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APPLICATIONS FOR HUMAN NEURAL STEM CELL-DERIVED MOTOR NEURONS IN AMYOTROPHIC LATERAL SCLEROSIS: CELL REPLACEMENT THERAPY AND DISEASE MODELING

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by

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Dedication

I wish to dedicate this dissertation to my family; my wife, Susan Thonhoff, my parents, Bob and Kim Thonhoff and my brother, Travis Thonhoff. Their love and support have been infinite throughout this tremendous endeavor. I could not have reached this feat without the countless sacrifices they have made over the course of their lives.

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APPLICATIONS FOR HUMAN NEURAL STEM CELL-DERIVED MOTOR NEURONS IN AMYOTROPHIC LATERAL SCLEROSIS: CELL REPLACEMENT THERAPY AND DISEASE MODELING

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Amyotrophic lateral sclerosis (ALS) is an incurable neurological disease characterized by the selective degeneration of spinal and upper motor neurons. One approach in the development of therapies for ALS is to explore the potential of human fetal neural stem cells (hNSCs) to replace lost motor neurons. The therapeutic efficacy of stem cell transplantation would depend greatly on the survival of grafted stem cell-derived motor neurons in the microenvironment of the spinal cord in ALS. Previously, we reported that hNSCs could be instructed to differentiate into motor neurons both *in vitro* and *in vivo*. Here, we report that the transplantation of primed hNSCs into the spinal cords of transgenic ALS rats only slightly delayed disease progression. Morphological analyses of the transplantation sites revealed that the grafted hNSCs differentiated into motor neurons, but were degenerated and showed signs of nitroxidative damage at the disease end-stage. Using an *in vitro* coculture system, we provided evidence that human mutant $SOD1^{G93A}$-expressing primary microglia, isolated after disease onset, were directly toxic to hNSC-derived motor neurons. Additionally, normal astrocytes not only lost their protective capacity toward hNSC-derived motor neuron survival *in vitro*, but also exhibited toxic features, when cocultured with mutant $SOD1^{G93A}$ microglia. Using inhibitors of inducible nitric oxide synthase and NADPH oxidase as well as scavengers for reactive oxygen and nitrogen species (ROS/RNS), we showed that microglia-generated nitric oxide, superoxide and peroxynitrite, at least, partially contributed to motor neuron loss and astrocyte dysfunction in this coculture paradigm. In summary, ROS/RNS released from overactivated microglia directly damage motor neurons and reduce the neuroprotective capacity of astrocytes, collectively dooming motor neuron survival in ALS.

These data provide evidence that treating ALS with motor neuron cell-replacement therapies will not be efficacious unless the toxic milieu created by endogenous overactivated microglia in the spinal cord of ALS is dramatically altered. Outcomes from these studies should aid in the development of novel combined therapies using stem cells to treat patients with ALS.

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List of Abbreviations

ALS	amyotrophic lateral sclerosis
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
BMC	bone marrow cell
cAMP	cyclic adenosine monophosphate
ChAT	choline acetyltransferase
CMAP	compound muscle action potential
CNTF	ciliary neurotrophic factor
CNS	central nervous system
CSF	cerebrospinal fluid
CXCR4	chemokine (C-X-C motif) receptor 4
EAAT2	excitatory amino acid transporter 2
EGF	epidermal growth factor
ESC	embryonic stem cell
FACS	fluorescent activated cell sorting
FBS	fetal bovine serum
FCS	fetal calf serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein

GSK3	glycogen synthase kinase 3
IGF-1	insulin-like growth factor 1
HD	homeodomain
hMSC	human mesenchymal stem cell
hNPC	human neural progenitor cell
hNSC	human neural stem cell
HNu	human nuclear antigen
hUCBC	human umbilical cord blood cell
iNOS	inducible nitric oxide synthase
iPS	induced pluripotent stem
L1CAM	L1 cell adhesion molecule
LPS	lipopolysaccharide
MAP2	microtubule-associated protein 2
mBMC	mouse bone marrow cell
MN	motor neuron
mNSC	mouse neural stem cell
mOB-NPC	mouse olfactory bulb neural precursor cell
MSC	mesenchymal stem cell
NeuN	neuronal nuclei
NF	neurofilament
NGF	nerve growth factor
NOX	NADPH oxidase
NPC	neural progenitor cell
NSC	neural stem cell
NT3	neurotrophin 3

NT4	neurotrophin 4
PGD2	prostaglandin D2
pMN	motor neuron progenitor
RA	retinoic acid
rAAV	recombinant adeno-associated virus
rMSC	rat mesenchymal stem cell
RNS	reactive nitrogen species
ROS	reactive oxygen species
SC	spinal cord
Shh	sonic hedgehog
SOD1	superoxide dismutase 1
SVZ	subventricular zone
TF	transcription factor
Tuj1	antibody against β-III tubulin
VEGF	vascular endothelial growth factor
VZ	ventricular zone
YFP	yellow fluorescent protein

CHAPTER 1:

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease caused by the selective loss of both spinal and upper motor neurons. One strategy in treating ALS is to use stem cells to replace lost spinal motor neurons. However, transplanted stem cell-derived motor neurons may not survive when exposed to the harsh microenvironment in the spinal cord of ALS. In particular, dysfunctional astrocytes and overactivated microglia in ALS may limit the survival of motor neurons generated from cell replacement therapy. On the other hand, stem cells may provide large quantities of motor neurons that can be used for studying glia-mediated toxic mechanisms and potential therapies in ALS. Here we will review methods and molecular factors for directed differentiation of stem cells into spinal motor neurons as well as the potential uses of these models for dissecting the mechanisms underlying glia-induced motor neuron degeneration and screening for new therapeutics aimed at protecting motor neurons in ALS.

1.1. RATIONALE FOR MOTOR NEURON REPLACEMENT THERAPY IN ALS

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease in adults with an average age of disease onset being in the sixth decade of life. Most ALS patients develop limb weakness initially, which progresses gradually to generalized

muscle atrophy and paralysis. Death often occurs within 5 years, usually due to respiratory failure (Bruijn et al., 2004). Unfortunately, ALS is diagnosed very late in the course of disease progression. At the time of diagnosis, symptoms have manifested as a large number of motor neurons have already been lost or degenerated. Thus, in order to restore muscle function and recover from ALS, lost motor neurons will ultimately need to be replaced.

Most cases of ALS are sporadic and not associated with known risk factors. However, 5-10% of cases are inherited (Bruijn et al., 2004). Although many genes have been linked to familial ALS (Kunst, 2004), mutations in superoxide dismutase 1 (SOD1) are the most common primary causes and represent 1-2% of total ALS patients (Rosen et al., 1993; Gaudette et al., 2000). Studies on transgenic rodent models overexpressing human mutant SOD1 (Gurney et al., 1994; Nagai et al., 2001; Howland et al., 2002; Matsumoto et al., 2006) have significantly advanced our understanding of ALS disease mechanisms and allowed testing of a variety of therapeutic strategies in animal models (Turner and Talbot, 2008). Despite our improved understanding of ALS pathogenesis from the use of transgenic animal models, there are still no effective treatments or preventive strategies in humans. Many potential therapies for ALS, ranging from drugs for anti-inflammation, anti-oxidation and anti-apoptosis, to providing trophic factors, have been unsuccessful in human clinical trials (Nirmalananthan and Greensmith, 2005). The only FDA-approved medicine for ALS, Riluzole, acts as an anti-excitotoxicity agent and provides a marginal effect by prolonging lifespan for approximately two to three months. As such, ALS patients are still in desperate need of new therapies.

Recent advances in stem cell differentiation (for motor neuron differentiation methods, see Table 1.1) and transplantation techniques combined with the need of ALS patients for new therapies prompted the exploration of stem cells for ALS (Nayak et al., 2006; Hedlund et al., 2007). Many types of stem cell therapies have been tested and provided some benefit in transgenic ALS rodent models, including fetal human neural stem and progenitor cells, human umbilical cord blood stem cells, human mesenchymal stem cells, rodent bone marrow and mesenchymal stem cells, mouse olfactory bulb neural progenitor cells and mouse neural stem cells (for details on stem cell therapy in ALS rodent models, see Table 1.2). The moderate improvement in motor function and slightly delayed disease progression by stem cell transplantation in some of these pre-clinical studies are hypothesized to be, at least, partially due to neuroprotection of endogenous motor neurons through the release of trophic factors that directly promote survival or decrease inflammation (Nayak et al., 2006; Xu et al., 2006; Hedlund et al., 2007; Martin and Liu, 2007).

Several clinical trials with stem cells have also been reported. Initially, two small trials using autologous peripheral blood cells administered intrathecally or mesenchymal stem cells injected intraspinally showed few or no adverse effects, but the efficacy of such transplantation was not determined (Janson et al., 2001; Mazzini et al., 2003). More recently, another pilot study confirmed the potential for the autologous transplantation of peripheral blood stem cells that were mobilized with granulocyte-colony stimulating factor, collected and then reinfused into ALS patients (Cashman et al., 2008). Although the procedure was well-tolerated, no therapeutic benefits were observed. Another clinical trial allografted hematopoietic stem cells in six sporadic ALS patients, but like the

autologous transplant study, no clinical benefits were observed (Appel et al., 2008). However, transplanted cells in this study did locate into areas of degenerating motor neurons, which suggests their potential to be genetically modified and used to deliver factors to modulate motor neuron injury pathways in ALS patients. Finally, autologous transplantation of mesenchymal stem cells intraspinally into the thoracic cord of nine ALS patients was well tolerated and four patients actually exhibited a slowing in the decline of their forced vital capacity (Mazzini et al., 2008). The exact underlying mechanisms remain unknown. Although neuroprotection may be a viable approach to delaying disease progression in ALS, the ultimate goal of stem cell therapy is still to replace lost motor neurons in order to improve muscle function.

Hence, additional applications of stem cells for treating ALS include replacing dysfunctional astroglia, overactivated microglia or degenerated and lost upper and spinal motor neurons. For the latter, the therapeutic efficacy of stem cell replacement would depend greatly on the survival of grafted stem cell-derived motor neurons in the microenvironment of the ALS spinal cord. Furthermore, transplanted stem cell-derived astrocytes or microglia may become activated and dysfunctional in the inflammatory and oxidative environment of the ALS cord, thus, rendering them toxic to endogenous motor neurons. The environment in ALS degenerating areas, especially after disease onset, may be hostile to grafted stem cell-derived motor neurons and glia due to progressive and exacerbated neuroinflammation, oxidative stress and glutamate excitotoxicity. Moreover, already arduous tasks such as incorporation into the neural circuitry, targeted axonal growth and reinnervation of denervated muscle fibers may be further hampered in ALS where transplanted stem cell-derived motor neurons may degenerate rather than prosper.

The remainder of this review will discuss glial cells and particularly oxidative stress in ALS that create a hostile microenvironment to both endogenous and transplanted motor neurons. It will then focus on the current knowledge of spinal motor neuron generation during development and from stem cells. Finally, potential uses and challenges of stem cell-derived motor neurons for dissecting ALS disease mechanisms and screening novel drug-based therapeutics aimed at protecting motor neurons in ALS will be discussed.

1.2. AMYOTROPHIC LATERAL SCLEROSIS

1.2.1. PATHOGENESIS AND MOTOR NEURON SUSCEPTIBILITY

Mutations in SOD1 result in a toxic gain of function for which the exact mechanisms remain unclear. However, several hypotheses have been proposed for the pathogenesis of mutant SOD1-mediated ALS, many of which may also be applicable to non-SOD1 linked familial and sporadic ALS, since all forms of ALS share striking similarities in pathology and clinical symptoms. These include mitochondrial dysfunction, oxidative damage, glutamate excitotoxicity, protein aggregation, proteasome dysfunction, cytoskeletal and axonal transport defects and inflammation (Cleveland and Rothstein, 2001; Bruijn et al., 2004; Bendotti and Carri, 2004; Cheroni et al., 2005; Strong et al., 2005; Boillee et al., 2006a). These factors may be linked in that one factor could be the cause or consequence of the other factors. Furthermore, multiple cell types have been implicated in mutant SOD1-mediated ALS pathogenesis. The latest evidence suggests that mutant SOD1-mediated ALS is initiated due to mutant SOD1 expression

within motor neurons and other yet unidentified non-motor neuron cells excluding astrocytes, microglia and oligodendrocytes (Boillee et al., 2006b; Yamanaka et al., 2008a), while disease progression is accelerated by dysfunction or activation of surrounding mutant SOD1-expressing non-neuronal cells such as astrocytes and microglia (Clement et al., 2003; Yamanaka and Cleveland, 2005; Boillee et al., 2006b; Yamanaka et al., 2008b).

The selective motor neuron degeneration observed in ALS pathogenesis is speculated to be, at least, partially due to the vulnerability of motor neurons. These cells may be at a higher risk due to their high metabolic activity, low levels of reduced glutathione and high levels of unsaturated lipids on their large membrane surfaces along axons. These risk factors presumably contribute to an increased susceptibility to oxidative damage. In addition, high levels of glutamate input are also present partly due to a decreased expression of the excitatory amino acid transporter 2 (EAAT2) in astrocytes, the main mediator of extracellular glutamate removal, in combination with high levels of AMPA-receptor expression on motor neurons. This increase in glutamate input is accompanied by low levels of intracellular calcium-binding proteins, which may result in a toxic level of intracellular calcium (Alexianu et al., 1994; Appel et al., 2000; Appel et al., 2001; Beers et al., 2001). Although still controversial, motor neuron death in ALS seems to be attributed to caspase-mediated apoptosis (Pasinelli et al., 2000; Vukosavic et al., 2000; Hensley et al., 2002; Sathasivam and Shaw, 2005). The susceptibility of motor neurons to the hostile microenvironment generated by overactivated astrocytes and microglia during disease progression becomes particularly relevant in terms of post-transplant survival and function of stem cell-derived motor neurons.

1.2.2. ASTROGLIA AND MICROGLIA INVOLVEMENT IN ALS

Activated microglia and astroglia are found in the degenerating areas in both ALS patients and human mutant SOD1 transgenic mice (Kawamata et al., 1992; Schiffer et al., 1996; Hall et al., 1998b; Almer et al., 1999; Henkel et al., 2004; Turner et al., 2004). It is known that these glial cells play important roles in progressive motor neuron degeneration in transgenic ALS rodent models (Boillee et al., 2006b; Yamanaka et al., 2008b). Although astrogliosis and microgliosis are pathological hallmarks of ALS (Barbeito et al., 2004; Sargsyan et al., 2005; Weydt and Moller, 2005), the critical roles of microglia and astroglia in ALS pathogenesis have only recently been revealed. Accumulated evidence shows that the ALS phenotype is only induced by the expression of human mutant SOD1 in all cell types in the central nervous system (CNS), but not by targeted expression within motor neurons, astroglia or microglia alone (Gong et al., 2000; Pramatarova et al., 2001; Lino et al., 2002; Beers et al., 2006). However, high levels of neuron-specific mutant SOD1 expression in transgenic mice resulted in motor neuron degeneration, likely due to the mutant SOD1 aggregation-induced disruption of the cytoskeletal structure and trafficking within dendrites (Jaarsma et al., 2008). Furthermore, selectively decreasing the expression of human mutant SOD1 in motor neurons delays the onset of the disease whereas selectively decreasing the expression within astroglia or microglia delays the progression of the disease after disease onset (Boillee et al., 2006b; Yamanaka et al., 2008b). In addition, the presence of wild-type non-motor neuronal cells in chimeric mice expressing mutant SOD1 in all motor neurons and oligodendrocytes also results in a significant delay in disease onset (Yamanaka et al., 2008a). Cell types other than motor neurons that may determine disease onset, but have not yet been tested,

include interneurons, Schwann cells, and the endothelial cells that make up the vasculature of the brain-spinal cord barrier (Yamanaka et al., 2008a). Interestingly, there is evidence that mutant SOD1 causes endothelial damage and disruption of the blood-spinal cord barrier prior to disease onset (Zhong et al., 2008). Accordingly, mutant SOD1-mediated ALS progresses through a non-cell autonomous mechanism in which the disease is initiated by mutant SOD1-acquired damage and protein aggregation (Higgins et al., 2002; Liu et al., 2004; Deng et al., 2006; Hervias et al., 2006; Martin, 2006; Boillee et al., 2006b; Martin, 2007; Martin et al., 2007; Sasaki and Iwata, 2007; Shaw and Valentine, 2007; Yamanaka et al., 2008a) within motor neurons as well as other unidentified non-motor neurons, while disease progression is accelerated by dysfunction or activation of the surrounding mutant SOD1-expressing non-neuronal astroglia and microglia (Clement et al., 2003; Beers et al., 2006; Boillee et al., 2006b; Yamanaka et al., 2008b). In other words, mutant SOD1-aquired damage within motor neurons and other non-motor neuronal cell types may cause initial degeneration and retraction of axons from neuromuscular junctions, thus resulting in disease onset, while overactivated astroglia and microglia hasten disease progression by producing a microenvironment toxic to motor neurons through increased inflammation, oxidative damage and glutamate excitotoxicity.

Under normal conditions, microglia, which are the principal immune cells in the CNS, protect neural cells against invading pathogens and neoplastic cells, while astroglia support neurons by providing neurotrophic factors, regulating glutamate levels and synaptic transmission, secreting reduced glutathione and providing precursors for reduced glutathione synthesis in motor neurons. However, in ALS, astrocytes and microglia may

be pathologically activated by proinflammatory cytokines and increased oxidative stress. Overactivated astroglia and microglia then produce high levels of neurotoxins, including proinflammatory factors (Alexianu et al., 2001; Hensley et al., 2002; Yoshihara et al., 2002; Hensley et al., 2003; Weydt et al., 2004; Weydt and Moller, 2005; Hensley et al., 2006b), reactive oxygen species and nitrogen species (ROS/RNS), and glutamate (Zhao et al., 2004; Xiao et al., 2007), which exacerbate inflammation and perpetuate a vicious cycle that results in the degeneration and loss of motor neurons. Reactive astroglia may also lose their ability to regulate synaptic transmission, take up excess glutamate in the cerebrospinal fluid, maintain normal glutathione levels in motor neurons (Chi et al., 2007) and provide neurotrophic support, which may all contribute to motor neuron degeneration. In addition, mutant SOD1-expressing astroglia secrete toxic factors, including prostaglandin D2, which selectively kill motor neurons (Di Giorgio et al., 2007; Nagai et al., 2007; Di Giorgio et al., 2008).

Activated microglia also accelerate ALS disease progression. Their activation in ALS is accompanied by increases of inflammatory cytokines such as TNFα, IL-1β and IL-6 (Elliott, 2001; Nguyen et al., 2001; Hensley et al., 2002; Yoshihara et al., 2002; Hensley et al., 2003; Xie et al., 2004; Hensley et al., 2006b). Furthermore, microglial activation is initiated before significant motor neuron loss and disease onset (Alexianu et al., 2001; Hensley et al., 2002). The broad suppression of microglial activation is neuroprotective as it delays disease onset and prolongs lifespan in transgenic ALS models (Kriz et al., 2002; Tikka et al., 2002; Van Den et al., 2002; Zhu et al., 2002; West et al., 2004). However, conditioned media from microglia derived from neonatal ALS mice show no toxicity to mouse primary or embryonic stem cell-derived motor neurons (Nagai

et al., 2007). On the other hand, brain microglia from neonatal ALS mice caused a significant loss of primary mouse motor neurons when the cells were cocultured (Xiao et al., 2007).

Activated astroglia and microglia contribute not only to the degeneration of endogenous motor neurons, but may also underlie the degeneration of grafted stem cell-derived motor neurons. It has previously been shown that human neural stem cell (NSC)-derived motor neurons, when grafted into an axotomy model of motor neuron degeneration, innervated peripheral muscles and improved motor functions (Gao et al., 2005). However, human NSC-derived motor neurons showed signs of oxidative damage and degeneration when grafted into ALS rat spinal cords, indicating that an oxidative harsh environment in the spinal cord may affect the survival and maturation of transplanted cells (see Chapter 4). This result correlates with reports that neonatal astroglia from ALS mice are toxic to embryonic stem cell-derived motor neurons (Di Giorgio et al., 2007; Nagai et al., 2007; Di Giorgio et al., 2008), and that adult astroglia and microglia isolated from ALS rats after disease onset kill human NSC-derived motor neurons (see chapter 5). Further studies are required to determine the mechanisms underlying ALS microglial and astroglial neurotoxicity specifically targeting motor neurons from both endogenous and exogenous sources.

1.2.3. ALS GLIAL CELLS AND OXIDATIVE STRESS

One of the main consequences of microglial overactivation in ALS is the production of oxidative stress. The elevation of reactive oxygen and nitrogen species (ROS/RNS) beyond endogenous antioxidant capacities plays a role in neurodegeneration

by destroying cells through oxidation of proteins (protein carbonyl and protein nitration), lipids (toxic aldehydes) and DNA (mutation). Evidence of redox perturbation has been revealed in both ALS patients and transgenic animal models, including increased protein carbonyl levels (Shaw et al., 1995; Andrus et al., 1998), protein nitration (Beal et al., 1997; Ferrante et al., 1997b; Tohgi et al., 1999; Casoni et al., 2005), and lipid peroxidation (Ferrante et al., 1997a; Ferrante et al., 1997b; Keller and Mattson, 1998; Smith et al., 1998; Hall et al., 1998a; Shibata et al., 2001; Simpson et al., 2004; Perluigi et al., 2005). Oxidative stress has also been linked to excitotoxicity in ALS. The lipid peroxidation product, 4-hydroxynonenal, may interfere with normal transport of the astrocyte glutamate transporter (Pedersen et al., 1998). The impaired glutamate transport may then contribute to excitotoxicity and the ensuing degeneration of motor neurons.

Despite the indisputable presence of oxidative stress in ALS, the initiating cause of the increased ROS/RNS production is ambiguous. The expression and activity of inducible nitric oxide synthase (iNOS), which stimulates nitric oxide production, and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase or NOX), which generates superoxide, in activated astroglia and microglia are increased in the spinal cords of transgenic ALS animal models and patients (Almer et al., 1999; Phul et al., 2000; Sasaki et al., 2000; Barbeito et al., 2004; Wu et al., 2006). ALS astroglia and microglia also show enhanced iNOS expression and nitric oxide production in response to inflammatory signals (Barbeito et al., 2004; Beers et al., 2006; Hensley et al., 2006b; Xiao et al., 2007). Thus, RNS may play a critical role in ALS microglial neurotoxicity. Increased levels of ROS, including hydrogen peroxide and hydroxyl radicals, have also been observed in the spinal cords of transgenic ALS animals (Liu et al., 1999). The most

abundant source of ROS in the CNS is generated from the respiratory burst system of activated microglia (Colton and Gilbert, 1987; Klegeris and McGeer, 1994). This system is an assembly of 5 subunits of the NADPH oxidase complex, which includes $gp91^{phox}$ (NOX2), $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac1/2 (Sumimoto et al., 2005). Cytokines stimulate normal microglia to produce superoxide (Chao et al., 1995a; Chao et al., 1995b), while microglia from transgenic ALS mice are more sensitive and thus, release more superoxide upon proinflammatory stimulation (Beers et al., 2006; Xiao et al., 2007). Increased expression of NADPH oxidase and production of superoxide in the spinal cords of ALS patients and transgenic ALS mice indicate the potential involvement of microglia-generated ROS in the pathogenesis of ALS (Wu et al., 2006). Furthermore, inhibiting NADPH oxidase in transgenic ALS mice dramatically prolongs their lifespan (Harraz et al., 2008; Boillee and Cleveland, 2008). As such, ALS microglial toxicity due to increases in ROS and RNS may contribute to a hostile ALS environment detrimental to both endogenous motor neurons and stem cell therapies aimed at replacing motor neurons.

Astroglia are critical in maintaining reduced glutathione levels in neurons (Sagara et al., 1993; Drukarch et al., 1997; Drukarch et al., 1998). Glutathione, a tripeptide containing glutamate, cysteine and glycine, is synthesized by glutamate cysteine ligase and glutathione synthetase. It is the most abundant non-protein thiol and the main antioxidant in the CNS (Dringen et al., 1999). Astrocytes release reduced glutathione into the extracellular fluid to protect neurons from oxidative stress by ultimately providing the cysteine necessary for neurons to synthesize reduced glutathione (Sagara et al., 1993; Bolanos et al., 1996; Sagara et al., 1996; Dringen et al., 1999; Iwata-Ichikawa et al.,

1999; Wang and Cynader, 2000; Gegg et al., 2003). Reduced glutathione is decreased in the spinal cords of ALS transgenic mice and depleting reduced glutathione in a motor neuron cell line results in motor neuron death (Chi et al., 2007), but the administration of reduced glutathione was shown not to be effective in a small, randomized clinical trial in ALS patients (Chio et al., 1998). Furthermore, it is unknown whether the glutathione deficiency is due to the inability of dysfunctional ALS astroglia to maintain sufficient levels of extracellular glutathione, thus, decreasing their capacity to protect motor neurons from increases in oxidative stress. Actually, the factors rendering ALS astroglia dysfunctional and toxic to motor neurons have not been completely elucidated. Some evidence in astrocytes extracted from transgenic ALS rats suggested that mutant SOD1 caused mitochondrial dysfunction and enhanced superoxide generation in these astrocytes, which resulted in motor neuron toxicity through unidentified secreted factors, and that this toxicity was prevented by mitochondrial-targeted antioxidants (Cassina et al., 2008). On the other hand, interactions between ALS microglia and astroglia have not been thoroughly explored, and it is possible that microglia-generated oxidative stress and proinflammatory cytokines could also cause or enhance astroglia toxicity *in vitro* (Chapter 5). Furthermore, it remains to be determined whether ALS astrocytes exert toxicity through these *in vitro* mechanisms in the transgenic ALS animal models and patients.

In summary, both primary and stem cell-derived motor neurons have been shown to be susceptible to mutant SOD1-expressing astroglia- and microglia-induced toxicity *in vitro*, irrespective to mutant SOD1 expression within motor neurons. These findings indicate that after disease onset has occurred in which toxicity driven by non-neuronal

microglia and astroglia becomes crucial, transplanted stem cell-derived motor neurons may also be vulnerable to the toxic microenvironment that ensues in the ALS spinal cord. Based on observations that stem cell-derived motor neurons are susceptible to toxic ALS glia, *in vitro* models designed to mimic the ALS microenvironment together with stem cell-derived motor neurons may be used for studying interactions between ALS glial cells and motor neurons. Such models would also allow high-throughput screening for novel ALS therapeutics. Developing methods for generating large quantities of motor neurons from stem cells is the first step in designing experiments to test mechanisms of motor neuron degeneration and potential ALS therapeutics. Identifying causes and mechanisms underlying stem cell-derived motor neuron death will allow us to develop strategies to prevent endogenous motor neuron degeneration and enhance the therapeutic efficacy of replacing lost motor neurons using stem cells in ALS.

1.3. SPINAL MOTOR NEURON DIFFERENTIATION

1.3.1. GENERATION OF SPINAL MOTOR NEURONS DURING DEVELOPMENT

The specification of cell fate in the CNS is largely dictated by both rostrocaudal and dorsoventral signaling systems (Lumsden and Krumlauf, 1996; Jessell, 2000). According to their position along these two axes, neural progenitor/stem cells (NP/SCs) are exposed to different concentrations of morphogens, which modify their transcriptional profile (Lewis, 2006; Lupo et al., 2006). While the rostrocaudal signaling establishes the main subdivisions of the CNS (the forebrain, midbrain, hindbrain and

spinal cord), the dorsoventral signaling system determines the cell types within each of these rostrocaudal subdivisions. Our current understanding of spinal motor neuron generation during embryonic development is based largely on studies in chicks and mice, and has previously been reviewed in details (Jessell, 2000; Lee and Pfaff, 2001; Briscoe and Novitch, 2008). Here, we provide a brief review of motor neuron development with the focus on the extrinsic morphogens and intrinsic transcription factors important for motor neuron specification during development *in vivo*, since they are likely to be involved in the fate determination of motor neurons from stem cells *in vitro*.

In the spinal cord, the ventral half of the neuroepithelium gives rise to the floor plate and five populations of neurons: V0, V1, V2 and V3 interneurons and motor neurons (Briscoe and Novitch, 2008). Two important gradient molecules influence the fates of these cells: Sonic hedgehog (Shh) that is initially produced by the notochord and later by the floor plate, and retinoic acid (RA) released from the paraxial mesoderm (Briscoe and Novitch, 2008). Shh signaling induces the more ventral class II homeodomain (HD) transcription factors (TFs) such as Nkx6 and Nkx2.2 and represses the more dorsal class I TFs such as Pax6, Irx3 and Dbx2 (Lee and Pfaff, 2001). In contrast, RA signaling induces the expression of class I TFs. Class-I and Class-II HD TFs cross repress each other, establishing boundaries that define the different cell types along the dorsoventral axis of the spinal cord (Jessell, 2000). Thus, the cells that express Pax6 and Nkx6 become primed to induce the basic helix-loop-helix (bHLH) TF Olig2, which is required for motor neuron differentiation.

Olig2 allows motor neuron progenitors (pMNs) to become motor neurons by inducing the expression of the TFs Ngn2 and Lhx3 (Novitch et al., 2001). However,

Olig2 also antagonizes the premature expression of motor neuron genes such as Hb9, while Ngn2 counteracts this effect (Lee et al., 2005). Thus, when Olig2 levels are high the cells are maintained in a pMN state, whereas increasing the levels of Ngn2 favors the conversion of pMNs into post-mitotic motor neurons. Ngn2 is a bHLH TF that regulates the commitment of progenitor cells to both pan-neuronal and specific motor neuron fates (Mizuguchi et al., 2001). Ngn2 promotes neurogenesis by increasing the expression of neurogenic genes such as NeuroD and β-III tubulin. In addition, Ngn2 possesses two conserved serine residues (S231 and S234) that, when mutated to alanines, impair motor neuron differentiation without affecting general neurogenesis (Ma et al., 2008). These serines are phosphorylated by glycogen synthase kinase 3 (GSK3). Serine phosphorylation facilitates the interaction of Ngn2 with LIM HD TFs such as Lhx3 and Isl1, which in a complex activate the transcription of motor neuron-specific genes such as Hb9 (Ma et al., 2008; Lee et al., 2008).

In addition to a general motor neuron fate specification, spinal motor neurons also acquire discrete columnar identities as a function of their position along the rostrocaudal axis of the spinal cord (Carpenter, 2002). Motor neurons in each column innervate particular target muscles, which is required for proper locomotor function. This patterning of motor neurons into columns is influenced by extrinsic morphogens. Recent evidence indicates that a gradient of fibroblast growth factor 8 (FGF8) is critical for the specification of columnar motor neuron subtypes through FGF8-mediated induction of various Hox-c genes (Dasen et al., 2003). The posterior Hox genes inhibit the expression of the anterior Hox genes and vice versa. Thus, cell autonomous repressor and activator functions of Hox-c proteins define the boundaries of MN columns. In addition, the

forkhead domain transcription factor Foxp1, through its activity as a Hox accessory factor, is required to establish the pattern of LIM-HD protein expression that defines the columnar identity of motor neurons (Dasen et al., 2008; Rousso et al., 2008).

1.3.2. GENERATION OF SPINAL MOTOR NEURONS FROM STEM CELLS

Stem cells are cells that self-renew, give rise to differentiated progenies and maintain these properties over a long period of time. For the generation of motor neurons, two major types of stem cells have been used as starting undifferentiated cell sources: embryonic stem cells (ESCs) and neural stem cells (NSCs). ESCs are pluripotent cell lines obtained from the inner cell mass of the blastocyst and have the potential to differentiate into cells of all three embryonic germ layers (i.e. endoderm, mesoderm, and ectoderm). On the other hand, NSCs are multipotent cell lines isolated from nervous tissue, commonly from fetal and adult brains or spinal cords of rodents and humans. These cells have the potential to generate neural cells, including neurons, oligodendrocytes and astrocytes (McKay, 1997; Gage, 2000; Donovan and Gearhart, 2001). Here, the studies thus far on the generation of spinal motor neurons from both ESCs and NSCs will be reviewed (see Table 1.1).

Table 1.1 Spinal Motor Neurons Differentiated from Stem Cells

Type of Cells	Species	Methods to Generate Spinal Motor Neurons	Efficiency of Spinal Motor Neuron Generation	References
Embryonic stem cells (ESC)	Mouse	Embryoid bodies treated with RA for 6 days	0.5-0.8% of stem cells expressed neurofilament, Isl1 and Hb9 *in vitro*	(Renoncourt et al. 1998)
	Mouse	Embryoid bodies treated with RA plus Shh or Shh agonist for 5 days	20-30% of stem cells expressed Hb9 and NeuN/ TuJ1 *in vitro* Following graft into stage 15-17 chick spinal cords, differentiated into $Hb9^+$ motor neurons, formed synaptic connections with intercostal muscles	(Wichterle et al. 2002)
	Mouse	ESCs cocultured with MS5 stromal cells, treated with Shh and RA for 4 days, followed by further differentiation for 6 days	60% of $TuJ1^+$ cells expressed Hb9 *in vitro*	(Barberi et al. 2003)
	Human	bFGF-differentiated ESC rosettes treated with Shh and RA for 7 days, followed by further differentiation for 1-2 weeks.	21% of rosette cells expressed Hb9 and ChAT, formed synapses on cocultured myotubes *in vitro*	(Li et al. 2005)
	Human	bFGF-differentiated ESC rosettes treated with bFGF, Shh and RA for 7 days, followed by further differentiation for 2 weeks	20-30% of rosette cells expressed Isl1, TuJ1 and ChAT *in vitro*	(Shin et al. 2005)
	Mouse	pHb9-eGFP-transfected ESCs treated with a Shh agonist and RA for 5 days, followed by further differentiation in GDNF/CNTF for 4 days	96% of Hb9-$eGFP^+$ motor neurons were $Lhx3^+$ Following graft into stage 17 chick neural tubes, projected to epaxial muscles	(Soundararajan et al. 2006)
	Human	Embryoid bodies treated with bFGF, RA and Shh for 2 weeks, followed by further differentiation for 3 weeks	58% of stem cells expressed Hb9 *in vitro*	(Lim et al. 2006)
	Mouse	ESCs treated with RA for 4 days, treated with BDNF/CNTF/NT3 and cAMP before grafting	Following graft into adult rat spinal cords, differentiated into Hb9 and ChAT motor neurons, sent axons into muscles and formed neuromuscular junctions	(Deshpande et al. 2006)
	Human	MS5 stromal cell-induced neural rosettes treated with Shh and RA for 15 days, followed by further differentiation in GDNF/BDNF/AA for 10 days	20% of rosette cells expressed Hb9 *in vitro* Following graft into rat and chick spinal cords, differentiated into Hb9 or ChAT motor neurons, sent axons into ventral root	(Lee et al. 2007)

Type of Cells	Species	Methods to Generate Spinal Motor Neurons	Efficiency of Spinal Motor Neuron Generation	References
	Human, Primate	bFGF-differentiated neuroepithelial cells treated with RA for 1 week, then RA plus Shh or purmorphamine for 1 week, followed by further differentiation in BDNF/GDNF/IGF-1	50% of rosette cells expressed Hb9 *in vitro*	(Li et al. 2008)
Neural stem cells (NSC)	Human	8-week forebrain-derived neurospheres primed with bFGF/heparin/laminin for 4-5 days followed by further differentiation for 9-10 days	20-50% of cells expressed Hb9 and ChAT *in vitro* Following graft into adult rat spinal cords, differentiated into $Hb9^+/ChAT^+$ motor neurons, sent myelinated axons into peripheral nerves and formed neuromuscular junction	(Wu et al. 2002) (Gao et al. 2005) (Jordan et al. 2008)
	Mouse	E16-18 spinal cord-derived neurospheres differentiated in BDNF/CNTF/GDNF for 7-10 days	Some cells expressed ChAT and Isl1	(MacDonald et al., 2003)
	Rat	E14 spinal cord-derived neurospheres treated with bFGF for 3 days, followed by RA agonists for 2 days	Activation of RA receptor β increasedIsl1 expression and $Hb9^+$ cells	(Goncalves et al., 2005)
	Mouse	E13.5 or adult spinal cord-derived cells, with high aldehyde dehydrogenase activity, differentiated in low bFGF, RA, Shh, cAMP and NGF	27.6% of E13.5 NSCs expressed Hb9, 7.4% of adult NSCs expressed Hb9	(Corti et al. 2006)
	Mouse	LeX+/CX+ adult mouse brain-derived neurospheres treated with bFGF/heparin/laminin plus Shh and RA for 5 days, followed by differentiation in 2% FBS plus BDNF/GDNF/CNTF/IGF1/NT3	22.5% of sphere cells expressed Hb9, coexpressed Isl1 and ChAT Following graft into adult mouse spinal cords, differentiated motor neurons sent axons to ventral roots	(Corti et al. 2007)
NSCs from organisms with motor neuron diseases	Human	Cortical NSCs derived from patients with lethal congenital contracture syndrome, treated with Shh agonist plus 2% FCS or IGF1 for 2 weeks	Isl1+ or Hb9+ cells derived from NSCs *in vitro*	(Pakkasjarvi et al. 2007)
	Mouse	Adult olfactory bulb-derived neurospheres from transgenic mice expressing either wild-type or mutant human SOD1 gene, proliferated in bFGF	Following transplantation into spinal cords, differentiated into large $ChAT^+$ neurons, formed myelinated axons and grew into peripheral nerves	(Martin et al. 2007)
	Human	pHb9egfp-transfected ESCs treated with Shh and RA for 4-5 days, sorted and differentiated in BDNF/GDNF/NT4/CNTF	90.1% of sorted cells expressed Hb9/Tuj1, 88.6% expressed Isl1/ChAT *in vitro*	(Roy et al. 2005)

Type of Cells	Species	Methods to Generate Spinal Motor Neurons	Efficiency of Spinal Motor Neuron Generation	References
Genetically modified stem cells	Human	Adult olfactory NPCs transfected with Hb9 plus Olig2 or Ngn2, then treated with Shh, RA and forskolin	Over 95% of cells expressed Hb9, 30-40% of cells expressed Isl1/2 *in vitro* Formed synapses on cocultured chicken muscle cells *in vitro*	(Zhang et al. 2006)
	Rat	Fetal spinal cord NPCs transduced with Hb9, Nkx6.1 and Ngn2, treated with Shh and RA	4-8% of cells expressed ChAT, formed contacts with myotubes *in vitro* Following graft into adult rat spinal cords, differentiated into $ChAT^{+}$ neurons, sent axons into ventral root	(Bohl et al. 2008)
	Human	ALS patient skin fibroblast-derived induced pluripotent stem cells transduced with Oct4, Sox2, Klf4 and c-myc, treated with Shh and RA	20% of embryoid body cells expressed Hb9 *in vitro*	(Dimos et al. 2008)

BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; cAMP, cyclic adenosine monophosphate; ChAT, choline acetyltransferase; CNTF, ciliary neurotrophic factor; ESC, embryonic stem cell; FBS, fetal bovine serum; FCS, fetal calf serum; GDNF, glial cell line-derived neurotrophic factor; IGF-1, insulin-like growth factor 1; NGF, nerve growth factor; NPC, neural progenitor cell; NSC, neural stem cell; NT3, neurotrophin 3; NT4, neurotrophin 4; RA, retinoic acid; Shh, sonic hedgehog; SOD1, superoxide dismutase 1; TuJ1, antibody against βIII tubulin.

Generation of spinal motor neurons has been reported first from mouse ESCs, which apparently recapitulates the embryonic development of motor neurons that requires two steps: general neural induction and motor neuron specification (Wichterle et al., 2002). Under non-adhesive growth conditions, mouse ESCs aggregate forming small spheres called embryoid bodies. Efficient generation of neurons from mouse embryoid bodies requires RA, which promotes neural and represses mesodermal gene expression (Bain et al., 1996). Further motor neuron specification from mouse embryoid bodies requires the neuralizing and caudalizing activity of RA together with the ventralizing action of Shh (Wichterle et al., 2002). When treated with RA, but not Shh, less than 0.5% of mouse ESCs differentiate into $Hb9^{+}/Isl1^{+}$ motor neurons (Renoncourt et al., 1998). In contrast, treating mouse ESCs with a combination of RA and Shh or a Shh agonist generates spinal motor neurons at 20-30% efficiency (Wichterle et al., 2002; Barberi et al., 2003; Soundararajan et al., 2006). When transplanted into embryonic chick or adult rat spinal cords, mouse ESC-derived motor neurons are able to extend axons into the periphery and form neuromuscular junctions (Wichterle et al., 2002; Deshpande et al., 2006).

Generation of spinal motor neurons from human ESCs has also been successful with approaches both similar to and different from those used for mouse ESCs. In terms of the initial neural induction, human ESCs, unlike mouse counterparts, require additional signals such as FGF2 or those generated from cocultured feeder cells (Li et al., 2005; Shin et al., 2005; Lee et al., 2007). Neuroepithelial cells derived from human ESCs are then treated with a combination of Shh and RA to generate spinal motor neurons. These neuroepithelial cells show two developmental stages during their differentiation: an early

primitive stage where the columnar epithelial cells express several neuroectodermal TFs, but not the definitive neuroectodermal TF Sox1 and a later definitive stage where Sox1 is expressed (Zhang, 2006). It is only in the early stage when neuroepithelial cells are more responsive to RA caudalization and Shh ventralization and thus generate motor neurons efficiently. RA at concentrations from 0.1 to 1 μM induces the expression of Hox genes and downregulates the expression of the rostral TF Otx2. Shh acts on the caudalized neuroepithelial cells to activate the expression of the TFs Olig2 and Hb9 through modulation of other TFs (e.g. Pax6, Nkx6). Using similar protocols with minor variations, several groups reported the generation of $Hb9^{+}/ChAT^{+}$ motor neurons from human ESCs with a 20-58% efficiency (Li et al., 2005; Shin et al., 2005; Lim et al., 2006; Lee et al., 2007). Most recently, a 50% efficiency of motor neuron differentiation through treating human and primate ESCs with purmorphamine, a small molecule that activates the Shh pathway, has been reported (Li et al., 2008). These human ESC-derived motor neurons form synapses on cocultured myoblasts/myotubes and are electrophysiologically functional (Li et al., 2005).

As with ESCs, NSCs have also been used to generate motor neurons *in vitro* and *in vivo*. During development, NSCs are found initially in the neural plate and then in the ventricular zone (VZ) of the neural tube where they are thought to acquire a radial glia phenotype (Anthony et al., 2004). NSCs are also found in the peripheral nervous system such as neural crest stem cells (Crane and Trainor, 2006). In the adult brain, NSCs are mainly found in two regions, the subventricular zone (SVZ) of the lateral ventricles where they adopt an astrocyte-like phenotype and the subgranular zone of the hippocampal dentate gyrus (Merkle and Alvarez-Buylla, 2006). NSCs can also be found

in the spinal cord (Weiss et al., 1996) and in areas outside the CNS such as the olfactory epithelium (Calof et al., 1998). Spinal motor neurons can be generated not only from spinal cord NSCs that naturally produce this type of neuron during embryonic development, but also from NSCs derived from the regions that do not usually give rise to spinal motor neurons.

Several groups have previously reported the successful generation of spinal motor neurons from rodent embryonic and adult spinal cord NSCs. The isolated NSCs are usually expanded as neurospheres in the presence of basic fibroblast growth factor (bFGF) and/or epidermal growth factor (EGF). By withdrawing growth factors, some of the mouse embryonic spinal NSCs differentiate into $ChAT^+/Isl1^+$ motor neurons (MacDonald et al., 2003). Additional treatment with RA seems to increase $Hb9^+$ motor neuron differentiation from rat embryonic spinal NSCs via the activation of RA receptor β (Goncalves et al., 2005). Another method to obtain motor neurons is to add both Shh and RA in the culture medium. Using this protocol, mouse embryonic spinal NSCs generated significantly more $ChAT^+$ motor neurons (27.6%) than those derived from adult spinal cords (Corti et al., 2006). However, the efficiency of generating motor neurons from human spinal cord NSCs has yet to be determined.

Interestingly, spinal motor neurons can also be produced from NSCs isolated from other regions of the CNS, where there is no development of spinal motor neurons *in vivo*. Human fetal brain-derived NSCs, when primed with bFGF, heparin, and laminin for 4-5 days and further differentiated in B27, generated cholinergic motor neurons *in vitro* (Wu et al., 2002). Activation of FGF receptors (FGFR) by bFGF during priming is required to generate a high percentage (50%) of $Hb9^+/ChAT^+$ spinal motor neurons (Jordan et al.,

2008b). Unlike spinal NSCs and ESCs, RA and Shh are not required for caudalizing and ventralizing human brain-derived NSCs toward a spinal motor neuron phenotype. Instead, FGF signaling seems sufficient to guide these NSCs to change their fate (Jordan et al., 2008b). Using a similar priming technique, but adding RA and Shh, 25% $Hb9^+$ motor neurons were generated from a subpopulation of cortical NSCs that were isolated from adult mouse brain and expressed the cell surface markers, Lewis X and chemokine receptor CXCR4 (Corti et al., 2007). When grafted into adult rodent spinal cords, human and mouse cortical NSCs as well as NSCs derived from the adult mouse olfactory bulb differentiated into $Hb9^+$ and/or $ChAT^+$ spinal motor neurons (Wu et al., 2002; Gao et al., 2005; Corti et al., 2007; Martin and Liu, 2007). Interestingly, NSCs derived from patients or animal models with motor neuron disease have also been shown to generate spinal motor neurons. These include fetal cortical NSCs derived from patients with lethal congenital contracture syndrome (Pakkasjarvi et al., 2007) and adult olfactory bulb NSCs from transgenic mice expressing the human mutant SOD1 gene (Martin and Liu, 2007).

In addition to the use of mitogens and other factors on wild-type stem cells, genetic modifications have taken place in attempts to increase the efficiency of spinal motor neuron generation. Fetal rat spinal cord neural precursor cells have been genetically engineered to coexpress the transcription factors Hb9, Ngn2 and Nkx6.1, which make them responsive to Shh and RA, and direct their differentiation into cholinergic motor neurons at 4-8% efficiency. *In vitro*, these motor neurons project axons that form contacts with cocultured myotubes (Bohl et al., 2008). When transplanted into the injured adult rat spinal cord, the engineered cells transiently proliferated, reached the ventral horn, projected their axons into the ventral root and expressed motor neuron

markers. Using a similar strategy, enforcing the expression of Hb9 together with either Olig2 or Ngn2 drove human adult olfactory neuroprogenitor cells to become Isl1/2^{+} and ChAT^{+} motor neurons (Zhang et al., 2006). Furthermore, these stably transfected neuroprogenitors uniformly expressed Hb9 (>95%), among which 40-60% coexpressed NeuN and Isl1/2. Genetic modifications have also been used to reprogram human fibroblasts from two elderly ALS patients to generate motor neurons. The genes Klf4, Sox2, Oct4, and c-Myc were introduced into the fibroblasts by retroviral transduction. The forced expression of these genes reprogrammed the fibroblasts to form induced pluripotent stem (iPS) cells. In culture, these iPS cells aggregated into embryoid bodies, which were subsequently differentiated into motor neurons by treatment with Shh and RA (Dimos et al., 2008). Finally, enrichment of the motor neuron population from ESCs has been achieved through the stable transfection of human and mouse ESCs with Hb9 promoter-driven green fluorescent protein genes, and a further isolation of the green cells by fluorescence-activated cell sorting (FACS) (Roy et al., 2005).

In summary, stem cell-derived motor neurons or their progenitors may be used for therapeutic purposes. However, in order to choose the right type of stem cells, both the advantages and limitations involved in their applications must be taken into consideration. ESCs display unlimited growth in culture, an undifferentiated state and great differentiation potentials. However, the risk of these cells to form teratomas is a major concern. Fetal brain NSCs can be expanded for long term *in vitro*, exhibit multiple differentiation potential and do not form teratomas. Unlike ESCs and fetal NSCs, adult stem cells can be used without ethical and immunological constraints, and thus, allow for autologous transplantation. However, their potential to become spinal motor neurons

remains to be elucidated. Induced pluripotent stem cells also circumvent the issue of immune rejection. The drawback of these genetically modified cells is the high risk of cancer formation, mainly due to the lack of control on where the transgenes integrate when using retrovirus as the gene delivery method. To reduce the cancerous risk, techniques such as homologous recombination could be used to gain control of the integration sites. In addition, iPS cells should be fully characterized for a period of time during their *in vitro* growth before being transplanted. Another alternative would be to use plasmids instead of viruses to deliver the transgenes, so that these genes would be transiently expressed and thus would not integrate (Okita et al., 2008).

1.4. ROLES FOR STEM CELL-DERIVED MOTOR NEURONS IN ALS

1.4.1. STEM CELL TRANSPLANTS IN TRANSGENIC ALS ANIMAL MODELS

Stem cell transplantation may be used to treat ALS by replacing lost motor neurons or protecting endogenous motor neurons through the replacement of dysfunctional non-neuronal glial cells such as astrocytes and microglia. Stem cells modified to secrete vital factors aimed at preserving surviving motor neurons through neurotrophic support or ameliorating inflammation is also an attractive strategy (Nayak et al., 2006; Hedlund et al., 2007; Suzuki and Svendsen, 2008). Several groups have transplanted stem cells into transgenic ALS animal models and reported various outcomes (see Table 1.2).

Table 1.2 Stem Cell Transplants in Transgenic ALS Animal Models

Stem Cell Therapy	Mutant SOD1 Model and Age at Grafting	Transplant Parameters	Morphological Analysis	Effect on Disease Progression	References
$mBMC^{GFP}$, isolated from transgenic GFP-expressing mice	$SOD1^{G93A}$ mouse, 6 weeks, 950-1100 rads irradiated	$5x10^6$ cells, intravenous	GFP^+ cells found in SC, GFP^+-$F4/80^+$ microglia (83%), GFP^+-$CD11b^+$ microglia (28%)	No effect on survival or rotarod performance	(Solomon et al. 2006)
mBMCs	$SOD1^{G93A}$ mouse, 8 weeks, 8.0 Gy irradiated	$5x10^6$ cells, retro-ocular	Not performed	Extended lifespan (~11 days)	(Ende et al. 2000)
mBMCs, isolated from β-Actin-eGFP-, neuron-specific Thy1-YFP or $SOD1^{G93A}$ adult mice	$SOD1^{G93A}$ mouse, 4 weeks, 8.0 Gy irradiated	$30x10^6$, intraperitoneal	YFP^+ cells in brain, some GFP^+ and YFP^+ cells colabeled with Tuj1, NeuN and NF possibly through cell fusion, no MNs	Extended lifespan (10-13 days), prolonged rotarod performance (~14 days), protected endogenous MNs	(Corti et al. 2004)
$rMSC^{GFP}$, isolated from transgenic GFP-expressing rats	$SOD1^{Leu126delTT}$ mouse, 14 weeks	$3\text{-}4x10^5$ cells, intrathecal into fourth ventricle	Some cells found in brain and SC, no analysis on differentiation	No effect on lifespan or disease onset (extended lifespan in females only), no delay in weight loss, no delay in decline of extension score or stride length	(Morita et al. 2008)
hMSCs	$SOD1^{G93A}$ mouse, 8 weeks, 6.0 Gy irradiated	$3x10^6$ cells, intravenous	Few HNu^+ cells colabled with Tuj1 and GFAP in brain, brain stem and SC, no MNs	Extended lifespan (~18 days), delayed disease onset (~14 days), prolonged rotarod performance, protected endogenous MNs, preserved sciatic nerve CMAP amplitude	(Zhao et al. 2007)

Stem Cell Therapy	Mutant SOD1 Model and Age at Grafting	Transplant Parameters	Morphological Analysis	Effect on Disease Progression	References
hMSCs or hMSC-derived neural stem-like cells (hMSC-NSCs)	$SOD1^{G93A}$ mouse, 45 days	10^5 cells, intrathecal into cisterna magna	Widespread cell migation within the subarachnoid space 10 days after transplant, cells found in brain parenchyma, no morphological analysis performed	No effect on lifespan, disease onset, endogenous MN survival and performance on running wheel	(Habisch et al. 2007)
hMSCs	$SOD1^{G93A}$ mouse, 28 weeks	10^5 cells, intraspinal at L1-L2	GFAP (<1%), MAP2 (<1%), no MNs	Delayed motor score decline, protected endogenous MNs (35%), decreased microgliosis, decreased astrogliosis, prolonged rotarod performance	(Vercelli et al. 2008)
$hMSC^{GFP}$-GDNF, modified with retrovirus-GFP and lentivirus-GDNF	$SOD1^{G93A}$ rat, 80 days	$1.2x10^5$ cells/injection, 3 injections at 1 week intervals, intramuscular	Cells survived, but differentiation not determined	Extended lifespan (~28 days), no effect on disease onset, maintained large endogenous motor neurons, delay denervation from neuromuscular junction, delayed decline in BBB	(Suzuki et al. 2008)
hUCBCs, combined from 11 donors according to blood type	$SOD1^{G93A}$ mouse, 8 weeks, 8.0 Gy irradiated	$34.2\text{-}35.6x10^6$, retro-ocular	Not performed	Extended lifespan (~21 days)	(Ende et al. 2000)
hUCBCs, pooled from donors according to blood type	$SOD1^{G93A}$ mouse, 7 weeks, 8.0 Gy irradiated	$70.2\text{-}73.3x10^6$ cells, retro-ocular	Not performed	Extended lifespan (~30-39 days)	(Chen and Ende 2000)
hUCBCs	$SOD1^{G93A}$ mouse, 9.5 weeks	10^6 cells, intravenous	HNu^+ cells colabeled with CD45 (leukocyte antigen), Nestin, Tuj1 and GFAP in brain and lumbar and cervical SC, morphology typical of neuronal and glial cells	Extended survival (~3 weeks), delayed weight loss, preserved hindlimb extension, delayed motor decline on footprint test	(Garbuzova-Davis et al. 2003)

Stem Cell Therapy	Mutant SOD1 Model and Age at Grafting	Transplant Parameters	Morphological Analysis	Effect on Disease Progression	References
hUCBCs	$SOD1^{G93A}$ mouse, 7-8 weeks	$10x10^6$, $25x10^6$, or $50x10^6$ cells, intravenous	$Nestin^+$ cells found in cervical and lumbar SC	Optimal dose $25x10^6$: extended survival (~14 days), delayed weight loss, maintained hindlimb extension, prolonged motor performance on rotarod, decreased levels of proinflammatory cytokines in the lumbar SC, decreased microglial cell density in cervical and lumbar SC	(Garbuzova-Davis et al. 2008)
$hUCBC^{VEGF-L1CAM}$, modified with human VEGF and mouse L_1CAM by electroporation	$SOD1^{G93A}$ mouse, 22-25 weeks	10^6 cells, retro-orbital	Cells found in blood vessel walls of lumbar SC, $CD34^+$ human endothelial cells	Not described	(Rizvanov et al. 2008)
hUCBCs or hUCBC-derived neural stem-like cells (hUCB-NSCs)	$SOD1^{G93A}$ mouse, 45 days	10^5 cells, intrathecal into cisterna magna	Widespread cell migration within the subarachnoid space 10 days after transplant, cells found in brain parenchyma, no morphological analysis performed	No effect on lifespan, disease onset, endogenous MN survival and performance on running wheel	(Habisch et al. 2007)
$hNPC^{GDNF}$, isolated from 22 week human fetus and modified with lentivirus-GDNF, treated with CNTF	$SOD1^{G93A}$ rat, 80 days	$1.2x10^5$ cells/injection, intraspinal bilaterally at L1 and L3	HNu^+ cells colabeled GFAP (~5%), no Tuj1, most $Nestin^+$, no MNs	No effect on lifespan and BBB scores, upregulation in ChAT expression, surviving $ChAT^+$ cells had larger soma, no effect on $ChAT^+$ cell survival, increased GDNF in SC	(Klein et al. 2005)
$hNPC^{GDNF}$, isolated from 15 week human fetus and modified with lentivirus-GDNF	$SOD1^{G93A}$ rat, 70 days	$1.2\text{-}1.8x10^5$ cells/injection, 4 injection sites, intraspinal at L1-4 unilaterally	Nestin (>95%), GFAP (<10%), no neurons	No effect on lifespan, disease onset or BBB score, protected endogenous MNs, no effect on denervation	(Suzuki et al. 2007)

Stem Cell Therapy	Mutant SOD1 Model and Age at Grafting	Transplant Parameters	Morphological Analysis	Effect on Disease Progression	References
hNSCs, isolated from cervical spinal cord of 8 week human fetus	$SOD1^{G93A}$ rat, 62 days	$5x10^4$ cells/injection, 8 injection sites, 4/side bilaterally intraspinal at L4-5	HNu^+ cells colabeled with Tuj1 (~70%), Nestin (~19%), GFAP (~1%)	Extended lifespan (~11 days), delayed disease onset (~7 days), protected endogenous MNs (~50%), delayed weight loss, prolonged performance on BBB score and inclined plane test, increased GDNF and BDNF in CSF and lumbar SC	(Xu et al. 2006)
hNSCs, isolated from cervical spinal cord of 8 week human fetus	$SOD1^{G93A}$ rat, 2 months	$2x10^4$ cells/injection, 4 injection sites, 2/side bilaterally intraspinal at L4-5	HNu^+ cells colabeled with Tuj1, some Nestin, rarely GFAP, no O4, evidence of graft integration into host circuitry	Cells with a combined immunosuppressive regimen prolonged lifespan (~2-3 weeks), delayed disease onset (~1-2 weeks) and delayed motor score decline compared to dead cell grafts and live cell grafts with FK506 monotherapy	(Yan et al. 2006)
mNSCs, Lewis $X^+/CXCR4^+$, isolated from β-Actin-GFP- or Hb9-GFP-expressing adult mice	$SOD1^{G93A}$ mouse, 70 days	$2x10^4$ cells, intraspinal at L4-5	β-actin-GFP: ChAT (~20%), MAP2 (~45%), GFAP (~26%), O4 (~4%); Hb9-GFP: ChAT (~77%)	Extended lifespan (~23 days), delayed disease onset by prolonging rotarod performance (~21 days), protected endogenous MNs (~75%)	(Corti et al. 2007)

Stem Cell Therapy	Mutant SOD1 Model and Age at Grafting	Transplant Parameters	Morphological Analysis	Effect on Disease Progression	References
mOB-NPCs, isolated from adult mouse olfactory bulb	$SOD1^{G93A}$ mouse, 70 days	$1.5x10^5$ cells/injection, 4 injection sites, lumbar intraspinal	ChAT (~30%), interneurons, GFAP (~15%), O4 (~5%)	Extended lifespan (~30 days), delayed disease onset (~27 days), protected endogenous MNs (32%), delayed weight loss, prolonged performance on running wheel and inclined plane test	(Martin and Liu, 2007)
$hNSC^{GFP}$, isolated from 8 week human fetus and modified with rAAV-GFP	$SOD1^{G93A}$ rat, 4 months	10^5 cells/injection, 4 injection sites, Intraspinal at L4-5 bilaterally, and C4-5 bilaterally	Majority GFP^+ cells colabeled with ChAT, few GFP positive axons observed in L5 ventral root	Extended lifespan (~17 days), delayed disease onset (~8 days), delayed weight loss, delayed motor score decline, prolonged performance on wire mesh ascending test and in a photobeam activity system	(Chapter 4)

BDNF, brain-derived neurotrophic factor; ChAT; choline acetyltransferase; CMAP, compound muscle action potential; CNTF, ciliary neurotrophic factor; CSF, cerebrospinal fluid; CXCR4, chemokine (C-X-C motif) receptor 4; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; hMSC, human mesenchymal stem cell; hNPC, human neural progenitor cell; hNSC, human neural stem cell; HNu, human nuclear antigen; hUCBC, human umbilical cord blood cell; L1CAM, L1 cell adhesion molecule; MAP2, microtubule-associated protein 2; mBMC, mouse bone marrow cell; MN, motor neuron; mOB-NPC, mouse olfactory bulb neural precursor cell; NeuN, neuronal nuclei; NF, neurofilament; O4, oligodendrocyte marker; rAAV, recombinant adeno-associated virus; rMSC, rat mesenchymal stem cell; SC, spinal cord; SOD1, superoxide dismutase 1;Tuj1, antibody against β-III tubulin; VEGF, vascular endothelial growth factor; YFP, yellow fluorescent protein

A few studies have examined the effect of rodent bone marrow cells (BMCs) on the disease progression when transplanted at pre-symptomatic ages in irradiated ALS mice. The cell delivery routes varied in each study, including intravenous and intraperitoneal injections. The delivery of mBMCs into the tail vein showed no significant effects on survival or motor performance (Solomon et al., 2006), although many transplanted mBMCs migrated to the spinal cord and most of these cells expressed markers typical of microglia at the disease end-stage. However, when mBMCs were delivered intravenously through a retro-ocular injection, an increase in lifespan was observed, but evaluation of cell fate and differentiation in the CNS was not performed (Ende et al., 2000). Mouse BMCs, transplanted intraperitoneally with 6 times more cells than the intravenous/retro-ocular delivery method, also showed an improvement in lifespan and motor performance (Corti et al., 2004). Furthermore, the mBMCs protected endogenous motor neurons and maintained axons exiting the ventral root. The majority of the cells differentiated into microglia/macrophages, while a few cells expressed neuronal markers possibly through a fusion process with endogenous neurons. No transplant-derived cells in any study showed motor neuron morphology or extension of processes down ventral roots. Similarly, mesenchymal stem cells (MSCs) extracted from transgenic GFP-expressing rats were delivered intrathecally into the fourth ventricle in pre-symptomatic $SOD1^{Leu126delTT}$ mice (Morita et al., 2008). Although there was no delay in disease parameters, except when evaluating the female population only, transplanted cells were found in the brain and spinal cord parenchyma. Thus, intrathecal transplantation into the fourth ventricle may represent a suitable delivery route for potentially more efficacious grafts of stem cells either specifically modified to secrete vital trophic factors

or primed to differentiate into specific lost or dysfunctional cell types. The beneficial effects on disease progression in two of these studies may be due to the replenishment of the microglia population in the ALS animals with non-mutant SOD1-expressing, and thus non-compromised microglia. The mBMCs may have also exerted a positive effect through secretion of factors influencing motor neuron survival directly or indirectly through ameliorating the inflammatory process ensuing in the ALS spinal cord. The lack of effect when cells were given through the tail vein or intrathecally is unknown, but may be due to a low number of stem cells, particularly transplant-derived non-mutant SOD1-expressing microglia, actually reaching pathological areas of the CNS. Positive benefits of mBMC transplantation, on the other hand, were not due to motor neuron or neuronal replacement.

Several studies have also evaluated the effect of human mesenchymal stem cells (hMSCs) on disease progression after transplantation into pre-symptomatic transgenic ALS animal models. Human MSCs injected intravenously into irradiated mice delayed the disease onset, extended lifespan, prolonged motor performance and protected endogenous motor neurons (Zhao et al., 2007). Implanted cells were located in the brain, brainstem and spinal cord, but only a few expressed neuronal or astroglial markers. Surprisingly, when hMSCs or hMSC-derived neural stem-like cells (hMSC-NSCs) were delivered intrathecally into the cisterna magna to pre-symptomatic ALS mice, no efficacious effects on lifespan, disease onset, motor performance and endogenous motor neuron survival were observed (Habisch et al., 2007). However, widespread cell migration within the subarachnoid space was evident shortly after transplantation and transplanted hMSCs were found in the brain parenchyma. The differentiation fate of the

transplanted cells was not determined. Thus, cell survival and migration to degenerating areas in the CNS are suggested to be a major concern when cells are injected intrathecally as compared to the intravenous route (Habisch et al., 2007). On the other hand, direct injection of hMSCs into specific lumbar spinal segments did delay the disease progression (Vercelli et al., 2008). Furthermore, the transplanted cells seemed to prevent the microgliosis and astrogliosis typically seen in ALS cords, while preserving endogenous motor neurons in the transplanted areas. Less than 1% of hMSCs differentiated into neurons or astrocytes and differentiation into microglia was not observed. There was also no evidence of motor neuron differentiation. Data from this study supports the hypothesis that intraspinal hMSC transplantation provides efficacy not through replacing motor neurons, but through neuroprotection by limiting microgliosis and astrogliosis and possibly direct neurotrophic support, although this was not verified in their study. Finally, hMSCs have been genetically modified to secrete glial cell line-derived neurotrophic factor ($hMSC^{GDNF}$) and transplanted into muscles affected in the transgenic ALS rats, including the tibialis anterior, forelimb triceps brachii and long muscles of dorsal trunk (Suzuki et al., 2008). No effect on disease onset was observed, but endogenous motor neurons as well as muscular innervations were maintained and lifespan was significantly extended. The differentiation fates of these cells were not determined, although the survival rate was high throughout the disease process. When assessing the potential of hMSC therapy based on these studies, intramuscular transplantation with hMSCs designed to secrete neurotrophic factors is an attractive approach that may be combined with intravenous or intraspinal hMSC therapy. In this manner, both axonal projections and connections with neuromuscular junctions will be

maintained by the intramuscular transplant, while transplanted cells, potentially also modified to secrete neurotrophic factors, in the degenerating areas of the spinal cord will protect the cell bodies of surviving motor neurons. Intrathecal administration of hMSCs shows less promise than intravenous or intraspinal delivery. Although stem cells reach the CNS more quickly when delivered directly into the cerebrospinal fluid (CSF), the cell survival, homing capability and migration to pathological areas seem to be inferior compared to stem cells placed into the circulatory system.

Human umbilical cord blood cell (hUCBC) transplantation has also revealed mixed outcomes in regards to efficacy depending on the route of transplantation and number of cells transplanted. Human UCBCs administered into the venous system through a retro-ocular injection to pre-symptomatic irradiated ALS mice at relatively large doses extended the lifespan of the animals in a dose-dependent manner (Ende et al., 2000; Chen and Ende, 2000). Thus, when administering hUCBCs intravenously, the number of cells transplanted directly affects the rate of the disease progression. Histological analysis in these studies was not performed to verify the presence and differentiation of transplanted hUCBCs in the CNS, but hypothetically, the more transplanted cells reaching the affected areas of the CNS, the better the improvement will be. Whether improvement in these studies is due to replacement of cells or a trophic effect on surviving motor neurons directly or indirectly through ameliorating inflammation is not known. In another study, hUCBCs were injected intravenously into pre-symptomatic mice and also showed a delay in disease progression and extension in lifespan (Garbuzova-Davis et al., 2003). Upon morphological analysis, transplanted hUCBCs were found to express leukocytic CD45, uncommitted Nestin, neuronal Tuj1

and astroglial GFAP in the brain as well as the lumbar and cervical spinal cord. A few cells showed morphology typical of neuronal and glial cells. The optimal therapeutic dose of hUCBCs administered intravenously has also been determined by the same group to be approximately 25 million cells (Garbuzova-Davis et al., 2008). Surprisingly, the highest dose tested of 50 million hUCBCs did not provide an efficacious outcome in this study, which possibly indicates that the beneficial effects on neuroprotection may be overturned by toxic effects produced by introducing a large number of potentially new inflammatory cells into the host. One strategy in improving the efficacy of hUCBC transplantation without increasing the total number of grafted cells to potentially toxic levels is through the genetic modification of these cells to secrete neuroprotective trophic factors. Human UCBCs have previously been transiently transfected with VEGF and L_1CAM, a cell adhesion molecule, through electroporation and were transplanted retro-orbitally into pre-symptomatic mice (Rizvanov et al., 2008). These modified grafted cells showed homing capability to the lumbar spinal cord and differentiation into endothelial cells within the lumbar cord blood vessel walls. Finally, when hUCBCs and hUCB-derived neural stem-like cells (hUCB-NSCs) were intrathecally administered into the cisterna magna of pre-symptomatic mice, no effect on disease parameters was observed (Habisch et al., 2007). These cells migrated within the subarachnoid space shortly after the transplant, but were not found in the brain or spinal cord. As in the case of intrathecally administered hMSCs and mBMCs, the survival and migration of hUCBCs after intrathecal administration seems to be very poor in affected areas of the CNS. Similarly to mBMCs and hMSCs, the hUCBCs showed potential for an efficacious treatment for ALS. However, the beneficial effects were more than likely due to

neuroprotection rather than replacement of motor neurons and dependent upon a sufficient number of surviving cells that homed into degenerated regions of CNS.

The therapeutic potential of neural stem and progenitor cells, both mouse and human, have also been tested in ALS animal models. Two studies have stereotaxically transplanted genetically modified human neural progenitor cells (hNPCs) that secrete GDNF ($hNPC^{GDNF}$) into specific lumbar regions of the spinal cord (Klein et al., 2005; Suzuki et al., 2007). In both studies, there was no effect on disease progression, but $hNPC^{GDNF}$ transplants caused an upregulation in ChAT expression and an increased soma size in surviving $ChAT^{+}$ motor neurons (Klein et al., 2005), and protected endogenous motor neurons, but did not prevent denervation (Suzuki et al., 2007). Most of the transplanted cells were $Nestin^{+}$ and only 5-10 % of the cells expressed astroglial GFAP in these studies. There were no motor neurons or neurons of any type that had differentiated from these progenitor cells. Thus, this approach of using genetically modified stem cells that secrete neurotrophic factors to save the cell bodies of surviving motor neurons may be useful in ALS patients when combined with cell therapies, as described above, aimed at maintaining neuromuscular junctions from these surviving motor neurons. Two additional studies have examined the effects of intraspinally injecting human NSCs isolated from the cervical spinal cord of an 8 week human fetus, without genetic modifications, into the lumbar region of pre-symptomatic ALS rats (Xu et al., 2006; Yan et al., 2006). These human spinal NSC transplants extended the lifespan and delayed disease onset with FK-506 immunosuppression therapy (Xu et al., 2006), but prolonged the lifespan considerably longer when grafted in combination with multiple immunosuppressive drugs or CD4 antibodies (Yan et al., 2006). Furthermore, the human

NSCs protected endogenous motor neurons possibly through the secretion of GDNF and BDNF in the CSF and lumbar spinal cord (Xu et al., 2006). In both studies, the majority of the human NSCs differentiated into $Tuj1^+$ neurons, although motor neuron specification was not observed. Some $Nestin^+$ grafted cells were present and $GFAP^+$ astroglial differentiation was rarely seen. This efficacious effect on lifespan is quite astounding considering the restriction of the grafted cells mainly to the lumbar area of the spinal cord. The secretion of neurotrophic factors from grafted human NSCs, and thus neuroprotection, may underlie the beneficial outcome in both studies. Evidence of grafted neuronal cell integration into the host circuitry was also observed (Yan et al., 2006), however, the effect of which on the beneficial delay in disease progression is not known. Insights from these studies do demonstrate, however, the potential for strategic intraspinal injections of human NSCs into pathological areas of the spinal cord to protect endogenous motor neurons and differentiate into new neurons that may aid in replacing affected neuronal connections.

Since abnormal astrocytes contribute to disease progression in ALS, it is logical to test the efficacy of replacing the dysfunctional astroglial cells surrounding endogenous motor neurons with human NSC-derived normal astrocytes. Interestingly, however, none of the human NSCs or NPCs provided a significantly higher population of $GFAP^+$ astrocytes when grafted intraspinally (Klein et al., 2005; Xu et al., 2006; Yan et al., 2006; Suzuki et al., 2007). Conversely, when the transplantation of lineage restricted glial progenitor cells into the cervical spinal cord, which gives rise to the phrenic nerve and regulates diaphragm muscle function, was tested in pre-symptomatic $SOD1^{G93A}$ rats, significant lifespan extension was observed (Lepore et al., 2008). Although there was no

effect on disease onset, weight loss or hindlimb grip strength, the cervical transplant delayed the decline in forelimb grip strength, maintained the phrenic nerve compound muscle action potential amplitude, protected endogenous motor neurons, attenuated the loss of the astroglial glutamate transporter, GLT1, and limited the microglial response in the cervical spinal cord. The positive effects of the grafted glial progenitor cells were likely due to the replacement of dysfunctional astrocytes and their actions at synaptic sites particularly in removing excess glutamate, rather than neuronal or microglial replacement or the secretion of neurotrophic factors such as BDNF, IGF-1 and VEGF. This study, thus, represents a proof of principle that stem cell therapy aimed to provide normal astrocytes is a feasible strategy for treating ALS.

As discussed in the studies above, replacing overactivated microglial cells or dysfunctional astrocytes and providing trophic support through stem cell transplants are only likely to have short-term benefits in delaying the progression of ALS. In order to reverse paralysis and regain muscle strength, motor neurons will need to be replaced. To this end, several groups have generated cholinergic spinal motor neurons from embryonic and neural stem cells (see Table 1.1 for details). These methods of generating relatively high quantities of spinal motor neurons may prove useful when attempting to replace motor neurons in ALS animal models and hopefully, ALS patients. Three groups, thus far, have demonstrated stem cell therapies that show $ChAT^+$ spinal motor neuron differentiation in the transgenic ALS spinal cord. The first group used mouse NSCs (mNSCs) isolated from adult transgenic β-actin-GFP-expressing mice or spinal motor neuron specific Hb9-GFP expressing mice, which were further subjected to fluorescent activated cell sorting (FACS) to purify Lewis X^+/$CXCR4^+$ stem cells (Corti et al., 2007).

These cells were primed in the presence of RA and Shh among other factors to enhance their capacity for spinal motor neuron differentiation. Intraspinally injecting these cells into the lumbar region of the spinal cord of pre-symptomatic ALS mice resulted in the extension of lifespan, a delay in disease onset and an increase in endogenous motor neuron survival possibly due to IGF-1 and VEGF secretion. On morphological analysis, 45 % of the β-actin-GFP$^+$ cells became MAP2$^+$ neurons, 26 % GFAP$^+$ astrocytes, 4 % O4$^+$ oligodendrocytes and 20 % ChAT$^+$ motor neurons. On the other hand, 77 % of the Hb9-GFP$^+$ transplanted cells expressed ChAT, indicating a spinal motor neuron phenotype. Furthermore, based on stereological analysis, the investigators estimated that approximately 20% of the surviving motor neurons in the transplanted area were graft-derived motor neurons, many of which exhibited neuritic outgrowth. The improved outcome is thought to be due to not only trophic support and alteration of the toxic environment, but also the addition of a new cell population that may have integrated into the host spinal cord circuitry within degenerating areas (Corti et al., 2007). In this study, the transplanted spinal motor neurons adopted typical motor neuron morphology and seemed to have large somata at the end-stage of the disease. The second group used multipotent neural precursor cells (NPCs) from the mouse olfactory bulb (OB-NPCs) and showed that bFGF-cultured OB-NPCs became motor neuron-like cells when transplanted into the lumbar spinal cord of pre-symptomatic ALS mice (Martin and Liu, 2007). Transplantation of these cells extended lifespan, delayed disease onset and maintained motor performance by protecting endogenous motor neurons. Grafted OB-NPCs showed a 30 % differentiation into ChAT$^+$ motor neurons, 15 % GFAP$^+$ astrocytes and 5 % O4$^+$ oligodendrocytes. Integration of grafted GFP$^+$ cells in host neural circuitry and extension

of processes down ventral roots and into the sciatic nerve were also observed. However, injecting a retrograde tracer into the hindlimb muscle did not reveal any retrogradely labeled grafted stem cell-derived motor neurons. Upon evaluation of the neuromuscular junctions, grafted GFP^+ cell axons showed no signs of innervation and in fact, displayed irregular swelling and dystrophy at axon terminals. Thus, the beneficial therapeutic effects in this experiment may be due to a variety of factors including neuroprotection through amelioration of excitotoxicty by forming new EAAT2-expressing astrocytes, reducing inflammation through immunomodulatory effects, direct neurotrophic effects on motor neuron survival and integration of potential spinal interneurons into the neural network within the host environment. Delays in disease progression were not due to reinnervation of distal muscle targets by new stem cell-derived motor neurons, however. This is the first *in vivo* evidence illustrating that transplanted motor neurons could not form new connections at neuromuscular junctions and exhibited morphology of degenerating distal axons after transplantation into the hostile ALS microenvironment. Evidence of stem cell-derived motor neuron susceptibility to the toxic ALS microenvironment was observed by our group when human NSC-generated motor neurons were transplanted into the lumbar and cervical spinal cord regions of pre-symptomatic ALS rats (see Chapter 4). Prior to transplantation, human NSCs were primed in the presence of bFGF to enhance their capacity to differentiate into spinal motor neurons *in vivo* (Wu et al., 2002; Gao et al., 2005; Jordan et al., 2008b). ALS rats receiving the human NSC transplants displayed a prolonged lifespan as well as delays in the decline of weight and motor function. Although a majority of the grafted human cells expressed ChAT, most of the surviving $ChAT^+$ grafted cells showed a degenerated

morphology indicated by a small soma size at the end-stage of the disease. Furthermore, almost every grafted cell within the ventral horn showed protein nitration damage and some of the transplanted cells had undergone lipid peroxidation as well. A few axons were found in the L5 ventral root at disease end-stage, but not to the same degree as described in other animal models (Wu et al., 2002; Gao et al., 2005). This study showed that human $ChAT^+$ motor neurons can be generated from NSC transplants in the ALS spinal cord, but adopt a degenerated, shrunken shape rather than the typical morphology of the motor neuron soma. The degeneration of human NSC-derived motor neurons is possibly due to the harsh oxidative environment that ensues in the spinal cord during the progression of the disease. Thus, stem cell-derived motor neurons may be susceptible to the same toxic cues that drive disease progression as endogenous motor neurons. To combat the potential vulnerability of stem cell-derived motor neurons to the ALS toxic environment, other approaches, either pharmacological or cell-mediated, will need to be developed to significantly change or even halt the persistent toxic ALS environment prior to initiating a motor neuron replacement therapy.

When assessing the affects of each of the studies described above on lifespan, each stem cell type showed a similar beneficial effect, regardless of the route of delivery or number of stem cells. The lone exception was intrathecal transplantation, which did not show an efficacious effect in any of the studies, but was only tested by two groups. Thus, neuroprotection through an alteration of the ALS environment most likely represents the major beneficial mechanism in most, if not all, of the studies that reported an efficacious outcome on lifespan. In addition, all transplants described in these pre-clinical studies were initiated well before observable motor deficits, which would not

currently be relevant in the vast majority of human ALS cases where disease onset is unpredictable due to the lack of diagnostic biomarkers. Thus, when a cell therapy could be initiated in most ALS patients, many motor neurons have already been lost and an unknown percentage of motor neuron axons have already retracted from neuromuscular junctions. Furthermore, at this time, the microenvironment in the cord is toxic due to persistent inflammation, oxidative stress and excitotoxicity. The effect of this hostile ALS environment on the long-term efficacy of any cellular therapy is currently unknown. It is possible that cells transplanted into the pathological areas of the spinal cord at disease onset may not work as predicted, since stem cell-derived motor neurons may degenerate and stem cell-derived microglia or astroglia may become overactivated and dysfunctional. Enhancing endogenous motor neuron survival and maintaining axonal connections with muscle through the use of modified trophic factor secreting stem cells may be the most appropriate therapeutic approaches until the microenvironment in degenerating areas can be altered well enough to halt the progression of the disease, at which time a cellular therapy to replace lost motor neurons and irreversibly dysfunctional non-neuronal cells can be initiated.

1.4.2. STEM CELL-DERIVED MOTOR NEURONS AND *IN VITRO* COCULTURE SYSTEMS

Glial cells are posited to create a hostile environment that contributes to motor neuron toxicity in transgenic ALS animal models and as such, may also adversely affect the survival and maturation of transplanted stem cell derived-motor neurons. In support, several studies have recently shown that stem cell-derived motor neurons are susceptible

to toxicity arising in the spinal microenvironment of ALS animals in organotypic slice cultures and from primary ALS glial cells in coculture (Kim et al., 2006; Di Giorgio et al., 2007; Nagai et al., 2007). Based on these results, stem cells may provide an excellent source of motor neurons to study the contribution of ALS glial cells to disease mechanisms and screen potential therapeutics aimed to protect motor neurons from glial-mediated toxicity. Furthermore, since primary human motor neurons are unattainable, stem cells can be utilized to provide insights into the vulnerability as well as cell injury mechanisms specifically of human motor neurons in an *in vitro* ALS disease setting.

1.4.3. MICROGLIAL TOXICITY TO MOTOR NEURONS *IN VITRO*

ESC-derived motor neurons from Hb9-GFP expressing transgenic mice were first shown to be susceptible to the ALS microenvironment after they were transplanted onto tissue slices isolated from pre-symptomatic mutant SOD1 transgenic mice (Kim et al., 2006). After 7 days in culture on mutant slices, no surviving GFP-expressing transplanted motor neurons could be found, whereas many motor neurons survived in cultures on wild-type spinal cord and mutant SOD1 and wild-type hippocampal organotypic slices. Hence, motor neuron-sensitive toxic factors, a lack of trophic factors or a combination thereof must persist in the microenvironment of the ALS spinal cord. When mouse ESC-derived motor neurons were cultured with mutant slices across a semi-permeable membrane, secreted soluble factors caused neurite shortening after 3-5 days and contributed to the death of motor neurons after 7 days. Further assays confirmed that mutant slices secreted much higher levels of nitric oxide, IL-1β, IL-6, IL-12p70 and

lower levels of VEGF. Not surprisingly, culturing ESC-derived motor neurons on mutant spinal cord slices in combination with a treatment consisting of neutralizing antibodies to all three proinflammatory cytokines, a nitric oxide scavenger and exogenous VEGF provided significantly more neuroprotection than any one treatment alone. These data indicate that not only will a combination treatment that blocks multiple cell injury pathways leading to motor neuron death be needed to protect endogenous motor neurons in ALS, but a combination therapy will also be needed to reduce the hostile nature of the ALS spinal cord in order to increase the efficacy of stem cell therapies aimed to replace lost motor neurons. To determine whether mutant SOD1-expressing microglia may contribute to ESC-derived motor neuron toxicity, a microglia cell line, BV-2, was stably transfected with inducible mutant SOD1 or wild-type SOD1 (Kim et al., 2006). Cocultures across a semi-permeable membrane did not induce motor neuron toxicity. However, when the microglia were stimulated with lipopolysaccharide (LPS), the mutant SOD1-expressing microglia dramatically increased the secretion of proinflammatory cytokines and nitric oxide, and induced a significant shortening of motor neuron neurite length compared to controls. Another study found that LPS-activated primary mutant SOD1-expressing microglia isolated from adult transgenic $SOD1^{G93A}$ mice, but not neonatal transgenic mice, released more TNF-α and less IL-6 compared to nontransgenic controls (Weydt et al., 2004). Thus, microglia that express mutant SOD1 are more responsive to inflammatory signals and their overactivation during disease progression may underlie the hostile oxidative and inflammatory environment contributing to motor neuron toxicity that is observed in the ALS spinal cord of mutant SOD1 transgenic animals.

As further evidence for microglial toxicity to motor neurons in ALS, several studies have indicated that isolated primary microglia become toxic to primary motor neurons when activated with either proinflammatory LPS or IgG immune complexes isolated from human ALS patients (Zhao et al., 2004; Beers et al., 2006; Zhao et al., 2006; Xiao et al., 2007). Nontransgenic microglia activated by these stimulatory factors initiate motor neuron death through nitric oxide and superoxide generation as well as glutamate release (Zhao et al., 2004). Treatment of these activated nontransgenic microglia with an anti-inflammatory cytokine, IL-4, suppressed nitric oxide and superoxide generation and provided neuroprotection in coculture, which indicates that suppressing the overactivated microglia in ALS may preserve motor neurons by ameliorating microglia-mediated toxicity (Zhao et al., 2006). Furthermore, primary motor neurons cocultured in direct contact, particularly with primary mutant SOD1-expressing microglia, showed decreased survival and shortened neurite length (Beers et al., 2006; Xiao et al., 2007). One mechanism is that inherent overexpression of mutant SOD1, as opposed to wild-type SOD1, specifically caused primary microglia to secrete more superoxide and nitric oxide and less IGF-1. Such higher concentrations of mutant SOD1-expressing microglia-generated nitric oxide have been correlated with a further reduction in motor neuron survival in coculture. In addition, stimulation of mutant SOD1-expressing microglia with the proinflammatory LPS caused enhanced nitric oxide and superoxide release, decreased IGF-1 secretion and greater motor neuron toxicity, which was partially abrogated by inhibiting microglial iNOS (Beers et al., 2006; Xiao et al., 2007). This indicates that mutant SOD1-expressing microglia inherently possess increased reactivity to proinflammatory stimulation. On the other hand, when conditioned

media from primary mutant SOD1-expressing microglia was added to the culture medium for ESC-derived motor neurons (Nagai et al., 2007) or primary mutant SOD1-expressing microglia were cocultured in transwells 1 mm away from NSC-derived motor neurons (see Chapter 5), a substantial toxic effect to motor neurons was not observed. Thus, mutant SOD1-expressing microglia require either direct contact or close proximity to exert a toxic effect.

Based on these data that primary motor neurons are susceptible to mutant SOD1-expressing microglia-mediated toxicity, it is important to determine the vulnerability of human stem cell-derived motor neurons to ALS microglia prior to initiating a stem cell treatment to replace motor neurons in ALS. Indeed, human NSC-derived motor neurons are susceptible to primary mutant SOD1-expressing microglia-mediated toxicity and human motor neuron death is partially ameliorated through inhibition of both microglial iNOS and NADPH oxidase (see Chapter 5). One potential mechanism through which mutant SOD1 directly increases ALS microglial toxicity to motor neurons is through regulating NADPH oxidase by binding and inhibiting the GTPase activity of the Rho-GTPase subunit, Rac1, and thus, maintaining NADPH oxidase in a persistently activated and superoxide-generating state (Harraz et al., 2008). Inhibiting NADPH oxidase not only protects human NSC-derived motor neurons in coculture (see Chapter 5), but also dramatically prolongs the lifespan of transgenic $SOD1^{G93A}$ mice when administered in their drinking water (Harraz et al., 2008).

1.4.4. ASTROGLIAL TOXICITY TO MOTOR NEURONS *IN VITRO*

Primary mutant SOD1-expressing astrocytes also exhibit toxic properties to both primary and stem cell-derived motor neurons *in vitro*. However, the identity of all the astrocytic toxic factors involved has remained elusive. First, mutant SOD1-expressing astroglia inherently display a "neuroinflammatory phenotype" (Hensley et al., 2006a). Even when these astrocytes are isolated from mice at young and thus pre-symptomatic ages, the basal expression levels of proinflammatory cytokines, eicosanoids, iNOS and protein carbonylation are significantly increased. Upon proinflammatory stimulation, mutant SOD1-expressing astroglia were more responsive and became overactivated in regards to these inflammatory factors and protein carbonylation damage as compared to nontransgenic controls. It will be important to discern the mechanisms of how this "neuroinflammatory phenotype" leads to selective motor neuron degeneration in ALS. For this purpose, stem cell-derived motor neurons can be used to dissect whether inhibiting these astroglia inflammatory processes prevents motor neuron death in cocultures. Reactive astrocytes that were activated with either LPS or peroxynitrite treatment secreted significantly more nerve growth factor (NGF), which contributed to primary motor neuron death through $p75^{NTR}$ activation only in the presence of low levels of nitric oxide (Pehar et al., 2004). Furthermore, mutant SOD1 may induce oxidative damage and dysfunction of astroglial mitochondria leading to enhanced superoxide radical release and development of an astroglial neurotoxic phenotype that requires nitric oxide synthase activation (Cassina et al., 2008). Mitochondria-specific antioxidants reduced mitochondria-generated superoxide, reversed mitochondrial respiration abnormalities and abrogated primary mutant SOD1-expressing astroglial toxicity to

primary motor neurons. On the other hand, uncoupling the mitochondria electron transport chain in nontransgenic, normal astroglia resulted in astroglia-acquired toxicity to primary motor neurons (Cassina et al., 2008). Functional consequences induced in motor neurons, irrespective to neuronal mutant SOD1 expression, by primary mutant SOD1-expressing astrocyte-released substances included a reduced mitochondrial redox state, decreased resting mitochondria membrane potential, elevated mitochondrial Ca^{2+} levels and diminished cytoplasmic Ca^{2+} levels, which may cumulatively enhance motor neuron vulnerability to potential toxic factors that have been implicated in ALS disease progression (Bilsland et al., 2008). Stem cell-derived motor neurons, specifically human motor neurons, should be tested in coculture to determine whether these abnormalities described in ALS astroglia and primary motor neurons are relevant to human motor neuron degeneration. As such, drug-screening can be initiated in coculture paradigms to determine whether inhibiting these potential cell injury pathways promotes human motor neuron survival.

Two reports first indicated that mouse ESC-derived motor neurons were susceptible to soluble toxic factors secreted from primary mutant SOD1-expressing astroglia (Di Giorgio et al., 2007; Nagai et al., 2007). In the first study, ESCs were isolated from Hb9-GFP expressing rats that either overexpressed mutant SOD1 or wild-type SOD1 and were differentiated into motor neurons by treating the embryoid bodies with RA and Shh (Di Giorgio et al., 2007). Both ESC-mutant SOD1- and ESC-wild-type SOD1-derived motor neurons died over a time period of weeks when cocultured with primary mutant SOD1-expressing astrocytes as compared to wild-type SOD1 astrocytes. Additionally, the toxicity of mutant SOD1-expressing astrocytes was greater for mutant

SOD1-expressing motor neurons than for wild-type motor neurons. Hence, autonomous mechanisms caused by mutant SOD1 expression increased the vulnerability of motor neurons to non-cell autonomous toxicity mediated by mutant SOD1-expressing astrocytes. In a similar study, nontransgenic primary motor neurons and motor neurons derived from ESCs, isolated from transgenic Hb9-GFP expressing mice, were lost when cocultured on mutant SOD1-expressing primary astrocyte monolayers as compared to nontransgenic or wild-type astrocytes (Nagai et al., 2007). Furthermore, conditioned medium from mutant SOD1 astrocytes killed both primary and ESC-derived motor neurons, which indicated that mutant SOD1-expressing astrocyte-mediated toxicity involved soluble and stable toxic factors. Mutant astrocyte toxicity was shown to be specific for motor neurons, since primary GABAergic neurons, dorsal root ganglion neurons and mouse stem cell-derived interneurons were not vulnerable when cocultured with primary mutant SOD1-expressing astrocytes. Moreover, inhibiting Bax-mediated apoptosis prevented motor neuron death in coculture.

In developing a stem cell therapy for ALS patients, it is important to determine whether human motor neurons are also vulnerable to ALS astroglial toxicity. Indeed, motor neurons generated from human ESCs have also recently been shown to be susceptible to mutant SOD1-expressing astroglia-secreted toxic factors (Di Giorgio et al., 2008; Hedlund and Isacson, 2008; Marchetto et al., 2008). Human ESC-derived motor neurons were lost when cocultured in direct contact with mutant SOD1-expressing astroglia or cultured in conditioned media from mutant SOD1-expressing astroglia for 10-20 days, but spared in cocultures with wild-type or nontransgenic astroglia, or mutant SOD1-expressing fibroblasts (Di Giorgio et al., 2008). Interneurons derived from human

ESCs were unaffected in cocultures with ALS astroglia, which indicated that the toxic factors were selective for human motor neurons. A microarray analysis was performed, which specified numerous genes that were upregulated in ALS astroglia as compared to nontransgenic and wild-type astroglia (Di Giorgio et al., 2008). Many of these genes are involved in inflammatory pathways. Astroglia were then treated with selected upregulated inflammatory factors and human ESC-derived motor neurons were used to screen for a potential toxic phenotype induced by these factors in previously non-lethal astroglia. Pretreatment of wild-type astroglia with prostaglandin D2 (PGD2) resulted in astroglial-mediated human motor neuron toxicity. Sure enough, inhibiting the PGD2 receptor on mutant SOD1-expressing astroglia partially abrogated human motor neuron loss in coculture. In a related study, human ESC-derived motor neurons were shown to be susceptible to toxicity arising from human astrocytes transduced with mutant $SOD1^{G37R}$ using a lentiviral vector (Marchetto et al., 2008). Once again, human ESC-derived non-motor neurons were resistant to mutant SOD1-expressing astroglia-secreted toxic factors while transduced mutant SOD1-expressing fibroblasts did not exhibit toxicity to human motor neurons. Increased expression of several proinflammatory factors occurred, including iNOS and NOX2 expression, in human mutant SOD1-expressing astrocytes compared to wild-type astrocytes. A NOX2 inhibitor and two antioxidants were shown to reduce ROS levels in mutant astrocytes and NOX2 inhibition in coculture was able to save stem cell-derived human motor neurons. Our own studies have also shown that human NSC-derived motor neurons were susceptible to mutant SOD1-expressing astroglial-mediated toxicity (see Chapter 5). Additionally, primary mutant astroglia isolated from transgenic $SOD1^{G93A}$ rats after disease onset were significantly more toxic

to human NSC-derived motor neurons than mutant astroglia isolated from one month pre-symptomatic rats.

Based on the above studies, one can predict that in clinically relevant studies performed at the time of disease onset, human stem cell-generated motor neurons transplanted into the spinal cords of post-disease onset transgenic animals or ALS patients may encounter much higher levels of astroglia-secreted toxic factors and thus a more hostile environment. This may severely limit the maturation and survival capability of grafted stem cell-derived motor neurons compared to transplantations performed pre-symptomatically. These studies also indicate that stem cells can be utilized to generate an endless supply of motor neurons that can be used to elucidate non-cell autonomous mechanisms of motor neuron death and screen therapeutics rationally designed to protect motor neurons from injury cascades. These data also provide evidence that grafted stem cell-derived motor neurons will be at risk to an ALS microenvironment made hazardous by toxic astroglia. It will be important to identify other astrocyte-secreted lethal factors using these experimental paradigms prior to initiating a motor neuron replacement stem cell therapy, not only to save remaining endogenous motor neurons, but also to protect transplanted motor neurons. Given that stem cells provide a renewable and easily obtainable source of motor neurons, potential combination therapies with stem cell grafts can be rationally designed using *in vitro* coculture paradigms prior to initiating stem cell pre-clinical *in vivo* studies with the ultimate goal of developing a combined therapy to translate into human clinical trials.

1.4.5. INTERACTIONS BETWEEN MICROGLIA AND ASTROGLIA *IN VITRO*

In vitro paradigms using stem cell-derived motor neurons may be designed to test motor neuron death involving interactions between several cell types critical to ALS pathogenesis. Crucial to understanding ALS disease progression is dissecting the relationships between microglia, astroglia and motor neurons. Addition of astrocytes to cocultures between activated primary microglia and motor neurons ameliorated microglia-induced motor neuron death by taking up glutamate and possibly through the secretion of reduced glutathione or neurotrophic factors as astrocyte condition medium also exerted a minimal, albeit not significant, protective effect (Zhao et al., 2004). However, pre-treating astrocytes with hydrogen peroxide prevented their beneficial effect in cocultures with microglia and motor neurons by reducing their capability to take up glutamate. Furthermore, oxidative and excitotoxic insults in primary neuronal-glial cultures caused astrogliosis, a decrease in astroglial EAAT activity and motor neuron loss (Zagami et al., 2009). Increasing astrocytic antioxidant defense mechanisms by increasing glutathione synthesis through overexpression of Nrf2 prevented primary mutant SOD1-expressing astrocytes from developing toxicity to primary motor neurons *in vitro* and increased the lifespan of transgenic mutant $SOD1^{G93A}$ mice (Vargas et al., 2008).

Astrocytic activity is influenced by the pathological milieu in the ALS spinal cord. Increases in inflammatory factors, oxidative stress and glutamate levels may transform astrocytes from neuroprotective to neurotoxic phenotypes. It is possible that overactivated microglia may drive disease progression by modulating the function and activation of astrocytes through the release of inflammatory factors, ROS/RNS and

glutamate. In fact, coculturing primary mutant SOD1-expressing microglia with nontransgenic astroglia one day prior to initiating coculture with human NSC-derived motor neurons caused these previously non-toxic, normal astroglia to adopt ALS-like astroglial toxicity to human NSC-derived motor neurons through secreted toxic factors (see Chapter 5). This adopted neurotoxicity was partially blocked by inhibiting NADPH oxidase or iNOS during the microglia-astroglia coculture and throughout coculture with human motor neurons, which indicates that oxidative damage to astrocytes from overactivated microglia can convert normal astrocytes to lethal cells. Accumulated data from these studies indicate that astroglial functions are sensitive to the pathological microenvironment in the ALS spinal cord. Oxidative damage, either from intracellular sources such as mitochondria or exogenous sources such as microglia, increased glutamate levels and proinflammatory cytokines, may transform astrocytes rendering them dysfunctional and toxic. Thus, exposing transplanted stem cell-derived astrocytes to the ALS spinal cord milieu, especially after disease onset has occurred, may not be very efficacious for long-term treatment as grafted astrocytes may become dysfunctional and toxic in this harsh environment.

1.4.6. SUMMARY

In summary, stem cell-derived motor neurons provide a useful means to test whether ALS microglia or astroglia-mediated oxidative stress, proinflammatory cytokine release, glutamate release, NGF release, etc., is detrimental to motor neuron maturation, function and survival. High throughput screening of potential therapeutics to protect

motor neurons, especially human motor neurons, from injury and death cascades can easily be performed in these *in vitro* coculture systems. Interactions between several neural cell types can also be dissected to determine whether their detrimental impact on stem cell-derived motor neurons is through direct damage such as microglial-mediated oxidative damage and astroglial secretion of toxic factors or indirect effects such as microglial-generated superoxide and nitric oxide induced damage to astrocytes resulting in astrocytic production of toxic factors. The relative importance of blocking each potential injury pathway in motor neurons or overactivation mechanisms of glial cells can be determined and combination therapies inhibiting the multi-factorial pathogenesis of ALS can be developed *in vitro* prior to initiating pre-clinical experiments in animal models. Results from these types of studies have the potential to provide essential insights into ALS glia-mediated toxicity as well as motor neuronal susceptibility and ultimately lead to the development of novel combined therapies with stem cell-derived motor neurons in order to provide motor recovery in ALS patients.

1.5. RATIONALE AND HYPOTHESIS FOR THESIS

1.5.1. RATIONALE

ALS is characterized by the selective degeneration of motor neurons and there are currently no effective treatments. Human NSCs may be used to replace motor neurons and our lab has previously shown that hNSC-derived motor neurons, when grafted into an axotomy model of motor neuron degeneration, innervated peripheral muscles and

improved motor functions. However, hNSC-derived motor neurons showed signs of degeneration and nitroxidative damage when grafted into spinal cords of transgenic ALS rats (see Chapter 4), indicating a harsh microenvironment in the ALS cord may affect the survival and maturation of transplanted hNSC-derived motor neurons. Accumulated evidence strongly suggets that a non-cell autonomous mechanism underlies disease progression in ALS, in which both astroglia and microglia play critical roles in motor neuron loss. However, it is unclear whether host glial cells are toxic to the new motor neuorns derived from grafted hNSCs, whether and to what extent oxidative stress is involved in glia-mediated injury, and whether interactions between microglia and astroglia cause protective or destructive effects on hNSC-derived motor neurons. Outcomes from these studies should provide valuable insights into how ALS non-neuronal cells (microglia or astroglia) affect human NSCs and human motor neurons and should provide directions for the future development of novel combined stem cell therapies for ALS.

1.5.2. HYPOTHESIS

Our overall hypothesis is that overactivated microglia from transgenic ALS rats directly and indirectly reduce the survival of hNSC-derived motor neurons through oxidative stress. This hypothesis was tested in the following specific aims.

1.5.3. SPECIFIC AIMS

Specific Aim 1: To determine whether microglia from transgenic ALS rats directly affect the survival of hNSC-derived motor neurons *in vitro* by generating reactive oxygen/nitrogen species (ROS/RNS) (Chapter 5).

Exp. 1 – To determine whether microglia from transgenic ALS rats are detrimental to hNSC-derived motor neuron survival in direct contact coculture.

Exp. 2 – To determine whether microglia from transgenic ALS rats generate higher quantities of ROS/RNS *in vitro*

Exp. 3 – To determine whether microglia from transgenic ALS rats cause nitroxidative damage to differentiated hNSCs in direct contact coculture

Exp. 4 – To determine whether specific inhibiters/scavengers for ROS/RNS protect hNSC-derived motor neurons from mutant SOD1 micoglia-mediated toxicity in direct contact coculture

Specific Aim 2: To determine whether microglia from transgenic ALS rats indirectly affect the survival of hNSC-derived motor neurons *in vitro* through a noxious interaction with nontransgenic and mutant SOD1-expressing astrocytes (Chapter 5).

Exp. 1 – To determine whether microglia from transgenic ALS rats are detrimental to hNSC-derived motor neuron survival in non-contact coculture

Exp. 2 – To determine whether astrocytes from transgenic ALS rats maintain long-term survival of hNSC-derived motor neurons in non-contact coculture

Exp. 3 – To determine whether microglia from transgenic ALS rats, through the generation of ROS/RNS, alter the capacity of nontransgenic and mutant SOD1-expressing astrocytes to maintain long-term survival of hNSC-derived motor neurons in non-contact coculture

Exp. 4 – To determine whether nontransgenic astrocytes have a greater capacity for reduced glutathione (GSH) secretion than mutant SOD1 astrocytes

Exp. 5 – To determine whether nontransgenic and mutant SOD1 astrocytes, through the secretion of GSH, have the capacity to protect hNSC-derived motor neurons from mutant SOD1 microglia-mediated toxicity

CHAPTER 2:

MATERIALS AND METHODS

2.1. HUMAN NSC CULTURE: Human NSCs, K048, were isolated from the forebrain of an 8-week fetus (Svendsen et al., 1999). Cells were primarily cultured in 75 cm^2 flasks as neurospheres. Growth media contained a basic media consisting of DMEM (high glucose, L-glutamine)/Hams-F12 (3:1) (Invitrogen/GIBCO, Carlsbad, CA), 15 mM HEPES (Sigma, St. Louis, MO), 1.5% D-glucose (Sigma), 67 I.U./ml/67 μg/ml penicillin/streptomycin (Cellgro, Herndon, VA) and 2 mM L-glutamine (Sigma). The basic media was supplemented with N2 (Bottenstein and Sato, 1979), which included 25 μg/ml bovine insulin (Sigma), 100 μg/ml human transferring (Sigma), 100 μM putrescine (Sigma), 20 nM progesterone (Sigma) and 30 nM sodium selenite (Sigma). Growth media was further supplemented with 20 ng/ml recombinant human epidermal growth factor (EGF) (R&D Systems, Minneapolis, MN), 20 ng/ml recombinant human basic fibroblast growth factor (bFGF) (R&D Systems), 5 μg/ml heparin (Sigma) and 10 ng/ml recombinant human leukemia inhibitory factor (LIF) (Chemicon, Temecula, CA). Two-thirds of the medium was changed every 3-4 days. Cells were passaged every 10–11 days through mechanical and enzymatic dissociation in 0.025% trypsin (Sigma) plus 0.6 % D-glucose (Sigma) in calcium- and magnesium-free Dulbecco's phosphate buffered saline (CMF-dPBS) (CellGro, Manassas, VA) for 15 minutes at 37°C according to our previous description (Tarasenko et al., 2004). The reaction was halted with 1.2 mg/ml trypsin

inhibitor (Sigma) diluted in conditioned growth medium. Cells in growth media were incubated at 37°C with 8.5% CO_2 to maintain a pH 7.4–7.5.

2.2. HUMAN NSC PRIMING AND MOTOR NEURON DIFFERENTIATION: Priming was performed in 25 cm^2 flasks that were pre-treated with 0.01% poly-D-lysine (Sigma) in CMF-dPBS for 1 hour at 37°C and then coated with 1 μg/cm^2 laminin (LMN) (GIBCO) in CMF-dPBS overnight at 37°C. Three to four days after passage (passage #15-35), approximately 2-4 million hNSCs in neurospheres were resuspended in 4 ml priming medium and plated in PDL/LMN-coated flasks. Priming media consisted of the basic media and N2 described above, and was supplemented with 10 ng/ml bFGF, 2.5 μg/ml heparin and 1 μg/ml LMN. Cells in priming medium were incubated at 37°C with 8.5% CO_2. After 4 days, 3 ml of priming medium was removed and 4 ml of differentiation medium was added to each flask. Differentiation medium consisted of the basic medium described above supplemented with B27 (20 μl/ml) (GIBCO). Half the B27 medium was changed after 3-4 days. Cells in differentiation medium were incubated at 37°C with 5% CO_2 to maintain a pH 7.4–7.5. After 6-7 days, differentiated hNSCs were dissociated and detached from the flask through mechanical and enzymatic dissociation in 0.025% trypsin plus 0.6 % D-glucose in CMF-dPBS for 10 minutes at 37°C. The reaction was halted with 1.2 mg/ml trypsin inhibitor diluted in conditioned differentiation medium. Cells were counted on a hemocytometer by trypan blue exclusion and resuspended in fresh B27 medium. These differentiated cells were used in all coculture experiments described below.

2.3. TRANSGENIC ALS RAT MODEL: Male hemizygous NTac:SD-TgN(SOD1G93A)L26H rats (Taconic, Hudson, NY), originally developed by Howland *et al.* (Howland et al., 2002), were bred locally by crossing with normal female Wistar rats (Charles River Laboratories, Wilmington, MA). Male $SOD1^{G93A}$ progenies were used for further breeding to maintain the line. Human mutant SOD1-negative littermates were not used as breeders to ensure the consistency of the colony phenotype. The breeding protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at The University of Texas Medical Branch. Rats were housed in a centralized Animal Care Facility with a 12-hr light-dark cycle (light 8AM - 8 PM). Food and water were provided *ad libitum*. $SOD1^{G93A}$ progenies were verified by PCR genotyping. Chromosomal DNAs were extracted from tails of 3-week old pups and subjected to PCR amplification with the following primers: sodi3-f primer (GTGGCATCAGCCCTAATCCA) and sodE4-r primer (CACCAGTGT GCGGCCAATGA).

2.4. HUMAN NSC TRANSPLANTATION: Human NSCs were cultured in growth media and priming media as described above. However, half the priming medium was changed after 2 days. On day 3 in priming medium, hNSCs were transduced by a recombinant adeno-associated viral vector containing the enhanced green fluorescent protein gene, AAVegfp, according to our previous description (Wu et al., 2002; Gao et al., 2005). Cells were then cultured in the differentiation medium after 5-day priming. Two to three days later, cells were dissociated, resuspended in fresh B27 medium containing 3 nM FK506 (Alexis Biochemicals, Plymouth Meeting, PA) and 250 U/ml DNase (Sigma) and stored

on ice during the transplantation process. At 4 months of age, transgenic or nontransgenic rats received stereotactic transplantation of hNSCs into the ventral horns of spinal cords using a 5 μL Hamilton syringe with a 26-gauge needle according to our previously described protocol (Wu et al., 2002; Gao et al., 2005). For experiments performed bilaterally at C4-5 and L4-5, 2 x 10^4 hNSCs were injected per site (4 injection sites). For experiments in which transplantations were performed only at L4-5 bilaterally, 1 x 10^5 hNSCs were injected per site (2 injection sites). Briefly, rats were anesthetized under isofluorane using an E-Z Anesthesia system (Euthanex Corporation). The rats' backs were shaved and sterilized, and incisions were made through the skin and muscle exposing the vertebral columns. Laminectomies were performed specifically at spinal segments C4-5 and L4-5. Injection speed was 0.2 μL/min and controlled by a microinjection unit (David Kopf Instruments). The needle was gradually withdrawn 2 minutes after injection. Rats received intraperitoneal injections of 2-3 ml Lactated Ringer's Solution and were allowed to recover on a heated blanket. Rats were given 60,000 units of penicillin G potassium (Pfizer, New York, NY) intramuscularly and 0.05 mg/kg of Buprenex subcutaneously daily for 3 days post-surgery. All transplanted and control rats were immunosuppressed with NEORAL cyclosporine (Novartis Pharmaceuticals, East Hanover, NJ) at 100 μg/ml in drinking water beginning 3 days prior to transplantation surgery and then throughout the lifespan of the animals. All surgery protocols were approved by IACUC at the University of Texas Medical Branch and performed under aseptic conditions in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

2.5. MCF-10A CULTURE AND TRANSPLANTATION: Briefly, the human mammary epithelial cell line MCF-10A (ATCC, Manassas, VA) was cultured in 10 cm^2 dishes in growth media consisting of DMEM/F12 (1:1), 5% horse serum (Invitrogen), 20 ng/ml EGF, 0.5 μg/ml hydrocortisone (Sigma), 100 ng/ml cholera toxin (Sigma), 10 μg/ml insulin and 1X penicillin/streptomycin. Cells were passaged every 3 days in 0.25% trypsin-EDTA (GIBCO) in CMF-dPBS (1:5 dilution) and plated at a 1:5 ratio in 10 ml growth medium (Debnath et al., 2003). Cells were incubated at 37°C with 5% CO_2. For transplantation, passaged cells were resuspended in fresh B27 medium containing 3 nM FK506 and 250 U/ml DNase and stored on ice during the transplantation process. MCF-10A cells were injected bilaterally at C4-5 and L4-5, 2 x 10^4 cells/site. The procedure was performed exactly as described for hNSC transplantation.

2.6. MOTOR SCORE AND LIFESPAN: Symptomatic disease onset and post-symptomatic disease progression were assessed using a modified 5-point motor score system derived from the original motor score description (Matsumoto et al., 2006). This assay was performed once per week beginning at 70 days of age, then twice per week at 120 days of age until disease onset and finally, once per day until the disease end-stage. Rats were allowed to move freely in an open field and were scored blindly by an observer. A non-parametrical scoring system was used following these criteria: 5, normal movement; 4, loss of some weight bearing in the hindlimbs, or limping or dragging of any limb, but still able to stand on hindlimbs; 3, loss of most weight bearing in hindlimbs, dragging of lower body and inability to stand on hindlimbs; 2, complete loss of weight bearing in hindlimbs accompanied by forelimb weakness, unable to drag the lower body, but

righting reflexes present from both sides; 1, a righting reflex from only one side; 0, absent righting reflexes from both sides within 30 seconds (defined as the end-stage). Symptomatic disease onset was identified when the rats showed a score of 4. Lifespan was determined by the age of the rats at end-stage (score of 0), at which time the rats were euthanized.

2.7. BODYWEIGHT: Rats were weighed once per week beginning at 70 days of age, then twice per week at 120 days of age until symptomatic disease onset (determined by motor score, see above) and finally, once per day until disease end-stage, as defined by a lack of righting reflexes, i.e. inability to right oneself from both sides within 30 seconds.

2.8. WIRE MESH ASCENDING TEST: Rats were tested once per week beginning at 70 days of age, then twice per week beginning at 120 days of age until disease end-stage. This test was conducted according to a previous description with modifications (Tchekalarova et al., 2005). A 10 mm wire mesh (H and W: 45 cm x 15 cm) was placed at an angle of 70° in contact with a cardboard box at the top and the edge of a lab bench at the bottom. Rats were placed on the bottom of the wire mesh and motivated to ascend by placing their littermates in the box at the top. Each rat was pre-trained 3 times per day for 3 consecutive days to adapt to the system. Time to reach the top of the wire mesh was recorded for up to 5 seconds. If a rat was unable to ascend or fell off the wire mesh, a time of 5 seconds was recorded. Three runs were recorded for each rat on each testing day.

2.9. PHOTOBEAM ACTIVITY SYSTEM: Exploratory activity was measured by a photobeam activity system (PAS) once per week beginning at 17 weeks of age. Two activity chambers (40 × 40 × 40 cm) collected movement data analyzed by a PAS (San Diego Instruments, Inc., CA) according to our previous description (Tarasenko et al., 2007). The exploratory activity of each rat was assessed over three consecutive 5-minute intervals (total 900 seconds) by the time of photobeam obstruction in an axis-oriented grid system. There were 16 photobeams on each horizontal axis located 4 cm above the chamber floor. Interruption of these photobeams recorded the distance traveled. Obstruction of another set of photobeams located 12 cm above the chamber floor was recorded as rearing events. Resting time occurred when the rats were stationary for at least 1 second.

2.10. DIAPHRAGM ELECTROMYOGRAPHY: Rats were anesthetized with halothane and a pair of AgCl electromyography (EMG) recording electrodes was inserted into the diaphragm muscle. EMGs were recorded for 5 minutes in each rat and analyzed offline for integrated EMG activity, the peak amplitude of EMGs and the respiration rate. These recordings were performed in the laboratory of Dr. Jin Mo Chung, a collaborator on the UTMB campus and a member of the dissertation committee.

2.11. PRIMARY ASTROCYTE ISOLATION: Primary spinal cord astrocytes were generated from transgenic ALS rats and nontransgenic littermates according to previously described methods (Schwartz and Wilson, 1992; Jordan et al., 2008a) with modifications. Briefly, transgenic ALS rats after disease onset or 1 month of age and nontransgenic age/sex-

matched control rats were euthanized with CO_2. Spinal cords were dissected and immediately placed in CMF-dPBS with 0.2% glucose on ice. The meninges were removed and approximately 1 cm from the lumbar spinal cord was removed. Spinal cord sections were pooled from three rats and the lumbar cords were cut into small pieces using sterile blades. The cord pieces were placed into glia growth medium (GGM) consisting of DMEM/F12 (3:1) supplemented with 10% fetal bovine serum (FBS) (GIBCO), 15 mM HEPES, 1.5% D-glucose and 67 I.U./ml/67 μg/ml penicillin/streptomycin. The cord pieces were then dissociated mechanically using sterile fire-polished glass pipettes followed by repeated trituration gently through 16, 18, and 20 gauge sterile syringes. The dissociated cells with some remaining tissue pieces were plated into two 25 cm^2 flasks and incubated at 37°C with 5% CO_2. Half the GGM was changed every 3 days or when the media turned yellow and cells reached confluence after two weeks. At confluence, the flasks were washed with dPBS containing calcium and magnesium (dPBS). The mixed glial cells were split by adding 2 ml 0.25% trypsin-EDTA (GIBCO) in dPBS (1:4 dilution) for 20 minutes at 37°C according to a previously described method (Saura et al., 2003) with modifications. Many cells remained attached after 20 minutes (presumably microglia), but floating cells were collected and plated in GGM in new flasks at 0.5-1 x 10^6 cells/25 cm^2. Cytosine arabinofuranoside (10 μM) (Sigma) was added to the GGM overnight to inhibit any rapidly dividing cells (presumably fibroblasts). At confluence, cells were split by the same method and used for experiments from passage #2-5. Astrocyte purity was approximately 94% by immunofluorescent analyses of the astroglial marker glial fibrillary acidic protein (GFAP).

2.12. PRIMARY MICROGLIA ISOLATION: Pure microglia populations were isolated from combined whole brain and spinal cord tissue using a density gradient centrifugation method in Percoll solutions (Frank et al., 2006) with slight modifications. Briefly, transgenic ALS rats after disease onset or 1 month of age and nontransgenic age/sex-matched control rats were euthanized with CO_2. Whole brains and spinal cords were dissected and immediately placed in CMF-dPBS with 0.2% glucose on ice. The meninges were removed and tissue was pooled from three rats to increase the total number of cells after isolation. Sterile blades were used to cut the tissue into small pieces. Then, the tissue was transferred to and homogenized in a Ten Broeck Homogenizer. The homogenate was then passed through a 40 μm cell strainer into two 50 ml polystyrene conical tubes (BD Biosciences, San Jose, CA). The homogenate was centrifuged at 1000 x g for 10 minutes at room temperature. The large pellet was resuspended in Percoll (Amersham Biosciences, Uppsala, Sweden) diluted to 70% in CMF-dPBS (4 ml/rat). The resuspension was equally transferred to 15 ml polystyrene centrifuge tubes (4 tubes/rat) (VWR, West Chester, PA), then topped slowly with 2 ml 50% isotonic Percoll and finally 1 ml CMF-dPBS. Tubes were centrifuged at 1,200 x g for 45 minutes. Microglia were collected from the interface between 50% and 70% Percoll. Cells were counted and resuspended in microglial culture medium containing half GGM and half conditioned medium from 3-day post-passage L929 fibroblast cell culture, which was collected and stored at -80°C. L929 fibroblasts (ATCC) secrete macrophage-colony stimulating factor (M-CSF) (Pang et al., 2000), which is a growth factor for microglia (Ponomarev et al., 2005). L929 fibroblasts were cultured in DMEM with 5% FBS. Isolated microglia were immediately plated at 50,000 cells/1.8 cm^2 and the culture medium was changed daily for

three days after isolation. The yield of microglia was approximately 1-3 x 10^5 per rat, with the purity of 98% confirmed by immunofluorescent analyses using the microglial marker Iba1.

2.13. COCULTURES BETWEEN HUMAN NSCS AND GLIA: For all coculture experiments, cell numbers were 150,000 differentiated hNSCs, 50,000 microglia and 100,000 astrocytes. German glass coverslips (Carolina Biological Supply) in 24-well plates were pre-treated with 0.01% poly-D-lysine in CMF-dPBS for 1 hour at 37°C and then coated with 1 μg/cm^2 LMN in CMF-dPBS overnight at 37°C. In experiments with microglia in direct contact coculture with hNSCs, microglia were plated on PDL/LMN-coated coverslips immediately after isolation and cultured for four days to allow for recovery. Microglia were then washed in CMF-dPBS and differentiated hNSCs were plated on top of microglia in 500 μl B27 medium overnight. B27 medium was replaced with 500 μl basic medium supplemented with N2 (N2 medium) containing either vehicle (DMSO or dH_2O), apocynin (Sigma), N6-(1-iminoethyl)-L-lysine dihydrochloride (L-NIL) (Cayman Chemical, Ann Arbor, MI), Urate (Sigma), Carboxy-PTIO (Calbiochem, Gibbstown, NJ) or SOD (Sigma). In experiments with microglia and hNSCs in non-contact coculture, microglia were plated in 0.4 μm polyester membrane transwells (Corning, Corning, NY) and cultured for four days. Differentiated hNSCs were plated on PDL/LMN-coated coverslips in a 24-well plate with 500 μl B27 medium per well overnight. Microglia were washed in CMF-dPBS and cocultures were initiated in 700 μl N2 medium. Non-contact cocultures of hNSCs and astrocytes were performed in the same manner as microglia except astrocytes were plated in 3.0 μm polyester membrane transwells (Corning) and

allowed to recover in GGM overnight prior to coculture in N2 medium. If glutathione depletion was required, astrocytes were washed in CMF-dPBS and incubated in N2 medium with vehicle (DMSO) or ethacrynic acid (Sigma) for 24 hours prior to initiating the coculture. In experiments in which microglia and astrocytes were cocultured in direct contact with each other, microglia were first plated in 0.4 μm transwells or on PDL/LMN-coated coverslips and allowed to recover for four days. Microglia were then washed in CMF-dPBS and astrocytes were plated on top of microglia in GGM overnight. Cells were washed with CMF-dPBS and coculture was initiated in N2 medium. Half the N2 medium with appropriate drug concentrations was changed after 3 days in all experiments. Cells on coverslips were fixed in 4% paraformaldehyde for immunofluorescent analyses after 7 days in coculture.

2.14. IMMUNOFLUORESCENT STAINING: Immunocytofluorescent staining was performed according to our previous descriptions (Tarasenko et al., 2004; Jordan et al., 2008b). Cultured medium was removed, coverslips were washed with CMF-dPBS and cells were fixed for 20 minutes in 4% paraformaldehyde followed by post-fixation in 100% methanol at –20°C for 10 minutes. Coverslips were then washed in Tris-buffered saline (TBS) at room temperature and blocked with 5% normal goat serum/0.25% Triton X-100/0.3% bovine serum albumin (BSA) in TBS for 1 hour at room temperature. Primary antibodies, including monoclonal anti-Hb9 (1:100) (Developmental Studies Hybridoma Bank), rabbit polyclonal anti-microtubule associated protein 2 (MAP2) (1:500) (Chemicon), rabbit polyclonal anti-iNOS (1:1,000) (Chemicon), monoclonal anti-gp91^{phox} (1:1,000) (BD Biosciences), rabbit polyclonal anti-Iba1 (1:500) (Wako,

Richmond, VA), monoclonal anti-CD11b (1:100) (Chemicon) and rabbit polyclonal anti-Fas receptor (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA), were diluted in 0.1% Triton-X-100/0.3 % BSA in TBS and were added overnight at 4°C. Coverslips were washed in TBS and Alexa fluorophore-conjugated secondary antibodies (Molecular Probes, Carlsbad, CA), 568 goat anti-mouse (1:400) and 488 goat anti-rabbit (1:400), were added in 0.1% Triton-X-100/0.3 % BSA in TBS for 2.5 hours at room temperature in the dark. Coverslips were washed with TBS, counterstained with DAPI (1:1,000) and mounted onto slides with Fluoromount-G (Fisher Scientific, Pittsburgh, PA). Images were acquired with a Nikon 80i epifluorescent microscope using NIS-Elements imaging software.

Immunohistofluorescent staining was performed according to our previous descriptions (Wu et al., 2002; Gao et al., 2005). Briefly, rats were anesthetized with pentobarbital and transcardially perfused with 0.1 M PBS followed by 4% paraformaldehyde. Spinal cords were dissected and cord sections were postfixed in 4% paraformaldehyde at 4°C overnight, washed in PBS and infiltrated in 30% sucrose at 4°C for 48 hours. Sections were then embedded in Tissue-Tek® O.C.T. Compound (Sakura), sectioned at 45 μm on a cryostat and stored at -20°C. For immunostaining with choline acetyltransferase (ChAT), glial fibrillary acidic protein (GFAP), Iba1 and nitrotyrosine, sections were washed in PBS and blocked in 5% normal donkey or goat serum-0.3% BSA-0.25% Triton X-100 in PBS for 1 hour at room temperature. For immunostaining with 4-HNE, sections were first exposed to sodium borohydride (50 mM) and MOPS buffer (100 mM), pH 8.0, for 10 minutes prior to serum blocking. Goat polyclonal anti-ChAT (1:100) (Chemicon), rabbit polyclonal anti-GFAP (1:1,000) (Chemicon), rabbit

polyclonal anti-Iba1 (1:500) (Wako), rabbit polyclonal anti-nitrotyrosine (7.5 μg/ml) (Upstate Cell Signaling Solutions, Lake Placid, NY) or rabbit polyclonal anti-4-HNE (1:200) (Calbiochem) primary antibodies were diluted in 0.3% BSA-0.1% Triton-X-100 in PBS and added overnight at 4°C. After washing in PBS, 568 donkey anti-goat (1:400) or 568 goat anti-rabbit (1:400) Alexa fluorophore-conjugated secondary antibodies (Molecular Probes) were added for 2 hours at room temperature in the dark. Slides were washed with PBS, counterstained with DAPI (1:1,000) and glass coverslips were mounted with Fluoromount-G. Images were acquired with a Nikon D-Eclipse C1 Laser Scanning Confocal microscope.

2.15. TUNEL STAINING: Cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature. For TdT-mediated dUTP nick end labeling (TUNEL) staining, the TACS® 2 TdT-Fluor *In Situ* Apoptosis Detection Kit (Trevigen, Gaithersburg, MD) was used according to the manufacturer's instructions. Counterstaining with DAPI (1:1,000) was performed and coverslips were mounted with Fluoromount-G. Images were acquired with a Nikon 80i epifluorescent microscope using NIS-Elements imaging software.

2.16. IMMUNOCYTOCHEMISTRY: Cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature, washed with PBS and post-fixed with 0.3% H_2O_2 in methanol for 30 minutes at room temperature. The Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used for staining of 4-HNE Michael adducts. Cells were blocked in 1.5% normal serum and incubated with primary rabbit polyclonal anti-4-HNE (1:3,000) (Calbiochem) overnight at 4°C. Goat anti-rabbit IgG (1:200) (Vector

Laboratories) was incubated for 30 minutes at room temperature. The avidin-biotin-peroxidase complex was added for 30 minutes at room temperature. Cells were incubated with 0.025% diaminobenzidine tetrahydrochloride (DAB) (Sigma) and 0.01% H_2O_2 for 6 minutes at room temperature. Cells were rinsed with water and dehydrated with 50%, 70%, 95% and 100% ethanol. Coverslips were dipped in xylene and mounted with Permount. Images were taken by Nikon light microscopy.

2.17. WESTERN BLOT ANALYSIS: Western blot analysis was performed as described previously (Tarasenko et al., 2004; Jordan et al., 2008b). Primary glia were washed in CMF-dPBS and lysed with Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA) supplemented with PMSF (Cell Signaling Technology) in Molecular Grade Water (Cellgro). Lysates were collected from 25 cm^2 flasks with a cell scraper and transferred to microcentrifuge tubes. Samples were rocked at 4°C for 1 hour. Centrifugation was done at 14,0000 x g at 4°C for 10 minutes and the supernatant was transferred to new tubes. Protein lysates were quantified with the bicinchoninic acid (BCA) protein quantification kit (Pierce Biotechnology, Rockford, IL) and stored at -80°C. Protein (30 μg) was diluted in NuPAGE® LDS sample buffer and reducing agent (Invitrogen) and heated to 70°C for 10 minutes. Samples were loaded into 4-12% NuPAGE® Novex Bis Tris Gels (Invitrogen) and electrophoresis was performed at 150V for approximately 2 hours. Gels were transferred onto Hybond ECL nitrocellulose membrane (Amersham Biosciences) by electrophoretic transfer at 30V for 1.5 hours at 4°C. Membranes were blocked in 5% nonfat milk in 0.1%TBS-Tween for 1 hour at room temperature. Primary antibodies, including rabbit polyclonal anti-glutamate-cysteine ligase (1:100) (Anaspec, Fremont,

CA), rabbit polyclonal anti-iNOS (1:1,000) (Chemicon) and monoclonal anti-Fas ligand (1:200) (Santa Cruz Biotechnology), were diluted in 5% nonfat milk in 0.1%TBS-Tween and added to the membrane overnight at 4°C. Membranes were washed with 0.1% TBS-Tween and incubated for 1 hour at room temperature with donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:7500) (Amersham Biosciences). Membranes were washed and detection was performed using an ECL Western Blot Detection System (Amersham Biosciences) and ECL hyperfilm (Amersham Biosciences). Membranes were stripped for 30 minutes with Restore Western Blot Stripping Buffer (Pierce Biotechnology) and reprobed for the loading control mouse anti-β-actin (1:25,000) (Sigma) and snake anti-mouse horseradish peroxidase-conjugated secondary antibody (1:7500) by the same protocol.

2.18. SUPEROXIDE ASSAY: Superoxide release from glia was determined by the reduction of the WST-1 reagent (Dojindo Laboratories, Rockville, MD) (Tan and Berridge, 2000; Zhao et al., 2010). WST-1 is a tetrazolium salt that is reduced by superoxide to the colorimetric product, formazan. Addition of SOD inhibits the reaction. Briefly, primary microglia were grown on PDL/LMN-coated coverslips in 24-well plates. Microglia were washed with Hanks' Balanced Salt Solution (HBSS) (Cellgro) and WST-1 (300 μM) and catalase (10 U/ml) (Sigma) in HBSS were added to each well to a final volume of 300 μl. Phorbol myristate acetate (PMA) (800 nM) (Calbiochem) was added to initiate the reaction. After a 2-hour incubation at 37°C, 100 μl aliquots were collected and the absorbance was measured at 450 nm on an ELx800uv Universal Microplate Reader (Biotek Instruments, Inc., Winooski, VT).

2.19. NITRIC OXIDE ASSAY: Total nitric oxide production was determined through the assessment of total nitrate and nitrite in the culture medium using the Nitrate-Nitrite Assay Kit (Cayman Chemical) according to the manufacturer's instructions. Briefly, glia culture medium was collected and nitrate in the medium was converted to nitrite by nitrate reductase. Then, nitrite was reacted with the Griess reagents to produce a purple azo compound in which the absorbance was measured at 540 nm on an ELx800uv Universal Microplate Reader.

2.20. PROSTAGLANDIN D2 ASSAY: Prostaglandin D2 (PGD2) in the culture medium was quantified by an ELISA assay using the Prostaglandin D2 EIA Kit (Cayman Chemical) according to the manufacturer's protocol. Briefly, 100 µl aliquots of conditioned medium were collected from primary glia cultures at indicated time points and stored at -80°C. After thawing, samples were added to the ELISA plate, and N2 medium as well as kit components EIA buffer, PGD2 acetylcholinesterase linked tracer and PGD2 monoclonal antibody were added to appropriate wells. After a 2-hour incubation at room temperature, wells were rinsed with wash buffer and Ellman's Reagent was added to each well. The plate was incubated for 1 hour in the dark on an orbital shaker. The absorbance was measured at 405 nm on an ELx800uv Universal Microplate Reader and PGD2 concentration was calculated according to the manufacturer's instructions.

2.21. GLUTATHIONE ASSAY: The amount of reduced glutathione (GSH) in the culture medium was assayed using the BIOXYTECH® GSH/GSSG-412 kit (Oxis International,

Beverly Hills, CA) according to the manufacturer's protocol with modifications. Briefly, 100 µl aliquots of conditioned medium were collected from primary glia cultures at indicated time points and stored at -80°C. Samples were thawed and diluted (1:2-1:10) in assay buffer provided with the kit. Then, samples were reacted with kit components glutathione reductase, NADPH, and a chromogen. The absorbance was measured at 405 nm on an ELx800uv Universal Microplate Reader. The rate of change in absorbance was determined over a 4-minute period and GSH concentrations were calculated according to the manufacturer's instructions.

2.22. CELL VIABILITY ASSAY: Cell viability was assessed using a commercially available WST-1 kit (Roche). Mitochondrial dehydrogenases in live cells cleave WST-1, a tetrazolium salt, into a colorimetric product, formazan, which was assayed to determine the amount of viable cells. Cocultures were conducted between differentiated hNSCs and microglia as previously described in 96-well plates while maintaining a 3:1 ratio of hNSCs to microglia. After 7 days of coculture, the WST-1 assay was performed according to the manufacturer's instructions using a 1:10 dilution of WST-1 for 1.5 hours. The absorbance of the formazan produced by metabolically active cells in each sample was measured at a wavelength of 450 nm with a 630 nm reference using an ELx800uv Universal Microplate Reader.

2.23. STATISTICS: Statistical analyses were done using GraphPad Prism Version 4 software (GraphPad Software, San Diego, CA). The unpaired Student *t*-test was used when comparing two groups. A one-way ANOVA was used for comparing multiple

groups. Post-hoc Tukey and Dunnett tests were used on some sets of data. The repeated measures mixed model ANOVA was utilized when comparing behavior data between groups. *A p* value less than 0.05 was considered statistically significant. All data were expressed as means ± S.E.M. Lifespan and disease onset data were depicted using Kaplan-Meier survival curves.

CHAPTER 3:

IDENTIFICATION OF EARLY DISEASE PROGRESSION IN AN ALS RAT MODEL

3.1. ABSTRACT

Transgenic rat models of amyotrophic lateral sclerosis (ALS) have recently been developed. Most assays of ALS-symptoms in these models monitor disease onset accurately, but do not identify individuals that will develop these symptoms before the motor deficits become apparent. Peak bodyweight has recently been shown to indicate affected individuals before motor deficits become apparent. However, it must be determined retrospectively due to weight fluctuation. Here, we report that exploratory activities detected by a Photobeam Activity System (PAS) and wire mesh ascending test can be used to detect slight motor deficits in the early phase of ALS. Thus, these tests may be used in addition to peak bodyweight to monitor early disease progression and to assay efficacy of new therapeutic interventions.

3.2. INTRODUCTION

Amyotrophic lateral sclerosis (ALS) or Lou Gehrig's disease is a devastating neurological disease characterized by selective degeneration of motor neurons. Approximately 10% of patients inherit the disease (familial or FALS), but the rest are sporadic (SALS) with no known genetic link (Bruijn et al., 2004). Most ALS patients develop limb weakness initially, which progresses quickly to generalized muscle atrophy and paralysis. Death ultimately occurs within 5 years due to respiratory paralysis.

In 1993, a mutated gene coding for copper/zinc superoxide dismutase 1 (Cu/Zn-SOD1) was first associated with ALS, marking a major breakthrough in our understanding of ALS pathogenesis. It is now known that approximately 20-25% of the familial cases or 1-2% of total ALS cases are due to dominantly inherited mutations in Cu/Zn-SOD1 (Rosen et al., 1993; Gaudette et al., 2000). Due to the clinical and pathological similarities to human FALS and SALS, our understanding of ALS mechanisms has been enhanced by studies using hemizygous transgenic rodent animal models that express mutant human Cu/Zn-SOD1 genes (Gurney et al., 1994; Nagai et al., 2001; Howland et al., 2002).

Behavioral analyses have previously been used to evaluate potential therapies for transgenic ALS mice or rats. RotaRod tests were the most frequently used method to evaluate motor performance after ALS therapies in mice (Klivenyi et al., 1999; Andreassen et al., 2000; Van Den et al., 2002; Zhu et al., 2002; Wang et al., 2002; Chiba et al., 2006; Kiaei et al., 2006). Other assays included grip strength and wire hanging tests (Azari et al., 2003; Guo et al., 2003; Miana-Mena et al., 2005). Also, an automated

motion analyses system (SCANET) and inclined plane test have been used to evaluate disease progression in ALS rats (Matsumoto et al., 2006). Unfortunately, these tests in rats only identified individuals at or near symptomatic disease onset (obvious limb weakness defined as limping or paralysis), but failed to detect slight motor deficits during the early disease stage.

Thus, it was a break-through when Matsumoto *et al.* showed that peak bodyweight was a reliable index of "pre-symptomatic" disease onset, identifying individuals approximately 13 days prior to symptomatic onset (Matsumoto et al., 2006). However, due to fluctuations in weight, bodyweight peak must be determined retrospectively (Matsumoto et al., 2006). Thus, other assays to identify affected individuals before apparent symptoms develop would be desirable. Here, we report that measurements of exploratory activities in a Photobeam Activity System (PAS) improve early diagnosis by identifying early motor deficits. In addition, a simple wire mesh ascending test monitors both early and late disease progression.

3.3. MATERIALS AND METHODS

Please refer to CHAPTER 2 for the materials and methods used in this chapter.

3.4. RESULTS

3.4.1. THE WIRE MESH ASCENDING TEST DETECTS EARLY LOSSES IN MUSCLE STRENGTH AND LIMB COORDINATION IN TRANSGENIC ALS RATS

Bodyweight measurements monitor the extent of muscle wasting in ALS rats. The bodyweights of ALS rats peaked at 143.5 ± 12.9 days (Table 3.1) and began to decrease approximately 24 days prior to the average age of symptomatic disease onset (Fig. 3.1A). The wire mesh ascending test monitors muscle strength and limb coordination, both of which are affected in ALS. Transgenic ALS rats significantly increased the time spent climbing a distance of 45 cm as early as 145 days of age compared to their wild-type littermates (Fig. 3.1B). Note that a dramatic increase of ascending time occurred close to the average age of symptomatic disease onset.

Table 3.1. Summary of methods for identifying pre-symptomatic disease onset, symptomatic disease onset and lifespan.

Evaluation methods	Age in days (range)
Pre-symptomatic disease onset	
Peak bodyweight[a]	143.5 ± 12.9 (114-163)
Distance traveled in PAS[b]	151.3 ± 19.0 (118-176)
Ascending time[c]	159.6 ± 13.3 (138-173)
Symptomatic disease onset	
Observable motor deficits[d]	167.6 ± 15.8 (138-187)
Rearing events in PAS[e]	165.0 ± 15.5 (138-186)
Resting time in PAS[f]	174.3 ± 12.5 (156-191)
End-stage disease	
Lifespan[g]	188.7 ± 15.2 (165-207)

Mean ± SD. ALS transgenic rats (n=10). Male:female ratio is 1:1.
[a] Max weight prior to a constant decline
[b] Total distance traveled ≤ 6500 cm
[c] Average climbing time ≥ 1.8 sec.
[d] Motor score of 4
[e] Total rearing events ≤ 60 events
[f] Total resting time ≥ 450 sec.
[g] Motor score of 0

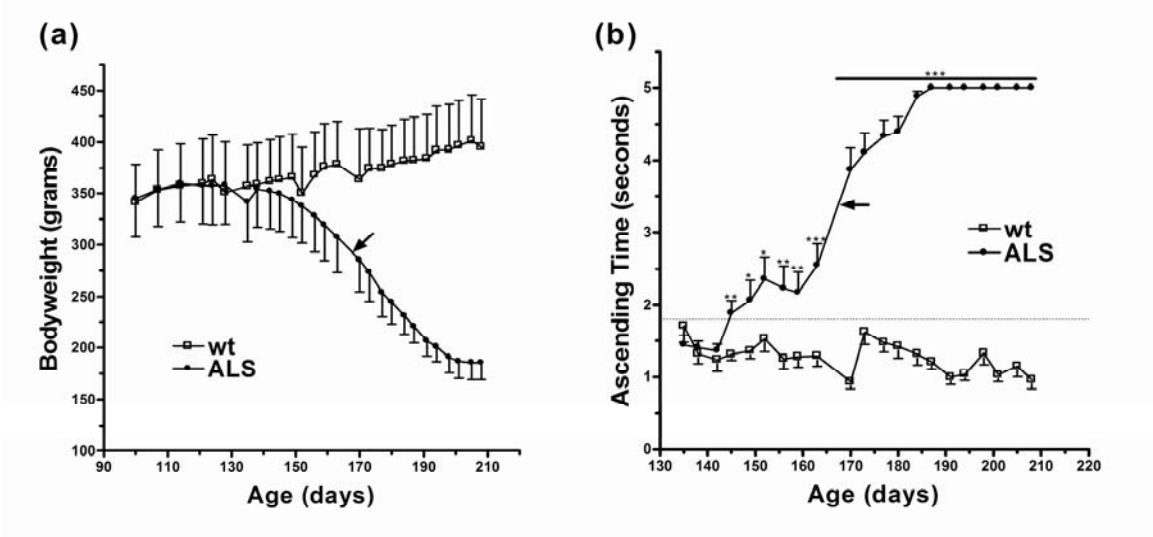

Fig. 3.1. Bodyweight measurements and time recordings on the wire mesh ascending test monitor pre-symptomatic disease progression.

Bodyweight measurements (a) and wire mesh ascending test time recordings (b). wt, wild-type littermates (n=6). ALS, transgenic ALS rats (n=10). Male:female ratio 1:1 in both groups. Black arrows represent the average age of symptomatic disease onset (167.6 days). Dot line depicts the first value in ALS rats that is significantly different from wild-type littermates. *$p < 0.05$. ** $p < 0.01$. ***$p < 0.001$.

3.4.2. A PHOTOBEAM ACTIVITY SYSTEM DETECTS EARLY DEFICITS IN MOTOR ACTIVITY IN TRANSGENIC ALS RATS

ALS pathogenesis causes a rapid decline in motor strength and activity. Several key parameters of motor function, including walking distance, rearing capability and resting time were evaluated using a Photobeam Activity System (PAS). The total distance traveled in the PAS showed a consistent and significant decrease in the transgenic ALS group compared to the wild-type littermates at 21 weeks of age (Fig. 3.2A). The number of rearing events significantly declined in consecutive weeks beginning at 23 weeks of age (Fig. 3.2B). However, the resting time did not significantly increase until 24 weeks of

age (Fig. 3.2C), which was near symptomatic disease onset determined by motor scoring. All three parameters showed a trend of pre-symptomatic disease progression, but only walking distance and rearing capability significantly declined prior to symptomatic disease onset (Table 3.1).

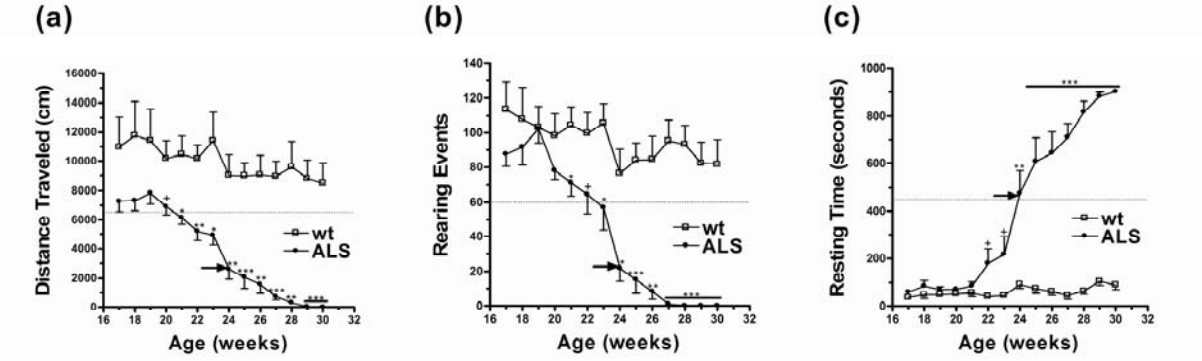

Fig. 3.2. Distance traveled and rearing capability, but not resting time, as measured in a Photobeam Activity System (PAS) identify pre-symptomatic motor deficits.

Total distance traveled (a), total number of rearing events (b) and total resting time (c) as determined over a 15-min time period in a PAS. wt, wild-type littermates (n=6). ALS, transgenic ALS rats (n=10). Male: female ratio 1:1 in both groups. Black arrows represent the average age of symptomatic disease onset (167.6 days). Dot line depicts the first value in ALS rats that is significantly different from wild-type littermates. *$p < 0.05$. ** $p < 0.01$. ***$p < 0.001$. $^{+}0.05 < p < 0.06$.

3.4.3. A MODIFIED MOTOR SCORE DETERMINES DISEASE ONSET WHEN VISIBLE SYMPTOMS ARISE

Symptomatic disease onset was subjectively determined through motor score assessment. The day of symptomatic disease onset was defined as the day when the motor score of a rat decreased from 5 to 4. Post-symptomatic disease progression was

subsequently evaluated through further assessments of motor score until each rat reached a motor score of 0. The day when the motor score reached 0 was considered the 'end-stage' of the lifespan for each rat. When averaging motor scores from all ALS rats on each assessment day, ALS rats first showed a significant decrease in motor score at 170 days of age, which was comparable to the average age of symptomatic disease onset (Fig. 3.3A). The Kaplan-Meier survival curves for symptomatic disease onset (DO) and end-stage (ES) are depicted in Fig. 3.3B. The interval between DO and ES was about 21 days (Table 3.1).

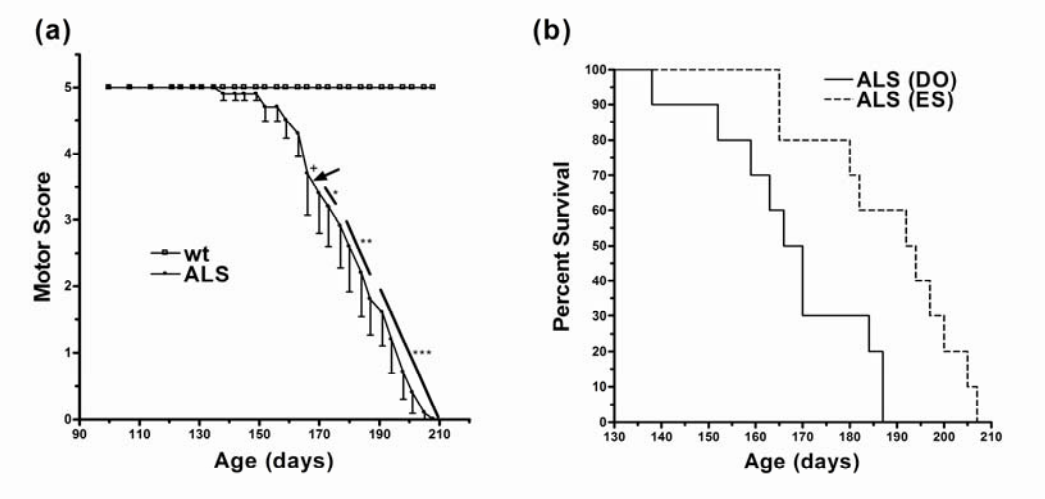

Fig. 3.3. Transgenic ALS rats show a delayed symptomatic disease onset and lifespan compared to previous reports.

Post-symptomatic disease progression based on a modified 5-point motor score (a). Kaplan-Meier survival curves depict the symptomatic disease onset (DO) and the end-stage (ES) (b). wt, wild-type littermates (n=6). ALS, transgenic ALS rats (n=10). Male: female ratio is 1:1 in both groups. Black arrow represents the average age of symptomatic disease onset (167.6 days). *$p < 0.05$. ** $p < 0.01$. ***$p < 0.001$. $^{+}p = 0.0703$.

3.5. DISCUSSION

The major thesis of the present study is that the earlier one can identify individuals that will develop ALS or ALS-like symptoms, the more effective therapy is likely to be. Accordingly, early diagnosis, particularly before substantial motor deficits can be recognized, is a matter of considerable importance. Most previously used tests, such as the motor score test used in this study, do an excellent job of monitoring the disease process, but they only indicate ALS-like individuals after motor symptoms become apparent (limping and paralysis). A real advance has been made recently in demonstrating that the peak weight of ALS rats identified individuals that will develop motor deficits with precision well before the onset of observable symptoms (Matsumoto et al., 2006). The difficulty with this assay is that rat weights vary enough that one cannot be sure when the peak is reached until a marked weight loss is seen, which is later than the time of peak weight. Therefore, further tests to identify individuals that will develop motor deficits are desirable.

In the present study, we tested the ability of a PAS and wire mesh ascending test to detect subtle motor abnormalities in transgenic ALS rats. These tests, in combination with bodyweight measurements, allowed us to objectively monitor pre-symptomatic disease onset more precisely than the use of peak bodyweight alone. Accordingly, the above tests should be combined with determinations of peak weight to obtain the most objective and earliest assessment of the onset of symptoms in ALS rats.

In more detail, the wire mesh ascending test first detected a significant difference between the transgenic ALS rats and their wild-type littermates at 145 days of age. Using

the threshold cutoff value of a 1.8-sec climbing time, the average age of the ALS rats was approximately 160 days. Thus, this test could detect motor deficits about 1 week prior to symptomatic disease onset (Table 3.1). Furthermore, the day each rat recorded an average climbing time of 3 seconds or more correlated well (r^2 = 0.8490) with the day of symptomatic disease onset. Better correlative values may have been obtained if this test was performed on a daily basis rather than twice per week. The conclusion is that the wire mesh ascending test was an adequate behavioral test to identify subtle pre-symptomatic motor deficits.

All three PAS parameters also indicated onset and monitored disease progression to detect signs of motor weakness too subtle to be visually observed in ALS rats, but total distance traveled was the best indicator of pre-symptomatic motor abnormalities. Using a threshold value of 6500 cm walking distance, this test detected motor abnormalities approximately 16 days prior to symptomatic disease onset. Rearing capability also dramatically decreased in the ALS rats. However, when a threshold of 60 events was used, rearing deficits were only detected 2-3 days prior to symptomatic disease onset. Similarly, total resting time drastically increased in the transgenic ALS rats, but resting time did not identify a pre-symptomatic deficit in motor activity using a cutoff value of 450 seconds. It is likely, however, that increasing the number of rats in the study would increase the capability of these three parameters to detect pre-symptomatic motor abnormalities.

In order to minimize litter and gender variations, the wild-type and transgenic groups each consisted of an assortment of rats from five and six litters, respectively, in a 1:1 ratio of males to females. However, a careful examination of potential effects of

gender matching and littermate pairing on wire mesh ascending time and PAS activity remains to be conducted in future studies with larger sample sizes. Furthermore, due to the large variation in disease progression in the ALS rodent model, trial experiments and a subsequent power analysis must be performed in order to determine the number of transgenic rats required to distinguish a significant treatment effect between groups when evaluating ascending time and PAS activities.

Finally, the symptomatic disease onset (168 days) and lifespan (189 days) in our colony as determined by a modified motor score (Table 3.1) were considerably delayed compared to what were earlier described, 115 days for disease onset and 126 days for lifespan, in the original transgenic line (Howland et al., 2002). The cause of these differences remains to be determined, but may indicate a decrease in mutant SOD1 expression in our colony or may be due to large variations in disease onset that characterize this model (Storkebaum et al., 2005).

In summary, we discovered that the exploratory activity measured in a PAS and the climbing time measured by a wire mesh ascending test, in combination with bodyweight measurements, can be used to better define early signs of disease progression in transgenic ALS rats. This is particularly useful in order to properly evaluate how potential therapies such as cell transplantation, intrathecal drug administration or gene therapy act to delay ALS disease progression.

CHAPTER 4:

TRANSPLANTED HUMAN NEURAL STEM CELL-DERIVED MOTOR NEURONS MARGINALLY DELAY DISEASE PROGRESSION AND SHOW SIGNS OF DEGENERATION IN AN ALS RAT MODEL

4.1. ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a devastating neurological disease resulting from selective degeneration of spinal and upper motor neurons. One approach in the development of therapies for ALS is to explore the potential of human neural stem cells (hNSCs) to replace lost motor neurons. The therapeutic efficacy of stem cell transplantation would depend greatly on the survival of grafted stem cell-derived motor neurons in the spinal cord in ALS. Here, we report that transplantation of hNSCs into the spinal cords of transgenic ALS rats slightly delays disease progression. Morphological analysis of the transplantation sites reveals that the grafted hNSCs differentiate into motor neurons, but are degenerated and show signs of nitroxidative damage at the disease end-stage. This study provides the first evidence that new stem cell-derived human motor neurons, like endogenous motor neurons, are susceptible to the harsh microenvironment that ensues within the spinal cord in ALS.

4.2. INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most common adult motor neuron disease and 1-2% of patients have familial ALS due to mutations in superoxide dismutase 1 (SOD1) (Bruijn et al., 2004). Current evidence, accumulated from transgenic rodent models expressing human mutant SOD1, indicates that mutant SOD1 expression within motor neurons determines the rate of disease onset (Boillee et al., 2006a; Boillee et al., 2006b; Yamanaka et al., 2008a). Disease progression after onset, on the other hand, is hastened by the activation and dysfunction of surrounding mutant SOD1-expressing microglia and astrocytes (Clement et al., 2003; Yamanaka and Cleveland, 2005; Beers et al., 2006; Boillee et al., 2006b; Yamanaka et al., 2008b). Microgliosis and astrogliosis in areas of motor neuron degeneration are pathological hallmarks of ALS (Kawamata et al., 1992; Schiffer et al., 1996). The overproduction of oxidative stress beyond endogenous antioxidant capabilities is also observed in ALS. Evidence of oxidative damage, including increased protein carbonylation (Shaw et al., 1995), protein nitration (Beal et al., 1997), 1997) and lipid peroxidation (Smith et al., 1998) has been described in both ALS patients and transgenic ALS animal models.

Due to the current lack of diagnostic biomarkers, ALS is often diagnosed very late in the disease course after numerous motor neurons have already been lost. Thus, in order to reinnervate muscle tissue and repair muscle function, motor neurons will need to be replaced. Many stem cell therapies have previously been tested in transgenic ALS rodent models (Nayak et al., 2006; Hedlund et al., 2007; Thonhoff et al., 2009). However, the slight benefit in some pre-clinical studies was most likely due to the neuroprotection of

endogenous motor neurons rather than motor neuron replacement. Advances in techniques for motor neuron differentiation from stem cells (Thonhoff et al., 2009) allows specifically for the exploration of motor neuron cell-replacement therapy in transgenic ALS models. Further, several recent studies have shown that stem cell-derived motor neurons are susceptible to toxicity in the neural microenvironment of transgenic ALS animals through organotypic slice cultures and cocultures with primary astrocytes (Kim et al., 2006; Di Giorgio et al., 2007; Nagai et al., 2007; Di Giorgio et al., 2008; Hedlund and Isacson, 2008; Marchetto et al., 2008). Thus, the hostile spinal microenvironment in ALS may be detrimental to the survival and maturation of transplanted stem cell-derived motor neurons.

Here, we sought to determine whether human neural stem cells (hNSCs) transplanted into the spinal cords of transgenic ALS rats would be efficacious in slowing the disease progression as well as whether the grafted cells could differentiate into motor neurons and survive within the harsh spinal microenvironment *in vivo*.

4.3. MATERIALS AND METHODS

Please refer to CHAPTER 2 for the materials and methods used in this chapter.

4.4. RESULTS

4.4.1. TRANSPLANTATION OF HNSCS PROLONGS LIFESPAN IN TRANSGENIC ALS RATS

In this context, "ALS" rats refer to those expressing transgenic mutant SOD1 and "normal" refers to nontransgenic matches. In order to determine whether hNSCs could extend survival in ALS rats, primed hNSCs were grafted bilaterally into strategic segments of the spinal cord: C4-5 (for diaphragm and forelimbs) and L4-5 (for hindlimbs) at age 4 months. The age at transplantation was prior to disease onset, but near an early disease stage characterized by motor weakness and weight loss as previously described (Thonhoff et al., 2007). The time point was chosen not only for its clinical relevance, but also for evaluating the potential for reinnervation of muscle targets, since it takes approximately 3 months for axons of grafted hNSC-derived motor neurons to reach the target gastrocnemius muscle in adult rats as we reported previously (Gao et al., 2005; Gao et al., 2007). Controls included ALS rats receiving no treatment or a transplant with MCF-10A cells, which is a mammary gland epithelial cell line, and normal rats. Although there was a trend that hNSC transplants delayed disease onset (ALS, 167 days; hNSCs, 175 days; MCF, 165 days), the delay was not significant (Fig. 4.1A, C). Lifespan was significantly prolonged in animals receiving hNSC transplants compared to ALS rats receiving no treatment (ALS, 187 days; hNSCs, 204 days; MCF, 188 days) (Fig. 4.1B, D). The time between disease onset and disease end-stage was also significantly prolonged by the hNSC transplants (ALS, 20 days; hNSCs, 29 days; MCF, 23 days) (Fig. 4.1E).

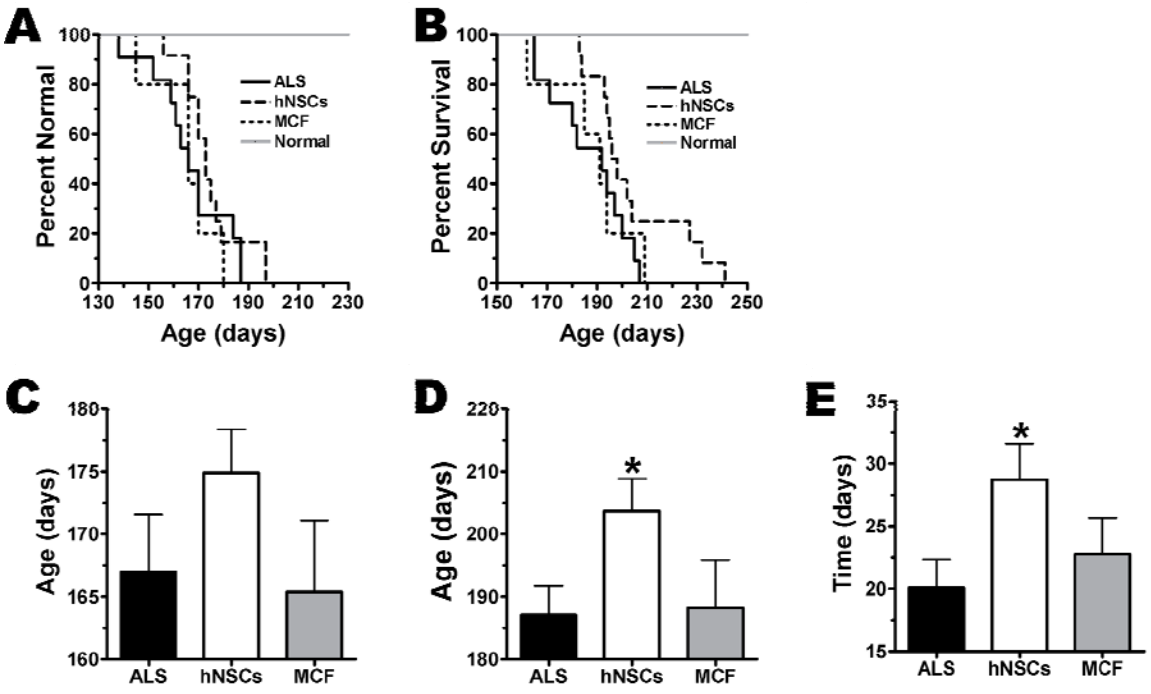

Fig. 4.1. Human neural stem cell (hNSC) transplants prolong lifespan in transgenic ALS rats.

A, Percent of group that has not reached disease onset (motor score of 4). **B**, Percent of group that has not reached disease end-stage (motor score of 0). ***C***, Average age at disease onset. **D**, Average age at disease end-stage. **E**, Average time between disease onset and disease end-stage. **A**-**E**, ALS rats receiving no treatment (ALS, n=11); ALS rats receiving hNSC transplants (hNSCs, n=12); ALS rats receiving control transplants, a human mammary epithelial cell line MCF-10A (MCF, n=5); and nontransgenic littermates (normal, n=6). Data expressed as mean ± SEM. *p<0.05, significantly different from ALS group.

4.4.2. TRANSPLANTATION OF HNSCS DELAYS DISEASE PROGRESSION PARAMETERS IN TRANSGENIC ALS RATS

The efficacy of hNSC transplantation was further determined by its effect in slowing the disease progression in the ALS animal model. Disease progression was characterized through analyses of motor score, bodyweight, performance on a wire mesh ascending test and exploratory activity in a photobeam activity system. Transplantation of hNSCs significantly delayed the decrease in motor score (Fig. 4.2A), the loss of bodyweight (Fig. 4.2B) and the decrease in performance on the wire mesh ascending test (Fig. 4.2C) compared to ALS rats receiving no treatment. Furthermore, hNSC grafts also significantly delayed the decrease in total distance traveled (Fig. 4.2D), the decrease in rearing capability (Fig. 4.2E) and the increase in total resting time (Fig. 4.2F) in a photobeam activity system. Interestingly, in addition to bodyweight, the performance on the wire mesh ascending test as well as all three parameters tested in the photobeam activity system declined prior to the visible observation of motor deficits (motor score of 4). This decline may represent a sign of early disease progression and subtle weakness in the ALS rats (Thonhoff et al., 2007). The rats in each group were also subjected to diaphragm electromyography (EMG) recordings at approximately 4 months of age (pre-symptiomatic stage) and 5.5 months of age (average disease onset). Protection of diaphragm muscle function, in regards to EMG amplitude (Fig. 4.2G) and frequency (data not shown), was not observed in transgenic ALS rats receiving hNSC transplants. However, the average amplitude of diaphragm muscle contractions also significantly declined during the second recording in nontransgenic littermates, indicating that the recording electrode may have injured the diaphragm muscle.

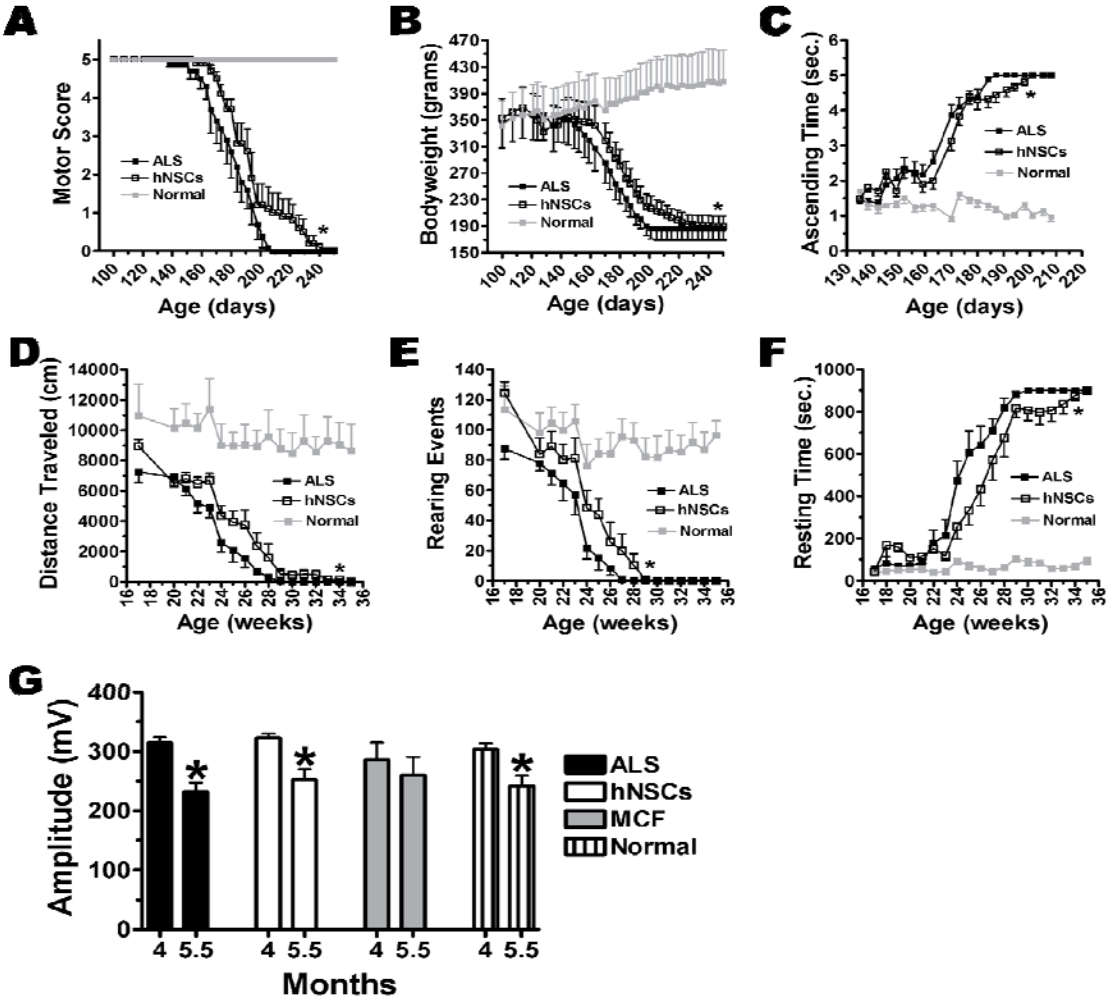

Fig. 4.2. Human neural stem cell (hNSC) transplants delay disease progression in transgenic ALS rats.

A, Disease progression assessed by motor score. **B**, Disease progression monitored through bodyweight measurements. **C**, Amount of time to complete the wire mesh ascending test. **D**, Total distance traveled in a photobeam activity system (PAS) within 15 minutes. **E**, Total number of rearing events in a PAS. **F**, Amount of time spent stationary in a PAS. **G**, Electromyography recordings showing the average amplitude of diaphragm muscle contractions at a pre-symptomatic age (4 months) and the average age of disease onset (5.5 months). **A-G**, ALS rats receiving no treatment (ALS, n=10); ALS rats receiving hNSC transplants (hNSCs, n=10); ALS rats receiving control transplants, a human mammary epithelial cell line MCF-10A (MCF, n=6); and nontransgenic littermates (normal, n=6). Data expressed as mean ± SEM. *$p<0.05$, significantly different from ALS group or **G**, significantly different within respective group.

4.4.3. TRANSPLANTED HNSCS DIFFERENTIATE INTO CHOLINERGIC CELLS AND DEGENERATE IN THE ALS CORD

Morphological analysis was performed at the lumbar spinal cord grafting sites in order to determine the fate of transplanted hNSCs. Three ALS rats received hNSC transplants at L4-5 unilaterally at age 4 months and were euthanized at the disease end-stage. Normal rats were simultaneously transplanted with hNSCs and sacrificed accordingly. Grafted GFP^+-hNSCs expressed choline acetyltransferase (ChAT), which indicated that hNSCs may become motor neurons (Fig. 4.3A, B). The $GFP^+/ChAT^+$ cells found in the spinal cord of ALS rats at the disease end-stage exhibited a small, degenerated morphology (Fig. 4.3B) compared to those observed in the normal spinal cord (Fig. 4.3A). $GFP^+/ChAT^+$ cells found in the ALS spinal cords exhibited a significantly smaller average maximum soma diameter (33 % decrease) than those grafted into the normal spinal cords (Fig. 4.3C). Furthermore, although grafted $GFP^+/ChAT^+$ cells showed a degenerated morphology in ALS spinal cords, some GFP^+ fibers had extended into the ventral white matter (Fig. 4.3D) and down the L5 ventral root (Fig. 4.3E), which indicated that hNSC-derived motor neurons have the potential to reinnervate distal muscle fibers in ALS rats.

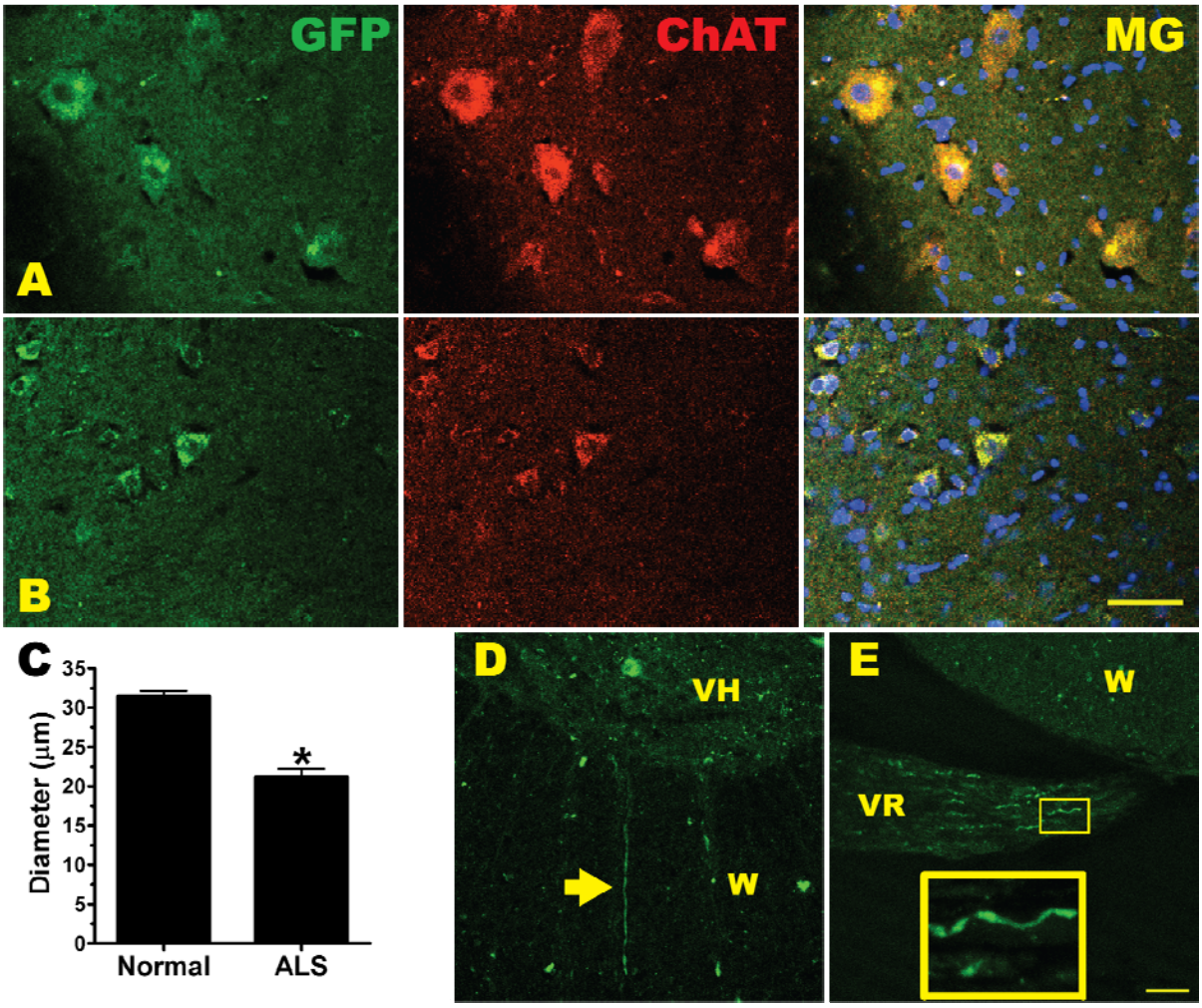

Fig. 4.3. Human NSCs degenerate in the spinal cord of ALS rats at the disease end-stage.

A, Normal spinal cord, hNSCs (labeled with green fluorescent protein, GFP) express choline acetyltransferase (ChAT) and merged image with DAPI shown in blue (MG). **B**, ALS spinal cord, hNSCs (GFP) express ChAT. **C**, Average maximum soma diameter of $GFP^+/ChAT^+$ derived from hNSCs in either normal or ALS spinal cords, n=3 rats/group. Data expressed as mean ± SEM. *$p<0.05$, significantly different from normal group. **D**, GFP^+ fiber (arrow) exiting ventral horn (VH) into the white matter (W) in ALS spinal cord. **E**, GFP^+ fibers, with magnified section (yellow box), coursing through the L5 ventral root (VR) from ALS spinal cord. **A-B** and **D-E**, scale bars = 50 μm.

4.4.4. TRANSPLANTED HNSCS UNDERGO NITROXIDATIVE DAMAGE IN THE ALS CORD

Human NSCs were transplanted into the L4-5 segment at 4 months of age. Animals were sacrificed at disease onset. Microgliosis, indicated by Iba1 (pan-microglia marker) (Fig. 4.4A, B), and astrogliosis, shown by intense GFAP immunoreactivity (Fig. 4.4C, D), were apparent at the grafting sites within the ventral horns in transgenic ALS rat spinal cords (Fig. 4.4A, C), but not nontransgenic cords (Fig. 4.4B, D). Microgliosis and astrogliosis in ALS cords were accompanied by increased 3-nitrotyrosine (NT) (Fig. 4.4E), a marker of protein nitration, and the 4-hyroxynonenal adduct (4-HNE) (Fig. 4.4G), a marker of lipid peroxidation, compared to those in nontransgenic control cords (Fig. 4.4F, H). In correlation with these results, most grafted GFP^+ hNSCs that were degenerated had incurred nitroxidative damage by the disease end-stage as shown by co-immunolabeling with NT (Fig. 4.4I) and 4-HNE (Fig. 4.4J).

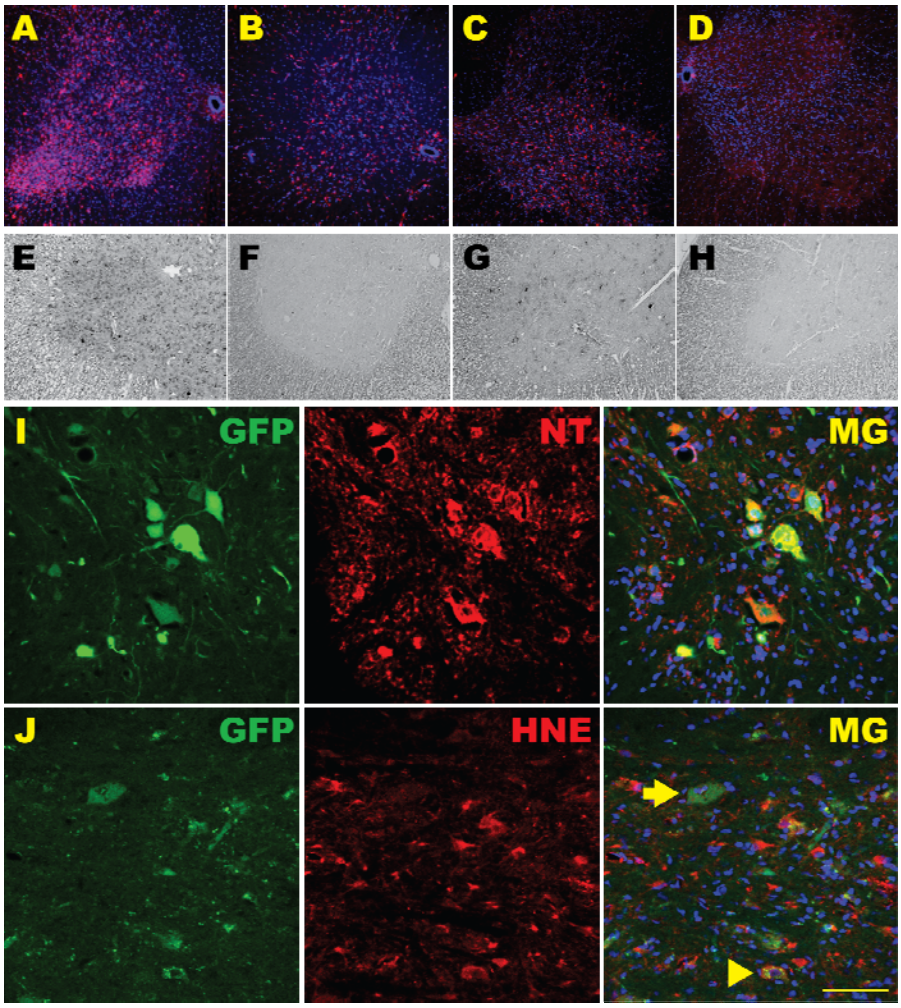

Fig. 4.4. Degenerated hNSCs exhibit nitroxidative damage in the spinal cord of ALS rats at the disease end-stage.

A, ALS spinal cord, microgliosis (Iba1, pan-microglia marker, red) in ventral horn at disease onset. **B**, Normal spinal cord, resident microglia (Iba1, red) in the ventral horn at the same age. **C**, ALS spinal cord, astrogliosis (intense GFAP, astroglia marker, red) in ventral horn at disease onset. **D**, Normal spinal cord, astrocytes (GFAP, red) in the ventral horn. **E**, ALS spinal cord, increased protein nitration shown by nitrotyrosine staining (black) in ventral horn at disease onset. **F**, Normal spinal cord, no observable protein nitration. **G**, ALS spinal cord, increased lipid peroxidation shown by 4-hydroxynonenal staining (black) in ventral horn at disease onset. **H**, Normal spinal cord, no observable lipid peroxidation. **I**, ALS spinal cord, hNSCs (GFP^+) exhibit nitrotyrosine (NT) immuno-labeling. **J**, ALS spinal cord, hNSCs (GFP^+) exhibit 4-hydroxynonenal (4-HNE) immuno-labeling (arrowhead). A few hNSCs shows no 4-HNE damage (arrow). **I-J**, scale bar = 50 μm.

4.5. DISCUSSION

The present study examined whether treatment with primed hNSCs would be efficacious in transgenic ALS rats through replacing lost endogenous motor neurons with human stem cell-derived motor neurons. We report that transplantation of hNSCs in transgenic ALS rats slightly delays disease progression and prolongs lifespan, but most likely not through replacement of motor neurons. Although we provide the first evidence that stem cells can produce human cholinergic neurons in the transgenic ALS spinal cord and emit processes that course through the ventral root, we also show that the microenvironment in the cord is unfavorable for human motor neuron maturation possibly through increased nitroxidative damage.

Due to the lack of effective treatments for ALS, a fatal disease, stem cells have become the arising hope to replace lost motor neurons. Several stem cell transplants from different sources have been tested in transgenic ALS animal models (Thonhoff et al., 2009), but only a few have produced cholinergic cells (Corti et al., 2007; Martin and Liu, 2007; Lopez-Gonzalez et al., 2009). Hence, the benefits observed in many of these stem cell therapies are thought to be due to neuroprotection of endogenous motor neurons rather than motor neuron replacement. We have previously shown that primed hNSCs differentiate into motor neurons *in vitro* and *in vivo* (Wu et al., 2002; Jordan et al., 2008b). When grafted into spinal cords, hNSC-derived motor neurons survive at least three months post-transplantation, emit processes that reach the gastrocnemius muscle and improve motor function in rats with axotomy-induced motor neuron deficit (Gao et al., 2005; Gao et al., 2007). In contrast to the long-term survival and function of human motor neurons in the axotomized rats, however, hNSC-derived motor neurons are degenerating in the ALS rat spinal cord, approximately two months after grafting.

Therefore, the benefits observed on disease progression after hNSC transplantation were possibly attributed to either 1) a neurotrophic effect on endogenous motor neurons through the secretion of glial cell line-derived neurotrophic factor (GDNF) (Xu et al., 2006), which is known to be secreted in high quantities from primed hNSCs (Gao et al., 2006) or 2) a decrease in relative mutant SOD1 expression within the ALS rats that received hNSCs and displayed an overall significant delay in disease progression.

In accordance with our data, an earlier study found that grafted mouse olfactory bulb-neural precursor cells differentiated into $ChAT^+$ cells and extended processes into the sciatic nerve in transgenic ALS mice, but processes did not reach distal muscle targets and a morphology indicating degenerating distal axons was observed (Martin and Liu, 2007). In addition, mouse embryonic stem cell-derived motor neurons did not survive long-term in ALS rat spinal cords (Lopez-Gonzalez et al., 2009). Collectively, these results indicate that stem cell-derived motor neurons, like endogenous motor neurons, are susceptible to a hostile microenvironment that ensues in the spinal cord in ALS. In these studies, stem cell-derived motor neurons were transplanted prior to disease onset in which differentiation and maturation occurred before drastic motor neuron toxicity developed. Thus, it is still questionable whether stem cell transplants could yield motor neurons when injected after disease onset, when many motor neurons have already been lost, as would be indicated in ALS patients.

In conclusion, the microenvironment after disease onset within degenerating areas in the transgenic ALS spinal cord is harmful to human motor neurons. Transplanted human motor neurons degenerate and undergo nitroxidative damage in the ALS cord. Whether and how diseased glial cells contribute to nitroxidative damage, degeneration and death of transplanted cells is unknown. In an *in vivo* environment, it is difficult to elucidate the differential effects of astrocytes and microglia on stem cell-derived motor

neurons. Thus, *in vitro* coculture paradigms can be utilized to determine the source and contribution of toxic mechanisms induced by each glial cell type. To this end, hNSCs represent a source for rapid generation of spinal motor neurons in higher quantities compared to other published methods using several stem cell sources (Thonhoff et al., 2009). Methods for protecting stem cell-derived motor neurons can also be screened and developed *in vitro* prior to translating potential therapies back into transgenic ALS animal models. To protect stem cell-derived motor neurons from the multi-faceted disease process in ALS and maximize their efficiency in motor neuron replacement, novel combined therapies with stem cell-derived motor neurons will ultimately need to be developed.

CHAPTER 5:

MUTANT SOD1 MICROGLIA-GENERATED NITROXIDATIVE STRESS PROMOTES TOXICITY TO HUMAN NEURAL STEM CELL-DERIVED MOTOR NEURONS THROUGH DIRECT DAMAGE AND NOXIOUS INTERACTIONS WITH ASTOCYTES

5.1. ABSTRACT

Amyotrophic lateral sclerosis (ALS) is an incurable neurodegenerative disease that selectively destroys spinal and upper motor neurons. Human NSCs (hNSCs) represent a source for rapid generation of high quantities of spinal motor neurons. Human NSC-derived motor neurons can be used to model disease processes and test for glia-mediated mechanisms of human motor neuron loss as well as screen for potential therapeutics in ALS. Using an *in vitro* coculture system, we provide evidence that human mutant $SOD1^{G93A}$-expressing primary microglia, isolated from transgenic rats after disease onset, are specifically toxic to hNSC-derived motor neurons. Additionally, nontransgenic astrocytes not only lose their protective capacity toward hNSC-derived motor neuron survival *in vitro*, but also exhibit toxic features when cocultured with mutant $SOD1^{G93A}$ microglia. Using inhibitors of inducible nitric oxide synthase (iNOS) and NADPH oxidase, we show that microglia-generated nitric oxide and superoxide partially contribute to motor neuron loss and astrocyte dysfunction in this coculture paradigm. We provide evidence that reactive oxygen/nitrogen species released from overactivated microglia in ALS may drive disease progression by directly damaging motor neurons as well as eliminating the neuroprotective capacities of astrocytes.

5.2. INTRODUCTION

Amyotrophic lateral sclerosis progresses through a complex mechanism that involves multiple neural cell types and several potentially lethal pathways, with the end-result being the selective death of motor neurons (Boillee et al., 2006a). Mutant SOD1 expression within motor neurons and other yet unidentified cell types determines disease onset (Boillee et al., 2006b; Yamanaka et al., 2008a) whereas disease progression is accelerated through mutant SOD1-expressing astrocytes and microglia (Clement et al., 2003; Yamanaka and Cleveland, 2005; Beers et al., 2006; Boillee et al., 2006b; Yamanaka et al., 2008b). At disease onset, approximately 50 % of endogenous motor neurons in transgenic ALS animals (Matsumoto et al., 2006) and an unknown number in ALS patients have already been lost. Stem cells represent a means to dramatically delay and potentially cure ALS through the restoration of muscle function. However, in designing a stem cell therapy to replace motor neurons in ALS, the newly grafted motor neurons would be subjected to a hostile microenvironment characterized by increased inflammation, oxidative stress and excitotoxicity. Reactive microglia and astrocytes already abound at the time of diagnosis in most ALS patients and considerably contribute to the encountered hostility. Hence, methods must be developed to defend transplanted stem cell-derived motor neurons from the toxic properties of these glial cells.

Human fetal neural stem cells provide a source for rapid and consistent generation of high quantities of spinal motor neurons that may be used *in vitro* to model disease mechanisms specifically with human motor neurons (Tarasenko et al., 2004; Jordan et al., 2008b; Thonhoff et al., 2009). Potential pitfalls in grafted motor neuron efficacy could be

identified and corrected in coculture systems with diseased glial cells prior initiating pre-clinical trials in transgenic ALS animals. In this manner, means to protect grafted stem cell-derived motor neurons as well as endogenous motor neurons could be ascertained. Previously, human embryonic stem cell-derived motor neurons have been shown to be vulnerable to mutant SOD1-expressing astrocyte toxicity *in vitro* (Di Giorgio et al., 2007; Nagai et al., 2007; Di Giorgio et al., 2008; Marchetto et al., 2008). Although the exact cause of toxicity has not been determined, the experiments have shown that elucidating mechanisms of motor neuron death in coculture paradigms with glial cells may be feasible. Utilizing such a system could allow for rapid and powerful screening of numerous therapeutic agents against motor neuron death *in vitro*.

Here, we sought to determine how microglia and astrocytes interact with each other to create such a toxic environment encountered in the spinal cord in ALS, and to what extent nitroxidative stress is involved in motor neuron toxicity.

5.3. MATERIALS AND METHODS

Please refer to CHAPTER 2 for the materials and methods used in this chapter.

5.4. RESULTS

5.4.1. ALS MICROGLIA AND ASTROCYTES ARE NOT CONDUCIVE TO LONG-TERM SURVIVAL OF HNSC-DERIVED MOTOR NEURONS *IN VITRO*

In this context, "ALS" rats or cells refer to those expressing transgenic mutant SOD1 and "normal" refers to nontransgenic matches. To determine whether microglia and/or astrocytes were specifically harmful to hNSC-derived motor neurons, direct contact cocultures were performed between differentiated hNSCs and mixed glial cell populations. Primed and differentiated hNSCs were seeded on top of primary astrocytes and microglia isolated from normal rats (Fig. 5.1A), astrocytes from normal rats and microglia from ALS rats post-disease onset (Fig. 5.1B), as well as astrocytes and microglia from ALS rats (Fig. 5.1C). Since Hb9 is a transcription factor specific for both premature and mature spinal motor neurons and MAP2 is a pan-marker for all mature neurons, spinal motor neurons *in vitro* were defined as $Hb9^+/MAP2^+$ cells. The survival of $Hb9^+/MAP2^+$ cells after one week in coculture was high in the presence of normal astrocytes and microglia (Fig. 5.1A, D). Replacing normal microglia with ALS microglia resulted in a 48 % decrease in survival (Fig. 5.1B, D). The presence of both ALS microglia and astroglia caused the greatest reduction (77 % decrease) in $Hb9^+/MAP2^+$ cell survival (Fig. 5.1C, D). Furthermore, $Hb9^+/MAP2^+$ cells exhibited morphology more characteristic of spinal motor neurons *in vivo* when cocultured with normal astrocytes and microglia (Fig. 5.1A) compared to cocultures with ALS glial cells (Fig. 5.1B, C).

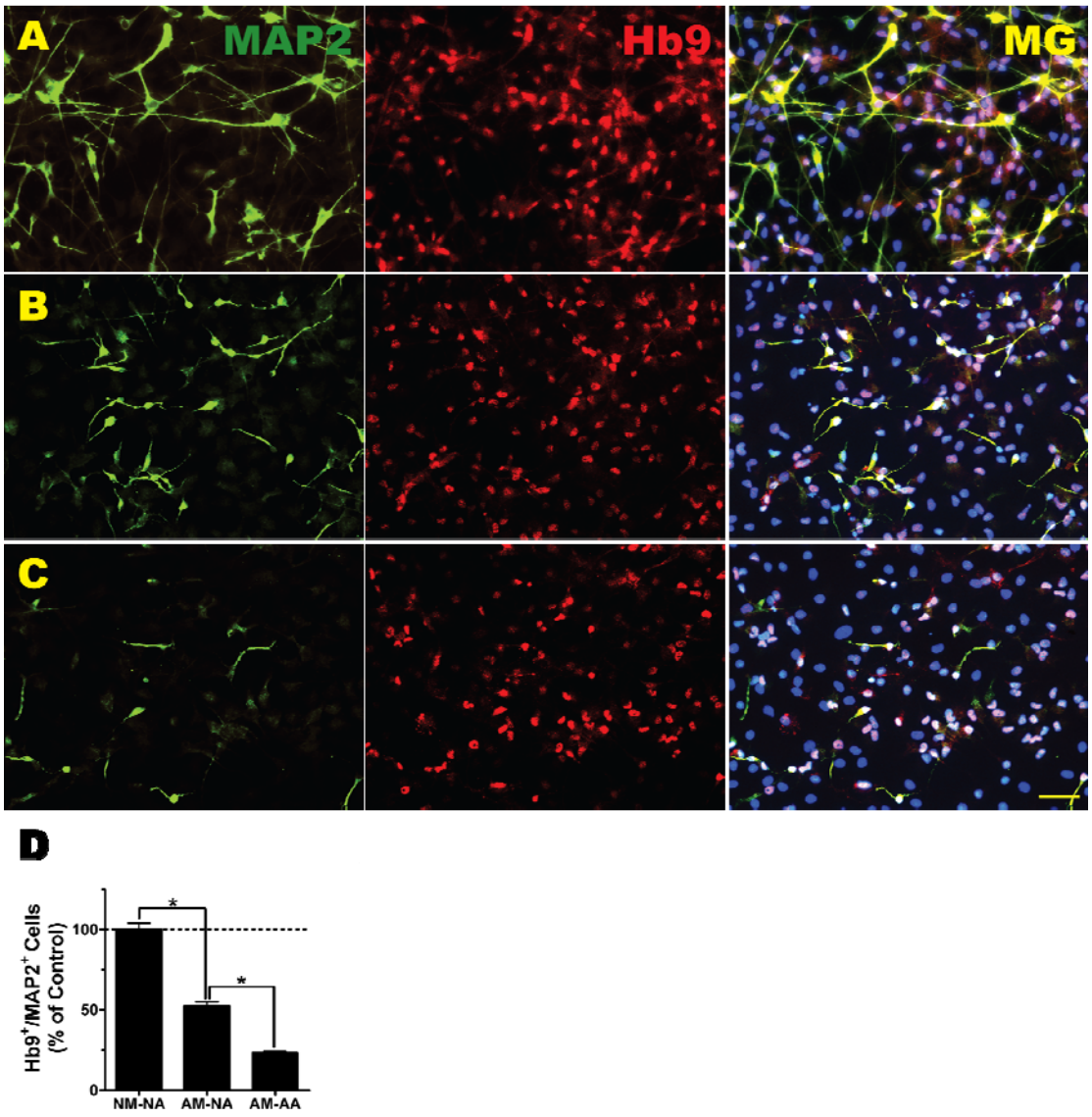

Fig. 5.1. ALS microglia and ALS astrocytes do not maintain survival of hNSC-derived motor neurons in vitro.

Human NSC-derived motor neurons, expressing microtubule associated protein 2 (MAP2, green, neuronal marker) and Hb9 (red, motor neuron transcription factor), were cocultured in direct contact with both microglia and astrocytes. Merged imaged with DAPI shown in blue (MG). **A**, Normal microglia and normal astrocytes. **B**, ALS microglia and normal astrocytes. **C**, ALS microglia and ALS astrocytes. **D**, Average relative percentages of $Hb9^{+}/MAP2^{+}$ cells in each group (NA = normal astrocytes, AA = ALS astrocytes and AM = ALS microglia). Data expressed as mean ± SEM (n=3, *p<0.05). **A-C**, scale bar = 50 μm.

5.4.2. ALS MICROGLIA ARE TOXIC TO HNSC-DERIVED MOTOR NEURONS THROUGH NITROXIDATIVE STRESS

Microglia isolated from normal (Fig. 5.2A, C) and ALS rats (Fig. 5.2B, D) eleven days post-isolation in culture medium were exceptionally pure (98 %) and expressed Iba1, a pan-microglial marker for a calcium binding protein. Qualitative differences were not observed in CD11b (Fig. 5.2A, B), a marker of quiescent microglia, and in CD68 (data not shown), a marker of activated microglia, through immunofluorescent staining analyses. Primary astroglia populations were not as pure (94 % by GFAP immunostaining), and were contaminated with microglia (data not shown). In order to determine whether ALS microglia were toxic to hNSC-derived motor neurons, cocultures were performed in direct contact in N2 media with differentiated hNSCs seeded on top of primary microglia. After 7 days of coculture, immunofluorescent staining with the Iba1 antibody was performed to detect either normal or ALS microglia. When cocultured with hNSC-derived motor neurons, most normal microglia exhibited a ramified morphology indicative of a resting state (Fig. 5.2C), whereas most ALS microglia displayed an activated/phagocytic morphology (Fig. 5.2D). The total number of cells was also reduced in cocultures with ALS microglia as shown by DAPI staining in the merged images, indicating increased cell death.

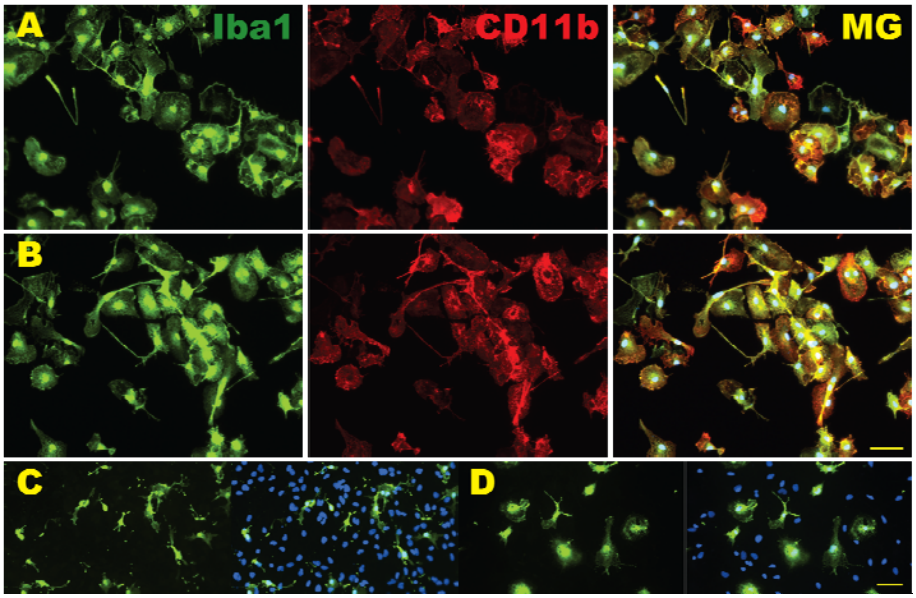

Fig. 5.2. ALS microglia display an activated phenotype in cocultures with differentiated hNSCs.

A, Normal microglia express Iba1 (pan-microglia marker, green) and CD11b (resting microglia marker, red). **B**, ALS microglia express Iba1 and CD11b. **C**, Normal microglia (Iba1, green) in direct contact coculture with hNSCs (DAPI, blue). **D**, ALS microglia post-disease onset (Iba1, green) in coculture with hNSCs (DAPI, blue). Note the difference in morphology between normal and ALS microglia. **A**-**D**, scale bars = 50 µm.

Immunofluorescent staining with the Hb9 motor neuron marker and MAP2 neuronal marker in coculture with normal (Fig. 5.3A) and ALS (Fig. 5.3B) rat microglia is also shown. Reduced immuno-labeled cell numbers and probable phagocytic microglia, which showed intense Hb9 and MAP2 staining, were observed in cocultures with ALS microglia (Fig. 5.3B). Cumulative data showed that ALS microglia significantly reduced

the numbers of $Hb9^{+}/MAP2^{+}$ cells (56 % decrease) (Fig. 5.3C). Furthermore, representative images of TUNEL staining for apoptotic cell death are shown in normal (Fig. 5.4A) and ALS (Fig. 5.4B) microglia cocultures. Increased TUNEL staining and reduced total cell number were observed in cocultures with ALS microglia (Fig. 5.4B). Cumulative data revealed that ALS microglia significantly increased the percentage of TUNEL positive cells (6.4-fold increase) (Fig. 5.4C). Further controls included differentiated hNSCs only in N2 medium as well as cocultures with microglia isolated from 1 month old pre-symptomatic ALS rats and normal rats, which showed no differences among groups (Fig. 5.3C, 5.4C). Interestingly, toxic effects were not observed when cocultures were performed in culture medium containing serum or B27 medium, which contains a variety of antioxidants, hormones and other pro-survival factors (data not shown).

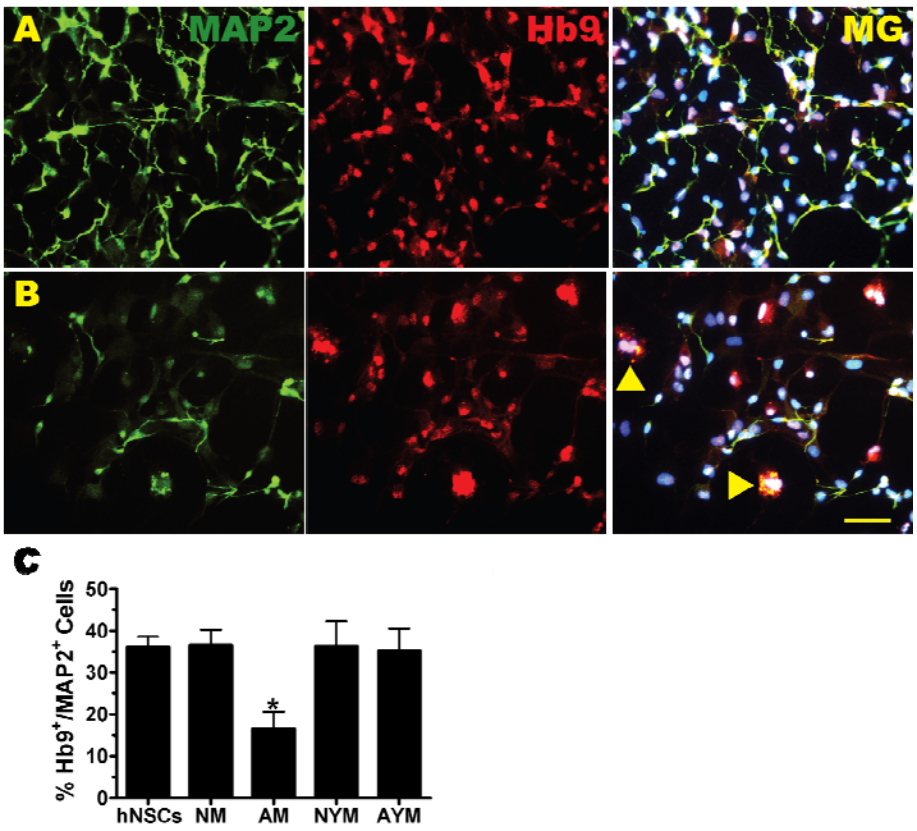

Fig. 5.3. ALS microglia are eliminate hNSC-derived motor neurons.

A, Human NSC-derived motor neurons, expressing microtubule associated protein 2 (MAP2, green, neuronal marker) and Hb9 (red, motor neuron transcription factor), cocultured with normal microglia. Merged image with DAPI shown in blue (MG). **B**, Human NSC-derived motor neurons ($Hb9^{+}/MAP2^{+}$) cocultured with ALS microglia. Presumably phagocytic/activated microglia shown by arrowheads. **C**, Average percentages of $Hb9^{+}/MAP2^{+}$ cells in each group. Data expressed as mean ± SEM (n=3). Human NSCs only (hNSCs), normal adult microglia (NM), post-disease onset ALS microglia (AM), normal young (1 month) microglia (NYM) and ALS young (1 month) microglia (AYM). *p<0.05, significantly different from hNSCs group. **A-B**, scale bar = 50 μm.

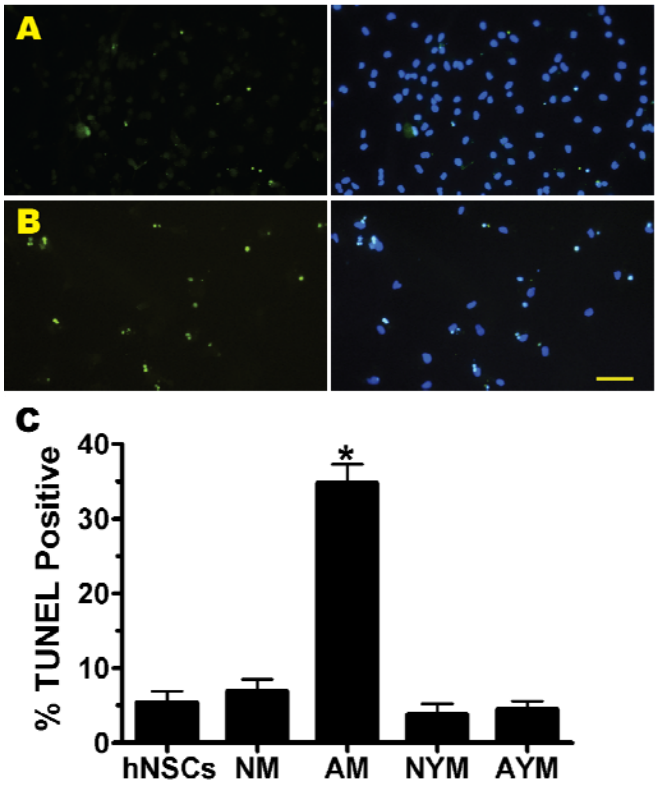

Fig. 5.4. ALS microglia induce apoptosis in differentiated hNSCs.

A, Apoptotic, TUNEL positive (green) cells in cocultures between normal microglia and hNSCs (DAPI, blue). **B**, TUNEL positive cells in cocultures between ALS microglia and hNSCs. **C**, Average percentages of TUNEL positive cells in each group. Data expressed as mean ± SEM (n=3). Human NSCs only (hNSCs), normal adult microglia (NM), post-disease onset ALS microglia (AM), normal young (1 month) microglia (NYM) and ALS young (1 month) microglia (AYM). *p<0.05, significantly different from hNSCs group. **A**-**B**, scale bar = 50 µm.

Although the presence of increased oxidative and nitrative damage in the degenerating areas of the spinal cord and motor cortex in ALS is indisputable, the exact source of reactive oxygen and nitrogen species is ambiguous. Given that many hNSCs

showed damage consisting of protein nitration and lipid peroxidation at the disease end-stage in the spinal cords of ALS rats whereas nitroxidative damage was not observed in normal spinal cords (Chapter 4), we aimed to test whether microglia from ALS rats post-disease onset could potentially contribute to nitroxidative damage to hNSC-derived motor neurons *in vitro*. Along this line, expression levels of crucial enzymes relevant to nitric oxide and superoxide production were first characterized through immunofluorescent staining. Normal microglia expressed lower levels of inducible nitric oxide synthase (iNOS) and $gp91^{phox}$ (Fig. 5.5A), the catalytic subunit of NADPH oxidase, than ALS microglia isolated after disease onset (Fig. 5.5B). In accordance with iNOS and NADPH oxidase expression levels, ALS microglia isolated after disease onset produced more nitric oxide (33 % increase) (Fig. 5.5C) and superoxide (46 % increase) (Fig. 5.5D) than normal adult microglia. Interestingly, microglia isolated from 1 month old rats generated significantly less nitric oxide (normal, 65 % decrease; ALS, 55% decrease) and superoxide (normal, 93 % decrease; ALS, 93 % decrease) than normal adult microglia. Furthermore, after coculture with differentiated hNSCs for 7 days, microglia from normal adult rats induced a slight increase in lipid peroxidation as shown by brownish immunostaining with the 4-HNE adduct (Fig. 5.6A). Cocultures with ALS microglia after disease onset caused more cell loss and small, dark brown cells indicative of increased cell death and lipid peroxidation, respectively (Fig. 5.6B). In contrast, hNSC-derived motor neurons, when cocultured with normal (Fig. 5.6C) or ALS (Fig. 5.6D) microglia from 1 month old rats, showed no apparent lipid peroxidation.

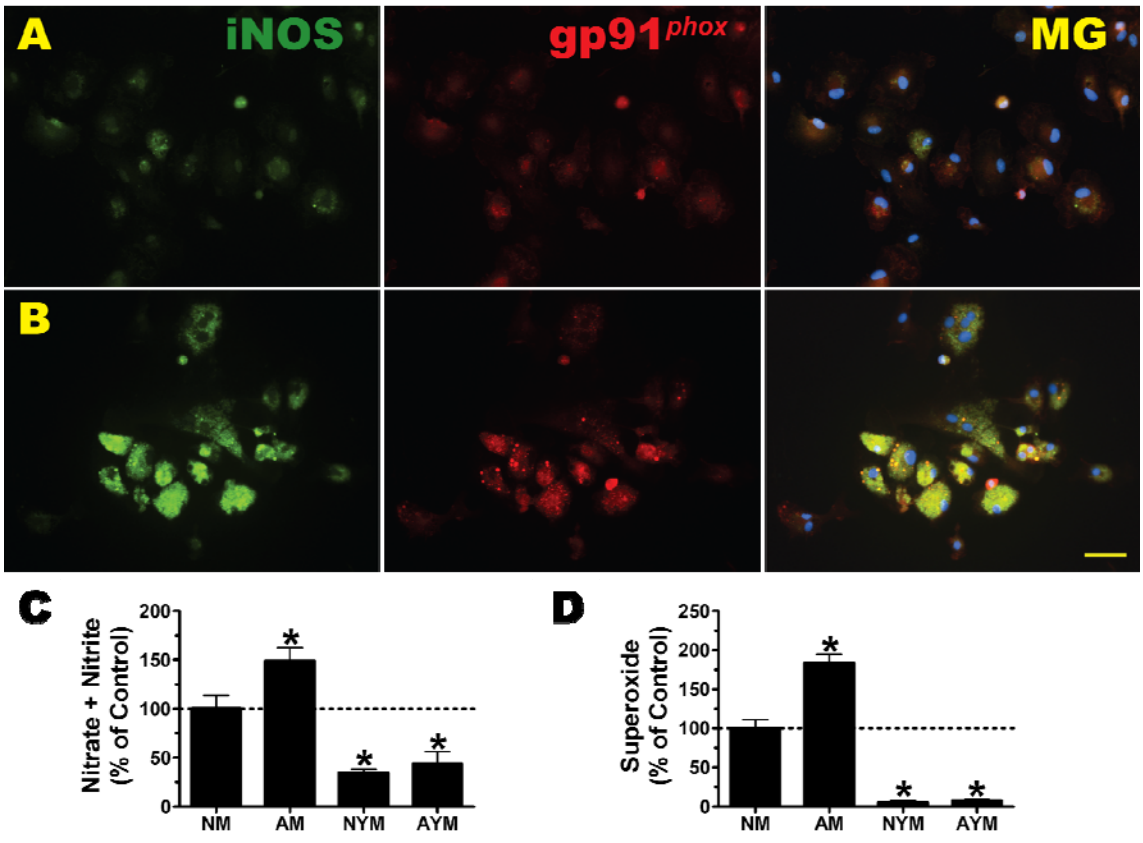

Fig. 5.5. ALS microglia release higher levels of nitric oxide and superoxide.

A, Normal microglia express lower levels of enzymes that produce nitric oxide and superoxide, including inducible Nitric Oxide Synthase (iNOS, green) and gp91^{phox} (catalytic subunit of NADPH oxidase, red), respectively. Merged image with DAPI shown in blue (MG). **C**, Relative average amounts of nitric oxide released into the medium from microglia (n=3-4). *p<0.05, significantly different from the NM group. **D**, Relative average amounts of superoxide released into the medium from microglia (n=3-5). *p<0.05, significantly different from the NM group. **A-B**, scale bar = 50 µm. **C-D**, Data expressed as mean ± SEM. Human NSCs only (hNSCs), normal adult microglia (NM), post-disease onset ALS microglia (AM), normal young (1 month) microglia (NYM) and ALS young (1 month) microglia (AYM).

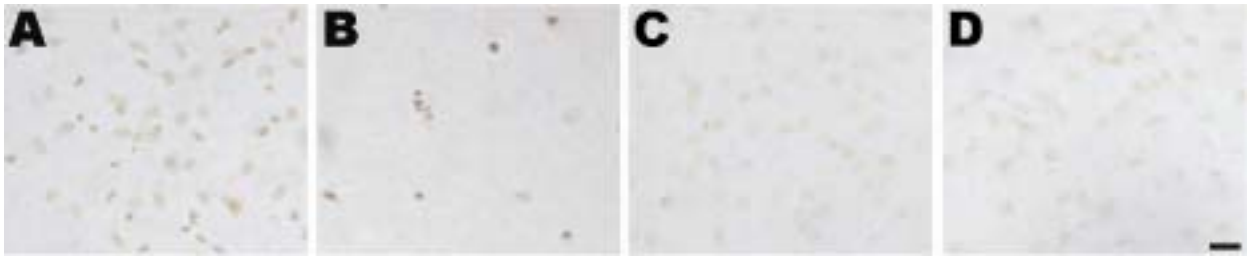

Fig. 5.6. ALS microglia induce more nitroxidative damage to differentiated hNSCs.

A, Normal adult microglia slightly increase lipid peroxidation, indicated by 4-hydroxynonenal staining, in direct contact coculture with hNSCs. **B**, ALS microglia post-disease onset dramatically increase lipid peroxidation. **C**, Normal young (1 month) microglia cause no observable lipid peroxidation. **D**, ALS young (1 month) microglia cause no observable lipid peroxidation. **A**-**D**, scale bar = 50 μm.

Selectively inhibiting NADPH oxidase and iNOS with apocynin (100 μM) and L-NIL (100 μM), respectively, throughout the duration of the coculture significantly increased the number of viable cells (Apocynin, 38 % increase, Fig. 5.7A; L-NIL, 31 % increase, Fig. 5.7B), partially ameliorated $Hb9^{+}/MAP2^{+}$ cell loss (apocynin, 43 % increase; L-NIL, 49 % increase) (Fig. 5.7C), and significantly reduced the number of TUNEL positive apoptotic cells (apocynin, 47 % decrease; L-NIL, 46 % decrease) (Fig. 5.7D). Scavengers for peroxynitrite (urate-100 μM), nitric oxide (carboxy-PTIO-20 μM)) and superoxide (SOD-100 U/ml) also provided partial protection to $Hb9^{+}/MAP2^{+}$ cells (urate, 39 % increase; c-PTIO, 31 % increase; SOD, 29 % increase) (Fig. 5.7C) in coculture with ALS microglia.

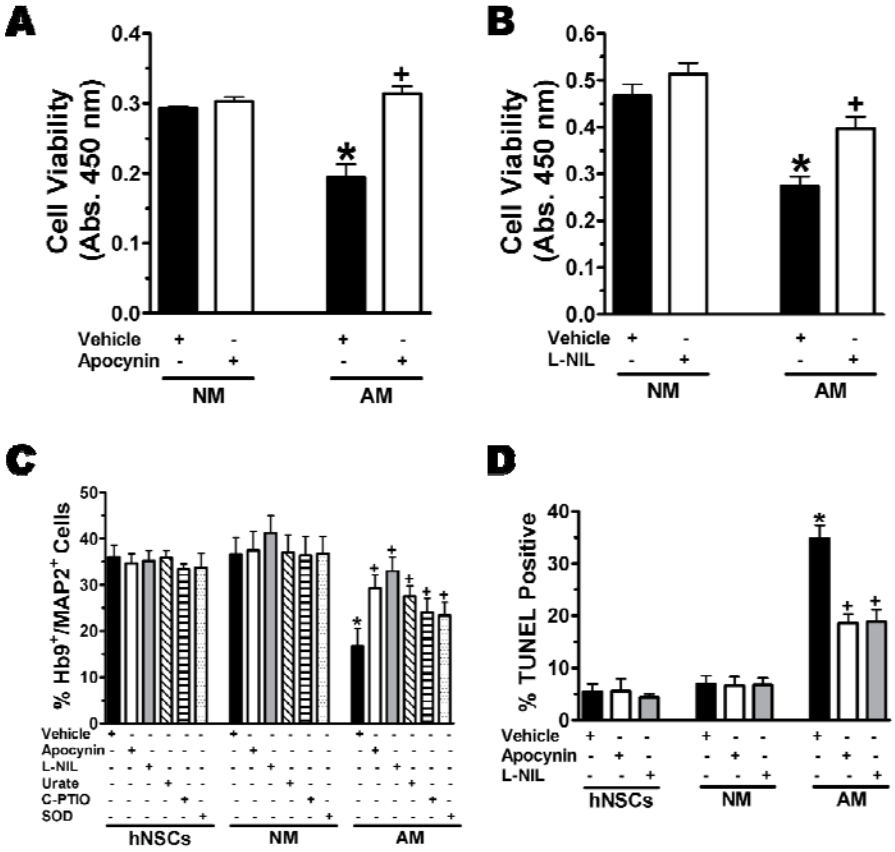

Fig. 5.7. ALS microglia eradicate hNSC-derived motor neurons through nitroxidative stress.

A, Total amount of viable cells in each group treated with vehicle or a NADPH oxidase inhibitor, apocynin (n=3). **B**, Total amount of viable cells in each group treated with vehicle or an iNOS inhibitor, L-NIL (n=6). **C**, Average percentages of $Hb9^+/MAP2^+$ cells in each group treated with vehicle (DMSO and dH_20 are combined; hNSCs, n=5; NM, n=9; AM, n=10); apocynin (n=3); L-NIL (n=3); a peroxynitrite scavenger, urate (n=3); a nitric oxide scavenger, c-PTIO (n=3); and a superoxide scavenger, SOD (n=3). **D**, Average percentages of TUNEL positive cells in each group treated with vehicle (DMSO and dH_20 are combined; hNSCs, n=6; NM, n=8; AM, n=8); apocynin (n=3); and L-NIL (n=3). **A-D**, Data expressed as mean ± SEM. $*p<0.05$, significantly different from NM with vehicle group in **A-B** and hNSCs with vehicle group in **C-D**. $^+p<0.05$, significantly different from AM with vehicle group. Human NSCs only (hNSCs), normal adult microglia (NM), post-disease onset ALS microglia (AM), normal young (1 month) microglia (NYM) and ALS young (1 month) microglia (AYM).

5.4.3. ALS ASTROCYTES LOSE NEUROPROTECTIVE CAPACITY AND EXERT TOXICITY TO HNSC-DERIVED MOTOR NEURONS

Recent evidence has shown that conditioned medium from and direct contact cocultures with primary astrocytes isolated from neonatal transgenic ALS mice are toxic to embryonic stem cell-derived motor neurons (Di Giorgio et al., 2007; Nagai et al., 2007; Di Giorgio et al., 2008; Marchetto et al., 2008). Our group has previously shown that normal adult astroglia conditioned medium and cocultures maintain long-term survival of hNSC-derived spinal motor neurons and that the secretion of bFGF from astrocytes was partially responsible for neuroprotection *in vitro* (Jordan et al., 2008a). In order to determine whether ALS astrocytes lose this protective capacity, primary astrocytes were seeded in transwells and cocultured with differentiated hNSCs. These cocultures were non-contact in nature, as the transwell bottoms were positioned 1 mm above the hNSCs, which were attached to glass cover slips. After 7 days in coculture in N2 medium, normal astrocytes derived from both adult and 1 month old rats provided significant long-term protection for $Hb9^{+}/MAP2^{+}$ cells (adult, 20 % increase; 1 month, 28 % increase) (Fig. 5.8A). On the other hand, astrocytes isolated from ALS rats did not display this same neuroprotective capacity and in fact, exhibited significant toxicity. Further, ALS astrocytes isolated after disease onset caused a greater decrease in the percentage of $Hb9^{+}/MAP2^{+}$ cells than astrocytes isolated from 1 month old ALS rats (disease onset, 47 % decrease; 1 month, 24 % decrease). Non-contact cocultures with ALS astroglia isolated after disease onset also resulted in a higher percentage of TUNEL positive cells (5.2-fold increase) than the differentiated hNSC only population (Fig. 5.8B). Additionally, prostaglandin D2 (PGD2) has been implicated in transgenic ALS astrocyte-mediated toxicity to embryonic stem cell-derived motor neurons (Di Giorgio et

al., 2008). Indeed, ALS astrocytes isolated after disease onset release significantly higher levels of PGD2 as detected by PGD2 ELISA (54 % increase) (Fig. 5.9A) and selectively inhibiting the PGD2 receptor with MK 0524 (10 μM) in non-contact cocultures partially prevents hNSC-derived motor neuron loss after 7 days (Fig. 5.9B). Toxicity arising in cocultures with ALS astrocytes was unlikely due to nitric oxide-mediated damage, since increases in ALS astrocyte iNOS expression by Western Blot analysis (Fig. 5.9C) as well as nitric oxide release into the medium (Fig. 5.9D) were not observed.

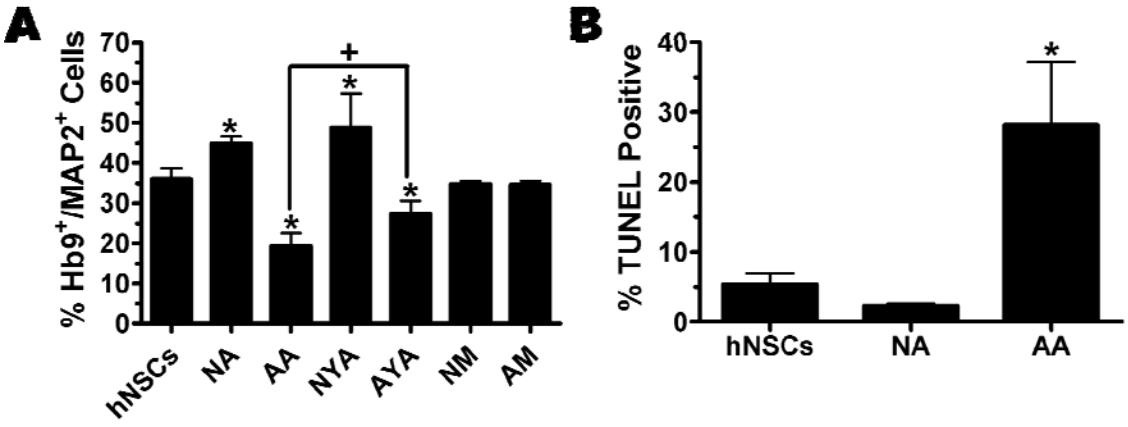

Fig. 5.8. ALS astrocytes lose neuroprotective capacity and exert toxicity to hNSC-derived motor neurons.

A, Average percentages of $Hb9^+/MAP2^+$ cells in each group of hNSCs in non-contact coculture with astrocytes or microglia (hNSCs, n=5; NA an AA, n=6; all other groups, n=3). *$p<0.05$, significantly different from hNSCs group; $^+p<0.05$, significantly different between two groups. **B**, Average percentages of apoptotic, TUNEL positive cells in each group of hNSCs in non-contact coculture with astrocytes (hNSCs, n=6; NA an AA, n=3). *$p<0.05$, significantly different from hNSCs group. **A**-**B**, Data expressed as mean ± SEM. Human NSCs only (hNSCs), normal adult astrocytes (NA), post-disease onset ALS astrocytes (AA), normal young (1 month) astrocytes (NYA), ALS young (1 month) astrocytes (AYA), normal adult microglia (NM) and post-disease onset ALS microglia (AM).

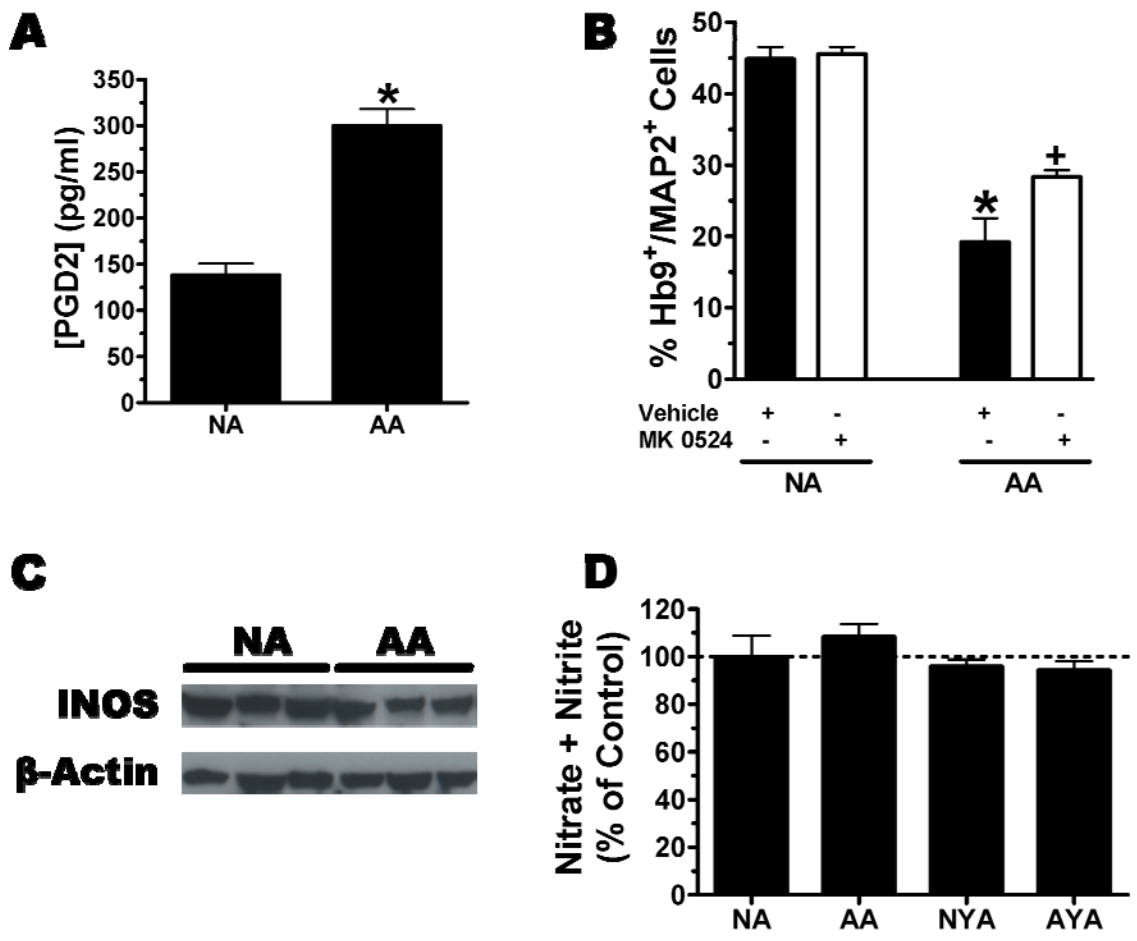

Fig. 5.9. ALS astrocyte-secreted prostaglandin D2, but not nitric oxide, contributes to hNSC-derived motor neuron loss.

A, Prostaglandin D2 (PGD2) release into the medium from astrocytes (n=6). *p<0.05, significantly different from NA group. **B**, Average percentages of $Hb9^{+}/MAP2^{+}$ cells in each group of hNSCs in non-contact coculture with astrocytes treated with vehicle or a PGD2 receptor antagonist, MK 0524 (n=3). *p<0.05, significantly different from NA with vehicle group. ^{+}p<0.05, significantly different from AA with vehicle group. **C**, Western blot analysis for iNOS and β-actin control. Note the similar to slightly decreased expression levels in ALS astrocytes (AA). **D**, Relative average amounts of nitric oxide released into the medium from astrocytes (n=8). **A**-**D**, Data expressed as mean ± SEM. Normal adult astrocytes (NA), post-disease onset ALS astrocytes (AA), normal young (1 month) astrocytes (NYA) and ALS young (1 month) astrocytes (AYA).

5.4.4. ALS MICROGLIA INDUCE A LOSS IN THE NEUROPROTECTIVE CAPACITY OF NORMAL ASTROCYTES

Interactions between microglia, astrocytes and motor neurons within the diseased setting in ALS have not been well characterized. Thus, we aimed to determine whether ALS microglia isolated after disease onset diminish the neuroprotective capacity of normal astrocytes and enhance the toxicity of ALS astrocytes to hNSC-derived motor neurons. The experimental design consisted of a direct contact coculture between microglia and astrocytes in transwells (0.4 μm pore size) for 24 hrs prior to being positioned 1 mm above and cocultured with differentiated hNSCs. Microglia were not observed on glass cover slips after being placed in transwells with the smaller pore size. Neither normal nor ALS microglia post-disease onset exhibited toxicity when independently placed in a transwell and cocultured in non-contact with differentiated hNSCs (Fig. 5.8A). Microglia isolated from ALS rats and normal rats also released PGD2, but the levels were not significantly different (Fig. 5.10A). However, when ALS microglia were cocultured in direct contact with normal adult astroglia for 7 days in N2 medium, a significant upregulation in PGD2 release was observed (40 % increase) (Fig. 5.10B). Furthermore, inhibiting NADPH oxidase with apocynin throughout the duration of the ALS microglia and normal astrocytes coculture resulted in a trend toward ameliorating the rise in PGD2 release (21 % decrease) (^ $p = 0.0605$). Inhibition of NADPH oxidase did, however, significantly reduce the amount of PGD2 release in cocultures with ALS microglia and ALS astrocytes (16 % decrease) (Fig. 5.10B). ALS microglia did not significantly enhance PGD2 release in cocultures with ALS astrocytes compared to cocultures between normal microglia and ALS astrocytes (Fig. 5.10B).

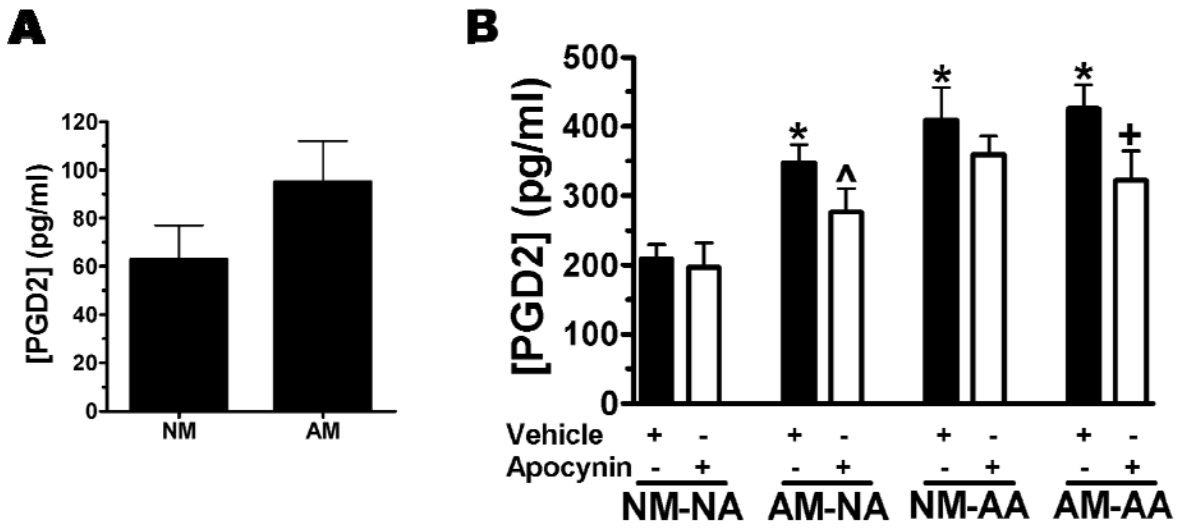

Fig. 5.10. ALS microglia enhance prostaglandin D2 secretion from normal and ALS astrocytes.

A, Prostaglandin D2 (PGD2) release into the medium from microglia (n=6). **B**, PGD2 release into the medium from astrocytes and microglia in direct contact coculture treated with vehicle or a NADPH oxidase inhibitor, apocynin (n=6). *$p<0.05$, significantly different from NM-NA with vehicle group. $^{\wedge}p=0.0605$, compared to AM-NA with vehicle group. $^{+}p<0.05$, significantly different from AM-AA with vehicle group. **A**-**B**, Data expressed as mean ± SEM. Normal adult microglia (NM), post-disease onset ALS microglia (AM), normal adult astrocytes (NA) and post-disease onset ALS astrocytes (AA).

To directly determine the effect of ALS microglia on the neuroprotective capacity of normal and ALS astrocytes, the survival of hNSC-derived motor neurons were evaluated after 7 days in non-contact coculture. Normal microglia and astrocytes maintained the survival of $Hb9^{+}/MAP2^{+}$ cells (Fig. 5.11A). However, replacing normal

microglia with post-disease onset ALS microglia resulted in a loss of the neuroprotective capacity of normal astrocytes shown by fewer $Hb9^+/MAP2^+$ cells (34 % decrease) (Fig. 5.11B). $Hb9^+/MAP2^+$ cells were lost in all cocultures with ALS astroglia irrespective to the presence of normal (54 % decrease) (Fig. 5.11C) or ALS (54 % decrease) microglia (Fig. 5.11D), indicating that normal microglia did not diminish the observed toxicity of ALS astrocytes. Quantitative analyses showed that $Hb9^+/MAP2^+$ cells were maintained if inhibitors of NADPH oxidase (23 % increase) or iNOS (21 % increase) were added throughout the duration of the direct coculture between ALS microglia and normal astrocytes (Fig. 5.11E). Conversely, NADPH oxidase and iNOS inhibitors had no effect on the toxicity exerted by ALS astrocytes to hNSC-derived motor neurons irrespective to the presence of microglial cells either from ALS or normal rats (Fig. 5.11E).

Similarly, non-contact cocultures with normal microglia and normal astrocytes showed reduced percentages of TUNEL positive cells in the differentiated hNSC population (2.1-fold decrease) (Fig. 5.12A, E) whereas cocultures with ALS microglia in combination with normal astrocytes resulted in an increase in TUNEL staining (2.7-fold increase) (Fig. 5.12B, E) compared to differentiated hNSCs only (Fig. 5.8B). Fewer cells and higher percentages of TUNEL positive cells were further observed in cocultures between ALS astrocytes and normal microglia (5.1-fold increase) (Fig. 5.12C, E) or ALS microglia (5.0-fold increase) (Fig. 5.12D, E) compared to differentiated hNSCs only (Fig. 5.8B).

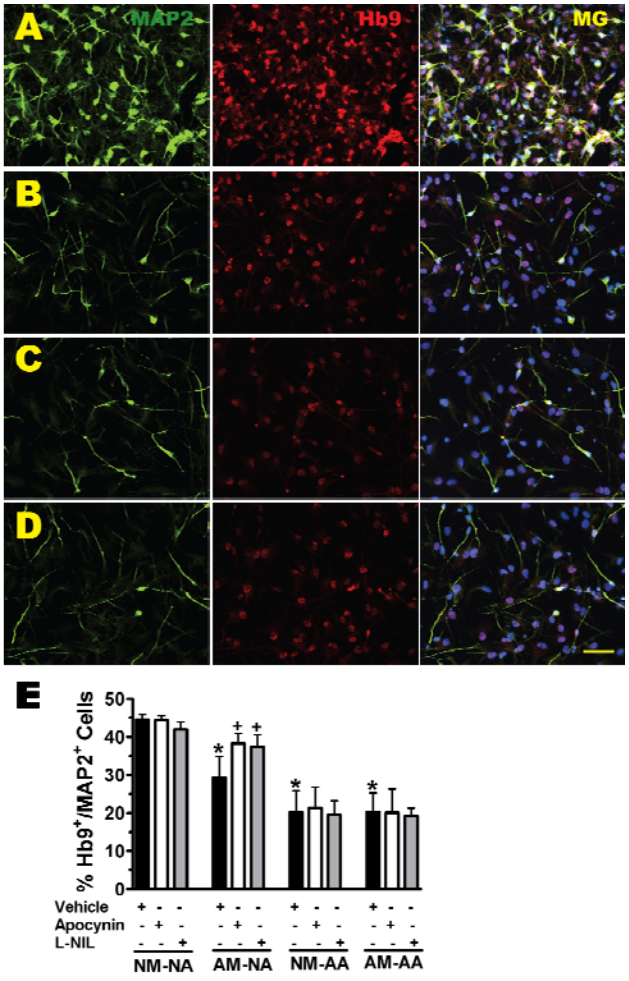

Fig. 5.11. ALS microglia induce a loss in the neuroprotective capacity of normal astrocytes through nitroxidative stress.

Microglia and astrocytes (adult or post-disease onset) were cocultered in transwells and then in non-contact with hNSC-derived motor neurons, expressing microtubule associated protein 2 (MAP2, green, neuronal marker) and Hb9 (red, motor neuron transcription factor). Merged image with DAPI shown in blue (MG). **A**, Normal microglia and normal astrocytes. **B**, ALS microglia and normal astrocytes. **C**, Normal microglia and ALS astrocytes. **D**, ALS microglia and ALS astrocytes. **E**, Average percentages of $Hb9^{+}/MAP2^{+}$ cells in each group treated with vehicle (DMSO and dH_20 are combined, n=6); a NADPH oxidase inhibitor, apocynin (n=3); and an iNOS inhibitor, L-NIL (n=3). Data expressed as mean ± SEM. *p<0.05, significantly different from NM-NA with vehicle group. ^{+}p<0.05, significantly different from AM-NA with vehicle group. Normal microglia (NM), normal astrocytes (NA), post-disease onset ALS microglia (AM) and post-disease onset ALS astrocytes (AA). **A**-**D**, scale bar = 50 µm.

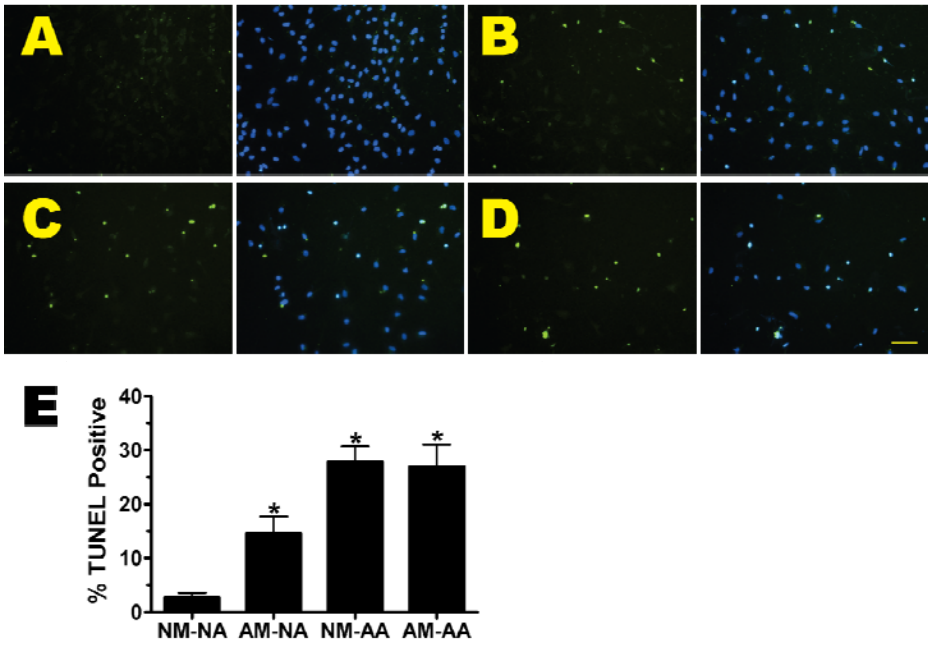

Fig. 5.12. ALS microglia trigger ALS-like cytotoxic properties in normal astrocytes.

Microglia and astrocytes (adult or post-disease onset) were cocultered in transwells and then in non-contact with hNSC-derived motor neurons. **A**, Apoptotic, TUNEL positive (green) cells in coculture with normal microglia and normal astrocytes. DAPI staining (blue) represents hNSC population. **B**, TUNEL positive cells in coculture with ALS microglia and normal astrocytes. **C**, TUNEL positive cells in coculture with normal microglia and ALS astrocytes. **D**, TUNEL positive cells in coculture with ALS microglia and ALS astrocytes. Note the decrease in total cell numbers in **B-D**. **E**, Average percentages of TUNEL positive cells in each group (n=3). Data expressed as mean ± SEM. **p*<0.05, significantly different from NM-NA group. Normal microglia (NM), normal astrocytes (NA), post-disease onset ALS microglia (AM) and post-disease onset ALS astrocytes (AA).**A-D**, scale bar = 50 μm.

5.4.5. ALS ASTROCYTES DO NOT LOSE THE CAPACITY TO RELEASE REDUCED GLUTATHIONE

In vivo, neurons critically rely on astrocyte release and breakdown of GSH into its components for maintaining intracellular reduced glutathione (GSH) levels, one of the most potent antioxidants (Dringen et al., 1999; Dringen et al., 2000). One potential mechanism by which motor neurons may be more susceptible to microglia-mediated nitroxidative damage in ALS would occur if astroglia sequestered GSH or lost capacity to synthesize GSH. In contradiction to this hypothesis, *in vitro*, primary ALS astrocytes showed increased expression levels of glutamate cysteine ligase (GCL), the rate limiting enzyme for GSH synthesis, as shown through Western blot analysis (Fig. 5.13A). Further, ALS astrocytes released significantly higher levels of GSH into the medium after one day in culture (35 % increase) (Fig. 5.13B). Astrocyte-secreted GSH was depleted in the presence ethacrynic acid (100 μM), a glutathione reductase inhibitor (normal astrocytes, 92 % decrease; ALS astrocytes, 92 % decrease) (Fig. 5.13B). Negligible quantities of GSH in the culture medium could be detected from normal and post-disease onset ALS microglia (Fig. 5.13C). Surprisingly, direct contact coculture of ALS microglia with normal adult or ALS astrocytes did not result in significant increases in GSH secretion after 7 days (normal microglia-normal astrocytes, 3.0 μM GSH; ALS microglia-normal astrocytes, 3.2 μM; normal microglia-ALS astrocytes, 3.4 μM; ALS microglia-ALS astrocytes, 3.5 μM) (Fig. 5.13D).

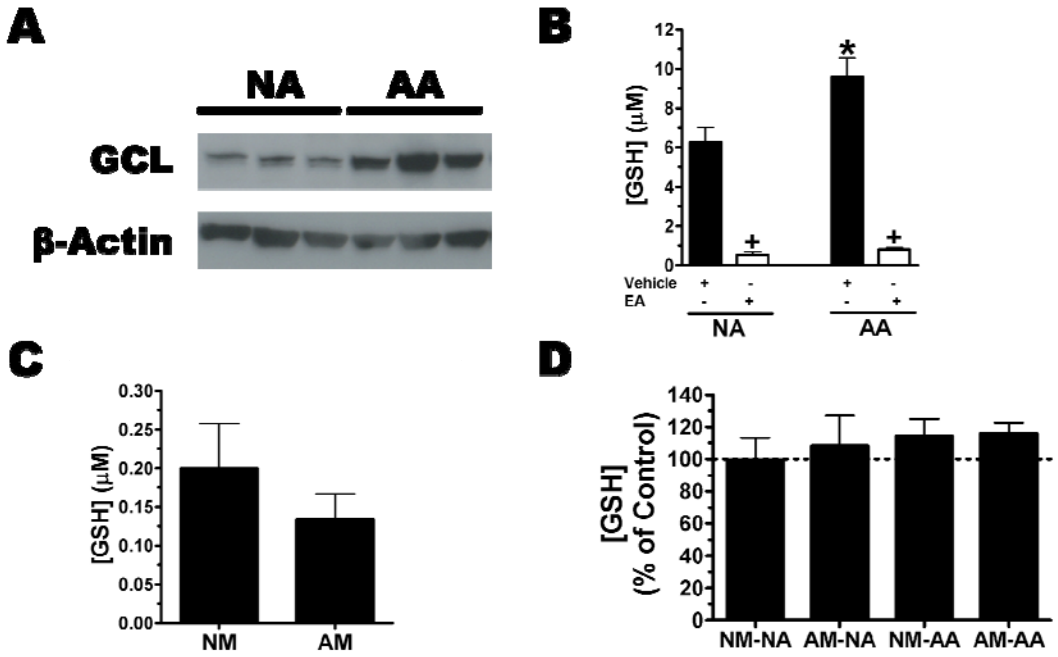

Fig. 5.13. ALS astrocytes release higher quantities of reduced glutathione.

A, Western blot analysis for the rate-limiting enzyme in glutathione synthesis, glutamate-cysteine ligase (GCL), and β-actin control. Note the increased GCL expression in ALS astrocytes (AA). **B**, Reduced glutathione (GSH) detected in the medium from astrocytes treated with vehicle or an inhibitor of glutathione reductase, ethacrynic acid (EA). *p<0.05, significantly different from NA with vehicle group. ^{+}p<0.05, significantly different from respective vehicle groups. **C**, Negligible amounts of GSH detected in the medium from microglia. **D**, GSH detected in the medium during direct contact coculture between microglia and astrocytes, which shows no significant differences among groups. **A**-**D**, Data expressed as mean ± SEM (n=3). Normal microglia (NM), normal astrocytes (NA), post-disease onset ALS microglia (AM) and post-disease onset ALS astrocytes (AA).

5.4.6. NORMAL ASTROCYTES MODERATELY PROTECT HNSC-DERIVED MOTOR NEURONS FROM ALS MICROGLIAL TOXICITY THROUGH GSH SECRETION

Since ALS microglia are directly toxic to hNSC-derived motor neurons through nitroxidative damage, we aimed to determine whether and to what extent astrocytes could provide protection through the secretion of GSH. In these experiments, primary microglia were cocultured in direct contact with differentiated hNSCs on glass cover slips, while astrocytes were placed in transwells and added to the coculture for 7 days in N2 medium. $Hb9^+/MAP2^+$ cells were maintained in cocultures with normal microglia and normal astrocytes (16 % increase) (Fig. 5.14A, E) compared to hNSCs only (Fig. 5.8A). Normal astrocytes moderately protected $Hb9^+/MAP2^+$ cells from ALS microglia-mediated loss (40 % increase) (Fig. 5.14B, E). $Hb9^+/MAP2^+$ cells were lost in cocultures with normal (38 % decrease) (Fig. 5.14C, E) and ALS (64 % decrease) microglia (Fig. 5.14D, E) in the presence of ALS astrocytes compared to hNSCs only (Fig. 5.8A). TUNEL staining results were inversely related with the percentages of $Hb9^+/MAP2^+$ cells. The lowest percentage of TUNEL positive cells was observed in the presence of normal microglia and normal astrocytes (3.1-fold decrease) (Fig. 5.15A, E) compared to hNSCs only (Fig. 5.8B). Normal astrocytes reduced the number of TUNEL positive cells in the presence of ALS microglia (46 % decrease) (Fig. 5.15B, E) compared to ALS microglia cocultures (Fig. 5.4C). Fewer total cells and higher numbers of TUNEL positive cells were observed in cocultures with ALS astrocytes plus normal (5.0-fold increase) (Fig. 5.15C, E) or ALS (6.3-fold increase) microglia (Fig. 5.15D, E) compared to hNSCs only (Fig. 5.8B).

To determine whether GSH plays a role in the neuroprotective effect of normal astrocytes, cocultures were challenged by GSH depletion. Normal astrocytes significantly

protected hNSC-derived motor neurons from ALS microglia-induced loss whereas this neuroprotective capacity was abolished when GSH was depleted in astrocytes with ethacrynic acid (100 μM) for 24 hrs prior to the initiation of coculture (35 % decrease) (Fig. 5.14E, 5.15E). Despite the increased secretion of GSH, ALS astrocytes did not exhibit the capability to protect hNSC-derived motor neurons from ALS microglia-mediated toxicity. On the contrary, a synergistic trend in increased $Hb9^{+}/MAP2^{+}$ cell loss and apoptosis was observed in cocultures with ALS microglia and ALS astrocytes. Depletion of GSH from ALS astrocytes did not exacerbate toxicity in cocultures with normal or ALS microglia (Fig. 5.14E, 5.15E).

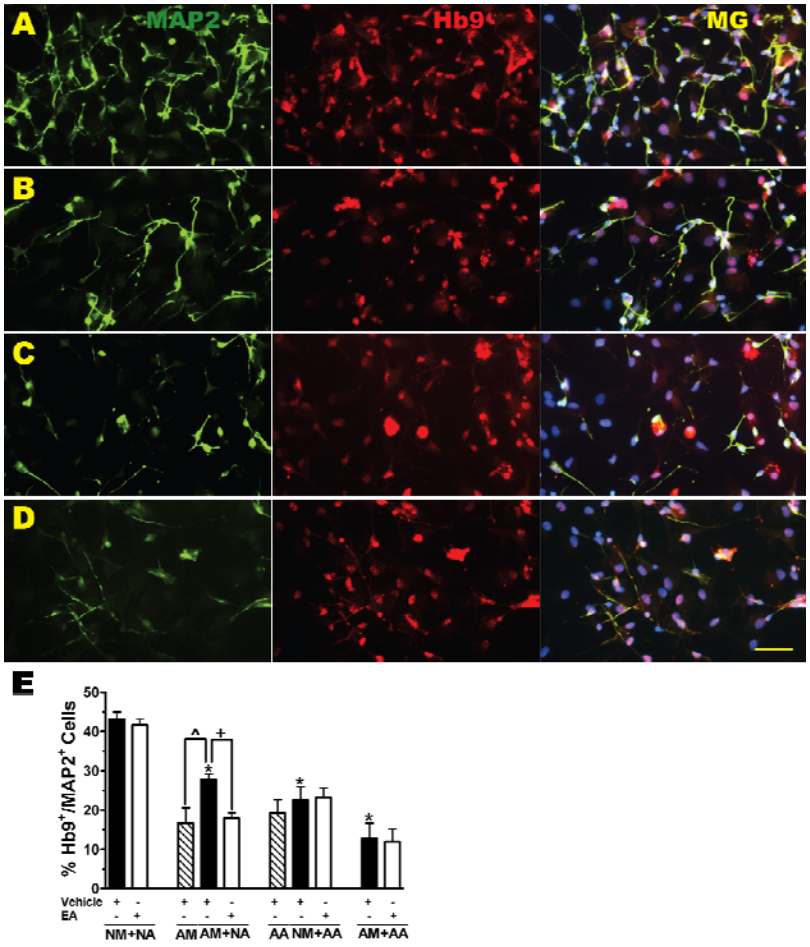

Fig. 5.14. Normal astrocytes moderately protect hNSC-derived motor neurons from ALS microglial-mediated loss through reduced glutathione secretion.

Microglia and astrocytes (adult or post-disease onset) were cocultured in direct contact and in transwells, respectively, with hNSC-derived motor neurons, expressing microtubule associated protein 2 (MAP2, green, neuronal marker) and Hb9 (red, motor neuron transcription factor). Merged images with DAPI shown in blue (MG). **A**, Normal microglia and normal astrocytes. **B**, ALS microglia and normal astrocytes. **C**, Normal microglia and ALS astrocytes. **D**, ALS microglia and ALS astrocytes. **E**, Average percentages of $Hb9^{+}/MAP2^{+}$ cells in each group in which astrocytes were pre-treated with vehicle (n=6) or a glutathione reductase inhibitor, ethacrynic acid (n=3). Data expressed as mean ± SEM. *p<0.05, significantly different from NM+NA with vehicle group. ^{+}p<0.05 and $^{\wedge}p$<0.05, significantly different between two groups. Normal microglia (NM), normal astrocytes (NA), post-disease onset ALS microglia (AM) and post-disease onset ALS astrocytes (AA).**A**-**D**, scale bar = 50 µm.

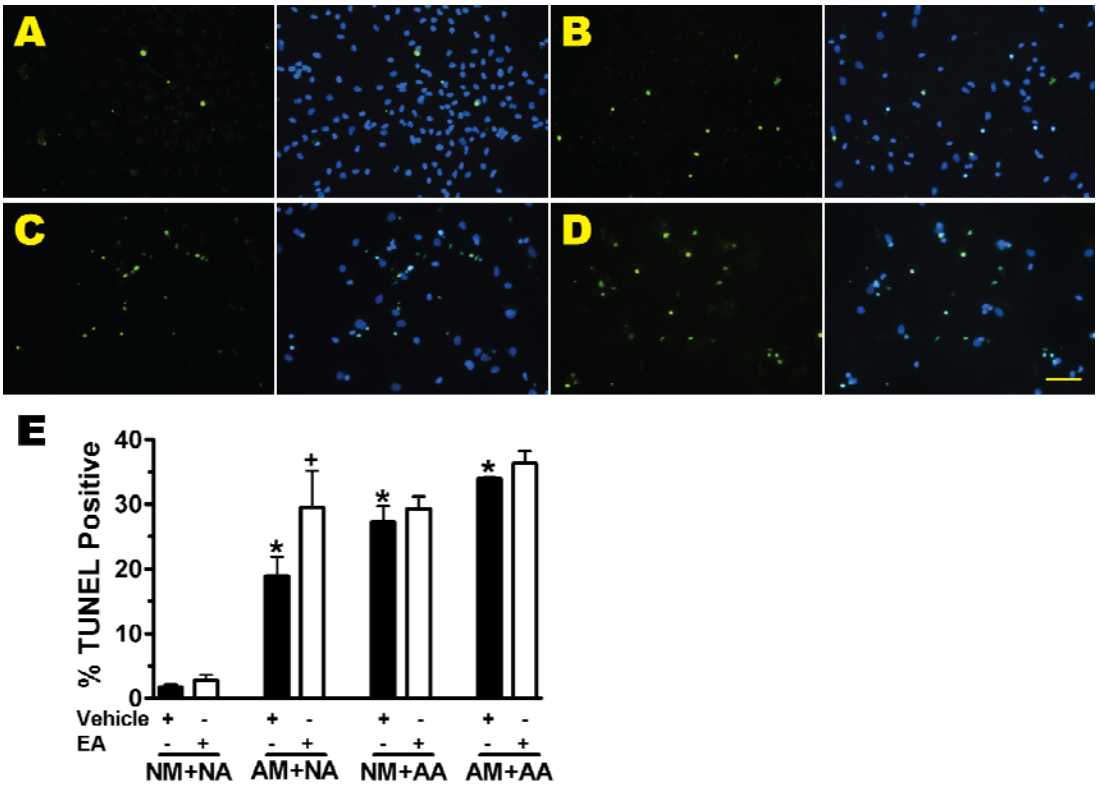

Fig. 5.15. Normal astrocytes ameliorate ALS microglial-induced apoptosis in differentiated hNSCs through reduced glutathione secretion.

Microglia and astrocytes (adult or post-disease onset) were cocultured in direct contact and in transwells, respectively, with hNSC-derived motor neurons. **A**, Apoptotic, TUNEL positive (green) cells in coculture with normal microglia and normal astrocytes. DAPI staining (blue) represents the mixed hNSC and microglia populations. **B**, TUNEL positive cells in coculture with ALS microglia and normal astrocytes. Note the increased apoptotic nuclei and reduced total cell numbers. **C**, TUNEL positive cells in coculture with normal microglia and ALS astrocytes. **D**, TUNEL positive cells in coculture with ALS microglia and ALS astrocytes. **E**, Average percentages of TUNEL positive cells in each group in which astrocytes were pre-treated with vehicle or a glutathione reductase inhibitor, ethacrynic acid (n=3). Data expressed as mean ± SEM. *p<0.05, significantly different from NM+NA with vehicle group. ^{+}p<0.05, significantly different from AM+NA with vehicle group. Normal microglia (NM), normal astrocytes (NA), post-disease onset ALS microglia (AM) and post-disease onset ALS astrocytes (AA). **A-D**, scale bar = 50 μm.

5.5. DISCUSSION

In the present study, we examined whether microglia and astrocytes extracted from the diseased setting in ALS were detrimental to hNSC-derived motor neurons through nitroxidative stress. Furthermore, we aimed to determine whether interactions between microglia and astrocytes affected hNSC-derived motor neurons and whether astrocytes could protect human motor neurons from nitroxidative damages through the enhancement of the main antioxidant defense pathway, glutathione. We found that microglia, isolated after disease onset in transgenic ALS rats, eradicate hNSC-derived motor neurons through direct nitroxidative damage. In addition, post-disease onset ALS microglia incapacitate normal astrocytes from performing neuroprotective roles for motor neurons, at least partially through releasing enhanced levels of ROS/RNS. Through the secretion of GSH, normal astrocytes confer moderate protection to human motor neurons from ALS microglial-induced nitroxidative injury. However, increased GSH secretion from ALS astrocytes is insufficient in providing protection, indicating that extra toxic factors or a lack of other neurotrophic factors contribute to a synergistic effect on human motor neuron death in this scenario. Overall, our data indicate that overactivated microglia in ALS may accelerate disease progression through the release of ROS/RNS, leading to degeneration and death of motor neurons by direct insults while obliterating the ability of astrocytes to support motor neuron survival.

In searching for the toxic sources to grafted hNSC-derived motor neurons in the ALS spinal cord, we turn to the host cells that are known to play critical roles in disease progression, astrocytes and microglia (Clement et al., 2003; Yamanaka and Cleveland,

2005; Beers et al., 2006; Boillee et al., 2006b; Yamanaka et al., 2008a; Yamanaka et al., 2008b). Previously, we have shown that normal adult astrocytes maintain the long-term survival of hNSC-derived motor neurons *in vitro* partially through basic FGF secretion (Jordan et al., 2008a). It was unknown whether ALS astrocytes would exhibit similar protective capacities for hNSC-derived motor neurons. Human embryonic stem cell-derived motor neurons have already been shown to be specifically vulnerable to neonatal ALS astrocyte-mediated toxicity in direct contact coculture and when cultured in astrocyte conditioned medium (Di Giorgio et al., 2007; Nagai et al., 2007; Marchetto et al., 2008). Additionally, prostaglandin D2 (PGD2) receptor activation in astrocytes has been implicated in contributing to motor neuron loss (Di Giorgio et al., 2008).

In agreement with these studies, we have shown that astrocytes isolated from transgenic ALS animals, especially after disease onset, lose neuroprotective capacity and exert added toxicity to hNSC-derived motor neurons. Observed increases in motor neuron loss with astrocytes post-disease onset may be associated with increased astrocyte dysfunction. For example, astrocytes from diseased animals may have acquired more mutant SOD1-related damage, particularly to the mitochondria (Cassina et al., 2008). Alternatively, exposure of these astrocytes to increased nitroxidative stress, or other stimuli, in the cord in ALS prior to isolation may have caused a phenotypic switch (Cassina et al., 2002). We found that astrocytes derived post-disease onset secreted higher levels of PGD2, but significant increases in iNOS expression or nitric oxide release were not detected. Human motor neuron vulnerability to ALS astrocytes in these models may simply be due to a decrease in the release of neurotrophic support while simultaneously depleting limited nutrients in the medium, which may lead to a more

rapid loss of stem cell-derived motor neurons. The possibility that ALS astrocytes secrete additional factor(s) exerting toxicity, however, cannot be excluded. In addition, primary astrocyte populations are usually contaminated with a small percentage of microglia, which can be responsible for effects that may be mistakenly attributed to astrocytes (Saura, 2007). Mutant SOD1-expressing microglia inherent to mutant SOD1-expressing astrocyte populations may negatively affect the function of astrocytes in motor neuron maintenance or directly cause toxicity to motor neurons as discussed below.

In this study, we found that mutant SOD1-expressing microglia, specifically after disease onset, contribute to human motor neuron toxicity through increased ROS/RNS production, while microglia isolated from transgenic ALS rats at a pre-symptomatic stage (age 1 month) did not cause hNSC-derived motor neuron loss. The latter is inconsistent with the previous reports showing that microglia isolated from neonatal transgenic ALS mice are toxic to primary embryonic rat motor neurons (Beers et al., 2006; Xiao et al., 2007). The discrepancy may be due to differences in the microglia isolation procedure, the type of motor neurons and experimental design. Using density gradient centrifugation, we were able to obtain pure microglia in less than 4 days for coculture experiments, as compared to 7-8 days required to shake off microglia from mixed primary glia cultures. Long-term i*n vitro* culture of microglia cells may result in artificial activation. Furthermore, the hNSC-derived motor neuron population contained approximately 10-30% $GFAP^+$ cells, presumably human astrocytes that may buffer the ALS microglia-mediated toxicity. It is unknown whether microglia acquire toxic properties through self-inflicted mutant SOD1-associated damage over time, or whether the stimuli received in the ALS microenvironment prior to isolation led to increased

reactivity and human motor neuron toxicity *in vitro*. Surprisingly, nontransgenic microglia from adult rats also displayed age-dependent changes through increases in nitric oxide and superoxide production compared to microglia from 1 month rats. Although increased, ROS/RNS release from nontransgenic adult microglia did not cross a threshold to induce significant motor neuron death. To increase the cell yield, microglia isolated from both brain and spinal cord tissues from three rats were pooled to perform these coculture experiments. Preliminary studies indicated that both spinal cord and brain-derived microglia from transgenic ALS rats after disease onset were toxic to hNSC-derived motor neurons (unpublished observation). However, it is possible that microglia specifically derived from degenerating areas within the spinal cord may exert more toxicity, and thus, the actual toxic effects of ALS microglia on grafted human motor neurons *in vivo* may be underestimated using the *in vitro* model with mixed brain and spinal cord microglia.

Our data indicate that ALS microglia derived after disease onset harmfully affect human motor neurons through noxious dialogue with normally neuroprotective astrocytes. Specifically nitroxidative stress, at least, partially inflicts a loss in the capacity of astrocytes to protect motor neurons and causes increases in PGD2 release, which has been implicated in promoting ALS astrocyte-mediated toxicity (Di Giorgio et al., 2008). Mutant SOD1-expressing microglia and astrocytes also display enhanced release of proinflammatory factors (Weydt et al., 2004; Hensley et al., 2006a) that may crosstalk and lead to a vicious cycle of exacerbated microglia overactivation and astrocyte dysfunction. To explore other potential mechanisms, related to nitroxidative stress, underlying the harmful interactions between ALS microglia and astrocytes, we examined

the secretion of GSH from normal and ALS astrocytes. GSH is synthesized, secreted and cleaved into components extracellularly by astrocytes, which represents the only source for motor neurons to utilize in synthesizing the potent antioxidant GSH (Dringen et al., 1999; Dringen et al., 2000). Previous evidence has indicated that GSH levels are decreased in transgenic ALS animals after disease onset (Chi et al., 2007) and activation of the transcription factor Nrf2 in astrocytes possibly confers protection to motor neurons in transgenic ALS animals by the enhancement of GSH release (Vargas et al., 2008). We found that although ALS astrocytes had an increased capacity for GSH synthesis and secretion *in vitro*, GSH depletion did not worsen ALS microglia-induced toxicity to human motor neurons. On the other hand, GSH secretion from normal astrocytes moderately protected human motor neurons, indicating that ALS astrocytes either fail to provide additional neurotrophic support or secrete additional factors that are toxic. Furthermore, there is evidence that GSH depletion may cause transformations in astrocytes that leads to neurotoxicity through the development of a neuroinflammatory phenotype (Lee et al., 2010). Based on our data, in developing therapies to maintain motor neurons *in vivo*, one approach would be through the upregulation of astrocyte antioxidant defense mechanisms against enhanced microglia-generated nitroxidative stress. Nrf2 activation may have also contributed to this purpose (Vargas et al., 2008). Thus, in addition to increased GSH secretion for motor neurons, spinal astrocytes may directly protect themselves from nitroxidative damage and preserve their capacity for motor neuron maintenance possibly through the secretion of neurotrophic factors as well as regulation of glutamate levels and synaptic transmission for a longer duration within the harsh microenvironment in ALS.

In conclusion, our data demonstrate that mutant SOD1-expressing microglia annihilate hNSC-derived motor neurons through enhanced release of ROS/RNS and direct nitroxidative damage. In addition, microglia-generated ROS/RNS, as well as other proinflammatory factors such as PGD2, lead to the dysfunction in the ability of normal astrocytes to protect hNSC-derived motor neurons. The possibility that ALS astrocytes secrete factor(s) directly toxic to motor neurons also exists. Essentially, in order for stem cell treatments designed to replace spinal motor neurons to succeed and allow for motor function restoration in ALS patients, the harsh microenvironment within the spinal cord must first be drastically improved.

CHAPTER 6:

SUMMARY AND FUTURE CHALLENGES: STEM CELL-DERIVED MOTOR NEURONS IN AMYOTROPHIC LATERAL SCLEROSIS

6.1. SUMMARY AND FUTURE DIRECTIONS

The major thesis of the present study was to determine the source for hNSC-derived motor neuron degeneration after transplantation into the spinal cords of transgenic ALS rats. We provide the first evidence that human NSCs produce cholinergic neurons in ALS spinal cords and that the microenvironment within the cord is detrimental to stem cell-derived motor neuron survival. Grafted motor neurons show degenerated phenotypes with small somas, similar to degenerating endogenous motor neurons during the initial injury and denervation in ALS disease progression. Regarding the source for the toxic spinal cord milieu in ALS, we reveal that microglia specifically after disease onset generate drastically higher levels of nitroxidative stress, which directly eliminates human motor neurons by apoptosis and leads to dysfunction in the motor neuron protective capacity of astrocytes. Microglia from ALS rats post-disease onset induce a transformation in normal astrocytes, in which the astrocytes become toxic to hNSC-derived motor neurons partially through enhanced prostaglandin D2 release. Interestingly, normal astrocytes also protect human motor neurons from ALS microglia-mediated toxicity through glutathione secretion. However, ALS astrocytes, albeit secreting a higher level of reduced glutathione, did not confer motor neuron protection from ALS microglial toxicity. In fact, astrocytes isolated post-disease onset led to a synergistic increase in motor neuron loss indicating that ALS astrocytes secrete either additional toxic factors or reduced neuroprotective factors compared to normal

counterparts. These data collectively support the hypothesis that ROS/RNS released from overactivated microglia in ALS directly damage human stem cell-derived motor neurons and reduce the neuroprotective capacities of astrocytes, collectively dooming motor neuron survival in ALS (illustrated in Fig. 6.1). A summary of the results from all coculture experiments between glia and hNSC-derived motor neurons is provided in Table 6.1.

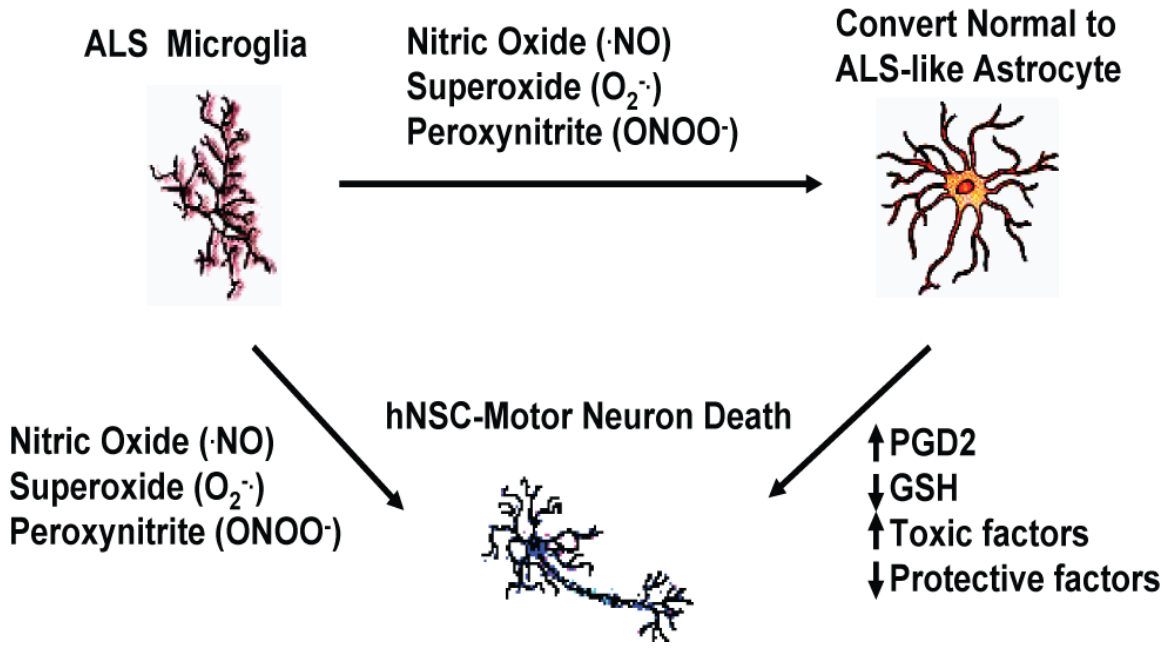

Fig. 6.1. ALS microglia-generated nitroxidative stress promotes toxicity to hNSC-derived motor neurons through direct damage and noxious interactions with astrocytes.

Table 6.1. Summary of *in vitro* coculture experiments between glial cells and hNSC-derived motor neurons.

Type of Coculture	Groups and Results			
Astrocytes in Non-contact	NA ↓	AA ↑↑	NYA ↓↓	AYA ↑
Microglia in Non-contact	NM –	AM –	ND	ND
Microglia in Direct Contact	NM –	AM ↑↑	NYM –	AYM –
Astrocytes & Microglia in Non-contact	NM + NA ↓	AM + NA ↑	NM + AA ↑↑	AM + AA ↑↑
Astrocytes & Microglia in Direct Contact	NM + NA ↓	AM + NA ↑	AM + AA ↑↑	ND
Microglia in Direct Contact & Astrocytes in Non-contact	NM + NA –	AM + NA ↑	NM + AA ↑↑	AM + AA ↑↑↑

NA = normal astrocytes (matched)
AA = ALS astrocytes (disease onset)
NYA = normal young astrocytes (1 month)
AYA = ALS young astrocytes (1 month)
NM = normal microglia (matched)
AM = ALS microglia (disease onset)
NYM = normal young microglia (1 month)
AYM = ALS young microglia (1 month)
↑ = relative increase in hNSC-derived motor neuron loss
↓ = relative decrease in hNSC-derived motor neuron loss
– = no change in hNSC-derived motor neuron survival
ND = not determined

In these studies, hNSC-derived motor neurons were transplanted into the ventral horns of transgenic ALS rats approximately one month prior to symptomatic disease onset. At this time, microglia have already migrated into areas of endogenous motor neuron injury. Microgliosis and astrogliosis occur simultaneously in the ventral horn and create a toxic environment for both endogenous and grafted motor neurons. The neuroinflammation observed in ALS also includes infiltrating $CD4^+$ T cells, which manipulate the activation status of microglia by promoting either neuroprotective or cytotoxic phenotypes (Henkel et al., 2009). When mutant SOD1 mice were crossed with mice lacking T cells, the disease was accelerated indicating that T cells promote neuroprotection in the ALS animal model (Beers et al., 2008). Further, bone marrow transplantations in T cell-deficient mutant SOD1 mice extended survival and caused increases in neurotrophic factors including IGF-1, GDNF and BDNF, increases in EAAT2 expression and the anti-inflammatory cytokine IL-4 as well as decreases in NOX2 expression and pro-inflammatory cytokines TNFα and IL-6 (Beers et al., 2008). Thus, T cells provide benefits in ALS animals possibly through the maintenance of neuroprotective properties in glia (Beers et al., 2008; Appel et al., 2010). All animals used in studies described in Chapter 4 were treated with cyclosporine A, which has been shown to extend survival in ALS animal models (Keep et al., 2001; Karlsson et al., 2004; Kirkinezos et al., 2004). If cyclosporine were not included in the experimental design, however, grafted hNSCs would not survive even in normal rat spinal cords (unpublished observation). Taken together, T cell activation is required for delaying ALS disease progression, while cyclosporine A protects grafted hNSCs through inhibition of T cell activation (Kirkinezos et al., 2004). Therefore, the paradoxical beneficial effect of

cyclosporine treatment in ALS animals is likely mediated through other yet unclear mechanisms.

Endogenous motor neurons are particularly susceptible to excitotoxicity in ALS, which develops due to decreased expression of the glutamate transporter, EAAT2, in reactive astrocytes (Howland et al., 2002). Under these circumstances, extracellular glutamate concentration increases and leads to persistent activation of amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainite receptors in motor neurons resulting in increased intracellular calcium levels (Appel et al., 2001). Motor neurons also express lower levels of calcium binding proteins including parvalbumin and calbindin-D_{28K}, which decreases their capability to handle increased calcium loads. Increased intracellular calcium ultimately results in motor neuron death (Appel et al., 2001). Oxidative and nitrosative stress also ensues in areas of microglia infiltration and motor neuron degeneration. However, whether damage from nitroxidative stress directly contributes to motor neuron death is uncertain. Some evidence indicates that activated microglia *in vitro* release increased ROS/RNS, which increases the susceptibility of motor neurons to glutamate-mediated cytotoxicity (Zhao et al., 2004). Furthermore, exogenous ROS/RNS has been shown to inhibit glutamate uptake in astroglia cultures *in vitro* (Miralles et al., 2001; Rao et al., 2003; Rao and Weiss, 2004). Preliminary data from our work has also indicated that ALS microglia induce a decrease in the expression of EAAT2 in normal astrocytes possibly through nitroxidative stress (unpublished observation). Our coculture studies indicate that microglia-generated ROS/RNS directly and indirectly contribute to hNSC-derived motor neuron death *in vitro*. When interpreting these results, however, it must be taken into consideration that atmospheric oxygen levels in the *in vitro*

experiments (~160 mm Hg) are much higher than tissue oxygenation levels *in vivo* (~40 mm Hg). Thus, it is expected that ROS/RNS generated *in vitro* is higher than *in vivo*, which may cause increased motor neuron injury and death. On the other hand, all cells in our experiments are treated under the exact same conditions, and our evidence clearly indicates that microglia from ALS rats have an increased capacity for generating ROS/RNS. However, in order to better simulate the oxygenation levels in the spinal cord microenvironment, coculture studies may be conducted in an oxygen chamber containing 5 % oxygen (~40 mm Hg).

Inhibiting ROS/RNS in direct cocultures with ALS microglia only provides partial protection to hNSC-derived motor neurons. Thus, it is likely that other factors also contribute to microglia-induced motor neuron death. Since motor neuron death is only observed in direct contact cocultures with ALS microglia, another possible cell death pathway, in addition to injury from short-lived ROS/RNS as well as glutamate-mediated AMPA/kainite overactivation, could occur through microglia-mediated activation of the Fas death receptor on hNSC-derived motor neurons. Our preliminary studies show that microglia derived specifically post-disease onset upregulate Fas ligand (FasL) (Fig. 6.2A) and differentiated hNSC-derived motor neurons express Fas receptor (Fig. 6.2B). Fas receptor antagonists could be administered *in vitro* during coculture to discern the contribution of Fas-mediated cell death. Previous evidence has indicated that primary embryonic mutant SOD1-expressing motor neurons are particularly vulnerable to nitric oxide-mediated death whereby exogenous nitric oxide stimulates the upregulation of FasL in cultured mouse motor neurons (Raoul et al., 2002; Raoul et al., 2006). FasL activates the Fas receptor leading to activation of downstream molecules including death

associated protein 6 (Daxx), p38 and neuronal NOS (nNOS). Endogenous nitric oxide synthesis then initiates a vicious feed-back loop promoting cell death. It remains to be determined whether overactivated ALS microglia, displaying upregulated iNOS, could also promote human motor neuron death by nitric oxide release and activation of Fas/FasL cell death pathway in human motor neurons through a similar mechanism. Despite *in vitro* evidence for Fas/FasL, nNOS and iNOS involvement in ALS pathogenesis, ablation of these factors by genetic knockout or pharmacologic inhibition had little to no effects on the disease progression in transgenic mutant SOD1 animals (Facchinetti et al., 1999; Upton-Rice et al., 1999a; Son et al., 2001; Petri et al., 2006). Taken together, these data indicate that inhibition of any one factor is unlikely to yield vast improvements in transgenic ALS animals due to the multitude of pathways that could contribute to motor neuron loss. Combined therapies targeting several implicated pathways must be utilized.

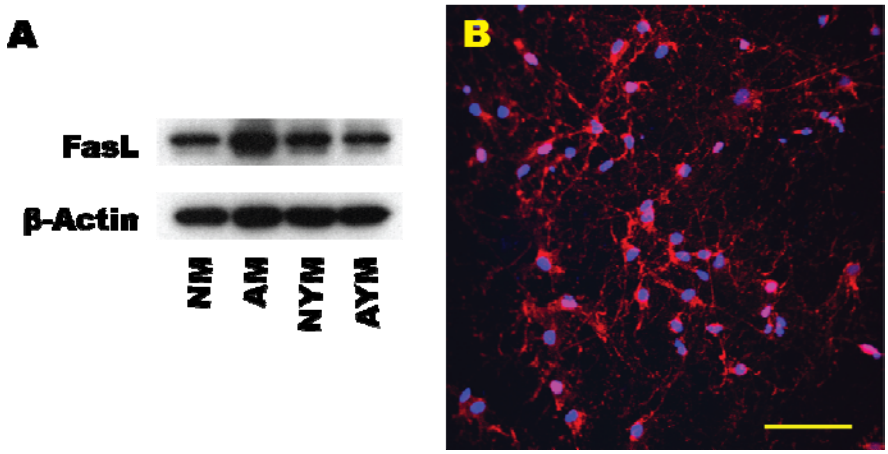

Fig. 6.2. ALS microglia may promote hNSC-derived motor neuron loss through activation of the Fas cell death receptor.

A, Western blot analysis for Fas ligand (FasL) and β-actin control in microglia. Note the upregulated FasL expression in ALS microglia post-disease onset (AM). Normal adult microglia (NM), normal young (1 month) microglia (NYM) and ALS young (1 month) microglia. **B**, Fas receptor (red) is expressed on differentiated hNSCs. Scale bar = 50 μm.

To identify additional candidate factors that may be associated with ALS microglia-induced neurotoxicity, we have conducted a microarray analysis to compare gene expression profiles between ALS microglia post-disease onset and normal adult microglia (data not shown). With further analyses of these preliminary data, potential toxic factors could be identified and tested in our *in vitro* coculture system. Upregulated factors in ALS microglia may be blocked while downregulated factors may be supplemented in cocultures with human motor neurons to test for conferred motor neuron protection. Factors known to be associated with inflammation, such as pro- or anti-inflammatory cytokines, nitroxidative stress, cell death pathways and excitotoxicity

should be principally investigated in the microglia-astrocyte-motor neuron paradigm. Cytokine cross-talk between microglia and astrocytes assuredly manipulates their distinct functions and those that directly promote or prevent toxicity may be determined. Similarly, microarray analyses comparing ALS neonatal astrocytes to normal and wild-type astrocyte controls have already been performed, but only genes overexpressed exclusively in ALS astrocytes were provided (Di Giorgio et al., 2008). It would be important to evaluate underexpressed ALS astrocyte genes as well, since decreased neurotrophic support could also contribute to the observed toxic properties to stem cell-derived motor neurons *in vitro*. Once again, several factors may be involved in astrocyte-mediated cell death as our group has shown that astrocyte-secreted basic FGF increased human motor neuron survival *in vitro* (Jordan et al., 2008a), but basic FGF provided no benefit in the transgenic ALS animals (Upton-Rice et al., 1999b). Through microarray analyses and drug screening in coculture paradigms, several potential therapeutic targets could be discovered and translated back into the transgenic ALS animal model in combination with hNSC-derived motor neuron transplants. The hope would be that a combination of several drugs could extend survival and alter the toxic microenvironment well enough to allow for grafted motor neuron survival, maturation and eventual reinnervation of muscle targets.

Our coculture data designate the need for microglial-specific iNOS and NADPH oxidase inhibition in combination with hNSC-derived motor neuron transplants in transgenic ALS animal models. Although individual iNOS inhibition in transgenic ALS mice provided no benefit (Upton-Rice et al., 1999a), NADPH oxidase inhibition exhibited a profound delay in disease progression (Harraz et al., 2008). Whether inhibition of both target enzymes simultaneously would exert additional benefits is unknown. Furthermore, it would be worthwhile to test dual transplants in which

transgenic ALS animals are irradiated and receive bone marrow transplants, thus, allowing for the infiltration of non-mutant SOD1-expressing microglia into degenerating areas of the spinal cord, while concurrently receiving hNSC-derived motor neuron grafts into the spinal cord. Non-mutant SOD1-expressing microglia show less reactivity to pro-inflammatory stimulation (Beers et al., 2006; Xiao et al., 2007). Also, since our experiments involve spinal cord transplantation and replacement of spinal motor neurons, cocultures with microglia isolated specifically from degenerating areas in the spinal cord may be more useful in evaluating potential toxic mechanisms. Methods must be developed to increase the microglia isolation yields from the spinal cord or to scale the coculture experiments down while maintaining the means to properly analyze the data. Finally, a potential intervention may be useful in preventing microglia infiltration and consequential acceleration of the disease process through blockade of the initiating signal. One initiation hypothesis is that mutant SOD1-expressing motor neurons incur insults over time related to mutant SOD1 aggregation, which causes initial degeneration/denervation and the release of signal(s) that are detected by neighboring astrocytes, resident microglia or both. One possible signal is the extracellularly released mutant SOD1, which has been shown to incite microgliosis through recognition involving toll-like receptors 2 and 4 as well as CD14 (Zhao et al., 2010). Interestingly, LPS also stimulates microglia through toll-like receptor 4 activation and thus, can be utilized in assessing the reactivity of microglia isolated from presymptomatic mutant SOD1 rats. Mutant SOD1 microglia isolated at presymptomatic ages may be more reactive to proinflammatory stimulation and could induce motor neuron injury under such circumstances. Other potential initiating signals may be determined using hNSC- or other stem cell-derived motor neurons. These cell lines may be transduced with mutant SOD1 and cultured with or without pre-symptomatic or normal microglia. If significant motor

neuron loss is encountered in mutant SOD1-expressing motor neurons cocultured with microglia compared to motor neuron cultures alone, then further analyses may be conducted to identify secreted factors that provoke microglial toxicity.

6.2. CHALLENGES TO STEM CELL THERAPY IN ALS

Several challenges must be overcome before stem cell treatment for replacing lost motor neurons in ALS patients becomes reality. Not only will combined therapeutic methods need to be developed to protect transplanted stem cell-derived motor neurons from the hazardous ALS spinal cord microenvironment, but effective means of preventing immune rejection of the grafts will also need to be implemented. Furthermore, if long term survival of the transplanted motor neurons is accomplished, axonal propagation into ventral roots and down appropriate pathways toward denervated muscle tissue will need to be achieved. In addition, the appropriate type and amount of stem cells as well as the segmental locations and number of graft sites will need to be optimized for attaining the most efficacious transplants possible. Low numbers of motor neurons transplanted into a few localized sites within the cervical or lumbar spinal cord, as in transgenic ALS animal models, may not prove to be as beneficial in patients. ALS is a result of a rather widely spread degeneration of upper motor neurons in the brain, and both motor neurons and interneurons throughout the spinal cord. It is questionable that replacing spinal motor neurons in a few segments of the cord alone will be effective without a new supply of upper motor neurons and interneurons. Along this line, the route of stem cell delivery becomes one of the most critical issues. Current techniques in obtaining a significant number of motor neurons from stem cells *in vivo* are limited to

those grafting the cells directly into the ventral horn of spinal cords (Table 1.1). One way to allow implanted stem cells to spread throughout the CNS is through intrathecal injection, which permits cells to circulate via the CSF. However, it is unknown whether stem cells grafted in this manner will efficiently generate neurons, particularly motor neurons, and locate to pathological areas in the CNS. Also, since glial cells may be irreversibly dysfunctional and overactivated, stem cell therapies aimed at incorporating functional glial cells around grafted motor neurons may need to be combined with motor neuron replacement. Finally, it is likely that each individual ALS patient will require a unique, personalized stem cell therapy based on areas of the spinal cord affected at disease onset and how wide-spread the disease has become when initiating the stem cell therapy.

Due to immune rejection of allogeneic ESC or NSC transplants, optimal immunosuppressive regimens for achieving and maintaining long-term efficacy of the grafts will need to be developed as has been shown in one pre-clinical study (Yan et al., 2006). Reduced immune rejection will limit the number of stem cell-derived motor neurons that need to be intraspinally transplanted. One strategy to circumvent the use of allogeneic transplants would be to transplant autologous iPS cells that are differentiated into motor neurons or glial cells. Somatic cells have been shown to change into iPS cells when transduced to overexpress critical genes essential for embryonic stem cells (Takahashi et al., 2007; Yu et al., 2007). In fact, somatic cells from an elderly patient with familial ALS have been induced into the pluripotent state and then differentiated into motor neurons (Dimos et al., 2008). This technique could be invaluable, not only in providing ALS patient-specific immune compliant stem cell-derived motor neurons or

glia for transplantation, but also for *in vitro* studies on drug screening, motor neuron death mechanisms and glial overactivation mechanisms. Bone marrow or mesenchymal stem cell transplants may also be useful in ALS patients to provide neuroprotection through the secretion of trophic factors or the replacement of overactivated microglial cells, especially since autologous bone marrow cells are easily obtained. A drawback to autologous transplantation, whether the cells are derived from the bone marrow or iPS cells, relies in the fact that cells that were originally predisposed to developing ALS will be transplanted into an ALS environment. Whether autologous stem cell-derived motor neurons and glial cells will reactivate ALS is unknown. One study has indicated that MSCs isolated from the bone marrow of transgenic mutant $SOD1^{G93A}$ rats displayed an impaired neuroprotective capacity, including a reduced ability to take up aspartate (Boucherie et al., 2008). Thus, it is possible that autologous stem cell-derived neural cells will not be very efficacious in providing protection. On the other hand, MSCs derived from the bone marrow of human donors and sporadic ALS patients did not have any apparent differences in proliferation rates, differentiation capacity or chromosomal appearance (Ferrero et al., 2008), but the neuroprotective capacity was not determined *in vitro* or in transgenic ALS animal models.

One strategy in lowering the number of stem cells needed to provide an efficacious number of motor neurons after transplantation is to develop techniques that increase the quantity of motor neuron differentiation after transplantation. This may require a pre-transplant priming stage in ESCs or NSCs to increase their motor neuron generation capacity. Higher quantities of human motor neurons following engraftment into the adult rat spinal cord have been generated by priming human NSCs in a cocktail

containing bFGF (Wu et al., 2002). The grafted human NSC-derived motor neurons were able to send axons through the ventral roots, innervated distal muscles by forming neuromuscular junctions and improved the motor function of rats with sciatic axotomy-induced motor neuron deficiency (Gao et al., 2005; Gao et al., 2007). The possibility of a future stem cell-based motor neuron replacement therapy for ALS has also been suggested by the finding that mouse ESC-derived pre-committed motor neurons sent axons through ventral roots, reached target muscle tissue and partially improved paralysis when combined with molecules that augmented axonal outgrowth in a rat model of virus-induced motor neuron death (Harper et al., 2004; Deshpande et al., 2006). These studies indicate that if immune rejection is prevented and long-term survival of stem cell-derived motor neurons is achieved, then motor neurons do have the potential to reach distal muscle targets. Since most ALS patients die within 3-5 years after disease onset, the effectiveness of transplanting stem cell-derived motor neurons into the spinal cord is questionable due to the long distances transplanted cells must grow to reach affected muscles. Even if the new motor neurons were resistant to the toxic ALS environment, it would theoretically take approximately 2-3 years for motor neurons to extend axons and form synaptic connections at neuromuscular junctions across these long distances based on an axonal growth of 1 mm per day. However, the hope is that combined therapies can be developed that will both protect transplanted motor neurons in the toxic ALS milieu and delay or even halt the disease progression, thus, allowing more time to establish muscle innervations from new motor neurons. It is, of course, also rational to implement stem cell therapeutic strategies aimed to regenerate the axons of surviving endogenous motor neurons potentially through the use of genetically modified stem cells that secrete

vital neurotrophic factors within the spinal cord to rescue motor neuron somas and from muscle targets to attract axons as mentioned above in pre-clinical studies. Such stem cell transplantations acting as biological trophic factor pumps seem essential to slowing the disease progression and maintaining as many viable motor neurons as possible prior to or in combination with commencing a motor neuron stem cell replacement therapy.

6.3. CONCLUSION

Stem cells provide the means to replace lost spinal motor neurons and potentially recover from paralysis in ALS. However, other therapies will need to be combined with motor neuron replacement to strengthen the therapeutic efficacy, since transplanted motor neurons are likely just as vulnerable to the toxic ALS microenvironment as endogenous motor neurons. *In vitro* coculture systems utilizing human stem cell-derived motor neurons together with diseased glial cells can be used to dissect both cell autonomous and non-cell autonomous mechanisms of ALS. Thus, stem cell-derived motor neurons provide an invaluable tool to develop combined therapies as drugs can be screened for their ability to prevent motor neuron death. It is likely that multiple drugs will be required to protect motor neurons due to the multi-factorial nature of ALS pathogenesis. Optimistically, combined therapies designed to protect human motor neurons *in vitro* will also save remaining endogenous motor neurons and slow or even halt the disease progression in transgenic ALS animal models and ALS patients. Once disease progression is significantly slowed, then hopefully, the spinal cord microenvironment will be amenable to motor neuron replacement using a variety of potential stem cell sources and ALS patients may begin their road toward recovery of motor function.

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VITA

Jason Robert Thonhoff was born on August 24, 1981, in Austin, Texas. His parents are Bob and Kim Thonhoff and his younger brother, Travis, was born in 1985. Jason graduated from James Bowie High School in Austin in 1999 and went on to earn a Bachelor of Science degree, majoring in Chemistry and Molecular Biology, at Texas Lutheran University in 2003. While at Texas Lutheran University, Jason participated in research projects pertaining to organometallic synthesis under the direction of his mentor, Dr. David Wasmund. He also gained clinical research experience working on a project to identify predictors of coronary in-stent restenosis during a medical internship with Dr. Ali Mortazavi at the Kelsey-Seybold Medical Center and St. Luke's Episcopal Hospital in Houston, Texas. Throughout high school and college, Jason developed a passion for biomedical research and medicine, and matriculated into the M.D./Ph.D. Program at UTMB in 2003. Jason later joined the Neuroscience Graduate Program in 2005 and in the laboratory of Dr. Ping Wu, he studied potential applications for human neural stem cells in modeling disease mechanisms, drug screening and therapy for Lou Gehrig's Disease. Jason received a Ruth L. Kirschstein National Research Service Fellowship from the National Institutes of Health to help fund this project. He also presented his research at Society for Neuroscience Annual Meetings, National Student Research Forums and Mission Connect Symposiums sponsored by The Institute for Rehabilitation and Research. Jason's future plans are to continue his medical training in a neurological field, while pursuing his research interests in the development of stem cell therapies for neurodegenerative diseases and neurological trauma.

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B.S., S*umma cum laude*, Chemistry and Molecular Biology, May 2003, Texas Lutheran University, Seguin, Texas.

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