LEBANESE AMERICAN UNIVERSITY

**The Regulation of RhoA in Focal Adhesions by StarD13 is
Essential for Astrocytoma Cell Motility**

By

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The Regulation of RhoA by StarD13 in Focal Adhesions is Essential for Astrocytoma Cell Motility

Bassem D. Khalil

ABSTRACT

Malignant astrocytomas are associated with poor prognosis and high morbidity and mortality rates despite intensive treatment. These tumors rarely metastasize into distant regions of the body; however, they are highly invasive into adjacent and distant regions of the normal brain, which renders them surgically and medically unmanageable and accounts for their fatal outcome. Invasion is a multistep process, which ultimately requires the cell to actively migrate through the ECM. Proper cell adhesion dynamics, involving the assembly and the disassembly of adhesion, is essential for the completion of the motility cycle. Rho-GTPases, mainly RhoA, Rac, and Cdc42, play a major role in the regulation of the processes that ultimately lead to cell migration. StarD13 is a RhoGAP that inhibits the function of RhoA and Cdc42.

We first aimed at determining the role of RhoA in the progression of astrocytic tumors, a topic that is still controversial in the literature. Our results showed that RhoA plays a positive role in this aspect.

Our current study also investigates the roles of RhoA, Rac1, and StarD13 in cell adhesion and cell migration. Our results showed that RhoA, Rac1, and StarD13 are essential for astrocytoma cell migration. Rac1 plays a role in the formation of focal complexes, which ultimately mature into focal adhesions under the action of RhoA. StarD13 plays a role in the inhibition of RhoA in focal complexes, a process that seems indispensable for astrocytoma cell migration.

Keywords: StarD13, Rho GTPases, RhoA, Rac1, Astrocytoma

TABLE OF CONTENTS

List of Figures	x
List of abbreviations	xi
1. Literature review	1-20
1.1. Brain tumors	1
1.1.1. Statistics	1
1.1.2. Classification	1
1.2. Astrocytoma	2
1.3. Glioma invasion	3
1.3.1. Glioma invasion is a multistep process	3
1.3.2. Factors involved in glioma invasion	3
1.4. Cell motility	5
1.5. Rho GTPases	7
1.5.1. General characteristics of Rho GTPases	7
1.5.2. Rho GTPases function as molecular switches	8
1.5.3. Regulation of Rho GTPases	9
1.5.4. Upstream signaling to Rho GTPases	10
1.5.5. Downstream effectors of Rho GTPases	11
1.6. Role of Rho GTPases in cancer and metastasis	14
1.6.1. The role of Rho GTPases in cell motility	14
1.6.2. Altered Rho GTPase expression and signaling in cancer and metastatic cells	15
1.7. Altered Rho GTPase signaling in brain tumors	16
1.8. StarD13	19
1.9. Purpose of the study	20

2. Materials and methods	21-26
2.1. Cell culture	21
2.2. Antibodies and reagents	21
2.3. Cell transfection with siRNA	21
2.4. Cell transfection with vectors	22
2.5. Western blotting	22
2.6. Förster Resonance energy transfer (FRET)	23
2.7. Immunohistochemistry	24
2.8. Immunostaining	24
2.9. Pull down assay	24
2.10. Wound healing assay	25
2.11. Motility assay	25
2.12. Adhesion assay	25
2.13. Statistical analysis	26
 3. Results	 28-47
3.1. RhoA plays a role in the progression of astrocytic tumors	28
3.1.1. RhoA is overexpressed in grade IV astrocytoma tissues	28
3.1.2. RhoA shows higher activation in malignant astrocytoma cell lines	28
3.2. Roles of RhoA and Rac1 in astrocytoma cell motility	30
3.2.1. RhoA knockdown inhibits cell motility	30
3.2.2. Dominant active RhoA inhibits cell motility	33
3.2.3. Rac1 knockdown inhibits cell motility	35
3.3. Roles of RhoA and Rac1 in cell adhesion dynamics	37
3.3.1. Rac localizes to and is active at the cell edge	37
3.3.2. Rac1 is needed for the formation of focal complexes	38
3.3.3. Rho is needed for the maturation of focal complexes into focal adhesions	39
3.3.4. Dominant active RhoA leads to the formation of large focal adhesions	39

3.4. RhoA undergoes cycles of activation and inactivation	41
3.5. StarD13 localizes to focal adhesions and inhibits the activity of RhoA	43
3.6. Role of StarD13 in astrocytoma cell motility and adhesion	45
3.6.1. StarD13 knockdown inhibits cell motility	45
3.6.2. StarD13 knockdown stabilizes focal adhesions and increases astrocytoma cell adhesion	47
4. Discussion	49-52
5. Conclusions	53-54
6. Bibliography	54-64

LIST OF FIGURES

Figure	Page
Figure 1: Local recurrence of glioblastoma	3
Figure 2: Mechanism of glioma invasion	5
Figure 3: The cell motility cycle	6
Figure 4: Roles of Rho, Rac, and Cdc42 in cell motility	7
Figure 5: Regulation of Rho GTPases by GEFs, GAPs, and GDIs	10
Figure 6: Downstream effectors of Rho in cell motility	12
Figure 7: Model for formation of focal adhesions induced by the Rho/Rho-kinase pathway	13
Figure 8: Downstream effectors of Rac and Cdc42 in cell motility	14
Figure 9: The RhoA FRET biosensor	27
Figure 10: Rho expression levels in different grades astrocytomas	29
Figure 11: RhoA activation in astrocytoma cell lines	30
Figure 12: RhoA is required for cell motility-1	31
Figure 13: RhoA is required for cell motility-2	32
Figure 14: Constitutively active RhoA inhibits cell motility	34
Figure 15: Constitutively active RhoA inhibits cell motility-2	35
Figure 16: Rac is needed for cell motility-1	36
Figure 17: Rac is needed for cell motility-2	37
Figure 18: Rac is needed for formation of focal complexes	38
Figure 19: Rho is needed for formation of focal adhesions	40
Figure 20: Rho undergoes cycles of activation and inactivation	42
Figure 21: StarD13 localizes to focal adhesions	44
Figure 22: StardD13 is a specific GAP for Rho and Cdc42	45
Figure 23: StardD13 is needed for cell motility-1	46
Figure 24: StardD13 is needed for cell motility-2	47
Figure 25: StarD13 knock down leads to larger adhesions	48
Figure 26: Proposed model for astrocytoma cell motility	54

LIST OF ABBREVIATIONS

WHO: World Health Organization

CNS: Central Nervous System

ECM: Extracellular matrix

NCAM: Neural cell adhesion molecule

ADAM: A disintegrin and metalloproteinase

MMP: Matrix metalloproteinase

MT-MMP: Membrane-type matrix metalloproteinase

uPA: Urokinase

uPAR: Urokinase receptor

GTPase: Guanosine triphosphatase

Rac1: Ras-related C3 botulinum toxin substrate 1

Cdc42: Cell division cycle 42

Rho A: Ras homologous member A

Arp2/3: Actin-related protein 2/3

PIP5-Kinase: Phosphatidylinositol-4-phosphate 5-kinase

PIP2: Phosphatidyl 4,5-bisphosphate

Ras: Rat sarcoma

Arf/Sar1: Adenosine diphosphate (ADP) ribosylation factor 1/ secretion-associated RAS-related protein 1

Rab/Ran: Ras-related protein Rab/Ras-related nuclear protein

GDP: Guanosine diphosphate

GTP: Guanosine triphosphate

GEF: Guanine Nucleotide Exchange Factor

GAP: GTPase Activating Protein

GDI: Guanine Nucleotide Dissociation Inhibitor

PH: Pleckstrin homology

DH: Dbl homology

PI3K: Phosphatidylinositol 3-kinase

PIP3: phosphatidylinositol (3,4,5)-triphosphate

FAK: Focal Adhesion Kinase

MLC: Myosin Light Chain

ROCK: Rho Kinase

mDia: Mammalian homolog of diaphanous

LIMK: LIM kinase

ERM: Ezrin-radixin-moesin

WASP: Wiskott-Aldrich Syndrome Protein

SCAR: Suppressor of Cyclic AMP

WAVE: WASP-Family Verprolin-Homologous

PAK: p21 activated kinases

MLCK: Myosin Light Chain Kinase

LPA: Lysophosphatidic acid

S1P: Sphingosine-1-phosphate

PTEN: Phosphatase and tensin

IQGAP1: IQ motif containing GTPase activating protein

ELMO1: Engulfment and Cell Motility 1

Dock180: Dedicator of cytokinesis 180

StarD13: StAR-related lipid transfer domain containing 13

START: STAR-related lipid transfer

DLC-2: Deleted in Liver Cancer 1

RT-PCR: Reverse Transcription Polymerase Chain Reaction

ERK1: Extracellular signal-regulated kinases 1

DMEM: Dulbecco's Modified Eagle Medium

FBS: Fetal Bovine Serum

siRNA: Small interfering Ribonucleic Acid

GFP: Green Fluorescent Protein

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

PVDF: Polyvinylidene fluoride

PBS: Phosphate Buffered Saline

ECL: Enhanced Chemiluminescence

FRET: Förster Resonance energy transfer

YFP: Yellow Fluorescent Protein

CFP: Cyan Fluorescent Protein

BSA: Bovine Serum Albumin

EGF: Epidermal Growth Factor

GST: Glutathione S-transferase

CRIB: Cdc42/Rac interactive binding

RBD: Rhotekin binding domain

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

ROI: Region of interest

DLC-1: Deleted in Liver Cancer 1

LITERATURE REVIEW

1.1. Brain tumors

1.1.1. Statistics

In 2010, The American Cancer Society reported 22,020 new brain and other nervous system cancer cases, accounting for approximately 1.4% of all the reported new cancer cases in the United States. It also reported that the estimated number of death cases from brain and other nervous system tumors in the United States in 2010 is 13,140, which corresponds to around 2.3% of the total estimated cancer death cases (American Cancer Society, 2010).

The incidence rate of primary malignant brain and central nervous system tumors is 6.4 cases per 100,000 person-years (Horner, et al., 2009). This rate of occurrence is higher in males than it is in females (7.6 compared to 5.4 per 100,000 person-years respectively). The lifetime risk of developing a primary malignant brain or a central nervous system tumor is 0.67% in males and 0.54% in females (Horner, et al., 2009). The chance of dying from a brain or a central nervous system tumor is 0.48% in males compared to 0.38% in females (Horner, et al., 2009).

1.1.2. Classification

The World Health Organization (WHO) bases its classification of primary brain tumors on their histological characteristics and their cellular origin. Accordingly, WHO divides primary brain tumors into five classes including, neuroepithelial tumors, tumors of the cranial and paraspinal nerves, tumors of the meninges, Lymphomas and hematopoietic neoplasms, and a fifth class which includes Germ cell tumors and tumors of the sellar region (Louis, Ohgaki, Wiestler, & Cavenee, 2007). Gliomas, which are neuroepithelial

brain tumors derived from astrocytes, oligodendrocytes, or ependymal cells, are the most common type of malignant primary brain tumors in humans accounting for 80% of the malignant cases (CBTRUS, 2011; Louis, et al., 2007). Gliomas can either be low-grade or high-grade (malignant). Malignant gliomas are pathologically, histologically, and genetically heterogeneous tumors and they are known to be invasive (DeAngelis, 2001).

1.2. Astrocytomas

Astrocytomas are gliomas that arise from astrocytes (Louis, et al., 2007). Based on the classification of the World Health Organization (WHO), astrocytomas are assigned into one of four prognostic grades: grade I (pilocytic astrocytoma), grade II (diffuse astrocytoma), grade III (anaplastic astrocytoma), and grade IV (glioblastoma), with grades III and IV considered malignant (Louis, et al., 2007). Malignant astrocytomas are usually associated with a poor prognosis and high morbidity and mortality rates despite intensive treatment. The median survival time is 12 to 14 months for patients with glioblastoma and 2 to 5 years for patients with anaplastic astrocytoma (Wen & Kesari, 2008). For unclear reasons, malignant astrocytomas rarely metastasize into distant regions of the body; however, these tumors are highly invasive into adjacent and distant regions of the normal brain. This renders them surgically and medically unmanageable and accounts for their high recurrence rate and fatal outcome (Figure 1) (Nakada, et al., 2007).

Figure 1: Local recurrence of glioblastoma. A right occipital glioblastoma (left) was operated upon, and a postoperative magnetic resonance imaging (MRI) scan confirmed complete removal (middle left). Chemotherapy and radiation therapy were administered. Twelve months later, a routine MRI showed a recurrent tumor immediately adjacent to the resection cavity (middle right). Seventeen months after the operation, the patient died (right). (Source: Nakada, et al., 2007)

1.3. Glioma invasion

1.3.1. Invasion is a multistep process

Invasion of glioma into normal regions of the brain is a complex process consisting of several steps that involve coordinated intracellular and extracellular interactions (Figure 2) (Giese, Bjerkvig, Berens, & Westphal, 2003; Nakada, et al., 2007). Initially, the glioma cells have to detach from the nascent tumor mass (Nakada, et al., 2007). The following step is the attachment of the cancer cells to the extracellular matrix (ECM). As the ECM represents a physical barrier to invading glioma cells, they secrete matrix proteinases and/or activators of matrix proteinases to promote the degradation of the ECM. The degradation of the ECM creates a space through which the invading glioma cells can actively migrate to complete the invasion process (Giese, et al., 2003; Nakada, et al., 2007).

1.3.2. Factors involved in glioma invasion

Detachment of the glioma cell requires the downregulation of adhesion molecules including neural cell adhesion molecules (NCAM) and E-cadherin (Teodorczyk & Martin-Villalba, 2010). NCAM, which belongs to the superfamily of glycoprotein immunoglobulin

receptors, mediates homophilic binding. The expression of NCAM was found to decrease with the progression of astrocytic tumors (Sasaki, et al., 1998). Another requirement for detachment is the cadherin switch suggested to occur during the transition from a benign to an invasive malignant tumor phenotype. This switch involves the downregulation of E-cadherin and the upregulation of N-cadherin (Teodorczyk & Martin-Villalba, 2010). This alters the adhesive state of cells by rendering them less adhesive to their neighboring epithelial cells and more adhesive to mesenchymal cells such as fibroblasts and vascular endothelial cells, which enhances the invasive and metastatic properties of cells (Hulit, et al., 2007; Nieman, Prudoff, Johnson, & Wheelock, 1999). Another adhesion molecule that is upregulated in human gliomas and that plays a role in the initiation of invasion is CD44 (Ranuncolo, et al., 2002; Teodorczyk & Martin-Villalba, 2010). CD44 is a transmembrane glycoprotein that belongs to the immunoglobulin receptor superfamily. CD44 has hyaluronic acid, which is a major constituent of the brain ECM, as a ligand (Nagano & Saya, 2004).

Attachment to ECM, the next step in the invasion process, is mainly mediated by integrins, which are transmembrane proteins that anchor the cells to the ECM (D'Abaco & Kaye, 2007). The local aggregation of integrin receptors on the cell surface form adhesion structures, referred to as focal complexes, that ultimately mature to form focal adhesions (Burrridge & Chrzanowska-Wodnicka, 1996).

The remodeling of the ECM is achieved by several classes of proteases including ADAMs (a disintegrin and metalloproteinase) and matrix metalloproteinases (MMPs) mainly MMP-2, MMP-9, and membrane-type MMP (MT-MMP). Another factor that is involved in this process is plasmin, which is required to cleave the inactive MMP pro-enzymes into their active forms. Plasmin needs to be generated from plasminogen by urokinase (uPA), which is by itself cleaved and activated following its binding to its receptor, uPAR (Rao, 2003).

The last step of invasion is the active migration of the glioma cell through the ECM in a regulated process that will be discussed in detail in the following section.

Figure 2: Mechanism of glioma invasion. Glioma invasion is a complex multistep process that involves detachment from original site, attachment to ECM, degradation of ECM, and active cell migration.
(Source: Nakada, et al., 2007)

1.4. Cell Motility

Chemotaxis is an essential cellular function that is central for many physiological and pathological processes, including embryogenesis, inflammation, wound healing and tissue regeneration, as well as cancer cell invasion and metastasis (Lauffenburger & Horwitz, 1996). There is a considerable growing interest in understanding the molecular basis of chemotaxis since this could lead to therapeutic strategies targeting tumor metastasis. Chemotaxis consists of a well-defined motility cycle that is initiated in response to the detection of a chemoattractant. This commits the cell to undergo actin polymerization transients in order to extend an actin-rich protrusion, such as lamellipodia or filopodia, towards the direction of the chemoattractant (Bailly, Condeelis, & Segall, 1998). The steps that follow to achieve the motility cycle include formation of adhesion structures that

stabilize the protrusion, development of contractile force that translocates the cell body forward, release of adhesion structures at the cell rear and finally retraction of the cell towards the direction of motility (Figure 3) (Condeelis, et al., 2001). These processes are mainly regulated by Rho family of small guanosine triphosphatases (GTPases) (Figure 4), which includes key enzymes that play a major role in the reorganization of the actin cytoskeleton (Ridley & Hall, 1992).

Figure 3: The cell motility cycle. In order to achieve the motility cycle, a cell must efficiently protrude, form adhesions to stabilize the protrusion, disassemble the old adhesion in the tail, and contract for the cell body to move forward.

(Source: Ananthakrishnan and Ehrlicher, 2007)

Figure 4: Roles of Rho, Rac, and Cdc42 in cell motility. The figure shows a migrating cell undergoing the sequential steps of migration. Cdc42 regulates the direction of migration. Rac induces membrane protrusion at the front of the cell through the stimulation of actin polymerization and focal complexes. Rho promotes actomyosin contraction (Source: El-Sibai, not published)

1.5. Rho GTPases

1.5.1. General Characteristics of Rho GTPases

Rho GTPases are small GTP-binding proteins, monomeric G proteins of a 20-40 kDa molecular mass, which belong to the Ras superfamily. The Ras gene was first discovered as the v-Ras oncogene of the Rous sarcoma virus around 1980. The Ras superfamily contains more than 130 members, which fall into the Ras, Rho, Arf/Sar1 and Rab/Ran-subfamilies (Takai, Sasaki, & Matozaki, 2001). The Rho gene was discovered as a homolog of the Ras gene in *Aplysia* in 1985. Rho homologues, RhoA, RhoB and RhoC were discovered in mammalian cells (Madaule & Axel, 1985). Other members of the Rho-subfamily were later identified, including human Cdc42, Rac1 and Rac2, which were found

to be distinct in function from the other Rho proteins (Ridley & Hall, 1992; Ridley, Paterson, Johnston, Diekmann, & Hall, 1992; Shinjo, et al., 1990).

The three most characterized and studied members of the Rho family are Rho, Rac, and Cdc42 (Etienne-Manneville & Hall, 2002). It was initially believed that Rho, Rac and Cdc42 regulate the formation of actin-myosin filaments, lamellipodia and filopodia respectively (Bishop & Hall, 2000). However, recent studies taking into consideration the different effects of Rho GTPases in different cell systems and the cross-talk between the signaling pathways regulated by Rho GTPases, have shown that this model is too simplistic. For instance, the role of RhoA during cell motility was initially thought to be restricted to the generation of contractile force and focal adhesion turnover needed for tail retraction; however, it was recently shown that Rho is active at the cell edge (Pertz, Hodgson, Klemke, & Hahn, 2006), and that this activation might coordinate the Cdc42 and Rac-1 regulation of the actin cytoskeleton (El-Sibai, et al., 2008). Moreover, in neutrophils, Rac activation was observed in the tail of the cells in addition to the leading edge (Gardiner, et al., 2002).

1.5.2. Rho GTPases function as molecular switches

Rho GTPases are found in two forms, a GDP-bound inactive and a GTP-bound active form (Bourne, Sanders, & McCormick, 1990). An upstream signal stimulates the dissociation of GDP and the binding of GTP. This leads to the conformational change of the effector-binding region of the GTPase so that this region interacts with downstream effectors. The GTP-bound form is then converted to the GDP-bound form by the action of the intrinsic GTPase activity, releasing the bound effector (Etienne-Manneville & Hall, 2002).

Rho GTPases function as molecular switches in a wide range of signal transduction pathways of eukaryotic cells to translate a variety of extracellular signals into intracellular events that ultimately induce changes in the organization of the actin cytoskeleton and gene transcription (Hall, 1998). Hence, these enzymes coordinate several essential cellular processes including cell polarity, membrane transport, cell cycle progression, and cell migration (Hall, 1998).

1.5.3. Regulation of Rho GTPases

As Rho GTPases govern a wide range of critical cellular functions, their function is tightly regulated by three classes of proteins, Guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). Their activity is mainly regulated by nucleotide binding and by subcellular localization (Wennerberg & Der, 2004). The rate-limiting step of the nucleotide exchange is the initial dissociation of GDP from the GDP-bound inactive form. This reaction is very slow and is stimulated by a guanine nucleotide exchange factor (GEF) that interacts with the GDP-bound form and releases bound GDP, forming a binary complex of a small G protein and GEF. The GEF is then replaced by GTP to form the GTP-bound form (Schmidt & Hall, 2002).

Rho GTPase activating proteins (GAPs), on the other hand, negatively regulate Rho GTPases by stimulating the intrinsic GTPase activity of Rho GTPases and promoting the formation of the inactive GDP-bound form (Moon & Zheng, 2003).

Finally, guanine nucleotide dissociation inhibitors (GDIs) inhibit Rho proteins by blocking nucleotide exchange (Olofsson, 1999). GDIs either sequester the inactive GTPase, preventing the dissociation of GDP and interaction with regulatory and effector molecules, or bind to the GTP-bound form of Rho GTPase in a way that prevents binding of GAPs or of effector molecules (DerMardirossian & Bokoch, 2005). The third activity of GDIs is to modulate the cycling of Rho GTPases between the membrane and the cytosol. This represents a mechanism through which the activation of Rho GTPases is restricted by its sub-cellular localization, since the function of Rho GTPases is critically dependent on their association with the membrane (Etienne-Manneville & Hall, 2002).

Figure 5: Regulation of Rho GTPases by GEFs, GAPs, and GDIs. PI3K produces PIP3, which recruits Rho GEFs. This promotes the GTP nucleotide exchange. GAPs inactivate Rho GTPases by stimulating the intrinsic GTPase activity leading to the GDP-bound inactive form of Rho. Rho GTPases are kept inactive by being sequestered by GDIs in the cytosol. (Source: El-Sibai, not published)

1.5.4. Upstream signaling to Rho GTPases

Since the activity of Rho GTPases must be tightly regulated, Rho GEFs are themselves tightly regulated. Almost all GEFs contain a PH (pleckstrin homology) domain C-terminal to a DH (Dbl homology) domain, which is necessary for GEF activity (Schmidt & Hall, 2002). The PH domain directly affects the catalytic activity of the DH domain (Zheng, Zangrilli, Cerione, & Eva, 1996). When bound to PI(4,5)P2, the PH domain strongly interacts with the DH domain and masks its catalytic activity. PI3K phosphorylates PI(4,5)P2 to produce PI(3,4,5)P3, which weakens the PH/DH interaction and alleviates the inhibition (Bustelo, 2000; Das, et al., 2000). Hence, PI3K plays an essential role in the activation of Rho GTPases through the recruitment and activation of GEFs.

Rho GTPases are also activated downstream of signaling pathways that are initiated by the cell adhesion to the ECM. Some of these pathways affect the activity of Rho

GTPases and cell migration, as part of the adhesion-protrusion coupling effect (DeMali & Burridge, 2003; Zamir & Geiger, 2001). One such pathway, and the best understood, is the activation of the focal adhesion kinase (FAK) (DeMali & Burridge, 2003). FAK, in turn, phosphorylates p130cas (Abecassis, Olofsson, Schmid, Zalcman, & Karniguian) and paxillin (Zamir & Geiger, 2001). These two proteins have been linked to Rac activation and lamellipodia extension (DeMali & Burridge, 2003). In addition to activating Rac, FAK leads to the inhibition of RhoA via the activation of the Rho GAP, p190RhoGAP (Ren, et al., 2000).

1.5.5. Downstream effectors of Rho GTPases

Rho is believed to induce focal adhesions by stimulating contractility, through the phosphorylation of myosin light chain (MLC) (Chrzanowska-Wodnicka & Burridge, 1996). This effect is mediated through a Rho effector, the serine/threonine kinase p160ROCK (Kimura, et al., 1996). ROCK elevates MLC phosphorylation through inhibiting MLC phosphatase, through the myosin binding subunit of the phosphatase (MBS) (Kimura, et al., 1996). ROCK can also directly phosphorylate MLC, at the same site as MLC kinase, thereby enhancing myosin activation (Amano, et al., 1997). However, the formation of normal stress fibers involves the cooperation of ROCK with another Rho effector, the mammalian homolog of diaphanous (mDia) (Watanabe, Kato, Fujita, Ishizaki, & Narumiya, 1999). LIM kinase is another important downstream effectors of Rho (Burridge & Wennerberg, 2004). LIMK phosphorylates the actin severing protein cofilin, inhibiting its function (Maekawa, et al., 1999). Additional downstream effectors from Rho involved in actin regulation include members of the ezrin-radixin-moesin (DerMardirossian & Bokoch) family of proteins (Takai, et al., 2001). ERM proteins are located in places where actin filaments are associated with the plasma membrane (Takaishi, Sasaki, Kameyama, Tsukita, & Takai, 1995).

Downstream effectors of Cdc42 and Rac include members of the WASP/SCAR/WAVE family of scaffold proteins, which are key nucleation promoting factors that activate the Arp2/3 complex leading to actin polymerization (Bishop & Hall,

2000). Another downstream effector that is activated by both Cdc42 and Rac is the serine/threonine kinase p65PAK (p21 activating protein) (Bishop & Hall, 2000). PAK phosphorylates and inactivates MLCK, thereby reducing MLC phosphorylation and contractility (Sanders, Matsumura, Bokoch, & de Lanerolle, 1999). PAK also phosphorylates LIMK, inducing its activity in vitro (Edwards, Sanders, Bokoch, & Gill, 1999). Thus, LIMK is activated by both ROCK and PAK, and is downstream of both Rho and Cdc42/Rac, leading to the phosphorylation and inhibition of cofilin (Burrige & Wennerberg, 2004). This potentially decreases severing, in order to stabilize actin polymerization at the leading edge (Burrige & Wennerberg, 2004).

Figure 6: Downstream effectors of Rho in cell motility. Explained in text in section 1.5.5.
(Source: El-Sibai, not published)

Figure 7: Model for formation of focal adhesions induced by the Rho/Rho-kinase pathway.

Source: (Kaibuchi, Kuroda, & Amano, 1999)

Figure 8: Downstream effectors of Rac and Cdc42 in cell motility. Explained in text in section 1.5.5.
(Source: El-Sibai, not published)

1.6. Role of Rho-GTPases in cancer and metastasis

1.6.1. The Role of Rho GTPases in cell motility

As previously mentioned, cell motility plays a major role in cancer invasion and metastasis (Lauffenburger & Horwitz, 1996). The first step in the cell motility cycle is the determination of the direction of movement. This is achieved by Cdc42, which is classically known to regulate the formation of filopodia, and is required for direction sensing during chemotaxis (Allen, Zicha, Ridley, & Jones, 1998; Nobes & Hall, 1999). Rac, on the other hand, induces the production of lamellipodia through the activation of the actin nucleator Arp2/3, the inhibition of cofilin, which stabilizes the actin filaments, and the activation of PIP 5-kinase, which produces PIP2 that inhibits actin-capping proteins. These pathways are also downstream of Cdc42. Accordingly, Cdc42 could potentially be important for the lamellipodia formation as well (El-Sibai, et al., 2007), consistent with the observation that Cdc42 can induce Rac-dependent lamellipodium extension (Hall, 1998).

After lamellipod extension, formation of focal complexes stabilizes the lamellipodium of migrating cells by binding to the ECM (Condeelis, et al., 2001). Rac stimulates the assembly of focal complexes (Nobes & Hall, 1995; Rottner, Hall, & Small, 1999), which are small punctate structures formed behind the front of the lamellipodium. These structures do not confer enough contractility needed for cell motility. In response to RhoA signaling pathway, focal complexes mature to form focal adhesions, which appear larger in size and less persistent (Kaverina, Krylyshkina, & Small, 2002). Focal adhesions confer mechanical strength needed for the cell body to contract and for the cell to slide over the ECM, in order to achieve the cycle of cell motility (Gupton & Waterman-Storer, 2006). After the cell lamella moves over them, focal complexes either disassemble or mature into Rho-induced focal adhesions in slowly migrating cells such as fibroblasts (Rottner, et al., 1999). Focal complexes/adhesions turnover is essential for cell migration as a high level of focal adhesions correlating with an increased level of Rho activation inhibits cell migration by strengthening the attachment of cells to the extracellular matrix (Cox, Sastry, & Huttenlocher, 2001).

Cell body contraction is dependent on actomyosin contractility (Mitchison & Cramer, 1996) and has been shown to be specifically regulated by Rho, which stimulates the formation of contractile actin–myosin filaments to form stress fibers (Ridley & Hall, 1992).

1.6.2. Altered Rho-GTPase expression and signaling in cancer and metastatic cells

Genetic screens of many human cancers have revealed altered expression of various Rho family GTPases (Ridley, 2004). *RhoC* mRNA level is increased in metastatic melanomas and Rac3 activity is increased in highly proliferative breast cancer cell lines (Ridley, 2004). A comparison of the gene expression pattern in a metastatic breast cancer cell line compared to its non-metastatic counterpart revealed that many genes encoding actin regulatory proteins are more highly expressed in metastatic cells (Wang, et al., 2002). Given the role of these Rho GTPases in the regulation of actin polymerization and cell motility in normal cells and their aberrant regulation in tumor cells (Wang, et al., 2003), it is most likely that they are involved in the invasive phenotype of tumor cells. Indeed, the role

of Rho GTPases in the regulation of cell motility has been shown to be required for tumor metastasis in some model systems. Increased Rho expression is sufficient to stimulate melanoma cell metastasis in a mouse model (Clark, Golub, Lander, & Hynes, 2000). RhoA and ROCK are required for the migrating tumor cells to cross the vascular epithelium during metastasis (Worthylake, Lemoine, Watson, & Burridge, 2001). Dominant interfering mutants that sequester Rho GEFs away from endogenous RhoA and Cdc42 prevent T-cell hybridomas from exiting the blood and colonizing the liver (Soede, Zeelenberg, Wijnands, Kamp, & Roos, 2001). Similarly, Y-27632, a specific inhibitor of the Rho effector ROCK, has been reported to have an effect on cancer metastasis. The inhibitor reduces the number of tumors in the lung following injection of tumor cells in the tail of mice (Itoh, et al., 1999). It also inhibits the metastatic growth of invasive prostate cancer cells in mice (Somlyo, et al., 2000). The activity of Cdc42 was also found to be essential for the protrusion and motility of MTLn3 carcinoma cells following the stimulation with EGF (El-Sibai, et al., 2007).

Hence, understanding the specific role of Rho GTPases and their effectors in the regulation of actin polymerization during cancer cell motility will provide a tool in anti-tumor therapeutics through the inhibition of metastasis and tumor cell invasion.

1.7. Altered Rho-GTPase signaling in Brain tumors

A study that aimed at classifying malignant gliomas based on their gene expression profiles showed that the genes of Rho-GTPases are highly correlated with glioblastoma (Nutt, et al., 2003). The expression of RhoA and RhoB was found to be significantly decreased in astrocytic tumors, and their levels were inversely proportional to the grade of the tumor. Moreover, the Rac1 expression level was reduced in astrocytic tumors of grades III and IV (Forget, et al., 2002). However, another study that was based on the analysis of clinical materials demonstrated that the expression of RhoA is increased in high-grade astrocytomas (Yan, Chour, Peh, Lim, & Salto-Tellez, 2006). Rac2 was also found to be underexpressed in 57.7% of brain tumors (Hwang, et al., 2005). However, another study has shown that Rac1 is overexpressed in medulloblastoma tumors, and that the use of small

interfering RNA to deplete Rac1 strongly inhibits medulloblastoma cell invasion (Senger, et al., 2002; Zavarella, et al., 2009). Furthermore, it has also been reported that the suppression of Rac1 inhibits the survival and induces apoptosis of glioma cells; however, this effect was not seen in normal primary human adult astrocytes. This effect was also shown to be independent of the mutational status of several factors including epidermal growth factor receptor, p16/p19, and p53 (Senger, et al., 2002). Additionally, a study has shown that 27% of analyzed brain tumors had base changes in the Rac1 gene. These mutations are thought to enhance the survival of brain tumors by enhancing the activity of Rac1 as they were found to be mainly localized in the putative effector-domain of the gene (Hwang, et al., 2004). In contrast, brain tumors show a low frequency of mutation and no hot spots of mutation in Rac2 gene (Hwang, et al., 2004).

The inhibition of Cdc42 by Slit2, a chemorepulsive factor that governs the migration of glial cells and neurons during development, inhibits the migration of glioma cells rendering them less invasive (Yiin, et al., 2009). Downregulation of Cdc42 and inhibition of migration by Slit2 were also observed in medulloblastoma (Werbowski-Ogilvie, et al., 2006). It has also been shown that PTEN inhibits glioma cell invasion by inhibiting the activity of Cdc42 and Rac (Furukawa, et al., 2006). Suppression of Cdc42 was also reported to inhibit, although not completely, LPA-induced migration in glioma cells (Malchinkhuu, et al., 2005).

It has been reported that Rho is rapidly activated following the attachment of human malignant astrocytoma cells to a substrate, and that this activation is responsible for the morphological changes that these cells exhibit upon attachment (Ding, et al., 2002). The increase in RhoA activity in glioblastoma cells has been linked to impaired cell migration through inducing profound morphological changes including the rearrangement of actin into stress fibers and the formation of focal adhesion. These changes, which are mainly linked to RhoA activity, rendered the cells immobile (Goldberg & Kluog, 2006). However, another study has reported that lysophosphatidic acid (LPA)-induced migration in glioma cells could be inhibited by blocking Rho activation. LPA is an agent that is able to strongly induce chemokinesis and chemotaxis in human glioma cells (Manning, Parker, & Sontheimer, 2000). LPA-induced migration in glioma cells could also be inhibited by

isoproterenol and sphingosine-1-phosphate (S1P). Both agents affect migration by inhibiting the activity of Rac, although S1P-induced down-regulation of Rac has been shown to be mediated by Rho (Malchinkhuu, et al., 2009).

Despite the fact that Rho GTPases commonly show altered expression and activity in many types of tumors, no mutations in any of the Rho GTPases have been reported in human tumors. This might indicate that the altered Rho GTPase signaling in tumors might be due to a dysfunction in the regulation of these proteins. In this context, several Rho GAPs and GEFs were implicated in the deregulation of Rho GTPases in brain malignancies. The overexpression of the RhoGAP domain or the full-length p200RhoGAP, a GAP that specifically inactivates RhoA and Rac1 and localizes at the cortical actin site in the undifferentiated state, induces the differentiation of N1E-115 neuroblastoma cells (Moon, Zang, & Zheng, 2003). In another study, the absence of IQ-domain GTPase-activating protein 1 (IQGAP1) was correlated with a long-term survival period in patients with glioblastoma multiforme (McDonald, et al., 2007). Unlike what the name implies, IQGAP1 binds to Rac1 and stabilizes it in the GTP-bound state to regulate several cellular processes including cell adhesion and migration. It has been shown that the knockdown of IQGAP1 decreases motility and invasion of breast cancer cells. In glioma, IQGAP1 is required for the activation of Rac1 in a signaling pathway that was shown to be crucial for the invasion of glioma cells (Hu, et al., 2009).

Several GEFs have also been shown to be involved in brain tumor progression and invasion. One study has shown that the members of the bipartite Rac1 GEF, Engulfment and cell motility 1 (ELMO1) and dedicator of cytokinesis (Dock180), show higher expression in invasive areas of primary human brain tumors as compared to non-invasive regions. This elevated expression is also detected in human glioma cell lines as compared to normal human astrocytes. This study has also reported that the inhibition of ELMO1/Dock180 significantly inhibits glioma cell invasion (Jarzynka, et al., 2007). Similarly another study has reported that the expression of the three GEFs, Trio, Ect2, and Vav3 is elevated in glioblastoma as compared to low-grade glioma. This expression is also correlated with poor prognosis. Moreover, while the depletion of the three GEFs reduces

migration and invasion of glioma cells, the depletion of Ect2 or Trio also inhibits proliferation of these cells (Salhia, et al., 2008).

1.8. StarD13

StarD13, which is also referred to as START-GAP2 or DLC2, was first described as a tumor suppressor gene product in hepatocellular carcinoma cells where it was found to be underexpressed (Ching, et al., 2003). This Rho-GAP, whose gene is located on the position 13q12.3, specifically inhibits the function of RhoA and Cdc42 and was demonstrated to inhibit, in cultured cells, the Rho-mediated assembly of actin stress fibers. Furthermore, overexpression of StarD13 is associated with a decrease in cell growth (Ching, et al., 2003). A study that aimed at examining the intracellular localization of StarD13 showed that it is found in focal adhesions and that it is anchored to tensin2, a component of focal adhesions, through a so-called focal adhesion targeting domain made of amino acids 318–472 in the N-terminal region (Kawai, Seike, et al., 2009). Another report that used cancer-profiling arrays indicated that StarD13 expression is down-regulated in several types of solid tumors including in renal, uterine, gastric, colon, breast, lung, ovarian, and rectal tumors (Ullmannova & Popescu, 2006). Moreover, they concluded that StarD13 deficiency plays a role in the metastasis of breast carcinoma into secondary sites after they detected, using quantitative RT-PCR, a significantly low expression of this gene in a high proportion of tumor samples taken from primary ductal carcinomas from patients with metastases into a regional lymph node (Ullmannova & Popescu, 2006). Furthermore, a Genome-Wide Analysis integrating a paired copy number and gene expression survey on glioblastoma samples concluded that StarD13 is a potential tumor suppressor gene that could be involved in the invasiveness of this tumor and its resistance to etoposide (de Tayrac, et al., 2009). A recent report has shown that StarD13 suppresses cell growth by inhibiting the Raf-1-ERK1/2-p70S6K signaling pathway (Leung, Yam, Chan, Ching, & Ng, 2010). StarD13 also plays a role in cell migration and attachment were its silencing was found to enhance the migration and decrease cell attachment in normal vascular endothelial cells (Lin, et al., 2010).

1.9. Purpose of the Study

Our first objective in the current study is to investigate the role of RhoA in the progression of astrocytic tumors, a topic that is still controversial in literature. We first aimed at determining the level of RhoA expression in patient astrocytoma tumor tissues of grades I and IV. Then we wanted to correlate the level of RhoA expression with the level of RhoA activation in cell lines representing the different grades of the tumor. For this purpose we used a RhoA FRET biosensor to directly measure the activity of RhoA.

We also aimed at investigating the role of RhoA along with StarD13, a Rho GAP that inhibits the function of RhoA, in astrocytoma cell motility. Since StarD13 localizes to focal adhesions, we also studied the involvement of both RhoA and StarD13 in the dynamic of cell adhesion. Furthermore, we examined the role of Rac in astrocytoma cell motility and adhesion since Rac also plays a role in cell motility and adhesion.

MATERIALS & METHODS

2.1. Cell Culture

Human astrocytoma cell lines (HS683, SF268, SW1088, U87, U251, T98G, A172, and SNB-19) were cultured in DMEM medium supplemented with 10% FBS and 100U penicillin/streptomycin at 37°C and 5% CO₂ in a humidified chamber.

2.2. Antibodies and reagents

Goat polyclonal anti-StarD13 antibody was obtained from Santa Cruz Biotechnology.

Mouse monoclonal anti-RhoA, mouse monoclonal anti-Rac1, and mouse monoclonal anti-paxillin antibodies were purchased from Upstate biotechnology, Lake Placid, NY.

Anti-goat and anti-mouse HRP-conjugated secondary antibodies were obtained from Promega.

Fluorescent secondary antibodies (AlexaFluor 488) were obtained from Invitrogen.

To visualize the actin cytoskeleton, cells were stained with Rhodamine phalloidin (Invitrogen). DAPI was also used to stain nuclei.

2.3. Cell transfection with siRNA

Goat FlexiTube siRNA for StarD13, RhoA, and Rac1 were obtained from Qiagen. The siRNAs used had the following target sequences: StarD13: 5'-CCCGCAATACGCTCAGTTATA-3', RhoA: 5'-TTCGGAATGATGAGCACACAA-3', and Rac1: 5'-ATGCATTTTCCTGGAGAATATA-3'. The cells were transfected with the

siRNA at a final concentration of 10 nM using HiPerfect (Qiagen) as described by the manufacturer. Control cells were transfected with siRNA sequences targeting GL2 Luciferase (Qiagen). After 72 hours, protein levels in total cell lysates were analyzed by western blotting using the appropriate antibodies or the effect of the corresponding knockdown was assayed.

2.4. Cell transfection with vectors

Cells were transfected with 5 µg GFP-StarD13, Dominant active RhoA, or control empty control vectors using Lipfectamine LTX with Plus reagent (Invitrogen) as described by the manufacturer. Cells were incubated with the transfection complexes for 4 hours then refed with DMEM supplied with 30% FBS. The experiments were carried on 24 hours following transfection.

The GFP-StarD13 and the RhoA constructs were generous gifts from respectively Dr. Hitoshi Yagisawa from the University of Hyogo, Japan and Dr. Hideki Yamaguchi from the Albert Einstein College of Medicine, New York, USA.

The constructs were transformed into One Shot TOP10 chemically competent E. Coli (Invitrogen), which were grown on a selective medium containing the appropriate antibiotic. The vectors were then extracted using MaxiPrep plasmid extraction kit from Qiagen.

2.5. Western blotting

Cell lysates were prepared by scraping the cells in a sample buffer consisted of 4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, and 0.125 M Tris-HCl at a pH of 6.8. The resulting lysates were boiled for 5 minutes. Protein samples were separated by SDS-PAGE on 8% (for StarD13) or 15% (for RhoA and Rac) gels and transferred to PVDF membranes overnight at 30V. The membranes were then blocked with 5% non-fat dry milk in PBS containing 0.1% Tween-20 for 1 hour at room temperature and incubated with primary antibody at a concentration of 1:100 for 2 hours at

room temperature. After the incubation with the primary antibody, the membranes were washed and incubated with secondary antibody at a concentration of 1:1000 for 1 hour at room temperature. The membranes were then washed, and the bands visualized by treating the membranes with western blotting chemiluminescent reagent ECL (GE Healthcare). The results were obtained on an X-ray film (Agfa Healthcare). The levels of protein expression were compared by densitometry using the ImageJ software.

2.6. Förster Resonance energy transfer (FRET)

Cells lines of grades II (HS683), III (SF268 and SW1088), and IV (U87, U251, T98G, A172, and SNB-19) were transfected with 1 µg of the RhoA fluorescence resonance energy transfer (FRET)-based biosensor plasmid. The biosensor consists of (from the N-terminus) the Rho binding domain (RBD) of the effector Rhotekin, a cyan fluorescent protein (CFP), a protease resistant 17-mer unstructured linker, a yellow fluorescent protein (YFP) domain, and a full length RhoA (Pertz, et al., 2006). FRET image sequences were obtained with an automated Olympus IX70 microscope equipped with filter wheels in the excitation and emission light path and coupled to a cooled SensiCam QE CCD camera (Cooke Corp., MI). CFP was excited using a S430/25 filter with a Sutter DG4 illuminator (Sutter Instruments, Novato, CA) and the fluorescence detected with a S470/30 (donor image) or S535/30 (FRET image) emission filter. YFP was imaged with exciter S500/20 and emitter S535/30 (YFP image). In all cases a dual-band dichroic mirror 86002v2bs was used (Chroma Technology Corp., VT). Images were background corrected and the YFP images were thresholded to generate a binary mask with values of 1 within the cell and 0 for the background. The increase in FRET signal due to activation of RhoA was detected by ratioing the FRET image (CFP excitation- YFP emission) to the donor image (CFP excitation- CFP emission). FRET signals were quantified by averaging the mean FRET ratio in regions of interest in cells. For the live FRET movies, the cells were transfected with the RhoA biosensor as described above, and images of the cells moving randomly in serum were taken at a 1 minute time interval for 1 hour. The images from each frame were analyzed as described above.

2.7. Immunohistochemistry

Frozen human astrocytoma tissues of grades I and IV were sectioned to 8 μm sections using a refrigerated microtome. Tissues were fixed with 4% paraformaldehyde for 10 minutes, and permeabilized with 0.5% Triton-X100 for 10 minutes. To decrease background fluorescence, tissues were rinsed with 0.1 M glycine then incubated with 0.1 M glycine for 10 minutes. For blocking, tissues were incubated 4 times with 1% BSA, 1% FBS in PBS for 5 minutes. Samples were stained with RhoA primary antibody for 2 hours and with a fluorophore-conjugated secondary antibody for 2 hours. Tissue fluorescent images were taken using a 10X objective on a confocal microscope. For image analysis, all digital images were imported in image J software (National Institutes of Health, MA). The total fluorescence intensity of a fixed area from at least 10 different frames from each tissue was determined.

2.8. Immunostaining

The cells were plated on cover slips, and the appropriate treatment was applied. Cells were fixed with 4% paraformaldehyde for 10 minutes, and permeabilized with 0.5% Triton-X100 for 10 minutes. To decrease background fluorescence, cells were rinsed with 0.1 M glycine then incubated with 0.1 M glycine for 10 minutes. For blocking, cells were incubated 4 times with 1% BSA, 1% FBS in PBS for 5 minutes. Samples were stained with primary antibodies for 2 hours and with fluorophore-conjugated secondary antibodies for 2 hours. Fluorescent images were taken using a 60X objective on a fluorescent microscope.

2.9. RhoA pull down assay

For experiments, cells were starved in low-glucose Opti-MEM medium without FBS for 3 hours, and stimulated with a final concentration of 10 nM Recombinant Human Epidermal Growth factor (Hu EGF) for various times (Invitrogen). Cells were then lysed and the pull-down assay performed using the RhoA/Rac1/Cdc42 Activation Assay Combo Kit (Cell BioLabs) following the manufacturer's instructions. Briefly, cell lysates were incubated with GST-RBD for 1 hour at 4 $^{\circ}\text{C}$ with gentle agitation. Then, the samples were centrifuged, and

the pellet washed for several times. After the last wash, the pellets were resuspended with sample buffer and boiled for 5 minutes. GTP-RhoA was detected by western blotting using the anti-RhoA antibody provided in the kit. Total RhoA was collected prior to the incubation with GST-RBD and used as a loading control.

2.10. Wound healing assay

Cells were grown to confluence on culture plates and a wound was made in the monolayer with a sterile pipette tip. After wounding, the cells were washed to remove debris and new medium was added. Phase-contrast images of the wounded area were taken at 0 and 24 hours after wounding. Wound widths were measured at 11 different points for each wound, and the average rate of wound closure was calculated in $\mu\text{m/hr}$.

2.11. Motility assay

For motility analysis, images of cells moving randomly in serum were collected every 60 seconds for 2 hours using a 20X objective. During imaging, the temperature was controlled using a Nikon heating stage which was set at 37 °C. The medium was buffered using HEPES and overlaid with mineral oil. The speed of cell movement was quantified using the ROI tracker plugin in the ImageJ software, which was used to calculate the total distance travelled by individual cells. The speed is then calculated by dividing this distance by the time (120 minutes) and reported in $\mu\text{m/min}$. The speed of at least 15 cells for each condition was calculated. The net distance travelled by the cell was calculated by measuring the distance travelled between the first and the last frames.

2.12. Adhesion assay

96-well plates were coated with collagen using Collagen Solution, Type I from rat tail (Sigma) overnight at 37 °C then washed with washing buffer (0.1% BSA in DMEM). The plates were then blocked with 0.5% BSA in DMEM at 37 °C in a CO₂ incubator for 1

hour. This was followed by washing the plates and chilling them on ice. Meanwhile, the cells were trypsinized and counted to 4×10^5 cell/ml. 50 μ l of cells were added in each well and incubated at 37°C in a CO₂ incubator for 30 minutes. The plates were then shaken and washed 3 times. Cells were then fixed with 4% paraformaldehyde at room temperature for 10 minutes, washed, and stained with crystal violet (5 mg/ml in 2% ethanol) for 10 minutes. Following the staining with crystal violet, the plates were washed extensively with water, and left to dry completely. Crystal violet was solubilized by incubating the cells with 2% SDS for 30 minutes. The absorption of the plates was read at 550 μ m using a plate reader.

2.13. Statistical analysis

All the results reported represent average values from three independent experiments. All error estimates are given as $\pm$ s.e.m. The p-values were calculated using SPSS software by ANOVA or chi-square tests depending on the experiment.

Figure 9: The RhoA FRET biosensor. The biosensor consists of (from the N-terminus) the Rho binding domain (RBD) of the effector Rhotekin, a cyan fluorescent protein (CFP), a protease resistant 17mer unstructured linker, a yellow florescent protein (YFP) domain, and a full length RhoA.

(Source: Pertz, et al., 2006)

RESULTS

3.1. RhoA plays a role in the progression of astrocytic tumors

Before studying the role of RhoA in astrocytoma cell motility, we first wanted to investigate the expression pattern of RhoA in human astrocytoma tissues and its activation in astrocytoma cell lines in an attempt to demonstrate its involvement in the progression of astrocytic tumors.

3.1.1. RhoA is overexpressed in grade IV astrocytoma tissues

We started by determining the expression level of RhoA in human astrocytoma tissues by immunohistochemistry. The tissues were stained using an anti-RhoA antibody (Figure 10A), and the mean fluorescence intensity was measured. The comparison of the mean fluorescence intensity reflecting the expression level of RhoA in grade IV tumor tissues to that of grade I tumor tissues, revealed a two-fold increase in the expression of RhoA in higher grade tumors (Figure 10B).

3.1.2. RhoA shows higher activation in malignant astrocytoma cell lines

Since our results showed that RhoA is overexpressed in high-grade astrocytic tumors, we wanted to compare the activation of RhoA in astrocytoma cell lines representing the different grades of the tumor. This was done using Fluorescence Resonance Energy Transfer (FRET) after transfecting grade II, grade III, and grade IV cell lines with a single chain CFP/YFP RhoA FRET biosensor (Pertz, et al., 2006) to directly measure the activity of RhoA in these cells. Representative images for each grade are shown in figure 11A. The mean FRET signal per cell for each grade was calculated, and the results showed higher activation of RhoA in malignant cell lines (grades III and

IV) as compared to low-grade cell lines of grade II; there was no significant difference in RhoA activity between grade III and grade IV cell lines (Figure 11B).

Figure 10: Rho expression levels in different grades astrocytomas. A) Representative confocal micrographs of frozen astrocytoma tissues of grade I (upper panels) and IV (lower panels) tumors were sectioned and immunostained with anti-Rho antibody (left panels) and stained with DAPI (middle panels). The two channels were merged in the right panels. **B)** Quantitation of the immunohistochemistry in A). The mean fluorescent intensity/pixel in the Rho channel was measured and expressed as fold increase in grade IV astrocytoma as compared to grade I. Data are the mean $\pm$ SEM from 3 different experiments (with 5 tissues each). The results were significant with $p < 0.001$.

Figure 11: RhoA activation in astrocytoma cell lines. **A)** Representative micrographs of cell lines of grades II, III, and IV astrocytoma that were transfected with the FRET RhoA biosensor and fixed. The cells were imaged in the FRET channel and the raw FRET images normalized to the CFP images. The images reflect the FRET ratio = Raw FRET image/CFP. The pseudo color scale is shown to the left where the dynamic range of activation is from 1.28 to 2.11. **B)** Cells in A) were quantitated for the mean FRET ratio/cell and the ratio expressed in arbitrary units. Data are the mean $\pm$ SEM. The results had borderline significance with $p=0.06$.

3.2. Roles of RhoA and Rac1 in astrocytoma cell motility

3.2.1. RhoA knockdown inhibits cell motility

After showing that RhoA is overexpressed in high-grade astrocytoma tissues and hyperactivated in high-grade astrocytoma cell lines, we aimed at investigating the role of RhoA in astrocytoma cell motility. We started by knocking down RhoA in astrocytoma cell lines, where an almost complete knockdown was achieved as determined by detecting

the expression level of RhoA by western blot in cells transfected with RhoA siRNA compared to that in control cells transfected with a non-specific siRNA (Figure 12A). RhoA knockdown resulted in the inhibition of astrocytoma cell motility as determined by both the effect on wound closure rate in the wound healing assay (Figure 12B) and the effect on cell speed and net speed migrated in the time-lapse assay. This was reflected by more than a two-fold decrease in the wound closure rate in the RhoA knockdown cells ($6.5 \mu\text{m/hr}$) as compared to controls ($15.5 \mu\text{m/hr}$) (Figure 12C). RhoA knockdown also decreased the average cell speed from $0.44 \mu\text{m/min}$ in controls to $0.20 \mu\text{m/min}$ (Figure 13B), and the net distance migrated from $8.50 \mu\text{m}$ in controls to $2.89 \mu\text{m}$ (Figure 13A and C).

Figure 12: RhoA is required for cell motility-1. Cells (T98G) were transfected with luciferase control siRNA or with RhoA siRNA for 72 hours. **A)** The cells were lysed and immunoblotted by western blot analysis for RhoA (upper gel) or for actin (lower gel) for loading control. **B)** The luciferase siRNA-transfected and RhoA siRNA-transfected cells were grown in a monolayer then wounded and left to recover the wound then imaged at the same frame after 24 hours (lower micrographs). **C)** Quantitation for B). Wound widths were measured at 11 different points for each wound, and the average rate of wound closure for the luciferase and the Rho siRNA-transfected cells was calculated in $\mu\text{m/hr}$. Data are the mean $\pm$ SEM from 3 wound closure movies. The results were significant with $p < 0.01$.

Figure 13: RhoA is required for cell motility-2. Cells (SF268) were transfected with luciferase control siRNA or with RhoA siRNA for 72 hours. **A)** The net paths of projected 120 frames from 2 hour long time lapse movies of cells undergoing random motility in serum (different colors represent different cells) **B)** Quantitation of the cell speed from A) expressed in $\mu\text{m}/\text{min}$. **C)** Quantitation of the net paths shown in A) expressed in μm . Data are the mean $\pm$ SEM from 15 cells. The results were significant with $p < 0.001$ for both cell speed and net distance.

3.2.2. Dominant active RhoA inhibits cell motility

After establishing that RhoA is overexpressed in astrocytic tumor and that its inhibition leads to the inhibition of cell motility, we were interested in determining the effect of the transfection of a dominant active form of RhoA on astrocytoma motility. Surprisingly, instead of enhancing the migratory ability of cells, dominant active RhoA significantly suppressed their motility. In the wound healing assay (Figure 14A), dominant active RhoA decreased cell motility from 7 $\mu\text{m/hr}$ to 2.7 $\mu\text{m/hr}$ (Figure 14B). Similarly, dominant active RhoA decreased the average cell speed from 0.34 $\mu\text{m/min}$ to 0.25 $\mu\text{m/min}$ (Figure 15B). However, what was more substantial is the decrease in the net path in the cells transfected with dominant active RhoA from 9.31 μm to 1.43 μm (Figure 15A and C).

Figure 14: Constitutively active RhoA inhibits cell motility. Cells (T98G) were left untransfected or transfected with a dominant active RhoA construct (RhoA DA). **A)** Cells were grown in a monolayer then wounded and left to recover the wound then imaged at the same frame after 24 hours (lower micrographs). **C)** Quantitation for A). Wound widths were measured at 11 different points for each wound, and the average rate of wound closure was calculated in $\mu\text{m/hr}$. Data are the mean $\pm$ SEM from 3 wound closure movies. The results were significant with $p=0.01$.

Figure 15: Constitutively active RhoA inhibits cell motility-2. Cells (SF268) were left untransfected or transfected with a dominant active RhoA construct (RhoA DA). **A)** The net paths of projected 120 frames from 2 hour long time lapse movies of cells undergoing random motility in serum (different colors represent different cells) **B)** Quantitation of the cell speed from A) expressed in $\mu\text{m}/\text{min}$. **C)** Quantitation of the net paths shown in A) expressed in μm . Data are the mean $\pm$ SEM from 15 cells. The results were significant with $p < 0.001$ for both cell speed and net path.

3.2.3. Rac1 knockdown inhibits cell motility

As Rac plays an indispensable role in cell motility, we were interested in determining the effect of Rac1 knockdown on astrocytoma cell motility. The efficiency of Rac1 knockdown in these cells was confirmed by western blot analysis (Figure 16A). Rac1 knockdown decreased the wound closure rate from $8.22 \mu\text{m}/\text{hr}$ to $1.76 \mu\text{m}/\text{hr}$ (Figure 16B and C). Likewise, there was a significant decrease in the cell speed from 0.44

$\mu\text{m}/\text{min}$ to $0.17 \mu\text{m}/\text{min}$ (Figure 17B) and the net distance migrated from $8.50 \mu\text{m}$ in controls to $1.7 \mu\text{m}$ (Figure 17A and C).

Figure 16: Rac is needed for cell motility-1. Cells (T98G) were transfected with luciferase control siRNA or with Rac siRNA for 72 hours. **A)** The cells were lysed and immunoblotted by western blot analysis for Rac (upper gel) or for actin (lower gel) for loading control. **B)** The luciferase siRNA-transfected and Rac siRNA-transfected cells were grown in a monolayer then wounded and left to recover the wound then imaged at the same frame after 24 hours (lower micrographs). **C)** Quantitation for B). Wound widths were measured at 11 different points for each wound, and the average rate of wound closure for the luciferase and the Rac siRNA-transfected cells was calculated in $\mu\text{m}/\text{hr}$. Data are the mean $\pm$ SEM from 3 wound closure movies. The results were significant with $p=0.001$.

Figure 17: Rac is needed for cell motility-2. Cells (T98G) were transfected with luciferase control siRNA or with Rac siRNA for 72 hours. **A)** The net paths of projected 120 frames from 2 hour long time lapse movies of undergoing random motility in serum (different colors represent different cells) **B)** Quantitation of the cell speed from A) expressed in $\mu\text{m}/\text{min}$. **C)** Quantitation of the net paths shown in A) expressed in μm . Data are the mean $\pm$ SEM from 15 cells. The results were significant with $p < 0.001$ for both cell speed and net distance.

3.3. Roles of RhoA and Rac1 in cell adhesion dynamics

3.3.1. Rac localizes to and is active at the cell edge

In order to determine the localization of Rac in astrocytoma cell lines, we immunostained the cells with an anti-Rac antibody. This showed that Rac shows high localization at the cell edge. Moreover, immunostaining using an antibody that only binds to the GTP-bound active form of Rac showed that Rac not only shows high localization at the cell edge, but also it is highly active in this region (Figure 18A).

3.3.2. Rac1 is needed for the formation of focal complexes

Rac has been shown to stimulate the assembly of focal complexes (Nobes & Hall, 1995; Rottner, et al., 1999). In order to confirm that Rac plays this role in astrocytoma cells, we knocked down Rac1 and immunostained the cells using an antibody against paxillin, a component of focal complexes and focal adhesions (Zaidel-Bar, Cohen, Addadi, & Geiger, 2004). Rac1 knockdown abolished any forms of adhesions. Moreover, due to the absence of evident paxillin staining at the cell edge, the cell edge was hard to identify (Figure 18B).

Figure 18: Rac is needed for formation of focal complexes. **A)** Representative micrographs of (T98G) cells that were fixed and immunostained for total (left) or active (right) Rac. **B)** Cells were transfected with luciferase or Rac siRNA and the immunostained with anti-paxillin antibody. Cells were imaged using a 60x objective.

3.3.3. RhoA is needed for the maturation of focal complexes into focal adhesions

After showing that Rac is indeed needed for the formation of focal complexes in astrocytoma cells, we wanted to verify that RhoA is essential for the maturation of focal complexes into focal adhesions similar to previous reports in other systems (Kaverina, et al., 2002). This was indeed verified, as the silencing of RhoA in astrocytoma cells led to the absence of mature focal adhesions. Only punctate small adhesions, which are most probably immature focal complexes, were evident (Figure 19A).

3.3.4. Dominant active RhoA leads to the formation of large focal adhesions

Following the verification of the role of RhoA in the maturation of focal complexes into focal adhesions, we aimed at addressing the question whether the inactivation of RhoA is essential for the disassembly of focal adhesions, which is in turn necessary for the completion of the motility cycle. The transfection of cells with the dominant active RhoA led to an increase in the size of focal adhesions (Figure 19B).

Figure 19: Rho is needed for formation of focal adhesions. **A)** Representative micrographs of (T98G) luciferase control cells (left panel) or RhoA siRNA-transfected cells (right) that were fixed and immunostained with paxillin. **B)** Cells were untransfected or transfected with DA RhoA and then immunostained with anti-paxillin. Cells were imaged using a 60x objective.

3.4. RhoA undergoes cycles of activation and inactivation

To validate that RhoA has to be inactivated at some point in order to complete the motility cycle, we stimulated the cells with EGF and directly measured the level of activation of RhoA by pull-down assay at 0, 0.5, 1, and 3 minutes following stimulation. The results showed that RhoA activity increases significantly after 30 seconds of stimulation and then decreases after 1 minute (Figure 20A).

The activation of RhoA was also spatiotemporally determined by analyzing FRET movies of cells transfected with the RhoA FRET biosensor and moving randomly in serum. These movies showed that RhoA shows high activation at the leading edge at all time points; however, RhoA undergoes cycles of activation and inactivation at the cell rear (Figure 20B).

A.

B.

C.

Figure 20: Rho undergoes cycles of activation and inactivation. A) Montage of SF268 cells transfected with the RhoA FRET biosensor undergoing motility in serum. **B)** T98G cells were transfected with Luciferase (left gels) or with StarD13 siRNA (right gels). The cells were then lysed and incubated with GST-RBD (Rhotekin binding domain) to pull down active RhoA (upper gels). The samples were then blotted with RhoA antibody. The lower gels in each panel are western blots for total RhoA. **C)** The bands from the active RhoA gels were quantitated using ImageJ and divided by the quantitation of their bands in the total RhoA gels.

3.5. StarD13 localizes to focal adhesions and inhibits the activity of RhoA and Cdc42 but not Rac in astrocytoma cells

It has been reported that StarD13 localizes to focal adhesions in HeLa cells (Kawai, Seike, et al., 2009). To confirm that StarD13 also localizes to focal adhesions in astrocytoma cells, we transfected the cells with a vector including GFP-StarD13 and determined its localization by fluorescence microscopy. Images showed that StarD13 indeed localizes to focal adhesions in astrocytoma cells (Figure 21).

As StarD13 was also described to act as a GAP for RhoA and Cdc42 but not for Rac (Ching, et al., 2003), we were interested in determining the effect of the overexpression of StarD13 on the activation of these Rho GTPases in astrocytoma cell lines. Hence, a pull-down assay was performed to detect the level of activation of RhoA, Cdc42, and Rac in cells transfected with the GFP-StarD13 vector and compare it to the level of activation in control cells transfected with an empty vector. The results showed higher RhoA and Cdc42 activation in control cells as compared to cells overexpressing StarD13 (Figure 22A and B). However the level of Rac activation was not affected (Figure 22C).

To further elucidate the effect of StarD13-mediated inhibition of RhoA activity in astrocytoma cells, we studied the effect of StarD13 overexpression on the formation of stress fibers. The formation of stress fibers is mainly mediated by the cooperative activity of ROCK and mDia, which are downstream effectors of RhoA (Watanabe, et al., 1999). The overexpression of StarD13 led to a complete inhibition of stress fiber formation as shown by fluorescence microscopy following the staining of actin with Rhodamine phalloidin (Figure 22D).

Moreover, we determined the effect of StarD13 knockdown on the activation of RhoA following the stimulation of cells with EGF. The results showed that the activation of RhoA at time 0 was much higher in cells with StarD13 knockdown as compared to the controls, which is consistent with the role of StarD13 as a GAP for RhoA. Moreover, it was evident that the activity of RhoA was high and almost consistent at all time points as

compared to the cycle of activation and inactivation of RhoA in control cells, which was previously discussed in section 3.4 (Figure 20B).

Figure 21: StarD13 localizes to focal adhesions. The montage to the left shows Z-sections (0.2 μm apart) of SNB-19 cells (grade IV) that were transfected with GFP-StarD13. The micrograph to the right is a 3D reconstruction of such Z-sections after deconvolution. Cells were imaged using a 60x objective.

Figure 22: StardD13 is a specific GAP for Rho and Cdc42. T98G cells were transfected with either GFP alone (right lanes) or with GFP-StarD13 (left lanes). The cells were then lysed and incubated with **A**) GST-RBD (Rhotekin binding domain), or with **B**) GST-CRIB (Cdc42 and Rac interactive binding domain) to pull down active Rho or Cdc42 and Rac, respectively. The samples were then blotted with Rho, Cdc42 and Rac antibodies. The lower gels in each panel are western blots for the total cell lysates for loading control. **D**) Cells were transfected with GFP alone (upper panels) or with GFP-StarD13 (lower panels), fixed and then stained with Rhodamin Phalloidin to look at actin (right panels). Cells were imaged with a 60 X objective.

3.6. Role of StarD13 in astrocytoma cell motility and adhesion

3.6.1. StarD13 knockdown inhibits cell motility

After showing that StarD13 inhibits the activity of RhoA and that dominant active RhoA inhibits the motility of astrocytoma cells, we aimed at investigating the role of StarD13 in astrocytoma cell motility. For this reason, we used a siRNA oligonucleotide targeting StarD13 mRNA in order to knockdown StarD13. The knockdown of StarD13 was confirmed by western blot (Figure 23A). The knockdown of StarD13 decreased the wound closure rate of cells by around 30% from 8.8 $\mu\text{m/hr}$ to 6.3 $\mu\text{m/hr}$ (Figure 23B and C). Furthermore, StarD13 knockdown significantly decreased the average speed of

individual cells from 0.44 $\mu\text{m}/\text{min}$ to 0.24 $\mu\text{m}/\text{min}$ (Figure 24C) and the net distance migrated from 8.5 μm to 3.02 μm (Figure 24B and D). Altogether these results show that the knockdown of StarD13 inhibits astrocytoma cell motility.

Figure 23: StarD13 is needed for cell motility-1. Cells (T98G) were transfected with luciferase control siRNA or with StarD13 siRNA for 72 hours. **A)** The cells were lysed and immunoblotted by western blot analysis for StarD13 (upper gel) or for actin (lower gel) for loading control. **B)** The luciferase siRNA-transfected and StarD13 siRNA-transfected cells were grown in a monolayer then wounded and left to recover the wound then imaged at the same frame after 24 hours (lower micrographs). **C)** Quantitation for B). Wound widths were measured at 11 different points for each wound, and the average rate of wound closure for the cells was calculated in $\mu\text{m}/\text{hr}$. Data are the mean $\pm$ SEM from 3 wound closure movies. The results were significant with $p < 0.01$.

Figure 24: StarD13 is needed for cell motility-2. Cells (SF268) were transfected with luciferase control siRNA or with StarD13 siRNA for 72 hours. **A)** A montage of a time-lapse movie (60 sec apart) showing StarD13 siRNA-transfected cells undergoing random motility in serum. **B)** The net paths of projected 120 frames from 2 hour long time lapse movies of undergoing random motility in serum (different colors represent different cells) **C)** Quantitation of the cell speed from B) expressed in $\mu\text{m}/\text{min}$. **D)** Quantitation of the net paths shown in A) expressed in μm . Data are the mean $\pm$ SEM from 15 cells. The results were significant with $p < 0.001$ for both cell speed and net path.

3.6.2. StarD13 knockdown stabilizes focal adhesions and increases astrocytoma cell adhesion to collagen

Since StarD13 localizes to focal adhesions, we suspected that StarD13 knockdown inhibits cell motility by altering the dynamics of cell adhesions. We started investigating this hypothesis by determining the effect of StarD13 knockdown on focal adhesions by immunostaining for paxillin. The images showed that StarD13 knockdown leads to the formation of larger focal adhesions, especially at the tail of the cells, as compared to control cells (Figure 25A). Then, we studied the effect of StarD13 knockdown on the

adhesion of astrocytoma cells to collagen. The results showed more than two-fold increase in the adhesion of cells with the StarD13 knockdown as compared to control cells (Figure 25B and D).

Figure 25: StarD13 knock down leads to larger adhesions. **A)** Representative micrographs of (T98G) luciferase control cells (left panel) or StarD13 siRNA-transfected cells (right) that were fixed and immunostained with paxillin. **B)** Representative micrographs of cells fixed and stained with crystal violet to detect adhesion (as described in methods). **C)** Quantitation of B) Crystal violet was solubilized and the absorption of the plates was read at 550 μm using an ELISA plate reader. Data is measured in arbitrary units and normalized to the luciferase control. Data are the mean $\pm$ SEM from 3 experiments. The results were significant with $p=0.007$.

DISCUSSION

RhoA has been reported to be upregulated in a variety of cancer types including colon, breast, lung, ovarian, testicular, and bladder cancers (Fritz, Just, & Kaina, 1999; Horiuchi, et al., 2003; Kamai, Arai, Tsujii, Honda, & Yoshida, 2001; Kamai, et al., 2003). However, whether RhoA expression increases as astrocytoma tumors progress, or the opposite, is still a debatable issue in the literature. Two reports have addressed this matter, the first one reported a significantly increased expression of RhoA in high-grade astrocytomas (WHO grades III and IV) as compared to low-grade astrocytomas (WHO grades I and II) (Yan, et al., 2006); however, the second study showed that RhoA expression is significantly decreased in astrocytic tumors as compared to normal brain, and that this expression decreases as the degree of tumor malignancy increases (Forget, et al., 2002). Hence, in order to further investigate this, we measured the level of RhoA expression in astrocytoma tumors by immunohistochemistry. Our results showed that the expression of RhoA is higher in grade IV as compared to grade I astrocytoma. This supports the notion that RhoA expression increases as the degree of astrocytoma tumor malignancy increases in accordance with the results of Yan et al. (2006).

As Rho GTPases cycle between an inactive GDP-bound state and an active GTP-bound state (Bourne, et al., 1990), it was important to study the activation status of RhoA in astrocytoma cell lines and correlate it with the expression level in tumors. The data from FRET analysis of RhoA activation showed higher activation of RhoA in malignant cell lines of grades III and IV as compared to low-grade cell lines. Hence, RhoA is not only upregulated in high-grade astrocytoma, but it also exhibits higher activation. Taken together these results show that RhoA plays a positive role in the progression of astrocytic tumors.

RhoA also plays a positive role in the regulation of cell motility. Indeed, the inhibition of the RhoA or its downstream effector ROCK inhibits cell motility and invasion in many cell systems (Hakuma, et al., 2005; Ridley, 2001; Sahai & Marshall, 2003; Takaishi, et al., 1993;

Worthylake, et al., 2001). Therefore, it was of great interest to us to determine the effect of the knockdown of RhoA on the motility of astrocytoma cell lines. Our data showed that the inhibition of RhoA in astrocytoma causes a significant decrease in cell motility. These results might indicate that RhoA plays a role in the progression of astrocytic tumors through enhancing the motility of cells rendering them more invasive. These results are in accordance with a previous study that showed that the inhibition of the RhoA signaling pathway leads to a significant inhibition of glioblastoma cell migration (Manning, et al., 2000). However, contradictory results have also been reported, where the inhibition of ROCK, a downstream effector of Rho, lead to an increase in astrocytoma cell migration and invasion (Salhia, et al., 2005).

The silencing of RhoA seems to inhibit astrocytoma cell motility by preventing the maturation of focal complexes into focal adhesions, which are needed to provide the cell with the mechanical strength needed for the cell body to contract and move forward (Gupton & Waterman-Storer, 2006). Moreover, the cells where RhoA is knocked down seem to lose polarity as they become round in shape and lack any type of directional protrusions. This shows that RhoA plays the major role in the maturation of focal adhesions, which are needed for the effective stabilization of protrusions. However, in order for RhoA to be able to drive the conversion of focal complexes into focal adhesions, Rac must first induce the formation of these focal complexes. This is supported by our results showing the absence of any type of adhesions in the Rac1 knockdown cells. Hence, Rac is needed for the formation of focal complexes that initiate the stabilization of protrusions at the cell edge where it in fact shows high localization and activation. Indeed, similar to RhoA knockdown, Rac1 knockdown also causes the cells to lose polarization and protrusions, which renders them much smaller in size than control cells.

After establishing the involvement of RhoA in the progression of astrocytic tumors and in the motility of astrocytoma cells in particular, the second part of our study focused on determining the role of StarD13, a Rho GAP that inhibits the activity of RhoA, in astrocytoma cell motility. Previous studies have shown its inhibitory effect against RhoA in hepatocellular carcinoma cells (Ching, et al., 2003) and its localization to focal adhesions in HeLa cells (Kawai, Seike, et al., 2009). However, neither its role nor its intracellular localization has been studied in

astrocytoma cells. We showed, directly through the decrease of RhoA activation in cells overexpressing StarD13 and indirectly through the complete absence of stress fibers in these same cells, that StarD13 indeed functions as a GAP for RhoA in astrocytoma cells. We also confirmed that StarD13 localizes to focal adhesions in astrocytoma cells. Therefore, these data collectively demonstrate that StarD13 is a Rho GAP that inhibits the activity of RhoA in focal adhesions in astrocytoma cells.

As both RhoA and focal adhesions play indispensable roles in cell motility, it was highly intriguing to investigate the role of StarD13 in astrocytoma cell motility. The knockdown of StarD13 decreased the migration of astrocytoma cells. The role of StarD13 in motility has only been described in one report where its silencing caused an increase in cell migration in contrast to our results (Lin, et al., 2010); however, this study was done in normal vascular endothelial cells and not in cancerous cell lines, which might explain the discrepancy as cancer cell lines have altered signaling pathways. Our lab previously showed that although StarD13 functions as a tumor suppressor in astrocytoma, it shows higher expression in grade IV astrocytoma tissues as compared to grade I (data not published). The results in our current study, which show that StarD13 is needed for astrocytoma cell motility, might explain this increase in StarD13 expression in Grade IV tumors. Hence, StarD13 might be playing a role in the invasiveness of these tumors. In fact, a recent paper showed that the focal adhesion localization of DLC1, which is a tumor suppressor that belongs to the same family of proteins that contain (STAR)-related lipid transfer (START) domain as StarD13 or DLC2, is essential for astrocytoma motility (Kawai, Iwamae, et al., 2009). Hence, this might indicate that while these proteins function as tumor suppressors, they are needed for cell motility.

In order to determine the mechanism of the decrease in motility upon StarD13 knockdown, we started investigating the hypothesis that the knockdown of StarD13 leads to the constitutive activation of RhoA in focal adhesions, which leads to the stabilization of focal adhesions by preventing their disassembly and ultimately inhibiting cell migration. We suspected that this phenomenon occurs at the cell edge since the time-lapse movies of the StarD13 knockdown cells showed that a large proportion of cells were unable to move forward due to their inability to retract their tail, although these cells were able to protrude efficiently (Figure 15A). Our results verified this hypothesis, as StarD13 silencing induced the formation of larger

and thicker focal adhesions especially at the tail of cells. This was also supported by the fact that the cells with StarD13 knockdown were more than two times more adhesive to collagen than control cells. The increase in adhesion could have prevented the retraction of the tail, which we suspect is the major event that impeded cell motility.

For this hypothesis to hold true, the constitutive activation of RhoA in cells must have the same effect on motility as the knockdown of StarD13 since the latter is an inhibitor of RhoA. Therefore, we transfected the cells with a vector containing a dominant active form of RhoA, and the analysis of the effect on motility revealed that the constitutive activation of RhoA does indeed inhibit the motility of astrocytoma cell lines. In fact, the decrease in cell motility after the transfection of cells with a dominant active RhoA has been reported before in other systems (Banyard, Anand-Apte, Symons, & Zetter, 2000; Li, et al., 2010; Tkach, Bock, & Berezin, 2005; Vial, Sahai, & Marshall, 2003). Moreover, similar to StarD13 knockdown, dominant active RhoA led to the formation of larger focal adhesions. This shows that StarD13 silencing mimics the effect of dominant active RhoA on cell motility and adhesion.

This hypothesis is highly reinforced by the results of the time-lapse RhoA pull down assay following EGF stimulation and the time-lapse FRET movies, which showed that RhoA undergoes cycles of activation and inactivation at the cell tail. Hence, the activation of RhoA as well as its inactivation probably by a whole orchestra of GEFs and GAPs respectively is essential for astrocytoma cell motility. This, coupled to our previously discussed results on the role of StarD13 in adhesion and migration, might implicate StarD13 in playing a role in astrocytoma cell motility by inhibiting the activity of RhoA in focal adhesions, a process that is essential for the disassembly of focal adhesions and the completion of the cell motility cycle.

Hence, Rho is active at the cell tail but then needs to get inactivated for the focal adhesions to dissolve and the cell to pull itself. This is why high levels of Rho, as in the cells transfected with dominant active RhoA or when StarD13 is knocked down, inhibits cell motility. Rho is also active at the leading edge for the focal complexes to mature and the cell to push forward. This is why when we inhibit Rho, the cells cannot move. Therefore, the cell must undergo a push and pull movement that is governed by Rho in order to migrate efficiently.

CONCLUSIONS

Our results from the RhoA IHC and the FRET experiments showed that RhoA expression and activation positively correlate with the progression of astrocytic tumors. Our results also showed that both RhoA and Rac1 play positive roles in cell motility since knocking them down by siRNA transfection inhibited cell motility. In this context, we showed that Rac plays a role in the formation of focal complexes, which mature into focal adhesions through the activity of RhoA. We expected that knocking down the Rho inhibitor, StarD13, would lead to an increase in cell motility. Surprisingly, when we knocked down StarD13 using siRNA we saw an inhibition of cell motility. This was mimicked by transfecting the cells with a dominant active RhoA construct. This suggested that RhoA activation needs to be tightly regulated with cycles of activation and inhibition. StarD13 mainly localized to adhesion structures, thus RhoA needs to be inhibited at the sites of adhesion. StarD13 knock down cells were unable to move and showed a phenotype of an elongated tail where large focal adhesions were observed. This showed us that Rho inhibition by StarD13 plays a role in the disassembly of focal adhesions at the cell rear, a process that is needed for the cell to detach its tail and to move forward. In order to summarize our results, we developed a model that is depicted in the following figure.

Figure 26: Proposed model for astrocytoma cell motility. **A)** Rac1 activation drives the formation of focal complexes. **B)** RhoA activation causes the maturation of focal complexes into focal adhesions and leads to the formation of stress fibers (upper panel) and the contraction of the cell body (lower panel). **C)** StarD13 plays a role in the inactivation of RhoA, driving the disassembly of focal adhesions and allowing tail detachment. **D)** The knockdown of StarD13 inhibits the inactivation of RhoA, which stabilizes old focal adhesions preventing their disassembly and impeding the detachment of the tail.

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