The Tumor-Suppressive Properties of Neural Stem Cells

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THE TUMOR-SUPPRESSIVE PROPERTIES OF NEURAL STEM CELLS

BY

Ryan Passino

THESIS

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The Tumor-Suppressive Properties of Neural Stem Cells

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ABSTRACT

THE TUMOR-SUPPRESSIVE PROPERTIES OF NEURAL STEM CELLS

By

Ryan Passino

Glioblastoma multiforme (GBM) is an aggressive central nervous system malignancy that commonly causes immune suppression in patients to avoid immune recognition and clearance. This complicates treatment options and limits the effectiveness of immunotherapy strategies. Interestingly, GBM formation can stimulate the neurogenic subventricular zone of the cerebral cortex and causes the proliferation and migration of neural stem cells (NSCs) towards the tumor. This migration reflects the NSC wound repair response following CNS injury. Studies using NSCs surgically implanted into GBM tumors showed decreased tumor growth and increased animal survival in mice; however, the mechanisms underlying these anti-tumor properties of NSCs are unknown. Here we performed co-culture proliferation assays in combination with gene expression analysis to show that there is a two-way communication between the NSCs and glioma cells that results in decreased glioma proliferation and changes to survival and apoptotic-related gene expression. These changes correlated with increased expression of tumor necrosis factor (TNF) superfamily death receptor ligands by the NSCs. NSCs also were found to express many immune-related cytokines and chemokines involved in immune signaling, suggesting a potential role in mediating anti-tumor immune responses. These results provide the first mechanistic evidence of NSC-mediated tumor suppression.
# TABLE OF CONTENTS

List of Figures ........................................................................................................................................... v
List of Tables ........................................................................................................................................... vi
Literature Review and Background ........................................................................................................... 1
  Introduction ........................................................................................................................................... 1
  Neural stem cells ................................................................................................................................ 1
  NSC Migration ...................................................................................................................................... 3
  Tumor Suppression ............................................................................................................................... 6
  Mechanisms of Suppression .................................................................................................................. 8
  Tumor Immunology .............................................................................................................................. 14
  Summary ............................................................................................................................................... 19
Objectives and Hypotheses ....................................................................................................................... 21
Methods .................................................................................................................................................. 23
  Cell Culture ......................................................................................................................................... 23
  Proliferation Assay .............................................................................................................................. 24
  Real-time PCR ..................................................................................................................................... 26
Results .................................................................................................................................................... 28
  NSCs suppress GL261 proliferation under normoxic but not hypoxic conditions .................................... 28
  Suppressive mechanisms by gene expression analysis ........................................................................ 31
  NSCs up-regulate tumor necrosis factors ............................................................................................. 34
  NSCs and immune signaling .................................................................................................................. 36
Discussion ............................................................................................................................................... 40
Mode and Mechanism.................................................................41

GL261 suppression by NSCs..........................................................41

Hypoxia promotes GL261 survival..................................................46

NSCs in immune signaling and activation.......................................49

Supplemental materials..................................................................52
LIST OF FIGURES

Figure 1: GL261 Proliferation (Normoxic Co-culture).................................................................30
Figure 2: GL261 Proliferation (Hypoxic Co-culture).................................................................30
Figure 3: GL261 Proliferation (Normoxic/hypoxic).................................................................31
Figure 4: Normalized GL261 gene expression (apoptosis/survival)........................................32
Figure 5: Normalized NSC gene expression (TNFsf ligands).................................................35
Figure 6: Normalized NSC gene expression).............................................................................37
Figure 7: Normalized NSC gene expression (immune-related).................................................39
Figure 8: Model for intercellular interactions.............................................................................52
Supplemental Figure 1: Preliminary proliferation conditions).................................................53
Supplemental Figure 2: Alamarblue preliminary time testing)...............................................53
LIST OF TABLES

Table 1: GL261 gene expression- NMX co-culture (NMX).................................32
Table 2: GL261 gene expression- HPX co-culture (HPX)................................32
Table 3: GL261 gene expression- HPX (NMX)................................................34
Table 4: NSC gene expression- NMX co-culture (NMX).................................35
Table 5: NSC gene expression- HPX co-culture (HPX)................................35
Table 6: NSC gene expression- NMX co-culture (NMX).................................37
Table 7: NSC gene expression- HPX co-culture (HPX)................................38
Table 8: NSC gene expression- NMX co-culture (immune-related)...............39
Table 9: NSC gene expression- HPX co-culture (immune-related)...............39
Supplemental table 1: List of target genes)..................................................53
LITERATURE REVIEW AND BACKGROUND

INTRODUCTION

Glioblastoma multiforme (GBM) is classified as a grade IV astrocytoma, the most aggressive form of brain tumor in human with a poor prognosis due to the high probability of recurrence and few effective therapeutic treatments available. Standard treatment consisting of surgery, radiation, and chemotherapy results in a median survival of less than one year (Fine et al., 1993). The aggressiveness of GBMs is attributed to many mechanisms including increased metastasis, resistance to chemo and radiotherapy, and immune suppression. New treatment options are desperately needed to improve patient outcomes and recent attention to the role of endogenous neural stem cells as potential mediators of wound repair suggests that the CNS might have intrinsic abilities to suppress tumor growth. However, the mechanisms behind NSC-mediated tumor suppression are unclear at this time and require further investigation.

NEURAL STEM CELLS

Although it had previously been thought that the adult brain was incapable of generating new neurons, it is now accepted that neural stem cells (NSCs) continue to exist within the adult brain for cell replacement throughout life (Ming and Song, 2005). Reynolds and Weiss were the first to demonstrate the neurogenic capability of the postnatal mouse brain by isolating NSCs from the striatum and expanding them in cultures containing epidermal growth factor (EGF) and fibroblast growth
factor (FGF). These cells were proliferative, self-renewing and expressed the
intermediate filament marker nestin that is commonly expressed by neuroepithelial
cells. These NSCs were also multipotent and able to generate new neurons and glial
cells in vitro (Reynolds and Weiss, 1992). Further research has recognized that NSC
populations reside in two main neurogenic regions of the brain - the subventricular
zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the
hippocampal dentate gyrus (Lois and Alvarez-Buylla, 1994) (Palmer et al., 1997).
NSCs in the SVZ are located adjacent to the ependymal cells that line the lateral
ventricles. These self-renewing NSCs (type B) are the primary neural precursors
that give rise to transit-amplifying progenitor cells (type C), which further divide to
generate immature neuroblasts (type A) (Doetsch et al., 1999). These neuroblasts
migrate via the rostral migratory stream towards the olfactory bulb, where they
continuously differentiate and replace granule and periglomerular interneurons
that participate in these olfactory networks (Lois and Alvarez-Buylla, 1994). Adult
neurons produced in the SGZ integrate directly into the granule layer of the dentate
gyrus and facilitate long-term potentiation and the stimulation of learning and
memory (Imayoshi et al., 2008). NSCs also participate in wound repair following
brain insults such as trauma or disease. Following injury, the neurogenic regions are
stimulated, resulting in NSC proliferation and redirected migration towards the
damaged areas where they promote neurogenesis (Kernie and Parent, 2010) (Dash
et al., 2001). Similar patterns of wound repair occur in models of traumatic brain
injury and stroke, as well as neurological conditions such as multiple sclerosis.
Multiple studies are now examining the role of NSCs during wound repair within the CNS and their potential use in cell replacement therapies.

**NEURAL STEM CELL MIGRATION**

Interestingly, glioblastoma models in mice suggest that GBM tumors also stimulate NSC activity within the SVZ and show that NSCs are diverted from their normal migratory path and exhibit strong tropism for the tumor. Aboody et al. first reported on the strong tropic capacity of NSCs for intracranial tumors by demonstrating that NSCs implanted at distant locations in the mouse brain could preferentially target the tumor mass located in the frontal lobe. LacZ-expressing NSCs implanted into either the contralateral hemisphere or the cerebral ventricles were observed migrating through normal tissue and dispersing throughout the tumor. NSCs also ‘tracked down’ individual glioma cells that had infiltrated beyond the edges of the tumor mass and into normal tissue. Surprisingly, NSCs could even target the tumor from within systemic circulation. When injected into circulation through the tail vein, the labeled NSCs were found distributed throughout the intracerebral tumor mass after four days (Aboody et al., 2000).

Work by Glass et al. further supports the idea that NSCs have tumor tropic capabilities and also potential tumor-suppressive properties. Using transgenic mice expressing green fluorescent protein labeled nestin (GFP-nestin) along with DsRed-labeled GL261 GBM cells to distinguish between nestin-positive NSCs and the cancer cells, they first demonstrated that endogenous NSCs could migrate from the SVZ to
the tumors. Nestin is a specific cell marker used for labeling NSCs and is primarily expressed within the neurogenic regions of the brain. Furthermore, the fluorescent dye Dil was also administered into the ventricle to ensure that nestin-positive cells arriving at the tumor only originated from the SVZ. Two weeks post-operation, the tumor was completely surrounded by a dense layer of Dil/Nestin-positive cells, and many close interactions between the NSCs and the glioma cells were observed (Glass et al., 2005). In support of this data, Ehtesham et al. showed that NSC tropism for glioblastomas is mediated by CXCR4/SDF-1 and many additional factors produced by the GBM, including FGF and vascular endothelial growth factor (VEGF) (Ehtesham et al., 2004). Along with chemotactic proteins and growth factors, cancerous cells themselves appear to induce NSC migration. When glioma cells were fractionated to test their chemotactic properties, the cellular membrane induced stem cell migration at levels comparable to FGF and serum (Spaeth et al., 2008). Taken together, this work strongly supports the idea that the endogenous NSCs possess the ability to respond and move toward GBM tumors 	extit{in vivo}.

NSC migration towards the tumor appears to reflect a wound repair response that may be influenced by the conditions that arise from tumor formation, including inflammation, hypoxia, and tissue necrosis. The tumor microenvironment experiences reduced oxygen concentrations (hypoxia) as a result of the rapid proliferation rate of cancer cells leading to insufficient blood and oxygen supply. This hypoxia regulates the expression of hypoxia-inducible factors (HIF)- dimeric transcription factors comprised of the hypoxic response factor HIF-1α and the
constitutively expressed aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-1 is regulated by a prolyl hydroxylase domain (PHD), which under normoxic conditions uses oxygen to selectively hydroxylate the proline residues of HIF-1. This form of HIF-1 can interact with Von Hippel-Lindau (VHL) protein, which causes HIF-1 to become ubiquitylilated and targeted for proteosomal degradation. Under hypoxic conditions, HIF is stabilized and can up-regulate the transcription of target genes including VEGF for angiogenesis, lactate dehydrogenase-A (LDH-A) for increasing glycolysis, and glucose transporter-1,3 (GLUT1, GLUT3) for increased glucose uptake (Harris, 2002).

NSC maintenance and migration are also influenced by hypoxia-inducible factors. The hypoxic stem cell niche enhances survival and proliferation of NSCs along with maintaining multipotency through HIF-2α regulation of Oct4 (Covello et al., 2006; Pistollato et al., 2007) (Mohyeldin et al., 2010). CXCR4 expression is increased after exposing NSCs to hypoxia (Zhao et al., 2008) and CXCR4 up-regulation correlates with enhanced migration to glioma-conditioned media and also to ischemic areas in vivo, where stromal cell-derived factor (SDF-1) is increased by HIF-1 overexpression (Ceradini et al., 2004). Knocking down HIF-1 with interfering RNA blocked the hypoxia-induced migration of NSCs (Zhao et al., 2008).

Along with inducing the migration of NSCs through hypoxia-inducible factors and chemotactic receptors, the hypoxic tumor microenvironment also causes cellular necrosis and the release of damage-associated molecular patterns (DAMPs) that act
as danger signals and have chemotactic properties on stem cells and immune cells to promote an immune response. One of the more important DAMPs released from necrotic tissue is the high-mobility group box-1 protein (HMGB1). HMGB1 is a non-histone DNA-binding protein normally located within the nucleus. HMGB1 can be released from cells stimulated with bacterial endotoxin and is a ligand for the receptor for advanced glycation end products (RAGE) (Taguchi et al., 2000) (Wang et al., 1999). Release of HMGB1 from necrotic cells stimulates the proliferation and migration of stem cells (Pistoia and Raffaghello, 2011) (Lotfi et al., 2011; Meng et al., 2008). These functions are lost upon HMGB1 oxidation, although it is thought that the hypoxic conditions of the tumor microenvironment preserve its functionality (Lotfi et al., 2011). Along with signaling injury, HMGB1 also serves as a tumor antigen that mediates antitumor immune responses, as discussed later.

**TUMOR SUPPRESSION**

The discovery that NSCs migrate strongly towards intracranial tumors suggests a new strategy for delivering anti-tumor drugs with genetically modified NSCs. Benedetti et al. isolated neural precursors from C57BL6J mice and overexpressed immune-related interleukin-4 (IL-4) cDNA or lysosomal enzyme galactocerebrosidase (GALC) as a control. IL-4 was used for its reportedly strong antitumor and dendritic cell induction effect (Benedetti et al., 1999) (Sallusto and Lanzavecchia, 1994). Mice receiving IL4-NSCs along with GL261 glioma cell injection showed a significant increase in survival compared to control mice not receiving NSCs. Interestingly, mice receiving unmodified control NSCs co-injected
with GL261 cells also experienced a significant increase in survival and a reduction in tumor size relative to controls. It was then concluded that NSCs could possess intrinsic antitumor properties and might release factors that can suppress tumor cell growth (Benedetti et al., 2000) (Noble, 2000). NSCs respond to the tumor microenvironment by proliferating and expressing high levels of the nuclear protein Ki-67, which is associated with cell cycle progression and proliferation of NSCs (Glass et al., 2005). This response does not require direct contact between cells as it was shown by Venugopal et al. that NSCs treated with GBM-conditioned media in vitro were transiently transformed, resulting in larger neurosphere formation, increased cell proliferation and increased self-renewal capacity (Venugopal et al., 2012).

An important study by Glass et al. demonstrated that the association of NSCs with glioblastomas results in reduced tumor size and increased survival in mouse models. These antitumor effects were first studied in vitro using co-culture assays and they found that NSCs decreased GBM proliferation rates and strongly induced GBM apoptosis (Glass et al., 2005). They also found a correlation between mouse age and tumor aggression. It was observed that younger mice had significantly higher survival rates than older mice. These results corresponded with data showing decreasing levels of nestin-positive cells surrounding the tumor as the age of the mice increased. The older P180 mice had significantly fewer tumor-associated NSCs (20%) and tumor volumes 2.5 times larger than the younger P25 mice. In another study, Staflin et al. showed that embryonic hippocampal NSCs prolonged
animal survival and completely inhibited the outgrowth of glioma cells in 25% of cases. The delayed tumor outgrowth was observed when the NSCs were inoculated into a developed tumor and also when first mixed and then co-injected (Staflin et al., 2004).

It is known that the activity of neurogenesis in the adult brain declines with increasing age (Hallbergson et al., 2003) (Maslov et al., 2004) and that age is an important prognostic factor in predicting the survival from glioblastoma (Kleihues et al., 1993). To determine whether the correlation between increasing age and tumor aggressiveness is due to a weakened NSC response, Glass et al. supplemented the tumor cell inoculant with NSCs (1:3) for the P180 age group. The survival time of these new subjects was significantly increased compared to the control group and similar to the younger mice (Glass et al., 2005). Taken together, this data suggests that NSCs have tumor-suppressive properties but the effect is attenuated as the NSC population declines with age.

MECHANISMS OF SUPPRESSION

While multiple studies have reported on the tumor-suppressive properties of NSCs, the mechanisms behind this suppression are currently unknown. We propose that NSC migration and tumor suppression represents a wound repair response that is influenced by the conditions of the tumor microenvironment such as inflammation, hypoxia, and tissue necrosis. Coincidently, cancer has been described by early pathologists as an aberrant wound that never completely heals (Dvorak, 1986). This
analogy suggests that a proper repair response towards the tumor could effectively eliminate it. Therefore, the wound repair process will be reviewed in attempt to apply this model towards NSC-mediated tumor suppression.

Aside from cell replacement, NSCs are reportedly involved in modulating the immune response during CNS wound repair (Martino et al., 2011). Following brain or spinal cord injury, local astrocytes and microglia respond to molecules released from damaged or necrotic cells and initiate the innate immune responses by producing cytokines and chemokines that promote inflammation and recruit peripheral immune cells to the injury site (Aarum et al., 2003) (Olson and Miller, 2004). Damaged tissues also stimulate the neurogenic regions of the brain, prompting the proliferation and migration of NSCs towards the damaged tissue. The activation of NSCs could also result from cross talk with microglia and the signaling molecules they produce. Aaurum et al. found that soluble factors present within microglial-conditioned media induce NSC migration and Belmadani et al. later reported that tumor necrosis factor-α (TNFα) and interferon-γ (IFN-γ) produced by activated astrocytes and microglia caused this migratory response (Aarum et al., 2003) (Belmadani et al., 2006). NSCs within the SVZ express the chemotactic receptors and adhesion molecules needed for immune signaling, including CXCR4, CCR2, CCR5, and CX3CR1 (Ji et al., 2004). This suggests that NSCs possess the necessary cellular machinery to respond to the signals released from the damaged tissues and the ability to move towards the injury site. Not surprisingly, many of the
same molecules used in recruiting innate immune cells to damaged tissue are also found within the tumor microenvironment (Spaeth et al., 2008).

At the site of injury, damage-associated molecular patterns (DAMPs)- endogenous molecules released from damaged cells- act as danger signals and are recognized by macrophages/microglia and immune cells via toll-like receptors (TLRs). TLRs are integral membrane glycoproteins containing an extracellular domain with leucine-rich-repeats motifs and a cytoplasmic Toll/IL-1R (TIR) signaling domain, named for its homology to the interleukin 1 receptor (IL-1R). The expression of these receptors is dynamic and can be rapidly increased in response to pathogens, cytokines, and environmental stressors. The effector response following TLR activation is normally related to immune function (Akira et al., 2006). There are multiple TLR subtypes that bind different damage and pathogen-associated molecular patterns and produce different effector responses. For example, TLR1, TLR2, and TLR6 recognize lipids, whereas TLR7, TLR8, and TLR9 recognize nucleic acids. TLR4 is more divergent and can recognize lipopolysaccharides (LPS), fibronectin, and heat-shock proteins (Akira et al., 2006). Damage-associated HMGB1 protein, commonly released from damaged tissue and necrotic tumor cells, can bind and activate both TLR2 and TLR4 (Apetoh et al., 2007; Curtin et al., 2009). TLRs are highly expressed by macrophages and dendritic cells and also throughout the CNS by microglia, astrocytes, and oligodendrocytes (Bsibsi et al., 2002). Importantly, NSCs also express TLR2 and TLR4 and they are reported to be important for neurogenesis (Akira et al., 2006) (Rolls et al., 2007). TLR2 is primarily involved in
cell-fate decisions while TLR4 affects both cellular proliferation and differentiation (Rolls et al., 2007). Activation of TLRs leads to the recruitment of the intracellular adaptor protein myeloid differentiation factor 88 (MyD88) and its association with protein kinases IRAK and TRAF6. The IRAK1-TRAF6 complex activates the downstream protein kinase TAK1 complex, resulting in the phosphorylation of IKK and the subsequent activation of nuclear factor-kappa-B (NF-κB) (Akira et al., 2006).

NF-κB is a family of master transcription factors that regulate the expression of many immune-related genes involved with inflammatory responses, cytokines/chemokines, stress response, survival, apoptosis, proliferation and differentiation. The dimeric transcription factors include NF-κB1/p50, NF-κB2/p52, RelA/p65, and RelB. The Rel factors mediate dimerization, nuclear localization, DNA binding and interactions with the I-κB inhibitory proteins. These inhibitors bind to Rel homology domains (RHD) on the Rel components to mask the nuclear location sequences, thereby restricting the NF-κB dimers inactive in the cytoplasm.

Activating signals including exogenous and endogenous ligands along with physical and chemical stresses can direct the degradation of I-κB through the activation of IKK (IkB kinase). These regulatory kinases are comprised of a heteromer of two related IkB kinase domains and a regulatory NEMO domain (Hayden and Ghosh, 2008; Wajant et al., 2003).

Activated macrophages/microglia and dendritic cells respond to TLR and downstream NF-κB activation by initiating the pro-inflammatory response through
cytokine production and immune cell recruitment. Key pro-inflammatory cytokines involved include tumor necrosis factor-α (TNFα), interferon-γ (INF-γ), interleukin-6 (IL-6), and IL-1β (Carpentier and Palmer, 2009) and important chemokines for immune cell recruitment include RANTES (CCL5), MIP-2 (CXCL2), MIP-1a (CCL3), monocyte chemo-attractant protein 1 (MCP-1) (CCL2), and interleukin-8 (CXCL8) (Spaeth et al., 2008). This early pro-inflammatory response is important for recruiting innate and adaptive immune cells, activating reactive gliosis, clearing cellular debris, up-regulating co-stimulatory signals and MHC II protein expression, and activating the adaptive immune responses to eliminate potential pathogens (Aarum et al., 2003) (Akira et al., 2006) (Babcock et al., 2003) (Bsibsi et al., 2002) (Olson and Miller, 2004).

The effects of pro-inflammatory cytokines have been tested on NSCs to determine how they respond to the conditions of the wound during the repair process. Widera et al. demonstrated that TNFα has a positive influence on NSC proliferation, indicated by neurosphere volume and the incorporation of bromodeoxyuridin (BrdU) into the proliferating NSCs. They also found that TNFα up-regulates cyclin D1 expression through the NF-κB signaling pathway and that inhibition of NF-κB with IκB-AA1 strongly attenuated this effect (Widera et al., 2006). Another important study by Covacu et al. tested the responsiveness of NSC to cytokines by measuring the expression levels of TLR proteins following treatment with macrophage supernatants, pro-inflammatory cytokines IFN-γ and/or TNFα, and agonists for TLR2 and TLR4. TLR2 expression was increased by activated
macrophage supernatant and by TNFα alone and synergistically with IFN-γ. TLR4 expression was increased by IFN-γ but was decreased by TNFα (Covacu et al., 2009). Neural stem cells not only respond positively to inflammatory cytokines, but they can also contribute to the immune signaling by producing TNFα when stimulated with TLR agonists. NSCs have also been found to produce additional pro-inflammatory (IL-1α, IL-1β, IL-6) along with anti-inflammatory cytokines (TGF-β1, and β2) when examined in vitro using RT-PCR and ELISA assays (Klassen et al., 2003). Taken together, these data suggests an important role for NSCs in promoting and regulating immune responses during wound repair.

Multiple in vivo studies have investigated the therapeutic potential of NSCs for the treatment of neurological disorders such as ischemic stroke, spinal cord injury, and multiple sclerosis. Most studies suggest that the effects of NSCs appear to be context-dependent and are mediated through a bystander effect, where the NSCs provide neuroprotection through trophic support and immune regulation rather than providing cellular replacement or engraftment. Transplanted NSCs were found to support host axonal regeneration after spinal cord injury by producing neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and glial cell-derived neurotrophic factor (GDNF) (Lu et al., 2003). In addition to the production of soluble paracrine factors, secreted microvesicles serve as important mediators in cellular communication. Composed of small membrane vesicles derived from larger multivesicular bodies, these microvesicles can transport proteins and genetic
materials such as messenger and micro-RNAs to surrounding cells and tissues. Because microvesicles are produced by stem cells, they represent a novel mechanism by which NSCs may promote wound repair within the CNS (Drago et al., 2013) (Sabin and Kikyo, 2014).

Along with immune signaling and trophic support, there is evidence that NSCs could promote regeneration through their ability to regulate immune cell function. Injuries to the CNS are often found to have unregulated chronic inflammation that prevents complete regeneration. It was reported that NSC transplantation can improve locomotor function following spinal cord injury and that this recovery was attributed to NSC-immune cell communication and regulation (Cusimano et al., 2012). Moreover, it was found that NSC transplantation during the acute phase of early inflammation following injury led to significant recovery by regulating levels of inflammatory messenger RNA in activated macrophages (Cusimano et al., 2012). The effect of NSCs in the tumor microenvironment with regards to immune regulation has not been studied. This is of particular interest because of the well-documented effect of immune suppression caused by the tumor that complicates and limits the effectiveness of treatments.

TUMOR IMMUNOLOGY

Immune surveillance for cancerous cells is another important role of the immune system. The concept of immunoediting describes the balance between tumor elimination and tumor escape (Swann and Smyth, 2007). Lymphocytes survey the
brain for regular maintenance and homeostasis and tumor-specific antigens can be recognized and eliminated (Engelhardt and Ransohoff, 2005). In cases where tumor cells are not completely eliminated, a state of equilibrium can develop between the immune system and the developing tumor whereby the selective pressures of immune attack foster changes and evolution of DNA mutations and tumor-specific antigens. This can continue until the tumor cell variants are capable of resisting and suppressing the antitumor response (Swann and Smyth, 2007). In this way, immunoediting can lead to the development of a tumor that will not be recognized and eliminated by the immune system.

Immune suppression is another hallmark characteristic of glioblastomas and the degree of immune suppression positively correlates with tumor size (Morford et al., 1997). Despite studies demonstrating the presence of tumor infiltrating immune cell populations and the presence of glioma-specific tumor antigens (Kurpad et al., 1995), cellular activation and immunological functions are reportedly attenuated in many aspects (Dix et al., 1999). Early observations of impaired cell-mediated immunity in GBM patients include reduced and anergic T-cells populations, reduced delayed-type hypersensitivity responses to common antigens and abnormal proportions of helper and regulatory T-cell subsets (Dix et al., 1999). These immune defects are attributed to tumor-suppressive mechanisms since immune functions are restored following surgical resection of the tumor (Brooks et al., 1981). The decreased T-cell counts could result from decreased proliferation following activation or cell death from tumor-induced apoptosis. T-cell fractions isolated from
GBM patients were shown to be unresponsive to mitogen stimulation and unable to enter G1 phase of the cell cycle (Elliott et al., 1984). Patient-derived T-cells also have signaling defects in interleukin-2 (IL-2) production and secretion (Elliott et al., 1984). IL-2 is a cytokine involved in T cell proliferation and activation. Following antigen presentation and the co-stimulatory signal, IL-2 and its receptor are transcriptional up-regulated and begin signaling in an autocrine manner to increase proliferation. The reduced IL-2 production by T-cells from GBM patients likely results from down-regulation of IL-2 along with the IL-2 receptor since the addition of recombinant IL-2 to lymphocyte cultures did not restore proliferation defects (Elliott et al., 1984). This inability to produce and respond to IL-2 signaling results in T-cell anergy. Along with T-cell anergy, GBM patients were shown to have a higher percentage of apoptotic peripheral T-cells and a separate study demonstrated that glioma cells can directly induce programmed cell death in T-cells through the Fas-ligand receptors (Morford et al., 1999). The Fas receptor is normally expressed by activated T-cells following immune response. This is an important regulatory mechanism for preventing chronic inflammation and autoimmune disease. (Lynch et al., 1995). GBM cells advantageously express Fas-ligand and its receptor and have been shown to induce apoptosis in Fas-expressing T-cells in culture (Saas et al., 1997).

Additional mechanisms employed by the tumor that contribute to immune escape include the production of immunosuppressive cytokines IL-10 and TGF-β, down-regulation of MHC molecules and decreased co-stimulatory signals from mediators
of the antitumor immune response (Zou et al., 1999) (Bodmer et al., 1989) (Hussain et al., 2006). Microglia/macrophages are important contributors to the activation of adaptive immune responses through cytokine production along with antigen presentation and expression of the co-stimulatory signals. Hussain et al. examined tumor-infiltrating microglia/macrophages from GBM tumors and found that pro-inflammatory cytokines expression levels were low or absent and they lacked co-stimulatory molecules CD86, CD80 and CD40 necessary for T-cell activation (Hussain et al., 2006). These findings suggest that GBMs can directly impair antitumor responses to avoid immune detection and promote tumor progression.

Due to the variation between anti-tumor immunity and adapted immune evasion by the tumor, it is important to study glioblastoma tumors obtained from long term glioblastoma survivors in order to understand the mechanisms resulting in successful tumor elimination. A study by Donson et al. examined high-grade astrocytomas from long-term survivors using gene expression microarray analysis to identify prognostic biomarkers associated with long-term survival. They found that increased expression of immune function-related genes was the predominant biological factor that positively correlated with longer survival. Gene sets associated with innate and adaptive immunity were highly expressed. A particularly large number of genes expressed by T cells (CD3D, CD3E, CD3G, CD8B, TRAC, TRAT1, VAV1, and ZAP70), microglia/macrophages (AIF1, CD68, CD86, CIITA, HLA-DOA, HLA-DQB2, HLA-DRB1, HLA-DRB6, NOD2), and also several toll-like receptors (TLR 2, 3, 5, 6, 7, 8) were associated with long-term survival. No positive immune-related
genes were found to be negatively correlated with survival. Furthermore, there were no negative immune regulators (TGF-β, FOXP3, STAT3) associated with long-term survival. However, the immunosuppressive cytokine IL-4 was found to be associated with shorter survival. Classifier gene sets for specific lymphoid lineages were made to identify important cell types. Genes designated as markers for CD4 helper and CD8 cytotoxic T cells and NK cells were significantly enriched, while myeloid lineage subsets approached significance. Histological analysis was also performed and demonstrated significant cytotoxic T-cell infiltration within the tumors of long-time survivors, while the proportion of microglia/microphage fraction approached significance (Donson et al., 2012).

Immunotherapy attempts have been made to circumvent the immune suppressive mechanisms deployed by GBMs to elicit a more effective immune response against the tumor. Vaccination strategies using peptide-pulsed dendritic cells have been attempted with some success to improve tumor antigen presentation (Yu et al., 2004). In this case, patient-derived hematopoetic stem cells were isolated and expanded ex vivo into dendritic cells, cultured with surface peptides from the GBM tumors, and then used to vaccinate GBM patients. Follow up analysis found significant T-cell cytotoxicity against autologous GBMs in four patients and two of four patients undergoing secondary tumor operation showed extensive cytotoxic and memory T-cell infiltration of the tumor mass (Yu et al., 2004). Apetoh et al. found that the antitumor response and the cross presentation of tumor antigen by dendritic cells depends on TLR4 and the release of the damage-associated HMGB1
protein from dying tumor cells as an immunogenic signal. This signaling is dependent on MyD88 signaling and downstream NF-κB activation (Apetoh et al., 2007). Curtin et al. followed up this finding by using adenoviral delivery system to introduce the Fms-like tyrosine kinase 3 ligand (Flt3L) and thymidine kinase into the tumor microenvironment in order to activate dendritic cells and promote tumor cell necrosis, respectively. Using this therapy in a mouse model, they showed significant increase in survival that was accompanied by increased dendritic cell infiltration and dependent on CD4 and CD8 T-cell activation. They confirmed that this antitumor response was due to the release of tumor HMGB1 and dependent on TLR signaling (Curtin et al., 2009).

SUMMARY

Cancer has been described as an aberrant wound in regards to structural composition and environmental conditions such as inflammation, hypoxia, and tissue necrosis. Similar to a wound repair response, tumor formation stimulates NSC proliferation and migration towards the tumor. This migration is mediated by factors derived from the tumor such as SDF-1 and HMGB1 and also inflammatory cytokines from tumor-infiltrating macrophages. The interactions that take place between NSCs and GBM cells within the tumor microenvironment are unknown but can result in reduced tumor growth and increased survival in mice models. The expression of TLR2 and TLR4 by NSCs could allow a mechanism for GBM recognition considering that GBMs release the HMGB1 protein that binds and activates these receptors. TLR signaling leads to downstream NF-κB activation and
the expression of many genes involved in stress response and immune signaling.

While NSCs have been shown to participate in immune signaling during CNS wound repair, they have never been examined in the context of tumor immunology. There are many unknown aspects of NSC-mediated tumor suppression as this is a relatively unexplored area of research. Therefore, examination of the mode and mechanism underlying the anti-tumor properties of NSCs could uncover interesting insight for new treatment options.
The objective of this project was to study the mode and mechanism of NSC-mediated tumor suppression. Many studies have characterized the migration of NSCs towards glioblastoma tumors and there is reported evidence on their tumor-suppressive properties. However, the mode and mechanism behind this anti-tumor response is not well understood. This interaction could be contact-dependent or mediated through releasable factors and/or microvesicles. In this study, the mode of NSC-mediated tumor suppression was determined by measuring the proliferation of GBM cells co-cultured with NSCs using a transwell system that separates the two cell types but allows for intercellular communication through releasable factors. Reduced GBM proliferation following NSC co-culture using this system indicates that there are factors produced by the NSCs that influence GBM proliferation and/or viability. Potential targets include factors of the tumor necrosis factor super family that are commonly involved in inducing programmed cell death. Gene expression analysis of these suspected targets was performed with RT-PCR on NSCs following GBM co-culture. Furthermore, the mechanism underlying this suppressive effect will be determined by examining GBM gene expression changes related to stress response, apoptosis and survival genes. Because these anti-tumor properties of NSCs could be influenced by the conditions of the tumor microenvironment such as hypoxia, the co-culture proliferation assays and the gene expression analysis will be performed under both normoxic and hypoxic conditions.
Hypothesis 1: NSCs can directly suppress tumor proliferation through releasable factors and this suppression is influenced by the conditions of the tumor microenvironment.

(Aim 1a) Measure GBM proliferation following co-culture with NSCs in a transwell system under normal and hypoxic conditions. (1b) Examine GBM gene expression changes related to apoptotic/anti-apoptotic and stress response genes following NSC co-culture. (1c) Examine NSC gene expression changes related to TNF death receptor ligand signaling.

Another important consideration is an indirect mode of NSC-mediated tumor suppression that could potentially involve immune signaling and the activation of anti-tumor immune responses. NSCs migration appears to reflect a wound repair response and NSCs have been shown to participate in immune signaling. Since immune suppression is a common mechanism of tumor progression, we propose that NSC-mediated tumor suppression involves a process comparable to wound repair that involves immune cell recruitment and activation that could subsequently result in an anti-tumor immune response. Gene expression analysis of immune-related genes following co-culture with GBM cells will provide insight into the potential immune contributions of NSCs.

Hypothesis 2: NSCs can indirectly mediate tumor suppression through immune signaling. (Aim 2) Examine NSC gene expression changes related to immune response/cytokine genes following co-culture with GBMs.
METHODS

CELL CULTURE

Mouse glioblastoma cell line GL261 was maintained in DMEM with 10% FBS at 37 degrees and 5% CO₂ in normal humidified conditions. Mouse embryonic neural stem cells (NSCs) were purchased from Stem Cell Technologies (Cat. #00330). NSCs were initially started as suspended neurosphere cultures at 5x10^6 cells/20mL in a T-75 cm² flask using Neurocult proliferation media (StemCell Tech, Cat. #05702) supplemented with EGF (Peprotech, 20 ng/ml) (referred to as stem cell media). NSC cultures were converted to monolayers once the neurospheres reached approximately 150-200μm in diameter. Accutase (StemCell Tech, Cat. #07920) was used for dissociation and cells were then plated on plates coated with a combination of poly-D-lysine (PDL, Sigma P7280) and laminin (Sigma L2020). Prior to plating, monolayer plates were prepared accordingly: PDL was first added to a normal tissue culture 6-well plate at 100μg/ml for 2 hours at 37 degrees. PDL was removed and the flask was washed once with PBS before adding laminin at 10μg/ml for 2-hours at 37 degrees. Coated plates were prepared new each time the NSCs were passaged. NSC monolayers were initially plated at 8x10^4 cells/cm² in 3ml/well until reaching 60-80% confluency. At that point the NSCs were dissociated and replated at 2x10^4 cells/cm² in a newly coated 6-well plate.

Co-cultures were setup using 24-well transwell inserts (Greiner Bio-one, cat. #662610). GL261s were first plated in the 24-well plate at 50,000 cells/well in 1ml
of DMEM with 10% FBS. The NSCs were initially plated in a separate plate inside the transwell inserts at 25,000 cells/well in 200ul of Neurocult media with EGF. The cells from each plate were then incubated and allowed to resume growth overnight.

On the following day the co-cultures were setup by first washing each well and insert once with PBS. The NSC inserts were then placed inside the wells containing the GL261 cells and then Neurocult with EGF media was added to the wells to make a 2:1 co-culture. GL261 proliferation was compared between the DMEM with FBS and the neurocult with EGF (Supplemental figure 1).

**PROLIFERATION ASSAY**

GL261 proliferation was measured using the alamarblue viability assay (Life technologies Cat. #DAL1025). Cell number was measured over the duration of 72 hours at 24-hour intervals. A plate was prepared with all of the controls, another plate for all the normoxic co-cultures and separate plates for the hypoxic co-cultures. Each designated time point had 3 co-culture wells. The normoxic co-culture plates were prepared with one column (three wells) containing blank wells with only neurocult media for blanking the spectrometer and then four following columns for the T0, 24, 48, 72 time points (three co-cultures each). The hypoxic plate was prepared with each time point on a separate plate (control and co-culture). This was done to not disturb the other time points when moving the plate to normoxia for alamarblue addition and incubation.
At each measuring interval the co-culture was disassembled by removing the transwell insert and removing the used media. This conditioned media was saved for future analysis. New media was added along with 10% alamarblue reagent. Once the alamarblue was added the plate was incubated for 6 hours in normoxia to allow for metabolic reduction of the reagent and colorimetric change. This incubation duration was predetermined experimentally (Supplemental figure 2) because the proper duration varies with each cell type and cell density. After the 6-hour incubation a 100ul sample from each well was transferred to a 96-well plate for reading with the spectrometer.

Alamarblue is a metabolic indicator that becomes reduced during cellular respiration and undergoes a colorimetric change from a blue oxidized form to a red reduced form. Based on its reduction potential, it is an intermediate between the cytochromes of the electron transport chain and the final reduction of oxygen. Therefore, it does not disrupt the flow of electrons and is nontoxic to cell culture, in contrast to MTT, which has a reduction potential lower than the cytochromes.

Absorbance was monitored at 570nm (reduced) and 600nm (oxidized) with the spectrometer and the percent reduction from the blue oxidized form was computed using the equation below. Relative percent reduction numbers were calculated by subtracting the baseline percent reduction at T0 from the following time points. Statistical analysis was performed using the student t-test to compare the relative percent reduction at each time point between groups.
\[
\frac{(\varepsilon_{\text{OX}})\lambda_2 A \lambda_1 - (\varepsilon_{\text{OX}}) \lambda_1 A \lambda_2}{(\varepsilon_{\text{RED}})\lambda_1 A' \lambda_2 - (\varepsilon_{\text{RED}})\lambda_2 A' \lambda_1} \times 100
\]

Where
- \( C_{\text{RED}} \) = concentration of reduced form alamarBlue® (RED)
- \( C_{\text{OX}} \) = oxidized form of alamarBlue® (BLUE)
- \( \varepsilon_{\text{OX}} \) = molar extinction coefficient of alamarBlue oxidized form (BLUE)
- \( \varepsilon_{\text{RED}} \) = molar extinction coefficient of alamarBlue reduced form (RED)
- \( A \) = absorbance of test wells
- \( A' \) = absorbance of negative control well. The negative control well should contain media + alamarBlue but no cells.
- \( A^0 \) = absorbance of positive growth control well
- \( \lambda_1 \) = 570nm (540nm may also be used)
- \( \lambda_2 \) = 600nm (630 may also be used)

REAL-TIME PCR

Gene expression analysis was performed on the NSCs and the GL261s following coculture for 72 hours using the RT² Profiler PCR array (Qiagen PAMM-225Z). This array includes a panel of 96 target genes of the NF-κB pathway including housekeeping genes for normalizing the data (Supplemental table 1). The housekeeping genes include actin-beta (Actb), beta-2 microglobulin (B2m), glyceraldehyde-3-phosphate dehydrogenase (Gapdh), glucuronidase-beta (Gusb), and heat shock protein 90-alpha (Hsp90ab1).

Separate plates for RNA collection were prepared for the normoxic controls and cocultures and the hypoxic controls and cocultures. Twenty (20) wells were setup for each plate. After the 72-hour time point the co-cultures were disassembled and the cells were lysed with RLT buffer and the lysates were consolidated for RNA purification and first strand synthesis. RNA was purified using the RNeasy mini kit.
cDNA was synthesized using the RT² first strand kit that included a genomic DNA elimination mix and the reverse transcription mix with the RE3 enzyme.

The real-time PCR reaction was performed using the RT² SYBR green mastermix (Qiagen #330512). The PCR mix was loaded into the array plate using an 8-channel micropipettor and the reaction was started using the MyiQ real-time PCR thermocycler. The cycler was programmed to begin with 1 cycle at 95°C for 10 min to activate the HotStart DNA Taq Polymerase and then 40 cycles starting at 95°C for 15 seconds and decreasing to 60°C for 1 min. The threshold cycle (Ct) was calculated by choosing the automatic baseline option and then setting the threshold value to 10 from the log view amplification plot. The Ct values were then exported to Excel and analyzed using the SABiosciences web-based software. Gene expression was analyzed using the comparative Ct method (Schmittgen and Livak, 2008). This involved comparing the Ct values from the co-cultured cells to the controls after normalization to B2m, Gusb, and Hsp90ab1. Fold change \(2^{\Delta\Delta Ct}\) is the normalized gene expression \(2^{\Delta Ct}\) in the test sample divided by the normalized gene expression \(2^{\Delta Ct}\) in the control sample. Fold change values greater than one indicate a positive up-regulation and the fold regulation is equal to the fold change. Fold change values less than one indicate a negative down-regulation and the fold regulation is the negative inverse of the fold change. This method was used to examine gene expression changes between the co-culture and control groups under normoxia and hypoxia for GL261s and NSCs.
RESULTS

NEURAL STEM CELLS SUPPRESS GL261 PROLIFERATION UNDER NORMAL BUT NOT HYPOXIC CONDITIONS

The cellular interactions between NSCs and GL261 glioma cells were studied using an \textit{in vitro} transwell co-culturing system that separates the two cell types but allows for intercellular communication via releasable factors transmitted through the semi-permeable insert. GL261s were plated at 50,000 cells/well in a 24-well plate and NSCs were added to the insert at 25,000 cells/well for a 2:1 co-culture. GL261 proliferation was measured over the course of 72 hours at 24-hour intervals using the alamarblue assay to determine the metabolic activity in correlation with cell number. Reduction of the metabolic indicator causes a colorimetric change that can be measured from the absorbance and then converted to percent reduction. A higher percent reduction therefore indicates a higher metabolic activity and more proliferation in the culture.

Under normoxic conditions there was a significant decrease in the reduction of alamarblue by the GL261s co-cultured with NSCs compared to the normoxic control, indicating a decrease in proliferation and/or cell survival (Figure 1). The percent reduction difference between the co-culture and the control was 5.4 percent (p=0.001) after 48 hours and 6.8 percent (p=0.005) after 72 hours. Furthermore, the density of GL261 cells was decreased in the area directly below the NSC insert.
compared to the outside perimeter, based on visual observation, suggesting either
cell death or migration away from the NSCs (no data shown).

The proliferation assay was also performed under hypoxia to represent the
conditions of the tumor microenvironment (Figure 2). In contrast to the normoxic
culture, GL261 proliferation was not affected by the NSCs under these conditions.
This suggests that either hypoxia affects the survival of GL261s or that NSCs
respond differently under hypoxic conditions.

When we compared the proliferation of the GL261 hypoxic control to the normoxic
control, we found that proliferation under hypoxia was comparable to the normoxic
group throughout the first 48 hours but then decreased after 72 hours (p=0.0003)
(Figure 3). The relatively normal proliferation rate throughout the first 48 hours
under hypoxia might suggest increased survival mechanisms that could counteract
the stresses of hypoxia. Many survival mechanisms implicated in cancer progression
have been attributed to hypoxia (Harris, 2002). Furthermore, the reduced
proliferation rate between 48-72 hours could have minimized the suppressive effect
observed from the NSCs. Overall this data shows that NSCs can directly suppress
GL261 proliferation in a contact-independent mechanism under normoxic but not
hypoxic conditions. Further analysis is required to determine the mechanism of this
NSC-mediated suppression.
Figure 1: GL261 cell proliferation measured by alamarblue reduction following 2:1 co-culture with NSCs in transwell system. Relative percent reduction equals the difference in reduction between each time point and time zero. NMX represents the normoxic group and the asterisks indicate statistical significance (<0.05).

Figure 2: GL261 cell proliferation measured by alamarblue reduction under hypoxic conditions following 2:1 co-culture with NSCs in transwell system. HPX represents the hypoxic group.
SUPPRESSIVE MECHANISMS BY GENE EXPRESSION ANALYSIS

GL261 gene expression was examined following co-culture with NSCs in attempt to determine how GL261 cells respond to NSC interaction and the cause of the suppressed proliferation. The genes selected for examination include NF-κB target genes related to stress response, survival and apoptosis. Real-time PCR was performed on GL261 lysates following NSC co-culture for 72 hours under either normal or hypoxic conditions and gene expression comparisons were made relative to control cultures under the same conditions without the NSCs. Therefore the only variable affecting GL261 gene expression was the interactions with NSCs.

Figure 3: GL261 cell proliferation measured by alamarblue reduction under normoxic and hypoxic conditions. These groups represent the controls for each co-culture experiment.
**Figure 4:** Gene expression changes represented by fold change relative to corresponding controls. The NMX co-culture changes are relative to the normoxic control and HPX co-cultures changes are relative to the hypoxic control. Genes 1-4 are characterized as pro-apoptotic and genes 5-13 are characterized as survival or anti-apoptotic.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Fold Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Map2k5</td>
<td>Apoptosis - activation of p38-MAPK pathway</td>
<td>5.3</td>
</tr>
<tr>
<td>Bcl2</td>
<td>Survival - negatively regulates BH123 apoptotic proteins</td>
<td>-9.0</td>
</tr>
</tbody>
</table>

**Table 1:** Gene expression analysis of RT-PCR data from GL261 cells co-cultured with NSCs under normoxic conditions. Expression changes are relative to the normoxic (NMX) control. Fold regulation was calculated as the inverse of fold change for negative values.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Fold Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adm</td>
<td>Survival/proliferation</td>
<td>4.1</td>
</tr>
<tr>
<td>Bcl2</td>
<td>Survival- negatively regulates BH123 apoptotic proteins</td>
<td>2.7</td>
</tr>
<tr>
<td>Gadd45b</td>
<td>Survival- negatively regulates JNK activation</td>
<td>2.4</td>
</tr>
<tr>
<td>Tnfsf6 (Fasl)</td>
<td>Death receptor ligand</td>
<td>3.5</td>
</tr>
</tbody>
</table>

**Table 2:** Gene expression analysis of RT-PCR data from GL261 cells co-cultured with NSCs under hypoxic conditions. Expression changes are relative to hypoxic (HPX) control.
The mitogen-activated protein kinase (MAP2k6) that is involved in activating pro-apoptotic p38-MAPK was found to be up-regulated 5.3 fold and anti-apoptotic gene Bcl2 involved in mitochondrial stability was down-regulated 0.11 fold or -9 fold-regulation in the normoxic co-culture relative to the normoxic control (Figure 4, Table 1). This gene expression data corresponds with the decreased GL261 proliferation data from the normoxic co-culture (Figure 1). The addition of hypoxia to the co-culture had opposite effects on GL261 cell proliferation and also gene expression levels of survival and pro-apoptotic genes (Table 2). We found a 4-fold increase in adrenomedullin (Adm) expression in the hypoxic co-culture compared to the hypoxic control. Adm is a multifunctional pro-survival gene with mitogenic and angiogenic functions in glioblastomas and has been shown to be regulated by hypoxia (Larrayoz et al., 2014). Our data supported these previous findings; Adm was also found up-regulated 8.1 fold in the hypoxic control relative to the normoxic control (Table 3), confirming that hypoxia alone can regulate Adm expression in GL261 cells. However, the increased Adm expression in the hypoxic co-culture was relative to the normally high expression of the hypoxic control, suggesting that the interactions of NSCs can further increase this pathway.

In contrast to the normoxic co-culture, Bcl2 was found to be up-regulated in the hypoxic co-culture. However, this up-regulation was relative to the hypoxic control where Bcl2 was found to be down-regulated by hypoxia (Tables 2-3). This down-regulation of anti-apoptotic Bcl2 correlates with the decreased proliferation rate in the hypoxic control after 72 hours (Figure 3). Tumor necrosis factor-6 (Fas-ligand)
that is involved in mediating programmed cell death upon binding to its receptor on
the target cell was up-regulated relative to the hypoxic control (3.5-fold), along with
Growth arrest and DNA damage-inducible protein (Gadd45b). Gadd45b has been
shown to block apoptosis by negatively regulating TNF-induced JNK signaling (De
Smal et al., 2001). These finding correspond with the increased proliferation
and/or survival of GL261s in hypoxic co-culture compared to the normoxic co-
culture (Figures 1-2).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Fold Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adm</td>
<td>Survival/proliferation</td>
<td>8.1</td>
</tr>
<tr>
<td>Gadd45b</td>
<td>Survival- negatively regulates JNK activation</td>
<td>1.5</td>
</tr>
<tr>
<td>Bcl2</td>
<td>Survival- negatively regulates BH123 apoptotic proteins</td>
<td>-16.4</td>
</tr>
</tbody>
</table>

Table 3: Gene expression analysis of RT-PCR data from GL261 cells cultured alone under hypoxic
conditions. Expression changes are relative to normoxic (NMX) control.

Taking together the proliferation data and the gene expression data from the
normoxic NSC co-culture, we show that there is a correlation between the decreased
GL261 proliferation rate and the increase in the pro-apoptotic Map2k6 and decrease
in the Bcl2 anti-apoptotic pathways. The addition of hypoxia to the co-culture
prevented the NSC-mediated suppression of GL261 proliferation while also up-
regulating survival pathways.

NEURAL STEM CELLS UP-REGULATE TUMOR NECROSIS FACTORS
NSC gene expression was also examined to determine how the NSCs respond to
interacting with the glioma cells and to determine the underlying mechanism of
GL261 suppression. The genes selected for examination included NF-κB target genes
related to tumor necrosis factor super family ligands, cytokines/chemokines and immune-related genes involved with innate and adaptive immune responses. Real-time PCR was performed on NSC lysates following the 72-hour co-culture with GL261 cells under either normal or hypoxic conditions. Gene expression comparisons were made relative to control cultures consisting of only NSCs under the same conditions.

![Normalized NSC gene expression](image)

**Figure 5:** Gene expression changes represented by fold change relative to corresponding control. The NMX co-culture changes are relative to the normoxic (NMX) control and HPX co-cultures changes are relative to the hypoxic (HPX) control.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Fold Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tnfa</td>
<td>Death receptor ligand, pro-inflammatory cytokine</td>
<td>3.2</td>
</tr>
<tr>
<td>Tnfsf6 (Fasl)</td>
<td>Death receptor ligand</td>
<td>3.9</td>
</tr>
</tbody>
</table>

**Table 4:** Gene expression analysis of RT-PCR data from NSCs co-cultured with GL261s under normoxic conditions. Changes are relative to normoxic control group.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Fold Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tnfsf6 (Fasl)</td>
<td>Death receptor ligand</td>
<td>4</td>
</tr>
</tbody>
</table>

**Table 5:** Gene expression analysis of RT-PCR data from NSCs co-cultured with GL261s under hypoxic conditions. Changes are relative to hypoxic control group.
Members of the tumor necrosis super family were examined in search of a mechanism behind NSC-mediated GL261 suppression. These mediators are known to regulate inflammatory processes and also apoptosis pathways through TNF super family receptors. Interestingly, NSCs had up-regulated TNFα expression in the normoxic co-culture and up-regulated Tnfsf6 (Fas-ligand) in the normoxic and hypoxic co-culture (Tables 4-5). These genes were not influenced by hypoxia alone, but only upon interactions with the GL261 cells.

**NEURAL STEM CELLS IN IMMUNE SIGNALING**

We then examined the expression of genes involved in immune cell signaling to determine if NSCs can initiate immune cell recruitment following glioma cell interactions (Figure 6). We found that NSCs co-cultured with GL261s under both normoxic and hypoxic conditions showed a similar increase in the expression of multiple chemokines with the CXC motif including CXCL1 and CXCL10 along with the C-C motif ligands CCL5 (RANTES) and CCL12. Cell adhesion molecules lcam1 and Vcam1 that are important in cellular migration and invasion were also commonly up-regulated in the NSCs (Tables 6-7). Specific changes to the normoxic co-culture group included the up-regulation of chemokines CXCL3 and CXCL9 whereas the hypoxic co-culture group specifically up-regulated CCR5. This data indicates that NSCs are involved in immune signaling by producing chemokines that are known to recruit innate and adaptive immune cells during wound repair. It also shows that this immune signaling is less influenced by hypoxia and more influenced by the interactions with GL261 cells and the factors they release.
Figure 6: Gene expression changes represented by fold change relative to corresponding control. The NMX co-culture changes are relative to the normoxic (NMX) control and HPX co-cultures changes are relative to the hypoxic (HPX) control.

Table 6: Gene expression analysis of RT-PCR data from NSCs co-cultured with GL261s under normoxic conditions. Changes are relative to normoxic control group. Bold names indicate genes that are similarly up-regulated in the normoxic co-culture and the hypoxic co-culture.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Function (Cytokines/Chemokines)</th>
<th>Fold Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL5</td>
<td>Chemokine</td>
<td>3</td>
</tr>
<tr>
<td>CCL12</td>
<td>Chemokine</td>
<td>2.7</td>
</tr>
<tr>
<td>Csf1</td>
<td>Chemokine</td>
<td>2.4</td>
</tr>
<tr>
<td>CCR5</td>
<td>Chemokine receptor</td>
<td>12.5</td>
</tr>
<tr>
<td>CXCL1</td>
<td>Chemokine</td>
<td>11</td>
</tr>
<tr>
<td>CXCL10</td>
<td>Chemokine</td>
<td>11.3</td>
</tr>
<tr>
<td>Icam1</td>
<td>Adhesion molecule</td>
<td>8.9</td>
</tr>
<tr>
<td>Vcam1</td>
<td>Adhesion molecule</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Table 7: Gene expression analysis of RT-PCR data from NSCs co-cultured with GL261s under hypoxic conditions. Changes are relative to hypoxic control group.

We also examined genes involved in immune cell activation and found up-regulation of many genes involved in the adaptive immune response (Figure 7). The cytokine IL6 was up-regulated in the normoxic co-culture (4.7 fold) and the hypoxic co-culture (7.8 fold), whereas interferon-γ (IFNG) was highly up-regulated in the hypoxic co-culture (20 fold) but down-regulated in the normoxic co-culture (-3.4 fold) (Tables 8-9). Furthermore, genes involved in T-cell activation and co-stimulation, including CD40 and CD80, were also up-regulated in the co-culture but not the controls. This suggests an important and novel mechanism for activating adaptive immune processes and anti-tumor responses.
Figure 7: Gene expression changes represented by fold change relative to corresponding control. The NMX co-culture changes are relative to the normoxic control and HPX co-cultures changes are relative to the hypoxic control.

Table 8: Gene expression analysis of RT-PCR data from NSCs co-cultured with GL261s under normoxic conditions. Changes are relative to normoxic control group.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Function (Immune-related)</th>
<th>Fold Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD40</td>
<td>Adaptive immunity- costimulatory signal</td>
<td>3</td>
</tr>
<tr>
<td>CD80</td>
<td>Adaptive immunity- costimulatory signal</td>
<td>2.2</td>
</tr>
<tr>
<td>Icam1</td>
<td>T-cell receptor accessory ligand</td>
<td>7.1</td>
</tr>
<tr>
<td>IL6</td>
<td>Cytokine</td>
<td>4.7</td>
</tr>
<tr>
<td>IFNG</td>
<td>Cytokine</td>
<td>-3.4</td>
</tr>
</tbody>
</table>

Table 9: Gene expression analysis of RT-PCR data from NSCs co-cultured with GL261s under hypoxic conditions. Changes are relative to hypoxic control group.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Function (Immune-related)</th>
<th>Fold Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD40</td>
<td>Adaptive immunity- costimulatory signal</td>
<td>5</td>
</tr>
<tr>
<td>CD80</td>
<td>Adaptive immunity- costimulatory signal</td>
<td>3.2</td>
</tr>
<tr>
<td>Icam1</td>
<td>T-cell receptor accessory ligand</td>
<td>8.9</td>
</tr>
<tr>
<td>IFNG</td>
<td>Cytokine</td>
<td>20</td>
</tr>
<tr>
<td>IL6</td>
<td>Cytokine</td>
<td>7.8</td>
</tr>
</tbody>
</table>
Taking together the expression data from the tumor necrosis family ligands along with the cytokine/chemokine and immune signaling molecules, it can be concluded that NSCs respond to GL261 interactions by up-regulating ligands involved in inducing apoptosis and also by up-regulating many immune-related genes that are involved in immune cell recruitment and activation.

**DISCUSSION**

Although the migratory nature of NSCs towards glioblastoma tumors has been well characterized, the actions of NSCs within the tumor microenvironment and the intercellular relationship between NSCs and gliomas in regard to tumor progression is not well studied. Glass et al. (2005) found that NSCs can prolong survival in animal models of GBM and further investigation found that NSCs could directly suppress and induce apoptosis in GBM co-cultures. However, the mechanism behind this suppressive effect has not been studied. This is the first study to examine the intercellular interactions between NSCs and GBM cells at the genetic level and the subsequent influence on gene expression. The objectives here were to study the mode and mechanism behind NSC-mediated tumor suppression. We performed co-culture proliferation assays in combination with gene expression analysis to show that there is a two-way communication between the NSCs and the GL261s that results in decreased GL261 proliferation and changes to survival and apoptotic-related gene expression.
MODE AND MECHANISM

To study the mode of intercellular communication we utilized in vitro culturing inserts that separate the two cell types but allow for the exchange of factors through the semi-permeable membrane. This co-culture system was advantageous in that it allowed for us to examine the specific interactions between the NSCs and GBMs while excluding many other cell types of the heterogeneous tumor microenvironment. Our findings suggest that these interactions are contact independent and are transmitted through soluble factors or microvesicles. The gene expression data has provided information on the suspected factors involved in this communication. An important finding was the increased expression of TNFα and Fas-ligand by the NSCs. This correlated with the decreased GL261 proliferation and/or viability and the up-regulation of stressed-activated MAP2k6 and the down-regulation of anti-apoptotic BCL2 in the GL261s from the co-cultures but not the controls.

GL261 SUPPRESSION BY NEURAL STEM CELLS

TNFα and Fas-ligand are proteins of the tumor necrosis factor super family that are involved in the pro-inflammatory immune response and also programmed cell death. TNFα has a cytotoxic effect on many tumor cell lines and can cause tumor necrosis in many animal models (Old, 1988). The production of tumor necrosis factor (TNF) ligands by NSCs has been demonstrated in other circumstances but never before in the context of tumor suppression. Klassen et al. found that NSCs constitutively produce TNFα under normal culturing conditions and Covacu et al.
found that this production is mediated through toll-like receptor (TLR) activation (Klassen et al., 2003) (Covacu et al., 2009). Therefore, this finding suggests a possible mechanism for NSC-mediated tumor suppression whereby NSC signaling through TLRs leads to TNFα production and GBM cell death.

TNFα is produced as a transmembrane protein arranged into homotrimers that get released as a soluble cytokine through the proteolytic cleavage by the TNF alpha-converting enzyme (TACE) (Wajant et al., 2003). This ligand can then interact with the TNF-receptors that are expressed on most cell types including glioblastoma (Tada et al., 1994). TNF has a diverse range of biological functions, including involvement in development and regeneration but also tissue destruction. The differential outcome has been attributed to cell type, physiological context, receptor subtype, timing and duration (Wajant et al., 2003).

Activation of the TNF-receptor (TNF-R1) signals to the intracellular death domain (DD) and initiates the coupling of the death receptor to caspase activation and apoptosis (Tartaglia et al., 1993). Downstream signaling can lead to the activation of NF-κB and stress-induced MAPK pathways through the TRAF2 (TNF receptor-associated factor-2) adaptor protein or it can lead to caspase activation through the Fas-associated death domain protein (FADD) (described below). Signaling through TRAF2 activates the MAP3K apoptosis signal-regulated kinase-1 (ASK1), leading to the downstream activation of stress-induced p38-MAPK and the c-Jun N-terminal kinase (JNK). These kinase pathways are simultaneously activated in response to
cellular and environmental stresses and can respond by modulating the cell cycle
and initiating programmed cell death. p38-MAPK is directly activated by MKK6
(MAP2K6). JNK is directly activated by MKK4/7 and induces programmed cell death
mainly through the activation of the AP-1 transcription factor component c-Jun and
also by the phosphorylation and inhibition of anti-apoptotic protein BCL2 (Wada
and Penninger, 2004; Wajant et al., 2003).

We found MAP2K6 to be up-regulated in the GL261 cells following NSC co-culture
and this corresponded with the increased TNFα expression by the NSCs. In another
study, fibroblasts treated with TNFα were shown to induce apoptosis through ASK1-
mediated MAP2K6 and JNK activation (Ichijo et al., 1997). This could be the case in
our experiment, however the kinase activation status needs to be determined along
with downstream p38 signaling targets to confirm a connection with decreased cell
viability. Furthermore, the results from the alamarblue assays on GL261s were
initially interpreted as a decrease in cell proliferation due to the decreased
alamarblue reduction. However, the reduced reduction rate could also reflect a
decrease in cell number caused by cell death. These findings would support the
results from the Glass et al. study where they showed NSC-induced glioma
apoptosis. Viability assays will need to be performed to confirm this outcome.

Fas-ligand was also found up-regulated by NSCs in the co-culture relative to
controls. The Fas/Fas-ligand system is the prototypic mechanism for extrinsic cell
death that is involved in cell-mediated cytotoxicity, immune regulation, and also
counterattack by many cancer cells (Choi and Benveniste, 2004). This suggests another NSC-mediated mechanism to decrease GL261 viability through programmed cell death. Fas-ligand (Tnfsf6) is a 40-kDa protein and another member of the tumor necrosis factor super family. Fas-ligand binds to its transmembrane glycoprotein counterpart Fas/APO-1 on target cells to initiate apoptosis through caspase activation. Similarly to TNFα, Fas-ligand can function as a transmembrane ligand or as a soluble form following cleavage by several matrix metalloproteinases. Activated T cells and NK cells are the major cells that express Fas-ligand as part of their cytotoxic machinery, however neurons, astrocytes, and neural stem cells have been shown to express Fas-ligand for participation in immune regulation in the CNS (Choi and Benveniste, 2004) (Carpentier and Palmer, 2009). GBM cells have also been shown to express Fas-ligand and the in vivo expression has been positively correlated with the malignancy grade in CNS tumors. This is associated with their ability to kill infiltrating lymphocytes (Saas et al., 1997). Glioblastomas also express relatively high levels of Fas/APO-1 and are sensitive to Fas-mediated apoptosis in vitro, although their sensitivity is variable.

Signaling by these tumor necrosis factor family death receptors leads to caspase activation and cellular apoptosis. Common to most death receptors is the FADD adaptor protein, which associates with the intracellular domain of TNF-R1 through TRADD (TNF receptor-associated death domain protein) and also with DISC (death-inducing signaling complex) following Fas/APO-1 activation to mediate the recruitment and activation of initiator procaspase-8 and 10, which then continue
downstream to activate executioner procaspases for initiation of apoptosis (Wajant et al., 2003). Fas activation can also transduce stress signals through adaptor protein Daxx to activate ASK1 and the downstream JNK pathway to induce apoptosis (Choi and Benveniste, 2004).

In addition to extrinsic death receptor ligands in the co-culture experiment, we also found changes in the intrinsic pathways that regulate apoptosis in response to stressful stimuli. Intrinsic apoptotic pathways involve triggering the release of mitochondrial protein cytochrome C from the outer membrane. It then enters the cytosol and binds to a procaspase-activating adaptor protein called the apoptotic protease activating factor-1 (Apaf1). Apaf1 oligomerizes into an apoptosome heptamer that recruits and activates initiator procaspase protein 9 to facilitate the downstream activation of executioner caspases. This intrinsic apoptotic pathway is regulated by a class of BCL2 proteins with both pro-apoptotic and anti-apoptotic effects by controlling the release of cytochrome C and other intermembrane mitochondrial components (Alberts et al., 2008). The pro-apoptotic BH123 proteins include mitochondrial-bound Bak and cytosolic Bax. Following intrinsic apoptotic stimuli these proteins form oligomers in the mitochondrial outer member to promote the release of cytochrome C. The anti-apoptotic BCL2 proteins include BCL2 itself and BCL-XL. These proteins act to stability the mitochondrial membrane by binding and inhibiting Bak and Bak from oligomerizing (Alberts et al., 2008). Nakasu et al. found that many CNS malignancies including low and high-grade astrocytomas express a relatively high level of BCL2 compared to their non-
malignant astrocytic counterparts (Nakasu et al., 1994). However, as stated before, TNF-induced JNK activation has the ability to inhibit BCL2 by phosphorylation to suppress its inhibitory effect. Interestingly, our findings show that BCL2 expression is significantly down-regulated in GL261s co-cultured with NSCs under normoxic conditions. This corresponds with the decreased GL261 proliferation and/or viability and the increase in death-related ligands by the NSCs, although the connection between TNF signaling and BCL2 transcriptional regulation has not been determined.

Taken together, we found that NSCs were capable of suppressing GL261 proliferation and/or viability through releasable factors that are suspected to be TNFα and Fas-ligand based on gene expression data. These factors could be regulated through toll-like receptor activation and NF-κB-regulated cytokine production. These tumor necrosis factor proteins are suspected to suppress GL261 cell cycle regulation and programmed cell death through Fas/APO-1 and TNF-receptors. Follow up experiments will confirm the TLR2/4 activation on NSCs following GL261 co-culture along with a western blot using the conditioned media from the co-culture to confirm the presence and up-regulation of TNFα and Fas-ligand proteins.
HYPOXIA PROMOTES GL261 SURVIVAL

Interestingly, the NSC-mediated GL261 suppression only occurred under normoxic conditions. No changes in proliferation were observed in the hypoxic co-culture and this corresponded with a different gene expression profile. This could be due to the decreased expression of TNFα by NSCs in the hypoxic co-culture, despite similar levels of Fas-ligand. These contrasting results also could be due to survival mechanisms initiated by the hypoxic culturing conditions. Hypoxia is an important aspect of cancer biology and has been associated with tumor progression, metastasis, and resistance to therapy. It has also been associated with poor survival in patients with CNS malignancies as well as other cancer types (Harris, 2002) (Hockel and Vaupel, 2001). Cancer cells show increased levels of HIF-1a in the cytoplasm and nucleus of many tumors and the overexpression of HIF-1a was associated with increased cell proliferation (Talks et al., 2000) (Zhong et al., 1999). Many hypoxia-inducible genes regulate processes such as cell proliferation, metabolism, and migration (Rankin and Giaccia, 2008). Hypoxia has also been shown to promote survival through NF-κB target genes. Hypoxia causes the activation of NF-κB through phosphorylation and degradation of I-κB, leading to increased NF-κB DNA binding and transcriptional activity (Koong et al., 1994).

Importantly, NF-κB is also activated downstream of TNF-R and Fas/APO-1 signaling and has a role in regulating the TNF-induced programmed cell death (Wajant et al., 2003). In addition to the TNF-signal transduction through the TNF-R1/FADD intracellular death domain that leads to apoptosis through caspase activation, TNF-
R1 signaling can also lead to NF-κB activation through TRAF2. TRAF2 is involved in recruiting IKK into the TNF-R1 signaling complex and along with RIP (receptor-interacting kinase) mediates its activation by interacting with the NEMO domain, resulting in I-κB degradation and NF-κB activation.

Fibroblasts with a RelA deletion have an inability to transcriptionally activate TNF target genes. However, this compromised TNF-mediated NF-κB activation shows increased sensitivity towards TNF-R apoptosis. This suggested that NF-κB could regulate protective mechanisms against the cytotoxic effect of TNFα. It was also shown that the anti-apoptotic protein A20 was normally induced in wildtype expressing RelA cells but not RelA-negative cells following TNFα treatment (Beg and Baltimore, 1996).

RelA-negative fibroblasts were also used to investigate the effect of NF-κB activation on TNF-induced caspase and JNK activity. NF-κB target gene Gadd45b, a member of the Gadd45 family of inducible factors associated with cell-cycle control and DNA repair, was found to be strongly induced by TNFα in wildtype fibroblasts but not RelA-negative cells. Expressing Gadd45b into the mutant cells restored viability following TNF treatment and suppressed caspase activity in NF-κB deficient cells. They also showed that JNK activity was suppressed by Gadd45b. Normally, JNK activity is only transiently increased following TNF treatment in wildtype cells, but it is prolonged in NF-κB deficient cells. The overexpression of Gadd45 impaired and
suppressed this prolonged activity, confirming that Gadd45b mediated the inhibitory effect of NF-κB on JNK activity (De Smaele et al., 2001).

From our gene expression analysis we found increased levels of Gadd45b in the Gl261 cells under hypoxic co-culture conditions. The increase in this anti-apoptotic gene could have provided protection and counteracted the apoptotic signal from Fas-ligand produced from the NSCs. It is proposed here that the death receptor activation and signaling is shifted in GL261 cells under the different culturing conditions. Under normoxic conditions, TNF-R and Fas/APO-1 activation favors the FADD signaling route to caspase activation and apoptosis. However, under hypoxic conditions, HIF-1 and NF-κB activity promote survival mechanisms through anti-apoptotic target genes. The mechanisms of NF-κB in cell survival and oncogenesis have been well documented (Baldwin, 2001). These in vitro experiments have demonstrated the cell-autonomous effects of glioma cells in responding to stressful conditions to resist programmed cell death and to promote tumor progression.

NEURAL STEM CELL IMMUNE SIGNALING AND ACTIVATION

Our survival/apoptosis data has shown a direct mode of intercellular communication between NSCs and GL261 glioma cells based on the in vitro co-culturing system. The advantages of this system were mentioned above but there are also disadvantages of this culturing system such as exclusion of the immune system and the known role of stem cells in immune signaling. However, gene
expression analysis has provided some insight into the cytokine profiles produced by NSCs and another potential mechanism for tumor suppression.

Similar to the immune signaling involved during the wound repair process, we found that many cytokines and chemokines associated with immune cell recruitment and activation were up-regulated by NSCs in the co-culture experiment. These changes were primarily influenced by the interactions with GL261s rather than the oxygen levels since the expression profiles were similar between the normoxic and hypoxic co-culturing conditions. These chemokines are known to recruit both innate and adaptive immune cells. CXCL1 and CXCL3 are ligands for receptor CXCR2 that is expressed primarily by monocytes and neutrophils, whereas CXCL9 and CXCL10 are ligands for receptor CXCR3 that is expressed primarily by T-cells. Chemokines such as CCL5 (ligand for CCR3) and CCL2 (ligand for CCR2) target a more broad range of immune cells, including macrophages, dendritic cells, and T-cells. Additional immune-related genes up-regulated by NSCs include IL-6, IFNG, and also co-stimulatory molecules involved in T-cell activation. IL-6 is an important pro-inflammatory cytokine involved in the innate immune response. It was found highly expressed by NSCs in both co-cultures. IFNG is more involved in the adaptive immune response by activating T-cells and increasing antigen processing and MHC1 expression. It was found highly expressed in the hypoxic co-culture.

Interestingly, hematopoietic stem cells (HSCs) were found to serve a similar role in pathogen detection and immune signaling. Zhao et al. found that HSCs are actively
engaged in converting danger signals such as lipopolysaccharide (LPS) into cytokine signals to regulate stress-induced hematopoiesis. This detection was mediated through TLR signaling and NF-κB activation, resulting primarily in IL-6 cytokine production (Zhao et al., 2014).

Our data also suggest a role of NSCs in adaptive immune support through the up-regulation of co-stimulatory molecules CD40 and CD80. These molecules are primarily expressed by antigen-presenting cells and used to provide a second co-stimulatory signal for T-cell activation. These findings are preliminary and need to be confirmed. However, there is some evidence that NSCs can express co-stimulatory molecules in response to inflammatory stimuli. In mouse models of multiple sclerosis, increased expression of CD80 was observed in the subventricular zone and further analysis showed that CD80 and CD86 were up-regulated by NSCs in response to inflammatory cytokines TNF and IFNG. It was also found that these NSCs could interact with and co-stimulate allogenic T-cells in vitro (Imitola et al., 2004). Taken together, we propose that another important mode in NSC-mediated tumor suppression could involve an indirect role in mediating an anti-tumor immune response. NSCs have been shown to participate in immune signaling during wound repair and our evidence here suggests a similar mechanism.

In summary, we have demonstrated two potential mechanisms for NSC-mediated tumor suppression (Figure 8). The first mechanism involves interaction between NSCs and GL261s that we showed using a co-culture system. This resulted in
reduced GL261 viability that was independent of cell-cell contact, suggesting that this mechanism was mediated by factors released from NSCs. This reduced viability correlated with the up-regulation of TNF-α and Fas-ligand by NSCs. TNF-α and Fas-ligand have cytotoxic effects on many different tumor cell lines in vitro and necrotic effects when administered in vivo (Old, 1988). Therefore, we suspect that the production of these cell death ligands could be a mechanism for NSC-mediated tumor suppression. However, this effect was not observed when co-cultured under hypoxic conditions, where we found changes to GL261 gene expression and the up-regulation of many survival genes, including Adrenomedullin, Bcl2, and Gadd45b. This is consistent with many studies reporting on the importance of hypoxia in cancer progression (Harris, 2002).

We also propose a second mechanism of NSC-mediated tumor suppression based on our findings from the NSC gene expression array. We found many immune-related genes up-regulated in NSCs following GL261 co-culture, including several pro-inflammatory cytokines and chemokines that are important for recruiting and activating immune cells. Therefore, we propose an indirect mechanism involving immune signaling that could potentially lead to an anti-tumor immune response. Further studies into the communication between NSCs, immune cells, and glioma cells are needed in order to better understand the involvement of NSCs in mediating this immune response and the potential effectiveness of promoting a stronger endogenous NSC population and the beneficial use of exogenous NSCs modified to better facilitate the immune response.
Figure 8: Model of intercellular interactions between the tumor cells, NSCs, and immune cells.
Supplemental figure 1: Preliminary alamarblue testing to compare GL261 proliferation rates with normal DMEM/10% FBS media and neurocult/EGF media (SCM) under normoxic and hypoxic conditions.

Supplemental figure 2: Preliminary alamarblue testing to determine the proper alamarblue incubation duration.
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<td>RTC</td>
<td>Reverse Transcription Control</td>
</tr>
<tr>
<td>H08</td>
<td>RTC</td>
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</tr>
<tr>
<td>H09</td>
<td>RTC</td>
<td>Reverse Transcription Control</td>
</tr>
<tr>
<td>H10</td>
<td>PPC</td>
<td>Positive PCR Control</td>
</tr>
<tr>
<td>H11</td>
<td>PPC</td>
<td>Positive PCR Control</td>
</tr>
<tr>
<td>H12</td>
<td>PPC</td>
<td>Positive PCR Control</td>
</tr>
</tbody>
</table>

**Supplemental table 1:** List of target gene included in RT-PCR array. H01-H05 lists the housekeeping genes.
REFERENCES


