THERAPEUTIC POTENTIAL OF RADIAL GLIAL RG3.6 CELLS IN RAT SPINAL CORD INJURY

By

YU-WEN CHANG

A Dissertation submitted to the Graduate School-New Brunswick

Rutgers, The State University of New Jersey

And

The Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in Cell Biology and Neuroscience

Written under the direction of

Dr. Martin Grumet

and approved by

_____

_____

_____

_____

_____

New Brunswick, New Jersey

October 2007

ABSTRACT OF THE DISSERTATION

Therapeutic potential of radial glial RG3.6 cells in rat spinal cord injury

by YU-WEN CHANG

Dissertation Director:

Martin Grumet

Spinal cord injury (SCI) triggers a cascade of pathophysiological changes that lead to secondary tissue damage after the mechanical insult. Early after SCI, cells are disrupted and excitotoxic amino acids (e.g. glutamate) are released. Inflammatory cytokines and chemokines are quickly induced upon injury, followed by leukocyte infiltration (e.g. neutrophil and macrophage). Neurons and glia undergo massive necrotic cell death and apoptosis. Axons become progressively degenerated and extracellular substrate deposits in the lesion site, which leads to cyst and scar formation.

Cell-based therapies have been widely applied during SCI sub-acute phase and at least one mechanism associated with behavioral improvement is promotion of axonal remyelination and regeneration. However, the efficacy of cell transplantation at acute SCI and associated mechanism has rarely been studied.

Radial glial cells are essential in guiding and supporting neuronal migration during central nervous system development. They are neural stem cells and have trophic functions under certain conditions. In this thesis, our goal is to understand whether acute transplantation of radial glia can mitigate deleterious responses in the injured spinal cord tissue and promote functional recovery. RG3.6 is a stable neural stem cell clone derived from E13.5 cortex and has radial glial phenotypes including bipolar shape, expression of BLBP and GLAST, and support of neurite growth. Transplanted in normal spinal cord,

RG3.6 retained radial glial phenotype and actively migrated along rostral-caudal axis of spinal cord and integrated nicely with host tissue. In SCI, acute transplantation of RG3.6 significantly improved locomotion recovery as early as 2 days post injury. The functional recovery may be associated with fewer infiltrating macrophages, less CSPG deposition, and more axonal preservation. At the molecular level, RG3.6 cell enhanced the expression of genes that are involved in tissue defense and stem cell development.

In summary, radial glia in acute SCI modulated host responses to the injury by mediating certain gene expressions at early times, suppressed neuroinflammation, and supported and reorganized axons during later phases. Our results provide different perspectives of cell-based therapy for acute SCI.

Preface

There are about 2.5 million people living with spinal cord injury (SCI) in the world, a quarter of a million are in the United States. Each year, over 130,000 new injuries occur in the world with 10,000 in the United States (Thuret, Moon et al. 2006). SCI is a severe central nervous system (CNS) trauma, which causes tremendous emotional and financial hardship to the patients, their loved ones and our society. The injury usually is not restricted to the traumatized site, as the pathology changes progress and tissue remodeling occurs over months to years. Tissue distal to the injury site also undergoes degeneration. Therefore, pathological changes in SCI also make it a degenerative disease which affects a broad array of cell types. Improved medical technology and the introduction of high-dose of methylprednisolone (MP) acutely following injury have greatly increased survival rate for SCI patients. With advanced primary care and routine physical rehabilitation, SCI patients can still maintain life quality and normal life-span. Although surgical stabilization of the vertebral column and application of MP acutely following injury can preserve some sensory and motor function, there is still a great unmet need for SCI patients to lead a satisfying life. Scientists and clinicians are still on a mission to find more effective treatments that significantly restore functions following the injury.

This thesis is focused on studying the efficacy of radial glial cell therapy on acute SCI using rats as animal model. Histological, behavioral, and gene expression changes associated with acute radial glia treatment are documented.

Acknowledgement

This work was performed from October 2002 to March 2007 in the W. M. Keck Center for Collaborative Neuroscience at Rutgers University. The completion of this thesis offers me an opportunity to express my sincere appreciation and gratitude to those who have helped me in science and life for the past almost 5 years in New Jersey.

Advisor & mentors

My first thanks go to Dr. Martin Grumet, my thesis advisor, for introducing me to the field of radial glial cells in spinal cord injury research. I always had unspeakable faith in stem cells, and it was the training and studying in his lab decoded the rationale behind my belief in stem cells. I appreciate the opportunity he gave me to work on the project and his openness allowing me to devote my own thoughts in developing the project. I am also grateful to him for assigning two superb scientists, Drs. Osamu Ikeda and Hedong Li, as my mentors in establishing my background in spinal cord and stem cell research. I was fortunate to have Osamu to teach me spine surgeries, behavior and histology analysis. I enjoyed our discussion about SCI research and his experience of treating patients with SCI. I am grateful to him for his willingness and patience in coaching my scientific endeavors; for his encouragement in guiding me build up an optimistic attitude in this business. I thank Hedong for teaching me how to work with stem cells from the very beginning and for his wonderful personality in calming the atmosphere when working around him. Also, for inviting me to many enjoyable Christian celebrations.

Committee members

I want to express my gratitude to my committee members for their insightful suggestions on my thesis project over the years.

-Dr. Wise Young was the reason I came to Rutgers. For me, he is the inspiration in SCI research. I am thankful to him for his advices on my thesis project and his great spirit in promoting the awareness of spinal cord injury and stem cell research in the society.

- to Dr. Bonnie Firestein for graciously giving me suggestions on my research projects and guidance in searching lab for postdoc training.

-Special thanks are due to Dr. Richard Nowakowski who has become one of the influential people in my budding research career. If it wasn't for his intellectual lectures and our great hours of conversations about "real" identity of radial glia, I would not have enjoyed my study this much. I appreciate his time and words of wisdom in guiding me to find the field I really like.

-to Dr. Rajiv Ratan from Burke Institute for serving on my outside examiner for my thesis defense.

Colleagues

-to Dr. Ronald Hart and Loyal Goff for their assistance on microarray analysis.

-I have great thanks to people in animal facility in taking care of my SCI animals and documenting BBB scores; these people are Hock Ng, Sean O'leary, Ryan Kirchoff, Judith Stugus, Bor Tom Ng, Pui Tom.

-to Dr. Patricia Morton for giving me the opportunity to tour and meet SCI families in the center and special appreciation to her effort in making Keck center a family-like environment.

-to Dr. Crista Adamson for her wonderful personality and her comments on my thesis.

-to Dr. Tsutomu Iseda for teaching me biostatistics and for his brightest smile in lighting up the bench area.

-to Dr. Noriko Kane-Goldsmith for introducing me confocal microscopy and image analysis; and for teaching me how to make authentic Japanese Sushi. Also for the emergency food she gave me over the years.

-to Joanne Babiarz for being a resourceful lab mate and great office neighbor.

-to Sayantani Basak for our conversations about science and life.

Friends

-to Christopher Ricupero, my best friend in Keck, for all our conversations (silly and intellectual) and our great friendship; also for fixing my English when needed.

-to Jessica Lam, for her generous support and kindest friendship.

-to Jonathan Davila for bringing up the spirit in the room and introducing me to wonderful Puerto Rican food.

-to Kevin Thomson for exchanging information about computer and refreshing my passion to Physics.

-to Elina Tzatzalos for bringing in new air in the lab and introducing Greek culture to me.

-to Xin Yue for our lunch-encouragement sessions and our friendship.

-to Shulun Chang for being the greatest roommate during the time I wrote my thesis.

-to Guey-Shin Wang for being my best scientific sibling and for the time we discussed science and life over MSN.

-to Jonathan Zhiyong Pan for teaching me real-time PCR, his comments on my thesis drafts and his companionship for the past 4 and half years.

Family

My biggest acknowledgment goes to my family for their endless love, support, encouragement, and believing in me. In particular, I am truly grateful to my mother,

Guey-In Chung, for her magic power in making me believe that I can take on any tasks in the world; and for praying to God for me and for my animals. To my father, Kuang-Hsung Chang, for teaching me the importance of being a humble person. To my big sister Mei-Hui, for her warmest care and the joy her two adorable girls have been bringing to our family. To my second sister, Mei-Fang, for introducing "English" to my world and widening my view with English literature and Greek Mythology. To my third sister, Li-Wen, for being my faithful listener and for our endless conversation about things in the world: from literature to politics to science to family to dogs in the neighborhood. To my dearest brother, Bruce, for being so supportive and informative about almost everything. To our dog DoDo for bringing so much laughter in Chang's family.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter Three

LIST OF TABLES

Introduction

Part I. Pathophysiology of Spinal cord injury

Section A. Acute Phase

- From primary injury to initiation of secondary injury

Spinal cord injury is usually induced by direct bone fracture, dislocation of spinal disc and ligament in forms of compression or contusion. Blood vessels are damaged, which leads to hemorrhage and ischemia at the injury epicenter within minutes after injury (Balentine 1978; Balentine 1978; Gale, Kerasidis et al. 1985; Noble and Wrathall 1985). Physical disruption of tissue causes membrane depolarization and results in massive glutamate release to activate NMDA and AMPA receptors to toxic levels. These events not only cause the first wave of necrotic cell death to neurons but also trigger apoptotic signals in neighboring cells (Hall and Springer 2004). Rapid calcium influx induces lactate accumulation, nitric oxide production, lipid peroxidation and release of arachidonic acid. Arachidonic acid will be transformed into prostaglandin and leukotriene by cyclooxygenase and lipsoxygenase respectively (Bartholdi and Schwab 1997; Resnick, Graham et al. 1998; Liu, Li et al. 2001; Lucas, Wheeler et al. 2002; Nakahara, Yone et al. 2002). These serial anatomical, cellular and chemical shocks during the acute phase spreads centripetally and causes prolonged tissue ischemia/edema that abolishes regulation of blood flow, normal circuitry in the spinal cord, as well as networks between spinal cord and other organs (Hausmann 2003).

Like a rolling snow ball, a single physical insult to spinal cord tissue triggers self-amplifying cascades of destructive cellular changes. Disrupted spinal cord tissue and

necrotic cells release pro-inflammatory cytokines and other toxic chemicals. These changes all contribute to the secondary injury that leads to more cell death in the spinal cord.

Section B. Sub-acute phase

- Prolonged inflammatory response

Secondary injury of SCI is characterized by increased tissue inflammation, myelin degeneration, and glial cell death at the injury epicenter and adjacent areas. Infiltrating leukocytes repair the tissue by removing debris of dead cells and secreting cytokines that recruit more leukocytes if needed. Leukocytes also promote angiogenesis, cell growth, and reconstitute connective tissue (Nathan 1987; Jones, McDaniel et al. 2005). However, for spinal cord trauma, the beneficial effects of leukocyte infiltration are controversial (Bethea 2000; Hauben and Schwartz 2003; Popovich and Jones 2003).

The primary insult initiates an immune response of local microglia and recruits peripheral neutrophils, followed by macrophages and T-lymphocytes, to the injury site (Popovich, Wei et al. 1997; Zhang, Krebs et al. 1997; Carlson, Parrish et al. 1998). Both pro-and anti-inflammatory cytokines and chemokines are present in the injured spinal cord.

Resident microglia are activated by injury and become phagocytic, which resemble infiltrating macrophages morphologically and functionally (Kreutzberg 1996). Activated microglia secrete pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumor necrotic factor alpha (TNF-α). In contused rat spinal cord, these cytokines peak 6-12 hours after injury and remain elevated until fourth day following injury (Giulian, Baker et al. 1986; Kreutzberg 1996; Bethea 2000; Jones, McDaniel et al. 2005). These cytokines

further stimulate endothelial cells to produce chemokines, including IL-8 (human), monocyte chemotactic protein (MCP, rat), and macrophage inflammatory protein (MIP) for the recruitment of neutrophils and monocytes (macrophages) to the injury site (McTigue, Tani et al. 1998; Jones, McDaniel et al. 2005). Endothelial cells also synthesize adhesion molecules, such as intracellular adhesion molecule (ICAM)-1, P selectin, E-selectin and integrins in response to elevated IL-1, IL-8 and TNF-α. These molecules facilitate neurophil migration to the injury site (Hamada, Ikata et al. 1996; Danton and Dietrich 2003). Neutrophils stay in the injury site transiently (1-2 days) (McTigue, Popovich et al. 2000) but their short presence attracts more leukocytes (mostly macrophages) to the spinal cord for days after injury. The infiltrating leukocytes and activated microglia clear up debris of dead cells. This process produces large amounts of nitric oxide (NO) and TNF-α (Anderson 1995; Taoka, Okajima et al. 1997; Carlson, Parrish et al. 1998).

T-lymphocytes (T-cells) invade the injury site around 3 days post injury and remain in considerable numbers for more than 6 weeks following SCI (McTigue, Popovich et al. 2000; Sroga, Jones et al. 2003). T-cells work closely with other leukocytes and have a biphasic function in the lesion site, where they secret IL-10 to suppress inflammation but also produce interferon (IFN) γ to activate macrophages (Lodge and Sriram 1996). Some T-cells become reactive to myelin basic protein (MBP) and destroy myelin together with IFNγ activated macrophages (Popovich, Horner et al. 1996; Gimsa, Peter et al. 2000).

- Apoptosis in glial cells

Massive cytokines released during sub-acute phase of SCI eventually initiate a series of apoptosis in all cell types in the spinal cord. Shortly after injury, neurons undergo necrotic cell death. Surprisingly, white matter remains intact during the first few hours following injury (Rosenberg and Wrathall 1997). However, about 8 days post-injury, many apoptotic cells can be observed adjacent to myelin sheath. These cells are positively immunolabeled by CC1 antibody suggesting they are oligodendrocytes (Crowe, Bresnahan et al. 1997; Shuman, Bresnahan et al. 1997). The degeneration of oligodendrocyte progresses centripetally to regions far from the primary injury site, which may be due to lack of trophic support after axonal loss and/or apoptosis triggered by overloaded pro-inflammatory cytokines (Barres, Jacobson et al. 1993; Barres and Raff 1993; Crowe, Bresnahan et al. 1997). Apoptosis also occurs in microglia and invading macrophages (Dusart and Schwab 1994; Popovich, Wei et al. 1997; Shuman, Bresnahan et al. 1997). This second wave (first wave is neuronal death in acute phase) of cell death of oligodendrocyte and microglia predominantly happens in the white matter tracts, which impairs ascending and descending spinal tracts that connect the spinal cord to rest of the body. This is known as Wallerian degeneration following SCI (Fu and Gordon 1995; Fu and Gordon 1995). Myelin debris and degenerating oligodendrocyte carry several inhibitory molecules such as NOGO-A, myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgp), semaphorin, and CSPG versican, each of which inhibits endogenous axonal regeneration (Fawcett 2006; Harel and Strittmatter 2006).

Pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) activate several signal transduction pathways that mediate programmed cell death. Signaling through IL-1 or

TNF-α receptors activates NFκB, mitogen activated protein kinase (MAPKs) including p38, c-Jun N-terminal kinase (JNK), and IκB kinase. These signal transductions also alter activity of caspase 3 and caspase 8 to trigger apoptosis (Keane, Davis et al. 2006). Delivering IL-1 receptor 1alpha (IL-1ra) antagonist to contused spinal cord significantly suppresses caspase 3 activation and reduces cell apoptosis (Nesic, Xu et al. 2001). TNF-α is the most potent cytokine in apoptosis initiation when bound to TNF-α receptor 1 (TNFR1) and CD95/Fas (Casha, Yu et al. 2001; Keane, Davis et al. 2006). Activation of CD95/Ras initiates cell apoptosis either through apoptosome formation (complex of cytochrome c, Apaf1,and caspase 9) or activation of caspase 8 signaling (Keane, Davis et al. 2006). Neutralizing CD95 ligand following SCI significantly decreases the number of apoptotic cells, increases survival of neurons and oligodendrocytes, and improves behavioral recovery (Demjen, Klussmann et al. 2004). Minocycline has been shown to improve functional recovery in contusive SCI partly by reducing cytochrome c and apoptosis (Teng, Choi et al. 2004). The decrease in cytochrome c may be attributed to suppression of CD95/Fas signaling.

IL-6 belongs to family proteins of ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF). IL-6 is involved in B-cell differentiation, T-cell maturation and activation (Hirano and Kishimoto 1992). Activation of IL-6 receptor (IL6R) initiates pathways of JAK (Janus family of tyrosine kinase) and STAT (signal transducer and activator of transcription) (Schindler and Darnell 1995), that are important in regulating stem cell differentiation into astroglia fate and will be described later.

- Anti-inflammatory cytokine and trophic factors

Anti-inflammatory cytokines are also present in the injured spinal cord to antagonize pro-inflammatory cytokine and terminate immune response. These cytokines include IL-4, IL-5, IL-10, IL-13 and transforming growth factor β (TGF-β), etc. (Bethea 2000). IL-10 is produced by T-cells, microglia/macrophages, and astrocytes (Moore, de Waal Malefyt et al. 2001). IL-10 exhibits potent anti-inflammatory effect in reducing the production of cytokines and chemokines, such as IL-1, IL-6, TNF-α, MCP1, MIP1/2 and even IL-10 itself. Therefore, IL-10 may suppress leukocyte infiltration to the injury site (Moore, de Waal Malefyt et al. 2001). Besides attenuating inflammatory signals, activation of IL-10 receptor prevents apoptosis by up-regulating anti-apoptosis protein Bcl-2 (Levy and Brouet 1994). Several studies have reported that administration of IL-10 following SCI can promote functional recovery by reducing inflammation and preserving cells (Bethea, Nagashima et al. 1999; Brewer, Bethea et al. 1999; Jackson, Messinger et al. 2005). However, IL-10 renders tissue protection only when it is administered during SCI acute phase; no significant improvement was found if IL-10 was applied later than 72 hours post injury (Bethea 2000). In view of highly concentrated cytokines (both pro- and anti-inflammatory) are accumulated in the injured spinal cord at 3 to 4 days post SCI (Popovich, Wei et al. 1997; McTigue, Popovich et al. 2000), the anti-inflammatory effect of IL-10 may not overcome the sluiced immune response.

TGF-β is another potent anti-inflammatory cytokine that is synthesized by almost every type of leukocyte, including macrophage, lymphocyte, and dendritic cell. TGF-β is known to reduce T-cell proliferation through inhibiting IL-2 production (Cottrez and Groux 2001; Li, Wan et al. 2006) and suppress T-cells recruitment to the injury site. TGF-β also has several anti-apoptotic features, which include inhibiting TNF-α receptor

CD95/Fas signaling (Li, Wan et al. 2006), enhancing NF-κB production (Zhu, Culmsee et al. 2004), and inactivating pro-apoptotic protein Bad (Zhu, Yang et al. 2002).

SCI-induced immune activity has both beneficial and detrimental properties. On one hand, it activates tissue inflammation and causes cell death; on the other hand, it clears up dead cells to facilitate recovery and triggers endogenous repair mechanism by producing trophic factors. Activated microglia and macrophages produce brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and glial cell line-derived neurotrophic factor (GDNF) in response to injury (Batchelor, Liberatore et al. 1999; Dreyfus, Dai et al. 1999; Ikeda, Murakami et al. 2001). Although IL-1 recruits immune cells to the injury site and often initiates toxic levels of tissue inflammation after SCI it promotes tissue repair by stimulating growth factor production, e.g. insulin like growth factor (IGF) and platelet derived growth factor (PDGF), that are known to induce proliferation and differentiation of astrocyte or oligodendrocyte (Silberstein, De Simone et al. 1996; Mason, Ye et al. 2000). Moreover, while promoting excess microglia/macrophage proliferation in the injured spinal tissue, IL-6 is crucial in preventing oligodendrocyte apoptosis during sub-acute phase of SCI (Kerr and Patterson 2005).

Immune response is indeed a double-edged sword in SCI (Bethea 2000). Keeping inflammatory response in balance at the right time, right place and right magnitude may be the key to restore tissue from primary insult.

Section C. Chronic phase

- Scar formation and CSPG deposition

The pathology changes during chronic phase of SCI are characterized by glial scar formation and progressive axon and myelin degeneration, which are developed in association with earlier immune response. Inflammation is an intrinsic mechanism to employ immune effectors (e.g. leukocytes) to clean dead cells and repair tissue. However, excess immune activity results in prolonged inflammation that can promote accumulation of activated macrophages at the injury site even months after SCI (Sroga, Jones et al. 2003; Silver and Miller 2004). Cytokines secreted from these immune cells, such as IL-1, activate proliferation and reactivity of astrocyte (Herx and Yong 2001; Liberto, Albrecht et al. 2004). Cytokine IL-6 can promote differentiation of endogenous neural stem cell and/or neural precursor cell into GFAP-positive astrocyte through activating STAT/JAK signaling (Bonni, Sun et al. 1997; Taga and Fukuda 2005). These reactive astrocytes, together with some oligodendrocyte precursor cells (OPCs) and macrophages gradually seal off the injury site by forming tenacious scar tissue in order to prevent more leukocytes invading to the injury site (Fawcett 2006).

Ablation of scar forming reactive astrocyte in traumatic brain injuries (TBI, e.g. stab or contusive injury) and SCI causes massive leukocytes infiltrating to the injury site, poor blood-brain-barrier (BBB) repair, increased neuronal death and myelin degeneration (Bush, Puvanachandra et al. 1999; Faulkner, Herrmann et al. 2004; Myer, Gurkoff et al. 2006). In addition, without reactive astrocyte, locomotion recovery is fully abolished (Faulkner, Herrmann et al. 2004). These studies suggest that scar formation is extremely important in regulating tissue inflammation, protecting neuron and oligodendrocyte, and reconstituting the BBB. However, the scar tissue becomes a physical and biochemical barrier for future regenerating axons which then form dystrophic endbulbs in front of the

scar (Beattie, Hermann et al. 2002; Silver and Miller 2004). Many inhibitory factors have been identified in the scar tissue, including members in the family of chondroitin sulphate proteoglycans (CSPGs), eph/ephrin, and semaphorins (Morgenstern, Asher et al. 2002; Tang, Davies et al. 2003; Benson, Romero et al. 2005; Fawcett 2006). Members of CSPGs include aggrecan, brevican, neurocan, phosphocan, versican, and NG2, all of which have chondroitin sulphate side chains (Morgenstern, Asher et al. 2002). The sulphated glycosaminoglycan (GAG) side chain of CSPG is inhibitory for axon growth (Properzi, Asher et al. 2003; Properzi, Carulli et al. 2005). Enzymatic digesting GAG chains by chondroitinase ABC has been applied in several TBI and SCI models, which showed significant axonal re-growth/ regeneration (Moon, Asher et al. 2001; Bradbury, Moon et al. 2002; Yick, Cheung et al. 2003; Caggiano, Zimber et al. 2005; Barritt, Davies et al. 2006). The mechanism by which the GAG chains inhibit axon growth is still unclear. Some studies reported that CSPGs or their GAG chains mask and/or interfere with axon-favorable adhesion molecules, such as laminin, L1, N-CAM and form a non-permissive environment for axon growth (Grumet, Flaccus et al. 1993; Friedlander, Milev et al. 1994; Condic, Snow et al. 1999; Sugahara, Mikami et al. 2003). Besides GAG chains, core proteins of some CSPGs also have inhibitory properties that cannot be removed by chondroitinase ABC; for examples core proteins of versican and NG2 (Dou and Levine 1994; Schmalfeldt, Bandtlow et al. 2000).

NG2 is one of the CSPGs that is highly up-regulated in the scar tissue after brain and spinal cord injury (Levine 1994; Jones, Yamaguchi et al. 2002; Tang, Davies et al. 2003). NG2 is expressed mostly by oligodendrocyte precursors and activated macrophages and some by reactive astrocytes (Nishiyama, Chang et al. 1999; Jones,

Yamaguchi et al. 2002). Several studies have shown that NG2 inhibits neurite outgrowth in vitro and in vivo (Dou and Levine 1994; Jones, Yamaguchi et al. 2002; Ughrin, Chen et al. 2003). Digestion of NG2 with chondroitinase ABC, however, does not eliminate its inhibition to axon growth, suggesting that the core structure of NG2 alone can effectively inhibit axons (Dou and Levine 1994). In addition to GAG chains located at extracellular domain (ECD) 2 of NG2, NG2 has other ECD domains (1 and 3) that can mediate most of the inhibition of neurite growth (Ughrin, Chen et al. 2003; Tan, Zhang et al. 2005). In a recent study of dorsal hemi-section injury model, neutralizing NG2 by applying antibodies against NG2 following SCI successfully induced sensory axonal regeneration (Tan, Colletti et al. 2006).

The strategy of reducing CSPG or NG2 deposition by chondroitinase ABC or antibody respectively in TBI and SCI has provided a more permissive environment for axon growth, however, significant functional recovery (motor and sensory) has not been reported from these treatments (Bradbury, Moon et al. 2002; Yick, So et al. 2004; Barritt, Davies et al. 2006). Modulating scar tissue alone may not be sufficient to attain prominent behavioral improvement. Combination approaches will be more effective for SCI in the chronic phase (Busch and Silver 2007).

Part II. Cell-based therapy in spinal cord injury research

Cell-based therapy has been extensively studied in various SCI animal models and shown to reduce tissue damage, restore neuronal activity and improve behavioral recovery following injury. The possible mechanisms underlying the recovery include tissue protection from secondary injury, bridging scaffold for axon growth, replacement of myelin forming cells, and promotion of axonal regeneration.

Cell transplantation in the acute or chronic phase of SCI encounters different advantages and challenges, which may accordingly affect activity of these implanted cells and their efficacy in promoting functional recovery.

Section A. Cell transplantation in SCI acute phase

The biggest challenge of applying cells to acutely injured spinal cord is to maintain their survival in the extremely complicated microenvironment that contains excitatory glutamate, reactive oxygen species, destructive enzymes and pro-inflammatory cytokines (Okano, Ogawa et al. 2003; Hall and Springer 2004). Transplantation of neural stem cells (NSCs) to spinal cord immediately following injury showed poor survival of implanted cells (Cao, Zhang et al. 2001; Hasegawa, Chang et al. 2005). However, more cells survived with an increased cell density during injection, and the majority of them differentiate into astrocyte in injured spinal cord (Cao, Zhang et al. 2001; Okano, Ogawa et al. 2003; Hasegawa, Chang et al. 2005). Astroglial fate of implanted and endogenous NSCs may be promoted by increased levels of cytokines IL-1β and IL-6. IL-6 activates JAK/STAT signaling that is required for astrocyte differentiation (Bonni, Sun et al. 1997; Okano, Ogawa et al. 2003; Taga and Fukuda 2005).

The time window for effectively modulating tissue inflammation may be restricted to the acute phase of SCI. Cell transplantation at acute SCI holds great potential in preserving axons and suppressing the progression of secondary injury (Hasegawa, Chang et al. 2005). Acute transplantation of NSCs may protect host cells against cytokine induced apoptosis by secreting trophic factors or mediate the inflammatory process (Martino and Pluchino 2006). Moreover, acute transplantation of NSCs or radial glial cells to injured spinal cord show active migration in the white matter and extensive integration into host tissue, which may be important for directing and supporting axon growth during recovery (Cao, Zhang et al. 2001; Hill, Proschel et al. 2004; Hasegawa, Chang et al. 2005).

Section B. Cell transplantation in SCI sub-acute phase

In contusive SCI, tissue inflammation lasts up to 7 days after injury (Popovich, Wei et al. 1997). Cell transplantation during SCI sub-acute phase (7-14 d post injury) can avoid exposure of transplants to pro-inflammatory mediators at the injury area and greatly increase graft survival (Plant, Christensen et al. 2003; Pearse, Pereira et al. 2004; Hofstetter, Holmstrom et al. 2005). The better survival of implanted cells may be also attributed to trophic factors secreted from regenerated and/or repaired blood vessels, since angiogenesis progresses actively around 7- 14 days following SCI (Casella, Marcillo et al. 2002). When transplanting NSCs to injured spinal cord at 7-9 days post SCI, they differentiate into neurons and oligodendrocytes, suggesting that the environment in the sub-acute phase is more favorable for neurogenesis and oligodendrocyte differentiation (McDonald, Liu et al. 1999; Ogawa, Sawamoto et al. 2002; Hofstetter, Holmstrom et al. 2005).

There are also disadvantages applying cells sub-acutely after SCI, including losing the critical time window to protect neurons/axons and reduce secondary injury. Moreover, as the scar starts to form during this phase, it becomes more difficult for implanted cells to incorporate into the host tissue. Cell integration and migration in sub-acute phase may not be as prominent as acute transplantation (Hofstetter, Schwarz et al. 2002; Lee, Bulte et al. 2004; Cao, Xu et al. 2005).

Myelination and axonal regeneration are the two major mechanisms believed to underlie functional recovery from transplantation of Schwann cells, olfactory ensheathing cells (OEC) or stem cell (ES or NSC) derived oligodendrocytes in SCI sub-acute phase (Takami, Oudega et al. 2002; Cao, Xu et al. 2005; Hofstetter, Holmstrom et al. 2005; Keirstead, Nistor et al. 2005; Karimi-Abdolrezaee, Eftekharpour et al. 2006). These studies show proof of concept that cell transplants can be used to improved recovery sub-acutely after SCI. It is possible that a more prominent functional recovery can be expected if sub-acute cell therapy is combined with suppression of tissue inflammation in acute phase. Acute administration of methylprednisolone and IL-10 following injury synergized locomotion recovery on transplantation of Schwann cells and OECs together at 7 days post injury, however it did not work on transplantation of Schwann cell alone (Pearse, Marcillo et al. 2004).

Section C. Cell transplantation in SCI chronic phase

Several weeks after contusion, visible cyst develops and glial scar forms. These anatomical and pathological changes make the environment inhospitable for cell-based therapy. Challenges in studying cell-based therapy during chronic phase include increased surgical difficulty and extremely non-permissive environment for transplant

integration into host tissue. However, it is the phase that clinicians and SCI patients hold greatest hope for developing possible reparative and regenerative therapies.

To achieve significant functional recovery, combination therapy is recommended for chronic phase of SCI. Combining trophic factors with delayed transplantation of fetal spinal cord tissue, the animals showed axonal regeneration and improved behavioral recovery than those that received transplants alone (Coumans, Lin et al. 2001). Treatment of chondroitinase infusion and delay transplantation of NSCs, successfully reduced CSPG deposition and promoted axonal regeneration, however, no behavioral improvement was reported (Ikegami, Nakamura et al. 2005). As any strategy to reduce CSPG alone can only improve anatomic and histological reconstruction with no significant effect on behavioral improvement, combination of treatments with different mechanisms is in great need for chronic SCI. More studies employing such strategies should be carried out to enhance our understanding of the mechanism and help the search for new therapies for clinical trails.

Part III. Using radial glial transplants in SCI

Section A. Discovery of radial glia

Radial glial cells play an indispensable role during the development of central nervous system (CNS), where they form a basic architectural frame and guide immature neurons migrating to appropriate future cortical layers. Derived from neuroepithelial cells, radial glial cells exist ubiquitously and behave actively during corticogenesis. The most recognizable feature of radial glia is their unique morphology of oval soma located close to the ventricular zone and bipolar fiber extended to the pial surface. These specialized cells were discovered back in mid-1880s by Magini, who was the first person to use Golgi impregnation and reveal elongated radial cells along with developing nerve cells (Bentivoglio and Mazzarello 1999). Ramón Cajal also applied Golgi impregnation method and further suggested that these radial cells transformed into astrocytes (Bentivoglio and Mazzarello 1999; Rakic 2003). Combining Golgi impregnation and electron microscopy (EM), Rakic confirmed observations of those pioneers by studying radial cells in primate embryos. He ascertained the astroglial fate of these radial cells, and finally named them "radial glia" (Rakic 1971; Rakic 2003). Ultrastructural features of radial glial cells include lamellate expansion on the fiber, terminal endfeet attached to pial surface forming basement membrane; along radial glial fibers are bipolar migrating neurons which exhibit voluminous leading processes filled with darker cytoplasm and much slender trailing processes apposing intimately with radial glial fibers (Rakic 1972).

The astroglial identity of radial glia was validated by their morphogenetic transformation into astrocytic phenotypes, presence of glycogen granules in the cytoplasm and expression of glial fibrilary acidic protein (GFAP) (Rakic 1978; Levitt and

Rakic 1980; Voigt 1989; Rakic 2003). It should be noted that radial glial cells in Rakic's studies were from primates, radial glial cells in rodents, however, do not express GFAP until late stage of corticogenesis (Dahl, Rueger et al. 1981; Cameron and Rakic 1991).

Several antibodies have been used to identify radial glia in rodents, including vimentin, RC1, RC2, glutamate transporter (GLAST), brain lipid binding protein (BLBP), Tenascin C etc (Dahl, Rueger et al. 1981; Feng, Hatten et al. 1994; Feng and Heintz 1995; Shibata, Yamada et al. 1997; Hartfuss, Galli et al. 2001). The concept of using these antibodies to label radial glia is mostly derived from the findings that the antibody expression coincidently matches the appearance and disappearance of radial glial cells. In addition, functional antagonization of the protein *in vivo* blocks radial glial activity and neuronal migration.

Section B. Radial glia and migrating neurons

As a scaffold for neuronal migration, each radial glial cell spans the neural tube with its elaborated process where it ends at basal membrane of pial surface. Neuroblasts (immature neurons) wind their cell body and process around radial glial fiber while migrating to their post-mitotic position. In order to maintain the migration route, migrating neurons regulate the bipolar morphology of radial glia and prevent their differentiation through activating Notch and ErbB signaling pathways by ligands Delta/Jagged and neuregulin respectively (Anton, Marchionni et al. 1997; Gaiano, Nye et al. 2000; Chambers, Peng et al. 2001; Patten, Peyrin et al. 2003).

Several extracellular matrix proteins and adhesion molecules have been identified that are involved in maintaining neuron-radial glia physical interaction or letting neuron departure to its final destination. Cell-surface glycoproteins, such as astrotactin and $\alpha 3$

integrin, are important in establishing neuron-glia adhesion (Edmondson, Liem et al. 1988; Zheng, Heintz et al. 1996; Anton, Kreidberg et al. 1999). Blocking their activity greatly retards neuronal migration and lamina formation (Anton, Kreidberg et al. 1999; Adams, Tomoda et al. 2002). Radial glial surface antigen SPARC (secreted protein acidic and rich in cysteine)-like 1, has been identified as a terminators for neuronal migration, which signals neuron to detach from radial glial fiber and translocate to its final position in the developing brain (Gongidi, Ring et al. 2004). Extracellular matrix protein reelin is another terminator for neuronal migration. Reelin is synthesized and secreted by Cajal-Retzius cells when they detach from radial glial scaffold to locate at the marginal zone in the developing cortex (Derer and Derer 1990; Frotscher 1998; Soriano and Del Rio 2005). Reelin breaks the neuron-glia adhesion by interacting with integrin-mediated cellular adhesion thereby inhibits/stops neuronal migration (Dulabon, Olson et al. 2000; Schmid, Jo et al. 2005).

There is much evidence showing that defects in radial glial cell morphology, maturation, maintenance and differentiation result in deprived neurogenesis, abnormal neuronal migration, and an underdeveloped cortex (Stoykova, Gotz et al. 1997; Heins, Malatesta et al. 2002; Hartfuss, Forster et al. 2003; Stoykova, Hatano et al. 2003; Weiss, Johanssen et al. 2003; Hack, Sugimori et al. 2004; Hatakeyama, Bessho et al. 2004).

Section C. Radial glial cells are neural stem cells

After neurogenesis is complete, radial glial cells disappear while some of them retract their process from ventricular zone and pial surface and then transform into astrocytes (Levitt, Cooper et al. 1981; Voigt 1989). Despite their name, radial glia not only support neuronal migration but actively generate daughter radial glia and

neuroblasts during corticogenesis (Noctor, Flint et al. 2001; Malatesta, Hack et al. 2003; Anthony, Klein et al. 2004; Noctor, Martinez-Cerdeno et al. 2004).

The identity of radial glial cells has been studied using transgenic mice or retrovirus or DiI for tracking. Such studies reveal a great heterogeneity of radial glial cells that give rise to virtually all cortical projection neurons (except interneurons in the ventral telecephalon), astrocytes and oligodendrocytes (Malatesta, Hartfuss et al. 2000; Miyata, Kawaguchi et al. 2001; Noctor, Flint et al. 2001; Malatesta, Hack et al. 2003; Anthony, Klein et al. 2004).

Radial glia are not present in adult brain, except Bergmann glia in the cerebellum and Müller glia in the retina. However, descendant astroglia of radial glia in the subventricular zone (SVZ) have been recognized as adult neural stem cells that can generate neurons and glia when cultured in vitro or in micro-lesion model in vivo (Palmer, Markakis et al. 1999; Magavi, Leavitt et al. 2000; Sanai, Tramontin et al. 2004).

Section D. Radial glia in SCI

In the injured spinal cord, axons are instantly damaged and undergo progressive degeneration, depending on injury type and severity. In rat contusive SCI model, axons of corticospinal tract die back and form retraction bulbs acutely post injury (Hill, Beattie et al. 2001; Beattie, Hermann et al. 2002). Axonal sprouting is one of anatomical reorganization that spinal cord attempts to repair the injured tissue (Hill, Beattie et al. 2001; Hasegawa, Chang et al. 2005). However, without support and guidance, these axons are randomly trapped in the injury cyst (Hasegawa, Chang et al. 2005). In view of multiple functions of radial glial cells in the developing CNS, these specialized cells hold great potential in supporting injured axon integrity and growth after SCI.

Previously, C6 glioma derived radial glia-like cell line C6R in culture were transplanted into adult brain and spinal cord. C6R cells support neuronal migration in vitro, retain their bipolar morphology and migrate extensively in spinal cord white matter (Friedlander, Brittis et al. 1998; Hormigo, McCarthy et al. 2001). However, they form tumors when implanted in the injured spinal cord (Hasegawa and Grumet 2003). To resolve this, we prepared radial glial cells from embryonic cortex and immortalized them by introducing v-myc. Radial glial cell RG3.6 is a stable clone after selection, that does not form tumors when transplanted in normal or injured spinal cord (Hasegawa, Chang et al. 2005).

In this thesis, we study RG3.6 cells and their therapeutic potential in mitigating acutely injured spinal cord. Basic characteristics of RG3.6 in culture and in normal spinal cord are described in Chapter One. The efficacy of RG3.6 in treating acute injured spinal cord is described in Chapter Two. Finally, the protective mechanism of RG3.6 in our treatment paradigms is explored at the gene expression level and the findings are reviewed and discussed in Chapter Three.

Chapter One

Characterization of a radial glial clone RG3.6 cells in vitro and in vivo

Introduction

Neuronal migration is a major feature during CNS development. Radial glia provide a cytoarchitectural network to guide migration of newborn neurons from proliferative zones to their final destinations (Rakic 1971; Rakic 1972; Sidman and Rakic 1973; Hatten 1999; Hartfuss, Galli et al. 2001). Radial glia are derived from neuroepithelial cells and appear very early during neural development (Hatten, 1999). They are characterized by (1) their unique bipolar morphology with their cell body located at the ventricular zone and processes extending to the pial surface of the brain and (2) their astroglial lineage when neuronal migration is complete (Levitt and Rakic 1980; Voigt 1989). During corticogenesis, radial glia have multiple functions in generating daughter cells and neuroblasts simultaneously, and forming a scaffold for migration of newborn cells to the intermediate zone for further expansion or to its final destination, cortical plate (Noctor, Flint et al. 2001; Doetsch 2003; Malatesta, Hack et al. 2003; Anthony, Klein et al. 2004; Noctor, Martinez-Cerdeno et al. 2004). A plethora of data in the literatures have shown that defects of radial glia in the developing CNS can result in poor neuronal migration and cortex formation (Gotz, Stoykova et al. 1998; Noctor, Palmer et al. 1999; Heins, Malatesta et al. 2002; Hartfuss, Forster et al. 2003; Stoykova, Hatano et al. 2003; Hatakeyama, Bessho et al. 2004).

Typically, most radial glia transform into astrocytes at late stage of corticogenesis (Sidman and Rakic 1973; Eckenhoff and Rakic 1984). The mechanism underlying the

transformation remains unknown. Radial glial cell share several important features with astrocytes, such as intracellular glycogen granules (Gotz, Hartfuss et al. 2002), expression of L-glutamte/L-aspartate transporter (GLAST) (Hartfuss, Galli et al. 2001), glutamine synthetase (Akimoto, Itoh et al. 1993), brain lipid binding protein (BLBP) (Anthony, Klein et al. 2004), intermediate filament protein vimentin (Dahl, Rueger et al. 1981), and glial fibrillary acidic protein (GFAP) in mammals, but not in rodents (Levitt and Rakic 1980; Sancho-Tello, Valles et al. 1995).

Unlike cortex development, radial glial cells in other regions transform into specialized astroglia that still retain radial glial features including bipolar morphology, expression of GFAP and vimentin. These cells include Bergmann glia in the cerebellum, tanycytes in hypothalamus, and Müller glia in retina (Rakic 1971; Robinson and Dreher 1989; Gould, Howard et al. 1990; Robinson and Dreher 1990). Interestingly, recent emerging evidence have shown that Müller and Bergmann glia, specialized descendants of radial glia, possess similar neurogenic and multipotent features as fetal radial glia (Fischer and Reh 2001; Sottile, Li et al. 2006).

In addition to breakthroughs in basic sciences, clinical potential of radial glia and their descendant cells in neurodegenerative disease and CNS trauma has been actively explored. Müller glia are reported to protect retinal neurons by reducing excitotoxicity and are able to differentiate into retinal neurons in response to injury (Heidinger, Hicks et al. 1999; Kawasaki, Otori et al. 2000; Fischer and Reh 2001; Fischer and Reh 2003). Glial-restricted precursor cells (GRPs) also have been found to protect motor neurons when seeded onto organotypic spinal cord in a chronic excitotoxicity model (Llado, Haenggeli et al. 2004; Maragakis, Rao et al. 2005). Immature astrocytes and GRPs have

been applied to injury models of brain and spinal cord. They provide a less inhibitory environment and promote axonal regeneration (Smith and Silver 1988; Smith and Miller 1991; Han, Liu et al. 2004; Hill, Proschel et al. 2004; Iseda, Nishio et al. 2004; Lepore, Bakshi et al. 2005; Lepore and Fischer 2005; Davies, Huang et al. 2006).

As radial glia are essential in neuronal migration during CNS development and their descendant cells (Müller glia, GRP, and immature astrocyte) are effective in reducing excitotoxicity in ALS (amyotrophic lateral sclerosis) or SCI models, it is of great interest to examine the therapeutic potential of radial glia in contusive SCI. ***Therefore, we hypothesize that implantation of radial glial cells can mitigate the toxic environment at early stage of SCI and support injured axon re-growth at later stage.*** Nevertheless, it should be noted that radial glial cells exist transiently during CNS development. It may be thus essential to maintain the radial glial phenotype long enough to take the full advantages of their biological function in translational study. To achieve this goal, we immortalized cortical radial glial cells by infection with a v-myc expressing virus to delay their differentiation (Villa, Snyder et al. 2000).

In this chapter, RG3.6, a radial glial clone, was characterized both in culture and in vivo. Radial glial properties of RG3.6 were validated to facilitate further transplantation study in SCI. RG3.6 fulfilled the criteria of radial glia and neural stem cell morphologically and antigenically. When co-cultured with cerebellar granule neurons, RG3.6 cells aligned and supported neurite outgrowth. Moreover, RG3.6 cells expressed mRNAs of trophic factors, such as GDNF, NGF, BDNF and NT3. Finally, RG3.6 cells were implanted in normal spinal cord to examine their capacity in migration and differentiation. RG 3.6 cells were distributed and/or migrated extensively in the white

matter and many of them remained undifferentiated after 4 weeks in vivo. This suggests that v-myc insertion restrained RG3.6 differentiation and the radial glial features were well maintained even weeks after implantation. The efficacy of RG3.6 cells in treating injured spinal cord will be described in details in Chapter II.

Materials and Methods

Cell culture

Transgenic Sprague Dawley rats carrying enhanced green fluorescent protein (EGFP) were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and established by Okabe group in University of Osaka (Ito, Suzuki et al. 2001). Radial glial cells were obtained from embryonic 13.5 day cortical tissue of GFP transgenic rat and expanded as neurospheres in culture with daily 10 ng/ml basic FGF replenishment. For differentiation, RG3.6 neurospheres were dissociated and plated onto laminin (20μg/ml) coated coverslips. Basic FGF was withdrawn and replaced with 1% FBS and 20 μm retinoic acid. Cells were fixed with 4% paraformaldehyde after 3 days of differentiation and immunostained for antibodies of interest. For glutamate metabolism study, different doses of glutamate were prepared from stock glutamate solution (100 mM in 1N HCl) and added to RG3.6 culture.

Immunofluorescence

4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 was used as fixative in all experiments conducted in this chapter. Cells were fixed for 15 min at room temperature and washed with PBS several times before incubation of primary antibodies (listed in Table 1) in 10% normal goat serum and 0.3% Triton X-100 in phosphate buffer saline (0.01 M PBS, pH 7.4) for 2 hours at room temperature. After washing, cells were incubated with appropriate secondary antibodies (listed in Table 1) for 1 hour at room temperature. Finally, nuclei were labeled with Hoechst 33342 at 1:2000 (Sigma-Aldrich, St. Louis, MO) and coverslips were mounted with Gel/Mount (Biomedia, Foster city,

CA). Images were acquired on a Zeiss Axiophot or Zeiss LSM 510 confocal microscope and were analyzed in Adobe Photoshop 7.0.

Cell transplantation in normal spinal cord

Adult Sprague Dawley female rats (Taconic, Germantown, NY) weighing 220-250 g were used in this study. Rats were anesthetized by an intraperitoneal (i.p.) injection of sodium pentobarbital (35 mg/kg). Under aseptic conditions, spinal thoracic level T9 and T10 spinal cord were exposed by laminectomy. Spinal cord was secured by holding spines of T8 and T11. 10^5 cells/μl of RG3.6 or GFP primary NSCs were prepared in DMEM+F12 medium and injected slowly during a period of 10 min into ventral column (at ~ 2 mm depth) of T10 using a sterile glass tip with a diameter of 50 μm connected to a 5-μl Hamilton syringe (4 rats for RG3.6 and 3 rats for NSCs). Following transplantation, the muscle and skin were sutured in layers. Cefazolin (25 mg/kg) was administered for 7 days after surgery to prevent infection and cyclosporine (10 mg/kg) was given daily via subcutaneous injection throughout the survival period to suppress immuno-rejection. Animal surgery and post-operation care were carefully followed by protocols approved by Rutgers University.

Tissue processing, immunostaining and image analysis

Four weeks after transplantation, rats were deeply anesthetized by sodium pentobarbital and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Spinal cords were carefully removed and post-fixed overnight at 4ºC. Spinal cords were then placed in 25% sucrose for cryoprotection before frozen sectioning. Spinal cords were embedded in OCT compound and sectioned horizontally at 20 μm on a cryostat.

Sections were washed with PBS and blocked with 10% normal goat serum and 0.3% Triton X-100 for 2 hours at room temperature. Following blocking, sections were incubated with primary antibody of GFAP (R401 from Dr. Martin Grumet) at 1:200 or nestin (Developmental Hybridoma bank) at 1:50 for overnight at 4ºC. Sections were washed thoroughly with PBS and incubated with secondary antibody of either goat-anti-rabbit or goat anti-mouse (Alexa568, molecular clone) at 1:400 for 2 hours at room temperature. After washing, sections were incubated with Hoechst nuclei staining at 1:2000 for 5 min before gel-mounting. Images were taken from a Carl Zeiss LSM 510 confocal microscope. More than 300 GFP+ cells were counted. Double positive cells of GFP+/GFAP+ or GFP+/nestin+ were quantitated.

Quantitative RT-PCR of trophic factor genes in RG3.6

Total RNA was prepared from RG3.6 cells or NSCs homogenates according to Qiagen RNeasy protocol. 1 μg of RNA was reverse-transcribed to cDNA using SuperScript II (Invitrogen, Carlsbad, CA) and oligo dT primers. Primer sequences for growth factors of interest are listed in table 1. RNAs from normal rat spinal cord tissue were used as control. Quantitative reverse transcription PCR (Q-RT-PCR) was carried out using 10 ng of cDNA, 50 nM of each primer, and SYBR green master mix (Applied Biosystems, Foster city, CA) in 10 μl reaction on an Applied Biosystems model 7900HT system. A pooled cDNA sample from RG3.6, NSCs, and spinal cord tissues was used to make standard curves for quantification.

Assay of GDNF production by ELISA

Conditioned medium was obtained from $2x10^6$ of RG3.6 cells or primary NSCs in 2 ml medium. The amount of GDNF secreted by RG3.6 or primary NSCs in vitro was

measured by enzyme-linked immunosorbent assay (ELISA) using the GDNF Emax ImmunoAssay system (Promega, Madison, WI). According to manufacture's instruction, the plates were coated with anti-GDNF monoclonal antibody (pH 8.2) overnight at 4ºC and blocked for 1 h at room temperature. GDNF standards and conditioned medium were than incubated at room temperature for 6 h on a shaker. The plates were then incubated sequentially with chicken anti-human GDNF polyclonal antibody overnight at 4ºC overnight, horseradish peroxidase (HRP)-conjugated anti-chicken antibody at room temperature for 2 h. Colormetric product was measured and sample values were calculated from the standard curve in the linear range.

Results

RG3.6 is a GFP radial glial clone that exhibits neural stem cell features

Primary neural stem cells were isolated from E13.5 cortices of GFP transgenic rats. After stabilizing the cells with v-myc retrovirus and selection with G418, we screened clones that have radial glia morphology and express BLBP. RG3.6 was one of the clones which has self-renewal capacity and can be expanded as neurospheres. When plated on laminin-coated plate, RG3.6 cells exhibited typical radial glial features including bipolar shape, expression of nestin and BLBP (figure 1). Upon withdrawal of FGF2 from the culture medium and addition of 1% FBS with 10 μM retinoic acid, RG3.6 cells differentiated into ßIII tubulin, GFAP or GALC positive cells indicating their potential of giving rise to neurons, astrocytes and oligodendrocytes in vitro (figure 1).

RG3.6 cells express glutamate transporter and glutamine synthetase

Like astrocytes, radial glial cells can metabolize glutamate through their glutamate transporter and glutamine synthetase. Therefore it is important to identify if RG3.6 cells can carry out this specific property. GLAST and glutamine synthetase expression by immunostaining was examined in RG3.6 cells that were exposed to various doses of glutamate (0, 10, 50 and 200 μM). During 24 hours incubation period, it was found that RG3.6 cells express glutamine synthetase in a dose response manner, while no significant difference in GLAST expression was observed (data not shown). Further, GLAST and glutamine synthetase expression were also evaluated at different time points (0, 1, 6, 24 hours) using 200 μM of glutamate (figure 2). Glutamine synthetase expression exhibited a time-dependent response, with strong expression at 24 hours of incubation (figure 2).

Although gradual increase of intensity of GLAST can be observed in RG3.6 cells, the difference is not as significant as glutamine synthetase.

RG3.6 cells express trophic factor mRNAs and secret GDNF in vitro

Several lines of evidence suggest that delivery of trophic factors by direct infusion or genetic modified cells promotes neuronal survival and axonal growth after spinal cord injury (Kim, Gutin et al. 1996; Nakahara, Gage et al. 1996; Ikeda, Murakami et al. 2002; Tobias, Shumsky et al. 2003). It was also reported that neural stem cells promote survival of injured motor neurons by secreting neurotrophic factors in glutamate excitotoxicity models (Llado, Haenggeli et al. 2004).

RG3.6 cells express mRNAs of glial-cell-line derived neuroteophic factor (GDNF) and neurotrophin genes including nerve growth factor (NGF), neurotrophin 3 (NT3) and brain derived neurotrophic factor (BDNF). The mRNA levels encoding for each of these molecules are all significantly higher in RG3.6 than primary neural stem cell using quantitative RT-PCR measurement (figure 3). This suggests that RG3.6 cells have potential to secret these growth factors in vitro and in vivo.

Among these trophic factors studied in animal models, GDNF has been reported to specifically support survival of cholinergic neurons and motor neurons (Bohn 2004). The efficacy of its protective effect on motor neurons has been extensively investigated in animal models of SCI (Blesch and Tuszynski 2001), ALS (Klein, Behrstock et al. 2005), and Parkinson's disease (Behrstock, Ebert et al. 2006) through either direct infusion or transplanting GDNF expressing stem cells to degenerating areas. Since preservation of motor neurons is essential for locomotor recovery after SCI, it is important to examine if

RG3.6 can help to increase GDNF level. GDNF was detected in supernatants of RG3.6 and the level was higher than that of primary NSCs (figure 3).

The survival, migration, and differentiation of RG3.6 in the normal spinal cord

One ultimate goal of studying this radial glial clone is to examine whether they can benefit the injured spinal cords, promote axonal growth and functional recovery. Before applying to the injured spinal cord, RG3.6 cells were first injected to normal spinal cord to be assessed for their behavior in intact spinal tissue, such as cell survival, differentiation, migration and compatibility with host tissue. Primary NSCs from E13.5 cortex were used as control. Four weeks after transplantation, both cell types showed extensive rostral-caudal migration in the white matter (figure 4). Significant number of RG3.6 cells remained as nestin positive while only a few differentiated into GFAP positive cells. In contrast, many NSCs lost nestin and became GFAP positive cells (figure 4). This observation is consistent with the work in Dr. Whittemore's group that primary NSCs tend to acquire astroglial fate in spinal cord (Cao, Zhang et al. 2001). It further validates that viral introduction of v-myc restrains RG3.6 cells from differentiation and allows longer maintenance of radial glial phenotypes.

Discussion

As our hypothesis is that radial glial cells can support axonal regeneration in injured spinal cords, we hope to maintain the radial glial phenotype in the implanted cells long enough in vivo to align and support injured axons. To approach this goal, we obtained a radial glial clone RG3.6 by introducing v-myc into cortical neural stem cells to prolong radial glial phenotype and function.

Results in this chapter confirmed that RG3.6 cell exhibited properties of radial glia, including morphologic and antigenic features. In addition, they expressed neuronal and glial markers when differentiated in serum containing medium for just 3 days, suggesting that they are multipotent neural stem cell under certain condition.

RG3.6 cells expressed glutamate transporter GLAST and glutamine synthetase. The glutamine synthetase expression was found to be dose and time-dependent in response to glutamate administration. Glutamate is an important excitatory neurotransmitter in the mammalian CNS, however, excess glutamate can be cleared by glutamate transporter to reduce its concentration below neutotoxic levels. Intracellular glutamate will then be converted into non-toxic glutamine by glutamine synthetase. Glutamate uptake is mainly mediated by astroglial glutamate transporter GLAST and GLT1 (Haugeto, Ullensvang et al. 1996; Rothstein, Dykes-Hoberg et al. 1996). Microglia also take up small amount of extracellular glutamate (van Landeghem, Stover et al. 2001; Persson, Sandberg et al. 2006). In rat SCI, cell depolarization after mechanical insult induces massive glutamate release as early as 15 min after injury, and the level remains high for one hour before returning to normal levels over the next 1.5 hours (Liu, Thangnipon et al. 1991; McAdoo, Xu et al. 1999). Glutamate transporter

GLT1 and GLAST are increased in glial cells after contusive SCI, probably for extracellular glutamate clearance (Rothstein, Dykes-Hoberg et al. 1996; Vera-Portocarrero, Mills et al. 2002). However, not only are local neurons destroyed, but glial cells are also impaired in spinal cord contusion. Therefore, destruction of large number of glial cells after injury would significantly reduce the capacity of host tissue to remove the excessive glutamate. The ability to express glutamate transporter and glutamine synthetase likely makes RG3.6 cell a good exogenous vehicle to assist extracellular glutamate reduction by acute transplantation. It will be important to assess whether RG3.6 cells can uptake glutamate in vitro to validate the functionality of GLAST and Glutamine synthetase expression.

Another rationale for using RG3.6 cells in SCI is their expression of mRNAs encoding for growth factor genes and secretion of GDNF. It indicates that RG3.6 cells may modulate the injured environment to favor survival of host neurons or glia. It has been widely accepted that trophic factors are beneficial for motor neuron survival and axonal regeneration in SCI. RG3.6 cells secreted more GDNF than primary NSCs. Although the basal level of GDNF secreted by RG3.6 cells is not as high as the mouse neural stem cell clone C17.2 (Lu, Jones et al. 2003), RG3.6 cells may be potentiated to produce more growth factors in response to injured environment.

Characterization of RG3.6 in normal spinal cord suggests that RG3.6 cells survive after implantation, retain bipolar shape for as long as four weeks, maintain GFP signal for tracing, and integrate with host tissue. Since more RG3.6 cells than NSCs remained nestin positive/ GFAP negative in intact spinal tissue, it meets our goal to obtain a cell type with prolonged radial glial phenotype. Acute transplantation of RG3.6 in injured

spinal cord and histological and behavioral results will be discussed in the following chapters.

Table 1. Antibodies used in this chapter

Primary antibody	Conc.	Isotype	Source	Secondary Antibody	Conc.	Source
Nestin	1 : 50	Mouse Monoclonal IgG	DSHB	Alexa568 Goat-anti-mouse IgG (H and L)	1: 400	Molecular Probe
BLBP	1 : 1000	Rabbit Polyclonal IgG	Dr. Nathaniel Heintz	Alexa647 (Cy5) Goat-anti-rabbit IgG (H and L)	1: 400	Molecular Probe
GFAP	1 : 200	Rabbit Polyclonal IgG	Dr. Martin Grumet	Alexa568 Goat-anti-rabbit IgG (H and L)	1: 400	Molecular Probe
TuJ1	1 : 200	Mouse Monoclonal IgG	Chemicon	Alexa568 Goat-anti-mouse IgG (H and L)	1: 400	Molecular Probe
GalC	1 : 50	Mouse Monoclonal IgM	Dr. Randall McKinnon	Alexa568 Goat-anti-mouse IgM (H and L)	1: 400	Molecular Probe
GLAST	1 : 5000	Guinea pig Polyclonal IgG	Chemicon	Cy3 conjugated Goat-anti-guinea pig IgG (H+L)	1: 400	Chemicon
Glutamine Synthetase	1 : 400	Mouse Monoclonal IgG	Chemicon	Alexa568 Goat-anti-mouse IgG (H and L)	1: 400	Molecular Probe

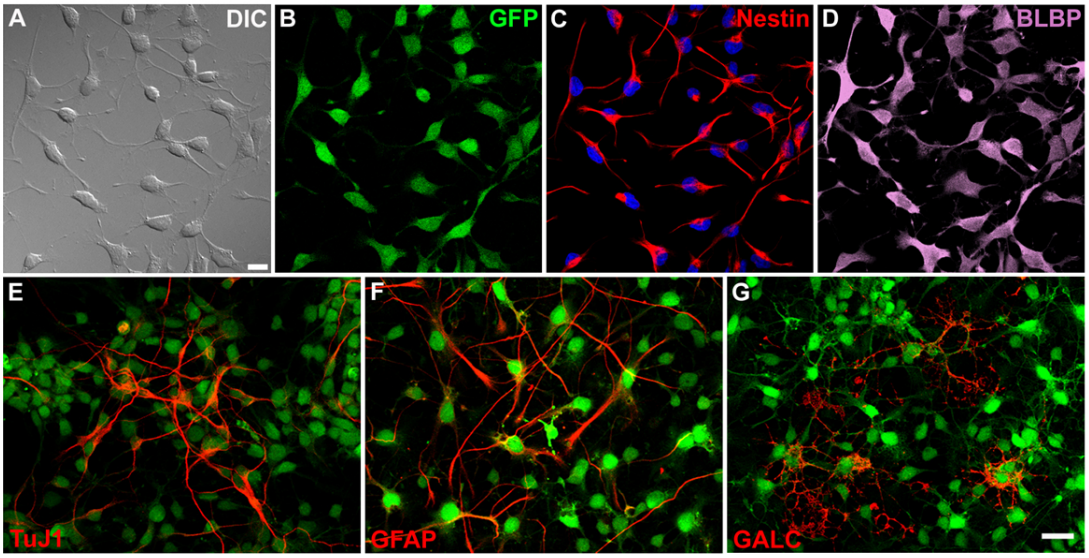


Figure 1. RG3.6 cells exhibit features of both radial glia and neural stem cells. Confocal microscopic images of the same field of RG3.6 cells showing cellular morphology by differential interference contrast (DIC) (A), GFP (B), nestin (red) revealing radial morphology and nuclei (DAPI, blue) (C), and radial glial marker BLBP (D). After withdrawal of bFGF and incubation in 1% fetal bovine serum with 10 μM retinoic acid for 3 days, RG3.6 gave rise to cells expressing the neuronal marker TuJ1 (E), astrocyte marker GFAP (F) and oligodendrocytes marker GALC (G). Scale bar is 20 μm in A-D and 40 μm in E-G.

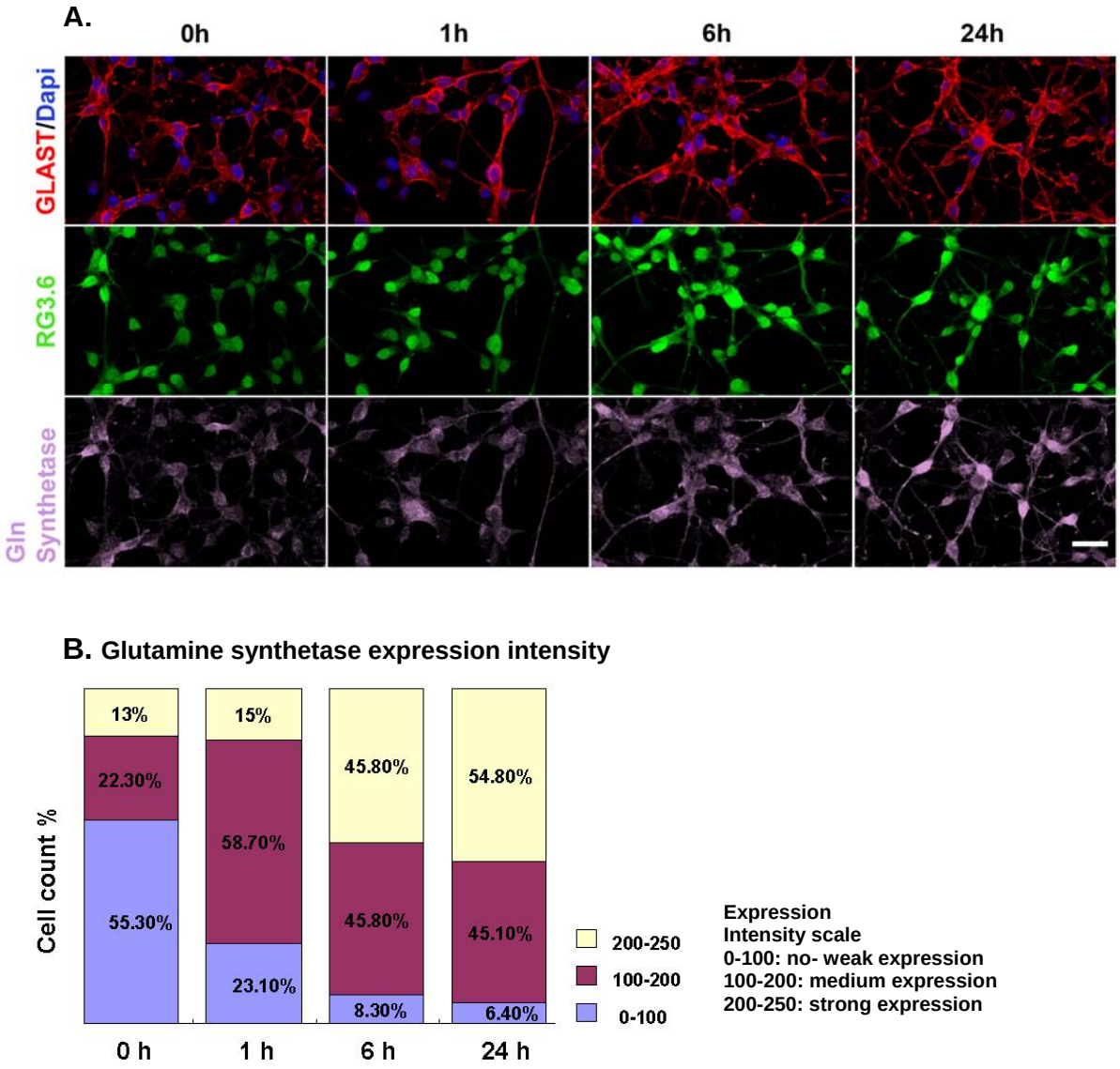


Figure 2. RG3.6 cells express glutamate transporter (GLAST) and glutamine synthetase. Glutamate (200 µM) administration increases expression of GLAST and glutamine synthetase in RG3.6 (A). The expression of glutamine synthetase is time-dependent as the intensity is stronger progressively after administration of glutamate (A-B). Expression intensity was measured using threshold histogram in Zeiss LSM program, scale from 0 to 250 (B). Cells were counted according to their expression intensity ranged from 0-100, 100-200, and 200-250. Scale is 20 µm in (A)

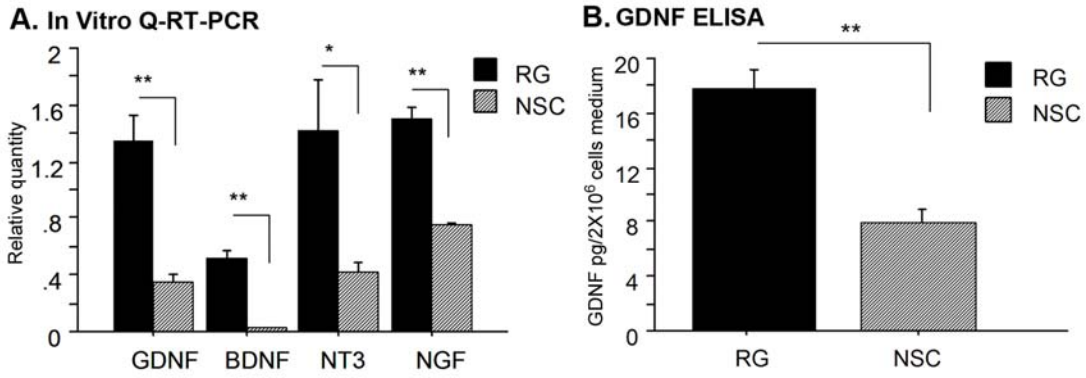


Figure 3. Trophic factor expression in RG3.6 cells in vitro. RG3.6 cells expressed significantly higher mRNA levels of GDNF and neurotrophin BDNF, NT3, and NGF than primary NSCs. The expression level was normalized to β-actin (A). Conditioned medium from 2 million RG3.6 cells or NSCs/day was collected and subjected to GDNF ELISA measurement. Results represent mean value of three individual preparations. Basal level of GDNF secreted from RG3.6 is significantly higher than NSCs (B). Values are means ± SEM, * $p < 0.05$, ** $p < 0.01$, t-test.

Table 2. Primers for quantitative RT-PCR

Gene		Forward	Reverse
NM_019139	GDNF	5'GGTCACCAGATAAACAAGCGG3'	5'GCCGGTTCCTCTCTCTTCG3'
NM_012513	BDNF	5'AGGCACTGGAACTCGCAATG3'	5'AAGGGCCCGAACATACGATT3'
NM_031073	NT3	5'GATATTTTGGCCGGAGGGAA3'	5'CCTCAAAAGGGCTGGGTTCT3'
NM_031523	rNGF	5'TGCTCCTGCATGCCTGTTAC3'	5'CAGGGCGAGGAACAGGATC3'
NM_031144	β-actin	5'CGTAGCCATCCAGGCTGTGT3'	5'CCAGTGGTACGACCAGAGGC3'
NM_017008	GAPDH	5'AAATGATACCCCACCGTGTGA3'	5'GCTGGCACTGCACAAGAAGAT3'

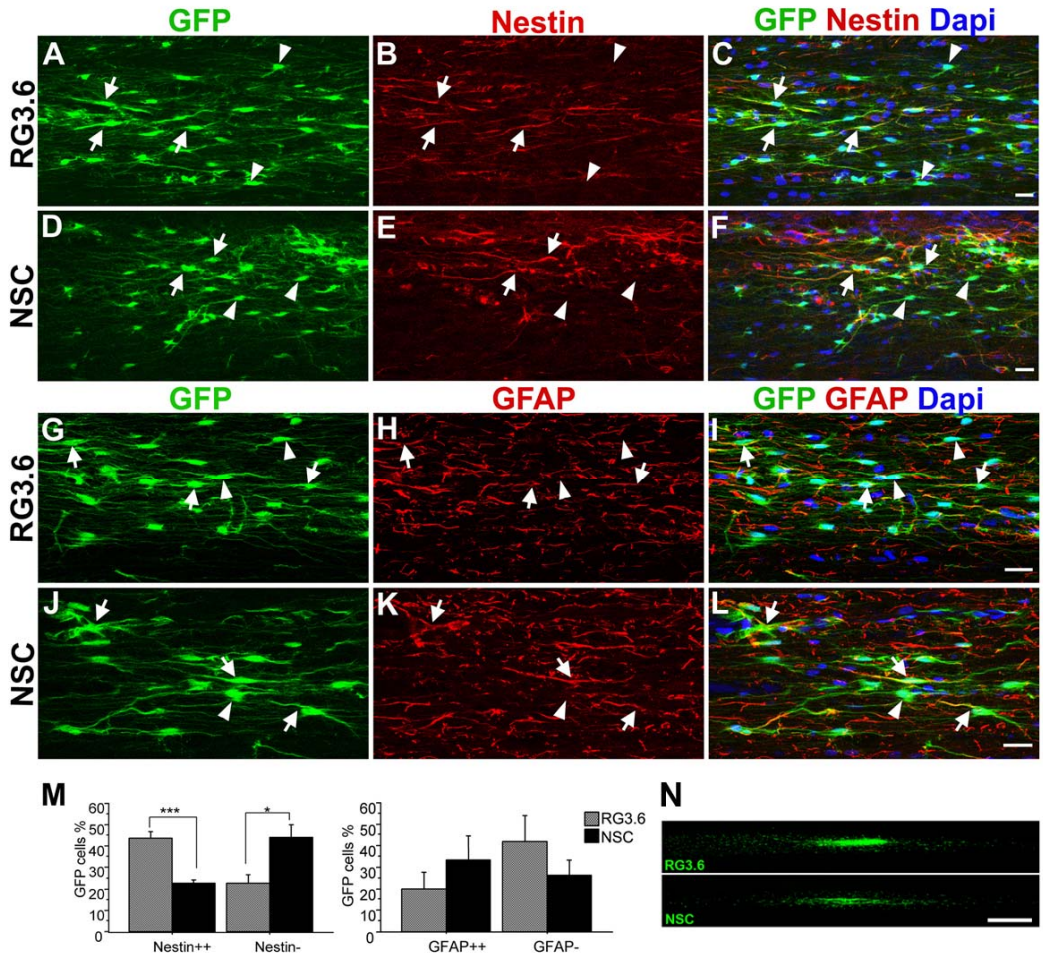


Figure 4. Differentiation of RG3.6 is slower than NSC in normal spinal cord. GFP (A, D, G, J) fluorescence was compared with staining for nestin (B and E) or GFAP (H and K) 4 weeks after transplantation of RG3.6 cell (A-C, G-I) and NSC (D-F, J-L). We counted non-differentiated GFP+/ nestin++ cells (arrows) and differentiated GFP+/nestin– cells (arrowheads) (A-F), and astrocytes as GFP+/ GFAP++ cells (arrows) and GFP+ /GFAP– cells (arrowheads) (G-L) in different rats injected with RG3.6 and NSC cells. C, F, I, L are merged confocal images <1 mm from the injection site. Scale bar is 20 μm. Quantitative results (M) showed that more RG3.6 cells remained nestin ++ while more NSCs remained GFAP++. ++ Strong positive, + weak positive, - negative expression. Percentages do not sum to 100% because weakly positive cells were omitted as their percentages did not differ significantly. RG3.6 cells and NSCs migrate extensively in normal spinal cord white matter 4 weeks after implantation (N). Scale bar is 1 mm (N). Values are means ± SEM. * $p < 0.05$, *** $P < 0.001$, t-test.

Chapter Two

Acute transplantation of RG3.6 into contused spinal cord

Introduction

The discovery of inhibitory or non-permissive environment after CNS injury, including oligodendrocyte derived factors, chondroitin sulfate proteoglycan (CSPG) deposition, astrocyte hypertrophy and proliferation, lacking of trophic factors and bridging substrates, has allowed scientists to design appropriate therapies to potentially promote functional recovery. Current strategies in developing treatments for spinal cord injury include the following approaches: (1) to **protect** neurons and prevent glial cell death early after injury (2) to **bridge** lesion site with cell transplants or synthesized biomaterials to provide permissive environment and/or substrate for axon regrowth (3) to encourage **remyelination** by myelin forming cells and (4) to **combine therapies** to target different destructive mechanisms after injury.

Among all the therapeutic interventions, cell-based therapy has been extensively studied in various SCI models and shown to improve functional recovery. Candidates for cell transplantation include Schwann cells (Chen, Xu et al. 1996; Takami, Oudega et al. 2002; Plant, Christensen et al. 2003; Pearse, Pereira et al. 2004), neural stem cells (NSC) (McDonald, Liu et al. 1999; Liu, Qu et al. 2000; Hofstetter, Holmstrom et al. 2005), glia-restricted precursor cells (GRPs) (Davies, Tang et al. 2004; Hill, Proschel et al. 2004; Cao, Xu et al. 2005; Mitsui, Shumsky et al. 2005; Davies, Huang et al. 2006), fetal spinal tissue (Reier, Bregman et al. 1986; Broude, McAtee et al. 1999) , olfactory ensheathing glia (OEG) (Lu, Feron et al. 2002; Plant, Christensen et al. 2003), embryonic stem (ES)

cell-derived oligodendrocytes (Keirstead, Nistor et al. 2005; Nistor, Totoiu et al. 2005), bone marrow stromal cells (Ankeny, McTigue et al. 2004; Lu, Jones et al. 2006), and genetically modified fibroblasts (Tuszynski, Murai et al. 1997; Blesch and Tuszynski 2003; Tobias, Shumsky et al. 2003; Tuszynski, Grill et al. 2003).

Axonal remyelination by implanted cells is a well studied mechanism that is associated with functional recovery of some cell-based therapy for SCI. These implanted cells can be myelin forming cells before transplantation or differentiated into oligodendrocytes after entering the injured environment (McDonald, Liu et al. 1999; Pearse, Pereira et al. 2004; Hofstetter, Holmstrom et al. 2005; Keirstead, Nistor et al. 2005; Karimi-Abdolrezaee, Eftekharpour et al. 2006). Given that remyelination takes place during the sub-acute phase of injury, noticeable locomotion recovery is often observed several weeks after injury. It is still unknown whether these myelin forming cells have other functions in addition to myelintating host axons.

It has not been addressed whether the functional improvement in cell-based therapy is involved in tissue protection and/or neuroprotection, although such mechanisms have been studied in combining cell therapy with anti-inflammatory drugs (e.g. methylprednisolone) to SCI (Nash, Borke et al. 2002; Pearse, Marcillo et al. 2004). In our group, we emphasize early intervention that has potential to alleviate cell loss by diminishing secondary damage thereby more neuron and white matter will be preserved for better locomotion recovery. We are particularly interested in radial glial cell and its therapeutic potential in acute SCI because its multiple functions during CNS development. Moreover, it has been reported that descendants of radial glia, immature astrocytes and glial restricted precursors (GRP) are beneficial to the injured spinal tissue

by their trophic function, reducing inhibitory CSPG and supporting axon growth (Hill, Proschel et al. 2004; Iseda, Nishio et al. 2004; Cao, Xu et al. 2005; Davies, Huang et al. 2006).

In this chapter, we applied a radial glia clone RG3.6 to spinal cord immediately following contusive injury. Histological and behavioral changes were evaluated accordingly. Two parallel experiments were carried out to compare the efficacy of RG3.6 in SCI to either medium (DMEM+F12) or fibroblast. RG3.6 cells survived, migrated and integrated into host tissue. Early locomotion improvement was the most promising outcome from acute RG3.6 transplantation, which can be observed as early as 2 - 7 days post injury, suggesting that RG3.6 cells render tissue protection against secondary damage. The tissue protection mechanism was associated with suppressed neuroinflamamtion as fewer infiltrating macrophages and/or reactive microglia were observed in the injured spinal cord with RG3.6 transplants. In addition, much less inhibitory extracellular matrix CSPG and NG2 was produced in RG3.6 treated rats. RG3.6 cells preserved more spared white matter perhaps by integrating into host tissue to support both myelinated and non-myelinated axons. In contrast, control rats that received medium showed intense neuroinflammatory response, great amount of CSPG/ NG2 deposition and poor tissue preservation. Fibroblast treated rats displayed fewer macrophages infiltrating, yet the grafts deteriorated remaining axons and yielded poor functional recovery. Neither of these two controls showed significant locomotion improvement compared to RG3.6 cells group.

Mechanisms underlying RG3.6 cells' beneficial effect in contused spinal cord at gene expression level are described in Chapter 3.

Materials and Methods

Spinal cord injury and acute transplantation

Animal used in Chapter Two

Time	RG3.6		Medium		Fibroblasts	
	Chap. 2	Pre.	Chap. 2	Pre	Chap. 2	Pre.
6 weeks	18* (10+8)	10	14**(10+4)		10	2
2 weeks	3		3			
1 weeks	3		3			
3 days	2	2	2	2		
1 day	3	1	3	2		

Chap. 2: animals used in this chapter. Pre.: animals used in preliminary experiments to optimize cell dose in vivo. * 10 rats were used by Yu-Wen Chang, 8 rats by Koichi Hasegawa. ** 10 rats were used by Yu-Wen Chang for histology purpose, 4 rats were used by Koichi Hasegawa for behavioral test.

Adult female Sprague-Dawley rats (Taconic, Germantown, NY) weighing 200–250 g were used in this study. Rats were anesthetized with 40 mg/kg of pentobarbital (Besse Medical Supply/ASD Specialty Healthcare, Louisville, KY) and the spinal thoracic T9-10 was exposed by laminectomy. Contusive injury was produced by dropping a 10 gm rod onto the exposed spinal cord from a height of 12.5 mm using MASCIS (Multicenter Animal Spinal Cord Injury Study) impactor (Gruner 1992; Young 2002). Within 10 minutes after injury, three points injection of RG3.6 cells at the concentration of 2×10^5 /μl in DMEM+F12 medium were performed at the injury epicenter (2 μl), 2 mm rostral (1 μl) and caudal (1 μl) to the epicenter. A concentration of 1-2 $x10^5$ /μl of fibroblasts formed masses in the injured spinal cord in our pilot study thus we reduced the dose to 5×10^4 /μl to perform 3-points injection following spinal cord injury. For vehicle medium controls, a total of 4 μl (2 μl at the center of the contusion site and 1 μl each at 2 mm rostral and caudal sites from the center) of DMEM+F12 was injected to dorsal column following injury. Each injection was conducted slowly during a period of 10 min into dorsal column (~ 1 mm depth) of T10 using a sterile glass tip with a diameter of 50 μm

connected to a 5-μl Hamilton syringe. Following injections, muscles and skin were closed separately. Cefazolin (25 mg/kg) was administered for 7 days after surgery and cyclosporine (10 mg/kg) was given daily via subcutaneous injection throughout the survival period. Animal surgery and post-operation care were carefully followed by protocols approved by Rutgers University.

Open field locomotor test

Hindlimb movement was evaluated using open field locomotor BBB scale (Basso, Beattie et al. 1996) by two separate BBB scoring teams that were unaware of experimental treatments. The score was recorded starting at 2 days post injury and once a week thereafter for 6 weeks. BBB scores of 0–7 represent hindlimb movements with no weight support; a score of 8 signifies sweeping or plantar placement without weight support, while a score of 9 indicates plantar placement weight support in stance only with no plantar stepping. A score of 10 indicates occasional weight supported plantar steps without forelimb coordination while a score of 11 requires consistent weight-supported plantar stepping with no forelimb–hindlimb coordination. Scores of 12-14 represent frequent to consistent weight supported plantar steps with occasional forelimb–hindlimb coordination. The highest score we recorded from our animals is 14 in this moderate contusive spinal cord injury model.

Tissue processing

Animals were euthanized with an intraperitoneal injection of sodium pentobarbital at 40 mg/kg, followed by transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer after vascular washout with PBS. The spinal cords were removed, post-fixed overnight in the same fixative, cryoprotected with 25% sucrose overnight, and

embedded in OCT compound (Fisher Scientific, Pittsburgh, PA) for frozen sectioning. Spinal cords taken from 6 weeks post injury were cut into serial sagittal sections at 20 μm on a cryostat (Hacker) and mounted on Superfrost Plus Microscope Slides (Fisher Scientific). Spinal cords taken from other time points were centered at the contusion site and cut transversely at 20 μm and every third section was collected onto slides. These coronal sections were divided into rostral 10 slides, injury 10 slides and caudal 10 slides. An average of 8 serial sections covered each segment of either rostral, injury or caudal spinal cord.

Immunofluorescence (immunostaining)

Antibodies used in this chapter are listed in table 1. Briefly, sections were blocked with 10% normal goat serum / 0.3% Triton X-100 in PBS for 2 h at room temperature and incubated with primary antibodies for overnight at 4°C (Table 1). Sections were then washed with PBS and incubated with appropriate secondary antibodies (Table 1) for 1 h at room temperature. After washing with PBS, sections were counterstained with Hoechst 33342 at 1: 2000 and mounted with Gel/Mount. Image analysis was performed using Zeiss 510 confocal laser scanning microscope (LSM). Intact spinal cords were imaged with a Cool Snap Pro camera (Media Cybernetics) using a Zeiss Stemi II microscope equipped with fluorescence optics.

Quantitation for immunofluorescence

(1) Iba 1 (7d post injury)

Six sections (2 from rostral, 2 from injury center, and 2 from caudal segment) were chosen from each animal for quantitation. Four regions of the section which included dorsal gray matter (DGM), dorsal lateral white matter (DLWM), ventral gray matter

(VGM) and ventral lateral white matter (VLWM) (illustrated in figure 3D) were photographed using LSM 510 confocal microscope 25x optic. Signal thresholds were determined by mean value of all selected images. Areas with intensities higher than thresholds were recorded.

(2) ED1 (14d post injury)

Eighteen coronal sections (6 from rostral, 6 from injury center, and 6 from caudal segment) were chosen from each animal for quantitation. Each cross section image was a montage of 16 individual pictures (X: 4, Y: 4) tiled using LSM 510 confocal microscope. Images were then inverted into black (ED1 positive) and white images in NIH Image J program for measuring positive pixels. Regions of meninges and roots (dorsal + ventral) were excluded for quantitation. Positive pixels in sections within the same segment (e.g. rostral segment, injury segment, or caudal segment) were summed to measure ED1 expression.

(3) CS56/NG2 (42d post injury)

Three sagittal sections closest to the mid-line were used for CS56 and NG2 expression quantitation. A tiled image composed of thirty (X: 10, Y: 3) individual pictures was made for each chosen section using LSM 510 confocal microscope. Staining intensity thresholds for CS56 or NG2 were determined after all images were acquired to optimize the signal to noise ratio for the antibody. Areas with intensities higher than thresholds were recorded and normalized to the total areas that were measured. Regions of meninges and roots (dorsal + ventral) were excluded for quantitation.

(4) Neurofilament (42d post injury)

Quantification of NF staining was performed similarly to CS56/NG2. 1-mm length of dorsal and ventral white matter were outlined as indicated in figure 8, excluding meninges, roots, cysts, and central fibroblast masses where cysts typically form. White matter regions were identified by light microscopy and verified in certain cases by Luxol fast blue staining.

White matter sparing

To measure spared white matter, sections were dried on a warm plate over night before incubation in 0.1% Luxol fast blue at 56°C for 2 hours. Excess stain was rinsed off in distilled water. Sections were differentiated in 0.05% lithium carbonate for 5 min followed by 70% alcohol for 1 min or the reaction was stopped when white matter turned into contrast bluish color (Carson 1997). Sections were washed with distilled water and dehydrated before coverslip mounting. Bright field images were acquired using Zeiss Axiophot microscope.

Statistical analysis

Data in each analysis are expressed as the mean ± SEM. One-way ANOVA followed by Fisher's protected least significant difference (PLSD) was used to determine statistical differences of BBB score between RG3.6 and medium or fibroblasts controls. Immunostaining quantitation was analyzed by student t-test.

Results

RG3.6 cells promoted locomotion recovery

(I) RG3.6 vs. Fibroblast

Trophic factor secreting fibroblasts have been reported to make the injured spinal tissue into a more permissive environment for axonal regrowth. Transplantation of fibroblasts into spinal cord dorsal column lesion model showed that the injured axons were attracted toward the implants and the animals had some behavioral improvement (Grill, Murai et al. 1997; Grill, Blesch et al. 1997; Tuszynski, Murai et al. 1997; Jones, Sajed et al. 2003). We used skin-derived fibroblast as cellular control to evaluate whether radial glial RG3.6 cells can ameliorate contusive SCI in rats.

When transplanted into normal spinal cord, RG3.6 showed good survival and migration at the concentration of 1-2 x 10^5 cells /μl, which was described in Chapter 1. Immediately following spinal cord contusion by MASCIS impactor, we applied the same intraspinal dose of RG3.6 cells at 3 points (2 μl at the injury epicenter and 1 μl each at 2 mm rostral and caudal, figure 1). Given that the fibroblast cell body is ~10 times larger than RG3.6 cell (figure 11) cellular density of transplants was optimized in preliminary studies (figure 1). Acute transplantation of fibroblasts at the concentration of 1-2 x 10^5 cells/μl exhibited graft expansion aat the injury site (figure 1). The bulging of fibroblasts through the dorsal surface of the spinal cord was reduced with a lower concentration (5 × 10^4 cells /μl) of cells.

Similar to that observed in normal spinal cord (Chapter 1), RG3.6 cells survived and migrated extensively along the rostral-caudal axis of the white matter following SCI. On the contrary, fibroblast grafts remained at the injury site without integrating into host

tissue. Both RG3.6 cells and dermal fibroblasts retained strong GFP signals at 6 weeks after injury and transplantation.

Hind-limb movement was examined using BBB scoring system, starting at 2 days post injury and once a week thereafter. Rats that received RG3.6 showed plantar stepping (BBB score 8) as early as 7 days post injury while control rats only had joints movement with no weight support or stepping (figure 1). The locomotion improvement was consistently and statistically higher in rats with RG3.6 transplant throughout the survival period (42 days). Considering that axonal regeneration and/or remyelination takes place several weeks after injury, the early recovery in our experiment should be attributed to another mechanism, such as tissue protection by RG3.6 cells.

(II) RG3.6 vs. Medium

A parallel experiment was carried out by Dr. Koichi Hasegawa, former postdoctoral fellow, to examine if acute transplantation of RG3.6 cells promotes functional recovery compared to medium controls. RG3.6 cells survived and migrated in the injured spinal cord as indicated by their GFP signals (figure 2). Whole mount images showed better tissue preservation in RG3.6 treated spinal cord than medium control. Early locomotor improvement was also observed in this experiment with ~2 points higher in BBB score in rats with RG3.6 transplants. This early recovery further suggests that RG3.6 cells may protect the contused spinal cord from secondary injury.

RG3.6 cells suppress neuroinflammation

The only clinically approved drug for SCI patients is the glucocorticosteroid methylprednisolone (MP). When given within 8 hours after injury, approximately 20%

of the motor function can be preserved in SCI patients (Bracken, Shepard et al. 1990; Young and Bracken 1992). MP has strong anti-inflammatory properties, which can prevent activation of microglia and infiltration of leukocytes to the lesion site. As a result, of MP treatment in rat SCI less pro-inflammatory cytokines were produced (Hsu and Dimitrijevic 1990; Bartholdi and Schwab 1995).

To examine if RG3.6 cells promote early locomotion recovery through reducing tissue inflammation, reactive microglia and infiltrating macrophages were immunolabeled after SCI and cell transplantation. Microglia were immunostained with Iba1 (ionized calcium-binding adaptor molecule-1) antibody and showed significantly reduce signals in RG3.6 treated spinal cord at 7 days post injury (figure 3A). In medium control rats, the Iba1 labeled microglia displayed stronger and thicker filaments suggesting a more activated state than Iba1 positive cells in RG3.6 transplanted spinal cords (Ito, Tanaka et al. 2001; Wu, Miyamoto et al. 2005) (figure 3B).

In contusive SCI, infiltration of macrophages peaks at 2 weeks after injury in Sprague-Dawley rats (Popovich, Wei et al. 1997; Sroga, Jones et al. 2003). Using ED1 (CD68) antibody to label macrophages at 2 weeks post injury, there was a significant reduction in ED1 labeling at the injury site in RG3.6 treated spinal cords compared to medium controls (figure 4). The suppression of ED1 was further evident at 6 weeks post injury in rats with RG3.6 transplants, while medium controls showed intense expression at the injury epicenter cavity (figure 4-5). The results suggest that RG3.6 cells modulate host tissue to suppress macrophage infiltration. Interestingly, rats with fibroblast grafts also reduced ED1 expression significantly. There appeared to be complementary distribution between ED1 positive cells and RG3.6 or fibroblasts, suggesting that the transplanted

cells inhibit ED1 macrophage infiltration. It is also possible that the reduction of ED1 macrophages is partly associated with space competition as fibroblasts can fill in the lesion site and prevent cyst formation (figure 5D).

RG3.6 cells reduced CSPG deposition

Extracellular matrix CSPGs are up-regulated in response to CNS injury and they inhibit axon growth and/or regeneration (Meiners, Powell et al. 1995; Davies, Fitch et al. 1997; Fitch and Silver 1997; Davies, Goucher et al. 1999; Jones, Yamaguchi et al. 2002; Morgenstern, Asher et al. 2002; Jones, Sajed et al. 2003). The inhibitory role of CSPGs is further evidenced by disruption of CSPGs using Chondroitinase ABC to improve axonal regeneration (Yick, Wu et al. 2000; Bradbury, Moon et al. 2002; Yick, Cheung et al. 2003; Barritt, Davies et al. 2006). NG2, a member of the CSPG family is highly up-regulated in the scar tissue after brain and spinal cord injury (Levine 1994; Jones, Yamaguchi et al. 2002; Tang, Davies et al. 2003). NG2 acts as a non-permissive substrate and inhibits neurite/axonal growth in vitro and in vivo (Dou and Levine 1994; Chen, Negra et al. 2002; Jones, Yamaguchi et al. 2002; Ughrin, Chen et al. 2003). CSPG and NG2 are reported to be the major component of the glial scar. They are produced by infiltrated macrophages, activated microglia, reactive astrocytes and oligodendrocyte precursors clustering in the lesion site (Davies, Fitch et al. 1997; Davies, Goucher et al. 1999; Jones, Yamaguchi et al. 2002). In our work, CSPG (which was labeled by CS56 antibody) and NG2 were both found to be significantly reduced in RG3.6 or fibroblasts treated spinal cords, as compared to medium controls (figure 6). Immature astrocytes and GRPs are reported to reduce scar formation and CSPG deposition following

transplantation to injured brain or spinal cord (Smith and Miller 1991; Hill, Proschel et al. 2004). As precursors to the above-mentioned cells, radial glia may also carry this function. Earlier we showed that macrophage infiltration (by ED1 immunostaining) and microglia reactivity (by Iba1 immunostaining) were significantly decreased in rats with RG3.6 transplants (figure 3-6). The significant reduction of CSPG and NG2 may result from RG3.6 cells suppressing the source of CSPG/NG2 producing cells (e.g. macrophages and reactive microglia) accumulated at the injury site.

Neuronal preservation and white matter sparing following SCI

One of the most important mechanisms for behavioral improvement is white matter sparing, which has been used to correlate with functional recovery after SCI (Farooque, Isaksson et al. 2001; Young 2002; Iannotti, Ping Zhang et al. 2004). As more white matter is preserved, more descending tracts remain functional for better behavioral recovery. To examine if white matter sparing correlates with early locomotion improvement in rats that received RG3.6 cells, we measured myelin preservation by Luxol fast blue staining at 2 weeks and 6 weeks post injury. At the 2 week time points, there is slight increase of spared white matter with no statistical significance in RG3.6 treated spinal cord as compared to medium control (data not shown) Significant amount of white matter was preserved by RG3.6 treatment at 6 weeks after injury (figure 7). Although rats treated with medium also showed some preserved white matter at the rim of injury epicenter, the positive area was restricted to the edge of ventral white matter. Interestingly, no cavity was observed in rats with fibroblast grafts because the grafts

filled up the injury site and blocked cyst formation. There was little spared white matter in fibroblasts treated controls.

To assess if spared white matter corresponds to neuronal and/or axonal preservation, we labeled and quantitated neurons and axons using immunostaining with antibody NeuN and neurofilament respectively. NeuN positive cells were quantitated within a 1.5-cm segment centered at the injury site at 7 days after injury. However, there was no significant difference between RG3.6 treated rats and medium controls (data not shown). Significant axonal preservation (labeled by neurofilament antibody) was observed in the white matter of RG3.6 treated spinal cord (figure 8-9). In addition to the increased expression of neurofilament, the presence of RG3.6 cells appeared to remodel the preserved axons in a very organized manner along the rostral-caudal orientation (figure 9D-E). In contrast, rats with medium treatment exhibited limited neurofilament positive fibers that were less organized in the injury site (figure 9C). There were some neurofilament positive axons inside fibroblasts grafts, however, very few axons were preserved in either dorsal or ventral white matter tracks (figure 8C). Without preserving axons in the injured spinal cord, fibroblast transplants therefore cannot promote significant functional recovery.

Higher magnification photomicrographs reveal that RG3.6 cells co-aligned with neurofilament axons, indicating a cell-cell contact relationship between transplant and host tissue (figure 9F-G). The cell-cell interaction was also identified in the coronal sections that were double-immunostained with neurofilament and myelin basic protein antibodies, showing that RG3.6 cells integrated into host tissue surrounding both myelinated and non-myelinated axons (figure 10a). The close cell-cell interaction can be

observed as early as 3 days post injury (figure 10b), suggesting that RG3.6 cell support axons at very early stages perhaps preventing further axonal degeneration, that may account for more axon preservation at later times (e.g. 6 weeks).

Cerebellar granule neuron neurite outgrowth on RG3.6 monolayer

An in vitro model of co-culture of RG3.6 cells and granule neurons was performed to test if RG3.6 cells provide a permissive environment for axon growth. Granule neurons grew and extended neurites on the monolayer of RG3.6 cells. RG3.6 fibers closely aligned with neurites of granule neurons suggesting there are some adhesion proteins on RG3.6 cells that interact with neuronal fibers to support and/or guide their movement (figure 11A). In contrast, granule neurons formed aggregates and appeared to avoid the fibroblasts monolayer (figure 11B) reflecting our in vivo observations that poor integration of fibroblasts into host tissue (figure 2AB, 8C).

Discussion

The novel observation in this chapter is that acute transplantation of radial glia protects tissue from secondary damage and contributes to early locomotion recovery following SCI. The tissue protection is associated with reduced infiltration of macrophages and/or suppressed micorglia reactivity. In addition, RG3.6 cells bridge the injury site, preserve white matter and support host axons. Similar to GRPs and immature astrocytes in other SCI studies (Hill, Proschel et al. 2004; Davies, Huang et al. 2006), radial glia RG3.6 cells suppress CSPG deposition and thereby may provide a more permissive environment for axonal growth/regeneration.

Most of the cell-based research for SCI emphasizes applying cells in sub-acute phase in order to obtain better survival of implanted cells. Survival and cell differentiation are two major challenges when transplanting cells acutely in the injured spinal cord where implanted cells are exposed to excess glutamate, reactive oxygen species and various inflammatory cytokines. Increased pro-inflammatory cytokines including TNF-α, IL-1, and IL-6, etc. (Pan, Ni et al. 2002; Pineau and Lacroix 2007) have been shown to promote neural stem cell differentiation into astrocytes (Bonni, Sun et al. 1997; Cao, Zhang et al. 2001). It is still unclear if differentiating implanted cells into astroglia in injured spinal cord is beneficial for axonal regeneration. Therefore studies aiming at utilizing implanted cells to remyelinate axons tend to transplant cells during sub-acute phase to avoid toxic and pro-astroglial differentiation environment in the acutely injured spinal cord.

Despite the challenges of cell-based treatment during acute SCI, our results suggest that interventions at acute phase can protect tissue and suppress the progression of

secondary injury. Like other groups, we also found poor survival of RG3.6 cells when directly transplanted into the contusion site following injury. Therefore we developed a 3-point injection protocol with two additional sites rostral and caudal to the injury epicenter, which greatly increased survival of RG3.6 cells in the injured spinal cord. Moreover, surviving RG3.6 cells migrated extensively in the host white matter.

Here we showed the therapeutic potential of radial glia neural stem cells in acute SCI. Our results demonstrated that v-myc stabilized radial glial cells promoted early locomotion recovery that cannot be attributed to remyelination, which strongly suggests that this treatment preserves and/or protects major spinal tracks to execute correct muscle/ joint innervation. The early improvements are most likely attributed to cellular and biochemical changes from RG3.6 cells to the host tissues.

Immediately after SCI, trophic factors including NGF, BDNF, NT3, and CNTF were transiently up-regulated in response to the injury (Dreyfus, Dai et al. 1999; Widenfalk, Lundstromer et al. 2001), however the levels secreted are not sustained and sufficient for spontaneous axonal regeneration. RG3.6 cells also express neurotrophic factors (see Chapter 1), thus implanting them into injury site may increase total amount of trophic factors available to protect host tissue. It will be important to measure trophic factor levels in the presence of RG3.6 cells in the injured spinal cord using ELISA assay.

Another possible pathway for RG3.6 cells to protect tissue is their ability to transport excess extracellualr glutamate and convert it into non-toxic glutamine. Astrocytes and some microglia are important in maintaining extracellular glutamate in balance through their glutamate transporters (Sonnewald, Westergaard et al. 1997; Nakajima, Tohyama et al. 2001). Immunofluorescent labeling of GLAST showed co-

localization with RG3.6 cells at the injected dorsal column (figure 12), suggesting that RG3.6 cells may modulate glutamate toxicity in part through providing additional transporter GLAST. It will require complicated experimental procedures, e.g. microdialysis and HLPC, to prove whether RG3.6 cells reduce extracelluar glutamate in the injured spinal cord.

The initial trauma to the spinal cord activates immune reactions, recruits leukocytes to clear dead or dying cells. In contusive injury, massive pro-inflammatory cytokines are quickly and consistently released from the injury site (by both local and infiltrated cells) resulting in a prolonged status of inflammation that recruits more macrophages to the injury site. Consequently immune response activates more cell death at regions adjacent to the lesion site. Infiltration of macrophages and reactivity of microglia have been used as an index for neuroinflammation after SCI (Popovich, Wei et al. 1997). Popovich and colleagues have reported that peripheral macrophages may employ dorsal and ventral roots of spinal cord as migration routes to the injury site (Popovich, Wei et al. 1997). Acute RG3.6 treatment significantly suppressed infiltration of macrophages and reactivity of microglia (figure 3-4). Interestingly, we found very little ED1 expression at the ventral roots of RG3.6 treated spinal cord while intense expression was observed in medium treated controls at 2 weeks post injury (figure 4). These results indicate that RG3.6 cells may interfere with the trauma cascades early after injury by modulating the activities of inflammatory cells (e.g. microglia and macrophages) at the lesion site. Suppression of ED1 expression was also observed in rats that received fibroblasts. As fibroblasts have significant trophic function, it is possible that acute transplantation of fibroblasts protects host tissue and reduces inflammation. In view of fibroblast grafts

aggregated in the lesion site with no cyst formation, it is also possible that fibroblasts prevent macrophage infiltration by filling the injury area to compete for space. When macrophages were detected in fibroblasts treated spinal cord, they were in a complementary pattern with implanted cells, which is an example of space competition.

Macrophages and reactive glia produce inhibitory proteoglycan CSPG following spinal cord injury (Davies, Fitch et al. 1997; Fitch and Silver 1997; Jones, Yamaguchi et al. 2002). The strong reduction of CSPG in both RG3.6 and fibroblasts implanted spinal cords is likely correlated to fewer macrophages infiltrating into the injury site. Another possible mechanism for the reduction of CSPG deposition is the presence of decorin, a small, leucin-rich, dermatan/chondroitin sulfate proteoglyacan found in the extracellular matrix of many tissues (Hocking, Shinomura et al. 1998). Decorin has been reported to degrade CSPGs and inhibit their synthesis (Davies, Tang et al. 2004; Davies, Tang et al. 2006). Interestingly, etiology research in Duchenne Muscular Dystrophy has identified that fibroblast is the source of decorin (Fadic, Mezzano et al. 2006). Additional work in the future is needed to determine whether radial glia and fibroblast can produce decorin and its association with CSPG degradation following SCI.

Successful integration with host tissue is another important factor that RG3.6 cells may help to promote functional recovery. RG3.6 cells formed interactive networks within the injured host tissue to surround myelinated and non-myelinated axons as early as 3 days post injury (figure 10). By virtue of radial glial cells' trophic function and their role in the developing CNS to support and guide neurons, RG3.6 cells may protect injured axons by secreting growth factors and cell-cell contact support. Preservation of axons by RG3.6 cell was demonstrated at 6 weeks post injury, when RG3.6 cell not only

saved significant amount of neurofilament fibers but also organized them in a rostral-caudal orientation (figure 8-9). In contrast to the positive affinity between radial glia RG3.6 cells and host axons, fibroblasts grafts showed no integration with host tissue yet expanded to deteriorate the tissue, especially the white matter (figure 8). Very limited neurofilament fibers or white matter were preserved in spinal cord with fibroblast transplants. Although fibroblast reduced macrophage infiltration to the injury site and has been a good vehicle for trophic factor delivery, the poor integration with host cells and the damage to host tissue made fibroblast a less desirable candidate for cell-based therapy for SCI.

In this chapter, we described behavioral and histological changes following spinal cord injury and acute radial glia transplantation. Radial glia RG3.6 cells protected injured spinal tissue from secondary tissue damage and supported host axons. Although acute cell transplantation may not yet be feasible clinically, we demonstrate another therapeutic potential to use neural stem cell in CNS trauma. It is important to understand the mechanisms underlying RG3.6 cells mediated tissue protection in this study, which may facilitate the development of more practical treatment for human clinical trials.

Table 1. Antibodies used in this chapter

Primary antibody	Conc.	Isotype	Source	Secondary Antibody	Conc.	Source
Iba1	1: 1000	Mouse Monoclinal IgG	Wako	Alexa568 Goat-anti-mouse IgG (H and L)	1: 400	Molecular Probe
ED1 (CD68)	1: 300	Mouse Monoclinal IgG	Serotec	Alexa568 Goat-anti-mouse IgG (H and L)	1: 400	Molecular Probe
CS56	1: 400	Mouse Monoclinal IgG	Sigma	Alexa568 Goat-anti-mouse IgG (H and L)	1: 400	Molecular Probe
NG2	1: 500	Rabbit Polyclonal IgG	Dr. Joel Levine	Alexa647 Goat-anti-mouse IgG (H and L)	1: 400	Molecular Probe
NF	1: 500	Mouse Monoclinal IgG	Sigma	Alexa568 or 647 Goat-anti-mouse IgG (H and L)	1: 400	Molecular Probe
MBP (SMI99) (SMI94)	1: 1000	Mouse Monoclonal IgG	Sternberger	Alexa568 Goat-anti-mouse IgG (H and L)	1: 400	Molecular Probe
Nestin	1: 50	Mouse, Monoclonal IgG	DSHB	Alexa488 Goat-anti-mouse IgG (H and L)	1: 400	Molecular Probe
TuJ1	1: 500	Rabbit Polyclonal IgG	Covance	Alexa568 Goat-anti-mouse IgG (H and L)	1: 400	Molecular Probe
GLAST	1: 5000	Guinea Pig Polyclonal IgG	Chemicon	Cy3 conjugated Goat-anti-guinea pig, IgG (H+L)	1: 400	Chemicon

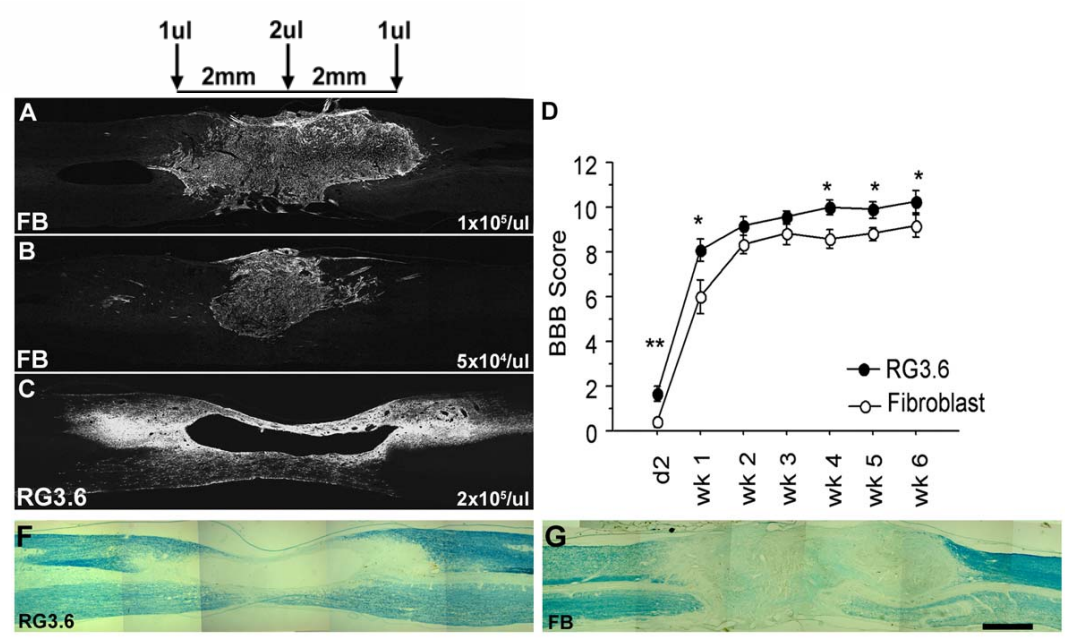


Figure 1. Transplantation of RG3.6 cells improved behavioral recovery and preserved myelin after injury. GFP fluorescence shows fibroblasts and RG3.6 cells 6 weeks after injection at three sites indicated by arrows above the micrographs (A-C). Note that the fibroblasts expanded locally often to the dura with accumulations that were dose-dependent (A vs. B). In contrast, RG3.6 cells migrated rostro-caudally and surrounded the cystic cavities that formed (C). BBB open field behavioral tests were recorded weekly for each animal (D). Statistically significant differences were observed at day 2 and several subsequent time points between the two groups with RG3.6 (●) showing consistently better performance than the fibroblast (O) transplanted group. n=10 per group. Data represent means ± SEM. (*$p < 0.05$, **$p < 0.01$, ANOVA). Luxol Fast Blue staining showed better preservation of myelin in RG3.6 (F) than in fibroblast (G) transplanted rats. Note the continuity across the injury site of myelinated bridges with RG3.6 but not with fibroblasts. Bar = 1 mm

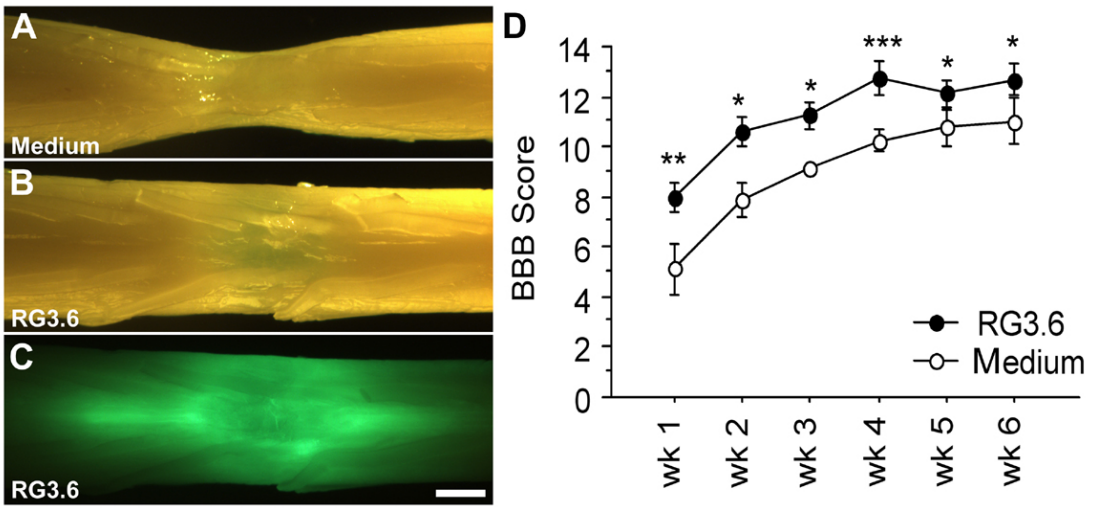


Figure 2. Transplantation of RG3.6 cells improved tissue preservation and behavioral recovery. Whole mounts of spinal cords dissected from rats injected with medium (A) or RG3.6 cells (B, C) visualized by light (A, B) and fluorescence microscopy (C). BBB open field behavioral tests were performed weekly. RG3.6 (●) showed consistently higher scores than medium (O) transplanted group. n=8 for RG3.6, n=4 for medium. Data represent mean ± SEM. *p < 0.05, **p < 0.01, ***P < 0.001, ANOVA. (By Koichi Hasegawa)

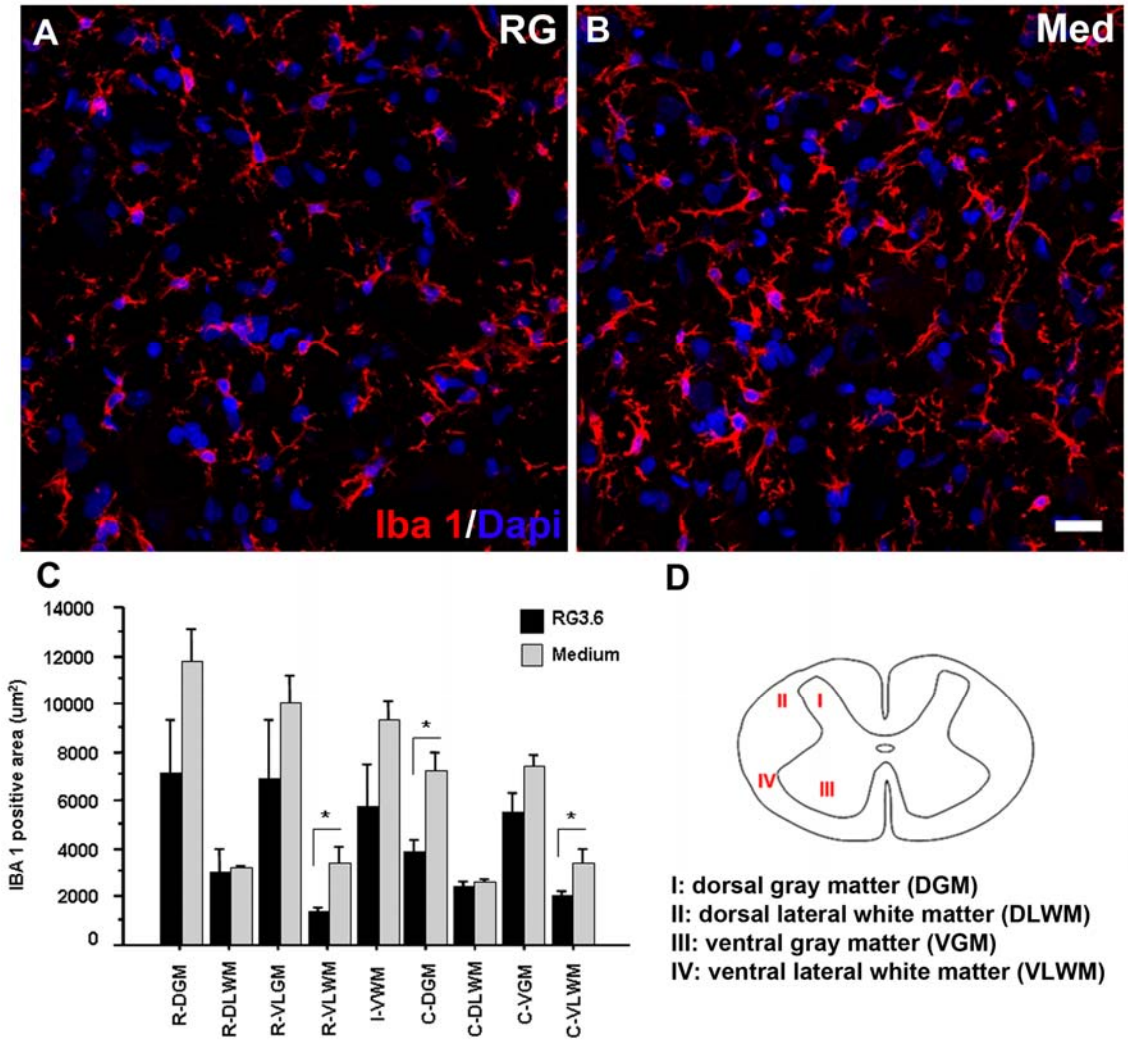


Figure 3. Microglia were immunolabeled by Iba1 antibody at 7 days post injury. (A-B) Images were taken from 5 mm caudal to the injury epicenter. Similar amount of Iba1 positive cells were detected in RG3.6 cell (A) and medium (B) treated spinal cords, but the expression was significantly stronger in medium controls (B). Serial sections from a 1.5 cm spinal segment centered at the injury site were used to quantitate Iba1 expression. Regions illustrated in the schematic cross section (D) were photographed for Iba1 quantitation. RG3.6 cells reduced microglia activity at rostral and caudal regions, mostly ventral lateral white matter (VLWM) (C). Scale bar is 20 μm. Data represent mean ± SEM. *$p < 0.05$, t-test.

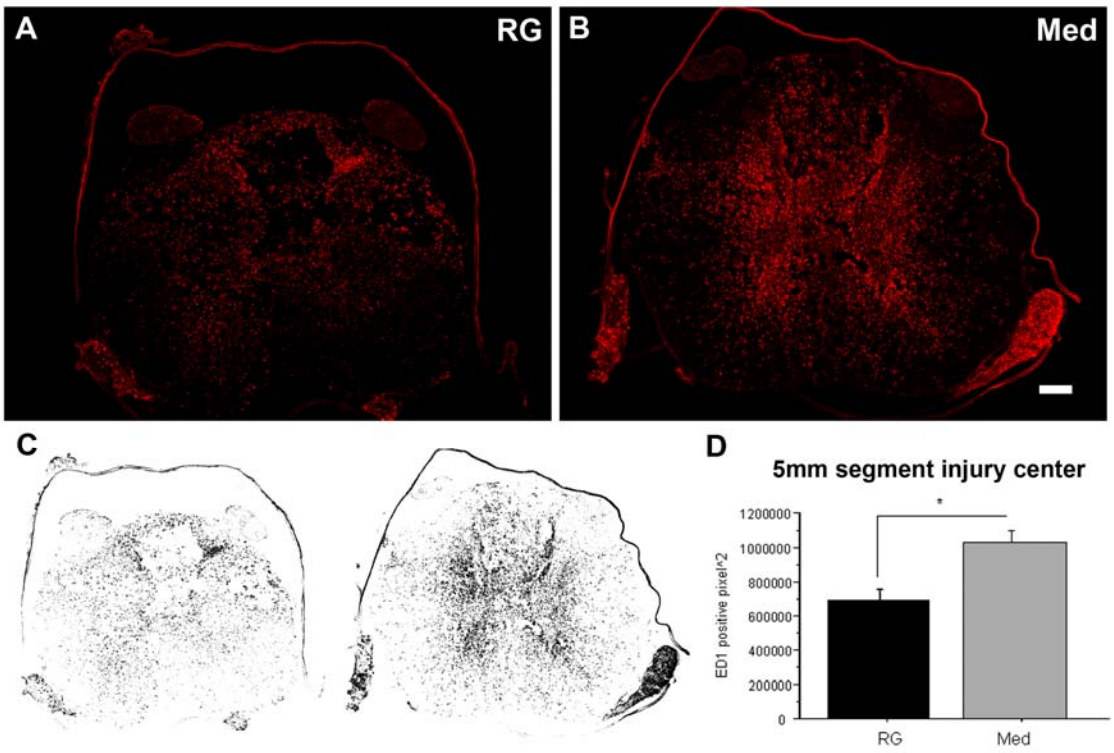


Figure 4. ED1 expression at the injury epicenter at 14 days post injury. Macrophages were immunolabeled by ED1 antibody and the expression was quantitated into pixel area by NIH Image J program on inverted images (C). Roots and meninges were excluded. ED1 expression was significantly reduced in RG3.6 treated spinal cords but remained strong in medium controls (A-B). Inverted images show positive staining and strong signal in the ventral root of medium treated controls (C). Serial sections from a 5-mm long segment centered at the injury were used for quantitation; summation of positive pixels indicates RG3.6 cells suppressed macrophages infiltration (D). Scale bar is 200 μm. Data represent mean ± SEM. *P <0.01, t-test.

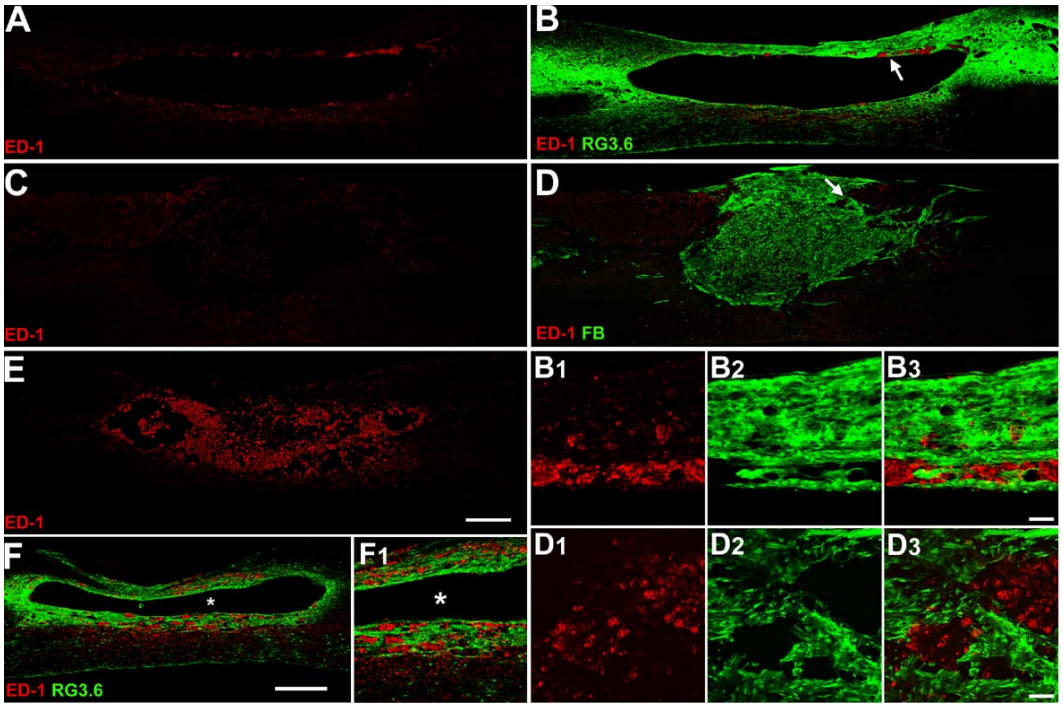


Figure 5. RG3.6 reduced macrophage accumulation at 6 weeks after SCI. ED1 staining was performed in midsagittal sections of RG3.6 (A-B), fibroblast (FB) (C-D), or medium treated groups (E). Twenty-four 10x images were tiled to cover the injury site using Zeiss LSM software. Panels B1-3 and D1-3 are higher power views of regions marked with arrows in panels B and D, respectively that illustrate complementary distributions of the transplanted cells with ED1 macrophages. Macrophages were much more prevalent in medium controls (E). A lateral section of an RG3.6 transplant illustrates a higher density of macrophages in this region (F, F1) than medial section (A-B). Scale bar is 500 μm in A-F and 50 μm in other panels.

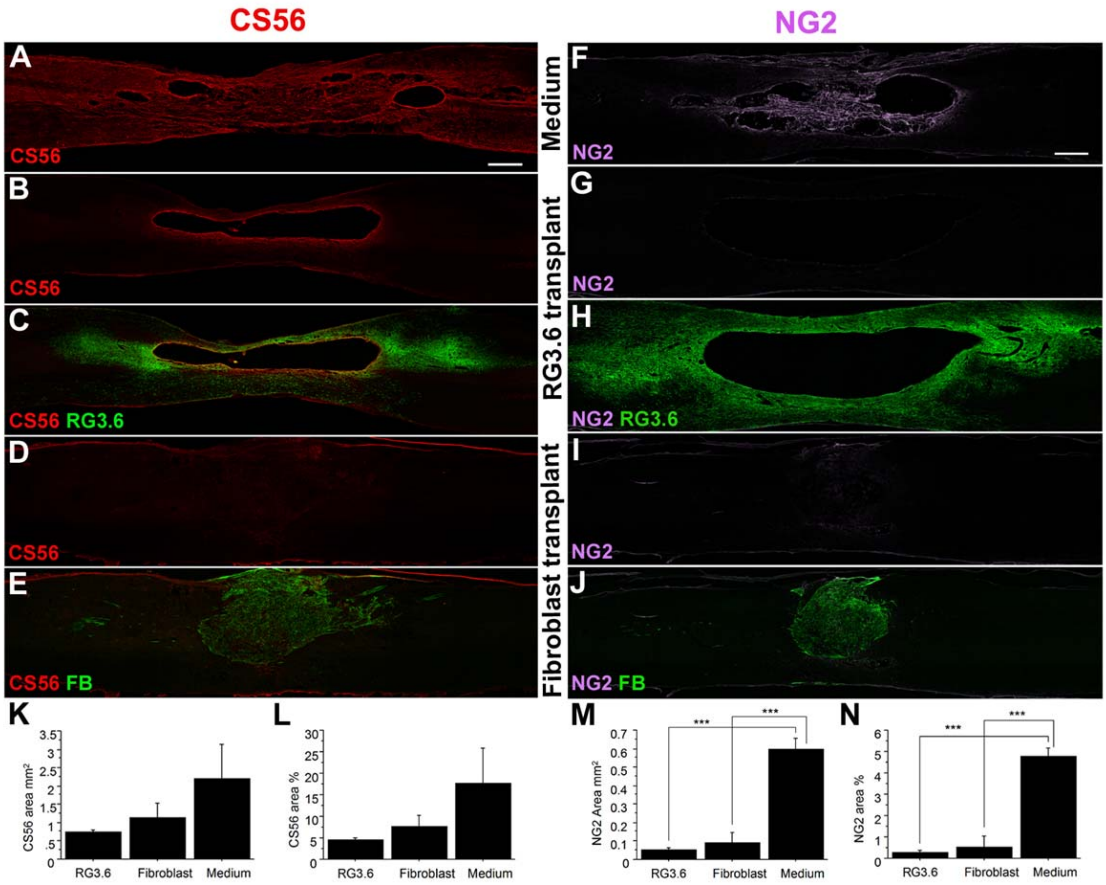


Figure 6. RG3.6 reduced deposition of CSPG and NG2 at 6 weeks after SCI. Mid-sagittal sections of control medium (A and G), RG3.6 (B, C, H and I), and fibroblast (FB) (D, E, I and J) treated groups were immunostained for CS56 (A-E) and NG2 (F-J). Thirty (10x) images were tiled (X: 10, Y: 3) to cover the injury site (9 mm x 3 mm) using Zeiss LSM software. Areas with intensity above threshold values for CS56 or NG2 were taken from each tile scan (K and M). The expression percentage was obtained by normalizing actual area above threshold to total area measured (L and N). Scale bar is 500 μm. Data represent means ± SEM. ***p <0.001, ANOVA.

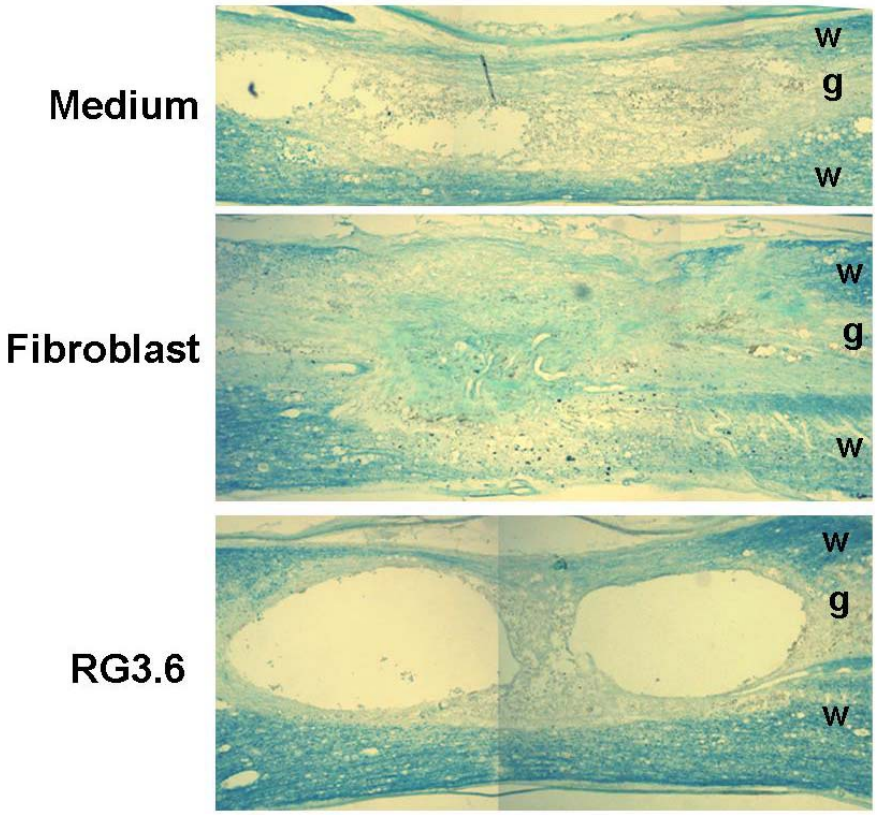


Figure 7. Spared white matter of mid-sagittal sections was labeled by Luxol fast blue at 6 weeks post SCI. RG3.6 cells preserve more spared dorsal and ventral white matter than medium or fibroblasts treated spinal cords. Medium treated control preserved some ventral white matter while fibroblast transplants blocked host spinal cord with extremely limited white matter remained at the lesion site despite no cavity was found. (g, gray matter; w, white matter)

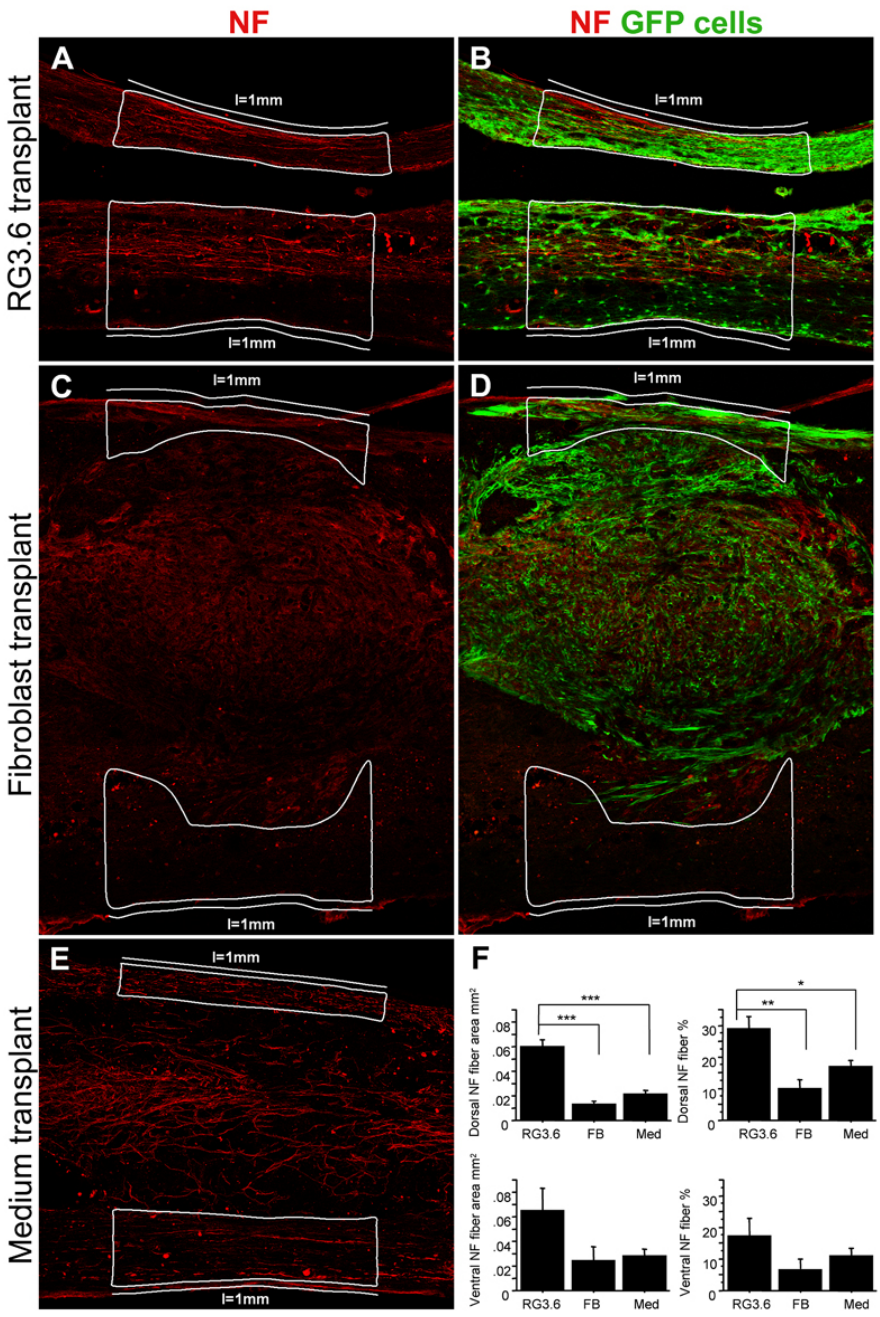


Figure 8. RG3.6 preserved host axons. Sections immunostained for neurofilament (NF) were shown in rats transplanted with RG3.6 (A-B), fibroblasts (C-D) and medium (E) 6 weeks after SCI. Spared dorsal and ventral fiber tracts were outlined (A-E) within 1 mm from the dorsal and ventral surfaces of the spinal cord in mid-sagittal sections and then measured using Zeiss confocal LSM software (F). Percentages of dorsal and ventral NF+ areas were normalized to the outlined areas (F). Data shown are means ± SEM. *p<0.05, **p<0.01, ***P<0.001, ANOVA.

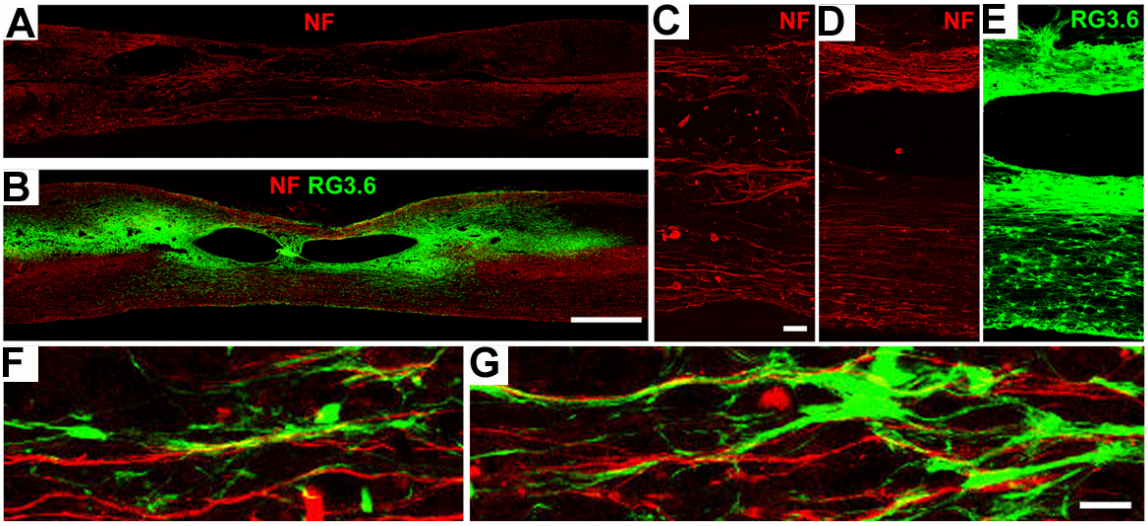


Figure 9. NF staining in sagittal sections of contused spinal cord injected with medium (A,C) or RG3.6 cells (B,D,E). Panels F and G are higher magnifications of the center of injury sites showing disorganized NF in medium treated rats by comparison to the more longitudinally organized NF patterns in RG3.6 treated rats (panel E shows the RG3.6 cells corresponding to panel D). Scale Bar is 1 mm in B, and 100 μm in C.
(By Koich Hasegawa and Yu-Wen Chang)

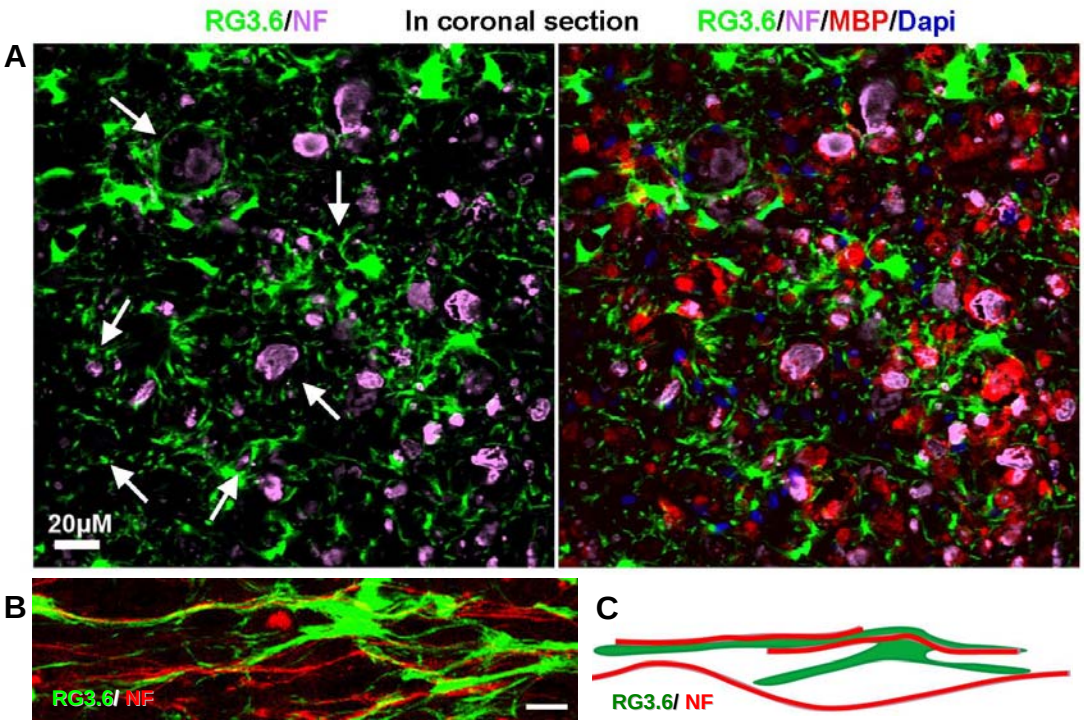


Figure 10a. RG3.6 cells developed network integrating into host tissue at 6 weeks post injury. Processes of RG3.6 cells surrounded host axons which are either myelinated (NF+/MBP+) or non-myelinated axons (NF+/MBP-) (A). In Sagittal sections, RG3.6 fibers aligned with NF+ axons. This is the same image as fig 2-9G (B). (C) Schematic illustration shows interaction between RG3.6 and NF in (B).

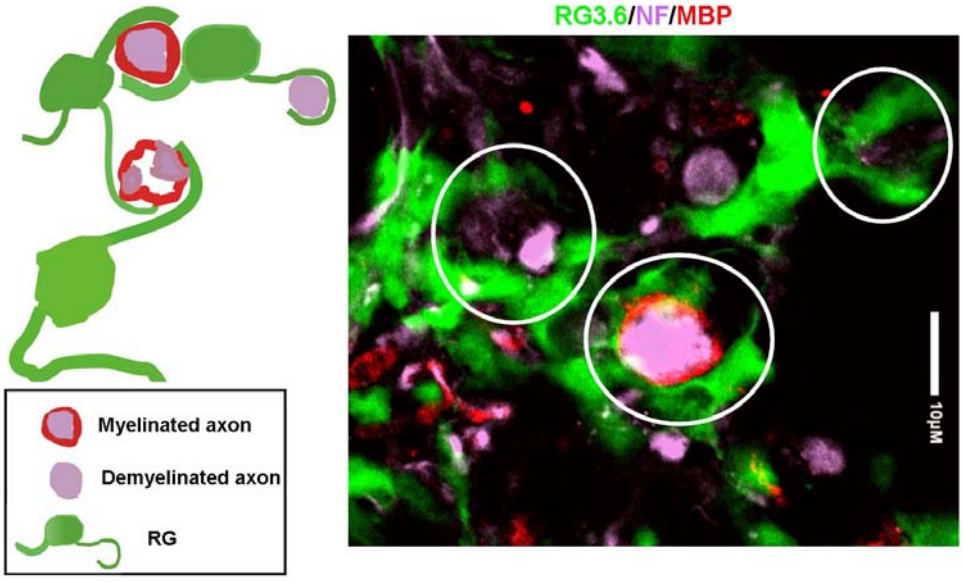


Figure 10b Schematic illustration of radial glial network surrounding axons. RG3.6 cells network can be seen as early as 3 days post injury. RG3.6 cells showed strong affinity to host axons to encircled NF+ axons and myelinated (MBP+) NF axons.

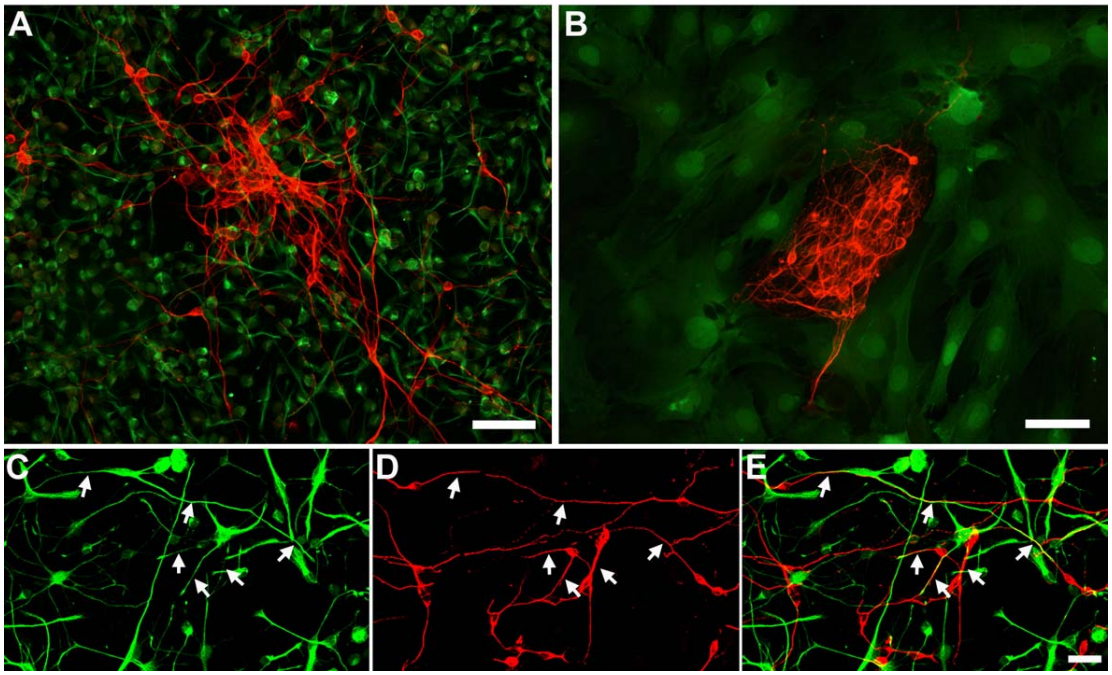


Figure 11. Coculture of cerebellar granule neurons on monolayers of RG3.6 or fibroblast. Aggregates of cerebellar granule neurons were grown on monolayers of RG3.6 (A and C–E) or fibroblast (B). Immunostaining with TuJ1 antibody (red) revealed that neuronal fiber outgrowth was much more extensive on RG3.6 cells than on fibroblasts, and higher magnification views showed very close association of the neurites along the radial glial processes (arrows) that are seen in the overlay (E) of the RG3.6 cells (C) and the neurites (D). Scale bar is 50 μm in A and B, and 20 μm in E.

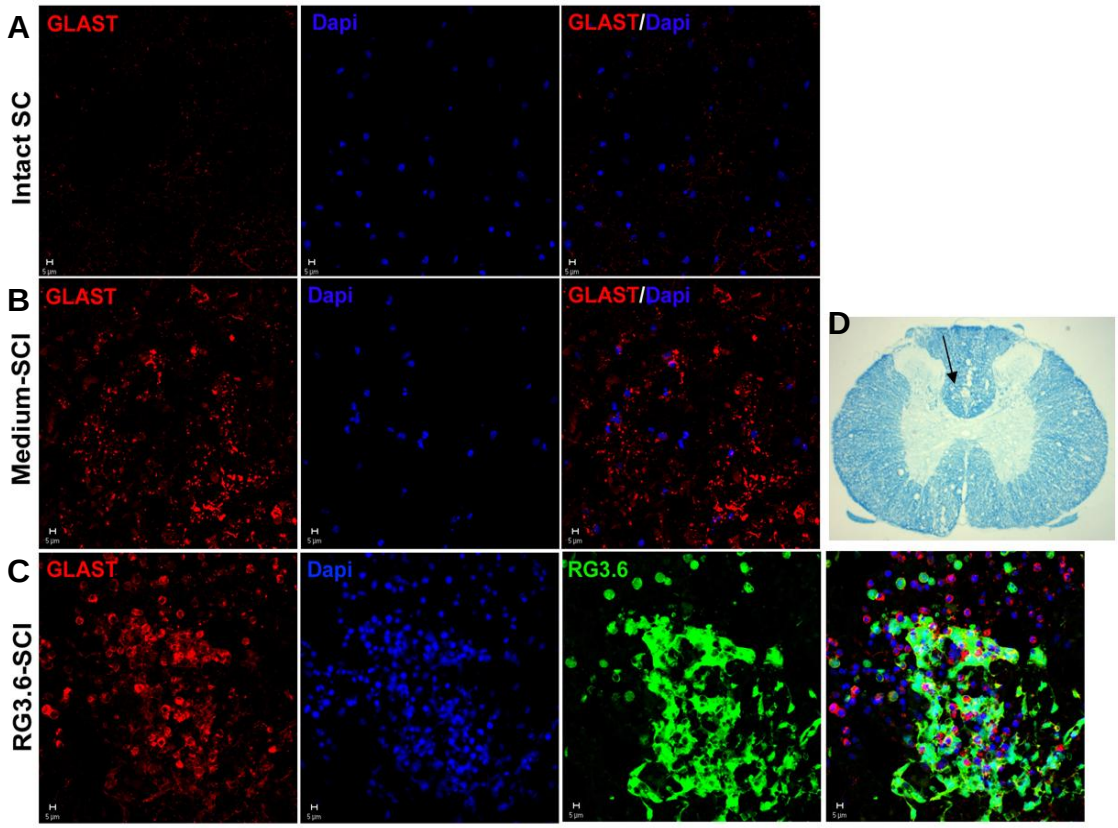


Figure 12. Glutamate transporter GLAST expression at dorsal column of injured spinal cord. GLAST expression was low in sham animal (A) but increased after SCI (B-C). Some GLAST is co-localized with RG3.6 in the spinal cord at 24 hrs post injury (C). Images were taken from dorsal column where cells were injected as illustrated in a luxal fast blue labeled spinal cord image (D). Scale bar is 5μm.

Chapter Three

Tissue protective mechanism by RG3.6 transplantation following SCI

Introduction

Spinal cord injury (SCI) triggers a series of pathophysiology changes that lead to progressive tissue damage and deterioration of neurological functions. The genome responses have been analyzed to understand the cellular and molecular changes after SCI using advanced microarray technology. Distinct patterns of gene expression at different injury areas and time points were identified and revealed characteristic tissue loss and degenerative events (Carmel, Galante et al. 2001; Di Giovanni, Knoblach et al. 2003; Aimone, Leasure et al. 2004; De Biase, Knoblach et al. 2005). Early after SCI, inflammatory and transcriptional genes were induced quickly, whereas genes coding for neuronal structural proteins and ion transport protein were suppressed (Carmel, Galante et al. 2001; Nesic, Svrakic et al. 2002); at later time points, growth factors, cell proliferation, and angiogenesis related genes were up-regulated (Bareyre and Schwab 2003; Velardo, Burger et al. 2004), suggesting tissue repair mechanisms have been initiated.

Gene expression profiles have been widely used to evaluate the efficacy of treatments for SCI, such as anti-inflammatory drugs (e.g. Cox2 inhibitor, MP, and MK801) or antibody IN-1 application (Plunkett, Yu et al. 2001; Bareyre, Haudenschild et al. 2002; Nesic, Svrakic et al. 2002; Bareyre and Schwab 2003; Pan, Jornsten et al. 2004). The application of microarray, however, has not been extended to study cell-based therapy for SCI. While most of the cell-based therapies emphasize behavioral and

histological changes associated with axonal regeneration and/or remyelination, which often take place during sub-acute phase of SCI, the underlying mechanism at gene expression level has not yet been examined. Previously we have shown that acute transplantation of a radial glial clone RG3.6 cells promoted locomotion recovery during very early phase of SCI (Hasegawa, Chang et al. 2005), yet mechanisms of this early improvement remain unknown. It has been shown that microglia activation and/or infiltration of macrophages to the injury site were significantly suppressed by RG3.6 cells treatment (figure 3 in Chap. 2). The behavioral and histological changes indicated that acute RG3.6 transplantation may protect tissue through modulating the magnitude of inflammatory signals. In an attempt to identify potential molecular mechanism of RG3.6's tissue protection function, we measured several pro-inflammatory genes e.g. IL-6, MCP-1, and MIP-2 by quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR). However, the mRNA expression of these genes was not significantly changed by RG3.6 transplantation. Genes that are associated with protective stress response and anti-oxidation, e.g. Hsp70, Hsp27 and HemeOx1, were also examined using Q-RT-PCR. The expression of Hsp70 was specifically enhanced in rats that transplanted with RG3.6 cells, and the up-regulation peaked at 12 hours and went down by 24 hours. Given little or no GFP expression was found in the proximal and distal segments during 6 to 12 hours post injury, RG3.6 cells did not migrate out of the injury site during this time. Therefore the enhanced up-regulation of Hsp70 at proximal and distal regions should be associated primarily with host responding to injury and signals secreted from RG3.6 transplants. Our current hypothesis is that RG3.6 cells at the injury site signal to surrounding tissue and promote favorable host responses that protect tissue from secondary injury.

To focus on host response, distal (caudal) segments at 12 hours post-injury were subjected to Affymetrix Rat 230.2 gene chip analysis. After data normalization and statistical analysis, levels of 30,000 gene transcripts were measured and compared in RG3.6 treated group over medium treated controls. Filtering with T test with less than 5% false discovery rate suggests that 60 genes were significantly changed by at least 1.5 fold. Among these, several genes involved in cell proliferation and stem cell development, such as Foxg1, Lef1, Top2a, Sox11, vimentin, Nkx2.2 were significantly increased in spinal cords with RG3.6 transplants at 12 hours after injury. Selected candidates of these genes were confirmed using Q-RT-PCR and/or immunohistochemistry.

These results indicate that acute radial glia transplantation helps to improve functional recovery, likely by remodeling the local injury environment. The protective effect may be due to enhancement of self-defense mechanisms, activation and promotion of cell proliferation/activation and stem cell differentiation, which may lead to tissue survival and repair.

Materials and methods

Spinal cord injury and acute transplantation

Animal used in Chapter Three

Time	RG3.6				Medium				Fibroblast				NSCs				RG3.6 in Sham				Sham			
	RNA			His.	RNA			His.	RNA			His.	RNA			His.	RNA			His.	RNA			His.
	P1	I	D1		P1	I	D1		P1	I	D1		P1	I	D1		P1	I	D1		P1	I	D1	
6 h	3				3				3															
12 h	4			2	3			1	3			1	3				3							
24 h	3			4	3			4	3			1									3			2

Animals used in this chapter. Some animals were prepared for RNA extraction (RNA), and some for histology examination (His.).

Adult female Sprague-Dawley rats (Taconic, Germantown, NY) weighing 200–250 g were used in this study. Surgery procedures and post-operation care were described in Chapter 2. For microarray or Q-RT-PCR study, animals were anesthetized with 40 mg/kg pentobarbital i.p. and their spinal columns were quickly removed and frozen by dry ice powder. A 5-mm spinal cord segment centered at injury epicenter (contusion site) was collected and labeled "I" for injury site. The adjacent 5-mm segments were collected and labeled "P1" for proximal site (rostral to epicenter) and "D1" for distal site (caudal to epicenter). Total cellular RNA was extracted from P1, I and D1 segments of each animal separately, and the procedure will be described later. Animals for histology study were anesthetized and then perfused with 4% paraformaldehyde. Tissue processing and sectioning were described in Chapter 1-2.

Total cellular RNA preparation and Q-RT-PCR

Spinal segments were homogenized in ice-cold Trizol. Chloroform was added to Trizol homogenate and aqueous phase was collected after centrifugation. RNA was prepared following Qiagen RNeasy Mini protocol (Qiagen, Valencia, CA). The column was washed and RNA eluted following the manufacturer's recommendations. RNA was

submitted for quality control testing on Agilent Bioanalyzer (Agilent, Palo Alto, CA) and all samples demonstrated sharp ribosomal RNA bands (not shown). 1 µg of total RNA was converted to first-strand cDNA using SuperScript II (Invitrogen, Carlsbad, CA) reverse transcriptase primed by oligo dT. The PCR reaction was performed on 10 ng of cDNA, 50 nM of primers, and SYBR Green master mix (Applied Biosystems, Foster city, CA) in 10 µl reactions using Applied Biosystems 7900HT machine. The PCR products were quantified based on standard curve which was developed from pooled samples' cDNA. The expression value of each gene was normalized to the amount of GAPDH cDNA to calculate a relative amount of RNA present in each sample. One-way ANOVA followed by Fisher's protected least significant difference (PLSD) was used to determine statistical differences.

Microarray data analysis

Data from 6 Affymetrix Rat 230.2 chips (3 for RG, 3 for Med) were imported into R (http://www.r-project.org) and preprocessed using several packages from Bioconductor (http://www.bioconductor.org), an open-source bioinformatics package. Briefly, data were background corrected and normalized using the Robust Multi-Array Average (RMA) technique. A Student's T-test was conducted, assuming equal variances, to select differentially expressed genes between the two groups. P-values were adjusted for multiple comparisons using the Benjamin-Hochberg method.

The resulting list contained 845 genes at a p-value false discovery rate (FDR) cutoff $\leq 5\%$. Among the 825 genes, sixty genes showed fold changes more than 1.5 folds and were exported to Ingenuity Pathway Analysis (http://www.ingenuity.com) for classification/functional and pathway analysis.

Immunofluorescence (immunostaining)

Antibodies used in this chapter

Primary antibody	Conc.	Isotype	Source	Secondary Antibody	Conc.	Source
Hsp70	1: 100 **	Mouse Monoclonal IgG	Santa Cruz	Alexa568 Goat-anti-mouse IgG (H and L)	1: 400	Molecular Probe
Foxg1	1: 1000	Rabbit Polyclonal IgG	Dr. Lorenz Studer	Alexa568 Goat-anti-rabbit IgG (H and L)	1: 400	Molecular Probe
Nkx2.2	1: 20 *	Mouse Monoclonal IgG	DSHB	Alexa568 Goat-anti-mouse IgG (H and L)	1: 400	Molecular Probe
NG2	1: 500	Rabbit Polyclonal IgG	Dr. Joel Levine	Alexa647 (Cy5) Goat-anti-rabbit IgG (H and L)	1: 400	Molecular Probe
Vimentin	1: 20	Mouse Monoclonal IgM	DSHB	Alexa568 Goat-anti-mouse IgM	1: 400	Molecular Probe

** Incubation at room temperature for two over night. * Incubation at room temperature over night.

Antibodies used in this chapter are listed above. Briefly, sections were blocked with 10% normal goat serum/0.3% Triton X-100 in PBS for 2 h at room temperature and incubated overnight at 4°C with primary antibodies (some antibodies require longer incubation at room temperature). Sections then were washed with PBS and incubated with appropriate secondary antibodies at 1:400 dilution for 1 h at room temperature. After washing with PBS, sections were counterstained with Hoechst 33342 at 1:2000 and mounted with Gel/Mount. Image analysis was performed using Zeiss 510 confocal laser scanning microscope (LSM).

Quantitation for Nkx2.2 immunofluorescence

Animals were sacrificed at 24 hours following SCI and transplantation. Three para-sagittal sections close to the mid-line were used for quantitation. The white matter (WM) was divided into 6 regions as follows: rostral dorsal WM, rostral ventral WM, injury dorsal WM, injury ventral WM, caudal dorsal WM, and caudal ventral WM. Two representative images (25x optic of LSM 510 confocal microscope) were chosen from

each region of the section, thus a total six images were used for Nkx2.2 counting for one specific region. Differences between RG3.6 and medium treated spinal cords were analyzed by student t-test.

Results

RG3.6 cells enhanced tissue protection genes with no effect on pro-inflammatory gene expression

The characteristic changes in gene expression after acute SCI include induction of pro-inflammatory mediators and decrease of neuronal genes for structural proteins, such as cytoskeleton, ionic transporter, and synaptic transmission (Carmel, Galante et al. 2001; Bareyre and Schwab 2003). The levels of these inflammatory genes peak between 12-24 hours post injury, then gradually return to baseline as in sham animal (Carmel, Galante et al. 2001; Aimone, Leasure et al. 2004). As we have observed tissue protection after early RG3.6 transplantation, it is of particular interest to see how transplants interfere with the acute destructive biological cascade after SCI. We examined the level of pro-and anti-inflammatory genes after RG3.6 transplantation following SCI using Q-RT-PCR. Interleukin 6 (IL-6), macrophages inflammatory protein 2 (MIP-2), monocyte chemoattractant protein 1 (MCP-1), heat shock protein 27, 70, (Hsp27, Hsp70) and heme oxygenase 1(HemeOx1) were measured in spinal cord tissue at the injury site, proximal (rostral), and distal (caudal) segments at 12 hours post injury.

- ***(I) Pro-inflammatory gene response after SCI***

IL-6, MIP-2 and MCP-1 are inflammatory molecules important for leukocyte proliferation and activation. They are highly up-regulated following SCI (Carmel, Galante et al. 2001; Jones, McDaniel et al. 2005). As our histological observations indicated that macrophage and/or microglia activity was reduced significantly in RG3.6 treated spinal cords (figure 3-4 in Chap. 2), we predicted a lower expression of pro-inflammatory mediators in comparison with medium treated controls. However, these

genes exhibited no significant change in mRNA expression at 12 hours post injury in all injury conditions that we examined (figure 1).

- (II) Genes associated with protective stress response after SCI

Genes associated with tissue protection are also up-regulated following SCI (Carmel, Galante et al. 2001; Bareyre and Schwab 2003). Heat shock proteins (HSP) are a family of conserved proteins induced in response to stress (e.g. heat, toxins, ischemia, and inflammation). Several HSPs are up-regulated after SCI, including Hsp70, Hsp27, and Hsp32 (also known as Heme oxygenase 1, HemeOx1) (Sharp, Massa et al. 1999; Mautes, Bergeron et al. 2000; Mautes and Noble 2000; Carmel, Galante et al. 2001). In ischemia/reperfusion models, brief induction of HSPs significantly protects cells from subsequent insults and increases the tolerance to stress (Yenari 2002). The protective function of HSP is attributed to refolding damaged protein and prevention of apoptosis (Gething and Sambrook 1992; Parsell and Lindquist 1993; Beere, Wolf et al. 2000; Beere and Green 2001). Previously, we have shown that RG3.6 cells promoted early locomotion recovery after acute transplantation to injured spinal cords. The early behavioral improvement may be in part related to up-regulation of HSPs. To test that idea, we performed Q-RT-PCR reaction of Hsp70, Hsp27 and HemeOx1 at 12 hours post injury and found that all the treatment conditions exhibited increase of Hsp70, and surprisingly the increase was significantly and specifically enhanced in rats that were transplanted with RG3.6 cells (figure 1). The up-regulation of Hsp70 by RG3.6 transplant was injury-dependent since we did not see changes in normal spinal cords that received RG3.6 cells (figure 1). Primary neural stem cells (NSCs) which share many similar properties with RG3.6 cells, however, did not show difference in Hsp70

expression as compared to medium treated spinal cords. This suggests that RG3.6 has unique properties in modulating injured environment. We also found statistically increased HemeOx1 expression in both fibroblast and RG3.6 treated spinal cords (figure 1). However the increase of HemeOx1 was not as prominent as Hsp70 in RG3.6 treated spinal cords. There was no significant change in Hsp27 in all treatments for injured spinal cords, as compared to medium controls at 12 hours post injury.

Hsp70 and HemeOx1 mRNA expression was also evaluated at 6, 12 and 24 hours post injury using Q-RT-PCR. Levels of Hsp70 started to increase significantly as early as 6 hours, peak at 12 hours and decline at 24 hours post injury. The difference in Hsp70 increase between RG3.6 treatment and medium control was more dramatic at 6 and 12 hours compared to 24 hours post injury. HemeOx1 expression also peaked during 12 to 24 hours post injury, with more of an increase in RG3.6 than medium treated spinal cords at 12 hours. No significant difference was detected at either 6 or 24 hours post injury (figure 2).

Rationale for studying gene expression in the distal segment at 12 hours post injury and data processing using multiple statistical analyses

RG3.6 cells were injected into 3 spots within 4mm of the injury epicenter quickly after the spinal contusion. The distribution of RG3.6 cells following the injection was measured based on GFP expression using Q-RT-PCR. Since only RG3.6 has intrinsic GFP expression, levels of GFP should correlate with the presence of RG3.6 cells at the injury site or in nearby tissues. At 6 and 12 hours GFP signals were detected almost exclusively in the injury site while at 24 hours they were also found proximally and

distally. This suggests that the majority of RG3.6 cells remained at the injury site (injection sites) at 6-12 hours post injury, and did not prominently migrate into P1 and D1 segment until 24 hours post injury (figure 3). Therefore, mRNA measures of spinal tissues at the injury site represent the changes in both host and implanted RG cells at 6-12 hours post injury. However, mRNA expression changes at both P1 and D1 were most likely due to changes in host tissues response to injury and signals generated from RG3.6 transplants.

In order to identify more gene expression changes that are associated specifically with host response to signals of RG3.6 cells, we subjected samples from D1 segments at 12 hours post injury to microarray analysis. These parameters were chosen based on the RT-PCR results (i.e. Hsp70 and GFP) to maximize changes occurring in host tissues resulting from the transplants. Data were imported into GeneSpring and program R, and processed by multiple normalizations. A total of 825 genes were obtained after t-test with multiple corrections (FDR p-cut-off ≤ 0.05), of which 60 genes have more than 1.5 fold changes in RG3.6 treated samples over medium controls (figure 4, table 1). Functions and classifications of these 60 genes were further analyzed using Ingenuity Pathway Analysis (IPA) (www.ingenuity.com).

Among these 60 genes, 40% (23 genes) can be classified into functional networks (I, II, III, IV in figure 4), 40% (25 genes) cannot be identified into any network and 20% (12 genes) were transcribe locus (table 1). Genes in the network I (52%, 12 genes) and network II (38%, 9 genes) are involved in cell proliferation and tissue development. Genes of interest were selected from network I and II and their expression was confirmed by Q-RT-PCR (spatially and temporally) and in some cases by immunostaining.

- RG3.6 activated genes involved in cell proliferation and development

Initially we predicted that microarray results might reveal that RG3.6 cells suppress pro-inflammatory genes since behavioral and histological results suggested that RG3.6 cells may protect tissue through anti-inflammatory pathways. After a series of stringent statistical analyses, the expression of pro-inflammatory cytokines were not changed in RG3.6 treated spinal cord compared to medium controls. However, RG3.6 treatment showed specific changes in genes that are involved in cell proliferation and stem cell development. These genes include foxg1, lef1, top2a, sox11 etc., and their biological functions are discussed as follows.

Foxg1 (also known as brain factor-1, BF1) is a winged-helix transcription factor expressed in neuroepithelial cells in the developing telecephalon and is important in the process of neurogenesis through regulating cytostatic and differentiation signals to the cells (Tao and Lai 1992; Xuan, Baptista et al. 1995). Foxg1 regulates neuroepithelial cell proliferation and differentiation via two distinct pathways, one that is DNA-binding independent and the other is DNA-binding dependent (Dou, Li et al. 1999; Hanashima, Shen et al. 2002).

It has been reported that Foxg1 blocks FoxO-Smad complex binding to the promoter of growth inhibitory *p21Cip1*, and promotes neuroepithethial cell proliferation; this action of Foxg1 does not require DNA-binding (Seoane, Le et al. 2004). The DNA-binding property of Foxg1 is involved in negative-regulating neuronal differentiation of neuroepithelial cells via executing transcriptional repression with the complex proteins of Hairy/enhancer of split-1 (Hes1) and Groucho (Gro)/ transducin-like enhancer of split (TLE) (Yao, Lai et al. 2001; Hanashima, Shen et al. 2002; Marcal, Patel et al. 2005)

Mice lacking Foxg1 show strong expression of p21Cip1, reduced size of cerebral hemispheres and premature neuronal differentiation (Xuan, Baptista et al. 1995; Seoane, Le et al. 2004).

The microarray results indicated that foxg1 was significantly up-regulated in distal segments of RG3.6 treated spinal cord at 12 hours following SCI (table 1). The pattern of expression was further confirmed by Q-RT-PCR in all injury segments (P1, I and D1) at 6, 12 and 24 hours post injury (figure 5). As early as 6 hours post injury, Foxg1 was specifically increased in RG3.6 treated spinal cord. Since the majority of RG3.6 cells remained at the I segments at 6-12 hours post injury (figure 2), the up-regulation of Foxg1 at P1 and D1 suggested that RG3.6 cells secreted signals to change host response to injury (figure 5). Twenty-four hours post injury, Foxg1 exhibited strong intracellular expression by immunofluorescence in the dorsal column white matter in RG3.6 treated spinal cord while very little expression was found in medium control (figure 5). Note the complementary patterns of Foxg1 expression and GFP indicating that they are located in different cells. Since Foxg1 promotes neuroepithelial cell proliferation through DNA-binding independent pathway, the intracellular expression of Foxg1 suggests that some cells may be activated and/or proliferate in response to injury and RG3.6 transplants.

In addition to Foxg1, topoisomerase 2a (**Top2a**) was significantly increased in spinal cords that received RG3.6 transplants. As Top2a has been considered as a proliferation marker in normal and neoplastic cells (Heck, Hittelman et al. 1988; Watanabe, Tsutsui et al. 1994; Holden and Townsend 1999), the increase of Top2a with RG3.6 treatment suggests a state of active DNA synthesis and/or cellular division (Kuan, Schloemer et al. 2004) in the injured spinal cord. In both microarray and Q-RT-PCR

analyses, the level of Top2a mRNA was highly induced in RG3.6 treatment and remained strong from 6 hours to 24 hours post injury (figure 5). In medium control, however, the level of Top2a did not increase until 24 hours after injury with lower magnitude (figure 5). This suggests that the mechanisms for cell or tissue repair are enhanced by RG3.6 transplants in SCI.

Lymphoid enhancing factor 1 (**Lef1**) is another gene involved in regulating cell proliferation and/or differentiation that was significantly up-regulated in RG3.6 treated spinal cord. Lef1 is a high-mobility-group (HMG)-box transcription factor, which often functions as a down-stream effector of Wnt signaling via forming a complex with T cells factor -1 (Lef-1/TCF-1) (Arce, Yokoyama et al. 2006). Lef1 activation is required for B- and T-lymphocyte proliferation and maturation (Reya, Okamura et al. 1999; Reya, O'Riordan et al. 2000; Wu and Strasser 2001). In addition to immune cell development, Lef1 regulates cell proliferation and neurogenesis through regulating c-myc and cyclin D1 in association with β-catenin (Galceran, Miyashita-Lin et al. 2000; Coyle-Rink, Del Valle et al. 2002; Lee, Wu et al. 2006). Lef1 is expressed abundantly in the telecephalon and mesencephalon during brain development (van Genderen, Okamura et al. 1994). In the postnatal brain, Lef1 can be found in the cytoplasm of neurons and nuclei of astrocytes (Coyle-Rink, Del Valle et al. 2002). As the neonatal brain matures, the expression level of Lef1 gradually declines (Aimone, Leasure et al. 2004).

Our microarray analyses revealed that Lef1 was significantly up-regulated in the D1 segment of RG3.6 treated spinal cord at 12 hours after SCI (table 1). Q-RT-PCR analyses indicated that RG3.6 treatment exhibited a slight down-regulation of Lef1 at 6 hours followed by a delayed yet significant up-regulation at 12 hours post injury (figure

5). Lef1 expression was not changed in the injured spinal cord treated with medium since the level was similar to sham animal. In light of the specificity of Lef1 in the developing brain, the increased Lef1 suggests that cells undergo activation and/or proliferation in response to injury and RG3.6 transplants.

SRY-box containing gene 11 (**Sox11**), similar as Lef1, is also a HMG-box containing transcription factor that was significantly increased in RG3.6 transplanted spinal cord following SCI. Sox11 is expressed throughout developing central nervous system and involved in cell fate determination during differentiation. The expression is reduced during late embryogenesis and not detectable in adult stage (Uwanogho, Rex et al. 1995; Kuhlbrodt, Herbarth et al. 1998). Sox11 is activated by proneural basic helix-loop-helix (bHLH) transcription factors (e.g. Ngn2, Ngn1 and Mash1) to induce pan-neuronal protein expression in the developing chick spinal cord (Bergsland, Werme et al. 2006). It has recently been reported to be crucial for neuronal survival and neurite growth (Jankowski, Cornuet et al. 2006). Sox11 is also involved in glial differentiation to synergize with Brn-1 protein in differentiating neural precursors into oligodendrocytes (Kuhlbrodt, Herbarth et al. 1998; Cheung, Abu-Elmagd et al. 2000).

Sox11 has been classified as one of the regeneration-associated genes (RAGs) and is up-regulated in the dorsal root ganglia after transection of sciatic nerves (Tanabe, Bonilla et al. 2003). In our microarray analysis, sox11 expression was significantly increased in RG3.6 treated spinal cord at 12 hours post injury. Q-RT-PCR also verified this up-regulation as early as 6 hours following SCI in all segments that we tested (figure 6). As Sox11 was also high in the medium control, it suggests that RG3.6 transplants

may contribute to the reinforcement of intrinsic host self-repair mechanism, by stimulating proliferation or regeneration of neural precursor cells.

It is conceivable that up-regulation of genes associated with cell proliferation, may occur concomitantly with the increase of genes associated with stem cells and/or progenitor cells. Several lines of evidence have shown that injury activates endogenous neural stem cells and/or neural progenitor cells in the adult spinal cords (Zai and Wrathall 2005; Horky, Galimi et al. 2006). Therefore we looked at **vimentin** expression following SCI since it has been widely used to label neural stem cells and radial glia (Alvarez-Buylla, Buskirk et al. 1987; Noctor, Flint et al. 2002). Q-RT-PCR of vimentin showed transient increase in both RG3.6 and medium treated spinal cords, with higher levels in RG3.6 treatment in all injury segments at 12 hours post injury (figure 6). There was more vimentin immunoactivity in the ventromedial white matter and gray matter that surrounds the central canal (i.e. ependymal region) and dorsal column in RG3.6 treated spinal cord than comparable regions of medium control at 24 hours post injury (figure 7). These vimentin immuno-positive cells may represent activation of neural stem cells and/or remnant radial glia in the adult spinal cord.

Another gene identified is homeodomain transcription factor **Nkx2.2**, which has multiple biological roles in the CNS. Nkx2.2 regulates differentiation of neurons and glia during CNS development (Shimamura, Hartigan et al. 1995; Briscoe, Sussel et al. 1999; Marquardt and Pfaff 2001; Zhou, Choi et al. 2001). It promotes proliferation of glial-restricted precursor (GRP) (Han, Liu et al. 2004; Talbott, Loy et al. 2005) and differentiation of oligodendrocyte progenitor in the injured spinal cord (Ohori, Yamamoto et al. 2006). Consistent with other work (Talbott, Loy et al. 2005), we also

found up-regulation of Nkx2.2 following SCI by Q-RT-PCR, with higher levels in RG3.6 treated spinal cord than medium control at 12 hours post injury (figure 6). Moreover, the number of Nkx2.2 immuno-positive cells was significantly higher in RG3.6 transplanted spinal cords than that of medium controls at 24 hours post injury (figure 8). NG2 chondroitin sulfate proteoglycan has been recognized as a marker for oligodendrocyte progenitors and NG2 positive cells were reported to proliferate actively after SCI (McTigue, Wei et al. 2001; Zai and Wrathall 2005; Horky, Galimi et al. 2006). We found more than 50% of the Nkx2.2 positive cells are double-positive for NG2 (figure 8), suggesting that implantation of RG3.6 cells may stimulate oligodendrocyte precursor proliferation and/or differentiation as early as 24 hours following SCI.

Trophic factor gene expression in the injured spinal cord

RG3.6 cells express trophic factor genes and secret GDNF (Chapter 1), but it is unclear if RG3.6 cells changed host microenvironment in injured tissue by mediating trophic factor production. We studied gene expression of trophic factors (e.g. GDNF, BDNF, NT3, and NGF) at 12 hours post injury using Q-RT-PCR. The mRNA expression levels of these trophic factors were increased in both RG3.6 and medium treated spinal cords, especially in the I segments (figure 9). Since the majority of RG3.6 transplants stayed at the injury site at 12 hours post injury, the increased expression of trophic factors may be contributed by both RG3.6 cells and host tissue. This suggests that RG3.6 transplants may directly increase the levels of growth factors by secretion and/or indirectly increase the levels by enhancing the production from host tissue. However, the levels of trophic factors were not significantly increased in the P1 and D1

segments of RG3.6 treated spinal cords, suggesting that RG3.6 transplants located at the I segment may not stimulate host tissue to produce more trophic factors. Therefore, we believe the increased signal of trophic factors in the injury sites was derived from RG3.6 cells. Since very limited numbers of RG3.6 cells migrated to P1 or D1, host tissue in these regions would be the source for trophic factors. This could explain why there were no differences in the levels of trophic factor mRNA in both P1 and D1 segments between RG3.6 and medium treatments.

Discussion

Previously we reported that acute transplantation of radial glia RG3.6 cells promoted functional recovery possibly through early tissue protection (Hasegawa, Chang et al. 2005). However, the mechanism remains unclear. The theme of this chapter is to explore mechanisms underlying RG3.6 cells' beneficial effect to acutely injured spinal cord at the level of gene expression. Q-RT-PCR and Microarray measurements were performed in the acute phase of SCI in attempts to examine whether RG3.6 transplantation changes genes involved in tissue protection. RG3.6 was not found to suppress pro-inflammatory genes, such as IL-6, MIP2, or MCP1 within 24 hours after SCI (figure 1).

Short term in vitro chemotaxis of macrophage was carried out using ChemoTx ® co-culture system (Neuro Probe, Gaithersburg, MD) (Frevert, Wong et al. 1998), and RG3.6 cells did not have direct effect on macrophage migration. However RG3.6 cells may have had an indirect effect on inhibiting the migration with their conditioned medium (data not shown). Nevertheless, significant reduction of microglia activation and macrophage infiltration were observed in RG3.6 treated spinal cord at 1-2 weeks post injury using immunohistochemistry (figure 3-4, Chap. 2). Thus, although we were unable to detect rapid (6-24h) changes by RG3.6 transplants on immune regulatory genes, the RG3.6 transplants suppressed the activation of microglia and macrophages at later times (1- 6 wks, figure 3-5 in Chap. 2)

Prolonged tissue inflammation is harmful to spinal cord in sub-acute to chronic phases of SCI. However, up-regulation of pro-inflammatory cytokines/chemokines is important to recruit immune cells for clearing up debris of dead cells during acute SCI

phase. It remains controversial if pro-inflammatory mediators during acute SCI phase are beneficial or detrimental to the tissue. In future study, it would be interesting to include more time points to study these pro-inflammatory cytokines/chemokines expression both at message and protein levels in a spatial and temporal spectrum. This may provide clues how RG3.6 cells decrease immune response in SCI.

With no obvious early effect on pro-inflammatory gene expression, RG3.6 transplants may render tissue protection by significantly increasing other mediators, such as heat shock proteins. Q-RT-PCR confirmed that Hsp70 was highly up-regulated in RG3.6 treated spinal cord as early as 6 hours following SCI (figure 1-2). Since RG3.6 cells did not migrate to proximal (rostral) or distal (caudal) segments by 6-12 hours post injury, the increased signals at P1 and D1 segments are likely attributed to host tissue response to RG3.6 transplants in injured spinal tissue. The enhanced up-regulation of Hsp70 mRNA by RG3.6 cells was transient, since no significant difference was found at 24 hours post injury. Moreover, there was no difference in Hsp70 expression between surgical sham animals (laminectomy only) treated with RG3.6 and those without. This suggests that RG3.6 cells require signals from the injured environment in order to release some signals and in return modulate certain gene expression in the host tissue (e.g. defense gene Hsp70). Hsp70 protein expression was also verified using immunohistochemistry and western blot, however we did not detect significant changes between RG3.6 and medium treated spinal cords (data not shown). Hsp70 immuno-positive cells exhibited morphological phenotype of oligodendrocytes and astrocytes (figure 3). Some of these Hsp70 positive cells also express NG2, which is a marker for oligodendrocyte precursors (figure 3).

It is unclear whether this transient enhancement of Hsp70 mRNA increase by RG3.6 can be efficaciously translated into functional protein and whether the insignificance of Hsp70 expression at protein level is due to low protein concentration or fast degradation in the tissue. In future studies, more time points after injury should be screened to look for the peak of Hsp70 protein expression in the temporal expression profile. In addition, the cell type of Hsp70 immuno-positive cells should be further identified in RG3.6 treated spinal cords.

HemeOx1 (Hsp32) is synthesized mostly in microglia in response to heat, heme, ischemia, and oxidative stress (Sharp, Massa et al. 1999). The up-regulation of HemeOx1 in RG3.6 treatment was enhanced at 12 hours post injury, suggesting that RG3.6 cells may modulate the activity of microglia in response to the injury. The histological observation of less activated microglia (Iba1 immunostaining) in RG3.6 treated spinal cord (figure 3 in Chap. 2) may be associated with early interaction between RG3.6 transplant and local microglia partly through regulating HemeOx1 expression.

To understand host response to injury and RG3.6 transplants, we studied gene expression at D1 segments using microarray at 12 hours post injury, during which the majority of RG3.6 cells remained at the injury site. After normalizing expression of RG3.6 treated samples over that of medium controls, we further performed serial stringent statistic analyses (i.e. t-test with multiple corrections, FDR p-cut-off ≤ 0.05) before we obtained the 825 genes that were specifically changed in the RG3.6 treated spinal cords. Although some genes of particular interest (e.g. Hsp70, HemeOx1, Thromobodulin, Nkx2.2, vimentin) did not appear in the list of 825 genes, their expressions were significantly changed, when measured by Q-RT-PCR. Due to the

stringency of the statistical analyses, false negative rate in our analysis may be high. Since there are only three biological replicates in each treatment, the power of microarray analysis to detect any mRNA changes (especially small changes) may not be sufficient.

Among the 825 genes, 60 genes showed more than 1.5-fold changes, which were submitted to Ingenuity for functional network analysis. Interestingly, genes involved in cell proliferation and stem cell development were major components of the networks (I and II) that were generated from Ingenuity. Genes from both microarray list and selected interesting pathways were confirmed by Q-RT-PCR and some also by immunohistochemistry.

RG3.6 transplants enhanced cell proliferation genes Top2a, Foxg1 and Lef1 in the injured spinal cord. Foxg1 expression is restricted to neuroepithelial cells in the telencephalon and its derivative regions in the brain (Xuan, Baptista et al. 1995). Immunostaining of Foxg1 showed strong expression in dorsal column and was not co-localized with RG3.6 cells at 24 hours post SCI (figure 5). Very little expression of Foxg1 was detected in the dorsal column of medium treated spinal cord (figure 5). Since Foxg1 staining exhibited intracellular expression, it remains to be clarified whether the positive cells are localized in the spinal cord or represent responses axons descending from the brain. However, the rapidity of the response (within 6h) suggests that most of the mRNA changes are local response. Nevertheless, more work should be done to identify what cells express Foxg1 in the spinal cord after injury and RG3.6 treatment. Lef1 is greatly expressed in the developing brain and the level is decreased in the postnatal brain (van Genderen, Okamura et al. 1994; Aimone, Leasure et al. 2004). The up-regulation of Foxg1 and Lef1 in RG3.6 treated spinal cord strongly suggests that host

environment was remodeled to promote cell activation and/or proliferation as in the developing brain. This specific change was not found in the medium treated controls. Interestingly, RG3.6 cells were derived from embryonic cortices thereby they may influence spinal cord to react like brain. More effort is needed to decipher factors of RG3.6 transplants that change host spinal cord, e.g. proteomic study of distal segments of RG3.6 treated spinal cords.

Transcription factor Sox11 is expressed in the embryonic central nervous system and is involved in regulating neuronal and glial differentiation; little or no Sox11 is found in adult spinal cord (Uwanogho, Rex et al. 1995; Kuhlbrodt, Herbarth et al. 1998). Neural precursors express intense Sox11 before they differentiate into mature neurons or oligodendrocytes (Cheung, Abu-Elmagd et al. 2000). Nkx2.2 is also involved in oligodendrocyte differentiation of neural precursor cells and was increased in RG3.6 treated spinal cord. Up-regulation of these neural precursor transcription factors strongly suggests that the presence of RG3.6 in the injured spinal cord tissue modulates host response to revitalize the regenerative, proliferative or protective potential of the tissue.

It is still unclear if growth factors are involved in RG3.6 mediating tissue protection, although we found expression of many trophic factors at RG3.6 transplanted sites. They appear to be contributed at least in part directly by the RG3.6 cells. It will require detail proteomic analyses (2D-gel or protein array, immunoblot and ELISA) to unravel factors of RG3.6 transplants that remodel injured spinal cord tissue.

Recently it has been proposed that application of stem cells may have multiple functions in neurodegenerative disease models or CNS trauma besides cell replacement by differentiation into specific cell types (Goldman 2005). Although it is a developing

concept, it is believed that stem cells may help host tissue by modulating its immune response and secreting wide range of trophic factors (Pluchino, Zanotti et al. 2005; Martino and Pluchino 2006). It is possible that RG3.6 cells promoted functional recovery by early activation of host stem/progenitor cells (e.g. Foxg1, Lef1, Sox11) and tissue protective mechanisms (e.g. Hsp70, HemeOx1), and those activated stem cell population (host and implant) suppressed immune reactions thereby reducing microglia/macrophage activation in the injury site.

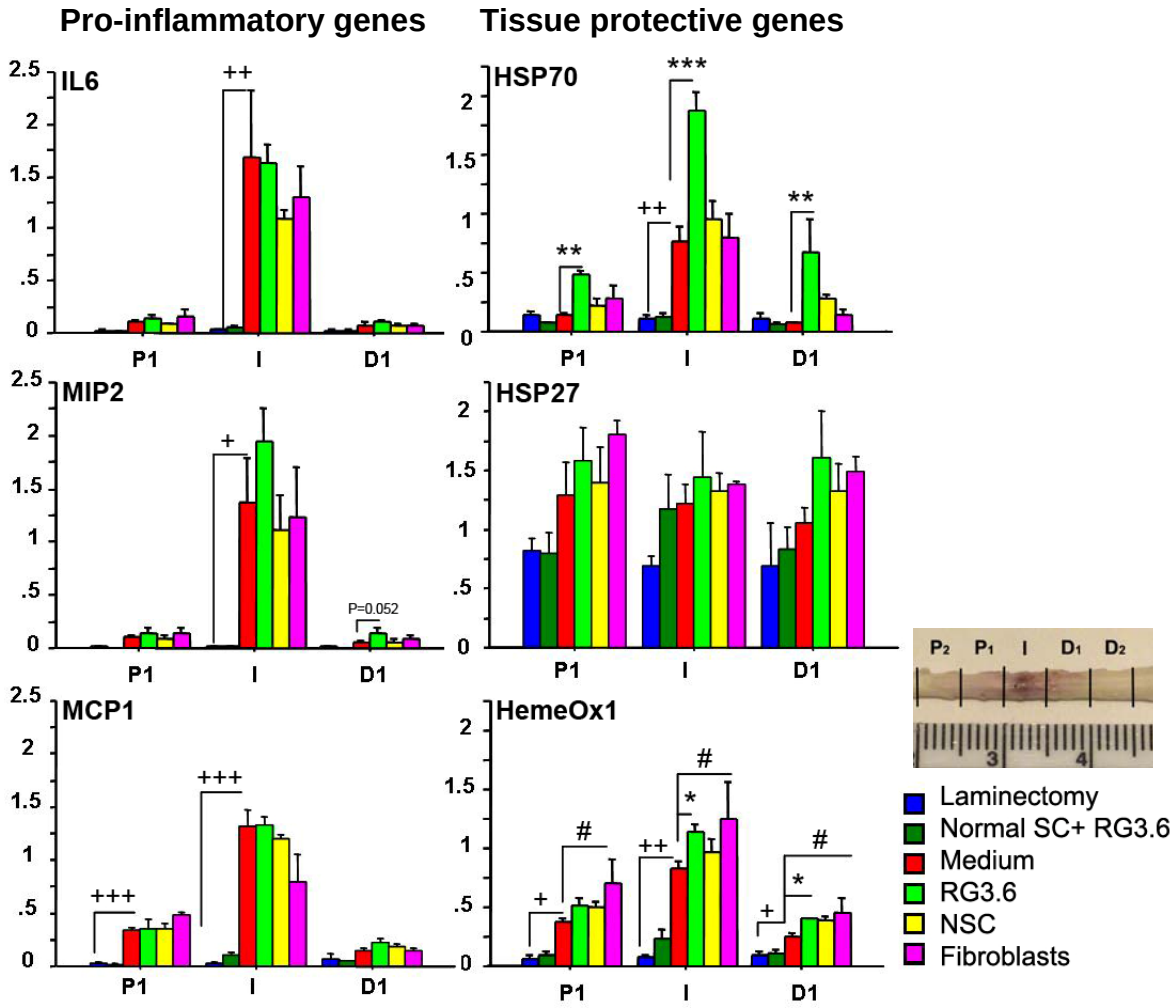


Figure 1. Pro-inflammatory and tissue protective genes were examined in the spinal cord segments (P1, I and D1) at 12 hours following SCI using Q-RT-PCR. Pro-inflammatory mediators were increased in all injury conditioned, especially in the I segments. RG3.6 treated spinal cords did not show differences in these genes from medium treated controls. Tissue protective Heat shock proteins Hsp70, Hsp27 and Hsp32 (Heme Oxygenase 1: HemeOx1) were also examined at 12 hours after injury using Q-RT-PCR. RG3.6 treated spinal cords showed significant increase at P1, I and D1 segments in comparison with other conditions. Hsp27, however, showed similar up-regulation in all injury conditions. Fibroblasts or RG3.6 treated spinal cords showed increased up-regulation of HemeOx1 in comparison with medium treated controls. Scale represents fold change after normalizing expression to GAPDH. Values are means ± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$, # $p < 0.05$. *: RG3.6 transplant vs. Medium, +: Medium vs. Sham, #: Fibroblast transplants vs. Medium, ANOVA.

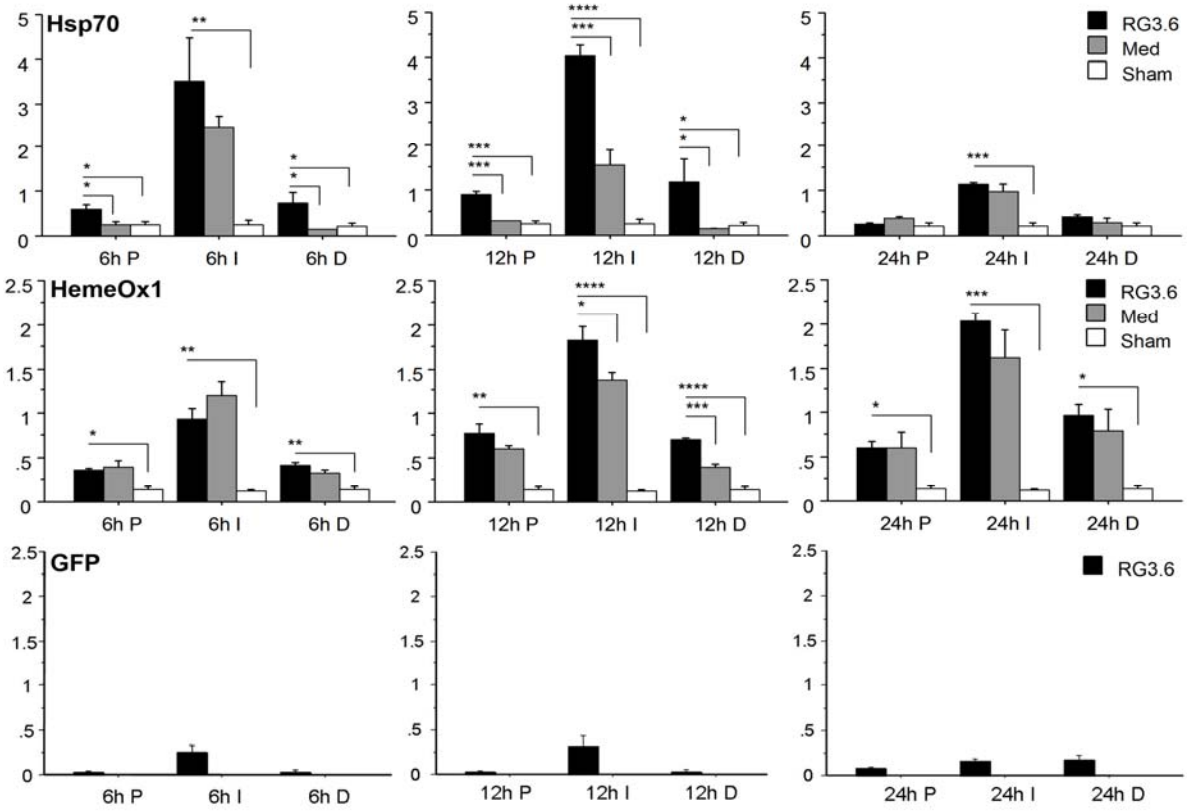


Figure 2. Spatial and temporal expression of Hsp70, HemeOx1 and GFP following SCI using Q-RT-PCR. **Hsp70** expression was measured in the P1, I and D1 segments at 6, 12, and 24 hours post injury. RG3.6 treated spinal cord showed significant up-regulation of Hsp70 as early as 6 hours following SCI; the up-regulation was declined at 24 hours after injury. **HemeOx1** expression was also measured and showed increased up-regulation at I and D1 segments of RG3.6 treated spinal cord compared to medium controls. The distribution of RG3.6 cells was measured by GFP expression. Very limited number of RG3.6 cells migrated to P1 or D1 segments at 6-12 hours following SCI, but detectable amount of migrated cells were found at P1 and D1 segments at 24 hours after injury. Scale represents fold change after normalizing expression to GAPDH. Values are means ± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ANOVA.

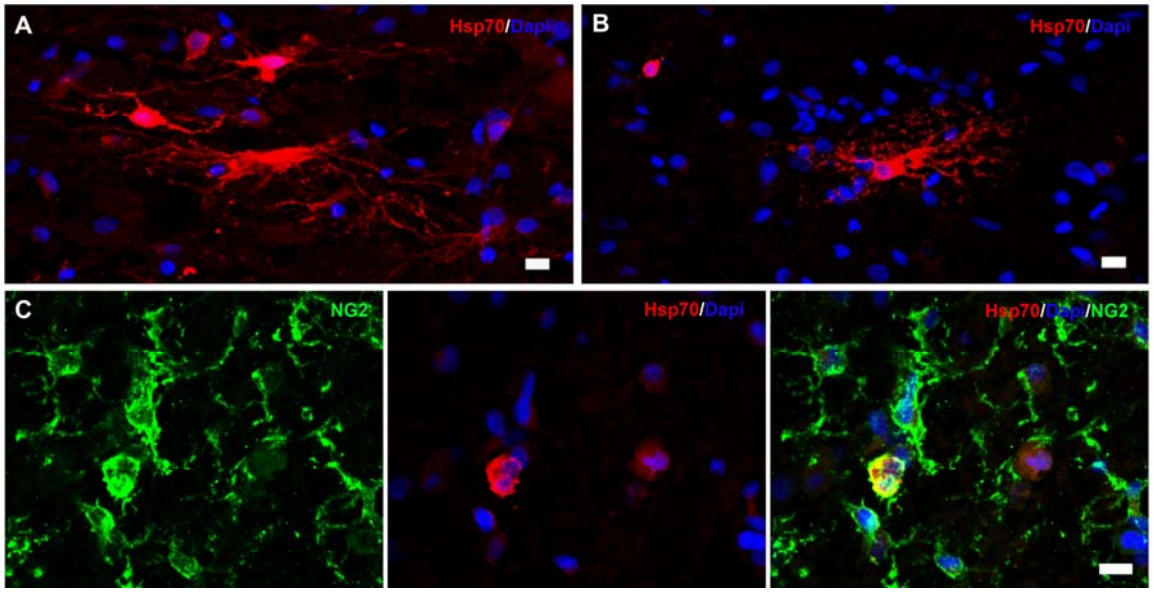


Figure 3. Hsp70 immunostaining at ~2.5-mm caudal to the injury epicenter at 24 hours post injury. Hsp70 was expressed in cells with morphology like astrocyte (A) and oligodendrocyte (B). Hsp70 was also expressed in NG2 positive cells (C). Scale bar is 10 μm.

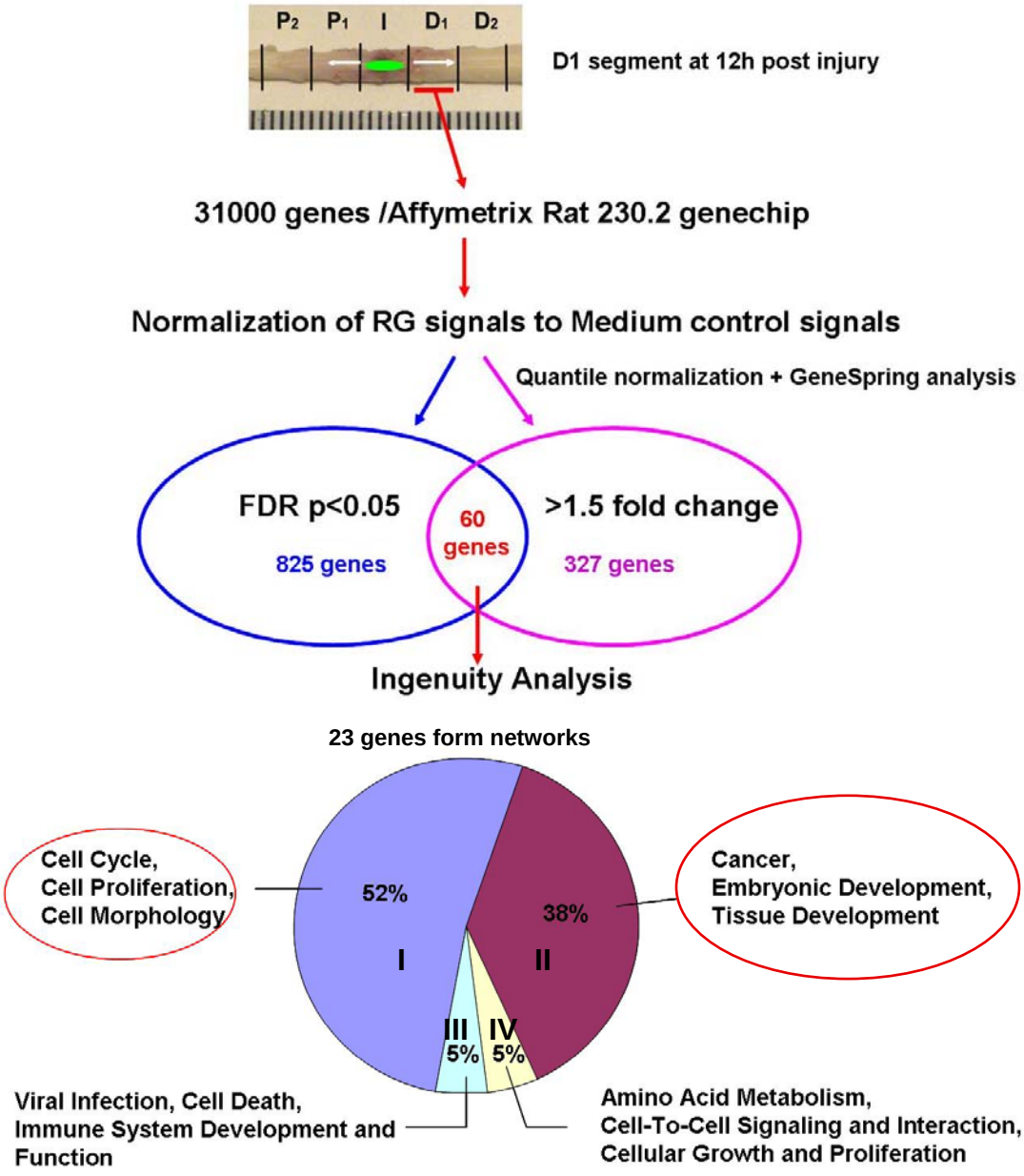


Figure 4. Distal spinal cord segments at 12 hours post injury were subjected to Affymetrix Rat230.2 genechips. After data normalization, signals from RG3.6 treated samples were compared to medium controls for differential expression. A total of 825 genes reached statistical significance after unpaired t-test with multiple corrections and 60 of them showed >1.5 fold changes (listed in table 1). After Ingenuity pathway analysis (IPA), four biological networks (I, II, III, IV) were identified with two highlighted i.e. genes involved in cell proliferation (I) and tissue development (II). Expression of genes of interest was further confirmed using Q-RT-PCR.

Table 1. Gene List after FDR p-cut-off ≤ 0.05 and differential expression ≥ 1.5 fold change

Genbank ID	RG/Med ratio	Common	Description	* Network
NM_012560	15.50	BF1A; Foxo1;	forkhead box G1(Foxg1)	**I**
BM385445	3.43	Top2a	Topoisomerase (DNA) 2 alpha	**II**
BF284168	3.09		Transcribed locus	
BE107070	3.03	Sox11	SRY-box containing gene 11	**II**
AA944326	2.88		EST199825 Normalized rat embryo, Bento Soares Rattus sp. cDNA clone REMAF43 3' end, mRNA sequence.	
AI012949	2.84		Transcribed locus, moderately similar to XP_489350.1 RIKEN cDNA 2700063P19 [Mus musculus]	
AI172110	2.82		EST218105 Normalized rat muscle, Bento Soares Rattus sp. cDNA clone RMUBV06 3' end, mRNA sequence.	
AW524041	2.33	Zbtb9	Zinc finger and BTB domain containing 9	
BF554576	2.27	Sox11	SRY-box containing gene 11	**II**
BF408872	2.25	Imp1	Insulin-like growth factor 2, binding protein 1	**II**
BE107098	2.19		Transcribed locus	
BE113173	2.16	Tubb2b	microtubule-based movement protein polymerization Tubulin, beta 2b	**II**
BM385870	2.13	Zbtb 10	Zinc finger and BTB domain containing 10	**III**
AW144239	2.09	Fstl3	Follistatin-like 3	**I**
BI285065	2.07	Tgfb1i4	Transforming growth factor beta 1 induced transcript 4	**II**
AA956727	1.99		Splicing factor, arginine/serine-rich 3 (SRp20) (predicted)	**I**
AI180454	1.94		Similar to IGF-II mRNA-binding protein 2 (predicted)	**II**

* Network I: Cell cycle, Cell proliferation, morphology. II: Cancer, Embryo and Tissue development.
III: Cell death, Immune response. IV: Cell-cell signal, Cell growth and Proliferation.

Table 1. Continued.

M24024	1.91	RT1Aw2	RT1 class Ib, locus Aw2 (RT1-Aw2)	**I**
BE113443	1.89		Kinesin family member 23 (predicted)	**II**
AF030088	1.85	Vesl-1; HOMER1F	homer homolog 1 (Drosophila) (Homer1)	**I**
BF398677	1.84	Arhgef12	Rho guanine nucleotide exchange factor (GEF) 12	**I**
BF409715	1.81		Similar to 106 kDa O-GlcNAc transferase-interacting protein (predicted)	
AA800639	1.80		Transcribed locus	
AI058451	1.80		Similar to IGF-II mRNA-binding protein 2 (predicted)	**II**
BI289386	1.78		Similar to BCoR protein (BCL-6 corepressor)	
AI598485	1.78		Transcribed locus	
AI712582	1.77		Glutamine and serine rich 1 (predicted)	
BE119432	1.77		Transcribed locus	
AA956336	1.73		Anaphase promoting complex subunit 10 (predicted)	
AI072161	1.73		UI-R-C2-mz-f-11-0-UI.s1 UI-R-C2 Rattus norvegicus cDNA clone UI-R-C2-mz-f-11-0-UI 3', mRNA sequence.	
U06434	1.71	Scya4; Mip1-b	chemokine (C-C motif) ligand 4 (Ccl4)	**I**
BE114260	1.70		Transcribed locus	
BF408816	1.68		UI-R-BT1-bne-g-03-0-UI.s1 UI-R-BT1 Rattus norvegicus cDNA clone UI-R-BT1-bne-g-03-0-UI 3', mRNA sequence.	
BI276223	1.64		Similar to mKIAA0215 protein	
BG374178	1.64	Kitl	Kit ligand	**I**
AI639117	1.64	cBf	complement factor B	**II**
BF419397	1.63		Transcribed locus	
BI282932	1.62	C1qr1	Complement component 1, q subcomponent, receptor 1; Lymphocyte antigen 68	**IV**
BI275740	1.62		Similar to triggering receptor expressed on myeloid cells-like 1	

102

Table 1. Continued.

AI044343	1.62		Similar to cofactor required for Sp1 transcriptional activation Subunit 2, 150kDa(predicted)	
AA963477	1.60	TSP-2	Thrombospondin-2	**I**
BE107208	1.60		UI-R-BS1-ayr-b-04-0-UI.s1 UI-R-BS1 Rattus norvegicus cDNA clone UI-R-BS1-ayr-b-04-0-UI 3', mRNA sequence.	
BF404982	1.59	Pnpt1	Polyribonucleotide nucleotidyltransferase 1	
AI172172	1.59	Aprin	Androgen-induced proliferation inhibitor (predicted)	
BF416058	1.58		UI-R-CA0-bkh-f-11-0-UI.s1 UI-R-CA0 Rattus norvegicus cDNA clone UI-R-CA0-bkh-f-11-0-UI 3', mRNA sequence.	
AA892240	1.58		Hypothetical LOC361786	
AI169080	1.57		Transcribed locus	
AA858605	1.56		Similar to Wolf-Hirschhorn syndrome candidate 1 protein isoform 1	
AW434912	1.54		Transcribed locus	
AA893192	1.54		EST196995 Normalized rat kidney, Bento Soares Rattus sp. cDNA clone RKIBD36 3' end, mRNA sequence.	
BI274401	1.54	P4ha1	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide	**I**
BF564309	1.53		UI-R-C4-aln-a-11-0-UI.r1 UI-R-C4 Rattus norvegicus cDNA clone UI-R-C4-aln-a-11-0-UI 5', mRNA sequence.	
AI175791	1.52		Transcribed locus	
BE117291	1.52		Similar to chromosome X open reading frame 23	
AI072424	1.51		Synaptotagmin binding, cytoplasmic RNA interacting protein	
AI010254	1.51	Cachd_1	Cache domain containing 1 (predicted)	
BF546659	1.50	Syncrip	Synaptotagmin binding, cytoplasmic RNA interacting protein	
BE115061	1.50	Lef1	Lymphoid enhancer binding factor 1	**II**
AI111816	0.66		Transcribed locus	
BF556812	0.31		Transcribed locus	

103

Table 2. Genes of interest and their expression at D1 segment at 12 hours SCI: Analysis by genechips and Q-RT-PCR

GenBank ID	common name	Genechip RG/Med ratio	Genechip t-test FDR5%	Q-PCR RG/Med ratio	Q-PCR, ANOVA $p<0.05$
NM_012560	Foxg1	15.5	+	146	+
BM385445	Top2a	4.6	+	5.4	+
BE115061	Lef1	1.5	+	1.54	+
NM_031140	Vim	1.3	-	1.7	+
AA925143	Nkx2.2	1.3	-	1.45	+
BE107070	Sox11	3	+	5.2	+
NM_031971	hspa1a (Hsp70)	3.5	-	2.8	+
NM_012580	hmox1	1.4	-	1.8	+

+: $p \leq 0.05$, -: $p > 0.05$.

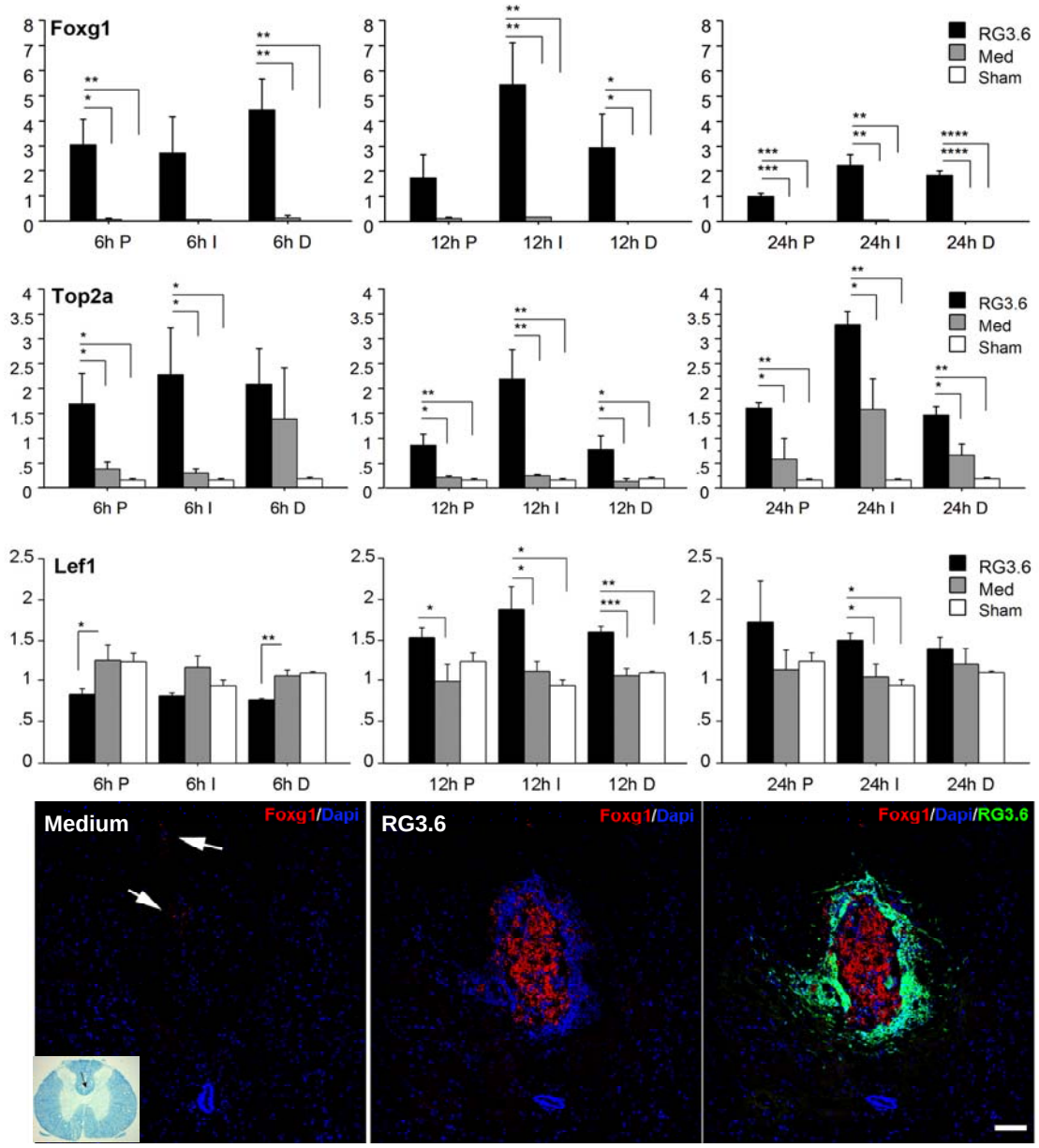


Figure 5. Spatial and temporal expression of Foxg1, Top2a and Lef1 following SCI by Q-RT-PCR. **Foxg1** expression was measured in the P1, I and D1 segments at 6, 12, and 24 hours post injury and showed almost exclusively up-regulation in RG3.6 treated spinal cord (except very limited expression was found at I segment as 12 hours post injury). **Top2a** expression was also measured in a spatial-temporal spectrum and showed significantly up-regulation in RG3.6 treated spinal cord. **Lef1** expression in RG3.6 treated spinal cord was significantly higher than medium controls at 12 hours but not at 6 hours or 24 hours post injury. Expression levels represent fold change after normalizing to GAPDH. Values are means ± SEM, * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, **** $p< 0.0001$, ANOVA. Foxg1 immunostaining showed strong expression at the dorsal white matter (~3 mm distal to injury epicenter) and did not overlap with RG3.6 cells. Medium treated spinal cord showed very weak Foxg1 expression (arrows). Scale is 100μm. Luxol fast blue stained cross section illustrates foxg1 staining region.

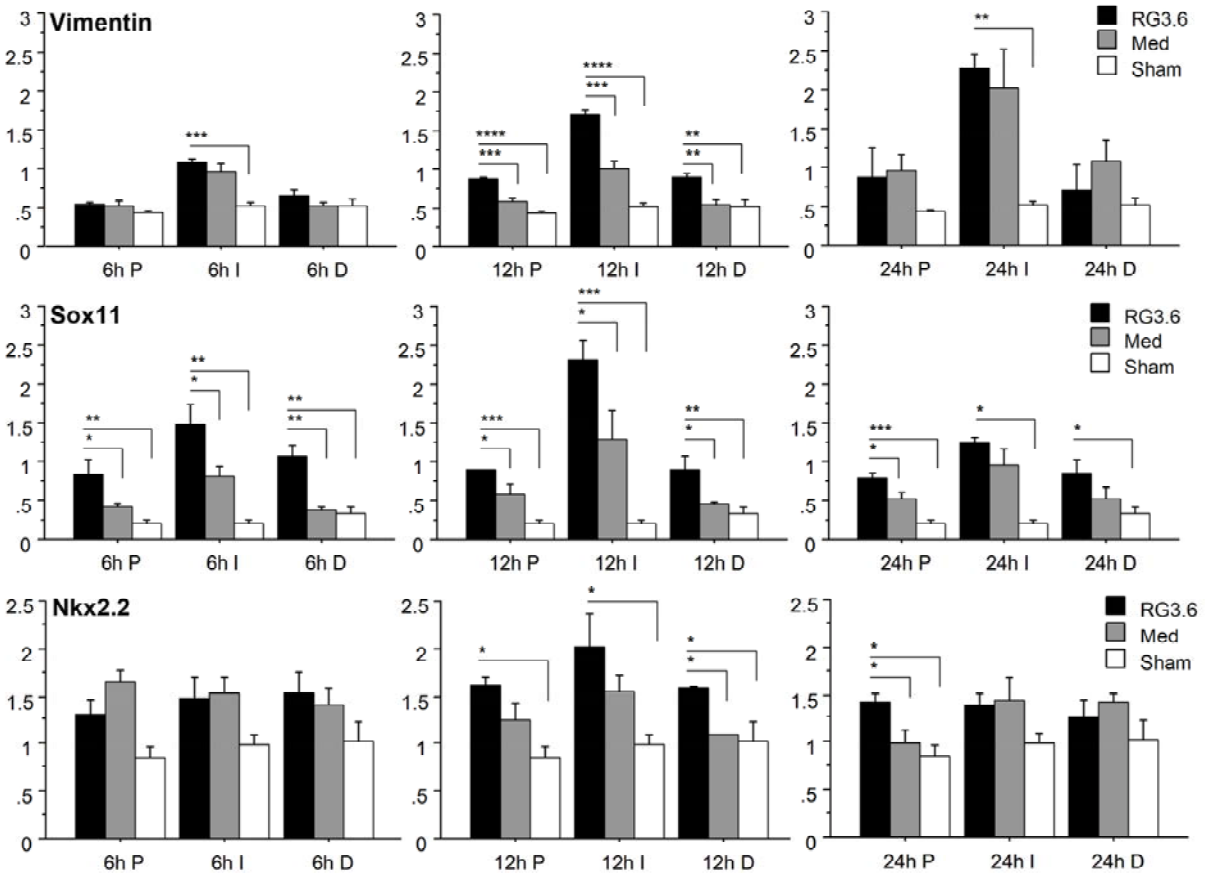


Figure 6. Spatial and temporal expression of vimentin, Sox11 and Nkx2.2 following SCI by Q-RT-PCR. **Vimentin** expression was measured in the P1, I and D1 segments at 6, 12, and 24 hours post injury and showed significantly up-regulation in RG3.6 treated spinal cord at 12 hours post injury. **Sox11** expression was also examined in a spatial-temporal spectrum and showed significantly increased signals in RG3.6 treated spinal cord at all segments measured. **Nkx2.2** expression in RG3.6 treated spinal cord was higher than medium control only at 12 hours post injury, and the up-regulation was not as significant as sox11 or vimentin. Expression levels represent fold change after normalizing to GAPDH. Values are means ± SEM, * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, **** $p< 0.0001$, ANOVA.

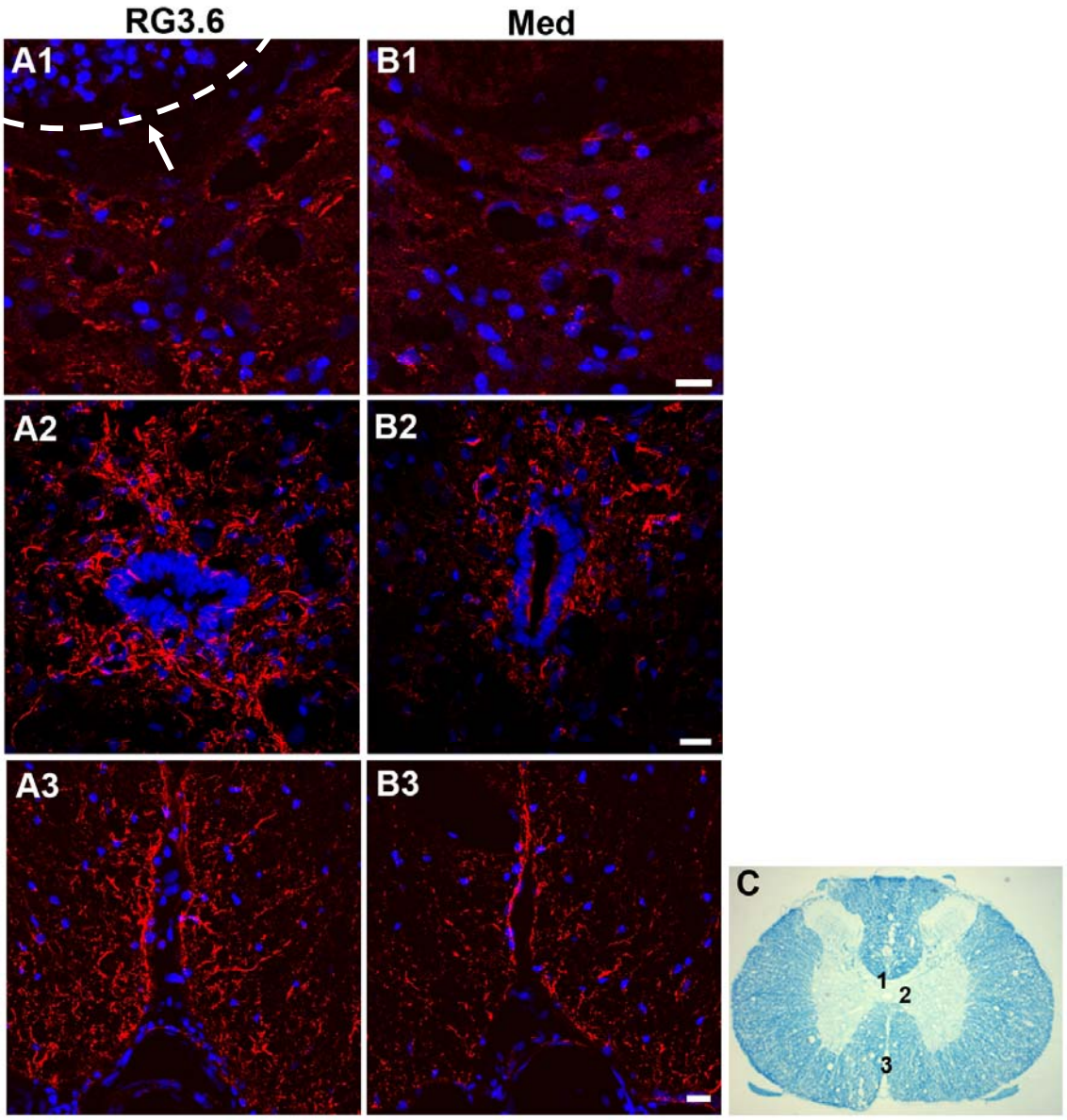


Figure 7. Vimentin expression at region 2.5-mm caudal to the epicenter at 24h SCI. RG3.6 cells treated spinal cords (A1-3) show more vimentin (red) positive fiber than medium controls (B1-3) at gray matter close to dorsal column (A1, B1) and ventral canal (A2, B2) and white matter at the ventral midline (ventral medial white matter, A3 and B3). Images were taken from regions indicated in the luxol fast blue stained section (C). Nuclei (Dapi in blue) at the dorsal column white matter (a1 in arrow) are RG3.6 cells (green channel was omitted to show better contrast of vimentin expression). Scale bar is 20μm.

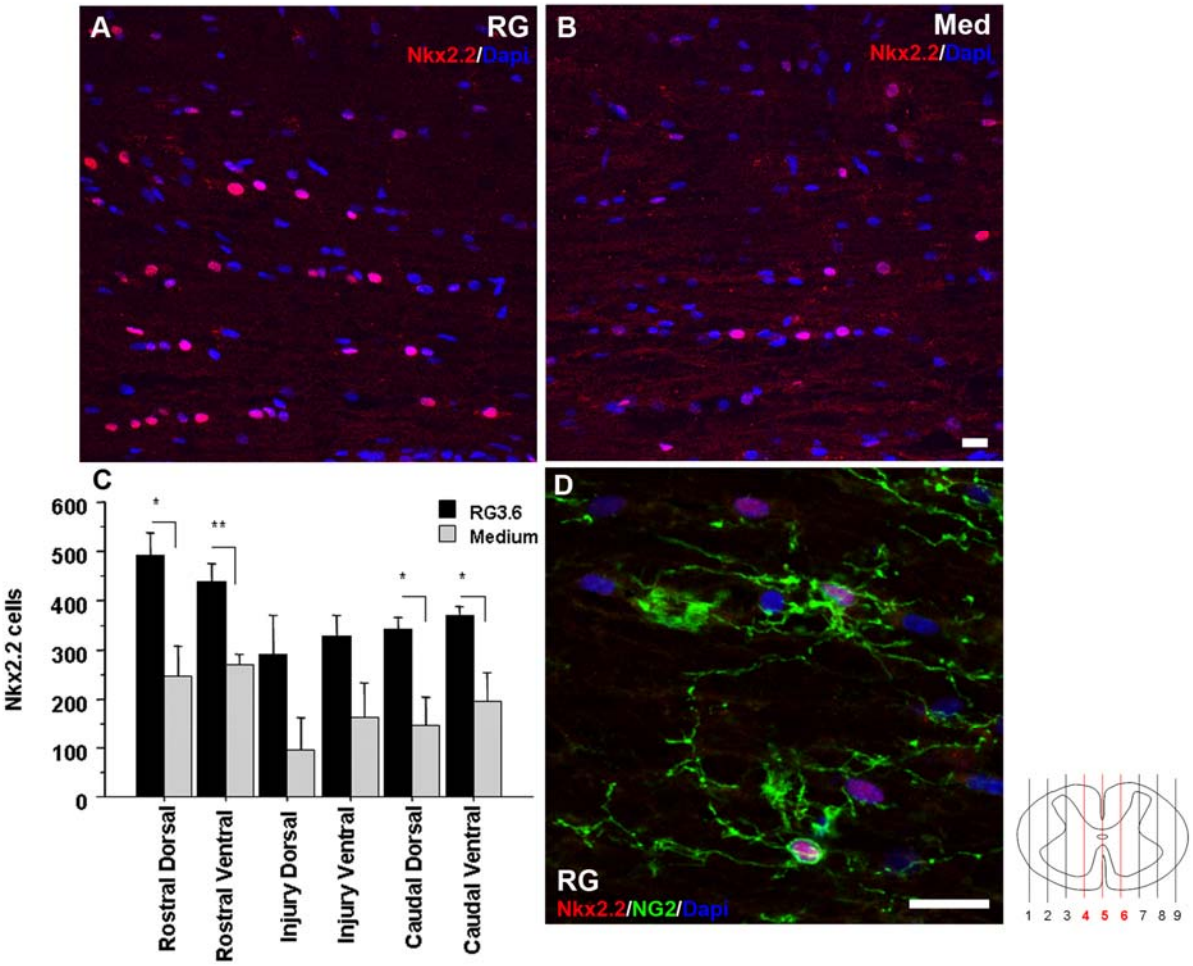


Figure 8. Nkx2.2 expression at 24 hours post SCI. Nkx2.2 expression was measured and quantitated from para-sagittal sections around midline (Red lines 4, 5, 6 in the schematic illustrated spinal cord). Data presented positive cells in the white matter. Rostral ventral white matter shows more Nkx2.2 positive cells in RG3.6 treated spinal cord (A) than medium controls (B). 2-cm long para-sagittal sections close to the midline centered at the epicenter were divided into rostral, injury and caudal regions for quantitation (C). Nkx2.2 positive cells were counted in both dorsal and ventral white matter. Spinal cords with RG3.6 transplants show significant number of Nkx2.2 cells in rostral and caudal white matter (C). Values are means ± SEM, * $p< 0.05$, ** $p< 0.01$, t-test (C). Nkx2.2 positive cells were double-labeled with NG2 (Cy5 shown in green) (D). Scale bar is 20μm.

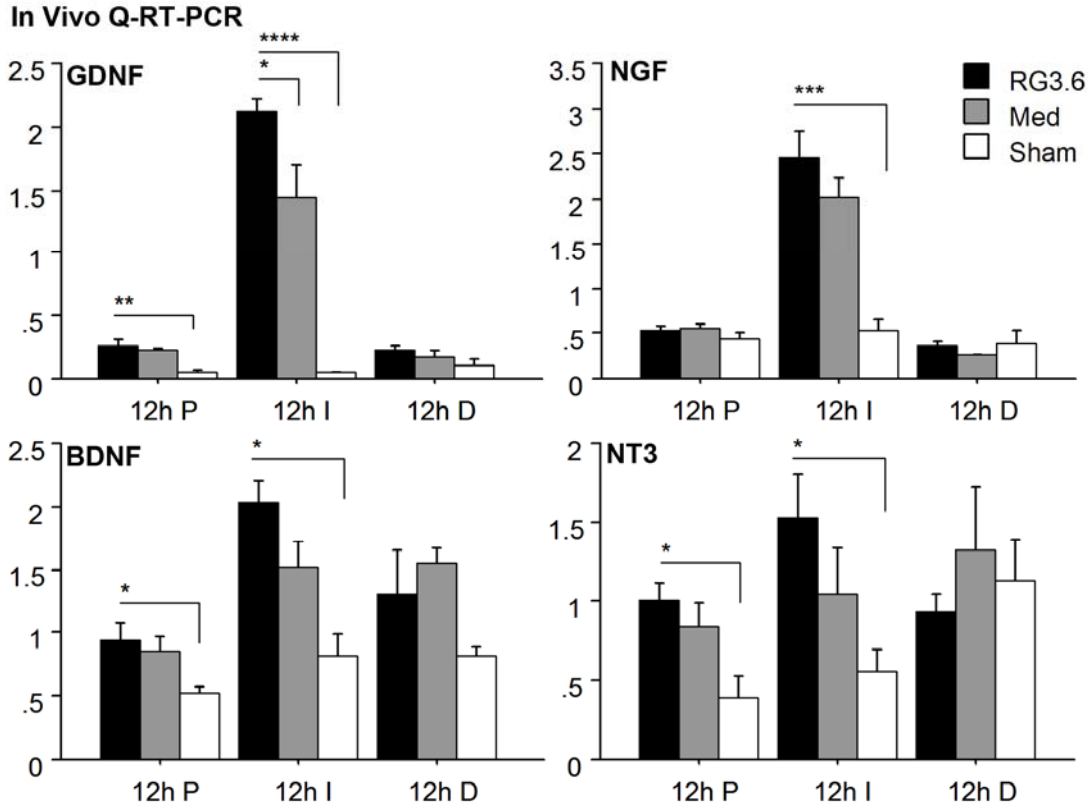


Figure 9. Spatial gene expression of trophic factors at 12 hours post SCI using Q-RT-PCR. GDNF, NGF, BDNF, and NT3 expression was measured in the P1, I and D1 segments at 12 hours post injury. Injury induced up-regulation of trophic factors especially in the injury epicenters. GDNF showed significantly up-regulation in RG3.6 treated injury segment. Expression levels represent fold change after normalizing to GAPDH. Values are means ± SEM, * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, **** $p< 0.0001$, ANOVA.

Table 3. Primers used for Q-RT-PCR in this chapter

Gene ID		Forward	Reverse
NM_017008	GAPDH	5'AAATGATACCCCACCGTGTGA3'	5'GCTGGCACTGCACAAGAAGAT3'
U55762	EGFP	5'CTGAGCAAAGACCCCAACGA3'	5'GAACTCCAGGACCATGTG3'
NM_012589	IL-6	5'ATTCTGTCTCGAGCCCACCA3'	5'CTGAAGGGCAGATGGAGTTGA3'
U45965	MIP-2	5'ACCTCAACGGGCAGAATCAA3'	5'GCTTCCTGGGTGCAGTTTGT3'
M57441	MCP-1	5'CTTCCTCCACCACTATGCAGG3'	5'TGAACAACAGGCCCAGAAGC3'
NM_031971	Hsp70	5'TTCAATATGAAGAGCGCCGTG3'	5'GCTGATCTTGCCCTTGAGACC3'
NM_031970	Hsp27	5'TGCCCAAAGCAGTCACACAA3'	5'CGAAAGTGACCGGAATGGTG3'
NM_012580	HemeOx1	5'CCATCCCTTACACACCAGCC3'	5'CCTCGTGGAGACGCTTTACG3'
BM_385445	Top2a	5'GGGAGGCAGGACTGTTGACA3'	5'ACGGATCACACTTCCTGGCT3'
NM_012560	Foxg1	5'CGTTCAGCTACAACGCGCT3'	5'TCTCGGGACTCTGCCTGATG3'
BE_115061	Lef1	5'GACGGATTGCCAAACGTGAC3'	5'AAACAGGTAGCCAAGCCCACT3'
NM_031140	Vimentin	5'GAGCACCCTGCAGTCATTCA3'	5'CGTGCCAGAGAAGCATTGTC3'
AA_925143	Nkx2.2	5'GGCGACACAGGCCCATC3'	5'TCGCTAGTGATCATCGTTGCC3'
BE_107070	Sox11	5'GCTGATGTCTTCTATGCATCCG3'	5'TTTTTCAAGCTCCCTGCAGTTTA3'
NM_019139	GDNF	5'GGTCACCAGATAAACAAGCGG3'	5'GCCGGTTCCTCTCTCTTCG3'
NM_012513	BDNF	5'AGGCACTGGAACTCGCAATG3'	5'AAGGGCCCGAACATACGATT3'
NM_031073	NT3	5'GATATTTTGGCCGGAGGGAA3'	5'CCTCAAAAGGGCTGGGTTCT3'
NM_031523	rNGF	5'TGCTCCTGCATGCCTGTTAC3'	5'CAGGGCGAGGAACAGGATC3'

Conclusion

Cell based therapy has great potential for SCI in animal models or even in human clinical trials. Most paradigms are to apply stem cells during sub-acute phase after SCI to avoid the unfavorable injury microenvironment in local spinal tissue. We studied the efficacy of acute radial glia treatment in SCI by behavioral test, histological observation and gene expression analysis. Acute transplantation of radial glial RG3.6 cells induced early locomotion recovery and significantly reduced neuroinflammation. Moreover, RG3.6 transplants modulated host responses to contusive injury by enhancing expression of genes associated with tissue protection, cell activation, and stem cell differentiation as early as 6 hours post injury. Like radial glia that can span the neuroepithelium in the developing CNS, RG3.6 cells bridged the injury site and supported injured axons in an organized rostral-caudal orientation.

In future studies, radial glial treatment should be examined whether it also promotes axonal remyelination and regeneration when applied during SCI sub-acute phase. It is also of great interest to study whether combining radial glia transplantation with other interventions can induce synergistic functional recovery. Finally, although it doesn't seem feasible to apply a v-myc stabilized embryonic radial glial clone in human clinical trials, it is important to understand how radial glia ameliorate injured spinal tissue in animal models in attempts to learn and develop practical interventions for SCI patients.

Appendix

Combination of acute anti-inflammatory treatment with RG3.6 transplantation following spinal cord injury

Traumatic injury to the spinal cord initiates a cascade of degenerative processes, known as secondary injury, which include various inflammatory reactions. Anti-inflammatory drug Methylprednisolone (MP) has been a standard treatment to SCI patients in acute phase and shown to preserve some neurological function if administered within 8 hours after the injury. The mechanism underlying MP's protective function has been associated with reduced lipid peroxidation and pro-inflammatory. Minocycline, a tetracycline derivative, has also been proved to be neuroprotective in animal models of neurodegenerative diseases, ischemia, and CNS injury. The major effect of minocycline is to block cell death pathway and to reduce inflammation.

Previously we have demonstrated that acute transplantation of radial glial clone RG3.6 promotes early functional recovery and suppresses neuroinflammation following spinal cord contusion. In this section, we combined acute administration of methylprednisolone or minocycline with acute or sub-acute transplantation of RG3.6 cells in an attempt to understand if acute suppression of inflammatory response synergizes the treatment efficacy of radial glial transplants in injured rat spinal cord.

In experiment I, we performed acute administration of anti-inflammatory drug MP or minocycline after spinal cord contusion followed by a 9-day delayed RG3.6 transplantation to the lesion site. Hindlimbs locomotor was documented using BBB score system and only showed a slight increase in rats that received MP or MC at day 2

post-SCI (data not shown). White matter preservation was analyzed by Luxol fast blue staining at 16 days after spinal cord injury. MP or MC treated rats preserved more white matter in rostral regions to epicenter (figure 1). In addition to increased spared white matter, MP or MC treated rats displayed more CNPase positive oligodendrocytes (figure 2). The histological observations indicate this combination therapy may protect oligodendrocytes and prevent Wallerian degeneration.

In an attempt to increase locomotor recovery by combination therapy of anti-inflammatory drug and RG3.6 transplantation, in experiment II, we optimized the time window for applying RG3.6 cells to the injured spinal cord. We modified our experiment design from 9 day delay RG3.6 transplantation to 0-2 day sub-acute transplantation with single injection of MP following contusive spinal cord injury. We did not continue any minocycline study because of severe liver fibrosis problem was found in some rats in experiment I, which may affect our evaluation on behavioral recovery after spinal cord injury. In view of BBB scoring system can not represent as a whole recovery index, we also conducted footprint and grid walking testes to evaluate animals' walking improvement. Significant improvement was observed in rats received acute MP and sub-acute RG3.6 transplantation. From footprints and grid walking results, we also found that 0-48 hours post injury is an effective window for radial glial transplantation.

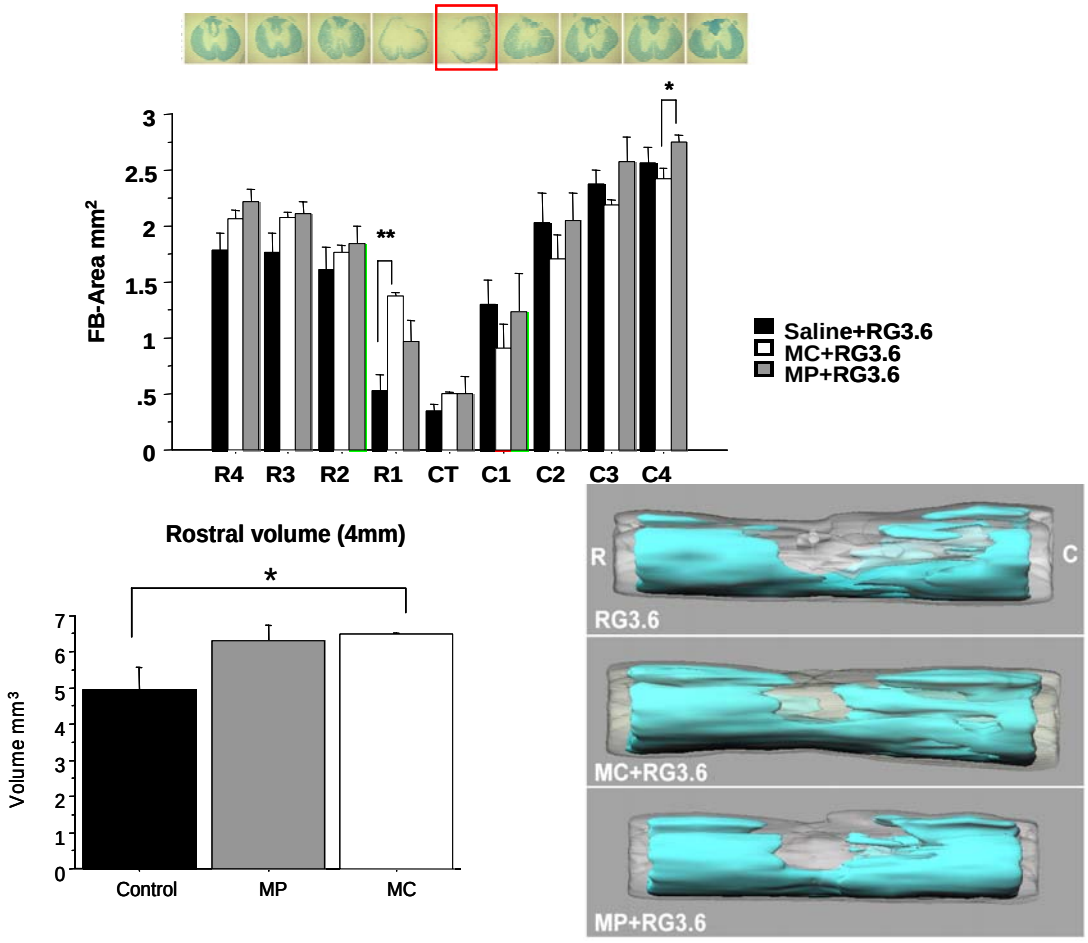


Figure 1. Spared white matter was stained by Luxol fast blue and quantitated by Zeiss LSM software. 1 cm segment of the spinal cord with the injury site in the center was frozen sectioned at 20 μm and every 5th section was taken. MC and MP both showed a trend to preserve more spared white matter at rostral regions, and only MC rats showed statistical significance at 1 mm rostral to lesion center (A). Spared white matter volume was reconstructed and showed that MC preserved more rostral spinal cord segment (R4-CT 4mm) than controls (B). 3D reconstruction of Luxol fast blue stained regions was obtained by Z-stack images of 2D bright-field images taken with Zeiss Axiophot and processed through 3D rendering using Imaris software (C). (R: rostral, C: caudal, and CT: lesion center. N=3 per group, data present mean ± SEM)

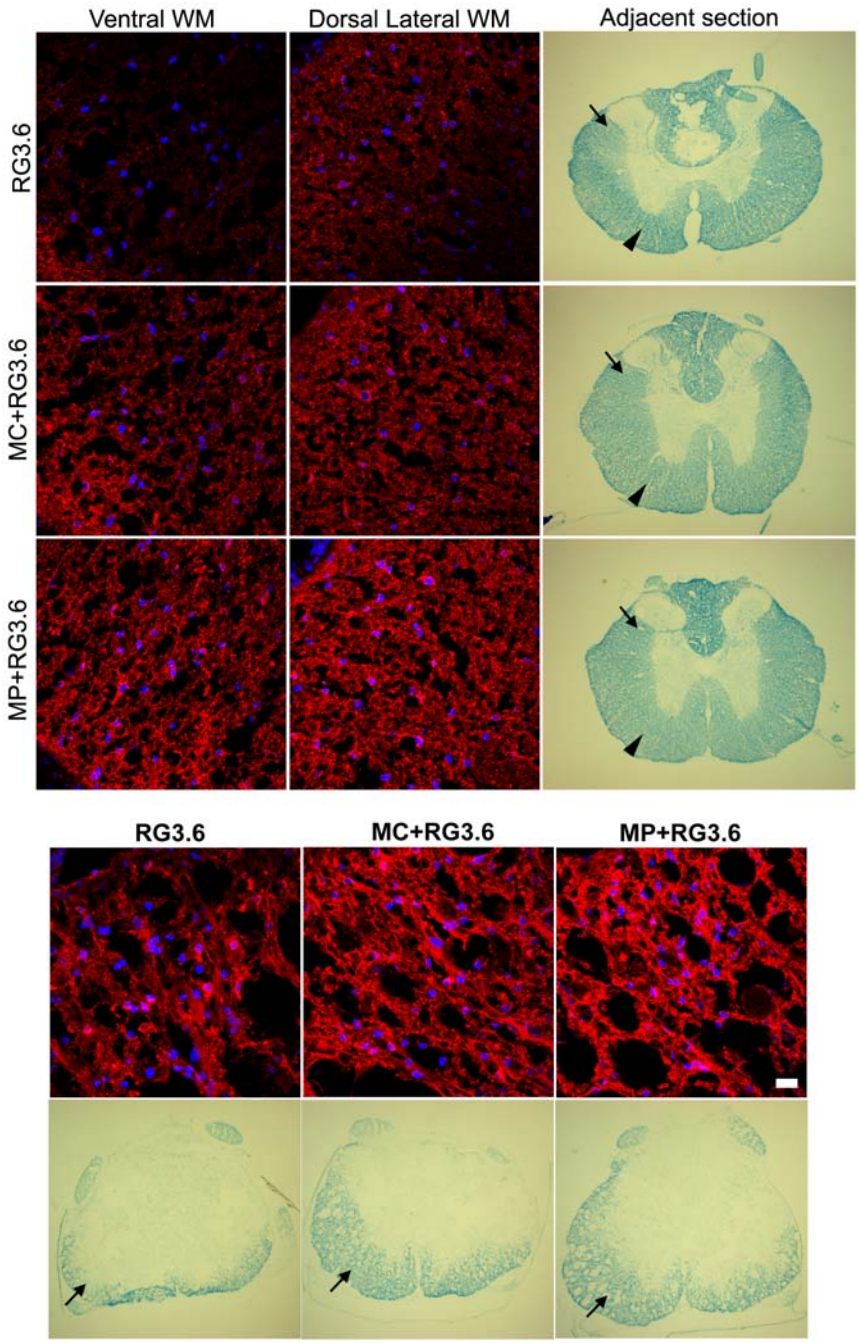
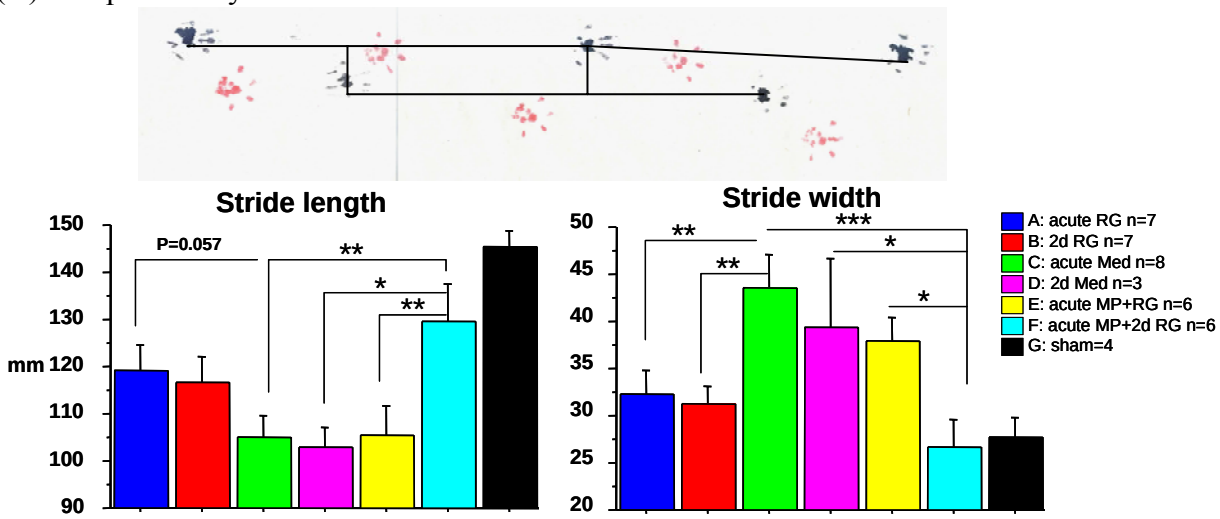


Figure 2. Delayed oligodendrocytes death occurs about 2 weeks after injury. We used CNPase to immunostain olidodendrocytes to determine if treatment preserve more oligodendrocytes. Rostral sections were used in (A) and showed more CNPase positive staining in the ventral white matter (WM) which is indicated by the arrow in the adjacent luxol fast blue stained section, and dorsal lateral WM by the arrowhead. Lesion centers (Shown in B) also showed more CNPase positive cells in the ventral WM. Immuostained areas were indicated by the arrow in the adjacent sections in the lower panel. Scale = 20μm.

(A) Footprint analysis



(B) Grid walking error counts

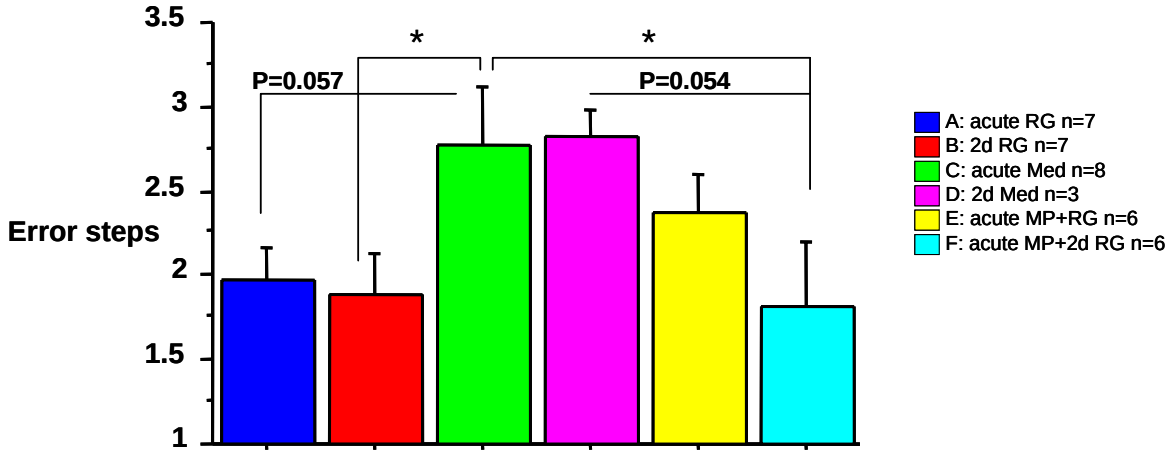


Figure 3. (A) Animals were placed on a 1-m long chamber, paws were inked and footprints were recorded on white paper strip. Animals with acute MP injection and sub-acute (2 d post injury) radial glia transplantation (Group F) have significant improved stepping, which is similar to naïve rats with long stride length and short stride width. Acute radial glia transplantation (Group A) also showed better stepping but this beneficial effect was reduced when combined with acute MP administration (Group E). The recovery of stepping is also improved in sub-acute RG3.6 transplantation (Group B) and was enhanced by acute MP (Group F). Animals treated with medium DMEM+F12 did not have functional recovery (Group C, D).
(B) Animals were placed on a horizontal ladder with irregular gaps and failed steps were counted. Rats received acute or sub-acute RG3.6 transplantation show significant recovery with fewest dropped steps. *P<0.05, **P<0.01, ***P<0.0001, Fisher's PLSD.

References

Adams, N. C., T. Tomoda, M. Cooper, G. Dietz and M. E. Hatten (2002). "Mice that lack astrotactin have slowed neuronal migration." Development **129**(4): 965-72.

Aimone, J. B., J. L. Leasure, V. M. Perreau and M. Thallmair (2004). "Spatial and temporal gene expression profiling of the contused rat spinal cord." Exp Neurol **189**(2): 204-21.

Akimoto, J., H. Itoh, T. Miwa and K. Ikeda (1993). "Immunohistochemical study of glutamine synthetase expression in early glial development." Brain Res Dev Brain Res **72**(1): 9-14.

Alvarez-Buylla, A., D. R. Buskirk and F. Nottebohm (1987). "Monoclonal antibody reveals radial glia in adult avian brain." J Comp Neurol **264**(2): 159-70.

Anderson, R. (1995). "The activated neutrophil--formidable forces unleashed." S Afr Med J **85**(10): 1024-8.

Ankeny, D. P., D. M. McTigue and L. B. Jakeman (2004). "Bone marrow transplants provide tissue protection and directional guidance for axons after contusive spinal cord injury in rats." Exp Neurol **190**(1): 17-31.

Anthony, T. E., C. Klein, G. Fishell and N. Heintz (2004). "Radial glia serve as neuronal progenitors in all regions of the central nervous system." Neuron **41**(6): 881-90.

Anton, E. S., J. A. Kreidberg and P. Rakic (1999). "Distinct functions of alpha3 and alpha(v) integrin receptors in neuronal migration and laminar organization of the cerebral cortex." Neuron **22**(2): 277-89.

Anton, E. S., M. A. Marchionni, K. F. Lee and P. Rakic (1997). "Role of GGF/neuregulin signaling in interactions between migrating neurons and radial glia in the developing cerebral cortex." Development **124**(18): 3501-10.

Arce, L., N. N. Yokoyama and M. L. Waterman (2006). "Diversity of LEF/TCF action in development and disease." Oncogene **25**(57): 7492-504.

Balentine, J. D. (1978). "Pathology of experimental spinal cord trauma. I. The necrotic lesion as a function of vascular injury." Lab Invest **39**(3): 236-53.

Balentine, J. D. (1978). "Pathology of experimental spinal cord trauma. II. Ultrastructure of axons and myelin." Lab Invest **39**(3): 254-66.

Bareyre, F. M., B. Haudenschild and M. E. Schwab (2002). "Long-lasting sprouting and gene expression changes induced by the monoclonal antibody IN-1 in the adult spinal cord." J Neurosci **22**(16): 7097-110.

Bareyre, F. M. and M. E. Schwab (2003). "Inflammation, degeneration and regeneration in the injured spinal cord: insights from DNA microarrays." Trends Neurosci **26**(10): 555-63.

Barres, B. A., M. D. Jacobson, R. Schmid, M. Sendtner and M. C. Raff (1993). "Does oligodendrocyte survival depend on axons?" Curr Biol **3**(8): 489-97.

Barres, B. A. and M. C. Raff (1993). "Proliferation of oligodendrocyte precursor cells depends on electrical activity in axons." Nature **361**(6409): 258-60.

Barritt, A. W., M. Davies, F. Marchand, R. Hartley, J. Grist, P. Yip, S. B. McMahon and E. J. Bradbury (2006). "Chondroitinase ABC promotes sprouting of intact and injured spinal systems after spinal cord injury." J Neurosci **26**(42): 10856-67.

Bartholdi, D. and M. E. Schwab (1995). "Methylprednisolone inhibits early inflammatory processes but not ischemic cell death after experimental spinal cord lesion in the rat." Brain Res **672**(1-2): 177-86.

Bartholdi, D. and M. E. Schwab (1997). "Expression of pro-inflammatory cytokine and chemokine mRNA upon experimental spinal cord injury in mouse: an in situ hybridization study." Eur J Neurosci **9**(7): 1422-38.

Basso, D. M., M. S. Beattie and J. C. Bresnahan (1996). "Graded histological and locomotor outcomes after spinal cord contusion using the NYU weight-drop device versus transection." Exp Neurol **139**(2): 244-56.

Batchelor, P. E., G. T. Liberatore, J. Y. Wong, M. J. Porritt, F. Frerichs, G. A. Donnan and D. W. Howells (1999). "Activated macrophages and microglia induce dopaminergic sprouting in the injured striatum and express brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor." J Neurosci **19**(5): 1708-16.

Beattie, M. S., G. E. Hermann, R. C. Rogers and J. C. Bresnahan (2002). "Cell death in models of spinal cord injury." Prog Brain Res **137**: 37-47.

Beere, H. M. and D. R. Green (2001). "Stress management - heat shock protein-70 and the regulation of apoptosis." Trends Cell Biol **11**(1): 6-10.

Beere, H. M., B. B. Wolf, K. Cain, D. D. Mosser, A. Mahboubi, T. Kuwana, P. Tailor, R. I. Morimoto, G. M. Cohen and D. R. Green (2000). "Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome." Nat Cell Biol **2**(8): 469-75.

Behrstock, S., A. Ebert, J. McHugh, S. Vosberg, J. Moore, B. Schneider, E. Capowski, D. Hei, J. Kordower, P. Aebischer and C. N. Svendsen (2006). "Human neural progenitors deliver glial cell line-derived neurotrophic factor to parkinsonian rodents and aged primates." Gene Ther **13**(5): 379-88.

Benson, M. D., M. I. Romero, M. E. Lush, Q. R. Lu, M. Henkemeyer and L. F. Parada (2005). "Ephrin-B3 is a myelin-based inhibitor of neurite outgrowth." Proc Natl Acad Sci U S A **102**(30): 10694-9.

Bentivoglio, M. and P. Mazzarello (1999). "The history of radial glia." Brain Res Bull **49**(5): 305-15.

Bergsland, M., M. Werme, M. Malewicz, T. Perlmann and J. Muhr (2006). "The establishment of neuronal properties is controlled by Sox4 and Sox11." Genes Dev **20**(24): 3475-86.

Bethea, J. R. (2000). "Spinal cord injury-induced inflammation: a dual-edged sword." Prog Brain Res **128**: 33-42.

Bethea, J. R., H. Nagashima, M. C. Acosta, C. Briceno, F. Gomez, A. E. Marcillo, K. Loor, J. Green and W. D. Dietrich (1999). "Systemically administered interleukin-10 reduces tumor necrosis factor-alpha production and significantly improves functional recovery following traumatic spinal cord injury in rats." J Neurotrauma **16**(10): 851-63.

Blesch, A. and M. H. Tuszynski (2001). "GDNF gene delivery to injured adult CNS motor neurons promotes axonal growth, expression of the trophic neuropeptide CGRP, and cellular protection." J Comp Neurol **436**(4): 399-410.

Blesch, A. and M. H. Tuszynski (2003). "Cellular GDNF delivery promotes growth of motor and dorsal column sensory axons after partial and complete spinal cord transections and induces remyelination." J Comp Neurol **467**(3): 403-17.

Bohn, M. C. (2004). "Motoneurons crave glial cell line-derived neurotrophic factor." Exp Neurol **190**(2): 263-75.

Bonni, A., Y. Sun, M. Nadal-Vicens, A. Bhatt, D. A. Frank, I. Rozovsky, N. Stahl, G. D. Yancopoulos and M. E. Greenberg (1997). "Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway." Science **278**(5337): 477-83.

Bracken, M. B., M. J. Shepard, W. F. Collins, T. R. Holford, W. Young, D. S. Baskin, H. M. Eisenberg, E. Flamm, L. Leo-Summers, J. Maroon and et al. (1990). "A randomized, controlled trial of methylprednisolone or naloxone in the treatment of acute spinal-cord injury. Results of the Second National Acute Spinal Cord Injury Study." N Engl J Med **322**(20): 1405-11.

Bradbury, E. J., L. D. Moon, R. J. Popat, V. R. King, G. S. Bennett, P. N. Patel, J. W. Fawcett and S. B. McMahon (2002). "Chondroitinase ABC promotes functional recovery after spinal cord injury." Nature **416**(6881): 636-40.

Brewer, K. L., J. R. Bethea and R. P. Yezierski (1999). "Neuroprotective effects of interleukin-10 following excitotoxic spinal cord injury." Exp Neurol **159**(2): 484-93.

Briscoe, J., L. Sussel, P. Serup, D. Hartigan-O'Connor, T. M. Jessell, J. L. Rubenstein and J. Ericson (1999). "Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling." Nature **398**(6728): 622-7.

Broude, E., M. McAtee, M. S. Kelley and B. S. Bregman (1999). "Fetal spinal cord transplants and exogenous neurotrophic support enhance c-Jun expression in mature axotomized neurons after spinal cord injury." Exp Neurol **155**(1): 65-78.

Busch, S. A. and J. Silver (2007). "The role of extracellular matrix in CNS regeneration." Curr Opin Neurobiol **17**(1): 120-7.

Bush, T. G., N. Puvanachandra, C. H. Horner, A. Polito, T. Ostenfeld, C. N. Svendsen, L. Mucke, M. H. Johnson and M. V. Sofroniew (1999). "Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice." Neuron **23**(2): 297-308.

Caggiano, A. O., M. P. Zimber, A. Ganguly, A. R. Blight and E. A. Gruskin (2005). "Chondroitinase ABCI improves locomotion and bladder function following contusion injury of the rat spinal cord." J Neurotrauma **22**(2): 226-39.

Cameron, R. S. and P. Rakic (1991). "Glial cell lineage in the cerebral cortex: a review and synthesis." Glia **4**(2): 124-37.

Cao, Q., X. M. Xu, W. H. Devries, G. U. Enzmann, P. Ping, P. Tsoulfas, P. M. Wood, M. B. Bunge and S. R. Whittemore (2005). "Functional recovery in traumatic spinal cord injury after transplantation of multineurotrophin-expressing glial-restricted precursor cells." J Neurosci **25**(30): 6947-57.

Cao, Q. L., Y. P. Zhang, R. M. Howard, W. M. Walters, P. Tsoulfas and S. R. Whittemore (2001). "Pluripotent stem cells engrafted into the normal or lesioned adult rat spinal cord are restricted to a glial lineage." Exp Neurol **167**(1): 48-58.

Carlson, S. L., M. E. Parrish, J. E. Springer, K. Doty and L. Dossett (1998). "Acute inflammatory response in spinal cord following impact injury." Exp Neurol **151**(1): 77-88.

Carmel, J. B., A. Galante, P. Soteropoulos, P. Tolias, M. Recce, W. Young and R. P. Hart (2001). "Gene expression profiling of acute spinal cord injury reveals spreading inflammatory signals and neuron loss." Physiol Genomics **7**(2): 201-13.

Carson, F. L. (1997). Histotechnology: A Self-Instructional Text. Chicago, ASCP Press.

Casella, G. T., A. Marcillo, M. B. Bunge and P. M. Wood (2002). "New vascular tissue rapidly replaces neural parenchyma and vessels destroyed by a contusion injury to the rat spinal cord." Exp Neurol **173**(1): 63-76.

Casha, S., W. R. Yu and M. G. Fehlings (2001). "Oligodendroglial apoptosis occurs along degenerating axons and is associated with FAS and p75 expression following spinal cord injury in the rat." Neuroscience **103**(1): 203-18.

Chambers, C. B., Y. Peng, H. Nguyen, N. Gaiano, G. Fishell and J. S. Nye (2001). "Spatiotemporal selectivity of response to Notch1 signals in mammalian forebrain precursors." Development **128**(5): 689-702.

Chen, A., X. M. Xu, N. Kleitman and M. B. Bunge (1996). "Methylprednisolone administration improves axonal regeneration into Schwann cell grafts in transected adult rat thoracic spinal cord." Exp Neurol **138**(2): 261-76.

Chen, Z. J., M. Negra, A. Levine, Y. Ughrin and J. M. Levine (2002). "Oligodendrocyte precursor cells: reactive cells that inhibit axon growth and regeneration." J Neurocytol **31**(6-7): 481-95.

Cheung, M., M. Abu-Elmagd, H. Clevers and P. J. Scotting (2000). "Roles of Sox4 in central nervous system development." Brain Res Mol Brain Res **79**(1-2): 180-91.

Condic, M. L., D. M. Snow and P. C. Letourneau (1999). "Embryonic neurons adapt to the inhibitory proteoglycan aggrecan by increasing integrin expression." J Neurosci **19**(22): 10036-43.

Cottrez, F. and H. Groux (2001). "Regulation of TGF-beta response during T cell activation is modulated by IL-10." J Immunol **167**(2): 773-8.

Coumans, J. V., T. T. Lin, H. N. Dai, L. MacArthur, M. McAtee, C. Nash and B. S. Bregman (2001). "Axonal regeneration and functional recovery after complete spinal cord transection in rats by delayed treatment with transplants and neurotrophins." J Neurosci **21**(23): 9334-44.

Coyle-Rink, J., L. Del Valle, T. Sweet, K. Khalili and S. Amini (2002). "Developmental expression of Wnt signaling factors in mouse brain." Cancer Biol Ther **1**(6): 640-5.

Crowe, M. J., J. C. Bresnahan, S. L. Shuman, J. N. Masters and M. S. Beattie (1997). "Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys." Nat Med **3**(1): 73-6.

Dahl, D., D. C. Rueger, A. Bignami, K. Weber and M. Osborn (1981). "Vimentin, the 57 000 molecular weight protein of fibroblast filaments, is the major cytoskeletal component in immature glia." Eur J Cell Biol **24**(2): 191-6.

Danton, G. H. and W. D. Dietrich (2003). "Inflammatory mechanisms after ischemia and stroke." J Neuropathol Exp Neurol **62**(2): 127-36.

Davies, J. E., C. Huang, C. Proschel, M. Noble, M. Mayer-Proschel and S. J. Davies (2006). "Astrocytes derived from glial-restricted precursors promote spinal cord repair." J Biol **5**(3): 7.

Davies, J. E., X. Tang, J. C. Bournat and S. J. Davies (2006). "Decorin promotes plasminogen/plasmin expression within acute spinal cord injuries and by adult microglia in vitro." J Neurotrauma **23**(3-4): 397-408.

Davies, J. E., X. Tang, J. W. Denning, S. J. Archibald and S. J. Davies (2004). "Decorin suppresses neurocan, brevican, phosphacan and NG2 expression and promotes axon growth across adult rat spinal cord injuries." Eur J Neurosci **19**(5): 1226-42.

Davies, J. E., X. Tang, T. Phoenix, M. Mayer-Proschel and S. J. Davies (2004). Transplantation of glial restricted precursor-derived type-1 astrocytes promotes axon growth across acute spinal cord injuries. SFN 2004 meeting, San Diego, CA, Society for Neuroscience.

Davies, S. J., M. T. Fitch, S. P. Memberg, A. K. Hall, G. Raisman and J. Silver (1997). "Regeneration of adult axons in white matter tracts of the central nervous system." Nature **390**(6661): 680-3.

Davies, S. J., D. R. Goucher, C. Doller and J. Silver (1999). "Robust regeneration of adult sensory axons in degenerating white matter of the adult rat spinal cord." J Neurosci **19**(14): 5810-22.

De Biase, A., S. M. Knoblach, S. Di Giovanni, C. Fan, A. Molon, E. P. Hoffman and A. I. Faden (2005). "Gene expression profiling of experimental traumatic spinal cord injury as a function of distance from impact site and injury severity." Physiol Genomics **22**(3): 368-81.

Demjen, D., S. Klussmann, S. Kleber, C. Zuliani, B. Stieltjes, C. Metzger, U. A. Hirt, H. Walczak, W. Falk, M. Essig, L. Edler, P. H. Krammer and A. Martin-Villalba (2004). "Neutralization of CD95 ligand promotes regeneration and functional recovery after spinal cord injury." Nat Med **10**(4): 389-95.

Derer, P. and M. Derer (1990). "Cajal-Retzius cell ontogenesis and death in mouse brain visualized with horseradish peroxidase and electron microscopy." Neuroscience **36**(3): 839-56.

Di Giovanni, S., S. M. Knoblach, C. Brandoli, S. A. Aden, E. P. Hoffman and A. I. Faden (2003). "Gene profiling in spinal cord injury shows role of cell cycle in neuronal death." Ann Neurol **53**(4): 454-68.

Doetsch, F. (2003). "The glial identity of neural stem cells." Nat Neurosci **6**(11): 1127-34.

Dou, C. L. and J. M. Levine (1994). "Inhibition of neurite growth by the NG2 chondroitin sulfate proteoglycan." J Neurosci **14**(12): 7616-28.

Dou, C. L., S. Li and E. Lai (1999). "Dual role of brain factor-1 in regulating growth and patterning of the cerebral hemispheres." Cereb Cortex **9**(6): 543-50.

Dreyfus, C. F., X. Dai, L. D. Lercher, B. R. Racey, W. J. Friedman and I. B. Black (1999). "Expression of neurotrophins in the adult spinal cord in vivo." J Neurosci Res **56**(1): 1-7.

Dulabon, L., E. C. Olson, M. G. Taglienti, S. Eisenhuth, B. McGrath, C. A. Walsh, J. A. Kreidberg and E. S. Anton (2000). "Reelin binds alpha3beta1 integrin and inhibits neuronal migration." Neuron **27**(1): 33-44.

Dusart, I. and M. E. Schwab (1994). "Secondary cell death and the inflammatory reaction after dorsal hemisection of the rat spinal cord." Eur J Neurosci **6**(5): 712-24.

Eckenhoff, M. F. and P. Rakic (1984). "Radial organization of the hippocampal dentate gyrus: a Golgi, ultrastructural, and immunocytochemical analysis in the developing rhesus monkey." J Comp Neurol **223**(1): 1-21.

Edmondson, J. C., R. K. Liem, J. E. Kuster and M. E. Hatten (1988). "Astrotactin: a novel neuronal cell surface antigen that mediates neuron-astroglial interactions in cerebellar microcultures." J Cell Biol **106**(2): 505-17.

Fadic, R., V. Mezzano, K. Alvarez, D. Cabrera, J. Holmgren and E. Brandan (2006). "Increase in decorin and biglycan in Duchenne Muscular Dystrophy: role of fibroblasts as cell source of these proteoglycans in the disease." J Cell Mol Med **10**(3): 758-69.

Farooque, M., J. Isaksson and Y. Olsson (2001). "White matter preservation after spinal cord injury in ICAM-1/P-selectin-deficient mice." Acta Neuropathol (Berl) **102**(2): 132-40.

Faulkner, J. R., J. E. Herrmann, M. J. Woo, K. E. Tansey, N. B. Doan and M. V. Sofroniew (2004). "Reactive astrocytes protect tissue and preserve function after spinal cord injury." J Neurosci **24**(9): 2143-55.

Fawcett, J. W. (2006). "Overcoming inhibition in the damaged spinal cord." J Neurotrauma **23**(3-4): 371-83.

Feng, L., M. E. Hatten and N. Heintz (1994). "Brain lipid-binding protein (BLBP): a novel signaling system in the developing mammalian CNS." Neuron **12**(4): 895-908.

Feng, L. and N. Heintz (1995). "Differentiating neurons activate transcription of the brain lipid-binding protein gene in radial glia through a novel regulatory element." Development **121**(6): 1719-30.

Fischer, A. J. and T. A. Reh (2001). "Muller glia are a potential source of neural regeneration in the postnatal chicken retina." Nat Neurosci **4**(3): 247-52.

Fischer, A. J. and T. A. Reh (2003). "Potential of Muller glia to become neurogenic retinal progenitor cells." Glia **43**(1): 70-6.

Fitch, M. T. and J. Silver (1997). "Activated macrophages and the blood-brain barrier: inflammation after CNS injury leads to increases in putative inhibitory molecules." Exp Neurol **148**(2): 587-603.

Frevert, C. W., V. A. Wong, R. B. Goodman, R. Goodwin and T. R. Martin (1998). "Rapid fluorescence-based measurement of neutrophil migration in vitro." J Immunol Methods **213**(1): 41-52.

Friedlander, D. R., P. A. Brittis, T. Sakurai, B. Shif, W. Wirchansky, G. Fishell and M. Grumet (1998). "Generation of a radial-like glial cell line." J Neurobiol **37**(2): 291-304.

Friedlander, D. R., P. Milev, L. Karthikeyan, R. K. Margolis, R. U. Margolis and M. Grumet (1994). "The neuronal chondroitin sulfate proteoglycan neurocan binds to the neural cell adhesion molecules Ng-CAM/L1/NILE and N-CAM, and inhibits neuronal adhesion and neurite outgrowth." J Cell Biol **125**(3): 669-80.

Frotscher, M. (1998). "Cajal-Retzius cells, Reelin, and the formation of layers." Curr Opin Neurobiol **8**(5): 570-5.

Fu, S. Y. and T. Gordon (1995). "Contributing factors to poor functional recovery after delayed nerve repair: prolonged axotomy." J Neurosci **15**(5 Pt 2): 3876-85.

Fu, S. Y. and T. Gordon (1995). "Contributing factors to poor functional recovery after delayed nerve repair: prolonged denervation." J Neurosci **15**(5 Pt 2): 3886-95.

Gaiano, N., J. S. Nye and G. Fishell (2000). "Radial glial identity is promoted by Notch1 signaling in the murine forebrain." Neuron **26**(2): 395-404.

Galceran, J., E. M. Miyashita-Lin, E. Devaney, J. L. Rubenstein and R. Grosschedl (2000). "Hippocampus development and generation of dentate gyrus granule cells is regulated by LEF1." Development **127**(3): 469-82.

Gale, K., H. Kerasidis and J. R. Wrathall (1985). "Spinal cord contusion in the rat: behavioral analysis of functional neurologic impairment." Exp Neurol **88**(1): 123-34.

Gething, M. J. and J. Sambrook (1992). "Protein folding in the cell." Nature **355**(6355): 33-45.

Gimsa, U., S. V. Peter, K. Lehmann, I. Bechmann and R. Nitsch (2000). "Axonal damage induced by invading T cells in organotypic central nervous system tissue in vitro: involvement of microglial cells." Brain Pathol **10**(3): 365-77.

Giulian, D., T. J. Baker, L. C. Shih and L. B. Lachman (1986). "Interleukin 1 of the central nervous system is produced by ameboid microglia." J Exp Med **164**(2): 594-604.

Goldman, S. (2005). "Stem and progenitor cell-based therapy of the human central nervous system." Nat Biotechnol **23**(7): 862-71.

Gongidi, V., C. Ring, M. Moody, R. Brekken, E. H. Sage, P. Rakic and E. S. Anton (2004). "SPARC-like 1 regulates the terminal phase of radial glia-guided migration in the cerebral cortex." Neuron **41**(1): 57-69.

Gotz, M., E. Hartfuss and P. Malatesta (2002). "Radial glial cells as neuronal precursors: a new perspective on the correlation of morphology and lineage restriction in the developing cerebral cortex of mice." Brain Res Bull **57**(6): 777-88.

Gotz, M., A. Stoykova and P. Gruss (1998). "Pax6 controls radial glia differentiation in the cerebral cortex." Neuron **21**(5): 1031-44.

Gould, S. J., S. Howard and L. Papadaki (1990). "The development of ependyma in the human fetal brain: an immunohistological and electron microscopic study." Brain Res Dev Brain Res **55**(2): 255-67.

Grill, R., K. Murai, A. Blesch, F. H. Gage and M. H. Tuszynski (1997). "Cellular delivery of neurotrophin-3 promotes corticospinal axonal growth and partial functional recovery after spinal cord injury." J Neurosci **17**(14): 5560-72.

Grill, R. J., A. Blesch and M. H. Tuszynski (1997). "Robust growth of chronically injured spinal cord axons induced by grafts of genetically modified NGF-secreting cells." Exp Neurol **148**(2): 444-52.

Grumet, M., A. Flaccus and R. U. Margolis (1993). "Functional characterization of chondroitin sulfate proteoglycans of brain: interactions with neurons and neural cell adhesion molecules." J Cell Biol **120**(3): 815-24.

Gruner, J. A. (1992). "A monitored contusion model of spinal cord injury in the rat." J Neurotrauma **9**(2): 123-6; discussion 126-8.

Hack, M. A., M. Sugimori, C. Lundberg, M. Nakafuku and M. Gotz (2004). "Regionalization and fate specification in neurospheres: the role of Olig2 and Pax6." Mol Cell Neurosci **25**(4): 664-78.

Hall, E. D. and J. E. Springer (2004). "Neuroprotection and Acute Spinal Cord Injury: A Reappraisal." Neurorx **1**(1): 80-100.

Hamada, Y., T. Ikata, S. Katoh, K. Nakauchi, M. Niwa, Y. Kawai and K. Fukuzawa (1996). "Involvement of an intercellular adhesion molecule 1-dependent pathway in the pathogenesis of secondary changes after spinal cord injury in rats." J Neurochem **66**(4): 1525-31.

Han, S. S., Y. Liu, C. Tyler-Polsz, M. S. Rao and I. Fischer (2004). "Transplantation of glial-restricted precursor cells into the adult spinal cord: survival, glial-specific differentiation, and preferential migration in white matter." Glia **45**(1): 1-16.

Hanashima, C., L. Shen, S. C. Li and E. Lai (2002). "Brain factor-1 controls the proliferation and differentiation of neocortical progenitor cells through independent mechanisms." J Neurosci **22**(15): 6526-36.

Harel, N. Y. and S. M. Strittmatter (2006). "Can regenerating axons recapitulate developmental guidance during recovery from spinal cord injury?" Nat Rev Neurosci **7**(8): 603-16.

Hartfuss, E., E. Forster, H. H. Bock, M. A. Hack, P. Leprince, J. M. Luque, J. Herz, M. Frotscher and M. Gotz (2003). "Reelin signaling directly affects radial glia morphology and biochemical maturation." Development **130**(19): 4597-609.

Hartfuss, E., R. Galli, N. Heins and M. Gotz (2001). "Characterization of CNS precursor subtypes and radial glia." Dev Biol **229**(1): 15-30.

Hasegawa, K., Y. W. Chang, H. Li, Y. Berlin, O. Ikeda, N. Kane-Goldsmith and M. Grumet (2005). "Embryonic radial glia bridge spinal cord lesions and promote functional recovery following spinal cord injury." Exp Neurol **193**(2): 394-410.

Hasegawa, K. and M. Grumet (2003). "Trauma-induced tumorigenesis of cells implanted into the rat spinal cord." J Neurosurg **98**(5): 1065-71.

Hatakeyama, J., Y. Bessho, K. Katoh, S. Ookawara, M. Fujioka, F. Guillemot and R. Kageyama (2004). "Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation." Development **131**(22): 5539-50.

Hatten, M. E. (1999). "Central nervous system neuronal migration." Annu Rev Neurosci **22**: 511-39.

Hauben, E. and M. Schwartz (2003). "Therapeutic vaccination for spinal cord injury: helping the body to cure itself." Trends Pharmacol Sci **24**(1): 7-12.

Haugeto, O., K. Ullensvang, L. M. Levy, F. A. Chaudhry, T. Honore, M. Nielsen, K. P. Lehre and N. C. Danbolt (1996). "Brain glutamate transporter proteins form homomultimers." J Biol Chem **271**(44): 27715-22.

Hausmann, O. N. (2003). "Post-traumatic inflammation following spinal cord injury." Spinal Cord **41**(7): 369-78.

Heck, M. M., W. N. Hittelman and W. C. Earnshaw (1988). "Differential expression of DNA topoisomerases I and II during the eukaryotic cell cycle." Proc Natl Acad Sci U S A **85**(4): 1086-90.

Heidinger, V., D. Hicks, J. Sahel and H. Dreyfus (1999). "Ability of retinal Muller glial cells to protect neurons against excitotoxicity in vitro depends upon maturation and neuron-glial interactions." Glia **25**(3): 229-39.

Heins, N., P. Malatesta, F. Cecconi, M. Nakafuku, K. L. Tucker, M. A. Hack, P. Chapouton, Y. A. Barde and M. Gotz (2002). "Glial cells generate neurons: the role of the transcription factor Pax6." Nat Neurosci **5**(4): 308-15.

Herx, L. M. and V. W. Yong (2001). "Interleukin-1 beta is required for the early evolution of reactive astrogliosis following CNS lesion." J Neuropathol Exp Neurol **60**(10): 961-71.

Hill, C. E., M. S. Beattie and J. C. Bresnahan (2001). "Degeneration and sprouting of identified descending supraspinal axons after contusive spinal cord injury in the rat." Exp Neurol **171**(1): 153-69.

Hill, C. E., C. Proschel, M. Noble, M. Mayer-Proschel, J. C. Gensel, M. S. Beattie and J. C. Bresnahan (2004). "Acute transplantation of glial-restricted precursor cells into spinal cord contusion injuries: survival, differentiation, and effects on lesion environment and axonal regeneration." Exp Neurol **190**(2): 289-310.

Hirano, T. and T. Kishimoto (1992). "Molecular biology and immunology of interleukin-6." Res Immunol **143**(7): 723-4.

Hocking, A. M., T. Shinomura and D. J. McQuillan (1998). "Leucine-rich repeat glycoproteins of the extracellular matrix." Matrix Biol **17**(1): 1-19.

Hofstetter, C. P., N. A. Holmstrom, J. A. Lilja, P. Schweinhardt, J. Hao, C. Spenger, Z. Wiesenfeld-Hallin, S. N. Kurpad, J. Frisen and L. Olson (2005). "Allodynia limits the usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome." Nat Neurosci **8**(3): 346-53.

Hofstetter, C. P., E. J. Schwarz, D. Hess, J. Widenfalk, A. El Manira, D. J. Prockop and L. Olson (2002). "Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery." Proc Natl Acad Sci U S A **99**(4): 2199-204.

Holden, J. A. and J. J. Townsend (1999). "DNA topoisomerase II-alpha as a proliferation marker in astrocytic neoplasms of the central nervous system: correlation with MIB1 expression and patient survival." Mod Pathol **12**(12): 1094-100.

Horky, L. L., F. Galimi, F. H. Gage and P. J. Horner (2006). "Fate of endogenous stem/progenitor cells following spinal cord injury." J Comp Neurol **498**(4): 525-38.

Hormigo, A., M. McCarthy, J. M. Nothias, K. Hasegawa, W. Huang, D. R. Friedlander, I. Fischer, G. Fishell and M. Grumet (2001). "Radial glial cell line C6-R integrates preferentially in adult white matter and facilitates migration of coimplanted neurons in vivo." Exp Neurol **168**(2): 310-22.

Hsu, C. Y. and M. R. Dimitrijevic (1990). "Methylprednisolone in spinal cord injury: the possible mechanism of action." J Neurotrauma **7**(3): 115-9.

Iannotti, C., Y. Ping Zhang, C. B. Shields, Y. Han, D. A. Burke and X. M. Xu (2004). "A neuroprotective role of glial cell line-derived neurotrophic factor following moderate spinal cord contusion injury." Exp Neurol **189**(2): 317-32.

Ikeda, O., M. Murakami, H. Ino, M. Yamazaki, M. Koda, C. Nakayama and H. Moriya (2002). "Effects of brain-derived neurotrophic factor (BDNF) on compression-induced spinal cord injury: BDNF attenuates down-regulation of superoxide dismutase expression and promotes up-regulation of myelin basic protein expression." J Neuropathol Exp Neurol **61**(2): 142-53.

Ikeda, O., M. Murakami, H. Ino, M. Yamazaki, T. Nemoto, M. Koda, C. Nakayama and H. Moriya (2001). "Acute up-regulation of brain-derived neurotrophic factor

expression resulting from experimentally induced injury in the rat spinal cord." Acta Neuropathol (Berl) **102**(3): 239-45.

Ikegami, T., M. Nakamura, J. Yamane, H. Katoh, S. Okada, A. Iwanami, K. Watanabe, K. Ishii, F. Kato, H. Fujita, T. Takahashi, H. J. Okano, Y. Toyama and H. Okano (2005). "Chondroitinase ABC combined with neural stem/progenitor cell transplantation enhances graft cell migration and outgrowth of growth-associated protein-43-positive fibers after rat spinal cord injury." Eur J Neurosci **22**(12): 3036-46.

Iseda, T., T. Nishio, S. Kawaguchi, M. Yamanoto, T. Kawasaki and S. Wakisaka (2004). "Spontaneous regeneration of the corticospinal tract after transection in young rats: a key role of reactive astrocytes in making favorable and unfavorable conditions for regeneration." Neuroscience **126**(2): 365-74.

Ito, D., K. Tanaka, S. Suzuki, T. Dembo and Y. Fukuuchi (2001). "Enhanced expression of Iba1, ionized calcium-binding adapter molecule 1, after transient focal cerebral ischemia in rat brain." Stroke **32**(5): 1208-15.

Ito, T., A. Suzuki, E. Imai, M. Okabe and M. Hori (2001). "Bone marrow is a reservoir of repopulating mesangial cells during glomerular remodeling." J Am Soc Nephrol **12**(12): 2625-35.

Jackson, C. A., J. Messinger, J. D. Peduzzi, D. C. Ansardi and C. D. Morrow (2005). "Enhanced functional recovery from spinal cord injury following intrathecal or intramuscular administration of poliovirus replicons encoding IL-10." Virology **336**(2): 173-83.

Jankowski, M. P., P. K. Cornuet, S. McIlwrath, H. R. Koerber and K. M. Albers (2006). "SRY-box containing gene 11 (Sox11) transcription factor is required for neuron survival and neurite growth." Neuroscience **143**(2): 501-14.

Jones, L. L., D. Sajed and M. H. Tuszynski (2003). "Axonal regeneration through regions of chondroitin sulfate proteoglycan deposition after spinal cord injury: a balance of permissiveness and inhibition." J Neurosci **23**(28): 9276-88.

Jones, L. L., Y. Yamaguchi, W. B. Stallcup and M. H. Tuszynski (2002). "NG2 is a major chondroitin sulfate proteoglycan produced after spinal cord injury and is expressed by macrophages and oligodendrocyte progenitors." J Neurosci **22**(7): 2792-803.

Jones, T. B., E. E. McDaniel and P. G. Popovich (2005). "Inflammatory-mediated injury and repair in the traumatically injured spinal cord." Curr Pharm Des **11**(10): 1223-36.

Karimi-Abdolrezaee, S., E. Eftekharpour, J. Wang, C. M. Morshead and M. G. Fehlings (2006). "Delayed transplantation of adult neural precursor cells promotes remyelination and functional neurological recovery after spinal cord injury." J Neurosci **26**(13): 3377-89.

Kawasaki, A., Y. Otori and C. J. Barnstable (2000). "Muller cell protection of rat retinal ganglion cells from glutamate and nitric oxide neurotoxicity." Invest Ophthalmol Vis Sci **41**(11): 3444-50.

Keane, R. W., A. R. Davis and W. D. Dietrich (2006). "Inflammatory and apoptotic signaling after spinal cord injury." J Neurotrauma **23**(3-4): 335-44.

Keirstead, H. S., G. Nistor, G. Bernal, M. Totoiu, F. Cloutier, K. Sharp and O. Steward (2005). "Human embryonic stem cell-derived oligodendrocyte progenitor cell

transplants remyelinate and restore locomotion after spinal cord injury." J Neurosci **25**(19): 4694-705.
Kerr, B. J. and P. H. Patterson (2005). "Leukemia inhibitory factor promotes oligodendrocyte survival after spinal cord injury." Glia **51**(1): 73-9.
Kim, D. H., P. H. Gutin, L. J. Noble, D. Nathan, J. S. Yu and R. P. Nockels (1996). "Treatment with genetically engineered fibroblasts producing NGF or BDNF can accelerate recovery from traumatic spinal cord injury in the adult rat." Neuroreport **7**(13): 2221-5.
Klein, S. M., S. Behrstock, J. McHugh, K. Hoffmann, K. Wallace, M. Suzuki, P. Aebischer and C. N. Svendsen (2005). "GDNF delivery using human neural progenitor cells in a rat model of ALS." Hum Gene Ther **16**(4): 509-21.
Kreutzberg, G. W. (1996). "Microglia: a sensor for pathological events in the CNS." Trends Neurosci **19**(8): 312-8.
Kuan, C. Y., A. J. Schloemer, A. Lu, K. A. Burns, W. L. Weng, M. T. Williams, K. I. Strauss, C. V. Vorhees, R. A. Flavell, R. J. Davis, F. R. Sharp and P. Rakic (2004). "Hypoxia-ischemia induces DNA synthesis without cell proliferation in dying neurons in adult rodent brain." J Neurosci **24**(47): 10763-72.
Kuhlbrodt, K., B. Herbarth, E. Sock, J. Enderich, I. Hermans-Borgmeyer and M. Wegner (1998). "Cooperative function of POU proteins and SOX proteins in glial cells." J Biol Chem **273**(26): 16050-7.
Lee, I. H., J. W. Bulte, P. Schweinhardt, T. Douglas, A. Trifunovski, C. Hofstetter, L. Olson and C. Spenger (2004). "In vivo magnetic resonance tracking of olfactory ensheathing glia grafted into the rat spinal cord." Exp Neurol **187**(2): 509-16.
Lee, J. E., S. F. Wu, L. M. Goering and R. I. Dorsky (2006). "Canonical Wnt signaling through Lef1 is required for hypothalamic neurogenesis." Development **133**(22): 4451-61.
Lepore, A. C., A. Bakshi, S. A. Swanger, M. S. Rao and I. Fischer (2005). "Neural precursor cells can be delivered into the injured cervical spinal cord by intrathecal injection at the lumbar cord." Brain Res **1045**(1-2): 206-16.
Lepore, A. C. and I. Fischer (2005). "Lineage-restricted neural precursors survive, migrate, and differentiate following transplantation into the injured adult spinal cord." Exp Neurol **194**(1): 230-42.
Levine, J. M. (1994). "Increased expression of the NG2 chondroitin-sulfate proteoglycan after brain injury." J Neurosci **14**(8): 4716-30.
Levitt, P., M. L. Cooper and P. Rakic (1981). "Coexistence of neuronal and glial precursor cells in the cerebral ventricular zone of the fetal monkey: an ultrastructural immunoperoxidase analysis." J Neurosci **1**(1): 27-39.
Levitt, P. and P. Rakic (1980). "Immunoperoxidase localization of glial fibrillary acidic protein in radial glial cells and astrocytes of the developing rhesus monkey brain." J Comp Neurol **193**(3): 815-40.
Levy, Y. and J. C. Brouet (1994). "Interleukin-10 prevents spontaneous death of germinal center B cells by induction of the bcl-2 protein." J Clin Invest **93**(1): 424-8.
Li, M. O., Y. Y. Wan, S. Sanjabi, A. K. Robertson and R. A. Flavell (2006). "Transforming growth factor-beta regulation of immune responses." Annu Rev Immunol **24**: 99-146.

Liberto, C. M., P. J. Albrecht, L. M. Herx, V. W. Yong and S. W. Levison (2004). "Pro-regenerative properties of cytokine-activated astrocytes." J Neurochem **89**(5): 1092-100.

Liu, D., L. Li and L. Augustus (2001). "Prostaglandin release by spinal cord injury mediates production of hydroxyl radical, malondialdehyde and cell death: a site of the neuroprotective action of methylprednisolone." J Neurochem **77**(4): 1036-47.

Liu, D., W. Thangnipon and D. J. McAdoo (1991). "Excitatory amino acids rise to toxic levels upon impact injury to the rat spinal cord." Brain Res **547**(2): 344-8.

Liu, S., Y. Qu, T. J. Stewart, M. J. Howard, S. Chakrabortty, T. F. Holekamp and J. W. McDonald (2000). "Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation." Proc Natl Acad Sci U S A **97**(11): 6126-31.

Llado, J., C. Haenggeli, N. J. Maragakis, E. Y. Snyder and J. D. Rothstein (2004). "Neural stem cells protect against glutamate-induced excitotoxicity and promote survival of injured motor neurons through the secretion of neurotrophic factors." Mol Cell Neurosci **27**(3): 322-31.

Lodge, P. A. and S. Sriram (1996). "Regulation of microglial activation by TGF-beta, IL-10, and CSF-1." J Leukoc Biol **60**(4): 502-8.

Lu, J., F. Feron, A. Mackay-Sim and P. M. Waite (2002). "Olfactory ensheathing cells promote locomotor recovery after delayed transplantation into transected spinal cord." Brain **125**(Pt 1): 14-21.

Lu, P., L. L. Jones, E. Y. Snyder and M. H. Tuszynski (2003). "Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury." Exp Neurol **181**(2): 115-29.

Lu, P., L. L. Jones and M. H. Tuszynski (2006). "Axon regeneration through scars and into sites of chronic spinal cord injury." Exp Neurol.

Lucas, J. H., D. G. Wheeler, Z. Guan, Z. Suntres and B. T. Stokes (2002). "Effect of glutathione augmentation on lipid peroxidation after spinal cord injury." J Neurotrauma **19**(6): 763-75.

Magavi, S. S., B. R. Leavitt and J. D. Macklis (2000). "Induction of neurogenesis in the neocortex of adult mice." Nature **405**(6789): 951-5.

Malatesta, P., M. A. Hack, E. Hartfuss, H. Kettenmann, W. Klinkert, F. Kirchhoff and M. Gotz (2003). "Neuronal or glial progeny: regional differences in radial glia fate." Neuron **37**(5): 751-64.

Malatesta, P., E. Hartfuss and M. Gotz (2000). "Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage." Development **127**(24): 5253-63.

Maragakis, N. J., M. S. Rao, J. Llado, V. Wong, H. Xue, A. Pardo, J. Herring, D. Kerr, C. Coccia and J. D. Rothstein (2005). "Glial restricted precursors protect against chronic glutamate neurotoxicity of motor neurons in vitro." Glia **50**(2): 145-59.

Marcal, N., H. Patel, Z. Dong, S. Belanger-Jasmin, B. Hoffman, C. D. Helgason, J. Dang and S. Stifani (2005). "Antagonistic effects of Grg6 and Groucho/TLE on the transcription repression activity of brain factor 1/FoxG1 and cortical neuron differentiation." Mol Cell Biol **25**(24): 10916-29.

Marquardt, T. and S. L. Pfaff (2001). "Cracking the transcriptional code for cell specification in the neural tube." Cell **106**(6): 651-4.

Martino, G. and S. Pluchino (2006). "The therapeutic potential of neural stem cells." Nat Rev Neurosci **7**(5): 395-406.

Mason, J. L., P. Ye, K. Suzuki, A. J. D'Ercole and G. K. Matsushima (2000). "Insulin-like growth factor-1 inhibits mature oligodendrocyte apoptosis during primary demyelination." J Neurosci **20**(15): 5703-8.

Mautes, A. E., M. Bergeron, F. R. Sharp, S. S. Panter, M. Weinzierl, K. Guenther and L. J. Noble (2000). "Sustained induction of heme oxygenase-1 in the traumatized spinal cord." Exp Neurol **166**(2): 254-65.

Mautes, A. E. and L. J. Noble (2000). "Co-induction of HSP70 and heme oxygenase-1 in macrophages and glia after spinal cord contusion in the rat." Brain Res **883**(2): 233-7.

McAdoo, D. J., G. Y. Xu, G. Robak and M. G. Hughes (1999). "Changes in amino acid concentrations over time and space around an impact injury and their diffusion through the rat spinal cord." Exp Neurol **159**(2): 538-44.

McDonald, J. W., X. Z. Liu, Y. Qu, S. Liu, S. K. Mickey, D. Turetsky, D. I. Gottlieb and D. W. Choi (1999). "Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord." Nat Med **5**(12): 1410-2.

McTigue, D. M., P. G. Popovich, L. B. Jakeman and B. T. Stokes (2000). "Strategies for spinal cord injury repair." Prog Brain Res **128**: 3-8.

McTigue, D. M., M. Tani, K. Krivacic, A. Chernosky, G. S. Kelner, D. Maciejewski, R. Maki, R. M. Ransohoff and B. T. Stokes (1998). "Selective chemokine mRNA accumulation in the rat spinal cord after contusion injury." J Neurosci Res **53**(3): 368-76.

McTigue, D. M., P. Wei and B. T. Stokes (2001). "Proliferation of NG2-positive cells and altered oligodendrocyte numbers in the contused rat spinal cord." J Neurosci **21**(10): 3392-400.

Meiners, S., E. M. Powell and H. M. Geller (1995). "A distinct subset of tenascin/CS-6-PG-rich astrocytes restricts neuronal growth in vitro." J Neurosci **15**(12): 8096-108.

Mitsui, T., J. S. Shumsky, A. C. Lepore, M. Murray and I. Fischer (2005). "Transplantation of neuronal and glial restricted precursors into contused spinal cord improves bladder and motor functions, decreases thermal hypersensitivity, and modifies intraspinal circuitry." J Neurosci **25**(42): 9624-36.

Miyata, T., A. Kawaguchi, H. Okano and M. Ogawa (2001). "Asymmetric inheritance of radial glial fibers by cortical neurons." Neuron **31**(5): 727-41.

Moon, L. D., R. A. Asher, K. E. Rhodes and J. W. Fawcett (2001). "Regeneration of CNS axons back to their target following treatment of adult rat brain with chondroitinase ABC." Nat Neurosci **4**(5): 465-6.

Moore, K. W., R. de Waal Malefyt, R. L. Coffman and A. O'Garra (2001). "Interleukin-10 and the interleukin-10 receptor." Annu Rev Immunol **19**: 683-765.

Morgenstern, D. A., R. A. Asher and J. W. Fawcett (2002). "Chondroitin sulphate proteoglycans in the CNS injury response." Prog Brain Res **137**: 313-32.

Myer, D. J., G. G. Gurkoff, S. M. Lee, D. A. Hovda and M. V. Sofroniew (2006). "Essential protective roles of reactive astrocytes in traumatic brain injury." Brain **129**(Pt 10): 2761-72.

Nakahara, S., K. Yone, T. Setoguchi, I. Yamaura, Y. Arishima, S. Yoshino and S. Komiya (2002). "Changes in nitric oxide and expression of nitric oxide synthase in spinal cord after acute traumatic injury in rats." J Neurotrauma **19**(11): 1467-74.

Nakahara, Y., F. H. Gage and M. H. Tuszynski (1996). "Grafts of fibroblasts genetically modified to secrete NGF, BDNF, NT-3, or basic FGF elicit differential responses in the adult spinal cord." Cell Transplant **5**(2): 191-204.

Nakajima, K., Y. Tohyama, S. Kohsaka and T. Kurihara (2001). "Ability of rat microglia to uptake extracellular glutamate." Neurosci Lett **307**(3): 171-4.

Nash, H. H., R. C. Borke and J. J. Anders (2002). "Ensheathing cells and methylprednisolone promote axonal regeneration and functional recovery in the lesioned adult rat spinal cord." J Neurosci **22**(16): 7111-20.

Nathan, C. F. (1987). "Secretory products of macrophages." J Clin Invest **79**(2): 319-26.

Nesic, O., N. M. Svrakic, G. Y. Xu, D. McAdoo, K. N. Westlund, C. E. Hulsebosch, Z. Ye, A. Galante, P. Soteropoulos, P. Tolias, W. Young, R. P. Hart and J. R. Perez-Polo (2002). "DNA microarray analysis of the contused spinal cord: effect of NMDA receptor inhibition." J Neurosci Res **68**(4): 406-23.

Nesic, O., G. Y. Xu, D. McAdoo, K. W. High, C. Hulsebosch and R. Perez-Pol (2001). "IL-1 receptor antagonist prevents apoptosis and caspase-3 activation after spinal cord injury." J Neurotrauma **18**(9): 947-56.

Nishiyama, A., A. Chang and B. D. Trapp (1999). "NG2+ glial cells: a novel glial cell population in the adult brain." J Neuropathol Exp Neurol **58**(11): 1113-24.

Nistor, G. I., M. O. Totoiu, N. Haque, M. K. Carpenter and H. S. Keirstead (2005). "Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation." Glia **49**(3): 385-96.

Noble, L. J. and J. R. Wrathall (1985). "Spinal cord contusion in the rat: morphometric analyses of alterations in the spinal cord." Exp Neurol **88**(1): 135-49.

Noctor, S. C., A. C. Flint, T. A. Weissman, R. S. Dammerman and A. R. Kriegstein (2001). "Neurons derived from radial glial cells establish radial units in neocortex." Nature **409**(6821): 714-20.

Noctor, S. C., A. C. Flint, T. A. Weissman, W. S. Wong, B. K. Clinton and A. R. Kriegstein (2002). "Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia." J Neurosci **22**(8): 3161-73.

Noctor, S. C., V. Martinez-Cerdeno, L. Ivic and A. R. Kriegstein (2004). "Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases." Nat Neurosci **7**(2): 136-44.

Noctor, S. C., S. L. Palmer, T. Hasling and S. L. Juliano (1999). "Interference with the development of early generated neocortex results in disruption of radial glia and abnormal formation of neocortical layers." Cereb Cortex **9**(2): 121-36.

Ogawa, Y., K. Sawamoto, T. Miyata, S. Miyao, M. Watanabe, M. Nakamura, B. S. Bregman, M. Koike, Y. Uchiyama, Y. Toyama and H. Okano (2002). "Transplantation of in vitro-expanded fetal neural progenitor cells results in neurogenesis and functional recovery after spinal cord contusion injury in adult rats." J Neurosci Res **69**(6): 925-33.

Ohori, Y., S. Yamamoto, M. Nagao, M. Sugimori, N. Yamamoto, K. Nakamura and M. Nakafuku (2006). "Growth factor treatment and genetic manipulation stimulate

neurogenesis and oligodendrogenesis by endogenous neural progenitors in the injured adult spinal cord." J Neurosci **26**(46): 11948-60.

Okano, H., Y. Ogawa, M. Nakamura, S. Kaneko, A. Iwanami and Y. Toyama (2003). "Transplantation of neural stem cells into the spinal cord after injury." Semin Cell Dev Biol **14**(3): 191-8.

Palmer, T. D., E. A. Markakis, A. R. Willhoite, F. Safar and F. H. Gage (1999). "Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS." J Neurosci **19**(19): 8487-97.

Pan, J. Z., R. Jornsten and R. P. Hart (2004). "Screening anti-inflammatory compounds in injured spinal cord with microarrays: a comparison of bioinformatics analysis approaches." Physiol Genomics **17**(2): 201-14.

Pan, J. Z., L. Ni, A. Sodhi, A. Aguanno, W. Young and R. P. Hart (2002). "Cytokine activity contributes to induction of inflammatory cytokine mRNAs in spinal cord following contusion." J Neurosci Res **68**(3): 315-22.

Parsell, D. A. and S. Lindquist (1993). "The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins." Annu Rev Genet **27**: 437-96.

Patten, B. A., J. M. Peyrin, G. Weinmaster and G. Corfas (2003). "Sequential signaling through Notch1 and erbB receptors mediates radial glia differentiation." J Neurosci **23**(14): 6132-40.

Pearse, D. D., A. E. Marcillo, M. Oudega, M. P. Lynch, P. M. Wood and M. B. Bunge (2004). "Transplantation of Schwann cells and olfactory ensheathing glia after spinal cord injury: does pretreatment with methylprednisolone and interleukin-10 enhance recovery?" J Neurotrauma **21**(9): 1223-39.

Pearse, D. D., F. C. Pereira, A. E. Marcillo, M. L. Bates, Y. A. Berrocal, M. T. Filbin and M. B. Bunge (2004). "cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury." Nat Med **10**(6): 610-6.

Persson, M., M. Sandberg, E. Hansson and L. Ronnback (2006). "Microglial glutamate uptake is coupled to glutathione synthesis and glutamate release." Eur J Neurosci **24**(4): 1063-70.

Pineau, I. and S. Lacroix (2007). "Proinflammatory cytokine synthesis in the injured mouse spinal cord: Multiphasic expression pattern and identification of the cell types involved." J Comp Neurol **500**(2): 267-85.

Plant, G. W., C. L. Christensen, M. Oudega and M. B. Bunge (2003). "Delayed transplantation of olfactory ensheathing glia promotes sparing/regeneration of supraspinal axons in the contused adult rat spinal cord." J Neurotrauma **20**(1): 1-16.

Pluchino, S., L. Zanotti, B. Rossi, E. Brambilla, L. Ottoboni, G. Salani, M. Martinello, A. Cattalini, A. Bergami, R. Furlan, G. Comi, G. Constantin and G. Martino (2005). "Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism." Nature **436**(7048): 266-71.

Plunkett, J. A., C. G. Yu, J. M. Easton, J. R. Bethea and R. P. Yezierski (2001). "Effects of interleukin-10 (IL-10) on pain behavior and gene expression following excitotoxic spinal cord injury in the rat." Exp Neurol **168**(1): 144-54.

Popovich, P. G., P. J. Horner, B. B. Mullin and B. T. Stokes (1996). "A quantitative spatial analysis of the blood-spinal cord barrier. I. Permeability changes after experimental spinal contusion injury." Exp Neurol **142**(2): 258-75.

Popovich, P. G. and T. B. Jones (2003). "Manipulating neuroinflammatory reactions in the injured spinal cord: back to basics." Trends Pharmacol Sci **24**(1): 13-7.

Popovich, P. G., P. Wei and B. T. Stokes (1997). "Cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats." J Comp Neurol **377**(3): 443-64.

Properzi, F., R. A. Asher and J. W. Fawcett (2003). "Chondroitin sulphate proteoglycans in the central nervous system: changes and synthesis after injury." Biochem Soc Trans **31**(2): 335-6.

Properzi, F., D. Carulli, R. A. Asher, E. Muir, L. M. Camargo, T. H. van Kuppevelt, G. B. ten Dam, Y. Furukawa, T. Mikami, K. Sugahara, T. Toida, H. M. Geller and J. W. Fawcett (2005). "Chondroitin 6-sulphate synthesis is up-regulated in injured CNS, induced by injury-related cytokines and enhanced in axon-growth inhibitory glia." Eur J Neurosci **21**(2): 378-90.

Rakic, P. (1971). "Guidance of neurons migrating to the fetal monkey neocortex." Brain Res **33**(2): 471-6.

Rakic, P. (1971). "Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A Golgi and electronmicroscopic study in Macacus Rhesus." J Comp Neurol **141**(3): 283-312.

Rakic, P. (1972). "Mode of cell migration to the superficial layers of fetal monkey neocortex." J Comp Neurol **145**(1): 61-83.

Rakic, P. (1978). "Neuronal migration and contact guidance in the primate telencephalon." Postgrad Med J **54 Suppl 1**: 25-40.

Rakic, P. (2003). "Elusive radial glial cells: historical and evolutionary perspective." Glia **43**(1): 19-32.

Reier, P. J., B. S. Bregman and J. R. Wujek (1986). "Intraspinal transplantation of embryonic spinal cord tissue in neonatal and adult rats." J Comp Neurol **247**(3): 275-96.

Resnick, D. K., S. H. Graham, C. E. Dixon and D. W. Marion (1998). "Role of cyclooxygenase 2 in acute spinal cord injury." J Neurotrauma **15**(12): 1005-13.

Reya, T., R. Okamura and R. Grosschedl (1999). "Control of lymphocyte differentiation by the LEF-1/TCF family of transcription factors." Cold Spring Harb Symp Quant Biol **64**: 133-40.

Reya, T., M. O'Riordan, R. Okamura, E. Devaney, K. Willert, R. Nusse and R. Grosschedl (2000). "Wnt signaling regulates B lymphocyte proliferation through a LEF-1 dependent mechanism." Immunity **13**(1): 15-24.

Robinson, S. R. and Z. Dreher (1989). "Evidence for three morphological classes of astrocyte in the adult rabbit retina: functional and developmental implications." Neurosci Lett **106**(3): 261-8.

Robinson, S. R. and Z. Dreher (1990). "Muller cells in adult rabbit retinae: morphology, distribution and implications for function and development." J Comp Neurol **292**(2): 178-92.

Rosenberg, L. J. and J. R. Wrathall (1997). "Quantitative analysis of acute axonal pathology in experimental spinal cord contusion." J Neurotrauma **14**(11): 823-38.

Rothstein, J. D., M. Dykes-Hoberg, C. A. Pardo, L. A. Bristol, L. Jin, R. W. Kuncl, Y. Kanai, M. A. Hediger, Y. Wang, J. P. Schielke and D. F. Welty (1996). "Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate." Neuron **16**(3): 675-86.

Sanai, N., A. D. Tramontin, A. Quinones-Hinojosa, N. M. Barbaro, N. Gupta, S. Kunwar, M. T. Lawton, M. W. McDermott, A. T. Parsa, J. Manuel-Garcia Verdugo, M. S. Berger and A. Alvarez-Buylla (2004). "Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration." Nature **427**(6976): 740-4.

Sancho-Tello, M., S. Valles, C. Montoliu, J. Renau-Piqueras and C. Guerri (1995). "Developmental pattern of GFAP and vimentin gene expression in rat brain and in radial glial cultures." Glia **15**(2): 157-66.

Schindler, C. and J. E. Darnell, Jr. (1995). "Transcriptional responses to polypeptide ligands: the JAK-STAT pathway." Annu Rev Biochem **64**: 621-51.

Schmalfeldt, M., C. E. Bandtlow, M. T. Dours-Zimmermann, K. H. Winterhalter and D. R. Zimmermann (2000). "Brain derived versican V2 is a potent inhibitor of axonal growth." J Cell Sci **113 (Pt 5)**: 807-16.

Schmid, R. S., R. Jo, S. Shelton, J. A. Kreidberg and E. S. Anton (2005). "Reelin, integrin and DAB1 interactions during embryonic cerebral cortical development." Cereb Cortex **15**(10): 1632-6.

Seoane, J., H. V. Le, L. Shen, S. A. Anderson and J. Massague (2004). "Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation." Cell **117**(2): 211-23.

Sharp, F. R., S. M. Massa and R. A. Swanson (1999). "Heat-shock protein protection." Trends Neurosci **22**(3): 97-9.

Shibata, T., K. Yamada, M. Watanabe, K. Ikenaka, K. Wada, K. Tanaka and Y. Inoue (1997). "Glutamate transporter GLAST is expressed in the radial glia-astrocyte lineage of developing mouse spinal cord." J Neurosci **17**(23): 9212-9.

Shimamura, K., D. J. Hartigan, S. Martinez, L. Puelles and J. L. Rubenstein (1995). "Longitudinal organization of the anterior neural plate and neural tube." Development **121**(12): 3923-33.

Shuman, S. L., J. C. Bresnahan and M. S. Beattie (1997). "Apoptosis of microglia and oligodendrocytes after spinal cord contusion in rats." J Neurosci Res **50**(5): 798-808.

Sidman, R. L. and P. Rakic (1973). "Neuronal migration, with special reference to developing human brain: a review." Brain Res **62**(1): 1-35.

Silberstein, F. C., R. De Simone, G. Levi and F. Aloisi (1996). "Cytokine-regulated expression of platelet-derived growth factor gene and protein in cultured human astrocytes." J Neurochem **66**(4): 1409-17.

Silver, J. and J. H. Miller (2004). "Regeneration beyond the glial scar." Nat Rev Neurosci **5**(2): 146-56.

Smith, G. M. and R. H. Miller (1991). "Immature type-1 astrocytes suppress glial scar formation, are motile and interact with blood vessels." Brain Res **543**(1): 111-22.

Smith, G. M. and J. Silver (1988). "Transplantation of immature and mature astrocytes and their effect on scar formation in the lesioned central nervous system." Prog Brain Res **78**: 353-61.

Sonnewald, U., N. Westergaard and A. Schousboe (1997). "Glutamate transport and metabolism in astrocytes." Glia **21**(1): 56-63.

Soriano, E. and J. A. Del Rio (2005). "The cells of cajal-retzius: still a mystery one century after." Neuron **46**(3): 389-94.

Sottile, V., M. Li and P. J. Scotting (2006). "Stem cell marker expression in the Bergmann glia population of the adult mouse brain." Brain Res **1099**(1): 8-17.

Sroga, J. M., T. B. Jones, K. A. Kigerl, V. M. McGaughy and P. G. Popovich (2003). "Rats and mice exhibit distinct inflammatory reactions after spinal cord injury." J Comp Neurol **462**(2): 223-40.

Stoykova, A., M. Gotz, P. Gruss and J. Price (1997). "Pax6-dependent regulation of adhesive patterning, R-cadherin expression and boundary formation in developing forebrain." Development **124**(19): 3765-77.

Stoykova, A., O. Hatano, P. Gruss and M. Gotz (2003). "Increase in reelin-positive cells in the marginal zone of Pax6 mutant mouse cortex." Cereb Cortex **13**(6): 560-71.

Sugahara, K., T. Mikami, T. Uyama, S. Mizuguchi, K. Nomura and H. Kitagawa (2003). "Recent advances in the structural biology of chondroitin sulfate and dermatan sulfate." Curr Opin Struct Biol **13**(5): 612-20.

Taga, T. and S. Fukuda (2005). "Role of IL-6 in the neural stem cell differentiation." Clin Rev Allergy Immunol **28**(3): 249-56.

Takami, T., M. Oudega, M. L. Bates, P. M. Wood, N. Kleitman and M. B. Bunge (2002). "Schwann cell but not olfactory ensheathing glia transplants improve hindlimb locomotor performance in the moderately contused adult rat thoracic spinal cord." J Neurosci **22**(15): 6670-81.

Talbott, J. F., D. N. Loy, Y. Liu, M. S. Qiu, M. B. Bunge, M. S. Rao and S. R. Whittemore (2005). "Endogenous Nkx2.2+/Olig2+ oligodendrocyte precursor cells fail to remyelinate the demyelinated adult rat spinal cord in the absence of astrocytes." Exp Neurol **192**(1): 11-24.

Tan, A. M., M. Colletti, A. T. Rorai, J. H. Skene and J. M. Levine (2006). "Antibodies against the NG2 proteoglycan promote the regeneration of sensory axons within the dorsal columns of the spinal cord." J Neurosci **26**(18): 4729-39.

Tan, A. M., W. Zhang and J. M. Levine (2005). "NG2: a component of the glial scar that inhibits axon growth." J Anat **207**(6): 717-25.

Tanabe, K., I. Bonilla, J. A. Winkles and S. M. Strittmatter (2003). "Fibroblast growth factor-inducible-14 is induced in axotomized neurons and promotes neurite outgrowth." J Neurosci **23**(29): 9675-86.

Tang, X., J. E. Davies and S. J. Davies (2003). "Changes in distribution, cell associations, and protein expression levels of NG2, neurocan, phosphacan, brevican, versican V2, and tenascin-C during acute to chronic maturation of spinal cord scar tissue." J Neurosci Res **71**(3): 427-44.

Tao, W. and E. Lai (1992). "Telencephalon-restricted expression of BF-1, a new member of the HNF-3/fork head gene family, in the developing rat brain." Neuron **8**(5): 957-66.

Taoka, Y., K. Okajima, M. Uchiba, K. Murakami, S. Kushimoto, M. Johno, M. Naruo, H. Okabe and K. Takatsuki (1997). "Role of neutrophils in spinal cord injury in the rat." Neuroscience **79**(4): 1177-82.

Teng, Y. D., H. Choi, R. C. Onario, S. Zhu, F. C. Desilets, S. Lan, E. J. Woodard, E. Y. Snyder, M. E. Eichler and R. M. Friedlander (2004). "Minocycline inhibits contusion-triggered mitochondrial cytochrome c release and mitigates functional deficits after spinal cord injury." Proc Natl Acad Sci U S A **101**(9): 3071-6.

Thuret, S., L. D. Moon and F. H. Gage (2006). "Therapeutic interventions after spinal cord injury." Nat Rev Neurosci **7**(8): 628-43.

Tobias, C. A., J. S. Shumsky, M. Shibata, M. H. Tuszynski, I. Fischer, A. Tessler and M. Murray (2003). "Delayed grafting of BDNF and NT-3 producing fibroblasts into the injured spinal cord stimulates sprouting, partially rescues axotomized red nucleus neurons from loss and atrophy, and provides limited regeneration." Exp Neurol **184**(1): 97-113.

Tuszynski, M. H., R. Grill, L. L. Jones, A. Brant, A. Blesch, K. Low, S. Lacroix and P. Lu (2003). "NT-3 gene delivery elicits growth of chronically injured corticospinal axons and modestly improves functional deficits after chronic scar resection." Exp Neurol **181**(1): 47-56.

Tuszynski, M. H., K. Murai, A. Blesch, R. Grill and I. Miller (1997). "Functional characterization of NGF-secreting cell grafts to the acutely injured spinal cord." Cell Transplant **6**(3): 361-8.

Ughrin, Y. M., Z. J. Chen and J. M. Levine (2003). "Multiple regions of the NG2 proteoglycan inhibit neurite growth and induce growth cone collapse." J Neurosci **23**(1): 175-86.

Uwanogho, D., M. Rex, E. J. Cartwright, G. Pearl, C. Healy, P. J. Scotting and P. T. Sharpe (1995). "Embryonic expression of the chicken Sox2, Sox3 and Sox11 genes suggests an interactive role in neuronal development." Mech Dev **49**(1-2): 23-36.

van Genderen, C., R. M. Okamura, I. Farinas, R. G. Quo, T. G. Parslow, L. Bruhn and R. Grosschedl (1994). "Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice." Genes Dev **8**(22): 2691-703.

van Landeghem, F. K., J. F. Stover, I. Bechmann, W. Bruck, A. Unterberg, C. Buhrer and A. von Deimling (2001). "Early expression of glutamate transporter proteins in ramified microglia after controlled cortical impact injury in the rat." Glia **35**(3): 167-79.

Velardo, M. J., C. Burger, P. R. Williams, H. V. Baker, M. C. Lopez, T. H. Mareci, T. E. White, N. Muzyczka and P. J. Reier (2004). "Patterns of gene expression reveal a temporally orchestrated wound healing response in the injured spinal cord." J Neurosci **24**(39): 8562-76.

Vera-Portocarrero, L. P., C. D. Mills, Z. Ye, S. D. Fullwood, D. J. McAdoo, C. E. Hulsebosch and K. N. Westlund (2002). "Rapid changes in expression of glutamate transporters after spinal cord injury." Brain Res **927**(1): 104-10.

Villa, A., E. Y. Snyder, A. Vescovi and A. Martinez-Serrano (2000). "Establishment and properties of a growth factor-dependent, perpetual neural stem cell line from the human CNS." Exp Neurol **161**(1): 67-84.

Voigt, T. (1989). "Development of glial cells in the cerebral wall of ferrets: direct tracing of their transformation from radial glia into astrocytes." J Comp Neurol **289**(1): 74-88.

Watanabe, M., K. Tsutsui and Y. Inoue (1994). "Differential expressions of the topoisomerase II alpha and II beta mRNAs in developing rat brain." Neurosci Res **19**(1): 51-7.

Weiss, K. H., C. Johanssen, A. Tielsch, J. Herz, T. Deller, M. Frotscher and E. Forster (2003). "Malformation of the radial glial scaffold in the dentate gyrus of reeler mice, scrambler mice, and ApoER2/VLDLR-deficient mice." J Comp Neurol **460**(1): 56-65.

Widenfalk, J., K. Lundstromer, M. Jubran, S. Brene and L. Olson (2001). "Neurotrophic factors and receptors in the immature and adult spinal cord after mechanical injury or kainic acid." J Neurosci **21**(10): 3457-75.

Wu, D., O. Miyamoto, S. Shibuya, M. Okada, H. Igawa, N. A. Janjua, H. Norimatsu and T. Itano (2005). "Different expression of macrophages and microglia in rat spinal cord contusion injury model at morphological and regional levels." Acta Med Okayama **59**(4): 121-7.

Wu, L. and A. Strasser (2001). ""Decisions, decisions.": beta-catenin-mediated activation of TCF-1 and Lef-1 influences the fate of developing T cells." Nat Immunol **2**(9): 823-4.

Xuan, S., C. A. Baptista, G. Balas, W. Tao, V. C. Soares and E. Lai (1995). "Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres." Neuron **14**(6): 1141-52.

Yao, J., E. Lai and S. Stifani (2001). "The winged-helix protein brain factor 1 interacts with groucho and hes proteins to repress transcription." Mol Cell Biol **21**(6): 1962-72.

Yenari, M. A. (2002). "Heat shock proteins and neuroprotection." Adv Exp Med Biol **513**: 281-99.

Yick, L. W., P. T. Cheung, K. F. So and W. Wu (2003). "Axonal regeneration of Clarke's neurons beyond the spinal cord injury scar after treatment with chondroitinase ABC." Exp Neurol **182**(1): 160-8.

Yick, L. W., K. F. So, P. T. Cheung and W. T. Wu (2004). "Lithium chloride reinforces the regeneration-promoting effect of chondroitinase ABC on rubrospinal neurons after spinal cord injury." J Neurotrauma **21**(7): 932-43.

Yick, L. W., W. Wu, K. F. So, H. K. Yip and D. K. Shum (2000). "Chondroitinase ABC promotes axonal regeneration of Clarke's neurons after spinal cord injury." Neuroreport **11**(5): 1063-7.

Young, W. (2002). "Spinal cord contusion models." Prog Brain Res **137**: 231-55.

Young, W. and M. B. Bracken (1992). "The Second National Acute Spinal Cord Injury Study." J Neurotrauma **9 Suppl 1**: S397-405.

Zai, L. J. and J. R. Wrathall (2005). "Cell proliferation and replacement following contusive spinal cord injury." Glia **50**(3): 247-57.

Zhang, Z., C. J. Krebs and L. Guth (1997). "Experimental analysis of progressive necrosis after spinal cord trauma in the rat: etiological role of the inflammatory response." Exp Neurol **143**(1): 141-52.

Zheng, C., N. Heintz and M. E. Hatten (1996). "CNS gene encoding astrotactin, which supports neuronal migration along glial fibers." Science **272**(5260): 417-9.

Zhou, Q., G. Choi and D. J. Anderson (2001). "The bHLH transcription factor Olig2 promotes oligodendrocyte differentiation in collaboration with Nkx2.2." Neuron **31**(5): 791-807.

Zhu, Y., C. Culmsee, S. Klumpp and J. Krieglstein (2004). "Neuroprotection by transforming growth factor-beta1 involves activation of nuclear factor-kappaB through phosphatidylinositol-3-OH kinase/Akt and mitogen-activated protein kinase-extracellular-signal regulated kinase1,2 signaling pathways." Neuroscience **123**(4): 897-906.

Zhu, Y., G. Y. Yang, B. Ahlemeyer, L. Pang, X. M. Che, C. Culmsee, S. Klumpp and J. Krieglstein (2002). "Transforming growth factor-beta 1 increases bad phosphorylation and protects neurons against damage." J Neurosci **22**(10): 3898-909.

Curriculum Vita

Yu-Wen Chang

Education

1997 B.S. Medical Technology, Chung Shan Medical University, Taichung, Taiwan
2000 M.S. Neuroscience, National Yang Ming University, Taipei, Taiwan
2007 Ph.D. Neuroscience, Rutgers University, New Jersey, U.S.A.

Experience

1998-2001 Graduate assistant, Institute of Neuroscience, National Yang Ming University
2002 Graduate fellow, Neuroscience program, Rutgers University
2003-2004 Graduate assistant, W. M. Keck center for Neuroscience, Rutgers University
2004-2005 Graduate fellow, New Jersey Commission on Spinal Cord Research
2006-2007 Graduate assistant, W. M. Keck center for Neuroscience, Rutgers University

Publication

Hasegawa K, Chang YW (co-first author), Li H, Ikeda O, Kane-Glodsmith N, Grumet M Embryonic radial glia bridge spinal cord lesions, protect tissue architecture, and promote functional recovery following spinal cord injury. Exp Neurol. 2005 Jun; 193 (2):394-410