Generation of Dopaminergic Neurons from Human Embryonic Stem Cells

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Stockholm 2008

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Stockholm 2008

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Printed by Universitetsservice US-AB
Drottning Kristinas väg 53B
SE-100 44 Stockholm
Sweden

ISBN 978-91-7415-136-7

TRITA-BIO Report 2008:25

ISSN 1654-2312

Tandis Vazin (2008): **Generation of Dopaminergic Neurons from Human Embryonic Stem Cells.** School of Biotechnology, Royal Institute of Technology (KTH), Stockholm, Sweden

"*Never regard study as a duty, but as the enviable opportunity to learn to know the liberating influence of beauty in the realm of the spirit for your own personal joy and to the profit of the community to which your later work belongs*"

Albert Einstein

Abstract

Since the first successful derivation of human embryonic stem cells (hESC), rapid progress has been attained in the development of strategies in differentiation of these cells into various neural lineages, with the fundamental objective of using these cells for replacement and repair of damaged neuronal circuits in the central nervous system (CNS). Of particular interest are midbrain dopaminergic (mDA) neurons, which play a central role in regulation of voluntary movement. Degeneration or loss of function of mDA neurons in the nigrostriatal pathway is associated with Parkinson disease (PD).

Stromal-Derived Inducing Activity (SDIA) is recognized as one of the most efficient methods in restricting ESC differentiation to a dopaminergic lineage, and refers to the property of mouse stromal cell lines such as PA6 or MS5 to cause ESC to differentiate to DA neurons. Although this strategy has been extensively used to generate mDA neurons from hESC, the biochemical nature of SDIA is yet unknown.

In the present study mDA neurons were generated from the BG01V2 hESC line by SDIA. To examine whether SDIA exerts its effect directly on hESC and is responsible for early dopaminergic induction, neural progenitor cells (NPC) were enyzmatically isolated from the co-cultures and allowed to differentiate in feeder-free conditions. The isolated cells were committed to a mesencephalic neural lineage, and were capable of maintaining their phenotype and developing into postmitotic mDA neurons in feeder-free conditions. The mDA neurons showed neuronal excitability and dopamine transporter function. The *in vitro* proliferation and differentiation of the NPC was also investigated by a BrDU incorporation assay.

Next, the maintenance of cellular memory and capacity for proliferation of the mesencephalic NPC was assessed. The NPC could be expanded *in vitro* by five-fold as neurospheres for up to two weeks while retaining their DA differentiation potential, but did not retain a stable phenotype over extended periods of time. Preliminary transplantation experiments of neurospheres in striatal lesioned animals indicated, however, that these cells could survive and conserve their phenotype *in vivo*.

To gain additional insight into the biochemical role of SDIA in early dopaminergic induction of hESC, the separate contributions of cell surface activity and secreted factors were examined. The data revealed that the PA6 cell surface activity promoted cell survival and was mainly responsible for enhanced neurogenesis of hESC, whereas secreted factors provided DA lineage-specific instructions.

In order to identify the soluble factors responsible for the DA phenotype-inducing component of SDIA, the gene expression profile of PA6 cells was compared to that of cell lines lacking the DA-inducing property. A number of soluble factors known to be associated with CNS development that were highly expressed in PA6 cells were identified as potential DA differentiation-inducing candidates. These differentially-expressed genes included stromal cell-derived factor 1 (SDF-1/CXCL12), pleiotrophin (PTN), insulin-like growth factor 2 (IGF2), and ephrin B1 (EFNB1). When these factors, termed SPIE, were applied to the hESC, they induced dopaminergic neuronal differentiation of hESC line, BG01V2 and other karyotypically normal hESC lines *in vitro*. Thus, it appears that SPIE comprises the DA phenotype-inducing property of SDIA. This may provide a simple and direct means of differentiating hESC to form DA neurons in a single step, without a requirement for co-culture, animal cell lines, or animal products.

List of publications

This dissertation is based on the following articles

I. **Vazin, T.,** Chen, J., Spivak C.E., Amable, R., Gabitzsch, E., et al. (2008). Dopaminergic neurons derived from BG01V2, a variant of human embryonic stem cell line BG01. *Restor. Neurol. Neuros.* (In press)

II. **Vazin, T.,** Chen, J., Wang Y., Harvey, B. K., Gabitzsch, E., Worden, L., and Freed, W.J., Limited proliferation capacity of mesencepahlic neural progenitor cells derived from human embryonic stem cells. (Manuscript)

III. **Vazin, T.,** Chen, J., Le, C.T., Amable, R., and Freed, W.J., (2008). Assessment of Stromal-Derived Inducing Activity in the Generation of Dopaminergic Neurons from Human Embryonic Stem Cells. *Stem Cells*. **26,** 1517-25.

IV. **Vazin, T.,** Becker, K.G., Zhang, Y., Worden, L., and Freed, W.J., The Molecular Nature of Stromal-Derived Inducing Activity in Dopaminergic Differentiation of Human Embryonic Stem Cells. (Manuscript)

Publications not included in this dissertation

I. Chen, J., Tsai, S.Y., Harvey, **Vazin, T.,** B. K., Coggiano, M., Shen, H., et al., Human embryonic stem cells which express hrGFP in the undifferentiated state and during dopaminergic differentiation. (Submitted)

II. Freed, W.J., Chen, J., Bäckman, C.M., Schwartz, C.M., **Vazin, T.,** Cai, J., et al. (2008). Gene expression profile of neuronal progenitor cells derived from hESCs: activation of chromosome 11p15.5 and comparison to human dopaminergic neurons. *PLoS ONE*. **9**, e1422.

III. Freed, W.J., Sanchez, J.F., Lee, C.T., **Vazin, T.,** Amable, R., et al. (2007). Human embryonic stem cells, dopaminergic neurons, and pathways for developing a Parkinson's disease therapy. In D. Emerich, C. Halberstad, (Eds.), *Cellular Transplantation: From Laboratory to Clinic* (pp. 523-544). Burlington: Academic Press.

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Abbreviations

AA	ascorbic acid
AADC	aromatic L-amino acid decarboxylase
AAV	adeno-associated virus
AP	anteroposterior
AS	α-synuclein
ASMA	α-smooth muscle actin
ATII	alveolar epithelial type II
BDNF	brain-derived neurotrophic factor
Bf1	brain factor-1
bFGF	basic fibroblast growth factor
BIRC5	baculoviral IAP repeat-containing 5
BMP	bone morphogenetic protein
BrDU	bromodeoxyuridine
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
CM	conditioned media
CNS	central nervous system
CNTF	ciliary neurotrophic factor
COMT	catechol-O-methyltransferase
CXCL	chemokine (C-X-C motif) ligand
D	dimensional
DAT	dopamine transporter
dbcAMP	dibutyryl-cyclic AMP
DBH	dopamine beta hydroxylase
DV	dorsoventral
EB	embryoid body
ECM	extracellular matrix
EFNB1	ephrin B1
EGF	epidermal growth factor
En-1	engrailed-1
ESC	embryonic stem cells
FatiGO	fast assignment and transference of information using gene ontology
FGF	fibroblast growth factor
FOXA1	forkhead box A1
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
Girk2	inwardly rectifying K+ channel
GTP	guanosine triphosphate
HA	hyaluronic acid
hESC	human embryonic stem cells
HGF	hepatocyte growth factor
HOX	homeobox
6-OHDA	6-hydroxydopamine
ICM	inner cell mass
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
L-DOPA	L-dihydroxy-L-phenylalanine
LB	lewy body
Lmx1	LIM homeobox transcription factor 1
MAO	monoamine oxidase
MAP2	microtubule-associated protein 2
MAPK	mitogen-activated protein kinase

Mash1	achaete scute homolog 1
mDA	midbrain dopaminergic
MPSS	massive parallel signature sequencing
MPTP	methyl-phenyl-tetrahydropyridine
Msx1	msh homeobox 1
NADH	nicotinamide adenine dinucleotide
Ngn2	neurogenin 2
NPC	neural progenitor cells
NT4	neurotrophin-4
Nurr1	nuclear receptor-related 1
Otx2	orthodenticle homolog 2
Pax	paired box
PD	Parkinson's disease
PDGF	platelet-derived growth factor
PGSA	poly-glycerolco-sebacate-acrylate
PI3K	phosphoinositide 3-kinase
PINK1	PTEN induced putative kinase 1
Pitx3	paired-like homeodomain transcription factor 3
PSA-NCAM	polysialylated neural cell adhesion molecule
PTCH	Patched
PTN	pleiotrophin
RA	retinoic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SCID	severe combined immunodeficient
SDF-1	stromal cell-derived factor 1
SDIA	stromal-derived inducing activity
SHH	sonic hedgehog
SMO	smoothened
SNMs	spherical neural masses
Sox	sry-related high mobility group box
SPIE	SDF-1, PTN, IGF2, EFNB-1
SSEA	stage-specific embryonic antigen
SNpc	substantia nigra pars compacta
STN	subthalamic nucleus
TGF-β	transforming growth factor-beta
TH	tyrosine hydroxylase
THFA	tetrahydrofolic acid
UCH-L1	carboxyterminal hydrolase L1
UCM	unconditioned media
VMAT2	vesicular monoamine transporter 2
VTA	ventral tegmental area

Introduction

1. Embryonic stem cells

Embryonic stem cells (ESC) are pluripotent cells which give rise to the three primary germ layers: ectoderm, endoderm and mesoderm that subsequently differentiate to all somatic cell types in an embryo. ESC are a valuable tool in understanding the complex mechanisms in development of specialized cells and establishment of organ structures. Because these cells can also self replicate indefinitely, they have awakened a great deal of interest and research effort. The unlimited self-renewal ability and plasticity of ESC potentially allows for *in vitro* generation of unlimited number of distinct cell types and has opened new avenues in regenerative medicine.

1.1 Derivation of ESC

Following fertilization of an egg and formation of a diploid zygote, a structure referred to as a blastocyst is generated by multiple mitotic cell divisions during early embryogenesis. The blastocyst consists of the embryoblast and trophoblast. The trophoblast, also referred to as the outer cell mass, forms the extra-embryonic tissue, which eventually gives rise to the placenta, chorion, and the umbilical cord, whereas the embryoblast also known as the inner cell mass (ICM) develops into the embryo [1]. Early studies of development of mouse blastocysts by Sherman et al in 1975 [2] examined the growth and differentiation of trophoblast cells as well as the proliferation of the inner cell mass in long-term cultures. Four cell lines were obtained and maintained for more than a year. However, these lines contained cell types other than undifferentiated ESC, were not able to differentiate to all the three germ layers *in vivo* and eventually developed chromosomal abnormalities. In 1981, established cultures of embryonal carcinoma stem cells were used to develop appropriate culture conditions and determine the optimal stage of isolation of pluripotent embryonic stem cells. These optimizations led to the successful derivation of the first stable mouse embryonic stem cell lines [3-5].

The pioneering work on mouse ESC, and later advances in culturing techniques that were developed to culture nonhuman primate ESC lines [6, 7, 8] eventually led to the first successful generation of human ESC lines by Thompson et al. and Reubinoff et al. in 1998 [9,10]. The hESC were derived from human embryos that were produced by in vitro fertilization for clinical purposes. HESC lines described by Thompson and coworkers retained their pluripotency, were karyotypically normal when grown on mouse embryonic fibroblast (MEF) feeders, and fulfilled all the criteria for ESC including having the capability to generate large germ cell tumors that can contain several different types of tissue (teratomas) when grafted to severe combined immunodeficient (SCID) mice [11]. As the SCID mouse lacks both B and T cells it is also an excellent model to study the behavior of ESC *in vivo* without the need for immunosuppressant drugs.

To date, hundreds of hESC lines have been generated from donated embryos. Isolation of the ICM from the trophectoderm at the blastocyst-stage has, for the most part, been achieved by immunosurgery or mechanical dissection. The first hESC lines were established using the immunosurgical method, which requires the use of animal-derived products including anti-human serum antibodies and guinea pig complement [10-12]. Exposure to animal-derived products would prevent the later use of hESC for transplantation therapies, due to possible transfer of pathogens

which would potentially initiate the patient's innate immune mechanisms leading to an increased risk of graft rejection. Therefore, mechanical or enzymatic isolation of the ICM from the trophectoderm in a manner that avoids contact between the ICM and animal products during the derivation procedure would be advantageous for future clinical applications [13-16]. In addition, laser beams have been successfully used in derivation of hESC lines by creating a small opening at the zona pellucida that encapsulates the blastocyst, followed by laser-assisted isolation of the ICM [17].

Generation of hESC lines from the inner cell mass at the blastocyst stage has thus far obligated the destruction of the embryo, which has raised ethical and political concerns. In order to address this issue, much work has been devoted to isolating cells from earlier stages of embryonic development without destruction of the embryo. Initial attempts in removal of one cell at the 8-cell or morula stage resulted in variable success rates and required co-culture of isolated blastomeres with established hESC lines [18-20]. Blastomere differentiation to ICM was highly inefficient because the blastomere-derived aggregates mostly gave rise to trophectoderm-like vesicles. To circumvent this problem and increase the efficiency of hESC derivation, a modified approach using culture media supplemented with laminin was employed [21]. This strategy was almost as efficient as conventional methods used to derive hESC lines from whole blastocysts. The authors rationalized that the essential role of laminin involved recreation of the natural ICM niche, which prevented polarization of the blastomeres into ICM and trophectoderm. In addition, optimization of culture conditions in this new procedure allowed successful generation of blastomere-derived hESC in feeder-free conditions, eliminating the need for co-cultures with animal-derived feeder layers or previously established hESC lines.

1.2 Growth and maintenance of hESC *in vitro*

Indefinite self-renewal is a fundamental hallmark of successful hESC culture. When the first hESC lines were derived, MEF feeder layers were used to support the propagation of hESC in the primitive undifferentiated state [10, 11]. Ever since, in effort to move toward a total xeno-free hESC culturing systems, different approaches using human-derived cell types including fibroblast feeder cells derived from fallopian tube epithelium [22], fetal foreskin, muscle [23, 24], bone marrow [25], or amniotic epithelium [26], have been established. Alternatively, hESC can be maintained in feeder-free environments in the presence of extracellular matrices such as matrigel and fibronectin-containing hydrogels. Nevertheless, media conditioned by feeder fibroblast cells and supplementation with basic fibroblast growth factor (bFGF) are required to maintain hESC in an undifferentiated state in such feeder-free conditions [27, 28].

In feeder-free culture systems, hESC often give rise to fibroblast or stromal-like cells that may serve as supportive cells in maintaining the undifferentiated growth of hESC. Studies examining the nature of these feeder cells provided evidence that feeder cells derived from hESC can be used to support their own growth [29, 30]. Although these cells fulfill the growth requirements of hESCs, they are not immortal and will senesce after several passages, thereby limiting their continual use. Derivation of new feeder cells can be cumbersome and may result in highly variable culture systems.

Thus, additional efforts are required to completely eliminate the need of feeder cells and establish a well-defined environment for hESC growth. Studies focused on secreted factors released from MEF feeder layers that maintain self-renewal divisions of hESC have identified a number of factors responsible for maintenance of hESC pluripotency [31-34]. In addition, high concentrations

of bFGF and repression of bone morphogenetic protein (BMP) signaling by noggin have been suggested to sustain undifferentiated proliferation of hESC in serum-freed media [34-37]. Other multifaceted exogenous treatments of hESC with cocktails of human recombinant proteins and signaling molecules including Activin A and transforming growth factor-beta 1 (TGF-β1) have also been introduced as new approaches in hESC culture [37-39].

On the other hand, there is evidence suggesting that maintaining hESC in feeder-free culture systems can decrease their stability and predispose them to developing genetic abnormalities [40]. Also, feeder-free conditions may not be optimal for a wide range of hESC lines [41]. Even though feeder-free and serum-free defined conditions provide a basic platform and clues in characterization of hESC self-renewal, further investigations need to be undertaken to determine factors contributing to maintenance of the pluripotent phenotype and stability of all existing hESC lines worldwide.

1.3 Methods for expansion of hESC

HESC have a high capacity for self renewal and need to be frequently passaged and transferred to freshly-prepared cultures upon confluency. Typically, hESC in co-culture with various murine or human cell types are isolated from their supportive feeder cell layer by enzymatic treatment or mechanical dissection. Commonly used animal-derived enzymes include collagenase IV, dispase, and trypsin [42]. In view of potential clinical applications, recombinant animal protein-free enzymes and human collagenase have also been used for hESC culture [43]. Enzymatic passaging as compared to mechanical isolation is less labor intensive and can easily be applied on a large scale, providing a more defined and reproducible culture system. However, enzymatic methods in propagation of hESC have been strongly linked to occurrence of genetic abnormalities [40, 44, 45]. To address concerns associated with enzymatic passaging, a number of groups have established enzymatic techniques involving bulk passaging and single cell dissociation without compromising the pluripotency or genetic stability over extended culture periods (more that 100 passages) [46, 47]. Furthermore an automated method has also been developed for robust mechanical large-scale expansion of undifferentiated hESC [48].

1.4 Characterization of hESC

HESC grow in tightly packed colonies and maintain a defined border at the periphery of colonies. High nucleus to cytoplasm ratio and prominent nucleoli are typical features of individual hESC within colonies. hESC are also characterized by expression of a number of cell surface markers and transcription factors including stage-specific embryonic antigen-4 (SSEA-4), SSEA-3, TRA antigens, Oct3/4, Nanog, and absence of hESC negative markers, such as SSEA-1[49-53].

Analysis of self-renewal capacity of hESC is often evaluated by telomere length and telomerase activity measurement after prolonged *in vitro* cultivation [54, 55]. In general, human somatic cells have a limited lifespan and can only escape replicative senescence upon acquisition of genetic alterations [56].In contrast, it has been established that the intrinsic mechanisms in hESC responsible for their immortality are not caused by genetic alterations or inactivation of the well-known p53 and Rb tumor suppressor pathways [55, 57].

It is, however, evident that hESC acquire karyotypic abnormalities during prolonged maintenance *in vitro*, which may be correlated to tumorigenic events that occur *in vivo* [40, 44, 45,

58]. Chromosomal aberrations are believed to be a result of *in vitro* adaptation and selection because abnormal rapidly-dividing cells gradually predominate and eventually take over the entire diploid culture [58]. Also, the most recurrent chromosomal changes in hESC *in vitro* reflect over-expression of genes associated with pluripotency, cell proliferation, and anti-apoptosis, which are highly comparable to genetic changes seen in common human cancers [58]. Consequently, for therapeutic use of hESC, in addition to traditional Giemsa-stained karyotyping that detects large chromosomal changes, fluorescent in situ hybridization, DNA microarray, whole-genome single nucleotide polymorphism, and short tandem repeat analysis are carried out to ensure maintenance of a normal genetic signature at a high level of resolution [40, 44, 45, 59, 60].

Functional confirmation of the multipotent nature of hESC is generally achieved by examining their potential to differentiate into all three germ layers (ectoderm, mesoderm and endoderm) *in vitro* as well as *in vivo*. *In vitro*, hESC are allowed to randomly differentiate as embryoid bodies (EBs), which are aggregates of cells grown in suspension culture, followed by immunocytochemical analysis, or measurement of expression of genes associated with the three germ layers by RT-PCR. The *in vivo* test for pluripotency of hESCs is teratoma formation in SCID mice [10, 11].

1.5 Three-dimensional culture of hESC

Ideal culture conditions for hESC should recapitulate the niche and physical environment in which hESC naturally reside. Generally, hESC are grown in colonies which must remain within a narrow size range during passaging procedures to prevent differentiation. Thus, it is clear that cell-cell interactions as well as paracrine or autocrine signals within colonies play an essential role in maintaining hESC in an undifferentiated state.

There is a profound difference in cell signaling, gene expression and organization of cells in two dimensional cultures (2D) as compared the three dimensional (3D) culture systems, which more closely resemble the *in vivo* hESC environment [61, 62]. A study by Nur-E-Kamal et al. [63] showed that physical and mechanical cues are also crucial in emulating the native microenvironment of mouse ESC, as 3D culture greatly enhanced cell proliferation and self-renewal in comparison with growth on 2D tissue culture surfaces. The authors used synthetic polyamide matrix scaffolds to mimic the fibrillar network of the basement membrane. The resultant gain in proliferative ability was correlated with the cellular mechanotransduction by activation of the small GTPase Rac, the activation of phosphoinositide 3-kinase (PI3K) pathway and enhanced expression of Nanog [63].

Another type of polymeric fibrous scaffold is made of cellulose acetate that creates a meshwork permitting surface immobilization of various extra cellular matrix (ECM) molecules and growth factors. This manually constructed 3D matrix has been used in the study of uncommitted human embryonic germ cell derivatives, which share some properties with hESC. The enhanced interaction of cells in 3D cultures led to maintenance of multipotency and elevated proliferation and cell survival [64].

Encapsulation of hESC has also been carried out in biocompatible poly-glycerolco-sebacate-acrylate (PGSA) elastomers, which are photo-polymerized to form porous scaffolds. Although hESC continued to proliferate under these conditions, the cells formed differentiating EB structures within the pores after seven days of culture and did not remain in an undifferentiated state [65]. Thus far, the most physiologically relevant matrix developed for culture of undifferentiated hESC is composed

of hyaluronic acid (HA) hydrogel. The HA hydrogel replicates major components of the ECM that is abundant in embryos and stem cell niches and can maintain hESC in undifferentiated state while preserving their full differentiation capacity [66].

Similar to PGSA scaffolds, structure and chemistry of synthetic hydrogels can be modified by external stimuli to manipulate the temporal and spatial availability of bioactive molecules. Addition of components such as growth factors and ECM proteins can also be used in inducing differentiation. Development of methods capable of controlling cell-cell interactions in scalable culture by encapsulation of hESC in size-specified scaffolds is indeed highly desirable; however, the release of cells from these 3D structures requires enzymatic digestion. For example, release of hESC from HA hydrogel is achieved by addition of hyaluronidase into the growth medium, and it is uncertain how the enzymatic treatment affects hESC in long-term cultures.

2. Directed *in vitro* differentiation of hESC

Theoretically, hESC have the capability to give rise to all somatic cell types present in an embryo. Although hESC exhibit this great potential, for development of new therapeutic strategies the challenge still remains in understanding the intricate mechanisms that govern stem cell growth, cell fate decisions, tissue generation and organogenesis. Systematic studies of the developmental biology of various cell types are leading to increasing success in lineage-specific differentiation of hESC.

Aggregation of hESC into 3D EB structures has a general inductive influence, and is frequently used as a first step for producing *in vitro* differentiation of many cell lineages. Spontaneous differentiation produced by EB formation or allowing increased confluency of hESC cultures generally results in a small fraction of cells with any particular phenotype. Therefore, considerable efforts are being made in identifying exogenous biochemical supplementation that can direct cell-specific differentiation, and results in enrichment of desired cell types.

2.1 Cell-specific endodermal differentiation

Examples of major cell types that have been derived from the endoderm using hESC include hepatocytes [67-69], insulin-producing β-cells [70-73], and lung epithelium [74-76] for development of potential cell replacement therapy in acute liver failure/hepatitis, type I diabetes, and pulmonary diseases.

The isolation of hepatocytes from randomly-differentiated hESC is relatively difficult, because many markers characteristic of liver cells are also expressed in the extraembryonic endoderm, which contributes to the formation of the yolk sac [67]. Selective induction and isolation of definitive endoderm can be achieved by activin A treatment in combination with fluorescence-activated cell sorting (FACS) of cells that express lineage-specific cell-surface proteins including CXCR4 [68, 69].

Hepatocytic induction is then accomplished by sequential addition of inducing factors FGF4, BMP2, and hepatocyte growth factor (HGF). The highest reported yield of albumin secreting hepatocytes is about 70% [69]. Additionally, transplantation of hepatocyte precursors or mature hepatocytes in animals with tetrachloride-induced liver injury has resulted in successful integration and participation in liver repair.

The signaling pathways in the developing pancreas are very complex and include the TGF-β, notch, hedgehog, FGF, and epidermal growth factor (EGF) pathways [70]. A study by Assady et al. [71] suggested that pancreatic β-cells could be produced by random differentiation of hESC as EBs. However, only 1–3% of cells within EBs were insulin-positive β -cell. Segev et al. [72] used a more efficient methodology for derivation of immature pancreatic cells involving growth in insulin-transferrin-selenium-fibronectin medium, followed by supplementation with N2, B27, and bFGF. Nicotinamide and decreased glucose concentrations in the media were also used to improve insulin secretion in response to glucose. Recently, co-transplantation of randomly differentiated hESC with mouse embryonic dorsal pancreas has also been found to result in differentiation of β-cell–like cell clusters capable of insulin synthesis [73].

Another target of hESC differentiation is the alveolar epithelial type II (ATII) cells that constitute the primary lung-lining tissue. These cells might possibly be used for treatment of lung chronic respiratory disease as well as pulmonary genetic disorders. Generation of mature ATII cells from hESC has been carried out via EB formation in small airway growth medium [74], or by co-culture with pulmonary mesenchyme [75]. Both of these strategies gave rise to a small percentage of cells with the pulmonary epithelial/ATII phenotype. Wang and colleagues [76] have recently achieved remarkable success in deriving an almost pure population of ATII cells from hESC by genetic selection. ATII cells are characterized by their unique ability to synthesize and secrete surfactant protein C. Wang et al. [76] used nonviral transfection of hESC with a neomycin transgene under the control of a surfactant protein C promoter and were thereby able to produce an almost homogenous population of surfactant protein C expressing ATII cells under neomycin selection.

2.2 Cell-specific mesodermal differentiation

Bone and cartilage cells are of therapeutic interest because of their potential use for the treatment of osteoporosis and osteoarthritis. Mesodermal differentiation of mesenchymal precursors has been achieved by co-culturing hESC with murine OP9 stromal cells, followed by FACS using antibodies against the lineage-specific cell-surface protein CD73. In this paradigm, mesenchymal precursors gave rise to chondrocytes, osteocytes, and skeletal myoblasts under defined conditions [77]. A more selective system for osteogenic derivation from hESC was reported by Sottile et al. [78], where mineralization of EB-derived cells was induced by an osteogenic supplement consisting of ascorbic acid phosphate, β-glycerophosphate and dexamethasone.

Major advances have been made in the treatment of ischemic heart disease and cardiomyopathy by heart transplantation. The number of patients waiting for cardiac transplantation continues, however, to rise, and the possibility of using cardiomyocytes derived from hESC is a promising alternative. In 2001, Kehat et al. [79] demonstrated that hESC differentiation as EBs can commit hESC to form mesodermal cells, and eventually contracting cardiac tissue. Recently, a technique was reported for enhancing the derivation and purity of cardiomyocytes directly from hESC, by treatment of high-density undifferentiated monolayer cultures with activin A and BMP4. Most importantly, the authors demonstrated that transplantation of these cells into damages rat heart in combination with insulin-like growth factor 1 (IGF-1) and a multicomponent prosurvival cocktail can results in improvement in heart function [80].

Generation of two additional cell types, blood-forming hematopoietic cells, and endothelial cells from hESC- derived mesoderm precursors has also been established. These cell types could potentially be used for blood transfusion, analyzing hematopoietic and vascular defects, or for cell transplantation in the treatment of immunodeficiency disorders. It has been suggested that the cytokines, interleukin 3 and interleukin 6 in combination with BMP4 strongly promote differentiation of hESC into hematopoietic cells subsequent to EB formation [81]. The use of this combination of factors resulted in conversion of the majority of treated EBs to hematopoietic cells, as indicated by the expression of the hematopoietic progenitor marker CD45. Alternatively, a co-culturing system with the mouse stromal cell lines S17 and OP9, or the yolk sac endothelial cell line C166, can be used to derive hematopoietic-like cells expressing CD34 [82, 83].

Isolation of endothelial cells from randomly-differentiated EBs has been carried out by flow cytometry cell sorting using antibodies against platelet endothelial cell-adhesion molecule-1 [84]. In this study, endothelial cell transplantation experiments using biodegradable scaffolds showed formation of functional vessel-like structures that were integrated with the animal's vascular network [84].

2.3 Cell-specific ectodermal differentiation

The ectoderm derivatives include external ectoderm, neural crest, and neural tube. These structures give rise to cells of the epidermis, external sense organs, and the peripheral and central nervous system [1].

2.3.1 Non-neuronal derivatives of the ectoderm

Retinoic acid (RA) and BMP signaling has been found to act synergistically to enhance the induction of hESC to keratinocytes, which are the major component of skin. Temporal addition of low concentrations (1 μM) of RA strongly upregulated expression of factors involved in epidermal morphogenesis and ectodermal specification, while repressing early neural induction [85]. Production of keratinocyte precursors from hESC can be improved by a series of mesenchymal inducers, including fixed PA6 stromal feeder cells, BMP4, and collagen [86]. In terms of evaluation of these cells for of suitability for cell transplantation therapy, hESC-derived keratinocytes have been expanded while retaining a normal karyotype, and transplanted into mice without generating teratomas.

Age-related macular degeneration is one of the leading causes of human blindness, and is mainly caused by loss and dysfunction of the retinal pigment epithelium. Transplantation studies indicate that donor retinal pigment epithelium can support photoreceptor survival and restore visual function in animals with degenerated retinal pigment epithelium [87]. The first retinal pigment epithelium cells were isolated from hESC by randomly differentiating colonies of hESC followed by mechanical isolation of epithelial-like clusters, or by transferring differentiated EBs to gelatin-coated substrates [88]. Enhanced conversion of hESC to retinal pigment epithelial cells has been achieved by co-culturing hESC with a human retinal pigment epithelial cell line, or with human basement membranes of the choriocapillaris endothelium and retinal pigment epithelium [89].

2.3.2 Differentiation of the neuroectoderm

Generating functional neurons from hESC with the aim of treating neurodegenerative diseases is the subject of intensive investigation. Shortly after derivation of the first hESC lines, Reubinoff et al. [90] described isolation of expandable neural progenitor cells from hESC that had been cultivated for four to seven weeks at a high density *in vitro*. The authors illustrated that the neuroepithelium containing areas of differentiating hESC colonies, identified by expression of the embryonic polysialylated neural cell adhesion molecule (PSA-NCAM), had distinct morphological features. These areas were mechanically dissected and expanded as neural aggregates or spheres in serum-free media. Neural induction was achieved by plating the spheres on coverslips coated with poly-D-lysine and laminin, which resulted in emergence of cells expressing the neuronal markers, β-tubulin and microtubule-associated protein 2 (MAP2). They also identified a subset of the neuronal cells as being glutaminergic and GABAergic neurons, as shown by the expression of glutamate and glutamic acid decarboxylase (GAD).

Later, the same group, Reubinoff and coworkers [91] optimized the expansion of hESC-derived NPC by addition of B27 supplement, human recombinant epidermal factor EGF and the mitogen bFGF. Furthermore, the expanded NPC were capable of differentiating into all three major neural lineages (neurons, astrocytes, oligodendrocytes) *in vitro* as well as *in vivo*. Lineage tracing studies showed that NPC grafted to the ventricles of newborn mice differentiated in a region-specific manner, according to normal developmental patterning signals. For example, neuronal differentiation was specifically detected in the olfactory bulb, where postnatal neurogenesis occurs [91].

At about the same time, Zhang et al. [93] used a different protocol with similar success in generating enriched populations of NPC from hESC. In that study, differentiating EBs were treated with insulin, transferrin, progesterone, heparin, and bFGF. A continuous exposure to bFGF led to formation of monolayers of neural tube–like rosettes that were isolated by dispase enzymatic treatment. Similar to the study by Reubinoff et al. [91], the generated NPC were capable of generating oligodendrocytes, astrocytes, and mature neurons both *in vitro* and after transplantation into mice.

These observations confirming the multi-lineage differentiation potential of hESC-derived NPC, and promising indications of survival and integration of these cells *in vivo,* set the stage for future developments of methods for selective differentiation of different neuronal phenotypes that could potentially be used to treat various central nervous system disorders. Indeed, studies examining the signals and factors that govern the proliferation and cell fate specification of neural progenitors are accumulating rapidly.

Generation of transplantable motor neurons from hESC could have potential for treating victims of spinal cord injuries, or degenerative diseases such as amyotrophic lateral sclerosis. The first functional motor neurons originating from hESC were described by Li et al. in 2005 [94]. In that study, Li and colleagues used the method described by Zhang et al. [93] to generate NPC cells that were subsequently induced to become motor neurons through specification by addition of RA to the culture medium. Further maturation to postmitotic motorneuron was induced by the ventralizing morphogenic protein SHH. An interesting aspect of this study was the specific temporal effect of RA on motor neuron induction. It was clearly illustrated that RA could only induce early, but not late neuroectodermal cells to differentiate into motorneuron [94].

This occurred through upregulation of expression of HOX genes that are involved in assigning the rostrocaudal positional identity of spinal motor neurons. The functionality of the generated motor neurons was confirmed by electrophysiological experiments and establishment of neuromuscular transmission in motorneuron-myotube co-cultures [94].

In another study, directed differentiation of motor neurons was also achieved by addition of RA/SHH extrinsic cues to cultures of differentiating NPC generated from hESC [95]. With respect to clinical potential, transplantation of hESC-derived motor neurons in developing chick embryo spinal cord showed that these cells were capable of survival and long distance directed axonal growth [95]. On the other hand, transplantation in adult rats did not result in any axonal outgrowth from the CNS. It should be pointed out that, although caudal and ventral patterning was achieved by RA and SHH, the initial neural induction of hESC was obtained by co-culture with MS5 mouse stromal cells, which prevents the use of motorneurons generated by this strategy for any type of human transplantation therapy for motorneuron diseases [95].

In a later study by Li et al. [96], neural induction medium containing heparin and cyclic adenosine monophosphate (cAMP) in addition to RA and SHH was successful in generating a nearly homogenous population of ventral spinal progenitor cells, with highly efficient generation of motor neurons, thus eliminating the need for any feeder cells.

Promoting remyelination is another approach for treatment of neurologic disorders caused by demyelination of motor neurons. One of the strategies used to promote remyelination involves transplantation of oligodendrocytes, which produce the myelin sheath of motor neurons and are essential for normal signal conduction. Transplantation of hESC-derived oligodendrocytes produced by stromal cell co-cultures and bFGF, EGF, and RA treatment, has led to remyelination of motorneurons, and recovery of motor function after spinal cord injury [97].

A number of additional studies have reported the production of multiple neuronal subtypes, including cholinergic, serotonergic, GABAergic, and dopaminergic neurons, from hESC [101-104]. As previously mentioned, protocols used for neural conversion of hESC generally give rise to a mixture of neuronal phenotypes. Previous studies of neural differentiation of mouse ESC (mESC) have established protocols for growth factor-mediated lineage selection and survival-promoting factors of neuronal cells [98-100]. As a general strategy for obtaining selective neuronal differentiation, factors with effects on the anteroposterior (AP) or the dorsoventral (DV) neuronal patterning in combination with specific neurotrophins are used at specific stages during *in vitro* ESC differentiation. In a comprehensive study of neural development of mouse ESC, Barberi et al. used a stromal feeder-based differentiation system to generate early ectodermal cells (6 days co-culture) and identified various combination of factors that govern neural and neuronal subtype specification [100].

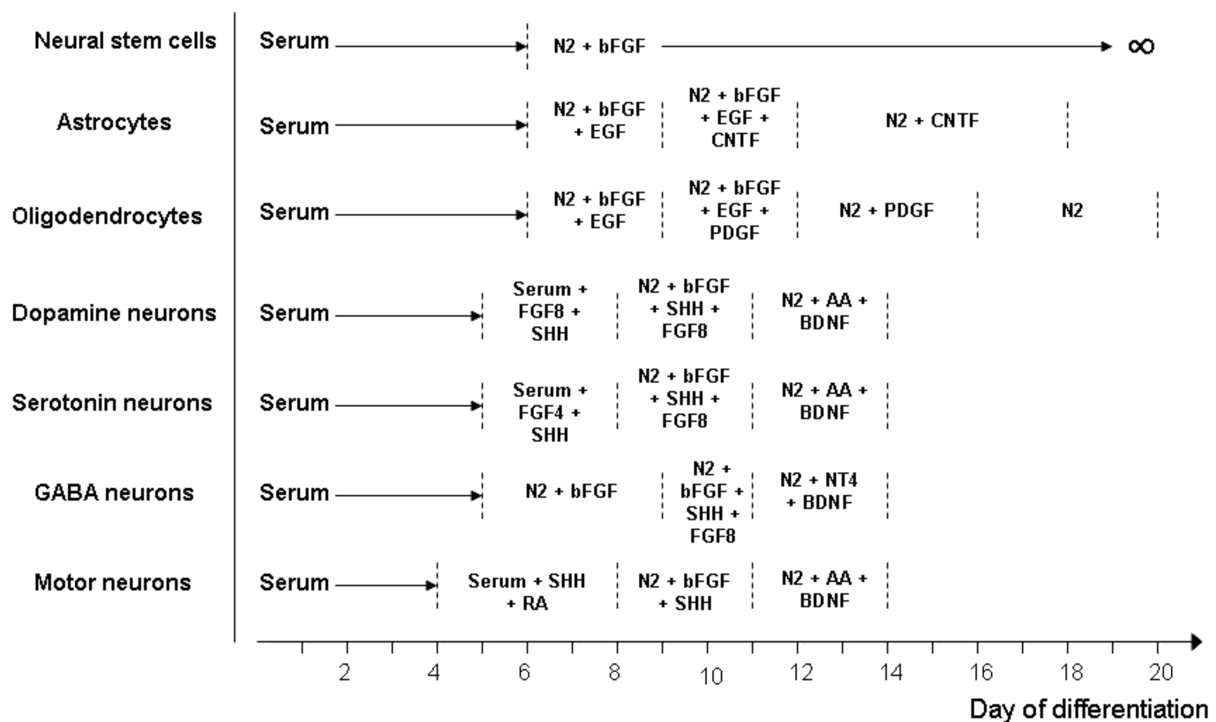

Figure 1. Neural subtype specification from neural progenitors derived from mouse ESC using various combinations of inducing factors. AA, ascorbic acid; bFGF, basic fibroblast growth factor; BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; EGF, epidermal growth factor; FGF4, fibroblast growth factor 4; FGF8, fibroblast growth factor 8; NT4, neurotrophin-4; PDGF, platelet-derived growth factor; RA, retinoic acid; SHH, sonic hedgehog. {Adapted from Barberi et al. (2003), [100]}

3. Dopaminergic differentiation of hESC

To date, the majority of studies on neural differentiation of hESC have been focused on generation of dopamine producing neurons of the midbrain subtype, due to their potential application in cell replacement therapy for PD. Established protocols for the *in vitro* induction of hESC fall into two distinct categories. The first strategy is based on previous findings in mESC, which involved exposing neural progenitor cells obtained by random differentiation of ESC, first to early midbrain patterning factors, and later to neurotrophic factors that contribute to the selective survival of mDA neurons. The second DA inductive technique involves the use of feeder cells, of animal or human origin, which possess a neural and dopaminergic cell fate specification property. In addition, specific culturing conditions appropriate for DA differentiation of hESC have also been developed.

3.1 DA induction by biochemical patterning cues

For successful derivation of functional, and midbrain specific DA neurons, it is imperative to develop *in vitro* models that can mimic the *in vivo* scenario. Although it is unlikely that a structure comparable to the developing neural tube can be recreated in ES cell cultures, it is important to mimic the induction events associated with different regions of the brain that lead to the development of midbrain specific cell populations.

SHH and FGF8

The secreted morphogens SHH and FGF8 possess important signaling activities and have been shown to play an essential role in neural tube patterning and specification of different cell types during early stages of development.

SHH is one of the three human homologs of the drosophila hedgehog (HH) gene. The role of the HH family was first discovered in the famous studies of developmental biology by Ed Lewis, Christiane Nüsslein-Volhard and Eric Wieschaus (winners of the Nobel Prize in Medicine in 1995) where large numbers of mutations affecting embryogenesis were identified. The name originated from HH mutant fly embryos that were short and covered in tiny spines resembling a hedgehog, while the specific name of ″sonic hedgehog″ was derived from a video game character. In drosophila, signaling by hedgehog family proteins was identified as one of the essential pathways responsible for DV patterning [106, 107]. Expression patterns of SHH was found to be associated with polarizing activities in vertebrate embryos in tissues including the notochord and floor plate of the neural tube [108, 109]. Subsequent studies of mouse SHH mutants revealed that disruption of the SHH gene results in malformation of the ventral neural tube leading to defects in the midbrain and forebrain [110].

The notochord consists of a rod-like structure of mesodermal cells situated on the ventral side of the neural tube. The SHH protein, secreted from the notochord and the floorplate, becomes distributed in a gradient across the ventral neural tube, where it acts in a concentration-dependent manner to specify a range of distinct neuronal cell types [111]. In addition, a recent study has shown that cellular responses to SHH can also depend on the duration of SHH signaling. In fact, changing the concentration or duration of exposure to SHH had a similar effect on intracellular signaling initiated by SHH [112].

SHH elicits its biological effects by binding to the cell-surface transmembrane protein patched (PTCH) [113]. The receptor PTCH prevents expression and activity of a seven membrane spanning receptor called smoothened (SMO). Upon ligand binding, PTCH is inhibited, allowing SMO to accumulate. Accumulation of SMO leads to the activation of the GLI transcription factors, the activators Gli1 and Gli2 and the repressor Gli3 [114-117]. The Gli proteins acting as transcriptional activators or repressors, suggests that the polarizing activity of SHH could be a result of the specific distribution and functional properties of each of the Gli proteins. The few developmental regulatory genes that have been identified as direct targets of the SHH-PTCH-GLI pathway include Wnt genes, BMP4, and the transcription factor, Hepatocyte Nuclear Factor-3β [118-120]. Notably, continuous exposure to SHH also leads to a negative feedback by transcriptional activation of PTCH itself, that will gradually desensitize the cells to SHH signaling [121].

FGF8 was formerly named androgen-induced growth factor. This protein is expressed in the isthmus and acts as an AP organizer in the midbrain-hindbrain boundary. Fibroblast growth factor (FGF) signals are known to interact with tyrosine kinase receptors that activate the mitogen-activated protein kinase (MAPK) and PI3 K pathways, which mediate growth and development [124]. The temporal expression of FGF8 in organizing centers of the CNS during early embryogenesis suggests an involvement of FGF8 in the neuronal development [125, 126]. The role of FGF8 signaling in specifying positional identity in the brain was further supported in a study by Crossely and colleagues [127] that clearly demonstrated that implanted FGF8-coated beads acted similarly to grafted isthmus tissue in transforming chick caudal forebrain into midbrain. In 1998,

localization of SHH and FGF8 along the AP and DV axis of the neural tube between rat embryonic days 11–15 showed that DA neurons develop at sites where the signals of both molecules intersect. In addition, SHH and FGF8 function-blocking experiments provided solid evidence that these two extracellular signaling factors are necessary and sufficient for the induction of DA neurons in the midbrain and forebrain [128].

DA induction of hESC by SHH and FGF8

Like their mouse counterparts, hESC can be directly induced to differentiate to a dopaminergic phenotype by SHH and FGF8 instructive signals. In 2005, Yan and coworkers [129] demonstrated neural induction and of hESC and DA specification of neurons in the absence of any type of feeder cells. This protocol involved differentiation of the hESC lines H1 or H9 in suspension as EBs for 4 days, followed by expansion as adhesive cultures in the presence of bFGF for an additional 14 to 16 days. This procedure generated neuroepithelial cells, which were then dissociated and differentiated in the presence to SHH and FGF8. The authors suggested that for generation of DA neurons with midbrain neuronal phenotypes, early exposure of FGF8 was necessary. When FGF8 was added to cultures before Sox1 expression, at day 10 of expansion instead of day 18, expression of early transcription factors expressed in the forebrain, including orthodenticle homolog 2 (Otx2) and brain factor-1 (Bf1) was suppressed, whereas expression of the midbrain specific transcription factor engrailed 1 (En-1) was induced. Addition of FGF8 to neural progenitor cells that had already begun to express Sox1 resulted in generation of DA neurons with mostly forebrain phenotypes. After three weeks of differentiation, the dissociated NPC that were treated with FGF8 at earlier stages gave rise to neurons positive for both tyrosine hydroxylase (TH) and En-1, which comprised 50%–60% of the total neuronal population. They further illustrated that the obtained midbrain TH+ neurons were biologically and electrophysiologically active.

Recent technical advances based on the same strategy used by Yan et al. [129] have allowed generation a more pure population of dopaminergic neurons from hESC [130]. For this improved differentiation procedure, the hESC lines SNUhES1, SNUhES3, and SNUhES16 were cultured as EBs for seven days and then expanded in NP serum-free selection media for four days to form neural rosettes. The neural rosettes were mechanically isolated and further expanded in suspension in the presence of bFGF and N2 supplements to form large aggregates referred to as spherical neural masses (SNMs).For neuronal induction, the SNMs were transferred to matrigel-coated substrates after 10 days and further cultured under the influence of SHH and FGF8 and ascorbic acid (AA).

In contrast to the protocol developed by Yan et al. [129], the procedure used by Cho and coworkers [130] involved addition of FGF8 and SHH at a much later stage, after the cells displayed neuronal morphologies (four days after differentiation of SNMs was initiated). Notably, the late exposure to FGF8 did not result in NPC with a forebrain character. After 14 days of differentiation, approximately 66% of the total cells were TH+ and EN-1 expression was also confirmed in the majority of the TH+ cells. The SNMs could also be expanded for long periods (>four months) without losing their capability to differentiate into functional midbrain TH + neurons.

The hESC line hES1 has also been differentiated to functional dopamine producing neurons by EB formation in insulin–transferrin–selenium fibronectin media (7-10 days), NPC expansion in N2 media supplemented with EGF, bFGF (10 days), and DA induction and differentiation by SHH and FGF8 treatment (10-15 days).

The majority of cells in these cultures were neuronal cells and about 40% of the β-III tubulin + neurons were also positive for TH [131].

DA induction of hESC in feeder-free conditions without SHH and FGF8

Differentiation of BG01 and BG03 hESC cell lines to midbrain dopaminergic neurons have been observed in a serum-free suspension system in the absence of added neural-inducing or instructive factors [132]. In this report, the authors generated EBs for one month in differentiation medium, and demonstrated that these aggregates were able to differentiate into TH+ neurons in adherent cultures in appropriate medium. It was suggested that the specification of the DA lineage was driven by cell-to-cell signaling within the suspension aggregates. They also hypothesized that the large cellular aggregates may have provided a low-oxygen environment which was previously shown to enhance differentiation of DA neurons from mESC [133].

The majority of protocols developed for DA differentiation of hESC involve the use of neurotrophic factors to enhance survival of TH+ neurons. Glial cell line-derived neurotrophic factor (GDNF) is a selective neurotrophic factor for midbrain dopaminergic neurons and has been shown to be neuroprotective in rodent and primate models of PD [134-136]. GDNF enhances the yield of TH+ neurons from fetal mesencephalic NPC as well as hESC [137, 138, 140]. Brain-derived neurotrophic factor (BDNF) is also known to contribute to the survival and differentiation of DA neurons during development [139, 140]. Additional survival factors for DA neurons are dibutyryl-cyclic AMP (dbcAMP) that accelerates the onset of the survival effects of GDNF and BDNF [141-143], as well as PTN [144-146], and conserved dopamine neurotrophic factor [147].

A study of the effect of transforming growth factor-α on the transition of hESC-derived NPC cells to DA neurons has reported that transforming growth factor-α, when used in concert with bFGF in cell-free induction systems, yields a higher percentage of dopaminergic neurons (≈ 20%) from hESC [141].

During ectodermal differentiation, epidermis is induced by BMPs, whereas neural fates arise by default following BMP inhibition by Noggin or other BMP inhibitors such as chordin and follistatin [149-152]. Inhibition of BMP signaling can be used to enhance induction of NPC from hESC [153, 154]. More recently, support systems for enhanced induction of DA neurons from hESC have been developed by the use of noggin in the presence of type IV human collagen, which is the major protein of the basal lamina extra cellular matrix during CNS development. Combined treatment with noggin during NPC expansion, and dbcAMP during neuronal differentiation significantly increased the number of dopaminergic neurons derived from hESC, as compared to untreated cultures [155].

3.2 DA differentiation of hESC on cellular substrates

When hESC are grown in co-culture systems with various feeder cells, the feeders are generally considered to provide signal transduction through two major mechanisms, promoting differentiation towards specific cellular phenotypes. First, the cellular substrate provides direct cell–cell contact, which may convey signaling that reflects the natural niche for survival and lineage specification of differentiating hESC. Secondly, cells secrete various soluble factors, such as growth factors and cytokines that stimulate the proliferation and differentiation of hESC. Cell-surface bound factors might also guide hESC towards specific lineages.

3.2.1 Role of extracellular matrix molecules (ECM) during CNS development

Numerous studies have provided evidence supporting a role of intercellular adhesion governed by ECM components and their receptors during CNS development.The first demonstration of the role of cell adhesion in embryonic cell sorting was provided more that 50 years ago by Townes and Holtfreter [156]. They hypothesized that ectodermal and mesodermal cells sort into two distinct germ layers due to their distinct cell adhesion properties. Indeed, later it was shown that the neural tube is separated from the surface ectoderm, because the neural tube-forming cells that initially express the cell adhesion molecule epithelial cadherin stop producing this protein, and instead synthesize another subtype of the cadherin protein, neural-cadherin, as well as NCAM, as the neural tube develops. Cell-type specific transient expression of different types of cadherins during the neural tube formation is also important for the closure of the neural tube [157, 158].

The expression of ECM and their receptors are highly regulated and contribute to controlling cell migration, differentiation, survival, and axon guidance during development of the nervous system [159-163]. Extracellular matrices are also known to interact strongly with many growth factors and modulate their biological activity [164]. Although differential expression of genes which encode ECM molecules is known to occur during the formation and regionalization of the neural tube, the specific role of individual ECM molecules and their receptors during neuronal specification is still under investigation [165-167].

Laminin, fibronectin and collagen are among the main ECM molecules that regulate neuronal development [159, 163].The mechanisms for attachment to extracellular matrices for neural cells predominantly involve binding to the integrin cell surface receptors, which mediate commitment and lineage determination during early embryogenesis [159-161, 163]. A large number of proteoglycans are also expressed in the developing nervous system. Aside from being a part of the ECM network, the potential functions of proteoglycans include roles as receptors, modulators of axon guidance cue function and regulators of the biological activity of a variety of growth factors [163, 166].

Interaction with ECM molecules leads to activation of intracellular second messenger pathways through activation of protein kinases including the focal adhesion kinase, a tyrosine kinase associated with integrins in focal contacts [153, 164]. Focal adhesion kinase initiates a cascade of intracellular signals and works in concert with signaling initiated by growth factors during development [172-175].

An important role of the extracellular environment in determining programmed differentiation of stem cells has recently been established [176-179]. Particularly, loss of β1 integrin function during ESC differentiation resulted in enhanced neuroectodermal differentiation, paralleled by over-expression of Wnt-1 and decreased BMP-4 mRNA levels [176-178]. Laminin-coated substrates have also been reported to enhance general neuronal differentiation of hESC and can affect neuronal subtype specification and electrophysiological properties of the resultant neurons [179]. Additionally, a more recent study utilizing mechanically-tunable synthetic biomaterials as a substrate for growth and differentiation of NPC illustrated that substrate rigidity influences self-renewal and lineage specification of NPC during differentiation [180].

3.2.2 DA differentiation induced by SDIA

Mouse stromal cells are feeder cells which are frequently used to induce differentiation of ESC to a DA lineage. Kawasaki and colleagues in Japan [181] examined the role of various feeder cells including MEF and NIH3T3 embryonic fibroblast cells, MDCK, and COS kidney epithelial cell lines, and two stromal cell lines OP9, and PA6 in differentiation of mESC. Using these co-culture systems, a surprising neural and dopaminergic-inducing activity of PA6 stromal cells, termed "stromal-derived inducing activity" (SDIA) was discovered.

The bone marrow stroma consists of a variety of different cell types including endothelial cells, adipocytes, smooth muscle cells, reticular cells, osteoblasts and stromal fibroblasts. Bone marrow stromal cells form the connective tissue that provides the structural and physiological support for hematopoietic cells [182]. These cells are rich in a variety of ECM molecules, including different types of collagen and laminin, and secrete many growth factors as part of their hematopoietic supporting role [183, 183]. They also have the ability to differentiate towards osteogenic, chondrogenic and adipogenic lineages [185, 186].

The PA6 cells were derived in 1982, from skull bone marrow of newborn mouse calvaria and were originally referred to as the MC3T3-G2/PA6 preadipose cell line because of their capacity to convert to adipose cells [187]. These cells were initially used to support *in vitro* proliferation of hematopoietic stem cells and hemopoiesis [188, 189].

In contrast to previous protocols for generation of mDA neurons from mESC, the co-culturing method does not require formation of embryoid bodies, selection of neural precursor cells, or addition of patterning cues or growth factors. In the initial study by Kawasaki et al [181], after 12 days of co-culture with PA6 cells 92% of ES cell colonies contained NPC and differentiated neurons characterized by positive staining for nestin, NCAM, β-tubulin, and MAP2. Less than 2% of the colonies contained cells that had differentiated to glial fibrillary acidic protein + (GFAP+) glial or mesodermal lineages. In this paradigm, GABAergic, cholinergic, and serotonergic neurons were observed in 43%, 28%, and 7% of colonies, respectively. The percentage of TH-expressing colonies was, however, the highest at 92%. Approximately one-third of neuronal cells had committed to a dopaminergic lineage, and the yield of TH+ neurons was 16% of the total cells. The resulting TH+ neurons expressed the midbrain markers nuclear receptor-related 1 (Nurr1) and paired-like homeodomain transcription factor 3 (Pitx3), were capable of synthesizing dopamine, and survived when transplanted to mouse 6-hydroxydopamine (6-OHDA) treated striatum (~ 20% survival rate). This study [181] also discovered that low doses of BMP4 after the onset of nestin expression suppressed the neural inducing activity of PA6 cells, but was not able to completely abolish the effect. The temporally limited effect of BMP indicated that SDIA may exert other neural inducing signals at earlier stages, before the cells can respond to BMP signaling. RT-PCR analysis did not show expression of BMP inhibitors, suggesting that SDIA may antagonize BMP signaling by a different mechanism. In this study, PA6 cells were reported to retain neural-inducing activity after being fixed with paraformaldehyde. In contrast, medium conditioned by PA6 cells for 3 days did not induce significant neural differentiation of ESC. Furthermore, the authors illustrated that SDIA activity was not mimicked by FGF8/SHH, or Wnt signaling [181].

This technically simple and efficient method for DA differentiation of ESC has been very widely used, and was successfully applied to primate ESC, resulting in 35% TH+ neurons after

selective cell sorting. In addition to TH+ neurons, the SDIA-treated primate ES cells gave rise to 20% GABAergic, 5% cholinergic, and 1% serotonergic neurons. In contrast to mESC that generated 92% TH+ colonies, primate ESC gave rise to only 45% TH+ colonies. The survival rate of generated primate TH+ neurons *in* vivo after transplantation was also somewhat less than TH+ neurons derived from mESC. Also, one unpredicted finding from the primate study was the appearance of pigmented epithelial cells in approximately 8% of the colonies [190].

Monitoring of the temporal regulation of SDIA-induced neural differentiation of primate and mouse ESC, has led to approaches in which ESC are co-cultured with PA6 cells and subsequently cultured as neurospheres. These approaches can produce cultures with enriched populations of NPC, which can be used to obtain enhanced transplantation efficiency of dopaminergic neurons [191-193]. For instance, Morizane and colleagues [192] reported that the survival of TH+ cells after transplantation in an animal model of PD was highest when mouse ESC were treated with SDIA for 12 days before transplantation. The proportion of TH+ neurons derived from proliferating neurospheres can also be increased by exposure to PA6 cell conditioned medium [191], or by addition of FGF20 [192] which is a neurotrophic factor preferentially expressed in the substantia nigra [193].

The effect of SDIA on hESC differentiation was tested in 2003 in Dr. W. Freed's laboratory [194]. When the hESC line BG01 was cultured on PA6 cells for three weeks, approximately 87% of hESC colonies contained large numbers of TH+ cells. The first appearance of TH+ cells was observed after 8-10 days of co-culture with PA6 cells, and the number of TH+ neurons within colonies reached a maximum at approximately day 18. In contrast, less than 10% of colonies contained GABAergic neurons. The TH+ neurons generate by PA6 cell induction had midbrain characteristics, as determined by expression of Nurr1 and Pitx3 transcription factors that are strongly associated with midbrain dopaminergic neurons. The dopaminergic nature of the TH+ neurons was further confirmed by the lack of expression of the noradrenergic neuronal marker, dopamine beta hydroxylase (DBH), and by release of dopamine. In this study, initial attempts were also made to identify potential PA6 cell-released neural inducing factors. A cytokine growth factor–focused array was used to identify genes that were highly expressed in PA6 cells, as compared to MEF cells which completely lack SDIA activity. Several growth factors including hepatocyte growth factor, vascular endothelial growth factor and FGF7 were differentially expressed in PA6 cells. Addition of these growth factors to hESC cultures was, however, not sufficient to induce differentiation of hESC to form TH+ colonies.

A parallel study of DA induction of hESC by Perrier and colleagues in 2004 [104], described multistep neural and dopaminergic induction by initial stromal feeder-mediated differentiation followed by sequential addition of SHH and FGF8 patterning molecules, ascorbic acid, and various neurotrophic factors including BDNF, GDNF, TGF-β3, dcAMP. In this work, differentiation of hESC lines H1, H9 and HE-3, as well as two monkey ESC lines, was carried out on MS5 or S2 stromal cell lines for 28 days. At this stage, rosette-like structures were mechanically isolated and further cultured in the presence of SHH, FGF8, AA, and BDNF. These cultures were passaged twice at seven to nine day intervals and exposed to GDNF and dcAMP in the absence of SHH and FGF8 to obtain terminal differentiation to dopaminergic neurons. Addition of SHH and FGF8 from day 12 to day 20 of differentiation, while in co-culture with stromal cells, increased the number of TH+ neurons by three fold. Nonetheless, continuous exposure to SHH and FGF8 until day 42 was necessary for development of midbrain specific TH+ neurons.

Perrier et al. [104] hypothesized that the continuous responsiveness of the neural precursor cells to patterning cues in their differentiation paradigm was due to expansion in the absence of FGF2. The electrophysiological and dopamine releasing properties of TH+ neurons were also highly dependent on exposure to SHH and FGF8. At day 50 of differentiation, 30-50% of the total cell population belonged to a neuronal lineage, and the neurons expressed TH at a frequency of 64-79%. Very small percentages of GABAergic and serotonergic neurons were also detected. The time frame for DA differentiation in this study was analogous to development of human midbrain DA development. Notably, however, even after more than 50 days of differentiation, TH+ neurons did not express dopamine transporter (DAT), indicative of their immature state [104].

Similar to this study, an investigation by Park et al. [195] identified a more efficient dopaminergic induction procedure of hESC based on co-culture with stromal cells, followed by expansion of the resulting neural precursor cells. In contrast to the protocol described by Perrier's group [104], this study [195] used co-cultures with stromal PA6 cells, or PA6 cells stably overexpressing SHH (PA6-SHH) for 14 days, followed by terminal differentiation for an additional 14 days in feeder-free conditions. This study confirmed that early exposure to SHH and FGF8 (after one week of induction) increased the yield of TH+ neurons, and was crucial in commitment of TH+ cells to a midbrain phenotype [195]. Stromal cell-induced DA differentiation of hESC has also been found to be enhanced by addition of the BMP inhibitor noggin [154]. Furthermore, it has also been reported that mesencephalic-restricted neuroectodermal cells produced by the SDIA method, can be expanded *in vitro* for extended periods, without any loss of their dopaminergic differentiation ability [196].

Despite the numerous studies that have used SDIA in DA differentiation of ESC, the only thorough characterization of SDIA that has been reported involves the temporal aspect of SDIA activity [197, 198]. Although, factors secreted from PA6 cells have been suggested to be involved in the neural and dopaminergic induction of ESC [199, 200], the exact role of the physical and molecular properties of SDIA remains unknown.

4. The dopaminergic system of the brain

4.1 Dopamine signal transduction

Dopamine acts as a neurotransmitter and has hormone-like effects in the brain.Dopamine is synthesized from its amino acid precursor, L-tyrosine. The conversion of L-tyrosine to L 3,4-dihydroxy-L-phenylalanine (L-DOPA) is the first and the rate-limiting step of dopamine biosynthesis and is catalyzed by TH. L-DOPA is then converted to dopamine by aromatic l-amino acid decarboxylase (AADC). Coenzymes that are also required in the formation of dopamine include tetrahydrofolic acid (THFA; for L-tyrosine to L-dopa), pyridoxal phosphate (for L-dopa to dopamine), and nicotinamide adenine dinucleotide (NADH; for the formation of THFA and pyridoxal phosphate). After synthesis, dopamine is packaged into vesicles in dopaminergic nerve terminals, and then released in response to the presynaptic action potential [201]. Upon release into the synaptic cleft, dopamine signal transmission is mediated by interaction of dopamine with postsynaptic receptors of several types, principally D1 or D2-type G-protein coupled receptors [202]. Released dopamine can also bind to presynaptic D2 autoreceptors which modulate dopamine synthesis and release [203].

4.2 Dopaminergic pathways

The major mechanism of inactivation of dopaminergic neurotransmission occurs via uptake by dopamine-specific transporters [204, 205]. The recycled dopamine is either re-packaged in synaptic vesicles by vesicular monoamine transporter 2 (VMAT2), or enyzmatically degraded. The catabolism of dopamine is mediated by monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) which convert dopamine to 3,4 dihydroxyphenylacetic acid and 3-methoxytyramine, respectively [201].

There are four specific pathways in the CNS that employ the transmitter dopamine but are diverse with respect to neuroanatomy and function. The nigrostriatal pathway is comprised of A9 subtype of DA neurons of the substantia nigra pars compacta (SNpc) that project to the neostriatum (caudate nucleus-putamen).This system is primarily concerned with sensory stimuli and regulation of motor function. The cell bodies of the A10 group of DA neurons are located in the ventral tegmental area (VTA), and comprise the mesocortical and mesolimbic pathways by innervations of the limbic system (medial and the ventral striatum) and the neocortex. The mesocortical/mesolimbic pathways are involved in the control of motor and emotional behavior as well as mediation of brain reward and addiction. The dopamine releasing neurons (A8 group) in the fourth pathway, known as the tuberoinfundibular system, are located in the arcuate nucleus of the hypothalamus and send their axonal projections to the median eminence. Dopamine released from these neurons regulates secretion of hormones from the anterior pituitary gland. DA neurons are also found in the diencephalic and hypothalamic brain regions (areas A11 to A15), periglomerular regions in the olfactory bulb (A16 cell group) and the retina (A17 cell group) [206-209].

In addition to the anatomical distinction of the dopaminergic systems, the two major DA types of neurons in the SNpc and VTA can be differentiated based of morphology and expression of specific markers [210-217]. A selective marker for the vast majority of the A10 neurons is the calcium binding protein calbindin [213, 214], whereas the G-protein-gated inwardly rectifying K^+ channel Girk2 is only expressed in the A9 neurons [215, 216]. Ephrin B2 and its receptor EphB1 have also been proposed to contribute to the establishment of the distinct DA neural pathways, based on data showing that these molecules selectively induce loss of the substantia nigra DA neurons by apoptosis, but not of VTA neurons *in vitro* [217].

4.3 Development and maturation of mesencephalic DA neurons

If stem cell therapy is to be used for neuron replacement in treatment of PD in the future, it is important to examine the course of *in vitro* DA differentiation of hESC to ascertain that any TH-expressing neurons that are produced also express region-specific transcription factors and possess other distinctive midbrain specific properties, which would be expected to increase the ability of these cells to function *in vivo*.

As previously discussed, the initial anatomical localization of DA neurons along the AP and DV axes of the developing brain is determined by the interaction of the two instructive factors SHH and FGF8 that are expressed in the ventricular zone and at the midbrain–hindbrain border, respectively. SHH and FGF8 are also known to initiate signaling cascades leading to activation of other molecules and transcription factors that lead to functional differentiation of DA neurons. The Otx2 homeogene is required for specification and regionalization of forebrain and midbrain regions

[218, 219]. It has also been reported that Otx2 has a role in cell-fate decisions during mid and late brain development by regulating the spatial patterns of SHH and FGF8 in a dose-dependent manner [220].

Signaling cascades downstream of SHH/FGF8, and upstream of the transcription factors that are required for specification and phenotype development of mDA neurons, have recently been identified by Andersson and colleagues at the Karolinska institute [221]. Their study demonstrated that the two homedomain transcription factors Lmx1a and Msx1 were dependent on SHH signaling, and were selectively expressed in dopamine progenitor cells during a time period corresponding with early specification of DA precursors. By using retroviral-mediated Lmx1a gene delivery into the developing chick midbrain, the authors were able to induce generation of dopaminergic neurons. Lmx1a knock down in the developing chick embryo dramatically reduced the number of postmitotic DA neurons, which confirmed that Lmx1a plays a critical role in midbrain DA neuronal development. It was further illustrated that Lmx1a is required to induce the expression of Msx1. The expression of Msx1 in turn repressed expression of Nkx6.1 gene which is associated with induction of motor neuron differentiation, while inducing expression of the proneural protein neurogenin 2 (Ngn2). Ngn2 has recently been identified as an important factor in DA development and in differentiation of mDA neurons [222, 223]. In addition, Andersson and colleagues [221] showed that selective expression of Lmx1a in nestin expressing neural progenitor cells derived from mESC induced expression of Msx1/2 and robust generation of TH+ neurons after 8 days of *in vitro* culture.

Studies on Lmx1b knockout mice have shown that the midbrain of Lmx1b -/- mice developed abnormally, thus providing evidence for the requirement of this factor for neuronal specification and maintenance of mDA neurons [224]. TH+ neurons that were found in the midbrain of Lmx1b-/- embryos failed to express the midbrain-specific paired homeodomain transcription factor Pitx3, and were eventually lost. Besides this role in regulating Pitx3 expression [224, 225], Lmx1b is also known to induce expression of Fgf8 and Wnt1 and other transcription factors critical for mDA development [226, 227].

Similar to SHH, expression of TGF-β in early embryonic structures such as notochord and floor plate, as well as areas of mDA development, suggested a role in midbrain cell-fate specification. In addition to the neurotrophic role of TGF-β, *in vivo* and *in vitro* studies have confirmed that SHH and TGF- β function synergistically in the induction of ventral midbrain dopaminergic neurons [228-230].

One of the first steps during gastrulation and early neurulation involves complex interactions of several signaling molecules that belong to the Wnt family of secreted glycoproteins [231-234]. Loss-of-function studies of Wnt-1, which is highly expressed in the developing midbrain, have been associated with failure of midbrain and hindbrain development [235, 236]. It has also been proposed that Wnt1 predominantly regulates specification of mDA progenitors through maintenance of expression of engrailed 1 (EN-1) [237], and Otx2 [238] genes. In addition, activation of the Wnt1 signaling cascade leads to increased mDA progenitor proliferation and differentiation of mDA precursors into mature mDA neurons [238, 239]. Two other members of the Wnt family, Wnt-3a and Wnt-5a are expressed in the dorsal and ventral midbrain, respectively [239, 241].

Previous *in vitro* studies in rat primary VM progenitor cell have confirmed a role of Wnt3a in enhancing the proliferation of DA neuron precursors, while inhibiting DA differentiation [239, 241].

Conversely, Wnt5a did not have a significant effect on proliferation of midbrain NPC, but promoted differentiation and maturation of mDA precursors into DA neurons [239, 240].

The paired box (Pax) transcription factors Pax2 and Pax5 are expressed at the midbrain-hindbrain boundary with different spatiotemporal patterns [242, 243]. Onset of Pax2 expression occurs earlier that Pax5 (about 1 day earlier in mouse), and it has been suggested that Pax2 initiates the expression of Pax5 [244]. The cooperative role of Pax2 and Pax5 in brain development has been established by targeted mutations, as both midbrain and hindbrain structures were deleted by progressive inactivation of Pax2 and Pax5 alleles [245]. Although single Pax2 or Pax5 mutations resulted in variable outcomes, the midbrain and cerebellum were completely deleted in Pax2/ Pax5 double-mutant embryos [246].

The developmental fate of midbrain dopaminergic neurons is also under the control of engrailed homeodomain transcription factors, En-1 and En-2. En-2 is expressed in a minority of mDA neurons, whereas En-1 is widely expressed in all midbrain TH+ cells [247]. The results from En-1/En-2 double mutant [248] and En1+/- studies in mice [249] indicate that the engrailed transcription factors are required for the maintenance and survival of DA neurons, as opposed to having an early role in mDA specification. Expression of En1 and En2 induces continued production of FGF8, which is important in maintaining the dopaminergic phenotype [250].

As the mDA precursor cells begin their radial migration along scaffolds of radial glial processes from the ventricular zone towards the marginal zone (E15 to E18 in developing rat brain) [251], they begin to express the Nuclear receptor 1 (Nurr1) [252]. The activation of Nurr1 expression drives the midbrain NPC to exit the cell cycle and initiates DA phenotype development by activating the expression of factors which are required for dopamine synthesis and signal transduction, in particular TH, VMAT2, and DAT [253, 254]. In addition to Nurr1, achaete scute homolog 1 (Mash1) has also been suggested to have a role in DA maturation [223, 254]. Overexpression of Nurr1 in combination with Mash1 in NSC isolated from rat cortex (E13–E14) resulted in induction of substantial numbers of TH+ neurons [255].

Recently, a new group of transcription factors, the forkhead/winged helix transcription factors Foxa1 and Foxa2, were found to be important during dopaminergic specification [256]. Foxa1 was first identified as being highly expressed in early DA neurons sorted from mouse primary embryonic mesencephalic tissue in a cDNA library [257]. Genetic studies of Foxa1 and Foxa2 single- and double-mutants by Ferri and coworkers showed that Foxa1/2 positively regulate the expression of Nurr1 and En1 in immature mDA neurons and enhance the expression of Ngn2. Foxa1/2 were also required for the expression of TH and AADC during differentiation and maturation of mDA neurons [256].

When the neurons arrive at their final ventral position, they adopt the full DA phenotype by expressing TH and paired-like homeodomain transcription factor 3 (Pitx3). Pitx3 is a terminal differentiation marker for mDA neurons and is exclusively expressed in mDA neurons [258]. Loss-of-function experiments have shown that Pitx3 is required for the terminal differentiation and survival of mDA neurons, mainly those of the SN [259, 260].

The regulation of the migratory processes of mDA neurons, in terms of establishing specific connectivity and axonal pathfinding, is not completely understood. It has been suggested that neuronal fibers extending from the SN and VTA innervate overlapping areas in the striatum in a

nonspecific fashion [261, 262], and that the distinct organization of the mesostriatal and mesolimbic pathways occurs through the selective elimination of misdirected projections at a later stage [262]. Also, several signaling molecules have been related to mdDA neuron migration and axonal projections. The netrin and Slit family of proteins are chemotropic molecules which guide cell migration during CNS development [263-265]. The netrin ligand and its receptors DCC are highly expressed in both SN and VTA during development [265]. DCC deficiency in mice has been correlated with altered DA organization of DA circuitry and function [266]. *In vitro* studies of fetal and mESC-derived DA neurons have also shown Slit-mediated navigation of DA axon growth mediated through Robo receptors [267, 268] which are also highly expressed in the developing midbrain [269].

The Ephrin family receptor tyrosine kinases and their ligands have been implicated in guiding axons during the development of the nervous system [270, 271].The EphB1 receptor and its ligand Ephrin-B2 have also been reported to play a role in the development of the nigrostriatal and mesolimbic pathways. This receptor-ligand pair selectively inhibits neurite outgrowth from the substantia nigra DA neurons which, as previously mentioned, led to loss of these cells [217]. In addition, dopaminergic signaling has shown to regulate expression of the EphB1 receptor [273].

5. Parkinson’s disease

The PD syndrome was first described in the “Essay on the shaking palsy” published in 1817 by the British physician James Parkinson. Involuntary tremulous motion, lessened voluntary muscle power, incomplete and slow movement (Bradykinesia), stiffness of the muscles, and postural instability are among the symptoms experienced by patients suffering from this disease [273, 274]. During the late 1950s, Dr. Oleh Hornykiewicz at the University of Vienna performed autopsies on deceased patients with PD, and discovered that PD was associated with depigmentation of the SN in the ventral midbrain [275]. Later discovery of decreased levels of dopamine in the substantia nigra suggested that PD was caused by decreased dopamine levels in the brain [275, 276].

PD primarily affects older individuals, over the age of 55, but Parkinsonian symptoms are occasionally seen in people in their 30's and 40's as well. Various studies have suggested that the incidence of PD varies by geographical location, and may be more common in men [278, 279]. Hereditary forms of PD account for a small percentage of reported cases of this neurodegenerative disorder, whereas most cases of PD occur sporadically [280, 281].

Parkinson's disease: Cause and Treatment

A number of neurodegenerative disorders are believed to be caused by accumulation of missfolded protein aggregates and formation of inclusion bodies known as amyloid fibrils [282]. Although the molecular pathology of PD is not completely understood, large inclusions referred to as Lewy bodies (LBs) are a prominent neuropathological hallmark of PD. LBs were first discovered in 1912 by Frederick Lewy, who examined the postmortem brains of patients with PD and observed abnormal structures in deteriorating nerve cells in the midbrain [283]. More recently, LBs have been found to be composed of predominantly abnormally polymerized α-synuclein (AS) protein [284, 285]. AS is principally a presynaptic protein, which may act as a molecular chaperone in folding and unfolding of synaptic proteins critical for neurotransmitter release [286, 287].

AS does not have an ordered structure, and remains in an unfolded state under normal physiological conditions. Animal studies have suggested that aggregation of AS can be induced by increased concentration, or by inhibition of mitochondria function and proteolytic activity of the proteasome [288-300]. Impairment of proteasomal and mitochondrial function leads to generation of reactive oxygen species (ROS), which leads to detrimental oxidative stress in cells. Indeed, several studies have shown a direct correlation between oxidative stress and formation of AS aggregates and inclusions [291-295]. Environmental toxins (e.g., paraquat, rotenone, and MPTP) that generate reactive intermediates are also believed to be risk factors for PD, apparently by damaging the mitochondrial transport chain, inducing AS aggregation [296].

Oxidative metabolism of dopamine itself also generates ROS (superoxide and hydrogen peroxide) in nigral neurons that may contribute to subsequent neurodegeneration [296, 297]. Recent observation have provided evidence that endogenous dopamine and AS work in synergy to induce selective degeneration of dopaminergic neurons. One hypothesis is that AS leads to intracellular release of dopamine through disruption of vesicular dopamine storage, or permeabilization of the cell membrane resulting in sequestration of exogenous dopamine, which is also toxic to cells [298-301].

Genetic mutations that have been linked to early and late onset of reported cases of PD are mainly found in genes that are involved in protein aggregation, stress-response, mitochondrial function, or the ubiquitin- proteasome system [302]. In fact, discovery of mutations in the gene coding for AS (SNCA) in rare cases of familial forms of PD led to the discovery of AS as the principal component of LBs [303]. The parkin and ubiquitin carboxyterminal hydrolase L1 (UCH-L1) genes are examples of mutated genes contributing to dysfunction in the ubiquitin–proteasome pathway [304, 305], whereas mutations in PINK1and HtrA2 genes affect mitochondrial function [306]. Each of these mutations can predispose patients to developing PD.

Currently, there is neither a cure for PD, nor any treatment which can arrest disease progression. Highly individualized medication treatment regimens that are available today can reduce disability by improving motor function, and help the patient to cope with symptoms. Levodopa or L-dopa (Larodopa, Dopar), is the drug most commonly used for treatment of PD, and was discovered as a result of studies by the Swedish scientist Arvid Carlsson, who identified the neurotransmitter properties of dopamine [307]. Unlike dopamine, levodopa can cross the blood brain barrier (BBB), and is converted to dopamine in the brain. Levodopa is often combined with a dopa-decarboxylase inhibitor, carbidopa (Sinemet), in order to inhibit peripheral metabolism of levodopa before in reaches the brain. In addition to L-DOPA, COMT inhibitors, MAO-B inhibitors, dopamine agonists, and anticholinergics are also used to alleviate the symptoms of PD.

Although L-DOPA is remarkably effective in treating PD, its effect diminishes over time in most patients. Long-term L-DOPA therapy is also associated with numerous adverse effects and motor complications including abnormal involuntary dyskinetic movements and psychiatric complications [308-311]. The undesired motor dyskinesias have been proposed to be due to DA receptor supersensitization [312-316]. Another theory is that the loss or insufficiency of the L-dopa converting enzyme, AADC, is responsible for the L-dopa induced involuntary movements [317-319]. Adeno-associated virus (AAV)-mediated AADC gene delivery into primates with PD has shown restoration of AADC activity [319, 320], and currently this gene therapy approach is being tested in clinical trials [321]. In addition to AADC, striatal gene transfer of the trophic factor nurturin is also being assessed in Phase II clinical trials.

The subthalamic nucleus (STN) plays a key role in the circuitry of the basal ganglia and in the pathophysiology of Parkinson's disease. In patients with PD, activity of the STN is increased mainly because of reduced GABAergic input from the globus pallidus [322, 323]. There is also evidence that disinhibition of the STN signaling in the basal ganglia is involved in L-DOPA-induced dyskinesia [324, 325]. In some cases, surgical interventions including lesions of the thalamus (thalamotomy) or, more recently, deep brain stimulation of the STN are effective in counteracting Parkinsonian symptoms, resulting in reduced need for L-DOPA and in turn reduction in the development of dyskinesia [326-330].

The activity of the STN can also be normalized by gene delivery of GAD, which is a key enzyme in synthesis of the inhibitory neurotransmitter, γ-aminobutyric acid (GABA). In hemiparkinsonian rats, transfer of GAD via AAV vectors has led to progressive motor improvement [331]. These observations provided the theoretical support for clinical studies of GAD gene transfer that are currently in progress [320, 332, 333].

5.1 Transplantation of fetal tissue in treatment of PD

Starting in 1979 and the 1980s, preclinical studies demonstrated that implanted fetal mesencephalic DA neurons could survive, reinnervate the striatum, manifest normal electrophysiological activity, and improve motor function in parkinsonian model rodents and primates [334-338]. On the other hand, since animals do not develop PD naturally, the parkinsonian symptoms were mimicked by destroying dopamine-producing cells in the SN with neurotoxins such as 6-hydroxydopamine (6-OHDA) or MPTP. Therefore, concerns were raised regarding uncertainties associated with animal models matching the exact symptoms of the human condition and whether the same effect could be reproduced in human patients with PD [339]. In addition, the efficacy of fetal transplantation procedure was limited in several respects, raising doubts concerning whether these procedures could be translated directly to human subjects [340].

In 1981, two young PD patients received adrenal medulla autografts in the caudate nucleus. This procedure was the first attempted cell-based therapy in treatment of PD [341]. The first trials of fetal nigral dopamine neurons transplantation occurred a few years later. In 1990, a Swedish group, led by Dr. Olle Lindvall, and Dr. Curt Freed's group at the University of Colorado in Denver reported moderate clinical improvement in single PD patients that received intrastriatal fetal mesencephalic cell transplants (7- 9 weeks gestational age) [342, 343]. Subsequent follow-up studies have documented long-term improvements in a few individual patients [344].

Based on these encouraging observations, numerous clinical and animal studies have been carried out to improve the survival of grafted cells in the human brain, and produce sustained symptomatic relief in idiopathic PD patients [345-351]. Nonetheless, results of double-blind controlled trials have been somewhat disappointing. The largest clinical study, reported in 2001 by Dr. Curt Freed [352], included 40 patients with advanced Parkinson's disease. Half of the patients received mesencephalic tissue transplants into the putamen bilaterally, while the rest underwent sham surgery. Improvement in motor coordination for at least one year after surgery was observed in 9 patients that received cell transplantation therapy, whereas the remaining 11 patients and the patients who had the placebo surgery showed little or no improvements of their symptoms. An important observation in this study was that patients that obtained a benefit from the transplantation

therapy were primarily among the younger group, which was under the age of 60. Another double-blind, placebo-controlled trial showed robust survival of dopamine neurons and increased dopamine in the grafted areas, but the neurochemical changes were accompanied by severe cases of dyskinesia in 56% of the treated patients [352]. Development of dyskinesia had also been previously observed in a subset of patients with embryonic dopaminergic tissue implants [352-355].

The variable outcomes from clinical studies of fetal DA neuron transplantation for PD are a clear indication that cell transplantation strategies for treatment of PD are at an experimental stage. Although complete restoration of the nigrostriatal pathway has not yet been achieved by current grafting strategies, evidence for survival and integration of fetal dopaminergic grafts is of high value, suggesting potential future efficacy. Our knowledge today illustrates that successful cell transplantation may not be achievable simply by transferring dopamine-producing cells to PD patient's brains. Possible improvements might involve refinement of surgical procedures, or the use of neurotrophic factors to enhance the therapeutic potential of cell transplantation in the future. In order to enhance survival and functional integration of the grafts and to avoid dyskinesias, it may also be essential to develop technologies to engineer the microenvironment so that grafted cells can be controlled following implantation. Nonetheless, assessment of the final efficacy of any neurotransplantation approach that is developed will require clinical trials, which face the obstacle of limited availability of human fetal tissue [356].

5.2 Transplantation of ESC-derived cells in animal models of PD

Stem cells have been proposed as ultimately better candidates for cell replacement therapy in PD because they have an unlimited life span and can be propagated and genetically engineered *in vitro*. Recent studies suggest that hESC-derived mesencephalic NPC and DA neurons can survive *in vivo*, but their functional integration in animal models of PD is still far from optimal. This is not surprising considering the differences in chemical and mechanical cues provided in various culturing procedures versus the actual *in vivo* environment. In addition, other major barriers that currently prevent the progression of hESC based therapies towards clinical use are the genetic instability of hESC, the possibility of teratoma formation, and immune rejection [357, 358].

Although new technologies are emerging to improve continuous control in regards to genetic and epigenetic normality of the cells, it is essential to derive new hESC in order to prevent prolonged cultures of individual hESC lines that inevitably will lead to genetic abnormalities. As previously discussed, strategies such as cell sorting, using specific cell surface markers, and incorporation of lineage-specific selection markers by genetic manipulation have been developed in effort to produce homogenous cell populations and eliminate the presence of hESC and precursors, which can potentially form teratomas. Prolonged pre-differentiation of hESC *in vitro* will naturally decrease the presence of undifferentiated cells; however, this also results in development of neuronal cells into a more mature state, which may not be optimal for survival upon grafting [359].

In most cases, the transplantation of cells or organs results in generation of a immune response that frequently leads to graft rejection. This response is directed towards the cell surface major histocompatibility complex antigens, which are encoded by the human leukocyte antigen genes. The human leukocyte gene sequences are highly variable and it is extremely difficult to obtain donors with precise matches. Currently, this rejection response is controlled by the administration of immunosuppressive drugs that are accompanied by serious side effects associated with suppression of the entire immune system. Grafts of neuronal tissue to the brain are generally at least somewhat

less susceptible to rejection, as compared to peripheral organ grafts, but this protection, termed ˝ immunological privilege˝ is not absolute [360, 340]. Some clinical trials of fetal tissue transplantation have obtained graft survival without immunosuppressive treatment [351].

Various approaches by which the immune response against transplanted hESC-derived cells could be reduced or eliminated are currently under investigation. Examples of such methods are generation of isogenic hESC lines by somatic nuclear transfer to an enucleated oocyte, from which hESC lines can be derived, and initiation of hematopoietic chimerism. The latter strategy is based on conditioning the patient's immune response to foreign hESC-derived cells by introducing hematopoietic cells from the same hESC line used to generate the cell type of interest prior to transplantation [360].

Transplantation of DA precursors or neuronal cells is still at the stage where survival and integration needs to be optimized. Numerous studies have demonstrated that transplantation of hESC-derived DA cells can induce reversal of biochemical and locomotor deficits produced by lesions in the nigrostriatal system in both rodents and primates. On the other hand, there are several reports that have failed to demonstrate any significant survival of dopaminergic neurons in grafts derived from hESC.

In one study, the hESC of the BG01 line were differentiated on PA6 cells for 8 days or 22 days, and then grafted into the striatum of rats that had received unilateral lesions with 6-OHDA. Five weeks after transplantation, only small numbers of TH+ cells were found in the grafted cell population [194]. In another study, where neuronal cells derived from the hESC lines HSF-6 and SNU-hES-3, by co-culture with PA6/PA6-SHH cells for 14 days, were transplanted into the striatum of hemiparkinsonian rats, no TH+ neurons were detected in the grafts. Approximately $5x10^5$ cells were transplanted in the form of single cells or cell aggregates, and cell survival was assessed two weeks after cell transplantation. Although TH+ neurons were not present in the grafts, this group illustrated that implantation of cell aggregates in contrast to single cells resulted in enhanced overall cell survival [195]. In a somewhat different SDIA-mediated differentiation paradigm, H7 and H9 hESCs were cultured on the mouse stromal cell line MS5-Wnt with noggin for three weeks and then grown in feeder-free conditions with factors supporting DA cell development. At day 42 of differentiation, $10x10^4$ cells were implanted into the striatum of 6-OHDA lesioned rats and the animals were sacrificed for immunohistochemical analysis after 12 weeks. Interestingly, the group of animals that received H7 hESC-derived cells showed behavioral improvement, whereas motor function was completely unaltered in the group of rats that received differentiated cells from H9 hESC. The number of surviving TH+ neurons were small in the majority of animals used in this study, and large teratoma formation occurred in two animals. In one animal where 560 TH+ neurons were detected, 94% behavioral recovery was observed [154].

Brederlau and coworkers [361] investigated the survival and differentiation of hESC that were induced by SDIA for various durations, in a rat model of PD. Differentiated hESC of line SA002.5 that had been grown on PA6 cells for 16, 20, or 23 days were enyzmatically isolated and dissociated into single cell suspensions. At each time point, the right striatum of each rat was implanted with $10x10^4$ cells. Cells survival assessment, performed two weeks after transplantation in four animals from each group, revealed that hESC cultured on PA6 cells for 16 or 23 days did not survive the grafting procedure. Thirteen weeks after engraftment, all animals that received cells after 16 days of differentiation, and 50% of animals with cells from 20 days of *in vitro* culture died because of teratoma formation. Disregarding animals that developed teratomas, only a small number TH+ cells

were found in the grafts (10-50/graft), and there was no significant difference between the two groups [361].

In addition, Martinat and colleagues [367] reported a study in which the H9 hESC were differentiated by co-culture with MS5 stromal cells followed by co-transduction with lentiviral vectors carrying Nurr1 and Pitx3 genes. The combination of Nurr1 and Pitx3 effectively potentiated the maturation of midbrain DA neurons while repressing the generation of non-dopaminergic neurons from hESC *in vitro*. Nonetheless, when the Nurr1/Pitx3 transduced neural precursors generated by EB formation were transplanted into mice that had been unilaterally lesioned with 6-OHDA, the TH+ cells found within the grafts did not mature and failed to extend processes.

These studies indicate that DA precursors or neurons derived from hESC by SDIA-mediated strategies alone do not differentiate or survive well *in vivo* in rodent models of PD. Conversely, when primate ESC-derived NPC, induced by SDIA and treated with FGF20, were transplanted in MPTP-treated monkeys, a substantial number of functional TH+ cells (more than 4000/brain) were found in the grafts, which consequently resulted in stable behavioral improvements [362].This suggests that DA cells derived from hESC by SDIA may require additional factors, including instructive and neurotrophic molecules for survival and development into functional DA cells *in vivo* that may not be provided by the rodent brain environment.

A study by Roy et al. [363] on transplantation of DA progenitors derived from the H1 and H9 hESC lines by EB formation followed by induction with immortalized human fetal midbrain astrocytes in the presence of SHH and FGF8 has provided more encouraging results. Ten weeks after engraftment of DA progenitors (at day 24 of differentiation), about 21% of the total number of transplanted cells (5 x10^5 cells) were TH+ in the rat striatal area. A substantial and long-lasting behavioral recovery was also observed in animals that received cell implants. Notably, the number of TH+ cells was higher in the peripheral area of the graft, as compared to the center of the cell cluster. The authors argued that this effect was due to the inducing effect of the host's surrounding midbrain astrocytes. Further evidence of this theory was provided by increased number of TH+ cells in rats with fewer engrafted cells, possibly because of a greater proximity and exposure to host astrocytes [363].

A recent study by Yang et al. [364] has shown that a hESC-derived population of DA cells, treated with a number of factors with DA inducing or mitogenic effects, can survive for prolonged periods (five months) after transplantation, and reverse the behavioral deficits seen in a parkinsonian rat model. Spontaneous neural induction was initiated in H9 hESC colonies in adherent cultures, followed by exposure to SHH and FGF8, and treatment with AA, B27, cAMP, TGFβ3, GDNF, and BDNF. In addition, the DA precursors differentiated for four weeks including two-week treatment with FGF8 and SHH were expanded in the presence of Wnt3a for 20 days prior to further differentiation and transplantation. Four weeks following transplantation, from the 2 x10^5 cells that were transplanted, an average of 361 TH-expressing neurons was detected per graft. This number increased to an average of 1,273 TH+ neurons per graft after an additional four months *in vivo* [364].

Transplantation of enriched uncommitted NPC derived from the HES-1 cell line without the use of any external dopaminergic inducing signaling has provided some interesting results [365]. These NPC were obtained by culturing hESC on MEF feeders for prolonged periods in the presence of noggin followed by mechanical isolation. When NPC that were expanded for 6 weeks as neurospheres were grafted into striatum of rats, 102–630 TH+ neurons per brain were identified

(~0.2% of the total cells transplanted), 12 weeks after transplantation. There was also a close correlation between the number of TH+ neurons present in the grafts and the degree of amelioration of behavioral deficits [365]. This indicates that NPC may have an intrinsic mechanism which allows a fraction of the cells to differentiate to DA neurons without any additional manipulation. There is also a possibility that the neurospheres were influenced to differentiate into TH+ neurons by the midbrain microenvironment.

In conclusion, the highly variable outcomes of the various transplantation studies of hESC-derived DA cells is likely to reside in the utilization of different differentiation paradigms, the nature of the hESC line, cell preparation techniques, as well as the level of maturity of cells at the time of transplantation. A greater understanding of the molecular mechanisms that control DA neuron differentiation, as well as their functional integration *in vivo*, is likely to ultimately contribute to the development of novel hESC-based replacement therapies for PD.

Present investigations

6. Results and conclusions

6.1 SDIA-mediated differentiation of hESC

In this study midbrain-restricted NPC were generated from the hESC line BG01V2 by a co-culturing technique utilizing SDIA activity. The BG01V2 line is a variant of the normal hESC line BG01. The BG01 cells retained a normal karyotype of 46, XY for 44 passages, but subsequently converted to trisomy 17. The BG01V2 cell line has shown an enhanced ability to generate DA neurons in the PA6-hESC culture system, as compared to several other karyotypically normal hESC lines. We periodically performed karyotype analysis to monitor the genetic integrity of the hESC in continuous culture. The BG01V2 cell line sustained a consistent trisomy 17 until at least passage 80, with no development of additional karyotype abnormalities. Experiments described in this report were carried out using passages 71-80 of the BG01V2 line. Description of the SDIA-induced neural and DA induction of hESC is provided below.

Differentiation of hESC in co-culture with PA6 cells

Small clusters of hESC were seeded on layers of PA6 cells (Fig. 2A). Shortly thereafter, the clusters attached and formed colonies. The colonies increased in size rapidly during the first few days (Fig 2B). The morphology of the cells composing the colonies changed from a compact stem cell-like shape to a more elongated shape over the course of a few days, as the cells differentiated towards an ectodermal lineage. The hESC were co-cultured with PA6 cells for 10-12 days (Fig 2C). At this time, the neural precursor markers nestin and PSA-NCAM were expressed in the majority of colonies (Fig 2 D-E). A subset of cells within some colonies was positive for the undifferentiated ESC marker Oct3/4, whereas the majority of colonies contained TH+ neurons (Fig. 2F). This clearly indicates the presence of cells in various stages of differentiation in co-cultures.

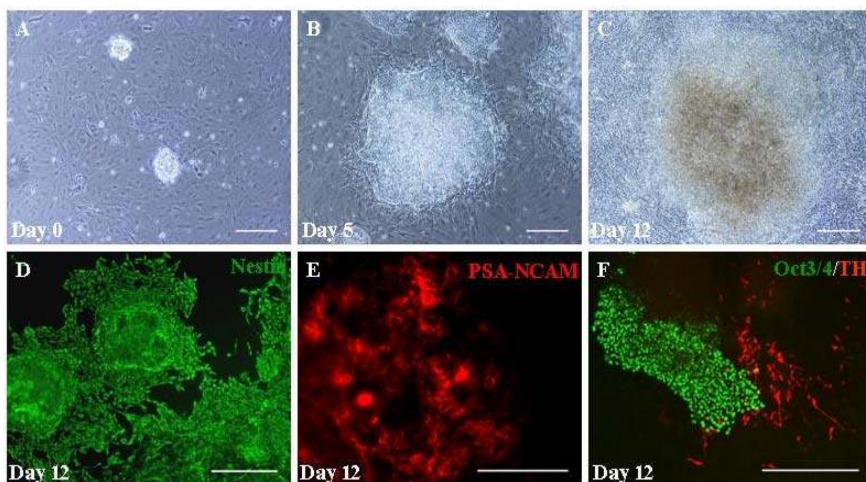

Figure 2. Dopaminergic induction of BG01V2 hESC line by the SDIA method. **(A)** Phase-contrast image of small clusters of BG01V2 seeded on a layer of confluent PA6 stromal cells. A colony after **(B)** 5 days and **(C)** 12 days of co-culture. Immunofluorescence analysis ofexpression of **(D)** nestin **(E)** PSA-NCAM and **(F)** Oct3/4 and TH after 12 days of co-culturewith PA6 cells. Expression of nestin, PSA-NCAM and TH was detected in majority of colonies at days 12 of co-culture. Expression of ESC marker Oct3/4 was detected in approximately 40% of colonies, although significantly reduced within each colony.Scale bars=200 µm

Differentiation of isolated NPC to dopaminergic neurons

After isolation from PA6 cells, NPC colonies were dissociated into small cell clusters and plated onto poly-L-lysine/laminin-coated dishes in the presence of SHH, FGF8 and GDNF (Fig. 3A). Under these conditions, the isolated cells attached to the plates and formed rosette-like structures after 3-5 days (Fig. 3B). Neuronal cell morphology could be observed in the center of the rosettes (Fig. 3C). As the differentiation of the NPC progressed, increased numbers of cells with neuronal morphology developed and occupied a larger fraction of the rosettes (Fig 3D). The differentiated rosettes were stained for the dopaminergic marker TH. After 18-20 days, approximately 34% of the cells were TH+ (Fig. 3E, F).

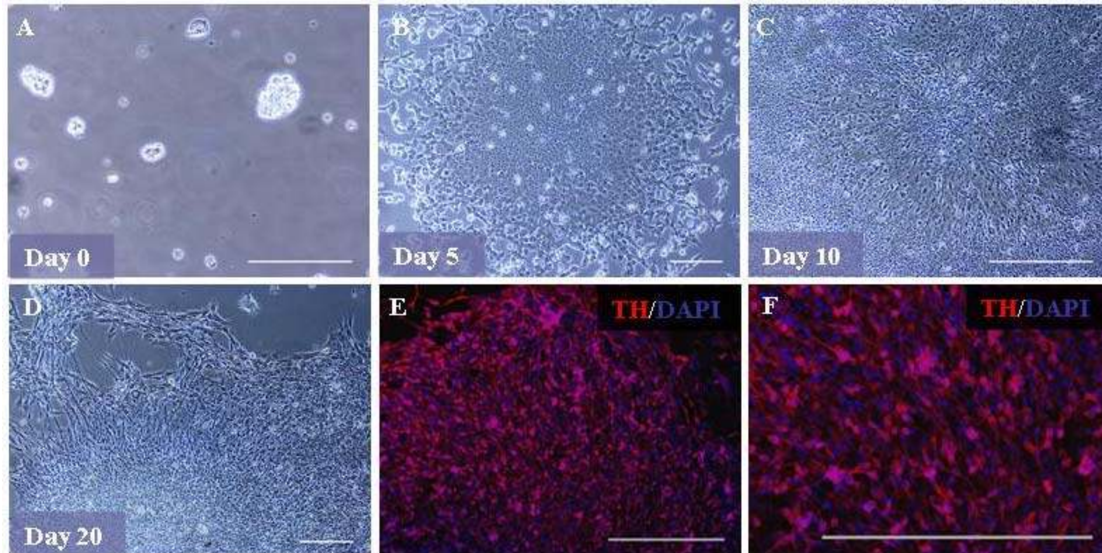

Figure 3. Differentiation of isolated cells. (A-D) Phase contrast images of cells isolated from PA6 cells after 12 days of co-culture. **(A)** Small clusters of partially differentiated BG01V2 cells were plated on poly-L-lysine/laminin-coated dishes. The cluster of cells attached to the plates and formed colonies within 48 hrs. **(B)** A colony after 5 days of differentiation, containing cells with typical neuroectodermal morphology. **(C)** After 10 days the neuroectodermal cells differentiated into cells with neuronal morphology in the center of the colonies. **(D)** By day 20, neuron-like cells migrated from the center to the periphery of colonies. **(E, F)** A colony differentiated for 20 days, immunostained for TH (red) and nuclear counterstaining with DAPI (blue). Scale bars=200 µm

Immunocytochemical identification of cell types in hESC-derived cultures

Approximately 46% of cells exhibited a neuronal morphology and expressed MAP2 (Fig. 4A). Double staining with TH and MAP2 revealed that about 80% of the MAP2+ cells co-expressed TH (Fig 4B, C). A large fraction of the rosettes expressed GFAP, consistent with differentiation into astroglia (Fig. 4D, E). In contrast, only a small number of cells (<1%) differentiated into GalC+ cells (Fig. 4F). To investigate differentiation of hESC towards non-dopaminergic neuronal phenotypes, we stained for dopamine DBH, choline acetyltransferase, glutamate decarboxylase (GAD), serotonin (5-HT), or GABA. Less than 1% of cells stained positive for GABA (Fig. 4G), while expression of the other neurotransmitter markers was absent. GABA and TH were never detected in the same cells by double staining.

Our previous investigations demonstrated α-smooth muscle actin (ASMA) positive cells of mesodermal origin in brain grafts from transplanted hESC after differentiation in PA6 co-cultures [194]. Therefore, we investigated the potential of hESC to differentiate into cells of mesodermal lineage by using an antibody against ASMA. The immunocytochemical analysis indicated that hESC generated a small subpopulation (<5%) of smooth muscle cells in vitro, as judged by expression of ASMA and morphology (Fig. 4H). An interesting observation regarding the population of ASMA+ cells was their segregation from the cells of ectodermal origin. As shown in Figure 4I, the ASMA+ cells were situated outside the borders of rosettes or colonies containing a large number of TH+ neurons.

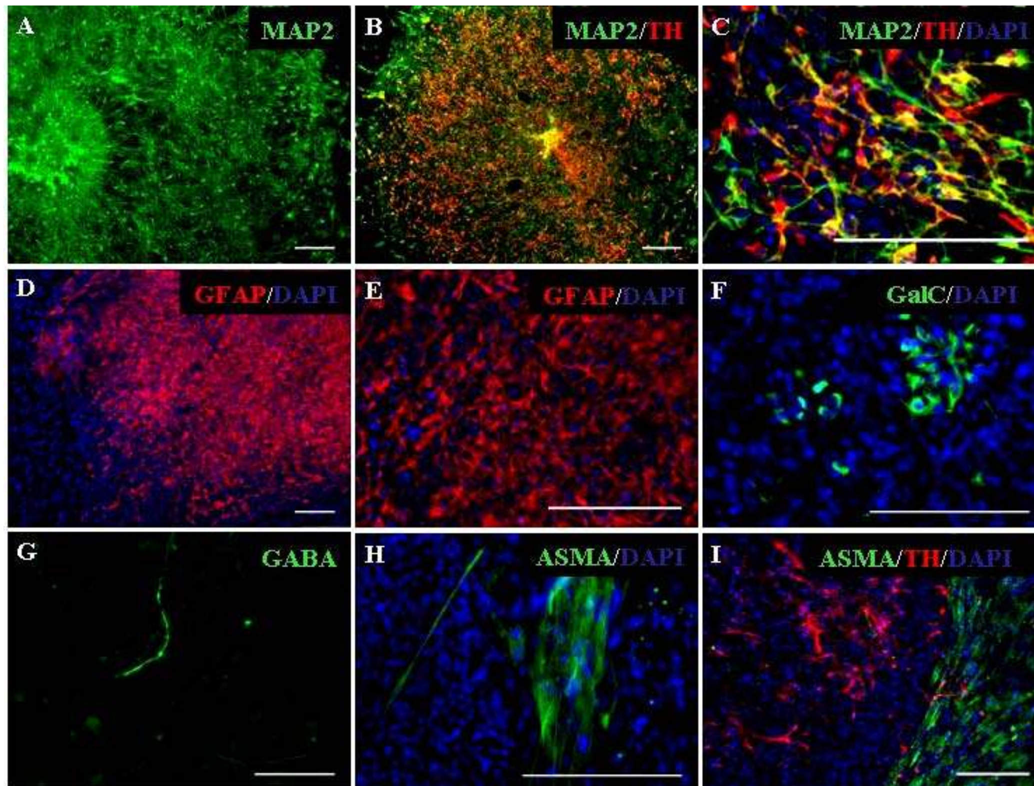

Figure 4. Cellular composition of differentiated cultures in feeder-free conditions. Marker expression in cultures after 19 additional days of differentiation in the presence of SHH, FGF8 and GDNF, following removal from the PA6 cell feeder layer. **(A)** Expression of the neuronal marker MAP2. **(B, C)** Co-expression of MAP2 and TH. Nuclei are counterstained with DAPI. **(D, E)** The glial marker GFAP **(F)** the oligodendrocytic marker GalC. GalC+ cells comprised less than 1% of the cell population. **(G)** Expression of GABA was seen in a small number of cells. **(H)** Expression of the mesodermal marker ASMA was detected in a subpopulation of cells, but **(I)** was rare in colonies containing large numbers of TH+ cells and was rarely seen in close proximity to TH+ cells. Scale bars=200 μm

6.2 Characterization of NPC and DA neurons derived by SDIA *(Paper I)*

In order to ascertain that NPC derived from the BG01V2 line by SDIA had a midbrain specific nature, we investigated the expression of key transcription factors involved in early specification of ventral mesencephalic precursors including Pax2, Pax5, Otx2 and Msx-1. Following enzymatic

isolation, as shown in Figure 5A and B, expression of Pax5, Otx2 and Msx1 was detected in the majority of cells within colonies.

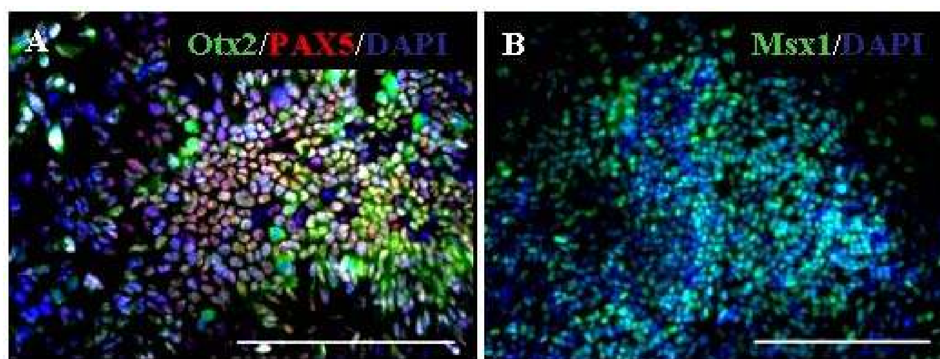

Figure 5. Expression of midbrain specific markers by isolated neural progenitor cells. Cells isolated from PA6 cell layers were differentiated for 7 days and immunostained with antibodies against **(A)** Otx2 and Pax5 and **(B)** Msx1. Scale bars=200 µm

Next, we investigated *in vitro* proliferation and differentiation of these NPC using a BrDU incorporation assay, and monitored the cell population and viability of TH+ neurons over an extended period of time. After isolation from feeders, the cultures were analyzed for BrDU incorporation, and stained with antibodies against BrDU and TH every three days. At day three, the majority of cells (85 ± 2%) were proliferating, while a small percentage of cells (4 ± 1%) had began to acquire a dopaminergic phenotype, as indicated by expression of TH. BrDU incorporation showed a gradual decline over time, while the number of TH+ neurons reached a maximum of 17 ± 3% of the total cells at day 18. Thirty days after isolation from the feeder cells cell division, as indicated by BrDU incorporation, ceased in 91 ± 0.4 % of cells. The absence of BrDU in TH+ cells at all stages of differentiation also showed that the dopaminergic neurons produced from the BG01V2 line were postmitotic. The relationship between the percentages of proliferating BrDU+ cells and TH+ neurons during 30 days of culture is presented in line graphs in Figure 6 at the indicated time points.

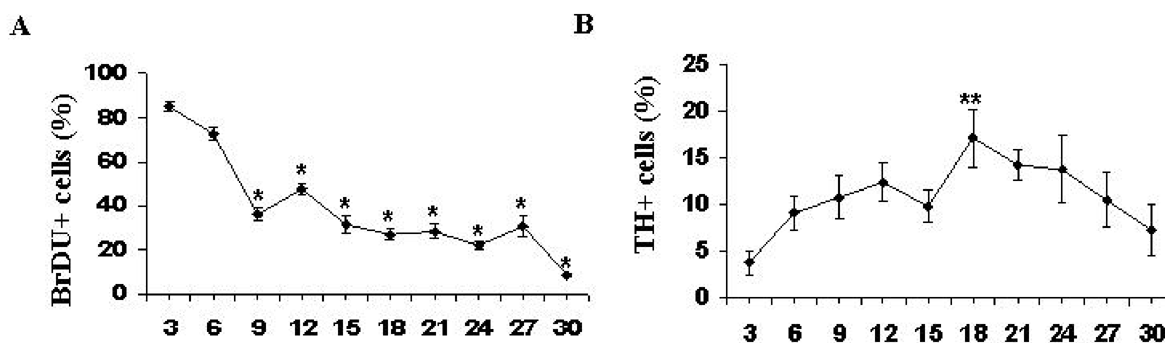

Figure 6. Examination of proliferation capacity of isolated neural progenitor cells and viability of TH+ neurons derived from hESC. Line graph illustrating the relationship between the mean values of percentages of **(A)** BrDU-labeled and **(B)** TH+ cells as a function of time (means ± SEM). The overall effect of time was statistically significant for the BrDU+ cells ($P < 0.0001$) and TH+ neurons ($P = 0.0287$). In addition, percentages of BrDU+ cells at days 9-30 versus day 3 were significantly different ($P < 0.001$). The percentage of TH+ neurons was significantly different ($P < 0.05$) at day 18 versus day 3. *=$P<0.001$, **=$P<0.05$

In addition, long-term monitoring of cultures was carried out to search for tumor cells with uncontrolled cell growth that may have arisen from the karyotypically-abnormal BG01V2 cell line. In cultures maintained for up to 49 days, there was no sign of transition of any cell type to tumor-like colonies with uncontrolled growth and cell viability was progressively decreasing at this time.

After 18 days of differentiation, the majority of neuronal cells in the cultures expressed the dopamine transporter DAT, which is exclusively found in dopamine neurons (fig. 7A). The DA neurons also exhibited functional DAT activity, evidenced by a concentration-dependent displacement of the binding of the radiolabeled DAT specific inhibitor [^{3}H] WIN 35428 by the unlabeled WIN 35428 (Fig. 7B). The concentration of unlabeled ligand that displaced half of the radioligand (IC_{50}) was 7.82 ± 0.56 µM.

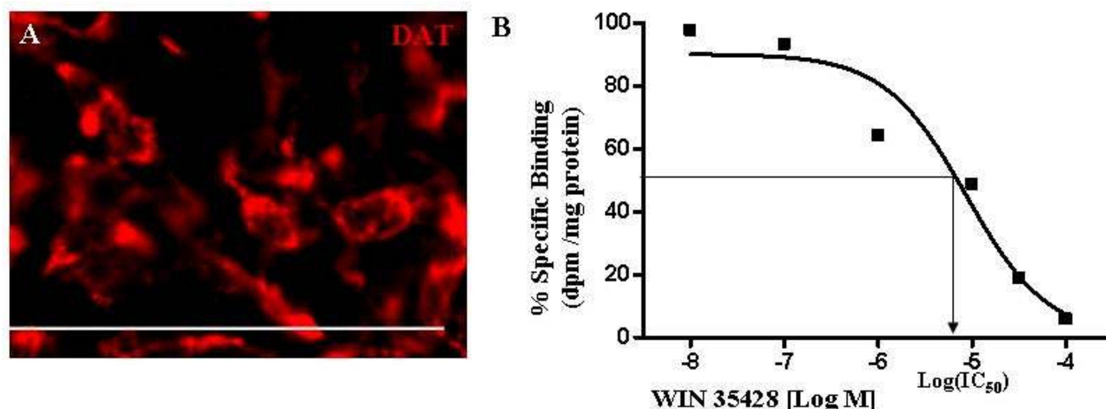

Figure 7. Assessment of dopamine transporter (DAT) function and cell surface expression. (A) Illustration of dopamine transporter (DAT) expression, mainly seen in plasma membranes, in terminally differentiated cultures by immunocytochemistry 18 days after isolation. **(B)** Saturation curve for [3H] WIN 35428 illustrating displacement binding inhibition values. The points represent the means of experimental data from three independent experiments. The IC50 was 8.66 uM. Scale bas=200 µm

Electrophysiological investigation of neuronal cells with a fusiform perikarya morphology typical of dopaminergic neurons at day 12 and 25 of differentiation in feeder-free conditions showed that sodium currents contributing to membrane excitability were present at early stages of differentiation (i.e., 12 days after isolation), and appeared to increase in magnitude in cells differentiated for 25 days, indicating progressive neuronal maturation (Figure 8).

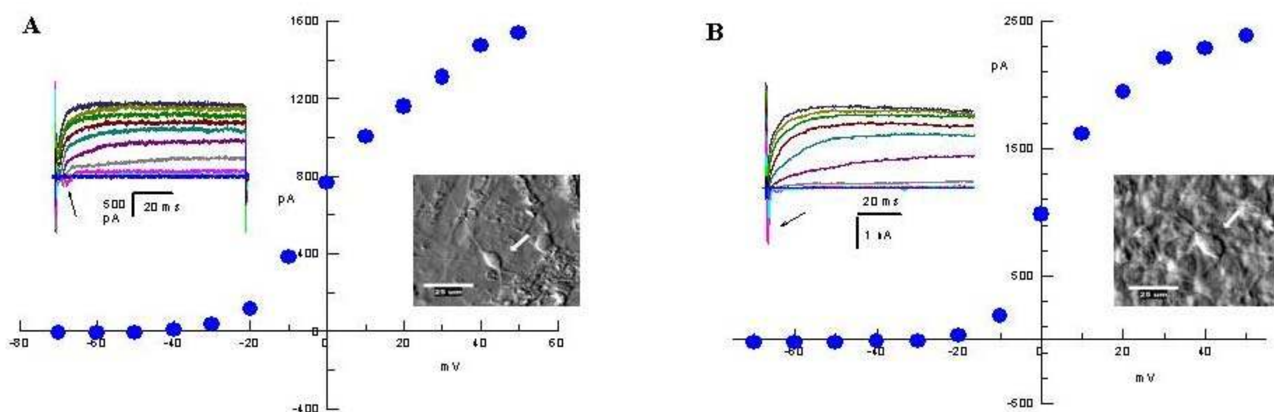

Figure 8. Functional maturation of hESC-derived neurons in feeder free cultures. Examples of whole-cell currents obtained under voltage clamp conditions showing **(A)** weak sodium current with a clear delayed rectifier current in a relatively immature neuron at day 12, and **(B)** increased sodium currents and stronger outward rectification in a more mature neuron at day 25, as indicated by arrows. Cells were held at –60 mV and stepped in 10 mV increments for 100 ms from –70 to +50 mV. The cell from which the data presented were recorded is shown under Hoffman contrast as an inset to its current-voltage plot for each condition. Scale bars=25 µm

In summary, NPC derived from the BG01V2 hESC line are midbrain-specific DA precursors, as indicated by the expression of midbrain specific marker genes Otx2, Pax5, Msx-1. In addition, data obtained from analysis of neuronal excitability and dopamine transporter function of the DA neurons derived from these NPC signified that these cells share many essential biological characteristics with normal DA neurons, allowing them to be used in models for the advancement of cell replacement therapy, as well as for *in vitro* studies of molecular mechanisms controlling development and functions of these neurons.

6.3 Proliferation of SDIA-derived mesencepahlic NPC *(Paper II)*

In order to assess the proliferation capacity and maintenance of cellular fate associated with DA differentiation in NPC generated by the SDIA approach, isolated NPC from PA6 cells were expanded in the form of neurospheres for up to six weeks in the presence of SHH, FGF8 and bFGF. We assessed the lineage restriction of differentiating hESC in co-culture with PA6 stromal cells and determined that the highest yield of undifferentiated mesencephalic restricted NPC could be obtained from co-cultures after seven days of induction. NPC were enyzmatically isolated from co-cultures and propagated as aggregates or neurospheres in suspension culture for six weeks. Neurospheres were passaged weekly, and a portion of the passaged cell population was allowed to differentiate in adherent cultures in the presence of SHH, FGF8, and GDNF for 14 days.

Cell proliferation was examined by quantifying the number of cells in culture directly after isolation from PA6 cells, and prior to passaging each week. As illustrated in Figure 8, the number of cells increased by more than two-fold after seven days and continued to increase up to three weeks (total of five-fold increase in cell number). From the beginning of the fourth week, cell division ceased in the majority of neurospheres, and the number of cells in cultures progressively declined (Fig. 9).

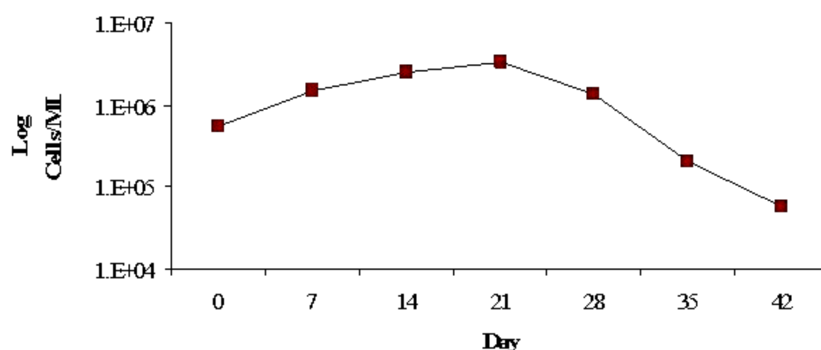

Figure 9. Effect of time on proliferative capacity of SDIA-derived neurospheres. Representation of the decline in proliferation rate of the neurosphere population over time, passaged by mincing with a razor blade showing the log cell number quantified every seven days.

Weekly investigation of DA differentiation ability of expanded NPC for various durations revealed that SDIA- derived NPC could be expanded by five-fold over the course of two weeks, while retaining their DA differentiation potential, but began to lose their ability to differentiate into glial, neuronal and dopaminergic lineages. When NPC were differentiated directly after isolation from PA6 layers, MAP2+ and TH+ cells comprised 27 ± 4 % and 38 ± 5 % of the total cell population, respectively. The GFAP+ glial population accounted for 46 ± 18% of the total cells in cultures. One week of expansion of the NPC resulted in 23 ± 4% TH+, 35 ± 1 % MAP2+, and 29 ± 4% GFAP+ cells after 14 days of differentiation. The percentages of TH+ and MAP2+ neurons did not change in cultures derived from secondary neurospheres (two weeks of expansion), whereas the percentage of GFAP+ glial cells increased to 35 ± 11. Cultures differentiated from tertiary neurospheres (three weeks of expansion) contained 50% fewer TH+ neurons (12 ± 1%), whereas the MAP2+ neuronal cells accounted for the same percentage of the total cell population as compared to cultures from 1 and 2 weeks of expansion. In tertiary neurospheres, the number of GFAP+ cells, as compared to cultures expanded for shorter time periods did not decrease, comprising 39 ± 11% of the total cell population. At passage four, there was a substantial decrease in the percentages of TH+ and MAP2+ cells representing 1 ± 1 % and 9 ± 5 % of the total number of cells. In these cultures, GFAP+ cells accounted for 16 ± 1% of the total cell population. Percentages of cells expressing TH, MAP2, and GFAP in differentiated cultures following each passage are shown in Figure 10.

Tertiary neurospheres exhibited enhanced GABAergic differentiation as compared to cultures derived from NPC after two weeks of expansion. Cultures processed from NPC cultured for one and two weeks contained about 200 GABA immunoreactive neurons (total per well), whereas neurospheres grown for three weeks gave rise to more than 200 GABA+ neurons, comprising approximately 1% of the total cell population. GABAergic neurons were completely absent in cultures differentiated from quaternary neurospheres (four weeks of expansion). Attempts in differentiating neurospheres beyond four weeks of expansion were in most cases unproductive, as survival of cells during differentiation was very limited.

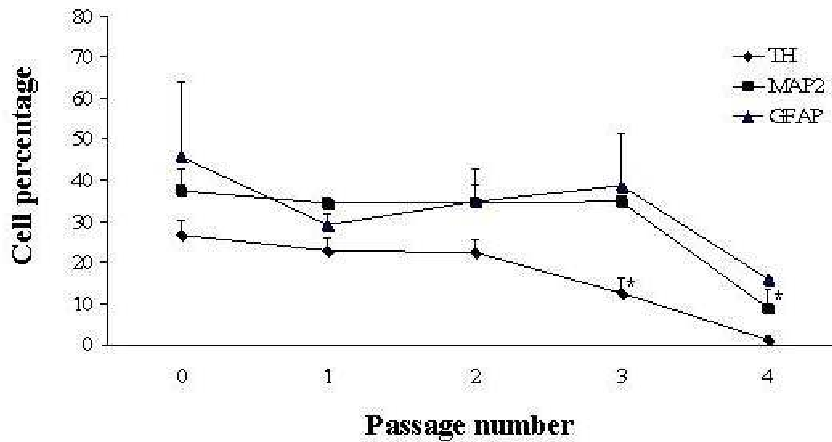

Figure 10. The effect of time on cellular fate. Line graft representation of percentages of dopaminergic (TH+), neuronal (MAP2+) and glial (GFAP+)cells in differentiated cultures following each passage. The percentages of TH+ cells at passage three and MAP2+ cells at passage four were significantly different ($P < 0.05$) as compared to direct differentiation following isolation from PA6 feeders (Passage number 0). *=$P<0.05$.

To investigate cell survival and behavior of the SDIA-derived neurospheres *in vivo* , NPC were expanded for three days after isolation from PA6 feeder cells and then transplanted to the striatum of rats unilaterally lesioned with 6-OHDA. Preliminary data, obtained from immunocytochemical studies three weeks after transplantation, showed that the majority of the grafted cells expressing human specific-nuclei antigen survived in the striatum and continued to express the mesencephalic progenitor markers Msx1 and Otx2. At this time, the midbrain neural progenitor cells had not differentiated to TH+ neurons in the host striatum.

In conclusion, the NPC could be expanded by five-fold as neurospheres for up to 2 weeks *in vitro* while retaining their DA differentiation potential, without a substantial loss of viability. Although cells were continuously maintained under the influence of the midbrain patterning factors SHH and FGF8, they progressively lost their ability to differentiate to DA neurons and maintain a stable phenotype *in vitro*. Preliminary transplantation studies indicated that NPC expanded as neurospheres for three days were able to maintain their phenotype *in vivo* up to three weeks, but did not differentiate into TH+ neurons at that time.

6.4 Assessment of PA6 cell surface activity and secreted molecules on DA differentiation *(Paper III)*

To gain a better understanding of the nature of SDIA, the specific contribution of various factors secreted from PA6 cells and surface activity of the PA6 cells was examined in differentiation of hESC. Induction of hESC differentiation was carried out by these two elements separately. We first examined whether cell surface activity of fixed PA6 cells was sufficient for neural or DA induction of hESC, as had been previously reported [181] by using fixed PA6 cells. Expression of Oct3/4, β-III-tubulin and TH was examined after 12 days of co-culture. Similar to untreated PA6 cells, inactivated PA6 cells were effective in promoting a decline in Oct3/4 expression. Fixed PA6 cells also behaved similarly to normal PA6 cells in inducing hESC to adopt a neuronal lineage as

measured by expression of β-III-tubulin. Expression of TH, representing the DA-inducing activity of PA6 cells, was most markedly affected by fixation of PA6 cells. On fixed PA6 cells, 18 ± 8% of colonies contained TH+ neurons, as compared to 70 ± 15% of colonies grown on untreated PA6 cells. Other co-culturing conditions using PA6 cells that had been mitotically inactivated by irradiation or mitomycin-c treatment also resulted in a significant reduction of colonies containing TH+ neurons. Fixation, irradiation or mitomycin-c treatment of PA6 cells each had less effect on the neural induction of hESC (Fig. 11).

Next, we investigated whether DA differentiation of hESC could be induced by factors secreted from the PA6 cells by introducing media conditioned by PA6 cells to hESC cultures and monitoring their differentiation. HESC were allowed to differentiate in PA6 conditioned media (CM) or unconditioned media (UCM) for 12 days in the absence of PA6 cells. Survival of colonies was considerably improved by CM, but the neural induction of hESC was not significantly enhanced by the CM. When hESC were grown in the presence of CM, the percentage of colonies positive for β-III-tubulin was 60 ± 13 versus 52 ± 8 in cultures containing UCM. Overall differentiation was also increased in cultures containing UCM, versus cultures under the influence of CM, as indicated by loss of Oct3/4 expression. TH expression was not found in cultures containing UCM, while cultures grown in CM gave rise to TH expression in 12 ± 3% of colonies (Fig. 11).

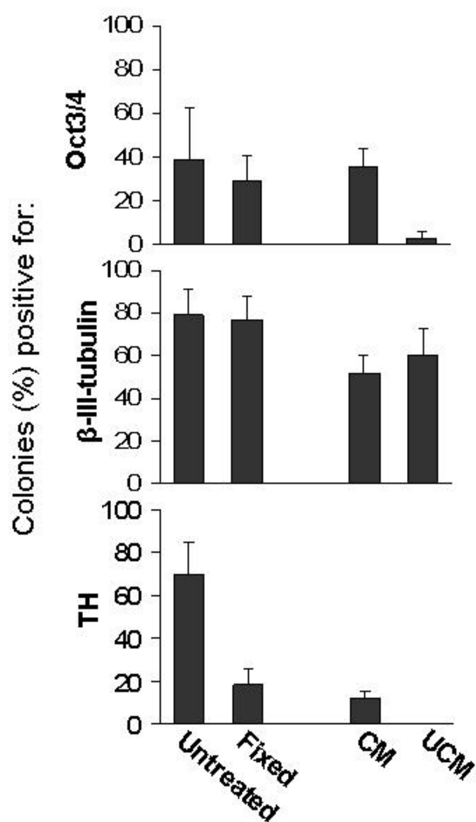

Figure 11. Influence of PA6 cell surface activity and factors secreted from PA6 cells on neural and DA differentiation of hESC. Quantitative analysis of expression of Oct3/4, β-III-tubulin and TH by immunocytochemistry in differentiated colonies cultured on untreated or fixed PA6 cells and in the presence or absence of PA6 cell conditioned media after 12 days of culture. Data represent 60 colonies from three independent experiments. The overall effect of PA6 cell fixation was statistically significant for TH ($P<0.0001$) expressing colonies as compared to the untreated condition. The effect of CM treatment was statistically significant for Oct3/4 ($P<0.0039$), but not for β-III-tubulin or TH. CM; Conditioned media, UCM; unconditioned media.

The overall viability of cells differentiated on inactivated PA6 cells or in the presence of UCM or CM was reduced upon subculture, as compared to cells co-cultured with untreated PA6 cells. As presented in Table 1, NPC that were isolated from co-cultures with inactivated PA6 cells had a

significantly reduced potential to develop into β-III-tubulin+ and MAP2+ neuronal and TH+ dopaminergic cells. Differentiation towards a glial lineage was also reduced by more that 50% in all conditions involving PA6 cell inactivation, as compared to NPC co-cultured with untreated PA6 cells.

hESC co-cultured with	Percent of cells expressing			
	TH	β-III-tub	MAP2	GFAP
Untreated PA6 cells	34 ±6	48±17	46 ±8	44±13
Mit-c treated PA6 cells	8±1	18 ±14	13 ±4	9±5
Fixed PA6 cells	7±2	5±1	12±2	19±13
Irradiated PA6 cells	3±2	11±8	4±2	12±11

Table 1. Quantitative analysis of marker expression in differentiated progenitor cells 18 days after isolation from co-culture with untreated or inactivated PA6 cells. The percentages of cells expressing GFAP, β-III-tubulin, MAP2, and TH (mean ± SD).

Addition of heparin to factors secreted from PA6 cells (CM) increased the percentage of TH+ colonies and number of TH expressing cells within each colony, as compared to cultures with CM alone. After 18 days of differentiation in feeder-free conditions in the presence of CM plus heparin, 54 ± 6% of colonies contained a large number of TH+ neurons, while co-cultures with PA6 cells yielded 81 ± 1% TH+ colonies (Fig. 12).

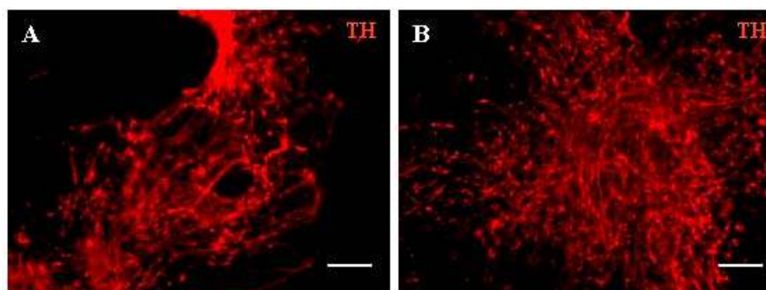

Figure 12. Effects of CM and heparin on dopaminergic induction of hESC after 18 days of culture. Comparison of a colony of hESC cultured in **(A)** CM plus heparin in the absence of PA6 cells to **(B)** co-culture with PA6 cells, illustrating a relatively less effective dopaminergic phenotype-inducing effect as indicated by decreased numbers of TH+ cells within a colony grown in CM and heparin. Scale bars=200 µm

Synergistic effects of PA6 cell surface activity and secreted molecules were also confirmed by increased dopaminergic induction of hESCs cultured on fixed PA6 cells in the presence of PA6 cell CM and heparin.

The current study clarifies the contributions of cell surface and soluble factors to the overall effect of SDIA, by showing that the PA6 cell surface promotes cell survival and cell adhesion, and is responsible for enhanced overall neurogenesis of hESC, rather than providing lineage-specific instructions. The specific dopaminergic-inducing effect appears to reside in soluble factors secreted by PA6 cells. Nevertheless, the presence of cell surface material enhances the survival of hESC during differentiation, thus markedly increasing the overall yield of dopaminergic cells.

6.5 Identification of the molecular nature of SDIA *(Paper IV)*

From the previous study, we concluded that PA6 cell surface activity enhanced cell survival and neurogenesis, whereas secreted factors were primarily responsible for the dopaminergic-inducing effect of SDIA. In attempt to identify soluble factors secreted from PA6 cells with a potential role in the dopaminergic induction of hESC we carried out gene expression profiling of PA6 cells using DNA microarrays, and compared the PA6 gene expression profile to that of a number of mouse cell lines lacking SDIA activity. The cell types used for comparison included MEF, a mouse kidney cell line MM55K, and sub-types of PA6 and MS5 lines that lack dopaminergic inducing activity. The potent PA6 cell line will be referred to as PA6-DA, and PA6 sub-types as PA6-X1 and PA6-X.

First, the lack of SDIA activity was confirmed by co-culturing hESC with these various cell lines for 12 days and examining the cultures for expression of Oct3/4, nestin, β-III-tubulin and TH by immunocytochemistry. Differentiation of hESC in co-culture with cell lines PA6-X1, PA6-X, and MS5 could not be assessed as these cell lines did not support survival of hESC for 12 days of co-culture. Expression of the stem cell marker Oct3/4 was detected in more than half of the colonies grown of MEF, while 82% of colonies cultured on MM55K and PA6-DA contained Oct3/4+ cells. The early neuronal marker nestin was expressed in the majority of colonies in co-culture with MEF, MM55K, and PA6-DA cells. The presence of dopaminergic neurons, identified by expression of TH, was found in 2% of colonies in MEF co-cultures and in less than 1% of colonies grown on MM55K cells. The majority of colonies cultured on PA6-DA cells contained large numbers of TH+ neurons. Percentages of colonies expressing Oct3/4, nestin, β-III-tubulin and TH after 12 days of co-culture are shown in Figure 13.

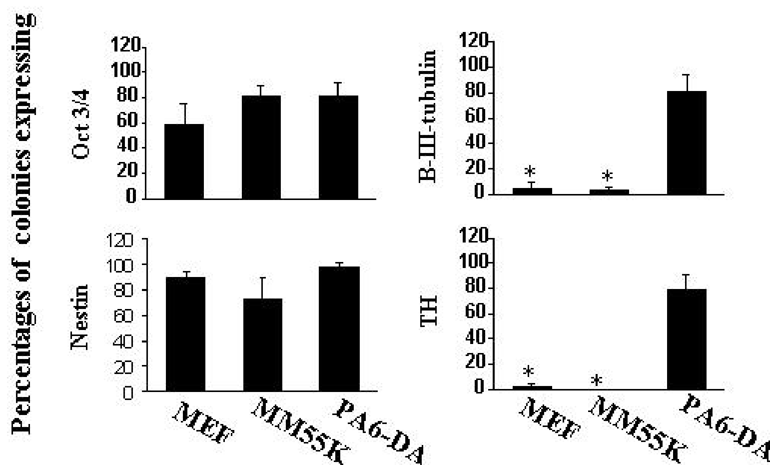

Figure 13. Cell feeder-induced differentiation of hESC. Marker expression analysis of differentiating hESC grown on mouse embryonic feeders (MEF); mouse kidney cell line, MM55K; and PA6 cells with potent SDIA activity was performed after 12 days of co-culture. Quantification results of percentages of colonies expressing Oct3/4, Nestin, β-III-tubulin and TH on MEF, MM55K or PA6-DA feeder cells are presented in bar graphs with error bars indicating standard deviations. Data represent 60 colonies from three independent experiments. The overall difference in TH and β-III- tubulin expressing colonies was statistically significant in conditions with MEF and MM55K cells ($P<0.0001$), as compared to PA6-DA co-cultures.

Gene expression analysis of the PA6-DA cell line, as compared to the five cell lines lacking SDIA activity, PA6-X1, PA6-X, MS5, MEF and MM55K, identified 288 genes preferentially expressed (Z ratio$\geq$ 3.0, $P < 0.05$) in PA6-DA cells as compared to the PA6-X cell subtype. The majority of these genes were also significantly up-regulated in PA6-DA cells as compared to the transformed stromal cell lines, PA6-X1, PA6-X, MS5, and the MM55K and MEF cells. In order to classify genes predominantly involved in biological processes and pathways related to CNS development among the 288 up-regulated genes, the platform gene ontology FatiGO (Fast Assignment and Transference of Information using Gene Ontology) was used to categorize these genes with respect to gene function including biological process (Table 2).

Gene Ontology Biological Process at level 6	Gene	UniGene Accession	Z-ratios	
			PA6-DA/ PA6-X	PA6-DA/ MM55K
Neurogenesis	IDB4	NM_031166.1	3.16	2.57
	EFNB1	NM_010110.2	8.56	7.43
	CXCL12	NM_013655.2	16.27	14.34
	TIMP2	NM_011594.2	3.32	1.66
	THY1	NM_009382.2	9.62	5.18
	MYH10	NM_175260.1	3.15	2.90
	RUNX1	NM_009821.1	4.28	4.71
	NOTCH1	NM_008714.2	3.02	3.99
Central nervous system development	OTX1	NM_011023.2	3.22	2.04
	IDB4	NM_031166.1	3.16	2.57
	CXCL12	NM_013655.2	16.27	14.34
	MYH10	NM_175260.1	3.15	2.90
	NOTCH1	NM_008714.2	3.02	3.99
Tissue development	MGLAP	NM_008597.2	9.42	1.40
	PTN	NM_008973.1	13.50	14.60
	SOX9	NM_011448	11.62	6.33
	OTOR	NM_020595	4.04	4.61
	NOTCH1	NM_008714.2	3.02	3.99
Wnt receptor signaling pathway	SFRP1	NM_013834.1	14.24	14.53
	SFRP2	NM_009144.1	3.16	3.09
	FRZB	NM_011356.2	3.44	2.56
	PPAP2B	NM_080555.1	3.37	7.36
Cellular morphogenesis during differentiation	EFNB1	NM_010110.2	8.56	7.43
	CXCL12	NM_013655.2	16.23	14.34
	THY1	NM_009382.2	9.62	5.18
	NOTCH1	NM_008714.2	3.02	3.99
Neural crest cell differentiation	EFNB1	NM_010110.2	8.56	7.43
	SEMA3F	NM_011349.2	4.66	0.40
	SOX9	NM_011448	11.62	6.33
Regulation of cell migration	CXCL12	NM_013655.2	16.23	14.34
	THY1	NM_009382.2	9.62	5.18
Cell fate specification	SOX9	NM_011448	11.62	6.33
	NOTCH1	NM_008714.2	3.02	3.99
Neural tube development	LTAP	NM_033509.2	3.21	2.18

Table 2. Presentation of gene clusters categorized into biological processes at FatiGO specificity level six with relevance to various aspects of brain development and maintenance of central neurological processes. Expression levels of genes in PA6-DA as compared to PA6-X and MM55K cells are presented as Z-ratios.

Focusing on soluble secretory molecules and gene products with a possible role in CNS development and regulation of neurogenesis, we identified stromal cell-derived factor 1 (SDF-1/CXCL12), pleiotrophin (PTN), insulin-like growth factor 2 (IGF2), insulin-like growth factor binding protein 4 (IGFBP4), and ephrin B1 (EFNB1) as factors potentially responsible for the dopaminergic inducing activity. These five factors were eventually reduced to four, SDF-1, PTN, IGF2, and EFNB1, based on *in vitro* functional analysis. This combination of factors was given the acronym "SPIE".

These genes were among the most up-regulated in PA6-DA as compared to PA6-X or MM55K cells, each having a seven-fold or greater Z-ratio. In addition, differences in these transcripts as determined by RT-PCR reproduced the results of the cDNA microarray for all four genes. Relative expression of these genes in PA6-DA versus each cell type is presented in Table 3.

Genes	UniGene Accession	Z-ratios				
		PA6-DA/ PA6-X	PA6-DA/ PA6-X1	PA6-DA/ MS5	PA6-DA/ MM55K	PA6-DA/ MEF
CXCL12	NM_013655.2	16.27	16.92	15.08	14.34	9.96
EFNB1	NM_010110.2	8.56	9.26	8.40	7.43	7.01
PTN	NM_008973.1	13.50	14.58	12.17	14.60	5.70
IGF-II	NM_010514.1	13.19	13.83	11.63	13.52	3.28

Table 3. Relative expression of the five candidate genes, illustrated as Z-ratios, in the PA6-DA cell line versus the cell lines PA6-X, PA6-X1, MS5, MM55K, and MEF.

Next, the effect of these candidate molecules on dopaminergic differentiation of BG01V2 and other karyotypically normal hESC lines BG02 and BG03 was examined. hESC maintained in an undifferentiated state under feeder-free conditions were dissociated and differentiated in suspension cultures for 2-4 days as EBs in hESC medium and subsequently transferred to poly-L ornithine/laminin coated tissue culture plates, and treated with the selected factors. EBs generated from the BG01V2 line exhibited poor survival in the absence of the candidate molecules (Fig. 14A), whereas EBs that were maintained in cultures containing the selected factors survived and differentiated into midbrain-specific NPC and TH+ neurons after 10 days of culture (Fig. 14B, C). The majority of BG01V2-derived colonies contained large numbers of TH+ neurons, although the center of colonies remained in a relatively undifferentiated state with expression of the midbrain neural progenitor marker, Msx1 (Fig. 14C). EBs derived from the BG03 cell line in the untreated cultures differentiated into colonies with a uniform appearance (Fig. 14D), whereas BG03-derived EBs that were exposed to SPIE developed into rosette-like structures in the center of colonies (Fig. 14E), morphologically similar to those seen in SDIA-induced cultures.

The overall survival of BG03 cell was significantly higher than the karyotypically abnormal BG01V2 cells, for both untreated and treated conditions. When EBs derived from the BG03 cell line were allowed to differentiate for 10 days, more that 90% of colonies were Msx1+ with extensive networks of TH+ neuronal cells (Fig. 14F).

In contrast to the BG03 cell line, BG02- derived EBs displayed poor survival in untreated conditions and SPIE did not exert a substantial survival-promoting effect. Due the progressive loss of cell viability, the BG02 cultures were examined after five days of culture. In the absence of SPIE, the majority of differentiating BG02 cells had an epithelial morphology (Fig. 14G), and although a few β-III-tubulin+ neurons were detected after five days of differentiation, TH+ neurons were completely absent. In the presence of SPIE, however, colonies of differentiating BG02 cells contained cells with a recognizable neuronal morphology three days after the EBs were transferred to adherent cultures (Fig. 14H). Immunocytochemical analysis after an additional two days showed that the majority of these cells were positive for TH (Fig. 14I). Surprisingly, the differentiated BG02 colonies did not express Msx1, highlighting the substantial variability in the properties of various hESC lines.

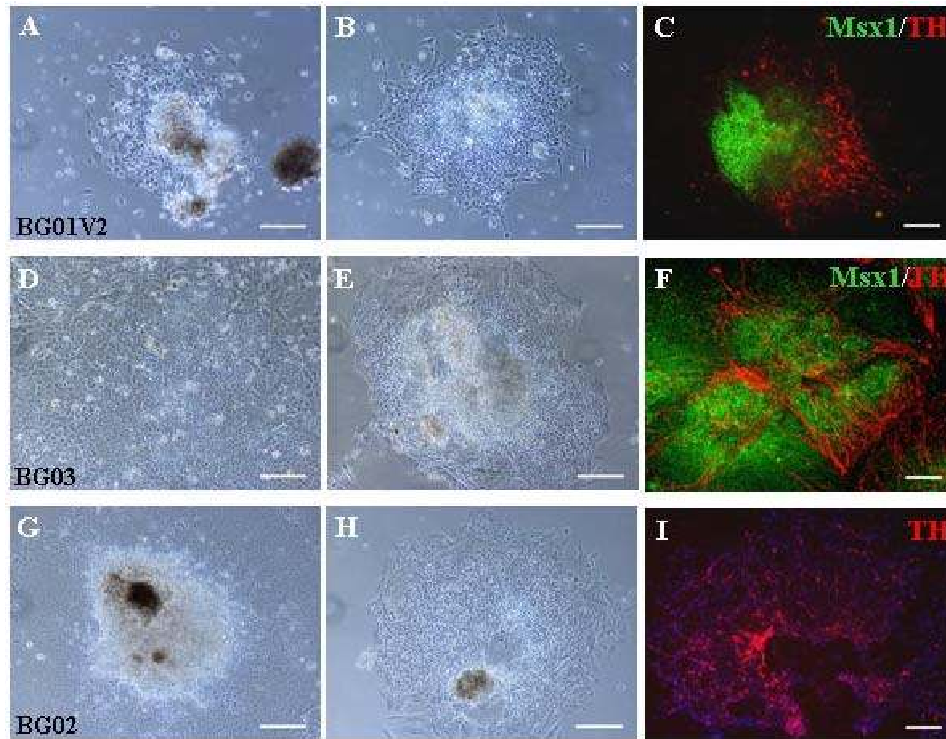

Figure 14. Effect of candidate molecules on differentiation of hESC. Differentiation of EBs derived from the BG01V2 (A-C), BG03 (D-F), and BG02 (G-I) hESC lines. Following EB formation, the hESC were transferred to poly-L-ornithine/laminin coated dishes and were cultured in the absence or presence of SDF-1, IGF2, IGFBP4, PTN, EFNB1, and heparin . Phase contrast images of differentiated EBs generated from (A) BG01V2 (D) BG03, and (G) BG02 in the absence of any factors after three days of culture. Panels B, E and H show EBs derived from BG01V2, BG03, and BG02, respectively, in the presence of the candidate molecules, SDF-1, IGF2, IGFBP4, PTN, EFNB1 after three days of differentiation. After 10 days of culture, Msx1 and TH-immunoreactive cells were found in BG01V2 (C) and BG03 (F) cultures treated with the five candidate molecules. EBs generated from the BG02 cell line generated TH+ neurons, but expression of Msx1 was completely absent from these cultures.
Scale bars=200 μm

It should be mentioned that all treated conditions also contained IGFBP4. We initially selected this factor as a potential DA inducing element, and speculated that it might have a role in regulation of IGF2 activity. To further characterize the activity by which the five molecules SDF-1, PTN, IGF2, IGFBP4, and EFNB1 exerted a DA inducing effect on hESC, we separated the role of the candidate molecules in dopaminergic induction by exposing the EB cultures to various combinations of inducing factors followed by immunocytochemical marker analysis after 10 days of differentiation. These preliminary data indicated that IGFBP4 was not required for SDIA activity, and that IGFBP4 in fact seemed to reduce the survival of proliferating midbrain NPC. In contrast, IGF2 was essential for survival of the proliferating NPC, whereas presence of SDF-1 and EFNB-1 was required for dopaminergic induction of hESC. PTN, as anticipated, increased the yield of TH+ neurons.

In summary, this study has identified the molecular nature of SDIA and demonstrates that the combination of SDF-1, PTN, IGF2, and EFNB1, termed ***SPIE***, can mimic SDIA activity and produce a high yield of TH+ neurons from hESC in the presence of heparin. Nevertheless, optimal culture conditions for enhancing survival and maturation of differentiating midbrain specific NPC into functional DA neurons, and adaptation for the use of SPIE for a variety of hESC lines, remain to be developed.

7. Discussion and future perspectives

In the research presented here, the activity of SDIA for neural and dopaminergic induction from hESC was examined. Experiments were designed to study the temporal effect of SDIA on hESC differentiation, and characterize NPC and dopaminergic neurons produced by this approach. The proliferative capacity of generated NPC, and the effect of time on their ability to adopt a dopaminergic phenotype was also assessed. SDIA is one of the most simple and efficient methods currently employed to generate DA neurons from hESC. Midbrain DA neurons derived from hESC offer a valuable *in vitro* model to study neurological disorders, such as schizophrenia, drug abuse, and PD that have been associated with dysfunction of the midbrain DA system, and to provide a functional cell-replacement strategy to treat Parkinson's disease. However, SDIA requires the culture of hESC on mouse stromal feeder cells, and because the use of animal cells or animal derived components does not meet safety requirements for clinical application, we intended to elucidate the activity of SDIA by identifying unknown inductive molecules that are produced by PA6 stromal cells. This would allow development of culture conditions with a chemically-defined media that would mimic the DA-inducing activity of SDIA.

Temporal aspects of SDIA-mediated DA induction

Previous studies that have adapted the SDIA method for generation of human DA neurons demonstrated a robust neural induction of hESC cells on PA6 or MS5 stromal cell lines [194-196]. Nonetheless, the co-culturing period of hESC with stromal cells differs between these studies. For instance, a study by Perrier et al. [104] reported that dopaminergic differentiation of hESC could be achieved by co-culturing hESC with MS5 or S2 stromal cell lines for 28 days followed by further differentiation for several weeks in the presence of growth factors and SHH and FGF8. Generally, 28 days is a sufficient period of time to allow for nearly complete differentiation of dopaminergic neurons by SDIA alone [194], however, the SDIA effect of PA6 and MS5 cells is quite variable. In the current study it was determined that SDIA is required only for the initial stages of differentiation. The first TH+ neurons appeared after 8 days of co-culture and assessment of lineage restriction of differentiating hESC in co-culture with PA6 stromal cells revealed that the highest yield of undifferentiated mesencephalic-restricted NPC expressing Pax2, Pax5, Otx2 and Msx-1 could be obtained from co-cultures after only 7 days of induction. When hESC of the BG01V2 line were co-cultured with PA6 cells for only 10 days, and subsequently transferred to feeder-free culture conditions and cultured in the presence of SHH, FGF8 and GDNF, the yield of TH+ neurons was approximately 34% after 18 additional days of differentiation. Nearly 80% of all MAP2+ neurons were TH+, which demonstrates the highly specific DA-inducing effect of SDIA. Investigation of effects and timing of patterning signals during SDIA induced differentiation of mouse ESC have also showed a similar time-window for DA induction, also suggesting that SDIA is responsible for early specification of dopaminergic phenotype [198]. It is also curious that the time span for SDIA-induced DA differentiation is similar for mouse and human ESC, despite the large differences in the duration for normal DA development if the two species.

Development of SDIA-derived midbrain NPC into functional DA neurons

We employed the SDIA method to generate dopaminergic neurons from a variant of BG01, here termed BG01V2, which carries a chromosome 17 trisomy. It has been suggested that long-term maintenance of hESC can cause chromosomal abnormalities, which may be associated with

enzymatic methods in propagation of hESC [40, 44, 45]. Interestingly, several laboratories have reported the gain of chromosome 17 as one of the most common alterations in late passages of hESC [40, 45, 58, 59]. Although it is suggested that chromosome 17 shows a higher incidence of aneuploidy, it has not yet been determined whether this chromosomal aberration is associated with adaptation to *in vitro* culture. Nevertheless, recent studies have identified possible candidate genes on this chromosome that may be involved in adaptation of hESC to *in vitro* culture. BIRC5 (survivin), an inhibitor of apoptosis, is among these genes, and is selectively over-expressed in common human cancers [368, 369].

We discovered that the BG01V2 cell line had a higher efficiency of DA neuron differentiation, as compared to other karyotypically normal hESC lines. Therefore, we asked whether TH-expressing neurons derived from the BG01V2 cells were post-mitotic, displaying phenotypic characteristics of normal midbrain dopaminergic neurons.

The obtained data from characterization studies of these cells indicated that the BG01V2-derived TH+ neurons displayed functional maturity in terms of electrical excitability. Several studies have demonstrated maturation of neuronal cells as a function of insertion and clustering of voltage-dependent sodium channels [370, 371]. Our observations were consistent with these studies, and showed an increase in sodium currents as the neurons matured. The TH+ cells also exhibited functional DAT activity as confirmed by competitive DAT binding experiments. The IC_{50} value from the binding assay was in the micromolar range, whereas dopamine transporter in a neuronal cell line and cell lines overexpressing DAT have displayed much higher binding affinities [372]. The electrophysiological studies demonstrated that many neurons in our cultures were not fully mature. This would possibly explain the low binding affinity of dopamine analog to DAT, which may be dependent on the state of neuronal maturation.

It should be noted that we do realize that a karyotypically abnormal cells line has no or limited applications in cell replacement therapy, and that ideally hESC should be differentiated in cultures free of any xenogeneic material. However, hESC-based transplantation studies are at an experimental stage. Thus, a model for efficient phenotypic differentiation of DA neurons could be of clinical interest in investigating the genetics and molecular mechanisms controlling the functions of these neurons *in vitro* and *in vivo*, and potentially for understanding and treating neurodegenerative and psychiatric diseases. Furthermore, the genetic integrity of hESC is often assessed by classical cytogenetic methods which are very limited in resolution. High resolution DNA analysis have revealed that presumably normal hESC, with an unaltered karyotype, carry high frequencies of genetic abnormalities including mutations that could predispose them to forming tumors if they would be transplanted in human subjects [57, 59]. Thus, before clinical utility of even karyotypically normal hESC can be realized, culturing techniques and efficient high-resolution genomic analysis would need to be developed to preserve and monitor the genetic integrity of hESC.

Proliferation capacity of NPC derived from hESC by SDIA

Several reports have demonstrated long-term survival, differentiation, and morphological integration of fetal- derived NPC from various brain regions including the nigro-striatal system [373-375]. Expansion of NPC as cellular aggregates in suspension cultures, also referred to as neurospheres, was initially developed for rodent NPC [376] and has recently been adapted for the long-term growth of human fetal NPC [373, 377]. Recently it has been reported that Wnt ligands (Wnt3a and Wnt5b) and receptor transcripts are upregulated in hESC-derived neurospheres and that

Wnt3a promotes the expansion of neurospheres derived from noggin-treated hESC [378]. On the other hand, little is known about the signaling factors controlling the ability of hESC-derived midbrain-specific NPC to undergo self-renewal and maintain cellular memory for extended periods of time as neurospheres.

In this study, enyzmatically isolated NPC from co-cultures were propagated as neurospheres in suspension cultures for six weeks. Weekly investigation of neurosphere proliferation and DA differentiation ability of expanded NPC for various durations revealed that SDIA- derived NPC could be expanded by five-fold while retaining their DA differentiation potential. Nevertheless, the NPC progressively lost their ability to multiply and develop into DA neurons *in vitro*. The decrease in neuronal differentiation of NPC over time *in vitro* is consonant with the course of neural differentiation in development of the mammalian CNS, where the majority of neurons arise during the embryonic period from multipotent progenitors which are progressively more biased toward a glial fate as the brain develops [379]. A previous published report has also showed a gradual decrease in DA differentiation of fetal NPC derived from embryonic rodent ventral mesencephalon with increasing age of donor tissue [380].

In final stages of our study, a study by Hong and coworkers that used the SDIA-inducing strategy to generate NPC from other hESC lines was published [184]. According to their study, the generated NPC could be passaged as clusters or single cells without losing their proliferative capacity or potential to differentiate to neurons, astrocytes, and oligodendrocytes as well as TH+ neurons during 12 weeks of expansion.

It is interesting that the NPC in the Hong et al. [196] study maintained their ability to differentiate for at least 12 weeks, even though they were first differentiated for three times as long as the NPC in the present study. Notable dissimilarities between our protocol and the protocol employed by Hong and coworkers include using different stromal cell lines, culturing techniques, and medium to achieve neural induction of hESC. In addition, Hong and coworkers used hESC lines H1 and HFS-6, whereas we used the BG01V2 line. The discrepancy between our study and the study of Hong and colleagues implies that the mechanisms involved in maintenance of cellular memory and differentiation potential of hESC-derived NPC might depend on culturing techniques employed in generation or propagation of these cells, or the nature of the hESC line they are derived from. Indeed, a recent study by Wu and colleagues [381] compared different hESC lines, including the HSF6 line that was used by Hong et al. [183], and illustrated a clear distinction between the genetic, epigenetic, and cellular properties of hESC and their derivative neurons. In particular, the HSF6 line was found to be more prone to generating midbrain and hindbrain neurons but failed to produce forebrain neurons. Comparison of the NPC in the study by Hong et al. [196], which showed a surprising lack of replicative senescence, to the BG01V2-derived NPC with limited proliferation capacity in our report, may be useful for investigating intrinsic mechanisms controlling self renewal and the transition of highly replicating neural stem cells to neural progenitor cells with a more limited life span. We should also note that a lack of replicative senescence is not necessarily a desirable property for hESC-derived cells.

Preliminary transplantation studies of neurospheres which retained their potential to differentiate towards a DA phenotype suggested that these cells could survive and maintain their phenotype *in vivo* for up to three weeks. However, no TH+ neurons were detected in the grafts at this time. The failure of grafted NPC to differentiate into DA neurons in the host striatum has been observed in several studies [382, 383]. This raises the question of whether the pre-differentiation into

DA neurons is necessary before transplantation. In a study where adult neural stem cells from the SVZ of adult mice were transplanted into various regions of the mouse brain including the striatum, differentiation into mature neurons of grafted cells were only observed in the olfactory bulb, suggesting that the adult striatum may be a non-neurogenic area [384]. Other studies have reported that a small percentage of human NPC derived from hESC or fetal tissue have the ability to different to TH+ neurons *in vivo* [361, 367, 365, 385], but additionally neural stem cells seem to possess the ability to rescue dysfunctional neurons [385, 386] and migrate towards the site of injury [386] in animal models of PD.

Identification of SDIA

To identify the nature of SDIA, we focused first on clarifying the effect of SDIA on neural and DA induction of hESC. At this time there was a controversy in the literature regarding the role of stromal cells in dopaminergic differentiation of ESC. The initial description of SDIA by Kawasaki et al. [181] suggested that SDIA was resistant to fixation and resided on the PA6 cell surface. Subsequent data (Schwartz et al. [199] and Yamazoe et al. [200]) have, in contrast, suggested that soluble secreted factors are involved. We attempted to determine the separate contributions of these two components, and found that the PA6 cell surface activity was sufficient for neural induction, but the secreted factors from the PA6 cells were the DA-inducing element of SDIA. We also found that the number of TH+ neurons was substantially increased when heparin was added to feeder-free cultures containing medium conditioned by PA6 cells.

Heparin is a highly-sulfated glycosaminoglycan and is found on cell surface and in the extracellular matrix. Heparan-sulfate glycosaminoglycans interact with various proteins and form proteoglycans [387, 388]. Although the role of these glycosaminoglycans is still to be fully determined, they are known to interact strongly with several growth factors, and modulate their biological activity, thereby playing a crucial regulatory role in embryogenesis and CNS development [389, 390].

In order to identify soluble factors that would potentially reproduce the DA inducing activity of SDIA, we compared gene expression of the potent PA6-DA cell line to cell lines largely lacking SDIA activity, including PA6-X1, PA6-X, MS5, MEF and MM55K. We thereby identified a set of candidate genes including SDF-1, PTN, IGF2, and EFNB1as potential dopaminergic inducing factors, and examined the role of molecules encoded by these genes in dopaminergic induction of hESC *in vitro*

The chemokine SDF-1 was originally identified in the immune system and induces leukocyte chemotaxis [391]. In addition to its T-cell chemotactic activity, SDF-1 is widely expressed in the developing nervous system [392, 393], interacting with Wnt pathway in neural development [394] and mediating sonic hedgehog-induced proliferation of cerebellar granule cells [395]. The temporal expression pattern of SDF-1 and CXCR4 implies roles in early neurogenesis. Indeed, genetic disruption of CXCR4 and SDF-1 in the mouse has resulted in defective fetal cerebellar [396] and hippocampal dental gyrus [397] development. SDF-1 has also been implicated in proliferation of NPC isolated from rat cortex by activating the ERK1/2 and PI-3 kinase pathways [388]. An up-regulation of SDF-1 and its receptor CXCR4 has also been reported during differentiation of neural stem cells to more restricted precursors [399]. Also, in a previous study by our group, gene expression profiling by a focused human stem cell microarray showed expression of CXCR4 in neurons generated from hESC by the SDIA method [194]. Interestingly, a very recent study by

Edman and colleagues [400] demonstrated expression of two other α-chemokines, CXCL1 and CXCL6, in developing rodent ventral midbrain and suggested that these factors have a regulatory role in the development of the midbrain DA cell population. The application of CXCL1 (ortholog of human CXCL8), CXCL6, and human CXCL8 to primary ventral midbrain precursor cultures demonstrated that CXCL1, CXCL6 and CXCL8 have a role in promoting DA neurogenesis, enhancing development of Nurr1+ cells into DA neurons, and increasing proliferation of DA progenitors, respectively.

The secreted growth factor pleiotrophin (PTN), also referred to as heparin-binding growth factor 8, exerts a mitogenic effect on cells that express its receptor, transmembrane protein tyrosine phosphatase [401-403]**.** PTN and its receptor are expressed during CNS development and as previously mentioned, PTN has a trophic effects on DA neurons and increases the yield of TH+ neurons differentiated from mouse ESC [144, 145, 404].

IGFs and their carriers, IGF-binding proteins (IGFBPs) are widely expressed throughout the CNS [405, 406]. IGF2 has been suggested to have neurotrophic effects promoting survival and differentiation of neuronal cells [407, 408]. Recently expression of the receptor for IGF2, IGF1R, has been reported in hESC, and it has also been proposed that IGF2 has a role in hESC survival and self-renewal [409].

Indication of the involvement of IGF2 in differentiation of mesencepahlic neural progenitor cells from hESC was recently obtained by a massively parallel signature sequencing (MPSS) analysis of gene expression in PSA-NCAM+ neuronal precursors derived by SDIA. Abundance of the IGF2 transcript was among the highest of the 11912 distinct sequences detected. IGF2 expression was also found in laser-captured dopamine neurons from human postmortem brain [410].

Eph receptors and their ephrin ligands are essential for migration of many cell types during embryonic development [270]. Transmembrane B-class ephrins promiscuously bind EphB receptors, whereas A-class ephrins, which are tethered to the cell surface by a glycosylphosphatidylinositol linkage, preferentially bind EphA receptors. Ephrin B1 acts both as a ligand and as a receptor in a tissue-specific manner during embryogenesis and is critically important in many biological processes including axon guidance, neural crest migration [270, 271, 411]. Yet Ephrin B1 has not been considered before in the context of DA neurogenesis. However, as previously discussed, another member of the ephrin family, ephrin B2, which signals through its receptor EphB1, have been reported to constitute one of the signaling mechanisms responsible for specification of distinct DA neural pathways [217].

A number of other genes that were also highly expressed in PA6-DA cells, but to a lesser extent than those studied, also have possible DA-inducing or neurotrophic effects. These included members of the TGF-β family and factors involved in the Wnt signaling pathway. Although we did not test the effect of these factors in our study, we cannot exclude the possibility that these factors might enhance the induction and survival of DA neurons derived from hESC.

When hESC of line BG01V2 and BG03 were stimulated by SDF-1, PTN, IGF2, and EFNB1, termed SPIE, they differentiated into Msx1+ midbrain NPC and TH+ neurons after 10 days of induction. We chose to generate EBs for 2-4 days prior to treatment because this procedure generally enhanced the viability of hESC. Although SPIE does not seem to have a sufficient survival-promoting effect to allow studies on fully undifferentiated hESC, the hESC were differentiated as

EBs only for 2-4 days in these experiments. We therefore believe that SPIE has a role in early DA induction of hESC. In addition, SPIE was not sufficient to direct lineage specific differentiation of midbrain NPC from the BG02 line. Similar differences in efficiency of DA neuron differentiation between hESC lines induced by SDIA have been observed in our laboratory and by other groups [154, 194, 195]. It may be that varying concentrations of SPIE components may be required to obtain differentiation of various hESC lines.

Surprisingly, about one month ago (August 13, 2008), a report studying expression of numerous receptors for growth factors in corticospinal motor neurons was published. This study showed that that corticospinal motor neurons express high levels of IGF2, CXCL12 (SDF-1) and PTN receptors. This group also demonstrated that the combination of these factors significantly enhanced the survival of corticospinal motor neurons [412].

Concluding remarks

We have identified a novel combination of four factors, SDF-1, PTN, IGF2, and EFNB1, termed SPIE, termed SPIE, which efficiently induce hESC to develop into midbrain specific NPC capable of differentiating into TH+ neurons. Future studies including investigation of the signaling mechanisms initiated by each of these four molecules may provide insights into novel pathways controlling DA development from hESC. Additional studies are required to determine whether these cells can mature into physiologically functional dopamine producing neurons, and whether they would be able to survive and integrate into the host brain circuitry in animal models of PD. Aside from having a theoretical value, we believe that our findings identifying early DA inducing factors can be utilized to efficiently produce DA neurons from hESC in cultures free from animal cells and products, which would ultimately contribute to the advancement of cellular replacement strategies for treatment of PD.

8. Popular Scientific summary

One of the most common age-related neurological diseases is Parkinson's disease, which causes a progressive impairment of the ability to control body movements. Parkinson's disease is mainly caused by deterioration of dopamine-producing neurons in a region at the base of the brain called substantia nigra.

One approach to treatment of Parkinson's disease is replacement of defective dopamine-producing neurons with healthy new cells, by transplanting cells into the brain. Previous studies using fetal tissue rich in dopamine neurons for transplantation have provided proof-of-concept that Parkinson's disease patients can benefit from cell replacement therapy. Results of clinical trials using fetal tissue grafts in patients with Parkinson's disease have, however, been highly variable, and in some cases there have been severe adverse effects. These observations indicate that current surgical strategies for dopamine cell replacement therapy need to be improved. Currently the limited availability of fetal tissue is restraining the development of appropriate cell transplantation procedures, because these procedures require large amounts of fetal tissue.

Human embryonic stem cells (hESC) are cells derived from embryos at a very early age. These cells can be grown in culture indefinitely and have the capability to develop into a variety of cell types including dopamine neurons. Embryonic stem cells are therefore a promising source of cells for transplantation therapies for treatment of Parkinson's disease. The major challenge in hESC research is to control and direct the stem cells toward specific cell types of interest.

Methods used to generate dopamine neurons from hESC include allowing spontaneous differentiation of hESC, followed by addition of specific molecules, or by culturing hESC on so called "feeder cells", which are cells commonly obtained from animal sources that have the ability to direct hESC to become dopamine neurons. The latter approach is more efficient and straightforward, but the use of animal cells hinders any clinical application due to possible transfer of animal cells and pathogens. Therefore it is of both theoretical and clinical importance to understand the activity of these feeder cell systems. By understanding how these feeder cells work it may be possible to develop defined *in vitro* differentiation approaches so that dopamine neurons can be generated from hESC without risk of contamination.

Feeder cells which can cause hESC to become dopamine neurons are generally a type of cells derived from bone marrow called "stromal cells". The aim of the studies presented here was to determine exactly how stromal cells cause this cellular transformation. First, we found that stromal cells cause hESC to differentiate by secreting chemical factors. The chemical factors produced by stromal cells were analyzed leading to identification of a novel combination of four factors that is responsible for conversion of hESC to dopamine neurons.

These studies, as well as many others highlighting the involvement of new factors in development of dopamine neurons, define a molecular scheme for inducing hESC differentiation, leading to generation of dopamine neurons. The key challenge in neuronal transplantation will be obtaining functionally effective cells which would integrate with the host endogenous neuronal circuitry after transplantation, leading to improved function. Also, hESC have the potential to form tumors, known as teratomas after transplantation. Thus there are safety issues to be considered in stem cell transplantation therapy. In the future, progress in hESC science and the world of bioengineering may enhance the clinical efficacy of hESC-derived dopamine neurons. For example, selective differentiation of dopamine neurons may be enhanced by genetic engineering of hESC, while development of culturing conditions more accurately mimicking the *in vivo* environment would potentially increase the function and stability of these neurons.

Acknowledgments

First and foremost, I would like to thank my advisor **Dr. William Freed** for his superb mentorship and the opportunity for professional and personal growth as a graduate student. Dr. Freed, it has been a gratifying experience to work in your laboratory. I have taken great pleasure in learning from you and would like to thank you not only for your guidance through every problem making this work possible, but also for your tremendous support, encouragement, and kind nature. Thank you for letting me work independently and believing in me.

I would also like to extend cordial thanks to **Dr. Barry Hoffer** for giving me this opportunity and providing me with support and career advice. Dr. Hoffer, thank you so much for introducing me to the exciting world of scientific research.

Professor Joakim Lundeberg, thank you for being so accepting of me enrolling into the graduate partnership program between KTH and NIH. You have been so gracious and accommodating. Thank you very much for your continuous support.

General thanks are extended to all members of Dr. Freed's laboratory for being extremely helpful and making my time at the laboratory very pleasant. **Dr. Jia Chen**, I highly appreciate your help, especially in performing the DAT and transplantation studies. It has been a pleasure to work with you. **Dr. Chung Ting (David) Lee**, **Ms. Lila Worden**, **Ms. Emily Gabitzsch,** and **Ms. Rose Amable,** thank you for culturing the human embryonic stem cells and your contributions to my studies.

Our collaborators, **Dr. Kevin Becker** (gene expression analysis), **Dr. Carl Lupica**, **Dr. Charles Spivak** (electrophysiological recordings), and **Drs. Yun Wung** and **Brandon Harvey** (cell transplantation studies) are gratefully acknowledged.

I would also like to express my sincere gratitude to **Dr. Sharon Milgram**, the director of the graduate partnership program at NIH. Dr. Milgram, your kind words and encouragement have meant the world to me.

In addition, I am grateful to **Ms. Cindy Ambriz** for her generous help with manuscript organization and submissions, and to **Ms. Kristina Burestad** for her assistance in printing this work.

Friends and Family

To my friends in California, thanks for standing by me and encouraging me to do my best. I love you dearly and would not have been able to accomplish this without you.

To Ms. Mary Anne Esposito, or simply my Mimi and her family, thank you for your tremendous love and kindness and for always believing in me. You have truly been a second family to me and you will always have a very special place in my heart.

To my dearest parents (**Maman va Pedar**), I would not have been here without your selfless support and sacrifices you have made in life. Words alone cannot express how thankful I am for your endless love, and the pride you express in having me as a daughter. This work is dedicated to you.

References

1. Gilbert, S. F. (2006). *Developmental biology (8th ed.)*. Sunderland, Mass.: Sinauer Associates, Inc. Publishers.

2. Sherman, M.I. (1975). Long term culture of cells derived from mouse blastocysts. *Differentiation*. **3**, 51-67.

3. Evans, M.J., and Kaufman, M. (1981). Establishment in culture of pluripotential stem cells from mouse embryos. *Nature*. **292**, 151-156.

4. Martin, G.R. (1980). Teratocarcinomas and mammalian embryogenesis. *Science*. **209**, 768-776.

5. Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA*. **78**, 7634-7638.

6. Thomson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., Becker, R.A., et al. (1995). Isolation of a primate embryonic stem cell line. *Proc Natl Acad Sci USA*. **92**, 7844-7848.

7. Thomson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., and Hearn, J.P. (1996). Pluripotent cell lines derived from common marmoset (Callithrix jacchus) blastocysts. *Biol Reprod*. **55**, 254-259.

8. Suemori, H., Tada, T., Torii, R., Hosoi, Y., Kobayashi, K., Imahie, H., et al. (2001). Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. *Dev Dyn*. **222**, 273-279.

9. Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., et al. (1998). Embryonic stem cell lines derived from human blastocysts. *Science*. **282**, 1145-1147.

10. Reubinoff, B.E., Pera, M.F., Fong C.Y., Trounson A., and Bongso A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotechnol.* **18**, 399-404.

11. Bosma, G.C., Custer, R.P., and Bosma, M.J. (1983). A severe combined immunodeficiency mutation in the mouse. *Nature*. **301**, 527-530.

12. Cowan, C.A., Klimanskaya, I., McMahon, J., Atienza, J., Witmyer, J., Zucker, J.P., et al. (2004). Derivation of embryonic stem-cell lines from human blastocysts. *N Engl J Med*. **350**, 1353-1356.

13. Amit, M., and Itskovitz-Eldor, J. (2002). Derivation and spontaneous differentiation of human embryonic stem cells. *J Anat*. **200**, 225-232.

14. Genbacev, O., Krtolica, A., Zdravkovic, T., Brunette, E., Powell, S., Nath, A., et al. (2005). Serum-free derivation of human embryonic stem cell lines on human placental fibroblast feeders. *Fertil Steril*. **83**, 1517-1529.

15. Strom, S., Inzunza, J., Grinnemo, K.H., Holmberg, K., Matilainen, E., Stromberg, A.M., et al. (2007). Mechanical isolation of the inner cell mass is effective in derivation of new human embryonic stem cell lines. *Hum Reprod*. **22**, 3051-3058.

16. Ellerstrom, C., Strehl, R., Moya, K., Andersson, K., Bergh, C., Lundin, K., et al. (2006). Derivation of a xeno-free human embryonic stem cell line. *Stem Cells*. **24**, 2170-2176.

17. Turetsky, T., Aizenman, E., Gil, Y., Weinberg, N., Shufaro, Y., Revel, A., et al. (2008). Laser-assisted derivation of human embryonic stem cell lines from IVF embryos after preimplantation genetic diagnosis. *Hum Reprod*. **23**, 46-53.

18. Strelchenko, N., Verlinsky, O., Kukharenko, V., and Verlinsky, Y. (2004). Morula-derived human embryonic stem cells. *Reprod Biomed Online*. **9**, 623-629.

19. Klimanskaya, I., Chung, Y., Becker, S., Lu, S.J., and Lanza, R. (2006). Human embryonic stem cell lines derived from single blastomeres. *Nature*. **444**, 481-485.

20. Klimanskaya, I., Chung, Y., Becker, S., Lu, S.J., and Lanza, R. (2007). Derivation of human embryonic stem cells from single blastomeres. *Nat Protoc*. **2**, 1963-1972.

21. Chung, Y., Klimanskaya, I., Becker, S., Li, T., Maserati, M., Lu, S.J., et al. (2008). Human embryonic stem cell lines generated without embryo destruction. *Cell Stem Cell*. **2**, 113-117.

22. Bongso, A., Fong, C.Y., Ng, S.C., and Ratnam, S.S. (1994). Isolation and culture of inner cell mass cells from human blastocysts. *Hum Reprod*. **9**, 2110-2117.

23. Richards, M., Fong, C.Y., Chan, W.K., Wong, P.C., and Bongso, A. (2002). Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. *Nat Biotechnol*. **20**, 933-936.

24. Amit, M., Margulets, V., Segev, H., Shariki, K., Laevsky, I., Coleman, R., et al. (2003). Human feeder layers for human embryonic stem cells. *Biol Reprod*. **68**, 2150-2156.

25. Cheng, L., Hammond, H., Ye, Z., Zhan, X., and Dravid, G. (2003). Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. *Stem Cells*. **21**, 131-142.

26. Miyamoto, K., Hayashi, K., Suzuki, T., Ichihara, S., Yamada, T., Kano, Y., et al. (2004). Human placenta feeder layers support undifferentiated growth of primate embryonic stem cells. *Stem Cells*. **22**, 433-440.

27. Xu, C., Inokuma, M.S., Denham, J., Golds, K., Kundu, P., Gold, J.D., et al. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol*. **19**, 971-974.

28. Van Hoof, D., Braam, S.R., Dormeyers, W., Ward-van Oostwaard, D., Heck, A.J., Krijgsveld, J., et al. (2008). Feeder-free Monolayer Cultures of Human Embryonic Stem Cells Express an Epithelial Plasma Membrane Protein Profile. *Stem Cells*. [Epub ahead of print].

29. Wang, Q., Fang, Z.F., Jin, F., Lu, Y., Gai, H., and Sheng, H.Z. (2005). Derivation and growing human embryonic stem cells on feeders derived from themselves. *Stem Cells*. **23**, 1221-1227.

30. Stojkovic, P., Lako, M., Stewart, R., Przyborski, S., Armstrong, L., Evans, J., et al. (2005). An autogeneic feeder cell system that efficiently supports growth of undifferentiated human embryonic stem cells. *Stem Cells*. **23**, 306-314.

31. Chin, A.C., Fong, W.J., Goh, L.T., Philp, R., Oh, S.K., and Choo, A.B. (2007). Identification of proteins from feeder conditioned medium that support human embryonic stem cells. *J Biotechnol*. **130**, 320-328.

32. Lim, J.W., and Bodnar, A. (2002). Proteome analysis of conditioned medium from mouse embryonic fibroblast feeder layers which support the growth of human embryonic stem cells. *Proteomics*. **2**, 1187-1203.

33. Cai, J., Chen, J., Liu, Y., Miura, T., Luo, Y., Loring, J.F., et al. (2006). Assessing self-renewal and differentiation in human embryonic stem cell lines. *Stem Cells*. **24**, 516-530.

34. Xu, R.H., Peck, R.M., Li, D.S., Feng, X., Ludwig, T., and Thomson, J.A. (2005). Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. *Nat Methods*. **2**, 185-190.

35. Wang, G., Zhang, H., Zhao, Y., Li, J., Cai, J., Wang, P., et al. (2005). Noggin and bFGF cooperate to maintain the pluripotency of human embryonic stem cells in the absence of feeder layers. *Biochem Biophys Res Commun*. **330**, 934-942.

36. Pera, M.F., Andrade, J., Houssami, S., Reubinoff, B., Trounson, A., Stanley, E.G., et al. (2004). Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin. *J Cell Sci*. **117**, 1269-1280.

37. Beattie, G.M., Lopez, A.D., Bucay, N., Hinton, A., Firpo, M.T., King, C.C., et al. (2005). Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers. *Stem Cells*. **23**, 489-495.

38. James, D., Levine, A.J., Besser, D., and Hemmati-Brivanlou, A. (2005). TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. *Development*. **132**, 1273-1282.

39. Lu, J., Hou, R., Booth, C.J., Yang, S.H., and Snyder, M. (2006). Defined culture conditions of human embryonic stem cells. *Proc Natl Acad Sci USA*. **103**, 5688-5693.

40. Draper, J.S., Smith, K., Gokhale, P., Moore, H.D., Maltby E., Johnson, J., et al. (2004). Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. *Nat Biotechnol*. **22**, 53-54.

41. Rajala, K., Hakala, H., Panula, S., Aivio, S., Pihlajamäki, H., Suuronen, R., et al. (2007). Testing of nine different xeno-free culture media for human embryonic stem cell cultures. *Hum Reprod*. **22**, 1231-1238.

42. Hoffman, L.M., and Carpenter, M.K. (2005). Characterization and culture of human embryonic stem cells. *Nat Biotechnol*. **23**, 699-708.

43. Crook, J.M., Peura, T.T., Kravets, L., Bosman, A.G., Buzzard, J.J., Horne, R., et al. (2007). The generation of six clinical-grade human embryonic stem cell lines. *Cell Stem Cell*. **1**, 490-494.

44. Mitalipova, M.M., Rao, R.R., Hoyer, D.M., Johnson, J.A., Meisner, L.F., Jones, K.L., et al. (2005). Preserving the genetic integrity of human embryonic stem cells. *Nat Biotechnol*. **23**, 19-20.

45. Brimble, S.N., Zeng, X., Weiler, D.A., Luo, Y., Liu, Y., Lyons, I.G., et al. (2004). Karyotypic stability, genotyping, differentiation, feeder-free maintenance, and gene expression sampling in three human embryonic stem cell lines derived prior to August 9, 2001. *Stem Cells Dev*. **13**, 585-507.

46. Suemori, H., Yasuchika, K., Hasegawa, K., Fujioka, T., Tsuneyoshi, N., and Nakatsuji, N. (2006). Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. *Biochem Biophys Res Commun*. **345**, 926-932.

47. Ellerström, C., Strehl, R., Noaksson, K., Hyllner, J., and Semb, H. (2007). Facilitated expansion of human embryonic stem cells by single-cell enzymatic dissociation. *Stem Cells*. **25**, 1690-1696.

48. Joannides, A., Fiore-Hériché, C., Westmore, K., Caldwell, M., Compston, A., Allen, N., et al. (2006). Automated mechanical passaging: a novel and efficient method for human embryonic stem cell expansion. *Stem Cells*. **24**, 230-235.

49. Carpenter, M.K., Rosler, E., and Rao, M.S. (2003). Characterization and differentiation of human embryonic stem cells. *Cloning Stem Cells*. **5**, 79-88.

50. Heins, N., Englund, M.C., Sjöblom, C., Dahl, U., Tonning, A., Bergh, C., Lindahl, et al. (2004). Derivation, characterization, and differentiation of human embryonic stem cells. *Stem Cells*. **22**, 367-376.

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

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

53. Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., et al. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell*. **95**, 379-91.

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

55. Brandenberger, R., Khrebtukova, I., Thies, R.S., Miura, T., Jingli, C., Puri, R., et al. (2004). MPSS profiling of human embryonic stem cells. *BMC Dev Biol*. **4**, 10.

56. Hayflick, L., and Moorhead, P.S. (1961). The serial cultivation of human diploid cell strains. *Exp Cell Res*. **25**, 585-621.

57. Rosler, E.S., Fisk, G.J., Ares, X., Irving, J., Miura, T., Rao, M.S., et al. (2004). Long-term culture of human embryonic stem cells in feeder-free conditions. *Dev Dyn*. **229**, 259-274.

58. Baker, D.E., Harrison, N.J., Maltby, E., Smith, K., Moore, H.D., Shaw, P.J., et al. (2007). Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. *Nat Biotechnol*. **25**, 207-215.

59. Maitra, A., Arking, D.E., Shivapurkar, N., Ikeda, M., Stastny, V., Kassauei, K., et al. (2005). Genomic alterations in cultured human embryonic stem cells. *Nat Genet*. **37**, 1099-1103.

60. Josephson, R., Sykes, G., Liu, Y., Ording, C., Xu, W., Zeng, X., et al. (2006). A molecular scheme for improved characterization of human embryonic stem cell lines. *BMC Biol*. **4**, 28.

61. Postovit, L.M., Seftor, E.A., Seftor, R.E., and Hendrix, M.J. (2006). A three-dimensional model to study the epigenetic effects induced by the microenvironment of human embryonic stem cells. *Stem Cells*. **24**, 501-505.

62. Lee, G.Y., Kenny, P.A., Lee, E.H., and Bissell, M.J. (2007). Three-dimensional culture models of normal and malignant breast epithelial cells. *Nat Methods*. **4**, 359-365.

63. Nur-E-Kamal, A., Ahmed, I., Kamal, J., Schindler, M., and Meiners, S. (2006). Three-dimensional nanofibrillar surfaces promote self-renewal in mouse embryonic stem cells. *Stem Cells*. **24**, 426-433.

64. Yim, E.K., and Leong, K.W. (2005). Proliferation and differentiation of human embryonic germ cell derivatives in bioactive polymeric fibrous scaffold. *J Biomater Sci Polym Ed*. **16**, 1193-1217.

65. Gerecht, S., Townsend, S.A., Pressler, H., Zhu, H., Nijst, C.L., Bruggeman, J.P., et al. (2007). A porous photocurable elastomer for cell encapsulation and culture. *Biomaterials*. **28**, 4826-4835.

66. Gerecht, S., Burdick, J.A., Ferreira, L.S., Townsend, S.A., Langer, R., and Vunjak-Novakovic, G. (2007). Hyaluronic acid hydrogel for controlled self-renewal and differentiation of human embryonic stem cells. *Proc Natl Acad Sci USA*. **104**, 11298-11303.

67. Lavon, N., and Benvenisty, N. (2005). Study of hepatocyte differentiation using embryonic stem cells. *J Cell Biochem*. **96**, 1193-1202.

68. Cai, J., Zhao, Y., Liu, Y., Ye, F., Song, Z., Qin, H., et al. (2007). Directed differentiation of human embryonic stem cells into functional hepatic cells. *Hepatology*. **45**, 1229-1239.

69. Agarwal, S., Holton, K.L., and Lanza, R. (2008). Efficient differentiation of functional hepatocytes from human embryonic stem cells. *Stem Cells*. **26**, 1117-1127.

70. Edlund, H. (1999). Pancreas: how to get there from the gut? *Curr Opin Cell Biol*. **11**, 663-668.

71. Assady, S., Maor, G., Amit, M., Itskovitz-Eldor, J., Skorecki, K.L., and Tzukerman, M. (2001). Insulin production by human embryonic stem cells. *Diabetes*. **50**, 1691-1697.

72. Segev, H., Fishman, B., Ziskind, A., Shulman, M., and Itskovitz-Eldor, J. (2004). Differentiation of human embryonic stem cells into insulin-producing clusters. *Stem Cells*. **22**, 265-274.

73. Brolén, G.K., Heins, N., Edsbagge, J., and Semb, H. (2005). Signals from the embryonic mouse pancreas induce differentiation of human embryonic stem cells into insulin-producing beta-cell-like cells. *Diabetes*. **54**, 2867-2874.

74. Samadikuchaksaraei, A., Cohen, S., Isaac, K., Rippon, H.J., Polak, J.M., Bielby, R.C., et al. (2006). Derivation of distal airway epithelium from human embryonic stem cells. *Tissue Eng*. **12**, 867-875.

75. Van Vranken, B.E., Romanska, H.M., Polak, J.M., Rippon, H.J., Shannon, J.M., and Bishop, A.E. (2005). Coculture of embryonic stem cells with pulmonary mesenchyme: a microenvironment that promotes differentiation of pulmonary epithelium. *Tissue Eng*. **11**, 1177-1187.

76. Wang, D., Haviland, D.L., Burns, A.R., Zsigmond, E., and Wetsel, R.A. (2007). A pure population of lung alveolar epithelial type II cells derived from human embryonic stem cells. *Proc Natl Acad Sci USA*. **104**, 4449-4454.

77. Barberi, T., Willis, L.M., Socci, N.D., and Studer, L. (2005). Derivation of multipotent mesenchymal precursors from human embryonic stem cells. *PLoS Med*. **2**, e161.

78. Sottile, V., Thomson, A., and McWhir, J. (2003). In vitro osteogenic differentiation of human ES cells. *Cloning Stem Cells*. **5**, 149-155.

79. Kehat, I., Kenyagin-Karsenti, D., Snir, M., Segev, H., Amit, M., Gepstein, A., et al. (2001). Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest*. **108**, 407-414.

80. Laflamme, M.A., Chen, K.Y., Naumova, A.V., Muskheli, V., Fugate, J.A., Dupras, S.K., et al. (2007). Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat Biotechnol*. **25**, 1015-1024.

81. Chadwick, K., Wang, L., Li, L., Menendez, P., Murdoch, B., Rouleau, A., et al. (2003). Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. *Blood*. **102**, 906-915.

82. Kaufman, D.S., Hanson, E.T., Lewis, R.L., Auerbach, R., and Thomson, J.A. (2001). Hematopoietic colony-forming cells derived from human embryonic stem cells. *Proc Natl Acad Sci USA*. **98**, 10716-10721.

83. Vodyanik, M.A., Bork, J.A., Thomson, J.A., and Slukvin, I.I. (2005). Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. *Blood*. **105**, 617-626.

84. Levenberg, S., Golub, J.S., Amit, M., Itskovitz-Eldor, J., and Langer, R. (2002). Endothelial cells derived from human embryonic stem cells. *Proc Natl Acad Sci USA*. **99**, 4391-4396.

85. Metallo, C.M., Ji, L., de Pablo, J.J., and Palecek, S.P. (2007). Retinoic acid and bone morphogenetic protein signaling synergize to efficiently direct epithelial differentiation of human embryonic stem cells. *Stem Cells*. **26**, 372-380.

86. Aberdam, E., Barak, E., Rouleau, M., de LaForest, S., Berrih-Aknin, S., Suter, D.M., et al. (2008). A pure population of ectodermal cells derived from human embryonic stem cells. *Stem Cells*. **26**, 440-444.

87. Coffey, P.J., Girman, S., Wang, S.M., Hetherington, L., Keegan, D.J., Adamson, P., et al. (2002). Long-term preservation of cortically dependent visual function in RCS rats by transplantation. *Nat Neurosci*. **5**, 53-56.

88. Klimanskaya, I., Hipp, J., Rezai, K.A., West, M., Atala, A., and Lanza, R. (2004). Derivation and comparative assessment of retinal pigment epithelium from human embryonic stem cells using transcriptomics. *Cloning Stem Cells*. **6**, 217-245.

89. Gong, J., Sagiv, O., Cai, H., Tsang, S.H., and Del Priore, L.V. (2008). Effects of extracellular matrix and neighboring cells on induction of human embryonic stem cells into retinal or retinal pigment epithelial progenitors. *Exp Eye Res*. **86**, 957-965.

90. Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A., and Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotechnol*. **18**, 399-405.

91. Reubinoff, B.E., Itsykson, P., Turetsky, T., Pera, M.F., Reinhartz, E., Itzik, A., et al. (2001). Neural progenitors from human embryonic stem cells. *Nat Biotechnol*. **19**, 1134-1140.

92. Kornack, D.R., and Rakic, P. (2001). The generation, migration, and differentiation of olfactory neurons in the adult primate brain. *Proc Natl Acad Sci USA*. **98**, 4752-4757.

93. Zhang, S.C., Wernig, M., Duncan, I.D., Brustle, O., and Thomson, J.A. (2001). In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotechnol*. **19**, 1129-1133.

94. Li, X.J., Du, Z.W., Zarnowska, E.D., Pankratz, M., Hansen, L.O., Pearce, R.A., et al. (2005). Specification of motoneurons from human embryonic stem cells. *Nat Biotechnol*. **23**, 215-221.

95. Lee H., Shamy G.A., Elkabetz Y., Schofield C.M., Harrsion N.L., Panagiotakos G., et al. (2007). Directed differentiation and transplantation of human embryonic stem cell-derived motoneurons. *Stem Cells* **25**, 1931-1939.

96. Li, X.J., Hu, B.Y., Jones, S.A., Zhang, Y.S., Lavaute, T., Du, Z.W., et al. (2008). Directed differentiation of ventral spinal progenitors and motor neurons from human embryonic stem cells by small molecules. *Stem Cells*. **26**, 886-893.

97. Keirstead, H.S., Nistor, G., Bernal, G., Totoiu, M., Cloutier, F., Sharp, K., et al. (2005). Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. *J Neurosci*. **25**, 4694-4705.

98. Okabe, S., Forsberg-Nilsson, K., Spiro, A.C., Segal, M., and McKay, R.D. (1996). Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. *Mech Dev*. **59**, 89-102.

99. Lee, S.H., Lumelsky, N., Studer, L., Auerbach, J.M., and McKay, R.D. (2000) Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nat Biotechnol*. **18**, 675-679.

100. Barberi, T., Klivenyi, P., Calingasan, N.Y., Lee, H., Kawamata, H., Loonam, K., et al. (2003). Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice. *Nat Biotechnol*. **21**, 1200-1207.

101. Benzing, C., Segschneider, M., Leinhaas, A., Itskovitz-Eldor, J., and Brustle, O. (2006). Neural conversion of human embryonic stem cell colonies in the presence of fibroblast growth factor-2. *Neuroreport*. **17**, 1675-1681.

102. Gerrard, L., Rodgers, L., and 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.

103. Erceg, S., Laínez, S., Ronaghi, M., Stojkovic, P., Pérez-Aragó, M.A., Moreno-Manzano, V., et al. (2008). Differentiation of human embryonic stem cells to regional specific neural precursors in chemically defined medium conditions. *PLoS ONE*. **3**, e2122.

104. Perrier, A.L., Tabar, V., Barberi, T., Rubio, M.E., Bruses, J., Topf, N., et al. (2004). Derivation of midbrain dopamine neurons from human embryonic stem cells. *Proc Natl Acad Sci USA*. **101**, 12543-12548.

105. Yan, Y., Yang, D., Zarnowska, E.D., Du, Z., Werbel, B., Valliere, C., et al. (2005). Directed differentiation of dopaminergic neuronal subtypes from human embryonic stem cells. *Stem Cells*. **23**, 781-790.

106. Nusslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. *Nature*. **287**, 795-801.

107. Ingham, P.W. (1995). Signaling by hedgehog family proteins in Drosophila and vertebrate development. *Curr Opin Genet Dev*. **5**, 492-498.

108. Echelard, Y., Epstein, D.J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J.A., et al. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell*. **75**, 1417-1430.

109. Ekker, S.C., Ungar, A.R., Greenstein, P., Von Kessler, D.P., Porter, J.A., Moon, R.T., et al. (1995). Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. *Curr Biol*. **5**, 944-955.

110. Chiang, C., Litingtung, Y., Lee, E., Young, K.E., Corden, J.L., Westphal, H., et al. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature*. **383**, 407-413.

111. Briscoe, J., and Ericson, J. (2001). Specification of neuronal fates in the ventral neural tube. *Curr Opin Neurobiol*. **11**, 43-49.

112. Dessaud, E., Yang, L.L., Hill, K., Cox, B., Ulloa, F., Ribeiro, A., et al. (2007). Interpretation of the sonic hedgehog morphogen gradient by a temporal adaptation mechanism. *Nature*. **450**, 717-720.

113. Marigo, V., Davey, R.A., Zuo, Y., Cunningham, J.M., and Tabin, C.J. (1996). Biochemical evidence that patched is the hedgehog receptor. *Nature*. **384**, 176-179.

114. Apionishev, S., Katanayeva, N.M., Marks, S.A., Kalderon, D., and Tomlinson, A. (2005). Drosophila Smoothened phosphorylation sites essential for Hedgehog signal transduction. *Nat Cell Biol.* **7**, 86-92.

115. Rahnama, F., Shimokawa, T., Lauth, M., Finta, C., Kogerman, P., Teglund, S., et al. (2006). Inhibition of GLI1 gene activation by Patched1. *Biochem J.* **394***,* 19-26.

116. Litingtung, Y., and Chiang, C. (2000). Specification of ventral neuron types is mediated by an antagonistic interaction between Shh and Gli3. *Nat Neurosci*. **3**, 979-985.

117. Pan, Y., Bai, C.B., Joyner, A.L., and Wang, B. (2006). Sonic hedgehog signaling regulates Gli2 transcriptional activity by suppressing its processing and degradation. *Mol Cell Biol*. **26**, 3365-3377.

118. Mullor, J.L., Dahmane, N., Sun, T., and Ruiz i Altaba, A. (2001). Wnt signals are targets and mediators of Gli function. *Curr Biol*. **11**, 769-773.

119. Sasaki, H., Hui, C., Nakafuku, M., and Kondoh, H. (1997). A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. *Development*. **124**, 1313-1322.

120. van den Brink, G.R., Hardwick, J.C., Tytgat, G.N., Brink, M.A., Ten Kate, F.J., Van Deventer, S.J., et al. (2001). Sonic hedgehog regulates gastric gland morphogenesis in man and mouse. *Gastroenterology*. **121**, 317-328.

121. Chen, Y., and Struhl, G. (1996). Dual roles for patched in sequestering and transducing Hedgehog. *Cell*. **87**, 553-563.

122. Crossley, P.H., and Martin, G.R. (1995). The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development*. **121**, 439-451.

123. Crossley, P.H., Martinez, S., and Martin, G.R. (1996). Midbrain development induced by FGF8 in the chick embryo. *Nature*. **380**, 66-68.

124. Powers, C.J., McLeskey, S.W., and Wellstein, A. (2000). Fibroblast growth factors, their receptors and signaling. *Endocr Relat Cancer*. **7**, 165-197.

125. Heikinheimo, M., Lawshé, A., Shackleford, G.M., Wilson, D.B., and MacArthur, C.A. (1994). Fgf-8 expression in the post-gastrulation mouse suggests roles in the development of the face, limbs and central nervous system. *Mech Ddev*. **48**, 129-138.

126. Crossley, P.H., and Martin, G.R. (1995). The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development*. **121**, 439-451.

127. Crossley, P.H., Martinez, S., and Martin, G.R. (1996). Midbrain development induced by FGF8 in the chick embryo. *Nature*. **380**, 66-68.

128. Ye, W., Shimamura, K., Rubenstein, J.L., Hynes, M.A., and Rosenthal, A. (1998). FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell*. **93**, 755-766.

129. Yan, Y., Yang, D., Zarnowska, E.D., Du, Z., Werbel, B., Valliere, C., et al. (2005). Directed differentiation of dopaminergic neuronal subtypes from human embryonic stem cells. *Stem Cells*. **23**, 781-790.

130. Cho, M.S., Lee, Y.E., Kim, J.Y., Chung, S., Cho, Y.H., Kim, D.S., et al. (2008). Highly efficient and large-scale generation of functional dopamine neurons from human embryonic stem cells. *Proc Natl Acad Sci USA*. **105**, 3392-3397.

131. Geeta, R., Ramnath, R.L., Rao, H.S., and 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.

132. Schulz, T.C., Noggle, S.A., Palmarini, G.M., Weiler, D.A., Lyons, I.G., and Pensa, K.A. (2004). Differentiation of human embryonic stem cells to dopaminergic neurons in serum-free suspension culture. *Stem Cells*. **22**, 1218-1238.

133. Studer, L., Csete, M., Lee, S.H., Kabbani, N., Walikonis, J., Wold, B., et al. (2000). Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen. *J Neurosci*. **20**, 7377-7383.

134. Lin, L.F., Doherty, D.H., Lile, J.D., Bektesh, S., and Collins, F. (1993). GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science*. **260**, 1130-1132.

135. Choi-Lundberg, D.L., Lin, Q., Chang, Y.N., Chiang, Y.L., Hay, C.M., Mohajeri, H., et al. (1997). Dopaminergic neurons protected from degeneration by GDNF gene therapy. *Science*. **275**, 838-841.

136. Gash, D.M., Zhang, Z., Ovadia, A., Cass, W.A., Yi, A., Simmerman, L., et al. (1996). Functional recovery in parkinsonian monkeys treated with GDNF. *Nature*. **380**, 252-255.

137. Roussa, E., and Krieglstein, K.(2004). GDNF promotes neuronal differentiation and dopaminergic development of mouse mesencephalic neurospheres. *Neurosci Lett*. **361**, 52-55.

138. Buytaert-Hoefen, K.A., Alvarez, E., and Freed, C.R. (2004). Generation of tyrosine hydroxylase positive neurons from human embryonic stem cells after coculture with cellular substrates and exposure to GDNF. *Stem Cells*. **22**, 669-674.

139. Hyman, C., Hofer, M., Barde, Y.A., Juhasz, M., Yancopoulos, G.D., Squinto, S.P., et al. (1991). BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. *Nature*. **350**, 230-232.

140. Sautter, J., Meyer, M., Spenger, C., Seiler, R.W., and Widmer, H.R. (1998). Effects of combined BDNF and GDNF treatment on cultured dopaminergic midbrain neurons. *Neuroreport*. **9**, 1093-1096.

141. Rolletschek, A., Chang, H., Guan, K., Czyz, J., Meyer, M., and Wobus, A.M. (2001). Differentiation of embryonic stem cell-derived dopaminergic neurons is enhanced by survival-promoting factors. *Mech Dev*. **105**, 93-104.

142. Engele, J., and Franke, B. (1996). Effects of glial cell line-derived neurotrophic factor (GDNF) on dopaminergic neurons require concurrent activation of cAMP-dependent signaling pathways. *Cell Tissue Res*. **286**, 235-240.

143. Murer, M.G., Raisman-Vozari, R., Yan, Q., Ruberg, M., Agid, Y., and Michel P.P. (1999). Survival factors promote BDNF protein expression in mesencephalic dopaminergic neurons. *Neuroreport*. **10**, 801-805.

144. Hida, H., Jung, C.G., Wu, C.Z., Kim, H.J., Kodama, Y., Masuda, T., et al. (2003). Pleiotrophin exhibits a trophic effect on survival of dopaminergic neurons in vitro. *Eur J Neurosci*. **17**, 2127-2134.

145. Jung, C.G., Hida, H., Nakahira, K., Ikenaka, K., Kim, H.J., and Nishino, H. (2004). Pleiotrophin mRNA is highly expressed in neural stem (progenitor) cells of mouse ventral mesencephalon and the product promotes production of dopaminergic neurons from embryonic stem cell-derived nestin-positive cells. *FASEB J*. **18**, 1237-1239.

146. Mourlevat, S., Debeir, T., Ferrario, J.E., Delbe, J., Caruelle, D., Lejeune, O., et al. (2005). Pleiotrophin mediates the neurotrophic effect of cyclic AMP on dopaminergic neurons: analysis of suppression-subtracted cDNA libraries and confirmation in vitro. *Exp Neurol*. **194**, 243-254.

147. Lindholm, P., Voutilainen, M.H., Laurén, J., Peränen, J., Leppänen, V.M., Andressoo, J.O., et al. (2007). Novel neurotrophic factor CDNF protects and rescues midbrain dopamine neurons in vivo. *Nature*. **448**, 73-77.

148. Park, S., Lee, K.S., Lee, Y.J., Shin, H.A., Cho, H.Y., Wang, K.C.,et al. (2004). Generation of dopaminergic neurons in vitro from human embryonic stem cells treated with neurotrophic factors. *Neurosci Lett*. **359**, 99-103.

149. Hogan, B.L. (1996). Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev*. **10**, 1580-1594.

150. Groppe, J., Greenwald, J., Wiater, E., Rodriguez-Leon, J., Economides, A.N., Kwiatkowski, W., et al. (2002). Structural basis of BMP signalling inhibition by the cystine knot protein Noggin. *Nature*. **420**, 636-642.

151. Di-Gregorio, A., Sancho, M., Stuckey, D.W., Crompton, L.A., Godwin, J., Mishina, Y., et al. (2007). BMP signalling inhibits premature neural differentiation in the mouse embryo. *Development*. **134**, 3359-3369.

152. Zimmerman, L.B., De Jesus-Escobar, J.M., and Harland, R.M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell*. **86**, 599-606.

153. Itsykson, P., Ilouz, N., Turetsky, T., Goldstein, R.S., Pera, M.F., Fishbein, I., et al. (2005). Derivation of neural precursors from human embryonic stem cells in the presence of noggin. *Mol Cell Neurosci*. **30**, 24-36.

154. Sonntag, K.C., Pruszak, J., Yoshizaki, T., van Arensbergen, J., Sanchez-Pernaute, R., and 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.

155. Iacovitti, L., Donaldson, A.E., Marshall, C.E., Suon, S., and Yang, M. (2007). A protocol for the differentiation of human embryonic stem cells into dopaminergic neurons using only chemically defined human additives: Studies in vitro and in vivo. *Brain Res*. **1127**, 19-25.

156. Townes and Holtfreter (1955): directed movements and selective adhesion of embryonic amphibian cells. *J Exp Zoolog A Comp Exp Biol*. **301**, 701-706.

157. Redies C. (2000). Cadherins in the central nervous system. *Prog Neurobiol*. **61**, 611-648.

158. Colas, J.F., and Schoenwolf, G.C. (2001). Towards a cellular and molecular understanding of neurulation. *Dev Dynamics*. **221**, 117-145.

159. Kreis T.,Vale R. (1999). *Guidebook to the extracellular matrix, anchor, and adhesion proteins*, *2nd edition.* Oxford; New York: Sambrook & Tooze Publication at Oxford University Press.

160. Fässler, R., Georges-Labouesse, E., and Hirsch, E. (1996). Genetic analyses of integrin function in mice. *Curr Opin Cell Biol*. **8**, 641-646.

161. Hogeweg, P. (2000). Evolving mechanisms of morphogenesis: On the interplay between differential adhesion and cell differentiation. *J Theor Biol*. **203**, 317-333.

162. Steindler, D.A. (1993). Glial boundaries in the developing nervous system. *Annu Rev Neurosci*. 16, 445-470.

163. Venstrom, K.A., and Reichardt, L.F. (1993). Extracellular matrix: Role of extracellular matrix molecules and their receptors in the nervous system. *FASEB J*. **7**, 996-1003.

164. Ruoslahti, E., and Yamaguchi, Y. (1991). Proteoglycans as modulators of growth factor activities. *Cell*. **64**, 867-869.

165. Wen, X., Fuhrman, S., Michaels, G.S., Carr, D.B., Smith, S., Barker, J.L., et al. (1998). Large-scale temporal gene expression mapping of central nervous system development. *Proc Natl Acad Sci USA*. **95**, 334-339.

166. Colas, J.F., and Schoenwolf, G.C. Differential expression of two cell adhesion molecules, Ephrin-A5 and Integrin alpha6, during cranial neurulation in the chick embryo. *Dev Neurosci*. **25**, 357-365.

167. Daniloff, J.K., Chuong, C.M., Levi, G., and Edelman, G.M. (1986). Differential distribution of cell adhesion molecules during histogenesis of the chick nervous system. *J Neurosci*. **6**, 739-758.

168. Gall, C.M., Pinkstaff, J.K., Lauterborn, J.C., Xie, Y., and Lynch, G. (2003). Integrins regulate neuronal neurotrophin gene expression through effects on voltage-sensitive calcium channels. *Neuroscience*. **118**, 925-940.

169. De Arcangelis, A., and Georges-Labouesse, E. (2000). Integrin and ECM functions: Roles in vertebrate development. *Trends Genet*. **16**, 389-395.

170. Hynes, R.O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell*. **110**, 673-687.

171. Davy, A., and Robbins, S.M. (2000). Ephrin-A5 modulates cell adhesion and morphology in an integrin-dependent manner. *EMBO J*. **19**, 5396-5405. 19:5396-5405.

172. Guan, J.L. (1997). Role of focal adhesion kinase in integrin signaling. *Int J Biochem Cell Biol*. **29**, 1085-1096.

173. Schlaepfer, D.D., Hanks, S.K., Hunter, T., and van der Geer, P. (1994). Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature*. **372**, 786-791.

174. Schlaepfer, D.D., and Hunter, T. (1997). Focal adhesion kinase overexpression enhances ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src. *J Biol Chem*. **272**, 13189-13195.

175. Comoglio, P.M., Boccaccio, C., Trusolino, L. (2003). Interactions between growth factor receptors and adhesion molecules: breaking the rules. *Curr Opin Cell Biol*. **15**, 565-571.

176. Fässler, R., Rohwedel, J., Maltsev, V., Bloch, W., Lentini, S., Guan, K., et al. (1996). Differentiation and integrity of cardiac muscle cells are impaired in the absence of β1 integrin. *J Cell Sci*. **109**, 2989-2999.

177. Fässler, R., and Meyer, M. (1995). Consequences of lack of beta 1 integrin gene expression in mice. *Genes Dev*. **9**, 1896-1908.

178. Czyz, J., and Wobus, A. (2001). Embryonic stem cell differentiation: the role of extracellular factors. *Differentiation*. **68**, 167-174.

179. Goetz, A.K., Scheffler, B., Chen, H.X., Wang, S., Suslov, O., Xiang, H., et al. (2006). Temporally restricted substrate interactions direct fate and specification of neural precursors derived from embryonic stem cells. *Proc Natl Acad Sci USA*. **103**, 11063-11068.

180. Saha, K., Keung, A., Irwin, E., Li, Y., Little, L., Schaffer, D., et al. (2008). Substrate modulus directs neural stem cell behavior. *Biophys J*. [Epub ahead of print]

181. Kawasaki, H., Mizuseki, K., Nishikawa, S., Kaneko, S., Kuwana, Y., Nakanishi, S., et al. (2000). Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. *Neuron*. **28**, 31-40.

182. Owen, M. (1988). Marrow stromal stem cells. *J Cell Sci Suppl*. **10**, 63-76.

183. Perkins, S., and Fleischman, R.A. (1990). Stromal cell progeny of murine bone marrow fibroblast colony-forming units are clonal endothelial-like cells that express collagen IV and laminin. *Blood*. **75**, 620-625.

184. Majumdar, M.K., Thiede, M.A., Haynesworth, S.E., Bruder, S.P., and Gerson, S.L. (2001). Human marrow-derived mesenchymal stem cells (MSCs) express hematopoietic cytokines and support long-term hematopoiesis when differentiated toward stromal and osteogenic lineages. *J Hematother Stem Cell Res*. **9**, 841-848.

185. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., et al. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science*. **284**, 143-147.

186. Kuznetsov, S.A., Krebsbach, P.H., Satomura, K., Kerr, J., Riminucci, M., Benayahu, D., et al. (1997). Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. *J Bone Miner Res*. **12**, 1335-1347.

187. Kodama, H., Amagai, Y., Koyama, H., and Kasai, S. (1982). Hormonal responsiveness of a preadipose cell line derived from newborn mouse calvaria. *J Cell Physiol*. **112**, 83-88.

188. Kodama, H., Hagiwara, H., Sudo, H., Amagai, Y., Yokota, T., Arai, N., et al. (1986). MC3T3-G2/PA6 preadipocytes support in vitro proliferation of hemopoietic stem cells through a mechanism different from that of interleukin 3. *J Cell Physiol*. **129**, 20-26.

189. Kodama, H., Sudo, H., Koyama, H., Kasai, S., and Yamamoto, S. (1984). In vitro hemopoiesis within a microenvironment created by MC3T3-G2/PA6 preadipocytes. *J Cell Physiol*. **118**, 233-240.

190. Kawasaki, H., Suemori, H., Mizuseki, K., Watanabe, K., Urano, F., Ichinose, H., et al. (2002). Generation of dopaminergic neurons and pigmented epithelia from primate ES cells by stromal cell-derived inducing activity. *Proc Natl Acad Sci USA*. **99**, 1580-1585.

191. Morizane, A., Takahashi, J., Shinoyama, M., Ideguchi, M., Takagi, Y., Fukuda, H., et al. (2006). Generation of graftable dopaminergic neuron progenitors from mouse ES cells by a combination of coculture and neurosphere methods. *J Neurosci Res*. **83**, 1015-1027.

192. Takagi, Y., Takahashi, J., Saiki, H., Morizane, A., Hayashi, T., Kishi, Y., et al. (2005). Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model. *J Clin Invest*. **115**, 102-109.

193. Ohmachi, S., Watanabe, Y., Mikami, T., Kusu, N., Ibi, T., Akaike, A., et al. (2000). FGF-20, a novel neurotrophic factor, preferentially expressed in the substantia nigra pars compacta of rat brain. *Biochem Biophys Res Commun*. **277**, 355-360.

194. Zeng, X., Cai, J., Chen, J., Luo, Y., You, Z.B., Fotter, E., et al. (2004). Dopaminergic differentiation of human embryonic stem cells. *Stem Cells*. **22**, 925-940.

195. Park, C.H., Minn, Y.K., Lee, J.Y., Choi, D.H., Chang, M.Y., Shim, J.W., et al. (2005). In vitro and in vivo analyses of human embryonic stem cell-derived dopamine neurons. *J Neurochem*. **92**, 1265-1276.

196. Hong, S., Kang, U.J., Isacson, O., and Kim, K.S. (2008). Neural precursors derived from human embryonic stem cells maintain long-term proliferation without losing the potential to differentiate into all three neural lineages, including dopaminergic neurons. *J Neurochem*. **104**, 316-324.

197. Roybon, L., Brundin, P., and Li, J.Y. (2005). Stromal cell-derived inducing activity does not promote dopaminergic differentiation, but enhances differentiation and proliferation of neural stem cell-derived astrocytes. *Exp Neurol*. **196**, 373-380.

198. Parmar, M., and Li, M. (2007). Early specification of dopaminergic phenotype during ES cell differentiation. *BMC Dev Biol*. **7**, 86.

199. Schwartz, C.M., Spivak, C.E., Baker, S.C., McDaniel, T.K., Loring, J.F., Nguyen, C., et al. (2005). NTera2: a model system to study dopaminergic differentiation of human embryonic stem cells. *Stem Cells Dev*. **14**, 517-534.

200. Yamazoe, H., Murakami, Y., Mizuseki, K., Sasai, Y., and Iwata, H. (2005). Collection of neural inducing factors from PA6 cells using heparin solution and their immobilization on plastic culture dishes for the induction of neurons from embryonic stem cells. *Biomaterials*. **26**, 5746-5754.

201. Squire LR: Fundamental neuroscience, 2nd. Amsterdam, Academic Press, 2003

202. Missale, C., Nash, S.R., Robinson, S.W., Jaber, M., and Caron, M.G. (1998). Dopamine receptors: from structure to function. *Physiol Rev*. **78**, 189-225.

203. Elsworth, J.D., Roth, R.H. (1997). Dopamine synthesis, uptake, metabolism, and receptors: relevance to gene therapy of Parkinson's disease. *Exp Neurol*. **144**, 4-9.

204. Amara, S.G., Arriza, J.L. (1993). Neurotransmitter transporters: three distinct gene families. *Curr Opin Neurobiol*. **3**, 337-344.

205. Floresco, S.B., West, A.R., Ash, B., Moore, H., and Grace, A.A. (2003). Afferent modulation of dopamine neuron firing differentially regulates tonic and phasic dopamine transmission. *Nat Neurosci*. **6**, 968-973.

206. Joel, D., and Weiner, I. (2000). The connections of the dopaminergic system with the striatum in rats and primates: an analysis with respect to the functional and compartmental organization of the striatum. *Neuroscience*. **96**, 451-474.

207. Nelson, E.L., Liang, C.L., Sinton, C.M., and German, D.C. (1996). Midbrain dopaminergic neurons in the mouse: computer-assisted mapping. *J Comp Neurol*. **369**, 361-371.

208. Williams, S.M., Goldman-Rakic, P.S. (1998). Widespread origin of the primate mesofrontal dopamine system. *Cereb Cortex*. **8**, 321-345.

209. Blumenfeld H: Neuroanatomy through clinical cases, Sunderland, Mass., Sinauer Associates, 2002

210. Arsenault, M.Y., Parent, A., Séguéla, P., and Descarries, L. (1988). Distribution and morphological characteristics of dopamine-immunoreactive neurons in the midbrain of the squirrel monkey (Saimiri sciureus). *J Comp Neurol*. **267**, 489-506.

211. Cossette, M., Lecomte. F., and Parent, A. (2005). Morphology and distribution of dopaminergic neurons intrinsic to the human striatum. *J Chem Neuroanat*. **29**, 1-11.

212. Thompson, L., Barraud, P., Andersson, E., Kirik, D., and Björklund, A. (2005). Identification of dopaminergic neurons of nigral and ventral tegmental area subtypes in grafts of fetal ventral mesencephalon based on cell morphology, protein expression, and efferent projections. *J Neurosci*. **25**, 6467-6477.

213. Rogers, J.H. (1992). Immunohistochemical markers in rat brain: colocalization of calretinin and calbindin-D28k with tyrosine hydroxylase. *Brain Res*. **587**, 203-210.

214. McRitchie, D.A., Hardman, C.D., and Halliday, G.M. (1996). Cytoarchitectural distribution of calcium binding proteins in midbrain dopaminergic regions of rats and humans. *J Comp Neurol*. 364, 121-150.

215. Schein, J.C., Hunter, D.D., and Roffler-Tarlov, S. (1998). Girk2 expression in the ventral midbrain, cerebellum, and olfactory bulb and its relationship to the murine mutation weaver. *Dev Biol*. **204**, 432-450.

216. Inanobe, A., Yoshimoto, Y., Horio, Y., Morishige, K.I., Hibino, H., Matsumoto, S., et al. (1999). Characterization of G-protein-gated K+ channels composed of Kir3.2 subunits in dopaminergic neurons of the substantia nigra. *J Neurosci*. **19**, 1006-1017.

217. Yue, Y., Widmer, D.A., Halladay, A.K., Cerretti, D.P., Wagner, G.C., Dreyer, J.L., et al. (1999). Specification of distinct dopaminergic neural pathways: roles of the Eph family receptor EphB1 and ligand ephrin-B2. *J Neurosci*. **19**, 2090-2101.

218. Acampora, D., Gulisano, M., Broccoli, V., and Simeone, A. (2001). Otx genes in brain morphogenesis. *Prog Neurobiol*. **64**, 69-95.

219. Simeone, A., Puelles, E., and Acampora, D. (2002). The Otx family. *Curr Opin Gene. Dev*. **12**, 409-415.

220. Puelles, E., Acampora, D., Lacroix, E., Signore, M., Annino, A., Tuorto, F., Filosa, S., et al. (2003). Otx dose-dependent integrated control of antero-posterior and dorso-ventral patterning of midbrain. *Nat Neurosci*. **6**, 453-460.

221. Andersson, E., Tryggvason, U., Deng, Q., Friling, S., Alekseenko, Z., Robert, B., et al. (2006). Identification of intrinsic determinants of midbrain dopamine neurons. *Cell*. **124**, 393-405.

222. Andersson, E., Jensen, J.B., Parmar, M., Guillemot, F., and Björklund, A. (2006). Development of the mesencephalic dopaminergic neuron system is compromised in the absence of neurogenin 2. *Development*. **133**, 507-516.

223. Kele, J., Simplicio, N., Ferri, A.L., Mira, H., Guillemot, F., Arenas, E., et al. (2006). Neurogenin 2 is required for the development of ventral midbrain dopaminergic neurons. *Development*. **133**, 495-505.

224. Smidt, M.P., Asbreuk, C.H., Cox, J.J., Chen, H., Johnson, R.L., and Burbach, J.P. (2000). A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b. *Nat Neurosci*. **3**, 337-341.

225. Matsunaga, E., Katahira, T., and Nakamura, H. (2002). Role of Lmx1b and Wnt1 in mesencephalon and metencephalon development. *Development*. **129**, 5269-5277.

226. O'Hara, F.P., Beck, E., Barr, L.K., Wong, L.L., Kessler, D.S. and Riddle, R.D. (2005). Zebrafish Lmx1b.1 and Lmx1b.2 are required for maintenance of the isthmic organizer. *Development*. **132**, 3163-3173.

227. Guo, C., Qiu, H.Y., Huang, Y., Chen, H., Yang, R.Q., Chen, S.D., et al. (2007). Lmx1b is essential for Fgf8 and Wnt1 expression in the isthmic organizer during tectum and cerebellum development in mice. *Development*. **134**, 317-325.

228. Roussa, E., Farkas, L.M., and Krieglstein, K. (2004). TGF-beta promotes survival on mesencephalic dopaminergic neurons in cooperation with Shh and FGF-8. *Neurobiol*. **16**, 300-310.

229. Farkas, L.M., Dünker, N., Roussa, E., Unsicker, K., and Krieglstein, K. (2003). Transforming growth factor-beta(s) are essential for the development of midbrain dopaminergic neurons in vitro and in vivo. *J Neurosci*. **23**, 5178-5186.

230. Roussa, E., and Krieglstein, K. (2004). Induction and specification of midbrain dopaminergic cells: focus on SHH, FGF8, and TGF-beta. *Cell Tissue Res*. **318**, 23-33.

231. Parr, B.A., Shea, M.J., Vassileva, G., and McMahon, A.P. (1993). Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. *Development*. **119**, 247-261.

232. Megason, S.G., and McMahon, A.P. (2002). A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development*. **129**, 2087-2098.

233. Veeman, M.T., Axelrod, J.D., and Moon, R.T. (2003). A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. *Dev Cell*. **5**, 367-377.

234. Logan, C.Y., and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol*. **20**, 781-810.

235. McMahon, A.P., and Bradley, A. (1990). The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell*. **62**, 1073-1085.

236. Thomas, K.R., and Capecchi, M.R. (1990). Targeted disruption of the murine int-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature*. **346**, 847-850.

237. Danielian, P., and McMahon, A. (1996). Engrailed-1 as a target of the Wnt-1 signalling pathway in vertebrate midbrain development. *Nature*. **383**, 332-334.

238. Prakash, N., Brodski, C., Naserke, T., Puelles, E., Gogoi, R., Hall, A., et al. (2006). A Wnt1-regulated genetic network controls the identity and fate of midbrain-dopaminergic progenitors in vivo. *Development*. **1331**, 89-98.

239. Castelo-Branco, G., Wagner, J., Rodriguez, F.J., Kele, J., Sousa, K., Rawal, N., et al. (2003). Differential regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a. *Proc Natl Acad Sci USA*. **100**, 12747-12752.

240. Schulte, G., Bryja, V., Rawal, N., Castelo-Branco, G., Sousa, K.M., and Arenas, E. (2005). Purified Wnt-5a increases differentiation of midbrain dopaminergic cells and dishevelled phosphorylation. *J Neurochem*. **92**, 1550-1553.

241. Castelo-Branco, G., and Arenas, E. (2006). Function of Wnts in dopaminergic neuron development. *Neurodegener Dis*. **3**, 5-11.

242. Nornes, H.O., Dressler, G.R., Knapik, E.W., Deutsch, U., and Gruss, P. (1990). Spatially and temporally restricted expression of Pax2 during murine neurogenesis. *Development*. **109**, 797-809.

243. Asano, M., and Gruss, P. (1992). Pax-5 is expressed at the midbrain-hindbrain boundary during mouse development. *Mech Dev*. **39**, 29-39.

244. Pfeffer, P.L., Bouchard, M., and Busslinger, M. (2000). Pax2 and homeodomain proteins regulate a 435 bp enhancer of the mouse Pax5 gene at the midbrain-hindbrain boundary. *Development*. **127**, 1017-1028.

245. Urbánek, P., Fetka, I., Meisler, M.H., and Busslinger, M. (1997). Cooperation of Pax2 and Pax5 in midbrain and cerebellum development. *Proc Natl Acad Sci USA* . **94**, 5703-5708.

246. Schwarz, M., Alvarez-Bolado, G., Urbánek, P., Busslinger, M., and Gruss, P. (1997). Conserved biological function between Pax-2 and Pax-5 in midbrain and cerebellum development: evidence from targeted mutations. *Proc Natl Acad Sci USA*. **94**, 14518-14523.

247. Davis, C.A., and Joyner, A.L. (1988). Expression patterns of the homeo box-containing genes En-1 and En-2 and the proto-oncogene int-1 diverge during mouse development. *Genes Dev*. **2**, 1736-1744.

248. Simon, H.H., Saueressig, H., Wurst, W., Goulding, M.D., and O'Leary, D.D. (2001). Fate of midbrain dopaminergic neurons controlled by the engrailed genes. *J Neurosci*. **21**, 3126-3134.

249. Sonnier, L., Le Pen. G., Hartmann. A., Bizot. J.C., Trovero. F., Krebs. M.O., et al. (2007). Progressive loss of dopaminergic neurons in the ventral midbrain of adult mice heterozygote for Engrailed1. *J Neurosci*. **27**, 1063-1071.

250. Shamim, H., Mahmood, R., Logan, C., Doherty, P., Lumsden, A., and Mason, I. (1999). Sequential roles for Fgf4, En1 and Fgf8 in specification and regionalisation of the midbrain. *Development*. **126**, 945-959.

251. Shults, C.W., Hashimoto, R., Brady, R.M., and Gage, F.H. (1990). Dopaminergic cells align along radial glia in the developing mesencephalon of the rat. *Neuroscience*. **38**, 427-436.

252. Castro, D.S., Hermanson, E., Joseph, B., Wallen, A., Aarnisalo, P., Heller, A., et al. (2001). Induction of cell cycle arrest and morphological differentiation by Nurr1 and retinoids in dopamine MN9D cells. *J Biol Chem*. **276**, 43277-43284.

253. Saucedo-Cardenas, O., Quintana-Hau, J.D., Le, W.D., Smidt, M.P., Cox, J.J., De Mayo, F., et al. (1998). Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. *Proc Natl Acad Sci USA*. **95**, 4013-4018.

254. Smits, S.M., Ponnio, T., Conneely, O.M., Burbach, J.P., and Smidt, M.P. (2003). Involvement of Nurr1 in specifying the neurotransmitter identity of ventral midbrain dopaminergic neurons. *Eur J Neurosci*. **18**, 1731-1738.

255. Park, C.H., Kang, J.S., Kim, J.S., Chung, S., Koh, J.Y., Yoon, E.H., et al. (2006). Differential actions of the proneural genes encoding Mash1 and neurogenins in Nurr1-induced dopamine neuron differentiation. *J Cell Sci*. **119**, 2310-2320.

256. Ferri, A.L., Lin, W., Mavromatakis, Y.E., Wang, J.C., Sasaki, H., Whitsett, J.A., et al. (2007). Foxa1 and Foxa2 regulate multiple phases of midbrain dopaminergic neuron development in a dosage-dependent manner. *Development*. **134**, 2761-2769.

257. Barrett, T., Xie, T., Piao, Y., Dillon-Carter, O., Kargul, G.J., Lim, M.K., et al. (2001). A murine dopamine neuron-specific cDNA library and microarray: increased COX1 expression during methamphetamine neurotoxicity. *Neurobiol Dis*. **8**, 822-833.

258. Smidt, M.P., van Schaick, H.S., Lanctôt, C., Tremblay, J.J., Cox, J.J., van der Kleij, A.A., et al. (1997). A homeodomain gene Ptx3 has highly restricted brain expression in mesencephalic dopaminergic neurons. *Proc Natl Acad Sci USA*. **94**, 13305-13310.

259. Nunes, I., Tovmasian, L.T., Silva, R.M., Burke, R.E., and Goff, S.P. (2003). Pitx3 is required for development of substantia nigra dopaminergic neurons. *Proc Natl Acad Sci USA*. **100**, 4245-4250.

260. Smidt, M.P., Smits, S.M., Bouwmeester, H., Hamers, F.P., van der Linden, A.J., Hellemons, A.J., et al. (2004). Early developmental failure of substantia nigra dopamine neurons in mice lacking the homeodomain gene Pitx3. *Development*. **131**, 1145-1155.

261. Lynd-Balta, E., and Haber, S.N. (1994). The organization of midbrain projections to the striatum in the primate: sensorimotor-related striatum versus ventral striatum. *Neuroscience*. **59**, 625-640.

262. Hu, Z., Cooper, M., Crockett, D.P., and Zhou, R. (2004). Differentiation of the midbrain dopaminergic pathways during mouse development. *J Comp Neurol*. **476**, 301-311.

263. Kennedy, T.E. (2000). Cellular mechanisms of netrin function: long-range and short-range actions. *Biochem Cell Biol*. **78**, 569-575.

264. Mueller, B.K. (1999). Growth cone guidance: first steps towards a deeper understanding. *Annu Rev Neurosci*. **22**, 351-388.

265. Livesey, F.J., and Hunt, S.P. (1997). Netrin and netrin receptor expression in the embryonic mammalian nervous system suggests roles in retinal, striatal, nigral, and cerebellar development. *Mol Cell Neurosci*. **8**, 417-429.

266. Flores, C., Manitt, C., Rodaros, D., Thompson, K.M., Rajabi, H., Luk, K.C., et al. (2005). Netrin receptor deficient mice exhibit functional reorganization of dopaminergic systems and do not sensitize to amphetamine. *Mol Psychiatry*. **10**, 606-612.

267. Lin, L., Rao, Y., and Isacson, O. (2005). Netrin-1 and slit-2 regulate and direct neurite growth of ventral midbrain dopaminergic neurons. *Mol Cell Neurosci*. **28**, 547-555.

268. Lin, L., and Isacson, O. (2006). Axonal growth regulation of fetal and embryonic stem cell-derived dopaminergic neurons by Netrin-1 and Slits. *Stem Cells*. **24**, 2504-2513.

269. Marillat, V., Cases, O., Nguyen-Ba-Charvet, K.T., Tessier-Lavigne, M., Sotelo, C., and Chédotal, A. (2002). Spatiotemporal expression patterns of slit and robo genes in the rat brain. *J Comp Neurol*. **442**, 130-155.

270. Flanagan, J.G., and Vanderhaeghen, P. (1998). The ephrins and Eph receptors in neural development. *Annu Rev Neurosci*. **21**, 309-345.

271. Flanagan, J.G., Vanderhaeghen, P., Palmer, A., and Klein, R. (2003). Multiple roles of ephrins in morphogenesis, neuronal networking, and brain function. *Genes Dev*. **17**, 1429-1450.

272. Halladay, A.K., Yue, Y., Michna, L., Widmer, D.A., Wagner, G.C., and Zhou, R. (2000). Regulation of EphB1 expression by dopamine signaling. *Brain Res Mol Brain Res*. **85**, 171-178.

273. Parkinson, J. (2002). An essay on the shaking palsy. *J Neuropsychiatry Clin Neurosci*. **14**, 223-236.

274. Kempster, P.A., Hurwitz, B., and Lees, A.J. (2007). A new look at James Parkinson's essay on the shaking palsy. *Neurology*. **69**, 482-485.

275. Ehringer, H., and Hornykiewicz, O. (1960). Distribution of noradrenaline and dopamine (3-hydroxytyramine) in the human brain and their behavior in diseases of the extrapyramidal system. [Article in German] *Klin Wochenschr*. **38**, 1236-1239.

276. Hornykiewicz, O. (1963). The tropical localization and content of noradrenalin and dopamine (3-hydroxytyramine) in the substantia nigra of normal persons and patients with Parkinson's disease. *Wien Klin Wochenschr*. **75**, 302-312.

277. Hornykiewicz, O., and Kish, S.J. (1987). Biochemical pathophysiology of Parkinson's disease. *Adv Neurol*. **45**, 19-34.

278. Twelves, D., Perkins, K.S., and Counsell, C. (2003). Systematic review of incidence studies of Parkinson's disease. *Mov Disord*. **18**, 19-31.

279. Dluzen, D.E., and McDermott, J.L. (2000). Gender differences in neurotoxicity of the nigrostriatal dopaminergic system: implications for Parkinson's disease. *J Gend Specif Med*. **3**, 36-42.

280. Payami, H., and Zareparsi, S. (1998). Genetic epidemiology of Parkinson's disease. J *Geriatr Psychiatry Neurol*. **11**, 98-106.

281. Forno, L.S. (1996). Neuropathology of Parkinson's disease. *J Neuropathol Exp Neurol*. **55**, 259-272.

282. Ross, C.A., and Poirier, M.A. (2004). Protein aggregation and neurodegenerative disease. *Nat Med*. **10**, S10-S17.

283. Lewy, F.H., (1912). Paralysis agitans: Pathologische Anatomie. *Handbuch der Neurologic Band III* (pp. 920–933). Berlin: Springer–Verlag.

284. Spillantini, M.G., Schmidt, M.L., Lee, V.M., Trojanowski, J.Q., Jakes, R., and Goedert, M. Alpha-synuclein in Lewy bodies. *Nature*. **388**, 839-840.

285. Spillantini, M.G., Crowther, R.A., Jakes, R., Hasegawa, M., and Goedert, M. (1998). alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. *Proc Natl Acad Sci USA*. **95**, 6469-6473.

286. Clayton, D.F., and George, J.M. (1998). The synucleins: a family of proteins involved in synaptic function, plasticity, neurodegeneration and disease. *Trends Neurosci*. **21**, 249-254.

287. Abeliovich, A., Schmitz, Y., Fariñas, I., Choi-Lundberg, D., Ho, W.H., Castillo, P.E., et al. (2000). Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. *Neuron*. **25**, 239-252.

288. Jin, J., Meredith, G.E., Chen, L., Zhou, Y., Xu, J., Shie, F.S., et al. (2005). Quantitative proteomic analysis of mitochondrial proteins: relevance to Lewy body formation and Parkinson's disease. *Brain Res Mol Brain Res*. **134**, 119-138.

289. Trimmer, P.A., Borland, M.K., Keeney, P.M., Bennett, J.P. Jr, and Parker, W.D. Jr. (2004). Parkinson's disease transgenic mitochondrial cybrids generate Lewy inclusion bodies. *J Neurochem*. **88**, 800-812.

290. McNaught, K.S., and Olanow, C.W. (2006). Proteasome inhibitor-induced model of Parkinson's disease. *Ann Neurol*. **60**, 243-247.

291. Jenner, P. (1991). Oxidative stress as a cause of Parkinson's disease. *Acta Neurol Scand Suppl*. **136**, 6-15.

292. Jenner, P. (1993). Altered mitochondrial function, iron metabolism and glutathione levels in Parkinson's disease. *Acta Neurol Scand Suppl*. **146**, 6-13.

293. Hashimoto, M., Hsu, L.J., Xia, Y., Takeda, A., Sisk, A., Sundsmo, M., et al. Oxidative stress induces amyloid-like aggregate formation of NACP/alpha-synuclein in vitro. *Neuroreport*. **10**, 717-721.

294. Jenner, P. (1994). Oxidative damage in neurodegenerative disease. *Lancet*. **344**, 796-798.

295. Sherer, T.B., Betarbet, R., Stout, A.K., Lund, S., Baptista, M., Panov, A.V., et al. An in vitro model of Parkinson's disease: linking mitochondrial impairment to altered alpha-synuclein metabolism and oxidative damage. *J Neurosci*. **22**, 7006-7015.

296. Nagatsu, T. Amine-related neurotoxins in Parkinson's disease: past, present, and future. *Neurotoxicol Teratol*. **24**, 565-569.

297. Olanow, C.W. (1990). Oxidation reactions in Parkinson's disease. *Neurology*. **40** Suppl, 32-39.

298. Xu, J., Kao, S.Y., Lee, F.J., Song, W., Jin, L.W., and Yankner, B.A. (2002). Dopamine-dependent neurotoxicity of alpha-synuclein: a mechanism for selective neurodegeneration in Parkinson disease. *Nat Med*. **8**, 600-606.

299. Tabrizi, S.J., Orth, M., Wilkinson, J.M., Taanman, J.W., Warner, T.T., Cooper, J.M., et al. Expression of mutant alpha-synuclein causes increased susceptibility to dopamine toxicity. *Hum Mol Genet*. **19**, 2683-2689.

300. Zhou, Z.D., Yap, B.P., Gung, A.Y., Leong, S.M., Ang, S.T., and Lim, T.M. (2006). Dopamine-related and caspase-independent apoptosis in dopaminergic neurons induced by overexpression of human wild type or mutant alpha-synuclein. *Exp Cell Res*. **312**, 156-170.

301. Moussa, C.E., Wersinger, C., Tomita, Y., and Sidhu, A. (2004). Differential cytotoxicity of human wild type and mutant alpha-synuclein in human neuroblastoma SH-SY5Y cells in the presence of dopamine. *Biochemistry*. **43**, 5539-5550.

302. Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., et al. (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science*. **276**, 2045-2047.

303. Dawson, T.M., and Dawson, V.L. (2003). Rare genetic mutations shed light on the pathogenesis of Parkinson disease. *J Clin Invest*. **111**, 145-151.

304. Lücking, C.B., Dürr, A., Bonifati, V., Vaughan, J., De Michele, G., Gasser, T., et al. (2000). Association between early-onset Parkinson's disease and mutations in the parkin gene. *N Engl J Med*. **342**, 1560-1567.

305. Liu, Y., Fallon, L., Lashuel, H.A., Liu, Z., and Lansbury, P.T. Jr. (2002). The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility. *Cell*. **111**, 209-218.

306. Plun-Favreau, H., Klupsch, K., Moisoi, N., Gandhi, S., Kjaer, S., Frith, D., et al. (2007). The mitochondrial protease HtrA2 is regulated by Parkinson's disease-associated kinase PINK1. *Nat Cell Biol*. **9**, 1243-1252.

307. Carlsson, A., Lindqvist, M., and Magnusson, T. (1957). 3,4-Dihydroxyphenylalanine and 5-hydroxytryptophan as reserpine antagonists. *Nature*. **180**, 1200.

308. Gelb, D.J., Oliver, E., and Gilman, S. (1999). Diagnostic criteria for Parkinson disease. *Arch Neurol*. **56**, 33-39.

309. Hughes, A.J., Daniel, S.E., and Lees, A.J. (1993). The clinical features of Parkinson's disease in 100 histologically proven cases. *Adv Neurol*. **60**, 595-599.

310. Rajput, A.H., Rozdilsky, B., Rajput, A., and Ang, L. (1990). Levodopa efficacy and pathological basis of Parkinson syndrome. *Clin Neuropharmacol*. **13**, 553-558.

311. Hughes, A.J., Colosimo, C., Kleedorfer, B., Daniel, S.E., and Lees, A.J. (1992). The dopaminergic response in multiple system atrophy. *J Neurol Neurosurg Psychiatry*. **55**, 1009-1013.

312. Groppetti, A., Ceresoli, G., Mandelli, V., and Parenti, M. (1990). Role of opiates in striatal D-1 dopamine receptor supersensitivity induced by chronic Ldopa treatment. *J Pharmacol Exp Ther*. **253**, 950-956.

313. Bezard, E., Ferry, S., Mach, U., Stark, H., Leriche, L., Boraud, T., et al. (2003). Attenuation of levodopa-induced dyskinesia by normalizing dopamine D3 receptor function. *Nature Med*. **9**, 762-767.

314. Hossain, M.A., and Weiner, N. (1994). Dopaminergic functional supersensitivity: effects of chronic L-dopa and carbidopa treatment in an animal model of Parkinson's disease. *J Pharmacol Exp Ther*. **267**, 1105-1111.

315. Kostrzewa, R.M., Nowak, P., Kostrzewa, J.P., Kostrzewa, R.A., and Brus, R. (2005). Peculiarities of L-DOPA treatment of Parkinson's disease. *Amino Acids*. **28**, 157-164.

316. Marsden, C.D., and Parkes, J.D. (1977). Success and problems of long-term levodopa therapy in Parkinson's disease. *Lancet*. **1**, 345-349.

317. Neff, N.H., and Hadjiconstantinou, M. (1995). Aromatic L-amino acid decarboxylase modulation and Parkinson's disease. *Prog Brain Res*. **106**, 91-97.

318. Fiandaca, M., Forsayeth, J., and Bankiewicz, K. (2008). Current status of gene therapy trials for Parkinson's disease. *Exp Neurol*. **209**, 51-57.

319. Bankiewicz, K.S., Snyder, R., Zhou, S.Z., Morton, M., Conway, J., and Nagy, D. (1996). Adeno-associated (AAV) viral vector-mediated gene delivery in non-human primates. *Soc Neurosci Abstr*. **22**, 768.

320. During, M.J., Samulski, R.J., Elsworth, J.D., Kaplitt, M.G., Leone, P., Xiao, X., et al. (1998). In vivo expression of therapeutic human genes for dopamine production in the caudates of MPTP-treated monkeys using an AAV vector. *Gene Ther*. **5**, 820-827.

321. Eberling, J.L., Jagust, W.J., Christine, C.W., Starr, P., Larson, P., Bankiewicz, K.S., et al. (2008). Results from a phase I safety trial of hAADC gene therapy for Parkinson disease. *Neurology*. **70**, 1980-1983.

322. Hornykiewicz, O. (2001). Chemical neuroanatomy of the basal ganglia-normal and in Parkinson's disease. *J Chem Neuroanat*. **22**, 3-12.

323. François, C., Savy, C., Jan, C., Tande, D., Hirsch, E.C., and Yelnik, J. (2000). Dopaminergic innervation of the subthalamic nucleus in the normal state, in MPTP-treated monkeys, and in Parkinson's disease patients. *J Comp Neurol*. **425**, 121-129.

324. Rascol, O., Brooks, D.J., Korczyn, A.D., De Deyn, P.P., Clarke, C.E., and Lang, A.E. (2000). A five-year study of the incidence of dyskinesia in patients with early Parkinson's disease who were treated with ropinirole or levodopa. *N Engl J Med*. **342**, 1484-1491.

325. Jenner, P. (2008). Molecular mechanisms of L-DOPA-induced dyskinesia. *Nat Rev Neurosci*. **9**, 665-677.

326. Benabid, A.L., Pollak, P., Louveau, A., Henry, S., and de Rougemont, J. (1987). Combined (thalamotomy and stimulation) stereotactic surgery of the VIM thalamic nucleus for bilateral Parkinson disease. *Appl Neurophysiol*. **50**, 344-346.

327. Benabid, A.L., Pollak, P., Hommel, M., Gaio, J.M., de Rougemont, J., and Perret, J. (1989). Treatment of Parkinson tremor by chronic stimulation of the ventral intermediate nucleus of the thalamus. *Rev Neurol (Paris)*. **145**, 320-323.

328. Benabid, A.L., Pollak, P., Gross, C., Hoffmann, D., Benazzouz, A., Gao, D.M., et al. (1994). Acute and long-term effects of subthalamic nucleus stimulation in Parkinsons disease. *Stereotact Funct Neurosurg*. **62**, 76-84.

329. Benazzouz, A., Gross, C., Feger, J., Boraud, T., and Bioulac, B. (1993). Reversal of rigidity and improvement in motor performance by subthalamic high-frequency stimulation in MPTP-treated monkeys. *Eur J Neurosci*. **5**, 382-389.

330. Breit, S., Schulz, J.B., Benabid, A.L. (2004). Deep brain stimulation. *Cell Tissue Res*. **318**, 275-288.

331. Luo, J., Kaplitt, M.G., Fitzsimons, H.L., Zuzga, D.S., Liu, Y., Oshinsky, M.L., et al. (2002). Subthalamic GAD gene therapy in a Parkinson's disease rat model. *Science*. **298**, 425-429.

332. During, M.J., Kaplitt, M.G., Stern, M.B., and Eidelberg, D. (2001). Subthalamic GAD gene transfer in Parkinson disease patients who are candidates for deep brain stimulation. *Hum Gene Ther*. **12**, 1589-1591.

333. Kaplitt, M.G., Feigin, A., Tang, C., Fitzsimons, H.L., Mattis, P., Lawlor, P.A., et al. (2007). Safety and tolerability of gene therapy with an adeno-associated virus (AAV) borne GAD gene for Parkinson's disease: an open label, phase I trial. *Lancet*. **369**, 2097-2105.

334. Sladek, J.R., Redmond, D.E., Collier, T.J., Haber, S.N., Elsworth, J.D., Deutch, A.Y., et al. (1987). Transplantation of fetal dopamine neurons in primate brain reverses MPTP induced Parkinsonism. *Prog Brain Res*. **71**, 309-323.

335. Perlow, M.J., Freed, W.J., Hoffer, B.J., Seiger, A., Olson, L., and Wyatt, R.J. (1979). Brain grafts reduce motor abnormalities produced by destruction of nigrostriatal dopamine system. *Science*. **204**, 643-647.

336. Freed, W.J., Perlow, M.J., Karoum, F., Seiger, Å., Olson, L., Hoffer, B.J., et al. (1980). Restoration of dopaminergic function by grafting of foetal rat substantia nigra to the caudate nucleus: long term behavioral, biochemical, and histochemical studies. *Ann. Neurol.*, **8**, 510–519.

337. Björklund, A., and Stenevi, U. (1979). Reconstruction of the nigrostriatal dopamine pathway by intracerebral nigral transplants. *Brain Res*. **177**, 555-560.

338. Dunnett, S.B., Björklund, A., Stenevi, U., and Iversen, S.D. (1981). Behavioural recovery following transplantation of substantia nigra in rats subjected to 6-OHDA lesions of the nigrostriatal pathway. I. Unilateral lesions. *Brain Res*. **215**, 147-161.

339. Lane, E., and Dunnett, S. (2008). Animal models of Parkinson's disease and L-dopa induced dyskinesia: how close are we to the clinic? *Psychopharmacology (Berl)*. **199**, 303-312.

340. Freed, W.J. (2000). *Neural transplantation : an introduction*, Cambridge, Mass.: MIT Press.

341. Backlund, E.O., Granberg, P.O., Hamberger, B., Knutsson, E., Mårtensson, A., Sedvall, G., et a. (1985). Transplantation of adrenal medullary tissue to striatum in parkinsonism. First clinical trials. *J Neurosurg*. **62**, 169-173.

342. Lindvall, O., Brundin, P., Widner, H., Rehncrona, S., Gustavii, B., Frackowiak, R., et al. (1990). Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. *Science*. **247**, 574-577.

343. Freed, C.R., Breeze, R.E., Rosenberg, N.L., Schneck, S.A., Wells, T.H., Barrett, J.N., et al. (1990). Transplantation of human fetal dopamine cells for Parkinson's disease. Results at 1 year. *Arch Neurol*. **47**, 505-512.

344. Freed, C.R., Breeze, R.E., Rosenberg, N.L., Schneck, S.A., Kriek, E., Qi, J.X., et al. (1992). Survival of implanted fetal dopamine cells and neurologic improvement 12 to 46 months after transplantation for Parkinson's disease. *N Engl J Med*. **327**, 1549-1555.

345. Spencer, D.D., Robbins, R.J., Naftolin, F., Marek, K.L., Vollmer, T., Leranth, C., et al. (1992). Unilateral transplantation of human fetal mesencephalic tissue into the caudate nucleus of patients with Parkinson's disease. *N Engl J Med*. **327**, 1541-1548.

346. Lindvall, O., Sawle, G., Widner, H., Rothwell, J.C., Björklund, A., Brooks, D., et al. (1994). Evidence for long-term survival and function of dopaminergic grafts in progressive Parkinson's disease. *Ann Neurol*. **35**, 172-180.

347. Kordower, J.H., Freeman, T.B., Snow, B.J., Vingerhoets, F.J., Mufson, E.J., Sanberg, P.R., et al. (1995). Neuropathological evidence of graft survival and striatal reinnervation after the transplantation of fetal mesencephalic tissue in a patient with Parkinson's disease. *N Engl J Med*. **332**, 1118-1124.

348. Freeman, T.B., Olanow, C.W., Hauser, R.A., Nauert, G.M., Smith, D.A., Borlongan, C.V., et al. (1995). Bilateral fetal nigral transplantation into the postcommissural putamen in Parkinson's disease. *Ann Neurol*. **38**, 379-388.

349. Wenning, G.K., Odin, P., Morrish, P., Rehncrona, S., Widner, H., Brundin, P., et al. (1997). Short- and long-term survival and function of unilateral intrastriatal dopaminergic grafts in Parkinson's disease. *Ann Neurol*. **42**, 95-107.

350. Brundin, P., Pogarell, O., Hagell, P., Piccini, P., Widner, H., Schrag, A., et al. (2000). Bilateral caudate and putamen grafts of embryonic mesencephalic tissue treated with lazaroids in Parkinson's disease. *Brain*., **123**, 1380-1390.

351. Freed, C.R., Greene, P.E., Breeze, R.E., Tsai, W.Y., DuMouchel, W., Kao, R., et al. (2001). Transplantation of embryonic dopamine neurons for severe Parkinson's disease. *N Engl J Med*. **344**, 710-719.

352. Olanow, C.W., Goetz, C.G., Kordower, J.H., Stoessl, A.J., Sossi, V., Brin, M.F., et al. (2003). A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease. *Ann Neurol*. **54**, 403-414.

353. Hagell,P., Piccini, P., Bjorklund, A., Brundin, P., Rehncrona, S., and Widner, H., et al. (2002). Dyskinesias following neural transplantation in Parkinson's disease. *Nat Neurosci* **5**, 627–628.

354. Ma, Y., Feigin, A., Dhawan, V., Fukuda, M., Shi, Q., Greene, P., et al. (2002). Dyskinesia after fetal cell transplantation for parkinsonism: a PET study. *Ann Neurol*. **52**, 628-634.

355. Lindvall, O., and Björklund, A. (2004). Cell therapy in Parkinson's disease. *NeuroRx*. **1**, 382-393.

356. Freed, W.J., Sanchez, J.F., Lee, C.T., Vazin, T., Amable, R., et al. (2007). Human embryonic stem cells, dopaminergic neurons, and pathways for developing a Parkinson's disease therapy. In D. Emerich, C. Halberstad, (Eds.), *Cellular Transplantation: From Laboratory to Clinic* (pp. 523-544). Burlington: Academic Press.

357. Choong, C., Rao, M.S. (2007). Human embryonic stem cells. *Neurosurg Clin N Am*. **18**, 1-14.

358. Moon, S.Y., Park, Y.B., Kim, D.S., Oh, S.K., and Kim, D.W. (2006). Generation, culture, and differentiation of human embryonic stem cells for therapeutic applications. *Mol Ther*. **13**, 5-14.

359. Freeman, T.B., and Brundin, P. (2006). Important aspects of surgical methodology for transplantation in Parkinson's disease. *Restorative therapies in Parkinson's disease* (p. 365). New York: Springer.

360. Drukker, M., and Benvenisty, N. (2004). The immunogenicity of human embryonic stem-derived cells. *Trends Biotechnol*. **22**, 136-141.

361. Brederlau, A., Correia, A.S., Anisimov, S.V., Elmi, M., Paul, G., Roybon, L., et al. (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.

362. Takagi, Y., Takahashi, J., Saiki, H., Morizane, A., Hayashi, T., Kishi, Y., et al. (2005). Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model. *J Clin Invest*. **115**, 102-109.

363. Roy, N.S., Cleren, C., Singh, S.K., Yang, L., Beal, M.F., and Goldman, S.A. (2006). Functional engraftment of human ES cell-derived dopaminergic neurons enriched by coculture with telomerase-immortalized midbrain astrocytes. *Nat Med*. **12**, 1259-1268.

364. Yang, D., Zhang, Z.J., Oldenburg, M., Ayala, M., and Zhang, S.C. (2008). Human embryonic stem cell-derived dopaminergic neurons reverse functional deficit in parkinsonian rats. *Stem Cells*. **26**, 55-63.

365. Ben-Hur, T., Idelson, M., Khaner, H., Pera, M., Reinhartz, E., Itzik, A., et al. (2004). Transplantation of human embryonic stem cell-derived neural progenitors improves behavioral deficit in Parkinsonian rats. *Stem Cells*. **22**, 1246-1255.

366. Freed, C.R., Breeze, R.E., Schneck, S.A., Bakay, R.A.E, Ansari A.A. (1995). Fetal neural transplantation for Parkinson's disease. In R.R. Rich, (Ed.), *Clinical immunology: principles and practice* (pp. 1677-87). New York: Mosby.

367. Martinat, C., Bacci, J.J., Leete, T., Kim, J., Vanti, W.B., Newman, A.H., et al. (2006). Cooperative transcription activation by Nurr1 and Pitx3 induces embryonic stem cell maturation to the midbrain dopamine neuron phenotype. *Proc Natl Acad Sci USA*. **103**, 2874-2879.

368. Azuhata, T., Scott, D., Takamizawa, S., Wen, J., Davidoff, A., Fukuzawa, M., et al. (2001). The inhibitor of apoptosis protein survivin is associated with high-risk behavior of neuroblastoma. *J Pediatr Surg*. **36**, 1785-1791.

369. Storlazzi, C.T., Brekke, H.R., Mandahl, N., Brosjö, O., Smeland, S., Lothe, R.A, et al. (2006). Identification of a novel amplicon at distal 17q containing the BIRC5/SURVIVIN gene in malignant peripheral nerve sheath tumours. *J Pathol*. **209**, 492-500.

370. Kaplan, M.R., Cho, M.H., Ullian, E.M., Isom, L.L., Levinson, S.R., and Barres, B.A. (2001). Differential control of clustering of the sodium channels Na(v)1.2 and Na(v)1.6 at developing CNS nodes of Ranvier. *Neuron*. **30**, 105-119.

371. Couraud, F., Martin-Moutot, N., Koulakoff, A., and Berwald-Netter, Y. (1986). Neurotoxin-sensitive sodium channels in neurons developing in vivo and in vitro. *J Neurosci*. **6**, 192-198.

372. Zhang, L., Elmer, L.W., and Little, K.Y. (1998). Expression and regulation of the human dopamine transporter in a neuronal cell line. *Brain Res Mol Brain Res*. **59**, 66-73.

373. Carpenter, M.K., Cui, X., Hu, Z.Y., Jackson, J., Sherman, S., Seiger, A., et al. (1999). In vitro expansion of a multipotent population of human neural progenitor cells. *Exp Neurol*. **158**, 265-278.

374. Svendsen, C.N., Caldwell, M.A., Shen, J., ter Borg, M.G., Rosser, A.E., Tyers, P., et al. (1997). Long-term survival of human central nervous system progenitor cells transplanted into a rat model of Parkinson's disease. *Exp Neurol*. **148**, 135-46.

375. Moses, D., Drago, J., Teper, Y., Gantois, I., Finkelstein, D.I., and Horne, M.K. (2008). Fetal striatum- and ventral mesencephalon-derived expanded neurospheres rescue dopaminergic neurons in vitro and the nigro-striatal system in vivo. *Neuroscience*. **154**, 606-620.

376. Reynolds, B.A., Tetzlaff, W., and Weiss, S. (1992). A multipotent EGF responsive striatal embryonic progenitor cell produces neurons and astrocytes. *J Neurosci*. **12**, 4565-4574.

377. Svendsen, C.N., ter Borg, M.G., Armstrong, R.J., Rosser, A.E., Chandran, S., Ostenfeld, T., et al. (1998). A new method for the rapid and long term growth of human neural precursor cells. *J Neurosci Methods*. **85**, 141-152.

378. Davidson, K.C., Jamshidi, P., Daly, R., Hearn, M.T., Pera, M.F., and Dottori, M. (2007). Wnt3a regulates survival, expansion, and maintenance of neural progenitors derived from human embryonic stem cells. *Mol Cell Neurosci*. **36**, 408-415.

379. Temple, S. (2001). The development of neural stem cells. *Nature*. **414**, 112-117.

380. O' Keeffe, G.W., and Sullivan, A.M. (2005). Donor age affects differentiation of rat ventral mesencephalic stem cells. *Neurosci Lett*. **375**, 101-106.

381. Wu, H., Xu, J., Pang, Z.P., Ge, W., Kim, K.J., Blanchi, B., et al. (2007). Integrative genomic and functional analyses reveal neuronal subtype differentiation bias in human embryonic stem cell lines. *Proc Natl Acad Sci USA*. **104**, 13821-13826.

382. Wang, X., Lu, Y., Zhang, H., Wang, K., He, Q., Wang, Y., et al. (2004). Distinct efficacy of pre-differentiated versus intact fetal mesencephalon-derived human neural progenitor cells in alleviating rat model of Parkinson's disease. *Int J Dev Neurosci*. **22**, 175-183.

383. Carvey, P.M., Ling, Z.D., Sortwell, C.E., Pitzer, M.R., McGuire, S.O., Storch, A., et al. (2001). A clonal line of mesencephalic progenitor cells converted to dopamine neurons by hematopoietic cytokines: a source of cells for transplantation in Parkinson's disease. *Exp Neurol*. **171**, 98-108.

384. Herrera, D.G., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. (1999). Adult-derived neural precursors transplanted into multiple regions in the adult brain. *Ann Neurol*. **46**, 867-877.

385. Ourednik, J., Ourednik, V., Lynch, W.P., Schachner, M., and Snyder, E.Y. (2002). Neural stem cells display an inherent mechanism for rescuing dysfunctional neurons. *Nat Biotechnol*. **20**, 1103-1110.

386. Bjugstad, K.B., Teng, Y.D., Redmond, D.E. Jr., Elsworth, J.D., Roth, R.H., Cornelius, S.K., et al. (2008). Human neural stem cells migrate along the nigrostriatal pathway in a primate model of Parkinson's disease. *Exp Neurol*. **211**, 362-369.

387. Fransson, L.A. (1989). Heparan sulphate proteoglycans: structure and properties. In D.A. Lane & U. Lindhal (Eds), *Heparin. Chemical and Biological Properties and Clinical Applications* (p. 303). Londin: Edward Arnold.

388. Sasisekharan, R., and Venkataraman, G. (2000). Heparin and heparan sulfate: biosynthesis, structure and function. *Curr Opin Chem Biol*. **4**, 626-631.

389. Yayon, A., Klagsbrun, M., Esko, J.D., Leder, P., and Ornitz, D.M. (1991). Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell*. **64**, 841-848.

390. Lin, X. (2004). Functions of heparan sulfate proteoglycans in cell signaling during development. *Development*. **131**, 6009-6021.

391. Bleul, C.C., Fuhlbrigge, R.C., Casasnovas, J.M., Aiuti, A., and Springer, T.A. (1996). A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J Exp Med*. **184**, 1101-1109.

392. McGrath, K.E., Koniski, A.D., Maltby, K.M., McGann, J.K., and Palis, J. (1999). Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. *Dev Biol*. **213**, 442-456.

393. Tham, T.N., Lazarini, F., Franceschini, I.A., Lachapelle, F., Amara, A., and Dubois-Dalcq, M. (2001). Developmental pattern of expression of the alpha chemokine stromal cell-derived factor 1 in the rat central nervous system. *Eur J Neurosci*. **13**, 845-856.

394. Luo, Y., Cai, J., Xue, H., Mattson, M.P., and Rao, M.S. (2006). SDF1alpha/CXCR4 signaling stimulates beta-catenin transcriptional activity in rat neural progenitors. *Neurosci Lett*. **398**, 291-295.

395. Klein, R.S., Rubin, J.B., Gibson, H.D., DeHaan, E.N., Alvarez-Hernandez, X., Segal, R.A., et al. (2001). SDF-1 alpha induces chemotaxis and enhances Sonic hedgehog-induced proliferation of cerebellar granule cells. *Development*. **128**, 1971-1981.

396. Ma, Q., Jones, D., Borghesani, P.R., Segal, R.A., Nagasawa, T., Kishimoto, T., et al. (1998). Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc Natl Acad Sci USA*. **95**, 9448–9453.

397. Lu, M., Grove, E.A., and Miller, R.J. (2002). Abnormal development of the hippocampal dentate gyrus in mice lacking the CXCR4 chemokine receptor. *Proc Natl Acad Sci USA*. **99**, 7090-7095.

398. Gong, X., He, X., Qi, L., Zuo, H., and Xie, Z. (2006). Stromal cell derived factor-1 acutely promotes neural progenitor cell proliferation in vitro by a mechanism involving the ERK1/2 and PI-3K signal pathways. *Cell Biol Int*. **30**, 466-471.

399. Luo, Y., Cai, J., Liu, Y., Xue, H., Chrest, F.J., Wersto, R.P., et al. (2002). Microarray analysis of selected genes in neural stem and progenitor cells. *J Neurochem*. **83**, 1481-1497.

400. Edman, L.C., Mira, H., Erices, A., Malmersjö, S., Andersson, E., Uhlén, P., et al. (2008). Alpha-chemokines regulate proliferation, neurogenesis, and dopaminergic differentiation of ventral midbrain precursors and neurospheres. *Stem Cells*. **26**, 1891-1900.

401. Li, Y.S., Milner, P.G., Chauhan, A.K., Watson, M.A., Hoffman, R.M., Kodner, C.M., et al. (1990). Cloning and expression of a developmentally regulated protein that induces mitogenic and neurite outgrowth activity. *Science*. **250**, 1690-1694.

402. Kinnunen, T., Raulo, E., Nolo, R., Maccarana, M., Lindahl, U., and Rauvala, H. (1996). Neurite outgrowth in brain neurons induced by heparin-binding growth-associated molecule (HB-GAM) depends on the specific interaction of HB-GAM with heparan sulfate at the cell surface. *J Biol Chem*. **271**, 2243-2248.

403. Meng, K., Rodriguez-Peña, A., Dimitrov, T., Chen, W., Yamin, M., Noda, M., et al. (2000). Pleiotrophin signals increased tyrosine phosphorylation of beta beta-catenin through inactivation of the intrinsic catalytic activity of the receptor-type protein tyrosine phosphatase beta/zeta. *Proc Natl Acad Sci USA*. **97**, 2603-2608.

404. Marchionini, D.M., Lehrmann, E., Chu, Y., He, B., Sortwell, C.E., Becker, K.G., et al. (2007). Role of heparin binding growth factors in nigrostriatal dopamine system development and Parkinson's disease. *Brain Res*. **1147**, 77-88.

405. Rotwein, P., Burgess, S.K., Milbrandt, J.D., and Krause, J.E. (1988). Differential expression of insulin-like growth factor genes in rat central nervous system. *Proc Natl Acad Sci USA*. **85**, 265-269.

406. Bondy, C., Werner, H., Roberts, C.T. Jr., amd LeRoith, D. (1992). Cellular pattern of type-I insulin-like growth factor receptor gene expression during maturation of the rat brain: comparison with insulin-like growth factors I and II. *Neuroscience*. **46**, 909-923.

407. Feldman, E.L., Sullivan, K.A., Kim, B., and Russell, J.W. (1997). Insulin-like growth factors regulate neuronal differentiation and survival. *Neurobiol Dis*. **4**, 201-214.

408. Russo, V.C., Gluckman, P.D., Feldman, E.L., and Werther, G.A. (2005). The insulin-like growth factor system and its pleiotropic functions in brain. *Endocr Rev*. **26**, 916-43.

409. Bendall, S.C., Stewart, M.H., Menendez, P., George, D., Vijayaragavan, K., Werbowetski-Ogilvie, T., et al. (2007). IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro. *Nature*. **448**, 1015-1021.

410. Freed, W.J., Chen, J., Bäckman, C.M., Schwartz, C.M., Vazin, T., Cai, J., et al. (2008). Gene expression profile of neuronal progenitor cells derived from hESCs: activation of chromosome 11p15.5 and comparison to human dopaminergic neurons. *PLoS ONE*. **9**, e1422.

411. Davy, A., Aubin, J., and Soriano, P. (2004). Ephrin-B1 forward and reverse signaling are required during mouse development. *Genes Dev*. **18**, 572-583.

412. Dugas, J.C., Mandemakers, W., Rogers, M., Ibrahim, A., Daneman, R., and Barres, B.A. (2008). A novel purification method for CNS projection neurons leads to the identification of brain vascular cells as a source of trophic support for corticospinal motor neurons. *J Neurosci*. **28**, 8294-8305.