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HUMAN FETAL NEURAL STEM CELLS: PROLIFERATION AND DIFFERENTIATION IN RESPONSE TO GROWTH FACTORS AND ROLE IN LOCOMOTOR RECOVERY AFTER SPINAL CORD CONTUSION INJURY

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“It is only with the heart that one can see rightly; what is essential is invisible to the eye.”
– Antoine de Saint -Exupery

To my family…

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HUMAN FETAL NEURAL STEM CELLS: PROLIFERATION AND DIFFERENTIATION IN RESPONSE TO GROWTH FACTORS AND ROLE IN LOCOMOTOR RECOVERY AFTER SPINAL CORD CONTUSION INJURY

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Human fetal neural stem cells (hNSCs) may be useful for developing a cell-based therapy to treat spinal cord injury (SCI). In these studies we examined the effects of epigenetic mitogens on proliferation and differentiation of hNSCs in vitro and the outcome of hNSC grafting into contusion injured rat spinal cords in vivo.

Cells were cultured in seven regimens with basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and leukemia inhibitory factor (LIF), either alone or in combinations. We found that a combination of bFGF, EGF and LIF expanded hNSCs more efficiently than any other treatment. Differentiation patterns of hNSCs expanded under different conditions were also analyzed. Cells expanded under different mitogen regimens varied in their phenotypic differentiation patterns and also in their response to a priming treatment with a combination of bFGF, heparin and laminin (FHL). Particularly, significant generation of cholinergic cells was observed only in hNSCs expanded with EGF/bFGF or EGF/bFGF/LIF, but not with other treatment regimens.

Subsequently, we examined the effect of temporal transplantation of hNSCs into contusion injured rat spinal cords. FHL-primed or unprimed hNSCs were grafted into the epicenter of injured spinal cords on either the same day, three or nine days after a moderate contusion injury. Histological analyses of the spinal cord revealed that stem cells survived three months post engraftment only in animals that received grafts at 9-day post injury. The survival rates of such cells were significantly lower than those grafted into the intact cord. Both primed and unprimed hNSCs differentiated into neurons;

however, only primed cells gave rise to cholinergic neurons. Functional assessment based on the BBB score and exploratory activity three months after grafting showed that hindlimb function and/or trunk stability improved significantly in only the group that received primed hNSC transplants on the ninth day post contusion.

Our results indicate that 1) hNSCs are highly plastic with their proliferation and differentiation potential dependent upon different growth factor treatments; and 2) *in vitro* stem cells priming is beneficial to achieve the desired differentiating phenotypes *in vivo* and help to attenuate locomotor deficits after SCI.

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LIST OF ABBREVIATIONS

7-AAD	7-amino-actinomycin-D – nuclear stain
AAV	Adeno-associated virus
BBB	Basso, Beattie, Bresnahan locomotor rating scale
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
BrdU	Bromodeoxyuridine
CGRP	Calcitonin gene related peptide – neuropeptide
ChAT	Choline acetyl transferase – motor neuron marker
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CsA	Cyclosporin-A
CST	Corticospinal tract
dMCAo	Distal middle cerebral artery occlusion
EGF	Epidermal growth factor
bFGF	Basic fibroblast growth factor – FGF2
GABA	γ-aminobutyric acid
GDNF	Glial derived nerve growth factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GLAST	Glial glutamate transporter EAAT1
GLT-1	Glial glutamate transporter EAAT2
hNPCs	Human neural progenitor cells
hNSCs	Human neural stem cells
hTERT	Human telomerase reverse transcriptase
IGF-1	Insulin like growth factor
IR/IF	Immunoreactivity/immunofluorescence

ITR	Inverted terminal repeats
LIF	Leukemia inhibitory factor
LMN	Laminin
MAP2	Microtubule-associated protein 2 – immature neuronal marker
NeuN	Neuronal Nuclei - neuron-specific nuclear protein
NF	Neurofilament – neuroblast and neuronal marker
NG2	Chondroitin sulfate proteoglycan – oligodenrdocyte precursor marker
NT3	Neurotrophin 3
NTF	Neurotrophic factor
OX42	Rat integrin αM [CD11b] – marker for macrophages, microglia and dendritic cells.
PBS	Phosphate-buffered saline
P450scc	Cytochrome P450 side chain cleavage enzyme – rat specific - marker for endogenous neural populations in rat spinal cord
SCI	Spinal cord injury
TBS	Tris-buffered saline
TuJ1	Neuronal class III β-tubulin – neuronal marker
WST-1	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt

CHAPTER 1: INTRODUCTION

1.1 SPINAL CORD INJURY

Spinal cord injury (SCI) is a traumatic event with a myriad of devastating consequences incurable by current medical approaches. According to the National Spinal Cord Injury Association, the majority of the victims are young adults: 82% are males ages of 16-30 injured in motor vehicle accidents (NSCIA, 2004). Every year, 11,000 new cases add to the estimated 250,000-400,000 people in the US alone who live with SCI caused dysfunctions. Less than half of SCI victims are able to maintain their employment; they incur extensive medical expenditures, and suffer losses in their personal lives. While SCI takes its physical and mental toll, there is no efficient medical treatment and cure.

The trauma to the spinal cord results in sensory and motor function deficits, distal to the injury site, consequent to extensive acute, secondary, and chronic events resulting in tissue loss due to direct damage to neural cells and disruption of the descending and ascending axonal pathways (Ribotta and Privat, 1998; Schwab and Bartholdi, 1996; Tator, 1995; McDonald, 1999; Hulsebosch, 2002).

In a spinal cord contusion injury, after the primary insult, the area of tissue damage progressively expands. In general, the spinal primary lesion is directly caused by adjacent vertebral disks and/or bone fragments. These structures are displaced during the impact and exert a direct compressive force causing mechanical damage to the underlying tissues. The trauma ruptures neural cells, severs axons and blood vessels, and disrupts the blood brain barrier. Then rapid edematization of the spinal cord at the injury site follows, reducing blood flow and producing local ischemia. On a cellular level, tissular hypoxia or ischemia leads to a rapid ATP depletion due to high metabolic demands and limited

intrinsic energy stores in the CNS. Subsequent to ATP depletion, the function of active Na,K-ATPase pumps is decreased. Malfunction of ATP-dependent ion transporters disturbs ionic homeostasis of the cells and changes the intracellular ion concentrations. Intracellular calcium concentrations rise due to open voltage-gated channels in depolarized cell membrane. Influx of calcium facilitates release of excitatory amino acids such as glutamate. Electrolytic changes, cell death-triggered release and a limited reuptake, are all factors that significantly contribute to the extracellular accumulation of glutamate reaching toxic levels. In addition, "exitotoxicity" further increases intracellular calcium to lethal levels. Elevated intracellular calcium enhances mitochondrial production of free radicals, as well as activation of lipid peroxidase and nitric oxide synthase, leading to the production and activation of reactive oxygen species (ROS) (Beckman and Koppenol, 1996; Dykens, 1994). Together, the consequent destabilization of membranes, impaired energy production, cytoskeletal disruption and DNA damage, resultant from excitotoxic cascades, lead to the extensive secondary cell death. The initial necrosis and subsequent apoptosis, cause substantial cellular loss and disruption of descending and ascending neuronal pathways, which require replacement in order to restore structure and function of the spinal cord.

Interestingly, some groups have reported functional recovery as a result of initial sprouting (Weidner et al., 2001). Notwithstanding this initial sprouting, extensive axonal regeneration does not occur in the spinal cord after injury. Any sprouting needs a substrate and a permissive environment containing the necessary neurotrophic and neurotropic cues to provide directional growth with the purpose of reestablishing the damaged or lost pathways.

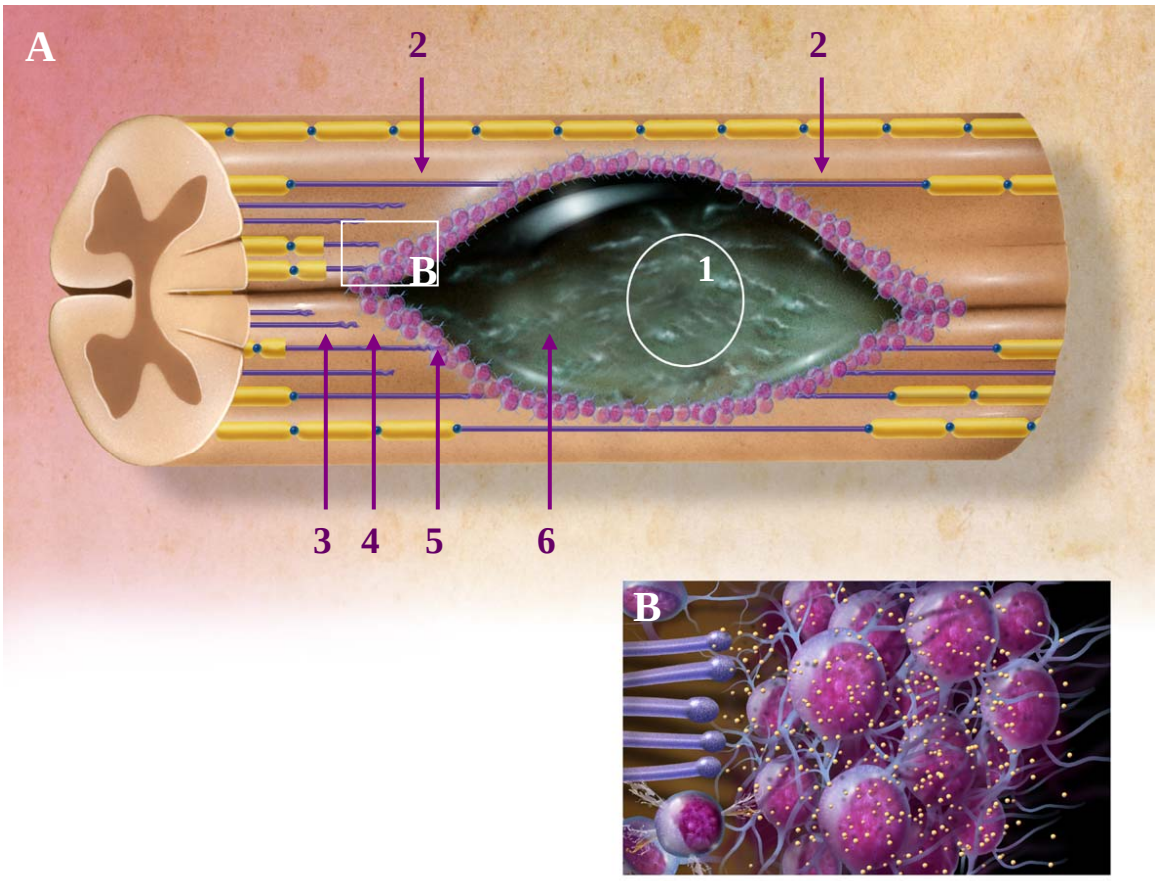

Figure 1.1: Schematic representation of a spinal cord injury and interventions in treatment. (A) Restoration of function following a spinal cord injury will necessitate 1) prevention of necrotic and apoptotic cell death secondary to excitotoxicity by administration of anti-excitotoxic compounds and anti-apoptotic treatments with growth factors, 2) remyelination of surviving axons to improve conduction, 3) support for regeneration by providing growth factors such as NT3, BDNF and GDNF, 4) elimination of inhibitors of regeneration, 5) prevention and reduction of the glial scar, 6) cell replacement therapies that deliver stem cells that are capable to replace lost cells and provide neurotrophic support. (B) An insert depicts the glial scar on the border of the syrinx that develops over time after the SCI. The physical barrier of the glial scar and chemical barrier of the inhibitory compounds inhibit axonal regeneration through the site of injury. Adapted from McDonald et al 2002 with permission of Alexander and Turner Studio, FL © Edmond Alexander

Restoration of spinal cord after SCI most likely depends on adequately preventing or limiting secondary necrotic processes, apoptotic cell loss, and removal of regeneration

inhibitory signals. Thus promoting remyelination by oligodendrocytes, restoration of signal conduction, facilitating axonal regeneration and directed sprouting, providing cellular bridges that allow axonal growth through the site of injury, replacing lost cells by transplanting *in vitro* modified stem cells that would be directed toward specific lineages, and providing trophic support to re-establish the functional architecture of the spinal cord and its neurocircuitry (Hulsebosch, 2002; McDonald and Sadowsky, 2002).

A variety of regenerative approaches are under investigation as potential therapies for spinal cord injury with subsequent functional improvement (McDonald et al., 1999; Ogawa et al., 2002; Takami et al., 2002); multipotential stem cells that provide neural progenitor cells represent an attractive intervention for spinal cord repair. This work focuses on the use of human neural stem cells as substrates for cell replacement therapy for spinal cord repair after the contusion injury.

1.2 CELL REPLACEMENT THERAPY

Considering the limited ability of the central nervous to regenerate and repair itself (Ramón y Cajal, 1928) and that the regrowth of endogenous neuronal populations is hindered by the inhibitors such as Nogo and myelin-associated neurite growth inhibitory proteins (Thallmair et al., 1998; Caroni and Schwab, 1988; Schnell and Schwab, 1990; Schwab, 2004), using transplantable stem cells is an attractive approach for cell replacement therapy. The cell based therapies rely on the potential of the grafts to remyelinate (Liu et al., 2000; McDonald et al., 2004), establish cellular bridges across the site of injury (Wirth, III et al., 2001; Bunge, 2002; Zompa et al., 1997), and provide neurotrophic support (Jones et al., 2001; Lu et al., 2003).

Transplantation of nonproliferative cells that are preprogrammed *in vivo* to differentiate predictably and consistently into specific neural phenotypes, thus replacing

the lost cell populations at and near the site of injury, is an extremely attractive strategy for treatment of spinal cord injury. For instance, *in vitro* modified stem cells grafted into the chronically injured spinal cord of adult rats survive, differentiate, integrate into spinal cord architecture and enhance functional recovery by remyelinating axons through their differentiation into oligodendrocytes (Liu et al., 2000; McDonald et al., 1999)

Furthermore, grafted stem cells may provide neurotrophic and neurotropic factors that support the endogenous cell populations by secreting such compounds at the site of injury, allowing precise targeting and exposure, thus enhancing regeneration (Whittemore et al., 1997; McTigue et al., 1998; Lu et al., 2003)

Human neural stem cells (hNSCs) have the potential to augment recovery from injury to the CNS. The cytokines following injury are in part responsible for regulation of neural stem cell proliferation and differentiation from the inert state (Liberto et al., 2004). NSCs serve as rebuilding blocks for regeneration and cell replacement. Cytokine activated astrocytes after injury secrete factors such as basic fibroblast growth factor (bFGF) that stimulate neurogenesis and promote neuron survival (Albrecht et al., 2002; Song et al., 2002; Nakayama et al., 2003).

Additionally, there is a possibility that transplanted cells play a role in attenuation of glutamate exitotoxicity by increasing the glutamate uptake. It was shown previously that glial restricted progenitor derivatives expressed functional glutamate transporters (Maragakis et al., 2004). Excess glutamate is sequestered by astrocytes via two transporters GLT-1 and GLAST. The levels of GLT-1 and GLAST are upregulated in astrocytes by cytokines (Aronica et al., 2003), injury (Krum et al., 2002), *in vitro* addition of EGF (Zelenaia et al., 2000) and FGF, as well as by EGF and IGF-1 (Suzuki et al., 2001) treatment. Incidentally, EGF and bFGF are part of the proliferation media for hNSCs employed in this study (discussed later in the text). Therefore, it is possible that this heterogeneous mix of neuronal and glial precursors are stimulated to express

glutamate transporters *in vitro* and subsequently *in vivo* after transplantation into contusion injured spinal cord. Thus the grafted hNSCs may provide neuroprotective effects to the endogenous cell population via glutamate sequestering and thus reduce excitotoxicity.

1.3 HUMAN NEURAL STEM CELLS

Stem cells sparked considerable enthusiasm within the scientific community as a potential donor source for transplantation therapy in various fields including SCI. Considering their pivotal roles during development and maintenance of tissues, stem cells possess two valuable features, including the ability to self-renew and to differentiate into multiple lineages. The range of cell types generated by a stem cell is defined as its potency, which is variable depending upon the type of the stem cell. Under optimal conditions totipotent stem cells (zygote derivatives) can generate the entire organism, organs or tissues. The inner cell mass of a blastocyst contains pluripotent stem cells that are unlimited in differentiation potential, however, cannot create an entire organism. Further lineage restrictions in differentiated progeny apply to precursors and progenitors, which contain multipotent stem cells that are limited to one or a few specific lineages belonging to a specific tissue type. Multipotential stem cells have been isolated from human fetal (Svendsen et al., 1998; Carpenter et al., 1999; Vescovi et al., 1999; Flax et al., 1998) and adult tissues (Palmer et al., 2001; Kirschenbaum et al., 1994). These cells can be successfully cultured long-term *in vitro* obtaining large numbers and differentiating into the major neural lineages. The clinically relevant advantage of multipontential stem cells is that they can express foreign genes (Martinez-Serrano and Bjorklund, 1997) and respond to the environment. However, loss of properties and limited differentiation potential was recorded after prolonged passaging and under the injury conditions *in vivo*(Quinn et al., 1999). Several factors determine the plasticity of the stem cells: when the stem cells were isolated during stages of development; regions of

the isolation; response to mitogens and differentiation factors; and the subsequent differentiation pattern in intact and injured spinal cord. These factors remain to be fully elucidated. One of the important issues to address is the optimal culturing conditions of the stem cells that would enable long term stable expansion of these stem cells lines *in vitro* while maintaining stable differentiation potentials.

Neural stem/progenitor cells have been isolated from embryonic and adult central nervous system (Reynolds and Weiss, 1992; Svendsen et al., 1998; Arsenijevic et al., 2001; Vescovi et al., 1999; Svendsen et al., 1998; Uchida et al., 2000). Neural progenitor cells have also been successfully isolated and propagated from human postmortem tissues and surgical specimens (Palmer et al., 2001), which proposes a possible solution to countless ethical dilemmas associated with stem cell research in general and donor sources in particular. While the efficacy of the cells based on their source is an ongoing debate, specific culturing conditions play an integral and perhaps pivotal role in stem cells preparation for clinical applications. Culturing neural stem/progenitor cells from adult and fetal tissues has become routine practice (Palmer et al., 1997; Reynolds and Weiss, 1992; Svendsen et al., 1998; Fricker et al., 1999; Gage, 2000). These cells have been successfully propagated under specific conditions for up to 2 years *in vitro* (Svendsen et al., 1999; Vescovi et al., 1999; Carpenter et al., 1999). However, culture conditions differ as do phenotypic outcome after long term culture. One of the goals of this investigation is to elucidate systematically the effect of various long term culture conditions on the human neural stem cells.

Currently, genetic and epigenetic modifications of stem cells *in vitro* are employed to promote proliferation of stem cells *in vitro*. Immortalization of stem cells constitutes genetic manipulation of the cells by transduction and chromosomal integration of immortalizing genes such as v-myc, SV40 and hTERT (Cattaneo and Conti, 1998; Martinez-Serrano and Bjorklund, 1997; Rao and Anderson, 1997; Onifer et al., 1997b; Whittemore and Onifer, 2000; Roy et al., 2004). Immortalized neural stem cells can be

continuously expanded thus providing infinite source of grafting material. Genetic manipulation can also be used to induce neural stem cells to deliver therapeutic transgenes to the site of injury after grafting. However, genetic modifications do raise the issue of clinically undesirable chromosomal aberrations (Whittemore, 1999). Considering this potentially adverse result of genetic manipulation, we chose to use the epigenetic method of expansion in this study.

Epigenetic expansion of stem cells entails application of exogenous mitogens to culture medium to maintain stem cell proliferation. Human neural stem cells have been expanded with either bFGF (Amit et al., 2000; Flax et al., 1998; Zhang et al., 2001); EGF (Reynolds and Weiss, 1996); bFGF and EGF (Arsenijevic et al., 2001; Caldwell and Svendsen, 1998; Vescovi et al., 1999); EGF and LIF (Svendsen et al., 1998; Wright et al., 2003); or EGF, bFGF and LIF (Carpenter et al., 1999; Uchida et al., 2000; Wu et al., 2002a). Several of these studies reported a synergistic effect of these growth factors and cytokines on proliferation of human neural stem cells (Caldwell and Svendsen, 1998; Carpenter et al., 1999; Wright et al., 2003). Developmentally, both EGF and bFGF play important roles in the regulation of stem cell proliferation. Effects of these growth factors on stem cells are particularly dependent on cell origin and stage of differentiation. During early embryonic development, neural stem cells express bFGF receptors (Qian et al., 1997; Tropepe et al., 1999) and respond to the proliferative effects of bFGF. The EGF receptor is expressed later in embryonic development (Kalyani et al., 1999) leading neural stem cells to an EGF-responsive fate (Ciccolini and Svendsen, 1998). Furthermore, EGF receptors have been shown to mediate the induction of the astrocyte phenotype by LIF (Viti et al., 2003). Although EGF supports proliferation and differentiation of astrocytes, it does not limit stem cell potential (Johe et al., 1996). Both growth factors, bFGF and EGF, were used to expand human neural stem cells in this study.

Neurotrophic factors affect survival and differentiation of neural progenitors (Caldwell et al., 2001). Ciliary neurotrophic factor (CNTF) directs human neural progenitor cells (hNPCs) toward astrocyte lineage and increases the number of GABA-ergic phenotype neurons. Brain derived neurotrophic factor (BDNF) (Chow et al., 2000), neurotrophins 3 and 4 (NT3 and NT4) (Caldwell et al., 2001) direct hNPC toward neuronal lineage by blocking apoptosis and promoting neuronal survival. Although, some neurotrophic effects on differentiation and maturation have been elucidated, much remains to be clarified, particularly effects of NTFs on hNPCs *in vivo* and effects of combined NTF and their interactions.

Neural stem cells have distinct growth factor requirements, express neural precursor markers, generate neurons and glia *in vitro* and have neural and non-neural lineage potential *in vivo*. The quality of neural stem cells differentiate into various cell phenotypes such as neurons and glia carries great promise for clinical applications (Shihabuddin et al., 1999; Gage, 2000; Lindvall et al., 2004). Transplanted fetal stem cells (Zompa et al., 1997; Roy et al., 2004; Kelly et al., 2004; Quinn et al., 1999; Wu et al., 2002a), embryonic (Kerr et al., 2003; McDonald et al., 1999; Cao et al., 2002; Harper et al., 2004; Ogawa et al., 2002) and adult neural stem/precursor cells (Shihabuddin et al., 2000; Takami et al., 2002; Vroemen et al., 2003) have survived and differentiated into neurons, astrocytes and oligodendrocytes after grafting (Table 1 Graft Review). Consistent with the idea of applying stem cell graft to treat SCI, enhanced improvement in motor function in spinal cord injured rats was observed (Kerr et al., 2003; McDonald et al., 1999; Takami et al., 2002; Ogawa et al., 2002). However, the majority of cells differentiated from transplants in injured spinal cord were not neuronal. Therefore, *in vitro* maintenance and modification prior to transplantation is pivotal to direct hNPC differentiation toward neuronal lineage and will be extremely valuable for replacement of lost neurons of a specific lineage (Wu et al., 2002a; Caldwell et al., 2001; Gage, 2000; McDonald et al., 1999; Shihabuddin et al., 1999).

Cell type and Source	In Vitro Modification Method	Model	Graft Time and Method	Survival time and Detection markers	Function	Reference
Human NRPs Fetal SC	Retroviral immortalization with hTERT bFGF 10 ng/ml	Rat E 17 brain SC Contusion T9	3 days Transuterine injection SC 0.5-1 mm caudal	5-24 weeks MAP2^{+}, TuJ1^{+}, GFAP^{-}, GAD^{+} and GABA^{+}	Not tested	Roy et al., 2004
Human EBD Embryonic germ cell derivatives	5% serum, bFGF, EGF, VEGF, IGF1 collagen 1I biomatrix	Rat raNSV Sindbid virus paralyses	8 days after raNSV Cannula	12-24 weeks CNPase^{+}, O4^{+}, TuJ1^{+}, GFAP^{+}, ChAT^{+}, MAP2^{+}, synaptophysin^{+} BrdU^{+}	BBB DFG Supine righting (+)	Kerr et al., 2003
Mouse ES	RA	Rat SC Contusion T9	9 days Epicenter	2-5 week APC CC1^{+} - 43%, GFAP^{+}-19% NeuN^{+}- 8% M2	BBB (+)	McDonald et al., 1999
Rat Schwann cells OEG	Purified	Rat SC Contusion T9	7 days Epicenter	12 weeks GFAP^{+}, CSPG^{+}, Tissue spearing	BBB (+)	Takami et al., 2002
Rat NRP E14 SC	bFGF and NT3 RA	Rat Contusion T8	10 days T7 and T9 segments	2w, 1m, 2m Normal: ChAT^{+}, Glut^{+}, GAD^{+}, TuJ1 Contused: Nestin+, MAP2+, TuJ1+	Not tested	Cao et al., 2002
Mouse ES	Shh, RA, astrocyte co-culture	Rat raNSV Sindbid virus paralyses	14-17 days Lumbar enlargement	GFP^{+}, NF^{+}, neural agrin^{+}, synaptophysin^{+}, agrin, ChAT^{+}	Not tested	Harper et al., 2004
Human Fetal brain hCNS	EGF, LIF, bFGF20ng/ml	Rat dMCAo Ischemia	7 days Peri-infarct area	TuJ1^{+}, GFAP^{+}, NG2^{-}, doublecortin^{-}	Not tested	Kelly et al., 2004
Rat NPC E14.5 SC	bFGF 20 ng/ml	Rat SC Contusion C4-C5	9 days Intraspinal cavity	5 weeks BrdU^{+}, Hu^{+}, GFAP^{+}, CNP^{+}	Pellet test (+)	Ogawa et al., 2002
Rat NPC Adult SC	Retroviral GFP bFGF 20 ng/ml	Rat Dorsal CST Transection	Same day Epicenter	3 weeks BrdU^{+}, NG2^{+}, BLBP^{+}, GFAP^{+}, GalC^{+}, TuJ1^{-}	Not Tested	Vroemen et al., 2003

Table 1: Stem Cell Transplantation in Spinal Cord and Test of Function

1.4 NEUROTROPHIC FACTORS

Neurotrophic factors regulate development of the mammalian nervous system by inducing proliferation, survival and differentiation of cell populations within the nervous system. Neurotrophic factors delivered into the injured spinal cord stimulate neuronal survival and regeneration (Widenfalk et al., 2001; Blesch and Tuszynski, 2001; Lu et al., 2001; Grill et al., 1997; Tuszynski et al., 1997; Schnell et al., 1994) Lack of trophic support after SCI hinders spontaneous sprouting with reported partial regeneration of the adult spinal cord (Widenfalk et al., 2001). Also expression of NTFs post SCI in adult and neonate spinal cord is different (Nakamura and Bregman, 2001). Transplantation of nerve tissue and delivery of different neurotrophic factors to promote functional recovery after spinal cord injury have been attempted in the past (Tatagiba et al., 1997; Tuszynski et al., 1997). Exogenous neurotrophic factors and neurotransplantation have been successful (Lacroix and Tuszynski, 2000; Bregman et al., 1998), however, responses to neurotrophic factors differ among diverse neuronal subpopulations. Survival and regeneration of corticospinal neurons *in vivo* is promoted by administration of BDNF, NT3, GDNF (Schnell et al., 1994). GDNF also supports phenotype of injured motor neurons, promotes axon growth and modulates CGRP (growth related neuropeptide), therefore facilitating motor neuron regeneration (Blesch and Tuszynski, 2001). IGF-1 also promotes motor neuron survival (Arsenijevic and Weiss, 1998; Kaspar et al., 2003). BDNF has a greater neuroprotective effect rather than neurotropism (Lu et al., 2001). It has been reported that BDNF seems to block neuroprotection mediated by NT3 (Novikova et al., 2000).

Furthermore, the capacity of NTFs to stimulate and guide stem/progenitor cells toward a specific lineage is of great importance for cell replacement and restoration of tissue loss after the SCI. Neurotrohpic factors and retinoic acid promote neurogenesis in neural stem cell cultures (Takahashi et al., 1999). CNTF has been reported to induce differentiation of astrocytes from stem/progenitor cells, as well as increase percentage of

GABA-ergic neurons in the neuronal sub-population (Caldwell et al., 2001). For these reasons, it is important to reveal NTF interactions *in vivo* prior to designing combination therapy with neurotrophic factors.

Human NPC can be genetically modified to produce and secrete neurotrophic factors (Flax et al., 1998). Delivery of exogenous NTFs will establish an environment within the spinal cord that is favorable for neuronal survival, remyelination, sprouting and axonal regeneration if these factors were delivered in suitable combinations, concentrations and proper sequences (perhaps via inducible promoters). Our previous study (Tarasenko and Wu, 2002) tested the endogenous neurotrophic factors expression in hNSCs and also delivery of insulin-like growth factor (IGF-1) using a recombinant adeno-associated viral vector carrying an IGF-1 transgene (AAV-IGF-1).

1.5 RECOMBINANT AAV DELIVERY SYSTEM

The recombinant Adeno-Associated Viral (rAAV) vector presents one of the most promising gene delivery vehicles for NTF transduction into hNSC. One of the main advantages of AAV is its ability to integrate into both dividing and non-dividing cells and express the programmed transgene in a stable and efficient manner. The linear, single stranded genome (4680bp) of the AAV is reduced by excising viral regulatory (rep) and structural (cap) genes. The remaining two inverted terminal repeats (ITR) sequences (145bp) are vital for replication, integration and packaging of NTFs transgenes. Since 96% of the viral genome is removed, AAV does not provoke cytotoxicity and host immune responses (Muzyczka, 1992; Monahan and Samulski, 2000a). Furthermore, wild type AAV has not been associated with any human disease, therefore making it the safest viral vector for human clinical use. Although, the small size of the AAV vector is a limitation on the length of the gene construct that is feasible to package for transduction delivery, it is not a limitation for small NTF transgenes. Once the transgenes are

delivered into the cells the expression takes place within 2-3 weeks. It is speculated that during this time-lapse, a second strand of DNA is synthesized in the transduced cells prior to NTF transgene expression (Monahan and Samulski, 2000b; Peel and Klein, 2000). The transduction efficiency varies between cells/neurons (Bartlett et al., 1998). The entry of recombinant AAV into cells is dependent on the presence of specific receptor molecules such as sulfate proteoglycans and co-receptors such as FGF receptor 1 (Bartlett et al., 2000; Peel and Klein, 2000; Bartlett et al., 1998). Changes in purification methods can increase rAAV infectious titer and yield (Zolotukhin et al., 1999), which will benefit transduction with rAAV. Furthermore, the addition of a posttranscriptional regulatory element may enhance transgene expression after transduction into mammalian cells (Zufferey et al., 1999). Studies showed that rAAV is able to efficiently transduce and integrate into CNS neurons *in vitro* and *in vivo* (Wu et al., 1998; Kaplitt et al., 1994). Furthermore, rAAV is capable of transducing both dividing and differentiating hNPCs and, subsequently, induce transgene expression. It was shown that hNPCs have survived, differentiated into neurons and glia, and expressed the marker transgenes (EGFP) delivered by rAAV (Wu et al., 2002b). Even of greater significance is the survival of these cells after transplantation into spinal cord and expression of rAAV delivered marker transgenes at and near the transplantation site.

1.6 SUMMARY AND EXPERIMENTAL DESIGN

The use of a contusion injury model for spinal cord injury will allow assessment of the feasibility of using stem cell based therapy to treat the injured spinal cord and facilitate recovery of function. Interventions using *in vitro* expanded and primed human neural stem cells require thorough analyses of the conditions favorable for these stem cells expansion and desired consistency of differentiating phenotypes. In this study, *in vitro* treatment of the human neural stem cells with growth factors and their effect on proliferation and consequent differentiation fates was examined. In order to apply these

in vitro expanded and primed human neural stem cells in a spinal cord contusion injury model we examined the efficacy of their use in SCI treatment at various time points after lesion induction. To examine the alterations in locomotor recovery, the Basso, Beattie and Bresnahan (BBB) open field test scale and a photobeam activity monitoring system were used to measure the behavioral activity changes. It is our aim to contribute to the understanding of stem cell based therapy for treatment of SCI by focusing on the modification and analyses of human neural stem cells *in vitro,* and subsequent assessment of the culture conditions on survival, differentiation and effect of these transplants on the restoration of function in a contused spinal cord. Viable stem cell therapy based interventions are a desirable goal for improved treatment results in SCI patients. We chose to elucidate the use of hNSCs in cell based therapy in a rat model of the spinal cord contusion injury and their potential for the future clinical efficacy.

***Hypothesis 1*:** Long term expansion of human neural stem cells (hNSCs) requires a combination treatment of EGF, bFGF and LIF in order to achieve optimal proliferation of hNSCs. (CHAPTER 3).

Specific Aim 1: To show that EGF, bFGF and LIF combined are required for optimal hNSC expansion *in vitro*.

Experiment 1.1: Trypan Blue cell exclusion cell counts were performed to assess viable cell numbers during long term expansion.

Experiment 1.2: Cell viability assay using a WST-1 reagent was carried out to assess long term and short term affect of growth factors on hNSC.

Experiment 1.3: BrdU incorporation assay was carried out to analyze hNSCs proliferation rates during long term expansion.

Hypothesis 2: Human NSCs expanded under different growth factors respond to priming procedure differently in terms of the differentiation pattern. (CHAPTER 3)

Specific aim 2: To elucidate the effect of proliferation conditions on cell differentiation with and without priming of hNSCs.

> **Experiment 2.1:** Western Blot analysis of hNSC phenotypes after long term proliferation was performed, quantified, and compared between groups.
>
> **Experiment 2.2:** After proliferation under various combination treatments and subsequent differentiation, hNSCs were analyzed immunocytochemically for phenotypic outcome with and without the priming procedure.

Hypothesis 3: Human NSCs survive and differentiate after grafting into uninjured and contused spinal cord and have an effect on locomotor recovery after the SCI. (CHAPTER 4).

Specific aim 3.1: To determine whether grafted hNSCs survive and differentiate in uninjured and contused spinal cord.

> **Experiment 3.1.1** Identify primed and unprimed hNSCs grafted into injured spinal cord and distinguish transplanted cells from the endogenous cell population.
>
> **Experiment 3.1.2** Assessing primed and unprimed cell survival using immunohistochemical analyses of hNSCs three months after grafting into injured spinal cord on the same day, 3 days and 9 days after the contusion injury.
>
> **Experiment 3.1.3:** Assessing primed and unprimed cell differentiation using immunohistochemical analyses of hNSCs three months after grafting into injured spinal cord on the same day, 3 days and 9 days after the contusion injury.

Specific aim 3.2: To determine whether engraftment of hNSCs enhance locomotor recovery after SCI.

Experiment 3.2.1 Behavior assessment of whether locomotor function and/or recovery after SCI was altered by hNSCs after SCI using the BBB locomotor rating scale.

Experiment 3.2.2: Assessment of spontaneous exploratory behavior after SCI and hNSCs grafts using the open field activity system.

CHAPTER 2: MATERIALS AND METHODS

2.1 CULTURE OF HUMAN NEURAL STEM CELLS

2.1.1 Cell source and expansion

Human fetal neural stem cells (line K048, generously provided by C.N. Svendsen, University of Wisconsin) were isolated from an 8-week fetal forebrain. Cells were cultured as free floating "neurospheres" in 75cm^2 flasks with growth medium containing DMEM (high glucose, L-glutamine)/Hams-F12 (3:1) (Invitrogen/GIBCO, Grand Island, N.Y., USA), and supplemented with 15 mM HEPES (Sigma), 1.5% D-glucose (Sigma), penicillin/streptomycin (67 I.U./ml/67 μg/ml; CellGro), N2 [(Bottenstein and Sato, 1979); 25 μg/ml bovine insulin, 100 μg/ml human transferrin, 100 μM putrescine, 20 nM progesterone, 30 nM sodium selenite (Sigma)], 20 ng/ml recombinant human EGF (R&D Systems), 10 ng/ml recombinant human bFGF (R&D Systems), 2.5 μg/ml heparin (Sigma), 10 ng/ml recombinant human LIF (Chemicon), and 2 mM L-glutamine (Sigma). Insulin stock was stored at 4^{o}C for up to 6 weeks and a mixture of other components of N2 at -80^{o}C for 3-4 months. Use of these reagents after longer storage resulted in a decline of activity and slower proliferation.

Several dissociation methods have been tested in our laboratory to expand hNSCs *in vitro*, including chopping (Svendsen et al., 1998), sieving (passing cells through a 0.38 mm stainless steel mesh), and a combination of mechanical and enzymatic dissociation. The latter has been used as our routine passaging method because of relative simplicity and minimized risk of contamination. Cells were passaged every 10-12 days. Cultured spheres were pelleted by centrifugation at 100 x g for 5 min, resuspended in 0.025% trypsin plus 0.6% glucose (Sigma) dissolved in calcium- and magnesium-free Dulbecco's phosphate buffered saline (CMF-dPBS, CellGro), and incubated for 15 min at 37^{o}C with periodic trituration. Occasionally, 250 units/ml DNAse were added to break down

aggregated DNA in rare cases of excessive cell lysis. The reaction was stopped using 1.2 mg/ml of trypsin inhibitor (Sigma) diluted in conditioned medium that was spared from the original cell culture. Cells were triturated with a 5 ml serological pipet and sometimes followed by trituration through a fine tip fire-polished glass pipet to assure separation of individual cells into a single cell suspension. Cell quantification was performed using trypan blue exclusion and hemacytometer counting. After passage, 5-6 x 10^6 cells were seeded into a 75cm^2 flask in 15 ml of conditioned and fresh medium (1:2-3) and incubated at 37^{o}C with 8.5% CO_2 to maintain pH 7.4-7.5. Prior to inoculating cells, new flasks were treated with conditioned medium (3-5ml/75cm^2) at 37^{o}C for at least one hour which helped prevent initial adhesion of cells to the bottom of new culture flasks.

2.1.2 Priming and differentiation

Human NSC differentiation was carried out by plating hNSCs onto German glass coverslips (Carolina Biological Supply) in 24-well plates. Coverslips were pre-treated with 0.01% Poly-D-lysine (PDL; Sigma) in CMF-dPBS for at least one hour at 37^{o}C, and then coated with 1 μg/cm^2 of laminin (LMN; GIBCO) diluted in CMF-dPBS overnight at 37^{o}C. Allowing 2-3 days recovery time after passaging, uniform small clusters of cells were plated onto PDL/LMN-coated glass coverslips at 2 x 10^5cells/1.8cm^2 in a defined serum-free medium including DMEM/F12 (3:1) supplemented with 15 mM HEPES, 1.5% D-glucose, penicillin/streptomycin (67 I.U./ml/67 μg/ml) and B27 (GIBCO). Cells were split into two groups: one was subjected to a priming step (Wu et al., 2002) prior to differentiation in B27 and the other was immediately plated in B27 supplemented medium. Priming involved treating cells with a mixture of 20 ng/ml bFGF, 5 μg/ml heparin and 1 μg/ml laminin for 5 days followed by an 8-day differentiation in B27 supplemented medium. All cultures were fed by renewing one half of either the priming or differentiation medium three times per week.

2.2 CELL PROLIFERATION ASSAYS

2.2.1 Cell growth curves

To evaluate the effect of EGF, bFGF, and LIF alone or in combinations on cell proliferation, 7 treatment groups were established: E (EGF), F (bFGF), L (LIF), EF, EL, FL, and EFL (Tarasenko et al., 2004b). Each treatment was maintained for over 6 passages. Cells were fed by changing two thirds of the growth medium twice weekly and passaged every 10-12 days using the enzymatic and mechanical protocol described above. Viable cells were counted with hemacytometer. Cell growth curves were generated by counting cells from a sampled flask of each treatment at each passage, and then plotting yields with the assumption that all cells from the sampled flask were seeded for further cultivation. Briefly, 5 x 10^6 cells per treatment group were seeded into 75cm^2 flasks in 15ml medium in duplicate at the beginning of each passage. At the end of each growth period (10-12 days), total cell numbers were obtained by counting live cells using the trypan blue exclusion assay. The total number of cells [N_P] was then divided by the 5 x 10^6 cells [N_{seeded}] to obtain a multiplying coefficient, which was used to multiply the total cell number [N_{P+1}] at the following passage to estimate the potential total cell number [N_{ET}] if all rather than only one flask was plated:

$$\mathbf{N_{ET} = N_P / N_{seeded} \times N_{P+1}}$$

2.2.2 WST-1 assay

Cell number and viability were further assessed using the reagent WST-1 (Roche). This colorimetric assay measures the metabolic activity of viable cells based on cleavage of the tetrazolium salt WST-1 into formazan by mitochondrial dehydrogenase in live cells. Cells under different treatments (n = 17 of each group) were plated at 1x10^4 cells/0.32cm^2 in a 96-well plate, and were maintained for 48 hours at 37°C with 8.5%

CO_2. This was followed by incubation with WST-1 reagent at a dilution of 1:10 in the original conditioned media for four hours. To generate baseline controls, $1x10^4$ cells/0.32cm^2 were plated and immediately exposed to WST-1 without the 48 hr. growth period. After thorough shaking, the formazan produced by the metabolically active cells in each sample was measured at a wavelength of 450 nm by an ELx800uv Universal Microplate Reader (Biotek Instruments Inc). Absorbance readings were normalized against control wells with medium alone. Statistical significance of the doubling rate between groups was determined using one way ANOVA and Tukey-Kramer Multiple Comparison tests.

2.2.3 BrdU incorporation and DNA content flow cytometry

Cells were cultured as free floating neurospheres at an approximate density of 2 x 10^6 cells/4ml/25cm^2 and maintained in various combinations of treatments through 2 passages (21 days total). For BrdU pulse labeling, cells were incubated for one hour with bromodeoxyuridine (BrdU) at a final concentration of 10 μM in culture medium. Subsequently, cells were trypsinized, fixed, permeabilized, and stained with anti-BrdU fluorescent marker using a BrdU Flow Kit (BD Pharmingen, San Diego, CA). For two-color flow cytometric analyses to enumerate and characterize cell cycle, hNSCs were subjected to a nuclear staining with 7-amino-actinomycin-D (7-AAD) and immunofluorescent staining with a BrdU antibody. The FlowCytometric readings of 2 $x10^4$ cells per treatment were analyzed using a flow cytometer (Becton-Dickinson FACS-Scan). This assay was repeated three times.

2.3 WESTERN BLOT ANALYSIS

Following PBS wash, cells were lysed with SDS lysis buffer (2% w/v SDS, 5mM EDTA, 50mM Tris base, 1mM each DTT and PMSF, and 1X protease inhibitor cocktail) (all from Sigma, St. Louis, MO), and then sonicated briefly before centrifugation at 20,000 xg for 10 minutes at 4°C. Supernatant was removed and quantified using BCA protein quantification kit (Pierce, Rockford, IL). Thirty μg total protein was diluted in 4X NuPAGE® LDS sample buffer and 10X NuPAGE® reducing agent (both from Invitrogen, Carlsbad, CA) and heated to 70ºC for 10 min. Samples were briefly vortexed, centrifuged and loaded onto 4-12% Nu PAGE® Novex Bis Tris Gels and electrophoresed on ice at 150V for 2 hrs. Gels were transferred onto Hybond ECL nitrocellulose membrane (Amersham Biosciences, UK) on ice by electrophoretic transfer at 30V for 1h. 5% nonfat milk (w/v in 0.1%TBS-tween) was used for blocking overnight at 4ºC, followed by a 1-hour incubation at room temperature with mouse anti-β III tubulin (Covance, Berkeley, CA), rabbit anti-glial fibrillary acidic protein (GFAP), or mouse anti-human Nestin (both from Chemicon, Temecula, CA) primary antibodies. Membranes were then washed 6X, 10 minutes each, with 0.1% TBS-Tween and incubated for 1 hour at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, UK). Membranes were again washed 6X, 10 minutes each, and immunoreactive bands were detected using a chemiluminescent Western blot detection kit (Amersham Biosciences, UK). Blots were exposed to ECL hyperfilm (Amersham Biosciences, UK), for 30 seconds to 5 minutes prior to developing. All blots were re-probed for mouse anti-β actin (Sigma, St. Louis, MO) using a similar protocol. Films were subsequently scanned, and densitometry analyzes were performed using an AlphaEase FC™ Software program (Alpha Innotech). All data were normalized against β-actin. Statistical significance between groups was determined using one way ANOVAs with Tukey post hoc tests.

2.4 IMMUNODETECTION

2.4.1 Immunocytochemistry

Cells differentiated on PDL/LMN-coated coverslips were fixed for 20 min with 4% paraformaldehyde in phosphate buffered saline (PBS) at room temperature and rinsed three times with 0.1 M PBS (pH 7.4). Cells were subsequently permeablized for 30 min at room temperature with 0.1-0.25% Triton X-100 (Sigma) and blocked by applying 5% bovine serum albumin (BSA, Sigma) with 2% normal goat serum (NGS) or 5% normal donkey serum (NDS) depending on the secondary antibody. Primary antibodies (see Table 2.1) were diluted in 1% BSA at the following working concentrations: neuronal class III β-tubulin (TuJ1) 1:2000 (BAbCO), glial fibrillary acidic protein (GFAP) 1:1000 (Chemicon), anti-brain-derived neurotrophic factor (BDNF) 1:200, anti-glial cell line derived neurotrophic factor (GDNF) 1:200, anti- neurotrophin 3(NT3) 1:200, anti-Insulin like growth factor 1 (IGF-1) 1:100 and anti-M2 1:200, γ-aminobutyric acid (GABA) 1:1000 (Sigma), glutamate 1:4000 (Sigma), human nestin 1:100 (provided by Conrad Messam, NIH), and choline acetyltransferase (ChAT) 1:100 (Chemicon).

Table 2.4.1 Immunocytochemical markers for hNSC differentiation.

Cells	Marker	Dilution	Manufacturer
Neural progenitors	Nestin	1:100	C.A. Messam, NIH
Glial precursors	Glial fibrillary acidic protein (GFAP)	1:1000	Chemicon
Neurons	Neuronal class III β-tubulin (TuJ1)	1:2000	Babco
GABAergic neurons	γ-aminobutyric acid (GABA)	1:1000	Sigma
Glutamatergic neurons	Glutamate	1:4000	Sigma
Cholinergic neurons	Choline acetyltransferase (ChAT)	1:100	Chemicon
	Glial cell line-derived neurotrophic factor (GDNF)	1:200	Chemicon
	Brain derived neurotrophic factor (BDNF)	1:200	Chemicon
	Neurotrophin 3 (NT3)	1:200	Chemicon
	Insuline Growth Factor 1 (IGF-1)	1:100	Chemicon
	M2 flag marker	1:200	Sigma

Cells were incubated with primary antibodies overnight at 4^{o}C and rinsed with PBS prior to secondary antibody application. Alexa Fluor 594 – conjugated secondary antibodies including goat anti-mouse, goat anti-rabbit, or donkey anti-goat (Molecular Probes), were diluted 1:200 in 1% BSA/PBS and applied to cells for one hour at room temperature in the dark. Cells were subsequently washed in PBS and cell nuclei were counterstained with 1 μg/ml DAPI (Sigma) in PBS. Coverslips with cells were mounted onto glass slides with Fluoromount-G (Fisher). For quantitative analyses, ten fields (more than 200 cells) per phenotype were randomly selected from 3-4 coverslips with cells cultured in a monolayer. Percent positive staining for any particular antibody was calculated by dividing the number of positive cells by the total number of DAPI-stained nuclei.

2.4.2 Immunohistochemistry

To assay the survival and differentiation of grafted hNSCs, spinal cords were processed for histological analyses. The subjects were anesthetized with pentobarbital and transcardially perfused with 0.1 M phosphate buffered saline (PBS, pH 7.4) followed with 4% paraformaldehyde. Graft-containing spinal cord segments were postfixed in 4% paraformaldehyde and cryo-protected with a sucrose gradient of 5%, 10% and stored in 30% sucrose at 4^{o}C overnight. Segments were embedded in OCT compound, longitudinally sectioned at 45μm on a cryostat and stored at -80 ^{o}C. Prior to immunofluorescent staining, tissue sections were washed with tris-buffered saline (TBS), permeablized for 30 min at room temperature with 0.25% Triton X-100 (Sigma) and blocked by applying 0.3% bovine serum albumin (BSA, Sigma) with 5% normal goat or donkey serum depending on the secondary antibodies. Primary antibodies (Table 2.2) were diluted in 0.3% BSA/TBS at the following working concentrations: polyclonal anti-microtubule associated protein-2 1:1000 (MAP2, Chemicon), mouse anti-lysosome associated membrane protein-2 1:1000 (LAMP2, Developmental Studies Hybridoma Bank, Iowa City, Iowa), goat anti-choline acetyltransferase 1:100 (ChAT, Chemicon), rabbit anti-glial fibrillary acidic protein 1:1,000 (GFAP, Chemicon), mouse anti-human Nestin 1:200 (C.A. Messam, NIH) and mouse anti-Ox-42 1:100 (Chemicon).

Table 2.4.2 Immunohistochemical markers for grafted hNSC differentiation

Cells	Marker	Dilution	Manufacturer
Neural progenitors	Nestin	1:200	C.A. Messam, NIH
Human NSC	Lysosome associated membrane protein-2 (LAMP2)	1:1000	Developmental Studies Hybridoma Bank
Glial precursors	Glial fibrillary acidic protein (GFAP)	1:1000	Chemicon
Microglia	Ox-42 1:100 (Chemicon)	1:100	Chemicon
Neurons	Microtubule associated protein-2 (MAP2)	1:1000	Chemicon
Cholinergic neurons	Choline acetyltransferase (ChAT)	1:100	Chemicon

Tissue sections were incubated with primary antibodies overnight at 4^{o}C and rinsed thrice with TBS prior to secondary antibody application. Alexa Fluor 568 and 488 conjugated secondary antibodies including goat anti-mouse, goat anti-rabbit, or donkey anti-goat (Molecular Probes) were diluted 1:300 in 0.3% BSA/TBS and applied to cells for three hours at room temperature in the dark. Tissues were subsequently washed in TBS and cell nuclei were counterstained with 1 μg/ml DAPI (Sigma) in TBS. The sections were preserved with anti-fade Fluoromount-G (Fisher) under coverslips. Imaging was conducted using fluorescent Nikon Eclipse 800, Zeiss Axiophot, LSM 510 microscope and a BioRad confocal system.

2.5 CONSTRUCTION OF RECOMBINANT AAV VECTOR PLASMID.

Transgenes carrying neurotrophic factors were constructed as described (Wu et al., 2002b). Briefly, the transgene flanked by Flag (synthetic marker sequence) and ligation sites was PCR amplified using a pair of primers: 5' – CGC GGA TCC ACC ATG GGG AAA ATC AGC AGT - 3' and 5'– GGA CTA GTC TAC TTG TCA TCG

TCG TCC TTG TAG TCT TTG TGT TCT TCA AGT GTA CTT CC - 3'. The transgene cassette was then released from the plasmid using enzymatic restriction digest by BamH1 and SpeI and inserted into a plasmid with a chimeric promoter with CMV immediate early enhancer and a chick b-actin promoter, and a polyadenilation signal (pA). The plasmid was transformed into Top 10 bacteria for expansion, and then extracted and purified using EndoFree Plasmid Maxi Kit (Qiagen Inc.). The functionality of the transgenes was assayed by transfecting human fibroblasts and detecting transgenes with flag immunocytochemically. A recombinant AAV vector was prepared by using three plasmid cotransfection of human embryonic kidney cells (293 cell line) based on previous protocols (Wu et al., 2002b). The three plasmid co-transfection method developed by Xiao involved an AAV helper plasmid (pXX2) and an AV helper plasmid (pXX6). Human embryonic kidney cell line 293 was cultured in 150 mm dishes DMEM/10% FBS at 37°C, 5% CO_2. When cells reached 80% confluence, calcium phosphate precipitation method was used for co-transfection with pCW-IGF-1, pXX2 and pXX6. Following a brief rinse with DMEM, OptiMEM (Life Technologies)/10% FBS/120 μM chloroquine was added to the cells. Then 2.5 ml of DNA–calcium phosphate solution was added per plate. The solution contained three plasmids at the molar ratio of 7:2:4, 125 mM $CaCl_2$ and 1 × HBS (2.5 M NaCl, 0.25 M HEPES, 75 mM Na_2HPO_4, pH 7.1). Cells were cultured with 5% CO_2 at 37°C for 18 h, and changed with OptiMEM/10% FBS. Two days after co-transfection, cells and medium were collected, centrifuged at 1140 *g* for 15 min, and then resuspended in 150 mM NaCl/20 mM Tris pH8.0 at 5×10^6 cells/ml. The cell suspension was further treated with 0.54% deoxycholate (Sigma) and 50 U/ml Benxonase (Sigma) at 37°C for 1 hour. Following centrifugation at 3000 *g* at room temperature for 20 min, supernatants were subjected to a cycle of freeze–thaw, and then centrifuged again at 10000 *g* at 4°C for 30 min. The supernatant was collected, filtered through a 1-μm disk filter (Fisher, Pittsburgh, PA, USA), and then run by gravity through a heparin agarose type I column (Sigma) pre-equilibrated with phosphate buffer saline/1 mM $MgCl_2$/2.5 mM KCl (PBS-MK). After

four washes with 5 ml PBS-MK each, rAAV viruses were eluted by 9 ml of 1 M NaCl/PBS-MK. The first 2 ml was discarded. The following 7 ml was collected, desalted by running through a Centricon Plus-20/Biomax-100 (Fisher) with four changes of lactated Ringer's solution, and concentrated by centrifugation at 3000 *g* at room temperature till reaching a desired volume. The 7 ml of elution was collected.

2.6 ENDOGENOUS AND EXOGENOUS EXPRESSION OF GDNF, BDNF, NT3 AND IGF-1

Human NPCs under proliferation or differentiation as described above were exposed to rAAV vectors carrying transgene at various dosages. For differentiation studies, the cells were plated either in small neurospheres (3-4 days after passage) or dissociated single cells onto 0.01 % Poly-D-Lysine and 0.5 mg/cm2 laminin coated German Glass cover slips. Six days after plating, the cells were either analyzed immunocytochemically in their proliferating stage or differentiated further in DMEM:F12 supplemented with B27 for additional 9 days as outlined in Table 2.3.

Table 2.6. Cell culture paradigms for proliferating and differentiating hNSCs to test endogenous and exogenous expression of neurotrophins.

	Endogenous
Proliferating	FHL 6 days IF
Differentiating	FHL 6 days B27 8 days IF
	Exogenous
Proliferating	FHL 3 days rAAV 3 days IF
Differentiating	FHL 3 days rAAV 3 days B27 9 days IF
	FHL 3 days B27 3 days rAAV 7 days IF

Media were changed every 3 days. Expression of transgene was observed using immunocytochemistry as described above and Nikon epifluorescent microscope.

2.7 SPINAL CORD CONTUSION INJURY

Male Sprague-Dawley rats, 200-250g [Harlan Sprague-Dawley, Inc] were maintained according to the procedures outlined by the NIH Guide for the Care and Use of Laboratory Animals. Subjects were divided into four groups: sham (contusion injury only n= 13), spinal injury + vehicle, spinal injury + unprimed cell graft, spinal injury + primed cell graft. The latter three groups were further divided into subgroups based on various time intervals between grafting and contusion injury: PCD0 (Post Contusion Day 0), PCD3 (post contusion day 3) and PCD9 (post contusion day 9). Specifically, 25 rats (PCD0 n=8, PCD 3 n=7, PCD 9 n=10) received vehicle injection, another 25 rats (PCD0 n=7, PCD 3 n=11 and PCD 9 n=7) received unprimed hNSCs grafts and 28 rats (PCD0 n=8, PCD 3 n=10, PCD 9 n=10) were grafted with primed hNSCs. A moderate spinal cord contusion injury was produced as described previously (Gruner 1992, Hulsebosch 2000). Subjects were anesthetized with an intraperitoneal delivery of pentobarbital (40mg/kg). The absence of flexor response to noxious stimulus was an indication of complete anesthesia. After the subjects' backs were shaved and sterilized, incision through skin and muscle exposed the vertebral column, and a laminectomy was performed at spinal segment T10. The spinal column was then secured with clamps, with the spinal cord exposed, and a contusion injury was produced using the Kentucky impactor (Infinite Horizons, Inc.) with a force of 130 kDynes. Following the contusion injury, muscle and fascia were sutured and the skin incision was closed with autoclips. Subjects received intraperitoneal (i.p.) injection of 1 ml lactate Ringer solution for rehydration, subcutaneous (s.c.) injection of 30mg/kg Baytril as a prophylactic antibiotic and recovered from surgery on a thermal pad. Bladders of all injured rats were expressed manually twice daily until bladder function was restored within two weeks post injury. Post contusion analyses of actual force of impact and tissue displacement indicated no significant differences between groups that might account for behavioral differences.

2.8 TRANSPLANTATION

2.8.1 Cell preparation for grafting

Human fetal neural stem cells (line K048, provided by C.N. Svendsen, University of Wisconsin) were isolated from an 8-week fetal forebrain and expanded in the presence of epigenetic mitogens including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and leukemia inhibitory factor (LIF) in the basal media as described previously (Tarasenko et al., 2004b). These cells were cultured as free floating "neurospheres" at 37^{o}C with 8.5% CO_2 in 75cm^2 flasks with growth medium containing DMEM (high glucose, L-glutamine)/Hams-F12 (3:1) (Invitrogen/GIBCO, Grand Island, N.Y., USA), and supplemented with 15 mM HEPES (Sigma), 1.5% D-glucose (Sigma), penicillin/streptomycin (67 I.U./ml/67 μg/ml; CellGro), N2 (Bottenstein and Sato, 1979); 25 μg/ml bovine insulin, 100 μg/ml human transferrin, 100 μM putrescine, 20 nM progesterone, 30 nM sodium selenite (Sigma)], 20 ng/ml recombinant human EGF (R&D Systems), 10 ng/ml recombinant human bFGF (R&D Systems), 2.5 μg/ml heparin (Sigma), 10 ng/ml recombinant human LIF (Chemicon), and 2 mM L-glutamine (Sigma).

Cells were passaged enzymatically every 10-12 days using 0.025% trypsin plus 0.6% glucose (Sigma) dissolved in calcium- and magnesium-free Dulbecco's phosphate buffered saline (CMF-dPBS, CellGro) and 250 units/ml DNAse. The reaction was stopped using 1.2 mg/ml of trypsin inhibitor (Sigma) diluted in conditioned medium that was saved from the original cell culture. For priming, 2 x10^6 cells were maintained for 7 days in PDL/LMN coated 25 cm^2 flask in 4 ml of media supplemented with 20 ng/ml bFGF, 5 μg/ml heparin and 0.5 μg/ml laminin (FHL), whereas unprimed cellswere not exposed to the FHL priming procedure. Both primed and unprimed cells were transduced using recombinant adeno-associated viral vector, AAVegfp (Wu et al., 2002b) three days after plating and incubated for an additional four days prior to transplantation.

2.8.2 Cell Transplantation

Rats were immunosuppressed with NeOral cyclosporine (Novartis Pharmaceuticals) at 100 μg/ml in drinking water 3 days prior to engraftment surgery and thereafter for the duration of the experiment. Primed and unprimed cells and vehicle injections were performed at three time points: immediately after contusion injury (PCD 0), three (PCD 3) and nine days (PCD 9) after injury. Same day graft recipients were injected with hNSC immediately after the contusion injury under pentobarbital anesthesia. PCD 3 and PCD 9 groups were anesthetized with intraperitoneal administration of ketamine (90 mg/kg) and xylazine (10 mg/kg), and the contusion injury site was re-exposed by removing autoclips from the skin, sutures from muscle and fascia and a small incision was made in the dura. Dissociated primed or unprimed AAVegfp-labeled hNSCs (2×10^5 in 2 μl) were grafted stereotactically into the spinal cord (ML: + 0.5mm from the midline and DV: -1.5mm from the dura) at the lesion epicenter. The same post-surgical treatment was administered as described above. The subjects survived three months after transplantation, underwent behavioral assessment tests described below and were sacrificed for immunohistochemical analyses.

2.9 BEHAVIORAL ANALYSES

2.9.1 Locomotor function

Locomotor function was assessed using an open-field locomotor test and the subjects' hindlimbs were scored according to the Basso, Beatie, and Bresnahan rank scale (BBB) (Basso et al., 1995). The BBB rank scale uses an ordinal non-linear 21 point scale for locomotor evaluation. Scores from 0 (no hindlimb movement) to 7 reflect the return of the isolated movements in the joints of the hip, knee, and ankle. Scores 8 through 13, indicate improvement in paw placement and coordination with the forelimbs. Scores 14 through 21 indicate improvement in toe clearance, paw position, trunk stability, and tail position. The subjects were scored with the BBB scale prior to surgery (score of 21 as a

baseline), then daily until two weeks after transplantation and weekly for three months after surgery. Due to the nonlinear nature of the BBB scale, measurements are reported as medians. Left and right hindlimbs were scored individually, and subjects with hindlimb score differences of greater than 3 points on day 1 post contusion were eliminated from the study in order to standardize the extent and symmetry of the injury. Reported BBB scores were averaged from both hindlimbs for individual subjects.

2.9.2 Spontaneous behavioral Activity

Behavioral activity was also measured using the Photobeam Activity System (PAS) with a FlexField software (San Diego Instruments, Inc.). Movements along the x and y planes were recorded based on the obstruction of 16 photobeams in the "X" direction and 16 photobeams in the "Y" direction 4 cm above the chamber floor. Obstruction of a second set of photobeams positioned 12 cm above the chamber floor allowed recording of movements along the "Z" axis and generated data on rearing events. The PAS records movements of a subject in an activity chamber (40x40x40cm) within a set period of time by recording the number of times the photobeams are obstructed in the X,Y, and Z axis oriented grid system. Six parameters of spontaneous behavioral activity were collected: active time, rest time, distance traveled, counts (number of beams obstructed), rearing events and rearing time. Behavioral activity was recorded at the same time of day, at the beginning of the dark cycle (7pm-9pm), both before surgery (baseline) and three months after transplantation. The subjects were most active during the first 15 min in the activity chamber (Mills 2001) and exploratory behavior subsequently subsided. Therefore, data were recorded only during the first 15 min for each subject in the activity chamber and collected in 5 min intervals. In order to eliminate olfactory stimuli such as urine from the previous test subjects, the chamber was cleaned with Cavicide and alcohol between each test.

2.10 QUANTITATIVE AND STATISTICAL ANALYSES

2.10.1 Statistical tests used to analyze hNSC *in vitro*

One-way analyses of variance (ANOVA) were used for statistical analyses. The InStat (GraphPad Software) and SigmaStat (SPSS Inc.) programs with Tukey-Kramer multiple comparison tests were used to determine statistical significance of variations.

2.10.2 Quantitative analyzes of hNSC *in vivo*

Cell survival was stereologically assessed by counting grafted cell profiles labeled with a human specific LAMP2 marker (Coggeshall, 1992). Five longitudinal 45-μm sections of each spinal cord were selected using systematic random method in which the first section was determined randomly and every fifth section was systematically sampled. Optical dissectors with upper and lower boundaries were determined with confocal planes using a Bio-Rad confocal system with a 20X objective. All grafted cell profiles were counted per section. To determine the percent of surviving cells, the estimated total number of cells per subject was divided by the total number of grafted cells (2×10^5). The percent of specific phenotypes that differentiated from grafted hNSCs was determined by dividing the estimated numbers of neuronal or astroglial immunoreactive cells by the total number of surviving hNSC immunopositive to a human marker. Given that AAVegfp did not achieve 100% transduction efficiency, anti-human LAMP2 marker was used to trace grafted cells of human origin after engraftment *in vivo*.

2.10.3 Statistical tests used to analyze hNSC *in vivo* and behavior

One-way analysis of variance (ANOVA) was performed to evaluate changes between groups. The Kruskal-Wallis test was used for nonparametric analyses of BBB scores between groups. The Holm Sidak *post hoc* test for pairwise comparison was used to analyze average rearing time data. An alpha level of significance of 0.05 was used for

all statistical tests. Data are expressed as means ± standard error of the mean (SEM). BBB scores are reported as medians.

CHAPTER 3: EFFECT OF GROWTH FACTORS ON PROLIFERATION AND PHENOTYPIC DIFFERENTIATION OF HUMAN FETAL NEURAL STEM CELLS[1]

Human fetal neural stem cells (hNSCs) can be expanded *in vitro* by mitogens or growth factors such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and/or leukemia inhibitory factor (LIF). Their effects on proliferation rate and differentiation pattern of hNSCs, however, have not been fully characterized. In this study, we cultured hNSCs in seven regimens including bFGF, EGF and LIF, either alone or in combinations. Cells were maintained as neurospheres in treatment media for various periods of time up to six passages. A combination of bFGF, EGF and LIF expanded hNSCs more efficiently than any other treatment as determined by counting total cell numbers using a trypan blue exclusion assay, a WST cell viability assay, and a BrdU incorporation flow cytometric analysis. Differentiation patterns of hNSCs expanded under different conditions were also analyzed. We reported previously that hNSCs primed *in vitro* with a combination of bFGF, heparin and laminin (FHL) induced neuronal differentiation toward a cholinergic phenotype. In this study, we show that the FHL priming increases neuronal differentiation while decreasing astroglial generation in all treatment groups as determined by immunostaining. However, cells proliferated under different growth factor conditions do vary in their phenotypic differentiation patterns. Particularly, significant generation of cholinergic cells was observed only in hNSCs expanded with EGF/bFGF or EGF/bFGF/LIF, but not with other treatment regimens even when they are exposed to the same priming procedure. Our results indicate that hNSCs are highly plastic with their proliferation and differentiation potential dependent upon different growth factor treatments.

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[1] Tarasenko, et al. *J Neurosci Res.* 78(5):625-36. 2004.

3.1 INTRODUCTION

The emergence of neural stem cell biology offers a prospect to investigate neural cell development and its potential for clinical applications. Multipotent (Carpenter et al., 1999; Villa et al., 2000; Vescovi et al., 1999) human neural stem cells (hNSCs) have been successfully isolated and expanded *in vitro,* short- and long-term, to generate neurons and glia (McKay, 1997; Palmer et al., 1997; Reynolds and Weiss, 1992; Svendsen et al., 1997; Svendsen et al., 1998; Vescovi et al., 1999). These cells hold great promise for a wide range of clinical applications from cell replacement to gene delivery systems. One of the key issues in stem cell research is finding optimal conditions for expanding hNSCs in clinically relevant numbers while maintaining normal karyotype and consistent differentiation capacities. Currently there are two ways to achieve stem cell expansion: by genetic modification via chromosomal integration of a propagating gene such as v-myc or SV40 that establishes an immortalized cell line (Martinez-Serrano and Bjorklund, 1997; Onifer et al., 1997a) or by application of exogenous mitogens (Carpenter et al., 1999; Gage, 2000; Svendsen et al., 1998; Villa et al., 2000). We have chosen the latter with the consideration that genetically immortalized cells may have significant undesirable chromosomal aberrations (Whittemore, 1999)

Previously, human neural stem cells have been expanded with either bFGF (Amit et al., 2000; Flax et al., 1998; Zhang et al., 2001); EGF (Reynolds and Weiss, 1996); bFGF and EGF (Arsenijevic et al., 2001; Caldwell and Svendsen, 1998; Vescovi et al., 1999); EGF and LIF (Svendsen et al., 1998; Wright et al., 2003); or EGF, bFGF and LIF (Carpenter et al., 1999; Uchida et al., 2000; Wu et al., 2002a). Several of these studies reported a synergistic effect of these growth factors and cytokines on proliferation of human neural stem cells (Caldwell and Svendsen, 1998; Carpenter et al., 1999; Wright et al., 2003).

Developmentally, both EGF and bFGF play important roles in the regulation of stem cell proliferation. Effects of these growth factors on stem cells are particularly dependent on cell origin and stage of differentiation. During early embryonic development, neural stem cells express bFGF receptors (Qian et al., 1997; Tropepe et al., 1999) and respond to the proliferative effects of bFGF. The EGF receptor is expressed later in embryonic development (Kalyani et al., 1999) leading neural stem cells to an EGF-responsive fate (Ciccolini and Svendsen, 1998). Furthermore, EGF receptors have been shown to mediate the induction of an astrocyte phenotype by LIF (Viti et al., 2003). However, the majority of the studies on the effects of these growth factors on neural stem cells are based on rodent neural progenitor cells, and it is important to determine whether human neural stem/progenitor cells have similar or different requirements.

Although various growth factors have been used to stimulate growth of human neural stem cells *in vitro*, there are no systematic studies that compare the effects of bFGF, EGF, and LIF on hNSC expansion or subsequent differentiation patterns. Long-term expansion of hNSCs may change their phenotypes, differentiation pattern, and subsequent responses to a priming procedure (Wu et al., 2002a). In this study we focus on elucidating the effects of EGF, bFGF, and LIF individually and in combinations on hNSCs' short- and long-term proliferation, differentiation, and response to FHL priming.

3.2 MATERIALS AND METHODS

3.2.1 Cell Culture

Procedures for cell culture are outlined in CHAPTER 2: GENERAL MATERIALS AND METHODS.

3.2.2 Differential growth factor treatments and cell growth curves

Procedures for differential growth factor treatment and establishing growth curves for various mitogenic regimens are described in CHAPTER 2: GENERAL MATERIALS AND METHODS.

3.2.3 WST-1 assay

Procedures for cell viability analyses are described in CHAPTER 2: GENERAL MATERIALS AND METHODS. Briefly, cells under different treatments (n = 17 of each group) were assayed with WST-1 reagent for viability. Absorbance readings were normalized against control and statistical significance of the doubling rate between groups was determined using one way ANOVA and Tukey-Kramer Multiple Comparison tests.

3.2.4 BrdU incorporation and DNA content flow cytometry

Procedures for cell proliferation rate assessment are described in CHAPTER 2: GENERAL MATERIALS AND METHODS. Briefly, in three independent trials cells under different treatments were terminally pulsed labeled with BrdU. The FlowCytometric readings of 2 $x10^4$ cells per treatment were analyzed using a flow cytometer (Becton-Dickinson FACS-Scan). This assay was repeated three times.

3.2.5 Western blot analysis

Procedures for Western blot analyses are described in CHAPTER 2: GENERAL MATERIALS AND METHODS.

3.2.6 Priming and differentiation

Procedures for cell priming and differentiation is described in CHAPTER 2: GENERAL MATERIALS AND METHODS.

3.2.7 Immunocytochemistry

Procedures for immunocytochemical analyses are presented in CHAPTER 2: GENERAL MATERIALS AND METHODS.

3.2.8 Statistical analyses

One-way analyses of variance (ANOVA) were used for statistical analyses. The InStat (GraphPad Software, San Diego, CA) and SigmaStat (SPSS Inc., Chicago, IL) programs with Tukey-Kramer multiple comparison tests were used to determine statistical significance of variations.

3.3 RESULTS

3.3.1 Effects of growth factors on hNSC proliferation

Human NSCs were seeded into flasks at equal density (5 x 10^6 cells/15ml/75 cm^2) in duplicate and were proliferated as free-floating neurospheres in growth medium supplemented with EGF, bFGF and LIF individually or in combination. Cell expansion rates were monitored by hemacytometer cell counting during routine passaging, WST-1 metabolic assay, and BrdU incorporation flow cytometric analyses. Apparent differences in cell expansion were observed soon after different treatments (Figure 3.1).

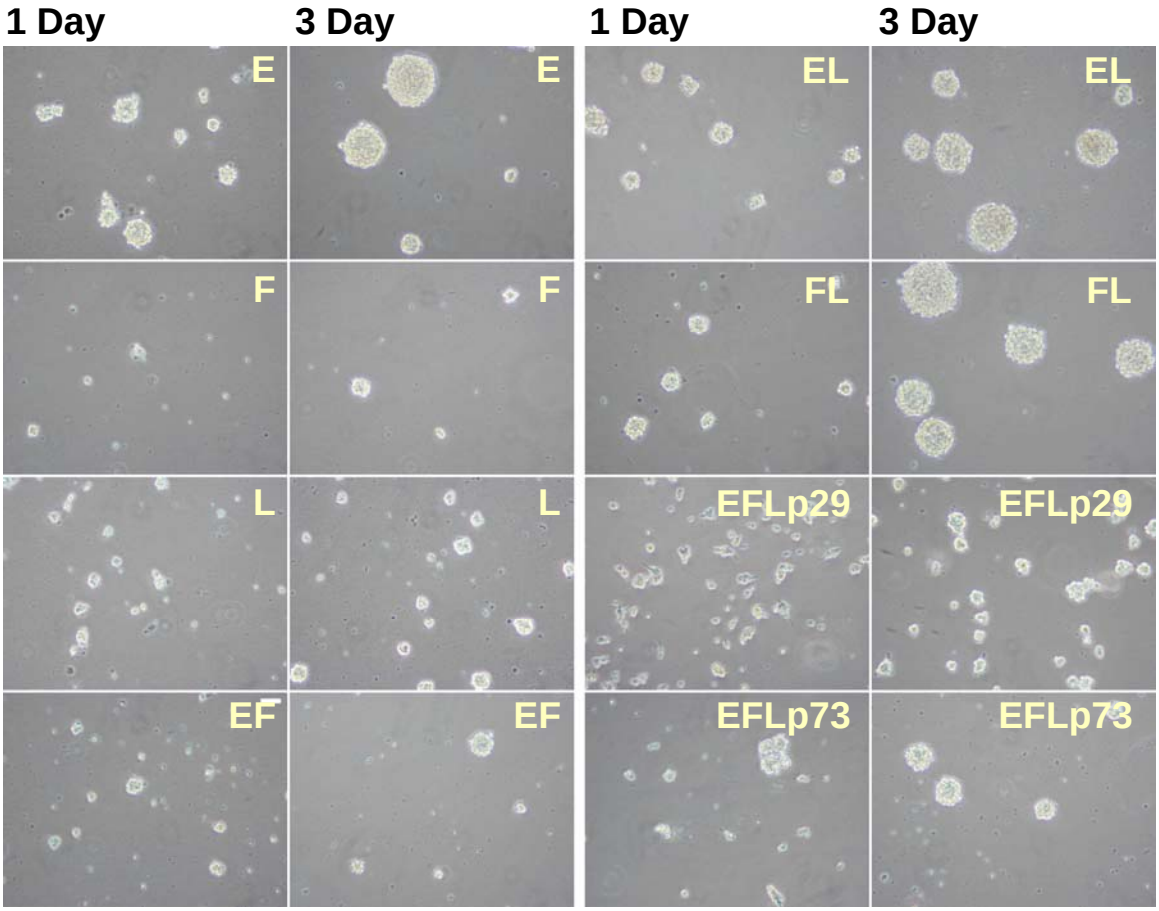

Figure 3.1 Human fetal neural stem cells expanded under various treatments of growth factors as free floating neurospheres. The phase-contrast images were taken at one or three days after passage. E, epidermal growth factor; F, basic fibroblast growth factor; L, leukemia inhibitory factor. Note that the combination treatments of EFL and EF show enhanced sphere size as early as 1 day after passage compared to single growth factor groups. Scale bar = 100 μm.

The effects of a single or combined growth factor treatment were observed as early as one day and became more robust three days after passage. Combination treatments with EF or EFL showed larger sphere sizes and more "hair-like" projections which are a sign of healthy growth. EFL treatment of early passage 29 (EFLp29) and late passage 73 (EFLp73) produced similar sphere size and appearance. Combined growth

factor treatment resulted in a larger sphere size as compared to a single growth factor treated culture. Cells treated with FL were smaller, more adhesive, and had a propensity to attach to the bottom of the flask. Treatment with L alone was not enough to sustain cell growth.

To monitor proliferation of hNSCs under different regimens for prolonged periods in a quantitative way, we traced the growth of hNSCs for 6 passages starting at passage 20 by directly counting the cell numbers at the end of each passage. To eliminate other variations, all treatment groups were passaged simultaneously and regularly once every 10-12 days. Cells were originally plated at 5×10^6 cells/15 ml/75 cm^2 flask and then counted using the trypan blue exclusion assay at the end of each 10 to 12-day growth period. Only 5×10^6 of these cells from each treatment were replated into one flask and allowed to continue growing. At the next passage, the cell number derived from that one flask was multiplied by the hypothetical number of flasks as if all cells under each treatment were plated during the preceding passage. Thus, a cumulative number of cells growing under that particular treatment was estimated and then plotted as a cell growth curve (Figure 3.2).

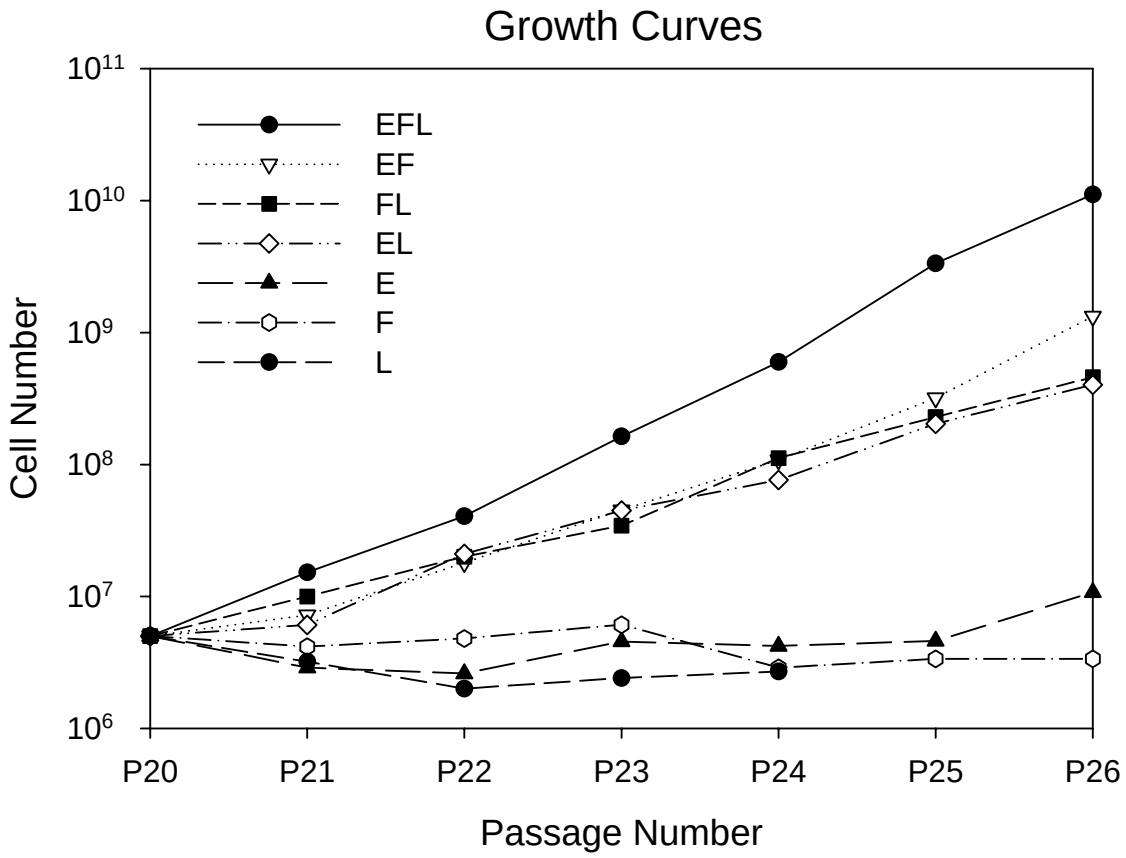

Figure 3.2 Growth curves of human fetal neural stem cells. Cultures were maintained in various combinations of E (EGF), F (bFGF) and L (LIF) for six passages (2 months) *in vitro*. Cell numbers were derived from trypan blue exclusion cell counts at each passage. Cells maintained in media containing single mitogens (E, F and L) have slower growth rates compared to those under combination treatments (EF, EL and FL). Particularly, a combination of all three mitogens (EFL) resulted in the fastest rate of cell growth. Population doubling time over 5 passages is the average of 2 samples that varies less than 10%.

It was apparent that growth medium containing a single mitogen was insufficient to maintain hNSCs in a proliferating stage. For example, cells maintained in medium with EGF, bFGF or LIF alone were found to remain in a static state with only very slight expansion (Figure 3.2). The LIF alone treatment group was eventually eliminated from the study because cell numbers were insufficient for further differentiation assays. Cell proliferation rates were greatest in groups treated with a combination of EGF and bFGF with or without LIF. Addition of LIF promoted exponential cell growth particularly at later stages of proliferation.

3.3.2 WST-1 Cells viability assessment

Proliferation of hNSCs under different growth factor treatments was also determined using a WST-1 colorimetric assay. This included cells under each growth factor regimen after acute or prolonged treatments with growth factors. Acute treatment was for 48 hours with a plating density of 10^4 cells/0.32 cm^2 in 96-well plates. All combined treatments resulted in a significantly higher mitochondrial enzymatic activity than those treated with a single factor (Figure 3A). For long-term studies, cells were maintained as free floating neurospheres (2 x10^6 cells/4ml/25cm^2) for two passages in different treatments, then dissociated and plated under the same conditions for 48 hours prior to the WST-1 assay. Results of the WST-1 assay suggested a synergistic effect of combined EGF, bFGF, and LIF on hNSC proliferation especially in the long-term treatment group (Figure 3.3 B). Cell groups treated with F alone or in combination with LIF (FL) had greater rates of proliferation compared to E alone (E) or combined with LIF (EL), which may have resulted from either enhanced mitochondrial enzymatic activity per cell, increased cell numbers, or both. Addition of LIF to either E (EL) or EF (EFL) medium resulted in a statistically significant increase in cell numbers ($P<0.001$) compared to EF or E groups without LIF in short- and long-term treatments.

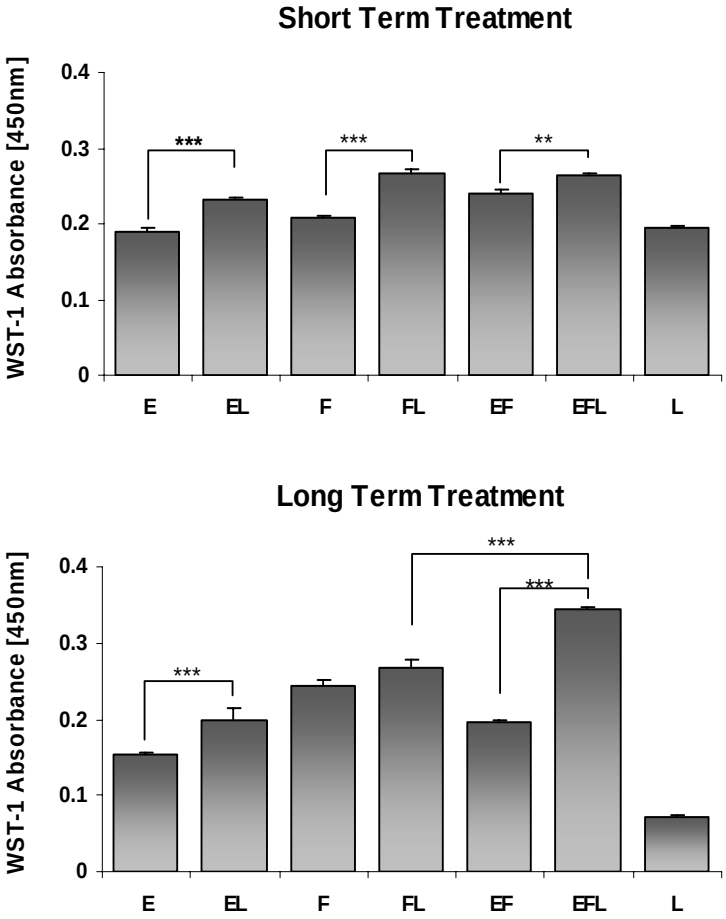

Figure 3.3 Effect of growth factors on human neural stem cell numbers evaluated by WST-1 assays. Cells were exposed to short (A) or long (B) term treatment with various combinations of EGF, bFGF and LIF. For short term study, cells originally expanded in EFL were dissociated and plated with various combination treatments for 48 hours prior to WST-1 assay. Treatments with 2 or 3 growth factors resulted in significantly higher absorbance, indicating higher numbers of viable cells, than those treated with a single factor ($n = 46$, *** $P < 0.001$ and ** $P < 0.01$, Tukey-Kramer Test). For long term study, cells were maintained as free floating neurospheres for two passages in different combination treatments, then dissociated and plated under the same conditions for 48 hours prior to WST-1 assay. Samples treated with L alone show the lowest absorbance indicating the least number, while EFL-treated samples have highest absorbance indicating the highest number of viable cells ($n = 18$, *** $P < 0.001$). Error bars, ± S.E.M.

3.3.3 BrdU incorporation and proliferation rate assessment

BrdU incorporation flow cytometry showed that combined treatments (FL, EL, EF and EFL) resulted in a greater percentage of cells undergoing S-phase (Figure 3.4). Proliferation rates of hNSCs under different growth conditions were measured by three independent trials, where cells were cultured as free floating neurospheres at 2 x 10^6 cells/4ml/25cm^2 in various combinations of treatments through 2 passages, and then BrdU was pulse-labeled for an hour at the termination of the experiment. The flow cytometry reading of 2 x10^4 cells per treatment group revealed that considerably more cells enter S-phase in combined EFL treatment (Figure 3.4A) as compared to groups cultured under single growth factor conditions such as E alone (Figure 3.4B). It is noteworthy that single mitogen groups had more counts with lower 7-AAD contents suggesting a greater amount of debris and/or dying cells. The percent of cells in S-phase, estimated as a ratio of $BrdU^+$ cells over all non-aggregates, was greater in combined treatments of EL, FL, EF, and particularly the EFL (early and late passage) as compared to E, F or L alone (Figure 3.4C).

3.3.4 Western blot analyses

Notably, all cells originated from an EFL-expanded population and then subjected to different growth factor treatments for several passages. An important question is then whether such prolonged exposures to altered mitogens changed cell phenotypes at a proliferating stage. To address this issue quantitatively, we applied Western blot analyses to determine the levels of nestin, GFAP and β-tubulin III expression in cells treated with different mitogens for 2-3 passages (about a month) as for the phenotypic neural stem/progenitor cells, astroglial and neuronal cells, respectively (Figure 3.5A). Three independent experiments showed that all cell populations expressed similar levels of nestin except for the EF-treated group (Figure 3.5B). Continuous passage in the presence

of E or F alone, or an EF combination reduced both GFAP (Figure 3.5C) and β-tubulin III (Figure 3.5D) expression significantly compared to their EFL counterpart ($P<0.05$).

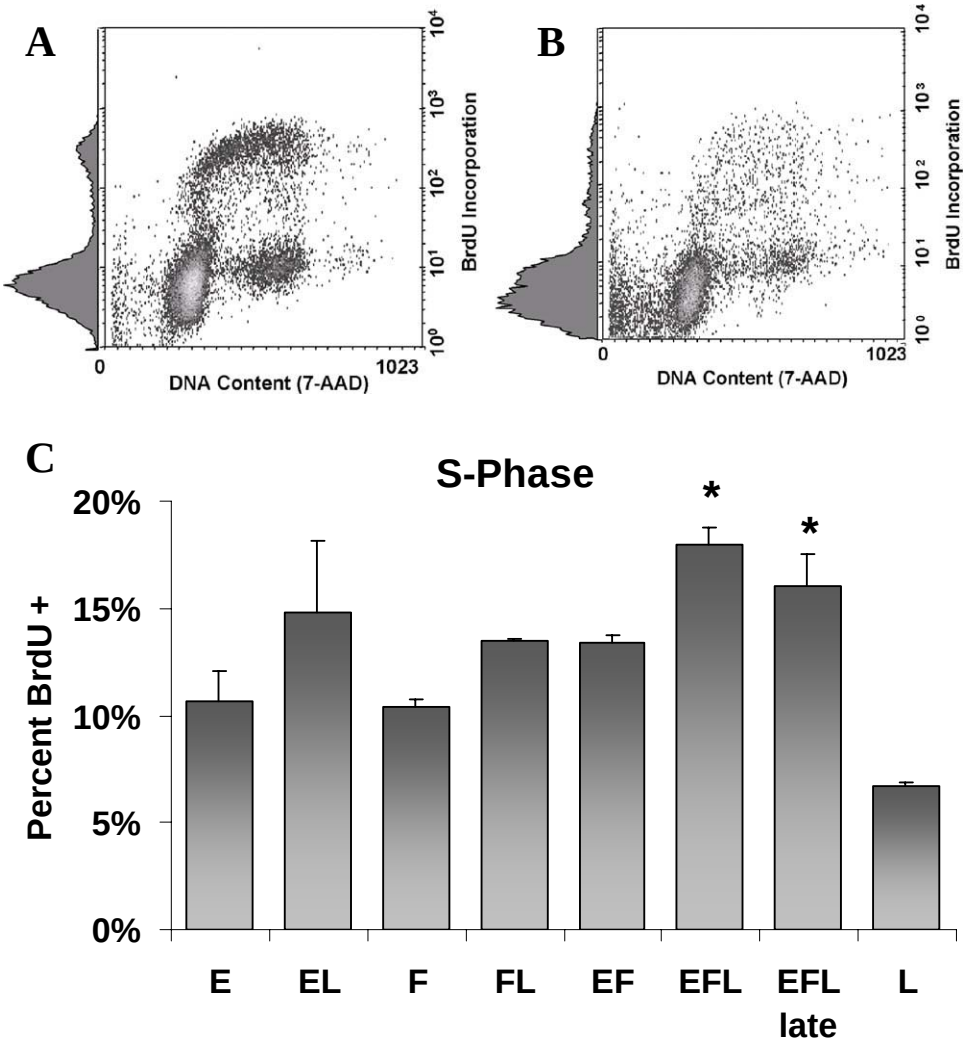

Figure 3.4. Proliferation of human neural stem cells determined by BrdU incorporation flow cytometry. In three independent trials, cells were cultured in various combinations through 2 passages and terminally pulse-labeled with BrdU for an hour. The representative density plots of EFL [A] and E [B] treatment groups are shown in A and B, respectively. Both early (p29) and late (p73) passages of EFL treatment groups show considerably more cells entering S-phase than all other treatment groups (C, *P<0.001). Percent of cells in S-phase was estimated as $BrdU^{+}$ cells/all non-aggregates.

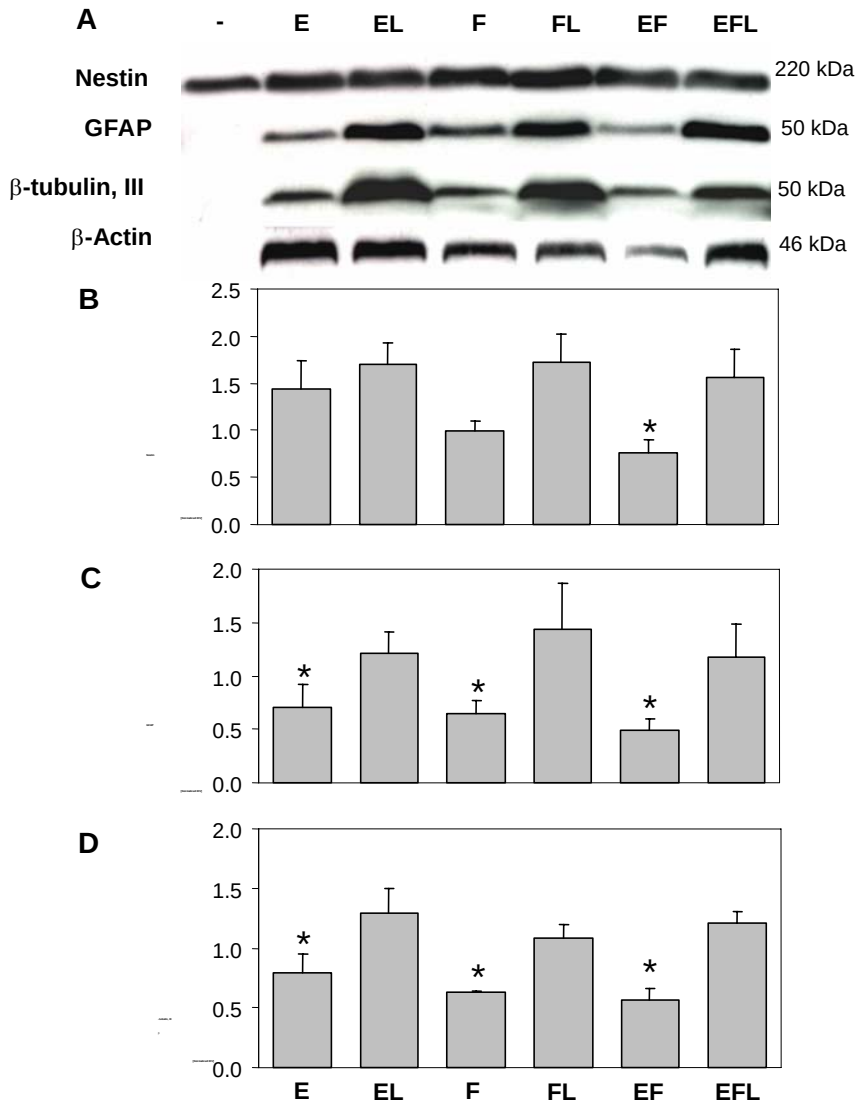

Figure 3.5. Expression of nestin, GFAP and β-tubulin in human neural stem cells during long-term proliferation under various culture conditions. (A) Representative gels of Western blot analyses. (B-D), Quantitative analyses of protein expression by densitometry. The expression levels are integrated density values (IDV) normalized against β-actin and are averages from three independent experiments. All cell populations showed nestin expression without significant differences except for EF-treated (B). Continuous passage in the presence of E or F alone or EF significantly reduces GFAP (C) and β-tubulin III (D) expression (*P<0.05, Tukey Test). Error bars, ± S.E.M.

3.3.5 Effect of growth factors on subsequent hNSC differentiation

To determine whether different mitogen treatments influence differentiation patterns of hNSCs and especially their responses to the FHL priming that we developed (Wu et al., 2002a), cells after 4-5 passages under various combinations of growth factors were subjected to a differentiation procedure with and without priming. Within hours after plating into differentiation medium, cells began to spread along the substratum creating a monolayer. Cells under priming conditions spread further on coverslips and form a monolayer faster than unprimed counterparts. Within two days, unprimed cells appeared as astrocytic/radial glial whereas primed cells also included bipolar and unipolar cells, a phenotype suggesting developing neurons.

Immunofluorescence revealed that unprimed cells generally produced 47-70% $GFAP^+$ cells regardless of the proliferation conditions (Figure 3.6A and C). In contrast, primed cells showed a decrease in GFAP expression overall (Figure 3.6B-C). This was especially apparent in the primed cells that were proliferated under EFL conditions (21.1 ± 1.7% $GFAP^+$), which was statistically different from the EF group (42.7 ± 4.6%), E (48.9 ±3.4%) or F (55.9 ± 5.0%) alone. Reduction in the $GFAP^+$ phenotype in EL (38.3 ± 3.5%) and FL (31.2 ± 2.6%) groups was also evident. Cells treated with E or F alone did not show differences in GFAP immunoreactivity under primed (E = 48.9 ±3.4%; F = 55.9 ± 5.0%) or unprimed (E = 59.2 ± 4.6%; F = 64.9 ± 5.3%) conditions. These data suggest that priming reduced differentiation into astrocytes (and/or radial glial cells), particularly in hNSCs cultured in the combination of EGF, bFGF and LIF.

Expression of the neuronal marker β-tubulin III was tested by immunostaining, and in some cases there were large differences between primed and unprimed groups (Figure 3.7 A and B). Quantitative analyses revealed that low percentages of β-tubulin III^+ neurons were differentiated from hNSCs proliferated in the presence of a single mitogen (E = 6.7 ± 1.1% or F = 11.0 ± 4.0%) without priming (Figure 3.7C). In contrast, unprimed cells propagated with a combination of mitogens gave rise more neurons

especially if all three growth factors were present (EFL = 47.1 ± 3.4%, Figure 3.7C). On the other hand, all proliferation groups showed a higher number of β-tubulin III cells after differentiation with a priming step.

Previously, we reported 27.8±4.2% $ChAT^+$ neurons in primed *in vitro* differentiated neurospheres (Wu et al., 2002). Here we show that combination treatment (EF and EFL) with a FHL priming step followed by differentiation in B27 were essential for $ChAT^+$ neurons to form (Figure 3.8B and C). Spheres expanded under EF or EFL conditions yielded 32.8 ± 3.25% and 38.9 ± 2.25% $ChAT^+$ neurons, respectively. All other proliferating regimens were not favorable for development of $ChAT^+$ neurons (Figure 3.8A and C).

We also examined GABA and glutamate cells differentiated from primed or unprimed groups that were treated with different mitogen regimes. There was a significant decline in glutamate and GABA expression in all groups after primed as compared to unprimed differentiation (data not shown). This change was most marked in $GABA^+$ cells in EFL primed (12.8 ± 3.2%) compared to unprimed EFL (51.2 ± 5.7%). At day 8 after differentiation in B27 supplemented medium only, the expression of nestin was significantly higher ($P<0.01$) in treatments with primed E (45.6 ± 5.6%) and F (43.3 ± 4.5%) compared to primed EF (27.3 ± 1.5%) and EFL (26.9 ± 3.4%) (data not shown); such differences among growth factor treatment groups were not observed during unprimed differentiation.

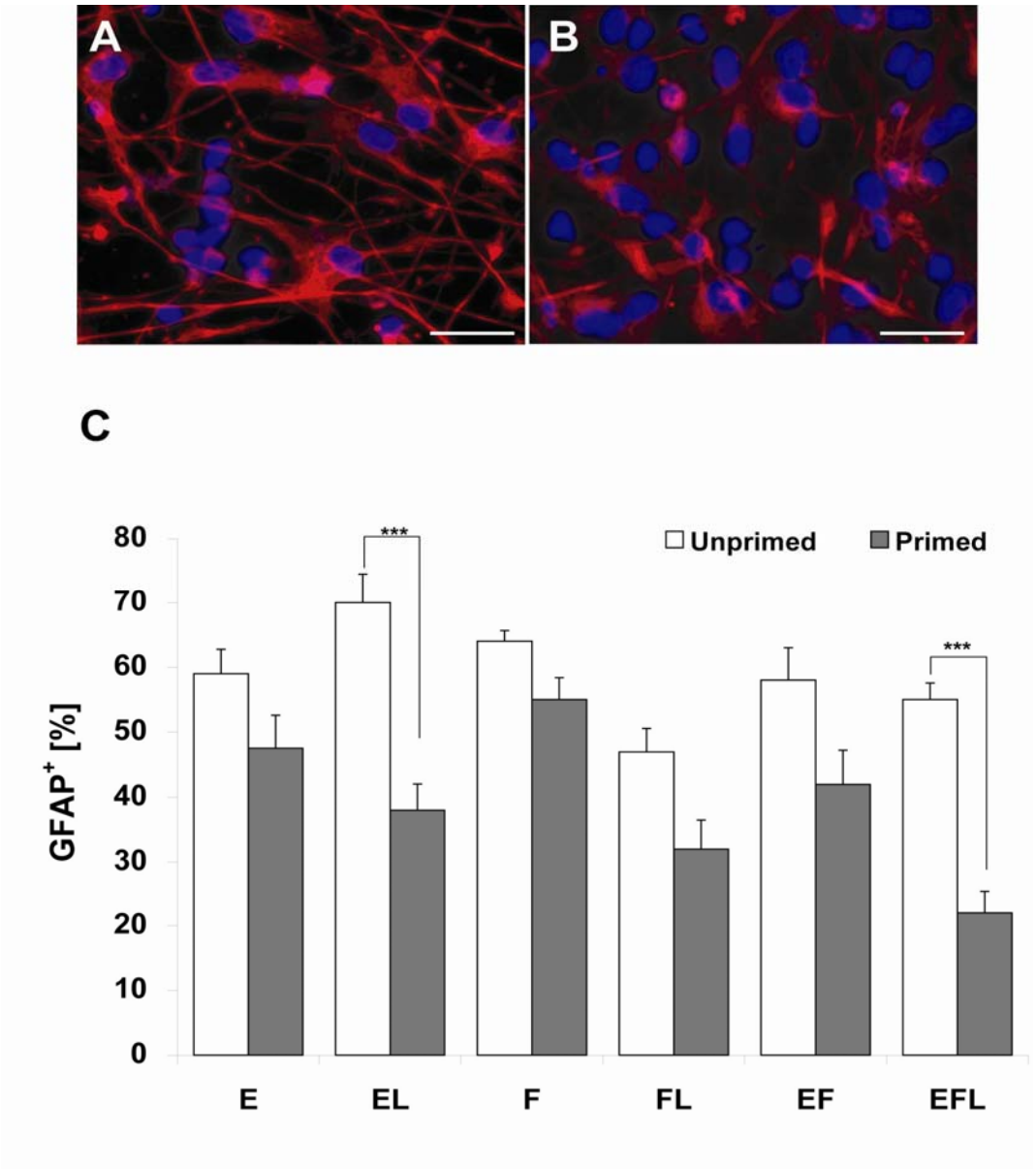

Figure 3.6. Effect of differential growth factor treatments on astroglial differentiation patterns of human neural stem cells. A (EFL treated and unprimed) and B (EFL treated and primed), immunofluorescent staining with a GFAP specific antibody. Scale bars = 40µm. C, quantitative analyses of GFAP positive cells over total number of cells. Note that priming reduced astroglial differentiation drastically in cells propagated with combined growth factors and to a much less extent in cells treated with single mitogens (*** P <0.001,* P<0.05 as compared to EFL-treated). Error bars, ± S.E.M (n = 10).

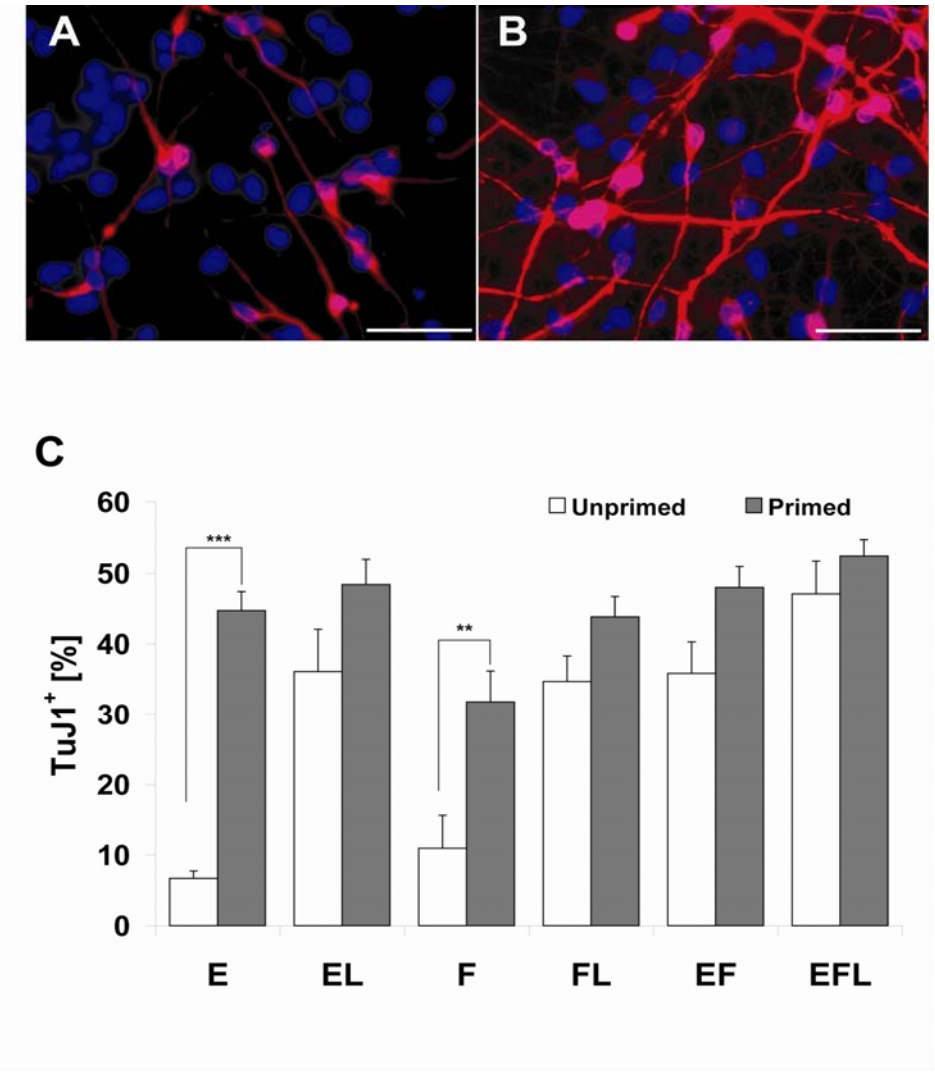

Figure 3.7. Effect of differential growth factor treatments on neuronal differentiation patterns of human neural stem cells. Cells expanded with different combinations of growth factors were either primed or unprimed, and then further differentiated in B27 supplemented medium for 8 days. A (EL treated and unprimed) and B (EFL treated and primed), immunofluorescent staining with a neuron specific antibody, TuJ 1, for β-tubulin III. Scale bars = 100µm. C, quantitative analyses of β-tubulin III positive cells (n=10). Without priming (A and C), cells expanded in a combination of mitogens gave rise to higher percentages of β-tubulin$^+$ neurons than those expanded with only one growth factor (*** $P < 0.001$ as compared to EFL). The priming procedure (B and C) resulted in significantly increased neuronal differentiation in all treatment groups to similar levels except for F (* $P < 0.05$ as compared to EFL). Error bars, ± S.E.M (n = 9-12).

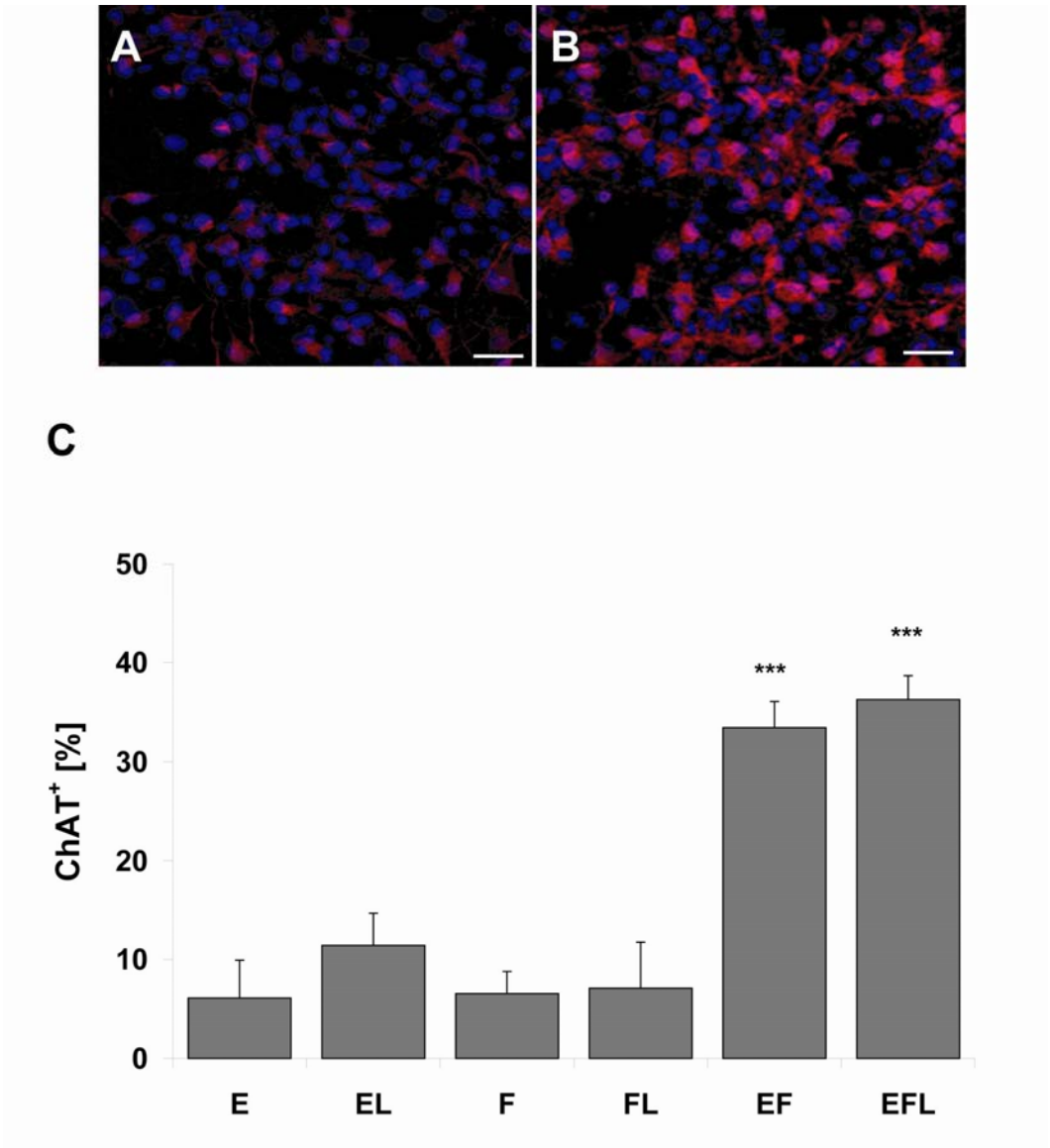

Figure 3.8. Effect of differential growth factor treatments on cholinergic neuronal differentiation patterns of primed human neural stem cells. A (FL treated and primed) and B (EFL treated and primed), immunofluorescent staining with a cholinergic neuron specific antibody, choline acetyltransferase (ChAT). Scale bars = 50μm. C, quantitative analyses of ChAT positive cells. Human neural stem cells expanded in media containing EGF and bFGF (EF or EFL) resulted in a much higher percentages of $ChAT^+$ cells after the priming step, which were statistically significant compared to other groups (***$P<0.001$). Error bars, ± S.E.M (n=10).

3.4 DISCUSSION

This study provides systematic analyses of correlation between proliferation conditions and subsequent differentiation phenotypes with and without priming of hNSCs *in vitro*. Particularly, we show the differential effects of mitogens EGF, bFGF and LIF on long-term proliferation rates of human forebrain neural stem cells, and their subsequent effect on the differentiation pattern of these cells with an emphasis on priming-induced cholinergic neuronal specification. The ability to fine-tune proliferating and differentiating conditions in order to achieve a specific subpopulation of neuronal phenotypes is of great importance for both understanding of stem cell biology and development of stem cell therapy.

Previously, several groups, including ours, have reported *in vitro* expansions of hNSCs using various combinations of mitogens including EGF, bFGF and LIF. The differences in mitogens may in part result in the variations in the life spans of human fetal NSCs (Carpenter et al., 1999; Reubinoff et al., 2001; Svendsen et al., 1998; Vescovi et al., 1999; Wu et al., 2002a). To determine an optimal way for cultivation of hNSCs *in vitro*, we compared various combinations of growth factors in regards to their short- and long-term effects on hNSC proliferation and found that the combination of EGF, bFGF and LIF synergistically promoted robust cell expansion compared to other treatment groups. Despite earlier reports of senescence after continuous long-term passaging (Jain et al., 2003; Ostenfeld et al., 2000; Wright et al., 2003), we did not, observe such declines while maintaining hNSCs in EGF, bFGF and LIF for at least 110 passages (Wu et al., unpublished observation). Furthermore, these long-term *in vitro* expanded hNSCs retain a normal karyotype, consistent proliferation rates and differentiation patterns (Wu et al., 2002a). Obviously, additional studies are needed to examine phenotypes of these hNSCs in greater detail since long-term mitogen-expanded NSCs might evoke mutations (Rothstein and Snyder, 2004).

The role of LIF has long been considered to be maintenance of cells in an undifferentiated state (Smith et al., 1988), which would be conducive to greater self-renewal and therefore expansion. However, a recent study indicated that LIF-enhanced hNSC growth is independent of its inhibitory effect on differentiation that is found for other cell types (Wright et al., 2003). In this study, we found that the addition of LIF actually promotes exponential cell growth by carrying more hNSCs to enter the S phase, consistent with previous findings (Caldwell and Svendsen, 1998). Furthermore, we found that this proliferating effect of LIF was only apparent when LIF was used in combination with the other growth factors (EGF or bFGF). LIF by itself is insufficient to maintain hNSCs in a proliferating stage. Although recent experiments revealed effects of LIF on hNSC's gene profiles (Wright et al., 2003), exact mechanisms of LIF-enhanced cell expansion remain to be elucidated.

Characterized by high plasticity and multipotential capability, human fetal NSCs might be expected to change their gene profiles when propagated with different growth factors or their combinations. Such changes may also affect their subsequent responses to priming and differentiation patterns. Along this line, we found that addition of LIF to proliferation media containing EGF or bFGF alone or EF in combination enhanced expression of three neural lineage markers, nestin (stem/progenitor), GFAP (astroglial) and β-tubulin III (neuronal), in a mixed population of hNSCs during the proliferating stage. In other words, hNSCs propagated under different mitogen treatments are different in their phenotypes and/or gene expression profiles even before differentiation. The expression patterns of the lineage markers we tested in proliferating hNSCs were partially conserved for β-tubulin III in both unprimed and primed cells, and completely reversed for GFAP and nestin in primed cells following differentiation. Nevertheless, priming in general, when compared to their unprimed counterparts, increased β-tubulin III^+ neuronal differentiation while decreasing percentages of $GFAP^+$ cells in all hNSCs cultivated with different mitogens. The priming procedure involves addition of bFGF and laminin, and withdrawal of LIF and EGF from the combination treatment groups. Developmentally,

bFGF has been known to play a critical role in proliferation and differentiation of neural stem/progenitor cells into neuronal progeny (Palmer et al., 1999; Qian et al., 1997; Panchision and McKay, 2002), but, higher concentrations of bFGF promote glial differentiation (Qian et al., 2000). On the other hand, although EGF is known to facilitate glial cell differentiation (Mujtaba et al., 1999), it is not essential for gliogenesis (Tropepe et al., 1999). More complicated, recent studies have shown that bFGF is not only conducive to neuronal differentiation (Zhu et al., 1999) but also induces elevated expression of epidermal growth factor receptors (EGFRs) (Lillien and Raphael, 2000), which then modulate the responsiveness of cortical neural progenitors to LIF resulting in astrocyte induction via the JAK/STAT pathway (Bonni et al., 1997; Viti et al., 2003). Moreover, withdrawal of LIF results in a decrease of STAT3, STAT1 and GFAP, with a simultaneous increase of EGFR (Wright et al., 2003). Therefore, adding bFGF and simultaneous withdrawal of LIF and/or EGF during the priming step lead to decreases in $GFAP^+$ and increases in $TuJ1^+$ phenotypes in combined mitogen-expanded hNSCs.

Another focus of this study is to determine how hNSCs cultured with different mitogens respond to the priming procedure that leads to cholinergic neuronal differentiation as we reported previously (Wu et al., 2002a). No cholinergic neurons were detected without priming under any kind of mitogen combinations, while priming induced a significant generation of cholinergic neurons in cells expanded in EF or EFL, and to a much less degree under other growth conditions. Notably, hNSCs propagated with different mitogen combinations respond to priming and differentiation differently, while the pattern of cholinergic specification is uncorrelated with the β-tubulin III expression pattern observed in hNSCs at both proliferating and differentiated stages.

In summary, we show that proliferation conditions of human neural stem cells can change their gene expression profiles and affect their phenotypic specification in response to priming and subsequent differentiation. Particularly, a combination of EGF, bFGF and LIF not only produces higher rates of hNSC growth, but also enhances

differentiation toward a ChAT $^{+}$ phenotype when such cells are primed prior to differentiation. Further characterization of the underlying mechanisms of these growth factors at a molecular level is desirable for both understanding of hNSC specific differentiation and the application of *in vitro* expanded and modified stem cells for clinical applications.

CHAPTER 4: SURVIVAL AND DIFFERENTIATION OF HUMAN NEURAL STEM CELLS IN CONTUSION-INJURED RAT SPINAL CORD

Spinal cord injury results in a devastating loss of neurons, and replacement of these lost cells may help to alleviate functional deficits. Previously, we have shown that primed human neural stem cells (hNSCs) can differentiate into cholinergic neurons in an intact spinal cord. In this study, we tested the fate of primed hNSCs transplanted into a contusion injury model. The hNSCs were expanded *in vitro* under a combination of growth factors, transduced to express green fluorescent protein using an adeno-associated viral vector, and divided into two groups: primed and unprimed. The primed group was exposed to bFGF, heparin and laminin for six days, while the unprimed group was treated with basic medium supplemented with B27. Primed and unprimed hNSCs were grafted into the spinal cords of adult male rats on either the same day, three or nine days after a moderate contusion injury. Histological analyses of the spinal cord revealed that stem cells survived three months post engraftment only in animals that received grafts at 9-day post injury. The survival rates of such cells were significantly lower than those grafted into the intact cord. Both primed and unprimed hNSCs differentiated into neurons; however, only primed cells gave rise to cholinergic neurons. Tumorogenesis was not observed. Functional assessment based on the BBB score and exploratory activity three months after grafting showed that hindlimb function and/or trunk stability improved significantly in the group that received primed hNSC transplants on the ninth day post contusion.

4.1 INTRODUCTION

The dogma that the central nervous system is unable to regenerate with restoration of function after injury has been challenged by considerable advances in the biomedical research field, including stem cells technology. Their capacity to proliferate long-term *in vitro*, self-renew and the pluripotency of differentiation makes stem cells an appealing source for cell-based therapies as well as developmental biology investigations. The multipontential differentiation capacity gives great promise for treating spinal cord injuries and other central nervous system (CNS) injuries as well as degenerative diseases. For instance, stem cells may be used to fill cavities, replace lost neurons or oligodendrocytes and provide trophic factors to enhance the survival of remaining host cells following injury (McDonald and Sadowsky, 2002; McDonald, 1999; Hulsebosch, 2002; Zompa et al., 1997; Whittemore, 1999).

Several studies reported some degree of locomotor improvement after various types of stem cells were grafted into animal models of traumatically injured spinal cord. These include mouse embryonic stem (ES) cells pre-differentiated toward a neural lineage (McDonald et al., 1999), rat neural stem/progenitor cells (Ogawa et al., 2002; Zompa et al., 1997), and rat or mouse bone marrow stem cells (Koshizuka et al., 2004; Hofstetter et al., 2002; Chopp et al., 2000). However, despite functional improvement, the majority of grafted stem cells differentiated mainly into glial cells, although a few studies reported that a small percentage of stem cells become neurons (McDonald et al., 1999; Ogawa et al., 2002). Still others provided evidence indicating that differentiation of stem cells engrafted into nonneurogenic or injured adult CNS was restricted to glial lineages (Shihabuddin et al., 2000; Cao et al., 2001; Fricker et al., 1999; Vroemen et al., 2003). Therefore, the partial recovery of function in the contusion model of spinal cord injury observed in these studies has been attributed to either remyelination (Liu et al., 2000; McDonald et al., 1999) or trophic factors released from grafted stem cells that facilitated survival and regeneration of host neurons.

Several important questions arise toward the ultimate goal of developing stem cell therapy to replace lost neurons: 1) how to maintain these cells in culture, 2) how to enhance neuronal differentiation from stem cells grafted into the adult spinal cord, and 3) how to direct stem cells toward a specific neuronal phenotype, such as cholinergic motor neurons. Recently, several laboratories were able to successfully obtain cholinergic neurons *in vitro* from mouse ES cells (Renoncourt et al., 1998; Wichterle et al., 2002; Barberi et al., 2003; Harper et al., 2004), immortalized human fetal spinal cord neuronal progenitors (Roy et al., 2004), and human fetal cortical neural stem cells (Wu et al., 2002a). However, so far cholinergic neuronal differentiation of stem cells grafted into the adult spinal cord is much less satisfactory (Kerr et al., 2003). Grafted human embryonic germ cell derivatives differentiated mainly into astrocytes and mature neurons with the rare cholinergic phenotype in spinal cord of adult rats with Sindbis virus-induced motor neuron injury (Kerr et al., 2003). The mechanisms that influence the fate of transplanted stem cells are unclear at present. It is conceivable that both the microenvironment of the host tissue and intrinsic cell properties guide phenotypic differentiation of neural stem cells after transplantation (White et al., 2001). Therefore, an *in vitro* induction of stem cells toward a specific lineage prior to grafting while still maintaining some degree of plasticity may be necessary in order to achieve cell replacement specificity *in vivo* (Harper et al., 2004; Lindvall et al., 2004; Liu et al., 2000; Wu et al., 2002a; Bregman et al., 2002; Rothstein and Snyder, 2004). In pursuit of this goal, we developed an *in vitro* priming technology that induces over half of the human fetal neural stem cells to acquire a cholinergic phenotype when grafted into intact adult spinal cord (Wu et al., 2002a). However, it is unknown whether *in vitro* primed hNSCs can survive and differentiate into cholinergic neurons in the damaged spinal cord. In the present study we report our novel findings that human neural stem cells primed *in vitro* and grafted into a contusion injured spinal cord of adult rat survived, differentiated into neurons, including cholinergic neurons, and enhanced locomotor recovery.

4.2 MATERIALS AND METHODS

4.2.1 Cell Culture

Methods for routine cell culture and priming are detailed in CHAPTER 2: MATERIALS AND METHODS.

4.2.2 Contusion Injury

Procedures for spinal cord contusion injury are delineated in CHAPTER 2: MATERIALS AND METHODS.

4.2.3 Cell Transplantation

Procedures for cell preparation prior to grafting and transplantation are detailed in CHAPTER 2: MATERIALS AND METHODS.

4.2.4 Immunohistochemistry

Procedures for tissue staining are detailed in CHAPTER 2: MATERIALS AND METHODS.

4.2.5 Locomotor Function

Procedures for using the BBB open field Locomotor Rating Scale are detailed in CHAPTER 2: MATERIALS AND METHODS.

4.2.6 Spontaneous Behavioral Activity

Procedures for assessing behavioral activity using the Photobeam Activity System (PAS) with a FlexField software is outlined in CHAPTER 2: MATERIALS AND METHODS.

4.2.7 Quantitative and Statistical Analyses

Quantitative and statistical analyses are detailed in CHAPTER 2: MATERIALS AND METHODS.

4.3 RESULTS

4.3.1 Survival of human neural stem cells in the contused spinal cord.

Grafted hNSCs were detected using immunohistochemical staining for the human specific lysosomal marker LAMP2 in the contused rat spinal cord three months after transplantation. We examined all histological sections and found no morphological evidence of tumor formation. Survival of hNSCs grafted on the same (PCD 0) and the third day (PCD 3) after contusion injury was minimal. In contrast, 0.47 ± 0.11% of unprimed and 0.75 ± 0.18% of primed hNSCs grafted into the epicenter of adult spinal cord on PCD 9 survived three months after transplantation (Figure 5.1). This is considerably less compared to our earlier reported 5.1 ± 0.5% primed and 4.8 ± 0.6% unprimed hNSCs that survived in intact spinal cord (Wu et al., 2002a). The 2×10^5 grafted cells were insufficient to completely fill the formed cavity, and surviving cells were mainly observed near the lesion site with many migrating into the adjacent white and gray matter. There was no colocalization of human specific marker LAMP2 and microglial marker OX-42, suggesting that these survival estimates were not affected by host microglia or macrophages that engulfed the debris and/or dead LAMP2-labeled hNSCs (Figure 5.2).

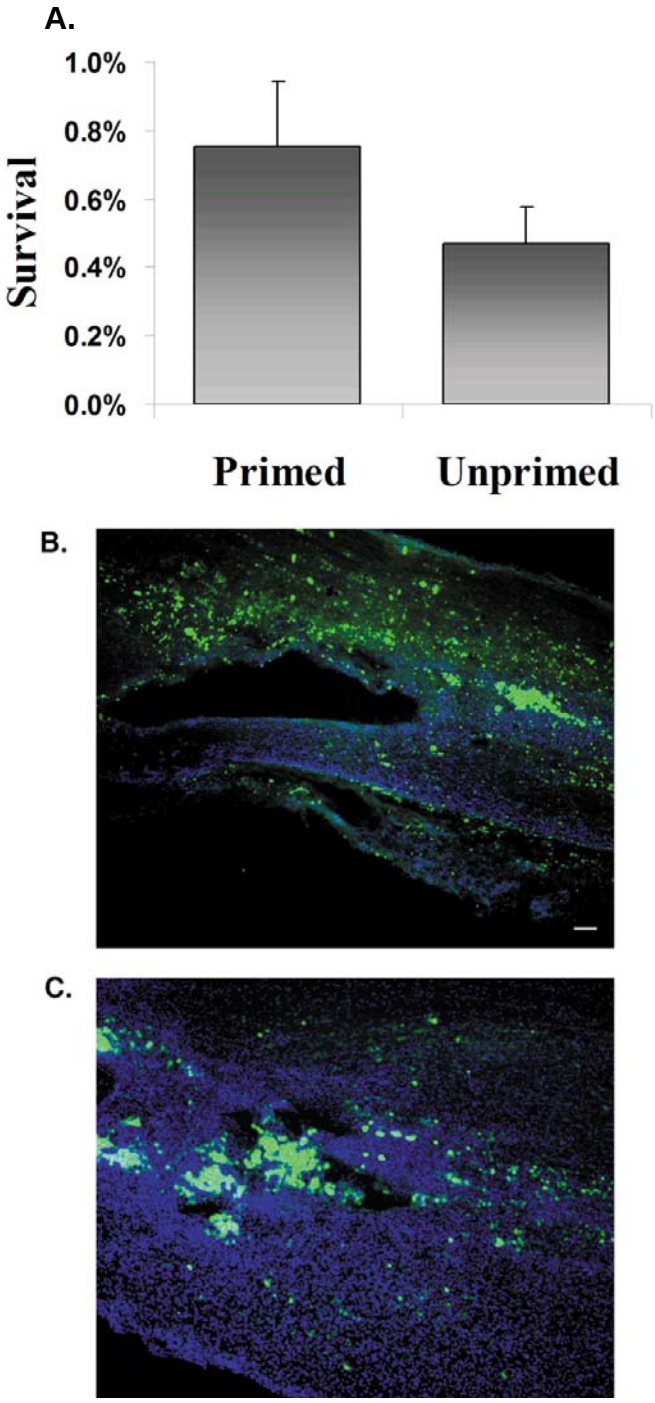

Figure 4.1 Survival of grafted hNSCs in contused rat spinal cord. Cell survival was determined by estimating the number of cells labeled with human specific marker LAMP2 (green) and dividing by the total number of grafted hNSCs (2.0x 10^5). Approximately 0.47 ± 0.11% unprimed (A and B) and 0.75 ± 0.18% primed (A and C) hNSCs were detectable 3 months after grafting on post-contusion day-9 into contused spinal cord. Error bars, ± S.E.M. Scale bar, 200 μm.

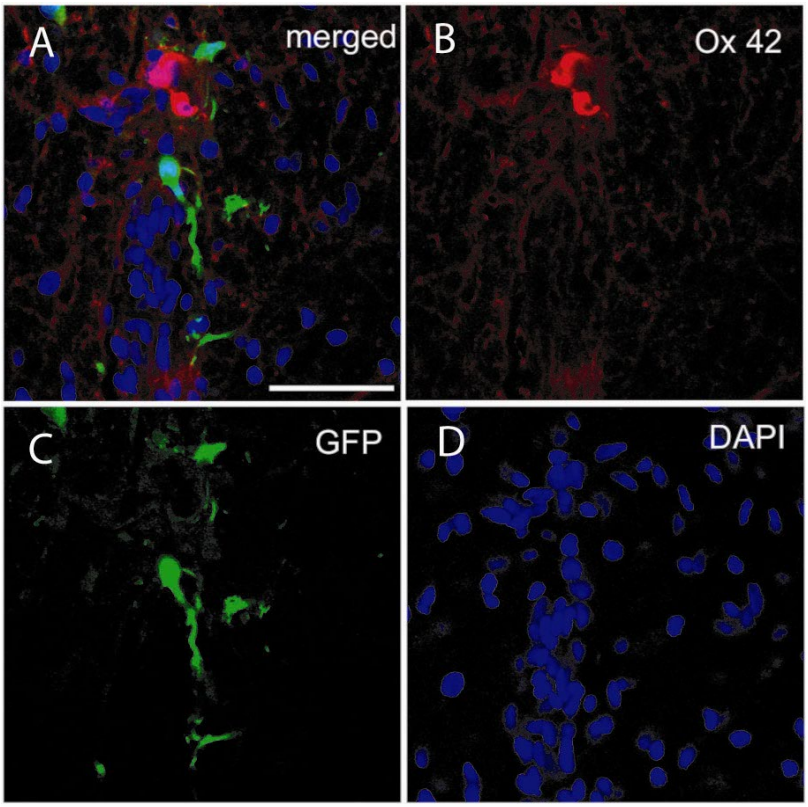

Figure 4.2 Immunofluorescent staining with microglia specific antibody OX-42 on tissue sections containing GFP-labeled hNSC grafts. Note the lack of co-localization with OX-42 (red, A and B) and GFP (green, A and C). D, DAPI nuclear counterstain. Scale bars = 40μm.

4.3.2 Differentiation of human neural stem cells in the contused spinal cord.

Human NSCs grafted on PCD 9 differentiated into both MAP2 positive and GFAP positive cells *in vivo* (Figure 5.3). There were not sufficient numbers of surviving cells in the PCD 0 and PCD 3 groups to analyze differentiation patterns *in vivo*. No statistically significant differences were observed in the differentiation patterns of unprimed (37.03 ± 0.03% $GFAP^+$ astroglia and 27.70 ± 0.18% $MAP2^+$ neurons) and primed (26.26 ± 0.08% $GFAP^+$ astroglia and 37.82 ± 0.058% $MAP2^+$ neurons) cells grafted on PCD 9 into injured spinal cord (Figure 5.4 and 5.5). However, a few primed hNSCs grafted on PCD9 differentiated into $ChAT^+$ neurons and survived for at least 3 months after grafting (Figure 5.6), which was not observed in the unprimed group. Furthermore, a few GFP^+ fibers were detected in the PCD 9 spinal cords of subjects grafted with primed hNSCs (Figure 5.4 E) but none in animals with unprimed hNSCs.

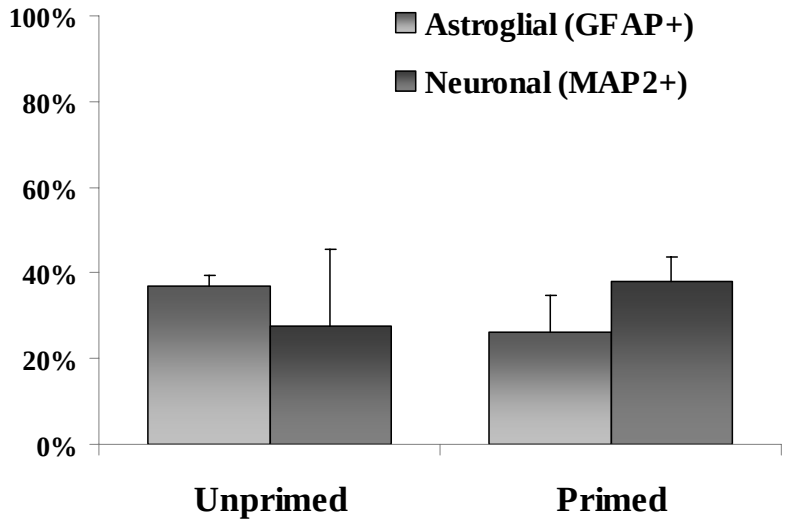

Figure 4.3 Human NSCs grafted on the post-contusion day-9 differentiated into neurons and astroglia *in vivo*. There were no significant differences in differentiation patterns of unprimed (37.03 ± 0.03% $GFAP^+$ astroglia and 27.70 ± 0.18% $MAP2^+$ neurons, P=0.57) and primed (26.26 ± 0.08% $GFAP^+$ astroglia and 37.82 ± 0.058% $MAP2^+$ neurons, P=0.33) cells. Error bars, ± S.E.M.

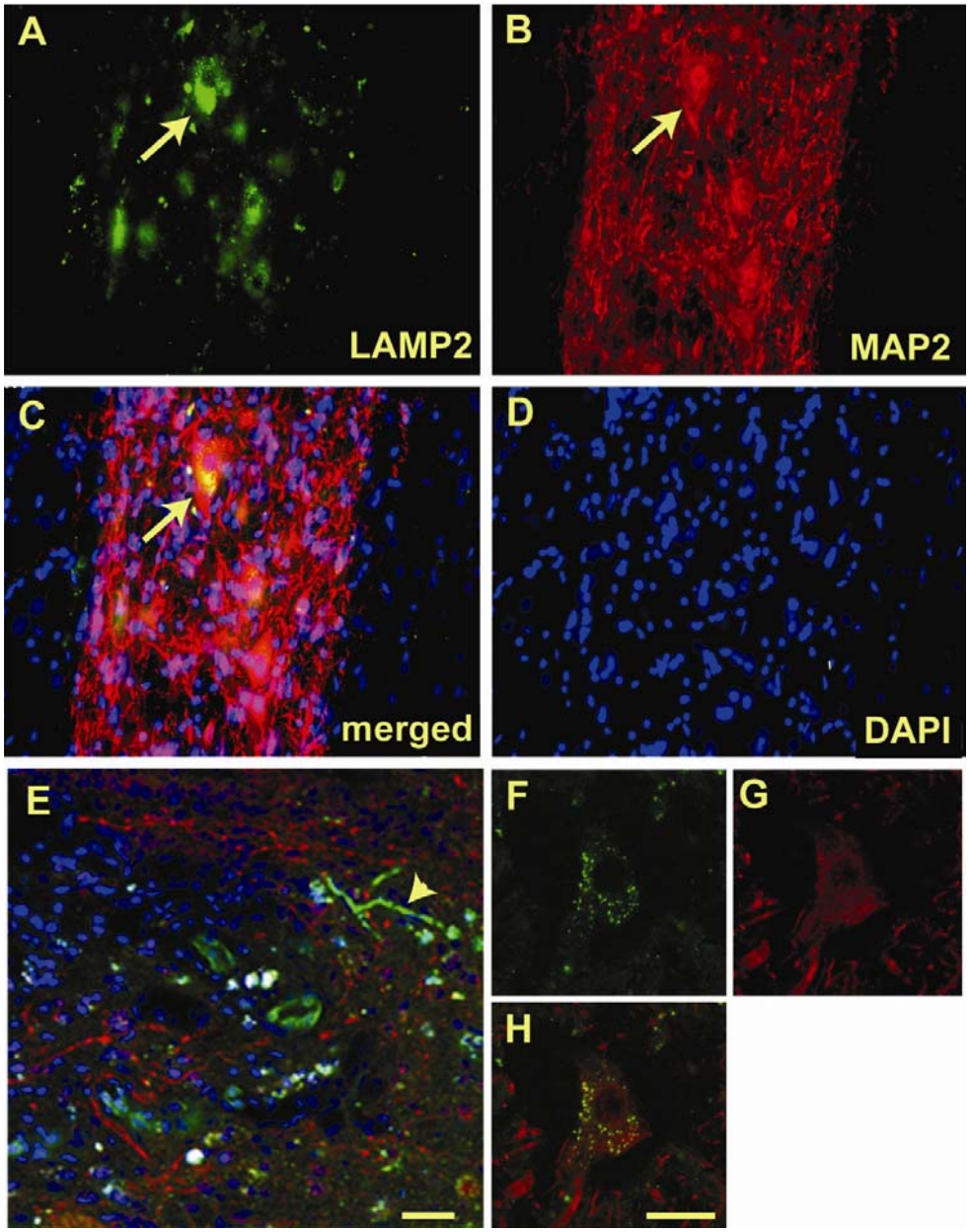

Figure 4.4 Human NSCs grafted on post-contusion day-9 differentiated into neurons, as detected by neuron specific MAP2 and human specific LAMP2 markers. Representative immunofluorescent images of spinal cord longitudinal sections from subjects with hNSC grafts show cells double-labeled with LAPM2 (green, A,C and F) and MAP2 (red, B, G and H). Nuclei are counterstained with DAPI (blue, D and E). A few GFP^{+} fibers can be detected in the spinal cord of primed hNSCs graft recipients (H). Scale bars = 40µm.

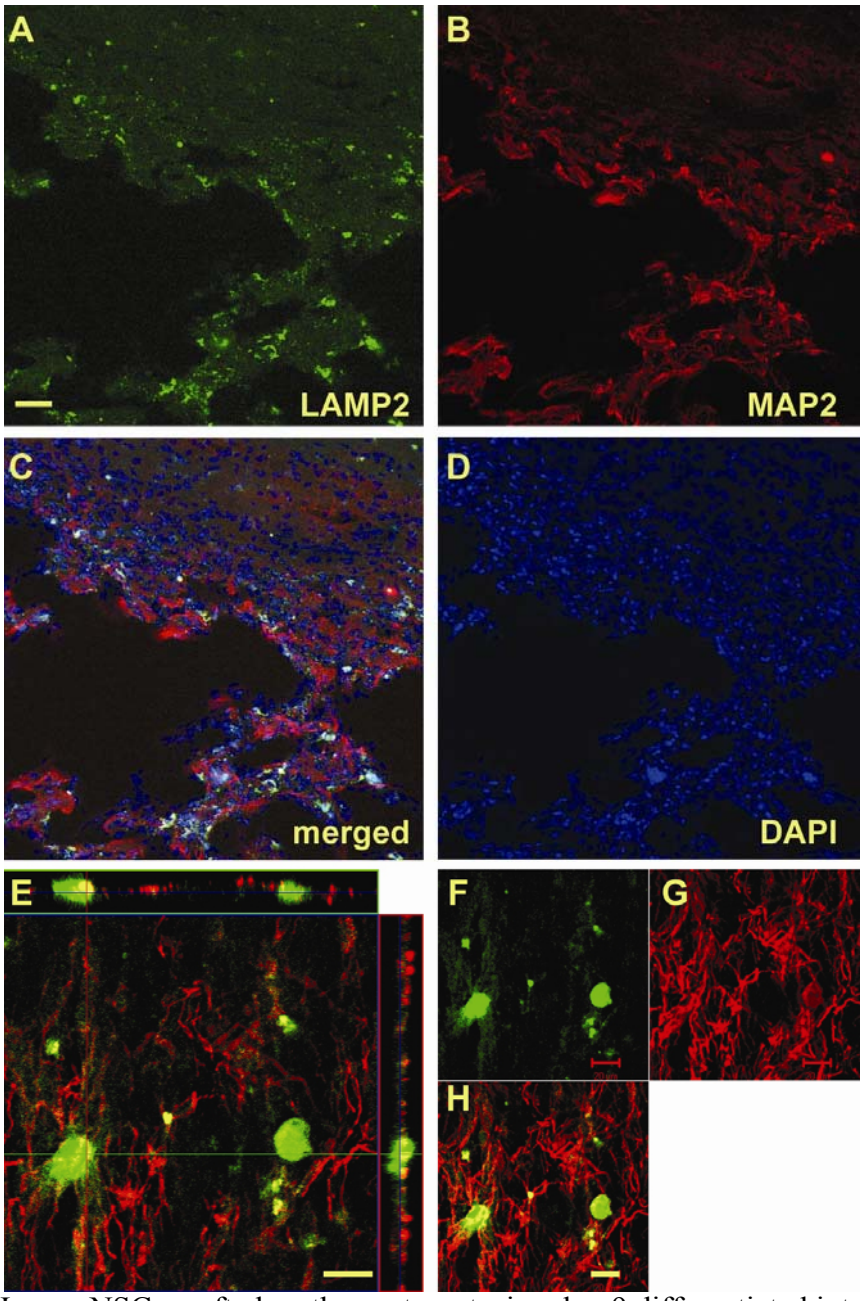

Figure 4.5 Human NSCs grafted on the post-contusion day-9 differentiated into astroglia in contusion injured spinal cord as revealed by GFAP and human specific LAMP2 or GFP markers. Representative immunofluorescent images of spinal cord longitudinal sections from subjects with hNSC grafts show cells double-labeled with either LAMP2 (green, A and C) or GFP (green, E, F and H) and GFAP (red, B, C, E, G and H). Nuclei are counterstained with DAPI (blue, D). Scale bars = 40μm.

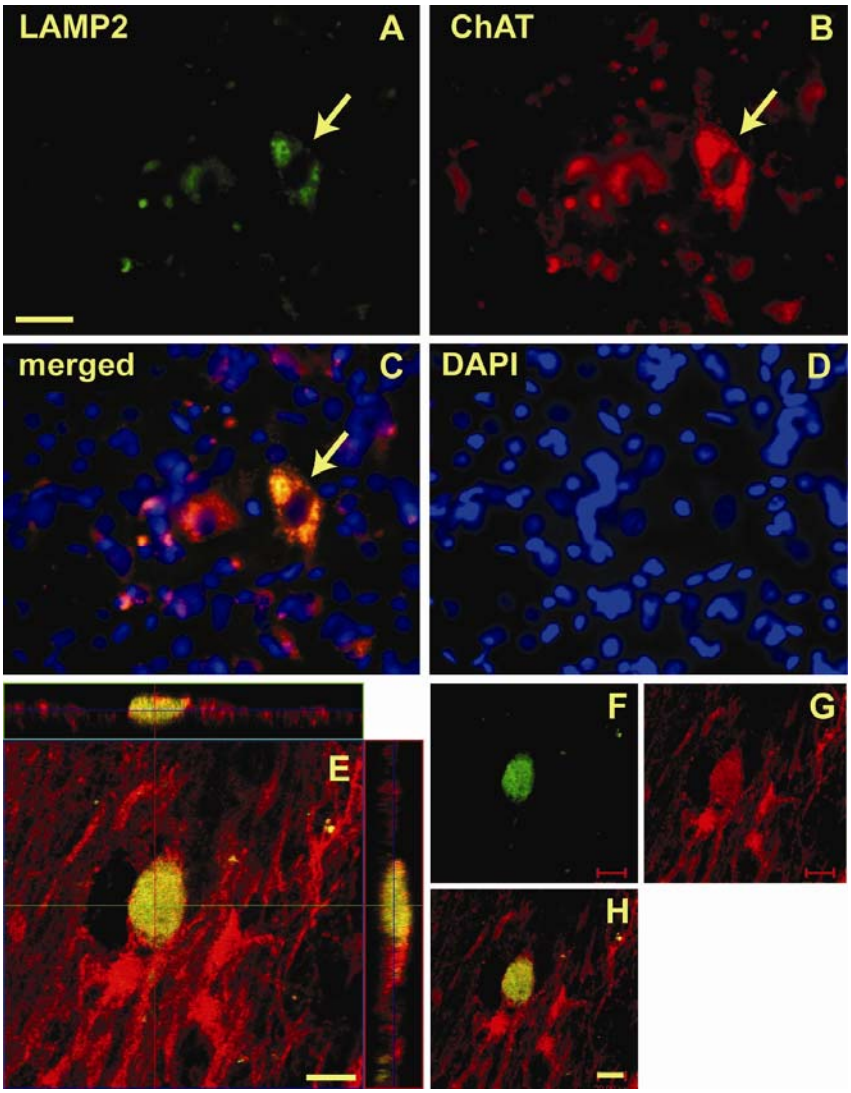

Figure 4.6 A few primed hNSCs grafted on the 9th day differentiated into ChAT^{+} neurons. Representative images from the longitudinal sections of the spinal cord from PSD 9 primed hNSCs graft recipients. Immunofluorescent staining shows cells double-labeled with human specific LAMP2 (arrow, green, A and C) or GFP (green, E, F and H) with choline acetyltransferase (ChAT, red, B-C, E, G and H). Nuclei are counterstained with DAPI (blue, B). Scale bars = 40 μm.

4.3.3 Locomotor assessment using the BBB scale

Preoperative baseline BBB scores of 21.0 ± 0.0 were established for all groups. In general, there were no significant differences among groups that received grafts of unprimed and primed hNSCs on PCD 0 and PCD3 (Figure 5.7). BBB scores of the PCD 0 graft recipients reached a plateau after PCD 49 and showed no significant differences among primed cells (18.75 ± 2.25), unprimed cells (18.25 ± 0.95) and vehicle (17.00 ± 0.5) injected subjects. Although the group with primed hNSCs grafted on PCD 3 showed a greater BBB score of 20.0 ± 0.37 compared to the unprimed group (19.0 ± 0.47) and vehicle (17.0 ± 0.95) three months post graft, the differences were not statistically significant. However, the BBB score of 20.00 ± 0.37 at the 3-month post-graft time point for PCD 9 primed graft recipients was statistically significantly higher ($P < 0.05$ Kruskal-Wallis) compared to the PCD 9 vehicle controls (a combined BBB score of 17.00 ± 0.93).

4.3.4 Spontaneous behavior activity

Spontaneous behavioral activity was measured three months post hNSCs grafting. There were no statistically significant differences between the groups in mean values collected for the distance traveled, number of spontaneous activity events, rest time or active time. To examine trunk musculature and hindlimb function, rearing events were recorded as average time per rearing event during which a subject was able to sustain its own body weight which requires both trunk and hindlimb muscles for stability (Figure 5.8). The contusion injured group showed an average rearing time per rearing event (8.0 ± 0.79 sec) that was significantly ($p < 0.05$, one way ANOVA with Holm-Sidak *post hoc* test) lower compared to the uninjured group (12 ± 1.46 sec). Administration of vehicle, unprimed or primed hNSCs on the same day (PCD 0) of injury or on PCD 3 did not alter the behavioral outcome measured by the average rearing duration per event (Figure 5.8 A and B). In contrast, grafting of primed hNSCs on PCD 9 promoted significant improvement ($p <0.05$) of hindlimb function (13 ± 1.4 sec) as determined by the average

duration of rearing per rearing events when compared to groups with untreated contusion (8.0 ± 0.79 sec), vehicle injectioned (8 ± 0.8 sec) or unprimed hNSCs grafts (10 ± 1.2 sec) (Figure 5.8 C). In terms of the number of rearing events, there were no significant differences among subjects in all groups.

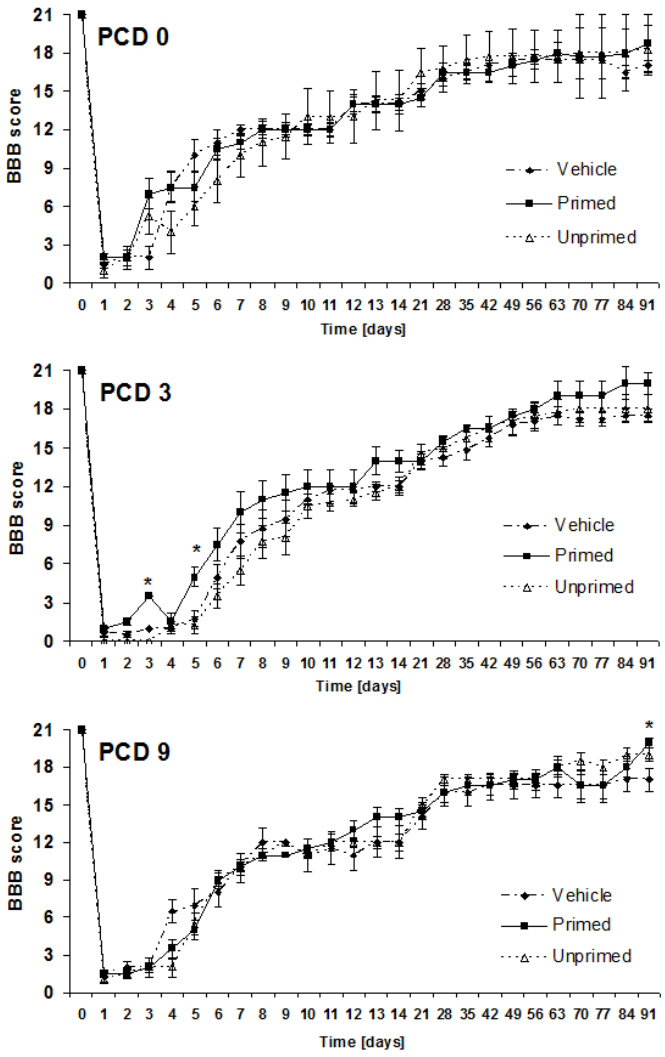

Figure 4.7 Locomotor assessment using median BBB scores. The tests were performed following SCI and hNSCs grafting. Animals were divided into 9 groups receiving vehicle (n=25), unprimed (n=25) and primed (n=28) hNSCs grafts administered at three time points: same day (A), 3rd day (B) and 9th day (C) after SCI. Scores are combined from both hindlimbs (collected daily for two weeks and weekly thereafter) and plotted with post contusion days (PCD) on the x-axis with -1 representing the day prior to contusion injury. The locomotor function decreased significantly immediately after the contusion injury and slowly increased over time in all groups. The only statistically significant improvement in recipients of primed hNSCs graft occurred on PCD 3 immediately after graft and in recipients of primed hNSCs graft on PCD 9 at the end of three months period (compared to vehicle controls; Kruskal-Wallis * $p<0.05$).

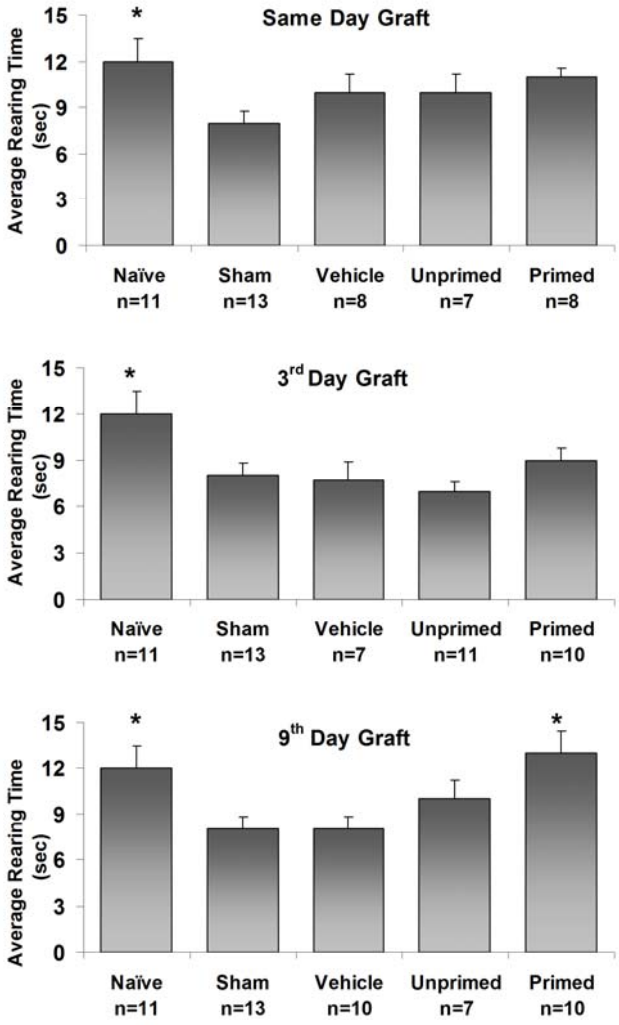

Figure 4.8 Average rearing time per rearing event over the first 15 minutes measured in an activity chamber three months post graft. Vehicle, unprimed and primed hNSCs administration on the same day (A) and PCD 3 (B) did not affect average rearing time compared to sham group (contusion injury without treatment). The group that received a graft of primed hNSCs on PCD 9 showe a statistically significant locomotor improvement (C) in terms of average rearing time per rearing event compared to the untreated group (Holm-Sidak * $P<0.05$). Error bars, ± S.E.M.

4.4 DISCUSSION

In this study, we evaluated the temporal effect of human fetal neural stem cells (hNSCs) on restoration of function of the contusion-injured spinal cord of adult rats by grafting unprimed and primed hNSCs into the injury site at various time points post injury. Our data show that a low percentage of human NSCs survive and differentiate into neurons and astrocytes after grafting into contused spinal cord at 9 days post injury. Furthermore, *in vitro* priming prior to grafting increased cell survival, promoted differentiation of cholinergic neurons in the injured spinal cord, and enhanced functional recovery.

This temporal study demonstrated that hNSCs grafted on the same day or three days post injury resulted in no or only a few surviving cells. In contrast, we found that grafting cells on day 9 after contusion injury enhanced cell survival, which is in agreement with the previously proposed concept of a window of opportunity for a cell replacement therapy and spinal cord repair (Bunge, 2001). Considering the inflammatory response immediately post injury and detrimental effect of inflammatory cytokines (Allan and Rothwell, 2001) on grafted cells, our findings confirm that time of grafting is important for graft survival (Lindvall et al., 2004; Kelly et al., 2004). On the other hand, the survival rates (0.47-0.75%) of hNSCs grafted at 9-days post injury are still much lower than those grafted into intact spinal cord (5%) (Cao et al., 2002; Wu et al., 2002a). This indicates that combined approaches including, but not limited to, antioxidative, anti-apoptotic and/or anti-excitotoxic treatments may be necessary to enhance graft survival and to facilitate spinal cord regeneration post injury (Blight, 2002; McDonald and Sadowsky, 2002; Hulsebosch, 2002; McDonald, 1999).

The ultimate goal of stem cell based therapy for treatment of spinal cord injury is to improve recovery through neuron and oligodendrocyte replacement, delivery of neurotrophic factors and reestablishment of networks that are damaged due to immediate and secondary tissue loss following traumatic injury. In this study we show that human NSCs were able to differentiate into neurons and astrocytes in the hostile environment of the contused spinal cord. Noticeably, 38% neuronal differentiation of primed hNSCs in the injured spinal cord, is significantly lower than the 95% neuronal differentiation in the intact cord (Wu et al., 2002a). This indicates that neuronal differentiation of primed hNSCs may be inhibited when grafted into the epicenter of a spinal cord injury. A similar phenomenon was reported earlier by Cao et al. using rat neuronal restricted precursors (Cao et al., 2001; Cao et al., 2002). On the other hand, it was surprising that unprimed hNSCs differentiated into 27% neurons in injured cord whereas almost no neuronal differentiation was observed from similarly treated hNSC in intact cord (Wu et al., 2002a). Recently, Kelly and colleagues also reported that undifferentiated human fetal neural stem cells, when grafted into ischemic rat cortex but away from the injury site, migrated toward the lesion site and differentiated into neurons near the site of injury (Kelly et al., 2004). Taken together, these studies suggest that the fate of grafted stem cells is determined by both intrinsic cell properties (such as types of cells and stages of development) and the host microenvironment.

The importance of intrinsic and extrinsic influences on stem cell fate determination became evident upon observation of a few hNSC-derived cholinergic neurons in injured spinal cords in the recipients of primed hNSC grafts but not in those with unprimed hNSCs. After primed hNSCs grafts, the cholinergic neurons were detected in the grey matter of spinal cords away from the epicenter of injury. Although only a few hNSCs differentiated into ChAT positive neurons, it is encouraging evidence indicating that the *in vitro* priming procedure promotes subsequent cholinergic neuronal differentiation even in acutely injured spinal cord. On the other hand, we noticed that more than half of similarly primed hNSCs become cholinergic neurons if transplanted

into intact (Wu et al., 2002a) or chronically degenerated spinal cord (Gao et al., 2005), indicating probable environmental cues in directing neuronal differentiation. Given that cholinergic differentiation occurred only away from the injury site, our data suggest that injecting hNSC directly into the epicenter may not be optimal. Therefore, it is possible that the minimal cholinergic differentiation from grafted hNSCs may be due in part to the hostile environment of the acutely injured spinal cord being unsupportive to the maturation of hNSC-derived cholinergic neurons. Further studies are required to determine how to enhance differentiation and survival of such neurons, and more importantly, whether these derivatives are in fact functional spinal motoneurons.

Functional improvements were also observed only in the recipients of primed hNSCs grafts nine days after injury which is concurrent with the survival and differentiation of grafted hNSCs in that group. Since inflammation that results from the contusion is subsiding at that time, this is also consistent with the previous studies that indicated an enhanced efficacy of grafts when applied after the peak of inflammation following acute injury (Bunge, 2001; Ogawa et al., 2002; Bregman et al., 2002). However, the functional improvements assayed by BBB scoring showed only slight differences between primed, unprimed and vehicle transplant groups, although there was a small but significant improvement in primed hNSCs graft recipients at the end of the examination period (91 days post grafting). This result is discrepant from previous reports that showed moderate but significant improvements of BBB scores at much earlier time points (1-5 weeks) after bone marrow stem cell grafting (Chopp et al., 2000; Hofstetter et al., 2002)A probable reason for the discrepancy is that a mild lesion at T10, such is in our case, would be expected to interfere more with trunk than limb movements. In support of this idea, a complete recovery of rearing activity limited to subjects receiving primed hNSCs was found. Since rearing activity would monitor trunk stability, this measure would seem to be more suitable to model behavioral improvements in our model.

As to why statistically significant improvement of rearing activity and BBB locomotor function were only observed in subjects with primed- but not unprimed-hNSCs grafts, one possible explanation is that primed hNSCs have a higher degree of survival and neuronal differentiation, specifically into cholinergic neurons. However, it is unlikely that these few hNSC-derived cholinergic neurons could completely account for restoring the rearing function by replacing endogenous cells that were lost due to injury. Rather, it is possible at least to some extent that the grafted hNSCs provided trophic support such as GDNF and/or BDNF (unpublished observations) for surviving endogenous neurons against secondary damage. Further studies are necessary to verify these hypotheses and to reveal possible mechanisms underlying functional recovery elicited by the hNSC graft. In conclusion, in order to successfully apply stem cells as cell replacement therapy in spinal cord injury, intrinsic cell properties as well as the microenvironment need to be elucidated and then manipulated for the most desirable survival rates, differentiation phenotypes and subsequent functional improvements.

CHAPTER 5: SUMMARY AND CONCLUSIONS

5.1 SUMMARY

In these studies we examined the epigenetic modification of human neural stem cells and consequent grafting into normal and contusion injured spinal cord. Our main objective was to develop a cell-based therapy to treat locomotor dysfunction secondary to SCI. Specifically, culture conditions were studied to determine their effects on *in vitro* expanded hNSCs proliferation rate and phenotypic differentiation. In order to accomplish this objective, we modified hNSCs *in vitro* to direct neural stem cell differentiation prior to grafting into contused and normal spinal cord for the purpose of cell replacement as potential therapy for SCI. Our approach evolved from previous reports indicating that stem cell acquisition of neuronal (Ogawa et al., 2002; Roy et al., 2000; Svendsen et al., 1998; Ostenfeld and Svendsen, 2004; Caldwell et al., 2001), astroglial (Cao et al., 2001; Vroemen et al., 2003; McDonald et al., 1999) and oligodendrial (Chandran et al., 2003; Liu et al., 2000; McDonald et al., 1999) phenotypic characteristics was largely dependent on the conditions of the graft development environment, both *in vivo* and *in vitro*.

Interestingly, grafting epigenetically modified hNSCs after SC contusion injury, during the optimal engraftment window (post contusion day nine in this study (Bunge, 2001)), without additional administration of methylprendisolone, resulted in partial augmentation of locomotor function (Tarasenko et al., 2004a). However, differentiation of grafted hNSCs into a cholinergic phenotype was hindered, possibly, by the milieu of contused compared to normal spinal cord (Cao et al., 2002; Wu et al., 2002a; Tarasenko et al., 2004a). Despite the lack of cholinergic phenotype, locomotor improvement may be attributed to the ability of the neural stem cell transplant to provide neurotrophic support

to the endogenous cell populations (Blesch et al., 2002; Bregman et al., 2002; Llado et al., 2004; Lu et al., 2003); thus, preventing the extent of tissue loss characteristic to the contusion injury to the cord.

Neurotrophic factors play a pivotal role in modulating neuronal survival and regeneration in the CNS (Widenfalk et al., 2001; Bregman et al., 1997; Blesch et al., 2002; Schnell et al., 1994). Neural stem cells have been shown to secrete neurotrophic factors, wicj may provide neuroprotection to the endogenous cell populations after injury (Llado et al., 2004). Specifically, the neurotrophic effect of GDNF was reported previously in a moderate spinal cord contusion model (Iannotti et al., 2004). Exogenous administration of GDNF decreased total lesion volume, increased white matter sparing and showed neuroprotective effect on the subset of proprio- and supraspinal axons following injury (Iannotti et al., 2004). GDNF has been detected immunohistochemically *in vivo* in human fetal brain (Koo and Choi, 2001) and in hNSCs *in vitro* (unpublished observations). Therefore, future studies should focus on the expression of trophic factors from grafted hNSCs into the spinal cord after contusion injury in addition to their effect on guidance of cellular differentiation (Johe et al., 1996; Caldwell et al., 2001; Takahashi et al., 1999). Understanding the interplay among growth factors and their effects on endogenous and transplanted cells may elucidate a mechanistic bases for neuroprotection.

The development of therapeutic interventions with neuroprotective potential is a necessity in conditions such as traumatic brain and spinal cord injury. Central traumatic injury results in progressive damage and functional loss of neural cell populations and pathways. Furthermore, the nonpermisive regeneration environment and limited ability of the CNS to repair itself after injury leads to devastating sensory and motor impairment. Restoration of spinal cord architecture through cell replacement and neuroprotection is a plausible intervention that may be able to prevent the lost neurocircuitry and facilitate reconstruction, thereby attenuating locomotor deficits. Therefore, stem cells can be genetically modified to deliver neurotrophic factors to provide neuroprotection to the

endogenous cell populations in addition to epigenetic modification of stem cells *in vitro*, which will direct subsequent differentiation *in vivo* while maintaining the cell plasticity and adaptability to the environment. *In vitro* modified neural stem cell-based therapy is an attractive approach in developing successful clinical applications which may provide specialized and purified cells for replacement as well as neuroprotection to the site of injury.

5.2 CAVEATS AND CONSIDERATIONS

Notwithstanding the results of the present investigation, many questions remain to be answered prior to a complete understanding of the experimental problems and meaningfulness of the results.

For instance, knowing that cellular β-actin levels may fluctuate during the cell cycle under different conditions, thus the use of actin as a control in western blot analyses of differentiating phenotypes may not yield meaningful results. Therefore, densitometry analyses of the normalized protein expression may not be precise. Alternative loading controls could be considered: glycolytic enzyme glyceraldehyde 3 phosphate dehydrogenase (GAPDH), mitochondrial enzyme cytochrome C Oxidase (COX IV), structural protein alpha tubulin and mitochondrial protein Porin (voltage-dependent anion channel VDAC) are good alternative to β-actin. Given that mitochondrial function and structural proteins may fluctuate during the cell cycle and under certain conditions, more than one control could be employed to maximize the efficacy of the controls in data interpretation.

A second consideration is that application of immunoreactivity to identify and quantify differentiating phenotypes from hNSCs is limited by the antibody specificity and availability. The rapid development of stem cell research will lead to advances in the identification and development of markers for neural stem cells during different stages of differentiation. Recently it was suggested that established markers for specific lineages,

such as the astroglial marker GFAP and neuronal marker beta-tubulin, may be coexpressed during an intermediate stage of differentiation (Leywell et al., 2003). This transient cell type that shares the morphology and antigenic profile of both neurons and astrocytes was detected in long-term suspension cultures of postnatal mouse cerebellum cells maintained at high density in serum-dependent conditions(Leywell et al., 2003) and clonogenic, multipotent cells isolated from medulloblastoma and other CNS tumors (Ignatova et al., 2002). During the proposed intermediate stages, stem cells may express both markers as evidence of their multipotentiality and transdifferentiation potential. Therefore, it would be prudent to utilize multiple, as well as neurotransmitter specific, markers in order to identify specific differentiating neuronal phenotypes. In relation to our investigation, identification of cholinergic differentiating phenotype from hNCSs needs to rely on immunoreactivity to cholinergic markers such as enzymes ChAT and VAChT, LIM homeobox transcription factors, terminal motoneuron marker Islet-1, as well as electrophysiological evidence of action potentials and formation of functional synapses with muscle.

Another aspect of this study that could be improved relates to the number of hNSCs and the site of injection, in relation to lesion size, in a contused spinal cord. In our studies, the amount of cells was inadequate to fill the volume of the cavity that formed secondary to the contusion injury. Grafted cell survival may be improved by avoiding injections directly into the lesion site, but rather rostrally to the lesion epicenter. Also utilizing bioengineered scaffolding would be beneficial in providing a foothold substrate for grafted cells (Holmes et al., 2000; Semino et al., 2004). Furthermore, post injury glial scarring should be addressed with administration of chondroitinases or other anti-scar strategies (Bradbury et al., 2002; Okada et al., 2004; Blight, 2002; Caggiano et al., 2005; Okada et al., 2004; Snow et al., 1990).

Furthermore, the role of standard administration of methylprednisolone at the time of grafting will need to be evaluated. Anti-inflammatory corticosteroid is currently

the standard of care for patients with SCI. Given that glucocorticoids interfere with the cell cycle of the activated lymphoid cells as well as have some effect on fetal development (Katzung, 1998), their effect on partially differentiated hNSCs will be important to understand prior to clinical application of stem cell-based therapies. Other important issues that need to be addressed include evaluation of grafted hNSCs survival, differentiation, immune reactivity, production and secretion of trophic factors and effectiveness of cell grafts at late time points, such as one year post-contusion.

The results and conclusions presented here require careful consideration with regard to realistic outcomes and expectation of stem cells application to treat human SCI. Collected data demonstrate a significant, although only slight, functional improvement in locomotor function. Although encouraging, future multifaceted behavior evaluations are paramount to determine graft efficacy. Complete restoration of function to the pre-injury state in human patients is presently not feasible; however, small improvements may be considered achievements on the road to recovery.

5.3 CONCLUSION

Cumulatively, these studies provide a comparative study and characterization of optimized cultured conditions for hNSCs and analyses of their consequent effects on long-term proliferation and differentiation. Temporal transplantation of *in vitro* unprimed and primed hNSCs into intact and injured spinal cords of adult rat demonstrated that *in vitro* stem cell priming is essential to achieve the desired differentiating phenotypes *in vivo*, and to help attenuate locomotor deficits after SCI. Understanding the general stem cell requirements for differentiation will allow development of cell-based therapies aimed to ameliorate the deficits that result from trauma and neurodegeneration of the central nervous system.

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VITA

Yevgeniya Igorevna "Zhenia" Tarasenko was born on October 3, 1976 to Natalia Nicolaevna Boykova and Igor Alexeevich Tarasenko. In 1994 she began undergraduate work at Texas A&M University at Corpus Christi, Texas, where she attained a Bachelor of Science degree with Honors in Biology during the spring of 1998. From 1994 through 1998 Zhenia was employed as an Administrative Assistant to Dr. Russell Miget at the Sea Grant College Program, Marine Advisory Service and also as a Research Assistant in the laboratory of Dr. Suzzette Chopin at the Texas A&M University – Corpus Christi. In August of 1998 she matriculated into the Cell Biology Program within the Graduate School of Biomedical Sciences at the University of Texas Medical Branch at Galveston, Texas and began her doctoral research under the tutelage of Dr. Ping Wu. Zhenia was awarded the Who is Who in American Universities and Colleges and a Christina Fleischmann Travel Award to attend the International Society for Stem Cell Research meeting. Her work was featured in media release (2002) and was included in the annual press release book at the Society for Neuroscience Annual Meeting.

During the course of her studies Zhenia gained significant teaching experience. She served as a Laboratory Instructor for the Gross Anatomy, Microanatomy, Neuroanatomy and Human Behavior, Molecules, Cells and Tissues and GI Histology courses taught at the UTMB School of Medicine, the UTMB Graduate School of Biomedical Science, and the UTMB School of Allied Health Sciences. Additionally, Zhenia represented the Graduate School as a Senator at Student Government Association, served as a Vice President for Graduate Student Organization, chaired GSBS student recruitment committee and represented GSBS at the UTMB Professionalism and Honor Code Committee.

Zhenia has accepted a postdoctoral research position in the laboratory of Dr. Nicolas G. Bazan at the Neuroscience Center of Excellence, at the Louisiana State University in New Orleans, LA.

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