified supplement B27 for neuronal cultures. J Neurosci Methods 171: 239-247.

Cho MS, Hwang DY, Kim DW (2008): Efficient derivation of functional dopaminergic neurons from human embryonic stem cells on a large scale. Nat Protoc 3: 1888-1894.

Chu K, Kim M, Park KI, Jeong SW, Park HK, Jung KH, Lee ST, Kang L, Lee K, Park DK, Kim SU, Roh JK (2004): Human neural stem cells improve sensorimotor deficits in the adult rat brain with experimental focal ischemia. Brain Res 1016: 145-153.

Chung S, Shin BS, Hedlund E, Pruszak J, Ferree A, Kang UJ, Isacson O, Kim KS (2006): Genetic selection of sox1GFP-expressing neural precursors removes residual tumorigenic pluripotent stem cells and attenuates tumor formation after transplantation. J Neurochem 97: 1467-1480.

Cizkova D, Rosocha J, Vanicky I, Jergova S, Cizek M (2006): Transplants of human mesenchymal stem cells improve functional recovery after spinal cord injury in the rat. Cell Mol Neurobiol 26: 1167-1180.

Cloutier F, Siegenthaler MM, Nistor G, Keirstead HS (2006): Transplantation of human embryonic stem cell-derived oligodendrocyte progenitors into rat spinal cord injuries does not cause harm. Regen Med 1: 469-479.

Colognato H, Galvin J, Wang Z, Relucio J, Nguyen T, Harrison D, Yurchenco PD, Ffrench-Constant C (2007): Identification of dystroglycan as a second laminin receptor in oligodendrocytes, with a role in myelination. Development 134: 1723-1736.

Coutts M, Keirstead HS (2008): Stem cells for the treatment of spinal cord injury. Exp Neurol 209: 368-377.

Crook J, Peura T, Kravets L, Boesman A, Buzzard J, Horne R, Hentze H, Dunn N, Zweigerdt R, Chua F, Upshall A, Colman A (2007): The generation of six clinical-grade human embryonic stem cell lines. Cell Stem Cell 1: 490-494.

Crowe MJ, Bresnahan JC, Shuman SL, Masters JN, Beattie MS (1997): Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys. Nat Med 3: 73-76.

Cummings BJ, Uchida N, Tamaki SJ, Salazar DL, Hooshmand M, Summers R, Gage FH, Anderson AJ (2005): Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. Proc Natl Acad Sci U S A 102: 14069-14074.

D'Arena G, Musto P, Cascavilla N, Carotenuto M (1998): Thy-1 (CDw90) and c-kit receptor (CD117) expression on CD34+ hematopoietic progenitor cells: a five dimensional flow cytometric study. Haematologica 83: 587-592.

Dahlke MH, Larsen SR, Rasko JE, Schlitt HJ (2004): The biology of CD45 and its use as a therapeutic target. Leuk Lymphoma 45: 229-236.

Darsalia V, Kallur T, Kokaia Z (2007): Survival, migration and neuronal differentiation of human fetal striatal and cortical neural stem cells grafted in stroke-damaged rat striatum. Eur J Neurosci 26: 605-614.

Davis SF, Hood J, Thomas A, Bunnell BA (2006): Isolation of adult rhesus neural stem and progenitor cells and differentiation into immature oligodendrocytes. Stem Cells Dev 15: 191-199.

Di Ianni M, Del Papa B, De Ioanni M, Moretti L, Bonifacio E, Cecchini D, Sportoletti P, Falzetti F, Tabilio A (2008): Mesenchymal cells recruit and regulate T regulatory cells. Exp Hematol 36: 309-318.

Ding WJ, Fang JY, Chen XY, Peng YS (2008): The expression and clinical significance of DNA methyltransferase proteins in human gastric cancer. Dig Dis Sci 53: 2083-2089.

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A (1999): Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97: 703-716.

Draper JS, Pigott C, Thomson JA, Andrews PW (2002): Surface antigens of human embryonic stem cells: changes upon differentiation in culture. J Anat 200: 249-258.

Durston AJ, Timmermans JP, Hage WJ, Hendriks HF, de Vries NJ, Heideveld M, Nieuwkoop PD (1989): Retinoic acid causes an anteroposterior transformation in the developing central nervous system. Nature 340: 140-144.

Ebert AD, Svendsen CN (2005): A new tool in the battle against Alzheimer's disease and aging: ex vivo gene therapy. Rejuvenation Res 8: 131-134.

Ellerstrom C, Strehl R, Moya K, Andersson K, Bergh C, Lundin K, Hyllner J, Semb H (2006): Derivation of a xeno-free human embryonic stem cell line. Stem Cells 24: 2170-2176.

Emgard M, Holmberg L, Samuelsson EB, Bahr BA, Falci S, Seiger A, Sundstrom E (2009): Human neural precursor cells continue to proliferate and exhibit low cell death after transplantation to the injured rat spinal cord. Brain Res 1278: 15-26.

Erceg S, Lainez S, Ronaghi M, Stojkovic P, Perez-Arago MA, Moreno-Manzano V, Moreno-Palanques R, Planells-Cases R, Stojkovic M (2008): Differentiation of human embryonic stem cells to regional specific neural precursors in chemically defined medium conditions. PLoS ONE 3: e2122.

Erceg S, Ronaghia M, Stojkovica M (2009): Human Embryonic Stem Cell Differentiation Toward Regional Specific Neural Precursors. Stem Cells 27: 78-87.

Erceg S, Ronaghi M, Oria M, Rosello MG, Arago MA, Lopez MG, Radojevic I, Moreno-Manzano V, Rodriguez-Jimenez FJ, Bhattacharya SS, Cordoba J, Stojkovic M (2010a): Transplanted oligodendrocytes and motoneuron progenitors generated from human embryonic stem cells promote locomotor recovery after spinal cord transection. Stem Cells 28: 1541-1549.

Erceg S, Ronaghi M, Zipancic I, Lainez S, Rosello MG, Xiong C, Moreno-Manzano V, Rodriguez-Jimenez FJ, Planells R, Alvarez-Dolado M, Bhattacharya SS, Stojkovic M (2010b): Efficient differentiation of human embryonic stem cells into functional cerebellar-like cells. Stem Cells Dev 19: 1745-1756.

Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998): Neurogenesis in the adult human hippocampus. Nat Med 4: 1313-1317.

Farrer RG, Quarles RH (1999): GT3 and its O-acetylated derivative are the principal A2B5-reactive gangliosides in cultured O2A lineage cells and are down-regulated along with O-acetyl GD3 during differentiation to oligodendrocytes. J Neurosci Res 57: 371-380.

Faulkner J, Keirstead HS (2005): Human embryonic stem cell-derived oligodendrocyte progenitors for the treatment of spinal cord injury. Transpl Immunol 15: 131-142.

Fawcett JW (2006): Overcoming inhibition in the damaged spinal cord. J Neurotrauma 23: 371-383.

Feron F, Perry C, Cochrane J, Licina P, Nowitzke A, Urquhart S, Geraghty T, Mackay-Sim A (2005): Autologous olfactory ensheathing cell transplantation in human spinal cord injury. Brain 128: 2951-2960.

Filbin MT (1996): The muddle with MAG. Mol Cell Neurosci 8: 84-92.

Finzsch M, Stolt CC, Lommes P, Wegner M (2008): Sox9 and Sox10 influence survival and migration of oligodendrocyte precursors in the spinal cord by regulating PDGF receptor alpha expression. Development 135: 637-646.

Fortunel NO, Otu HH, Ng HH, Chen J, Mu X, Chevassut T, Li X, Joseph M, Bailey C, Hatzfeld JA, Hatzfeld A, Usta F, Vega VB, Long PM, Libermann TA, Lim B (2003): Comment on " 'Stemness': transcriptional profiling of embryonic and adult stem cells" and "a stem cell molecular signature". Science 302: 393; author reply 393.

Franklin RJ, Barnett SC (2000): Olfactory ensheathing cells and CNS regeneration: the sweet smell of success? Neuron 28: 15-18.

Freed CR, Greene PE, Breeze RE, Tsai WY, DuMouchel W, Kao R, Dillon S, Winfield H, Culver S, Trojanowski JQ, Eidelberg D, Fahn S (2001): Transplantation of embryonic dopamine neurons for severe Parkinson's disease. N Engl J Med 344: 710-719.

Friedman B, Hockfield S, Black JA, Woodruff KA, Waxman SG (1989): In situ demonstration of mature oligodendrocytes and their processes: an immunocytochemical study with a new monoclonal antibody, rip. Glia 2: 380-390.

Frost EE, Buttery PC, Milner R, ffrench-Constant C (1999): Integrins mediate a neuronal survival signal for oligodendrocytes. Curr Biol 9: 1251-1254.

Fu H, Qi Y, Tan M, Cai J, Takebayashi H, Nakafuku M, Richardson W, Qiu M (2002): Dual origin of spinal oligodendrocyte progenitors and evidence for the cooperative role of Olig2 and Nkx2.2 in the control of oligodendrocyte differentiation. Development 129: 681-693.

Geeta R, Ramnath RL, Rao HS, Chandra V (2008): One year survival and significant reversal of motor deficits in parkinsonian rats transplanted with hESC derived dopaminergic neurons. Biochem Biophys Res Commun 373: 258-264.

Geffner LF, Santacruz P, Izurieta M, Flor L, Maldonado B, Auad AH, Montenegro X, Gonzalez R, Silva F (2008): Administration of autologous bone marrow stem cells into spinal cord injury patients via multiple routes is safe and improves their quality of life: comprehensive case studies. Cell Transplant 17: 1277-1293.

Gerrard L, Rodgers L, Cui W (2005): Differentiation of human embryonic stem cells to neural lineages in adherent culture by blocking bone morphogenetic protein signaling. Stem Cells 23: 1234-1241.

Gil JE, Woo DH, Shim JH, Kim SE, You HJ, Park SH, Paek SH, Kim SK, Kim JH (2009): Vitronectin promotes oligodendrocyte differentiation during neurogenesis of human embryonic stem cells. FEBS Lett 583: 561-567.

Gilbertson JA, Sen A, Behie LA, Kallos MS (2006): Scaled-up production of mammalian neural precursor cell aggregates in computer-controlled suspension bioreactors. Biotechnol Bioeng 94: 783-792.

Glaser T, Perez-Bouza A, Klein K, Brustle O (2005): Generation of purified oligodendrocyte progenitors from embryonic stem cells. Faseb J 19: 112-114.

Goldman S (2005): Stem and progenitor cell-based therapy of the human central nervous system. Nat Biotechnol 23: 862-871.

Goldman SA, Windrem MS (2006): Cell replacement therapy in neurological disease. Philos Trans R Soc Lond B Biol Sci 361: 1463-1475.

Gomes WA, Mehler MF, Kessler JA (2003): Transgenic overexpression of BMP4 increases astroglial and decreases oligodendroglial lineage commitment. Dev Biol 255: 164-177.

Gopalan A, Dhall D, Olgac S, Fine SW, Korkola JE, Houldsworth J, Chaganti RS, Bosl GJ, Reuter VE, Tickoo SK (2009): Testicular mixed germ cell tumors: a morphological and immunohistochemical study using stem cell markers, OCT3/4, SOX2 and GDF3, with emphasis on morphologically difficult-to-classify areas. Mod Pathol 22: 1066-1074.

Gould E, Woolley CS, McEwen BS (1990): Short-term glucocorticoid manipulations affect neuronal morphology and survival in the adult dentate gyrus. Neuroscience 37: 367-375.

Gould E, Cameron HA, Daniels DC, Woolley CS, McEwen BS (1992): Adrenal hormones suppress cell division in the adult rat dentate gyrus. J Neurosci 12: 3642-3650.

Gould E, Tanapat P (1997): Lesion-induced proliferation of neuronal progenitors in the dentate gyrus of the adult rat. Neuroscience 80: 427-436.

Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ (1999): Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci 2: 260-265.

Grinspan JB, Coulalaglou M, Beesley JS, Carpio DF, Scherer SS (1998): Maturation-dependent apoptotic cell death of oligodendrocytes in myelin-deficient rats. J Neurosci Res 54: 623-634.

Guest JD, Hiester ED, Bunge RP (2005): Demyelination and Schwann cell responses adjacent to injury epicenter cavities following chronic human spinal cord injury. Exp Neurol 192: 384-393.

Guillaume DJ, Zhang SC (2008): Human embryonic stem cells: a potential source of transplantable neural progenitor cells. Neurosurg Focus 24: E3.

Guzman R, De Los Angeles A, Cheshier S, Choi R, Hoang S, Liauw J, Schaar B, Steinberg G (2008): Intracarotid injection of fluorescence activated cell-sorted

CD49d-positive neural stem cells improves targeted cell delivery and behavior after stroke in a mouse stroke model. Stroke 39: 1300-1306.

Hains BC, Yucra JA, Hulsebosch CE (2001): Reduction of pathological and behavioral deficits following spinal cord contusion injury with the selective cyclooxygenase-2 inhibitor NS-398. J Neurotrauma 18: 409-423.

Hajihosseini M, Tham TN, Dubois-Dalcq M (1996): Origin of oligodendrocytes within the human spinal cord. J Neurosci 16: 7981-7994.

Hall ED, Springer JE (2004): Neuroprotection and acute spinal cord injury: a reappraisal. NeuroRx 1: 80-100.

Hall PE, Lathia JD, Miller NG, Caldwell MA, ffrench-Constant C (2006): Integrins are markers of human neural stem cells. Stem Cells 24: 2078-2084.

Hamann A, Arndt R, Klein P, Thiele HG (1980): Isolation and characterization of the thymus-brain antigen (analogous to thy-1 antigen) from human brain. Biochem J 187: 403-412.

Hardy R, Reynolds R (1991): Proliferation and differentiation potential of rat forebrain oligodendroglial progenitors both in vitro and in vivo. Development 111: 1061-1080.

Hargus G, Cooper O, Deleidi M, Levy A, Lee K, Marlow E, Yow A, Soldner F, Hockemeyer D, Hallett PJ, Osborn T, Jaenisch R, Isacson O (2010): Differentiated Parkinson patient-derived induced pluripotent stem cells grow in the adult rodent brain and reduce motor asymmetry in Parkinsonian rats. Proc Natl Acad Sci U S A 107: 15921-15926.

Hart IK, Richardson WD, Heldin CH, Westermark B, Raff MC (1989): PDGF receptors on cells of the oligodendrocyte-type-2 astrocyte (O-2A) cell lineage. Development 105: 595-603.

Hedlund E, Pruszak J, Lardaro T, Ludwig W, Vinuela A, Kim KS, Isacson O (2008): Embryonic stem cell-derived Pitx3-enhanced green fluorescent protein midbrain dopamine neurons survive enrichment by fluorescence-activated cell sorting and function in an animal model of Parkinson's disease. Stem Cells 26: 1526-1536.

Heikkilä TJ, Ylä-Outinen L, Tanskanen JM, Lappalainen RS, Skottman H, Suuronen R, Mikkonen JE, Hyttinen JA, Narkilahti S (2009): Human embryonic stem cell-derived neuronal cells form spontaneously active neuronal networks in vitro. Exp Neurol 218: 109-116.

Heins N, Englund MC, Sjoblom C, Dahl U, Tonning A, Bergh C, Lindahl A, Hanson C, Semb H (2004): Derivation, characterization, and differentiation of human embryonic stem cells. Stem Cells 22: 367-376.

Heinz M, Huang CA, Emery DW, Giovino MA, LeGuern A, Kurilla-Mahon B, Theodore P, Arn JS, Sykes M, Mulligan R, Down JD, Sachs DH, Goodell MA (2002): Use of CD9 expression to enrich for porcine hematopoietic progenitors. Exp Hematol 30: 809-815.

Heiskanen A, Satomaa T, Tiitinen S, Laitinen A, Mannelin S, Impola U, Mikkola M, Olsson C, Miller-Podraza H, Blomqvist M, Olonen A, Salo H, Lehenkari P, Tuuri T, Otonkoski T, Natunen J, Saarinen J, Laine J (2007): N-glycolylneuraminic acid xenoantigen contamination of human embryonic and mesenchymal stem cells is substantially reversible. Stem Cells 25: 197-202.

Henderson JK, Draper JS, Baillie HS, Fishel S, Thomson JA, Moore H, Andrews PW (2002): Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. Stem Cells 20: 329-337.

Hendriks WT, Ruitenberg MJ, Blits B, Boer GJ, Verhaagen J (2004): Viral vector-mediated gene transfer of neurotrophins to promote regeneration of the injured spinal cord. Prog Brain Res 146: 451-476.

Hentze H, Soong PL, Wang ST, Phillips BW, Putti TC, Dunn NR (2009): Teratoma formation by human embryonic stem cells: Evaluation of essential parameters for future safety studies. Stem Cell Res 2: 198-210.

Herzenberg LA, Sweet RG, Herzenberg LA (1976): Fluorescence-activated cell sorting. Sci Am 234: 108-117.

Herzenberg LA, Parks D, Sahaf B, Perez O, Roederer M, Herzenberg LA (2002): The history and future of the fluorescence activated cell sorter and flow cytometry: a view from Stanford. Clin Chem 48: 1819-1827.

Hexige S, Guo J, Ma L, Sun Y, Liu X, Ma L, Yan X, Li Z, Yu L (2005): Expression pattern of growth/differentiation factor 3 in human and murine cerebral cortex, hippocampus as well as cerebellum. Neurosci Lett 389: 83-87.

Holford LC, Case P, Lawson SN (1994): Substance P, neurofilament, peripherin and SSEA4 immunocytochemistry of human dorsal root ganglion neurons obtained from post-mortem tissue: a quantitative morphometric analysis. J Neurocytol 23: 577-589.

Horan PK, Slezak SE, Poste G (1986): Improved flow cytometric analysis of leukocyte subsets: simultaneous identification of five cell subsets using two-color immunofluorescence. Proc Natl Acad Sci U S A 83: 8361-8365.

Horiuchi M, Lindsten T, Pleasure D, Itoh T (2010): Differing in vitro survival dependency of mouse and rat NG2+ oligodendroglial progenitor cells. J Neurosci Res 88: 957-970.

Horky LL, Galimi F, Gage FH, Horner PJ (2006): Fate of endogenous stem/progenitor cells following spinal cord injury. J Comp Neurol 498: 525-538.

Hovatta O, Mikkola M, Gertow K, Stromberg AM, Inzunza J, Hreinsson J, Rozell B, Blennow E, Andang M, Ahrlund-Richter L (2003): A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. Hum Reprod 18: 1404-1409.

Hsieh J, Aimone JB, Kaspar BK, Kuwabara T, Nakashima K, Gage FH (2004): IGF-I instructs multipotent adult neural progenitor cells to become oligodendrocytes. J Cell Biol 164: 111-122.

Hu BY, Du ZW, Li XJ, Ayala M, Zhang SC (2009a): Human oligodendrocytes from embryonic stem cells: conserved SHH signaling networks and divergent FGF effects. Development 136: 1443-1452.

Hu Z, Li T, Zhang X, Chen Y (2009b): Hepatocyte growth factor enhances the generation of high-purity oligodendrocytes from human embryonic stem cells. Differentiation 78: 177-184.

Hulsebosch CE (2002): Recent advances in pathophysiology and treatment of spinal cord injury. Adv Physiol Educ 26: 238-255.

Hwang DH, Kim BG, Kim EJ, Lee SI, Joo IS, Suh-Kim H, Sohn S, Kim SU (2009): Transplantation of human neural stem cells transduced with Olig2 transcription factor improves locomotor recovery and enhances myelination in the white matter of rat spinal cord following contusive injury. BMC Neurosci 10: 117.

in 't Anker PS, Noort WA, Scherjon SA, Kleijburg-van der Keur C, Kruisselbrink AB, van Bezooijen RL, Beekhuizen W, Willemze R, Kanhai HH, Fibbe WE (2003a): Mesenchymal stem cells in human second-trimester bone marrow, liver, lung, and spleen exhibit a similar immunophenotype but a heterogeneous multilineage differentiation potential. Haematologica 88: 845-852.

in 't Anker PS, Scherjon SA, Kleijburg-van der Keur C, Noort WA, Claas FH, Willemze R, Fibbe WE, Kanhai HH (2003b): Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. Blood 102: 1548-1549.

Inzunza J, Gertow K, Stromberg MA, Matilainen E, Blennow E, Skottman H, Wolbank S, Ahrlund-Richter L, Hovatta O (2005): Derivation of human embryonic stem cell lines in serum replacement medium using postnatal human fibroblasts as feeder cells. Stem Cells 23: 544-549.

Irioka T, Watanabe K, Mizusawa H, Mizuseki K, Sasai Y (2005): Distinct effects of caudalizing factors on regional specification of embryonic stem cell-derived neural precursors. Brain Res Dev Brain Res 154: 63-70.

ISSCR (2008): Guidelines for the Clinical Translation of Stem Cells.

Itoh K, Tezuka H, Sakoda H, Konno M, Nagata K, Uchiyama T, Uchino H, Mori KJ (1989): Reproducible establishment of hemopoietic supportive stromal cell lines from murine bone marrow. Exp Hematol 17: 145-153.

Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, Soreq H, Benvenisty N (2000): Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med 6: 88-95.

Itsykson P, Ilouz N, Turetsky T, Goldstein RS, Pera MF, Fishbein I, Segal M, Reubinoff BE (2005): Derivation of neural precursors from human embryonic stem cells in the presence of noggin. Mol Cell Neurosci 30: 24-36.

Iwanami A, Kaneko S, Nakamura M, Kanemura Y, Mori H, Kobayashi S, Yamasaki M, Momoshima S, Ishii H, Ando K, Tanioka Y, Tamaoki N, Nomura T, Toyama Y, Okano H (2005): Transplantation of human neural stem cells for spinal cord injury in primates. J Neurosci Res 80: 182-190.

Ivanisevic M, Segerer S, Rieger L, Kapp M, Dietl J, Kammerer U, Frambach T (2010): Antigen-presenting cells in pregnant and non-pregnant human myometrium. Am J Reprod Immunol 64: 188-196.

Izrael M, Zhang P, Kaufman R, Shinder V, Ella R, Amit M, Itskovitz-Eldor J, Chebath J, Revel M (2007): Human oligodendrocytes derived from embryonic stem cells: Effect of noggin on phenotypic differentiation in vitro and on myelination in vivo. Mol Cell Neurosci 34: 310-323.

Jang S, Cho HH, Cho YB, Park JS, Jeong HS (2010): Functional neural differentiation of human adipose tissue-derived stem cells using bFGF and forskolin. BMC Cell Biol 11: 25.

Jeong SW, Chu K, Jung KH, Kim SU, Kim M, Roh JK (2003): Human neural stem cell transplantation promotes functional recovery in rats with experimental intracerebral hemorrhage. Stroke 34: 2258-2263.

Jones LL, Yamaguchi Y, Stallcup WB, Tuszynski MH (2002): NG2 is a major chondroitin sulfate proteoglycan produced after spinal cord injury and is expressed by macrophages and oligodendrocyte progenitors. J Neurosci 22: 2792-2803.

Jones LL, Margolis RU, Tuszynski MH (2003): The chondroitin sulfate proteoglycans neurocan, brevican, phosphacan, and versican are differentially regulated following spinal cord injury. Exp Neurol 182: 399-411.

Jones TB, McDaniel EE, Popovich PG (2005): Inflammatory-mediated injury and repair in the traumatically injured spinal cord. Curr Pharm Des 11: 1223-1236.

Kaiser S, Hackanson B, Follo M, Mehlhorn A, Geiger K, Ihorst G, Kapp U (2007): BM cells giving rise to MSC in culture have a heterogeneous CD34 and CD45 phenotype. Cytotherapy 9: 439-450.

Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P, Woltjen K (2009): Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature 458: 771-775.

Kang SM, Cho MS, Seo H, Yoon CJ, Oh SK, Choi YM, Kim DW (2007): Efficient induction of oligodendrocytes from human embryonic stem cells. Stem Cells 25: 419-424.

Kannagi R, Cochran NA, Ishigami F, Hakomori S, Andrews PW, Knowles BB, Solter D (1983): Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. Embo J 2: 2355-2361.

Katz AJ, Tholpady A, Tholpady SS, Shang H, Ogle RC (2005): Cell surface and transcriptional characterization of human adipose-derived adherent stromal (hADAS) cells. Stem Cells 23: 412-423.

Kawasaki H, Suemori H, Mizuseki K, Watanabe K, Urano F, Ichinose H, Haruta M, Takahashi M, Yoshikawa K, Nishikawa S, Nakatsuji N, Sasai Y (2002): Generation of dopaminergic neurons and pigmented epithelia from primate ES cells by stromal cell-derived inducing activity. Proc Natl Acad Sci U S A 99: 1580-1585.

Kehler J, Tolkunova E, Koschorz B, Pesce M, Gentile L, Boiani M, Lomeli H, Nagy A, McLaughlin KJ, Scholer HR, Tomilin A (2004): Oct4 is required for primordial germ cell survival. EMBO Rep 5: 1078-1083.

Keirstead HS, Nistor G, Bernal G, Totoiu M, Cloutier F, Sharp K, Steward O (2005): Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. J Neurosci 25: 4694-4705.

Kelly S, Bliss TM, Shah AK, Sun GH, Ma M, Foo WC, Masel J, Yenari MA, Weissman IL, Uchida N, Palmer T, Steinberg GK (2004): Transplanted human fetal neural stem cells survive, migrate, and differentiate in ischemic rat cerebral cortex. Proc Natl Acad Sci U S A 101: 11839-11844.

Kempermann G, Kuhn HG, Gage FH (1997): More hippocampal neurons in adult mice living in an enriched environment. Nature 386: 493-495.

Kempermann G, Jessberger S, Steiner B, Kronenberg G (2004): Milestones of neuronal development in the adult hippocampus. Trends Neurosci 27: 447-452.

Kempermann G, Chesler EJ, Lu L, Williams RW, Gage FH (2006): Natural variation and genetic covariance in adult hippocampal neurogenesis. Proc Natl Acad Sci U S A 103: 780-785.

Keyoung HM, Roy NS, Benraiss A, Louissaint A, Jr., Suzuki A, Hashimoto M, Rashbaum WK, Okano H, Goldman SA (2001): High-yield selection and extraction of two promoter-defined phenotypes of neural stem cells from the fetal human brain. Nat Biotechnol 19: 843-850.

Kil SH, Krull CE, Cann G, Clegg D, Bronner-Fraser M (1998): The alpha4 subunit of integrin is important for neural crest cell migration. Dev Biol 202: 29-42.

Kim I, Yilmaz OH, Morrison SJ (2005): CD144 (VE-cadherin) is transiently expressed by fetal liver hematopoietic stem cells. Blood 106: 903-905.

Kim K, Doi A, Wen B, Ng K, Zhao R, Cahan P, Kim J, Aryee MJ, Ji H, Ehrlich LI, Yabuuchi A, Takeuchi A, Cunniff KC, Hongguang H, McKinney-Freeman S, Naveiras O, Yoon TJ, Irizarry RA, Jung N, Seita J, Hanna J, Murakami P, Jaenisch R, Weissleder R, Orkin SH, Weissman IL, Feinberg AP, Daley GQ (2010): Epigenetic memory in induced pluripotent stem cells. Nature 467: 285-290.

Kim SU, de Vellis J (2009): Stem cell-based cell therapy in neurological diseases: a review. J Neurosci Res 87: 2183-2200.

Kishi Y, Tanaka Y, Shibata H, Nakamura S, Takeuchi K, Masuda S, Ikeda T, Muramatsu S, Hanazono Y (2008): Variation in the incidence of teratomas after the transplantation of nonhuman primate ES cells into immunodeficient mice. Cell Transplant 17: 1095-1102.

Klassen H, Schwartz MR, Bailey AH, Young MJ (2001): Surface markers expressed by multipotent human and mouse neural progenitor cells include tetraspanins and non-protein epitopes. Neurosci Lett 312: 180-182.

Knoblach SM, Huang X, VanGelderen J, Calva-Cerqueira D, Faden AI (2005): Selective caspase activation may contribute to neurological dysfunction after experimental spinal cord trauma. J Neurosci Res 80: 369-380.

Knoepfler PS (2009): Deconstructing stem cell tumorigenicity: a roadmap to safe regenerative medicine. Stem Cells 27: 1050-1056.

Knoller N, Auerbach G, Fulga V, Zelig G, Attias J, Bakimer R, Marder JB, Yoles E, Belkin M, Schwartz M, Hadani M (2005): Clinical experience using incubated autologous macrophages as a treatment for complete spinal cord injury: phase I study results. J Neurosurg Spine 3: 173-181.

Koivisto H, Hyvärinen M, Strömberg A-M, Inzunza J, Matilainen E, Mikkola M, Hovatta O, Teerijoki H (2004): Cultures of human embryonic stem cells - serum replacement medium or serum-containing media and the effect of basic fibrolast growth factor. Reproductive BioMedicine Online 9: 330-337.

Kozubenko N, Turnovcova K, Kapcalova M, Butenko O, Anderova M, Rusnakova V, Kubista M, Hampl A, Jendelova P, Sykova E (2009): Analysis of in vitro and in vivo characteristics of human embryonic stem cell-derived neural precursors. Cell Transplant.

Kudoh T, Wilson SW, Dawid IB (2002): Distinct roles for Fgf, Wnt and retinoic acid in posteriorizing the neural ectoderm. Development 129: 4335-4346.

Kuhn HG, Dickinson-Anson H, Gage FH (1996): Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci 16: 2027-2033.

Kuleshova LL, Tan FC, Magalhaes R, Gouk SS, Lee KH, Dawe GS (2009): Effective cryopreservation of neural stem or progenitor cells without serum or proteins by vitrification. Cell Transplant 18: 135-144.

Lajer H, Daugaard G, Andersson AM, Skakkebaek NE (2002): Clinical use of serum TRA-1-60 as tumor marker in patients with germ cell cancer. Int J Cancer 100: 244-246.

Lanier LL, Testi R, Bindl J, Phillips JH (1989): Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. J Exp Med 169: 2233-2238.

Lappalainen RS, Salomaki M, Yla-Outinen L, Heikkila TJ, Hyttinen JA, Pihlajamaki H, Suuronen R, Skottman H, Narkilahti S (2010): Similarly derived and cultured hESC lines show variation in their developmental potential towards neuronal cells in long-term culture. Regen Med 5: 749-762.

LaVaute TM, Yoo YD, Pankratz MT, Weick JP, Gerstner JR, Zhang SC (2009): Regulation of neural specification from human embryonic stem cells by BMP and FGF. Stem Cells 27: 1741-1749.

Lee H, Shamy GA, Elkabetz Y, Schofield CM, Harrsion NL, Panagiotakos G, Socci ND, Tabar V, Studer L (2007a): Directed differentiation and transplantation of human embryonic stem cell-derived motoneurons. Stem Cells 25: 1931-1939.

Lee HJ, Kim KS, Park IH, Kim SU (2007b): Human neural stem cells over-expressing VEGF provide neuroprotection, angiogenesis and functional recovery in mouse stroke model. PLoS One 2: e156.

Lee KH, Suh-Kim H, Choi JS, Jeun SS, Kim EJ, Kim SS, Yoon do H, Lee BH (2007c): Human mesenchymal stem cell transplantation promotes functional recovery following acute spinal cord injury in rats. Acta Neurobiol Exp (Wars) 67: 13-22.

Leung CT, Coulombe PA, Reed RR (2007): Contribution of olfactory neural stem cells to tissue maintenance and regeneration. Nat Neurosci 10: 720-726.

Levine AJ, Brivanlou AH (2006): GDF3, a BMP inhibitor, regulates cell fate in stem cells and early embryos. Development 133: 209-216.

Levine JM, Reynolds R, Fawcett JW (2001): The oligodendrocyte precursor cell in health and disease. Trends Neurosci 24: 39-47.

Li XJ, Du ZW, Zarnowska ED, Pankratz M, Hansen LO, Pearce RA, Zhang SC (2005): Specification of motoneurons from human embryonic stem cells. Nat Biotechnol 23: 215-221.

Li XJ, Hu BY, Jones SA, Zhang YS, Lavaute T, Du ZW, Zhang SC (2008): Directed differentiation of ventral spinal progenitors and motor neurons from human embryonic stem cells by small molecules. Stem Cells 26: 886-893.

Lian Q, Lye E, Suan Yeo K, Khia Way Tan E, Salto-Tellez M, Liu TM, Palanisamy N, El Oakley RM, Lee EH, Lim B, Lim SK (2007): Derivation of clinically compliant MSCs from CD105+, CD24- differentiated human ESCs. Stem Cells 25: 425-436.

Lima C, Escada P, Pratas-Vital J, Branco C, Arcangeli CA, Lazzeri G, Maia CA, Capucho C, Hasse-Ferreira A, Peduzzi JD (2010): Olfactory mucosal autografts and rehabilitation for chronic traumatic spinal cord injury. Neurorehabil Neural Repair 24: 10-22.

Lindvall O, Kokaia Z (2006): Stem cells for the treatment of neurological disorders. Nature 441: 1094-1096.

Litvinov SV, Balzar M, Winter MJ, Bakker HA, Briaire-de Bruijn IH, Prins F, Fleuren GJ, Warnaar SO (1997): Epithelial cell adhesion molecule (Ep-CAM) modulates cell-cell interactions mediated by classic cadherins. J Cell Biol 139: 1337-1348.

Livak KJ, Schmittgen TD (2001): Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 4: 402-408.

Looijenga LH, Stoop H, de Leeuw HP, de Gouveia Brazao CA, Gillis AJ, van Roozendaal KE, van Zoelen EJ, Weber RF, Wolffenbuttel KP, van Dekken H, Honecker F, Bokemeyer C, Perlman EJ, Schneider DT, Kononen J, Sauter G, Oosterhuis JW (2003): POU5F1 (OCT3/4) identifies cells with pluripotent potential in human germ cell tumors. Cancer Res 63: 2244-2250.

Lowdell MW, Theocharous P (1997): "Less is More": The Role of Purging in Hematopoietic Stem Cell Transplantation. Oncologist 2: 268-274.

Lu QR, Sun T, Zhu Z, Ma N, Garcia M, Stiles CD, Rowitch DH (2002): Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. Cell 109: 75-86.

Lu TY, Lu RM, Liao MY, Yu J, Chung CH, Kao CF, Wu HC (2010): Epithelial cell adhesion molecule regulation is associated with the maintenance of the undifferentiated phenotype of human embryonic stem cells. J Biol Chem 285: 8719-8732.

Ludwig T, Levenstein M, Jones J, Berggren W, Mitchen E, Frane J, Crandall L, Daigh C, Conard K, Piekarczyk M, Llanas R, Thomson J (2006): Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol 24: 185-187.

Luo YC, Zhang HT, Cheng HY, Yang ZJ, Dai YW, Xu RX (2010): Differentiation of cryopreserved human umbilical cord blood-derived stromal cells into cells with an oligodendrocyte phenotype. In Vitro Cell Dev Biol Anim 46: 585-589.

Ma W, Tavakoli T, Derby E, Serebryakova Y, Rao MS, Mattson MP (2008): Cell-extracellular matrix interactions regulate neural differentiation of human embryonic stem cells. BMC Dev Biol 8: 90.

Mackay-Sim A, Feron F, Cochrane J, Bassingthwaighte L, Bayliss C, Davies W, Fronek P, Gray C, Kerr G, Licina P, Nowitzke A, Perry C, Silburn PA, Urquhart S, Geraghty T (2008): Autologous olfactory ensheathing cell transplantation in human paraplegia: a 3-year clinical trial. Brain 131: 2376-2386.

Mallon BS, Park KY, Chen KG, Hamilton RS, McKay RD (2006): Toward xeno-free culture of human embryonic stem cells. Int J Biochem Cell Biol 38: 1063-1075.

Martin MJ, Muotri A, Gage F, Varki A (2005): Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat Med 11: 228-232.

Martins AA, Paiva A, Morgado JM, Gomes A, Pais ML (2009): Quantification and immunophenotypic characterization of bone marrow and umbilical cord blood mesenchymal stem cells by multicolor flow cytometry. Transplant Proc 41: 943-946.

Mayer M, Bhakoo K, Noble M (1994): Ciliary neurotrophic factor and leukemia inhibitory factor promote the generation, maturation and survival of oligodendrocytes in vitro. Development 120: 143-153.

McBride JL, Behrstock SP, Chen EY, Jakel RJ, Siegel I, Svendsen CN, Kordower JH (2004): Human neural stem cell transplants improve motor function in a rat model of Huntington's disease. J Comp Neurol 475: 211-219.

McKinnon RD, Matsui T, Dubois-Dalcq M, Aaronson SA (1990): FGF modulates the PDGF-driven pathway of oligodendrocyte development. Neuron 5: 603-614.

McMorris FA, Dubois-Dalcq M (1988): Insulin-like growth factor I promotes cell proliferation and oligodendroglial commitment in rat glial progenitor cells developing in vitro. J Neurosci Res 21: 199-209.

Mehler MF, Mabie PC, Zhu G, Gokhan S, Kessler JA (2000): Developmental changes in progenitor cell responsiveness to bone morphogenetic proteins differentially modulate progressive CNS lineage fate. Dev Neurosci 22: 74-85.

Mekki-Dauriac S, Agius E, Kan P, Cochard P (2002): Bone morphogenetic proteins negatively control oligodendrocyte precursor specification in the chick spinal cord. Development 129: 5117-5130.

Mendez I, Vinuela A, Astradsson A, Mukhida K, Hallett P, Robertson H, Tierney T, Holness R, Dagher A, Trojanowski JQ, Isacson O (2008): Dopamine neurons implanted into people with Parkinson's disease survive without pathology for 14 years. Nat Med 14: 507-509.

Mikkelsen TS, Hanna J, Zhang X, Ku M, Wernig M, Schorderet P, Bernstein BE, Jaenisch R, Lander ES, Meissner A (2008): Dissecting direct reprogramming through integrative genomic analysis. Nature 454: 49-55.

Milner R, Campbell IL (2002): The integrin family of cell adhesion molecules has multiple functions within the CNS. J Neurosci Res 69: 286-291.

Miltenyi S, Muller W, Weichel W, Radbruch A (1990): High gradient magnetic cell separation with MACS. Cytometry 11: 231-238.

Mimeault M, Hauke R, Batra SK (2007): Stem cells: a revolution in therapeutics-recent advances in stem cell biology and their therapeutic applications in regenerative medicine and cancer therapies. Clin Pharmacol Ther 82: 252-264.

Mitalipov S, Wolf D (2009): Totipotency, pluripotency and nuclear reprogramming. Adv Biochem Eng Biotechnol 114: 185-199.

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S (2003): The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113: 631-642.

Mizuguchi M, Otsuka N, Sato M, Ishii Y, Kon S, Yamada M, Nishina H, Katada T, Ikeda K (1995): Neuronal localization of CD38 antigen in the human brain. Brain Res 697: 235-240.

Mohle R, Bautz F, Rafii S, Moore MA, Brugger W, Kanz L (1998): The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. Blood 91: 4523-4530.

Moran DT, Rowley JC, 3rd, Jafek BW, Lovell MA (1982): The fine structure of the olfactory mucosa in man. J Neurocytol 11: 721-746.

Morshead CM, Craig CG, van der Kooy D (1998): In vivo clonal analyses reveal the properties of endogenous neural stem cell proliferation in the adult mammalian forebrain. Development 125: 2251-2261.

Moviglia GA, Fernandez Vina R, Brizuela JA, Saslavsky J, Vrsalovic F, Varela G, Bastos F, Farina P, Etchegaray G, Barbieri M, Martinez G, Picasso F, Schmidt Y, Brizuela P, Gaeta CA, Costanzo H, Moviglia Brandolino MT, Merino S, Pes ME, Veloso MJ, Rugilo C, Tamer I, Shuster GS (2006): Combined protocol of cell therapy for chronic spinal cord injury. Report on the electrical and functional recovery of two patients. Cytotherapy 8: 202-209.

Munz M, Kieu C, Mack B, Schmitt B, Zeidler R, Gires O (2004): The carcinoma-associated antigen EpCAM upregulates c-myc and induces cell proliferation. Oncogene 23: 5748-5758.

Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N, Yamanaka S (2008): Generation of induced pluripotent

stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol 26: 101-106.

Nakahara J, Seiwa C, Tan-Takeuchi K, Gotoh M, Kishihara K, Ogawa M, Asou H, Aiso S (2005): Involvement of CD45 in central nervous system myelination. Neurosci Lett 379: 116-121.

Nakamura Y, Handa K, Iwamoto R, Tsukamoto T, Takahasi M, Mekada E (2001): Immunohistochemical distribution of CD9, heparin binding epidermal growth factor-like growth factor, and integrin alpha3beta1 in normal human tissues. J Histochem Cytochem 49: 439-444.

Narkilahti S, Rajala K, Pihlajamaki H, Suuronen R, Hovatta O, Skottman H (2007): Monitoring and analysis of dynamic growth of human embryonic stem cells: comparison of automated instrumentation and conventional culturing methods. Biomed Eng Online 6: 11.

Nat R, Nilbratt M, Narkilahti S, Winblad B, Hovatta O, Nordberg A (2007): Neurogenic neuroepithelial and radial glial cells generated from six human embryonic stem cell lines in serum-free suspension and adherent cultures. Glia 55: 385-399.

Nesic O, Xu GY, McAdoo D, High KW, Hulsebosch C, Perez-Pol R (2001): IL-1 receptor antagonist prevents apoptosis and caspase-3 activation after spinal cord injury. J Neurotrauma 18: 947-956.

Ng VY, Ang SN, Chan JX, Choo AB (2010): Characterization of epithelial cell adhesion molecule as a surface marker on undifferentiated human embryonic stem cells. Stem Cells 28: 29-35.

Ni HT, Hu S, Sheng WS, Olson JM, Cheeran MC, Chan AS, Lokensgard JR, Peterson PK (2004): High-level expression of functional chemokine receptor CXCR4 on human neural precursor cells. Brain Res Dev Brain Res 152: 159-169.

Nicolay DJ, Doucette JR, Nazarali AJ (2007): Transcriptional control of oligodendrogenesis. Glia 55: 1287-1299.

Nilsson L, Astrand-Grundstrom I, Anderson K, Arvidsson I, Hokland P, Bryder D, Kjeldsen L, Johansson B, Hellstrom-Lindberg E, Hast R, Jacobsen SE (2002): Involvement and functional impairment of the CD34(+)CD38(-)Thy-1(+) hematopoietic stem cell pool in myelodysplastic syndromes with trisomy 8. Blood 100: 259-267.

Nishihira S, Okubo N, Takahashi N, Ishisaki A, Sugiyama Y, Chosa N (2010): High-cell density-induced VCAM1 expression inhibits the migratory ability of mesenchymal stem cells. Cell Biol Int. [Epub ahead of print].

Nishiyama A, Lin XH, Giese N, Heldin CH, Stallcup WB (1996): Co-localization of NG2 proteoglycan and PDGF alpha-receptor on O2A progenitor cells in the developing rat brain. J Neurosci Res 43: 299-314.

Nishiyama A, Komitova M, Suzuki R, Zhu X (2009): Polydendrocytes (NG2 cells): multifunctional cells with lineage plasticity. Nat Rev Neurosci 10: 9-22.

Nistor GI, Totoiu MO, Haque N, Carpenter MK, Keirstead HS (2005): Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation. Glia 49: 385-396.

Ohneda O, Ohneda K, Arai F, Lee J, Miyamoto T, Fukushima Y, Dowbenko D, Lasky LA, Suda T (2001): ALCAM (CD166): its role in hematopoietic and endothelial development. Blood 98: 2134-2142.

Okano H (2010): Neural stem cells and strategies for the regeneration of the central nervous system. Proc Jpn Acad Ser B Phys Biol Sci 86: 438-450.

Okita K, Ichisaka T, Yamanaka S (2007): Generation of germline-competent induced pluripotent stem cells. Nature 448: 313-317.

Ozdinler PH, Macklis JD (2006): IGF-I specifically enhances axon outgrowth of corticospinal motor neurons. Nat Neurosci 9: 1371-1381.

Paratore C, Goerich DE, Suter U, Wegner M, Sommer L (2001): Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling. Development 128: 3949-3961.

Park CH, Minn YK, Lee JY, Choi DH, Chang MY, Shim JW, Ko JY, Koh HC, Kang MJ, Kang JS, Rhie DJ, Lee YS, Son H, Moon SY, Kim KS, Lee SH (2005): In vitro and in vivo analyses of human embryonic stem cell-derived dopamine neurons. J Neurochem 92: 1265-1276.

Parker GC, Acsadi G, Brenner CA (2009): Mitochondria: determinants of stem cell fate? Stem Cells Dev 18: 803-806.

Peh GS, Lang RJ, Pera MF, Hawes SM (2009): CD133 expression by neural progenitors derived from human embryonic stem cells and its use for their prospective isolation. Stem Cells Dev 18: 269-282.

Peng H, Kolb R, Kennedy JE, Zheng J (2007): Differential expression of CXCL12 and CXCR4 during human fetal neural progenitor cell differentiation. J Neuroimmune Pharmacol 2: 251-258.

Perrier AL, Tabar V, Barberi T, Rubio ME, Bruses J, Topf N, Harrison NL, Studer L (2004): Derivation of midbrain dopamine neurons from human embryonic stem cells. Proc Natl Acad Sci U S A 101: 12543-12548.

Pevny L, Placzek M (2005): SOX genes and neural progenitor identity. Curr Opin Neurobiol 15: 7-13.

Piao JH, Odeberg J, Samuelsson EB, Kjaeldgaard A, Falci S, Seiger A, Sundstrom E, Akesson E (2006): Cellular composition of long-term human spinal cord- and forebrain-derived neurosphere cultures. J Neurosci Res 84: 471-482.

Pincus DW, Keyoung HM, Harrison-Restelli C, Goodman RR, Fraser RA, Edgar M, Sakakibara S, Okano H, Nedergaard M, Goldman SA (1998): Fibroblast growth factor-2/brain-derived neurotrophic factor-associated maturation of new neurons generated from adult human subependymal cells. Ann Neurol 43: 576-585.

Poncet C, Soula C, Trousse F, Kan P, Hirsinger E, Pourquie O, Duprat AM, Cochard P (1996): Induction of oligodendrocyte progenitors in the trunk neural tube by ventralizing signals: effects of notochord and floor plate grafts, and of sonic hedgehog. Mech Dev 60: 13-32.

Portmann-Lanz CB, Schoeberlein A, Huber A, Sager R, Malek A, Holzgreve W, Surbek DV (2006): Placental mesenchymal stem cells as potential autologous graft for pre- and perinatal neuroregeneration. Am J Obstet Gynecol 194: 664-673.

Portner R, Nagel-Heyer S, Goepfert C, Adamietz P, Meenen NM (2005): Bioreactor design for tissue engineering. J Biosci Bioeng 100: 235-245.

Pranke P, Hendrikx J, Debnath G, Alespeiti G, Rubinstein P, Nardi N, Visser J (2005): Immunophenotype of hematopoietic stem cells from placental/umbilical cord blood after culture. Braz J Med Biol Res 38: 1775-1789.

Pruszak J, Sonntag KC, Aung MH, Sanchez-Pernaute R, Isacson O (2007): Markers and methods for cell sorting of human embryonic stem cell-derived neural cell populations. Stem Cells 25: 2257-2268.

Pruszak J, Ludwig W, Blak A, Alavian K, Isacson O (2009): CD15, CD24, and CD29 define a surface biomarker code for neural lineage differentiation of stem cells. Stem Cells 27: 2928-2940.

Qi Y, Cai J, Wu Y, Wu R, Lee J, Fu H, Rao M, Sussel L, Rubenstein J, Qiu M (2001): Control of oligodendrocyte differentiation by the Nkx2.2 homeodomain transcription factor. Development 128: 2723-2733.

Raff MC, Mirsky R, Fields KL, Lisak RP, Dorfman SH, Silberberg DH, Gregson NA, Leibowitz S, Kennedy MC (1978): Galactocerebroside is a specific cell-surface antigenic marker for oligodendrocytes in culture. Nature 274: 813-816.

Rajala K, Hakala H, Panula S, Aivio S, Pihlajamaki H, Suuronen R, Hovatta O, Skottman H (2007): Testing of nine different xeno-free culture media for human embryonic stem cell cultures. Hum Reprod 22: 1231-1238.

Rajala K, Lindroos B, Hussein SM, Lappalainen RS, Pekkanen-Mattila M, Inzunza J, Rozell B, Miettinen S, Narkilahti S, Kerkela E, Aalto-Setala K, Otonkoski T, Suuronen R, Hovatta O, Skottman H (2010): A defined and xeno-free culture method enabling the establishment of clinical-grade human embryonic, induced pluripotent and adipose stem cells. PLoS One 5: e10246.

Ramon-Cueto A, Valverde F (1995): Olfactory bulb ensheathing glia: a unique cell type with axonal growth-promoting properties. Glia 14: 163-173.

Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A (2000): Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 18: 399-404.

Reubinoff BE, Itsykson P, Turetsky T, Pera MF, Reinhartz E, Itzik A, Ben-Hur T (2001): Neural progenitors from human embryonic stem cells. Nat Biotechnol 19: 1134-1140.

Richards M, Fong CY, Chan WK, Wong PC, Bongso A (2002): Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat Biotechnol 20: 933-936.

Richards M, Tan S, Fong CY, Biswas A, Chan WK, Bongso A (2003): Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells. Stem Cells 21: 546-556.

Rivera FJ, Kandasamy M, Couillard-Despres S, Caioni M, Sanchez R, Huber C, Weidner N, Bogdahn U, Aigner L (2008): Oligodendrogenesis of adult neural progenitors: differential effects of ciliary neurotrophic factor and mesenchymal stem cell derived factors. J Neurochem 107: 832-843.

Roisen FJ, Klueber KM, Lu CL, Hatcher LM, Dozier A, Shields CB, Maguire S (2001): Adult human olfactory stem cells. Brain Res 890: 11-22.

Rosner MH, Vigano MA, Ozato K, Timmons PM, Poirier F, Rigby PW, Staudt LM (1990): A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. Nature 345: 686-692.

Rowitch DH (2004): Glial specification in the vertebrate neural tube. Nat Rev Neurosci 5: 409-419.

Roy NS, Wang S, Harrison-Restelli C, Benraiss A, Fraser RA, Gravel M, Braun PE, Goldman SA (1999): Identification, isolation, and promoter-defined separation of mitotic oligodendrocyte progenitor cells from the adult human subcortical white matter. J Neurosci 19: 9986-9995.

Roy NS, Benraiss A, Wang S, Fraser RA, Goodman R, Couldwell WT, Nedergaard M, Kawaguchi A, Okano H, Goldman SA (2000a): Promoter-targeted selection and isolation of neural progenitor cells from the adult human ventricular zone. J Neurosci Res 59: 321-331.

Roy NS, Wang S, Jiang L, Kang J, Benraiss A, Harrison-Restelli C, Fraser RA, Couldwell WT, Kawaguchi A, Okano H, Nedergaard M, Goldman SA (2000b): In vitro neurogenesis by progenitor cells isolated from the adult human hippocampus. Nat Med 6: 271-277.

Roy NS, Cleren C, Singh SK, Yang L, Beal MF, Goldman SA (2006): Functional engraftment of human ES cell-derived dopaminergic neurons enriched by coculture with telomerase-immortalized midbrain astrocytes. Nat Med 12: 1259-1268.

Saberi H, Moshayedi P, Aghayan HR, Arjmand B, Hosseini SK, Emami-Razavi SH, Rahimi-Movaghar V, Raza M, Firouzi M (2008): Treatment of chronic thoracic spinal cord injury patients with autologous Schwann cell transplantation: an interim report on safety considerations and possible outcomes. Neurosci Lett 443: 46-50.

Sackstein R, Merzaban JS, Cain DW, Dagia NM, Spencer JA, Lin CP, Wohlgemuth R (2008): Ex vivo glycan engineering of CD44 programs human multipotent mesenchymal stromal cell trafficking to bone. Nat Med 14: 181-187.

Sahni V, Kessler JA (2010): Stem cell therapies for spinal cord injury. Nat Rev Neurol 6: 363-372.

Salazar DL, Uchida N, Hamers FP, Cummings BJ, Anderson AJ (2010): Human neural stem cells differentiate and promote locomotor recovery in an early chronic spinal cord injury NOD-scid mouse model. PLoS One 5: e12272.

Salewski RP, Eftekharpour E, Fehlings MG (2010): Are induced pluripotent stem cells the future of cell-based regenerative therapies for spinal cord injury? J Cell Physiol 222: 515-521.

Santos-Benito FF, Ramon-Cueto A (2003): Olfactory ensheathing glia transplantation: a therapy to promote repair in the mammalian central nervous system. Anat Rec B New Anat 271: 77-85.

Schmelzer E, Wauthier E, Reid LM (2006): The phenotypes of pluripotent human hepatic progenitors. Stem Cells 24: 1852-1858.

Schulz TC, Noggle SA, Palmarini GM, Weiler DA, Lyons IG, Pensa KA, Meedeniya AC, Davidson BP, Lambert NA, Condie BG (2004): Differentiation of human embryonic stem cells to dopaminergic neurons in serum-free suspension culture. Stem Cells 22: 1218-1238.

Schwartz PH, Bryant PJ, Fuja TJ, Su H, O'Dowd DK, Klassen H (2003): Isolation and characterization of neural progenitor cells from post-mortem human cortex. J Neurosci Res 74: 838-851.

Schwob JE (2002): Neural regeneration and the peripheral olfactory system. Anat Rec 269: 33-49.

Sen A, Kallos MS, Behie LA (2004): New tissue dissociation protocol for scaled-up production of neural stem cells in suspension bioreactors. Tissue Eng 10: 904-913.

Sharp J, Frame J, Siegenthaler M, Nistor G, Keirstead HS (2009): Human Embryonic Stem Cell-Derived Oligodendrocyte Progenitor Cell Transplants Improve Recovery after Cervical Spinal Cord Injury. Stem Cells 28: 152-163.

Sharp J, Frame J, Siegenthaler M, Nistor G, Keirstead HS (2010): Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants improve recovery after cervical spinal cord injury. Stem Cells 28: 152-163.

Shin S, Sun Y, Liu Y, Khaner H, Svant S, Cai J, Xu QX, Davidson BP, Stice SL, Smith AK, Goldman SA, Reubinoff BE, Zhan M, Rao MS, Chesnut JD (2007): Whole genome analysis of human neural stem cells derived from embryonic stem cells and stem and progenitor cells isolated from fetal tissue. Stem Cells 25: 1298-1306.

Shine HD, Readhead C, Popko B, Hood L, Sidman RL (1992): Morphometric analysis of normal, mutant, and transgenic CNS: correlation of myelin basic protein expression to myelinogenesis. J Neurochem 58: 342-349.

Silver J, Miller JH (2004): Regeneration beyond the glial scar. Nat Rev Neurosci 5: 146-156.

Singh RP, Shiue K, Schomberg D, Zhou FC (2009): Cellular epigenetic modifications of neural stem cell differentiation. Cell Transplant 18: 1197-1211.

Skottman H, Dilber S, Hovatta O (2006): The derivation of clinical-grade human embryonic stem cell lines. FEBS Letters 580: 2875-2878.

Skottman H, Narkilahti S, Hovatta O (2007): Challenges and approaches to the culture of pluripotent human embryonic stem cells. Regen Med 2: 265-273.

Skottman H (2010): Derivation and characterization of three new human embryonic stem cell lines in Finland. In Vitro Cell Dev Biol Anim 46: 206-209.

Soldner F, Hockemeyer D, Beard C, Gao Q, Bell GW, Cook EG, Hargus G, Blak A, Cooper O, Mitalipova M, Isacson O, Jaenisch R (2009): Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. Cell 136: 964-977.

Solly SK, Thomas JL, Monge M, Demerens C, Lubetzki C, Gardinier MV, Matthieu JM, Zalc B (1996): Myelin/oligodendrocyte glycoprotein (MOG) expression is associated with myelin deposition. Glia 18: 39-48.

Sonntag K-C, Pruszak J, Yoshizaki T, van Arensbergen J, Sanchez-Pernaute R, Isacson O (2007): Enhanced Yield of Neuroepithelial Precursors and Midbrain-Like Dopaminergic Neurons from Human Embryonic Stem Cells Using the Bone Morphogenic Protein Antagonist Noggin. Stem Cells 25: 411-418.

Sordi V, Malosio ML, Marchesi F, Mercalli A, Melzi R, Giordano T, Belmonte N, Ferrari G, Leone BE, Bertuzzi F, Zerbini G, Allavena P, Bonifacio E, Piemonti L (2005): Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. Blood 106: 419-427.

Stolt CC, Rehberg S, Ader M, Lommes P, Riethmacher D, Schachner M, Bartsch U, Wegner M (2002): Terminal differentiation of myelin-forming oligodendrocytes depends on the transcription factor Sox10. Genes Dev 16: 165-170.

Storstein A, Knudsen A, Bjorge L, Meri S, Vedeler C (2004): Heterogeneous expression of CD59 on human Purkinje cells. Neurosci Lett 362: 21-25.

Sun Y, Pollard S, Conti L, Toselli M, Biella G, Parkin G, Willatt L, Falk A, Cattaneo E, Smith A (2008): Long-term tripotent differentiation capacity of human neural stem (NS) cells in adherent culture. Mol Cell Neurosci 38: 245-258.

Suss-Toby E, Gerecht-Nir S, Amit M, Manor D, Itskovitz-Eldor J (2004): Derivation of a diploid human embryonic stem cell line from a mononuclear zygote. Hum Reprod 19: 670-675.

Suzuki M, McHugh J, Tork C, Shelley B, Klein SM, Aebischer P, Svendsen CN (2007): GDNF secreting human neural progenitor cells protect dying motor neurons, but not their projection to muscle, in a rat model of familial ALS. PLoS One 2: e689.

Swistowski A, Peng J, Han Y, Swistowska AM, Rao MS, Zeng X (2009): Xeno-free defined conditions for culture of human embryonic stem cells, neural stem cells and dopaminergic neurons derived from them. PLoS One 4: e6233.

Sykova E, Homola A, Mazanec R, Lachmann H, Konradova SL, Kobylka P, Padr R, Neuwirth J, Komrska V, Vavra V, Stulik J, Bojar M (2006): Autologous bone marrow transplantation in patients with subacute and chronic spinal cord injury. Cell Transplant 15: 675-687.

Takahashi K, Yamanaka S (2006): Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126: 663-676.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007): Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131: 861-872.

Tamaki S, Eckert K, He D, Sutton R, Doshe M, Jain G, Tushinski R, Reitsma M, Harris B, Tsukamoto A, Gage F, Weissman I, Uchida N (2002): Engraftment of sorted/expanded human central nervous system stem cells from fetal brain. J Neurosci Res 69: 976-986.

Tator CH (1995): Update on the pathophysiology and pathology of acute spinal cord injury. Brain Pathol 5: 407-413.

Tator CH (1996): Experimental and clinical studies of the pathophysiology and management of acute spinal cord injury. J Spinal Cord Med 19: 206-214.

Tator CH (1998): Biology of neurological recovery and functional restoration after spinal cord injury. Neurosurgery 42: 696-707; discussion 707-698.

Taussig DC, Pearce DJ, Simpson C, Rohatiner AZ, Lister TA, Kelly G, Luongo JL, Danet-Desnoyers GA, Bonnet D (2005): Hematopoietic stem cells express multiple myeloid markers: implications for the origin and targeted therapy of acute myeloid leukemia. Blood 106: 4086-4092.

Taylor CT, Johnson PM (1996): Complement-binding proteins are strongly expressed by human preimplantation blastocysts and cumulus cells as well as gametes. Mol Hum Reprod 2: 52-59.

Teng YD, Lavik EB, Qu X, Park KI, Ourednik J, Zurakowski D, Langer R, Snyder EY (2002): Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells. Proc Natl Acad Sci U S A 99: 3024-3029.

Terstappen LW, Nguyen M, Lazarus HM, Medof ME (1992): Expression of the DAF (CD55) and CD59 antigens during normal hematopoietic cell differentiation. J Leukoc Biol 52: 652-660.

Thomson A, Wojtacha D, Hewitt Z, Priddle H, Sottile V, Di Domenico A, Fletcher J, Waterfall M, Corrales NL, Ansell R, McWhir J (2008): Human embryonic stem cells passaged using enzymatic methods retain a normal karyotype and express CD30. Cloning Stem Cells 10: 89-106.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998): Embryonic stem cell lines derived from human blastocysts. Science 282: 1145-1147.

Thuret S, Moon LD, Gage FH (2006): Therapeutic interventions after spinal cord injury. Nat Rev Neurosci 7: 628-643.

Tocci A, Forte L (2003): Mesenchymal stem cell: use and perspectives. Hematol J 4: 92-96.

Tong J, Gordon MS, Srour EF, Cooper RJ, Orazi A, McNiece I, Hoffman R (1993): In vivo administration of recombinant methionyl human stem cell factor expands the number of human marrow hematopoietic stem cells. Blood 82: 784-791.

Tricot G, Gazitt Y, Leemhuis T, Jagannath S, Desikan KR, Siegel D, Fassas A, Tindle S, Nelson J, Juttner C, Tsukamoto A, Hallagan J, Atkinson K, Reading C, Hoffman R, Barlogie B (1998): Collection, tumor contamination, and engraftment kinetics of highly purified hematopoietic progenitor cells to support high dose therapy in multiple myeloma. Blood 91: 4489-4495.

Tsuji O, Miura K, Okada Y, Fujiyoshi K, Mukaino M, Nagoshi N, Kitamura K, Kumagai G, Nishino M, Tomisato S, Higashi H, Nagai T, Katoh H, Kohda K, Matsuzaki Y, Yuzaki M, Ikeda E, Toyama Y, Nakamura M, Yamanaka S, Okano H (2010): From the Cover: Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury. Proc Natl Acad Sci U S A 107: 12704-12709.

Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV, Tsukamoto AS, Gage FH, Weissman IL (2000): Direct isolation of human central nervous system stem cells. Proc Natl Acad Sci U S A 97: 14720-14725.

Unger C, Skottman H, Blomberg P, Dilber MS, Hovatta O (2008): Good manufacturing practice and clinical-grade human embryonic stem cell lines. Hum Mol Genet 17: R48-53.

van Praag H, Christie BR, Sejnowski TJ, Gage FH (1999): Running enhances neurogenesis, learning, and long-term potentiation in mice. Proc Natl Acad Sci U S A 96: 13427-13431.

Wang S, Sdrulla AD, diSibio G, Bush G, Nofziger D, Hicks C, Weinmaster G, Barres BA (1998a): Notch receptor activation inhibits oligodendrocyte differentiation. Neuron 21: 63-75.

Wang S, Wu H, Jiang J, Delohery TM, Isdell F, Goldman SA (1998b): Isolation of neuronal precursors by sorting embryonic forebrain transfected with GFP regulated by the T alpha 1 tubulin promoter. Nat Biotechnol 16: 196-201.

Wang WH, Sun XF (2005): Human embryonic stem cell lines are contaminated: what should we do? Hum Reprod 20: 2987-2989.

Vazin T, Chen J, Lee CT, Amable R, Freed WJ (2008): Assessment of stromal-derived inducing activity in the generation of dopaminergic neurons from human embryonic stem cells. Stem Cells 26: 1517-1525.

Vedeler C, Ulvestad E, Bjorge L, Conti G, Williams K, Mork S, Matre R (1994): The expression of CD59 in normal human nervous tissue. Immunology 82: 542-547.

Wernig M, Tucker KL, Gornik V, Schneiders A, Buschwald R, Wiestler OD, Barde YA, Brustle O (2002): Tau EGFP embryonic stem cells: an efficient tool for neuronal lineage selection and transplantation. J Neurosci Res 69: 918-924.

Vescovi AL, Gritti A, Galli R, Parati EA (1999a): Isolation and intracerebral grafting of nontransformed multipotential embryonic human CNS stem cells. J Neurotrauma 16: 689-693.

Vescovi AL, Parati EA, Gritti A, Poulin P, Ferrario M, Wanke E, Frolichsthal-Schoeller P, Cova L, Arcellana-Panlilio M, Colombo A, Galli R (1999b): Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. Exp Neurol 156: 71-83.

Vierbuchen T, Ostermeier A, Pang ZP, Kokubu Y, Sudhof TC, Wernig M (2010): Direct conversion of fibroblasts to functional neurons by defined factors. Nature 463: 1035-1041.

Wilson HC, Scolding NJ, Raine CS (2006): Co-expression of PDGF alpha receptor and NG2 by oligodendrocyte precursors in human CNS and multiple sclerosis lesions. J Neuroimmunol 176: 162-173.

Wineman JP, Gilmore GL, Gritzmacher C, Torbett BE, Muller-Sieburg CE (1992): CD4 is expressed on murine pluripotent hematopoietic stem cells. Blood 80: 1717-1724.

Winstead W, Marshall CT, Lu CL, Klueber KM, Roisen FJ (2005): Endoscopic biopsy of human olfactory epithelium as a source of progenitor cells. Am J Rhinol 19: 83-90.

Vogel H, Butcher EC, Picker LJ (1992): H-CAM expression in the human nervous system: evidence for a role in diverse glial interactions. J Neurocytol 21: 363-373.

Vogel W, Grunebach F, Messam CA, Kanz L, Brugger W, Buhring HJ (2003): Heterogeneity among human bone marrow-derived mesenchymal stem cells and neural progenitor cells. Haematologica 88: 126-133.

Woltjen K, Michael IP, Mohseni P, Desai R, Mileikovsky M, Hamalainen R, Cowling R, Wang W, Liu P, Gertsenstein M, Kaji K, Sung HK, Nagy A (2009): piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature 458: 766-770.

Vose JM, Bierman PJ, Lynch JC, Atkinson K, Juttner C, Hanania CE, Bociek G, Armitage JO (2001): Transplantation of highly purified CD34+Thy-1+ hematopoietic stem cells in patients with recurrent indolent non-Hodgkin's lymphoma. Biol Blood Marrow Transplant 7: 680-687.

Wright LS, Prowse KR, Wallace K, Linskens MH, Svendsen CN (2006): Human progenitor cells isolated from the developing cortex undergo decreased neurogenesis and eventual senescence following expansion in vitro. Exp Cell Res 312: 2107-2120.

Wu P, Tarasenko YI, Gu Y, Huang LY, Coggeshall RE, Yu Y (2002): Region-specific generation of cholinergic neurons from fetal human neural stem cells grafted in adult rat. Nat Neurosci 5: 1271-1278.

Wynn RF, Hart CA, Corradi-Perini C, O'Neill L, Evans CA, Wraith JE, Fairbairn LJ, Bellantuono I (2004): A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. Blood 104: 2643-2645.

Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK (2001): Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 19: 971-974.

Yamamoto S, Yamamoto N, Kitamura T, Nakamura K, Nakafuku M (2001): Proliferation of parenchymal neural progenitors in response to injury in the adult rat spinal cord. Exp Neurol 172: 115-127.

Yang CC, Shih YH, Ko MH, Hsu SY, Cheng H, Fu YS (2008): Transplantation of human umbilical mesenchymal stem cells from Wharton's jelly after complete transection of the rat spinal cord. PLoS One 3: e3336.

Yang H, Lu P, McKay HM, Bernot T, Keirstead H, Steward O, Gage FH, Edgerton VR, Tuszynski MH (2006): Endogenous neurogenesis replaces oligodendrocytes and astrocytes after primate spinal cord injury. J Neurosci 26: 2157-2166.

Yang X, Skoff RP (1997): Proteolipid protein regulates the survival and differentiation of oligodendrocytes. J Neurosci 17: 2056-2070.

Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG, Olweus J, Kearney J, Buck DW (1997): AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 90: 5002-5012.

Yoon SH, Shim YS, Park YH, Chung JK, Nam JH, Kim MO, Park HC, Park SR, Min BH, Kim EY, Choi BH, Park H, Ha Y (2007): Complete spinal cord injury treatment using autologous bone marrow cell transplantation and bone marrow stimulation with granulocyte macrophage-colony stimulating factor: Phase I/II clinical trial. Stem Cells 25: 2066-2073.

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin, II, Thomson JA (2007): Induced pluripotent stem cell lines derived from human somatic cells. Science 318: 1917-1920.

Yu J, Hu K, Smuga-Otto K, Tian S, Stewart R, Slukvin, II, Thomson JA (2009): Human induced pluripotent stem cells free of vector and transgene sequences. Science 324: 797-801.

Zaehres H, Lensch MW, Daheron L, Stewart SA, Itskovitz-Eldor J, Daley GQ (2005): High-efficiency RNA interference in human embryonic stem cells. Stem Cells 23: 299-305.

Zaka M, Rafi MA, Rao HZ, Luzi P, Wenger DA (2005): Insulin-like growth factor-1 provides protection against psychosine-induced apoptosis in cultured mouse oligodendrocyte progenitor cells using primarily the PI3K/Akt pathway. Mol Cell Neurosci 30: 398-407.

Zambidis ET, Peault B, Park TS, Bunz F, Civin CI (2005): Hematopoietic differentiation of human embryonic stem cells progresses through sequential hematoendothelial, primitive, and definitive stages resembling human yolk sac development. Blood 106: 860-870.

Zeigler FC, Bennett BD, Jordan CT, Spencer SD, Baumhueter S, Carroll KJ, Hooley J, Bauer K, Matthews W (1994): Cellular and molecular characterization of the role of the flk-2/flt-3 receptor tyrosine kinase in hematopoietic stem cells. Blood 84: 2422-2430.

Zhang PL, Izrael M, Ainbinder E, Ben-Simchon L, Chebath J, Revel M (2006a): Increased myelinating capacity of embryonic stem cell derived oligodendrocyte precursors after treatment by interleukin-6/soluble interleukin-6 receptor fusion protein. Mol Cell Neurosci 31: 387-398.

Zhang SC, Ge B, Duncan ID (2000): Tracing human oligodendroglial development in vitro. J Neurosci Res 59: 421-429.

Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA (2001): In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol 19: 1129-1133.

Zhang SC (2006): Neural subtype specification from embryonic stem cells. Brain Pathol 16: 132-142.

Zhang X, Cai J, Klueber KM, Guo Z, Lu C, Qiu M, Roisen FJ (2005): Induction of oligodendrocytes from adult human olfactory epithelial-derived progenitors by transcription factors. Stem Cells 23: 442-453.

Zhang YW, Denham J, Thies RS (2006b): Oligodendrocyte progenitor cells derived from human embryonic stem cells express neurotrophic factors. Stem Cells Dev 15: 943-952.

Zhou H, Wu S, Joo JY, Zhu S, Han DW, Lin T, Trauger S, Bien G, Yao S, Zhu Y, Siuzdak G, Scholer HR, Duan L, Ding S (2009): Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell 4: 381-384.

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REGULAR ARTICLE

CD marker expression profiles of human embryonic stem cells and their neural derivatives, determined using flow-cytometric analysis, reveal a novel CD marker for exclusion of pluripotent stem cells

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Abstract Human embryonic stem cells (hESCs) are pluripotent cells that can differentiate into neural cell lineages. These neural populations are usually heterogeneous and can contain undifferentiated pluripotent cells that are capable of producing teratomas in cell grafts. The characterization of surface protein profiles of hESCs and their neural derivatives is important to determine the specific markers that can be used to exclude undifferentiated cells from neural populations. In this study, we analyzed the cluster of differentiation (CD) marker expression profiles of seven undifferentiated hESC lines using flow-cytometric analysis and compared their profiles to those of neural derivatives. Stem cell and progenitor marker CD133 and epithelial adhesion molecule marker CD326 were more highly expressed in undifferentiated hESCs, whereas neural marker CD56 (NCAM) and neural precursor marker (chemokine receptor) CD184 were more highly expressed in hESC-derived neural cells. CD326 expression levels were consistently higher in all nondifferentiated hESC lines than in neural cell derivatives. In addition, CD326-positive hESCs produced teratomas in SCID mouse testes, whereas CD362-negative neural populations did not. Thus, CD326 may be useful as a novel marker of undifferentiated hESCs to exclude undifferentiated hESCs from differentiated neural cell populations prior to transplantation.

Introduction

Human embryonic stem cells (hESCs) are pluripotent cells derived from the inner cell mass of preimplantation embryos

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that can be expanded in large amounts in culture in an undifferentiated state (Reubinoff et al., 2000; Thomson et al., 1998). The expression of specific markers is linked to the maintenance of hESC pluripotency and self-renewal. Such markers include the transcription factors Oct-4 and Nanog (Hart et al., 2004; Reubinoff et al., 2000) and various cell surface markers, such as the stage-specific embryonic glycolipid antigens (SSEA) 3 and 4, the keratan sulfate-related antigens TRA-1-60 and TRA-1-81, and alkaline phosphatase (Inzunza et al., 2005; Reubinoff et al., 2000; Thomson et al., 1998). SSEA-3 and SSEA-4, however, are not critical for maintaining hESC pluripotency (Brimble et al., 2006). The results of a recent large study by the International Stem Cell Initiative (ISCI) indicated that a significant proportion of hESC lines do not express SSEA-3 (ISCI, 2007). Also, SSEA-4 is expressed by a subset of dorsal root ganglion cells (Holford et al., 1994), by dissociated fetal forebrain and spinal cord tissues (Piao et al., 2006), and by early neuroepithelial cells in the developing forebrain (Barraud et al., 2007).

Human ESCs can differentiate into all cell types of the human body. By following established differentiation protocols, hESCs may be induced to differentiate into cells of neuroectodermal lineage, producing cells of neural origin (Carpenter et al., 2001; Reubinoff et al., 2001; Zhang et al., 2001). Early neural differentiation is characterized by the expression of the neuroectodermal transcription factors Pax6 and Sox1, the intermediate filament protein nestin, and the neural cell adhesion molecule (NCAM, CD56) (Nat et al., 2007; Reubinoff et al., 2001; Zhang et al., 2001). Maturing neurons are distinguished by morphologic features and express the neural markers β-tubulin$_{III}$, microtubule-associated protein-2 (MAP-2), and neurofilament proteins, among others (Nat et al., 2007; Reubinoff et al., 2001; Zhang et al., 2001). Upon transplantation, hESC-derived neural progenitor cells positive for the markers Pax6, nestin, and NCAM integrate into the host brains of newborn mice, migrate, and differentiate into neurons, astrocytes, and oligodendrocytes (Reubinoff et al., 2001; Zhang et al., 2001). Thus, neural cells derived from hESCs are a possible donor source for cell transplants to be used in the treatment of various neurodegenerative injuries and diseases (Reubinoff et al., 2001; Zhang et al., 2001). Due to the risk of teratoma formation, however, the transplanted neural population should not contain any undifferentiated hESCs.

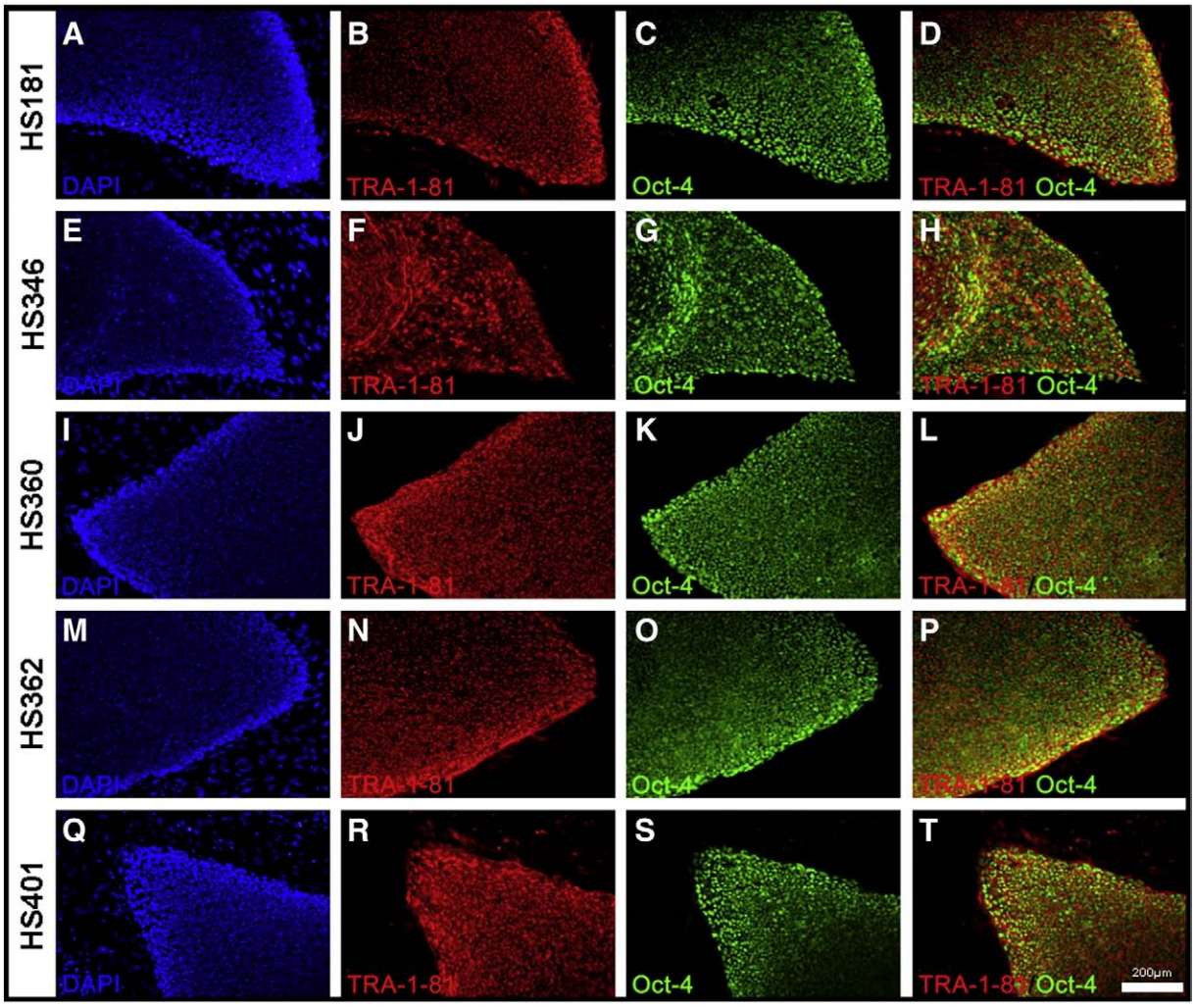

Figure 1 Immunofluorescent staining to characterize undifferentiated hESCs. hESCs expressing (B, F, J, N, R) TRA-1-81 and (C, G, K, O, S) Oct-4 and (D, H, L, P, T) merged TRA-1-81 and Oct-4 images. (A, E, I, M, Q) Nuclear staining is shown in blue (DAPI). (A–D) HS181, (E–H) HS346, (I–L) HS360, (M–P) HS362, (Q–T) HS401. Scale bar, 200 μm.

Cluster of differentiation (CD) markers are surface proteins that belong to several different classes, such as integrins, adhesion molecules, glycoproteins, and receptors. Antibodies recognizing CD markers are frequently used to identify and characterize various cell populations. The CD markers associated with pluripotent hESCs are CD9, CD24, and CD133 (Assou et al., 2007; Bhattacharya et al., 2004; Carpenter et al., 2004; Lian et al., 2006; Skottman et al., 2005; Zambidis et al., 2005). In addition, hESCs express markers such as CD29, CD90, and CD117 (Carpenter et al., 2004; Draper et al., 2002; Lian et al., 2006; Xu et al., 2001). These and other markers are also associated with neural stem cells (NSCs) and neural precursor cells (NPCs) and with mature neurons and/or glial cells in the adult human central nervous system (CNS) and peripheral nervous system (Supplemental Table 1).

Thus, CD markers are a useful tool for studying the differentiation of living cells (Pruszack et al., 2007). Furthermore, these markers can be used to isolate specific cell populations based on their surface marker expression profile using techniques such as immunopanning, magnetic cell sorting, and fluorescence-activated cell sorting (FACS). For example, $CD133^{+}$ human CNS stem cells isolated from fetal brain tissue using FACS differentiate into neural cells when engrafted into the brains of immunodeficient newborn mice (Tamaki et al., 2002; Uchida et al., 2000). The hESC-derived neural populations must be carefully characterized and purified prior to transplantation to avoid

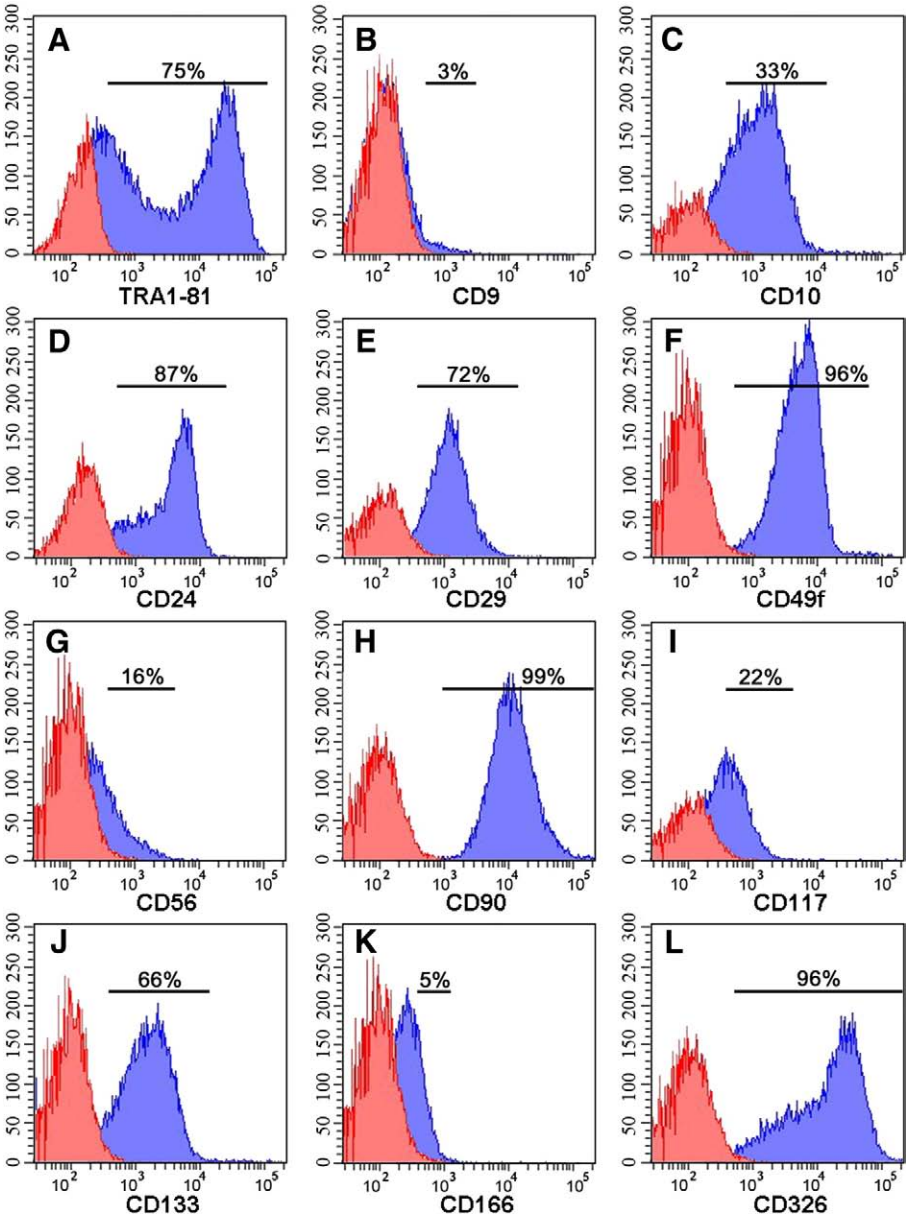

Figure 2 Flow-cytometric analysis of undifferentiated hESCs. Representative FACS plots of hESC line HS181 expressing (A) TRA-1-81, and CD marker expression by undifferentiated TRA-1-81-positive hESCs: (B) CD9, (C) CD10, (D) CD24, (E) CD29, (F) CD49f, (G) CD56, (H) CD90, (I) CD117, (J) CD133, (K) CD166, and (L) CD326.

tumor formation *in vivo* caused by undifferentiated hESCs. To characterize the cells, the expression patterns of specific markers in undifferentiated hESCs need to be validated.

In this study, the expression of a panel of CD markers, some of which were previously associated with hESCs, was compared in five hESC lines derived and cultured similarly (Hovatta et al., 2003, Inzunza et al., 2005). An expanded panel consisting of 30 surface markers was used to characterize undifferentiated TRA-1-81-positive hESCs and their neural derivatives. The expression patterns of the most interesting CD markers were further studied in two hESC lines derived and cultured in-house.

Results

hESC culture and neural differentiation

The undifferentiated state of hESCs was confirmed by positive staining for Nanog, Oct-4, and TRA-1-81 (Figs. 1 and 4), and SSEA-3 (data not shown), and by expression of *Nanog* and *Oct-4* (Supplemental Fig. 1). The neural cultures were monitored and characterized morphologically using the Cell-IQ online monitoring culture platform (Supplemental Movie 1) and immunocytochemically using MAP-2 (Supplemental Fig. 2), nestin, β-tubulin$_{III}$, BLBP, and GFAP (data not shown here, see Nat et al., 2007). The expression of *Pax6, nestin, Mash1,* and *MAP-2* was confirmed in neural cultures (Supplemental Fig. 1).

Surface protein expression profiles of hESCs

The hESCs (from lines HS181, HS346, HS360, HS362, and HS401) were labeled with TRA-1-81 to identify undifferentiated cells and colabeled with 11 CD markers (CD9, CD10, CD24, CD29, CD49f, CD56, CD90, CD117, CD133, CD166, and CD326). Representative FACS plots are presented in Fig. 2. The CD marker expression in undifferentiated hESCs from five hESC lines is shown in Fig. 3. The expression profile was categorized as follows: 0–2%, negative; 3–20%, weak; 21–50%, medium; 51–80%, medium high; >80%, high (Fig. 3). For analysis details see Supplemental Table 2.

Flow-cytometric analysis of undifferentiated hESCs (HS lines) showed high expression of CD24, CD90, and CD326 in all hESC lines (Fig. 3). As CD326 has not been previously associated with hESCs, its expression was confirmed immunocytochemically (Fig. 4). Staining showed that CD326 was expressed on the surface of Oct-4 and Nanog-positive hESCs (Fig. 4). Also, the hESC lines derived in REGEA expressed CD326 (Figs. 4 and 5). As Fig. 3 shows, expression of CD49f and CD133 was also high or medium high in all five hESC lines. Expression of CD10 was medium or medium high in all hESC lines, while that of CD9, CD56, CD117, and CD166 was weak or absent. Expression of marker CD29 was heterogeneous in the five hESC lines analyzed, varying from 13 to 72%.

Surface protein expression profiles of hESCs and their neural derivatives

The HS181 line was chosen for a broader surface marker study in which we compared the expression of 30 CD markers on TRA-1-81-positive hESCs and their neural derivatives. The surface protein expression profiles are shown in Fig. 5. Similar to HS181 hESCs, neural cells expressed high or medium high levels of CD24, CD29, CD49f, CD59, and CD90. Expression of CD10, CD133, and CD326 was substantially higher (>20%) in HS181 hESCs compared to the neural derivatives. The most prominent difference was in the expression of CD326, which was high in undifferentiated hESCs (>90%) and weak in neural cells (<20%). Compared to the hESCs, hESC-derived neural cells had higher expression of CD49b, CD56, CD117, CD166, CD184, and CD271. Of these markers, CD49b, CD146, and CD166 have not been previously associated with human NSCs or NPCs (for comparisons, see Fig. 5A and Supplemental Table 1). Supplemental Fig. 3 shows representative FACS plots of CD marker expression levels that were substantially increased in hESC-derived neural cells. Differentially expressed CD markers between hESCs and neural cells were further profiled in four other hESC lines: HS360, HS362, Regea040/06, and Regea023/08. This analysis showed that CD133 and CD326 were expressed at substantially higher levels in undifferentiated hESCs

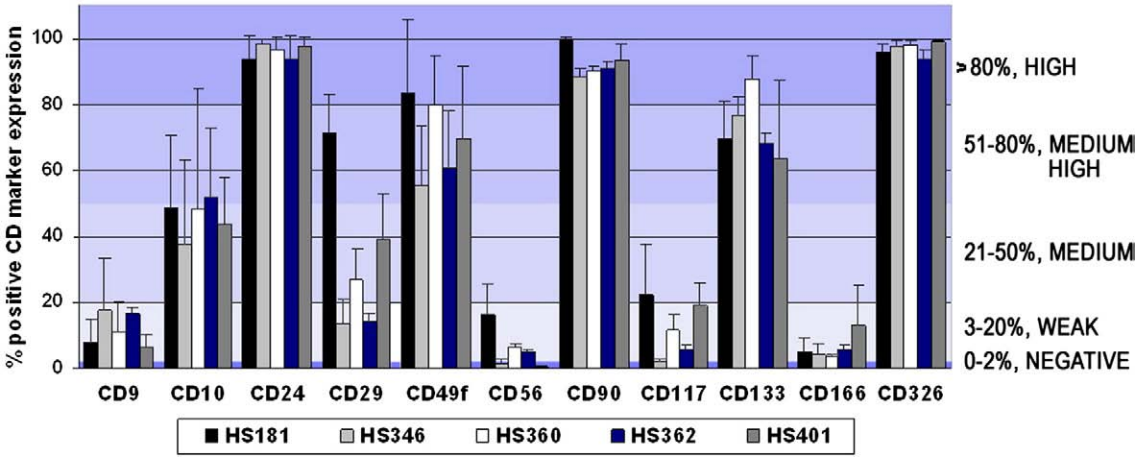

Figure 3 CD marker expression in five hESC lines. Expression of CD markers in TRA-1-81-positive hESCs in hESC lines HS181, HS346, HS360, HS362, and HS401, as determined by flow cytometry.

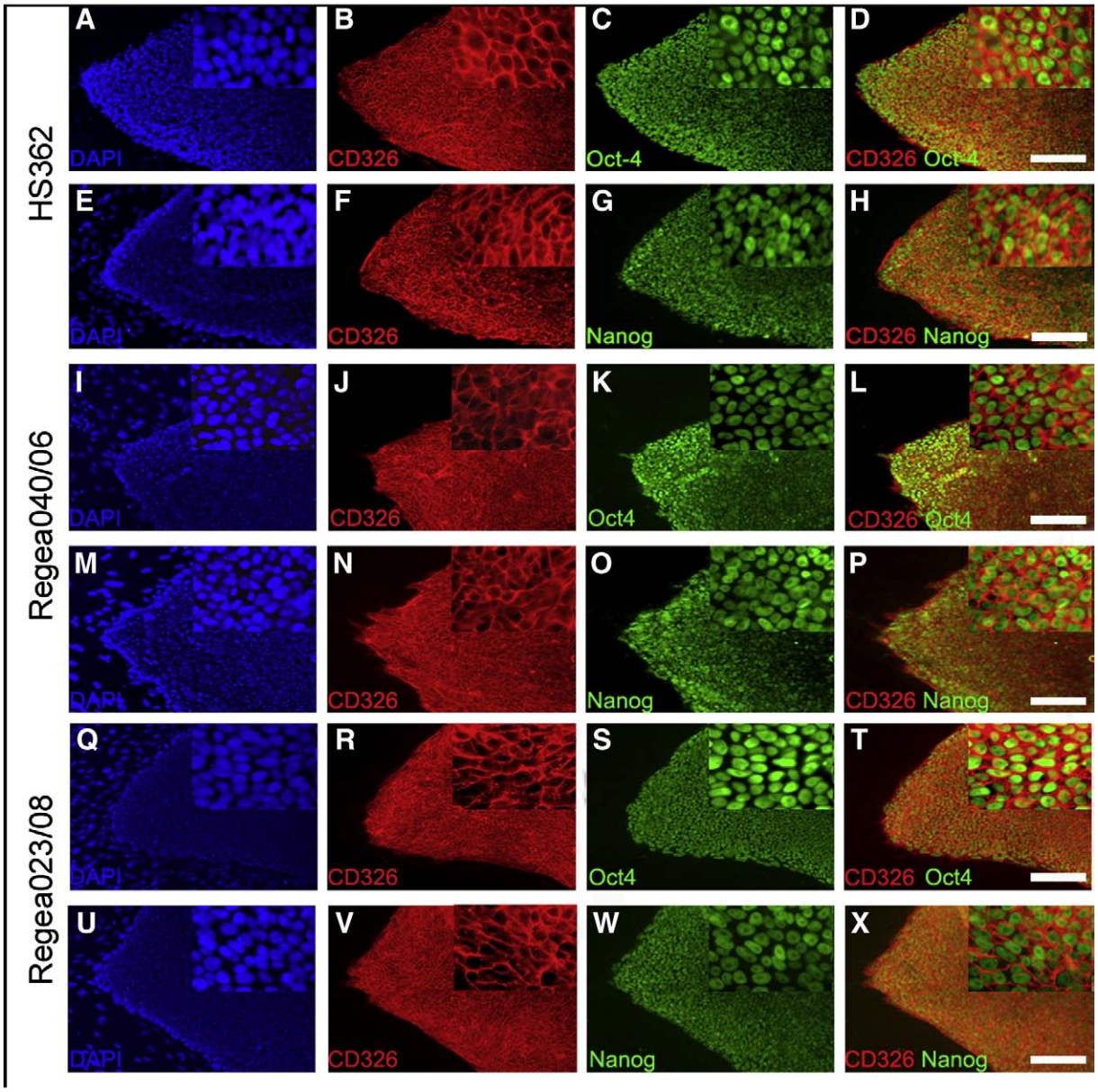

Figure 4 Immunofluorescent staining of hESC colonies expressing CD326, Oct-4, and Nanog. hESC lines: (A–H) HS362, (I–P) Regea040/06, and (Q–X) Regea023/08. hESCs expressed (B, F, J, N, R, V) CD326, (C, K, S) Oct-4, and (G, O, W) Nanog. (D, H, L, P, T, X) Merged CD326/Oct-4 and CD326/Nanog images. Each image also includes higher magnifications obtained with a 40× objective. (A, E, I, M, Q, U) Nuclear staining with DAPI. Scale bar, 100 μm.

compared to their neural derivatives, while CD56 and CD184 expression was higher in neural cells (Fig. 5).

Neural subpopulations and FACS sorting

Double labeling showed that CD56- (NCAM) and MAP-2-positive neural populations were not positive for CD326, a finding confirmed by combinatorial FACS analysis (Fig. 6). Subpopulations of CD56- and MAP-2-positive neural cells were positive for CD184 that especially localized to neurites in MAP-2-positive neurons (Fig. 6). Combinatorial FACS analysis showed that 48.8% of neural cells were $CD56^+/CD184^+$ (Fig. 6).

Neural cell sorting was performed using either positive selection for CD56, CD117, CD133, CD166, CD184, and CD271 or negative selection for CD326. The viability and neuronal morphology of sorted cells was monitored for 3 days after subcultivation (Supplemental Movie 2) and the results showed that cells in the original neural populations and in $CD326^-$, $CD56^+$, and $CD184^+$ populations resembled typical neurons (Fig. 6). FACS-sorted cells were 34 to 64% positive for MAP-2 (Supplemental Fig. 2). Especially, $CD56^+$ and $CD184^+$ cell populations were highly (>60%) positive for MAP-2. No GFAP-positive cells were identified.

Additional teratoma formation experiments with SCID mice revealed that undifferentiated hESCs formed teratomas, whereas neural cells and CD326-negative neural

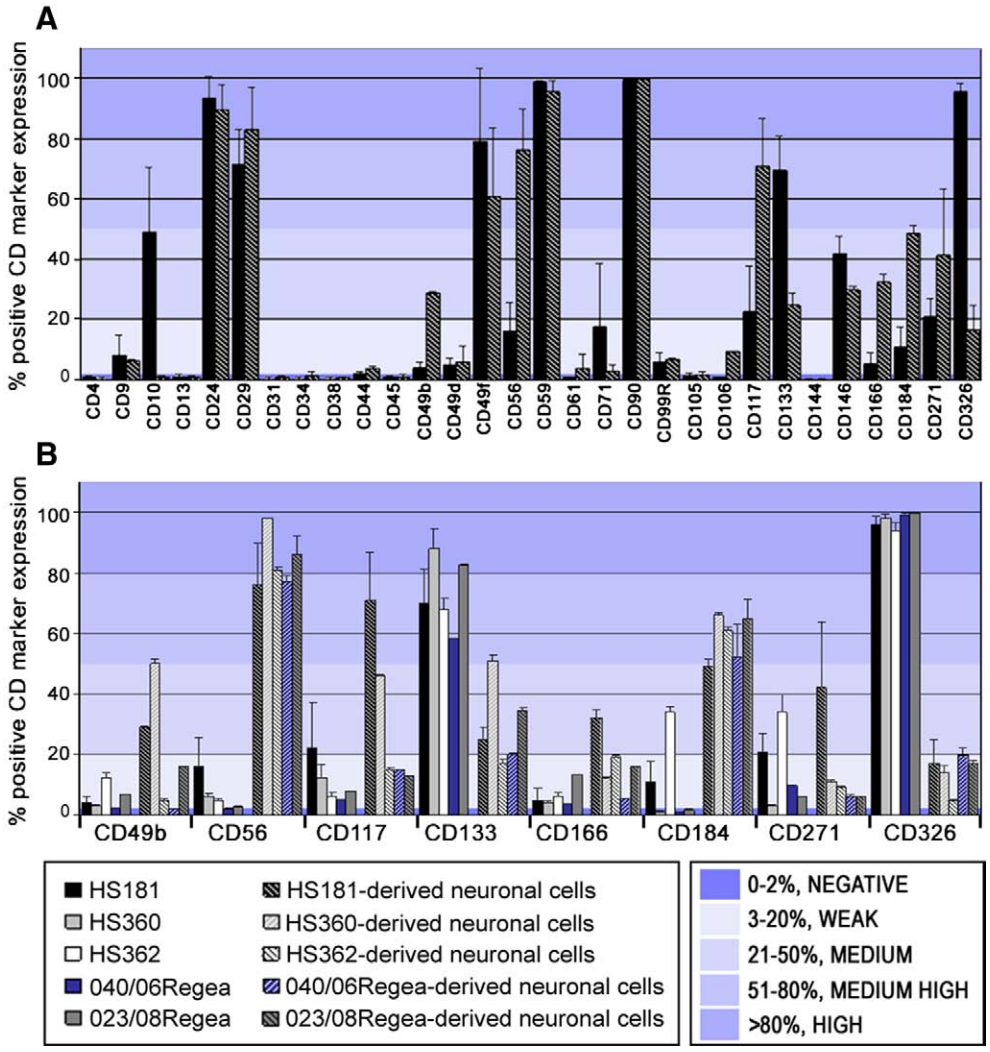

Figure 5 CD marker expression in hESCs and their neural derivates. (A) Expression of 30 different CD markers in TRA-1-81-positive hESCs (HS181) and neural derivatives determined by flow cytometry. (B) The expression of CD49b, CD56, CD117, CD133, CD166, CD184, CD271, and CD326 in TRA-1-81-positive hESCs and hESC-derived neural cells, determined by flow cytometry.

cells did not show any tumor formation after 2 months (Supplemental Fig. 4).

Downregulation of pluripotent markers and upregulation of neural markers during differentiation

Next, we studied the more detailed expression profiles of specific markers during the differentiation of hESCs toward neural cells. The expression of *Oct-4* and *Nanog* was gradually downregulated during the first 4 weeks of differentiation at which time the endo- and mesodermal markers also vanished. Expression of *Pax-6* and *nestin* was constant, while expression of *Mash1*, *MAP2*, and *CXCR4* appeared after 1 week of differentiation and remained quite constant thereafter (Supplemental Fig. 1).

The surface expression of the pluripotency marker TRA-1-81 was downregulated in parallel with CD326 during 3 weeks of neural differentiation. The expression of CD133 varied but was downregulated in 6 weeks' time. CD56 was highly upregulated already during the first week of differentiation and remained high during the 6-week time course. CD184 expression followed the expression curve of CD56 at a lower level (Fig. 7).

The combinatorial FACS analysis revealed that 88% of $CD133^+$ cells were positive for CD326 in hESCs (day 0). After 3 weeks, less than 8% of $CD133^+$ cells were CD326 positive. Interestingly, the $CD184^+$ subpopulation in $CD56^+$ cells increased from 28 up to 46% during 6 weeks of differentiation, further confirming that CD184 is expressed in differentiating neural cell populations (Fig. 7).

Discussion

In this study, the expression of 30 CD surface markers on TRA-1-81-positive hESCs and their neural derivatives was characterized. The CD expression profiles of seven hESC lines were analyzed and compared between undifferentiated hESCs and their neural derivatives. Here, we showed that similarly derived and cultured hESC lines had relatively

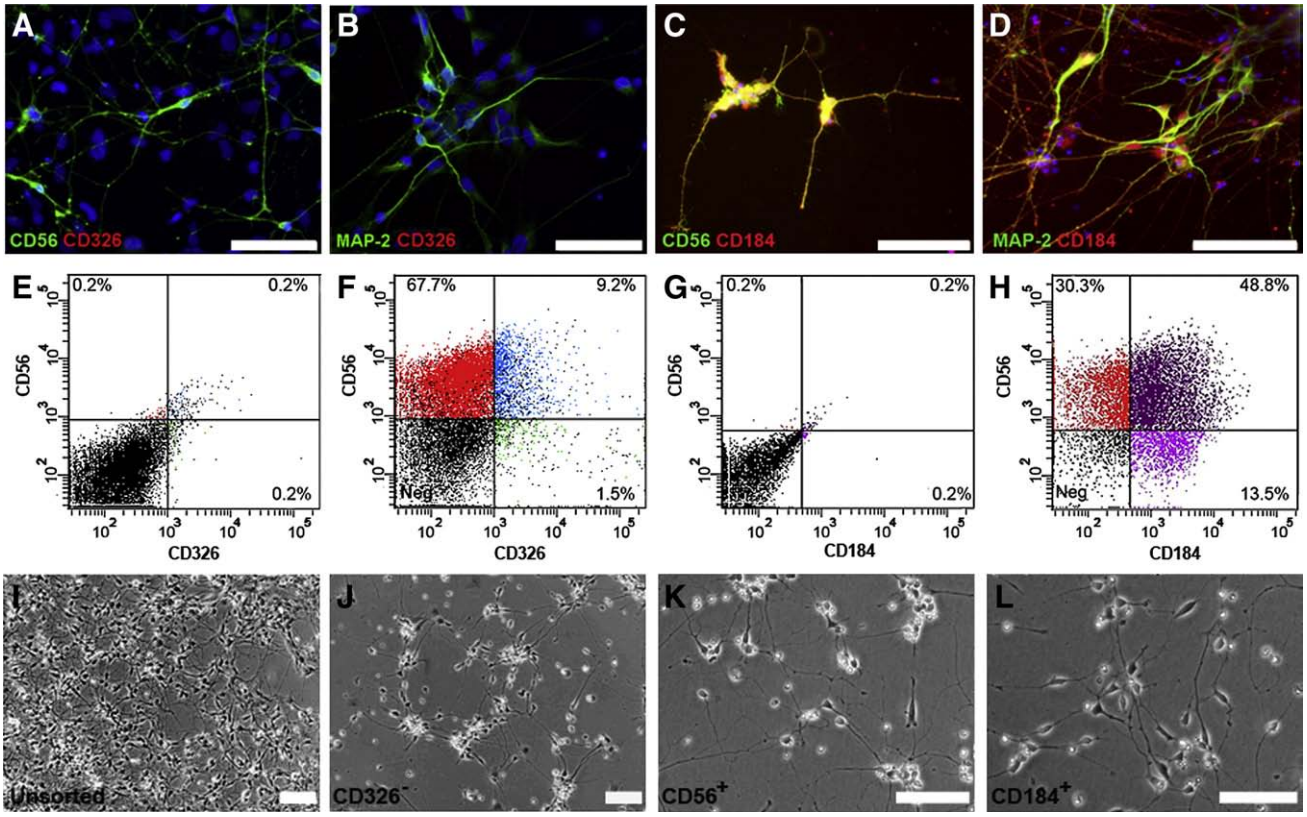

Figure 6 Immunofluorescent staining of neural populations showed that cells positive for (A) CD56 (NCAM, green) and (B) MAP-2 (green) were not positive for CD326 (red). (C, D) CD56- and MAP-2-positive cells coexpressed CD184 (red). Combinatorial FACS analysis showed that (F) the majority of neural cells were $CD56^+$ (~77%) and only a minor population was $CD56^+/CD326^+$ (~9%), (H) whereas ~49% of cells were $CD56^+/CD184^+$. (E, G) Unlabeled neural cells were used for population determination. The morphology of (I) unsorted neural cells and (J) $CD326^-$-, (K) $CD56^+$-, and (L) $CD184^+$-sorted populations is presented. Scale bar, 100 μm.

constant CD marker expression levels, whereas neural populations derived from different hESC lines had more variable CD marker expression levels. The expression of CD133 and CD326 was strong in undifferentiated hESCs and low in neural derivatives, and the expression of CD56 and CD184 was increased in hESC-derived neural cells. These CD marker expression results were similar in hESCs derived in two laboratories.

A detailed characterization of undifferentiated hESCs and their derivatives is important for many reasons. First, it is important to determine the similarity of hESCs derived and cultured by different laboratories, as recently investigated by the ISCI (ISCI, 2007). For example, their analysis of 59 hESC lines revealed large variability between different hESC lines in the expression of the surface markers SSEA-3 and SSEA-4 (ISCI, 2007). Here, we used TRA-1-81, which is more consistently expressed by undifferentiated hESCs (ISCI, 2007), as a comarker in CD marker screening for the selection of undifferentiated hESCs. Second, novel surface markers that are highly and consistently expressed in all undifferentiated hESC lines would enable separation of undifferentiated hESCs from their neural derivatives. In heterogeneous populations, this would enable the exclusion of pluripotent cells that can form teratomas *in vivo*. This negative selection may be needed to produce safe neural cell grafts for transplantation therapies. Further, negative-selected populations do not contain surface-bound antibodies, in contrast to positive-selected populations, a difference that may have a large impact on graft survival.

Here, we showed that all five of the undifferentiated hESC lines analyzed had a consistently high expression of CD24, CD90, and CD133, markers that were previously associated with hESCs. In this study, more than 80% of hESCs expressed CD24 and CD90, whose gene and protein products have been identified in hESCs (Draper et al., 2002; Skottman et al., 2005). CD24 can be used to distinguish hESCs from fibroblasts in culture (Assou et al., 2007) and to distinguish mouse multipotent fetal stem cells from neural progenitors and postmitotic neurons (Panchision et al., 2007). Pruszack and co-workers suggested that CD24 is a specific surface marker that is upregulated during neural differentiation (Pruszack et al., 2007). In contrast, our study shows that hESCs and hESC-derived neural cells expressed CD24 at equal levels (90% vs 94%, respectively). Also, high (>90%) expression of CD90 was detected in both undifferentiated hESCs and hESC-derived neural cells, which is consistent with the results of previous studies (Schwartz et al., 2003; Draper et al., 2002; Hamann et al., 1980). Hence, our data exclude the potential use of CD24 and CD90 as specific markers for undifferentiated hESCs or differentiating hESC-derived neural cells. In contrast, CD133 marker expression was over 65% in all undifferentiated hESC lines and was downregulated in hESC-derived neural cells. This finding is consistent with the literature and suggests that

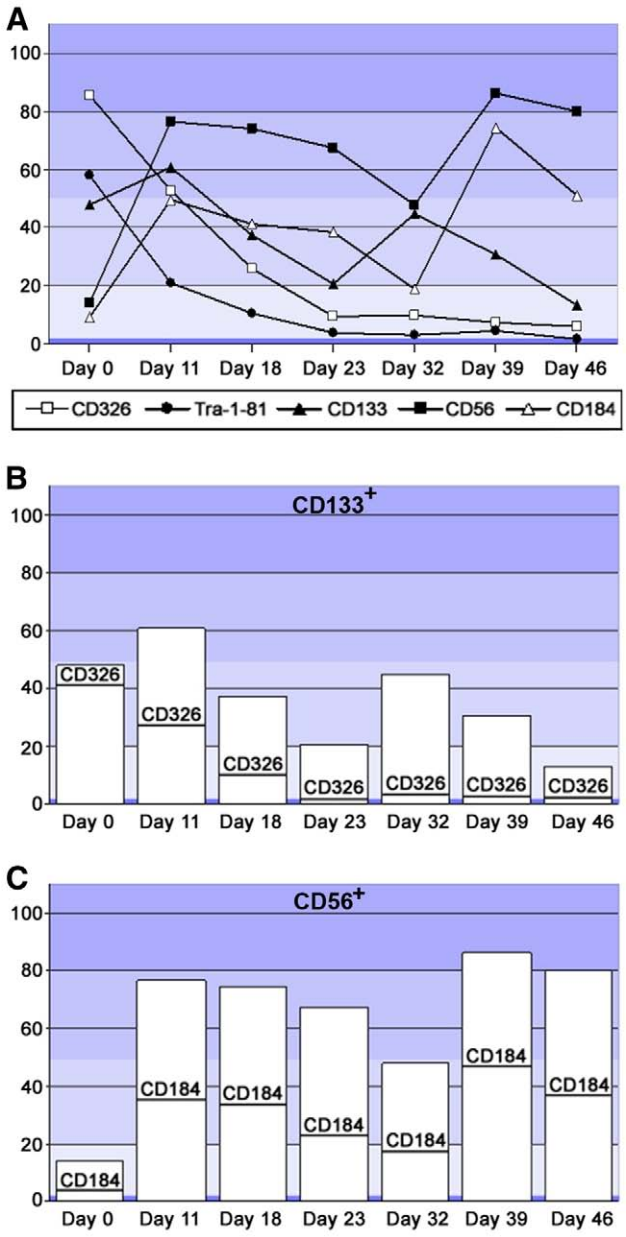

Figure 7 Time-point analysis of hESC (HS360) neural differentiation. (A) Expression curves of TRA-1-81$^+$, CD326$^+$, CD133$^+$, CD56$^+$, and CD184$^+$ populations from 0 to 46 days of neural differentiation. (B) CD326 expression in the CD133$^+$ subpopulation and (C) CD184 expression in the CD56$^+$ subpopulation.

CD133 is a reliable marker for stem and progenitor cells (Carpenter et al., 2004; Schwartz et al., 2003; Yin et al., 1997). CD133 can also be used as a selection marker for proliferating neural stem and precursor populations from postmitotic neurons (Uchida et al., 2000). Moreover, we suggest that the downregulation of CD133 expression in differentiating neural cells indicates their decreasing proliferation capacity compared to undifferentiated hESCs.

Most interestingly, we found a novel surface marker, CD326, consistently expressed in over 94% of the undifferentiated cells in all seven hESC lines studied. This result was constant between hESC lines derived in two independent laboratories. CD326, also known as epithelial cell adhesion molecule (EpCAM), is a glycoprotein involved in cell adhesion (Litvinov et al., 1997). The high expression of CD326 by hESCs might explain the tight cell-to-cell contacts of hESCs in colonies. CD326 is associated with stem cells in hepatic cell lineages (Schmelzer et al., 2006). CD326/EpCAM is also part of the same epithelial adhesion molecule family as E-cadherin, which is expressed in undifferentiated hESC colonies but not in differentiated embryoid bodies (Cai et al., 2005; Ullmann et al., 2007). In carcinoma cells, CD326/EpCAM affects upregulation of the proto-oncogene c-myc and cyclin A/E and thereby has direct impact on the cell cycle and proliferation (Munz et al., 2004). Thus, this molecule might also affect the ability of hESCs to proliferate efficiently. Importantly, both flow-cytometric and immunocytochemical analyses showed that hESC-derived neural populations did not contain CD362-positive cells. Moreover, expression of CD362 was downregulated in parallel with TRA-1-81 on hESC-derived populations during the first 4 weeks of neural differentiation. Further, combinatorial FACS analysis showed that the CD133$^+$/CD326$^+$ population was downregulated during differentiation. Thus, we propose that CD326 can be used as a novel marker for undifferentiated hESCs to facilitate the removal of undifferentiated hESCs from differentiated neural cell populations before cell transplantation.

Some markers previously associated with hESCs, such as CD9, CD29, and CD117 (Assou et al., 2007; Bhattacharya et al., 2004; Carpenter et al., 2004; Draper et al., 2002; Lian et al., 2006; Xu et al., 2001; Zambidis et al., 2005) were only weakly or heterogeneously expressed by our undifferentiated hESCs. CD marker expression during neural differentiation of hESCs appears to vary depending on both the differentiation protocol used and the initial expression profile in hESCs. Previously, Pruszack and co-workers performed flow-cytometric analysis and sorting on hESCs and neural cells differentiated in a coculture system (Pruszak et al., 2007). The expression levels of stem-cell-related markers CD24, CD29, and CD133 were less than 15% in the hESC lines HI and H7 (Pruszak et al., 2007), compared to much higher expression in our undifferentiated hESCs. These differences may reflect the different derivation and culturing methods of hESCs and further imply that universal, constantly highly expressed markers for hESCs are needed.

There are currently many differentiation protocols for neural differentiation of hESCs utilizing adherent, suspension, and cocultures with various supplements (Hoffman and Carpenter, 2005). Thus, the neural cell cultures are often heterogeneous, impure, and not necessarily comparable with one another. Here, we used simple methods for neural differentiation and compared the CD profiles in these cultures to the undifferentiated hESCs. In our study, the neural cells derived from five hESC lines had increased expression of CD56 and CD184 compared to undifferentiated hESCs, whereas the expression of CD49b, CD177, CD166, and CD271 was heterogeneously increased. CD56 is a marker of early neuroectodermal differentiation (Reubinoff et al., 2001) and has been used to identify neural differentiation of hESCs by flow-cytometric analysis and sorting (Pruszak et al., 2007). CD184, also known as the chemokine receptor CXCR4, is characterized as a receptor protein modulating cell growth and migration of neural cells in the CNS (Ni et al., 2004). Interestingly, in our study the hESC-derived neural cells had quite similar expression levels of CD184 compared

to NPCs derived from human fetal brain tissues (Ni et al., 2004), suggesting that these cell populations might have similar migration capacities.

The more detailed analysis of the differentiating neural populations revealed that both gene and surface protein markers for pluripotent cells disappeared during the first 4 weeks of differentiation. These time curves for TRA-1-81 and CD326 expression were very similar to those described for TRA-1-81, TRA-1-60, SSEA-3, and SSEA-4 in a coculture system (Pruszak et al., 2007). In addition, the expression of CD56 was rapidly upregulated in differentiating neural populations and remained high during 6 weeks of differentiation. These results are in line with a previous study (Pruszak et al., 2007), suggesting that CD56 can be used to monitor neural specification. Moreover, our neural cell population did not produce teratomas when transplanted into SCID mice. This implies that our hESC-derived neural cell cultures are valid populations for further production of more specialized neural cells or for transplantation.

As expression of CD184 was increased in parallel with CD56, we performed combinatorial FACS analysis with CD56 and CD184. These results showed that during 5 to 6 weeks of differentiation up to 50% of CD56-positive cells were CD184 positive. Further, CD184 localized in soma and neurites of $CD56^+$ cells and especially in MAP-2-positive neurons. FACS-sorted $CD184^+$ neural cells, followed with time-lapse imaging, matured into viable neurons. These results are in line with a previous study by Peng and co-workers (Peng et al., 2007), who showed that human fetal brain tissue-derived NPCs also express CD184 at high levels when differentiated into neuronal cells. Altogether, these results suggest that CD184 is an important factor in neurogenesis and in maturation of neuronal cells and should be studied in more detail in the future.

Fluorescence-activated cell sorting has been considered as a possible method for producing pure neural subpopulations to be used in transplantation therapies (Chung et al., 2006; Guzman et al., 2008). Here, we showed that neural subcultivation is possible after sorting with various CD markers, CD56, CD117, CD133, CD166, CD184, and CD271. Especially, by sorting with CD56 and CD184 viable neuronal populations can be produced. Moreover, pluripotent cells can be eliminated by negative sorting for CD326 if more purification of the neural population is needed prior to transplantation. Although there are milder and more sensitive ways to sort with FACS using a bigger nozzle (100 μm) and low sorting setups for fragile cells, this method needs to be better optimized before separating populations for cell grafting. For these types of studies, microfluidics-based cell sorting techniques may be a future prospect (Studer et al., 2004; Chen et al., 2008). In any case, FACS is a usable and reliable method for studies of neural subpopulations and their characteristics.

In conclusion, surface marker expression in previously established hESC lines varies to a great extent universally, whereas hESC lines derived and cultured similarly have quite constant CD marker profiles. Even though there are many commonly used markers for hESCs (SSEA-3, SSEA-4, TRA-1-60, TRA-1-81), their expression levels vary markedly in different hESC lines. In contrast, CD326 showed consistently high expression in all undifferentiated hESCs compared to their neural derivatives. CD326 has not, to our knowledge, been previously associated with hESCs, and therefore we consider this surface protein to be a novel marker for undifferentiated hESCs. As our transplantation experiments showed no teratoma formation with hESC-derived CD326-negative selected neural cells, we conclude that this marker might be useful for the production of differentiated, hESC-free neural populations.

Materials and methods

hESC culture

Five hESC lines were used in this study: HS181, HS346, HS360, HS362, and HS401. All hESC lines were derived from the inner cell mass of supernumerary blastocyst-stage embryos at the Karolinska Institute, Karolinska University Hospital Huddinge, Sweden, after approval by the ethics committee of the Karolinska Institute. The procedures for the derivation, characterization, and culture of these hESC lines were described previously (Hovatta et al., 2003). Studies utilizing the hESC lines derived at the Karolinska Institute and performed at REGEA, Institute for Regenerative Medicine, University of Tampere, Finland, were approved by the ethics committee of the Hospital District of Pirkanmaa (Hovatta, R05051). In REGEA, two novel hESC lines, Regea040/06 and Regea023/08, were derived and cultured (H. Skottman et al., unpublished data). REGEA has a supportive statement from the ethics committee of the Pirkanmaa Hospital district to derive and expand new human ESC lines from surplus embryos, which cannot be used in the infertility treatment of the donating couples (Skottman, R05116). All the hESCs were cultured on commercially available, mitotically inactivated human foreskin fibroblasts (CRL-2429, ATCC, Manassas, VA, USA) in hESC medium consisting of knockout Dulbecco's modified Eagle's medium (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 20% knockout SR (Gibco Invitrogen), 2 mM GlutaMax (Gibco Invitrogen), 0.1 mM minimal essential medium nonessential amino acids (Cambrex Bio Science, Karlskoga, Sweden), 0.1 mM β-mercaptoethanol (Gibco Invitrogen), 50 U/ml penicillin/streptomycin (Cambrex), and 8 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, USA) at 37 °C in 5% CO_2 in a humidified atmosphere. The hESC colonies were passaged at 5- to 7-day intervals by mechanical splitting and replated on fresh feeder cells.

Neural differentiation of hESCs

HS181, HS360, HS362, Regea040/06, and Regea023/08 colonies were mechanically dissected and differentiated into neural cells in adherent or suspension culture using a modified protocol by Nat and co-workers (Nat et al., 2007). The neural proliferation medium contained Dulbecco's modified Eagle's medium/F-12 and neurobasal medium (1:1) supplemented with 1× B27, 1× N2, 2 mM GlutaMax (all from Gibco Invitrogen), 25 U/ml penicillin/streptomycin, and 20 ng/ml bFGF. Briefly, differentiating neural cells formed rosette-like structures in adherent culture (CellBIND Surface, Corning, Inc., Corning, NY, USA) or in suspension culture in proliferation medium after 7 to 10 days. These rosette structures were dissected and plated on laminin-coated (10 μg/ml; Sigma Chemical Co., St. Louis, MO, USA)

culture well plates (Nunc, Roskilde, Denmark) in the absence of bFGF (neural differentiation medium) for further adherent culturing for 6 to 8 weeks. In suspension culture, the spheres were mechanically split once a week and cultured for 6 to 8 weeks. Thereafter, spheres were dissected and replated on laminin-coated well plates for 1 week. bFGF (4 ng/ml) and brain-derived neurotrophic factor (5 ng/ml; Gibco Invitrogen) were added to cultures to support the growth and survival of maturing neural cells. All cultures were maintained in a humidified atmosphere at 37 °C and 5% CO_2.

Automated monitoring of hESC-derived neural cells

The growth and morphologic characteristics of hESC-derived neural cells were monitored prior to immunocytochemical and flow-cytometric analysis using an online cell culture platform (Cell-IQ, Chip-Man Technologies, Tampere, Finland) equipped with phase-contrast microscope optics (10×) and a CCD camera, as described previously for hESCs (Narkilahti et al., 2007). This software allows time-lapse imaging of 500 × 670-μm areas in the culture wells. Captured images (JPEG) were saved in separate folders and converted into movie format using Cell-IQ analysis software.

Immunocytochemical analysis of hESCs and hESC-derived neural cells

Subsets of hESCs and hESC-derived neural cells were fixed using 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.01 M PBS, pH 7.4) for 20 min at room temperature and washed twice with PBS. The cells were permeabilized and blocked with 10% normal donkey serum (NDS; Sigma) and 1% bovine serum albumin (BSA; Sigma) in PBS containing 0.1% Triton X-100 for 45 min at room temperature. Primary antibodies were applied overnight at 4 °C in 1% BSA, 1% NDS in PBS containing 0.1% Triton X-100. Cells were washed three times and incubated with fluorescent secondary antibodies in 1% BSA in PBS for 60 min at room temperature in the dark. Cells were then washed two times with PBS and two times with phosphate buffer and mounted using Vectashield containing 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining (Vector Laboratories, Inc., Burlingame, CA, USA). The primary antibodies used for hESCs were mouse anti-epithelial-specific antigen IgG (EpCAM/CD326, 1:200; Chemicon, Temecula, CA, USA), goat anti-Nanog IgG (1:200; R&D Systems), goat anti-Oct-4 IgG (1:100; R&D Systems), and mouse anti-TRA-1-81 IgM (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Primary antibodies used for neural cells were mouse anti-CXCR4 IgG (1:2000; R&D Systems), sheep anti-GFAP IgG (1:600; R&D Systems), rabbit anti-MAP-2 IgG (1:600–1:800; Chemicon), and rabbit anti-NCAM IgG (1:800; Chemicon). Secondary antibodies used were Alexa Fluor 488 donkey anti-goat IgG (1:800), Alexa Fluor 488 donkey anti-rabbit IgG (1:400), Alexa Fluor 488 donkey anti-sheep IgG, and Alexa Fluor 568 goat anti-rabbit IgG (1:400) (all from Molecular Probes Invitrogen) and rhodamine red-conjugated donkey anti-mouse IgM and IgG (1:400; Jackson ImmunoResearch Europe Ltd., Cambridgeshire, UK). For negative controls, the primary antibodies were omitted from the immunostaining, which resulted in the disappearance of all staining. Stained cells were viewed and photographed using an Olympus IX51 phase-contrast microscope equipped with fluorescence optics and an Olympus DP71 camera.

Sample preparation for flow cytometry

The hESC cultures were washed once with ice-cold sterile PBS and trypsinized for 10 min at 37 °C (trypsin–EDTA; BioWhittaker, Fisher Scientific, Leicestershire, UK) and inactivated with 2% fetal bovine serum (FBS; Gibco Invitrogen) in PBS. Cell colonies were dissociated into single-cell suspensions by trituration, centrifuged, resuspended in 2% FBS in PBS, and counted using trypan blue exclusion to identify viable cells. Aliquots of 100,000 viable cells per sample were used for flow-cytometric analysis. hESC-derived neural cells were washed twice, trypsinized for 5 min at 37 °C, and inactivated with 5% human serum (HS) in PBS. Cells were dissociated into a single-cell suspension by trituration, centrifuged, resuspended in 5% HS in PBS, and filtered using 50-μm cell strainers (CupFilcons; BD Biosciences, Franklin Lakes, NJ, USA) prior to trypan blue counting. Aliquots of 100,000 viable cells per sample were used for flow-cytometric analysis.

Surface antigen expression analysis

To identify undifferentiated cells, hESCs were labeled for TRA-1-81 and colabeled with fluorochrome-conjugated anti-human CD markers. Briefly, hESCs were incubated for 15 min at 4 °C with TRA-1-81 antibody, followed by incubation with fluorescent secondary antibody and antibodies recognizing CD markers for 15 min at 4 °C in the dark. Cells were washed twice with 2% FBS in PBS between each step. Thereafter, labeled cells were suspended in 2% FBS in PBS. At least three parallel samples for each TRA-1-81 and CD marker combination were analyzed. hESC-derived neural cells were directly labeled with anti-human CD markers and suspended in 5% HS in PBS. Two to four parallel samples for each CD marker were analyzed.

The primary antibody used was mouse anti-TRA-1-81 IgM (1:200; Santa Cruz Biotechnology) with secondary antibody anti-mouse Alexa Fluor 488 IgM (1:6500; Molecular Probes Invitrogen) or anti-mouse phycoerythrin (PE) IgM (1:500; Caltag Invitrogen). Antibodies recognizing CD markers were CD4–fluorescein isothiocyanate (FITC), CD9–FITC, CD13–PE, CD24–FITC, CD31–FITC, CD34–allophycocyanin (APC), CD38–PE, CD44–FITC, CD49b–FITC, CD56–PE, CD59–FITC, CD61–FITC, CD71–PE, CD99R–FITC (ImmunoTools, Friesoythe, Germany); CD45–FITC, CD117–APC, CD133–PE, CD271–FITC, CD326–APC (Miltenyi Biotech, Bergisch Gladbach, Germany); CD9–PE, CD10–PE–Cy7, CD29–APC, CD49d–PE, CD49f–APC, CD90–APC, CD106–PE–Cy5, CD146–PE, CD166–PE, CD184–PE–Cy5 (BD Biosciences); and CD105–PE and CD144–PE (R&D Systems). Antibody concentrations were chosen according to the manufacturer's protocol or adjusted to the optimal concentration when necessary. Background fluorescence was excluded using unlabeled cells or isotype controls or by incubation with secondary antibodies only. Analyses were performed using FACSAria equipment with 488- and 633-nm lasers, a standard filter set, and FACSDiva software (BD Biosciences). The cell population of interest was determined and dead cells were excluded using forward-

and side-scatter parameters. For each sample run, 10,000 to 20,000 events were recorded and analyzed.

Fluorescence-activated cell sorting and subcultivation of hESC-derived neural cells

HS181-derived neural cell samples (400,000–1,000,000 cells/sample) were prepared as described above and labeled for CD56, CD117, CD133, CD166, CD184, or CD271. Cell sorting was performed using a FACSAria with a 100-μm nozzle, 20.00 psi sheath pressure, sort precision mode set for purity, flow rate 2, and plates voltage 5.0 (sort setup: low). Live cells were gated using forward- and side-scatter parameters, and cells positive for each CD marker were collected into 5-ml polystyrene tubes (BD Biosciences) in neural differentiation medium. Cells were centrifuged and resuspended in medium containing 4 ng/ml bFGF and 5 ng/ml brain-derived neurotrophic factor and plated onto laminin-coated culture plates. After 3 days, the FACS-sorted cells were monitored with CellIQ for 24 h, fixed, and stained using antibodies for MAP-2 and GFAP, as described above.

Teratoma formation

To study *teratoma formation, hESCs and hESC-derived neural cell populations* were transplanted into SCID mouse testes as previously described (Hovatta et al., 2003; Inzunza et al., 2005). After the cell injections of hESCs, 200,000 cells/testis ($n=3$); neural cells, 1,000,000 cells/testis ($n=7$); and CD326-negative neural cells, 1,000,000 cells/testis ($n=3$), the animals were followed for 2 months for teratoma formation.

Time-point analysis of specific markers during neural differentiation

The more detailed time-point analyses of specific markers were performed with HS360 during the neural differentiation. The cells were first grown in suspension for 0, 7, 14, 19, 28, 35, or 42 days. Thereafter, they were *in vitro* differentiated on laminin-coated wells for 4 days as described above. Thus, the samples for gene expression (RT-PCR) and FACS analysis were collected at days 0, 11, 18, 23, 32, 39, and 46.

For RT-PCR, the expression of the pluripotent markers *Nanog* and *Oct4;* the endodermal marker *α-fetoprotein;* the mesodermal marker *brachyury;* the neural markers *MAP-2, Mash1, nestin,* and *Pax6; C-X-C chemokine receptor type 4;* and the housekeeping gene *GAPDH* was analyzed. For RT-PCR, RNA was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany). A total of 50 ng of RNA was used for cDNA synthesis (Sensiscript RT Kit; Qiagen) according to the manufacturer's instructions. Each PCR contained 700 ng of cDNA, 0.25 μM forward and reverse primers, 1× Taq buffer (−MgCl, +KCl) (Fermentas, Leon-Rot, Germany), 2.5 mM dNTP (Fermentas), 25 mM MgCl (Fermentas), dH_2O, and 0.6 U Taq DNA polymerase enzyme (Fermentas). PCR program parameters were denaturation at 95 °C for 3 min followed with 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min and a final extension for 5 min in 72 °C.

For FACS analysis, the expression of TRA-1-81, CD326, CD133, CD56, and CD184 was analyzed. In addition, combinatorial analyses for CD133/CD326, CD56/CD326, and CD56/184 were performed. All FACS analyses were performed as described above.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.scr.2008.08.001.

References

Assou, S., Lecarrour, T., Tondeur, S., Strom, S., Gabelle, A., Marty, S., Nadal, L., Pantesco, V., Reme, T., Hugnot, J.P., Gasca, S., Hovatta, O., Hamamah, S., Klein, B., De Vos, J., 2007. A meta-analysis of human embryonic stem cells transcriptome integrated into a web-based expression atlas. Stem Cells 25, 961–973.

Barraud, P., Stott, S., Mollgard, K., Parmar, M., Bjorklund, A., 2007. In vitro characterization of a human neural progenitor cell coexpressing SSEA4 and CD133. J. Neurosci. Res. 85, 250–259.

Bhattacharya, B., Miura, T., Brandenberger, R., Mejido, J., Luo, Y., Yang, A.X., Joshi, B.H., Ginis, I., Thies, R.S., Amit, M., Lyons, I., Condie, B.G., Itskovitz-Eldor, J., Rao, M.S., Puri, R.K., 2004. Gene expression in human embryonic stem cell lines: unique molecular signature. Blood 103, 2956–2964.

Brimble, S.N., Sherrer, E.S., Uhl, E.W., Wang, E., Kelly, S., Merrill Jr., A.H., Robins, A.J., Schulz, T.C., 2006. The cell surface glycosphingolipids SSEA-3 and SSEA-4 are not essential for human ESC pluripotency. Stem Cells 25, 54–62.

Cai, J., Olson, J.M., Rao, M.S., Stanley, M., Taylor, E., Ni, H.T., 2005. Development of antibodies to human embryonic stem cell antigens. BMC Dev. Biol. 5, 26.

Carpenter, M.K., Inokuma, M.S., Denham, J., Mujtaba, T., Chiu, C.P., Rao, M.S., 2001. Enrichment of neurons and neural precursors from human embryonic stem cells. Exp. Neurol. 172, 383–397.

Carpenter, M.K., Rosler, E.S., Fisk, G.J., Brandenberger, R., Ares, X., Miura, T., Lucero, M., Rao, M.S., 2004. Properties of four human embryonic stem cell lines maintained in a feeder-free culture system. Dev. Dyn. 229, 243–258.

Chen, P., Feng, X., Du, W., Liu, B.F., 2008. Microfluidic chips for cell sorting. Front. Biosci. 13, 2464–2483.

Chung, S., Shin, B.S., Hedlund, E., Pruszak, J., Ferree, A., Kang, U.J., Isacson, O., Kim, K.S., 2006. Genetic selection of sox1GFP-expressing neural precursors removes residual tumorigenic pluripotent stem cells and attenuates tumor formation after transplantation. J. Neurochem. 97, 1467–1480.

Draper, J.S., Pigott, C., Thomson, J.A., Andrews, P.W., 2002. Surface antigens of human embryonic stem cells: changes upon differentiation in culture. J. Anat. 200, 249–258.

Guzman, R., De Los Angeles, A., Cheshier, S., Choi, R., Hoang, S., Liauw, J., Schaar, B., Steinberg, G., 2008. Intracarotid injection of fluorescence activated cell-sorted CD49d-positive neural stem

cells improves targeted cell delivery and behavior after stroke in a mouse stroke model. Stroke 39, 1300–1306.
Hamann, A., Arndt, R., Klein, P., Thiele, H.G., 1980. Isolation and characterization of the thymus-brain antigen (analogous to thy-1 antigen) from human brain. Biochem. J. 187, 403–412.
Hart, A.H., Hartley, L., Ibrahim, M., Robb, L., 2004. Identification, cloning and expression analysis of the pluripotency promoting Nanog genes in mouse and human. Dev. Dyn. 230, 187–198.
Hoffman, L.M., Carpenter, M.K., 2005. Characterization and culture of human embryonic stem cells. Nat. Biotechnol. 23, 699–708.
Holford, L.C., Case, P., Lawson, S.N., 1994. Substance P, neurofilament, peripherin and SSEA4 immunocytochemistry of human dorsal root ganglion neurons obtained from post-mortem tissue: a quantitative morphometric analysis. J. Neurocytol. 23, 577–589.
Hovatta, O., Mikkola, M., Gertow, K., Stromberg, A.M., Inzunza, J., Hreinsson, J., Rozell, B., Blennow, E., Andang, M., Ahrlund-Richter, L., 2003. A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. Hum. Reprod. 18, 1404–1409.
Inzunza, J., Gertow, K., Stromberg, M.A., Matilainen, E., Blennow, E., Skottman, H., Wolbank, S., Ahrlund-Richter, L., Hovatta, O., 2005. Derivation of human embryonic stem cell lines in serum replacement medium using postnatal human fibroblasts as feeder cells. Stem Cells 23, 544–549.
ISCI (International Stem Cell Initiative)Adewumi, O., Aflatoonian, B., Ahrlund-Richter, L., Amit, M., Andrews, P.W., Beighton, G., Bello, P.A., Benvenisty, N., Berry, L.S., Bevan, S., Blum, B., Brooking, J., Chen, K.G., Choo, A.B., Churchill, G.A., Corbel, M., Damjanov, I., Draper, J.S., Dvorak, P., Emanuelsson, K., Fleck, R.A., Ford, A., Gertow, K., Gertsenstein, M., Gokhale, P.J., Hamilton, R.S., Hampl, A., Healy, L.E., Hovatta, O., Hyllner, J., Imreh, M.P., Itskovitz-Eldor, J., Jackson, J., Johnson, J.L., Jones, M., Kee, K., King, B.L., Knowles, B.B., Lako, M., Lebrin, F., Mallon, B.S., Manning, D., Mayshar, Y., McKay, R.D., Michalska, A.E., Mikkola, M., Mileikovsky, M., Minger, S.L., Moore, H.D., Mummery, C.L., Nagy, A., Nakatsuji, N., O'Brien, C.M., Oh, S.K., Olsson, C., Otonkoski, T., Park, K.Y., Passier, R., Patel, H., Patel, M., Pedersen, R., Pera, M.F., Piekarczyk, M.S., Pera, R.A., Reubinoff, B.E., Robins, A.J., Rossant, J., Rugg-Gunn, P., Schulz, T.C., Semb, H., Sherrer, E.S., Siemen, H., Stacey, G.N., Stojkovic, M., Suemori, H., Szatkiewicz, J., Turetsky, T., Tuuri, T., van den Brink, S., Vintersten, K., Vuoristo, S., Ward, D., Weaver, T.A., Young, L.A., Zhang, W., 2007. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. Nat. Biotechnol. 25, 803–816.
Lian, Q., Lye, E., Yeo, K.S., Tan, E.K., Salto-Tellez, M., Liu, T.M., Palanisamy, N., El Oakley, R.M., Lee, E.H., Lim, B., Lim, S.K., 2006. Derivation of clinically compliant MSCs from CD105$^+$, CD24$^-$ differentiated human ESCs. Stem Cells 25, 425–436.
Litvinov, S.V., Balzar, M., Winter, M.J., Bakker, H.A., Briaire-de Bruijn, I.H., Prins, F., Fleuren, G.J., Warnaar, S.O., 1997. Epithelial cell adhesion molecule (Ep-CAM) modulates cell–cell interactions mediated by classic cadherins. J. Cell Biol. 139, 1337–1348.
Munz, M., Kieu, C., Mack, B., Schmitt, B., Zeidler, R., Gires, O., 2004. The carcinoma-associated antigen EpCAM upregulates c-myc and induces cell proliferation. Oncogene 23, 5748–5758.
Narkilahti, S., Rajala, K., Pihlajamäki, H., Suuronen, R., Hovatta, O., Skottman, H., 2007. Monitoring and analysis of dynamic growth of human embryonic stem cells: comparison of automated instrumentation and conventional culturing methods. Biomed. Eng. Online 12 (6), 11.
Nat, R., Nilbratt, M., Narkilahti, S., Winblad, B., Hovatta, O., Nordberg, A., 2007. Neurogenic neuroepithelial and radial glial cell generated from six human embryonic stem cell lines in serum-free adherent and suspension cultures. Glia 55, 385–399.
Ni, H.T., Hu, S., Sheng, W.S., Olson, J.M., Cheeran, M.C., Chan, A.S., Lokensgard, J.R., Peterson, P.K., 2004. High-level expression of functional chemokine receptor CXCR4 on human neural precursor cells. Brain Res. Dev. Brain Res. 152, 159–169.
Panchision, D.M., Chen, H.L., Pistollato, F., Papini, D., Ni, H.T., Hawley, T.S., 2007. Optimized flow cytometric analysis of central nervous system tissue reveals novel functional relationships among cells expressing CD133, CD15, and CD24. Stem Cells 25, 1560–1570.
Peng, H., Kolb, R., Kennedy, J.E., Zheng, J., 2007. Differential expression of CXCL12 and CXCR4 during human fetal neural progenitor cell differentiation. J. Neuroimmune Pharmacol. 2, 251–258.
Piao, J.H., Odeberg, J., Samuelsson, E.B., Kjaeldgaard, A., Falci, S., Seiger, A., Sundstrom, E., Akesson, E., 2006. Cellular composition of long-term human spinal cord- and forebrain-derived neurosphere cultures. J. Neurosci. Res. 84, 471–482.
Pruszak, J., Sonntag, K.C., Aung, M.H., Sanchez-Pernaute, R., Isacson, O., 2007. Markers and methods for cell sorting of human embryonic stem cell-derived neural cell populations. Stem Cells 25, 2257–2268.
Reubinoff, B.E., Itsykson, P., Turetsky, T., Pera, M.F., Reinhartz, E., Itzik, A., Ben-Hur, T., 2001. Neural progenitors from human embryonic stem cells. Nat. Biotechnol. 19, 1134–1140.
Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A., Bongso, A., 2000. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat. Biotechnol. 18, 399–404.
Schmelzer, E., Wauthier, E., Reid, L.M., 2006. The phenotypes of pluripotent human hepatic progenitors. Stem Cells 24, 1852–1858.
Schwartz, P.H., Bryant, P.J., Fuja, T.J., Su, H., O'Dowd, D.K., Klassen, H., 2003. Isolation and characterization of neural progenitor cells from post-mortem human cortex. J. Neurosci. Res. 74, 838–851.
Skottman, H., Mikkola, M., Lundin, K., Olsson, C., Strömberg, A.-M., Tuuri, T., Otonkoski, O., Hovatta, O., Lahesmaa, R., 2005. Gene expression signatures of seven individual human embryonic stem cell lines. Stem Cells 23, 1343–1356.
Studer, V., Jameson, R., Pellereau, A., Pepin, A., Chen, Y., 2004. A microfluidic mammalian cell sorter based on fluorescence detection. Microelectron. Eng. 73–74, 852–857.
Tamaki, S., Eckert, K., He, D., Sutton, R., Doshe, M., Jain, G., Tushinski, R., Reitsma, M., Harris, B., Tsukamoto, A., Gage, F., Weissman, I., Uchida, N., 2002. Engraftment of sorted/expanded human central nervous system stem cells from fetal brain. J. Neurosci. Res. 69, 976–986.
Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., Jones, J.M., 1998. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145–1147.
Uchida, N., Buck, D.W., He, D., Reitsma, M.J., Masek, M., Phan, T.V., Tsukamoto, A.S., Gage, F.H., Weissman, I.L., 2000. Direct isolation of human central nervous system stem cells. Proc. Natl. Acad. Sci. USA 97, 14720–14725.
Ullmann, U., In't Veld, P., Gilles, C., Sermon, K., De Rycke, M., Van de Velde, H., Van Steirteghem, A., Liebaers, I., 2007. Epithelial-mesenchymal transition process in human embryonic stem cells cultured in feeder-free conditions. Mol. Hum. Reprod. 13, 21–32.
Xu, C., Inokuma, M.S., Denham, J., Golds, K., Kundu, P., Gold, J.D., Carpenter, M.K., 2001. Feeder-free growth of undifferentiated human embryonic stem cells. Nat. Biotechnol. 19, 971–974.
Yin, A.H., Miraglia, S., Zanjani, E.D., Almeida-Porada, G., Ogawa, M., Leary, A.G., Olweus, J., Kearney, J., Buck, D.W., 1997. AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 90, 5002–5012.
Zambidis, E.T., Peault, B., Park, T.S., Bunz, F., Civin, C.I., 2005. Hematopoietic differentiation of human embryonic stem cells progresses through sequential hematoendothelial, primitive, and definitive stages resembling human yolk sac development. Blood 106, 860–870.
Zhang, S.C., Wernig, M., Duncan, I.D., Brustle, O., Thomson, J.A., 2001. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat. Biotechnol. 19, 1129–1133.

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Markers of Pluripotency and Differentiation in Human Neural Precursor Cells Derived From Embryonic Stem Cells and CNS Tissue

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Cell transplantation therapies for central nervous system (CNS) deficits such as spinal cord injury (SCI) have been shown to be effective in several animal models. One cell type that has been transplanted is neural precursor cells (NPCs), for which there are several possible sources. We have studied NPCs derived from human embryonic stem cells (hESCs) and human fetal CNS tissue (hfNPCs), cultured as neurospheres, and the expression of pluripotency and neural genes during neural induction and in vitro differentiation. mRNA for the pluripotency markers *Nanog*, *Oct-4*, *Gdf3*, and *DNMT3b* were downregulated during neural differentiation of hESCs. mRNA for these markers was found in nonpluripotent hfNPC at higher levels compared to hESC-NPCs. However, Oct-4 protein was found in hESC-NPCs after 8 weeks of culture, but not in hfNPCs. Similarly, SSEA-4 and CD326 were only found in hESC-NPCs. NPCs from both sources differentiated as expected to cells with typical features of neurons and astrocytes. The expressions of neuronal markers in hESC-NPCs were affected by the composition of cell culture medium, while this did not affect hfNPCs. Transplantation of hESC-NPC or hfNPC neurospheres into immunodeficient mouse testis or subcutaneous tissue did not result in tumor formation. In contrast, typical teratomas appeared in all animals after transplantation of hESC-NPCs to injured or noninjured spinal cords of immunodeficient rats. Our data show that transplantation to the subcutaneous tissue or the testes of immunodeficient mice is not a reliable method for evaluation of the tumor risk of remaining pluripotent cells in grafts.

Key words: Neural precursor cells; Embryonic stem cells; Pluripotency; Spinal cord injury; Transplantation; Tumor

INTRODUCTION

Central nervous system (CNS) injuries often lead to severe functional deficits with no curative treatment available. Much effort is spent on developing therapies for CNS injuries using cell replacement strategies (13, 31). Stem cell therapy could be beneficial by 1) replacing damaged or dead cells, 2) reconnecting injured neurons, 3) secreting neurotrophic factors to support survival and regeneration of remaining cells, 4) remyelinating damaged axons. Neural precursor cells (NPCs) for transplantation therapies can be derived from embryonic stem cells, embryonic or fetal CNS, or adult CNS tissue, and several methods have been developed for neural differentiation and long-term culture of these cells (9,39).

Due to their pluripotency and potential to differentiate into any cell type, human embryonic stem cells (hESCs) are a unique source for various types of cell therapies, including treatment of CNS insults and neurodegenerative diseases (13,23,26). Human ESCs are usu-

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ally derived from the inner cell mass of blastocyst stage embryos. They can be maintained in pluripotent state in culture and differentiated into tissues of all three germ layers (28,44). Following established differentiation protocols, hESCs can be differentiated into neuroectodermal cells, and into mature neuronal and glial cells (8,47). The ability of these neural cells to regenerate damaged areas of the CNS has been studied intensively. For example, hESC-derived oligodendrocyte precursors cells have been shown to remyelinate axons in spinal cord injury (SCI) animal model (23). Still, further optimization is needed for controlled differentiation and purification of hESC-derived neural cell populations for transplantation purposes (15,34).

A crucial and largely unresolved problem is the necessary elimination of remaining pluripotent cells, because even a few pluripotent cells may be sufficient to give rise to tumors (16). Efficient protocols for phenotype induction, as well as reliable markers of pluripotency to be used in elimination of undifferentiated hESCs, are needed in order to achieve clinically acceptable methods for safe cell therapy. A number of markers for pluripotent cells such as Nanog, Oct-4, DNMT3b, SSEA-4, Tra1-81, and CD326 (1,3,41) have been suggested, but their specificity and selectivity need to be further evaluated.

Human NPCs can also be isolated from the human embryonic or fetal CNS, or the adult CNS, and expanded in vitro for prolonged periods (36,45). Although these cells may have more limited differentiation potential compared to hESC-derived neural precursors, they can give rise to neurons, oligodendrocytes, and astrocytes in vitro (33,45). NPCs derived from the human embryonic and fetal CNS (hfNPCs) have been transplanted in SCI animals (21). These cells survive for months after transplantation to injured rat spinal cord, and differentiate into neurons and astrocytes (2). Importantly, whatever the cell type used for future clinical applications, large amounts of cells have to be produced under Good Manufacturing Practice (GMP) quality standards, which can be accomplished by modifying previously published protocols (7,36,42). However, the main concern of hESC-derived cells in cell therapy is the risk of tumor formation due to remaining pluripotent cells.

Here, we studied the differences between hESC-NPCs and hfNPCs cultured under equivalent conditions. We compared the expression of pluripotency, neural and glial markers, and the propensity for tumor formation after transplantation to immunodeficient animals. Our results show that there are important differences between the two cell types with respect to expression of pluripotency markers, that these differences are associated with the presence of pluripotent cells among hESC-NPCs, but that this cannot be reliably assessed in standard teratoma tests.

MATERIALS AND METHODS

Human ESC Cultures

Human ESC lines (HS181 passage 65, HS360 passage 12) were derived from the inner cell mass of supernumerary blastocyst-stage embryos at the Karolinska Institute, Karolinska University Hospital Huddinge, Sweden. Approval for these procedures was obtained from the Regional Ethics Vetting Board in Stockholm. The ethics committee of the Hospital District of Pirkanmaa, Finland (Hovatta, R05051 and Skottman, R05116) has given approval to perform research utilizing the hESC lines derived at the Karolinska Institute. Human ESCs were cultured as previously described (37). Briefly, hESCs were cultured on top of mitotically inactivated human foreskin fibroblasts (CRL-2429, ATCC, Manassas, CA) in hESC medium containing knockout Dulbecco's modified Eagle's medium (DMEM) (Gibco Invitrogen, Carlsbad, CA), 20% knockout-SR (Gibco Invitrogen), 2 mM GlutaMax (Gibco Invitrogen), 0.1 mM minimal essential medium nonessential amino acids (Cambrex Bio Science, Karlskoga, Sweden), 0.1 mM β-mercaptoethanol (Gibco Invitrogen), 50 U/ml penicillin/streptomycin (Cambrex Bio Science), and 8 ng/ml human basic fibroblast growth factor (bFGF, R&D Systems, Minneapolis, MN). Human ESC colonies were passaged by mechanical splitting at 5–7-day intervals and replated on fresh fibroblasts. Karyotypic normality was confirmed after every 25–30 passages.

Neural Induction of hESCs in Suspension Culture

HS181 and HS360 colonies were mechanically dissected and differentiated into neural cells in suspension culture (29,41). The neural differentiation was performed in two different media: neural differentiation medium (NDM) and neural stem cell medium (NSM). NDM contained DMEM/F-12 and neurobasal medium (1:1) supplemented with $1 \times$ B27, $1 \times$ N2, 2 mM GlutaMax (Gibco Invitrogen), 25 U/ml penicillin/streptomycin (Cambrex Bio Science), and 20 ng/ml bFGF. The NSM contained DMEM/F-12 supplemented with $1 \times$ N2, 2 mM GlutaMax (Gibco Invitrogen), 0.6% glucose, 25 U/ml penicillin/streptomycin supplemented (Cambrex Bio Science) with 20 ng/ml human epithelial growth factor (EGF, R&D Systems), 20 ng/ml bFGF (R&D Systems), and 10 ng/ml human ciliary neurotrophic factor (CNTF, R&D Systems). The neurospheres were mechanically split once a week and cultured up to 20 weeks. All cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2. Unless otherwise specified, analyses of hESC-NPCs were carried out in intact neurospheres.

Fetal Neural Precursor Cells

Cultures of hfNPCs derived from human first trimester CNS tissue (6–10 weeks of gestation) were established as previously described (2,33). The procedure was approved by the Regional Ethics Vetting Board, Stockholm, Sweden. Briefly, fetal tissue was retrieved from clinical first trimester routine abortions with informed consent by the woman undergoing termination of pregnancy. Identified CNS tissue dissected from the spinal cord and forebrain was homogenized with a glass-Teflon homogenizer and cultured at 100,000–200,000 cells/ml in NSM or NDM. The cultures of free-floating neurospheres were passaged every 7–14 days by enzymatical dissociation with TrypLE Express (Invitrogen) and gentle trituration, and fresh medium added twice a week. All cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2. Unless otherwise specified, analyses of hfNPCs were carried out in intact neurospheres.

RNA Extraction and cDNA Synthesis

Samples were collected from hESC-NPCs and hfNPCs. RNA was extracted using RNeasy® Micro kit (Qiagen, Hilden, Germany). A total of 50 ng of RNA was used for cDNA synthesis (Sensiscript RT kit, Qiagen) according to the manufacturer's instructions.

PCR

Each PCR reaction contained 1 μl of cDNA, 0.25 μM of forward and reverse primers, 1× Taq-buffer (−MgCl, +KCl) (Fermentas, Leon-Rot, Germany), 2.5 mM dNTP (Fermentas), 25 mM $MgCl_2$ (Fermentas), dH_2O, and 0.6 U Taq-DNA-polymerase enzyme (Fermentas). The PCR program included: denaturation in 95°C for 3 min followed with 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. The following primers were used for 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*, *MAP-2*, β-*tubulin III*; f) astrocytes: *glial fibrillary acidic protein* (*GFAP*); g) oligodendrocytes: *Olig1*, *Olig2*; h) antiapoptosis: *Birc5*; and i) housekeeping gene: *GAPDH*. See Table 1 for primer sequences. Time points for PCR sample collections were 2, 4, 6, 8, and 12 weeks in culture.

Quantitative RT-PCR

Quantitative RT-PCR (qPCR) was performed with FastStart Universal SYBR Green Master Mix (ROX, Roche) for the genes *Oct-4*, *DNMT3b*, *Gdf3*, *GAPDH* and with a TaqMan assay (Applied Biosystems, Foster City, USA) for *Nanog*. 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 done 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. Quantification was done using the ΔΔCt-method as described previously (27). Undifferentiated hESCs (line HS181) were used as a reference sample and *GAPDH* as reference gene. The Ct values were calculated using the 7500 Fast System software, version 1.3.1. Data analysis was done with Microsoft Excel.

Immunohistochemistry

Neurospheres derived from hESCs and human fetal CNS tissue were collected at different time points, fixed in 4% paraformaldehyde (PFA), treated with 30% sucrose over night at 4°C, mounted in Tissue-Tek mounting medium (Sakura Finetek, Zoeterwoude Netherlands), and frozen on dry ice. The neurospheres were cryostat sectioned 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. Primary antibodies used were towards β-tubulin type III (1:800, Sigma), BLPB (1:500, Chemicon, Temecula, CA), GFAP (1:500, DAKO), MAP-2 (1:50, Chemicon), nestin (1:200 Chemicon). Secondary antibodies used were Cy3-conjugated AffiniPure goat anti-mouse IgG (1:2400, Jackson Immuno Research Laboratories, Baltimore, MD, USA), Alexa Fluor 488-conjugated goat anti-mouse IgG, and Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1200, both from Molecular Probes, Leiden, the Netherlands). 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. Analysis was performed with hESC-NPCs cultured in NSM and NDM (HS360 and HS181), and hfNPCs cultured in NSM (spinal cord: 566sc and 569sc, forebrain: 575for and 558for) at time points of 2, 4, 8, 12, and 20 weeks. Samples were evaluated semiquantitatively using the fluorescence microscope (Zeiss, Axiophot).

Table 1. Primer Sequences

Gene	Primer Sequence	Size (bp)
ActivinA	F: CTCTGGATCTGGCTCTGGTC R: TCAGCAGCAATGAAACCAAG	240
AFP	F: AGAACCTGTCACAAGCTGTG R: GACAGCAAGCTGAGGATGTC	672
Birc5	F: ACCTGAAAGCTTCCTCGACA R: TAACCTGCCATTGGAACCTC	184
BLBP	F: CGCTCCTGTCTCTAAAGAGGGG R: TGGGCAAGTTGCTTGGAGTAA	594
GAPDH	F: GTTCGACAGTCAGCCGCATC R: GGAATTTGCCATGGGTGGA	229
GFAP	F: GCTCGATCAACTCACCGCCAACA R: GGGCAGCAGCGTCTGTCAGGTC	430
Doublecortin	F: AAGCTTAGGTGCCTGCGTTA R: AAGGGGCACTTGTGTTTGTC	156
DNMT3b	F: TTGAATATGAAGCCCCCAAG R: GGTTCCAACAGCAATGGACT	179
MAP-2	F: AATAGACCTAAGCCATGTGACATCC R: AGAACCAACTTTAGCTTGGGCC	132
Mash1	F: GTCTCCCGGGGATTTTGTAT R: TCTCCATCTTGGCAGAGCTT	199
Musashi	F: AGCTTCCCTCTCCCTCATTC R: GAGACACCGGAGGATGGTAA	161
Nanog	F: TGCAAATGTCTTCTGCTGAGAT R: GTTCAGGATGTTGGAGAGTTC	286
NCAM	F: GCCAGGAGACAGAAACGAAG R: GGTGTTGGAAATGCTCTGGT	164
Nestin	F: CAGCTGGCGCACCTCAAGATG R: AGGGAAGTTGGGCTCAGGACTGG	208
Oct-4	F: CGTGAAGCTGGAGAAGGAGAAGCTG R: AAGGGCCGCAGCTTACACATGTTC	245
Olig1	F: TTGCATCCAGTGTTCCCGATTTAC R: TGCCAGTTAAATTCGGCTACTACC	389
Olig2	F: CAGAAGCGCTGATGGTCATA R: TCGGCAGTTTTGGGTTATTC	208
Pax-6	F: AACAGACACAGCCCTCACAAACA R: CGGGAACTTGAACTGGAACTGAC	274
Sox2	F: ACACCAATCCCATCCACACT R: GCAAACTTCCTGCAAAGCTC	224
T	F: GCTTCAAGGAGCTCACCAAT R: CACCGCTATGAACTGGGTCT	425
β-*Tubulin III*	F: CAGATGTTCGATGCCAAGAA R: GGGATCCACTCCACGAAGTA	164

Flow Cytometry

Human ESC- and fetal-derived neurospheres were dissociated into single cells with TrypLe Express (Invitrogen) and labeled with antibodies diluted according to the manufacturer's instructions: mouse IgM anti-A2B5 (R&D Systems), mouse IgG1 anti-CD133-PE (Miltenyi Biotech, Bisley, Surrey, UK), mouse IgM CD15-PerCP (Beckman Coulter, Fullerton, CA), mouse IgM anti-PSA-NCAM (Chemicon), mouse IgG1 anti-CD44-PE (BD Pharmingen, San Diego, CA), mouse IgG anti-CD326 (EP-CAM, Chemicon), anti-CD56-PE-Cy7™ (NCAM, BD Pharmingen, San Diego, CA), and mouse IgG anti-SSEA4 (Millipore, St Charles, MO). Unconjugated antibodies were stained with the following secondary antibodies: FITC-conjugated goat anti-mouse IgM (Jackson ImmunoResearch Laboratories) and Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen). Cells were incubated with primary antibodies for 30 min in the dark on ice and washed once with PBS followed by incubation with the appropriate secondary antibody for 30 min in the dark on ice and washed twice with PBS. Flow cytometry was run on a FACsort flow cytometer (Beckton Dickinson) using the software Cell Quest. Isotype controls were used to determine nonspecific staining and gates were set using directly conjugated isotype controls and/or unconjugated isotype controls and the corresponding secondary antibodies as negative control. Human ESC-NPCs and hfNPCs were cultured in vitro for 8 weeks prior to flow cytometry analysis. For analysis of hfNPCs, cells from five different cases were used and the average presented.

Teratoma Formation Analysis

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 (19,20). Equivalent subcutaneous transplantations of 10–12 neurospheres (~100,000 cells) in the left groin were also performed in the same animals. Undifferentiated hESC 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 were 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 postfixed for 4 h in PFA, and then transferred to 10% sucrose for at least 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 above. Human cells were detected using human nuclear marker HuNu (1:250, Chemicon) and hESC-NPCs with β-tubulin III (1:800, Sigma).

Spinal Cord Injury In Vivo Model

A total of 24 adult immunodeficient female rats (170&–200 g, HsdHan:RNU-rnu, Harlan, UK) were included in the study. After a laminectomy of thoracic vertebra 9, a spinal cord contusion injury was produced using the IH Spinal Cord Impactor (Precision Systems & Instrumentation) set at a force of 150 kilodynes, with no dwell time. The lesioned spinal cords were covered with one layer of meningeal substitute (Lyoplant, B/Braun Aesculap) prior to suture of the wound as previously described (2). Eight days later the animals were reanaesthetized and 10–12 hESC-derived 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-NPC cultured in NDM (HS360), b) hESC-NPC cultured in NSM (HS360), c) hESC-NPC cultured in NDM (HS181). One group of rats with a laminectomy but without contusion injury (6 animals/group = sham rats) was injected with hESC-NPC cultured in NDM (HS360). Animals were followed up to 12 weeks after injury and testing of hind limb motor function was performed 1 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 (4), with 21 as the score for normal hind limb function, and 0 for complete paralysis. Functional testing was performed by an experienced investigator blinded to the experimental design. 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 postfixed 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 as described above.

RESULTS

Gene Expression Profiles of hESC-NPCs and hfNPCs

Gene expression analysis revealed that the expression of pluripotency markers *Oct-4*, *Nanog*, and *DNMT3b* was downregulated already 2 weeks after initiation of neural induction in hESC-NPCs while quite surprisingly these markers were found at comparable levels in hfNPCs during the 12 week follow-up time (Fig. 1). Expression of neural markers *sox2*, *nestin*, *Pax-6*, *double-*

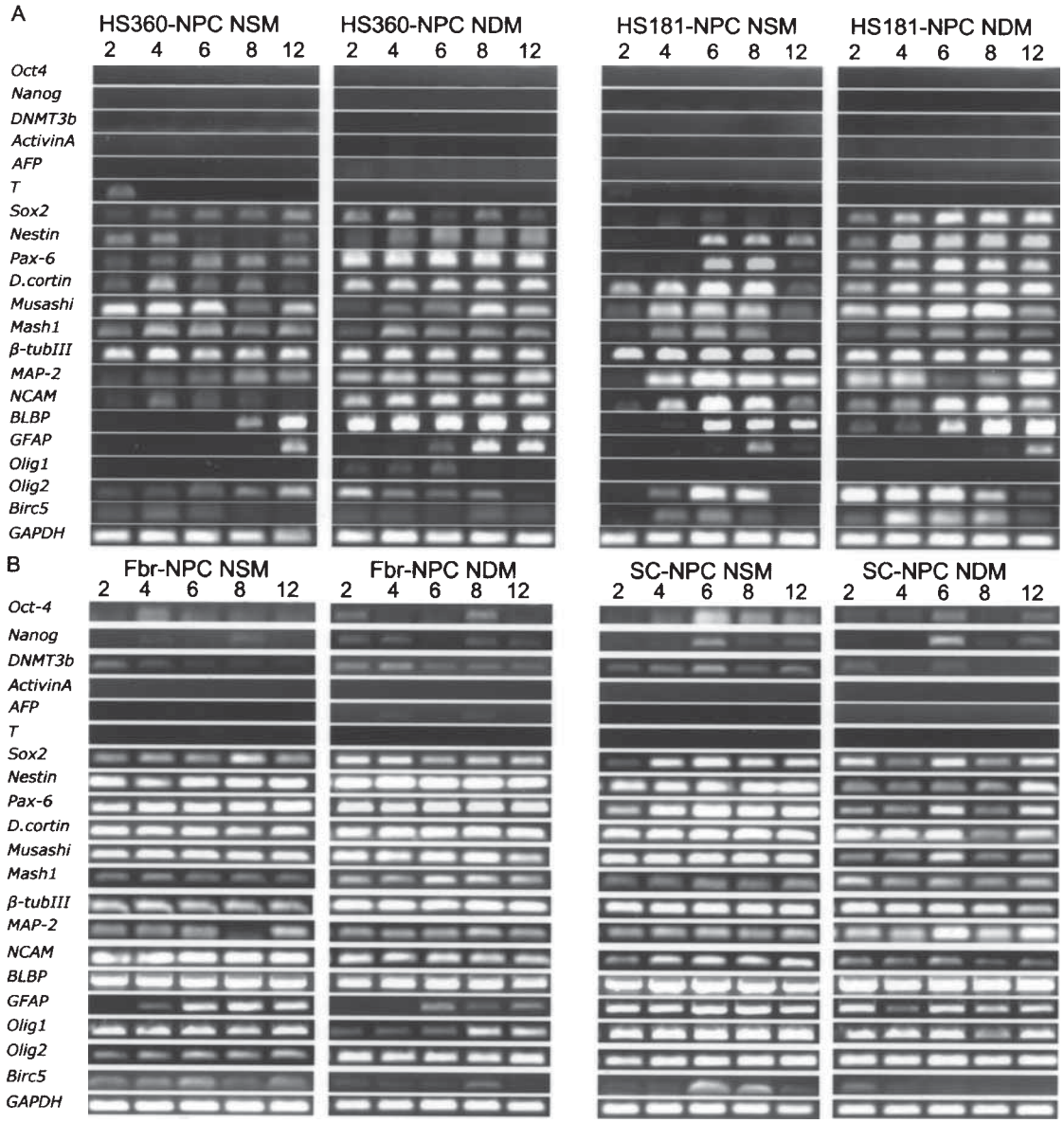

Figure 1. Gene expression profiles of hESC-NPCs and hfNPCs. (A) Representative images of agarose gels of RT-PCR products from hESC-NPC derived from hESC-lines HS181 and HS360. (B) Representative images of agarose gels of RT-PCR products from hfNPC derived from spinal cord (SC) or forebrain (Fbr). Cells were harvested at 2, 4, 6, 8, and 12 weeks of in vitro culture. NPCs were cultured in NSM or NDM.

cortin, *Musashi*, *Mash1*, β-*tubulin III*, *MAP-2*, and *NCAM* increased in hESC-NPCs during differentiation while hfNPCs expressed these markers constantly during 2–12 weeks follow-up. The expression levels of the astrocyte marker *GFAP* increased in hESC-NPCs and forebrain-derived hfNPCs during later time points of culture, while *GFAP* expression in spinal cord-derived hfNPCs remained constant from 2 weeks (Fig. 1). In hESC-NPCs *Olig1* was expressed weakly, and *Olig2* expression varied, while both of them were expressed at constant levels in hfNPCs. In addition, the expression of antiapoptosis marker BIRC5 was detected in varying levels in both cell types (Fig. 1).

We also compared cells cultured in NSM and NDM with respect to the gene expression levels. hESC-NPCs cultured in NDM expressed neuronal markers *MAP-2* and *NCAM*, and radial glial marker *BLPB*, already after 2 weeks of culturing, whereas hESC-NPCs cultured with NSM expressed these markers at lower levels and after longer time in culture. In contrast, hfNPCs expressed all

of these markers at similar levels regardless of medium used (NSM vs. NDM).

Quantitative RT-PCR Analysis of Pluripotency Genes

The results of the semiquantitative RT-PCR with pluripotency genes were confirmed using quantitative PCR. The mRNA levels for *Nanog*, *Oct-4*, *DNMT3b*, and *Gdf3* were significantly lower in hESC-NPCs after 2 weeks of neural induction compared to undifferentiated hESCs, and decreased during further neural differentiation (Fig. 2A). In hfNPCs the levels of *Nanog*, *DNMT3b*, and *Gdf3* mRNAs were higher at 2 weeks after the initial derivation, compared to hESC-derived NPCs (Fig. 2B) and fluctuated during the 12 weeks of study. Two weeks after differentiation, hESC-NPCs cultured with NDM had higher mRNA levels of *Nanog* and *Oct-4* than hESC-NPCs cultured at NSM (Fig. 2A). In hfNPCs, the mRNA levels of the pluripotency markers did not show any consistent differences with respect to the region of origin or cell culture medium used (Fig. 2B).

Protein Expression Analysis

According to the mRNA analysis, expression of pluripotency markers was almost completely suppressed in hESC-NPCs after 8 weeks of neural differentiation. Protein expression in NPCs at this time point was analyzed using flow cytometry and immunocytochemistry. A small proportion of hESC-NPCs expressed the pluripotency markers SSEA4 and CD326 after 8 weeks of differentiation in vitro, while hfNPCs were negative for these proteins. The other early stem/progenitor cell markers CD133 and CD15 (SSEA1) was found in hfNPCs although at lower frequency than hESC-NPCs cultured in NDM. Cells expressing markers for neural stem/progenitor cells, neuronal and glial cells were common among hESC-NPCs cultured in NDM while less so among hfNPCs and hESC-NPCs cultured in NSM (Fig. 3). The most pronounced difference concerned the neuronal marker PSA-NCAM which occurred on twice as many hESC-NPCs as hfNPCs.

Neurospheres were also analyzed with immunohistochemistry. In accordance with flow cytometry (CD326) and PCR (Oct-4) analysis, occasional cells expressing these pluripotency markers were found in hESC-NPCs. No one of these markers could be detected in hfNPCs (Fig. 4). Neuronal markers β-tubulin III and MAP2, glial marker GFAP, and radial glia marker BLBP all increased in sectioned neurospheres of hESC-NPCs during the neural differentiation in NDM. The increase in β-tubulin III did not appear during differentiation in NSM. In hfNPCs the immunoreactivity for these markers remained the same during the study period except for

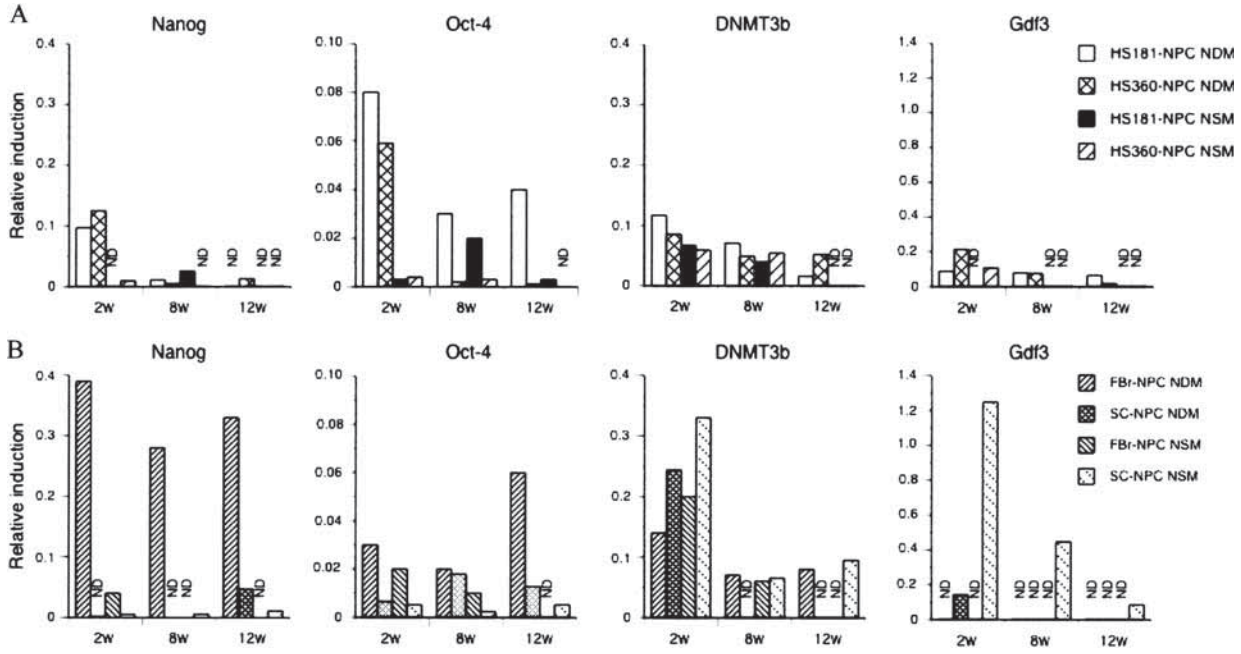

Figure 2. Quantitative mRNA analysis of *Nanog*, *Oct-4*, *DNMT3b*, and *Gdf3*. (A) hESC-NPCs (HS181, HS360) at 2, 8, or 20 weeks of in vitro culture. NPCs were cultured with NSM or NDM. The results are presented as relative fold-induction compared to undifferentiated hESCs. ND: mRNA expression was not detected. (B) hfNPCs (SC and Fbr) at 2, 8, 12, or 20 weeks of in vitro culture. NPCs were cultured with NSM or NDM. The results are presented as relative fold-induction compared to undifferentiated hESCs. ND: mRNA expression was not detected.

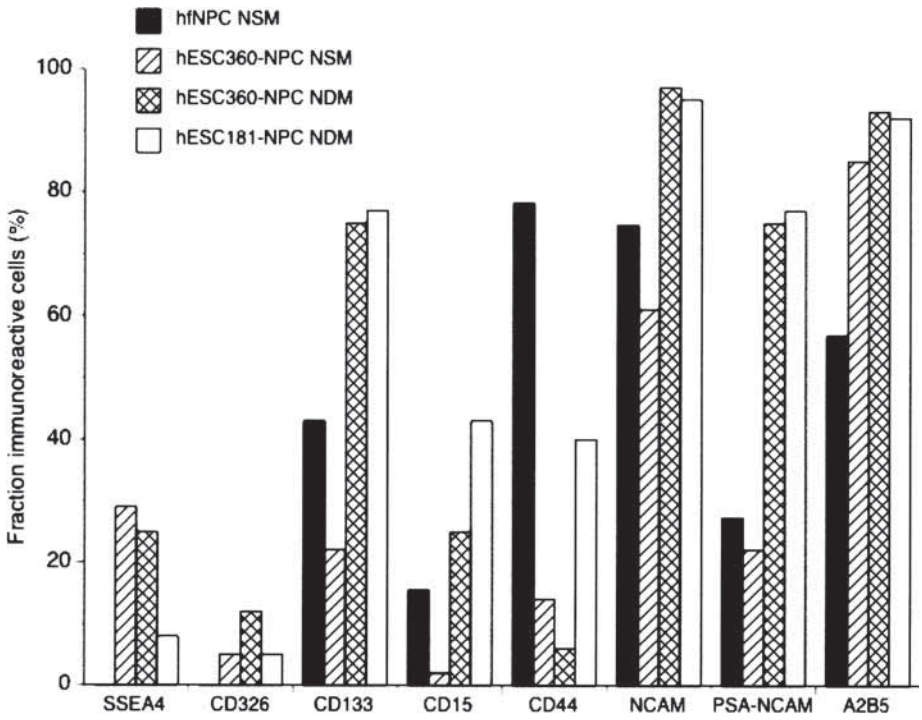

Figure 3. Flow cytometry analysis of hESC-NPCs and hfNPCS. Protein expression of SSEA-4, CD326, CD133, CD15, CD44, CD56, PSA-NCAM, and A2B5 in hESC-NPCs cultured with NDM or NSM (HS181 and HS360) and hfNPCs (SC) cultured in NSM for 8 weeks.

GFAP, which increased in spinal cord-derived hfNPCs, while a progressive decrease was seen in forebrain-derived hfNPCs (data not shown).

hESC-NPC Transplantation to SCID Mice Testis and Subcutaneous Tissue

Initially, a pilot experiment was performed in which dissociated hESC-NPCs, derived from HS181 and HS360, and differentiated for 8 weeks in vitro, were injected into the testis of 8 SCID mice. As these cells did not give rise to any macroscopical teratomas, we transplanted hESC-NPC neurospheres grown in NDM into the lesioned spinal cord of 10 immunodeficient rats. Unexpectedly, the majority of these rats developed tumors, and histological analysis of a few of these animals verified that the tumors were teratomas.

For the analysis of pluripotent cells and teratoma formation, we reasoned that the lack of teratomas in the first testis transplantation experiment may have been due to the injection of dissociated cells, while intact hESC-NPC neurospheres were transplanted to the spinal cord, according to the procedures we previously used for hfNPCs. We therefore transplanted intact hESC-NPC neurospheres into the testis and the subcutaneous tissue of the groin of a total of 10 immunodeficient SCID mice. However, again the hESC-NPCs did not give rise to any macroscopical tumor formation after 12 weeks of in vivo observation, whereas undifferentiated hESC used as positive control did (data not shown). On microscopic examination, the hESC-NPC testis grafts consisted of homogenous tissue with a histological appearance resembling neuropil. There was no invasive growth into the seminiferous tubuli. No cartilage, muscle fibers, pigmented cells, fat, or glands, typical of the teratomas found after transplanting undifferentiated hESCs, could be seen in the grafts (Fig. 5B). hfNPCs transplanted to SCID mice testes also survived and developed into a tissue with neural appearance (Fig. 5A).

To verify that hESC-NPC neurospheres were indeed successfully transplanted to the mice testes, immunohistochemical evaluation using human-specific antibody (HuNu) was applied to cryosections of the tissue. The specificity of the HuNu antibody allowed an efficient screening of the serially sectioned testes in fluorescence microscope, with reliable detection also of small groups of human cells. In 9 out of 10 testes, a human graft could be identified in the testis parenchyma (Fig. 5c). Some transplanted cells were positive for both HuNu and β-tubulin III, with a neuron-like morphology indicating neuronal maturation of the transplanted hESC-NPCs (Fig. 5d).

hESC-NPC Transplantation to Rat Spinal Cord

In parallel with the testis and subcutis transplantation experiments, we transplanted the same hESC-NPCs as neurospheres into sham or injured spinal cords of immunodeficient rats. In sham rats, the median BBB score was 21 up to 10 weeks postsurgery (Fig. 6A). Experimental groups with an SCI initially had a reduction in the median BBB score from a prelesion score of 21 to 7–12 at 1 week postlesion. The hind limb function thereafter improved up to 6 weeks after injury, reaching BBB scores around 15. However, between 6 and 12 weeks postinjury, hind limb function of all rats gradually declined, including the sham-operated rats transplanted with hESC-NPCs (Fig. 6A).

At postmortem analysis, the spinal cords were swollen and semitranslucent at the site of the injury, suggestive of tumor masses with extensive cyst formation (Fig. 6B). Microscopically, hematoxylin-eosin-stained sections showed the typical appearance of teratomas, dominated by large cystic cavities surrounded by tissue representing all three germ layers (Fig. 7).

DISCUSSION

In the present study, we have investigated the differences between hESC-derived NPCs and fetal CNS-derived hfNPCs—two types of cells with potential for future clinical use—in terms of neural differentiation, pluripotency, and risk of teratoma formation. Both hESC-NPCs and hfNPCs were cultured at similar culture conditions and their gene and protein expression were compared. Thereafter, the teratoma formation capacity of NPCs was tested in testis and subcutaneous tissue of immunodeficient animals. Finally, we performed a long-term safety study of hESC-NPCs a rat SCI model.

hESC-NPCs and hfNPCs were cultured for prolonged time (over 12 weeks) in neural differentiation medium (NDM) and neural stem cell medium (NSM) and their gene expression profile for pluripotency and neural markers were studied. As expected, we found a rapid downregulation of the pluripotency genes *Nanog*, *Oct-4*, *DNMT3b*, and *Gdf3* (25,29,41) in hESC-NPCs compared to undifferentiated hESCs. Surprisingly, hfNPCs

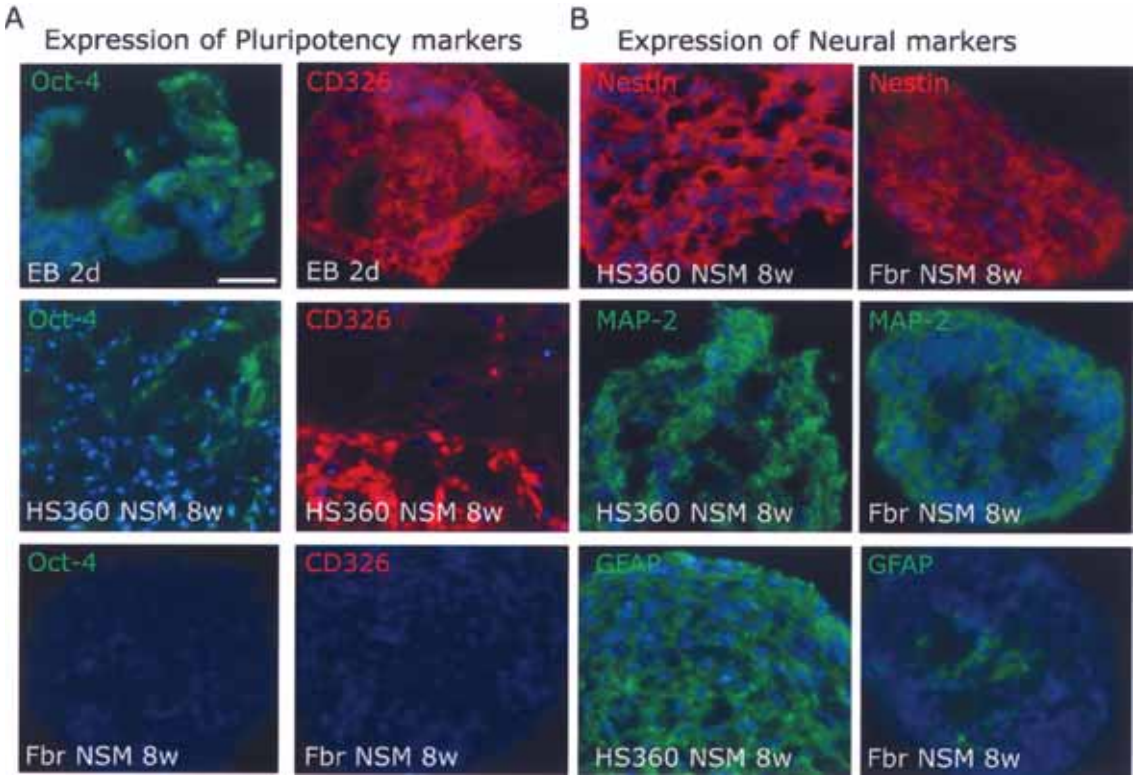

Figure 4. Immunocytochemical analysis of hESC-NPCs and hfNPCS. (A) Expression of pluripotency markers. Embryoid bodies (2 DIV) were used as a positive staining control for pluripotency markers Oct-4 (green) and CD326 (red). A proportion of cells in hESC-NPCs, differentiated for 8 weeks, were immunoreactive for Oct-4 and for CD326. hfNPCs did not show any immunoreactivity for these markers. (B) Expression of neural markers. Both hESC-NPCs and hfNPCs were positive for neural markers nestin (red), MAP-2 (green), and GFAP (green). Same magnification in all pictures. Scale bar: 100 μm.

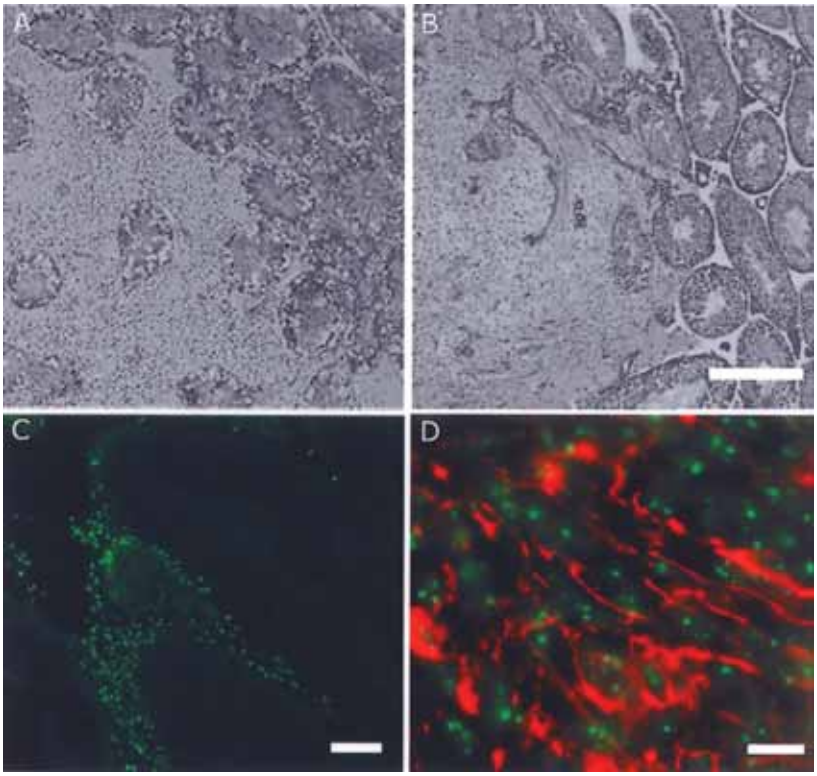

Figure 5. Testis transplantations. (A) Hematoxylin-eosin staining shows the hfNPC graft (left) displacing the normal testicular tissue (right). Scale bar: 500 μm. (B) Hematoxylin-eosin staining shows the hESC-NPC graft (left) displacing the normal testicular tissue (right). Scale bar: 500 μm. (C) HuNu (green) expression in testis confirms the human origin of cells in the graft (hESC-NPCs). Scale bars: 100 μm. (D) Double staining with HuNu (green) and β-tubulin III (red) demonstrates neuronal differentiation of grafted hESC-NPC. Scale bar: 20 μm.

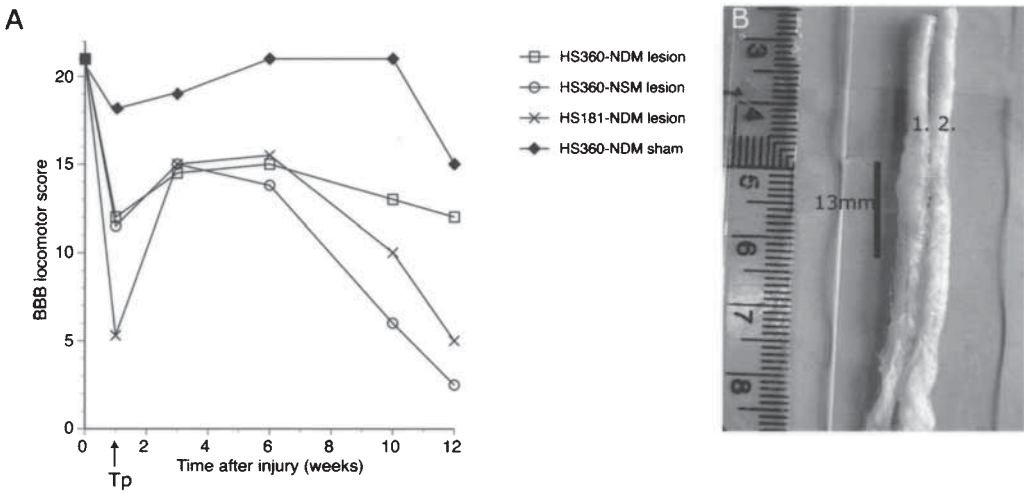

Figure 6. Outcome of hESC-NPCs to rat model of spinal cord injury. (A) Hind limb motor function after hESC-NPC transplantation to the spinal cord. hESC-NPCs were injected into intact or traumatized spinal cords (Tp) and rats were followed for 12 weeks thereafter. BBB locomotor scores of hind limb motor function show that during first 6 weeks the rats' hind limb function either remained stable or improved. However, thereafter the function gradually was reduced in all the transplanted experimental groups. (B) Macroscopically, expanding tumor masses could be seen in the spinal cords 12 weeks after transplantation (1), while no tumors were seen in the shrunken, non-transplanted injured spinal cord (2).

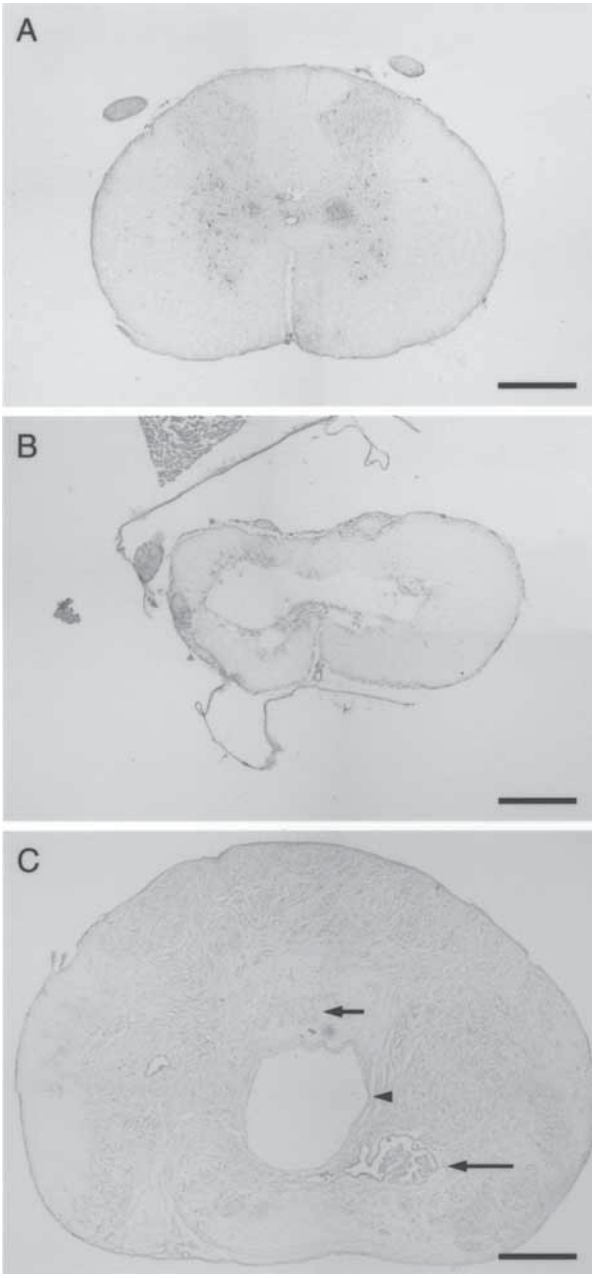

Figure 7. Intraspinal teratoma after hESC-NPCs transplanted to the injured rat spinal cord. Bright-field micrographs of hematoxylin-eosin-stained cross sections of the spinal cord. Scale bars: 500 μm. (A) Normal thoraco-lumbar spinal cord of a noninjured rat. (B) In the traumatized spinal cord, the degenerated gray matter is replaced by a large central lesion cavity. (C) In animals transplanted with hESC-NPC, several millimeters of the injured spinal cord consists of expanding teratomas, displaying examples of the different germ layers. In this section, connective tissue and fat cells (short arrow) and typical exocrine gland tissue (long arrow) are seen. The large cystic cavity (arrowhead) is most likely the pathologically widened central canal.

expressed mRNA for *Nanog*, *Oct-4*, *DNMT3b*, and *Gdf3* at levels that were even higher than in hESC-NPCs, according to quantitative PCR analysis. To eliminate false-positive results, primers for the qPCR analyses of *Nanog*, *Oct-4*, *DNMT3b*, and *Gdf3* were designed to span two exons to avoid the risk of contamination with genomic DNA. In addition, the primers used were validated with undifferentiated hESCs. In accordance with our results on hfNPCs, *Oct-4* is expressed in nonpluripotent NPCs isolated from adult rhesus macaque brain (10), and *Gdf3* has been found in the human cerebral cortex, hippocampus, and cerebellum (17), as well as in other somatic cell types (22). Importantly, in a recent study on dorsal root ganglia-derived neural stem cells, a large number of cells were immunoreactive to Oct-4, as well as to DNMT1 (40). However, we are not aware of any previous reports on expression of *Nanog* in nonpluripotent cells. Despite the low mRNA levels in hESC-NPCs, we could also detect low protein expression of pluripotency markers Oct-4, SSEA4, and CD326 in these cells. Using a slightly different protocol, Kozubenko and collaborators generated neural precursor cells from hESC which they transplanted as dissociated cells to an ischemic brain injury. Interestingly, according to their flow cytometry data at P5 these cells contained between 2% and 5% cells identified as SSEA-4-, TRA-1-60-, and Nanog-immunoreactive cells, although classified as "negative reaction" by the authors. These P5 cells resulted in teratomas in 5/11 animals while P8 cells did not give rise to a single case. This contrasts to the fraction of cells immunoreactive to the three suggested pluripotency markers, which did not change during this time, and for SSEA-4 increased to 16.5% at P10 (25).

According to our results, in contrast to hESC-NPCs the hfNPCs did not express detectable levels of these proteins. Thus, it seems that even relatively low expression levels of pluripotency mRNAs results in protein translation in hESC-NPCs but not in hfNPCs. It is possible that mRNA for pluripotency markers such as Oct-4 is not translated into proteins in hfNPCs, due to micro RNAs that posttranscriptionally modulate genes, and may be responsible for repressing translation (43). The biological significance of this is not clear, but we hypothesize that the hfNPCs expressing mRNA for these pluripotency genes are the least differentiated precursor cells (i.e., the minor subpopulation of "core" neural stem cells of the fetal neurospheres).

During neural differentiation of hESCs, the expression of the neural markers *sox2*, *nestin*, *Pax-6*, *doublecortin*, *Musashi*, *Mash1*, β-*tubulin III*, *MAP-2*, and *NCAM* as expected increased with time. hfNPCs expressed these markers already soon after establishment as neurospheres, and this expression remained stable during the 12 weeks of culture. This agrees with a previ-

ous study showing that hfNPCs have a more pronounced expression of genes such as *GAP43* (growth associated protein 43) and *NNAT* (neuronatin) compared to hESC-NPCs (39). It is important to note, however, that the two cell types studied in this analysis were not cultured under similar conditions, which reduces the accuracy of their comparisons, and also prevents a more conclusive comparison with our data. Here, both NPCs derived from hESCs and human fetal CNS expressed neural markers at protein level after 8 weeks of culture. As we have described previously, expression of neural proteins in hESC-NPC increases over time (41), while their expression is relatively constant in hfNPC s during in vitro culture (33).

When comparing different medium compositions, we found that hESC-NPCs cultured in NDM expressed neuronal markers *MAP-2* and *NCAM*, and radial glial marker *BLPB*, already after 2 weeks of culturing, whereas these markers appeared later and at lower levels in hESC-NPCs cultured in NSM. Flow cytometry analysis showed that NDM also promoted expression of neural stem cell/precursor markers CD133, CD15 as well as neuronal markers CD56 and PSA-NCAM. In contrast, hfNPCs expressed all of these markers at similar levels regardless of medium used (NSM vs. NDM). Thus, while composition of the medium critically affects the cell fate of hESC-NPCs, the neural commitment of hfNPCs is independent of the cell culture medium used. NDM consists of neurobasal medium with B27 supplement, which are known factors to induce neuronal differentiation (11,29), while NSM contains N2 supplement in DMEM/F12, which are less permissive to neural differentiation (11). Compared to hESC-NPCs cultured in NDM, hfNPCs expressed lower amounts of CD133, CD15, and PSA-NCAM in NSM but higher amounts of CD44. In addition, when fetal CNS tissue is the source of NPCs, the regional differences between forebrain- and spinal cord-derived NPCs can affect the expression levels of neural markers (32,46). Here, the spinal cord-derived NPCs expressed astrocyte marker GFAP constantly and at higher levels compared to forebrain-derived NPCs, as shown previously at the protein level (33). Hence, both the medium composition and the origin of the cells are important factors to take into account when differentiating NPCs either from hESCs or fetal tissue.

Intratesticular injections of cells (38) followed by analysis of teratoma formation are generally considered a reliable assay for identifying the presence of pluripotent cells in cell preparations. Recent studies have shown that there are differences between target organs with regard to the propensity of transplanted hESC to develop teratomas (16), and also between different brain regions (14). In the present study we found even more pronounced differences than previously reported. In repeated experiments with hESC-NPCs transplanted as small neurospheres, a total of 100,000 cells consistently gave rise to teratomas in the rat spinal cord, while we could not detect a single case of tumor growth after intratesticular or subcutaneous transplantations in mice. To ensure that there was sufficient time for the teratomas to develop, the transplanted animals were kept for 12 weeks. During this time, the hind limb motor function of SCI rats recovered to some extent according to the BBB rating, reaching similar levels that are shown in other studies at 6 weeks time point (23). However, during the later part of the 12 weeks follow-up time, functional deterioration and extensive growth of teratomas in spinal cords were seen in 26/26 rats with hESC-NPCs transplants. In contrast, hESC-NPC spheres grafted to testis survived in 9/10 mice and differentiated into neural tissue, with no signs of expansive tumor growth. By using undifferentiated hESC as positive controls, we confirmed that the procedures used were appropriate, as described previously (19). The survival of transplanted hESC-NPCs was also confirmed.

We want to emphasize that we do not claim that cells giving rise to intraspinal tumors are unable to develop into tumors in testis or other locations. The hESC-NPCs may develop into teratomas in testis if sufficient numbers are transplanted. We used no more than 100,000 cells for these grafts, because this is a number of cells that previously have been used in SCI transplantation experiments; 100,000 undifferentiated hESCs were sufficient for generating tumors in the positive controls, as has also been shown previously (24). Intraspinal transplantation of a suspension of 1 million cells or more—typically used in teratoma experiments—is not compatible with the small tissue volume of the rat spinal cord. We conclude that with the number of cells used, there are surprisingly large differences in the permissiveness for tumor formation between the rat spinal cord and SCID mouse testis and subcutis. A consequence of this finding is that there may be similar differences between the human spinal cord and the models commonly used to evaluate tumorigenesis; that is, neural cells derived from pluripotent cells may appear as safe in routine teratoma tests and lead to tumor growth after transplantation to human SCI patients. Whether the spinal cord is a CNS region uniquely permissive to growth of pluripotent cells is to our knowledge not known, because comparisons have only been made between regions of the brain, excluding the spinal cord (14).

There are several examples of protocols for neural differentiation of hESC that apparently eliminate pluripotent cells (30,47) and thus the risk of teratoma growth, also after transplantation to the spinal cord (23). It has

been shown that the presence of pluripotent cells and the associated risk of teratoma formation decreases over time under differentiating conditions (5) but it is not a guarantee for complete elimination of all pluripotent cells. We found a large downregulation of pluripotent genes in hESC-NPCs differentiated for 8 weeks before transplantations, but these genes are according to our results not specific for pluripotent cells. The number of pluripotent cells in hESC-derived neurospheres was apparently sufficient for generating teratomas after transplantation to the rat spinal cord, but did not exceed the number needed for growth of teratomas in SCID mouse testis (16).

A factor that may influence the teratoma formation capacity of transplanted cells is the use of intact neurospheres versus dissociated neural cells. Dissociated neural cells survive poorly after transplantation to the rodent CNS (18) while cell death after transplantation of intact neurospheres is low (12). Hence, in transplanted neurospheres pluripotent cells may survive better. In agreement with this, Kishi and colleagues (24) found that pluripotent cells transplanted as cell aggregates more likely survived and generated teratomas than dissociated cells.

Our results illustrate a number of important issues with regard to the use of hESC derivatives for cell therapy, including the use of appropriate protocols for production of differentiated neural populations and the need of cell purification using methods such as magnetic beads (6) or fluorescence-activated cell sorting (35,41).

In summary, this study emphasizes the importance of proper characterization of cell grafts in means of gene and protein expression levels and the importance of using reliable animal models for preclinical transplantation studies to measure the safety and efficacy. First, protein expression of pluripotency genes is a better predictor of tumorigenicity than mRNA, but there are problems with specificity and sensitivity. Second, reliable evaluation of the safety and efficacy of cell therapies should be assessed in the expected target of the clinical application, and include extended observation times. Taken together, many aspects including the graft composition and characterization, transplantation site, and follow-up time are all important factors that influence the reliability of safety transplantation studies. This needs to be taken into account especially in hESC-associated studies.

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REFERENCES

1. Adewumi, O.; Aflatoonian, B.; Ahrlund-Richter, L.; Amit, M.; Andrews, P. W.; Beighton, G.; Bello, P. A.; Benvenisty, N.; Berry, L. S.; Bevan, S.; Blum, B.; Brooking, J.; Chen, K. G.; Choo, A. B.; Churchill, G. A.; Corbel, M.; Damjanov, I.; Draper, J. S.; Dvorak, P.; Emanuelsson, K.; Fleck, R. A.; Ford, A.; Gertow, K.; Gertsenstein, M.; Gokhale, P. J.; Hamilton, R. S.; Hampl, A.; Healy, L. E.; Hovatta, O.; Hyllner, J.; Imreh, M. P.; Itskovitz-Eldor, J.; Jackson, J.; Johnson, J. L.; Jones, M.; Kee, K.; King, B. L.; Knowles, B. B.; Lako, M.; Lebrin, F.; Mallon, B. S.; Manning, D.; Mayshar, Y.; McKay, R. D.; Michalska, A. E.; Mikkola, M.; Mileikovsky, M.; Minger, S. L.; Moore, H. D.; Mummery, C. L.; Nagy, A.; Nakatsuji, N.; O'Brien, C. M.; Oh, S. K.; Olsson, C.; Otonkoski, T.; Park, K. Y.; Passier, R.; Patel, H.; Patel, M.; Pedersen, R.; Pera, M. F.; Piekarczyk, M. S.; Pera, R. A.; Reubinoff, B. E.; Robins, A. J.; Rossant, J.; Rugg-Gunn, P.; Schulz, T. C.; Semb, H.; Sherrer, E. S.; Siemen, H.; Stacey, G. N.; Stojkovic, M.; Suemori, H.; Szatkiewicz, J.; Turetsky, T.; Tuuri, T.; van den Brink, S.; Vintersten, K.; Vuoristo, S.; Ward, D.; Weaver, T. A.; Young, L. A.; Zhang, W. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. Nat. Biotechnol. 25: 803–816; 2007.
2. Akesson, E.; Piao, J. H.; Samuelsson, E. B.; Holmberg, L.; Kjaeldgaard, A.; Falci, S.; Sundstrom, E.; Seiger, A. Long-term culture and neuronal survival after intraspinal transplantation of human spinal cord-derived neurospheres. Physiol. Behav. 92:60–66; 2007.
3. Babaie, Y.; Herwig, R.; Greber, B.; Brink, T. C.; Wruck, W.; Groth, D.; Lehrach, H.; Burdon, T.; Adjaye, J. Analysis of Oct4-dependent transcriptional networks regulating self-renewal and pluripotency in human embryonic stem cells. Stem Cells 25:500–510; 2007.
4. Basso, D. M.; Beattie, M. S.; Bresnahan, J. C. A sensitive and reliable locomotor rating scale for open field testing in rats. J. Neurotrauma 12:1–21; 1995.
5. Brederlau, A.; Correia, A. S.; Anisimov, S. V.; Elmi, M.; Paul, G.; Roybon, L.; Morizane, A.; Bergquist, F.; Riebe, I.; Nannmark, U.; Carta, M.; Hanse, E.; Takahashi, J.; Sasai, Y.; Funa, K.; Brundin, P.; Eriksson, P. S.; Li, J. Y. Transplantation of human embryonic stem cell-derived cells to a rat model of Parkinson's disease: Effect of in vitro differentiation on graft survival and teratoma formation. Stem Cells 24:1433–1440; 2006.
6. Campanelli, J. T.; Sandrock, R. W.; Wheatley, W.; Xue, H.; Zheng, J.; Liang, F.; Chesnut, J. D.; Zhan, M.; Rao, M. S.; Liu, Y. Expression profiling of human glial precursors. BMC Dev. Biol. 8:102; 2008.
7. Carpenter, M. K.; Cui, X.; Hu, Z. Y.; Jackson, J.; Sherman, S.; Seiger, A.; Wahlberg, L. U. In vitro expansion of a multipotent population of human neural progenitor cells. Exp. Neurol. 158:265–278; 1999.

8. Carpenter, M. K.; Inokuma, M. S.; Denham, J.; Mujtaba, T.; Chiu, C. P.; Rao, M. S. Enrichment of neurons and neural precursors from human embryonic stem cells. Exp. Neurol. 172:383–397; 2001.
9. Coutts, M.; Keirstead, H. S. Stem cells for the treatment of spinal cord injury. Exp. Neurol. 209:368–377; 2008.
10. Davis, S. F.; Hood, J.; Thomas, A.; Bunnell, B. A. Isolation of adult rhesus neural stem and progenitor cells and differentiation into immature oligodendrocytes. Stem Cells Dev. 15:191–199; 2006.
11. Dhara, S. K.; Stice, S. L. Neural differentiation of human embryonic stem cells. J. Cell. Biochem. 105:633–640; 2008.
12. Emgard, M.; Holmberg, L.; Samuelsson, E. B.; Bahr, B. A.; Falci, S.; Seiger, A.; Sundstrom, E. Human neural precursor cells continue to proliferate and exhibit low cell death after transplantation to the injured rat spinal cord. Brain Res. 1278:15–26; 2009.
13. Goldman, S. A.; Windrem, M. S. Cell replacement therapy in neurological disease. Philos. Trans. R. Soc. Lond. B Biol. Sci. 361:1463–1475; 2006.
14. Harkany, T.; Andang, M.; Kingma, H. J.; Gorcs, T. J.; Holmgren, C. D.; Zilberter, Y.; Ernfors, P. Region-specific generation of functional neurons from naive embryonic stem cells in adult brain. J. Neurochem. 88:1229–1239; 2004.
15. Hedlund, E.; Pruszak, J.; Lardaro, T.; Ludwig, W.; Vinuela, A.; Kim, K. S.; Isacson, O. Embryonic stem cell-derived Pitx3-enhanced green fluorescent protein midbrain dopamine neurons survive enrichment by fluorescence-activated cell sorting and function in an animal model of Parkinson's disease. Stem Cells 26:1526–1536; 2008.
16. Hentze, H.; Soong, P. L.; Wang, S. T.; Phillips, B. W.; Putti, T. C.; Dunn, N. R. Teratoma formation by human embryonic stem cells: Evaluation of essential parameters for future safety studies. Stem Cell Res. 2:198–210; 2009.
17. Hexige, S.; Guo, J.; Ma, L.; Sun, Y.; Liu, X.; Ma, L.; Yan, X.; Li, Z.; Yu, L. Expression pattern of growth/differentiation factor 3 in human and murine cerebral cortex, hippocampus as well as cerebellum. Neurosci. Lett. 389:83–87; 2005.
18. Hicks, A. U.; Lappalainen, R. S.; Narkilahti, S.; Suuronen, R.; Corbett, D.; Sivenius, J.; Hovatta, O.; Jolkkonen, J. Transplantation of human embryonic stem cell-derived neural precursor cells and enriched environment after cortical stroke in rats: Cell survival and functional recovery. Eur. J. Neurosci. 29:562–574; 2009.
19. Hovatta, O.; Mikkola, M.; Gertow, K.; Stromberg, A. M.; Inzunza, J.; Hreinsson, J.; Rozell, B.; Blennow, E.; Andang, M.; Ahrlund-Richter, L. A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. Hum. Reprod. 18: 1404–1409; 2003.
20. Inzunza, J.; Gertow, K.; Stromberg, M. A.; Matilainen, E.; Blennow, E.; Skottman, H.; Wolbank, S.; Ahrlund-Richter, L.; Hovatta, O. Derivation of human embryonic stem cell lines in serum replacement medium using postnatal human fibroblasts as feeder cells. Stem Cells 23:544–549; 2005.
21. Iwanami, A.; Kaneko, S.; Nakamura, M.; Kanemura, Y.; Mori, H.; Kobayashi, S.; Yamasaki, M.; Momoshima, S.; Ishii, H.; Ando, K.; Tanioka, Y.; Tamaoki, N.; Nomura, T.; Toyama, Y.; Okano, H. Transplantation of human neural stem cells for spinal cord injury in primates. J. Neurosci. Res. 80:182–190; 2005.
22. Jones, C. M.; Simon-Chazottes, D.; Guenet, J. L.; Hogan, B. L. Isolation of Vgr-2, a novel member of the transforming growth factor-beta-related gene family. Mol. Endocrinol. 6:1961–1968; 1992.
23. Keirstead, H. S.; Nistor, G.; Bernal, G.; Totoiu, M.; Cloutier, F.; Sharp, K.; Steward, O. Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. J. Neurosci. 25:4694–4705; 2005.
24. Kishi, Y.; Tanaka, Y.; Shibata, H.; Nakamura, S.; Takeuchi, K.; Masuda, S.; Ikeda, T.; Muramatsu, S.; Hanazono, Y. Variation in the incidence of teratomas after the transplantation of nonhuman primate ES cells into immunodeficient mice. Cell Transplant. 17:1095–1102; 2008.
25. Kozubenko, N.; Turnovcova, K.; Kapcalova, M.; Butenko, O.; Anderova, M.; Rusnakova, V.; Kubista, M.; Hampl, A.; Jendelova, P.; Sykova, E. Analysis of in vitro and in vivo characteristics of human embryonic stem cell-derived neural precursors. Cell Transplant. 19:471–486; 2010.
26. Lindvall, O.; Kokaia, Z. Stem cells for the treatment of neurological disorders. Nature 441:1094–1096; 2006.
27. Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 4:402–408; 2001.
28. Mitalipova, M.; Calhoun, J.; Shin, S.; Wininger, D.; Schulz, T.; Noggle, S.; Venable, A.; Lyons, I.; Robins, A.; Stice, S. Human embryonic stem cell lines derived from discarded embryos. Stem Cells 21:521–526; 2003.
29. Nat, R.; Nilbratt, M.; Narkilahti, S.; Winblad, B.; Hovatta, O.; Nordberg, A. Neurogenic neuroepithelial and radial glial cells generated from six human embryonic stem cell lines in serum-free suspension and adherent cultures. Glia 55:385–399; 2007.
30. Nistor, G. I.; Totoiu, M. O.; Haque, N.; Carpenter, M. K.; Keirstead, H. S. Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation. Glia 49:385–396; 2005.
31. Okano, H. Neural stem cells and strategies for the regeneration of the central nervous system. Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. 86:438–450; 2010.
32. Ostenfeld, T.; Joly, E.; Tai, Y. T.; Peters, A.; Caldwell, M.; Jauniaux, E.; Svendsen, C. N. Regional specification of rodent and human neurospheres. Brain Res. Dev. Brain Res. 134:43–55; 2002.
33. Piao, J. H.; Odeberg, J.; Samuelsson, E. B.; Kjaeldgaard, A.; Falci, S.; Seiger, A.; Sundstrom, E.; Akesson, E. Cellular composition of long-term human spinal cord- and forebrain-derived neurosphere cultures. J. Neurosci. Res. 84:471–482; 2006.
34. Pruszak, J.; Ludwig, W.; Blak, A.; Alavian, K.; Isacson, O. CD15, CD24 and CD29 define a surface biomarker code for neural lineage differentiation of stem cells. Stem Cells 27:2928–2940; 2009.
35. Pruszak, J.; Sonntag, K. C.; Aung, M. H.; Sanchez-Pernaute, R.; Isacson, O. Markers and methods for cell sorting of human embryonic stem cell-derived neural cell populations. Stem Cells 25:2257–2268; 2007.
36. Quinn, S.; Walters, W.; Vescovi, A.; Whittemore, S. Lineage restriction of neuroepithelial precursor cells from fetal human spinal cord. J. Neurosci. Res. 57:590–602; 1999.
37. Rajala, K.; Hakala, H.; Panula, S.; Aivio, S.; Pihlajamaki,

H.; Suuronen, R.; Hovatta, O.; Skottman, H. Testing of nine different xeno-free culture media for human embryonic stem cell cultures. Hum. Reprod. 22:1231–1238; 2007.
38. Reubinoff, B. E.; Pera, M. F.; Fong, C. Y.; Trounson, A.; Bongso, A. Embryonic stem cell lines from human blastocysts: Somatic differentiation in vitro. Nat. Biotechnol. 18: 399–404; 2000.
39. Shin, S.; Sun, Y.; Liu, Y.; Khaner, H.; Svant, S.; Cai, J.; Xu, Q. X.; Davidson, B. P.; Stice, S. L.; Smith, A. K.; Goldman, S. A.; Reubinoff, B. E.; Zhan, M.; Rao, M. S.; Chesnut, J. D. Whole genome analysis of human neural stem cells derived from embryonic stem cells and stem and progenitor cells isolated from fetal tissue. Stem Cells 25:1298–1306; 2007.
40. Singh, R. P.; Shiue, K.; Schomberg, D.; Zhou, F. C. Cellular epigenetic modifications of neural stem cell differentiation. Cell Transplant. 18:1197–1211; 2009.
41. Sundberg, M.; Jansson, L.; Ketolainen, J.; Pihlajamaki, H.; Suuronen, R.; Skottman, H.; Inzunza, J.; Hovatta, O.; Narkilahti, S. CD marker expression profiles of human embryonic stem cells and their neural derivatives, determined using flow-cytometric analysis, reveal a novel CD marker for exclusion of pluripotent stem cells. Stem Cell Res. 2:113–124; 2009.
42. Swistowski, A.; Peng, J.; Han, Y.; Swistowska, A. M.; Rao, M. S.; Zeng, X. Xeno-free defined conditions for culture of human embryonic stem cells, neural stem cells and dopaminergic neurons derived from them. PLoS One 4:e6233; 2009.
43. Tay, Y.; Zhang, J.; Thomson, A. M.; Lim, B.; Rigoutsos, I. MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. Nature 455: 1124–1128; 2008.
44. Thomson, J. A.; Itskovitz-Eldor, J.; Shapiro, S. S.; Waknitz, M. A.; Swiergiel, J. J.; Marshall, V. S.; Jones, J. M. Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147; 1998.
45. Varghese, M.; Olstorn, H.; Berg-Johnsen, J.; Moe, M. C.; Murrell, W.; Langmoen, I. A. Isolation of human multipotent neural progenitors from adult filum terminale. Stem Cells Dev. 18:603–613; 2009.
46. Watanabe, K.; Nakamura, M.; Iwanami, A.; Fujita, Y.; Kanemura, Y.; Toyama, Y.; Okano, H. Comparison between fetal spinal-cord- and forebrain-derived neural stem/progenitor cells as a source of transplantation for spinal cord injury. Dev. Neurosci. 26:275–287; 2004.
47. Zhang, S. C.; Wernig, M.; Duncan, I. D.; Brustle, O.; Thomson, J. A. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat. Biotechnol. 19:1129–1133; 2001.

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REGULAR ARTICLE

Production and isolation of NG2$^+$ oligodendrocyte precursors from human embryonic stem cells in defined serum-free medium

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Abstract Human embryonic stem cells (hESCs) are a promising source of oligodendrocyte precursor cells (OPCs) and oligodendrocytes. These cells can be used to repair myelin in central nervous system deficits such as multiple sclerosis or traumas such as spinal cord injury. Here, we introduce a novel differentiation method for the production of OPCs from hESCs. OPCs were differentiated as spheres in defined serum-free medium supplemented with recombinant human growth factors. A broad gene expression analysis revealed that this OPC population expressed *Olig1/2*, *Sox10*, *PDGFR*, *Nkx2.2*, *Nkx6.2*, *oligodendrocyte-myelin glycoprotein*, *myelin basic protein (MBP)*, and *proteolipid protein (PLP)*. According to quantitative RT-PCR analyses addition of ciliary neurotrophic factor (CNTF) upregulated the *Olig2* mRNA levels in the OPC population. According to the flow cytometry analyses the OPC population was >90% NG2-positive, >80% PDGFR-positive, and >60% CD44-positive, and further matured into O4- (45%) and GalC- (80%) positive oligodendrocyte populations when cultured on top of human extracellular matrix proteins, which were used instead of Matrigel. In addition, OPCs matured into myelin-forming cells when cocultured with neuronal cells. The multilayered myelin sheet formation around axons was detected with transmission electron microscopy in cocultures. Further, the OPC populations could be purified with sorting of NG2$^+$ cells. These NG2$^+$ cells reformed spheres that remained stable during prolonged culturing (7 weeks), and matured into GalC-positive oligodendrocytes. Importantly, these NG2$^+$ spheres were free of pluripotent Tra1-81, Oct-4, and CD326-positive hESCs. Thus, this method is suitable for the efficient production of OPCs and in the future for therapeutic graft production.

Introduction

Oligodendrocyte precursor cells (OPCs) develop from neural stem cells through various genetic and morphologic stages (Nicolay et al., 2007). OPCs migrate from the germinal zones into the brain parenchyma where they mature into oligodendrocytes (Miller, 2002; Spassky et al., 2001). OPCs develop from bipolar cells and express genes such as *Olig1/2*, *Sox10*, and *Nkx2.2* (Hu et al., 2009), and specific proteins, such as chondroitin sulfate proteoglycan (NG2) and platelet-derived growth factor receptor-α (PDGFRα) (Levine et al., 2001; Nishiyama et al., 1996). Maturing OPCs express the myelination-related genes *Nkx6.2*, *myelin basic protein (MBP)*, and *proteolipid protein (PLP)*, and proteins such as galactocerebroside (GalC), O4, and MBP (Nicolay et al., 2007). The major function of oligodendrocytes is to form myelin sheets around axons to protect and enhance neuronal signaling

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(Grinspan, 2002). Oligodendrocyte death or atrophy causes demyelination in central nervous system (CNS) deficits such as multiple sclerosis, spinal cord injury (SCI), and brain injury. There are currently no effective treatments for these CNS deficits. Oligodendrocyte-based transplantation represents a potential treatment option.

Human OPCs can be obtained from aborted fetuses (Zhang et al., 2000), olfactory biopsies of the neuroepithelium (Zhang et al., 2005), and differentiated human embryonic stem cells (hESCs) (Hu et al., 2009; Izrael et al., 2007; Nistor et al., 2005; Zhang et al., 2006b). hESCs are clinically the most attractive material for OPC production due to their high proliferation potential and differentiation capacity (Nistor et al., 2005; Thomson et al., 1998). Compared to mouse ESC-derived OPCs (Brustle et al., 1999; Glaser et al., 2005; Zhang et al., 2006a), the production of hESC-derived OPCs is less straightforward and most attempts to transfer mouse ESC methods to hESC cultures have failed (Izrael et al., 2007). Nevertheless, hESCs do differentiate into OPCs and these cells have been shown to restore locomotor function and increase myelination in SCI rats (Keirstead et al., 2005) and restore myelination in the shiverer mouse brain (Izrael et al., 2007). Thus, the development of methods to efficiently differentiate hESCs into OPCs especially for clinical purposes is urgently needed to expand treatment options for patients with CNS disorders.

Current protocols for OPC differentiation from hESCs use animal-derived components like Matrigel and animal-derived growth supplements (Izrael et al., 2007; Nistor et al., 2005; Zhang et al., 2006b). In addition, most hESC lines are cultured on mouse fibroblasts, which may contaminate the cultures and cause immunologic responses (Heiskanen et al., 2007; Martin et al., 2005). In addition, commonly used enzymatic passaging of hESCs tends to increase the incidence of abnormal chromosomal mutations (Thomson et al., 2008), which can lead to development of cancer cells. Some neural differentiation methods utilize either stromal cell lines (MS5 and PA6) or Matrigel (Carpenter et al., 2001; Nistor et al., 2005; Reubinoff et al., 2001; Zhang et al., 2001), both of which increase the risk of pathogen cross-transfer. Despite this, the first Phase I clinical trial on spinal cord injury patients using hESC-derived oligodendrocyte progenitor cells (GRNOPC1) will be conduct by Geron in the near future. The cells differentiation protocol is based on the Nistor and colleagues method (2005) and contains several animal derived components and Matrigel (Nistor et al., 2005). Currently the study has been placed on clinical hold by the FDA pending data from a nonclinical animal study which showed cystic formation in spinal cords of the transplanted animals (www.geron.com). This implies that obtaining hESC-derived cell populations for clinical trials is a time-consuming and complicated process where the safety of the cell grafts must be clearly proved. Thus, differentiation methods that aim for xeno-free cell production with detailed cell population characterizations and purification steps are crucial en route to clinics.

Here, we introduce a new method for OPC differentiation from hESCs. First, the hESCs used for OPC production were cultured on human feeder cells, passaged mechanically, and grown in serum-free media. Second, OPCs were differentiated in suspension culture and passaged mechanically in the presence of human recombinant growth factors and mitogens in serum-free media. Third, the cells were matured without Matrigel on human extracellular matrix (ECM) proteins. Fourth, OPC populations were purified by fluorescence-activated cell sorting using NG2-antibody.

Results

HESC oligodendrocyte differentiation

Two hESC lines, HS360 and HS362, were used to develop the oligodendrocyte differentiation protocol. The entire three-stage differentiation protocol took 13 weeks to complete (Fig. 1). In Stage 1 hESCs differentiated into neural precursors in neural spheres in suspension culture. These neural spheres were cultured for 4 to 8 weeks in the presence of bFGF, EGF, and CNTF. In Stage 2, the OPCs were differentiated in suspension culture for 3 weeks in the presence of bFGF, EGF PDGF-AA, laminin, ± CNTF, or ± IGF-1. In Stage 3, the OPCs were matured in suspension culture for 2 weeks in the presence of T3, ± CNTF, or ± AA.

Gene expression profiles of developing OPCs

The hESC-derived differentiating OPCs were analyzed with semiquantitative RT-PCR at 0, 4, 8, 11, and 13 weeks (Figs. 2A–C). Gene expression analysis showed that the undifferentiated hESCs (HS360 and HS362) expressed the pluripotent gene *Oct-4* which was downregulated during differentiation. Expression of the neural precursor gene *Nestin* was constant during Stages 1 and 2 (0–11 weeks; Fig. 2A), whereas expression of the neural precursor gene *Pax-6* was downregulated in Stage 2 (11 weeks; Fig. 2A). *Sox10* and *Olig2* were expressed during OPC differentiation Stages 1 to 3 (4–13 weeks; Fig. 2B). Especially at Stage 3 *Olig2* was expressed in cells cultured in Medium 3.2, containing T3, CNTF, and AA (13 weeks; Fig. 2B). More detailed gene expression analysis of the hESC line HS362 revealed that during Stage 1 (4 weeks), neural precursor gene *Mash1*, and OPC gene *Olig1*, and *oligodendrocyte-myelin glycoprotein (OMG)* were expressed (Fig. 2C). Up to 8 weeks was required, however, before a neural to glial cell switch was confirmed by the upregulation of *Ngn3* and *Sox9*. Also, the expression of *PDGFR*, *Nkx2.2*, *Nkx6.2*, and *MBP* was detected and the expression of *Sox8* was upregulated after 8 weeks (Fig. 2C). After Stage 2 (11 weeks), the gene expression patterns in the OPC populations varied to some extent depending on the growth factor composition of the medium. *Olig1* and *Nkx2.2*, however, were upregulated in cells grown in medium containing IGF-1 and CNTF (Medium 2.2.). When cells grown in this medium were advanced to Stage 3 for further maturation, and cultured in growth factor-reduced media containing T3, ± CNTF, or ± AA, the gene expression patterns changed considerably (13 weeks; Fig. 2C). In particular, *Gli1*, *Gli2*, *Sox9*, *Sox10*, *Olig1*, *Nkx2.2*, and *Nkx6.2* were strongly upregulated in the presence of T3, CNTF, and AA-treated cells (Medium 3.2, Fig. 2C), indicating that the combination of these factors supports OPC maturation. According to qRT-PCR analysis at Stage 2 (11 weeks), the *Olig2* expression increased in OPCs treated with CNTF (Media 2.1 and 2.2) compared to cells

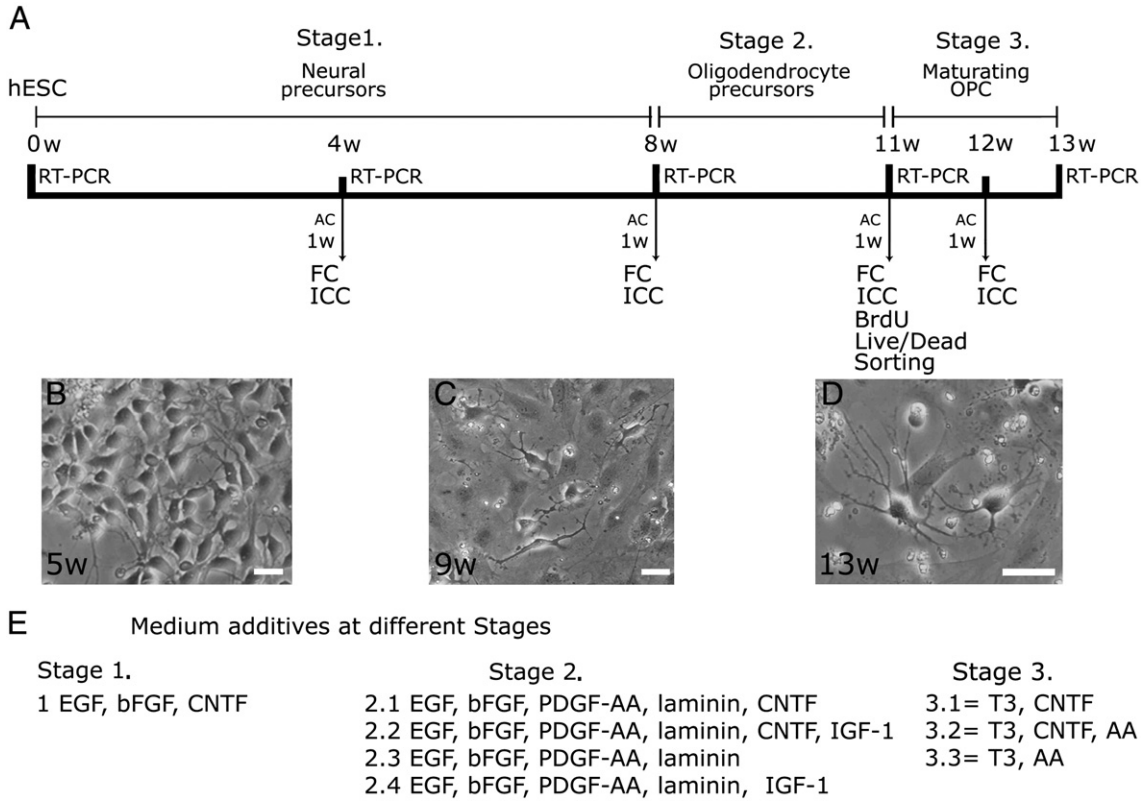

Figure 1 A schematic representation of the differentiation of hESC-derived OPCs (A). RT-PCR analysis of the differentiating spheres was performed at Stage 1 (4 and 8 weeks), Stage 2 (11 weeks), and Stage 3 (13 weeks). The morphologic analysis and collection of samples for the flow cytometric (FC, time points 5, 9, 12, and 13 weeks), immunocytochemistry (ICC; 5, 9, 12, and 13 weeks), cell proliferation (5′-bromo-2-deoxyuridine, BrdU, 12 weeks), cell viability (Live/Dead, 12-weeks), and cell sorting (12 weeks) were performed after 1 week of adherent culturing (AC). Morphology of cells during differentiation stages is presented (B–D, scale bar = 50 μm). The medium additives at different stages are presented in (E). In Stage 1, the hESCs differentiated to neural precursors in suspension culture in the presence of EGF (epidermal growth factor), bFGF (basic fibroblast growth factor), and CNTF (ciliary neurotrophic factor) = Medium 1. In Stage 2, the cells were induced to differentiate into OPCs in the presence of bFGF, EGF, PDGF-AA (platelet-derived growth factor-AA), laminin, ± CNTF, and ± IGF-1 (insulin-like growth factor-1) = Media 2.1–2.4. In Stage 3, the OPCs differentiated further in the presence of T3 (3,3′,5-triiodo-L-thyronine), ± AA (L-ascorbic acid 2-phosphate), and ± CNTF = Media 3.1–3.3 (E). In the adherent cultures, cells were grown in the presence of bFGF, EGF, PDGF-AA, laminin, CNTF, and IGF-1 (5-, 9-, and 12-week samples) or in the presence of T3, AA, and CNTF (13-week samples).

cultured without CNTF (Media 2.3–2.4, Figs. 2D and E). At Stage 3 (13 weeks), in the presence of T3, CNTF, and AA, *Olig2* expression was increased (Medium 3.2, Figs. 2D and E). *Olig2* induction was confirmed with two different hESC lines (HS360 and HS362, Figs. 2D and E).

Immunocytochemical characterization of developing OPCs

Immunocytochemical staining patterns showed that OPCs (Stage 2, 12 weeks) expressed NG2, Olig2, PDGFR-α, (Figs. 2F–H). These OPC populations were proliferative and stained positive for BrdU marker (Fig. 2H). Prematuring GalC-positive cells were dividing and expressed Ki67 (Fig. 2I). At Stage 3 (13 weeks), the maturing OPCs coexpressed NG2 and O4 (Fig. 2J) and were highly positive for GalC (Figs. 2K–M) and coexpressed MBP (Fig. 2M), a major protein involved in the myelination process. Further, the produced population had typical morphologic characteristics of oligodendrocytes (Supplemental Movie).

Effects of IGF-1 on OPC proliferation and survival *in vitro*

IGF-1 is shown to be crucial for OPC survival (Cui et al., 2005; Ness et al., 2004; Pang et al., 2007). Here, we studied the effect of IGF-1 on the proliferation and survival of OPCs at Stage 2 (12 weeks). After 3 weeks of culturing in the presence or absence of IGF-1, the OPCs were stained for NG2 and BrdU (Figs. 3A–D), and the number of colabeled cells was counted for each medium. The results indicated that IGF-1 significantly increased cell proliferation by 24 percentage unit ($P<0.01$, HS362) and 20 percentage unit ($P<0.05$, HS360) in the presence of CNTF (Medium 2.2) but did not have constant effect without CNTF (Medium 2.4, Fig. 3E). Then we evaluated the effect of IGF-1 on cell survival. The numbers of live and dead cells were counted for each medium (Figs. 4F–I). IGF-1 increased the number of living cells from 16 to 23 percentage unit in the presence of CNTF (16 percentage unit, $P<0.05$, HS360-derived OPCs and 23 percentage unit $P<0.01$, HS362-drived OPCs; Fig. 3J). No

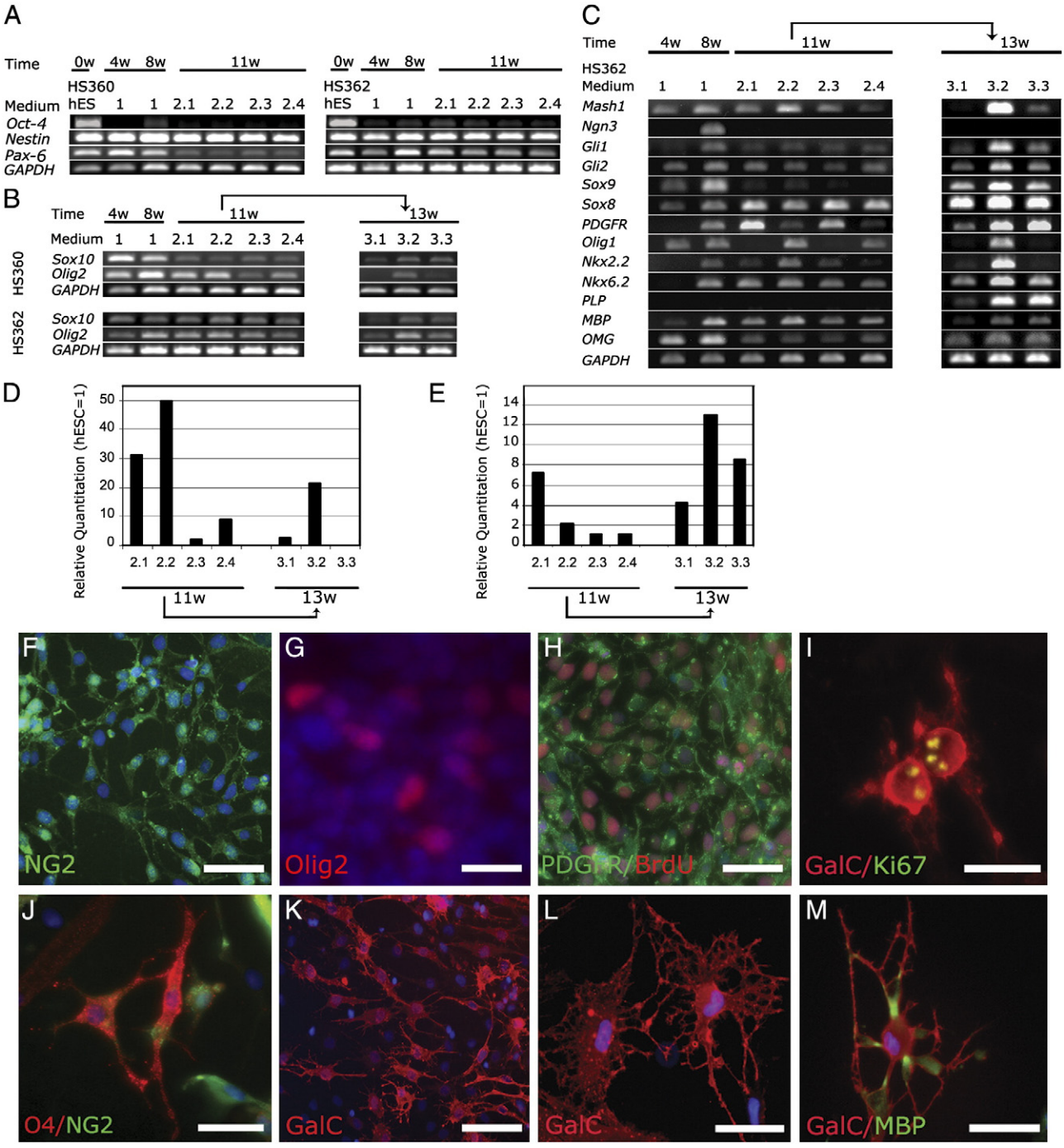

Figure 2 Gene expression analyses in hESC lines HS360 and HS362 during OPC differentiation (A, B). Pluripotent hESC marker *Oct-4* was expressed in undifferentiated hESCs, neural markers *Nestin* and *Pax-6* were expressed constantly during differentiation, and *GAPDH* was used as a housekeeping gene (A). *Sox10* and *Olig2* (OPC markers) were expressed during differentiation Stages 1–3 (B). Wider gene expression analysis of OPC differentiation of hESCs (HS362) in Stages 1–3 is presented (C). Quantitative RT-PCR analysis of *Olig2* showed upregulation of *Olig2* in the presence of CNTF in Stage 2 and in Stage 3. Undifferentiated hESCs were used as a reference sample and *GAPDH* as a reference gene (hESC lines HS360 and HS362, D, E). Medium additives: hES = bFGF; Medium 1 = EGF, bFGF, CNTF; Medium 2.1 = EGF, bFGF, PDGF-AA, laminin, CNTF; Medium 2.2 = EGF, bFGF, PDGF-AA, laminin, CNTF, IGF-1; Medium 2.3 = EGF, bFGF, PDGF-AA, laminin; Medium 2.4 = EGF, bFGF, PDGF-AA, laminin, IGF-1; 3.1 = T3, CNTF; Medium 3.2 = T3, CNTF, AA; Medium 3.3 = T3, AA. Immunocytochemical characterization of hESC-derived OPCs and oligodendrocytes (hESC line HS360, F–M). OPCs in Stage 2 were positive for NG2 (green, F) and Olig2 (red, G). Proliferating OPCs were positive for PDGFR (green) and BrdU (red, H) and positive for GalC (red) and Ki67 (green, I). Maturing oligodendrocytes in Stage 3 were positive for O4 (red, J). Mature oligodendrocytes were positive for GalC (red, K–M) and expressed MBP (green, M), which accumulated in the branches of oligodendrocytes. Scale bars: 100 μm (F), 50 μm (G), 50 μm (H), 50 μm (I), 50 μm (J), 100 μm (K), and 50 μm (L–M).

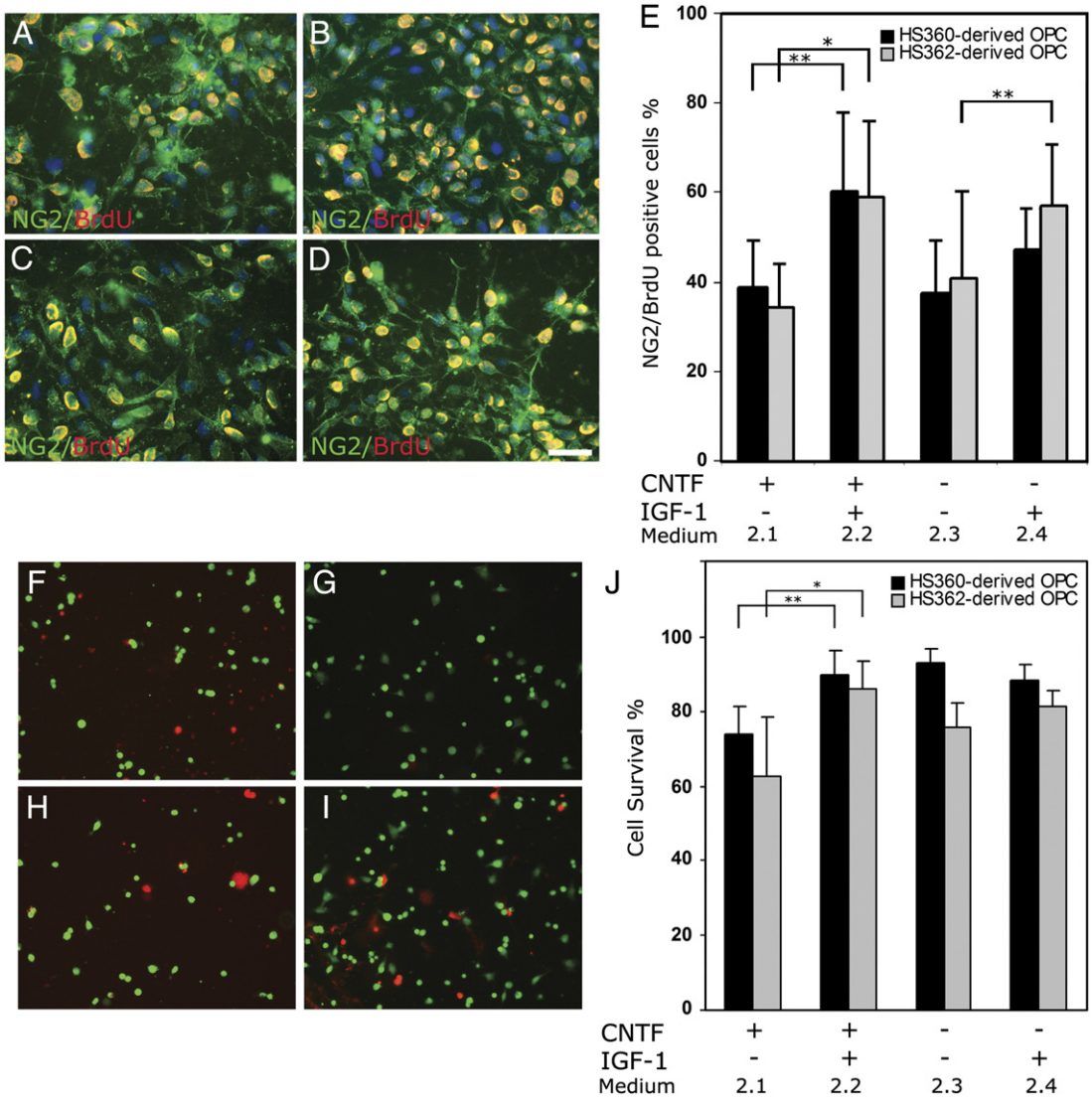

Figure 3 Effects of IGF-1 on cell proliferation at Stage 2 (12 weeks) were studied with hESC lines HS360 and HS362 (A–E). BrdU-positive cells (red) in a NG2-positive cell population (green); cells were cultured with +CNTF/–IGF-1 (Medium 2.1, A), +CNTF/+IGF-1 (Medium 2.2, B), –CNTF/–IGF-1 (Medium 2.3, C), and –CNTF/+IGF-1 (Medium 2.4, D). Cell proliferation significantly increased after IGF-1 stimulation (* $P<0.01$, ** $P<0.05$, results are means ± SD, $n=5–18$/group) (E). Effect of IGF-1 on cell survival at Stage 2 (12 weeks, F–J). Fluorescent pictures of live cells (green) and dead cells (red), cells were treated with +CNTF/ –IGF-1 (Medium 2.1, F), +CNTF/+IGF-1 (Medium 2.2, G), –CNTF/–IGF-1 (Medium 2.3, H), and –CNTF/+IGF-1 (Medium 2.4, I). Cell viability study indicated that cell survival increased when cultures were treated with IGF-1 and CNTF (* $P<0.01$, ** $P<0.05$, results are means ± SD, $n=8–10$/group, J). Scale bar, 100 μm.

significant differences were detected in cell cultures treated with IGF-1 in the absence of CNTF (Fig. 3J). These results indicated that Medium 2.2 containing both IGF-1 and CNTF provided the best support for OPC proliferation and survival.

Expression of oligodendrocyte-specific proteins during OPC differentiation

The protein expression analysis of differentiating OPC cultures was performed using immunocytochemical cell counts and flow cytometry. Prior to analyses, cells from Stages 1 (8 weeks) and 2 (11 weeks) were cultured for 1 week in adherent cultures in Medium 2.1, 2.2, 2.3, or 2.4. Immunocytochemical cell counts revealed that after 1 week of differentiation (9 weeks) cells were 43 to 55% positive for NG2 (Supplemental Table 2) and 90 to 93% NG2 positive in Stage 2 (12 weeks, Supplemental Table 2). Expression of PDGFRα increased from 28 to 73% in Stage 1 (9 weeks) and from 86 to 88% in Stage 2 (12 weeks; Supplemental Table 2). After Stage 2 the OPC population could be cryo-preserved and after thawing remained viable and were >90% NG2 positive (data not shown). Importantly, astrocytes and neurons were either not detected or present in low numbers during the OPC differentiation (Supplemental Table 2, Figs. 4B and D). For further OPC differentiation Medium 2.2 was chosen and Figure 4A shows the upregulation of NG2 (>90%) and CD44 (>60%) expressions in HS360- and HS362-derived OPCs at

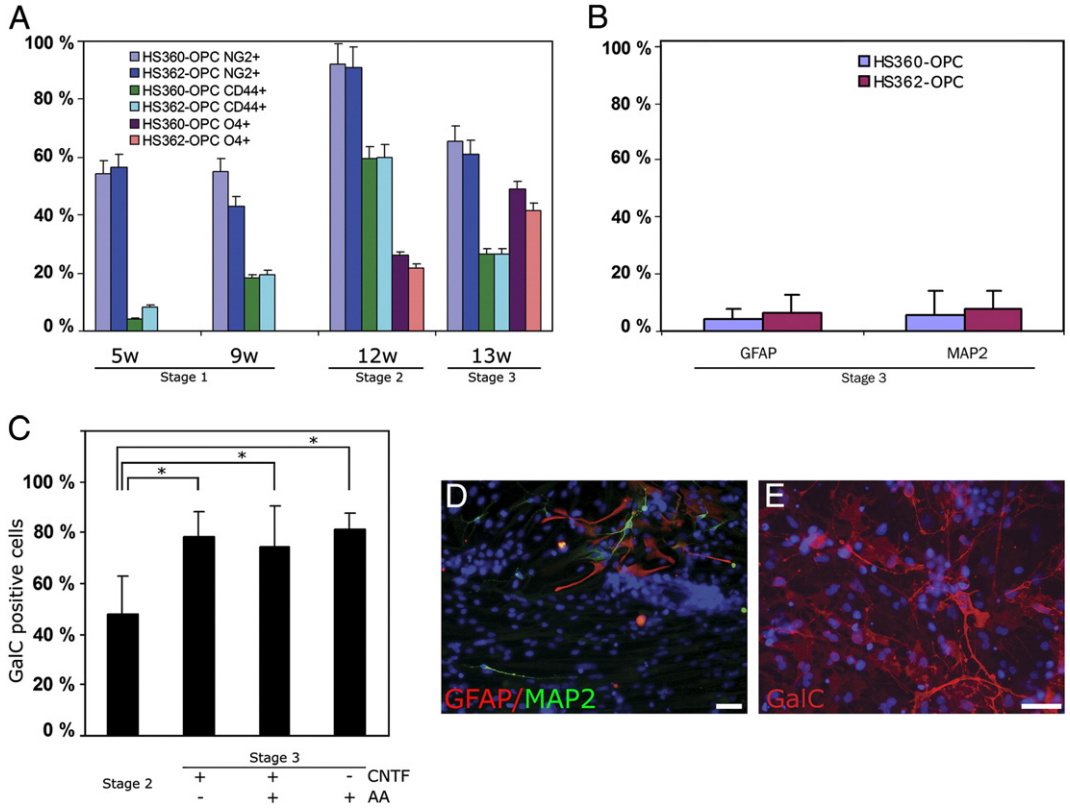

Figure 4 Flow cytometry analysis of CD44, NG2, and O4 expression during OPC differentiation from two independent experiments of two hESC lines (HS360 and HS362, A). Expression of GFAP and MAP2 during OPC differentiation (hESC line HS362, B). Expression of GalC-positive cells during OPC differentiation in Stages 2 and 3. Results are combined means ± SD from two cell lines studied (HS360 and HS362, $n = 12–16$/group, C). At Stage 3, only a few cells expressed GFAP (red) and MAP2 (green, D) whereas most of the cells were GalC-positive (red, E) (hESC line HS362). Scale bar, 100 μm.

Stage 2 (Fig. 4A). In the maturing OPCs at Stage 3 the expression of NG2 and CD44 was decreased, while expression of O4 increased up to ~45% compared to ~20% expression at Stage 2 (Fig. 4A). Similarly, the amount of GalC-positive cells significantly increased up to ~80% in the maturing OPC population at Stage 3 compared to Stage 2 ($P < 0.001$, Figs. 4C and E). Detailed expression of GalC at Stage 3 is presented in Supplemental Table 3.

Expression of CD44 during oligodendrocyte differentiation

As the flow cytometric analysis indicated relatively high expression of CD44 at Stage 2 during OPC differentiation (~60%, Fig. 4A), it was examined in more detail. The combinatorial flow cytometric analysis revealed that during further differentiation at Stage 3, the CD44-positive cells coexpressed NG2 (Supplemental Fig. 1A), whereas only a few of O4-positive cells were CD44-positive (Supplemental Fig. 1C). On the other hand, NG2-positive cells coexpressed O4 (Supplemental Fig. 1B). The coexpression of CD44 and NG2 was confirmed by immunocytochemical staining (Supplemental Fig. 1D). In addition, PDGFRα and Olig2-positive OPCs, BLBP-positive radial glial cells, and GFAP-positive astrocytes expressed CD44 to some extent (Supplemental Figs. 1E–I). On the other hand, the maturing GalC-positive oligodendrocytes did not express CD44 (Supplemental Fig. 1G). These results suggest that CD44 is expressed in radial glial cells, astrocytes, and OPCs, but not in maturing oligodendrocytes.

Myelination capacity of OPCs

OPCs from Stage 2 were cocultured with GFP neurons. The OPCs differentiated into pre- and myelinating oligodendrocytes, positive for GalC which aligned along neurites (Supplemental Fig. 2A). Further, the oligodendrocytes attached to the neurons (Supplemental Fig. 2B) and formed MBP-positive wraps around the neurites (Supplemental Fig. 2C). Scanning electron microscopy analysis revealed ramification of cell branches of maturing oligodendrocytes in single and cocultures (Figs. 5A and B). Myelination capacity of oligodendrocytes (from Stage 3) was detected with transmission electron microscopy, which showed myelin sheets around multiple axons (Figs. 5C–E). Higher magnification revealed the formation of multilayered myelin sheath around axons (Figs. 5D and E).

Sorting of NG2$^+$ OPC populations

After Stage 2 (11 weeks), OPCs positive for NG2 were sorted (Fig. 6A and B). After 1 week of subculturing and the NG2$^+$

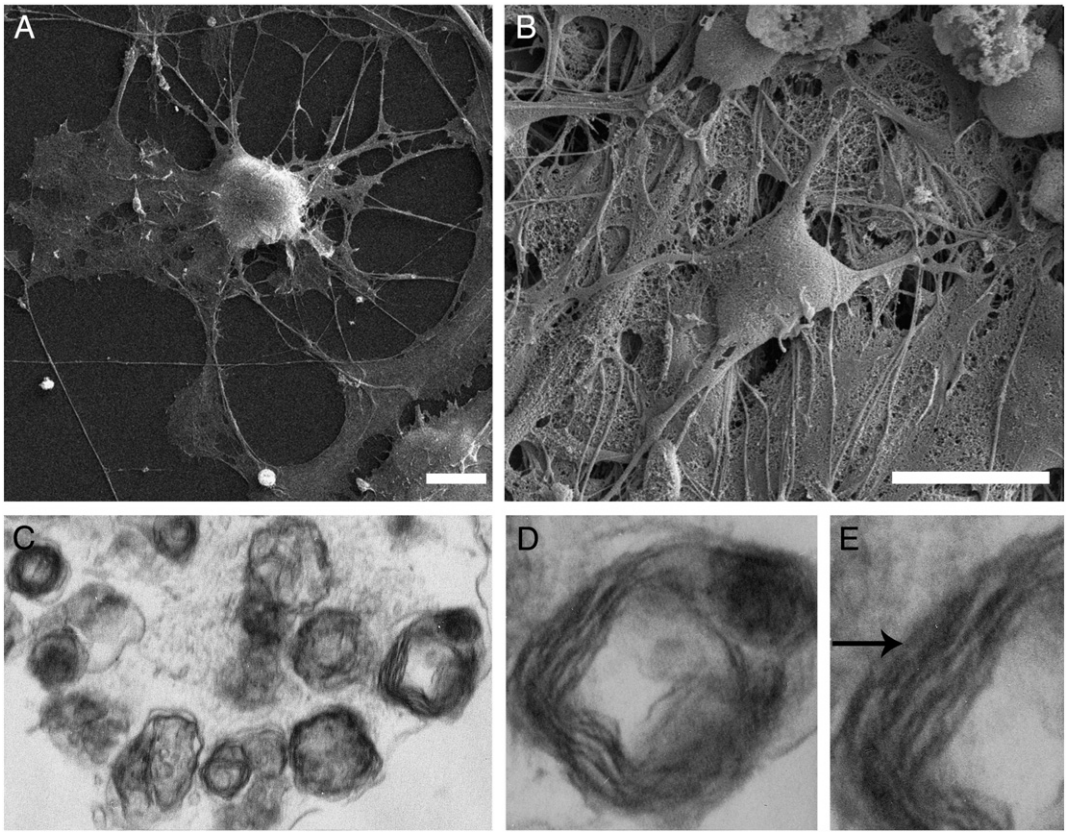

Figure 5 Scanning electron micrographs (SEM) of maturing hESC-derived OPCs (A), and coculture of OPCs with neurons (hESC line Regea 06/040, B). The transmission electron micrograph illustrates myelination of axons in cocultures (C); higher magnification of multilayered myelin sheaths around the axons (D, E). Arrow indicates the myelin layers (E). Scale bar, 10 μm.

population showed a higher proliferation capacity (48% vs 40% of Ki67-positive cells) when cultured with a B27 supplement (Figs. 6C and D). In addition, NG2$^+$ cells after 7 weeks of subculturing expressed higher levels of NG2 and O4 when cultured with B27 compared to cells cultured without it (Fig. 6G). Thus, for prolonged subculturing and sphere formation B27 was added to media as described earlier (Jiang et al., 2009). The NG2$^+$ cells aggregated into spheres already at 4 to 8 days after sorting and were mechanically passaged once a week thereafter (Fig. 6E). The NG2$^+$ spheres had a stable oligodendrocyte-related gene expression profile during the 2- to 6-week follow-up time, and expression of *Oct4* could not be detected (Fig. 6F). Compared to unsorted cell population, 2 and 4 weeks after sorting the expressions of *NG2, PDGFR, Olig2, Nkx6.2*, and PLP were stronger in NG2$^+$ cells (Fig. 6F). Flow cytometry analysis revealed that the sorted cell population remained NG2$^+$ during 7 weeks of subculturing compared to an unsorted cell population at the same time point (Fig. 6G). In addition, NG2$^+$ cells expressed >50% O4 (Fig. 6G), whereas unsorted cells were <20% O4 positive. Importantly, NG2$^+$ cells were negative for pluripotency markers Tra1-81 and Oct-4 (Fig. 6H) and for CD362 (Fig. 6I). After 7 weeks of subculturing NG2$^+$ cells differentiated into GalC-positive cells (Fig. 6K). The amount of proliferating cells decreased after prolonged subcultivation of NG2$^+$ spheres, compared to 1 week after sorting (Figs. 6L and C). All cultures were free on neuronal cells, whereas few GFAP-positive astrocytes were detected (Fig. 6M).

Discussion

To date, the protocols developed for OPC differentiation from hESCs contain animal-derived substances and therefore are not designed as such for clinical grade production (Izrael et al., 2007; Kang et al., 2007; Nistor et al., 2005). Despite this, attempts to transform these methods for graft production for clinical trials are ongoing (Geron Corporation, USA), although contents and safety issues of these products are not fully characterized. Here, we introduced a novel method for human OPC production from hESCs using human recombinant growth factors and ECM proteins. This protocol comprised 3 stages in same basal medium with different growth factor cocktails. Further, this method was based on the sphere formation in suspension culture with mechanical passaging. Mechanical passaging allows large-scale OPC production and cell amplification *in vitro*. In the future, it will be possible to transfer these suspension cultures to bioreactor cultivation, where gas flows, medium changes, and cell passaging are performed under invariable conditions under computer monitoring (Gilbertson et al., 2006; Portner et al., 2005; Sen et al., 2004). The entire differentiation procedure took 13 weeks to complete and during Stage 2 the proliferating

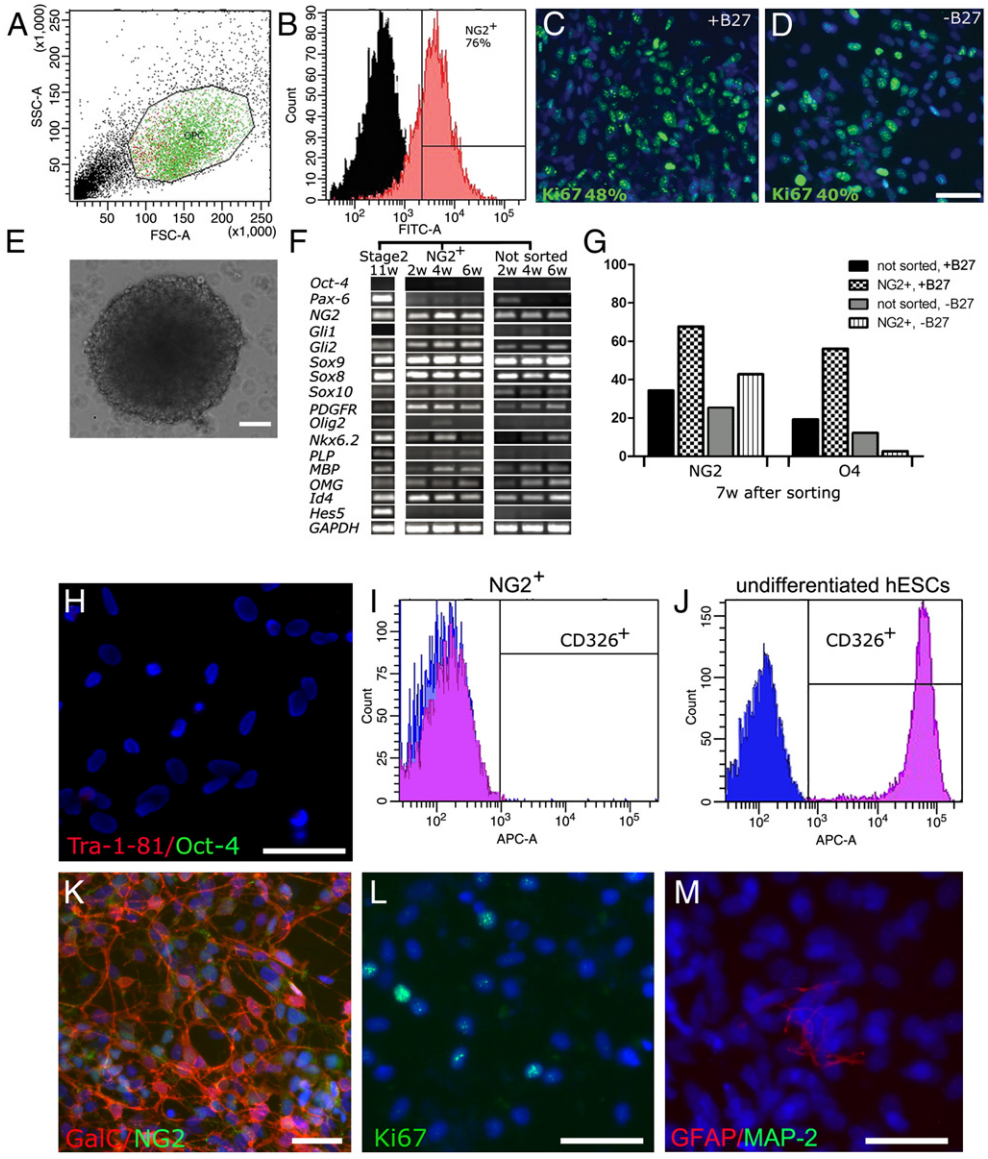

Figure 6 Characterization of sorted NG2$^+$ cells (hESC lines Regea 06/040 and Regea 08/023). FSC/SSC dot plot shows determination of OPC population (A). FITC-histogram shows that 76% of cells were NG2$^+$ (red); unstained cells were used for background determination (black, B). Subcultured NG2$^+$ cells expressed Ki67; 48% if cultured with B27 (green, C), and 40% if cultured without B27 (green, D), in Medium 2.2. Eight days after sorting, NG2$^+$ cells formed round and solid spheres in suspension culture (E). Gene expression analyses of cells before sorting (Stage 2, 11 weeks), NG2$^+$ cells (2, 4, and 6 weeks after sorting), and not sorted cells (2, 4, and 6 weeks) are presented (F). Seven weeks after sorting the NG2$^+$ and not sorted cell population, cultured with or without B27, were analyzed with flow cytometry for expression of NG2 and O4 (G). NG2$^+$ cells were negative for Tra-1-81 (red, H) and Oct-4 (green, H) and only 0.4% CD326-positive after 7 weeks of subculturing in Medium 2.2 (I). Undifferentiated hESCs were used as a positive control for the staining with pluripotency marker CD326 (J), and unstained cells were used for background determination (blue, I, J). *In vitro* differentiated NG2$^+$ cells (7 weeks time point) expressed GalC (red K), NG2 (green, K), Ki67 (green, L), and GFAP (red, M), but did not express MAP-2 (green, M). Scale bars, 100 μm.

OPCs were produced in high purity. Importantly, we demonstrated that the sorted OPCs formed pure NG2$^+$ spheres that remained stable during prolonged culturing.

Previously published methods for hESC-derived OPC differentiation comprise 5 to 6 stages (6 to 7 weeks long) with growth factor stimulation and withdrawal (Izrael et al., 2007; Kang et al., 2007; Nistor et al., 2005) and thus cannot be directly compared with the three-stage 13-week-long protocol described here. Our protocol contains, however, a long neural induction stage (8 weeks) for effective cell population amplification which can be shortened by half to 4 weeks. Recently, Hu and colleagues (2009) described also a 13-week-long protocol for human OPC differentiation (Hu et al., 2009). As human developmental processes are much more complex than in rodents, for example, longer protocols are required for human OPC production. Compared to previously published methods describing hESC-derived OPC production our medium composition is most similar to that of

Kang and co-workers, who used DMEM/F12 with N2 and FGF for the initial neural differentiation (Kang et al., 2007). In addition to FGF, we added EGF and CNTF for the initial neural differentiation which are known factors to increase differentiation of both neural and glial cells (Kulbatski and Tator, 2009). Sphere formation was initiated directly in neural medium, without initial embryoid body (EB) formation, as previously described for hESCs in neuronal induction (Nat et al., 2007). For OPC production at stage 2, in addition to PDGF-AA, EGF, and bFGF used by Kang and co-workers (2007), we added CNTF and IGF-1. CNTF is known to enhance differentiation and maturation of OPC cultures (Rivera et al., 2008) and IGF-1 increases the survival, proliferation, and differentiation of OPCs and oligodendrocytes in rodents (Barres et al., 1993; Hsieh et al., 2004; Zaka et al., 2005). In the present study, we showed that CNTF induced *Olig2* expression in OPCs at Stage 2, which is a novel finding of CNTF effects on OPC differentiation. *Olig2* expression has shown to be essential for OPC specification (Hu et al., 2009). In addition, we showed that at Stage 2 IGF-1 increased the amount of proliferating NG2-positive cells and, together with CNTF, increased cell survival, as previously demonstrated in rodent OPCs (Barres et al., 1992; Carson et al., 1993; McMorris and Dubois-Dalcq, 1988). Further, IGF-1 and CNFT together increased the expression of *Nkx2.2*. We did not use exogenous sonic hedgehog (Shh) in our protocol as previously reported (Hu et al., 2009). Despite this, we detected upregulation of the Shh mediator *Gli1* (Dakubo et al., 2008) and thus we suggested that developing OPCs have endogenous Shh signaling. In the last stage of differentiation, T3, CNTF, and AA were added to the cultures. It is known that T3 as well as CNTF enhances the maturation of rodent OPCs (Dugas et al., 2007; Rivera et al., 2008), and AA upregulates the expression of the myelination-related genes *PLP* and *MAG* in glioma cell line (Laszkiewicz et al., 1992). According to our results, a combination of all these 3 factors together enhanced expression of oligodendrocyte-related genes in maturing OPCs and increased the amount of GalC-positive cells up to 80% in Stage 3 compared to 40% expression in Stage 2.

In contrast to all of the previous methods (Izrael et al., 2007; Nistor et al., 2005) we derived OPCs without Matrigel adhesion. Instead, the differentiation was performed on a human ECM protein surface containing laminin, collagen IV, and nidogen-1. This composition was chosen because laminin receptors are important for OPC survival, maturation, and myelination (Colognato et al., 2002, 2007) and nidogen-1 acts as a bridge between laminin-1 and collagen IV and facilitates laminin-1 signaling (Pujuguet et al., 2000). We suggest that this human ECM protein cocktail is sufficient for OPC culturing and differentiation and thus enables removal of Matrigel, an animal-derived component, from the protocol.

To monitor hESC-derived OPC differentiation, we performed a wider oligodendrocyte-related gene expression analysis compared to any previous study (Izrael et al., 2007; Kang et al., 2007; Nistor et al., 2005; Zhang et al., 2006b). Consistent with previous studies (Izrael et al., 2007; Kang et al., 2007), the expression of *PDGF-R, Nkx2.2, Sox10, Olig1/2, MBP,* and *PLP* was upregulated. In addition, the expressions of *Ngn3, Sox9, Sox8, Gli1, Gli2, Nkx6.2,* and *oligodendrocyte-myelin glycoprotein,* genes crucial for oligodendrocyte development (Nicolay et al., 2007), were also upregulated in hESC-derived OPCs. Recently, Hu and colleagues reported the importance of *Olig2* in OPC development (Hu et al., 2009), and in line with this, we showed that the developing OPCs were positive for *Olig2* using quantitative RT-PCR. Further, *Sox9, Sox8,* and *Sox10* were upregulated in our cells. *Sox9* promotes the switch from neurogenesis to oligodendrogenesis with *Sox8* and *Sox10* (Stolt et al., 2003). *Sox10* expression was detected in neural precursor and in maturating OPCs as shown previously by Izrael and co-workers (2007). Further, the mediators of Shh signaling in neural stem cell development, *Gli1* and *Gli2* (Matise and Joyner, 1999; Qi et al., 2003), were constantly expressed in developing OPCs. The myelination-related genes *PLP* and *MBP* were expressed in the final stage of our differentiation protocol, consistent with a previous report (Kang et al., 2007). Also, the expression of *Nkx2.2* and *Nkx6.2,* known controllers of *PLP* expression (Awatramani et al., 2000; Qi et al., 2001), were detected in the final stage. In particular, *Nkx6.2* activates myelin gene expression by binding *MBP* promoter regions, and is required for normal myelin formation (Southwood et al., 2004). Thus, the expression of these genes suggests that the maturing OPC population is capable of forming myelin.

The hESC-derived OPCs were positive for NG2 and PDGFRα, two markers routinely used to identify OPCs (Levine et al., 2001; Nishiyama et al., 1996). According to flow cytometry and immunocytochemical analysis, the OPC population after Stage 2 was more than 80% positive for NG2 and more than 80% positive for PDGFRα. Further, OPC from Stage 2 could be cryo-preserved without influencing their phenotype. In the final stage, Stage 3, the maturing OPC population coexpressed to some extent precursor proteins and proteins related to maturation. Thus, we suggest that there exists a transition stage of prematuring OPCs before finally maturated oligodendrocytes, and the expression of certain precursor markers is not strictly downregulated but they are slowly fainting during differentiation. At Stage 3, the cells were more than 45% positive for O4, and more than 80% positive for GalC. These results are quite similar to those of previous study (Kang et al., 2007). Taken together, the gene expression, immunocytochemical, and flow cytometry analyses confirmed the efficiency of our protocol for OPC production.

Previously, the myelination capacity of human OPCs has been demonstrated *in vivo* with shiverer mice that do not contain compact myelin (Hu et al., 2009; Izrael et al., 2007; Nistor et al., 2005). Here, we demonstrated *in vitro* in cocultures with neurons that the GalC-positive pre- and myelinating oligodendrocytes aligned along neurites and expressed MBP. Electron microscopical analysis revealed that these cells had highly branched cell processes and the morphology of mature oligodendrocytes. More importantly, we showed that maturing oligodendrocytes myelinated axons with multilayered myelin sheets in cocultures with neurons. Together, these results confirmed that OPCs developed according to our method are capable of differentiate into oligodendrocytes and myelinate neurons.

To produce a pure OPC population, we sorted OPCs with $NG2^+$ selection. In the earlier studies, rats OPCs were purified using A2B5-antibody (Barres et al., 1992; Robinson and Miller, 1996) and cultured for long time in the precursor state, using

PDGF-AA and FGF2. However, there are debates among researchers about the specificity of A2B5 antibody for OPC detection, because it is also expressed in astrocyte precursor cells (Mi and Barres, 1999). NG2 is a chondroitin sulfate proteoglycan, a surface marker detected in OPCs in different species (Nishiyama et al., 2009; Wilson et al., 2006). According to previous study, $NG2^+$ cells can give rise to GABAergic neurons, olfactory interneurons, or oligodendrocytes depending on region-specific cues in mouse brain (Aguirre and Gallo, 2004; Aguirre et al., 2004). Importantly, rodent glial cell populations sorted for $NG2^+$ and subcultured in OPC specific medium did not give rise to neurons *in vitro* (Horiuchi et al., 2009). Similar to a previous study with rodent cells, we chose NG2-antibody for purification of OPC population and cultured these sorted $NG2^+$ cells for prolonged periods in our OPC-inducing medium. According to our study, the $NG2^+$ cells were proliferative after sorting, which is important when large-scale production of cells is needed. The sorted $NG2^+$ cells expressed genes and proteins specific for OPC development. Importantly, the $NG2^+$ cell population was free from pluripotent cells as they were negative for Tra1-81, Oct-4, and CD326, known markers for pluripotency (Adewumi et al., 2007; Ng et al., 2010; Sundberg et al., 2009). This is crucial for safe nontumorgenic cell population production. The sorted $NG2^+$ population matured into GalC-positive cells and only very few astrocytes were detected. We suggest that sorting of $NG2^+$ cells is reliable way for OPC population purification. Thus, this is the first report describing purification of human $NG2^+$ OPCs, their amplification, prolonged culturing, and differentiation into oligodendrocytes.

In conclusion, we developed an efficient method for differentiating human OPCs from hESCs. These OPCs were differentiated in serum free-media with human-derived growth factors, mitogens, and human ECM proteins. These OPCs were able to myelinate neurons *in vitro*. Importantly, the formation of $NG2^+$ spheres allowed for amplification of the pure OPC population in suspension culture, which remained stable during 7 weeks of culturing *in vitro* and further differentiated into GalC-positive cells. Our OPC differentiation method is suitable for studying the development of human oligodendrocytes and an initial start point for developing xeno-free protocols for future cell graft production for demyelination disorders of the CNS.

Materials and methods

hESC cultures

The hESC lines (HS360, p59 and p69; and HS362, p93, p100, and p118) used in the study were kindly provided by Dr. Outi Hovatta, Karolinska Institute, Sweden. The procedures used to derive, characterize, and culture the hESC lines were described previously (Hovatta et al., 2003; Inzunza et al., 2005). In addition, we used two hESC lines derived at Regea–Institute for Regenerative Medicine; Regea 06/040 (p35 and p44) and Regea 08/023 (p73) both listed in European Human Embryonic Stem Cell Registry (www.hescreg.eu). All the hESCs were cultured as previously described (Rajala et al., 2007; Sundberg et al., 2009). See details in supplemental information.

Differentiation of OPCs from hESCs

The hESCs were differentiated into oligodendrocytes as spheres in a suspension culture in three stages (Fig. 1). The basal N2 medium used throughout the experiment contained DMEM/F-12 supplemented with 1X N2 supplement and 2 mM GlutaMax (both from Gibco Invitrogen), 0.6% glucose, 5 mM Hepes, 2 μg/ml heparin (all from Sigma, St. Louis, MO), and 25 U/ml penicillin and streptomycin. The suspension culturing was performed in ultralow cell cluster 6-well plates (Costar, Corning Inc., Corning, NY) using a three-stage protocol. *Stage 1: Neural precursor production.* In the beginning, hECS colonies were mechanically cut into small clusters and directly transferred into neural inductor medium as described earlier (Nat et al., 2007). Differentiating neural cell aggregates were cultured for 4 or 8 weeks in N2 medium supplemented with 10 ng/ml human ciliary neurotrophic factor (CNTF), 20 ng/ml human epithelial growth factor (EGF), and 20 ng/ml bFGF (all purchased from R&D Systems Europe, Abingdon, UK). *Stage 2: Oligodendrocyte precursor production.* Cells were cultured for 3 weeks in N2 medium supplemented with 10 ng/ml CNTF, 20 ng/ml EGF, 10 ng/ml bFGF, 100 ng/ml insulin-like growth factor-1 (IGF-1, Sigma), 20 ng/ml platelet-derived growth factor-AA (PDGF-AA, Peprotech Inc. Rocky Hill, NJ), and 1 μg/ml laminin (Sigma). *Stage 3: Oligodendrocyte precursor maturation.* OPCs were cultured for 2 weeks in N2 medium supplemented with 200 μM L-ascorbic acid 2-phosphate (AA, Sigma), 10 ng/ml CNTF, and 40 ng/ml 3,3′,5-triiodo-L-thyronine (T3, Sigma). Throughout the experiment, the spheres were dissected mechanically once a week. The medium was changed 3 times per week. All cultures were maintained in a humidified atmosphere at 37 °C and 5% CO_2. Fig. 1 shows the experimental design, sample collection points and used analyses, and description of the used medium additives.

Gene expression analysis

RNA samples were collected directly from the suspension cultures at Stage 1 (0, 4, and 8 weeks), Stage 2 (11 weeks), and Stage 3 (13 weeks; Fig. 1). RT-PCR was done as previously described (Sundberg et al., 2009). See supplemental information and Supplemental Table 1 for analyzed genes and their primer sequences. Quantitative RT-PCR (qRT-PCR) was performed with a TaqMan assay (Applied Biosystems, Foster City, CA, USA) for *Olig2 and GAPHD* using the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems). See supplemental information for details.

Cell proliferation

Adherent cultures of OPCs from Stage 2 (11 weeks) were incubated with 10 μM 5′-bromo-2-deoxyuridine (BrdU, Sigma) in Medium 2.1, 2.2, 2.3, or 2.4 for 12 h. Thereafter, cells were fixed with 4% paraformaldehyde (PFA) for 20 min, washed with PBS, treated with 2 M HCl at room temperature for 30 min, and neutralized with 0.1 M sodium borate (pH 8.5) for 2 min. See immunocytochemical characterization section for details.

Cell viability assay

OPC spheres from Stage 2 (11 weeks) were dissected into single cell suspensions with 0.05× trypsin-EDTA (BioWhittaker, Fisher Scientific Inc., Leicestershire, UK) and plated on poly-D-ornithine- (50 μg/ml in PBS) coated 24-well plates in Medium 2.1, 2.2, 2.3, or 2.4. After 2 to 3 h, calcein-AM and ethidium homodimer-1 were added to the medium and incubated with cells for 30 min; the protocol was modified from manufacturer's instructions (LIVE/DEAD viability/cytotoxicity Kit for mammalian cells, Molecular Probes, Invitrogen). See supplemental information.

Immunocytochemical characterization

Differentiating oligodendrocytes in adherent cultures at Stages 1–3 (4, 8, 11, and 12 weeks) were characterized immunocytochemically. Briefly, cells were fixed with 4% PFA for 10 min at room temperature and washed twice with PBS. For blocking 10% NDS and 1% BSA in PBS (containing 0.1% Triton X-100 for detection of intracellular proteins) were used. Primary antibodies were diluted in 1% BSA and 1% NDS in PBS (containing 0.1% Triton X-100 for detection of intracellular proteins) and incubated with cells overnight at 4 °C. Secondary antibodies were diluted in 1% BSA in PBS and incubated with cells for 1 h at room temperature in the dark. Cells were then washed with PBS and with phosphate buffer and mounted with Vectashield containing DAPI. See list of used antibodies and description of cell counting in supplemental information.

Flow cytometric analysis

Flow cytometric analysis of differentiating OPCs was performed at Stages 1 (4 or 8 weeks), 2 (11 weeks), and 3 (12 weeks; see Fig. 1). Sample preparation was performed using a modified method from (Sundberg et al., 2009); see supplemental information for details.

Coculture of OPCs with hESC-derived neurons

After 11 weeks of differentiation, the OPCs from Stage 2 were cocultured with green fluorescent protein (GFP)-labeled hESC neurons differentiated as described previously (Sundberg et al., 2009). The neuronal cells were transduced with LV-PGK-GFP at a multiplicity of infection of 30 for 5 h at 37 °C (a kind gift from Professor Seppo Ylä-Herttuala, University of Kuopio, Finland). After 1 week, the mechanically split hESC-derived OPC spheres were plated with GFP-labeled neurons and cultured in Medium 2.1 for 2 weeks. Cultures were double-stained for GalC and MBP as described above. The HS360 line was used for these analyses. For electron microscopy analyses, OPCs (Stage 3) and neurons were cocultured on 4-well Lab-Tek Chamber Slides (Nunc Corporation, Denmark) in Medium 3.2 supplemented with 1X B27 for 2 weeks. See supplemental information for detail sample preparation.

$NG2^+$ cell sorting, subculturing, and analysis

Cell sorting of $NG2^+$ cells (Stage 2, 11 weeks) with FACSAria was performed as previously described (Sundberg et al., 2009); see supplemental information. After sorting $NG2^+$ cells were subjected to proliferation assay, sphere formation assay, and for *in vitro* differentiation. The sorted cells were characterized using RT-PCR, flow cytometry, and immunocytochemistry. See supplemental information for details.

Statistical analysis

SPSS for Windows (v. 16.0, SPSS Inc., Chicago, IL) was used for statistical analysis. Nonparametric Kruskal-Wallis test followed by Mann-Whitney *U* test were used for all analyses. *P* values of less than 0.05 were considered statistically significant.

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Appendix A. Supplementary data

Supplementary data for this article may be found, in the online version, at doi:10.1016/j.scr.2010.04.005.

References

Adewumi, O., Aflatoonian, B., Ahrlund-Richter, L., Amit, M., Andrews, P.W., Beighton, G., Bello, P.A., Benvenisty, N., Berry, L.S., Bevan, S., et al., 2007. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. Nat. Biotechnol. 25, 803–816.

Aguirre, A., Gallo, V., 2004. Postnatal neurogenesis and gliogenesis in the olfactory bulb from NG2-expressing progenitors of the subventricular zone. J. Neurosci. 24, 10530–10541.

Aguirre, A.A., Chittajallu, R., Belachew, S., Gallo, V., 2004. NG2-expressing cells in the subventricular zone are type C-like cells and contribute to interneuron generation in the postnatal hippocampus. J. Cell Biol. 165, 575–589.

Awatramani, R., Beesley, J., Yang, H., Jiang, H., Cambi, F., Grinspan, J., Garbern, J., Kamholz, J., 2000. Gtx, an oligodendrocyte-specific homeodomain protein, has repressor activity. J. Neurosci. Res. 61, 376–387.

Barres, B.A., Hart, I.K., Coles, H.S., Burne, J.F., Voyvodic, J.T., Richardson, W.D., Raff, M.C., 1992. Cell death in the oligodendrocyte lineage. J. Neurobiol. 23, 1221–1230.

Barres, B.A., Schmid, R., Sendnter, M., Raff, M.C., 1993. Multiple extracellular signals are required for long-term oligodendrocyte survival. Development 118, 283–295.

Brustle, O., Jones, K.N., Learish, R.D., Karram, K., Choudhary, K., Wiestler, O.D., Duncan, I.D., McKay, R.D., 1999. Embryonic stem cell-derived glial precursors: a source of myelinating transplants. Science 285, 754–756.

Carpenter, M.K., Inokuma, M.S., Denham, J., Mujtaba, T., Chiu, C. P., Rao, M.S., 2001. Enrichment of neurons and neural precursors from human embryonic stem cells. Exp. Neurol. 172, 383–397.

Carson, M.J., Behringer, R.R., Brinster, R.L., McMorris, F.A., 1993. Insulin-like growth factor I increases brain growth and central nervous system myelination in transgenic mice. Neuron 10, 729–740.

Colognato, H., Baron, W., Avellana-Adalid, V., Relvas, J.B., Baron-Van Evercooren, A., Georges-Labouesse, E., ffrench-Constant, C., 2002. CNS integrins switch growth factor signalling to promote target-dependent survival. Nat. Cell Biol. 4, 833–841.

Colognato, H., Galvin, J., Wang, Z., Relucio, J., Nguyen, T., Harrison, D., Yurchenco, P.D., French-Constant, C., 2007. Identification of dystroglycan as a second laminin receptor in oligodendrocytes, with a role in myelination. Development 134, 1723–1736.

Cui, Q.L., Zheng, W.H., Quirion, R., Almazan, G., 2005. Inhibition of Src-like kinases reveals Akt-dependent and -independent pathways in insulin-like growth factor I-mediated oligodendrocyte progenitor survival. J. Biol. Chem. 280, 8918–8928.

Dakubo, G.D., Beug, S.T., Mazerolle, C.J., Thurig, S., Wang, Y., Wallace, V.A., 2008. Control of glial precursor cell development in the mouse optic nerve by sonic hedgehog from retinal ganglion cells. Brain Res. 1228, 27–42.

Dugas, J.C., Ibrahim, A., Barres, B.A., 2007. A crucial role for p57 (Kip2) in the intracellular timer that controls oligodendrocyte differentiation. J. Neurosci. 27, 6185–6196.

Gilbertson, J.A., Sen, A., Behie, L.A., Kallos, M.S., 2006. Scaled-up production of mammalian neural precursor cell aggregates in computer-controlled suspension bioreactors. Biotechnol. Bioeng. 94, 783–792.

Glaser, T., Perez-Bouza, A., Klein, K., Brustle, O., 2005. Generation of purified oligodendrocyte progenitors from embryonic stem cells. FASEB J. 19, 112–114.

Grinspan, J., 2002. Cells and signaling in oligodendrocyte development. J. Neuropathol. Exp. Neurol. 61, 297–306.

Heiskanen, A., Satomaa, T., Tiitinen, S., Laitinen, A., Mannelin, S., Impola, U., Mikkola, M., Olsson, C., Miller-Podraza, H., Blomqvist, M., et al., 2007. *N*-Glycolylneuraminic acid xenoantigen contamination of human embryonic and mesenchymal stem cells is substantially reversible. Stem Cells 25, 197–202.

Horiuchi, M., Lindsten, T., Pleasure, D., Itoh, T., 2009. Differing in vitro survival dependency of mouse and rat NG2(+) oligodendroglial progenitor cells. J. Neurosci. Res. 88, 957–970.

Hovatta, O., Mikkola, M., Gertow, K., Stromberg, A.M., Inzunza, J., Hreinsson, J., Rozell, B., Blennow, E., Andang, M., Ahrlund-Richter, L., 2003. A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. Hum. Reprod. 18, 1404–1409.

Hsieh, J., Aimone, J.B., Kaspar, B.K., Kuwabara, T., Nakashima, K., Gage, F.H., 2004. IGF-I instructs multipotent adult neural progenitor cells to become oligodendrocytes. J. Cell Biol. 164, 111–122.

Hu, B.Y., Du, Z.W., Li, X.J., Ayala, M., Zhang, S.C., 2009. Human oligodendrocytes from embryonic stem cells: conserved SHH signaling networks and divergent FGF effects. Development 136, 1443–1452.

Inzunza, J., Gertow, K., Stromberg, M.A., Matilainen, E., Blennow, E., Skottman, H., Wolbank, S., Ahrlund-Richter, L., Hovatta, O., 2005. Derivation of human embryonic stem cell lines in serum replacement medium using postnatal human fibroblasts as feeder cells. Stem Cells 23, 544–549.

Izrael, M., Zhang, P., Kaufman, R., Shinder, V., Ella, R., Amit, M., Itskovitz-Eldor, J., Chebath, J., Revel, M., 2007. Human oligodendrocytes derived from embryonic stem cells: effect of noggin on phenotypic differentiation in vitro and on myelination in vivo. Mol. Cell. Neurosci. 34, 310–323.

Jiang, X., Gwye, Y., McKeown, S.J., Bronner-Fraser, M., Lutzko, C., Lawlor, E.R., 2009. Isolation and characterization of neural crest stem cells derived from in vitro-differentiated human embryonic stem cells. Stem Cells Dev. 18, 1059–1070.

Kang, S.M., Cho, M.S., Seo, H., Yoon, C.J., Oh, S.K., Choi, Y.M., Kim, D.W., 2007. Efficient induction of oligodendrocytes from human embryonic stem cells. Stem Cells 25, 419–424.

Keirstead, H.S., Nistor, G., Bernal, G., Totoiu, M., Cloutier, F., Sharp, K., Steward, O., 2005. Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. J. Neurosci. 25, 4694–4705.

Kulbatski, I., Tator, C.H., 2009. Region-specific differentiation potential of adult rat spinal cord neural stem/precursors and their plasticity in response to in vitro manipulation. J. Histochem. Cytochem. 57, 405–423.

Laszkiewicz, I., Wiggins, R.C., Konat, G., 1992. Ascorbic acid upregulates myelin gene expression in C6 glioma cells. Metab. Brain Dis. 7, 157–164.

Levine, J.M., Reynolds, R., Fawcett, J.W., 2001. The oligodendrocyte precursor cell in health and disease. Trends Neurosci. 24, 39–47.

Martin, M.J., Muotri, A., Gage, F., Varki, A., 2005. Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat. Med. 11, 228–232.

Matise, M.P., Joyner, A.L., 1999. Gli genes in development and cancer. Oncogene 18, 7852–7859.

McMorris, F.A., Dubois-Dalcq, M., 1988. Insulin-like growth factor I promotes cell proliferation and oligodendroglial commitment in rat glial progenitor cells developing in vitro. J. Neurosci. Res. 21, 199–209.

Mi, H., Barres, B.A., 1999. Purification and characterization of astrocyte precursor cells in the developing rat optic nerve. J. Neurosci. 19, 1049–1061.

Miller, R.H., 2002. Regulation of oligodendrocyte development in the vertebrate CNS. Prog. Neurobiol. 67, 451–467.

Nat, R., Nilbratt, M., Narkilahti, S., Winblad, B., Hovatta, O., Nordberg, A., 2007. Neurogenic neuroepithelial and radial glial cells generated from six human embryonic stem cell lines in serum-free suspension and adherent cultures. Glia 55, 385–399.

Ness, J.K., Scaduto Jr., R.C., Wood, T.L., 2004. IGF-I prevents glutamate-mediated bax translocation and cytochrome C release in O4+ oligodendrocyte progenitors. Glia 46, 183–194.

Ng, V.Y., Ang, S.N., Chan, J.X., Choo, A.B., 2010. Characterization of epithelial cell adhesion molecule as a surface marker on undifferentiated human embryonic stem cells. Stem Cells 28, 29–35.

Nicolay, D.J., Doucette, J.R., Nazarali, A.J., 2007. Transcriptional control of oligodendrogenesis. Glia 55, 1287–1299.

Nishiyama, A., Komitova, M., Suzuki, R., Zhu, X., 2009. Polydendrocytes (NG2 cells): multifunctional cells with lineage plasticity. Nat. Rev. Neurosci. 10, 9–22.

Nishiyama, A., Lin, X.H., Giese, N., Heldin, C.H., Stallcup, W.B., 1996. Co-localization of NG2 proteoglycan and PDGF alpha-receptor on O2A progenitor cells in the developing rat brain. J. Neurosci. Res. 43, 299–314.

Nistor, G.I., Totoiu, M.O., Haque, N., Carpenter, M.K., Keirstead, H. S., 2005. Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation. Glia 49, 385–396.

Pang, Y., Zheng, B., Fan, L.W., Rhodes, P.G., Cai, Z., 2007. IGF-1 protects oligodendrocyte progenitors against TNFalpha-induced damage by activation of PI3K/Akt and interruption of the mitochondrial apoptotic pathway. Glia 55, 1099–1107.

Portner, R., Nagel-Heyer, S., Goepfert, C., Adamietz, P., Meenen, N.M., 2005. Bioreactor design for tissue engineering. J. Biosci. Bioeng. 100, 235–245.

Pujuguet, P., Simian, M., Liaw, J., Timpl, R., Werb, Z., Bissell, M.J., 2000. Nidogen-1 regulates laminin-1-dependent mammary-specific gene expression. J. Cell Sci. 113 (Pt 5), 849–858.

Qi, Y., Cai, J., Wu, Y., Wu, R., Lee, J., Fu, H., Rao, M., Sussel, L., Rubenstein, J., Qiu, M., 2001. Control of oligodendrocyte differentiation by the Nkx2.2 homeodomain transcription factor. Development 128, 2723–2733.

Qi, Y., Tan, M., Hui, C.C., Qiu, M., 2003. Gli2 is required for normal Shh signaling and oligodendrocyte development in the spinal cord. Mol. Cell. Neurosci. 23, 440–450.

Rajala, K., Hakala, H., Panula, S., Aivio, S., Pihlajamaki, H., Suuronen, R., Hovatta, O., Skottman, H., 2007. Testing of nine different xeno-free culture media for human embryonic stem cell cultures. Hum. Reprod. 22, 1231–1238.

Reubinoff, B.E., Itsykson, P., Turetsky, T., Pera, M.F., Reinhartz, E., Itzik, A., Ben-Hur, T., 2001. Neural progenitors from human embryonic stem cells. Nat. Biotechnol. 19, 1134–1140.

Rivera, F.J., Kandasamy, M., Couillard-Despres, S., Caioni, M., Sanchez, R., Huber, C., Weidner, N., Bogdahn, U., Aigner, L., 2008. Oligodendrogenesis of adult neural progenitors: differential effects of ciliary neurotrophic factor and mesenchymal stem cell derived factors. J. Neurochem. 107, 832–843.

Robinson, S., Miller, R., 1996. Environmental enhancement of growth factor-mediated oligodendrocyte precursor proliferation. Mol. Cell. Neurosci. 8, 38–52.

Sen, A., Kallos, M.S., Behie, L.A., 2004. New tissue dissociation protocol for scaled-up production of neural stem cells in suspension bioreactors. Tissue Eng. 10, 904–913.

Southwood, C., He, C., Garbern, J., Kamholz, J., Arroyo, E., Gow, A., 2004. CNS myelin paranodes require Nkx6-2 homeoprotein transcriptional activity for normal structure. J. Neurosci. 24, 11215–11225.

Spassky, N., Olivier, C., Cobos, I., LeBras, B., Goujet-Zalc, C., Martinez, S., Zalc, B., Thomas, J.L., 2001. The early steps of oligodendrogenesis: insights from the study of the plp lineage in the brain of chicks and rodents. Dev. Neurosci. 23, 318–326.

Stolt, C.C., Lommes, P., Sock, E., Chaboissier, M.C., Schedl, A., Wegner, M., 2003. The Sox9 transcription factor determines glial fate choice in the developing spinal cord. Genes Dev. 17, 1677–1689.

Sundberg, M., Jansson, L., Ketolainen, J., Pihlajamaki, H., Suuronen, R., Skottman, H., Inzunza, J., Hovatta, O., Narkilahti, S., 2009. CD marker expression profiles of human embryonic stem cells and their neural derivatives, determined using flow-cytometric analysis, reveal a novel CD marker for exclusion of pluripotent stem cells. Stem Cell Res. 2, 113–124.

Thomson, A., Wojtacha, D., Hewitt, Z., Priddle, H., Sottile, V., Di Domenico, A., Fletcher, J., Waterfall, M., Corrales, N.L., Ansell, R., et al., 2008. Human embryonic stem cells passaged using enzymatic methods retain a normal karyotype and express CD30. Cloning Stem Cells 10, 89–106.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., Jones, J.M., 1998. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145–1147.

Wilson, H.C., Scolding, N.J., Raine, C.S., 2006. Co-expression of PDGF alpha receptor and NG2 by oligodendrocyte precursors in human CNS and multiple sclerosis lesions. J. Neuroimmunol. 176, 162–173.

Zaka, M., Rafi, M.A., Rao, H.Z., Luzi, P., Wenger, D.A., 2005. Insulin-like growth factor-1 provides protection against psychosine-induced apoptosis in cultured mouse oligodendrocyte progenitor cells using primarily the PI3K/Akt pathway. Mol. Cell. Neurosci. 30, 398–407.

Zhang, P.L., Izrael, M., Ainbinder, E., Ben-Simchon, L., Chebath, J., Revel, M., 2006a. Increased myelinating capacity of embryonic stem cell derived oligodendrocyte precursors after treatment by interleukin-6/soluble interleukin-6 receptor fusion protein. Mol. Cell. Neurosci. 31, 387–398.

Zhang, S.C., Ge, B., Duncan, I.D., 2000. Tracing human oligodendroglial development in vitro. J. Neurosci. Res. 59, 421–429.

Zhang, S.C., Wernig, M., Duncan, I.D., Brustle, O., Thomson, J.A., 2001. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat. Biotechnol. 19, 1129–1133.

Zhang, X., Cai, J., Klueber, K.M., Guo, Z., Lu, C., Qiu, M., Roisen, F.J., 2005. Induction of oligodendrocytes from adult human olfactory epithelial-derived progenitors by transcription factors. Stem Cells 23, 442–453.

Zhang, Y.W., Denham, J., Thies, R.S., 2006b. Oligodendrocyte progenitor cells derived from human embryonic stem cells express neurotrophic factors. Stem Cells Dev. 15, 943–952.