DIFFERENTIATION OF DOPAMINERGIC NEURONS FROM HUMAN EMBYRONIC STEM CELL DERIVED NEURAL PROGENITORS USING GLIAL CELL-LINE DERIVED NEUROTROPHIC FACTOR: A DEVELOPMENTAL AND PARKINSON'S DISEASE MODEL

by

AMBER YOUNG

(Under the Direction of Steven L. Stice)

ABSTRACT

Parkinson's disease, the second most common neurodegenerative disease, causes the degeneration of the dopaminergic neurons projecting from the substantia nigra to the basal ganglia. Current treatments for Parkinson's disease become progressively less effective or cause serious side effects. Human neural progenitor cells derived from human embryonic stem cells provide a source for modeling the development of dopaminergic neurons, a source for drug screens of potential Parkinson's disease drugs or a potential source for a cell therapy for Parkinson' disease. Glial cell-line derived neurotrophic factor is a known dopaminergic neurotrophic factor, which has been shown in rodent and non-human primate models to protect dopaminergic neurons from 6-OHDA and MPTP. In addition, glial cell-line derived neurotrophic factor has been used in human clinical trials; however, these trials were halted due to unexpected side effects. We previously derived a stable, adherent monolayer culture of human neural progenitor cells that can be maintained as proliferative cells or differentiated into neurons, astrocytes or oligodendrocytes. In this dissertation, we determined the human neural progenitors to have functional ion channels and ionotrophic receptors. These human neural progenitors also had a basal level of dopamine transporter expression that we were able to enhance using glial cell-line derived neurotrophic factor. The addition of 25ng/ml glial cell-line derived neurotrophic factor advanced the human neural progenitor cells from dopamine

progenitors through a dopamine specification stage to a dopaminergic-like neuron as marked by NURR1, EN1, TH, PITX3, VMAT2 and DAT expression. The pathway through which glial cell-line derived neurotrophic factor enhanced dopaminergic-like neurons from human neural progenitor cells occurred through RET receptor activation of the ERK and p38MAPK pathways as well as the mTOR pathway. The results from these studies provide a novel one-step mechanism for obtaining dopaminergic-like neurons from human embryonic stem cell derived neural progenitor cells. These dopaminergic-like neurons progress developmentally through the stages of dopaminergic differentiation providing a model for dopaminergic differentiation. Additionally, these cells provide a human neural model to screen future drugs, which may treat Parkinson's disease.

INDEX WORDS: Dopaminergic Neuron, Differentiation, Human Embryonic Stem Cells, Human Neural Progenitors, Glial Cell-line Derived Neurotrophic Factor, Mitogen Activatied Protein Kinase, Phosphoinositide 3-kinase, Parkinson's Disease, Cell Therapy

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B.S., Emory University, 2006

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2010

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December 2010

ACKNOWLEDGEMENTS

Without the sources of inspiration, motivation and guidance, the work within this dissertation and throughout my time in graduate school would not be possible. I would like to thank Dr. Steve Stice for his support and the continual guidance without which I would not be the scientist I am today. I would also like to thank the members of the Stice Lab who allowed me to come to them for advice with experiments, help with new techniques or just as a sounding board.

These past years would not be possible without great friends who have stood by even when I was not the best person I could be and who provided me with some great memories. To Melissa, thanks for the many  years of friendship and support. I am glad to know you are always there. To Paige and Erin, I know our friendship can survive anything after surviving living together! Thanks for seeing past my immature years and for being there in the years since. To all my other friends, too many to name, without the comic relief, entertainment, and support, this work would not be possible.

Thanks to Andrew for putting up with my mood swings, the many late nights and long weekends I spent working and for doing your best to make it as easy as it could be for me.

“The only rock I know that stays steady, the only institution I know that works is the family” Lee Iacocca. Mom and Dad, your unwavering support of my desires to be a scientist and your continued pride in me even when I messed up are a great example of the kind of parent I want to be someday, I hope I can be half the parent you were to me. Eric, thank you for always being there when I needed you, especially when my car broke down in the middle of nowhere.

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# CHAPTER 1

## INTRODUCTION

Parkinson's disease (PD) affects approximately 1% of the American population with debilitating motor symptoms [1]. These motor symptoms are caused by the disintegration of the dopaminergic neurons that project from the substantia nigra (SN) to the basal ganglia [2]. Current treatments often cause side effects worse than the symptoms of the disease itself or are only effective for short periods of time [3]. Derivation of dopaminergic neurons from human embryonic stem cells (hESCs) or human neural progenitors (hNPs) provides a potential source of a human model to study PD, to screen future drug therapies for PD or to use as a cell replacement therapy for PD.

Attempts at dopaminergic differentiation from hESCs followed attempts at dopaminergic differentiation in mouse embryonic stem cells (mESCs) [4,5]. Original reports made use of stromal cell-derived inducing activity (SDIA) to generate 79% of neurons expressing tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis [6]. Since this report, several others have differentiated dopaminergic neurons from hESCs using either the SDIA method or a 5-stage differentiation protocol [7-9]. While a higher percentage of dopaminergic neurons are seen with the SDIA method, the use of an animal co-culture system prevents future clinical use. The 5-stage method does not require a contaminating feeder layer; however, the time to differentiation is much longer (~49 days compared with ~14 days). In the 5 years since the first report of dopaminergic neurons from hESCs, little progress has been made in achieving a simple and effective method for deriving dopaminergic neurons.

Glial cell-line derived neurotrophic factor (GDNF) was discovered as a neuroprotectant for dopaminergic neurons in 1993 [10]. Since that discovery, GDNF has been investigated for use as a neuroprotectant in PD patients, as a treatment option for PD patients and as a factor to

enhance the differentiation of dopaminergic neurons from hESCs [11-13]. Trials involving the delivery of GDNF into human striatal cortex have encountered complications due to inefficient delivery and due to difficulty of injection into the correct location [14]. Successful neuroprotection of dopaminergic neurons in animal models of PD occurs after GDNF is administered.

**Specific Aims**

This dissertation tests the hypothesis that the addition of GDNF to hNPs will enhance the differentiation of dopaminergic neurons. Currently, the pathway through which GDNF enhances dopaminergic differentiation is unknown; however, the role of the c-Jun N-terminal kinase (JNK) pathway in neural survival and the role of the Src pathway in lipid raft recruitment of the Rearranged in Transfection (RET) receptor for GDNF has been established. This dissertation tests the hypothesis that the mitogen activated protein kinase (MAPK) pathway and the phosphoinositide 3-kinase (PI3K) pathway are involved in the role of GDNF in enhancing differentiation of hNPs towards a dopaminergic-like neuron through activation of downstream factors as well as through crosstalk between the two pathways.

*Specific Aim 1:*

To confirm the ability of the hNPs and differentiated hNPs to be differentiated into functional dopaminergic-like neurons, the ionotrophic receptor profile of the hNPs and differentiated hNPs will be tested with RT-PCR. Additionally, basal expression of DAT will be measured with RT-PCR.

*Specific Aim 2:*

To enhance the differentiation of dopaminergic-like neurons from hNPs though the use of GDNF, a known dopaminergic neuroprotectant. This will be measured through developmental progression of dopaminergic protein markers as determined with immunocytochemistry and flow cytometry.

*Specific Aim 3:*

To elucidate the pathways through which GDNF acts to enhance differentiation of dopaminergic-like neurons from hNPs through examining genetic markers of the MAPK and PI3K pathway. Roles of these genetic changes will be analyzed for functional changes in cellular processes to confirm their roles in enhancing dopaminergic-like neuron differentiation from hNPs.

If successful, these studies will provide a model system for studying dopaminergic differentiation and development, for screening PD drugs, as well as providing a system through which the processes of differentiation in hESCs can be studied.

Abbreviations and Nomenclature

GDNF – Glial Cell-line Derived Neurotrophic Factor

GLC – Germ-like Cells

hESC – Human Embryonic Stem Cell

hNP – Human Neural Progenitor

JNK – c-Jun N-terminal Kinase

MAPK – Mitogen Activated Protein Kinase

mESC – Mouse Embryonic Stem Cell

PD – Parkinson's Disease

PI3K – Phosphoinositide 3-kinase

RET – Rearranged in Transfection

SDIA – Stromal Cell-line Derived Inducing Activity

SN – Substantia Nigra

TH – Tyrosine Hydroxylase

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# CHAPTER 2

# LITERATURE REVIEW

## DOPAMINERGIC NEURONS DERIVED FROM HUMAN EMBYRONIC STEM CELL DERIVED NEURAL PROGENITORS: BIOLOGICAL RELEVANCE AND APPLICATION[1]

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[1]Young, A. and Stice, S.L. Accepted by Embryonic Stem Cells. Reprinted here with permission of publisher, 11/6/2010

Dopaminergic neurons are studied at length for their role in Parkinson's disease (PD), schizophrenia and addiction [1] . While these commonly known roles for dopamine involve a similar neural subtype, the brain areas and identifying genetic markers involved in each pathway differ. These differences lead to selective involvement of each pathway (nigrostriatal, mesolimbic and mesocortical), allowing for derivation of dopaminergic neurons from human embryonic stem cells (hESCs) as well as from human neural progenitors (hNPs) that can be used for drug development or for cell therapy in PD.

Ever since their isolation in 1998, hESCs have been touted as potential cell therapies, drug development assays and a source for studying human development [2]. Due to PD effecting 1% of the American population over 65 as well as the specificity of the cell type affected, PD presents as a neurodegenerative disease with potential to be helped with hESCs [3]. In 2004, the first report of tyrosine hydroxylase (TH) positive neurons derived from hESCs demonstrated that obtaining dopaminergic neurons would be possible in humans [4]. The stromal cell-derived inducing activity (SDIA) method enhanced dopaminergic differentiation through co-culture with mouse derived stromal cells, which secreted factors that directed differentiation towards a dopaminergic phenotype. Following the SDIA method, a 5-stage method for deriving dopaminergic neurons from hESCs that did not require co-culture with contaminating feeder layers obtained fewer dopaminergic neurons from hESCs [5]. Since this work, there has been limited success in obtaining high levels of TH+ neurons without the addition of feeder layers.

A selective dopaminergic neuron neuroprotectant was discovered in 1993, glial cell-line derived neurotrophic factor (GDNF) [6]. The potential for this neurotrophic factor to protect those cells affected in PD was explored, and in rat models of PD, GDNF administration was effective in protecting substantia nigra dopaminergic neurons as well as in protecting neural cells transplanted into lesioned rat midbrains [7,8]. Methods for administering GDNF into human patients have been developed and clinical trials utilizing GDNF as a protectant for dopaminergic

neurons have proceeded with unfavorable results due to difficulty with localization of GDNF administration as well as invasiveness of the surgery required [9-12]. Methods for GDNF administration other than through lesions have not lead to successful results. However, GDNF has potential as a dopaminergic neuroprotection agent in the differentiation of dopaminergic neurons from hESCs or hNPs.

In this chapter, we intend to cover dopaminergic development in the mouse and human brain in order to understand more fully dopaminergic derivation from hESCs and hNPs. We also intend to examine the processes of dopaminergic derivation that have been used as well as the role the GDNF plays in this process. Finally, we intend to cover the potential applications for hNP derived dopaminergic neurons.

**Parkinson's disease**

*Epidemiology*

PD is a progressive neurodegenerative disease that is second in prevalence only to Alzheimer's disease [3]. While typically thought of as a disorder only affecting the elderly population, early onset PD (appearance of symptoms between 45 and 65) currently accounts for 10% of the diagnosed cases of Parkinson's [13]. There are two main subtypes of PD, idiopathic and secondary. Idiopathic forms of PD can be either sporadic (90% of cases) or genetic. Most often seen in young onset PD, the most common genetic mutation is in the *Park8* (*Lrrk2*) gene and the second most common is in the *Park1* gene which encodes for the alpha synuclein protein [14]. Sporadic PD has no clear etiology but may be caused by environmental factors, toxins or aging. Secondary PD is caused by medications, infection or metabolic disorders [15,16].

Diagnosis usually begins with the presentation of motor symptoms which fall into four categories: 1) resting tremor, 2) bradykinesia, 3) rigidity and 4) postural instability [3]. Younger patients present with tremor as their primary symptom and older patients present with bradykinesia as their primary symptom [15]. The resting tremor appears unilaterally and moves

bilateral as the disease progresses. Most often, the tremor is seen in the distal portion of the limbs in the hands or leg. Bradykinesia, the inability to initiate movement, leads to the shuffling gait associated with PD. Most often this is noticed in the slowness and difficulty a PD patient has when walking, but it can also lead to difficulty in turning in bed or rising from a chair [15]. Rigidity, stiffness of the muscles in the limbs and trunk, often leads to postural instability [15]. Postural instability, the inability to maintain balance and coordination, occurs in the most advanced stages of PD [15]. This affliction often leads to the falls that can lead to rapid decline in a person's quality of life. In addition to decreasing quality of life, postural instability has very little response to the current treatments for PD [3].

In addition to the motor symptoms, PD patients are affected by non-motor symptoms. This is due to the large involvement of other neurons in the limbic area of the brain, the compensation for the loss of dopaminergic neurons by other neurons and the connections between the basal ganglia and the frontal cortex. The most prevalent non-motor symptom is depression beyond which would be expected for the average population affected by a debilitating disorder with between 20 and 45% of people with PD being diagnosed with depression post diagnosis of PD [17]. The second most common non-motor symptom is psychosis, most often manifesting in hallucinations [17]. Cognitive decline is seen as the disease progresses with memory loss, attention impairment and executive function deficits reported most often [18]. The co-morbidity of these non-motor symptoms with the motor symptoms paints an image of PD as a whole body and mind disorder not just as a motor disorder [17,19].

The first effective treatment for PD and still the leading treatment is a dopamine precursor that crosses the blood brain barrier (BBB) and is converted into dopamine in the brain known as levo-dopa (L-dopa) [15]. However, L-dopa often produces serious side effects. In addition, over time, patients require higher and higher dosages to be effective, a concern with younger patients [13]. The final hallmark of PD, postural instability, is resistant to L-dopa

treatment. Another common treatment option is dopamine agonists, which can be used in mono-therapy or in combination with L-dopa. Argument for their use alone as a first treatment is to delay L-dopa treatment slowing the wearing off of L-dopa [13]. However, due to the lack of robustness of dopamine agonists, almost all patients will require L-dopa at some point. In the many years since the beginning of a search for treatment, the lack of progress demonstrates the complexity of the disease [14].

*Pathophysiology*

The earliest and most studied cause of PD is the degeneration of the dopaminergic neurons in the substantia nigra (SN). In the normal brain, dopaminergic neurons are found in three main areas, the olfactory bulb, the hypothalamus and the midbrain, which consists of the SN and the ventral tegmental area (VTA). From the midbrain, there are three main projections of the dopaminergic neurons. The mesolimbic pathway projects dopaminergic axons from the VTA to the nucleus accumbens, plays a role in addiction and reward and is the pathway most often affected in schizophrenia [20]. The mesocortical pathway projects axons from the VTA to the frontal cortex and is most often associated with motivation and memory [20]. The nigrostriatal pathway projects from the SN to the basal ganglia (BG) and is associated with motor control [21]. This pathway is involved in PD, and will be the focus of this review.

In the normal brain, dopaminergic projections from the substantia nigra pars compacta (SNc) synapse on the striatum, which consists of the caudate nucleus and the putaman (Figure 2.1) [22]. From the striatum, a direct or an indirect pathway leads to the substantia nigra pars reticula (SNr) [22]. The direct pathway sends inhibitory GABA and substance P axons to the globus pallidus internal (GPi)/SNr [17,23]. The indirect pathway projects inhibitory GABA and enkephalin axons to the globus pallidus external (GPe) which then sends GABAergic projections to the subthalamic nucleus (STN) which then sends glutamatergic (excitatory) outputs to the GPi/SNr [23] The projections to the SNr proceed to the thalamus. From the thalamus, glutamatergic projections head toward the cortex or GABAergic projections proceed

to the brain stem and from the brain stem axons project back to the SNc completing the loop [23]. Both pathways lead to activation of muscle movement and control. Through the activation of the motor cortex or the brain stem as well as through feedback loops within the basal ganglia, fine motor movements can be controlled [19,23], the decision to move can be separated from the movement itself and other outside inputs can be factored into muscle movement decision.

In PD patients, the dopaminergic projections to the striatum deteriorate. The decline in dopaminergic modulation of the basal ganglia leads to problems in controlling muscle movements and to the symptoms seen in PD (Figure 2.1). Often the symptoms do not present until approximately 60% of the dopaminergic cells in the SNc have died suggesting a compensating mechanism for controlling movement [19]. Proposed mechanisms for this redundancy include the feedback loops located within the basal ganglia as well as movement of serotonergic neurons located nearby the basal ganglia [22]. These mechanisms from the serotonergic neurons may be responsible for some of the early non-motor symptoms [17]. Additionally, the relationship of the basal ganglia with the frontal cortex may be responsible for cognitive decline [17]. The dopaminergic neurons in the SNc preferentially deteriorate in PD, leaving the dopaminergic neurons in the rest of the brain intact and not leading to symptoms typically seen in other dopaminergic disorders.

## Human Embryonic Stem Cells and Derivatives

### *Human Embryonic Stem Cells*

In 1998, James Thomson and colleagues derived hESCs from the inner cell mass of discarded blastocysts [2]. From 14 inner cell masses collected, five embryonic stem cell lines could be created. Thomson and colleagues established early characteristics of hESCs which included high nuclear to cytoplasmic ratio, prominent nucleoli, the formation of a distinct colony, high telomerase activity, the ability to form cells from all three germ layers, and teratoma formation in addition to embryonic markers SSEA-3, SSEA-4, Tra-1-60, Tra-1-81 and alkaline

phosphytase (AP) [2]. Mouse embryonic fibroblast (MEF) feeder layers support pluripotency of hESCs [2].

Mouse embryonic stem cells (mESCs) can be maintained in an undifferentiated state using leukemia inhibitory factor (LIF) alone without feeder layers. LIF activates the signal transducer *gp130* there by activating STAT3, the state of self-renewal in mESCs is maintained (Figure 2.3) [24]. Bone morphogenic proteins (BMPs) can be used in the place of serum in addition to LIF to maintain pluripotency through the activation of *Id* genes [24-26]. This has not been the case for hESCs as LIF does not maintain the pluripotency of hESCs and is not necessary for maintenance of self-renewal [27]. Initial attempts at feeder free culture of hESCs expanded upon the knowledge that hESC populations express $\alpha$6 and β1 integrins leading to successful culture on laminin and Matrigel as extracellular matrices for hESCs in MEF conditioned media with differentiation results similar to what was found in previous studies [28]. Basic fibroblast growth factor (bFGF) has been used to maintain clonally derived hESCs suggesting potential in a feeder free, serum free culture [29]. Differentiation studies in which BMPs were blocked in hESC culture initiated neural differentiation. Taking these two together, Xu and colleagues used bFGF and BMP to maintain hESC self-renewal in the absence of MEFs or MEF conditioned media [27].

Our lab derived three lines from discarded embryos in 2001. These lines were isolated from the inner cell mass of 19 embryos and resulted in four cell lines. These cell lines were maintained in a pluripotent state on MEF feeder layers [30]. Two of these cell lines (BG01 and BG02) have the ability to form EBs and to differentiate into neural cells and cardiac cells [30].

*Neural Progenitor Cells*

Directing the differentiation of hESCs towards neural cells allows for a controlled culture system to develop specific neural subtypes including motor neurons, dopaminergic neurons and forebrain neurons. Several groups have attempted to establish a proliferative population of multipotent hNPs, which can be differentiated to neurons, astrocytes or oligodentrocytes.

Differentiation of hESCs toward hNPs occurs through either an embyroid body (EB) or a monolayer culture system. In EB differentiation, hESCs are grown in suspension and allowed to form masses of cells, which develop into a mixed population that includes hNPs [31,32]. From these masses, the neural cells were selected and used in further proliferation or differentiation experiments. In monolayer differentiation, hESCs are induced with various morphogens in the tissue culture dish and neural rosette structures are allowed to form. From these structures, neural cells are selected, re-plated and allowed to proliferate or differentiate [33].

Each type of differentiation (EB or monolayer) requires various morphogens to direct the differentiation toward a neural multipotent cell. Retinoic acid (RA) plays a role in neural patterning and neural differentiation in the developing embryo [34-36]. Bone morphogenetic proteins are often inhibited by the antagonist Noggin, which leads to development of the neural phenotype in the mouse [37,38]. bFGF signaling maintains the proliferative capacity of neural cells as well as involvement in induction and patterning [39]. bFGF was used in a neural differentiation protocol for its known caudalizing factors. Originally, bFGF was shown to be important in the brain as a neural growth factor that maintained the pluripotency of immortalized NSCs (Figure 2.3) [40]. Later, bFGF has been shown to be a caudalizing factor within the neural plate and the neural floor [39]. Epidermal growth factor (EGF) is a mitogen that was used in many hESC neural differentiation protocols to maintain self-renewal potentially through crosstalk with Notch or through EGF's suppression of apoptosis [35,41]. LIF is another factor known to maintain proliferation of neural cells [33]. Several reports of hNP differentiation have used varying combinations of these factors to achieve hNP differentiation from hESCs.

Differentiation of these hNPs into specific neural subtypes creates neural cells that are better models for specific developmental patterns, disease progression or transplantation. Differentiation of motor neurons has required the addition of RA, sonic hedgehog (SHH) and bFGF [42,43]. Forebrain differentiation has required *Otx1*, *Otx2* and *Bf1* expression and is thought to involved WNT signaling [41]. Serotonergic neuron differentiation requires SHH and

fibroblast growth factor 4 (FGF4) [44]. Differentiation toward a dopaminergic fate begins with SHH and fibroblast growth factor 8 (FGF8) and will be further explored in this review [4].

In 2006, our lab derived hNPs from hESCs using a monolayer culture system. Neural derivation media was used to induce neural rosette structures from which neural cells were selected and transferred to a monolayer culture [33]. The combination of bFGF and LIF added to the culture media allowed for the maintenance of neural progenitor cells in a monolayer that could be continually cultured for several (>40 passages) while maintaining a stable karyotype [33]. We have demonstrated the ability to differentiate these hNPs to motor neurons with the addition of RA [43] and to dopaminergic neurons with the addition of GDNF [45].

**Factors Involved in Dopaminergic Differentiation**

*Sonic Hedgehog*

In the developing embryo, signaling factors in the developing nervous system control the movement of the different types of neurons in the brain and spinal cord to their correct position. In dopaminergic neuron development, sonic hedgehog (SHH) modulates the dorsal/ventral placement of the midbrain dopaminergic neurons [46]. SHH is secreted from the notochord to induce floor plate cells through a decreasing gradient and to signal for the ventral forebrain and midbrain development of serotonergic and dopaminergic neurons [46,47]. SHH signaling is regulated closely to ensure proper enlargement of the midbrain area and is turned off to allow for post-mitotic differentiation. Dopaminergic neurons will arise from the pool of neuroepithelial progenitors found in the ventricular floor plate [47]. *Wnt* causes a down regulation of SHH signaling allowing for the end of neural proliferation and the beginning of neurogenesis [48]. In 1995, SHH was discovered to be important for dopaminergic neural development through its activation of cAMP and PKA [46]. Transplantation of floor plate tissue to other areas or induced expression of SHH in other brain areas will cause ventralization of those areas. Over expression of the SHH target Gli1 causes the same effects as SHH itself, further confirming SHH's role in dopaminergic neuron development [49]. The ability for floor plate tissue combined with FGF8

beads to induce the formation of midbrain dopaminergic neurons further added increased evidence for SHH in the midbrain/hindbrain organization. SHH activates Patched (Ptc), releasing its negative control on Smoothened (Smo) and activating downstream transcription factors Gli1, Gli2 and Gli3 [49]. Each Gli has distinct actions; Gli1 acts to increase SHH activation. Gli2 acts to modulate *Wnt*, *Brachyury*, *Xhox3* and *Bcl-2* genes. Gli3 activates Ptc as a negative control of SHH signaling [49]. The decreasing gradient outward from the ventral midbrain signals the beginning of neurogenesis, which is suppressed by Wnt signaling. SHH interacts with FGF8 to induce the correct size pool of dopaminergic neurons [48].

*Fibroblast Growth Factor 8*

In combination with SHH, FGF8 controls the boundaries of the midbrain-hindbrain organizer (MHO) which direct the area in which dopaminergic neurons will be expressed. FGF8 expression originates at the isthmus and radiates anterior/posterior [50]. The size of the MHO is determined by outside induction factors including the Hox genes at the anterior edge and FGF4 which signals with SHH for serotonergic neuron development. FGF8 interacts with other early regulatory genes involved in dopaminergic neuron development (*Otx2*, *Gbx2*, *En1*, *En2*, *Pax2* and *Pax5*) to maintain and regulate the dopaminergic field of development [51]. If ectopically applied, FGF8 and SHH induce a two-dimensional system of midbrain neural precursor cells [47].

*Leukemia Inhibitory Factor*

Leukemia inhibitory factor (LIF) is a member of the interleukin 6 family of cytokines and supports cell growth and development. LIF has known function in maintaining the pluripotency of mESCs [52]. This function does not carry over into hESCs, as the addition of LIF to the culture media for hESCs does not maintain their pluripotency [27]. In the non-dividing cells of the neural crest, LIF induces sensory neuron development [53]. Later it was discovered that LIF also promoted proliferation of the progenitor pool found in the olfactory bulb and in fetal neural stem cells [54,55]. This is thought to occur through the *gp130* receptor regulation of Notch

signaling which controls neural stem cell proliferation. LIF has been used to maintain pluripotency in hNPs derived from hESCs as well as in NSC cultures [56].

While it was known that LIF supported glial cell differentiation, in 2003 it was discovered that LIF acts through the ERK pathway to decrease the expression of dopamine beta hydroxylase (DβH; Figure 2.2) [57]. Mouse and rat mesencephalic derived progenitors were differentiated into dopaminergic neurons using both LIF and GDNF [58]. These differentiated dopaminergic neurons were maintained in culture for extended periods as well as used for deriving a clonal line [58]. LIF has also been used in a rat model of PD to increase the number of mesencephalic dopaminergic neurons. Support for the use of LIF as a factor to enhance dopaminergic differentiation from hNPs in this chapter comes from the suppression of DβH by LIF (Figure 2.2) in addition to the known success in a rat and mouse model of dopaminergic differentiation with GDNF and LIF [59,60].

*Glial Cell-line Derived Neurotrophic Factor*

GDNF was discovered as a neurotrophic factor for dopaminergic neurons in 1993 in rat glial cell cultures [6]. Since this time, its use as a potential treatment for PD has been explored in several animal models (rat, mouse, non-human primate), cell culture models (rat, mouse, non-human primate, mESCs, hESCs, human fetal tissue) and in human drug trials. GDNF was first tested as a recovery factor in animal models of PD [61,62]. Rats lesioned with 6-OHDA and then injected with GDNF showed increase in TH expression and a reduction in apomorphine induced turning [61,62]. Retrograde tracing studies show that GDNF injected into the midbrain was transported back to the SN [63]. In C57/B1 mice, injection of GDNF in the SN protects from degeneration and aids in the recovery of dopaminergic neurons in an MPTP model [7]. The first study of GDNF in a non-human primate, a rhesus monkey, showed recovery in bradykinesia, rigidity and postural instability in an MPTP lesioned striatum that was maintained with injections of GDNF every 4 weeks [64]. As injections into the brain are not desired for a potential

treatment option for PD, fetal mesencephalic neurons from rat brains that excrete GDNF were injected into 6-OHDA lesioned rats and an increase in TH expression and expansion of neurite tracts were seen postmortem [65-67]. Adenovirus' created to promote GDNF expression were tested by several groups for their ability to protect the SN from 6-OHDA neurotoxicity with limited results and lack of long-term effectiveness [68,69]. Most of the data has shown limited results with long-term data unavailable; however, due to the known protective role for GDNF in DA neurons, enthusiasm is still high.

A 12-week study of GDNF injection into the ventricle of a 6-OHDA lesioned rat showed an increase in response to amphetamine to those seen in sham lesioned rats [70]. Injections into the SN are found to be protective where as injections into the putamen are not [71]. Long-term adenovirus vector administration in the SN led to re-innervation of the striatum after 6 months of administration [72]. Lentiviral administration of GDNF into the striatum of both mice and non-human primate that were lesioned 2 weeks later protected TH neurons in both young and old mice and non-human primates [73,74]. GDNF administration in a mouse $\alpha$-synuclein model did not protect the SN from neurodegeneration presenting a complication that remains for GDNF as a therapy for PD [75].

GDNF's role in protecting and recovering neurons in the SN that degenerate in PD led to research into its use as a growth molecule in hESCs. hESCs differentiated towards dopaminergic neurons have been touted as a potential cell therapy in PD. The use of GDNF along with co-culture with PA6 cells increased the number of TH positive cells produced over the number of TH+ cells produced with PA6 co-culture alone [76]. Using hESC derived dopaminergic neurons in a model of PD, GDNF provided protection against MPTP toxicity [77].

Another method of improving delivery systems involves genetically altering neural stem cells or astrocytes to release GDNF and injecting these into the brain, which has protected from parkinsonian motor responses in mouse models of PD [78,79]. Injection of hNPs modified to secrete GDNF into MPTP monkeys increased axon fibers that express both TH and VMAT2;

however, these cells remained at the area of injection and did not travel to the area of need [80]. The neuroprotection of GDNF in rat, non-human primate and hESC models of PD demonstrates its robustness as a useful tool for developing future therapies.

Further expanding on the rodent research, intracerebral injections of GDNF into MPTP treated rhesus monkeys induced a 20% increase in dopamine levels and functional recovery with GDNF injections every four weeks [64]. When GDNF was administered along with the most commonly prescribed L-dopa drug in parkinsonian rhesus monkeys, a significant functional improvement in PD symptoms was seen as well as a decrease in the side effects typically accompanying L-dopa drugs [78]. Further study between the relationship of GDNF administration and functional recovery indicates a role for GDNF in modulating dopamine plasticity in the striatum. Safety and efficacy studies in non-human primates demonstrated that the injections do not cause any negative histological effects in the injected brain and that the most notable side effect of GDNF delivery was weight loss [12]. These studies advanced the field towards using GDNF in human clinical trials.

Following determination that human PD patient brains maintained expression of the RET receptor for GDNF, a male patient received intracerebroventricular injections of GDNF which resulted in severe side effects and no functional recovery. Several years later, a randomized double-blind study of intracerebroventricular monthly injections of various dosages of GDNF lead to no parkinsonian symptom improvement with GDNF but an increase in adverse effects including significant weight loss potentially because the GDNF did not reach the target tissues [10]. Targeted injections of GDNF into the putamen resulted in significant improvements in PD quality of life scores, dopamine uptake and dyskinesia [81]. In a two-year follow up study, the patients continued to improve with no added side effects. Withdrawal of GDNF injections caused a complete reversal to pre-injection levels of quality of life and symptomatic scores [9]. At this stage, GDNF is still being evaluated as a potential treatment for PD but the route of administration and side effect profile are holding back major advances in the field.

## Genes Involved in Dopamine Development

*Nuclear Receptor Related 1*

Nuclear receptor related 1 (NURR1) is a member of the NUR family of proteins which are involved in cell growth and apoptosis. Beginning expression at E10.5 in the mouse, NURR1 is required for normal dopaminergic development (Table 2.1) [82]. NURR1 was discovered to be similar to NUR77 already found in the olfactory bulb, cortex, hippocampus, SN and VTA of the mouse [83,84]. Mice administered 6-OHDA not only show a loss in dopaminergic neurons but also in NURR1 expression. *Nurr1* knockout (*Nurr1*-/-) mice do not express TH in brain areas A8 (retrorubral nucleus), A9 (SN) and A10 (VTA) [82]. Discovery of the need for NURR1 to regulate TH expression led to further examination of the role that NURR1 plays in maintenance of other proteins important for a properly functional dopaminergic neuron (Table 2.1). In *Nurr1*-/- mice, aromatic L-amino acid decarboxylase (AADC), the enzyme responsible for converting L-dopa to dopamine or 5-hydroxytryptophan to serotonin, was found to be absent in dopaminergic neurons only; however, paired-like homeodomain transcription factor 3 (PITX3), a gene found only in SN dopaminergic neurons, expression was unaffected in *Nurr1*-/- mice (Table 2.1) [85]. As discussed earlier, GDNF signals through co-receptor GFR$\alpha$1 binding to RET (Table 2.1). *Nurr1* knockouts are deficient in RET but not in GFR$\alpha$1 suggesting the importance of NURR1 not only in pathways involved in dopamine production but in neuron maintenance and support [86].

NURR1 is not only important for embryonic development of dopaminergic neurons but also for the maintenance of these neurons in the postnatal and adult brain. Conditional knockouts induced by *Cre* ablation of NURR1 at E13.5 to E15.5 show a loss of TH and the dopamine transporter (DAT) expression in postnatal rats while adult ablation leads to reduction in TH expression in the SN preferentially over the VTA (Table 2.1) [87]. Overall, NURR1 plays a role in activating and maintaining the expression of AADC, TH, RET and DAT. With such an

importance in dopaminergic neurons, finding a decrease in NURR1 in PD patients as well as a base pair insertion mutation is not surprising. Further expansion on the role of NURR1 in PD patients may lead to future treatment options.

*Engrailed 1*

Engrailed 1 (EN1) is part of a family of homeobox genes consisting of *En1* which is expressed in the VTA and SN and *En2* which is only expressed in a subset of dopaminergic neurons and begins to be expressed later in development than *En1* [88]. EN1 is a developmental regulation protein that is expressed in mouse around day E7.5 and plays a role in the development of dopaminergic neurons and the maintenance of those neurons (Table 2.1) [88]. EN1 is expressed in the neuroepithelium of the ventral midbrain around the isthmus, which is responsible for controlling the midbrain/hindbrain boundary [89]. Induction of EN1 by FGF8 maintains the area of the brain that will consist of the dopaminergic neurons. *En1* knockout mice lose the expression of all dopaminergic neurons by birth [90]. A gain of function study in mice demonstrated that EN1 would induce the midbrain/hindbrain expression in any area in which it was expressed (Table 2.1) [91]. Both EN1 and EN2 are necessary for proper induction of midbrain dopaminergic neurons, and they can partially compensate for each other [91]. The other role of EN1 is in maintaining dopaminergic neurons post-mitotically in the midbrain (Table 2.1) [92]. *En1* conditional knockout mice lose their dopaminergic neurons in the midbrain due to caspase 3 induction and apoptosis [92,93]. Heterogeneous *En*+/- mice will progressively lose their dopaminergic neurons in a pattern that is similar to that seen in PD patients [92,93].

*Tyrosine Hydroxylase*

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in dopamine synthesis, making it the main marker for dopaminergic neurons. In the production of catecholamines, L-tyrosine is converted to L-dopa by TH. Aromatic L-amino acid decarboxylase (AADC) then converts the L-dopamine into dopamine. In dopaminergic neurons, the process stops there. In noradrenergic neurons, dopamine is converted into norephinephine by dopamine β hydroxylase (DβH). If the

neuron releases epinephrine, then norephinephine is converted to epinephrine by phenylethanolamine N-methyltransferase (PMNT) (Table 2.1) [94]. As TH has an important role in several neural subtypes, it cannot be the sole marker for a dopaminergic neuron even if it is an important one. In the midbrain, TH expression occurs at E11.5 in the mouse immediately prior to PITX3 expression (Table 2.1). TH expression is driven by NURR1, which began its expression at E10.5 [95].

*Paired-like Homeodomain Transcription Factor 3*

Paired-like homeodomain transcription factor 3 (PITX3) is a homeodomain protein which is found only in dopaminergic neurons in the midbrain of the central nervous system. Expression of PITX3 is found outside of the CNS transiently in the eye lens. Expression of PITX3 begins at E11.5 in the mouse, immediately following expression of TH (Table 2.1) [96]. PITX3 expression completely overlaps the areas of TH expression in the SN and only a subset of the TH+ neurons in the VTA; additionally, the TH promoter has a PITX3 binding site. *Aphakia* mutant mice (*Pitx3*-/-) lack SN neurons exclusively starting around E12.5, but not VTA neurons suggesting the molecular mechanism of PITX3 in the VTA differs from that in the SN and this mechanism may provide insight into the selective degeneration of dopaminergic neurons in the SN in PD [97-99]. Retrograde tracing studies confirm that the absence of dopaminergic neurons in the SN in *apkakia* mice leads to a lack of the normal connections to the caudate putamen as is seen in PD [97-99]. The absence of PITX3 in *aphakia* mice does not lead to an absence in many of the other genes involved in dopaminergic neuron development and maintenance (*Nurr1*, *Lmx1b*, *En1*, *En2* and *Ret*) [100]. ADH2 expression is affected causing a decrease in retinoic acid (RA; Table 2.1) [101]. Restoring the levels of RA can counteract the developmental deficits seen in these mice suggesting that PITX3's role in dopaminergic neural maintenance is through regulation of RA expression. However, this is only required during early development and does not account for the continued deficits seen in *aphakia* mice [101]. A possible role for PITX3 in

continued deficits is in its control of VMAT2 and DAT expression [102]. *Aphakia* mice lack both VMAT2 and DAT as seen by both in situ hybridization and PCR (Table 2.1) [103].

*Dopamine Transporter*

The dopamine transporter (DAT) is the protein responsible for removing dopamine from the synapse post release and taking it back into the neuron. This allows for recycling of the neurotransmitter as well as halting the activation of the post-synaptic neuron. DAT activity depends on sodium moving down its concentration gradient, dopamine and chloride ions being recognized outside the transporter, dopamine and chloride ion translocation into the cell and unloading, and the transporter returning to its original state [104]. *Dat* mRNA is denser in the SN relative to the VTA suggesting a possible role for DAT in the pathology of PD. Over expression of *Dat* led to excitotoxicity and loss of dopaminergic neurons [105]. Due to MPP+ entering the dopaminergic neuron through the DAT transporter, *Dat* knockout mice are insensitive to MPTP toxicity (Table 2.1) [105]. Variable number tandem repeats (VNTRs) found in DAT also occur in patients with various neurological disorders including PD [106]. These VNTRs seem to occur prior to symptomology of the disease suggesting that these VNTRs pre-dispose the dopaminergic neurons to susceptibility of the disease [106].

*Vesicular Monoamine Transporter 2*

Vesicular monoamine transporter 2 (VMAT2) is a protein which is responsible for packaging monoamines (dopamine, serotonin, norephinephine) into vesicles in the cytosol for transmission out of the cell [107]. VMAT2 also packages several neurotoxins such as MPTP to prevent them from causing harm to the neuron (Table 2.1) [107]. VMAT2 expression starts at E11 in the telencephalon and is seen in the caudate putamen and nucleus accumbens at P1 [108]. At E18, expression is found in the SN and VTA (Table 2.1). *Vmat2* -/- mice die shortly after birth; however, *Vmat2* +/- mice or blockage of the transporter yield results on the transporter function [108]. *Vmat2*+/- mice have a drastic decrease in dopamine despite compensation inside the neuron by more than doubling synthesis [109]. MPTP destruction is

more than twice that in normal mice through greater accumulation of the toxin to remain in the cytosol where it can cause damage to the neuron [107]. Animals that lack VMAT2 do not cause depletion the neurons themselves, just the monoamines; cells eventually die through lack of use via the caspase 3 and caspase 9 pathways [109]. Brains of PD patients examined postmortem express 88% less VMAT2 in the putamen, 83% less in the caudate and 70% less in the nucleus accumbens compared to brains of people who were not diagnosed with PD (Table 2.1) [110].

**Dopaminergic Differentiation**

Due to the lack of success in developing a new therapeutic for PD over the last 30 years combined with the specificity of the cells that deteriorate in PD, differentiating dopaminergic neurons from hESCs for use in cell therapy or drug discovery for PD has been a research focus for many years with the first successful attempt by Perrier and colleagues in 2004. Discovered in 2000 for its ability to induce midbrain dopaminergic neurons from mESCs, stromal cell-derived inducing activity (SDIA) refers to the factors secreted from or imbedded in the cell membrane of PA6 cells or other bone marrow cells which have been shown to promote dopaminergic differentiation [111]. Studies on fixed PA6 cells and on mitomycin c treated and irradiated cells show a reduction in ability to differentiate to dopaminergic neurons [112]. Microarray studies examining the factors secreted from these cells have suggested 8 possible categories (IGF, FGF, Notch, PDGF, SHH, TGFβ, VEGF, Wnt) for potential secreted factors [113]. Utilizing SDIA, hESCs were co-cultured with stromal cells, SHH and FGF8 to differentiate them towards a neural fate [4]. Removal of SHH and FGF8 and replacement with brain derived neurotrophic factor (BDNF) and ascorbic acid (AA) induced 60-70% TH positive/TUJ positive cells [4]. The dopaminergic phenotype of these cells was further confirmed by VMAT2 and EN1 staining [4]. GDNF, used in co-culture with SDIA, doubled the number of TH positive cells seen with SDIA activity alone [114]. Another hESC line, SA002.5 was differentiated on PA6 cells resulting in up to 37% TH positive/TUJ1 positive neurons. These neurons were transplanted into the nigral-stratial pathway with negative consequences including proliferation following

transplantation and terotoma formation [115]. Differentiation of H9 hESCs on a SHH secreting M5S stromal feeder layer with bFGF lead to no teratoma formation when transplanted into the SN, but few TH+ cells survived [116]. Attempts at differentiation with a bone marrow stromal cell feeder layer and FGF8/SHH lead to 40% TH+ cells but no cells survived the graft. In an effort to differentiate a line that would be post mitotic after injection, H9 and H1 cells were co-cultured with rat astrocytes; however, transplanted cells that survived were still undifferentiated mitotic cells [117].

Following induction using the SDIA method, focus on a method using only growth factors and no co-culture methods began and was reported in 2005 by Yan and colleagues. hESCs were differentiated to neural progenitors through an embyroid body (EB) stage. Dopaminergic induction began with 7 days of FGF8 culture followed by 7 days of FGF8 and SHH culture [118]. Progression to biologically functional dopaminergic neurons required 14 days of culture with dopamine survival factors (GDNF, BDNF), dopamine inducing factor ascorbic acid (AA), neural specification factor cyclic AMP (cAMP) in addition to the FGF8 and SHH. The dopaminergic neurons expressed 31% TH positive neurons after 5 weeks of differentiation [118]. Another report using all of the above factors plus a dopamine induction factor, TGFβ lead to 43% TH positive cells; however, transplantation resulted in few surviving TH+ post mitotic cells and primarily neural precursors that continued to proliferate [119]. The first report of hESCs differentiated towards a dopaminergic phenotype being transplanted that resulted in significant improvements in rotational and forepaw stepping also resulted in the formation of tumors [119].

The field progressed to promoter systems that express genes known to be involved in dopaminergic development. *Lmx1*a is induced at E7.5 in mice by *Otx2* [120]. *Lmx1a* helps to induce a midbrain dopaminergic neuron identity through controlling NURR1 and PITX3 expression [121]. An *Lmx1a* promoter was used in hESCs to promote differentiation of 10 to 20% TH+ neurons [120]. Efforts to improve the derivation of dopaminergic neurons have included formation of spherical neural masses (SNMs) instead of EBs prior to differentiation

[122,123]. Elucidating the factors expressed in and secreted by stromal cells used to differentiate dopaminergic neurons included microarray studies. One study found that the cell membrane of stromal cells expressed FGF7, hepatocyte growth factor and vascular endothelial growth factor, which were sufficient to induce dopaminergic differentiation [112]. A microarray examining the mRNA expression of PA6 cells found IGF2 and several IGF binding proteins, *Fgf10*, *Dlk1, Ngf*, *Shh, Tgf3*$\beta$, *Vegf* and *Wnt* RNAs to be highly expressed in PA6 cells [113]. Additionally, receptors for these genes were more highly expressed in NSCs compared to hESCs. A study using various combinations of factors that activated these receptors or replicated the factors expressed by PA6 cells determined that the combination of factors termed SPIE (SDF-1, PTN, IGF2, and EFNB1) was most effective at differentiating hESCs towards dopaminergic neurons [122,123]. However, a highly efficient and effective method of dopaminergic differentiation has not been obtained.

In an attempt to improve on the 5 stage method and to remove the feeders from the co-culture system, En-Stem A cells were differentiated with PA6 conditioned media for 4 weeks resulting in 18% TH+ cells compared with 26%TH+ cells derived from H9 derived hNPs cultured in PA6 conditioned media [113]. The time of exposure to PA6 conditioned media was important. Cells exposed to PA6 conditioned media at the neural stem cell stage produced more TH+ neurons than did cells exposed as hESCs or cells exposed later in neural differentiation. Differentiation with FGF-20, a novel neurotrophic factor found to be expressed in the SN of rat brains, on PA6 feeder cells lead to a 5-fold increase (3% to 15%) in TH+ cells and reduced overall cell death via the caspase 8 and BAX pathways [124]. *Foxa2* ventralizes neural progenitors in the developing brain and leads to cell cycle arrest of ventral midbrain cells to promote differentiation over proliferation. Additionally, *Foxa2* acts in an auto regulatory loop with SHH to promote dopaminergic neurons and to inhibit GABAergic differentiation [125]. In order to promote *Foxa2*+ progenitor cells that mark ventral mesencephalic dopaminergic neurons, a high activity form of SHH and the FGF8a isoform induced dopaminergic neurons [126] Currently

research remains ongoing working to improve upon the differentiation protocol used to derive dopaminergic neurons.

**GDNF and its Mechanism of Action within the Neuron**

GDNF belongs to the transforming growth factor-β (TGF-β) superfamily. Within the superfamily is the GDNF family of ligands, which include neurturin (NRTN), artemin (ARTN), persephin (PSPN) and GDNF [127]. Each of these ligands bind preferentially to GDNF-family receptor-α (GFR-α) co-receptors (GDNF to GFR-α1; NRTN to GFR-α2; ARTN to GFR-α3; PSPN to GFR-α4) prior to binding to receptor tyrosine kinase (RET) protein which is attached to the plasma membrane with a glycosyl phophatidylinositol (GPI) anchor [127]. In order to activate downstream pathways, the RET-GFRα complex must become associated with a lipid raft, recruited by SRC and FRS2 [127]. This binding activates the PI3K and MAPK pathway involved in neuron survival and neurite outgrowth (Figure 2.4) [127]. The GDNF interaction with GFRα1-RET promotes dopamine neuron survival, axon growth and hypertrophy (Figure 2.4) [127].

*Mitogen Activated Protein Kinase Pathway*

The mitogen activated protein kinase (MAPK) pathway consists of a network of kinases that are involved in cell survival, differentiation, proliferation, apoptosis, growth and involved in GDNF signaling (Figure 2.4) [128], . There are currently three well known MAPK pathways: the c-JUN N-terminal kinase (JNK)/stress activated protein kinase (SAPK), the extracellular signal-regulated kinase (ERK1/2 and ERK5), and the p38 MAPK pathway (Figure2.4) [129]. MAPKKK1-4 will activate MAPKK 4 and 7, which in turn activates JNK 1, 2 and 3. The JNK pathway is involved in retinoic acid neurogenesis in *Jnk* knockout mESCs [130]. The JNK pathway regulates cellular survival and neuronal migration (Figure2.4) [131]. The ERK pathway divides into the ERK1/2 pathway and the ERK5 pathway [132]. The ERK1/2 pathway is activated by MAPKK1/2, which is turned on by ARAF, BRAF or CRAF [130]. The ERK pathway regulates cellular survival [131]. ERK stimulates transcription factors such as *Elk* and *c-Myc* and

protein kinases such as ribosomal S6 kinase (RS6K; Figure 2.4) [129]. ERK5 is involved in cell survival and proliferation through activation of MAPKKK 1-4 which triggers MAPKK5 [132]. In vivo mouse models have demonstrated that ERK5 signaling is involved in both cardiovascular and neural development [129]. The final MAPK pathway, p38 MAPK pathway, is stimulated by MAPKKK1-4 activation of MAPKK3/6 (Figure 2.4) [129]. In embryonic development, there are two peaks of p38 activity [129]. The first acts as a switch between cardiovascular and neural development. The later peak modulates neurite formation and neural survival.

The mechanism through which GDNF acts to promote dopaminergic neural survival and differentiation is not entirely known, but it is thought that the MAPK pathway may play a role in promoting neural survival, differentiation or neurite outgrowth [133,134]. Cultured embryonic rat cortical cells exposed to GDNF increased arborization and neurite outgrowth through activation of the p42/p44 MAPK pathway (Figure 2.4) [133,134]. RET coupling with the SHC/GRB2 domains leads to downstream activation of the MAPK pathway (Figure 2.4) [133,134]. Further research needs to be done to determine the involvement of the MAPK pathway in dopaminergic differentiation after RET activation.

*Phosphoinositide 3-kinase Pathway*

The phosphoinositide 3-kinase (PI3K) pathway is activated by cytokines, growth factors and hormones and is involved in downstream regulation of cell survival, proliferation, apoptosis and regulation of transcription factors, PI3K exerts action on AKT, which acts in cellular functions such as survival, protein synthesis, proliferation, glucose metabolism, and neural signaling through its triggering of several other factors [135]. AKT inhibits pro-apoptotic signals BAD and the Forkhead family thus increasing cell survival (Figure2.4) [136]. Regulation of glucose metabolism occurs through glycogen synthase kinase 3 (GSK3) activation (Figure 2.4) [136]. Finally, AKT neural involvement occurs through regulation of the GABA receptor, ataxin-1 and huntingtin in addition to interaction with TGF-β signaling (Figure 2.4) [136]. Acting along with AKT signaling are pathways involved in translation control (eIF4E and p70 S6K), cell

growth and survival (mTOR) and cell cycle regulation [phosphatase and tensin homolog (PTEN) (Figure 2.4)] [137]. mTOR is found in two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 integrates signals to encourage cell growth or catabolic processes depending on which condition is more favored. The p70 S6K pathway controls phosphorylation of ribosomal protein S6 that is important for cell size and glucose homeostasis (Figure 2.4) [137]. mTORC2 promotes cellular survival and cytoskeletal maintenance [138]. Mutations in mTOR signaling are involved in cancer, cardiovascular disease and metabolic disorders [139]. PTEN is a tumor suppressor through its regulation of cell cycle, cell division and negative regulation of the PI3K/AKT pathway [140].

In the mouse dopaminergic cell line, MN9D, the PI3K inhibitor LY294002 was administered prior to GDNF addition. In these studies, GDNF failed to protect the viability of the neurons exposed to 6-OHDA. In rat primary cultures, GDNF administration phosphorylates AKT [141]. This phosphorylation was completely blocked by pre-incubating the cells with Wortmannin, a PI3K inhibitor (Figure 2.4) [141]. When RET complexes with GAB1 and stimulates CREB, GDNF activates the PI3K pathway preferentially to the MAPK pathway (Figure 2.4) [142].

*Src*

Src was the first discovered tyrosine kinase located in the cytoplasm. The family of SRC tyrosine kinases (SFK) consists of FYN, LYN, HCK, c-YES, BLK, FGR, and LCK. SFKs play roles in cell growth, differentiation and survival, as well as cellular adhesion and synaptic transmission (Figure 2.4) [143]. When GDNF binds to its co-receptor GFR$\alpha$1, the glycosyl phosphatidylinositol (GPI) that anchors the GFR$\alpha$1 to the membrane recruits RET to the lipid raft and allows for activation of cellular signaling pathways that increase neural survival and differentiation (Figure 2.4) [144]. RET activation can occur in *cis* or *trans*. *Cis* activation occurs when a GPI anchored GFR$\alpha$1 co-localizes on the same cell as the RET and allows for

recruitment of a lipid raft in that cell [144]. When the GPI anchored GFRα1 is on an adjacent cells (such as a glial cell), the lipid raft is recruited in *trans* (Figure 2.4) [143]. *Trans* activation of RET is not sufficient to activated downstream pathways such as MAPK and PI3K. It is not known the reason for the availability of *trans* activation as it leads to decreased differentiation and decreases neural survival [143]. RET activation of Src has been shown to increase axon sprouting of dopamine [145].

*c-Jun N-terminal Kinase (JNK) Pathway*

The c-Jun N-terminal kinase (JNK) pathway is a subfamily of the MAPK pathway. This pathway plays a role in stress response in the cell and is activated by cytokines and environmental stresses (Figure 2.4) [146]. MAPK phosphatases (MKP) negatively regulates the JNK pathway and these MKPs can be inhibited by reactive oxygen species, which causes increased activation of the JNK pathway and can lead to cellular death [146]. There are 3 JNK genes (*Jnk1-3*), but only *Jnk3* activates neuronal cell death [146,147]. JNKs also include a group of scaffold proteins (JIP1-4) which interact with the mechanisms for vesicular transport, axon growth and axon repair after damage [146]. Both RAC1 and CDC42 activate the JNK pathway (Figure 2.4). Through this activation, they modulate cytoskeletal organization within the neuron as well as aid in neural migration [147].

GDNF also activates the JNK pathway. Through GDNF and its co-receptor GFR$\alpha$1 activating RET, JNK has been shown to modulate neurite outgrowth and extension in dopaminergic neurons (Figure 2.4) [148]. Additionally, this JNK activation causes a cell cycle delay at G2/M to allow actin reorganization to improve cell viability (Figure 2.4) [149].

**Conclusion**

The high prevalence of PD in the American population combined with the increasing percentage of aging population presents a need for improving upon the treatments available for the disease. Currently, the treatments available have not changed from the first largely available drug and the side effects obtained from this compound combined with the lack of long-term response

suggest a need for a better treatment option. The models that have been used thus far have been animal models that do not offer a direct comparison to human physiology. hESC derived hNPs that are differentiated to dopaminergic neurons provide an optimal tool for studying the basic biology of dopaminergic neurons as well as for researching new drug options. The methods for deriving these neurons needs to be improved upon in order to provide better treatment options for PD.

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**Table 2.1 Proteins Expressed in Dopamine Neurons**

| Protein | Expression (mouse) | Role in Dopaminergic Neurons | References |
|---|---|---|---|
| NURR1 | E10.5 | • Drive expression of TH, AADC, RET, VMAT2, DAT<br>• Support development of DA neurons<br>• Maintain post-mitotic DA neurons | [82,85,86] |
| EN1 | E7.5 | • Expressed in neuroepithelium<br>• Secreted to maintain mid-/hindbrain boundary<br>• Induced by FGF8<br>• Maintain post-mitotic DA neurons | [89,92] |
| TH | E11.5 | • Driven by NURR1<br>• Rate limiting enzyme in DA synthesis | [94,95] |
| PITX3 | E11.5 | • Drive expression of VMAT2, DAT and RA<br>• Maintain SN neurons | [96,97,100,101] |
| DAT | E13.5 | • Gives MPTP access to DA neurons<br>• Denser in SN neurons<br>• Removes DA from synapse | [105,150] |
| VMAT2 | E18 | • Package MPTP to prevent it from damaging cell<br>• Less VMAT2 expression in PD brains | [107,108,110] |

AADC - Aromatic L-Amino Acid Decarboxylase, DA - Dopamine, DAT – Dopamine Transporter, EN1 – Engrailed 1, FGF8 – Fibroblast Growth Factor 8, MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, NURR1 - Nuclear Receptor Related 1, PD – Parkinson's Disease, PITX3 - Paired-like Homeodomain Transcription Factor 3 , RA – Retinoic Acid, RET – Rearranged in Transfection, SN – Substantia Nigra, TH – Tyrosine Hydroxylase, VMAT2 - Vesicular Monoamine Transporter 2

**Figure 2.1 – Dopamine Signaling to the Basal Ganglia in Normal and Parkinson's Disease State** In a normal state, dopaminergic neurons from the substantia nigra pars compacta (SNc) project onto the striatum. Activation of the striatum leads to motor movement modulation through the direct pathway (globus pallidus internal, GPi) or the indirect pathway (globus pallidus external, GPi). Both pathways lead to the thalamus and then to the cortex and brainstem [22]. In the Parkinson's disease (PD) state, the dopaminergic neurons from the SNc are absent. This lack of input prevents the inhibitory signaling to the indirect and direct pathways, which causes a disruption in motor control [151].

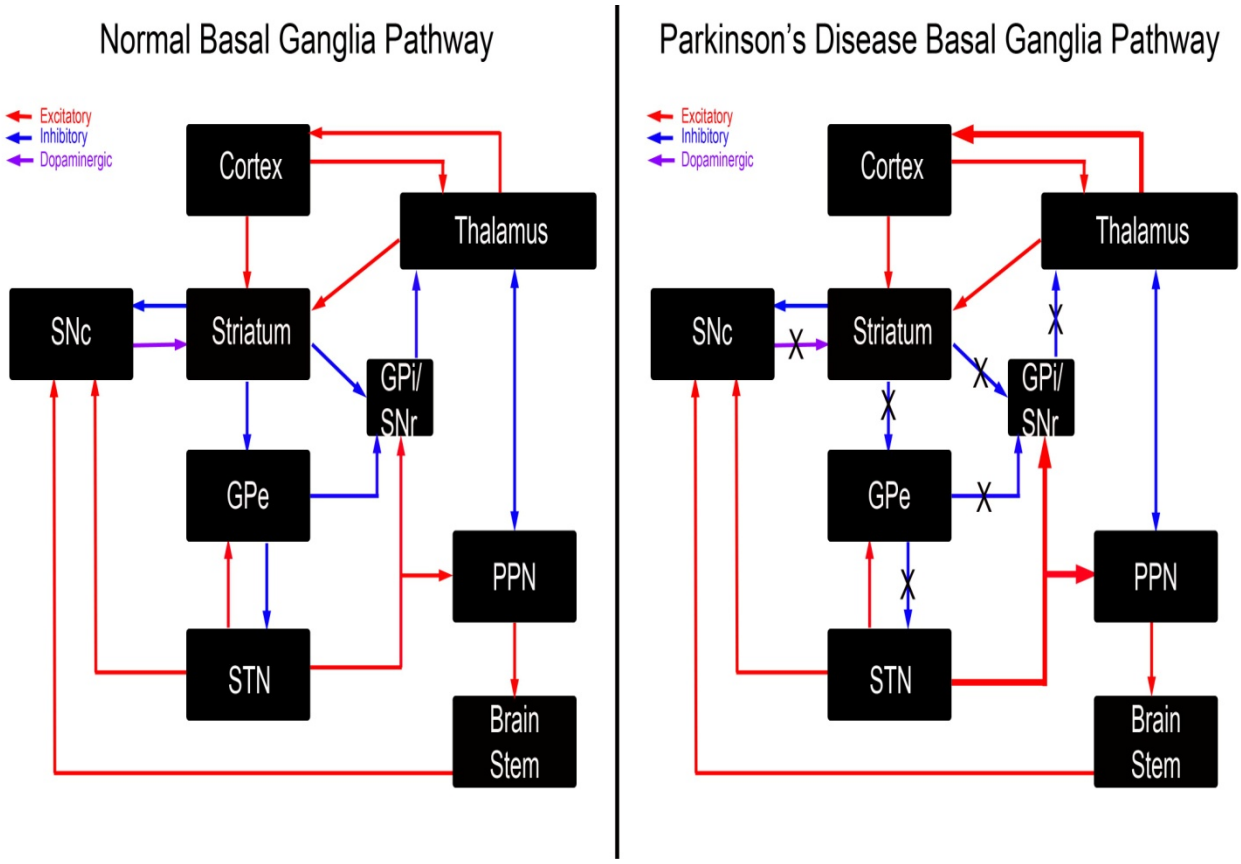

Normal Basal Ganglia Pathway
Excitatory
Inhibitory
Dopaminergic
Cortex
Thalamus
SNc
Striatum
GPi/
SNr
GPe
PPN
STN
Brain
Stem
Parkinson's Disease Basal Ganglia Pathway
Excitatory
Inhibitory
Dopaminergic
Cortex
Thalamus
SNc
Striatum
GPi/
SNr
GPe
PPN
STN
Brain
Stem

**Figure 2.2 – Leukemia Inhibitor Factor action in dopaminergic and non-dopaminergic neurons** Leukemia inhibitory factor (LIF) binds to the gp130 receptor on the cell surface causing activation of the JAK/STAT pathway and the MAPK pathway [152]. Activation of the JAK/STAT pathway modulates self-renewal in neurons other than dopaminergic [152]. The MAPK pathway is activated by LIF in dopaminergic neurons to suppress dopamine beta hydroxylase production, which would lead to production of norephinephine instead of dopamine [57]. General neural pathway refers to any neural subtype other than dopaminergic while dopaminergic specific refers to dopaminergic neurons similar to those derived in this dissertation.

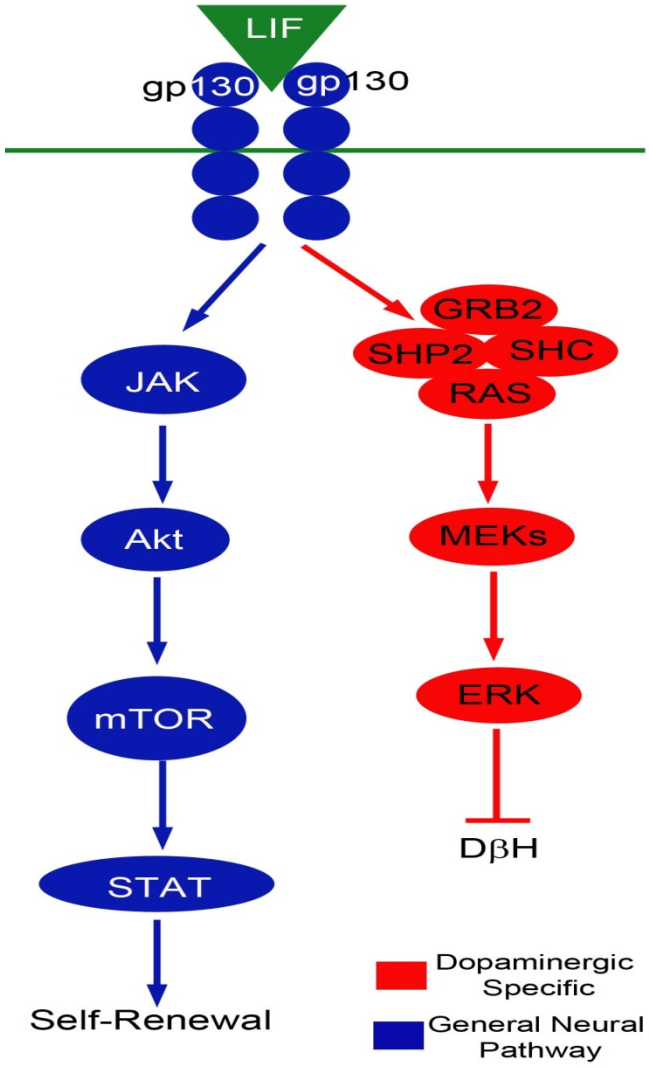

LIF
gp130
gp130
GRB2
SHP2
SHC
RAS
JAK
Akt
mTOR
STAT
Self-Renewal
MEKs
ERK
DβH
Dopaminergic Specific
General Neural Pathway

**Figure 2.3 – Fibroblast Growth Factor 2 Induces Caudalization and Prevents Apoptosis**

Fibroblast growth factor 2 (FGF2) binds to the fibroblast growth factor receptor in developing neurons to activate the PI3K pathway or the MAPK pathway. In neural progenitor (hNPs) and neural stem cells (NSCs), FGF2 activates AKT, which blocks BAD signaling to prevent apoptosis [153]. MAPK activation of the transcription factor CREB increases proliferation in hNPs and NSCs [153]. These pathways are general neural pathways seen in all neurons prior to specification and represented by the general neural pathway. Additionally, FGF2 activates HOX genes in a gradient within the developing brain to caudalize neurons, which is the action of FGF2 in the dopaminergic neurons differentiated in this dissertation and represented by the dopaminergic specific pathway [154].

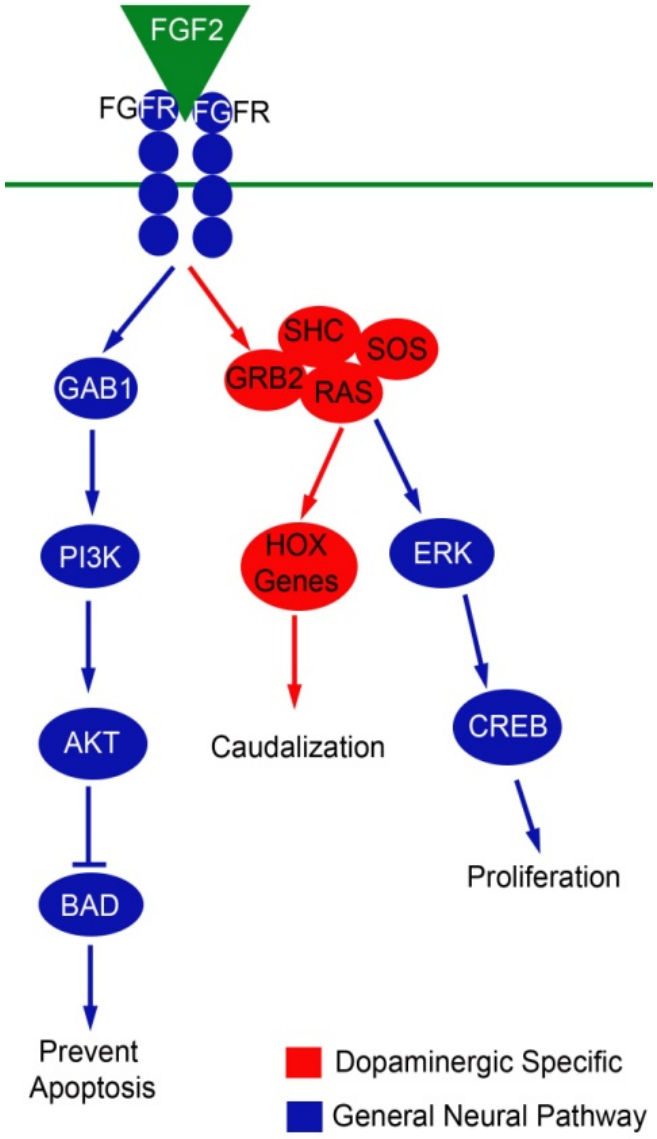
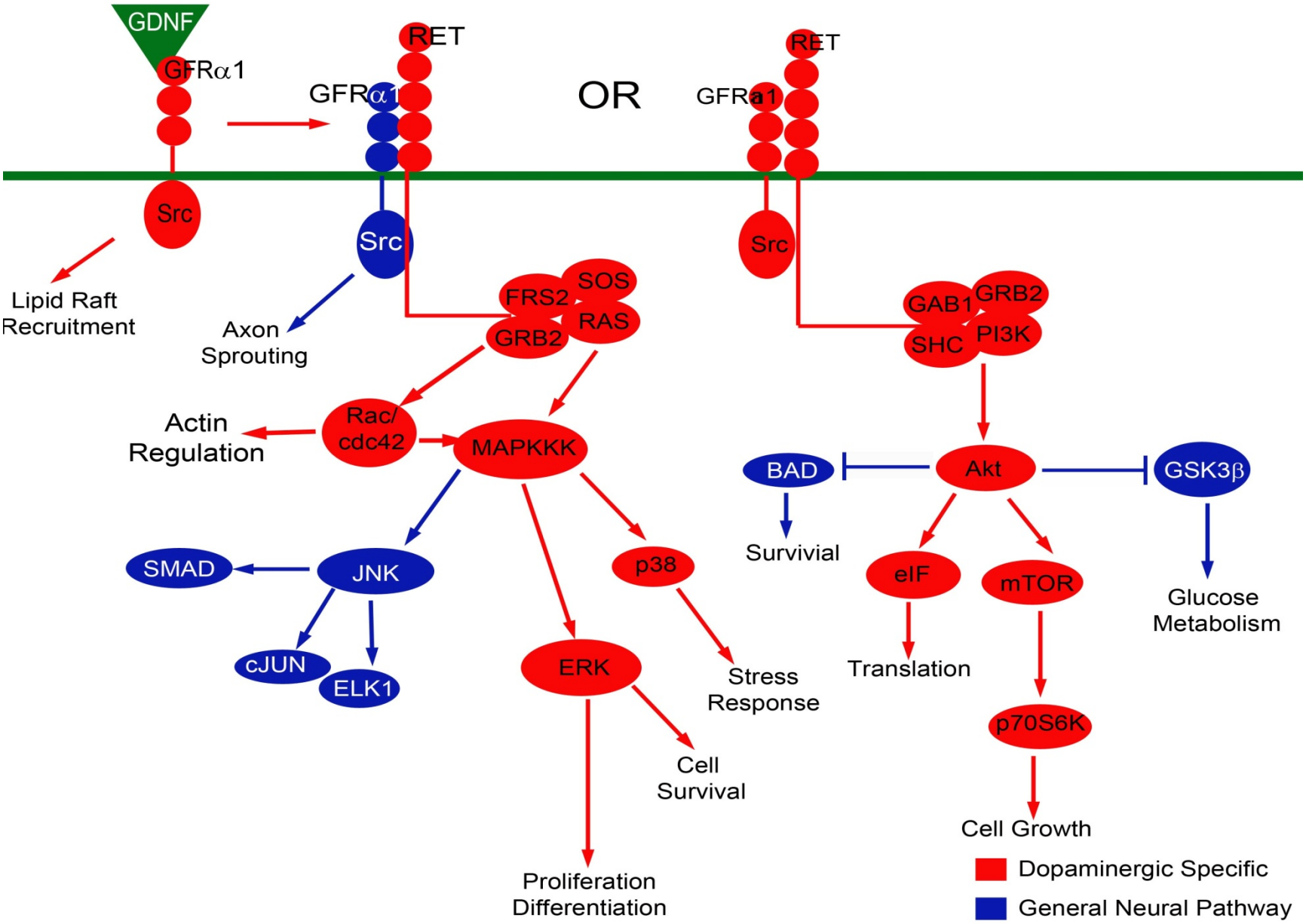

FGF2
FGFR
FGFR
GAB1
SHC
SOS
GRB2
RAS
PI3K
HOX Genes
ERK
AKT
Caudalization
CREB
Proliferation
BAD
Prevent Apoptosis
Dopaminergic Specific
General Neural Pathway

**Figure 2.4 – Glial Cell-line Derived Neurotrophic Factor Supports Dopaminergic Differentiation through Activation of Several Pathways** Glial cell-line derived neurotrophic factor (GDNF) binds to its co-receptor GFR$\alpha$1 which then binds to the RET receptor to activate the MAPK, PI3K, JNK and Src pathways. Src recruits RET to the lipid raft for binding [127,155]. MAPK activation of the JNK pathway regulates actin in its enhancement of the dopaminergic differentiation in this dissertation. Other parts of the JNK pathway activated by GDNF not involved in dopaminergic differentiation include SMAD, cJUN and ELK1 [127,155]. MAPK activation of the ERK pathway leads to enhancement of dopaminergic differentiation and cell survival in the cells in this dissertation. The AKT pathway activated by GDNF in the dopaminergic neurons in this pathway leads to cell growth through the mTOR pathway, translation factor activation through the eIF pathway and cell survival through inhibition of BAD. Not involved in the dopaminergic differentiation of the cells in this dissertation is activation of GSK3$\beta$ [127,155].


GDNF
GFRα1
RET
GFRα1
OR
RET
GFRa1
Src
Src
Src
Lipid Raft
Recruitment
Axon
Sprouting
FRS2
SOS
GRB2
RAS
GAB1
GRB2
SHC
PI3K
Actin
Regulation
Rac/
cdc42
MAPKKK
BAD
Akt
GSK3β
Survivial
SMAD
JNK
p38
eIF
mTOR
Glucose
Metabolism
cJUN
ELK1
ERK
Stress
Response
Translation
p70S6K
Cell
Survival
Cell Growth
Proliferation
Differentiation
Dopaminergic Specific
General Neural Pathway

CHAPTER 3

# ION CHANNELS AND IONOTROPIC RECEPTORS IN A HUMAN EMBRYONIC STEM CELL DERIVED NEURAL PROGENITORS[1]

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[1]Young, Amber, Machacek, Dave W., Dhara, Sujoy, MacLeish, Peter R., Benveniste, Morris, Dodla, Mahesh C., Sturkie, Carla D., Stice, Steven L. To be submitted to *Neuroscience*.

**Abstract**

Human neural progenitors differentiated from human embryonic stem cells offer a potential cell source for studying neurodegenerative diseases and for drug screening assays. These differentiated neural progenitors have previously been screened for $\alpha$-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor agonists in which they performed the same as the mouse embryonic stem cells in 17 of the 21 compounds tested. Confirming the usefulness of these differentiated human neural progenitors requires determining the ionotropic receptor subunit expression. Real-time PCR was performed to determine the ionotropic receptor subunit expression profile while tetrodotoxin and vertridine were used to determine the functionality of the differentiated neural progenitors. Differentiated neural progenitors expressed markers of a post-mitotic phenotype, MAP2 and TUJ1 in addition to glial cell markers and neural developmental markers (*Cdh2*, *Gbx2*). Differentiated neural progenitors express subunits of glutamatergic, GABAergic, nicotinic, purinergic and transient receptor potential receptors. Additionally, sodium and calcium channel subunits were expressed. These results suggest a developmentally mature source of neural cells that can be easily obtained and maintained for use in drug assay screens.

## Introduction

Human embryonic stem cells (hESCs) differentiated into neural cells are thought to be representative of early central nervous system (CNS) tissue. Therefore, these cells should possess a developmentally and physiologically repertoire of genes expressed in these tissues. Of particular interest to the field of drug development are the ion channel and ionotropic receptor subtypes. Having normal early expression and early physiological properties could make these cells ideal for screening pharmacological compounds [1]. The presence of the proper ion channel and ionotropic receptor subtypes is required for normal functional neural activity. In vivo neural differentiation requires hNPs to migrate, develop transmitter specificity, be electrically excitable and produce the outgrowth of axons and dendrites to form functional connections. Ion channels and ionotropic receptors, though generally recognized for their roles in regulating the electrical excitability of cells, are proteins that have also been shown to play an essential role in many aspects of neural development [2]. Ionotropic glutamate receptors are critical for neural migration [3], synaptogensis [4] and neural survival [5]. Expression of ion channels and ionotropic receptor subunits during prenatal development has been demonstrated in mammals; however, characterization in human tissue is lacking [6,7]. Despite the essential role of these proteins in development, very little is known about the expression of ion channels in hESC derived neural tissue. Although fully functional neurons have been derived from mouse embryonic stem cells (mESCs), other studies have reported mESC derived neural tissue with the morphology and biochemical markers of differentiated neurons and synaptic contacts, but lacking voltage-dependent sodium channels required for functional synaptic transmission [8]. Thus, it seems that in vitro neural differentiation could provide an excellent model to examine the mechanisms by which endogenous populations of ion channels and ionotropic receptors are regulated throughout development. Of particular interest is the role of ionotropic glutamate receptors in mediating excitotoxity [9]. An improper balance of glutamate and gamma-aminobutyric acid (GABA) receptor activity and responsiveness can lead to excitotoxicity and

apoptosis [10]. The glutamatergic ionotropic receptors have been implicated in excitotoxicity [9] since normal glutamate receptors maintain a balance of ion exchange in neurons. Excitotoxicity occurs in a wide range of maladies including but not limited to stroke, traumatic brain injury, seizure and could be associated with neurological diseases such as Huntington's disease, amyotrophic lateral sclerosis (ALS) or Parkinson's disease.

Well-characterized hNPs may provide an in vitro human model system for these and other neurological diseases and the ability to examine the effects of excitotoxic insult on specific ion channel subunits. Previously we derived a karotypically normal, stable, adherent monolayer of human neural progenitors (hNPs) from WA09 hESCs [11]. These cells have been characterized for their ability to maintain multipotency and for their expression of neural stem cell marker NESTIN and lack of expression of stem cell marker POU5F1 [12]. The hNPs have also been previously differentiated into all three subtypes of the neural lineage, neurons, oligodendrocytes and astrocytes [11]. Upon further differentiation in basal conditions with leukemia inhibitory factor (LIF) alone, greater than 90% of the cells were beta III tubulin and MAP2 positive [11,13]. The differentiated cells were then used to identify compounds that target and potentiate agonists to specific ionotropic glutamate receptors [14]. Ionotropic glutamate receptors are broken into three subtypes, N-methyl-D-aspartic acid (NMDA), kainate and $\alpha$-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) so called for their preferential binding to drugs by the same name [7,15]. We found that differentiated hNPs were indeed useful in screening for specific potentiators of AMPA activity [14]. However, this work assumed the endogenously expressed glutamate receptors on neuronal cells derived from hNPs function like native receptors [14]. In order to determine whether these hNPs can be used in a range of compound screening assays, it is important to characterize directly the hNP's ion channel subtype expression and electrical properties. In addition to glutamate receptors, other ionotropic receptors found in the CNS include but are not limited to nicotinic receptors, purinergic

receptors and transient receptor potential (TRP) channel receptors. We would expect expression of multiple receptors in differentiated human neural tissue.

Here, we demonstrate that post-mitotic differentiated hNPs express developmental regionalization genes as well as markers of functional neural cells including the dopamine transporter (*Dat*), serotonin transporter (*Sert*) and synaptophysin. These differentiated hNPs can evoke action potentials that can be blocked with tetrodotoxin (TTX) as well as increase calcium response when exposed to AMPA receptor potentiator, cyclothiazide. GABAergic and glutamatergic ionotropic receptors expression was found to be up regulated as early as after two weeks of hNP differentiation. These results suggest that these differentiated hNPs are capable of eliciting responses and may prove to be a human in vitro model for excitotoxicity associated with various CNS diseases and injuries.

**Experimental Procedures:**

*hESC Cultures*

WA09 hESCs (WiCell Research Institute, Madison, WI) were cultured on mouse embryonic fibroblast (MEF; Harlan, Indianapolis, IN) feeders inactivated by mitomycin C (Sigma-Aldrich, St. Louis, MO) in 20% knockout serum replacement (KSR) media consisting of Dulbecco's modified Eagle medium (DMEM)/F12 medium (Gibco, Carlsbad, CA) supplemented with 20% KSR (Gibco), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), 0.1 mM non-essential amino acids (Invitrogen), 50 units/ml penicillin (Invitrogen), 50 µ/ml streptomycin (Invitrogen), 0.1mM β-mercaptoethanol (Sigma-Aldrich) and 4 ng/ml basic fibroblast growth factor (bFGF; R&D, Minneapolis, MN). They were maintained in 5% $CO_2$ and at 37°C. Cells were passaged every 3 days by mechanical dissociation, re-plated on fresh feeders to prevent undirected differentiation with daily media changes as previously described [16].

*hNP Cultures*

Human neural progenitor (hNP) cells were derived from hESC line WA09 by our laboratory as previously described [11]. Briefly, after one week of culture on MEF layers, WA09

hESCs were grown with derivation medium containing DMEM/F12 medium (Gibco) supplemented with 2mM L-glutamine, 2 U/mL penicillin, 2µg/mL streptomycin, N2 (Gibco), and 4 ng/ml bFGF for 7 days. Rosettes were selected with hook passaging from culture dishes and re-plated on polyornithine (Sigma-Aldrich, St Louis, MO) and laminin (Sigma-Aldrich) coated dishes. These rosettes were propagated for 3 days on polyornithine and laminin coated dishes in neurobasal medium supplemented with 2mM L-glutamine, 2 U/mL penicillin, 2 µg/mL streptomycin, 1X B27 (Gibco), 20 ng/mL bFGF, and 10ng/mL leukemia inhibitory factor (LIF; Millipore, Billerica, MA). Media were changed every other day and cells were passaged every fourth day or as needed. Cells used for this experiment were passage 22-39.

*hNP Differentiation*

hNP cells were grown on polyornithine/laminin coated 100mm tissue culture treated plates (BD Bioscience, Bedford, MD) in growth medium consisting of neural basal medium, 1X penicillin/streptomycin, 2mM L-glutamate, 1X B27, 10ng/mL LIF and 20ng/mL bFGF. After 24 hours, the media were changed to neural differentiation media, which consisted of growth medium without bFGF. Media were changed every three days. Cells were collected at 14 days, 35 days and 125 days for analysis.

*Cell Proliferation Analysis by Carboxyfluorescein succinimidyl ester (CFSE)*

hNPs and differentiated hNPs were analyzed for cell proliferation using CellTrace™ CFSE Cell Proliferation Kit (Invitrogen) following manufacturer's instructions. Briefly, cells were incubated for 10 minutes in 10 µM of CFSE solution at 37°C. CFSE staining was quenched with ice-cold media, cells were washed and re-suspended in fresh hNP media and plated at a density of $1x10^6$ cells per plate in a 35mm plate. Cells were incubated for 0, 24 and 48 hours. At each time point, cells were harvested and washed 2 times with PBS+/+ (phosphate buffered saline with calcium and magnesium; ThermoScientific, Whatham, MA). Cells were analyzed on Dako CyAn (Beckman Coulter, Brea, CA) with a 488nm laser. Non-stained hNPs and

differentiated hNPs were used as a control. CFSE data was analyzed using the FlowJo software (TreeStar, Ashland, OR) proliferation model.

*Immunocytochemistry*

Cells were fixed with 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS+/+ for 20 minutes and processed for immunocytochemistry. Cells were washed in PBS+/+ 3 times followed by 3 washes for 5 minutes each with permeabilization buffer consisting of 25 µL (0.5%) Tween 20 (EMD Chemicals, Gibbstoen, NJ) in 50mL of high salt buffer consisting of 1M Tris base (Sigma-Aldrich), 0.25M NaCl (Sigma-Aldrich) in distilled water up to 1L. Cells were then blocked in 6% goat serum (JacksonImmuno, West Grove, PA) for 45 minutes. The following primary antibodies were used: mouse anti NESTIN (1:200, Neuromics, Edina, MN), mouse anti TUJ1 (1:200, Neuromics) and mouse anti MAP2 (1:500, Millipore). Reaction was revealed using AlexaFluor goat 488 or 594 secondary antibodies (1:1000, Molecular Probes, Carlsbad, CA). Cell nuclei were stained using 4',6-diamidino-2-phenylindole DAPI (Invitrogen). Fluorescence was visualized using spinning disk confocal microscope (Olympus, Center Valley, PA).

*Real Time Polymerase Chain Reaction (RT-PCR)*

RNA was extracted using the Qiashredder and RNeasy kits (Qiagen, Valencia, CA) according to manufacturer's instructions. The RNA quality and quantity were verified using a RNA 600 Nano Assay (Agilent Technologies, Santa Clara, CA) and the Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA (5 µg) was reverse-transcribed using the cDNA Archive Kit (Applied Biosystems Inc., Foster City, CA) according to manufacturer's instructions. Reactions were initially incubated at 25$^{o}$C for 10 minutes and subsequently at 37$^{o}$C for 120 minutes. Quantitative real-time PCR (Taqman, Applied Biosystems, Inc.) assays were chosen for the transcripts to be evaluated from Assays-On-Demand™ (Applied Biosystems Inc.), a pre-validated library of human specific QPCR assays, and incorporated into a 384-well Micro-Fluidics Cards. From the cDNA samples, 2 µl was mixed with 50 µl of 2X PCR master mix, then

loaded into respective channels on the microfluidic cards followed by centrifugation. The card was sealed and real-time PCR and relative quantification was carried out on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Inc). All failed (undetermined) reactions were excluded and ΔCt values were calculated. For calculation of relative fold change values, initial normalization was achieved against endogenous 18S ribosomal RNA using the ΔΔCT method of quantification (Applied Biosystems Inc.) [17]. Average fold change from four independent runs were calculated as $2^{\Delta\Delta CT}$. Significance was determined by running a 2-way ANOVA and Tukey's Pair-Wise (Statistical Analysis Software, SAS, Cary NC) comparisons for each gene. Treatments where a *p*-value was <0.05 were considered to be significantly different.

Primers were selected using Primer Blast (National Center for Biotechnology Information, Bethesda, MD) and were as follows: *Nes*: sense CAGGAGAAACAGGGCCTACA antisense TAAGAAAGGCTGGCACAGGT(GeneID: 10763), *Pax6* sense CCGGCAGAAGATTGTAGAGC antisense CTAGCCAGGTTGCGAAGAAC (GeneID: 5080), *Vim* sense CCTTGAACGCAAAGTGGAAT antisense GCTTCAACGGCAAAGTTCTC (GeneID: 7431), *S100β* sense GCCCTCATCGACGTTTTCCA antisense AAGAGTCCCTGGGGCCAGTC (GeneID: 6285), *Cntfr* sense ACCATTGTGAAGCCTGATCC antisense GGAGGAGAAATCGGATGTGA (GeneID: 1271), *Il6r* sense CTCCTGCCAGTTAGCAGTCC antisense TGTCGCATTTGCAGAATCTC (GeneID: 3570), *Sc1* sense TGTGACGTCTGGTTCCATGT antisense GTGATGCCACCAAGAACCTT (GeneID: 6941), *Blbp* sense AAGGATGGTGGAGGCTTTCT antisense ACAGCAACCACATCACCAAA (GeneID: 2173), *Glast* sense TGCTGGGGAATTCACCTCGT antisense CGCATTCCCATCTTCCCTGA (GeneID: 6507), *Glul* sense CCCTGCCTCAGGGTGAGAAA antisense TGGCGCTACGATTGGCTACA (GeneID: 2752), *Aqp4* sense AGCCTGGGATCCACCATCAA antisense CTCCCCGGTCAACGTCAATC (GeneID: 361), *Gapdh* sense GAGTCAACGGATTTGGTCGT antisense TTGATTTTGGAGGGATCTCG (GeneID: 12597), *Mapk* sense TTCCAAGGGCTACACCAAGT antisense CAGTCCTCTGAGCCCTTGTC

(GeneID: 5594), *Akt* sense AACACCATGGACAGGGAGAG, antisense CAAACTCGTTCATGGTCACG (GeneID: 207), *Ncam* sense CAGGTCATTGTGAATGTGCC antisense TGCCCATCCAGAGTCTTTTC (GeneID: 4684), *Src* sense AGCACAACCTGACCATCCTC antisense CCACCAGTCTCCCTCTGTGT (GeneID: 6714), *Cdh2* sense CTCCGCGGCCCGCTATTTGT antisense CCAGAAGCCTCTACAGACGCCTGA (GeneID: 1000), *Neurod* sense CTAACGCCCGGGAGCGGAAC antisense TGCGGCGGAGGCTTAACGTG (GeneID: 4760), *Gbx2* sense CGAGCGCGTCTATGAGCGCA antisense GACAGCCCCGACGAGCGAAG (GeneID: 2637), *Foxg1* sense ACGAGAAGCCGCCGTTCAGC antisense TTGAAGGCCAGCTTGGCCCG (GeneID: 2290). cDNA, primers, GoTaq Green (Promega, Madison, WI) and distilled water were added to PCR tubes (Invitrogen) and incubated at 95$^{o}$C for 3 minutes and then 35 cycles at 57$^{o}$C for 30 seconds and then 72$^{o}$C for 30 seconds. Finally, the reactions were incubated at 72$^{o}$C for 10 minutes. The PCR reactions were run on a 2% agarose gel (Bio-Rad, Hercules, CA) with ethidium bromide (Promega) for 45 minutes at 100V. Gel was visualized on Ugenius (Syngene, Frederick, MD).

*Fluorometric Imaging Plate Reader (FLIPR) Assay*

FLIPR assay was performed using Molecular Devices Calcium 4 assay kit (Molecular Devices, catalog number R8142) following manufacturer's directions. Briefly, buffer is prepared containing 145 mM NaCl (Sigma-Aldrich), 10 mM Glucose (Sigma-Aldrich), 5mM KCl (Sigma-Aldrich), 1 mM $MgSO_4$ (Sigma-Aldrich), 10 mM HEPES (Sigma-Aldrich) and 2 mM $CaCl_2$ (Sigma-Aldrich). Dye is prepared at a 2X concentration of 11 mls buffer/vial of dye. Each plate uses 14.4 ml of 1X dye (150 μl/ well). Equal volume of 2X solution was added to Locke's buffer to make 1X solution. Media was removed from wells and 200 μl of dye incubation media was added to each well of the plate and incubated for 1 hour at 37$^{o}$C and 5% $CO_2$. After incubation, plates were brought to room temperature over the course of 30 minutes. Flexstation 3

(Molecular Devices) was used to analyze calcium responses to 100 μM AMPA (Sigma-Aldrich), 100 μM kainic acid (Sigma-Aldrich), 100 μM NMDA (Sigma-Aldrich), half-log dilutions of cyclothiazide with the highest dose being 32 μM (Sigma-Aldrich) and/or half-log dilutions dose curve of cyclothiazide with the highest dose being 32 μM veratridine (Sigma-Aldrich). AMPA, kainic acid or NMDA are added to the cell plates using the Flexstation 3 plate reader (Molecular Devices). 50 μl of AMPA, NMDA or kainic acid was added first and recorded. 50 μl of cyclothiazide or veratridine was then added to the cell plates and again recorded. Buffer only and DMSO control wells were used as controls while the high dose of cyclothiazide or veratridine was used as 100% response. Area under the curve was calculated for response to agonist challenge. Dose response curves were fit to a three-parameter model with a Hill coefficient of 1 using Prism software (GraphPad, La Jolla, CA). Total area under the curve for responses were normalized such that the average response to 100 μM AMPA in the presence of 32 μM cyclothiazide was defined as 100 and the average response to buffer plus DMSO was defined as 0. Significance was determined by running a 2-way ANOVA and Tukey's Pair-Wise (SAS) comparisons for each gene. Treatments where a *p*-value was <0.05 were considered to be significantly different.

*Whole Cell Patch Clamp*

hNPs were grown in the absence of bFGF on a substrate of laminin (1 mg/ml) for up to one month. hNPs with significant neurite growth 23 days after removal of bFGF were subjected to whole cell voltage clamp utilizing an Axopatch 200B amplifier (Axon Instruments, Forest City, CA) and pClamp 9.2 data acquisition software (Axon Instruments) for electrophysiology. The extracellular solution consisted of 139 mM NaCl (Sigma-Aldrich), 3mM KCl (Sigma-Aldrich), 16 mM glucose (Sigma-Aldrich), 1.8 mM $CaCl_2$ (Sigma-Aldrich), 0.5 mM $MgSO_4$ (Sigma-Aldrich), 0.5 mM $NaH_2PO_4$ (Sigma-Aldrich), 1 mM $NaHCO_3$ (Sigma-Aldrich), 2mM HEPES (Invitrogen), 1 mM NaPyruvate (Invitrogen), 0.1 mM Choline Chloride (Sigma-Aldrich), and phenol red

(Affymetrix). The pH was titrated at 7.25 with NaOH (Sigma-Aldrich) and the osmolarity was 300 mOsm. The intracellular solution consisted of: 135 mM Kgluconate (Sigma-Aldrich), 0.1 mM $CaCl_2$ (Sigma-Aldrich), 10 mM $MgCl_2$ (Sigma-Aldrich), 10 mM HEPES (Invitrogen), 1 mM EGTA (Sigma-Aldrich), 2 mM MgATP (Sigma-Aldrich), 0.4 mM NaGTP (Sigma-Aldrich),, titrated to pH 7.29 and had an osmolarity of 281 mOsm. In some cases, an equimolar amount of Csgluconate was substituted for Kgluconate in order to block currents through potassium channels. Experiments were carried out at 30$^o$ C in a humidified atmosphere of 5% $CO_2$/ 95% $O_2$. In voltage clamp experiments, the holding potential was kept at -60 mV, but was hyperpolarized to -100 mV, 50 ms prior to the depolarization step. Current clamp experiments were done with some minimal current injection to maintain a membrane potential of approximately -70 mV prior to injection of the depolarizing pulse. Drugs were locally perfused around the cell utilizing glass pipets with a 500 μm diameter opening positioned 100 μm from the cell.

**Results**

*hESC-derived hNP differentiation*

hNPs were maintained on poly-ornithine/laminin coated plates in a karotypically stable, adherent monolayer and were characterized with neural stem cell marker NESTIN (Figure 3.1A). These hNP cells differentiated into mature neuronal cells with the removal of bFGF from the culture while the LIF concentration remained constant. Immunocytochemical analysis indicated that at 14 days after removal of bFGF, immature neural cell marker TUJ1 was expressed (Figure 3.1B) and neural marker microtubule-associated protein 2 (MAP2, Figure 3.1C) expression was observed 21 days after removal of bFGF. Terminal differentiation is marked by the transition from a proliferating cell type to a post-mitotic cell type. Carboxyfluorescein succinimidyl ester (CFSE) dye can be absorbed by the parent cell and have approximately half of the fluorescence expressed in each daughter cell with each cell division. Total population doubling was represented by a decrease in relative fluorescent units (RFU) by 50% of the parent cells. Proliferation was measured in hNPs in the presence of bFGF every 8

hours for 48 hours with addition of the CFSE dye (Figure 3.1D). After bFGF was removed from the culture medium for 14 days, proliferation was measured with the CFSE dye every 24 hours for 96 hours (Figure 3.1E). Half mean fluorescence for the hNP population occurred at 24 hours (535 RFU at 0 hours and 241 RFU at 24 hours; expected would be 267 RFU, not significantly different) and again at 48 hours (191 RFU; expected would be 134 RFU, not significantly different) but not in cells following the removal of bFGF (674 RFU at time 0, 694 RFU at 24 hours and 664 RFU at 48 hours; expected would be 337 RFU at 24 hours and 168 RFU at 48 hours, significantly different from observed). In summary, when bFGF was removed from the culture medium the cell cycle of hNPs was arrested followed by an increase in pan neuronal marker expression.

The following transcripts were significantly up regulated ($p<0.05$) upon removal of bFGF from hNP cultures for 14, 35 and 125 days: dopamine transporter (*Dat)*, vesicular acetylcholine transporter (*Vacht*), serotoninergic transporter (*Sert)* and glutamate decarboxylase 1 (*Gad1*; Figure 3.2A). Real-time PCR also showed significant ($p<0.05$) expression changes in ionotropic receptor channels – *hERG* (potassium voltage gated channel), *Kcc2* (potassium chloride transporter) and *Kir4.1* (potassium inwardly rectifying channel; Figure 3.2A). Synaptic markers *synaptophysin* and *syntaxin 1A* were also significantly ($p<0.05$) up regulated (Figure 3.2A). Transcripts for endosomal markers *Rab5a* and *CD146* were not significantly altered during differentiation (Figure 3.2A). Results were normalized to GAPDH expression.

The developmental marker gastrulation brain homeobox 2 (*Gbx2*) was expressed in hNPs and in hNPs without bFGF at 14 and 35 days but not 125 days of culture (Figure 3.2B). Telecephalon developmental marker forkhead box G1 (*Foxg1*) was present at day 125 after bFGF removal but not at other time points (Figure 3.2B). Cadherin 2 (*Cdh2*), calcium dependant cell to cell adhesion marker was seen at all time points and in hNPs (Figure 3.2B). Neural differentiation maker *Neurod* was expressed at 14 and 21 days post differentiation (Figure 3.2B). Regionalized differentiation and signaling pathway genes were expressed in the

undifferentiated hNPs and the neuronal cells. *Mapk* is a signaling factor important for neural synaptic plasticity and hESC self-renewal and cell cycle maintenance [18,19] and transcripts were present in both hNPs and differentiated hNPs (Figure 3.2C). *Akt*, a signaling factor involved in axon elongation and neuron polarity [20,21], *Ncam*, a cell adhesion molecule involved in directed growth of axons in neural development, presynaptic function [22] and in triggering neurite outgrowth through intracellular signaling cascades [23] and *Src*, important in fully developed neurons for up regulating ion channel expression and in gating synaptic plasticity and potentiation [24] were only expressed in day 14 differentiated hNPs (Figure 3.2C).

*hNPs and differentiated hNPs express glutamatergic receptor transcripts*

The three ionotropic receptor subtypes that transmit glutamatergic signaling are NMDA, kainate and AMPA receptors. The hNPs and differentiated hNPs cultured in the absence of bFGF for 2 weeks were analyzed for mRNA expression of subunits of each glutamate receptor subtype relative to hESCs. Significant increases ($p<0.05$) in *Grin2b* were seen in hNPs (20 fold) and differentiated hNPs (25 fold) relative to hESCs (Figure 3.3A). Additionally, *Grin1* and *Grin2d* (Figure 3.3A) were significantly up regulated ($p<0.05$) in differentiated hNPs relative to hESCs (Figure 3.3A). Of the kainate receptors, *Girk4* and *Girk5* were significantly ($p<0.05$) up regulated in hNPs relative to hESCs (Figure 3.3B), whereas, *Girk2* was significantly ($p<0.05$) up regulated in 14 day after bFGF removal differentiated hNPs (Figure 3.3B). Up regulated, though not significantly, in 14 day differentiated hNPs relative to hESCs were *Girk1, Girk 4* and *Girk5* (Figure 3.3B). AMPA receptor subunits were examined also. *Gria1* and *Gria4* were up regulated in hNPs relative to hESCs (Figure 3.3C). Two week differentiated hNPs showed significant ($p<0.05$) up regulation of *Gria2* and *Girk4* relative to hESCs (Figure 3.3C).

To determine functionality, a FLIPR assay was performed on day 14 differentiated hNPs grown in a 96 well plate. Activity of the AMPA, kainate and NMDA receptors was determined through intracellular calcium release (Figure 3.3G) and potentiated with 50 $\mu$M of cyclothiazide, a positive allosteric modulator with preferential actions which minimize desensitization in the

AMPA receptor subunits with a lesser effect on kainate receptors and none with NMDA (Figure 3.3G). A dose dependant increase in calcium influx was detected in the presence of cyclothiazide in calcium activity as measured with the FLIPR assay in a 96 well plate (Figure 3.3H).

*hNPs and differentiated hNPs are responsive to calcium and sodium*

Voltage dependant calcium and sodium channels play a role in regulating the membrane excitability of neurons as well as synaptic transmission. The hNPs and day 14 differentiated hNPs in this study were evaluated for calcium and sodium channel subunit expression using real-time PCR. The following subunits were significantly ($p<0.05$) up regulated in hNPs relative to hESCs: *Cacna1h*, *Cacnb3 Cacnb4* (Figure 3.3D). Subunits significantly ($p<0.05$) up regulated in day 14 differentiated in hNPs relative to hESCs were *Cacna1b*, *Cacn1c*, *Cacna1h*, *Cacnb3* and *Cacnb4* (Figure 3.3D). Sodium subunits *Nav1.2*, *Nav1.4, Nav1.7*, *Nav1.9* were significantly ($p<0.05$) up regulated in both hNPs and day 14 differentiated hNPs relative to hESCs (Figure 3.3E). Additionally, *Nav2.3* was significantly ($p<0.05$) up regulated in hNPs relative to hESCs (Figure 3.3E). In support of this, increasing concentrations of a sodium channel activator veratridine in a FLIPR assay on differentiated hNPs show an increasing calcium response (Figure 3.3F).

Sodium channel activity in differentiated hNPs was measured using whole cell voltage clamp. 81 total hNPs differentiated for 4 to 27 days were successfully patched. Of these, 34 exhibited no fast inward currents in response to a step depolarization indicating the absence of functional voltage gated sodium channels. The remaining cells yielded between 0.04 - 1.5 nA of inward current in response to the step depolarization (Figure 3.4B and 3.4G). These currents inactivated rapidly in all cases (Figure 3.4B and 3.4C).These currents could be abolished with the addition of 1 μM TTX (n = 3 cells; Figure 3.4C).Voltage-dependent steady state inactivation (n = 11 cells; Figure 3.5D) and recovery from fast inactivation (n = 5 cells; Figure 3.4E) were also observed on several positive cells. Differentiated hNPs were functionally positive for

voltage-gated sodium channel activation. Action potentials were observed under current clamp upon current injection on 8 or 10 cells for which the prerequisite was a voltage dependent inward current elicited a step depolarization to -10 mV (Figure 3.4F). In support of this, increasing concentrations of a sodium channel activator veratridine in a FLIPR assay on differentiated hNPs show an increasing calcium response (Figure 3.4H).

The 58% hit rate for voltage-gated sodium channel function (Figure 3.4G), does not reflect the true proportion of sodium channel positive cells in our differentiated hNPs, but rather our ability to morphologically distinguish these cells from negative cells by eye. An example of the morphology of a sodium channel positive cell is shown in Figure 3.4A.The positive cells were phase bright with a few long processes.

All cells examined with a potassium gluconate intracellular solution exhibited voltage-dependent outwardly rectifying currents (Figure 3.4B).These currents were not observed for cells in which a cesium gluconate based intracellular solution was used. hNPs also exhibited outward potassium currents.

*Expression of ionotropic receptors in hNPs and differentiated hNPs*

In addition to the main excitatory transmitter in the CNS, other neurotransmitters and ionotropic receptors are of interest. We used real-time PCR to measure the relative expression levels of nicotinic receptor subunits. Relative to hESCs, *Chrna3*, *Chrna5*, *Chrnb4* and *Fam7a3* were significantly ($p<0.05$) up regulated in differentiated hNPs relative to hESCs (Figure 3.5A). *Chrna7*, *Chrna3*, *Chrna5* and *Fam7a3* were significantly ($p<0.05$) up regulated in hNPs relative to hESCs. One subunit, *Chrna10* is significantly ($p<.05$) down regulated in both hNPs and day 14 differentiated hNPs relative to hESCs (Figure 3.5A).

Purinergic receptors are ionotropic receptors, which activate ATP and regulate cellular secretions and sensory transmission. *P2rx5* expression was significantly ($p<0.05$) higher in hNPs relative to hESCs (Figure 3.5B); however, hNPs show a significant ($p<0.05$) down regulation of *P2rx2* relative to hESCs (Figure 3.5B). Day 14 differentiated hNPs also show an up

regulation of *P2rx5* relative to hESCs (Figure 3.5B), but a down regulation of *P2rx4* relative to hESCs (Figure 3.5B).

TRP channels are ion channels, which have varying selectivity for sodium and calcium ions. *Trpv1* and *Trpm7* subunit were both significantly ($p<0.05$) higher in hNPs relative to hESCs (Figure 3.5C). While the *Trpv1* subunit is also significantly ($p<0.05$) up regulated in differentiated hNPs relative to hESCs, the *Trpv3* subunit is significantly down regulated ($p<0.05$, Figure 3.5C).

In early development, the GABA neurotransmitter acts as an excitatory signal. As the CNS matures, GABA becomes the primary inhibitory neurotransmitter in the CNS. GABRA3 and GABRB3 were significantly ($p<0.05$) down regulated in differentiated hNPs relative to hESCs (Figure 3.5D).

**Discussion**

The overall goal of this study was to examine a cohort of important ionotropic receptors found in the CNS and their subunit expression levels during basal in vitro neuronal differentiation of developmentally representative hNPs derived from hESCs. Developmentally, it is important to determine regionalization of the neural cells if they are ever to be used to represent relevant CNS models. Early in brain development, transcriptional factors are important for establishing boundary areas from which neurons located within these boundaries can receive the signaling factors necessary to develop into the neural subtypes found within these locations. The differentiating hNPs expressed different regional developmental markers as they proceeded through differentiation. Early regulation of the midbrain-hindbrain boundary and the development of the midbrain and the cerebellum are controlled by *Gbx2* [25], which was expressed in hNPs as well as in cultures after bFGF was removed for 14 and 35 days. The differentiation of the telencephalon is regulated by *Foxg1*'s modulation of brain morphogenic protein and fibroblast growth factor 8 signaling [26,27]. *Foxg1* null mice have a severely underdeveloped telecephalon and die prior to birth highlighting its importance in neural

differentiation [26]. *Foxg1* expression occurs at day 125 after bFGF was removed. The factors expressed in the hNPs differentiated for 35 days in culture are representative of first midbrain/hindbrain development (*Gbx2*) then later (125 days after bFGF removal) of proper telencephalon development (*Foxg1*). Following regional organization, *Cdh2* is involved in regulation of the cortex and in regulation of cortical neuron differentiation in the subventricular zone [28] and was expressed in hNPs and differentiated hNPs at day 14, 35 and 125 of differentiation. *Cdh2* controls cortical neuron placement through modulating neuron movement along radial glial cells [29]. *NeuroD*, expressed early in neural differentiation but not in hNPs, is part of a regulatory pathway that controls early neural differentiation and glutamatergic neurogenesis [30-32]. The time dependent presence of these transcripts during in vitro differentiation suggests that hNPs are a potential model for temporal associated events such as regionalization in the human CNS areas of development.

The family of GRIN subunits are expressed pre- and postnatally in most areas of the brain [33,34]. *Grin1* is the site of glycine binding and has an important role in axon refinement. *Grin1* mutant mice exhibited reduced social behavior suggesting that *Grin1* associated glutamate activity has a role in schizophrenia [35-37] These behaviors can be reversed with glycine site functional agonists indicating that gycine binding site on *Grin1* is involved with schizophrenia [36]. This gene was up regulated in the differentiated hNPs suggesting the potential for using differentiated hNPs to study glycine agonists. The *Grin2* subunits are expressed postnatally (*Grin2a* and *Grin2c*) [7] or embryonically (*Grin2b* and *Grin2d*) [7] in mice and are the site for glutamate binding [38,39]. In the differentiated hNPs used in these studies, we see up regulation in expression of the *Grin2b* and *Grin2d* subunits but not the *Grin2a* or *Grin2c* subunits. Up regulated in both hNPs and 14 day differentiated hNPs, *Grin2b* controls receptor recycling [40,41] and a mutated *Grin2b* subunit increases the calcium permeability of the NMDA receptor [42]. *Grin2b*, *Grin2d*, which were also significantly up regulated in differentiated hNPs, helps to modulate dopamine neurogenesis [43]. *Grin3b* expression begins

late prenatal and continues early postnatal [7] and in this study, we see slight up regulation expression of the *Grin3b* subunit. As neurons develop postnatally, *Grin2b* subunit is swapped for the *Grin2a* subunit [44]. The NMDA subunits are important for the pruning of developing synapses through increased activity at those neurons which receive input from more than one synapse [4]. Additionally, NMDA receptors are important for mediating neural motility in development with high glutamate expression seen at E13 and glutamate stimulated motility beginning at E17 in mice [3]. Previously we demonstrated that hESC derived hNPs express NURR1, a protein important for dopaminergic neurogenesis, and could form dopaminergic cells at a high level (> 50% TH+ and PITX3+) when LIF and GDNF are added to the cultures [45]. Together, the expression of GRIN subunits such as *Grin2b* and *Grin2d* in combination with growth factor supplement could potentially affect the phenotype of in vitro differentiated hNPs.

AMPA and kainite receptor subunits are highest during synpatogenesis and synapse remodeling [46] as well as being expressed endogenously throughout the adult nervous system [47]. *Gria4* and *Grik1* beginning expression at E10 with more defined expression by E12 in both rats and mice where as *Grik4* and *Girk5* begin expression at E14 [46]. *Gria1-4* expression becomes segregated into distinct brain regions at E14 and *Gria1* expression increases in the cortex and decreases in the striatum at E15 [46]. Here we showed up regulation of kainate receptor subunits *Girk1* and *Girk2* and AMPA receptor subunits *Gria2* and *Gria4* in hNPs and differentiated hNPs relative to hESCs suggesting that these cells might be used to identify human neural glutamatergic modulators. Expression of Girk4 begins at E12 while Girk1 peaks around birth and Girk2 is only expressed prenatally [7]. AMPA potentiating compounds could serve as cognitive enhancers in human subjects. In a related study, we determined that day 14 differentiated hNPs demonstrated increased calcium levels activated by AMPA in the presence of cyclothiazide and when compared to mouse neural cells, generated a similar $EC_{50}$ for 17 of 21 compounds screened. However, there was a greater than 10 fold difference in response for 19% of the compounds tested in both rodent and human cells. Therefore, compounds tested in

human neural cells generated from hNPs may avoid species translation issues when these compounds are moved into clinical trials [14].

*Gria2* is the most commonly affected AMPA subunit in the neurodegenerative disease amyotrophic lateral sclerosis [7,47]. In ALS, the AMPA receptor mediates excitotoxicity. Normally the *Gria2* subunit is impermeable to calcium; however, ALS patients have been shown to have a defect in pre-mRNA editing of *Gria2* which changes the permeability of the AMPA channel to allow calcium to pass through [9]. In addition to the proper regulation of AMPA subunits, the proper assembly of calcium channel subunits are required to maintain calcium homeostasis in ALS patients [48]. Death of motor neurons in ALS has been linked to increased calcium intake mediated by AMPA receptor calcium influx as well as NMDA receptor calcium influx [49]. *Cacna1b*, *Cacna1c*, *Cacna1h*, *Cacnb1* and *Cacnb4* subunit transcripts were up regulated in hNPs and day 14 differentiated hNPs. Since these hNPs derived from hESCs have also been differentiated towards a motor neuron fate, these same channel subunits might in the future be specifically studied in a hNP derived motor neuron phenotype [50].

Sodium channels consist of alpha and beta subunits, which are only permeable to sodium ions. The subunits up regulated in the hNPs and differentiated hNPs used in this study (*Nav1.6, Nav1.7*, *Nav1.9* and *Nav2.3*) all have roles in modulating neural differentiation, neural excitability and neural development [51,52]. There are two classes of sodium subunits, those sensitive to TTX (*Nav 1.1-1.5* and *Nav 1.7*) and those insensitive to TTX (*Nav1.5*, *Nav1.8* and *Nav 1.9*) [51]. Here electrophysiological results indicate that differentiated hNPs can generate inward currents that were blocked with TTX. *Nav1.6*, expressed only in the CNS, and *Nav1.7*, expressed predominately in differentiated neurons and increasing postnatally, are both up regulated in neural differentiation and known to accompany morphological changes that occur in during differentiation to various neural subtypes [29]. *Nav1.2* is constitually expressed throughout development while *Nav1.9* expression begins at E17 [51]. *Nav1.4* is only expressed in the skeletal muscles [53] and is not seen in the hNPs and differentiated hNPs used in this

study. *Nav1.7* expression occurs in soma, neurite terminals and growth cone of differentiated cells increasing action potentials as neurons develop [29]. The expression levels of sodium channel subunits seen in this study correspond with the expression seen in rat and mice development.

Other ionotropic receptors with important roles in signal transduction, development and disease pathways are nicotinic, purinergic, TRP and GABAergic. GABA is the primary inhibitory neurotransmitter found in the brain; however, early in embryogenesis it acts as an excitatory neurotransmitter [54] and has been demonstrated to evoke glutamate release [10]. $GABA_A$ receptors are ligand gated chloride channels that are heteropentameric and consist of 2 $\alpha$, 2 $\beta$ and either a $\gamma$, $\delta$ or $\varepsilon$ subunit [55]. GABA receptor regulation can be altered by sodium currents regulated by the *Nav* subunits as sodium and chloride ions are transported into GABA neurons via a sodium/chloride co-transporter. bFGF has been shown to increase the expression of glutamate receptor subunits; however, it has no effect on GABA receptor subunit expression [56]. *Gabrb2*, *Gabrb3* and *Gabrg2* subunits are expressed throughout development while *Gabrg1* and *Gabrg3* expression drops in development [7]. *Gabaa* receptor expression has been shown to reduce the proliferation of neuroblasts and stem cells and to reduce migration [5]. In excitotoxicity models, there is a persistent up regulation in sodium current, which leads to over excitation [10]. Particularly affected is the *Gabaa1* subunit, which are found to be absent or reduced in motoneurons [57]. The differentiated hNPs, which express all of the affected subunits, might be a viable model for excitotoxicity in various diseases.

The TRP channels that transmit sensory information involved in hot/cold sensation, modulation of pain and protection of neurons from oxidative stress, which are generally associated with the peripheral nervous system, were down regulated in hNPs and differentiated cultures. The data suggests that hNPs may not be used under basal conditions to generate differentiated cells representative of the peripheral nervous system. TRP channels are

associated with $Ca^{2+}$ transient expression in the growth cones of axons [2]. Purinergic receptors are lesser known receptors that associate with ATP inducing fast synaptic potentials [58]. The potency of ATP in response to a purinergic receptor depends on the subunits, which compromise the receptor. While different subunits are located in various parts of the CNS, *P2x4* and *P2x7* subunits have been shown to be up regulated in models of excitotoxicity [59]. The *P2x4* and *P2x7* subunits are expressed in microglia; their role in protecting motoneurons is in maintaining the glutamatergic balance to prevent excitotoxicity [59]. When these subunits become up regulated, ATP release is increased as is neural excitation [59], Down regulation of *P2rx4* and lack of change in expression of *P2rx7* suggests the differentiated hNPs used in this study are not currently excitotoxic but have the potential to model excitotoxicity and disease states. Nicotinic receptors are most commonly associated with their role in nicotine addiction smoking behavior and the neuromuscular junction. The standard neural subunits are $\alpha$2-10 and $\beta$2-4 and these subunits typically form heteromeric channels in various combinations of $\alpha$ and $\beta$ subunits, however, $\alpha$7 and $\alpha$9 can combine to form homomeric channels. All neural nicotinic subunits begin expression around E11 in the rat and the expression level remains the same in adult tissue. The $\alpha$5, $\alpha$3 and $\beta$4 subunits, which were expressed in differentiated hNPs are all responsible for nicotine addiction [60] and are involved in motoneuron signaling between the neuron and the muscle at the neuromuscular junction [61]. Nicotinic receptors have a protective effect on motoneurons, which can be blocked with $\alpha$4 and $\alpha$7 inhibitors [61] suggesting that receptor subunits on the human cells in the cultures could serve as targets in neural protective drug development models. Understanding important receptor subunit transcript levels in hNPs and the differentiated derivatives of hNPs provide some insight in how they could be used to screen therapeutic drug targets that affect neurological diseases and injury.

## Conclusion

As hypothesized, hESC derived neural tissue expressed a large repertoire of endogenously expressed ion channels which can result in functional responses; therefore, it seems that hNPs and their differentiated products can provide a relevant model for development and drug discovery. As these hNPs are differentiated, they orient themselves toward developmental organization that is seen in vivo. Ionotropic receptors are important for electrical transduction of signals within neurons and are often targets for drug therapies. These differentiated hNPs could provide human neuronal cultures for studying cellular activity associated with deleterious events such as excitotoxicity. As these differentiated hNPs express ion channel subunits whose regulation is implicated in responses to insult, these cultures should provide an in vitro model to study the mechanisms and changes that occur with glutamate signaling and glutamate receptor mediated pathologies.

## Acknowledgements

We thank Julie Nelson and the Center for Tropical and Emerging Global Diseases Flow Cytometry Facility for her technical expertise. Funding was provided by NIH R41N5053272-01 and DoD # N000140810989.

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**Figure 3.1:** Human neural progenitor cells differentiate towards a mature neuronal population hNPs maintained in proliferation media express neural stem cell marker NESTIN (green, A). When differentiated in neural differentiation media for 14 days, differentiated hNPs express immature neural marker TUJ1 (green, B) and after 21 days of differentiation differentiated hNPs express interfillament marker MAP2 (green, C). Halting of proliferation is a marker of post-mitotic neurons. hNPs measured for proliferation with CFSE on FlowJo proliferation model show a 24 hour cell doubling (D, F). Differentiated hNPs measured on the same proliferation model do not have a population doubling over 96 hours (E,F). The population doubling is supported by the reduction in fluorescence from 524 RFU to 241 RFU at 24 hours and from 241 RFU to 191 RFU at 48 hours (F). This was not seen in the differentiated hNPs. Scale bars 10μm. hNP- human neural progenitor cell; CFSE - carboxyfluorescein succinimidyl ester; RFU – relative fluorescence unit.

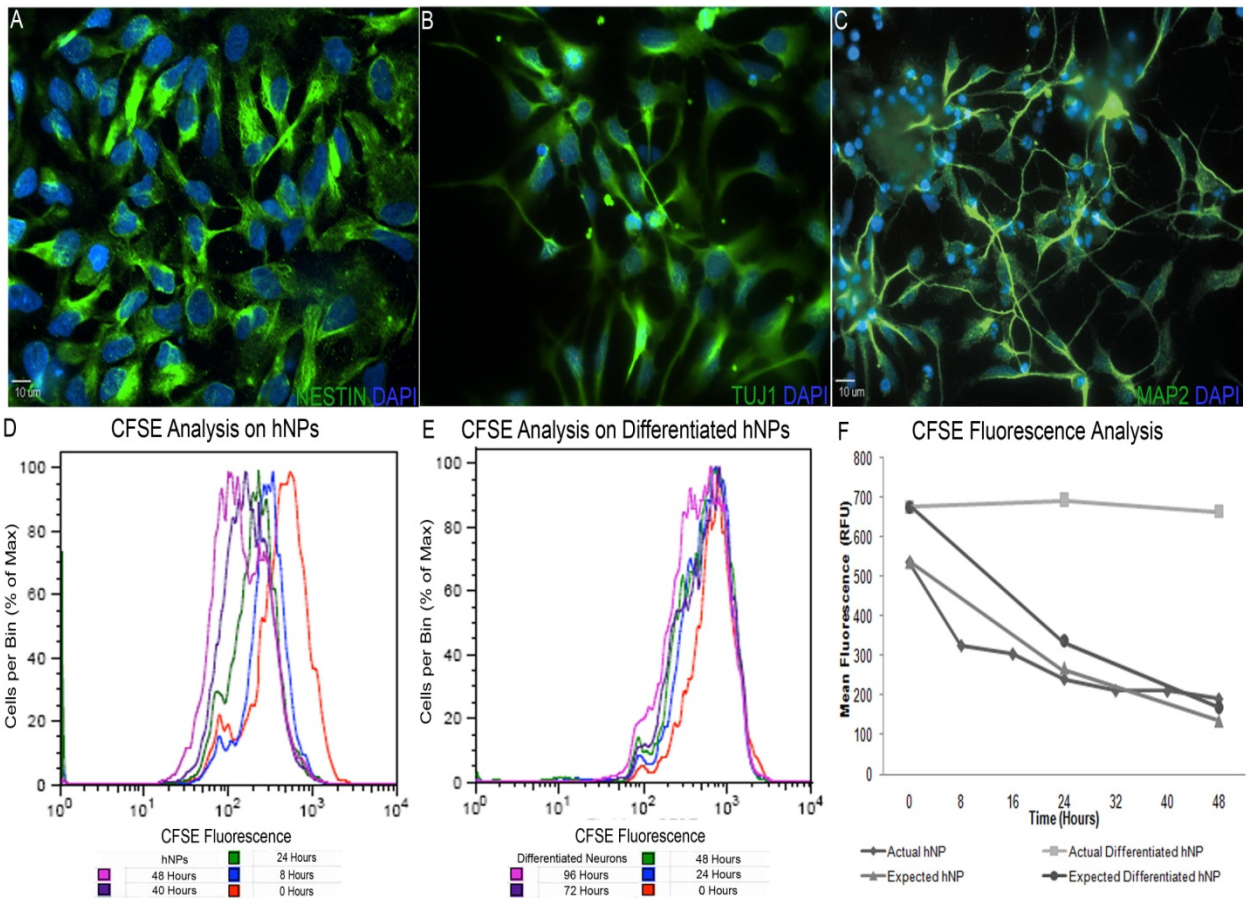

A
10 um
NESTIN DAPI
B
TUJ1 DAPI
C
10 um
MAP2 DAPI
D
CFSE Analysis on hNPs
Cells per Bin (% of Max)
CFSE Fluorescence
hNPs
24 Hours
48 Hours
8 Hours
40 Hours
0 Hours
E
CFSE Analysis on Differentiated hNPs
Cells per Bin (% of Max)
CFSE Fluorescence
Differentiated Neurons
48 Hours
96 Hours
24 Hours
72 Hours
0 Hours
F
CFSE Fluorescence Analysis
Mean Fluorescence (RFU)
Time (Hours)
Actual hNP
Actual Differentiated hNP
Expected hNP
Expected Differentiated hNP

**Figure 3.2:** Differentiated neural progenitor cells express markers of developmental progression Differentiated hNPs at 28 days post differentiation express *S100β* and *Cntfr* markers of glial differentiation (A). At 49 days post differentiation, other glial markers *Sc1* and *Aqp4* are expressed (A). After differentiation of hNPs for 14, 35 or 125 days, there is an increase in presynaptic receptors and proteins necessary for functional neurotransmitter release, *Dat, Sert, Herg, Kcc2, synaptophysin, syntaxin1A* and *Kir4.1* (B). Embryonic neural development markers *Chd2* and *Gbx2* are see in hNPs and differentiated hNPs at 14, 35 and 125 days (*Chd2*) or 35 days (*Gbx2*, C). Differentiation marker *NeuroD* is expressed at 14 and 35 days of differentiation but not in hNPs, while telencephalic neurogenesis marker *Foxg1* is seen in 125 day differentiated hNPs (C). Pathways involved in neural development include *Mapk* and *Pi3k*. Markers of these pathways, *Mapk, Akt*, *Ncam* and *Src* are expressed in 14 day differentiated hNPs and *Mapk* is expressed in hNPs (D). hNP – human neural progenitor cells.

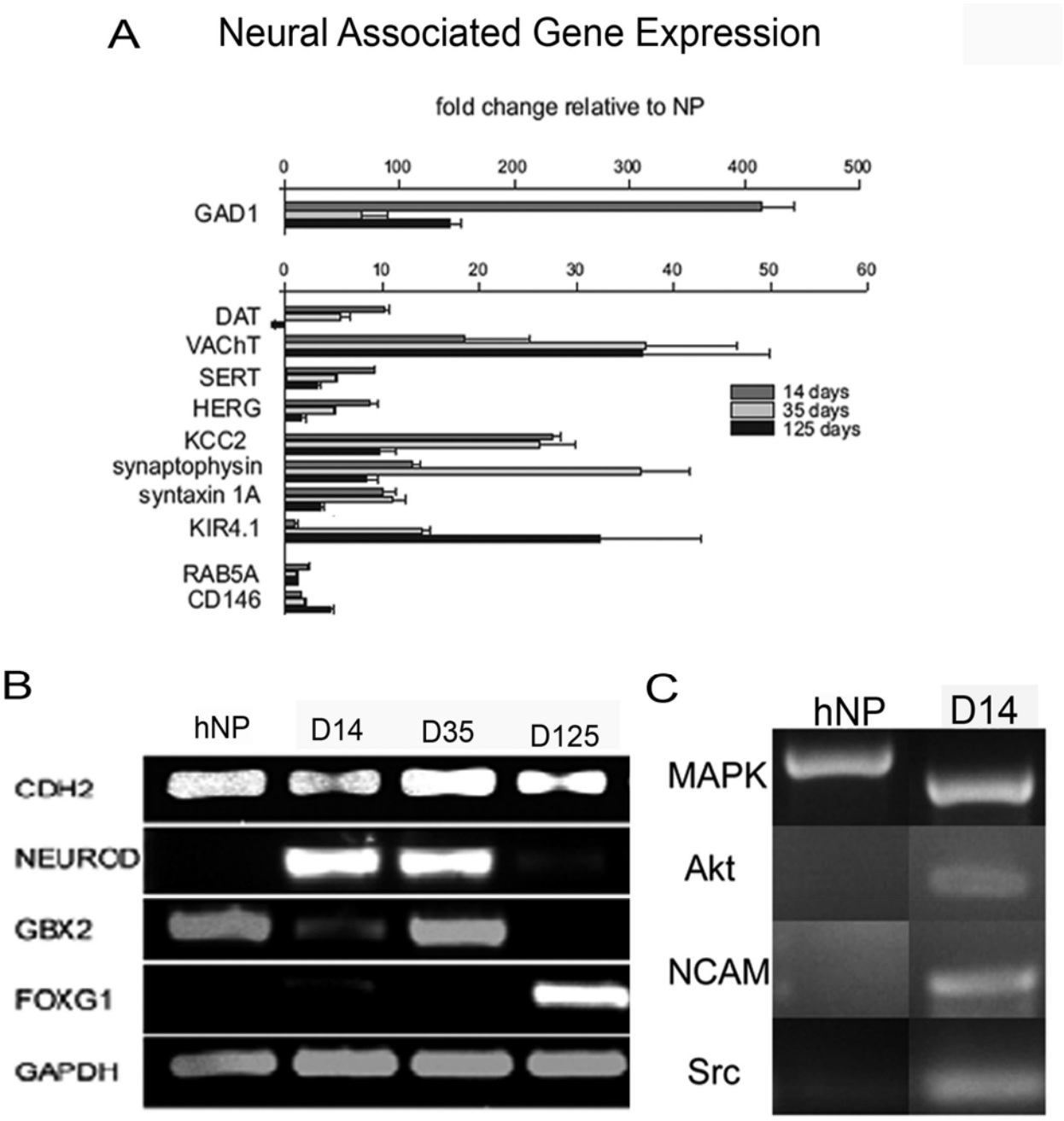

A
Neural Associated Gene Expression
fold change relative to NP
0
100
200
300
400
500
GAD1
0
10
20
30
40
50
60
DAT
VAChT
SERT
HERG
KCC2
synaptophysin
syntaxin 1A
KIR4.1
RAB5A
CD146
14 days
35 days
125 days
B
hNP
D14
D35
D125
CDH2
NEUROD
GBX2
FOXG1
GAPDH
C
hNP
D14
MAPK
Akt
NCAM
Src

**Figure 3.3:** Expression of ionotropic glutamatergic receptor subunits, sodium and calcium channels in hNPs and differentiated hNPs

Differentiated hNPs show up-regulation of *Grin1*, *Grin2b* and *Grin2d* NMDA subunits relative to hESCs while hNPs only show up-regulation of *Grin2d* (A) relative to hESCs. hNPs have up-regulated expression of kainate subunits *Grik4* and *Grik5* relative to hESCs, while differentiated hNPs expression the *Grik2* subunit relative to hESCs (B). The AMPA receptor subunits *Gria1* and *Gria4* are up regulated relative to hESCs in hNPs, while *Gria2* and *Gria4* are up-regulated relative to hESCs in differentiated hNPs. Expression of various calcium channel subunits (A) and sodium channel subunits (B) make these hNPs and differentiated hNPs good models for studying signal transduction. AMPA - α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate, NMDA - N-methyl-D-aspartic acid, hESCs – human embryonic stem cells; hNPs – human neural progenitor cells, PCR – polymerase chain reaction.

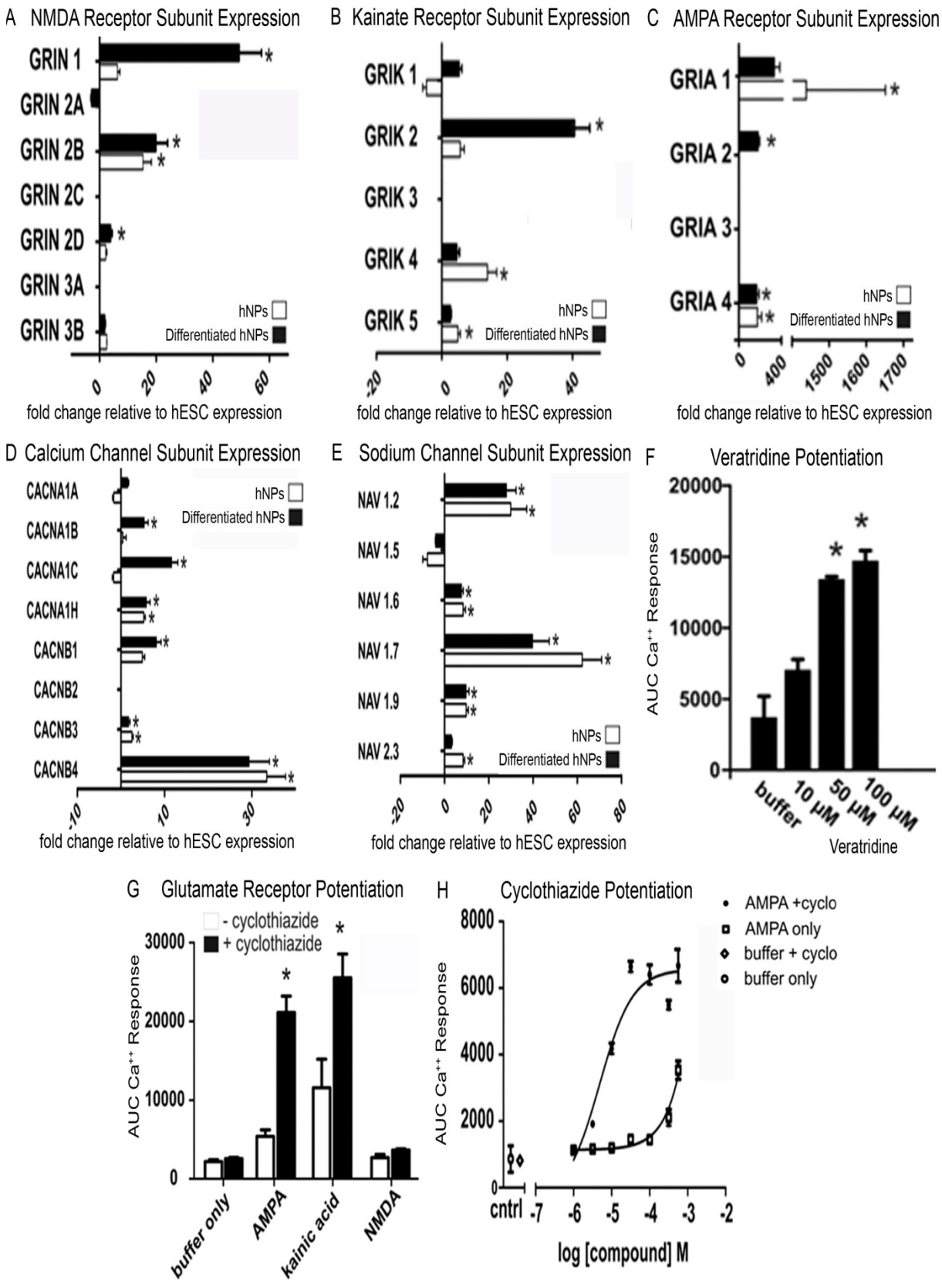

A NMDA Receptor Subunit Expression
GRIN 1
GRIN 2A
GRIN 2B
GRIN 2C
GRIN 2D
GRIN 3A
GRIN 3B
hNPs
Differentiated hNPs
0
20
40
60
fold change relative to hESC expression
B Kainate Receptor Subunit Expression
GRIK 1
GRIK 2
GRIK 3
GRIK 4
GRIK 5
hNPs
Differentiated hNPs
-20
0
20
40
fold change relative to hESC expression
C AMPA Receptor Subunit Expression
GRIA 1
GRIA 2
GRIA 3
GRIA 4
hNPs
Differentiated hNPs
0
400
1500
1600
1700
fold change relative to hESC expression
D Calcium Channel Subunit Expression
CACNA1A
CACNA1B
CACNA1C
CACNA1H
CACNB1
CACNB2
CACNB3
CACNB4
hNPs
Differentiated hNPs
-10
10
30
fold change relative to hESC expression
E Sodium Channel Subunit Expression
NAV 1.2
NAV 1.5
NAV 1.6
NAV 1.7
NAV 1.9
NAV 2.3
hNPs
Differentiated hNPs
-20
0
20
40
60
80
fold change relative to hESC expression
F Veratridine Potentiation
AUC Ca++ Response
20000
15000
10000
5000
0
buffer
10 µM
50 µM
100 µM
Veratridine
G Glutamate Receptor Potentiation
- cyclothiazide
+ cyclothiazide
AUC Ca++ Response
30000
20000
10000
0
buffer only
AMPA
kainic acid
NMDA
H Cyclothiazide Potentiation
AMPA +cyclo
AMPA only
buffer + cyclo
buffer only
AUC Ca++ Response
8000
6000
4000
2000
0
cntrl
-7
-6
-5
-4
-3
-2
log [compound] M

**Figure 3.4 :** Expression of nicotinic, purinergic, transient receptor potential and GABAergic subunits

Ionotropic receptors are responsible for elicting response to several stimuli. Nicotinc receptors (A) are most commonly associated with nicotine addiction. Purinergic receptors (B) are newly discovered and are linked to ATP activation. TRP channels (C) are sensory channels which transmit hot/cold responses, mechanical response or mineral regulation. GABA is the primary inhibitory neurotransmitter in the brain and has a role in modulating signals transmitted in the brain (D). TRP – tranisent receptor potential, GABA - gamma-aminobutyric acid.

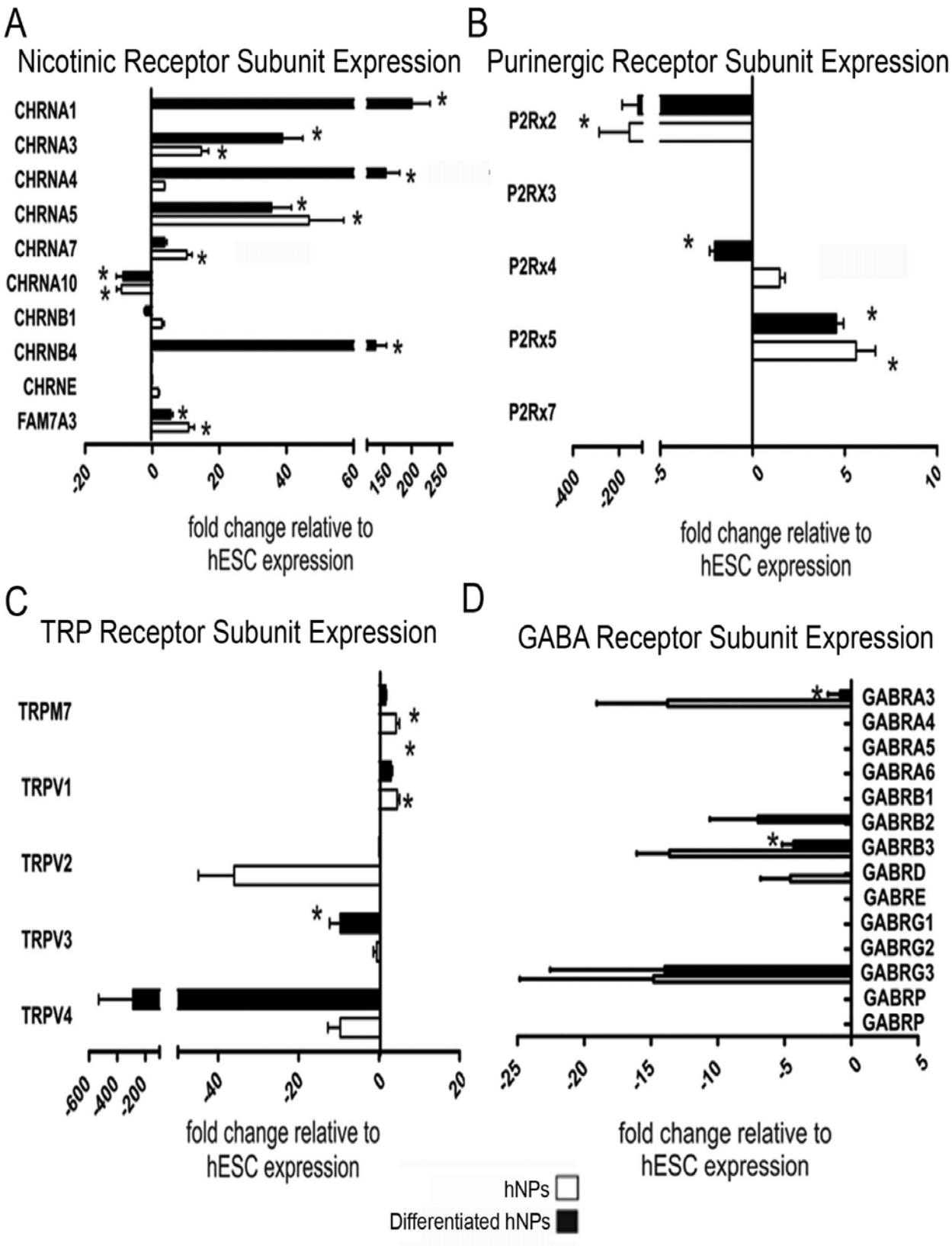
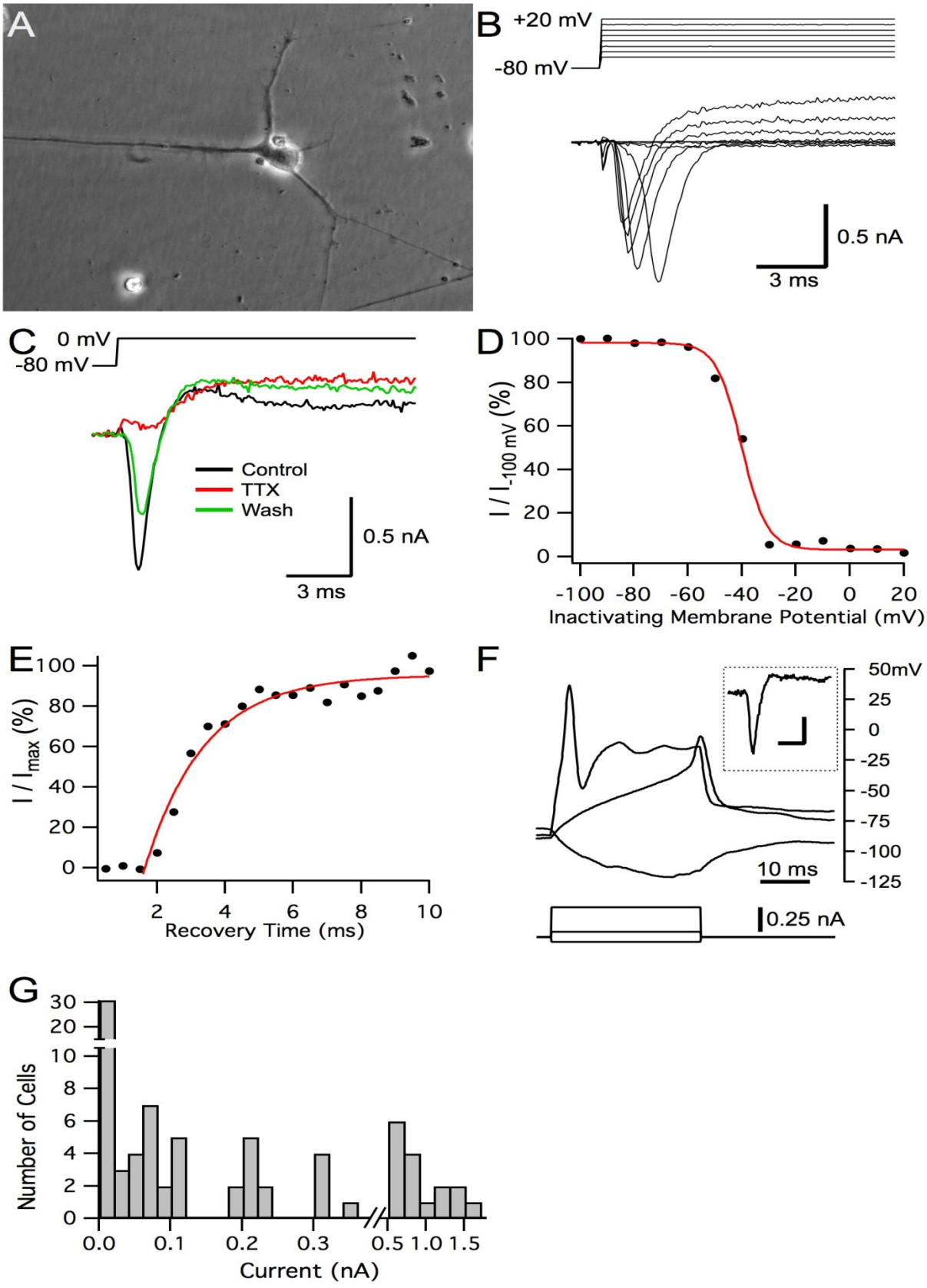

A
Nicotinic Receptor Subunit Expression
CHRNA1
CHRNA3
CHRNA4
CHRNA5
CHRNA7
CHRNA10
CHRNB1
CHRNB4
CHRNE
FAM7A3
-20
0
20
40
60
150
200
250
fold change relative to hESC expression
B
Purinergic Receptor Subunit Expression
P2Rx2
P2RX3
P2Rx4
P2Rx5
P2Rx7
-400
-200
-5
0
5
10
fold change relative to hESC expression
C
TRP Receptor Subunit Expression
TRPM7
TRPV1
TRPV2
TRPV3
TRPV4
-600
-400
-200
-40
-20
0
20
fold change relative to hESC expression
D
GABA Receptor Subunit Expression
GABRA3
GABRA4
GABRA5
GABRA6
GABRB1
GABRB2
GABRB3
GABRD
GABRE
GABRG1
GABRG2
GABRG3
GABRP
GABRP
-25
-20
-15
-10
-5
0
5
fold change relative to hESC expression
hNPs
Differentiated hNPs

**Figure 3.5:** Differentiated hNPs exhibit voltage-dependent sodium channel function and can produce inward currents that generate action potentials.

Differentiated hNP's grown on a substrate of laminin 1 mg/ml for up to one month and differentiated hNPs with significant neurite growth were isolated after 23 days of differentiation (A). This cell was subjected to whole cell voltage clamp utilizing a potassium gluconate based intracellular solution and elicited both voltage gated inward and outward currents in response to depolarizing voltage steps (B, C). Inward currents from another cell (potassium gluconate intracellular) were abolished by local application of 1 μM TTX (red trace) while outward currents remained. Inward current recovered as TTX washed out of the region (green trace; D). A different cell exhibited classic sodium channel steady state inactivation by showing voltage activated inward currents that inactivated in response to a 50 ms pre-pulse at different membrane potentials. The experiment was done 27 days after the removal of bFGF. A cesium gluconate based intracellular solution was used for this experiment to block outward potassium currents. The membrane potential for half maximal inactivation by standard Boltzman fitting (red line) was -40.1 mV with a slope of 4.7 (E). Recovery from fast inactivation utilizing a paired pulse protocol in the same cell as C. The single exponential time constant for recovery of inactivation was 1.7 ms (red line; F). A different cell elicited an overshooting action potential upon current injection under whole cell current clamp utilizing a potassium gluconate based intracellular solution. Inset: Response of the same cell under voltage clamp to a change in membrane potential from -80 mV to -10 mV elicited a peak current of 457 pA. Scale bars for inset: 5 ms, 0.2 nA (G). Histogram of maximum peak current amplitudes elicited on depolarization from all successfully patched differentiated hNPs under voltage-clamp. Left-most bar indicates cells exhibiting no inward current in response to the depolarization. TTX – tetrodotoxin; hNP – human neural progenitor.


A
B
+20 mV
-80 mV
0.5 nA
3 ms
C
0 mV
-80 mV
Control
TTX
Wash
0.5 nA
3 ms
D
I / I-100 mV (%)
Inactivating Membrane Potential (mV)
E
I / Imax (%)
Recovery Time (ms)
F
50mV
10 ms
0.25 nA
G
Number of Cells
Current (nA)

CHAPTER 4

# GLIAL CELL-LINE DERIVED NEUROTROPHIC FACTOR ENHANCES IN VITRO DIFFERENTIATION OF MID-/HINDBRAIN NEURAL PROGENITOR CELLS TO DOPAMINERGIC-LIKE NEURONS[1]

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[1]Young, Amber, Assey, Kristin S., Sturkie, Carla D., West, Franklin D., Machacek, David W., Stice, Steven L. 2010. *Journal of Neuroscience Research*. 88(15):3222-32. Reprinted here with permission of publisher.

**Abstract**

Parkinson's disease (PD) affects the motor system through the degeneration of the dopaminergic neurons of the substantia nigra. The use of human embryonic stem cell (hESC) derived human neural progenitor (hNP) cells provides a potential cell source for cell therapies and drug screens for future treatments. Glial cell-line derived neurotrophic factor (GDNF) is a known dopaminergic neuroprotectant agent; however, its potential role in neural differentiation remains largely unknown. Addition of 25ng/ml of GDNF to hNP cell differentiation media, over a 21 day period, induced a significantly ($p<0.05$) greater portion of hNP cells to differentiate into dopaminergic neurons than non-GDNF cultures, 50% compared to 2.9% of cells expressing tyrosine hydroxylase (TH), respectively. The hNP cells exposed to GDNF selectively expressed dopamine receptors 1, 4 and 5 and were evoked to release dopamine with KCl. This is the first report of GDNF and LIF enriching hESC derived hNP cells towards dopaminergic-like neurons.

## Introduction

Parkinson's disease (PD), a debilitating disease manifesting in motor symptoms, affects the mesencephalic dopaminergic neurons of the substantia nigra and the planning of movement [1]. In people affected by PD, these neurons typically degenerate over a course of several years beginning around age 50 [2]. Endogenous levels of glial cell-line derived neurotrophic factor (GDNF) regulate the dopaminergic neurons in the substantia nigra in vivo [3-9]; however, little is known about GDNF's potential in vitro role in enrichment and differentiation of neural cells towards a dopaminergic fate. GDNF bound GFR$\alpha$1 activates the Rearranged in Transfection (RET) receptor and leads to a downstream intracellular cascade that promotes survival, proliferation and differentiation of parasympathetic, sympathetic, motor, enteric and dopaminergic neurons [10-16]. Through this activation of downstream pathways, increases in axonal outgrowth, target innervations and protection occurs in dopaminergic neurons of the striatum [17-20]. Evidence of GDNF's role in dopaminergic neuron projections has come from studies of conditional GDNF knockout mice that demonstrate decreased expression of GDNF in the striatum, expression of RET and GFR$\alpha$1 in dopaminergic neurons, low survival and reduced fiber outgrowth [21,22].

Previously, human embryonic stem cells (hESCs) have been differentiated towards a dopaminergic fate using various means including stromal cell-line derived induction activity (SDIA) [23] and, more recently, a five stage differentiation protocol using numerous growth factors [24]. The efficiencies of these systems range from 25 to 86% tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine synthesis, positive (+) neurons [25,26]. Besides producing variable results, the length of time in culture to generate dopaminergic cells from hESC varies from 2 to 7 weeks [25,27]. This could be due in part to the intrinsic propensity of hESCs to differentiate spontaneously; therefore, some studies may have begun with less uniform hESC starting populations than others [28]. A hESC derived neural progenitor population responsive to

growth factors capable of consistent and quantifiable differentiation to a dopaminergic fate without multiple steps or feeder cells will be a valuable resource for PD research.

Neural stem cells (NSCs) cultured from the adult human central nervous system (CNS) have limited potential for becoming multiple types of neurons with most tending to become glial cells [29]. Human neural progenitor (hNP) cells derived from hESCs have demonstrated an ability to produce various cell types. Previous studies suggest that midbrain dopaminergic cells have been difficult to derive from propagated NSCs given the culture conditions needed for proliferation [30]. Propagated hNP cells derived from hESCs have a posterior cell fate, potentially due to the propagation of cells in the presence of retinoic acid (RA) [29,31]. Shin et al. 2005 previously show that hNP cells form motor neurons when induced with RA and sonic hedgehog (SHH); however, propagated hNP cells that become dopaminergic neurons have not been previously demonstrated [27,32].

The objective of this study was to determine whether previously established hESC derived, continually cultured and expanded hNP cell lines could be a source of dopaminergic neurons and to determine the role of GDNF in this differentiation process. Removal of bFGF from hNP culture media induced differentiation of TH+ neurons, which was further enhanced by the addition of GDNF to hNP cells, obtaining 50.5% TH+ cells after 21 days in culture. After additional differentiation, dopaminergic-like cells, when stimulated, released dopamine into culture media, indicating these cells could produce and release dopamine. In this study, the derivation of dopaminergic-like cells did not require the use of a feeder layer and allowed for a shorter time to differentiation than previous reports using hESC as the starting population [27]. Together these data suggest that propagated hNP cell lines are capable of forming dopaminergic-like neurons in vitro.

## Materials and Methods

*hNP Cultures*

Human neural progenitor (hNP) cells were derived from hESC line WA09 by our lab as previously described [33]. Briefly, after one week of culture on mouse fibroblast feeder layers, WA09 hESCs were grown with derivation medium containing Dubecco's modified Eagle medium (DMEM)/F12 medium (Gibco) supplemented with 2mM L-glutamine (Gibco), 2 U/mL penicillin (Gibco), 2µg/mL streptomycin (Gibco), N2 (Gibco), and 4 ng/ml basic fibroblast growth factor (bFGF; R&D) for 7 days. Rosettes were selected with hook passaging from culture dishes and re-plated on polyornithine (Sigma) and laminin (Sigma) coated dishes. These rosettes were propagated for 3 days on polyornithine and laminin coated dishes in neurobasal medium (Gibco) supplemented with 2mM L-glutamine, 2 U/mL penicillin, 2 µg/mL streptomycin, 1X B27 (Gibco), 20 ng/mL bFGF, and 10ng/mL leukemia inhibitory factor (LIF; Millipore). Media were changed every other day and cells were passaged every fourth day or as needed. Cells used for this experiment were passage 22-39.

*Dopaminergic Neural Differentiation*

hNP cells were grown on polyornithine/laminin coated 35mm plates (Falcon) for flow cytometry or on polyornithine/laminin coated 4 well slides (Falcon) for immunocytochemistry staining. Cells were plated in growth medium consisting of neural basal medium, 1X penicillin/streptomycin, 2mM L-glutamate, 1X B27, 10ng/mL LIF and 20ng/mL bFGF. After 24 hours, the media were changed to neural differentiation media, which consisted of growth medium without bFGF or neural differentiation medium plus 25ng/ml GDNF (Neuromics). Media were changed every three days. Cells were harvested at Day 0, 3, 7, 14 and 21 for further analysis.

*Immunocytochemistry and Cell Quantification*

Cells were fixed with 2% paraformaldehyde (PFA; Electron Microscopy Sciences) in PBS with calcium and magnesium (PBS+/+) (Thermo Scientific) for 20 minutes and processed

for immunocytochemistry. Cells were washed in PBS with calcium and magnesium (PBS+/+) 3 times followed by 3 washes for 5 minutes each of permeabilization buffer consisting of 25μL (0.5%) Tween 20 (EMD Chemicals) in 50mL of high salt buffer. Cells were then blocked in 6% goat serum (JacksonImmuno) for 45 minutes. The following primary antibodies were used: mouse anti Tuj1 (1:200, Neuromics), chicken anti TH (1:100), rat anti DAT (1:1000), and rabbit anti VMAT2 (1:500), anti Pitx3 (1:100) (all from Millipore, Inc), anti Nurr1 (1:100, R&D), and anti EN1 (1:250, SantaCruz). Reaction was revealed using AlexaFluor goat 488, 594, or 633 secondary antibodies (1:1000, Molecular Probes). Cell nuclei were stained using DAPI (Invitrogen). Fluorescence was visualized using spinning disk confocal microscope (Olympus). Negative controls included human mesenchymal cells and secondary only staining. Cell counting was performed using Image Pro software (Media Cybernetics). Five random visual fields were selected and counted in triplicate. Data are presented as mean ±SD. Values of $p<.05$ was considered significant using ANOVA and Tukey's Pair-Wise test (Statistical Analysis Software, SAS Institute).

*Flow Cytometry*

Cells were fixed with 4% PFA in PBS without calcium and magnesium (PBS-/-) for 10 minutes. Cells were washed in PBS with calcium and magnesium (PBS+/+) 3 times followed by 3 washes for 5 minutes each of permeabilization buffer consisting of 25μL (0.5%) Tween 20 in 50mL of high salt buffer. Cells were blocked in 6% goat serum for 45 minutes. The following primary antibodies were used: mouse anti Tuj1 (1:200, Neuromics), chicken anti TH (1:100), rat anti DAT (1:1000), rabbit anti VMAT2 (1:500) rabbit anti Pitx3 (1:100) (all from Millipore), mouse anti Nurr1 (1:100, R&D), and rabbit anti EN1 (1:250, SantaCruz). Reaction was revealed using AlexaFluor goat 488 or 633 secondary antibodies (1:1000, Molecular Probes). Cells were quantified on Dako Cyan (Beckman Coulter). Negative controls were secondary only and cell only staining. Cell quantification was done using FlowJo (TreeStar) software. Each experiment

was run in triplicate. Data are presented as mean ±SD. Values of $p<.05$ was considered significant using ANOVA and Tukey's Pair-Wise (Statistical Analysis Software, SAS Institute).

*Polymerase Chain Reaction (PCR)*

RNA was extracted using the Qiashredder and RNeasy kits (Qiagen) according to manufacturer's instructions. The RNA quality and quantity was verified using a RNA 600 Nano Assay (Agilent Technologies) and the Agilent 2100 Bioanalyzer. Total RNA (5 µg) was reverse-transcribed using the cDNA Archive Kit (Applied Biosystems Inc.) according to manufacturer's instructions. Reactions were initially incubated at 25$^{o}$C for 10 minutes and subsequently at 37$^{o}$C for 120 minutes. Primers were selected using Primer Blast (National Center for Biotechnology Information) and were as follows: DRD1 sense: TCTCGAAAGGAAGCCAAGAA antisense: TTCCCCAAATAAAGCACTG, DRD2 sense: GCTCCACTAAAGGGCAACTG antisense: TTCTCCTTCTGCTGGGAGAG, DRD3 sense: TACCTGGAGGTGACAGGTGG antisense: CTATGGTGGGACTCAGGGAA, DRD4 sense TCGTCTACTCCGAGGTCCAG antisense: AGCACACGGACGAGTAGACC, DRD5 sense: CCATCTCTTCCTCGCTCATC antisense: CCCAGACAGACTCAGCAACA, DBH sense: CACCCAGCTGGCACTGCCTC antisense: TGGAAGCGGACGGCTGAGGA PMNT sense: GCAACAACTACGCGCCCCCT antisense: TGCCAGCATTCCCCCTTGCC CHAT sense: GCCCTGCTGGACAGCCACTC antisense: TCCGCCGTCAAGGAGCTGGA TPH1 sense: TGACCTGGACCATTGTGCCAACA antisense: TGGCAGGTATCTGGCTCTGGGG GIRK2 sense: CCCGTCCGCAGCCAGGAAAA antisense: GGCACAGTTTCCCATCCCGCA GAD1 sense: ACGATACCTGGTGCGGCGTG antisense: TGGATATGGCGCCCCCAGGA. cDNA, primers, GoTaq Green (Promega) and water were added to PCR tubes and incubated at 95$^{o}$C for 3 minutes and then 35 cycles at 57$^{o}$C for 30 seconds and then 72$^{o}$C for 30 seconds. Finally, the reactions were incubated at 72$^{o}$C for 10 minutes. The PCR reactions were run on a 2% agarose gel (Bio-Rad) with ethidium bromide (Promega) for 45 minutes at 100V. Gel was visualized on Ugenius (Syngene).

*HPLC*

hNP cells, hNP cells differentiated for 21 days, and hNP cells differentiated for 21 days with GDNF were exposed to 56mM KCl (Sigma) for 30 minutes. Media were collected and acidified with HCl (Sigma) and stored at -80$^{o}$C until HPLC was performed. Media were sonicated with 0.2 ml of ice-cold buffer/mobile phase (0.1 mM $NaHSO_4$/0.1 mM EDTA/0.2 mM octanesulfonic acid/6.5% acetonitrile, pH 3.1). The homogenate was centrifuged at 4°C at 16,100´ *g* for 30 min, and then subjected to the same centrifugation at 16,100 ´ *g* for 30 min in a 0.22-mm spin column. The resulting supernatant (20 ml) was injected by using a Waters 717 autoinjector (Whatman, Milford, MA) and run through a $C_{14}$X-mm max reverse-phase column (4-mm, 80-Å silica; 150 ´ 4.6 mm; Phenomenex, Torrance, CA) and an electrochemical (HPLC-EC) detector (cells maintained at 0.5 nA), where dopamine and dopac were analyzed. Samples were delivered at a constant rate of 1 ml/min (retention times: DA, 8.32 min; DOPAC, 13.05 min). The position and height of DA and DOPAC peaks were compared with reference standard solutions (Sigma-Aldrich, St. Louis, MO). Peak areas were quantified by Millennium$^{32}$ software (Waters).

**Results**

*hNP cells can be induced and specified towards a dopaminergic neuron*

hNP cells used in this study have previously been characterized to be immunoreactive for the immature neural marker NESTIN as well as the neural stem cell marker SOX2 while at the same time being nonreactive to the stem cell marker POU5F1 [33,34]. These hNP cells were evaluated in this study for the receptor involved in GDNF activation, the RET receptor, which is expressed in the substantia nigra of mice [35]. While human mesenchymal cells (hMSCs; Figure 4.1B) and hESCs did not express the RET receptor, hNP cells (Figure 4.1C), differentiated neurons (Figure 4.1D) and neurons differentiated with GDNF (Figure 4.1E) expressed RET at the cell membrane suggesting an active site for GDNF signaling.

Neither hNP cells nor hMSC (data not shown) expressed RET, EN1, TH, PITX3, DAT or VMAT2 at the protein level. However, hNP cells did express NURR1, a transcription factor

necessary for regulating TH, DAT and VMAT2 expression in dopaminergic neurons, as did GDNF differentiation cultures and cultures with differentiation medium only through day 21 (Figure 4.2A, B). While hNP cells did not express EN1 (Figure 4.2D), differentiation of hNP cells with GDNF induced expression of EN1 at day 3 through 21 (Figure 4.2E). Flow cytometry further confirmed immunocytochemistry results, the hNP cells and differentiated neurons with GDNF were NURR1+ (Figure 4.2G, H) and differentiated neurons with GDNF were EN1+ (Figure 4.2I, J). The expression of NURR1 increased significantly ($p<.05$) at day 7 with GDNF (68.0 ±4.0) compared with neurons differentiated without GDNF (46.0 ± 2.0; Figure 4.2C). As previously reported, NURR1 and EN1 were localized to the perinuclear space [(Figure 4.2B, E) [36,37]]. Also at day 7, flow cytometry indicated that there was a significant ($p<.05$) increase in EN1 expression with GDNF exposure (36.3 ± 4.0) compared to cells differentiated without GDNF (21.4 ± 5.7; Figure 4.2F). These data indicated the presence of early induced and specified NURR1 stage cells in the hNP cell population that increased significantly ($p<.05$) when GDNF was present (62.9 ±6.0; Figure 4.2C) and the EN1+ population was increased at day 21 with GDNF (74.0 ± 1.0) compared with neurons differentiated without GDNF (37.7 ± 5.7; Figure 4.2F).

*hNP cells differentiate toward dopaminergic progenitors*

hNP cells differentiated with GDNF for 21 days expressed TH (Figure 4.3A, B), the rate limiting enzyme for dopamine synthesis, and PITX3 (Figure 4.3D, E), a transcription factor expressed only in substantia nigra dopaminergic neurons. Similar to previous studies, TH expression was in the cytoplasm (Figure 4.3B), while PITX3 expression was in the nucleus (Figure 4.3E; [37,38]).Flow cytometry further confirmed the immunocytochemistry results (Figure 4.3G-J). The percentage of cells expressing PITX3 was significantly higher ($p<.05$) at day 14 (28.4 ± 4.6) and 21 (65.7 ± 2.0) with GDNF when compared to the percentage of PITX3+ cells (0%) in groups without GDNF at either time point (Figure 4.3C). At day 21, there was a

significant increase in the percentage of TH expressing cells in the GDNF cultures (51.0± 2.0) relative to cells differentiated without GDNF (2.9 ± 0.1; Figure 4.3F).

*Mature dopaminergic neurons express functional markers*

hNP cells differentiated with GDNF for 21 days expressed the dopamine transporter (DAT; Figure 4.4A, B) and vesicular monoamine transporter 2 (VMAT2; Figure 4.4D, E). Similar to a previous report, DAT and VMAT2 was localized to the cytoplasm (Figure 4.4B,E; [37]. Flow cytometry confirmed immunocytochemistry results (Figure 4.4G-J). DAT expression significantly increased ($p<.05$) at day 14 in the treated cells (16.5 ± 3.1) compared with neurons differentiated without GDNF (5.4 ± 1.7; Figure 4.4C). At day 14, there was a significant ($p<.05$) increase in VMAT2 expression at day 14 with or without GDNF exposure (58.0 ± 25.2; 61.8 ± 9.5; Figure 4.4F).

*Differentiated hNP cells release dopamine*

We examined whether the differentiated hNP cells expressed members of the dopamine receptor 1 (D1) family, which consists of D1 and dopamine receptor 5 (D5), and of the dopamine receptor 2 (D2) family, which consists of D2, dopamine receptor 3 (D3), and dopamine receptor 4 (D4). Receptors in the D1 family are associated with neural development; whereas, those receptors in the D2 family are more closely deregulated in neurodegenerative diseases and schizophrenia [39]. hNP cells, differentiated neurons and neurons differentiated with GDNF expressed the D1, D4, and D5 mRNA in all three cell types, while D2 and D3 mRNA expression was not seen in any cell type (Figure 4.5A).

PCR for markers of other neural cell types showed no expression for G protein-coupled inwardly rectifying potassium channel (GIRK) and tryptophan hydroxylase 1 (TPH1) with expression in differentiated neurons for CHAT. Neurons differentiated with GDNF also expressed choline acetyltransferase (CHAT), glutamate dehydroxylase (GAD), phenylethanolamine-N-methyl transferase (PMNT) and dopamine beta hydroxylase (DBH). DBH was expressed in hNP cells, differentiated neurons and neurons differentiated in the presence

of GDNF (Supplementary Figure 4.1). To confirm that the cells that were reactive for TH were not also reactive for DBH, immunocytochemistry was performed on hNP cells differentiated for 14 days with GDNF. Separate populations, one which expressed DBH only, one which expressed TH only, and one which expressed DBH and TH, were found within the differentiated cultures (Supplementary Figure 4.1).

To determine if the dopamine-like neurons were active, dopamine release was evoked with 56 mM KCl for 30 minutes and measured with HPLC. Dopamine-like neurons showed a significant ($p<.05$) increase in dopamine release (0.98ng/ml ± 0.05) when differentiated with GDNF compared to hNP cells (0.96ng/ml ± 0.02; Figure 4.5B). In addition, neurons differentiated with GDNF had a significant ($p<.05$) increase of L-dopa (0.18ng/ml ± 0.05) compared with differentiation without GDNF (0.04ng/ml ± 0.02; Figure 4.5C).

**Discussion**

Pluripotent hESCs differentiated toward a dopaminergic phenotype offer a potential source of cells to study PD in vitro, for developing PD specific cell based assays for drug discovery and eventually a cell source for therapy [27,40]. The goal of this study was to examine the progressive in vitro differentiation of hESC derived propagated hNP cells to a dopaminergic fate and to determine whether hNP cells were responsive to a one-step dopaminergic differentiation process using LIF alone or in combination with GDNF. Here we demonstrate for the first time a method of deriving dopaminergic neurons from a starting hNP cell population without the use of feeder cells. The resulting population produced up to 50.5% TH+ cells when GDNF was added to the differentiation culture containing LIF, and corresponded with an increase in PITX3 expression. This population of TH and PITX3 positive cells expressed the dopamine receptors D1, D4 and D5 and released dopamine as measured by HPLC in comparison with the levels seen by other groups [25]. Previously, hESCs have been differentiated to dopaminergic neurons using the five-stage method and the SDIA method. hESCs are co-cultured with stromal cells, sonic hedgehog (SHH) and fibroblast growth factor 8

(FGF8) to induce neural differentiation. Removal of SHH and FGF8 and extended culture with brain derived neurotrophic factor (BDNF) and ascorbic acid (AA) produced 60-70% tyrosine hydroxylase (TH) positive Tuj positive cells [27]. Other studies use alternative growth factor cocktails without stromal cell co-culture methods. Following neural differentiation with SHH and FGF8, GDNF, BDNF, AA and cyclic AMP (cAMP) were utilized to obtain 31% TH positive neurons after 5 weeks of differentiation [41]. The five-stage method has proven to be less efficient than the SDIA method. However, the SDIA method involves the use of contaminating animal feeder layers, preventing their eventual movement to clinical trials. Cho and coworkers obtained 86% TH+ neurons from a starting hESC population, and unlike the present study, required extended cultures incorporating a neurosphere stage with the resulting neurons not expressing PITX3, a protein exclusively found in substantia nigra dopaminergic cells [26]. Buytaert-Hoefen and coworkers also used GDNF to obtain TH+ neurons; however, they utilized a co-culture system as well as only examined TH and not PITX3 [42]. In the present study, the hNP cells progressed through the dopaminergic specification stage marked by co-expression of NURR1 and EN1. Unique to this study, we started with hESC derived hNP cells expressing NURR1 and were POU5F1 negative [33,43] suggesting that these hNP cells are potential pre-dopaminergic progenitors and that the administration of GDNF helps to promote the expression of midbrain dopaminergic neuron proteins. Further differentiation in the presence of GDNF and LIF led to the dopamine progenitors expressing TH and PITX3 confirming these differentiated hNPs as committed dopaminergic progenitors. These progenitors became phenotypically mature dopaminergic neurons (DAT and VMAT2 positive). The expression of mRNA for CHAT, PMNT, DBH and GAD is expected due to the mixed population seen in the GDNF differentiated group. The combination of factors which confirm the dopaminergic phenotype, in addition to the distinctive DBH+, TH+, and DBH/TH+ populations shown by immunocytochemisty expression, indicates that there is a subpopulation of dopaminergic neurons in the GDNF differentiated group. This study suggests that continually cultured populations of hNP cell can differentiate to

dopaminergic-like fate. These cells express traits similar to their in vivo counterparts and GDNF enhances the in vitro process potentially through hNP cells, which were primed toward the posterior neural fate, to become dopaminergic neurons. We hypothesize that under these conditions, caudalization was likely induced through bFGF and LIF effects on propagated hNP cells. The removal of bFGF and the addition of GDNF to the LIF containing differentiation medium induced the hNP cells to a dopaminergic phenotype.

In this study, all hNP cells were exposed to bFGF and LIF during hNP cell propagation. Previously, bFGF increased the expression of HOX genes in hESCs, suggesting that bFGF can caudalize the neural cells differentiated from the hESCs [44]. Our use of bFGF prior to dopaminergic cell differentiation may have had a caudalizing priming effect on hNP cells. In addition, others established NP cells using epidermal growth factor (EGF) and bFGF but not LIF [45,46]. Since our hNP cells were established in an adherent monolayer continuous culture with LIF and bFGF and without EGF, these conditions potentially led to a population of hNP cells that were primed for differentiation toward a dopaminergic fate. We found that under these conditions hNP cells expressed NURR1 potentially eliminating the need to induce a dopaminergic specification in these NP cell cultures.

Similar to our findings with hNP cells, when LIF was used in addition to GDNF to differentiate mESCs, increased TH expression was observed, suggesting a role for LIF and GDNF in differentiation and neuroprotection of dopaminergic neurons [47]. Murine substantia nigra derived NP cells exposed to LIF were protected from 6-OHDA damage, which selectively affected the cells of the dopaminergic system [48]. In addition, rat primary cervical ganglia exposed to LIF have decreased dopamine beta-hydroxylase (DβH) expression through LIF's suppression of the noradrenergic properties of neural cells [49,50]. LIF used in combination with GDNF in rat fetal mesencephalic neural progenitor cells (NPCs) increased differentiation towards dopaminergic neurons as shown by increased TH expression in the cultures [48,51]. Therefore, we confirmed and added to these previous studies using a human ESC derived

source (hNP cells) by demonstrating that LIF and GDNF together have a synergistic and potentially additive effect on differentiation and survival of dopaminergic cells in vitro.

This is the first report of a one-step process for dopaminergic derivation from a primed source of proliferative hNP cells. This work differed from previous dopaminergic differentiation studies by utilizing GDNF's effect on primed hNP cells that express the GDNF receptor RET without the addition of dopaminergic induction factors or feeder cells. Previous work in hESCs [27,52] first induced early neural differentiation and then used the midbrain/hindbrain organizing factors SHH and fibroblast growth factor 8 (FGF8). These two factors were involved in organizing the borders of midbrain dopaminergic development [53,54]. Previously, hESCs have been differentiated to dopaminergic cells using GDNF and other factors such as brain cell derived neurotrophic factor (BDNF) or as a co-culture with SDIA using lengthy intermediary stages including neurosphere formation and did not directly examine the effects of GDNF in these cultures. [42]. In contrast, the hNP cells used in this study were predetermined to a neural lineage based on previous work showing expression of neural markers and absence of pluripotent markers including POU5F1 [33,43]. The hNP cells were NURR1 positive suggesting that they were primed to become dopaminergic neurons. The most effective dopaminergic enhancement occurred when LIF and GDNF were used on these LIF and bFGF primed hNP cells: LIF tended to repress the noradrenergic fate in the mouse and bFGF induced caudalization [49,53,55]. The hNP cells express the RET receptor; whereas, hESCs or the hMSCs do not. Expression of the RET receptor suggests an active binding site for GDNF with its co-receptor GFRα1. The use of bFGF in propagation and LIF and GDNF in differentiation leading to an increase in TH and PITX3 positive neurons suggests that the hNPs used in this study are primed for dopaminergic differentiation. This population of proliferative primed adherent hNP cells provides a novel cell source for study of dopaminergic differentiation and PD drug development as well as indicates that LIF and GDNF are effective growth factors involved in the in vitro differentiation of hNP cells towards a dopaminergic fate.

**Acknowledgements**

This work was supported by the GRA Eminent Endowment and DOD #N000140810989. We thank Julie Nelson and the Center for Tropical and Emerging Global Diseases Flow Cytometry Facility for her technical expertise. Thanks also go to Kimberly Freeman for her help with the HPLC.

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**Figure 4.1** – Dopaminergic differentiation in vivo and in vitro

In vitro development shown in this paper differs from in vivo mouse development in earlier expression of Nurr1 (A). In vitro cells progress from hNP cells to a neural induction stage to a dopaminergic specification stage to mature dopaminergic neurons (A). While meschynchemal stem cells (B) do not express the RET receptor for GDNF, hNP cells (C), differentiated neurons (D), and dopamine progenitors (E) show RET protein reactivivty as a active site for GDNF based on immunofluorescence shown here in red with DAPI in blue. TH – tyrosine hydroxylase; EN1 – engrailed1; SHH – sonic hedgehog; FGF8 – fibroblast growth factor 8; RET – REarranged in Transfection; GDNF – glial cell-line derived neurotropic factor; Pitx3 - paired-like homeodomain transcription factor 3; Nurr1 - Nuclear receptor related 1; TGF - Transforming growth factor; Tuj – Beta III Tubulin; Scale bars 10 μM.

A

## In Vitro Developmental Timeline

| Stage | Markers | Reference |
|---|---|---|
| Neural Progenitor Cell | Nestin | Sonntag, et al., 2007 |
| Neural Progenitor Induction Stage | Tuj Nurr1 | Smidt,et al., 2003; Prakash & Wurst, 2006; von Bohlen und Halbach, 2007 |
| Dopamine Specification Stage | Nurr1 EN1 | Alavian, et al., 2008; Smidt,et al., 2003 |
| Dopamine Progenitor Stage | TH Pitx3 EN1Nurr1 | Smidt,et al., 2003; Prakash & Wurst, 2006 |
| Mature Dopamine Neuron | VMAT2 DAT TH Pitx3 EN1 Nurr1 | Meng, et al., 1999; Hansson, et al., 1998 |

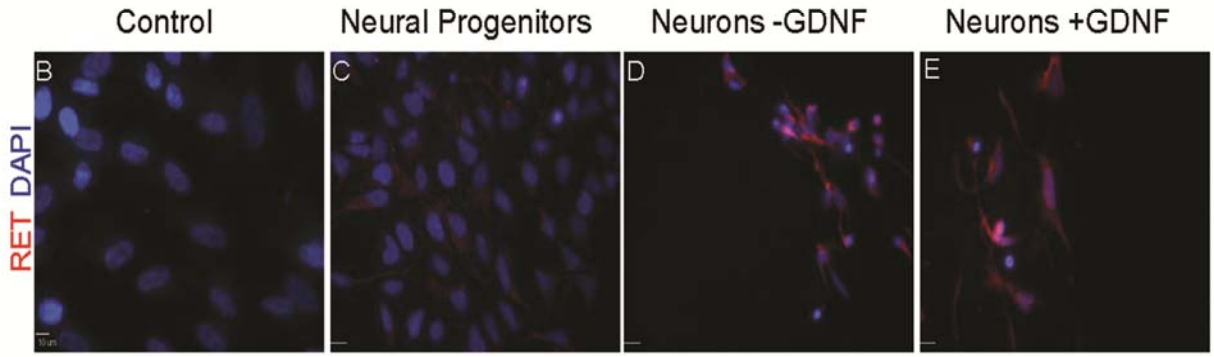


**Figure 4.2** – hNP cells can be induced and specified

hNP cells express NURR1 (A), shown here in red with DAPI in blue, and continue to express NURR1 through 21 days of differentiation with GDNF (B). Immunocytochemistry demonstrates no EN1 immunoreactivity in hNP cells (D); however, EN1 protein immunoreactivity with 21 days of differentiation with GDNF (E) shown here in green with DAPI in blue. The NURR1 and EN1 expression becomes significantly different at day 7 with GDNF differentiation (C, F) suggesting a progression to the dopamine progenitor stage. Flow cytometry analysis demonstrates a population of cells positive for Nurr1 and EN1 at day 14 with GDNF differentiation (G-J). Nurr1 - Nuclear receptor related 1; EN1 – engrailed1; GDNF – glial cell-line derived neurotropic factor; Scale bars 10 μM; # significantly different from differentiated neurons without GDNF; * significantly different from day 0; + significantly different from day 3; † significantly different from day 7.

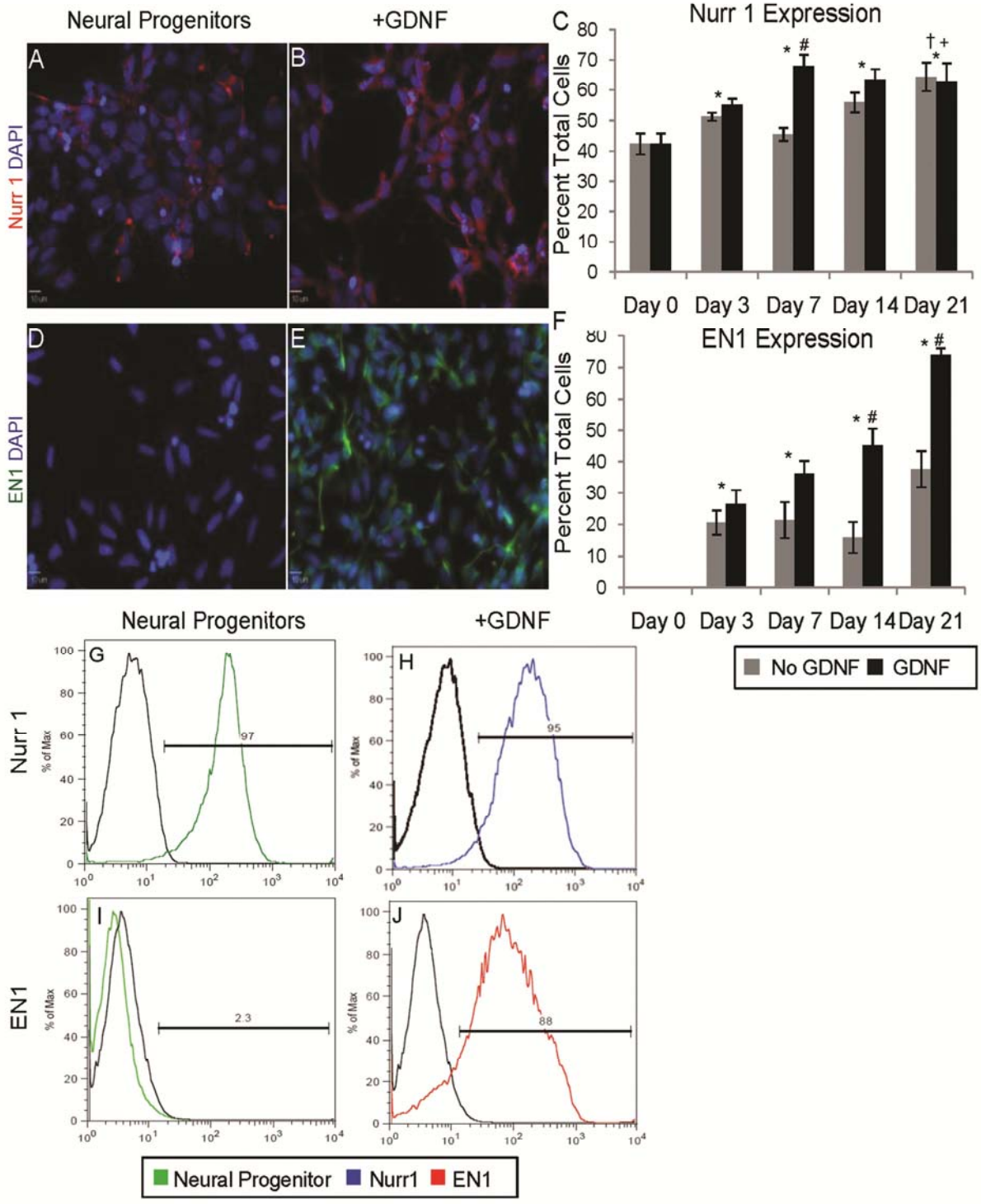

Neural Progenitors
+GDNF
A
B
Nurr 1 DAPI
C
Nurr 1 Expression
Percent Total Cells
Day 0
Day 3
Day 7
Day 14
Day 21
D
E
EN1 DAPI
F
EN1 Expression
Percent Total Cells
Day 0
Day 3
Day 7
Day 14
Day 21
No GDNF
GDNF
Neural Progenitors
+GDNF
Nurr 1
G
H
% of Max
97
95
EN1
I
J
% of Max
2.3
88
Neural Progenitor
Nurr1
EN1

**Figure 4.3** – hNP cells can be differentiated to dopaminergic progenitors

Differentiation of hNP cells with GDNF demonstrates TH immunoreactivity shown in red with DAPI in blue and Tuj in green(B) and PITX3 shown in red with DAPI in blue and Tuj in green (E). After 14 days of GDNF differentiation, PITX3 protein immunoreactivity increases significantly demonstrating a progression to a dopamine progenitor stage (F). This is further confirmed by the significant reactivity of TH at day 21 with GDNF differentiation (C). Flow cytometry demonstrates a population of TH and Pitx3 positive neurons at day 21 with GDNF differentiation (G-J). TH – tyrosine hydroxylase; Pitx3 - paired-like homeodomain transcription factor 3; GDNF – glial cell-line derived neurotropic factor; Scale bars 10 μM; # significantly different from differentiated neurons without GDNF; * significantly different from day 0; † significantly different from day 7.

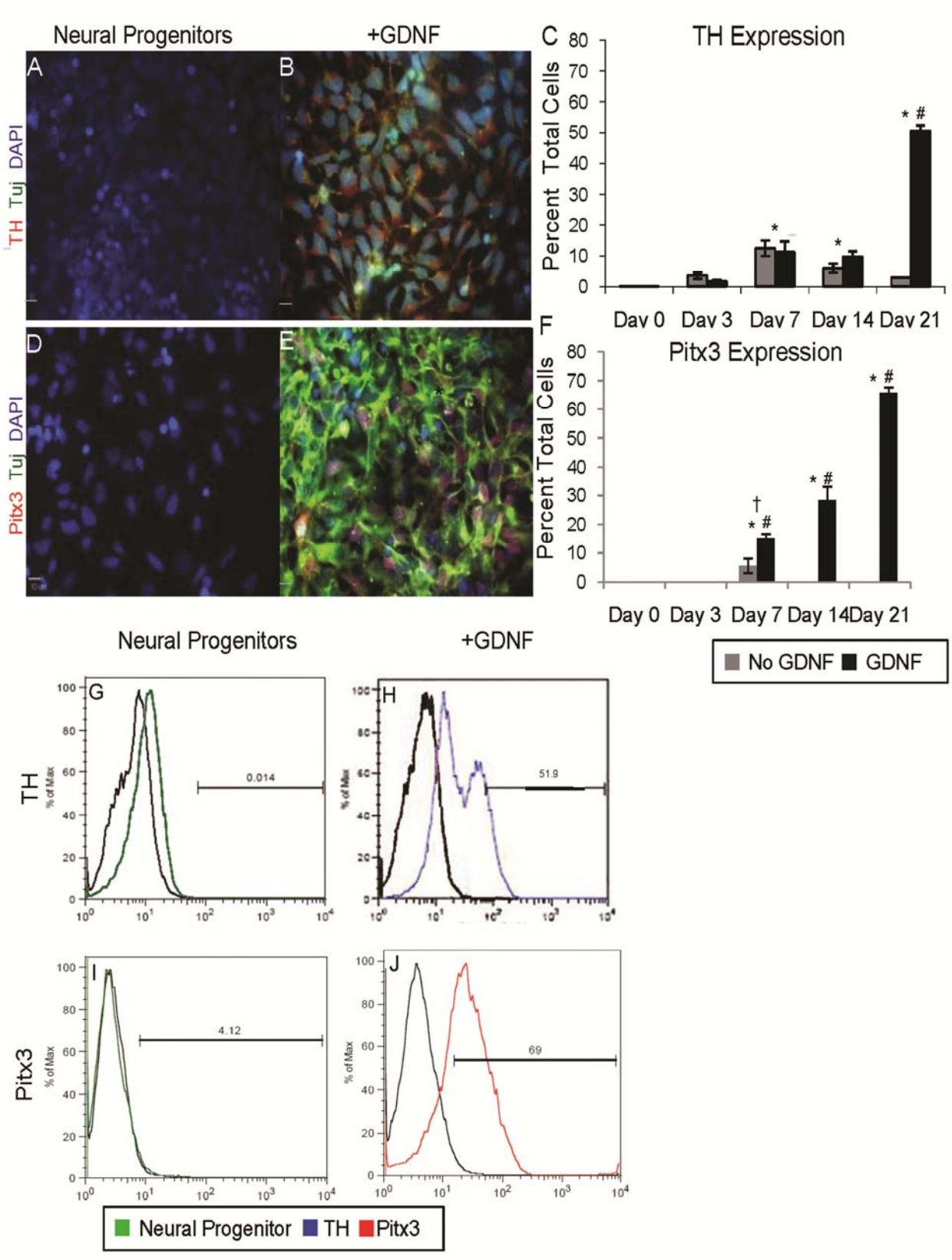

Neural Progenitors
+GDNF
A
B
C
TH Expression
Percent Total Cells
Dav 0
Dav 3
Dav 7
Dav 14
Dav 21
TH Tuj DAPI
D
E
F
Pitx3 Expression
Pitx3 Tuj DAPI
Day 0
Day 3
Day 7
Day 14
Day 21
No GDNF
GDNF
Neural Progenitors
+GDNF
G
H
TH
% of Max
0.014
51.9
I
J
Pitx3
4.12
69
Neural Progenitor
TH
Pitx3

**Figure 4.4** – Mature dopaminergic neurons express functional markers

Dopamine progenitors progress to mature dopaminergic neurons as immunocytochemistry for DAT shown in green with DAPI in blue (B) and VMAT2 shown in pink with DAPI in blue (E) demonstrates at day 21 of GDNF differentiation compared to the lack of immunoreactivity in hNP cells for DAT (A) and VMAT2 (D). VMAT2 immunoreactivity (F) increases significantly at day 21 with or without GDNF and DAT immunoreactivity (C) increases significantly with GDNF at day 14. Flow cytometry demonstrates a population of cells that express VMAT2 at day 21 (I, J) and DAT at day 21(G, H). DAT – dopamine transporter; VMAT2 – vesicular monoamine transporter 2; GDNF – glial cell-line derived neurotropic factor; Scale bars 10 μM; # significantly different from differentiated neurons without GDNF; * significantly different from day 0; + significantly different from day 3; † significantly different from day 7; ++ significantly different from day 14.

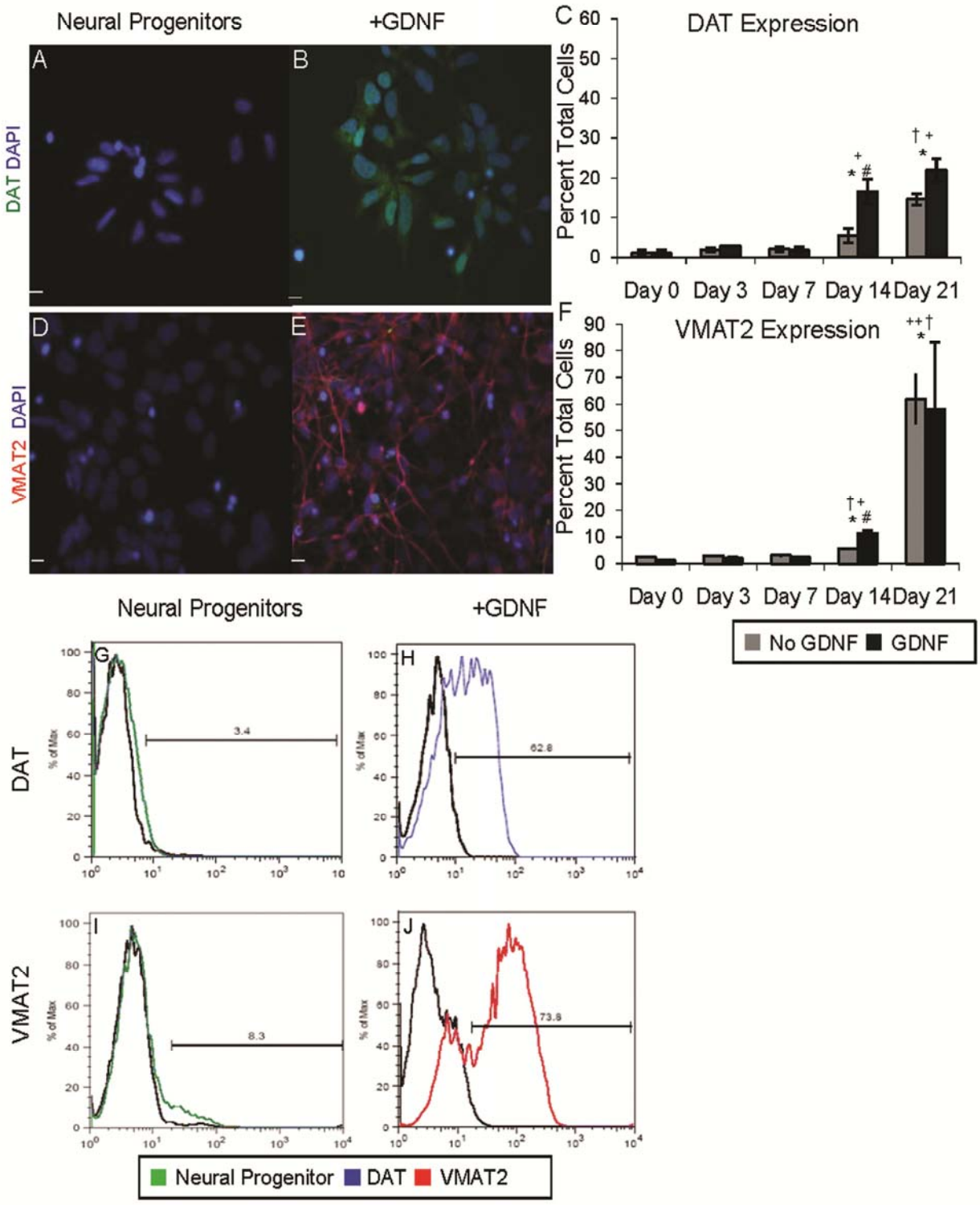
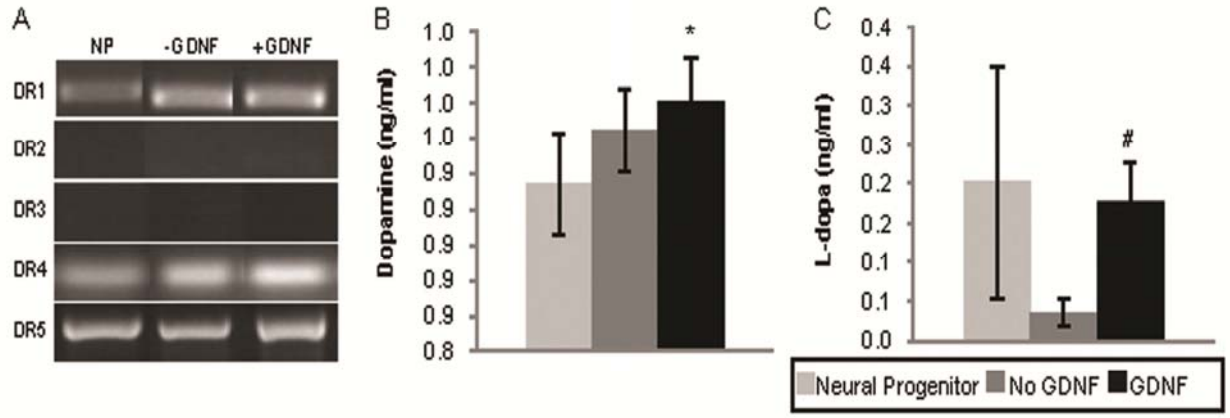

Neural Progenitors
+GDNF
A
B
DAT DAPI
C
DAT Expression
Percent Total Cells
Day 0 Day 3 Day 7 Day 14 Day 21
D
E
VMAT2 DAPI
F
VMAT2 Expression
Percent Total Cells
Day 0 Day 3 Day 7 Day 14 Day 21
No GDNF GDNF
Neural Progenitors
+GDNF
DAT
G
H
3.4
62.8
% of Max
VMAT2
I
J
8.3
73.8
% of Max
Neural Progenitor DAT VMAT2

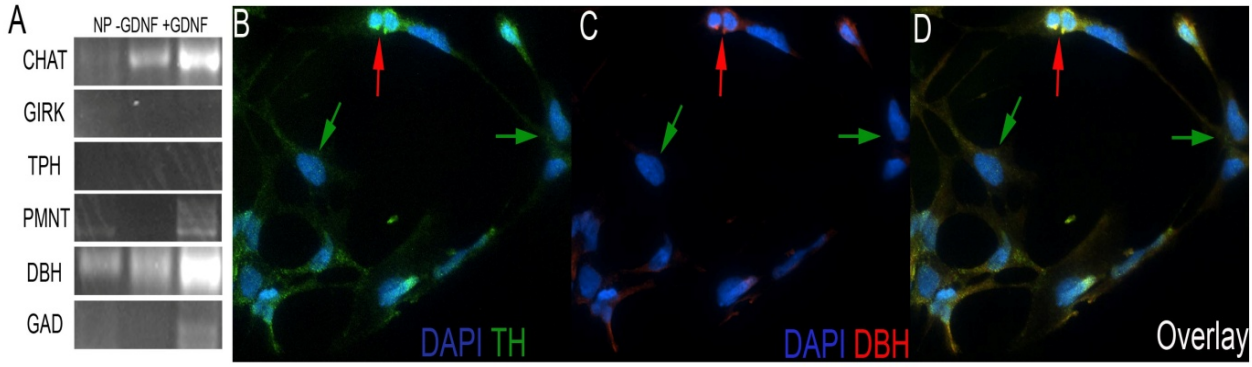
**Figure 4.5** – Evoked differentiated hNP cells release dopamine

PCR for the 5 dopamine receptors (A) demonstrates expression of the D1, D4, and D5 receptors inhNP cells, differentiated neurons, and neurons differentiated with GDNF. HPLC demonstrates increased expression of dopamine (B) and L-dopa (C) at day 21 with GDNF exposure . DR1 – dopamine receptor 1; DR2 – dopamine receptor 2; DR3 – dopamine receptor 3; DR4 – dopamine receptor 4; DR5 – dopamine receptor 5; GDNF – glial cell-line derived neurotropic factor; Scale bars 10 μM; * significantly different from hNP cells; # significantly different from neurons differentiated without GDNF.


A
NP
-GDNF
+GDNF
DR1
DR2
DR3
DR4
DR5
B
Dopamine (ng/ml)
1.0
1.0
1.0
0.9
0.9
0.9
0.9
0.9
0.8
*
C
L-dopa (ng/ml)
0.4
0.4
0.3
0.3
0.2
0.2
0.1
0.1
0.0
#
Neural Progenitor
No GDNF
GDNF

**Supplementary Figure 4.1** – Neurons differentiated with GDNF express distinct neural subtype populations

CHAT, a marker for cholinergic neurons, gene expression was seen in neurons differentiated with or without GDNF for 21 days (A). Neurons differentiated with GDNF for 21 days also expressed PMNT, a marker for adrenergic neurons (A), DBH, a marker for noradrenergic neurons, (A) and GAD, a marker for gluatmatergic neurons (A). DBH was also expressed in hNPs as well as in differentiated neurons without GDNF for 21 days (E). Markers for gabaergic neurons (GIRK, A) and serotenergic neurons (TPH, A) were not seen in any cell type. Immunocytochemistry for DBH and TH in neurons differentiated for 14 days with GDNF demonstrate a subpopulation that express TH only (B, C, D green arrows) and a subpopulation that express DBH and TH (B, C, D red arrows). CHAT - choline acetyltransferase; GIRK - G protein-coupled inwardly rectifying potassium channel; TPH - tryptophan hydroxylase 1; PMNT - phenylethanolamine-N-methyl transferase; GAD - glutamate dehydroxylase; GDNF - glial cell-line derived neurotropic factor; DBH – dopamine beta hydroxylase; TH – tyrosine hydroxylase; GDNF – glial cell-line derived neurotrophic factor. Scale bars 10μM.


A
NP -GDNF +GDNF
CHAT
GIRK
TPH
PMNT
DBH
GAD
B
DAPI TH
C
DAPI DBH
D
Overlay

# CHAPTER 5

## GLIAL CELL-LINE DERIVED NEUROTROPIC FACTOR: ITS ROLE IN DOPAMINERGIC DEVELOPMENT IN HUMAN EMBYRONIC STEM CELL DERIVED NEURAL PROGENITORS[1]

[1]Young, Amber, West, Franklin D., Jordan, Erin, Stice, Steven L. To be submitted to *BMC Developmental Biology*.

**Abstract**

Glial cell-line derived neurotrophic factor (GDNF) was discovered in 1993 for its use in protecting dopaminergic neurons. Since its discovery, GDNF has been used as both a neuroprotectant in dopaminergic neurons and a recovery factor for dopaminergic neurons in Parkinson's disease mouse, rat and non-human primate models. Additionally, clinical trials testing GDNF in Parkinson's patients have improved quality of life scores; however, the side effects and difficulty in administration has lead to the halting of these trials. The usefulness of GDNF as a neuroprotectant for dopaminergic neurons has lead to its use in differentiating dopaminergic-like neurons from human embryonic stem cell derived neural progenitors with ~50% TH and PITX3+ neurons previously derived from human neural progenitors after 21 days of differentiation with GDNF in differentiation media. Understanding the mechanisms through which this differentiation occurs could greatly improve the efficacy of differentiation and lead to RT-PCR was used to analyze genetic changes as well as apoptosis and proliferation studies to analyze functional changes in the MAPK and PI3K pathway when human neural progenitors were differentiated with GDNF compared to without GDNF. Up regulation was seen in the ERK and p38 MAPK pathways as well as the mTOR pathway with GDNF, while no change was seen in the RAC1 and GSK3$\beta$ pathway suggesting the ERK, p38 and mTOR pathways are involved in GDNF differentiation of dopaminergic-like neurons. These results provide evidence towards mechanisms that can be used to improve the yield of dopaminergic-like neurons obtained from human neural progenitors as well as mechanisms that could potentially improve the treatments for Parkinson's disease.

## Introduction

Glial cell-line derived neurotrophic factor (GDNF) was discovered in 1993 for its role in promoting dopamine uptake into midbrain dopaminergic neurons [1]. The potential of GDNF as a treatment for Parkinson's disease (PD) was evident due to the link between the motor symptoms in PD being caused by the degeneration of the dopaminergic neurons in the substantia nigra (SN) [2]. At the time of GDNF's discovery, current PD treatment options did not protect the remaining dopaminergic neurons from degenerating leading to progressive increase in symptoms and further decline in the quality of life [3]. Since its discovery, GDNF has been tested in vitro and in vivo as a treatment for PD without having reached a definitive treatment option. This is due in part to lack of understanding as to how GDNF protects dopamine neurons. GDNF was first tested for recovery of dopaminergic neurons in PD rat models with success demonstrated by increased TH expression and behavioral recovery [4]. From there, GDNF was used as a preventative measure in rats [5] and tested in non-human primates with positive results [6]. This lead to human trials which failed due to side effects, lack of long term recovery, and difficulty with route of administration [7,8].

Human neural progenitors (hNPs) derived from human embryonic stem cells (hESCs) provide a suitable model system for studying the pathway through which dopaminergic neurons can be protected or differentiated with GDNF. Our lab has previously derived hNPs from hESCs that were maintained in a stable, adherent monolayer culture system [9]. These hNPs remain continually proliferative for many passages and maintain a stable karotype in addition to being able to differentiate into the three main types of cells found in the nervous system, neurons, oligodendrites and astrocytes [9]. Our lab has successfully enriched a population of the hNPs to become dopaminergic neurons with the addition of GDNF to the differentiation media. Currently, approximately 50% of the differentiated neurons were tyrosine hydroxylase (TH) positive [10]. Understanding the mechanism through which GDNF enhances the differentiation of the hNPs to dopaminergic neurons would allow for elucidating a potential mechanism through which to

increase the percentage of dopaminergic neurons obtained. Additionally, it would allow for an understanding of the basic science behind GDNF's mechanism of action for its protection of midbrain dopaminergic neurons allowing for research in potential mechanisms for designing future treatments for PD.

Currently, the actions of Src protein tyrosine kinases and the c-Jun N-terminal kinase (JNK) pathways in GDNF's neuroprotective role in midbrain dopamine neurons have been elucidated while the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase **(**PI3K) pathways have been implicated in GDNF's role but not fully understood. Src has known roles in cell growth, differentiation and survival [11]. When GDNF binds to its co-receptor GDNF family receptor alpha 1 (GFR$\alpha 1$), it activates rearranged in transcription (RET) receptors which are brought to the cellular membrane with the assistance of Src [12]. When GDNF is present and active, Src causes the up regulation of RET receptors to lipid rafts allowing for increased binding of the GDNF-GFR$\alpha 1$ complex to the RET receptor [13,14]. The JNK pathway is a subfamily of the MAPK pathway. When GDNF and its co-receptor GFRα1 bind to the RET receptor, JNK modulates dopaminergic neurite outgrowth as well as initiating a delay at G2/M to allow for actin reorganization within the neuron [15,16].

The mechanisms through which the MAPK/ERK and PI3K pathway promote dopaminergic neuronal survival and differentiation are elusive. Rat cortical cells have shown that GDNF increases neurite outgrowth through the MAPK/ERK pathway [17]. In the rat dopaminergic cell line MD90, inhibiting the PI3K pathway prevents GDNF from protecting the dopaminergic neurons from 6-hydroxydopamine induced death [18].

The objectives of this study were to establish the involvement of the MAPK and PI3K pathway in dopaminergic enrichment of the hNPs as previously reported and to evaluate the mechanisms through which this enrichment occurred. Inhibiting both the MAPK and the PI3K pathway prevented the establishment of dopaminergic neurons in the differentiated hNP

population when differentiated with GDNF. Additionally, *Rac* genes involved in neurite extension were up regulated in dopaminergic-like neurons relative to differentiated hNPs. Inhibition of the MEK, ERK and p38 affected the dopaminergic-like neurons while the GSK3β pathway did not affect the dopaminergic-like neurons. The data in this study provides insight into the changes that occur when GDNF enhances dopaminergic-like neuron differentiation.

**Materials and Methods**

*hESC Cultures*

WA09 (H9) hESCs were cultured on mouse embryonic fibroblast (Harlan) feeders inactivated by mitomycin C (Sigma-Aldrich) in 20% knockout serum replacement media consisting of Dulbecco's modified Eagle medium/F12 medium (Gibco) supplemented with 20% knockout serum replacement, 2mM L-glutamine, 0.1 mM non-essential amino acids, 50 units/ml penicillin/50µ/ml streptomycin (Invitrogen, Carlsbad, CA), 0.1mM β-mercaptoethanol (Sigma-Aldrich) and 4ng/ml basic fibroblast growth factor (bFGF ; R&D). They were maintained in 5% CO2 and at 37°C. Cells were passaged every 3 days by mechanical dissociation, re-plated on fresh feeders to prevent undirected differentiation with daily media changes as previously described [19].

*hNP Cultures*

Human neural progenitor (hNP) cells were derived from hESC line H9 by our lab as previously described [9]. Briefly, after one week of culture on mouse embryonic fibroblast layers, H9 hESCs were grown with derivation medium containing Dulbecco's modified Eagle medium /F12 medium (Gibco) supplemented with 2mM L-glutamine (Gibco), 2 U/mL penicillin (Gibco), 2µg/mL streptomycin (Gibco), N2 (Gibco), and 4 ng/ml bFGF (R&D) for 7 days. Rosettes were selected with hook passaging from culture dishes and re-plated on polyornithine (Sigma-Aldrich) and laminin (Sigma-Aldrich) coated dishes. These rosettes were propagated for 3 days on polyornithine and laminin coated dishes in neurobasal medium (Gibco) supplemented with 2mM L-glutamine, 2 U/mL penicillin, 2 µg/mL streptomycin, 1X B27 (Gibco), 20 ng/mL bFGF, and

10ng/mL leukemia inhibitory factor (Millipore). Media were changed every other day and cells were passaged every fourth day or as needed. Cells used for this experiment were passage 22-32.

*hNP Differentiation*

hNP cells were grown on polyornithine/laminin coated 100mm plates in growth medium consisting of neural basal medium, 1X penicillin/streptomycin, 2mM L-glutamate, 1X B27, 10ng/mL LIF and 20ng/mL bFGF. After 24 hours, the media were changed to neural differentiation media, which consisted of growth medium without bFGF. Media were changed every three days. Cells were collected at 21 days post differentiation for analysis.

*hNP Dopaminergic Differentiation*

hNPs were differentiated towards dopaminergic-like neurons as described previously. Briefly, hNP cells were grown on polyornithine/laminin coated 35mm plates (Falcon) for flow cytometry or on polyornithine/laminin coated 4 well slides (Falcon) for immunocytochemistry staining. Cells were plated in growth medium consisting of neural basal medium, 1X penicillin/streptomycin, 2mM L-glutamate, 1X B27, 10ng/mL LIF and 20ng/mL bFGF. After 24 hours, the media were changed to neural differentiation media, which consisted of growth medium without bFGF or neural differentiation medium plus 25ng/ml GDNF (Neuromics). Media were changed every three days. Cells were harvested at day 21 for further analysis.

*Immunocytochemistry and Cell Quantification*

hNPs were differentiated towards dopaminergic-like neurons as described above. In parallel, hNPs were differentiated towards dopaminergic-like neurons as described above with inhibitors to GDNF (GDNF antibody, R&D; 1ng/ml, 10ng/ml, 100ng/ml), RET (SU-5416, Sigma-Aldrich; 1ng/ml, 10ng/ml, 100ng/ml) MAPK (PD98059, BioMol; 1ng/ml, 10ng/ml, 100ng/ml) and PI3K (LY-294002, BioMol; 1ng/ml, 10ng/ml, 100ng/ml) for 21 days. Cells were fixed with 2% paraformaldehyde (Electron Microscopy Sciences) in PBS with calcium and magnesium (PBS+/+) (Thermo Scientific) for 20 minutes and processed for immunocytochemistry. Cells

were washed in PBS with calcium and magnesium (PBS+/+) 3 times followed by 3 washes for 5 minutes each of permeabilization buffer consisting of 25µL (0.5%) Tween 20 (EMD Chemicals) in 50mL of high salt buffer. Cells were then blocked in 6% goat serum (JacksonImmuno) for 45 minutes. The following primary antibodies were used: mouse anti Tuj1 (1:200, Neuromics), chicken anti TH (1:100; all from Millipore, Inc). Reaction was revealed using AlexaFluor goat 488 or 633 secondary antibodies (1:1000, Molecular Probes). Cell nuclei were stained using DAPI (Invitrogen). Fluorescence was visualized using spinning disk confocal microscope (Olympus). Negative controls included secondary only staining. Cell counting was performed using Image Pro software (Media Cybernetics). Five random visual fields were selected and counted in triplicate. Data are presented as mean ±SD. Values of $p<.05$ was considered significant using ANOVA and Tukey's Pair-Wise test (Statistical Analysis Software, SAS Institute).

*Quantitative Polymerase Chain Reaction (qPCR)*

RNA was extracted using the Qiashredder and RNeasy kits (Qiagen) according to manufacturer's instructions. The RNA quality and quantity was verified using a RNA 600 Nano Assay (Agilent Technologies) and the Agilent 2100 Bioanalyzer. Total RNA (5 µg) was reverse-transcribed using the cDNA Archive Kit (Applied Biosystems Inc.) according to manufacturer's instructions. Reactions were initially incubated at 25$^{o}$C for 10 minutes and subsequently at 37$^{o}$C for 120 minutes. RT-PCR ($RT^2$ Profiler PCR Array, SABiosciences) assays were used for the MAPK pathway (Human MAP Kinase Signaling Pathway, SABiosciences) and PI3K pathway (Human PI3K-AKT Signaling Pathway, SABiosciences) to analyze the expression of 84 genes for each pathway. The cDNA samples were diluted in 91µL of $ddH_2O$. From the cDNA samples 120µL were mixed with 550µl of 2X $RT^2$ SYBR Green qPCR Master Mix (SABiosciences) and 448µL of $ddH_2O$, then loaded into respective channels on the microfluidic cards followed by centrifugation. The card was sealed and real-time PCR and relative quantification was carried out on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Inc). All failed

(undetermined) reactions were excluded and ΔCt values were calculated. For calculation of relative fold change values, the software provided with the assays on the SABioscience's website was used ($RT^2$ Profiler PCR Array Data Analysis, SABiosciences). All failed (undetermined) reactions were excluded and ΔCt values were calculated. For calculation of relative fold change values, initial normalization was achieved against endogenous 18S ribosomal RNA using the ΔΔCT method of quantification. Average fold change from three independent runs were calculated as $2^{\Delta\Delta CT}$. Significance was determined by running a 2-way ANOVA and Tukey's Pair-Wise (SAS) comparisons for each gene. Treatments where there was a fold change of greater than 4-fold were considered significant following manufacturer's recommendation.

*Cell Cycle Analysis*

H9s, hNPs, differentiated hNPs and dopaminergic-like neurons were analyzed for cell cycle effects using propidium iodide (Invitrogen). Differentiated hNPs and dopaminergic-like neurons were differentiated for 21 days before inhibitors for insulin-like growth factor 1 (IGF1; Tyrphostin AG-1024, Enzo Life Sciences; 1ng/ml), cyclin-dependent kinase 2 (CDK2; AG-494, Enzo Life Sciences; 1ng/ml), G1 (CI898, Tocris; 10ng/ml), G1 (Daidzein, Tocris; 10ng/ml), glycogen synthase kinase 3 beta (GSK3β; Indirubin, Tocris; 1ng/ml), Rac1 (NSC23766, Tocris; 10ng/ml), mammalian target of rapamycin (mTOR ;Rapamycin, EMD Biosciences; .3ng/ml), MAPK (PD98059, Enzo Life Sciences; 10ng/ml), ERK (PD035901, Cayman Chemicals; 10ng/ml), p38 (SB202190, Enzo Life Sciences; 10ng/ml) and Shc (Sclerotiorin, Cayman Chemicals; 10ng/ml) were added for 24 hours. H9s and hNPs were grown as described previously and then media were changed to media containing inhibitors as listed above for 24 hours before analysis. After 24 hours, cells were harvested and washed in PBS-/- before being fixed in cold 70% ethanol at 4°C for 30 minutes. Cells were then washed 2 times in PBS-/- before adding 200μL of 50μg/ml PI. Cells were quantified on Dako Cyan (Beckman Coulter). Negative controls were secondary only and cell only staining. Cell quantification was done using

FlowJo (TreeStar) software. Each experiment was run in triplicate. Data are presented as mean ±SD. Values of $p<.05$ was considered significant using ANOVA and Tukey's Pair-Wise (Statistical Analysis Software, SAS Institute). Each treatment was normalized to the non-treated control cells and background staining.

*Apoptosis Assay*

Differentiated hNPs and dopaminergic-like neurons were analyzed for apoptosis effects using Caspase Glo 3/7 assay (Promega). Differentiated hNPs and dopaminergic-like neurons were differentiated for 21 days before transfer to a 96 well plate and inhibitors for IGF1 (Tyrphostin AG-1024, Enzo Life Sciences; 1ng/ml), CDK2 (AG-494, Enzo Life Sciences; 1ng/ml), G1 (CI898, Tocris; 10ng/ml), G1 (Daidzein, Tocris; 10ng/ml), GSK3β (Indirubin, Tocris; 1ng/ml), Rac1 (NSC23766, Tocris; 10ng/ml), mTOR (Rapamycin, EMD Biosciences; .3ng/ml), MAPK (PD98059, Enzo Life Sciences; 10ng/ml), ERK (PD035901, Cayman Chemicals; 10ng/ml), p38 (SB202190, Enzo Life Sciences; 10ng/ml) and Shc (Sclerotiorin, Cayman Chemicals; 10ng/ml) were added for 24 hours.. H9s and hNPs were grown as described previously and then transferred to a 96 well plate and media were changed to media containing inhibitors as listed above for 24 hours before analysis. Caspase Glo 3/7 assay (Promega) was used following manufacturer's directions to analyze apoptosis. The buffer was added to the substrate and the substrate dissolved. 100µL of this mix was added to each well of the 96 well plate and incubated for 1 hour at RT protected from light. The plate was then analyzed on the Flexstation 3 (Molecular Devices). Data are presented as mean ±SD. Values of $p<.05$ was considered significant using ANOVA and Tukey's Pair-Wise (Statistical Analysis Software, SAS Institute). Each treatment was normalized to the non-treated control cells and background staining.

*Proliferation Assay*

Differentiated hNPs and dopaminergic-like neurons were analyzed for proliferation effects using Click-iT EdU High Content Screen kit (Invitrogen). Differentiated hNPs and

dopaminergic-like neurons were differentiated for 21 days before transfer to a 96 well plate and inhibitors for IGF1 (Tyrphostin AG-1024, Enzo Life Sciences; 1ng/ml), CDK2 (AG-494, Enzo Life Sciences; 1ng/ml), G1 (CI898, Tocris; 10ng/ml), G1 (Daidzein, Tocris; 10ng/ml), GSK3β (Indirubin, Tocris; 1ng/ml), Rac1 (NSC23766, Tocris; 10ng/ml), mTOR (Rapamycin, EMD Biosciences; .3ng/ml), MAPK (PD98059, Enzo Life Sciences; 10ng/ml), ERK (PD035901, Cayman Chemicals; 10ng/ml), p38 (SB202190, Enzo Life Sciences; 10ng/ml) and Shc (Sclerotiorin, Cayman Chemicals; 10ng/ml) were added for 24 hours.. H9s and hNPs were grown as described previously and then transferred to a 96 well plate and media were changed to media containing inhibitors as listed above for 24 hours before analysis. Click-iT EdU High Content Screen kit (Invitrogen) was used following manufacturer's instructions to analyze proliferation. EdU expression level was measured on the Flexstation 3 (Molecular Devices). Cell nuclei were stained using DAPI (Invitrogen). Fluorescence was visualized using spinning disk confocal microscope (Olympus). Negative controls included secondary only staining. Data are presented as mean ±SD. Values of $p<.05$ was considered significant using ANOVA and Tukey's Pair-Wise (Statistical Analysis Software, SAS Institute). Each treatment was normalized to the non-treated control cells and background staining.

**Results**

*Blocking GDNF and its Downstream Pathways Inhibits Dopaminergic Enhancement*

Differentiating hNPs in normal differentiation media produces 11% TH+ neurons. This process is enhanced by the addition of 25ng/ml GDNF to the differentiation media significantly ($p<.05$) increasing the number of TH+ neurons to 52%. An antibody to GDNF which has been shown to neutralize GDNF activity and prevent it from binding to the RET receptor decreased TH expression significantly ($p<.05$) to 19% at 1ng/ml, 3% at 10ng/ml (Figure 5.1A) and removed all expression at 100ng/ml after 21 days in culture (Figure 5.1A, B, C). SU-5416, a competitive inhibitor of the RET receptor, decreased TH expression significantly ($p<.05$) to 8% at 1ng/ml and killed all cells at dosages higher than that (Figure 5.1D, E, F). LY294002, a reversible

inhibitor of PI3K, only slightly decreased the TH expression to 41% with 1ng/ml of the compound added to the differentiation media; cells cultured with greater than that concentration did not survive (Figure 5.1G, H, I). PD-98059, an inhibitor which prevents mitogen-activated protein kinase kinase 1 (MEK1) from activating downstream pathways by binding to the receptor and preventing MEK1 activation, reduced TH expression significantly ($p<.05$) to 8% with 1ng/ml of inhibitor in the differentiation media. 10ng/ml of inhibitor reduced expression of TH completely while higher dosages killed the cells (Figure 5.1J, K, L). Following evidence supporting the inhibition of GDNF and its downstream signaling pathways inhibited dopaminergic enhancement, genes involved in the MAPK and PI3K pathway were analyzed by RT-PCR.

*Changes in MAPK Pathway Affect Dopaminergic Differentiation*

The MAPK pathway expression changes with the differentiation of differentiated hNPs to dopaminergic-like neurons. Specific to the changes in GDNF enhancement of dopaminergic neurons, dopaminergic-like neurons relative to differentiated hNPs up regulate significantly ($p>0.05$) *Creb1* (360 fold ± 3), *Mapk8* (5 fold ± 4), *Mapk13* (5 fold ± 2) and *Mef2c* (54 fold ± 3; Figure 5.2A).

Examination of the functional changes that occur because of inhibition of these pathways occurred through measurement of apoptosis and proliferation. Inhibition of MEK with PD98059 decreased apoptosis significantly ($p<0.05$) in dopamine-like neurons 1.1 fold relative to differentiated hNPs with inhibitor and decreased apoptosis 24 fold relative to dopamine-like neurons cultured without inhibitor (Figure 5.2B). Differentiated hNPs cultured with inhibitor decrease significantly ($p>0.05$) 9.1 fold relative to differentiated hNPs cultured without inhibitor in caspase levels (Figure 5.2B). Dopamine-like neurons decreased in proliferation 104.7 fold relative to differentiated hNPs and 2.7 fold to dopamine-like neurons without inhibitors when cultured with PD98059 (Figure 5.2B).

PD035901 inhibits ERK in the MAPK pathway. Addition of PD035901 to culture of dopamine-like neurons decreased apoptosis significantly ($p<0.05$) 1.2 fold relative to

differentiated hNPs with inhibitor and decreased apoptosis level significantly ($p>0.05$) 8.1 fold relative to dopamine-like neurons cultured without inhibitor (Figure 5.2C). Differentiated hNPs cultured with inhibitor decreased in apoptosis significantly ($p>0.05$) 8.8 fold relative to differentiated hNPs without inhibitor. Proliferation of dopamine-like neurons with inhibitor increased 16 fold relative to differentiated hNPs with inhibitor (Figure 5.2C).

Inhibiting the p38 pathway with SB202190 significantly ($p<0.05$) decreased apoptosis of dopamine-like neurons 17 fold compared to differentiated hNPs cultured with inhibitor while significantly ($p<0.05$) decreasing proliferation in dopamine-like neurons 3.6 fold relative to differentiated hNPs cultured with inhibitor (Figure 5.2D). Dopamine-like neuron apoptosis is decreased significantly ($p >0.05$) 6.1 fold when cultured with inhibitor relative to culture without inhibitor and proliferation is significantly ($p >0.05$) decreased 1.1 fold when cultured with inhibitor relative to culture without inhibitor (Figure 5.2D). The mechanism through which the MEK and ERK pathways are activated by GDNF is outlined in Figure 5.2E and the mechanism through which p38 is activated by GDNF is shown in Figure 5.2F.

Dopaminergic-like neuron e expression of *Pak1* (71 fold) and *Rac1* (33 fold) expression is significantly (>4 fold) up regulated relative to differentiated hNPs while *Cdc42* expression is unchanged (Figure 5.3A). NSC23766, a RAC1 inhibitor, decreased apoptosis on dopaminergic-like neurons cultured with inhibitor 1.2 fold relative to differentiated hNPs cultured with inhibitor (Figure 5.3B). Apoptosis significantly ($p>0.05$) increased in dopaminergic-like neurons cultured with inhibitor 1.5 fold relative to dopaminergic-like neurons cultured without inhibitor and proliferation significantly ($p>0.05$) decreased 1.2 fold in differentiated hNPs cultured with inhibitor relative to differentiated hNPs cultured without inhibitor (Figure 5.3B). Proliferation of dopaminergic-like neurons increased 1.75 fold relative to differentiated hNPs when cultured with inhibitor (Figure 5.3B). The mechanism through which the RAC1 pathway is activated by GDNF is outlined in Figure 5.3C.

*Downstream Targets of PI3K Pathways Unchanged in GDNF Cultured hNPs*

Dopaminergic-like neurons show no change in expression of GSK3β pathway genes APC and GSK3β (Figure 5.4A). Indirubin, an inhibitor of GSK3β, decreased apoptosis 1.2 fold in dopamine-like neurons relative to differentiated hNPs while proliferation increased 2880 fold in dopamine-like neurons relative to differentiation hNPs (Figure 5.4B). The mechanism through which GDNF activates the GSK3β pathway is outlined in the pathway in Figure 5.4C. Relative to differentiated hNPs, *Pten* (60 fold) is significantly (>4 fold) up regulated while *Tsc1* (55 fold) and *Tsc2* (34 fold) are down regulated (Figure 5.4D). Inhibition of the mTOR pathway with rapamycin decreased dopamine-like neuron apoptosis 3.7 fold relative to differentiated hNPs cultured with rapamycin (Figure 5.4E). Proliferation decreased significantly ($p<0.05$) 5.6 fold in dopaminergic-like neurons cultured with rapamycin relative to differentiated hNPs (Figure 5.4E). The mechanism through which GDNF activates the mTOR pathway is outlined in the pathway in Figure 5.4F.

*Cell Cycle in Dopaminergic-like Neurons*

IGF1, a cell growth factor, is significantly ($p>0.05$) down regulated 11 fold in dopaminergic-like neurons relative to differentiated hNPs. *Shc1*, responsible for modulating IGFR insertion into the membrane, is significantly (>4 fold) down regulated 13 fold in dopaminergic-like neurons relative to differentiated hNPs (Figure 5.5A). The mechanism through which SHC modulated IGF1 insertion into the membrane is outlined in Figure 5.5B. hNPs were significantly (> 4 fold) up regulated in *Ccnb1* (6 fold), *Ccnd1* (42 fold), *Ccnd3* (6 fold), *Cdkn1a* (51 fold), *Cdkn1b* (16 fold), *Cdkn1c* (9 fold; Figure 5.5C). Differentiated hNPs were significantly (>4 fold) up regulated in Ccnb1 (9 fold), *Ccnd1* (19 fold), *Ccnd3* (20 fold), *Cdkn1a* (65 fold), *Cdkn1b* (10 fold), Cdkn1c (7 fold) relative to hNPs (Figure 5.5C). Dopaminergic-like neurons were significantly (>4 fold) down regulated in *Ccnb1* (12 fold), *Ccnd1* (19 fold), *Ccnd3* (20 fold), *Cdkn1a* (55 fold), *Cdkn1b* (19 fold), *Cdkn1c* (25 fold) relative to hNPs

(Figure 5.5C). CI-898, an inhibitor of cell cycle that causes cell cycle arrest at late G1 phase or early S phase, was cultured in H9s, hNPs, differentiated hNPs and dopamine-like neurons at 10ng/ml. In H9s, CI-898 arrested the cells in late G1, increasing the number of cells in G1 when measured with propidium iodide (PI; Figure 5.5D). hNPs cultured with CI-898 were also arrested at late G1 increasing the G1 phase and number of cells in the phase (Figure 5.5E). There was no effect on the cell cycle of differentiated hNPs and dopaminergic-like neurons (Figure 5.5F, G).

**Discussion**

The goal of this study was to examine the pathway through which GDNF enhances dopaminergic differentiation from hNPs derived from hESCs. Inhibition of GDNF and its receptor RET led to complete abolishment of TH expression when cultured for 21 days. Additionally, inhibitors to the MAPK (MEK inhibitor) and PI3K (AKT inhibitor) pathway caused complete abolishment of TH expression. This led us to examine further the potential signaling pathways that could be causing enhancement of dopaminergic differentiation through culture with GDNF. The MAPK pathway can be divided into three sub-pathways, the ERK pathway, the JNK pathway and the p38 pathway. The JNK pathway responds to growth factors, cellular stress, cytokines, oxidative stress and G protein coupled receptors [20]. The JNK pathway sends stress signals through the MAP3K1-4 to MAP2K4/7 to activate cellular growth, differentiation, survival or apoptosis through regulating transcription factors [21]. The p38 MAPK pathway is activated by G protein coupled receptors, DNA damage, oxidative stress, cytokines and transforming growth factor beta TGF-β [22]. MAP3K1-4 activates MAP2K3/6 or MAP2K4, which leads to activation of transcription factors within the nucleus that increase cytokine production and apoptosis [22]. Regulation of the p38MAPK pathway is important for the control of apoptosis and response to cellular stresses [23]. The ERK pathway can be activated by G protein coupled receptors, integrins, and ion channels leading to activation of MAP2K1/2 and ERK1/2 [24].

ERK1/2 activates transcription factors, which modulate growth and development within the cell [25].

When GDNF is cultured with the hNPs in this study for 21 days, genes involved in the ERK and p38 aspects of the MAPK pathway were up regulated relative to differentiated hNPs cultured without GDNF including *Creb1*, *Mapk8*, *Mapk13* and *Mef2c*. The MEK activation of ERK leads to transcription of *Creb1*. A suggested mechanism for this activation is through promoting survival of dopaminergic neurons through ERK's known role in promoting TH protein expression [26]. The decreased level of apoptosis in hNPs differentiated with GDNF compared with the level seen when hNPs are differentiated without GDNF when inhibited with a MEK and an ERK inhibitor suggests that GDNF does act through the MEK and ERK pathways to promote dopaminergic survival in this mechanism (Figure 5.6B). The p38 pathway activates *Mef2c* to promote dopamine neuron survival through selective synaptic pruning [27,28]. In those neurons which are primed to become dopaminergic, GDNF enhances the synaptic connections between neurons which will improve their survival. The p38 pathway increases apoptosis of the non-primed dopaminergic neurons as is suggested by the decrease in apoptosis in the hNPs differentiated with GDNF when cultured with a p38 inhibitor. An increase in non-dopaminergic neuron apoptosis would be seen during the enhancement with GDNF through the p38 pathway; however, with that pathway blocked, that selective enhancement would not occur (Figure 5.6A). RAC1 regulates actin within the neuron as well as the morphology of the neuron [29]. The increase in *Rac1* and *Pak1* expression with differentiation of hNPs with GDNF suggests activation of this pathway in dopaminergic-like neurons used in this study. The lack of significant change in apoptosis and proliferation when this pathway is inhibited further suggests the role of the RAC1 pathway in GDNF enhancement of dopaminergic-like neurons lies outside of apoptosis and proliferation and might lay in the known roles of RAC1 in actin regulation and morphology modulation [30].

The mammalian target of rapamycin (mTOR) acts downstream of AKT in the PI3K pathway and can be activated by RAS homolog enriched in brain and blocked by TSC1/TSC2 [31]. MTORC1, which can be activated by growth factors, acts to activate eukaryotic translation initiation factor 4EBP1, which leads to mRNA translation that increases cell growth. mTORC1 also suppresses autophagy and signals for ribosome biogenesis [32]. mTORC2 acts to control actin organization and cellular survival [33,34]. GDNF activation of RET also leads to PI3K activation of the mTOR pathway which activates eukaryotic initiation factors that regulate synaptic plasticity and dopamine neural survival (Figure 5.6C). The inhibition of TSC1/TSC2 on mTOR was lifted with the decrease in genetic expression of TSC1/TSC2 in differentiated hNPs with GDNF increasing the expression of *eif4b* and *eif4g.*Dopaminergic-like neurons relative to differentiated hNPs up regulate PTEN, which has been shown to regulate neural growth [35,36], potentially slowing neural growth as the hNPs differentiate. TSC1 and TSC2 complexes regulate neural growth and are down regulated in dopamine-like neurons relative to differentiated hNPs [37]. Rapamycin, an inhibitor of the mTOR pathway has been shown to prevent PD cell death [38]. The dopaminergic-like neurons used in this study decreased in apoptosis when inhibited with rapamycin confirming these results (Figure 5.6C).

GSK3β signaling, activated by growth factors or WNT signaling through the Frizzled receptor, modulated glucose metabolism and glycogen synthesis through activation of the enzyme necessary for glycogen synthesis [39]. Additionally, GSK3β modulates cell cycle through blocking Cyclin D1 and p21Cip, which lead to an increase in proliferation and growth. Activation of β-Catenin leads to activation of transcription factors [40,41]. GSK3β activation has less of a role in GDNF differentiation and more of a role in neural progenitor cell growth and development [42]. Blocking GSK3β increases proliferation in dopaminergic-like neurons suggesting that with the lack of glucose synthesis, the dopaminergic-like neurons revert to an

immature fate. The enhancement of GDNF on dopaminergic-like neuron differentiation likely does not occur through the GSK3β pathway (Figure 5.6C).

SHC is a protein involved in receptor trafficking within the cell. SHC binds to the IGFR and controls its insertion into the membrane [43,44]. IGF1 plays a role in neural growth and development early in embryogenesis [45,46].The IGF pathway and its downstream activation of GSK3β has less involvement in the GDNF activation of RET and dopaminergic differentiation but more to do with the growth and proliferation of the neural progenitor cells. *Igf1* and *Shc* are down regulated in dopaminergic-like neurons relative to differentiated neurons suggesting a post-mitotic neuron. Supporting the movement of differentiated hNPs with and without GDNF towards a post-mitotic neuron is the decrease in cell cycle genes as the hNPs differentiate. Additionally, the cell cycle in differentiated hNPs and dopaminergic-like neurons resembles that of a post-mitotic neuron. The increased decrease in *Igf1* and *Shc* in dopaminergic-like neurons relative to differentiated hNPs suggests that GDNF acts to establish a more mature neural phenotype.

**Conclusion**

GDNF has been shown to enhance dopamine differentiation of hNPs [10]; this differentiation is completely blocked when GDNF and its receptor RET are blocked. The MEK and ERK pathways support dopaminergic neural survival while the p38MAPK pathway modulates dopamine enhancement through pruning of non-primed dopaminergic neurons. RAC1 is involved in neurite outgrowth. While inhibiting the mTOR pathway helped to support the maintenance of the dopaminergic-like neurons through up regulating eukaryotic initiation factors that improve synaptic plasticity and dopamine neural survival, GSK3β has no role in GDNF enhancement of dopaminergic-like neurons. This study has further elucidated the mechanisms through which GDNF helps to support dopaminergic neurons and to enhance the differentiation of dopaminergic-like neurons from hNPs allowing for potential future targets for PD therapies.

**Acknowledgements**

This work was supported by DOD #N000140810989 and the GRA Eminent Endowment. We thank Julie Nelson and the Center for Tropical and Emerging Global Diseases Flow Cytometry Facility for her technical expertise.

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**Figure 5.1** – GDNF enhancement of differentiation of dopaminergic-like neurons blocked with inhibitors to MAPK and PI3K

Inhibiting GDNF with an antibody that binds all GDNF found in the cell decreased TH expression significantly ($p<.05$) at 1ng/ml and 10ng/ml and prevented TH expression completely at 100ng/ml (A). Immunocytochemistry images show the difference in differentiation without inhibitor (B) and with inhibitor (C). Blocking the RET receptor also decreased TH expression with 1ng/ml but completely killed all cells at 10ng/ml and 100ng/ml (D). Immunocytochemistry images show dopaminergic-like neurons without inhibitor (E) and with (F). Examination of the PI3K pathway with an inhibitor to prevent its activation killed all cells at 10ng/ml and 100ng/ml but only slightly decreased TH expression with 1ng/ml (G). Similar immunocytochemistry images are shown without inhibitor (H) and with (I). Inhibition of MEK at a dosage of 1ng/ml and 10ng/ml significantly reduced TH expression while 100ng/ml killed all cells (J). Immunocytochemistry showed TH expression without inhibitor (K) and no TH expression with (L). Scale bars = 10μM.

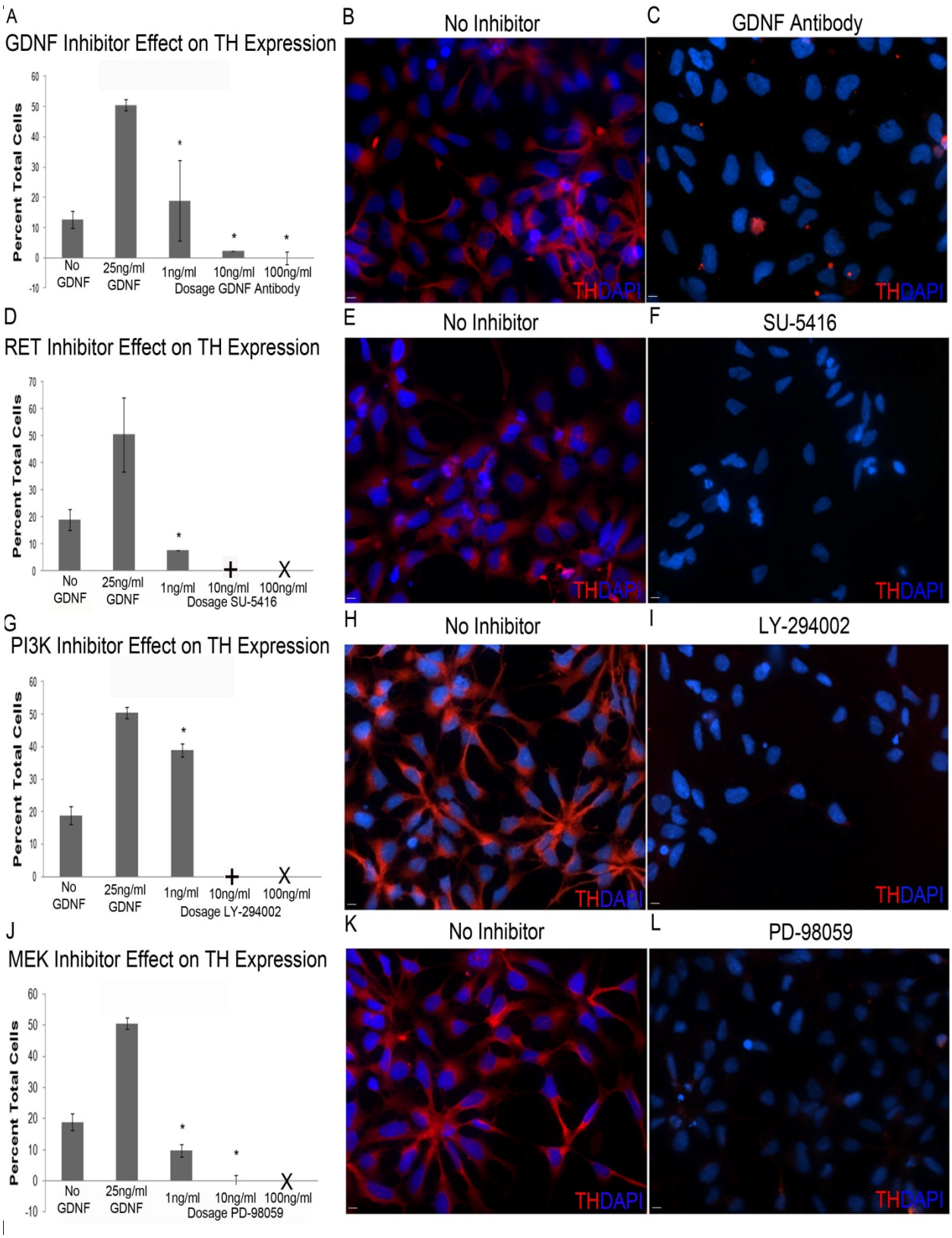
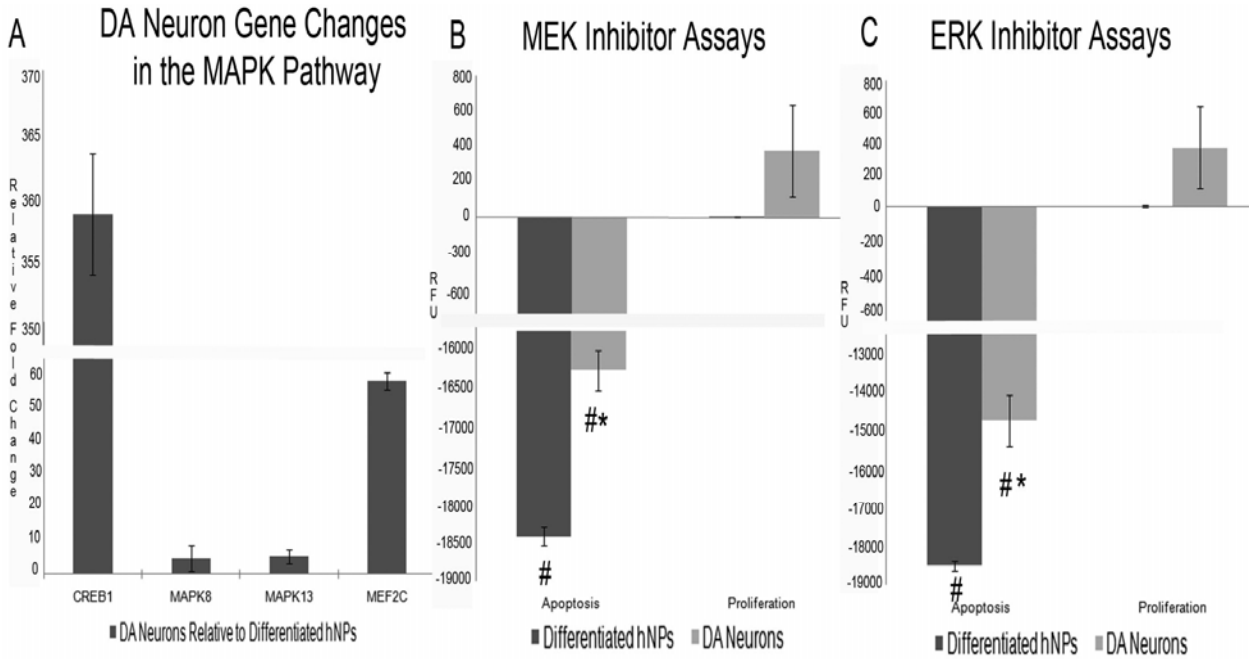
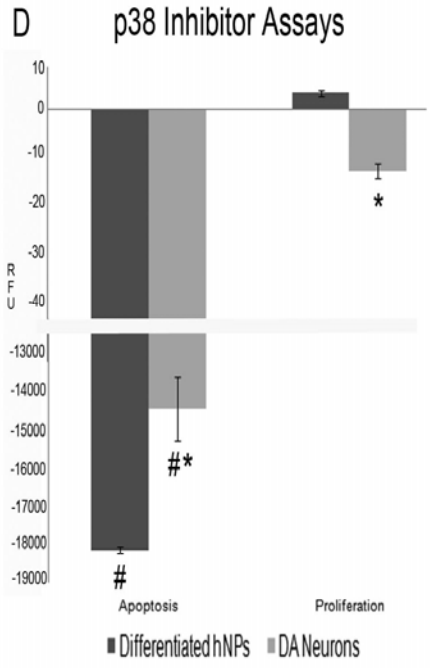
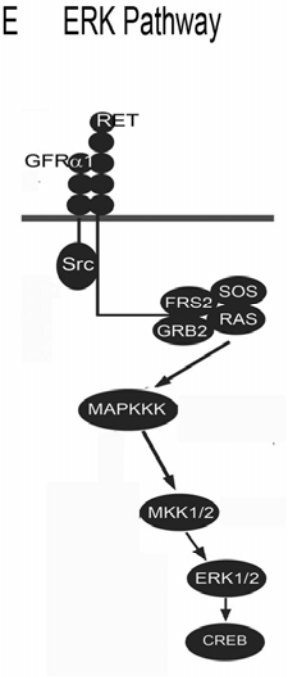

A
GDNF Inhibitor Effect on TH Expression
Percent Total Cells
No GDNF
25ng/ml GDNF
1ng/ml
10ng/ml
100ng/ml
Dosage GDNF Antibody
B
No Inhibitor
C
GDNF Antibody
THDAPI
D
RET Inhibitor Effect on TH Expression
Dosage SU-5416
E
No Inhibitor
F
SU-5416
G
PI3K Inhibitor Effect on TH Expression
Dosage LY-294002
H
No Inhibitor
I
LY-294002
J
MEK Inhibitor Effect on TH Expression
Dosage PD-98059
K
No Inhibitor
L
PD-98059

**Figure 5.2** – Changes in the MAPK Pathway with GDNF Enhance Dopamine-like Neurons Relative to differentiated hNPs, dopaminergic-like neurons express higher levels of *Creb1*, *Mapk8*, *Mapk13* and *Mef2c* aspects of the ERK and p38 pathways (A). Inhibition of the MEK pathway lead to a greater decrease in apoptosis in differentiated hNPs than in dopaminergic-like neurons with no significant change in proliferation (B). These results were similar to those found with inhibition of ERK pathway (C). Inhibiting the p38 pathway leads to a greater decrease in apoptosis in differentiated hNPs relative to dopaminergic-like neurons and a decrease in proliferation in dopaminergic-like neurons (D). The mechanisms for the GDNF activation in the ERK pathway are outlined in (E) and those for the p38 pathway are outlined in (F). hNP – human neural progenitors; MEK – mitogen activated kinase kinase; ERK – extracellular signal related kinase; RET – rearranged in transcription; GFR$\alpha$1 – glial cell-line derived neurotrophic factor family receptor alpha 1; RFU – relative fluorescence unit; DA – dopamine; * significant ($p>0.05$) relative to differentiated hNPs, # significant ($p>0.05$) relative to culture with no inhibitor.


A
DA Neuron Gene Changes in the MAPK Pathway
Relative Fold Change
CREB1
MAPK8
MAPK13
MEF2C
DA Neurons Relative to Differentiated hNPs
B
MEK Inhibitor Assays
RFU
Apoptosis
Proliferation
Differentiated hNPs
DA Neurons
C
ERK Inhibitor Assays
RFU
Apoptosis
Proliferation
Differentiated hNPs
DA Neurons


D
p38 Inhibitor Assays
RFU
Apoptosis
Proliferation
Differentiated hNPs
DA Neurons


E
ERK Pathway
RET
GFRα1
Src
FRS2
SOS
GRB2
RAS
MAPKKK
MKK1/2
ERK1/2
CREB

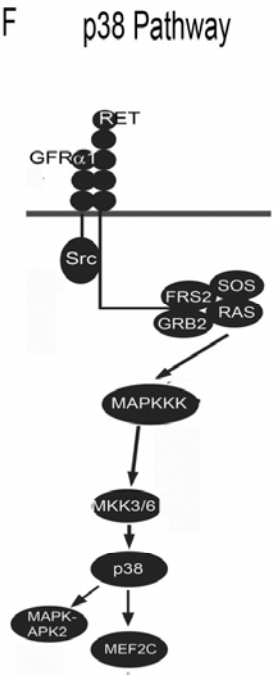
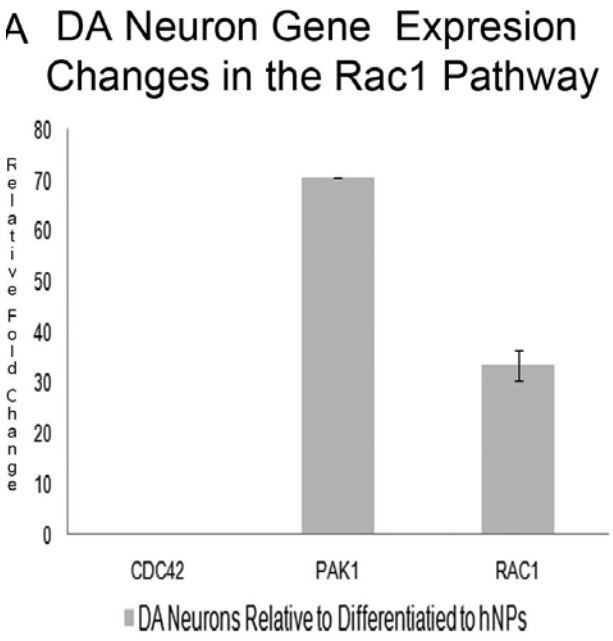
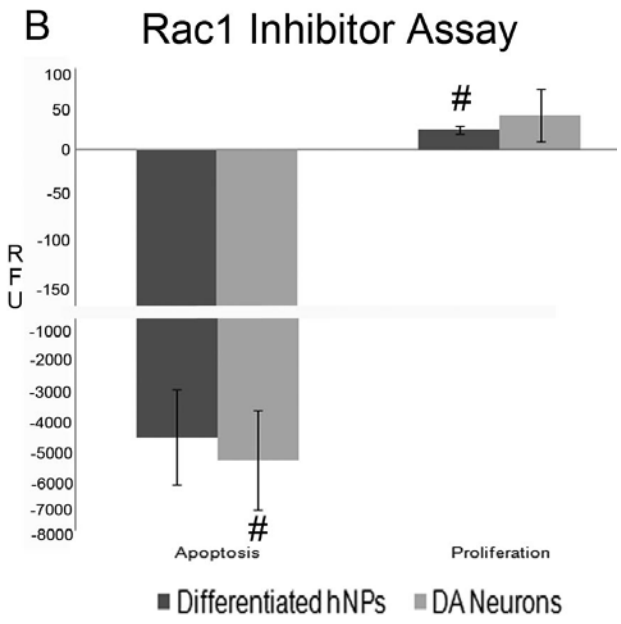

F
p38 Pathway
RET
GFRα1
Src
FRS2
SOS
GRB2
RAS
MAPKKK
MKK3/6
p38
MAPK-APK2
MEF2C

**Figure 5.3** – Role of the Rac1 Pathway in Dopaminergic-like Neurons

Dopamine-like neurons express higher levels of *Pak1* and *Rac1* relative to differentiated hNPs with no chance in CDC42 expression (A). Apoptosis in dopaminergic-like neurons cultured with inhibitor to RAC1 decreased significantly ($p>0.05$) relative to dopaminergic-like neurons without inhibitor while proliferation in differentiated hNPs with inhibitor increased significantly ($p>0.05$) relative to differentiated hNPs without inhibitor with no significant changes in apoptosis or proliferation between differentiated hNPs and dopaminergic-like neurons (B). The mechanism through which GDNF activates the RAC1 pathway is outlined in (C). RET – rearranged in transcription; GFR$\alpha$1 – glial cell-line derived neurotrophic factor family receptor alpha 1; RFU – relative fluorescence unit; DA – dopamine; # significant ($p>0.05$) relative to culture with no inhibitor.


A DA Neuron Gene Expresion Changes in the Rac1 Pathway
Relative Fold Change
80
70
60
50
40
30
20
10
0
CDC42
PAK1
RAC1
DA Neurons Relative to Differentiatied to hNPs


B Rac1 Inhibitor Assay
RFU
100
50
0
-50
-100
-150
-1000
-2000
-3000
-4000
-5000
-6000
-7000
-8000
#
#
Apoptosis
Proliferation
Differentiated hNPs
DA Neurons

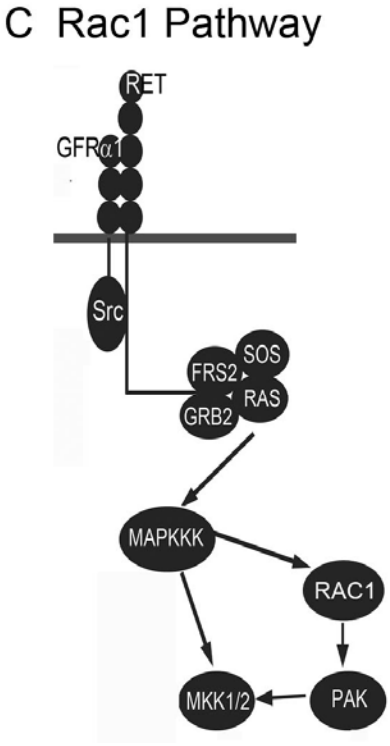

C Rac1 Pathway
RET
GFRα1
Src
SOS
FRS2
RAS
GRB2
MAPKKK
RAC1
MKK1/2
PAK

**Figure 5.4** – PI3K Sub-pathway Effects on Dopaminergic-like Neurons

*Gsk3β* and *Apc* expression is not changed in dopaminergic-like neurons relative to differentiated hNPs (A). There was no change in apoptosis and proliferation when differentiation hNPs or dopaminergic-like neurons when cultured with inhibitor (B). The mechanism of action of GSK3β is shown in (C). *Pten* expression is up regulated in dopaminergic-like neurons relative to differentiated hNPs while *Tsc1* and *Tsc2* expression is down regulated in dopaminergic-like neurons relative to differentiated hNPs (D). There is a significant increase in apoptosis in dopaminergic-like neurons inhibited with an mTOR inhibitor relative to differentiated hNPs inhibited (B) with an mTOR inhibitor suggesting the role of mTOR in dopaminergic-like neuron enhancement with GDNF is through changes in transcription factors as outlined in (C). RET – rearranged in transcription; GFRα1 – glial cell-line derived neurotrophic factor family receptor alpha 1; RFU – relative fluorescence unit; mTOR – mammalian target of rapamycin; GSK3β - glycogen synthase kinase 3 beta; DA – dopamine; * significant ($p>0.05$) relative to differentiated hNPs.

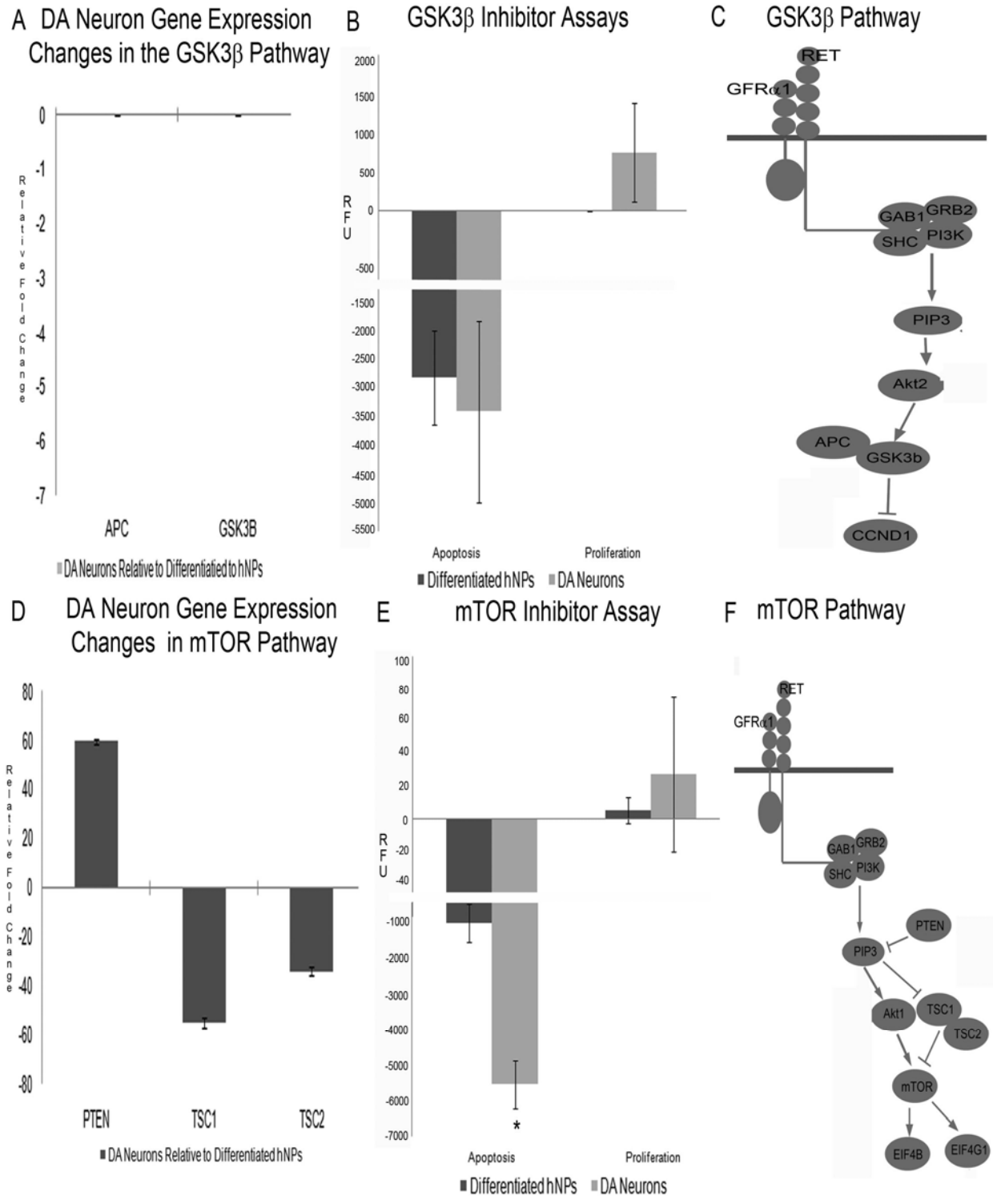

A DA Neuron Gene Expression Changes in the GSK3β Pathway
Relative Fold Change
0
-1
-2
-3
-4
-5
-6
-7
APC
GSK3B
DA Neurons Relative to Differentiated hNPs
B GSK3β Inhibitor Assays
RFU
2000
1500
1000
500
0
-500
-1500
-2000
-2500
-3000
-3500
-4000
-4500
-5000
-5500
Apoptosis
Proliferation
Differentiated hNPs
DA Neurons
C GSK3β Pathway
RET
GFRα1
GAB1
GRB2
SHC
PI3K
PIP3
Akt2
APC
GSK3b
CCND1
D DA Neuron Gene Expression Changes in mTOR Pathway
Relative Fold Change
80
60
40
20
0
-20
-40
-60
-80
PTEN
TSC1
TSC2
DA Neurons Relative to Differentiated hNPs
E mTOR Inhibitor Assay
RFU
100
80
60
40
20
0
-20
-40
-1000
-2000
-3000
-4000
-5000
-6000
-7000
*
Apoptosis
Proliferation
Differentiated hNPs
DA Neurons
F mTOR Pathway
RET
GFRα1
GAB1
GRB2
SHC
PI3K
PTEN
PIP3
Akt1
TSC1
TSC2
mTOR
EIF4B
EIF4G1

**Figure 5.5** – Changes in cellular functions with inhibitors to PI3K pathway targets

*Igf1* and *Shc* expression decreased in dopamine-like neurons relative to differentiated hNPs (A). The mechanism through which SHC acts to modulate IGFR insertion in the membrane is outlined in (B). Cell cycle genes are up regulated in hNPs relative to H9s while cell cycle genes are down regulated in differentiated hNPs relative to hNPs and in dopaminergic-like neurons relative to hNPs (C). Inhibition of H9 (D) and hNP (E) and cell cycle increases the number of cells in G1. Inhibition of differentiated hNP (F) and dopaminergic-like neuron (G) cell cycle had no effect on the G1 stage and the cell cycle inhibits that of post-mitotic neurons. hNPs – human neural progenitors; IGF1 – insulin growth factor 1; DA – dopamine.

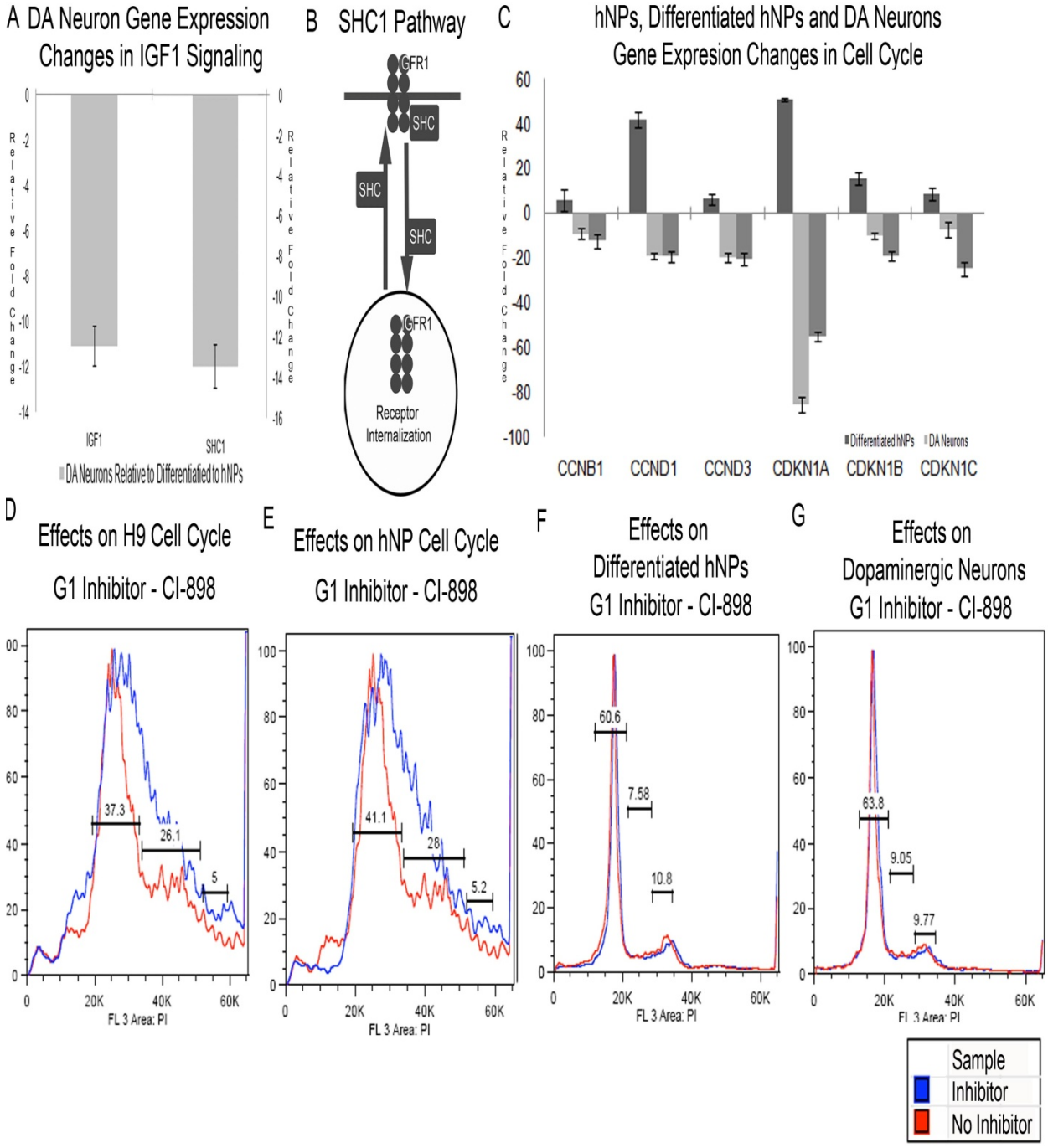
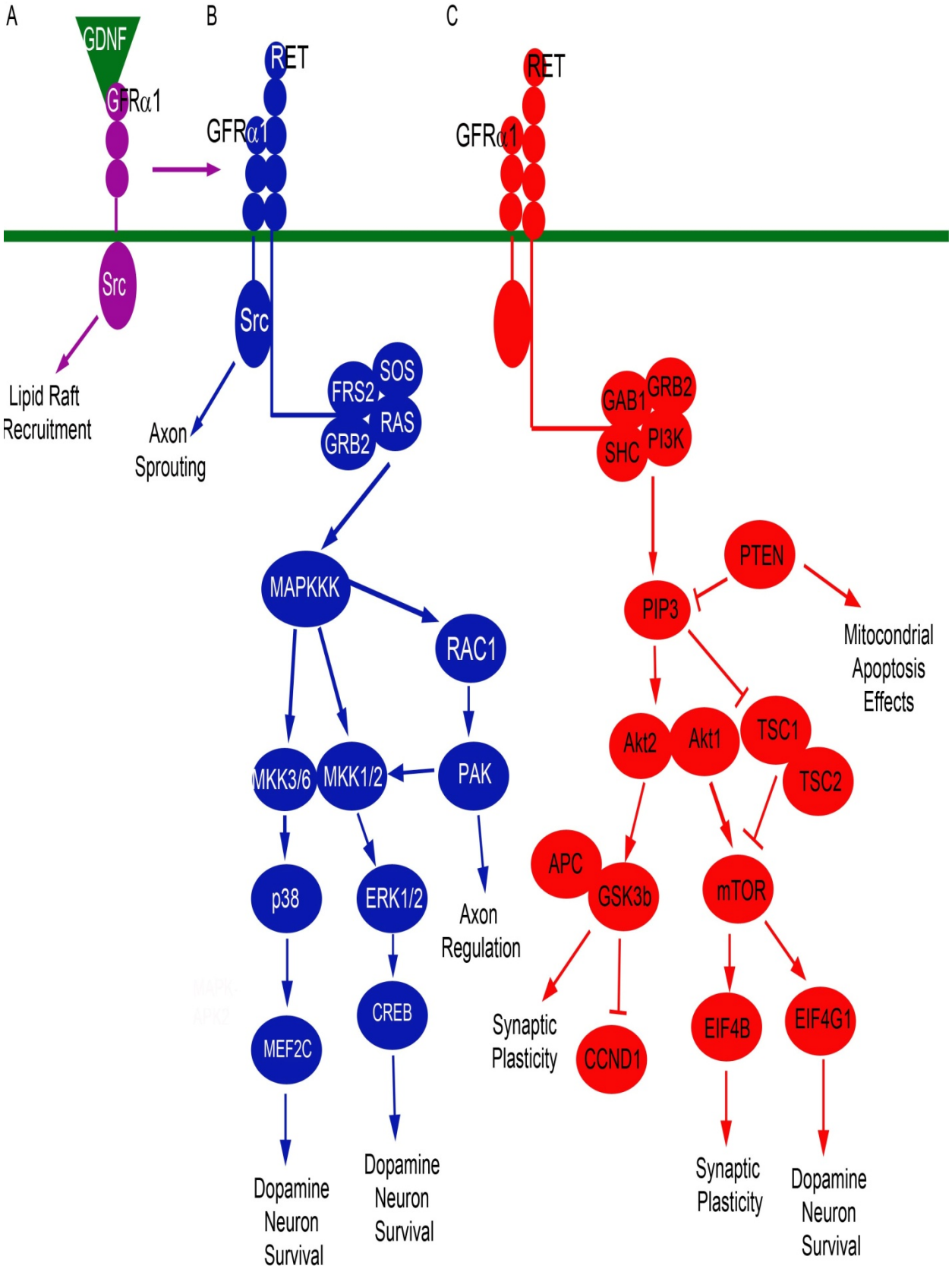

A DA Neuron Gene Expression Changes in IGF1 Signaling
Relative Fold Change
IGF1
SHC1
DA Neurons Relative to Differentiated to hNPs
B SHC1 Pathway
IGFR1
SHC
SHC
SHC
IGFR1
Receptor Internalization
C
hNPs, Differentiated hNPs and DA Neurons Gene Expresion Changes in Cell Cycle
Relative Fold Change
Differentiated hNPs
DA Neurons
CCNB1
CCND1
CCND3
CDKN1A
CDKN1B
CDKN1C
D
Effects on H9 Cell Cycle
G1 Inhibitor - CI-898
37.3
26.1
5
FL 3 Area: PI
E
Effects on hNP Cell Cycle
G1 Inhibitor - CI-898
41.1
28
5.2
FL 3 Area: PI
F
Effects on Differentiated hNPs
G1 Inhibitor - CI-898
60.6
7.58
10.8
FL 3 Area: PI
G
Effects on Dopaminergic Neurons
G1 Inhibitor - CI-898
63.8
9.05
9.77
FL 3 Area: PI
Sample
Inhibitor
No Inhibitor

**Figure 5.6** – GDNF Pathway in Dopaminergic Enhancement

Src binds to the GFR$\alpha$1 co-receptor when GDNF binds to its co-receptor and helps to bring RET to the membrane to allow for further cell signaling (A). GDNF binding to the RET receptor activates the MAPK pathway (B). The ERK pathway activation leads to dopamine neural survival, p38 activation leads to dopamine neural survival and RAC1 activation leads to axon regulation (B). Activation of the PI3K pathway with GDNF leads to the activation of the mTOR pathway and dopamine neural survival and synaptic plasticity through the eukaryotic initiation factors (C). The GSK3$\beta$ pathway is not involved in GDNF enhancement of dopamine neurons (C). GDNF – glial cell-line derived neurotrophic factor; GFR$\alpha$1 – glial cell-line derived neurotrophic factor family receptor alpha 1.


A
B
C
GDNF
GFRα1
RET
GFRα1
RET
GFRα1
Src
Src
Lipid Raft
Recruitment
Axon
Sprouting
FRS2
SOS
RAS
GRB2
GAB1
GRB2
SHC
PI3K
MAPKKK
RAC1
PTEN
PIP3
Mitocondrial
Apoptosis
Effects
Akt2
Akt1
TSC1
TSC2
MKK3/6
MKK1/2
PAK
APC
GSK3b
mTOR
p38
ERK1/2
Axon
Regulation
CREB
MEF2C
Synaptic
Plasticity
CCND1
EIF4B
EIF4G1
Dopamine
Neuron
Survival
Dopamine
Neuron
Survival
Synaptic
Plasticity
Dopamine
Neuron
Survival

# CHAPTER 6

# CONCLUSION

The goal of this thesis was to determine if the addition of GDNF to hNPs derived from hESCs would enhance the differentiation of dopaminergic-like neurons from hNPs. If differentiation was enhanced with GDNF, the involvement of the MAPK and PI3K pathways in this differentiation was to be determined. Dopaminergic neurons are the cells that become depleted in PD patients. The dopaminergic neurons in the SN project their axons to the basal ganglia and help to modulate control of movement in normal brains; however, the lack of dopaminergic projections from the SN to the basal ganglia in PD patients lead to the tremor, ridgity, bradykinesia and postural instability [1]. Due to PD affecting 1% of the American population over 60 with the percentage increasing with the increasing aging population and the lack of an effective treatment which does not cause severe side effects or lose efficacy overtime, a more effective treatment would greatly benefit the population [2].

GDNF is a known dopaminergic neural protectant [3]. Research on the mouse nigrostriatal pathway has shown that injection of GDNF, fetal neurons that secrete GDNF, or adenoviruses that express GDNF protect the dopaminergic neurons from MPTP injury [4-7]. Recovery in non-human primate behavioral response was seen in MPTP lesioned rhesus monkeys when GDNF was injected into the striatum [8]. Success in animal models led to GDNF drug trials in humans. Initial reports were successful, but long-term studies resulted in increased side effects including weight loss [9-11]. The side effects combined with the difficulty of administration into the human brain has stalled human trials of GDNF as a PD treatment but has not dampened desire for GDNF with potential use in PD.

Following hESC derivation in 1998 [12], interest has included directing these hESCs towards specific lineages including neural cells. Our lab derived a continually proliferative, stable hNP line that can be grown in a monolayer [13]. The potential use of these cells as a

neural developmental model, drug assay screening technique or as a cell therapy relies on the ability to differentiate these hNPs towards specific cell fates. hESC derived hNPs provide a source for studying drug effects or developmental pathways in a human system as well as providing an easily attainable source of neural cells.

In these studies, we first established the biological functionality of the hNPs when differentiated toward neural cells. In order to use the differentiated hNPs in disease models such as a PD model, their similarity to normal CNS tissue must first be established. The differentiated hNPs were examined for pre- and postsynaptic receptor expression and ionotrophic receptors with RT-PCR, while functionality of the receptors was established with calcium response FLIPR assays. We established expression of glutamatergic, GABAergic, nicotinic, purinergic, sodium and calcium ionotrophic receptors in the differentiated hNPs that are found in functional CNS neural tissue. Potentiators of AMPA and sodium channel response tested in a calcium FLIPR assay confirmed responsiveness of these ionotrophic receptors. Additionally, basal level of expression of DAT, a marker for dopaminergic neurons, established the potential for these differentiated hNPs to become dopaminergic-like neurons.

Following establishment of basal expression, we enhanced the differentiation of dopaminergic-like neurons from hNPs with the addition of GDNF to the differentiation media to 50% TH+ neurons [14]. We also examined the developmental progression of dopaminergic differentiation in a human model with immunocytochemistry and flow cytometry. The hNPs were primed to become dopaminergic-like neural cells in the first report of hNPs expressing NURR1 [14]. Following 7 days of differentiation, a dopaminergic specification stage was reached with an increase in EN1+ and NURR1+ neural cells. Dopaminergic progenitors were established after 21 days of differentiation as marked by continued NURR1 and EN1 expression and an increase in TH+ and PITX3+ expression. Finally, mature dopaminergic-like neurons were established with the expression of functional markers DAT and VMAT2 in addition to TH, PITX3, EN1 and NURR1. HPLC analysis of dopamine metabolites demonstrated the ability of these cells to

produce dopamine, while PCR analysis showed the expression of D1, D4 and D5 receptors further establishing a mature dopaminergic-like neuron. This developmental progression of dopaminergic markers in hNPs in addition to the enhancement of dopaminergic differentiation in a one-step protocol including GDNF had not been done previously [14].

The mechanism through which GDNF promoted enhancement of dopaminergic-like neurons was examined in the final study. It was known the role that Src and JNK had in GDNF's role in dopaminergic neural protection [15,16], but, while the MAPK and PI3K pathways had been implicated, their role had not been established [17,18]. We examined the expression of genes involved in these pathways with RT-PCR. Up regulation of aspects including ERK1/2, p38 and mTOR  and lead to further studies in the functional results of these pathways. p38 increased survival of dopaminergic-like neurons, while the mTOR pathway maintained normal dopaminergic functional gene translations and ERK promoted dopaminergic-like neuron survival. The demonstration of the roles for specific parts of the PI3K and MAPK pathway in GDNF's effect on neural protection and enhancement of dopaminergic differentiation had not been done previously.

**Future Studies**

While the advancements made in this thesis addresses many of the issues in the mechanism of GDNF neural protection and advance the field of hNP derivation of dopaminergic-like neurons, there is still much work that remains. One concern is in the heterogeneous population of dopaminergic-like neurons. In order to use these cells in a drug screen or a cell replacement therapy, having a homogenous population would be ideal. One potential mechanism for increasing the number of TH+ cells in the population would be FACS cell sorting of TH+ cells only. This presents an issue with differentiated hNP cells in that breaking their neurite connections and establishing a single cell population decreases the survivability of the differentiated hNPs. This drawback would have to be modulated before FACS sorting could proceed. An additional mechanism for enhancing the homogeneousness of the population is

through examining the prime timing for GDNF exposure. Increasing the differentiation with GDNF or beginning GDNF exposure at earlier or later times in differentiation may alter the percentage of TH+ cells in the dopaminergic-like neuron population. A final mechanism for increasing the number of TH+ cells in the population is with a reporter system. Using a TH promoter driven GFP reporter system would allow for the selection of only those cells, which express TH. These cells could then be sorted with FACS or selected for with antibiotic resistance. Using a TH promoter system would allow for several sorts until a homogenous population was established allowing for a gradual advancement towards a pure population. The establishment of a pure population through any of these methods allows for the creation of a better system for studying dopaminergic neural development or cell-based assays.

In addition, these studies examined specific aspects of the MAPK and PI3K pathway, which are influenced by GDNF's addition to differentiation media. These parts of the pathways are thought to be the mechanisms through which GDNF enhances dopamine-like neuron differentiation. Further manipulations on these pathways may help explain the mechanisms through which dopaminergic neurons are injured in PD. Studies with inhibitors to these pathways and the effects on reactive oxygen species (ROS) will help to determine those pathways that are susceptible to ROS. Additionally, examining the effects on TH expression can help with determining which pathways directly affect dopamine cell differentiation. Inhibiting aspects of the PI3K and MAPK pathway while measuring the markers for dopamine specification, dopamine progenitors and dopaminergic-like neurons will help to determine the mechanisms that regulate each aspect of dopaminergic neuron development.

The use of these cells in drug screening assays needs to be validated prior to moving forward with using these cells as a screen for PD drugs. In this thesis, these cells were used in apoptosis and proliferation assays; therefore, they should be tested with known PD causative agents to verify their ability to respond similarly to biological CNS tissue. MPTP, pesticides and herbicides are known environmental toxins that cause symptoms similar to PD. Verifying the

effects of these known toxins on the dopaminergic-like cells in this thesis through measures such as ROS, TH expression or apoptosis would further confirm the use of these cells in drug screens.

Finally, the dopaminergic-like neurons derived in this thesis need to be further characterized as a PD model. The expression of genes known to be involved in PD such as *Lrrk2*, *Dj1*, *Parkin* and *αsynuclein* should be measured with RT-PCR to establish baseline level of expression. Knocking out or over expression of the genes found to be expressed would allow for study of the effects of these genes on dopaminergic-like neuron survival and differentiation. The MAPK and PI3K pathway can be examined to determine the roles of these genes in changing the pathway actions allowing for mechanistic study of these genes on dopaminergic neurons in PD. ROS and TH expression can be studied in knockout or over expression models.

GDNF was used to establish a dopaminergic-like neuron, which can be easily established from stable, adherent monolayer cultures. These cells provide a cell source for use to study the mechanisms that cause PD as well as to use as a drug screen for future PD drugs which could be more effective than those currently available. More work remains to be done to establish the functionality of these dopaminergic-like neurons as a PD model, but the possibilities for providing treatment options for those suffering with PD are great.

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APPENDIX

# APPENDIX A

## PROLIFERATION AND PRIMORDIAL GERM CELL MAINTENANCE BY THE MAPK AND PI3K PATHWAY IN GERM-LIKE CELS DERIVED FROM HUMAN EMBYRONIC STEM CELLS[1]

[1]Young, Amber, West, Franklin D., Jordan, Erin, Stice, Steven L. To be submitted to *Human Reproduction.*

**Abstract**

The MAPK and PI3K cell cycle pathways have been shown to be important in Drosophila, Xenopus and mouse germ cell development. The MAPK pathway is important in normal gameotgenesis, normal mitotic and meiotic division, heat stress and heat resistance and the maintenance of spermatozoa through the ERK, p38 and JNK pathway. The PI3K sub-pathways mTOR and GSK3β play roles in sperm cell self-renewal, sperm motility enhancement and aid in capacitation within the acrosome. Recent work into hESC derived germ cells has demonstrated the ability to derived germ-like cell from hESCs, which provide a useful model for studying human germ cell development. Our lab has recently isolated from hESCs a >90% pure germ-like cell line which is DDX4/POU5F1+. The factors such as FGF2, which are useful in deriving these germ-like cells, activate the MAPK and PI3K pathways and studying those pathways may prove useful in understanding germ-like cell development. RT-PCR was used to analyze genetic changes as well as apoptosis and proliferation studies to analyze functional changes in the MAPK and PI3K pathway in germ-like cells compared to their parent hESC population. We found up regulation in ERK, JNK and p38 gene expression as well as increases in apoptosis with MAPK, ERK and p38 pathway inhibition in germ-like cells relative to hESCs. Additionally, the mTOR and GSK3β pathway genes are up regulated while inhibitions lead to functional changes in proliferation and apoptosis. Finally, IGF1 and IGFR genes were up regulated as well as cell cycle and proliferation increased in germ-like cells relative to hESCs supporting an increase in proliferation in these cells. The genetic expression changes support that of primordial germ cells and provide evidence for the involvement of the MAPK and PI3K pathway in germ-like cells derived from hESCs.

## Introduction

Mitogen activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K) pathways are critical in the proliferation, differentiation and maintenance of germ cells with inhibition of these pathways resulting in the failure to properly form gametes or loss of derived germ cells in Drosophila, Xenopous and the mouse [1-5]. The MAPK signaling is critical throughout normal gametogenesis affecting mitotic division, meiosis, heat stress and a number of key developmental processes in differentiation. ERK signaling, part of the MAPK pathway, increases proliferation in germ cells and maintains early germ cells [6,7]. Apoptosis is an important aspect of germ cell development with a number of germ cells undergoing apoptosis throughout development. The MAPK p38 pathway is activated in cases of stress to modulate apoptosis of incorrectly developed sperm [8]. The MAPK pathway also plays a role in germ cell heat resistance preventing apoptosis and increasing the survival of germ cells [9]. Mouse meiotic spindle activity requires activation by ERK1/2 in order to proceed properly [10]. During meiosis, the MAPK pathway is inactivated as active MAPK prevents meiotic spindle formation and M phase cell cycle exit [11,12]. Previous research has shown inhibitors to the MAPK pathway can halt meiotic division through modulation of calcium signaling within the spermatid [3]. When the JNK pathway, a sub-pathway of the MAPK, is inhibited, the scaffold protein JNK-associated leucine zipper protein (JLP) dysfunction causes reduced fertility in male mice [13]. In germ cells, the defects within the JNK pathway lead to a decline in fertility and an inability to maintain spermatozoa [14]. Additionally, the RHO/RAC proteins within the MAPK pathway are expressed in the acrosome and play an important role in enabling sperm cells to penetrate the zona pellucid, a protective outer membrane of the ovum, and fertilize oocytes [15]. These previous reports suggest that the MAPK pathway may also play a role in GLCs derived from hESCs.

PI3K pathway signaling has also proven to be critical to germ cell development in differentiation and functional activities. Activation of the PI3K sub-pathway mTOR has been

shown to be involved in sperm expansion and proper cell division, while activation of GSK3β leads to meiotic and post-meiotic sperm cells [16,17]. Additionally, in early sperm cell development, AKT phosproylation leads to increased self-renewal of spermatogonial stem cells [18]. Later in development, androgen hormones modulate the activity of the PI3K pathway with low doses activating PI3K and high doses inactivating PI3K and activating a negative regulator of the PI3K pathway, PTEN with high doses causing apoptosis of sperm cells [19]. Interactions between PKA, PKC and PI3K pathways are important regulation of spermatozoa motility [20]. Inhibition of the PI3K pathway by LY-294002 enhances sperm motility; however, another PI3K inhibitor wortmannin does not cause this same effect potentially due to the inhibitors differential effects on Ca2+ signaling [21,22]. Polymerization which occurs during capacitation of the spermatozoa is driven by the PI3K pathway [23]. However, inhibition of the PI3K at the time of fertilization does not affect sperm/oocyte interaction implying that the role for PI3K is in earlier stages of sperm cell development [24]. The role of the PI3K pathway in sperm cell development is a complex one involving modulation of several inputs and specific timing.

Investigation of early human germ cell differentiation has been hindered by the inaccessibility of these cells, due in part to ethical and technical issues, as they can only be isolated from fetal tissue, and the lack of a developmentally competent human model. However, recent germ cell differentiation studies of human embryonic stem cells (hESCs) have demonstrated that hESCs may be capable of providing a relevant human germ cell model through derived germ-like cells (GLCs) which express a myriad of gene and protein markers and show functional competency [25-28]. Pioneering studies utilizing an embryoid body (EB) three dimensional germ cell differentiation system resulted in a small subset of cells that became germ like with the expression of the definitive germ cell marker DDX4 [29]. However, the EB system resulted in heterogeneous populations with low numbers of GLCs preventing cells from being isolated and making them difficult to study. These challenges have in part been overcome with novel adherent two dimensional differentiation systems that allow for

homogeneous GLC populations to be derived and for easier isolation and study of GLCs [25-28]. GLCs derived in these adherent systems have been shown to undergo complex developmental processes including epigenetic reprogramming, resetting of imprinting gene methylation patterns and meiosis, hallmarks of normal germ development. Yet certain disadvantages remain such as the inability to propagate continually GLCs, the confounding variable of mixed populations and the high amount of variability in developmental potential (e.g. ability to reset imprinting genes or undergo meiosis) between derived GLC populations. The need to re-derive continuously GLCs has resulted in populations that are different characteristically in gene and protein expression and in functional ability causing significant variability in results [25-28]. This variability adds an additional layer of complexity to the already challenging task of identifying key signaling events that orchestrate normal germ cell differentiation.

To address these challenges, we have recently isolated homogenous GLC lines in which >90% of cells are DDX4/POU5F1+ [30]. These cells are capable of maintaining germ cell morphological features, gene expression and developmental potential for >50 passages. Under advanced differentiation conditions, these cells are capable of synchronized entry into meiosis with >70% of cells expressing the prophase I meiotic markers SYCP3 and MLH1and forming haploid gametes. Comparing these cells to parent hESC populations, we identified key changes in gene expression that may shed light on the hESC to GLC differentiation process. Additionally, in a previous study, we have shown that fibroblast growth factor 2 (FGF-2) was important in the differentiation of hESCs into GLCs [26]. FGF-2 is a known proliferation, anti-apoptotic and differentiation factor that acts through the MAPK and PI3K pathways indicating that these two pathways may play a central role in the differentiation of GLCs [31,32]. Improving our understanding of the potential involvement of the MAPK and PI3K in germ cell differentiation from hESCs not only could improve hESC to GLC differentiation cultures, providing a human model for study, but also could provide a tool to identify targets for novel infertility treatments

and to understand the mechanisms behind conditions related to abnormal gametogenesis (e.g. Down's and Kleinfelter's Syndromes)..

The objective of this study was to determine changes in MAPK and PI3K pathway signaling in hESC derived GLCs relative to their parent hESC population to understand better the potential mechanisms orchestrating differentiation and development of human germ cells. We demonstrated up regulation of genes relative to hESCs previously shown in in vivo counterparts to be important to primordial germ cell development including MAP2K2, MAPK14, GSK3β and eukaryotic initiation factors. In addition, down regulation or absence of embryonic markers *Atf2*, *Rhoa*, *Rheb* and *Elk1* and later-stage germ cell markers *Mapk1*, *Pten*, *Grb2*, *Rac1*, *Mos* and *Foxo3* further confirmed that these GLCs were in a primordial germ cell state. Additionally, we demonstrate that inhibition of the MAPK pathway greatly increases apoptosis and decreases proliferation of GLCs while this effect is limited in hESCs suggesting a role for the MAPK pathway in preventing apoptosis and increasing proliferation of GLCs. Finally, results showed a significant increase in apoptosis when the GSK3β pathway is inhibited as well as an increase in GSK3β gene expression suggests that GLC rapid expansion occurs through this mechanism, while the mTOR pathway is involved in genetic changes often seen in GLC expansion.

**Methods**

*hESC Cultures*

BG01 hESCs were cultured on mouse embryonic fibroblast (Harlan, Indianapolis, IN, USA) feeders inactivated by mitomycin C (Sigma-Aldrich, St. Louis, MO, USA) in 20% knockout serum replacement media consisting of Dulbecco's modified Eagle medium/F12 medium (Gibco, Carlsbad, CA, USA) supplemented with 20% knockout serum replacement (Gibco), 2mM L-glutamine (Gibco), 0.1 mM non-essential amino acids (Gibco), 50 units/ml penicillin/50μ/ml streptomycin (Invitrogen, Carlsbad, CA, USA), 0.1mM β-mercaptoethanol

(Sigma-Aldrich, St. Louis, MO, USA) and 4ng/ml basic fibroblast growth factor (bFGF; R&D, Minneapolis, MN, USA). They were maintained in 5% CO2 and at 37°C. Cells were passaged every 3 days by mechanical dissociation, re-plated on fresh feeders to prevent undirected differentiation with daily media changes as previously described [33].

*Germ-like Cell Culture*

Homogeneous GLC lines were derived from BGO1 (XY) hESCs as previously described [30]. GLCs were cultured on mouse feeders (Harlan) inactivated by mitomycin C (Sigma-Aldrich). Cells were cultured in 20% KSR stem cell media consisting of Dulbecco's modified Eagle medium (DMEM)/F12 supplemented with 20% KSR (Gibco), 2 mM L-glutamine (Gibco), 0.1 mM non-essential amino acids (Gibco), 50 111 units/ml penicillin (Gibco), 50 µg/ml streptomycin (Gibco), 0.1mM β-mercaptoethanol (Sigma-Aldrich) and 4 ng/ml bFGF (Sigma-Aldrich and R & D Systems). They were maintained in 5% CO2 and at 37°C. Cells were enzymatically passaged using 0.05% trypsin (Gibco).

*Quantitative Polymerase Chain Reaction (qPCR)*

RNA was extracted using the Qiashredder and RNeasy kits (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. The RNA quality and quantity was verified using a RNA 600 Nano Assay (Agilent Technologies, Santa Cruz, CA, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA (5 µg) was reverse-transcribed using the cDNA Archive Kit (Applied Biosystems Inc., Carlsbad, CA, USA) according to manufacturer's instructions. Reactions were initially incubated at 25$^{o}$C for 10 minutes and subsequently at 37$^{o}$C for 120 minutes. RT-PCR ($RT^2$ Profiler PCR Array, SABiosciences, Frederick, MD, USA) assays were used for the MAPK pathway (Human MAP Kinase Signaling Pathway, SABiosciences, Frederick, MD, USA) and PI3K pathway (Human PI3K-AKT Signaling Pathway, SABiosciences, Frederick, MD, USA) to analyze the expression of 84 genes for each pathway. The cDNA samples were diluted in 91µL of $ddH_2O$. From the cDNA samples 120µL were mixed with 550µl of 2X $RT^2$ SYBR Green qPCR Master Mix (SABiosciences, Frederick, MD, USA) and 448µL of

dd$H_2O$, then loaded into respective channels on the microfluidic cards followed by centrifugation. The card was sealed and real-time PCR and relative quantification was carried out on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Inc, Carlsbad, CA, USA). All failed (undetermined) reactions were excluded and ΔCt values were calculated. For calculation of relative fold change values, the software provided with the assays on the SABioscience's website ($RT^2$ Profiler PCR Array Data Analysis, SABiosciences, Frederick, MD, USA). All failed (undetermined) reactions were excluded and ΔCt values were calculated. For calculation of relative fold change values, initial normalization was achieved against endogenous 18S ribosomal RNA using the ΔΔCT method of quantification. Average fold change from three independent runs were calculated as $2^{\Delta\Delta CT}$. Significance was determined by running a 2-way ANOVA and Tukey's Pair-Wise (Statistical Analysis Software, SAS Institute, Cary, NC, USA) comparisons for each gene. Treatments where there was a fold change of greater than 4-fold were considered significant following manufacturer's recommendation.

*Cell Cycle Analysis*

hESCs and GLCs were analyzed for cell cycle effects using propidium iodide (Invitrogen). hESCs and GLCs were differentiated for 21 days before inhibitors for insulin-like growth factor 1 (IGF1; Tyrphostin AG-1024, Enzo Life Sciences, Farmingdale, NY, USA; 1ng/ml), cyclin-dependent kinase *2* (CDK2; AG-494, Enzo Life Sciences, Farmingdale, NY, USA; 1ng/ml), G1 (CI898, Tocris, Ellisville, MI, USA; 10ng/ml), G1 (Daidzein, Tocris, Ellisville, MI, USA; 10ng/ml), glycogen synthase kinase 3 beta (GSK3β; Indirubin, Tocris, Ellisville, MI, USA; 1ng/ml), Rac1 (NSC23766, Tocris, Ellisville, MI, USA; 10ng/ml), mammalian target of rapamycin (mTOR ;Rapamycin, EMD Biosciences, Gibbstown, NJ, USA; .3ng/ml), MAPK (PD98059, Enzo Life Sciences, Farmington, NY, USA; 10ng/ml), ERK (PD035901, Cayman Chemicals, Ann Arbor, MI, USA; 10ng/ml), p38 (SB202190, Enzo Life Sciences, Farmingdale, NY, USA; 10ng/ml) and Shc (Sclerotiorin, Cayman Chemicals, Ann Arbor, MI, USA; 10ng/ml) were added for 24 hours. After 24 hours, cells were harvested and washed in PBS-/- before

being fixed in cold 70% ethanol at 4°C for 30 minutes. Cells were then washed 2 times in PBS-/- before adding 200µL of 50µg/ml PI. Cells were quantified on Dako Cyan (Beckman Coulter, Brea, CA, USA). Negative controls were secondary only and cell only staining. Cell quantification was done using FlowJo (TreeStar, Ashland, OR, USA) software. Each experiment was run in triplicate. Data are presented as mean ±SD. Values of $p<.05$ was considered significant using ANOVA and Tukey's Pair-Wise (Statistical Analysis Software, SAS Institute, Cary, NC, USA). Each treatment was normalized to the non-treated control cells and background staining.

*Apoptosis Assay*

hESCs and GLCs were analyzed for apoptosis effects using Caspase Glo 3/7 assay (Promega, Madison, WI, USA). hESCs and GLCs were transferred to a 96 well plate and inhibitors for IGF1 (Tyrphostin AG-1024, Enzo Life Sciences, Farmingdale, NY, USA; 1ng/ml), CDK2 (AG-494, Enzo Life Sciences, Farmingdale, NY, USA; 1ng/ml), G1 (CI898, Tocris, Ellisville, MI, USA; 10ng/ml), G1 (Daidzein, Tocris, Ellisville, MI, USA; 10ng/ml), GSK3β (Indirubin, Tocris, Ellisville, MI, USA; 1ng/ml), Rac1 (NSC23766, Tocris, Ellisville, MI, USA; 10ng/ml), mTOR (Rapamycin, EMD Biosciences, Gibbstown, NJ, USA; .3ng/ml), MAPK (PD98059, Enzo Life Sciences, Farmingdale, NY, USA; 10ng/ml), ERK (PD035901, Cayman Chemicals, Ann Arbor, MI, USA; 10ng/ml), p38 (SB202190, Enzo Life Sciences, Farmingdale, NY, USA; 10ng/ml) and Shc (Sclerotiorin, Cayman Chemicals, Ann Arbor, MI, USA; 10ng/ml) were added for 24 hours.. Caspase Glo 3/7 assay (Promega, Madison, WI, USA) was used following manufacturer's directions to analyze apoptosis. The buffer was added to the substrate and the substrate dissolved. 100µL of this mix was added to each well of the 96 well plate and incubated for 1 hour at RT protected from light. The plate was then analyzed on the Flexstation 3 (Molecular Devices, Sunnyvale, CA, USA). Data are presented as mean ±SD. Values of $p<.05$ was considered significant using ANOVA and Tukey's Pair-Wise (Statistical Analysis Software,

SAS Institute, Cary, NC, USA). Each treatment was normalized to the non-treated control cells and background staining.

*Proliferation Assay*

hESCs and GLCs were analyzed for proliferation effects using Click-iT EdU High Content Screen kit (Invitrogen, Carlsbad, CA, USA). hESCs and GLCs s were transferred to a 96 well plate and inhibitors for IGF1 (Tyrphostin AG-1024, Enzo Life Sciences, Farmingdale, NY, USA; 1ng/ml), CDK2 (AG-494, Enzo Life Sciences, Farmingdale, NY, USA; 1ng/ml), G1 (CI898, Tocris, Ellisville, MI, USA; 10ng/ml), G1 (Daidzein, Tocris, Ellisville, MI, USA; 10ng/ml), GSK3β (Indirubin, Tocris, Ellisville, MI, USA; 1ng/ml), Rac1 (NSC23766, Tocris, Ellisville, MI, USA; 10ng/ml), mTOR (Rapamycin, EMD Biosciences, Gibbstown, NJ, USA; .3ng/ml), MAPK (PD98059, Enzo Life Sciences, Farmingdale, NY, USA; 10ng/ml), ERK (PD035901, Cayman Chemicals, Ann Arbor, MI, USA; 10ng/ml), p38 (SB202190, Enzo Life Sciences, Farmingdale, NY, USA; 10ng/ml) and Shc (Sclerotiorin, Cayman Chemicals, Ann Arbor, MI, USA; 10ng/ml) were added for 24 hours. were added for 24 hours. Click-iT EdU High Content Screen kit (Invitrogen) was used following manufacturer's instructions to analyze proliferation. EdU expression level was measured on the Flexstation 3 (Molecular Devices, Sunnyvale, CA, USA). Cell nuclei were stained using DAPI (Invitrogen, Carlsbad, CA, USA). Fluorescence was visualized using spinning disk confocal microscope (Olympus, Center Valley, PA, USA). Negative controls included secondary only staining. Data are presented as mean ±SD. Values of $p<.05$ was considered significant using ANOVA and Tukey's Pair-Wise (Statistical Analysis Software, SAS Institute, Cary, NC, USA). Each treatment was normalized to the non-treated control cells and background staining.

**Results**

*Up Regulation of MAPK Pathway Gene Expression in Germ-like Cells*

MAPK and PI3K signaling has proven to be critical in normal development of germ cells in vivo and are expected to play an important role in hESC derived germ cells. To determine the

role of MAPK and PI3K signaling in GLCs, changes in gene expression of both signaling pathways were determined by comparing GLCs to their parent hESC population. Relative to hESCs, GLCs demonstrated up regulation of the p38 and ERK pathway genes *Braf* (5 fold), *Map3k4* (7 fold), *Map2k1* (23 fold), *Mapk7* (33 fold), *Mapk10* (160 fold) and *Mapk13* (11 fold) (Figure A.1A). Additionally, MAPK genes involved in germ cell differentiation were up regulated significantly (> 4 fold) relative to hESCs and include *Crebbp* (6 fold), *Map2k2* (27 fold), *Mapk14* (88 fold) and *Max* (9 fold; Figure A.1B). Involved in later spermatogenesis, *Mapk1* is significantly (>4 fold) down regulated 12 fold in GLCs relative to hESCs (Figure A.1B).

RT-PCR Analysis of MAPK, mTOR, GSK3 and IGF1 gene expression demonstrated significant up regulation of a number of genes in GLCs, most of which have been previously shown to modulate proliferation and apoptosis. To determine whether these signaling pathways play an important role in proliferation and apoptosis, pathways were inhibited with compounds specific to each. PD98059, a common inhibitor which prevents MEK from binding and activating its receptor, was cultured with hESCs and GLCs. Inhibition of MEK with PD98059 resulted in a significant ($p<0.05$) 11 fold increase in apoptosis in GLCs relative to hESCs with inhibitor and GLCs cultured without inhibitor (Figure A.1C). Caspase levels in hESCs cultured with inhibitor decreased significantly ($p>0.05$) relative to hESCs cultured without inhibitor (Figure A.1C). GLCs significantly ($p<0.05$) decreased in proliferation by 29 fold relative to hESCs and to GLCs without inhibitors when cultured with PD98059 (Figure A.1C). To identify further the particular MAPK sub-pathway that effects GLC differentiation, the MAPK sub-pathway ERK was inhibited utilizing the PD035901 ERK inhibitor, which acts through suppressing the phosphorylation of ERK. Addition of PD035901 to GLC cultures significantly ($p<0.05$) increased apoptosis by 14 fold or greater relative to hESCs with inhibitor and GLCs cultured without inhibitor (Figure A.1D). Proliferation of GLCs with inhibitor decreased significantly ($p<0.05$) by 16 fold relative to hESCs with inhibitor and relative to GLCs without inhibitor (Figure A.1D). Inhibiting the p38 pathway with SB202190, which binds to the ATP binding pocket of the p38 receptor to prevent its

activation, significantly ($p<0.05$) increased apoptosis of GLCs by 5.3 fold compared to hESCs cultured with inhibitor, while significantly ($p<0.05$) decreasing proliferation in GLCs by 21 fold relative to hESCs cultured with inhibitor (Figure A.1E). GLC apoptosis is up regulated significantly ($p >0.05$) when cultured with inhibitor relative to culture without inhibitor and proliferation is significantly ($p >0.05$) down regulated when cultured with inhibitor relative to culture without inhibitor (Figure A.1E). Apoptosis increased significantly ($p> 0.05$) in hESCs cultured with inhibitor compared to hESCs cultured without inhibitor (Figure A.1E). The proposed mechanism through which the MAPK pathway is activated in GLCs is outlined in Figure A.1F.

*Aspects of PI3K pathway up Regulated in Germ-like Cells*

Genes that are involved in the mTOR pathway are significantly (> 4 fold) up regulated in GLCs relative to hESCs including *Eif4e* (29 fold), *Eif4g1* (14 fold) and *Eif2ak2* (14 fold; Figure A.2A). *Pten*, a factor inactivated in primordial germ cells, is significantly (> 4 fold) down regulated (19 fold; Figure A.2A). *Gsk3*$\beta$ is significantly (> 4 fold) up regulated 14 fold relative to hESCs and *Smad4* is significantly (> 4 fold) up regulated 6 fold relative to hESCs (Figure A.2B). The spermatozoa marker *Grb2* is significantly down regulated (>4 fold) relative to hESCs (4 fold; Figure A.2B).

Inhibition of the mTOR pathway with rapamycin, which binds to mTOR's receptor to prevent mTOR activation, significantly ($p<0.05$) decreased GLC apoptosis by 6 fold relative to hESCs cultured with rapamycin (Figure A.2C). hESC apoptosis increased significantly ($p>0.05$) when cultured with inhibitor relative to culture without inhibitor (Figure A.2C). Proliferation decreased significantly ($p<0.05$) by 28 fold in GLCs cultured with rapamycin relative to hESCs and relative to GLCs cultured without inhibitor (Figure A.2C). Indirubin, an inhibitor of GSK3$\beta$ which prevents the phosphorylation of the GSK3$\beta$ inhibitor, significantly ($p<0.05$) increased apoptosis by 5 fold in GLCs relative to hESCs while proliferation decreased

significantly ($p<0.05$) by 8 fold in GLCs relative to hESCs (Figure A.2D). Culture of GLCs with indirubin significantly ($p<0.05$) increased apoptosis relative to GLCs cultured without inhibitor while proliferation significantly ($p>0.05$) decreased in GLCs with inhibitor relative to GLCs without inhibitor (Figure A.2D). The proposed mechanisms through which GLCs activate the AKT pathway are outlined in the pathway in Figure A.2E.

*Genes Involved in Germ Cell Development Affected in Germ-like Cells*

While *Cdc42*, a cell polarity modulator [34], is significantly (> 4 fold) up regulated by 32 fold relative to hESCs, proteins involved in acrosome reaction, cell proliferation, cell motility and endocytosis [35] *Rac1* (27 fold) and *Pak1* (4 fold) are significantly (> 4 fold) down regulated in germ-like cells relative to hESCs (Figure A.3A). NSC23766, a RAC1 inhibitor which prevents guanine nucleotide exchange factors (GEFs) from interacting with RAC1, significantly ($p<0.05$) increased proliferation in GLCs cultured with inhibitor by 19 fold relative to hESCs cultured with inhibitor (Figure A.3B). Apoptosis significantly ($p>0.05$) increased in GLCs cultured with inhibitor relative to GLCs cultured without inhibitor and proliferation significantly ($p>0.05$) decreased in GLCs cultured with inhibitor relative to GLCs cultured without inhibitor (Figure A.3B). Proliferation of GLCs decreased significantly ($p<0.05$) by 19 fold relative to hESCs when cultured with inhibitor while metabolism of hESCs increased significantly ($p<0.05$) by four fold relative to GLCs (Figure A.3B). The potential mechanism through which the RAC1 pathway is activated in GLCs is outlined in Figure A.3C.

*Germ Cells Proliferate at a Higher Rate than hESCs*

Relative to hESCs, GLC expression of IGF1 (8 fold ± 2 fold) and IGFR (6 fold ± 2 fold) increased significantly (> 4 fold; Figure A.4A). SHC traffics IGFR into and out of the cell membrane as needed. Inhibiting this trafficking protein would prevent further activation of IGF through presenting new receptors being inserted into the membrane. SHC is inhibited by Sclerotiorin through its prevention of SHC interaction with GRB2. While apoptosis in GLCs significantly ($p<0.05$) increased by 5 fold relative to hESCs when cultured with inhibitor,

proliferation decreased significantly ($p<0.05$) by 26 fold in GLCs relative to hESCs when cultured with sclerotiorin (Figure A.4B). Apoptosis in GLCs cultured with inhibitor increased significantly ($p>0.05$) relative to GLCs cultured without inhibitor (Figure A.4B) and proliferation in GLCs cultured with inhibitor decreased significantly ($p>0.05$) relative to GLCs cultured without inhibitor (Figure A.4B). The mechanism through which SHC modulated IGF1 receptor insertion into the membrane is outlined in Figure A.4C.

Early in mouse germ cell development, primordial germ cells are highly proliferative starting with an initial population of ~45 cells at embryonic day 7 (E7) and expanding to ~ 50,000 cells at E13 [36,37]. This suggests that GLCs similar to PGCs should express high levels of proliferation genes. Gene expression analysis showed significant (>4 fold) up regulation in GLCs relative to hESCs of proliferation genes cyclins CCNA1 (5 fold) and CCNB2 (7 fold; Figure A.5A). Additionally, cell cycle regulators CDKN2A (7 fold), CDKN2B (24 fold), CDKN2D (11 fold) and E2F1 (23 fold) are also up regulated significantly (>4 fold) in GLCs relative to hESCs (Figure A.5A). Gene expression data was supported by the EdU proliferation assay, which showed that GLCs proliferated 8 times faster than hESCs (Figure A.5B).

**Discussion**

In this study, we demonstrated key differences in the MAPK and PI3K signaling pathways between hESCs and derived germ-like cells that suggest GLCs are highly similar to in vivo germ cells. Additionally, GLCs showed significant changes in proliferation and apoptosis in inhibitor studies, even more so than in hESCs. This suggests that MAPK and PI3K signaling may play a more critical role in germ cell development than in hESCs. When MEK and its sub-pathways ERK and p38 are inhibited with their respective inhibitors (PD-98059, PD035901 and SB202190) apoptosis in GLCs significantly ($p>0.05$) increases relative to apoptosis in hESCs and to GLCs without inhibitor. Additionally, inhibition of these pathways causes a significant decrease in proliferation relative to hESCs and to GLCs without inhibitor. In the PI3K pathway, genes associated with mTOR and GSK3$\beta$ are up regulated in GLCs relative to hESCs. Inhibition

of the GSK3β pathway leads to an increase in apoptosis in GLCs while inhibition of the mTOR pathway leads to a decrease in apoptosis. Finally, increase in IGF1 and IGFR gene expression combined with increases in proliferation in GLCs relative to hESCs suggest increased division in the GLCs that would be expected of primordial germ cells.

The goal of this study was to determine the pathways that distinguish GLCs from hESCs in order to improve the characterization and differentiation of hESC derived GLCs for use in infertility models and a human germ cell differentiation model. Previously, the cells used in this study have been confirmed to express markers indicative of the migratory stage of germ cell development. There was no expression of late stage markers *Mos* and *Foxo3* or embryonic markers *Atf2* and *Rhoa* [38-41]. Additional studies on genes seen in late stage germ cells showed a down regulation of *Rac1* and *Pak1* seen in the acrosome [42] (Figure A.3A). Inhibition of RAC1, which has been shown to be involved in late stage germ cells in addition to being upstream to the MAPK pathway, increased apoptosis and decrease proliferation suggesting a role for RAC1 in earlier stage germ cells in maintaining proliferation through the MAPK pathway (Figure A.3B). The results of this study confirm the expression of primordial germ cell markers and lack of expression of later stage germ cell markers and embryonic stem cell markers.

The MAPK pathway can be divided into 3 main sub-pathways ERK, JNK and p38. The JNK pathway is involved in cell mediated stress response as well as scaffolding that aids in the transport of proteins within the cell and in maintaining the integrity of the cell [13]. The p38 MAPK pathway protects germ cells from heat-activated stress leading to apoptosis in those cells affected by heat-activated stress and decreasing the chance of mutated DNA to be passed to future generations [9]. Additionally, the p38 pathway maintains the mitotic spindle in DNA division during the rapid proliferation that occurs in primordial germ cells [43] (Figure A.5B). The ERK pathway is associated with the proliferation of germ cells, in ejaculated spermatids, RHO/RAC combine with SHC, GRB2 and ERK to maintain the acrosome cap and the germ cell enclosure [44] (Figure A.6B). In this study, we found up regulation of genes involved in the ERK

pathway including *Braf*, *Map2k1* and *Mapk10* in GLCs relative to the expression seen in hESCs (Figure A.1A). Additionally, germ cell specific genes in the ERK pathway were up regulated in GLCs relative to hESCs including *Map2k2* and *Creb* (Figure A.1B). These genetic changes confirm that aspects of the ERK pathway are involved in maintaining early germ cells and in proliferating primordial germ cells (Figure A.6B). Inhibition of ERK in GLCs and in hESCs decreased the proliferation of the GLCs while having no effect on the proliferation of the hESCs confirming the role for the ERK pathway in GLC proliferation. Apoptosis increased in GLCs over hESCs when ERK was inhibited suggesting a role for the ERK pathway in maintaining GLCs (Figure A.1D). A MEK inhibitor, which would block not only the ERK sub-pathway but also the p38 sub-pathway, decreased proliferation of GLCs with no change in hESCs (Figure A.1C). Apoptosis in the inhibited GLC inhibited with PD98059, a MEK inhibitor, increased more than what was seen with the ERK inhibitor suggesting the use of the p38 pathway in modulating apoptosis stress response in these germ cells. Additionally, the apoptosis level in GLCs inhibited with a p38 inhibitor increased more than the level seen with just the ERK inhibitor but less than that seen with the MEK inhibitor (Figure A.1E). This is balanced by a decline in proliferation in p38-inhibited GLCs suggesting that blocking the p38 pathway removes the cellular modulation to stress which is normally imparted by the p38 pathway thereby increasing apoptosis and decreasing proliferation [9]. These studies on the MAPK pathway confirm the role for ERK in proliferation, the role of p38 in apoptosis and suggest that these GLCs are primordial germ cells.

The PI3K/AKT pathway can be broken into several sub-pathways. Of importance to germ cells are the mTOR and the GSK3β pathways (Figure A.6A). PI3K has been shown to be important in spermatogenesis and spermatagonial expansion [45]. Additionally, through the Src family the PI3K pathway germ cell proliferation is increased [46]. PTEN, which inhibits AKT, is inactivated in primordial germ cells and acts to prevent pluripotency [16,47]. In this study, we found the up regulation of eukaryotic transcription factor genes in GLCs relative to hESCs which

are involved in germ cell expansion and chromatin building in germ cells including *Eif4e*, *Eif4g1* and *Eif2ak2* (Figure A.2A). Additionally, *Pten* is down regulated in GLCs relative to hESCs suggesting that these GLCs are primordial germ cells (Figure A.2A). Inhibition of the mTOR pathway decreased GLC proliferation while having no effect on hESC proliferation supporting the role for mTOR in germ cell expansion. While hESC apoptosis increased with mTOR inhibition, GLC apoptosis decreased (Figure A.2C). mTOR's role in germ cells is in chromatin building thereby allowing for correct DNA translation in cell division and without that corrective control, the GLCs proceed through apoptosis instead of dividing. *Gsk3*$\beta$ is up regulated in GLCs over hESCs, which is more highly expressed in primordial germ cells over hESCs (Figure A.6A). *Grb2* is down regulated in GLCs relative to hESCs and *Grb2* is activated in later stage germ cells in the acrosome [44]. Inhibition of GSK3$\beta$ increased apoptosis in GLCs while it decreased it in hESCs. The decrease in apoptosis is suggestive of the role GSK3$\beta$ in spermatogenesis and is further supported by the decrease in proliferation in GLCs with inhibition of GSK3$\beta$ [16] (Figure A.2D). These results suggest the role of the AKT pathway in spermatogenesis and chromatin modulation through the mTOR and GSK3$\beta$ pathways.

The increased proliferation of GLCs relative to hESCs suggested by the genetic markers from the PI3K and MAPK pathway is supported by the up regulation of cell cycle genes in GLCs relative to hESCs and results from the alamar blue proliferation assay (Figure A.5A, B). Additionally, cell growth factor, IGF1, and its receptor, IGFR, are up regulated in GLCs relative to hESCs (Figure A.4A). Inhibited SHC, involved in IGF receptor trafficking, shows a similar increase in apoptosis and decrease in proliferation suggesting the role for SHC in early germ cells is in maintaining the proliferation of the primordial germ cells [44,46].

In conclusion, examination of the MAPK pathway through genetic and functional analysis suggests a role for the pathway in GLCs for the rapid proliferation seen in primordial germ cells. The PI3K pathway maintains the proliferation and differentiation of primordial germ cells from

hESCs. The genetic expression profile shows up regulation of primordial germ cell genes, down regulation of hESC and late germ cell genes. This study provides evidence for the usefulness of these cells as a human germ cell model.

**Acknowledgements**

We thank Julie Nelson and the Center for Tropical and Emerging Global Diseases Flow Cytometry Facility for her technical expertise. This work was supported by DOD #N000140810989 and the GRA Eminent Endowment.

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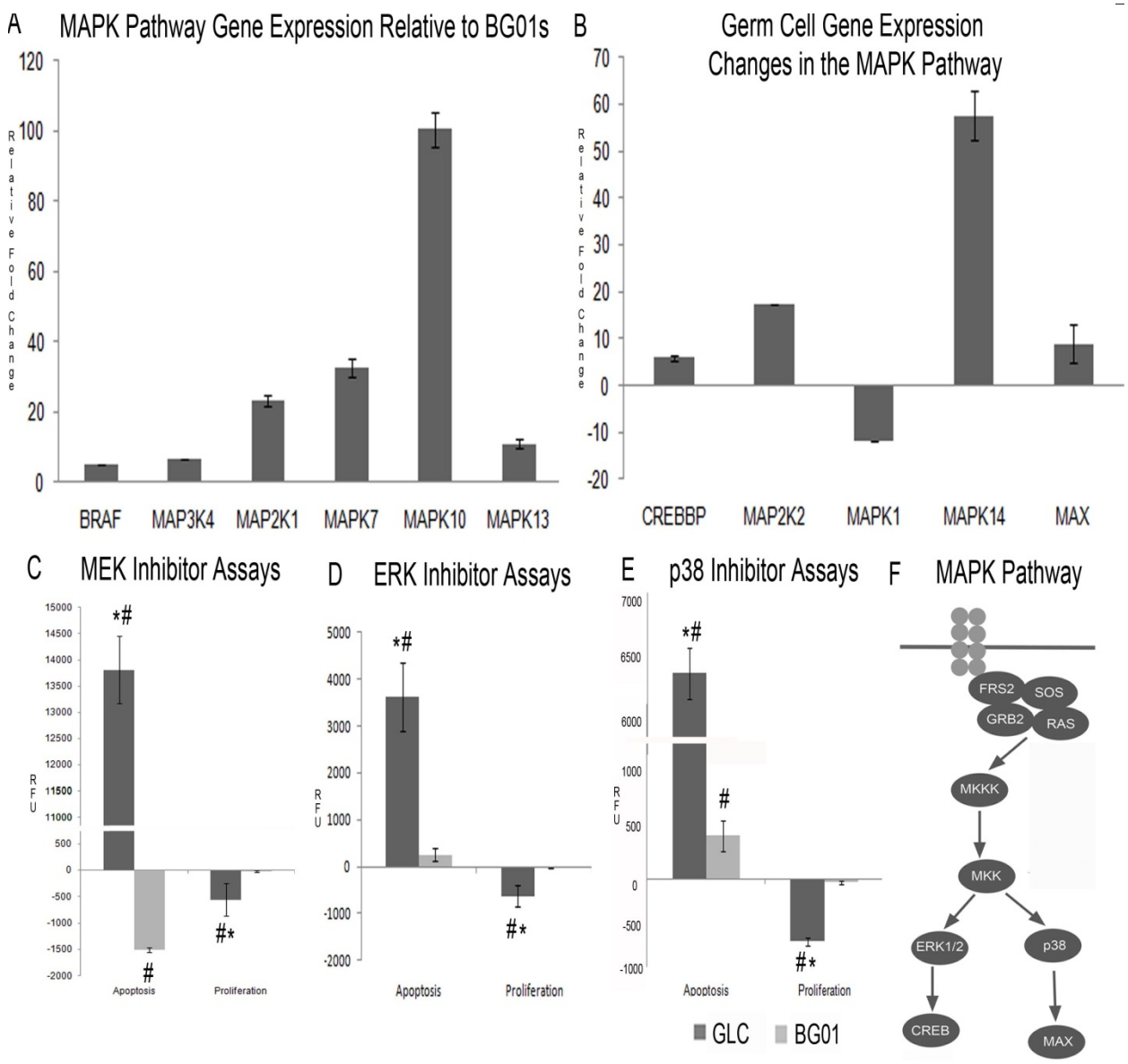
**Figure A.1:** The MAPK Pathway Maintains Proliferation in GLCs.

The MAPK pathway is up regulated in GLCs relative to hESCs, including the p38 and ERK sub-pathways (A). Additionally, ERK and p38 genes involved in germ cell proliferation were up regulated while *Mapk1* involved in late stage germ cells and *Elk1* seen in neural cells were down regulated (B). Inhibition of the p38 pathway suggests a role for p38 in modulating germ cell response to stress (E). ERK inhibition suggests a role for the ERK pathway in germ cell proliferation (D) and MEK inhibition suggests that MEK activates both the ERK and p38 pathway in early germ cells (C). GLCs are maintained and proliferated through the MAPK pathway (F) GLC – germ-like cells; ERK – extracellular-signal related kinase; * significantly ($p>0.05$) different from hESCs, # significantly ($p>0.05$) different from no inhibitor.


A
MAPK Pathway Gene Expression Relative to BG01s
Relative Fold Change
120
100
80
60
40
20
0
BRAF
MAP3K4
MAP2K1
MAPK7
MAPK10
MAPK13
B
Germ Cell Gene Expression Changes in the MAPK Pathway
Relative Fold Change
70
60
50
40
30
20
10
0
-10
-20
CREBBP
MAP2K2
MAPK1
MAPK14
MAX
C
MEK Inhibitor Assays
RFU
15000
14500
14000
13500
13000
12500
12000
11500
11000
500
0
-500
-1000
-1500
-2000
*#
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#*
Apoptosis
Proliferation
D
ERK Inhibitor Assays
RFU
5000
4000
3000
2000
1000
0
-1000
-2000
*#
#*
Apoptosis
Proliferation
E
p38 Inhibitor Assays
RFU
7000
6500
6000
1000
500
0
-500
-1000
*#
#
#*
Apoptosis
Proliferation
GLC
BG01
F
MAPK Pathway
FRS2
SOS
GRB2
RAS
MKKK
MKK
ERK1/2
p38
CREB
MAX

**Figure A.2:** mTOR and GSK3β Pathways Cause Proliferation and Differentiation of Primordial Germ Cells

In GLCs, transcription factors downstream of mTOR are up regulated while *Pten*, a factor which is inactivated in primordial germ cells, is down regulated (A). *Gsk3β*, involved in primordial germ cell differentiation from hESCs and spermatogenesis, is up regulated in GLCs. *Smad4* is up regulate din GLCs and involved in genetic expression of germ cell factors (B).*Grb2*, a late stage germ cell factor, is down regulated in GLCs (B). Inhibition of the mTOR pathway confirms the role for it in sperm expansion (C). GSK3β inhibition confirms its role in spermatogenesis (D). GLC – germ-like cells; mTOR – mammalian target of rapamycin; GSK3β - glycogen synthase kinase 3 beta; * significantly ($p>0.05$) different from hESCs, # significantly ($p>0.05$) different from no inhibitor.

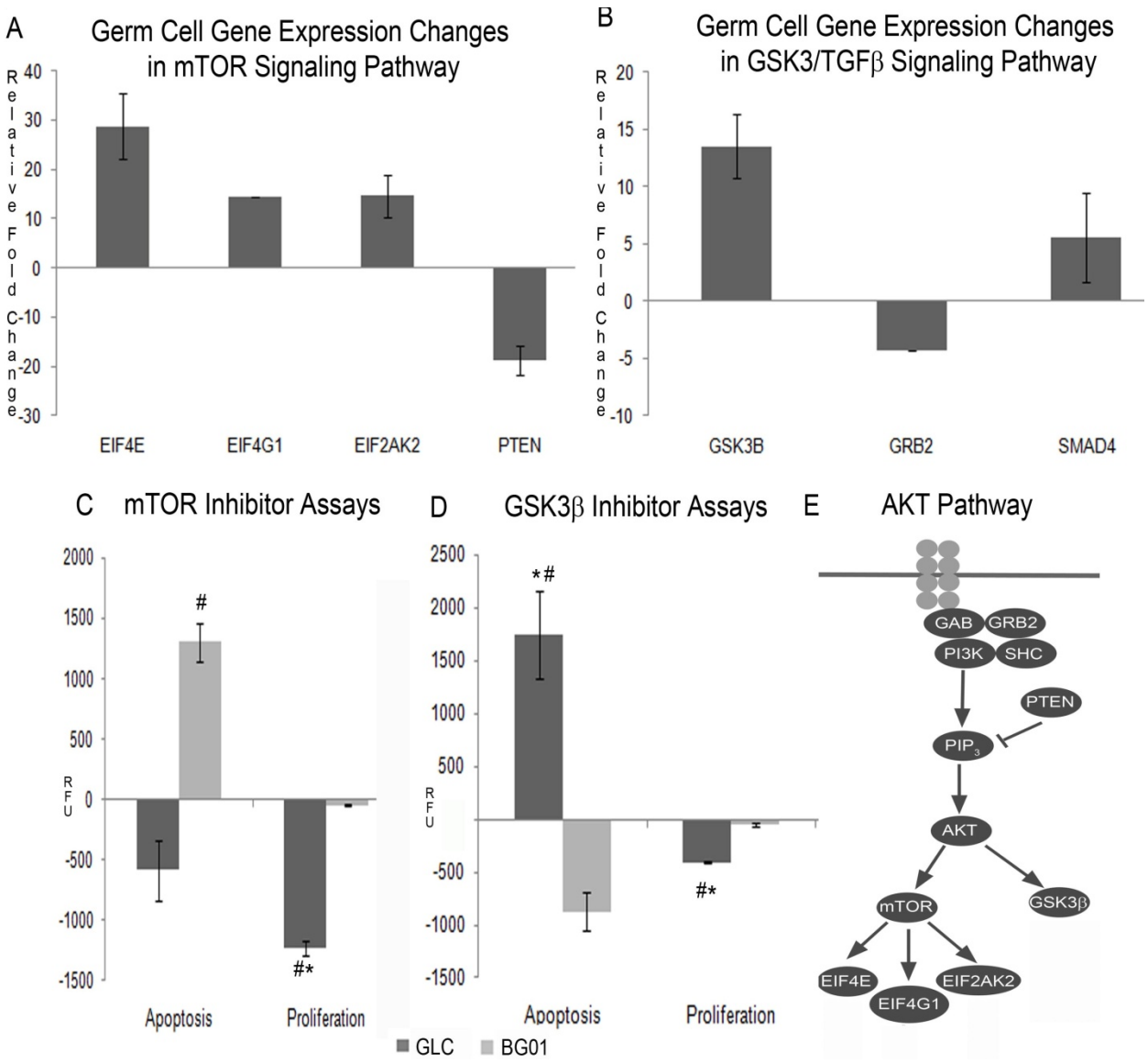

A
Germ Cell Gene Expression Changes in mTOR Signaling Pathway
Relative Fold Change
40
30
20
10
0
-10
-20
-30
EIF4E
EIF4G1
EIF2AK2
PTEN
B
Germ Cell Gene Expression Changes in GSK3/TGFβ Signaling Pathway
Relative Fold Change
20
15
10
5
0
-5
-10
GSK3B
GRB2
SMAD4
C
mTOR Inhibitor Assays
RFU
2000
1500
1000
500
0
-500
-1000
-1500
#
#*
Apoptosis
Proliferation
D
GSK3β Inhibitor Assays
RFU
2500
2000
1500
1000
500
-500
-1000
-1500
*#
#*
Apoptosis
Proliferation
GLC
BG01
E
AKT Pathway
GAB
GRB2
PI3K
SHC
PTEN
PIP3
AKT
mTOR
GSK3β
EIF4E
EIF2AK2
EIF4G1

**Figure A.3:** Late Stage Germ Cell Markers Down Regulated in GLCs

*Pak1* and *Rac1*, late stage germ cell markers, are down regulated in GLCs while Cdc42, is up regulated (A). Inhibition of Rac1 suggests a role for these genes in the survival of germ cells (B). The pathway through which Rac1 acts within the MAPK pathway is outlined in (c). GLC – germ-like cells; * significantly ($p>0.05$) different from hESCs, # significantly ($p>0.05$) different from no inhibitor.

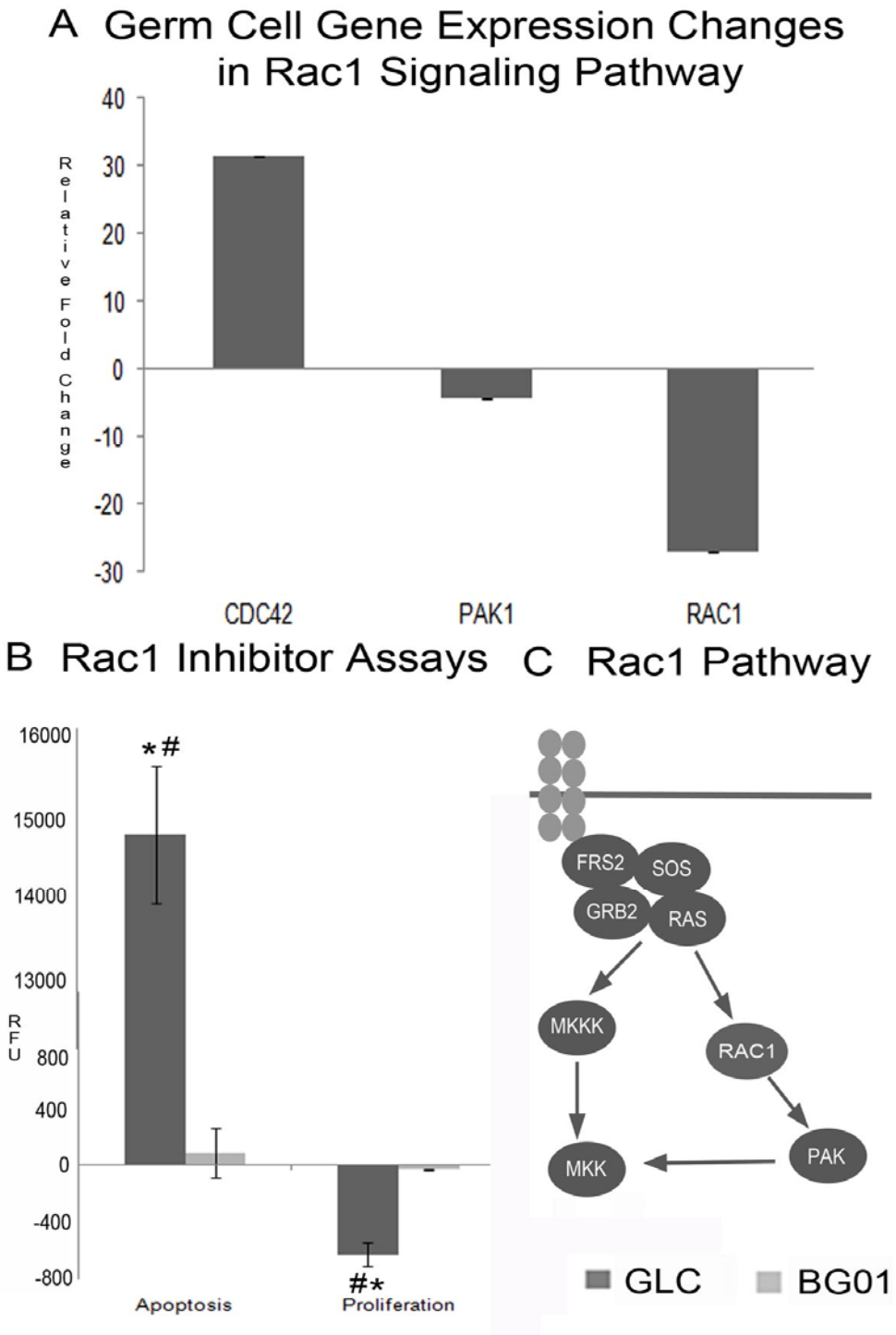

A Germ Cell Gene Expression Changes in Rac1 Signaling Pathway
Relative Fold Change
40
30
20
10
0
-10
-20
-30
CDC42
PAK1
RAC1
B Rac1 Inhibitor Assays
C Rac1 Pathway
16000
15000
14000
13000
RFU
800
400
0
-400
-800
*#
#*
Apoptosis
Proliferation
FRS2
SOS
GRB2
RAS
MKKK
RAC1
MKK
PAK
GLC
BG01

**Figure A.4:** Cell Growth Genes Up Regulated in GLCs

IGF1 and IGFR gene expression is up regulated in GLCs relative to hESCs suggesting an increase in cell growth relative to hESCs (A). Inhibition of SHC, the protein involved in receptor trafficking of IGFR increases apoptosis in GLCs relative to GLCs without inhibition while proliferation was significantly down regulated (B). (C) shows the pathway through which SHC modulated IGFR insertion into the membrane. IGF1 – insulin growth factor 1, IGFR – insulin growth factor receptor, GLC – germ-like cell; * significantly ($p>0.05$) different from hESCs, # significantly ($p>0.05$) different from no inhibitor.

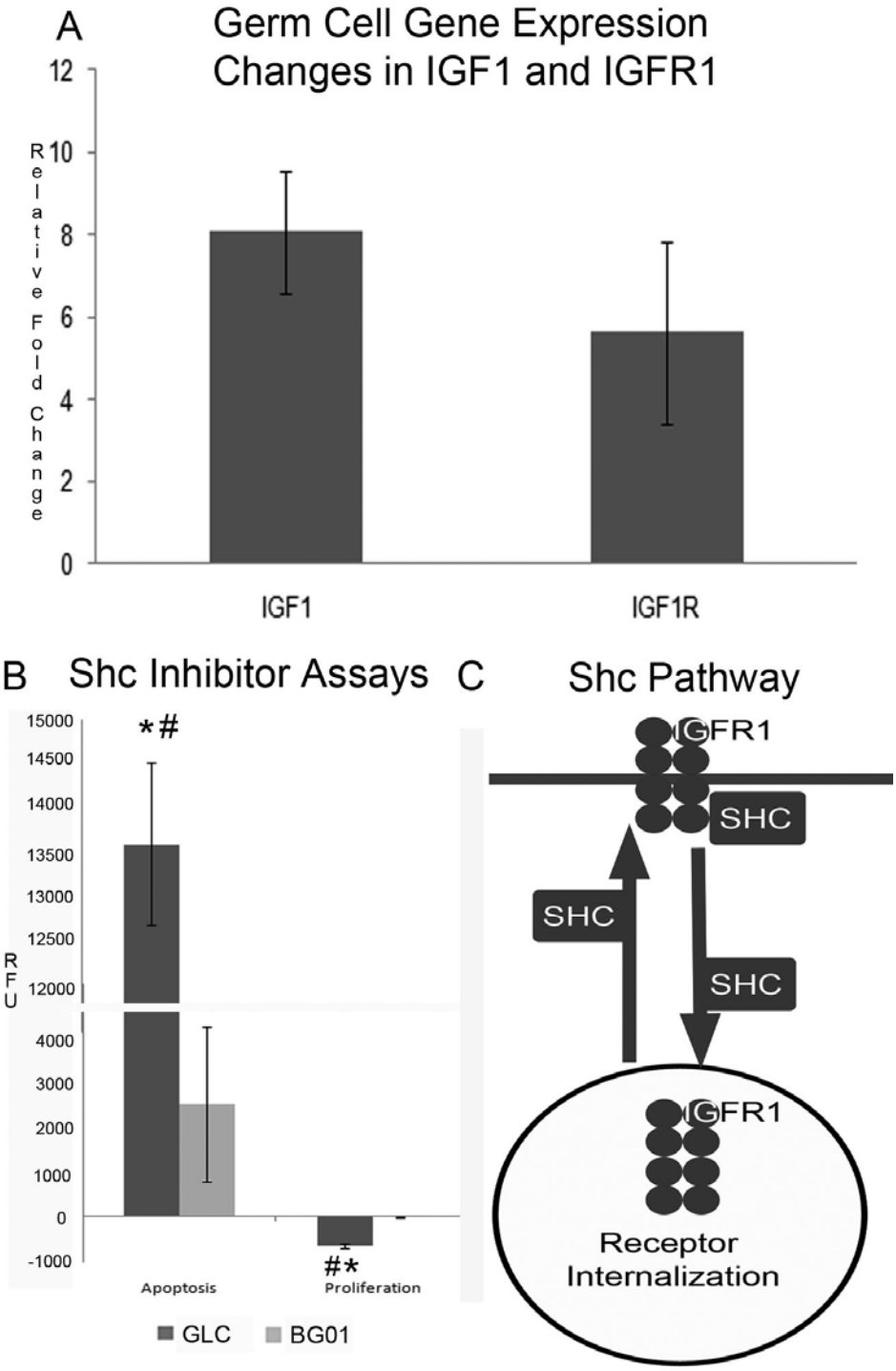

A
Germ Cell Gene Expression
Changes in IGF1 and IGFR1
Relative Fold Change
12
10
8
6
4
2
0
IGF1
IGF1R
B Shc Inhibitor Assays
C Shc Pathway
RFU
15000
14500
14000
13500
13000
12500
12000
4000
3000
2000
1000
0
-1000
*#
#*
Apoptosis
Proliferation
GLC
BG01
IGFR1
SHC
SHC
SHC
IGFR1
Receptor
Internalization

**Figure A.5:** Cell Cycle Genes are Up Regulated in GLCs

As a marker of the increased proliferation in germ cells compared to hESCs, cell cycle genes were analyzed with RT-PCR. Cyclin genes, *Ccna1* and *Ccnb2* were up regulated. Additionally cyclin modulators, *Cdkn2*a, *Cdkn2b*, *Cdkn2d* and *E2f1* are also up regulated (A). Proliferation as measured by EdU increased in GLCs relative to hESCs (B). hESCs – human embryonic stem cells; GLCs – germ-like cells; RFU – relative fluorescence unit; * significantly ($p>0.05$) different from hESCs, # significantly ($p>0.05$) different from no inhibitor.

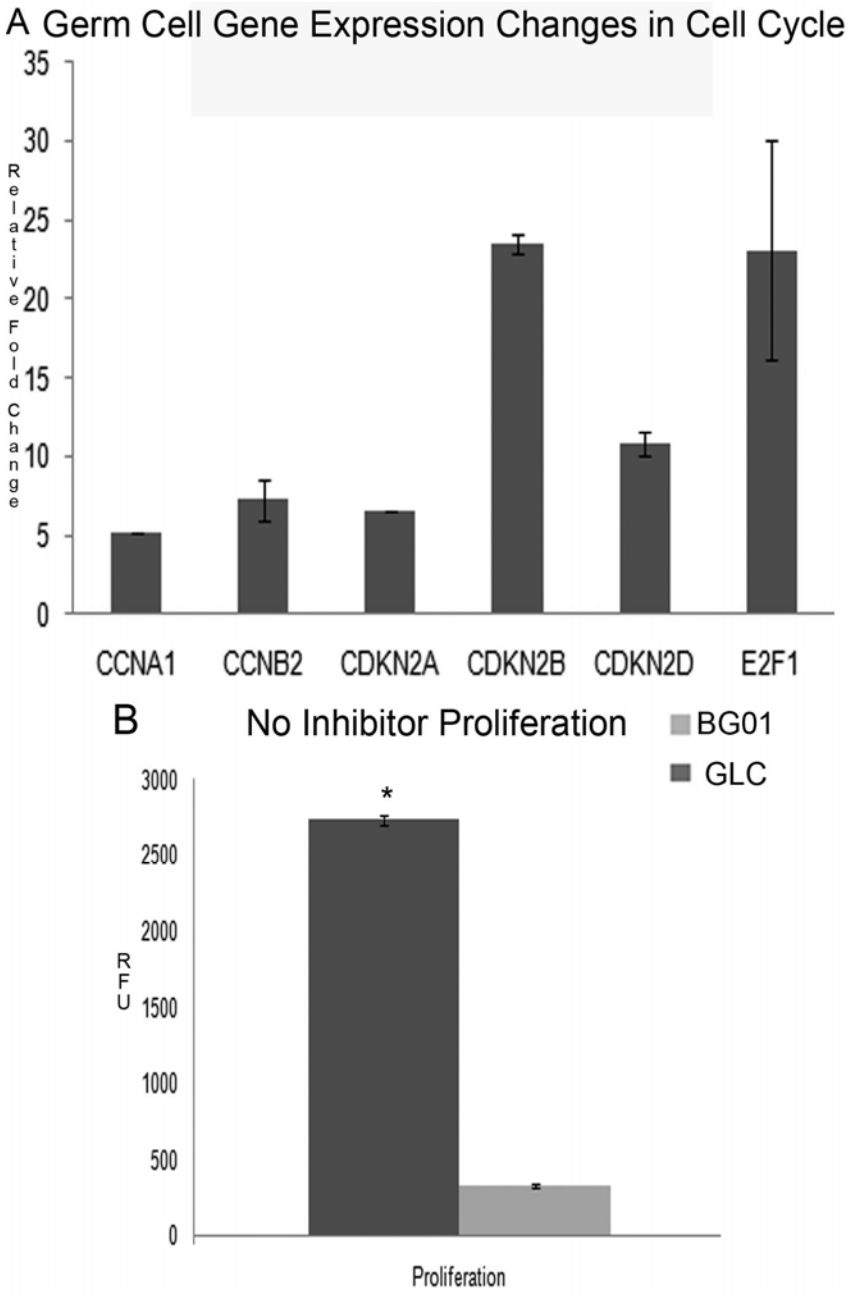
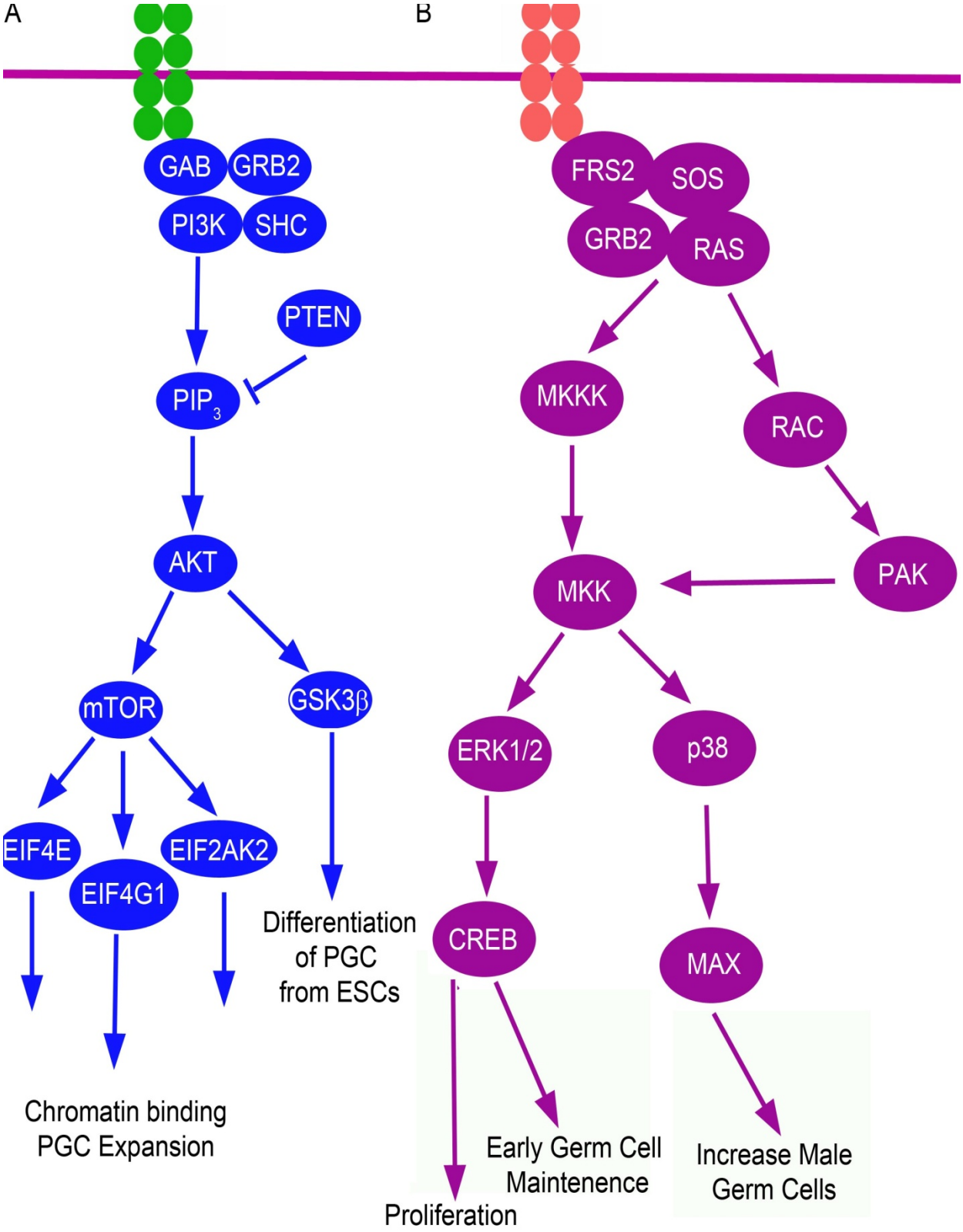

A Germ Cell Gene Expression Changes in Cell Cycle
Relative Fold Change
35
30
25
20
15
10
5
0
CCNA1
CCNB2
CDKN2A
CDKN2B
CDKN2D
E2F1
B
No Inhibitor Proliferation
BG01
GLC
RFU
3000
2500
2000
1500
1000
500
0
*
Proliferation

**Figure A.6:** Pathways involved in GLCs

The PI3K pathway in involved in GLCs through the mTOR and GSK3β sub-pathways. Activation of the mTOR pathway leads to sperm expansion and chromatin modulation while the GSK3β pathway is involved in spermatogenesis and in the differentiation of primordial germ cells from hESCs (A). Finally, PTEN is inactivated in primordial germ cells, and in late stage germ cells, it is responsible for controlling the rapid proliferation (A). Secondly, the MAPK sub-pathways ERK and p38 are activated in GLCs. The ERK pathway activation leads to CREB activation, which increases proliferation (C). The p38 pathway leads to MAX activation, which is involved in male germ cells (C). ELK1, involved in neural differentiation, is down regulated in GLCs (C). RAC1 and PAK1, commonly associated with late stage germ cells, are down regulated in GLCs (C). GLC – germ-like cells; TGFβ - transforming growth factor beta; MAPK – mitogen activated protein kinase; GSK3β - glycogen synthase kinase 3 beta.


A
B
GAB
GRB2
PI3K
SHC
PTEN
PIP3
AKT
mTOR
GSK3β
EIF4E
EIF4G1
EIF2AK2
Chromatin binding
PGC Expansion
Differentiation
of PGC
from ESCs
FRS2
SOS
GRB2
RAS
MKKK
RAC
PAK
MKK
ERK1/2
p38
CREB
MAX
Proliferation
Early Germ Cell
Maintenence
Increase Male
Germ Cells