BONE MARROW-DERIVED MESENCHYMAL STEM CELLS AS AN ALTERNATE DONOR CELL SOURCE FOR TRANSPLANTATION IN TISSUE-ENGINEERED CONSTRUCTS AFTER TRAUMATIC BRAIN INJURY

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Wisdom is the awareness

that while you are defined by what lies within your sphere,

you are infinitely influenced by what lies beyond it.

-Anonymous

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LIST OF SYMBOLS AND ABBREVIATIONS

3-D	Three-Dimensional
MSC	Mesenchymal Stem Cell
NSC	Neural Stem Cell
TBI	Traumatic Brain Injury
ECM	Extracellular Matrix
CNS	Central Nervous System
CCI	Cortical Contusion Injury
CIM	Cell Isolation Media
CEM	Cell Expansion Media
GFP	Green Fluorescent Protein
bFGF	Fibroblast Growth Factor
MC	Methocellulose
LN	Laminin
EGF	Epidermal Growth Factor
GFAP	Glial Fibrilary Acidic Protein
SEM	Scanning Electron Microscope
MSC-PA	Mesenchymal Stem Cells – Plastic Adherence Isolation Method
MSC-LD	Mesenchymal Stem Cells – Low Density Isolation Method
RT-PCR	Reverse Transcriptase Polymerse Chain Reaction
NGF	Nerve Growth Factor
BDNF	Brain Derived Neurotrophic Factor
NT-3	Neurotrophin-3
DIV	Days *in vitro*

SUMMARY

The incidence and long-term effects of traumatic brain injury (TBI) make it a major healthcare and socioeconomic concern. Cell transplantation may be an alternative therapy option to target prolonged neurological deficits; however, safety and efficacy of the cells must be determined. Bone marrow-derived mesenchymal stem cells (MSCs) are an accessible and expandable cell source which circumvent the many of the accessibility and ethical concerns associated with fetal tissues. A major impediment to recent clinical trials for cell therapies in the central nervous system has been the lack of consistency in functional recovery where some patients receive great benefits while others experience little, if any, effect (Watts and Dunnett 2000; Lindvall and Bjorklund 2004). There are many possible explanations for this patient-to-patient variability including genetic and environmental factors, surgical techniques, and donor cell variability. Of these, the most easily addressable is to increase the reproducibility of donor cells by standardizing the isolation and pre-transplantation protocols, which is the central goal of this dissertation. First, we present an animal study in which transplants of MSCs and neural stem cells (NSCs) were given to brain-injured mice, however, the efficacy of the treatment had high variability between individual subjects. Second, we designed a method to produce MSC-spheres and characterize them *in vitro.* Last, we employed an *in vitro* 3-D culture testbed as a pre-transplant injury model to assess the effects of the MSC-spheres on neural cells. The electrophysiological function of the uninjured testbed was assessed, and then MSC-spheres were injected into the testbed and apoptosis of the host cells were measured. The results of this study contribute to our understanding of how extracellular context may influence MSC-spheres and develop MSCs as a donor cell source for transplantation.

CHAPTER 1

INTRODUCTION

1.1 Motivation

Approximately 1 in 50 United States citizens are currently living with a permanent TBI-related disability (CDC 1999). The lack of clinically successful therapies is due at least in part to the number of different cell populations that are compromised upon injury. Reconstructing the original heterogeneous organization has proved difficult so far however; the multipotential and adaptable nature of stem cells suggests that they might provide therapeutic value for the multiple pathological events in TBI over a sustained period. The harvest of adult stem cells from bone marrow provides a potential graft source with minimal ethical, immunological or accessibility concerns. Preceding the clinical implementation of MSC therapy, cell-specific transplant parameters must be determined including adopting standardized, reproducible donor cell isolation and expansion protocols. In addition, low donor cell survival need to be addressed. One approach to provide MSCs with the signals and support they need to survive and influence host neural tissue is transplantation of the stem cells as multicellular aggregates within bioactive tissue-engineered constructs. Once transplant parameters of donor cells are assessed, neural transplantation will be one step closer to clinical reality.

1.2 Background

Traumatic Brain Injury

Epidemiology

Traumatic brain injury (TBI) is a major contributor to morbidity and mortality in our society. In the United States alone, over 50,000 people are killed and nearly another 80,000 are significantly disabled by TBI each year with the highest incidence occurring in the 15 to 24 year age group (CDC 1999). Rehabilitation efforts may be extensive as many TBI survivors are physically disabled, suffer memory loss, or acquire psychological disorders (Thurman et al. 1999). In 1995, TBI-related injuries had an estimated annual expense of $56 billion per year in direct and indirect costs (e.g. lost work hours, etc) (Thurman 2001). Since TBI has been named the hallmark injury of modern day war, the incidence and associated costs are projected to rise (Warden 2006). TBI has been identified as a critical public health concern, and the Public Health Service has made the decrease of TBI hospitalizations a focused objective in Healthy People 2010, an annual report which outlines national health and disease prevention goals in the United States.

Treatment Options for TBI

Current Treatment Strategies

Although the health and socioeconomic impact of TBI is great, current treatment options are limited. Most current clinical treatments are pharmaceutical in nature and focus on reduction of acute sequelae, such as controlling intracranial pressure and bleeding. Several pharmacological treatments have been explored for TBI treatment including compounds which aim to attenuate excitotoxity, decrease calcium release, scavenge

reactive oxygen species, decrease inflammation, inhibit pro-apoptotic cascades, correct neuroendocrine and neurotransmission abnormalities, increase neurotrophins, reduce seizures, or reduce ischemia (for review see (Marklund et al. 2006)). Unfortunately, none of the Phase III clinical trials to date demonstrate a significant improvement in outcome following TBI (Marklund et al. 2006), and the current standard of care following TBI is primarily supportive and palliative. After a meta-analysis of the recent current research, *The 2007 Guidelines for the Management of Severe Traumatic Brain Injury* recommend maintaining adequate blood pressure and monitoring intercranial pressure. Elevated intercranial pressure should be reduced by the use of mannitol, hyperosmolar saline, barbiturates, sedatives, or hyperventilation (2007). Several other therapies including prophylactic hyperthermia, antibiotics, or hyperventilation have been used with variable results, however pooled data suggest that none of these result in a decrease in overall mortality (2007). While there has been an overall improvement in prognosis for TBI victims in recent years, none the most common current interventions used clinically have been shown to reliably reduce morbidity and mortality after severe TBI (Roberts et al. 1998; Marklund et al. 2006) nor do they address the loss of cells in the brain directly.

Therapeutic Considerations

Difficulty in creating clinically effective treatment options stems partly from the fact that the injury is multifaceted. Besides the acute effects from primary brain deformation, secondary cascades are complex and may increase inflammatory response, alter cell signaling and gene expression, and cause cell death in surrounding tissues (McIntosh et al. 1998; Verma 2000). This secondary cascade, which results in sustained complications including inflammation, excitotoxicity, and breakdown of the blood-brain barrier, may result in cell death for months after the initial traumatic event (Conti et al.

1998; Bramlett and Dietrich 2002). In addition, axonal degeneration has been observed by histological analysis up to a year post-injury in the cortex, striatum, and corpus callosum following fluid percussion injury (Pierce et al. 1998). While much of the cell death immediately following the primary injury is necrotic, the post-injury environment also activates apoptotic pathways (Wong et al. 2005; Zhang et al. 2005; Byrnes and Faden 2007). The resulting secondary cascades may promote a progressive, degenerative condition in the brain following TBI. Treatment strategies designed to attenuate the progressive cell loss and encourage regeneration are likely to be broad in nature and, therefore, may promote long-term recovery more than selective pharmacological targets.

Cell Transplantation

One attractive treatment option is the delivery of cells to the injury site (Schouten et al. 2004). Transplanted cells may facilitate recovery by counteracting the deleterious effects within the injury environment, providing continuous trophic support to injured cells, participating in pro-survival cell-cell signaling, and/or replacing neural cells lost during the injury. Clinical trials of transplanted cells have shown some promise for the treatment of severe brain injury (Seledtsov et al. 2005); however, cell therapy has an inherent variability. Although host individuality certainly plays a role, the donor cell contribution to this variability cannot be ignored, and may even supercede host contribution in terms of influence on donor cells survival, phenotype, and function (Watts and Dunnett 2000; Lindvall and Bjorklund 2004). Therefore, the choice of a cell source, preparation of the cells in culture, and parameters of delivery warrant closer investigation.

Donor Cell Sources for Transplantation

Fetal Tissue Transplantation

Transplantation of fetal tissues into animals receiving central nervous system (CNS) trauma and neurodegeneration has shown some functional recovery (for review see (Koutouzis et al. 1994; Subramanian 2001)). In brain trauma specifically, fetal cortical tissue transplants improved functional recovery following frontal cortical lesions (Labbe et al. 1983) or fluid percussion injury (Sinson et al. 1996). Despite these promising results, there are several ethical and practical issues which limit the clinical applicability of fetal tissue grafts (for review see (Stein 1991; Stein and Glasier 1995)). One of the major limitations is the availability of fetal tissue and ethical concerns surrounding its procurement. Another practical consideration is the invasive intercranial surgery required to place such transplants and the short time window that brain tissue remains viable *ex vivo*. Therefore, a more feasible option may be an injectable suspension of non-fetal cells.

Stem Cell Transplantation

Transplantation of cell suspensions may have several advantages over tissue grafts, however the choice of a donor cell source is significant, and there are several options with advantages and limitations to each.

Embryonic Stem Cells

Embryonic stem cells have the advantage of having the potential to differentiate into multiple phenotypes, including neurons, given the correct signals (Guan et al. 2001; Kim et al. 2007). Transplantation of embryonic stem cells in animals following TBI has

improved motor and cognitive recovery, however tumors were also found in multiple subjects (Riess et al. 2007). Clinical trials of embryonic stem cell therapy in Parkinson's disease has had some success in individual patients, but has also had high patient-to-patient variability and late developing immune responses, which have curbed enthusiasm (Bjorklund 2005; Winkler et al. 2005). In addition, because embryonic stem cells are embryonically-derived, and the ethical procurement and use of embryos is fiercely debated and tissue is difficult to obtain in sufficient quantity, therefore, alternative donor cell sources are an attractive option.

Neural Stem Cells

The use of neural stem cells (NSCs) for neurotransplantation has been well-studied in animal models for multiple neurological disorders including neurodegenerative diseases, stroke, and trauma (for review see (Lim et al. 2007)). NSCs are more committed than embryonic stem cells and give rise to the three major cell types in the central nervous system: neurons, astrocytes, and oligodendrocytes (Kallos et al. 2003). While neural stem cells can be isolated from multiple brain regions and ages (for review see (Kornblum 2007)), most are still harvested from embryonic or fetal tissues. In addition, since they are isolated from interior regions of the brain, the harvest is highly invasive and allows limited potential for autologous transplantation (Taupin 2006).

Adult Stem Cells

Besides avoiding most of the ethical dilemmas surrounding embryonic tissues, studies have shown that adult stem cells may retain much of the multipotentiality of their embryonic and fetally-derived counterparts. There are many sources for adult stem cells

including the umbilical cord, adipose tissue, and certain regions of the adult brain; however, the most accessible and least invasive source is bone marrow.

Bone Marrow-Derived Mesenchymal Stem Cells

Definition of MSCs

Within the bone marrow there are many cell types including hematopoetic stem cells and mesenchymal stem cells (MSCs). Hematopoetic stem cells are responsible for re-populating the bone marrow and blood. MSCs are non-hematopoetic stem-like cells capable of differentiating into multiple cell types of mesenchymal and non-mesenchymal origin (Prockop 1997; Mezey et al. 2000; Herzog et al. 2003). While there is not a single marker to identify MSCs, they are generally defined by a combination of physical, phenotypic, and functional properties including the lack of hematopoetic markers and ability to differentiate into osteoblasts and adipocytes. While there are multiple similar definitions of MSCs and related cell types isolated from the bone marrow, this is one we will use here.

Advantages to Adult Stem Cells

Recent advances in stem cell research suggest that adult sources of tissue grafts retain the plasticity necessary for regenerative medicine applications while avoiding many of the ethical, immunological, and accessibility concerns surrounding embryonic tissues or xenografts. Bone marrow harvests and transplants have been successful for decades, and autologous transplants, as well as allogenic transplants, are performed regularly. Post-mortem analysis of human brain tissue has found transgender neurons and astrocytes after sex-mismatched bone marrow transplants (Mezey et al. 2003; Cogle et

al. 2004; Sostak et al. 2007) and similar results were obtained in uninjured mice (Brazelton et al. 2000; Mezey et al. 2000) suggesting that bone marrow may be a natural source of neural regeneration. Furthermore, MSCs may have multiple desirable immunomodulatory effects as neither undifferentiated nor differentiated MSCs elicit significant lymphocyte proliferation (Bartholomew et al. 2002; Le Blanc et al. 2003; Liu et al. 2006a) and may even inhibit T-cell activation (Glennie et al. 2005). In addition, MSC transplantation in severe graft-verses-host-disease has shown significant improvement (Le Blanc et al. 2004) implying that although autologous MSC transplants are optimal, allogenic transplants may also be possible without significant immune rejection, increasing clinical flexibility for graft sources in individual patients. Together, these studies suggest the feasibility and flexability for clinical applications of MSCs may surpass embryonic or neural stem cells.

Transplantation of MSCs in the Central Nervous System

Bone marrow-derived MSCs have been shown to be capable of self-proliferation and differentiation into multiple cell types including neural progenitor cells (Prockop 1997; Mezey and Chandross 2000; O'Connor et al. 2000b; Jiang et al. 2002a; Verfaillie 2002; Herzog et al. 2003). MSCs may be related to another type of bone marrow-derived cell, multipotent adult progenitor cells or MAPCs, which have also been shown to possess multipotenitality (Jiang et al. 2002a; Jiang et al. 2002b; Verfaillie 2002). Transplanted MSCs in animal models of stroke, TBI, spinal cord injury, and neurodegenerative disorders have shown improved functional recovery on behavioral tests (for review see ((Corti et al. 2003; Dezawa 2006; Tang et al. 2007)); however, the variability in long-term outcomes is often great. There are many sources of variability within behavioral outcome studies in animal models and clinical trials (Watts and Dunnett 2000; Lindvall

and Bjorklund 2004). While patient-to-patient variability certainly plays a role, variability of the donor cells may also contribute in large part. MSCs are a small proportion of the heterogeneous population of bone marrow from which they are harvested, and isolation protocols can yield anywhere from a fairly pure population of stem cells to a heterogeneous mix. Variability may be improved by using a more stringent isolation method and reproducible culture conditions to obtain a more homogeneous population of cells prior to transplantation.

Extracellular Influences to Mesenchymal Stem Cell Fate

The cellular differentiation process has not been fully explained, however, it is evident that complex cell-cell and cell-ECM interactions contribute to the development and function of MSCs (Fried et al. 1996; Gronthos et al. 2001), and studies by many investigators suggest survival, proliferation, and differentiation are the result of multiple soluble factors and substrate signals (for review see (Corti et al. 2003)). Most MSC transplantations to date have been of single cell suspensions in a saline solution; however, with the overall goal to increase MSC survival and function post-transplant, it may be beneficial to transplant cells in multicellular aggregates within a bioactive matrix to stimulate pro-survival, migration, and differentiation signals.

Extracellular Matrix

Tissue-engineered constructs may increase cell survival and promote differentiation by providing transplanted cells with attachment sites similar to those normally found in the extracellular matrix (ECM). The availability of attachment sites has been shown to improve cell survival and integration of neural cells (Saporta et al. 1997; Chauhan et al. 1999). This may be mediated in part by membrane bound receptors such as integrins

which play a role in regulating survival, proliferation, and differentiation (Danen and Yamada 2001). One study showed significant difference in MSC proliferation on tissue culture dishes coated with laminin, fibronectin, collagen, or Matrigel over those plated on poly-D-lysine (Qian and Saltzman 2004). In another study, the combination of fibronectin-coated dishes and soluble fibroblast growth factor and retinoic acid resulted in 40% of the cells expressing neurofilament-M (Kim et al. 2002).

Furthermore, transplanting cells within a thermal-reversible polymer that is a viscous liquid at room temperature but becomes a soft gel at 37ºC may have advantages over cells injected in solution including 1) buffering of detrimental factors from surrounding tissues, 2) the ability to fill irregularly-shaped cavities, and 3) a decreased tendency to leak out of the injection path. Intercellular signaling cascades are complex, yet developmental biology has shown that cells receive signals from soluble factors and their immediate extracellular environment that affect survival, migration, and differentiation. However, the interaction between MSCs and their ECM has not been fully characterized, particularly in the brain.

CHAPTER 2

BONE MARROW-DERIVED MESENCHYMAL STEM CELLS AS AN ALTERNATE DONOR CELL SOURCE FOR TRANSPLANTATION INTO TRAMATICALLY INJURED BRAINS

2.1 Introduction

Traumatic brain injury (TBI) has widespread socioeconomic effects and causes long-term damage to affected individuals (CDC 1999; Thurman 2001). The injury is difficult to treat as the insult usually results in the loss of multiple cell types within the brain, and no clinically effective treatments have been identified that ameliorate the long-term effects of TBI (Roberts et al. 1998). One attractive treatment option is the transplantation of stem cells.

Tissue-engineered constructs consisting of cells within a scaffold may increase cellular survival following transplant by providing increased cell-cell and cell-matrix cellular adhesion points. A scaffold which is inherently bioactive separate from the transplanted cells may also provide some benefit to the host such as buffering host tissue from secondary injury cascades and inflammatory response. A thermo-reversible scaffold which gels *in situ* also has the added benefit of trapping the cells during transplantation to minimize leakage back through the needle track. Laminin promotes neurite outgrowth in the developing brain and has been used in CNS scaffolds (Yu et al. 1999; Stabenfeldt et al. 2006). Scaffolds containing laminin and collagen type I have shown increased cellular survival of NSCs when transplanted into the injured brain compared to cells in

suspension (Tate et al. 2002). Therefore, we utilized a laminin-containing scaffold for transplantation.

Neural stem cell (NSC) transplantation has shown promising results as a treatment option in multiple brain disorders including TBI (Shear et al. 2004), however since NSCs are fetally-derived, the cell source is a point of concern for clinical application. Bone marrow-derived mesenchymal stem cells (MSCs) have recently been shown to retain the plasticity necessary for regenerative medicine while avoiding many of the accessibility, political, and ethical issues surrounding fetally-derived graft sources(Prockop 1997; Bjornson et al. 1999). In addition, the technology required for bone marrow transplants has progressed to the point where autografts and allografts of bone marrow cells are routinely preformed. The bone marrow may also be a natural source of regeneration as bone marrow-derived cells have been found post-mortem in the brains of bone marrow transplant recipients who have also suffered head trauma (Mezey et al. 2003; Cogle et al. 2004; Sostak et al. 2007) and similar results have been found in mice (Brazelton et al. 2000; Mezey et al. 2000).

In order to adopt a new cell source for transplantation, it must first be compared to the "gold standard" cell source to determine if the feasibility and effectiveness of the new donor cell source. In our laboratory, we have used NSCs as donor cells for transplantation into a traumatically-injured mouse and have observed long-term behavioral recovery (Tate et al. 2002; Shear et al. 2004; Tate et al. 2004). We have also developed extracellular matrix protein-containing scaffolds for delivery of the donor cells (Tate et al. 2001; Stabenfeldt et al. 2006). Here, we compare NSCs to MSCs as potential donor cells for injection in a laminin-containing scaffold following traumatic brain injury.

Experimental Model - Controlled Cortical Impact Injury

The controlled cortical impact (CCI) device delivers a controlled mechanical compression to the cortical surface of the brain of adult animals which produces histopathology consistent with cortical contusion in human TBI (Dixon et al. 1991). This well-characterized experimental model produces consistent and reproducible injuries in adult animals, and motor and cognitive deficits of injured animals mimic certain aspects of human TBI patients including acute subdural hematoma, blood brain barrier disruption, and edema (Fox et al. 1998; Shear et al. 2004). Using the CCI model, the investigator can control velocity, depth, duration, and angle of impact, although injury severity can best be predicted by velocity and depth of impact (Cernak 2005; Morales et al. 2005). Parameters for a moderate injury (6.0 m/s; 1 mm depth) were used here, which causes cortical and subcortical damage considered moderate for a mouse model, and produces sustained sensorimotor and spatial learning deficits (Fox et al. 1998). The CCI injury model used in this work is a custom-built pneumatically-driven impactor device (**see Figure 2.1**) with the following moderate injury parameters: 6.0 m/s velocity, I.0 mm below dura depth, 150 ms duration, and a 15° angle from vertical.

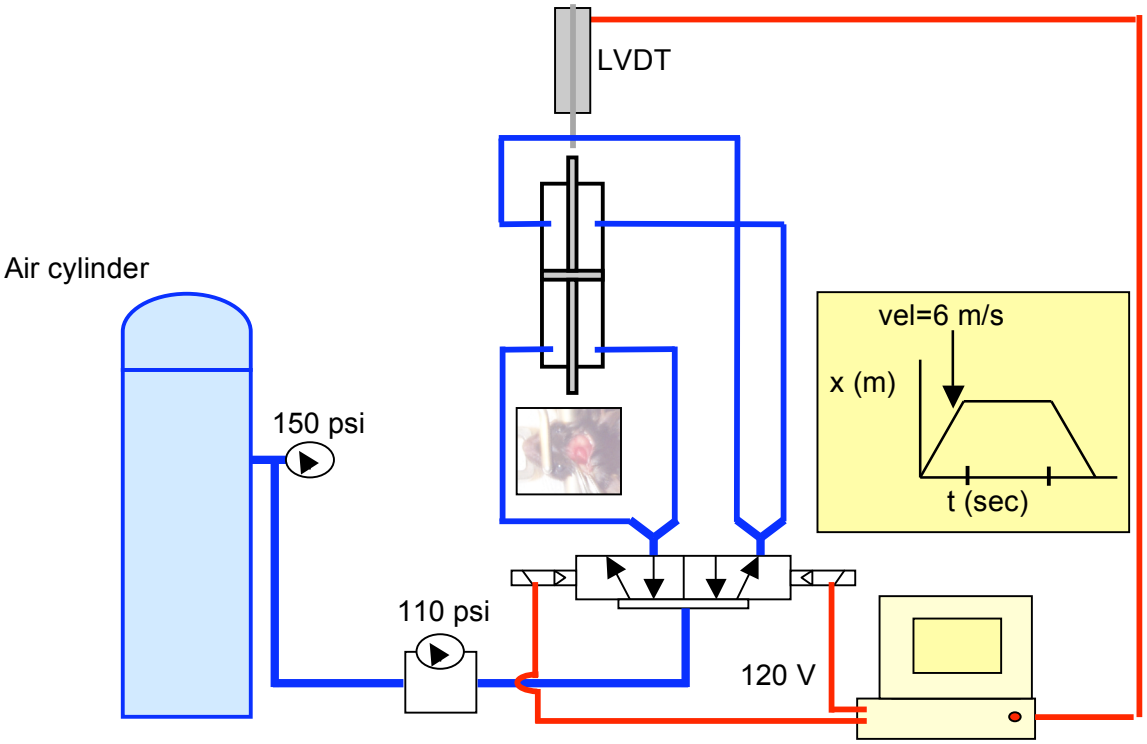

Figure 2.1: Schematic of controlled cortical contusion device for producing traumatic injuries in mice. This custom built device uses compressed air at 110 psi to deliver a controlled impact to the exposed mouse cortex with the dura intact. The compressed air from the cylinder drives the piston down and then up as it moves though two distinct port on the double solenoid valve. The device is controlled by LabVIEW software (National Instruments, Austin, TX) that also gives a reading of the duration and velocity from the linear voltage displacement transducer (LVDT). (Schematic provided by Ciara Tate.)

2.2 Methods

Mesenchymal Stem Cell Culture

Harvest and Isolation of MSCs

MSCs were isolated and harvested as described before (Peister, et al. 2004) with slight modifications. Whole bone marrow was isolated from the femurs and tibias of 8-10 week old male GFP+ transgenic C57BL/6-Tg(cAG-EGFP)C14-701-FM1310sb (C57BL6 background) mice bred in house. Mice were sacrificed, and bones were dissected out and cleaned of muscle and connective tissue. One end of the bone was clipped and the bone marrow extracted by centrifugation. The pellet from each bone was re-suspended in cell isolation media (CIM; RPMI-1640, 10% fetal bovine serum (FBS), and 10% horse serum), mechanically dissociated with a 22-gauge needle, filtered through a 70 μm filter, and plated in a T175 flask. At 24 hours, non-adherent cells were removed, cells were rinsed with saline, and fresh CIM was added. Cells were fed every 3-4 days. After 4 weeks, cells were trypsinized for 2 minutes and re-plated in CIM in a T175 flask. Cells that did not detach in 2 minutes were discarded. After 2 weeks, cells were trypsinized for 2 minutes and re-plated in 100 mm tissue culture dishes at a density of 50-500 cells/cm^2 in cell expansion media (CEM; IMDM, 10% FBS, and 10% horse serum). Once passage 3 cells were confluent, they were either expanded further in 100mm dishes or frozen (FBS + 10%DMSO). Cells were used at passages 4-10.

Priming of MSCs for Transplantation

MSCs were expanded to 80-90% confluency in 100 mm tissue culture dishes. Cells were trypsinized, and plated in NeuroBasal A (Invitrogen, Carlsbad, CA) supplemented with serum substitute B27 (Invitrogen), insulin (25 μg/mL), transferrin (100 μg/mL),

putrescine (60 μM), sodium selenite (30 nM), progesterone (20 nM), and L-glutamine (2 mM) in T25 tissue culture flasks. Human recombinant basic fibroblast growth factor (bFGF; 20 ng/mL; PreproTech, Rocky Hill, NJ) was added to the media every 2-3 days. As cells began to rise from the culture surface, flasks were knocked sideways against the lab bench to dislodge cell aggregates.

Neural Stem Cell Culture

Harvest and Isolation of NSCs

Neural stem cells were harvested from transgenic C57BL/6-Tg(cAG-EGFP)C14-701-FM1310sb mice (C57BL6 background) mice bred in house. Pregnant mice were sacrificed by guillotine and embryos were isolated by Caesarian section at gestational day 14.5. The skull was removed and parasagital cuts were made to expose the germinal layer. Using a dissection microscope, the ganglionic eminence of the germinal zone was separated. Tissue was mechanically dissociated by a flame-narrowed Pasteur pipette in Hank's balanced salt solution (HBSS). Cells were cultured as neurospheres in tissue culture flasks in Dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM/F12, 1:1; Invitrogen) supplemented with insulin (25 μg/mL), transferrin (100 μg/mL), putrescine (60 μM), sodium selenite (30 nM), progesterone (20 nM), and glucose (0.3%). Human recombinant basic fibroblast growth factor (bFGF; 20 ng/mL; PreproTech) was added to the media every 2-3 days. Cells were passaged every 7-10 days with fresh media and bFGF. All cell culture reagents were from Sigma-Aldrich (St. Louis, MO) unless specified otherwise. Experiments were preformed using NSCs passages 3-5.

Traumatic brain injury model

Controlled Cortical Impact Injury

Unilateral contusions were generated using a custom-built controlled cortical impactor (CCI) device as described previously (Dixon et al. 1991; Tate et al. 2001). Adult male C57BL6 mice (8-12 weeks old; Jackson Laboratory, Bar Harbor, MN) were anesthetized with isoflurane (induction 3%; maintenance 1-2%) and placed in a stereotaxic frame. The skull was exposed and a tissue-punch craniotomy (4 mm diameter) was preformed on the exposed skull over the left frontoparietal cortex (center: 2.0 mm posterior to bregma; 2.0 mm lateral to the midline). The impactor rod was angled perpendicular to the tangential plane of the brain surface (15º from vertical). The injury was delivered in a controlled manner by a pneumatically-operated metal impactor (3 mm diameter) at a velocity of 6.0 m/s to a depth of 1.0 mm below the dura mater for a duration of 150 ms. A linear variable displacement transducer (LVDT) connected to the impactor recorded velocity and duration to verify consistency among the injuries. Following injury, the incision was sutured and animals were allowed to recover on warm pads. Sham surgeries for uninjured controls involved anesthesia, incision, crainectomy, and suturing. The mechanical impact leads to cell death in the impacted area and surrounding tissue. A cavity forms at the impact site by one week post-CCI. All mice were housed in a temperature and humidity-controlled environment with a 12:12 hour light:dark cycle with food and water available ad libitum.

All procedures conformed to guidelines set forth by the Guide for the Care and Use of Laboratory Animals and were approved by the Georgia Tech Institutional Animal Care and Use Committee (IACUC).

Transplantation of Stem Cells

Seven days following the CCI injury, animals were anesthetized with isoflurane (3% induction, 1% maintenance) and placed in a sterotaxic frame. The incision site for injury or sham was re-opened and MSCs (passage 5) or NSCs (passage 3) (6.125×10^4 cells in 7μL HBSS; 8.75×10^3 cell/μL) were transplanted through a Hamilton syringe with a 27-guage needle (Hamilton, Reno, NV) into the injury cavity. Transplants were delivered into the center of the 4 mm crainiectomy circle at a 1.1 mm depth below the dura mater over 10 minutes (given at a constant rate of >1μL/min using a syringe pump (Sage Instruments) to press the piston of the Hamilton syringe). After injection, the needle was left in place for an additional 5 minutes and then withdrawn slowly over 2 minutes to minimize leakage through the needle track. Incisions were sutured and animals were kept on a heating pad for recovery. Vehicle control animals received 7 μL HBSS without cells. Animals receiving the sham surgery (injured controls, sham, and naïve animals) were anesthetized and the incision was re-opened and sutured, but no transplant or vehicle injection was given. Groups for transplant were as follows: MSCs + vehicle (n=8), MSCs + scaffold (n=9), NSCs + vehicle (n=6), NSCs + scaffold (n=8), vehicle only (n=3), scaffold only (n=8), injured controls (no transplant; n=5), shams (no injury or transplant; n=4), naïve (anesthesia and suturing only; n=3).

Behavioral Analysis

Beam Walk Motor Functional Test

The beam walk task was used as a measure of motor ability. A narrow beam was used to measure fine motor coordination as described previously (Fox et al. 1998). Mice were trained one week prior to injury surgery and were tested for baseline levels one day prior to injury surgery. The mice were placed at the start point near the end of a narrow beam

(12 mm width for the first 50 cm; 6 mm width for the second 50 cm; 25 cm above a padded surface) and were allowed to traverse the beam into a darkened goal box. The CCI injury to the left frontoparietal cortex results in deficits in motor control for the right hindlimb, so the outcome measure was the percentage of right hindlimb steps where the foot slipped 5 mm or more below the beam surface. Mice were given three trials per time point, and the mean value was used in data analysis. Mice were allowed to rest for 30 seconds in the goal box between trails for both training and testing. Testing occurred at 1 day prior to CCI-injury, at 6 days post-injury (1 day prior to transplant), and weekly until 8 weeks post-transplant. All researchers involved in the beam walk data collection and analysis were blinded.

Statistical Analysis

Data were analyzed by parametric analysis of variance (ANOVA) followed by Tukey's pairwise comparisons ($p<0.05$). Means were considered to be statistically significant by using an alpha value of 0.05. Data is reported as mean ± standard deviation. A post-hoc power analysis was calculated to determine the minimal number of subjects needed to have 80% power ($\alpha = 0.05$, $\beta = 0.2$; assuming 100% prevalence) at detecting a 15 - 30% improvement effect.

Histological Analysis

Tissue Harvest and Processing

Animals were sacrificed at 8 weeks post-transplant for histological analysis. Mice were anesthetized with an intraperitoneal injection of pentobarbital (80 mg/kg) and were perfused with PBS (0.1M; pH 7.4) followed by 4% paraformaldehyde in PBS. Brains

were harvested, post-fixed (4% paraformaldehyde; 4°C; 12 hours), and cryoprotected (phosphate-buffered 30% sucrose). Then, brains were embedded in optimal cutting temperature (OCT) medium (Sakura, Torrence, CA), frozen, and stored at -80°C. Brains were cut into 10 µm thick coronal sections on a Microtome Cyrostat (Richard-Allan Scientific; Kalamazoo, MI) and mounted in sequential series on gelatin-coated glass slides.

Lesion Volume

Serial sections were stained for Nissl with cresyl violet (0.5%; pH 2.5; Sigma-Aldrich, St. Louis, MO). Briefly, slides were dipped successively in acetone (5 minutes), air dried (5 minutes), dipped in dH2O (1 minute), stained in cresyl violet (3 minutes), dipped in 95% ethanol twice (10 seconds each), dipped in 100% ethanol three times (30 seconds, 1 minute, and 2 minutes respectively), dipped in xylene twice (2 minutes each), and coverslipped with Permount. The Cavalieri volume estimator (Rosen and Harry 1990) with Stereo Investigator software (Microbrightfield Bioscience, Williston, VT) was used to determine the volume of the injury cavity (sections at 80 µm increments per brain).

2.3 Results

Cortical Contusion Impact Produces a Deficit

Motor behavior was assessed weekly for eight weeks post-transplant using the beam walk task. The CCI-injury to the frontoparietal cortex resulted in an injury deficit throughout the testing period for all injury groups compared to sham or naïve control groups for both the 6 mm (**Figure 2.2**) and the 12 mm (**Figure 2.3**) beam walk tasks (two-way ANOVA, n=6-10; $p<0.05$). There was no statistical difference between sham and naïve groups, and these groups were combined for further analyses.

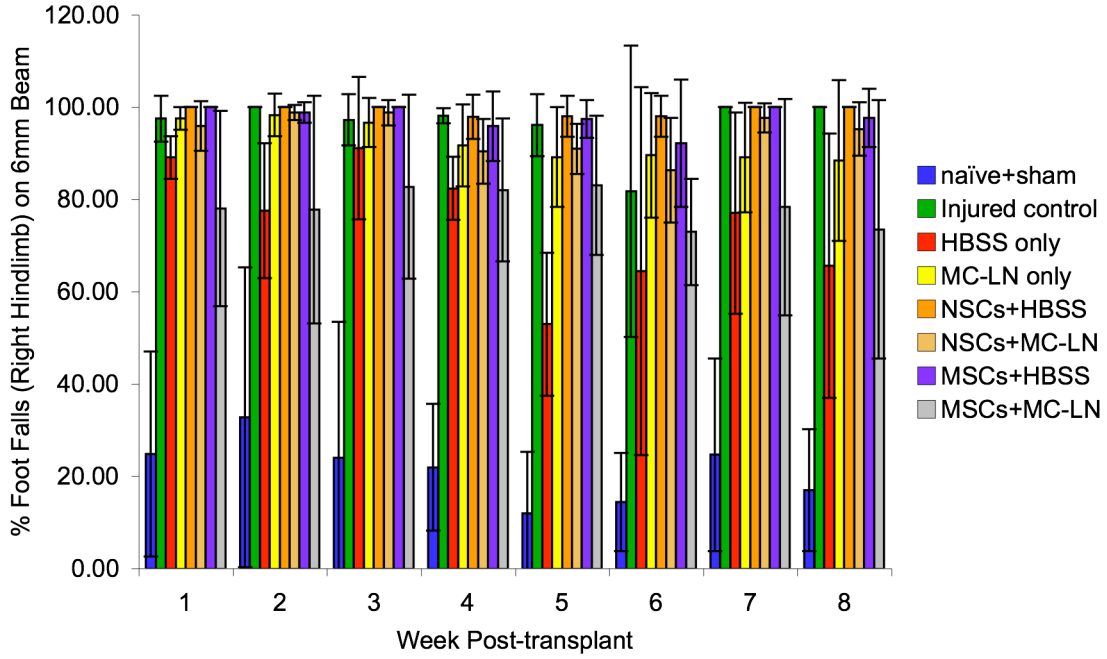

Figure 2.2: Behavioral assessment of motor coordination on the 6 mm beam walk following cortical contusion injury and transplant. There was a significant injury deficit in all injured groups compared to sham and naïve animals, however, no statistical differences were seen in any of the treatment groups. (Two-way ANOVA, n=6-10; $p<0.05$).

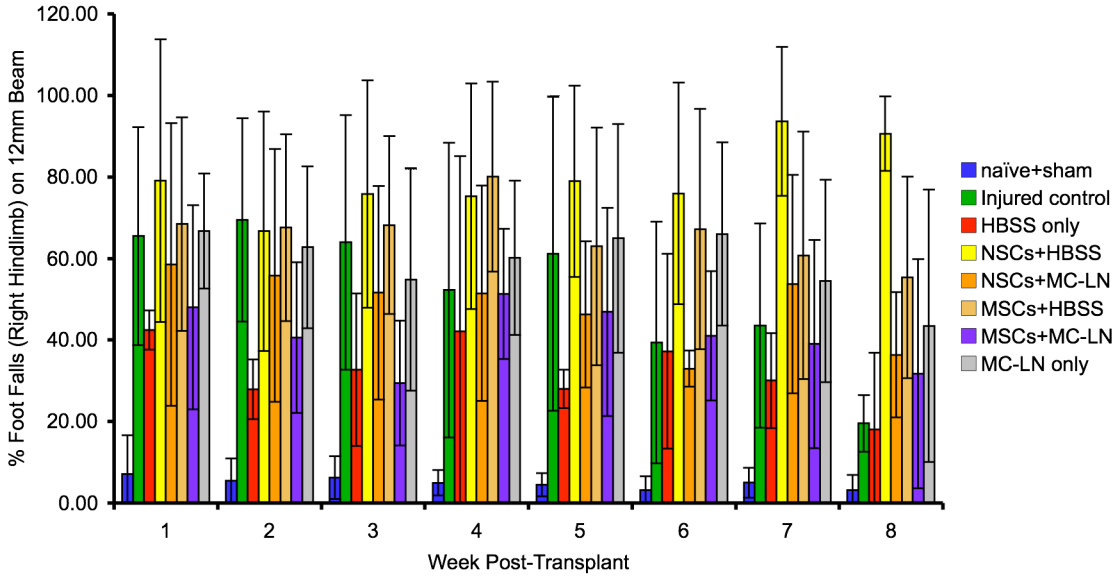

Figure 2.3: Behavioral assessment of motor coordination on the 12 mm beam walk following cortical contusion injury and transplant. There was a significant injury deficit in all injured groups compared to sham and naïve animals for weeks 1, 2, 3, and 5 post-transplant. (Two-way ANOVA, n=6-10; $p<0.05$).

At the 8 week time point for the 6 mm beam walk task (**Figure 2.4**), there is still a statistical difference between the sham/naïve group and all groups which received a CCI injury. Certain individual animals showed some functional recovery within the treatment groups including the MSC and MSC+scaffold groups, however, there was no statistical difference.

In the 12 mm beam walk task, the mice had spontaneously recovered by 8 weeks to the point where there was no longer a difference between the injured groups and the sham/naïve group (**Figure 2.5**). However, there was considerable variability in the individual animal outcomes. The difference between the 6 mm and 12 mm deficits suggests that the 12 mm beam walk task may not be sensitive enough to detect lingering injury deficits at 8 weeks since injured mice still have significant deficits with the 6 mm beam walk task.

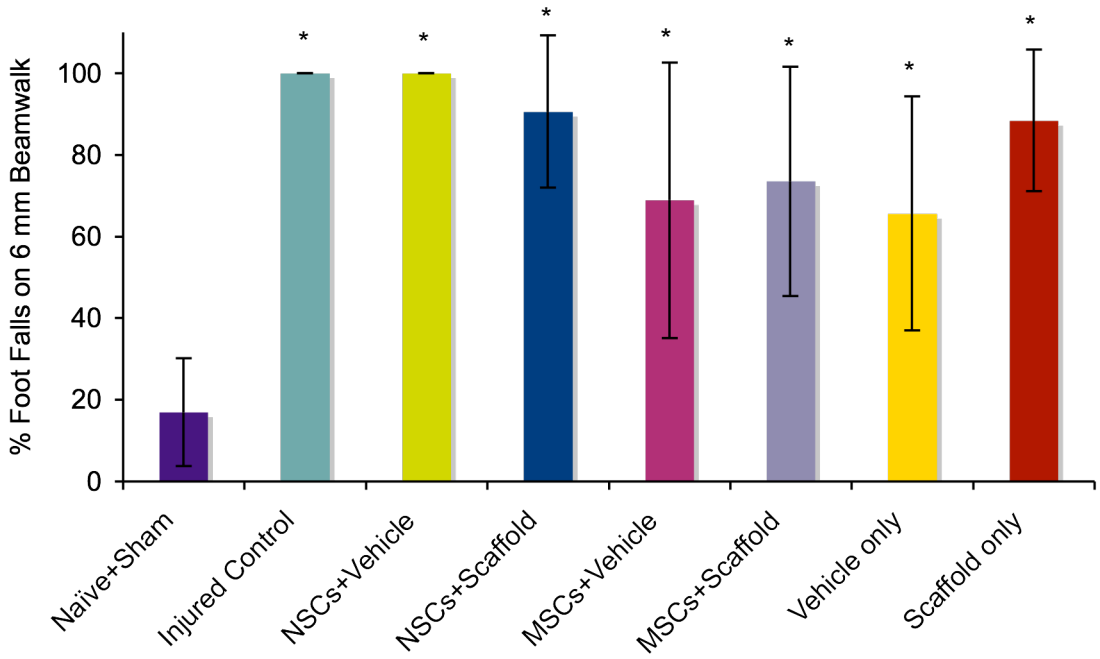

Figure 2.4: Behavioral assessment of motor coordination on the 6 mm beam walk at 8 weeks post-transplant. There was a significant injury deficit in all injured groups compared to sham and naïve animals (* significant difference verses sham/naïve group; $p>0.05$). While there are no statistical differences between treatment groups and injured controls, some individual animals showed improvement; however, there was considerable variability in the outcome.

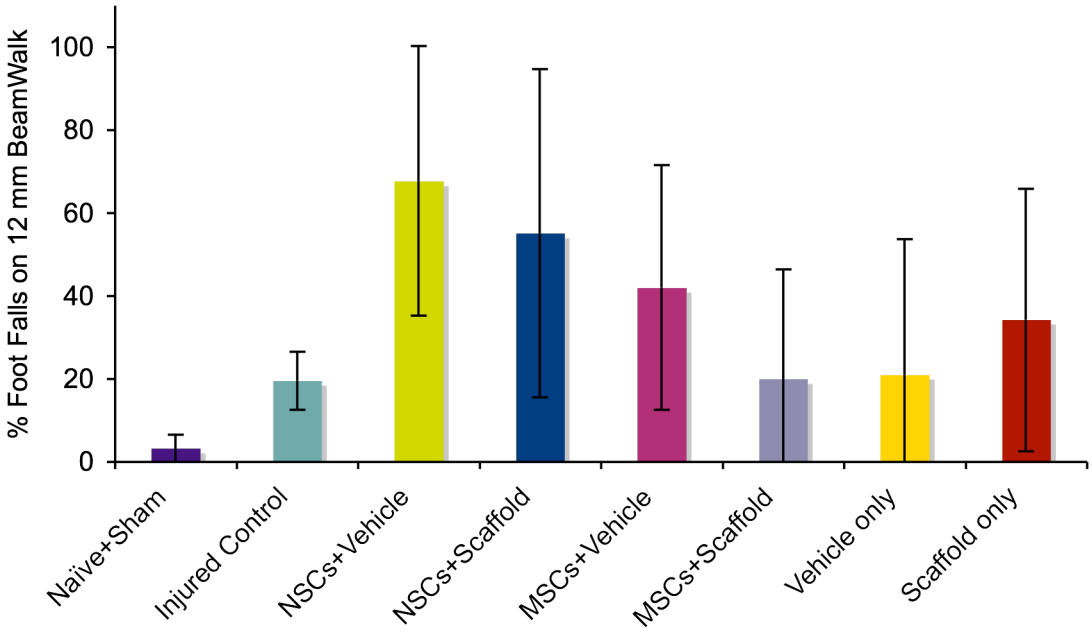

Figure 2.5: Behavioral assessment of motor coordination on the 12 mm beam walk at 8 weeks post-transplant. There is no statistical difference in the injured groups and the sham/naïve group suggesting that this test may not be sensitive enough to detect lingering injury deficits at this late time point since injured mice still have significant deficits with the 6 mm beam walk task.

Post-hoc power analysis

6 mm beam walk task

A post-hoc power analysis was performed on the beam walk task to determine sample size that would have been needed to observe a difference using the means and standard deviations in this study using the same two-way ANOVA statistical analysis. For the 6 mm beam walk task, assuming a confidence interval of 95% and a power of 0.80, a sample size of 12 animals per group would have been required to observe a difference of 20% improvement in foot falls and a sample size of 22 animals per group would have been required to detect a 30% improvement in foot falls.

12 mm beam walk task

On the 12 mm beam walk task, the difference observed between the sham/naïve group and the injured control at 8 weeks was 15% difference in footfalls. Using this as the effect and the largest standard deviation of any treatment group, a sample size of 53 would have been required to detect a difference. Since animal transplant studies requiring more than 400 subjects (8 groups) are not practical, this suggests that this motor function task is not sensitive enough at 8 weeks and that another motor task should be used.

Mesenchymal stem cells may result in a smaller injury cavity

The lesion volume resulting form the CCI injury and MSC or NSC transplants was calculated by Stereo Investigator from Nissl-stained brain sections at 8 weeks post-transplant. The average lesion volume for brains transplanted with MSCs was 3.85 ± 0.05 mm^3 (n=2) while the lesion volume for NSC transplantation was 4.60 mm^3

(n=1)(**Figure 2.6**). While the small number of samples did not allow for statistical analysis, there is a trend that the brains receiving MSCs had less brain tissue lost than the brain receiving NSCs. For comparison from a previous study, injured brains which did not receive a treatment had a lesion volume of ~8 mm while treatment groups had similar reduction as seen with the NSCs and MSCs (Tate, C, unpublished results). This is also consistent with published results (Lu et al. 2002).

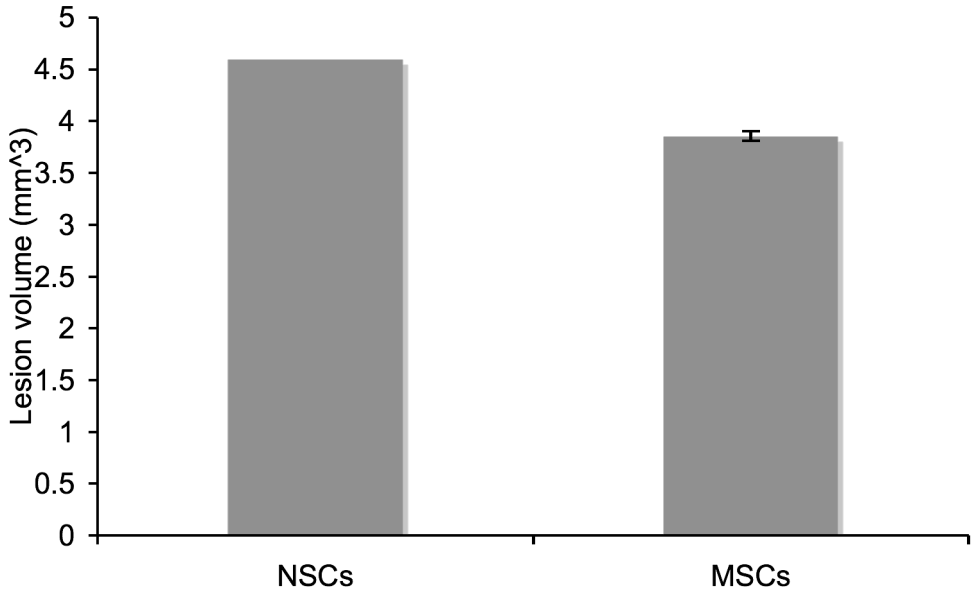

Figure 2.6: Lesion volume of traumatically injured brains transplanted with either NSCs or MSCs at 8 weeks post-transplant. Average lesion volume for brains transplanted with MSCs was 3.85 ± 0.05 mm^3 (n=2) while the lesion volume for NSC transplantation was 4.60 mm^3 (n=1).

2.4 Discussion

Transplantation of stem cells following TBI is a potential treatment option, however both the source of donor cells and delivery parameters need to be studied in a controlled manner. Here we present a transplantation study comparing NSCs and bone-marrow MSCs transplanted with a laminin-containing scaffold or vehicle. Behavioral analysis of motor coordination by the 6mm beam walk task showed a significant injury deficit in injured groups compared to naïve or sham groups, however the effects of the transplants are inconclusive. We also present preliminary data on lesion volume in which transplants of MSCs yield a smaller lesion then after a NSC transplant.

Transplantation of MSCs showed a trend of decreasing motor deficits although at a level that was not statistically significant. The level at which the MSCs decreased the motor deficit was comparable to the level of the NSCs. In addition, transplantation within the scaffold appeared to further enhance the effect of either the cells or the scaffold alone in both MSCs and NSCs.

While behavioral recovery is the optimal assessment of a treatment, there are several inherent limitations of behavioral tests that can lead to inconsistent and inconclusive results. While past experiments in our laboratory of transplanted NSCs have shown behavioral recovery with the beam walk task at 8 weeks (Tate, unpublished results), it is possible that the duration of testing did not extend long enough to show statistically significant results or that another motor task (potentially involving a more complicated task) may have shown motor recovery sooner.

In addition, this study involved fewer transplanted cells than some of the previous studies, adding to the possibility that they may have required a longer time frame to show effects or that the number of cells given was below the effective dose. There has also been a correlation between the number of surviving cells within the brain and functional recovery (Tate, unpublished observations); however since the MSCs decreased their GFP+ expression significantly following transplantation, there was difficulty in distinguishing donor cells from host, so the correlation of donor cell survival to functional recovery could not be completed. Alternately, it may be possible to correlate the number of degenerating neurons as identified with Fluoro-Jade staining to the behavioral results (currently under quantification).

From the post-hoc power analysis, future studies should increase the sample size to at least 12 subjects per group for the 6 mm beam walk task. In addition, another more sensitive task should be considered in place of the 12 mm beam walk task. Furthermore, attempts should be made to decrease variability as this study had higher variability than is usually observed. There are many sources of variability within behavioral outcome studies in animal models and clinical trials (Watts and Dunnett 2000; Lindvall and Bjorklund 2004). While patient-to-patient variability certainly plays a role, variability of the donor cells may contribute in large part. MSCs are a small proportion of the bone marrow from which they are harvested and isolation protocols can yield anywhere from a fairly pure population of stem cells to a heterogeneous mix. One potential area to decrease variability is to use a more stringent isolation method and reproducible culture conditions to obtain a less heterogeneous population of cells prior to transplantation.

In addition, since the extracellular context influences cell survival and differentiation, priming the cells either prior to transplant or with a delivery scaffold may increase their effect on host recovery. Priming the cells *in vitro* may include incubating them with growth factors, growing them on extracellular matrix proteins, or exposing them to mechanical forces. Transplanting cellular aggregates would allow cells to be transplanted at greater densities in the cavity and may increase cell survival by influencing intercellular signaling through cell-cell interactions. Another mechanism to potentially increase cell survival and decrease variability is to inject cells within a scaffold to which they can adhere and migrate. Mice transplanted with MSCs injected within the laminin-containing scaffold had fewer footfalls on average than those transplanted with MSCs in a saline vehicle. Although the results are not statistically significant, it was a fairly consistent trend across the 8 weeks. However, a scaffold that was designed for NSCs may not be the most suitable scaffold for the transplantation of MSCs, so it may be helpful to examine other potential scaffold constituents such as fibronectin or collagen.

Although the data for this behavioral study are inconclusive with respect to the efficacy of MSCs as a treatment for brain injury, there are prior reports of beneficial MSC transplantation (Lu et al. 2001; Mahmood et al. 2004; Mahmood et al. 2006) and the presence of MSCs do not appear to be detrimental to endogenous recovery mechanisms. In addition, the decrease in lesion volume observed with MSCs is encouraging. Therefore, there remains potential for MSCs to be used as a donor cell source for transplantation. However, it will be important to find a method to refine MSC culture methods and to characterize the cells prior to transplantation. It may also be potentially beneficial to prime the cells prior to transplantation and/or find an appropriate

scaffold and transplantation regime that may differ from that used for the NSC transplants.

Acknowledgements

Animal experiments were performed with Sarah Stabenfeldt. Sarah Stabenfeldt also provided the NSCs and laminin-containing scaffold. Vishnu Ambur and Morgan Wolfe provided assistance with tissue sectioning. Scott Medway assisted in Nissl and Fluoro-Jade staining and with Stereo Investigator. Previous work by Matt Tate and Ciara Tate led to the injury and transplant parameters and the beam walk apparatus used for behavioral assessment.

CHAPTER 3

EXTRACELLULAR INFLUENCES ON BONE MARROW-DERIVED MESENCHYMAL STEM CELL-SPHERES

3.1 Introduction

Within the bone marrow there are many cell types including hematopoetic stem cells which are responsible for repopulating the blood and mesenchymal stem cells (MSCs). MSCs are non-hematopoetic stem-like cells capable of differentiating into multiple cell types of mesenchymal and non-mesenchymal origin (Prockop 1997; Mezey et al. 2000; Herzog et al. 2003). While there is not a single marker to identify MSCs, they are generally defined by a combination of physical, phenotypic, and functional properties including the lack of hematopoetic markers and ability to differentiate into osteoblasts and adipocytes. While there are multiple similar definitions of MSCs and related cell types isolated from the bone marrow, this is one we will use here.

Bone-marrow derived mesenchymal stem cells have been shown to be capable of self-proliferation and differentiation into multiple types including neural progenitor cells (Prockop 1997; Mezey et al. 2000; Jiang et al. 2002a; Herzog et al. 2003; Verfaillie et al. 2003). Transplantation of MSCs has shown some promise for treatment in animal models of stroke, traumatic brain injury, spinal cord injury, and neurodegenerative diseases (for review see (Corti et al. 2003; Dezawa 2006; Tang et al. 2007)). However, in clinical trials there has been difficulty in determining the effectiveness of stem cell transplants in part due to the high variability of outcomes. One source of variability within these studies is the composition and properties of the donor cells.

MSCs are low in abundance within the heterogeneous bone marrow especially in the adult, therefore during stem cell culture, it is important to find methods by which to isolate and expand the stem cells from other interfering cell types. In addition, methods for MSC isolation are not standardized, and therefore, it is difficult to draw conclusion among multiple investigators when the starting material may vary greatly depending on the culture protocol.

In the context of transplantation into the brain, priming MSCs towards a neuroprogenitor lineage *in vitro* prior to transplantation may improve transplant survival and efficacy. Neural stem cells (NSCs), which can differentiate into neurons, astrocytes, and oligodendrocytes, are cultured as floating clonal colonies in a defined, serum-free medium. NSCs isolated from the subventricular region of the brain are FGF-responsive and/or EGF-responsive depending in part on the age of harvest (Ciccolini and Svendsen 1998; Ciccolini 2001). NSCs express the neuroprogenitor marker, nestin, but do not express mature neural markers such as NeuN, GFAP, or NG2 until growth factors are withdrawn. Many investigators have described *in vitro* neural induction methods for MSCs, however, induction protocols vary widely, are sometimes contradictory, and only a few produce cell aggregates which resemble neurospheres (Lu et al. 2002; Hermann et al. 2004).

Cell aggregates or spheres may have several advantages for transplantation. Spheres of cells are densely-packed allowing the transplantation of high numbers of cells in a small volume. In addition, cells in spheres may be expressing increased cell-cell and cell-matrix receptors which may influence migration once the cells have been delivered. Cell aggregates for transplantation are ideally fast growing, homogeneous in size,

reproducible, viable throughout the interior, and sized to an overall diameter that can easily be injected through a Hamilton syringe.

Transplantation of tissue-engineered constructs consisting of multipotential stem cells and a bioactive extracellular matrix (ECM)-based scaffold may increase graft survival and migration. However, an optimal scaffold preparation has not yet been fully established for transplantation of MSCs into the brain. Determining the interactions of donor cells with ECM proteins (including adhesion, migration, and additional ECM production) will help evaluate and predict appropriate scaffold components

Here we present a novel method to induce murine bone marrow-derived mesenchymal stem cells (MSCs) to form highly homogeneous nestin-positive spheres which can be produced in multiple diameters depending on culture conditions. We also begin to explore the interaction of the MSC-spheres with the ECM and changes to the MSCs which occur during or following sphere formation.

3.2 Methods

Harvest and Isolation of MSCs

Low Density Method

MSCs were isolated and harvested as described before (Peister et al. 2004) with slight modifications. Whole bone marrow was isolated from the femurs and tibias of 8-10 week old male GFP+ transgenic C57BL/6-Tg(cAG-EGFP)C14-701-FM131Osb mice (C57BL6 background) mice bred in house. Mice were sacrificed, and bones were dissected out and cleaned of muscle and connective tissue. One end of the bone was clipped and the bone marrow extracted by centrifugation in modified microcentrifuge tubes. The pellet from each bone was re-suspended in Cell Isolation Media (CIM; RPMI-1640, 10% FBS, and 10% Horse Serum), mechanically-dissociated by a 22-gauge needle, filtered through a 70 μm filter, and plated in a T175 flask. At 24 hours, non-adherent cells were removed, cells were rinsed with saline, and fresh CIM was added. Cells were fed every 3-4 days. After 4 weeks, cells were trypsinized for 2 minutes and re-plated in CIM in a T175 flask. Cells that did not detach in 2 minutes were discarded. After 2 weeks, cells were trypsinized for 2 minutes and re-plated in 100 mm tissue culture dishes at a density of 50-500 cells/cm^2 in Cell Expansion Media (CEM; IMDM, 10% FBS, and 10% Horse Serum). Once passage 3 cells were confluent, they were either expanded further in 100 mm dishes or frozen (FBS + 10%DMSO). Cells were used between passages 4-10.

Plastic Adherence Method

Bone marrow was removed as described above. Bone marrow from a single bone was re-suspended in 3mL DMEM + 20% FBS and placed in a 6 well plate. Non-adherent

cells were removed at 24 hours and cells were expanded into T75 flasks based on confluency. Cells were used between passages 3-5.

Adipogenesis and Osteogenesis of MSCs

Cells were plated in 100 mm tissue culture dishes with CEM for 10 days and then switched into adipogenic induction media (AIM; IMDM, 10% FBS, 10% HS, 12 mM L-glutamine, 5 μg/mL insulin, 50 μM indomethacin, $1x10^{-6}$ M dexamethasone, 0.5 μM 3-isobutyl-1-methylxanthine, and 100 U/mL penicillin/streptomycin) or osteogenic induction media (OIM; IMDM, 10% FBS, 10% HS, 12 mM L-glutamine, 20 μM β-glycerol phosphate, 50 ng/mL thyroxine, 1 nM dexamethasone, 0.5 μM ascorbate-2-phosphate, and 100 U/mL penicillin/streptomycin) for 2-3 weeks (media was changed every 3-4 days.) Cells were fixed with 10% formalin for 20 minutes at room temperature and then either 0.5% Oil Red O (Sigma) in methanol or Alizarin Red S (Sigma) for 20 minutes at room temperature and visualized on a Nikon TE300.

Cell Surface Marker Analysis

Low density method MSCs (passage >3), plastic adherence method MSCs, and freshly-isolated whole bone marrow (from a littermate or closely related mouse of the same age) were incubated with the following antibodies to CD45-PE (Becton Dickinson 553081; leukocyte common antigen, Ly-5) and CD11b-APC/Cy7 (BD 557657; macrophage and granulocyte marker, Mac-1, Integrin αM chain) for 20 minutes at room temperature in the dark. Cells were analyzed by flow cytometry on a Becton Dickinson LSR Flow Cytometer with FACS Diva software.

MSC-sphere Induction

MSCs were expanded to 80-90% confluency in 100 mm tissue culture dishes. Cells were trypsinized, and plated in NeuroBasal A (Invitrogen, Carlsbad, CA) supplemented with serum substitute B27 (Invitrogen), insulin (25 μg/mL), transferrin (100 μg/mL), putrescine (60 μM), sodium selenite (30 nM), progesterone (20 nM), and L-glutamine in 100 mm Petri dishes or tissue culture dishes. Cell seeding density was 500,000-750,000 cells/dish unless stated otherwise. Cells were incubated on a rotary shaker at speeds of 0, 25, 40, or 55 rpm for up to 14 days. Human recombinant basic growth factor (bFGF; 10 ng/mL; PreproTech) and/or EGF (10 mg/mL) was added to the media every 2-3 days.

Sphere Size Determination

MSC-spheres were induced and photographs were taken at specified timepoints on a Nikon TE300 microscope. Using Image Pro Plus software, the circle tool (which draws a circle and calculates the area, circumference, and related parameters) was used to encircle each individual sphere in 10X and 20X images. The software calculates the radius from the known magnification based on the pixels in the image which is a known quantity from the objective and imaging software used. Data were exported to Excel and the average sphere radius for each condition and timepoint was calculated. Data were averaged for at least 50 spheres per condition per timepoint for radius calculations. Data was imported to Igor (Wavemetrics), binned into even radius ranges, and graphed as histograms. Outliers, which were defined as more than two standard deviations from the mean, were excluded. Standard error of mean (SEM) and/or standard deviations were calculated where appropriate, and two-tailed, paired Students t-test was used to determine statistical significance ($p < 0.05$).

Viability Analysis

MTT Viability Assay

Viability was quantified by the MTT Assay Kit (Sigma) according to the manufacturer's instructions. MTT is a reagent that is cleaved by the mitochondria of active cells forming insoluble purple crystals. Briefly, cells were incubated with MTT solution for 2-4 hours at 37°C in an incubator. MTT solublization solution was added and the plate was rocked and/or triturated to dissolve formed crystals. Absorbance was taken at 570nm/690nm. Results were averaged for 4-6 wells per condition. Average absorbance was corrected for the number of spheres per well.

Live/Dead Staining

Viability throughout the spheres was examined by Live/Dead staining of representative spheres with Calcein Blue (live cells) and ethidium homodimer (dead cells) and imaged on a Zeiss LSM 510 Confocal Microscope for 3-color channels. Ethanol-treated positive controls were also imaged using the same settings.

Histology

Histological Processing

Cells were fixed in suspension with ethanol and glacial acetic acid for 20 minutes. Cells were suspended in Histogel and paraffin embedded, and sectioned (7 μm) on a microtome. Slides were deparaffinized, and antigen retrieval was preformed with Proteinase K (1 μg/mL) for 10 minutes at room temperature.

Immunocytochemistry

Cells were permeablized with 0.3% Triton-X100 and 4% goat serum in PBS for 20 minutes at room temperature. Cultures were blocked with 4% goat serum. Primary antibodies were incubated overnight at 4º C against the following proteins: Nestin Rat-401 (Chemicon MAB353; 1:200), fibronectin (Chemicon AB5032; 1:200), integrin $\alpha 5\beta 1$ (Chemicon MAB2514; 1:100), neurocan (MAB 5212; 1:500), L1 (Chemicon MAB5272; 1:200), GFAP (Chem MAB360; 1:200), N-cadherin (Zymed 33-3900; 1:200), vimentin (Chem MAB3400; 1:300), integrin αv (Chemicon AB1930; 1:1000), CD-44 (Zymed 13-5500; 1:300), O4 (Chem MAB 345; 1:300), integrin $\beta 3$ (Chem AB1932; 1:80), SSEA-1 (Chem MAB353, 1:200), integrin $\beta 4$ (Chem AB1922; 1:200), NeuN (Chem MAB377; 1:200), NG2 (Chem AB5320; 1:300). Spheres were counterstained with nuclear stain Hoechst 33258. NSCs were used as a positive control from nestin, fibronectin, laminin, GFAP, and NG2 (data not shown). For remaining stains, no positive control was found. Secondary-only controls were used to set imaging parameters. Secondary anti-mouse-Alexa 546 (1:500), anti-rabbit-Alexa 633 (1:500), and anti-rat IgG-FITC (1:200) antibodies was incubated for 2 hours at room temperature. Cells were imaged on a LSM Confocal Microscope.

Phalloidin Staining

Adherent MSCs (MSC-LD) were grown for 3 days on coverslips. MSC-spheres were grown for 14 days as described above. Cells were rinsed with PBS, and fixed in 3.7% formalin for 15 minutes at room temperature. Cells were then rinsed with PBS and permeabilized with 0.1% Triton-X-100 in PBS for 15 minutes at room temperature.

Cells were stained with Hoechst 33258 (25 μg/mL) and phalloidin-Texas Red (2U/mL) for 20 minutes at room temperature in the dark. Cells were rinsed twice for 15 minutes in PBS and mounted on slides in Gel Mount.

Radial Migration Assay

Tissue culture treated plates (24-well) were incubated with known concentrations of extracellular matrix proteins overnight. Protein solution was removed, and MSC-spheres were seeded in the wells and incubated at 37°C. MSC-spheres were then photographed on a Nikon TE300 and the radial migration distance was measured. To measure migration, a circle was drawn to the edge of the sphere and eight radiating lines were drawn at 45° angles around the circle. Migration in that direction was measured as the farthest distance a cell has traveled along that line. Migration distances along the eight lines were averaged for a given sphere. At least 6-10 spheres were measured for each condition and results were averaged.

Data Analysis

Data were exported to Excel (Microsoft) or Igor Pro (Wavemetrics), and average sphere radius was calculated. Averages of sphere radii represent at least 50 sample spheres per condition. Outliers that were greater then two standard deviations from the mean were excluded. Standard error of mean (SEM) and/or standard deviations were calculated where appropriate, and Students t-test was used to determine statistical significance ($p>0.05$)

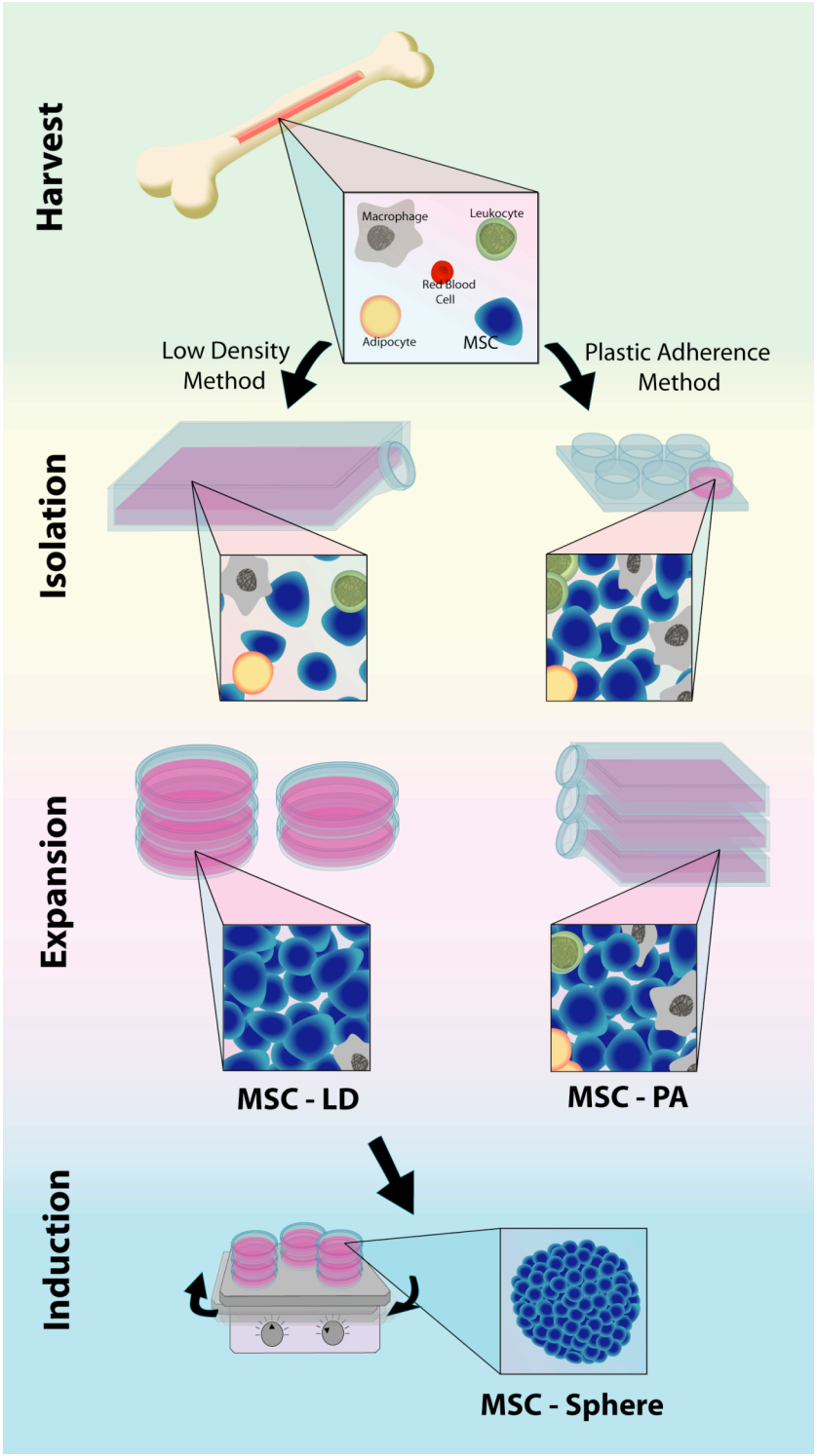

Figure 3.1: MSC harvest, isolation, expansion, and induction methods.

3.3 Results

Low density isolation produces more consistent MSC cultures

MSCs isolated from the low density method described by Peister, et al. (Peister et al. 2004) (MSCs-LD) differed from MSCs isolated by the traditional plastic adherence method (MSCs-PA) based on several criteria. By passage 3, MSCs-LD were a less heterogeneous population of large (~15 μm diameter) cells with prominent nuclei (**Figure 3.2A**). MSC-PA were in general smaller (6-10 μm diameter) and were a heterogeneous population ranging from small spindle shaped-cells to large flattened cells (**Figure 3.2B**).

Low density isolation selects multipotential cells

MSCs-LD and MSCs-PA were analyzed for their ability to differentiate into adipocytes and osteoblasts through phenotypic-specific induction media. MSCs were seeded into 6-well plates with adipogenic or osteogenic induction media for 2 weeks and 3 weeks respectively. Cells were then fixed and stained with Oil Red O for lipids or Alizarin Red S for calcium phosphate deposits. Representative pictures of cultures show approximately 90% of MSCs-LD in adipogenic media stained positive with Oil Red O (**Figure 3.2C**). On the other hand, only about 10% of MSC-PA in adipogenic media stained positive with Oil Red O (**Figure 3.2D**). In osteogenic media, approximately 50% of MSC-LD had some staining with Alizarin Red (**Figure 3.2E**) and approximately 20% of MSCs-PA had a similar level of staining (**Figure 3.2F**). Although the staining was lighter than typically observed with osteoblasts, the cells had some positive areas suggesting the cells were in early stages of differentiation which is consistent with observations that MSCs isolated from C57BL6 mice take longer to differentiate into osteoblasts ((Peister et

al. 2004) and personal communication with Alex Peister). MSCs-LD and MSCs-PA incubated in non-inductive expansion media had less than 5% positive cells for either Oil Red O or Alizarin Red (data not shown).

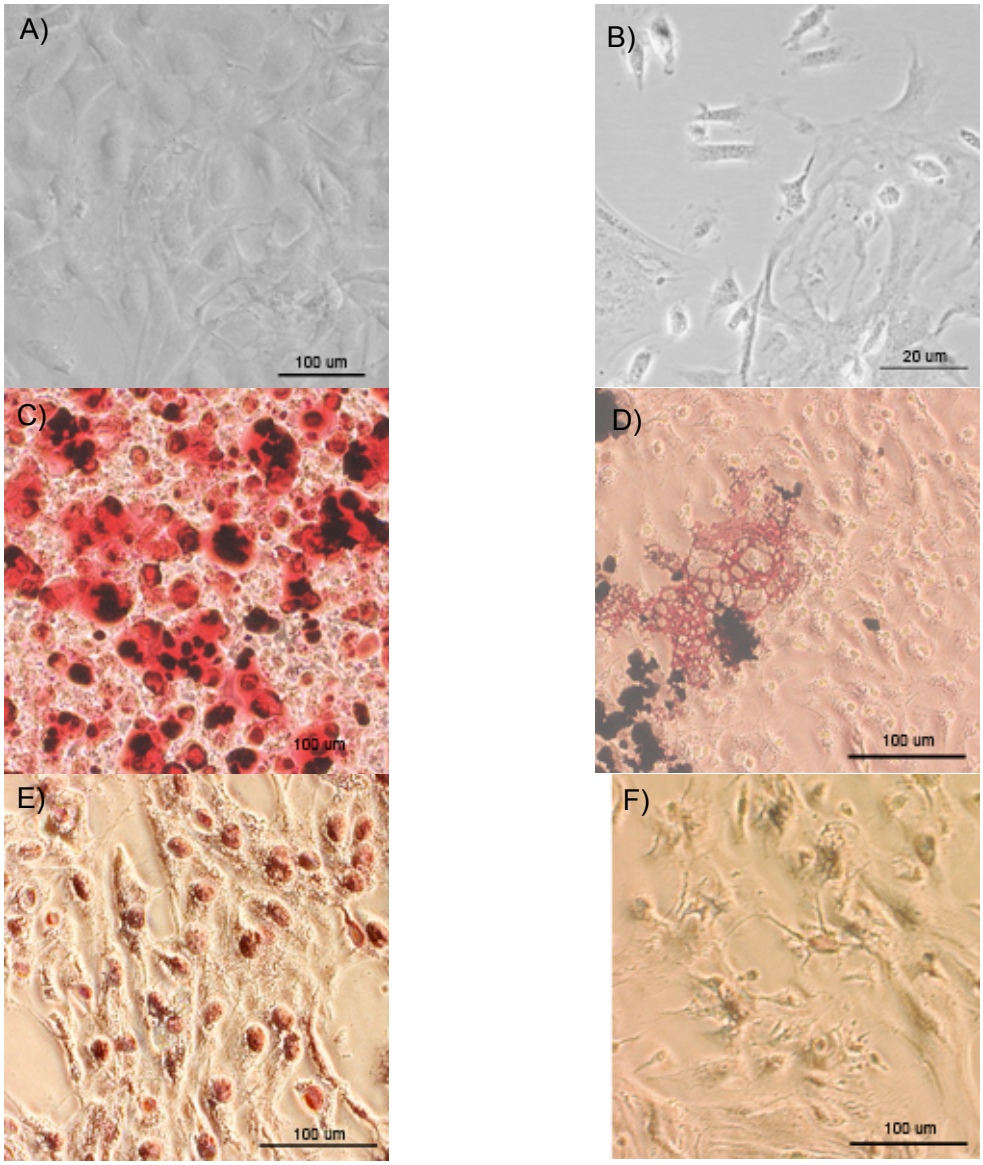

Figure 3.2: Morphology and differentiation potential of MSCs isolated by the low-density method and plastic adherence methods. Phase contrast images of **A)** MSC-LD and **B)** MSC-PA. MSC-LD cells are more homogeneous in size and morphology than MSC-PA. Differentiation potential of MSC-LD and MSC-PA. **C)** MSC-LD and **D)** MSC-PA cultured in adipogenic induction media and stained with Oil Red O. **E)** MSC-LD and **F)** MSC-PA cultured in osteogenic induction media and stained with Alizarin Red S.

Low density isolation selects non-hematopoetic cells

MSCs-LD, MSCs-PA, and whole bone marrow from congenic mice were analyzed by flow cytometry for cell surface marker CD45 (PE) which is found on hematopoetic cells including leukocytes and CD11b (APC-Cy7) which is expressed by macrophages, microglia, and activated neutrophils. Nearly all cells within the whole bone marrow expressed high levels of CD45 and a portion of cells was high for both CD45 and CD11b (**Figure 3.3A**). MSC-PA had a different profile with a population expressing low levels of both CD45 and CD11b and a population with high levels of both (**Figure 3.3B**). The majority of MSCs-LD had lower level of CD45 than either bone marrow or MSC-PA and only a few which were medium-high for CD11b (**Figure 3.3C**). Noticeably absent in the MSC-LD group was the population of high CD45 and high CD11b cells. Cells were gated based on size to exclude red blood cells and small debris.

Together these results suggest that MSC-LD have a higher percentage of cells than MSCs-PA which fit the criteria traditionally used to define MSCs (multipotential and low expression of CD45 and CD11b), and therefore may be a more homogeneous population of stem cells. Unless stated otherwise, MSCs-LD were used in all subsequent experiments and are referred to from this point forward as MSCs.

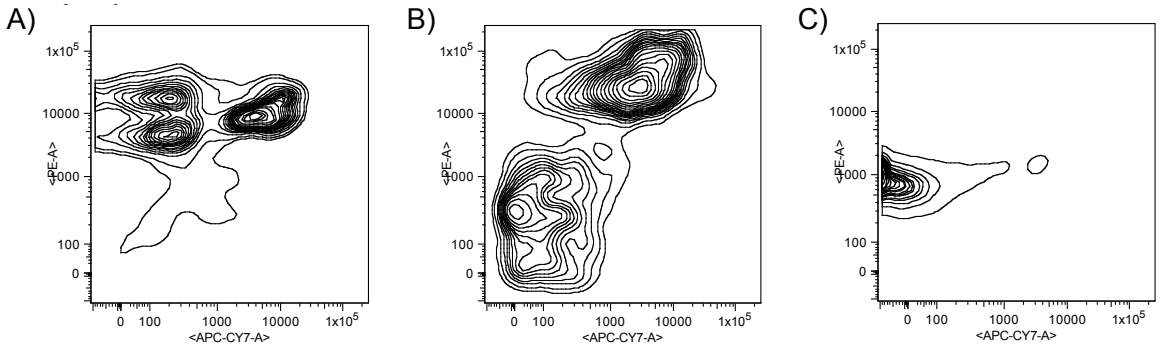

Figure 3.3: MSC cell surface marker expression. A) Whole bone marrow, **B)** MSC-PA, and **C)** MSC-LD were incubated with CD45-PE (y-axis) and CD11b-APC-Cy7 (x-axis) antibodies. Expression levels were measured by flow cytometry. Noticeably absent in the MSC-LD sample is the population with high levels of both CD45 and CD11b. Cells were gated to exclude red blood cells and small debris. (n=5 per condition)

MSCs can be induced to form multicellular spheres

MSCs isolated by the low density method (passage 3-8) were induced to form spherical cell aggregations by seeding them in sphere induction media in 100 mm non-tissue culture treated Petri dishes on rotary shaker in an incubator at speeds of 0, 25, 40, or 55rpm or in 100 mm tissue culture treated dishes. Cells were supplemented with bFGF (10 ng/mL) every 2-3 days. By this method, MSCs formed mutlicellular aggregates (MSC-spheres) within one week (**Figure 3.4**). Other media formulations or growth factor additions did not induce sphere formation (see Appendix A). MSCs isolated by the MSC-PA method did not form spheres by the method described here in static or rotary conditions.

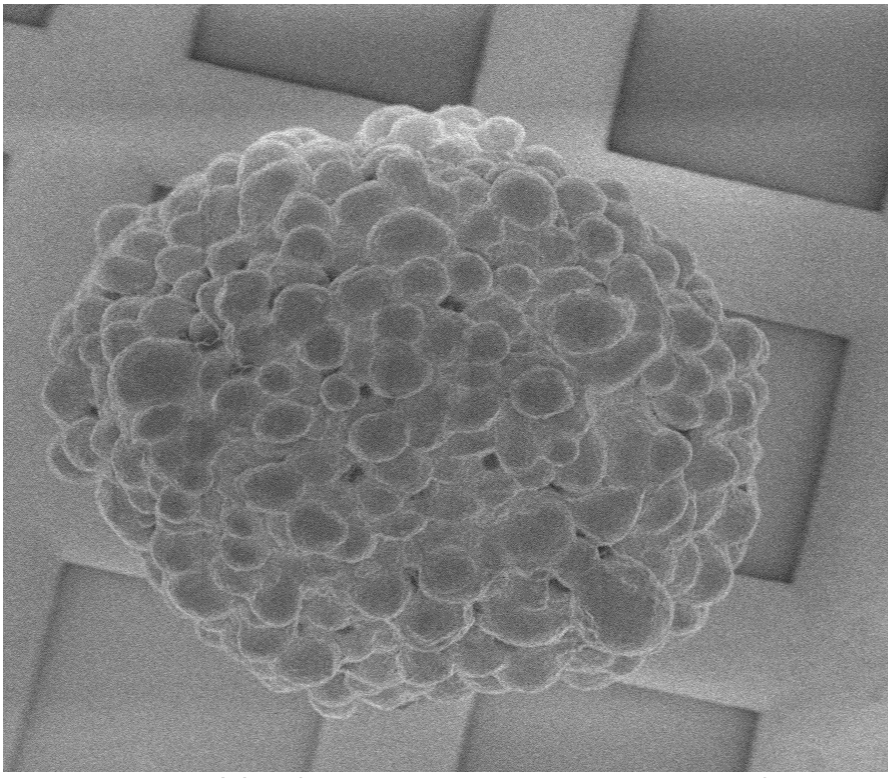

Figure 3.4: MSCs form multicellular aggregates following induction. Scanning electron micrograph of MSC-sphere 7 days post-induction on 40 rpm rotary shaker. MSCs were seeded at 75,000 cells/mL in induction media supplemented with bFGF and EGF every 2-3 days. Interior of grid is 55 μm square.

MSC-sphere radius is dependent on rotary speed

On tissue culture treated dishes, MSCs first adhered to the bottom of the dish, and then after 2-3 days aggregated into small clumps of cells which formed raised, rounded, conglomerates of cells. After 7-10 days, a subset of these spheres detached from the surface and floated in the media. Sphere size was highly variable as was the shape with most appearing more oval than round. Static cultures in Petri dishes (non-tissue culture treated) formed spheres of variable size similar to those in the tissue culture treated dishes, but they also had a smaller portion of cells attaching to the bottom. Conversely, on the rotary shakers, within 24 hours, MSCs were forming multicellular aggregations (MSC-spheres). With rotary motion, spheres were more consistent in size and shape.

The size of MSC-spheres was dependent on rotary speed. MSCs were induced on rotary shakers at speeds of 0 rpm, 25 rpm, 40 rpm, and 55 rpm with a seeding density of 75,000 cells/mL. At 7 days after induction, sphere size was measured. The average radius of spheres formed at 25 rpm was 70.5 ± 9.0 μm while the average radius formed at 40 rpm was 36.0 ± 4.8 μm. MSC-spheres formed at 55 rpm had a radius of 11.7 ± 3.87 μm. The static (0 rpm) culture yielded spheres that were 12.7 ± 5.0 μm. In **Figure 3.5** the distribution of sphere radii for the different speeds is plotted as a histogram with a representative photo of each rotary speed.

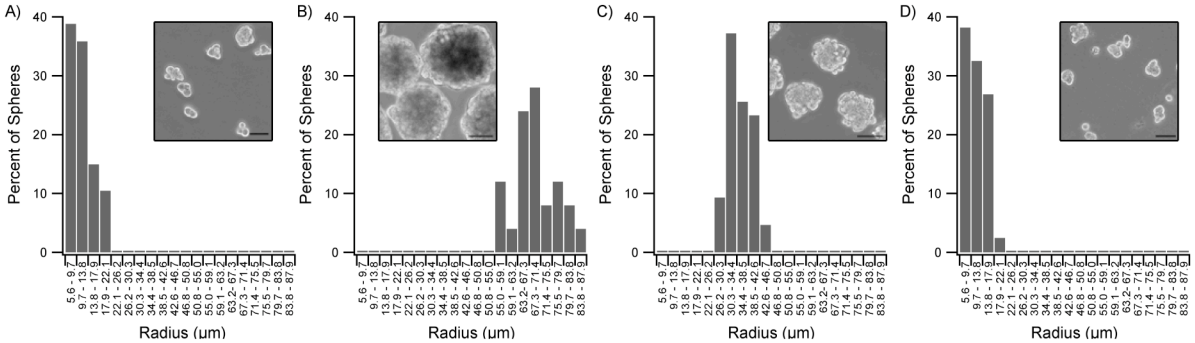

Figure 3.5: MSC-sphere size is dependent on rotary speed during induction. Induction rotary speed of A) 0 rpm, B) 25 rpm, C) 40 rpm, and D) 55 rpm produced significantly different sized spheres. All MSCs were seeded at 50,000 cells/mL with bFGF and measured at 7 days post-induction. n ≥ 50 spheres/speed.

MSC-sphere radius is dependent on cell seeding density.

MSC-sphere size is also dependent on seeding density. MSCs were seeded at 10,000 cells/mL, 50,000 cells/mL, 100,000 cells/mL, and 200,000 cells/mL and incubated on the 40 rpm rotary shaker. At 4 days, spheres from the 10,000 cells/mL dish had an average radius of 8.8 ± 1.3 μm (n=32) while spheres from the 50,000 cells/mL dish had an average radius of 13.1 ± 2.2 μm (n=49). Seeding the plate at 100,000 cells/mL and 200,000 cells/mL produced spheres with an average radius of 31.3 ± 7.2 μm and 18.3 ± 5.4 μm respectively. However, spheres with 200,000 cells/mL had formed several clumps of spheres (several of which exceeded 500 μm) which were excluded from the measurements since they were too large to capture even in the 4X objective. Also because of the clumping, there was a large variation in size in the 200,000 cells/mL group. **Figure 3.6** presents a histogram of the sphere radii by seeding density and representative photos of each. Spheres in the 100,000 cells/mL and 50,000 cells/mL groups produced spheres with a radius of 30-50 μm at 7 days (data not shown) with the most consistent sized spheres. Therefore, MSC-spheres were chosen to be produced on the 40 rpm rotary shaker at a seeding density of 75,000 cells/mL for future studies.

MSC-spheres are viable though the sphere interior

To verify that the interior of the spheres were not necrotic, a Live/Dead assay was performed with Calcein Blue which fluoresces blue in living cells and ethidium homodimer which stains dead cells red. The spheres were then imaged on a confocal microscope. Although sphere size differed with rotary speed, dead cells were not observed in the center of the spheres at any sphere except the ethanol-treated control. (**Figure 3.7**).

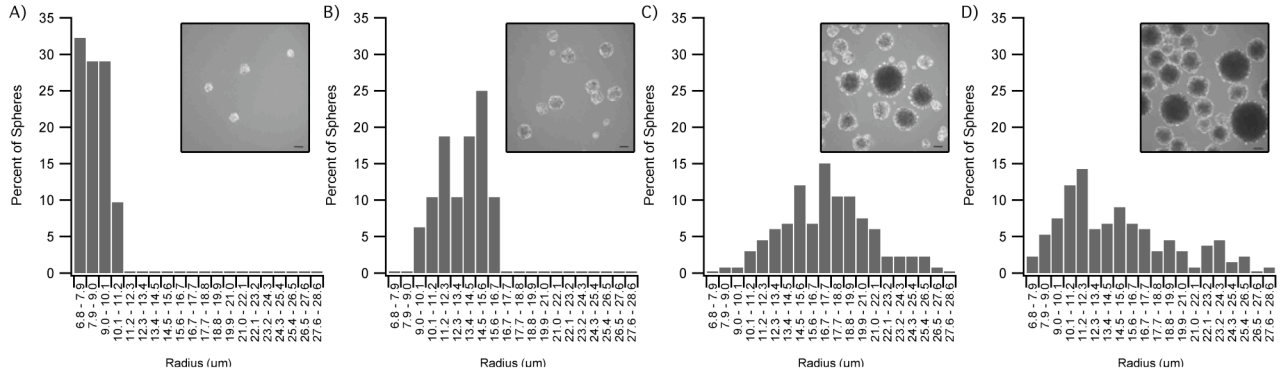

Figure 3.6: MSC sphere radius is determined by the seeding density. MSCs were seeded at A) 200,000 cells/mL, B) 100,000 cells/mL, C) 50,000 cells/mL, and D) 10,000 cells/mL at 40 rpm with bFGF. After 4 days, the spheres had noticeable size differences.

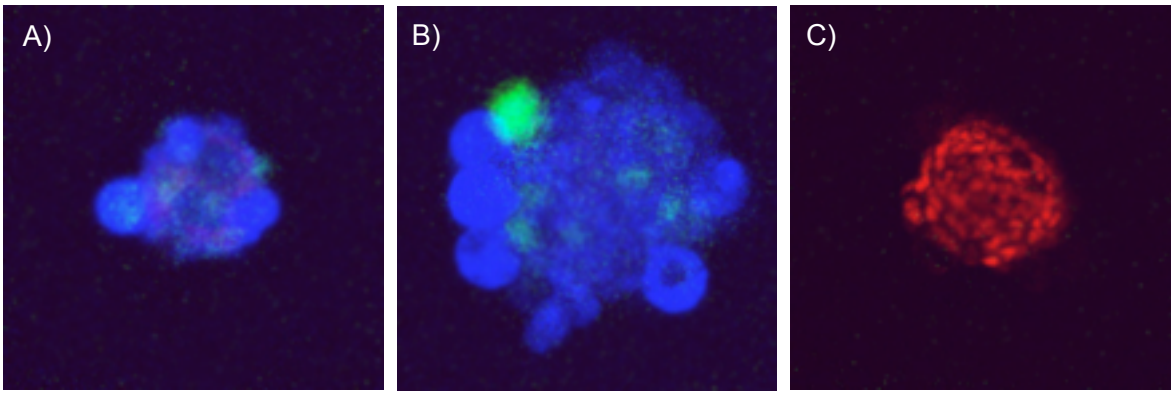

Figure 3.7: Viability of MSC-spheres. A) Static Tissue Culture B) 40 rpm C) ethanol-treated control MSC-spheres were stained with calcein blue (live cells) while ethidium homodimer (red; dead cells) and viewed on a confocal microscope.

MSC-spheres are bFGF and EGF responsive

Formation of MSC-spheres is dependent on growth factor addition

Sphere formation was dependent on the presence of bFGF in the induction media. Spheres did not form if bFGF was not added during the first day of induction, however, the maintenance of spheres was less sensitive to additional supplements of bFGF added between 2-5 days without obvious differences in the timing (data not shown). Although, the addition of EGF influenced sphere size and viability (data below), EGF was not sufficient for sphere formation in the absence of bFGF (data not shown).

Growth factor addition influences sphere size and viability

The addition of EGF during MSC-sphere induction increased both the average sphere radius at 7 days and the viability measured by the MTT assay. The average radius of the spheres without EGF was 21.8 ± 6.5 μm while the average radius with EGF was 35.2 ± 7.8 μm (**Figure 3.8A**). The cleavage of the tetrazolium ring of MTT is a measure of the mitochondrial activity in living cells. The average reduction of MTT normalized per sphere was significantly increased $5.0 \times 10^{-3} \pm 0.7 \times 10^{-3}$ (n=5 cultures) for the MSC-spheres with bFGF and EGF compared to $4.0 \times 10^{-3} \pm 0.6 \times 10^{-3}$ (n=5 cultures) for the MSC-spheres with only bFGF (*t*-test p=0.04) (**Figure 3.8B**).

The cleavage of MTT is a measure of mitochondrial activity and not a direct measure of cell viability or cell number, however, taken with the live/dead staining and the larger sphere radius of the EGF supplemented spheres, it suggests that the spheres with EGF have more cells per sphere. This was in agreement with our observations, however, quantifying the number of cells per sphere directly proved difficult.

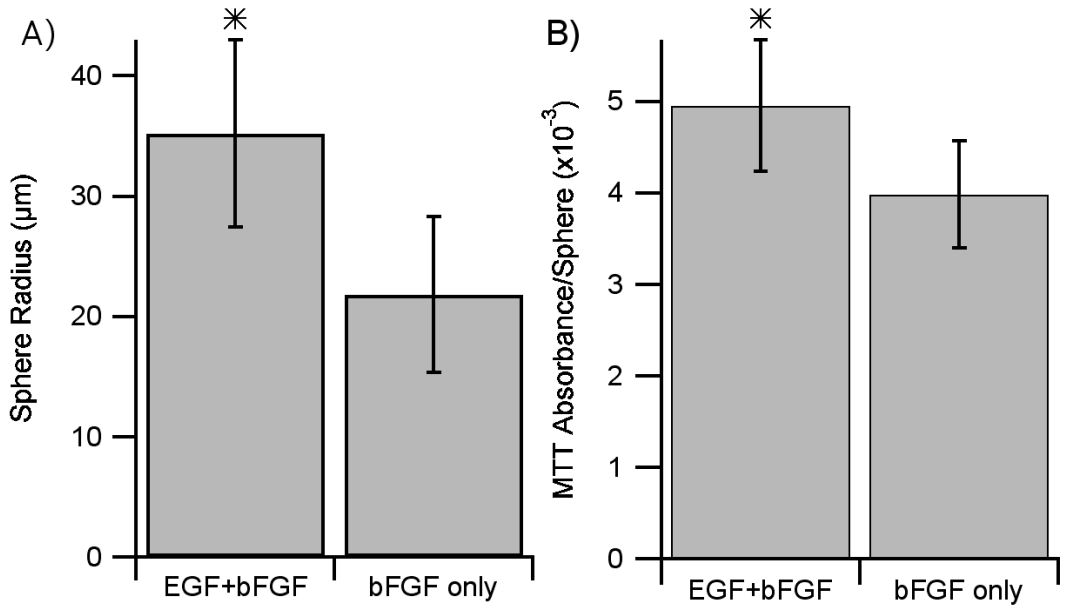

Figure 3.8: Effect of EGF addition during sphere induction. A) Addition of EGF significantly increased the average sphere radius from 21.8 ± 6.5 μm to 35.2 ± 7.8 μm. B) Addition of EGF during induction significantly increased the reduction of MTT by formed MSC-spheres. The average reduction of MTT normalized per sphere was 5.0 x 10^{-3} ± 0.7 x 10^{-3} for the MSC-spheres with bFGF and EGF and was 4.0 x 10^{-3} ± 0.6 x 10^{-3} for the MSC-spheres with only bFGF. * t-test p-value ≤ 0.5

Growth rate of MSC-spheres is influenced by EGF

MSC-spheres were induced at 75,000 cells/mL on tissue culture plastic, and at 25 rpm and 40 rpm on the rotary shaker with or without EGF. MSC-spheres were measured for their growth over two weeks post-induction. The addition of EGF influenced the rate at which spheres began to form and hence their average size at 7 days, but did not affect their size at 14 days when growth had begun to plateau (**Figure 3.9**). This effect was especially apparent on the static cultures.

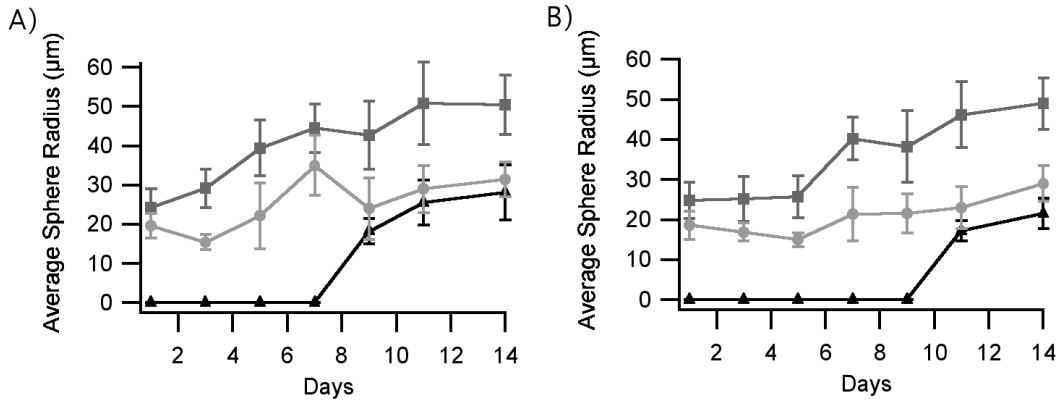

Figure 3.9: MSC-sphere size growth over two weeks with and without EGF. A) with bFGF and EGF (10 ng/mL) and B) bFGF alone without EGF (■ 25 rpm, ● 40 rpm, ▲ Static Tissue Culture Plastic). The addition of EGF influenced the rate at which spheres began to form and hence their average size at 7 days, but did not affect their size at 14 days when growth had begun to plateau. All plates were seeded at 75,000 cells/mL.

MSC-spheres expression of neural markers

MSC-spheres were stained fixed, paraffin embedded, sectioned, and stained by immunocytochemistry for neural markers and examined on a confocal microscope. MSC-spheres express neural precursor marker, nestin, but do not express significant amounts of GFAP, NG2, or NeuN. This suggests that the MSC-spheres are remaining in an undifferentiated state while expressing the neural precursor marker, nestin. (**Figure 3.10**)

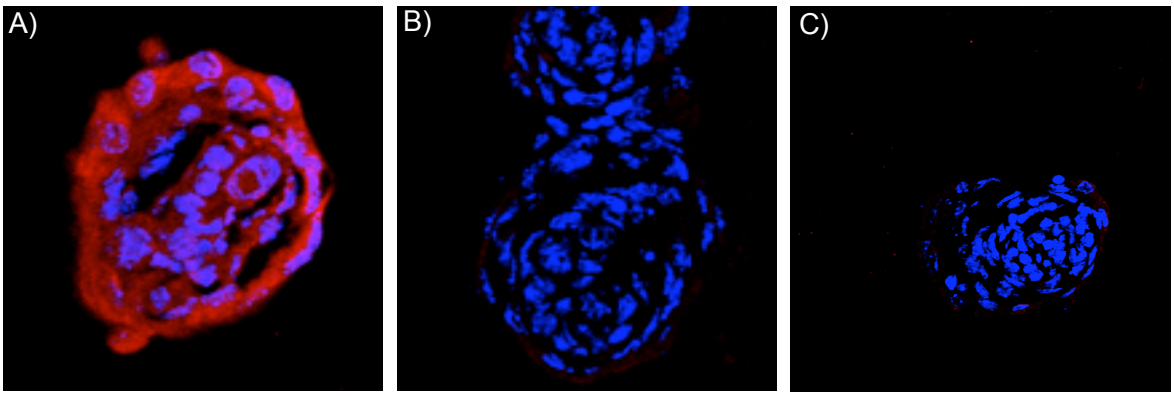

Figure 3.10: Expression of neural markers by MSC-spheres. MSC-spheres express A) nestin, but do not express significant amounts of B) GFAP, C) NG2, or NeuN (not shown). All neural markers are red with nuclear counterstain Hoechst 33258 in blue.

MSC-spheres migrate on extracellular matrix proteins

Migration is enhanced on laminin and collagen type IV

MSC-spheres were plated in a 48-well plate that had been coated overnight with extracellular matrix proteins laminin-1, collagen type IV, and fibronectin at concentrations of 10 μg/mL and 5 μg/mL and on control wells for poly-L-lysine (10 μg/mL) and tissue culture plastic. At 36 hours, the radial migration distance was measured. Radial migration distance was significantly increased on all ECM proteins verses poly-L-lysine or tissue culture plastic (*t*-tests $p<0.05$). The migration of MSC-spheres is greatest on laminin-1 and collagen type IV (**Figure 3.11**). In addition, fibronectin showed a concentration-dependent increase of migration distance while laminin and collagen type IV did not.

Centrifugation of spheres increases migration

Interestingly, it appears that centrifugation prior to plating for migration enhances migration distance (**Figure 3.12**) to the point that cells which have been centrifuged will migrate on laminin, collagen type IV, fibronectin, and tissue culture plastic equally (data not shown). Differences in migration substrate were only significant when spheres were allowed to settle rather than being centrifuged. Also interestingly, MSC-spheres migrate very little if plated in new MSC-S media, rather, they migrate to a much greater distance in conditioned media either from themselves, cultured astrocytes, or neural stem cells (data not shown).

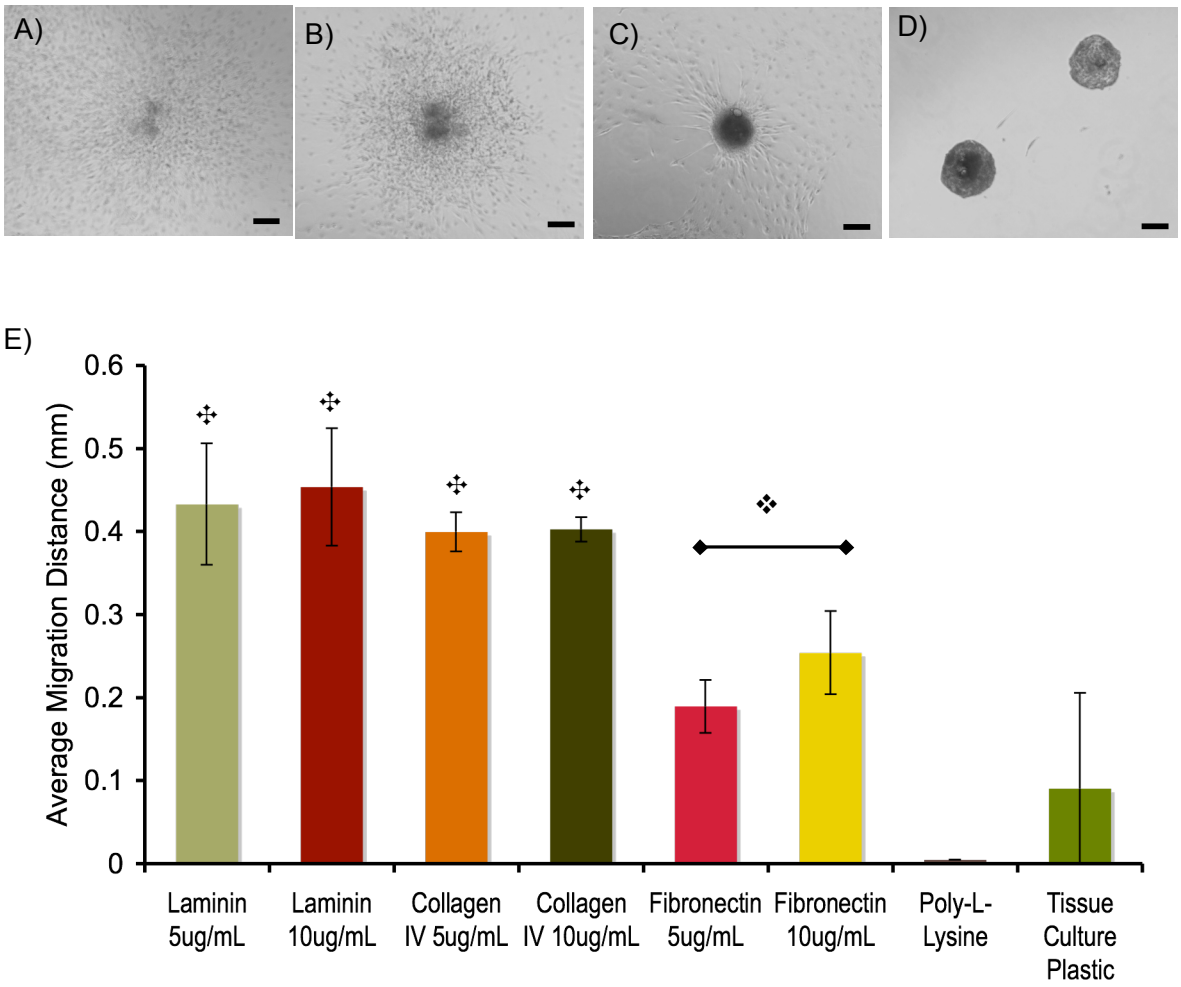

Figure 3.11: Migration of cells from MSC-spheres on extracellular matrix proteins. **A)** laminin, **B)** collagen type IV, **C)** fibronectin, and control **D)** poly-L-lysine. **E)** Average radial migration distance for each condition. Spheres migrated on laminin-1 and collagen type IV significantly more than fibronectin, poly-L-lysine, or tissue culture plastic (✣ t-test; p-values <0.05). In addition, there was a significant effect of coating concentration (5 μg/mL vs 10 μg/mL) for fibronection (❖ t-test; p-value <0.05). Scale bars in pictures are 50μm.

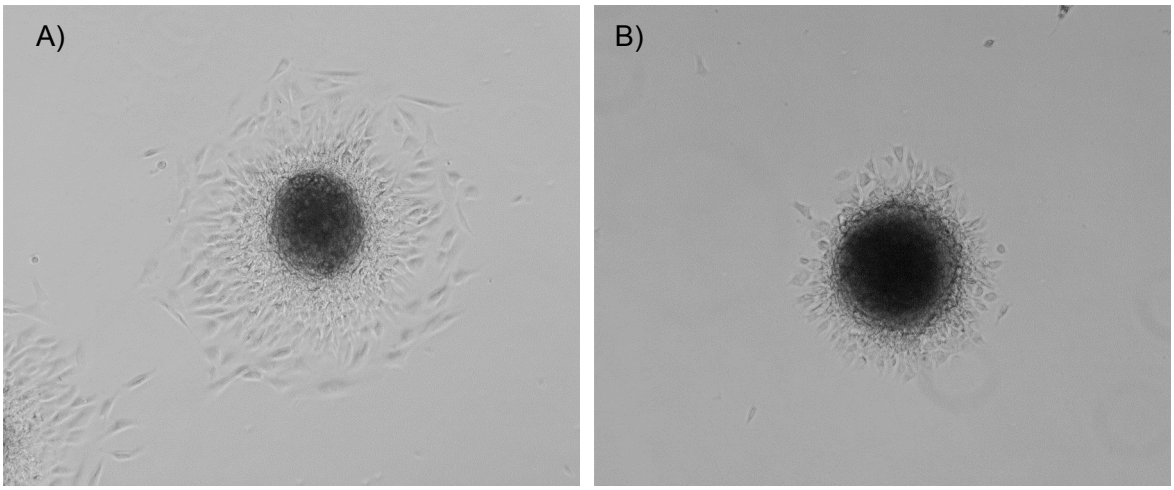

Figure 3.12: Migration of MSC-spheres is stimulated by centrifugation. Prior to plating on tissue culture plastic, MSC-spheres were **A)** centrifuged at 1000 rpm for 3 minutes or **B)** allowed to gravity settle in a 15 mL conical tube. Spheres were plated with their own conditioned media. Migration was photographed at 18 hours and representative photos shown. (1000 rpm x 3 min)

MSC-spheres contain ECM proteins and cell adhesion receptors

In addition to migrating on ECM proteins, MSC-spheres stained positive for ECM proteins within the sphere. MSC-spheres were fixed, paraffin embedded, sectioned, and stained with immunocytochemistry for ECM proteins. The MSC-spheres contained fibronection, laminin, vimentin, and neurocan, and to a lesser degree, they also stained positive for collagen IV (**Figure 3.13**). In addition, we stained for cell-cell and cell-matrix adhesion molecules. The MSC-spheres contained integrin $\alpha 5\beta 1$, integrin αv, L1, and to a lesser degree N-cadherin and CD-44. Therefore, the cells appear to be producing their own ECM and are expressing several cell-cell and cell-matrix adhesion markers. Interestingly, much of the matrix produced appears to be in the interior of the sphere.

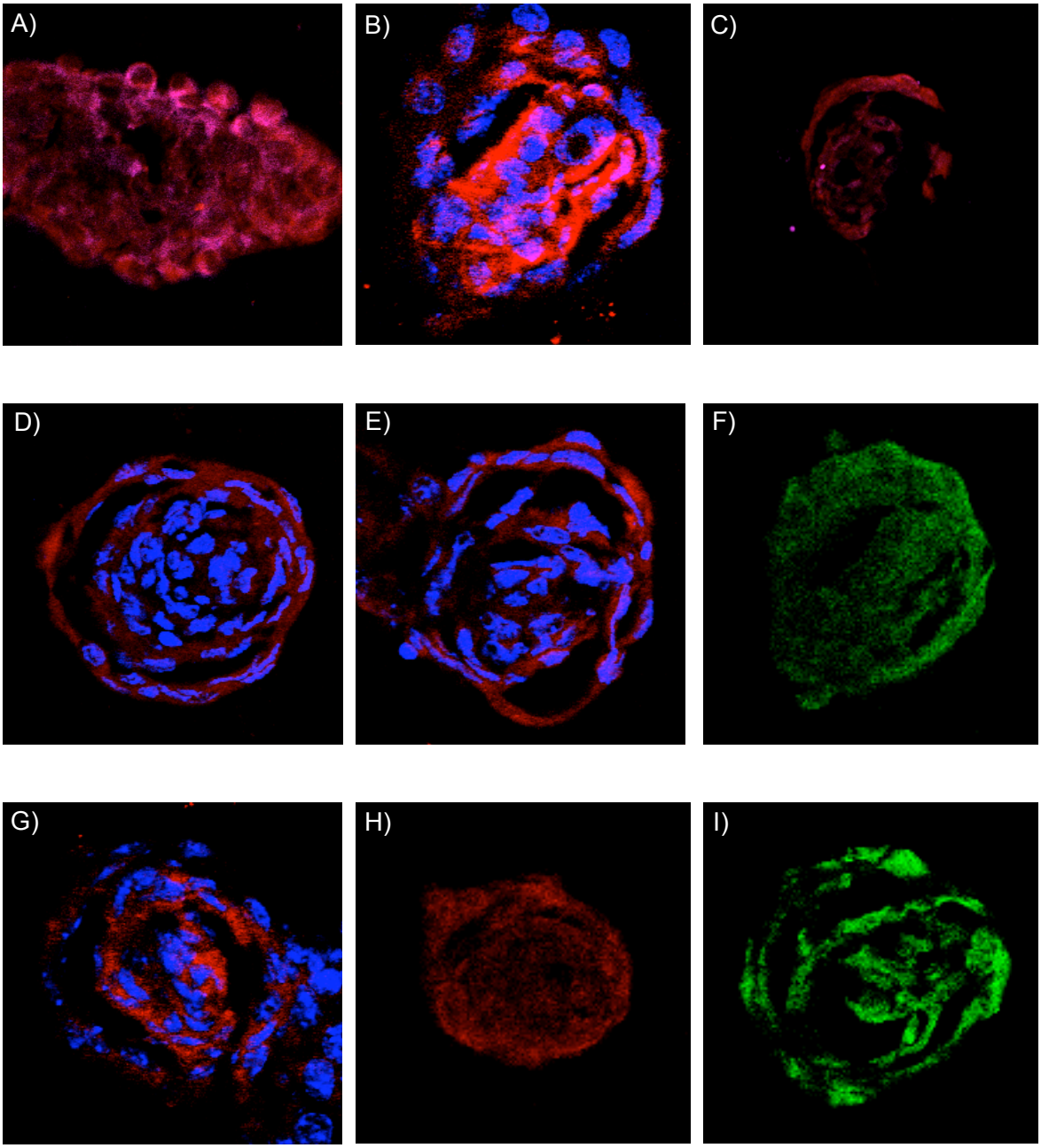

Figure 3.13: MSC-spheres stain positive for ECM proteins and cell adhesion proteins. A) Laminin B) Fibronectin C) Collagen IV D) Neurocan E) Vimentin F) Integrin $\alpha 5\beta 1$ G) Integrin αv H) N-cadherin I) L1. Immunostains are either red or green with nuclear counterstain Hoeschst 33258 in blue in some images.

MSC-spheres alter their cytoskeletal configuration

Interestingly, although isolated from a GFP+ transgenic mouse under a β-actin promotor, MSCs have reduced GFP fluorescence as they are passaged compared to a fresh bone marrow isolation. In addition, when they become MSC-spheres, the GFP fluorescence is even dimmer. Therefore, we looked for the expression of β-actin in the spheres by staining for β-actin by immunocytochemistry in paraffin-embedded sections and by staining fixed cultures with phalliodin conjugated to Texas Red. Prior to MSC-sphere induction, MSCs have obvious β-actin structure (**Figure 3.14A**) however they appear to decrease β-actin when in MSC-spheres (**Figure 3.14B**).

To quantify these results, quantitative RT-PCR was performed for β-actin and normalized to GAPDH. The MSC-spheres appear to have decreased production of β-actin compared to the pre-sphere adherent MSCs (**Figure 3.15**). Although surprising, this is consistent with our observed differences in phalliodin staining.

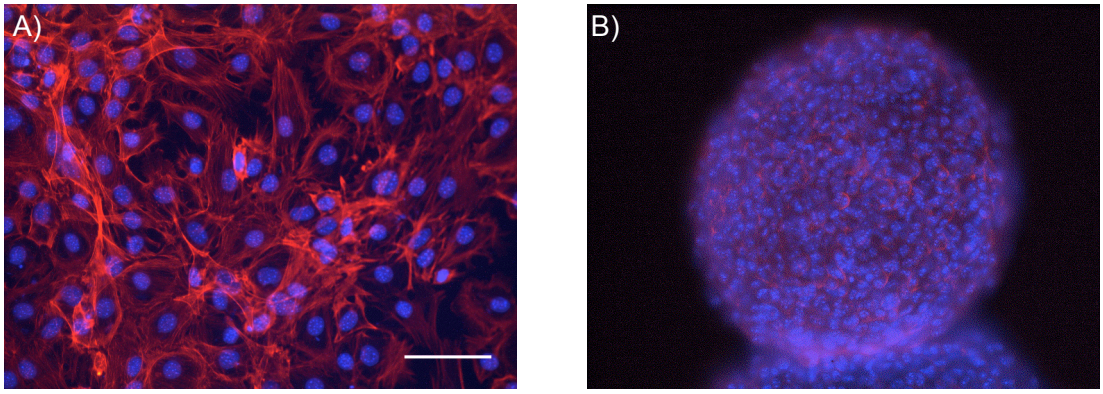

Figure 3.14: MSC-spheres alter their cytoskeletal structure. A) MSCs in expansion culture and B) MSC-spheres were fixed and stained with phalliodin-Texas Red and counterstained with Hoescht. In the sphere, MSCs decrease their cytoplasm and β-actin. Photos are the same magnification; scale bar is 25μm.

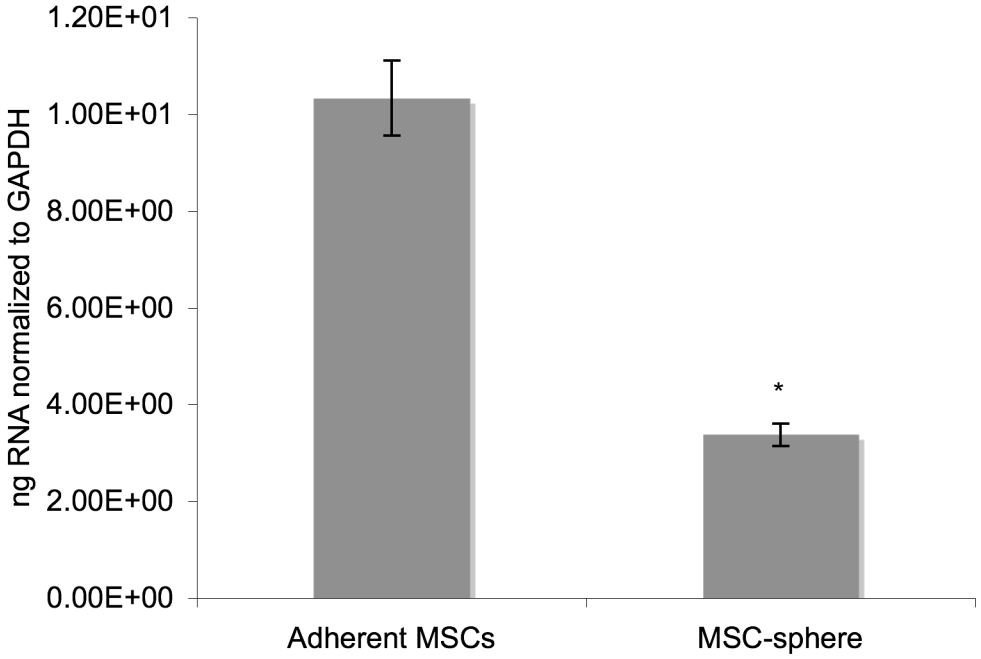

Figure 3.15: MSC-spheres significantly downregulate β-actin compared to the pre-sphere MSCs. Quantitative RT-PCR for β-actin was performed and normalized to the expression of GAPDH. (n=5 each condition; p=5.88x10^{-5} paired students t-test)

MSC-sphere induction may alter gene expression

During MSC-sphere induction, neurotrophins (brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), or neurotrophin-3 (NT-3)) were added every 2-3 days with bFGF and EGF and cultured on the 40 rpm rotary shaker. MSCs were plated at 75,000 cells/mL One week after induction, RNA was isolated and quantitative RT-PCR was performed for Nanog and nestin. Nanog expression was decreased in MSC-spheres compared to MSCs prior to sphere induction (**Figure 3.16A**). The addition of neurotrophins during induction did not appear to alter this effect. While there appears to be a trend in nanog expression, it is important to note that nanog expression in all cases is quite low in all samples. The expression of nestin in MSC-spheres was enhanced by the addition of neurotrophins during induction (**Figure 3.16B**). However, all adherent MSCs and MSC-spheres were still below the level of nestin expression by NSCs.

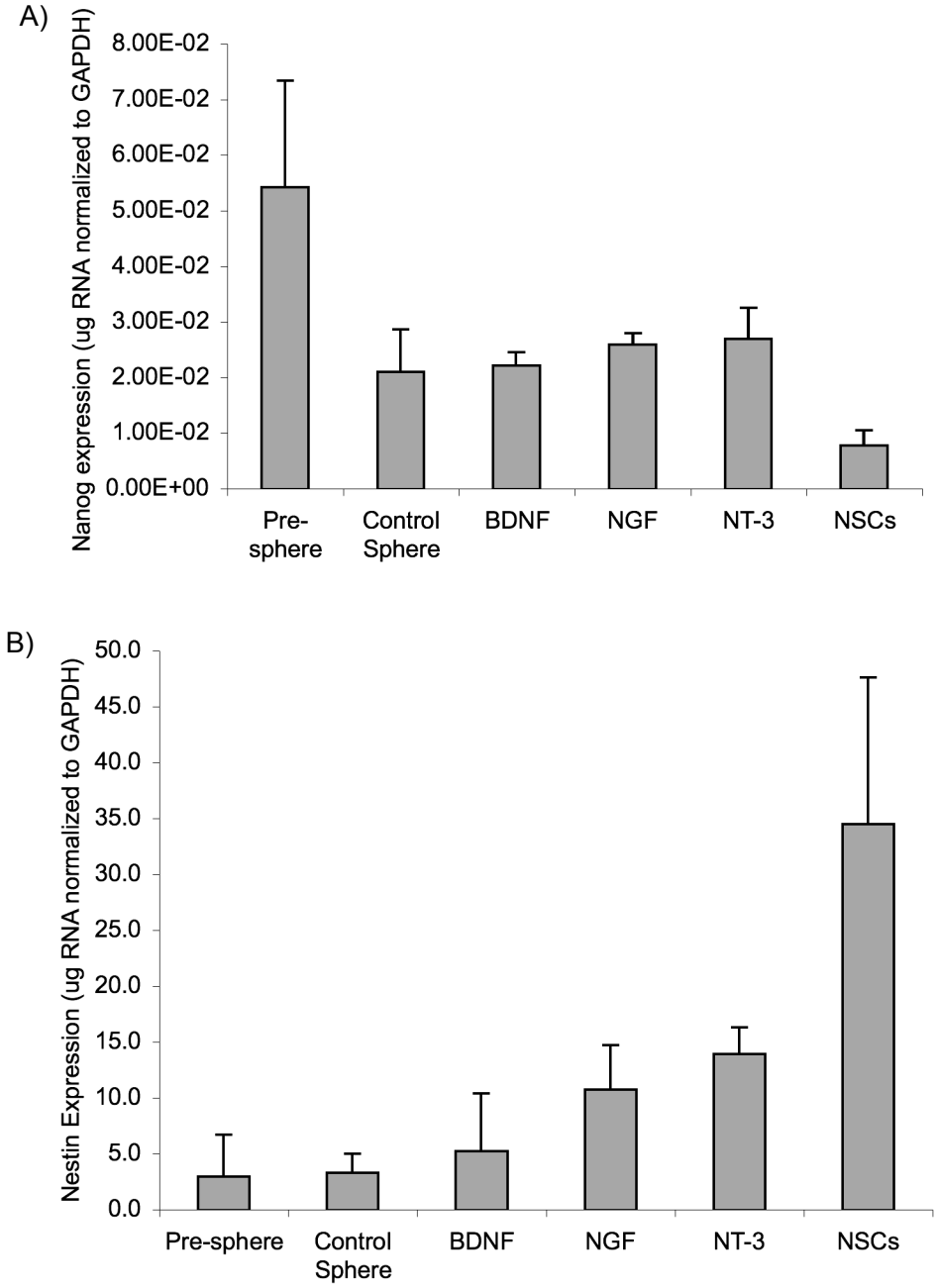

Figure 3.16: Expression of Nanog and nestin in MSC-spheres. A) MSC-spheres have decreased nanog expression compared to MSCs prior to sphere induction. The addition of neurotrophin does not appear to affect the expression further. B) The addition of neurotrophins during MSC-sphere induction increases nestin expression in comparison to controls, however, the expression is still below that of NSCs.

3.4 Discussion

Bone marrow-derived mesenchymal stem cells have been shown to be a potentially useful source of donor cells for neurotransplantation; however high variability has been observed in donor cells which may influence transplant outcomes. Therefore, a more defined population of cells to transplant may lead to more consistent results. We have designed a novel method to yield multicellular MSC-spheres in reproducible sizes based on the culture parameters. These MSC-spheres are bFGF and EGF-responsive and express nestin similar to neural stem cells. They do not appear to express high levels of GFAP, NeuN, or NG2 in their MSC-sphere state. In addition, they migrate on laminin and collagen type IV, and to a lesser extent fibronectin. MSC-spheres also contain these ECM proteins within the sphere as well as neurocan and vimentin, and express several cell adhesion receptors. Interestingly, MSC-spheres also downregulate β-actin and nanog expression compared to the pre-sphere MSCs. The addition of neurotrophins during the sphere induction also increases nestin expression. Once characterized, these MSC-spheres may be a useful donor cell source for future neurotransplantation studies.

To produce donor cells for transplantation into the brain, we identified several desirable criteria, including a less heterogeneous population of cells and a reproducible method by which to culture donor cells in large numbers. The donor cells should also be able to be delivered through a syringe in a densely packed manner so that the transplantation does not induce further damage to the brain by its volume. It may also be beneficial to begin with a population of cells which are migratory and possess some of the characteristics of neuroprogenitor cells. Together these criteria may improve donor cell survival and influence regeneration in the injured brain.

Using the low density method, we were able to isolate a reproducible population of cells which were then induced to form multicellular MSC-spheres. MSC-PA that were isolated by the plastic adherence method did not form spheres by this method. There may be several explanations for this observation, including that the MSC-PA may have had more residual hematopoetic cells which do not usually from large multicellular aggregates. Alternately, by the low density method, we may have selected for a cell type which forms spheres more readily.

The use of the rotary shaker in the MSC-sphere induction adds an interesting dynamic to the culture technique. While we characterized more extensively the effect of varying the rotary speed and density of plating, there are several other parameters which also influenced the cells in this environment. The forces exerted on the spheres by the rotary motion are quite complex but are influenced by several factors including, but not limited to, the size of the dish, the amount of media in the dish, the distance the sphere is located from the center of the dish, the viscosity of the liquid, and whether the dish is kept at a constant velocity. While the mechanics involved are beyond the scope of this manuscript, it is important to acknowledge that there are many other potential mechanisms by which to control the sphere formation.

Other methods of sphere formation exist including the hanging drop method; however, the rotary shaker produces many more spheres and is less labor intensive to produce enough spheres for transplantation. One variation on the rotary method which may be worth exploring would be the use of an orbital shaker rather than the planar rotary shaker. Other future directions include the use of larger culture volumes.

The definition of a neurosphere is somewhat vague in that it is usually defined as a nestin-positive cell aggregation that can give rise to the three types of neural cells: neurons, astrocytes, and oligodendrocytes. There are different stages of neural progenitor development and several different cell populations have been isolated at different ages from the brain with FGF-responsive cells being found earlier than EGF-responsive cells (Ciccolini and Svendsen 1998; Ciccolini 2001). While the addition of EGF to the MSC-sphere cultures enhances their growth and viability, EGF is not sufficient or necessary to form MSC-spheres. On the other hand, FGF is both necessary and sufficient to produce MSC-spheres.

The downregulation of β-actin expression in the MSC-sphere is interesting, and may have multiple possible interpretations. One potential explanation is simply the shape of the cells within the sphere does not require as much β-actin in their cytoskeleton. Therefore, the cells may not make as much β-actin in this configuration. It is also possible that the MSCs are expressing another type of actin or other cytoskeletal protein (such as vimentin which is a major intermediate filament in mesenchymal cells) instead when they are in the MSC-sphere conformation. Alternately, it is possible that there is simply less cytoplasm and therefore less cytoskeleton in general in the MSC-spheres since they appear smaller in size in the MSC-spheres than in adherent culture. While the cause is interesting, one practical implication of the downregulation of β-actin is that another method of tracking the MSCs will be required in order to visualize the cells following transplantation.

The downregulation of nanog expression and upregulation of nestin expression may suggest that the cells are differentiating to some extent in the spheres. Nanog is

expressed in embryonic stem cells and thought to be a key factor in maintaining pluripotency. The decrease in nanog expression when the MSCs are cultured as spheres may suggest that the sphere configuration is contributing to their differentiation. However it is important to note that while the MSCs have a greater expression of nanog than the NSCs, both cell types have low levels of expression in comparison to level normally seen in embryonic stem cells. Likewise, the increased expression of nestin in the MSC-spheres which are cultured in the presence of neurotrophins may also suggest that MSC-spheres are becoming more neuroprogenitor-like. However, the MSCs are still expressing less nestin than NSCs grown in neurospheres. Regardless, together this may be a viable avenue of investigation on inducing the MSCs to a more neuroprogenitor-like fate.

Here we have described a novel method by which to induce MSCs to form MSC-spheres with the use of induction media and a rotary shaker. These MSC-spheres are reproducible and share some characteristics of neural progenitor cells, and may therefore, represent an alternate donor cell source for transplantation. However, before they can be used for this purpose, it will be useful to determine how other parameters influence sphere size and MSC differentiation and assessing the efficacy of the MSC-spheres to rescue neural tissue.

Acknowledgements

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CHAPTER 4

BONE MARROW-DERIVED MESENCHYMAL STEM CELL SPHERES DECREASE APOPTOSIS IN INJURED 3-D CO-CULTURE TESTBEDS

4.1 Introduction

We utilized an *in vitro* injury testbed, consisting of primary neurons and astrocytes in a 3-D construct, to evaluate the effect of MSC-spheres on degenerating neurons. These constructs were developed in our lab and have been characterized previously with neural stem cells (Cullen *(in press)-b*). Here, we first tested the electrophysiological properties of the cells within the constructs which adds to the strength of the testbed as a model which supports healthy, functional neurons. Then, we use the testbed to inject bone-marrow derived mesenchymal stem cells (MSCs) which have been primed *in vitro* to form MSC-spheres into an injured environment. By using the testbed, we are able to test cells and delivery vehicles in a controlled manner prior to transplantation in an animal, thereby optimizing some aspects of the transplant *in vitro* and decreasing the number of animal subjects required.

3-D Co-culture Testbeds

Cells are influenced by complex environmental stimuli, central to which is the local microenvironment, as extracellular context profoundly affects cell behavior. For example, the matrix surrounding cells has been shown to have widespread effects on cellular behavior for a variety of cell types (Cukierman et al. 2001; Yamada et al. 2003; Schindler et al. 2006) including neural cells (Venstrom and Reichardt 1993; Kiryushko et

al. 2004; Loers and Schachner 2007). Cell-cell and cell-matrix interactions are inherently different in the intact organism than in monolayer cell culture (Cukierman et al. 2001; Yamada et al. 2003), therefore the projection of cell culture-based results to *in vivo* systems is not without numerous caveats. Cells cultured in a 3-D environment have been shown to better represent *in vivo* cellular behavior than cells cultured in a monolayer in several cell types (Schindler et al. 2006; Smalley et al. 2006). For example, planar culture of chondrocytes results in de-differentiation, whereas 3-D chondrocyte cultures maintain the expression of chondrogenic markers aggrecan and collagen type II (Benya and Shaffer 1982; Kimura et al. 1984; Buschmann et al. 1992). Similarly, cardiomyocytes in 3-D culture maintain more *in vivo*-like molecular and electrophysiological characteristics than those cultured in monolayers (Bursac et al. 2003). A growing body of evidence suggests that cells grown in planar cultures do not have the same morphology (Friedl et al. 1998; Cukierman et al. 2001), proliferation rates (Hindie et al. 2006; Willerth et al. 2006), migration (Friedl et al. 1998), gene expression (Birgersdotter et al. 2005; Liu et al. 2006b; Yefang et al. 2007), differentiation (Chun et al. 2006; Willerth et al. 2006; Yefang et al. 2007), cellular signaling (Pedersen and Swartz 2005), or pathological susceptibility (Behravesh et al. 2005; Hindie et al. 2006; Smalley et al. 2006) as in 3-D culture or *in vivo*. These findings raise questions about the appropriateness of employing monolayer culture models to study whole organism phenomena.

In the context of recording cellular electrical activity, alterations in ion channel expression, membrane permeability, and the cellular microenvironment (including ion concentrations and pH) can affect the static and dynamic electrophysiological properties of cells. For example, in 3-D culture, Na+/H+ exchangers have been shown to have polarized expression to the apical membrane, which is difficult to maintain in monolayer

cultures (Castillon et al. 2002). In addition, neurons in 2-D have been reported to have exaggerated Ca^{2+} dynamics in comparison to 3-D (Mao and Kisaalita 2004; Desai et al. 2006; Xu et al. 2006). Therefore, the establishment of a 3-D culture system for the study of electrophysiological properties may provide insight into cellular behaviors that more closely mimic those seen in the intact CNS.

Here we describe a novel 3-D construct of neurons and astrocytes within a bioactive scaffold consisting of extracellular matrix-derived proteins and proteoglycans. These culture conditions support considerable process outgrowth, complex 3-D morphologies, and neurons remain viable over several weeks in culture. Neurons within these cultures demonstrate spontaneously electrical activity and exhibit signs of functional synapse formation and network properties. We propose that these constructs may be better platform for a variety of neural research avenues than the current 2-D monolayer culture, as they more closely mimic *in vivo* cytoarchitecture and function.

3-D Co-cultures as a Testbed for Apoptosis in the Injured Environment

A traumatic insult to the brain results in immediate death and dysfunction of neural cells and also triggers a secondary cascade which results in sustained complications including inflammation, excitotoxicity, and breakdown of the blood-brain barrier which may result in cell death for months after the initial traumatic event (Conti et al. 1998; Bramlett and Dietrich 2002). While much cell death that occurs immediately following the primary injury is necrotic, the post-injury environment also activates apoptotic pathways (Wong et al. 2005; Zhang et al. 2005; Byrnes and Faden 2007).

Since the effects of the injury are sustained and multifactorial, it may be beneficial to provide a treatment approach which can act over a prolonged period of time. Stem cell transplantation may facilitate recovery by playing a role in counteracting secondary damage, reconstructing damaged tissue, providing continuous trophic support, mediating cell-cell repair, and/or replacing neural cells (for review see (Longhi et al. 2005). Bone marrow-derived mesenchymal stem cells (MSCs) have been reported to decrease apoptosis in injured neurons most likely through secreted factors (Chen et al. 2002; Crigler et al. 2006). MSC-spheres which have been induced to form cell aggregates may also decrease apoptosis of host cells.

Optimization of donor cells, delivery vehicles, and concentrations will likely require an iterative approach and therefore, the utilization of an *in vitro* testbed could speed this process and reduce the number of animal subjects required. Therefore, we have used this testbed model to assess the effect of MSC-sphere injection has on apoptosis in injured neural cells.

4.2 Methods

3-D Neuronal-Astrocytic Co-culture Constructs

Neurons were isolated from embryonic day 17 - 18 Sasco Sprague-Dawley rats (Charles River Labs) or GFP+ transgenic C57BL/6-Tg(cAG-EGFP)C14-701-FM1310sb mice (C57BL6 background) mice bred in house. Cerebral cortices were isolated and digested in Hank's buffered salt solution (HBSS) with trypsin (0.25% + 1mM EDTA) at 37ºC for 10 minutes, followed by mechanical trituration with DNase I (0.15mg/mL). The cells were centrifuged at 1000 rpm for 3 minutes and resuspended in co-culture medium (Neurobasal + 2% B-27 + 1% G-5 + 500μM L-glutamine). Astrocytes were separately harvested separately from postnatal day 1 Sprague-Dawley pups. Isolated cortices were minced, digested in trypsin (0.25% + 1mM EDTA) for 3-5 minutes at 37°C, followed by DNase I (0.15 mg/ml) treatment and gentle mechanical trituration. Cells were plated in DMEM/F12 with 10% fetal bovine serum. Mechanically agitation was used to detach less adherent cell types at 24 and 72 hours and the primary astrocyte culture (>95% type I astrocytes) was passaged upon reaching ~90% confluency for four weeks. Astrocytes were used between passages 4-12 for plating in 3-D culture.

Cultures were plated in custom-made cell culture chambers consisting of a glass coverslip below a silicone-based elastomer mold (Sylgard 184 and 186, Dow Corning; Midland, MI; cross-sectional area = 2 cm^2). Prior to plating, the chambers were pre-treated with 0.05 mg/mL poly-L-lysine (PLL, Sigma) followed by Matrigel™ (0.5 mL/well at 0.5 mg/mL, Becton Dickinson Biosciences; Bedford, MA) in Neurobasal™ medium (each treatment was >4 hours). 3-D neuronal-astrocytic co-cultures were plated at a 1:1 initial neuron:astrocyte ratio at a density of 2500 cells/mm^3 in Matrigel™ matrix (500-750 μm thick; final concentration 7.5mg/mL). Following Matrigel™ gelation, co-culture

medium was added. Co-cultures were maintained at 37°C and 5% CO_2-95% humidified air and medium was replaced at 24 hours and every 2 days thereafter. Experiments were initiated at 21 days *in vitro* (DIV). All procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the Georgia Institute of Technology. All cell reagents were obtained from Invitrogen (Carlsbad, CA) or Sigma (St. Louis, MO) unless otherwise noted.

Cell Viability

Cell cultures were incubated with 4 μM ethidium homodimer-1 (EthD-1) and 2 μM calcein AM (both from Molecular Probes, Eugene, OR) at 37° C for 30 minutes and then rinsed with 0.1M Dulbecco's phosphate-buffered saline (Invitrogen). The percentage of viable cells was calculated by counting the number of live cells (fluorescing green by AM-cleavage) and the number of cells with compromised membranes (nuclei fluorescing red by EthD-1). At 21 DIV, co-culture viability was assessed (n = 18).

Immunocytochemistry

Cultures were fixed in 3.7% formaldehyde (Fisher, Fairlawn, NJ) for 30 min, rinsed in PBS and permeabilized using 0.3% TritonX100 (Kodak, Rochester, NY) + 4% goat serum (Invitrogen) for 20 minutes. Primary antibodies were added (in PBS + 4% serum) at 18-24°C for 4 hours. Secondary fluorophore-conjugated antibodies (FITC or TRITC-conjugated IgG (Jackson Immuno Research, West Grove, PA) or Alexa 488 or 546-conjugated IgG (Molecular Probes) were added (in PBS + 4% serum) at 18-24°C for 2 hours. Co-cultures were immunostained using primary antibodies recognizing a combination of the following intracellular proteins: 1) tau-5 (MS247P, 1:200,

NeoMarkers, Fremont, CA), a microtubule-associated protein expressed predominantly in neurons, and 2) MAP-2 (rabbit polyclonal antibody; AB5622; 1:200; Chemicon) 3) synapsin I (A6442, 1:200, Molecular Probes), a synaptic vesicle protein localized in presynaptic specializations (Fletcher et al. 1991). Counterstaining was performed using Hoechst 33258 (1:1000, Molecular Probes).

Fluorescence Microscopy

Cells were viewed using confocal laser-scanning microscopy (LSM 510, Zeiss, Oberkochen, Germany) or epifluorescence microscopy (Nikon TE-300, Melville, NY). Images were analyzed using LSM Image Browser (Zeiss) or Image-Pro Plus (Media Cybernetics, Silver Spring, MD).

Image Processing

All processing was performed in ImageJ (U. S. National Institutes of Health, Bethesda, Maryland) with built-in with custom libraries and with the VolumeJ plugin (Abramoff and Viergever 2002). Briefly, z-stacks (512X512X18; optical slice thickness of 4.3 mm) were converted from RGB to 8-bit grayscale. Stacks were thresholded in intensity (to accentuate thin dendrites) using the middle-z-axis plane as a reference point. Histograms were summed along the z-axis and normalized (full-range grayscale). Stacks were linearly interpolated in the z-direction. Image was volume rendered with ray-casting algorithm using a Levoy depth-cueing classifier (makes voxels more opaque the closer their intensity is to a user-defined threshold and the higher their surface gradient with voxel brightness decreasing with distance). Color mapping was determined from a grayscale intensity-ramped index volume created with the same interpolation parameters

used on the stacks. Volume-rendered images were despeckled using a median filter with 2-pixel radius. Histograms for volume-rendered images were then normalized.

Scanning Electron Micrograph

Cell morphology and the spatial distribution of cell-cell and cell-matrix interactions were qualitatively assessed through low voltage, high resolution scanning electron microscopy (LVHR-SEM). Co-cultures were fixed using 2.5% EM-grade glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) at 4°C for 24 hours, washed in di-H_2O, and then post-fixed with 1% OsO_4 in 0.1M cacodylate buffer (pH 7.4) for 1 hour followed by several rinses. Co-cultures were dehydrated using ethanol (30, 50, 70, 3 x 100%, 15 min each), wrapped in parafilm, and loaded into a Polaron E-3000 critical point dryer (CPD) and exchange with CO_2 was made while monitoring the exhaust gas rate. The CPD was thermally regulated to the critical temperature and pressure and, following phase transition, the CO_2 gas was released at a constant flow rate. Prior to LVHR-SEM, all specimens were sputter coated with 3 nm of chromium.

Whole-cell Patch Clamp

For all patch clamp recordings, borosilicate glass electrodes with filaments (external diameter 1.5 mm; inner diameter 0.75 mm; Sutter Instruments, Novato, CA) were pulled on a laser puller (P-2000; Sutter Instruments) with a 7-10 MΩ resistance. Electrodes were back-filled with internal solution (140mM K-gluconate, 10mM HEPES, 5mM Kcl, 0.1mM K-EGTA, 3mM Na-ATP, 0.3mM GTP; pH 7.3; 315-325 mOsm). The external solution used was artificial cerebral spinal fluid (aCSF) containing 119mM NaCl, 2.5mM KCl, 1.3mM $MgCl_2$, 2.5mM $CaCl_2$, 1mM NaH_2PO_4, 26.2 $NaHCO_3$, 11mM glucose (pH 7.4 by bubbling with CO_2/O_2 (95/5) mixture; 320 mOsm) made daily and gravity perfused. A

separate perfusion tube was used to add the high K^+ solution (20nM in external solution) which flowed into the bath solution at 1-2 mL/min. The normal perfusion system continued to flow during high K^+ influx and efflux experiments. An AxoPatch 1D (Axon Instruments) patch-clamp amplifier coupled with a Digidata acquisition PCI card, and WinWCP software (University of Strathclyde, Glasgow, U.K.; http://www.bio-logic.info/electrophysiology/winwcpandwinedr.html) was used for data acquisition, recording and stimulating. Recordings were displayed on Tektronix Oscilloscopes (TDS 3014B and 7603) and analyzed offline in WinWCP and Igor Pro (WaveMetrics).

Mesenchymal Stem Cell Culture

Harvest and Isolation of MSCs

MSCs were isolated and harvested as described before (Peister et al. 2004) with slight modifications. Whole bone marrow was isolated from the femurs and tibias of 8-10 week old male GFP+ transgenic C57BL/6-Tg(cAG-EGFP)C14-701-FM1310sb mice (C57BL6 background) mice bred in house. Mice were sacrificed, and bones were dissected out and cleaned of muscle and connective tissue. One end of the bone was clipped and the bone marrow extracted by centifugation in modified microcentrifuge tubes. The pellet from each bone was resuspended in Cell Isolation Media (CIM; RPMI-1640, 10% FBS, and 10% Horse Serum), mechanically-dissociated by a 22-gauge needle, filtered through a 70 μm filter, and plated in a T175 flask. At 24 hours, non-adherent cells were removed, cells were rinsed with DPBS, and fresh CIM was added. Flasks were fed every 3-4 days. After 4 weeks, cells were trypsinized for 2 minutes and re-plated in CIM in a T175 flask. Cells that did not detach in 2 minutes were discarded. After 2 weeks, cells were trypsinized for 2 minutes and re-plated in 100 mm tissue culture dishes at a density of 50-500 cells/cm^2 in Cell Expansion Media (CEM; IMDM, 10% FBS, and 10%

Horse Serum). Once Passage 3 cells were confluent, they were either expanded further in 100 mm dishes or frozen (FBS + 10%DMSO). Cells were used at passages 4-10.

MSC-sphere Induction

MSCs were expanded to 80-90% confluency in 100 mm tissue culture dishes. Cells were trypsinized, and plated in NeuroBasal A (Invitrogen, Carlsbad, CA) supplemented with serum substitute B27 (Invitrogen), insulin (25 μg/mL), transferrin (100 μg/mL), putrescine (60 μM), sodium selenite (30 nM), progesterone (20 nM), and L-glutamine in 100 mm Petri dishes or tissue culture dishes. Cell seeding density was 500,000-750,000 cells/dish unless stated. Cells were incubated on a rotary shaker. Human recombinant basic growth factor (bFGF; 10 ng/mL; PreproTech) was added to the media every 2-3 days.

Injury of 3-D Co-culture Constructs

3-D co-culture constructs were injured at 21 days *in vitro* in a cell shearing device described previously (LaPlaca et al. 2005; Cullen and LaPlaca 2006; Cullen et al. 2007a; Cullen et al. 2007b) at 0.50 E and 30 s^{-1}. Media was collected before injury and new media was replaced following the injury. Control non-injured cultures were removed from the incubator for the same length of time and received a media change. All cultures were returned to the incubator for 48 hours.

Injection of MSC-spheres into the Co-culture Testbed

At 48 hours post-injury, MSC-spheres were injected though a Hamilton syringe into the center of the 3-D injury testbed. Cells were injected in 2.5 μL of HBSS at a

concentration of 6.0 x 10^3 cells/μL (1.5 x 10^4 cell total per culture)(n=3-4 per condition). Cultures were returned to the incubator for 72 hours following injection.

Apoptosis Assay

Caspase levels were quantified at 3 days post-injection by the Caspatag 3,7 Assay (Chemicon APT523). Briefly, the reagent was reconstituted according to the instructions (dissolved lyophilized reagent with 50μL DMSO; 1:150 stock; then diluted with media) and 250 μL was added to each culture for 1 hour @ 37°C. Caspatag reagent was removed and cultures were counterstained with Hoechst. Cultures were imaged on a confocal microscope, and z-stacks were quantified for the number of caspase-positive cells.

Statistical Analysis

For viability and immunocytochemical assays, three to five randomly selected regions per culture were counted and averaged. Standard deviations were calculated when appropriate. Statistical values were calculated by paired, 2-tailed *t*-tests with p-value < 0.05 considered significant. For the caspae assay, a two-way ANOVA was performed with Tukey's pairwise comparison. Results with p-value < 0.05 were considered significant.

4.3 Results

3-D Co-culture Constructs are Electrophysiologically Active

We have developed a novel neural 3-D co-culture construct to be used as an *in vitro* model of neural tissue. These constructs consist of cerebral cortical neurons and astrocytes within a Matrigel™ matrix, which is biologically active for neural cells through the matrix components (e.g., laminin, collagen IV, entactin, heparan sulfate proteoglycan) (Kleinman et al. 1986) and cytokine-related interactions (Vukicevic et al. 1992). Constructs were maintained using a defined, serum-free, Neurobasal™ medium for up to 60 days. The 3-D neural constructs contract and rise from the culture surface during the first week *in vitro*, likely due reactive forces on matrix fibrils resisting tension associated with process outgrowth, a phenomenon observed in similar 3-D collagen (e.g., fibroblasts(Grinnell et al. 2006)) and collagen-glycosaminoglycan matrices (e.g., peripheral nerve explants(Spilker et al. 2001)). This contraction results in a ~50% reduction in culture diameter (to 7-9 mm), a ~25% reduction in thickness (to 500-600 μm), with a concomitant 3.5-fold increase in 3-D cell density (to ~9000 cells/mm^3). Following this contraction, the constructs are similar in size and opacity to acute cortical slices of rodent brains (**Figure 4.1A**) and cells can be visualized to a depth of approximately 100 μm with differential interference contrast (DIC) and near-infrared optics.

The cultures can be plated in a variety of neuron-to-astrocyte ratios (1:1 in the present study) that have >95% viability at 21 days (Cullen *(in press)-a*). We have maintained viable 3-D neural constructs for over 60 days *in vitro* using this method. This method utilizes separate neuronal and astrocytic harvests, such that neurons isolated from GFP+ transgenic mice, mixed with wild-type astrocytes, greatly enhance the ability to

distinguish neurons for electrophysiological assessment. Immunocytochemistry reveals that neurons within the constructs express the neuronal-specific proteins MAP-2 and tau-5 (**Figure 4.1B**). The initial neuron-to-astrocyte ratio, coupled with modest levels of astrocyte proliferation influence the relative neuronal presence in mature cultures. For instance, an initial 1:1 neuron-to-astrocyte ratio yields cultures that are ~10% neuronal by 21 days *in vitro* (data not shown). Extensive processes extending from cells are visible under bright field microscopy by 7 days and become more pronounced as the culture matures. Morphologically, neurons within the constructs exhibit spherical somata with processes extending throughout 3-D space. Processes within 2-D cultures are constrained to an approximately planar region, while those in 3-D constructs traverse much longer z-distances (**Figure 4.2**), which is more representative of *in vivo* cytoarchitecture.

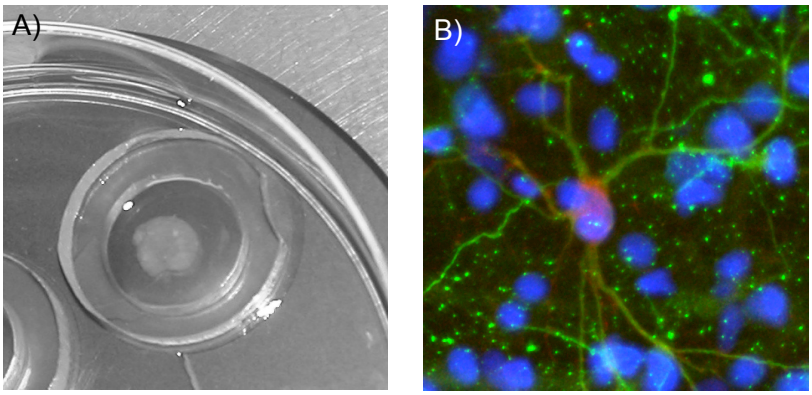

Figure 4.1: Gross and micro-morphology of 3-D constructs. A) Within a few days of plating, 3-D constructs contract and lift off the culture surface taking on a shape similar to an acute slice. **B**) Morphology of a fluorescently-labeled neuron in a 3-D culture extending processes in all directions. Neurons within the constructs express the neuronal-specific proteins MAP-2 and tau-5. *(Submitted Irons, et al. 2007)*

The 3-D constructs are similar in size and shape to acute brain slices, which allowed the use of a modified acute slice patch clamp rig and perfusion system for electrophysiological recording. We found unique challenges to recording from cells within 3-D cultures including an increased susceptibility to mechanical vibrations than acute slices, which required additional anchoring. In addition, the construct was more adhesive and denser than acute slices from young rodent brains. Consequently, it was more difficult to penetrate the extracellular matrix with an electrode; however, by maintaining strong positive pressure on the electrode and a diagonal approach angle, successful recordings were achieved. While there was some difficulty establishing intracellular access for sub-surface cells, this challenge was not unique to our constructs and was overcome with a modified approach. Similar challenges have been reported with 3-D collagen I cultures (O'Shaughnessy et al. 2003); however, in those cultures, the cells were seeded on top of partially formed gels and hence the cells recorded from were at or near the surface of the gel. In contrast, we were able to access cells substantially below the surface. The ability to access cells at increasing depths may be improved by altering the concentration or constituents of the matrix; however, decreasing the Matrigel concentration below 5.0 mg/mL resulted in more fragile cultures and increased their susceptibility to mechanical vibration during recording.

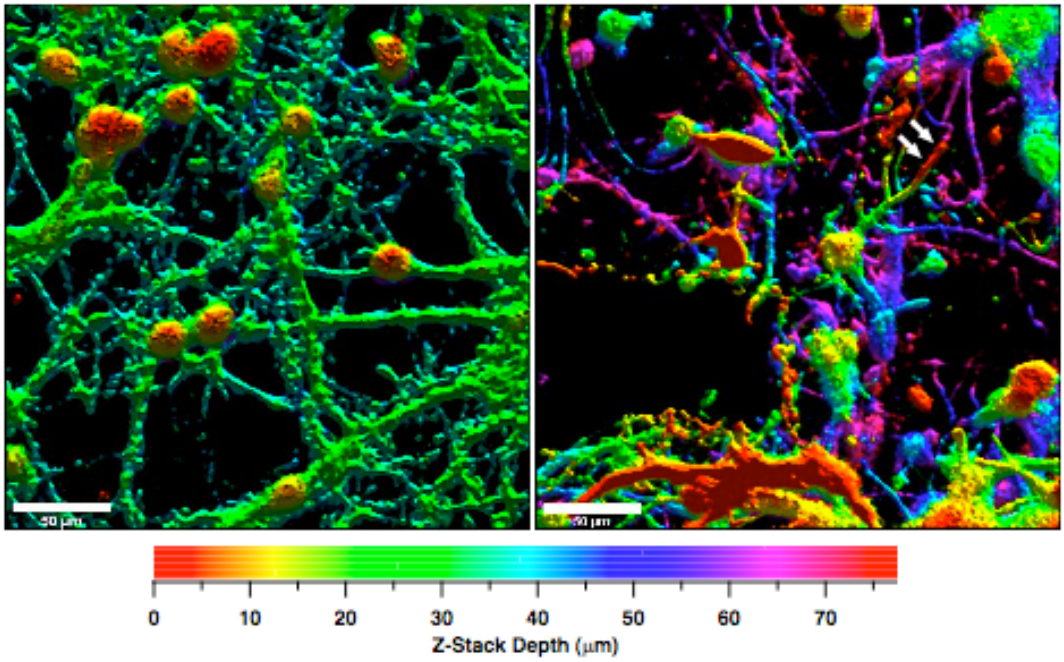

Figure 4.2: Comparison of 2-D (*left*) **and 3-D** (*right*) **cultures.** Volume-rendered z-stacks are color-coded for depth from the objective lens. While most of the 2-D culture neurites are restricted to a planar region, in the 3-D cultures they traverse much larger z-distances (see arrows). *(Submitted Irons, et al. 2007)*

Using techniques modified from traditional acute slice whole-cell patch clamp recordings, we successfully recorded from cells within the 3-D co-culture constructs (**Figure 4.3A**). Neurons within the 3-D constructs exhibited excitable membrane properties including spontaneous and evoked action potentials. Cells within the 3-D constructs were observed to have typical resting membrane potentials (–56.2 ± 11.5 mV; n=28) which were not statistically different from neurons in similar 2-D cultures (–52.8 ± 6.1 mV; n=12; *t*-test p=0.35). In addition, action potentials of neurons within the 3-D constructs had large action potential amplitudes (70.3 ± 9.9 mV) similar to mature neurons. Neurons within the 3-D cultures exhibited depolarization and repetitive spiking with an increased discharge frequency in response to an increase in extracellular K^+ concentration (**Figure 4.3B**). Membrane potential and firing rate returned to original levels within 3 minutes after K+ washout (data not shown). Together, these data suggest the neurons within the 3-D constructs express functional voltage-gated ion channels and exhibited action potentials similar to those observed in acute slices, with the added ability to control cell density and types.

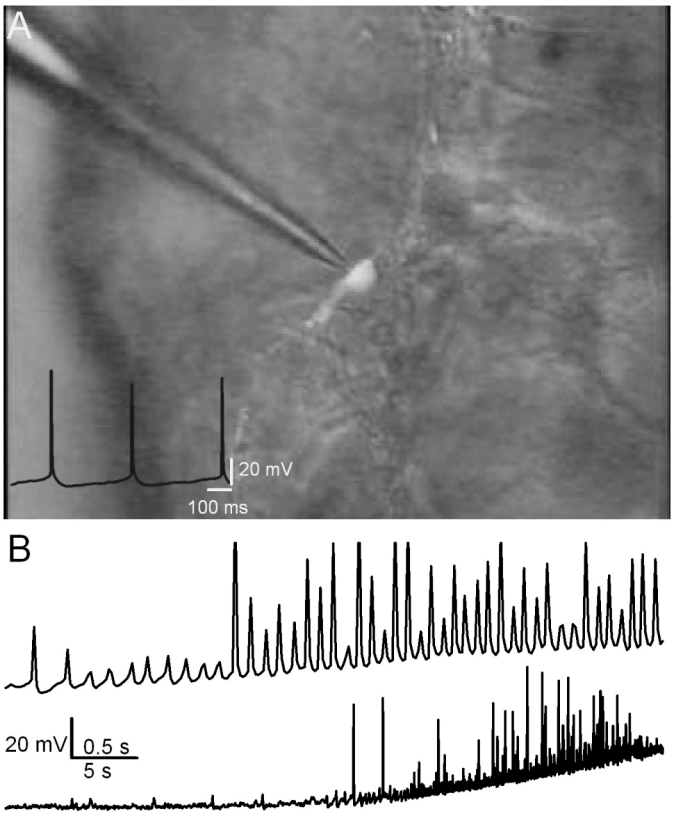

Figure 4.3: Whole-cell patch clamp recording in 3-D constructs. A: Patch clamp electrode in GFP^+ neuron within a 3-D construct. Using modified techniques normally used with acute slice whole-cell patch clamp recording, we were able to record from cells within the 3-D co-cultures. Neurons isolated from a GFP^+ transgenic mouse were cultured with wildtype astrocytes to facilitate identification of neurons within the culture. (*inset*) Spontaneous action potentials from a neuron within a 3-D co-culture. **B:** Active response of a neuron to a 20 pA/s current ramp (100 pA maximum; top) and to high, extracellular K^+. Extracellular K^+ perfusion (20mM) evoked an increase in spiking and an elevated resting membrane potential. *(Submitted Irons, et al. 2007)*

We also observed evidence of functional synapses and network activity within the 3-D co-culture constructs. Fluorescent immunocytochemistry of representative cultures at 21 days *in vitro* revealed abundant synapsin I expression (**Figure 4.4A; green puncta**) along with the neuronal marker tau-5 (red). Electrophysiologically, spontaneous synaptic activity was evident (**Figure 4.4B; black circles**), as was network-driven burst activity. Additionally, through scanning electron microscopy, we observed processes with the characteristic appearance of neurites extending across somata and culminating in synaptic terminals (**Figure 4.4C**). Together, these observations provide significant evidence of functional synapses and network properties within the 3-D co-culture constructs.

A B C

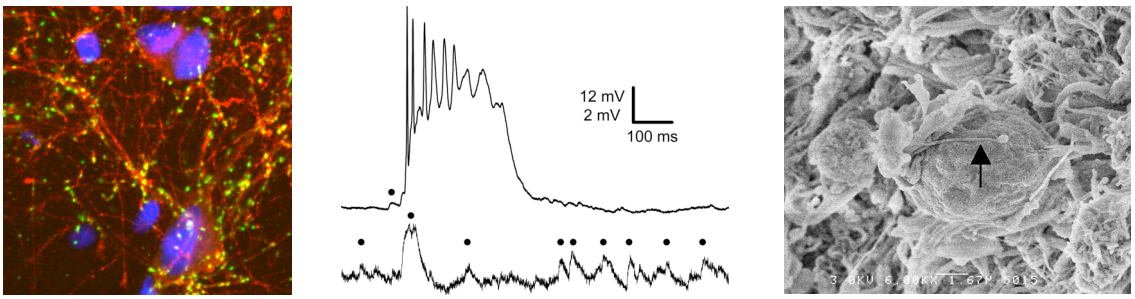

Figure 4.4. Evidence of functional synapses and network activity in 3-D constructs. A: Immunofluorescent photomicrographs of representative cultures at 21 days in vitro. Triple-labeled for neuronal marker tau (red), synaptic marker synapsin (green puncta), and nuclear marker Hoechst 33258 (blue). **B**: Spontaneous synaptic activity was evident above the baseline noise (black circles) as was network-driven burst activity. **C**: Scanning electron microscope image of a synapse (arrow) within a 3-D culture. (Submitted Irons, et al. 2007)

MSC-spheres injected into injured 3-D testbed decrease apoptosis

MSC-spheres were injected into 3-D co-culture construct testbeds 48 hours after *in vitro* cell shearing injury. At 3 days post-injection, caspase levels were quantified in the culture. We observed a significant increase in caspase-positive injured construct cells verses non-injured controls (n=3-4; p-value <0.05 by two-way ANOVA). The injection of MSC-spheres into the injured constructs decreased the host caspase level by a statistically significant amount (**Figure 4.5**)(n=3-4 per condition; p-value <0.05 by two-way ANOVA).

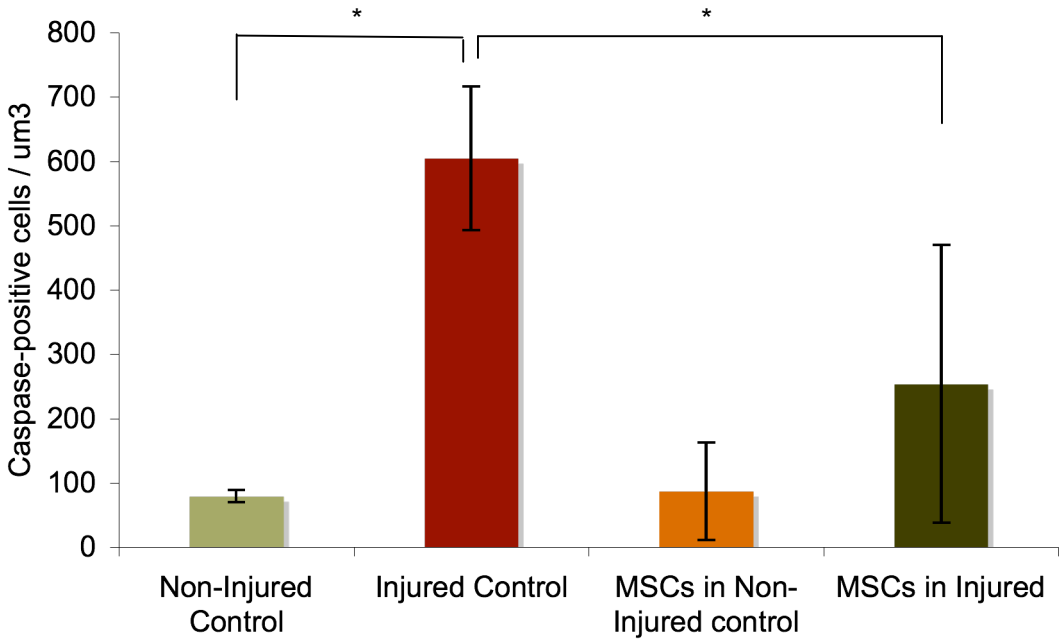

Figure 4.5: MSC-spheres decreased host caspase levels at 5 days post-injury in a 3-D co-culture testbed when MSC-spheres were injected 48 hours following an *in vitro* shear injury. (* p-value < 0.05; n = 3-4 cultures per condition)

4.4 Discussion

3-D Co-culture Constructs are Electrophysiologically Active

We have engineered 3-D co-culture constructs as a model of neural tissue composed of neurons and astrocytes within a bioactive matrix. Cells within these cultures exhibit *in vivo*-like somatic morphologies with extensive process outgrowth, high viability, spontaneous excitable properties, and evidence of functional network connections. These 3-D constructs are similar in size and opacity to acute slices and also have the distinct advantage of being able to be reliably reproduced with a variety of initial cell types and ratios, thus presenting a novel model for neural tissue studies that benefit from reliable electrophysiological measurements as outcome metrics.

Cells *in vivo* are intimately connected to each other and the surrounding extracellular matrix forming a complex microenvironment that is not reproduced in traditional culture systems. Suitable 3-D microenvironments must support neural cell attachment while allowing sufficient diffusion of nutrients to keep the culture viable for long-term studies. In addition, the microenvironment should sufficiently support neurite outgrowth and the formation of functional synapses. Of previously existing 3-D primary neural cell cultures, many incorporate scaffolds of collagen (Coates et al. 1992; O'Connor et al. 2000a), agarose (Yu et al. 1999; Luo and Shoichet 2004) or fibrin (Willerth et al. 2006) with varying viability. Survival of primary neurons in agarose is poor beyond ten days (O'Connor et al. 2001), however neurite outgrowth can be improved by the addition of collagen type IV (Cullen et al. 2007a) or laminin (Yu et al. 1999). Collagen I matrices, either alone or mixed with agarose, have also shown some improvement for the growth of neurons, astrocytes, and neural progenitor cells (O'Connor et al. 2000a), however the addition of bioactive ECM constituents more representative of those found in the

developing or mature brain (e.g. laminin, hyaluronan, or proteoglycans) may further increase the survival, function, and relevance of cultured neural cells.

Hydrogels such as Matrigel™ have several advantages as scaffolds for 3-D cultures. Several groups have reported the use of hydrogel-based 3-D neural cultures of either agarose or collagen type I; however, although commonly used as a scaffold for many other cell types, collagen I may not be the most appropriate scaffold for neural cells. We utilized Matrigel™ matrix, a reconstituted basement membrane derived from the Engelbreth-Holm-Swarm mouse sarcoma, which is biologically active for neural cells through matrix (e.g., laminin, collagen IV, entactin, heparan sulfate proteoglycan)(Kleinman et al. 1986) and cytokine-related interactions (Vukicevic et al. 1992). Despite the clear benefits for Matrigel™ in a 3-D culture system, alternative biomimetic scaffolds, such as methylcellulose (Stabenfeldt et al. 2006) or agarose (Cullen et al. 2007a) may allow more control over the presentation of extracellular matrix ligands while continuing to provide the support necessary for neuronal function.

In addition, the presence of astrocytes may also influence electrophysiological properties. Specifically, astrocytes serve as substrates for neurite outgrowth, provide trophic factors and metabolic precursors, and regulate neuromodulation, synaptic efficacy, and synapse number (Tsacopoulos and Magistretti 1996; Ullian et al. 2001). The intimate interaction between neurons and astrocytes across three-dimensions is essential to recapitulate phenomena observed *in vivo*. Since transformed cell lines have been shown the have incomplete synapse formation (Pancrazio et al. 1999), altered ion-channel expression (Pancrazio et al. 1999) or abnormal resting membrane potentials (< -40 mV)(Desai et al. 2006; Wu et al. 2006), primary cell cultures incorporating both neurons and astrocytes may be more requisite for electrophysiological studies. An

additional advantage of building 3-D co-culture constructs from dissociated cells is that neurons and glial cells from different transgenic sources can be incorporated. In this work, we have used neurons isolated from GFP+ or YFP+ transgenic mice mixed with wild-type astrocytes, which allows easier identification of neurons. This flexibility opens the door to a wide range of applications with different transgenic sources to address specific mechanistic questions.

Although morphology and neuronal marker expression are valid indicators of phenotypic maturity, the ultimate attribute of healthy neurons is the ability to generate action potentials and form synapses. Neurons within the 3-D cultures exhibited spontaneous excitable properties along with evidence of synaptic currents and network activity. These traits allow the cultures to be used in studies of intercellular properties given the ability to interface with multiple electrodes within a culture.

In summary, the differences between 3-D, ECM-based cultures and monolayer cultures cannot be ignored and may impact cellular responses significantly. Physiologically relevant models of neural tissue require prioritization among complexity, control, and reproducibility. Although typical *in vitro* cell preparations (e.g., planar cultures) have yielded important neurophysiological information, the conditions used in these studies vary significantly from those found in the highly complex system of the whole organism and therefore may influence individual cellular functions. 3-D culture models consisting of multiple neural cell types are capable of maintaining many positive aspects of *in vitro* modeling while approximating the cytoarchitecture of the brain to a greater extent than traditional planar cultures (Bass et al. 1971; Williams and Herrup 1988). The 3-D neural co-cultures presented here represent an important step towards more physiologically-

conformant neural tissue models having many distinct advantages for electrophysiological applications.

MSC-spheres reduce host caspase levels in injured testbeds

The injection of MSC-spheres into injured 3-D co-culture testbeds significantly reduced the host apoptosis following *in vitro* injury. The effect seen in the MSC-sphere injected groups may be due to a variety of factors. MSCs have been reported to decrease cell death through secreted factors including neurotrophins, for example brain-derived neruotrophic factor (BDNF) and nerve growth factor (NGF) (Chen et al. 2002; Gao et al. 2005; Crigler et al. 2006). BDNF and NGF have many survival regulatory roles in the brain both in development and in the adult brain (for review see (Hempstead 2006)).

Therefore, it will be interesting to assess whether MSC-spheres are secreting neurotrophins while in culture. Preliminary experiments indicate that they are producing some amount of BDNF during induction. If this is the case, it may be possible to prime the MSC-spheres to produce more BDNF prior to transplantation through incubation with NGF or neurotrophin-3 which have been shown to influence each other's secretion in cortical neurons in a complex pathway (Patz and Wahle 2004). Experiments to determine the expression of neurotrophins by MSCs are ongoing in our laboratory and may provide insight into one area that MSCs may be influencing the host response.

Other explanations are also possible for the observed decrease in apoptosis. Since macrophages and microglia are potential components and/or differentiation products of MSC-spheres, it is possible that the addition of MSCs is decreasing the host caspase level by the destruction of dying cells rather than preventing apoptosis. However, the

level of CD11b (marcophage marker) observed in MSCs cultures by our method is low as is the total cell number injected, so macrophage activity would be expected to have a more modest effect. Nevertheless, it cannot be ruled out without further experimentation.

The observed decrease in apoptosis in the injured testbeds is encouraging for the use of MSC-spheres as a donor cell source. The next step towards this end will be aimed at assessing if MSC-spheres have a similar effect once transplanted into the injured brain, and by what method they are providing benefit to the host cells.

Acknowledgements

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The incidence and long-term effects of traumatic brain injury make it a major healthcare and socioeconomic concern. Cell transplantation may be an alternative therapy option to target prolonged neurological deficits; however, in order to be used clinically, the safety and efficacy must be determined. Of the currently-used cell types available, bone marrow-derived mesenchymal stem cells (MSCs) are an easily accessible and expandable cell type which also circumvents the ethical dilemmas surrounding the use of fetal tissues. However, like all other cell types, they must also be proven efficacious. A major disappointment of other central nervous system cell therapies in clinical trials has been the lack of consistency in functional recovery where some patients receive great benefits while other experience little if any effect (Watts and Dunnett 2000; Lindvall and Bjorklund 2004). There are many possible explanations for this variability including genetic and environmental factors, surgical techniques, and donor cell phenotype. Of these, one of the most easily addressable is the homogeneity of donor cells.

Here, we present data from an animal study which similar to many clinical trials was inconclusive. A few individual mice that received transplants made impressive behavioral recoveries while others did not. While many groups are exploring other cell types to use, few focus to standardize and improve the isolation and pre-transplantation culture protocols. Our goal was to produce a reproducible population of MSCs and to induce them *in vitro* to form cell aggregates which resemble neurospheres.

We began by comparing isolation and expansion protocols of MSCs. Two of the most common isolation techniques are the plastic adherence method where cells are cultures in medium to high density in 20% serum. After 1-3 days, the media and all non-adherent cells are removed. At this point, the cells are expanded and used at various passages. While this method effectively removes red blood cells and some other cell types, it does not select for MSCs as many other cell types in the bone marrow are adherent. As the cells are passaged, they probably become less heterogeneous by simple selection, but cells in long-term culture and many passages may have altered gene expression patterns. We compared the plastic adherence method to the low-density method in their production of MSCs. The low density method used a minimal nutrient media with the intent to essentially starve off the non-MSC cell types leaving a purer population. We compared the efficiency of these MSC isolation methods in their ability to isolate cells which fit two of the criteria normally associated with MSCs: 1) the lack of hematopoietic cell surface markers and 2) the ability to differentiate into osteoblasts and adipocytes. The MSCs cultured at low density were more homogeneous in morphology, expressed lower levels of hematopoetic cell surface markers, and had a greater percentage of cells which differentiated into adipocytes and osteoblasts. Therefore, the low density isolation method was used in the rest of the experiments.

By using a combination of media, cell culture constituents, and growth factors normally used to culture neural stem cells (NSCs) and primary neurons, we were able to produce multicellular aggregates of MSCs (referred to as MSC-spheres). When grown in standard tissue culture plates, the spheres require 7-10 days to form and have a range of sizes. However, by adding dynamic rotary motion MSC-spheres formed more quickly and were more consistent in size and shape.

The speed and the seeding density are two parameters we identified that influenced the size and quantity of the MSC-spheres. By using this method, MSC-spheres can be produced reliably at a consistent range of radii. This level of control may be advantageous in the future if a particular sphere size is desired. For our purposes, we chose 40 rpm because it produced consistent spheres that were able to be transplanted through a 10 μL syringe. Larger spheres were eliminated as they tended to clog the syringe. However, larger cell spheres may be helpful in experiments involving differentiation or to compare MSC-spheres to embryoid bodies as the larger size may influence differentiation signals. However, it will be important to assess the diffusion limits of nutrients and oxygen into the spheres if larger spheres are used to avoid a necrotic core.

We also explored the interaction of MSCs with their extracellular environment. We found that MSCs migrated on extracellular proteins laminin and collagen IV to a greater extent than fibronectin. Interestingly, by immunocytochemistry, the spheres appear to produce more fibronectin than collagen type IV. Another interesting observation is that the MSC-spheres varied in their migratory distance based on whether they were centrifuged before being plated. Those spheres that were centrifuged migrated farther and faster suggesting that the centrifuge may be stimulating migration possibly through a mechanotransduction signaling mechanism. Alternately, it is possible that by allowing the spheres to settle we are selecting against a population of slowly migrating cells, however this seems less likely as the spheres are relatively the same size and settling is a function of size and density.

For reasons that are not clear, the MSCs appear to express low levels of GFP despite being isolated from a homozygous transgenic mouse. The GFP transgene is on an β-

actin promotor and is normally expressed constitutively. The lack of GFP in the cells is puzzling as they appear to express β-actin in the adherent expansion stage, but decrease expression in the MSC-spheres. However, the MSC-spheres appear dense with nuclei and comparatively little cytoplasm, so it is possible that the decreased β-actin level is a result of their conformation. It is also possible they are using a different structural protein such as vimentin. Either way, another cell tracking mechanism will need to be used to track the cells following transplantation as GFP expression from the β-actin promoter may be unreliable.

The MSC-spheres share some characteristics of neural stem cells (NSCs). Neural stem cells are often cultured in suspension in neurospheres, however the MSC-spheres are significantly larger than NSCs (and smaller than embryoid bodies). MSC-spheres also resemble the NSCs by their dependence on bFGF for sphere formation. NSCs harvested at earlier timepoints in development, are also EGF-responsive. While the MSC-spheres are not dependent on EGF for sphere formation, EGF influences both viability and early sphere formation. In addition, MSC-spheres produce nestin, a neuroprogenitor marker. Nestin expression in increased by the induction of MSC-spheres in the presence of neruotrophins. Interestingly, the expression of nanog (a marker of pluripotency) decreases in MSC-spheres compared to adherent MSCs, however, it is only produced at low levels even in the adherent MSCs.

Once the MSC-spheres had been characterized, we injected them into an *in vitro* injury testbed consisting of a 3-D culture of neurons and astrocytes in Matrigel injured in a cell shearing device . After injury *in vitro*, the testbed reproduces some aspects of the injury environment including apoptosis of host cells (Cullen *(in press)-b*). When MSC-spheres

were injected into the injured environment of the testbed we detected a significant decrease in caspase activation of host cells compared to the injured control.

In the end, this work set the groundwork for many questions about stem cells and their role in injury. What adhesion molecules are at work in the MSC-spheres? What different genes are being expressed during sphere formation compared to adherent culture? How do MSC-spheres compare to other cell aggregates such as NSC neurospheres and embryoid bodies both *in vivo* and *in vitro*? Can we use this system to upregulate trophic factor production to improve the potential beneficial effect on injured cells? If so, what factors should be target? Can MSC-spheres cultured by this new method improve functional recovery to a greater extent than we saw previously?

In conclusion, we determined that MSCs may be an alternative cell donor cell type for cell therapy after traumatic brain injury. We also learned that MSCs have the ability to form spheres in culture and that the spheres size can be controlled by the culture system. The MSC-spheres share some characteristics of NSCs and may decrease host cell apoptosis in the injury environment. Although we are far from understanding the signals that contribute to stem cell phenotype and function, each piece brings us closer to being able to harness the potential they posses for regenerative medicine and bring effective cell therapies one step closer to the clinic.

APPENDIX A

UNSUCCESSFUL INDUCTION METHODS

Many induction methods were attempted before a successful method was found to induce MSC-sphere formation. Some less successful methods are listed here.

Media Formulations

1) Dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM/F12, 1:1; Invitrogen) supplemented with insulin (25 μg/mL), transferrin (100 μg/mL), putrescine (60 μM), sodium selenite (30 nM), progesterone (20 nM), and glucose (0.3%). Human recombinant basic fibroblast growth factor (bFGF; 20 ng/mL; PreproTech) added to the media every 2-3 days.
2) DMEM:F12 supplemented with insulin (25 μg/mL), transferrin (100 μg/mL), putrescine (60 μM), sodium selenite (30 nM), progesterone (20 nM), glucose (0.3%) and L-glutamine. Human recombinant basic fibroblast growth factor (bFGF; 20 ng/mL; PreproTech) added to the media every 2-3 days.
3) Neuralbasal media with insulin (25 μg/mL), transferrin (100 μg/mL), putrescine (60 μM), sodium selenite (30 nM), progesterone (20 nM), glucose (0.3%) and L-glutamine. Human recombinant basic fibroblast growth factor (bFGF; 20 ng/mL; PreproTech) added to the media every 2-3 days
4) Neuralbasal A media with serum substitute B27 (Invitrogen), EGF (20ng/mL), NGF (20 ng/mL), and FGF (20 ng/mL).
5) DMEM media with serum substitute B27 (Invitrogen), EGF (20ng/mL), NGF (20 ng/mL), FGF (20 ng/mL), and retinoic acid (RA).

6) 50:50 mix of DMEM:F12 and Neuralbasal supplemented with insulin (25 μg/mL), transferrin (100 μg/mL), putrescine (60 μM), sodium selenite (30 nM), progesterone (20 nM), and glucose (0.3%).
7) 50:50 mix of DMEM:F12 and Neuralbasal A supplemented with insulin (25 μg/mL), transferrin (100 μg/mL), putrescine (60 μM), sodium selenite (30 nM), progesterone (20 nM), and glucose (0.3%).

Culture Dishes

On the rotary shaker, 6-well plates, 12-well plates, and 35mm, 60mm, and 150mm dishes did not work to induce cells. The cells in smaller dishes clumped into one large cell clump. Tissue culture plastic coated dishes were not detrimental to sphere formation, however, the MSC-spheres did not form as fast as the cells plated in Petri dishes.

Growth Factors

The growth factor bFGF is required for MSC-sphere formation. Cultures that did not receive bFGF during induction, did not form spheres and many cells died within a few days. The addition of retinoic acid (RA) during induction prevented sphere formation, however, it did not break up MSC-spheres which were already formed. The addition of nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), or epidermal growth factor (EGF) either alone or in combination was not sufficient to form MSC-spheres. Only when bFGF was added did MSC-spheres form in large numbers.

Conditioned media

MSCs induced in conditioned media from NSCs formed MSC-spheres, but since conditioned media is variable based on the culture it is obtained from, we decided to use a more defined, reproducible media. MSC-spheres were plated in conditioned media from primary neurons, astrocytes, and co-cultures, but for the same reason, this line of experiments was discontinued.

Extracellular Matrix Proteins

While MSC-spheres adhered to fibronectin, laminin, collagen I, and collagen IV, this did not increase their sphere formation and may have even inhibited it.

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