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# The Role Of The Sparc Acidic Domain And Egf-Like Module In Glioma Migration, Invasion, And Signaling

Heather M. Mcclung  
*Wayne State University*

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**THE ROLE OF THE SPARC ACIDIC DOMAIN AND EGF-LIKE MODULE IN  
GLIOMA MIGRATION, INVASION, AND SIGNALING**

by

**HEATHER M. MCCLUNG**

**DISSERTATION**

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2011

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Advisor

Date

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## TABLE OF CONTENTS

Acknowledgements. . . . .	ii
List of Figures. . . . .	iv
Chapter I: Introduction. . . . .	1
Chapter II: Materials and Methods . . . . .	23
Chapter III: SPARC Upregulates MT1-MMP Expression, MMP-2 Activation, and the Secretion and Cleavage of Galectin-3 in U87MG Glioma cells . . . . .	34
Introduction. . . . .	34
Results. . . . .	37
Discussion . . . . .	45
Chapter IV: Deletion of the SPARC Acidic Domain or EGF-like Module Reduces SPARC-Induced Migration and Signaling Through the p38 MAPK/HSP27 Signaling Pathway. . . . .	51
Introduction. . . . .	51
Results. . . . .	55
Discussion . . . . .	79
Chapter V: Future Directions. . . . .	91
Appendix: Previously Published Data. . . . .	93
References . . . . .	94
Abstract. . . . .	117
Autobiographical Statement . . . . .	119

## LIST OF FIGURES

Figure 1: Structure of SPARC . . . . .	7
Figure 2: SPARC signaling. . . . .	21
Figure 3: Increased SPARC Expression correlates with increased MMP-2 expression and activity . . . . .	38
Figure 4: SPARC increases expression of MT1-MMP . . . . .	40
Figure 5: MT1-MMP cleaves Galectin-3 . . . . .	42
Figure 6: SPARC increases Galectin-3 secretion and cleavage. . . . .	44
Figure 7: Schematic of the deletion constructs. . . . .	56
Figure 8: Expression of the constructs in the selected clones . . . . .	58
Figure 9: Levels of expression and secretion of the constructs . . . . .	59
Figure 10: Intracellular processing of the constructs. . . . .	61
Figure 11: Internalization of the constructs. . . . .	63
Figure 12: Deletion of the Acidic Domain increases adhesion . . . . .	67
Figure 13: SPARC-induced migration on FN is reduced by deletion of the Acidic Domain or EGF-like Module . . . . .	69
Figure 14: $\Delta$ Acidic and $\Delta$ EGF eliminate SPARC-induced migration through 8- $\mu$ m pores . . . . .	71
Figure 15: Deletion of the Acidic Domain or EGF-like Module reduces SPARC-mediated signaling through the p38 MAPK/HSP27 signaling pathway . . . . .	73
Figure 16: The deletions show a trend to increase invasion through Matrigel . . . . .	75
Figure 17: The deletions show a trend to alter MMP-2 activation. . . . .	77
Figure 18: Working model. . . . .	87

## CHAPTER I INTRODUCTION

### Gliomas

Gliomas are the most common primary brain tumor in adults. Gliomas are tumors derived from glial cells or neural progenitor cells (Wen and Kesari, 2008) and include ependymomas (< 10% of all gliomas), oligodendrogliomas (10 – 30%), and astrocytomas (60 – 70%) (Lefranc et al., 2005). Gliomas are classified by the World Health Organization as Grades I – IV. Grade I is relatively rare, indicated by low proliferative potential and the possibility of cure by surgical resection alone (Louis et al., 2007). Grade II gliomas are characterized by infiltration into the normal brain and low proliferative index. These tumors include diffuse astrocytomas, oligodendrogliomas, and oligoastrocytoma, which often recur and progress to higher grade tumors (Louis et al., 2007). Anaplastic astrocytoma, anaplastic oligodendroglioma, and anaplastic oligoastrocytoma are classified as Grade III gliomas and are considered malignant. They are characterized by increased cellular density and increased mitotic activity with abnormal nuclei (Louis et al., 2007; Wen and Kesari, 2008). Glioblastoma Multiforme (GBM) is considered a Grade IV glioma and can arise from the transformation of lower grade gliomas or can develop *de novo* (Lefranc et al., 2005; Wen and Kesari, 2008). Despite genetic differences between the primary GBMs that arise *de novo* and those that are secondary to a lower grade glioma (reviewed in Wen and Kesari, 2008), they are morphologically indistinguishable.

Even with newer therapies, the median survival time in patients with GBM is about 15 months after diagnosis (Demuth and Berens, 2004).

GBMs contain areas of microvascular proliferation and necrosis, and show much more peritumoral edema than Grade III gliomas (Wen and Kesari, 2008). GBMs contain a central necrotic core, surrounded by a highly cellular rim, and a peripheral infiltrative edge (Rempel and Mikkelsen, 2006). The peripheral cells that are able to migrate away from the tumor mass are the primary cause for dismal prognosis in GBM patients (Lefranc et al., 2005; Rempel and Mikkelsen, 2006). Most recurrences are near the site of the original tumor, indicating that tumor cells had already infiltrated the adjacent brain by the time the initial tumor was removed. Surgery and radiotherapy are effective in targeting the tumor center, but not as effective against the invading cells (Louis, 2006).

In brain tumors, increased proliferation is often due to the increased expression of growth factors or growth factor receptors, or both. This generally occurs early in tumorigenesis (Rempel, 2001; Louis, 2006). Some of the known growth factors associated with CNS tumors include the Platelet Derived Growth Factors (PDGFs) and their receptors, Vascular Endothelial Growth Factor (VEGF) and its receptors, as well as activating mutations of the Epidermal Growth Factor Receptor (EGFR) (Rempel, 2001; Louis, 2006; Wen and Kesari, 2008). The increase in growth factor receptor activation appears to be sufficient to induce proliferation as mutations in the signaling molecules involved in the mitogenic pathways have not been found. However, molecules that are involved in cell cycle control, such as the tumor suppressor genes including p27 and



PTEN, are frequently mutated in gliomas. Mutations in these molecules allow the cell cycle to proceed and cells continue to proliferate in response to the excess growth factors available (Rempel, 2001). Growth factors may also be released from the extracellular matrix if it is undergoing digestion by proteases (Rempel, 2001).

### **Cell Migration and Invasion**

For many cell types, there are situations where active motility is a normal function, such as in embryonic development, wound healing and immune response. Cell motility is otherwise tightly regulated. Often in cancer, however, this regulation is lost (Demuth and Berens, 2004). Glioma cells can invade regionally as single cells or small clusters of cells into the adjacent brain parenchyma, along white matter tracts, around nerve cells, and along blood vessels. Cells can also migrate to sites distant from the primary tumor, which occurs along distinct pathways such as the fornix or the corpus callosum (Bellail et al., 2004; Rempel and Mikkelsen, 2006). Motility of normal cells is restricted on white matter (Belien et al., 1999) and brain tumors that arise from other cancers and metastasize to the brain invade only short distances from the tumor mass (Bellail et al., 2004). This distinction indicates that glioma cells may be especially well adapted for migration on CNS matrix. While they migrate along the blood vessels, they do not invade into blood vessel walls or into bone and they rarely metastasize outside the brain (Bellail et al., 2004).

Tumor cell invasion is a complex process that involves the coordination of several factors, including changes in cell adhesion, actin cytoskeleton reorganization, and degradation of extracellular matrix (ECM) components (Lefranc et al., 2005; Le Mercier et al., 2010). Cell adhesion involves cell-cell and cell-matrix interactions and is dependent upon the interplay between adhesive proteins and de-adhesive proteins. A shift in the balance of these proteins can induce an intermediate state of adhesion. It is thought that cells in an intermediate state of adhesion are the most migratory (Greenwood and Murphy-Ullrich, 1998). Reorganization of the actin cytoskeleton includes rearrangement of stress fibers and formation of extensions at the leading edge including filopodia and lamellopodia (Demuth and Berens, 2004; Lefranc et al., 2005). Degradation of ECM proteins provides room for the cells to migrate. ECM degradation can be achieved by a number of proteases including matrix metalloproteases (MMPs), some members of the cathepsin family and plasmin (Rempel, 2001; Louis, 2006).

### **The Matricellular Proteins**

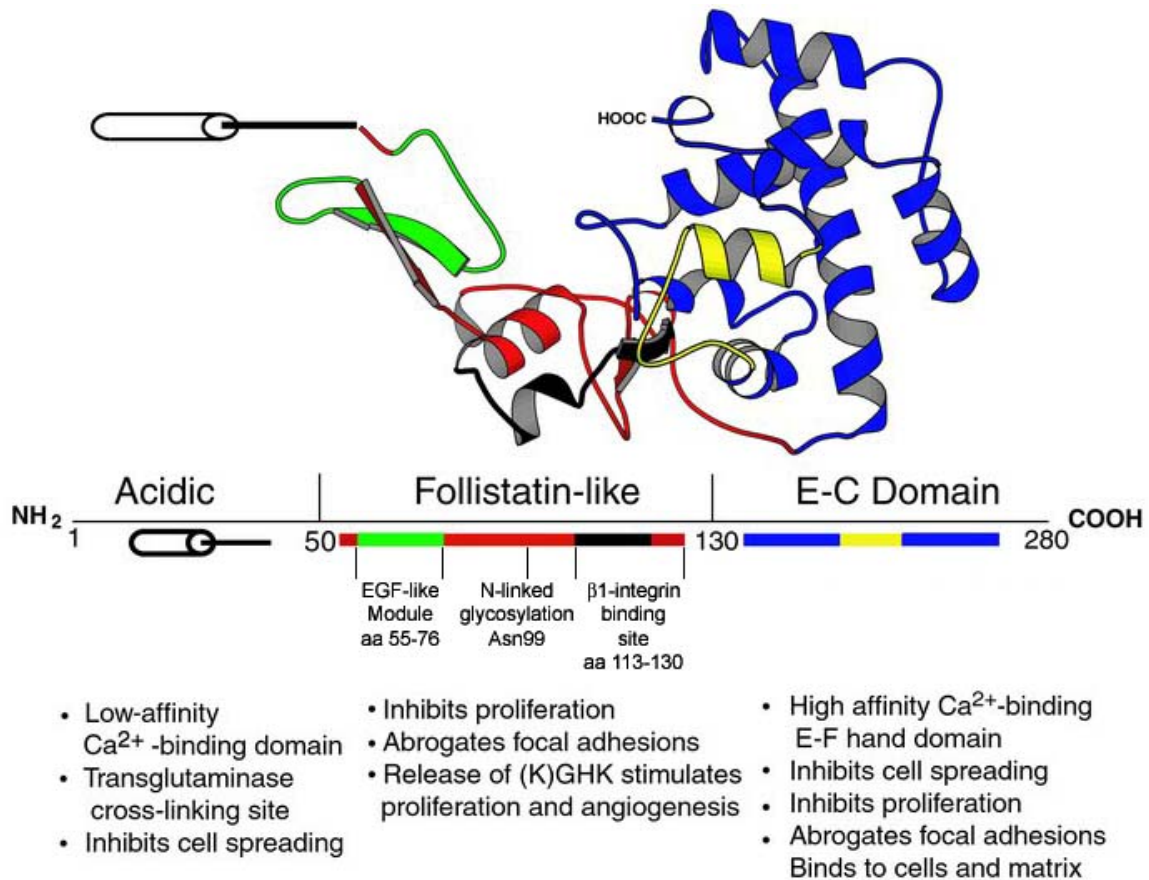
The matricellular proteins are a group of structurally unrelated proteins that are secreted into the extracellular matrix. The term “matricellular” refers to proteins that are found in the extracellular matrix, but do not contribute to the ECM structure and are distinguished from bioactive proteins such as growth factors, cytokines, and proteases (Rani et al., 2010). They are grouped based on their ability to mediate cell-matrix interactions, which in turn affects cell function

(Rempel and Mikkelsen, 2006). The matricellular proteins include Secreted Protein Acidic and Rich in Cysteine (SPARC) and its homolog hevin/SC1, tenascins-C and -X, the family of Small Integrin-Binding Ligand N-linked Glycoproteins (SIBLINGs), the CCN1-6 family of proteins, thrombospondins-1 and -2 and some of the galectins (Rempel and Mikkelsen, 2006; Chiodoni et al., 2010). These proteins are highly expressed during development; however, targeted disruption of these genes in mice results in normal or near normal phenotype (Bornstein, 2009), suggesting that they have overlapping functions. Matricellular proteins are also highly expressed in response to injury (Rempel and Mikkelsen, 2006; Bornstein, 2009; Chiodoni et al., 2010). They mediate de-adhesion, which is characterized by restructuring of focal adhesions and actin stress fibers, resulting in intermediate adherence (Murphy-Ullrich, 2001). This suggests that one purpose for the de-adhesive effects caused by these proteins may be to facilitate cell migration (Murphy-Ullrich, 2001). In addition to their counter-adhesive effects, these proteins may also affect proliferation, apoptosis, angiogenesis, and ECM degradation through their influence on signaling cascades and cytoskeletal changes (Rempel and Mikkelsen, 2006).

### **Secreted Protein Acidic and Rich in Cysteine (SPARC)**

SPARC (Sage et al., 1984), also known as osteonectin (Termine et al., 1981) or the 40 kDa Basement Membrane protein (BM-40) (Mann et al., 1987) is a 32 kDa secreted glycoprotein; however, post-translational modifications result in an apparent molecular weight of approximately 43 kDa. It is developmentally

regulated and highly expressed during vascular morphogenesis and in tissues undergoing remodeling and repair (Lane and Sage, 1994; Sage, 1997). SPARC is secreted into the extracellular matrix where it can modulate cell adhesion, motility, proliferation, and ECM production (Framson and Sage, 2004). SPARC is composed of a 17 amino acid signal sequence, an N-terminal Acidic Domain, followed by a Follistatin-like Domain, and a C-terminal Extracellular Calcium (EC) Binding Domain (figure 1). This signal sequence is necessary for secretion of the protein, but is cleaved prior to secretion. The Acidic domain contains several glutamic acid residues and binds 5-8 calcium ions with low affinity ( $K_d = 5 - 10$  mM) (Lane and Sage, 1994). It is believed that this domain has a well defined structure only in the presence of high amounts of calcium (Maurer et al., 1992). When added exogenously to fibroblasts and endothelial cells in culture, the Acidic Domain prevents cell spreading (Lane and Sage, 1990). Follistatin-like Domains are common among matricellular proteins; they contain five disulfide bonds and a hydrophobic core (Hohenester et al., 1997). The 5' region of the Follistatin-like Domain is an Epidermal Growth Factor (EGF)-like Module, which is highly twisted by 2 of the disulfide bonds which link Cysteines 1 to 3 and 2 to 4 (Hohenester et al., 1997). The EGF-like Module has been implicated in both the anti-proliferative and counter-adhesive activities of SPARC (Lane et al., 1994; Lane and Sage, 1994; Sage et al., 2003). EGF-like motifs within proteins are often involved in protein-protein interactions, though this function typically requires calcium binding within the domain (Rao et al., 1995). The EGF-like Module in SPARC does not bind calcium and it is not known whether this part of



**Figure 1. Structure of SPARC.** SPARC is characterized by three domains. At the N-terminus is the Acidic Domain, followed by the Follistatin-like Domain, which contains the EGF-like Module (shown in green). The Extracellular Calcium Binding Domain (or E-C Domain) is at the C-terminus. SPARC is glycosylated at Asparagine 99 as indicated in the schematic. Several regions of the protein have been extensively studied. Results from these studies are summarized at the bottom of the figure and are described in detail in Chapter I of this report. (Adapted from Bradshaw and Sage, 2001; reprinted with permission)

the protein is involved in any of the protein-protein interactions known for SPARC. It has been suggested that Follistatin-like Domains may be involved in growth factor binding (Patthy and Nikolics, 1993). While SPARC is known to bind some growth factors, it is not known whether this binding occurs through the Follistatin-like Domain. Some Follistatin Domains may serve as protease inhibitors (Hohenester et al., 1997); however no protease inhibitor activity has been found for SPARC. The E-C Domain contains two nearly identical EF-hands and binds two calcium ions cooperatively and with high affinity ( $K_d = 170 \text{ nM}$ ) (Hohenester et al., 1996; Busch et al., 2000). EF-hands are highly conserved  $\text{Ca}^{2+}$  binding motifs that act as calcium sensors in the cytosol. Since extracellular calcium levels are high, the EF-hands of SPARC are constitutively bound to calcium and so it is presumed that they play a structural role (Hohenester et al., 1996). This domain appears to be essential for proper folding of the protein as some constructs with point mutations to this domain were not secreted (Busch et al., 2000). This domain binds to collagens and is involved in de-adhesion and slowing of cell cycle progression (Lane and Sage, 1990; Mayer et al., 1991; Motamed and Sage, 1998).

There have been many reports that SPARC may directly or indirectly regulate cell proliferation. This function appears to be carried out through signal transduction, involving a G protein-coupled receptor, which has not yet been identified (Motamed and Sage, 1998). SPARC inhibited cell cycle progression in bovine aortic endothelial (BAE) cells, in which cell cycle arrest occurred in early  $G_1$  phase. In fibroblasts, the effects of SPARC were biphasic; at low levels,

SPARC arrested cells at the G<sub>2</sub>M phase whereas high levels of SPARC arrested the cells in G<sub>0</sub>/G<sub>1</sub> phase (Funk and Sage, 1993). It has been reported that SPARC may inhibit cell growth by interfering with growth factor-receptor interactions. SPARC can bind VEGF and PDGF and prevent them from binding to their respective receptors (Raines et al., 1992; Kupprion et al., 1998). SPARC also antagonizes bFGF induced proliferation; however, SPARC does not bind to bFGF or interfere with the binding of bFGF to its receptor (Motamed et al., 2003). SPARC can also translocate to the nucleus, however its function there is unknown (Gooden et al., 1999).

When added to normal cells in culture, SPARC functions as a counter-adhesive protein (Lane and Sage, 1994). Two mechanisms have been proposed for these effects. The first is that SPARC binds to a receptor to initiate signaling, resulting in focal adhesion disassembly. Stabilin-1 has been proposed as a putative receptor for SPARC. In human macrophages, stabilin-1 binds to SPARC and internalizes it (Kzhyshkowska et al., 2006). The mechanisms and signaling by which stabilin-1 mediates the effects of SPARC remain to be elucidated. The second proposed mechanism suggests that SPARC binds ECM components or integrins to directly disrupt cell-matrix interactions, which would influence intracellular signaling through integrin-associated proteins. SPARC binds  $\beta$ 1 integrin and this binding involves the 3' region of the Follistatin-like Domain (Weaver et al., 2008). Barker et al., (2005) demonstrated that SPARC binds to Integrin-Linked Kinase (ILK), though the mechanism for this interaction has not yet been determined as ILK is an intracellular protein and SPARC is presumed to

act extracellularly. This study also found that SPARC was required for fibronectin-induced activation of ILK. Collectively, these studies indicate that SPARC may influence cell attachment and signaling by modulating integrin signaling. Changes in integrin signaling can lead to changes in cell adhesion and cell shape, and can induce cell spreading and locomotion (Clark and Brugge, 1995), which will be discussed in more detail later in this chapter.

### **SPARC in Cancer**

While SPARC has an important role in the adhesion and proliferation of normal cells, much data suggest a role for SPARC in several cancers. It has been demonstrated that aberrant expression of SPARC contributes to malignancies such as melanoma (Ledda et al., 1997a; Massi et al., 1999), colon (Porte et al., 1995), breast (Bellahcene and Castronovo, 1995; Gilles et al., 1998), prostate (Jacob et al., 1999; Thomas et al., 2000), and ovarian cancers (Mok et al., 1996; Yiu et al., 2001), as well as glioblastoma (Rempel et al., 1998), and neuroblastoma (Chlenski et al., 2002). However, the role for SPARC in each of these cancers, appears to be cell-type specific or may depend on interactions with the tumor microenvironment (Bos et al., 2004; Podhajcer et al., 2008; Tai and Tang, 2008; Chlenski and Cohn, 2010). SPARC expression is inversely correlated with the degree of malignant progression in neuroblastoma (Chlenski et al., 2002). SPARC expression in ovarian cancer is low and exposure to SPARC inhibits proliferation in ovarian cancer cells (Socha et al., 2009). In a breast cancer cell line, infection with SPARC-expressing adenovirus inhibited



tumor growth and reduced invasion of these cells through Matrigel (Koblinski et al., 2005). However, others have shown that SPARC increases invasion of breast cancer and prostate cancer cells (Jacob et al., 1999). Increased expression of SPARC is correlated with increased malignancy in other cancers as well. In malignant melanoma, decreased SPARC expression by transfection with antisense RNA resulted in the loss of the ability for these cells to adhere and invade (Ledda et al., 1997b). Knockdown of SPARC with siRNA also decreased glioma invasion (Shi et al., 2007). Fibroblasts and/or inflammatory cells in the tumor microenvironment often express SPARC, which may contribute to malignancy in some cancers (Sangaletti et al., 2008), or may be a type of normal wound healing response to the presence of the tumor (Chiodoni et al., 2010). The therapeutic approach to SPARC, either as a therapy or as a therapeutic target, will depend on the specific role for SPARC in each cell type (Bos et al., 2004).

In cancers where increased SPARC correlates with increased malignancy, there are considerable data implicating it in the invasion process. As described earlier, there are three major components to invasion: ECM degradation, adhesion, and cytoskeletal reorganization. SPARC is associated with upregulation of proteinases, including MMP-1, MMP-3, MMP-9 (Tremble et al., 1993), and MMP-2 (Gilles et al., 1998). SPARC also mediates focal adhesion disassembly. This function is calcium-dependent (Hasselaar and Sage, 1992) and involves a tyrosine phosphorylation-dependent pathway (Yan and Sage, 1999). Finally, SPARC induces reorganization of actin stress fibers (Yan and

Sage, 1999). Therefore, SPARC elicits the necessary components for invasion and in fact, it is associated with metastatic tumors (Bradshaw and Sage, 2001).

In gliomas, our lab has shown that high SPARC expression is associated with tumor cells in the less cellularly dense areas of the tumor specimen and with the endothelial cells of the neovessels (Rempel et al., 1998). SPARC expression is also associated with increased expression of Membrane Type 1 – Matrix Metalloprotease (MT1-MMP) and Matrix Metalloprotease-2 (MMP-2) in glioma cells (Golembieski and Rempel, 2002). As in fibroblasts, SPARC has a biphasic effect on cell cycle in gliomas. At lower levels, SPARC arrests cells in G<sub>2</sub>M phase and at higher levels, cells are arrested in G<sub>0</sub>/G<sub>1</sub> phase (Rempel et al., 2001). SPARC slows cell cycle progression by mechanisms that decrease cyclins D1, D3, A, and B (Golembieski and Rempel, 2002). It is thought that cells may temporarily exit the cell cycle to facilitate migration (Rempel and Mikkelsen, 2006). SPARC also induces changes in cytoskeletal structure that result in elongated morphology, which is conducive to migration (Golembieski et al., 2008). These observations suggest a role for SPARC in the invasive aspect of gliomas. Indeed, our lab has demonstrated that SPARC promotes glioma cell invasion both *in vitro* (Golembieski et al., 1999) and *in vivo* (Schultz et al., 2002; Thomas et al., 2010). SPARC may mediate glioma invasion through suppression of cell cycle, modulation of focal adhesions, changes in cytoskeletal structure, and changes in expression of MT1-MMP and MMP-2.

### **Matrix Metalloproteases**

The matrix metalloproteases (MMPs) are a homologous family of zinc-dependent endopeptidases, which have broad specificity for ECM proteins such as collagen, fibronectin, and laminin (Kessenbrock et al., 2010). MMPs are important in many cellular processes including tissue remodeling, organ development, and regulation of inflammation. They are also involved in chronic inflammatory diseases and cancer (Kessenbrock et al., 2010). MMPs are synthesized as zymogens, which are held inactive by the Propeptide Domain until it is enzymatically removed by plasmin, furin, trypsin, cathepsins or other active MMPs (Sawaya et al., 1996; Kessenbrock et al., 2010). The gelatinases, MMP-2 and MMP-9 are upregulated in many cancers including gliomas. There is a low level of MMP-9 expression in normal brain, but this expression is localized to endothelial cells. There is no expression of MMP-2 in normal brain (Tews, 2000). In glioma, MMP-2 and MMP-9 expression increases with tumor malignancy. Expression of the gelatinases is heterogeneous throughout the tumor and is primarily expressed by tumor cells invading into the adjacent brain (Tews, 2000). MMP-2 may be more important in the invasive properties of gliomas since it is expressed most intensely by the tumor cells, whereas MMP-9 is primarily expressed by proliferating endothelial cells and therefore may be more important in angiogenesis (Forsyth et al., 1999; Raithatha et al., 2000). The tumor microenvironment, including the neovasculature, and tumor-associated fibroblasts and microglia, may also contribute to MMP expression at the site of the tumor (Sawaya et al., 1996; Sameshima et al., 2000; Markovic et al., 2009). In addition to degrading the ECM, MMPs can also cleave other extracellular

proteins including SPARC. It is thought that differential effects of SPARC observed among different cancers may be due to the peptides resulting from the cleavage of SPARC (Tai and Tang, 2008). It is well documented that MMPs are responsible for the cleavage of galectin-3 (Ochieng et al., 1994; Shekhar et al., 2004; Toth et al., 2005) and this cleavage product has recently been proposed as a marker for the presence of MMP activity in some cancers (Nangia-Makker et al., 2007).

### **Galectin-3**

The galectins are a family of evolutionarily conserved, structurally related animal lectins, which have high affinity for  $\beta$ -galactosides. The 15 galectins that have been characterized to date each contain either one or two Carbohydrate Recognition Domains (CRDs), which are responsible for  $\beta$ -galactoside binding. Galectins are localized to multiple compartments in the cell and some are secreted, presumably through a non-classical pathway since they lack the signal sequence required for the classical secretory pathway. Inside the cell, their protein-protein interactions are carbohydrate independent. Extracellularly, the galectins interact with the cell surface and the ECM (Le Mercier et al., 2010).

Galectin-3 is a mono-CRD galectin, which has a short proline, glycine, and tyrosine – rich N-terminal domain fused to the CRD. This allows for the formation of oligomers, which makes it unique among the mono-CRD galectins. Secreted galectin-3 binds to the integrin receptor  $\alpha_1\beta_1$ . This interaction may regulate cell adhesion by preventing the interaction of  $\alpha_1\beta_1$  integrin with the ECM (Ochieng et

al., 1998). In normal brain, galectin-3 expression is limited to the vasculature (Bresalier et al., 1997; Tews, 2000); however in gliomas, galectin-3 is expressed by the tumor cells and the level of expression increases with tumor grade (Bresalier et al., 1997). Galectin-3 is also associated with increased resistance to chemotherapy. This may be related to its anti-apoptotic effects. Galectin-3 contains the NGWR anti-death motif found in members of the Bcl-2 family and has been linked to decreased apoptosis induced by cisplatin and etoposide (Le Mercier et al., 2010).

### **Integrin Signaling**

Integrins are a family of cell surface receptors. Integrin receptors are heterodimers, composed of  $\alpha$  and  $\beta$  subunits, which mediate cell attachment to the ECM (Clark and Brugge, 1995; Juliano, 2002) and relay specific information between the ECM and the cell (Kuphal et al., 2005). There are at least 24 different integrin heterodimers, which recognize different ECM components; however there is some overlap between the integrins and the ligands they recognize. Upon ligand binding, integrins cluster on the cell surface, which leads to the formation of focal adhesions. The cytoplasmic domains of integrins couple with other cytoplasmic proteins, forming large protein complexes, termed Cell-Matrix Adhesion Complexes (CMACs), which include cytoskeletal proteins as well as signaling proteins (Clark and Brugge, 1995; Juliano, 2002; Bellail et al., 2004; Lock et al., 2008). CMACs mediate signals from inside the cell to the ECM, termed “inside-out-signaling”, through conformational changes in the integrins,

which modifies the affinity of integrin for the ECM proteins. They also mediate signals from the ECM to the inside of the cell, “outside-in-signaling”, which depends on the interactions between the integrins and the ECM proteins (Kuphal et al., 2005; Lock et al., 2008). Integrins can also couple with other receptors such as tyrosine kinase receptors, G protein-coupled receptors, and cytokine receptors and can modulate the signaling pathways that are activated by these receptors (Juliano, 2002). The interactions of integrins with a large variety of structural and signaling molecules allow integrins to influence many facets of cell function including cell adhesion, morphology, differentiation, proliferation, survival, and locomotion (Clark and Brugge, 1995; Juliano, 2002; Bellail et al., 2004).

Many of the proteins that are associated with CMACs are involved in the dynamics of the actin cytoskeleton including vinculin, paxillin, talin, and tensin, among others. They can also interact with proteins involved in actin dynamics such as Arp2/3 and formins. The recruitment and/or activation of these proteins can determine whether the actin filaments will polymerize and extend, will be capped to prevent further polymerization, or whether a branching event will occur. Branching and/or extension of the actin filaments and the subsequent branches are important events in the formation of lamellipodia, filopodia and cell migration (Pichon et al., 2004; Chhabra and Higgs, 2007; Le Clainche and Carlier, 2008). Cell migration requires the formation of new adhesion complexes at the leading edge of the cells and also the disassembly of adhesions at the

trailing end. Disassembly involves the dispersal of CMAC components and internalization of integrins (Le Clairche and Carlier, 2008; Lock et al., 2008).

Focal Adhesion Kinase (FAK) is an important mediator of integrin signaling. Upon integrin engagement and clustering, FAK is recruited and activated by autophosphorylation at tyrosine 397. FAK binds to adaptor proteins through Src Homology (SH) domains, which assemble large protein complexes, linking FAK and the integrins to the cytoskeleton and signaling molecules (Clark and Brugge, 1995; Juliano, 2002). FAK activates Mitogen Activated Protein Kinase (MAPK) pathways and is involved in cell survival and motility (Juliano, 2002). Increased FAK activity is associated with focal adhesion turnover and increased cell migration (Zachary, 1997; Webb et al., 2002). SPARC-induced invasion in glioma is mediated in part through activation of FAK (Shi et al., 2007).

Integrin signaling can also be mediated through Integrin Linked Kinase. ILK is a serine/threonine kinase that interacts directly with the cytoplasmic domains of  $\beta 1$  and  $\beta 3$  integrins, and modulates cell-cell and cell-matrix interactions as well as cell contraction, spreading and migration. ILK forms molecular complexes with proteins including Particularly Interesting Cysteine-Histidine-rich protein (PINCH), CH-ILKBP, affixin, paxillin and parvin. These complexes serve as signaling platforms for integrins and growth factors and provide a link between the ECM and the actin cytoskeleton and several signaling pathways that impact actin (Wu and Dedhar, 2001; Legate et al., 2006). ILK can activate or inactivate signaling pathways directly by phosphorylation of signaling proteins including AKT and GSK3 (Glycogen Synthase Kinase) (Wu and Dedhar,

2001). ILK can also activate Myosin Light Chain directly by phosphorylating it and indirectly by phosphorylating inhibitors of Myosin Light Chain Phosphatase (Deng et al., 2002), which may account for the contractile activity of ILK. Under stress conditions, SPARC-induced activation of ILK is mediated through the binding of SPARC to  $\beta 1$  integrin (Weaver et al., 2008). SPARC is also required for fibronectin-induced ILK activation and the downstream effects of ILK on cell contractile signaling (Barker et al., 2005).

### **The p38 MAPK/HSP27 Signaling Pathway**

Mitogen Activated Protein Kinases (MAPKs) are a group of conserved enzymes, which are activated by dual phosphorylation of the Thr-X-Tyr motif. MAPKs can phosphorylate specific serine and threonine residues on many proteins, which activate signaling pathways involved in gene expression, proliferation, survival, differentiation, inflammation, stress response, and migration (Huang et al., 2004; Wagner and Nebreda, 2009). There are three groups of MAPKs, the ERKs, JNKs, and p38s, all of which are involved in cell migration, but through different mechanisms (Huang et al., 2004). There has been much recent evidence for p38-mediated cell migration in many cell types (Rousseau et al., 1997; Esfandiarei et al., 2010; also reviewed in Huang et al., 2004), including gliomas (Demuth et al., 2007; Golembieski et al., 2008). P38 MAPK mediates cell migration through paxillin, p16 Arc, and also through the activation of MK2/3 and HSP27 (Huang et al., 2004).



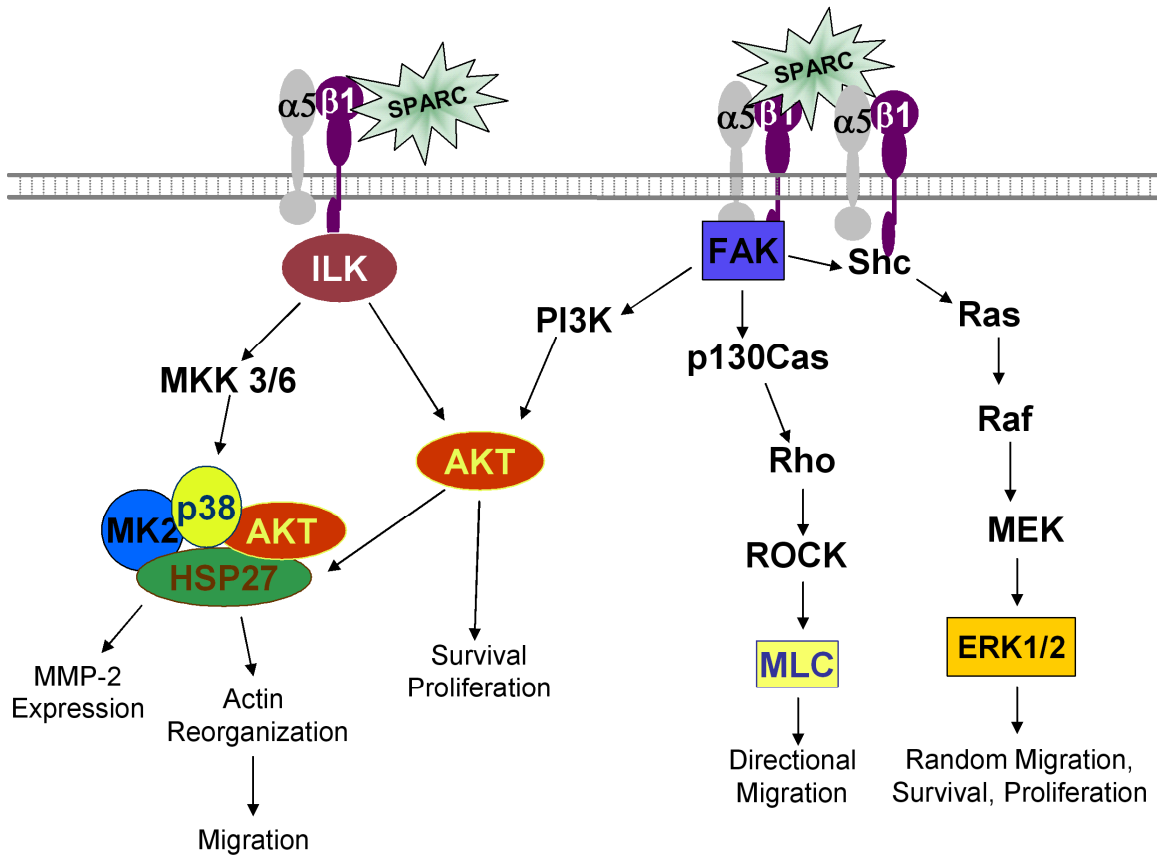
HSP27 is a member of the family of heat shock proteins, which were first identified by their upregulation in response to heat shock. They are known for their ATP-dependent molecular chaperone activity in response to heat shock or oxidative stress, in which they mediate the proper folding of proteins or traffic them to the proteasome for degradation (Ciocca and Calderwood, 2005; Kostenko and Moens, 2009). In some cancers, HSP27 is correlated with resistance to chemotherapy. HSP27 may confer this resistance by repairing proteins damaged by cytotoxic chemicals and thereby protecting tumor cells from apoptosis, making HSP27 an attractive target for therapy (Ciocca and Calderwood, 2005).

In the unphosphorylated form, HSP27 forms multimers and can also complex with other cytosolic proteins including p38 MAPK, MK2 and AKT (Zheng et al., 2006; Kostenko and Moens, 2009). Several proteins have been shown to phosphorylate HSP27 including MK2, MK3, MK5, AKT, Protein Kinase A (PKA), PKD, and PKG (cGMP-dependent Protein Kinase). Phosphorylation of HSP27 can occur at serine-15, -78, and -82 by most of these kinases, but can also occur at threonine-143 by PKG (Kostenko and Moens 2009). Unphosphorylated HSP27 can localize to the barbed ends of actin filaments and act as an actin capping protein, inhibiting actin polymerization (Huang et al., 2004; Pichon et al., 2004). Phosphorylated HSP27 does not localize to the leading edge of lamellipodia (Pichon et al., 2004). HSP27 phosphorylation and the uncapping of actin filaments at the leading edge is critical to cell migration as the overexpression of

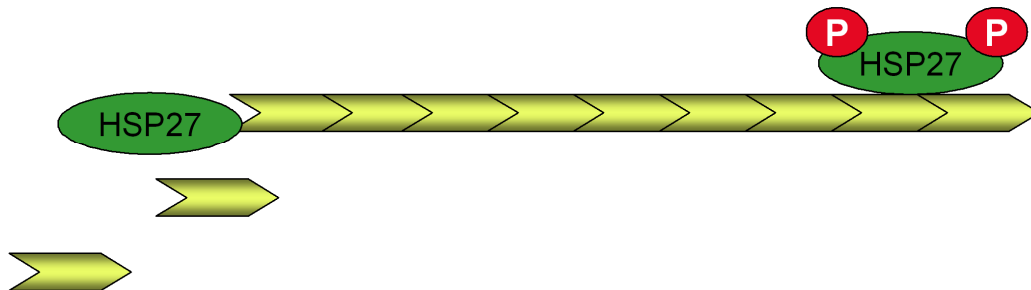
a non-phosphorylatable mutant inhibited microfilament dependent extensions (Piotrowicz et al., 1998).

We have previously shown that SPARC alters glioma morphology and enhances migration on fibronectin and that these changes are mediated by the activation of the p38 MAPK/HSP27 signaling pathway. We also demonstrated coincident localization of SPARC and HSP27 in invading cells in xenografts of implanted primary human tumor GBM cells (Golembieski et al., 2008). Others have also shown that gliomas express HSP27 (Hermisson et al., 2000) and that it increases with tumor grade (Hitotsumatsu et al., 1996). Esfandairei et al., (2010) demonstrated in mouse aortic smooth muscle cells that activation of ILK by PDGF caused increased cell migration through the activation of p38 MAPK. While the migration was attributed to the transient activation of cofilin, a known actin binding protein that enhances actin reorganization, and not to HSP27, the ILK-mediated activation of p38 supports our working model, as follows (fig. 2A). SPARC, through the binding of integrin  $\beta 1$ , activates ILK. ILK then activates p38 MAPK, possibly through MAPK Kinase 3 or 6, which are known to directly activate p38 in gliomas (Demuth et al., 2007). This results in activation of MK2 and then HSP27 phosphorylation. Phosphorylation of HSP27 reduces actin capping and facilitates actin polymerization and cell migration (fig. 2B). In addition, activation of the p38 MAPK/HSP27 pathway increases expression of MMP-2 (Xu et al., 2006), which degrades the ECM to aid in cell invasion. The Acidic Domain and the EGF-like Module of SPARC, which have been shown to modulate cell adhesion (described earlier in the section Secreted Protein Acidic

A



B



**Figure 2. SPARC Signaling.** **A.** SPARC signals at the cell surface, presumably through integrin  $\beta 1$ , and activates ILK and/or FAK, which then activate multiple signaling pathways including the p38 MAPK/HSP27 pathway (Esfandairei et al., 2010). Note: the signaling molecules investigated in this study are highlighted. **B.** Unphosphorylated HSP27 acts as an actin capping protein, which stabilizes the barbed end of the actin filaments, preventing actin polymerization. When phosphorylated, HSP27 can stabilize actin filaments at the base of the lamellipodia and facilitate polymerization (Pichon et al., 2004), which promotes cytoskeletal structure, conducive to cell migration.

and Rich in Cysteine), may contribute to the effects of SPARC-induced signaling and cell migration and invasion. The deletion of these domains of SPARC may increase cell adhesion and reduce cell migration.

### **Overall Hypothesis and Aim of Study**

Our hypothesis is that SPARC increases glioma migration and invasion through the activation of the p38 MAPK/HSP27 signaling pathway and the increased expression and activation of MT1-MMP and MMP-2 and these effects are mediated, at least in part, through the Acidic Domain and EGF-like Module.

The aims of this study are 1) to confirm, at the protein level, our previous results from cDNA array, that SPARC increases MT1-MMP and MMP-2 expression and determine the effects of SPARC on MMP-2 activation and 2) determine the effects of deleting the Acidic Domain and EGF-like Module on SPARC induced migration, invasion, and signaling. The information obtained from this study will further define the multiple functions of SPARC and give insight into targeting SPARC as a glioma therapy.

## **CHAPTER II MATERIALS AND METHODS**

### **Cell Maintenance**

All cells were maintained in a humidified chamber at 37°C and 5% CO<sub>2</sub>. U87MG cells and the U87D8 clone were maintained in DMEM + 10% Fetal Bovine Serum (FBS) + 1% Penicillin/Streptomycin (Pen/Strep [1:1]) + 5 µg/ml Gentamicin. Derivation of the doxycycline-regulatable U87T2 and U87-SPARC-transfected clones A2b2 and A2bi, have been previously described (Golembieski et al., 1999). The SPARC-transfectants (A2b2 and A2bi) and empty vector control cells (B1b2 and C2a2) were maintained in DMEM + 10% FBS + 1% Pen/Strep + 400 µg/ml Geneticin + 1 µg/ml puromycin. The clones transfected with GFP and the GFP-fusion constructs were maintained in DMEM + 10% FBS + 1% Pen/Strep + 5 µg/ml Gentamicin + 400 µg/ml Geneticin (G418). Cell culture reagents were purchased from Invitrogen (Grand Island, NY).

### **Western Blot Analyses**

For collection of lysates and conditioned media, clones were plated 2 x 10<sup>5</sup> or 4 x 10<sup>5</sup> on plastic or fibronectin (FN [Millipore, Temecula, CA]) in DMEM + 10% FBS +1% Pen/Strep overnight. Clones were washed twice with PBS and media were changed to Serum-free (SF) OptiMEM (Invitrogen, Grand Island, NY). Media were collected at 24 hr or 48 hr, centrifuged to remove loose cells and debris and frozen at -20°C. For collection of lysates at 3 hr, 6 hr, or 24 hr, cells were washed twice with ice cold Phosphate Buffered Saline (PBS) and lysed with single detergent lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-

100) + 5 mM NaVO<sub>4</sub> + 10 mM NaF + Easy mini protease inhibitor cocktail tablet (Roche, Indianapolis, IN). The lysed cells were scraped, vortexed and centrifuged. Supernatants were stored at -80°C.

For Galectin-3 studies lysates and media were collected as above. Then media were concentrated approximately 25-fold using Centricon Plus-20 centrifugal filters Ultracel PL-10 (Millipore, Bedford, MA). The resulting media volumes were measured by pipette and media concentrations were then equalized by adding SF OptiMEM.

For collection of lysates from cells exposed to conditioned medium, conditioned media were collected as described above. U87D8 cells were plated  $2 \times 10^5$  on plastic in DMEM +10% FBS +1% Pen/Strep overnight. Cells were washed twice with PBS and media were replaced with conditioned media. Lysates were collected at 10 min, 30 min, 1 hr, 3 hr and 24 hr as described above.

Protein concentration in the lysates was determined by BCA protein assay (Thermo, Rockford, IL) and 8 – 20 µg protein in lysates or 20 µl media were subject to sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, using 10% polyacrylamide gels. The prestained molecular weight ladder Precision Plus Dual Color Protein Standards (Bio Rad, Hercules, CA) or Magic Mark XP Western Standard (Invitrogen, Carlsbad, CA) was loaded on each gel and molecular weight markings on all Western blots in this report are given from these markers. Gels were transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford,

MA). Membranes were dried for at least 20 min and were wetted with methanol, washed twice with Tris buffered saline (TBS) and blocked with 5% milk in TBS + 0.05% Tween-20 (TBST) for one hour at room temperature for the detection of most of the proteins or overnight at 4°C. Membranes were probed with the respective primary antibodies, diluted in 1 - 5% milk or 5% BSA according to the manufacturers instructions for 1 hr at room temperature or 4°C overnight. Primary antibodies include SPARC (1:6,000, Haematologic Technologies, Essex Junction, VT), MT1-MMP Ab815 (1:5,000, Chemicon, Temecula, CA) and MT1-MMP Cytoplasmic tail (1:10,000, Triple Point Biologics, Forest Grove, OR). The Galectin-3 antibody (1:500) was a generous gift from Dr. Avraham Raz. Other primary antibodies include GFP (1:2,000, Invitrogen, Eugene, OR), HSP27 and actin (each 1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA), p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), pHSP27 Ser82 (each 1:1,000, Cell Signaling Technology, Danvers, MA), pHSP27 Ser15 and pHSP27 Ser78 (each 1:2,000, Assay Designs, Ann Arbor, MI). Membranes were washed three times with TBST and were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 2.5% or 5% milk. Membranes were washed with TBST and subject to Enhanced Chemiluminescence or Super Signal West Femto Enhanced Chemiluminescence reagents (Thermo, Rockford, IL). Membranes were stripped using Antibody Stripping Solution (Alpha Diagnostics, San Antonio, TX) or Restore Western Blot Stripping Buffer (Thermo, Rockford, IL). Films were scanned using a Hewlett-Packard 8300 series scanner and images captured using Photoshop Software.

Densitometry was measured using Image J software (National Institutes of Health, Bethesda, MD) and values were normalized to actin. Values represent average of three independent experiments and indicate the fold change versus the average of the values for the two GFP-control clones.

### **Gelatin Zymography**

Cells were plated  $\pm$  FN (50  $\mu$ g/ml)  $5 \times 10^5$  each in one well in six-well plates in DMEM + 10% FBS + 1% Pen/Strep overnight. Media were changed to SF OptiMEM. Conditioned media and lysates were collected after 3 days using an NP-40 cell lysis buffer + EZ mini EDTA-free protease inhibitor cocktail tablet (Roche, Indianapolis, IN). Lysates were subject to BCA protein assay and 25 – 50  $\mu$ g lysate was loaded on 10% SDS-PAGE gels containing 0.1% gelatin. Gels were incubated in renaturing buffer for 30 min, rinsed 2x with deionized water and incubated in zymogram developing buffer for 30 min. Developing buffer was changed and gels were incubated overnight at 37°C. Gels were stained with 0.5% Coomassie Brilliant Blue (Invitrogen, Grand Island, NY) for 1 hr and destained for 30 min – 2 hr. Gels were scanned using a Hewlett-Packard 8300 series scanner and images captured using Photoshop software.

### **Methods for Chapter III**

#### **RT-PCR**

Cells ( $2 \times 10^5$ ) were plated for 3 days, and RNA was isolated using the Tri-reagent kit (MRC, Inc., Cincinnati, OH) according to the manufacturer's



protocol. Intact RNA was verified by 1.1% formaldehyde gel electrophoresis, quantitated, and first strand cDNA synthesis and RT-PCR were performed as previously reported (Golembieski and Rempel, 2002) using primers for MT1-MMP (forward 5'-ATAAACCCAAAAACCCCACC-3' and reverse 5'-ACACCCAATGCTTGTCTCC-3') and for GAPDH (forward 5'-CGTCTTCACCACCATGGAGA-3' and reverse 5'-CAGGGGTCTTACTCCTTGGA-3'). GAPDH was coamplified as a normalizing control.

### **Galectin-3 Processing by MT1-MMP**

Recombinant human galectin-3 (from Dr. Avraham Raz) was incubated at 37°C with a recombinant catalytic domain of human MT1-MMP (Calbiochem, San Diego, CA) in a 1:10 molar ratio of MT1-MMP to galectin-3 in 50 mM Tris/HCl pH 7.5 buffer supplemented with 150 mM NaCl, 5 mM CaCl<sub>2</sub> and 0.02% Brij 35. At various times, an aliquot of the reaction containing ~40 ng of galectin-3 was collected and mixed with SDS-sample buffer. Samples were then resolved by reducing 12% SDS-PAGE followed by immunoblot analysis using anti-galectin-3 antibody recognizing full length and cleaved galectin-3.

## **Methods for Chapter IV**

### **Vector Constructs, Transfection, and Clone Selection**

The SPARC-GFP fusion construct was created previously in our lab (Golembieski et al., 2008). The deletion mutants were created using the

QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The PAGE-purified primers for the site-directed mutagenesis for the deletion of the Acidic Domain (forward 5'-GGGAGGGCCTTGGCAAATCCCTGCCAGAAC-3' and reverse 5'-GTTCTGGCAGGGATTTGCCAAGGCCCTTCCC-3') and for the deletion of the EGF-like Module (forward 5'-GTGGCGGAAAATCCCGTGTGCCAGGACCCC-3' and reverse 5'-GGGGTCCTGGCACACGGGATTTTCCGCCAC-3') were purchased from Invitrogen Life technologies (Carlsbad, CA). The PCR products were treated with the restriction enzyme *Dpn* I to digest the parental plasmids. The resulting deletion-mutant plasmids were amplified in bacteria and purified by miniprep or maxiprep (Qiagen, Valencia, CA). Mutations were verified by enzyme digestion and also by dye terminator sequencing (Applied Genomics Technology Center, Wayne State University).

U87MG cells were transfected by electroporation using the nucleofector program X-01 and solution T (Amaxa, Gaithersburg, MD). Cells were subject to G418 selection in DMEM containing 400 µg/ml G418 for 10 days. Cells were diluted, plated in 100-mm dishes and allowed to grow for several days. Colonies were examined for expression of the fluorescent constructs using an Olympus IX50 fluorescence microscope. Fluorescent colonies were circled and then individually transferred to 24-well plates using cloning discs (Labcor Products, Frederick, MD) soaked in trypsin. Selected colonies were observed over several days for fluorescence to ensure a pure fluorescent clone. The clone selection process was repeated as necessary until several clones for each construct were

obtained. Clones were examined for expression levels by Western blot analysis as described above and clones expressing similar levels of the constructs were chosen. Two clones expressing each of the constructs were chosen. The clones expressing GFP are called GFP14 and GFP72. The two clones expressing SPARC-GFP are SPARCB8 and SPARC83. The deletion mutants include  $\Delta$ AcidicG3,  $\Delta$ AcidicE61,  $\Delta$ EGF1.3, and  $\Delta$ EGFC1. Throughout this report they will be referred to as GFP, SPARC,  $\Delta$ Acidic, and  $\Delta$ EGF, respectively. In assays where data from all clones is shown, such as Western Blots and zymograms, note that the loading order for all data is the same throughout.

Selection of the U87D8 clone was performed using the cloning discs as described above. Several clones were chosen and analyzed for levels of endogenous SPARC and the ability to internalize SPARC-GFP by Western blot. For this the clones were exposed to SF OptiMEM or conditioned medium from one of the SPARC-GFP-expressing clones for 24 hr. From these analyses, U87D8 was chosen for its low endogenous SPARC and ability to internalize SPARC-GFP.

### **Immunofluorescence and Confocal Microscopy**

For intracellular localization and actin cytoskeleton studies, 1,500 cells were plated on coverslips coated with 50  $\mu$ g/ml FN in 24 well plates in DMEM + 10% FBS + 1% Pen/Strep overnight. Clones were rinsed twice with PBS and media were changed to SF OptiMEM for 24 hr. Cells were washed once with PSB, fixed with 3% paraformaldehyde for 20 min, and permeabilized with 0.05%

Triton X-100 for 5 min. Cells were washed twice with PBS, blocked with 1% bovine serum albumin (BSA) for 30 min, and were incubated with TGN46 antibody (1:200, AbD Serotec, Oxford, UK) or phospho-Y307 FAK (1:250, BD Biosciences, San Diego, CA). Cells were washed three times and incubated with Cy3-labeled goat ant-rabbit secondary antibody (1:1,000, Jackson ImmunoResearch Laboratories, West Grove, PA) or Cy5-labeled goat anti-mouse secondary antibody (1:200, Jackson ImmunoResearch Laboratories, West Grove, PA). For cytoskeletal structure, cells were incubated in Rhodamine-labeled phalloidin (Invitrogen, Eugene, OR) for 30 min, following secondary antibody. Cells were washed three times with PBS, once with deionized water and mounted to slides using VECTASHIELD Hard-Set mounting medium (Vector Laboratories, Burlingame, CA).

For colocalization studies of the constructs taken up by naïve U87D8 cells, clones were plated at  $1.8 \times 10^5$  cells per well in 6-well plates and conditioned OptiMEM was collected as described above for Western blotting. U87D8 cells were plated on FN-coated coverslips (2,000 cells) overnight and media were replaced with conditioned media for 3 hr. Immunostaining was performed as described above, using EEA1 primary antibody (1:500, BD Biosciences, San Diego, CA) and Cy3-conjugated goat anti-mouse secondary antibody (1:1,000, Jackson Laboratories, West Grove, PA).

Cells were imaged using a Nikon Confocal Microscope C1 System at 60X magnification. Images were captured in 0.5  $\mu$ M sections to generate single-slice images or whole-cell built images using Nikon EZC1 2.30 software.

**Adhesion Assay**

Ninety-six-well plates were coated with FN as indicated, then blocked with 1% BSA for 1 h at room temperature. Wells were washed with PBS and cells were plated in triplicate, 5,000 cells per well in OptiMEM, and were kept on ice for 30 min to allow the cells to settle. Cells were incubated at 37°C for 24 hr. Non-attached cells and loosely attached cells were removed by shaking the plates on an orbital shaker at 350 rpm for 6 min. Wells were washed with PBS and adherent cells were fixed with 1% glutaraldehyde for 30 min. Cells were washed 3 times with PBS and stained with 0.1% crystal violet for 10 min. Cells were washed 3 times with PBS. The color from the stained cells was solubilized in 1% SDS and quantified by reading absorbance at 540 nm.

**Wound Migration Assay**

Cells were plated  $3 \times 10^5$  in 60 mm dishes on 50  $\mu\text{g/ml}$  FN in DMEM + 10 % FBS + 1% Pen/Strep and allowed to grow for 3 days to confluence. Wounds were made with  $\frac{3}{4}$  inch razor blades by pressing the blade into the plate to mark the starting line and gently scraping away the cell layer. The wounds were examined to confirm that the cell layer had been removed completely and the plates were washed twice with PBS to remove cell debris. Media were replaced with SF OptiMEM. Four of 6 wounds were chosen based on optimal clearing of cells. After 20 hours, two 10x fields were imaged per wound. For each field, the distance the cells migrated past the wounding line was the average of the

distance measured at three locations across each image using Advanced SPOT imaging software (Diagnostic Instruments, Sterling Heights, MI). For each construct the distance is the average of two images per eight wounds.

### **Growth Curve**

For each time point, cells were plated in triplicate on FN (50  $\mu\text{g/ml}$ ) in DMEM + 10% FBS + 1% Pen/Strep overnight (10,000 cells per well) in 6-well plates. Cells in triplicate wells for day 0 were counted and media were changed on the remaining wells to SF OptiMEM. Triplicate wells were stained with 0.4% trypan blue (Invitrogen, Grand Island, NY) and counted using a hemacytometer at 24 hr and 48 hr.

### **Transwell Migration and Invasion Assays**

For migration assays,  $5 \times 10^4$  cells were plated in triplicate in SF OptiMEM, in Corning transwell inserts with 8- $\mu\text{M}$  pores (Fisher Scientific, Pittsburg, PA). Cells were allowed to settle to the bottom of the transwell for 15 min at 37°C; then the lower chambers were filled with OptiMEM containing 10% FBS to stimulate migration. Migration was stopped after two hours. For invasion assays, transwell inserts were coated with 50  $\mu\text{g/well}$  Matrigel (BD Biosciences, Bedford, MA) for one hour at 37°C. Cells were plated  $5 \times 10^4$  cells per well in SF OptiMEM and lower chambers were filled with OptiMEM + 10% FBS. Invasion assays were stopped after 24 hr. Cells and Matrigel were removed from the top of the filters with cotton swabs. Cells on the underside of the filters were fixed

with 10% neutral buffered formalin for 3 min, rinsed with deionized water, and stained with hematoxylin for 6 min. The filters were rinsed in tap water, blued in ammonia water and rinsed again. Filters were stored in tap water until imaged.

Cells were imaged using an Olympus IX81 microscope attached to a DP70 digital camera. Five fields per filter were imaged at 10X for migration or 12 fields at 20X for invasion. Nuclei were counted and the number of cells per field was averaged for each well. Results represent average number of cells per field  $\pm$  standard deviation for three experiments.

### **Statistical Analyses**

For the wound and transwell assays, the means for each construct were compared using generalized linear mixed models, which adjusted for the variability between clones expressing the same construct. One-way analysis of variance was used for the Western Blots. The growth curve and the adhesion assay were analyzed using the Student's t-test. Differences were considered significant when  $p < 0.05$ .

## **CHAPTER III SPARC UPREGULATES MT1-MMP EXPRESSION, MMP-2 ACTIVATION, AND THE SECRETION AND CLEAVAGE OF GALECTIN-3 IN U87MG GLIOMA CELLS**

### **Introduction**

Tumor cell invasion involves the coordination of changes in cell adhesion, actin cytoskeleton reorganization, and degradation of extracellular matrix components (Lefranc et al., 2005; Le Mercier et al., 2010). Proteases including matrix metalloproteases (MMPs), some members of the cathepsin family and plasmin are often upregulated in tumor cells in order to facilitate invasion (Rempel, 2001). High levels of SPARC have been correlated with the increased expression of specific MMPs. Exposure to SPARC increased expression of MMP-1 (collagenase), MMP-3 (stromelysin), and MMP-9 (gelatinase B) in cultured rabbit synovial fibroblasts (Tremble et al., 1993). In addition, SPARC has been correlated with tumor invasion in several different types of cancer (Bradshaw and Sage, 2001; Framson and Sage, 2004), where ECM and basement membrane degradation during tumor cell invasion may be one mechanism enabling enhanced invasion. SPARC was found to increase MMP-2 activation in breast cancer cell lines (Gilles et al., 1998). In addition, using a genetic model of glioma invasion, SPARC expression was associated with increased levels of MMP-3 and MMP-9, but inhibitor studies identified MMP-3 as the major protease involved in promoting glioma invasion through a Matrigel substrate (Rich et al., 2003).



Interestingly, MMPs appear to regulate the function of SPARC as well. MMPs, including collagenase-3 (MMP-13), MMP-2, MMP-9, matrilysin (MMP-7), and stromelysin-1 (MMP-3) can cleave SPARC, and the cleaved product shows an increased affinity for collagen (Sasaki et al., 1997). Furthermore, proteolytic cleavage of SPARC has been observed *in vivo*, suggesting a specific biological function for its cleavage products (Sasaki et al., 1997).

Galectin-3 is a 31-kDa member of the family of  $\beta$ -galactoside binding lectins (Houzelstein et al., 2004) that modulates normal development (Ochieng et al., 2004), and several biological processes involved in cancer, including apoptosis (Nakahara et al., 2005), tumor invasion, and metastasis (Califice et al., 2004; Krzeslak and Lipinska, 2004). Galectin-3 has been localized to the nucleus and cytosol, but is also secreted into the ECM where it mediates cell-cell and cell-matrix interactions. Galectin-3 is made up of two domains. The C-terminal domain is the carbohydrate binding domain (CRD). The N-terminal domain, which is important for galectin-3 dimerization (Shekhar et al., 2004), is made up of Gly-X-Tyr repeats, which are characteristic of collagen, and make the protein susceptible to proteolysis by MMP-2, MMP-9 (Ochieng et al., 1994; Shekhar et al., 2004) and the MT1-MMP soluble fragment (Toth et al., 2005). Thus, cleavage of galectin-3 is considered to be an indicator of MMP-2, -9, and MT1-MMP activity (Toth et al., 2005; Nangia-Makker et al., 2007). The processing of galectin-3 results in the generation of 22-kDa and/or 27-kDa degradation products that include the CRD and maintain carbohydrate binding activity (Ochieng et al., 1994; Shekhar et al., 2004; Toth et al., 2005). Thus, cleavage of

galectin-3 by MMPs may be an additional function of the proteinases to regulate galectin-3 activity (Ochieng et al., 1994).

Our laboratory has demonstrated that SPARC is highly expressed in invading brain tumors (Rempel et al., 1998; Rempel et al., 1999; Vajkoczy et al., 2000) and that SPARC induces tumor invasion *in vitro* (Golembieski et al., 1999) and *in vivo* (Schultz et al., 2002). cDNA array analysis indicated that upregulation of SPARC caused an increase in MMP-2 and MT1-MMP transcript abundance, 2.2- and 2.3- fold respectively, when transfected into U87MG glioma cells (Golembieski and Rempel, 2002). Therefore, to confirm these findings at the mRNA and protein level and to determine whether SPARC also contributes to increased MMP activity, we used RT-PCR and Western blot analysis to assess the levels of MT1-MMP and gelatin zymography to assess the levels of latent and active MMP-2. We also examined galectin-3, a target of MT1-MMP and MMP-2, as a marker of MMP activity.

## **Results**

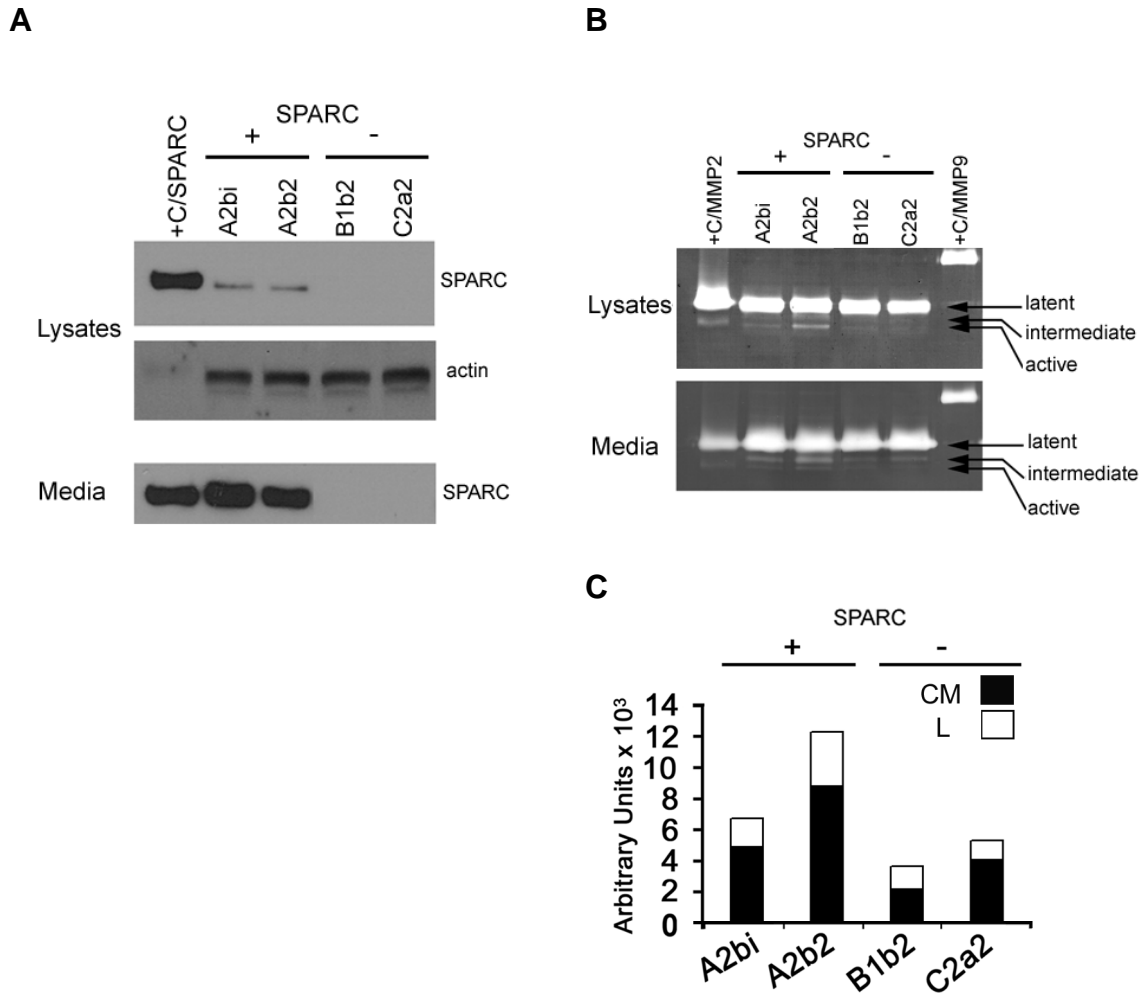
### **SPARC Expression is Associated with Increased MMP-2 Levels and Activity**

We performed Western blot analysis of equally plated cells (fig. 3A) to measure the level of expression of SPARC in the cell lysates and in the conditioned media of the SPARC-expressing A2bi and A2b2 cell lines and the control vector-transfected cell lines, B1b2 and C2a2. The blots indicate that A2bi and A2b2 indeed express and secrete greater levels of SPARC than the control cells. The actin signal demonstrates equal loading of the protein for the cell lysates.

Gelatin zymography (fig. 3B) of the lysates and conditioned media of SPARC-transfected and control vector-transfected cells indicates that the SPARC-transfected cells, A2bi and A2b2, express more latent and active species of MMP-2. The control cells, B1b2 and C2a2, expressed mainly the latent species of MMP-2, with low to undetectable levels of active MMP-2. The levels of active MMP-2 in cell lysates as well as the levels of the intermediate bands in the conditioned medium were quantified by densitometry (levels of active MMP-2 in the conditioned media were too weak to quantify). Figure 3C shows the total amount of processed MMP-2 in lysates and conditioned medium and further illustrates the increase in activation of MMP-2 in the presence of SPARC.

### **SPARC Expression is Associated with Increased MT1-MMP Levels**

We next examined the expression of MT1-MMP because we had observed increased transcript abundance by cDNA array analysis (Golembieski

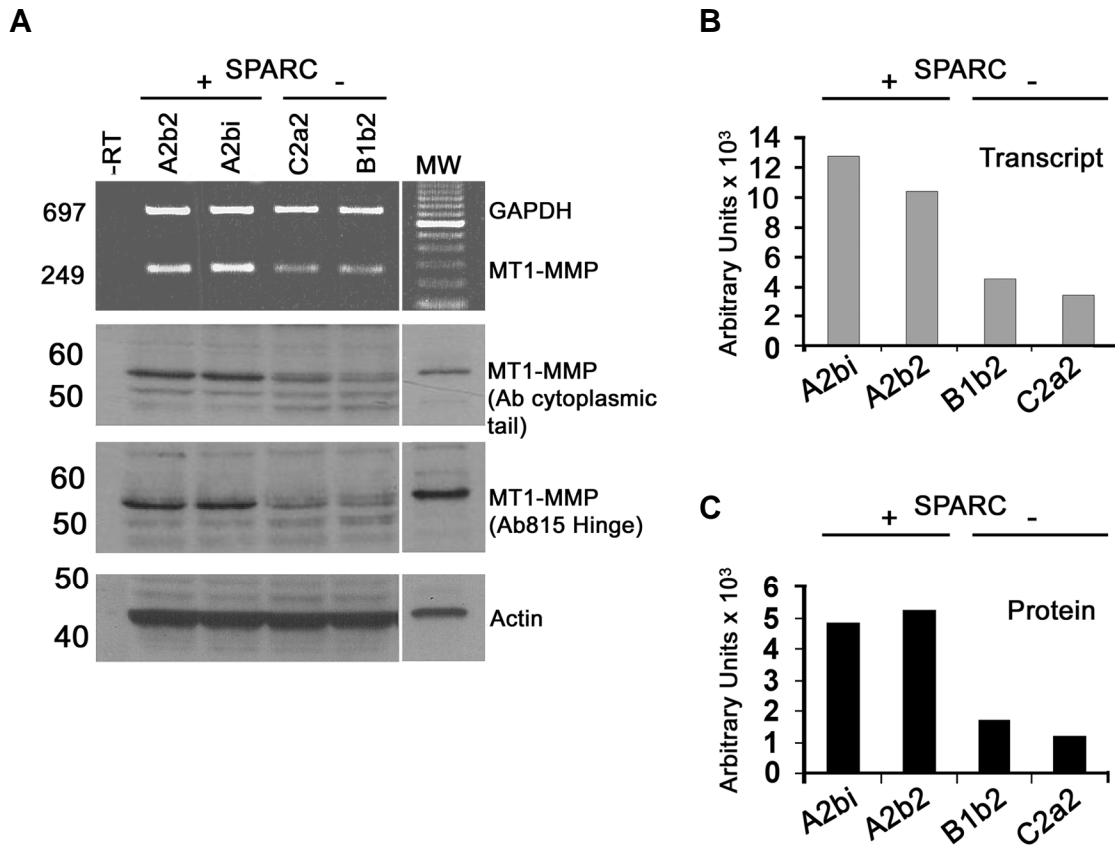


**Figure 3. Increased SPARC correlates with increased MMP-2 expression and activity.** (A) Western blot analysis demonstrating increased SPARC in lysates and conditioned media of SPARC-(A2bi, A2b2) versus control-(B1b2, C2a4) transfected cells. +C/SPARC indicates SPARC positive control. Actin was used as a loading control. (B) Gelatin zymography demonstrating increased MMP-2 expression and activation in lysates and conditioned media of SPARC-versus control-transfected cells. +C/MMP2 and +C/MMP9 indicate MMP-2 and MMP-9 positive controls. The latent, intermediate, and active forms are indicated. (C) Densitometric analysis indicates increased active MMP-2 in the lysates (L) and intermediate MMP-2 in the conditioned medium (CM) of the SPARC- versus control-transfected cells (the active MMP-2 signals in CM were too weak to quantitate). Representative results from  $n = 3$  experiments are shown.

and Rempel, 2002), and because MT1-MMP, along with TIMP-2, is the physiological activator of MMP-2. Using RT-PCR, increased levels of MT1-MMP transcript were observed in SPARC-transfected cells compared to control-transfected cells (fig. 4A). We further confirmed the increase in MT1-MMP with SPARC at the protein level using Western blot analysis. The protein was detected using both the anti-MT1-MMP antibody, which detects the cytoplasmic tail, and the Ab815 MT1-MMP antibody that detects the hinge region (fig. 4A). The membranes were reprobed with an anti-actin antibody, which indicates that there was equal loading among the lysate samples (fig. 4A). Note: The lane for the protein standard was moved closer from another region of the same blot. MT1-MMP protein levels as detected with both antibodies were higher in the SPARC-transfected cells, A2bi and A2b2, compared to control cells, B1b2 and C2a2. Densitometry results indicated that the SPARC-transfected cells had > 2-fold increase in MT1-MMP transcript (fig. 4B) and protein (fig. 4C) in SPARC-transfected cells when compared to control cells.

### **Galectin-3 Processing by MT1-MMP**

Galectin-3 is cleaved by MMP-2 and MMP-9 at the Ala<sup>62</sup>-Tyr<sup>63</sup> peptide bond generating a ~22-kDa fragment (Ochieng et al., 1994). Toth et al. (2005) reported increased galectin-3 processing in the presence of MT1-MMP. This processing was attributed to MT1-MMP activity since it was inhibited by the addition of TIMP-2, but not TIMP-1. However, the kinetics and cleavage site of

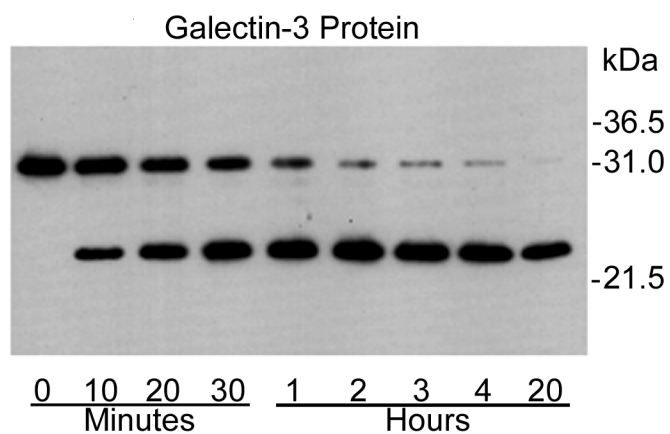


**Figure 4. SPARC increases expression of MT1-MMP.** (A) RT-PCR indicates an increase in MT1-MMP transcript in SPARC-(A2b1, A2b2) versus control-(B1b2, C2a2) transfected cells. GAPDH serves as the internal control. Western blot analysis shows increased MT1-MMP in SPARC- versus control-transfected cells using the AB-815 antibody to the hinge region or the antibody to the cytoplasmic tail. Actin was used as a loading control (only one actin blot is illustrated). Note: The lanes for the protein standard were moved closer from another region of the same blots. Densitometric analyses, demonstrating increased MT1-MMP transcript (B) and protein [densitometry for the cytoplasmic tail antibody shown (C)] in the SPARC- versus control-transfected cells. Representative results from  $n = 3$  experiments. Base pairs and molecular weights are marked at the left of (A).

MT1-MMP-dependent galectin-3 degradation were not reported. Here we examined the kinetics of galectin-3 degradation by MT1-MMP in a purified system and determined the N-terminal sequence of the cleaved product. To this end, recombinant galectin-3 was incubated with a recombinant catalytic domain of human MT1-MMP and aliquots of the reaction were collected at various times for immunoblot analysis. As shown in figure 5, MT1-MMP cleaved galectin-3 (31-kDa) to a ~22-kDa product in a time-dependent manner. The ~22-kDa degradation product was readily detected after 10-min incubation and after two hours most of the galectin-3 was converted to the degradation product. N-terminal sequencing of the 22-kDa fragment revealed an N-terminus starting with Tyr<sup>63</sup> consistent with a cleavage at the Ala<sup>62</sup>-Tyr<sup>63</sup> peptide bond. Taken together, these results establish galectin-3 as an MT1-MMP substrate via a cleavage site that is also targeted by MMP-2 and MMP-9 (Ochieng et al., 1994).

### **SPARC Expression is Associated with Increased Galectin-3 Secretion and Cleavage**

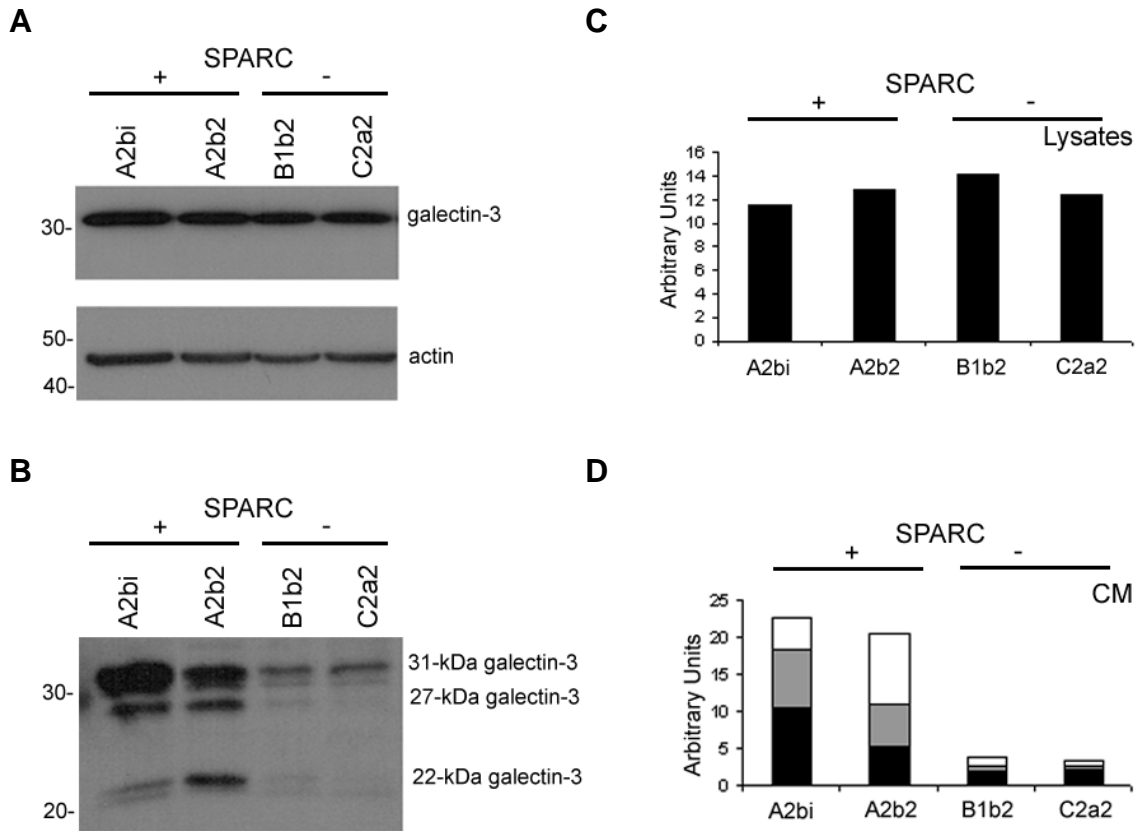
Since galectin-3 is a target of MMP-2 and MT1-MMP, we next examined protein levels of galectin-3, including the 31-kDa and the proteolytically processed forms. Western blot analysis of cell lysates show similar levels of galectin-3 in the SPARC-transfected and control vector-transfected cells, and



**Figure 5. MT1-MMP cleaves galectin-3.** Recombinant human galectin-3 was incubated with a recombinant catalytic domain of human MT1-MMP in a 1:10 molar ratio of MT1-MMP to galectin-3. Samples were taken at the indicated time points and resolved by reducing 12% SDS-PAGE followed by Western blot analysis using the anti-galectin-3 antibody recognizing full-length and cleaved galectin-3. Molecular weights are marked at the right of the blot.



only the 31-kDa unprocessed galectin-3 is present (fig. 6A). Actin shows equal loading of the lysates (fig. 6A). Densitometric analysis shows no change in galectin-3 in lysates associated with different SPARC protein levels (fig. 6C). In contrast, Western blot analysis of conditioned media indicates the presence of the 31-kDa galectin-3, as well as the 27-kDa and 22-kDa cleaved forms (fig. 6B). The densitometry analysis illustrates the levels of total galectin-3, with relative levels of the 31-kDa galectin-3 (black), the 27-kDa cleaved form (grey), and the 22-kDa form (white) illustrated for each cell line (fig. 6D). The Western blot and densitometric analyses show increased secretion of full-length galectin-3 and of the 27-kDa and 22-kDa forms in the SPARC-transfected cells versus control cells. The increase in secretion of the full-length galectin-3 does not completely account for the increase in galectin-3 processing, as there is a greater percentage of the cleaved forms in the SPARC-transfected cells than in the control cells (fig. 6D), thus indicating an increase in protease activity with increased SPARC expression.



**Figure 6. SPARC increases galectin-3 secretion and cleavage.** (A) and (C) Western blot and densitometric analysis demonstrating no change in galectin-3 levels in lysates of SPARC-(A2bi, A2b2) versus control-(B1b2, C2a2) transfected cells. Actin (A) is the loading control used for normalization in (C). (B) and (D) Western blot and densitometric analysis showing the increase of full-length and both cleaved form of galectin-3 in conditioned media from SPARC- versus control-transfected cells. The relative levels of the 31-kDa (black), the 27-kDa (gray), and the 22-kDa (white) proteins are illustrated (D). The bars in total indicate relative levels of total secreted galectin-3 by each cells line. Representative results from  $n = 3$  experiments. Molecular weights are given at the left of each blot.

## Discussion

Our laboratory and others have shown that increased SPARC expression correlates with glioma cell invasion *in vitro* (Golembieski et al., 1999) and *in vivo* (Schultz et al., 2002; Rich et al., 2003). *In vivo*, we observed increased SPARC expression promoted tumor invasion along blood vessels, and as individual cells invading the corpus collosum and adjacent brain tissue (Schultz et al., 2002). Our cDNA array analysis suggested that SPARC may promote invasion by altering the expression of proteases involved in extracellular matrix degradation, particularly MT1-MMP and MMP-2 (Golembieski and Rempel, 2002). In this study, we confirm that SPARC expression induces upregulation of MT1-MMP, increased expression and activation of MMP-2, and increases in the secretion and cleavage of galectin-3, a target of both MT1-MMP and MMP-2. Although the exact means whereby SPARC increases the expression of these proteins is unknown, these data provide a mechanism whereby SPARC promotes glioma invasion by increasing the degradation of the surrounding ECM via MMP activation and/or altering tumor cell adhesion and motility via galectin-3 secretion and/or cleavage.

The role of MMPs in glioma invasion is well documented (Rao, 2003; Bellail et al., 2004; Demuth and Berens, 2004). MMP-9 and MMP-2 have been studied extensively. Differential roles have been suggested whereby MMP-9 contributes primarily to invasion along established blood vessels, but MT1-MMP and MMP-2 may regulate both invasion and angiogenesis (Forsyth et al., 1999). In gliomas, MT1-MMP expression correlates with tumor grade (Nakada et al.,

1999; Nuttall et al., 2003) and invasiveness (Guo et al., 2005). MT1-MMP promotes invasion of C6 glioma cells on the normally restrictive central nervous system white matter (Belien et al., 1999). Activation of pro-MMP-2 requires MT1-MMP and TIMP-2 (Gingras et al., 2000) and transfection of U251 cells with MT1-MMP displayed prominent activation of MMP-2 and increased invasive growth *in vitro* (Nakada et al., 2001).

SPARC has been implicated in the upregulation of MMP-2. Gilles et al. (1998) reported that increased SPARC levels correlated with increased activation of MMP-2 in breast cancer cell lines. They did not see a change in the steady state levels of MT1-MMP mRNA or protein; however, they did detect a decrease in the levels of TIMP-2 protein in the media of SPARC-expressing cells. This is in contrast to our study, where increased SPARC expression was associated with an increase in MT1-MMP transcript and protein levels, but TIMP-2 levels in the media did not correlate with SPARC expression (data not shown). The presence of both the activating enzyme and the endogenous inhibitor must be considered, since both are involved in the regulation of MMP-2 activity (Kessenbrock et al., 2010). Differences may be attributed to the different cell lines used in these studies.

Interestingly, Xu et al., (2006) demonstrated that TGF- $\beta$  upregulates MMP-2 through increased activation of the p38 MAPK/HSP27 signaling pathway. We have demonstrated that SPARC increases glioma invasion through activation of the p38 MAPK/HSP27 pathway (Golembieski et al., 2008). Others have shown that SPARC regulates TGF- $\beta$  signaling through Smad-2 and JNK by binding to

the TGF- $\beta$ /receptor complex (Francki et al., 2004). While it is not known whether SPARC and TGF- $\beta$  cooperate in activation of the p38 MAPK/HSP27 signaling pathway, this does provide a possible mechanism by which SPARC upregulates MMP-2 expression.

The increase in MMP-2 we observed in the SPARC-transfected U87MG cells was also observed in another genetically defined model of SPARC-induced glioma invasion (Rich et al., 2003). SPARC expression in low-grade astrocytoma cells harboring SV40 large T Ag, hTERT, and oncogenic Ha-Ras was associated with increased MMP-9, MMP-3, and a modest increase in MMP-2. Invasion through Matrigel was attributed to MMP-3. The use of a non-specific MMP-2/MMP-9 protease inhibitor suggested that invasion was not mediated by either of these proteases. However, the effects of the modest increase in MMP-2, and its effects on invasion may have been clouded by the expression of the other MMPs. That study did not evaluate MT1-MMP or TIMP-2 expression.

A benefit to the U87MG cell line is the lack of MMP-9 expression, thereby eliminating any confounding effects of its expression, especially with respect to cleavage of galectin-3 (Ochieng et al., 1994). Our data suggest that SPARC-induced upregulation and activation of MT1-MMP and MMP-2 may indeed contribute to SPARC-induced invasion, by degradation of surrounding ECM, and possibly through the modulation of tumor cell adhesion and/or motility by the increased secretion and cleavage of galectin-3.

Although the secreted lectin galectin-3 has been implicated in the regulation of growth, invasion, and metastasis of human tumors, differences have

been observed depending on the tumor type. For example, galectin-3 expression is downregulated in prostate, ovarian, and breast cancers, but upregulated in gastric cancers and hepatocellular carcinoma (Califice et al., 2004). However, as described for breast cancer, the overall decrease in galectin-3 expression was accompanied by a localized expression to peripheral tumor epithelial cells that are associated with the acquisition of the invasive phenotype (Shekhar et al., 2004). Therefore, increased expression in this subset of tumor cells correlated with tumor progression.

Galectin-3 is expressed in a number of glioma cells lines (Lahm et al., 2001). However, reports on the expression of galectin-3 in gliomas have also been conflicting. Bresalier et al., (1997) reported that the level of galectin-3 expression increases during glioma progression. Subsequent studies have found a global decrease in expression (Gordower et al., 1999; Camby et al., 2001). However, higher galectin-3 expression was also associated with the invasive regions *in vivo*, and GBM cells exhibited greater motility on galectin-3 *in vitro*, suggesting that increased galectin-3 promoted invasion (Camby et al., 2001). In addition, galectin-3 expression is not restricted to the tumor cells. Indeed, galectin-3 expression from other normal cell sources must also be considered, as heterogeneous expression within the tumors results from various cell types including microglia and endothelial cells (Strik et al., 2001).

Data relating to effects of galectin-3 on adhesion and motility are also conflicting. Debray et al., (2004) demonstrated that inhibition of galectin-3 expression did not alter U373 adhesion on several ECM proteins or invasion

through Matrigel, and only increased migration on laminin. In contrast, John et al. (2003) found that treatment of breast cancer cells with a recombinant N-terminally truncated galectin-3, which functions as a dominant-negative inhibitor of galectin-3-induced cell adhesion, decreased tumor growth and metastasis in an *in vivo* model of breast cancer. Whether these differences reflect cell type-specific differences remains to be determined.

Differences in the reports correlating galectin-3 in gliomas and other cancer types may also be due to the different antibodies used to detect galectin-3, and their ability to detect the cleaved fragment. The increasing expression of MMP-2 and MMP-9 associated with glioma and other cancer progression could result in more cleavage of galectin-3. Antibodies incapable of detecting the cleaved fragment would underestimate the amount of galectin-3 fragment present. Of significance, none of these studies differentiated between the native and cleaved forms of the protein. Since the cleaved fragment is present and stable *in vivo* and it can compete for cell surface or ECM binding, it has been speculated that cleavage would result in change in biological function such as alterations of cell adhesion and motility (Ochieng et al., 1998). Our results show that there is an increase in galectin-3 secretion by the more invasive SPARC-expressing cells, and much of the secreted protein is cleaved. Galectin-3 cleavage in these cells is likely to be mediated by MMP-2 and/or MT1-MMP. Here we confirmed that MT1-MMP can readily accomplish the degradation of galectin-3 to the ~22-kDa product by hydrolyzing the same peptide bond cleaved by gelatinases.

In summary, our results show that SPARC plays a role in expression of MT1-MMP and MMP-2. Galectin-3 secretion and cleavage is also increased in cells expressing high levels of SPARC. Taken together, increased MMP activities, increased galectin-3 secretion, and the subsequent increase in the proteolytic processing of galectin-3 provide mechanisms by which SPARC increases tumor cell invasion.



## **CHAPTER IV DELETION OF THE SPARC ACIDIC DOMAIN OR EGF-LIKE MODULE REDUCES SPARC-INDUCED MIGRATION AND SIGNALING THROUGH THE P38 MAPK/HSP27 PATHWAY**

### **Introduction**

There is much evidence that SPARC plays different roles in different cancers (reviewed in Bos et al., 2004; Tai and Tang, 2008; Chlenski and Cohn, 2010). It is believed that some of the differences may be due to differential processing in the microenvironment of different tumor types. SPARC can be proteolytically cleaved by MMPs and plasmin and the cleavage products have altered affinity for collagen and exhibit differential effects on proliferation and migration (Lane et al., 1994; Sasaki et al., 1997; Sage et al., 2003). There have been numerous studies investigating the specific roles of SPARC peptides in various cell types (fig. 1).

A peptide corresponding to a portion of the Acidic Domain (fig. 1) inhibited cell spreading and an antibody to this peptide blocked the anti-spreading activity of wt-SPARC (Lane and Sage, 1990). This peptide also caused a partial decrease in focal adhesion-positive endothelial cells (Murphy-Ullrich et al., 1995). The N-terminal peptide also contributes to SPARC-mediated changes in gene expression. When added to angiogenic endothelial cells in culture, this peptide, like wt-SPARC, down-regulated thrombospondin and FN and induced the expression of PAI-1 (Lane et al., 1992). This peptide also induced MMP-2 activation in breast cancer cells (Gilles et al., 1998). The N-terminal peptide had no effect on endothelial cell proliferation (Funk and Sage, 1991). The Acidic

Domain is absent or truncated in lower organisms and it is predicted that it evolved to facilitate interactions with ECM components (Koehler et al., 2009). These data indicate that this region of the protein is necessary for some of the activities of SPARC.

The N-terminal region of the Follistatin-like Domain includes an EGF-like Module (fig. 1). Peptides mimicking the EGF-like Module caused focal adhesion disassembly when added to endothelial cells in culture (Murphy-Ullrich et al., 1995). This peptide inhibited endothelial cell proliferation (Funk and Sage, 1991) and had a biphasic effect on fibroblast proliferation (Funk and Sage, 1993). A similar peptide derived from the digestion of SPARC by MMP-3, had a biphasic effect on endothelial cell proliferation (Sage et al., 2003). The EGF-like Module peptide was also a more potent competitor for SPARC binding to the cell surface of endothelial cells than the wt-SPARC protein (Yost and Sage, 1993). This region also inhibited angiogenesis induced by neuroblastoma cells in a dose-dependent manner (Chlenski et al., 2004). Another region of the Follistatin-like Domain of SPARC, which is downstream of the EGF-like Module, was shown to stimulate proliferation of both endothelial cells and fibroblasts (Funk and Sage, 1993). This C-terminal region of the Follistatin-like domain is also the region of SPARC that binds to  $\beta$ 1 integrin (Weaver et al., 2008).

A peptide corresponding to the second EF-hand in the E-C Domain (fig. 1) disrupted focal adhesions in endothelial cells (Murphy-Ullrich et al., 1995). This peptide also inhibited cell spreading and showed calcium-dependent binding to collagens (Lane and Sage, 1990). This EF-hand has been shown to bind to the

endothelial cell surface with similar affinity as wt-SPARC (Yost and Sage, 1993) and inhibit proliferation of endothelial cells, which was also calcium dependent (Sage et al., 1995). This domain is also important in the proper folding of SPARC, as some constructs containing point mutations were not secreted (Busch et al., 2000).

We have previously shown that SPARC expression increases glioma cell migration on FN *in vitro* (Golembieski et al., 2008). The increased migration of these cells is not simply due to the counter-adhesive properties of SPARC since SPARC does not significantly alter glioma cell attachment to fibronectin (Rempel et al., 2001). We have demonstrated that SPARC increases migration through the activation of the p38/HSP27 signaling pathway (Golembieski et al., 2008). As shown in figure 2, at the cell surface, secreted SPARC binds to integrin  $\beta 1$  (Nie et al., 2008; Weaver et al., 2008), which then activates ILK. Activation of ILK affects several pathways, including p38 MAPK (Esfandiarei et al., 2010). P38 exists in a complex with MK2, AKT and HSP27, in their inactive states. These signaling complexes often occur in cells and it is thought that the purpose of the complex is to facilitate rapid activation of the pathways (Zheng et al., 2006). Upon activation, p38 can phosphorylate MK2, which then can phosphorylate HSP27 (Guay et al., 1997). MK2 can also activate AKT in this complex (Rane et al., 2001; Wu et al., 2007) and AKT can activate HSP27 directly (Zheng et al., 2006). Once HSP27 is phosphorylated, it dissociates from the complex (Rane et al., 2001; Zheng et al., 2006). HSP27, in its unphosphorylated form, participates in actin capping to prevent actin polymerization (Guay et al., 1997). When HSP27

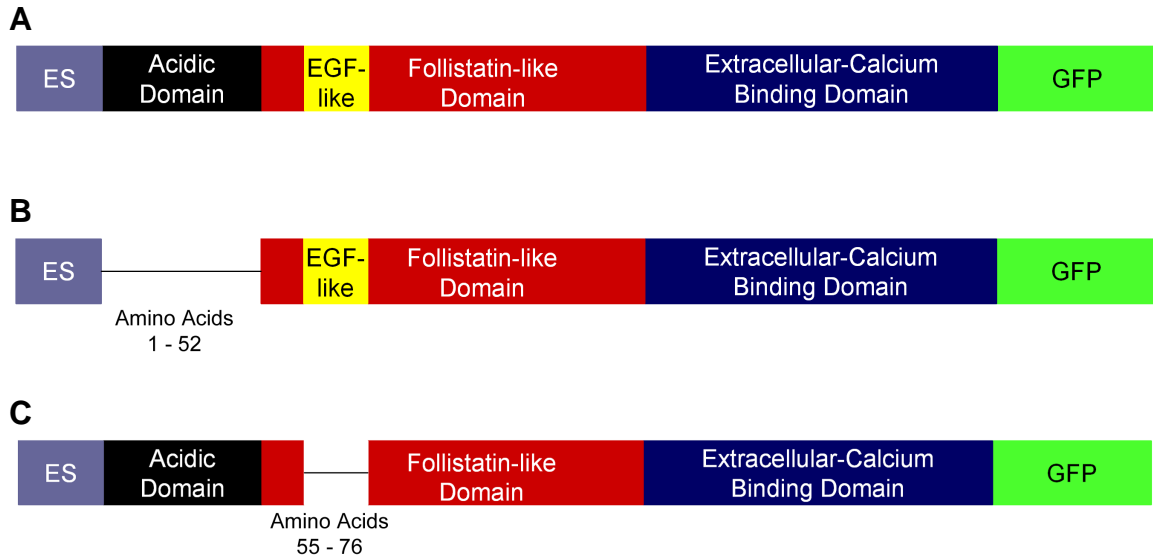
is phosphorylated, it no longer localizes to the leading edge of the lamellipodia, but localizes further back, where it stabilizes the actin filaments and prevents depolymerization in order to facilitate migration (Guay et al., 1997; Schafer et al., 1999). Expression of SPARC in glioma increases p38 MAPK activation and also increases the expression and phosphorylation of HSP27. Inhibition of HSP27 phosphorylation using a p38 MAPK selective inhibitor or knockdown of HSP27 with siRNA results in decreased migration and/or invasion of glioma cells *in vitro* (Golembieski et al., 2008).

These studies prompted us to ask what specific roles the Acidic Domain and the EGF-like Module have in glioma migration, invasion and signaling. Since SPARC expression is high in gliomas (Rempel et al., 1998), we expressed deletion mutant constructs of SPARC in glioma cells as opposed to exposing the glioma cells to the purified proteins. Using deletion mutants of a SPARC-GFP fusion construct, we investigated the effects of deleting these regions of SPARC on glioma migration, invasion, MMP-2 activity, and signaling. Based on our studies and previously reported peptide studies, the two deletion mutant constructs were predicted to increase cell adhesion due to a loss or decrease in the ability to disrupt focal adhesions. Furthermore, changes in the interactions of these mutants with integrins would alter SPARC-induced signaling through integrin-mediated pathways, as outlined in figure 2, including ILK and FAK and their downstream signaling pathways, p38 MAPK/HSP27, SHC/RAF/ERK, and/or RHO/ROCK/MLC. Altered signaling through these pathways would result in changes in SPARC-induced migration and invasion.

## Results

### Transfection, Expression, and Secretion of the Constructs in U87MG Cells

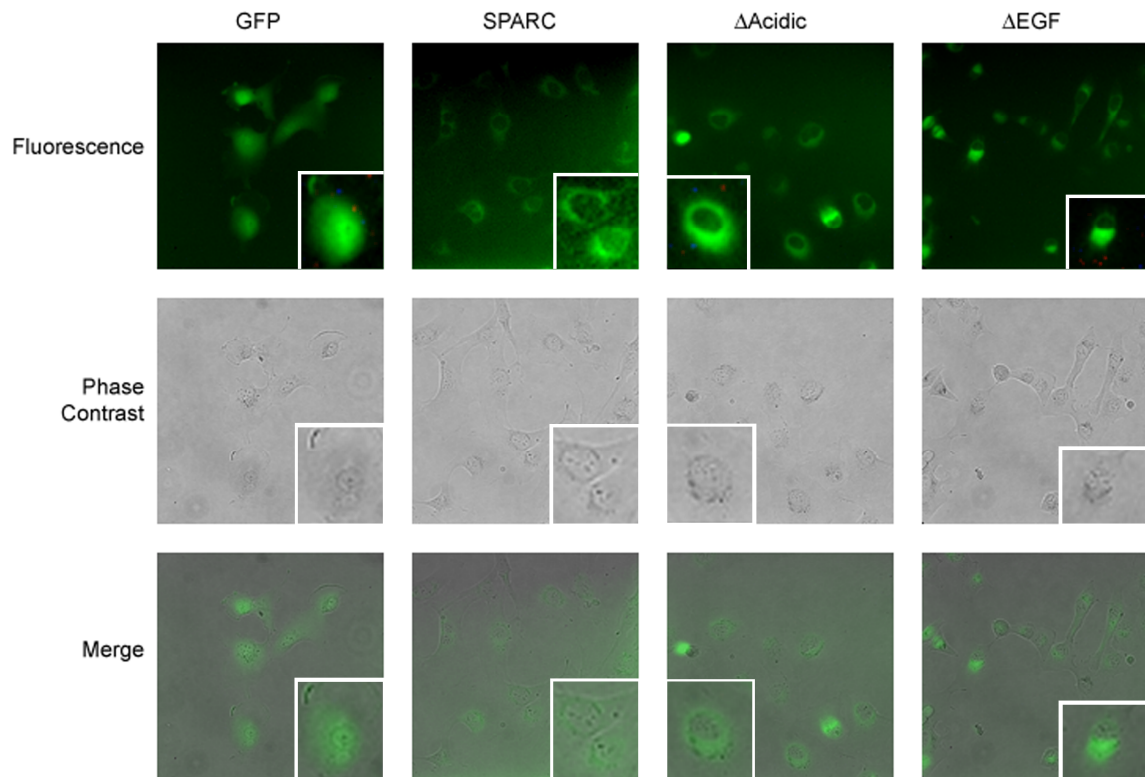
While the second EF-hand of SPARC is important in SPARC function (Framson and Sage, 2004), it is also important in the proper folding of the protein (Busch et al., 2000). Therefore, this study focuses on the Acidic Domain and the EGF-like Module portion of the Follistatin-like Domain. Since most of the effects of SPARC are presumed to occur extracellularly, the 17 amino acid N-terminal signal peptide was also left intact to ensure that the constructs would be secreted. The SPARC-GFP fusion plasmid was created previously in our lab (Golembieski et al., 2008). The deletion mutant constructs (fig. 7) were created from this plasmid using site-directed mutagenesis. The Acidic Domain deletion mutant ( $\Delta$ Acidic) was created by deleting base pairs 209 – 264, corresponding to amino acids 1 – 52. The deletion of this entire domain was expected to have little effect on the proper folding of the construct, since it is loosely folded in native SPARC. For the EGF-like Module deletion mutant construct ( $\Delta$ EGF), base pairs 271 – 336 were deleted, corresponding to amino acids 55 – 76. This deletion includes four cysteines, which in native SPARC, form two disulfide bonds within this region (Cys 1 – 3 and 2 – 4) (Hohenester et al., 1997). The deletion of the four cysteines, which participate in these two disulfide bond pairs, was intended to promote proper folding of the remaining part of the protein since the protein contains ten additional cysteines; all of which are disulfide bonded (5 – 9, 6 – 8, 7 – 10, 11 – 12, and 13 – 14) (Hohenester et al., 1996; Hohenester et al., 1997).



**Figure 7. Schematic of the deletion constructs.** Wildtype SPARC-GFP (A) contains the Acidic Domain (black), Follistatin-like Domain (red) which includes the EGF-like Module (yellow) and the E-C Domain (dark blue). ES (light blue) indicates the export signal, which is not part of the mature protein, and GFP (green) refers to the C-terminal GFP tag. (B) The Acidic Domain deletion mutant construct ( $\Delta$ Acidic) has amino acids 1 – 52 of the mature protein deleted. (C) The EGF-like Module deletion mutant construct ( $\Delta$ EGF) has amino acids 55 – 76 of the mature protein deleted.

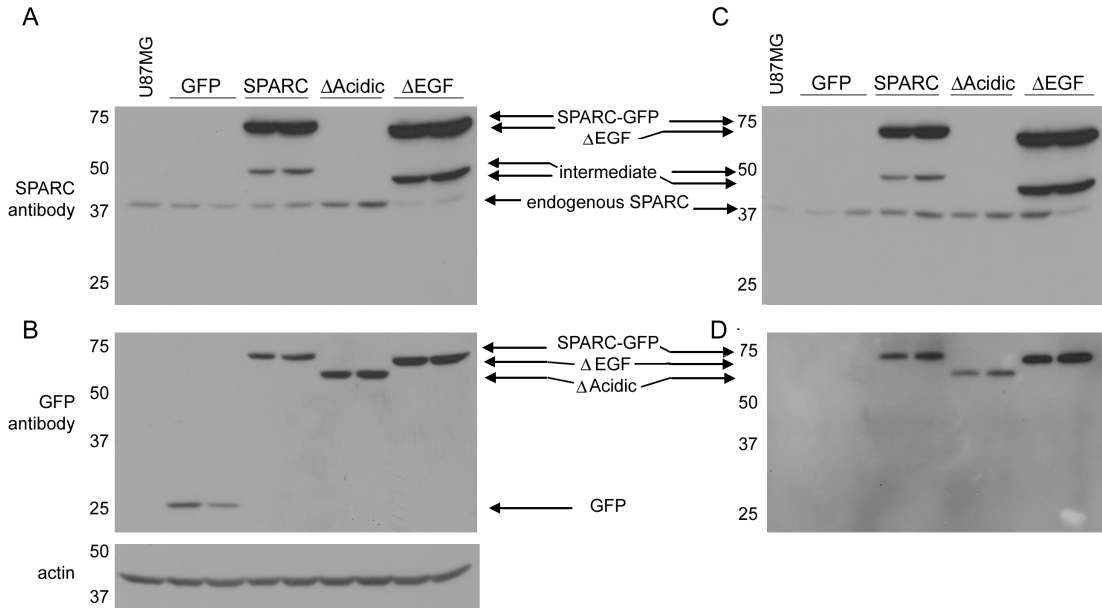
The GFP empty vector (GFP), wt-SPARC-GFP (herein referred to as SPARC or SPARC-GFP), and the deletion constructs ( $\Delta$ Acidic and  $\Delta$ EGF) were transfected into U87MG cells and stable clones were selected. Expression was verified by fluorescence microscopy (fig. 8). The fluorescence images show that cells expressing GFP show fluorescence throughout the cell, while in cells expressing SPARC-GFP the fluorescence appears perinuclear, which is expected since SPARC is secreted through the classical pathway (Mason et al., 1986). Both of the deletion mutant constructs demonstrate similar intracellular localization to the wild-type SPARC-GFP.

Two clones were chosen for each construct based on the level of expression and secretion. The level of expression of the respective constructs in the lysates and conditioned medium, as well as the low level of endogenous SPARC are indicated by Western blotting in figure 9. Western blots in figure 9A and C were probed with an anti-SPARC antibody and indicate the level of expression and secretion of the SPARC-GFP and the  $\Delta$ EGF construct as well as the levels of endogenous SPARC expressed (fig. 9A) and secreted (fig. 9C) by the clones. The epitope that the anti-SPARC antibody recognizes is within the Acidic Domain; therefore the  $\Delta$ Acidic construct is not recognized by the SPARC antibody. Expression and secretion levels of all four constructs can be seen when the blots are probed using an anti-GFP antibody (fig. 9B and D). Expression levels of the constructs in the clones are similar. GFP is not secreted as indicated (fig. 9D). We expected that both deletion mutant constructs would be secreted since the export signal was not disrupted in either deletion. However,



**Figure 8. Expression of the constructs in the selected clones.** Top panels show 40X fluorescence images (one clone for each construct is shown) indicating that the clones express the constructs and that the constructs have a functional GFP-tag. Middle panels are phase contrast images of the same field. Bottom panels are merged images of the top and middle panels indicating the fluorescence within the cells. Insets show 160X zoomed images for clarity.





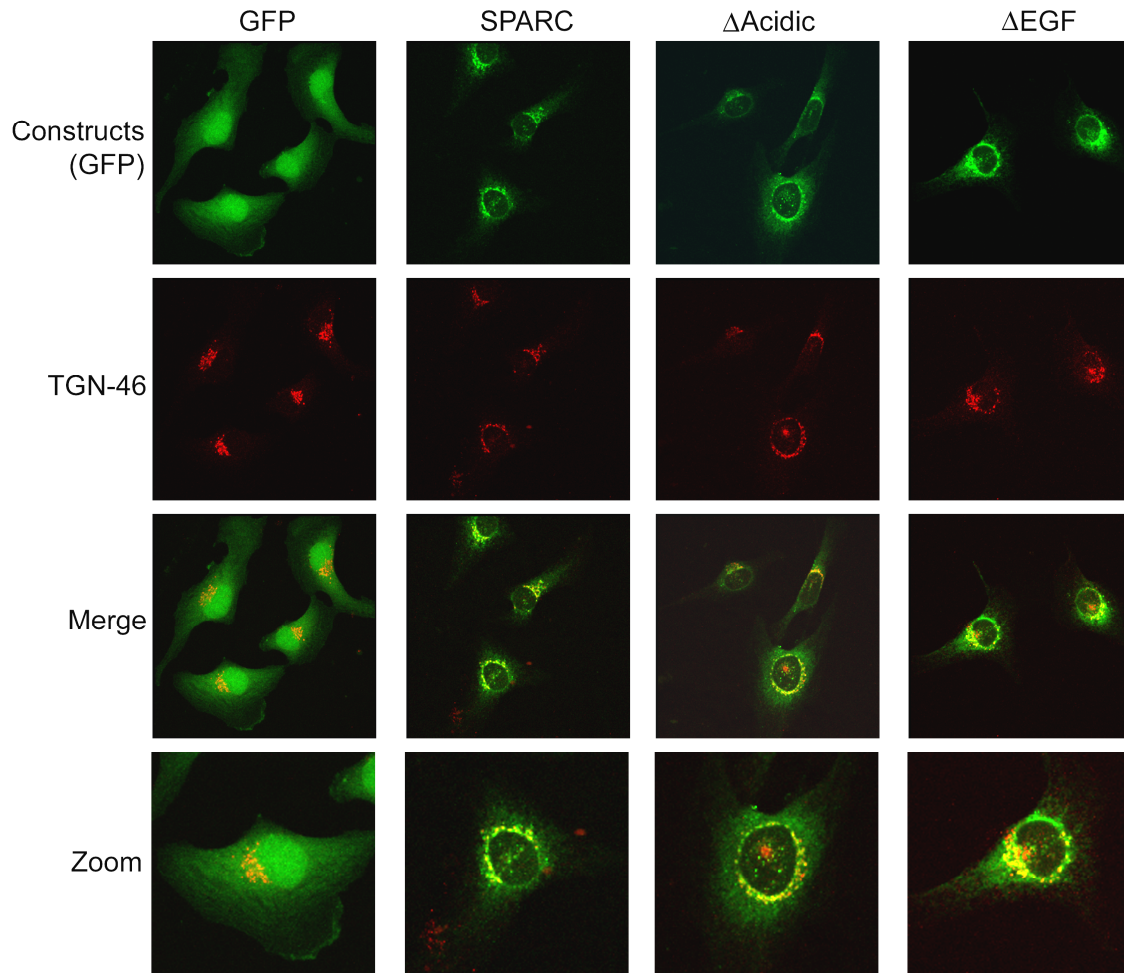
**Figure 9. Levels of expression and secretion of the constructs.** Western blot analyses of cell lysates (A and B) and conditioned medium (C and D) indicate the level of expression and secretion of the constructs as well as the levels of endogenous SPARC in each of the clones. The parental cell line, U87MG, is represented in lane 1 of each blot. Blots were probed using an anti-SPARC antibody (A and C), which shows the levels of endogenous SPARC, SPARC-GFP and  $\Delta$ EGF, but does not detect  $\Delta$ Acidic. Blots were stripped and reprobed using an anti-GFP antibody (B and D), which detects all of the constructs. The intermediate bands observed in A and C are specifically detected by the SPARC antibody and are believed to be due to alternate processing or proteolytic cleavage. Actin indicates equal loading of cell lysates. Molecular weights are shown at the left of each blot.

while the expression levels, in the lysates, of  $\Delta$ Acidic were similar to SPARC and  $\Delta$ EGF, the levels in the conditioned medium were consistently lower than that for SPARC and  $\Delta$ EGF. Since the Acidic Domain is in close proximity with the export signal, deleting this region may affect secretion.

In creating deletion mutant constructs, proper folding of the constructs was a concern. Since SPARC does not have specific catalytic activity or activation state, there is not a method to clearly prove that effects of the deletion mutants on the cells are not due to misfolding of the constructs. However, both deletion mutant constructs are secreted and run at the expected size on Western blot, which suggests that they are glycosylated and properly folded. The next experiments, in addition to characterizing the deletion mutants, provide further evidence for proper folding.

### **Perinuclear Localization of SPARC-GFP and the Deletion Mutants**

To better demonstrate the intracellular localization of the constructs, cells were fixed and immunostained using an anti-TGN-46 antibody (fig. 10). TGN-46 is an integral membrane glycoprotein found in the trans Golgi network. Since GFP is a cytosolic protein and is localized diffusely throughout the cell, it does not co-localize with TGN-46. SPARC is secreted through the classical pathway and therefore is processed in the Endoplasmic Reticulum and the Golgi Complex (Mason et al., 1986). Both of the deletion mutant constructs are secreted (fig. 9C and D), presumably through the same mechanism as wt-SPARC. Additionally, SPARC and the deletion mutants co-localize with TGN-46 (fig. 10). This

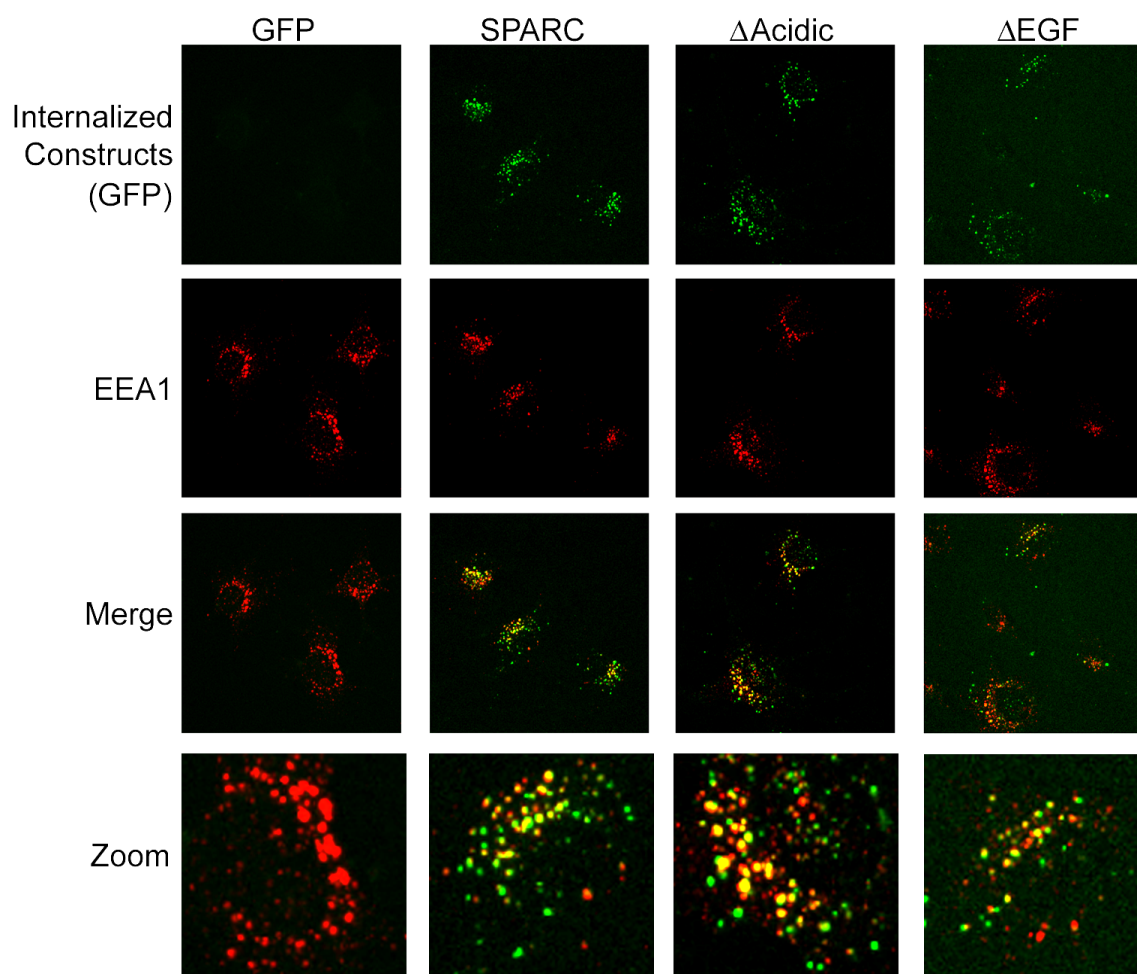


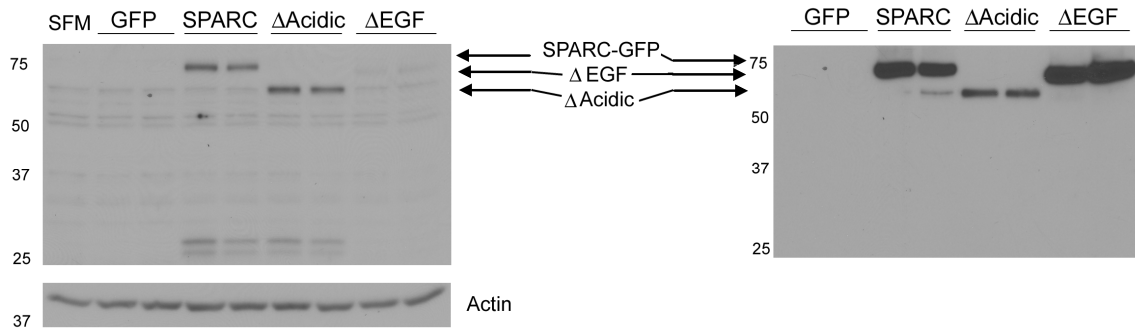
**Figure 10. Intracellular processing of the constructs.** Built confocal images showing one clone expressing each of the constructs indicate the intracellular processing of the constructs. The top panels showing GFP in each of the clones indicate that the control GFP is localized diffusely throughout the cells, while SPARC-GFP and the deletion mutants are localized perinuclear. The second set of panels show expression of the trans Golgi marker TGN-46. The merged images (bottom two rows of panels) indicate the constructs SPARC-GFP and both deletion mutants, but not GFP co-localize with TGN-46, confirming their localization to the Golgi complex. The bottom panels are zoomed images, to better demonstrate the co-localization between the SPARC constructs and TGN-46 and lack of co-localization between GFP and TGN-46. Images in the top three sets of panels were captured at 60X. Zoomed images are 240X.

perinuclear localization for all three constructs was expected since they are all secreted, but this colocalization to the trans Golgi network supports proper expression and processing for secretion of the deletion mutants.

### **Internalization of the Constructs by Naïve Cells**

Studies have shown that SPARC is taken up by cells in culture (Gooden et al., 1999; Kzhyshkowska et al., 2008; Zhang et al., 2009). Uptake is mediated by the receptor stabilin-1 in macrophages (Kzhyshkowska et al., 2006; Zhang et al., 2009) and occurs through the binding of SPARC to  $\alpha 5\beta 1$  integrin in adipose stromal cells (Nie et al., 2008). The latter may be a mechanism for uptake of SPARC in other cells, as integrins are widely expressed. SPARC is taken up by U87MG cells (Rempel unpublished observations). We have found that the non-clonal U87MG cell line does not internalize SPARC uniformly and so for these experiments we used a clone derived from U87MG, termed U87D8. This clone is denoted as naïve because of its very low expression of endogenous SPARC and because it does not express or secrete the SPARC-GFP-derived constructs. The U87D8 cells were exposed to conditioned medium collected from clones expressing each of the constructs. For visualization of uptake of the constructs by confocal microscopy (fig. 11A), cells were exposed to conditioned medium for three hours. Cells were then fixed and immunostained for EEA1, a protein involved in early endosome trafficking. Since GFP is not secreted, there is none in the conditioned medium to be taken up by cells as shown in figure 11A. Fluorescence imaging demonstrates that SPARC-GFP and both deletion mutant

**A**

**B**

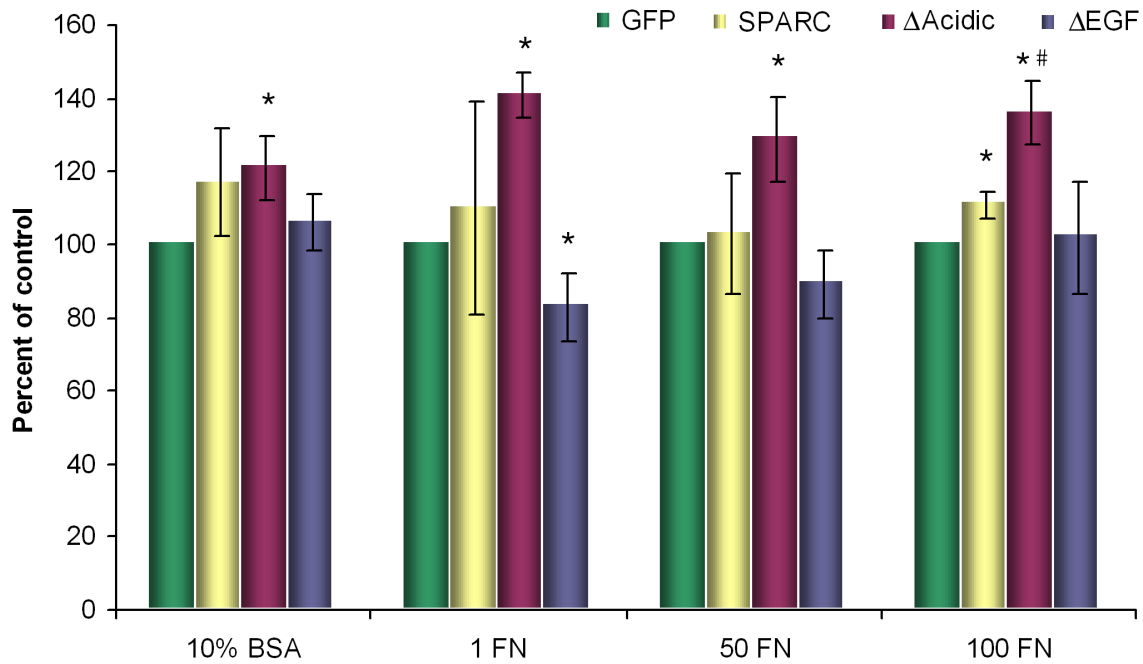
**Figure 11. Internalization of the constructs by naïve cells.** U87D8 cells, plated on FN-coated coverslips, exposed to conditioned medium from each of the clones for 3 hr, were immunostained for the early endosomal marker EEA1. 60X built confocal images are shown. (A) Top panels show the internalized constructs. Note that GFP is not secreted and so there is no GFP in the conditioned medium to be taken up by the cells. The second set of panels show the EEA1 staining. The third set of panels consists of merged images showing colocalization of SPARC-GFP and the two deletion mutants, indicating that they are internalized into the endosomes. The bottom panels are zoomed-in images (240X) of the merged images. (B) Western blot analysis of lysates (left panel) collected from U87D8 after 6 hr exposure to conditioned media or SF OptiMEM (denoted as SFM) shows that SPARC-GFP and both deletion mutants are present in cell lysates of the naïve cells. The blot is labeled according to the conditioned media to which the U87D8 cells were exposed. Actin indicates equal loading of cell lysates. The level of SPARC-GFP or deletion mutant constructs present in the conditioned media that the cells were exposed to is indicated in the right panel. Molecular weights are indicated at the left of each blot.

constructs were internalized by the U87D8 cells, as merged images of the green fluorescent constructs and the EEA1 staining show co-localization, indicating localization to the early endosome, which also points to proper folding of the deletion mutants. The uptake of the EGF-like Module by the U87D8 cells appeared less than SPARC and  $\Delta$ Acidic, and so to verify this observation, we performed Western blot analysis (fig. 11B) of lysates from U87D8 cells exposed to conditioned medium. Lysates were collected at several time points up to 24 hr. Very low levels of the constructs were seen taken up as early as within 10 min of exposure to the conditioned medium (data not shown); however, uptake was best observed by Western blot at 6 hr as shown in figure 11B or at later time points (data not shown). The Western blots suggest that SPARC,  $\Delta$ Acidic, and  $\Delta$ EGF are all internalized by the U87D8 cells; however, when comparing the levels of the constructs in the conditioned medium to the levels of the constructs that were internalized by the U87D8 cells, at the time points tested (3 hr, 6 hr, and 24 hr),  $\Delta$ EGF was taken up to a lesser extent, and  $\Delta$ Acidic was taken up to a greater extent than SPARC, suggesting domain-specific interactions with a cell-surface receptor. Interestingly, degradation products of the SPARC and  $\Delta$ Acidic constructs, which are presumed to be the GFP tag since it is approximately 29-kDa and is detectable with the GFP antibody, were detectable as early as 3 hr. However, there was no degradation product observed in the U87D8 cells exposed to  $\Delta$ EGF conditioned medium at any time point tested. Despite the lack of a detectable degradation product,  $\Delta$ EGF did not accumulate within the cells any more than SPARC-GFP or  $\Delta$ Acidic.

### **$\Delta$ Acidic Increases Cell Adhesion on Fibronectin**

After determining that the constructs are not likely to be misfolded, we next determined the effects of the deletions on cell adhesion. Adapting a method described by Geise et al., (1994) clones were plated on increasing concentrations of fibronectin with 10% BSA as a control. Adhesion was determined 24 hr after plating (fig. 12). It should be noted that the results in figure 12 are shown as a percent of the absorbance of the control cells on each matrix and that all cells were more adherent on all levels of FN than on 10% BSA. When plated on 10% BSA,  $\Delta$ Acidic cells were slightly more adherent than GFP control cells, but were not significantly different from SPARC or  $\Delta$ EGF. SPARC did not affect adhesion when plated on the lower concentrations of FN compared to GFP control cells, but SPARC increased adhesion when cells were plated on 100  $\mu$ g/ml FN. Our results are in contrast to previous studies of SPARC in normal cells, which indicated SPARC decreases adhesion (see Chapter I). However, these previous studies used normal cells that were not plated on a matrix. Our results may reflect a difference in tumor versus normal cells and/or matrix-specific effect of SPARC. Additionally, our results only slightly differ from previous studies in our lab, which showed that SPARC expression did not alter attachment on FN (Rempel et al., 2001) and differences observed may be an effect of the different time points examined. Deletion of the Acidic Domain increased cell adhesion on all levels of FN compared to GFP control cells and  $\Delta$ EGF cells. These cells were also more adherent than SPARC-expressing cells on the highest level of FN. Deletion of the EGF-like Module showed a trend for





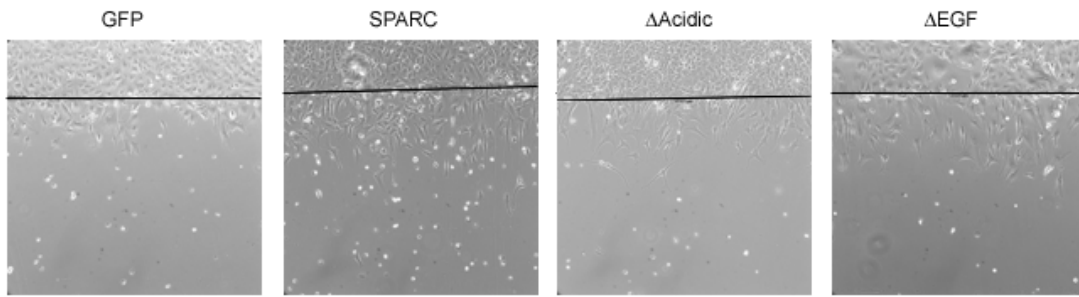
**Figure 12. Deletion of the Acidic Domain increases cell adhesion.** Adhesion was measured 24 hr after plating. SPARC-expressing cells were more adherent than GFP cells on the highest level of fibronectin ( $p = 0.0062$ ). Deletion of the Acidic Domain increased adhesion compared to control on all levels of FN and on BSA ( $p \leq 0.036$ ). This deletion also increased adhesion on 100  $\mu\text{g/ml}$  FN compared to SPARC-expressing cells ( $p = 0.0095$ ). Deletion of the EGF-like Module decreased adhesion compared to control cells only on the lowest level of FN ( $p = 0.03$ ). These cells were not significantly different from SPARC on any concentration of FN; however, they were significantly less adherent than  $\Delta\text{Acidic}$  on all concentrations of FN ( $p \leq 0.03$ ). \* = Significantly different from GFP ( $p \leq 0.036$ ), # = significantly greater than SPARC ( $p = 0.0095$ ).

decreasing adhesion.  $\Delta$ EGF cells were less adherent than GFP cells on the lowest level of FN, but were not significantly less adherent than SPARC cells on any FN concentration. However,  $\Delta$ EGF cells were less adherent than  $\Delta$ Acidic cells on all levels of FN.

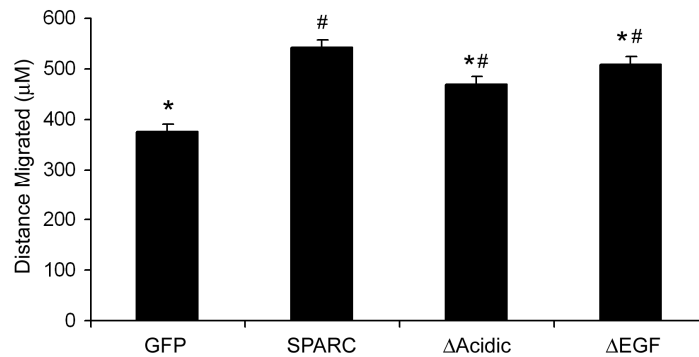
### **Deletion of the Acidic Domain or EGF-like Module Reduces SPARC-Induced Migration**

We used a wound assay, which allowed us to measure the distance that the cells migrated from the start of the wound. Figure 13A shows representative images of one clone for each of the constructs (a line has been drawn to clarify the start of migration). Figure 13B indicates the average distance migrated by cells expressing each of the constructs. SPARC-expressing cells migrated a significantly greater distance over 20 hr than the GFP-expressing cells, which is consistent with our previous data (Golembieski et al., 2008). The SPARC-expressing cells also migrated significantly farther than the cells expressing either of the two deletion mutant constructs. However, neither of the two deletion mutants was able to reduce the SPARC-induced migration to control levels. The deletion of the Acidic Domain did have a greater effect on SPARC-induced migration than the deletion of the EGF-like Module, as cells expressing  $\Delta$ Acidic migrated significantly less than  $\Delta$ EGF-expressing cells. To show that the cells that are present in the wound area were a result of migration and not due to increased proliferation, cell proliferation was measured over 48 hr. Approximately twice the amount of time allowed for migration, was used in order to amplify

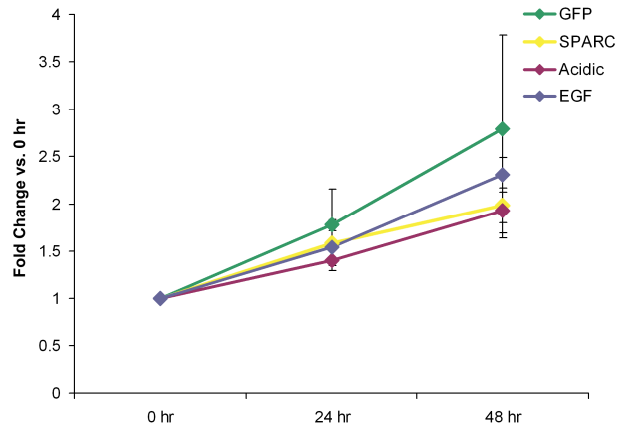
A



B



C



**Figure 13. SPARC-induced migration on fibronectin is reduced by the deletion of the Acidic Domain or the EGF-like Module.** (A) Representative 10X images of one clone expressing each of the constructs indicate cell migration from the start of the wound after 20 hr. (B) Average distance migrated for two clones expressing each of the constructs. SPARC significantly increased migration over control. Migration was significantly reduced by deletion of the Acidic Domain or the EGF-like Module when compared to SPARC-expressing cells. However, both deletion mutants migrated significantly more than control cells. \* = Significantly less than SPARC ( $p \leq 0.033$ ), # = significantly greater than GFP ( $p < 0.01$ ). (C) Fold change in number of cells in SF OptiMEM at 24 and 48 hr relative to 0 hr, indicating that increased migration is not due to proliferation of cells into the wound area.

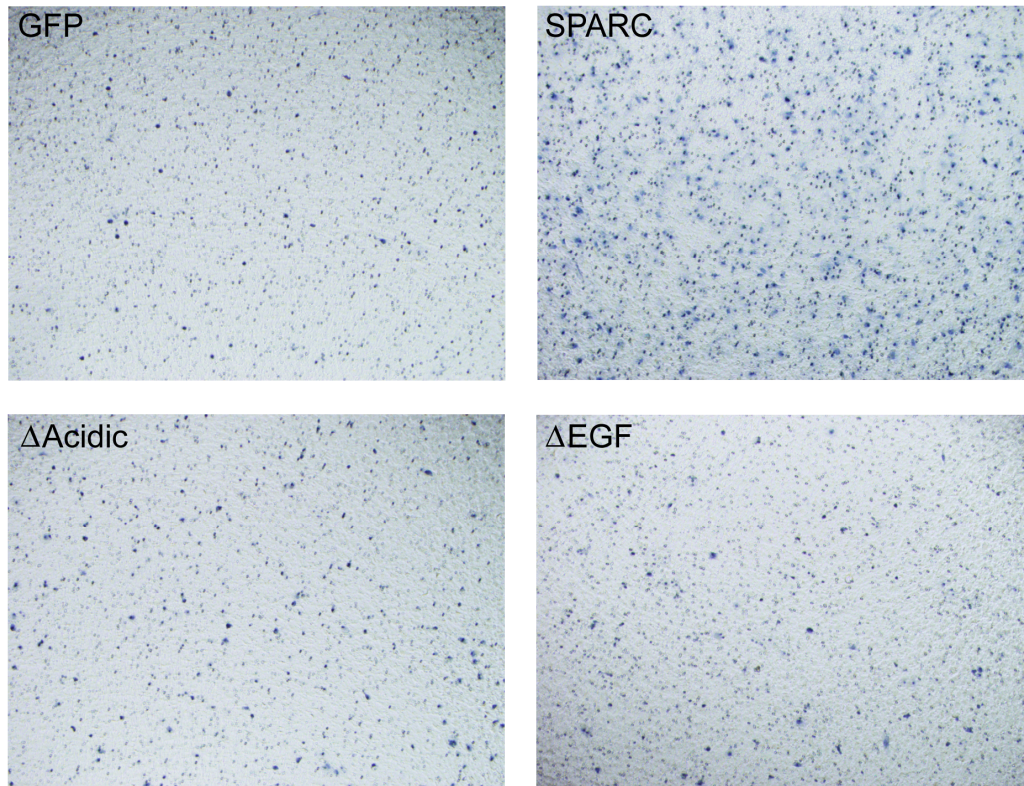
subtle changes in proliferation during the time allowed for migration. As shown in figure 13C, there was no difference in proliferation among the eight clones over this period of time.

To further confirm the effects of the deletions on cell migration, the transwell assay was used to assess migration by the number of cells in SF-OptiMEM that migrated through 8- $\mu$ m pores. A chemoattractant of Opti-MEM containing 10% serum is added to the lower chamber to induce migration through the pores. Cells that migrated through to the bottom side of the transwell filters within 2 hr were fixed, stained and imaged. Representative 10x images from each of the four clones used in this assay are shown (fig. 14A). Figure 14B indicates the average number of cells per field. As in the previous migration assay, SPARC significantly increased cell migration compared to the GFP-control cells and both of the deletion mutants. However, in contrast to the wound assay, both deletion mutants reduced SPARC-induced migration to levels similar to the GFP-control cells. The differences observed between wound and transwell migration assays indicated that the ECM may influence the activity of SPARC and the deletion mutants.

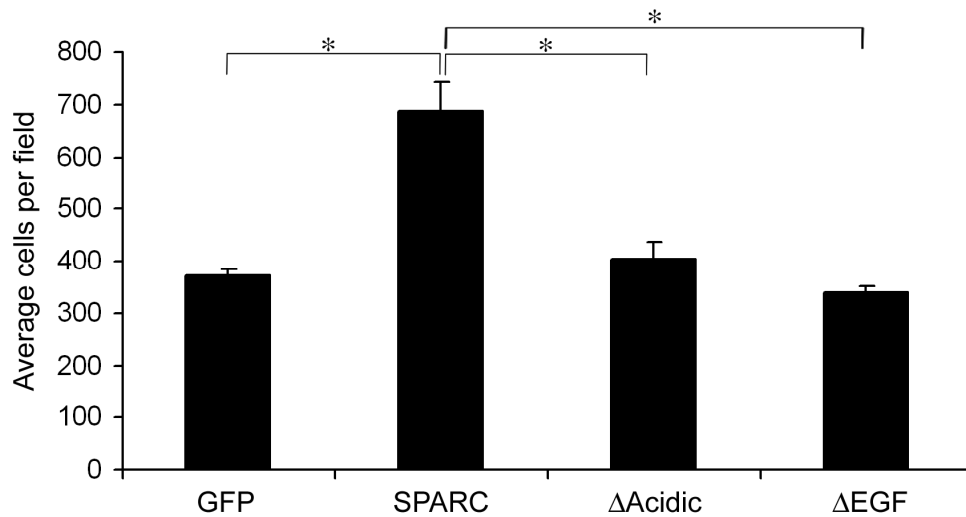
### **$\Delta$ Acidic and $\Delta$ EGF Reduce SPARC-Induced Activation of the p38 MAPK/HSP27 Pathway**

We have previously demonstrated that p38 MAPK and HSP27 mediate SPARC-induced migration (Golembieski et al., 2008). We used Western blot analysis to determine the effects of the deletions on this signaling pathway (fig.

A



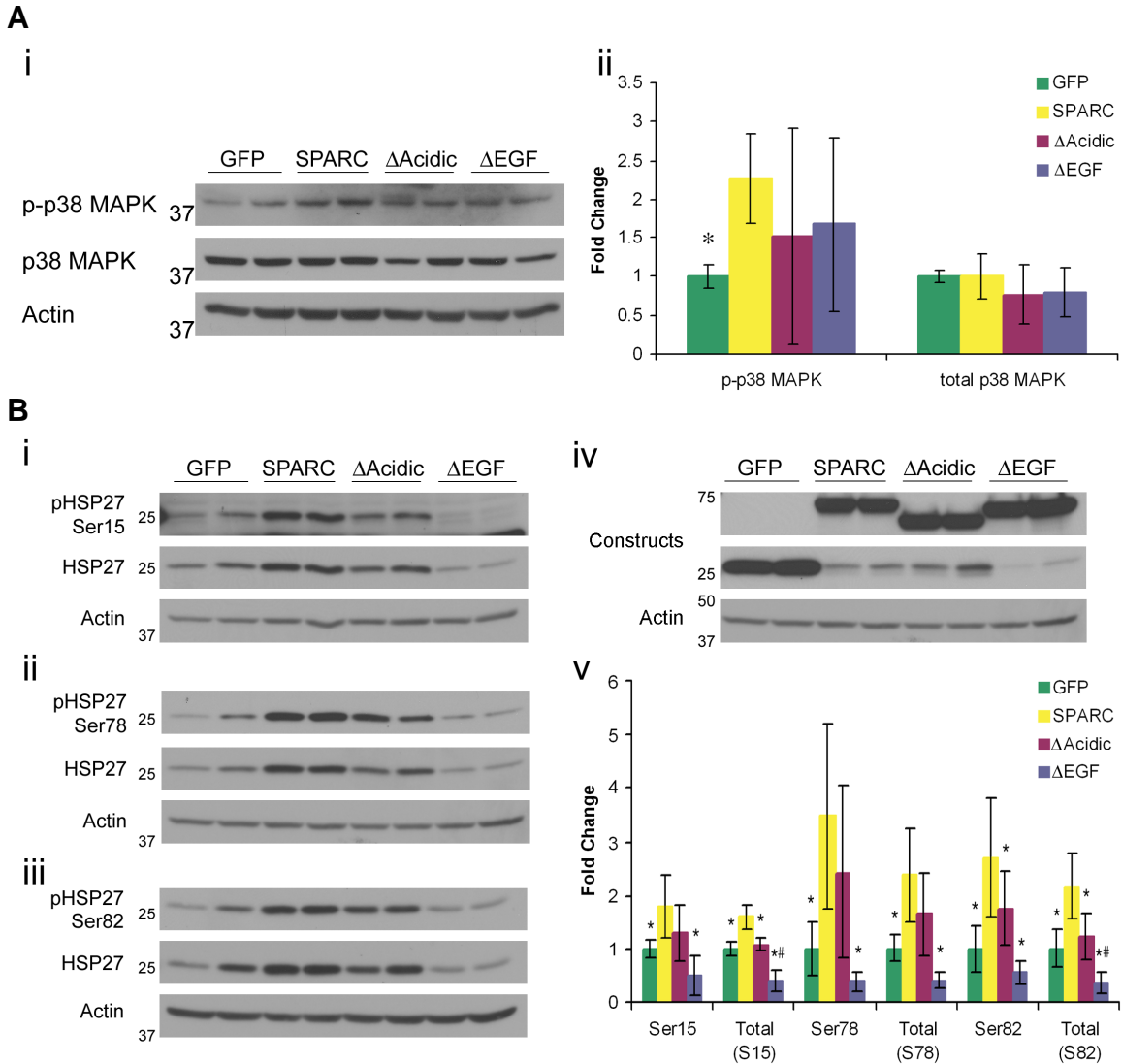
B



**Figure 14.  $\Delta$ Acidic and  $\Delta$ EGF reduce SPARC-induced migration through 8- $\mu$ m pores.** (A) Representative 10X images of one clone expressing each of the constructs after cells migrated through transwell filters with 8- $\mu$ m pores for 2 hr. (B) Average number of cells per field is shown. Expression of SPARC-GFP increases cell migration compared to control cells. Deletion of the Acidic Domain or the EGF-like Module reduces migration to control levels. \* indicates significantly less than SPARC  $p \leq 0.001$

15). As in our previous study, SPARC expression increased p38 MAPK phosphorylation (fig. 15A). The increased p38 MAPK activation is transient and was only significantly different at 3 hr and when the cells were plated on the highest level of FN tested (100  $\mu$ g/ml), indicating that the SPARC-induced activation of this pathway may be mediated through integrins. SPARC also increased the expression of HSP27 versus GFP controls with a corresponding increase in phosphorylation of HSP27 at all three serine residues (fig. 15Bi, ii, iii, and v). Deletion of the Acidic domain or the EGF-like module showed a trend for intermediate activation of p38 MAPK when compared to SPARC and GFP (fig. 15A). Deletion of the Acidic Domain resulted in a slight reduction in expression of HSP27 compared to SPARC; however, phosphorylation of HSP27 was only significantly less at Serine 82. HSP27 expression and phosphorylation was not significantly different between  $\Delta$ Acidic and GFP cells. The deletion of the EGF-like Module reduced expression of HSP27 compared to SPARC and  $\Delta$ Acidic cells; there was even a decrease in HSP27 expression compared to GFP cells. Phosphorylation of HSP27 in the  $\Delta$ EGF cells was significantly less than SPARC and  $\Delta$ Acidic cells, with a trend to decrease phosphorylation at all three sites compared to GFP cells.

Because the level of expression and phosphorylation of HSP27 in the  $\Delta$ EGF cells did not correlate with the level of migration in the wound assay and the level of expression and phosphorylation of HSP27 in  $\Delta$ Acidic cells did not correlate with the very low level of migration in the transwell assay, we looked at alternate signaling pathways that are known to be involved in migration to



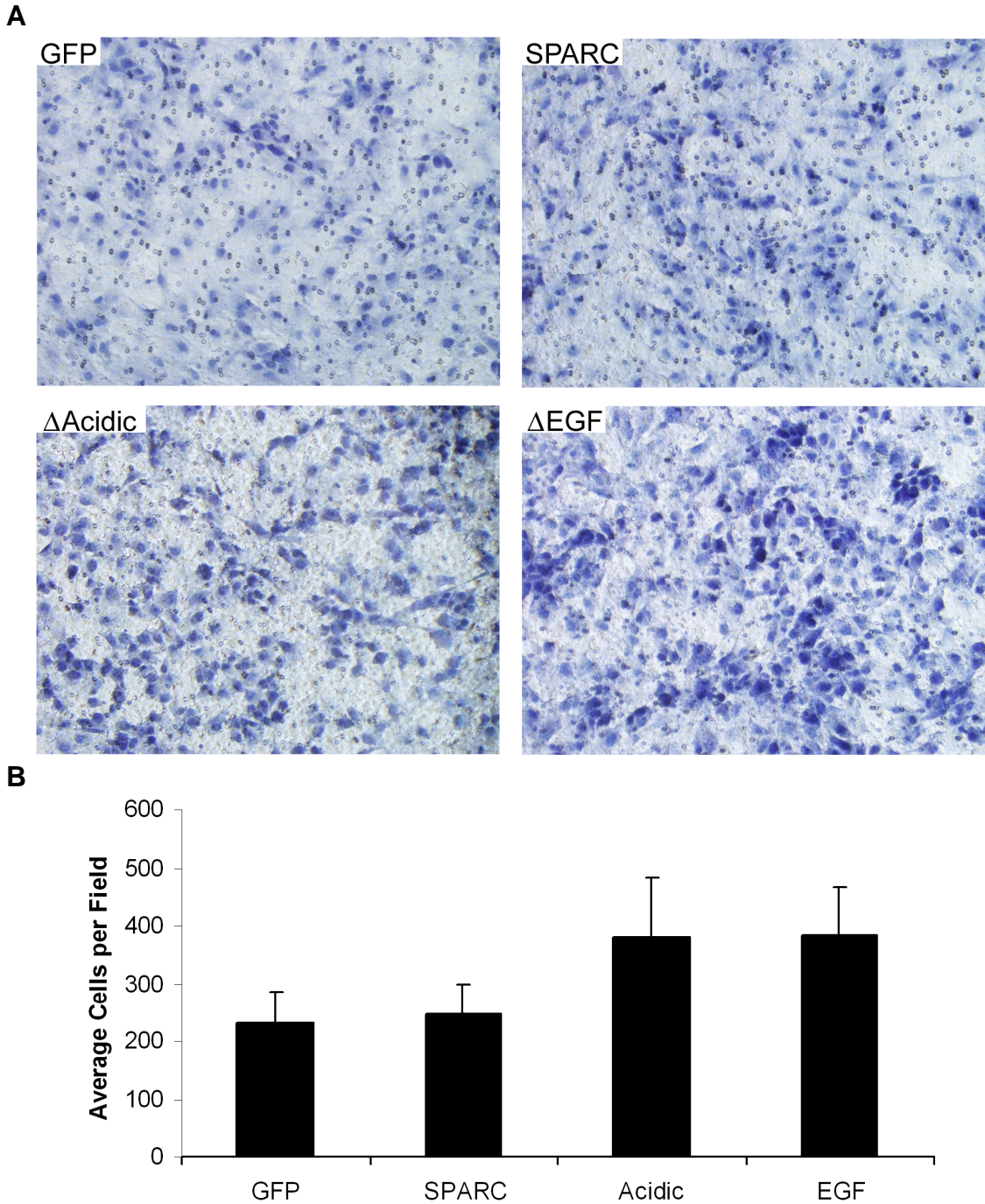
**Figure 15. Deletion of the Acidic Domain or EGF-like Module reduces SPARC-mediated signaling through the p38 MAPK/HSP27 signaling pathway.** (A i) Western blot analysis of expression and activation of p38 MAPK. (A ii) Densitometric analysis indicates that SPARC increases phosphorylation of p38 MAPK. The deletion mutants showed a trend for increased p38 activation, but were not significantly different from GFP- or SPARC-expressing cells. (B i-iii) Western blot analysis of HSP27 expression and phosphorylation at Serine 15 (i), Serine 78 (ii), and Serine 82 (iii). (B iv) Western blot confirming equal expression of the constructs. (B v) Densitometric analysis shows SPARC increases HSP27 expression and phosphorylation at all three Serines. Deletion of the Acidic Domain reduces HSP27 expression and phosphorylation at Serine 82. Total or phosphorylated HSP27 in  $\Delta$ Acidic was not significantly different from control cells. Deletion of the EGF-like Module decreased HSP27 expression and phosphorylation to, or below control levels. \* = Significantly less than SPARC ( $p < 0.05$ ), # = Significantly less than GFP ( $p < 0.016$ ). Molecular weights are indicated at the left of each blot.

determine whether they could be preferentially activated by one or both of the deletions. Others have shown that SPARC-induced migration is mediated in part through the activation of FAK (Shi et al., 2007). We examined members of FAK signaling pathways by Western blot and confocal imaging. We did not see consistent effects on FAK activity by SPARC or either of the deletion mutants under the conditions tested (data not shown). At the time points examined, there was also no change in the intracellular localization of phosphorylated FAK in the SPARC-expressing cells or in the deletion mutants compared to the GFP control cells as determined by confocal imaging (data not shown). Western blot analysis also indicated no change in the expression or activation of ERK or Myosin Light Chain, which are downstream effectors of FAK, under the conditions tested (data not shown).

### **SPARC-GFP and the Deletion Mutants Have No Effect on Glioma Cell Invasion Through Matrigel**

The decrease in migration by the deletion mutants compared to SPARC-expressing cells prompted us to investigate the effects of the deletions on cell invasion using transwell filters coated with Matrigel. While Matrigel is composed of different ECM proteins than was used in the wound assay and does not well recapitulate the ECM present in the brain, it is a standard *in vitro* model to assess invasion. We have previously reported that SPARC increases glioma invasion through Matrigel (Golembieski et al., 2008). Cells plated on Matrigel-coated



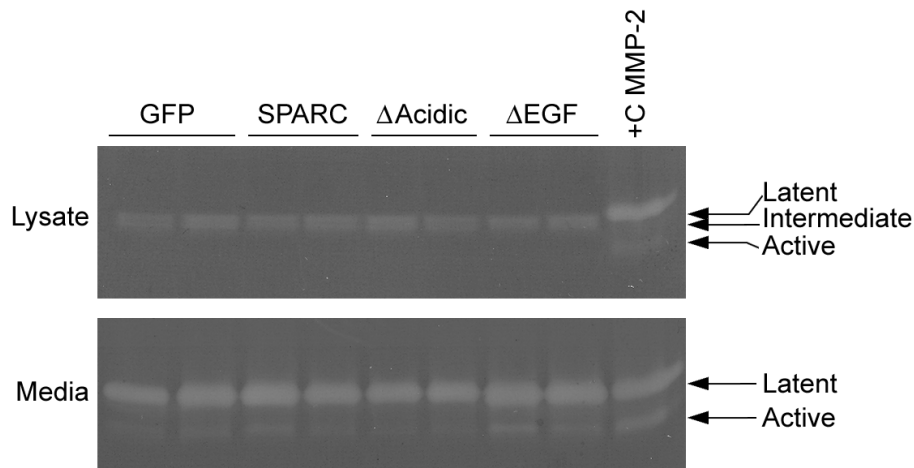
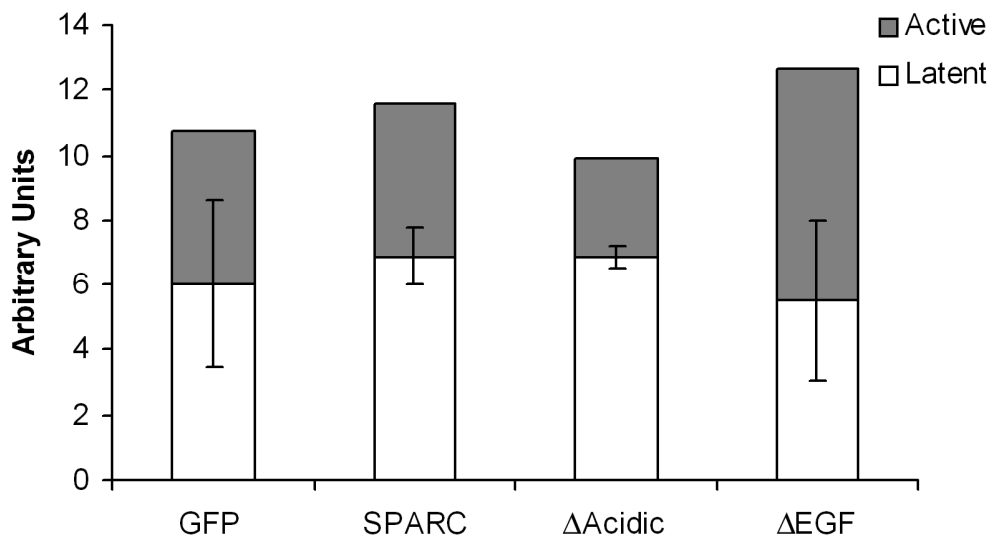


**Figure 16. Deletion Mutants show a trend to increase invasion through Matrigel.** (A) Representative 20X images of cells 20 hr after plating on Matrigel. (B) SPARC-GFP cells showed similar invasion to GFP-expressing cells. Both  $\Delta$ EGF and  $\Delta$ Acidic showed a trend for increased invasion. Results indicate the average of two experiments, 6 images per well, 5 wells per experiment.

transwell filters were allowed to invade for 20 hr. In contrast to our previous data, SPARC expression did not increase invasion compared to control cells under the conditions tested (fig. 16). The deletion mutants did, however, show a trend to increase invasion compared to GFP- and SPARC-expressing cells (fig. 16).

### **SPARC-GFP and the Deletion Mutants Did Not Change MMP-2 Expression or Activation**

We demonstrated in figure 3 (Chapter III) that wt-SPARC (without the GFP tag) increased expression and activation of MMP-2 when expressed in U87MG cells. Gilles et al., (1998) showed that a peptide corresponding to a portion of the Acidic Domain was involved in the SPARC-induced activation of MMP-2 in breast cancer. Therefore, we were interested in the effects of the deletion mutants on MMP-2 expression and activation. We performed gelatin zymography of cell lysates and conditioned medium. SPARC-GFP did not increase MMP-2 activity compared to GFP-expressing cells (Figure 17A and B). While this is in contrast to data shown in Chapter III, it is consistent with other observations in our lab using conditioned medium from clones expressing GFP and SPARC-GFP (Rempel unpublished observations). It is apparent that a tag as large as GFP, or its location at the C-terminus of SPARC, may interfere with some of the activity of SPARC. The gelatin zymography was performed several times; however the levels of MMP-2 in the lysates and the levels of active MMP-2 in the conditioned medium were too low to quantitate by densitometry except in one experiment each. There was no change in the levels of MMP-2 in the cell lysates between

**A****B**

**Figure 17. Deletion mutants show a trend to alter activation of MMP-2.** (A) The top panel indicates the levels of MMP-2 in cell lysates and indicates that there are no detectable levels of active MMP-2 in the cell lysates. The lower panel indicates the levels of latent and active MMP-2 in the conditioned medium. The zymogram shown is the only zymogram where active MMP-2 was measurable. (B) Quantitation of MMP-2 in the conditioned medium. Open bars represent the latent MMP-2 and are the average of three experiments. The gray bars indicate the levels of active MMP-2 in one zymogram (A).

any of the clones (quantitation not shown). The levels of latent MMP-2 in the conditioned medium in three experiments were quantifiable and are shown in figure 17B, but there was no significant change in any of the clones. The active MMP-2 in the conditioned medium shown in figure 17A was the only zymogram in which we were able to quantitate the levels of active MMP-2 (shown in 17B).  $\Delta$ Acidic shows a trend to decrease the levels of active MMP-2 and  $\Delta$ EGF shows a trend to increase active MMP-2; however, since only one zymogram had measurable levels of active MMP-2 and it appears that the GFP tag may be interfering with the activity of SPARC on MMP-2 regulation, additional experiments must be done to reliably determine the effects of the deletion mutants.

## Discussion

The evidence that different domains of SPARC may be involved in different functions of SPARC prompted us to question whether the deletion of specific domains would alter adhesion and/or signaling so as to reduce or eliminate SPARC-induced glioma cell migration. SPARC is highly expressed in gliomas and so the control, SPARC-GFP, and the two deletion mutant constructs that we created were expressed in U87MG glioma cells, which express low levels of SPARC. Deletion of the Acidic Domain or the EGF-like Module of SPARC did not appear to significantly affect the processing and secretion of the protein, as assessed by fluorescence (fig. 8) and confocal (fig. 10) imaging and Western blot analyses (fig. 9). Both deletion mutants were localized to the trans Golgi network (fig. 10) and were secreted (fig. 9), suggesting that they are glycosylated like wt-SPARC. These data suggest that the constructs could be used to examine the biological effects of the deletions on SPARC function in cell adhesion, migration, invasion, and signaling.

Previous studies of SPARC peptides involving cell adhesion (discussed in Chapter IV Introduction) prompted us to investigate the effects of the deletion mutants on adhesion. Cell adhesion can be affected by the type and concentration of ECM, expression levels of integrins, and expression of de-adhesive proteins such as the matricellular proteins. To assess adhesion, we examined  $\beta 1$  integrin expression and the affects of increasing levels of FN on adhesion. FN is not a major component of the matrix in the brain except in blood vessel basement membranes (Mahesparan et al., 2003). We chose to use FN for

these studies because gliomas tend to migrate along blood vessels (Bellail et al., 2004; Rempel and Mikkelsen, 2006). FN is required for the deposition of other ECM proteins and for the proper organization of the ECM (Sottile and Hocking, 2002; Velling et al., 2002). FN is secreted by gliomas (Ohnishi et al., 1998) and gliomas express FN receptors (Ohnishi et al., 1998; Rempel and Mikkelsen, 2006). Tumor cells may also induce host cells to produce ECM proteins including FN (Mahesparan et al., 2003). In addition, the activity of SPARC is carried out, at least in part, through integrins (Barker et al., 2005), prompting us to use a matrix that engages integrins.

In this study, we demonstrated that SPARC increases adhesion on high levels of FN compared to GFP control cells. Deletion of the Acidic Domain increased adhesion compared to GFP- and  $\Delta$ EGF-expressing cells on all levels of FN and increased adhesion to a greater extent than SPARC on the highest level of FN tested. Deletion of the EGF-like Module showed a trend for decreased adhesion on all concentrations of FN compared to SPARC and GFP cells and were significantly less adherent than GFP cells on the lowest level of FN. The data suggest that on FN, the binding of SPARC with FN and with  $\beta$ 1 integrin may act to reinforce the integrin binding to the matrix; however, the Acidic Domain has de-adhesive properties (Lane and Sage, 1990; Murphy-Ullrich et al., 1995). Therefore, the balance between these two characteristics causes the cells to be similarly adherent to the GFP-expressing cells (fig. 12), but the effects of the Acidic Domain can be overpowered by increasing the concentration of FN, resulting in increased adhesion. This is also observed by the deletion of

the EGF-like Module. In this construct the Acidic Domain is positioned closer to the  $\beta 1$  integrin binding site on SPARC and so, at least at lower levels of FN, it may have a greater ability to disrupt the interaction between  $\beta 1$  and FN, resulting in decreased adhesion. As with SPARC, this effect is overcome by increasing the concentration of FN. Deletion of the Acidic Domain results in increased adhesion due to the loss of the de-adhesive domain. The Acidic Domain is also involved in the suppressive effect of SPARC on FN production (Lane et al., 1992), and so deletion of this domain would result in greater deposition of FN compared to SPARC and  $\Delta$ EGF, resulting in increased adhesion. Additionally, a construct lacking the Acidic Domain showed increased affinity for Collagen IV (Maurer et al., 1995). Our data suggest that  $\Delta$ Acidic may also have increased affinity for FN, causing these cells to be more adherent than GFP-expressing cells.

Studies have shown that SPARC modulates adhesion through the decreased cell surface expression of  $\beta 1$  and  $\alpha v$  integrins (Said et al., 2007), both of which can participate in FN binding. We found no change in the expression of  $\beta 1$  integrin in whole cell lysates in our cells (data not shown); however, levels of  $\alpha v$  and the localization of integrins were not investigated. The mechanism for the SPARC-mediated decrease in integrin expression is not known; however, if the Acidic Domain is involved, then deleting the Acidic Domain may result in an increase in integrin  $\alpha v$ , which could result in increased attachment to FN compared to SPARC-expressing cells. However, it is more likely that the deletions affect adhesion through changes in the interaction between SPARC

and  $\beta 1$  integrin. This hypothesis is further supported by the differences observed in uptake of the deletion mutants by naïve cells.

In adipose stromal cells, SPARC is internalized by binding to  $\alpha 5\beta 1$  integrin (Nie et al., 2008). While the mechanism for uptake of SPARC in glioma cells is unknown,  $\alpha 5\beta 1$  integrin is a candidate receptor as U87MG cells express  $\alpha 5\beta 1$  (Maglott et al., 2006). Uptake of SPARC and the deletion mutants may be through  $\beta 1$  integrin since both deletion mutants have the region of SPARC that binds to  $\beta 1$  integrin (Weaver et al., 2008); however, uptake of  $\Delta$ EGF was reduced, while  $\Delta$ Acidic was enhanced compared to SPARC (fig. 11). The EGF-like Module is in the same domain as the  $\beta 1$  integrin binding site and is in close proximity to it in the secondary structure (fig.1). The data suggest that while the EGF-like Module is not required for binding and internalization of SPARC, it may increase the affinity of SPARC for  $\beta 1$  integrin. This agrees with an observation by Yost and Sage (1993), who demonstrated that a peptide mimicking the EGF-like Module was a more potent competitor for cell surface binding than wt-SPARC. Therefore, the deletion of the EGF-like Module would likely decrease binding to a cell surface receptor such as  $\beta 1$  integrin. Deletion of the Acidic Domain, which places the EGF-like Module at the N-terminus of the mature protein (fig. 7), resulted in enhanced uptake compared to SPARC, which further suggests that the EGF-like Module promotes the binding of SPARC to  $\beta 1$  integrin.

While only  $\Delta$ Acidic increased adhesion, both deletion mutants decreased migration when compared with wt-SPARC-GFP-expressing cells. The extent of the effects on migration was dependent on the assay used to measure migration.



In the wound assay, cells were plated on FN and migration was along a flat surface, without spatial constraints (fig. 13A). In the transwell assay, there was no matrix added to the inserts, but the cells had to move through the pores in the filters (fig. 14A), which would provide spatial constraint. The deletion mutants reduced migration compared to SPARC; however the cells migrated significantly more than the control cells in the wound assay (fig. 13), but similarly to control cells in the transwell assay (fig. 14). The differences between the two assays indicate that the actions of the deletion mutants are dependent on the matrix or that they are involved in SPARC signaling to Myosin II. Myosin II is required for glioma migration through the pores of transwell filters (Beadle et al., 2008) and both deletions reduced migration to control levels in the transwell migration assay. While we did not see changes in phosphorylated Myosin Light Chain by Western blot under the conditions tested, others have shown that SPARC activates Myosin through ILK-mediated activation of Myosin Light Chain or inhibition of Myosin Light Chain Phosphatase, which is an inhibitor of Myosin Light Chain (Barker et al., 2005). However, we have previously demonstrated that SPARC induced migration in the wound assay as well as invasion through Matrigel coated transwell filters is inhibited by HSP27 siRNA (Golembieski et al., 2008), indicating that SPARC-induced migration, whether on a surface or through a pore, is mediated by HSP27. Additionally, in the invasion assay, which also uses the same transwell filters as in the transwell migration assay, both deletion mutants showed a trend to increase invasion compared to SPARC-GFP-expressing cells, indicating that the mutations do not compromise the Myosin-

mediated migration that cells employ when they are under spatial constraint. Based on these results and the changes in binding of the constructs to  $\beta$ 1 integrin discussed above, it is more likely that the differences observed between the two assays are due to the presence or absence of FN.

We have previously shown that SPARC-induced migration is mediated through the increased expression and phosphorylation of HSP27. In cells expressing  $\Delta$ Acidic, the decreased migration (figs. 13 and 14) correlated with decreased expression of HSP27 and phosphorylation at serine 82 (fig. 15B) compared to SPARC-expressing cells. The  $\Delta$ Acidic cells were not significantly different from the GFP cells in the level of HSP27 expression and phosphorylation (fig. 15), which correlates well with the similar level of migration as measured by the transwell assay (fig. 14). Additionally, there was a trend for an increase in phosphorylation of HSP27 at serines 78 and 82 in  $\Delta$ Acidic compared to GFP cells, which may explain the slight increase in migration in the wound assay (fig. 13). Considering the evidence that this construct is able to bind to integrins, the data suggest that the Acidic Domain is important in the activation of SPARC-induced signaling through the p38 MAPK/HSP27 pathway.

Despite the low levels of HSP27, clones expressing  $\Delta$ EGF migrated a greater distance than the  $\Delta$ Acidic and GFP cells in the wound assay (fig. 13). Deletion of the EGF-like Module resulted in a reduction in the expression of HSP27 to less than GFP-control levels; however, the levels of phosphorylated HSP27 in the  $\Delta$ EGF cells were not significantly less than GFP cells. The relative levels of phosphorylated and unphosphorylated HSP27 may be critical in the

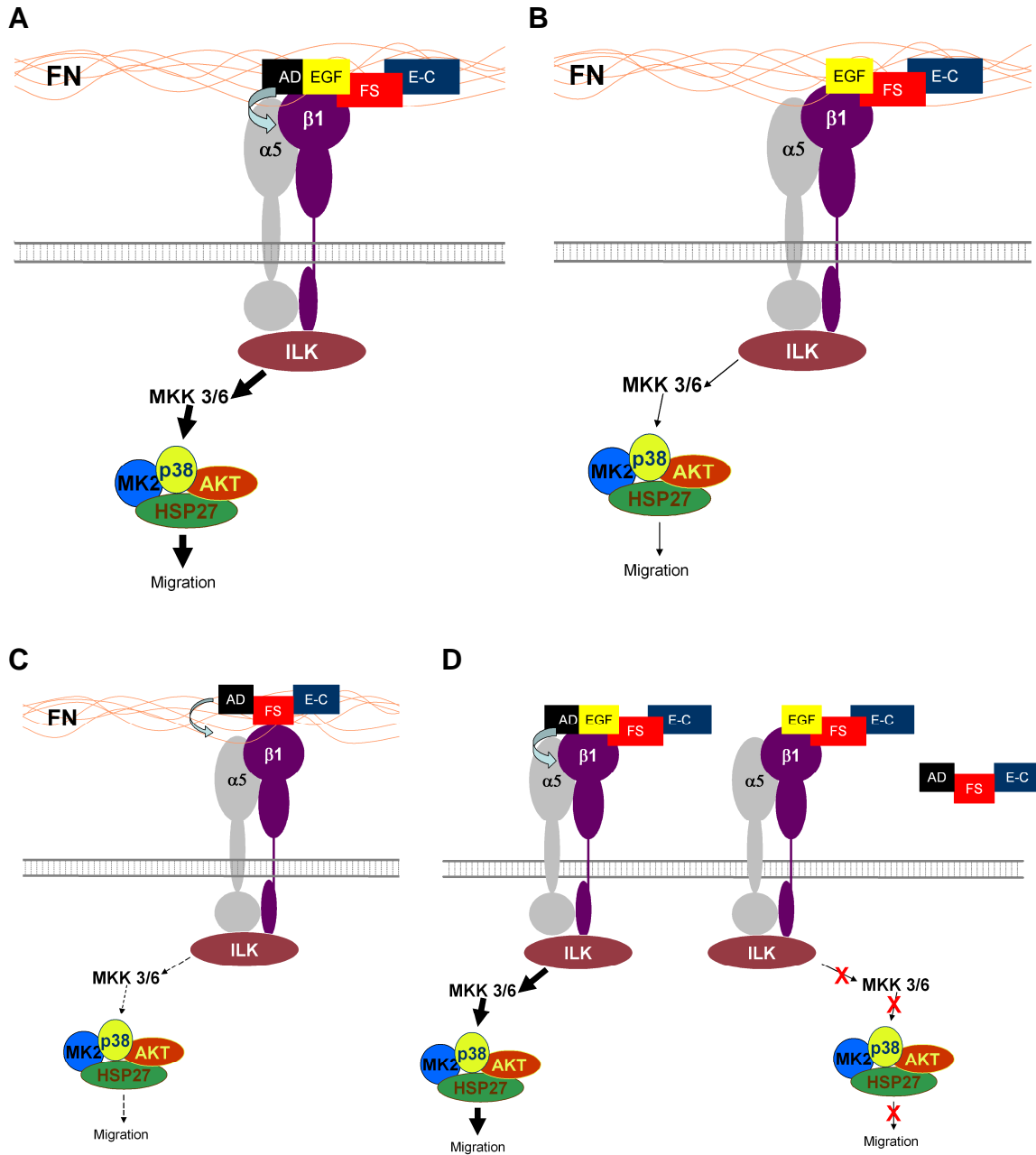
function of this protein. Unphosphorylated HSP27 acts as an actin capping protein, preventing polymerization and migration, while phosphorylated HSP27 stabilizes the actin filaments at the base of the lamellipodia, preventing depolymerization and facilitating migration (Pichon et al., 2004). The high degree of phosphorylation relative to the low level of total HSP27 present in the  $\Delta$ EGF cells may be able to promote migration due to a low availability of unphosphorylated HSP27 to contribute to actin capping. The mechanism by which SPARC upregulates HSP27 is unknown. It is clear from our studies that the EGF-like Module is essential in SPARC-mediated upregulation of HSP27. Further studies of this construct may yield insight into the SPARC – HSP27 relationship.

In contrast to results shown in Chapter III, there was no change in MMP-2 expression or activation in control versus SPARC expressing cells (fig. 17). An independent investigation with other U87MG-derived clones expressing GFP and SPARC-GFP fusion constructs confirmed that these results may be due to the presence of the GFP-tag (Rempel, unpublished observations). However, the deletion mutants did affect MMP-2 activation. While only one zymogram had measurable levels of active MMP-2 in the conditioned medium, this preliminary data suggest that the Acidic Domain may promote the activation of MMP-2. When the Acidic Domain is deleted, MMP-2 activation decreased compared to GFP- and SPARC-expressing cells, which is consistent with data reported by Gilles et al., (1998). When the EGF-like Module was deleted, MMP-2 activation

increased; however, additional studies must be done to determine the consistency and significance of these results.

There was also no change in invasion through Matrigel between SPARC and GFP cells (fig. 16). This is consistent with the level of MMP-2 activity, but is not consistent with our previous results (Golembieski et al., 2008), in which the expression of SPARC-GFP increased invasion compared to GFP-expressing cells. While this may be due to clonal variability, as the transfections were done at a different time, using different methods and into a different passage number of U87MG cells, it is more likely due to differences in Matrigel preparations (Hughes et al., 2010).

Based on our results, we propose the following model (fig. 18). SPARC binds FN through the E-C Domain and binds  $\beta 1$  integrin through the C-terminal region of the FS Domain. Binding to  $\beta 1$  is enhanced by the EGF-like Module. The interaction of these SPARC domains with  $\beta 1$  and FN supports integrin – FN binding; however, the Acidic Domain interferes with the binding of the integrin with FN. Therefore, in this complex, there is a balance between the de-adhesive Acidic Domain and the FS and E-C Domains which are helping to tether the integrin to the matrix. However, the effects of the Acidic Domain can be overcome at higher levels of FN, increasing adhesion. The Acidic Domain is also involved in the activation of ILK and downstream signaling pathways, which may also result in the induction of MMP-2 and a decrease in FN production. Therefore, deletion of the Acidic Domain results in a construct that can bind to the integrin very well, but does not disrupt the adhesion complex and cannot



**Figure 18. Working model.** The constructs are illustrated by the sum of the domains/module; AD = Acidic Domain (black), EGF = EGF-like Module (yellow), FS = Follistatin-like Domain (red), EC = Extracellular Calcium Binding Domain (blue) and are illustrated without the GFP-tag for simplicity. Note that the size of the arrows indicates relative level of activation or change. (A-C) When cells are plated on FN, (A) WT-SPARC binds to FN and  $\beta$ 1 integrin. The interaction with  $\beta$ 1 integrin activates p38 MAPK and HSP27, most likely through ILK, resulting in cell migration. (B) Deletion of the Acidic Domain results in a construct with increased ability to bind FN and integrins, which results in increased cell adhesion, but without the Acidic Domain it has less ability to activate ILK and downstream signaling pathways. (C)  $\Delta$ EGF binds to FN, which localizes the construct near integrins, but it has reduced capacity to bind the integrins. The presence of the Acidic Domain reduces adhesion when FN is low and also can induce signaling; however, since HSP27 is reduced, cells expressing this deletion have reduced migration compared to SPARC. (D) When FN is not present, SPARC and  $\Delta$ Acidic can still bind to  $\beta$ 1 integrin, but only SPARC can activate signaling pathways that induce migration. Without FN,  $\Delta$ EGF is not localized to the integrins and so does not bind and migration is reduced to control levels.

activate ILK and downstream signaling pathways such as p38 MAPK/HSP27 as effectively as SPARC. This construct also does not suppress FN production, which contributes to the observed increase in adhesion. The decreased p38 MAPK/HSP27 signaling combined with the increased adhesion, results in decreased migration. Deletion of the EGF-like Module yields a construct that does not bind to the integrin efficiently; however, because the construct binds FN, it can be brought into close proximity with integrins. The small amount that does bind has an enhanced ability to disrupt the adhesion complex because when the EGF-like Module is deleted, the de-adhesive Acidic Domain is placed closer to the  $\beta 1$  binding site on SPARC. However this deadhesive property is eliminated by increasing the concentration of FN. The reduced ability for this construct to bind to the integrin results in loss of p38 MAPK/HSP27 signaling and decreased migration.

In the absence of matrix, as in the transwell assay, SPARC is still able to bind to integrins and induce signaling for migration.  $\Delta$ Acidic can also bind to integrins, but does not induce the signaling required for migration, resulting in reduced migration compared to SPARC. The binding of  $\Delta$ EGF is even further reduced in the absence of FN because there is no matrix for the construct to bind and therefore  $\Delta$ EGF is not recruited to the integrins, resulting in reduced migration.

Studies have implicated SPARC or SPARC peptides as a therapy for cancers in which SPARC proves a positive prognostic marker (Atorrasagasti et al., 2010; Chlenski and Cohn, 2010). Expression of the  $\Delta$ Acidic construct

increased glioma cell adhesion and reduced SPARC-induced migration and signaling through HSP27, though the effects on invasion are unclear. While some of the effects of this deletion mutant must be examined in more detail, the  $\Delta$ Acidic construct may prove effective in the treatment of cancers where SPARC is associated with good or poor prognosis. Others have shown that peptides that mimic all or part of the EGF-like Module were effective in blocking angiogenesis associated with neuroblastoma (Chlenski et al., 2004; Chlenski and Cohn, 2010).  $\Delta$ Acidic has an intact EGF-like Module and the absence of the Acidic Domain places the EGF-like Module at the N-terminus of the protein, resulting in a protein that can compete for SPARC binding with limited induction of signaling. Therefore this construct may retain this anti-angiogenic activity. Further analysis of these deletion mutants will give more insight into the effects on SPARC-expressing tumors.



## CHAPTER V FUTURE DIRECTIONS

To completely appreciate the effects of these deletions on SPARC activity, it will be necessary to create the constructs without the GFP tag. It may also be beneficial to express the constructs in cells that do not express SPARC. This would be possible using SPARC siRNA as the 3' untranslated region that the siRNA binds is not present in our constructs; however the effects of the transfection reagents may add another level of complexity to the interpreting the results. Additional studies such as cross-linking and/or co-immunoprecipitation of the constructs with  $\beta$ 1 integrin will give better insight into the interactions of SPARC and the integrin. In addition, since current *in vitro* models do not adequately recapitulate the brain parenchyma, studying these deletion mutants *in vivo*, in a rat xenograft model as previously described by our lab (Schultz et al., 2002, Thomas et al., 2010), would provide information about how the deletions affect glioma invasion in the brain.

Since deletion of the Acidic Domain causes increased adhesion, decreased migration, and potentially reduced MMP-2 activity, compared to SPARC, it would be beneficial to examine whether this deletion mutant can compete for binding with wt-SPARC. If it does indeed compete for binding, and can reduce the invasive capacity of the tumor cells, this deletion mutant could prove to be a valuable therapy in tumor expressing high levels of SPARC.

Further studies of the  $\Delta$ EGF clones may also provide information on the mechanism by which SPARC upregulates HSP27. Since HSP27 is also involved

in survival pathways, information about its regulation by SPARC may also yield a potential target for therapy in SPARC-expressing tumors.

**APPENDIX**  
**PREVIOUSLY PUBLISHED DATA**

The image shown in figure 1 was adapted from a figure in the following manuscript:

Bradshaw AD and Sage EH (2001) SPARC, a matricellular protein that functions in cellular differentiation and tissue response to injury. *J Clin Invest* 107:1049-1054.

The content contained in Chapter III including figures and the methods pertaining to those experiments were previously published in the following manuscript:

McClung HM, Thomas SL, Osenkowski P, Toth M, Menon P, Raz A, Fridman R and Rempel SA (2007) SPARC upregulates MT1-MMP expression, MMP-2 activation, and the secretion and cleavage of galectin-3 in U87MG glioma cells. *Neurosci Lett* 419:172-177.

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**ABSTRACT**  
**THE ROLE OF THE SPARC ACIDIC DOMAIN AND EGF-LIKE MODULE IN  
GLIOMA MIGRATION, INVASION, AND SIGNALING**

by

**HEATHER M. MCCLUNG**

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**Advisor:** Sandra A. Rempel, Ph.D.

**Major:** Pharmacology

**Degree:** Doctor of Philosophy

We have previously shown that Secreted Protein Acidic and Rich in Cysteine (SPARC) is upregulated in all astrocytoma grades and increases tumor cell migration and invasion. It is thought that different domains within the protein may regulate SPARC functions, suggesting domain-specific targeting to inhibit invasion. To enhance our understanding of SPARC-mediated invasion, we first confirm, at the protein level, our previous cDNA array results, that SPARC increases expression of the matrix metalloproteases (MMPs) MT1-MMP and MMP-2. We also demonstrate that SPARC increases MMP-2 activation and the secretion and processing of galectin-3, a known target of MMPs. To investigate the roles of specific domains, we used a SPARC-GFP fusion protein and deletion mutant constructs of SPARC-GFP with deletions of either the Acidic Domain ( $\Delta$ Acidic) or EGF-like Module ( $\Delta$ EGF). We confirm our previous findings that SPARC-GFP increased migration and activation of p38 MAPK and HSP27 signaling compared to GFP control cells.  $\Delta$ Acidic increases cell adhesion and

reduces SPARC-induced migration and p38 MAPK/HSP27 signaling.  $\Delta$ EGF decreases SPARC-induced migration and dramatically decreases the expression and phosphorylation of HSP27. The extent to which the deletions reduced migration was dependent upon the presence of extracellular matrix. Preliminary data also suggest that the deletions affect invasion and MMP-2 activation. In conclusion, both regions of interest regulate SPARC-induced migration and signaling through the p38 MAPK/HSP27 signaling pathway. Importantly, their impact on migration is influenced by the presence or absence of extracellular matrix. This and future studies of the deletion mutants will provide valuable insight into new strategies that effectively target invasion in SPARC-expressing tumors.

**AUTOBIOGRAPHICAL STATEMENT****HEATHER M. MCCLUNG****EDUCATION**

Wayne State University, Detroit, MI

Doctor of Philosophy, Pharmacology (2011)

University of Detroit Mercy, Detroit, MI

Bachelor of Science, Biology (2003)

Portland High School, Portland, MI (1999)

**PUBLICATIONS**

Golembieski WA, Thomas SL, Schultz CR, Yunker CK, **McClung HM**, Lemke N, Cazacu S, Barker T, Sage EH, Brodie C and Rempel SA (2008) HSP27 mediates SPARC-induced changes in glioma morphology, migration, and invasion. *Glia* 56:1061-1075.

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