Role and mechanisms of brevican, a major component of the neural microenvironment, to promote invasion in malignant gliomas

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Hosung Sim

Graduate Program in Molecular, Cellular and Developmental Biology

The Ohio State University

2012

Dissertation Committee:

Mariano S. Viapiano, PhD, Advisor

Balveen Kaur, PhD

John Oberdick, PhD

Tsonwin Hai, PhD
Abstract

Gliomas are the most aggressive primary tumors of the central nervous system (CNS). The median survival of patients with high-grade gliomas remains at approximately one year and has not changed over the past decade, even though these patients are treated with aggressive therapies that include improved surgery and adjuvant radio-chemotherapy. A major cause of poor prognosis for glioma is the characteristic invasive ability of the malignant cells, which facilitates tumor scattering and recurrence, making gliomas impossible to manage in the long term.

Novel and more effective therapies for glioma must address the invasive nature of these tumors and target the molecular mechanisms that support tumor invasion. Glioma invasion is a unique process of adaptation to the neural microenvironment and, in particular, to the unique composition and structure of the neural extracellular matrix (ECM). The neural ECM is devoid of fibrillar proteins but is rich in hyaluronic acid (HA) and HA-binding chondroitin sulfate proteoglycans (CSPGs) that usually inhibit cell motility and axonal extension.

Brevican is a neural-specific CSPG and the most abundant HA-binding CSPG in normal brain. Although brevican behaves as a traditional inhibitory proteoglycan in normal CNS, it exhibits a paradoxal role in glioma, promoting tumor cell invasion. The unique expression of brevican in the brain and gliomas, the existence of glioma-specific isoforms of brevican, and the pro-invasive role of this CSPG make brevican an appealing
and accessible target in the tumor microenvironment of gliomas. However, the mechanisms by which brevican promotes tumor invasion have remained largely unknown. In this thesis, I investigate the molecular mechanisms of brevican that promote glioma cell motility and the role of brevican as a pro-invasive signal in gliomas and in the tumor microenvironment.

In chapter 2 of this thesis, I investigate the interaction of brevican with HAPLN4, a small glycoprotein of the HAPLN family, in the normal brain and gliomas. HAPLNs are the canonical partners of the HA-binding CSPGs and contribute to the structure and stability of the neural ECM. The major hypothesis in this chapter is that the association of brevican and HAPLN4 may explain the pro-invasive role of brevican in gliomas. However, results in this chapter show that HAPLN4 is in fact absent in gliomas and likely not associated to brevican. In addition, when HAPLN4 is reintroduced in glioma cells it does not appear to interact with brevican and both proteins behave in a complementary manner, promoting tumor cell motility. Overall, the results invalidate our initial hypothesis that brevican's effects in gliomas could be in part explained by its interaction with HAPLNs.

In chapter 3 of this thesis I focus on the activation of EGFR by brevican, which is the only signaling mechanism ever described for this CSPG in glioma. The major hypothesis in this chapter is that activation of EGFR may be necessary and sufficient to explain the pro-invasive role of brevican in gliomas. In agreement, I demonstrate a novel mechanism by which a bioactive fragment of brevican, B50, binds to sulfatides in the cell membrane and activates Src kinase, therefore trans-activating EGFR and promoting cell migration. This is the first demonstration of a novel signaling pathway triggered by a
CSPG and provides a novel understanding of these proteins as signaling molecules in the neural ECM.

In chapter 4, I study a novel mouse genetic model with a dominant inactivating mutation in EGFR (Velvet mice) and analyze the effects of the Velvet microenvironment for tumor formation, invasion, and the pro-invasive effect of brevican. My hypothesis in this chapter is that absence of EGFR activity in the brain may affect glioma biology even when the tumor is driven by its endogenous EGFR overexpression. Results in this chapter show for the first time the characterization of Velvet astrocytes and their lack of response to glioma cells. Surprisingly, I also demonstrate that intracranial tumors grow in Velvet mice in the same manner as in wild-type animals, but their invasion is reduced. In addition, I demonstrate that the pro-invasive effect of brevican is lost when glioma cells invade through Velvet brain tissue, showing that the effects of brevican are not restricted to glioma cells but also extend to the tumor microenvironment.

Finally, in chapter 5 I discuss the implications of the findings above and how they improve our understanding of brevican functions and mechanisms in glioma. In addition, I provide preliminary data showing that the molecular signaling of brevican may be even more complex and may affect other families of receptors, such as PDGFR. In sum, results in this thesis show typical "structural" molecules in the neural ECM under a new role as signaling molecules that can trigger multiple pathways in the tumor and the tumor microenvironment to promote glioma invasion. These results underscore the possible relevance of targeting brevican and the mechanisms triggered by this CSPG for future therapeutic anti-invasive strategies in glioma.
Acknowledgements

I would like to thank my advisor, Dr. Mariano Viapiano, for his guidance and enormous support during my graduate studies at The Ohio State University. He encouraged me to think creatively and gave me many ideas to advance my research.

I would like to thank the members of my committee, Dr. Balveen Kaur, Dr. John Oberdick, and Dr. Tsonwin Hai for their time and advice for my graduate research. I also thank Dr. Sean Lawler, who was a former collaborator and member of the committee.

I would also like to thank all former and present members of the Viapiano lab, in particular Dr. Bin Hu, who provided me help and advice to improve my research in many occasions. Dr. Nandhu Mohan is a great colleague that helped me through the last year of my research. Colleen Pineda was an excellent student working with me and creating the constructs needed for my research.

I thank Dr. Lyn Jakeman for the collaboration that we established and that helped the last section of my studies. I also thank Kent Williams and Paul Gruenbacher, students from Dr. Jakeman, for their kind help with my research.

I thank Dr. Taewan Kim and Dr. Jongkuk Park for their friendship and support since we started the graduate school together at OSU. I specially thank Youngjun Jeon for his support, advice and friendship over many years in Korea as well as in Ohio.

Finally, I would like to thank my parents and my younger brother for their love even though I have been far away from home for many years. Without their support, it
would have been very difficult for me to finish my graduate study. I am very happy to be
finishing my graduate studies, which will be a good present for my parents and at my
brother’s wedding this year.
Vita

2006…………………………………………………………………...Bachelor of Science

SungKyunKwan University

2006-Present………………………………………………....Graduate Research Associate

Molecular, Cellular and Developmental Biology Graduate Program,

The Ohio State University

Fields of Study

Major Field: Molecular, Cellular and Developmental Biology

Neuro-oncology

Publications


• Sim H., Hu B. Pineda C. A., Yoon S.O., Viapiano, M.S. Brevican transactivates the Epidermal Growth Factor Receptor via Src kinase and cell-surface sulfatides to promote glioma cell motility (Second submission to the Journal of Biological Chemistry)

• Sim H., Gruenbacher P., Jakeman L., Viapiano, M.S. Reduced Epidermal Growth Factor Receptor activity in the brain does not affect glioma growth but inhibits tumor invasion (In preparation for J. Neuroscience)
Table of contents

<table>
<thead>
<tr>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>- Acknowledgements</td>
<td>v</td>
</tr>
<tr>
<td>- Vita</td>
<td>vi</td>
</tr>
<tr>
<td>- List of Tables</td>
<td>ix</td>
</tr>
<tr>
<td>- List of Figures</td>
<td>x</td>
</tr>
<tr>
<td>- Abbreviations</td>
<td>xiii</td>
</tr>
<tr>
<td>- Chapters:</td>
<td></td>
</tr>
<tr>
<td>1. Introduction</td>
<td></td>
</tr>
<tr>
<td>1.1 Gliomas</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Glioma invasion</td>
<td>3</td>
</tr>
<tr>
<td>1.3 The neural extracellular matrix</td>
<td>5</td>
</tr>
<tr>
<td>1.4 The lectican family of chondroitin sulfate proteoglycans</td>
<td>6</td>
</tr>
<tr>
<td>1.5 The CSPG brevican and its roles in gliomas</td>
<td>8</td>
</tr>
<tr>
<td>1.6 The family of hyaluronan binding and proteoglycan link proteins</td>
<td>10</td>
</tr>
<tr>
<td>1.7 Epidermal Growth Factor Receptor signaling in gliomas</td>
<td>12</td>
</tr>
<tr>
<td>1.8 Conclusions and major hypothesis</td>
<td>14</td>
</tr>
<tr>
<td>Figures of chapter 1</td>
<td>16</td>
</tr>
<tr>
<td>2. Characterization of HAPLNs and their interaction with brevican in</td>
<td></td>
</tr>
<tr>
<td>malignant gliomas</td>
<td></td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>22</td>
</tr>
<tr>
<td>2.2 Materials and methods</td>
<td>24</td>
</tr>
<tr>
<td>2.2.1 Cells and antibodies</td>
<td>24</td>
</tr>
<tr>
<td>2.2.2 Human tissue processing</td>
<td>26</td>
</tr>
<tr>
<td>2.2.3 Constructs and cell transfection</td>
<td>26</td>
</tr>
<tr>
<td>2.2.4 Cell adhesion and motility assays</td>
<td>27</td>
</tr>
<tr>
<td>2.2.5 Protein solubilization from brain membranes</td>
<td>28</td>
</tr>
<tr>
<td>2.2.6 Western blotting and RT-PCR</td>
<td>29</td>
</tr>
<tr>
<td>2.2.7 Microarray meta-analysis</td>
<td>30</td>
</tr>
<tr>
<td>2.3 Results</td>
<td>31</td>
</tr>
<tr>
<td>2.3.1 HAPLN4 is the predominant member of the link protein family in</td>
<td>31</td>
</tr>
<tr>
<td>human brain cortex</td>
<td></td>
</tr>
<tr>
<td>2.3.2 Expression of brain-specific HAPLNs is strongly reduced</td>
<td></td>
</tr>
</tbody>
</table>
in high grade gliomas

2.3.3. Expression of HAPLN4 increases the motility of glioma cells

2.3.4 HAPLN4 retention in brain cell membranes cannot be replicated in cultured glioma cells

2.4 Discussion

Figures of chapter 2

3. Brevican trans-activates the epidermal growth factor receptor via Src kinase and cell-surface sulfatides to promote glioma cell motility

3.1 Introduction

3.2 Materials and methods

3.2.1 Cells and antibodies

3.2.2 Purified proteins and cleavage of full-length brevican

3.2.3 cDNA constructs and cell transfection

3.2.4 Purification of biotinylated B50

3.2.5 Western blotting and quantitative RT-PCR

3.2.6 Sulfatide analysis

3.2.7 Cell adhesion and motility assays

3.3 Results

3.3.1 Cleavage of brevican is necessary for activation of EGFR and Erk

3.3.2 B50 activates EGFR at a Src-dependent phosphorylation site

3.3.3 B50 activates Src kinase to phosphorylate EGFR

3.3.4 B50 binds to sulfatides to activate Src kinase

3.3.5 Src inhibition abolishes the pro-adhesive and pro-migratory effects of B50

3.4 Discussion

Figures of chapter 3

4. Reduced EGFR activity in the brain does not affect glioma growth but reduces tumor invasion and prevents the pro-invasive role of brevican

4.1 Introduction

4.2 Materials and methods

4.2.1 Cells, antibodies and purified proteins

4.2.2 Biochemical assays and immunocytochemistry

4.2.3 Cell viability and proliferation

4.2.4 Cell migration and invasion assays

4.2.5 Tumor xenografts, immunohistochemistry and tumor invasion

4.2.6 Statistics

4.3 Results
4.3.1 Velvet astrocytes lack EGFR activity but do not exhibit compensatory ErbB expression

4.3.2 Velvet astrocytes do not show increased growth or viability in presence of an EGFR ligand

4.3.3 EGFR-dependent motility of Velvet astrocytes is significantly impaired

4.3.4 Velvet astrocytes show reduced attraction towards glioma cells

4.3.5 The tumor microenvironment of Velvet mice does not affect the intrinsic growth of malignant glioma

4.3.6 The tumor microenvironment of Velvet mice reduces tumor invasion

4.3.7 B50 cannot enhance glioma cell invasion through Velvet brain tissue

4.4 Discussion

Figures of chapter 4

5 Conclusions and future directions: Relevance of brevican for glioma progression and potential therapeutic strategies

5.1 Focusing on brevican

5.2 The molecular partners of brevican in the brain and glioma

5.3 EGFR activation as the key promigratory trigger of brevican

5.4 Brevican requires EGFR in the tumor microenvironment to promote glioma invasion

5.5 Other signaling pathways triggered by brevican? The case for PDGFR

5.6 Conclusions: Brevican as a therapeutic target in the tumor microenvironment

Figures of chapter 5

Bibliography
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Major isoforms of the CSPGs of the lectican family</td>
</tr>
<tr>
<td>5.1</td>
<td>cDNA primers used to quantify gene expression by quantitative RT-PCR</td>
</tr>
<tr>
<td>5.2</td>
<td>Antibodies used for Western blotting and immunohistochemistry</td>
</tr>
</tbody>
</table>
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 General structure of the neural ECM</td>
<td>16</td>
</tr>
<tr>
<td>1.2 The lectican family of CSPGs</td>
<td>17</td>
</tr>
<tr>
<td>1.3 Multiple isoforms of brevican</td>
<td>18</td>
</tr>
<tr>
<td>1.4 Chromosomal arrangement of CSPGs and HAPLNs</td>
<td>19</td>
</tr>
<tr>
<td>1.5 Schematic of EGFR signaling in gliomas</td>
<td>20</td>
</tr>
<tr>
<td>2.1 HAPLN4 is the predominant member of the link protein family in adult human brain</td>
<td>45</td>
</tr>
<tr>
<td>2.2 Expression of the HAPLN mRNAs is highly reduced in malignant gliomas</td>
<td>46</td>
</tr>
<tr>
<td>2.3 HAPLN proteins are absent in malignant gliomas</td>
<td>48</td>
</tr>
<tr>
<td>2.4 HAPLN4s are expressed in astrocytes but are downregulated in glioma cells</td>
<td>49</td>
</tr>
<tr>
<td>2.5 Overexpression of HAPLN4 does not affect cell viability</td>
<td>50</td>
</tr>
<tr>
<td>2.6 HAPLN4 increases glioma cell adhesion in a substrate-dependent manner</td>
<td>51</td>
</tr>
<tr>
<td>2.7 HAPLN4 increases glioma cell motility</td>
<td>52</td>
</tr>
<tr>
<td>2.8 HAPLN4 and brevican increase the motility of glioma cells cultured in a wound-healing assay</td>
<td>53</td>
</tr>
<tr>
<td>2.9 HAPLN4 does not associate to the surface of cultured glioma cells</td>
<td>54</td>
</tr>
<tr>
<td>2.10 HAPLN4 does not associate to HA-producing glioma cells</td>
<td>55</td>
</tr>
<tr>
<td>2.11 HAPLN4 is strongly associated to cell membranes of neural tissue</td>
<td>56</td>
</tr>
<tr>
<td>3.1 The time-course of EGFR activation coincides with brevican cleavage by glioma cells</td>
<td>75</td>
</tr>
<tr>
<td>3.2 Preparation of purified B50</td>
<td>77</td>
</tr>
<tr>
<td>3.3 Purified B50 activates EGFR at a Src-dependent phosphorylation site</td>
<td>78</td>
</tr>
<tr>
<td>3.4 B50 activates EGFR in a Src-dependent manner</td>
<td>79</td>
</tr>
<tr>
<td>3.5 B50 can activate EGFR in a ligand-insensitive EGFR-deficient model</td>
<td>80</td>
</tr>
<tr>
<td>3.6 B50 to cell-surface sulfatides to activate Src</td>
<td>81</td>
</tr>
<tr>
<td>3.7 Src inhibition abolishes the effect of B50 on cell adhesion and migration</td>
<td>82</td>
</tr>
<tr>
<td>3.8 Proposed model of action of brevican in glioma cells</td>
<td>83</td>
</tr>
<tr>
<td>4.1 Immunochemical characterization of astroglial cultures</td>
<td>105</td>
</tr>
<tr>
<td>4.2 EGFR activity is almost completely abolished in Velvet astrocytes</td>
<td>106</td>
</tr>
</tbody>
</table>
4.3 Expression of EGFR is not compensated by other ErbB receptors in Velvet astrocytes 107
4.4 Velvet astrocytes showed reduced proliferation and viability even in presence of TGF-alpha 108
4.5 EGFR activity is sufficient to enhance the migration of astrocytes in absence of other growth factors 109
4.6 Velvet astrocytes show reduced formation of lamellipodia and activation of VASP 110
4.7 Velvet astrocytes show reduced attraction towards glioma cells 111
4.8 Total growth of non-invasive glioma is not different between wild-type and Velvet mice 112
4.9 Expression of basal-lamina antigens is comparable in tumors developed in wild-type and Velvet animals 113
4.10 Invasive tumors show reduced dispersion in Velvet tissue 114
4.11 Tumor invasion away from a compact core is reduced in Velvet mice 115
4.12 Expression of MMP2 is highly reduced in Velvet astrocytes 116
4.13 The pro-invasive effect of B50 is lost in cells dispersing through Velvet tissue 117

5.1 Brevican activates PDGFR signaling in glioma cells and oligodendrocyte precursors in the brain 130
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACAN</td>
<td>aggrecan</td>
</tr>
<tr>
<td>ADAMTS</td>
<td>A Disintegrin And Metalloproteinase with ThromboSpondin Motifs</td>
</tr>
<tr>
<td>BCAN</td>
<td>brevican</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSPG</td>
<td>chondrotin sulfate proteoglycan</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamino-tetraacetate</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronic acid</td>
</tr>
<tr>
<td>HAPLN</td>
<td>hyaluronan binding link protein</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's buffered saline solution</td>
</tr>
<tr>
<td>NCAN</td>
<td>neurocan</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCAN</td>
<td>phosphacan</td>
</tr>
<tr>
<td>PDGFR</td>
<td>platelet derived growth factor receptor</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>TME</td>
<td>tumor microenvironment</td>
</tr>
<tr>
<td>VASP</td>
<td>Vasodilator-stimulated phosphoprotein</td>
</tr>
<tr>
<td>VCAN</td>
<td>versican</td>
</tr>
</tbody>
</table>
Chapter 1:

Introduction

1.1. Gliomas

Gliomas are the most common tumors in the central nervous system (CNS) and strike approximately 15,000 people every year in the US (Central Brain Tumor Registry of the United States, 2011). Although they are not as common as other solid tumors, their rapid progression and extremely poor prognosis (overall survival <3% at 5 years post-diagnosis) makes them one of the most deadly types of solid cancer that causes more years of life lost compared to any other tumors (Schwartzbaum et al., 2006).

Current therapy for glioma includes surgical resection in all operable cases, followed by an aggressive course of 6-weeks of radiotherapy and up to six months of recurring chemotherapy with the alkylating drug temozolomide (Temodar™) (Wen and Kesari, 2008). Novel therapeutic modalities are being developed and include drug combinations, in particular with anti-angiogenic agents. However, the prognosis of these brain tumors has remained dismal despite considerable technological advances in surgery and radiotherapy, and the median survival for high-grade glioma (glioblastoma) remains at less than 14 months (Wen and Kesari, 2008). This underscores the need for a further understanding of the biology of these tumors, their intimate relationship with the neural microenvironment, and the identification of novel molecular targets for effective
therapeutic strategies.

Gliomas originate from undifferentiated or glial-committed neural precursors (Germano et al., 2010), and the resulting tumor cells resemble astrocytes, oligodendrocytes, or ependymal cells (Louis, 2006), usually characterized by nuclear atypia. Therefore, gliomas have been historically classified as astrocytoma, oligodendroglioma, or ependymoma based on their morphology (Zimmermann, 1969). In addition, gliomas can be classified as grade I-IV according to WHO criteria for malignancy (Louis et al., 2007). The most common and malignant gliomas, which are grade IV highly undifferentiated astrocytomas, are known as glioblastoma multiforme (Holland, 2000).

Recently, glioblastomas have also been classified into four molecularly defined subgroups, (neural, proneural, classical, and mesenchymal) according to transcriptome analysis (Phillips et al., 2006). Further studies have tried to simplify this analysis and reduce it to a minimum subset of genes sufficient to identify molecular subgroups with differential prognosis, with variable levels of success (Carro et al., 2010; de Tayrae et al., 2011; Kawaguchi et al., 2012).

Aberrant gene expression (gene deletion or duplications) has been a more reliable indicator of glioma prognosis and has also been utilized to establish a classification of these tumors (Louis et al., 2001). Interestingly, a novel relationship between molecular signatures and major underlying gene deletions/amplifications is now emerging. For example, classical-type gliomas show EGFR amplification and deletion of the tumor suppressor genes p16/INK4A and PTEN. In contrast, mesenchymal-type gliomas are characterized by inactivating NF1, p53, and CDKN2A mutations. Finally, proneural-type
gliomas usually have amplification of PDGFRA, CDK4, and CDK6 (Huse et al., 2011). Therefore, specific patterns of mutations, which are easier to identify than transcriptomic signatures, have been proposed as a basis to establish prognostic or therapeutic information about these tumors.

1.2. Glioma invasion

The poor prognosis of gliomas is a result of the resistance of these tumors to conventional therapy and the rapid recurrence of residual tumor after the initial treatment (Louis, 2006). A major cause of recurrence is the invasion of gliomas into surrounding brain tissue, resulting in a general dispersion of cells beyond the limits of detection and treatment (Maher et al., 2001). Glioma cells are able to infiltrate through white matter tracts like the corpus callosum and the thalamo-reticular tract, despite the presence of myelin inhibitors of cell motility in these white matter tracts (Fournier et al., 2002). In addition, tumor cells are able to migrate along blood vessels, non-myelinated axons, and under the pia mater in the brain (Louis, 2006), showing a remarkable ability to find dispersion avenues from the central tumor core.

At the cellular level, glioma invasion is a multiple-step process that involves cell adhesion to a non-fibrillar scaffold (described below), extracellular matrix (ECM) degradation and a unique type of myosin II-based cell migration. Adhesion of glioma cells in vitro is mediated by multiple cell adhesion molecules, including the cadherins, Neural Cell Adhesion Molecule (NCAM), integrins, and the receptor for hyaluronic acid, CD44, among others (Demuth and Berens, 2004). Some of these molecules are involved in migration through cell-cell adhesion mechanisms (cadherins and NCAM) while
integrins and CD44 mediate association of glioma cells to the HA-based, non-fibrous components of the neural ECM, as well as to the fibronectin- and laminin-based basal lamina of the blood vessels and subpial surface (Viapiano and Lawler, 2009).

ECM degradation is thought a critical process for glioma cells to invade the brain parenchyma (Claes et al., 2007), although most models of matrix degradation have been adapted from other solid tumor models and mimick invasion through basal lamina or a collagen-based stroma, none of which exist in the brain (Viapiano and Lawler, 2009). In agreement with this "classical" view of matrix degradation, research in the field has largely focused on studying and targeting MMPs and ADAMTSs, two major families of secreted metalloproteases that promote degradation of ECM molecules in several tissues, including the brain (Levicar et al., 2003; Rao, 2003). Another major protease secreted by glioma cells is the serine-protease urokinase-type plasminogen activator (uPA), which activates plasminogen to plasmin and pro-MMPs to MMPs (Rao, 2003). MMP2 and MMP9 are in fact highly upregulated in high-grade gliomas (Levicar et al., 2003) and MMP inhibition reduces tumor growth, although its effects on tumor invasion in vivo have been less evident (Viapiano and Lawler, 2009). The view of invasive glioma cells as highly aggressive cells contrasts, however, with the fact that these tumors never breach the blood vessels in the brain to metastasize (see below), suggesting that glioma invasion could be largely supported by different mechanisms.

Indeed, there is considerable research showing that tumor cells may invade without using proteolytic mechanisms (Friedl and Wolf, 2003; Sabeh et al., 2009). This "interstitial migration" is largely dominated by the rigidity of the matrix and the resistance to cell motility (Gritsenko et al., 2012). Because gliomas migrate through a
unique type of soft ECM in the CNS (see below) it is likely that their invasion may be at least in part independent of matrix degradation. Instead, it has been shown that glioma cells are able to "squeeze" through intercellular pores in the ECM using a myosin-II dependent mechanism (Beadle et al., 2008) and use this mechanism to achieve cell dispersion \textit{in vivo}.

The most remarkable feature of glioma invasion is that it is a striking process of adaptation of these tumor cells to their surrounding microenvironment. Gliomas almost never metastasize and do not even breach the basal lamina of brain blood vessels (Bernstein and Woodard, 1995; Bohm et al., 2003). Moreover, if glioma cells are implanted peripherally they do not invade non-neural tissues (Pilkington, 1997). At the same time, most solid tumors that metastasize to the brain can grow within neural tissue but cannot invade it (Subramanian et al., 2002). Overall, these results underscore the importance of the relationship between invading glioma cells and the surrounding ECM for brain tumor invasion (Bellail et al., 2004; Gladson, 1999; Viapiano and Lawler, 2009).

\textbf{1.3. The neural extracellular matrix}

The neural ECM occupies approximately 20\% of the total volume of the CNS (Sykova and Nicholson, 2008). Despite being a major component of the neural microenvironment, the role of the neural ECM in neurophysiology was largely ignored due to technical difficulties to visualize it, fix it, and study its protein components (Celio and Blumcke, 1994). Indeed, the neural ECM is non-fibrillar, devoid of collagens, extremely compliant (soft), largely soluble, and composed of molecules that form complexes that can be extracted with relatively mild conditions from the tissue (Viapiano
and Matthews, 2006). The major components of neural ECM are hyaluronic acid (HA), chondroitin sulfate proteoglycans (CSPGs), and small glycoproteins (Ruoslahti, 1996) (Figure 1.1).

HA is the major structural molecule forming the scaffold of the neural ECM. This protein-free polysaccharide is a very large molecule \((10^5-10^6 \text{ kDa in Mw})\), containing a linear chain of 10,000 or more repeated disaccharides (glucuronic acid and \(N\)-acetylglucosamine) (Park et al., 2008; Ruoslahti, 1996). HA is very hygroscopic and occupies an extremely large volume for its size, forming a compliant and highly porous scaffold where cells can easily grow (Hascall and Esko, 2009). Although HA is a major component of soft cartilage tissue in the body, where it is tightly bound to aggrecan and the link protein HAPLN1, in the CNS HA is associated with a specific set of proteins that form soluble complexes and give unique properties to this matrix (Viapiano and Matthews, 2006).

1.4. The Lectican family of chondroitin sulfate proteoglycans

The proteoglycans carrying the glycosaminoglycan polysaccharide chondroitin sulfate (chondroitin sulfate proteoglycans or CSPGs) are the major protein components of the neural ECM (Viapiano and Matthews, 2006). Most high-Mw CSPGs belong to the lectican family, characterized by their binding to HA. This family is composed of the CSPGs aggrecan, versican, neurocan, and brevican (Figure 1.2) (Yamaguchi, 2000). Of these proteoglycans, neurocan and brevican are of special interest in neuroscience because they are restricted to the neural ECM (Viapiano and Matthews, 2006). The core protein of all lecticans consists of three major domains (Yamaguchi, 2000). The N-
terminal domain of the lecticans binds to HA, while the C-terminal domain is a lectin-like domain that binds to membrane lipids and tenascins (Yamaguchi, 2000), forming anchoring complexes that associate the cell membrane to the ECM (Figure 1.1). These two domains are connected by a central, stretched domain containing a sequence where the chains of CS are covalently attached. The total number of CS chains is highly variable and can range from 0-3 chains/molecule in brevican to >100 chains/molecule in aggrecan.

The lecticans constitute a very complex family because although there are only four essential members, each lectican exhibits multiple isoforms produced by protein cleavage, differential splicing, and differential glycosylation (Viapiano and Matthews, 2006), as summarized in Table 1.1. The role of this structural variability is largely unknown and it could be associated with structural plasticity of the ECM as well as the involvement of specific isoforms in signaling pathways, as we will describe in this dissertation.

At the functional level, the lectican CSPGs have been consistently described as the major molecular barrier in neural ECM for cell motility and axonal extension (reviewed in (Viapiano and Matthews, 2006)). This effect was originally attributed to their CS chains (reviewed in (Busch and Silver, 2007)). In agreement, treatments of injured spinal cord with the enzyme chondroitinase ABC were shown to promote the extension of axons past the sites of glial scar that contain elevated CSPG expression, promoting partial functional recovery (Bradbury et al., 2002; Massey et al., 2008). However, it is known that the core proteins can also inhibit cell or axonal motility (Viapiano and Matthews, 2006). This suggests that interactions of the CSPG proteins with specific ligands may underlie their inhibitory effect while chondroitinase treatment
results perhaps in the removal of CSPGs from injured tissue, therefore terminating these inhibitory interactions (Crespo et al., 2007).

Despite this classical role of the CSPGs in the central nervous system, at least two CSPGs have been shown to exert opposite effects in brain tumors. Different versican isoforms have been shown to regulated cell motility and proliferation in several tumors including gliomas (Arslan et al., 2007; Du et al., 2010; Wu et al., 2001; Zheng et al., 2004). Similarly, brevican has been identified as a unique pro-oncogenic lectican in gliomas.

1.5. The CSPG brevican and its role in gliomas

Brevican is the most abundant member of the lectican CSPG family in the adult brain (Yamaguchi, 2000). In agreement with the overall structure of the lecticans, the N-terminal domain of brevican binds to HA while the C-terminal domain has been shown to bind tenascin-R, tenascin-C, and sulfolipids in the cell membrane. Therefore, brevican has been proposed to act as a “bridge” attaching neural cells to the ECM in the normal CNS (Viapiano and Matthews, 2006). In addition, to these ligands, brevican was proposed to interact with a small glycoprotein that binds HA, known as hyaluronan and proteoglycan link protein-4 (HAPLN-4) (Bekku et al., 2003) (See section below on HAPLNs). However, this interaction was hypothesized from co-localization of these proteins in the brain and was never confirmed by protein association studies (Sim et al., 2009).

As indicated above, there are several isoforms of brevican originated from differential splicing, glycosylation, and cleavage (Figure 1.3) (Matthews et al., 2000;
Seidenbecher et al., 1995; Viapiano et al., 2003). The differential splicing of brevican is unique in that it generates a variant without the C-terminal lectin domain; instead, this isoform is covalently retained on the cell surface through a GPI anchor, thus being the only lectican that exhibits a GPI-bound variant (Seidenbecher et al., 1998). The spatial distribution of this variant is different from full-length brevican, being highly expressed in white matter tracts (Jaworski et al., 1995).

In addition, brevican is an unusual proteoglycan because it was the first CSPG that could be natively detected without CS chains, thus being a "facultative" CSPG (Yamaguchi, 1996). Moreover, the full-length form of brevican shows differential glyco-variants in brain tumors compared to normal brain, including forms with over-sialylation and under-glycosylation (Viapiano et al., 2005). The relevance of these glyco-variants is not understood although the under-glycosylated form of brevican is unique to brain tumors, suggesting that it may be a product of a deficient glycosylation process in these tumor cells (Viapiano et al., 2005).

Finally, brevican is known to be cleaved both by ADAMTS and MMPs (Nakamura et al., 2000). MMP-dependent fragments of brevican have transient and variable expression in the brain, and are downregulated in a mouse model of Alzheimer’s compared to normal mice (Ajmo et al., 2010). Although the functional relevance of this finding is unclear, it suggests a possible correlation between AD pathology and the remodeling of CSPGs.

The major, stable cleavage products of brevican are generated by ADAMTS-4 and ADAMTS-5 (Seidenbecher et al., 1995), resulting in two fragments of approximately 50-kDa (N-terminal) and 90-kDa (C-terminal). Both full-length brevican and ADAMTS-
generated fragments are highly upregulated in experimental and clinical gliomas (Viapiano et al., 2005), where brevican shows its most interesting and paradoxal role.

Almost immediately after the discovery of this protein, brevican expression was observed highly upregulated in gliomas (Jaworski et al., 1996), in agreement with previous data showing that brevican was also upregulated during periods of developmental gliogenesis (Jaworski et al., 1995). Overexpression of this protein was shown to be more than a passenger effect in gliomas: In fact, overexpression of brevican or the N-terminal fragment of this proteoglycan was demonstrated to be sufficient to promote tumor growth and reduce animal survival (Nutt et al., 2001; Zhang et al., 1998). Further results showed that brevican required ADAMTS cleavage to promote tumor growth and invasion (Viapiano et al., 2008) and that preventing its cleavage resulted in a non-functional protein that didn't even increase cell adhesion or migration in vitro (Hu et al., 2008).

The molecular mechanisms underlying this paradoxal of brevican as a pro-invasive protein in gliomas started to be unraveled in 2008 when Hu et al. demonstrated that brevican was able to activate EGFR in glioma cells, leading to increased secretion of fibronectin and enhanced glioma cell adhesion (Hu et al., 2008). Despite this seminal observation, the molecular mechanisms explaining the connection between brevican upregulation, EGFR activation, and glioma cell motility remained unknown and form part of the studies in this thesis.

1.6 The family of hyaluronan-binding and proteoglycan-link proteins

The HAPLNs form a very poorly studied family of proteins that resemble the N-
terminal domain of the lecticans, and in fact may be the evolutionary predecessors of these proteoglycans (Spicer et al., 2003). There are four HAPLNs (or link proteins), HAPLN1 to HPLN4, which are distributed in the same chromosomes as the lecticans and in adjacent positions (Figure 1.4), remarking their common origin. Two of these HAPLNs (HAPLN2 and HAPLN4) are adjacent to the genes of the neural lecticans neurocan and brevican, and their expression is also restricted to the CNS (Bekku et al., 2003; Oohashi et al., 2002).

The functions of the HAPLNs are essentially unknown. These proteins can bind HA directly but their most common role has been as a partner of the lecticans, enhancing the affinity of these CSPGs for HA. This is particularly true for the prototypical pair aggregcan:HAPLN1, and it is thought that the formation of these complexes contributes to the overall structure and stability of the HA-based matrix (Binette et al., 1994; Matsumoto et al., 2003).

The interaction of lecticans and HAPLNs seem to be highly specific, with four major pairs identified to date: aggregcan:HAPLN1 (prototypical and most studied), versican:HAPLN2, neurocan:HAPLN3 and brevican:HAPLN4. This last pair was postulated based on the detection of co-localization of brevican and HAPLN4 around the same cells in the CNS (Bekku et al., 2003; Carulli et al., 2006) but has not been experimentally confirmed. Brevican and HAPLN4 are thought to associate to form perineuronal nets around brainstem and cerebellar neurons (Bekku et al., 2012). These nets are a specialized form of dense neural ECM detected only around fast-firing neurons, thought to regulate neuronal activity and synaptic plasticity (Morris and Henderson, 2000).
Apart from the purely structural role of HAPLNs as "clasps" between lecticans and HA in the neural ECM, any other functions of these proteins are unknown. In particular, the expression of HAPLNs in gliomas and their possible mechanisms and association to brevican have not been investigated and form part of this dissertation.

1.7. Epidermal Growth Factor Receptor signaling in gliomas

EGFR is the prototypical member of the ErbB family, which is one of the major families of receptor tyrosine kinases (RTKs). The ErbB (also known as HER) family consists of four members: ErbB1/EGFR; ErbB2/Neu; ErbB3; and ErbB4 (Huang et al., 2009b). EGFR is expressed at very low levels in the normal adult CNS but increases rapidly in neurons and astrocytes following neural injury and other inflammatory neuropathologies (Liu and Neufeld, 2007; White et al., 2011). The role of EGFR activation on neural recovery following traumatic injury has been subject of controversy (Berry et al., 2011), but it is largely accepted that pathologic activation of this receptor in neurons impairs axonal regeneration and functional recovery (Koprivica et al., 2005).

EGFR is, in addition, highly expressed in malignant gliomas (Hatanpaa et al., 2010), with approximately 40% of clinical glioma samples showing EGFR gene amplification and overexpression (Gan et al., 2009). In addition, gliomas frequently exhibit expression of EGFRvIII, which is a constitutively-active deletion variant lacking the extracellular domain that is unique to malignant cells (Gan et al., 2009; Huang et al., 2009a).

Activation of EGFR triggers, in turn, multiple downstream mechanisms, of which two major pathways are those activated by phosphoinositide 3-kinases (PI3Ks) and
mitogen-activated kinases (MAPKs) (Huang et al., 2009b) (Figure 1.5). Activation of
PI3K activates AKT, which drives glioma cell survival and proliferation through the
mammalian Target of Rapamycin (mTOR) and S6 kinase (McLendon et al., 2007). On
the other hand, activation of MAPK promotes glioma progression through mitogenic
signaling (Jiang et al., 2006; Sonoda et al., 2001). MAPK activation also activates Erk
1/2, which regulates the secretion of ECM proteins (Knott et al., 1998; Zhang et al.,
2005) and levels of MMPs, and is a potential mechanism underlying glioma invasion.

Due to its aberrant expression and activity, EGFR has been a major therapeutic
target in gliomas and has been targeted both by small molecule inhibitors and blocking
antibodies. Inhibitors of EGFR, such as Gefitinib and Erlotinib, block the tyrosine kinase
activity of the receptor and have been used in trials and combination therapies in
recurrent gliomas (Omuro et al., 2007). Inhibition of EGFR by these molecules induces
apoptosis and blocks cell cycle progression in tumor cells (Moyer et al., 1997). In
addition, Erlotinib selectively down-regulates genes induced by EGFRvIII that promote
glioma invasion (Lal et al., 2002).

As a separate strategy, antibody-based therapies against EGFR have been largely
based on the humanized monoclonal antibody Cetuximab, a blocking antibody against
wild-type EGFR that decreases cell proliferation and increases apoptosis in vivo (Eller et
al., 2002). Alternative therapies have also been developed with antibodies against
EGFRvIII, resulting in a more specific targeting of the mutant variant in glioma cells
(Kuan et al., 2001; Sampson et al., 2008a). A major limitation of these inhibitory
therapies against EGFR is the development of drug resistance (Huang et al., 2009b). A
few alternatives are actively pursued such as the development of immunotherapy
(vaccination) against EGFRvIII (Sampson et al., 2009), but immunological escape has also been observed (Sampson et al., 2010). Therefore, further understanding of the mechanisms that regulate EGFR signaling in gliomas and identification of novel molecular targets may contribute to the design of more efficient therapeutic strategies. Because brevican was discovered to activate EGFR (Hu et al., 2008), the identification of the mechanisms by which brevican regulates the receptor form part of this thesis and may be particularly relevant for the future therapy of gliomas.

1.8 Conclusions and major hypotheses

Glioma is a deadly brain tumor with a poor prognosis due mainly to tumor recurrence. As indicated, a major cause of recurrence is the invasion of glioma cells in the surrounding brain. Invasion of glioma cells through the brain parenchyma seems a unique process that may depend on the interactions of the tumor cells with unique molecules of the neural ECM.

In this work, we have focused on the neural-specific CSPG brevican with the goal of understanding how this proteoglycan may enhance glioma adhesion. We have hypothesized that brevican may exhibit an interaction with link proteins in gliomas different from their interaction in normal brain, which may lead to changes in the ECM structure around the motile glioma cells facilitating migration. In addition, we have hypothesized that brevican or its cleavage products, which are upregulated in gliomas, may act as a trigger of EGFR signaling to promote glioma cell migration. Finally, we have investigated whether EGFR in the tumor microenvironment may be critical for glioma invasion and for the pro-invasive effects of brevican. The overarching goal of this
work is to elucidate the molecular mechanisms of brevican in gliomas and determine if this neural-specific protein could have a prognostic or therapeutic relevance in these tumors.
**Figure 1.1** General structure of the neural ECM. This cartoon depicts the major components of the neural ECM (HA, CSPGs, and glycoproteins such as the link proteins and tenascins), their interactions, and their association to cell-surface receptors. The multimolecular complexes formed by CSPGs, tenascins and/or HAPLNs are major anchoring complexes of the neural cells to the ECM. *SGGLs*: sulfo-glucuronyl glycolipids.
Figure 1.2 The lectican family of CSPGs. This schematic diagram shows the structure of the four lectican CSPGs. All lecticans are characterized by an N-terminal HA binding domain, a central CS-attachment domain, and a C-terminal selectin-like domain. The HA binding domain ("link domain") is composed by an immunoglobulin-like and two link protein-like repeats and is duplicated in aggrecan. The selectin domain is formed by an EGF-like repeat, a calcium-dependent C-type lectin motif, and a complement regulatory protein-like domain motif. (Drawings adapted from (Yamada et al., 1994)).
Figure 1.3 Multiple isoforms of brevican. This cartoon represents the multiple forms of brevican that can be found in the CNS: A) Alternative splicing generates a shorter, GPI-anchored form of brevican (unique among the lecticans); B) Multiple glycosylation variants can be found such as variants with and without CS and with different extension of O-glycosylation; C) Cleavage of the full-length protein by the metalloproteases ADAMTS-4/5 result in two stable fragments that can be detected in the CNS. (Drawings adapted from (Viapiano and Matthews, 2006)).
Figure 1.4 Chromosomal arrangement of CSPGs and HAPLNs. The cartoon depicts the chromosomal distribution of the lectican CSPGs and their associated HAPLNs in human cells. The blue arrows represent the CSPG genes while the orange arrows indicate the HAPLNs. The direction of the arrows indicates the direction of each gene's open reading frame. This gene distribution indicates a clear common evolutionary origin of the CSPGs and HAPLN arising from gene duplication. Interestingly, despite their chromosomal arrangement the pairs that have been postulated or validated experimentally so far are aggrecan:HAPLN1; versican:HAPLN2; neurocan:HAPLN3; and brevican:HAPLN4. (Drawings adapted from (Spicer et al., 2003)).
Figure 1.5 Schematic of EGFR signaling in gliomas. Both TGF-alpha and EGF are ligands of EGFR that can trigger signaling cascades involving phosphoinositide 3-kinase (PI3K), mitogen-activated kinase (MAPKs), and the signal transducer and activator of transcription 3 (STAT3). These three major downstream pathways underlie the most widely described effects of EGFR on cell proliferation, differentiation, and migration. (Drawings adapted from (Huang et al., 2009a)).
### Table 1.1: Major isoforms of the CSPGs of the lectican family

<table>
<thead>
<tr>
<th>Proteoglycan</th>
<th>Differential splicing</th>
<th>Differential glycosylation</th>
<th>Cleavage isoforms</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Neurocan</strong></td>
<td>Not studied</td>
<td>Not studied</td>
<td>MMP-generated fragments of 130 and 180 kDa are predominant in adult CNS, the full-length protein is predominant in the newborn [6]</td>
<td>6: Rauch U. <em>Cell Mol Life Sci.</em> 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter 2:
Characterization of HAPLNs and their interaction with brevican in malignant gliomas

2.1. Introduction

As described in chapter 1, the extracellular matrix (ECM) of the adult central nervous system lacks most fibrous proteins (collagens, fibronectin and laminins) that are present in the matrices of other tissues, and is formed instead by a scaffold of hyaluronic acid (HA) with associated glycoproteins (Ruoslhti, 1996), including the proteoglycan brevican that is the major focus of this thesis.

Brevican binds both to HA and to cell-surface receptors (Viapiano and Matthews, 2006), regulating the cross-linking and compressibility of the matrix scaffold, and therefore modulating many neural processes including cell motility during development, axonal navigation, and the stabilization of synapses (Galtrey and Fawcett, 2007). A second family of HA-binding proteins expressed in the central nervous system are the HA- and proteoglycan-link proteins (HAPLNs) that bind both to HA and to the lecticans, forming ternary complexes (Binette et al., 1994; Zimmermann and Dours-Zimmermann, 2008). The structure of the HAPLNs is remarkably similar to the N-terminal region of the lecticans, therefore the highly homologous HA-binding domains from HAPLNs and lecticans are indistinctly known as proteoglycan tandem repeats or link-protein modules.

1 Results in this chapter were published in: Sim et al., J. Biol. Chemistry (2009) 284:26547
Two of the HAPLNs, HAPLN2 and HAPLN4 have only been detected in neural tissue and their genes are adjacent to the neural-specific proteoglycans, brevican and neurocan, respectively (Spicer et al., 2003). Both HAPLN2 and HAPLN4, also known as Brain-specific link protein (Bral)-1 and Bral-2, are upregulated in the adult central nervous system and match the temporal expression profile of brevican, which is the most abundant CSPG in adult neural tissue (Bekku et al., 2003; Hirakawa et al., 2000).

Current evidence suggests that the HAPLNs may be key components in the organization of the HA-based matrix scaffold. HAPLN1, the best studied member of the family, increases the affinity of the lecticans for HA (Rauch et al., 2004; Shi et al., 2004) and stabilizes lectican-HA matrix aggregates (Binette et al., 1994; Neame and Barry, 1993). Moreover, the increased expression of lecticans and HAPLNs in the adult central nervous system correlates temporally and spatially with changes in ECM solubility and with appearance of dense ECM aggregates around subsets of neurons, known as 'perineuronal nets'. These changes have been associated with restricted cellular motility and decreased synaptic plasticity (Rauch, 2004).

In addition to its clear structural functions in the normal CNS, brevican has been object of much interests in the field of brain cancer for being a CNS-specific molecule upregulated in glioma, secreted by gliomas, and with a clear pro-tumoral role (Viapiano and Lawler, 2009). Still, a major aspect of the biology of this lectican that has not been addressed is the binding partners that associate with brevican in glioma and the molecular mechanisms that are triggered by this proteoglycan in these tumors.

While the role of brevican in brain tumors is starting to become better defined, the most obvious and canonical partners of this lectican, i.e., HAPLNs, have never been
analyzed in human brain or in neuropathologies. Therefore, we still have a highly incomplete picture not only of the molecular changes that occur in the tumor ECM but also of how those changes could affect brevican signaling and critical aspects of glioma biology such as invasion of the surrounding tissue.

For the work described in this chapter, our initial hypothesis was that the gain of function of brevican in gliomas could be, at least in part, caused by changes in the levels of specific HAPLNs in gliomas or in the molecular association between brevican and HAPLNs. Therefore, our hypothesis was that the functions of brevican could be explained as a consequence of overall changes in the ECM structure/composition in the tumor. To test this hypothesis, we studied here the expression and biochemical properties of the HAPLN family in human normal brain and glioma tissue, as well as the effects of co-expressing HAPLNs and brevican in glioma cells.

The results in this chapter provide the first biochemical characterization of the brain-specific human HAPLN4, and, in addition, show that both neural-specific link proteins HAPLN2 and HAPLN4, which are abundant in adult brain, are virtually absent from the ECM of malignant gliomas. More importantly, our results show that brevican does not associate to HAPLNs in gliomas in the same manner as it does in normal CNS and therefore these proteins are unlikely to explain the pro-invasive mechanisms of brevican in malignant brain tumors.

2.2. Materials and Methods

2.2.1. Cells and antibodies

The human glioma cell lines U251MG and U87MG (American Type Culture
Collection, Manassas, VA) were grown at 5% CO\textsubscript{2} in DMEM supplemented with 10% fetal bovine serum (FBS). Quantitative RT-PCR and Western blotting were used to identify the major CSPGs produced endogenously by these cells. Non-transformed human fetal astrocytes (Clonetics Lonza, Allendale, NJ) were cultured in supplemented astrocyte basal medium provided by the manufacturer. Primary glioma-derived neurospheres, prepared from fresh clinical specimens as described (Lee et al., 2006) were characterized and kindly provided by Drs. Sean Lawler and E. Antonio Chiocca (Department of Neurological Surgery, The Ohio State University) These neurospheres were cultured in DMEM/F-12 supplemented with 2 µM glutamine, 20 ng/ml epidermal growth factor (Peprotech, Rocky Hill, NJ), 20 ng/ml basic fibroblast growth factor (Peprotech) and 1x B27 supplement (Invitrogen, Carlsbad, CA). Culture medium in all cases was supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin.

Human HAPLNs were detected with mouse monoclonal antibodies against HAPLN1 (clone 8A4, NICHD Developmental Studies Hybridoma Bank, Iowa City, IA), HAPLN2 (Abnova, Taiwan) and HAPLN4 (R&D Systems, Minneapolis, MN). Human CSPGs were detected with antibodies against versican (clone 12C5, NICHD DSHB), phosphacan (clone 3F8 against the core protein, NICHD DSHB), and brevican (rabbit polyclonal B6 and B5 that we have previously characterized (Viapiano et al., 2005)). Chondroitin sulfate epitopes were detected after treating CSPGs samples with chondroitinase ABC, using the antibodies anti-chondroitin-4-sulfate (clone BE123, Millipore, Temecula CA) and anti-6-sulfate (clone 3B3, Seikagaku, Japan). Actin and the V5-epitope tag were detected with mouse monoclonal antibodies from Sigma-Aldrich and Invitrogen, respectively.
2.2.2. Human tissue processing

All studies involving human tissue specimens were performed in compliance with the guidelines of the Human Investigations Committee at The Ohio State University College of Medicine. Pathologically graded fresh-frozen surgical specimens of high-grade adult gliomas (patient age range: 37 to 74 years old) were obtained through the NCI Cooperative Human Tissue Network. Human brain cortex tissue (parietal and temporal) from age-matched controls and other developmental stages was obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland (Baltimore, MD). Tissues were individually homogenized in 20 mM TrisHCl, pH 7.4, containing 320 mM sucrose and a cocktail of protease inhibitors (Complete, Roche Applied Science, Indianapolis, IN). Total homogenates were subjected to subcellular fractionation as previously described (Viapiano et al., 2005) and further processed for protein electrophoresis.

2.2.3. Constructs and cell transfection

Clones containing the complete coding sequence of human HAPLN4 (NM_023002), human brevican (NM_021948) and the HA-synthase HAS2 (NM_005328) were subcloned by PCR into the vector pcDNA3.1-V5.6xHis (Invitrogen) to produce V5/His-tagged proteins. Cells were transfected with Lipofectamine-2000 (Invitrogen) according to the manufacturer protocols. cDNA dosage was controlled by qRT-PCR and cells were selected for further experiments when their stable expression levels were comparable with those in neural tissue (for HAPLN4) or glioma tissue (for
brevican), as previously described (Viapiano et al., 2008). To confirm that protein overexpression was compatible with normal ER function, we analyzed the expression of the ER stress sensor protein GRP78 and observed that this protein was not increased in the transfection conditions we used for our experiments (shown in Figure 2.5 below). Control transfections were performed with the original pCDNA3.1 vector or, alternatively, with pDsRed2 (Beckton Dickinson Biosciences, Palo Alto, CA) carrying the cDNA for red fluorescent protein. Transiently transfected cells employed for biochemical experiments were routinely changed to serum-free Opti-MEM culture medium (Life Technologies, Gaithersburg, MD) 24 hours after transfection, followed by medium collection 24-48 hours later. Stably transfected cells were selected with Geneticin (G418, Invitrogen) as previously described (Viapiano et al., 2008).

2.2.4. Cell adhesion and motility assays

48-well plates were precoated for 2 hours at room temperature with the following substrates: human fibronectin (5 µg/mL, Becton-Dickinson, San Jose, CA), high-Mw poly-L-lysine (50 µg/mL, Sigma-Aldrich, St Louis, MO) and high-Mw hyaluronic acid (200 µg/mL, Calbiochem, La Jolla, CA). Non-specific binding sites were subsequently blocked with 1% bovine serum albumin in Dulbecco's PBS (DPBS). Glioma cells were dissociated in DPBS/2mM EDTA, washed in fresh culture medium and plated on the precoated well (at 50,000 cells/well) for 30 minutes. Adhered cells were fixed and quantified by crystal violet staining as described (Hu et al., 2008).

To analyze cell migration, we used a radial-dispersion assay to mimic cell dispersion away from the tumor bulk (Chicoine and Silbergeld, 1995), a predominant
feature of malignant brain tumors. Glioma cells were resuspended at a density of 100,000 cells/ml and seeded on 1% agar plates during 48h to form floating aggregates. Aggregates were manually applied to the center of individual pre-coated wells and cells were followed by time-lapse microscopy to analyze detachment and migration as described (Stein et al., 2007). The dispersion index (= total area occupied by cells at each time divided by the original area of the cell aggregate) was plotted against time and analyzed by two-way ANOVA for repeated measures. As independent confirmation of cell motility we tested the cells in a 'wound-healing' assay, using culture inserts with pre-formed gaps of 500 µm (Ibidi GmbH, Germany). Cells were plated at 60,000 cells/insert and allowed to form monolayers around the gaps overnight. Inserts were subsequently removed and the residual gap area was measured at 0h and 24h to quantify cell dispersion. Results were analyzed by one-way ANOVA. All experiments in both motility models were repeated three times with 3 to 6 replicates per experimental condition.

2.2.5. Protein solubilization from brain membranes

Microsomal fractions obtained from subcellular fractionation of human brain specimens were pooled and used as source of 'total brain membranes' at a protein concentration of 1-2 mg total protein/ml. Total membranes were resuspended in 25 mM TrisHCl buffer, pH 7.4, in the presence or absence of 10 mM EDTA or 1 % w/v Triton X-100, for 1h at 4 ºC. Alternatively, the membranes were resuspended in 100 mM sodium carbonate buffer (pH 11.0) for 30 min at 4 ºC.

To test whether HAPLN4 solubilized with Triton X-100 effectively partitioned with the aqueous phase, membranes were instead solubilized with cold 2% w/v Triton X-
114 (Sigma-Aldrich) as previously described (Viapiano et al., 2003). The resulting solution was warmed at 37 ºC, causing the separation of aqueous and detergent-containing phases. All samples were subsequently diluted to reduce the Triton-X114 concentration before preparation for protein electrophoresis.

To investigate the effect of glycosaminoglycan removal on protein solubilization, membranes were resuspended in 50 mM TrisHCl buffer, pH 7.0, containing 10 mM sodium acetate and protease inhibitors (CH buffer), and treated with 1mU/µl protease-free chondroitinase-ABC (EC 4.2.2.4 Seikagaku) or 0.5mU/µl hyaluronidase SD (EC 4.2.2, Seikagaku) for 4h at 37 ºC. To investigate the possible involvement of a GPI anchor in HAPLN4 retention, membranes were resuspended in CH buffer and treated with 1mU/µl protease-free chondroitinase-ABC and 1U/ml phosphatidylinositol-specific phospholipase C (EC 3.1.4.10, Sigma-Aldrich) for 8h at 37 ºC.

After any of these incubations the suspended membranes were centrifuged at 25,000 g x 15min. Released proteins were recovered in the supernatant and the membranes were washed twice in 25 mM TrisHCl and re-centrifuged as before. Samples that had not been pre-treated with chondroitinase were then further treated with 1 mU/ml chondroitinase ABC for 4h at 37 ºC to improve the resolution of CSPGs in blots. Samples were finally processed for protein electrophoresis.

2.2.6. Western blotting and RT-PCR

Subcellular fractions from brain tissue were prepared for electrophoresis using standard protocols. Cultured cells were first lysed in 25 mM TrisHCl, pH 7.4, containing 150 mM NaCl, 1% w/v CHAPS and a cocktail of protease inhibitors (Complete).
Conditioned, serum-free, culture medium was concentrated and, if required, treated with chondroitinase ABC as previously described (Viapiano et al., 2005). Cell and tissue samples containing 15-20 µg total protein were electrophoresed on 4-10% gradient polyacrylamide gels and analyzed by Western blotting.

Samples processed for mRNA analysis were snap-frozen immediately after dissection or culture, followed by extraction in Trizol (Invitrogen). Residual DNA was degraded using Turbo-DNA Free (Applied Biosystems, Foster City, CA), and total RNA was processed for RT-PCR PCR using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. When possible, primers were designed to span gene introns and thus detect contamination with genomic DNA. Primers are listed in Table 5.1.

2.2.7. Microarray meta-analysis

mRNA expression data for the members of the lectican and HAPLN families was obtained from 295 grade II-IV glioma specimens and 30 controls stored in the NCI Repository for Molecular Brain Neoplasia Data (REMBRANDT, http://rembrandt.nci.nih.gov, accessed between January and December 2008). Expression values were collected for 'unified gene' probesets corresponding to the different splice-forms of each gene (Madhavan et al., 2009). Values were plotted as the fold-level (i.e., log₂ ratio) of each tumor to control samples, and analyzed by one-way ANOVA for each gene. To normalize the expression of each gene using neuronal markers, values were recalculated relative to the expression level of the genes ENO2 (neuron-specific enolase) and TUBB4 (neuron-specific beta-tubulin III) for each sample.
2.3. Results

2.3.1. HAPLN4 is the predominant member of the link protein family in human brain cortex

Previous studies have confirmed the expression of the link proteins HAPLN1, 2 and 4 in rodent neural tissues (Bekku et al., 2003; Galtrey et al., 2008; Oohashi et al., 2002), but no comparison of these link proteins has yet been made in human central nervous system. To determine which members of the HAPLN family were expressed in human brain cortex we first compared their expression by quantitative RT-PCR in adult tissue (Figure 2.1A). Our results indicated that the neural-specific HAPLN4 was the largely predominant member of this family in human brain cortex, in agreement with what has been observed in rat and mouse brain. Processing of additional tissue samples revealed that the ranking HAPLN4 >> HAPLN2 >> HAPLN1 >> HAPLN3 was consistent in different regions of the brain and spinal cord (not shown). This prompted us to focus mostly on HAPLN4 as the most abundant, but largely unexplored, member of this family in human central nervous system.

Western blotting analysis of the developmental profile of HALPN4 in human brain cortex showed a low expression of this protein in fetal tissue, which increased significantly during the first postnatal year (Figure 2.1B). HAPLN4 was not found at developmental ages earlier than 19 weeks of gestation and not all 19-week samples yielded positive results (not shown), suggesting that this could be the starting point for expression of the protein.

In rodents, the postnatal time-course of HAPLN4 follows closely that of brevican,
the predominant lectican in adult neural tissue (Hagihara et al., 2000). In agreement, brevican and HAPLN4 followed very similar postnatal expression profiles in human brain cortex, including a peak during adolescence and late fall in adulthood that have been previously observed for brevican in human but not in rat brain (Gary et al., 2000). However, our results indicated that brevican protein appeared in human brain at earlier developmental stages than HAPLN4 (Figure 2.1B), matching the early embryonic expression of this lectican in the rat (Jaworski et al., 1995; Viapiano et al., 2003). Remarkably, while brevican started as a largely soluble protein and remained partially soluble throughout human brain development (Viapiano et al., 2005), HAPLN4 became aggregated and/or strongly membrane-associated within the first year of postnatal life and there was virtually no soluble form of this protein in the adult brain (see further analysis below).

2.3.2. Expression of brain-specific HAPLNs is strongly reduced in high-grade gliomas

Next, we compared the expression of the link proteins in normal adult brain versus high-grade malignant gliomas, which are known for their high expression of two members of the lectican family, versican and brevican (Viapiano and Matthews, 2006). For this, we first analyzed microarray data from glioma specimens stored in the NCI Repository for Molecular Brain Neoplasia Data (REMBRANDT), which is used to identify specific patterns of gene expression in primary brain tumors (Madhavan et al., 2009). This analysis disclosed a strong decrease in mRNA levels for the neural specific link proteins HAPLN2 and HAPLN4 in high-grade gliomas, a notorious contrast against the upregulation of the lecticans versican and brevican in the same types of tumors
(Figure 2.2A). When the expression patterns were expressed relative to the levels of two neuron-specific markers in each sample (Figure 2.2B-C), both HAPLN2 and HAPLN4 remained unchanged in tumor versus non-tumor tissue, suggesting that their decrease matched the death of adult neurons in tissue invaded by glioma.

To validate our analysis of microarray data, we quantified the expression of the proteins HAPLN1, 2 and 4 in total homogenates of normal brain cortex and glioma tissue by Western blotting (Figure 2.3A-B). Results indicated that both HAPLN2 and HAPLN4 were virtually absent from the tumor parenchyma compared to normal brain. Remarkably, HAPLN1, which had not appeared significantly downregulated at the mRNA level, was also strongly reduced at the protein level. We further analyzed and characterized the expression of CSPGs and HAPLNs in glioma cell lines and found that, although expression of some CSPGs (by qRT-PCR) was maintained, link proteins were absent in glioma cells (Figure 2.4A-B). However, we were able to detect both HAPLN2 and HAPLN4 mRNA in short-term primary cultures of normal human astrocytes (Figure 2.4C). Moreover, we detected HAPLN4 by RT-PCR (not shown) and Western blotting (Figure 2.4D) in several cultures of primary-glioma derived neurospheres, which have been characterized as a source of glioma initiating cells that can express both neuronal and glial markers (Lee et al., 2006). Even though the detected levels were much lower than in adult brain, the results suggested that HAPLNs might not be completely restricted to adult neurons and could be expressed in minor populations of glial/glioma cells.

2.3.3. Expression of HAPLN4 increases the motility of glioma cells.

Because lecticans and HAPLNs co-localize and are thought to associate in the
ECM scaffold of normal neural tissue, our results suggested a remarkable dissociation between these two kinds of molecules in the parenchyma of malignant gliomas. We hypothesized that absence of HAPLNs could correlate to the pro-motility gain-of-function of CSPGs in gliomas. Thus, we next asked whether introduction of HAPLN in glioma cells could affect cell behavior by itself or modulate the effects of the lecticans secreted by glioma cells.

Previous studies by our laboratory have shown that upregulation of the lectican brevican in glioma cells increases cell adhesiveness and motility, and could underlie in part the ability of these cells to invade the neural parenchyma (Hu et al., 2008). We then hypothesized that HAPLN might disturb the pro-adhesive/pro-migratory effects of brevican, and perhaps impair cell adhesion directly, as previously observed for HAPLN1 (Yang et al., 1998). To test this hypothesis, we generated stable transfectants in several glioma cell lines that are used as typical models to study the molecular mechanisms of glioma cell adhesion and migration.

Expression of HAPLN4, alone or in combination with brevican, did not affect significantly cell morphology (not shown), expression of other ECM or intracellular markers (Figure 2.5A-B) or proliferation in vitro (Figure 2.5C). However, when we tested the adhesive ability of glioma cells overexpressing HAPLN we found that they had enhanced attachment to substrates enriched in the glioma ECM, such as HA and fibronectin (Hu et al., 2008) (Figure 2.6A). We have already reported a pro-adhesive effect of brevican on these substrates, and shown that it is likely caused by brevican-dependent upregulation and recruitment of fibronectin to the surface of glioma cells (Hu et al., 2008). However, we did not observe an increase of fibronectin on cells
overexpressing HAPLN4 (Figure 2.5), suggesting a different pro-adhesive mechanism. In agreement, co-expression of HAPLN4 and brevican resulted in separate increase of the integrin subunit beta-1 and partially additive effects regarding glioma cell adhesion on all substrates tested (Figure 2.6B).

Because increased adhesion may result in either increased or decreased motility (DiMilla et al., 1991), we next tested whether HAPLN4 expression in glioma cells could specifically affect cell motility. We used a radial-dispersion model in which a cell aggregate is plated on a pre-coated surface and acts as a 'tumor bulk' from where cells detach and migrate radially (Figure 2.7A) (Chicoine and Silbergeld, 1995). Cell migration in this model is a result of cell-substrate versus cell-cell adhesive forces, mimicking the detachment and invasive properties of malignant brain tumor cells (Chicoine and Silbergeld, 1997). Results from these assays indicated that expression of HAPLN4 resulted in a net increase in cell motility (measured as the increase in area occupied by migrating cells) on surfaces coated with HA but not on uncoated surfaces, in agreement with our adhesion tests (Figure 2.7B). Again, co-expression of HAPLN4 and brevican caused a partial additive effect on cell motility, indicating that introduction of HAPLN4 not only did not disrupt but in fact enhanced the motogenic role of the lectican. The pro-migratory effects of brevican and HAPLN4 (although not their additive effect) were confirmed using an independent 'wound-healing' assay (Figure 2.8).

2.3.4. HAPLN4 retention in brain cell membranes cannot be replicated in cultured glioma cells.

Although the functional effects of HAPLN expression have never been previously
studied in tumor cells, the motogenic effect observed with HAPLN4 was somewhat surprising because HAPLNs have been shown to reduce cell adhesion in other cell types (Yang et al., 1998) and have been proposed as restrictive to cell and axonal motility in the central nervous system (Carulli et al., 2006). Thus, we investigated whether the expression of HAPLN4 in our glioma cell cultures was actually reproducing the expression profile of the native protein, in particular its distinguishing, strong association with cell membranes that we had previously observed in neural tissue (Figure 2.1).

Partition of secreted ECM proteins with ‘insoluble’ microsomal-containing fractions is commonly observed in adult neural tissue and is thought to reflect the aggregation and membrane-association of matricellular complexes during neural development and maturation (Bignami et al., 1993; Viapiano et al., 2003). However, most ECM proteins can still be found in both soluble and particulate subcellular fractions. Indeed, we observed that HAPLN1 and HAPLN2, as well as the lecticans brevican and versican, distributed as both soluble and membrane-associated proteins in the same brain tissue specimens in which HAPLN4 was found only associated to membranes (Figure 2.9A).

Strikingly, transient overexpression or stable expression of HAPLN4 in glioma cell lines resulted in this protein being largely secreted to the culture medium (Figure 2.9B). The remaining HAPLN4 detected in cell lysates lacked attached carbohydrates (Viapiano laboratory, unpublished observations) and could not be detected on the cell surface by live-cell staining but was detected intracellularly (Figure 2.9C). This suggested that secreted HAPLN4 in cultured glioma cells was largely soluble rather than retained on the cell surface, in contrast to the previous description of the native protein.
(Bekku et al., 2003).

To determine if the lack of association of HAPLN4 to glioma cell membranes was due to absence of its possible molecular partners, i.e., lecticans or HA, we studied the soluble versus cell-associated distribution of HAPLN4 in U251MG glioma cells after co-expression with the lectican brevican and/or the HA-synthase enzyme HAS2. We confirmed that HAS2 expression resulted in large pericellular coats of HA using a standard particle-exclusion assay with fixed red blood cells (Maleski and Hockfield, 1997) (Figure 2.10A). However, our results indicated that neither expression of brevican, HAS2 or a combination of both proteins altered the relative proportions of HAPLN4 in the culture medium and cell lysates (Figure 2.10B).

Therefore, to further characterize the association of native HAPLN4 to brain cortex membranes, we assayed different extraction conditions from native tissue (Figure 2.11A). HAPLN4 was resistant to mild solubilization conditions unless the membranes themselves were solubilized with detergents. Because HAPLN4 has a hydrophobic C-terminal sequence that could be a potential lipid-anchoring site (see discussion below) we also tested the possibility of an integral association of this protein with cell membranes. However, HAPLN4 remained in the aqueous phase after solubilization with Triton X-114 (Figure 2.11A) and was not affected by phospholipase-C (Figure 2.11B), suggesting that no lipid anchors were attached to it. Moreover, we were able to extract HAPLN4 with alkaline carbonate buffer, which finally suggested a strong, but still peripheral, association to the cell membrane.

Most notoriously, HAPLN4 was highly resistant to treatment of cell membranes with the glycosaminoglycan-degrading enzymes chondroitinase ABC (Figure 2.11B) and
hyaluronidase SD (Figure 2.11C), which readily solubilized other HA-binding proteins such as HAPLN1, brevican and versican. Taken together, these results argued for a strong, HA-independent and lectican-independent association of HAPLN4 to brain membranes that could not be reproduced in glioma cells.

2.4. Discussion

Although the first link protein (HAPLN1) was identified shortly after the first aggregating proteoglycan (aggrecan) in the early 70s', the properties and functions of these HA-binding glycoproteins have remained much less investigated than those of their lectican partners. The major function identified for these proteins, as stabilizers of HA-lectican complexes and therefore of ECM aggregates, is certainly critical for survival and normal development of the skeleton and cartilaginous tissue (Watanabe and Yamada, 1999). However, the high expression of HAPLNs in the central nervous system, the existence of neural-specific HAPLNs that follow the expression pattern of neural-specific lecticans, and the observed association of HAPLNs with lecticans in perineuronal nets and nodes of Ranvier strongly suggest additional functions of these glycoproteins in neural development, synaptic plasticity, and the organization of the neural extracellular matrix (Carulli et al., 2006; Oohashi et al., 2005).

To further understand the molecular interactions of brevican we focused in this chapter on the expression and biochemical properties of its potential partner HAPLN4, which is the predominant member of the HAPLN family in the adult mammalian brain. This protein has high homology to the other members of the family but exhibits two differences worth noting: it contains a 10-aa long glycine-rich insertion within the first
HA-binding domain, which could affect its HA-binding activity, and has a 30-aa long, unique C-terminal sequence. This C-terminal sequence is a hydrophobic, low-complexity motif rich in glycine, triptophane and proline, and has ~95% identity between human, bovine, rat and mouse HAPLN4, suggesting a strong conservation of function. Two prediction algorithms (Koh-GPI at http://gpi.unibe.ch/ and Big-PI predictor at http://mendel.imp.ac.at/sat/gpi/gpi_server.html) assigned a high probability of cleavage in that sequence for addition of GPI anchor, although our solubilization results suggest that this is not the case in native HAPLN4.

Previous studies of HAPLN4 have described the expression of this protein in different regions of rodent central nervous system, including brain cortex (mouse), cerebellum (mouse and rat) and spinal cord (rat) (Bekku et al., 2003; Carulli et al., 2006; Carulli et al., 2007). These descriptions have indicated a late appearance of HAPLN4, from postnatal day 7 to postnatal day 20 depending on the central nervous system region and methodology used, followed by maintenance of the protein levels throughout adulthood (Galtrey et al., 2008). In contrast, we detected HAPLN4 as early as midgestation in the human brain cortex (Figure 2.1). This pattern matched the prenatal expression of brevican, which is also expressed earlier in human than in rodent brain development (Viapiano et al., 2005), suggesting that the neural-specific components of the brain ECM appear at earlier developmental stages in humans. In addition, HAPLN4 levels decreased in the mature brain cortex, following again the temporal profile of brevican in human cortex, which differs slightly from the one observed in the mature central nervous system of the rat.

Both HAPLN4 and brevican switched progressively during development from a
highly soluble subcellular localization to a membrane-associated one, likely reflecting their gradual accumulation in aggregates and further insolubilization (Viapiano et al., 2003). However, while other lecticans and HAPLNs remained partially soluble in adult brain cortex, HAPLN4 became completely membrane-associated and could not be detected in the soluble fraction even after attempting enrichment by immunoprecipitation (data not shown).

The initial characterization of HAPLN4 in normal brain led us to investigate the expression of this and other HAPLNs in malignant gliomas, because these tumors are thought to remodel the neural ECM as glioma cells disperse away from the tumor mass (Bellail et al., 2004). This remodeling is in part a result of overexpression of ECM proteins by the tumor cells, including matrix proteins specifically produced by neural cells as well as mesenchymal proteins not found in nervous tissue (Bellail et al., 2004; Tso et al., 2006). Some members of the lectican family that are abundant in normal brain (e.g., brevican) are even more highly expressed in malignant gliomas and directly promote glioma invasion in the brain (Viapiano and Matthews, 2006). We thus investigated whether the HAPLNs would perhaps be expressed in these tumors following the expression pattern of their putative lectican partners.

However, our results indicated that none of the HAPLN members were upregulated in malignant gliomas and, in fact, both HAPLN2 and HAPLN4 that predominate in normal brain were strongly reduced in the tumor parenchyma (Figure 2.2 and 2.3). This decrease mirrored that of neuronal-specific markers, suggesting that it could have been largely caused by the neuronal death in tissue invaded by the tumor. Interestingly, pre-cancer inflammation in peripheral tissues causes increased methylation
of the genomic region containing HAPLN4 (Hahn et al., 2008), raising the possibility that this ECM protein could have also been downregulated in neural cells by inflammatory events occurring during tumor infiltration. Finally, it is worth mentioning that, although adult neurons are thought to be the major source of the neural link proteins (Galtrey et al., 2008), we could detect HAPLN expression in cultures of fetal astrocytes and primary glioma-derived neurospheres, suggesting that these proteins are also expressed, albeit in low amounts, by subsets of cells from the glial lineage.

The decrease of the neural HAPLNs in the tumor parenchyma suggests that lecticans such as versican and brevican, which are highly expressed in gliomas, would be largely non-associated to link proteins in these tumors and could perhaps interact with other matrix or cell-surface ligands absent in normal brain and expressed in the tumor. For example, the Viapiano lab has shown that brevican associates with fibronectin produced by glioma cells and that this mesenchymal ECM protein is necessary for the pro-migratory effect of brevican in glioma cells (Hu et al., 2008).

The absence of HAPLNs in gliomas prompted us to investigate the effect of reintroducing these proteins in glioma cells. Expression of HAPLN1 has been shown to disrupt cell adhesion (Yang et al., 1998), which led us to hypothesize that HAPLN4 could have a similar "anti-tumoral" effect on glioma cells. However, introduction of HAPLN4 in cultured glioma cells did not perturb the pro-adhesive and pro-migratory effects of the lectican brevican and moreover increased net adhesion and migration of glioma cells in a manner partially additive to brevican's.

While we do not have a conclusive explanation for this general increase in adhesion/motility, we observed that expression of HAPLN4 results in increased
expression of beta1-integrin in U251MG cells (Figure 2.6). Interestingly, we had previously demonstrated that brevican increases the expression of beta3- in glioma cells (Hu et al., 2008). It is possible that co-expression of HAPLN4 and brevican may have resulted in joint upregulation of several classes of integrins, resulting in the observed increase of cell adhesion and motility. These results led us to investigate more closely whether HAPLN4 in glioma cells was showing the same properties of native HAPLN4 in normal brain.

A distinctive feature of native HAPLN4 in brain tissue was its strong association with membrane-containing subcellular fractions, contrasting with the more widespread distribution of all other neural ECM proteins tested. On the other hand, HAPLN4 secreted by cultured glioma cells was almost exclusively soluble, even in cells stably expressing this protein and cultured undisturbed for several days.

Most ECM proteins expressed in cultured cells are secreted to the culture medium and appear largely soluble, but can also be detected in membrane-containing fractions. For example, brevican retains a cell surface-associated isoform in cultures (Viapiano et al., 2005; Viapiano et al., 2003) and the proteoglycan released to the medium can be induced to accumulate on the surface of glioma cells by addition of exogenous fibronectin of HA (Figure 2.10 and unpublished observations). In contrast, HAPLN4 remained largely soluble even after co-expression with a lectican (brevican) and an HA-source (the HA-synthase HAS2), which are considered the necessary components for the formation of cell-associated aggregates in the neural ECM (Carulli et al., 2007; Galtrey et al., 2008). These results suggested that, at least in our culture conditions, HAPLN4 had little, if any, interaction with both brevican and cell-surface associated HA in glioma cells.
Attempts to co-immunoprecipitate HAPLN4 and brevican from the conditioned medium of glioma cells were unsuccessful (Viapiano laboratory, unpublished observations).

In comparison, native HAPLN4 in brain tissue was not only strongly associated to cell membranes but also difficult to solubilize, and was only extracted after membrane solubilization with non-ionic detergents, or harsh alkaline treatment with sodium carbonate. Mild treatments that detached other membrane-associated HAPLNs or lecicans did not work with HAPLN4. In particular, HAPLN4 could not be released by enzymatic treatment that degraded HA or chondroitin sulfate, which partially released all other HAPLNs and lecicans tested. This suggests that, while HAPLN4 could associate with HA in adult brain tissue (Bekku et al., 2003), it also seems retained on neural cell membranes by a strong, HA-independent interaction. This result differs from the previous observation by Bekku et al. that HAPLN4 immunoreactivity decreases in tissue sections after hyaluronidase treatment (Bekku et al., 2003), probably due to solubilization of the protein. Our different observation could be largely a result of using different tissue sources and methods to evaluate the association of HAPLN4 to neural tissue. Moreover, we have observed that long-term treatment of tissue samples with hyaluronidase reduces overall protein content due to residual protease activity in the enzyme preparation (Viapiano laboratory unpublished observation), which prompted us to use shorter incubation times and excess of protease inhibitors in our experiments.

In sum, our results demonstrate that HAPLN4 is the predominant member of the HAPLN family in human brain and disclose the significant reduction of the neural HAPLNs in the parenchyma of high-grade malignant gliomas. In addition, we have shown that HAPLN4 exhibits a strong, HA-independent association to membranes of
neural cells, which is not reproduced in glioma cells *in vitro* and may in part underlie the unusual motogenic effect of recombinant HAPLN4 in cultured cells. Moreover, our results suggest that brevican is not associated to HAPLNs in gliomas and therefore these proteins are likely not the molecular partners of brevican that can help explain the functions of this proteoglycan in the tumors.

Overall, our results strongly suggest that the glioma ECM is rich in lecticans that are not associated to HAPLNs, in stark contrast with the proposed association of these molecules in the normal neural ECM (Carulli et al., 2006). This dissociation could contribute to the matrix remodeling caused by glioma cells as they invade the neural tissue.
Figures of chapter 2

**Figure 2.1** *HAPLN4 is the predominant member of the link protein family in adult human brain.*

A) Comparison of HAPLN mRNA expression by qRT-PCR, using total RNA from four independent samples of normal human brain cortex (range: 45 to 65 years old). Measurements were repeated two times in triplicate for each individual tissue sample. HAPLN4 was in all cases largely predominant over all other members of the family. GAPDH was used as normalization control. 

B) Total homogenates from human brain cortex (16 gestational weeks (gw) to 71 years (y) old) were separated by subcellular fractionation into high-density nuclear (*n*), membrane-containing (*m*), and soluble (*s*) fractions. Equal amounts of total protein from each fraction (15 µg) were treated with chondroitinase ABC, and processed for Western blotting to detect HAPLN4 and the lectican brevican. The asterisk indicates a possible cleavage product of HAPLN4. Note the strong association of HAPLN4 to membrane-containing fractions in all postnatal stages.
Figure 2.2 Expression of the HAPLN mRNAs is highly reduced in malignant gliomas. A) Comparison of mRNA expression levels for the members of the link protein (LP) and lectican families in different types of high-grade gliomas, using microarray data from the NCI Repository for Molecular Brain Neoplasia Data. Each column represents the level of expression (fold-over-control, mean ± S.E.M.) for each tumor type compared to normal brain tissue. Results for each gene were analyzed by one-way ANOVA (* p< 0.01 or lower, additional asterisks were removed for clarity). The results show strong reduction of the expression of HAPLN2 and HAPLN4 that contrasts with the upregulation of brevican and versican in gliomas. ACAN, aggrecan; BCAN, brevican; NCAN, neurocan; VCAN, versican. B-C) Data were re-normalized using the neuronal markers beta-tubulin (TUBB4, B) and neuron-specific enolase (ENO2, C) to test the correlation of HAPLN2 and HAPLN4 to the abundance of neurons in the tumor parenchyma. Notice that HAPLN2 and HAPLN4, which are highly reduced in tumor tissue (A), appear unchanged from control values (median control=1) when expressed relative to neuronal makers.
Figure 2.2
Figure 2.3 *HAPLN* proteins are absent in malignant gliomas. A) Total homogenates from high-grade gliomas (two grade III astrocytomas and seven grade IV glioblastomas, of which five are shown) and age-matched controls were probed for HAPLN1, HAPLN2, HAPLN4, and actin as loading control. B) Quantification of normalized results from (A); HAPLN2 and HAPLN4 are expressed at very low levels in the tumor parenchyma (white bars) compared to normal brain (black bars). *I.O.D.*, integrated optical density.
Figure 2.4 HAPLN2s are expressed in astrocytes and but are downregulated in glioma cells. A) Expression of CSPGs and the neural HAPLN2s was quantified by qRT-PCR in cultured U251MG glioma cells compared against normal adult human forebrain. Only the lectican versican (VCAN) and the non-lectican CSPG phosphacan (PCAN) were expressed in these cells, whereas all other CSPGs and HAPLN2s were essentially undetectable. Similar results were obtained in U87MG cells (not shown). ACAN: aggrecan; NCAN: neurocan; BCAN: brevican. V0 to V3 indicate
different splice isoforms of versican. B) Detection of versican, phosphacan, and full-length brevican in the conditioned medium of brevican-transfected U251MG cells. Blots were probed with antibodies against each specific CSPG or against the epitopes chondroitin-4-sulfate (∆4s) and chondroitin 6-sulfate (∆6s) that are revealed after treatment of CSPGs with chondroitinase. Results indicated that U251MG cells in culture retain the ability to add and modify GAG chains in endogenous (VCAN, PCAN) or transfected (BCAN) CSPGs. C) Detection of HAPLN2 and HAPLN4 by RT-PCR, using total RNA from low-passage cultures of human fetal astrocytes. The same procedure yielded negative results with five different human glioma cell lines tested (U87MG, U118MG, U251MG, U373MG and A172, not shown). RT: presence (+) or absence (-) of reverse transcriptase in the mixture. E) Detection of HAPLN4 by Western blot in the conditioned medium from primary glioma-derived neurospheres (identified as G2 to G12). Cultures with detectable expression of HAPLN4 also expressed high levels of brevican (not shown).
Figure 2.5 Overexpression of HAPLN4 does not affect cell viability. A-B) To confirm that our transfection conditions were compatible with normal ER function, we analyzed (A) and quantified (B) the joint expression of HAPLN4 and the ER stress sensor protein GRP78 (Li and Lee, 2006). We observed that HAPLN4 was always predominantly soluble (and GRP78 was not increased) in the range of transfection conditions used in our experiments. In addition, expression of the ECM protein fibronectin (FN) was not affected by overexpression of HAPLN4. We concluded that our transfections did not likely generate artifacts of ECM protein processing or secretion.  
C) U251MG human glioma cells were transiently transfected for expression of HAPLN4 and/or brevican, and cultured in multiwell plates during 6 days to analyze cell proliferation rates. No significant differences were observed in overall cell morphology (not shown) or cell proliferation.
**Figure 2.6** *HAPLN4 increases glioma cell adhesion in a substrate-dependent manner.* A) U251MG human glioma cells transiently transfected for expression of HAPLN4 and/or brevican were plated on multiwell plates coated with fibronectin (FN), high-MW hyaluronic acid (HA), or left uncoated and blocked with albumin (BSA), as described in the methods section. Adherent cells were fixed and quantified by crystal violet staining. All experiments were repeated at least three times with 3-6 replicates per condition. Data (mean ± S.E.M.) were analyzed by two-way ANOVA and Bonferroni’s post-hoc test (** p<0.01; *** p< 0.001). Essentially the same results were observed with the human glioma cell line U87MG and rat glioma cell line CNS-1 (not shown). Results on poly-L-lysine-coated wells were undistinguishable from those on uncoated surfaces. B) Overexpression of HAPLN4 and brevican resulted in increased expression of beta1-integrin (beta1-ITG) in these cells, explaining in part the observed increase in cell adhesion.
Figure 2.7 HAPLN4 increases glioma cell motility. A) Representative images of U251MG cells detaching from a cell aggregate and migrating on a pre-coated culture well. Images were captured by time-lapse microscopy every 15 minutes, for a total time of 14h. Pictures in the second row show the result of applying contrast- and edge-enhancing algorithms to measure the total area of dispersion, as previously described (Chicoine and Silbergeld, 1997). B) The average dispersion index (i.e., area occupied by cells at each time point divided by area occupied by the original aggregate) was plotted against elapsed time for glioma cells migrating on non-coated (BSA, upper graph) or HA-coated (HA, lower graph) surfaces. Brevican increased net cell motility, in agreement with our previous observations (Hu et al., 2008). HAPLN4 did not affect the motility of cells plated on non-coated surfaces, but promoted cell motility independently of brevican when cells were plated on HA-coated surfaces. Total areas covered by migrating cells at the end of the experiment, a measure of net cell migration, were compared by two-way ANOVA for repeated measures (**p< 0.01; *** p<0.001). Bars = 200 µm.
Figure 2.8 HAPLN4 and brevican increase the motility of glioma cells cultured in a wound-healing assay. A) Representative images of U251MG cell monolayers formed within square culture inserts with preformed 500-µm wide gaps (Ibidi GmbH #80209). Images were captured by time lapse microscopy and analyzed using an algorithm for edge-enhancement and quantification of gap area (NIH ImageJ). B) Residual gap area (i.e., area not covered by migrating cells) was calculated at t=24h as percentage of initial gap area and plotted for cells migrating on non-coated (BSA) or HA-coated (HA) plates. Results were analyzed by one-way ANOVA, followed by post-hoc Dunnett's test to compare the different treatments against control cells (* p<0.05; ** p<0.01; *** p< 0.001). Bar = 200 µm.
Figure 2.9 *HAPLN4 does not associate to the surface of cultured glioma cells.* A) Subcellular fractionation of human adult brain cortex, followed by probing of the nuclear-enriched (n), membrane-enriched (m) and soluble (s) fractions with antibodies against versican (*VCAN*), brevican (*BCAN*), HAPLN1, HAPLN2 and HAPLN4. Only HAPLN4 appeared tightly associated to membrane-containing fractions whereas all the other ECM proteins distributed in the soluble and particulate fractions. B) Transient transfection of HPLN4 in cultured human (U87MG and U251MG) and rat (CNS-1) glioma cell lines, followed by preparation of conditioned medium (*CM*) and total cell lysates (*Ly*) for Western blotting; equal amounts of total protein (10 µg) were loaded for all samples. HAPLN4 appeared largely secreted to the medium and little protein was found in the cell lysates. C) U251MG cells, transiently transfected with brevican or HAPLN4, were cultured on glass coverslips and processed for immunofluorescence as previously described (Viapiano et al., 2005). Primary antibodies were added to unfixed, unpermeabilized cells (*Live staining*) or to post-fixed and permeabilized cells (*Post-fixed*). Both proteins were detected in the cells after fixation but only brevican was detected associated to the cell surface of non-permeabilized cells.
Figure 2.10 *HAPLN4 does not associate to HA-producing glioma cells* A) U251MG glioma cells were transfected with control RFP or co-transfected with a plasmid carrying the HA-synthase enzyme HAS2, which is the predominant HA synthase isoform in the central nervous system. Two days after transfection, live cells were incubated with fixed red-blood cells as described (Maleski and Hockfield, 1997) and imaged by fluorescence (to reveal the cytoplasm) and phase-contrast (to reveal their pericellular coats). Bars= 40 µm. B) U251MG cells expressing HAPLN4 alone (*none*), or co-expressed with brevican (*BCAN*), HAS2, or brevican+HAS2 (*BCAN+HAS2*), were collected and processed for Western blotting as in (B). None of the co-expressed proteins significantly affected the distribution of secreted HAPLN4. Brevican accumulated in the cell lysates in presence of HA coats synthesized by HAS2 as shown in (A) Equal protein loading in culture media and lysates was controlled by detection of total albumin in the medium and actin in the cell lysates.
Figure 2.11 HAPLN4 is strongly associated to cell membranes of neural tissue. A) Total membranes (M) obtained from subcellular fractionation of adult human brain cortex were resuspended in 25mM Tris-HCl buffer, pH 7.4 (Tris); Tris buffer containing 10mM EDTA (EDTA); Tris buffer containing 0.5% w/v Triton X-100 (Tx100); or 100 mM sodium carbonate buffer, pH 11.0 (CO₃). After incubation, membranes were centrifuged and the resulting supernatant (s) and pellet (p) were processed for Western blotting. To verify whether released HAPLN4 was effectively water-soluble, membranes were extracted with 2% Triton X-114 as indicated in the methods section and subsequently separated by temperature-dependent partition in an insoluble pellet (Ins), aqueous phase (Aq) and Triton-containing phase (Tx). All the results suggested that HAPLN4 was strongly, but peripherally, associated to cell membranes. B) Brain cortex membranes resuspended in Tris-HCl buffer containing 10mM sodium acetate were treated with chondroitinase ABC (CHase) or chondroitinase plus phosphatidylinositol-specific phospholipase-C (PLC). After incubation, membranes were processed as indicated in (A). C) Brain cortex membranes resuspended in Tris-HCl buffer were treated with hyaluronidase (HAsE) for 4h, separated into supernatant (s) and pellet (p) fractions as indicated, and processed for Western blotting for versican (VCAN), brevican (BCAN), HAPLN4 and HAPLN1. Only HAPLN4 was insensitive to hyaluronidase treatment.
Chapter 3:

Brevican transactivates the Epidermal Growth Factor Receptor via Src kinase and cell-surface sulfatides to promote glioma cell motility\textsuperscript{2}

3.1. Introduction

As described in the introduction of this thesis, the overwhelming majority of malignant gliomas recur after surgery due to their highly invasive nature, which facilitates the scattering of malignant cells throughout the neural parenchyma (Louis, 2006). Moreover, invasive cells are highly resistant to chemo- and radiotherapy through poorly understood mechanisms (Johannessen et al., 2009; Lefranc et al., 2005) and current data suggest that invasion of the residual tumor may in fact be stimulated by adjuvant therapies (Miletic et al., 2009; Zhai et al., 2006). Therefore, anti-invasive strategies are urgently needed in the field, both to contain tumor spread and to potentially sensitize already scattered cells to cytotoxic therapies (Viapiano and Lawler, 2009).

The mechanisms that guide cell invasion and the microenvironmental signals that trigger motility in these cells are still poorly understood. Gliomas can efficiently invade the neural parenchyma (neuropil) and scatter en route to major elongated structures for tumor dispersion such as the outer surface of blood vessels and myelinated axons (Louis, 2006; Viapiano and Lawler, 2009). As previously described, this mechanism of dispersion is restricted to the CNS (Pilkington, 1997) and represents an exquisite example of

\textsuperscript{2} Results in this chapter are currently under review in J. Biol. Chemistry.
adaptation to the tumor microenvironment (TME). This adaptation suggests that CNS-specific molecules produced or processed by glioma cells could play a major role as signals driving or supporting glioma invasion (Viapiano and Lawler, 2009).

Of the potential neural-specific molecules that promote tumor invasion, brevican has been at the forefront of research since the moment it was discovered because of its restricted expression in the CNS, consistent upregulation in gliomas, and pro-invasive role (Viapiano and Matthews, 2006). In a previous chapter, we investigated the potential interaction of brevican with its canonical partner, HAPLN4, in an attempt to understand the molecular mechanisms of brevican in glioma. However, expression of brevican and HAPLN4 was highly different in gliomas compared to normal brain tissue and our results suggested that the proteins were not associated in these tumors. These observations were unexpected and opposite to our original hypothesis, which had focused on the expected canonical partners of brevican to explain the mechanisms of this lectican in glioma.

Because these results did not advance our understanding of the molecular partners of brevican and the molecular mechanisms underlying its pro-invasive effects, we abandoned the approach to understand brevican as a structural component of the tumor matrix and moved our focus to the only signaling mechanism that had been previously identified in glioma cells, namely, the activation of EGFR by brevican.

In previous work, the Viapiano laboratory demonstrated that the pro-tumoral role of brevican in gliomas requires cleavage of the full-length protein by metalloproteases of the ADAMTS family (ADAMTS-4 and -5) (Viapiano et al., 2008). Additional work in vitro demonstrated that cleaved brevican was sufficient to activate the EGFR pathway and induce fibronectin secretion, therefore increasing glioma cell adhesion and migration.
Given the relevance of the EGFR pathway as one of the predominant signaling pathways dysregulated in gliomas (Hatanpaa et al., 2010), these initial results were of particular relevance because they highlighted a potentially novel mechanism by which this receptor could be activated by ECM components highly upregulated in the matrix of invading gliomas. However, the transduction mechanism connecting brevican cleavage, EGFR activation, and glioma cell motility, is still unknown.

In this chapter, we demonstrated that following cleavage, a bioactive fragment of brevican triggers Src kinase activation, which is necessary to activate EGFR and to increase cell adhesion and migration. Moreover, we identify a novel interaction of the N-terminal domain of brevican with cell-surface sulfatides, which is required to activate Src kinase. Combined with previous work in the field, these results provide a more complete view of brevican as a novel and relevant signaling molecule in glioma. Moreover, these results give new insight on the signaling mechanisms that can be triggered by neural ECM molecules, which can be explored in multiple neuropathologies.

3.2. Materials and Methods

3.2.1. Cells and antibodies

Glioma cell lines (U87, U251 and U373) and HEK293 cells were obtained from American Type Culture Collection (ATCC) and grown at 5% CO2 in DMEM supplemented with 10% FBS. Mouse astroglial cultures were prepared from neonatal C57Bl/6J wild-type or Velvet mice, kindly provided by Dr. L. Jakeman (Dept. of Physiology and Cell Biology, The Ohio State University). The Velvet mutation is a dominant EGFR mutation (Asp^{833}Gly) that reduces kinase activity in the receptor and
prevents activation by soluble ligands such as EGF and TGF-α (Du et al., 2004). Astrocytes were maintained in astrocyte complete medium (Lonza) supplemented with 2% FBS. All cultures were supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin.

Full-length brevican and its N-terminal fragment were detected with the rabbit polyclonal antibodies B5 (N-terminal and full-length protein) B6 (full-length protein) and Be50 (N-terminal fragment), which have been previously described (Hu et al., 2008). Signaling molecules were detected with the following antibodies against total and phosphorylated (p-) proteins: rabbit polyclonal anti-Src kinase and p-Tyr\textsuperscript{416}Src kinase; rabbit polyclonal anti-EGFR, p-Tyr\textsuperscript{845}EGFR and p-Tyr\textsuperscript{1173}EGFR; rabbit polyclonal anti-p-Thr\textsuperscript{202}/Tyr\textsuperscript{204} Erk 1/2 (all from Cell Signaling); rabbit polyclonal anti-Erk1/2 (from Santa Cruz Biotechnology); mouse monoclonal anti-β-tubulin; and mouse monoclonal anti-β-actin (both from Sigma-Aldrich).

3.2.2. Purified proteins and cleavage of full-length brevican

Recombinant full-length human brevican was purchased from R&D Systems Inc. A recombinant active form of the metalloprotease ADAMTS-4 (fragment Phe\textsuperscript{213-}Cys\textsuperscript{685}) was obtained from the same vendor and frozen in aliquots in 50 mM Tris-HCl buffer, pH 7.4, containing bovine serum albumin (100 µg/ml) to preserve activity. Protein purity was routinely confirmed by SDS-PAGE.

To completely cleave purified full-length brevican \textit{in vitro}, 1 µg brevican was incubated with 50 ng ADAMTS-4 in 100 µl of reaction buffer (50 mM TrisHCl, 5 mM CaCl\textsubscript{2}, pH 7.4) for 8h at 37 °C. Purified full-length or pre-cleaved brevican were added to
cultured cells at a calculated final concentration of 200 ng/ml.

3.2.3. cDNA constructs and cell transfection

A clone of human full-length brevican cDNA (Met\textsuperscript{1}Pro\textsuperscript{911}, GenBank BC010571) was obtained commercially and subcloned into pcDNA3.1(+) (Invitrogen). The N-terminal fragment of human brevican (B50, Met\textsuperscript{1}Glu\textsuperscript{400}) was generated by PCR and subcloned into pcDNA3.1(+).

To purify recombinant B50, a biotin acceptor peptide (\textit{BAP: GGLNDIFEAQKIEWH}) was inserted by PCR between the signal peptide (Met\textsuperscript{1}Ala\textsuperscript{23}) and the remaining sequence of B50 (Asp\textsuperscript{24}Glu\textsuperscript{400}). The resulting construct (\textit{BAP-B50}) was co-transfected with the biotin transferase enzyme BirA (Verhaegen and Christopoulos, 2002) engineered to be retained in the endoplasmic reticulum (Barat and Wu, 2007). This recombinant form of B50 was biotinylated at a single specific site (\textit{-EAQK\textsubscript{biot}IEW-}) and could be purified from culture medium for subsequent assays. BirA cDNA was kindly provided by Dr. Anna Wu from the UCLA School of Medicine.

All constructs were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Stable transfectants were generated by selection with geneticin (800 µg/ml, B50 constructs) and zeocin (800 µg/ml, BirA) according to standard protocols (all antibiotics from Invitrogen). Levels of the ER stress sensor protein GRP78 were regularly compared in the transfectants and naïve cells to confirm that protein overexpression was compatible with normal cellular function (Sim et al., 2009).

3.2.4. Purification of biotinylated B50
BAP-B50 and BirA cDNAs were used to generate stably co-transfected HEK293 cells. To induce biotinylation of BAP-B50, cells were cultured overnight in serum free medium containing 100 µg/ml biotin (Sigma-Aldrich). Serum free conditioned medium was collected and concentrated using 10 kDa-MWCO centrifugal concentrators (Millipore). BAP-B50 was enriched by affinity chromatography using low-affinity monomeric avidin-agarose (Pierce) and extensively washed with 50 mM phosphate buffer, pH 7.4, containing 150 mM NaCl (PBS). Non-specific contaminants were removed by washing the column with 50 mM phosphate buffer, pH 7.4, containing increasing NaCl (300 and 500 mM), and BAP-B50 was finally eluted using PBS containing 4 mM biotin. Eluted BAP-B50 was dialyzed overnight against PBS and concentrated by ultrafiltration.

3.2.5. Western blotting and quantitative RT-PCR

All cell cultures analyzed for protein phosphorylation were cultured overnight in serum-free medium before incubation with full-length brevican, cleavage products, or purified BAP-B50. After incubation, cells were washed, flash-frozen and lysed in 20 mM Tris-HCl, pH 8.0, containing 137 mM NaCl, 1% w/v NP-40, 1 mM MgCl₂, 10% w/v glycerol, 0.5 mM EDTA, and a mixture of protease (Complete EDTA-free) and phosphatase (PhosStop) inhibitors (both from Roche Applied Science). 50 µg of each cell extract was electrophoresed on 7% SDS-polyacrylamide gels and analyzed by Western blotting. For kinase inhibition experiments the Src kinase inhibitor PP2 (150 nM, Calbiochem) was added to the cells 2h before incubation with brevican.

To analyze mRNA expression, frozen cells were extracted using Trizol (Invitrogen) and residual DNA was removed using Turbo-DNA Free (Applied
Biosystems). Reagents for semiquantitative RT-PCR (iQ SYBR Green Supermix, Bio-Rad) were used according to the manufacturer’s instructions.

3.2.6. Sulfatide analysis

The interaction between B50 and cell membrane lipids was analyzed by dot blotting using nitrocellulose membranes with pre-spotted polar lipids (SphingoStrips, Echelon Biosciences). Membranes were blocked using 50 mM phosphate buffer, pH 7.4, containing 150 mM NaCl, 0.05% w/v Tween-20 and 3% fatty-acid free bovine serum albumin (Sigma-Aldrich). Membranes were subsequently probed with purified BAP-B50, which was detected using Bc50 antibody. Competition studies were performed using porcine brain sulfatides (Avanti Polar Lipids, 20 µM final concentration) or the Ca$^{++}$ chelating agent EDTA (5-20 mM final concentration). Competing agents were pre-incubated with BAP-B50 for 1h.

To confirm the requirement of cell-membrane sulfatides for B50 signaling, glioma cells were cultured in serum-free medium and treated with 100 U/ml of Helix pomatia aryl-sulfatase type H2 (Sigma-Aldrich) for 1h, before adding B50 to the cultures.

3.2.7. Cell Adhesion and Motility Assays

Cell adhesion assays were performed essentially as previously described (Hu et al., 2008), with minor modifications. Briefly, cells overexpressing B50 or a control cDNA were lightly trypsinized, loaded with calcein-AM (1 µg/mL, Invitrogen) and seeded at 50,000 cells/well on 96-well plates coated with bovine plasma fibronectin (10 µg/ml). Cells were washed after 30 min using phosphate buffered saline containing 1 mM CaCl$_2$
and MgCl$_2$ to remove non-adhered cells. Adhered cells were lysed in 20 mM Tris-HCl, pH 8.0, containing 137 mM NaCl and 1% w/v NP-40 and quantified by measuring calcein fluorescence.

For migration assays, cells were lightly trypsinized and seeded at 150,000 cells/ml on hard-agar plates to form floating spheroids in low (0.1%) serum. After 48h, spheroids were seeded on fibronectin-coated wells and migration was quantified by analyzing the increase in area and perimeter covered by the cells dispersing radially from the core aggregate (Sim et al., 2009).

To inhibit Src activation during adhesion and migration experiments, the inhibitor PP2 (250 nM) was pre-incubated with the cells for 3h before measuring adhesion, or co-incubated during the migration experiments. All adhesion and migration experiments were repeated in triplicate with at least four independent replicates per experiment.

3.3. Results

3.3.1. Cleavage of brevican is necessary for activation of EGFR and Erk

In previous work it was shown that overexpression of brevican in glioma cells resulted in activation of EGFR, leading to increased fibronectin synthesis and cell adhesion (Hu et al., 2008). These results contributed to understanding the pro-motility effects of brevican but the mechanism by which the secreted proteoglycan activated EGFR was unknown.

Interestingly, preliminary experiments in this thesis indicated that short-term incubation (less than 1h) of glioma cells with purified brevican was insufficient to activate EGFR (data not shown), suggesting that an additional process was required for
this proteoglycan to trigger EGFR signaling. Full-length brevican is cleaved by ADAMTS metalloproteases into stable fragments of 50-kDa (N-terminal fragment) and 90-kDa (C-terminal) (Matthews et al., 2000; Nakada et al., 2005), which are significantly upregulated in gliomas and may account for the functions of brevican in these tumors (Viapiano et al., 2005). Therefore, we hypothesized that brevican was likely cleaved in culture before a fragment of this protein could activate EGFR.

To test this hypothesis, we first incubated purified brevican with conditioned medium from U87 glioma cells and confirmed that the full-length protein was cleaved by ADAMTS activity present in the medium, with almost complete processing by 24h (Figure 3.1A). Incubation of purified brevican with recombinant ADAMTS-4 reproduced this effect, with complete cleavage of brevican within 8h (Figure 3.1B). Next, we incubated U87 cells with purified (non-cleaved) brevican, purified brevican that had been pre-cleaved with ADAMTS-4, or a prototypical EGFR ligand (epidermal growth factor). The results showed that incubation with full-length brevican resulted in a slow kinetics of EGFR activation, matching the time course of brevican cleavage (Figure 3.1C-D). In contrast, incubation with pre-cleaved brevican resulted in much faster EGFR activation peaking at 15 min, matching the profile of EGF. Erk1 (MAPK p44), a downstream target of EGFR involved in cell adhesion and migration, was also activated by full-length or cleaved brevican with essentially the same kinetics. Erk2 (MAPK p42) was also activated but at a much lower level (data not shown).

The results above were confirmed using two additional glioma cell lines, U251 and U373: short-time (30 min) incubation with full-length brevican was insufficient to activate EGFR but incubation with pre-cleaved brevican for the same amount of time
resulted in robust increase of phospho-EGFR (Figure 3.1E). Together, these results indicated that cleavage of brevican was a necessary step to trigger EGFR and Erk activation.

3.3.2. B50 activates EGFR at a Src-dependent phosphorylation site

Previous work has shown that expression of the N-terminal fragment of brevican (B50) in glioma cells enhances their adhesion and motility, while transfection with the C-terminal fragment lacks any defined effect on glioma cell growth or invasion (Hu et al., 2008) and unpublished results). Therefore, we focused on B50 as the potential signaling fragment of brevican following cleavage of the full-length protein.

In order to assay the effects of B50 on cultured cells, we produced a biotin-tagged, recombinant B50 construct (BAP-B50) that could be purified from conditioned medium by affinity chromatography. Preliminary tests of BAP-B50 indicated that it was secreted to the medium in amounts equivalent to unmodified B50 and was biotinylated as expected (Figure 3.2).

Incubation of U87 cells with BAP-B50 showed that this purified fragment of brevican was sufficient to trigger the phosphorylation of EGFR in a time-dependent manner, with the effect being considerably reduced by 1h (Figure 3.3A). A similar assay in a second glioma line (U373) confirmed that BAP-B50 was able to activate EGFR and Erk1/2 for at least 30 minutes (Figure 3.3B). Most interestingly, this experiment revealed that BAP-B50 was able to trigger EGFR phosphorylation not only at Tyr1173, which is involved in downstream MAPK activation (Zwick et al., 1999), but also at Tyr845, a site phosphorylated by Src kinases (Biscardi et al., 1999).
3.3.3. *B50 activates Src kinase to phosphorylate EGFR*

The surprising result above suggested that Src kinase could be a possible mediator in the activation of EGFR by brevican. EGFR and Src kinase are known to have bi-directional signaling and EGFR can be transactivated by activation of other receptors triggering Src kinase activity (Fischer et al., 2003). Therefore, we next analyzed the expression and activity of Src kinases in our model.

First, we confirmed that short-term incubation of U87 glioma cells with BAP-B50 was sufficient to induce Src phosphorylation (using a pan-Src antibody), concurrent with phosphorylation of EGFR at Tyr$^{845}$ (*Figure 3.4A*). Incubation of the cells with purified full-length brevican for the same amount of time did not induce phosphorylation of Src, or EGFR at Tyr$^{845}$, as expected.

We then analyzed the expression of Src kinases in glioblastoma cells and found that the cell lines used in our assays expressed several members of this family (Lyn, Fyn and c-Src) at comparable levels (*Figure 3.4B*), prompting us to use a pharmacological strategy to globally inhibit Src activity in the cells.

Using the pan-Src inhibitor PP2 we confirmed that Src inhibition was sufficient to abolish the effect of BAP-B50 on EGFR (*Figure 3.4C*). The effect of PP2 was specific for Src-mediated transactivation of EGFR, because inhibition of Src did not prevent direct activation of EGFR by its own ligand (EGF). This result indicated that B50 activates EGFR in a Src-dependent manner.

While the result above indicated that Src activity was necessary for EGFR activation by B50, it did not indicate if a transactivating mechanism was sufficient for
B50 to trigger EGFR activation. To test this hypothesis we evaluated the effect of B50 in primary cultures of neonatal astrocytes from Velvet mice (described in further detail in chapter 4). The Velvet mutation is a dominant inactivating mutation in EGFR that prevents direct activation by the EGFR specific ligand TGF-α, but does not prevent transactivation of the receptor (Du et al., 2004).

Neonatal astrocytes from wild-type and Velvet mice were cultured in serum-free medium in the presence of TGF-α or BAP-B50, and analyzed for activation of EGFR and Src. The results showed that TGF-α failed to activate EGFR in Velvet astrocytes, as expected (Figure 3.5A). In contrast, B50 showed similar effects in normal and velvet astrocytes: Although the overall sensitivity of Velvet astrocytes to B50 was reduced compared to wild type cells, B50 activated Src kinase as well as EGFR in both cell types (Figure 3.5B). These results suggested that a transactivation mechanism was sufficient to explain the activation of EGFR by B50.

3.3.4. B50 binds to sulfatides to activate Src kinase.

We next investigated how the B50 fragment could activate Src kinase. B50 contains one N-terminal immunoglobulin-like domain and two hyaluronic acid (HA)-biding domains upstream of the ADAMTS cleavage site. This configuration makes B50 a homologue of the extracellular HA-binding proteins of the link-protein family (Sim et al., 2009), for which there is no membrane receptor identified. Repeated attempts to identify a cell-surface protein receptor for B50, including protein crosslinking and affinity chromatography, yielded negative results (data not shown).

Interestingly, there is abundant literature showing the regulation of Src and EGFR
activity by membrane lipids, particularly gangliosides involved in lipid raft organization (Patra, 2008). Therefore, we used a dot-blot method to explore the possible binding of B50 to cell membrane lipids that could explain its mechanism.

Results from dot blotting showed a strong and specific interaction of B50 with sulfo-galactosylceramides (sulfatides, Figure 3.6A). Competition with excess sulfatides confirmed this interaction (Figure 3.6B). We did not observe interaction with ceramide, suggesting that the binding was restricted to the galactose-3-sulfate polar head of the sulfatides.

Interestingly, all members of the lectican family contain a selectin-like domain close to the C-terminus. This domain can bind sulfoglycolipids, including sulfatides, in a Ca\textsuperscript{2+}-dependent manner (Miura et al., 1999). In stark contrast, attempts to displace the binding of B50 with EDTA (up to 20 mM) were unsuccessful (Figure 3.6B), suggesting a novel lectin-like mechanism for the binding of this brevican fragment to sulfatides.

To confirm if the interaction of B50 with sulfatides was relevant to the activation of Src and EGFR, we treated U87 cells in culture with a sulfatase enzyme before incubation with purified B50, to remove the sulfate from the polar head of sulfoglycolipids (Li et al., 2005). Sulfatase treatment did not prevent activation of EGFR by its ligand EGF (Figure 3.6C). However, the treatment abolished the enhancing effect of B50 on Src and EGFR activation (Figure 3.6D). These results indicated that binding of B50 to cell-surface sulfatides was likely the starting point for B50 signaling leading to Src activation and EGFR transactivation.

3.3.5. Src inhibition abolishes the pro-adhesive and pro-migratory effects of B50.
The results above strongly suggested that Src activity would be critical for the downstream cellular effects of brevican, and more specifically, its B50 fragment. Since this fragment is sufficient to promote glioma cell adhesion and migration (Hu et al., 2008), we tested if the pro-motility effect of B50 in glioma cells could be explained by a Src-dependent mechanism.

To this end, two glioma cell lines were transiently transfected with B50 or control cDNAs and tested in cell adhesion and cell migration assays in the presence or absence of the Src inhibitor PP2. In short-term adhesion assays we found that PP2 did not reduce the baseline level of cell adhesion, but essentially abolished the enhancing effect of B50 (Figure 3.7A). Similarly, in 24h-long migration assays PP2 limited the dispersion of glioma cells and suppressed the effects of B50 (Figure 3.7B-C). These differences were more marked at longer times (48h) but also likely affected by reduced viability of the cells under prolonged incubation with PP2 (not shown). Overall, these results indicated that inhibition of Src activity was sufficient to abolish the pro-migratory effect of B50 in glioma cells.

3.4. Discussion

The EGFR signaling pathway is one of the most commonly altered pathways in high-grade glioma and a major driving force in the pathogenesis and development of these tumors (Hatanpaa et al., 2010; Watanabe et al., 1996). Amplification and overexpression of EGFR can be detected in 40% to 60% of primary glioblastomas and has been consistently linked to the process of gliomagenesis and sustained tumor growth (Mimeault and Batra, 2011). Indeed, EGFR amplification is considered the leading
molecular signature of the classical or proliferative type of glioma (Phillips et al., 2006; Verhaak et al., 2010). In addition it is common to detect mutant variants of the receptor, of which the most common is the constitutively active EGFRvIII deletion variant that is more tumorigenic than the wild-type receptor (Nishikawa et al., 1994).

The role of the EGFR pathway in glioma cell migration and invasion has been addressed less extensively. However, it has been shown that EGFR signaling upregulates downstream targets such as metalloproteases, mesenchymal ECM proteins, and novel nucleotide binding factors (e.g. GBP1) that support increased invasion (Hu et al., 2008; Lal et al., 2002; Li et al., 2011). In vivo models have also shown that constitutive EGFR signaling can increase dispersion of normal neural stem cells in the brain cortex (Boockvar et al., 2003) and that specific targeting of EGFR results in less invasive brain tumors (Martens et al., 2008).

Direct activation of EGFR by molecules of the extracellular matrix independently of EGF has been rarely described. Previous work with the CSPG versican demonstrated that the C-terminal fragment of this proteoglycan can bind directly to EGFR, leading to increased EGFR signaling in neurons but, surprisingly, reduced EGFR signaling and cell migration in glioma cells (Wu et al., 2004; Xiang et al., 2006). In contrast, our previous and current work with brevican has shown that this CSPG clearly activates EGFR signaling in glioma cells. We have demonstrated that activation of EGFR by brevican leads to accumulation of pro-adhesive fibronectin on the cell surface (Hu et al., 2008) and subsequent increase in cell adhesion and motility. Of the two lecticans, brevican shows a much higher upregulation than versican across all kinds of gliomas (Sim et al., 2009).

Brevican is highly upregulated in high-grade gliomas compared to normal brain
(Sim et al., 2009; Viapiano et al., 2005) and its overexpression in experimental models results in a marked increase of tumor growth and invasion (Jaworski et al., 1996; Viapiano et al., 2008). This is an unusual or "paradoxal" role for this CSPG because the upregulation of brevican is common in inflammation- and trauma-related neuropathologies, but correlates instead with reduced motility of glial cells and extension of neurites (Viapiano and Matthews, 2006). However, previous work has shown that the expression of brevican in glioma is not as critical as the cleavage of the full-length proteoglycan by ADAMTS metalloproteases. The resulting soluble fragments, but not the ECM-associated full-length protein, are necessary and sufficient for the pro-invasive role of brevican (Hu et al., 2008; Viapiano et al., 2008).

In agreement with a pro-motility role of cleaved brevican, the bioactive fragment B50 is increased by over 2-fold in human glioblastomas compared to normal brain tissue (Viapiano et al., 2005), while this increase is never seen in experimental models of spinal or brainstem injury (Andrews et al., 2011; Massey et al., 2008). Taken together, this evidence highlights the role of the soluble fragments of brevican that are abundant in glioma as potential signaling molecules that exacerbate invasion as glioma cells degrade the neural ECM. Moreover, it allows us to predict that upregulation of brevican would likely not be an indicator of clinical relevance unless analyzed together with the extracellular processing of the protein.

In this study, we have demonstrated that activation of Src kinases is the key transduction mechanism underlying the activation of EGFR and Erk by brevican and the consequent increase in cell motility. The role of Src kinases in promoting brain tumor growth and invasion has been well documented (Angers-Loustau et al., 2004; Lu et al.,
and these proteins have become recent targets of single or combined pharmacological strategies (Ahluwalia et al., 2010; Milano et al., 2009; Premkumar et al., 2010).

Src kinases have been implicated both as downstream effectors of EGFR as well as upstream mediators of EGFR intracellular transactivation by other receptor tyrosine kinases or G-protein coupled receptors (Fischer et al., 2003). Moreover, recent work has shown that Src activity mediates the transactivation of EGFR by Toll-like receptors to increase the motility of glioblastoma cells (Thuringer et al., 2011). Together, these results highlight the intracellular transactivation of EGFR independent of ErbB ligands or classic integrin-mediated signaling as a novel and potentially major mechanism underlying glioma invasion.

The studies with the CSPG versican mentioned above, which demonstrated direct interaction with EGFR in neural cells, also suggested that Src was the downstream effector of this signaling pathway (Wu et al., 2004). In contrast, our results are the first to demonstrate that a CSPG can transactivate EGFR by upstream activation of Src kinase, leading to an increase in cell motility that can be abolished by suppressing Src activity. These results suggest that Src kinase inhibitors may have a high potential as anti-invasive drugs in glioma by reducing the pro-invasive signals derived from the ECM, and could therefore assist in chemosensitizing strategies (Ahluwalia et al., 2010).

The co-distribution and activation of Src and EGFR in the membrane of cancer cells has been associated with the transient formation of lipid rafts that are involved in cell transformation and tumor growth (Patra, 2008). Src and EGFR can be activated in the rafts not only by EGF or transactivating receptors, but also by direct engagement of cell-
surface glycolipids (Tanikawa et al., 2008). For example, it has been shown that binding of laminin to cell-surface sulfatides is sufficient to induce lipid raft formation and Src activation (Li et al., 2005). In addition, a modeling study has shown that the sulfogalactose moiety of sulfatides can mimic a phospho-tyrosine moiety and bind to the SH2 domain of c-Src to activate this enzyme (Lingwood et al., 2005). Taken together, this evidence suggests that a direct interaction between B50 and sulfatides could be sufficient to physically modify Src- and EGFR-containing lipid rafts, triggering a signaling pathway from Src to EGFR to Erk1/2, leading to subsequent fibronectin synthesis (Zhang et al., 2005).

In sum, our results characterized for the first time a signaling pathway for a CSPG in glioma, as proposed in Figure 3.8, explaining the involvement of these molecules in cell adhesion and motility. Moreover, these results suggest that similar transduction mechanisms could be responsible for the physiological effects of other lectican CSPGs in pathologies where ADAMTS-mediated cleavage is exacerbated, as it is the case with aggrecan in arthritic disease (Stanton et al., 2011). Finally, these results suggest potential ways to tailor therapies to uncouple EGFR signaling from ECM signals, reducing the microenvironmental stimuli that promote tumor invasion.
Figures of chapter 3

Figure 3.1 The time course of EGFR activation coincides with brevican cleavage by glioma cells.
A) Purified recombinant brevican was incubated with conditioned medium from the glioma cell line U87. Samples were collected at the specified times and processed for Western blotting. Results show the time-course of brevican cleavage, which is almost complete by 24h. B90 and B50 are the resulting 90- and 50-kDa stable cleavage fragments. B) Purified brevican was incubated with recombinant ADAMTS-4 (ATS4) for 8h. Western blotting using the antibody B5 shows complete cleavage of the full-length protein and accumulation of the stable B50 fragment (the antibody does not detect B90). C) U87 cells were incubated with purified brevican (bcan), purified brevican that had been cleaved with ADAMTS-4 as in (B) (cleaved bcan), or epidermal growth factor (EGF, 10 ng/ml), and processed for Western blotting at the specified times. Representative blots show the time course of activation of EGFR and Erk1/2. C) Quantification of immunoblots indicates that activation of EGFR and Erk1 (MAPK p44) by cleaved brevican peaks in less than 1h, resembling the activation profile observed with EGF. In contrast, activation of EGFR and Erk1 by full-length brevican is much slower likely follows the cleavage of brevican by the cells. D) Similar results as in (B) were observed with U251 and U373 glioblastoma cells treated with vehicle, brevican, cleaved brevican (cl. bcan), or ADAMTS4 for 30 minutes. EGFR activation was triggered by cleaved brevican but not by the full-length protein or ADAMTS-4.
Figure 3.1
Figure 3.2 Preparation of purified B50. A) The N-terminal end of B50 was modified by PCR to include a biotin acceptor peptide (BAP). BAP-B50 was expressed in HEK293 cells engineered to retain bacterial biotinyltransferase BirA in the endoplasmic reticulum (ER lumen). BAP-B50 was biotinylated in the ER, secreted, and recovered from the culture medium. B) Typical results from HEK293 transfected with unmodified B50 cDNA or with BAP-B50 (two independent purifications). BAP-B50 could be detected with an antibody against the C-terminus of B50 (Bc50) and with peroxidase-conjugated streptavidin (Str-HRP).
Figure 3.3 *Purified B50 activates EGFR at a Src-dependent phosphorylation site.* A) U87 cells were incubated with purified BAP-B50 and processed for Western blotting; the results show time-dependent activation of EGFR by BAP-B50, as previously observed with pre-cleaved brevican. B) Western blotting using U373 cells confirmed the ability of purified BAP-B50 to activate EGFR (phosphorylation at Tyr^{1173}) and Erk1/2. In addition, these results disclosed the activation of EGFR at the Tyr^{845} Src-dependent phosphorylation site.
Figure 3.4 B50 activates EGFR in a Src-dependent manner. A) Western blotting of U87 cells treated with BAP-B50. The results show time-dependent activation of Src kinase (using a pan-Src antibody) and the corresponding Tyr^{845}-phosphorylated EGFR. In contrast, full-length brevican was unable to elicit the same activation of EGFR and Src. B) Comparative expression of Src kinase family members by qRT-PCR in glioblastoma cells. The results show similar expression levels of multiple Src kinases (especially in U251 and U373 cells). C) Western blotting of U87 cells pre-treated with the pan-Src inhibitor PP2 (150 nM), or vehicle, before incubation with BAP-B50. Inhibition of Src abolished the activation of EGFR by B50; however, direct activation of EGFR by its ligand (EGF) was not affected, suggesting that B50 transactivates EGFR in glioblastoma cells in a Src-dependent manner.
Figure 3.5 B50 can activate EGFR in a ligand-insensitive, EGFR-deficient model. Velvet mice have a dominant mutation that renders EGFR insensitive to activation by its specific ligand TGF-α. A) Primary cultures of wild-type (WT) or Velvet (Ve) astrocytes from neonatal cortex were serum-starved and treated with TGF-α (5 ng/ml) for 30 min. EGFR (arrow) was not activated in Velvet cells in response to the ligand. B) In contrast, incubation of the same cells with purified B50 for 30 minutes resulted in activation of Src and EGFR, suggesting that Src activation by B50 was sufficient for EGFR transactivation.
Figure 3.6 B50 binds to cell-surface sulfatides to activate Src. A) Purified BAP-B50 or conditioned medium from HEK293 cells (negative control) were used to probe nitrocellulose membranes containing the following lipids: 1, sphingosine; 2, sphingosine-1-phosphate; 3, phytosphingosine; 4, ceramide; 5, sphingomyelin; 6, sphingosylphosphorylcholine; 7, lysophosphatidic acid; 8, myriocin; 9, monosialoganglioside GM1; 10, disialoganglioside GD3; 11, sulfatide; 12 psychosine; 13, cholesterol; 14, lysophosphatidylcholine; 15, phosphatidylcholine; 16, blank (solvent). Results show specific detection of sulfatides (arrow) and lack of binding of B50 to the non-sulfated ceramide backbone or to any other lipids in the array. The asterisk marks a non-specific spot. B) Binding of purified B50 to spotted sulfatides was specifically displaced by pre-incubation with sulfatides (sulfogalactosylceramide, SGC, 20 µM), but could not be displaced with EDTA (10 mM). C) Pre-treatment of U87 cells in culture with sulfatase (100 U/ml, 1h) did not prevent activation of the EGFR by the ligand EGF. D) In contrast, the same treatment of U87 cells with sulfatase abolished the activating effect of B50 on Src and EGFR.
Figure 3.7 *Src inhibition abolishes the effect of B50 on cell adhesion and migration.* A) U87 and U251 cells transfected with B50 or control cDNAs were treated with the pan-Src kinase inhibitor (250 nM) for 3h and tested for adhesion to fibronectin-coated plates as described (Hu et al., 2008). Inhibition of Src kinase activity abolished the enhancing effect of B50 on cell adhesion. B) Cells were treated as before and tested in a radial migration assay on fibronectin-coated plates. B50 effect on cell migration was also suppressed by the pan-Src inhibitor. C) Representative images of cells dispersing from core aggregates after 24h. The black lines indicate the size of the original aggregate applied to the well. Bars= 250 µm. Results in (A) and (B), significant differences versus control cells treated with vehicle at **p<0.01; ***p<0.001, two-way ANOVA and Bonferroni's post-hoc tests.
Figure 3.8 Proposed model of action of brevican in glioma cells. Brevican is produced both by normal glia and glioma cells and accumulates in the ECM. In addition, glioma cells secrete ADAMTS proteases (expressed at much lower levels in normal brain) that cleave brevican and generate the fragments B50 and B90. B50 engages sulfatides on the cell surface of glioma cells, leading to EGFR transactivation, MAPK activation and downstream fibronectin synthesis that increase cell adhesion and motility. This model is based on results from the present study and from reference (Hu et al., 2008). The C-terminal domain of brevican has been shown to bind sulfated lipids and tenascins but it does not promote glioma cell motility (Hu et al., 2008).
Chapter 4:

Reduced EGFR activity in the brain does not affect glioma growth but reduces tumor invasion and prevents the pro-invasive role of brevican

4.1. Introduction

As described elsewhere in this dissertation, a major component for the extremely poor prognosis of malignant gliomas is the highly infiltrative nature of glioma cells, which makes these tumors recurrent and therefore essentially incurable with conventional therapies (Louis, 2006).

Signaling by receptor tyrosine kinases (RTKs), including receptors from the ErbB, Met, and PDGF families, has been shown to contribute not only to tumor growth but also to the invasion of these cells (Chu et al., 2009; Kapoor and O'Rourke, 2003). The epidermal growth factor receptor (EGFR) is the most commonly mutated RTK in gliomas and exhibits genomic amplification, mRNA upregulation, and expression of constitutively active variants in over a third of all gliomas (Wykosky et al., 2011). EGFR is a central regulator of glioma progression by activating multiple signaling pathways driven by mitogen-activated protein kinases (MAPKs) and the transcription factor STAT-3 (Huang et al., 2009a) among others. In turn, EGFR can be activated not only by direct ligand binding but also by transactivation of intracellular active sites (Shah et al., 2003). As we showed in the previous chapter, one of the mechanisms of brevican in glioma
involves the regulation of EGFR via Src-transactivation of the receptor.

In agreement with the central role of EGFR in gliomas, multiple therapeutic strategies have been pursued against EGFR, including the use of blocking monoclonal antibodies (Kapoor and O'Rourke, 2003), small-molecule inhibitors (Huang et al., 2009b), and the recent development of immunotherapy against the constitutively active deletion variant EGFRvIII that is overexpressed in glioma (Sampson et al., 2008b). These strategies have shown a variable level of efficacy in different patient populations (Sampson et al., 2010) and, most importantly, have frequently elicited the development of chemoresistance (Huang et al., 2009b).

While the effects of EGFR targeting have been extensively studied in glioma cells, the consequences of EGFR suppression in the tumor microenvironment have been virtually ignored. EGFR expression in the normal adult CNS is usually very low and restricted to neurons (White et al., 2011). However, under neural injury and pro-inflammatory conditions, such as the ones that can be found during tumor progression, EGFR is highly upregulated and activated in reactive astroglial cells and contributes to the pro-inflammatory glial response (Zhang and Neufeld, 2005). Since normal glial cells are genetically stable and therefore unlikely to develop resistance to EGFR inhibition, it is particularly relevant to identify the consequences of suppressing EGFR signaling in this compartment of the tumor stroma.

The Velvet mouse is a nitrosourea-induced mutant strain that expresses a dominant hypomorphic form of EGFR (Du et al., 2004). The mutation Asp$^{833} \rightarrow$Gly in the tyrosine kinase domain of EGFR causes almost complete loss of activity of the receptor and in
particular prevents the activation by external canonical ligands such as EGF and TGF-α. Velvet mice have been shown to exhibit a reduced glial response towards neural injury, including reduced or absent formation of a protective glial scar (White et al., 2011) in a spinal cord injury model.

Here, we have characterized for the first time the use of Velvet mice and astroglial cultures derived from these animals to study how EGFR deficiency in the tumor microenvironment may affect tumor development. In addition, we have used this model to determine if the pro-invasive effects of brevican in glioma, which are mediated by EGFR, are maintained in EGFR-expressing glioma cells within an EGFR-deficient microenvironment. The results described in this chapter show that EGFR-deficient Velvet astrocytes exhibit poorer response towards glioma cells when compared with the wild-type counterparts. Interestingly, this does not affect total tumor growth in vivo and syngeneic tumors develop equally well both in Velvet and wild-type mice. However, our results also suggest that tumors become less invasive when they grow within the EGFR-deficient microenvironment. More interestingly, the bioactive fragment of brevican, B50, does not promote dispersion of glioma cells when those cells grow in the EGFR-deficient brain parenchyma. Taken together, these results are very relevant to understand the signaling effects of brevican in the tumor microenvironment and the role of stromal EGFR for tumor invasion.

4.2. Materials and Methods

4.2.1. Cells, antibodies and purified proteins

The mouse glioma cell line GL261 was kindly provided by Dr. Russell Mathews.
(Dept. Neuroscience and Physiology, SUNY Upstate Medical University) while the human glioma cell line U251MG was obtained from the American Type Culture Collection. Both cell types were grown in DMEM supplemented with 10% FBS. The rat glioblastoma cell line CNS1 was characterized and kindly provided by Dr. William Hickey (Dept. Pathology, Dartmouth Medical School) and growing in high-glutamine RPMI supplemented with 10% FBS. The mouse glioblastoma cell line Mut4 was derived from a transgenic model of glioma (GFAP-Cre; Nf1loxP/+ Trp531/2 PTENloxP/+) (Alcantara Llaguno et al., 2009) and kindly provided by Dr. Chang-Hyun Kwon (Dept. Neurological Surgery, OSU). Tumor initiating Mut4 cells were cultured as floating neurospheres in DMEM/F12 supplemented with 20 ng/ml of EGF, 20 ng/ml bFGF, 2 µM glutamine and B27 supplement (Invitrogen). Primary cortical astroglial cultures from wild-type and Velvet mice were prepared following standard protocols and maintained in astrocyte basal medium (Lonza) supplemented with 3% FBS and a growth kit provided by the manufacturer (EGF, insulin, glutamine, gentamicin and amphotericin). Astrocytes used for biochemical tests were cultured in basal medium lacking FBS and the growth kit. All cultures were supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin.

To investigate the status of EGFR signaling in astrocytes, the following antibodies were used: rabbit polyclonal anti-EGFR, anti-phosphoTyr1173-EGFR and anti-phosphoTyr1068-EGFR (Cell Signaling); rabbit polyclonal anti-Akt and anti-phosphoSer473-Akt (Cell Signaling); rabbit polyclonal anti-Erk1/2 (Santa Cruz Biotechnology) and anti-phosphoThr202/Tyr204-Erk (Cell Signaling). Additional antibodies used included: rabbit polyclonal anti-VASP and anti-phosphoSer157-VASP (Cell Signaling), mouse monoclonal anti-Nestin (Becton Dickinson), mouse monoclonals
anti-GFAP and anti-GAPDH (Sigma Aldrich), and mouse monoclonal anti-tubulin (Sigma Aldrich). All growth factors used in this work were from Peprotech.

4.2.2. Biochemical assays and immunocytochemistry

Cells assayed for protein phosphorylation were cultured overnight in serum-free medium before incubation with TNF-alpha (5 ng/ml) for 20min. Cells were subsequently lysed in 20 mM Tris-HCl, pH 8.0, containing 137 mM NaCl, 1% w/v NP-40, 1 mM MgCl2, 10% w/v glycerol, 0.5 mM EDTA, and a cocktai l of protease (Complete EDTA-free) and phosphatase (PhosStop) inhibitors (both from Roche Applied Science). 50 µg of each cell extract was electrophoresed on 7% SDS-polyacrylamide gels and analyzed by Western blotting.

To analyze mRNA expression, total RNA was extracted from frozen cells using Trizol reagent (Invitrogen) according to the manufacturer instructions. Residual DNA was removed using Turbo-DNA Free (Applied Biosystems) and RNA was processed for semiquantitative RT-PCR using iQ SYBR Green Supermix (Bio-Rad).

Glial cells processed for immunocytochemistry were seeded in 4-well chambers (LabTek) at 30,000 cells/well and cultured for three days. Cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature and blocked with PBS containing 0.1% Tween-20 and 3% bovine serum albumin for 1 hour. Primary antibodies were applied at 4°C overnight in blocking buffer and the cells were subsequently washed and incubated with fluorescent secondary antibodies for 1 hour at room temperature. Nuclei were counterstained using 4’,6-diamidino-2-phenylindole (DAPI).
4.2.3. Cell viability and proliferation

Cells were plated at 5,000 cells/well in 96-well plates and cultured for 24 h in astrocyte growth medium. The following day the medium was changed to basal medium containing TNF-alpha (20 ng/ml) or its vehicle. Cell viability was measured every other day using a conventional redox assay based in the reduction of a soluble tetrazolium compound (MTS Cell-Titer kit, Promega), as previously described (Hu et al., 2008).

To quantify cell proliferation, cells treated as above were fixed each day and stained with sulforhodamine-B to assess total cell biomass as previously described (Skehan et al., 1990).

4.2.4. Cell migration and invasion assays

To assess astrocyte motility, wild type or Velvet astrocytes were seeded in cell-culture inserts (Ibidi) and 25,000 cells/side. The following day the inserts were removed, leaving a 400-μm wide gap to be crossed by motile cells. Migration assays were performed in presence of complete growth medium or basal medium containing TNF-alpha (20 ng/ml) or its vehicle. Gap closure by migrating cells was imaged every day and quantified using the software ImageJ (v. 1.45).

To assess astrocyte motility towards glioma cells, astrocytes were seeded as before on one side of the culture inserts and GL261 glioma cells were seeded on the opposite side at 35,000 cells. After two days, the inserts were removed and GL261 cells formed a compact layer with little to no motility. Gap-closure caused by astrocyte motility towards glioma cells was analyzed as indicated above.

Invasion of CNS1 glioma cells was assessed using the brain slice invasion assay.
as previously described (Hu et al., 2008). Briefly, coronal brain slices (300 µm thick) were prepared from neonatal wild type or Velvet mice and cultured on Millicell organotypic cell culture inserts (Millipore) on top of Neurobasal-A:HBSS: high-glucose DMEM (50:25:25) containing 1 mM sodium pyruvate, 2 µM glutamine, 5 mM HEPES, and the supplements B27 and G5 (Invitrogen). CNS1 cells stably transduced to express Green Fluorescent Protein were transfected with brevican B50 or control cDNAs and the next day transferred to agar-coated plates. Cells were allowed to form aggregated spheres for 48h, which were manually seeded on the tissue slices. Cell dispersion was analyzed by fluorescence microscopy and the area occupied by dispersed cells over time was quantified using ImageJ.

4.2.5. Tumor xenografts, immunohistochemistry and tumor invasion

All animal experiments were approved by the Institutional Animal Care and Use Committee at The Ohio State University. GL261 or Mut4 were stably transduced with lentiviruses carrying GFP and RFP, respectively, to detect the cells intracranially. Tumor cells were resuspended at 50,000 cells/ul in HBSS and implanted stereotactically in the right striatum according to standard procedures (coordinates: 1 mm frontal from bregma / 2 mm right lateral from bregma / 3.4 mm deep from skull border).

After three weeks, animals were perfused with PBS followed by buffered 4% paraformaldehyde and the brains were dissected and prepared for cryosectioning. Brains were cryosectioned the coronal plane at 20 µm. Tumor sections were imaged by fluorescence microscopy and serial sections were measured by a researcher blinded to the experimental conditions. Total tumor volumes were calculated using a modification of
Cavalieri's estimation procedure (Rosen and Harry, 1990).

To quantify tumor dispersion, tumor sections were imaged and thresholded to extract binary (black and white) information. The core of the tumor was identified using ImageJ and a variation of Sholl analysis was applied to estimate tumor dispersion. Briefly, concentric circles were drawn from the core and the proportion of total tumor cells enclosed by circles of increasing radius was quantified using ImageJ. Tumor invasiveness was assessed as a function of total amount of dispersed tumor detected as a function of the distance to the core. This approach has been used previously in similar analyses of glioma invasion (Hu et al., 2009; Zhang et al., 1998). In an alternative model, a "dual-color" tumor was injected by combining poorly-invasive GFP:GL261 (80% cells) and highly-invasive RFP:Mut4 (20% cells). Dispersion of Mut4 away from the core of GL261 was calculated using the same imaging procedure.

Tissue sections processed for immunohistochemistry were rehydrated and probed with antibodies against GFAP and the basal lamina proteins laminin-B and collagen-IV. Histochemistry was performed in collaboration with Dr. Lyn Jakeman (Dept. Physiology and Cell Biology, The Ohio State University).

4.2.6. Statistics

All biochemical and cell culture experiments were performed at least in triplicate with three independent replicates (up to eight independent neurospheres were analyzed in brain slice invasion assays). Tumor models were analyzed with N=4-7 per condition. Quantitative results were analyzed by Student's t-test or ANOVA followed by post-hoc Boferroni's test based on the experimental design. In all cases a value of p<0.05 was
accepted to indicate statistically significant differences.

4.3. Results

4.3.1. Velvet astrocytes lack EGFR activity but do not exhibit compensatory ErbB expression

Because this is the first time that the Velvet model was used to analyze neural-derived cells, we first characterized the astroglial cultures obtained from neonatal Velvet mice. Astroglial cultures were prepared following standard procedures and yielded a cell population highly enriched in Nestin-positive and GFAP-positive cells (Figure 4.1). No differences were observed between cultures derived from wild-type and Velvet mice regarding cell density or the proportion of GFAP-positive and Nestin-positive cells in the cultures. However, cultures from Velvet mice took longer to become established and reach confluence.

Next, we evaluated the expression of EGFR and phosphor-EGFR in these cultures following serum depletion (Figure 4.2) The phosphorylated form of EGFR, pTyr\textsuperscript{1068}-EGFR, which reflects ligand-dependent activation, was absent in Velvet astrocytes and could not be increased in presence of TGF-alpha. Basal levels of pTyr\textsuperscript{1173}-EGFR, which reflects total EGFR activation, were similar in wild-type and Velvet cultures but there was no increase in Velvet cells after exposure to TGF-alpha. Together, this suggested an almost complete activity of EGFR in these cells. In agreement, signaling molecules downstream of EGFR, such as Akt and MAPK were also poorly or not responsive to TGF-alpha in Velvet cells (Figure 4.2).

Because these results suggested almost complete suppression of EGFR signaling
in Velvet cells, we analyzed if other members of the ErbB family could be upregulated to compensate for lack of EGFR (Figure 4.3). Results from qRT-PCR comparing each ErbB receptor to EGFR showed that relative expression of ErbB2 (Neu), ErbB3, and ErbB4 was similar in Velvet and wild-type cells in normal growth conditions (Figure 4.3A). Under starvation, there was only a small increase in ErbB2 both in wild-type and Velvet cells (Figure 4.3B). Therefore, the expression pattern of the ErbB receptors did not seem different in either cell type. When the expression of Velvet receptors was normalized to wild-type we observed that total ErbB1 and ErbB2 mRNAs were expressed at comparable levels in both genotypes. Only ErbB3 and ErbB4 were significantly lower in Velvet cells in both growth and starvation conditions (Figure 4.3C-D). Overall, this suggested that there was no evident increase in the expression of other Velvet ErbB receptors that could compensate for lack of EGFR activity. It is not impossible, however, that other RTKs may have exhibited different regulation in Velvet cells. For example, expression of total PDGFRA followed very different patterns under growth and starvation conditions in Velvet cells compared to wild-type.

4.3.2. Velvet astrocytes do not show increased growth or viability in presence of an EGFR ligand

Because EGFR signaling is critical for cell proliferation and survival both in glioma (Huang et al., 2009a) and glial cells (White et al., 2011), we next investigated if lack of EGFR activity affected growth or viability of Velvet astrocytes. To this end, we cultured wild-type and Velvet astrocytes under starvation conditions to prevent activation of other RTK pathways, in presence of TGF-alpha or its vehicle for up to five days.
The results demonstrated that wild-type astrocytes were highly sensitive to the EGFR ligand and showed increased cell growth (Figure 4.4A), as observed using a sulforhodamine-binding assays that measures the increase in cell biomass (Vajkoczy et al., 2000). Similarly, these cells showed enhanced viability in presence of TGF-alpha, as measured by reduction of a tetrazolium compound (Figure 4.4B). This enhanced viability was nevertheless lost after five days due to the over-stressing culture conditions of primary cells without any other growth factors or serum.

In contrast, Velvet astrocytes were completely insensitive to TGF-alpha, in agreement with our previous molecular results. This observation agreed with previous work showing that blockade of EGFR activation caused the inhibition of cell cycle progression (Yang et al., 2011). Overall, our results suggested that, in Velvet astrocytes, EGFR signaling is effectively eliminated as suggested in the literature (Du et al., 2004).

4.3.3. EGFR-dependent motility of Velvet astrocytes is significantly impaired.

Our next experiment evaluated the motility of Velvet astrocytes compared to wild-type cells, as well as the dependence on EGFR for cell motility. Astrocytes have slow but measurable rates of cell "stretching" and migration and their motility in vitro is best assessed using the conventional "scratch assay" or "gap assay" (White et al., 2011).

We first evaluated cell motility in presence of full growth medium containing serum and growth factors. In these conditions, both wild-type and Velvet astrocytes exhibited high motility and the cells were able to cover the gap in less than 48h. More importantly, there were no significant differences between the genotypes (Figure 4.5A-B).

In absence of serum, wild-type astrocytes showed no motility in basal conditions
but were still able to move and close the gap completely within 5-6 days in presence of TGF-alpha (Figure 4.5C-D). In contrast, Velvet astrocytes not only did not move but also did not respond to TGF-alpha. These results showed that EGFR signaling was critical to promote astroglial cell motility in starvation conditions.

Cell migration in two-dimensions in vitro is essentially dependent on the formation of adhesion complexes and the mechanisms of cytoskeletal rearrangement that permit cell crawling. Therefore, we hypothesized that the deficiency in migration of Velvet astrocytes could be due to either or both of those processes being disrupted. Because Velvet astrocytes exhibited a less "stretched-out" (flat) morphology (Figure 4.6A), we investigated if the molecular mechanisms that regulate actin polymerization and turnover would be involved in the observed deficiency in cell motility. Specifically, we analyzed the expression and activation of the small protein VASP (vasodilator-stimulated phosphoprotein). VASP regulates the polymerization of actin (Harbeck et al., 2000) and phosphor-Ser\(^{157}\)-VASP is known to localize preferably at focal adhesion points (Benz et al., 2009). As expected, we found that the activating phosphorylation of VASP in Ser\(^{157}\) was significantly reduced in Velvet astrocytes, compared to wild-type cells in presence of TGF-alpha (Figure 4.6B), suggesting that lack of EGFR activation in these cells results in a subsequent deficit in their ability to regulate cell motility.

4.3.4. Velvet astrocytes show reduced attraction towards glioma cells

The mechanisms by which gliomas invade over neighboring normal tissue may involve tumor cell-specific mechanisms as well as mechanisms by which signals from the tumor cells activate and recruit normal cells in the stroma (Cirri and Chiarugi, 2011). In
this regard, previous work has shown that glioma cells in vitro may induce the production of metalloproteases in astrocytes, which could be necessary to promote tumor invasion (Le et al., 2003). Since local hyperplasia and migration are typical features of activated astrocytes being recruited to sites of injury and inflammation, we next asked whether Velvet astrocytes would be attracted towards glioma cells in a manner different to wild-type astrocytes.

To test this hypothesis, we took advantage of pre-formed gap-assay inserts (Ibidi LLC) that permit the seeding of two different cell types separated by a gap of specific width (400 µm). Using these culture inserts we co-cultured astrocytes and glioma cells GL261 (derived from C57Bl/6 mice). As expected, glioma cells grew faster than astrocytes and reached an over-confluent layer with little motility. This facilitated our detection of astrocyte motility towards the side with glioma cells.

Analysis of astrocyte migration under starvation conditions showed that wild-type astrocytes were strongly attracted towards GL261 cells even in absence of growth factors, and this effect was significantly enhanced by TGF-alpha. Conversely, Velvet astrocytes showed very little migration towards glioma cells during the first three days, and this effect was not modified by incubation of the cells with TGF-alpha (Figure 4.7A-B). Overall, this suggested that EGFR signaling was also necessary in astrocytes to respond to signals secreted by glioma cells.

4.3.5. The tumor microenvironment of Velvet mice does not affect the intrinsic growth of malignant glioma

The results above suggested an evident lack of response of EGFR-deficient
astrocytes towards syngeneic glioma cells. Therefore, we next asked if GL261-derived tumors would grow differently in wild-type versus Velvet mice.

To test this hypothesis, we implanted GFP-expressing GL261 cells intracranially to allow development of orthotopic gliomas. After three weeks, mice were euthanized and their brains perfused, sectioned, and stained for multiple antigens and for tumor morphometric reconstruction.

Interestingly, visualization of the resulting GL261-derived tumors and volumetric reconstruction (Figure 4.8A-B) suggested that the total volume of these tumors in Velvet animals was not significantly different from those tumors in wild-type controls. Immunohistochemistry for GFAP (Figure 4.8C) suggested a comparable amount of astroglial response around the tumor border in both genotypes. In addition, immunohistochemistry for two markers of blood vessel basal lamina (collagen-IV and laminin-B) also showed comparable expression in tumors developed in wild-type or Velvet mice (Figure 4.9), suggesting similar tumor vascularization. Overall, these results suggested that the response from the microenvironment to intrinsic tumor growth had not been different despite the EGFR deficiency in Velvet brains.

4.3.6. The tumor microenvironment of Velvet mice reduces tumor invasion

The results above suggested that total tumor growth in the GL261 model was essentially independent of EGFR signaling in the tumor microenvironment. Because serum-grown tumor cell lines have become adapted to conditions that promote proliferation and may not reproduce the complex phenotype of malignant gliomas, including their invasive ability, we next tested a highly invasive cell line to investigate
the influence of the tumor microenvironment on tumor invasion.

Using Mut4 glioma initiating cells we repeated the orthotopic tumor model and analyzed the resulting tumors after three weeks. These tumors were much smaller than GL261-derived tumors because of their slower rate of growth, but exhibited a core and considerable dispersion of tumor cells and formation of tumor "islets" away from the core (Zhang et al., 1998) (Figure 4.10A). Quantification of tumor dispersion showed that Mut4 cells and islets were less dispersed from the core in Velvet animals compared to wild-type animals (Figure 4.10B), suggesting that the Velvet microenvironment had affected tumor dispersion.

To validate these initial results we used a second model of tumor invasion where two cell types (one invasive and one non-invasive) are injected together and the dispersion of the invasive cells is visualized compared to the core of non-invasive cells (Dr. Sean Lawler, Dept. Neurosurgery, personal communication about methodology). When the combination of RFP:Mut4 and GFP:GL261 was coinjected and analyzed we observed that Mut4 dispersed farther away from the GL261 tumor in wild-type animals than in Velvet animals (Figure 4.11). These results confirmed the initial test with Mut4 cells alone.

Previous work has shown that glioma cells can regulate the activity of proteases in astrocytes, which could help glioma invasion (Le et al., 2003). Therefore, we studied the expression of the metalloprotease MMP2 in cultures of astroglial cells treated with conditioned medium from human U251 glioma cells. Our results (Figure 4.12) indicate that total MMP2 expression was significantly lower in Velvet astrocytes. Although expression of MMP2 mRNA increased when astrocytes were cultured with the medium
from glioma cells, the total expression in Velvet astrocytes was 2-fold lower compared to the expression in wild-type cells, which could explain in part the reduced invasion observed in the intracranial tumors.

4.3.7. B50 cannot enhance glioma cell invasion through Velvet brain tissue

Finally, we wanted to explore the potential role of brevican in the Velvet model. Because brevican is secreted by glioma cells and activates EGFR in glioma cells and astrocytes (Figure 3.1 and 3.5) we tested whether brevican's pro-invasive ability would remain when the tumor microenvironment was deficient in EGFR activity.

To this end, we tried to generate Mut4 cells expressing the bioactive fragment of brevican B50 using lentiviral transduction, but observed very poor transduction efficiency and maintenance of B50 expression over time. Therefore we switched to rat glioblastoma CNS-1 cells. This is a very well-characterized invasive model where the pro-invasive effects of brevican and B50 have been extensively described (Hu et al., 2008; Nutt et al., 2001; Viapiano et al., 2008).

Because CNS-1 cells are not syngeneic with Velvet mice we could not perform intracranial injections and used a brain-slice invasion assay instead. This is a model extensively validated by several laboratories (Hu et al., 2009; Palfi et al., 2004) to study glioma cell invasion under conditions that accurately reproduce the natural barriers to cell motility in the brain. Using this model, we observed that CNS-1 cells expressing B50 showed significantly higher invasion over control cells seeded on wild-type brain slices (Figure 4.13), as expected. In stark contrast, the effect of B50 disappeared when the cells
were seeded under the same conditions on Velvet brain slices. These results strongly suggested that the pro-invasive effects of brevican were not limited to activating EGFR in glioma cells but extended to cellular components of the tumor microenvironment that were necessary for glioma invasion.

4.4. Discussion

As in any other tumors, gliomas are surrounded with multiple cell types that form the different cellular components of the tumor microenvironment (TME). However, the TME of gliomas show some unique features compared to the TME of other epithelial tumors, such as the overall absence of fibroblastic stromal cells and the presence of multiple cell types from the glial lineage (e.g., astrocytes, oligodendrocytes, and their different precursor cells). In addition, the ECM of the CNS exhibits unique features that set it apart from the stromal matrix that is transversed by other epithelial tumors. Most notably, the absence of fibrillar proteins that promote adhesion in the neural ECM suggests that gliomas may use different mechanisms of tumor invasion (Beadle et al., 2008; Wolf and Friedl, 2011) compared to other tumors even when the intracellular signaling pathways are the same.

Studies focused on the role of the neural TME for glioma growth and progression are still in their infancy and have largely focused on the influence of immune cells. For example, multiple studies from the Kettenmann group have shown that microglia promotes tumor cell invasion and overall upregulation of the protease MMP2 in the tumor (Markovic et al., 2005; Markovic et al., 2009; Sliwa et al., 2007). Studies of
astrocytes have been less common, but Le et al. demonstrated that astroglia co-cultured with glioma cells showed increased expression and activity of urokinase-type plasminogen activator and MMP2, promoting glioma invasion in an in vitro Matrigel invasion assay (Le et al., 2003).

Because astrocytes are the major source of brevican in normal brain (Viapiano and Matthews, 2006) and cleaved brevican can activate EGFR signaling in these cells as well as in glioma cells, we wanted to analyze if the influence of brevican on tumor dispersion was autonomous to the tumor or extended to the TME. To this end, we characterized in this chapter the Velvet mouse, a recent spontaneous genetic model that is deficient in EGFR activity (Du et al., 2004). This is a lethal mutation when homozygous and even heterozygous animals show an almost complete absence of phosphorylated EGFR in their tissues (Du et al., 2004).

Overall, our results using cultured astrocytes derived from these animals showed that EGFR activation was critical for cell proliferation, viability and migration in absence of other growth factors. Although the key role of EGFR signaling in cell proliferation is well known, the mechanisms triggered by EGFR that regulate cell motility are less understood and could be different in different tumor models. For example, EGFR is known to stimulate migration in keratinocytes through phosphorylation of STAT1 and triggering of JAK/STAT signaling (Andl et al., 2004). However, the major pathway that appears triggered by EGFR in glioma leading to increased cell migration is the MAPK pathway (Hu et al., 2008), which in turn promotes fibronectin secretion and adhesion to the ECM.

Independently of the mechanisms involved, it is interesting to note that EGF is
known to increase cell migration by promoting actin dynamics and formation of lamellipodia (Harms et al., 2005). In this regard, it is particularly relevant that the small actin regulator VASP was significantly less active in Velvet astrocytes compared to normal cells in response to TGF-alpha. Because Ser\textsuperscript{157} is the major phosphorylation site of VASP by PKA (Smolenski et al., 1998), lack of VASP activation at that site may provide a new insight on the molecular mechanisms triggered by EGFR that regulate astroglial motility and potential recruitment to tumor growth sites.

Despite the observed differences between wild-type and Velvet astrocytes cultured \textit{in vitro} in absence of growth factors, the phenotypic differences essentially disappeared in full growth medium, suggesting that there are compensation mechanisms that attenuate the Velvet phenotype and obviously permit the survival of the heterozygous animals. These compensation mechanisms may have supported normal tumor growth when GL261 were implanted in Velvet animals, and explained the absence of differences in total tumor volume, vascularization, astroglial response, and even expression of some markers of ECM response to injury (e.g., neurocan, not shown).

While tumor growth is largely driven by the proliferative activity of the tumor cells and is largely limited by the lack of nutrient supply in the TME, tumor invasion and metastasis are highly dependent on the interactions between tumor cells, stromal cells, and ECM, resulting in effective cell adhesion, motility and/or degradation of ECM barriers (Wolf and Friedl, 2011). For example, recent evidence has shown that tumor cell invasion is in many cases guided by macrophages, which facilitate invasion through the ECM even when MMP expression is abolished in the tumor cells (Guiet et al., 2011). The significantly reduced dispersion of glioma cells through Velvet brain compared to wild-
type brain strongly suggests that specific cell types in the Velvet TME were unable to respond to pro-invasive signals from the tumor cells and therefore did not help tumor invasion.

In this chapter, we did not pursue the identity of the TME cell type that was likely promoting tumor invasion through normal brain, although previous evidence from several laboratories has attributed important roles to both microglia and astroglia. We propose that one or both of these cell types likely responds to paracrine signals from tumor cells either secreting ECM proteins to favor tumor adhesion or proteases that promote tumor invasion. In agreement, previous work has shown that glioma cells produce TGF-alpha that may activate EGFR in astroglia, while ErbB2 and ErbB3 are non-responsive to glioma cells (Hoelzinger et al., 2007).

Finally, it is particularly interesting to observe that the pro-invasive effect of B50 was lost when glioma cells were cultured on Velvet brain slices. As previously demonstrated in the literature and elsewhere in this dissertation (chapters 2 and 3), the bioactive fragment of brevican B50 can increase glioma cell adhesion and migration in an autonomous manner, through a mechanism that involves EGFR activation in glioma cells. However, glioma cells must effectively invade brain tissue slices in order to disperse, suggesting that the pro-invasive effects of brevican could be a result of activating mechanisms not in glioma cells but in TME cells. Lack of EGFR activity in normal cell types in the brain slice would therefore explain the lack of additional factors secreted by those cells in response to brevican and necessary for the enhanced invasion of brevican-expressing glioma cells.

Interestingly, we showed in chapter 3 that brevican is able to trans-activate EGFR
in cultured Velvet astrocytes via Src-kinase (Figure 3.5). This makes more difficult to explain how brevican may have lost its effect, since it would be expected that it could activate EGFR both in normal and Velvet cells in the slice tissue. However, although we have characterized the sulfatide-Src-EGFR pathway in glioma cells (and partially in astrocytes), we do not know if that is effectively the pathway activated by brevican in the TME. Velvet cells may lack activity in a different pathway also sensitive to brevican (as we discuss in chapter 5 below) and necessary to promote invasion of glioma cells. Moreover, not all cell types survive equally well on dissected brain slices. While microglia, astrocytes and neonatal neurons can survive culture in brain slices for several days, other cell types (most notably endothelium, and oligodendroglial cells) could have been particularly affected in brain slices from Velvet mice, thus resulting in different cellular compositions in wild-type and Velvet slices that could underlie the lack of effect of brevican.

In sum, results in this chapter described for the first time a novel model of EGFR deficiency in the neural TME and show that EGFR activity in brain tissue is critical to promote invasion of malignant glioma cells. More importantly, the pro-invasive effects of brevican that have been described in multiple occasions appear critically dependent on effects of B50 not on glioma cells but in the neural TME. These results highlight the multiple roles of brevican in promoting tumor invasion (both in glioma and normal cells) and underscore the relevance of targeting this proteoglycan for future anti-invasive strategies.
Figure 4.1  *Immunohistochemical characterization of astroglial cultures*. Astroglial cultures derived from wild-type or Velvet mice were prepared as indicated in the methods section and processed for ICC when they reached approximately 30% confluence (cultures were processed before their third passage). Results show a high enrichment in GFAP- and Nestin-positive cells in both cases, confirming that the cultures were enriched in cells of the astrocytic lineage.
**Figure 4.2** EGFR activity is almost completely abolished in Velvet astrocytes. Wild-type and Velvet astrocyte cultures were starved for 24h to reduce RTK activity to basal levels, followed by treatment with TGF-alpha (10 ng/ml) for 20 minutes. Samples were immediately harvested, lysed in presence of phosphatase inhibitors and processed for Western blotting. Results show that, although total levels of EGFR were not affected the ligand-dependent (Tyr\(^{1068}\)) and total (Tyr\(^{1173}\)) activation of the receptor were significantly downregulated. In agreement, downstream pathways triggered by EGFR (in particular Akt) were less or no sensitive to TGF-alpha in Velvet cultures.
Figure 4.3 Expression of EGFR is not compensated by other ErbB receptors in Velvet astrocytes. 
A-B) Expression of ErbB receptors was quantified by qRT-PCR and shown in comparison to EGFR for each cell type. Results of cells grown in complete medium (A) or starvation conditions (B) indicate that the expression profile of ErbB receptors was essentially the same in Velvet and wild-type cells. 
C-D) Same results as (A-B) but each ErbB receptor in Velvet astrocytes is shown in comparison to the expression levels of the same receptor in wild-type cells. Results of cells grown in complete medium (C) or starvation conditions (D) indicate that total EGFR mRNA did not change in Velvet astrocytes while other ErbB receptors were downregulated compared to wild-type cells. These results suggest that downregulation of EGFR activation is probably not compensated by overexpression of other ErbB receptors. However, expression profiles of other RTKs differed considerably between Velvet and wild-type cells. For example, PDGFRα mRNA expression was reduced in Velvet astrocytes compared to wild-type cells cultured in complete medium, but showed a significant increase in starvation conditions. Results were analyzed by Student's t-test for each receptor (* p<0.05; ** p<0.01; *** p<0.001)
Figure 4.4 Velvet astrocytes show reduced proliferation and viability even in presence of TGF-alpha. A) Cells were plated on 96-well plates and cultured in starvation conditions in presence of TGF-alpha (20 ng/ml, added on day 0) or its vehicle. Total cell proliferation measured by sulforhodamine staining showed that Velvet cells did not respond to TGF-alpha stimulation until the third day and the magnitude of this response was much smaller than for wild-type cells (# p<0.05; ** p<0.01; *** p<0.001, by two-way ANOVA. Post-hoc tests compared proliferation for each cell type treated with TGF-alpha versus vehicle). B) The same cultures as in (A) were run in parallel and analyzed for viability using a the MTS-reduction (redox) reaction. Results show that Velvet cells were again much less responsive to TGF-alpha compared to wild-type astrocytes (# p<0.05; ** p<0.01; *** p<0.001, by two-way ANOVA. Post-hoc tests compared viability for each cell type treated with TGF-alpha versus vehicle). Loss of viability at five days was an expected result of culturing primary cells in starvation conditions for so long.
Figure 4.5 *EGFR activity is sufficient to enhance the migration of astrocytes in absence of other growth factors.* Wild-type and Velvet astrocytes were cultured in cell-culture inserts to form "gaps" and the gap closure was measured after removal of the inserts, using imaging software. A-B) Quantitative results (A) and representative images (B) of cell migration in complete growth medium indicate that both cell types migrated in the same manner, suggesting that lack of EGFR can be compensated by other migratory mechanisms in Velvet cells. C-D) In starvation conditions, both cell types showed almost absent migration (C). However, migration of wild-type cells was rescued by activation of EGFR using TGF-alpha (20 ng/ml), indicating that this receptor is sufficient to promote astrocyte motility. As expected, Velvet astrocytes were not responsive to stimulation with TGF-alpha. D) Representative image of cells under starvation conditions treated with TGF-alpha. Notice the polarization of wild-type astrocytes crossing the gap and their different morphology compared to the same cells in complete growth medium. (**p < 0.001 by two-way ANOVA)
Figure 4.6 Velvet astrocytes show reduced formation of lamellipodia and activation of VASP. A) Representative image of the expanding border of a monolayer of astrocytes after 48h of starvation and treatment with TGF-alpha (20 ng/ml). Notice the absence of stretched out or flattened cells in Velvet cultures. B) The protein VASP regulates actin polymerization and formation of lamellipodia. Astrocyte cultures starved and treated with TGF-alpha were collected and processed to detect activation of VASP. Results indicated that Velvet astrocytes have a very reduced phosphorylation of VASP in Ser\textsuperscript{157} (isoform associated to focal adhesions) compared to wild-type cells.
Figure 4.7 Velvet astrocytes show reduced attraction towards glioma cells. A) Astrocytes and GL261 glioma cells were cultured in opposite sides of cell-culture inserts and the migration of astrocytes (gap closure) was quantified after removal of the inserts. Results of cells cultured in starvation conditions show that wild-type astrocytes are rapidly attracted towards GL261 cells in a manner that is enhanced by TGF-alpha (20 ng/ml). In contrast, Velvet astrocytes show much slower migration towards glioma cells that is not modified by TGF-alpha during at least the first 72h of the cultures. Results at 120h are likely a combination of total migration and proliferation of both cell types in the cultures (** p<0.01; *** p<0.001 by two-way ANOVA). B) Representative image of astrocyte-glioma co-cultures in growing in starvation conditions in presence of TGF-alpha. Notice the much higher density and restricted motility of GL261 cells, as well as the lack of attraction of Velvet astrocytes towards these cells.
Figure 4.8 Total growth of non-invasive gliomas is not different between wild-type and Velvet mice. GL261 cells were implanted intracranially and the resulting tumors were processed after three weeks and analyzed by immunohistochemistry or morphometric reconstruction using imaging software. A) Representative images of GFP-expressing GL261-derived tumors showed similar tumor extent in the brain. B) Morphometric reconstruction indicated that the total volume of these tumors was not different between wild-type and Velvet animals. C) Immunohistochemistry for GFAP in adjacent sections suggested that the astroglial response around the tumor was also comparable between wild-type and Velvet animals.
Figure 4.9 Expression of basal-lamina antigens is comparable in tumors developed in wild-type and Velvet animals. GL261 tumor sections (shown in Figure 4.8) were processed for immunohistochemistry with antibodies against the basal-lamina antigens collagen-IV and laminin-B, which identify basal lamina of blood vessels in the brain. Results from IHC suggested that there were no evident differences in intensity or distribution of these antigens, suggesting that the vascularization was likely similar in GL261-derived tumors developed in wild-type versus Velvet animals (IHC procedures and analysis were performed in collaboration with Dr. Lyn Jakeman, Dept. Physiology and Cell Biology, OSU)
Figure 4.10 Invasive tumors show reduced dispersion in Velvet tissue. RFP-expressing Mut4 cells were implanted intracranially in wild-type or Velvet animals and allowed to develop tumors during three weeks. Tumors were processed for histology in a similar manner as GL261 tumors shown in previous figures. Tumor dispersion was analyzed using a variation of Sholl analysis to quantify the proportion of tumor enclosed at different distances from the core. A) Representative images of Mut4 tumors, their B&W images generated with imaging software and the concentric circles expanding from the identified tumor core (only the initial fitting circle is shown for the Velvet tumor). B) Quantitative analysis showed that tumors developed in Velvet animals reached full enclosure faster than in wild-type littermates (* p<0.05 by two-way ANOVA) suggesting a decrease in total dispersion.
Figure 4.11 Tumor invasion away from a compact core is reduced in Velvet mice. RFP-expressing Mut4 cells (invasive, 20%) combined with GFP-expressing GL261 cells (core, 80%) were injected intracranially and the resulting tumors were processed and analyzed after two weeks as shown in Figure 4.10 (using the GFP core to calculate dispersion of RFP cells). Results confirmed that most invasive cells (Mut4) were found at shorter distances from the core in Velvet tissue compared to wild-type, suggesting that the Velvet TME was less conducive to tumor invasion (*p<0.05, p<0.01 by two-way ANOVA).
Figure 4.12 Expression of MMP2 is highly reduced in Velvet astrocytes. Wild-type and Velvet astrocytes were incubated alone or in presence of conditioned culture medium from human glioma U251 cells for 24h and then processed for qRT-PCR. Quantitative results showed that expression of total MMP2 mRNA was significantly reduced in Velvet cells compared to wild-type astrocytes. Treatment with U251 medium increased significantly the expression of MMP2 in both cell types but total expression in Velvet astrocytes was still less than half of the expression in wild-type astrocytes (** p<0.01; *** p< 0.001 by two-way ANOVA)
Figure 4.13 The pro-invasive effect of B50 is lost in cells dispersing through Velvet tissue. A) Schematic representation of the procedure to prepare brain slices and culture glioma cell aggregates to measure ex-vivo invasion (Hu et al., 2008). B) B50-expressing CNS1 cells showed increased dispersion in wild-type brain slices compared to control cells, in agreement with previous results from the literature. In contrast, control and B50-expressing CNS1 cells showed the same basal dispersion in Velvet brain slices, suggesting that the pro-invasive effect of brevican disappeared in cells invading through the Velvet microenvironment (* p<0.05; *** p<0.001 by two-way ANOVA for repeated measures)
Chapter 5:
Conclusions and Future directions:

Relevance of brevican for glioma progression and potential therapeutic strategies

5.1 Focusing on brevican

Gliomas are malignant tumors of glial origin that are characterized by local infiltration of brain tissue. Despite this invasive ability, gliomas remain restricted to the CNS and almost never metastasize outside the brain and spinal cord (Pilkington, 1997). In contrast, peripheral tumors that metastasize into the brain almost never invade it diffusely (Subramanian et al., 2002). This perplexing contrast between gliomas (which can invade only the brain) and metastatic tumors (that are invasive but cannot invade the brain) highlights the unique relationship of malignant brain tumors with their microenvironment and strongly suggests that understanding this relationship will help us develop better therapeutic strategies against glioma.

A starting point to understand the interaction of glioma cells with their TME is to understand the composition of this TME and in particular those molecular components that are unique to the CNS. Brevican is the most abundant proteoglycan in the ECM of the CNS and its expression is completely restricted to neural tissue throughout development and adulthood (Jaworski et al., 1994; Jaworski et al., 1995). In addition, this proteoglycan is highly expressed during periods of developmental gliogenesis (Jaworski
et al., 1995) as well as in the white matter, where cycling glial precursors that could be a major source of gliomas have been described (Canoll and Goldman, 2008). Since 1996, brevican has been known to be highly upregulated in gliomas (Jaworski et al., 1996), in particular oligodendrogliomas, and has been used as a characteristic marker of these tumors (Liang et al., 2005; Phillips et al., 2006).

The relationship of brevican with malignant gliomas is not only correlative. Increased expression of brevican in experimental models of glioma has been repeatedly shown to promote tumor progression and reduce animal survival (Nutt et al., 2001; Viapiano et al., 2008). More importantly, further research has shown that overexpression of brevican is not sufficient, but it must be followed by cleavage and release of its N-terminal fragment, B50 (Nutt et al., 2001; Viapiano et al., 2008; Zhang et al., 1998). In agreement, in vitro analysis of brevican in gliomas has shown that the N-terminal, but not the full-length uncleaved protein, is able to promote fibronectin secretion, glioma cell adhesion, and invasion (Hu et al., 2008).

In order to advance our understanding and potential targeting of this unique component of the glioma microenvironment, the goal of this doctoral research project was to further understand the molecular mechanisms of brevican and to characterize its mode(s) of action. Interestingly, although the effects of brevican in brain tumors and neural injury models had been consistently described for the past decade, the known molecular partners of this proteoglycan included a tiny set of molecules (HA, tenascin-R, and cell-membrane sulfatides bound by the C-terminus of the protein) that had not been studied in order to understand brevican's functions. Therefore, this dissertation had to deal largely with characterizing the possible molecules in the neural TME that were
relevant partners underlying the effects of brevican in glioma.

5.2 The molecular partners of brevican in the brain and glioma

Brevican belongs to the lectican family of CSPGs, which are characterized by their binding to HA. Although the specific properties of brevican binding to HA have not been published, several groups have used HA-affinity columns to purify brevican (Yamada et al., 1997). In addition, brevican binds the ECM protein tenascin-R, which in turn binds integrins and anchors cells to the ECM (Yamaguchi, 2000). Furthermore, the C-terminal lectin domain of brevican has been shown to bind a subset of sulfolipids (sulfatides) in a Ca$^{++}$-dependent manner (Miura et al., 1999). Overall, this previous body of work has shown brevican as a pro-adhesive molecule that would promote the association of cells to the matrix and underscore the "structural" role of brevican as one of the major scaffolding proteins in the neural ECM. However, this work has been unable to explain the functional roles of brevican such as its inhibitory effects on axonal extension (Heron et al., 2007) and its promoting effects on glioma cell migration.

Although the inhibitory mechanisms of brevican are unknown, they agree with the overall inhibitory effects of all lecticans in the CNS and are likely to be explained by a same molecular mechanism common to all these proteins in the family. In contrast, the pro-migratory role of brevican in glioma is a "paradoxal" effect that does not seem to be replicated by the closest lectican (versican) that has also been studied in these tumors (Wu et al., 2005). This made us focus first on any possible "unique" molecular interactions of brevican with a binding partner.

The HAPLNs are the canonical binding partners of the lecticans and share a
common evolutionary origin by gene duplication (Spicer et al., 2003). The association of the lecticans and HAPLNs enhances the formation of ternary complexes with HA and could underlie a pro-migratory effect in tumor cells that must navigate the HA-rich neural ECM. Previous evidence has suggested that there is a one-to-one lectican-HAPLN interaction, with HAPLN4 being the putative binding partner of brevican (Bekku et al., 2003), although this association had never been demonstrated. In this dissertation, we investigated the possible association of brevican and HAPLN4 in normal tissue and more importantly in glioma, trying to establish if HAPLN4 would be a molecular partner of brevican in these tumors.

Our results showed that although brevican and HAPLN4 shared a similar developmental profile in normal brain, HAPLN4 was essentially absent in gliomas, which made it an unlikely partner of brevican in the tumor tissue. This prompted us to analyze HAPLN4 as a possible "tumor-suppressor" of brevican, but functional assays showed that overexpression of HAPLN4 enhanced tumor cell adhesion and dispersion in vitro. Moreover, HAPLN4 showed a very different behavior compared to brevican when co-expressed in gliomas, suggesting that they would not even associate in these tumors. Overall, our results increased our understanding of a severely under-studied component of the ECM (HAPLN4) but also indicated that this protein had most likely no relation with the effects of brevican in gliomas.

5.3 EGFR activation as the key pro-migratory trigger of brevican

Previous work has shown that brevican is normally cleaved by ADAMTS-4/5 at a specific site, releasing the fragments B50 (N-terminal) and B90 (C-terminal) which can
be detected in rodent brain throughout development (Viapiano et al., 2003). In human brain, the expression of these fragments is very reduced compared to rodents. However, the cleavage fragments are significantly upregulated in human gliomas, reflecting overall cleavage of brevican by ADAMTS (Viapiano et al., 2005). More importantly, cleavage of brevican is critical for the role of this proteoglycan in glioma because uncleaved brevican cannot promote tumor growth or invasion and is essentially silent (Viapiano et al., 2008). Recent evidence from the Viapiano lab investigated this effect at the molecular level, showing that B50 was able to activate EGFR, leading to increased fibronectin secretion and adhesion of glioma cells. However, a major question that remained unanswered was: how was brevican able to trigger EGFR?

Initial work during this dissertation dedicated considerable time to trying to find a membrane protein that could explain the connection between B50 and EGFR. Direct association of B50 with EGFR was almost immediately discarded after several co-precipitation and other protein bindings assays. We next attempted to identify any other potential binding partners using protein crosslinking, co-immunoprecipitations, and even full mass-spectrometry of whole mixtures of co-IP proteins. Unfortunately, we were unable to find any relevant partner of B50 because the protein seemed essentially soluble. In fact, previous binding experiments from the Viapiano lab suggested that, while full-length brevican could easily bind to cell membranes, the association of B50 was much weaker and/or transient.

As we have described in chapter 3 of this dissertation, B50 has a unique mechanism of activation of EGFR that involves binding to cell-surface sulfatides and triggering EGFR transactivation via Src-kinase. This mechanism is particularly
interesting since B50 can trigger it while full-length brevican does not. This suggests the possible exposure of a neo-epitope in B50 that is somehow hidden in the full-length molecule. Interestingly, the sequence immediately upstream of the ADAMTS cleavage site is a short ~40-aa long coiled-coil that extends between a cleavage site by MMPs (Ala$^{361}$-Ile$^{362}$) and the ADAMTS cleavage site (Glu$^{400}$-Ser$^{401}$). This region offers high flexibility to the "head" of brevican and could be hidden or folded in the full-length molecule. In fact, a similar region in the lectican aggrecan serves as a hinge allowing the folding of the N-terminal domain of aggrecan over the C-terminal domain for proper secretion (Zheng et al., 1998). It is very likely that this region contains the triggering sequence that binds the sulfatides since a shorter version of B50 lacking this sequence does not trigger EGFR phosphorylation (recent unpublished result, in process).

Our discovery that brevican (a CNS-specific protein) can trans-activate EGFR via sulfatides (which are particularly enriched in the CNS) is of special relevance because it suggests a CNS-specific mechanism by which this pathway can be triggered not only in glioma cells but also normal neural cells. Indeed, upregulation of EGFR activity following neural injury is known to mediate the inhibitory role of the lecticans against axonal regeneration (Koprivica et al., 2005), by poorly understood mechanisms. We suggest that the mechanisms described in chapter 3 of this dissertation may explain, at least, in part, the effect of CSPGs on EGFR activation and axonal inhibition. In agreement, sulfatides have been recently identified as major components in myelin that prevent axon regeneration (Winzeler et al., 2011). Based on our identification of the sulfatide-Src kinase-EGFR signaling axis activated by B50 we propose that Src inhibitors could therefore inhibit this pathway and attenuate the axon inhibitory effects following
neural injury. Given the relevance of novel experimental Src-kinase inhibition therapies in glioma (Ahluwalia et al., 2010), this is a promising field for the development of novel pharmacological therapies in the near future.

5. 4 Brevican requires EGFR in the tumor microenvironment to promote glioma invasion

The recognition that the environment of a tumor is critical for the development and progression of the malignancy dates back to Dr. Stephen Paget's work in the nineteen century and his "seed and soil" hypothesis that recognized that cancer cells develop differently in different microenvironments (Fidler, 2003). In the field of brain cancer, the study of the TME has been hampered by attempts to mimic the neural TME with the TME from other solid tumors and by the lack of sufficient recognition of key neural components such as the different glial lineages and the structure of the neural ECM. Nevertheless, some authors have recognized the importance of the unique structure of the neural tissue and its micro-heterogeneity in promoting glioma growth (Bellail et al., 2004).

In this dissertation, we have focused largely on the molecular mechanisms and role of brevican in promoting glioma dispersion. Because the bioactive fragment of brevican, B50, is produced extracellularly and is therefore a diffusible factor, we hypothesized that B50 could play an important role not only by activating EGFR signaling in glioma cells but also by triggering this pathway in brain cells. The availability of an EGFR-deficient model through our recent collaboration with Dr. Lyn Jakeman at OSU presented an excellent opportunity to test this hypothesis.
In the results described in chapter 4 of the dissertation, we analyzed for the first time the cellular and molecular mechanisms present in cultured astrocytes from Velvet mice. As indicated in that chapter, EGFR was critical for the growth, viability, and motility of those cells in vitro. More importantly, lack of EGFR reduced the ability of Velvet astrocytes to target glioma cells in vitro and resulted in reduced levels of MMP2 expressed in astrocytes co-cultured with glioma cells.

Despite these interesting results in vitro, experiments in vivo showed that lack of EGFR in the TME would not affect tumor development. This result suggested that tumor development is largely autonomous from EGFR signaling in the brain, which suggests that any future anti-EGFR therapeutic strategies should emphasize localized targeting of EGFR and development of resistance in the tumor and not focus in the TME as a potential anti-tumoral pharmacological target for this pathway.

Nevertheless, the most interesting result was the observation that tumor invasion, which results from the interplay between the tumor cells and the cellular and acellular components of the TME, was significantly reduced in the Velvet microenvironment. Moreover, the pro-invasive effect of brevican was abolished when Velvet brain slices were used to measure invasion, suggesting that the pro-adhesive and pro-migratory effects of B50 (chapter 2 and 3) may be glioma cell-autonomous but its pro-invasive effects require a target in the neural TME.

These results are particularly relevant from a future therapeutic standpoint. As previously described in this dissertation, tumor invasion is both a hallmark of gliomas and their most challenging aspect that makes them essentially incurable over the long term (Louis, 2006). Results from this dissertation suggest that the TME may be a novel
source of molecular targets to reduce or prevent invasion, and global targeting of the TME could provide a future paradigm for anti-invasive therapies.

5.5 Other signaling pathways triggered by brevican? The case for PDGFR

The studies in this dissertation have largely focused on the molecular mechanisms of brevican that explain its promoting effects in gliomas. Although the EGFR pathway emerges as a central target of brevican, our results in chapter 4 raise the possibility that other pathways, specially in the TME, could also be triggered by the bioactive fragment of this lectican.

In this regard, it is interesting to notice that brevican is predominantly upregulated in oligodendrogliomas (Figure 2.2) and is produced by cells of the oligodendroglial lineage during early development (Jaworski et al., 1995). Because one of the major pathways regulating oligodendrocyte growth is the PDGFR pathway (Fruttiger et al., 1999), and because PDGFR is also one of the major RTK pathways upregulated in gliomas (Ozawa et al., 2010), we have initiated preliminary investigations on PDGFR as a future development of our research on brevican in gliomas.

Using cultures of glioblastoma cells transfected with brevican or B50 fragment, we observed that both molecules were in fact able to activate PDGFRA (Figure 5.1A). Several binding and co-IP experiments allowed us to rule out the direct interaction of B50 with PDGFRA or PDGFRB; however, we detected a novel interaction: a direct association of B50 with the PDGFR ligand PDGFaa (Figure 5.1B). This is particularly interesting because CSPGs are usually used as negative proteoglycan controls demonstrating that they do not bind growth factors compared to HSPGs (Taipale and

3 This work was awarded a two-year Pelotonia Graduate Research Fellowship, 2010-2012
Keski-Oja, 1997). Again, the use of B50 instead of the full proteoglycan may be uncovering here a novel type of molecular interaction.

In addition, using cultures of immortalized oligodendrocyte precursor cells (OPCs) we demonstrated that exogenous brevican is able to activate PDGFRA in these cells in a time-dependent manner (compatible with ADAMTS cleavage, Figure 5.1C). Finally, we have recently implanted intracranial tumors overexpressing B50 and observed that those tumors had a more diffuse border compared to controls and were surrounded by a much higher number of NG2-positive putative OPCs (Figure 5.1D).

Taken together, these initial results underscore the potential signaling complexity of brevican and highlight how this protein may trigger multiple pathways both in glioma cells and the TME. These studies are a beginning of new research projects that will continue unravelling the role of brevican in the normal CNS and in neuropathologies.

5.6 Conclusions: Brevican as a therapeutic target in the tumor microenvironment

Since the first detection of CNS-specific brevican in glioma (Jaworski et al., 1996) there has been considerable interest in this protein as a marker and potential target in gliomas. Molecular analysis at the mRNA level has shown that brevican is listed in the top-100 upregulated genes in all gliomas (Tso et al., 2006) and can be used to identify molecular subclasses of glioma (Liang et al., 2005; Phillips et al., 2006) with differential prognosis. At the protein level, previous work from Viapiano et al. (Viapiano et al., 2005) demonstrated that brevican exhibits unique glycosylation patterns in glioma, which can also be used to identify a subset of indolent, non-recurring tumors. However, despite the descriptions of brevican as a putative glioma biomarker, there have been no projects
focused on the potential targeting of brevican, mostly due to the fact that the molecular mechanisms of this lectican were unknown.

In this regard, results in this dissertation are particularly relevant because they combine with preliminary work from the Viapiano lab (Hu et al., 2008) and provide for the first time a more complete picture of brevican signaling. The newly identified pathway from sulfatides to EGFR, and then potentially to Erk1/2 and fibronectin (Hu et al., 2008) is a source of novel molecular targets. Based on our work, we can propose that ADAMTS-4/5 inhibitors as well as Src-kinase inhibitors are two promising areas for pharmacological research. Src-kinase inhibitors (e.g. Dasatinib) are currently employed for the treatment of leukemia and are a major focus of large pharmacological industries (Ahluwalia et al., 2010). On the other hand, ADAMTS inhibitors have been for years a focus of pharmacological research in anti-inflammation, in particular in diseases where inflammation is caused by aggrecan degradation in cartilage (arthritis) (Stanton et al., 2011).

In addition to these areas of research, novel experimental strategies may develop from our research. For example, sulfatides are interaction partners of P-selectin in colon adenocarcinoma (Garcia et al., 2007), but removal of the sulfate group by arylsulfatase is sufficient to disrupt this interaction and reduce tumor metastasis. This enzymatic treatment in vivo, as well as the inhibitors cited above, could prevent brevican's effects and render the proteoglycan function-less, therefore reducing tumor invasion and potentially extending overall survival.

In sum, the present body of work shows that brevican, which is a major structural component of the neural ECM, is at the same time a complex signaling molecule that
triggers novel mechanisms in gliomas and their TME. Future research should focus in further understanding the molecular mechanisms of brevican and developing strategies to disrupt the role of this lectican in pathologies such as malignant brain tumors and neural injury.
**Figures of chapter 5**

**Figure 5.1** Brevican activates PDGFR signaling in glioma cells and oligodendrocyte precursors in the brain. A) Two glioma cell lines (U87 and U251) expressing full-length brevican or the fragment B50 show increased expression and phosphorylation of PDGFRA. B) The purified fragment B50 binds to the growth factor PDGFaa (and in lesser amount to PDGFbb) in a dot-blot assay. C) Immortalized oligodendrocyte precursor cells (OPCs) treated with purified brevican show increased phosphorylation of PDGFRA over time in a manner coincident with possible cleavage of the full-length molecule and release of B50. D) B50-expressing U87 tumors implanted intracranially show more diffuse borders and significant increase in OPCs around the tumor border compared to control tumors. Together these results establish the base for new investigations of the molecular signaling of brevican in gliomas and the components of the neural microenvironment.
Table 5.1: cDNA primers used to quantify gene expression by quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
</table>
| ACAN (aggrecan) variant 1 | Forward 5'-TGATGATCTGGCACAGGAGAAG  
Reverse 5'-GCTTCTGTAGTCTGGCTTG |
| ACAN (aggrecan) variant 2 | Forward 5'-TGATGATCTGGCACAGGAGAAG  
Reverse 5'-GCTTCTGTAGTCTGGCTTG |
| BCAN (brevican)    | Forward 5'-CACCACAGGAATGACAACA  
Reverse 5'-GCTGCAAGGGTAAAGAGACAG |
| NCAN (neurocan)    | Forward 5'-CCCAGCAAGCAAAAATTTTC  
Reverse 5'-GCCATTAGATCATGCACTGG |
| VCAN (versican) V0 | Forward 5'-CTGATAGCAGATTTGATGTC  
Reverse 5'-AGGACGAATGCTGGACTTT |
| VCAN (versican) V1 | Forward 5'-CCCAGCAAGCAAAAATTTTC  
Reverse 5'-ACAGGTGCATACGTAGGAAG |
| VCAN (versican) V2 | Forward 5'-CTGATAGCAGATTTGATGTC  
Reverse 5'-ACAGGTGCATACGTAGGAAG |
| VCAN (versican) V3 | Forward 5'-CCCAGCAAGCAAAAATTTTC  
Reverse 5'-ACAGGTGCATACGTAGGAAG |
| PTPRZ1 (phosphacan) | Forward 5'-TCCCTGAATTTGAATGACTGG  
Reverse 5'-TCTGGGGTTCCTTTTTCCTG |
| HAPLN1             | Forward 5'-CAGGCAAGCAAGGGTGGTTT  
Reverse 5'-ATGGATCTCTGAGCCAAATG |
| HAPLN2 (Bral-1)    | Forward 5'-CCCAGCAAGCAAAAATTTTC  
Reverse 5'-GCCATTAGATCATGCACTGG |
| HAPLN3             | Forward 5'-CTGATAGCAGATTTGATGTC  
Reverse 5'-GGGGGACTCGTGAAAGGAAG |
| HAPLN4 (Bral-2)    | Forward 5'-CCCAGCAAGCAAAAATTTTC  
Reverse 5'-GGGTTAGGGAAGAAAAGCAC |
| c-SRC              | Forward 5'-AGGGGAATGTTGCTGGACTTT  
Reverse 5'-ACGTAATCCCATCGCTCCAC |
| FYN                | Forward 5'-CTCAGCAGTACCCCGTGCTTC  
Reverse 5'-ACACCTCCAAAGACGGTGAG |
| LYN                | Forward 5'-GTGGCAAACAATCTCAACCCTT  
Reverse 5'-ATTTCTCTTGCTGGCAAAAGC |
| ErbB1              | Forward 5'-CTTCTGAGCCTGAGGAGAT  
Reverse 5'-GTGGAGGGCAGAATGAGCAG |
| ErbB2              | Forward 5'-TGGGCAATGAGCACCCTCCA  
Reverse 5'-CCGGAGAAGGGGCTTCCTCATCA |
| ErbB3              | Forward 5'-GTCAATCCTGCTGCCACGCCCA  
Reverse 5'-TGTAATCCCTGCTGCCACGCCCA |
| ErbB4              | Forward 5'-GCCGTCTGATGTCGCTTCCA  
Reverse 5'-CAGGTTCCGGTTCATGGCATG |
| PDGFRA             | Forward 5'-TGCCATGATGTCGCTTCCA  
Reverse 5'-TGGTCTCGGCTGCATCCTG |
| PDGFRB             | Forward 5'-GCCGTCTGCTGCTGCTGCTG  
Reverse 5'-TCATGATGTCGCTGCTGCTG |
| MMP2               | Forward 5'-ACACTGGGACCTGTGCTTCCA  
Reverse 5'-TGTCATGATGTCGCTGCTGCTT |
| MMP9               | Forward 5'-CATTGCCGATGGAATAGGAGT  
Reverse 5'-TCACACGGCAGAAAGAATTTG |
| GAPDH              | Forward 5'-AGTTGCGATGCTGCCACCGCAC  
Reverse 5'-ATGACCTTGCCACAGCCTTG |
Table 5.2: Antibodies used for Western blotting and immunohistochemistry

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Catalog number</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAPLN1</td>
<td>8-A-4</td>
<td>NICHD Developmental Studies Hybridoma Bank</td>
</tr>
<tr>
<td>HAPLN2</td>
<td>H00060484-A01</td>
<td>Abnova</td>
</tr>
<tr>
<td>HAPLN4</td>
<td>MAB4058</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>Versican</td>
<td>12C5</td>
<td>NICHD Developmental Studies Hybridoma Bank</td>
</tr>
<tr>
<td>Phosphocan</td>
<td>3F8</td>
<td>NICHD Developmental Studies Hybridoma Bank</td>
</tr>
<tr>
<td>Brevican</td>
<td>B5 and B6</td>
<td>Custom antibodies (*)</td>
</tr>
<tr>
<td>Brevican B50</td>
<td>B50</td>
<td>Custom antibody against neo-epitope (*)</td>
</tr>
<tr>
<td>Chondroitin-4-sulfate</td>
<td>MAB2030</td>
<td>Millipore</td>
</tr>
<tr>
<td>Chondroitin-6-sulfate</td>
<td>270433</td>
<td>Seikagaku</td>
</tr>
<tr>
<td>V5-epitope tag</td>
<td>R961-25</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Erk1/2</td>
<td>SC-94</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>p-Thr^{302}/Tyr^{308}Erk 1/2</td>
<td>4377</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>EGFR</td>
<td>2232</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>p-Tyr^{845}EGFR</td>
<td>2231</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>p-Tyr^{1173}EGFR</td>
<td>4407</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>AKT</td>
<td>4691</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>p-Ser^{473}-AKT</td>
<td>4058</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>Src kinase</td>
<td>2109</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>p-Tyr^{416}Src kinase</td>
<td>2101</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>VASP</td>
<td>3132</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>p-Ser^{157}-VASP</td>
<td>3111</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>αβ-Tubulin</td>
<td>2148</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>β-Actin</td>
<td>AC-74</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>

(*) Developed in the Hockfield laboratory (Yale University School of Medicine) and described for the first time in (Viapiano et al., 2003).


21. Bradbury, E. J., Moon, L. D., Popat, R. J., King, V. R., Bennett, G. S., Patel, P. N.,


motility in vivo and in vitro. JNeurosurg 82, 615-622.
38. Du, W. W., Yang, B. B., Shatseva, T. A., Yang, B. L., Deng, Z., Shan, S. W., Lee,


Biochem Biophys Res Commun 276, 982-989.


of extracellular matrix components in the normal brain by invading glioma cells.
Int J Cancer 75, 864-872.


82. Lee, J., Kotliarova, S., Kotliarov, Y., Li, A., Su, Q., Donin, N. M., Pastorino, S., Purow, B. W., Christopher, N., Zhang, W., et al. (2006). Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 9, 391-403.


111. Nakada, M., Miyamori, H., Kita, D., Takahashi, T., Yamashita, J., Sato, H., Miura,


protein gene family whose members are physically linked adjacent to chondroitin sulfate proteoglycan core protein genes: the missing links. J Biol Chem 278, 21083-21091.


