Rho A versus Rho C: Role in invadopodia formation and invasion of astrocytoma cells

By
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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Molecular Biology

School of Arts and Sciences
May 2014
LEBANESE AMERICAN UNIVERSITY
School of Arts and Sciences; Byblos Campus

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Thesis Title: Rho A versus Rho C: Role in invadopodia formation and invasion of astrocytoma cells

Program: Master of Science in Molecular Biology

Department: Natural Sciences

School: Arts and Sciences

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DEDICATION

To my friend Belinda Allen
You will always be remembered
ACKNOWLEDGEMENTS

First of all, I would like to express my deepest and most sincere gratitude to Dr. Mirvat El- Sibai who has not only been a dedicated and supportive advisor but also a mentor and a friend. I am very grateful for her continuous support, encouragement, and belief in my potentials. I am extremely lucky to have had the opportunity to work with such a talented and candid scientist.

I would also like to convey my appreciation to the Dean of Arts and Sciences Dr. Nashaat Mansour, the Chairperson of the Biology department Dr. Constantine Daher, the faculty members of the Biology department Dr. Sima Tokajian, Dr. Roy Khalaf, Dr. Ralph Abi-Habib, Prof. Fuad Hashwa, Dr. George Khazen, Dr. Brigitte Wex, Dr. Sandra Rizk-Jamati, to the committee members Dr. Ralph Abi Habib and Dr. Sandra Rizk-Jamati.

I am also thankful to Ms. Maya Farah, Ms. Helena Bou Farah and Mr. Jean Karam for their technical support and continuous help.

I would like to thank my colleagues in the Master’s program especially my colleagues in Dr. El Sibai’s lab. A special appreciation goes to Bechara Saykali, Gilbert Salloum and Emile Lakis.

Finally a special thanks goes to my family and friends for supporting me all the way.
Rho A versus Rho C: Localization, role in invadopodia formation and invasion of astrocytoma cells
Saleh B. AL Dimassi

ABSTRACT
Malignant astrocytomas are associated with high mortality rates; these tumors are highly invasive into adjacent areas of the normal brain. It has been established that Rho family of GTPases play a central role in regulation of cell migration and invasion. The Rho family is ras-related, consisting of the Rho, Rac, and CDC42 subfamilies. The Rho Protein has subtypes designated as RhoA, RhoB, RhoC, RhoD, RhoE, and RhoH genes. In this study we aim to look at the role both Rho A and Rho C play in protrusion formation where we found that upon RhoA knockdown protrusions are no longer formed, using 2D time lapse microscopy that shows the depletion of RhoA and RhoC leads to the decrease in 2D motility, RhoA knockdown decreases adhesion of cells in contrast RhoC knockdown increases adhesion of astrocytoma, and FRET based biosensors. In addition to study the role both proteins play in invadopodia formation which is not RhoA dependent according to our data, and cellular invasion where our results shows an increase in cell invasion upon both RhoA and RhoC knockdown. This study examines for the first time, the differential roles of RhoA and RhoC isoforms in astrocytoma motility and invasion.

Keywords: RhoA, RhoC, Invadopodia, Invasion, Fret, motility.
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List of Abbreviations

WHO: World Health Organization
CNS: Central Nervous System
ECM: Extracellular matrix
MMP: Matrix metalloproteinase
MT-MMP: Membrane-type matrix metalloproteinase
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
GDP: Guanosine diphosphate
GTP: Guanosine triphosphate
GEF: Guanine Nucleotide Exchange Factor
GAP: GTPase Activating Protein
GDI: Guanine Nucleotide Dissociation Inhibitor
PI3K: Phosphatidylinositol 3-kinase
MLC: Myosin Light Chain
ROCK: Rho Kinase
Y-27632: ROCK inhibitor
PMA: phorbol 12-myristate 13-acetate
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
DMEM: Dulbecco's Modified Eagle Medium
L15: Leibovitz’s L15
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
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

CAFs: cancer associated fibroblasts
Chapter 1

Literature Review

1.1 Cancer Hallmarks:

Cancer is a complex group of diseases. It is caused by several factors that are divided into extrinsic factors such as tobacco, viruses and radiation, and intrinsic factors such as hereditary gene mutations. Coupling of these factors will increase the risk of cancer (American Cancer Society, 2014).

Cancer cells develop tumors by a series of mutations that occur in critical sites of the genome, more specifically in oncogenes and tumor suppressor genes (Al Dimassi, Abou Antoun, & El sibai, 2014). These cells have certain characteristics that differentiate them from normal cells such as uncontrolled growth, escape from growth suppressors, activation and increase in cell motility and invasion, promotion of angiogenesis, and inhibition of apoptosis (Hanahan & Weinberg, 2011).
Fig. 1. Several mutations must occur in the cell genome to develop cancer. Mutation in normal cells (light gray) result in an increase in proliferation rate. Mutated cells (light blue) these cells and because of their faster growth of normal cells may undergo more mutations resulting in a faster growth, when enough mutation is accumulated formation of tumor (blue cells), more mutations need to occur to convert this tumor to malignant (red cells) and even to metastatic one to reach other organs. (Source: Jay D. Hunt, LSU Health Sciences Center)

1.2. Cancer statistics:

Cancer is considered as one of the leading causes of deaths worldwide. Every year around 13 million new cases of cancer are reported (Baskar, Lee, Yeo & Yeoh, 2012).

According to the American cancer society, a total of 1,660,290 new cancer cases for both sexes will be reported in United States by the end of 2013. Unfortunately one third of these cases will die, with an estimated 580,350 deaths by the end of same year (Siegel, Naishadham & Jemal, 2013). Based on National Cancer Institute an average of 1600 patient will die per day because of cancer in 2013 (American Cancer society, 2013).

An approximate of 40,000 brain tumor cases were reported between 1973 and 2001. Half of the cases were diagnosed and confirmed as glioblastoma multiform (Deorah, Lynch, Sibenaller & Ryken, 2006).
1.3. Brain Cancer:

1.3.1. Glioblastomas and Astrocytomas:

According to the world health organization, malignant tumors are classified into four different grades, from I to IV, where I has the lower aggressiveness with an increasing aggressiveness to reach the most with grade IV (Collins, 2004).

Brain tumors become more malignant with time and are classified into 4 different grades, including (grade II) astrocytomas with an occurrence peak above 25 years of age, anaplastic astrosytomas (grade III), and the most aggressive type glioblastomas (grade IV) with occurrences peak above 45 years of age (Biernat, Tohma, Yonekawa, Kleihues & Ohgaki, 1997; Deimling, et al., 1993). Survival rate of patients with brain tumors differs depending on the aggressiveness and the grade of the tumor. For example patients with grade II astrocytomas may survive up to 7 years, as for patients with grade III anaplastic astrocytomas survive up to 3 to 4 years. Grade IV is the most aggressive and lethal type of brain tumor, with a survival period of less than 1 year (McCormack, Miller, Budzilovich, Voorhees & Ransohoff, 1992; Simpson, et al., 1993). It’s reported that around 9000 new cases of giloblastoma are diagnosed every year in the States (Perry, et al., 2012). This tumor
is outlined histologically by cellular atypical, necrotic foci with peripheral cellular pseudopalisading, and microvascular hyperplasia. These features differentiate this high grade of brain tumor from lower grade astorsyomas (Tso, et al., 2006).

1.3.2. Diagnosis:

Brain tumors represent an overwhelming and unsolved clinical problem despite the remarkable advancements in therapies of other types of cancer (Koo, et al., 2006). Several methods are used to diagnose brain tumors such as biopsy, CT scan, PET, ultrasound and MRI. These recent imaging techniques do not determine the definite tumor size due to the presence of edema around the tumor site. This makes it hard to exactly define the boundaries of the tumor and thus to quantify it (Nimsky, Ganslandt, Kober, Buchfelder, & Fahlbusch, 2001).

1.3.3. Treatment:

Brain tumors are basically treated via surgery followed by chemotherapy, radiation therapy and photodynamic therapy (Ullrich & Pomeroy, 2003). Each of these treatments has limiting factors, Surgery is very invasive to the brain: it is in fact impossible to remove the tumor mass without destroying brain tissues and causing damage. In addition, it is difficult to remove the whole tumor mass without leaving some of the cancerous cells in the site (Albayrak, Samdani & Black, 2004). The
remaining three types of therapies are considered as non-invasive (Armstrong, et al., 2002). Chemotherapy is not selective and can affect both normal and cancerous cells, in addition to the development of multidrug resistance system in cancer cells resulting in the rejection of the drug by tumor cells (Gatmaitain & Arias, 1993). Unlike chemotherapy, radiation therapy targets narrower and more specific areas of the tumor. Unfortunately tumor develops resistant mechanisms resulting in tumor recurrence, cell survival and inhibition of apoptosis (Begg, Stewart & Vens, 2011).

1.3.4. Brain cancer and Invasion:

Glioblastoma invades the surrounding brain sites. This type of invasion is highly reliant on growth factors and signaling pathways that regulate cell motility (Demuth, et al., 2000). The highly invasive type of the glioblastoma leads to the failure of the treatments used to target this cancer (Giese, Bjerkvig, Berens & Westpal, 2003). Glioblastoma cells invade as single cells; their invasion is either through white matter of the brain or through blood vessel walls and subpial glial space. Grade IV gliomas do not metastasize outside the brain, unless they are the result of other cancer cells that have translocated to the brain and formed a secondary tumor (Bellail, Hunter, Brat, Tan & Van Meiret, 2004).
1.4. **Cell migration and invasion:**

1.4.1. **Cell motility:**

Cell motility is a complicated process that is tightly coordinated by the cell with the help of various proteins. These proteins serve to determine the direction of cell movement, and to carry out the motility cycle. This cycle is driven and affected by actin polymerization and reorganization (Ananthakrishnan & Ehrlicher, 2007).

Cell movement plays a central role in tumor progression resulting in cancer spreading and invasion of other organs (Lauffenburger & Horwitz, 1996). The cell will first detect a chemoattractant and determine the direction of movement, the cell will then extend a protrusion towards the signal (Bailly, Condeelis & Segall, 1998). The next step is the stabilization of this protrusion by adhesion to the substratum resulting in the anchorage of this protrusion, which allows the cell to pull itself towards the stimulus direction. In the mean time the adhesion structures at the rear site of the cell start to disassemble resulting in the retraction of the cell tail and shifting of the cell position (Ananthakrishna & Ehrlicher, 2007; Condeelis, et al., 2001).
Fig. 2. 2D motility is a multistep process. The first step is formation of protrusion (1), followed by anchorage of this protrusion due to the formation of focal adhesions (2), then cell contract (3) using the previously formed anchorage resulting in cell movement after the disassembly of the focal adhesions at the tail end (step 4) (Ladoux & Nicolas, 2012).

1.4.2 Patterns of cells movement:

Single cells acting individually or by a group of cells can achieve 2D cell motility. Single cell migration is described above. In multiple cell movement, as is
the case in tumors, a leading cell forms a track by proteolytic activity leading to the degradation of the extracellular matrix, then the other cells in the group follow the leader resulting in increased proteolysis and widening of the previous track formed by the leading cell, causing enablement of multiple cells movement. The third pattern is collective migration where a group of cells acts a single mega cell (Fried & Alexander, 2011).

During cancer metastasis, cells migrate on a single cell basis or as a group of cells migrating. Single cell movement can be either of amoeboid or mesenchymal type. Both types lack cell-cell junctions. On the other hand, multicellular streaming follows the same type of movement as single cell migration, except the fact that in this case, cell-cell junctions are required for the efficient migration. (Friedl & Alexander, 2011).

<table>
<thead>
<tr>
<th>Cell migration type</th>
<th>Cell-cell junctions</th>
<th>Tumor type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoeboid</td>
<td>-</td>
<td>Leukemia, lymphoma, melanoma, renal cell carcinoma (renal cell carcinoma)</td>
</tr>
<tr>
<td>Mesenchymal</td>
<td>-</td>
<td>Stromal tumors, epithelial tumours, frank cell tumours</td>
</tr>
<tr>
<td>Amoeboid (multicellular)</td>
<td>+</td>
<td>All tumors developing additional single cell dissemination</td>
</tr>
<tr>
<td>Mesenchymal (multicellular)</td>
<td>(+)</td>
<td>Tumour cell-derived leading tumor cells</td>
</tr>
<tr>
<td>Cluster</td>
<td>++</td>
<td>Moderately differentiated epithelial tumours</td>
</tr>
<tr>
<td>Solid strand</td>
<td>++</td>
<td>Moderately differentiated epithelial tumours (PAN)</td>
</tr>
<tr>
<td>Strand (with lumen)</td>
<td>++</td>
<td>Differentiated epithelial tumours, vascular neoplasia</td>
</tr>
<tr>
<td>Strand (protrusive)</td>
<td>++</td>
<td>Moderately differentiated epithelial tumours (PAN)</td>
</tr>
<tr>
<td>Outward pushing tumor</td>
<td>++</td>
<td>All solid tumors</td>
</tr>
</tbody>
</table>

**Fig. 3. Different patterns of cancer cell movement.** Individual cell migration (amoeboid: leukemia and mesenchymal: stromal tumors) does not need any kind of cell cell junctions, as for multicellular streaming were some adhesions are needed as fibroblast leading tumors, as for the collective cell migration cell-cell junctions is highly required as in differentiated epithelial tumors (Friedl & Alexander, 2011).
1.4.3. Invasion /3D motility:

Invasion is an essential step of cancer progression and secondary tumor formation, by which cancer cells decide to attack and colonize nearby tissues and organs. It is believed that cancer cell invasion is a major cause behind the failure of treatment of many tumor types (De Wever & Mareel, 2003). In the seed and soil theory, Stephan Paget suggested that the cancer cell “seed” depends on the competency of secondary cancer site or organ “soil” (Paget, 1889). The site of formation of the secondary tumor is not randomly chosen; three types of colonization determinations are proposed, the first type is selective growth type where the cancer cells grow in organs and tissues that have the growth factors they need. The second type is selective adhesion to specific sites and locations at specific organ. The last type is selective chemotaxis were the secondary proposed cancer site secretes and produces chemicals and other factors that attract and direct these invading cancer cells to them (Bogenrieder & Heryln, 2003).

During invasion around one million cancerous cells leave the initial tumor and enter the circulatory system each day, with less than 0.1% of these cells that succeed to develop migration (Fidler, 1970; Mack & Marshall, 2010). Tumor cell invasion occurs in several steps including: local invasion of neighboring and adjacent tissue, transendothelial movement of cancer cells into blood vessels or intravasation, survival of cancer cells in the blood stream, and extravasation followed by invading and colonizing the targeted secondary tumor site (Van Zijil, Krupitza & Mikulits, 2011).
Fig. 4. **Invasion is a multistep process.** Growth of cells as benign tumors followed by invasion of basement membrane into blood (intravasation), travel of cells in blood and adhesion to capillary walls, finally extravasation and growth of cancer cells at secondary tumor site (Alberts, 2002).

1.4.4. **Invadopodia:**

Cancer cells need to degrade the extracellular matrix to be able to invade and intravasate and this is achieved by structures called invadopodia (Bravo-Cordero, et al., 2011). These protrusions formed and extended vertically to ECM are enriched with actin regulatory proteins (Condeelis, Singer & Segall, 2005); Polymerization of actin is necessary for maturation and formation of invadopodia (Oser, et al., 2009).
Invadopodia contain variable proteins that serve different functions such as adhesion molecules and matrix degradation proteases (Condeelis, Singer & Segall, 2005).

Fig. 5. Tumor cell invasive types. The three types are Plasma membrane blebs, invadopodia or pseudopodia. Protruding blebs totally lack actin, invadopodia are rich in actin and proteins that regulates actin, as for pseudopodia that depend on Wave protein to nucleate actin (Nürnberg, Kitzing & Grosse, 2011).

Invadopodia formation is divided into four stages. Primary gathering of cortactin and actin is the first step, which is known as invadopodia initiation. The same happens in second step. As for the third stage, the invadopodia maturation step MT1-MMP is accumulated and matrix degradation is observed. The final step is known as late invadopodia stage that is characterized by presence of MT1-MMP and matrix degradation with the absence of actin and cortactin (DesMarais, et al., 2009).
1.5. Rho GTPases:

RAS protein superfamily includes different protein sub families as Ras, Rab, ARf, Rho, and Ran families. The Rho family is characterized by the presence of Rho domain. Rho family of GTPases contains 20 members that are divided into 8 different subfamilies (Boureux, Vignal, Faure & Fort, 2007). These proteins, which vary in size between 20 KDa and 40 KDa, are considered as key players and regulators of cancer cell migration and invasion (Boettner & Van Aelst, 2002).

Rho proteins act as molecular switches. They cycle between two states, the active one that is bound to GTP and the inactive state that is bound to GDP (Jaffe & Hall, 2005). GEF, GAP, and GDI proteins tightly regulate this switching between the two states. Guanine nucleotide exchange factors ”GEFS” promote the activation of Rho proteins by facilitating the release of GDP and the binding of GTP, GAPs or GTPase activating protein, act opposite to GEFs resulting in the hydrolysis of GTP and the deactivation of Rho protein (Bos, Rehmann & Wittinghofer, 2007). As for “GDIs “ guanine nucleotide dissociation inhibitors, which can bind both, the GDP bound form and the GTP bound form by binding to the C-terminal group, preventing the membrane association of GTPases and insulating them in the cytoplasm, thus preventing their contact to down stream effector proteins and targets (Etienne-Manneville & Hall, 2002).
Fig. 6. The Activation and deactivation cycle of Rho GTPases. Rho proteins cycle between two forms the active one binding GTP that can stimulate downstream reactions and inactive form that bound to GDP. Three proteins regulates this switching GEF which promotes switching to the active form, GAP works opposite to GEF, and the third protein GDI that can hold both forms the active and in active one away from their membrane binding sites and stoping their function (Aktories & Barbieri, 2005).

1.5.1. Rho A and Rho C:


Studies have shown that Rho A plays a role in cancer cell proliferation and survival. It was found that Rho A promotes epithelial polarity in addition to junction assembly and function (Jaffe & Hall, 2005; Braga & Yap, 2005). Rho A is important for amoeboid and mesenchymal motility. Rho A and Rock cross talk leads to the
promotion of actomyosin dependent contractility resulting in amoeboid migration, in addition to the retraction of the tail in mesenchymal migration (Friedl & Wolf, 2003).

Previous study showed that overexpression or knocking down RhoA will lead to the decrease of 2D motility, it also effect the maturation and formation of focal adhesions. The same study shows that RhoA is responsible for protrusion formation and its localized at the edges of cell, and it undergo activation and deactivation cycle (Khalil, et al., 2013)

As for Rho C, its role in cancer is restricted to metastasis. It has been found that Rho C is up regulated in metastatic melanoma, whereby several studies correlate Rho C with invasion and motility, however the mechanism is still not .The difference between Rho C effect and Rho A is also of a lot of interest (Bellovin, et al., 2006; Pillé, et al., 2005;Clark, Golub, Lander & Hynes, 2000;Simpson, Dugan & Mercurio, 2004;Miles, Pruitt, Van Golen & Cooper, 2008).
1.1. **Purpose of the Study**

Our first objective in the current study is to investigate the distinctive roles that Rho A and Rho C play in the progression of astrocytomas, by studying how the absence of Rho A and Rho C affect the phenotype and morphology of astrocytic cancer cells. The second aim is to determine the roles of these two proteins in the regulation of 2D cell motility. We also investigate their effect on cell adhesion is considered a crucial and important step of cell invasion.

In addition we aim to study the different roles they play in invasion or 3D motility of astrocytomas, through the formation of invadopodia. This study is the first to distinguish between the roles of RhoA and RhoC isoforms in astrocytoma motility and invasion.
Chapter 2

MATERIALS & METHODS

2.1. Cell Culture

Human astrocytoma cell line SF268 was 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, Mouse monoclonal anti-Actin antibody, Rabbit polyclonal anti-Rock1 antibody and Rabbit polyclonal a, anti-Rock2 antibody were obtained from Santa Cruz Biotechnology. Mouse
monoclonal anti-RhoA, mouse monoclonal anti-Rac1, were purchased from Upstate biotechnology, Lake Placid, NY. Mouse polyclonal anti-RhoC obtained from abcam. Anti-goat, anti- Rabbit 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).

2.3. Cell transfection with siRNA

Goat FlexiTube siRNA for StarD13, RhoA, RhoC, Rock1, Rock2 and Rac1 were obtained from Qiagen. 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 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 reseeded with DMEM supplied with 30% FBS. The experiments were carried on 24 hours following transfection.

RhoA construct was a generous gift from Dr. Hitoshi Yagisawa from the University of Hyogo, Japan and Dr. Hideki Yamaguchi from the Albert Einstein College of Medicine, New York, USA.

The construct was 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 Maxi Prep plasmid extraction kit from Qiagen.

2.5. Western blotting

Cell lysates were prepared by scraping the cells in a sample buffer (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)

SF268 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, Hodgson, Kelmke & Hahan, 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. 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.

2.8. Motility assay/Analyzing 2D motility:
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 overlayed 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 µm/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.

2.9. **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. Washing the plates and chilling them on ice followed this. Meanwhile, the cells were trypsinized and counted to 4x105 cell/ml. 50 µl 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 µm using a plate reader.

2.10. Boyden Chamber/Invasion Assay:
Cells were transfected with either control or RhoA siRNAs, RhoC siRNAs, or both RhoA and RhoC siRNAs, and the invasion assay was performed 48 h following the treatment period using the collagen-based invasion assay (Millipore) according to the manufacturer's instructions. Briefly, 24 h prior to the assay, cells were starved with serum-free medium. Cells were harvested, centrifuged and then resuspended in quenching medium (without serum). Cells were then brought to a concentration of 1x10^6 cells/ml. In the meantime, inserts were prewarmed with 300 µl of serum-free medium for 30 min at room temperature. After rehydration, 250 µl of medium was removed from the inserts, and 250 µl of cell suspension was added. Inserts were then placed in a 24-well plate, and 500 µl of complete medium (with 10% serum) was added to the lower wells. Plates were incubated for 24 h at 37°C in a CO2 incubator. Following the incubation period, inserts were stained for 20 min at room temperature with 400 µl of cell stain provided with the kit. The stain was then extracted with extraction buffer (also provided). The extracted stain (100 µl) was then transferred to a 96-well plate suitable for colorimetric measurement using a plate reader. Optical density was then measured at 560 nm.
2.11. Invadopodia formation Assay:

The cells were plated on cover slips, and the appropriate treatment was applied. Prior to staining day cells was starved using L 15 media obtained from gibco with 0.35% BSA overnight, then immuno-staining as described before, using rhodamin phalloidin targeting actin. Induction of invadopodia was done either by PMA of 370mM for one hour prior to staining at 37 degrees and 5% CO2. Rock inhibition was performed using 30 µM of Rock inhibitor treatment 1 hour at 37 degrees and 5% CO2 prior to staining.

![Fig. 7. The RhoA FRET biosensor.](image)

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 fluorescent protein (YFP) domain, and a full length RhoA ((Pertz, Hodgson, Kelmke & Hahan, 2006).
Chapter 3

RESULTS

3.1. RhoA and RhoC Depletion using siRNA

Before studying the role of RhoA and RhoC in astrocytoma cell motility, we first wanted to study the expression level of RhoA and RhoC astrocytoma cells before and after the silencing of both proteins genes (Figure 8).

![RhoA and RhoC Depletion](image)

**Fig. 8. RhoA and RhoC Depletion.** A) Depletion of RhoA after 72 hours of transfecting cells with RhoAsiRNA. B) Depletion of RhoC after 72 hours transfection and usage RhoCsiRNA. In both experiments actin was used as a loading control.
3.2. **RhoA and RhoC depletion effect on cell morphology**

We started by transfecting cells with siRNAs targeting RhoA and Rho C separately, and after 72 hours we look at the morphology of astrocytoma cells in response to silencing. RhoA knockdown lead to the loss of cell polarity, decrease surface area and elongated, while RhoC knocking down lead to the increase in cell surface area and the loss of polarity. (Figure 9).

![Control](image1) ![RhoA Knock down](image2) ![RhoC Knockdown](image3)

**Fig. 9. Depletion of RhoA or RhoC leads to different changes in cell morphology.** Representative Phase contrast images of astrocytoma cells showing the morphological changes due to RhoA or RhoC knockdown, compared to control cells where the expression of these proteins is normal.

3.3. **RhoA and RhoC are necessary for 2D cell migration**

RhoA knock down results in the decrease of cell motility as observed by time-lapse microscopy, Same was observed for cells with RhoC knockdown. The presence of both proteins is essential for cell migration to occur,
quantitation of cell movement was done using image j software where every cell was tracked over the 120 frames, 11 cells for each time-lapse movie, and the average of the distance was measured (Figure 10).

Fig. 10. RhoA and RhoC positively regulate 2D cell motility. Depletion of RhoA or RhoC in astrocytoma cells decreased cell motility as compared to control cells. Data are the mean +/- SEM from 3 different experiments. The results were significant with p<0.001.

3.4. RhoA and Rho C play opposite roles in astrocytoma cells adhesion
Cells where plated on collagen coated 96 well plates, followed by staining of cells by crystal violet and then the absorption of the plates was read at 550 µm using a plate reader.

RhoC knock down results in increase of cell adhesion, while RhoA knockdown decreased the adhesion of astrocytoma cells (Figure 11).
Fig. 11. RhoA and RhoC play different roles in cell adhesion. A) Cells were transfected with RhoC siRNA or luciferase siRNA, and after 72 hours adhesion experiments was performed as described in chapter two. B) Cells were transfected with RhoA or luciferase siRNA this was followed by adhesion experiment after 72 hours. Data are the mean +/- SEM from 3 different adhesion experiments for each protein. The results were significant with p<0.01.
3.5. RhoA and RhoC depletion promotes 3D invasion in astrocytoma

After establishing the importance of RhoA and RhoC in cell adhesion, we wanted to test their effect in cell invasion as shown in figure 12, this was done by knocking down RhoA followed by boyden chamber, where cells invade toward rich media with growth factors after being starved and platted in porous inserts, the invading cells will be trapped in the pores after that they where stained and extracted and measured at 560 nm. Both RhoA and RhoC seem to attenuate cell invasion since knocking down either of these proteins or both led to an increase in cell invasion.
Fig. 12. RhoA and RhoC have the same effect on cell Invasion. Cells were transfected with RhoAsiRNA (A) or with RhoCsiRNA or both (B) and tested for invasion. Data are the mean +/- SEM from 3 experiments. The results were significant with p=0.007.
3.6. **Invadopodia is not RhoA dependent**

In response to PMA stimulation, cells produce invadopodia. Starving cells in serum free media followed by PMA stimulation and staining the cell with rhodamine phalloidin to look at Actin in invadopodia structures. These appeared to be completely RhoA independent since knocking down RhoA did not affect the formation of invadopodia and no decrease in dots or invadopodia as compared to cells expressing RhoA normally (Figure 13).

A.
Fig. 13. RhoA does not affect invadopodia formation. A) Cells were treated with PMA or left untreated and stained with Rhodamine phalloidin. B) Cells were transfected with luciferase or RhoAsiRNA, treated with PMA or left untreated and stained with rhodamine phalloidin. Data are the mean of +/- SEM of 3 different experiments. The result were significant with P<0.01.

3.7. Invadopodia is not starD13 dependent

Stard13 is known to be a negative regulator of RhoA, overexpression of starD13 leads to the deactivation of RhoA, we wanted to see if stard13 affect invadopodia formation. We knocked down stard13 in astrocytoma cells, and then we performed invadopodia assay as described before with /without PMA stimulation. Invadopodia was formed upon PMA treatment when the cells were treated with siRNA for stard13 (Figure 14).
Fig. 14. **Invadopodia formation doesn’t depend on StarD13.** A) Cells were transfected with luciferase or stard13siRNA, lysed and blotted using stard13 antibody. B) Cells were transfected with siRNA as in (A) treated with PMA or left untreated and then stained with rhodamine phalloidin.

3.8. **Invadopodia is ROCK independent**

Rock is known as down stream effector of RhoA, inhibition of rock using the Y26732 rock inhibitor, followed by invadopodia assay and PMA stimulation
revealing the absence of invadopodia. This is consistent with the fact that RhoA do not affect invadopodia formation (Figure 15).

Fig. 15. Invadopodia are rock independent. Cells were transfected by with Y27632 as described in chapter two or left untreated, cells were then fixed and stained with rhodamine phalloidin.

3.9. Localization of RhoA and its activation

We used the previously described RhoA biosensor to look and detect the localization of RhoA in Glioblastoma cell lines were our finding suggest that RhoA is localized at cell edges, in addition to that we examined the activation of RhoA in different grades of brain tumor cells that shows different activation ratios depending on the grade. This is consistent with the invadopodia. (Figure 16).
Fig. 16. **Localization and activation of RhoA in brain tumor.** A) Using RhoA FRET biosensor to detect the localization of RhoA in Brain Tumor cancer cells revealed the presence of active form at the edge of cell. **B)** Activation levels of RhoA in different grades of brain tumors.
3.10. Invadopodia is RhoC dependent

Upon the depletion of RhoC and PMA stimulation no invadopodia were observed as shown (Figure 17).

Fig. 17. RhoC expression is necessary for invadopodia formation. Cells were transfected with luciferase or RhoC siRNA, treated with PMA and or left untreated and stained with rhodamine phalloidin.
Invasiveness of brain carcinoma and its infiltration to the neighboring locations in brain limits the survival of patients from several years to several months, depending on the stage of this cancer. Rho Family of GTPases plays a central and important role in regulation and controlling cell motility and invasion. Based on that we wanted to determine the effect of the two-rho family members, RhoA and RhoC, on motility and invasion of astrocytoma.

We knocked down the two Rho proteins in astrocytoma cells using specific siRNA targeting RhoA and Rho C, and looked at the effect. Cells with low RhoA expression or RhoC expression tend to lose their normal shape. RhoA depleted cells lose their normal shape and are more extended and elongated. RhoC depletion showed a totally opposite phenotype where the surface area of cells is increased and cells look more flat than the normal cells. These results suggest that both proteins have different and distinctive roles in the regulation of down stream proteins resulting in the difference of the two observed phenotypes. The decrease in cell area detected in RhoA
knockdown cells suggests that RhoA play a role in stabilizing the protrusion, which is consistent with our previous studies done by (Khalil, et al., 2013) and with the localization of active RhoA to the cell edge is observed by FRET. The round phenotype in RhoC knock down cells is indicative that cells are losing polarity. This phenotype is the same as that observed in breast adenocarcinoma upon Rac is overly activated (Backer, et al., 2007). Current work in our lab showed that RhoC inhibits cdc42. RhoC could be also leading to Rac inhibition through cdc42, which would explain why when RhoC is knocked down, the cells mimic a phenotype of an increase in active Rac.

Previous studies showed that RhoA and RhoC play an important role in the regulation of cell migration. Therefore, it became of great interest to study the role RhoA and RhoC plays in regulating astrocytoma motility. Our data showed that depletion of both proteins results in the decrease of cellular motility. These results come in accordance to previous studies on prostate cancer (Vega, Fruhwirth & Ridley, 2011). Since adhesion is very important and crucial step in cell motility as described in chapter one, in addition to our data that shows the change of phenotype and decrease in motility upon the knockdown of RhoA and RhoC. The next target was to see what would be the effect of both RhoA and RhoC down regulation on astrocytoma cell adhesion. Our adhesion data showed a different role RhoA and RhoC perform in adhesion control, RhoA depleted cells results in an decrease of adhesion while RhoC depleted cells showed the opposite with an increase of adhesion. To correlate our findings with each other it is important to keep in mind
that cycling of adhesion at different sites of the cell is important to maintain healthy migration pattern, RhoA knock down leaded to decrease in 2D motility and with an extended phenotype and a decrease of adhesion this suggests that RhoA positively regulates adhesion, so once we lose adhesion by knocking down RhoA the cell can not adhere any more and thus can not perform efficient movement. On the other hand RhoC knockdown resulting in increase of adhesion, which means that the cell is now adhered well, and the cycle of formation adhesion structures and this protein interrupts the disassembly. This suggests that RhoC in contrary to RhoA negatively regulate adhesion. The balance between RhoA and RhoC expression in the cell is very important to regulate the adhesion cycling in cell, therefore regulation of cell motility.

After establishing that both RhoA and RhoC positively regulate 2D motility, the second part of the study focused on determining both proteins role in 3D motility of astrocytoma or invasion. The invasion results showed that after knocking down RhoA invasion increased, the same was observed with RhoC knockdown. This finding suggests that both proteins act on the same down stream proteins resulting in regulating invasion. In fact RhoC expression increase cellular invasion (Simpson, Dugan & Mercurio, 2004) this is contradictory to our RhoC invasion results, as for RhoA its role in invasion varies depending on cell type and system (Bellovin, et al., 2006). The contradiction between our results about the effect of RhoC in invasion and other studies in the literature can be explained by the possibility of that RhoC effect on invasion is cell and system dependent same as RhoA.
As both RhoA and RhoC negatively regulates invasion, the next aim was to study their role in the formation on invadopodia. Starting our investigation with knocking down stard13 an upstream regulator of RhoA, we found that invadopodia is still forming even with the lack of expression of this protein, same goes for RhoA and Rock. Our Data suggest that the formation of invadopodia is not RhoA dependent, or its upstream regulator stard13, or its down stream effector Rock. Surprisingly after knocking down RhoC and even with the presence of PMA no invadopodia was observed and formed, this finding leads us to conclude that invadopodia formation is RhoC dependent.
Chapter 5

CONCLUSIONS

In this study we tried to point at the distinctive roles that both proteins RhoA and RhoC serves in astrocytoma cells invasion, movement and invadopodia formation. Our results suggest that RhoA and Rho C expression is necessary for cell movement, RhoA expression increases cellular adhesion which is expected as described in previous studies done by our lab, the surprising results was when RhoC depletion led to the decrease of adhesion, further more both proteins presence decreases cellular invasion. Our results also showed that invadopodia formation is RhoA, Rock and StarD13 independent, while on the other hand it shows that is RhoC. Fret Results suggests that RhoA is localized at the cell edges, where the activation of RhoA is increased depending on the grade of brain tumor as the grades increases the activation is noticed more.


