To my beautiful wife and darling daughter, Melinda and Olivia
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>4</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>9</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>10</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>12</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>14</td>
</tr>
<tr>
<td>CHAPTER 1: BACKGROUND</td>
<td>16</td>
</tr>
<tr>
<td>Identity of the Neural Stem Cell</td>
<td>18</td>
</tr>
<tr>
<td>The Astrocytic Family</td>
<td>24</td>
</tr>
<tr>
<td>Neural Stem Cell Characteristics</td>
<td>27</td>
</tr>
<tr>
<td>The Relationship Between Boundary and Neurogenic Astrocytes</td>
<td>28</td>
</tr>
<tr>
<td>Reactive Neurogenic Astrocytes Following Injury or Disease</td>
<td>34</td>
</tr>
<tr>
<td>Abnormal Astrocytic Neurogenesis Following Oncogenic Transformation</td>
<td>37</td>
</tr>
<tr>
<td>Glioma Invasion and the Extracellular Matrix</td>
<td>44</td>
</tr>
<tr>
<td>CHAPTER 2: CHONDROITIN SULFATE PROTEOGLYCANS POTENTLY INHIBIT INVASION AND SERVE AS A CENTRAL ORGANIZER OF THE BRAIN TUMOR MICROENVIRONMENT</td>
<td>58</td>
</tr>
<tr>
<td>Results</td>
<td>59</td>
</tr>
<tr>
<td>CSPGs Discriminate Between Invasive and Non-Invasive Lesions</td>
<td>59</td>
</tr>
<tr>
<td>Specific CSPG Core Proteins Define the Non-Invasive Tumor ECM</td>
<td>60</td>
</tr>
<tr>
<td>Reactive Astrocyte Responses Differentiate Invasive from Non-Invasive Lesions</td>
<td>61</td>
</tr>
<tr>
<td>Microglial Responses Differentiate Between Invasive from Non-Invasive Lesions</td>
<td>63</td>
</tr>
<tr>
<td>Reducing CSPG-Mediated Inhibition Facilitates Brain Tumor Invasion</td>
<td>64</td>
</tr>
<tr>
<td>Expression of LAR Phosphatase Receptor Distinguishes Non-Invasive from Invasive Brain Lesions</td>
<td>67</td>
</tr>
<tr>
<td>Addition of CSPGs Helps to Restrict Diffuse Infiltration</td>
<td>68</td>
</tr>
<tr>
<td>MMP Profile of Invasive and Non-Invasive Lesions</td>
<td>71</td>
</tr>
<tr>
<td>CSPGs Discriminate Between Invasive and Non-Invasive Human Tumors</td>
<td>72</td>
</tr>
<tr>
<td>Methods</td>
<td>73</td>
</tr>
<tr>
<td>Cell Culture</td>
<td>73</td>
</tr>
<tr>
<td>Primary human cancer cell culture</td>
<td>73</td>
</tr>
<tr>
<td>Classic human cancer cell culture</td>
<td>75</td>
</tr>
<tr>
<td>Primary murine astrocyte cell culture</td>
<td>76</td>
</tr>
</tbody>
</table>
3 ZEB1 MEDIATES INVASION AND CHEMoresistance of GlIoblastoma

Results .................................................................................................................. 95
   Isolation and Characterization of Invasive Tumor Initiating Cells ............... 96
   Knockdown of ZEB1 Results in Decreased Invasion and Increased Chemoresistance .... 97
   Mechanism of ZEB1 Mediated Chemoresistance ........................................... 98
   The Complementary Nature of Invasion and Chemoresistance ....................... 99
Methods ........................................................................................................... 102
   Cell Culture ...................................................................................................... 102
   CFSE Loading and FACS ............................................................................... 102
   Cell Viability Assay ......................................................................................... 103
   Knockdown Experiments ................................................................................ 103
   Animal Experiments ....................................................................................... 103
   Immunohistochemistry and Immunocytochemistry ........................................ 104
   Image Acquisition and Data Analysis ............................................................. 105
   RNA Isolation and Quantitative Real-time PCR ............................................ 105
   Protein Isolation and Western Blotting ............................................................ 106
   Statistical Testing ............................................................................................. 106

4 Segregation of Human Brain Tumor-Initiating Cells and Genes 118

Results .................................................................................................................. 119
   Identification of Pertinent Brain Tumor Samples ............................................. 119
   Isolation and Expansion of Heterogeneous Target Cell Populations .............. 120
   Clonal Propagation and Comparative Analysis of Putative Target Cells ......... 122
   Segregation of Tumorigenicity Genes ............................................................... 123
Methods .............................................................................................................. 125
   Cell Culture ...................................................................................................... 125
   Immunocytochemistry ...................................................................................... 128
   Xenograft Experiments ..................................................................................... 129
   Molecular Biology ............................................................................................. 129
5 SYNTHESIS ...................................................................................................................................... 140
LIST OF REFERENCES ...................................................................................................................... 151
BIOGRAPHICAL SKETCH .................................................................................................................. 162
<table>
<thead>
<tr>
<th>Table</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>56</td>
</tr>
</tbody>
</table>
| Neural Stem-Like Astrocytes | }
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>An astrocytic neural stem cell derived neurosphere</td>
<td>48</td>
</tr>
<tr>
<td>1-2</td>
<td>The astrocytic identity of the neural stem cell</td>
<td>49</td>
</tr>
<tr>
<td>1-3</td>
<td>Stem cell characteristics</td>
<td>50</td>
</tr>
<tr>
<td>1-4</td>
<td>Cartoon depicting the four examples of astrocytic stem cells</td>
<td>51</td>
</tr>
<tr>
<td>1-5</td>
<td>Tenascin-C and CSPGs in the neurogenic subventricular zone</td>
<td>52</td>
</tr>
<tr>
<td>1-6</td>
<td>Boundary astrocytes are associated with developmentally regulated ECM proteins</td>
<td>53</td>
</tr>
<tr>
<td>1-7</td>
<td>Injury associated reactive astrocytes up-regulate developmentally regulated ECM proteins</td>
<td>54</td>
</tr>
<tr>
<td>1-8</td>
<td>Human gliomas express reactive and neurogenic astrocytic cell markers</td>
<td>55</td>
</tr>
<tr>
<td>2-1</td>
<td>CSPGs discriminate between diffusely invasive and non-invasive brain lesions</td>
<td>87</td>
</tr>
<tr>
<td>2-2</td>
<td>Reactive astrocytes respond differentially to invasive and non-invasive lesions</td>
<td>88</td>
</tr>
<tr>
<td>2-3</td>
<td>Microglial activation differs markedly between invasive and non-invasive brain lesions</td>
<td>89</td>
</tr>
<tr>
<td>2-4</td>
<td>Reducing CSPG-mediated inhibition facilitates brain tumor invasion</td>
<td>90</td>
</tr>
<tr>
<td>2-5</td>
<td>The CSPG receptor LAR differentiates invasive from non-invasive lesions</td>
<td>91</td>
</tr>
<tr>
<td>2-6</td>
<td>Co-transplantation of invasive and non-invasive tumors</td>
<td>92</td>
</tr>
<tr>
<td>2-7</td>
<td>CSPG expression inversely correlates with invasion in human clinical specimens</td>
<td>93</td>
</tr>
<tr>
<td>2-8</td>
<td>Putative model of CSPG-mediated invasion inhibition</td>
<td>94</td>
</tr>
<tr>
<td>3-1</td>
<td>Isolation and characterization of invasive tumor-initiating cells</td>
<td>107</td>
</tr>
<tr>
<td>3-2</td>
<td>Knockdown of ZEB1 results in decreased invasion and increased chemosensitivity</td>
<td>108</td>
</tr>
<tr>
<td>3-3</td>
<td>Mechanism of ZEB1 mediated chemoresistance</td>
<td>109</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
<td></td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein Succinimidyl Ester</td>
<td></td>
</tr>
<tr>
<td>Ch’ase ABC</td>
<td>Chondroitinase ABC</td>
<td></td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
<td></td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer Stem Cell</td>
<td></td>
</tr>
<tr>
<td>CS-GAG</td>
<td>Chondroitin Sulfate Glycosaminoglycan</td>
<td></td>
</tr>
<tr>
<td>CSPG</td>
<td>Chondroitin Sulfate Proteoglycan</td>
<td></td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective Tissue Growth Factor</td>
<td></td>
</tr>
<tr>
<td>EC</td>
<td>Ependymal Cell</td>
<td></td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
<td></td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-Mesenchymal Transition</td>
<td></td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
<td></td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma Multiforme</td>
<td></td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
<td></td>
</tr>
<tr>
<td>HABD</td>
<td>Hyaluronic Acid Binding Domain</td>
<td></td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic Stem Cell</td>
<td></td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparin Sulfate Proteoglycan</td>
<td></td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced Pluripotent Stem Cell</td>
<td></td>
</tr>
<tr>
<td>LAR</td>
<td>Leukocyte Antigen-Related Phosphatase Receptor</td>
<td></td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of Heterozygosity</td>
<td></td>
</tr>
<tr>
<td>MGMT</td>
<td>O-6-Methylguanine DNA Methyltransferase</td>
<td></td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
<td></td>
</tr>
<tr>
<td>NOD/SCID</td>
<td>Non-Obese Diabetic/Severe Combined Immunodeficiency</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>NSC</td>
<td>Neural Stem Cell</td>
<td></td>
</tr>
<tr>
<td>OPC</td>
<td>Oligodendrocyte Precursor Cell</td>
<td></td>
</tr>
<tr>
<td>PXA</td>
<td>Pleomorphic Xanthoastrocytoma</td>
<td></td>
</tr>
<tr>
<td>RMS</td>
<td>Rostral Migratory Stream</td>
<td></td>
</tr>
<tr>
<td>ROBO1</td>
<td>Roundabout Homologue 1</td>
<td></td>
</tr>
<tr>
<td>SEZ</td>
<td>Subependymal Zone</td>
<td></td>
</tr>
<tr>
<td>SGZ</td>
<td>Subgranular Zone</td>
<td></td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
<td></td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular Zone</td>
<td></td>
</tr>
<tr>
<td>TMZ</td>
<td>Temozolomide</td>
<td></td>
</tr>
<tr>
<td>WFA</td>
<td>Wisteria floribunda agglutinin</td>
<td></td>
</tr>
<tr>
<td>ZEB1</td>
<td>Zinc Finger E-Box Binding Homeobox 1</td>
<td></td>
</tr>
</tbody>
</table>
INVESTIGATIONS INTO THE BIOLOGY OF THE EXTRACELLULAR MATRIX IN NEUROGENESIS, BRAIN INJURY, AND CANCER

By

Daniel Joseph Silver

May 2012

Chair: Dennis A Steindler
Cochair: Brent A Reynolds
Major: Medical Sciences - Neuroscience

Despite tremendous effort, glioblastoma multiforme (GBM) remains the most pervasive and lethal of all brain malignancies. One factor that contributes to this exceedingly poor clinical prognosis is the highly invasive character of the tumor. Not a focused mass of cells, GBM is characterized by the microscopic infiltration of tumor cells throughout the otherwise healthy brain. Contrariwise, non-neural metastases to the brain, as well as select lower-grade gliomas can be relatively more treatable, specifically because they do not invade. Instead, these tumors develop as self-contained and clearly delineated lesions – in many respects, distinct and separate from the surrounding brain. With this dissertation, we first present evidence that the fundamental switch between these two distinct tumor pathologies – invasion and non-invasion – is mediated extrinsically, through the tumor extracellular matrix (ECM). Specifically, well-circumscribed, focused brain lesions are associated with a rich, heavily glycosylated, chondroitin sulfate proteoglycan (CSPG)-containing, tumor ECM, whereas no detectable CSPGs are associated with diffusely infiltrative brain tumors. Secondly, we extend our work to the intrinsic regulators of invasion and demonstrate that a
subpopulation of highly invasive and chemoresistant glioblastoma cells is maintained by the transcription factor ZEB1 (zinc finger E-box binding homeobox 1). ZEB1 is preferentially expressed in invasive glioblastoma cells, and its knockdown results in a dramatic reduction of tumor invasion as well as increased sensitivity to the chemotherapeutic agent Temozolomide (Temodar®, TMZ) *in vitro* and *in vivo*. Lastly, we present findings on the cellular identities of the stem-like tumor cells themselves that compose a single GBM. We examine the concept that GBM may not be a single disease, but rather the culmination of multiple, discrete stem cell pathologies that ultimately manifest as GBM. In all, this work represents several unique attempts to better understand the complex biology of human brain cancer.
CHAPTER 1
BACKGROUND

Most contemporary neuroscientists and neurosurgeons were educated according to the classical view that the central nervous system (CNS) is a peculiarly non-healing tissue. Severed nerve fibers generally do not regrow to form appropriate synaptic connections, and certainly no new neurons are generated after perinatal development is completed. This view is colorfully encapsulated by the oft-cited statement of Santiago Ramon y Cajal: (1928)

Once development was ended, the fonts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centers, the nerve paths are something fixed, and immutable: everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree.

The science of the future, many believe, has arrived in the form of the relatively recently discovered indigenous neural stem cells (NSC) that persist within the brain throughout life. While there was a small historical body of anti-canonical reports hinting at persistent adult neurogenesis, appreciation for the existence of NSC within the mature brain became widespread only after the publication of innovative methods to culture and expand these cells under highly specialized conditions in the form of clonal neurospheres (see Figure 1-1). Since then, there has accumulated substantial literature demonstrating both the presence of multipotent NSC across virtually all regions of the neuraxis, and persistent and functionally relevant neurogenesis within the subependymal zone (SEZ) and the hippocampal dentate gyrus in rodents and primates, including Man (reviewed in Taupin and Gage, 2002). In particular, the SEZ has been shown to generate mitotic neuronal precursors that undergo long-range migration within the rostral migratory stream (RMS) to the olfactory bulb (Luskin, 1993). While the
majority dies en route to or within the olfactory bulb (Morshead and van der Kooy, 1992), many survive to differentiate and functionally integrate as either granule cell or periglomerular cell interneurons that help modulate incoming olfactory sensory information from the nasal epithelium. Similarly, the subgranular zone (SGZ) of the dentate gyrus has been shown to contain mitotically active cells that give rise to neuroblasts that migrate out into the granule cell layer before differentiating and sending an axonal projection to the CA3 region (Seri et al., 2001; van Praag et al., 2002). Hippocampal neurogenesis is, of course, particularly germane to human neurological function, as it has been shown to be critically involved in learning and memory. Additionally, hippocampal neurogenesis has proven to be sensitive to a variety of modulating stimuli -both positive and negative- that can dramatically influence cognition (Kempermann et al., 1997; van Praag et al., 1999), suggesting that it may be possible to therapeutically augment this system to combat the memory decline that accompanies a variety of neurological diseases and insults.

The connection between NSC activity and higher-order brain function exemplified in the hippocampus, represents the root of the greatest hope associated with NSC; their potential use in cell replacement therapies. At some future point, such replacement may be accomplished either by in vitro expansion and transplantation, or by directing the migration of indigenous NSC pools towards areas of damage or cell loss. Achieving these future goals requires a deep understanding of the unique biology and activity of these cells, beginning with the most fundamental inquiries into their particular identity.

Investigations into the identity of adult NSC over the past decade have established with high confidence that a special type of astrocyte is primarily responsible for both the
persistent neurogenesis seen in vivo in the subependymal zone and hippocampus, and the formation of multipotent neurosphere clones seen in vitro. While there is still no foolproof method for prospectively identifying which astrocytes in the CNS have NSC attributes, the identification of the cell type responsible for these phenomena opens the door for detailed study of their intrinsic capacity for self-renewal and differentiation, as well as their ultimate suitability for therapeutic transplantation approaches and their potential role in the formation and pathogenesis of neoplasias. In this review, we will present a historical narrative of some of the early studies that identified the astrocyte as a likely NSC candidate. Next, we will address the largely semantic issue of whether the astrocytic NSC is a true “astrocyte”, or whether a new and distinct designation would be more appropriate. Then we will review the defining characteristics of NSC and compare these properties to those applied to the grandfather of all tissue-specific stem cells, the hematopoietic stem cell (HSC). Finally, we conclude with a discussion of the possible role of astrocytic stem cells in the genesis and progression of brain tumors.

Identity of the Neural Stem Cell

An enduring roadblock to the study of NSC biology is the lack of effective prospective markers leading to isolation strategies from either whole tissues or cell cultures. The original descriptions of techniques for culturing NSC relied on a retrospective approach. That is, a population of single cells was obtained from brain and cultured under optimizing conditions. Eventually, a percentage of these cells formed multipotent clones, proving in hindsight that the starting population harbored NSC. To prove self-renewal, this process was typically repeated with single-cell dissociates of these multipotent clones, and the formation of secondary multipotent clones was again retrospective evidence of the presence of NSC. While this approach
has been fruitful for localizing, in broad terms, the existence of NSC, prospective identification is needed if one wishes to understand and ultimately control the proliferation and differentiation of NSC and their progeny. For the sake of comparison, it is instructive to consider the HSC. Once recognized only retrospectively by their ability to reconstitute the bone marrow of myeloablated hosts, living HSC can now be prospectively identified on the basis of the selective expression of a battery of surface antigens. In fact, this method of prospective isolation now allows for the highly efficient transplantation of single HSC that are capable of fully reconstituting ablated bone marrow (Osawa et al., 1996).

As was true in the early search for the identity of the HSC, the initial investigations into the identity of the NSC began not by interrogating candidate NSC with antibodies against surface antigens, but rather by fractionating and testing subpopulations of previously identified cells within the system. Of cells within the mammalian CNS, the ciliated ependymal cell (EC) was perhaps the most “logical” NSC candidate. EC are immediately subjacent to the neurogenic SEZ, and seem, therefore, appropriately positioned to serve as NSC. More importantly, perhaps, EC have a phylogenetic history of functioning as NSC (Bruni, 1998). There is persuasive evidence that adult EC respond to injury in both reptiles and amphibians by dividing to generate replacement neurons throughout the CNS (see below for references). Moreover, EC have long been suspected of being responsible for the sporadic reports of adult mammalian neurogenesis following injury (Altman, 1962). Experimental evidence supporting the NSC role of EC in mammalian CNS was provided in one of the first functional tests of the NSC attributes of a prospectively identified neural cell type. Johansson and
colleagues (1999) examined the ability of EC to form multipotent neurospheres in vitro by pre-labeling them via diI infusion into the lateral ventricle. Upon dissociation and sorting, they then showed that labeled cells were capable of generating neurospheres with multilineage differentiation potential (i.e. neurons, astrocytes, and oligodendrocytes). Additionally, in support of their hypothesis that EC are the in vivo source of NSC, they showed that EC ringing the central canal of the spinal cord of adult rats became proliferative and generated astrocytes after spinal cord injury. These were very provocative findings, and caused quite a stir among the nascent NSC biology community. However, this paper was followed in rapid succession by a series of reports that immediately called into question the conclusion that EC are the NSC. Chiasson and colleagues, also in 1999, used mechanical microdissection to isolate the EC layer and showed that, while EC were capable of forming clonal structures, these structures were not true neurospheres since they were invariably unipotent, giving rise only to cells identified as astrocytes on the basis of GFAP antigenicity. Almost simultaneously, Alvarez-Buylla and colleagues repeated the diI labeling protocol used in the Johanssen study. Results from this study indicated that, while intraventricular diI does label the EC layer, the dye is apparently transferred to other cells either in vivo, or in vitro during the neurosphere-culturing step (Doetsch et al., 1999). Additional experiments reported in this same study, using cellular tracers that do not show evidence of intercellular transfer (i.e. rhodamine beads, and adenovirus), revealed that EC labeled via a contralateral ventricular injection of tracer failed to form neurospheres, and in fact disappeared from the cultures altogether. Neurospheres were obtained only from periventricular tissue obtained ipsilateral to the injection, and these injections invariably resulted in some
labeling of SEZ cells due to leakage of tracer from the needle penetration. Shortly thereafter, our laboratory published a study examining the ability of single dissociated EC, identified visually by their rhythmically beating cilia, to generate neurospheres (Laywell et al., 2000). Our results were concordant with those described in the Chiasson study –individual EC formed only unipotent clones of GFAP+ astrocytic cells. These negative findings, combined with the lack of persuasive confirmatory studies –even to this day- supporting the notion that EC have NSC attributes, strongly suggest that EC neither normally divide in vivo, nor generate multipotent neurospheres in vitro, and therefore are not likely to represent the NSC. These disparate results are attributable possibly to discrete transfer of dil from EC to cells of the SEZ either prior to or during the dissociation step of neurosphere culture. It is still not known, however, why Chiasson and we were able to show unipotent clone formation by cultured EC, while Doetsch –using similar culture techniques-was not.

If EC do not possess NSC attributes, the list of “logical” candidates can be reduced to the population of cells that inhabit the SEZ –the area of the greatest persistent neurogenesis and the highest density of neurosphere-forming cells (Weiss et al., 1996). Extensive ultrastructural analysis of the SEZ by the Alvarez-Buylla laboratory identified three major constituents of this region (Doetsch et al., 1997). The most abundant SEZ cells are the highly proliferative neuroblasts, termed type A-cells. Type-A cells are the neuronally committed precursors that migrate through the RMS to the olfactory bulb where a fraction of them differentiate into either granule or periglomerular interneurons. There is also a second highly proliferative cell with ambiguous phenotype, termed the type-C cell, which seems to function as a transit-amplifying intermediary
between the NSC and the migrating neuroblasts. Finally, there is a slowly dividing SEZ astrocyte, termed the type B-cell, which seems to display many characteristic of the NSC. In addition to expressing GFAP, B-cells were shown by ultrastructure to have irregular and invaginated nuclei, and their cytoplasm contained intermediate filaments and dense bodies. Subsequent experiments showed that the highly proliferative SEZ constituents can be ablated either by antimitotic drugs or ionizing radiation, but that the normal SEZ anatomy can regenerate after such depletion (Doetsch et al., 1999b; Marshall et al., 2005). By careful ultrastructural examination of the SEZ during ablation/regeneration, the Alvarez-Buylla group (Doetsch et al., 1999b) showed that the B-cell astrocyte survives the ablation, and divides to generate the C-cell. The C-cell in turn divides to generate the rapidly dividing type A-cells that migrate to the olfactory bulb. This, then, suggested that a special type of astrocyte residing within the SEZ might be the NSC responsible for both persistent neurogenesis in vivo, and the generation of multipotent neurospheres in vitro. This same group showed that cells expressing GFAP can give rise to neurons in vivo. Using a transgenic mouse that allows for the selective retroviral infection of GFAP-expressing cells with a constitutive reporter gene, these investigators showed that labeled astrocytes within the SEZ and SGZ give rise to olfactory bulb interneurons and hippocampal granule neurons, respectively (Doetsch et al., 1999a; Seri et al., 2001). At about the same time, we used the same transgenic system to show that astrocytes cultured from the adult mouse SEZ could form neurospheres capable of both glial and neuronal differentiation (Laywell et al., 2000). We also showed in this study that a subset of GFAP-expressing astrocytic cells obtained from cerebral cortex, cerebellum, and spinal cord are capable of forming
neurospheres, but only when obtained from animals younger than about postnatal day 12. Since this age corresponds closely to the disappearance of radial glia in most regions of the mouse CNS, and since the neurogenic B-cell astrocyte of Alvarez-Buylla maintains some radial glia-like morphological characteristics, we interpreted this latter result as evidence that radial glia or immature astrocytes are the in vivo representation of the NSC. Since these initial studies, there have been numerous confirmatory studies supporting the astrocytic identity of the NSC, at least in the adult, including human (Quinones-Hinojosa et al., 2006) brain. That is not to say, however, that cells with astrocyte phenotype are the only cells with apparent attributes of multipotent progenitors. Kondo and Raff (2000) published a study showing that cultured oligodendrocyte precursors (OPC) isolated from the early postnatal rat optic nerve could be induced to generate all three neural lineages by manipulation of the in vitro conditions, including the substrate and the presence of mitogens. These precursors were also able to form sphere-like structures when cultured under conditions of anchorage withdrawal, but the potency of these structures was not reported. One unusual and interesting aspect of this study is that the conversion of OPC to multipotent progenitor was observed only after the OPC were induced to differentiate into type-2 astrocytes by culturing in, and then withdrawing, serum, PDGF and BMP. Subsequently, another group reported that human white matter contains a glial progenitor cell, which can be prospectively enriched on the basis of CNP expression -a protein associated with oligodendroglial progenitor cells- and can be induced to generate neurons and multipotent neurospheres (Nunes et al., 2003). It may in fact be that, with enough experimental manipulation, many different types of cells can be
induced to acquire NSC properties. There is evidence that seemingly differentiated neurons can switch their phenotype to acquire astrocyte characteristics, including antigenic profile and membrane physiology (Okano-Uchida et al., 2004; Laywell, et al., 2005). And we have proposed elsewhere that cells exist developmentally along a continuum stretching from non-committed and multipotent to fully differentiated and functionally mature, but that there may be instances in which cell fate may move “backward” along this continuum, especially during early ontogeny (Steindler and Laywell, 2003). Therefore, it is perhaps not surprising that a variety of cells show NSC attributes in vitro. Nevertheless, a preponderance of the evidence suggests that the cell responsible for normal maintenance of the persistently neurogenic regions of the brain – the NSC - is a cell with apparent astrocytic phenotype.

The Astrocytic Family

Astrocytes that display adult NSC characteristics have persuasively been show to express the astrocytic cytoskeletal protein, GFAP (Imura et al., 2003; Garcia et al., 2004). However, these NSC also lack many of the classically accepted characteristics of astrocytes, particularly functional features such as regulation of extracellular neurotransmitter concentration. Thus, there is a growing chorus of researchers who are uncomfortable with the designation of these cells as astrocytes, and agree with the sentiment that, “It is far past time to stop calling every neural cell in the brain that is not a neuron, a glial cell” (Barres, 2003). It is true that allowing diverse and non-overlapping criteria to define a variety of astrocytic cells is unique for neural lineages. For instance, while there are many different subtypes of neurons, they can all be said to share the functional property of synaptic transmission. Similarly, a cardinal feature of oligodendrocytes is the production of myelin, and the ensheathment of axons. So has
the NSC been inappropriately identified as an astrocyte? Should we reconsider this classification, and perhaps develop new nomenclature to uniquely describe stem cells in the brain? We think not. For while we agree with Kimelberg (2003) that astrocytic identity is sometimes difficult to establish “...because it is unusually multifunctional”, we maintain that it is appropriate to expand the functional definition of the astrocyte family to include the subpopulation of cells that possess NSC characteristics for at least three reasons: phylogeny, ontogeny, and convention.

Phylogenetic analysis allows us to address the sometimes confusing phenotype of astrocytes by constructing an “astrocytic family” of cells in the mammalian brain that are functionally related to the ependymoglial cell of lower vertebrates (see Figure 1-2). In lizards and reptiles, the amazingly multifunctional ependymoglial cell is the predominant astrocytic cell type (Reichenbach and Robinson, 1994; Bruni, 1998). Ependymoglia are radially oriented throughout life, and are thought to guide the migration of neuroblasts generated near the periventricular germinal matrix. Additionally, these cells have been shown to divide to generate functional neurons following lesions of both spinal cord (Egar and Singer, 1972; Nordlander and Singer, 1978; Anderson and Waxman, 1985; Simpson and Duffy, 1994), and cerebral cortex (Molowny et al., 1995; Pérez-Cañellas and García-Verdugo, 1996; Font et al., 1997). Ependymoglia maintain ciliated connections with the ventricle, and are also responsible for phagocytic, injury response functions. Thus, it is clear that the functions encapsulated by the ependymoglial cell of lower vertebrates are subserved by an “astrocytic” family of distinct but related cells in the mammalian brain. Radial glia serve as migration scaffolds for newly-generated neurons during early development, and have themselves been shown to be neurogenic
Ependymal cells in the mammalian brain line and project cilia and microvilli into the ventricles (Spassky et al., 2005). Mature astrocytes can become “reactive” after injury, and perform some phagocytic and wound delimiting “scar” functions (Mazlo et al., 2004; Silver and Miller, 2004). Finally, as discussed above, a subpopulation of astrocytic cells within the secondary germinal matrices of the SEZ and hippocampus are persistently neurogenic throughout life.

Ontogenic analysis, too, reveals the interrelatedness of the major NSC candidates as members of the “astrocytic family”. Radial glia have long been recognized as antecedents of stellate astrocytes (Levitt and Rakic, 1980; Misson et al., 1991), and new evidence suggests that ependymal cells also develop from radial glia (Spassky et al., 2005). Expression of GFAP, at least during certain developmental stages or after injury, is also a feature shared by radial glia, astrocytes, and ependymal cells (Takahashi et al., 2003), and there is evidence that mouse ependymal cells of the choroid plexus can differentiate into GFAP-expressing astrocytes following transplantation to the injured spinal cord (Kitada et al., 2001). Additionally, we and others have shown that clones of GFAP+ cells developed from prospectively identified EC (Chiasson et al., 1999; Laywell et al., 2000).

Finally, convention suggests that we lump adult NSC features in with the multifunctionality of astrocyte, rather than split these cells into a separate category. The fact is that, prima facie, adult NSC in vitro and in vivo simply seem like astrocytes. Yes, yes, a picker of nits can and will point out this or that astrocyte feature that is missing from astrocytic NSC, but there has for years been a large umbrella over the diverse
cells that are considered “astrocytes” in the adult brain, and this has seemingly not cast a pall of confusion over the field. Type I and II astrocytes are clearly distinct morphologically and functionally, and yet both are considered to be astrocytes. Likewise, Bergmann glia of the cerebellum and Müller glia of the retina are also commonly subsumed under the astrocyte designation. We believe that the overall phenotypic gestalt of the adult NSC is that of an astrocyte, and for the sake of common understanding we should refer to it as such.

**Neural Stem Cell Characteristics**

In comparison to other tissue-specific stem cells, such as the HSC, the defining aspects of NSC are shrouded in ambiguity. This ambiguity can cause considerable confusion, and usually results in NSC biologist talking past researchers from other stem cell fields who generally have a much more rigorous definition of what constitutes a true stem cell (see Figure 1-3). In the initial glow of excitement over the descriptions of substantial adult neurogenesis and the persistence of neural stem-like cells, the actual fundamental characteristics of NSC were, for the most part, poorly delineated, and varied greatly between laboratories. For instance, within the persistent germinal matrices in vivo –the SEZ and hippocampal dentate gyrus- the overwhelming majority of stem cell progeny are neurons. Nevertheless, multilineage differentiation in the form of neurons, astrocytes, and oligodendrocytes is considered an indispensable in vitro characteristic of NSC. Self-renewal is also generally required for NSC designation, although there is considerable confusion regarding how extensive self-renewal should be in order to distinguish true NSC from long-term progenitor cells. Most investigators would accept that one round of self-renewal is not enough to determine “stemness.” But how many are required? By contrasting with the hematopoietic system, one can
see how “loose” is the definition of the functional attributes of NSC. While there is some intralaboratory variability in the antigenic profile used to select HSC from whole blood or bone marrow, there is general agreement that the HSC is defined functionally as: an extensively self-renewing cell capable of multilineage differentiation, that can fully reconstitute depleted bone marrow in a serial transplantation paradigm. This means that a single HSC transplanted into a myeloablated host must reconstitute all of the blood lineages for the duration of that animals’ life. Furthermore, it must be possible to again isolate a single HSC from such a recipient and use it to reconstitute the blood lineages of a second myeloablated host for life. Clearly, then, from the perspective of the hematopoietic field, the salient attributes of NSC may seem relatively inadequate and poorly defined. Nevertheless, while recognizing the need to clearly define the in vitro and in vivo properties that characterize NSC, we believe that each tissue-specific adult stem cell may require its own unique functional definition, and it may be inappropriate to force a simulacrum of the HSC definition onto the NSC. Indeed, to do so is to flirt with unnecessary scrupulosity given that the CNS is far more static that the hematopoietic system, and the constituent cells generally persist throughout life and are not replaced in toto.

**The Relationship Between Boundary and Neurogenic Astrocytes**

The subgranular zone (SGZ) of the hippocampal dentate gyrus and the subventricular zone, (SVZ) lining the lateral walls of the lateral ventricles, represent the only two regions within the adult mammalian brain that support ongoing neurogenesis throughout life. (Figure 1-4) Within these rare germinal niches, astrocytes, functioning as neural stem cells (Imura et al., 2003; Laywell et al., 2000), begin the cascade of events that continually renew the granule and periglomerular interneurons of the
olfactory bulb (Doetsch et al., 1999), and granule neurons of the adult hippocampus (Seri et al., 2001; van Praag et al., 2002). In vivo, the neurogenic process within the SVZ begins with a GFAP (glial fibrillary acidic protein) expressing astrocytic stem cell, or “B-cell” (Doetsch et al., 1999). These unique neurogenic astrocytes, or what we refer to as multipotent astrocytic stem cells (MASCs) (Laywell et al., 2000; Chiasson et al., 1999; Garcia et al., 2004; Zheng et al., 2006) are the direct descendants of embryonic radial glia (Merkle et al., 2004; Merkle et al., 2007) and unlike mature cortical astrocytes, maintain a thin process, including a cilium, tethering them to the lateral ventricular wall and ventricular cavity (Doetsch et al., 1997). The relatively rare and quiescent B-cells have been reported to give rise to a population of highly proliferative, however less-potent progenitor cells, referred to as transit-amplifiers or C-cells. Finally, from this putative intermediate progenitor pool, comes a population of young, immature neurons, or A-cells that migrate forward over a tremendous distance through the rostral migratory stream (RMS) before a select few mature and integrate into the olfactory bulb neural circuitry as new interneurons (Doetsch et al., 1999; Kishi, 1987; Petreanu and Alvarez-Buylla, 2002). Interestingly, a recent publication by Danilov and colleagues has challenged the canonical B → C → A cell genesis cascade, suggesting that the multipotent B-cell astrocyte may actually give rise to its neuroblast progeny directly, without the C-cell intermediate (Danilov et al., 2009). Coincidently, these results are in accord with our own in vitro model of SVZ neurogenesis, wherein cultured astrocytic stem cells are seen to robustly and directly generate a population of young neurons (Scheffler et al., 2005). Whether the B-cell astrocyte is directly neurogenic or requires a less-potent intermediary, its activity is limited to the specific regions within the adult
brain where the expression of the extracellular matrix (ECM) is greatest. Under normal circumstances, the ECM molecules CSPG, HSPG, and tenascin-C are all intensely expressed strictly within the SVZ in the adult brain. (Figure 1-4) Thus, the coincidence of enriched matrix and persistent cell genesis, although still largely un-quantified, must assuredly be of fundamental importance (Alvarez-Buylla and Lim, 2004; Kerever et al., 2007).

In the developing neonatal brain, neurogenesis is not quite so limited, yet it is still defined by a germinal ECM. Over 20 years ago, our laboratory and others described transient patterns of enriched ECM expression within the early postnatal rodent brain. These matrix patterns appeared to outline functionally different emerging brain structures and, as such, engendered significant controversy over their particular role during brain development (for review see Steindler, 1993). During the course of these investigations, a subpopulation of astrocytes was brought to light that were consistently found intimately associated with these patterns. Situated within the ECM boundary itself, these unique cells became known as boundary-associated astrocytes, or simply, “boundary astrocytes.” (Figures 1-4A and 1-6) Although the original finding characterized these astrocytes within the developing somatosensory cortical barrel field (Cooper and Steindler, 1986a; Cooper and Steindler, 1986b), (Figures 1-4A and 1-6) subsequent investigations ultimately revealed boundary astrocytes associated with patterning neostriatal striosomes and subcortical brainstem nuclei (Steindler et al., 1988), emerging olfactory glomeruli (Gonzalez Mde et al., 1993), the laminating optic tectum (Miskevich, 1999), as well as the roof plate of the embryonic spinal cord (Snow et al., 1990), suggesting that these interesting cells might be present extensively across
the entire neuraxis. At the time, we were unsure of the significance of these cells. With their characteristic hypertrophic GFAP-expressing cytoskeleton and accompanied expression of the neurite growth inhibitory molecules CSPG and tenasin-C, the boundary astrocyte intimated a strong resemblance to the so-called “reactive” astrocyte of the adult brain. Interestingly, as we will elaborate below – only now, in light of our current understanding of cytogenic astrocytes, have we begun to appreciate that the behavioral and molecular hallmarks of neural stem cells may be present in the boundary astrocyte population.

At first glance, boundary astrocytes may seem a bit out of place in the context of a stem cell story. They are certainly not, as yet, members of the neural stem cell canon. After all, although these unique cells express many of the same immunophenotypic markers commonly associated with neural stem and progenitor cells, they have not yet been isolated ex vivo and tested for their ability to repeatedly self-renew and generate clonal multipotent progeny. We believe that boundary astrocytes are potential stem cells: cells that typically do not function as stem cells but are capable of doing so under certain conditions (Potten and Loeffler, 1990). We’ve based this hypothesis on the following pieces of correlative evidence. First, similar to the B-cell of the adult mammalian SVZ (Merkle et al., 2004; Merkle et al., 2007), boundary astrocytes are directly descended from radial glia – the first genuine stem cells of the developing CNS (Hartfuss et al., 2001; Levitt and Rakic, 1980; Noctor et al., 2001). Evidence for this descent is clear based on our early morphological analyses of mouse whisker barrel boundary astrocytes. Very early on, observable only during the first weeks of postnatal development, these unique cells echo one of the hallmarks of radial glial morphology –
they secure a direct connection to the pial surface by way of a lengthy radial process. The direct descent from radial glia is significant because it hints at the possibility that the key set of intrinsic genetic and epigenetic factors required for stemness has been transferred more-or-less completely to boundary astrocytes (Sakakibara and Okano, 1997). Just as the direct descendant of the B-cell – the C-cell – is more primitive than its neuroblast progeny (Doetsch et al., 1999), might the boundary astrocyte also maintain a more primitive, stem cell-like quality than other more mature astrocytes? At this point, this aspect of our argument is more of a thought experiment than anything else. Although clearly not definitive, we are intrigued by this genealogical parallel and what it might mean regarding stemness in the population of boundary astrocytes.

It would be presumptive to state that simply having a lineage relationship with radial glia predicates stemness. After all, ventricular ciliated-ependymal cells are lineage associated with radial glia (Spassky et al., 2005) and both our own laboratory (Laywell et al., 2000) and others (Doetsch et al., 1999; Chiasson et al, 1999) have convincingly ruled-out the possibility of an ependymal neural stem cell within the brain. Interestingly, Frisen and collaborators have recently presented compelling evidence that ependymal cells, in contrast to the situation within the brain, may mediate a reactive cytogenesis following spinal cord injury (Meletis et al., 2008). Nonetheless, focusing specifically on the brain, additional evidence presented by Laywell and colleagues bolsters our argument that these unique cortical boundary astrocytes might have the capability of displaying neural stem-like character. Laywell and coworkers challenged astrocytes, isolated from various regions within the late embryonic, early postnatal, and adult mammalian CNS, with the neurosphere assay; an in vitro bioassay used to test
these tissues for the presence of neural stem-like cells (Rietze and Reynolds, 2006).
Remarkably, the authors found that until the close of the second postnatal week, the
cerebral cortex harbors a population of astrocytic cells, which display the neural stem-
cell attribute of neurosphere formation (Laywell et al., 2000). The expression of the
geriminal ECM undergoes a dramatic shift precisely in the same time period that
correlates with cortical neurosphere formation. Initially expressed broadly at high levels,
(Figure 1-5, lower inset) the matrix is progressively spatially restricted until it exclusively
defines the adult SVZ. (Figure 1-5) Put another way, cortical boundary astrocytes
retract their radial glial-like connections to the pia and undergo other aspects of terminal
differentiation in precisely the same interval of time that coincides with the end of the
critical window for cortical neurosphere formation and the withdrawal of the germinal
ECM into the adult germinal center (Cooper and Steindler, 1989). Is the boundary
astrocyte the neurosphere-forming cell of the neonatal cortex? As we stated above, the
boundary astrocyte has never been directly isolated and assessed for neural stemness;
however, taken together, these two lines of inquiry strongly suggest that given the
proper conditions, boundary astrocytes may represent a unique cortical / CNS potential
stem cell.

Above and beyond their radial glial ancestry, the idea that cortical boundary
astrocytes might be potential stem cells is motivated by the second key feature at the
center of this perspective – the highly unique makeup of the ECM environment in which
neural stem/progenitor cells are found. There is a growing body of literature that
suggests a connection between the stemness of a cell and the ECM environment in
which it is maintained (for review see Alvarez-Buylla and Lim, 2004; Nelson and Bissell,
For example, consider the adult mammalian SVZ and rostral migratory stream (RMS). The cells of the SVZ and RMS are confined within a uniquely matrix-rich environment, strongly expressing the ECM molecules HSPG and CSPG and glycoproteins such as tenascin-C (Kerever et al., 2007; Mercier et al., 2002; Thomas et al., 1996). Although the precise role played by environment in SVZ neurogenesis remains unknown, we contend that these surroundings serve to physically sequester the cells of the niche, preserving them in a state of perpetual immaturity. Similarly, boundary astrocytes of the early postnatal cortex themselves reside in an enriched matrix environment, strikingly similar to that of the adult germinal SVZ. Consider for example, early postnatal murine somatosensory cortical boundary astrocytes; these particular boundary astrocytes reside in the spaces, or septae, that will eventually demarcate the walls of the adult murine whisker barrel field. (Figures 1-4A and 1-6) In the neonatal brain, these highly patterned spaces are defined by their intense expression of certain lectin-binding glycoconjugates and confine the boundary astrocytes, as the neurogenic cells of the SVZ and RMS are confined. If the neural stem cell character of the SVZ B-cell is dependent, at least in part, on this unique primordial ECM environment, might such an environment support this same character in the boundary astrocyte? We concede that environment is, as yet, an unproven metric for stemness, especially in these days of the induced pluripotent stem (iPS) cell (Takahashi et al., 2007; Yu et al., 2007) where the intrinsic programming of a cell has cast a long shadow over less well-quantified extrinsic influences.

**Reactive Neurogenic Astrocytes Following Injury or Disease**

What becomes of the neonatal boundary astrocyte after the down-regulation of the ECM boundaries? Might there be a carry-over of these interesting cortical astrocytes in
the adult brain? One of the main hallmarks of a lesion to the adult CNS is the glial scar (for review see Silver and Miller, 2004). Interestingly, glial scarring is associated with an enriched ECM, highly reminiscent of the adult SVZ and to neonatal pattern-associated boundaries. Similar to boundary astrocytes, astrocytes within and surrounding the lesion, referred to as reactive astrocytes, are identified by exaggerated or hypertrophic GFAP-expressing processes. Additionally, reactive glia begin to express many of the same markers commonly associated with the neurogenic stem and progenitor cells of the SVZ, and to a lesser extent, the boundary astrocytes of the neonatal cortex. Further, a few – only those immediately adjacent to the lesion itself – begin to secrete the matrix proteins that define the lesion. At this point, it is crucial to note that this discussion pertains exclusively to a unique subpopulation of reactive astrocytes. Only those reactive astrocytes that directly circumscribe the lesion and adopt the aforementioned profile – cellular immaturity, proliferation, and ECM production – are germane. (Figures 1-4C and 1-7) Previously, it has been postulated that the re-expression of these primitive markers suggests these select reactive glia are potential stem cells (Steindler et al., 1998). Further, we propose that the boundary astrocytes and the specific reactive astrocytes of interest are similar, lineally related cell types. Both cell types present an immature astroglial phenotype within a common matrix environment, suggesting that both the intrinsic and extrinsic factors required for stemness are accounted for. The Götz laboratory (Buffo et al., 2008) has recently proven this postulate correct in an elegant article describing the neural stem-like attributes of reactive glia both in vitro and in vivo.
The notion of astroglial scar formation and the reappearance of boundary molecules intimated the possibility that brain injury or disease might re-induce normally highly controlled developmental programs for neurogenesis. Penetrating brain injuries result in astroglial scars that exhibit an enhanced expression of ECM proteins including tenascin-C and various proteoglycans (Laywell et al., 1992; McKeon et al., 1991; Brodkey et al., 1995). (Figure 1-7A and inset) It is now accepted that within the glial scar, there are cycling cells that exhibit an enhanced expression of astrocytic GFAP (Buffo et al., 2008). These recent results synergize with earlier studies showing reactive astrocytes exhibiting an enhanced expression of the neural stem cell marker nestin (Lin et al., 1995). There is now no question that following brain injury or disease, the release of growth factors, cytokines, and other factors from at-risk or dying cells, as well as vascular and immune-related elements, may support a reactive cyto- or neurogenesis.

In the report by Buffo and colleagues, the authors combined a powerful new conditional GLAST transgenic mouse as well as astrocyte-targeted lentiviral vectors. In doing so, they were able to discern cortical reactive astrocyte de-differentiation (“up-regulating developmental features”) in the injured adult mouse cortex. The authors showed that penetrating cortical lesions induced proliferation of the local, resident astrocyte population that contributed to reactive astrogliosis, do not proliferate, and that the adult cerebral cortex mature astrocytes lack expression of GFAP, nestin, vimentin, and tenascin-C. However, these proteins are contained in some reactive glial cells, some of which proliferate, and in more immature glia such as radial glia and postnatal glial progenitors. Because our fate mapping analysis now reveals that reactive astroglia derive from mature astrocytes, these data suggest that astrocytes exposed to injury
may indeed resume properties of glia present at earlier developmental stages (Buffo et al., 2008). Whether these cells are in fact lineage-associated with radial glia, boundary astrocytes, or B-cell astrocytes remains to be determined. However, their common developmental behaviors, including an ability to maintain proliferation and execute neurogenic programs, along with distinct developmental molecular expression patterns, suggests that they hold promise for successful CNS regeneration as well as exuberant growth, e.g. neoplasia (elaborated on below). We propose that the boundary astrocyte, the B-cell astrocyte, and the reactive astrocyte have similar if not identical phenotypes in a common matrix environment.

**Abnormal Astrocytic Neurogenesis Following Oncogenic Transformation**

Why should a cell that is responsible for protecting and facilitating neural function, e.g. by generating all of the cells of the brain during development or contributing to adult neurogenesis, change its job and contribute to tissue overgrowth? Stem cells do have an innate purpose of generating tissue, and there is no question that altered oncogene expression, loss of tumor suppressor genes, epigenetics and environmental carcinogens can result in such potent cells changing their nature and generating too much tissue. As discussed by Weissman and others (Passegue et al., 2003), it is also possible that cancer-initiating cells take on stem cell characteristics rather than starting out as stem cells gone awry, but as discussed above, certain astrocytic cells clearly exhibit a variety of structural and behavioral phenotypic changes during development and injury that creates the potential to become hyperplastic (Zheng et al., 2002).

Clonal neurospheres derived from the human brain exhibit a profound heterogeneity of stem and progenitor cells, when derived from the neurogenic SVZ and hippocampus (Suslov et al., 2002). Along with their ardent responsiveness to changes
in growth conditions, this could likewise lead to distinctive examples of cell lineage diversity of their progeny following oncogenic transformation; e.g. the varieties of cell phenotypes seen in the gliomas, especially glioblastoma. (Figures 1-4D and 1-8C, D) Glioma stem-like cells were originally discovered in our studies that exploited the same neurosphere culture approaches used for studying normal neural stem cells (Ignatova et al., 2002) and these findings were corroborated and expanded upon in numerous studies that followed showing the presence of stem cell-like, tumor-initiating cells in human GBM (Galli et al., 2004; Hemmati et al., 2003; Singh et al., 2004). In our study, as seen in studies of normal adult human brain stem/progenitor cells, cells expressed transcripts and proteins associated with stemness including tenascin-C, notch and nestin; a transcriptome indicative of common programs for growth and differentiation between normal SVZ B-cells, reactive astrocytes, and astrocytes found within gliomas. (Figure 1-8A, B) To this end, it is interesting that an anti-tenascin-C approach has been developed for treating human gliomas (Reardon et al., 2007).

In a general sense, the underlying function of any stem cell, be it a potential stem cell (Potten and Loeffler, 1990) (e.g. a boundary or reactive astrocyte) or an actual stem cell (e.g. a B-cell) is to generate tissue; however, concomitant with this function is an inherent vulnerability. Active cell division opens a cycling cell up to the possibility of accumulating mutations and aneuploid divisions – the consensus inroads to oncogenic transformation. With reactive glia, we are presented with a primitive, stem-like cell; a cell fully capable of driving cytogenesis (Brodkey et al., 1995) but outside of the self-governing confines of the adult neurogenic niches (Petreanu and Alvarez-Buylla, 2002).
In this final section, we explore the possible consequences of reactive astrocytic cytogenesis specifically related to brain cancers and the cancer stem cell hypothesis.

In 1858, the “Father of Cellular Pathology,” Rudolf Virchow began work on the Embryonal-Rest theory of cancer. Based only upon his own detailed histological analyses, Virchow appreciated similarities between the cell types present in certain adult tumors and those of the developing embryo. These likenesses lead to the conclusion that cancers were the inevitable byproducts of displaced embryonic tissues. In 1875, his student, Julius Cohnheim, extended and clarified this hypothesis. Cohnheim postulated that adult tissues harbor a residue of dormant embryonic cells, or embryonal-rests. These resting, otherwise silent cells, when made active, hold the potential to develop into cancers. These brilliantly intuitive ideas, first articulated a century and a half ago, speak directly to modern discussions of the cancer stem cell hypothesis.

There is debate within the cancer field concerning the existence and nature of so-called cancer stem cells. (for review see Passegue et al., 2003; Adams and Strasser, 2008; Vescovi et al., 2006) There are those who, similar to Virchow and Cohnheim, argue that cancer is the product of an initially stem-like cell gone awry. Although the current literature no longer refers to these cells as “embryonal-rests,” they are nonetheless resident, genetically aberrant tissue-specific stem cells. Alternatively, investigators have also entertained the idea of a once-mature, newly dedifferentiated cell that has in the process of aberrant dedifferentiation, acquired stem cell characteristics. However, considering this perspective on stem-like reactive glia, a third option emerges: rather than the novel acquisition of stem cell characteristics, in reactive
glia we may be dealing more accurately with the reacquisition of dormant stem cell properties. Further, because of this trait, we contend that aberrant reactive glia may serve as a novel biological substrate for brain cancer initiation and provides a yet unexplored therapeutic target for brain cancer research.

Bachoo and colleagues (Bachoo et al., 2002) have inadvertently provided strong evidence for a reactive astrocytic tumor-initiating cell. Working to elucidate the molecular mechanisms underscoring the most common and most devastating adult brain cancer – grade IV astrocytoma, or glioblastoma multiforme (GBM) – these investigators chanced upon a fundamental mechanism used by mature astrocytes to preserve terminal differentiation. The authors reasoned, based upon the genetic profile of an “average” GBM, that three commonly altered genes (the p16INK4a/p19ARF tumor suppressor and the EGF receptor) might be responsible for oncogenic cellular transformation and resultant gliomagenesis. Remarkably, beginning initially in mature astrocytes with a dual knockout, the authors found that the combined loss of p16INK4a and p19ARF resulted in dedifferentiation back to a more primitive, putatively stem-like state. When induced by the forced over-expression of the EGF receptor, INK4a / ARF − / − transgenic knockout mice developed a GBM-like tumor. Ironically, Bachoo and investigators perhaps wrongfully concluded that the driving force behind the tumor formation was the synergy of the three genetic mutations rather than the aberrant astrocyte cell-of-origin. As the Götz laboratory (Buffo et al., 2008) would not confirm reactive astrocytic cytogenesis for another six years we certainly cannot fault these authors such a conclusion, however, in light of current findings, we would like to offer a reinterpretation of these data. We challenge that Bachoo and coworkers essentially
generated an aberrant reactive astrocyte. They clearly established that p16 and p19 signaling plays a vital role in preserving terminal differentiation in mature glia. If these loci are compromised, the affected astroglia dedifferentiate back to a more primitive stem-like state. In the context of a lesion, the ECM surroundings presumably concentrate growth factors around these de-regulated reactive astrocytes. In conjunction, secondary EGFR expression activation exacerbates the proliferative potential of these cells, which in this case, becomes highly problematic. Ultimately, these cells are induced into a highly mitotic state, proliferating in an uncontrolled manner that ultimately manifests as glioma.

Our reinterpretation of the conclusions drawn by Bachoo and colleagues, namely that certain reactive astrocytes may serve as a substrate for oncogenic transformation, inflammatory as it may be, is not without precedent in the literature. The first line of support coincidently comes from an independent follow-up study to the Bachoo report. Using the same p16⁻ / p19⁻ / EGFR High genetic lesion model introduced in the Bachoo study, Ligon and colleagues demonstrated that ablating the basic helix-loop-helix transcriptional repressor protein Olig2 in these already genetically aberrant mice, successfully abrogated the onset of gliomagenesis (Ligon et al., 2007). Such a robust result is powerfully striking in and of itself, however, additional work championed by Chen and co-workers the following year brings these results in line with our aforementioned reinterpretation. Chen and colleagues presented a careful examination of the role played by Olig 2 in reactive astrogliosis after cortical injury (Chen et al., 2008). Strikingly, when these investigators ablated Olig 2 specifically from the GFAP+, reactive astrocyte compartment of the lesion, they noted a significant decrease in the
proliferation of these lesion-associated reactive glia. Moreover, the authors illustrate that the once proliferative reactive astrocytes affected by the Olig 2 ablation are the same nestin+, vimentin+ potentially stem-like reactive glia at the center of the argument presented here. Thus, the same genetic lesion that completely abolishes glioma tumor-formation in the p16− / p19− / EGFR High animal, confers a similar suppression of proliferation specifically to reactive astrocytes. Fraser and colleagues provided a second line of support for our re-analysis of the Bachoo findings (Fraser et al., 2004). The authors presented a careful analysis of another genetic lesion common to many cancers including glioblastoma – loss of the tumor suppressor gene PTEN. Remarkably, PTEN ablation specifically from within the GFAP-expressing astrocyte compartment generated primitive (nestin+, vimentin+), putatively stem-like reactive astrocytes with a significantly enhanced proliferative potential, along with hypertrophy of cells and enlarged brains. As noted by the authors, altering genes like PTEN can, “...render astroglial cells susceptible to neoplastic transformation or malignant progression...” Thus, reactive astrocytes have proven uniquely sensitive to many of the constellation of specific genetic aberrations commonly associated with glioma tumorigenesis, bolstering our reinterpreted hypothesis and presenting them as likely candidates for oncogenic transformation.

We have chosen to present the aforementioned study and our reinterpretation in the interests of scholarly provocation. It suggests the possibility that a reactive astrocyte, a cell that has been induced back to a more immature state, could acquire the constellation of mutations necessary for oncogenic transformation. Further, it hints at the possibility that such an aberrant reactive astrocyte could be bolstered by extrinsic
factors in its environment. However, it’s also somewhat artificial in that the glial reactivity and the acquisition of the cancer-initiating phenotype is genetically engineered rather than chanced upon spontaneously. There is an interesting clinical observation linking prior traumatic brain injury to the onset of certain brain cancers later in life. Presumably, a few of the reactive glia associated with such an injury could provide the biological substrate for transformation. Hasegawa and colleagues have made an instructive attempt to bring clarity and weight to such a clinical correlate of aberrant reactive astrocytic tumor initiation (Hasegawa and Grumet, 2003). The authors reasoned, just as we’ve done here with SVZ neurogenesis, reactive astrocytic cytogenesis should be the product of an interplay between intrinsic and extrinsic factors. As such, the output of a tumorigenic reactive astrocyte might be bolstered if presented with the germinal-like environment of a glial scar and dampened when presented with the unsupportive environment of a healthy CNS. The authors transplanted an astrocytic rodent glioma cell line (C6 glioma) into control and into previously contused rodent spinal cords. Invariably, within only 6 weeks of engraftment, the previously injured animals presented with hemorrhagic tumors whereas the uninjured controls did not. The reactive injury-associated environment thus provides a molecular niche that appears conducive for tumor growth, again containing cellular and molecular elements in common with the normal tissue-generating environment of CNS developmental (boundaries) and adult (SVZ and astroglial scar) neurogenesis.

In conclusion, we’ve presented a synthesis of the current neural stem and cancer stem cell literature while attempting to make the case for two interrelated hypothetical ideas. First, stemness is the result of an inextricable link between seed and soil —
between cell and environment. We’ve argued that tissue generation emerges from a combination of cell-intrinsic developmental programming and the primordial environment fostered by an enriched ECM. We’ve illustrated that this rare combination is present within neonatal CNS patterning boundaries, the adult SVZ, and the penumbra of an injury to the CNS – only those specific regions to which we attribute the presence of neural stem or stem-like cells, and the process of neurogenesis. Secondly, we’ve presented our interpretation of a possible consequence of reintroducing a tissue-generating cell back into the adult brain. More specifically, we’ve presented a possible ontogeny for glioblastoma; beginning from a sub-population of stem-like boundary astrocytes that may subsequently re-emerge as transformed stem-like reactive glia within the adult brain. Ultimately, the re-emergence of these immature, aberrant stem-like astrocytes may regrettably offer a substrate for neoplastic transformation. Thus, because these three cell types: the neonatal boundary astrocyte, the post-natal reactive astrocyte, and the astrocytic tumor initiator may actually be three separate manifestations of essentially the same cell, they may represent future targets for therapeutics aimed at encouraging appropriate reactive neurogenesis following injury or disease and limiting the extent of neoplastic transformation at the root of gliomagenesis.

**Glioma Invasion and the Extracellular Matrix**

Diffuse infiltration of tumor cells throughout the otherwise healthy brain is a central hallmark of the pathology of high-grade astrocytomas (such as glioblastoma multiforme, GBM, WHO grade IV astrocytoma) (Louis et al., 2007). In the most severe cases, microscopic insinuation of the tumor can involve large portions of the brain, spreading throughout the primary tumor-bearing and contralateral hemispheres. (DeAngelis, 2001) The products of this extensive invasion are diffuse collections of residual tumor
cells that go essentially untreated and remain to perpetuate the malignancy by contributing to treatment resistance and tumor recurrence (Glas et al., 2010). Conversely, certain lower-grade gliomas as well as non-neural metastases to the brain tend to form focal, space filling, but non-invasive lesions. The biological underpinnings for these two distinct behaviors – invasion versus non-invasion – remain poorly understood. The list of intrinsic regulators of invasion is extensive and includes factors related to cell-cell adhesion, proteolysis, cell-substrate attachment, and overall cellular motility that all contribute to the innate capacity of a cell to propel itself through tissue. However, the extrinsic elements involved, specifically the interplay between the invading cells and their surrounding microenvironment, are clearly also critical, however far less extensively studied. Such a complex feature of the overall tumor pathology as invasion is arguably, better thought of as a consequence of a mutual exchange between the motile cell populations within a tumor and their immediate microenvironment. Interestingly, the tumor extracellular matrix (ECM) is physically positioned at the junction between the tumor cells themselves and the cells within their surrounding environment, and as such, may be uniquely situated to serve as a critical regulator of these tumor-environmental interactions.

A central problem that hinders any investigation into invasion is the lack of pathologically accurate animal models of human glioma. The most commonly used models of high-grade, human astrocytoma rely on the transplantation of human glioma cells, which have been propagated in vitro extensively over the last 4 decades (Pontén and Macintyre, 1968). These classic cell lines have become the industry standard cellular models of human glioma both in vitro and in vivo, have been at the center of our
efforts to understand the basic biology of glioma invasion (Viapiano et al., 2005; Zhang et al., 1998), and unfortunately sorely underrepresent tumor invasion. Similar to non-neural metastases to the brain, xenografts of these “classic” cell lines (such as U-87MG, U-251MG, and the like) generate aggressively expanding, self-contained masses of cells that physically exclude resident neurons and glia, but do not invade into the adjacent tissue. In the absence of robust, pathologically accurate models of the human disease, the biological determinants underlying brain tumor invasion or non-invasion remain poorly understood. In response, our laboratory has developed a library of novel human GBM cell lines, which demonstrate remarkably invasive phenotypes in vivo. When transplanted into the brains of immune-compromised mice, the resulting gliomas circumscribe blood vessels, invade within the sub-pial space, and demonstrate a strong preference for long-distance, single-cell migration along myelinated fiber tracts. By comparing xenografts of these newly derived, invasive cell lines to “classic,” non-invasive glioma models, we have been able to re-examine a suite of cellular and molecular mechanisms, specifically associated with the tumor extracellular matrix (ECM), and overall tumor microenvironment that appears to have a profound influence over brain tumor invasion.

Chondroitin sulfate proteoglycans (CSPGs) are a diverse family of extracellular matrix (ECM) molecules. Each family member consists of a core protein, for which it is named, covalently linked to at least one long-chain chondroitin sulfate glycosaminoglycan (CS-GAG) polysaccharide (Galtrey and Fawcett, 2007). CSPGs play critical roles during central nervous system (CNS) development as arbiters of neural circuitry pattern formation. They are thought to serve as molecular barriers
against the movement of cells across the junction of two adjacent emerging structures. (Cooper and Steindler, 1986; Steindler et al., 1988; Snow et al., 1990) Additionally, CSPGs are a major component of the unique ECM microenvironment of the germinal centers of the embryonic and adult mammalian brain. They contribute to the specialized milieu that fosters the immense proliferation and cytogenesis required of the developing CNS, and maintain this association into adulthood within the rare, persistently cytogenic niches of the adult brain. (Gates et al., 1995; Thomas et al., 1996, Silver and Steindler, 2009) There is an indication that a unique variant of the core protein of one member – Brevican – of a subfamily of CSPGs referred to as the Lecticans (which also consists of Aggrecan, Versican, and Neurocan) may play a role in brain tumor invasion. (Zhang et al., 1998; Viapiano et al., 2005) However, the CS-sugar side chains, which decorate these molecules, and are responsible for their potently inhibitory properties during development, have never been addressed in the context of brain tumor invasion.
Figure 1-1. An astrocytic neural stem cell (NSC)-derived neurosphere. A fluorescent micrograph of a once free-floating neurosphere in transition to adherent culture conditions demonstrates the multipotential progeny of the sphere-forming NSC. Astrocyte and neuronal progeny are illustrated by Glial Fibrillary Acidic Protein (GFAP, Green) and β-III Tubulin (Red) immunostaining respectively. Cellular nuclei are visualized by the Hoechst stain (Blue).
Figure 1-2. A synopsis of the research that has lead to the current insight into the identity and activity of the astrocytic neural stem cell (NSC). A cartoon depiction of the relationships between the various members of the astrocytic family highlights each of the candidate astroglial cell types that have been considered as the neural stem cell. Also, from a developmental perspective, it is interesting to note that the diverse functionality of the stem-like ependymoglial cell of cold-blooded vertebrates appears to have been parceled off into individual astrocytic cells within the mammalian CNS.
Quick Reference: Comparison of the Hematopoietic Stem Cell (HSC) to the Neural Stem Cell (NSC)

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<th>Characteristic</th>
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<th>NSC</th>
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<td>Multipotential Progeny</td>
<td>Yes</td>
<td>Yes</td>
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<td>Extensive Self-Renewal</td>
<td>Yes</td>
<td>Yes</td>
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<td>Serial Reconstitution of the Germinal Niche</td>
<td>Yes</td>
<td>No (?)</td>
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<td>Consensus surface antigen profile for prospective screening and isolation</td>
<td>Yes</td>
<td>No (?)</td>
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Figure 1-3. Stem cell characteristics
Figure 1-4. Cartoon depicting the four examples of astrocytic stem cells, in vivo and in vitro, that are the focus of this review. (A) Developmental Boundary Astrocytes. “Boundary astrocytes” present in the somatosensory cortical whisker barrel system during early postnatal development. Even though it is predicted that these cells would be both multipotent and clonogenic, and give rise to neurospheres (arrow to the central culture dish), this is as yet unproven. (B) Adult Neurogenic Astrocytes. Multipotent astrocytic stem cells are present in the adult neurogenic niches of the subventricular zone around the lateral ventricle as well as in the hippocampus. These cells have been shown in numerous studies to exhibit stem cell characteristics including self-renewal, neurosphere-generation, and the ability to give rise to all three neural lineages: oligodendrocytes (black cell in center of figure); neurons (pink cell); and astrocytes (green cell). (C) Reactive Astrocytes. Injury-associated astrogial scar astrocytes attempt reactive neurogenesis following, in this case, a penetrating injury in the adult brain. These cells also exhibit multipotency, giving rise to neurospheres and all three neural lineages. (D) Tumorigenic Astrocytes. In gliomas, including in this example, a cortical glioblastoma, tumor-initiating astrocytic cells not only may give rise to the tumor mass, but also contribute to invasion and metastasis following their migration to disparate sites. Tumorigenic astrocytes also have been shown in numerous studies to give rise to multipotent neurospheres, thus exhibiting stem cell attributes including the contribution to neural cell lineage diversity as well as self-renewal.
Figure 1-5. Immunofluorescence for tenascin-C (main figure) and chondroitin sulfate proteoglycans (lower inset) in the neurogenic subventricular zone (SVZ) surrounding the lateral ventricle as seen in a coronal section through the adult mouse forebrain. In contrast to the late embryonic brain (E17) (lower inset) where chondroitin sulfate proteoglycans and other extracellular matrix (ECM) molecules including tenascin-C are intensely expressed in both the SVZ and ventricular zone (region between star in the lateral ventricle and arrow), as well as in the overlying cortex, there is little labeling in surrounding structures including the striatum, septum, and subcortical white matter. Upper Inset shows a single adult human brain neurosphere, derived from an SVZ multipotent astrocytic stem cell (MASC or B-cell) immunostained for tenascin-C. There is a dense tenascin-C matrix surrounding cells of this cultured neurosphere. (Figure adapted, with permission, from Gates et al., 1995; Thomas et al., 1996, and Suslov et al., 2002)
Figure 1-6. Boundary astrocytes and their expression of developmentally regulated molecules, e.g. tenascin-C, cordon-off developing structures and functional units throughout the neuraxis. In the example presented here, somatosensory cortical barrels are demarcated by boundary astrocytes and ECM molecules. (A) In a flattened tangential section through layer IV of the postnatal day 6 mouse somatosensory cortex, immunoperoxidase staining of the ECM glycoprotein tenascin-C reveals boundaries around all five rows of posteromedial and anterolateral subfield whisker barrels. Each barrel is 200-250 μm in diameter. (Adapted with permission from Steindler et al., 1989[66]) (B) GFAP immunolabeling of the postnatal day 6 barrel field reveals astrocytes and their processes that distribute in boundaries around forming barrel units. A single astrocyte can be seen (arrow) within a boundary (future inter-barrel septum) between two barrels. Barrel hollows (two transected blood vessels appear in two hollows) exhibit less GFAP staining, except for sparse transected radial glial processes that are more concentrated in the boundaries (appear as immunolabeled punctae). (Adapted, with permission, from Cooper and Steindler, 1986)
Brain lesions induce certain reactive astrocytes to proliferate and up-regulate developmentally regulated ECM proteins including tenascin-C in response to injury. (A) Immunofluorescence for the tenascin-C glycoprotein following a penetrating injury to the adult mouse brain (3 day survival) results in up-regulation of this ECM protein just around the injury cavity. Inset in (A) shows in situ hybridization for tenascin-C mRNA in the lesioned cortex 3 days following a stab injury, just around the injury cavity. (Adapted with permission from Laywell et al., 1992[12]) (B) Glial fibrillary acidic protein (GFAP) immunoperoxidase and tritiated thymidine autoradiography in the cerebral cortex of a postnatal week 3 mouse following a penetrating stab lesion (lower left side of the figure). In addition to birthday-identified vascular elements, GFAP+ reactive astrocytes (brown) near the injury site are also found to proliferate in response to the stab wound (black autoradiographic signal over their nuclei), that along with their expression of tenascin-C, (A) and (B) suggest possible attempts at reactive neurogenesis. (Adapted, with permission, from Laywell and Steindler, 1991)
Figure 1-8. Human gliomas, both in situ and in vitro, express reactive and neurogenic astrocytic cell markers. (A) and (B) Tenascin-C protein immunoperoxidase (A) and mRNA following in situ hybridization (B) show expression of the ECM protein in fixed tissue specimens from a human anaplastic astrocytoma. The dense areas of immunoperoxidase labeling in (A) presumably correspond to clusters of tenascin-C riboprobe-positive cells from the same tumor specimen shown in (B). (C) Two attached neurospheres derived from a human glioblastoma specimen indicate the diversity of cell types generated within and growing out from the attached spheres (β-III tubulin (green) and GFAP (blue)). (D) A single β-III tubulin+ cell from one of these spheres exhibits a morphology suggestive of migratory behavior as seen in invasive cells observed from these specimens (unpublished observations, and see migratory cells as depicted crossing the corpus callosum in Figure 1D). These glioblastoma-derived neurospheres also express tenascin-C, in addition to other markers associated with normal astrocytic stem cells. (Figure adapted, with permission, from Ignatova et al., 2002). Scale bar in C = 100 μm, D = 10 μm.
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<th>Cell Type</th>
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<tr>
<td>Boundary Astrocyte</td>
<td>A cell that expresses GFAP as well as developmentally regulated ECM molecules such as various proteoglycans (CSPG, HSPG) and glycoproteins (TN-C). Observed throughout the neuraxis at the interface between two functionally different structures during pattern formation.</td>
<td>Cooper and Steindler, 1986</td>
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<td></td>
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<td>Snow et al., 1990</td>
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<td>SVZ Neurogenic Astrocyte</td>
<td>A cell with morphological and immunophenotypic characteristics of astrocytes. Although a precise surface antigen profile is unknown, these cells are known to express nestin, GFAP, and certain ECM molecules. Reside within the SVZ and contribute to olfactory neurogenesis in vivo and give rise to clonal, self-renewing multipotent neurospheres in vitro.</td>
<td>Doetsch et al., 1999</td>
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<td>Chiasson et al., 1999</td>
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<td>Reactive Astrocyte</td>
<td>An astrocyte that up-regulates GFAP, nestin, and developmentally regulated ECM proteins in response to injury or disease. Can respond by proliferating and may become neurogenic/cytogenic following exposure to particular environmental conditions both in vivo and in vitro.</td>
<td>Reier and Houle, 1988</td>
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<td>Laywell et al., 1992</td>
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<td>Tumorigenic Astrocyte</td>
<td>A transformed astrocytic cell found within human glioma, transgenic mouse models of glioma, or in spontaneously transformed rodent SVZ astrocytes, that exhibits multipotency in vitro and contributes to tumorigenesis in vivo.</td>
<td>Uhrbom et al., 2002</td>
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<td>Quinones-Hinojosa et al., 2007</td>
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<td>Siebzenhrubl et al., 2008</td>
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Here, we present a re-examination of brain tumor invasion, with a specific emphasis on the microenvironment of the tumor, through a comparative study of non-invading glioma models with several, newly generated, diffusely infiltrating models, derived from primary human glioblastomas (hGBM L0, hGBM L1, and hGBM L19). We show that the expression of full-length, glycosylated CSPGs was strictly associated with non-invading tumors, whereas diffuse infiltration only occurred in the relative absence of proteoglycans. Moreover, we demonstrate that the abundantly present, tumor-derived CSPGs within the non-invading tumor mass induced dramatic cellular alterations and architectural rearrangements within the resident glia (astrocytes and microglia) surrounding and embedded within the lesion. These stromal cell alterations were essentially absent from the CSPG-deplete microenvironment of invasive brain tumors and may therefore serve as critical regulators of non-invasion. Importantly, we demonstrate that tumor invasion can be modulated by adjusting the amount of CSPGs that are present within the microenvironment of a brain lesion, and speculate on a novel mechanism, mediated through the ECM, and the newly identified CSPG-specific LAR phosphatase receptor, by which CSPG-expressing tumor cells are bound up within a CSPG-rich ECM and cordoned off by a dense wall of reactive glia. In total, our findings offer a novel clarification of the role played by the ECM in regulating brain tumor invasion and hint at the intriguing possibility that the tumor confining abilities of CSPG-stimulated reactive astrocytes might be harnessed in a unique and novel approach toward therapeutic intervention against glioma cell dissemination.
Results

CSPGs Discriminate Between Invasive and Non-Invasive Lesions

We compared the transplants of 50,000 cells from 2, non-invading, “standard” models (U-87MG and U-251MG) with 3 novel human GBM cell lines (hGBM L0, hGBML1, and hGBML19). As expected, the “standard” models generated a focused mass of cells with a very clear and well-defined edge. These self-contained lesions exerted a prominent mass effect, compressing and distorting the surrounding tissue, however, neither line produced any credible invasion beyond the rim of the tumor mass. Conversely, the three novel cell lines (hGBM L0, hGBM L1, and hGBM L19) each produced diffusely infiltrating tumors. In these cases, there was no discernable edge to the primary tumor mass. Rather, the tumor gradually faded at a microscopic, single-cell level into the surrounding tissue. Further, given sufficient time (4-6 weeks for hGBM L19, 10 weeks for hGBM L0, and 13 weeks for hGBM L1) each of these cell lines demonstrated long distance invasion, most commonly along myelinated fiber tracts, involving both the initially-transplanted and contralateral hemispheres, up to X mm away from the primary tumor mass. Superimposed upon the distinct cellular pathologies of these two separate brain tumor types, the extracellular matrix (ECM) associated with invasive and non-invasive tumors was markedly different. The non-invasive tumors (U-87MG and U-251MG) were each associated with an intense expression of chondroitin sulfate proteoglycans (CSPGs) within the immediate tumor microenvironment. In both cases, CSPGs precisely defined the tumor mass up to and including the sharp edge of the tumor but no further. Conversely, the invasive tumors (hGBM L0, hGBM L1, and hGBM L19) were all, surprisingly, devoid of CSPGs. (Figure 2-1) Aside from minimal (and inconsistent) expression within the necrotic cores of the primary tumor mass,
CSPGs were virtually absent from the microenvironment of the diffusely invasive tumors. Thus, these data attest to the development of a series of novel models of human GBM that each demonstrates a remarkable propensity for long-range, diffuse insinuation into the host brain tissue. Further, and to the best of our knowledge, these findings represent the first report of the near complete absence of tumor-associated matrix in the context of genuine brain tumor invasion.

**Specific CSPG Core Proteins Define the Non-Invasive Tumor ECM**

It is important to consider that, although CSPGs are commonly referred to collectively, as if it were a single molecule, in actuality CSPGs are a diverse family of proteins, related to one another by the lengthy CS-GAG polysaccharide side-chains that they all have in common. Further, although both the CS-56 antibody and WFA-lectin allow us to visualize the sulfated, CS-sugars, neither provides any information on the identity of the core proteins themselves. We therefore sought to better define the biochemical signature of the non-invasive tumor by defining the precise set of CSPG core proteins present within that unique tumor microenvironment. Using immunohistochemistry, we examined a series of 20-μm, thin tissue sections, bearing the non-invading human tumor U-87MG. The non-invasive tumors stained robustly for three of four lecticans – versican, neurocan, and brevican, as well as the CSPG, phosphacan. Aggrecan, however was markedly absent. In each case, the individual core proteins were expressed uniformly within the extracellular spaces throughout the entire tumor mass. The matching staining patterns also confirmed that the individual proteins were mixed, homogenously with one another. Further, in parallel with the CS-sulfated sugars, the staining for the core proteins abruptly stopped at the tumors outer edge. Interestingly, the absence of aggrecan represented a genuine, and unexpected
negative finding, not a failure of the antibody or the immunohistochemical reaction in general. The peri-neuronal nets (PNNs) that condensed around the cortical neurons of the contralateral, non-tumor bearing side stained brightly for the missing CSPG, confirming the validity of the immune reaction. Thus, although we will continue to refer to the CSPGs collectively, we must keep in mind that the non-invasive tumor is actually producing a constellation of overlapping proteoglycans, each with subtly different biochemical properties, which work in concert to define the non-invasive lesion.

**Reactive Astrocyte Responses Differentiate Invasive from Non-Invasive Lesions**

Interestingly, invasive and non-invasive brain lesions were associated with strikingly different responses from the astrocytes within their immediate vicinities. Astrocytes associated with non-invading tumors had been forced aside – excluded to the edge of the growing tumor. Non-invasive tumors therefore, featured a well-defined astrocyte circumscription of the tumor mass. Presumably repelled by the growing tumor, astrocytes had consequently enveloped the mass within a sphere of tightly interwoven astrocytic processes. Conversely, invasive brain tumors left the astrocytic architecture largely intact. These astrocytes formed an elaborate web throughout the entire tumor, yet by comparison, remained essentially where they had been prior to tumor implantation. (Figure 2-2) These data suggested that some factor (or factors) associated specifically with non-invading tumors – and absent from invasive lesions – potently repelled the resident astrocyte population to the outskirts of the tumor mass. Because the tumor-ECM is so astonishingly different between these separate tumor types, we reasoned that the tumor-ECM, specifically tumor-associated CSPGs, could be the factor responsible for astrocyte displacement away from a non-invading lesion. We therefore tested whether CSPGs, by themselves, could induce astrocyte repulsion. We
chose to address this concern in vitro, employing an adaptation of the spot assay first introduced as a neurite-outgrowth assay in the CNS regeneration literature. (Tom et al., 2004a) The spot assay begins with the generation of two opposing gradients of protein within the confines of a single, circular, substrate-bound spot. The growth-permissive protein laminin occupies the center of each spot and gradually tapers off approaching the edge. Conversely, the CSPG, aggrecan generates a potently inhibitory rim – defining the edge of the spot – and tapers off approaching the center. Finally, a uniform coating of laminin is applied to the remaining, unspotted growth surface. For these purposes, we have likened the spot assay to a cross section through the center of a non-invading brain lesion. Therefore, the inhibitory proteoglycan rim approximates the portion of the CSPG-rich tumor mass that makes direct contact with the surrounding astrocytes. We challenged primary, sub-cortical mouse astrocytes with spots, fashioned with progressively greater concentrations of CSPGs. Low concentrations of proteoglycan did not repel the cultured astrocytes; rather astrocytes freely crossed the rim of the spot. However, with increasing levels of CSPGs within the rim, fewer and fewer astrocytes crossed the inhibitory barrier. Effectively, abundant CSPGs induced astrocyte circumscription of the spot. It was important that we rule out the possibility that the astrocytes might be repelled not by CSPGs specifically, but merely by the growing abundance of protein at the edge of the spot. However, no astrocyte repulsion or circumscription was noted when aggrecan was substituted with equal concentrations of BSA, even at the greatest concentrations of total protein. Thus, these data suggest that CSPGs specifically, are sufficient to induce astrocyte repulsion, and by extension may explain the astrocyte phenotype surrounding a CSPG-enriched, focal tumor.
Microglial Responses Differentiate Between Invasive from Non-Invasive Lesions

We then turned our attention to the microglial component of the tumor microenvironment. Unlike astrocytes, which are spatially displaced by CSPG-rich, non-invasive brain tumors (described above), microglia uniformly populate both types of brain tumors – invasive and non-invasive. The difference here is in the activation state of the microglial cells themselves. Microglia associated with an invasive brain tumor are morphologically indistinguishable from their counterparts either in the naïve rodent brain or from the microglia that populate the healthy, non-tumor bearing regions of the transplanted host. These microglia, characterized by their ramified appearance, are considered resting, or at their lowest state of activation. Interestingly, this microglial population is clearly interwoven throughout the entire tumor, yet the abundance of surrounding tumor cells is not sufficient to promote any noticeable reaction from the cohabitant microglia. In counterpoint, the situation with non-invasive tumors is entirely the opposite. Microglia associated with non-invading lesions are all, uniformly induced to their highest state of activation. Each microglial cell within the confines of the tumor is characterized by the simplified and rounded morphology suggestive of active phagocytosis. Even more importantly, the edge of the non-invading tumor acts as a line of demarcation, separating the ramified microglia outside of the tumor, from the activated microglia within. (Figure 2-3) Thus, microglial activation derives from the non-invading tumor itself, rather than the tumor-bearing brain tissue in general. We therefore reasoned that some element, specifically associated with non-invading tumors – and essentially absent from healthy brain tissue or from diffusely infiltrating lesions – is at the center of this dramatic microglial phenotypic shift. As noted above, because the tumor-ECM is so drastically different between these separate tumor types, we
hypothesized that the tumor-ECM, specifically tumor-associated CSPGs, could be the factor responsible for microglial activation within a non-invading lesion. We therefore tested whether CSPGs, by themselves, could induce such robust and consistent microglial activation. We challenged primary, murine microglia with progressively greater concentrations of substrate-bound CSPGs deposited evenly across an in vitro growth surface. Low concentrations of proteoglycans maintained the cultured microglia in a state of rest. However, when introduced to increasingly greater levels of substrate-bound CSPGs, fewer and fewer ramified microglia could be appreciated. It could be argued that the cultured microglia were activated not by the progressively greater concentrations of proteoglycans specifically, but rather by the growing concentrations of substrate-bound protein in general. To control for this confounding factor, we replaced the CSPGs with escalating concentrations of BSA. The BSA had no discernible activating effects on the microglia, even at the highest concentrations. Thus, because CSPGs alone are sufficient to induce potent microglial activation, their strong presence within non-invading lesions, and relative absence from invasive lesions may explain the disparate microglial response between these two distinct tumor types.

**Reducing CSPG-Mediated Inhibition Facilitates Brain Tumor Invasion**

The near-complete absence of glycosylated CSPGs from infiltrative brain tumors prompted us to question whether the removal (or reduction) of CSPGs from the microenvironment of a non-invasive tumor could foster infiltration. Essentially, we sought to test whether the CSPGs themselves inhibit the escape of a potentially infiltrative tumor cell population. We initially addressed this question in vitro by capitalizing on the nature of cultured U-87MG cells to grow in spheroidal aggregates. During the initial growth phase of the U-87MG culture, the cells grow as an even
monolayer. However, above a certain critical density, and typically in the center of the growth surface, the cells coalesce into roughly spherical aggregates. We hypothesized that this tendency for self-aggregation in vitro is mediated by the presence of CSPGs and is analogous to the self-contained growth of a U-87MG tumor in vivo. Therefore, we cultured U-87MG in the presence of progressively increasing concentrations of the bacterial enzyme chondroitinase ABC (Ch’ase ABC). Ch’ase ABC selectively cleaves the long-chain carbohydrate moieties from the proteoglycan core protein and has been shown to dramatically reduce the inhibitory properties of these ECM molecules. In a dose-dependent manner, as we increased the Ch’ase ABC concentration, fewer and fewer aggregates coalesced in the U-87MG cultures. Conversely, U-87MG aggregation was unaffected by the negative control, Penicillinase – an alternative bacterial enzyme that has no biological substrate in the U-87MG culture system. (Figure 2-4) Thus, in our preliminary in vitro assessment, CSPGs directly inhibited the dispersal of the otherwise non-invasive cell line, U-87MG. These results suggest that CSPGs may actually bind the cells together into a self-contained mass. Further, this effect is specially mediated by the lengthy carbohydrate side-chains that decorate the proteoglycan core proteins.

These data indicated the intriguing possibility that CSPGs may actually constrain an otherwise infiltrative tumor cell population into a consolidated mass. Therefore, it was critical that we extend this line of questioning into the living animal. Unfortunately, the delivery of liquid Ch’ase ABC is technically impractical for long-term in vivo experimentation. The enzyme is exquisitely temperature sensitive and is rendered inactive in as little as three days at physiological temperatures. (Tester et al., 2007; Lee
et al., 2010) We therefore transduced U-87MG cells with lentiviral vectors encoding the genes for chondroitinase ABC (Jin et al., 2011) and/or enhanced green fluorescent protein (eGFP). Initially, we verified the activities of the lenti-Ch’ase ABC and lenti-eGFP expressing cells in vitro in the same aggregation assay as described above. Both transduced cell lines behaved as expected. Because of the continual production of new and active enzyme, cellular aggregation was completely abolished in the lenti-Ch’ase ABC cultures. Conversely, the control lenti-eGFP expressing cells exhibited a similar growth pattern to either non-transduced U-87MG, or the Penicillinase negative control (described above). To test the effect of Ch’ase ABC digestion on brain tumor invasion in vivo, two separate cohorts of immune-compromised, NOD/SCID animals received unilateral striatal transplants of either 50,000 U-87MG-Ch’ase ABC or U-87MG-eGFP cells (n = 6 each). After three weeks, the tumor-bearing brains were excised and examined with immunohistochemistry. The tumors derived from the lenti-eGFP expressing cells presented as self-contained, non-infiltrating lesions across the entire rostral-caudal axis of the tumor. Strikingly, the lenti-Ch’ase ABC expressing cells did exhibit diffuse infiltration at certain positions within the developing tumor. (Figure 2-4) The most robust invasion occurred at the caudal border of the malignancy; however this diffuse pathology did not extend to the main body of the tumor. This incomplete result gave us pause. What could explain this partial shift toward an invasive phenotype? We wondered whether invasion from the caudal extent of the mass was achieved, because it was the single region where the Ch’ase ABC digestion was able to outstrip the intrinsic production of new matrix by the growing tumor. Using the 2-B-6 antibody, which reveals sugar fragments left after digestion, we confirmed that the Ch’ase ABC
enzyme was active and functional in vivo. There was evidence of chondroitin sulfate “stubs” throughout the entire mass; however, only at the caudal-most position was an observable reduction of glycosylated CSPGs detected. Thus, although regionally limited, where the CSPG digestion was most successful, the resulting tumor invasion was considerable. Thus, these findings bolster the initial indications that CSPGs serve, at least in part, to constrain otherwise invasive tumor cell populations, into self-contained and focused lesions.

**Expression of LAR Phosphatase Receptor Distinguishes Non-Invasive from Invasive Brain Lesions**

We thought it interesting that the CSPGs derived from non-invasive brain lesions remained so perfectly aligned with the tumor cell mass. More specifically, we never observed the diffusion of these tumor-associated proteoglycans away from the outer boundary of a non-invasive lesion. This curious behavior stood in stark contrast with CSPGs that were directly injected into the NOD/SCID mouse brain. Intra-striatal injection of high concentrations (700 µg/mL) of aggrecan, revealed a relatively dim and widespread cloud of the CSPG. Over a three-week period – sufficient to develop a sizable non-invasive tumor – the protein had diffused extensively throughout the brain, favoring white matter pathways, moving across the midline, and deep into the subpallium along the subcortical white matter. Therefore, we wondered whether non-infiltrative tumor cells might bind themselves up within the CSPG-rich lesion through an active, receptor-mediated coupling. Using immunohistochemistry, we examined infiltrating and non-infiltrating tumors for the expression of the leukocyte common antigen-related (LAR) phosphatase receptor, a transmembrane receptor that binds CSPGs with high affinity, and contributes to the growth inhibitory properties of CSPGs.
on axon outgrowth. (Fisher et al., 2011) Interestingly, the LAR phosphatase receptor was intensely expressed by non-invading lesions, and was entirely absent from diffusely infiltrating tumors. (Figure 2-5) Further, expression of the LAR receptor was perfectly coincident in space with the CSPG expression pattern. Appropriate for a transmembrane protein, the outer membrane of each cell populating the mass could be discerned by its intense staining for the receptor, and the receptor was uniformly expressed throughout the entire non-invading tumor. Additionally, in the same way that the edge of the tumor precisely defined the space occupied by the CSPGs, the edge of the tumor also provided the outer limit for the expression of the LAR receptor.

Conversely, at immunohistochemical levels, diffusely infiltrating tumors – characterized by the absence of microenvironmental CSPGs – were also devoid of LAR receptor protein expression. Thus, these results suggest that that non-invasive tumor cells actively bind themselves to their own CSPG-rich ECM and in turn, may hold the CSPGs in place. However, these data hold greater implications for the pathogenicity of invasive brain tumors. These data intimate that an inhibitory matrix, providing that one could be established, may not actually be sufficient to constrain a diffusely infiltrative brain tumor.

**Addition of CSPGs Helps to Restrict Diffuse Infiltration**

Since removing CSPGs from a non-invasive lesion helped to facilitate invasion, we wondered whether the addition of proteoglycans could be used to constrain an invasive tumor. Although a potentially appealing option from a translational / therapeutic perspective, our aforementioned LAR phosphatase receptor data immediately brought this possibility into question. Without the appropriate receptor, how could the presence of additional matrix corral an otherwise invasive tumor cell population? We hypothesized that the reactive astrocytes that normally respond to and circumscribe a
non-invasive lesion might be recruited to constrain an invasive tumor. We initially attempted to establish a reactive astrocyte barrier around a developing invasive lesion by injecting the tumor cells suspended within a concentrated solution of CSPGs. 50,000 hGBM L19-eGFP cells, suspended in a 700-µg/mL-aggrecan solution were injected into the striata of four separate NOD/SCID mice. An additional three animals transplanted with hGBM L19-eGFP cells alone as well as three animals injected with the concentrated aggrecan solution alone were brought to bear as comparative controls. Unfortunately, this paradigm failed to elicit any change in the invasive pathology of the tumor. As described above, rather than remain in position at the engraftment site, the initially highly concentrated aggrecan solution diffused rapidly. This widespread dissemination greatly diminished the potency of the CSPGs at any given point, and as such clearly failed to establish a focus away from which astrocytes might have withdrawn. We therefore reasoned that such a drastic microenvironmental re-organization required a tightly focused point of intense CSPG concentration, a highly reactive point of induction, away from which astrocytes would retreat.

Coincidently, because the effect we were looking for was the natural consequence of a non-invasive lesion on the brain, we reasoned that co-transplanting the infiltrative tumor line, hGBM L19 along with the non-invasive tumor, U-87MG, might provide the right amount of matrix, in the right location, and help to clarify the role played by the enveloping reactive astrocyte population. It was important that we control for the possibility that these two tumor lines might proliferate at different rates within the living brain. This was not the case in vitro; however cell division rates in culture may or may not predict proliferation rates in vivo. Observing an invasive tumor cell population
strictly within the bounds of a larger, non-invasive mass might indicate that the non-invasive tumor is capable of sequestering the invasive cells. However, we would expect this same result if the growth rate of the infiltrative tumor was greatly outstripped by that of the non-invasive mass. Therefore, three separate cohorts of mice (n = 3 each) received intra-striatal transplants of 50,000 total tumor cells comprised of the invasive line hGBM L19, transduced with a lentiviral vector encoding firefly-luciferase (hGBM L19-f-Luc), in addition to the non-invasive tumor line U-87MG, expressing eGFP (U-87MG-eGFP). One cohort received equal parts invasive to non-invasive cells (1:1), the second cohort received double the number of invasive to non-invasive cells (2:1), and the third cohort received half the number of invasive to non-invasive cells (1:2). Additionally, we allowed enough time (3 weeks post-transplant) for the tumors to develop, such that an unencumbered invasive tumor would be capable of long-distance infiltration into the corpus callosum, approximately to the midline of the brain. Remarkably, in each case, the non-invading mass completely sequestered the invasive tumor cell population. Visualized using immunohistochemistry, a large, well-defined mass occupied the majority of the striatum. The f-Luc-expressing, invasive cells presented as collective tendrils of cells, swirling within the larger space-filling mass. These tendrils approached and, in some cases skirted the edge of the larger mass, however, we did not observe any cells actually escape the confines of the non-invasive lesion. (Figure 2-6) Because invasive brain tumors lack the appropriate receptor to bind CSPGs, these data suggests that the enveloping reactive astrocytes may not be mere bystanders in this situation. Rather, these observations imply that the enveloping
astrocytic barrier around the outer, non-invading lesion may actually serve as a physical barrier against the release of invasive cells beyond the edge of the tumor.

**MMP Profile of Invasive and Non-Invasive Lesions**

We are accustomed to thinking about tumor invasion from the perspective of epithelial cancers, which invade (and may subsequently metastasize) only after the breakdown of an initially present, inhibitory ECM. The idea that this barrier may be essentially absent from high-grade glioma represents a significant departure from the generally held view of tumor invasion. The matrix metalloproteinase (MMP) family of proteins, specifically MMPs-2, 9, and 14, are considered the agents responsible for the breakdown of this matrix barrier in the context of non-neural tumor invasion. (Wolf et al., 2007) Similarly, based largely on efforts involving “classic,” CSPG-rich experimental models, this same set of MMPs has been associated with the diffuse infiltration of high-grade gliomas. We wondered how these findings might translate to our diffusely infiltrative brain tumors, where the inhibitory matrix is essentially absent. Using gelatin zymography, we examined the soluble protein fraction of tumor-bearing hemispheres from animals previously transplanted with either invasive (hGBM L19) or non-invasive (U-87MG) tumor cells for functional MMP-2 and 9. In order to minimize the amount of potentially confounding rodent host protein present within the protein lysates, we harvested tumors strictly from long-term surviving animals, in which the tumors occupied large portions of the excised hemispheres. We also included protein isolated from the same hemisphere of a naïve, age-matched animal as an additional negative control. Interestingly, we found no detectable MMP-2 or 9 activity from protein isolated from either the diffusely infiltrating hGBM L19 tumor, or the naïve host brain; however, functional MMP-2 was evident within the CSPG-enriched, non-invasive U-87MG tumor.
We confirmed these findings using immunohistochemistry. Representative tumor bearing sections from invasive and non-invasive tumor transplants corroborated the strong presence of MMPs within the non-invasive U-87MG tumor as well as their absence from each of our diffusely infiltrating gliomas. These data suggest that unlike epithelial tumors and non-invasive glial tumors, diffusely infiltrative gliomas, which are intrinsically free of an inhibitory ECM, do not require MMP-mediated proteolysis for successful insinuation into the brain parenchyma. Additionally, these data rule out the possibility that CSPGs may have been produced by the tumor only to be immediately proteolyzed – and presumably, therefore undetected – by overly abundant and highly active MMPs.

**CSPGs Discriminate Between Invasive and Non-Invasive Human Tumors**

Because these data support a somewhat contrary model of glioma invasion – one without the prerequisite breakdown of a proteoglycan rich ECM – it was critical that we validate our experimental findings with genuine human, neuro-pathological specimens. We hypothesized that, similar to U-87MG, low-grade (WHO grades I or II), relatively non-invasive gliomas would present with a CSPG-rich tumor ECM and well-defined edges circumscribed by reactive astrocytes. Further, analogous to our experimental hGBM lines, infiltrative gliomas would present with negligible micro-environmental CSPGs and widely distributed reactive astrocytes throughout the tumor-bearing tissue. We examined thin sections of archival, paraffin-embedded samples of tumor-bearing tissue from neurosurgical resections using standard histology and immunohistochemistry. In parallel with our experimental findings, the low-grade benign glioma, pleomorphic xanthoastrocytoma (PXA, WHO grade II astrocytoma, n = 5) presented with clean borders and abundant reactivity for the CSPG-specific antibody
CS-56. CSPG immune-reactivity was distributed uniformly throughout the tumor mass, precisely defining the boundary of the PXA, and by default, established a line of demarcation separating the benign tumor from the healthy adjacent brain tissue. (Figure 2-7) We also observed a curious difference in the CSPG expression patterns between the human specimens and the experimental U-87MG xenografts. Within a given section, CSPGs were evident within the extracellular space surrounding each cell, uniformly spanning the entire cross-sectional area of the tumor. Within the PXAs however, the protein was expressed in ribbons, woven intricately throughout the tumor although not surrounding each and every hyperplastic cell. Additionally, in accord with our experimental results, CSPG expression in high-grade, aggressively infiltrative GBM (n = 5) samples was negligible. We did observe some subtle CS-56 immune-reactivity within pseudo-palisading necrotic sites; however, this staining was likely non-specific. Regardless, the intensity of the stain in the non-invasive PXAs far outstripped any questionable staining in the GBM samples. In total, these data help to validate our experimental findings and assert that our novel human glioma cell lines reflect the biology of brain tumor infiltration better than the standard, “classic” models, which dominate the literature. Additionally, these data provide critical support for the novel concept that genuine glioma invasion occurs in the absence of a proteoglycan-rich inhibitory ECM.

**Methods**

**Cell Culture**

**Primary human cancer cell culture**

hGBM L0 (derived from a 43-year old male) and hGBM L1 (derived from a 45-year old female), both classified as WHO grade IV astrocytomas, were initially established as
described by Deleyrolle and colleagues. (Deleyrolle et al., 2011) hGBM L19, a pediatric glioblastoma cell line, was derived from a 9-year old male, characterized as a WHO grade IV astrocytoma. The complete account of the isolation and cultivation of hGBM L19 from the original patient tumor specimen is beyond the scope of this report, but will be elaborated in a forthcoming article by Scheffler and colleagues. Briefly, the tumor specimen, collected in accordance with the University of Florida Institutional Review Board (IRB), was subdivided into multiple smaller micro-explants roughly 1-mm3. These micro-explants were then evenly distributed across a poly-L-ornithine (15 µg/mL, Sigma-Aldrich, St. Louis, MO) coated culture surface and sustained by a minimal volume — sufficient to suffuse the substrate without enabling the micro-explants to float — of N2 growth medium supplemented with fetal bovine serum (FBS, 5% v/v, Atlanta Biologicals, Lawrenceville, GA), the mitogens recombinant human epidermal growth factor (rhEGF, 10 ng/mL, R&D Systems, Minneapolis, MN), recombinant human fibroblast growth factor (rhFGF-basic, 10 ng/mL, R&D Systems), recombinant human leukemia inhibitory factor (rhLIF, 1 µL/mL, Millipore, Billerica, MA), and the glycoprotein natural mouse laminin (1µL/mL, Invitrogen, Carlsbad, CA). The N2 growth medium itself consists of Dulbecco’s Modified Eagle Medium with F-12 Nutrient Mixture (DMEM/F12, Invitrogen) bolstered by recombinant human Insulin (5 mg/L, Millipore), recombinant human Transferrin (100 mg/L, Millipore), Sodium Selenite (NaSel, 30 nM, Sigma-Aldrich), Progesterone (20 nM, Sigma-Aldrich), Putrescine (100 µM, Sigma-Aldrich), Bovine Pituitary Extract (BPE, 2.5 mL/L, Invitrogen), and antibiotic-antimycotic solution (abx, 1X, Invitrogen). Taking great care to preserve the attachment of the explanted tissue to the growth surface, the volume of medium was gradually increased
over an approximately weeklong period. During this time, tumor-derived cells began to emigrate from the micro-explants onto adjacent portions of the growth surface and proliferate. At weeks end, the tissue fragments were discarded and the growth medium was exchanged. Complete medium, including mitogens and laminin was exchanged every other day until the culture reached 85 – 90% confluence. The cells were then culled and cryopreserved, without further passaging, for future use in subsequent studies.

During the course of these studies, the three primary human cancer cell lines – hGBM L0, L1, and L19 were each propagated under the following adherent culture conditions. A single-cell suspension of 50,000 cells/mL was seeded evenly across a poly-L-ornithine-coated culture surface and nourished by N2 growth medium supplemented by FBS (5% v/v) and the mitogens rhEGF, rhFGF, and rhLIF. Note: the soluble laminin included as a supplement to N2 medium during the initial isolation of hGBM L19 was omitted during these investigations. Medium and supplements were exchanged every other day until the cultures reached 85 – 90% confluence. Approximately once every 7 days, cultures were passaged using trypsin-EDTA (0.25% trypsin, 1 mM EDTA, Atlanta Biologicals) and filtered through a 70-µm Nylon cell strainer (BD Biosciences, Bedford, MA) in order to re-establish a single-cell suspension before re-seeding at 50,000 cells/mL in N2 growth medium.

**Classic human cancer cell culture**

During the course of these studies, both U-87MG and U-251MG were propagated in Minimum Essential Eagle Medium (EMEM, ATCC) supplemented with FCS (10% v/v) and abx according to protocols established by ATCC with the following modification. Between each passage, the cellular suspension was filtered through a 70-µm Nylon cell
strainer in order to re-establish a single-cell suspension before re-seeding at 50,000 cells/mL in complete growth medium.

**Primary murine astrocyte cell culture**

The method employed herein for the isolation and cultivation of primary astrocytes was adapted from our previous efforts generating astrocyte monolayer cultures from the early postnatal rodent brain (Laywell et al., 2000). Briefly, a block of tissue containing the left ventricles, septum, and bilateral striata was prepared from neonatal (postnatal days P4 to P8) C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME). Typically, tissues from three to four animals were culled and processed collectively for a single astrocyte monolayer culture. A single-cell suspension was prepared from the collected blocks of tissue by way of a series of mechanical and chemical dissociation techniques. The tissue, minced roughly with a sterile razor blade, was incubated in trypsin-EDTA in a 37°C water bath for 5 minutes. The cell suspension was then thoroughly triturated through a series of progressively smaller diameter pipettes and filtered through a 70-µm Nylon cell strainer. Finally, cells were seeded evenly across a 25-cm² growth surface and nourished by N2 medium containing FBS (5% v/v) and the mitogens rhEGF and rhFGF. Medium and supplements were exchanged every other day until the cultures reached 85 – 90% confluence. Approximately once every 7 days, cultures were passaged using trypsin-EDTA and filtered through a 70-µm Nylon cell strainer in order to re-establish a single-cell suspension before re-seeding at 50,000 cells/mL in complete growth medium. Astrocytes were maintained in culture strictly through four subsequent passages from their initial isolation. Thus, the astroglial spot assays (see below) were all conducted using cells from these first four initial passages in vitro.
Primary murine microglial cell culture

The blocks of tissue used to generate the astrocyte monolayer cultures (described above) also contain a limited population of primary microglia. In order to expand and gain access to this initially minor cellular fraction, we apply a protocol, devised by Marshall and colleagues (Marshall et al., 2008), that employs the cytokine, Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) to selectively drive proliferation within the microglial cell pool. Briefly, either primary or first passage astrocyte cultures were grown to 95 – 100% confluence. At this point, the standard N2 growth medium was replaced with microglial proliferation medium (MPM). MPM consists of N2 growth medium supplemented by FCS (10% v/v), abx-solution, and GM-CSF (20 ng/mL, Stem Cell Technologies, Vancouver, BC). The MPM was exchanged every other day for an approximately weeklong period during which time, phase-bright, spherical microglia gradually accumulated either directly atop the astrocyte monolayer or floating within the culture medium. At weeks end, the culture flask was gently agitated at room temperature for 10 – 15 minutes to lose the cells that were directly attached to the astrocyte monolayer. Lastly, the detached microglia, suspended within the culture medium were culled and processed for further experimentation and analysis.

Genetic Modification of Cancer Cell Lines

Subsets of U-87MG were transduced with lentiviral vectors encoding the genes for either chondroitinase ABC (LV-Ch’ase ABC) or enhanced green fluorescent protein (LV-eGFP). (Generous gifts from Dr. George Smith, University of Kentucky and Dr. Lung-Ji Chang, University of Florida, respectively). 50,000 cells/mL were plated evenly across 6 wells of a sterile 6-well plate. Overnight incubation ensured the firm attachment of the cells to the culture surface while maintaining an appropriately low density (30 – 50%
confluence) for optimal transduction efficiency. The following day, the medium was exchanged with a minimal volume (500 µL/well) of virus-containing medium, which consisted of complete growth medium charged with 10µg/mL polybrene (Sigma) and the virus solution. Each well of the six was treated separately and two were reserved as experimental controls. The first control – a non-transduced control – was treated to standard growth conditions, whereas the other was used as a polybrene control. The other five received a progressively increasing viral titer, from 10 to 100 multiplicity of infection (MOI). The plate was removed from the incubator once every hour, for the following three hours for gentle agitation, then left alone overnight. The following day, additional growth medium was added without removing the initial virus-containing medium. The medium was finally exchanged with standard U-87MG growth medium on the third day and every other day thereafter until the culture reached 85 – 90% confluence. The single well of transduced cells with the highest MOI that demonstrated a growth rate approximately equivalent to the non-transduced control was carried forward and expanded for subsequent experimentation.

**Aggrecan – Laminin Spot Gradient Assay**

Tom and colleagues originally conceived the spot assay (Tom et al., 2004a) as an in vitro model of glial scarring and neuronal regeneration. In order to apply this assay to the astroglial compartment of the non-invasive brain tumor microenvironment, we have had to modify both the formulation of the spot solutions as well as the schema for interpreting and quantifying the results. First, regarding the basic assembly and structure of the assay, we began by treating poly-L-ornithine (15 µg/mL, Sigma) coated, 18-mm glass coverslips with a volatile solution of nitrocellulose (1.6 cm² fragment dissolved in methanol; Bio-Rad, Hercules, CA). As the solvent evaporates away, a
uniform coating of nitrocellulose is deposited across the culture surface, which in turn facilitates the binding of the aggrecan and laminin proteins into a tight circular spot. It is important to note that spots can be prepared without substrate-bound nitrocellulose; however, in these cases, the spots tend to dry with irregular, undulating edges, which can confound interpretation and downstream quantification. The opposing gradient spots themselves were established by placing 4, 3-µL droplets of an aggrecan (10 – 250 µg/mL, Sigma) and laminin (10 µg/mL, Invitrogen)-containing solution in DMEM/F12 onto the pre-treated glass substrate in a roughly square-shaped pattern and allowed to dry completely. Each aggrecan / laminin solution was used to prepare 12 redundant spots across three separate cover glasses. Additionally, negative control spots consisting of BSA (25 – 250 µg/mL, Sigma) and laminin (10 µg/mL) as well as laminin-alone (10 µg/mL) were also included and prepared in parallel with the experimental groups. Once the spots had dried, the entire culture surface was suffused with a solution of laminin (10 µg/mL) in DMEM/F12 and incubated at 37°C for 3 hours. Finally, the laminin bath was gently aspirated and 50,000 primary murine sub-cortical astrocytes suspended in standard astrocyte growth medium (described above), were distributed evenly across the spot-treated surface. The medium was exchanged every other day until the culture reached 85 – 90% confluence within the non-spotted portion of the cover glass. The completed assay was then fixed in warm 4% formaldehyde for 30 minutes and prepared for subsequent immunocytochemical staining. The assay was bathed in blocking solution consisting of fetal bovine serum (FBS; 10% v/v), goat serum (GS, 5% v/v, Vendor Information), horse serum (HS, 5% v/v, Vendor Information), and Triton X-100 (0.1% v/v, Vendor Information) dissolved in phosphate buffered saline.
(PBS, Vendor Information) during an initial 4°C, overnight incubation. The assay was then immuno-stained at 4°C, overnight for the antigens glial fibrillary acidic protein (GFAP; 1/800, Dako, Glostrup, Denmark) and CS-56 (1/200, Sigma). The primary antibodies were coupled to appropriate Alexa Fluor 488 (1/500, Invitrogen) or 555 (1/500, Invitrogen) – conjugated secondary antibodies during a final overnight incubation. Lastly, the assay was overspread with a blocking solution containing the nuclear dye 4’,6-diamidino-2-phenylindole (DAPI; 0.1µg/mL, Sigma) for 10 minutes at room temperature. The cover glasses were mounted to glass microscope slides within a fluorescence protecting mounting medium (Vectashield, Vector Laboratories, Burlingame, CA) and examined using a Leica DMLB standard fluorescent microscope. In order to quantify the spot assay, 6 images were captured around the CSPG-rich rim of each spot at 100X magnification at approximately the 1, 3, 5, 7, 9, and 11 o'clock positions. The total number of astrocytes with DAPI+ nuclei in the outer-spot region, which extended processes across the rim and into the penumbra of the spot were tallied using the Cell Counter plugin for the open-source software package ImageJ (National Institutes of Health, USA). It is important to note that, even at the highest concentrations of proteoglycan, a subset of astrocytes consistently grew within the core of the spot where the effective CSPG concentration is far lower than at the rim. As we never encountered this situation in vivo – astrocytes growing within the confines of a non-invasive tumor – these inner spot astrocytes were disregarded from quantification.

**Microglial Activation Assay**

We began assembly of the microglial activation assay by treating 18-mm glass coverslips with poly-L-ornithine (15 µg/mL, Sigma) at 37°C for no less than 18 hours. Once this initial coating was established, an additional coating – which served as the
direct substrate for the assay – of laminin (10 µg/mL) and the CSPG aggrecan (25 – 250 µg/mL in DMEM/F12) was added over a second 3 hour, 37°C incubation. Additionally, negative control conditions consisting of BSA (25 – 250 µg/mL, Sigma) and laminin (10 µg/mL) as well as laminin-alone (10 µg/mL) were included and prepared in parallel with the experimental groups. Three redundant cover glasses were prepared for each experimental and control condition. Once the topcoat was secure, these solutions were gently aspirated and replaced by 50,000 primary murine microglia per well suspended in microglial proliferation medium (detailed above). Media was exchanged every other day for 5 days before a 10-minute fixation in warm 4% formaldehyde. The completed assay was blocked in detergent-free blocking solution (10% FCS, 5%GS, 5%HS in PBS) overnight at 4°C before a 48-hour, 4°C immuno-staining for the microglial surface antigen CD11b (1/200, BD Pharmingen, San Diego, CA). The primary antibody was coupled to an appropriate Alexa Fluor 488 (1/500, Invitrogen) – conjugated secondary antibody during a final overnight incubation and finished with DAPI.

In order to quantify the microglial activation assay, an observer, blinded to the substrate conditions of the assay, first surveyed the entire culture surface of each cover glass with a series of 10 non-overlapping images, at 100X magnification. Employing the Cell Counter plugin for the open-source software package ImageJ (National Institutes of Health, USA), the blinded observer was then instructed to score each cell as either ramified or activated based on its unique cellular morphology. For these purposes, “ramified” microglia must have displayed a complex amoeboid structure with three or more elaborate peripheral extensions. By comparison, “activated” microglia must have
been essentially spherical, simple cells without elaborate processes. However, in any given visual field, a subset of microglia presented a morphology that was difficult to classify. These so-called “pseudo-ramified” cells fell into the center of the morphological spectrum between the extremes of microglial activation and rest. Because the incidence of incomplete microglial activation in vivo was negligible, these partially activated cells in vitro were designated as “activated” to maintain the objectivity of the scoring system.

**In Vitro Tumor Dispersal Assay**

50,000 cells/mL of U-87MG were plated uniformly across the wells of an untreated, sterile 6-well plate in standard growth media. Two redundant wells were seeded for each experimental and control condition. Immediately thereafter, either chondroitinase ABC (0.025 U/mL – 0.1U/mL) or the negative control enzyme penicillinase (0.025 U/mL – 0.1U/mL) was added to the culture media. Complete growth media, including the proper enzyme was exchanged every other day in order to compensate for the thermolability of Ch’ase ABC at 37°C. Cultures were maintained until the 0.1U/mL Ch’ase ABC-treated wells reached 85 – 90% confluence. Using a Leica DM IRB inverted microscope, outfit with a Leica DFC 300F digital camera, a series of two to four low magnification (25X), phase contrast images of the aggregate-occupied area of the growth surface of each well were captured. The Photomerge automation for Photoshop CS4 (Adobe Systems, Inc., San Jose, CA) was then used to reconstruct the entire aggregate-occupied area into a single image. Finally, the total number of visible, phase-dark aggregates was quantified using the Cell Counter plugin for the Image J open-source software package (National Institutes of Health, USA).
Intracranial Transplantation

Surgical procedures

All animal procedures were conducted in accordance with protocols previously vetted and approved by the University of Florida Institution Animal Care and Use Committee (IACUC). Adult female NOD/SCID mice (Charles River Laboratories, Wilmington, MA) were induced into a surgical plane of anesthesia using inhaled USP-grade isoflurane (2 – 2.5%; Halocarbon, North Augusta, SC) in oxygen (2L/min). Once the animal was transferred to the stereotaxic apparatus, a sterile field was established and the skull was demonstrated. A burr hole was drilled 0.5-mm rostral and 1.8-mm lateral of bregma. A 33-guage, stainless steel needle (Hamilton, Reno, NV) was lowered 2.5-mm beneath the surface of the brain and 50,000 cells suspended in 1 µL of sterile culture medium were slowly injected over the course of approximately 5 minutes. The needle was held in place for an additional 5 minutes before gentle and measured retraction from the brain. Finally, the scalp incision was closed with sterile staples.

Post-operative survival time varied for each tumor model under scrutiny. However, in each case, it was important that animals were culled before the tumor mass had become so large that the tumor microenvironment could no longer be appreciated. As such, animals transplanted with the “classic” glioma cell lines U-87MG and U-251MG survived in a University of Florida vivarium for no longer than 5 weeks, whereas the primary glioma cell cultures hGBM L19, hGBM L0, and hGBM L1 survived for 4 – 6, 12, and 13 weeks respectively.

Tissue preparation and immunohistochemistry

At the close of the post-operative period, all animals were anesthetized and transcardially perfused with cold 4% formaldehyde (Sigma) in PBS. The intact brain
was carefully removed and immediately transferred to a fresh volume of 4% formaldehyde for overnight post-fixation. The tissue was then immersed in a solution of 30% sucrose (Sigma) in PBS until equilibrium was achieved and the tissue had sunk to the bottom of its container. A second, overnight immersion in a 1:1 solution of 30% sucrose : O.C.T. Compound (Tissue-Tek, Torrance, CA) completed cryo-protection and readied the tissue for frozen sectioning. Finally, the tissue was embedded in O.C.T. Compound, frozen, and 20-µm coronal sections were prepared on a Leica CM 1850 or a Microm HM 505E cryostat. Subsets of these tissue sections were culled for immunohistochemical analysis against a battery of antigens specific to the experiment and question under study. Tissue sections were immuno-stained in detergent-containing blocking solution according to the procedure described above for the aggrecan – laminin spot gradient assay. A detailed list of the antibodies employed can be found in the supplementary data. In all cases, primary antibodies were coupled to appropriate Alexa Fluor 488 (1/500, Invitrogen) or 555 (1/500, Invitrogen) – conjugated secondary antibodies.

**Quantification of in vivo tumor invasion**

20-µm thin sections were initially immuno-stained according to the protocol described above. The xenografted human glioma cells were visualized in green, using an antibody raised against Human-specific Nestin (hNestin, 1/1000, Millipore, Billerica, MA). Additionally, in order to examine the tumor in context, the murine host tissue was stained in red with an antibody raised against β-III Tubulin (1/1000, Promega, Madison, WI) and nuclei were visualized with DAPI (0.1µg/mL, Sigma). The stained sections were mounted onto Colorfrost Plus microscope slides, (Fisher Scientific, Waltham, MA) overspread with a fluorescence protecting mounting medium, (Vectashield, Vector
Laboratories, Burlingame, CA) cover-slipped, and examined using a Leica DMLB standard fluorescent microscope equipped with a Spot RT3 CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Using Spot Advanced software, (Diagnostic Instruments, Inc.) a series of three-color images were captured at 50X magnification covering at least the tumor bearing hemisphere, if not the entire tissue section. The Photomerge automation for Photoshop CS4 (Adobe Systems, Inc., San Jose, CA) was then used to reconstruct the entire series into a single image. Photomerged images were then imported in Image J and separated by channel. The green channel, representing the hNestin+ glioma cells was then inverted – generating a black-on-white image – and the threshold was adjusted in order to distinguish the tumor from any non-specific background staining. Finally, the Analyze Particles function was used to quantify the total number of discrete particles contained within the tumor. We found that diffusely invasive tumors were made up of several thousand times (~ 2000 – 7300) more discrete, non-contiguous particles than non-invasive lesions.

**Gelatin-Substrate Zymography**

Zymography was performed on the soluble protein fraction isolated from in vivo tumor-bearing tissue from animals previously transplanted with either invasive (hGBM L19, X weeks survival) or non-invasive (U-87MG, X weeks survival) tumor cells. Novex Zymogram gels were used with the XCell SureLock Mini-Cell electrophoresis system as instructed by the manufacturer (Invitrogen). The gel was stained for 4 hours at RT with an aqueous solution of 0.1% Coomassie Brilliant Blue R250 (Serva Electrophoresis, Heidelberg, Germany) and 16% glacial acetic acid and de-stained for 10 minutes at RT in an aqueous solution of 30% EtOH and 10% glacial acetic acid. Areas of protease activity, which appear as clear bands against the dark blue background of the
coomassie blue stained gelatin, were imaged on a FluorChemQ Multi Image III system (Cell Biosciences, Santa Clara, CA) equipped with AlphaInnotech software version 1.0.1.1. The identity of the MMP was determined based on co-migration with purified recombinant human MMP positive controls as well as molecular weight standards.

**Human Tissue Collection and Processing**

All human tissue was acquired and processed in accordance with protocols previously vetted and approved by the University of Florida Gainesville, Health Science Center Institutional Review Board (UF-IRB-01). Briefly, 5-µm thin sections were prepared from de-identified blocks of paraffin-embedded human tumor specimens, which had been previously collected from the UF, Shands Department of Neuropathology tissue archives. After air-drying overnight at room temperature, the slides were sequentially de-paraffinized, rehydrated, and blocked for endogenous peroxidase activity. Optimal staining required 25 minutes of heat antigen retrieval in 10 mM Citrate buffer (pH 6.0). The tissue was then immuno-stained at 4°C, overnight for the antigen CS-56 (1/200, Sigma). Slides were stained using the ABC-Elite kit (Vector Labs, Burlingame, CA) following the manufacturer’s instructions, with endogenous biotin blocking steps included. Positive staining was detected with DAB (Vector Labs) as the chromogen and hematoxylin 560 (SurgiPath, Richmond, IL) as the nuclear counterstain.

**Statistical Analysis**

Data were analyzed using the GraphPad Prizm 5.0 (GraphPad Software, La Jolla, CA) software package. In all cases, a P value of <0.05 was deemed significant.
CSPGs discriminate between diffusely invasive and non-invasive brain lesions. The development of a series of novel human GBM cell lines revealed a startling disparity between the matrix content of invasive and non-invasive brain lesions. Representative, low (A) and high (B, B') magnification fluorescence micrographs of diffusely infiltrative hGBM xenografts reveals a paucity of the ECM protein CSPG. In contrast, (C, D, D') CSPGs are present in great abundance in non-invasive, strictly expansively growing lesions. (E) Quantification of the invasive character of a series of four human GBM cell lines illustrates the tremendous disparities between invasive and non-invasive tumor phenotypes. (mean ± SEM, one-way ANOVA, Bonferroni-Dunn post hoc test, *p < 0.0001, n = 6 / group) (F) Quantification of the fluorescence intensity of the CSPG-specific, WFA-Lectin reveals the stark dissimilarity in the proteoglycan content between invasive and non-invasive GBM transplants. (a.u. = arbitrary units, mean ± SEM, unpaired t test, *p < 0.0001, n = 18 / group) Scale bar = 50 µm.
Figure 2-2. Reactive astrocytes withdraw from the space occupied by CSPG-rich; non-invasive lesions but persist throughout regions occupied by invasive tumor cells. Representative low (A) and high (B, B’) magnification fluorescence micrographs illustrate the astrocytic circumscription of non-invasive, CSPG-heavy lesions. Note the scant GFAP+ processes remaining within the penumbra of the non-invasive mass. In contrast, representative low (C) and high (D, D’) magnification fluorescence micrographs exemplify widespread occupation of infiltrative lesions by reactive astrocytes. CSPG expression level may help to clarify this disparate astrocyte response. (E) Spot-gradient assays illustrate the strong aversion that reactive astrocytes demonstrate toward increasing concentrations (F, G) of substrate-bound CSPGs. (H) Quantification of the spot-gradient assay emphasizes the inhibitory effects of high levels of CSPGs on reactive astrocytes. (Mean ± SEM, one-way ANOVA, Tukey’s post hoc test, *p < 0.0001, n = 48 visual fields for 0 and 250 µg/mL CSPG groups, n = 72 visual fields for 5 – 100 µg/mL CSPG groups) Scale bar: A – D’, = 50 µm; E – G = 10 µm.
Figure 2-3. Microglial activation state differs markedly between invasive and non-invasive brain lesions. Representative low magnification fluorescence micrographs demonstrate that microglia uniformly populate both non-invasive (A) and invasive lesions, (D) however, (B, B’) only microglia within the confines of a CSPG-rich non-invasive lesion demonstrate the simple, spheroidal morphology associated with activation. In contrast, the (E, E’) ramified morphology of invasive-tumor associated microglia suggests a resting phenotype. (G, H) Microglial activation assays support a direct causal relationship between the microglial activation state and CSPG concentration. (I) Quantification of the microglial activation assay demonstrates the direct link between CSPG concentration and activated microglial as well as the concomitant indirect link between CSPG concentration and ramified microglia. (Mean ± SEM, two-way ANOVA, Bonferroni-Dunn post hoc test, *p < 0.0001, #p = < 0.0001, n = 30/group) Scale bar: A, D = 50 µm; B, C, E – H = 10 µm
Figure 2-4. Reducing CSPG-mediated inhibition facilitates brain tumor invasion. (A, B) Tumor dispersal assays demonstrated that U-87MG aggregation (A) is mediated through the potently inhibitory CS-side chains that decorate the proteoglycan core proteins. (C) In a dose dependent manner, addition of chondroitinase ABC, but not the negative control enzyme penicillinase, to the culture medium prevented aggregation and fostered the maintenance of an even monolayer (B) of cells. (Mean ± SEM, one-way ANOVA, Newman-Keul's post hoc test, *p < 0.05, n = 3 / group) (D) As opposed to eGFP-transduced negative control cells, (E) lentiviral transduction of U-87MG cells with chondroitinase ABC enables the diffuse infiltration of once non-invasive tumor cells. (F) Immunostaining for 2B6 antibody confirmed that U-87MG infiltration occurred within zones of digested CSPGs. (G) Quantification of chondroitinase ABC-mediated U-87MG invasion clarifies the inhibitory effects of CSPGs on tumor invasion. (a.u. = arbitrary units, mean ± SEM, unpaired t test, *p < 0.05, n = 7 for LV-eGFP; n = 15 for LV-Ch’ase ABC) Scale bar = 10 µm
Figure 2-5. The CSPG receptor LAR differentiates invasive from non-invasive lesions. Representative fluorescence micrographs of (A) non-invasive and (C) invasive lesions highlight the disparity in the expression of the CSPG-specific LAR phosphatase receptor. (B) Whereas LAR is uniformly expressed throughout the non-invasive tumor, (D) it is entirely absent from diffusely invasive lesions. (n = 6/group) Scale bar = 50 µm
Figure 2-6. Co-transplantation of invasive and non-invasive tumors sequesters the invasive tumor within the boundaries on the non-invasive tumor mass. (A) Panoramic fluorescence micrograph of co-transplanted U-87MG with firefly luciferase (f-Luc)-expressing hGBM L19 illustrates the restriction of invasion by entrapment within the confines of a non-invasive lesion. (A’’) f-Luc-labeled invasive tumor cells organize as sweeping tendrils within the (A’’’) larger, hNestin-expressing tumor mass. Invasive cells approach the edge of the mass (arrowheads) and appear to turn in accordance with the (A’) dense network of astrocyte fibers that circumscribe the lesion. (n = 9) Scale bar = 50 µm
Figure 2-7. Human clinical specimens recapitulate the inverse relationship between CSPG expression and diffuse invasion. (A) The grade II glioma, pleomorphic xanthoastrocytoma (PXA) typically presents as a discrete lesion with well-defined borders. (B) CSPGs are robustly expressed throughout the lesion and precisely define the edge of the tumor mass. (C, D) In contrast, the highly infiltrative grade IV glioma, GBM is largely devoid of glycosylated CSPGs aside from minimal staining specifically at sites of pseudo-palisading necrosis.
Figure 2-8. Putative model of CSPG-mediated invasion inhibition. When present, CSPGs induce dramatic rearrangements in the astrocyte and microglial populations associated with the tumor. These tumor cell-glial cell interactions, mediated through the tumor ECM, define the tumor microenvironment and presumably, participate actively and directly in the regulation of the invasive character of a brain lesion.
CHAPTER 3
ZEB1 MEDIATES INVASION AND CHEMORESISTANCE OF GLIOBLASTOMA

Despite intense research efforts, glioblastoma remains one of the most lethal types of cancer. In particular, tumor recurrence after surgical resection and radiation frequently occurs regardless of aggressive chemotherapy. This recurrence has been attributed to residual cancer cells that can re-initiate tumor growth. Here, we provide evidence that a subpopulation of highly invasive and chemoresistant glioblastoma cells is maintained by the transcription factor ZEB1 (zinc finger E-box binding homeobox 1). ZEB1 is preferentially expressed in invasive glioblastoma cells, and its knockdown results in a dramatic reduction of tumor invasion as well as increased sensitivity to the chemotherapeutic agent Temozolomide (Temozolomide, TMZ) in vitro and in vivo. We find that this activator of epithelial-mesenchymal transition (EMT) controls expression of the chemoresistance-mediating enzyme MGMT (O-6-Methylguanine DNA Methyltransferase) through the transcription factor c-Myb, as well as cell-cell adhesion pathways, thus linking chemoresistance and brain tumor invasion. Moreover, ZEB1 expression in glioblastoma patients correlates with tumor grade and is predictive of shorter survival. These results indicate that invasive glioblastoma cells are particularly sheltered from current therapeutic approaches, rendering them likely candidates for tumor recurrence. We thus identify ZEB1 as an important candidate molecule for glioblastoma recurrence, a potential marker of invasive tumor cells and a promising therapeutic target.

Results

With a low median survival of about 15 months, glioblastoma is the most frequent and most aggressive of all gliomas. A hallmark of glioblastomas is their high propensity
to invade the surrounding parenchyma, where single invasive tumor cells can be found far away from the primary site and frequently cross into the contralateral hemisphere. These cells cannot be isolated for surgical resection, or targeted by irradiation. The current standard of care therefore includes chemotherapy to ablate invasive cells. Here, we provide evidence for a subpopulation of tumor cells that invade as single cells and demonstrate enhanced resistance to the chemotherapeutic agent Temozolomide. Combined with their higher resistance to chemotherapy, these invasive cells leave the primary tumor site, invade deeply into the surrounding parenchyma, and thus evade all standard therapeutic regimens, rendering them likely candidates for glioblastoma recurrence. We further identify the EMT activator ZEB1 as a master regulator of both invasion and chemoresistance pathways. In solid tissue tumors outside the brain, ZEB1 initiates distant spread and metastasis, and while gliomas rarely metastasize, an EMT-like conversion has been speculated as a mechanism for their invasive phenotype. ZEB1 is a transcriptional repressor of cell-adhesion molecules, such as E-cadherin, miRNAs – particularly the miR-200 family – and cell polarity-associated genes, and has emerged as one of the master regulators for mesenchymal transition and metastasis, and plays a critical role in the initiation of distant tumors. We found that ZEB1 expression confers chemoresistance, as well as invasiveness, thus generating a highly malignant phenotype that may represent the basis for tumor recurrence. This is reflected in poorer clinical outcome of ZEB1 expressing tumors.

**Isolation and Characterization of Invasive Tumor Initiating Cells**

To better understand the cells responsible for invasion and treatment resistance, we postulated that invasive tumor cells are characterized by a lower proliferation index than other cancer cells and employed a paradigm that allowed us to separate cell
populations based on their proliferation rate. Primary tumor sphere cultures were loaded with the amine-reactive fluorescent dye carboxyflurescein succinimidyl ester (CFSE) that is diluted equally to the daughter cells. This enabled separation of high proliferating (HP) and low proliferating (LP) cell fractions based on fluorescence intensity proportional to dye dilution (Figure 3-1A and 3-5). We tested this hypothesis in an in vitro scratch assay and found that LP cells were more migratory than HP cells (Figure 3-1B). Next, we transplanted both cell populations into the striata of immunocompromised animals, where both LP and HP cells gave rise to fatal tumors with a median survival time of 153 and 177 days, respectively. Concomitant with LP cells being more migratory in vitro, we observed that LP cells gave rise to significantly more invasive tumors in vivo relative to the HP cohort (Figure 3-1C and 3-6). Reduced cell-cell adhesion in glioma correlates with invasiveness and N-cadherin expression is proportional to the proliferative index of gliomas. Immunohistochemistry revealed expression differences of cell-adhesion molecules between LP and HP derived tumors. While HP cells showed high levels of membrane associated N-cadherin and beta-catenin, no staining was found in LP cells (Figures 3-1D and 3-2). We concluded that loss of cell-cell contact facilitates invasion of LP cells. Immunoreactivity to ZEB1, known to regulate expression of cadherins in cancer was absent in HP-derived tumors, but prominent in LP-derived tumors, especially in the invasion zone (Figure 3-1F, arrows) and in single invading cells distant from the primary tumor mass (Figure 3-1G).

**Knockdown of ZEB1 Results in Decreased Invasion and Increased Chemo-Sensitivity**

We next tested whether LP cells are more resistant to the standard chemotherapeutic agent TMZ. We analyzed the susceptibility of LP and HP cells to TMZ
in a cell viability assay (Figure 3-2A), and found that LP cells displayed a significantly greater resistance to TMZ (concentration inhibiting viability by 90% (IC90) LP: 113 mM, IC90 HP: 1.1 mM, F-test, p=0.0111). This suggests that cell cycle kinetics (i.e. in LP cells) provide a link between tumor invasion and chemoresistance, but at a higher level than expected from actual proliferation differences (Figure 3-2). This link was supported by the observation that TMZ-resistant cells showed similar capacity for migration and invasion as LP cells (Figure 3-2B-D). These data are substantiated by the clinical observation that tumors frequently recur despite aggressive chemotherapy, and establish a specific glioblastoma cell subpopulation that is more invasive and chemoresistant, which exclusively expresses ZEB1.

**Mechanism of ZEB1 Mediated Chemoresistance**

We therefore tested whether knocking down ZEB1 in tumor cultures results in reduced invasion and chemoresistance (Figure 3-3A-F). ZEB1 knockdown cultures showed a prominent reduction in ZEB1 mRNA levels compared to control tumor cultures, both in sphere and adherent conditions (Figure 3-3A). ZEB1 knockdown was additionally accompanied by reduced migration (Figure 3-2), reduced invasion (Figure 3-3C), and increased expression of membrane-associated N-cadherin and beta-catenin (Figures 3-3D and 3-7). Cell viability analysis revealed that ZEB1 knockdown significantly increases the sensitivity to TMZ (Figure 3-4A). We tested whether knocking down ZEB1 increased TMZ sensitivity of the most resistant population (i.e. LP) in vivo. Indeed, injection of therapeutic concentrations of TMZ into tumor-bearing animals grafted with either shZEB1 LP or shGFP LP cells resulted in a reduction of tumor size in shZEB1, but not in shGFP animals (Figure 3-4B). TMZ had little or no effect on invasive
cells (shGFP), but prominently depleted the tumor mass (Figure 3-4B). Together, these data demonstrate that ZEB1 links tumor invasion and chemoresistance.

The Complementary Nature of Invasion and Chemoresistance

To elucidate the molecular pathways affected by ZEB1, we isolated proteins from adherent control and ZEB1 knockdown cultures and confirmed reduced expression levels of ZEB1 protein by western blot. N-cadherin expression is inversely correlated with tumor invasion, but we found no significant change in total N-cadherin protein levels (Figure 3-4C). Interestingly, rather than the expected reduction in total protein level, we found that the distribution of N-cadherin had changed after ZEB1 knockdown. Specifically, ZEB1 knockdown induced an enrichment of N-cadherin within the cell membrane, therefore enabling N-cadherin-mediated cell-cell attachment and retarded cellular motility (Figure 3-2). N-cadherin is connected to the cytoskeleton by alpha- and beta-catenin, and the axon guidance molecule ROBO1 (roundabout homolog 1) can sever this connection. ROBO1, though not its ligand Slit, is expressed in malignant glioma, and the ROBO1 sequence contains 3 binding sites for miR-203 20 (Figure 3-9). We showed that ROBO1 protein expression levels are reduced in ZEB1 knockdown cells, supporting the hypothesis that with active ZEB1, ROBO is expressed and disconnects N-cadherin from the cytoskeleton, which enables ZEB1-expressing cells to shed cell-cell contacts and invade as single cells (Figure 3-4C).

The enzyme MGMT (O-6-Methylguanine-DNA-Methyltransferase 21) confers resistance to TMZ, and MGMT expression in glioma is prognostic for chemotherapeutic success. We observed reduced expression of MGMT in ZEB1 knockdown cells, concomitant with increased sensitivity to TMZ in these cells (Figure 3-4C). The MGMT sequence does not contain binding sites for known ZEB1-regulated microRNAs, nor
does the MGMT promoter contain sequences that would predict direct regulation of MGMT expression by ZEB1. However, the MGMT promoter contains predicted c-MYB-regulatory elements, and one study found c-MYB associated with the MGMT promoter in breast cancer cells. c-MYB contains at least five predicted binding sites for miR-200c and miR-203 20 (Figure 3-9). Importantly, ZEB1-knockdown reduced expression of c-MYB (Figure 3-4C), supporting the hypothesis that MGMT expression is mediated by ZEB1 through its indirect regulation of c-MYB (Figure 3-4D). We explored this by modulating expression of the intermediate regulatory elements in this pathway, i.e. miR-200 and c-MYB. Induced expression of miR-200c resulted in decreased expression of c-MYB, MGMT and increased chemosensitivity, while antagonizing miR-200c resulted in increased expression of ZEB1, c-MYB, MGMT, and increased chemoresistance (Figure 3-4E). Knocking down c-MYB reduced, while forced expression increased, MGMT expression and chemoresistance, respectively, but had no effect on ZEB1 expression (Figure 3-4E). Expression of c-MYB in ZEB1 knockdown cells restored MGMT expression in these cells and rescued chemoresistance (Figure 3-4E). Our data identify ZEB1 as regulator of malignant glioma invasion, which is further corroborated by immunohistochemistry and immunoblotting in specimens of invasive and non-invasive tumors (Figure 3-10). Strikingly, ZEB1 expression correlated significantly with MGMT expression in glioblastoma samples (Figure 3-4C). Consequently, we found that ZEB1 expression also correlates with reduced survival of glioblastoma patients (median survival ZEB1+ 11.0 months (n=13), median survival ZEB1- 19.2 months (n=20), Log-rank test, p=0.0014, Figure 3-4). Furthermore, ZEB1 expression correlates with poorer outcomes in TMZ treated patients, which are reflected in both shorter survival and
shorter duration of successful TMZ therapy (Figure 3-8). These findings emphasize the relevance of ZEB1 for glioblastoma invasion and chemoresistance.

Expression of ZEB1 is associated with migratory and invasive cells that are further characterized by reduced cell cycle kinetics (Figure 3-1) and TMZ resistance (Figure 3-3). Low proliferating cells have been identified and correlated with poor prognosis in a number of cancers and ZEB1-mediated invasion and chemoresistance may contribute significantly to this phenomenon. In human brain tumor specimens, ZEB1 expression is correlated with invasion and MGMT expression (Figure 3-4). Knockdown experiments demonstrate the functional importance of ZEB1 in regulating invasion and chemoresistance and indicated that these processes are interrelated (Figure 3-8).

ZEB1 stands at the center of an intricate pattern of direct and indirect regulatory mechanisms, which is executed through the miR-200/-203 families (Figure 3-3). These microRNAs directly inhibit expression of the transcription factor c-MYB, which in turn activates MGMT expression (Figure 3-3E). MGMT is a suicide enzyme, and two MGMT monomers are covalently linked upon its DNA repair activity. Of note, we find protein levels of monomeric, active MGMT to be very low in glioblastoma cells, while the majority of cellular MGMT exists in the exhausted, dimeric form (Figures 3-4E and 3-11). In fact, we were only able to observe monomeric MGMT in LP cells (Figure 3-4C). This indicates (a) high levels of DNA repair in glioblastoma cells and (b) that minor changes in MGMT expression may have pronounced therapeutic consequences. Loss of cell adhesion is achieved by disconnecting N-cadherin from the cytoskeleton through ROBO1. ROBO1 expression may be regulated analogously by ZEB1 inhibiting miR-203-mediated interference. Hence, ZEB1 is a regulator of invasion and
chemoresistance in glioblastoma, a candidate agent for tumor recurrence, and may be a promising target for tumor therapy.

**Methods**

**Cell Culture**

Tumor cell lines were generated (Piccirillo et al., 2006) and maintained (Siebzehnrubl et al., 2009) as described. Briefly, 50,000 cells were seeded per ml of culture medium (N2, Invitrogen, Carlsbad, CA) in the presence of mitogens (20 ng/ml each of EGF and FGF2, Sigma, St. Louis, MO). Cells were propagated as spheres and passaged using Accutase (PAA, Cölbe, Germany) every 7 days. For experiments with adherent cells, spheres were dissociated and plated in N2 medium supplemented with 1% fetal bovine serum (FBS). For scratch assays, 2 x 106 cells were plated per well of a 6 well culture plate in N2 containing 1% FBS, and grown to confluence overnight. Confluent monolayers were scratched with a pipette tip, and imaged at the time of the lesion and 24 hours later. TMZ resistant cells were obtained by treating a sphere culture with 800 µM TMZ for 3 days. Medium was changed after that and surviving cells were grown up into a new culture. For sphere forming frequency assay, single cells were plated in 96-well cluster plates at a density of 1,000 cells/well. Three weeks after plating, the number of spheres > 50 µm was counted.

**CFSE Loading and FACS**

Fluorescent dye loading and FACS sorting of low and high proliferating cancer cells were performed as described (Deleyrolle et al., 2011), with the exception that the lowest 5% of CFSE+ cells were used as HP. Briefly, cells were incubated with CFSE (Invitrogen) during regular passaging, washed three times and plated at 50,000 cells/ml. Seven days after loading, CFSEhi (LP) and CFSElo (HP) cells were sorted on a BD
FACS\textsuperscript{A}ria\textsubscript{2} flow cytometer, collected in medium, counted and plated again at 50,000 cells/ml. These fractions were subcultured for 7 days before being used in subsequent in vitro or in vivo experiments.

**Cell Viability Assay**

The Methyltetrazolium bromide (MTT) assay was used as indicator of cell viability and performed as described (Holsken et al., 2006). Briefly, 10,000 cells were plated per well into 96-well cell culture plates and treated one hour after plating with varying concentrations of TMZ (ranging 5 µM – 5 mM, Tocris, Ellisville, MO). Five days after plating, cell viability was assessed by MTT dye conversion and analyzed on a plate reader (excitation wavelength 580 nm, reference wavelength 450 nm). Concentration-effect curves for TMZ treatment were generated by nonlinear regression analysis as described 33. All curve slopes were larger than unity (F-test, p>0.05); therefore pIC\textsubscript{50} and pIC\textsubscript{90} values were obtained from curves with variable slopes.

**Knockdown Experiments**

Plasmids for knockdown of ZEB1 and expression of hsa-miR-200c, as well as antago-miR-200c and control sequences are described in (Brabletz and Brabletz, 2010). Plasmids for knockdown of c-MYB were obtained from OpenBiosystems (Lafayette, CO). The plasmid for expression of human c-MYB (Clarke et al., 1988) was a kind gift of Dr. J.S. Lipsick (Stanford University). Cancer cells were transfected using Lipofectamine LTX (Invitrogen) according to the manufacturer’s instructions. Transfected cells were selected using puromycin (Sigma) before being used for subsequent experiments.

**Animal Experiments**

Adult female Fox-Chase SCID mice (Charles River, Wilmington, MA) were used for in vivo tumor transplants. All procedures were performed according to NIH and
institutional guidelines for animal care and handling. After animals were deeply anaesthetized using USP grade Isoflurane (Halocarbon, North Augusta, SC), an incision was made in the scalp, the skull demonstrated and a hole drilled at the coordinates Bregma -0.5 mm anterior and -1.5 mm lateral. A Hamilton syringe was lowered 2.5 mm into the burr hole, and 1 µl of a cell suspension was injected over 5 min before the needle was retracted. After the incision was closed with surgical staples the animal was allowed to recover before being returned to the cage. For survival and tumor end-stage analysis, animals were transplanted with 10,000 cells, and tumor-bearing animals were scored regularly for tumor-related symptoms. Moribund animals were anaesthetized and transcardially perfused with 4% paraformaldehyde in saline, the brains removed, postfixed and prepared for histology. For tumor invasion analysis, animals received grafts of 100,000 cells, and were perfused 12 weeks post injection. For in vivo TMZ treatment, animals received orthotopic grafts of 100,000 shGFP LP or shZEB1 LP cells. 14 weeks after transplantation, tumor-bearing animals were intraperitoneally injected with 20 mg/kg TMZ in saline (final DMSO concentration 25%). Animals received two injections per week, spaced 48 hours apart, for a total of six weeks. 48 hours after the last injection, animals were perfused.

**Immunohistochemistry and Immunocytochemistry**

Immunostainings were performed as described (Siebzehnrubl et al., 2009; Zheng et al., 2006). A table of employed antibodies, suppliers and dilutions can be found in the supplementary information. All secondary antibodies were obtained from Invitrogen and diluted 1:500.
Image Acquisition and Data Analysis

Low power fluorescent images were taken on a Leica DMLB epifluorescence microscope (Bannockburn, IL) equipped with a CCD camera (Spot Imaging Solutions, Sterling Heights, MI). To obtain full images of brain sections, multiple gray scale images were acquired per section using Spot Advanced software (Spot Imaging Solutions) and merged into a full image and inverted into black-on-white images using Photoshop CS4 (Adobe Systems, San Jose, CA). Photomerged images were imported into ImageJ and threshold levels were adjusted to distinguish tumor from background. Using the wand tool, all outlines of positively stained (black) tumor areas were outlined in each section and the perimeter and area were measured. The ratio of the squared perimeter distance over the area (P^2/A) was calculated and used to compare invasive properties of different tumors. Since P^2/A is a dimensionless number, the resulting figure is termed “invasion index”. A greater invasion index is indicative of a more dissociated tumor, whereas a lower invasion index represents a more spherical tumor. For in vivo TMZ treatment outcome, tumor area was used as indicator of overall tumor size.

High power images were taken on an Olympus BX-81 DSU spinning disc confocal microscope (Olympus, Center Valley, PA) and projection images of z-stacks were generated using Slidebook (Olympus) software.

RNA Isolation and Quantitative Real-time PCR

Total RNA was isolated from tumor sphere or adherent cultures using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instruction. RNA was quantified on a Nanodrop Spectrophotometer (Thermo, Wilmington, DE), and 1 µg of total RNA was used for cDNA synthesis as described (Siebzehnrubl et al., 2009). 25 ng of cDNA were used for quantitative PCR using the SYBR green PCR master mix
(Applied Biosystems, Carlsbad, CA) on an ABI 7900HT (Applied Biosystems) as previously described 32. Expression levels of ZEB1 were quantified in triplicate relative to beta-actin using the ΔΔCt method. Primer sequences and amplification times are described elsewhere (Wellner et al., 2009).

**Protein Isolation and Western Blotting**

Proteins were isolated from adherent cancer cell cultures and primary tumor specimens obtained from the Florida Center for Brain Tumor Research (FCBTR) as described (Siebzehnrubl et al., 2009). For western blotting, 20 µg of denatured protein were loaded on 4-12% Bis-Tris reducing gel (Invitrogen), separated and blotted onto a PVDF membrane (iBlot, Invitrogen). Blots were blocked and probed with respective primary and secondary antibodies (see supplement) as described 32, and developed using the ECL Plus kit (Amersham, Piscataway, NJ) on a FuorChemQ Multi Image III (Cell Biosciences, Santa Clara, CA) and Alphalnnotech software version 1.0.1.1. Band densitometry was performed using ImageJ.

**Statistical Testing**

All statistical analyses were performed in GraphPad Prizm 5.0 (GraphPad Software, La Jolla, CA). Statistical tests are indicated in the text. In all analyses, a P value of <0.05 was deemed significant. We used the D'Agostino-Pearson test for normal distribution of values. Observers were blinded to the patient data (including survival time) when performing the ZEB1 analysis.
Figure 3-1. Isolation and characterization of invasive tumor-initiating cells. Paradigm for the isolation of migratory (low proliferation, LP) and proliferative cells (high proliferation, HP). Top 5% CFSEhi (LP) and bottom 5% CFSElo (HP) fractions were separated 7 days after CFSE loading (A). In vitro scratch assays showed an increased capacity for migration in LP cells (B, mean +/- s.e.m., two-tailed t-test, p<0.0001, n=3). In vivo transplantation of LP and HP cells resulted in tumor formation with distinct invasive properties (C). Scale bars 10 µm. Human-specific nestin immunostaining reveals tumor invasion from LP cells (mean +/- s.e.m., two-tailed t-test, p<0.0001, n=5, a.u. arbitrary units). HP tumors stain for N-cadherin and beta-catenin, while LP tumors are negative (D). Scale bars 10 µm. Fluorescence micrographs in D are projection images of confocal z-stacks. While HP tumors are negative for ZEB1 (E), LP tumors show prominent reactivity at the invasion zone (arrowheads), which tapers off towards the tumor center (asterisk, F). Scale bars 20 µm. Single invading LP cells prominently express ZEB1 (G). Scale bar 10 µm.
Figure 3-2. Knockdown of ZEB1 results in decreased invasion and increased chemosensitivity. qPCR demonstrates upregulation of ZEB1 mRNA (A) during adhesion (adh) of control cells, but prominent downregulation in ZEB1 knockdown experiments (ANOVA, p<0.0001, n=3). ZEB1 knockdown results in significantly reduced migration in vitro (B, mean +/- s.e.m., two-way ANOVA, p<0.0001, n=3) as well as reduced invasion in vivo (C, mean +/- s.e.m., t-test, p<0.0001, n=5, scale bars 10 µm, a.u. arbitrary units). shZEB1-derived tumors are negative for ZEB1 (D), but show cell surface expression of beta-catenin and N-cadherin. Scale bars 10 µm. Projection images of confocal z-stacks.
Figure 3-3. Mechanism of ZEB1 mediated chemoresistance. MTT assays (A) show increased TMZ sensitivity in ZEB1 knockdown cells. IC50 shGFP: 152 µM, IC50 shZEB1: 15 µM, IC90 shGFP: 26 mM, IC90 shZEB1: 1.5 mM. IC50 and IC90 values differed significantly (F-test, p=0.0011). ZEB1 knockdown and control tumor-bearing animals were treated i.p. with either TMZ or vehicle (n=5 for each group, B). Fluorescence micrographs of representative tumors show that TMZ treatment affects the tumor mass (shZEB1), but not the invasive cell population (shGFP). Scale bars 100 µm. Tumor size was reduced after treatment in the ZEB1 knockdown group, but not in the control group (two-way ANOVA, p<0.0001). Dashed line represents vehicle treated tumor size used for normalization. Western blots (C) show reduction of ZEB1 in shZEB compared to control cells, as well as reduced levels of MYB, MGMT, and ROBO1. GAPDH serves as loading control. A putative model (D) links ZEB1 to invasion and chemoresistance; both pathways are regulated through intermediary factors (miR-200/-203, ROBO1 and MYB, respectively). (E) Immunoblot analysis of c-MYB and MGMT regulation. Forced expression of hsa-miR-200c reduces, while antagonizing miR-200c increases, protein levels of ZEB1, c-MYB and MGMT. Knockdown of c-MYB reduces, while forced expression increases, expression of MGMT. Expression of c-MYB in ZEB1 knockdown cells can rescue expression of MGMT and chemoresistance. Full-length blots are presented in Supplementary Figure 6. Bar graphs depict relative cell viability of respective cell cultures exposed to 1 mM TMZ for 96 hours (normalized to DMSO treated control cells, n=8, p<0.0001, one-way ANOVA with Bonferroni post test).
ZEB1 expression correlates with invasion in gliomas. Non-invasive tumors show no immunoreactivity for ZEB1 (pilocytic astrocytoma WHO I), but invasive gliomas are strongly positive (glioblastoma WHO IV, A). Scale bars 20 µm. Immunoblotting and densitometry demonstrate increased ZEB1 expression levels in glioblastoma (GBM) over lower grade pilocytic astrocytoma (Pilo, two-tailed t-test, p=0.0473, B). In the glioblastoma cohort, expression of ZEB1 correlates with MGMT expression in these tumors (C, Pearson correlation, p=0.0022; depicted is linear regression and 95% confidence interval). Full-length blots are presented in Supplementary Figure 7. Notably, not all glioblastomas express ZEB1 (red box), and ZEB1 negative tumors have a better prognosis (median survival ZEB1 positive 11.0 months, ZEB1 negative 19.2 months, Log-rank test, p=0.0014, D). In addition, survival intervals of TMZ treated ZEB1+ patients were significantly increased (ZEB1+ 12.8 +/- 2.1 months, ZEB1- 22.8 +/- 3.7 months, two-tailed t-test, p=0.0362), as was the average duration of TMZ treatment in these patients (ZEB1+ 2.54 +/- 0.73 months, ZEB1- 9.09 +/- 2.07 months, two-tailed t-test, p=0.0087). No significant age difference was found between the ZEB1+ and ZEB1- groups (average age ZEB1 positive 61.9 ± 15.8 years, ZEB1 negative 58.1 ± 11.7 years, Mann-Whitney test, p=0.15).

Figure 3-4.
Figure 3-5. CFSE loading and FACS paradigm. Cells were loaded on day 0 with CFSE and the 5% CFSEhi and 5% CFSElo fractions separated based on CFSE intensity 7 days after loading. CFSE intensity inversely correlates with cell proliferation [12,30]. Growth curves show differences in the expansion rate of LP and HP fractions, as well as these rates in relation to other paradigms employed in this study (unsorted, TMZR). Average expansion rates were 8.22 +/- 0.41 for unsorted cells, 9.25 +/- 0.82 for HP, 3.69 +/- 2.11 for LP, and 3.89 +/- 2.23 for TMZR. Bar graphs represent mean +/- s.e.m. A Kaplan-Meier curve shows that transplantation of 10k cells/animal of both HP and LP cells results in fatal tumors. Tumor presence was confirmed post-mortem. Median survival HP: 153 days, LP: 177 days. Knockdown of ZEB1 does not affect proliferation rate significantly (two-tailed t-test, p=0.43).
Figure 3-6. Fluorescence micrographs and threshold images of representative tumors derived from LP, HP, shGFP LP and shZEB1 LP cells. Boxes in the images represent areas of larger magnification used in Fig. 1C and Fig. 3C, D. Scale bars 500 µm. Fluorescence images were used to generate threshold images, which were used to measure invasion index (see methods). Bar graph demonstrates invasion index for a second tumor line with similar invasion patterns.
Figure 3-7. In vitro fluorescence micrographs of N-cadherin and ZEB1 immunostaining. HP and shZEB1 cells show high membrane association of N-cadherin, while LP and shGFP cells do not. Conversely, ZEB1 is absent in HP and shZEB1 cells, but present in LP and shGFP cells. Scale bars 20 µm.
Figure 3-8. Complementary nature of invasion and chemoresistance. Sigmoidal curve-fit of an MTT assay shows increased TMZ resistance in LP cells (A). IC50 LP: 53 µM, IC50 HP: 13 µM, IC90 LP: 113 mM, IC90 HP: 1.1 mM. IC50 and IC90 values differed significantly (F-test, p=0.0111). TMZ resistant cells (TMZR) migrate similar to LP cells in vitro (B, two-tailed t-test, p=0.38). In vivo analysis of TMZR derived tumors reveals similar invasion to LP derived tumors (C, mean +/- s.e.m., n=4, two-tailed t-test, p=0.733). Scale bar 200 µm. TMZR tumors are negative for N-cadherin and beta-catenin, but express ZEB1 (D). Scale bars 10 µm. Fluorescence micrographs in D are projection images of confocal z-stacks.
Figure 3-9. Gene regulation of c-MYB and ROBO1. Shown are predicted 20 miR-200c/miR-203 binding sites in the UTR of the respective genes. The MGMT promoter sequence reveals predicted c-MYB binding sites 23.
Figure 3-10. Full images of all western blots presented in Figure 3-3. Frames indicate excised bands. Arrows indicate monomeric (21 kDa) and dimeric (42 kDa) MGMT.
Figure 3-11. Full images of all western blots presented in Figure 3-4. Frames indicate excised bands.
CHAPTER 4
SEGREGATION OF HUMAN BRAIN TUMOR-INITIATING CELLS AND GENES

Despite exceptional efforts invested by academic research and the pharmaceutical industry, the prognosis of many brain tumor patients remains dismal. The recently introduced neoplastic stem cell theory may offer an intriguing explanation, as it is implied that a rare population of CSCs drives the maintenance, propagation and potentially also recurrence of some of the most malignant tumors. This hypothesis contrasts to the stochastic model that attributes the capability of self-renewal and continual growth to many of the tumor cells. Both models rely on the activity of clonal cell populations, and therefore, the most reasonable approach to study cancer would be the investigation of individual cells. However, there is a surprising lack of enabling technology that could be used to entertain this rationale. Today’s brain tumor research is rather centered on the study of heterogeneous mixtures of cells from individual tumor samples, or alternatively, on the investigation of populations of enriched target cells expressing predicted traits (e.g. the CD 133 antigen).

Here, we introduce a multi-step procedure tailored to facilitate (i) the identification of pertinent brain tumor samples, (ii) the unbiased separation of target cells, as well as (iii) their clonal propagation under adhesive culture conditions for comparative analysis and (iv) segregation of tumor-initiating cells and genes. Our data demonstrate that various CSC phenotypes can be present in one tissue specimen, and furthermore, that their respective tumorigenic profile cannot be predicted from analyzing the heterogeneous mixture of cells in tumor samples.
Results

Identification of Pertinent Brain Tumor Samples

Due to their infrequency, not every brain tumor biopsy may contain sufficient amounts of target cells, i.e. clonogenic cells with tumor-sustaining potential. To first identify tissue samples harboring clonally active populations, we applied a semi-solid, methylcellulose-based neurosphere assay (mcNSA) that is also suitable for quantification of neural stem/progenitor cells (see Methods). This assay was applied to specimens obtained from 19 pediatric and one adult neurosurgery patients in order to determine clonal cell frequency. Clonal cell frequency represents the number of cells within a heterogeneous population, capable of generating a clonal sphere. Sphere-forming cells are unquestionably rare. Depending on the particular tumor specimen examined, only 0.01 to 1.78% had sufficient proliferative potential to generate a clone (Figure 4-1A, B). Yet, frequency or morphology of primary neurospheres did not predict multipotency and self-renewal attributes. These properties were found in only two cases, an anaplastic ependymoma (AE, #018) and a glioblastoma multiforme (GBM, #019). In both cases, multipotent, long-term self-renewing sphere-forming cells continued to develop neurospheres for at least one year in culture (Figure 4-1C). Human brain tumor research currently does not distinguish between long-term and short-term activity in stem cell populations, but differing degrees of self-renewal are acknowledged in the hierarchy of hematopoietic stem cells. As there may be parallels to neuropoietic cells, this long term, self-renewing population could represent the top of the multipotent stem/progenitor cell lineages, potentially also responsible for sustaining the growth of brain tumor tissue. In this light, tissue cases 018 and 019 were selected for further discriminative studies.
The mcNSA is impractical for use in generating sufficient numbers of cells efficiently enough for subsequent investigations. During the period of one year in culture (15°/16° neurosphere stage, respectively), the fraction of SFCs increased just slowly from 0.24 to 1.21% and from 0.30 to 1.27% in cases 018 and 019, respectively (Figure 4-1D). Additionally, the average size of neurospheres generated after the fifth passage (three months in mcNSA conditions) remained stable (200-400 cells/neurosphere), suggesting that SFCs and their generated number of cellular progeny reached an assay-bound equilibrium (Figure 4-1E). Because of its limited practicality, the mcNSA was used only as an initial screening tool to identify specimens of particular interest; those containing a long-term self-renewing population. All additional analyses of these specimens were performed either directly on the resected tissue itself, or on cells derived from our adherent explant culture system (described below). (Figure 4-2).

**Isolation and Expansion of Heterogeneous Target Cell Populations**

In addition to long-term proliferative potential, the migratory/invasive character of certain brain cancers underscores their pathogenesis. In this light, we’ve formulated a novel explant culture system that has enabled us to isolate and analyze these migratory, potentially cancer stem-like cells. This system offers an unbiased mechanism by which tumor tissue may be separated and enriched into various subpopulations based on the cells migratory competence and preference to attach to particular substrates at the time of tissue extraction (Figure 4-4A). The procedure was performed in parallel on untreated plastic dishes as well as under the influence of various substrate coatings (Laminin/poly-L-ornithine, LPO; gelatine; fibronectin; Methods and 10) in analogy to earlier studies. Following a suitable time in culture (typically 7-10 days), the tissue explants were removed from the dishes, leaving behind
the separated, substrate-specific migratory cell populations (mig). The remainder tissue pieces were dissociated into a single cell suspension and propagated as a separate population in their specific substrate condition (dis). Consequently, up to eight distinguishable cell populations were available from each tissue specimen (Figures 4-2 and 4-3). Comparative analysis revealed highest frequencies of clonogenic, multipotent cells in LPOmig cultures, which were therefore were selected for further analysis (Figure 4-4B). Both, 018 and 019 LPOmig cultures were characterized by heterogeneous cell morphologies present throughout the expansion period lasting for at least 35 population doublings (Figure 4-4C). Their respective cellular expansion rates exceeded the rate of newly generated cells in the mcNSA by far. After 130±2 days in culture, the total number of 018LPOmig cells increased by a factor of 2.68E+06, and for 019LPOmig cells by a factor of 2.37E+10. In comparison, the total number of mcNSA cells after the same period of time was reduced in case 018 by a factor of 4.86, and in case 019 by a factor of 1.25. Thus, serially passaged adherent cells rather than their sphere-forming counterparts were selected for further analysis. To demonstrate the suitability of stably expandable LPOmig cultures for the study of target cells, we used passage 10 cells for molecular profiling and investigation of tumorigenic traits. Surprisingly, both 018 and 019 LPOmig populations revealed characteristic sets of neural stem/progenitor cell transcripts, (Figure 4-4D), but only 019LPOmig cells were tumorigenic. Not one of the 018LPOmig orthotopic xenograft experiments could recapitulate the features of an AE (n=0/8), while every engraftment of 019LPOmig cells developed into a tumor with GBM-specific characteristics (n=4/4; Figures 4-4E, F and 4-5). The failure of 018LPOmig cells to induce AE features in our animal model may be due to low frequencies of tumorigenic
cells and/or potential mismatches of donor and host cells in the xenograft environment. Additionally, while little is known of the clonality of tumor-sustaining AE cells, many recent reports predict these qualities as coinciding in GBM-CSC populations. Thus, further analysis along the aims of our study was restricted towards examination of 019LPOmig cells.

**Clonal Propagation and Comparative Analysis of Putative Target Cells**

To attribute the characteristics of multipotency, clonality, and tumorigenicity to individual cells and their progeny, heterogeneous 019LPOmig cultures were seeded at ultra-low densities in passage 5 (Figure 4-6A). As expected from the relatively low frequencies of SFCs found in the 019LPOmig cultures (0.39% at P+5, see Figure 4-4B), most adherent cells died under this condition within the first week. Yet, some individual cells survived, forming distinct colonies composed of uniform cells. From these, morphologically distinct colonies were selected, expanded for an additional five passages (P5+5), and subsequently evaluated in the mcNSA. Of the selected cell populations, two multipotent clones (CL6 and CL7) performed notably different from each other (Figure 4-6B, and 4-7). CL6 cells generated primary neurospheres at high frequency (16.4%) containing an average of 281 cells, while only 0.5% of the CL7 cells formed neurospheres with an average size of 99 cells. Because size and frequency of neurospheres could indicate biologically diverse subpopulations of stem-like cells (Reynolds and Rietze, 2005), we next determined if CL6 and CL7 cells represented distinct CSC entities. Molecular profiling revealed a remarkable overlap of expressed genes, with an extensive list of stem/progenitor-typic transcripts (Figure 4-6B, C). The exclusive expression of CD133 (Prominin 1) and the robust increase in MGMT expression (2.3 fold) within CL7 has provided two noteworthy contradictions. CD133 is
frequently used as a CSC indicator (Bao et al., 2006; Singh et al., 2004), and epigenetic silencing of the MGMT DNA repair gene (O6-methylguanine-DNA methyltransferase) by promoter methylation is associated with longer survival of GBM patients receiving alkylating chemotherapy. Nevertheless, not one CL7 xenograft (n=0/5) proliferated in situ, while every CL6 transplant (n=5/5) was tumorigenic in recipient NOD-SCID mouse brains. Thus, our data suggests co-existing stem cell phenotypes with distinct biological functions: CL6 as tumorigenic CD133-/MGMTlow cells, and CL7 as a non-tumorigenic CD133+/MGMT+ cell population (Figure 4-6E). Analysis of transcripts involved in major pathways regulating the self-renewal and the proliferation of stem- and cancer cells alike (Pardal et al., 2003), revealed furthermore a variety of differentially expressed receptors, ligands, and target genes particularly in the Notch-, WNT-, and TGFβ-pathways (Figure 4-6F). Compared to the profile of heterogeneous 019LPOmig cultures, patterns of differentially expressed CL6 and CL7 genes complemented each other, suggesting potential cellular interactions. This was further encouraged by preliminary in vitro studies demonstrating an interdependence of CL6 and CL7 cells for maintaining their ability to self-renew over prolonged periods of time (Figure 4-7). However, because these findings alone could not explain the discriminating tumorigenic potential of CL6 vs. CL7 cells, we were curious to evaluate their respective genomic configuration.

**Segregation of Tumorigenicity Genes**

Whole genome single nucleotide polymorphism (SNP) analysis was conducted on CL6 and CL7 cells (P5+5), using early and late stage 019LPOmig cultures (P+2 and P+10, respectively) for comparison. All samples showed GBM-specific alterations, including loss of heterozygosity (LOH) of tumor suppressor loci p53 and Rb1, as well as
amplification of the CDK6 and EGF receptor loci (not shown). Specifically, examination of SNPs revealed a total of 34 LOHs that were identically present in all four investigated cultures (Figure 4-8A). This strongly related CL6 and CL7 cells to the GBM pedigree of case 019 and additionally justified the use of our in vitro expansion protocol. The tumorigenic CL6 cells, however, showed additional distinctive LOHs located on chromosomes 10p (39.1Mb), 11c (9.1Mb), 13q (0.7Mb), and 16p (6.4Mb). These LOHs were copy-neutral (Figure 4-8B), potentially affecting a total of 482 gene products. Correlation with whole transcriptome data narrowed down the list of putative tumorigenicity genes to 81 that were actively expressed in CL6, CL7, and/or 019LPOmig cells (Figure 4-8C). 51 of these (63%) mapped to chromosome 10p, 27 (33%) to 16p, 2 to 11c, and none to the 13q locus. Among the identified candidates, several take active part in the process of cancer development (i.e. C10orf7, CUL2, EIF3S8, FUS, NET1), (n=60/81; 74%), and showed a comparable expression level in CL6 vs. CL7 cells. This finding corroborates that the four extra copy-neutral LOH loci reflect a uniparental disomy, in which altered expression of affected genes depends on epigenetic control and complex transcriptome interactions. Yet, 21 genes from the extra loci were exclusively or differentially expressed in CL6 vs. CL7 cells, and one candidate suited to explain the tumorigenic behavior of CL6 cells. The Kruppel-like transcription factor KLF6 (COPEB), a tumor suppressor gene frequently mutated in human prostate cancer, was exclusively downregulated. Mutations of this gene are also observed in human GBM. More frequently, however, the expression of KLF6 may be affected by genomic losses on chromosome 10 that characteristically occur during gliomagenesis. Kimmelman et al recently demonstrated the functional relevance of KLF6 expression for
suppressing tumorigenic activity in human GBM cell lines (Kimmelman et al., 2004). In their work, the authors also reported highly variable degrees of KLF6 expression in primary GBM tissue. Similarly, our 019LPOmig cells showed high degrees of KLF6 expression (Fig. 4c), likely due to the activity of other cell phenotypes in the heterogeneous population that were not affected by the specific genomic aberration on 10p. Failure of KLF6 expression in tumorigenic CL6 cells was therefore not predictable from heterogeneous 019 tumor cells. We conclude that for profiling of tumorigenicity cells and genes, the evaluation of individual tumor cells and their progeny is pivotal.

**Methods**

**Cell Culture**

Handling and transfer to the lab of human tissue derived from consented patients and/or their guardians at the University of Florida Department Neurosurgery, was performed according to institutionally approved protocols. Brain tissue was minced under sterile conditions into chunks of ~1-mm3 volume (Figure 4-2) and randomly divided into two equal parts. One part was fixed in 4% paraformaldehyde (PFA) and stored until further use; the other vital tissue pieces were equally assorted to the mcNSA and the adhesive cell derivation protocols.

Preparation of a single cell suspension for the mcNSA was conducted overnight in a 0.25% trypsin solution on a shaker at 4°C followed by adding five percent fetal calf serum (FCS, HyClone, Logan, UT, USA) and gentle dissociation using graded fire-polished glass pipettes the next day. Trypan blue exclusion confirmed >95% vital cells in each case. Dissociated cells (100,000/ml) were distributed into non-adhesive culture dishes in semi-solid mcNSA media comprising: 1% mc; 5% FCS; modified N2 components (100 μg/ml human apo-transferrin; 5 μg/ml human insulin; 6.29 ng/ml
progesterone, 5 ng/ml sodium selenite; 16.1 μg/ml putrescine); 35μg/ml bovine pituitary extract; 1X antibiotic-antimycotic solution (abx); and 1,000 units/ml human LIF in DMEM/F12. Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), each at a concentration of 40 ng/ml, were added the first day, and at 20 ng/ml every other day thereafter. Unless otherwise specified, media and growth factors are available from Sigma (St. Louis, MO, USA), Invitrogen (Carlsbad, CA, USA), Millipore (Billerica, MA, USA) and/or R&D systems (Minneapolis, MN, USA). Cell culture plastic ware was purchased from Corning (Corning NY, USA). At week 2-3 in culture, primary neurospheres were counted (6 wells for each experiment), collected, centrifuged at 330g, trypsinized, and filtered before counting using 70 μm nylon meshes (BD Biosciences, San Jose, CA, USA) to ensure a single cell suspension. For derivation of higher-degree neurospheres, the cells were re-distributed in non-adhesive culture dishes at densities of 50,000 cells/ml as described above. This passaging protocol was conducted for each case at intervals of 2-3 weeks, for as long as formation of neurospheres was observed (Figure 4-1). For analysis of multipotency, third degree neurospheres derived from the mcNSA were attached to Laminin/poly-L-ornithine (LPO)-coated glass coverslips and maintained without growth factors for 14-35 days in Neurobasal medium containing 1x B27 supplement, 2mM L-glutamine and abx. Cells were fixed in ice-cold 4% PFA for 20 minutes. The mcNSA protocol was also applied to a variety of adherent cell populations at passages 5, 10, and 20 as specified in the results.

For derivation of adhesive cell populations (Figure 4-2), the remaining tissue chunks were further teased into the smallest-possible fragments using a scalpel and
forceps. Approximately 50 micro-fragments were placed per 6cm culture dish, in individual drops (50μl) of media containing 10% FCS in DMEM/F12 + abx overnight. In addition to standard, uncoated plastic dishes, three defined surface coatings (i.e., LPO, FN, and GL; see10 for dish-coating protocols) were used for the simultaneous preparation of adherent cultures from each tissue case. During the course of one week, the volume of media around the adherent micro-fragments was gently increased to a total of 4ml/6cm dish, by daily additions of proliferative media (P-media) consisting of: DMEM/F12 supplemented with 5% FCS; modified N2 components; 1.1 x B27 supplement; 35μg/ml bovine pituitary extract; 1 x abx; and 1,000 units/ml human LIF. EGF and bFGF (each 40 ng/ml) were added the first day, and 20 ng/ml of each were added every other day thereafter. After 2-10 days, cells were observed to migrate out and proliferate around the vicinity of the adherent tissue (Figure 4A). At this time, all tissue fragments (containing cells that were unable to migrate out onto the respective culture dish surface) were removed, trypsinized, and distributed in P media into 6 cm dishes coated with the same substrate. Thus, for each adhesive condition, two types of culture preparations were made – one containing migratory active cells (mig), the other containing dissociated (diss), migratory-delayed, resident cells from the same tissue specimen (Figure 4-3). P-media was changed every four days; 1 μg/ml laminin was continuously present in LPO conditions. For banking of adherent populations, cells from all conditions were grown to confluence and frozen without further passaging (P+0) in two cryotubes / 6cm culture dish (2.5 - 5x105 cells/cryotube).

For preparation of clonal sub-populations, adhesive cultures (P+5) were distributed in P-media at densities of 2-20 cells/cm2 in LPO-coated 10cm dishes. 20
individual locations of adherent cells were circled with a pen on the bottom of the dish at 3 days after plating (Figure 4-6A) and regularly photodocumented from this time on. At days 30-60 after seeding, selected colonies were separated using 8 mm cloning rings, trypsinized, transferred to a 6cm LPO-coated culture dish, grown to confluency, and stored in liquid nitrogen (P5+1) until further use. Expansion of clones and analysis in the mcNSA assay was performed as described above for substrate-specific adhesive cell populations.

The proliferative capacity of adhesive cell populations was assessed expansion in P-media on 6cm dishes coated with the respective substrates. Cells were grown to confluency, trypsinized, counted, and passaged in ratios of 1:2 for up to 20 passages. Population doublings (PD's) were quantified using Hayflick’s formula: \( n = 3.32(\log \text{UCY} - \log I) + X \), where \( n \) is the final PD number at end of a given subculture; \( \text{UCY} \) is the cell yield at that point; \( I \) is the cell number used as inoculum to begin that subculture; and \( X \) is the doubling level of the inoculum used to initiate the subculture being quantified. Cells were photodocumented at least every fifth passage using a Leica (Bannockburn, IL, USA) DM IRB microscope equipped with a DFC 300F camera system.

**Immunocytochemistry.**

The basic immunolabeling buffer contained PBS, 10% FCS, and, for intracellular antigens, additionally 0.1% Triton X-100. After blocking nonspecific antibody activity for 20 min in 5% goat serum, primary antibodies (βIII tubulin, monoclonal mouse, 1:3000, Promega; GFAP, polyclonal rabbit, 1:400, DAKO; CNPase, monoclonal mouse, 1:250, Chemicon) were applied for 4 hours at room temperature. Antigens were visualized using corresponding secondary antibodies (Jackson ImmunoResearch, West Grove,
PA, USA; or Molecular Probes, Eugene, OR, USA). Cell nuclei were labeled for 10 min with 0.8 μg/ml DAPI (Sigma). Fluorescence microscopy was performed on a Leica DMLB upright microscope and images were captured with a Spot RT Color CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA).

**Xenograft Experiments.**

Animal experimentation was conducted according to institutional IACUC guidelines. Cells were trypsinized, concentrated to a density of 2-10 x 10^4/μl DMEM/F12, and one μl of cell suspension was injected into either the lateral ventricle or frontal cortex of adult (>90 days) immuno-compromised NOD-SCID mice (Charles River, Wilmington, MA, USA). The stereotactic coordinates were: 2.5/-0.5/1.2 and 1.5/2/1.3 (mm depth, A-P, lateral), respectively. Engrafted mice were monitored on a daily basis, and tumor-related behavioral abnormalities were videotaped. Animals were sacrificed and perfused transcardially with 4% PFA. Brains were removed and stored in 2% PFA until further use. MRI data were obtained using an 11-T magnet from fixed brains samples placed in PBS for the imaging session. For histological analysis and standard H&E staining, brains were placed in 2% PFA containing 30% sucrose (v/v) overnight, serially sectioned into 20 μm coronal sections on a freezing microtome, and stored in cryoprotectant. Scoring of tumor formation was based on evaluating every fifth section of the engrafted brains for the typical appearance of human tumor cells in the DAPI staining.

**Molecular Biology.**

Total cellular RNA was isolated from neurospheres and adherent cell samples using the RNeasy Mini Kit following the manufacturer’s recommendations (Qiagen, Valencia, CA, USA). Sample amplification and cDNA synthesis was performed using
100ng of total RNA for hybridization to the HumanRef-8 BeadChip according to the manufacturer's instructions (Illumina, Inc., San Diego, CA, USA; http://www.illumina.com/pages.ilmn?ID=197). Arrays were scanned at the microarray core facility at the Burnham Institute for Medical Research (La Jolla, CA, USA) with an Illumina Bead array Reader confocal scanner according to the manufacturer's instructions. Data processing, analysis, and generation of heat maps was conducted using Illumina BeadStudio software (Gene expression module v. 3.2.32). Background and rank invariant methods were used for normalization. Expression intensities with p-values below 0.01 in at least one of four samples were log10 transformed and normalized by z-scoring. Transcripts were defined as ‘differentially expressed’ when intensity values differed more than 2-fold.

For genome-wide single nucleotide polymorphisms (SNP) analysis, DNA was isolated using the Qiagen DNeasy kit according to the manufacturer’s suggestions. Samples were investigated for 561,466 SNPs on the Illumina HumanHap550 BeadChip according to the manufacturer’s Infinium II protocol. Data were analyzed using the Illumina BeadStudio 3.1 software including the Genotyping 3.1.12 and Genome Viewer 3.1.4 modules. Deletions were identified by examination of Log R ratio and B allele frequency. Gene lists were extracted using SNPper - Gene Finder (http://snpper.chip.org/bio/snpper-enter-gene). Multiple chromosomal regions showed characteristics of duplication events based on the intensity ratios of the two alleles. However, in most of these regions, the overall signal intensity (log R ratio) was not exceptionally high. This might be explained by an elevated intensity average due to the fact that a large proportion of the genome is duplicated or indicates that only a
subpopulation of the cells contains the duplications. Thus, due to higher validity, only deletions (resulting in LOH regions) were included in the analysis.
Figure 4-1. *It-SFCs in the mcNSA* (a) Single cell suspensions of aic’s exposed to mitogens in non-adherent, low-density growth conditions, formed multi-cellular spherical aggregates (neurospheres) within 21 ± 7 days (phase contrast, left panel). *1°* neurospheres were dissociated to a single cell suspension and plated to evaluate the generation of higher degree neurospheres (phase contrast, medium panels). For evaluation of multipotency, *3°* neurospheres were plated, outgrowing cells were allowed to differentiate for 21 ± 7 days, and subsequently assessed for expression of neuronal (βIII tubulin, red) and glial (GFAP, green) markers. (b) Most samples contained SFCs (respective frequencies, right), but *lt-SFCs were found in only two cases (arrows). (c) Increase of neurosphere- and total cell numbers were documented for one year in culture (up to the 16° neurospheres stage). Note declining cell- and neurosphere-numbers in sample 020 (a medulloblastoma). SFC fractions did not increase significantly after the 5° neurosphere culture stage (d), and average cells/neurosphere remained constant (e). AE, anaplastic ependymoma; DAPI, nuclear marker 4’,6-diamidino-2-phenylindole; GBM, glioblastoma multiforme; INF, sample lost due to infection; n.d., not determined; SFCs, sphere-forming cells. Scale bars: a (left, phase contrast), 300 μm; a (right, fluorescence), 50 μm.
Figure 4-2. Cell derivation protocol. (a) Surgical tissue specimens were minced into ~1mm³-sized chunks, and randomly selected pieces were allocated to two isolation protocols conducted in parallel. (b) For generating neurospheres in the mcNSA, chunks were dissociated to a single cell suspension and cultured for 2-3 weeks as described in the Methods section. Higher degree neurospheres were prepared by dissociation to a single cell suspension and re-plating of cells into identical mcNSA conditions. This procedure was continued for as long as neurospheres formation could be observed. (c) For separation of migratory active cells, tissue chunks were plated onto substrate-coated culture dishes and covered with individual drops of media. “Migratory” (mig) cultures formed from cells that actively traveled away from the plated tissue chunk within a week. At that time, chunks were removed, dissociated, and the single cell suspension was plated onto similar substrate-coated culture dishes for the generation of “dissociated” (diss) cultures. Both, mig and diss cells were grown to confluency and frozen as case- and substrate-specific adherent cultures in P+0.
Figure 4-3. Distinguishable cell populations result from the adhesive isolation protocol. Phase contrast pictures showing substrate-specific migratory (mig) and non-migratory (dis) cells of cases #018 and 019 at P+4 (population doubling 4-7). Individual cultures could be distinguished based on morphology and distinct population doubling times (upper left corner each image). Scale bar: 75 μm. Abbreviations: dis, migratory inactive (dissociated) subpopulation; FN, fibronectin; GL, gelatine; hr, doubling time of cell population in culture (hours); LPO, laminin/poly-L-ornithine; mig, migratory active subpopulation; no outgrowth, the tissue chunks of case 018 did not adhere on plastic dishes; PL, plastic.
Figure 4. Explant cultures generate target cell populations. (a) The photograph depicts an isolated tissue chunk (case 019) at 3 days in culture with migratory active cells moving away from the tissue (inset). (b) Comparative analysis of the various substrate-specific 018/019 mig- and dis-subpopulations in the mcNSA revealed that LPOmig cultures contained highest frequencies of SFCs (arrows). Their respective multipotency and continuing ability to form higher degree neurospheres could be demonstrated in all cases, with the exception of FNdis and GLdis cultures (stars), using 3° neurospheres in analogy to Figure 1a. Shown are the results from analysis of P+5 cells; similar findings were recorded from 018/019 LPOmig cells at expanded passages 10, 20, and 30 (not shown). (c) A stable expansion kinetic was recorded from both, 018 and 019 LPOmig cultures, with heterogeneous cellular morphologies present throughout the observation period (insets, phase contrast at P+10). (d) Transcript analysis of 018/019 LPOmig cultures at P+10 suggested a continuing presence of stem/progenitor cells. Note that CD133 (PROM 1) transcripts were not present in 018LPOmig cultures. (e) Coronal sequence of FLAIR- (left) and corresponding T2-weighted (right) MRI sections of a NOD-SCID mouse brain at 77 days after engraftment of 25,000 018LPOmig cells (P+10) into the lateral ventricle. No sign of tumor formation can be noted. (f) In contrast, the macroscopic appearance and a sequence of T2-weighted coronal MRI sections of a recipient mouse brain at 39 days after frontal cortex (asterisk) transplantation of 25,000 019LPOmig (P+10) cells. GBM-specific signs include typical T2-heterogeneity, a midline shift, and also a crossing of the midline with far distance spread along the rostro-caudal axis of the host brain. Scale bars: a, 300μm; a (inset), 30μm. Abbreviations: aic, acutely isolated cell; dis, migratory inactive (dissociated) subpopulation; FN, fibronectin; GL, gelatine; LPO, laminin/poly-L-ornithine; mig, migratory active subpopulation; NSA, neurospheres assay; SFC, (neuro-) sphere-forming cell.
Figure 4-5. 019LPOmig cells at early stages post-engraftment. (a) The H&E stained tissue section shows a graft located in the fimbria, 23 days after transplantation of 25,000 donor cells aimed at the lateral ventricle. At this stage, dense clusters of proliferative active donor cells were observed, with individual tumor cells infiltrating the surrounding host tissue, and also moving considerable distance across the midline (boxed area magnified left). (b) Frequently, multinucleated tumor giant cells (left) were observed, which was a characteristic finding of tumor cells present in the original 019 GBM tissue (not shown). Further typical traits of engrafted cells at this stage after engraftment included their accumulation around host capillaries (centre), and the presence of small necrotic areas within the tumor mass (right).
Figure 4-6. Generation of cell clones from 019LPOmig cultures. (a) Phase contrast micrographs of heterogeneous 019LPOmig cells and monomorphic CL6 and CL7 cells after clonal derivation. (b) The graph highlights the dissimilar performance of CL6 and CL7 cells (arrowheads) in the mcNSA. For comparison, data acquired from 019aic cells, as well as from early and late passage 019LPOmig cultures are presented. Two other clonal cell populations (not shown) performed similar to CL6 cells, and thus were not chosen for further analysis. (c) The graph clusters the number of genes expressed in CL6 and CL7 cells according to their respective ratio. Note that the expression of most genes overlap. (d) Expression profiling of CL6 and CL7 cells revealed the presence of stem/progenitor-typic transcripts in both populations. Cultures were investigated at five expansion steps after cloning (P5+5). Note the specific expression of CD133 (PROM1) and nestin in CL7 and CL6 cells, respectively (asterisks). (e) Table summarizing the constellation of most-relevant clinical characteristics of CL6 and CL7 cells. (f) Comparative analysis of differentially expressed genes in proliferation- and self-renewal-pathways (asterisks indicate receptors, ligands, and target genes). Scale bars in a, 30μm.
Figure 4-7. Preliminary studies on interdependence for long-term self-renewal of CL6 and CL7 cells. (a) Growth chart demonstrating stable expansion kinetics of CL6 and CL7 cells. The arrows indicate the culture stage cells were derived from for analysis in this study. (b) Phase contrast images (left) of morphologically distinguishable CL6- and CL7-neurospheres. In analogy to the procedure for aic cells, CL6 and CL7 neurospheres could be serially passaged in the mcNSA, and multipotency analysis was performed on 3° neurospheres. The respective 3° neurospheres generated cells of mostly plump morphologies expressing neuronal and/or glial marker proteins (βIII tubulin and/or GFAP). (c) The failure of CL6 and CL7 cells to self-renew for prolonged periods of time was observed in the mcNSA. Both populations seized to generate neurospheres beyond the 5° culture stage. However, when CL6 and CL7 cells were combined, neurosphere formation continued.
Figure 4-8. Segregation of tumorigenicity genes. (a) Graphic representation of all chromosomes and LOH loci recorded in the four investigated cell samples (chromosomal ideograms from Beadstudio software, Illumina). Note the overlap of 34 LOHs in all samples, as well as CL6-specific aberrations on chromosomes 10p, 13q, and 16p. The LOH on chromosome 11c was also evident in expanded 019LPOmig cells, suggesting a relative increase of CL6 cells over time. (b) Sample file of chromosome 10 SNP analysis. The CL6-selective 39.1Mb large LOH locus is highlighted in red. Note the copy-neutral LOH-typical unchanged gene dose in the log R ratio graph (for comparison, an apparent deletion on 10q is highlighted; arrow). (c) Comparative analysis of the 81 expressed genes from the four extra LOH loci. Of the total 482 potentially affected gene products, the array recognized 225; 144 of these were neither expressed in CL6 or CL7 cells. In CL6 cells, three genes (AKR1C1, AKR1C2, CUGBP2) were exclusively expressed; two genes (AKR1C3, PTPLA) were differentially upregulated; 10 genes (BMP1, PFKP, CLN3, RSU1, STX4A, COMMD3, NMT2, MAPK3, DNAJC1, VIM) were differentially downregulated, and 6 genes (CAMK1D, BAMBI, COPEB, ZNF25, OPTN, MGC3121) were exclusively not expressed. The asterisk demarcates COPEB (KLF6).
OUR laboratory has developed a series of novel human GBM cell lines, which unlike “classic” human glioma cell lines, each demonstrate remarkable, long-distance infiltration throughout the host brain upon intracranial transplantation. Further, the patterns of this microscopic insinuation have remained true to the hallmark corridors of invasion reflected in the human disease – the so-called Secondary Structures of Scherer – peri-vascular satellitosis, subpial spread within and inferior to the meninges, and intrafascicular spread within patterned white matter tracts. With this study we demonstrate, for the first time, that high-grade glioma infiltration into the brain occurs in the absence of a CSPG-rich, inhibitory matrix. In contrast, lesions that strictly grow expansively, such as metastases to the brain, certain low-grade gliomas, and the standard human glioma cell lines are characterized by a robust, CSPG-containing ECM that both defines the mass and profoundly effects the “stromal” cells around and within the tumor. Importantly, our findings suggest several, striking departures from the canon of common knowledge for glioma cell invasion. The role of reactive astrocytes, the association between activated microglia and non-invasive tumor types, and the absence of MMP-mediated proteolysis are among the topics in which our findings deviate from the widely accepted models of glioma cell invasion. In total, this finding support the assertion that tumor-associated CSPGs are sufficient to induce the massive glial reorganizations that characterize the non-invasive tumor microenvironment and the absence of these elements provides favorable conditions for the diffuse infiltration that typifies high-grade glioma and GBM.
Aside from brevican, relatively little is known about CSPGs and glioma invasion. The work that has been done in this area has focused specifically on the activity of the brevican core protein, not the CS-GAG side chains under scrutiny here. Convincing evidence has been brought to light associating diffuse infiltration with the presence of a variant of the brevican protein. This unique brevican derivative, referred to either as the hyaluronan-binding domain (HABD) fragment (Jaworski et al., 1996) or the B/bΔg isoform (Viapiano et al., 2005) has been identified across an array of primary human glioma specimens and is entirely absent from non-invasive brain lesions. These findings may appear at first to controvert our data. They have, in essence presented a credible link between glioma invasion and the presence of a CSPG, whereas we have clearly stated the opposite. However, when we consider the details, our separate investigations are in fact, quite complementary. From our position, staining with the CS-56 antibody or WFA-lectin reveals the GAG-side chains rather than the core proteins, or variants of the core proteins themselves. Therefore, our work identifies these side chains as the true mediators of invasion inhibition. Interestingly, although the B/bΔg isoform (or the HABD fragment as it was referred to originally) described by Hockfield and Matthews is clearly present and enriched in high-grade human glioma, these authors go further to demonstrate that this molecule is actually an under-glycosylated, or perhaps incompletely glycosylated variant of the full-length brevican molecule. (Viapiano et al., 2005) Thus, in both situations, diffuse infiltration corresponds to the relative absence of the otherwise inhibitory CS-GAG side chains. Moreover, using our methods, although presumably present in abundance, this unique under-glycosylated variant would likely go undetected. When taken together, rather than contradict one
another, our separate investigations have elucidated distinct and opposing roles for the proteoglycan core proteins and their CS-GAG side chain in the context of glioma invasion.

Astrocytes have been extensively researched in the context of tumor origination. Persuasive evidence indicates that, for at least certain subclasses of glioma, reactive or stem-like astrocytes may be the original tumorigenic seeds, initiating further tumor growth. (Scherer, 1940; Silver and Steindler, 2009; Hambardzumyan et al., 2011) Beyond these cell-of-origin studies however, there is very limited information regarding their role in the microenvironment of an established tumor and even less that speaks to their specific contributions to the invasive niche. Other than the work presented here, Edwards and colleagues has offered what may be the only other examination of human glioma infiltration and reactive astroglia in vivo. (Edwards et al., 2011) These investigators describe a molecular interaction between reactive astrocytes and an adjacent glioma cell mass, which culminates in the reactive astrocyte population inducing tumor cell dispersal. Our work suggests that the situation described by Edwards and colleagues may represent only one of potentially many reactive astrocyte-tumor cell interactions that ultimately regulate brain tumor invasion. We have demonstrated that reactive astrocytes may either nurture or constrain tumor cell infiltration depending on the presence of CSPGs and the corresponding state of the astrocyte population. On one hand, reactive astrocytes may foster microscopic tumor cell invasion – presumably employing the connective tissue growth factor (CTGF)-mediated mechanism detailed by Edwards and colleagues. On the other hand, in the presence of a robust, CSPG-containing matrix – as in the case of our co-transplantation
experiments – reactive astrocytes actually have the capacity to forcibly confine an otherwise invasive tumor cell population. Thus, our data suggests that the reactive astrocyte-brain tumor relationship is dynamic, allowing for both invasive and anti-invasive modes, modulated through the tumor ECM. Additionally, these findings present the intriguing possibility that the tumor confining abilities of CSPG-stimulated reactive astrocytes might be harnessed in a unique and novel approach toward therapeutic intervention against glioma cell dissemination.

Analogous to the situation with astrocytes, common knowledge informs us that microglia facilitate tumor cell dispersal and contribute to a pro-invasive microenvironment. (Charles et al., 2011) This work has largely been established with the GL261 rodent model of glioma, and suggests that tumor “expansion” is bolstered by the involvement of matrix metalloproteinase 14 (MMP-14 or MT1-MMP) bound to the membrane of tumor-associated microglia. These authors also suggest that the microglial-derived MMP-14 may activate glioma-derived pro-MMP-2 into active MMP-2, thereby adding to the invasive capacity of the tumor. (Markovic et al., 2009) Our data reaffirms the association between microglia and invasion, and also establishes a potentially novel concurrence of microglia with non-invasive tumor growth. To our knowledge, there has been no definitive evidence presented that speaks directly to microglial inhibition of invasion. However, because they are clearly present and active in the case of invasion, we reason that their predominance, coupled with their alternate morphology suggests that they may also actively participate in the pathogenesis of non-invasive tumors. Strikingly, our work provides an explanation for this dramatic morphological and putatively functional difference. Because the composition of the
ECM is so robustly different between these separate tumor types, we contend that these two, distinct cell states arise as a consequence of the microglial-matrix interaction. A conclusion supported by our in vitro microglial activation assay. The presence of tumor-associated CSPGs triggers microglial activation, whereas their absence preserves the microglia in a state of rest. Thus, a synthesis of our work with the preponderance of data that suggests that microglia are pro-invasive, suggests that the microglia-tumor interaction is not fixed. Rather, microglia may either foster or potentially inhibit tumor invasion depending on their specific activation state, which in turn is regulated through the tumor ECM.

The association between CSPGs and non-invasion is something of a paradox when considered from the standpoint of developmental biology. During neural development, CSPGs serve as inhibitory guidance molecules, delineating boundaries between functionally different emerging brain structures. (Cooper and Steindler, 1986; Steindler et al., 1988; Snow et al., 1990) Essentially, these matrix-rich outlines inhibit or even repel axon outgrowth away from a proteoglycan barrier. Therefore, it is something of a paradox that non-invasive tumor cells remain within an environment that is so rich with these classically inhibitory guidance molecules. By extension, when we suppress CSPG inhibition through Ch’ase ABC digestion, we have presumably generated an environment that would allow cells to remain in place, yet under these conditions the tumor cells disperse. Our observations with the LAR phosphatase receptor offer a uniquely simple explanation for these otherwise curious results. Essentially, the non-invasive phenotype is an active, receptor-mediated pathology, in which the tumor cells anchor themselves within the fibrils of a CSPG-rich ECM. The LAR receptor acts as a
tether, securing the cells to their secreted matrix and in turn preventing the matrix molecules from diffusion. In consequence, the non-invasive tumor develops as a precisely focused concentration of proteoglycan. In addition, the absence of LAR from the reactive astrocyte population helps to clarify their unique response to the developing lesion. Recall that the LAR staining pattern precisely defined the non-invasive tumor itself and was not seen in the surrounding reactive astrocytes. Thus, whereas the LAR-expressing tumor cells are held in place, the reactive astrocytes are potently repelled away to the boundaries of the CSPG-rich field of matrix. Importantly, this scheme is supported by our Ch’ase ABC-induced tumor invasion studies. Ch’ase ABC digestion removes the ligand for the LAR phosphatase receptor. Analogous to a function-blocking antibody or peptide, the absence of the ligand compromises receptor-mediated coupling to the proteoglycan-rich matrix. Consequently, the constraints on this otherwise non-invasive tumor cell population are lifted and we observe microscopic dissemination of the tumor.

The concept that glioma cell invasion might occur in the absence of a CSPG-rich inhibitory matrix is highly controversial. Numerous studies have indicated that the breakdown of an initially present inhibitory ECM is a required first step before invasion is possible. (Rao, 2003) Although this is clearly the case for epithelial tumors, our data suggests that CSPG-mediated inhibition of invasion may represent a minimal, or perhaps negligible barrier, from the very earliest stages of high-grade glioma development. However, we were concerned that diffusely infiltrative lesions may have initially produced CSPGs, only to be immediately proteolyzed by abundant and highly active proteases. Although our data did not support this notion – no evidence for
functional MMP-2 or 9 was detected in the invasive tumors – we cannot rule out the possibility that other proteolytic enzymes may contribute to the diffusely invasive phenotype and we do not intend to discount protease-mediated tumor invasion in general. Rather, our findings substantiate a need to place CSPGs, and matrix proteolysis in context within the overall tumor pathology. For instance, CSPGs are observable, albeit at relatively low levels, within and surrounding sites of pseudopalisading necrosis both within our animal models as well as human pathological specimens of glioma. Thus, these matrix-degrading mechanisms may apply to these specific zones, however may have little or no applicability to the tumor in general, and especially to the invasive edge where CSPGs are essentially absent. In this light, our two distinct tumor types may better represent the opposite ends of a spectrum of invasion – comprising CSPG-rich, expansive lesions on one side, and CSPG-absent, diffusely infiltrative tumors on the other – all parts of which may be present within a single, complete glioma.

In total, (Figure 2-8) we have presented the novel finding that the relative presence or absence of micro-environmental CSPGs correlates with the invasive character of human glioma. Using primary human glioma cell lines as well as pathological human specimens, we have demonstrated that high-grade, diffusely infiltrating lesions are associated with the relative absence of CSPGs, whereas their robust presence is associated with strictly expansive tumor growth. Further, the strong presence of these potently inhibitory molecules within the non-invasive tumor microenvironment induces sweeping morphological and/or architectural changes in tumor-associated astrocytes and microglia that may contribute to the active containment of the tumor cell population.
Conversely, the absence of these tumor-glial cell interactions preserves an environment that is uniquely conducive to widespread tumor cell insinuation. Importantly, amplifying or attenuating the levels of CSPGs within the tumor microenvironment can directly modulate tumor cell infiltration. This work presents a novel insight into the basic biology of the brain tumor microenvironment, the ECM, and their synergist influence over microscopic tumor cell invasion.

The findings from Chapter 4 address a series of considerations prevailing in the field of cancer research. First, the phenotypic and molecular profiles of rare cell populations that drive aggressive types of cancer are not necessarily apparent among the heterogeneous cells of a tumor sample. Second, a distinctive profile of tumorigenic cells remains difficult to predict based on existing theories; and third, more than one clonal cell population may be responsible for tumor maintenance.

In contrast to prospective isolation methods that aim for purification of target cells with predicted qualities, such as the expression of specific cell surface markers or the exclusion of Hoechst-dye by side populations, we initiated our experiments with a heterogeneous population. Because extensive self-renewal is regarded as a cardinal feature of stem cells and CSCs alike, analysis was restricted to tissue samples that showed a profound content of multipotent, clonally active, long-term self-renewing cells, (as determined in the mcNSA). The rationale of this approach was to access the highest possible degree of phenotypic diversity present among tumor-residing cells at the time of surgery for expansion and retrospective analysis of the tumor-sustaining cell fraction. To facilitate cell extraction from tumor tissue without bias, we offered a spectrum of culture substrates that attracted diverse subpopulations of tumor cells from tissue
explants, additionally enabling the separation of local migratory from non-migratory cell populations. Cells derived from this system were rapidly expandable as heterogeneous or as single cell-derived adherent cultures without losing the characteristic genomic traits of the initial starter cell population. Thus, a plentitude of material was made readily available that enabled the reliable evaluation of the tumorigenic and stem/progenitor qualities of the original specimen.

The successful use of this approach was exemplified on two individual, single cell-derived populations isolated from GBM tissue. Clonal activity was a rare feature among 019-GBM cells, however, characterization of two clonally expandable cancer stem-like clones (CL6 and CL7) showed largely overlapping genomic and transcriptome expression profiles when compared to the heterogeneous maternal culture and to each other. In accordance to the CSC hypothesis, both clones showed neural stem/progenitor traits upon analysis in the mcNSA, many of the expressed genes would be expected active in bona fide developing CNS neural stem cells, and the respective chromosomal aberrations were GBM-typic. Yet, CL6 and CL7 cells differed in their ability to induce tumors in orthotopic xenograft experiments. The characteristic loss of KLF6 expression due to a distinctive copy-neural LOH on chromosome 10p could explain this marked biological behavior of CL6 cells. These findings attenuate previous implications on tumor suppressing activities of KLF6 to an individual cell-derived level, and furthermore demonstrate the need to explore single cells and their progeny for classification of distinctive targets in malignant brain tumor diagnosis and therapy.

An intriguing observation of our study, echoing conclusions made by Beier et al., 2007, was that the ability for long-term propagation and the expression of CD133 – two
candidate characteristics of tumor sustaining cells according to existing theories, do not represent essential hallmarks of tumorigenicity. Although both cell lines robustly expressed CD133, neither heterogeneous 018-AE cultures, nor single cell-derived 019-GBM CL7 cells displayed tumorigenic potential. These findings weaken the argument within recent discussions relating to the specificity of CD133 as an indicator of tumorigenic cells (Beier et al., 2007; Shmelkov et al., 2008). Alternatively this result may be viewed in light of the potential pitfalls of xenograft experimentation. Just as Kelly et al., 2007 suggested, there might be a need to co-transfer additional accessory cells to enable complete mimicking of malignant disease. This thought is further driven by our preliminary observations of tumorigenic CL6 and non-tumorigenic CL7 cells relying on each other for prolonged self-renewal. Cellular interactions might involve components of the pleiotropic Notch-, WNT-, and TGFβ-pathways, for which partly complementary expression profiles were recorded. And consequently, a potential lack of cellular interactions could explain why, in our experiments, CL6 cells required more time to elicit neuro-behavioral abnormalities in xenografted animals than similar quantities of heterogeneous 019LPOmig cells (71±5 vs. 41±3 d, respectively. Lastly, the particular phenotypic constellation of CD133-/MGMTlow CL6 and CD133+/MGMT+ CL7 cells deserves attention, as MGMT-expressing GBM cells are considered less susceptible to alkylating chemotherapy.

Taken together, our data exemplify an alternative in vitro approach suited to study the cellular basis of heterogeneity typically observed in malignant tumors, such as GBM3. While, currently, the entire spectrum of clonally active cellular phenotypes cannot be anticipated, their co-existence needs to be considered, as well as their
interactions – with each other, and the surrounding tissue. Our method may help to interrogate cancers for the particular stem cell types at the root of tumor formation and tumor maintenance. Knowledge of the identity of these cells as well as a more complete understanding of their interactions with others cells within and without the tumor will help to direct our therapeutic efforts in targeting these unique cells. Respective analysis relating phenotypic, molecular, genetic and epigenetic traits to functional activity may pave the way for dissecting the hierarchical composition of human malignancies, facilitating their classification and leading to a more individualized diagnostic and therapeutic approach.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Daniel was born in Boston, Massachusetts, raised in Cleveland, Ohio and currently lives in Gainesville, Florida with his wife Melinda and daughter Olivia.

He attended Charles F. Brush High School in South Euclid, Ohio and graduated magna cum laude with a BS in chemistry from Cleveland State University. Daniel worked for several years after graduation as an organic synthesis chemist for the Cleveland biotech company Athersys, Inc. After leaving Athersys, Daniel went to work as a lab technician for his father Jerry, at Case Western Reserve University were he published his first papers and fell in love with biology.

While working at CWRU, Daniel met his mentor Dennis Steindler at a Society for Neuroscience meeting in San Diego, California in 2004. The following fall, Daniel began his doctoral training at the University of Florida with Dennis as his advisor. This dissertation represents the culmination of his time as a student with Dennis.

He and his family will leave Gainesville for New York City, where he has accepted a position as a postdoctoral fellow in Eric Holland’s laboratory at Memorial Sloan-Kettering Cancer Center.