The Dissertation Committee for Paivi Martina Jordan Certifies that this is the approved version of the following dissertation:

MOLECULAR AND CELLULAR REGULATION OF MOTONEURON DIFFERENTIATION OF HUMAN NEURAL STEM CELLS

Committee:

Ping Wu, M.D., Ph.D., Supervisor

Lisa A. Elferink, Ph.D.

David McAdoo, Ph.D.

Kathleen O'Connor, Ph.D.

Robert L. Tsai, Ph.D.

Dean, Graduate School

MOLECULAR AND CELLULAR REGULATION OF MOTONEURON DIFFERENTIATION OF HUMAN NEURAL STEM CELLS

by

Paivi Martina Jordan, BS

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas Medical Branch

March 2007

Dedication

This dissertation is dedicated to my friends and family who have supported me throughout this vast endeavor and to that special someone who has captured my heart and soul. The road to find you may have been long and winding, but had I not traveled that road, I would have never found you and my life would not be complete.

∞

The fact is that to do anything in the world worth doing we must not stand back shivering and thinking of the cold and danger, but jump in and scramble through as well as we can.

~Robert Cushing

Acknowledgements

First I would like to acknowledge the two women who have encouraged me through thick and thin; who have talked me off a ledge on many occasions; who listened to me; who supported me; who gave me hugs when I most needed them; and whose kind words and friendship mean more to me than anything: Erika and Janel, this Ph.D is as much my work as it is yours. A second big thank you goes out to the man who first introduced me to research, my undergraduate mentor, Dr. Phillip Senger. It was your voice I heard every time I wanted to quit, pushing me and telling me to continue on in my quest for knowledge no matter how difficult the road was to get there. Also a big thank you to my mentor, Dr. Ping Wu, who not only taught me how to be a scientist but also what it means to be a true mentor and a friend. When things were really difficult, I knew I could always count on her to get me through. A big thank you also to all of the faculty within the graduate school, especially within the neuroscience graduate program; your hard work is what allows students such as myself the honor of completing the Ph.D. Very heartfelt thanks to Pat Gazzoli whose technical assistance was absolutely essential in getting out my proposal, manuscripts and this dissertation. Your kindness, open heart and willingness to put in time outside of work to help are what true teaching is all about. Last but certainly not least, a very heartfelt thank you to the members of my committee, Dr.'s David McAdoo, Lisa Elferink, Kathleen O'Connor and Robert Tsai. Your guidance throughout this process has been instrumental in helping me to develop into the scientist and person that I am today.

MOLECULAR AND CELLULAR REGULATION OF MOTONEURON DIFFERENTIATION OF HUMAN NEURAL STEM CELLS

Publication No._____

Paivi Martina Jordan, Ph.D.

The University of Texas Medical Branch, 2007

Supervisor: Ping Wu, M.D., Ph.D.

One main goal of stem cell biologists is to efficiently direct stem cells into particular cell fates useful for the treatment or study of neurological disease. Towards that goal, our laboratory has previously developed an *in vitro* procedure consisting of basic fibroblast growth factor (bFGF), heparin and laminin (FHL), which directs human fetal neural stem cells (hNSCs) to differentiate into choline acetyltransferase (ChAT) motoneurons *in vitro* and after transplantation into the rodent spinal cord *in vivo*. However, the mechanisms surrounding FHL-induced motoneuron differentiation of hNSCs are unknown. Thus, the overall goal for this thesis was to further understand the molecular and cellular basis underlying FHL-primed hNSC differentiation. As a first step, we focused upon the growth factor bFGF. We demonstrate here that application of exogenous bFGF induced hNSCs towards the motoneuron lineage *in vitro.* In particular, FHL-primed hNSCs expressed motoneuron developmental transcription factors including Olig2, Ngn2, Islet1 and Hb9.

We also found that the motoneuron determinant, Hb9, was induced in hNSCs in bFGF-dose and time-dependent manner and that blockade of bFGF signaling reduced Hb9 expression. Furthermore, bFGF treatment did not significantly enhance cell survival and bFGF dependent proliferation was not required for Hb9 expression.

Because astrocytes are a known producer of bFGF and are also part of the post-transplantation environment, we further strove to understand the role of astrocytes and astrocytic-secreted factors (including astrocytic-secreted bFGF) upon hNSC differentiation and cell survival. In co-culture studies we found that astrocytes or astrocyte conditioned media (ACM) enhanced hNSC viability, decreased cell death and in particular, decreased apoptotic cell death. Interestingly, addition of astrocytes did not affect the overall neuron: glia ratio in long-term (LT) differentiated hNSCs. However, LT survival of a specific neuronal subtype, cholinergic neurons, was maintained in cultures with ACM but was lost in cultures differentiated without astrocytic factors. Addition of a FGF-receptor antagonist to ACM eliminated hNSC derived motoneurons from LT cultures suggesting that astrocyte secreted FGFs might modulate hNSC-derived cholinergic neuronal survival.

Together these studies demonstrate for the first time that bFGF is an important modulator of motoneuron differentiation and long-term *in vitro* maintenance of motoneurons differentiated from hNSCs.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

Stem cells have the potential to be useful clinical tools in the treatment of many neurological diseases including Alzheimer's disease and amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease), both of which are characterized by degeneration of cholinergic neurons. In Alzheimer's disease, cholinergic forebrain neurons are targeted; and in ALS, a subset of cholinergic cells, spinal motoneurons, are targeted for degeneration. Currently there are no efficacious treatments for these diseases. In regards to ALS, the average lifespan following the appearance of clinical symptoms is less than 5 years (Motoneuron Disease Association, http://www.mndassociation.org, 2006) and furthermore the quality of life for these patients is very poor. Although stem cells are a promising therapeutic, in order to adequately harness their therapeutic potentials for neurological disease treatment, we must first understand the molecular mechanisms guiding their cell type-specific differentiation and integration into the central nervous system.

The first objective of this dissertation is to understand the molecular mechanisms behind a bFGF, heparin and laminin (FHL) *in vitro* priming step which pushes human neural stem cells (hNSCs) towards a cholinergic phenotype (Chapter 3). After transplantation into rodent spinal cord, FHL-primed hNSCs differentiate into cholinergic cells that make connections with muscle targets and improve functional outcome after injury (Gao et al., 2005;Gao et al., 2006;Wu et al., 2002;Tarasenko et al., 2006). Because the transplantation environment is quite different from that found in the cell culture system and because bFGF is a molecule produced within the central nervous system mostly by astrocytic cells and is also utilized in our FHL priming procedure, my second objective was to understand whether astrocytic secreted bFGF might also influence hNSC cholinergic differentiation (Chapter 4).

In this chapter, I will first give a review on stem cells, including definitions and classifications, how these cells are cultured *in vitro* and the processes developed to direct differentiation of these cells particularly into cholinergic cells. Next, I will focus on background related to the potent mitogen and morphogen, bFGF. Finally, I will examine the role of other cell types (i.e. astrocytes) on the directed differentiation and survival of stem cells.

STEM CELLS

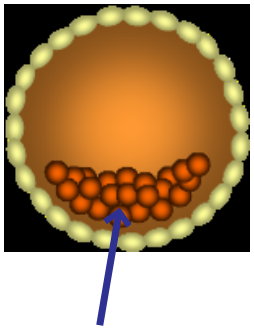
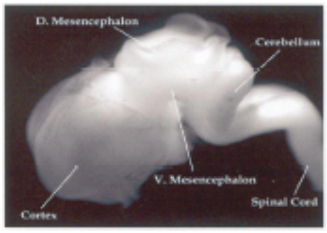
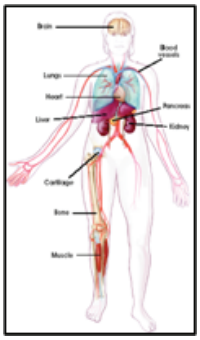
Definition of a stem cell

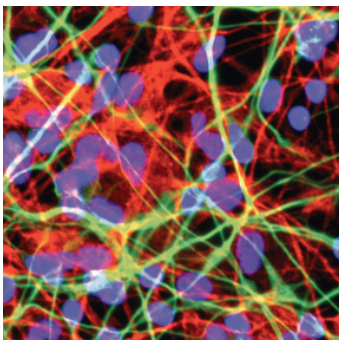
Stem cells are defined as any cell within a living organism, which can self renew indefinitely through cell division and retain the ability to differentiate into a variety of tissues (NIH Stem Cell Primer, http://stemcells.nih.gov, 2007). Stem cells can be derived from a variety of sources including embryonic, fetal and adult tissues. Embryonic tissues provide the largest reservoir of stem cells with the greatest ability to differentiate into diverse tissue types. In general, potency or the ability to differentiate into different tissues becomes more restricted as organisms age. Totipotent stem cells are the most primitive of cells having the unique ability to differentiate into any tissue within the body including the extra-embryonic (placental) tissues. These cells are found only within the first few divisions of the fertilized egg. Slightly more restricted are cells derived from the inner cell mass of the blastocyst. These pluripotent cells can become any of the three germ layers (endoderm, mesoderm or ectoderm) but can not form the extra-embryonic tissues. Embryonic stem cells (ESCs) typically fall in this category. A more restricted stem cell, such as one derived from the developing nervous system, is termed as multipotent, meaning it can develop into any of the cells within the nervous system. The most restricted of stem cells is the unipotent stem cell which is self-renewing but has the ability to only form one single cell type.

Cellular potency is not only restricted to age, however, location of stem cells or microenvironment is also an important factor. Within the central nervous system two areas retain pools of multipotent stem cells that are actively involved in neurogenesis well into adulthood. The two main neurogenic regions are the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricle. In addition to these neurogenic stem cells, adult stem cells can be found in almost all tissues, including bone marrow, cornea and retina, liver, skin, pancreas and tooth however, cells from these areas are less likely to participate in active neurogenesis (NIH Stem Cell Primer, http://stemcells.nih.gov, 2007).

The derivation of rodent (Evans and Kaufman, 1981) and human embryonic stem cells (mESCs and hESCs, respectively) (Thomson et al., 1998) have revolutionized our understanding of mammalian development, in particular the development of the central nervous system. Both rodent and human derived embryonic stem cells have been studied *in vitro* as a model to understand developmental biology and used *in vivo* in attempts to treat injury or disease. ES cells are the earliest form of stem cells and the only cell type which theoretically can remain indefinitely in culture without changes in karyotype, reduction in proliferative ability or potency changes; and when properly differentiated, can form all other types of stem cells (McDonald, 2001). ES cells are characterized by unlimited cellular division or self renewal *in vitro* while retaining expression of markers such as Oct-3/4, Sox2 and NANOG, transcription factors found in undifferentiated cells. Further characterization is typically done by examining cellular pluripotency via transplantation into immunocompromised mice and examining those animals for teratomas that contain all three germ layers. Although the ability to multiply indefinitely in culture and to form all cell types within the organism are benefits to using ES cells, the propensity to form tumors is one of the main problems that must be addressed when utilizing these cells *in vivo*. In order to avoid tumor formation, researchers often transform ES cells with inducible promoters or pre-differentiate cells prior to transplantation (McDonald, 2001). However,

Stem Cell Potency

Type of Cells	Embryonic Stem Cells	Fetal Neural Stem Cells	Adult Stem Cells
			
Area cells are derived from	Inner Cell Mass	Fetal brain mesencephalon, telencephalon etc.	Blood, neurogenic regions (i.e. Lateral Ventricles, etc)
% Stemness	100%	1-10%	0.01-0.1%
Potency	Pluripotent	Multipotent	Multipotent, Unipotent
Tumor Formation	Likely pre-differentiation is necessary	Unlikely	Highly unlikely



Neurons and Glia

Figure 1.1: Potency of differing types of stem cells. (Figure created in Adobe Illustrator with some images modified from http://stemcells.nih.gov/info/basics, 2007).

tumor formation is still a potential problem even with these genetic modifications. To avoid this issue, many groups including ours, have utilized cells derived from later stage embryos which retain multipotency but are less likely to be tumorigenic due in part to decreased activity of telomerase, an enzyme necessary to maintain telomere length (Figure 1.1). Telomerase is high expressed in both embryonic cells as well as in cancer cells but its activity is diminished in later derived stem cells such as neuronal stem cells (Hiyama and Hiyama, 2007).

Directed Differentiation of ES Cells

Using mouse embryonic stem cells, several groups have achieved high rates of specific neuronal differentiation. For example, Kawasaki et al. (2000) and Lee et al., (2000) utilized sonic hedgehog (SHH) and FGF8 to differentiate mES cells into a dopaminergic cell fate (Lee et al., 2000;Kawasaki et al., 2000). In particular, Lee et al. (2000) further enhanced dopaminergic differentiation by adding ascorbic acid in conjunction with FGF8 and SHH. Using this method they demonstrate that >33% of mES cells differentiate into dopaminergic neurons, and moreover that after depolarization that these cells release significant levels of dopamine as detected by reverse-phase-high performance liquid chromatography (RP-HPLC). In the same study, Lee et al. (2000) also showed that using either SHH alone or SHH with FGF8 also induced mES cells to differentiate into serotonergic expressing cells (~11%).

In regards to cholinergic cell types, sonic hedgehog and retinoic acid have been used to induce cholinergic differentiation from mES cells (Wichterle et al., 2002) and bFGF, sonic hedgehog and retinoic acid were utilized to derive cholinergic neurons from hES cells (Li et al., 2005). However, induction of cholinergic phenotype was limited in both of these studies to less than 30% (also see Table 1.1, and sub-section directed differentiation of stem cells into MNs *in vitro*).

Neural Stem Cells (NSCs)

Neural stem cells have been isolated from fetal (Svendsen et al., 1998;Villa et al., 2000;Carpenter et al., 1999) and adult (Temple and Alvarez-Buylla, 1999;Palmer et al., 1999;Reynolds and Weiss, 1992) central nervous systems. The hallmark features of these cells are self renewal and differentiation into all three cell types of the nervous system, i.e. astrocytes, neurons and oligodendrocytes. These features make hNSCs appealing sources for cell replacement therapy to treat many neurological disorders.

Neural stem cells can be expanded in culture as free floating aggregates termed neurospheres. Neurospheres are usually heterogeneous, including immature neurons, glia and stem/progenitor cells (Steindler et al., 2003;Reynolds et al., 1992), which can be phenotyped by specific markers such as beta tubulin type III, glial fibrillary acidic protein (GFAP) and nestin, respectively. It has been hypothesized that cells in each neurosphere grown in 3D undergo differentiation through a variety of stages, which may mimic cell growth and differentiation in the developing embryo. Thus the study of neurosphere biology could potentially reveal signals previously undiscovered during neuronal and glial development (Steindler et al., 2003).

Neurospheres are typically expanded *in vitro* under serum-free conditions in basal media supplemented with a cocktail of growth factors including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and/or leukemia inhibitory factor (LIF). Neurospheres derived from human cells have been expanded in bFGF alone (Amit et al., 2000;Zhang et al., 2001); EGF (Reynolds and Weiss, 1996); bFGF and EGF (Caldwell and Svendsen, 1998;Svendsen et al., 1998;Wright et al., 2003;Vescovi et al., 1999); EGF and LIF (Wright et al., 2003) or EGF, bFGF and LIF (Carpenter et al., 1999;Wu et al., 2002;Tarasenko et al., 2004). Many groups including ours have been able to maintain hNSCs under these *in vitro* conditions without loss of expression of undifferentiated markers or

karyotype changes for many passages (Wu et al., 2002;Carpenter et al., 1999;Uchida et al., 2000) while generating large numbers of cells potentially useful for clinical applications. Since these growth factors have different effects that are both cell type and developmental stage-dependent, it is not surprising that the addition of even low levels of growth factors such as bFGF changed phenotypic outcome in stem cell culture models (Qian et al., 1997;Gabay et al., 2003;Tsai and Kim, 2005). Thus when considering the results of any individual study it is important to take into consideration the factors and conditions used for cellular expansion.

In order to preserve phenotypic multipotency while maximizing expansion rate, we previously examined hNSCs cultured with EGF, bFGF and LIF either individually or in combination, and found that the combination of all three factors induced the greatest proliferative rate while retaining cellular multipotency (Tarasenko et al., 2004). In particular, cells grew faster and retained the ability to differentiate into the three cell types both *in vitro* and *in vivo* and were able to respond to local environmental cues after transplantation into intact and injured rodent spinal cord and brain (Wu et al., 2002;Tarasenko et al., 2006;Gao et al., 2005;Gao et al., 2006). Confirming the effect of bFGF specifically on cellular potency, Qian et al. (1997) eloquently demonstrated that addition of bFGF at low levels (0.1ng/ml) was sufficient to enhance the generation of multipotent precursors from rodent cortical derived cells and that increasing concentrations of bFGF increased the percentage of clones that produced both neurons and glial cells from <1% at 0.1ng/ml to >60% at 10ng/ml bFGF treatment (Qian et al., 1997). These data together suggest that bFGF is an important *in vitro* factor for the maintenance of multipotency of neural stem cells.

Directed Differentiation of Neural Stem Cells

Despite the successes in the expansion of NSCs, the generation of pure neuronal subtypes or glial populations from NSCs has proved challenging. In spite of almost a decade

of study, we still don't completely understand how to efficiently direct differentiation of these cells into specific neuronal or glial subtypes. In particular, which molecular and environmental cues are necessary and at which time points to guide these cells towards a strictly neuronal or glial fate. Toward this goal several groups have tried epigenetic or genetic modifications followed by phenotypic analyses either *in vitro* or after transplanting NSCs into animal models *in vivo*.

In regards to genetic modification, cell immortalization via transduction and chromosomal integration of SV40, hTERT and v-myc have been utilized to enhance cell survival (Roy et al., 2004;Rao and Anderson, 1997). Of particular interest are the findings of Rao and Anderson (1997) who eloquently demonstrated that genetically immortalized mouse neural crest cells can be efficiently directed into neurons using adherent culture conditions (Poly D Lysine (PDL) and fibronectin) combined with dibutyrl-cyclic AMP treatment *in vitro* and into glia with addition of serum (10%) and forskolin *in vitro* (Rao and Anderson, 1997). Another success in directed differentiation via epigenetic modification was reported by Wagner et al. (1999) who reported integration of a nuclear receptor (Nurr1) into an immortalized mouse NSC line to generate approximately 80% dopaminergic cells (Wagner et al., 1999). In a novel RNAi study, Chen et al. (2005) demonstrated that knockdown of a newly discovered gene with unknown function (AF11609) led to 80% neuronal differentiation of rodent neural stem cells (Chen et al., 2005). Despite the fact that detection of neuronal differentiation in this study was performed through loss of immunoreactivity of the stem cell marker nestin, coupled with phase contrast microscopy, however, neuronal phenotype was not fully analyzed in this study. Thus, it is unknown whether these cells were directed into a specific neuronal subtype.

In regards to human derived neural stem cells, the reality of directed neuronal differentiation, particularly differentiation into specific neuronal subtypes using genetic methods, has not yet been achieved. Nevertheless, groups are attempting this endeavor.

For example, in an attempt to treat Parkinson's disease, human fetal neural stem cells transfected with tyrosine hydroxylase (TH) cDNA were transplanted into 6-OHDA lesioned rats followed by behavioral and immunocytochemical analyses. Results of this study, however, were disappointing as only 3-4% of cells were successfully transfected with TH and very few of those cells survived transplantation. Interestingly though, despite the low level of differentiation, there was a significant behavioral effect 60d post engraftment which remained until the end of the study on day 180 (Hurley et al., 2001). Thus in this case, genetic modification of human neural stem cells did prove therapeutic. Finally, although promising, because genetic modification raises potential problems of chromosomal abnormalities, in the current study we chose to use an epigenetic approach to address the issue of directed differentiation of hNSCs.

Epigenetic modification includes the use of exogenous growth factors, morphogens, cytokines or other molecules to activate downstream cell signaling cascades allowing for differentiation of cells into specific phenotypes. Molecules such as neurotrophic factors (GDNF, BDNF, NT-3, IGF-1), growth factors or morphogens (EGF, bFGF, SHH, Wnt, retinoic acid), cytokines (LIF, IL-1, CNTF), and astrocyte or neuronal conditioned mediums have been used alone or in combination in an attempt to direct differentiation of stem cells into specific phenotypes. Effects of neurotrophic factors, growth factors and morphogens and cytokines on neural stem cells are briefly summarized below, while bFGF and astrocytes are the focuses of two other sections of this chapter.

Neurotrophic factors such as ciliary neurotrophic factor (CNTF), glial derived neurotrophic factor (GDNF), Neurotrophin-3 (NT-3) and 4 (NT-4) and platelet derived growth factor (PDGF) have been used to enhance cell survival and aid in directed differentiation of stem cells with some success. Particularly well studied is the effect of CNTF on glial fate induction (Ishii et al., 2006;Johe et al., 1996). CNTF treatment *in vitro* is well known to enhance the percentage of GFAP expressing cells (Kahn et al., 1997;Johe

et al., 1996). Consistent with this notion is the finding of Ishii and colleagues (2006) that blocking of CNTF signaling *in vivo* reduced transplanted NSC glial differentiation by >20% (Ishii et al., 2006). In contrast to CNTFs role as an inducer of glial phenotype, nerve growth factor (NGF) or BDNF treatments have shown beneficial effects on neuronal differentiation of neural precursor cells (NPCs) (Lachyankar et al., 1997) and treatment with NT-3, NT-4 and PDGF have also been reported to significantly enhance neuronal differentiation of NSCs via anti-apoptotic mechanisms (Caldwell et al., 2001). Furthermore, many groups add neurotrophic factor cocktails to their differentiation medium to aid in cell survival, particularly neuronal cell survival *in vitro* (Li et al., 2005;Vicario-Abejon et al., 1995)

Addition of other factors such as cAMP, SHH and retinoic acid (RA) have also had some success on directed neuronal differentiation of stem cells. For example, using human neural stem cells, Riaz et al (2001) found that addition of RA in combination with BDNF, dopamine, GDNF, and forskolin enhanced neuronal ($MAP2^+$) and specifically, tyrosine hydroxylase (TH) neuronal differentiation. Interestingly, BDNF, GDNF and forskolin without RA treatment also enhanced the percentage of TH^+ cells suggesting that RA may not be required for TH differentiation of hNSCs (Riaz et al., 2004;Riaz et al., 2002). It's also worthy to note that in the same study researchers found that the addition of serotonin, forskolin and bFGF were sufficient to enhance serotonergic neuronal differentiation and the authors suggest that it is the addition of the neurotransmitter of interest that allows hNSCs to choose which phenotype to adopt (i.e. add serotonin to the culture to get serotonergic neurons) rather than additional neurotrophic factors or morphogens. In other cellular models such as mouse neural stem cells, the addition of bFGF, SHH and FGF-8 allowed for serotonergic cell differentiation with a maximal differentiation of >60% after 8 weeks in culture (Ren-Patterson et al., 2005).

Cytokines such as Leukemia Inhibitory Factor (LIF) have also been used to direct stem cell fate. In particular, LIF treatment has been documented in both mouse and human

cells to enhance astroglial differentiation (Koblar et al., 1998;Galli et al., 2000). In contrast to these reports, in mouse embryonic stem cells, LIF has also been used to enhance neuronal differentiation by ~2X as compared to mESC's cultured without LIF (He et al., 2006). However, in this study glial differentiation was not fully examined and LIF was further shown to enhance proliferation, thus it is possible that glial cell number were also enhanced upon addition of LIF to the culture perhaps through a proliferative mechanism.

In regards to specific neuronal subtypes, other groups have used cytokines such as IL-1β to enhance dopaminergic differentiation of neural precursor cells. Using human neural precursor cells, Storch et al (2001) demonstrated that IL-1β, LIF and GDNF were sufficient to induce TH expression from human neural precursor cells, but limited to just 1% of the population (Storch et al., 2001). In a rat neural precursor model, Ling et al. (1998) found that following IL-1β stimulation, addition of IL-11, leukemia inhibitory factor (LIF), and glial cell line-derived neurotrophic factor (GDNF) increased the number of TH immunoreactive cells to ~20-25% of the whole cell population (Ling et al., 1998). Taken together, this data suggests that addition of cytokines have beneficial effects in regards to dopaminergic neuronal differentiation from both rodent and human derived stem cells.

The use of neurosteroids in directing stem cell fate has also been attempted. In one study, human neural stem cells grown in EGF and LIF plus the steroid dehydroepiandrosterone (DHEA) showed significantly greater proliferative capacity than those grown without DHEA. Unfortunately, however, DHEA did not enhance neuronal differentiation but did significantly increase GFAP expression at both the mRNA and protein level (Suzuki et al., 2004).

Despite promising progress being made toward directing fate specification of stem cells, thus far, no single or combination of factors has yet allowed for the directed differentiation of neural stem cells into pure populations of neuronal subtypes such as a purely dopaminergic or a purely cholinergic neuronal cell population. The benefits of

developing such a system include, but are not limited to, the ability to study particular cell type's responses to neurotoxic agents, to examine the effects of drugs aimed to enhance neuron survival, and to further characterize and thus better understand the developmental steps regulating specific neuronal differentiation. With that goal in mind, I will now focus upon the directed differentiation of stem cells into a specific subtype of cholinergic expressing neurons, motoneurons. First I will review the development of MNs *in vivo*, and then move into data regarding *in vitro* differentiation of stem cells into this subtype of neurons.

DEVELOPMENT OF MOTOR NEURONS *IN VIVO*

Motoneuron Differentiation: Lessons learned From *in vivo* Studies

Cholinergic motoneuron (MN) development in vertebrates has been well characterized *in vivo,* [for review see (Jessell, 2000;Briscoe and Novitch, 2007)] mainly from chick and mouse studies. It involves three major steps, each of which are initiated by a specific combination of different signals (morphogens) via subsequent regulation of specific transcription factors. First, cells are directed from a stem cell fate into a neural cell fate; second cells are caudalized; and third, cells are directed into a ventralized position by the graded secretion of the signaling molecule sonic hedgehog (SHH) produced by the developing notochord and floor plate (Ericson et al., 1996;Tanabe et al., 1998;Patten and Placzek, 2000). Each stage-specific step can be identified by the expression of a set of specific transcription factors. For example, early on, Sox1 and Nestin are highly expressed and indicate the presence of immature neuroectodermal cells (Pevny and Placzek, 2005;Kan et al., 2004;Dahlstrand et al., 1995). The initial formation of neural lineage is followed by caudalization via induction of the HoxC genes (HoxC5, C6, C8 and C9) (Dasen et al., 2003), and then SHH-induced ventralization through regulation of expression of Class

II transcription factors (Nkx6.1 and Nkx2.2) along with retinoic acid-activated Class I transcription factors (Olig2 and Pax6) (Ericson et al., 1997;Briscoe et al., 2000;Zhou et al., 2001;Briscoe and Novitch, 2007). At this stage, cells within this region of the developing CNS are termed pre motoneurons or pMN cells.

Within the developing spinal cord, pMN cells have the ability to differentiate into mainly two neural cell types, oligodendrocytes or motoneurons (Lu et al., 2002) and it has been suggested that there are several factors influencing which path cells will take. Examples of these influences include enhanced expression of the transcription factor, Nkx2.2 in oligodendrocytic progenitors (Zhou et al., 2001;Liu and Rao, 2004) and expression of two basic helix-loop-helix (bHLH) transcriptional regulators, Ngn2 and Olig2 in motoneuron progenitors (Lu et al., 2002;Zhou et al., 2001;Zhou and Anderson, 2002). In particular, a high level of Olig2 expression is believed to maintain cells in a proliferative state allowing them to remain as pMN cells or when expressed with Nkx2.2, to drive them down an oligodendrocytic fate path. In contrast, MN progenitors co-express Ngn2 with Olig2, the ratio of which is believed to push cells towards a final round of cellular division and a motoneuron cell fate identity (Mizuguchi et al., 2001;Lee et al., 2005). Thus, taken together, it is not surprising that Olig1/2 knockout animals do not develop MNs or oligodendrocytes but rather generate vast numbers of interneurons and astrocytes, while ectopic Olig2 expression induces neuronal differentiation believed to be moderated by the induction of Ngn2 (Zhou and Anderson, 2002;Novitch et al., 2001).

As pMNs undergo a final round of division and thus commit to becoming post-mitotic MNs they begin to express other proneural bHLH factors such as NeuroM, and begin to down-regulate Ngn2 expression (Lee and Pfaff, 2003). Additionally, expression of other homeodomain (HD) transcription factors such as Islet1 and Lim3 during this stage allow for the formation of a heteromeric complex with NLI (nuclear LIM interacting protein) (Thaler et al., 2002). Formation of this complex is believed to be necessary for activation

of the enhancer of the post-mitotic motoneuron factor, Hb9 (Lee et al., 2005;Thaler et al., 2002;Arber et al., 1999). Islet1 and Lim3 are believed to be essential components within this complex as knockout animals for either factor show a loss of motoneuron development (Allan and Thor, 2003;Pfaff et al., 1996).

The homeobox gene, Hb9, was first shown to play a critical role in mammalian MN differentiation in 1996 (Pfaff et al., 1996) and its close relative, MNR2 was discovered in chick studies in 1998 (Tanabe et al., 1998). Similar to the chick protein, MNR2, Hb9 is a basic protein with a molecular weight of approximately 54 kd and is highly homologous with other homeobox containing proteins (19/21 aa) with a highly conserved DNA binding domain (Deguchi and Kehrl, 1991). Its function is as a known transcriptional regulator during MN development in rodent and chick, however its exact molecular role during MN development is currently under investigation. Providing evidence for the importance of Hb9 during MN development, ectopic Hb9 expression leads to MN formation at the expense of nearby V2 interneurons in rodents (Arber et al., 1999). Interestingly, however, removal of Hb9 through knockout technology does not decrease MN differentiation, but rather leads to transient expression of V2 interneuron factors and later differentiation of atypical MNs. These MNs are not normal, though, as they show axon projection and migration pattern problems as well as abnormal muscle innervation (Li et al., 1999;Arber et al., 1999).

Directed Differentiation of Stem Cells Into MNs *in vitro*

Several agents have been used *in vitro* to mimic the *in vivo* cell signaling events leading to MN development. The most commonly utilized are retinoic acid (neuronal inducing agent) and sonic hedgehog (ventralizing agent) (Wichterle et al., 2002;Li et al., 2005;Calza et al., 2003;Soundararajan et al., 2006;Lim et al., 2006) and bFGF (Wu et al., 2002;Shin et al., 2005;Ren et al., 2006). The use of exogenous growth factors in different paradigms has yielded some success in the directed differentiation of hNSCs. In

particular, many groups including ours have reported an increase in neuronal phenotype with the addition of bFGF to stem cell cultures (Vescovi et al., 1993;Qian et al., 1997;Park et al., 2004). Notably, our group has developed an *in vitro* priming method which directs differentiation of hNSCs into a cholinergic phenotype both *in vitro* and after transplantation into the rat spinal cord (Wu et al., 2002). Priming consists of plating neurospheres onto poly-D-lysine and laminin coated culture dishes in serum-free medium supplemented with bFGF, heparin and laminin. Cells plated in this manner spread out to form a monolayer consisting of both neurons (immunolabeled for beta tubulin type III and MAP2) and glia (GFAP). In particular, approximately 36% of cells plated in this manner *in vitro* differentiate into immature neurons expressing cholineacetyltransferase (ChAT) (Tarasenko et al., 2004) and 50-60% differentiate into a $ChAT^+$ phenotype after transplantation into rodent spinal cord or medial septum (Wu et al., 2002). These data are comparable to the results reported by others using mitogenically expanded murine NSCs (~15% $ChAT^+$) (Ito et al., 2003), human embryonic stem cells (~21% $Hb9^+$) (Li et al., 2005) or mouse embryonic stem cells (25-30% $ChAT^+$) (Wichterle et al., 2002) treated with various growth factors and mitogens *in vitro* (See Table 1.1). Studies in our lab further demonstrated that cells primed without bFGF do not generate significant numbers of ChAT positive cells, suggesting that bFGF is one of the main players directing differentiation of hNSCs towards a cholinergic cell fate which is the focus for Specific Aim #1 of this thesis.

BASIC FIBROBLAST GROWTH FACTOR

Basic Fibroblast Growth Factor (bFGF)

Basic fibroblast growth factor (bFGF) is one of twenty two identified members of the fibroblast growth factor (FGF) superfamily (Reuss and Bohlen und, 2003;Wang and McKedhan, 2004). Ten of these members, including bFGF (or FGF2), have been found in

Directed Differentiation of Stem Cells into Cholinergic Expressing Neurons

Model system	Experimental Conditions	Results	Citation
Mouse embryonic stem cells	mES treated for 7d RA (2 µM) as hanging drops RA from different batches/companies yields different results	• 6-7d RA=RNA expression for ChAT, Hb9, Shh, GAD,TH • Two populations: 1.MNs 40-68% ($Isl1^+$) 2. Interneurons ($Isl1^-$) • $Islet1^+$ cells: ~12%= $Isl1^+/Hb9^+$, ~60% =$Isl1^+/Phox2b^+$	(Renoncourt et al., 1998)
Mouse embryonic stem cells	EBs grown w. hedgehog agonist (1 µM) + RA (100nM-2 µM) for 5d *In vitro* survival (3d) required NTF No FGF2 utilized	• *In vitro:* $Isl1^+/Hb9^+$ ~25%; $ChAT^+$ ~25-30% but req'd NTF. • *In vivo:* Most were $Lim3^+$> transplantation into chick sc w. many synaptic muscle connections • Functionally these cells show electrophysiological characteristics of MNs	(Wichterle et al., 2002;Miles et al., 2004;Soundararajan et al., 2006)
Human embryonic stem cells	Multi-step process: 1) EBs treated w. bFGF ~ 2 wks 2) +RA ~1 wk, 3) +Shh + RA ~ 1 wk 4) +Shh only ~1 wk, 5) Maturation with NTF for 1-2 wks. ~4-5 wks total. hES respond to lower conc of RA and Shh than mES (0.001 µM vs 2 µM RA and 500ng/ml vs 6000 ng/ml Shh)	• Early but not late EBs respond to RA & Shh to produce MNs • +RA+Shh ~21% $Hb9^+$; +RA only<5% $Hb9^+$; +Shh only <1% $Hb9^+$ • $Hb9^+$ also expressed $HoxC8^+$,$ChAT^+$, $vChAT^+$ • Functional analysis via electrophysiology	(Li et al., 2005)
Human embryonic stem cells	Multi-step process: 1) 4 ng/ml bFGF + Feeder (7d) 2) Remove feeder, adhesive+bFGF+Lif (3d) 3) trypisinze, +Shh + RA + bFGF (7d) 4) Differentiate in basal media (14d) ~4 wks total.	• FGF+RA+Shh yielded 8X increase in RNA expression of Hb9 as compared to FGF alone & ~4X vs FGF+RA. • ~20-30% FGF+RA+Shh were $ChAT^+$ via ICC	(Shin et al., 2005)
Human neural stem cells	Neurospheres plated onto adhesive culture (PDL/LMN) + bFGF (10 ng/ml), heparin & laminin (FHL) 4-6d & 10 d differentiation in B27	• *In vitro:* ~28% $ChAT^+$>4d FHL+ 10d B27 • *In vivo:* 20-50% $ChAT^+$ in basal forebrain and spinal cord	(Wu et al., 2002;Tarasenko et al., 2004)
Mouse neural stem cells (E14 telencephalic)	Growth:DMEM:F12+TPPS+FGF-2 (10 ng/ml) Differentiation: withdraw FGF, + 1% FBS ± NTF (NGF, BDNF, NT-3) DMEM:F12 + 1% B27 + EGF (20ng/ml), bFGF	• +NTF/FGF2 ~15% $ChAT^+$; +NTF/FGF2 ~8-15% TH^+ (ICC) • bFGF and heparin are required for ChAT differentiation • $ChAT^+$ ICC 7d: FHL: 30 ± 5% ; FH: 10 ± 2.5%; FL or LH:	(Ito et al., 2003)
Rat neural stem cells (E14 striatum)	(20 ng/ml), heparin (5 µg/ml) & laminin (1 µg/ml)	• 0.5 ± 0.3% • FHL = $ChAT^+$ RNA	(Ren et al., 2006)

Table 1.1: Summary of directed differentiation of stem cells into cholinergic neurons

the human central nervous system (Reuss and Bohlen und, 2003) where they are thought to be involved in CNS development, repair processes following nerve lesions and degenerative disorders (Reuss and Bohlen und, 2003), neuronal survival (Grothe et al., 1991), axonal growth (Klimaschewski et al., 2004), and learning and memory (Ishihara et al., 1992) . bFGF is synthesized primarily by astrocytes in the CNS while other FGFs are mainly synthesized by neurons (Bean et al., 1991) and are expressed ubiquitously throughout the brain (Ernfors et al., 1990). Members of the FGF family share a homologous core region separated by more heterogenous sequences and are all heparin binding polypeptides (Wang and McKedhan, 2004;Ornitz et al., 1996;Dono, 2003).

Unlike other FGF family members, bFGF has five molecular weight isoforms (18, 22, 22.5, 24 and 34 kDa) in human (Delrieu, 2000;Sorensen et al., 2006), all of which are expressed in the CNS and shown to be differentially regulated during development. Generation of these isoforms is through alternative start sites (Prats et al., 1989;Florkiewicz and Sommer, 1989;Kiefer et al., 1994) and different isoforms are further compartmentalized into separate sub-cellular locations (Florkiewicz et al., 1991;Renko et al., 1990). The cell signaling and functional role of each molecular weight isoform is still controversial. However, some data suggests that the high molecular weight (HMW) molecules can act as neurotrophic factors (Sorensen et al., 2006). For example, in studies using the neurotoxic agent 6-hydroxydopamine (Grothe et al., 2000) or high dose glutamate (Reuss and Bohlen und, 2003), HMW bFGF isoforms were shown to be neuroprotective. Further substantiating a neuroprotective role, the 23kd form of bFGF, has been shown to bind and activate the survival of motoneuron (SMN) protein (Claus et al., 2003) and to FGF-2 interacting factor (FIF), an important anti-apoptotic molecule (Van den et al., 2000). In regards to the lower molecular weight (LMW) isoforms, the most commonly studied is the 18kDa variant of bFGF. Like its HMW cousins, this molecule has also been shown to be important for cortical, spinal cord and hippocampal cell survival (Otto et al., 1987;Cheng and Mattson, 1991). In

addition to its survival role, the LMW isoforms are associated with neural progenitor cell proliferation both *in vitro* (Kitchens et al., 1994;Tropepe et al., 1999;Gritti et al., 1995;Gritti et al., 1996) and *in vivo* (Tao et al., 1996;Tao et al., 1997;Martens et al., 2002;Hagood et al., 2006) as well as for the maintenance of pluripotency in human embryonic stem cells (Wang et al., 2005;Xu et al., 2005). Interestingly, in addition to activating cell surface receptors, the 18kDa isoform also has been shown to localize to the nucleus where it is believed to act directly as a DNA binding factor (Sorensen et al., 2006).

Basic fibroblast growth factor is also known to be secreted by astrocytes both *in vivo* and *in vitro* (Gray and Patel, 1992;Le and Esquenazi, 2002). In particular, during early development stem cells express receptors only for bFGF but not for EGF and respond to bFGF in a proliferative manner (Qian et al., 1997;Tropepe et al., 1999). Following exposure to bFGF, cells become competent to respond to EGF and as such, up-regulate EGF receptors (Ciccolini and Svendsen, 1998). Furthermore, bFGF has been shown to influence stem cell decisions to develop into glia or neurons (Qian et al., 1997;Palmer et al., 1999;Tsai and Kim, 2005;Nelson and Svendsen, 2006).

FGF Receptors and FGF Mediated Cell Signaling Pathways In The CNS

FGFs bind with different affinities to four tyrosine kinase receptors (FGFR1-4) located on the cell surface. Activation of these receptors is associated with many intracellular signaling pathways including MAP kinase, PLC-gamma, and PI3 kinase pathways among others (Jin et al., 2005;Wang and McKedhan, 2004;Reuss and Bohlen und, 2003;Schlessinger, 2004). FGFRs are composed of three regions; an extracellular ligand binding domain, a single transmembrane domain and an intracellular tyrosine kinase domain (Ornitz and Itoh, 2001). To add to the complexity, the extracellular region contains three immunoglobulin like (IgG-like) regions of which the third domain can be alternatively spliced to create three forms of the FGF receptor in FGFRs 1-3 (e.g., FGFR1-

IIIa, FGFR1-IIIb, and FGFR1-IIIc). Each of these isoforms has unique binding properties for the different FGF family members and is expressed in a tissue specific manner (See Table 1.2). Although there are some exceptions, b exon (i.e. IIIb) expression is typically

Receptor subtype	Binding affinity for bFGF	Location
FRFR1 (IIIa)	N/S	Neurons and astrocytes. Adult CNS (cortex, hippocampus, cerebellum, retina) and kidney.
(IIIb)	++	
(IIIc)	++++	
FRFR2 (IIIa)	N/S	Glia only. Adult CNS (cortex, cerebellum, retina) and kidney.
(IIIb)	+	
(IIIc)	++	
FRFR3 (IIIb)	+	Glia only. Diffuse throughout adult brain
(IIIc)	+++	
FGFR4	++++	Developmentally expressed; retinal and kidney mainly.

(From Ornitz et al., 1996; Yazaki et al., 1994; Asai et al., 1993, Fuhrmann et al., 1999) (+ = low affinity, ++++ = highest affinity; N/S = not studied)

Table 1.2: FGF Receptor affinities for bFGF.

restricted to epithelial lineages while c forms (i.e. IIIc) are restricted to mesenchymal lineages (Ornitz et al., 1996). The only non-alternatively spliced FGFR is FGFR4.

Activation of FGFRs trigger many intracellular signaling pathways (Figure 1.2) including MAP kinase, src, PKC, PI3 kinase as well as Ras signaling cascades (Reuss and Bohlen und, 2003;Wang and McKedhan, 2004;Jin et al., 2005). Activation of each signaling pathway has been associated with multiple functions. For example, FGF signaling mediated by src is believed to be important for FGFR mediated cytoskeletal alterations (Reuss and Bohlen und, 2003) while PI3 kinase activation is believed to mediate at least in part, some of FGFs mitogenic properties (Jin et al., 2005). bFGF induced expression of the MAP kinase protein, ERK1/2 has also been shown to be an important modulator of the proliferative (Huang et al., 2004) as well as the differentiation capacity of this growth factor (Spector et al., 2005).

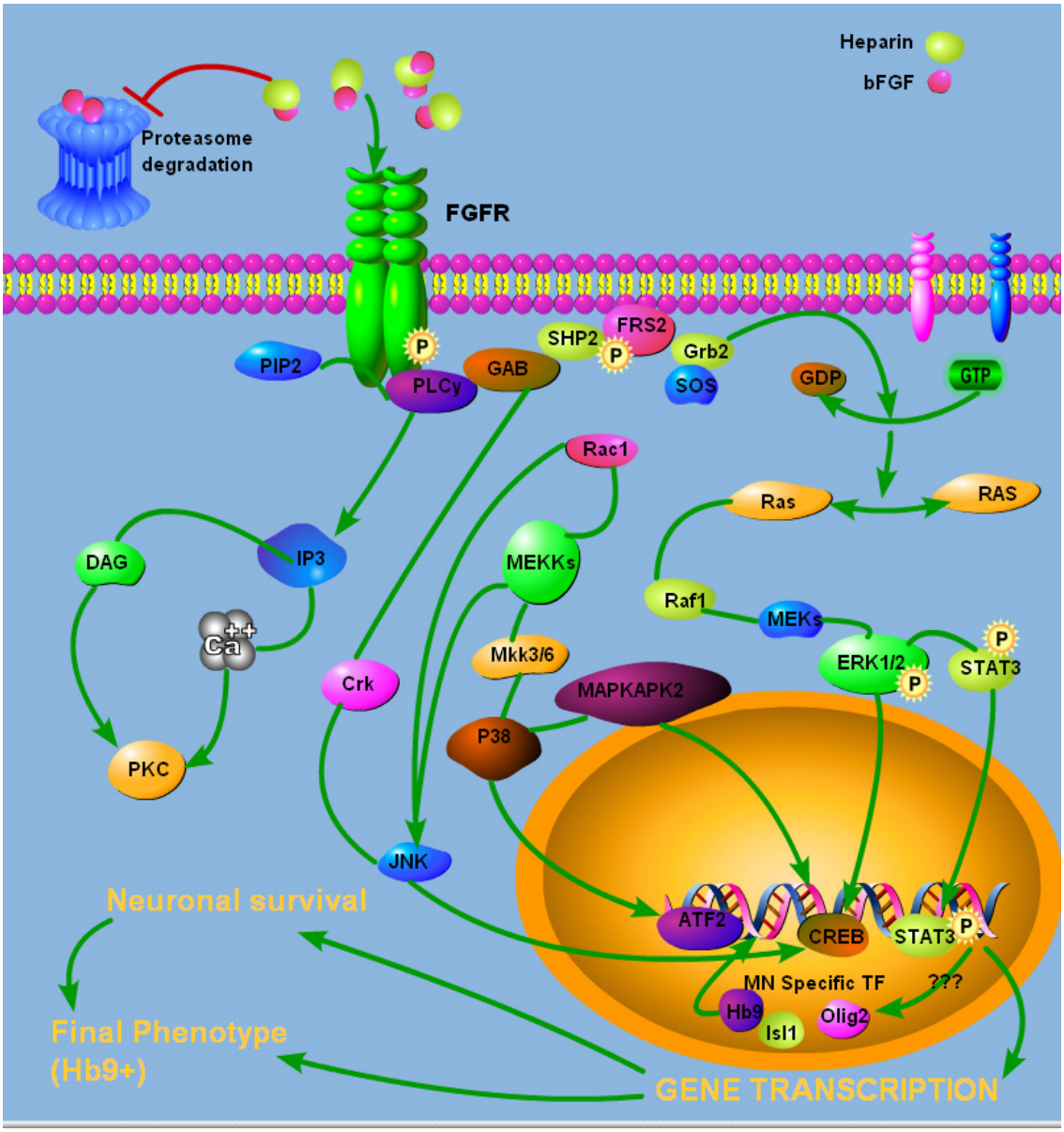


Figure 1.2: FGF signaling pathways. Image created in Pathway Builder, 2006.

Surprisingly, the main signaling difference between FGFRs is believed to be mediated by the strength of tyrosine kinase activation and not via differences in signaling cascades or target proteins (Reuss and Bohlen und, 2003;Schlessinger, 2004). In fact, many receptor tyrosine kinases (RTK) activate similar signaling pathways (Schlessinger, 2000). For example, EGF and bFGF share most, if not all, intracellular signaling molecules. Thus one unanswered question is how specificity is retained when intracellular signaling is so similar. One hypothesis is that compared with other RTK's which have direct cell signaling effects, bFGF signaling is mediated by the assembly of a multidocking protein complex and also involves additional layers of control both within and outside the cell (Schlessinger, 2004). For example, FGF receptor signaling is regulated beyond a simple phosphorylation event of a tyrosine residue on the receptor. In particular, FGFR signaling is mediated by recruitment of an additional docking protein, FRS2, which must also be tyrosine phosphorylated prior to recruitment of additional proteins Grb2 and Shp2. In contrast to this, under EGF signaling, no docking molecule is required. Rather, EGFRs are directly tyrosine phosphorylated and act as docking platforms for adaptor proteins such as Grb2, Nck and Shc (Schlessinger, 2004). As an additional layer of control, FRS2 is also associated with a second negative feedback loop from MAPK which is not present in EGF mediated signaling. The lack of these additional levels of control are hypothesized to be important for the development of cancer in EGFR over-expression models (Blume-Jensen and Hunter, 2001).

Heparan sulfate proteoglycans (HSPGs) are also known to be important regulators of FGFR activation (Ornitz and Itoh, 2001;Reuss and Bohlen und, 2003;Wang and McKedhan, 2004;Caldwell and Svendsen, 1998). It is hypothesized that one FGF, one FGFR and one heparin/heparan sulfate chain interact and then dimerize to activate the FGF signal pathways through a prototypical receptor tyrosine kinase (RTK) formation (Mohammadi et al., 2005). Interestingly, FGF bound to HSPG in the extracellular matrix

(ECM) is believed to provide a store of FGF molecules which are less likely to undergo degradation (Schlessinger, 2004;Caldwell et al., 2004) thus one can hypothesize that FGF signaling may be maintained for longer periods of time or at a higher strength depending upon the level of HSPGs available for storage of FGF.

bFGF's Role in Vertebrate CNS Development

In both rodents and humans, neurons are formed earlier than astrocytes during nervous system development (Qian et al., 2000;Dono, 2003). Correlated with neuronal development in rodents is a rise in bFGF levels (Qian et al., 1997;Dono, 2003;Dono, 2003) with a subsequent decrease during later developmental stages associated with the period of astrogenesis. Moreover, ventricular injection of bFGF into adult rodents has been shown to induce neurogenesis in hippocampus, cortex and the central canal of the spinal cord (Gage, 2000;Martens et al., 2002;Vaccarino et al., 1999a) and correspondingly, injection of anti-bFGF antibodies decreases both proliferation and neurogenesis in rodent newborn CNS (Tao et al., 1997). Furthermore, bFGF knockout mice show a decrease in neuronal formation in sensory and motor cortical areas while glial cell development appears normal (Dono et al., 1998;Ortega et al., 1998). In homozygous FGF-2 knockout mice a >50% reduction in cell number at E10.5 followed by a significant decrease in proliferative capacity led to a significant decrease in cortical cell number at birth as compared to wild type mice (Raballo et al., 2000). Interestingly, some brain regions seemed to be spared in FGF-2 knockouts. In particular, the basal ganglia of FGF-2 knockouts appeared normal while in frontal areas, FGF-2 knockouts lacked large projection neurons suggesting that FGF-2 effects may be cell type specific (Vaccarino et al., 1999a;Vaccarino et al., 1999b;Raballo et al., 2000). Taken together, these data suggest a significant role for bFGF during neuronal development.

In contrast to the *in vivo* evidence suggesting a role for bFGF in neuronal development and to a lesser degree during glial development, addition of exogenous bFGF has been

shown to induce either neurogenesis or gliogenesis in stem cell culture models (Buc-Caron, 1995;Palmer et al., 1999;Qian et al., 1997;Reimers et al., 2001;Song and Ghosh, 2004;Tsai and Kim, 2005) (For a summary of bFGF's role in directing stem cell fate, see Table 1.3). The addition of bFGF in combination with other growth factors such as EGF also elicits novel effects on stem cells not seen when factors are added individually (Kitchens et al., 1994;Vescovi et al., 1993). Further complicating the matter, concentration of bFGF appears to be very important in directing cell fate. In particular, high concentrations of bFGF have been demonstrated to be mitogenic and/or to induce astrogenesis or oligogenesis (Tsai and Kim, 2005;Qian et al., 1997;Nelson and Svendsen, 2006) while lower concentrations direct cells into a neuronal fate (Qian et al., 1997;Nelson and Svendsen, 2006). For example, in mouse E10 ventricular derived stem cells, 0.1ng/ml bFGF stimulation for 10 days induced an almost strictly neuronal population to develop (92% neuron only clones, 8% neuron-glia/mixed clones, <1% glia only clones) while cells treated with 10ng/ml bFGF showed an increase in heterogeneity with 69% of clones showing a mixed phenotype (neuron and glia immunoreactivity) while the remaining cells showed an approximately 50:50 neuron only:glial only clone ratio (15% total cells were neuron only, 16% glia only) (Qian et al., 1997). Thus from this study we can conclude that higher levels of bFGF increase the total number of glial cells *in vitro* from <10% (glial and neuron-glial clones together) at 0.1ng/ml to greater than 80% at 10ng/ml. Intriguingly, it was also suggested that other factors in addition to bFGF (i.e. astrocyte secreted factors found in astrocyte conditioned media) may be involved in cell fate decisions within glial clones (i.e. differentiation of oligodendrocyte or astrocytes) from these cultures (Qian et al., 1997).

In a study similar to Qian et al., (1997), Nelson and Svendsen (2006) found that low levels of bFGF (2 pg/ml) stimulated neurogenesis while high levels (20ng/ml) maintained a stem cell state in human neural precursor cells (Nelson and Svendsen, 2006). Furthermore, using CREB phosphorylation as a readout of FGF receptor activation, Nelson and Svendsen

Summary of bFGF's Role in Directing Stem Cell Fate

Model system	Experimental Conditions	Results	Citation
Rat hippocampal neurons E16	DMEM+10% FBS +5ng/ml bFGF or BSA (ctrl) Only compared acutely isolated and after 2 passages *in vitro* no long term culture done	• bFGF increases # of Nestin$^+$ cells, 2DIV to 30DIV Nestin remained >80% while BSA ctrls lost nestin expression after 1wk. • GFAP increased over time with bFGF treatment: 4DIV GFAP<5% to >25% 27DIV • MAP2$^+$ cells decreased with addition of 5ng/ml bFGF over 30 DIV (2 DIV~25% to 27 DIV<5%). • bFGF is a NTF for neurons w/o bFGF neurons die • bFGF is mitogenic, >7d DIV ~90% BrdU+, 14 DIV decrease 30%	(Vicario-Abejon et al., 1995)
Adult rat cortex (C), hippocampus (HC) & optic nerve (ON)	Growth: DMEM+F12 (1:1)+N2 +10%FBS +20ng/ml bFGF, adherent culture Differentiation: DMEM:F12 (1:1) +1%FBS +100ng.mlRA+1ng/ml bFGF	• Cortex derived cells gain competence to differentiate into neurons after exposure to bFGF (limited to ~10%) • HC derived cells increase neuronal differentiation with bFGF • Regions restricted to glial formation generate neurons after bFGF (ON derived cells +bFGF ~1% Tuj1+, w/o bFGF=0% Tuj1)	(Palmer et al., 1999)
Rat E15 neural stem cells	DMEM:F12 (1:1)+TPPS+EGF (20ng/ml) Neurospheres >5p, bFGF 10ng/ml 2h-10d	• bFGF treatment increased growth rate (4X) and oligodendrocyte differentiation (~1X). Tuj1 neurons decreased with bFGF	(Reimers et al., 2001)
Rat cortical E14.5 derived neural stem cells	Proliferate: Monolayer culture; DMEM+N2+bFGF (25ng/ml) Differentiate: +10% FBS ± 25ng/ml bFGF bFGF differentiation=F2, F8 (bFGF2d, bFGF8d) vs S2, S8 (serum2d, serum 8d)	• bFGF is anti-neurogenic • S8=15%Tuj1, 70%GFAP, 2%O4 vs F8=0%Tuj1, 85-90%GFAP, 1.8% O4 • Dose dependent: Tuj1$^+$~15, 3.5,0.5, 0.5, and 0% at 0, 2, 5, 10, 25ng/ml bFGF for 8d of differentiation • Proliferation, cell death & cell maturation are similar. Increased anti-neurogenic factors (i.e. HES-1) /earlier expression of gliogenic factors	(Tsai and Kim, 2005)
Human neural precursors	Proliferate: Neurospheres; 8-11wk post conception DMEM-F12 (3:1)+B27(1%)+EGF+FGF-2 (20ng/ml each)+heparin (5ng/ml) Differentiation: PLL+LMN+DMEM-F12+B27 (2%)	• Neurogenesis was inhibited by either high dose EGF/FGF-2 or AraC treatment during differentiation (no growth factor wd) • Neurospheres treated with EGF, FGF-2 or E+F for 8 wks & differentiated via growth factor withdrawal all showed ~28% Tuj1 • EGF, FGF-2 or E+F yields similar BrdU incorporation & phospho-Creb	(Ostenfeld and Svendsen, 2004)
Human embryonic stem cells	Multi-step process: 1) 4ng/ml bFGF + Feeder (7d) 2) Remove feeder, adhesive+bFGF+Lif (3d) 3) trypisinze, +Shh + RA + bFGF (7d) 4) Differentiate in basal media (14d) 4 wks total.	• FGF+RA+Shh yielded 8X increase in RNA expression of Hb9 as compared to FGF alone & ~4X vs FGF+RA. • ~20-30% FGF+RA+Shh were ChaT$^+$ via ICC	(Shin et al., 2005)
Human neural stem cells	Neurospheres plated onto adhesive culture (PDL/LMN) + bFGF (10 ng/ml), heparin & laminin (FHL) 4-6d & 10 d differentiation in B27	• *In vitro:* ~28% ChAT$^+$ >4d FHL+ 10d B27 • *In vivo:* 20-50% ChAT$^+$ in basal forebrain and spinal cord	(Wu et al., 2002;Tarasenko et al., 2004)
Mouse NSCs E14 telencephalic	Growth:DMEM:F12+TPPS+FGF-2 (10 ng/ml). Differentiation: -FGF, + 1% FBS ± NTF (NGF, BDNF, NT-3)	• +NTF/FGF2 ~15% ChAT$^+$; +NTF/FGF2 ~8-15% TH$^+$ (ICC)	(Ito et al., 2003)
Rat neural stem cells (E14 striatum)	DMEM:F12 + 1% B27 + EGF (20ng/ml), bFGF (20 ng/ml), heparin (5 µg/ml) & laminin (1 µg/ml)	• bFGF and heparin are required for ChAT differentiation • ChAT$^+$ ICC 7d: FHL: 30 ± 5% ; FH: 10 ± 2.5%; FL or LH: 0.5 ± 0.3% • FHL = ChAT$^+$ RNA	(Ren et al., 2006)
Mouse E10 ventricular derived cells	Single cell & clonal analysis. Serum Free DMEM-N2+B27 bFGF 0.1-10ng/ml replenished 50% bFGF every 48-72hr.	• Mitogenic effect: bFGF ED_{50} ~0.11ng/ml demonstrated by BrdU • Varying bFGF conc alters neuron-glia composition.0.1ng/ml ~92% neuron only (n), <1% glia only (g), 8% n or g VS 10ng/ml ~15% n, 16%g & 69% n or g. • FGFR1 IIIC, R2IIIc, R3IIIC found in E10, 12 and 14d cells.	(Qian et al., 1997)

Table 1.3: Summary of bFGF's role in stem cell differentiation and cell survival

(2006) noted that although higher concentrations of bFGF did not activate more cells, that immunostaining for activated CREB was significantly stronger in cultures stimulated with high concentrations of bFGF than those stimulated with low concentrations of bFGF (Nelson and Svendsen, 2006). As suggested by Schlessinger (2004) and Reuss and Bohlen und (2003), differences in FGF effects may be due to the strength of FGF induced intracellular cell signaling pathways. Extrapolating back to Nelsons study, perhaps cultures stimulated with high concentrations of bFGF might lead to maintenance of stemness via high level activation of FGF induced intracellular cell signaling proteins while low level stimulation might induce differentiation via activation of the same pathways at a lower level (Reuss and Bohlen und, 2003;Schlessinger, 2004). Interestingly, addition of a bFGF neutralizing antibody did not block neurogenesis in these cultures, suggesting that either endocrine bFGF signaling and/or other factors may also be important for neurogenesis in hNPCs. However, the exact mechanism behind concentration differences and differentiation or maintenance of hNPCs has yet to be determined.

Further validating the sensitivity of cells to low level bFGF stimulation, bFGF has also been implicated in dramatic changes in cell fate outcome at low concentrations. For example, as little as 0.2ng/ml bFGF added to spinal cord precursor cells has been recently shown to switch dorsal neural precursors into ventral precursors via induction of the basic helix loop helix (bHLH) transcription factor, Olig2 (Gabay et al., 2003). Thus taken together, it appears that even low levels of exogenous or endogenous bFGF may induce critical cell signaling changes which modify cell fate outcome in stem cell models.

Overall, the suggestion that low level bFGF stimulation is a powerful modulator of neurogensis *in vitro* is in contrast to the *in vivo* data suggesting that high levels of bFGF are essential for proper neurogenesis. For example, it has been shown that during the time of neurogenesis in rodent (E10-E18) that bFGF mRNA and protein levels rise while during gliogenesis (E15 to postnatal), bFGF levels significantly decrease (Powell et al.,

1991;Weise et al., 1993). One might speculate, however, that other FGF family molecules as well as interactions with a variety of other cellular proteins *in vivo* may have significant and potentially repressive effects not mimicked by the simple addition of exogenous bFGF *in vitro.* Additionally, unlike *in vivo* where all bFGF isoforms are present, in cell culture models, the 18kd isoform is most commonly used and is usually added in combination with an excess of heparan to the culture medium. Because heparin stabilizes un-bound bFGF (Caldwell and Svendsen, 1998;Caldwell et al., 2004), excess amount of heparin may allow for extended preservation of FGF mediated signaling for significantly longer periods than found *in vivo.*

Basic fibroblast growth factor has also been shown to have significant effects on cholinergic neuron activity and cell survival. For example, bFGF has been shown to significantly enhance choline acetyltransferase (ChAT) activity in embryonic chick or rat ventral spinal cord (Grothe et al., 1991;McManaman et al., 1989), in septal cholinergic cell cultures (Yokoyama et al., 1994), and to enhance the survival of enriched chick embryonic motoneurons (Arakawa et al., 1990) and embryonic ventral spinal cord neurons (Unsicker et al., 1987). In addition, a previous study by Gray and Patel (1992) demonstrated that astrocyte conditioned media strongly increased ChAT activity in rodent septal neurons, the effect of which was significantly reduced by application of an anti-bFGF antibody (Gray and Patel, 1992) thus suggesting a role for astrocyte secreted bFGF in the regulation of the cholinergic cell function. Moreover, in a brain injury model, Calza and colleagues (2003) eloquently demonstrated functional recovery from cholinergic basal forebrain lesions through osmotic mini-pump delivery of EGF and bFGF. Notably, they showed evidence for proliferation and migration of endogenous NSCs from neurogenic regions to injured areas and enhancement of ChAT activity in response to bFGF treatment (Calza et al., 2003). These results suggest that exogenous growth factor treatment may control the activity of endogenous neural stem cells aiding in brain repair. Thus in summary bFGF appears to

induce a wide variety of effects on stem cell differentiation both *in vivo* and *in vitro* in a concentration and stage dependent manner in the CNS.

ASTROCYTES AND STEM CELLS

The environment that stem cells are exposed to post-transplantation has a profound impact on cell survival, maturation and differentiation. In particular, transplantation of stem cells into the injured CNS is known to recruit several cell types to the site of grafting, the most plentiful of which are astrocytes (Cao et al., 2002). Astrocytes used to be known as simply the "support cells" of the nervous system, but in more recent years, they have been shown to do more than just support neurons. In fact, astrocytes have been implicated in directing differentiation of stem cells (Song et al., 2002a;Song et al., 2002b;Nakayama et al., 2003), influencing cell survival (Hans et al., 2002;Engele et al., 1991;Reuss and Unsicker, 2000), and modulating the formation and activity of synapses (Smith, 1998;Pfrieger and Barres, 1997;Ullian et al., 2004). Thus it is quite probable that astrocytes recruited to the site of stem cell transplantation may influence the differentiation, cell survival and maturation of transplanted stem/progenitor cells.

Several groups have used astrocytes or astrocyte conditioned media (ACM) to direct *in vitro* differentiation of primate and murine derived embryonic stem (ES) cells (Nakayama et al., 2003) and neural stem cells (Wagner et al., 1999;Song et al., 2002a;Song et al., 2002b;Chang et al., 2003). Of particular interest to our group is the use of astrocytes to direct stem cells into a neuronal cell fate. To this end, promising data from Nakayama et al. (2003) showed that astrocyte conditioned medium (ACM) induced the generation of a strictly neuronal population in mouse ES cells. Specifically, mouse ES cells cultured in the presence of ACM and other growth factors for ten days showed enhanced expression of phenotypic markers for dopaminergic (TH), cholinergic (ChAT) and GABAergic (GAD) cell fates. Surprisingly, researchers found no astrocytic or oligodendrocytic differentiation

within these cultures. Along the same line, Wagner et al. (1999) was able to generate approximately 80% dopaminergic neurons from an immortalized neural stem cell line exposed to ACM and induced to overexpress a nuclear receptor (Nurr1). In contrast to these data, however, Chang et al. (2003) found that rat cortical stem cells cultured in the presence of ACM differentiated preferentially into astrocytes while cells cultured in the presence of neuronal derived conditioned medium (NCM) developed primarily into neurons (Chang et al., 2003). This data would seem to indicate that astrocytes and neurons secrete molecules that direct stem cells into an astrocytic or neurogenic cell fate, respectively.

Unfortunately, no group has been able to pinpoint which secreted factor(s) or combination of factors was essential for directing stem cell fate in their systems. To this end, however, Barkho et al. (2006) demonstrated that astrocytes from different regions and/or developmental stages had differential expression of neurogenic and astrogenic genes, suggesting that astrocytic effects on stem cell differentiation might be different depending upon which area cells were derived from (Barkho et al., 2006). Historically, astrocytes are known to secrete several important cell signaling molecules including bFGF (Albrecht et al., 2002;Le and Esquenazi, 2002), ciliary neurotrophic factor (CNTF) (Rudge et al., 1992), glial derived neurotrophic factor (GDNF) (Schaar et al., 1993), laminin (Liesi et al., 1983;Wagner and Gardner, 2000)and cholesterol (Jung-Testas et al., 1989). Any number of these molecules or others could account for the changes in stem cell differentiation seen in these studies. As a whole, these data indicate that secreted factors from astrocytes play an important cell signaling role for the differentiation and survival of stem cells.

In direct contact *in vitro* cell culture models, Song et al. (2002) demonstrated that astrocytes could enhance neuronal cell fate in adult rat hippocampal stem cells. Researchers in this study found a robust increase in neuronal differentiation (ten-fold greater than control cultures) when stem cells were cultured directly on top of neonatal hippocampal derived astrocytes. Co-culture with adult hippocampal derived astrocytes exhibited a

similar phenomenon albeit not as vigorous. Interestingly, stem cells exposed to spinal cord derived astrocytes (neonatal or adult derived) did not exhibit significant neuronal generation (Song et al., 2002a). In an embryonic stem cell model, Bentz et al. (2006) found that co-culture of mouse embryonic cells with cerebral astrocytes induced an astrocytic cell fate while culture with astrocyte conditioned media induced both astrocytic and neuronal differentiation (Bentz et al., 2006). Taken together, data from stem cells cultured with ACM and direct co-culture models indicate that astrocytes from different areas (brain vs. spinal cord), at different stages of development (neonatal vs. adult) and via direct or indirect (ACM) contact can influence final phenotypic differentiation of stem cells.

In addition to their role in directing cell fate, many have proposed that astrocytes are essential in the development of functional synapses (Pfrieger and Barres, 1997;Fields and Stevens-Graham, 2002;Song et al., 2002a;Song et al., 2002b;Slezak and Pfrieger, 2003). In regards to stem cell maturation and synapse formation, Song et al. (2002b) showed that astrocytes not only enhanced neuronal differentiation but also influenced maturation and synapse formation. They documented formation of functional synapses with active recycling of synaptic vesicles and tetrodotoxin blocked action potentials in differentiated neural stem cells cultured in the presence of either adult or neonatal derived hippocampal astrocytes. Interestingly, neuronal synaptic number and synaptic strength has also been attributed to astrocytic cell signaling (Slezak and Pfrieger, 2003). Particularly in the retina, an increase in cytoplasmic calcium in astrocytes was shown to enhance synaptic strength in adjacent neuronal synapses in culture (Fields and Stevens-Graham, 2002). Not surprisingly, communication between astrocytes and neurons is not a one way street as neurons have also been shown to communicate with astrocytes. In particular, calcium responses in astrocytes can be seen in brain slice cultures when neurons are stimulated to fire (Fields and Stevens-Graham, 2002;Slezak and Pfrieger, 2003). Thus, there appears to be reciprocal communication between astrocytes and neurons both *in vitro* and *in vivo*.

Previously, we have shown that bFGF-primed hNSCs survive and differentiate into cholinergic neurons in rat spinal cord and make connections with the appropriate muscle targets (Wu et al., 2002;Gao et al., 2005;Tarasenko et al., 2006). Since transplantation surgery is known to induce astroglial infiltration into the transplantation site, one cell type that could be directing hNSC survival and cell fate in our transplantation model is astrocytes. In light of the evidence for astrocyte-neuron communication, particularly the directed differentiation of stem cells and survival effects of astrocytes and astrocyte conditioned medium, it will be interesting to determine whether astrocytes might influence human neural stem cell differentiation and long term survival *in vitro* (Hypothesis 2).

SUMMARY, RATIONALE AND HYPOTHESES

Human neural stem cells (hNSCs) have the potential to be very powerful therapeutic agents. In order to harness this power, however, we must first strive to understand how to direct their phenotypic cell fate both *in vitro* and *in vivo*. In light of this goal, in this dissertation I have attempted to understand the mechanism behind cholinergic phenotypic differentiation of hNSCs exposed to a simple *in vitro* priming procedure consisting of bFGF, heparin and laminin (FHL) and adhesive culture conditions. Since bFGF is a well known cell signaling molecule and cell survival agent, a potent mitogen and a powerful morphogen, I first targeted bFGF as the main candidate involved in FHL induced cholinergic differentiation. To assess this, I examined bFGF's role in driving expression of the early marker of the motoneuron/oligodendrocyte lineage, Olig2, as well as late motoneuron transcription factors, with particular focus on the motoneuron determinant, Hb9. Next, I assessed the time and dose dependence of FHL-primed hNSCs and the requirement of hNSCs for bFGF mediated cell signaling for Hb9 induction. Finally, I strove to understand the molecular basis for cholinergic differentiation of FHL primed hNSCs by analyzing cell survival/cell death, the potential requirement for cell proliferation and the activation of two

mediators of the MAP kinase cell signaling pathway, ERK1/2 and JNK in FHL-primed or unprimed hNSCs (Hypothesis 1: Specific Aims 1.1-1.5; Chapter 3).

Because bFGF is a molecule known to be secreted by astrocytic cells both *in vivo* and *in vitro* and FHL-primed hNSCs transplanted into rodent brain and spinal cord differentiated into cholinergic expressing cells *in vivo*, I next wanted to understand whether addition of astrocytes might influence hNSC fate determination and long term cell survival *in vitro*. Toward this goal, I used a co-culture system with semi-permeable membrane inserts to separate the two cell types (i.e. astrocytes and FHL primed hNSCs), as well as directly mixing astrocytes and FHL-primed hNSCs or adding conditioned media derived from astrocytes to FHL-primed hNSCs. In these sets of experiments I assessed cholinergic differentiation and long-term survival of cholinergic differentiated hNSCs in the presence or absence of astrocytic factors using immunocytochemistry, RT-PCR analysis and cell death/cell viability assays (LDH, WST-1 and TUNEL). I further analyzed bFGF secretion into conditioned media by astrocytes as well as the effect of blocking bFGF-mediated signaling on the differentiation and long-term survival of cholinergic differentiated hNSCs (Hypothesis 2; Specific Aims 2.1-2.3, Chapter 4).

Together the data presented in this thesis strongly suggests that bFGF is a necessary player in the differentiation of hNSCs into Hb9 and later into ChAT expressing cells *in vitro* and furthermore, that astrocyte-secreted bFGF mediates, at least in part, long term *in vitro* survival of cholinergic neurons derived from FHL primed hNSCs.

Hypothesis 1: bFGF Plays A Critical Role In Cholinergic Neuron Differentiation From FHL-Primed Human Fetal Neural Stem Cells *in vitro* (Chapter 3).

Specific Aim 1.1: To determine if FHL primed hNSCs express markers of developing motoneurons.

- **Experiment 1.1a:** To assess morphological differences between FHL, ELL and unprimed hNSCs using phase contrast microscopy.

- **Experiment 1.1b:** To assess cholinergic differentiation of FHL and ELL primed hNSCs using immunocytochemistry for choline acetyltransferase (ChAT).
- **Experiment 1.1c:** To assess expression of developmental motoneuron transcription factors Olig2, Neurogenin2, Islet1, Hb9 via RT-PCR analysis of unprimed (spheres), FHL-primed and hNSCs primed with EGF, Lif and Laminin (ELL).
- **Experiment 1.1d:** To thoroughly assess expression of the early motoneuron and oligodendrocytic transcription factor Olig2 and the late, definitive motoneuron marker, Hb9, in FHL, ELL and unprimed (spheres) hNSCs using both semi-quantitative RT-PCR and immunocytochemistry.

Specific Aim 1.2: To determine if hNSC differentiation into Hb9 expressing cells is time and bFGF dose dependent.

- **Experiment 1.2a:** To assess Hb9 transcript levels in FHL primed hNSCs at various time points from 0-96 hours and after further differentiation.
- **Experiment 1.2b:** To assess Hb9 transcript levels in hNSCs primed for 4 days with different amounts of bFGF.

Specific Aim 1.3: To determine if blockade of bFGF signaling or proliferation influences motoneuron induction in FHL primed hNSCs

- **Experiment 1.3a:** To assess the level of the motoneuron transcription factor, Hb9, using semi-quantitative RT-PCR of FHL primed hNSCs treated with the FGF receptor antagonist, PD173074 or in the presence of a bFGF-neutralizing antibody via semi-quantitative RT-PCR analysis.
- **Experiment 1.3b:** To assess whether proliferation of hNSCs is necessary for Hb9 expression of FHL primed hNSCs. This will be determined by treating FHL primed hNSCs with two cell cycle inhibitors followed by RNA

collection and RT-PCR analysis for Hb9. Proliferation will be assessed by BrdU long term labeling followed by immunocytochemistry.

Specific Aim 1.4: To determine whether cell death account for differences in transcript levels of Hb9 in primed hNSCs.

- **Experiment 1.4a:** To assess the level of cell death using three separate measurements, LDH release, TUNEL immunocytochemistry and caspase 3 activity in FHL, FHL plus the FGF receptor antagonist, PD173074 and ELL primed hNSCs.

Specific Aim 1.5: To determine if MAP kinase signaling pathways are involved in Hb9 induction following FHL priming of hNSCs.

- **Experiment 1.5a:** To assess by western blotting the protein level of two MAP kinase proteins, Erk1/2 and JNK/SAPK in FHL primed, ELL primed and FHL or ELL primed plus the FGF receptor antagonist, PD173074.
- **Experiment 1.5b:** To assess whether proliferation of hNSCs is necessary for Hb9 expression of FHL primed hNSCs. This will be determined by treating FHL primed hNSCs with two cell cycle inhibitors followed by RNA collection and RT-PCR analysis for Hb9. Proliferation will be assessed by BrdU long term labeling followed by immunocytochemistry.

Hypothesis 2: Long Term Survival of hNSCs is Enhanced in the Presence of Astrocytes or Astrocyte Secreted Factors (Chapter 4).

Specific Aim 2.1:To determine if astrocytes or astrocyte secreted factors enhance cholinergic differentiation of FHL primed hNSCs.

- **Experiment 2.1a:** To examine long term neuronal or glial phenotypic outcome of FHL primed hNSCs co-cultured under direct, indirect or in the presence of ACM using immunocytochemistry.

- **Experiment 2.1b:** To examine cholinergic differentiation of long term differentiated FHL primed hNSCs using immunocytochemistry and RT-PCR analysis for choline acetyltransferase.

Specific Aim 2.2: To determine if astrocytes or astrocyte secreted factors enhance long term hNSC survival, particularly cholinergic neuronal survival

- **Experiment 2.2a:** To examine the morphology of FHL primed hNSCs differentiated in the presence of either primary derived spinal cord astrocytes under direct contact or indirect (Transwell) culture or with astrocyte conditioned media using phase contrast microscopy.
- **Experiment 2.2b:** To assess the long term hNSC survival using LDH cell death assays, WST1 cell viability assays and TUNEL apopototic staining of FHL primed hNSCs differentiated in the presence or absence of astrocyte secreted factors.

Specific Aim 2.3: To determine the molecular basis for astrocytic effects on hNSC differentiation and cell survival.

- **Experiment 2.3a:** To assess whether immortalized astrocytes (CRL2005) secrete detectable levels of bFGF into the culture media which can be preserved and detected using a bFGF ELISA.
- **Experiment 2.3b:** To examine the role of astrocyte secreted bFGF on cell viability following addition of the FGF receptor antagonist, PD173074 or after addition of a bFGF-neutralizing antibody to long term cultured hNSCs in the presence of ACM.
- **Experiment 2.3c:** To examine the role of astrocyte secreted bFGF on long-term cholinergic neuronal cell survival using RT-PCR analysis for FHL primed hNSCs differentiated with ACM in the presence of the FGF receptor antagonist (PD173074) or with a bFGF-neutralizing antibody.

CHAPTER 2: MATERIALS AND METHODS

CELL CULTURE

Human Fetal Neural Stem Cell Culture

Human fetal NSCs (lines K048 and K054, generously provided by C.N. Svendsen, University of Wisconsin) were isolated from 8- and 9-week fetal forebrains, respectively. Cells were cultured as free-floating neurospheres in 75-cm^2 flasks as previously described (Tarasenko et al., 2004). All chemicals in this study were purchased from Sigma (St. Louis, MO) unless otherwise specified. Briefly, cells were grown in basic medium containing DMEM (high glucose, L-glutamine)/Ham's F12 (both from Cell-Gro) (3:1) supplemented with 67 IU/ml penicillin/67 µg/ml streptomycin (1% Pen Strep), 15 mM HEPES, N2 (Bottenstein and Sato, 1979) , 15 mM HEPES, 1.5% D-glucose and 1 mM L-glutamine. For proliferation as neurospheres the following growth factors were added to the above basic culture medium: 10 ng/ml recombinant human leukemia inhibitor factor (LIF, Chemicon, Temecula, CA), 20 ng/ml each of recombinant human EGF and bFGF (both from R&D Systems, Minneapolis, MN) and 2.5 µg/ml heparin. Cultures were maintained at 8.5% CO_2, 37°C, and a half volume of culture medium was replaced every 3.5 days. Cells were dissociated every 7-10 days using 0.25% trypsin diluted in Dulbecco's phosphate buffered saline (dPBS)/glucose for approximately 10-15 mins at 37°C. To facilitate cell detachment, spheres were periodically triturated with a 5 ml serological pipette. Following dissociation, trypsin inhibitor diluted in conditioned media [1:100] was added, cells were counted using a trypan blue exclusion assay and approximately $6x10^6$ live cells were re-seeded into T75 culture flasks which had been pre-treated to prevent cell adherence with 5 ml of conditioned media and then 10 ml of new media.

Cell Priming

For cell priming, ~$2x10^6$ hNSCs were plated as small spheres (3-4 days post passage, see Figure 2.1) in T25 culture flasks pre-coated with 0.01% Poly-D-Lysine (PDL) and 1 μg/cm^2 mouse laminin (Invitrogen/GIBCO; Carlsbad, CA), and incubated with either of two priming media as follows. FHL priming media (basic medium supplemented with 10 ng/ml bFGF, 2.5 μg/ml heparin and 1 μg/ml Laminin) (Wu et al., 2002) or ELL priming media (basic media containing 20ng/ml EGF, 10 ng/ml Lif and 1 μg/ml Laminin). Cells were incubated at 8.5% $C0_2$, 37°C for 4-5 days prior to harvesting for RNA or protein extraction. For immunofluorescent analysis, cells were plated at a density of 1-$1.5x10^5$ cells/well in 24-well plates containing PDL and laminin pre-coated German glass coverslips (Carolina Biological Supply) and cultured in the above priming medium.

Co-Culture Experiments: Primary Rat Astrocytes

Primary spinal cord astrocytes were generated from adult (8 weeks) Sprague Dawley rats according to the method described by Schwartz and Wilson (1992) with modifications (Schwartz and Wilson, 1992). Briefly, spinal cords were dissected from adult rats sacrificed with CO_2 and placed immediately into calcium-magnesium free-dPBS (CMF-dPBS) on ice. Meninges were removed under a dissecting scope (Nikon). Cords were cut into small pieces using sterile blades and then placed into astrocyte growth medium (AGM) consisting of DMEM supplemented with 10% FBS, 1.5% D-glucose, 1X Fungizone/penicillin-streptomycin on ice. Cells from 3-4 spinal cords were retrieved by mechanical dissociation using sterile fire-polished glass pipettes followed by repeated passage through 16, 18 and 20 gauge sterile syringes. Numbers of live cells were determined using the trypan blue exclusion assay and approximately one cord was plated per T25 culture flask. Occasionally, cells were further purified by passage through 60 μm nylon mesh filters. Cells were maintained in a humidified incubator supplemented with

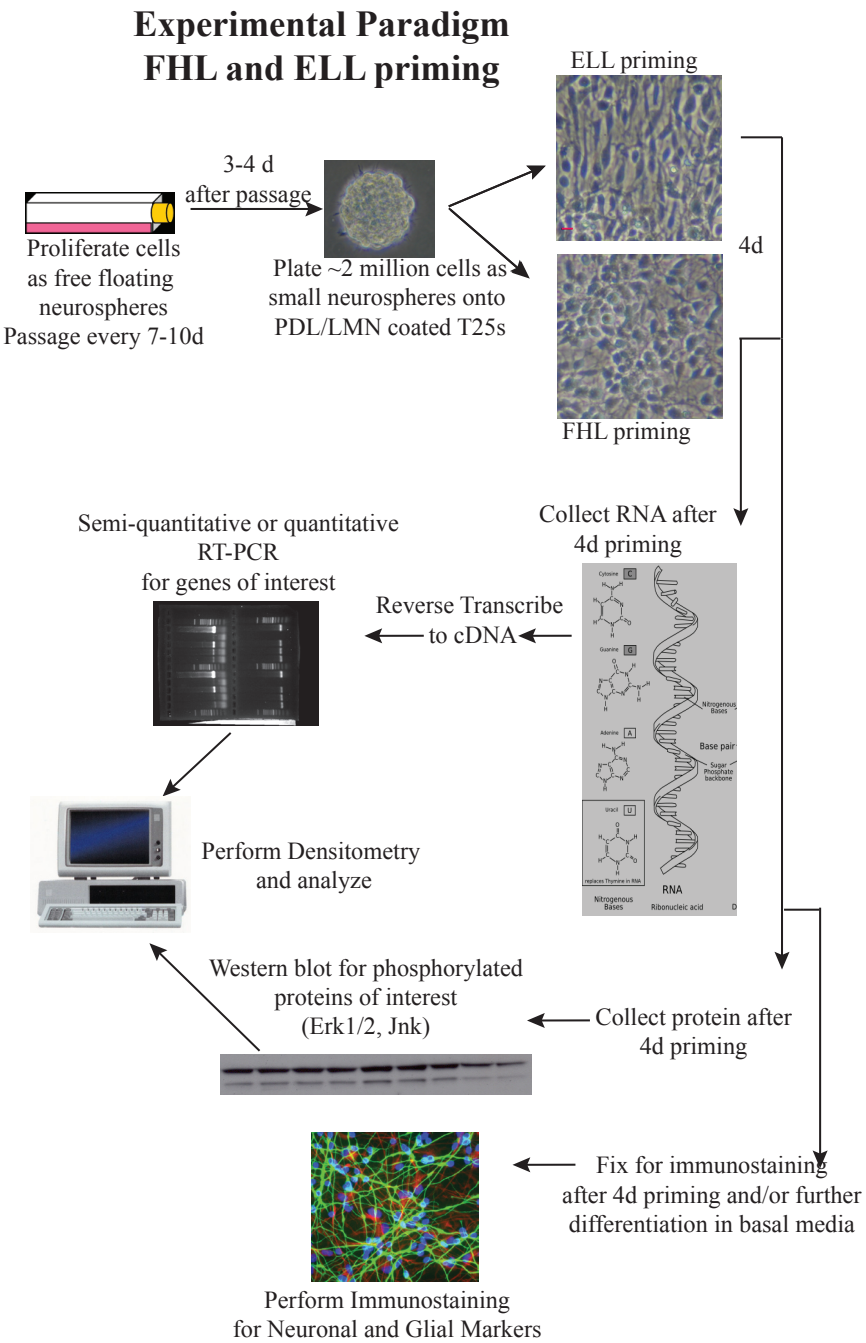


Figure 2.1: Experimental paradigm for ELL vs FHL priming experiments. Human neural stem cells were passaged every 7-10d and allowed to aggregate into small neurospheres (3-4d) prior to plating onto PDL and Laminin coated dishes for either ELL (EGF, Lif and Laminin) or FHL (bFGF, heparin and laminin) priming. Following 4 d of priming, cells were lysed for protein or RNA collection or fixed with 4% paraformaldehyde and samples were further analyzed via semi-quantitative RT-PCR, immunostaining or western blotting. Composite figure created in Adobe Illustrator. PC image courtesy of: http://www.cedmagic.com/history/ibm-pc-5150.html. RNA image from http://images.search.yahoo.com/images)

5% CO_2 for 7-14 days or until nearly confluent. At that time, cells were shaken for several hours to remove less adherent cells (neurons, microglia and oligodendrocytes) followed by removal of media, washed with CMF-dPBS and addition of new media. Following shaking, cells were allowed to proliferate to confluence, at which time they were passaged using 0.25% trypsin supplemented in CMF-dPBS and plated at approximately $2x10^6$ cells/T25. Immunohistochemical analyses (Figure 2.2) verified that cells purified using this method were over 94% positive for the astroglial marker glial fibrillary acidic protein (GFAP) prior to first passage. After passaging, nearly 100% of cells plated in either FBS or serum-free B27 medium expressed GFAP (Figure 2.2).

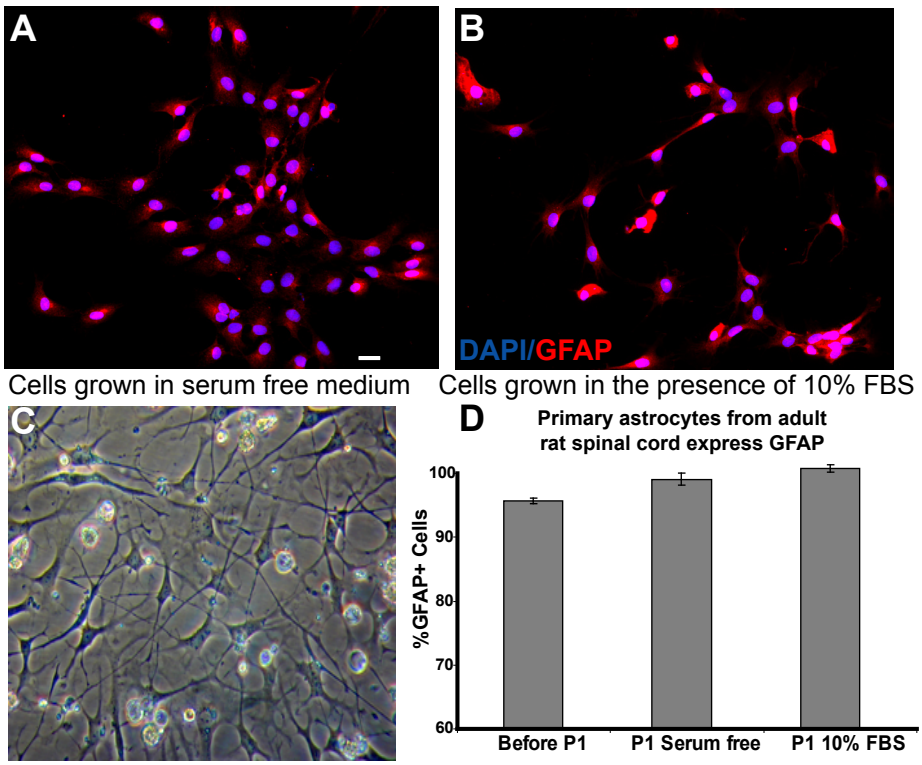


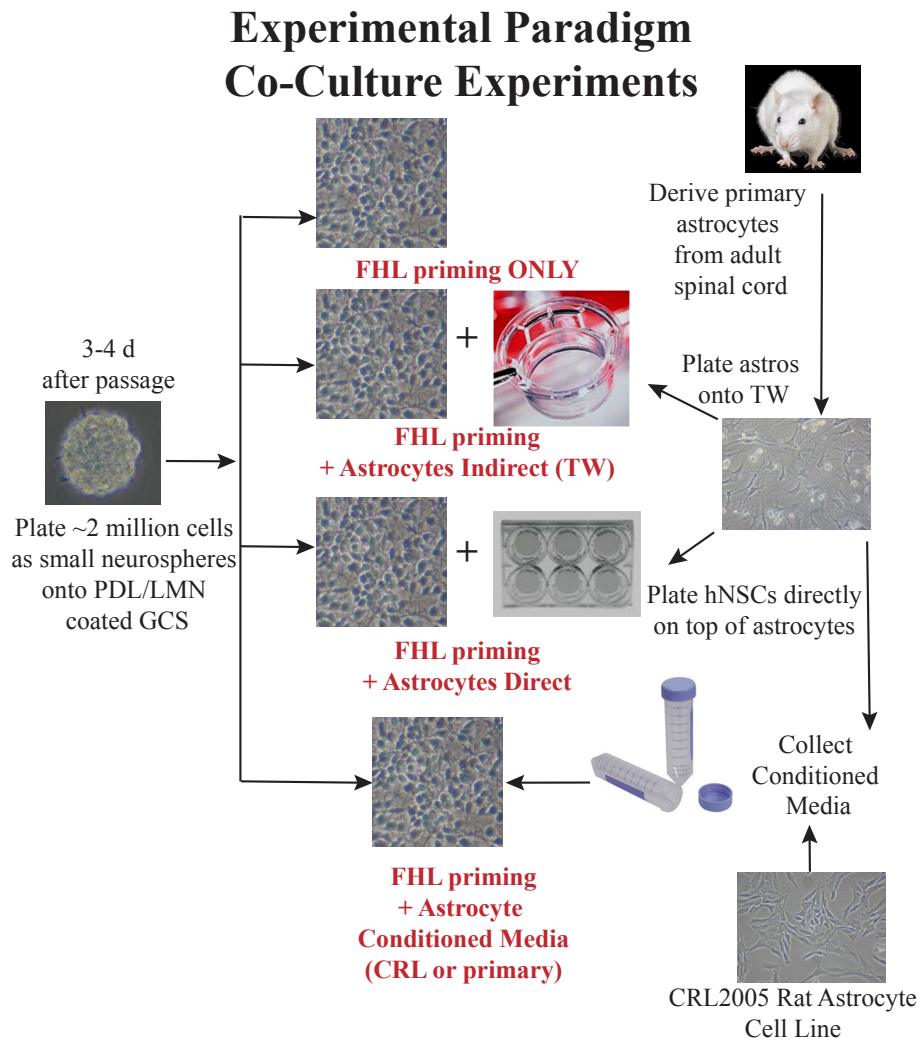
Figure 2.2: Astrocytes derived from adult rat spinal cord express GFAP. A and B) Nearly 100% of astrocytes grown in serum free (B27) media (A) or serum containing media (B) were immunopositive for GFAP (GFAP shown in red; blue=DAPI nuclear counter stain). Scale bar=10µm. C) Phase contrast image of astrocytes in culture show their unique morphology. D) Quantification of GFAP expressing cells under various culture conditions.

For direct co-culture studies, $1x10^5$ astrocytes/cm^2 were plated onto PDL/laminin coated glass coverslips, treated with 0.2 μM cytosine arabinoside (AraC) and allowed to adhere for 24 hours. Human NSCs were then added on top of astrocytes. For indirect culture, astrocytes were plated onto permeable inserts (Transwells; Corning-Fisher Scientific) pre-treated with poly-L-lysine (PLL) and laminin at a similar density and allowed to adhere for 12-24 hours. Inserts with astrocytes were then transferred to plates containing FHL-primed hNSCs. In this co-culture, hNSCs were exposed to secreted factors from astrocytes suspended above them in the transwells but lacked direct contact with the cells. To exclude any serum-derived effects on hNSC differentiation and survival, co-cultures were grown in serum-free (B27 supplemented) medium. Media were changed every 3-4 days for up to 30 days and some were collected for LDH assays at days 5, 10, 15, 20, 25 and 30. For an outline of all co-culture experiments see Figure 2.3.

For generation of astrocyte conditioned media (ACM), $0.5X10^6$ cells were plated into T75 flasks in AGM for 12 h at which time half of the medium was changed to serum-free B27 medium (B27:AGM 1:1). After a further 12 h (total time of 24 h post plating), all media were removed and cells were washed with dPBS and incubated with 15 ml of B27. Astrocyte conditioned medium (ACM) was then collected 24 h later. Floating cells/debris were removed by centrifugation and media were sterile filtered through a 0.2 μm filter, aliquoted and stored at -80°C for future experiments. ACM was used at 1:1 dilution with freshly prepared B27 media for all ACM experiments.

Immortalized Rat Astroglial Cell Line

An immortalized type 1 astroglial cell line derived from neonatal day-1 rat diencephalon (CRL2005-ATCC; Manassas, VA) were grown to produce astrocyte-conditioned medium (ACM). Cells were grown in astrocyte growth media (AGM). Every 3-4 days (approximately 80% confluent) cells were dissociated by applying 0.25% trypsin:



Assays Performed

1. **LDH Cell Death Assay**
2. **WST-1 Cell Survival Assay**
3. **Imunofluorescent staining: neurons and glia**
4. **Immunofluorescent staining: Cholinergic phenotype**
5. **RNA and RT-PCR analysis**

Figure 2.3: Experimental paradigm for co-culture studies. Small neurospheres were plated in one of five different paradigms: 1) under FHL priming alone 2) with astrocytes under indirect culture (i.e. with astrocytes plated onto transwells suspended above FHL primed hNSCs) 3) under direct co-culture (i.e.with astrocytes and hNSCs plated directly on top of one another) 4 & 5) with conditioned media from either primary astrocytes or from an astrocytic cell line (CRL2005).

EDTA for 5 minutes followed by trituration and addition of fresh medium. Cells were re-plated by splitting every confluent T75 into 6-10 T75 flasks (approximately 0.5-1x10^6cells/ T75). ACM was collected in the same manner as for primary astrocytes.

Inhibitors of Cell Proliferation

Cell proliferation was inhibited using two mechanisms. First, DNA synthesis was inhibited by adding the chemotherapeutic agent cytosine arabinoside (AraC). AraC is known to inhibit DNA synthesis through incorporation into the DNA causing replication of new DNA strands to terminate early. AraC was prepared in 1 mM stocks, stored at -20°C and utilized at 1 μM concentration in fresh culture media. In order to determine whether AraC at 1 μM concentration inhibited DNA synthesis, BrdU labeling (see BrdU labeling below) of AraC and non-AraC treated samples was utilized. Cultures treated with 1 μM AraC did not show any BrdU labeling while those grown in media without AraC showed extensive BrdU incorportation. Thus 1 μM concentration of AraC was an effective mitotic inhibitor for hNSCs.

To confirm AraC's effects, a second mitotic inhibitor which functions through a separate molecular mechanism was utilized. To progress through the cell cycle, a group of proteins termed cyclins and their associated kinases, the cyclin dependent kinases (Cdk), are required. Blockade of Cdk binding with its associated Cyclin inhibits cell cycle progression and thus halts cells in a particular phase of the cell cycle. For our study we chose to inhibit cells from progressing from G_1 to S phase by inhibiting Cdk II (type III; EMD Biosciences). To test the efficacy of this inhibitor, a dose response curve (0.5 μM to 10 μM) was generated using a similar BrdU labeling and detection paradigm as for AraC. In this experiment, it was found that a pulse of 2.5 μM concentration completely inhibited cell cycle progression as judged by a lack of BrdU staining in these cultures (Figure 2.4). Because cytotoxicity using mitotic inhibitors could be a problem, it is worthy to note that

cells treated with this concentration also maintained healthy cell morphologies with intact nuclei.

Bromodeoxyuridine (BrdU) Pulse Labeling

Bromodeoxyuridine (BD Biosciences, San Jose, CA) was purchased in 35mM DMSO stock solutions and kept frozen at -80°C until use. For long term BrdU labeling, 0.2 µM BrdU was added to cell culture media on d1 and cells were allowed to proliferate for 96 hrs at which time they were fixed for immunodetection of BrdU incorporation (see immunofluorescent detection of BrdU). For short term pulse labeling, 0.2 µM BrdU was added to cells 24 hours prior to fixation and detection as described under immunofluorescent staining.

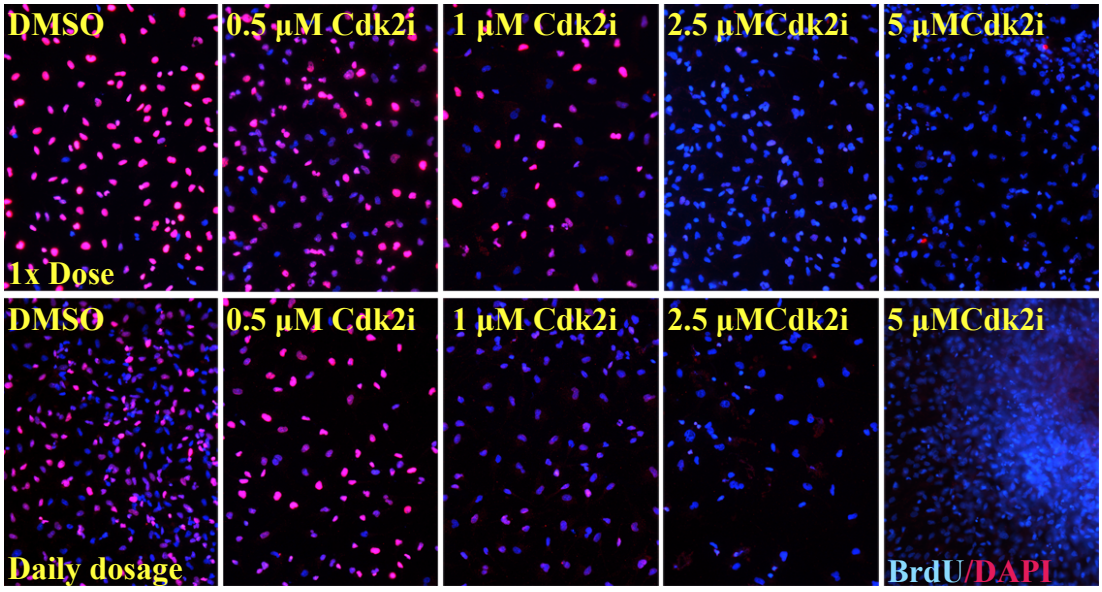


Figure 2.4: Optimization of a CDK2 inhibitor to block hNSC proliferation. Addition of CDK2i at 2.5 µM concentration completely blocks BrdU incorporation (lack of red staining). Adding CDK2i at either the time of plating (upper plan) or daily dosing (lower panel) does not influence efficacy. Blue=DAPI nuclear counterstain. Red=anti-BrdU; labels proliferating cells.

FGF Receptor Antagonist and bFGF Neutralizing Antibody

In order to analyze the effects of bFGF, a FGF receptor antagonist, PD173074 (a kind gift from Pfizer, New York), was utilized at 25-50 nM concentration (Bansal et al., 2003;Mohammadi et al., 1997;Mohammadi et al., 1998). PD173074 was prepared in sterile DMSO and stored in frozen stocks at -80°C until ready for use. To preserve effectiveness, fresh stocks were prepared every two weeks from the original lyophilized powder. To test the effectiveness of this inhibitor, three experiments were performed. First, a dose response was conducted using a WST-1 cell proliferation assay on small hNSC spheres stimulated with bFGF and different doses of PD173074. After 3d in culture, WST-1 assays were performed and a dose response curve was generated (Figure 2.5) (see also WST-1 cell proliferation assays below for details). From this data we chose to use 50nM concentration of PD170374 for our future experiments. Second, a specificity test was performed using similar methodology. Briefly, hNSCs grown either without mitogens (ctrl), in EGF and LIF (EL), or in EGF, LIF and bFGF (bFGF) were treated with PD173074 following which WST1 assays were again performed after 72 hrs in culture. In this set of experiments we found that PD173074 was an effective inhibitor of proliferation only in hNSCs grown in the presence of bFGF (Figure 2.5). In order to verify WST-1 results, a third experiment was performed in which FHL and ELL primed hNSCs treated with 25 or 50nM PD173074 were lysed and Western blots were performed for a bFGF downstream target (Erk1/2). 25-50nM were both found to effectively block activation of Erk1/2 in FHL primed hNSCs. Again confirming PD173074's specificity for bFGF signaling, ELL primed hNSCs treated with PD173074 (ELL+PD) showed little difference in Erk1/2 activation as compared to ELL primed alone (ELL) (Figure 2.6).

To further confirm bFGF's effects on hNSC differentiation, a bFGF neutralizing antibody (3 μg/ml working concentration) (US Biologicals, Swampscott, MA) was also

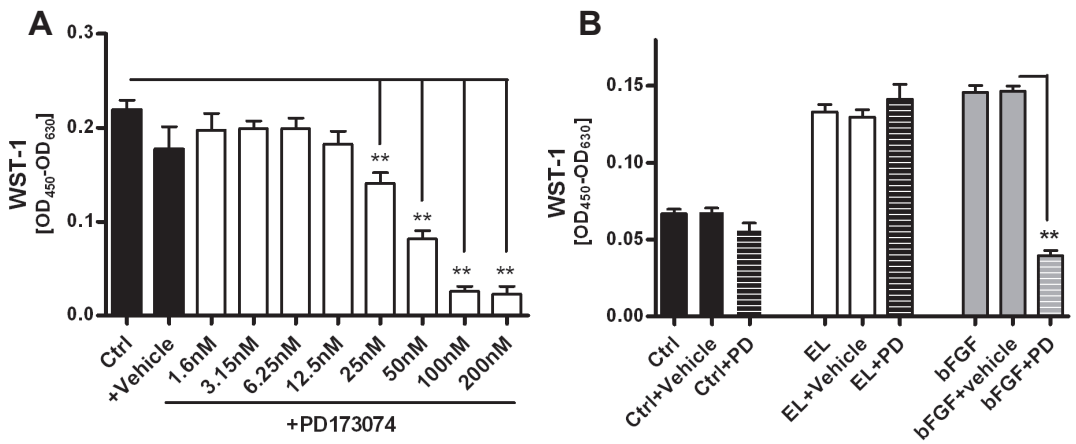


Figure 2.5: Optimization of PD173074 dose and specificity in hNSCs. A) Dose response of PD173074 in bFGF treated hNSCs. Note that at low concentrations there are no differences versus control. Vehicle=DMSO treatment only. Ctrl=cells treated with only bFGF. **p<0.01 vs control, one way ANOVA. B) Specificity of PD173074 for FGF signaling. WST-1 cell proliferation assays were performed on hNSCs grown without mitogens (ctrl), in EGF and LIF (EL) or in EGF, LIF and bFGF (bFGF) +/- Vehicle (DMSO) or PD173074, a FGF receptor antagonist for 3d. Only cells grown in the presence of bFGF show a decrease in proliferation in response to PD173074. **p<0.01 vs bFGF vehicle treated, unpaired students *t* test.

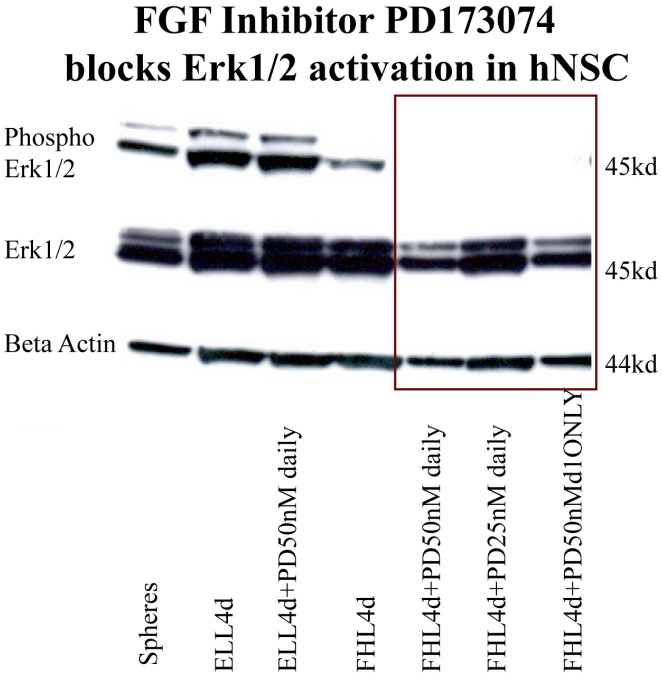


Figure 2.6: Western blot of hNSCs primed with various treatments including addition of a FGF receptor antagonist, PD173074. Treatment with PD173074 blocks activation of Erk1/2 in FHL treated hNSCs (see box). Note that ELL primed cells treated with PD173074 do not show down-regulation of activated Erk1/2.

utilized to inhibit bFGF signaling. Antibody stocks were maintained at -80°C and diluted antibody was allowed to bind bFGF in new media for 10 min prior to addition to cells. In some experiments in order to ensure long term efficacy, neutralizing antibody was added at several times points (every other day).

ASSESSMENT OF CELL DEATH AND CELL SURVIVAL

Lactate Dehydrogenase Assay (LDH)

The LDH cell death assay (Roche, Indianapolis, IN) was performed according to the manufacturer's directions. Briefly, conditioned media (CM) were collected and immediately placed on ice in sterile microcentrifuge tubes, centrifuged at 14,000 X g for 2 min to remove cellular debris and supernatant was transferred to clean tubes. After vortexing, 100 µl of CM from each sample was plated in triplicate in 96-well plates followed by the addition of 100 µl of LDH reagent/well. Plates were incubated at room temperature in the dark for 30 min prior to reading on an ELISA plate reader set at Abs_{490nm} with a background correction of Abs_{630nm}. hNSCs treated with 1% Triton X-100 were used as positive controls for cell death and for standardizing the assay between trials, and blank wells were used to correct for plate imperfections. [Abs_{490nm}-Abs_{630nm}] readings were used to calculate the degree of cell death per sample. Triplicate samples were averaged and each treatment group was compared to the control using an unpaired Student's *t* test with $p<0.05$ considered statistically significant.

Terminal-Deoxynucleotidyl-Transferase-mediated dUTP Nick End Labeling (TUNEL)

See immunofluorescent detection (below).

Caspase-3 activity Assay

Caspase-3 activity was detected using a commercially available kit (EnzChek®, Molecular Probes; Eugene, OR) per manufacturer's directions. Briefly, 20 µg of protein lysates were pipetted in triplicates into 96 well plates followed by the addition of a master mix containing reaction buffer, water, DTT (final concentration 5mM) and the fluorescently tagged substrate, Z-DEVD-R110 (final concentration 25 µM). Z-DEVD-R110 is a fluorescent molecule containing the specific caspase 3 cleavage site DEVD (i.e. Asp-Glu-Val-Asp). The fluorescent R110 tag is quenched when the molecule is intact, however, in the presence of active Caspase-3, DEVD is cleaved releasing the R110 fluorescent tag. For fluorescent detection, plates were read in a fluorescent plate reader with excitation 490nm, emission 520nm every minute for at least 60 minutes. A positive control for this experiment included treatment of hNSCs for 4 hours with the apoptotic agent, 1µM Staurosporine (Calbiochem). Values for each triplicate sample were averaged and a linear regression of fluorescence versus time per µg protein was generated with the slope of the line being used to compare caspase activity between samples. A one way ANOVA with Tukey post hoc tests were used to statistically analyze differences between the treatment groups.

WST-1 Cell Survival Assay

A commercially available kit was used for WST-1 cell proliferation assays (Chemicon) as per manufacturer's directions. Briefly, $1x10^4$ cells /per well were seeded as small spheres 1 day after passage into duplicate 96-well plates. After 4 days in FHL priming, 80% of medium was removed and replaced with B27 (1:50 in DMEM:F12) +/- ACM (1:1). Cells were allowed to differentiate for 14 or 30 days during which time half of the medium was changed every 3-4 days. On day 14 or 30, WST-1 reagent (1:10) was added and 2 hours later, OD readings were taken with an ELISA plate reader (Biotek Instruments) set at Abs_{450nm} with a background correction of Abs_{620nm}. Wells containing medium only (no

cells) were used to further correct for background differences. For example: [Treatment wells containing cells (Abs_{450nm} - Abs_{620nm})] – [Average of wells with media only (Abs_{450nm} - Abs_{620nm})]. Groups were averaged (n=16/treatment/time point) and compared using an unpaired Student's *t* test with $p<0.05$ considered statistically significant.

Manual Cell Counts (Trypan Blue Exclusion Assay)

To determine cell numbers following priming, trypan blue cell exclusion assays were performed after 2 and 4 days post priming. At each time point, culture media were removed and cells were washed 1X with Calcium-Magnesium Free (CMF) dPBS, following which 0.25% trypsin (Sigma) in dPBS/glucose (Invitrogen) was added. Cells were allowed to incubate in trypsin/dPBS/glucose for approximately 10 min at 37°C or until cells became loosely adherent to the bottom of the flask. At that time, trypsin inhibitor diluted in conditioned media [1:100] (Sigma) was added, cells were aspirated several times with a 5ml pipette and removed into a 15 ml centrifuge tube for cell counting. 5 µl of this mixture was removed and combined with 15 µl of trypan blue, 10ul of which was pipetted onto a hemocytometer and cells were manually counted under an upright phase contrast microscope. For each time point, at least 3 T25 flasks were treated in this manner and the average cell number was determined.

To determine proliferation rate, three aliquots of cells prior to cell priming (on d0 of priming) were trypsinized as above and the actual cell numbers were determined using the same trypan blue technique with an average value determined by dividing the total cell number in three aliquots by three. Cell proliferation was then calculated by dividing the number of cells on d2 or d4 determined as above by the number of cells plated down on d0. Averages for at least 3 T25s were calculated for each group using the following formula:

$$\mathbf{AVE\ [\#\ Cells_{day2} \div \#\ Cells_{day0}]}$$

IMMUNOFLUORESCENT AND TUNEL STAINING

Immunofluorescent Staining for Cytoplasmic and Cell Surface Markers

Culture media were removed, and coverslips were washed 1X with dPBS followed by fixation for 25 min in fresh ice-cold 4% paraformaldehyde. Coverslips were then washed 3-4X in TBS at RT and incubated with 5% normal goat serum and 0.25% Triton X-100 in 0.3% BSA/TBS for 30-60 min. Rabbit anti-GFAP [1:1000] (Chemicon; Temecula, CA) and monoclonal anti-beta III tubulin [1:5000] (Covance) were added and allowed to incubate overnight at 4°C. On day 2, cells were washed 3X, 10-15mins each in TBS followed by secondary Alexa Fluor® 568 goat anti-rabbit (568GAR) and Alexa Fluor® 488 goat anti-mouse (488GAM) [1:300] (Molecular Probes, Eugene, OR) for 3-4 h at room temperature. Coverslips were again washed 3X, 10-15 min each, counterstained with DAPI [1:1000] for 5 min and washed one more time prior to mounting onto glass slides with Fluoromount-G (Fisher; Fair Lawn, NJ). For choline acetyltransferase (ChAT) staining [1:100] (Chemicon), a similar procedure was used with the following changes: cells were blocked with normal goat serum and Alexa Fluor® 568 donkey anti-goat (568DAG) (Molecular Probes) secondary antibody was used for fluorescent detection. In all immunofluorescent experiments, cells stained with secondary antibody only were used as negative controls for each primary antibody.

Immunofluorescent Staining for Transcription Factors

Culture medium was removed and coverslips were washed 1X with dPBS followed by fixation in fresh 4% paraformaldehyde for 20 minutes at RT. Following several washes in PBS, cells were post fixed for 10 minutes with ice cold 100% Methanol at -20°C. Cells were then washed 3X in PBS, blocked with 5% normal goat serum/0.25% Triton-X/0.3% BSA/PBS (all from Sigma, St. Louis, MO) for 30 min to 1 hr, following which a monoclonal

anti-Hb9 antibody [1:20-50] (Developmental Studies Hybridoma Bank-DSHB, Iowa) was diluted in PBS and allowed to incubate overnight at 4°C. Secondary antibody labeling was done as for cytoplasmic and membrane bound immunofluroescent staining (above).

Bromodeoxyuridine Detection

For BrdU immunocytochemistry, cells previously pulse labeled with BrdU (see Bromodeoxyuridine pulse labeling) were washed 1X with dPBS, fixed with fresh 4% paraformaldehdye for 25 min on ice followed by 3 washes in dPBS. A second fixation in ice cold 100% Methanol for 20 min at -20°F was then performed followed by an additional 3 washes in dPBS. Cells were then permeabilized and DNA was uncoiled using 2N hydrochloric acid (HCl) for 20 min at 37°C. HCl was neutralized with two washes of Borate Buffer (pH 8.5), 10 min each at RT, followed by 3 washes in dPBS. Samples were then blocked with 1%BSA:0.5% Tween 20:5% NGS for 1 hr at RT followed by application of a monoclonal anti-BrdU antibody [1:1000] (Sigma) for 1 hr at RT. Cells were washed 3X with dPBS followed by either Alexa Fluor® 488 or 568 conjugated goat anti-mouse antibodies [1:300-400] (Molecular Probes, Eugene OR). Samples were washed 3X in dPBS, nuclei were counterstained with DAPI [1:1000] and coverslips mounted in Fluormount G (Fisher). In all experiments, cells stained with secondary antibody only and cells that did not receive BrdU pulse labeling were used as negative controls.

TdT-Mediated dUTP Nick End Labeling (TUNEL)

For TUNEL staining, a commercially available kit (ApopTag® Fluorescein *In Situ* Apoptosis detection kit; Chemicon; Temecula, CA) was utilized as per the manufacturer's directions. Briefly, cells were fixed with fresh 4% paraformaldehyde for 20 min. at RT followed by 3 washes in PBS. Cells were then post-fixed in pre-cooled Ethanol:Acetic Acid (2:1) at -20°C for 5 min followed by 3 PBS washes. PBS was then removed and 27 µl equilibration buffer/well (75 µl/5 cm^2) was added followed by 20 µl TdT enzyme diluted in

reaction buffer. Cells were then allowed to incubate at RT for 1 hour followed by addition of stop/wash buffer, 3 PBS washes and addition of anti-digoxigenen conjugated secondary antibody for 30 min at RT. Samples were then washed with PBS, counterstained with DAPI [1:1000], mounted with Fluoromount G (Fisher) and visualized using standard fluorescent microscopy. TUNEL positive cells were counted and the ratio of TUNEL/total cell numbers, the latter determined after DAPI nuclear staining, determined the % of apoptotic cells per treatment group.

Immunofluorescent Microscopy and Cell Counting

Images were taken using a Nikon epifluorescent microscope (e800i) and MetaMorph Software (Molecular Devices Corporation; Downington, PA). For quantitative analyses, 10 fields per treatment or at least 1000 total cells were randomly selected from 3-4 coverslips and cells were manually counted using MetaMorph imaging software. Percentage positive staining for any particular antibody was calculated by dividing the number of positive cells by the total number of DAPI-stained nuclei. Statistical analyses were performed using InStat (GraphPad Software, San Diego, CA) and SigmaStat (SPSS Inc., Chicago, IL) programs.

PCR ANALYSIS

RNA Collection and Generation of cDNA by Reverse Transcription

Total RNA was extracted using RNAqueous-4PCR kit (Ambion; Austin, TX) as per the manufacturer's directions. Briefly, media were removed and cells were rinsed with CMF-dPBS followed by the addition of 300-500 µl of lysis buffer. Cells were lysed on ice for up to 5 min followed by lysate collection using sterile cell scrapers. Samples were then vigorously vortexed followed by additional passage through sterile 25 gauge syringes. An equal volume of 64% ethanol was added and lysates were applied to columns, spun down at

10,000 x g and rinsed several times. RNA was eluted into sterile RNAse/DNAse-free tubes and DNAse I was added for 30 min at 37°C followed by inactivation with DNAse inactivation reagents. Samples were pelleted by centrifugation and supernatants were collected and stored at -80°C. RNAs were quantified and purity-checked using a spectrophotometer with an A260:A280 ratio between 1.9 and 2.1 being considered acceptable for further assay. Samples with ratios outside of this range were discarded. Reverse transcription (RT) reactions were performed using an RT kit (Applied Biosystems; Foster City, CA) as per manufacturer's directions. Briefly, 2 µg RNA was combined with RT buffer (10 µl), dNTPs (4 µl), random primers (10 µl), and RNAse free water to a total volume of 80 µl. Samples were cycled at for 10 min at 25°C and 120 min at 37°C. Following RT, cDNA was diluted in TE buffer to yield 10 ng/µl and frozen at -20°C.

Semi-Quantitative RT-PCR

10-20 ng of cDNA was used for each individual PCR reaction with the following primers and cycling conditions. **Olig2** Forward: 5'-AAG CTA GGA GGC AGT GGC TTC AAG TC-3'. Olig2 Reverse: 5'-CCG TCA CCA GTC GCT TCA TC-3'. Tm=60°C 10-20 ng cDNA, 28 cycles, product size = 312bp. **Neurogenin** 2 Forward: 5'-GAA GAC CCG TAG ACT GAA GGC-3'. Neurogenin 2 Reverse: 5'-GAA AGG GAA CCC ACT AAG GC-3'. Tm=54°C 20-40 ng cDNA, 40 cycles, product size = 537 bp. **Islet-1** Forward 5'-AGG ATG TGG AGG TAG TGA GA Islet-1 Reverse 5'-GGA GAT CTC AGT GGC TCT T ; Tm=54°C 20-40 ng cDNA, 40cycles, product size = 356bp. **Hb9** Forward 5'-AGC TGG GCG CCG GCA CCT TCC-3'. Hb9 Reverse 5'-CCG CCG CCG CCC TTC TGT TTC TC-3'; Tm=65°C 20 ng cDNA, 37-38 cycles, product size = 348bp. **ChAT** Forward: 5'-AAG ACG CCC ATC CTG GAA AAG-3'; ChAT Reverse:5'-TGA GAC GGC GGA AAT TAA TGA C-3'. Tm = 61°C; 40 cycles; product size = 650 bp. **GAPDH** Forward: 5'-GAA GGT GAA GGT CGG AGT C-3';GAPDH Reverse:5'-GAA GAT GGT GAT GGG

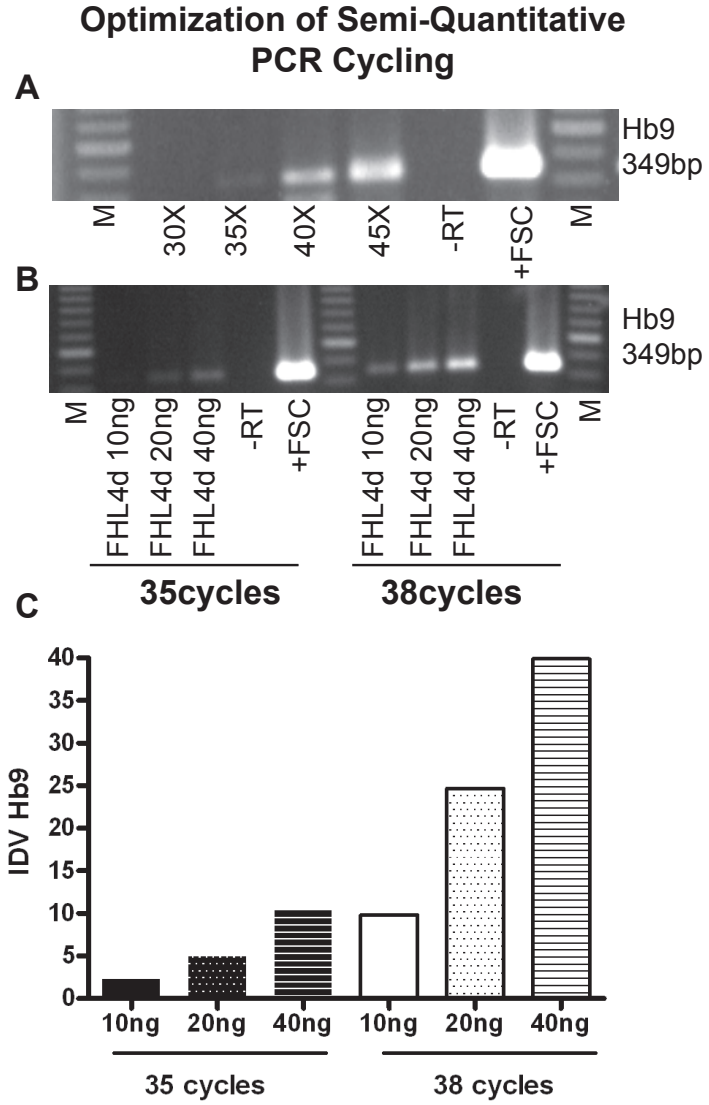


Figure 2.7: Optimization of semi-quantitative PCR parameters for Hb9. A) 20 ng of cDNA were run in quadruplicate tubes and each were pulled from the PCR reaction after 30, 35, 40 and 45 cycles. PCR products were electrophoresed and visualized after ethidium bromide (EtBr) staining. B) From A it appeared that between 35 and 40 cycles was optimal. To test this finding, triplicate PCR reactions were set up with 10, 20 and 40 ng of cDNA and PCR was performed for 25 and 38 cycles. PCR products were elecrophoresed and visualized using EtBr staining. C) Densitometry of PCR products from B indicate that 35 or 38 cycles of PCR are within the correct range for optimal semi-quantitative PCR. Note that 40 ng cDNA generates a PCR product, which is ~2X greater than 20 ng, which is ~2 fold greater than 10 ng. [M=Molecular marker. FSC=Fetal spinal cord, a positive control. -RT = negative control (i.e. samples run without reverse transcriptase)].

ATT TC-3'. Tm = 52°C; 20 ng cDNA; 21 cycles; product size = 226 bp. A master mix containing 2.5 µl Red Taq polymerase, 2.5 µl 10X buffer, 1 µl 25X dNTPs (all from Sigma) and 1 µl each of primers were utilized for all PCR reactions. For determination of semi-quantitative conditions, 20 ng cDNA was loaded into quadruplicate PCR reactions and PCR was performed for 25, 30, 35 and 40 cycles. 15 µl of each PCR product was then loaded onto 1.5% agarose gels and electrophoresed at 90V for 1.5 hrs. Gels were post stained with ethidium bromide, imaged using a Chemi-Imager 4400 v5.5 (Alpha Innotech Corporation) and densitometric analysis was performed using Alpha-Ease software (Alpha Innotech Corporation). The cycle number determined to be in the middle of the logarithmic phase of PCR was then utilized for further optimization via running triplicate PCR reactions again this time with 10, 20 and 40 ng of cDNA and utilizing the cycle number previously determined (Figure 2.7). Densitometry was again performed to verity that 40 ng of cNDA yielded ~2-fold increase in PCR products as compared to 20 ng cDNA and that 20 ng was ~2-fold greater than 10 ng. For each semi-quantitative reaction hence forth, two control samples were added: 1 with half volume of cDNA (typically 10 ng) and the second with the double amount (typically 20 ng). Densitomtery of PCR products was verified for each PCR reaction performed and reactions which did not show 10 ng > 20 ng densitometry were discarded from the analysis.

Real-Time Quantitative RT-PCR (qPCR)

Quantitative PCR was performed by the quantitative PCR core facility (UTMB) per established methods. Briefly, Applied Biosystems Assays-on-demand 20X assay mixture of primers and TaqMan MGB probe (FAM™ dye-labled) for the target genes (see below) and pre-developed 18S rRNA(VIC™-dye labled probe) TaqMan® assay reagent (P/N 4319413E) for endogenous control were utilized for these assays with universal PCR master mix reagent kit (P/N 4304437).

Assays-on-Demand™(P/N 4331182): HLXB9

Genebank accession #	Assay ID	Sequence
NM_005515.2,X56537.1,AF107457.1	Hs00232128_m1	ACTTCAACTCCCAGGCGCAGTCGAA

For relative quantitation of gene expression separate tubes (singleplex) real time PCR was performed with 30 ng cDNA for both target gene and endogenous control. Cycling parameters for real time PCR were: UNG activation 50°C, 2min, AmpliTaq activation 95°C for 10 min, denaturation 95°C for 15 sec and annealing/extension 60°C for 1 min (repeat 40 times) on an ABI7000 PCR machine. Duplicate C_T values were analyzed in Microsoft Excel using the comparative $C_T(\Delta\Delta C_T)$ method as described by the manufacturer (Applied Biosystems). The amount of targets ($2^{-\Delta\Delta CT}$) was obtained by normalized to endogenous reference (18s) and relative to a calibrator (one of the experimental samples).

IMMUNOBLOT ANALYSIS

Collection of Total Cell Protein

After rinsing in phosphate-buffered saline (PBS), cells were lysed with NP-40 Lysis Buffer [1% NP-40, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate] (all from Sigma; St. Louis, MO) with fresh 10 µg/ml Apoprotinin and 10 µg/ml Leupeptin added just prior to cell lysis. Approximately 200-350 µl lysis buffer was used per T25 ($2x10^6$ cells) and cells were allowed to lyse on ice for 5 min prior to removal of cell lysates to microcentrifuge tubes with a cell scraper. Cells were allowed to lyse for an additional 15-30 min rotating at 4°C prior to clarification by centrifugation at 14,0000 X g, 4°C for 10 min and removal of supernatant to a clean tube.

Protein lysates were quantified with a bicinchoninic acid (BCA) protein quantification kit (Pierce; Rockford, IL), aliquoted and stored at -80°C.

One Dimensional Gel Electrophoresis

For electrophoresis, 20-30 µg of total protein were diluted in 4X NuPAGE® LDS sample buffer and 10X NuPAGE® reducing agent (both from Invitrogen; Carlsbad, CA) and heated to 70°C for 1 min. Samples were briefly vortexed, centrifuged, loaded onto 4-12% NuPAGE® Novex Bis Tris Gels and electrophoresed at 150V for ~2 hrs. Gels were transferred onto Hybond ECL nitrocellulose membrane (Amersham Biosciences; U.K.) by electrophoretic transfer at 30V for 1-1.5 hr.

Western Blotting

Membranes were blocked with 5% nonfat milk (w/v in 0.1%TBS-Tween) for either 1 hr at RT or overnight at 4°C, followed by a 1-hr incubation at room temperature with the following primary antibodies: phospho-p44/42 (i.e. pErk1/2) (Thr202/Tyr204) [1:500]; p42/44 [1:500]; phospho-SAPK/JNK (Thr183-Tyr185) [1:1000]; SAPK/JNK [1:1000] (all from Cell Signaling Technologies; Danvers, MA), diluted in 5% nonfat milk. Membranes were washed 6X, 5 min each, with 0.1% TBS-Tween and incubated for 1 hr at RT with the appropriate anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody [1:5000-1:10,000] (Amersham Biosciences, U.K.). Membranes were again washed 6X, 5 min each, and immunoreactive bands were detected using a chemiluminescent Western blot detection kit (ECL-Amersham Biosciences, U.K.). Blots were exposed to ECL hyperfilm (Amersham Biosciences, UK) for 5 sec - 5 min prior to developing using a standard developer (Kodak). All blots were first probed with the phosphorylated protein of interest followed by stripping (Restore™; Pierce Biotechnological; Rockford, IL) and re-probing for the corresponding un-phosphorylated protein of interest and again for mouse

anti-β-actin [1:25-50,000] (Sigma; St. Louis, MO) as a loading control using a similar protocol.

Western Blot Analysis

Films were scanned using a digital scanner connected to a PC, and densitometry via integrated density value (IDV) analyses were performed with AlphaEase FC Software program (Alpha Innotech). For detection of phosphorylation status, the phosphorylated protein band value (IDV) was divided by the band from the un-phosphorylated protein (i.e. IDV phospho Erk ÷ IDV Erk) and then normalized using the loading control (β-actin). All IDVs were generated using automatic background control. For other proteins, data were normalized against β-actin alone. Statistical significance between groups was determined using one-way ANOVA with Tukey post hoc test or for single comparisons, Student's *t* test with $p<0.05$ considered significant.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

bFGF ELISA

A commercially available bFGF ELISA was utilized to detect secreted bFGF in astrocyte conditioned medium (bFGF DuoSet®; R&D Systems; Minneapolis, MN) per manufacturer's directions. ACM was generated as stated above with an additional centrifugation step to concentrate and remove small molecular weight molecules. For the concentration step, ACM was applied to Amicon® Ultra-15 Centricon filters with a 10,000 MWCO (Millipore) and centrifuged at 4,000 x g, 4C° for 30 mins. The volume of concentrated ACM was measured and aliquoted for ELISA.

Briefly for ELISA analysis, 96 well ELISA plates were coated with 100 µl of a 2 µg/ml stock of bFGF capture antibody, covered with plastic tape and left to hybridize overnight at room temperature. The next day, wells were washed 3X with 400 µl of wash

buffer (PBS+0.5% Tween) and blocked with 300 µl of blocking buffer (1% BSA diluted in dPBS) for at least 2h at room temperature. Blocking reagents were then removed, wells were washed as previously and replaced with 100 µl each of bFGF standards (1000 pg/ml to 0 pg/ml of recombinant human bFGF (rh bFGF) diluted in 1% BSA/dPBS) and conditioned media unknowns previously concentrated (see above). Samples were allowed to hybridize for 2-3h, shaking at 500 RPM @ RT following which all wells were washed again as above and 100 µl of a 0.5 µg/ml stock of secondary/detection antibody was added. Plates were again shaken at 500 RPM for 2-3h at RT after which samples were washed 3X with wash buffer. Streptavidin-HRP conjugated antibody was then added [1:200] and allowed to hybridize for 20 mins in the dark at RT and samples were again washed 3X as above. Following washing, 100 µl of detection reagent (A:B, 1:1) was added for 20 min in dark and the reaction was stopped by adding 20 µl of Stop Solution (2N H_2SO_4) to each well. Plates were immediately read in a standard plate reader at 450 nm with a correction of 570 nm. For analysis, all samples were run in triplicates and bFGF standards were used to generate a standard curve (r^2=0.98) utilized for bFGF concentration determination.

CHAPTER 3: BASIC FIBROBLAST GROWTH FACTOR INDUCES HB9 EXPRESSION IN LONG TERM EXPANDED HUMAN FETAL FOREBRAIN-DERIVED NEURAL STEM CELLS

ABSTRACT

Human neural stem cells (hNSCs) have the potential for use in the clinical treatment of neurological disorders including amyotrophic lateral sclerosis (ALS). Since cholinergic motoneurons (MNs) are targeted for degeneration in this disease, directed differentiation of stem cells into MNs could be a very useful therapeutic measure. We previously reported that fetal hNSCs primed with a cocktail of bFGF, heparin and laminin (FHL) pushed cells towards a cholinergic phenotype following further differentiation. In order to determine whether bFGF, an important developmental mitogen, was a key player in this induction, I examined the expression of several developmental markers of motoneurons in hNSCs primed with or without bFGF and/or treated with inhibitors of FGF signaling. In this study I found that FHL-primed cells exhibited enhanced levels of MN developmental transcription factors including Olig2, Islet1, Neurogenin 2 and the motoneuron determinant, Hb9. Furthermore, induction of Hb9 was found to be both time- and bFGF dose-dependent and inhibition of FGF signaling with either a bFGF neutralizing antibody or the FGF receptor antagonist PD173074, significantly down-regulated Hb9 transcripts. In addition to this, I found that proliferation was not required for Hb9 MN differentiation as blockade of proliferation in bFGF treated cells did not block Hb9 induction. In accordance with the inductive role of bFGF, proliferation mediated by treatment with other growth factors did not enhance Hb9 expression. To rule out a role for selective Hb9+ cell survival in response to bFGF, cell death assays were performed. Data from these assays demonstrated that cells treated without bFGF survived equally well as FHL-primed cells. Furthermore, data from western blot analysis of cell signaling pathways, Erk1/2 and JNK, did not substantiate a

claim for the role of either MAP kinase molecules in Hb9 induction. Finally, addition of either EGF or Leukemia Inhibitory Factor (LIF) after FHL-priming down-regulated Hb9 expression. Taken together these data suggest a novel role for bFGF as an inducer of MN differentiation in fetal forebrain-derived and long-term expanded hNSCs.

INTRODUCTION

Cholinergic motoneuron (MN) differentiation in vertebrates has been well characterized (for review see (Jessell, 2000;Briscoe and Novitch, 2007) and *in vivo* involves three major steps, each of which are regulated by specific transcription factors. First, cells must be directed from a stem cell fate into a neural cell fate. This is often demarcated by expression of markers of immature neuroectodermal cells such as Sox1 and/or Nestin (Kan et al., 2004). Second cells are caudalized, activating transcription factors such as the HoxC genes (Dasen et al., 2003). Finally, cells are directed into a ventralized phenotype by the graded secretion of sonic hedgehog (SHH) produced by the developing notochord and floor plate (Ericson et al., 1996;Patten and Placzek, 2000). At this stage cells show markers of ventral cell types such as Pax6 and Olig2 (Ericson et al., 1997;Mizuguchi et al., 2001;Lu et al., 2002) and upon further differentiation, cells within this region begin to express several homeodomain (HD) proteins such as Islet 1 and Lim3 (Lee and Pfaff, 2003;Thaler et al., 2004;Thaler et al., 2002). It is after expression of these HD proteins that cells are considered to be motor neuron progenitor cells or pre-motoneuron (i.e. pMN) cells which have the potential to differentiate into one of two final neural cell types, oligodendrocytes or motoneurons (Lu et al., 2002) and it has been suggested that there are several factors influencing which path cells will take. Examples of these influences include expression of the transcription factor Nkx2.2 in oligodendrocytic precursors (Zhou et al., 2001) and the expression ratio of two basic helix-loop-helix (bHLH) transcriptional regulators, Ngn2 and Olig2 (Lee et al., 2005;Mizuguchi et al., 2001) which has been suggested to drive the last

stages of MN differentiation. Upon expression of the bHLH factors Ngn2 and Olig2 and the HD protein Lim3, a complex is formed which stimulates transcription of the motoneuron determinant, Hb9 (Thaler et al., 2002). Similar to two important MN determinants in chicken, MNR2 and HLXB9, mammalian Hb9 is an essential regulator of MN induction in rodents, the importance of which has been demonstrated through both knockout (Thaler et al., 1999) and ectopic expression studies (Arber et al., 1999;William et al., 2003).

Several agents have been used *in vitro* to mimic the *in vivo* cell signaling events implicated in the induction of MN's during development. The most common utilized are retinoic acid (caudalizing agent) and sonic hedgehog (ventralizing agent) (Renoncourt et al., 1998;Wichterle et al., 2002;Li et al., 2005;Lim et al., 2006;Soundararajan et al., 2006), and bFGF (Wu et al., 2002;Shin et al., 2005;Ren et al., 2006). Notably, our group has generated an *in vitro* priming method consisting of plating human derived neurospheres onto poly D lysine and laminin coated culture dishes in serum-free medium supplemented with bFGF, heparin and laminin (FHL) which directs differentiation of hNSCs into a cholinergic phenotype (Wu et al., 2002;Tarasenko et al., 2004). Cells plated in this manner spread out to form a monolayer consisting of both neurons (immunolabeled for beta tubulin type III and MAP2) and glia (GFAP). In particular, approximately 36% of FHL-primed cells differentiate into neurons expressing choline acetyltransferase (ChAT) *in vitro* (Tarasenko et al., 2004) and 50% of FHL-primed hNSCs differentiate into a $ChAT^+$ phenotype after transplantation into rodent spinal cord or medial septum (Wu et al., 2002). What is currently lacking, however, is an understanding of the molecular mechanisms responsible for driving the differentiation of hNSCs into motoneurons. In order to further examine the mechanisms behind FHL- priming, I began by examining the role of one factor in the priming medium, the potent growth factor bFGF.

Basic fibroblast growth factor (bFGF) is one of 22 identified members of the fibroblast growth factor (FGF) signaling family (for review see (Reuss and Bohlen und, 2003). Ten

of these members, including bFGF (or FGF-2), have been identified in the human central nervous system where they are thought to be involved in repair processes following nerve lesions and degenerative disorders (Reuss and Bohlen und, 2003), in neuronal survival (Otto et al., 1987;Grothe et al., 1991;Walicke et al., 1986), in axonal growth (Sapieha et al., 2003;Klimaschewski et al., 2004) and in learning and memory (Ishihara et al., 1992). In regards to stem cells, bFGF is also important for neural progenitor cell proliferation both *in vitro* (Kitchens et al., 1994;Tropepe et al., 1999) and *in vivo* (Martens et al., 2002) as well as for the maintenance of pluripotency in human embryonic stem cells (Wang et al., 2005;Xu et al., 2005). A role for bFGF during human CNS development has been hypothesized and is indirectly supported by the finding that changes in bFGF expression levels correlate with neurogenesis and gliogenesis in rodents (Powell et al., 1991;Qian et al., 1997) and that bFGF knockout animals show reduced neuron numbers by >50% (Raballo et al., 2000) and impaired cortical development with a lack of large projection neurons (Dono et al., 1998;Vaccarino et al., 1999;Raballo et al., 2000). Because it is virtually impossible to study human neural development *in vivo*, the role of bFGF during human development is unclear. One way to better understand early human development is through the use of *in vitro* models that include human-derived stem cells.

Inferring from the *in vivo* and *in vitro* evidence above, it is likely that bFGF's effects on NSC differentiation are concentration, developmental stage and cell type specific. Thus it is not surprising that bFGF's roles in directing stem cell fate are somewhat controversial since several models and species are utilized for the study of stem cells. For example, Reimers et al. (2001) and Tsai and Kim (2005) both found that continuous bFGF treatment inhibited neurogenesis in rodent neural precursor/stem cells while Palmer et al. found that bFGF promoted neurogenesis via a re-programming of adult non-neuronal cells into neurons (Palmer et al., 1999). One can surmise that in addition to differences in cell handling, that differences in stage of derivation (adult vs neonatal) might offer at least a

partial explanation for differences in these studies. Interestingly, though, specific neuronal subtypes were not fully examined in these studies thus it is possible that specific neuronal subtypes may have been influenced by bFGF treatment. In light of this, we and others have found that bFGF treatment enhances differentiation of cholinergic expressing neurons in both human and rodent derived neural and embryonic stem cells (Wu et al., 2002;Tarasenko et al., 2004;Ren et al., 2006;Shin et al., 2005). Further substantiating a reprogramming effect for bFGF, Gabay et al (2003) demonstrated that even very low concentrations of bFGF (0.2ng/ml) induced a change in cell fate specification from dorsal to ventral cell types in rodent derived neural precursors (Gabay et al., 2003). Taken together this data suggests that bFGF acts as a very potent morphogen with the ability to influence cell fate decisions in both human and rodent derived stem cells.

In this study I show that bFGF acts to direct human neural stem cells into pre-motoneurons which express Olig2, Islet-1 and Hb9 *in vitro.* I demonstrate that bFGF-induced Hb9 expression is concentration and time dependent and that blockade of bFGF signaling down-regulates Hb9 expression. Furthermore, I provide evidence that enhanced cell survival and proliferation are not mechanisms that mediate bFGF induction of Hb9 expression in hNSCs. I also demonstrate that two mediators of the MAP kinase cell signaling pathway, ERK1/2 and JNK are not directly involved in this phenomenon. Finally, I show that while bFGF enhances Hb9 expression that two other growth factors, Epidermal Growth Factor (EGF) and Leukemia Inhibitory Factor (LIF), act to down-regulate Hb9 expression. Taken together these findings demonstrate that bFGF is an essential component in the directed differentiation of hNSCs into Hb9 expressing cells *in vitro.* These finding are important because a better understanding of the molecules which drive neuronal, particularly motoneuron differentiation of human derived cells will allow researchers to better direct neuronal differentiation *in vitro*. This information could lead to better cellular

models for the study and testing of treatments for motoneuron diseases such as Amyotrophic Lateral Sclerosis (ALS).

RESULTS

FHL-Priming Induces Expression of Transcription Factors of the Motoneuron Lineage

Previously, we reported that human neural stem cells primed with a cocktail of bFGF, heparin and laminin (FHL) spread out into a monolayer of large, homogenous shaped cells with many processes (Wu et al., 2002) (see also Figure 3.1A). In this study, I first compared the morphological appearance between FHL-primed cells and those primed with epidermal growth factor (EGF), leukemia inhibitory factor (LIF) and laminin (ELL). I found that ELL-primed cells did not show vigorous migration and remain heterogeneous in size and shape (Figure 3.1A). In particular, many ELL primed cells were spindle shaped and migrated out from the adherent sphere along distinct tracts. ELL primed cells did not spread out into a complete monolayer, rather, preferring to remain within the sphere structure. Interestingly, ELL primed and differentiated cells showed significant enhancement in overall cell number/ field after just four days of priming (Figure 3.1C). In regards to phenotypic differentiation, four days after priming and further differentiated for 14 days via withdrawal of growth factors, many ELL primed cells exhibited small neuron-like morphology showing strong phase bright with one (unipolar) or two processes (bipolar) (data not shown) and stained with the immature neuronal marker, beta III tubulin (Figure 3.1 D). FHL-primed cells also expressed markers of immature neurons (Beta III tubulin) and glia (GFAP) (Fig. 3.1E). Morphologically, ELL and FHL-primed and differentiated cells also showed differences. For example, many FHL-primed cells showed neurons with large cell bodies and extensive neurites while ELL-primed neurons were smaller in size with less neurite extensions (Fig 3.1 D and E). Interestingly, both ELL and FHL-primed cells showed similar percentages

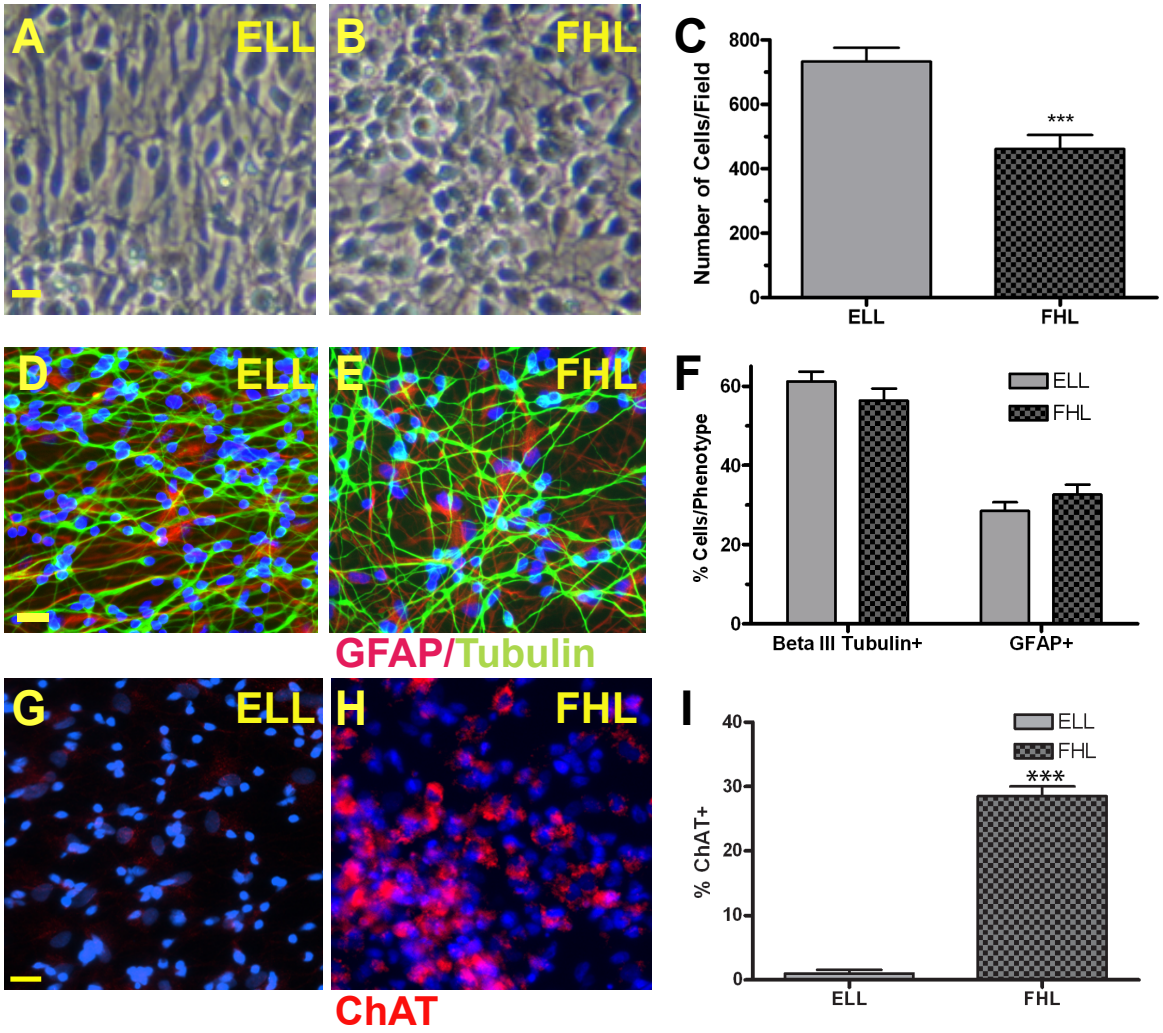


Figure 3.1: FHL-primed hNSCs express markers of developing motoneurons. (A & B) Phase contrast images 4 days after plating hNSCs primed with either EGF, Lif and laminin (ELL) or bFGF, heparin and laminin (FHL) onto adhesive substrate. (C) Overall cells/field are increased in ELL primed cultures as compared to FHL primed cells. ***p<0.001 vs ELL, unpaired students *t* test. (D & E) Glial and immature neuronal differentiation is similar between ELL and FHL primed hNSCs. Representative images of hNSCs primed with ELL (D) or FHL (E) for 4 days followed by further differentiation via withdrawal of growth factors for 14 days show immunoreactivity for both an immature neuronal marker (Beta III tubulin, green) and glia (GFAP, red). Blue = DAPI, nuclear counterstain. (F) Cell counts demonstrate that ~60% of either ELL or FHL primed hNSCs differentiate into immature neurons while ~40% differentiate into glia. (G & H) Only FHL primed hNSCs show expression of cholineacetyltransferase (ChAT, in red) after 4 days of priming followed by 14 days of differentiation in basal (B27) media. Blue=DAPI nuclear stain.. (I) Quantification of ChAT+ immunoreactive cells after ELL or FHL priming and further 14 day differentiation. ***p<0.001 vs ELL, unpaired students *t* test. Scale bar in all images = 20 µm.

of immature neurons and glial cells upon differentiation (Beta III Tubulin: ELL= 61 ± 2.5%, FHL=56 ± 3.0%. GFAP: ELL= 28 ± 2.3%, FHL= 33 ± 2.5%) (Figure 3.1F). In regards to neuronal subtype, however, FHL-primed cells were the only group to show extensive choline acetyltransferase immunoreactivity (ChAT, Figure 3.1 G -I), indicating their cholinergic phenotype.

Because FHL but not ELL-primed cells become ChAT immunoreactive cells and our previous studies showed cholinergic motoneuron differentiation of grafted FHL-primed hNSCs *in vivo* (Tarasenko et al., 2006;Gao et al., 2005), I next sought to determine whether FHL-primed cells *in vitro* expressed transcription factors known to play important roles during motoneuron development *in vivo*. Semi-quantitative RT-PCR analyses demonstrated that after four days of plating that FHL-primed hNSCs dramatically up-regulated markers associated with motoneuron development such as Olig2, Islet1, Neurogenin2, and Hb9 (Figure 3.2) as compared to neurospheres during routine cell expansion. In contrast, ELL-primed cells showed little or no increases in Olig2, Ngn2 or Hb9 transcript. ELL-primed cells did show some Islet1 transcript, however Islet1 is also associated with interneuron development and since ELL-primed cells do not develop into cholinergic expressing cells, I believe these cells are becoming interneurons.

Olig2 is Upregulated in FHL-Primed Cells

Olig2 is a transcription factor associated with ventralized cell types and developmentally found in oligodendrocytic and motoneuron precursors (Mizuguchi et al., 2001;Zhou and Anderson, 2002). Under FHL-priming conditions, hNSCs significantly upregulated Olig2 mRNA and protein expression (Figure 3.3). In particular, semi-quantitative RT-PCR analysis indicated that FHL-primed cells showed a 73% increase in Olig2 mRNA levels as compared to ELL primed and a 90% increase as compared to cells in neurospheres. At the protein level, Olig2 was also significantly up-regulated in

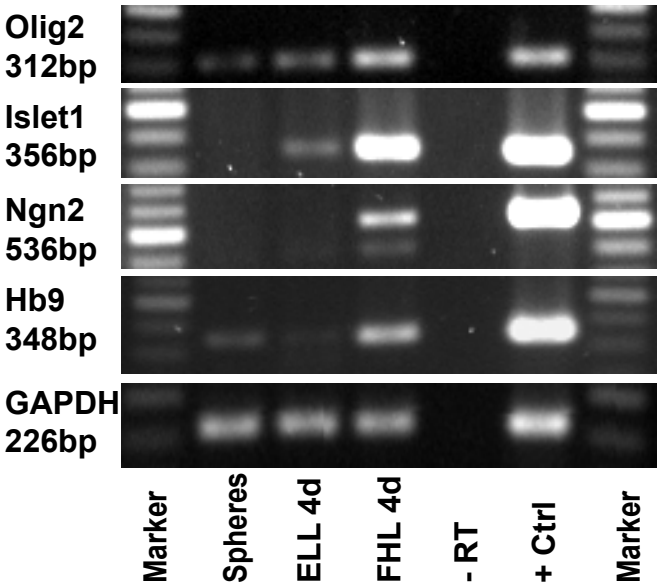


Figure 3.2: RT-PCR for early motoneuron transcription factors Olig2, Islet1, Neurogenin 2 (Ngn2), and Hb9 in neurospheres, ELL or FHL-primed hNSCs. – RT = negative control without reverse transcriptase. + Ctrl = fetal spinal cord, a positive control. GAPDH = glyceraldehyde phosphate dehydrogenase, an internal control. Marker = molecular weight marker/ladder.

FHL- primed cells. Cell counts showed a greater than 120% increase in Olig2 expressing cells in FHL-primed group when compared to ELL (FHL = 45.7 ± 2.9%; ELL = 20.6 ± 2.2%, unpaired Student's *t* test, $p<0.001$; $n \geq 10$ fields/group). Furthermore, many ELL primed cells only showed weak Olig2 nuclear labeling (arrows in Figure 3.3C) suggesting low levels of the transcription factor while most FHL-primed cells showed strong Olig2 immunoreactivity.

Hb9 is Up-Regulated in FHL-Primed Cells

Developmentally, two main phenotypic possibilities exist for Olig2 expressing cells, namely an oligodendrocytic or a motoneuron cell fate. In order to distinguish whether FHL-primed cells were going down the path to become motoneurons, I further analyzed expression of the motoneuron transcription factor, Hb9. Hb9 is a transcription factor well established as a motoneuron precursor marker (Arber et al., 1999). In figure

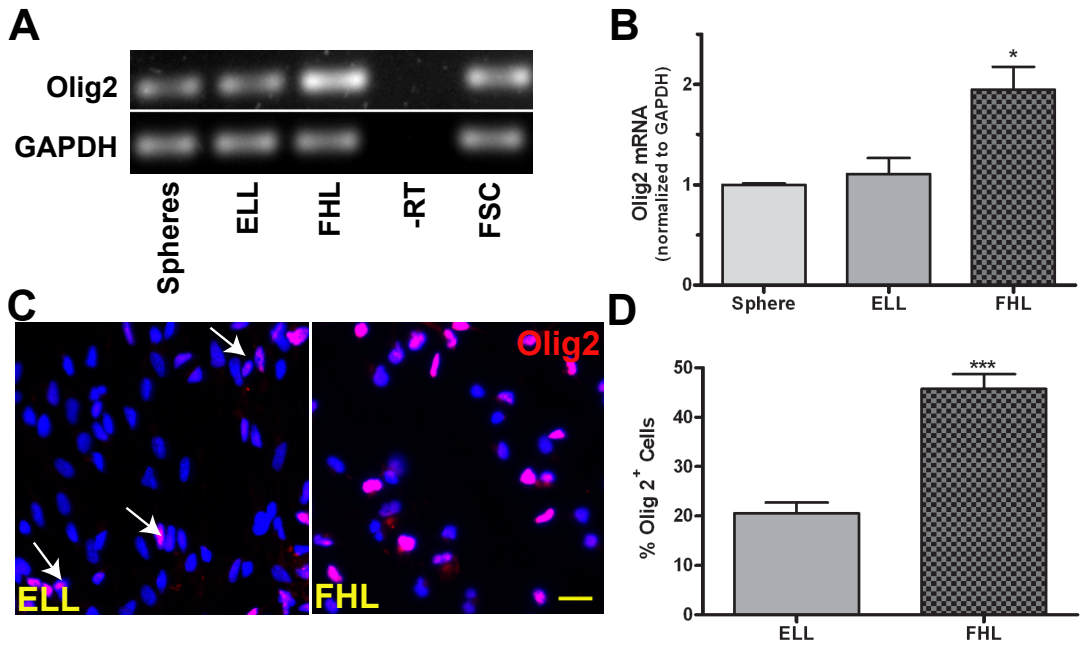


Figure 3.3: FHL-primed hNSCs upregulate Olig2 after 4 days of priming. A) Semi-quantitative RT-PCR for the bHLH transcription factor Olig2 in neurospheres, or hNSCs primed for 4 days with either ELL or FHL. – RT = negative control without reverse transcriptase. + Ctrl = fetal spinal cord RNA used as a positive control. GAPDH = glyceraldehyde phosphate dehydrogenase, an internal control. B) Densitometrical analysis of Olig2 RNA expression (n = 3-6/group; One way ANOVA, *p<0.05 vs spheres or ELL primed hNSCs.) All data are normalized to internal control, GAPDH transcript levels and to spheres = 1 unit. C) Olig2 (shown in red) nuclear protein levels are detected in ELL and FHL-primed hNSCs after 4 days of priming. Note that Olig2 nuclear expression is found at a low level in many ELL- primed cells (arrows). (Blue=DAPI nuclear stain.) D) Cell counts (>10 fields/group) demonstrate that significantly more FHL-primed hNSCs express Olig2 protein than ELL (Unpaired students *t* test; ***p<0.001). Scale bar = 20 µm.

3.4 I demonstrate that FHL-primed cells show both mRNA and nuclear protein expression for Hb9. In particular, semi-quantitative RT-PCR analysis indicated a greater than 8-fold increase in Hb9 mRNA in FHL-primed cells as compared to unprimed spheres and a 15-fold increase as compared to ELL primed cells (Figure 3.4A; FHL = 9.9 ± 0.9 units, ELL = 0.61 ± 0.32 units, sphere = 1 ± 0.55 units; all normalized to GAPDH. One-way ANOVA, Tukey post hoc test; p<0.001).

To confirm semi-quantitative Hb9 RT-PCR data, real time quantitative RT-PCR (qPCR) was also performed and indicated a similar induction of Hb9 mRNA in FHL-

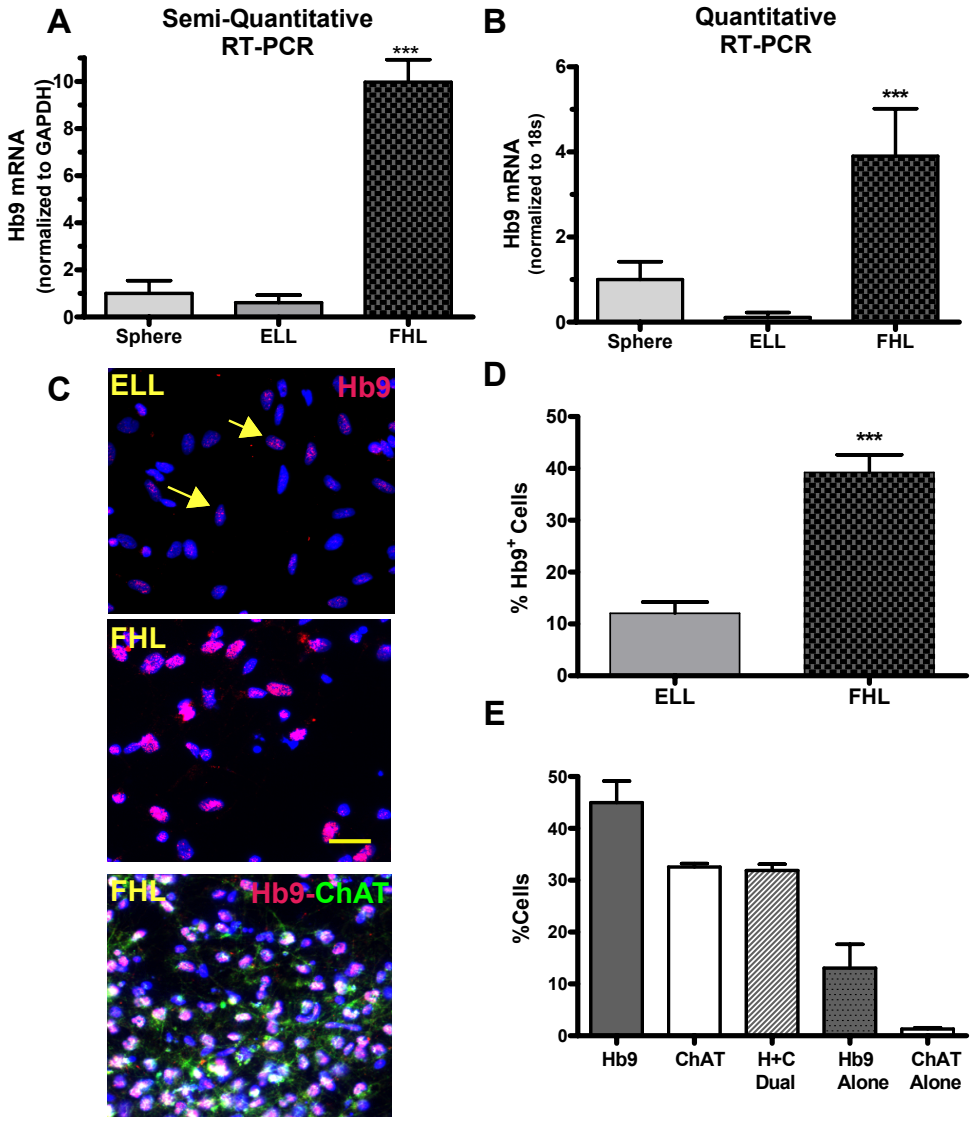


Figure 3.4: Hb9 expression is up-regulated in FHL-primed hNSCs. (A) Semi-quantitative RT-PCR for Hb9 transcript in neurospheres, ELL or FHL-primed cultures (One Way ANOVA; FHL vs spheres or vs ELL ***p<0.01 n=3/group). Semi-quantitative RT-PCR data are normalized to internal control, GAPDH transcript levels and to spheres = 1 unit. (B) Real-time quantitative RT-PCR for Hb9 was used to validate semi-quantitative findings in A. (One way ANOVA; FHL vs spheres or vs ELL, ***p<0.01, n=3-6/group). Quantitative RT-PCR was normalized to internal control, 18s RNA. (For further validation of semi-quantitative methods see supplemental materials). (C) Immunocytochemistry for Hb9 (red) and ChAT (green) protein in ELL or FHL primed hNSCs after 4 days of priming and 14 days of differentiation. Arrows indicate low level Hb9 protein expression in ELL-primed cells. (Blue, DAPI, nuclear stain). Scale bar = 20 µm. (D & E) Cell counts of ELL or FHL primed hNSCs immunostained for Hb9 and/or ChAT protein (Students *t* test, FHL vs ELL, ***P<0.001, n≥10 fields/group).

primed cells (Figure 3.4B). In particular, quantitative RT-PCR showed a 3-fold increase in Hb9 mRNA as compared to spheres and a 14-fold increase as compared to ELL primed cells (FHL = 3.9 ± 1.1 units, ELL = 0.1125 ± 0.08 units, sphere = 1 ± 0.42 units; data normalized to 18s RNA; one-way ANOVA, Tukey post hoc test; $p < 0.001$; $n \geq 3$/group). The slight discrepancies in fold change between semi-quantitative and quantitative results are likely attributed to normalization to different control genes (GAPDH vs 18s). Further validation of our semi-quantitative methods can be found in Chapter 2 under the PCR section (Chapter 2, Figure 2.6). Semi-quantitative RT-PCR analysis of RNA from another epigenetic expanded human fetal neural stem cell line (K054) confirmed an induction of Hb9 mRNA by FHL-priming (Figure 3.5A). Furthermore, time in culture (passage number) was found to have little effect on Hb9 induction as both early passages (<30, Figure 3.5A) and late passages (>90, Figure 3.5B) showed similar patterns of Hb9 expression levels in response to FHL and ELL-priming.

At the protein level, Hb9 was also significantly up-regulated in FHL-primed hNSCs. After 4 days of FHL or ELL-priming, there was little immunoreactivity for Hb9 in either group (data not shown). However after 4 days of priming and further differentiation in basal media (B27) for 8 days (data not shown) or 14d, Hb9 immunoreactivity was readily detectable in FHL-primed cells (Figure 3.4C; FHL = 39.21 ± 3.4%). In contrast to the strong nuclear staining seen in FHL-primed and differentiated cells, only 12.1 ± 2.2% of ELL-primed cells showed very weak immunolabeling with Hb9 (see arrows in Figure 3.4C). Untreated neurospheres did not show any specific Hb9 nuclear labeling (data not shown).

Double immunofluorescent staining of FHL-primed cells revealed that 31.9 ± 1.2% of FHL-primed and differentiated cells show double immunoreactivity for both Hb9 and ChAT (Figure 3.4C and 3.4E), indicating their motoneuron phenotype. Approximately 13.1% of $Hb9^{+}$ cells did not double label for ChAT and are presumably at a transition

phase in their development. Also ~1.3% of ChAT^{+} cells did not double label with Hb9 and are likely cholinergic interneurons which would not normally express motoneuron markers such as Hb9 (Hb9-ChAT: 31.9 ± 1.2%; ChAT Only 1.3 ± 0.23%; Hb9 Only 13.07 ± 4.6%). Because ELL primed cells expressed extremely low levels of Hb9 mRNA and protein and I have shown previously that ELL-primed cells do not express ChAT (Figure 3.1 and Wu

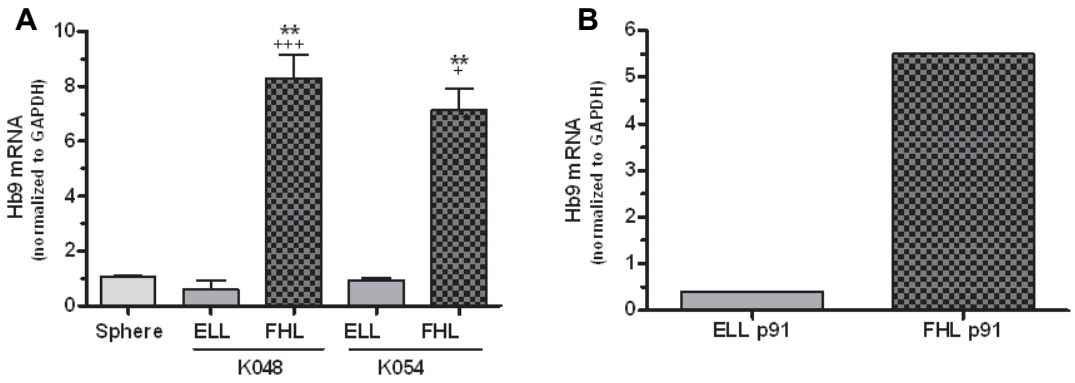


Figure 3.5: hNSCs derived from two different cell lines show up-regulation of Hb9 transcripts in response to FHL-priming. (A) hNSCs derived from an 8-week human fetal forebrain (cell line K048) and hNSCs derived from a 10-week fetal forebrain (cell line K054) both respond similarly to FHL priming by up-regulating Hb9 transcripts (One way ANOVA, **p<0.01 vs spheres, +++p<0.001 vs ELL of same cell line. N=3/group). Similar to K048 cells, K054 hNSCs also show low level activation of Hb9 under ELL priming conditions. (B) Early and late passage K048 hNSCs exposed to FHL priming for 4 days both show similar up-regulation of Hb9 transcript suggesting that time in culture has little effect on FHL priming effects. P = passage number. All data are normalized to internal control, GAPDH transcript levels and to spheres = 1 unit.

et al., 2002), double immunolabeling of ELL primed cells with Hb9 and ChAT was not performed.

bFGF Induces HB9 mRNA Expression in a Time and Dose Dependent Manner

In order to elucidate the mechanism behind Hb9 motoneuron induction in FHL-primed cells, I monitored Hb9 mRNA expression in FHL-primed cells over time and in

response to bFGF concentration. RNA was collected from FHL-primed hNSCs at various time points for Hb9 mRNA expression analysis. Semi-quantitative RT-PCR revealed that Hb9 expression was significantly up-regulated after 72 hours of FHL-priming and continued to increase over the four day priming procedure (Figure 3.6. FHL 72 hrs = 4.7 ± 0.68 units, FHL 96 hrs = 8.2 ± 0.39 units, all units normalized to GAPDH expression; $p<0.05$, $p<0.01$ respectively vs spheres; one way ANOVA, Dunnett post hoc test; n =4). Furthermore, Hb9 expression was maintained after differentiation of FHL-primed cells via withdrawal of

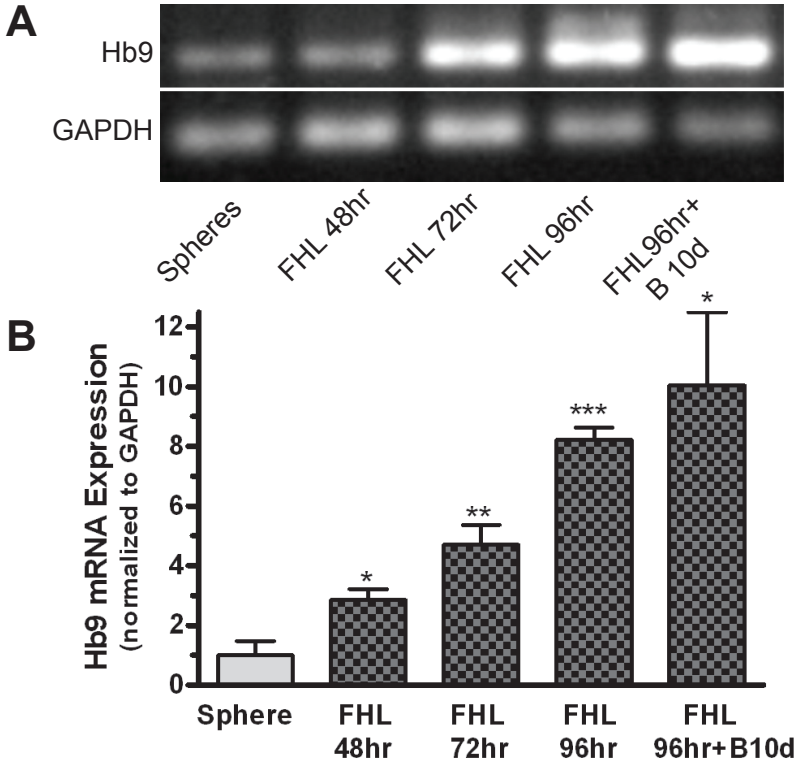


Figure 3.6: Hb9 expression is time dependent in FHL-primed hNSCs. A) Representative gel of Hb9 PCR products after semi-quantitative RT-PCR for Hb9 transcript. B) Densitometrical analysis of multiple gels for Hb9 transcripts in spheres, or FHL primed hNSCs after 48 hours, 72 hours, or 96 hours of FHL priming or 96 hours of FHL priming followed by further differentiation in basal (B27) media for 10 days (10d). (One way ANOVA, *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs spheres; $n\geq3$/group). All data are normalized to internal control, GAPDH transcript levels and to spheres = 1 unit.

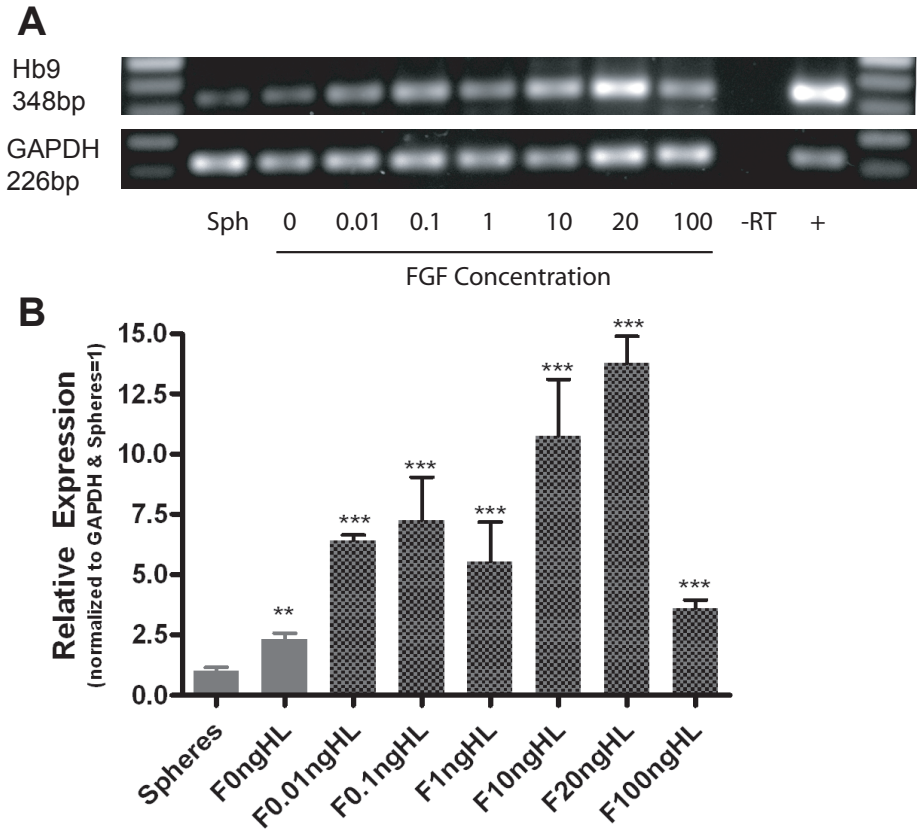


Figure 3.7: Hb9 expression is bFGF dose dependent. (A) Representative semi-quantitative (SQ) Hb9 RT-PCR gel demonstrating an enhancement of Hb9 PCR product with increasing bFGF concentrations. hNSCs were primed with increasing concentrations of bFGF (from 0.1 ng/ml to 100 ng/ml) and RNA was collected after 4 days of priming for SQ-RT-PCR analysis for Hb9 transcript. (B) Densitometrical analysis of Hb9 transcript levels in hNSCs primed with increasing bFGF concentrations for 4 days (one way ANOVA, ***$p<0.01$ vs spheres; $n\geq3$/group). Data normalized to internal control, GAPDH transcript levels and to spheres = 1 unit.

growth factors and differentiation in B27 media for 10 days (FHL 96 hr + B 10 d= 10.1 ± 2.4 units; One Way ANOVA, Dunnet post hoc test; $p<0.01$ vs sphere; $n\geq3$).

In order to understand whether FHL-induced Hb9 expression was bFGF-dependent, I performed a bFGF dose-response analysis for Hb9 mRNA expression. Addition of bFGF at a very low concentration (0.01 ng/ml) induced significant up-regulation of Hb9 mRNA as compared to either spheres or hNSCs primed with only heparin and laminin (F 0 ng/

ml) (Figure 3.7A-B). Hb9 expression continued to increase as bFGF dose increased with a maximum effect found at 20 ng/ml. At the highest bFGF dose tested (100 ng/ml), Hb9 expression was down-regulated. In addition, I also assessed the types of FGF receptors in hNSCs in response to FHL-priming by semi-quantitative RT-PCR analysis. I found expression of the four known FRF receptors in all groups (spheres, ELL and FHL-primed). Interestingly, I found significant up-regulation of FGFR2 and R3 following FHL-treatment

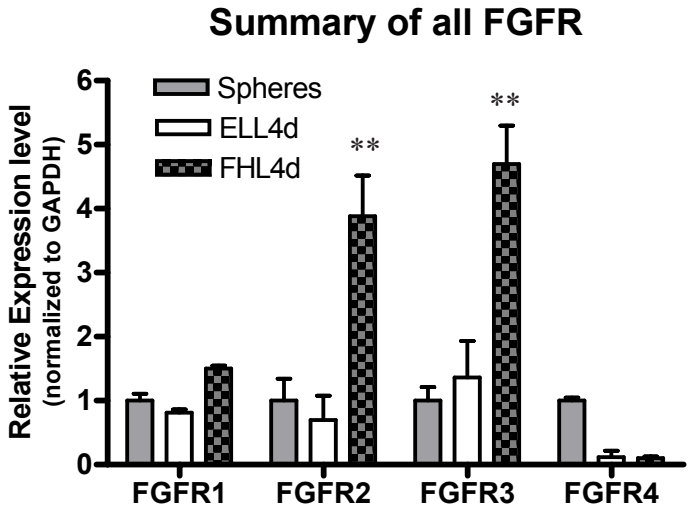


Figure 3.8: hNSCs express all four FGF receptor subtypes. Semi-quantitative RT-PCR for FGF receptor 1, 2, 3 and 4 (FGFR1-4) demonstrate that FHL primed cells have enhanced mRNA levels for FGFR2 and FGFR3. FGFR4 was significantly down-regulated in both ELL and FHL primed groups as compared to spheres (one way ANOVA, **$p<0.01$ vs spheres; n=3). All data are normalized to internal control, GAPDH transcript levels and to spheres = 1 unit.

as compared to either sphere or ELL-primed cells, while R1 expression was unaltered and R4 significantly decreased (Figure 3.8).

In order to confirm that bFGF was the factor inducing Hb9 expression in FHL-primed hNSCs, we next blocked bFGF signaling using two mechanisms. First, we blocked bFGF signaling at the receptor level by adding a specific FGF receptor antagonist that blocks all subtypes of FGF receptors, PD170374 (Mohammadi et al., 1998;Koziczak et

al., 2004); and second, we blocked endogenously secreted and exogenously added bFGF with a bFGF neutralizing antibody. After four days of FHL-priming with either the FGF receptor antagonist or neutralizing antibody, RNA was collected and semi-quantitative RT-PCR analysis for Hb9 was performed. The concentration of PD173074 chosen for these experiments (25-50nM) was based upon data from the literature suggesting that high doses of this antagonist could have antagonistic effects upon other cell signaling pathways (Mohammadi et al., 1998;Mohammadi et al., 1997). Results of these experiments revealed that both the bFGF neutralizing antibody and the FGF receptor antagonist, PD173074, significantly down-regulated Hb9 expression by approximately 40% as compared to IgG- and vehicle (DMSO) controls, respectively (Figure 3.9A-B) (FHL+DMSO = 9.0 ± 1.0 units vs FHL + PD173074 = 5.1 ± 0.6 units; FHL + IgG = 8.4 ± 0.5 units vs FHL + anti-bFGF =

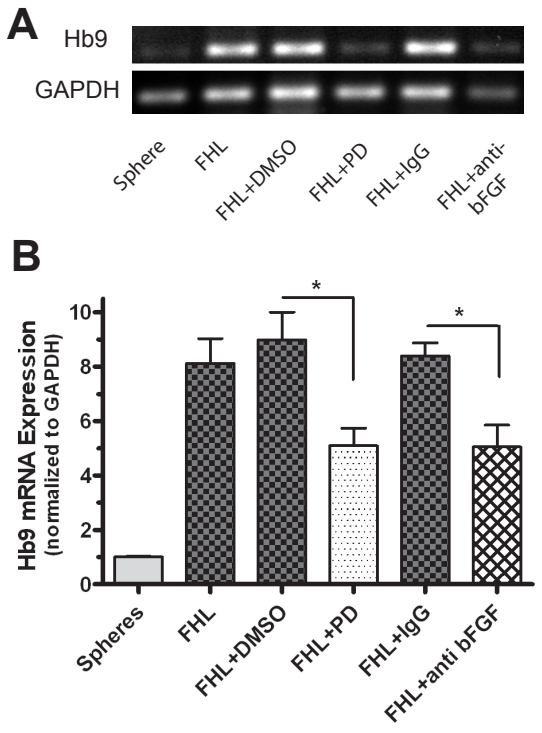


Figure 3.9: Hb9 expression in hNSCs is down-regulated upon blockade of bFGF signaling. (A) Representative semi-quantitative RT-PCR gel demonstrating that blockade of bFGF signaling decreases Hb9 transcript level in FHL primed hNSCs. (B) Statistical analysis of semi-quantitative RT-PCR for Hb9 in hNSCs treated with FGF signal cascade inhibitors. Addition of either the FGF receptor antagonist, 25nM PD173074 (PD) or 3 μg/ml neutralizing-bFGF antibody (anti-bFGF) significantly decreased Hb9 transcript levels as compared to controls [Unpaired Student's t Test, *$p<0.05$ FHL + PD vs FHL + DMSO (vehicle for PD173074) or FHL + anti-bFGF antibody vs FHL + IgG- (a negative control for anti-bFGF neutralizing antibody)]. Data normalized to internal control, GAPDH transcript levels and to spheres = 1 unit.

5.1 ± 0.8 units; Unpaired Student's *t* test, $p<0.05$; $n\geq3$). Together this data suggests a novel role for bFGF as an essential factor for Hb9 motoneuron differentiation in hNSCs.

Cell Proliferation Is Not Required for HB9 Expression

Our next question was how bFGF up-regulated Hb9 expression in FHL-primed hNSCs. In order to examine this question, I first addressed bFGF's role as a potent mitogen and asked whether bFGF-enhanced Hb9 expression was solely due to a mitogenic effect. Priming with FHL and ELL for 4 days resulted in an increase of total cell numbers by ~1.3 fold and ~2 fold, respectively (Figure 3.10). Addition of the FGF receptor antagonist, PD173074, to FHL-primed cells decreased cell # by ~20% compared to FHL-treated cells. Continuous bromodeoxyuridine (BrdU) labeling of FHL and ELL- primed hNSCs for four days demonstrated that both groups underwent significant proliferation with 60 ± 3.1 % of FHL and 71 ± 2.9 % of ELL primed cells showing BrdU incorporation (Figure 3.11A-B). Addition of the FGF receptor antagonist, PD173074, down-regulated proliferation in FHL-primed cells by more than 50% (FHL-PD = 28.4 ± 4.3%) and proliferation was abolished

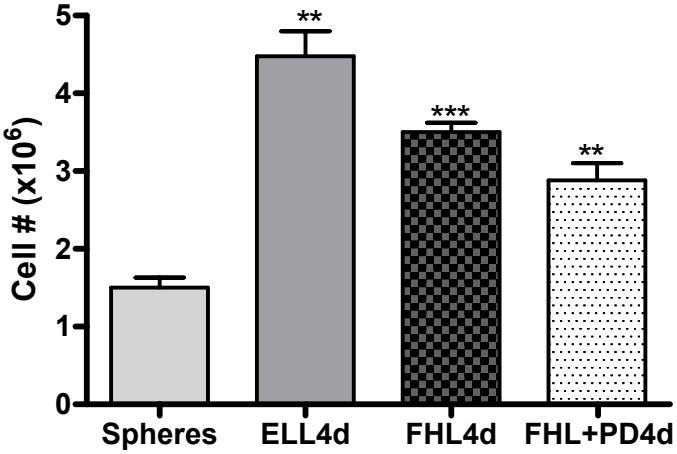


Figure 3.10: Total cell numbers derived after 4d of FHL or ELL priming. Sphere cell counts indicate the number of cells originally plated on d0. ELL primed cells proliferate to a greater degree than FHL primed cells and FHL primed cells treated with the FGF receptor antagonist decreased proliferation by about 20% compared to FHL alone; **$p<0.01$ or ***$p<0.001$ vs spheres, One way ANOVA, n=3/group.

in the presence of either the mitotic inhibitor, cytosine arabinoside (AraC) or an inhibitor of an essential cell cycle protein, CDK2 (CDK2i) (Figure 3.11A-B).

Using the mitotic inhibitor, AraC [1 μM], I found that FHL-enhanced Hb9 expression was significantly down-regulated in the presence of AraC (Figure 3.11C-D; FHL + AraC = 3.1 ± 0.3 units vs FHL + DMSO (vehicle control) = 9.1 ± 0.72 units; one way ANOVA, Tukey post hoc test, $p<0.01$; $n\geq3$). However, since AraC is known to be incorporated into the DNA of replicating cells and inhibits both DNA and RNA polymerases, there exists a possibility that AraC-mediated Hb9 down-regulation might be due to AraC-disrupted DNA transcription rather than inhibition of cell proliferation per se. In order to further determine whether FHL-upregulated Hb9 expression required cell proliferation, I sought out a second mitotic inhibitor which utilizes a different molecular mechanism. An inhibitor of cyclin dependent kinase 2 (CDK2), CDK2i, was thus used to halt dividing cells in G1 phase by blocking CDK2 binding to Cyclin E, which is required for cells to move from G1 to S phase. A dose response study was conducted to determine the minimal concentration to prevent cell cycle progression (See Chapter 2, Figure 2.). The optimal dose of CDK2i (2.5 μM) was then added during FHL-priming and RNA was collected four days later for semi-quantitative RT-PCR analysis for Hb9 expression. Surprisingly, I found that Hb9 expression was still strongly up-regulated in FHL-primed cells even after treatment with CDK2i (Figure 3.11C-D, CDK2i = 8.0 ± 0.6 units vs FHL + DMSO (vehicle control) = 9.1 ± 0.72 units; n=4). This is in contrast to the down-regulation of Hb9 found in AraC treated cells. Addition of the FGF receptor antagonist, PD173074 and AraC together (FHL + PD + AraC) did not further down-regulate Hb9 mRNA levels as compared to AraC alone suggesting that proliferation is not required for Hb9 induction. Taken together these data do not substantiate a critical role for a bFGF mitogenic effect on Hb9 expression in hNSCs.

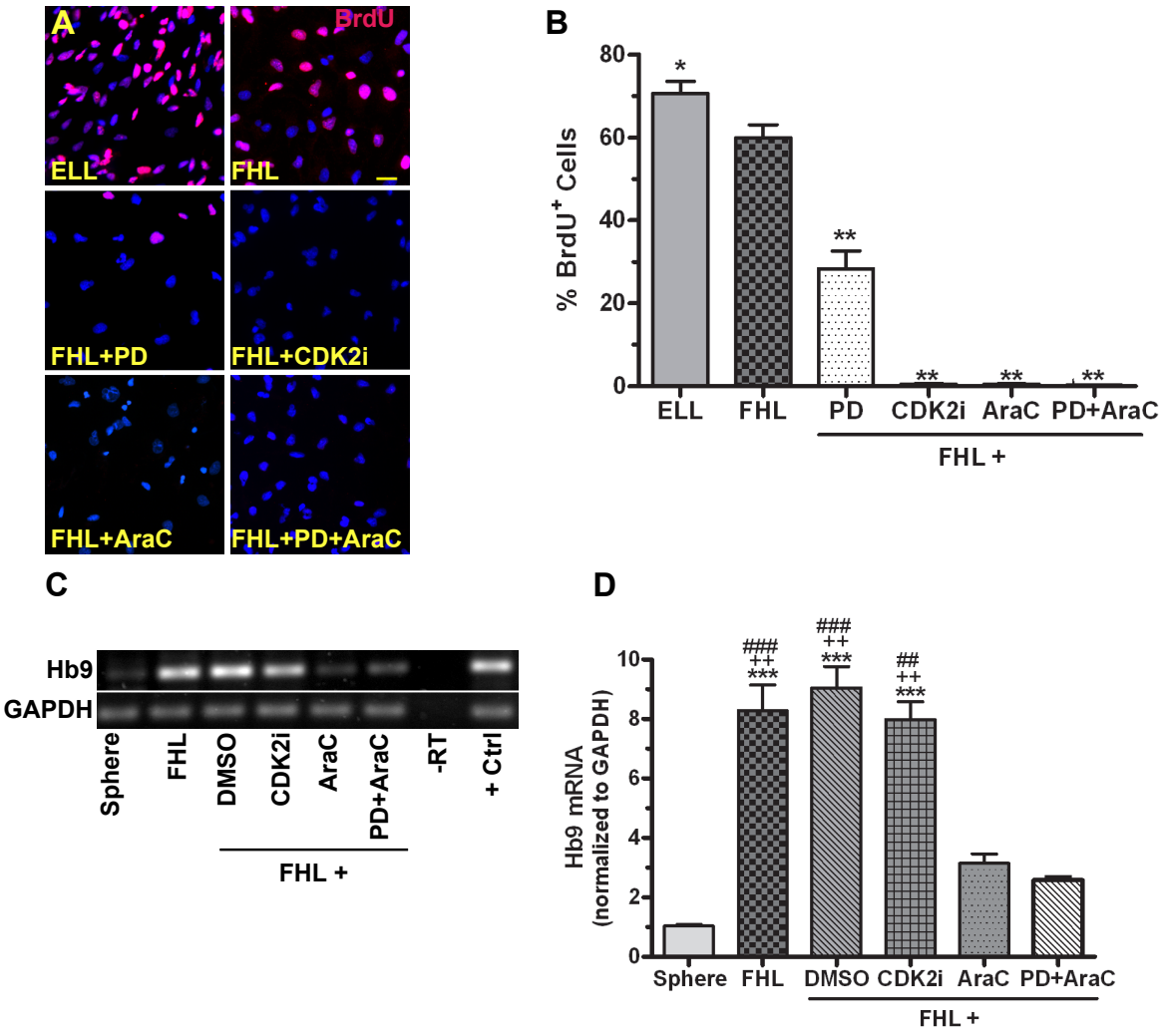


Figure 3.11: Proliferation may not be necessary for Hb9 induction in FHL-primed hNSCs. A) Representative images of BrdU labeled (shown in red) ELL, FHL, or FHL-primed hNSCs treated with the FGF receptor antagonist, PD173074 (FHL + PD), or mitotic inhibitors Cytosine Arabinoside (AraC) or with a Cyclin Dependent Kinase 2 inhibitor, (CDK2i) for 4 days. B) Cell counts of BrdU+ cells show the highest level of proliferation in ELL primed cells followed by FHL primed, FHL primed treated with Pd173074 and an absolution of proliferation with the addition of either AraC or CDK2i. (One way ANOVA, *p<0.05, **p<0.01 vs FHL; n≥10 fields/group). Blue = DAPI, nuclear stain. Scale bar = 20 µm. C) A representative gel of Hb9 PCR products from FHL primed cells treated with either vehicle (DMSO) or mitotic inhibitors AraC or CDK2i. -RT = RNA treated without reverse transcriptase, a negative control. + Ctrl = fetal spinal cord, a positive control. GAPDH = glyceraldehydes phosphate dehydrogenase, an internal control. D) Densitometrical analysis of RNAs after semi-quantitative RT-PCR analysis for Hb9 transcript. All data are normalized to GAPDH and to spheres = 1 unit. Addition of the mitotic inhibitor AraC significantly down-regulated Hb9 transcript levels as compared to controls (FHL alone or FHL+DMSO). Blockade of mitosis using the cell cycle inhibitor CDK2i did not down-regulate Hb9 transcript as compared to either FHL alone of FHL + DMSO (vehicle control). (One way ANOVA, *** p<0.001 vs spheres; ++ p<0.01 vs AraC; ### p<0.01 vs PD + AraC; n≥3/group).

Cell Survival is Not Related to bFGF-Enhanced HB9 Expression

A second possibility for bFGF-mediated Hb9 expression in hNSCs was through a selective survival effect on Hb9-expressing cells. In order to determine whether bFGF-mediated Hb9 expression was related to enhanced cell survival, a lactate dehydrogenase (LDH) assay was first performed (Figure 3.12A). The premise behind the LDH assay is through the conversion of the chemical formazan (colorless) into tetrazolium salt (red) by LDH secreted by dead/dying cells. The level of formazan is easily detected using a colorimetric plate reader and can be directly correlated to enzymatic (LDH) activity. LDH assay showed that spheres proliferated in the presence of EGF, bFGF and Lif (EFL), FHL-primed and ELL-primed hNSCs all exhibited only minimal cell death with no differences among groups (Figure 3.12A), which is probably due to trophic effects of the growth factors. Furthermore, FHL-primed cells treated with the FGF receptor antagonist, PD170374, did not show significantly enhanced cell death as compared to all other groups.

Since LDH is a measure of overall cell death, I next asked whether apoptotic cell death might be different between ELL- and FHL-primed hNSCs. To examine apoptosis, I performed two experiments: first I measured the activity of an apopototic enzyme, caspase 3; and second, I assessed apoptosis through TUNEL staining. Caspase 3 activity did not differ significantly among spheres, ELL- and FHL-primed cells at both 2 and 4 days post priming (Figure 3.12B; one way ANOVA, Tukey post hoc test, $p>0.05$, n=3/group). Moreover, TUNEL staining confirmed the finding that apoptosis was minimal with all groups showing less than 6% TUNEL+ cells. In particular, there were no significant differences in TUNEL positive cells between spheres, ELL- and FHL-primed hNSCs (Figure 3.12C-D; sphere = $1.9 \pm 0.1\%$, ELL = $2.3 \pm 0.3\%$, FHL = $1.7 \pm 0.3\%$; one way ANOVA, Tukey post hoc test, $p>0.05$; n=10 fields/group). FHL-primed hNSCs treated with the FGF receptor antagonist, PD173074, did show a ~2-fold increase in apoptotic cells compared to FHL-primed cells

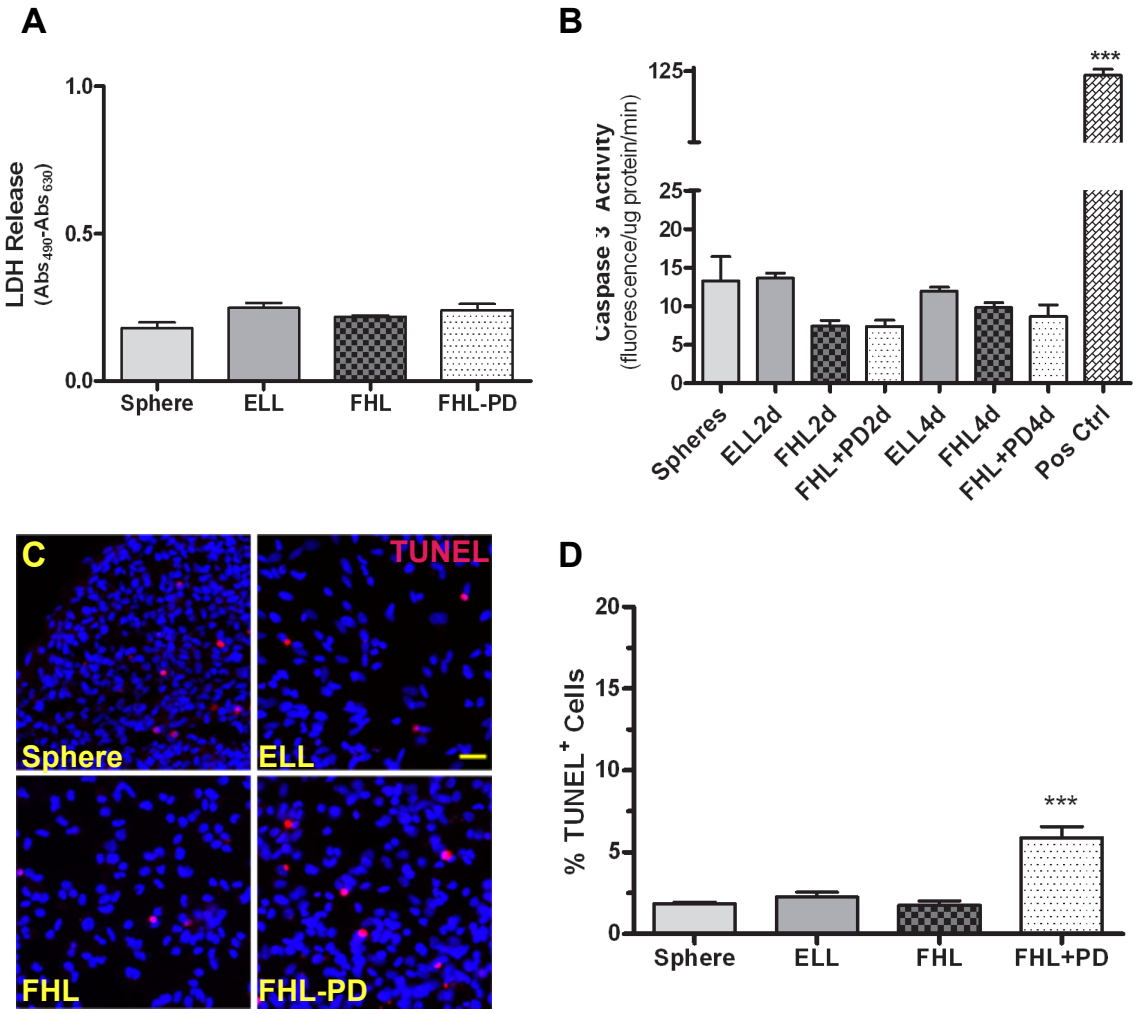


Figure 3.12: Enhanced cell survival does not account for Hb9 induction in FHL primed hNSCs. A) Lactate dehydrogenase (LDH), an enzyme released by dead/dying cells and assayed as a secreted factor in culture media, indicates that all groups exhibit relatively similar and low levels of cell death. B) A measure of apoptosis, caspase 3 activity assay indicates that at either 2 or 4 days post priming both ELL and FHL-primed hNSCs exhibit similar levels of cell death. Pos Ctrl=positive control, hNSCs treated for 4 hours with the apoptotic inducer, Staurosporine (One way ANOVA, Pos Ctrl vs all other groups, ***$p<0.001$; n=6/group except for spheres, n=3). C and D) Representative images and cell counts of another apoptotic index, TUNEL immunostaining (shown in red) of spheres, ELL, FHL or FHL-primed plus the FGF receptor antagonist, PD173074 confirms the presence of very few (<6%) apoptotic cells under any of the culture conditions tested (n≥10 fields/group. One way ANOVA,***$p<0.001$ vs spheres, ELL or FHL-primed hNSCs). Scale bar = 20 μm.

(FHL-PD = 5.9 ± 0.7%; one way ANOVA, Tukey post hoc test $p<0.001$ vs FHL). Thus using three measures of cell death, (LDH release, caspase 3 activity and TUNEL staining) I show that FHL-primed cells survive similarly as ELL-primed cells or spheres. Consequently, it seems unlikely that FHL-enhanced Hb9 expression is mediated through increased cell survival.

Activation of ERK1/2 and JNK Are Not Required For bFGF-Mediated HB9 Expression

Since all of the above data pointed to an essential role for bFGF in mediating Hb9 expression in hNSCs, I next asked what molecules in bFGF signaling pathways are responsible for bFGF-enhanced Hb9 expression. Among many molecules involved in bFGF cell signaling, those in the MAP kinase pathway were first chosen as candidates for this initial study. These include the extracellular signaling related kinase 1/2 (i.e. Erk1/2) and jun N-terminal kinase (i.e. JNK/SAPK), which are both known to play important roles in regulating gene expression and mediating growth factor signaling. Western blot analyses revealed the presence of phosphorylated Erk1/2 protein in FHL-primed hNSCs (Figure 3.13A-B). However, the levels of phospho-Erk1/2 in FHL-primed cells were significantly lower than those in spheres or ELL-primed, and almost completely abolished by the addition of either the FGF receptor antagonist PD173074 (Figure 3.13A-B; Student's *t* test; FHL + PD vs FHL, $p<0.05$; n=3) or by treatment with a bFGF neutralizing antibody (data not shown). In contrast, hNSCs primed with ELL showed similar levels of phophorylated Erk1/2 as compared to unprimed spheres (Figure 3.13A-B), Furthermore, Erk1/2 activation was not down-regulated in ELL primed cells treated with the FGF receptor antagonist, PD173074, demonstrating the specificity of this drug for FGF-mediated cell signaling.

Next I examined the activation of SAPK/JNK. FHL-primed hNSCs showed lower levels of phosphorylated JNK as compared to either spheres or ELL-primed cells (Figure 3.13C-D; one way ANOVA, Tukey post hoc test; $p<0.05$ vs sphere, $p<0.01$ vs ELL; n=3).

Application of the FGF receptor antagonist did not influence activation of JNK in FHL- or ELL- primed cells (FHL-PD vs FHL; ELL-PD vs ELL). Addition of a bFGF neutralizing antibody also did not further down-regulate phospho-JNK levels in FHL- primed cells (FHL vs FHL + anti-bFGF). Taken together these experiments suggest that neither Erk1/2 nor JNK are involved directly in bFGF-mediated Hb9 expression in hNSCs.

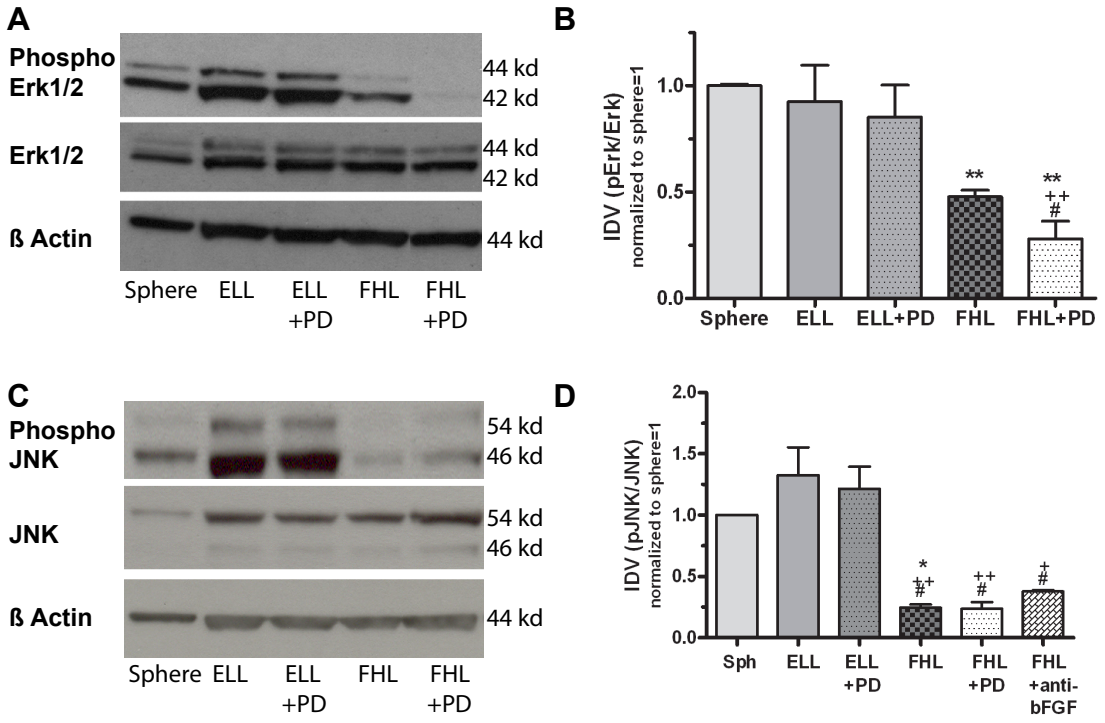


Figure 3.13: MAP Kinase cell signaling pathways can not account for Hb9 induction in FHL-primed hNSCs. A) Representative western blot image of spheres, ELL, FHL and ELL or FHL primed cells treated with the FGF receptor antagonist, PD173074 (ELL + PD or FHL + PD) immunoblotted for activated Erk1/2 (phosphorylated at Thr202/Tyr204), total Erk1/2 and loading control, β- actin. B) Densitometrical analysis of phosphorylated Erk1/2 levels in primed hNSCs indicate that FHL and FHL + PD primed hNSCs have decreased levels of activated Erk1/2 protein levels as compared to spheres (** $p<0.01$), ELL (+ $p<0.05$) or ELL + PD (#$p<0.05$) (One way ANOVA; n=3/group). For analysis, pErk1/2 is normalized to loading control and further to overall Erk1/2 protein levels. Note that ELL + PD treated cells show no difference in activated Erk1/2 protein from ELL alone samples indicating the specificity of the PD173074 inhibitor for FGF mediated cell signaling. C) Representative western blot of activated JNK (phosphorylated at Thr183-Tyr185) in primed or unprimed (spheres) hNSCs. D) Densitometrical analysis of activated JNK protein levels normalized to overall JNK levels and further normalized to loading control, β-actin (One way ANOVA, *$p<0.05$ vs spheres, +$p<0.05$b vs ELL, #$p<0.05$ vs ELL-PD). IDV=Integrated density value.

EGF and/or LIF Repress bFGF-Mediated HB9 Expression

Because both unprimed spheres and ELL-primed hNSCs showed very low levels of Hb9 expression and both are cultured in the presence of EGF and LIF, I next questioned if EGF and/or LIF down-regulated or repressed Hb9 expression (Figure 3.14). To test this hypothesis, hNSCs were exposed to FHL-priming conditions for four days followed by either continuation with FHL-priming for an additional 2 days or by withdrawal of FHL and replacement with EGF, LIF or EGF and LIF together (total time of priming = 6 days). As in our previous experiments, hNSCs primed for four days with FHL showed significant Hb9 up-regulation, which was maintained following 2 more days of FHL treatment (i.e. Hb9 expression in FHL 6d ≈ FHL 4d). In contrast, cells exposed to FHL for 4 days followed by withdrawal of FHL-priming and replacement with EGF or EGF plus LIF for an additional 2 days showed an approximately 65% or 78% down-regulation of Hb9 mRNA levels, respectively (Figure 3.14B FHL4d= 8.5 ± 1.3 units; FH4d + E2d= 3.0 ± 0.6 units; FHL4d + EL2d= 1.9 ± 0.5 units; one way ANOVA, Tukey post hoc test vs FHL4d; n=3). Thus it appears that Hb9 expression is reversible by application of the growth factors EGF and/or Lif.

DISCUSSION

In this study I found that addition of the growth factor bFGF in conjunction with heparin and laminin and adherent culture conditions induced Hb9 motoneuron differentiation of fetal brain derived human neural stem cells (hNSCs). *In vitro* bFGF has been shown to be an essential factor for the maintenance of undifferentiated embryonic stem cells (Wang et al., 2005;Xu et al., 2005), to act as a potent mitogen for neural stem/progenitor cells (Gritti et al., 1995;Gritti et al., 1996;Kitchens et al., 1994) and to influence cell fate decisions (Tsai and Kim, 2005;Vicario-Abejon et al., 1995;Ostenfeld and Svendsen, 2004;Palmer et al., 1999). In particular, bFGF has demonstrated the ability to induce a dorsal to ventral switch

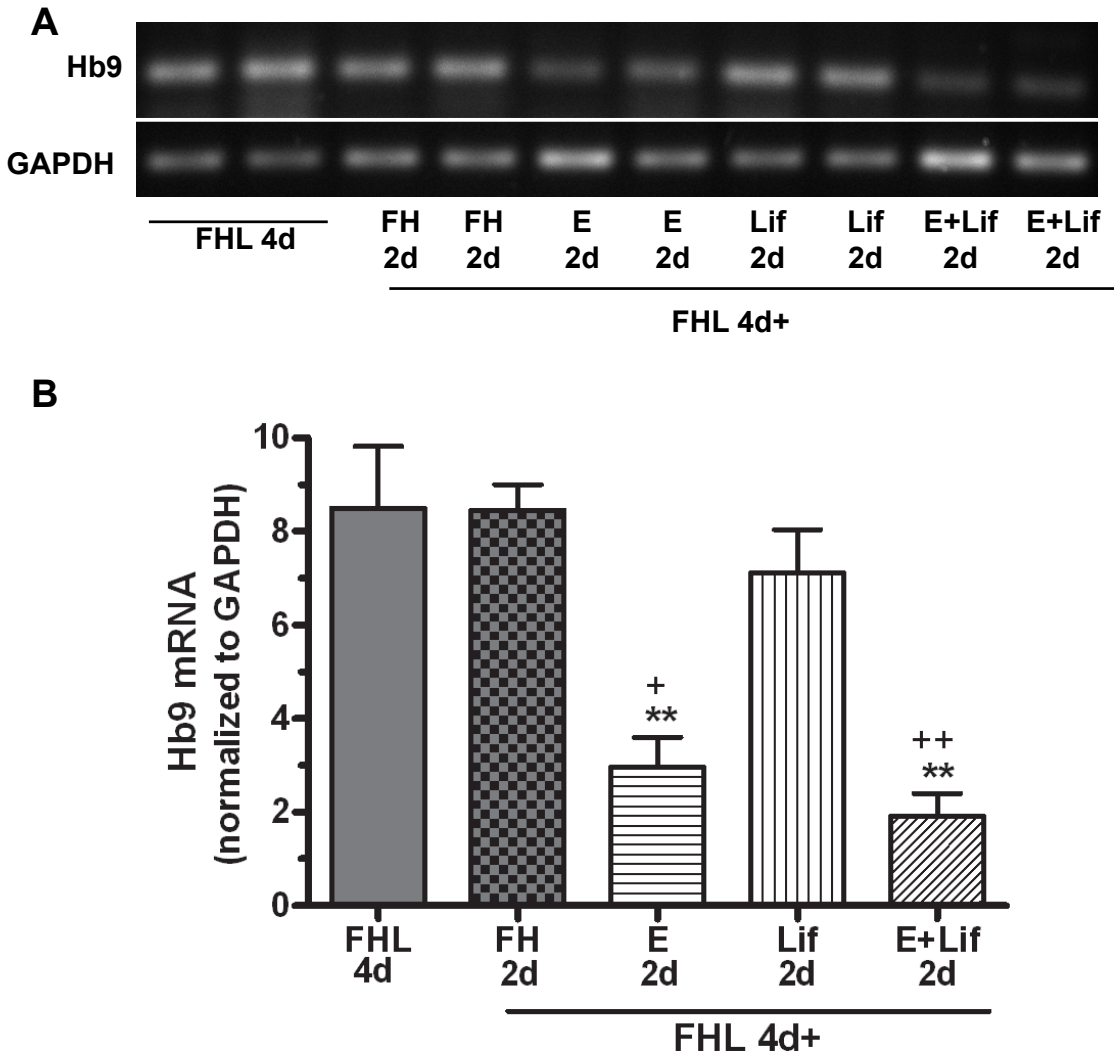


Figure 3.14: EGF inhibits Hb9 induction in hNSCs. A) hNSCs primed with FHL for 4 days show strong Hb9 RNA transcript levels while those exposed to FHL for 4 days followed by two days of either EGF alone or EGF + Lif treatments show a down-regulation of Hb9 transcript. A representative gel is shown here with duplicate samples, however for analysis, 3 samples/group from 3 independent experiments were analyzed. B) Densitometrical analysis of Hb9 mRNA in hNSCs primed with FHL for 4 days +/- 2 days of treatment with varying growth factors. Addition of EGF or EGF + Lif significantly down-regulated Hb9 transcript as compared to FHL 4 days (**$p<0.01$) or FHL4d + FH 2d (total of 6 days of FHL treatment). Addition of Lif alone after the 4 day FHL treatment (FHL4d + Lif 2d, vertical striped bar) had little effect on Hb9 transcript however, the combination of EGF and Lif significantly down-regulated Hb9 RNA levels (+$p<0.05$, ++$P<0.01$ vs FHL4d+Lif2d) suggesting that EGF may be the main factor down-regulating Hb9 expression. All data are normalized to GAPDH, an internal control.

in cell fate in rodent derived neural stem cells (Gabay et al., 2003), and a re-programming of adult derived non-neurogenic cells into neurons (Palmer et al., 1999). Furthermore *in vitro*, bFGF has been shown to have dual roles, as that of a glial induction factor (Tsai and Kim, 2005;Ostenfeld and Svendsen, 2004) or as a neuronal induction factor (Buc-Caron, 1995;Wu et al., 2002;Ren et al., 2006;MacDonald et al., 1996). Differences in cell type (rodent vs. human for example) and culture method (i.e. addition of other growth factors, neurosphere vs. adherent growth conditions, concentrations of bFGF, etc.) and stage of cell (embryonic vs. neonatal vs. adult) as well as region of cell derivation are all likely to play large roles in the specific effects of bFGF on stem cell differentiation. In order to better understand the role of bFGF in combination with heparin and laminin in a unique cell priming procedure developed in our lab (i.e. FHL-priming), I conducted experiments designed to test the hypothesis that bFGF was a required factor for hNSC motoneuron differentiation *in vitro.*

bFGF Drives hNSC Differentiation into Hb9 Expressing Cells

In our first set of experiments I found that hNSCs primed with FHL for 4 days showed robust increases in expression of transcription factors Islet1, Olig2, Ngn2 and Hb9, which are all known to be important factors in motoneuron development. In contrast, cells either unprimed (neurospheres) or primed with a cocktail consisting of EGF, LIF and Laminin (ELL) did not show up-regulation of these factors. Taken together these results suggest that adherent culture in combination with bFGF and likely withdrawal of EGF and Lif are required for motoneuron differentiation of hNSCs *in vitro*.

To further understand this phenomenon, I next examined expression of the bHLH transcription factor Olig2 in FHL, ELL and unprimed hNSCs. Four days of bFGF treatment significantly up-regulated Olig 2 mRNA and protein expression as compared to both unprimed and ELL primed hNSCs. Olig2 up-regulation in response to bFGF has been

reported in multiple cell types both *in vivo* and in *vitro* (Naruse et al., 2006;Dromard et al., 2007;Kessaris et al., 2004;Gabay et al., 2003) and has also been associated with a dorsal to ventral cell type switch in rodent neural progenitor cells (Gabay et al., 2003). It has been reported that at the time of Olig2 expression in spinal cord development, however, cells have the choice of two distinct cell fate pathways: that of oligodendrocytes or of motoneurons (Lu et al., 2002;Zhou and Anderson, 2002). In order to further understand whether hNSCs treated with bFGF were capable of choosing a motoneuron fate, I next examined expression of the motoneuron lineage marker, Hb9, and found that the priming procedure including bFGF did significantly enhance Hb9 expression in hNSCs.

Hb9 Expression is Time and bFGF Dose Dependent

In order to better understand the mechanism behind FHL-induced Hb9 expression, I next performed a bFGF dose and time response study for the motoneuron transcription factor, Hb9. In this set of experiments I found that even low levels of bFGF for four days (0.01ng/ml) induced significant Hb9 up-regulation and that higher levels of bFGF (10ng/ml) but for shorter time periods (48 hrs for example) also significantly up-regulated Hb9 expression. Together these data suggest that higher concentrations of bFGF can substitute for longer periods of time in culture with low levels of bFGF. Interestingly, hNSCs primed with very high concentrations of bFGF (100ng/ml) showed a down-regulation of Hb9 suggesting that receptor saturation and/or receptor endocytosis in response to high levels of growth factor may also be an important modulator of bFGF induced Hb9 expression.

It is also important to stress that a switch from free floating neurosphere culture to adherent culture is required for this phenomenon as differing concentrations of bFGF in neurosphere culture has little effect on Hb9 expression within neurospheres (data not shown). As has been reported by others (Caldwell et al., 2001;Steindler et al., 2003), most cells within neurosphere cultures in our laboratory remain undifferentiated and express

high levels of the neural precursor marker, Nestin (data not shown) and differentiation of hNSCs into cholinergic expressing cells requires adherent culture conditions (Wu et al., 2002). Furthermore, it is interesting to note that neurospheres in our laboratory are grown in the presence of multiple growth factors (EGF, LIF and bFGF), and that withdrawal of EGF and LIF and maintenance of bFGF levels for a period of time (4 days) in combination with heparin and laminin appear necessary for Hb9 induction. Thus it is possible that long term exposure to bFGF during cell expansion may also be important to maintain the capacity of hNSCs *in vitro* for later proper MN differentiation. In light of this, no other group has shown that long term bFGF treatment in either neurosphere or adherent cultures induced up-regulation of motoneuron determinants such as Islet1 or Hb9.

bFGF is required for Hb9 Induction

In order to confirm that bFGF was the driving force for Hb9 differentiation of FHL-primed hNSCs I utilized two separate inhibitors of bFGF signaling in conjunction with FHL-priming. First I chose the FGF receptor antagonist, PD173074, which has previously been shown to specifically inhibit activation of all FGF receptors and thereby block FGF mediated cell signaling (Mohammadi et al., 1997;Mohammadi et al., 1998;Bansal et al., 2003a). Unlike other FGF receptor antagonists such as SU5402, PD173074 shows little affinity for the PDGF receptor (Bansal et al., 2003b). Furthermore, our data also confirms that PD173074 is specific for FGF mediated signaling as addition of PD173074 to EGF and LIF treated samples had no effect on activation of downstream targets JNK or Erk1/2. Addition of PD173074 during FHL- priming significantly down-regulated Hb9 mRNA levels as compared to FHL+DMSO (vehicle) or FHL alone suggesting that bFGF is a potent mediator of Hb9 expression in hNSCs.

In order to confirm bFGF's role as an inductive factor for Hb9 differentiation, I also blocked bFGF mediated signaling using a bFGF neutralizing antibody. As was the

case with PD173074, blockade of bFGF signaling with anti-bFGF antibody treatment also significantly down-regulated Hb9 mRNA expression. Interestingly, both Hb9 transcript and activation of Erk1/2 were repressed ~40% by either PD173074 or bFGF neutralizing antibody treatment. This similar level of down-regulation may be more than coincidental, however, further study of Erk1/2 activation is required in order to show a direct role for this protein in Hb9 induction in hNSCs. Further complicating the story, other MAP kinase proteins (such as p38) which were not examined in this study as well as interaction(s) between these signaling molecules may also be important mediators of this phenomena.

Alternatively, other possibilities also exist for why complete Hb9 inhibition did not occur under conditions when bFGF signaling was down-regulated. First, bFGF could be produced but not secreted by hNSCs in culture. Endogenous non-secreted bFGF would not be inhibited by either FGF receptor blockade nor by neutralizing antibody treatment. It is also known that bFGF has a second mechanism of action outside of FGF receptor activation via nuclear localization and cell signaling through direct binding to DNA (Sorensen et al., 2006), effects of which would not be affected by either FGF blocking treatment. Furthermore, other studies have shown that nuclear localized bFGF has the ability to radically effect differentiation [for review see (Sorensen et al., 2006)]. For example, Sherman et al. (1993) eloquently demonstrated that nuclear bFGF induced trans-differentiation of avian neural crest-derived schwann cells into a melanocytic cell fate (Sherman et al., 1993). A role for nuclear bFGF in hNSC differentiation however, remains to be clarified and is the focus of future studies in the lab. Another possibility for incomplete Hb9 repression under bFGF signaling inhibition is that additional factors besides bFGF may also play a role in Hb9 cellular differentiation from hNSCs. In this scenario, Hb9 up-regulation by bFGF may be a combinatorial effect whereby bFGF interacts with other proteins or indirectly regulates other proteins necessary for Hb9 induction. This hypothesis, however, remains to be examined.

Proliferation Is Not Required for Hb9 Induction

Other groups have reported that a final round of division is necessary for neuronal differentiation of hNSCs (Ostenfeld and Svendsen, 2004). To examine whether cell division was required for Hb9 expression by FHL-priming, I utilized two mitotic inhibitors, AraC and an inhibitor of the cell cyclin dependent kinases, CDK2i. AraC was chosen because it is a well established, potent mitotic inhibitor first developed as a chemotherapeutic. One of its known mechanisms of action is through incorporation into the DNA thereby disrupting the DNA-DNA polymerase interaction and causing early strand termination (Momparler, 1982). Addition of AraC to FHL-primed hNSCs completely blocked proliferation and dramatically decreased Hb9 expression by approximately two fold as compared to FHL alone. Such negative effects of AraC on FHL-induced Hb9 expression might be explained as a requirement of bFGF-mediated proliferation for Hb9 expression. However, it is also possible that Hb9 reduction is the consequence of ArcC interfering gene transcription through its inhibition of RNA polymerase (Chuang and Chuang, 1976) and DNA incorporation (Momparler, 1982).

To further examine whether proliferation was required for Hb9 induction, we utilized a second mitotic inhibitor (CDK2i) that has a separate molecular mechanism from AraC and inhibit an essential cell cycle protein, CDK2. To progress from G1 to S phase, CDK2 is a required molecule. Addition of CDK2i potently inhibited cell cycle progression as determined by a lack of bromodeoxyuridine incorporation in the presence of this molecule. Surprisingly, hNSCs primed with FHL and treated with CDK2i showed similar mRNA expression of Hb9 as FHL-primed cells treated with vehicle (DMSO). Thus, unlike the AraC experiments, it appears from CDK2 inhibition experiments that proliferation is not a requirement for Hb9 induction. We hypothesize that the differences in Hb9 expression between AraC and CDK2i treated FHL-primed cells are due to the inhibition of transcription

by AraC incorporation or cytotoxic effects of AraC. The exact mechanism behind these differences, however, has yet to be determined.

bFGF Mediated Cell Survival and Hb9 Induction

bFGF is a well established neurotrophic factor both *in vivo* and *in vitro* (Unsicker et al., 1987;Otto et al., 1987;Walicke, 1988;McManaman et al., 1989;Gray and Patel, 1992;Albrecht et al., 2002). Application of bFGF has proved beneficial in many injury models including ischemic injury (Watanabe et al., 2004), neuronal cultures from deafferentated spinal cord (Yin et al., 1994), and axotomy induced cell death (Blottner and Herdegen, 1998). Furthermore, bFGF has been shown to be neuroprotective against the neurotoxic effects of 6-hydroxydopamine (Grothe et al., 2000) and glutamate induced cell death (Reuss and Bohlen und, 2003). In order to understand whether bFGF may be promoting cell survival in our system I examined total cell death using an LDH assay and apoptotic cell death using both TUNEL staining and a caspase 3 enzymatic activity assay. All three assays indicated that FHL-primed cells survive similarly as both ELL and undifferentiated cells (spheres). These results suggest that under our culture conditions, bFGF does not exert a potent neurotrophic effect that is higher than ELL-priming or EFL proliferation conditions. Thus it is highly unlikely that enhanced general cell survival can account for the potent 8- and 16-fold Hb9 induction seen in FHL-primed hNSCs compared to spheres and ELL primed hNSCs, respectively.

To further examine whether bFGF-enhanced Hb9 expression is due to a selective survival effect on Hb9-expressing cells, I also analyzed cell death in FHL-primed cells treated with the FGF receptor antagonist, PD173074 (FHL-PD). PD treatment did not increase overall cell death nor caspase 3 activity as compared to FHL-treated samples alone. On the other hand, TUNEL staining showed that apoptotic cell death was enhanced by approximately 2 fold in FHL-PD treated cells (total of 6%) as compared to FHL-primed

alone (2%). Thus this data suggests that FGF signaling seems to prevent hNSCs from apoptotic cell death during priming. However, whether bFGF's trophic effect accounts for Hb9 induction is questionable. First, PD reduces bFGF-mediated Hb9 mRNA expression by only 40% at the end of the 4-day priming, which is not correlated with its 2-fold increase in apoptotic cell death. Second, the number of PD-mediated apoptotic cells at the highest only counts for 6% of the total population, and this does not match the percentage of Hb9 immunoreactive cells, 45%, following the withdrawal of bFGF and further differentiation. Although a trophic role of bFGF for Hb9 enhancement cannot be ruled out completely, our accumulated data suggested that bFGF acts as an inducer for Hb9 expression in fetal brain-derived and long-term expanded hNSCs.

MAP Kinase Pathways and bFGF Induced Hb9 Expression

bFGF is a known activator of several MAP kinase cell signaling molecules including Erk1/2, JNK/SAPK and p38 (Schlessinger, 2000). I first chose to analyze Erk1/2 activity in primed and unprimed hNSCs. Erk1/2 has previously been shown to be an important modulator of both cell proliferation and differentiation. For example, bFGF induced phosphorylation of Erk1/2 has been shown to enhance proliferation of rodent stem cells via activation of Flk1 in co-operation with another growth factor, VEGF (Xiao et al., 2007). In other *in vitro* models, such as in an osteoblast differentiation model, bFGF signaling mediated by Erk1/2 is essential for proper cellular differentiation (Spector et al., 2005). In rat PC12 cells, FGF treatment induces neuronal differentiation mediated through FGFR1 and Erk1/2 activity (Hayashi et al., 2001;Lin et al., 1996). Interestingly, bFGF induced Erk1/2 activity has also been shown to be enhanced at the expense of p38 activity in erythroid progenitors (K2562 cells) (Huang et al., 2004). This suggests that multiple cell signaling molecules may be co-regulated and furthermore that individual cell signaling molecules may even influence the activity of other molecules leading to specific cell signaling events. Along these lines, it has

been suggested that specificity of signaling by receptor tyrosine kinases (RTKs) is in fact mediated by multiple cell signaling pathways. In particular, it is still unclear why activation of RTKs by FGF result in cell proliferation in fibroblasts and yet activation of the same RTKs in neuronal cultures induces differentiation (Schlessinger, 2000)?

In order to understand whether FHL-priming also activated MAP kinase cell signaling events I began by analyzing activity of two of these molecules: Erk1/2 and JNK/SAPK. Interestingly, I found that hNSCs exposed to 4d of FHL-priming showed a down-regulation of both phosphorylated Erk1/2 and JNK as compared to neurospheres and ELL primed cells. Addition of the FGF receptor antagonist, PD173074, further down-regulated activated/phosphorylated Erk1/2 (i.e. pErk) by about 40% but had no effect on JNK/SAPK phosphorylation (i.e. pJNK). Notably, FHL+PD treatment also reduced Hb9 expression by 40% as compared to FHL-priming, indicating the possibility of Erk1/2 pathway involvement in bFGF-mediated Hb9 expression. On the other hand, Erk1/2 proteins were highly activated in unprimed spheres and ELL-primed hNSCs, yet these cells showed minimal Hb9 expression. Taken together, our data suggests that the Erk1/2 MAPK pathway may be involved in bFGF-induced Hb9 expression but at a very tightly controlled level of activation, possibly through other auto-inhibitory mechanisms of the bFGF signaling pathway (Schlessinger, 2004). Further studies need to be done to confirm the exact role of Erk1/2 in bFGF's effect. Alternatively, Erk1/2 may not be directly involved in bFGF-induced Hb9 expression and other pathways of bFGF signaling, including p38 and phospholipase C gamma, need to be explored.

Hb9 Induction in hNSCs is Sonic Hedgehog (SHH) Independent

In vivo, SHH is a well characterized, required molecule for dorsal-ventral pattern formation and in particular, for the proper induction of MNs within the developing spinal cord ((Briscoe and Ericson, 1999;Liem, Jr. et al., 2000). Also for a review of SHH during

pattern formation see (Briscoe and Novitch, 2007)). Data supporting the necessity of SHH signaling *in vivo* comes from mutant models where either SHH or SMO (a G-protein coupled receptor and an essential SHH signaling molecule) were mutated leading to a neural tube lacking ventral cell types (Chiang et al., 1996;Zhang et al., 2001). Furthermore, SHH signaling has been implicated in proper homeodomain transcription factor expression leading to MN formation *in vivo* (Briscoe et al., 2000). *In vitro,* Li et al. (2005) and Wichterle et al. (2002) found that human and mouse ES cells, respectively, did not differentiate into Hb9 expressing cells without exogenous SHH stimulation (Li et al., 2005;Wichterle et al., 2002). In contrast to the situation found in ES cells, under our *in vitro* priming conditions, bFGF appears sufficient for Hb9 MN induction from hNSCs.

Recently, it has been suggested that *in vivo* SHH may be a required molecule for proper spatial regulation within the developing spinal cord rather than for the induction of specific progenitor domains (Briscoe and Novitch, 2007). There are at least two reasons why SHH may not be essential in our system. First, hNSCs, derived from 8-wk forebrain and expanded in EGF, bFGF and LIF *in vitro*, may be refractory to SHH stimulation due to lack of receptors and/or temporal regulation of molecules within the SHH signaling pathway. Secondly, spatial cues that are essential *in vivo* may not be required *in vitro*. In support of the first idea, within the developing spinal cord, early SHH expression induces up-regulation of factors such as Nkx 6.1 and Pax 6 which allow for Olig2 expression and finally for activation of homeodomain proteins such Lim3 and Islet1 (Briscoe and Novitch, 2007). If hNSCs cultured *in vitro* follow *in vivo* MN development, then it would not be surprising that SHH is not required for MN development from hNSCs because Olig2 is already expressed at high levels within cultured neurospheres. Thus it is possible that hNSC within neurospheres are already past the early essential SHH signaling phase.

CONCLUSIONS

In summary, I found that bFGF is a required factor for motoneuron differentiation of hNSCs *in vitro.* I demonstrate this through both semi-quantitative and quantitative RT-PCR and immunocytochemistry for the motoneuron lineage transcription factor, Hb9. Furthermore, we show that this phenomenon is not solely due to bFGF's ability to act as a neurotrophic nor as a mitogenic factor. Finally, I show that the Erk1/2 MAP kinase pathway may be involved in this induction process. This information furthers our understanding of human neuronal development and may also be important in drug screening of neuroprotective compounds to treat neurological diseases.

CHAPTER 4: ASTROCYTES ENHANCE LONG-TERM SURVIVAL OF CHOLINERGIC NEURONS DIFFERENTIATED FROM HUMAN FETAL NEURAL STEM CELLS

ABSTRACT

Establishment of an *in vitro* model of human cholinergic neurons is highly desirable to understand and develop treatment for Alzheimer's and motoneuron diseases. Previously we reported that the combination of basic fibroblast growth factor (bFGF), heparin and laminin directs human fetal neural stem cells to form cholinergic neurons. One problem, however, is that long term *in vitro* survival of these cells is low. My goal for this study was to determine whether astrocytes or their secreted factors enhance differentiation and survival of cholinergic neurons under long-term differentiation conditions. I demonstrate here that astrocytes or conditioned media did not enhance cholinergic differentiation but did increase the long-term survival of differentiated human neural stem cells, particularly cholinergic neurons. I further show that astrocytes protected long-term differentiated stem cells from apoptotic cell death, which is at least partially mediated by astrocyte-secreted bFGF. My findings indicate that long-term survival of human stem-cell derived cholinergic neurons requires trophic factors from non-neuronal cells. This data may provide insights into the development of an *in vitro* model of long-term cultured human cholinergic neurons useful for the understanding of the mechanisms of cholinergic differentiation and developing treatments for neurological diseases.

INTRODUCTION

Alzheimer's disease and amyotrophic lateral sclerosis (ALS) are both neurodegenerative diseases which target cholinergic neurons. Currently we have limited treatments for these diseases. One reason is because we lack *in vitro* models necessary for the study of cholinergic neurodegeneration. The benefits of such a model are many. For example, a cholinergic cell *in vitro* model derived from human cells could be used for pathologic studies of cholinergic neuron degeneration and associated screening of neuroprotective drugs.

Several groups reported previously the generation of cholinergic neurons from embryonic stem cells with a maximum yield of ~15-20% (Wichterle et al., 2002;Li et al., 2005). Similarly, using human fetal neural stem cells (hNSCs) we have shown that approximately 28% of hNSCs primed with a cocktail of bFGF, heparin and laminin differentiate into cholinergic cells *in vitro* (Wu et al., 2002). These cholinergic neurons, however, were not fully mature, as they did not show extensive neurite and axonal outgrowths and exhibited only single spike action potentials *in vitro*. Furthermore, many differentiated hNSCs did not survive for more than two weeks when cultured *in vitro* (Wu, unpublished observation). However, if transplanted into medial septum or spinal cord, not only did these cells survive, but 50-60% differentiated into cholinergic neurons and many made connections with muscle targets (Wu et al., 2002;Tarasenko et al., 2006;Gao et al., 2005a). Thus the discrepancies between the *in vitro* and *in vivo* systems seem to indicate that the host provides essential cues guiding the differentiation, maturation and survival of grafted hNSCs that we are lacking in our *in vitro* cultures.

One likely source of host cues are the non-neuronal support cells. Astrocytes were chosen as a likely candidate to provide these cues for several reasons. First, astrocytes improve neuron viability in injury models by taking up excitotoxic amino acids and

providing neurotrophic factors (Rosenberg and Aizenman, 1989;Rao et al., 2001;Yamagata et al., 2002;Zhao et al., 2004;Albrecht et al., 2002). For example, astrocytes and/or astrocyte-secreted molecules protect neurons against a variety of insults, including energy impairment (Ohgoh et al., 2000), hypoglycemic damage (Cheng and Mattson, 1991), excitotoxicity (Mattson and Rychlik, 1990) anoxia (Vibulsreth et al., 1987) and axotomy (Zhao et al., 2004;Yin et al., 1994). Second, astrocytes improve neuronal differentiation, maturation and synapse formation of rodent cells *in vitro* (Song et al., 2002a;Barkho et al., 2006). Despite this wealth of information in rodent systems, however, astrocytic effects on the differentiation and survival of human NSCs are yet to be elucidated.

The aim of this study is thus two fold: First, to determine whether the addition of astrocytes or their secreted factors improve cholinergic differentiation from primed hNSCs; and second, to determine whether astrocytes enhance long term survival of hNSC-derived cholinergic neurons *in vitro*. I report here that astrocytes do not enhance the proportion of hNSCs which differentiated into a cholinergic phenotype, but the addition of astrocytes or astrocyte conditioned media does significantly improve long-term (up to 30 days) survival of hNSC-derived cholinergic neurons. This survival effect is shown to be at least partially mediated through astrocyte secretion of bFGF.

Enhancement of long term *in vitro* survival of human derived cholinergic neurons may prove beneficial for drug screening of compounds for neuroprotective properties. In the future, this may lead to the development of better therapeutics for neurodegenerative diseases which target cholinergic cells. Furthermore, information presented here may be useful in the derivation of a more accurate *in vitro* model to study cholinergic cell pathology in response to neurotoxic stimuli, thus leading to a better understanding of causes of neuronal cell death.

RESULTS

Astrocytes Improve hNSC Survival During Long-Term Differentiation *in vitro*.

To determine the efficacy of astrocytes for improving long-term survival of differentiated hNSCs and to mimic the rat central nervous system environment into which hNSC were grafted in our earlier studies (Wu et al., 2002;Gao et al., 2005b;Tarasenko et al., 2006;Tarasenko et al., 2006), we used two types of astrocytes: primary cultured spinal cord derived astrocytes from adult rats and an immortalized rat astroglial cell line. Initially, we co-cultured FHL-primed hNSCs with primary rat astrocytes, either under direct contact with FHL primed hNSCs plated on top of an astrocyte feeder layer or under indirect co-culture with astrocytes plated in transwells suspended above primed hNSCs. Primed hNSCs cultured in B27 differentiation media alone served as controls, which showed cells with blebbing that had detached from other cells as well as increased debris in media over time (Fig. 4.1A-C). These became particularly apparent when cells were seeded at lower cell densities (data not shown). In contrast, the addition of primary rat astrocytes, either under indirect contact (i.e. with astrocytes plated onto transwells suspended above hNSCs), or direct contact improved hNSC viability (Fig 4.1 D&E; G-I). Overall, co-cultures of astrocytes and hNSCs showed preserved cells with a healthy morphology and diminished cell debris as compared to cultures without astrocytes.

Since direct and indirect hNSCs cultured together with astrocytes showed a similar survival phenotype, I hypothesized that secreted molecules may aid in stem cell survival. In order to further examine the effects of astrocyte-secreted factors, I tested the effects of supplementing our cultures with astrocyte conditioned medium (ACM). Long-term differentiated (30 day) hNSCs survived better when cultured in ACM collected from immortalized CRL2005 astrocytes (Fig. 4.1F) or from a primary cell line (data not shown).

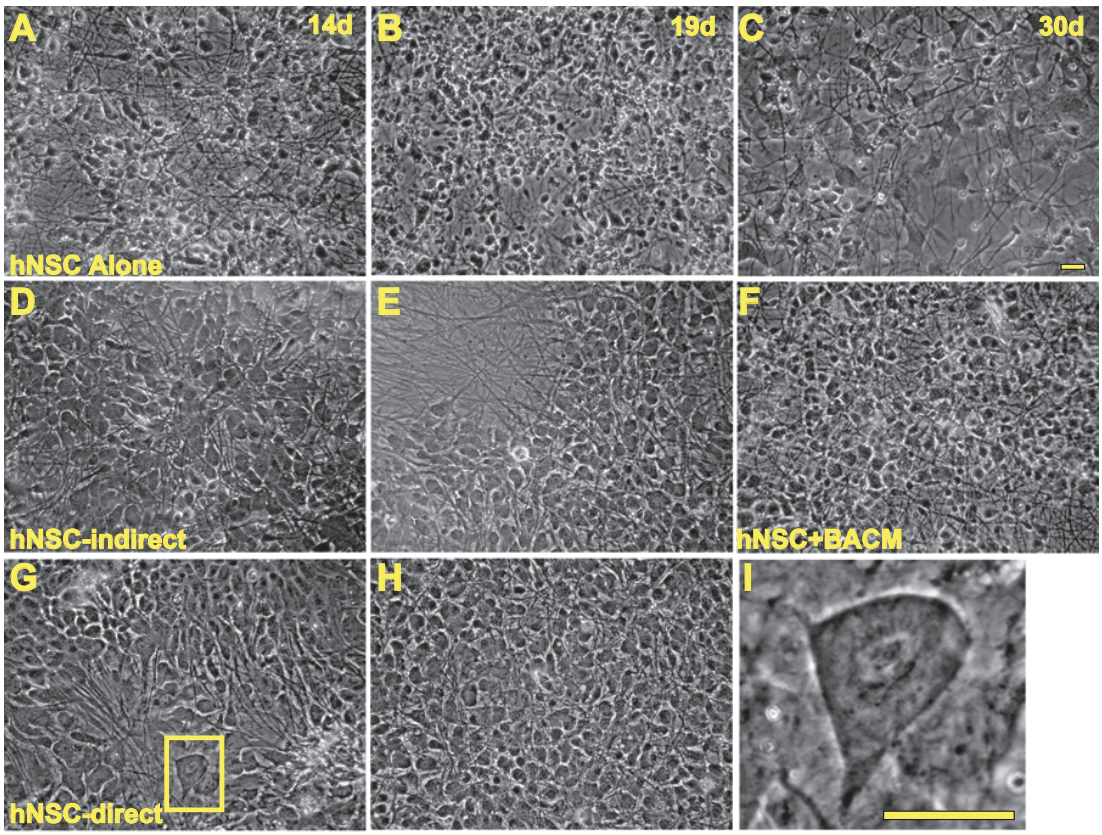


Figure 4.1: Cell morphology of hNSCs cultured with astrocytes or astrocyte conditioned medium (ACM). Phase contrast images of FHL-primed hNSCs differentiated in B27 alone (A-C), in the presence of primary adult rat spinal cord astrocytes through indirect co-culture (D-E), or with astrocyte conditioned media (F) or directly co-cultured with primary astrocytes (G-I). Images were taken at 14d (A, D, G), 19d (B, E, H) or 30d (C, F). The inset in G is shown enlarged in I. Scale bar = 20 µm.

This cell-protective effect was further confirmed by quantitative analyses using both WST-1 cell viability and LDH cytotoxicity assays (Fig 4.2). The WST-1 assay gives a read out of mitochondrial enzymatic activity via the conversion of a tetrazolium salt (colorless) to formazan (red). The amount of formazan can be directly correlated to the increase in red color and further to the number of healthy cells. As shown in Fig. 4.2A, hNSCs differentiated for either 14 or 30 days in the presence of ACM from an immortalized astrocyte cell line showed enhanced mitochondrial function and cell survival by approximately 28% as compared to

those without ACM (B27 alone) (d14: B = 1.464 ± 0.1708, BACM = 2.022 ± 0.8512; d30: B = 0.9811 ± 0.054, BACM = 1.368 ± 0.04986). Similar results were obtained using ACM from primary cells (data not shown). Lactate dehydrogenase (LDH), an enzyme released by dead/dying cells, was measured using a similar chemical assay. In this assay, LDH enzymatic activity was assessed through the conversion of tetrazolium salt to formazan with an increase in dead cells correlated to an increase in LDH enzymatic activity. In figure 4.2B I show that the addition of ACM significantly decreased LDH release from early (d5) and late (d30) but not middle (d15) time points of culture (Fig. 4.2B). Taken together, this data indicate that astrocytes enhanced the survival of long-term differentiated hNSCs through the secretion of trophic factors.

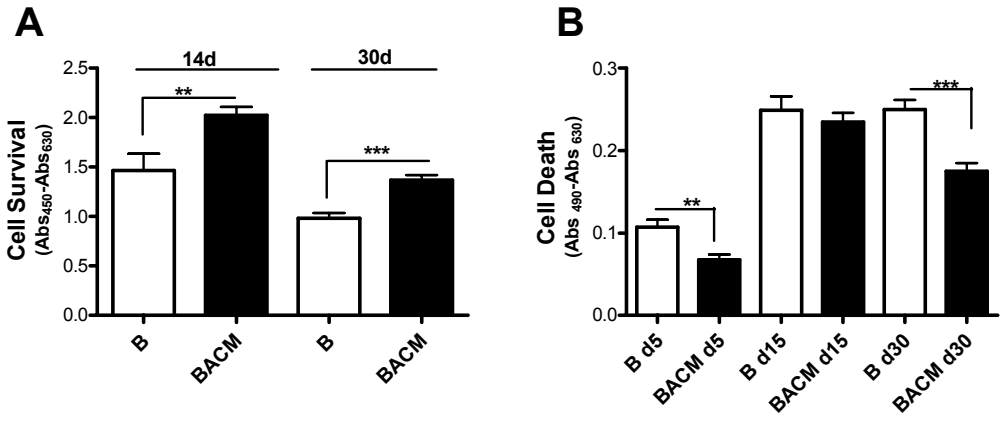


Figure 4.2: Cell survival of hNSCs differentiated in astrocyte-conditioned medium (ACM). (A) WST-1 cell survival assay of hNSCs differentiated in B27 alone (B) or B27 plus ACM (BACM). At both 14 and 30 days, cultures differentiated with ACM show enhanced cell survival (n = 16; students t test, **p<0.01, ***p<0.001) (B) Lactate dehydrogenase (LDH), an enzyme released by dead cells, was measured and quantified. hNSCs differentiated in the presence of ACM showed significantly less cell death at early (day 5) and late time points (30 days) (n = 6; Students t test, **p<0.01, ***p<0.001).

Astrocytes Enhance The Long-Term Survival Of hNSC-Differentiated Cholinergic Neurons *in vitro*

I then asked whether astrocytes protected or facilitated specific type(s) of cells differentiated from hNSCs. Immunostaining with specific neuronal (βIII-tubulin) and glial (GFAP) markers showed that although the total cell number was greatly enhanced in the ACM group as compared to the control (BACM=106.3±7.88 cells/0.148mm^2 vs B=71.55±4.825 cells/0.148mm^2), the relative percentage of astrocytes and neurons remained the same regardless of the addition of ACM (Fig. 4.3). In particular, ACM-treated and B27 control cells both exhibited approximately 40% neuronal differentiation (B=37.1±2.8%, BACM=38±2.7%) and 55% astroglial differentiation (B=54.4±2.7%, BACM+53±3.2%) after 30 days of culture. The remaining 5% of cells were unlabeled and may possibly represent oligodendrocytic cells.

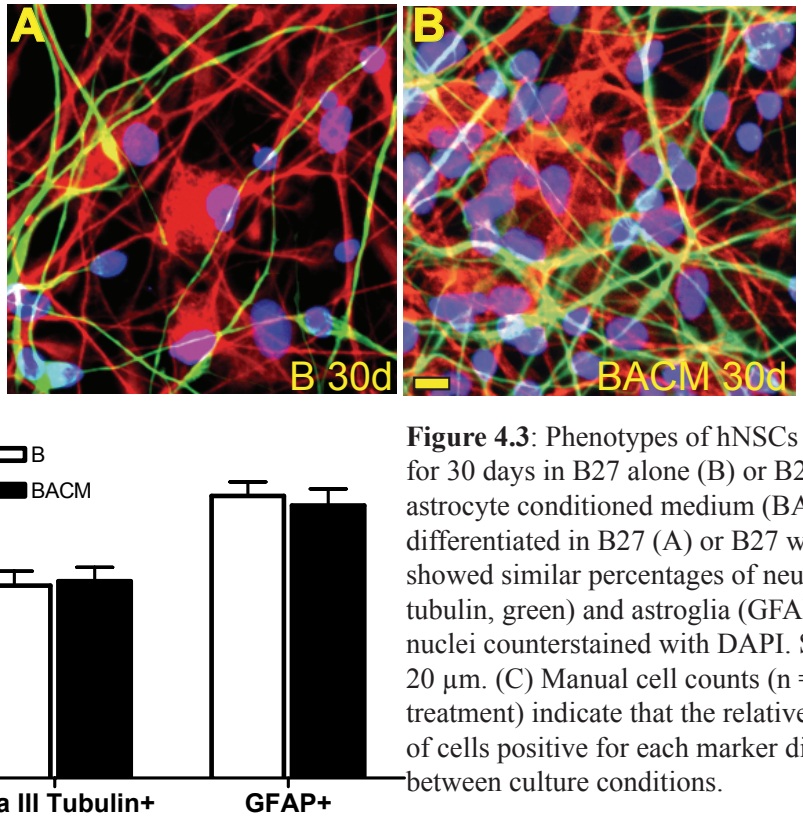


Figure 4.3: Phenotypes of hNSCs differentiated for 30 days in B27 alone (B) or B27 plus astrocyte conditioned medium (BACM). hNSCs differentiated in B27 (A) or B27 with ACM (B) showed similar percentages of neurons (Beta III tubulin, green) and astroglia (GFAP, red). Blue, nuclei counterstained with DAPI. Scale bar = 20 μm. (C) Manual cell counts (n = 10 fields/ treatment) indicate that the relative percentage of cells positive for each marker did not change between culture conditions.

To determine whether astrocytes facilitated and/or protected hNSC-differentiated cholinergic neurons, hNSCs co-cultured with astrocytes or treated with ACM were subjected to immunostaining with a choline acetyltransferase (ChAT) antibody. hNSCs plated in direct contact with astrocytes for 19 days formed "island" like clusters of cells with the stem cells located in the center and the feeder astrocytes surrounding the outside of the island (Fig. 4.4A-D). Cells within the center of this island stained strongly for ChAT with elongated neurites (red in Fig. 4.4A and D). Indirectly co-cultured hNSCs also showed ChAT positive staining (Fig. 4.4E). Cell counts demonstrated that ~10-20% of hNSCs

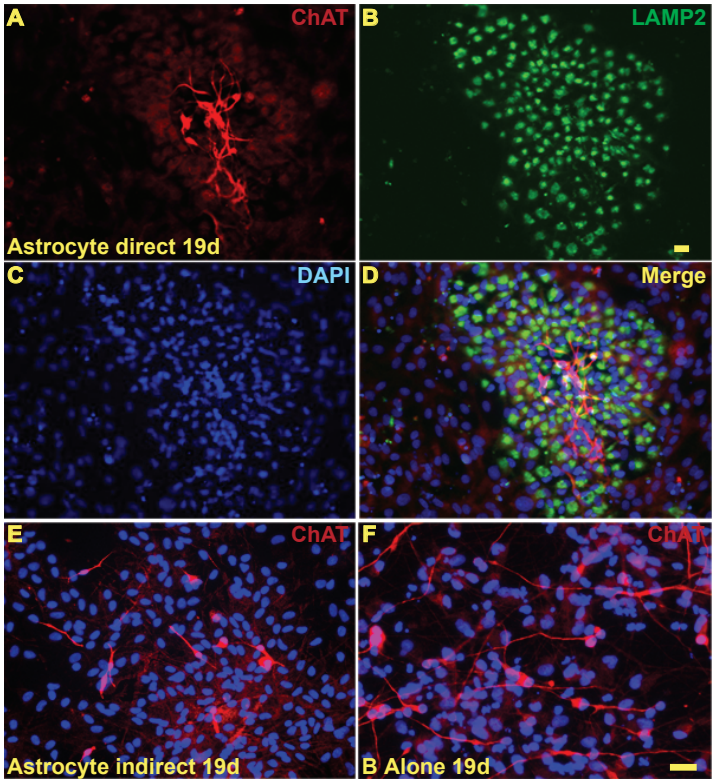


Figure 4.4: Cholinergic differentiation of hNSCs. hNSCs differentiated for 19 days under direct contact with primary astroglia from adult rat spinal cord (A-D) show strong immunoreactivity for choline acetyltransferase (ChAT, red). A human specific antibody, LAMP2 shown in green (B), was used to distinguish between hNSCs and rat-derived astrocytes. (D) Merged images of A-C. hNSCs differentiated under indirect astrocyte co-culture conditions (E) or without astrocytes (F) also show ChAT immunoreactivity. Red = ChAT. Green = LAMP2. Blue = nuclei counterstained with DAPI. Scale bar in B = 20 µm for A-D. Scale bar in F = 20 µm for E and F.

cultured directly or indirectly with astrocytes for 19 days differentiated into a cholinergic phenotype. Surprisingly, hNSCs differentiated without astrocytes for 19 days showed a similar level of ChAT positive cells (~17%) (Fig. 4.4F). Overall, this is a lower yield of cholinergic neurons than we have reported previously (~28% cholinergic differentiation, Wu et al. 2002) however, in this study, lower cell density likely led to a slightly decreased cholinergic differentiation potential.

Because both direct and indirect co-cultured astrocytes differentiated into a cholinergic phenotype, I next asked whether astrocytes might aid in long term cholinergic survival. After 30 days of culture, approximately 29% of hNSCs differentiated in the presence of astrocyte secreted factors maintained a cholinergic phenotype as detected by immunostaining with a ChAT antibody (Figure 4.5B). In contrast, less than 1% of hNSCs differentiated without ACM for 30 days demonstrated ChAT immunoreactivity (B=0.61±0.252%, BACM=29.4±3.0%). Furthermore, only 30 day cultures differentiated with ACM exhibited a detectable ChAT transcript (Figure 4.5A). Taken together this data suggests that astrocyte-secreted factors aid survival of hNSC-differentiated cholinergic cells under long-term *in vitro* culture.

Astrocytes Improve Long-Term Survival of hNSCs By Decreasing Apoptotic Cell Death

Since many of the dying hNSCs after long-term differentiation in basic B27 medium showed apoptotic morphology such as cell shrinkage, blebbing and detachment from healthy cells, I next asked whether astrocyte-secreted factors decreased apoptosis. Using a measurement of apoptosis (TUNEL), we observed that a significant number of hNSCs were TUNEL positive in 14-day and especially 30-day cultures with B27 media alone (Fig. 4.6). In contrast, in the presence of ACM derived from the CRL2005 astroglial cell line there were significant decreases in hNSC TUNEL staining of approximately 70%

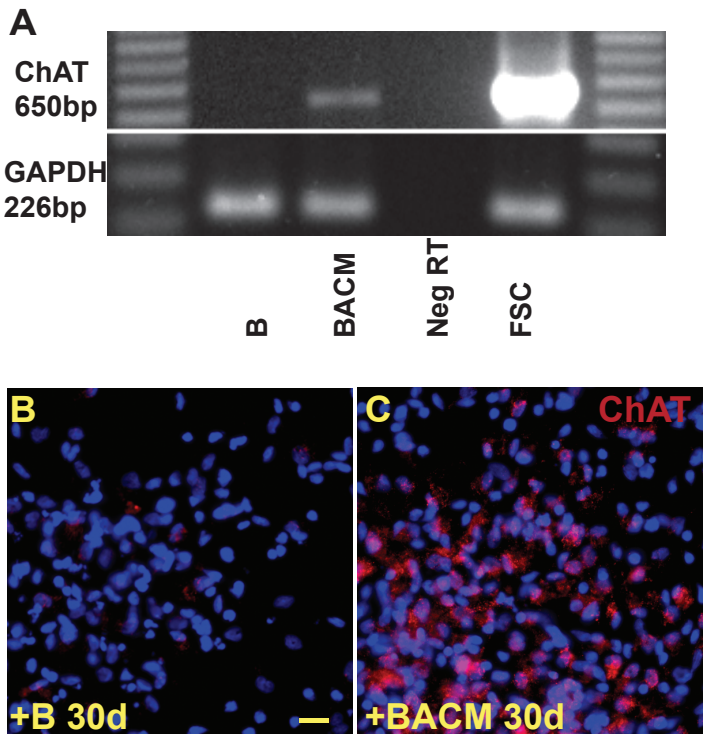


Figure 4.5: hNSCs cultured long term with astrocyte conditioned media maintain (ACM) a cholinergic phenotype. (A) ChAT transcripts in hNSCs differentiated with or without astrocyte conditioned medium (ACM). RNA was collected after 30 days of differentiation from hNSCs cultured with ACM (BACM) or without ACM (B). RNA was reverse transcribed and semi-quantitative RT-PCR was performed for detection of ChAT mRNAs. Neg RT, RNA without reverse transcriptase (negative control). FSC, fetal spinal cord (positive control). GAPDH, glyceraldehyde-3-phosphate dehydrogenase (internal control). (B-C) Cholinergic differentiation of hNSCs with or without ACM for 30 days. Cells differentiated in B27 alone (B) show diminished immunoreactivity for choline acetyltransferase (red, ChAT) as compared to those differentiated in the presence of ACM (BACM). Blue = DAPI, nuclear counterstain. Scale bar = 20 μm.

for both 14-day (d14: B = 15.3 ± 2.705, BACM = 4.6 ± 0.3728) and 30-day cultures of hNSCs (d30: B = 37 ± 4.183, BACM = 10.2 ± 4.93). The addition of ACM derived from primary rat spinal cord astrocytes affected cell survival similarly as CRL2005 ACM (data not shown).

Astrocytes Improve Long-Term Survival of hNSCs Through Astrocyte-Secreted bFGF

Under our culture conditions, hNSCs express all four FGF receptors (Wu and Jordan, see chapter 3) and respond to bFGF (Tarasenko et al., 2004;Wu et al., 2002). In

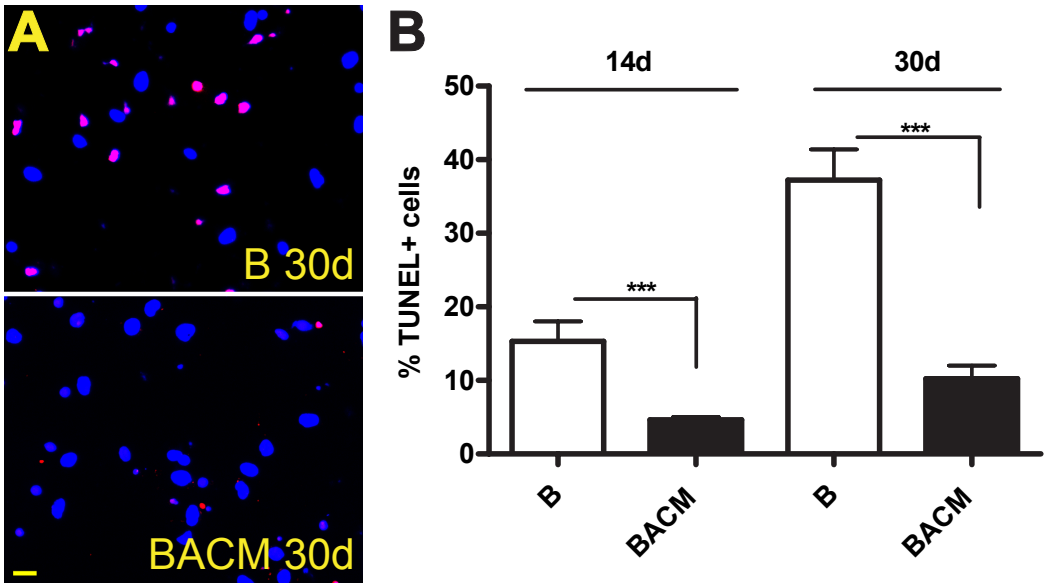


Figure 4.6: Apoptosis in differentiated hNSCs. hNSCs differentiated in B27 alone for 30 days (top panel in A) show extensive TUNEL labeling (red) while cells differentiated with ACM (lower panel A) show few TUNEL positive cells. Blue, nuclei counter stained with DAPI. Scale bar = 20 µm. (B) Quantification of manual cell counts (n = 10 fields/ treatment) of TUNEL positive cells after 14 and 30 days of differentiation (Students t test, **p<0.01, ***p<0.001).

addition, bFGF has been shown to have neurotrophic effects both *in vivo* and *in vitro* (Cheng and Mattson, 1991;Engele and Bohn, 1991;Caldwell et al., 2001;Grothe et al., 1991) . To determine whether bFGF plays a role in astrocyte-improved survival of long-term differentiated hNSCs *in vitro*, I first performed a bFGF ELISA to test secreted bFGF in ACM. As shown in Fig. 4.7A, approximately 122 pg/ml of bFGF was detected in ACM (n = 4) that was collected from 24-hr cultured astrocytes. To test whether bFGF within ACM had any effect on hNSC long-term survival, WST-1 cell survival assays were performed on hNSCs cultured with or without an FGF receptor antagonist, PD173074, which specifically blocks the tyrosine kinase activity of all four FGF receptors (Mohammadi et al., 1998;Koziczak et al., 2004). PD 173074 had little influence on ACM-improved hNSC survival at an early time point (d14) (Fig. 4.7B). However, after 30 days in culture,

the addition of the FGF receptor antagonist significantly blocked ACM-enhanced hNSC survival and decreased cell survival to similar levels as found in B27 control treated cells (Fig. 4.7B). Not surprisingly, hNSCs differentiated with PD173074 (i.e. without FGF signaling) for 30 days also did not show a ChAT transcript (Fig. 4.7C). In summary, we find that bFGF secreted by astrocytes and preserved in ACM aids in hNSC cholinergic survival during long-term *in vitro* differentiation.

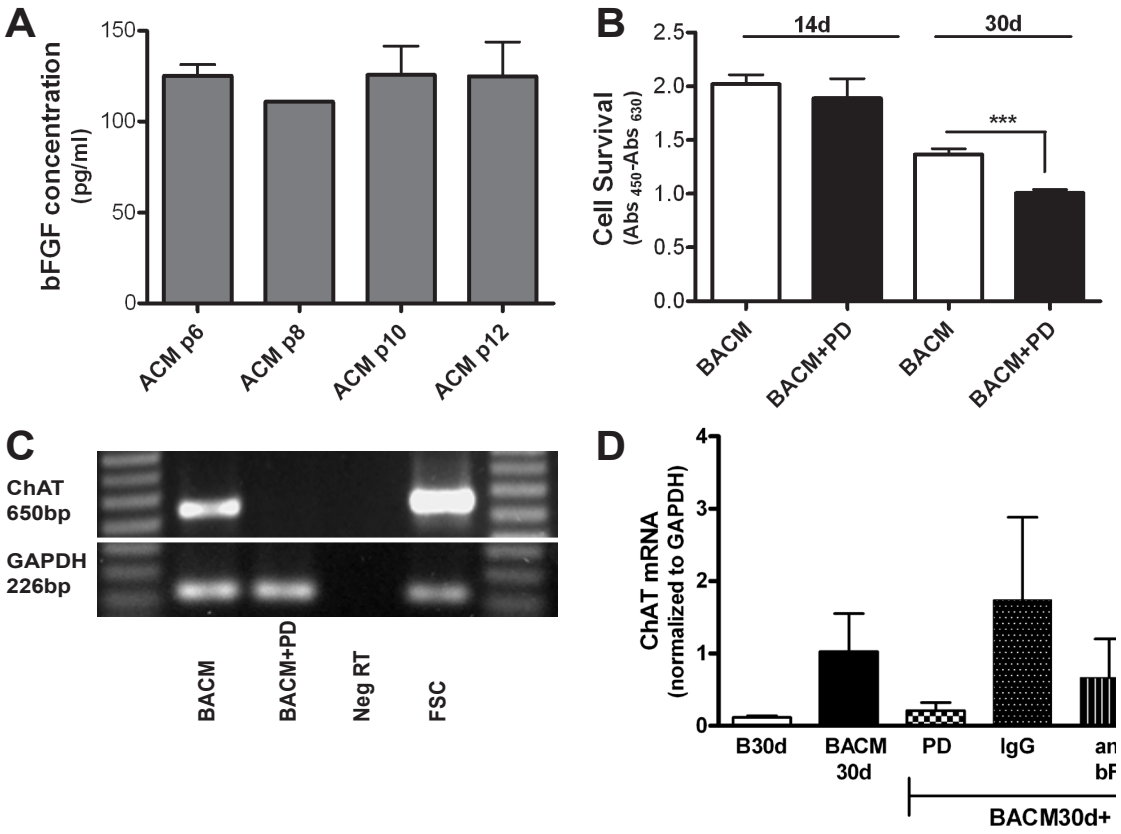


Figure 4.7: Astrocyte-secreted bFGF contributes to long-term survival of hNSC-derived cholinergic neurons. (A) Conditioned media (ACM) generated from a rat astrocytic cell line (CRL2005) was collected and subjected to a bFGF ELISA at various passages (p, passage number). (B) WST-1 cell survival test of hNSCs differentiated in the presence of ACM plus the FGF receptor antagonist, PD173074 (n = 16, Students t test, ***$p<0.001$). (C) Semi-quantitative RT-PCR of hNSCs differentiated for 30 days with ACM (BACM) or with ACM plus the FGF receptor antagonist (BACM+PD) for ChAT transcripts. Neg RT, RNA treated without reverse transcriptase (negative control). FSC, fetal spinal cord (positive control). GAPDH, glyceraldehyde-3-phosphate dehydrogenase (internal control).

DISCUSSION

The objective of this study was to examine whether astrocytes can enhance cholinergic neuron differentiation and survival from long-term cultured fetal human neural stem cells *in vitro*. Previously we reported that an *in vitro* cell priming procedure consisting of bFGF, heparin and laminin in addition to adhesive culture pushed hNSCs towards a cholinergic fate. In this study I found that adding astrocytes or astrocyte conditioned media did not increase overall neuronal differentiation but did improve long-term survival of differentiated hNSCs in general and specifically, cholinergic neuron survival. Such a neurotrophic effect is shown to be mediated through an anti-apoptotic mechanism, presumably by astrocyte-secreted bFGF.

Astrocytes play an important role in protecting mature rodent neurons from various insults, including energy impairment (Ohgoh et al., 2000), excitotoxicity (Mattson and Rychlik, 1990) and anoxia (Vibulsreth et al., 1987). Our finding of astrocyte-mediated maintenance of healthy human NSC-differentiated neurons for over 30 days indicates that astrocytes may also be crucial during development and maturation of human neurons from these stem cells.

Besides survival effects, I also tested whether rat astrocytes affected hNSC fate determination, particularly neuronal differentiation. I found that the addition of primary astrocytes derived from adult rat spinal cord or an immortalized astroglial cell line either through a direct contact co-culture or through secreted factors in conditioned medium did not influence the relative percentage of differentiating glia or neurons when compared to hNSC differentiation without astrocytes or ACM. This is in contrast to previous reports by several groups showing that astrocytes can direct or enhance *in vitro* neuronal differentiation of primate and murine embryonic stem (ES) cells (Nakayama et al., 2003) and rodent neural stem cells (Song et al., 2002a;Song et al., 2002b;Wagner et al., 1999). Of particular interest

are the findings of Nakayama et al. (2003) showing that conditioned medium from mouse fetal cerebral astrocytes induced the generation of a strictly neuronal population in mouse and primate ES cells. Specifically, ES cells cultured in the presence of ACM and growth factors for ten days showed enhanced expression of phenotypic markers for dopaminergic (TH), cholinergic (ChAT) and GABAergic (GAD) cell fates. Surprisingly, there was no astrocytic or oligodendrocytic differentiation within these cultures suggesting that ACM may actually inhibit glial cell fate in favor of neuronal cell fate. In a similar study, Wagner et al. (1999) was able to generate approximately 80% dopaminergic neurons from an immortalized mouse neural stem cell line (C17.2) exposed to ACM, but this had to be combined with a genetic modification of the NSCs through over-expression of a nuclear receptor (Nurr1). Song et al. (2002), also reported that astrocytes derived from neurogenic regions (adult or newborn hippocampus or newborn spinal cord) promoted neuronal differentiation of rodent adult neural stem cells (but limited to 20%) while astrocytes derived from non-neurogenic regions (adult spinal cord) promoted glial differentiation (Song et al., 2002). In contrast to the studies suggesting that ACM is neurogenic, Chang et al. (2003) found that rat E14.5 cortical neural stem cells cultured in the presence ACM from rat postnatal day 1 brain derived astrocytes differentiated mainly into astrocytes while cells cultured in the presence of neuronal derived conditioned medium (NCM) developed mainly into neurons (Chang et al., 2003). Further complicating the story, astrocytes derived from different regions (neurogenic vs. non-neurogenic) or different developing stages (e.g. neonatal vs. adult) may produce either neurogenic or gliogenic effects. Along this line, Barkho et al. (2006) recently reported, using microarray technology, that astrocytes found in different CNS regions at various developmental time points have very different gene expression patterns which may influence their effects on rat neural stem cell differentiation. More specifically, astrocytes from neurogenic vs. non-neurogenic regions have different levels

of neurogenesis promoting factors (such as IL-1β and IL-6) and neurogenesis-inhibiting factors (such as IGF-1 binding protein 6 and decorin) (Barkho et al., 2006). Admittedly, differences in species, cell type, developmental stage and *in vitro* culture condition of any given stem cell line may also affect differentiation. However, taken together, these studies suggest that various factors may mediate the astrocytic influence on stem cell fate determination.

Although astrocytes or ACM did not increase overall neuronal differentiation from long-term differentiated fetal hNSCs, we did find that cholinergic neurons were maintained in long-term differentiation (30 days) with ACM but were lost in cultures without ACM. While we did not look at other neuronal phenotypes besides ChAT, one can speculate that perhaps there might be differences in the specific neuronal subtypes maintained in the presence of astrocytes or astrocyte secreted factors.

Together this data suggest that astrocytes likely protect long-term differentiated hNSCs and cholinergic neurons through an anti-apoptotic mechanism. This is based on the nature of the long-term culture conditions, which do not impose a strong insult to cause necrosis but rather trigger programmed cell death, possibly due to lack of survival signals. Correspondingly, many apoptotic features were observed under long-term differentiation of hNSCs without astrocyte co-culture or conditioned medium, including cell shrinkage, membrane blebbing and DNA degradation detected by TUNEL. Astrocytes and their conditioned medium were found to block these apoptotic features and maintain hNSC in a healthy state. However, at this point we can not completely exclude a possible necrotic cell death occurring in long-term differentiated hNSCs without astrocytes, and the role of necrotic cell death remains to be determined.

Since both astrocytes and ACM protect long-term differentiated hNSCs, I hypothesized that astrocyte-secreted factors played an important role in their neuroprotective

effects. Astrocytes are known to secrete several important cell signaling molecules including bFGF (Hatten et al., 1988;Le and Esquenazi, 2002), ciliary neurotrophic factor (CNTF) (Rudge et al., 1992), nerve growth factor (NGF) (Yamakuni et al., 1987), glial derived neurotrophic factor (GDNF) (Schaar et al., 1993), laminin (Liesi et al., 1983) and cholesterol (Jung-Testas et al., 1989). I focused on bFGF as a first step to dissect possible mechanisms underlying astrocyte-mediated long-term survival of differentiated hNSCs. In this study I found that the astrocyte cell line CRL2005 secreted significant amounts of bFGF. Blockade of FGF receptor signaling using the specific receptor antagonist PD173074 inhibited the cell survival effect of ACM. Furthermore, ACM maintained ChAT expression in 30-day differentiated hNSCs while cells treated with ACM plus PD173074 did not show ChAT transcripts. These findings correlate well with a previous study by Gray and Patel (1992) who found that ACM strongly increased ChAT expression in rodent subcortical neurons, the effect of which was significantly reduced by application of an anti-bFGF antibody (Gray and Patel, 1992). Albrecht et al. (2002) also reported that astrocyte-mediated motoneuron survival was enhanced by stimulating secretion of bFGF from astrocytes (Albrecht et al., 2002). Taken together, astrocyte-provided bFGF seems to play an important role in maintaining cholinergic neurons both during normal development and after injury. However, given that many factors other than bFGF are also expressed and released from astrocytes, it is likely that any effects seen in co-culture or ACM studies on stem cell differentiation and survival are due to the presence or absence of several secreted factors. Further studies are required to explore other astroglial factors that are involved in mediating long-term survival of differentiated hNSCs.

In summary, astrocytes and astrocyte conditioned media enhanced the overall survival of long-term differentiated human neural stem cells. While not increasing neuronal differentiation, astrocytes had a particular protective effect on hNSC-derived cholinergic

neurons. Such neuroprotective effects are mediated through an anti-apoptotic mechanism and at least partially through astrocyte-secreted bFGF. Further studies to understand the interaction between astrocytes and hNSCs may be useful in order to establish a human motoneuron *in vitro* model, which could be beneficial for drug screening towards development of treatments for motoneuron diseases.

CHAPTER 5: CONCLUSIONS, CAVEATS AND FUTURE DIRECTIONS

The overall objective of this study was to examine the roles of exogenous bFGF and astrocytes on human neural stem cell differentiation and survival. In the previously described experiments I found that addition of exogenous bFGF induced hNSCs towards the motoneuron lineage *in vitro* expressing transcription factors associated with that phenotype, including OLIG2, Ngn2, Islet1 and Hb9. Furthermore, I found that Hb9 induction in hNSCs was both bFGF dose and time dependent and that blockade of bFGF signaling reduced but did not completely eliminate Hb9 expression. bFGF treatment did not significantly enhance cell survival when compared to other growth factors (EGF and LIF) under our *in vitro* conditions and bFGF dependent proliferation was not required for Hb9 expression. Finally, treatment of hNSCs with other growth factors (EGF and LIF) did not induce Hb9 expression; actually addition of EGF and/or LIF following bFGF treatment down-regulated Hb9 expression, suggesting that bFGF is a required factor for not only induction but also for maintenance of Hb9 transcript levels.

In the second set of studies, I examined the role of astrocytes and astrocytic factors, including astrocytic-secreted bFGF, on hNSC survival and neuronal differentiation. In co-culture studies I found that the addition of astrocytes or astrocyte secreted factors (in astrocyte conditioned media (ACM)) enhanced overall cell viability and decreased overall cell death and in particular, decreased apoptotic cell death. Interestingly, addition of astrocytes or ACM did not affect the overall neuron:glia ratio in long-term differentiated hNSCs. However, long-term survival (>30 days) of a specific neuronal subtype, cholinergic expressing cells, was maintained in ACM while that phenotype was lost in cultures differentiated without astrocytic factors. These data suggested that secreted factors from astrocytes promoted survival of a specific subtype of neurons, cholinergic neurons. Finally,

I found that addition of the FGF-receptor antagonist, PD173074, eliminated cholinergic expressing cells from long-term cultured hNSCs suggesting a role for FGF in cholinergic differentiation and survival of hNSCs. To follow up on that finding, I performed a bFGF ELISA using ACM and found that astrocytes secreted significant levels of bFGF which was maintained in ACM and potentially could be involved in cholinergic long-term cell survival.

Species and Cell Type Differences in Responses to Growth Factor Stimulation and Transcription Factor Activity

Many different paradigms exist for the culture of stem cells *in vitro*. For example, cells may be grown as a monolayer under adherent culture conditions or as free floating neurospheres, with various single growth factors (EGF, LIF or bFGF being the most common) or in combinations of those factors (i.e. EGF+LIF, EGF+bFGF+LIF). Cell handling can also radically affect the differentiation potential. Cumulatively, the variability in culture conditions coupled with individual handling differences can make data interpretation challenging.

Our current understanding of motoneuron development derives mainly from classical studies conducted in the chick embryo. Using this technology, scientists have identified many transcription factors and inductive agents (i.e. SHH and RA) necessary for proper MN development. Many of these factors appear to be essential in rodent MN development as well, suggesting that there may be few interspecies differences. For instance, both chick and rodent MN developmental studies indicate that MN differentiation requires concurrent NGN2 and OLIG2 activity (Mizuguchi et al., 2001;Lee et al., 2005). This line of logic has been further extended to human and rodent stem cell studies. For example, both hES and mES cells require SHH and RA for efficient MN differentiation (Wichterle et al., 2002;Li et al., 2005).

Despite the similarities, however, it is likely that inter-species differences also exist, especially in the spatiotemporal regulation of transcription factor activity. For example, rodent or human homologues to the chick essential MN transcription factor MNR2 have yet to be identified. It has been speculated, however, that another pre-MN transcription factor, OLIG2, may substitute for MNR2 in these species (Novitch et al., 2001;Briscoe and Novitch, 2007). OLIG2 shares many of the same characteristics of MNR2 in chick, including activation of the MN determinant, ISLET 1/2 (Novitch et al., 2001). The MN determinant, HB9, is also expressed only post-mitotically in chick while in rodent it appears prior to the final cellular division (Thaler et al., 1999). These findings together suggest that there may be some inherent species specific differences in MN development. In humans, primarily due to ethical issues, there has been little progress in MN developmental studies. However, with the derivation of human derived MNs from immortalized human spinal cords (Li et al., 2000), embryonic stem cells (Li et al., 2005) and neural stem cells (Ren et al., 2006;Wu et al., 2002), it may be possible now to better understand human MN development as well.

Sonic Hedgehog Independent Differentiation of hNSCs into Motoneurons

Because MNs and oligodendrocytes share a common developmental ancestor, we can glean insights into MN development from the knowledge surrounding the development of oligodendrocytes and vice verse. Interestingly, during oligodendrocyte development two separate developmental pathways have been described; one in which SHH is essential and one in which bFGF can substitute for SHH (i.e SHH independent). In early studies of the pMN and oligodendrocytic transcription factor OLIG2, expression of OLIG2 was believed to be regulated by SHH indirectly and both SHH and OLIG2 were thought to be essential components of oligodendrocytic development (Lu et al., 2000). Recently, however, it was found that bFGF can substitute for SHH during oligodendrocyte development and

thus that a SHH-independent pathway also exists for oligodendrocyte formation. In order to demonstrate this finding, in a series of *in vivo* studies, Naruse et al. (2006) micro-injected bFGF into the forebrain of developing rodents (E13) intra-utero and looked at the development of oligodendrocytic precursors (OLPs). At E15.5, they found enhanced numbers of OLP's (labeled with OLIG2 and PDGFR-α) in the bFGF treated group as compared to controls. Furthermore, induction of SHH receptors, Patched (PTC) and Smoothened (SMO), were not seen in bFGF-treated animals. When explants were taken from these embryos and cultured for several days in the absence of growth factor, mature oligodendrocytes also developed from both groups. SHH receptors were not induced during explant culture, thus it appeared that a SHH independent but bFGF dependent pathway also existed for oligodendrocyte differentiation. Confirming this finding, addition of a SHH antagonist, cyclopamine, during micro-injection of bFGF (i.e. bFGF+Cyclopamine) did not down-regulate OLP formation suggesting that exogenous bFGF could substitute for SHH within the developing rodent brain (Naruse et al., 2006).

Supporting this bFGF effect, it was previously shown in neurosphere cultures from rodent E14 spinal cord derived neural stem cells that bFGF could also induce OLP formation in the absence of exogenous SHH as well as in conjunction with the SHH antagonist, cyclopamine (Chandran et al., 2003). These findings were further corroborated in NSCs derived from SHH knockout mice, in which again bFGF induced OLP formation in the absence of both exogenous and endogenous SHH (Chandran et al., 2003).

Further substantiating a role for SHH independent oligodendrocyte formation is the work of Cai et al. (2005) who demonstrated a second wave of oligodendrocyte formation from **dorsal** spinal cord derived cells, which occurred around E14.5 (~2d after the ventral wave from pMN cells). Since dorsal cells typically do not receive much SHH signaling, it is not surprising that this second wave of oligodendrocytic differentiation was SHH-independent. Furthermore, in this study researchers also used mES cells derived from

SHH receptor knockout mice ($SMO^{-/-}$) to demonstrate that *in vitro,* SHH was dispensable for oligodendrocyte formation. This was shown through immunostaining for the oligodendrocytic marker, GalC, which was found to be similarly expressed between $SMO^{-/-}$ and WT cultures. Although the exact signals inducing dorsal oligodendrocyte formation are currently unknown, the authors speculate that FGFs, and particularly bFGF, might be important modulators (Cai et al., 2005). Thus, during oligodendrocyte development, at least two separate developmental pathways exist: One in which SHH is an essential component and the other, in which bFGF can substitute for SHH.

Because of the similarities in development between oligodendrocytes and MN's, I speculated that a SHH independent pathway may also exist for human MN development. Contrary to this notion, others have shown that sonic hedgehog (SHH) is an essential component of rodent MN development *in vivo* (Chiang et al., 1996;Tanabe et al., 1995) and in both rodent and human embryonic stem cells *in vitro* (Wichterle et al., 2002;Li et al., 2005). In contrast to these findings, however, Novitch et al. (2003) found that concurrent application of FGF and retinoids could substitute for SHH activity in neural precursor cells allowing for MN differentiation *in vitro* (Novitch et al., 2003). Similarly, in our hands, the development of cholinergic motoneurons from human neural stem cells *in vitro* does not require exogenous sonic hedgehog application. As such, studies in our lab indicate that FHL priming with or without SHH induces approximately 28% of cholinergic MNs *in vitro* and 50-60% after transplantation *in vivo* (SFHL ≈ FHL) (Wu et al., 2002;Tarasenko et al., 2004). Trial experiments in our lab, however, have shown that ELL, FHL and undifferentiated (spheres) cultured using our methods do express detectable mRNA levels of SHH receptor, PTC, and downstream SHH effectors, GLI 1 and 2 (Ojeda and Wu, unpublished observations). These data suggest that perhaps long-term expanded hNSCs produce and secrete endogenous SHH or have an activated SHH pathway, which might act in conjunction with bFGF, heparin and laminin to push hNSCs towards a MN cell fate.

Levels of SHH following transplantation are unknown, however, one can speculate that perhaps *in vivo,* FHL primed hNSCs which express SHH receptors might be influenced by endogenously produced SHH within the animal. Supporting this hypothesis is the finding of Traiffort et al. (1999) that adult rats produce significant levels of SHH within both the brain and spinal cord (Traiffort et al., 1999). Furthermore, SHH could potentially also be produced by hNSCs in culture and could either be secreted or act via an autocrine/non-secretory pathway to activate SHH signaling *in vitro*. SHH protein production by hNSCs, however, has yet to be verified and is one of the future focuses of this laboratory.

An alternative to SHH production from hNSCs is that bFGF might substitute for SHH as was the case for the development of oligodendrocytes (Chandran et al., 2003;Cai et al., 2005;Naruse et al., 2006). However, a definitive role for SHH signaling during FHL priming and in MN development from hNSCs has yet to be thoroughly investigated.

FGF vs EGF and Receptor Tyrosine Kinase Mediated Cell Signaling Pathways

In this study I demonstrated that both EGF, LIF and laminin (ELL) and bFGF, heparin and laminin (FHL) primed hNSCs activate three MAP kinase proteins (ERK1, ERK2 and JNK) at different levels. In particular, hNSCs primed with FHL for 4 days showed a lower level of activation of these MAP kinase proteins than did cells primed with ELL for 4 days or cells which were unprimed (neurospheres). Interestingly, addition of the FGF receptor antagonist (PD173074) to FHL-primed cells did decrease Erk1/2 protein levels but had little effect on JNK activated protein amount detectable via western blot as compared to FHL primed cells alone. Assuming that bFGF induced JNK or ERK1/2 were involved in Hb9 activation, one would expect that both ELL and FHL-PD treated cells, which both show low or no expression of Hb9 mRNA, would show similar levels of these proteins. What we found instead was that ELL primed groups showed high levels of both phosphorylated ERK1/2 and phosphorylated JNK while FHL-PD primed groups

showed low to no activity and FHL-primed showed intermediate levels of activity. Thus, ELL and FHL-PD treated cells exhibited opposite effects on JNK and Erk1/2 activated protein amounts. There are several explanations for this unexpected finding and ways we could further understand the involved cell signaling pathways better in the future.

First and foremost, we must remember that in utilizing these types of molecular techniques that we are looking at one snapshot of the cellular milieu at one time point. In order to really delve into the cell signaling pathways involved in Hb9 induction in response to bFGF treatment, we need to examine multiple time points after plating into the various treatment groups. In this case, a secondary assay, such as the SEAP assay (mentioned below) might be highly beneficial.

Secondly, using western blot analyses we are examining a whole population of cells, not just the Hb9 positive cells. Thus, any changes in protein expression within specific cell populations may be difficult to discern using this technique. In order to circumvent this, in the future we could transfect hNSCs with a GFP reporter gene driven by a specific Hb9 promoter, sort the cells based on GFP expression and then perform protein analysis in GFP^+ vs GFP^- cell populations. Potential problems with this approach include difficulty in efficiently transfecting hNSCs while maintaining their cellular potency, and that the addition of GFP to the cells may change their ability to respond to FHL-priming. In regards to GFP expression, we currently transduce hNSCs to express GFP via a recombinant adeno-assicated viral vector (AAV) technology for cell tracing after transplantation. Yet even with this relatively mild treatment, cells do not respond to growth factor treatment and proliferate well long-term *in vitro* (Ojeda and Wu, unpublished findings). Thus maintenance of long term expression of GFP may also be potentially difficult. Furthermore, FACS analysis can be harsh on the cells and cell viability following cell sorting may be low. In particular the cells we are most interested in (neurons) are unlikely to withstand this treatment well as we have shown previously that simple cell dissociation and cell plating following dissociation

kills many neurons (Gao and Wu unpublished findings). At this time, other members of the lab are currently investigating the possibility of establishing a Hb9 promoter-controlled GFP-expressing hNSC line and this technology will prove highly beneficial if it becomes feasible in the future. Finally, another alternative would be to perform immunostaining for Hb9 in conjunction with staining for activated ERK or JNK proteins (double labeling) at different time points during FHL priming. However, due to the difficulty in utilizing the single commercially available Hb9 antibody, it is likely that we would need to generate our own antibody which could be cost prohibitive.

Cellular location of each protein of interest could also yield interesting insight into hNSC differentiation. In regards to this, it may be beneficial to look at the compartmentalization of each protein (i.e. cytoplasmic vs mitochondrial vs nuclear). To simply state that a protein is phosphorylated does not mean that it is in a cellular compartment where it may be able to perform its function. Compartmentalization within cell organelles is one way in which cells regulate signaling effects and could potentially be involved in FHL-induced Hb9 induction in hNSCs. In order to further understand if this is occurring in our system, immunostaining coupled with fluorescence microscopy for phosphorylated MAP kinase members could be highly beneficial.

Specificity of FGF and EGF Cell Signaling Pathways

EGF and FGF activate a very similar complement of cell signaling proteins. This brings about the question as to what governs the differences in cell signaling behavior if the same intracellular signaling pathways are evoked with different growth factor treatments? Or how about in the case of the converse of this (i.e. that treatment with the same factor induces different effects in different cell types?) For example, stimulation of FGFR1 in fibroblasts leads to cell proliferation while stimulation of FGFR1 in neuronal cells induces cell survival and differentiation (Schlessinger, 2000). How can these very different effects

be induced by treatment with the same molecule coupled with activation of the same receptor? One explanation is that different cell types express different sets of cell-type specific effector proteins that mediate their differential effects. Because receptor tyrosine kinases (RTKs), including EGF and FGF, recruit many different molecules to the RTK-receptor complex, it is quite feasible to imagine that recruitment may be more efficient in one cell type versus another, or alternatively that recruited molecules may be more readily available in certain cell types than in others. These types of subtle changes would likely be undetectable using western blot analysis. It has also been hypothesized that different cell types might express antagonistic molecules which could compete for protein binding and thereby inhibit activation of certain effector molecules as well. In this case, detection of specific proteins would not indicate that they were functional. Furthermore, it is highly likely that signaling pathways are intertwined in a very complex pattern and that activity of a single molecule may regulate activities (both inhibitory and stimulatory) of many other molecules involved in its own, as well as other seemingly unrelated pathways.

Finally, it is worthy to note that cellular differentiation is a highly regulated and complex process. To believe that only one cell signaling pathway, much less only one effector molecule within that pathway, is involved in such a complicated biological process is unrealistic. Indeed, an entire network of cell signaling molecules, all differentially regulated and probably compartmentalized, are likely involved in this very complex cellular phenomenon. Furthermore, it is highly probable that critical cell signaling pathways are also prone to regulation via multiple mechanisms. This theme of cellular redundancy has been shown to be important in other cell models. For example, in the EGF system it has been demonstrated that when EGFR is activated, it recruits the adaptor protein Grb2 directly and indirectly via two cell signaling molecules (Shc and Gab1). Thus, in EGFR mutatnts, despite the inability of EGFR to induce Grb2 binding directly, EGFR mutants are still capable of recruiting Grb2 indirectly resulting in activation of the MAP kinase

signaling cascade (Schlessinger, 2000). Therefore, despite knocking out one important cellular function (i.e Grb2 binding), the cell is still able to utilize a secondary mechanism to compensate for that loss.

In regards to hNSC differentiation, it is quite possible that compensatory mechanisms are also at play in the complex differentiation of these cells into Hb9 expressing neurons. For example, blockade of FGF-signaling via treatment with the FGF- receptor antagonist, PD173074, does not fully block bFGF mediated differentiation into an Hb9 expressing phenotype. However, endogenous and non-secreted bFGF, which could act at the nuclear level, would not be influenced by PD173074 treatment and indeed, blockade with PD173074 reduces Hb9 transcript levels, but does not completely eliminate Hb9 expression. Thus the possibility remains that endogenous bFGF may also be playing a role in bFGF induced Hb9 expression. In addition to this, treatment of FHL-primed hNSCs with a neutralizing antibody against bFGF also did not completely eliminate Hb9 transcript. Together these two findings suggest a role for nuclear or non-secreted bFGF, or for other compensatory mechanisms, during FGF signaling blockade in the induction of an Hb9 phenotype from FHL-primed hNSCs. To further understand the importance of endogenous bFGF and/or nuclear localized bFGF on hNSC differentiation, one could utilize siRNA technology against bFGF, and/or apply exogenous mutant bFGF protein which does not activate nuclear signaling components (Sorensen et al., 2006). Utilization of these techniques would further our understanding of the molecular mechanism surrounding bFGF-induced Hb9 expression in hNSCs.

hNSCs and Astrocytes: Effects of Astrocytes and Astrocyte Secreted Factors on hNSC Differentiation

In my second set of studies, I found that co-culture of hNSCs with astrocytes improved hNSC long term survival, particularly survival of cholinergic expressing cells. I further demonstrated that this enhancement in cell survival was through an anti-apoptotic

mechanism mediated by astrocyte secreted factors and suggest that one factor, bFGF, preserved in ACM may be involved in this long term neuronal survival.

My first question for this study was whether astrocytes could enhance cholinergic neuronal differentiation and survival from fetal human neural stem cells *in vitro*. Using either indirect or direct contact co-culture studies with astrocytes or through the application of astrocyte conditioned media, I demonstrated that the addition of astrocytes to FHL-primed hNSCs did not increase overall neuronal or glial differentiation. Rather, after long term (30d) differentiation, I saw a similar percentage of glia and beta III tubulin positive neurons in groups treated with astrocyte secreted factors and those treated without (~40% neurons, 60% glia). These findings were in contrast to previous reports by several groups showing that astrocytes can direct or enhance *in vitro* neuronal differentiation of primate and murine embryonic stem (ES) cells (Nakayama et al., 2003) and rodent neural stem cells (Song et al., 2002a;Song et al., 2002b;Wagner et al., 1999).

In particular, Song et al. (2002) reported that astrocytes derived from neurogenic regions (adult or newborn hippocampus or newborn spinal cord) promoted neuronal differentiation of rodent adult neural stem cells (but limited to 20%) while astrocytes derived from non-neurogenic regions (adult spinal cord) promoted glial differentiation. However, in other studies, Chang et al. (2003) demonstrated that rat E14.5 cortical neural stem cells cultured in the presence ACM from rat postnatal day 1 brain astrocytes differentiated mainly into astrocytes while cells cultured in the presence of neuronal derived conditioned medium (NCM) developed mainly into neurons (Chang et al., 2003). Taken together, these studies suggest that various factors affect the final outcome of astrocytic influence on stem cell fate determination: i.e. either neurogenesis or gliogenesis. From the perspective of stem cells, this may be influenced by differences in species, cell type, developmental stage and *in vitro* culture condition of any given stem cell line. Under our *in vitro* conditions I did not test the ability of astrocytes from various regions or ages on hNSCs differentiation. Thus, in light

of other researchers findings, it would be highly beneficial and interesting to know if there are differential effects depending upon region of derivation (i.e. brain vs spinal cord for example), age of animal utilized (i.e. embryonic vs. neonate vs. adult) and culture method chosen (i.e. direct vs. indirect vs. utilization of conditioned media).

Astrocytes Protect Long-Term Differentiated hNSCs

Given that the relative percentage of total neurons was unchanged between hNSCs co-cultured with astrocyte conditioned media or without, I next wanted to assess whether specific subtypes of neurons may be influenced by the presence of astrocytes. In light of this question, I examined cholinergic neuronal differentiation and found that at early time points, cholinergic cells were present in both culture conditions (i.e. hNSCs ± astrocytes), but at late time points (d30), cholinergic cells were only found in groups differentiated in the presence of ACM. This suggested that astrocytes and/or astrocyte secreted factors aided in the survival/maintenance of cholinergic expressing neurons under long term *in vitro* culture conditions. This type of neurotrophic effect is not surprising since astrocytes are known to aid in the survival of many other cell types and to aid in cell recovery after injury. For example, astrocytes are known to play an important role in protecting mature rodent neurons from various insults, including excitotoxicity (Mattson and Rychlik, 1990), energy impairment (Ohgoh et al., 2000) and anoxia (Vibulsreth et al., 1987). This finding of astrocyte-mediated maintenance of healthy human NSC-differentiated cholinergic neurons for over 30 days indicates that astrocytes may also be crucial during development and maturation of human neurons. Granted, I did not examine hNSC differentiation into other neuronal subtypes besides ChAT expressing cells, thus it is possible that astrocytes or astrocyte secreted factors may also influence neuronal differentiation into other neuronal subtypes as well. To further elucidate astrocytic effects on hNSC differentiation, it would be beneficial to perform immunostaining for neuronal subtypes such as GABA, Glutamatergic

or Dopaminergic neurons in hNSCs differentiated in the presence or absence of astrocytic factors.

Astrocytes and hNSC Survival: the Potential Role of Astrocyte Secreted bFGF

Throughout these studies I also noticed that although the relative percentages of glia and neurons did not differ in the presence or absence of astrocytes, that the overall cell number per microscopic field was greater in the ACM group as compared to the non-ACM group. This suggested that either hNSCs differentiated in the presence of ACM were proliferating to a greater degree or that relatively more of those cells were surviving during long term *in vitro* differentiation. To test this hypothesis, I next examined cell survival and proliferation in the presence or absence of ACM. Cell survival was measured using several parameters including WST-1 cell viability assays, TUNEL immunostaining and LDH release. Results from WST-1 experiments indicated that cell viability was improved at all time points tested with the addition of ACM. Furthermore, BrdU pulse labeling confirmed that this effect was due to an enhancement in cell survival and not due to cell proliferation as proliferation was not significantly enhanced in ACM treated groups. Cell survival was further confirmed as a mediator of enhanced cell number verified through decreases in both TUNEL staining and LDH release in hNSC cultures differentiated in the presence of astrocytes or astrocyte secreted factors.

Because both ACM and direct cultures with astrocytes protected long-term differentiated hNSCs, I next hypothesized that astrocyte-secreted factors might play a neuroprotective role in the long-term survival of hNSC-derived cholinergic neurons. This role would not be unexpected since astrocytes are known to secrete several important cell signaling molecules including bFGF (Sensenbrenner et al., 1987;Hatten et al., 1988), ciliary neurotrophic factor (CNTF) (Rudge et al., 1992), nerve growth factor (NGF) (Yamakuni et al., 1987), glial derived neurotrophic factor (GDNF) (Schaar et al., 1993), laminin (Liesi et

al., 1983) and cholesterol (Pfrieger and Barres, 1997). Because our cells have previously been shown to respond to bFGF which is a known neurotrophic factor (Wu et al., 2002; Tarasenko et al., 2004), I focused on bFGF as a first step to dissect possible mechanisms underlying astrocyte-mediated long-term survival of differentiated hNSCs.

Using a bFGF ELISA, I found that astrocyte conditioned media contained significant amounts of bFGF which could be preserved even after freeze-thaw and further refrigeration of ACM. Furthermore, I found that this secreted bFGF may be important for long-term cholinergic neuronal survival because blockade of FGF-receptor signaling inhibited the cell survival effect of ACM. Furthermore, I found that treatment with ACM alone maintained ChAT and Islet-1 expression in 30-day differentiated hNSCs while cells treated with ACM plus FGF receptor antagonists did not show ChAT nor Islet-1 transcripts. These findings correlate well with a previous study by Gray and Patel (1992) who found that ACM strongly increased ChAT expression in rodent subcortical neurons, the effect of which was significantly reduced by anti-bFGF antibody treatment (Gray and Patel, 1992). Along these lines, Albrecht et al. (2002) also reported that CNTF enhanced astrocyte-mediated motoneuron survival by stimulating secretion of bFGF from astrocytes (Albrecht et al., 2002). Taken together, astrocyte-provided bFGF seems to play an important role in maintaining cholinergic neurons both during normal development and after injury. However, given that many factors other than bFGF are also expressed and released from astrocytes, it is likely that any effects seen in co-culture or ACM studies on stem cell differentiation and survival are due to the presence or absence of several secreted factors, one of which is likely to be bFGF.

In light of these findings, the hypothesis that astrocytic secreted bFGF might mediate hNSC derived cholinergic neuron long term cell survival could be strengthened by application of low levels of exogenous bFGF (i.e. in the range of ACM ~100pg/ml) over the 30d differentiation period followed by immunostaining for choline acetyltransferase

and/or collection of RNA for RT-PCR for this enzyme. The number of ChAT+ cells and/or level of ChAT transcript could then be compared between cultures with ACM, with basal media+low level bFGF stimulation and those without any treatment. However, as mentioned above, it is unlikely that bFGF is the only secreted factor involved in long term hNSC derived cholinergic cell survival, thus I would expect that addition of bFGF alone would not suffice to prevent cholinergic cell death. In order to determine the factors present in ACM that might also be involved in cholinergic long-term survival, it would be interesting to subject ACM to an entire panel of ELISAs against many growth and neurotrophic factors. This could lead insight into potential factors necessary to direct hNSC differentiation and long term survival of cholinergic neurons *in vitro.*

In summary, astrocytes and astrocyte conditioned media enhanced the overall survival of long-term differentiated human neural stem cells. While not increasing neuronal differentiation, astrocytes demonstrated a protective effect on hNSC-derived cholinergic neurons mediated through an anti-apoptotic mechanism and at least partially through astrocyte-secreted bFGF. Long term *in vitro* cholinergic neuronal survival is an important finding which may be beneficial for the development of models to test treatments for various neurological impairments which affect cholinergic expressing cells including Alzheimer's Disease or those which affect a subset of cholinergic cells, (i.e. cholinergic motoneurons) such as Lou Gehrigs Disease.

Caveats: Hb9 Induction via FHL Treatment

In the first study I demonstrate motoneuron differentiation in response to FHL-priming via induction of the homeodomain factor, Hb9 in hNSCs. From these analyses, I found that Hb9 mRNA and protein were up-regulated after 4d of FHL priming of hNSCs. Interestingly, hNSCs treated with only heparin and laminin alone (i.e. no exogenous bFGF) showed some Hb9 induction (Figure 3.7) as compared to hNSCs grown as neurospheres or

to ELL primed cells. However, because hNSCs up-regulate Hb9 in a dose responsive manner to bFGF treatment, this suggested that hNSCs might secrete low levels of endogenous bFGF. This hypothesis is not unexpected as astrocytic cells are known producers of bFGF (Sensenbrenner et al., 1987;Hatten et al., 1988) and astrocytes are a known cell type which differentiate from hNSCs after FHL-priming (Figure 3.2). Furthermore, in my second study, I found that astrocytes secreted pictogram levels of bFGF which was maintained in conditioned media (Figure 4.8). In preliminary experiments I also found that hNSCs expressed bFGF transcript which suggested that the cells could potentially secrete the growth factor as well and/or utilize it in an autocrine fashion (Jordan and Wu, unpublished observations). In order to test the hypothesis that bFGF is secreted by hNSCs following FHL priming, conditioned media from primed hNSCs could be utilized for a bFGF ELISA. One potential problem with this assay would be that since bFGF is "sticky" it may be difficult to remove exogenously applied bFGF completely from the culture prior to collection for ELISA and thus detection of secreted bFGF versus exogenously added bFGF (i.e. in FHL priming) could be problematic. Furthermore, any bFGF which was not secreted externally, but rather remained internally within the cell would not be detectable using this method. Finally, cells may pick up secreted bFGF quickly from the CM, thus accurate detection of bFGF levels might potentially be a problem as well.

For the majority of the first study, I utilized semi-quantitative and quantitative RT-PCR for Hb9 transcript following FHL priming. Although I present sufficient evidence to show that bFGF is an inductive factor for Hb9 expression in hNSCs, in order to further validate that bFGF is acting as an inductive signal and that Hb9 is not only present but is active, utilization of a secondary assay such as a SEAP (secreted alkaline phosphatase) transcription factor activation assay would be beneficial. In particular, this assay could utilize SEAP as a reporter gene under the control of the Hb9 promoter. Thus, when activated, Hb9-driven SEAP would be secreted, the amount of which could be directly correlated

to transcription factor (such as Hb9) activity. Furthermore, SEAP is easily assayed from conditioned media, following which cells can be harvested for protein or RNA analysis. This method, therefore, would allow for simultaneous analysis of protein or RNA levels and transcription factor activity within the same samples. Utilization of such a system would also be highly useful for checking transcription factor activity (especially at low levels) at different time points in culture as well as identifying other factors which might be important in activation of the Hb9 promoter. Although I did examine a time course of Hb9 activation in FHL-primed hNSCs (48hrs to 96 hrs after FHL priming, Figure 3.7), a SEAP assay would allow for a more sensitive detection of transcription factor activity and might lead insight into earlier events (prior to 48 hrs) that were undetectable using RT-PCR.

Reference List

1. Motoneuron disease statistics. http://www.mndassociation.org/life_with_mnd/what_is_mnd/risk_of.html. Motoneuron Disease Association. 12-15-2006.

2. NIH Stem Cell Primer. http://stemcells.nih.gov/info/basics .NIH website. 2-14-2007.

3. Albrecht PJ, Dahl JP, Stoltzfus OK, Levenson R, Levison SW (2002) Ciliary neurotrophic factor activates spinal cord astrocytes, stimulating their production and release of fibroblast growth factor-2, to increase motor neuron survival. Exp Neurol 173: 46-62.

4. Allan DW, Thor S (2003) Together at last: bHLH and LIM-HD regulators cooperate to specify motor neurons. Neuron 38: 675-677.

5. Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA, Itskovitz-Eldor J, Thomson JA (2000) Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol 227: 271-278.

6. Arakawa Y, Sendtner M, Thoenen H (1990) Survival effect of ciliary neurotrophic factor (CNTF) on chick embryonic motoneurons in culture: comparison with other neurotrophic factors and cytokines. J Neurosci 10: 3507-3515.

7. Arber S, Han B, Mendelsohn M, Smith M, Jessell TM, Sockanathan S (1999) Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. Neuron 23: 659-674.

8. Bansal R, Magge S, Winkler S (2003) Specific inhibitor of FGF receptor signaling: FGF-2-mediated effects on proliferation, differentiation, and MAPK activation are inhibited by PD173074 in oligodendrocyte-lineage cells. J Neurosci Res 74: 486-493.

9. Barkho BZ, Song H, Aimone JB, Smrt RD, Kuwabara T, Nakashima K, Gage FH, Zhao X (2006) Identification of astrocyte-expressed factors that modulate neural stem/progenitor cell differentiation. Stem Cells Dev 15: 407-421.

10. Bean AJ, Elde R, Cao YH, Oellig C, Tamminga C, Goldstein M, Pettersson RF, Hokfelt T (1991) Expression of acidic and basic fibroblast growth factors in the substantia nigra of rat, monkey, and human. Proc Natl Acad Sci U S A 88: 10237-10241.

11. Bentz K, Molcanyi M, Hess S, Schneider A, Hescheler J, Neugebauer E, Schaefer U (2006) Neural differentiation of embryonic stem cells is induced by signalling from non-neural niche cells. Cell Physiol Biochem 18: 275-286.

12. Blottner D, Herdegen T (1998) Neuroprotective fibroblast growth factor type-2 down-regulates the c-Jun transcription factor in axotomized sympathetic preganglionic neurons of adult rat. Neuroscience 82: 283-292.

13. Blume-Jensen P, Hunter T (2001) Oncogenic kinase signalling. Nature 411: 355-365.

14. Bottenstein JE, Sato GH (1979) Growth of a rat neuroblastoma cell line in serum-free supplemented medium. Proc Natl Acad Sci U S A 76: 514-517.

15. Briscoe J, Ericson J (1999) The specification of neuronal identity by graded Sonic Hedgehog signalling. Semin Cell Dev Biol 10: 353-362.

16. Briscoe J, Novitch BG (2007) Regulatory pathways linking progenitor patterning, cell fates and neurogenesis in the ventral neural tube. Philos Trans R Soc Lond B Biol Sci.E Pub. February 5, 2007, 1-15.

17. Briscoe J, Pierani A, Jessell TM, Ericson J (2000) A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell 101: 435-445.

18. Buc-Caron MH (1995) Neuroepithelial progenitor cells explanted from human fetal brain proliferate and differentiate in vitro. Neurobiol Dis 2: 37-47.

19. Cai J, Qi Y, Hu X, Tan M, Liu Z, Zhang J, Li Q, Sander M, Qiu M (2005) Generation of oligodendrocyte precursor cells from mouse dorsal spinal cord independent of Nkx6 regulation and Shh signaling. Neuron 45: 41-53.

20. Caldwell MA, Garcion E, terBorg MG, He X, Svendsen CN (2004) Heparin stabilizes FGF-2 and modulates striatal precursor cell behavior in response to EGF. Exp Neurol 188: 408-420.

21. Caldwell MA, He XL, Wilkie N, Pollack S, Marshall G, Wafford KA, Svendsen CN (2001) Growth factors regulate the survival and fate of cells derived from human neurospheres. Nature Biotechnology 19: 475-479.

22. Caldwell MA, Svendsen CN (1998) Heparin, but not other proteoglycans potentiates the mitogenic effects of FGF-2 on mesencephalic precursor cells. Exp Neurol 152: 1-10.

23. Calza L, Giuliani A, Fernandez M, Pirondi S, D'Intino G, Aloe L, Giardino L (2003) Neural stem cells and cholinergic neurons: regulation by immunolesion and treatment with mitogens, retinoic acid, and nerve growth factor. Proc Natl Acad Sci U S A 100: 7325-7330.

24. Cao QL, Howard RM, Dennison JB, Whittemore SR (2002) Differentiation of engrafted neuronal-restricted precursor cells is inhibited in the traumatically injured spinal cord. Exp Neurol 177: 349-359.

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

26. Chandran S, Kato H, Gerreli D, Compston A, Svendsen CN, Allen ND (2003) FGF-dependent generation of oligodendrocytes by a hedgehog-independent pathway. Development 130: 6599-6609.

27. Chang MY, Son H, Lee YS, Lee SH (2003) Neurons and astrocytes secrete factors that cause stem cells to differentiate into neurons and astrocytes, respectively. Mol Cell Neurosci 23: 414-426.

28. Chen F, Guo Q, Yang Y, Song H, Wen T (2005) Inhibition of AF116909 gene expression enhances the differentiation of neural stem cells. Neurol Res 27: 557-561.

29. Cheng B, Mattson MP (1991) NGF and bFGF protect rat hippocampal and human cortical neurons against hypoglycemic damage by stabilizing calcium homeostasis. Neuron 7: 1031-1041.

30. Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA (1996) Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature 383: 407-413.

31. Chuang RY, Chuang LF (1976) Inhibition of RNA polymerase as a possible anti-leukaemic action of cytosine arabinoside. Nature 260: 549-550.

32. Ciccolini F, Svendsen CN (1998) Fibroblast growth factor 2 (FGF-2) promotes acquisition of epidermal growth factor (EGF) responsiveness in mouse striatal precursor cells: identification of neural precursors responding to both EGF and FGF-2. J Neurosci 18: 7869-7880.

33. Claus P, Doring F, Gringel S, Muller-Ostermeyer F, Fuhlrott J, Kraft T, Grothe C (2003) Differential intranuclear localization of fibroblast growth factor-2 isoforms and specific interaction with the survival of motoneuron protein. J Biol Chem 278: 479-485.

34. Dahlstrand J, Lardelli M, Lendahl U (1995) Nestin mRNA expression correlates with the central nervous system progenitor cell state in many, but not all, regions of developing central nervous system. Brain Res Dev Brain Res 84: 109-129.

35. Dasen JS, Liu JP, Jessell TM (2003) Motor neuron columnar fate imposed by sequential phases of Hox-c activity. Nature 425: 926-933.

36. Deguchi Y, Kehrl JH (1991) Nucleotide sequence of a novel diverged human homeobox gene encodes a DNA binding protein. Nucleic Acids Res 19: 3742.

37. Delrieu I (2000) The high molecular weight isoforms of basic fibroblast growth factor (FGF-2): an insight into an intracrine mechanism. FEBS Lett 468: 6-10.

38. Dono R (2003) Fibroblast growth factors as regulators of central nervous system development and function. Am J Physiol Regul Integr Comp Physiol 284: R867-R881.

39. Dono R, Texido G, Dussel R, Ehmke H, Zeller R (1998) Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. EMBO J 17: 4213-4225.

40. Dromard C, Bartolami S, Deleyrolle L, Takebayashi H, Ripoll C, Simonneau L, Prome S, Puech S, Tran VB, Duperray C, Valmier J, Privat A, Hugnot JP (2007) NG2 and Olig2 expression provides evidence for phenotypic deregulation of cultured central nervous system and peripheral nervous system neural precursor cells. Stem Cells 25: 340-353.

41. Engele J, Bohn MC (1991) The neurotrophic effects of fibroblast growth factors on dopaminergic neurons in vitro are mediated by mesencephalic glia. J Neurosci 11: 3070-3078.

42. Engele J, Schubert D, Bohn MC (1991) Conditioned media derived from glial cell lines promote survival and differentiation of dopaminergic neurons in vitro: role of mesencephalic glia. J Neurosci Res 30: 359-371.

43. Ericson J, Morton S, Kawakami A, Roelink H, Jessell TM (1996) Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. Cell 87: 661-673.

44. Ericson J, Rashbass P, Schedl A, Brenner-Morton S, Kawakami A, van H, V, Jessell TM, Briscoe J (1997) Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. Cell 90: 169-180.

45. Ernfors P, Lonnerberg P, Ayer-LeLievre C, Persson H (1990) Developmental and regional expression of basic fibroblast growth factor mRNA in the rat central nervous

system. J Neurosci Res 27: 10-15.

46. Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292: 154-156.

47. Fields RD, Stevens-Graham B (2002) New insights into neuron-glia communication. Science 298: 556-562.

48. Florkiewicz RZ, Shibata F, Barankiewicz T, Baird A, Gonzalez AM, Florkiewicz E, Shah N (1991) Basic fibroblast growth factor gene expression. Ann N Y Acad Sci 638: 109-126.

49. Florkiewicz RZ, Sommer A (1989) Human basic fibroblast growth factor gene encodes four polypeptides: three initiate translation from non-AUG codons. Proc Natl Acad Sci U S A 86: 3978-3981.

50. Gabay L, Lowell S, Rubin LL, Anderson DJ (2003) Deregulation of dorsoventral patterning by FGF confers trilineage differentiation capacity on CNS stem cells in vitro. Neuron 40: 485-499.

51. Gage FH (2000) Mammalian neural stem cells. Science 287: 1433-1438.

52. Galli R, Pagano SF, Gritti A, Vescovi AL (2000) Regulation of neuronal differentiation in human CNS stem cell progeny by leukemia inhibitory factor. Dev Neurosci 22: 86-95.

53. Gao J, Coggeshall RE, Tarasenko YI, Wu P (2005) Human neural stem cell-derived cholinergic neurons innervate muscle in motoneuron deficient adult rats. Neuroscience 131: 257-262.

54. Gao J, Prough DS, McAdoo DJ, Grady JJ, Parsley MO, Ma L, Tarensenko YI, Wu P (2006) Transplantation of primed human fetal neural stem cells improves cognitive function in rats after traumatic brain injury. Exp Neurol 201: 281-292.

55. Gray CW, Patel AJ (1992) Characterization of a neurotrophic factor produced by cultured astrocytes involved in the regulation of subcortical cholinergic neurons. Brain Res 574: 257-265.

56. Gritti A, Cova L, Parati EA, Galli R, Vescovi AL (1995) Basic fibroblast growth factor supports the proliferation of epidermal growth factor-generated neuronal precursor cells of the adult mouse CNS. Neurosci Lett 185: 151-154.

57. Gritti A, Parati EA, Cova L, Frolichsthal P, Galli R, Wanke E, Faravelli L, Morassutti DJ, Roisen F, Nickel DD, Vescovi AL (1996) Multipotential stem cells from

the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. J Neurosci 16: 1091-1100.

58. Grothe C, Wewetzer w, Lagrange A, Unsicker K (1991) Effects of basic fibroblast growth factor on survival and choline acetyltransferase development of spinal cord neurons. Developmental Brain Research 62: 257-261.

59. Grothe C, Schulze A, Semkova I, Muller-Ostermeyer F, Rege A, Wewetzer K (2000) The high molecular weight fibroblast growth factor-2 isoforms (21,000 mol. wt and 23,000 mol. wt) mediate neurotrophic activity on rat embryonic mesencephalic dopaminergic neurons in vitro. Neuroscience 100: 73-86.

60. Hagood SK, McGinn MJ, Sun D, Colello RJ (2006) Characterizing the mitogenic effect of basic fibroblast growth factor in the adult rat striatum. J Neurotrauma 23: 205-215.

61. Hans G, Moonen G, Rigo JM (2002) Modulation of neuronal survival and excitability by an astroglia-derived factor. J Physiol Paris 96: 323-328.

62. Hatten ME, Lynch M, Rydel RE, Sanchez J, Joseph-Silverstein J, Moscatelli D, Rifkin DB (1988) In vitro neurite extension by granule neurons is dependent upon astroglial-derived fibroblast growth factor. Dev Biol 125: 280-289.

63. Hayashi H, Ishisaki A, Suzuki M, Imamura T (2001) BMP-2 augments FGF-induced differentiation of PC12 cells through upregulation of FGF receptor-1 expression. J Cell Sci 114: 1387-1395.

64. He Z, Li JJ, Zhen CH, Feng LY, Ding XY (2006) Effect of leukemia inhibitory factor on embryonic stem cell differentiation: implications for supporting neuronal differentiation. Acta Pharmacol Sin 27: 80-90.

65. Hiyama E, Hiyama K (2007) Telomere and telomerase in stem cells. Br J Cancer 96: 1020-1024.

66. Huang HM, Chang TW, Liu JC (2004) Basic fibroblast growth factor antagonizes activin A-mediated growth inhibition and hemoglobin synthesis in K562 cells by activating ERK1/2 and deactivating p38 MAP kinase. Biochem Biophys Res Commun 320: 1247-1252.

67. Hurley MJ, Gerard DJ, Jauniaux E, Stern GM, Uchida K, Bradford HF (2001) Cultured human foetal cerebral cortex, transfected with tyrosine hydroxylase cDNA, as a source of neural transplant material. J Neural Transm 108: 781-792.

68. Ishihara A, Saito H, Nishiyama N (1992) Basic fibroblast growth factor ameliorates

learning deficits in basal forebrain-lesioned mice. Jpn J Pharmacol 59: 7-13.

69. Ishii K, Nakamura M, Dai H, Finn TP, Okano H, Toyama Y, Bregman BS (2006) Neutralization of ciliary neurotrophic factor reduces astrocyte production from transplanted neural stem cells and promotes regeneration of corticospinal tract fibers in spinal cord injury. J Neurosci Res 84: 1669-1681.

70. Ito H, Nomoto H, Furukawa Y, Furukawa S (2003) Neurotrophins facilitate synthesis of choline acetyltransferase and tyrosine hydroxylase in cultured mouse neural stem cells independently of their neuronal differentiation. Neurosci Lett 339: 231-234.

71. Jessell TM (2000) Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat Rev Genet 1: 20-29.

72. Jin L, Hu X, Feng L (2005) NT3 inhibits FGF2-induced neural progenitor cell proliferation via the PI3K/GSK3 pathway. J Neurochem 93: 1251-1261.

73. Johe KK, Hazel TG, Muller T, Dugich-Djordjevic MM, McKay RD (1996) Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev 10: 3129-3140.

74. Jung-Testas I, Hu ZY, Baulieu EE, Robel P (1989) Neurosteroids: biosynthesis of pregnenolone and progesterone in primary cultures of rat glial cells. Endocrinology 125: 2083-2091.

75. Kahn MA, Huang CJ, Caruso A, Barresi V, Nazarian R, Condorelli DF, de Vellis J (1997) Ciliary neurotrophic factor activates JAK/Stat signal transduction cascade and induces transcriptional expression of glial fibrillary acidic protein in glial cells. J Neurochem 68: 1413-1423.

76. Kan L, Israsena N, Zhang Z, Hu M, Zhao LR, Jalali A, Sahni V, Kessler JA (2004) Sox1 acts through multiple independent pathways to promote neurogenesis. Dev Biol 269: 580-594.

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

78. Kessaris N, Jamen F, Rubin LL, Richardson WD (2004) Cooperation between sonic hedgehog and fibroblast growth factor/MAPK signalling pathways in neocortical precursors. Development 131: 1289-1298.

79. Kiefer P, Acland P, Pappin D, Peters G, Dickson C (1994) Competition between nuclear localization and secretory signals determines the subcellular fate of a single

CUG-initiated form of FGF3. EMBO J 13: 4126-4136.

80. Kitchens DL, Snyder EY, Gottlieb DI (1994) FGF and EGF are mitogens for immortalized neural progenitors. J Neurobiol 25: 797-807.

81. Klimaschewski L, Nindl W, Feurle J, Kavakebi P, Kostron H (2004) Basic fibroblast growth factor isoforms promote axonal elongation and branching of adult sensory neurons in vitro. Neuroscience 126: 347-353.

82. Koblar SA, Turnley AM, Classon BJ, Reid KL, Ware CB, Cheema SS, Murphy M, Bartlett PF (1998) Neural precursor differentiation into astrocytes requires signaling through the leukemia inhibitory factor receptor. Proc Natl Acad Sci U S A 95: 3178-3181.

83. Koziczak M, Holbro T, Hynes NE (2004) Blocking of FGFR signaling inhibits breast cancer cell proliferation through downregulation of D-type cyclins. Oncogene 23: 3501-3508.

84. Lachyankar MB, Condon PJ, Quesenberry PJ, Litofsky NS, Recht LD, Ross AH (1997) Embryonic precursor cells that express Trk receptors: induction of different cell fates by NGF, BDNF, NT-3, and CNTF. Exp Neurol 144: 350-360.

85. Le R, Esquenazi S (2002) Astrocytes mediate cerebral cortical neuronal axon and dendrite growth, in part, by release of fibroblast growth factor. Neurol Res 24: 81-92.

86. Lee SH, Lumelsky N, Studer L, Auerbach JM, McKay RD (2000) Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. Nat Biotechnol 18: 675-679.

87. Lee SK, Lee B, Ruiz EC, Pfaff SL (2005) Olig2 and Ngn2 function in opposition to modulate gene expression in motor neuron progenitor cells. Genes Dev 19: 282-294.

88. Lee SK, Pfaff SL (2003) Synchronization of neurogenesis and motor neuron specification by direct coupling of bHLH and homeodomain transcription factors. Neuron 38: 731-745.

89. Li H, Arber S, Jessell TM, Edlund H (1999) Selective agenesis of the dorsal pancreas in mice lacking homeobox gene Hlxb9. Nat Genet 23: 67-70.

90. Li R, Thode S, Zhou J, Richard N, Pardinas J, Rao MS, Sah DW (2000) Motoneuron differentiation of immortalized human spinal cord cell lines. J Neurosci Res 59: 342-352.

91. Li XJ, Du ZW, Zarnowska ED, Pankratz M, Hansen LO, Pearce RA, Zhang SC

(2005) Specification of motoneurons from human embryonic stem cells. Nat Biotechnol 23: 215-221.

92. Liem KF, Jr., Jessell TM, Briscoe J (2000) Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites. Development 127: 4855-4866.

93. Liesi P, Dahl D, Vaheri A (1983) Laminin is produced by early rat astrocytes in primary culture. J Cell Biol 96: 920-924.

94. Lim UM, Sidhu KS, Tuch BE (2006) Derivation of Motor Neurons from three Clonal Human Embryonic Stem Cell Lines. Curr Neurovasc Res 3: 281-288.

95. Lin HY, Xu J, Ornitz DM, Halegoua S, Hayman MJ (1996) The fibroblast growth factor receptor-1 is necessary for the induction of neurite outgrowth in PC12 cells by aFGF. J Neurosci 16: 4579-4587.

96. Ling ZD, Potter ED, Lipton JW, Carvey PM (1998) Differentiation of mesencephalic progenitor cells into dopaminergic neurons by cytokines. Exp Neurol 149: 411-423.

97. Liu Y, Rao MS (2004) Olig genes are expressed in a heterogeneous population of precursor cells in the developing spinal cord. Glia 45: 67-74.

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

99. Lu QR, Yuk D, Alberta JA, Zhu Z, Pawlitzky I, Chan J, McMahon AP, Stiles CD, Rowitch DH (2000) Sonic hedgehog--regulated oligodendrocyte lineage genes encoding bHLH proteins in the mammalian central nervous system. Neuron 25: 317-329.

100. MacDonald KP, Murrell WG, Bartlett PF, Bushell GR, Mackay-Sim A (1996) FGF2 promotes neuronal differentiation in explant cultures of adult and embryonic mouse olfactory epithelium. J Neurosci Res 44: 27-39.

101. Martens DJ, Seaberg RM, van der KD (2002) In vivo infusions of exogenous growth factors into the fourth ventricle of the adult mouse brain increase the proliferation of neural progenitors around the fourth ventricle and the central canal of the spinal cord. Eur J Neurosci 16: 1045-1057.

102. Mattson MP, Rychlik B (1990) Glia protect hippocampal neurons against excitatory amino acid-induced degeneration: involvement of fibroblast growth factor. Int J Dev Neurosci 8: 399-415.

103. McDonald JW (2001) ES Cells and Neurogenesis. In: Stem Cells and Development (Rao MS, ed), pp 207-261. New Jersey: Humana Press.

104. McManaman J, Crawford F, Clark R, Richker J, Fuller F (1989) Multiple neurotrophic factors from skeletal muscle: demonstration of effects of basic fibroblast growth factor and comparisons with the 22-kilodalton choline acetyltransferase development factor. J Neurochem 53: 1763-1771.

105. Mizuguchi R, Sugimori M, Takebayashi H, Kosako H, Nagao M, Yoshida S, Nabeshima Y, Shimamura K, Nakafuku M (2001) Combinatorial roles of olig2 and neurogenin2 in the coordinated induction of pan-neuronal and subtype-specific properties of motoneurons. Neuron 31: 757-771.

106. Mohammadi M, Froum S, Hamby JM, Schroeder MC, Panek RL, Lu GH, Eliseenkova AV, Green D, Schlessinger J, Hubbard SR (1998) Crystal structure of an angiogenesis inhibitor bound to the FGF receptor tyrosine kinase domain. EMBO J 17: 5896-5904.

107. Mohammadi M, McMahon G, Sun L, Tang C, Hirth P, Yeh BK, Hubbard SR, Schlessinger J (1997) Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. Science 276: 955-960.

108. Mohammadi M, Olsen SK, Ibrahimi OA (2005) Structural basis for fibroblast growth factor receptor activation. Cytokine Growth Factor Rev 16: 107-137.

109. Momparler RL (1982) Biochemical pharmacology of cytosine arabinoside. Med Pediatr Oncol 10 Suppl 1: 45-48.

110. Nakayama T, Momoki-Soga T, Inoue N (2003) Astrocyte-derived factors instruct differentiation of embryonic stem cells into neurons. Neurosci Res 46: 241-249.

111. Naruse M, Nakahira E, Miyata T, Hitoshi S, Ikenaka K, Bansal R (2006) Induction of oligodendrocyte progenitors in dorsal forebrain by intraventricular microinjection of FGF-2. Dev Biol 297: 262-273.

112. Nelson AD, Svendsen CN (2006) Low concentrations of extracellular FGF-2 are sufficient but not essential for neurogenesis from human neural progenitor cells. Mol Cell Neurosci 33: 29-35.

113. Novitch BG, Chen AI, Jessell TM (2001) Coordinate regulation of motor neuron subtype identity and pan-neuronal properties by the bHLH repressor Olig2. Neuron 31: 773-789.

114. Novitch BG, Wichterle H, Jessell TM, Sockanathan S (2003) A requirement for

retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification. Neuron 40: 81-95.

115. Ohgoh M, Shimizu H, Ogura H, Nishizawa Y (2000) Astroglial trophic support and neuronal cell death: influence of cellular energy level on type of cell death induced by mitochondrial toxin in cultured rat cortical neurons. J Neurochem 75: 925-933.

116. Ornitz DM, Itoh N (2001) Fibroblast growth factors. Genome Biol 2: (3). Epub 2001 Mar 9.

117. Ornitz DM, Xu J, Colvin JS, McEwen DG, MacArthur CA, Coulier F, Gao G, Goldfarb M (1996) Receptor specificity of the fibroblast growth factor family. J Biol Chem 271: 15292-15297.

118. Ortega S, Ittmann M, Tsang SH, Ehrlich M, Basilico C (1998) Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. Proc Natl Acad Sci U S A 95: 5672-5677.

119. Ostenfeld T, Svendsen CN (2004) Requirement for Neurogenesis to Proceed through the Division of Neuronal Progenitors following Differentiation of Epidermal Growth Factor and Fibroblast Growth Factor-2-Responsive Human Neural Stem Cells. Stem Cells 22: 798-811.

120. Otto D, Unsicker K, Grothe C (1987) Pharmacological effects of nerve growth factor and fibroblast growth factor applied to the transectioned sciatic nerve on neuron death in adult rat dorsal root ganglia. Neurosci Lett 83: 156-160.

121. Palmer TD, Markakis EA, Willhoite AR, Safar F, Gage FH (1999) Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. J Neurosci 19: 8487-8497.

122. Park S, Lee KS, Lee YJ, Shin HA, Cho HY, Wang KC, Kim YS, Lee HT, Chung KS, Kim EY, Lim J (2004) Generation of dopaminergic neurons in vitro from human embryonic stem cells treated with neurotrophic factors. Neurosci Lett 359: 99-103.

123. Patten I, Placzek M (2000) The role of Sonic hedgehog in neural tube patterning. Cell Mol Life Sci 57: 1695-1708.

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

125. Pfaff SL, Mendelsohn M, Stewart CL, Edlund T, Jessell TM (1996) Requirement for LIM homeobox gene Isl1 in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation. Cell 84: 309-320.

126. Pfrieger FW, Barres BA (1997) Synaptic efficacy enhanced by glial cells in vitro. Science 277: 1684-1687.

127. Powell PP, Finklestein SP, Dionne CA, Jaye M, Klagsbrun M (1991) Temporal, differential and regional expression of mRNA for basic fibroblast growth factor in the developing and adult rat brain. Brain Res Mol Brain Res 11: 71-77.

128. Prats H, Kaghad M, Prats AC, Klagsbrun M, Lelias JM, Liauzun P, Chalon P, Tauber JP, Amalric F, Smith JA, . (1989) High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. Proc Natl Acad Sci U S A 86: 1836-1840.

129. Qian X, Davis AA, Goderie SK, Temple S (1997) FGF2 concentration regulates the generation of neurons and glia from multipotent cortical stem cells. Neuron 18: 81-93.

130. Qian X, Shen Q, Goderie SK, He W, Capela A, Davis AA, Temple S (2000) Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. Neuron 28: 69-80.

131. Raballo R, Rhee J, Lyn-Cook R, Leckman JF, Schwartz ML, Vaccarino FM (2000) Basic fibroblast growth factor (Fgf2) is necessary for cell proliferation and neurogenesis in the developing cerebral cortex. J Neurosci 20: 5012-5023.

132. Rao MS, Anderson DJ (1997) Immortalization and controlled in vitro differentiation of murine multipotent neural crest stem cells. J Neurobiol 32: 722-746.

133. Rao VL, Dogan A, Bowen KK, Todd KG, Dempsey RJ (2001) Antisense knockdown of the glial glutamate transporter GLT-1 exacerbates hippocampal neuronal damage following traumatic injury to rat brain. Eur J Neurosci 13: 119-128.

134. Reimers D, Lopez-Toledano MA, Mason I, Cuevas P, Redondo C, Herranz AS, Lobo MV, Bazan E (2001) Developmental expression of fibroblast growth factor (FGF) receptors in neural stem cell progeny. Modulation of neuronal and glial lineages by basic FGF treatment. Neurol Res 23: 612-621.

135. Ren W, Guo Q, Yang Y, Chen F (2006) bFGF and heparin but not laminin are necessary factors in the mediums that affect NSCs differentiation into cholinergic neurons. Neurol Res 28: 87-90.

136. Ren-Patterson RF, Kim DK, Zheng X, Sherrill S, Huang SJ, Tolliver T, Murphy DL (2005) Serotonergic-like progenitor cells propagated from neural stem cells in vitro: survival with SERT protein expression following implantation into brains of mice lacking SERT. FASEB J 19: 1537-1539.

137. Renko M, Quarto N, Morimoto T, Rifkin DB (1990) Nuclear and cytoplasmic localization of different basic fibroblast growth factor species. J Cell Physiol 144: 108-114.

138. Renoncourt Y, Carroll P, Filippi P, Arce V, Alonso S (1998) Neurons derived in vitro from ES cells express homeoproteins characteristic of motoneurons and interneurons. Mech Dev 79: 185-197.

139. Reuss B, Bohlen und HO (2003) Fibroblast growth factors and their receptors in the central nervous system. Cell Tissue Res 313: 139-157.

140. Reuss B, Unsicker K (2000) Survival and differentiation of dopaminergic mesencephalic neurons are promoted by dopamine-mediated induction of FGF-2 in striatal astroglial cells. Mol Cell Neurosci 16: 781-792.

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

142. Reynolds BA, Weiss S (1996) Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev Biol 175: 1-13.

143. Riaz SS, Jauniaux E, Stern GM, Bradford HF (2002) The controlled conversion of human neural progenitor cells derived from foetal ventral mesencephalon into dopaminergic neurons in vitro. Brain Res Dev Brain Res 136: 27-34.

144. Riaz SS, Theofilopoulos S, Jauniaux E, Stern GM, Bradford HF (2004) The differentiation potential of human foetal neuronal progenitor cells in vitro. Brain Res Dev Brain Res 153: 39-51.

145. Rosenberg PA, Aizenman E (1989) Hundred-fold increase in neuronal vulnerability to glutamate toxicity in astrocyte-poor cultures of rat cerebral cortex. Neurosci Lett 103: 162-168.

146. Roy NS, Nakano T, Keyoung HM, Windrem M, Rashbaum WK, Alonso ML, Kang J, Peng W, Carpenter MK, Lin J, Nedergaard M, Goldman SA (2004) Telomerase immortalization of neuronally restricted progenitor cells derived from the human fetal spinal cord. Nat Biotechnol 22: 297-305.

147. Rudge JS, Alderson RF, Pasnikowski E, McClain J, Ip NY, Lindsay RM (1992) Expression of Ciliary Neurotrophic Factor and the Neurotrophins-Nerve Growth Factor, Brain-Derived Neurotrophic Factor and Neurotrophin 3-in Cultured Rat Hippocampal Astrocytes. Eur J Neurosci 4: 459-471.

148. Sapieha PS, Peltier M, Rendahl KG, Manning WC, Di Polo A (2003) Fibroblast growth factor-2 gene delivery stimulates axon growth by adult retinal ganglion cells after acute optic nerve injury. Mol Cell Neurosci 24: 656-672.

149. Schaar DG, Sieber BA, Dreyfus CF, Black IB (1993) Regional and cell-specific expression of GDNF in rat brain. Exp Neurol 124: 368-371.

150. Schlessinger J (2000) Cell signaling by receptor tyrosine kinases. Cell 103: 211-225.

151. Schlessinger J (2004) Common and distinct elements in cellular signaling via EGF and FGF receptors. Science 306: 1506-1507.

152. Schwartz JP, Wilson DJ (1992) Preparation and characterization of type 1 astrocytes cultured from adult rat cortex, cerebellum, and striatum. Glia 5: 75-80.

153. Sensenbrenner M, Labourdette G, Pettmann B, Perraud F, Besnard F (1987) Neuronal-derived factors regulating glial cell proliferation and maturation. J Physiol (Paris) 82: 288-290.

154. Sherman L, Stocker KM, Morrison R, Ciment G (1993) Basic fibroblast growth factor (bFGF) acts intracellularly to cause the transdifferentiation of avian neural crest-derived Schwann cell precursors into melanocytes. Development 118: 1313-1326.

155. Shin S, Dalton S, Stice SL (2005) Human motor neuron differentiation from human embryonic stem cells. Stem Cells Dev 14: 266-269.

156. Slezak M, Pfrieger FW (2003) New roles for astrocytes: regulation of CNS synaptogenesis. Trends Neurosci 26: 531-535.

157. Smith SJ (1998) Glia help synapses form and function. Curr Biol 8: R158-R160.

158. Song H, Stevens CF, Gage FH (2002a) Astroglia induce neurogenesis from adult neural stem cells. Nature 417: 39-44.

159. Song HJ, Stevens CF, Gage FH (2002b) Neural stem cells from adult hippocampus develop essential properties of functional CNS neurons. Nat Neurosci 5: 438-445.

160. Song MR, Ghosh A (2004) FGF2-induced chromatin remodeling regulates CNTF-mediated gene expression and astrocyte differentiation. Nat Neurosci 7: 229-235.

161. Sorensen V, Nilsen T, Wiedlocha A (2006) Functional diversity of FGF-2 isoforms by intracellular sorting. Bioessays 28: 504-514.

162. Soundararajan P, Miles GB, Rubin LL, Brownstone RM, Rafuse VF (2006)

Motoneurons derived from embryonic stem cells express transcription factors and develop phenotypes characteristic of medial motor column neurons. J Neurosci 26: 3256-3268.

163. Spector JA, Mathy JA, Warren SM, Nacamuli RP, Song HM, Lenton K, Fong KD, Fang DT, Longaker MT (2005) FGF-2 acts through an ERK1/2 intracellular pathway to affect osteoblast differentiation. Plast Reconstr Surg 115: 838-852.

164. Steindler C, Scheffler B, Laywell E, Suslov O, Zheng T, Reiniger T, Kukekov VG (2003) Neural Stem/Progenitor Cell Clones or "Neurospheres". In: Neural Stem Cells for Brain and Spinal Cord Repair (Zigova T, Snyder EY, Sanberg PR, eds), pp 183-202. New Jersey: Humana Press.

165. Storch A, Paul G, Csete M, Boehm BO, Carvey PM, Kupsch A, Schwarz J (2001) Long-term proliferation and dopaminergic differentiation of human mesencephalic neural precursor cells. Exp Neurol 170: 317-325.

166. Suzuki M, Wright LS, Marwah P, Lardy HA, Svendsen CN (2004) Mitotic and neurogenic effects of dehydroepiandrosterone (DHEA) on human neural stem cell cultures derived from the fetal cortex. Proc Natl Acad Sci U S A 101: 3202-3207.

167. Svendsen CN, ter Borg MG, Armstrong RJ, Rosser AE, Chandran S, Ostenfeld T, Caldwell MA (1998) A new method for the rapid and long term growth of human neural precursor cells. J Neurosci Methods 85: 141-152.

168. Tanabe Y, Roelink H, Jessell TM (1995) Induction of motor neurons by Sonic hedgehog is independent of floor plate differentiation. Curr Biol 5: 651-658.

169. Tanabe Y, William C, Jessell TM (1998) Specification of motor neuron identity by the MNR2 homeodomain protein. Cell 95: 67-80.

170. Tao Y, Black IB, DiCicco-Bloom E (1996) Neurogenesis in neonatal rat brain is regulated by peripheral injection of basic fibroblast growth factor (bFGF). J Comp Neurol 376: 653-663.

171. Tao Y, Black IB, DiCicco-Bloom E (1997) In vivo neurogenesis is inhibited by neutralizing antibodies to basic fibroblast growth factor. J Neurobiol 33: 289-296.

172. Tarasenko YI, Gao J, Nie L, Johnson KM, Grady JJ, Hulsebosch CE, McAdoo DJ, Wu P (2007) Human fetal neural stem cells grafted into contusion-injured rat spinal cords improve behavior. J Neurosci Res. 85(1):47-57.

173. Tarasenko YI, Yu Y, Jordan PM, Bottenstein J, Wu P (2004) Effect of growth factors on proliferation and phenotypic differentiation of human fetal neural stem cells. J Neurosci Res 78: 625-636.

174. Thaler J, Harrison K, Sharma K, Lettieri K, Kehrl J, Pfaff SL (1999) Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9. Neuron 23: 675-687.

175. Thaler JP, Koo SJ, Kania A, Lettieri K, Andrews S, Cox C, Jessell TM, Pfaff SL (2004) A postmitotic role for Isl-class LIM homeodomain proteins in the assignment of visceral spinal motor neuron identity. Neuron 41: 337-350.

176. Thaler JP, Lee SK, Jurata LW, Gill GN, Pfaff SL (2002) LIM factor Lhx3 contributes to the specification of motor neuron and interneuron identity through cell-type-specific protein-protein interactions. Cell 110: 237-249.

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

178. Traiffort E, Charytoniuk D, Watroba L, Faure H, Sales N, Ruat M (1999) Discrete localizations of hedgehog signalling components in the developing and adult rat nervous system. Eur J Neurosci 11: 3199-3214.

179. Tropepe V, Sibilia M, Ciruna BG, Rossant J, Wagner EF, van der KD (1999) Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. Dev Biol 208: 166-188.

180. Tsai RY, Kim S (2005) Fibroblast growth factor 2 negatively regulates the induction of neuronal progenitors from neural stem cells. J Neurosci Res 82: 149-159.

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

182. Ullian EM, Christopherson KS, Barres BA (2004) Role for glia in synaptogenesis. Glia 47: 209-216.

183. Unsicker K, Reichert-Preibsch H, Schmidt R, Labourdette G, Sensesnbrenner M (1987) Acidic and basic fibroboast growth factors have neurotophic functions for cultured peripheral and central nervous system neurons. Proc Natl Acad Sci U S A 84: 5459-5463.

184. Vaccarino FM, Schwartz ML, Raballo R, Nilsen J, Rhee J, Zhou M, Doetschman T, Coffin JD, Wyland JJ, Hung YT (1999a) Changes in cerebral cortex size are governed by fibroblast growth factor during embryogenesis. Nat Neurosci 2: 848.

185. Vaccarino FM, Schwartz ML, Raballo R, Rhee J, Lyn-Cook R (1999b) Fibroblast

growth factor signaling regulates growth and morphogenesis at multiple steps during brain development. Curr Top Dev Biol 46: 179-200.

186. Van den BL, Laurell H, Huez I, Zanibellato C, Prats H, Bugler B (2000) FIF [fibroblast growth factor-2 (FGF-2)-interacting-factor], a nuclear putatively antiapoptotic factor, interacts specifically with FGF-2. Mol Endocrinol 14: 1709-1724.

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

188. Vescovi AL, Reynolds BA, Fraser DD, Weiss S (1993) bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. Neuron 11: 951-966.

189. Vibulsreth S, Hefti F, Ginsberg MD, Dietrich WD, Busto R (1987) Astrocytes protect cultured neurons from degeneration induced by anoxia. Brain Res 422: 303-311.

190. Vicario-Abejon C, Johe KK, Hazel TG, Collazo D, McKay RD (1995) Functions of basic fibroblast growth factor and neurotrophins in the differentiation of hippocampal neurons. Neuron 15: 105-114.

191. Villa A, Snyder EY, Vescovi A, Martinez-Serrano A (2000) Establishment and properties of a growth factor-dependent, perpetual neural stem cell line from the human CNS. Exp Neurol 161: 67-84.

192. Wagner J, Akerud P, Castro DS, Holm PC, Canals JM, Snyder EY, Perlmann T, Arenas E (1999) Induction of a midbrain dopaminergic phenotype in Nurr1-overexpressing neural stem cells by type 1 astrocytes. Nat Biotechnol 17: 653-659.

193. Wagner S, Gardner H (2000) Modes of regulation of laminin-5 production by rat astrocytes. Neurosci Lett 284: 105-108.

194. Walicke P, Cowan WM, Ueno N, Baird A, Guillemin R (1986) Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. Proc Natl Acad Sci U S A 83: 3012-3016.

195. Walicke PA (1988) Basic and acidic fibroblast growth factors have trophic effects on neurons from multiple CNS regions. J Neurosci 8: 2618-2627.

196. Wang F, McKedhan W (2004) The Fibroblast Grwoth Factor (FGF) Signaling Complex. In: Handbook of Cell Signaling Vol 1 (Bradshaw, Dennis, eds), pp 265-270.

Elsevier Science.

197. Wang G, Zhang H, Zhao Y, Li J, Cai J, Wang P, Meng S, Feng J, Miao C, Ding M, Li D, Deng H (2005) Noggin and bFGF cooperate to maintain the pluripotency of human embryonic stem cells in the absence of feeder layers. Biochem Biophys Res Commun 330: 934-942.

198. Watanabe T, Okuda Y, Nonoguchi N, Zhao MZ, Kajimoto Y, Furutama D, Yukawa H, Shibata MA, Otsuki Y, Kuroiwa T, Miyatake S (2004) Postischemic intraventricular administration of FGF-2 expressing adenoviral vectors improves neurologic outcome and reduces infarct volume after transient focal cerebral ischemia in rats. J Cereb Blood Flow Metab 24: 1205-1213.

199. Weise B, Janet T, Grothe C (1993) Localization of bFGF and FGF-receptor in the developing nervous system of the embryonic and newborn rat. J Neurosci Res 34: 442-453.

200. Wichterle H, Lieberam I, Porter JA, Jessell TM (2002) Directed differentiation of embryonic stem cells into motor neurons. Cell 110: 385-397.

201. William CM, Tanabe Y, Jessell TM (2003) Regulation of motor neuron subtype identity by repressor activity of Mnx class homeodomain proteins. Development 130: 1523-1536.

202. Wright LS, Li J, Caldwell MA, Wallace K, Johnson JA, Svendsen CN (2003) Gene expression in human neural stem cells: effects of leukemia inhibitory factor. J Neurochem 86: 179-195.

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

204. Xiao Z, Kong Y, Yang S, Li M, Wen J, Li L (2007) Upregulation of Flk-1 by bFGF via the ERK pathway is essential for VEGF-mediated promotion of neural stem cell proliferation. Cell Res 17: 73-79.

205. Xu RH, Peck RM, Li DS, Feng X, Ludwig T, Thomson JA (2005) Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Methods 2: 185-190.

206. Yamagata K, Tagami M, Ikeda K, Tsumagari S, Yamori Y, Nara Y (2002) Differential regulation of glial cell line-derived neurotrophic factor (GDNF) mRNA expression during hypoxia and reoxygenation in astrocytes isolated from stroke-prone spontaneously hypertensive rats. Glia 37: 1-7.

207. Yamakuni T, Ozawa F, Hishinuma F, Kuwano R, Takahashi Y, Amano T (1987) Expression of beta-nerve growth factor mRNA in rat glioma cells and astrocytes from rat brain. FEBS Lett 223: 117-121.

208. Yin QW, Johnson J, Prevette D, Oppenheim RW (1994) Cell death of spinal motoneurons in the chick embryo following deafferentation: rescue effects of tissue extracts, soluble proteins, and neurotrophic agents. J Neurosci 14: 7629-7640.

209. Yokoyama M, Morrison RS, Black IB, Dreyfus CF (1994) Septal neuron cholinergic and GABAergic functions: differential regulation by basic fibroblast growth factor and epidermal growth factor. Brain Res Dev Brain Res 78: 201-209.

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

211. Zhang XM, Ramalho-Santos M, McMahon AP (2001b) Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R asymmetry by the mouse node. Cell 105: 781-792.

212. Zhao Z, Alam S, Oppenheim RW, Prevette DM, Evenson A, Parsadanian A (2004) Overexpression of glial cell line-derived neurotrophic factor in the CNS rescues motoneurons from programmed cell death and promotes their long-term survival following axotomy. Exp Neurol 190: 356-372.

213. Zhou Q, Anderson DJ (2002) The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. Cell 109: 61-73.

214. Zhou Q, Choi G, Anderson DJ (2001) The bHLH transcription factor Olig2 promotes oligodendrocyte differentiation in collaboration with Nkx2.2. Neuron 31: 791-807.

VITA

Paivi Martina Jordan (Roozen) was born on February 9th, 1977 in the small farming community of Mount Vernon, Washington. She was the second daughter, the eldest Mia born in 1970 and the youngest Leea born in 1978, to parents Jan and Ritva Roozen. Jan and Ritva immigrated first to Canada in 1969 from their native countries of the Netherlands and Finland, respectively, and later, in 1972 to the United States. Together they own and operate a small flower bulb business, Choice Bulb Farms, which specializes in rare and exotic cut flowers. Growing up on a farm, Paivi learned to love animals, becoming a skilled equestrian and later, taking that love of animals to college at Washington State University where she obtained her B.S. in Animal Sciences *summa cum laude*. She then spent two years working as a research assistant in a bacteriology lab within the Veterinary College at Washington State University. It was there, working in the field of epidemiology and bacteriology, that Paivi became excited about a post-graduate career and decided to further her education with a Ph.D. Thus she moved to Galveston in 2002 where she matriculated into the Graduate School of Biomedical Sciences at UTMB. She completed her preliminary coursework, joined the Neuroscience Graduate Program (NGP) and began working with Dr. Ping Wu in the spring of 2004. Since joining NGP, Paivi has been awarded the "Who's Who Among Students in American Universities" award, the University Federal Credit Union Scholarship, a travel award to attend MD Andersons Stem Cells and Cancer Cell Biology meeting and most recently, she was awarded top honors for her oral presentation on bFGF and Stem Cell Differentiation at the National Student Research Forum.

EDUCATION

B.S. *summa cum laude* Animal Sciences, May 2000. Washington State University, Pullman, WA.

PUBLICATIONS

Jordan, P.M. Thonhoff, J.R., Cain, L.D. and Wu, P. Astrocytes Enhance Long-Term Survival of Cholinergic Nurons Differentiated from Human Fetal Neural Stem cells. J. Neurosci Res. 2007 (June 2007, accepted).

Jordan, P.M. Thonhoff, J.R., Ojeda, L.O. and Wu, P. bFGF induces Hb9 expression in fetal human neural stem cells. Cell Stem Cell. 2007 (submitted).

Thonhoff J.R., Lou D.I, **Jordan P.M**, Zhao, X and Wu, P. Compatibility of human fetal neural stem cells with bioengineering hydrogels *in vitro*. J Neurosci Res. (submitted).

Thonhoff, J.R, **Jordan, P.M**, Karam J.R., Bassett B.L. and Wu, P. Identification of early disease progression in an ALS rat model. Neuroscience Letters. 2007. Mar 30; 415(3):264-8.

Thomas, M.S., Zhang, W., **Jordan, P.M**., Saragovi, H.U. and Taglialatela G. Signaling Pathways Mediating a Selective Induction of Cytotosolic Levels of Nitric Oxide Synthase II by Tumor Necrosis Factor alpha in Nerve Growth Factor-responsive Cells. J Neuroinflammation. 2005. Sep 6; 2(1):19.

Tarasenko,Y.I., **Jordan, P.M**., Yu, Y., Bottenstein, J.E. and Wu, P. Effect of growth factors on proliferation and phenotypic differentiation of human fetal neural stem cells. J Neurosci Res. 2004. Dec 1;78(5):625-36.

Rice, D.H., Hancock, D.D, **Roozen, P.M,** Szymanski, M.H, Scheenstra, B.C., Cady, K.M, Besser, T.E and P.A. Chudek. Household Contamination with *Salmonella enterica*. Emerging Infectious Diseases. 2003. 9(1):120-122