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The Role of Phosphoinositide Signaling in Breast Cancer Metastasis Suppressor 1-Mediated Metastasis Suppression of Human Breast Carcinoma Cells

Sitaram Harihar

Utah State University

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THE ROLE OF PHOSPHOINOSITIDE SIGNALING IN BREAST CANCER METASTASIS

SUPPRESSOR 1-MEDIATED METASTASIS SUPPRESSION OF HUMAN BREAST CARCINOMA CELLS

by

Sitaram Harihar

A dissertation submitted in partial fulfillment of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biology

Approved:

Daryll B. DeWald, Ph.D.                Jon Y. Takemoto, Ph.D.
Major Professor                Committee Member

Timothy A. Gilbertson, Ph.D.        Joan M. Hevel, Ph.D.
Committee Member                Committee Member

Glenn D. Prestwich, Ph.D.          Byron Burnham, Ed.D.
Committee Member                Dean of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2011
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ABSTRACT

The Role of Phosphoinositide Signaling in Breast Cancer Metastasis Suppressor 1-Mediated Metastasis Suppression of Human Breast Carcinoma Cells

by

Sitaram Harihar, Doctor of Philosophy

Utah State University, 2011

Major Professor: Dr. Daryll. B. DeWald
Department: Biology

Breast cancer is the most common non-skin cancer in women and the second most common cause of cancer-related death in U.S. women. Despite numerous advances in treatment strategies against breast cancer, the presence of undetected distant metastasis of the primary tumor remains the main cause of mortality. Current screening and detection methods such as mammograms are simply not sensitive enough to detect formation of metastasis. Further, currently available therapies against metastatic breast cancer do not provide a complete cure for the disease. Thus, understanding the biology and molecular factors involved in cancer metastasis will help aid in preventing the onset of metastasis and discovering an effective treatment for this deadly disease. My research focused on understanding the mechanism of action of one such factor, breast cancer metastasis suppressor 1 (BRMS1), a suppressor gene found deleted in late stage breast cancers. The goal of my dissertation was to investigate the
role of membrane signaling lipids phosphoinositides, specifically phosphatidylinositol(4,5)bisphosphate (PI(4,5)P₂) in BRMS1-mediated metastasis suppression in MDA-MB-435 and MDA-MB-231 human breast carcinoma cells. My studies revealed BRMS1 selectively reduced receptor tyrosine kinases (RTK) and G-protein coupled receptors (GPCR) expression and downstream signaling in human breast carcinoma cells. My observations are critical as many of these receptors are upregulated in metastatic breast cancer and PI(4,5)P₂ is a critical constituent for mediating their downstream signaling events. Further, using immunoblotting studies, I uncovered a possible compensatory mechanism in tumor cells to overcome downregulation of PI(4,5)P₂ by BRMS1 and maintain its downstream signaling. When studied for BRMS1 regulation of enzymes involved in PI(4,5)P₂ synthesis, I showed BRMS1 completely inhibits phosphatidylinositol 4-phosphate 5-kinase β (PIP5Kβ) expression. Using overexpression studies, I showed PIP5Kβ to be the major contributor to the cellular PI(4,5)P₂ pool required for agonist-induced intracellular calcium rise. Taken together, my dissertation research has identified some critical breast cancer markers and revealed signaling pathways altered by BRMS1 in human breast carcinoma cells that can be studied as potential therapeutic targets against breast cancer metastasis.
ACKNOWLEDGMENTS

First and foremost, I would like to thank my major advisor, Dr. Daryll DeWald, whose guidance and support have helped me shape my dissertation ideas, and grow as a scientist and more importantly as a person. He has been a mentor for me in many ways and has taught me a lot more than he realizes, for which I will be deeply indebted.

I would like to thank Dr. Jon Takemoto for letting me use his laboratory facilities and for his support and advice throughout my program here. He has been as much a mentor to me as he is to his students. I would like to thank Dr. Tim Gilbertson for letting me use his laboratory facilities, and for his support and advice throughout my Ph.D. program. I would like to thank Dr. Joanie Hevel and Dr. Glenn Prestwich for their support, advice, and constant encouragement throughout my Ph.D. program. I am especially grateful to Dr. Prestwich for taking time to come to Logan whenever requested.

Special thanks to Dr. Dane Hansen for teaching and helping me with RT-PCR and other valuable discussions we had. I am deeply indebted to Dr. Mie Jung Park for helping me get started with the lentiviral work; without her help this would not have been possible. I would like to thank Joseph Shope for helping me with the confocal microscopy. I would also thank our collaborator, Dr. Danny Welch, and his laboratory for providing me with the cells and other reagents.

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Finally, I would like to thank my parents and sister. They never understood what I was doing all this long time here, but in their world I am one of the best things to have happened. They have nurtured my dreams and ambitions and taught me to strive for the best. To them I dedicate this dissertation.

Sitaram Harihar
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LIST OF ABBREVIATIONS

ARF     ADP ribosylation factor
BCA     Bicinchoninic acid
BRMS1   Breast cancer metastasis suppressor 1
CaCl₂   Calcium chloride
cDNA    Complementary deoxyribo nucleic acid
ChIP    Chromatin immunoprecipitation
CK2     Caesin kinase 2
CMT     Charcot marie tooth
Cx      Connexin
DAG kinase Diacylglycerol kinase
DAG     Diacylglycerol
DMSO    Dimethyl sulfoxide
DNA     Deoxyribo nucleic acid
dNTP    Deoxynucleotide Triphosphate
ECL     Enhanced chemiluminescence
EDTA    Ethylenediaminetetraacetic acid
EGF     Epidermal growth factor
EGFP    Enhanced green fluorescent protein
EGFR    Epidermal growth factor receptor
ELISA   Enzyme-linked immune sorbent assay
EMT     Epithelial to mesenchymal transition
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<td>ENTH</td>
<td>Epsin N-terminal homology domain</td>
</tr>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>EST</td>
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<td>FYVE</td>
<td>Fab 1p, Yotb, Vac1p and EEA1</td>
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<tr>
<td>GAP</td>
<td>GTPase activating proteins</td>
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<tr>
<td>GJIC</td>
<td>Gap junctional intercellular communication</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter type 4</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GRP</td>
<td>Gastrin releasing peptide receptor</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3β</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>IP</td>
<td>Inositol phosphate</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol(1,4,5)trisphosphate</td>
</tr>
<tr>
<td>IP₃R</td>
<td>Inositol (1,4,5)trisphosphate receptor</td>
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<td>IP₆</td>
<td>Inositol hexaphosphate</td>
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<td>IκB</td>
<td>Inhibitor kappa B</td>
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<td>KAI1</td>
<td>Kangai 1</td>
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<tr>
<td>KCl</td>
<td>Potassium chloride</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
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<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption/ionisation-time of flight</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MDM2</td>
<td>Murine double minute 2</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal to epithelial transition</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>MTM</td>
<td>Myotubularin</td>
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<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<td>Myosin heavy chain 9</td>
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<td>Nuclear factor-kappa B</td>
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<td>Oculocerebrorenal syndrome of Lowe</td>
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<td>Osteopontin</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
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<td>PDK1</td>
<td>Phosphoinositide dependent kinase 1</td>
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<td>Plant homeo domain</td>
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<td>PI</td>
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<td>PI(3)P</td>
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PI(3,4)P₂  Phosphatidylinosito(3,4)bisphosphate
PI(3,4,5)P₃  Phosphatidylinosito(3,4,5)trisphosphate
PI(3,5)P₂  Phosphatidylinosito(3,5)bisphosphate
PI(4)P  Phosphatidylinosito(4)phosphate
PI(4,5)P₂  Phosphatidylinosito(4,5)bisphosphate
PI(5)P  Phosphatidylinosito(5)phosphate
PI3K  Phosphoinositide 3-kinase
PIP₄K  Phosphatidylinositol 5-phosphate 4-kinase
PIP₅K  Phosphatidylinositol 4-phosphate 5-kinase
PKA  Protein kinase A
PKB  Protein kinase B
PKC  Protein kinase C
PLC  Phospholipase C
PTEN  Phosphatase and tensin homolog
PVDF  Polyvinylidene fluoride
PX  Phox homology
RKIP  Raf kinase inhibitor protein
RNA  Ribonucleic acid
RNAi  RNA interference
RTK  Receptor tyrosine kinase
RT-PCR  Real time-polymerase chain reaction
SDS-PAGE  Sodium dodecyl sulfate polyacrylamide gel electrophoresis
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<td>SH2- containing inositol-5-phosphatases</td>
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<td>SIN3-HDAC</td>
<td>SWI-independent 3-histone deacetylase</td>
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<td>Star-PAP</td>
<td>Star-poly A polymerase</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
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<tr>
<td>XLMTM</td>
<td>X-linked myotubular myopathy</td>
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CHAPTER 1

INTRODUCTION

Rationale for the proposed research

Breast cancer is the most common non-skin cancer in women and the second most common cause of cancer-related death in U.S. women. Multiple factors ranging from family history, age, and obesity to environmental factors such as smoking, exposure to carcinogens have been shown to contribute to susceptibility to breast cancer [1,2]. The American Cancer Society estimates that more than 200,000 women will be diagnosed with breast cancer in 2010 and approximately 40,000 will die [3]. The primary cause of mortality is due to the presence of undetected distant metastasis of the primary tumor. Metastasis is the process where the cancer cells dissociate from the primary tumor and disseminate to secondary sites within the body. In cases where breast cancer remains localized, the five year survival rate is 98%; however, when a breast cancer has metastasized the five year survival rate drops to 26% [4]. Therefore understanding the biological biochemical and genetic mechanisms involved in the process of cancer metastasis holds the key towards finding a cure for cancer.

Metastatic cascade

The metastatic cascade involves multiple steps governed by several genes in tumor cells as well as cells in the surrounding microenvironment. The cascade begins with the formation of primary tumor which usually occurs due to a dominant gain of function of oncogenes or loss of function in tumor suppressor genes. This is followed by
angiogenesis or formation of new blood vessels which provide nutrition to the developing tumor and are also possible sites for entry of tumor cells into the circulatory system. The tumor cells then invade through the surrounding extracellular matrix components through the secretion of proteases such as matrix metalloproteinases [5]. The cells then intravasate into the circulatory system survive through the circulation in the blood vessels or lymphatic system, arrest in the secondary organs; extravasate, stay dormant or colonize by initiating growth within this secondary organ to form micrometastasis some of which may later develop into overt clinically detectable macrometastasis (Fig. 1.1) [6]. This entire process is highly inefficient, out of the $\sim 4 \times 10^6$ cells that enter the vascular compartment per gram of primary tumor per day $< 0.0001\%$ form metastasis [7,8]. This is because the inability of the cancer cell to complete any one of the above steps will render it non-metastatic. Thus identifying genes regulating the metastatic process could lead to the development of target specific drugs for therapeutic intervention against metastasis [9,10].

**Metastasis suppressor genes**

Distinct from the tumor suppressor genes, metastasis suppressor genes are an expanding group of family of genes that are capable of blocking the development of metastasis without affecting the primary tumor formation. The idea for the existence of metastasis suppressor genes has been supported by data from several laboratories where fusions of metastatic cancer cells with non-metastatic cells resulted in progeny that was non-metastatic. Later it was discovered that the metastatic ability of these fusion cells was restored through the loss of specific regions within chromosomes
implicating genes present in these regions as important for metastasis suppression. So far more than 25 of these candidate metastasis suppressor genes have been identified [11,12]. The mode of action of metastasis suppressor genes is dependent on their function as well as the type of cancer, with some genes suppressing metastasis of multiple types of cancer. These genes have been shown to inhibit steps in metastasis ranging from invasion to colonization at secondary sites with some genes inhibiting several steps in the process [13]. The proteins encoded by these genes have been shown to localize at the plasma membrane, cytoplasm, Golgi apparatus, cytoskeleton and nucleus and regulate signal transduction pathways, cytoskeletal reorganization, motility, invasion and transcription [14]. This complicated interplay of pathways affected by these genes has made it challenging in identifying their specific mechanisms of action.

In the mid 1980’s Nm23, the first metastasis suppressor gene in breast carcinoma cells was discovered and despite numerous studies to elucidate the mechanism of action, a biochemical mechanism has only recently been described for Nm23 [15,16]. Further studies have identified roles for metastatic suppressor genes Kangai 1 (KAI1), KISS1, Raf kinase inhibitor protein (RKIP) and Breast cancer metastasis suppressor 1 (BRMS1) in breast cancer metastasis [17-21]. These genes have been shown to act by reducing motility, invasion or colonization of tumor cells mainly by modulating signaling through the mitogen activated protein kinase (MAPK) or phosphoinositide 3-kinase (PI3K) pathways [22]. Further many of these genes have been shown to suppress metastasis in ovarian carcinomas and melanoma cell lines suggesting a much broader role in regulating metastasis [23,24]. My dissertation study focused on
understanding signaling events modulated by BRMS1 in human breast carcinoma cells. Towards that end, I discuss below some of our current understanding on role of BRMS1 as a metastasis suppressor.

**Breast cancer metastasis suppressor 1 (BRMS1)**

It was observed that loss of genetic material on chromosome 11q13-q14 frequently occurred in 40-65% of late-stage, metastatic breast carcinomas suggesting metastasis controlling genes are encoded there. This was confirmed when normal human chromosome 11 was introduced in MDA-MB-435 breast carcinoma cells, the resulting neo11/435 hybrids were suppressed for metastasis but were still tumorigenic [20,25]. Using differential display when the gene expression was compared between MDA-MB-435 and the neo11/435 cells BRMS1 was discovered [21]. The full length brms1 gene is coded by 738 nucleotides. BRMS1 protein contains 246 amino acids with a predicted molecular weight of 28.5 KD [26]. BRMS1 is widely expressed in normal human tissues and is present in multiple species [25]. BRMS1 orthologs are also highly conserved [27]. Conservation at the amino acid level is even higher, suggesting that it is biologically important.

BRMS1 is predominantly localized in the nucleus and has been shown to interact with SIN3-histone deacetylase (HDAC) complex [28-30]. The SIN3-HDAC complex deacetylates histones and condenses chromatin, thereby silencing transcription of several genes. This suggests a crucial role for BRMS1 in modulating cellular transcriptional machinery. The transcription factor nuclear factor-kappaB (NFkB) upregulates transcription of many proteins involved in invasion and metastasis [31].
BRMS1 decreases the nuclear translocation of NFκB by inhibiting the phosphorylation and degradation of the NFκB inhibitor termed inhibitor-kappaB (IκB) and thus reduces its activity resulting in suppression of metastasis [32]. BRMS1 has been found to restore the heterotypic gap junctional intercellular communication (GJIC) [33]. BRMS1 transfected MDA-MB-231 and 435 cells have upregulated connexin 43 (Cx43) and downregulated connexin 32 (Cx32) mRNA and protein expression [34,35]. In general, the connexins expression in BRMS1 transfectants has been restored to a pattern reminiscent of normal breast [36]. BRMS1 causes over 90% decrease in osteopontin (OPN) mRNA and protein expression, a protein critical in breast cancer progression and metastasis [37]. BRMS1 has been shown to upregulate the expression of microRNAs involved in metastasis suppression and downregulate metastasis promoting microRNAs (Fig 1.2) [38,39]. Previous studies from our laboratory showed that BRMS1 downregulates phosphoinositide signaling by specifically reducing the levels of phosphatidylinositol(4,5)bisphosphate (PI(4,5)P₂) [40]. Towards this end, my dissertation involved understanding signaling events modulated by BRMS1 downstream of PI(4,5)P₂ and identifying critical players regulating these actions.

**Phosphoinositides**

Phosphoinositides are lipid second messengers that are polyphosphorylated derivatives of phosphatidylinositol (PI), a phospholipid that resides almost entirely on the cytosolic surface of cell membranes. Differential phosphorylation at the hydroxyl groups 3, 4 and 5 of the inositol ring in PI generates seven distinct phosphoinositides contributing to their signaling diversity [41]. Phosphoinositides constitute about 10% of
the total phospholipids and thus are minor in composition but regulate major cellular processes like protein trafficking, cytoskeletal rearrangement, cell growth, proliferation and motility [42-46]. Aberrant phosphoinositide signaling has been implicated in numerous human diseases including cancer, neurological disorders, channelopathies and diabetes [47-49].

Hokin and Hokin first reported that phosphoinositides are metabolized in response to extracellular signals, laying the foundation for research in phosphoinositides as signaling molecules [50,51]. Later a critical pathway in phosphoinositide metabolism was discovered when studies demonstrated that agonist-stimulated hydrolysis of PI(4,5)P₂ by phospholipase C (PLC) generates two second messengers, inositol(1,4,5)trisphosphate (IP₃) and diacylglycerol (DAG), which transduce extracellular signals to elicit Ca²⁺ release and protein kinase C (PKC) activation [52,53]. Besides generating second messengers, phosphoinositides act as signaling molecules on their own and help define organelle identity and recruit protein regulators [54]. They fulfill their functional roles by associating with numerous coordinators, such as adaptors, protein kinases, and small guanosine triphosphatases (GTPases) that contain specialized phosphoinositide binding domains (e.g. PH, FYVE, PX, ENTH, FERM PHD, and Tubby domains) [55,56]. Each of the phosphoinositide has a distinct localization in the cell through which they carry out their specific function, although some overlap observed in their functions too. For example, PI(4,5)P₂ is especially enriched at the plasma membrane, while PI(3)P is found on early endosomes. PI(3,5)P₂ is found on late endocytic organelles, PI(4)P is predominantly found at the Trans Golgi network (TGN).
PI(3,4,5)P₃ is normally not seen in quiescent cells, but accumulates at the cell membrane on stimulation with extracellular agonists [42]. Nuclear phosphoinositide signaling has been an enigma to researchers for a long time; recent advances have helped understand the role of phosphoinositides in the nucleus [57,58]. There is a distinct phosphoinositide pool in the nucleus separate from the cytosolic pool and has been shown to be key to regulating cell cycle events, chromatin structure, DNA repair, RNA editing, transcription and mRNA metabolism. Also the presence of soluble inositol polyphosphates branching out from phosphoinositides regulates nuclear processes like mRNA splicing and nucleosome organization [59-64].

The distinct subcellular phosphoinositide pools are maintained by a combination of the enzymatic activities of phosphatidylinositol kinases and phosphatases. The promiscuous nature of these enzymes ensures the synthesis of multiple phosphoinositides. Moreover, within the same enzyme the presence of different isoforms allows regulated local production of phosphoinositides according to their cellular localization. The tight coupling of these enzymes ensures the production of appropriate levels of phosphoinositides. Because phosphoinositides have emerged as key regulators of cell function, interest in the regulation and cellular roles of the phosphoinositide-metabolizing enzymes has grown accordingly. In the following sections I will focus on the intracellular roles of two critical phosphoinositides PI(4,5)P₂ and PI(3,4,5)P₃, further the regulation of their metabolic enzymes will also be touched upon.
**PI(4,5)P₂ signaling**

PI(4,5)P₂ is the central component of the canonical phosphoinositide pathway and constitutes the highest percentage of the polyphosphorylated phosphoinositides along with PI(4)P. It is a signaling molecule on its own as well as a precursor for PI(3,4,5)P₃, IP₃ and DAG which further the signaling cascade (Fig. 1.3). The relative levels of PI(4,5)P₂ remain fairly constant, other than minor fluctuations during agonist stimulation [65]. This suggests that PI(4,5)P₂ signaling takes place due to its localized changes in the cell. PI(4,5)P₂ through its role in protein trafficking, cytoskeleton reorganization, focal adhesion assembly and disassembly, and as a second messenger regulates myriad of cellular processes like growth, division and proliferation. PI(4,5)P₂ is also involved in pre-mRNA splicing machinery and has been observed in nuclear speckles possibly modulating transcriptional processes [41,42,44-46,54].

PI(4,5)P₂ is synthesized by the action of two distinct but related enzymes of the phosphatidylinositol phosphate kinase (PIPK) family [66]. Phosphatidylinositol 4-phosphate 5-kinase (PIP5K) phosphorylates PI(4)P at the 5-position, whereas phosphatidylinositol 5-phosphate 4-kinase (PIP4K) phosphorylates PI(5)P at the 4-position of the inositol ring to form PI(4,5)P₂ [67]. Since the cellular levels of PI(4)P are more abundant than that of PI(5)P, it is thought that the major pathway for PI(4,5)P₂ synthesis is through PI(4)P. However it is possible that PIP4K may also make significant amounts of PI(4,5)P₂ at specific cellular locations. Apart from the lipid kinases the levels of PI(4,5)P₂ are also controlled by the action of the highly conserved phosphatases. Synaptojanin is a 5-phosphatase which hydrolyzes the 5-phosphate of PI(4,5)P₂, leading
to a decrease in PI(4,5)P₂ and theoretically in the production of PI(4)P. Extensive studies have revealed that synaptojanin resides in clathrin coats and plays a key role in clathrin-mediated endocytosis of synaptic vesicles on the plasma membrane [68].

The role of PI(4,5)P₂ in calcium signaling stems from the universal mechanism for calcium release from intracellular stores, termed the classical phosphoinositide cycle. Activation of receptor tyrosine kinases (RTK’s) (Fig. 1.4) or G-protein coupled receptors (GPCR) (Fig. 1.5) causes hydrolysis of PI(4,5)P₂ by PLC into IP₃ and DAG [69,70]. IP₃ binds to IP₃R in the endoplasmic reticulum (ER) causing opening of the ER Ca²⁺ channel, releasing Ca²⁺ from the ER to increase intracellular free Ca²⁺ levels in the cytosol. Ca²⁺ binds to PKC and initiates its translocation to the plasma membrane, where DAG binds to it and activates it. Ca²⁺ sensitive DAG kinase phosphorylates DAG to produce phosphatidic acid, which is a potent activator of PIP5K resulting in synthesis of PI(4,5)P₂ and the completion of the cycle. Increase in cytosolic free Ca²⁺ concentration is well-known to play critical roles in various physiological processes such as exocytosis, gene transcription and cell motility [69]. Further, many of the RTKs and GPCRs involved in calcium signaling are themselves receptors to potent agonists and play a critical role in controlling most fundamental cellular processes including cell growth, survival and proliferation. Deregulation on any of these controls leads to pathological conditions like cancer [71-75].

Since PIP5K are the enzymes involved in the major pathway for PI(4,5)P₂ synthesis, I will focus on the possible mechanisms that regulate their activity for the
localized synthesis of PI(4,5)P₂. In addition I will discuss the function of specific PIP5K isoforms, signaling pathways and their effectors.

**Insight into the PIP5K family of enzymes**

The PIP5K family of enzymes includes three isoforms, PIP5Kα, PIP5Kβ and PIP5Kγ. PIP5Kγ is further spliced into two main isoforms PIP5Kγ635, PIP5Kγ661 and the recently discovered third isoform PIP5Kγ688 [76-78]. All the isoforms have a conserved catalytic domain within which is the activation loop which dictates the kinase specificity and activity [76,77]. The PIP5K isoforms have distinct localizations in the cell dictated mainly by the cellular localization of their interacting proteins [66,79]. PIP5Kα is present in membrane ruffles and in discrete regions in the nucleus called nuclear speckles where it regulates the activity of Star-PAP a poly(A) polymerase that controls mRNA expression [64]. PIP5Kβ is observed mainly at the plasma membrane and is critical to receptor endocytosis and cell migration and PIP5Kγ has been shown to be present at the site of focal adhesions and adherens junctions and plays an important role in synaptic vesicle recycling [80-82]. Further all three PIP5K isoforms play significant role in actin remodeling events, crucial to many cellular functions such as protein trafficking, cell division and locomotion [83].

The PIP5K are regulated by the Rho family of G-proteins [84]. Rho, Rac and Arf stimulate PIP5K activity either directly or through activation of phospholipase D (PLD) which cleaves phosphatidylcholine to phosphatidic acid a potent activator of PIP5K [85]. These small G-proteins also influence the subcellular localization of PIP5K. Rho and Rac have been shown to target over expressed PIP5Kα to the plasma membrane [86]. PIP5K
activity can be directly regulated by phosphorylation of specific sites in the protein that allows binding partners to interact or dissociate with PIP5K [87]. PIP5Kβ is phosphorylated by PKA leading to a modest reduction in its activity, whereas lysophosphatidic acid (LPA) dephosphorylates PIP5Kβ leading to increase in its activity [88]. Phosphorylation of PIP5Kγ661 by Src directly increases its affinity for talin [89]. Considering the central position PI(4,5)P₂ occupies as a precursor of PI3K and PLC signaling whose downstream products activate tumor survival and anti-apoptotic pathways, PIP5K constitute interesting targets for regulating PI(4,5)P₂ levels. Development of pharmacological inhibitors against specific isoforms of PIP5K is underway, but the progress has been slow due to the high sequence and structure similarity of these isoforms. Understanding the mechanism for localized synthesis of PI(4,5)P₂ from PIP5K as well as how its regulation and interacting partners contribute to its function will aid in identifying more specific targets in clinical areas as a strategy for tumor therapy.

**Phosphoinositide 3-kinase signaling**

PI(3,4,5)P₃ is synthesized via the phosphorylation of PI(4,5)P₂ at the 3 position of the inositol ring by the action of class I phosphoinositide 3-kinase (PI3K). PI3K family is arguably the best studied of all phosphoinositide kinases [90]. In the past two decades, knowledge about PI3Ks has revolutionized our understanding of the signaling roles phosphoinositides play in normal physiology and disease pathogenesis and has far-reaching implications for disease treatment. PI3K signaling is modulated by two families of lipid phosphatases, PTEN (phosphatase and tensin homolog) and SHIP (SH2-
containing inositol-5-phosphatases). PTEN dephosphorylates PI(3,4,5)P₃ at position 3 of the inositol ring and thus directly reverses the actions of PI3K, leading to the downregulation of the PI3K signaling [91-93]. In contrast, the SHIP family proteins dephosphorylate PI(3,4,5)P₃ at position 5 of the inositol ring, generating PI(3,4)P₂ which is a second messenger by itself [94].

Class I PI3Ks are heterodimeric proteins composed of a catalytic subunit and regulatory subunit. Based on the different regulatory subunits, class I PI3Ks are further divided into two subclasses: class IA PI3K (α, β, δ) and class IB PI3Kγ. The catalytic subunits are p110α, p110β, p110δ and p110γ. Class IA PI3Ks use one of the five regulatory subunits, p85α, p55α, p50α, p85β, and p55γ which are collectively named p85. Any combination of p110 and p85 is possible and their interaction occurs by the binding of the adaptor binding domain of p110 to the inter-SH2 coiled coil region of p85 subunit [95]. The regulatory subunit of the class IB enzyme known as p101 shows no similarity to the class IA p85 subunits. The p110γ binding site has been mapped to the N-terminal region of p101. While p110α and p110β are ubiquitously expressed, p110δ and p110γ are found predominantly in leukocytes. In addition to their lipid kinase activities, all class I PI3Ks can also act as protein kinases. However, the physiological significance of their protein kinase activity is less well established, though autophosphorylation of PI3Ks like PIP5K has been suggested to regulate their activity [95].

Upon agonist activation, class I PI3K catalyze the formation of PI(3,4,5)P₃ which serves as the docking site for pleckstrin homology (PH) domain-containing proteins such as the AKT serine/threonine kinases (protein kinase B or PKB) and 3-phosphoinositide-
dependent protein kinase-1 (PDK1), leading to their recruitment to the membrane [90].

Once at the membrane, AKT is phosphorylated by PDK1 and becomes activated. AKT has multiple downstream targets including mTOR, tuberin, GSK3β, BAD, MDM2, caspase 9, and a subset of forkhead transcription factors [96]. The phosphorylation of these downstream molecules regulates a wide range of cellular processes such as cell proliferation, survival, and motility [97].

The critical role of the PI3K-AKT pathway in tumorigenesis has resulted in the generation of many pharmacological inhibitors against PI3K such as wortmannin and LY294002 [98]. However, they are non-specific inhibiting the activity of other enzymes such as PI4K [99]. Casein kinase 2 (CK2), a ubiquitous and constitutively active protein kinase involved in cell proliferation, was also shown to be inhibited by LY294002 at a concentration that was similar to that for PI3K [100]. Moreover even within the PI3K family they are broadly specific inhibiting the activity of all the isoforms. This assumes significance due the discovery of high frequency of p110α mutation in human cancers. Inhibition of p110α is regarded as a promising strategy for cancer treatment. Recently several small molecule inhibitors of PI3K have been reported that are isoform specific. SF1126 a small molecule inhibitor has entered phase I clinical trials and is showing to have anti-tumor activity [101]. Development of these isoform specific inhibitors will help increase the efficiency of the drug without the adverse side effects.

**Phosphoinositides and diseases**

Given that phosphoinositides regulate basic cellular processes it’s not surprising that alterations in phosphoinositide signaling is responsible for a number of human
diseases and developmental disorders. Mutations in genes encoding for proteins that regulate phosphoinositide metabolism and function are linked to many of these diseases. Moreover aberrant inositol phosphate signaling downstream of phosphoinositides contributes to a plethora of human diseases.

Lowe’s syndrome or oculocerebrorenal syndrome of Lowe (OCRL) is an X-linked disorder caused by mutations in ocr1 gene a PI(4,5)P_2 5-phosphatase, located in the trans Golgi network implying the role of PI(4,5)P_2 in this disease [102]. This disease is characterized by severe mental retardation, growth defects, renal Fanconi syndrome, and bilateral congenital cataracts ultimately causing blindness. Charcot-Marie-Tooth (CMT) and X-linked myotubular myopathy (XLMTM) are hereditary disorder associated with the myotubularin (MTM) family of phosphatases, caused, at least in part, by defects in phosphoinositide metabolism specifically in the PI(3)P and PI(3,5)P_2 pathway [103,104]. Type 2 diabetes and insulin resistance two important causes of obesity, involves PI3K and PI(3,4,5)P_3 by their regulation of the insulin signaling pathway [49,68]. Recent data have suggested that PI(5)P the newest phosphoinositide member may also play a role in insulin signaling by regulating GLUT4 translocation [105].

Aberrant PI(4,5)P_2 signaling also contributes to other human diseases such as cardiac hypertrophy. PI(4,5)P_2 also regulates the activity of many ion channels on the plasma membrane through either direct or indirect interaction [106]. Abnormal PI(4,5)P_2 signaling contributes to several channelopathies. Bacterial pathogens like salmonella and listeria have been shown to specifically manipulate phosphoinositide metabolism to gain entry into cells and enhance their virulence [49].
The numerous inositol phosphates (IPs) downstream of phosphoinositides have been shown to play crucial role in many human diseases especially due to their involvement in calcium metabolism. Although the cellular roles of these molecules are not fully understood, inositol phosphates have been shown to convey signals for a variety of hormones, growth factors, and neurotransmitters. The important IPs in terms of health and disease are IP₃ and IP₆. Perturbation of the IP₃/Ca²⁺ signaling pathway leads to neurological disorders, mainly bipolar affective disorder, Alzheimer’s disease and Parkinson’s disease. IP₆, also known as phytic acid, was found to inhibit neoplastic growth in many types of cancer by interfering with several signal transduction pathways and causes cell cycle arrest in these cancer cells [107-109].

**Phosphoinositides and cancer**

The transformation of a localized benign tumor to a malignant cancer occurs mainly due to the ability of tumor cells to undergo contact independent growth, acquire motility aiding in their passage across the basement membrane to form metastasis. Accomplishing these steps involves a combination of mutations in tumor cells as well as the ability of tumor cells to acquire signals from the surrounding stroma, such as signals from the endothelial cells helping in angiogenesis and immune cells helping in avoiding recognition and destruction of tumor cells. There is accumulating evidence that the important rate-limiting step of cancer metastasis is dictated by tumor cell response to exogenous signals, including growth and apoptosis signals from tissues at the secondary site [110]. Evidence suggests that several metastasis suppressor genes, including BRMS1, block metastasis by regulating growth at the secondary site [24].
Phosphoinositides (and downstream signaling molecules) have been implicated in several processes associated with cancer metastasis (e.g., adhesion, invasion, proliferation). Therefore, it is possible that phosphoinositide signaling is a critical regulator of the metastatic pathway. Signaling molecules downstream of phosphoinositides have precedent in controlling metastasis (e.g. AKT, PKC, Ca\(^{2+}\)) [97,111-117].

The most extensively studied phosphoinositide signaling pathway with respect to cancer is the PI3K/AKT pathway [118,119]. Aberrant PI3K signaling has been linked to various human cancers. Increased expression of the gene encoding p110\(\alpha\)-catalytic subunit of PI3K was found in ovarian, breast and colon cancers [120-123]. The discovery of PTEN as a tumor suppressor that is frequently mutated in late stage cancers confirmed that increased PI(3,4,5)P\(_3\) levels contributes to oncogenesis [124,125]. Further, somatic mutations in PTEN have been linked to three dominant inherited disorders: Cowden disease, Lhermitte-Duclos disease and Bannayan-Zonana syndrome [126]. Patients with these disorders are at an increased risk of developing cancers. Recent studies show mutations in SHIP1 cause acute myeloid leukemia [127,128]. Given the role of PI(4,5)P\(_2\) in regulating cellular events such as cytoskeletal organization and cell motility factors that contribute to tumor cell invasion and migration, it was not surprising when PIP5K and PI(4,5)P\(_2\) were recently implicated in breast cancer metastasis. Along with PIP5K isoforms, PI(4,5)P\(_2\) has been shown to play a role in the initiation and disassembly of focal adhesions in response to chemoattractants. Further their role in controlling adherens junction formation is critical in cancer as dissolution of
adherens junctions is a contributing event to dissociation of cells from primary tumor leading to metastasis [129].

Though, we have been able to gain a better understanding of phosphoinositide signaling in cancer, the intricate pathways and complex networks connecting these lipids to other cellular components needs to be investigated. Further due to the essential role of phosphoinositides in cell metabolism, therapeutic approaches targeting specific isoforms of enzymes involved in phosphoinositide signaling is required. This requires a greater understanding of their cellular roles. Recent development of isoform specific PI3K inhibitors is a positive step in that direction [130-132].

Dissertation outline

Our understanding on the role of phosphoinositides in cancer comes mainly from work done on the PI3K signaling pathway, whereas our knowledge on the other phosphoinositides is fairly limited. Towards this end, my dissertation research explores the role of phosphoinositides, specifically PI(4,5)P₂ signaling in BRMS1 mediated metastasis suppression in MDA-MB-435 and MDA-MB-231 human breast carcinoma cell lines. Both MDA-MB435 and MDA-MB-231 are highly metastatic and have been used as model cell lines for studying the biology of breast cancer [133]. Microarray expression studies showed MDA-MB-435 cells to cluster with many melanoma cell lines such as M14 leading to speculation that MDA-MB-435 cells may be melanoma rather than breast carcinoma in origin [134]. This created a controversy on the use of this cell line for breast cancer studies. Recent studies using karyotype analysis on MDA-MB-435 and M14 cells shows both cell lines to be identical but contrary to earlier observations
suggests that M14 cells may have been cross contaminated with MDA-MB-435 cells and therefore both the cells are breast carcinoma cells [135]. The credence for these observations comes from data showing female origin for both cell lines, whereas the original M14 cell line was isolated from a male patient [136]. Further MDA-MB-435 cells express milk proteins and have been shown to form primary tumors when injected into the mammary fat pad of mice, markers for breast origin of these cells [137,138]. MDA-MB-435 cells were also identified to be of the basal category of breast cancer cells many of which have genetic properties similar to melanocytic cells possibly explaining their proximity to many melanoma cell lines and wrongful categorization as a melanoma cell line [139].

Previous observations from our laboratory showed BRMS1 specifically reduces PI(4,5)P_2 levels in human breast carcinoma cells. Based on these results, in Chapter 2, I focused on understanding signaling events downstream of PI(4,5)P_2 modulated by BRMS1, specifically IP_3 mediated calcium signaling in response to RTK (EGF and PDGF) and GPCR (bombesin) agonists. Furthermore using immunoblotting, I studied how BRMS1 modulates expression of specific proteins involved in calcium signaling and PI(3,4,5)P_3 synthesis downstream of PI(4,5)P_2. Finally using pharmacological inhibitors I explored the role of PI3K and MAPK pathways in PDGF and bombesin mediated calcium signaling.

In Chapter 3, my research focused on understanding how BRMS1 regulates PI(4,5)P_2 synthesis. Using RT-PCR and immunoblotting I studied the expression of the different PIP5K isoforms in human breast carcinoma cells. Further using transient
overexpression studies and ELISA assay I studied the contribution of different PIP5K isoforms to restoring cellular PI(4,5)P2 levels downregulated by BRMS1 in MDA-MB-435 cells. Finally, using transient overexpression and ratiometric calcium imaging I studied the contribution of PIP5K isoforms to restoration of calcium signaling in response to PDGF and bombesin in BRMS1 expressing MDA-MB-435 cells. My data shows BRMS1 completely inhibits PIP5Kβ expression in MDA-MB-435 cells. Further I show PIP5Kβ isoform as the major contributor to cellular PI(4,5)P2 synthesis and calcium signaling in MDA-MB-435 cells.

Cytoskeletal organization is critical to accomplishing many of the steps in the metastatic cascade. In Chapter 4, I studied how BRMS1 modulates F-actin cytoskeletal organization in MDA-MB-435 and MDA-MB-231 cells. Further using in vitro wound healing and invasion assays I showed BRMS1 significantly reduces cell motility and invasion in MDA-MB-435 cells. Finally continuing from my observations in Chapter 3, in Chapter 4 I explored the role of PIP5Kβ in restoring some of the metastatic phenotypes suppressed by BRMS1 in MDA-MB-435 cells. After stably transforming 435/BRMS1 cells with a lentiviral vector for PIP5Kβ, I showed increasing PIP5Kβ and PI(4,5)P2 does not contribute alone to restoring cell migration and invasion suppressed by BRMS1 in MDA-MB-435 cells. A working model based on my observations of BRMS1 regulation of phosphoinositide signaling is shown in figure 1.6.

References


Figure 1.1: The metastatic cascade - The metastatic cascade comprises six distinct steps leading to the establishment of metastasis. Initially as the primary tumor grows it attracts angiogenic factors to promote vessel formation which furthers tumor cell growth and invasion into the circulatory system. Tumor cells then enter the blood vessel and migrate to a distant organ, where they extravasate. Cells then colonize and establish metastasis at the distant organ site where appropriate growth factors are provided. [140]
Figure 1.2: Schematic diagram showing cellular localization and known biochemical mechanisms for BRMS1 - BRMS1 has been found to localize mainly in the nucleus and interact with proteins involved in chromatin remodeling and transcriptional regulation such as SIN3-HDAC and SUDS3. BRMS1 decreases transcription factor NF-κB expression which in turn reduces expression of osteopontin (OPN) a protein implicated in breast cancer metastasis. BRMS1 also restores gap-junctional intercellular communication by regulating connexin 32 and 43 (Cx32 and Cx43) gap junctional proteins expression. BRMS1 also reduces phosphoinositide signaling by reducing PI(4,5)P₂ levels-a critical substrate in the PI3K-AKT cell survival pathway.
Figure 1.3: Model for the structures of phosphoinositides involved in the major pathway for PI(4,5)P$_2$ synthesis and signaling - Phosphatidylinositol (PI) is converted to phosphatidylinositol(4)phosphate (PI(4)P) by the action of phosphatidylinositol 4-kinase (PI4K). PI4P is phosphorylated at the 5-position of the inositol ring by phosphatidylinositol 4 phosphate 5-kinase (PIP5K) to phosphatidylinositol (4,5)bisphosphate (PI(4,5)P$_2$). On stimulation with extracellular signals (growth factors, small peptides), PI(4,5)P$_2$ forms the second messengers phosphatidylinositol(3,4,5)trisphosphate (PI(3,4,5)P$_3$) and inositol (1,4,5)trisphosphate (IP$_3$) by the action of phosphoinositide 3-kinase (PI3K) and phospholipase C (PLC) respectively.
Figure 1.4: Phosphoinositide signaling involved in calcium release by receptor tyrosine kinases (RTKs) - External stimulus such as a growth factor activates RTK, which recruits and activates PI3K and partially activates PLCγ1. PI3K then phosphorylates PI(4,5)P₂ to PI(3,4,5)P₃. PI(3,4,5)P₃ binds to PLCγ1 and increases its activity. Once activated PLCγ1 catalyzes hydrolysis of PI(4,5)P₂ into DAG and IP₃. IP₃ interacts with a calcium channel in the ER, releasing Ca²⁺ into the cytoplasm. The increase in Ca²⁺ activates PKC, which translocates to the membrane, associating with or binding to DAG.
Figure 1.5: Phosphoinositide signaling involved in calcium release after G-protein coupled receptor activation - External stimulus activates a G-protein-coupled receptor (GPCR). The G-protein activates PLCβ, which catalyzes hydrolysis of PI(4,5)P2 into DAG and IP3. The IP3 interacts with a calcium channel in the ER, releasing Ca^{2+} into the cytoplasm. The increase in Ca^{2+} levels activates PKC, which translocates to the membrane, anchoring to DAG.
Figure 1.6: Model for BRMS1 modulation of phosphoinositide signaling in MDA-MB-435 human breast carcinoma cells - Activation of EGFR or PDGFR (RTKs) or GRP (GPCR) leads to stimulation of specific isoforms of phospholipase C PLCγ1 and PLCβ1 respectively that catalyze hydrolysis of PI(4,5)P2 leading to the generation of two second messengers, DAG (not shown) and IP3. IP3 binds to IP3R on the endoplasmic reticulum and releases intracellular Ca2+. Further PI(4,5)P2 serves as a substrate for PI(3,4,5)P3 synthesis. BRMS1 reduced intracellular calcium rise by simultaneously reducing EGFR, GRP, PI(4,5)P2, PI(3,4,5)P3 and PLCβ1 ( ). BRMS1 also increased PLCγ1, IP3R and PI3K expression ( ) suggesting a compensatory mechanism in tumor cells to overcome the depletion of PI(4,5)P2 levels and maintain its signaling role. Further, BRMS1 inhibits cell survival pathways emanating from AKT due to reduction in PI(3,4,5)P3 production on growth factor stimulation.
CHAPTER 2

BRMS1 DIFFERENTIALLY MODULATES CALCIUM SIGNALING IN RESPONSE TO RTK AND GPCR AGONISTS IN HUMAN BREAST CARCINOMA CELLS

Abstract

Breast cancer metastasis suppressor 1 (BRMS1) suppresses metastasis by preventing colonization of tumor cells at secondary sites. We investigated signaling changes mediated by BRMS1 as a metastatic suppressor. Here we report that BRMS1 expression in metastatic human breast cancer cells leads to a selective regulation of receptor tyrosine kinases (EGFR and PDGFR) and G-protein coupled receptor (GRP) expression. Consistent with decreased PI(4,5)P2 levels observed, BRMS1 reduced intracellular calcium rise in these cells on treatment with RTK and GPCR agonists. In contrast, BRMS1 upregulated proteins involved in calcium mobilization and PI(3,4,5)P3 production suggesting a compensatory mechanism for reduction in PI(4,5)P2 signals. Addition of PI3K inhibitor LY294002 partially reduced calcium rise in MDA-MB-435 cells suggesting a role for PI(3,4,5)P3 in calcium rise in these cells. Thus, BRMS1 differentially attenuates cellular responses to mitogenic signals, not only dependent upon the specific signal received, but at varying steps within the same signaling cascade. Specific modulation of signaling responses received from the tissue microenvironment may ultimately dictate

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which environments are suitable for tumor cell growth and provide insights into the biology underlying metastasis.

**Introduction**

Breast cancer metastasis begins with dissemination of tumor cells from the breast, passage through the blood stream, colonization and proliferation at secondary sites such as the bone and lymph nodes [1]. For proliferation at secondary sites, tumor cells need to adapt themselves to the new microenvironment as this may not be similar to that available at the primary tumor site. This is probably why the propensity to metastasize at secondary sites varies depending on the type of cancer (e.g., breast cancer metastasizes mainly to lymph nodes and bones). This also implicates the final step in metastasis—survival and proliferation at secondary sites as a possible rate limiting step. The colonization step in the metastatic cascade has long been studied as a viable target for drug design to arrest cancer [2,3]. Metastasis suppressor genes commonly block this final step in the metastatic cascade.

Metastasis suppressor genes are a family of more than twenty proteins that, when re-expressed in metastatic cells, block metastasis without preventing tumor growth at orthotopic sites [4]. Their mechanisms of action have not been fully elucidated, but many appear to block growth at secondary sites by interfering with the tumor cells ability to respond to their microenvironment. Breast cancer metastasis suppressor 1 (BRMS1) - has been shown to suppresses metastasis in human breast carcinoma cells by preventing colonization at secondary sites [5-7]. This suggests that BRMS1 may selectively regulate particular environmental responses, such as responses
to exogenous signals at secondary sites. This can be due to alterations in the ability of cells to perceive these extracellular signals and through regulation of intracellular signaling intermediates.

Expression of BRMS1 has been shown to downregulate expression of many genes involved in metastasis such as osteopontin, alter NF-κB signaling and reduce phosphoinositide signaling, specifically the levels of PI(4,5)P$_2$ [8-10]. Apart from being a signaling molecule on its own, PI(4,5)P$_2$ acts as a substrate for the production of IP$_3$ and PI(3,4,5)P$_3$ [11]. IP$_3$ induced calcium rise plays an important role as an intracellular messenger downstream of stimulation with growth factors and other mitogens by binding to and regulating many proteins such as calmodulin dependent protein kinases family, protein kinase C family (PKC) and many transcription factors [12]. Cells tightly regulate their intracellular free calcium levels as deregulation of calcium contributes to diseases such as Alzheimer’s disease and cancer [13].

In the present study, we show that BRMS1 selectively regulates cell surface receptor expression in human breast carcinoma cells. Further, consistent with diminished PI(4,5)P$_2$ levels reduces cellular calcium signaling downstream of PI(4,5)P$_2$ in response to RTK and GPCR agonists. In addition, we show a possible compensatory mechanism in MDA-MB-435 cells to overcome the reduction in PI(4,5)P$_2$ levels by BRMS1, in order to maintain its signaling role.

Materials and methods

Cell lines and cell culture. MDA-MB-435 and 231 human breast carcinoma cells were transfected with a lentiviral vector construct expressing full length BRMS1 cDNA
under the control of a cytomegalovirus promoter [14]. Both the cell lines were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F-12) supplemented with 5% fetal bovine serum (HyClone, Logan, UT). Cells were cultured in 25 cm² Corning tissue culture dishes at 37 °C with 5% CO₂ in a humidified atmosphere. Cells were passaged at 80–90% confluency using 2 mM EDTA in Ca²⁺/Mg²⁺-free phosphate buffered saline (PBS, 0.01 M, and pH 7.4, Thermo Scientific, Logan, UT). Cell lines were confirmed to be free of mycoplasma contamination using PCR (TaKaRa, Madison, WI). No antibiotics or antimitotics were used during routine culture.

**Immunoblotting.** Cells were rinsed 2X with ice-cold PBS and lysed in a buffer containing 25 mM Tris-HCl (pH 7.4), 50 mM β-glycerol phosphate, 0.5 mM EDTA, 5% glycerol, 0.1% triton X-100, 1 mM sodium orthovanadate, 1 mM benzamidine, and protease inhibitor cocktail containing aprotinin, leupeptin, and phenylmethylsulfonyl fluoride (Roche, Indianapolis, IN). Protein concentration was determined using a BCA assay (Pierce, Rockford, IL). Protein was denatured with Laemmli’s buffer at 95°C for 5 minutes and lysate equal to 50 μg was loaded to each well. Proteins were separated using 10% SDS-PAGE gel electrophoresis and resolved proteins were transferred to PVDF membranes. Membranes were incubated in Tris-buffered saline containing 0.05% Tween-20 and 5% fat-free dry milk for 1 hour at room temperature. Membranes were incubated with primary antibodies [all of which were purchased from Cell Signaling, Danvers, MA, except GRP (Abcam, Cambridge, MA), IP₃R₃ (BD Biosciences, Bedford, MA), PLCβ1 (Santa Cruz Biotechnology, Santa Cruz, CA) and p85 (Millipore, Temecula,
CA]) overnight at 4°C and subsequently with HRP-conjugated secondary antibody at room temperature for 1 hour. Signals were visualized using ECL (Pierce, Rockford, IL) following manufacturer’s instructions.

**Ratiometric calcium Imaging.** Cells grown on coverslips were incubated in serum-free medium overnight prior to loading with 5 µM Fura-2 AM (Fura-2-acetoxyethyl ester; Molecular Probes, Eugene, OR) from a 5 mM DMSO stock solution for 1 hour in the dark. Cells were then rinsed and placed in medium to allow deesterification of the acetoxymethyl ester group from Fura-2. The coverslips were then mounted onto the chamber (RC-25F, Warner Instruments, Hamden, CT), placed on the stage of an inverted microscope (Nikon, Eclipse TS100, Japan) and perfused continuously with Tyrode’s a standard extracellular saline solution without CaCl₂ containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose, and 10 mM sodium pyruvate; pH 7.4. Cells were illuminated with a 100-watt xenon lamp and excitation wavelengths (340/380 nm) were delivered by a monochromator (Bentham FSM150, Intracellular Imaging Inc., Cincinnati, OH) at a rate of 20 ratios per minute. Fluorescence was measured by a CCD camera (pixelFly, Cooke, MI) coupled to a microscope and controlled by imaging software (Incyt Im2TM, Intracellular Imaging). The ratio of fluorescence (340 nm/380 nm) was directly converted to calcium concentrations using a standard curve generated for the imaging system using Fura-2 calcium imaging calibration kit (Molecular Probes, Eugene, OR).

Ratiometric data were initially collected to confirm a stable baseline. Then PDGF or bombesin (Sigma, St. Louis, MO) at desired final concentrations was applied
extracellularly with a bath perfusion system at a flow rate of 4 ml/minute permitting complete exchange of the extracellular solution in less than 20 seconds. For detection of cellular calcium mobilized on treatment with PLC, PI3K or MAPK inhibitors the cells were treated with U73122 (Sigma, St. Louis, MO) at 10 µM for 5 min, LY294002 (Sigma, St. Louis, MO) at 10 µM for 45 minutes and PD98059 (Sigma, St. Louis, MO) at 50 µM for 30 minutes, before stimulating the cells with PDGF or bombesin. The increase in cellular calcium concentration was analyzed by subtracting the rise in calcium concentration from the basal calcium concentration in each of the individual cells giving the resulting increase in cellular calcium levels.

For collection of ratiometric data for cells treated with EGF, the procedure was similar as stated above, except cells were illuminated with a Lambda DG-4 light, and images were collected with a Coolsnap HQ camera controlled by Metafluor imaging software (Molecular Devices, Sunnyvale, CA) and the excitation ratios recorded.

**PI(3,4,5)P3 mass assay.** PI(3,4,5)P3 levels were measured using the PI(3,4,5)P3 Mass Strip Kit (Echelon Biosciences Inc. Salt Lake City, UT) following manufacturer’s instructions. Briefly, after incubating MDA-MB-435 and 435/BRMS1 cells in serum free medium overnight the cells were stimulated with PDGF for 10 minutes, the medium was then aspirated and cellular material precipitated by the immediate addition of 0.5 ml of ice-cold 0.5 M TCA. After standing on ice for 5 minutes the cells were scraped off and the wells rinsed with additional TCA if required and the precipitate was pelleted. The pellet was then washed two times with 1 ml of 5% TCA and 1 mM EDTA. Neutral lipids were extracted from the pellet with 1 ml of methanol:chloroform 2:1 by vortexing three
to four times over a 10-minute period at room temperature. This extraction was repeated and the solvent supernatants were discarded. The acidic lipids were then extracted by addition of 750 μl chloroform:methanol:12 M HCl 40:80:1 to the pellet and vortexed over a 15 minute period at room temperature. A phase split was then carried out by the addition of 250 μl chloroform and 450 μl 0.1 M HCl followed by centrifugation to separate the organic and aqueous phases. The organic phase was collected into a clean tube and dried under nitrogen. The pellet containing the lipids was then resuspended by sonication in a water bath in 10 μl of chloroform:methanol:12 M HCl 40:80:1. The suspension was spotted on PI(3,4,5)P₃ strip at increments of 1 µl for a total of 5 µl. The strip was then probed using PI(3,4,5)P₃ detector for 1 hour at room temperature, followed by incubation with secondary and tertiary detector for 45 minutes each at room temperature. Signal was visualized using tetramethylbenzidine (TMB) developing solution. The amount of PI(3,4,5)P₃ present was estimated by plotting the pixel intensity signal vs pmol PI(3,4,5)P₃ on the standard curve.

**Statistical analysis.** The significant effects of all the treatments were determined by unpaired Student’s t-test (α=0.05) compared with their controls as described in the text. Data are presented as mean ± SEM, unless otherwise indicated.

**Results**

**BRMS1 decreases EGFR expression and downstream intracellular calcium mobilization in response to EGF.** Cells increase their intracellular calcium levels mainly via two mechanisms, one of which is through activation of cell surface receptors (RTK, GPCR) by stimulation from exogenous signals. PI(4,5)P₂ is critical intermediate of these
pathways. Among RTKs, EGFR signaling is mainly upregulated in breast cancers through activation of NF-κB activity [15], and BRMS1 has been shown to affect NF-κB activity and translocation [8]. To probe this link, we explored whether BRMS1 altered calcium signaling through EGFR. First, we measured EGFR expression by immunoblot in MDA-MB-435 and MDA-MB-231 parental and BRMS1-expressing cells. We found EGFR protein expression was ablated in 435/BRMS1 cells, while it was substantially reduced in 231/BRMS1 cells (Fig. 2.1a).

Previous work from our laboratory has shown BRMS1 expression leads to a selective downregulation of PI(4,5)P2 in MDA-MB-435 cells and a similar but not quite dramatic reduction in MDA-MB-231 cells [9]. Here we show that BRMS1 also reduces EGFR expression. To probe this further, we examined whether calcium signaling downstream of EGFR changed correspondingly. EGF treatment (50 ng/ml) induced intracellular calcium release in parental breast carcinoma cells (435-0.088 ± 0.012, 231-0.084 ± 0.03, Fig 2.1b) that was abrogated when BRMS1 was re-expressed (435/BRMS1-0.015 ± 0.003, p<0.01; 231/BRMS1- 0.014 ± 0.004, p=0.031, Fig. 2.1b). This suggests that attenuation of calcium signaling may be due to loss of EGFR expression, reduction in PI(4,5)P2 or a synergistic function of both.

**BRMS1 does not affect PDGFR expression but reduces downstream intracellular calcium mobilization in response to PDGF.** We examined the expression of another RTK- PDGFR, which has also been found to be upregulated in breast cancers [16] and check if BRMS1 downregulates its expression too. However, we found immunoblot studies revealed no changes in PDGFR protein expression in either 435 or
231 parental versus BRMS1 expressing cells (Fig. 2.2a). To explore if this findings are consistent with downstream calcium signaling, we examined calcium mobilization in response to PDGF (100 ng/ml) and we found PDGF-induced intracellular calcium release in parental breast carcinoma cells but calcium mobilization was abrogated in BRMS1 expressing MDA-MB-435 cells (435- 61.82 ± 10.8 nM, 435/BRMS1- 8.44 ± 3.1 nM, p = 0.01, Fig. 2.2b) and showed a partial reduction in MDA-MB-231 cells (231- 97.2 ± 25.3 nM, 231/BRMS1- 62.4 ± 1.3 nM, p = 0.3, Fig. 2.2b). This suggested a possibility that BRMS1 reduction in PI(4,5)P₂ levels is playing a prominent role in reduced calcium signaling in response to RTK agonists in these cells.

**BRMS1 differentially modulates GRP expression and downstream intracellular calcium mobilization in response to bombesin.** To examine the role of GPCR agonists in BRMS1-mediated calcium signaling, we studied the expression of gastrin releasing peptide receptor (GRP) in human breast carcinoma cells. GRP is the receptor for bombesin, one of the most potent agonists for IP₃ mediated calcium release in mammalian cells and has been correlated with tumor progression and metastasis [17,18]. We found BRMS1 significantly reduced GRP expression in MDA-MB-435 cells but no decrease was observed in MDA-MB-231 cells (Fig. 2.3a). Further, consistent with the changes in upstream GRP expression, BRMS1 abrogated calcium mobilization in MDA-MB-435 cells (435-202.2 ± 13.1 nM, 435/BRMS1-39.7 ± 5.1 nM, p < 0.01, Fig. 2.3b), while no decrease in intracellular calcium mobilization was observed in MDA-MB-231 cells following bombesin (50 nM) treatment (231-202.5 ± 7.7 nM, 231/BRMS1-192.4 ± 9.5, p = 0.4, Fig. 2.3b). The results from these studies suggest BRMS1 has evolved
different mechanisms to reduce intracellular calcium signaling in response to exogenous signals, underscoring the importance of cellular calcium in metastasis.

**BRMS1 expression alters cellular concentration of proteins that regulate PI(4,5)P₂ mediated calcium increase.** Stimulation of eukaryotic cells by exogenous signals, leads to hydrolysis of PI(4,5)P₂ to inositol (1,4,5) trisphosphate (IP₃) and diacylglycerol (DAG) by the action of phospholipase C (PLC). IP₃ binds to its receptor (IP₃R) on the endoplasmic reticulum (ER) causing the opening of its calcium channel leading to rise in intracellular calcium [19]. To further explore the events involved in BRMS1 reduction of calcium mobilization, we studied the expression of proteins involved in hydrolysis of PI(4,5)P₂ and subsequent calcium release. We found that BRMS1 upregulates PLCγ1 and two main isoforms of IP₃R- IP₃R1 and IP₃R3 [20], while it reduces PLCβ1 expression in MDA-MB-435 cells. In MDA-MB-231 cells, BRMS1 upregulates IP₃R1 expression while PLCγ1, PLCβ1 and IP₃R3 remain relatively unchanged (Fig. 2.4b).

PLCγ1 is involved in mediating signals downstream of RTK, while PLCβ1 is required for transmitting signals downstream of GPCR activation [13]. PLCγ1 has also been implicated in tumor progression and metastasis (Fig. 2.4a) [21]. Here when we studied the role of these enzymes and IP₃R in mediating calcium rise, we observed that though BRMS1 differentially regulated these proteins in MDA-MB-435 and 231 cells, there appeared to be a compensatory mechanism downstream of RTKs in MDA-MB-435 cells to overcome the reduction in PI(4,5)P₂ levels, as we observed an increase in both PLCγ1 and IP₃R in these cells. In contrast, in MDA-MB-231 cells, the expression of
proteins regulating PI(4,5)P₂ was relatively unchanged.

**BRMS1 reduced PI(3,4,5)P₃ production despite increasing PI3K expression in MDA-MB-435 cells.** PI3K and PTEN are two critical enzymes involved in the synthesis and breakdown of PI(3,4,5)P₃ [22]. PI3K synthesis PI(3,4,5)P₃ by using PI(4,5)P₂ as a substrate, while PTEN synthesis PI(4,5)P₂ by dephosphorylation of PI(3,4,5)P₃ (Fig. 2.5a). This implies by their activity these enzymes also contribute to regulation of PI(4,5)P₂ level. Using immunoblot, we checked for the expression of PI3K and PTEN in MDA-MB-435 and 231 cells. We found BRMS1 upregulates the expression of both p85-regulatory isoform and p110α-catalytic isoform of PI3K in MDA-MB-435 cells, in contrast p85 and p110α expression in MDA-MB-231 cells were relatively unchanged. PTEN expression remained relatively constant in both MDA-MB-435 and 231 cells (Fig. 2.5b). This again suggests a compensatory mechanism of BRMS1 in MDA-MB-435 cells to account for the decrease in PI(4,5)P₂ level.

To explore if this increased activity of PI3K contributes to PI(3,4,5)P₃ production in 435/BRMS1 cells despite reduced PI(4,5)P₂ levels, we stimulated MDA-MB-435 and 435/BRMS1 cells with 100 ng/ml PDGF, a known agonist for PI(3,4,5)P₃ production in mammalian cells. We found that despite higher PI3K expression, PI(3,4,5)P₃ levels were almost negligible in 435/BRMS1 cells (Fig. 2.5c). This suggests higher activity of PI3K is not sufficient to circumvent the lack of PI(4,5)P₂.

**Partial Involvement of PI3K pathway in PDGF induced calcium mobilization in MDA-MB-435 cells.** Alteration in PI3K and MAPK signaling pathways are integral to the development of many types of cancers. In order to understand the role of these two
pathways in BRMS1 mediated inhibition of calcium rise, we examined the effect of pharmacological inhibitors of PI3K and MAPK on calcium mobilization in response to PDGF in MDA-MB-435 cells. Activation of PLC in response to exogenous signals is one of the earlier events required for calcium mobilization. To confirm the involvement of PLC in cellular calcium rise, MDA-MB-435 cells were treated with U73122 a pharmacological inhibitor of PLC. Calcium mobilization in response to PDGF was ablated in the presence of U73122 (10.9 ± 1.5 nM p<0.01 Fig. 2.6b and 2.6e) in comparison to the control (83.43 ± 21 nM Fig. 2.6a and 2.6e).

When treated with LY294002, a pharmacological inhibitor of PI3K, there was a significant reduction in intracellular calcium rise in MDA-MB-435 cells (32.71 ± 7.52 nM p = 0.04 Fig. 2.6c and 2.6e), whereas intracellular calcium rise when treated with PD98059 a pharmacological inhibitor of MAPK was overall unaffected (53.98 ± 15.4 nM, p = 0.3 Fig. 2.6d and 2.6e). This suggests possible involvement of PI3K pathway in PDGF induced calcium mobilization in MDA-MB-435 cells.

To determine if inhibition of PI3K reduces intracellular calcium rise in response to bombesin, MDA-MB-435 cells were treated with LY294002 and observed for calcium mobilization when stimulated with bombesin. We found no significant reduction in response to bombesin when treated with PI3K inhibitor LY294002 (284.11 ± 12.5 nM p > 0.05 Fig. 2.7c and 2.7e) in comparison with control (319.68 ± 20.94 nM Fig. 2.7a and 2.7e). Further when probed to determine the involvement of MAPK pathway, we found intracellular calcium rise was unaffected when treated with MAPK inhibitor PD98059 in response to bombesin (248.86 ± 16.02 nM p > 0.05 Fig. 2.7d and 2.7e ). Treatment with
PLC inhibitor U73122 significantly reduced calcium rise in response to bombesin (77.26 ± 10.75 nM p < 0.01 Fig. 2.7b and 2.7e). This suggests PI3K signaling is not involved in bombesin and possibly GPCR induction of intracellular calcium rise in MDA-MB-435 cells.

**Discussion**

With improved imaging and diagnostic procedures, cancers are being diagnosed earlier and cure rates are improving. Yet, 25-30% of women with breast cancer develop metastasis months to years following primary tumor removal [23]. Tumor cells that had already disseminated had lain dormant during the interim, only to recommence growth. Therefore understanding signaling events regulating every step of the metastatic cascade is of utmost importance. In this context, metastasis suppressor genes play a crucial role as knowledge of their function will help get insight to the cellular events deregulated in metastasis. Keeping this in mind, the focus of the current study was to gain a greater understanding of the mechanism of BRMS1 action in human breast carcinoma cells. From previous observations it was known that BRMS1 inhibits metastasis by preventing colonization of tumor cells at ectopic sites and it is also known that BRMS1 significantly reduces PI(4,5)P2 levels in MDA-MB-435 cells [9,24,25]. Following up on these observations, we studied signaling changes downstream of PI(4,5)P2 with particular emphasis on intracellular calcium changes in these cells. Release of calcium from intracellular stores is important in modulation of the actin cytoskeleton for lamellipodial and filopodial formation during active processes, including motility and invasion and is also critical for cell survival at ectopic sites [26-28].
Although not an exhaustive panel, responses to two distinct growth factors – EGF and PDGF, both of which have been implicated in breast tumor and metastatic progression were analyzed [29,30]. Further, we analyzed responsiveness to bombesin, a potent GPCR agonist for release of calcium from intracellular stores. Specifically, BRMS1 signaling in MDA-MB-435 and 231 was modulated differently, depending on the stimulus. The muted calcium response to EGF in BRMS1-expressing cells can be attributed, in part, to reduced or complete loss of EGFR expression, while decreased calcium signaling downstream of PDGFR may be a consequence of reduced PI(4,5)P₂ levels. Whereas reduced calcium responses to bombesin in 435/BRMS1 cells may be attributed to a synergistic effect of reduced GRP expression, reduced PI(4,5)P₂ levels and loss of PLCβ1 expression. A possible compensatory mechanism was observed specifically in 435/BRMS1 cells to overcome reduction in PI(4,5)P₂ levels. Proteins involved in RTK induced calcium signaling downstream of PI(4,5)P₂ like PLCγ1 and IP₃R were upregulated in 435/BRMS1 cells. Expression of both the regulatory and catalytic subunits of PI3K was upregulated in 435/BRMS1 cells. None of these changes were able to restore intracellular calcium rise or PI(3,4,5)P₃ levels on par with that seen in the parental cells indicating a critical role for PI(4,5)P₂ in these processes.

In BRMS1-expressing cells, reduced calcium mobilization from RTK agonists could also be due to reduction in PI(3,4,5)P₃ production. The PI3K inhibitor LY294002 greatly reduced intracellular calcium rise in MDA-MB-435 cells in response to PDGF. For complete activation of PLCγ to hydrolyze PI(4,5)P₂ it needs to recruited to the cell membrane, this requires binding to both phosphorylated tyrosine residues on the RTK
through its SH2 domain and PI(3,4,5)P₃ through its PH domain [31]. Inhibiting PI3K would reduce PI(3,4,5)P₃ production resulting in an absence of binding sites for the PH domain of PLC-γ. This, in turn, would lead to incomplete activation of PLC-γ and a subsequent reduction in cytosolic calcium levels. This is also supported by previous findings showing delivery of PI(3,4,5)P₃ alone mobilizes calcium in mammalian cells [32]. However, recent findings showing non-specific action of LY294002, where it has been found to inhibit the activity of casein kinase 2 (CK2) which acts immediately upstream of PLCγ indicates the limitations of using chemical inhibitors in calcium analysis [33]. Future studies using RNAi directed against PI3K would help answer the role of PI3K and PI(3,4,5)P₃ in mobilizing cellular calcium.

Overall from our studies the picture emerging is BRMS1 regulation of signaling events in human carcinoma cells is complex with selective downregulation of receptors (EGFR and GRP in MDA-MB-435 cells) and signaling intermediates (PI(4,5)P₂ regulating enzymes and calcium signaling). Further we show there is a differential regulation by BRMS1 of signaling molecules between MDA-MB-435 and 231 cells. Since BRMS1 is mainly found in the nucleus [34] it is possible that there is variability in BRMS1 interaction with transcription factors in these two cell lines which is leading to differential changes in some signaling intermediates. Our knowledge of interacting partners to BRMS1 is limited [35], future studies aimed at identifying BRMS1 binding proteins will help us get a better understanding of the signaling events regulated by BRMS1.
References


Figure 2.1: BRMS1 expression decreases EGFR expression and downstream intracellular calcium mobilization in response to EGF - a. Receptor expression status for EGFR was examined by immunoblotting in MDA-MB-231, 231/BRMS1 and MDA-MB-435, 435/BRMS1 cells. β-Actin was used as the loading control. b. MDA-MB-231, 231/BRMS1 and MDA-MB-435, 435/BRMS1 cells were grown on coverslips and incubated with Fura-2/AM-containing medium. Cells were treated with 50 ng/ml EGF, (treatment time indicated by arrows) and an excitation ratio of 340/380 nm was obtained as described in materials and methods. * Statistically significant difference
Figure 2.2: BRMS1 expression does not significantly alter PDGFR expression but reduces downstream intracellular calcium mobilization in response to PDGF - a. Receptor expression status for PDGFR was examined by immunoblotting in MDA-MB-231, 231/BRMS1 and MDA-MB-435, 435/BRMS1 cells. β-Actin was used as the loading control. b. MDA-MB-231, 231/BRMS1 and MDA-MB-435, 435/BRMS1 cells were grown on coverslips and incubated with Fura-2/AM-containing medium. Cells were treated with 100 ng/ml PDGF (treatment time indicated by arrows), and an excitation ratio of 340/380 nm was obtained as described in materials and methods section. * Statistically significant difference
Figure 2.3: BRMS1 expression differentially modulates gastrin releasing peptide receptor (GRP) expression and downstream intracellular calcium mobilization in response to bombesin - a. Receptor expression status for GRP was examined by immunoblotting in MDA-MB-435, 435/BRMS1 and MDA-MB-231, 231/BRMS1 cells. β-Actin was used as the loading control. b. MDA-MB-435, 435/BRMS1 and MDA-MB-231, 231/BRMS1 cells were grown on coverslips and incubated with Fura-2/AM-containing medium. Cells were treated with 50 nM bombesin, (treatment time indicated by arrows) and an excitation ratio of 340/380 nm was obtained as described in materials and methods. * Statistically significant difference
Figure 2.4: BRMS1 expression alters cellular concentration of proteins that regulate PI(4,5)P₂ mediated calcium increase - a. Pathway for calcium release from intracellular stores b. Immunoblot analysis shows BRMS1 reduces PLCβ1 expression, but increases PLCγ₁, IP₃R1 and IP₃R3 expression in MDA-MB-435 cells, whereas in MDA-MB-231 cells, BRMS1 upregulates IP₃R1 while PLCγ₁, PLCβ1 and IP₃R3 expression remain largely unchanged.
Figure 2.5: BRMS1 expression decreases PI(3,4,5)P₃ concentrations despite increasing PI3K expression levels in MDA-MB-435 cells - a. Major pathway for PI(3,4,5)P₃ production in mammalian cells. b. Immunoblot analysis showed BRMS1 increases p85 and p110α expression in MDA-MB-435 cells, whereas their levels remain unchanged in MDA-MB-231 cells, while PTEN expression is relatively unchanged in both MDA-MB-435 and 231 cells. c. PI(3,4,5)P₃ production when measured using PI(3,4,5)P₃ strips showed approximately five fold reduction in 435/BRMS1 cells on treatment with 100 ng/ml PDGF. * Statistically significant difference
Figure 2.6: BRMS1 expression partially reduces calcium mobilization via alteration of PDGF-stimulated PI3K activity in MDA-MB-435 cells - PDGF BB elicited calcium mobilization in a. MDA-MB-435 control treated with b. U73122 and c. LY294002-pharmacological inhibitors of PLC and PI3K respectively, d. PD98059-a pharmacological MAPK inhibitor, e. Mean responses (intracellular rise in calcium concentration) + SEM on PDGF stimulation in control conditions, U73122, LY294002 and PD98059 treatments in MDA-MB-435 cells. Calcium mobilization was assayed as described in materials and methods. * Statistically significant difference
Figure 2.7: BRMS1 reduction of calcium mobilization by bombesin in MDA-MB-435 cells is independent of PI3K and MAPK pathway. Bombesin elicited calcium mobilization in a. MDA-MB-435 control treated with b. U73122 and c. LY294002—pharmacological inhibitors of PLC and PI3K respectively, d. PD98059—a pharmacological MAPK inhibitor, e. Mean responses (intracellular rise in calcium concentration) + SEM on bombesin stimulation in control conditions, U73122, LY294002 and PD98059 treatments in MDA-MB-435 cells. Calcium mobilization was assayed as described in materials and methods. * Statistically significant difference.
CHAPTER 3

PIP5Kβ IS THE MAJOR CONTRIBUTOR TO PI(4,5)P₂ SYNTHESIS AND PDGF-MEDIATED CALCIUM SIGNALING IN MDA-MB-435 HUMAN BREAST CARCINOMA CELLS.

Abstract

BRMS1 has been shown to selectively reduce PI(4,5)P₂ levels and subsequent calcium signaling in response to extracellular stimuli in human breast carcinoma cells. The predominant pathway for PI(4,5)P₂ synthesis is through the phosphorylation of PI(4)P at the 5-position of the inositol ring by phosphatidylinositol 4-phosphate 5-kinase (PIP5K). In this study, we show BRMS1 expression in MDA-MB-435 cells leads to a selective reduction of the PIP5K isoform-PIP5Kβ. On overexpression, PIP5Kβ contributed to a significant increase in cellular PI(4,5)P₂ levels in 435/BRMS1 cells. Further PIP5Kβ restored calcium signaling in response to PDGF stimulation in 435/BRMS1 cells. These results indicate calcium signaling in response to PDGF in MDA-MB-435 cells requires and possibly regulated by PI(4,5)P₂ produced primarily by PIP5Kβ.

Introduction

Breast cancer metastasis suppressor1 (BRMS1) belongs to the family of metastatic suppressor genes, many of whom suppress metastasis by inhibiting colonization of tumor cells at ectopic sites in the body [1]. BRMS1 is a nuclear protein and has been shown to associate with SIN3:histone deacetylase complexes which are...
involved in modulating chromatin structure and selective regulation of gene expression [2,3]. This suggests BRMS1 may be regulating the expression of metastasis-associated genes. Indeed, recent studies have shown BRMS1 regulates the expression of many metastasis promoting and suppressing micro RNAs (miRNAs) [4,5]. Further, BRMS1 selectively regulates expression of the EGFR, osteopontin, connexins, urokinase plasminogen activator and phosphoinositides specifically PI(4,5)P2 levels [6-10].

PI(4,5)P2 plays a critical role in regulating multiple cellular processes and is a central component of the classical phosphoinositide cycle [11]. Stimulation of PLC isoforms by G-protein coupled receptors (GPCR) and receptor tyrosine kinases (RTK), leads to hydrolysis of PI(4,5)P2. This is essential for Inositol (1,4,5) trisphosphate (IP3) mediated calcium rise and protein kinase C activation [12]. The critical role of calcium in mediating cellular metabolism and its association with many pathological conditions call for a greater understanding of this cellular pathway specifically the proteins regulating PI(4,5)P2 [13,14].

PI(4,5)P2 is predominantly localized at the plasma membrane, this makes it readily accessible to enzymes such as PLC and PI3K for the production of second messengers. In mammals the synthesis of PI(4,5)P2 from PI(4)P is accomplished by the action of the three distinct isoforms of phosphatidylinositol 4-phosphate 5-kinase (PIP5K)- α, β and γ, further the γ isoform is further alternatively spliced to PIP5Kγ635 and γ661 isoforms [15]. These isoforms selectively localize at different subcellular locations based on their interactions with specific proteins where they synthesize pools of PI(4,5)P2. PIP5Kα is mainly found in the nucleus, PIP5Kβ is seen at the plasma
membrane while PIP5Kγ is localized at focal adhesions and is the major isoform in the brain [16-19]. Despite our growing understanding of these kinases the relative roles of each isoform in generation of localized pools of PI(4,5)P₂ and subsequent calcium on cell stimulation is limited. This is further complicated by the promiscuous and overlapping functions of these kinases discovered through knock out studies [20-24].

In the present study, I found BRMS1 expression selectively reduced PIP5Kβ expression in MDA-MB-435 cells. Further overexpression studies showed PIP5Kβ to be the major contributor to PI(4,5)P₂ levels and PDGF-mediated calcium rise in MDA-MB-435 cells.

**Materials and methods**

**Cell lines and cell culture.** MDA-MB-435 and 231 human breast carcinoma cells were transfected with a lentiviral vector construct expressing full length BRMS1 cDNA under the control of a cytomegalovirus promoter [25]. Both the cell lines were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F-12) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT). Cells were cultured in 25 cm² corning tissue culture dishes at 37 °C with 5% CO₂ in a humidified atmosphere. Cells were passaged at 80–90% confluency using 2 mM EDTA in Ca²⁺/Mg²⁺-free phosphate buffered saline (PBS, 0.01 M, and pH 7.4, Thermo Scientific). Cell lines were confirmed to be free of mycoplasma contamination using PCR (TaKaRa, Japan). No antibiotics or antimycotics were used during routine culture.

**Transient transfection.** Transient transfection studies in 435/BRMS1 cells were performed using pEGFP-C1-PIP5Kα, PEGFP-C1-PIP5Kβ and pEGFP-C1-PIP5Kγ (Kindly
provided by Santos Manes, Centro Nacional de Biotecnología, Madrid, Spain). The transfections were performed using Fugene HD (Roche, Indianapolis, IN), as per the manufacturer's instructions. Briefly, 435/BRMS1 cells were plated on to 6-well tissue culture plates one day before the transfection, to achieve a confluence of 80–90%. The cells were transected using 2 μg of plasmid DNA/plate. The proteins and lipids were harvested after 24 hours for immunoblot and ELISA studies respectively. Localization of the three isoforms of PIP5K in 435/BRMS1 cells was detected using anti-GFP antibody (Cell Signaling, Danvers, MA).

**Taqman quantitative RT-PCR.** mRNA levels of the three isoforms of PIP5K-PIP5Kα, PIP5Kβ and PIP5Kγ including endogenous ribosomal S9, as an internal reference, were quantified using real-time PCR analysis (Taqman) on an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). Total cellular RNA was extracted using TRIzol, and ethanol-precipitated RNA was resuspended in 60 μl of diethyl pyrocarbonate-treated water. Amplification of specific PCR products was detected using fluorescent probes labeled with 6-carboxyfluorescein at the 5′ end. PCR was performed in a total reaction volume of 50 μl containing 4 mm MgCl₂, 200 μm each dNTP, 1.25 units of Taq polymerase, and 3 μl of total RNA. The relative mRNA expression of the target genes was quantified using the comparative cycle time method. Each sample was assayed in triplicate.

**Immunoblotting.** Cells were rinsed 2X with ice-cold PBS and lysed in a buffer containing 25 mM Tris-HCl (pH 7.4), 50 mM β-glycerol phosphate, 0.5 mM EDTA, 5% glycerol, 0.1% Triton X-100, 1 mM sodium orthovanadate, 1 mM benzamidine, and
protease inhibitor cocktail containing aprotinin, leupeptin, and phenylmethylsulfonyl fluoride (Roche, Indianapolis, IN). Protein concentration was determined using a BCA assay (Pierce, Rockford, IL). Protein was denatured with Laemmli’s buffer at 95°C for 5 minutes and lysate equal to 50 μg was loaded to each well. Proteins were separated using either 10% SDS-PAGE gels and resolved proteins were transferred to PVDF membranes. Membranes were incubated in Tris-buffered saline containing 0.05% Tween-20 and 5% fat-free dry milk for 1 hour at room temperature. Membranes were incubated with primary antibodies for PIP5Kα, PIP5Kβ (both from Santa Cruz Biotechnology, Santa Cruz, CA) PIP5Kγ (Cell Signaling, Danvers, MA), PIP5Kγ6 (gift from Dr. Pietro Di camilli, Yale University), GFP (Cell Signaling, Danvers, MA) and β-actin (Sigma, St. Louis, MO) overnight at 4°C and subsequently with HRP-conjugated secondary antibody at room temperature for 1 hour. Signals were visualized using ECL (Pierce, Rockford, IL) following manufacturer’s instructions.

**Immunolocalization.** Cellular localization of PIP5Kα was determined using immunocytochemistry. Cells plated on tissue culture treated cover slips (Fisher Scientific, Pittsburgh, PA) were washed with cold PBS, fixed with 4% para-formaldehyde (Electron microscopy sciences, Hatfield, PA) and permeabilized with 0.2% Triton X-100 in PBS. After blocking with 5% BSA in PBS, cells were incubated with either PIP5Kα (1:50 dilution) in 5% BSA solution overnight at 4°C. After washing thrice with PBS, Alexia Fluor 594-labeled anti-goat IgG (1:400 dilution, Molecular probes, Eugene, OR) was added and incubated at room temperature for 1 hour. After washing the cells thrice with PBS, the cover slips were mounted (Vector laboratories Inc, Burlingame, CA) and were observed
using a confocal microscope (model #MRC1024; Bio-Rad, Hercules, CA).

**PI(4,5)P$_2$ mass ELISA assay.** PI(4,5)P$_2$ levels were measured using the PI(4,5)P$_2$ mass ELISA kit (Echelon Biosciences Inc, Salt Lake City, UT) following manufacturer’s instructions. Briefly, media was aspirated from MDA-MB-435, 435/BRMS1 and 435/BRMS1 cells over expressing either PIP5K$\alpha$, PIP5K$\beta$ or PIP5K$\gamma$635, and cellular material precipitated by the immediate addition of 0.5 ml of ice-cold 0.5 M TCA. After incubation on ice for 5 minutes the cells were scraped off and the wells rinsed with additional TCA if required and the precipitate was pelleted. The pellet was then washed two times with 1 ml of 5% TCA 1 mM EDTA. Neutral lipids were extracted from the pellet with 1 ml of methanol:chloroform 2:1 by vortexing three to four times over a 10 minute period at room temperature. This extraction was repeated and the solvent supernatants were discarded. The acidic lipids were then extracted by addition of 750 μl chloroform:methanol:12 M HCl 40:80:1 to the pellet and vortexed over a 15 minute period at room temperature. A phase split was then carried out by the addition of 250 μl chloroform and 450 μl 0.1 M HCl followed by centrifugation to separate the organic and aqueous phases. The organic phase was collected into a clean tube and dried in under nitrogen. The pellet containing the lipids was then resuspended by sonication in a water bath in 200 μl of PBS. The PI(4,5)P$_2$ suspension was then incubated with a PI(4,5)P$_2$ detector protein, then added to the PI(4,5)P$_2$-coated plate for competitive binding. A peroxidase-linked secondary detection reagent and colorimetric substrate was used to detect PI(4,5)P$_2$ detector protein binding to the plate. The colorimetric signal observed was inversely proportional to the amount of PI(4,5)P$_2$ extracted from cells. The amount
of PI(4,5)P₂ present was estimated by recording the absorbance values for the extracted samples containing PI(4,5)P₂ on a standard curve.

**Ratiometric calcium Imaging.** MDA-MB-435, 435/BRMS1 and PIP5K expressing 435/BRMS1 cells grown on coverslips were incubated in serum-free media overnight prior to loading with 5 µM Fura-2 AM (Fura-2-acetoxymethyl ester; Molecular Probes, Eugene, OR) from a 5 mM DMSO stock solution for 1 hour in the dark. Cells were then rinsed and placed in medium to allow deesterification of acetoxymethyl ester group from Fura-2. The coverslips were then mounted onto the chamber (RC-25F, Warner Instruments, Hamden, CT), placed on an inverted microscope (Nikon, Eclipse TS100, Japan) and perfused continuously with Tyrode’s a standard extracellular saline solution without CaCl₂ containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose, and 10 mM sodium pyruvate; pH 7.4. Cells were illuminated with a 100-watt xenon lamp and excitation wavelengths (340/380 nm) were delivered by a monochromator (Bentham FSM150, Intracellular Imaging Inc., Cincinnati, OH) at a rate of 20 ratios per minute. Fluorescence was measured by a CCD camera (pixelFly, Cooke, MI) coupled to a microscope and controlled by imaging software (Incyt Im2TM, Intracellular Imaging). The ratio of fluorescence (340/380 nm) was directly converted to calcium concentrations using a standard curve generated for the imaging system using Fura-2 calcium imaging calibration kit (Molecular Probes, Eugene, OR).

Ratiometric data was initially collected to confirm a stable baseline. Then PDGF or bombesin (Sigma, St. Louis, MO) at desired final concentrations were applied extracellularly with a bath perfusion system at a flow rate of 4 ml per minute permitting
complete exchange of the extracellular solution in less than 20 seconds. For detection of cellular calcium mobilized by over expression of PIP5K isoforms, 435/BRMS1 cells expressing EGFP-PIP5Kα, EGFP-PIP5Kβ or EGFP-PIP5Kγ635 were selected for GFP expression, before stimulating the cells with PDGF or Bombesin. The increase in cellular calcium concentration was analyzed by subtracting the rise in calcium concentration from the basal calcium concentration in each of the individual cells giving the resulting increase in cellular calcium levels.

**Statistical analysis.** The significant effects of all the treatments were determined by unpaired Student’s t-test (α=0.05) compared with their controls as described in the text. Data are presented as mean + SEM, unless otherwise indicated.

**Results**

**BRMS1 differentially regulates PIP5K expression in MDA-MB-435 and MDA-MB-231 cells.** Expanding from my observations on the regulation of PI(4,5)P₂ levels by BRMS1 (Chapter 2), I examined the expression of PIP5K isoforms in human breast carcinoma cells. I began by measuring mRNA expression for all the three isoforms of PIP5K-PIP5Kα, PIP5Kβ and PIP5Kγ in MDA-MB-435 and 231 cells. I found BRMS1 drastically reduced PIP5Kβ mRNA in MDA-MB-435 cells by approximately 200-fold (p=0.0007) while no significant difference was observed in PIP5Kβ mRNA levels in MDA-MB-231 cells. PIP5Kα and PIP5Kγ mRNA expression was reduced by almost 2-fold in 435/BRMS1 cells, however the difference was not statistically significant (p>0.05). A similar trend was observed in MDA-MB-231 cells, BRMS1 reduced mRNA expression for PIP5Kγ by about 40%, but interestingly more than 2-fold increase in mRNA level for
PIP5Kα was observed in 231/BRMS1 cells (Fig. 3.1a). However, none of these alterations in mRNA levels induced by BRMS1 in MDA-MB-231 cells were found to be statistically significant (p>0.05).

When measured by immunoblot, the trend observed was similar; PIP5Kβ expression was undetectable in 435/BRMS1 cells, while no noticeable changes in PIP5Kβ was detected in 231/BRMS1 cells. Further, BRMS1 expression revealed no changes in PIP5Kα expression. However, when studied for PIP5Kγ635 expression, 435/BRMS1 showed a slower migrating diffuse band apart from the sharp band for PIP5Kγ635. The diffuse band has been shown to be hyperphosphorylated PIP5Kγ635, whose function is not clear [26] [23]. Further no significant differences were observed in PIP5Kγ661 isoform expression in either MDA-MB-435 or 231 cells (Fig. 3.1b).

**PIP5Kβ causes the major rise in PI(4,5)P₂ levels in 435/BRMS1 cells.** I next investigated the contribution of each of the PIP5K isoforms to PI(4,5)P₂ synthesis in 435/BRMS1 cells. To accomplish this, 435/BRMS1 cells were transiently transfected independently with plasmid vectors for PIP5Kα or PIP5Kβ or PIP5Kγ635. Protein expression was confirmed by immunoblotting using antibodies against the GFP tag (Fig. 3.2a). Cells were then stained with anti-GFP antibody to determine the percentage of cells expressing them and the locations of the proteins within the cells. PIP5K isoforms were effectively overexpressed after transient transfection (~50% efficiency). All three overexpressed isoforms were predominantly localized to the cell membrane (Fig. 3.2b).

After transfection, lipids were extracted and analyzed for PI(4,5)P₂ production by employing the PI(4,5)P₂ mass ELISA kit. The three isoforms of PIP5K increased PI(4,5)P₂
production to different extents. Overexpression of PIP5Kα, β and γ635 resulted in about a 76, 172 and 54% increase in PI(4,5)P2 levels respectively. However, only PIP5Kβ (1475.38 ± 248.97 pmol) caused a statistically significant increase in PI(4,5)P2 compared to 435/BRMS1 control cells (560.27 ± 32.88 pmol, p = 0.038, Fig. 3.2d Fig. 3.2d). This increase in PI(4,5)P2 by PIP5Kβ was about 59% to that observed in parental MDA-MB-435 cells (2603.8 ± 463.77 pmol, p = 0.068, Fig. 3.2c).

**PIP5Kα and PIP5Kβ restore PDGF mediated calcium signaling in 435/BRMS1 cells, but do not affect bombesin induced calcium responses.** PDGF binds to PDGFR, a receptor tyrosine kinase (RTK), activates PLCγ resulting in hydrolysis of PI(4,5)P2 which culminates in calcium release from intracellular stores in mammalian cells [27]. 435/BRMS1 cells show significantly reduced PI(4,5)P2 and intracellular calcium levels (11.46 ± 7.93 nM, p<0.01, Fig. 3.3b and 3.3f) when compared with parental MDA-MB-435 cells (92.51 ± 6.7 nM, Fig 3.3a and 3.3f). My previous results show overexpression of PIP5K isoforms increases cellular PI(4,5)P2 to different extents. I next investigated the contribution of the three PIP5K isoforms to increasing cellular calcium in 435/BRMS1 cells. Overexpression of PIP5Kα and PIP5Kβ restored cellular calcium to about 67 (62.13 ± 22.25 nM, p = 0.39, Fig. 3.3c and 3.3f) and 95% (87.80 ± 21.68 nM, p = 0.84, Fig. 3.3d and 3.3f) while PIP5Kγ635 caused a minimal increase in calcium of about 25% (23.2 ± 4.25 nM, p<0.01, Fig. 3.3e and 3.3f) to that observed in MDA-MB-435 cells.

PI(4,5)P2 signals are highly localized and generated in near proximity to its interacting proteins, for cells to mobilize calcium it requires the synthesis PI(4,5)P2 at the plasma membrane. Keeping this in mind, I explored the role of PIP5Kα
overexpression on calcium rise despite no significant reduction of its expression by BRMS1. I found PIP5Kα was mainly located in the nucleus close to the perinuclear space in both MDA-MB-435 and 435/BRMS1 cells, but when overexpressed its location changes dramatically to the plasma membrane (Fig. 3.3g). This may contribute to its role in calcium rise on PDGF stimulation in 435/BRMS1 cells.

435/BRMS1 cells (72.26 ± 7.78 nM, p<0.01, Fig. 3.4b and 3.4f) show reduced bombesin mediated calcium signaling compared to parental MDA-MB-435 cells (397.7 ± 7.74 nM, Fig. 3.4a and 3.4f). When further studied to understand the role PIP5K isoforms in calcium signaling on bombesin stimulation, I found overexpression of PIP5Kα (104.79 ± 15.51 nM, p<0.01, Fig. 3.4c and 3.4f) PIP5Kβ (89.69 ± 11.55 nM, p<0.01, Fig. 3.4d and 3.4f) or PIP5Ky635 (96.48 ± 16.8 nM, p<0.01, Fig. 3.4e and 3.4f) did not contribute to calcium rise 435/BRMS1 cells. This can be attributed to reduced GRP and PLCβ1 expression in 435/BRMS1 cells. This result also shows increased PLCγ1 expression cannot compensate the loss of PLCβ1 in 435/BRMS1 cells.

Discussion

The role played by the most abundant phosphorylated phosphoinositide-PI(4,5)P₂, in maintaining vital cellular activities such as cell growth, motility, protein trafficking and cell division is well known. Deregulation of PI(4,5)P₂ synthesis has been linked with numerous diseases such as cancer, cardiovascular disease, diabetes and many neurological disorders. Apart from being a signaling molecule on its own, PI(4,5)P₂ also serves as a substrate for second messengers such as calcium and PI(3,4,5)P₃ which are critical to many cellular functions and have been shown to be involved in tumor
progression [28,29]. These findings on PI(4,5)P₂ assume added significance, especially after our previous work where BRMS1 has been shown to significantly reduce PI(4,5)P₂ synthesis in human breast carcinoma cells.

Localized PI(4,5)P₂ synthesis is tightly controlled by the targeting and activation of the three isoforms of PIP5K- PIP5Kα, PIP5Kβ and PIP5Kγ to specific sites in the cell [30]. Overexpression and knock out studies have shown the contribution of PIP5K isoforms to cellular PI(4,5)P₂ varies depending on the type of tissue studied. PIP5Kα synthesis of PI(4,5)P₂ in the nucleus has been shown to regulate among many others the activity of a novel nuclear poly (A) polymerase termed Star-PAP possibly and regulates gene transcription [19,31]. PI(4,5)P₂ synthesized PIP5Kβ plays a major role in constitutive receptor-mediated endocytosis and is also critical for directional cell migration in neutrophils [17,32]. PI(4,5)P₂ generated by PIP5Kγ is critical for synaptic vesicle recycling and is required for formation of focal adhesions and trafficking of E-cadherin to the cell membrane [16,18,33]. Further, studies by Padron et al. and others show cells try to maintain an overall constant PI(4,5)P₂ level by compensating for the loss or constitutive activation of an isoform by altering the expression of the other isoforms [32,34].

I examined the expression of the three PIP5K isoforms in MDA-MB-435 and 231 cells, further I analyzed their contribution to restoring cellular PI(4,5)P₂ levels and subsequent agonist mediated calcium signaling downregulated by BRMS1. Specifically, BRMS1 expression resulted in complete loss of PIP5Kβ expression in MDA-MB-435 cells; while no significant changes were observed in PIP5Kα and γ expression neither at RT-
PCR nor immunoblot analysis.Interestingly, BRMS1 did not cause any noticeable reduction in the three isoforms of PIP5K in MDA-MB-231 cells.

Overexpression of the three PIP5K isoforms separately rescued cellular PI(4,5)P$_2$ levels to different extents in 435/BRMS1 cells. PIP5Kβ was the major contributor to PI(4,5)P$_2$ synthesis, though a similar trend was observed on PIP5Kα or PIP5Kγ635 overexpression, the increase in PI(4,5)P$_2$ levels were not as dramatic as seen for PIP5Kβ. Previous studies have shown all three isoforms of PIP5K play a role in calcium signaling depending on the type of stimulus applied [22,35,36]. When the role of the three PIP5K isoforms for restoring PDGF-mediated calcium signaling was explored, I found that both PIP5Kα and β caused a significant rise in intracellular calcium while no noticeable difference was observed on PIP5Kγ635 overexpression. Interestingly, overexpression of PIP5Kα dramatically altered its localization from the nucleus to the cell membrane in 435/BRMS1 cells. Previous studies have reported on the promiscuous nature of the PIP5K isoforms and the compensatory role played by each isoform in PI(4,5)P$_2$ synthesis. Alteration of cellular localization on overexpression of PIP5Kα suggests a possible compensatory mechanism by PIP5Kα to overcome or minimize the loss of PIP5Kβ in 435/BRMS1 cells. This is also consistent with my observation where PIP5Kβ was found to be localized at the cell membrane (data not shown).

Overall in this study, I show PIP5Kβ is the major contributor for restoring the PI(4,5)P$_2$ levels downregulated by BRMS1 in MDA-MB-435 cells. Further PIP5Kβ plays a critical role in rescuing calcium signaling in response to PDGF. My study also reveals differences but also some overlap in the function of PIP5K isoforms. Hence, although
each kinase has a special role in the control of specific PI(4,5)P₂ pools with important functional consequences on specific cellular functions, a significant level of mixing among these pools must also exist. Further, the differences in BRMS1 regulation of PIP5K isoforms in MDA-MB-435 and 231 cells though interesting underscores the need to decipher the exact mechanism through which BRMS1 downregulates PIP5Kβ expression and subsequent PI(4,5)P₂ level in MDA-MB-435 cells and if this reduction contributes to its metastasis suppression. To this end, in chapter 4 I present data showing the contribution of PIP5Kβ and PI(4,5)P₂ to partially restoring metastatic phenotypes by stably transforming 435/BRMS1 cells with PIP5Kβ lentiviral construct.

References


Figure 3.1: BRMS1 expression differentially regulates PIP5K expression in MDA-MB-435 and MDA-MB-231 cells. 

a. Quantitative RT-PCR analysis showing a significant reduction in mRNA expression of PIP5Kβ in MDA-MB-435/BRMS1 cells whereas no significant difference was observed in either PIP5Kα or PIP5Kγ (both PIP5Kγ635 and 661) mRNA levels. In MDA-MB-231 cells no significant difference in mRNA expression was observed in any of the PIP5K isoforms (error bars indicate S.E.M.).

b. Expression of three isoforms of PIP5K (including two splice variants of PIP5Kγ) in MDA-MB-435 and 231 cells as determined by immunoblotting described in materials and methods. * Statistically significant difference.
Figure 3.2: PIP5Kβ causes a significant increase in PI(4,5)P$_2$ levels in MDA-MB-435/BRMS1 cells - MDA-MB-435/BRMS1 cells were transfected separately with EGFP tagged PIP5Kα or β or γ635 isoform. Protein expression and localization was detected by a. immunoblot and b. immunofluorescence using anti-GFP antibody with arrows indicating the plasma membrane localization of the three PIP5K isoforms. Increase in PI(4,5)P$_2$ levels caused by each isoform was measured using a PI(4,5)P$_2$ ELISA kit. Averages of PI(4,5)P$_2$ levels in MDA-MB-435/BRMS1 cells expressing each of the three PIP5K isoforms are graphed with SE in comparison with c. MDA-MB-435 control cells and d. MDA-MB-435/BRMS1 control cells * Statistically significant difference
Figure 3.3: Overexpression of PIP5Kα or PIP5Kβ increase PDGF mediated calcium signaling in 435/BRMS1 cells - PDGF BB elicited calcium mobilization in a. MDA-MB-435 control b. MDA-MB-435/BRMS1 control c. 435/BRMS1-PIP5Kα d. 435/BRMS1-PIP5Kβ e. 435/BRMS1-PIP5Kγ635. Calcium mobilization was assayed as described in materials and methods f. Mean responses (change in intracellular Ca^{2+} concentration) + S.E.M. to PDGF in 435, 435/BRMS1 control cells and 435/BRMS1 cells expressing either α, β or γ635 isoforms of PIP5K. g. (Top) localization of native and overexpressed PIP5Kα detected using antibodies against PIP5Kα and GFP in 435 and 435/BRMS1 cells, (bottom) fluorescence intensity profiles showing extent of PIP5Kα localization as determined along the white line drawn along the breadth of a cell. * Statistically significant difference
Figure 3.4: Overexpression of PIP5K isoforms does not increase bombesin mediated calcium signaling in MDA-MB-435/BRMS1 cells - bombesin elicited calcium mobilization in a. 435 control b. 435/BRMS1 control c. 435/BRMS1-PIP5Kα d. 435/BRMS1-PIP5Kβ e. 435/BRMS1-PIP5Kγ635 Calcium mobilization was assayed as described in materials and methods f. Mean responses (change in intracellular Ca^{2+} concentration) + S.E.M. to bombesin in 435, 435/BRMS1 control cells and 435/BRMS1 cells expressing either α, β or γ635 isoforms of PIP5K. * Statistically significant difference
CHAPTER 4

OVEREXPRESSION OF PIP5Kβ DOES NOT CIRCUMVENT BRMS1-MEDIATED SUPPRESSION OF CELL MIGRATION AND INVASION IN MDA-MB-435 CELLS

Abstract

BRMS1 blocks metastasis by inhibiting colonization at secondary sites in the body. In the present study, we show BRMS1 also affects multiple steps preceding colonization in the metastatic cascade. BRMS1 inhibits cell migration and invasion through Matrigel in MDA-MB-435 human breast carcinoma cells and also dramatically alters their cytoskeletal architecture. Further, overexpression of PIP5Kβ restored PI(4,5)P₂ levels downregulated by BRMS1 in MDA-MB-435 cells. However, PIP5Kβ expression alone did not circumvent BRMS1 inhibition of cell migration and invasion in MDA-MB-435 cells.

Introduction

Breast cancer metastasis suppressor 1 (BRMS1) belongs to a class of genes termed metastasis suppressor genes [1]. Our understanding of the mechanism of action of BRMS1 is incomplete. BRMS1 has been shown to regulate transcriptional process through its association with histone deacetylases [2]. BRMS1 has also been shown to
downregulate EGFR signaling, restore gap-junctional intercellular communication, and reduce phosphoinositide signaling by specifically reducing PI(4,5)P_2 levels [3-6].

The membrane phosphoinositide PI(4,5)P_2 has been shown to regulate cell migration through its ability to bind to effector proteins involved in processes such as localized polymerization of actin and assembly of focal adhesions [7-9]. Due to the key role of cell migration in metastasis, regulation of PI(4,5)P_2 signaling is critical to the cell. PI(4,5)P_2 signals are highly localized and enzymes involved in its synthesis or degradation are tightly regulated. PI(4,5)P_2 is mainly synthesized by the enzymatic action of PIP5K on PI(4)P [10]. Apart from generation of PI(4,5)P_2 all three known isoforms of PIP5K-PIP5Kα, PIP5Kβ and PIP5Kγ have been linked with regulation of cytoskeletal organization and cell motility, but evidence for involvement in cancer metastasis is only available for PIP5Kγ [11-14].

Previous studies on BRMS1 demonstrate that it blocks metastasis by inhibiting colonization of tumor cells at ectopic sites [15]. In the present study we show BRMS1 also regulates migration and invasion steps in the metastatic cascade in MDA-MB-435 cells possibly by altering their cytoskeletal organization. Further, extending our observations from chapter 3 we show PIP5Kβ is the major contributor to cellular PI(4,5)P_2 pools in 435/BRMS1 cells but does not restore their migration or invasion profiles to that observed in metastatic MDA-MB-435 cells.

Materials and methods

Cell lines and cell culture. MDA-MB-435 and 231 human breast carcinoma cells were transfected with a lentiviral vector construct expressing full length BRMS1 cDNA
under the control of a cytomegalovirus promoter. Both the cell lines were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F-12) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT). Cells were cultured in 25 cm² corning tissue culture dishes at 37 °C with 5% CO₂ in a humidified atmosphere. Cells were passaged at 80–90% confluency using 2 mM EDTA in Ca²⁺/Mg²⁺-free phosphate buffered saline (PBS, 0.01 M, and pH 7.4, Thermo Scientific). Cell lines were confirmed to be free of mycoplasma contamination using PCR (TaKaRa, Japan). No antibiotics or antimycotics were used during routine culture.

**Immunofluorescent staining for F-actin localization.** To evaluate F-actin localization, MDA-MB-435 and 231 cells grown on coverslips for 24 hours were fixed using 4% para-formaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 20 minutes, and permeabilized using 0.1% Triton X-100 (Union Carbide Corporation, Texas City, TX) for 10 minutes. F-actin was stained using alexa fluor 488 Phalloidin (Invitrogen, Carlsbad, CA), nuclei were counter-stained using 4′,6-diamidino-2-phenylindole (Vector laboratories Inc, Burlingame, CA), and cells were viewed under an Olympus IX-70 inverted epifluorescence microscope. Representative images were obtained, combined, and processed using IPLab (v3.7) deconvolution software (Scanalytics, Rockville, MD).

**Wound healing assay.** MDA-MB-435 and 231 cells were plated in a 6-well tissue culture plate at 5X10⁴ cells/well and grown to 100% confluency. At 100% confluency, a linear wound was created by scraping across the center of the well using a 10-µl sterile pipette tip. Media were changed gently to remove any floating cells. Images of wound
cultures were collected at time intervals of 0 and 18 hours using an Olympus IX-70 inverted microscope (average doubling time is about 20 hours). The images were analyzed by measuring the mean distance from the wound edge of the cell sheet at the end of 18 hours to the original wound site in 5 independent fields per well. For experiment involving 435/BRMS1 cells transfected with lentiviral vector construct expressing EGFP-PIP5Kβ similar procedure of analysis was followed. Each test group was assayed in triplicate, and the results are expressed relative to MDA-MB-435 cell migration.

**In vitro invasion assay.** MDA-MB-435 and 231 cells were assessed for their invasive potential in vitro using the BioCoat Matrigel Invasion Chamber (BD Biosciences, Bedford, MA). The invasion chamber is a 24-well plate containing inserts of an upper Matrigel matrix layer and a lower polycarbonate membrane. The upper Matrigel layer acts as a reconstituted basement membrane *in vitro* and serves as a barrier that will allow invasive and non-invasive cells to be differentiated. The invasive cells are able to degrade the Matrigel layer and pass through the polycarbonate membrane, whereas passage of the non-invasive cells is blocked by the Matrigel layer. Control inserts containing only the polycarbonate membrane without the upper Matrigel layer were used as a control. Both control and Matrigel Inserts were rehydrated for 2 hours in DMEM/F-12 media and then placed on the wells containing DMEM/F-12 media with a chemoattractant (5% FBS). MDA-MB-435 and 231 cells were then seeded on the insert at a density 2.5 x 10^4 cells/insert. The invasion chamber was then incubated for 22 hours in a humidified tissue culture incubator at 37 °C. After incubation, the non-
invading cells were removed from the upper surface of the membrane by scrubbing using a cotton-tipped swab. The invaded cells on the lower membrane surface were fixed and stained using Diff-Quik kit (BD Biosciences, Bedford, MA). The stained cells were photographed and quantified using Olympus IX-70 inverted microscope. Data was expressed as the percent invasion through the matrigel matrix membrane relative to the migration through the control membrane. For experiments involving 435/BRMS1 cells transfected with lentiviral vector construct expressing EGFP-PIP5Kβ data was expressed as percent invasion through the Matrigel matrix and membrane relative to MDA-MB-435 cells. All experiments were assayed in triplicate.

**Generation of 435/BRMS1 cell line stably expressing EGFP-PIP5Kβ.** Full length human PIP5Kβ cDNA tagged with EGFP was cloned into a lentiviral construct under the control of a cytomegalovirus promoter. After screening for its presence the construct was purified using the SNAP column (Invitrogen, Carlsbad, CA). The construct was then transfected with the viral packaging mix to 293FT cells, viral stock was recovered and transfected into 435/BRMS1 cells to generate stably transformed cells expressing the lentiviral construct for EGFP-PIP5Kβ. The cells were then assayed for EGFP-PIP5Kβ expression by immunoblot and immunofluorescence using antibody directed against EGFP (cell signaling technologies, Danvers, MA).

**Radio labeling and lipid analysis.** MDA-MB-435, 435/BRMS1 and 435/BRMS1-PIP5Kβ cells were grown to 80% confluence in 60 cm² plates, washed with Ca²⁺/Mg²⁺-free PBS and labeled with myo-[2-³H]inositol (PerKin-Elmer, Waltham, MA) at a concentration of 20μCi/ml for 24 hours in inositol-free DMEM/F-12 1:1 (Hyclone, Logan,
UT) containing 5% fetal bovine serum. After 24 hours, the medium was replaced. Ice-cold trichloroacetic acid was added to the plates to a final concentration of 10% and incubated on ice for 1 hour before scraping and placing the liquid in 15 ml conical screw-cap centrifuge tubes. After centrifugation at 4000 rpm for 20 minutes, the cell pellet was resuspended in 5ml 5% trichloroacetate in a 1 mM EDTA solution before recentrifugation and lipid extraction.

Lipids were extracted from the cell pellet by resuspending cells in 0.75 ml chloroform/methanol/HCl (40:80:1 v/v/v) and vortexing vigorously. Then, 0.25 ml of chloroform and 0.45 ml of 0.1 M HCl were added to the cells and they were vortexed for 2 minutes, centrifuged, and the bottom, organic layer was transferred to another tube for continued processing. 50 μl of 1 M NH₄OH was added and the solutions in the tubes were dried.

The dried lipids were deacylated by resuspension in 0.5 ml of methylamine reagent (42.8% of 25% methylamine, 45.7% of methanol, 11.4% of n-butanol) followed by bath sonication and incubated at 53 °C for 50 minutes, and dried under reduced pressure. Deacylated lipids were suspended in 0.75 ml H₂O by sonication and extracted thrice with 0.5 ml n-butanol/petroleum ether/ethyl formate (20:4:1 v/v). The aqueous phase was dried under reduced pressure and suspended in 200 μl of H₂O. An aliquot (10 μl) of each sample was used to determine the radioactivity by liquid scintillation counting. For preparation of loading samples for HPLC, standardization was done using the ³H counts, which approximates phosphoinositide content.
Phosphoinositides were resolved with a mobile phase of ammonium phosphate (pH 3.8) using strong anion exchange Partisil 10 SAX (4.6 × 250 mm) columns (Whatman, Clifton, NJ). Anion-exchange columns were fitted with guard columns (SecurityGuard; Phenomenex, Torrance, CA) containing strong anion exchange inserts. The gradients for separation of glycerophosphoinositols (gPI): gPI(3)P, gPI(4)P, gPI(3,4)P₂, gPI(3,5)P₂, gPI(4,5)P₂, and gPI(3,4,5)P₃ were 5 ml of 10 mM, 60 ml of a linear gradient, 10 mM to 0.8 M, 2 ml of a linear gradient, 0.8 to 1 mM, 3 ml of 1 mM, respectively. Fractions (0.2 ml) were collected every 20 seconds, mixed with 2 ml of water-miscible scintillation cocktail, and counted in a liquid scintillation counter.

**Statistical analysis.** The significant effects of all the treatments were determined by unpaired Student’s t-test (α=0.05) compared with their controls as described in the text. Data are presented as mean ± SEM, unless otherwise indicated.

**Results**

**BRMS1 specifically alters cytoskeletal architecture in MDA-MB-435 cells**

**without affecting MDA-MB-231 cells.** For a cancer cell to successfully metastasize it must complete all the steps starting with detachment from the neighboring cells and extracellular matrix components at the primary tumor site followed by invasion through the basement membrane to successful colonization at the secondary site. While all steps are essential for the establishment of metastasis, the ability of the tumor cells to easily dissociate from the primary tumor and survive in an anchorage-independent manner is a critical step [16]. Metastatic cells undergo numerous biochemical and morphological changes in accomplishing this process; cytoskeletal reorganization is one
of them. Metastatic cells reorganize their F-actin filaments to minimize adhesion to substratum leading to easier dissociation from the substratum. To determine if BRMS1 alters cytoskeletal properties, MDA-MB-435 and 231 parental and BRMS1 expressing cells were stained with alexa fluor phalloidin for determining F-actin localization. It was observed that BRMS1 significantly alters F-actin organization in MDA-MB-435 cells. Fig. 4.1a shows the difference in F-actin organization in MDA-MB-435 and 435/BRMS1 at a multiple cell and single cell level. BRMS1 expressing cells had a markedly different shape and actin organization than the parental MDA-MB-435 cells. They were surprisingly more mesenchymal in nature and appeared elongated with long thick actin fibers that were aligned longitudinally along the entire length of the cell providing stronger adhesion to the substratum. MDA-MB-435 cells in contrast had an irregular shape resembling an epithelial morphology with short actin filaments that were mainly localized at the plasma membrane, suggesting easier dissociation from the substratum. They also showed the presence of antennae like actin protrusions called filopodia on the cell membrane, which are characteristics of highly motile cells. MDA-MB-435 cells also uniquely showed specks of actin surrounding the nucleus whose function is not known, these specks were completely absent in BRMS1 cells. While BRMS1 showed significant alteration in cytoskeletal organization in MDA-MB-435 cells, no prominent differences were observed in MDA-MB-231 cell line. Fig. 4.1b shows MDA-MB-231 and 231/BRMS1 at a multiple cell and single cell level. Both the parental and BRMS1 expressing cells were irregular in shape with short actin filaments that were mainly localized at the plasma membrane. These results suggest BRMS1 expression may be differentially
regulated in MDA-MB-435 and 231 cells and at the same time supports the concept that cytoskeletal organization determines the shape of the cell [17].

**BRMS1 reduces directional migration in MDA-MB-435 cells, but does not affect MDA-MB-231 cells.** To determine if the cytoskeletal changes induced by BRMS1 in MDA-MB-435 affects cell motility, both MDA-MB-435 and 231 parental and BRMS1 expressing cells were analyzed for their ability to migrate and heal the wound created at the center of a well in a 6-well plate. Images collected at 0 and 18 hours after wounding were analyzed for the distance migrated by the parental and BRMS1 expressing cells. The data collected was plotted as percentage of distance migrated by BRMS1 expressing cells relative to the parental cells. It was observed BRMS1 significantly reduced motility in MDA-MB-435 cells without affecting migration in MDA-MB-231 cells. Representative images collected at 0 and 18 hours in MDA-MB-435 and 435/BRMS1 cells shows that after 18 hours of incubation, the wound created by scraping with a pipette tip was almost completely healed in the MDA-MB-435 cells, whereas the migration activity for the 435/BRMS1 cells was considerably slower (Fig. 4.3a). When compared with the parental cells the migration was almost 50% less in 435/BRMS1 cells. In contrast, as observed with cytoskeletal organization BRMS1 expression did not significantly affect migration in MDA-MB-231 cells (Fig. 4.2b)

**BRMS1 reduces invasive properties of MDA-MB-435 cells, but does not affect MDA-MB-231 cells.** One of the critical components in metastasis is the ability of the tumor cells to invade through the extracellular matrix and enter the blood circulation to be disseminated to ectopic sites in the body. In order to verify if BRMS1 inhibits
invasiveness in breast carcinoma cells, MDA-MB-435 and MDA-MB-231 parental and
BRMS1 expressing cells were analyzed for their ability to invade through the a Matrigel
coated basement membrane like matrix. BRMS1 significantly blocks invasion in MDA-
MB-435 cells (Fig. 4.3a) without affecting invasion in MDA-MB-231 cells (Fig. 4.3b). The
number of cells that invaded through the Matrigel inserts relative to the control inserts
was employed to calculate the percent invasion for each cell line. Percent invasion was
determined to be 45.54% for 435 and 17.14% for 435/BRMS1 whereas percent invasion
was 37.54% in 231 and 34.6% in 231/BRMS1 cells. Interestingly, 435 and 435/BRMS1
cells revealed a marked difference in cellular characteristics as they invaded through the
membrane. The 435 cells were uniformly distributed both on the Matrigel and control
inserts. However, the 435/BRMS1 cells were primarily found at the periphery whereas
231 and 231/BRMS1 cells were uniformly distributed both on the Matrigel and control
inserts.

**Restoration of PIP5Kβ expression does not increase directional migration or
invasion in 435/BRMS1 cells.** Considering the central role played by PI(4,5)P₂ in
regulating cell migration—a critical step in cancer metastasis we investigated if reduction
in levels of PI(4,5)P₂ by BRMS1 in MDA-MB-435 cells is responsible for reduced
migration and invasion in these cells. Lentiviral vector construct for EGFP-PIP5Kβ was
designed (Figure 4.4a (i)) and expressed in 435/BRMS1 cells. Stable expression was
confirmed by immunoblot. Cells were stained with antibody against EGFP to determine
the location of the protein inside the cells. EGFP-PIP5Kβ was located primarily in the
plasma membrane (Fig. 4.4a (ii)). This stable cell line was designated as 435/BRMS1-EGFP-PIP5Kβ.

MDA-MB-435, 435/BRMS1 and 435/BRMS1-EGFP-PIP5Kβ cells were labeled with myo-[2-3H]inositol, and lipids were extracted and analyzed by anion exchange HPLC. Glycerophosphoinositols from MDA-MB-435 cells were detected in decreasing order of abundance: gPI(4)P > gPI(4,5)P2 > gPI(3)P. gPI(5)P, gPI(3,4)P2, gPI(3,5)P2 and gPI(3,4,5)P3 were not detected (Fig. 4.4b; Table 1). Unlike previous observations [4], the difference in phosphoinositide levels between MDA-MB-435 and 435/BRMS1 cells were not as drastic with 435/BRMS1 showing 60-65% of gPI(3)P, 80-90% of gPI(4)P and 60-70% of gPI(4,5)P2 compared to the parental cells. This may be due to the stable transformation of MDA-MB-435 cells with BRMS1 in this study compared to the previous study where BRMS1 was overexpressed using the pcDNA vector system. Expression of PIP5Kβ in 435/BRMS1 cells increased the levels of gPI(4,5)P2 to 110% of that of the parental cells. Interestingly PIP5Kβ expression also increased levels of gPI(4)P to around 140% of that seen in the parental 435 cells whereas not much difference was seen in the levels of gPI(3)P (data not shown). It is possible 435/BRMS1 cells are responding to increased PIP5Kβ expression by increasing the levels of its substrate PI(4)P.

We performed wound healing and invasion assays to determine if increased PIP5Kβ and PI(4,5)P2 levels restore the migration and invasion properties of 435/BRMS1 cells. Overexpression of PIP5Kβ did not significantly increase either motility or invasive properties of 435/BRMS1 cells (Fig 4.5a and 4.5b). This result suggests that though
PIP5Kβ restores PI(4,5)P₂ levels to that seen in parental 435 cells it may not be playing a role in restoring metastatic properties of these cells on its own.

**Discussion**

The mechanism of action of BRMS1 has not yet been completely deciphered; the available data so far indicates a complex role for BRMS1 in metastasis suppression regulating cellular events at transcriptional and physiological levels [18]. In Previous studies using *in vitro* assays assessing individual steps in the metastatic cascade show that BRMS1 functions are downstream of local invasion, where it inhibits colonization of tumor cells at secondary sites [15]. In this study using *in vitro* migration and invasion assays we show that BRMS1 significantly also reduces cell migration and invasion in MDA-MB-435 cells. Further we observed BRMS1 dramatically alters the cytoskeleton and inhibits the ability of the MDA-MB-435 cells to produce and activate matrix metalloproteinase-2 and -9 as detected by zymography [Baker SM et al.-unpublished observations]. However, we did not observe similar pattern of inhibition in migration and invasion or changes in cytoskeletal organization in MDA-MB-231 cells although BRMS1 inhibited colonization at secondary sites in both the cell lines. This suggests a differential action of BRMS1 in the steps preceding colonization in the metastatic cascade.

Release of calcium from intracellular stores is important in modulation of the actin cytoskeleton for lamellipodial and filopodial formation during active processes, including motility and invasion [19,20]. BRMS1 reduction of PI(4,5)P₂, a substrate for IP₃ mediated calcium rise may play a critical role in inhibiting the formation of lamellipodial
and filopodial extensions for cell migration in MDA-MB-435 cells [8]. Examining the expression levels of enzymes involved in the synthesis and breakdown of PI(4,5)P₂ (Chapter 2 and 3) showed a dramatic reduction in PIP5Kβ expression in 435/BRMS1 cells. Further restoration of PIP5Kβ expression in 435/BRMS1 cells increased PI(4,5)P₂ levels to 110% of that seen in MDA-MB-435 cells showing PIP5Kβ as the major PI(4,5)P₂ generating enzyme, interestingly there was also a dramatic increase in PI(4)P levels to 140% of that seen in MDA-MB-435 cells. Enzymes belonging to the PIP5K family have been shown to be promiscuous in their activity, but so far no study has reported PIP5K enzymes phosphorylating PI to PI(4)P. This suggests the increase in PI(4)P may be due to inherent increase in the substrate levels to cope with the increased enzyme activity. When probed if restoration of PI(4,5)P₂ levels increased migration and invasion levels we found increase in PI(4,5)P₂ levels alone does not contribute to restoring cell migration and invasion in 435/BRMS1 cells.

Metastasis is a complex cascade of cellular events carried out through the interplay of numerous genes. Here, in this study we have shown the role of BRMS1 as a metastasis suppressor extends to multiple steps in the metastatic cascade in MDA-MB-435 cells blocking cell migration and invasion apart from colonization. Further increasing PI(4,5)P₂ levels alone is not sufficient to circumvent the inhibition of BRMS1 on the migration and invasion properties of MDA-MB-435 cells. PI(4,5)P₂ signals are known to be highly localized and generated in close proximity to their binding proteins, to accomplish this all the three isoforms of PIP5K are localized at specific locations in the cell [12]. Therefore it is possible the metastatic phenotype in MDA-MB-435 cells is a
concerted action of all the three isoforms. Further the role of PI(3,4,5)P₃, a product of PI(4,5)P₂, cannot be discounted in development of cancer metastasis.

References


Figure 4.1: BRMS1 specifically alters cytoskeletal architecture in MDA-MB-435 cells without significantly affecting MDA-MB-231 cells - Cells were grown on coverslips for 24 hours; actin cytoskeleton was visualized by phalliodin staining. Multiple and single cell images were obtained for both a. MDA-MB-435 and b. MDA-MB-231 cells. Arrows indicate areas of formation of filopodia.
Figure 4.2: BRMS1 reduces motility in MDA-MB-435 cells but shows no significant affect in MDA-MB-231 cells - cell motility as measured by the ability of human breast carcinoma cells to migrate into a wound created on a cell monolayer after 18 hours. Representative photographs of wound healing assay and an analysis of comparative motility from the wound healing assay depicted as percent migration of the wild type for a. MDA-MB-435, and b. MDA-MB-231 cells (error bars indicate S.E.M., n=3 experiments). * Statistically significant difference.
Figure 4.3: BRMS1 reduces the invasive potential of human MDA-MB-435 cells but does not significantly affect the invasion potential of MDA-MB-231 cells - Representative photographs and quantification of chemoinvasion assay of a. MDA-MB-435 and b. MDA-MB-231 cells on matrigel inserts compared with migration on control inserts (error bars indicate S.E.M., n=3 experiments). * Statistically significant difference.
Figure 4.4: Stable PIP5Kβ expression in MDA-MB-435/BRMS1 increases both PI(4)P and PI(4,5)P₂ levels - a. i) Map of the lentiviral vector containing the construct for human EGFP-PIP5Kβ ii) lentiviral construct generated for human EGFP-PIP5Kβ was tested for expression with immunoblot and immunofluorescence using antibody directed against EGFP and b. 435, 435/BRMS1 and 435/BRMS1-EGFP-PIP5Kβ cells were radiolabeled to steady state with [³H]myo-inositol. Radiolabeled phospholipids were extracted, deacylated, and glycerophosphoinositols separated by HPLC. Representative plots(s) for the separation of specific glycerophosphoinositols is shown for 435 (black line), 435/BRMS1 (grey line) and 435/BRMS1-EGFP-PIP5Kβ (dashed line).
Figure 4.5: Restoration of PIP5Kβ alone does not significantly increase either cell proliferation or invasion in MDA-MB-435/BRMS1 cells - Motility as measured by the ability of human breast carcinoma cells to migrate into a wound created on a cell monolayer after 18 hours. Representative photographs and analysis of a. wound healing assay and b. invasion assay for 435, 435/BRMS1 and 435/BRMS1-PIP5Kβ cells (comparative motility and invasion are depicted as percent proliferation or invasion relative to the wild type, error bars indicate S.E.M., n=3 experiments). * Statistically significant difference.
Table 4.1: Stable expression of PIP5Kβ in MDA-MB-435/BRMS1 increases both PI(4)P and PI(4,5)P₂ levels - Total glycerophosphoinositol cpm was calculated by adding cumulative cpm found in each glycerophosphoinositol peak. The percentage of each glycerophosphoinositol was determined by dividing total glycerophosphoinositol cpm into cpm for individual glycerophosphoinositols.

<table>
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<th>Cell Line</th>
<th>gPI</th>
<th>gPI(4)P</th>
<th>gPI(4,5)P₂</th>
<th>gPI(4,5)P₂/gPI(4)P</th>
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<tr>
<td>MDA-MB-435</td>
<td>98.39</td>
<td>1.35</td>
<td>0.16</td>
<td>0.12</td>
</tr>
<tr>
<td>435/BRMS1</td>
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<td>1.11</td>
<td>0.11</td>
<td>0.10</td>
</tr>
<tr>
<td>435/BRMS1-PIP5Kβ</td>
<td>97.70</td>
<td>1.98</td>
<td>0.23</td>
<td>0.12</td>
</tr>
</tbody>
</table>
CHAPTER 5
SUMMARY AND FUTURE DIRECTIONS

The main aim of my dissertation study was to understand the role of phosphoinositides, in particular PI(4,5)P₂ signaling in BRMS1-mediated metastasis suppression in MDA-MB-435 and MDA-MB-231 human breast carcinoma cells. The premise behind this was preliminary data from our laboratory showed BRMS1 expression significantly reduced PI(4,5)P₂ levels in MDA-MB-435 cells, while a similar trend was observed in MDA-MB-231 cells, the reduction wasn’t as dramatic [1].

Extending these observations, in my dissertation I explored if this reduction in PI(4,5)P₂ affects its downstream signaling events, specifically I studied extracellular agonist stimulated IP₃-mediated calcium signaling and PI3K-mediated production of PI(3,4,5)P₃ (Chapter 2) in human breast carcinoma cells. Further, using quantitative RT-PCR and immunoblot, I studied how BRMS1 modulates the expression of proteins involved in PI(4,5)P₂ signaling (Chapters 2 and 3). Finally, using ratiometric calcium imaging and in vitro metastasis assays I addressed the question is PI(4,5)P₂ a specific downstream mediator of BRMS1 mediated metastasis suppression or the alteration in PI(4,5)P₂ levels merely correlative? (Chapters 3 and 4)

Toward this end, much of my research was carried out on the MDA-MB-435 cell line, the reason being the severity of alteration in PI(4,5)P₂ levels by BRMS1 and an expectation to see a more prominent effect of BRMS1 in these cells compared to MDA-MB-231 cells. However, due to the controversy on the origin of MDA-MB-435 cells (argument that it may be a melanoma rather than breast carcinoma cell line [2]), I
carried out a significant number of additional experiments on MDA-MB-231 cells, a bona
difie human breast carcinoma cell line as a test of my hypotheses [3-5]. Indeed, as
expected cellular changes induced by BRMS1 were much more pronounced in MDA-MB-
435 cells than in MDA-MB-231 cells. I show in my work and as I discuss further in this
chapter, that some of these changes observed are as a direct result of the reduced
Pi(4,5)P₂ synthesis by BRMS1.

**BRMS1 expression modulates critical players involved in Pi(4,5)P₂ signaling**

Both EGFR and PDGFR expression is highly upregulated in stage IV breast
cancers, which is the final stage in breast cancer progression where tumor cells have
metastasized [6,7]. Modulation in intracellular calcium levels is also critical to tumor
progression and metastasis. Using immunoblot and ratiometric calcium imaging, I
showed BRMS1 completely inhibits calcium signaling through EGFR and PDGFR in MDA-
MB-435 cells. The effect on EGFR signaling was compounded by the complete loss of
EGFR on BRMS1 expression. BRMS1 synergistic reduction in Pi(4,5)P₂ and receptor
levels (EGFR) may contribute significantly to metastasis suppression. Further support for
this hypothesis comes from my studies on bombesin, a potent agonist for IP₃-induced
calcium rise and a ligand for GRP a GPCR upregulated in many types of cancers [8].
BRMS1 inhibited bombesin induced calcium rise by simultaneously reducing GRP, PLCβ1
and Pi(4,5)P₂ levels in MDA-MB-435 cells. Further my studies show evidence for a
possible compensatory mechanism in MDA-MB-435 cells to overcome the depletion in
Pi(4,5)P₂ levels by BRMS1 and in order to maintain Pi(4,5)P₂ signaling. This is observed by
increased expression of PLCγ1, IP3R and PI3K, proteins involved in signaling downstream of PI(4,5)P2. This is plausible, as calcium is a critical regulator of many cellular events and cells modulate their intracellular calcium levels to mediate variety of signals.

MAPK and PI3K-AKT pathways are downstream mediators of receptor activation signals in mammalian cells and are frequently deregulated in many types of cancers [9]. Using pharmacological inhibitors to block the activity of PI3K and MAPK in MDA-MB-435 cells, I showed a critical role for PI3K signaling in RTK mediated intracellular calcium rise, while no significant role for the MAPK pathway was observed. Consistent with my observations, work by our colleagues revealed inhibition of AKT activity by BRMS1 following RTK stimulation in MDA-MB-435 cells, while MAPK signaling was only marginally reduced [10]. These results not only demonstrate differential signaling patterns, but also suggest that inhibition of the PI3K-AKT pathway might be relatively more relevant in BRMS1 metastasis suppression. For successful establishment of metastasis, the disseminated tumor cells must colonize at secondary sites requiring the cells to survive and respond to critical signals at these sites. Taken together, data from chapter 2 shows BRMS1 reduces the ability of tumor cells to respond to these signals providing evidence of BRMS1 mediated metastasis suppression at ectopic sites.

The role of phosphatidylinositol 4-phosphate 5-kinases

Phosphatidylinositol 4-phosphate 5-kinases (PIP5K) on their own and their product PI(4,5)P2 have been shown to contribute to numerous cellular processes many of which are deregulated in cancer metastasis [11,12]. Indeed, the recent identification of their role in breast cancer metastasis confirms them as potential targets in metastasis...
suppression. After studying the regulation of protein levels downstream of PI(4,5)P₂, my focus shifted to the role of PIP5K in BRMS1 metastasis suppression. In chapter 3 using quantitative RT-PCR and immunoblot studies I showed BRMS1 completely inhibits PIP5Kβ expression in MDA-MB-435 cells whereas PIP5Kα and γ isoforms expression remained largely unchanged. Further using ELISA, I showed PIP5Kβ was the major contributor to the PI(4,5)P₂ synthesis.

Previous studies have shown overexpression of either PIP5Kα, β or γ splice variants increased intracellular calcium levels based on the cell type, mode of activation (RTK or GPCR) and cellular localization of these isoforms [13-15]. My data shows PIP5Kβ is the major contributor to the PI(4,5)P₂ pool required for intracellular calcium rise in MDA-MB-435 cells. Interestingly, overexpression of PIP5Kα also contributed to calcium rise despite no significant reduction in its expression by BRMS1. This suggested a possible change in cellular localization. Indeed on overexpression, PIP5Kα localized to the cell membrane rather than the nucleus. Previous studies showed dynamic changes in PI(4,5)P₂ based on change in expression and localization of PIP5K isoforms, often a result of alterations in other isoforms. My studies show a differential localization of PIP5Kα, possibly to overcome the loss of PIP5Kβ by BRMS1 in MDA-MB-435 cells.

Do all metastatic cancer cells undergo epithelial to mesenchymal transition?

In Chapter 4, I showed BRMS1 dramatically alters cytoskeletal organization in MDA-MB-435 cells. BRMS1 expressing cells were more mesenchymal in appearance in comparison with the parental cells which were more epithelial. Further, using in vitro
assays I showed MDA-MB-435 cells were highly motile and invasive, while BRMS1 expression reduced both motility and invasive properties in these cells. These observations were interesting, considering the importance of epithelial to mesenchymal transition (EMT) in invasion and dissemination [16]. If EMT is critical to cell invasion the question arises why are the parental highly invasive MDA-MB-435 cells epithelial in appearance? And why are the non-invasive BRMS1 expressing cells mesenchymal?

Metastatic cells isolated from ectopic sites have been shown to have an epithelial appearance due to the reverse process of mesenchymal to epithelial transition (MET) shown to be critical to colonization of tumor cells at these sites [16]. Since MDA-MB-435 cells have been obtained from a metastatic tumor and appeared epithelial in origin, these cells may have undergone MET to establish themselves at secondary sites and BRMS1 expression prevents this transition from happening. The evidence for this hypothesis comes from studies showing BRMS1 mainly inhibits colonization of tumor cells at secondary sites in in vivo mouse models. The other viewpoint according to genetic background hypothesis is not all types of metastatic cancers undergo EMT or MET, a subset of primary tumor cells express a predictive-gene signature profile programmed to metastasize [17]. This suggests MDA-MB-435 cells originally were a subset of cells in the primary tumor inherently programmed to metastasize and BRMS1 expression inhibits metastasis by reducing their invasive and colonization properties by inducing among many others alterations in cytoskeletal organization.
Stably transforming 435/BRMS1 cells with PIP5Kβ did not restore motility and invasion profiles

Previous studies have shown PIP5Kβ plays a critical role in directional migration of neutrophils and also disassembles focal adhesions crucial for cell migration [18]. Expanding my observations in chapter 3 where I showed PIP5Kβ was a major contributor to PI(4,5)P₂ synthesis and intracellular calcium rise on stimulation with PDGF. I explored the role of PIP5Kβ in restoration of metastatic phenotypes using in vitro wound healing and invasion assays. Stably transforming 435/BRMS1 cells with lentiviral construct for PIP5Kβ showed no significant increase in cell motility or invasion.

Metastasis is a complex multistep cascade involving the interplay of numerous genes regulating every step in this cascade. In vitro metastasis assays that measure different steps in the metastatic cascade are not always true indicators of metastasis as the process not just involves the tumor cells themselves but also a plethora of other factors mainly interaction with surrounding stromal cells, breaking cell-cell and cell-matrix adhesion forces and change in gene expression patterns. However, they offer some insight before studies on expensive animal models. My studies show PIP5Kβ and its product PI(4,5)P₂ alone do not contribute to increasing cell motility and invasion. However, PIP5Kβ may be a part of a network of genes regulating these processes. Further work in understanding signaling pathways altered through BRMS1 downregulation of PIP5Kβ will shed more light on the role of this lipid kinase in metastasis.
The role of phosphoinostide 3-kinase signaling

My studies on phosphoinositide 3-kinase (PI3K) and PI(3,4,5)P3 signaling, though not exhaustive did underscore the importance of PI(3,4,5)P3 in calcium signaling. Using LY294002 a pharmacological inhibitor for PI3K in MDA-MB-435 cells, I showed a significant reduction in calcium rise on stimulation with RTK ligand PDGF. Calcium mobilization in response to bombesin a GPCR agonist was unaffected on treatment with LY294002. Stimulation of RTK and GPCR result in activation of PLCγ and PLCβ isoforms respectively for PI(4,5)P2 hydrolysis. In a recent study, it was shown that complete activation of PLCγ requires interaction with PI(3,4,5)P3 at the cell membrane [19]. My studies though not complete provide evidence in support of these observations.

When checking for the expression levels of the catalytic subunit of PI3K-p110α using immunoblot, I observed the monoclonal antibody for p110α recognized a protein band at around 220 KD much more significantly than p110α. Further this unknown band was highly expressed in metastatic MDA-MB-435 and 231 cells, but completely inhibited on BRMS1 expression in MDA-MB-435 cells (Fig. 5.1a). MALDI-TOF mass spectrometry identified it as nonmuscle myosin heavy chain 9 (MyH9), a motor protein mainly expressed in endothelial cells (Fig. 5.1b). MyH9 is one isoform of the class II myosin family - actin-based motor proteins which mediate a variety of cellular processes including protrusion, migration, and modulation of cell locomotion [20,21].

These observations are significant, especially after my earlier data where BRMS1 significantly reduces motility and invasion in MDA-MB-435 cells. Many of the attributed functions of MyH9 require its presence at the cell membrane, which is where PI3K
signaling is localized. The fact that MyH9 was distinctly recognized by the p110α antibody suggests MyH9 may bind to p110α. Further studies using strategies such as co-immunoprecipitation to identify potential binding proteins to p110α will help provide the link between PI3K signaling and MyH9. In addition, as shown in my model (Fig. 5.2) understanding how BRMS1 modulates the expression of MyH9 in MDA-MB-435 cells may provide a clue in understanding the mechanism behind BRMS1 inhibition of two key steps in the metastatic cascade-motility and invasion.

\[ \text{PI}(4,5)\text{P}_2 \] is the substrate for PI3K catalyzed \[ \text{PI}(3,4,5)\text{P}_3 \] production, hence their signaling events are very much intertwined. Significant reduction in \[ \text{PI}(4,5)\text{P}_2 \] levels, will result in reduced or no \[ \text{PI}(3,4,5)\text{P}_3 \] production despite increased PI3K activity as seen in BRMS1 expressing cells. From my studies I tried to decipher BRMS1 modulation of signaling events involved in regulating \[ \text{PI}(4,5)\text{P}_2 \] levels and their role in metastasis. Although my studies at this point are complete, future work using RNA interference and overexpression studies on PI3K will shed more light on its regulation by BRMS1.

**Why don’t we observe the same dramatic changes induced by BRMS1 in MDA-MB-231 cells?**

Although BRMS1 has been shown to suppress metastasis in both MDA-MB-435 and MDA-MB-231 cell lines, BRMS1 regulation of many signaling moieties ranged from having a similar or partially similar trend to quite contrasting effects. Even where the trends were similar a much more dramatic effect of BRMS1 was observed in MDA-MB-435 cells compared to the MDA-MB-231 cells (e.g. RTK signaling, PIP5Kβ expression and \[ \text{PI}(4,5)\text{P}_2 \]). While quite contrasting effects were observed for bombesin induced GPCR
signaling, the most interesting differences were observed in cytoskeletal organization, motility and invasion profiles. The question that arises is what is the cause of such dramatic differences observed on BRMS1 expression in MDA-MB-435 and 231 cells?

To start with, both cell lines though categorized as breast adenocarcinomas have been derived from different patients [3,22]. In recent years, argument about the classification of MDA-MB-435 as a breast carcinoma cell line has surfaced, its expression of milk proteins and it metastasizing from mammary fat pad but not subcutaneous tissue are consistent with breast carcinoma cell lines [23-26]. Further microarray analysis showed they have quite different gene expression patterns [2]. These genetic differences between cells may regulate how BRMS1 is expressed. We are still not totally clear on BRMS1 regulation of transcriptional events in the cell [27]. Identification of BRMS1 interactions to DNA in the nucleus using strategies such as chromatin immunoprecipitation (ChIP) will help get a better understanding of its regulation of various signaling pathways. This may give evidence for the differences in BRMS1 modulation of cellular events.

References


from various sources with those shown to have melanoma properties. Clin Exp Metastasis 21 (2004) 543-552.

Figure 5.1: MyH9 a putative PI3K binding protein is downregulated by BRMS1 in MDA-MB-435 cells. - a. Immunoblot using anti p110α antibody and SDS-PAGE analysis showing the unknown band at about 220KD b. Information from MALDI-TOF analysis identified the protein as Myosin heavy chain 9 (MyH9).
Figure 5.2: A revised model showing a possible role for MyH9 in BRMS1 mediated metastasis suppression of MDA-MB-435 human breast carcinoma cells. BRMS1 reduced intracellular calcium rise by simultaneously reducing EGFR, GRP, PI(4,5)P₂, PI(3,4,5)P₃ and PLCβ1. BRMS1 also increased PLCγ₁, IP₃R and PI3K expression, suggesting a compensatory mechanism in tumor cells to overcome the depletion of PI(4,5)P₂ levels and maintain its signaling role. Further, BRMS1 inhibits cell survival pathways emanating from AKT due to reduction in PI(3,4,5)P₃ production on growth factor stimulation. BRMS1 also inhibits MyH9 expression in MDA-MB-435 cells.
APPENDIX

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Utah State University
Logan, UT-84322-5305
435-512-4413
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Sitaram Harihar
Department of Biology
Utah State University
Logan, UT-84322-5305
435-512-4413
Sitaram.harihar@usu.edu

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Curriculum Vitae
SITARAM HARIHAR

Department of Biology     Phone: 435-797-0058 (Lab)
5305 Old Main Hill                435-512-4413 (Cell)
Utah State University             email: sitaram.harihar@usu.edu
Logan, UT 84322-5305

Education
B.S.  2000  Biology  Kakatiya University; India
M.S.  2002  Biotechnology  Osmania University; India
Ph.D.  2011  Cell and Molecular Biology  Utah State University; USA

Research Experience
• **Ph.D. Dissertation:** Role of phosphoinositide signaling in metastasis suppression by BRMS1 in human breast carcinoma cells.
• Study of ultrastructural and biochemical properties of human breast carcinoma cells.
• Changes in intracellular calcium levels induced by delivery of phosphoinositides in NIH 3T3 cells.
• The effect of chemical agonists of IP$_3$ and antagonists of IP$_3$R on intracellular calcium levels in human breast carcinoma and NIH 3T3 cells.
• Modeling EGFR pathway in mammalian cells.

Technical Skills
• Cell & Tissue culture
• Calcium Imaging
• Heterologous gene expression (transfection)
• HPLC
• Immunocytochemistry
• Lentiviral cloning
• RT-PCR, Western Blotting, ELISA
• Basic and advanced molecular biology techniques
• Thin layer chromatography
• Confocal microscopy
• Trained to use radioactive substances

Teaching Experience
Aug 2003–present, Teaching Assistant, Utah State University

Courses taught
• Methods in animal cell culture (BIOL 5160)
• General microbiology (BIOL 3300)
• Elementary microbiology (BIOL 2060)
• General biology (BIOL 1620)
• Human anatomy (BIOL 2320)

Awards and Accomplishments
• Junior research fellowship, CSIR-UGC, Government of India, 2002
• Joseph Greaves memorial fellowship, Biology, Utah State University, 2005-2006
• Graduate student senate travel award
• Reviewer for the journal clinical and experimental Metastasis

Publications


• Harihar S, Rzepecki PW, Chen D, Anderson CL, DeWald DB. Reduced Intracellular Calcium Mobilization by Novel Cyclopentyl Antagonists of IP3 Receptor (*Manuscript in preparation*)

• Harihar S, de Guzman G, Baker S, Welch DR and DeWald DB. Differential Regulation of Type I Phosphatidylinositol 4-Phosphate 5-kinase isoforms in MDA-MB-435 cells by BRMS1 (*manuscript in preparation*)

• Harihar S and DeWald DB. Phosphoinositides and Cancer Metastasis (*manuscript in preparation*)

Conference Presentations


Invited Presentations
• Breast Cancer Metastasis Suppressor 1 reduces phosphoinositide Signaling and invasive potential of MDA-MB-435 cells (2008). American Heart Association Roundtable, Utah State University, Logan, UT

• The Role of Phosphoinositide Signaling in BRMS1 Mediated Metastasis Suppression of MDA-MB-435 Human Breast Carcinoma Cells (2010) University of Virginia, Charlottesville, VA