Derivation, Enrichment and Characterization of Dopaminergic Neurons from Pluripotent Stem Cells

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CHARACTERIZATION OF
DOPAMINERGIC NEURONS FROM
PLURIPOTENT STEM CELLS

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“The uniformity of earth's life, more astonishing than its diversity, is accountable by the high probability that we derived, originally, from some single cell, fertilized in a bolt of lightning as the earth cooled.”

Cover:

On the cover is a picture of pluripotent stem cells differentiating towards dopaminergic neurons. Medium conditioned by the mouse stromal cell line PA6 directs dopaminergic differentiation of the pluripotent human embryonal carcinoma stem cell line, NTera2. Dopaminergic neurons express the neuronal protein β III tubulin (green) and the dopamine synthesis protein tyrosine hydroxolase (red). This thesis, among other subjects, studied the ability of secreted factors from PA6 cells to induce dopaminergic differentiation of pluripotent stem cells.
ABSTRACT

Parkinson’s disease (PD) is characterized by progressive degeneration of dopaminergic (DA) neurons residing in the substantia nigra and innervating the striatum. Current medical interventions provide initial symptomatic relief, but unfortunately do not slow or reversing the disease course. Pluripotent stem cell (PSC) based replacement therapies are an attractive solution, since in theory PSC serve as an indefinite source capable of generating any somatic cell type. In recent years, derivation and preclinical transplantation of DA neurons from PSC have generated great enthusiasm in the field of regenerative medicine. Prior to clinical application, improved efficiency and a comprehensive understanding of molecular mechanisms governing DA differentiation is necessary. This thesis establishes and employs the NTera2 cell line as a PSC system to study, characterize and explore mechanisms and methods directing DA differentiation.

In paper I, the NTera2 cell line was established as a model system to examine DA neuron differentiation of hESC and expedite basic research. We showed that in addition to expression of PSC markers, undifferentiated NTera2 cells were similar to multiple hESC lines in overall gene expression profiles. Following co-culture with the stromal cell line PA6, NTera2 cells expressed DA and neuronal markers in a similar time frame and expression profile to what has been reported for hESC. We established important simplifications to the PA6 co-culture system including the use of PA6 conditioned medium (PA6 CM) to generate DA neurons. In a proof of principle approach, we used flow cytometry to select neuronal progenitors capable of generating functional DA neurons upon further differentiation.

In the following study (paper II), we designed, generated and validated a focused glial-DA array for the purpose of evaluating derived populations. Among the assessed populations, we examined both undifferentiated NTera2 cells and NTera2 derived neuronal progenitors directed towards DA neurons. Derivation techniques optimized in the NTera2 cell line were extrapolated to select neuronal progenitors from hESC differentiated towards DA neurons and examine their subsequent detailed genomic expression profiles (paper III). Interestingly, we observed activation of the 11.15p.5
chromosome, and an up-regulation of IGF2 and CDKN1C in both neuronal progenitors directed toward DA neurons and human substantia nigra DA neurons.

In paper IV, we identified several components of PA6 CM that were responsible for DA neuron differentiation. PA6 CM induced DA neuron differentiation in both NTera2 (hESC line) and I6 cells (hESC line). We indentified candidate DA inducing factors through comparative microarray gene expression analysis and mass spectroscopy analysis of PA6 CM. Following the addition of candidate factors (SDF1\(\alpha\), sFRP1 and VEGFD) we observed an increase in DA neuron differentiation in both the NTera2 and I6 cell lines.

In paper V, we report that flow cytometry selection of neuronal progenitors resulted in a three-fold increase in the number of DA neurons generated in PA6 CM. However, this differentiation capacity was observed in PA6 CM and differentiation in defined medium resulted in a more than 10 fold reduction in the number of DA neurons. Global microarray gene expression allowed us to examine the characteristics of progenitors and their more mature progeny.

Taken together, our data provide important insight into the molecular mechanisms that promote the differentiation of DA neurons from PSC.
LIST OF PUBLICATIONS (INCLUDED IN THESIS)


A Focused Microarray to Assess Dopaminergic and Glial Cell Differentiation from Fetal Tissue or Embryonic Stem Cells. (2006) Stem Cells.


The Identified Stromal Factors SDF1α, sFRP1 and VEGFD Induce Dopaminergic Neuron Differentiation of Human Pluripotent Stem Cells. 
Manuscript.

V. Catherine M Schwartz, Mahendra S Rao, Ernest Arenas and Mark P Mattson. 
Characterization and Maintenance of Neural Progenitors Derived from Human Pluripotent Stem Cells Differentiated Towards Dopaminergic Neurons. 
Manuscript.


* denotes equal contribution
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<tr>
<td>AADC</td>
<td>aromatic amino acid decarboxylase</td>
</tr>
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<td>Aldh1</td>
<td>aldehyde dehydrogenase</td>
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<td>ALDH2</td>
<td>aldehyde dehydrogenase 2</td>
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<td>A-P</td>
<td>anterior-posterior</td>
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<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<td>BMP</td>
<td>bone morphomgenic protein</td>
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<td>conserved dopamine neurotrophic factor</td>
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<td>central nervous system</td>
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<tr>
<td>CNTF</td>
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<td>EGC</td>
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<td>Frizzled</td>
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<td>growth arrest-specific 6</td>
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<td>Gbx2</td>
<td>gastrulation brain homoebox 2</td>
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<td>GDF3</td>
<td>growth differentiation factor 3</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<td>-------------------------------------------------</td>
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<tr>
<td>GDNF</td>
<td>glial cell-derived neurotrophic factor</td>
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<td>GIRK-2</td>
<td>G-protein regulated inwardly rectifier potassium channel</td>
</tr>
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<td>Gli-1</td>
<td>glioma-associated oncogene homolog 1</td>
</tr>
<tr>
<td>hECSC</td>
<td>human embryonal carcinoma stem cells</td>
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<tr>
<td>hPSC</td>
<td>human pluripotent stem cells</td>
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<tr>
<td>ICM</td>
<td>inner cell mass</td>
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<td>IGF</td>
<td>insulin like growth factor</td>
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<td>inducible pluripotent stem cells</td>
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<td>Krueppel-like factor 4</td>
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<td>L-DOPA</td>
<td>L-dihydroxyphenylalanin</td>
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<td>Lmx1a</td>
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<td>LIM homeodomain transcription factor b</td>
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<td>MACS</td>
<td>magnetic-activated cell sorting</td>
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<tr>
<td>MANF</td>
<td>mesencephalic astrocyte-derived neurotrophic factor</td>
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<td>Map2</td>
<td>microtubule-associated protein 2</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
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<tr>
<td>mDA</td>
<td>midbrain dopaminergic</td>
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<tr>
<td>MEK</td>
<td>mitogen-activated and extracellular signal-regulated kinase</td>
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<td>mESC</td>
<td>mouse embryonic stem cells</td>
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<td>MHB</td>
<td>midbrain-hindbrain boundary</td>
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<td>MPSS</td>
<td>Massive Parallel Signature Sequencing</td>
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<td>Msx1</td>
<td>muscle segment homeobox drosophila homolog of 1</td>
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<td>Ngn2</td>
<td>Neurogenin 2</td>
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<td>Otx2</td>
<td>orthodenticle homologue 2</td>
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<td>p-75</td>
<td>low affinity nerve growth factor receptor</td>
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<td>Pax2</td>
<td>paired box gene 2</td>
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<td>PD</td>
<td>Parkinson's Disease</td>
</tr>
<tr>
<td>PGC</td>
<td>primordial germ cells</td>
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<td>Pitx1</td>
<td>Paired-like homeodomain transcription factor 1</td>
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<tr>
<td>Pitx2</td>
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</tr>
<tr>
<td>Pitx3</td>
<td>Paired-like homeodomain transcription factor 3</td>
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<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
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<tr>
<td>PSA-NCAM</td>
<td>polysialated neural cell adhesion molecule</td>
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<tr>
<td>PSC</td>
<td>pluripotent stem cells</td>
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<tr>
<td>PTN</td>
<td>pleiotrophin</td>
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<tr>
<td>RA</td>
<td>retinoic acid</td>
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<tr>
<td>R-NSC</td>
<td>rosette neural stem cell</td>
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<tr>
<td>RP</td>
<td>roofplate</td>
</tr>
<tr>
<td>SDF1-α</td>
<td>stromal cell derived factor 1-alpha</td>
</tr>
<tr>
<td>SDIA</td>
<td>stromal-derived inducing activity</td>
</tr>
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sFRP1  secreted frizzled related protein 1
sFRPs  secreted frizzled related proteins
Shh  sonic hedgehog
Smo  Smoothened
SMOC1  SPARC related modular calcium binding 1
SNpc  substantia niagra pars compacta
Sox2  sex determining region Y box 2
SSEA  stage-specific embryonic antigens
TDGF1  teratocarcinoma-derived growth factor 1
TGF  transforming growth factor
TGFb3  transforming growth factor -b3
TH  tyrosine hydroxylase
TRA  tumor recognition antigens
TrkB  tyrosine kinase or BDNF/NT-3 growth factors receptor
TuJ1  β III Tubulin
UTF1  undifferentiated embryonic cell transcription factor 1
VEGF  vascular endothelial growth factor
VEGFD  vascular endothelial growth factor-D, c-fos
VM  ventral mesencephalon
VMAT2  vesicular monoamine transporter 2
VTA  vegmental area
VZ  ventricular zone
1 INTRODUCTION

1.1 PARKINSON’S DISEASE

Parkinson’s disease (PD) is a chronic and progressive degenerative disorder of the central nervous system that affects one in 100 individuals over the age of 60. Although PD is typically classified as an age-related disorder, it has been diagnosed in patients as young as 18 years of age. Genetic abnormalities are typically associated with early onset of the disease; however, the majority of patients are diagnosed with sporadic PD.

The midbrain region contains three groups of DA neurons: the retrorubral field referred to as A8, the ventral tegmental area (VTA) referred to as A10 and the substantia nigra pars compacta (SNpc) referred to as A9. The main pathology of PD results from progressive loss of A9 DA neurons that innervate the striatum region of the brain. This selective neuronal loss leads to decreased dopamine production, primarily resulting in the symptomatic hallmarks of the disease including tremors, rigidity, bradykinesia and posture instability (Jankovic, 2005). The leading theories to explain why DA neurons degenerate in PD include intracytoplasmic inclusions known as Lewy bodies, mitochondrial dysfunction, oxidative stress and impairment of the ubiquitin-proteasome system (Dauer and Przedborski, 2003). In addition to cell death and Lewy body formation in the SNpc, other populations of neurons also degenerate including noradrenergic neurons of the locus ceruleus and motor vagal nucleus, cholinergic neurons residing in the pedunculopontine nucleus pars compacta and the nucleus basalis of Meynert, the serotonergic neurons of the raphe nuclei, and central and peripheral components of the autonomic nervous system (Jellinger, 1991; Olanow and Tatton, 1999). As a result patients often experience secondary symptoms including autonomic dysfunction, cognitive impairment, neuropsychiatric problems, decreased sensory responses and sleep disturbances (Jankovic, 2008a)

1.1.1 Current treatments

Current pharmacological therapies for treatment of PD have been unsuccessful in slowing the disease progression and focus on symptomatic relief. Pharmaceutical approaches primarily increase dopamine levels through the administration of the dopamine precursor L-dihydroxyphenylalanin (L-DOPA), dopamine agonists that stimulate the dopamine receptors, or monoamine oxidase B inhibitors that reduce the
breakdown of dopamine. However, all of these pharmacological approaches lose their efficacy as the disease progresses inexorably to death. Surgical treatments were largely abandoned following the implementation of L-DOPA treatment; however, recent successful symptomatic alleviation with deep brain stimulation has moved it to the forefront of potential treatment options (Kringelback et al., 2007). Deep brain stimulation involves the implantation of a pacemaker device that delivers electrical stimulation to either the subthalamic nucleus, the internal globus pallidus or the pedunculopontine nucleus (Fukuda et al., 2001; Kringelback et al., 2007). Although, the exact mechanism of action is unknown, stimulation of these areas is thought to either inhibit inhibitory neurons or stimulate neuronal activity (Gildenberg, 2005; Kringelbach et al 2007). Alternative therapies including strategies aimed at dietary and lifestyle modifications have resulted in some successful pre-clinical and clinical outcomes (Palmer et al., 1986; Duan and Mattson, 1999; Maswood et al., 2004 Jankovic, 2008a;). Although the precise mechanisms are uncertain, it is thought that neuroprotective and neurorestorative pathways are involved (Mattson et al., 2002). Despite these concerted efforts, current therapies have been unable to restore function to PD patients.

1.1.2 Cell replacement therapy

Cell replacement strategies are designed to be a one-time treatment with long-term benefits for PD patients. The idea is based on transplantation of dopamine-producing cells or their progenitors into the SNpc and/or striatum to restore the normal control of movement. The foundation of this approach relies on the fact that a minimum level of dopamine is required to achieve normal motor function. Clinical application of cell replacement therapies requires a precise understanding of mechanisms governing DA neuron differentiation and a detailed characterization of derived cells. Several approaches, including the transplantation of primary fetal tissue and pluripotent stem cells, have been used in either clinical trials or preclinical animal models in an attempt to restore nigro-striatal function and lessen Parkinsonian symptoms.

1.1.2.1 Fetal mibrain tissue

Clinical trials involving transplantation of human fetal mesencephalic tissue in PD patients were first performed approximately 20 years ago (Lindvall et al., 1989; Freed et al., 1990). Such cell replacement strategies have demonstrated that grafted DA neurons can survive, reinnervate the striatum, release DA and become functionally
integrated in the host neural circuits (Lindvall et al., 1990; Lindvall et al., 1994; Kordower et al., 1995; Piccini et al., 1999, Barker et al., 1999). Additionally, in several reported cases patients have been able to discontinue L-DOPA treatment and functional improvements have lasted for as long as 6-10 years post transplantation (Lindvall et al., 1994; Wenning et al., 1997; Hagell et al., 1999; Piccini et al., 1999, Barker et al., 1999). While encouraging, functional outcomes have been highly variable with patients showing no or minimal improvement, worsening symptoms, the onset of dyskinesias and the emergence of Lewy body formation in the grafted tissue (Olanow et al., 2003; Freed et al., 2001; Hagell et al., 2002; Winkler et al., 2005; Bjorklund et al., 2003; Kordower et al., 2008; Li et al., 2010). Several contributing factors including tissue quality and quantity, the age and stage of disease progression in PD patients and immunosuppression have emerged as pivotal aspects of success (Piccini et al., 2005). Additionally, major limitations with respect to the availability of tissue have limited rigorous clinical testing and widespread application; because approximately 6-8 fetuses are needed to treat a single PD patient.

### 1.1.2.2 Embryonic stem cells and inducible pluripotent stem cells

In an effort to bypass issues pertaining to variable quality and availability of fetal tissue, many recent efforts have focused on developing pluripotent stem cell-based strategies for cell replacement therapy. In theory, pluripotent stem cells (PSC), such as embryonic stem cells (ESC) and inducible pluripotent stem cells (iPSC), provide a limitless source that can be standardized and optimized to generate the appropriate cell population(s) to achieve maximum functional recovery. DA neurons have been efficiently generated from human ESC and iPSC (Odorico et al., 2001; Zeng et al., 2004; Cai et al., 2009b; Soldner et al., 2009; Chambers et al., 2009; Sacchetti et al., 2009). However, transplantations in animal models have been unsuccessful thus far and highlight risks associated with pluripotent stem cells including teratoma formation, neuroepithelial tumors and the presence of non-neural phenotypes within the grafted tissue (Schulz et al., 2004; Zeng et al., 2004; Brederlau et al., 2006; Roy et al., 2006; Cai et al., 2009b). Clinical application of hESC- or hiPSC-derived DA neurons will require a detailed understanding of transcription factors, secreted molecules and pathways governing DA neuron differentiation. Moreover, improvements in survival and integration of derived DA neurons, as well as prevention of undesired populations are additional impediments that need to be overcome prior to clinical trials.
1.2 PLURIPOTENT STEM CELL

1.2.1 Stem cell biology
A stem cell has the innate capacity to self-renew and generate one or more different cell types along a developmental path. Stem cells are present throughout the life cycle of many multi-cellular organisms including humans, from the fertilized egg to the adult, where most tissues have been shown to contain at least small numbers of stem cells. There are three major levels of stem cell classification distinguished by their capacity to differentiate. Totipotent stem cells possess the highest level of developmental potential and the ability to generate the embryo proper and trophectodermal tissue. Totipotent stem cells only exist in the zygote up to the 8-cell morula stage prior to compaction and cell-initiated polarization. Approximately 24 hours following the morula stage the blastocyst forms by the development of an inner fluid-filled cavity containing an aggregate of cells referred to as the inner cell mass (ICM). The ICM contributes to the embryo proper and extraembryonic endoderm. The cells in the ICM have the ability to generate all cells in the adult organism and are therefore PSC. Following implantation, the ICM continues to proliferate and undergoes gastrulation to produce the three germ lineages. Multipotent stem cells are the third level of stem cell classification that can be found in both fetal and adult tissues. These stem cells are restricted in their differentiation capacity to generate progenitors cells that progressively give rise to more differentiated cell types that ultimately terminally differentiate to a specific somatic cell type (Fuchs et al., 2000).

1.2.2 Characteristics of pluripotency
PSC share a common set of attributes independent of their origin that can be defined in both functional and molecular terms (Ralston et al., 2010). Functional pluripotency refer to the ability of a cell to give rise to cell types of all three embryonic germ layers: mesoderm, ectoderm and endoderm. Molecular pluripotency necessitates the identification of factors that support their functional properties. Although some molecular characteristics are not unique to PSC they serve as a means to separate and identify them from their somatic counterparts.

1.2.2.1 Functional pluripotency
All PSC can differentiate in vitro and in vivo into cell types representative of the three primary germ layers: mesoderm, endoderm and ectoderm. The developmental potential
of PSC is typically tested by three independent assays: 1) spontaneous differentiation in cell culture; 2) in vivo differentiation by the formation of tertomas or teratocarcinoma by injection into an immune compromised adult animal; and 3) in vivo differentiation by chimera incorporation when cells are introduced into the cavity of a developing blastocyst prior to implantation. Due to ethical concerns, chimera incorporation is not always performed on hPSC lines (Committee on Guidelines for Human Embryonic Stem Cell Research and National Research Council, 2005). Both in vivo and in vitro assessment typically involves the evaluation of derived cells for acquisition of a variety of mesoderm-, endoderm- and ectoderm-specific markers and loss of pluripotent markers.

1.2.2.2 Markers and molecular pluripotency

**Cell surface antigen expression:**
The globoseries oligosaccharide stage-specific embryonic antigens-3 and-4 (SSEA-3 and -4) and the keratan sulfate tumor recognition antigens (TRA) TRA-1-60 and TRA-1-81 were first used to identify early developmental stages and teratocarcinomas. Human and non-human primate PSC typically express SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 in the undifferentiated state (Thomson et al., 1995; Andrews et al., 1996; Thomson et al., 1998; Adewumi et al., 2007, Okita et al., Yu et al., 2007). The functions of these cell surface antigens are unknown; however, they have proven useful in cell selection strategies to enrich or eliminate PSC from mixed cell populations (Sundberg et al., 2009; Fong et al., 2009).

**Transcription Factors:**
There are many factors that are selectively expressed in PSC, including Lin28, GDF3, UTF1, DNMT3B, Rex1 and TDGF1 (Adewumi et al., 2007). However, Oct 4 (also known as Pou5f1), Nanog, and Sox2 are three transcription factors that participate in a regulatory network that is pivotal for the maintenance of pluripotency. These three transcription factors co-occupy putative enhancer elements of genes to promote pluripotency and repress differentiation (Boyer et al., 2005; Rodda et al., 2005; Lee et al., 2006; Loh et al., 2006).
Oct4, Nanog and Sox2 are homeobox transcription factors that were initially discovered in mESC. Early studies examining targeted deletions and conditional repression and expression in ESCs revealed that Oct4 is critical for pluripotency and can prevent differentiation into mesoderm, endoderm or trophectoderm (Nichols et al., 1998; Niwa et al., 2000; Hay et al., 2004). However, Oct4 alone was unable to maintain a pluripotent phenotype (Hay et al., 2004). Similar to Oct4, reduction in levels of Nanog expression resulted in trophoectoderm, mesoderm, endoderm and ectoderm differentiation (Hyslop et al., 2005; Hatano et al., 2005; Zaehres et al. 2005). Although Sox2 is not uniquely expressed in PSC, it is associated with maintaining the phenotypes of neural stem cells (NSC) and PSC (Li et al. 1998; Zappone et al., 2000; Avilion et al., 2003). Similar to both Oct4 and Nanog, disruption of Sox2 expression results in differentiation of PSC (Avilion et al., 2003). The central significance of Oct4, Nanog and Sox2 to PSC biology is confirmed by the requirement of these genes in maintaining pluripotency in early embryogenesis.

Epigenetics:
In recent years remarkable progress has been made in defining and manipulating pluripotency. Epigenetics refers to the underlying mechanisms of coordinated gene control that affect gene expression without changes in DNA sequence. Although epigenetic status may not provide a readout of gene expression, patterns of DNA methylation and chromatin modifications such as histone acetylation can provide insight into underlying molecular mechanisms that persist through cell division to effect pluripotency and differentiation (Jaenisch and Bird et al., 2003; Richards, 2006). Several epigenetic characteristics are typically associated with PSC including little or no DNA methylation at pluripotent gene promoter regions and a less compact chromatin structure. Recent advances allowing whole genome assessment of DNA methylation and histone modifications have found that profiles of human PSC were distinct from somatic cells, suggesting possible differences in regulatory mechanisms (Bibikova et al., 2006; Barski et al., 2007; Mikkelsen et al., 2008). It is likely that multiple epigenetic elements influence pluripotency and that a balance between these factors regulates lineage-specific differentiation (Bibikova et al., 2008).
1.2.3 Type of pluripotent stem cells (PSC)

PSC can be classified into four main cell types: embryonic stem cells (ESC), inducible pluripotent stem cells (iPSC), embryonic germ cells (EGC) and embryonal carcinoma stem cells (ECSC). Although these cell types have diverse origins, they all exhibit both functional and molecular pluripotency.

1.2.3.1 Embryonic stem cells (ESC)

Embryonic stem cells (ESC) have been derived from the ICM of human and various other mammalian pre-implantation blastocysts (Evans and Kaufman, 1981; Thomson et al., 1995; Thomson et al., 1998; Rossant, 2001). Established ESC lines display a remarkable proliferative capacity and the ability to generate a multitude of somatic cell types. Since their discovery, ESC have been recognized as a potential source of progenitor cells for restorative therapies to treat debilitating injuries and diseases. Although some argue that ESC are a result of continued cell culture and there is no in vivo counterpart that remains proliferative in an undifferentiated state (Smith, 2001; Rossant et al., 2001; Buehr and Smith, 2003), ESC are considered the gold standard of pluripotency by which other pluripotent cell lines are typically compared.

1.2.3.2 Inducible pluripotent stem cells (iPSC)

Unlike other pluripotent cell types, inducible pluripotent stem cells (iPSC) originate from a non-pluripotent cell through the forced expression of certain genes or exposure to specific proteins. iPSCs provide a remarkable tool for understanding molecular reprogramming resulting in self-renewal and pluripotency, since the somatic cells from which they are derived do not possess these unique properties. The first reports of iPSCs from fibroblasts involved the introduction of four genes (Oct4, Sox2, c-Myc and Klf4) by retrovirus (Takahashi and Yamanaka et al., 2006). Subsequent studies have used various gene combinations and delivery methods (Takahashi et al., 2007; Okita et al., 2007; Feng et al., 2009; Soldner et al., 2009). Molecular reprogramming results in genome-wide modifications resulting in epigenetic profiles that resemble pluripotent ESC rather than the somatic cells from which they are derived. iPSC provide a unique opportunity to derive disease-specific stem cells that provide a novel way in which to study disease progression and develop pharmaceuticals (Park et al., 2008).

Importantly, iPSC are a significant advancement in future clinical stem cell research allowing for patient-specific cell replacement therapies that could evade the adaptive immune response and lack the ethical implications of embryos. Although iPSC offer an
ethical and immunological advantage over ESCs, the technological limitations such as reprogramming efficiency and therapeutic safety require additional examination.

1.2.3.3  *Embryonic germ cells (EGC)*

Embryonic germ cells (EGC) are derived from the primodial germ cells (PGC) in the gonads of the developing embryo (Resnick et al., 1992; Shamblott et al., 1998). PGC undergo substantial epigenetic modifications resulting in erasure of imprints, genome wide demethylation, and X chromosome reactivation in female PGC. hEGC were derived several years prior to hESCs and viewed as a model to study human development.

1.2.3.4  *Embryonal carcinoma stem cells (ECSC)*

Embryonal carcinoma stem cells (ECSC) are the founding cells of teratocarcinomas or testicular germ cell tumors (Kleinsmith et al., 1964; Andrews et al., 1988). Teratocarinomas typically consist of a variety of tissues such as bone, cartilage, muscle, epithelium and neuroectoderm (Damjanov et al., 1979; Andrews et al., 1988). ECSC were the first recognized pluripotent cell type and thus have played a key role in establishing markers and culture system for ESC (Damjanov et al., 2005). Some ECSC lines have reported lineage-restricted differentiation that may limit their application in pluripotent differentiation (Bahrami et al., 2005).

1.2.3.5  *Alternative systems to study human embryonic stem cell biology*

Despite their promise, hESC are one of the more challenging culture systems. Typical methods for maintaining pluripotency involve culturing cells on fibroblast feeder cells supplemented with basic fibroblast growth factor (bFGF), in the absence of feeder cells and in medium conditioned by fibroblast cells or a defined medium containing high concentrations of bFGF and/or Noggin (Wang et al. 2005; Mallon et al 2006; Xu et al., 2005). These methods can be time consuming, expensive, and problematic for routine culture purposes, because hESC rapidly undergo differentiation when maintained in suboptimal conditions. Additionally, cells grow relatively slowly and physical isolation if often required to remove differentiated cells because enzymatic passaging has been linked to chromosomal abnormalities (Mitalipova et al., 2005). Current culture techniques need improvement and limit advances in stem cell research. Therefore, it becomes necessary to develop model systems that can serve to efficiently explore differentiation paradigms and mechanisms involved in cell type specification.
Model systems that have been used to expedite hESC studies include karyotypically abnormal variants of established hESC lines, non-human ESCs and teratocarcinoma derived hECSC lines. Karyotypically abnormal hESC lines, such as BG01V and BG02V, remain the closest model to hESC as they behave similarly to their normal counterparts (Zeng X et al. 2004; Plaia et al., 2006; Vazin et al., 2008a). However, for non-established variant hESC lines or variant hESC maintained for extended periods in culture, rigorous testing is necessary to determine retention of pluripotent characteristics and intact differentiation capabilities. Additionally, these variant lines require the same propagation techniques and their growth rates are not significantly faster than their karyotypically normal counterparts.

Mouse ESC (mESC) grow more rapidly due to a shorter cell cycle, some lines can be easily propagated without feeder cells and they share many common features with hESC (Sato et al., 2003). Although a great deal of our current knowledge of PSC biology and differentiation was founded on mESC, key differences in pathway regulation and pluripotent marker expression exist between hESC (Ginis et al., 2004; Rao, 2004; Sato et al., 2004; Koestenbauer et al., 2006). When grown in culture non-human primate derived ESC are more similar to hESC in terms of morphology and known regulatory pathways, although comprehensive comparisons have not been performed (Thomson et al., 1995; Nakatsuji, 2002). Similar to murine and human ESC, optimal pluripotency of non-human primate ESC is typically maintained by culture with fibroblast feeder cells. Teratocarcinoma derived hECSC lines are of germ cell origin and share many pluripotency characteristics with hESC (Przyborski et al., 2004; Schwartz et al., 2005). Unlike hESC and other proposed alternatives, NTera2, a human ECSC line, is most favorably grown in the absence of feeder cells, recovers easily from freeze-thaw, has a relatively short cell cycle, and can be routinely cultured to obtain large numbers while retaining a relative homogeneous phenotype. Therefore, the NTera2 cell line provides an ideal system to easily optimize methods and explore differentiation mechanisms of hESC in vitro.

1.2.3.6 NTera2 cell line, a pluripotent human embryonal carcinoma stem cell line

The TERA2 cell line is a widely studied pluripotent human ECSC line originally derived from a lung metastasis in a patient diagnosed with testicular teratocarcinoma.
A subline of the TERA2, the NTera2 cell line was derived from a xenograft tumor produced in an immune compromised mouse (Andrews et al., 1984a). The NTera2 cell line is hypotriploid containing on average 12 marker chromosomes with approximately 48% of the cells having 63 chromosomes and 24% having 62 chromosomes (Plaia et al., 2006; American Type Culture Collection, 2010). Most cells contain a normal Y chromosome and single copies of chromosome 1, 10, 11 and 13 while others chromosomes exist mainly as two to three copies per cell (American Type Culture Collection, 2010). Although NTera2 cells are genetically abnormal, they maintain a stable karyotype over prolonged passaging and differentiation (Trojanowski et al., 1993; Duran et al., 2001).

NTera2 cells express characteristic markers and display an epigenetic signature typical of PSC (Andrews et al., 1998; Schwartz et al 2005; Skotheim et al., 2005; Bibikova et al., 2006; Josephson et al., 2007). Pluripotent markers such as Oct 4, Nanog, SSEA-3, SSEA-4, TRA-1-81, TRA-1-60 are expressed in undifferentiated NTera2 cells (Draper et al., 2002; Przyborski et al., 2004; Schwartz et al, 2005; Josephson et al., 2007). Several global microarray gene expression analyses of NTera2 cells revealed overall gene expression profiles were somewhat similar to other pluripotent cell lines including hESC, karyotypically abnormal variant hESC lines and human germ cell tumors (Schwartz et al., 2005; Skotheim et al., 2005; Plaia et al., 2006; Josephson et al., 2007). Recent large scale comparisons have revealed that overall methylation patterns of NTera2 cells are similar to multiple hESC lines, however, different methylation status of several imprinted genes have also been observed (Bibikova et al., 2006; Plai et al., 2006). Such a pattern would be consistent with a PGC origin in which all inherited imprints are absent, and thus could potentially affect their differentiation capacity.

The NTera2 cell line has been used as an in vitro and in vivo tool to explore human neural development, as it follows similar pathways observed in neural ectoderm vertebrate development (Andrews et al., 1998). Single clones of the NTera2 cell line expressed characteristics similar to other hESC lines, such as 2102Ep and generated similar teratocarcinoma xenografts to the original TERA2 and NTera2 cell lines (Andrews et al., 1984a). Xenograft studies examining their differentiation capacity found various progenitor- and tissue-specific cell types of mesoderm, endoderm and ectoderm origin (Andrews et al., 1984a; Duran et al., 2001). Differentiation of NTera2 cells by embryoid body formation, an in vitro protocol used to induce spontaneous
differentiation in hESC, resulted in mesoderm, endoderm and ectoderm cell types (Pal et al., 2006). Following the addition of all-trans retinoic acid (RA), cells rapidly lost expression of pluripotent markers SSEA-3, SSEA-4 and TRA-1-60 (Fenderson et al., 1987) and began expressing neurofilament proteins (Andrews 1984b; Lee and Andrews, 1986). Remarkably these derived neurons displayed functional neuronal characteristics including electrical excitability responsivity to neurotransmitters and the ability to form functional synapses (Pleasure et al., 1992; Pleasure et al., 1993; Zeller and Strauss, 1995; Squires et al., 1996; Marchal-Victorion et al., 2003). Prior to the U.S. regulations resulting in tighter control of cellular products, several groups studied the regenerative potential of NTera2-derived neurons and found they were able to repair and restore function in preclinical animal neurodegenerative models and clinical trials in stroke patients (Kleppner et al., 1995; Borlongan et al., 1998; Philips et al., 1999; Kondziolka et al., 2000). Despite their malignant source, NTera2 cells are an useful pluripotent system to study human neural development.

Figure 1: Types of PSC and their potential biomedical applications.
1.3 DOPAMINERGIC NEURON DEVELOPMENT

The nervous system contains many distinct types of neurons defined by their morphology, connectivity, neurotransmitter phenotype and electrophysiological properties. Together these cells coordinate diverse functions such as movement, sensory response, cognition, memory and autonomic control. Neurogenesis is a highly evolved and regulated process that strictly follows precise stepwise formation in early development (Edlund and Jessell, 1999). Different regions of the nervous system generate different types of neurons in a temporal-spatial sequence generally conversed among species (Gilbert, 2003; Cepko et al., 1996). Postmitotic progeny of neural progenitors acquire distinct phenotypes that are typically determined within the mitotic progenitor prior to final cell division (Desai and McConnell, 2000). Progenitors integrate extrinsic signals from their environments with intrinsic information to ultimately decide the fate of their daughter cells. It is this signal integration that is ultimately responsible for generating and organizing the development of the nervous system. The focus of this thesis is DA neuron differentiation from PSCs. Therefore, I will describe in vivo DA development since these mechanisms are generally recapitulated in PSC differentiation.

1.3.1 Gastrulation through neural tube segmentation

The generation of the three germ layers, the mesoderm, endoderm and ectoderm, through the process of gastrulation is among the earliest and most fundamental events of organism development. The developing embryo undergoes a morphological rearrangement through a consecutive series of extensions and invaginations to form an internal endoderm, an intermediate mesoderm and an external ectoderm. Signaling events occurring between these regions distinguishes the neuroectoderm from the epidermis.

The neural induction default model proposes that the modulation of bone morphogenic protein (BMP) signaling induces and organizes neural tissue as a default fate, in the absence of any instructive signals (Hemmati-Brivanlou and Melton 1994; Levine and Brivanlou, 2007). BMP antagonists such as, noggin, chordin and follistatin are members of the transforming growth factor (TGF) β superfamily and regulate the activity of BMP at the extracellular level, while intracellular BMP signaling is controlled by receptor inactivation or interference with Smad complexes (Hemmati-
Neurulation begins when the ectoderm instructively receives signals from the underlying mesoderm and endoderm and begins to thicken to form the neural plate. These neural instructive signals consist of several steps and signaling pathways including inhibition of bone morphogenic protein (BMP) signaling (Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Reversade et al., 1995; Streit et al., 1998), and activation of signaling by retinoic acid (RA) (Sive et al., 1990; Altaba et al., 1991), fibroblast growth factors (FGFs) (Lamb et al., 1995; Launay et al., 1996; Streit et al., 2000; Delaune et al., 2005) and Wnts (Parr et al., 1993; Parr and McMahon et al., 1994a; Wilson et al., 2001), all of which are spatially and temporally controlled. However, BMPs are highly expressed in the dorsal midline and also play a critical role patterning the nervous system after neural induction through gene expression, cell proliferation and apoptosis (Furuta et al., 1997; Shimogori et al., 2004). There is evidence of crosstalk between BMP signaling and other major signaling pathways including Notch and insulin-like growth factor (IGF) (Pera et al., 2003; Dahlqvist et al, 2003; Ille et al., 2006; Machold et al., 2007). IGF signaling has been implicated in neural induction, downstream of BMP antagonists (Pera et al., 2001), however, the signal transduction mechanisms that might regulate IGF signaling during neural induction have not been analyzed.

Following neural induction the lateral edges of the neural plate begin to role up at the anterior region towards the dorsal midline of the embryo to form a tube-like structure. Subsequently, at the posterior end of the embryo, cells proliferate and extend along the trunk of the neural plate. The posterior end also begins to fold and fuses towards the dorsal midline to form the neural tube. At this stage the vertebrate ectoderm can be divided into distinct developmental regions: the externally positioned epidermis of the skin and the internally positioned neural tube and notochord. The neural tube and notochord eventually forms the central nervous system (CNS) and peripheral nervous system (PNS), and the neural crest cells located between the epidermis and neural tube give rise to mesodermal and PNS derivatives. After neural induction, the anterior region of the developing embryo further compartmentalizes along the anterior-posterior (A-P) axis into several vesicles representing the future forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon), while the posterior region forms the spinal cord (Lumsden and Krumlauf, 1996).
1.3.2 The midbrain

Following neural tube formation, axes and signaling centers secrete factors that intersect with cell-intrinsic factors to establish midbrain patterning and direct the temporal and spatial position of neurons. Midbrain DA (mDA) development results from the consecutive expression of determinate factors that permit the distinction of developmental stages into early patterning events, neuroepithelial progenitors, committed DA precursors, immature DA neurons and mature DA neurons.

1.3.2.1 Early development of the midbrain

An important event in early patterning of the vertebrate CNS is the establishment of organizational axes that are dependent on signaling centers. The A-P (also referred to as rostral-caudal) axis lies along the neural tube and reaches (from head to tail) and the dorso-ventral (D-V) axis (from back to belly). The midbrain is located posterior to the forebrain and anterior to the hindbrain. The integrative actions of two signaling centers, the isthmus and floorplate (FP), which lie along these axes, are critical for the positioning, organization and generation of mDA neurons.

In the developing neural tube, the isthmic organizer lies at the midbrain-hindbrain boundary (MHB) and is primarily responsible for patterning along the A-P axis. The organizing activity of the isthmus develops from the expression of two transcription factors: orthodenticle homologue 2 (Otx2), which is positioned rostrally to the isthmus, and gastrulation brain homeo box 2 (GbX2), which is positioned caudally to the isthmus. Otx2 expression is restricted to the forebrain and midbrain and accordingly mutant Otx2 mice display a loss of these primitive brain regions (Millet et al., 1999; Rhinn et al., 1999). Antagonistic repression of Otx2 and Gbx2 are necessary for defining the MHB. In the absence of Gbx2 expression, Otx2 expression is expanded caudally (Broccoli et al., 1999). The isthmus coordinates the expression of several soluble factors including fibroblast growth factor 8 (Fgf8) and Wnt1, which are critical for early midbrain patterning and development. Fgf8 regulates proliferation and maintains the A-P axis (Ye W et al., 1998; Crossley et al. 1996; Lee et al., 1997). The induction and initiation of Fgf8 is regulated by transcription factors paired box gene-2 (Pax 2) (Joyner, 1996; Ye et al., 2001) and LIM homeodomain transcription factor b (Lmx1b) (Guo et al., 2007), subsequent deletion of either factor results in loss of Fgf8 expression in the MHB. Wnt1 is expressed anterior to Fgf8 along the dorsal midline and is
essential for mid/hindbrain development (McMahon et al., 1992b). Together Wnt1 and FGF8 maintain the expression patterns of transcription factors including Engrailed 1 and 2 (En1/2) (Thomas et al., 1990; Wurst et al., 1994; Danielian and McMahon, 1996). Although Wnt signaling plays an important role in different stages of development, the activity of this pathway needs to be tightly regulated by Wnt antagonists such as secreted frizzled related proteins (sFRPs), which are required for embryonic patterning and specification (Satoh et al., 2006).

In the midbrain, the patterning of the D-V axis is established by soluble factors from two signaling centers located along the neural tube mid-line: the roof plate (RP) positioned dorsally and the floor plate (FP) positioned ventrally. BMPs are secreted from the RP and function as dorsalizing factors in an antagonistic fashion to the FP (Liem et al., 1995); however, dorsal patterning has mainly been studied in the hindbrain and spinal cord and little is known about its role in the midbrain (Liem et al., 1997; Arkell et al., 1997; Alexandre and Wassef, 2005). The glycoprotein sonic hedgehog (Shh) is required for the establishment of the D-V axis. Shh is a ventralizing signaling factor secreted in a gradient fashion from a subset of forkhead/winged helix transcription factor A2 (FoxA2) positive cells located in the ventral neuroepithelium of the FP (Hynes et al., 1995a; Yamada et al., 1991). Shh aids in the acquisition of ventral tissue identity and the induction of distinct neuronal phenotypes dependent on their position along the A-P axis: DA neurons in the midbrain, serotonergic neurons in the hindbrain and motor neurons in the spinal cord (Roelink et al., 1995; Ye et al., 1998). In addition to its role in DA induction, Shh plays a role in axonal guidance, regeneration and structural diversity in mDA neurons (Maden et al., 2007; Hammond et al., 2009). Similar to Shh, members of the transforming growth factor (TGF)-β superfamily are expressed in the FP and notochord (Flanders et al., 1991; Howard and Gershon, 1993). TGF-β cooperates with Shh to induce mDA neurons and also promotes their survival (Farkas et al., 2003).

1.3.2.2 The development of midbrain dopaminergic neurons
Following the proper establishment of the axes through the formation of the isthmus and soluble signals from the FP and RP, the midbrain is divided into longitudinal domains along the A-P axis. The generation of mDA progenitor cells begins close to the midline of the ventral mesencephalon (VM) as a result of the cooperative actions of secreted factors Fgf8, Shh, Wnt1 and TGF-β. Several locations have been implicated as
the origin of mDA progenitors including MHB progenitors (Zervas et al., 2004, Marchand and Poirier, 1983), the lateral FP (Hynes et al., 1995b), the diencephalon (Marin et al., 2005) and most recently radial glial cells residing in the FP (Bonilla et al., 2008; Hebsgaard et al., 2009; Joksimovic et al., 2009). The intersection between Shh expression from the FP and Fgf8 expression from the MHB results in DA neuron induction at E10.5 in mouse and E12.5 in rat (Hynes et al., 1995a; Hynes et al., 1995b; Ye et al., 1998). Shh signals are essential to mDA neuron identity, since ectopic expression of Shh or its downstream target Gli-1 results in DA induction (Hynes et al., 1997).

Early mDA progenitors cells residing in the ventricular zone (VZ) expand their progenitor pool and can be indentified by the expression of cell cycle marker phosphohistone H3 and the transcription factor Sox2 (Graham et al., 2003; Kele et al., 2006). Proliferating progenitors first express muscle segment homeobox drosophila homolog of 1 (Msx1), LIM homeodomain transcription factor-a (Lmx1a) and aldehyde dehydrogenase (Aldh1), which is an enzyme that functions in RA metabolism (Lindahl et al., 1984). mDA progenitors residing in the VZ, some of which are radial glia cells, express Neurogenin 2 (Ngn2) and undergo neurogenesis by giving rise to postmitotic precursors that migrate along radial glia cells to their final destination in the ventral region of the midbrain (Shults et al., 1990; Kawano et al., 1995; Hall et al., 2003). This process is marked by the down-regulation of Sox2 (Kele et al., 2006) and the expression of aromatic amino acid decarboxylase (AADC), nuclear receptor related 1 (Nurr1) (Zetterstrom et al., 1997) and En1/2 (Simon et al. 2001). Additionally, post-mitotic DA precursors and mature neurons express the markers β III Tubulin (TuJ1) and vesicular monoamine transporter 2 (VMAT2). DA differentiation terminates with the expression of the midbrain specific transcription factor Paired-like homeodomain transcription factor 3 (Pitx3) (Smidt et al., 2004), tyrosine hydroxylase (TH) (Kawano et al., 1995), the receptor tyrosine kinase c-Ret (Wallen et al., 2001) and finally dopamine transporter (DAT) (Smits et al., 2003).

Several secreted factors have been implicated in mDA progenitor and precursor differentiation. Wnt5a is involved in progenitor proliferation (Andersson et al., 2008) and postmitotic mDA precursor differentiation (Castelo-Branco et al., 2003, Castelo-Branco et al., 2006; Schulte et al., 2005). The expression pattern of sFRP1, a biphasic Wnt agonist/antagonist (Uren et al., 2000), in the early midbrain indicates that it may
serve to refine Wnt signaling during mDA development (Kele-Olovsson, 2007). Interestingly, stromal cell derived factor 1-α (SDF1-α) acts as a chemoattractant in the FP to regulate the migration of mDA precursors (Edman, 2009). Other factors that have been suggested to play additional roles in mDA development include Wnt1 (Castelo-Branco et al., 2003; Prakash et al., 2006), Wnt3a (Castelo-Branco et al., 2003), fibroblast growth factor-20 (Fgf20) (Ohmachi et al., 2003), α-chemokines chemokine (C-X-C motif) ligand (CXCL) -1, -8 and -6 (Edman et al., 2008a) and β-chemokines chemokine (C-C motif) ligand (CCL) -2 and -7 (Edman et al., 2008b). It is likely many other factors exist that aid in the orchestration of mDA precursor and progenitor development.

Several of the previously mentioned transcription factors involved in the development of mDA neurons are discussed in further detail below.

**FoxA2**

FoxA2 is an important factor in DA specification (Arenas et al., 2009). Shh from the notochord directly induces the expression of FoxA2 through Gli-1, and FoxA2 induces Shh in FP mDA progenitors (Sasaki et al., 1997; Jeong and Epstein, 2003; Nelander et al., 2009). Recently, an autoregulatory loop between Wnt1 – Lmx1a and Shh-FoxA2 in mDA development has been identified resulting in enhanced mDA progenitor differentiation (Chung et al., 2009). FoxA2 regulates mDA development by inhibiting Nkx2.2 expression, an alternate fate, and inducing Ngn2 expression followed by downstream DA genes (Ferri et al., 2007). In the adult, FoxA2 has been implicated in mDA neuronal survival and mutant studies revealed DA neuron loss and onset of PD like symptoms in heterozygous animals (Kittappa et al., 2007). Thus, FoxA2 has emerged as a mediator of Shh signaling and an important component in the mDA regulatory network.

**Lmx1b**

Lmx1b is broadly expressed prior to neural tube closure and subsequently restricted to mDA progenitors. Expression disappears shortly after the onset of TH expression (around E11 in mouse) and reappears at later stages of embryonic development (around E16 in mouse) in Pitx3- and TH-positive cells
and remains through adulthood. Lmx1b controls the expression of Wnt1 and Fgf8 in the isthmus, however is not sufficient to induce mDA neurons when ectopically expressed (Guo et al., 2007). Lmx1b null mice generate a small pool of mDA neurons that express Nurr1 and TH at mE10.5; however, these neurons lack Pitx3 and are lost by birth (Smidt et al., 2000). Similarly, deletion of Wnt1, a gene directly regulated by Lmx1b (Chung et al., 2009), also results in a loss of Pitx3 expression and of mDA neurons (Prakash et al., 2006).

**Lmx1a and Msx1**

Shh signaling was previously thought to induce the expression of two key determinant mDA transcription factors, Lmx1a and Msx1 (Andersson et al., 2006). However, a recent study (Chung et al., 2009) has indicated that the expression of Lmx1a is directly regulated by Wnt1 via β-catenin, but not Shh. Lmx1a is expressed by proliferating progenitors in ventral midline cells of the FP region and precedes Msx1 expression (Andersson et al., 2006). Ultimately, the signaling cascade inhibits Nkx6.1 expression, a negative regulator of neurogenesis, and broadly induces the proneural factor Ngn2 (Andersson et al., 2006). Lmx1a expression is maintained in ventral mDA neurons following DA terminal differentiation and persists in the adult, suggesting it could play a role in cell survival (Cai et al., 2009a).

**Nurr1**

Expression of Nurr1 is induced in postmitotic mDA precursors just prior to TH expression (Wallen et al., 2001). At this stage Nurr1 expression is restricted to mDA precursors and, subsequently, it is expressed in other brain regions including the hippocampus, cortex and cerebellum (Saucedo-Cardenas et al., 1998). Nurr1 is required for acquisition of mDA phenotype, because loss of Nurr1 results in lack of mature DA marker expression including, TH, VMAT2, c-Ret and DAT (Wallen et al., 2001). Additionally, Nurr-1 deficient mice display deficits in mDA migration, axonal innervations (Wallen et al., 1999) and survival in the adult (Saucedo-Cardenas et al., 1998).

**Pitx3**

The expression of Paired-like homeodomain transcription factor 3 (Pitx3) begins shortly after Nurr1 expression in mDA precursors and is required for
terminal differentiation of mDA neurons (Smidt et al., 1997; Smidt et al., 2004).
Pitx3 plays a role in the regulation of TH and DAT in mDA neurons (Cazorla et al., 2000). Pitx3 expression is restricted to mDA neurons, which persists through adulthood where it is exclusively expressed in the SNpc and VTA (Smidt et al., 1997; Martinat et al., 2006). Pitx3-deficient mice display progressive loss of mDA neurons and reduced axonal projections to the striatum (Hwang et al., 2003; Nunes et al., 2003; van der Munckhof et al., 2003; Smidt et al., 2004).

1.3.2.3 Functional maturation and survival of midbrain dopaminergic neurons

Newly born mDA must extend axonal and dendritic processes, innervate the target tissue, maintain appropriate connections, and survive in order to appropriately function.
in the adult. Following terminal differentiation, mDA neurons project axons rostrally and loop in the D-V plane through a process likely governed by extrinsic cues (Nakamura et al., 2000). Although the molecular mechanisms guiding axonal projections of mDA neurons remains largely speculative, it is likely governed by similar guidance cues that regulate neuronal projections in other brain regions including netrins, Slits, semaphorins, and ephrins (Dickson, 2002). Neuropilin-1, a co-receptor for the semaphorin family of ligands, is induced by Nurr1 expression (Hermanson et al., 2006). En1/2 can induce the expression of guidance molecule receptors ephrin-A2 and ephrin-A5 (Logan et al., 1996). Netrin and Slits and have been linked to target recognition, as such, mDA express their corresponding receptors and Slit-2 and Netrin-1 that cooperatively function to guide axons towards the primitive striatum (Lin et al., 2005). Ephrins have been linked to regulating the correct target innervation in mDA neurons (Yue et al., 1999). The addition of ephrin-B2, normally expressed in the striatum, to mDA neurons expressing the ephrinB1 receptor, resulted in an upregulation of Nurr1 (Calo et al., 2005). Additionally, SDF1-α is expressed in the meningeal tissue during mDA development where it promotes neurite outgrowth and target innervation in terminally differentiated mDA neurons (Edman, 2009). Following target innervation it is likely that mDA neuron axons compete to form synapses, followed by selective pruning in order to establish the proper functional connections (Burke, 2003).

Neurotrophic factors regulate a variety of functions in normal and pathological conditions including cell survival, synaptic plasticity and neurogenesis from endogenous neural stem cells. In the adult, mDA neurons are considered especially vulnerable to insult, and endogenous and exogenous neuroprotective agents can play an important role in the survival of mDA neurons. Several growth factors have been proposed as neuroprotective including, glial cell-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), bFGF, IGF1, IGF2, transforming growth factor -β3 (TGFβ3) and neurotrophins 4 and 5 (NT4/5) (Haag et al., 1998; Alexi et al., 2000). GDNF is currently considered the most effective neuroprotective agent for mDA neurons, thus its cytoprotective function and axonal regeneration capabilities have been widely studied (Gash et al., 1998). In animal models of PD, GDNF has shown to increase survival of mDA neurons, prevent cell death and induce axonal sprouting (Sauer et al., 1995; Akerud et al., 1999; Rosenblad et al., 1999). BDNF has also shown to have neuroprotective effects on mDA neurons in animal models of PD (Hyman et al., 1991; Levivier et al., 1995; Feng et al., 1999) as
well as broad neuronal protection in various other neuronal phenotypes (Sendtner et al., 1992; Morse et al., 1993). Nurr1 regulates BDNF expression in mDA neurons during midbrain development, which may continue in the adult as a protective mechanism (Volpicelli et al. 2007). Alternative neuroprotective strategies such as caloric restriction promote mDA survival, likely due to increased levels of GDNF and BDNF (Maswood et al., 2004). In recent years several novel growth factors including conserved dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF) have been identified as neuroprotective agents that protect against insults and restore DA neuronal function (Petrova et al., 2003; Lindholm et al., 2007).

Figure 3: Over lifespan, various extrinsic pathways contribute to the existence and maintenance of mDA neurons.
1.4 NEURAL DIFFERENTIATION OF PLURIPOTENT STEM CELLS, A FOCUS ON DOPAMINERGIC NEURONS

The main challenge in PSC research lies in efficiently directing undifferentiated cells towards terminally differentiated DA neurons. In order to accomplish this we need a thorough understanding of signals involved in neural, neuronal and DA neuron specification. Considering that cellular development is dependent on both extrinsic signals and intrinsic signal transduction programs (Edlund and Jessel, 1999), cell culture systems focus on mimicking in vivo developmental programs with regards to timing, spatial organization and signaling mechanisms. Below we describe current methods for differentiation of PSC towards DA neurons.

1.4.1 Neural and neuronal differentiation

The specification of region-specific neural progenitors is crucial to the generation of neuronal subtypes. Neural derivatives generated from PSCs are typically designated neural precursor or progenitor cells. The distinction between cell types is based on their differentiation stage and defined by their developmental capacity and marker expression. The first stage of neural development is the generation of neuroepithelial progenitors, which are considered the developmental equivalent of cells within the neural plate (O’Rahilly and Muller, 1994). Neuroepithelial progenitor cells orientate radially to form rosette-like structures reminiscent of the early neural tube. However, it has also been suggested that rosettes correspond to the neural plate/neural-fold stage since they lack markers that define the dorsal-ventral (D-V) domains (Elkabetz and Studer, 2008). Early neural rosettes, termed rosette neural stem cells (R-NSC), undergo extensive proliferation and can generate neurons, astrocytes and oligodendrocytes (Reubinoff et al., 2000; Zhang et al., 2001; Elkabetz and Studer, 2008). R-NSC exhibit anterior-posterior (A-P) polarity and can be further patterned to generate a broad range of neuronal fates (Elzabetz et al., 2008). R-NSC mimic neurulation and neural tube growth, gradually giving rise to more differentiated cells and lineage-restricted progenitors. Neuronal progenitors and glial progenitors are further limited in their differentiation capacity to exclusively generate neurons or glial cells, respectively (Mayer-Proschel et al., 1997; Lee et al., 2000).

Disruption of signaling involved in pluripotency promotes spontaneous and uncontrolled differentiation into multiple lineages. Spontaneous differentiation
protocols, such as withdrawal of growth factors, growth at high density or formation of suspension cell aggregates, are inefficient methods for generating terminally differentiated neurons. However, these protocols are typically used to initiate a general differentiation program and in recent years have been modified to include instructive factors or methods that direct more lineage-specific neuronal differentiation. Current protocols tend to either exploit our understanding of neural development or have little developmental significance and are used mainly due to their convenience and efficiency. Neural progenitors appear to have similar morphology and express similar markers despite their derivation technique; however, some methods favor neural progenitors with particular characteristics and differentiation capacities (Zhang et al., 2008). Neural progenitor cells produced from human PSC via several commonly used methods are described below.

### 1.4.1.1 Embryoid body (EB) formation and adherent aggregates

Early protocols in neural differentiation focused on spontaneous differentiation, since PSC (specifically ESC) have an innate nature to generate cells along the neural lineage (Tropepe et al., 2001; Schwartz et al., 2008). Spontaneous neural differentiation can be initiated through aggregation of hESC in suspension cultures termed embryoid bodies (EB) or overgrown hESC colonies in adherent culture (Carpenter et al., 2001; Reubinoff et al., 2001). EB form multilayer structures composed of neural cells as well as other ectoderm, mesoderm, and endoderm derivatives (Schuldiner et al., 2001). Cell-cell interactions commence a differentiation process that positions cells into an inside-out developmental pattern (the ectoderm resides interiorly, the endoderm resides on the exterior, and the mesoderm is in the middle), resulting in the generation of primitive ectoderm (Jiang et al., 2002). The methods for EB formation are straightforward, but have several drawbacks associated with prolonged cell culture. In extended cell culture necessary for neural induction, EB develop cysts that enclose fluid-filled cavities. At this point EB behave as distinct entities and acquire their own differentiation program such that high concentrations of extrinsic factors are required to penetrate the deepest layers and direct differentiation (Bain et al., 1995; Carpenter et al., 2001; Schuldiner et al., 2001; Wichterle et al., 2002). Adherent hESC differentiation or initial EB formation followed by adherent culture circumvent these issues while preserving cellular interactions that are important in early patterning events (Zhang et al., 2001; Shin et al., 2006). Additionally, these systems allow for more directed and controlled
differentiation through the addition of morphogens or growth factors to increase the efficiency and survival of neural derivatives.

1.4.1.2 Cues from development - BMPs, Retinoic acid (RA) and FGF signaling

The inefficiency of spontaneous differentiation has led to the use of various development-relevant cues to direct neural differentiation. The prevailing view on neural induction is that the neuroectoderm is generated from the ectoderm as long the TGF\(\beta\) pathway is inhibited; because of this the neuroepithelia is considered the default fate of the ectoderm (Stern, 2005). Noggin, a BMP inhibitor, enhances neuroectodermal differentiation of hESCs grown adherently (Pera et al., 2004; Gerrard et al., 2005; Baharvand et al., 2007; Sonntag et al., 2007) or as EB in suspension (Itsykson et al., 2005). Other members of the TGF\(\beta\) pathway affect differentiation. For example, follistatin prevents the formation of extra-embryonic ectoderm cells but fails to generate neural cells (Gerrard et al., 2005), whereas activin/nodal signaling promotes neuroectodermal differentiation in hESC (Smith et al., 2008). Direct dual-inhibition of BMP signaling by addition of Noggin and inhibition of SMAD signaling with the compound SB431542 (a Nodal receptor antagonist) resulted in complete conversion (greater than 80%) of hESC and hiPSC into neural progenitors (Chambers et al., 2009).

Members of the FGF family of proteins activate signal transduction cascades including Raf, mitogen-activated and extracellular signal-regulated kinase (MEK) and mitogen activated protein kinase (MAPK). Consequently, FGF signaling converges with several other pathways including TGF\(\beta\), Shh, Wnt, Notch, and IGF (Pera et al., 2001; Pera et al., 2003; Dahlqvist et al., 2003; Ille et al., 2006; Machold et al., 2007; Chen and Panchision, 2007). bFGF aids in survival and proliferation of primary neuroepithelial cells and is routinely used to isolate and maintain neural precursors derived from ESC (Carpenter et al., 2001; Shin et al., 2006; Dhara et al., 2008; Elkabetz et al., 2008) as well as to generate neurons in adherent cultures (Benzing et al., 2006; Axell et al., 2009).

In the developing embryo, RA works synergistically with Shh, FGF, and BMP pathways to determine specific neuronal fates. RA is the most commonly used factor to induce neural differentiation. RA is a morphogen and, as such, varied concentrations result in wide-ranging neuronal fates (Carpenter et al., 2001; Reubinoff et al., 2001;
Schuldiner et al., 2001; Zhang et al., 2001; Park et al., 2004, Baharvand et al., 2007; Erceg et al., 2008) at various stages in differentiation (Carpenter et al., 2001; Schuldiner et al., 2001).

1.4.1.3 Co-culture and stromal-derived inducing activity (SDIA)

Co-culture techniques with glial (Roy et al., 2006), meningeal (Hayashi et al., 2008) and stromal cells such as, PA6 (Zeng et al., 2004; Schwartz et al., 2005), MS5 (Perrier et al., 2004) and HepG2 (Schulz et al., 2003) promote neuroepithelial and neuronal differentiation from human PSC. Stromal cell lines are derived from mesodermal connective tissue and are the most widely used co-culture system to support neuronal differentiation via a mechanism termed stromal-derived inducing activity (SDIA). Although SDIA mainly generates anterior patterned neural progenitors (Lee H et al., 2007), a variety of neuronal phenotypes have been reported including midbrain DA neurons (see section 1.4.2) (Zeng et al., 2004), neural crest cells (Pomp et al., 2005; Lee G et al., 2007), PNS sensory neurons (Lee H et al., 2007) and motor neurons (Lee H et al., 2007).

The molecular mechanisms governing SDIA-induced neuronal differentiation are largely speculative and loosely based on the developmental requirement of mesoderm signaling in embryonic neural induction. The addition of BMPs blocked neural differentiation of mESC co-cultured with the mouse stromal cell line PA6 (Kawasaki et al., 2000), suggesting that BMP inhibition may be involved in SDIA. However, the involvement of BMP antagonists or inhibition of SMAD signaling has not been determined. Several studies found that co-culture with fixed PA6 cells resulted in efficient neuronal differentiation (Kawasaki et al., 2000; Vazin et al., 2008b), indicating a membrane or surface bound component is necessary for neural induction. However, other studies have reported neuronal differentiation following exposure to medium conditioned by PA6 stromal cells, suggesting secreted factors are responsible, at least in part, for neuronal differentiation (Schwartz et al., 2005; Hayashi et al., 2008; Vazin et al., 2008b). SDIA-mediated differentiation offers an efficient method for neuronal differentiation, but introduces unknown stromal factors of non-human origin, limiting their clinical use.
1.4.1.4 *Media Supplements*

Often neural cells derived from PSC are cultured in defined conditions in medium supplemented with B27 (Gibco/Invitrogen) or N2 (Gibco/Invitrogen). While the composition of these supplements is generally overlooked, they contain factors that influence neural differentiation and survival. B27 contains RA and when supplemented according to the manufacturer’s specification and various defined protocols (Shin et al., 2006, Dhara et al., 2008), medium contains over 10 μM RA, which is sufficient RA to induce neuronal differentiation. N2 contains some of the same components as B27, including insulin, which has been linked to neuronal differentiation (Pera et al., 2001; Vazin et al., 2009). Other components of B27 and N2 include transferrin, progesterone, putrescine and selenium have been implicated in neural progenitor differentiation (Zhang et al., 2001). Knock-out serum replacement (Gibco/Invitrogen) is a serum-free substitute for fetal bovine serum that is used mainly in maintaining undifferentiated hESC and in reduced percentages in some differentiation protocols associated with stromal cell co-culture (Zeng et al., 2004; Perrier et al., 2004). hESC derived neuronal progenitors exhibit enhanced proliferation in medium supplemented with knockout-serum replacement and non-essential amino acids (Dhara et al., 2008). Non-essential amino acids are typically used in many differentiation protocols and play an important role in neural differentiation and maintenance (Miranda-Contreras et al., 2002). Although the components of supplements are not of great importance, they are routinely used in the majority of defined protocols.

1.4.2 *Dopaminergic derivation strategies from pluripotent stem cells*

The generation of DA neurons from PSC is a complex process that requires sequential steps to recapitulate development in vitro, ultimately resulting in neurons capable of normal in vivo function. Despite this challenging undertaking, remarkable progress has been made at the proof of principle level towards the generation of mDA neurons for potential cell replacement therapy. Recently, differentiation protocols for PSC-derived DA neurons have focused on obstacles potentially impacting future clinical translation including the specification and yield of DA neurons and the avoidance of undesired contaminating cell populations including highly proliferative cells that may result in tumor formation.
1.4.2.1 Phenotyping midbrain dopaminergic neurons in culture
Identifying midbrain DA neurons typically involves the use of neuronal markers such as TuJ1 and microtubule-associated protein 2 (MAP2) co-expressed with TH, the rate-limiting enzyme in dopamine synthesis responsible for converting tyrosine to L-DOPA. Other catecholaminergic neurons also express TH, including noradrenergic neurons that express dopamine beta hydroxylase (DBH). mDA neurons will selectively express AADC and lack expression of DBH. Markers indicative of functionally mature DA neurons include DAT which is involved in dopamine recycling from the synaptic cleft to the presynaptic terminal, and VMAT2 which is responsible for packaging dopamine and other monoamines into vesicles for exocytosis. Neurons residing in the SNpc or A9 neurons, as opposed to VTA A10 neurons, express the G-protein regulated inwardly rectifier potassium channel GIRK-2 and aldehyde dehydrogenase 2 (ALDH2).

Biochemical markers are complemented with mDA transcription factors. Similar to midbrain development, mDA neurons generated from PSC should also express region-specific markers such as En1/2, Lmx1a, Lmx1b, Otx2, Nurr1 and Pitx3. Often assays are performed to reflect neuronal and dopaminergic function, such as electrophysiological recordings and measurements of dopamine release/re-uptake.

1.4.2.2 Differences between mouse and human embryonic stem cells affecting dopaminergic differentiation
The majority of differentiation protocols are based on initial studies in the mESC system and have only recently been applied to hESC. Despite differences between mESC and hESC with respect to pluripotency and self-renewal (Ginis et al., 2004, Sato et al., 2004), it appears that overlapping mechanisms govern DA neuron differentiation. DA neurons can be successfully generated using similar SDIA protocols from both mESC (Kawasaki et al., 2000) and hESC (Zeng et al., 2004). Additionally, many of the soluble factors used in mESC protocols such as Shh, Fgf8, BDNF, GDNF and TGFβ3 have been applied to hESC DA derivation protocols (Deierborg et al., 2008). However, developmental timing of PSC differentiation is crucial to optimize DA differentiation protocols. Differences in gestation and development rate between mouse and human establish intrinsic differences resulting in shorter differentiation protocols in mESC (Kawasaki et al., 2000) compared to hESC (Zeng et al., 2004) and hECSC (Schwartz et al., 2005). Another major difference between mESC and hESC is the emergence of neural rosettes with NSC properties (R-NSC) in hESC (Elkabetz et al., 2008). R-NSC are frequently observed during hESC differentiation, but either do not form or are too
transient to be observed in mESC. R-NSC express distinct characteristic markers and can be isolated, propagated, and patterned prior to terminal differentiation, making them an important stage in hESC DA protocols.

### 1.4.2.3 Pluripotent stem cell dopaminergic differentiation protocols

One of the major hurdles limiting stem cell based strategies from clinical use is inefficient differentiation into DA neurons. Numerous in vitro protocols have been developed to generate DA neurons from PSC that mainly involve either co-culture with stromal cells or EB based step-wise methods. The majority of these protocols tend to be similar; they use either stromal cells or EB formation for the initial step of neural induction, followed by the addition of soluble factors to increase efficiency and survival.

Stromal cell co-culture has the benefit of efficiency and simplicity. Signals from stromal cells tend to promote the differentiation of neural progenitors with mid/hindbrain characteristics (Lee H et al., 2007), whereas, EB formation tends to initiate a more general neural induction program. For this reason stromal cells may provide an additional advantage over EB aggregates; however, the signals that contribute SDIA are largely undefined. Several stromal cell lines have been used for DA neuronal differentiation of hESC including the PA6 (Zeng et al., 2004, Park et al., 2005; Brederlau et al., 2006) and the MS5 (Perrier et al., 2004; Sonntag et al., 2007) cell lines. In the absence of any extrinsic patterning molecules, co-culture with PA6 cells typically generate between 7-15% TH-positive cells or 80-90% TH-positive colonies (Zeng et al., 2004; Schwartz et al., 2005; Brederlau et al., 2006). Wnt-1 expressing MS5 cells have been used in several protocols, resulting in a modest increase in the differentiation capacity of the MS5 stromal cell line (Perrier et al., 2004; Sonntag et al., 2007).

In both EB and stromal cell co-culture protocols, the additions of similar exogenous factors are used to further enhance neural and DA differentiation. Shh and Fgf8 are typically added at an early stage in differentiation to aid in neural patterning (Perrier et al., 2004; Park et al., 2005; Roy et al., 2006). The addition of Noggin has also been instructive to generate neuroepithelial precursors, thus increasing the efficiency of DA derivation in both stromal co-culture and EB protocols (Ben-Hur T et al., 2004; Itsykson et al., 2005; Sonntag et al., 2007). Several protocols isolate neural rosette
structures followed by expansion, typically in the presence of Fgf8, Shh and bFGF, to
generate a pool of R-NSC from which to further derive DA neurons (Perrier et al.,
2004; Iacovitti et al., 2007; Sonntag et al., 2007). Factors that have been shown to
promote neurogenesis such as, oxysterols (Sacchetti et al., 2009) or to protect mDA
neurons in vivo (Branton et al.,1998; Krieglstein et al., 1998; Ling et al., 1998;
Rosenblad et al., 1999; Ohmachi S et al., 2000; Rolletschek et al., 2001) have been
added following initial neural differentiation including: ascorbic acid, BDNF, GDNF,
interleukin-1β, Neurturin, TGFβ3, dibutryl cAMP and fibroblast growth factor 20
(FGF20) (Perrier et al., 2004; Park et al., 2005; Yan Y et al., 2005; Roy et al., 2006;
Correia et al., 2007; Sonntag et al., 2007; Yang et al., 2008). Recently, these protocols
have been applied to hiPSC to derive mDA neurons (Cai et al., 2009b; Soldner et al.,
2009).

The mechanisms by which stromal cells promote DA differentiation are unclear;
however, it appears that soluble molecules and cell-cell interactions play important
roles. Stromal cells have similar effects on a variety of PSC types, including mESC,
hESC, non-human primate ESC and hECSC (Kawasaki et al., 2000; Zeng et al., 2004;
Takagi et al., 2005; Schwartz et al., 2005). However, neurospheres derived from rat
embryonic midbrain and forebrain did not generate DA neurons, and instead resulted in
increased astrocyte production (Roybon et al., 2005), suggesting DA differentiation
mechanisms are likely limited to earlier stages of neuronal commitment. Several
experiments point to the involvement of cell surface components in the neural inductive
aspect of SDIA. Fixed PA6 cells maintain their ability to generate neurons, however
lose their ability to induce TH positive DA neurons (Vazin et al., 2008b). PA6 cell
conditioned medium (PA6 CM) also generated DA neurons from PSCs (Schwartz et
al., 2005; Hayashi et al., 2008; Vazin et al., 2008b). Interestingly, the addition of
heparin in the preparation of conditioned medium is important for the generation of DA
neurons from PSC (Hayashi et al., 2008; Vazin et al., 2008b). In the absence of heparin
in the preparation of PA6 conditioned medium, hESC were unable to generate DA
neurons; however, NSC did generate DA neurons (Swistowska et al., 2010). This
suggests that a cell surface/heparin bound protein could be responsible for the early
neural induction, whereas a non-heparin bound protein may be involved in later stages
of DA neuron differentiation.
PA6 CM can also induce DA neuron differentiation and several factors have been identified in PA6 cells (Hayashi et al., 2008; Vazin et al., 2009; Swistowska et al., 2010). SDIA likely affects several stages of neural differentiation (Figure 4). Neural progenitors from hESC-derived EB can efficiently generate DA neurons in the presence of SDF-1α, pleiotrophin (PTN), IGF2, and ephrin B1 (EFNB1) (Vazin et al., 2009). In combination these factors generate a high percentage of colonies that expressed TH and Msx-1 (Vazin et al., 2009). NSC derived from hESC generated DA neurons in response to PA6 CM and addition of Shh antagonist cycloamine reduced the number of TH positive cells, suggesting that Shh (derived from the hESC and/or PA6 CM) is involved in DA differentiation of the NSC stage (Swistowska et al., 2010). Additionally, Shh can act at early stages to promote neural differentiation, which implies that Shh may be responsible for the observed stage-specific differentiation. The efficiency of DA differentiation was also enhanced in mESC treated with the Wnt5a, a factor that regulates the differentiation of mDA neurons (Andersson et al., 2008) and is present in stromal cells (Hayashi et al., 2008). In addition to specific factors, gene expression analysis has implicated several neural and DA developmental pathways in SDIA including, Shh, Wnt, TGFβ, IGF, vascular endothelial growth factor (VEGF), Notch and FGF (Hayashi et al., 2008; Vazin et al., 2009; Swistowska et al., 2010).

Figure 4: Stages of neural differentiation at which SDIA may exert an affect resulting in increased DA differentiation. SDIA factors including Shh, SDF1α, PTN, IGF2 and EFNB1 have been implicated in DA differentiation of hESC-derived progenitors.
1.4.2.4 Genetic approaches to specify DA neurons

Although extrinsic factors have a profound effect on DA differentiation and survival, intrinsic genetic strategies based on mDA development are being developed to further enhance the yield of mDA neurons from PSCs. Over-expression of the transcription factor Nurr1, enhanced the yield, function and transplantation of DA neurons derived from mESC (Kim et al., 2002; Chung et al., 2002; Kim et al., 2006). The combined action of over-expressing Pitx3 and Nurr1 enhanced the yield of hESC derived DA neurons (Martinat et al., 2006). Nurr1 overexpression has also been combined with FoxA2 in hESC to reduce proliferating neural progenitors that pose a risk of tumors and enhanced both the yield and survival of mDA neurons, but only modestly reversed motor deficits in animal PD models (Lee et al., 2009). Over-expression of Lmx1a drastically enhanced the yield of mDA neurons from mESC (Andersson et al., 2006) and hESC (Cai et al., 2009a; Frilling et al., 2009) and transplantation of mESC derived DA neurons improved functional recovery in animal models of PD, however, generated tumor/neural overgrowths (Frilling et al., 2009). Genetically modified hESC pose an additional concern with respect to clinical translation, but as a research tool they have thus far been more effective at promoting mDA differentiation than extrinsic factors alone.

1.4.2.6 Cell selection strategies

Neuronal differentiation protocols can generate DA neurons from hESCs and enrich for a particular subtype (Perrier et al., 2004; Yan et al., 2005). Standard protocols typically result in the generation of various non-neural phenotypes and cells at various stages of cellular maturity that limit their clinical and experimental use. Cells of mesodermal lineage have been reported in both in vitro differentiation protocols and in grafted cells in animal models (Zeng et al., 2004; Sonntag et al., 2006). Of clinical consequence, highly proliferative immature cells have resulted in tumor formation following transplantation of derived DA neurons from hESC (Zeng et al., 2004; Bredelau et al., 2006; Roy et al., 2006; Cai et al., 2009b). Although genetic engineering and optimized
differentiation protocols are currently focused on limiting immature cells, cell selection strategies offer a feasible and more direct alternative.

Flow cytometry or magnetic bead-based selection can be used to select a desired cell population or remove an unwanted population. Flow cytometry has been used to select hemopoietic cells, mesodermal progenitor and endothelial cells from ESC (Wang et al., 2004; Kouskoff et al., 2005; Kattman et al., 2006) and is routinely used in the clinical setting for hemopoietic stem cells (Morrison et al., 1995; Herzenberg et al., 2002). Although cell selection strategies are an attractive option to select or exclude cell populations, unlike the field of hemopoietic stem cells and immunology, the field of neurobiology lacks cell surface marker sets that define developmental maturity and fate specification. Difficulties with neural cell selection strategies are confounded by the fact that differentiated neural derivatives have difficulty surviving dissociation and cell selection. Despite these challenges, and in an effort to expedite cell selection progress, several investigators have focused on outlining markers and cell selection strategies to isolate neural cell populations from embryonic neural tissue (Maric and Barker, 2004; Biagioli et al., 2009) and differentiating hESCs (Pruszak et al., 2007; Pruszak et al., 2009). Cell selection strategies have been applied successfully to eliminate tumor-forming proliferative cells from ESC-derived neural populations using a either a single cell surface marker or combinations of markers (Chung et al., 2006; Pruszak et al., 2007; Wernig et al., 2008; Hedlund et al., 2008; Sundberg et al., 2009). Cell selection with Corin, a cell surface marker expressed in mDA progenitors in both the developing ventral midbrain and in ESC-derived DA cells, was used to select mesencephalic cells for transplantation resulting restored function in animal models of PD (Jonsson et al, 2009). Alternatively, genetically modified fluorescent markers can be used to select a desired population, such as Tau-GFP (Li et al., 2008) or Synapsin-GFP (Pruzak et al., 2007). Genetically modified Pitx3-enhanced green fluorescent protein (Pitx3-eGFP) knock-in blastocyst-derived mESC were used in conjunction with flow cytometry to select and purify mDA neurons that were then able to survive, innervate, and restore function in animal PD models (Hedlund et al, 2007). Cell selection protocols provide a way to select desired populations and remove unwanted populations as well as a unique opportunity to study human DA neuron development.
2 AIMS

PSC possess an unlimited proliferative capacity and can generate any cell type in the body. Therefore, PSC provide a potential source of cells for regenerative therapy and system in which to study mechanism controlling neuronal differentiation. Clinically feasible sources of PSC, including hESC and hiPSC, are limited with regards to cell numbers and the time-consuming nature of the current culture systems. This thesis examines DA neuron differentiation in PSC and establishes the NTera2 hECSC line as a model system for hESC in which DA neuron differentiation can be efficiently studied.

The specific aims of this study were to:

- Establish the NTera2 cell line as a model system to examine DA differentiation of hESCs (Paper I)
- Examine gene expression characteristics of PSC-derived DA neurons and progenitors (Paper II, Paper III and Paper V)
- Identify the molecular mechanisms of SDIA that govern DA neuron differentiation (Paper IV)
- Explore cell selection methods and markers to enrich DA neurons (Paper V)
3 RESULTS AND DISCUSSION

3.1 PAPER I: NTera2: A MODEL SYSTEM TO STUDY DOPAMINERGIC DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS

To date, hundreds of hESC lines have been derived, and even more with the advent of iPSC. However, maintaining and using these lines can be difficult, time consuming and expensive due to demanding cell culture procedures. Additionally, hESC and hiPSC are routinely and optimally maintained on supportive fibroblast feeders and rapidly differentiate if grown in suboptimal conditions. As a result, hESC model systems are beneficial for predicting cellular behavior and exploring mechanisms of PSC differentiation. Of the available model systems, the NTera2 cell line, a human ECSC, presents several advantages over other systems such as mESC, non-human primate ESC, EGC and karyotypically abnormal hESC. The NTera2 cell line is most favorably grown without feeder cells or expensive growth factor supplements, recovers rapidly from freeze thaw and can easily be propagated while retaining a relatively homogeneous phenotype. NTera2 cells share many similarities with hESC including expression of characteristic PSC markers, the ability to generate cells from all three germ layers and pluripotent epigenetic status (Andrews et al., 1984a; Andrews, 1988; Draper et al., 2002; Przyborski et al., 2004; Bibikova et al., 2006). Additionally, the NTera2 cell line has been used extensively to study cell cycle regulation, human neural development and properties of neurons (Andrews et al., 1984b; Pleasure et al., 1992; Pleasure et al., 1993; Squires et al., 1996; Walsh and Andrews, 2003; Bahrami et al., 2005). Therefore, we reasoned that the NTera2 cell line could serve as a model system for DA differentiation in order to more efficiently explore mechanisms and derivation techniques that could be rapidly translated to hESC. This possibility was examined in subsequent studies (Papers III and IV).

At the time of this study, although NTera2 cells were commonly used as an experimental model of hESC and human neural development, no large-scale gene expression comparison had been used to determine how closely they resemble hESC in overall gene expression. Therefore, we compared global gene profiles of undifferentiated NTera2 cells to multiple hESC lines (BG01, BG02 and BG03). We found a surprisingly high similarity in overall gene expression and the expression of
known pluripotent markers. Similar to hESCs, undifferentiated NTera2 cells expressed markers indicative of their pluripotent nature including, SSEA-4, SSEA-3, TRA-1-60, TRA-1-81, Sox2, Oct4 and Nanog.

To determine whether the NTera2 cell line could serve as a system to explore hESC DA differentiation, we differentiated NTera2 cells via PA6-co-culture, a method commonly used for DA derivation of hESC (Zeng et al., 2004; Perrier et al., 2004). Co-culture of NTera2 cells with PA6 cells generated a similar efficiency of TH and TuJ1 positive colonies to what was reportedly observed in hESC-PA6 co-culture (Zeng et al., 2004). Furthermore, flow cytometry analysis and RT-PCR for undifferentiated, DA and neuronal markers were also similar to hESC (Zeng et al., 2004) and consistent with differentiation towards a mDA neuronal phenotype. Since the NTera2 cell line offers an advantage in terms of readily and easily exploring DA differentiation, we reasoned we could use it in an attempt to simplify the PA6 co-culture system. We found that NTera2 cells co-cultured with mitotically arrested PA6 cells, or cultured without PA6 cells in the presence of PA6 CM, also generated DA neurons. Both of these systems offered the advantage of limiting exposure to proliferating PA6 cells. The inductive nature of PA6 cells is thought to accumulate at the cell surface as cell membrane- or surface-bound proteins (Kawasaki et al., 2000); however, our results suggested that factors secreted from PA6 cells could potentially contribute to SDIA (Paper IV). Additionally, we used the flow cytometry positive selection strategy with PSA-NCAM, a neuronal progenitor marker, as a proof of principle approach in several subsequent studies (Paper II, Paper III, Paper V). We reasoned that PSA-NCAM selection would eliminate contaminating populations including undifferentiated, non-neural and PA6 cells, as well as potentially increase the efficiency of DA derivation. PSA-NCAM selected cells were able to generate DA neurons following further differentiation on either PA6 co-culture or PA6 CM. Furthermore, whole-cell patch clamp electrophysiological recordings from PSA-NCAM-positive differentiated cells showed that they display functional characteristics of neurons.

In summary, our results suggest that the NTera2 cell line is a useful model system to study and explore DA differentiation of hESC. NTera2 cells differentiated by PA6 co-culture resembled hESC differentiation in timing, efficiency and expression of differentiated markers, suggesting that they share fundamental similarities and respond to differentiation cues via similar mechanisms. The simplified culture system offered
by the NTera2 cell line allowed us to rapidly and advantageously modify the PA6 co-culture system. Furthermore, flow cytometry isolation in the NTera2 system provides a proof of principle approach to neural selection protocols to remove unwanted populations. Collectively, we propose that the NTera2 cell line offers an easy and relevant system in which to explore DA differentiation of hESC.

3.2 3.2 PAPER II: A FOCUSED MICROARRAY TO ASSESS DOPAMINERGIC AND GLIAL CELL DIFFERENTIATION FROM FETAL TISSUE OR EMBRYONIC STEM CELLS

Various types of cells and stages of differentiation have been considered for potential cell replacement therapy including NSC, neuronal progenitors, glial progenitors and ESC (Tai et al., 2004). However, prior to transplantation populations need to be thoroughly characterized to determine the degree of contaminating populations, expression of appropriate markers and which signaling pathways are active. Additionally, addressing these issues has been challenging due to difficulties with respect to reaching a consensus on marker expression and the cost associated with assessing numerous markers. Despite the widespread use of global gene expression techniques, we set out to develop a focused microarray since it offers several advantages including the ability to rapidly and reliably assess the state of a given population and it is more cost effective for the majority of researchers. We reasoned that a focused array comprised of DA and glial genes as well as development-related signaling molecules would allow for rapid assessment of a given cell population prior to use in transplantation studies.

In designing the array we incorporated approximately 280 genes including genes associated with DA neurons, glial cells, neural progenitors, PSC, signaling molecules associated with neural differentiation, cytokines and chemokines, and their respective receptors, to provide insight into additional potential signaling pathways. Additionally, we included pluripotent markers such as Oct4 and Nanog and NSC/neural progenitor markers Sox2 and Nestin to determine the presence of immature populations, which have been linked to tumor formation following transplantation (Bredelau et al., 2006; Roy et al., 2006). We used samples from various sources including undifferentiated
hESC, hESC derived NSC and human SNpc RNA (commercially purchased) to show that the array is able to distinguish these populations in a quantitative manner. We also tested polysialated neural cell adhesion molecule (PSA-NCAM)-selected neural progenitors derived from the NTera2 cell line differentiated towards DA neurons (Paper I and Paper V), in order to determine their expression profiles as well as providing insight into signaling molecules regulating DA differentiation. Included in the focused array were over 100 genes with known roles in neural development aimed at elucidating molecular events occurring during differentiation. The molecules encoded by these genes belong mainly to the Wnt, TGF-β, FGF and BMP pathways, which play critical roles in neural development. PSA-NCAM-selected cells, as compared to undifferentiated NTera2 cells from which they were derived, had significantly higher levels of expression of several genes to have known roles in mDA neuron development including, TH, AADC, Nurr1 (Nr4a2), En1 and the Shh receptor Smo (Prakash and Wurst, 2006; Abeliovich and Hammond, 2007), suggesting that pathways similar to those activated during normal development are likely activated in PSC DA differentiation.

In summary, we set out to develop a focused microarray that could be used for routine monitoring of the process of differentiation. Our results showed that the gene array we designed was able to discriminate various neural populations, determine the degree of contaminating undifferentiated populations that pose tumor risks, and identify signaling pathways that may be involved in directing the process of differentiation.

3.3 PAPER III: GENE EXPRESSION PROFILE OF NEURONAL PROGENITOR CELLS DERIVED FROM HESCS: ACTIVATION OF CHROMOSOME 11P15.5 AND COMPARISON TO HUMAN DOPAMINERGIC NEURONS

Despite our best efforts to direct the developmental potential of hESC, the presence of various cell types and levels of cellular maturity resulted in heterogeneity that significantly limits the power of gene expression analyses. Since various cells existed in the hESC cultures, the resulting analyses could provide at best a composite view of gene expression, which may mask genes expressed at low levels, or changes associated with one cell population may be opposed by changes in another population. Therefore,
we decided to pursue gene expression studies using purified neuronal populations to study cell type-specific gene expression. Flow cytometry-based cell selection provides a way to enrich for a particular population of interest; therefore, several studies have successfully applied these techniques in combination with gene expression profiling to examine DA neuron development in the mammalian brain (Blass-Kampmann et al., 1994; Jorgensen et al., 2006; Baer et al., 2007). Previously, in a proof-of-principle approach we showed that PSA-NCAM could be used as a selection marker for neuronal progenitors during DA directed differentiation of NTera2 cells (Paper I). The purpose of the present study was to apply these cell selection techniques to hESC-derived neuronal progenitors and investigate their transcriptional profile.

In this study, we used flow cytometry to select PSA-NCAM-positive cells from hESC (the BG03 cell line) differentiating toward DA neurons in PA6-coculture, in order to obtain a purified precursor population with the potential of differentiating along the DA neuronal lineage. Following PSA-NCAM cell selection, RT-PCR determined that appropriate markers were expressed such as TH, Lmx1b and Pitx3, while pluripotent markers were either absent or expressed at low levels. Furthermore, electrophysiological recordings from selected cells that were further differentiated revealed that cells displayed functional neuronal properties including electrical excitability, neurotransmitter responsiveness and the presence of action potentials. These results suggested that we successfully selected neuronal progenitors that could be used further for gene analysis.

Using Massive Parallel Signature Sequencing (MPSS), a gene expression method based on the sequencing of 17-mer and 20-mer tags generated by the restriction enzyme DpnII, we obtained the gene profiles of PSA-NCAM-positive neuronal progenitors directed toward DA neurons. Of particular interest, several genes on chromosome 11p15.5, in the H19-IGF2 imprinting center were highly expressed. Of the (11, 912) genes expressed in PSA-NCAM-positive cells, 232 were highly expressed specifically in PSA-NCAM-derived population compared to undifferentiated hESC or hESC derived embryoid bodies. Notably, several transcription factors associated with neural and mDA development were highly expressed including Msx1, Pitx1, Pitx2, and many genes associated with the solute carrier family. Furthermore, examination of the cytogenetic map locations revealed that genes were clustered in particular chromosome regions. Most notably, we observed that five genes highly expressed in PSA-NCAM
selected cells were located on chromosome 11p15.5 in close proximity to the H19-IGF2 imprinting region, including H19, IGF2, CDKN1C, TSSC4 and HGB2.

To validate the MPSS gene analysis we used RT-PCR. We selected 18 genes differentially expressed in PSA-NCAM-positive cells including both novel and known genes (EMP3, SLC7A7, Pitx1, Msx1, Pitx2, SDF2L1, NPY and NINJ1, Hs.551588 (H19), Hs.19193, Hs.473109, Hs.109798, Hs.534052, Hs.446315, Hs.211282 and Hs.479491, TSSC4, and IGF2). With the exception of SDF2L1 and NINJ1, all of the selected genes were differentially expressed by PCR, suggesting that they could be specific markers for the DA/neuronal lineage. To further validate these results in a biologically relevant population, we examined H19, IGF2, and cyclin-dependent kinase inhibitor 1C (CDKN1C) (p57, Kip2) expression in laser-captured mDA neurons from a series of postmortem human brain samples from PD and control cases. H19 was expressed in the midbrain, but was not expressed in DA neurons. CDKN1C and one IGF2 transcript were expressed in mDA neurons, suggesting that in addition to their selective expression in hESC DA-directed progenitors; CDKN1C and IGF2 are present in mature DA neurons. Interestingly, in addition to its role in regulating cell cycle exit and differentiation (Zhang et al., 1997; Cunningham, et al., 2001), CDNK1C (p57KIP2) cooperates with Nurr1 to effect post-mitotic DA differentiation (Josephet al., 2003).

In summary, the data presented suggest that the H19-IGF2 imprinting region located on chromosome 11p15.5 is involved in DA differentiation of hESC. Notably, IGF2 and CDKN1C were highly expressed and may play an important role in DA differentiation.

3.4 PAPER IV: THE IDENTIFIED STROMAL FACTORS SDF1A, SFRP1 AND VEGFD INDUCE DOPAMINERGIC NEURON DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS

The PA6 mouse stromal cell line can generate DA neurons from various PSC following co-culture (Kawasaki et al., 2000; Morizane et al., 2002; Perrier et al., 2004; Zeng et al., 2004; Schwartz et al., 2005). PA6 cells, as well as other stromal cells such as MS5, S17 and HepG2, exert a DA neuron-inducing effect on ESC that has been termed SDIA (Kawasaki et al., 2000; Barberi et al., 2003; Perrier et al., 2004; Schulz et al., 2004). The initial study describing SDIA
proposed that components accumulate at the cell surface as heavily modified or tethered proteins (Kawasaki et al., 2000); however, subsequently several studies, including one from our lab (Paper I), showed that PA6 CM was sufficient to induce DA differentiation from PSC and derived progenitors (Schwartz et al., 2005; Yamazoe et al., 2005; Hayashi et al., 2008; Vazin et al., 2008b; Swistowska et al., 2010), implicating secreted factors in SDIA. We reasoned that identifying the soluble components of SDIA would: (1) lead to a greater understanding of DA differentiation; (2) possibly identify novel compounds involved in DA neuron induction, differentiation and/or survival; and (3) lead to further progress towards clinically relevant treatments that will rely on defined factors in differentiation protocols.

We first set out to extend our previous studies with the NTera2 cell line, and comparatively and quantitatively examined the effects of PA6 CM on DA differentiation of hECSC (NTera2 cell line) and hESC (I6 cell line). We monitored the differentiation of NTera2 and I6 cells in various conditions including with and without mouse embryonic fibroblast feeder (MEF) cells in the presence of either PA6 CM, MEF conditioned medium (MEF CM) or medium alone (Media). Following differentiation with PA6 CM, both NTera2 and I6 cells efficiently generated DA neurons compared to medium alone. We observed an increase in the number of TH- and TuJ1- positive colonies, as well as an increase in the total number of TH- and TuJ1-positive cells. PSC exposed to PA6 CM more closely resembled PA6 co-culture than MEF co-culture, indicating that PA6 co-culture and PA6 CM shared many of the same DA neuron-inducing components, likely secreted factors.

We were intrigued by the implied role of secreted factors in DA differentiation and sought to identify the components that contributed to our observed effect. In order to begin to explore the molecular mechanisms responsible for SDIA, we comparatively assessed microarray gene expression profiles of PA6 and MEF cells, focusing on differentially expressed genes that encoded secreted or cell surface-associated proteins. Four candidate factors, HGF, SDF1α, sFRP1 and VEGFD, were selected and confirmed by RT-PCR. We also noted up-regulation of several other genes (Dlk1, Gas6, IGFBP4, Fgf10, gremlin, chordin, SMOC1, CCL9, spondin-2 and neuritin-1) and pathways (TGFβ, Wnt, VEGF, FGF, Notch) that were novel or consistent with previously published findings (Hayashi et al. 2008; Vazin et al., 2009; Swistowska et al., 2010) or reportedly involved in embryonic development, neuronal survival and/or dendritic outgrowth (Naeve et al., 1997; Burstyn-Cohen et al., 1999; Feinstein et al., 1999; Newrzella et al., 2007; Gersdorff et al., 2006).
In addition to our microarray findings, several other studies also examined the gene expression profiles of PA6 cells. However, although the mRNAs for putative secreted factors were present in the PA6 cells, it was critical to establish that the encoded protein was secreted and exerted a biological effect on PSC. Therefore, we used two techniques to explore the protein secretome of PA6 cells: (1) mass spectroscopy of PA6 CM and MEF CM to prospectively identify proteins differential present in PA6 CM; and (2) immunoaffinity capillary electrophoresis to determine the concentration of a given protein in PA6 CM, MEF CM or media alone. Mass spectroscopy of PA6 CM identified several proteins associated with neuronal function including talin-1, Septin-7 and Nedd 4 (Morgan et al., 2004; Xie et al., 2007; Putz et al., 2008). Notably, we also indentified sFRP1 and IGF2 as components of PA6 CM. sFRP1 was also selected based on our microarray data and has been implicated in neural induction (Satoh et al., 2006) and potentially could promote DA differentiation through either activation or inhibition of Wnt or Frizzled (Fz) binding partners (Uren et al., 2000; Kele-Olovsson, 2007). IGF2 has known roles in neuronal differentiation and survival (Pera et al., 2001; Pera et al., 2003; Silva et al., 2009) and has recently been identified as a potential DA neuron-inducing component of SDIA (Vazin et al., 2009). Next we used immunoaffinity capillary electrophoresis to determine the presence of a given protein and its concentration in medium. Our comparative immunoaffinity capillary electrophoresis analysis of PA6 CM, MEF CM and media alone, showed that HGF, SDF1α, sFRP1 and VEGFD were present in significantly higher concentrations in PA6 CM. Additionally, since recent studies have either alluded to or examined the roles of IGFBP4, IGF2, Wnt5a, PTN, EFNB1 and Fgf10 in SDIA DA induction (Hayashi et al., 2008; Vazin et al., 2009; Swistowska et al., 2010), we also examined these proteins and found that IGFBP4, IGF2, Wnt5a, and Fgf10 were present in relatively high concentrations in PA6 CM.

Together these results suggested that PA6 cells not only expressed HGF, SDF1α, sFRP1 and VEGFD but also secreted these factors and they could potentially contribute to SDIA. Since we have previously established the NTera2 cell line as a system to efficiently explore DA differentiation of hESC (Paper I), we first examined the effects of candidate factors on the NTera2 cell line to determine if there was an affect and the appropriate concentration, followed by testing each factor on the hESC (I6 cell line). We determined that addition of SDF1α, sFRP1 and VEGFD increased the number of TuJ1- and TH-positive cells in the NTera2 and I6 cell lines, as well as an increase in mDA and neuronal markers including DAT, Lmx1b and Pitx3. In the NTera2 cell line we made several notable observations following the addition of sFRP1. Interestingly, we found an increased number of neural rosettes following addition of
sFRP1 in the NTera2 cell line, suggesting a role in neural induction. Following the addition of high concentrations of sFRP1, a biphasic Wnt agonist/antagonist, we observed massive cell death, possibly through complete ablation of Wnt signaling. However, our results showed that no single factor alone or in combination mimicked the effects of PA6 CM, suggesting that additional factors may be involved in SDIA.

In summary, our findings suggest that PA6 cells secrete SDF1α, sFRP1 and VEGFD, and these factors play a role in DA neuron differentiation. Although, the stage at which these factors specifically regulate DA differentiation is undetermined since SDIA components likely affect multiple stages of differentiation. Nevertheless, our identification of soluble factors contributing to SDIA advances our understanding of the mechanisms that regulate DA neuron differentiation. Moreover, the inclusion of SDF1α, sFRP1 and VEGFD in DA derivation protocols may contribute to the progress towards PSC-based cell replacement therapies for PD.

3.5 PAPER V: CHARACTERIZATION AND MAINTENANCE OF NEURAL PROGENITORS DERIVED FROM HUMAN PLURIPOTENT STEM CELLS DIFFERENTIATED TOWARDS DOPAMINERGIC NEURONS

Current differentiation protocols can generate DA neurons from hESC (Perrier et al., 2004; Zeng et al., 2004; Yan et al., 2005; Sonntag et al., 2007; Chambers et al., 2009); however due to the pluripotent nature of PSC the resulting cultures almost always contain non-neuronal cells, as well as varied stages of cellular maturity, both of which limit clinical and experimental value. Therefore, methods for enriching DA neurons or progenitors from cultures are needed for both the basic studies on stem cell neurobiology and potential therapeutic application. Cell selection approaches including fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) have been successfully applied for the positive selection of neural populations or for the purpose of excluding potential tumor forming PSC or non-neural populations (Chung et al., 2006; Hedlund et al., 2008; Pruszak et al., 2009; Sundberg et al., 2009). In addition to pre-clinical applications of cell selection methods, enriched populations provide a unique opportunity to study human DA neuron development, since heterogeneity severely limits the utility of certain studies, such as microarray gene expression. Based on our previous studies involving PSA-NCAM-isolated neural progenitors (Paper I, Paper II and

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Paper III), we reasoned that PSA-NCAM and other cell surface markers could potentially be used to isolate progenitors and further investigate DA differentiation.

In this study we set out to explore PSA-NCAM, tyrosine kinase or BDNF/NT-3 growth factors receptor (TrkB) and low affinity nerve growth factor receptor (p75) as potential cell selection markers for enriching DA neurons. Continuing our previous studies using PSA-NCAM-positive cell selection, we found that PSA-NCAM- and TrkB-selected cells, as compared to p75 selection, most efficiently generated DA neurons while limiting mesodermal and astrocytic lineages. In our system p75 selection did result in TH- and TuJ1-positive cells; however, the efficiency more closely resembled non-selection than PSA-NCAM or TrkB selection. This suggests that p75 selects an earlier stage than neuronal or neural progenitors, illustrating the heterogeneity of derived cell populations.

Given that we were able to isolate progenitors with PSA-NCAM or TrkB selection, we reasoned that we could use selection methods to specifically study characteristics of these cells since they are typically examined in heterogeneous cell populations. We therefore reasoned that neuronal progenitors selected by either PSA-NCAM or TrkB, could be further differentiated in a defined DA neuronal supportive medium supplemented with BDNF, GDNF and bFGF. However, selected cells cultured in defined medium led to a greater than 10-fold reduction in the number of TH/TuJ1-positive cells compared to PA6 CM. Additionally, in defined medium we observed an increase in the percentage of total cells positive for the noradrenergic marker DBH. RT-PCR results were similar and we observed comparative levels of neuronal markers such as Map2 and doublecortin in defined and PA6 CM conditions. These results indicate that PA6 CM contains additional DA inductive cues that affect neuronal progenitor cells.

The above-mentioned PSA-NCAM selection criteria followed by PA6 CM directed differentiation resulted in greater than 60% TH positive cells. Therefore, we examined the microarray expression profiles of PSA-NCAM positive cells and their differentiated progeny to provide insight into characteristics and genes governing this process. We first surveyed known genes associated with mDA and found genes such as, AADC, Msx1, Ngn2, Nurr1, Otx 2 and Pax5 were highly expressed in both PSA-NCAM and differentiated PSA-NCAM populations. We next examined genes with reported neural function including KCNQ2, EFNB2, Hes 4, DPYSL4, NRD1, FEZ1, RAB3A, DLL1
and ARNT2. Interestingly, KCNQ2 is a potassium channel subunit that has been reported to be necessary for dopamine release in DA neurons (Cooper et al., 2001; Martire et al., 2007). While Ephrin B2 (EFNB2) and its receptor EFB1 have a reported role in axon guidance and target innervation of DA neurons (Yue et al., 1999).

In summary, positive neuronal selection strategies using either PSA-NCAM or TrkB can be used to enrich for DA neurons. Furthermore, PA6 CM contains unknown instructive cues that are important for DA commitment in neuronal progenitors. Isolated progenitors provided a unique tool to study DA neuron differentiation without confounding heterogeneity.
4 CONCLUSIONS

The wide differentiation potential of PSC offer remarkable opportunities and pose significant challenges for regenerative medicine and neural development. For years researchers have relied on alternative systems to explore differentiation mechanisms in order to streamline strategies and circumvent challenges associated with hESC. Therefore, we first set out to investigate whether the NTera2 cell line could serve as a useful surrogate to study DA neuron differentiation, and second, apply that knowledge to improve the efficiency and explore mechanisms of DA neuron differentiation. Based on the work so far presented we concluded:

Paper I: NTera2 cell line can be used as a PSC model system to study DA neuron differentiation.

- NTera2 cells are similar to hESC in expression of PSC markers and overall gene expression profiles.
- NTera2 cells co-cultured with PA6 cells generate neurons expressing characteristic DA neuron markers
- Cell culture modifications, such as co-culture with mitotically inactivated PA6 cells or with medium conditioned by PA6 cells also generate DA neurons.
- In a proof of principle approach, flows cytometry selection of NTera2 derived neuronal progenitors followed by further differentiation yields functional DA neurons.

Paper II: A focused DA-glial microarray distinguishes various PSC derived neural populations and provides insight into mechanisms regulating differentiation.

- A focused DA-glial microarray was designed to include (1) genes characteristic of DA neurons and glial cells, (2) markers highly expressed in “contaminating” pluripotent and neural progenitor populations, and (3) relevant transcription
factors, extracellular matrix molecules, receptors and soluble factors associated with neural development and differentiation.

- Validation testing determined the array was able to selectively and quantitatively detect genes highly expressed in DA neurons, glial, hESC and hESC derived NSC.

- Application testing determined that flow cytometry selected neuronal progenitors derived from NTera2 cells differentiating towards DA neurons expressed DA neuron markers and revealed potential signaling pathways involved in DA neuron differentiation.

**Paper III:** Gene expression analysis of neuronal progenitors revealed a unique gene profile and an upregulation of CDNK1C and IGF2 associated with chromosome 11p15.5.

- PSA-NCAM selection of neuronal progenitors from hESC differentiating towards DA neurons expressed appropriate DA markers.
- Differentiated neuronal progenitors generated functional DA neurons.
- MPSS gene expression analysis showed a unique gene expression pattern with respect to undifferentiated hESC and activation of chromosome 11p15.5, in the H19-IGF2 imprinting region.
- Selected neuronal progenitors and human mDA neurons expressed CDNK1C and IGF2.

**Paper IV:** SDF1α, sFRP1 and VEGFD are factors secreted by PA6 stromal cells that induce DA neuron differentiation in both hECSC and hESC.

- PA6 CM generates cells expressing characteristic DA neuron markers in the NTera2 (hECSC) and the I6 (hESC) cell lines.
- Large-scale gene analysis of PA6 cells and proteome analysis of PA6 CM identifies factors potential responsible for SDIA.
- PA6 cells secret HGF, SDF1α, sFRP1, VEGFD, IGF2, IGFBP4, Wnt5a, Fgf10 and Shh-N.

- SDF1α, sFRP1, VEGFD increased the number of cells expressing characteristic DA neuron markers in both the NTera2 (hECSC line) and the I6 (hESC line).

*Paper V:* PSA-NCAM and TrkB cell selection increases the efficiency of DA neuron generation.

- PSA-NCAM, TrkB and p75 are co-expressed with TH and TuJ1 in NTera2 cells differentiated towards DA neurons, to varied extents.

- PSA-NCAM and TrkB positive cell selection increased the number of DA neurons generated (TH and TuJ1 positive cells) and limited the number of non-neuronal cells (GFAP and SMA positive cells).

- PA6 CM resulted in DA differentiation of PSA-NCAM or TrkB selected neuronal progenitors compared to defined medium.

- PSA-NCAM positive selection generated DA neurons with mature characteristics and global gene expression analysis provides insight into potential signaling mechanisms.
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