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Intracellular Signaling and Trafficking in Cancer: Role of Rab5-GTPase in Migration and Invasion of Breast Cells

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

INTRACELLULAR SIGNALING AND TRAFFICKING IN CANCER: ROLE OF
RAB5-GTPASE IN MIGRATION AND INVASION OF BREAST CANCER CELLS

A dissertation submitted in partial fulfillment of
the requirements for the degree of
DOCTOR OF PHILOSOPHY
in
BIOLOGY
by
Nicole C. Portner

2015

To: Dean Michael R. Heithaus
College of Arts and Sciences

This dissertation, written by Nicole C. Porthier, and entitled Intracellular Signaling and Trafficking in Cancer: Role of Rab5-GTPase in Migration and Invasion of Breast Cancer Cells, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend it to be approved.

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Florida International University 2015

DEDICATION

To my most patient husband and wonderful kids

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ABSTRACT OF THE DISSERTATION

INTRACELLULAR SIGNALING AND TRAFFICKING IN CANCER: ROLE OF RAB5-GTPASE IN MIGRATION AND INVASION OF BREAST CANCER CELLS

by

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Florida International University, 2015

Miami, Florida

Professor Manuel A. Barbieri, Major Professor

Metastasis is characterized pathologically by uncontrolled cell invasion, proliferation, migration and angiogenesis. Steroid hormones, such as estrogen, and growth factors, which include insulin growth factor I/II (IGF-1/IGF-2) therapy has been associated with most if not all of the features of metastasis. It has been determined that IGF-1 increases cell survival of cancer cells and potentiate the effect of E2 and other ligand growth factors on breast cancer cells. However not much information is available that comprehensively expounds on the roles of insulin growth factor receptor (IGFR) and Rab GTPases may play in breast cancer. The latter, Rab GTPases, are small signaling molecules and critical in the regulation of many cellular processes including cell migration, growth via the endocytic pathway. This research involves the role of Rab GTPases, specifically Rab5 and its guanine exchange factors (GEFs), in the promotion of cancer cell migration and invasion. Two important questions abound: Are IGFR stimulation and downstream effect involved the endocytic pathway in carcinogenesis? What role does Rab5 play in cell migration and invasion of cancer cells? The hypothesis is that growth factor signaling is dependent on Rab5 activity in mediating the

aggressiveness of cancer cells. The goal is to demonstrate that IGF-1 signaling is dependent on Rab5 function in breast cancer progression. Here, the results thus far, have shown that while activation of Rab5 may mediate increased cell proliferation, migration and invasion in breast cancer cells, the Rab5 GEF, RIN1 interacts with the IGFR thereby facilitating migration and invasion activities in breast cells. Furthermore, endocytosis of the IGFR in breast cancer cells seems to be caveolin dependent as the data has shown. This taken together, the data shows that IGF-1 signaling in breast cancer cells relies on IGF-1R phosphorylation, caveolae internalization and sequestration to the early endosome RIN1 function and Rab5 activation.

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LIST OF ABBREVIATIONS AND ACRONYMS

AT.....	Amoeboid Transition
Bad.....	Bcl2 Associated Death Promoter
Bax.....	Bcl2 associated X Protein
Bcl2.....	B cell Lymphoma 2
Bif1.....	Bad interacting factor-1
CDC42.....	Cell division control protein 42 homolog
CDK.....	Cyclin Dependent Kinase
CSF-1.....	Colony Stimulating Factor 1
DMEM.....	Dulbecco's Modified Eagle's medium
EEA1.....	Early Endosome Antigen 1
EGF.....	Epithelial Growth Factor
EGFR.....	Epithelial Growth Factor Receptor
EMT.....	Epithelial Mesenchymal Transition
ERK.....	Extracellular signal Regulated Kinase
FA.....	Focal Adhesion
FAK.....	Focal Adhesion Kinase
FYVE.....	Zinc finger domain named after cysteine rich proteins Fab1, YOTB, Vac1 and
GAP.....	GTPase Activating Protein
Gab2.....	Grb2 associated binding protein2
GDI.....	Rab GDP dissociation inhibitor
GDI.....	GDP dissociation Inhibitors
GDP.....	Guanosine di phosphate
GEF.....	Guanine nucleotide Exchange Factor
GFP.....	Green Fluorescent Protein
Grb2.....	Growth factor Receptor Bound Protein 2
GSK3B.....	Glycogen Synthase Kinase 3B
GTP.....	Guanosine Tri Phosphate

H-Ras.....	Harvey rat sarcoma viral oncoprotein
Ig	Immunoglobulin
IFN γ	Interferon gamma
IGF.....	Insulin Growth Factor
IGFR.....	Insulin Growth Factor Receptor
IL2.....	Interleukin -2
IR.....	Insulin Receptor
IRS.....	Insulin Receptor Substrate
K-Ras.....	Kirsten ras oncogene homolog
MAPK.....	Mitogen Activated Protein Kinase
MAT.....	Mesenchymal Amoeboid Transition
MET.....	Hepatocyte Growth Factor Receptor
MMP.....	Matrix Metalloproteinases
N-Ras.....	Neuroblastoma Ras Viral oncogene
P53.....	Tumor Protein p53
PBS.....	Phosphate Buffered Saline
PDGF.....	Platelet derived Growth Factor
PI3K.....	Phosphoinositide 3 Kinase
PI3P.....	Phosphatidyl inositol 3 phosphate
PIP3.....	Phosphatidyl inositol-3,4,5-triphosphate
PKC.....	Protein Kinase C
PTEN.....	Phosphatase and Tensin homolog protein
R-Ras.....	related RAS viral (r-ras) oncogene homolog
Rab.....	Ras Related in Brain
RABEX-5.....	Rabaptin-5 associated Exchange factor for Rab5
Rac1.....	Ras-related C3 botulinum toxin substrate 1
RAP6.....	Rab5 Activating Protein 6
RAS.....	Rat sarcoma
Rho.....	Ras homolog gene family

RIN1.....	Ras interference/interaction 1
RTK.....	Receptor Tyrosine Kinase
SDS.....	Sodium dodecyl sulfate
SDS-PAGE.....	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2.....	Src Homology 2
SHIP-1.....	SH2 containing Inositol Phosphatase-1
SNARE ..	Soluble N-ethylmaleimide-sensitive factor (NSF) Attachment protein Receptor
TAMs.....	Tumor Activated Macrophages
TGF β	Transforming Growth Factor beta
TNF- α	Tumor Necrosis Factor alpha
VEGF.....	Vascular Endothelial Growth Factor
VPS9.....	Vacuolar Sorting Protein 9
Wnt1.....	Wingless-type MMTV integration site family, member 1
WT.....	Wild Type

CHAPTER 1

The scope of this chapter is to introduce the concept of intracellular signaling cascade in normal and cancer cells with specific focus on early endocytosis and growth factor receptor activity upon ligand stimulation. The role of Rab GTPases, key metastatic traits (cell invasion and cell migration), and a short overview of the insulin growth factor pathway will be covered in cancer cells. Overall the information creates an outline for the research hypothesis.

1.1 Introduction

The last decade in cancer research has seen great strides in identifying the interplay between the stroma and tumor environment in cancer progression. Briefly, cancer has been long viewed as a disease consisting of transformed or mutated cells that acquire hyper-proliferative, invasive and limitless survival capacities. While this in itself is true, it was a limited approach as ensuing therapeutic cancer intervention strategies sought to target only the tumor cell. However, carcinogenesis and tumor progression are viewed not as an autonomous cell-centered condition, but rather as a disease involving complex multicellular (stromal cells) crosstalk within a newly formed tissue. This breast cancer tissue or the tumor matrix hijacks key cellular functions which have evolved as cornerstones of metastasis. The primary focus of this review will delve on the early endocytic pathway in modulating one of the most important hallmarks of cancer cell metastasis; cell migration and cell invasion in response to growth factor stimulation.

1.2 The Traits of Cancer

Hanahan and Weinberg published a landmark paper referred to as the ‘Hallmarks of Cancer’ that encapsulated the key stages from tumorigenesis to metastasis in cancer

progression⁵³. The six phases described, though distinctive in nature, complemented each other and worked in concert in the transformation a normal cell to a cancer cell. These hallmarks were later updated to ten key traits by the authors and include but not limited to tumor induced inflammation to the constitution and signaling interactions of the tumor microenvironment in the maintenance of cancer phenotypes. For this project, three hallmarks were studied: sustained proliferation, migration and invasion.

1.2.1. Always On: Growth Factor sustained proliferation

The multiplication of cells relies on biochemical molecules called growth factors¹⁴¹ that are highly bio-reactive despite their low copy number within tissues. Their primary functions include cell growth, differentiation and survival thereby making them targets for opportunistic pre-cancer cells. During normal body function, homeostasis is achieved by the regulation of the production and secretion of growth signals (mitogens) and the cells that respond and progress from a quiescent status to the growth and division cycle¹⁴¹.

Cancer cells, on the other hand, modulate these mitogenic signals and evolve as autonomous growth signal entities^{64,71,141}. They upset the delicate balance by promoting the binding of multiple growth factors to cell surface receptors, synthesize growth signals that can either bind to their own receptor forming an autocrine loop or influence the secretion of more growth factors by normal cells into the stroma^{12,25}, which then binds again to the receptors on the cancer cells, creating a paracrine feedback loop.

Within the cancer cell, activated receptors transduce signals via the intracellular signaling pathway resulting in increased cell proliferation, survival and size and

translation of more modified cell surface receptors which continue to feed into the self-sufficient, machinery created by cancer cells^{53,117}. These receptors may also be altered where they are permanently activated and hyper-sensitive to various stimuli or non-specific ligand binding.

Mutations within the signaling pathway (besides the receptor) can also mediate a sustained proliferative response in cancer cells independent of growth factor stimulus. For example, Ras is a known downstream signaling protein in the receptor mediated pathway and one in four (25%) of all human tumors have the mutant Ras gene switched on³⁶.

1.2.2. Metastasis

In cancer, a solid tumor cannot expand and spread without a series of synchronized events. Metastasis is a multistep process that encompasses the modulation of basement membrane permeability at the tumor site; decreased cell attachment, tumor cell invasion, cell migration and proliferation in response to survival and pro-angiogenic signals.³⁷ Though the classical belief regards metastasis as a late event in cancer progression, it is acknowledged that metastatic signaling events can be initiated during early tumorigenesis and progression. Specifically, cell origin and oncogenic alterations influence the molecular characteristics of a tumor and its malignancy potential.¹³⁴

While the metastatic potential is relatively small for tumor cells, there is an epigenetic influence that leads to the transformation from non-metastatic to aggressive tumor cell^{37,53}. It is not a passive event where cancerous cells are sloughed off a tumor and shuttled to distal sites for colonization. On the contrary, it is a highly active process that

involves chemotactic signals that drive cell motility, release of proteases and other enzymes that degrade the extracellular matrix (ECM), avoidance of the immune response and apoptotic, anoikis and anorectic signals, unresponsiveness to growth inhibitory signals and as mentioned earlier, growth factor independent proliferation^{53,117}. Furthermore changes in cell adhesion molecules (integrins, cadherin-catenins) and protease expression have been linked to tumor invasion and metastasis^{1,122}. Within recent years, interest has been generated regarding the role of endocytosis in tumor progression and metastasis, particularly the trafficking of metastatic factors that mediate two of the necessary stages that characterize metastasis; cell migration and cell invasion.^{67,97,122}

1.2.3. Characteristics of Invading cells

Cell migration and invasion are key components of metastasis where atypical epithelial cells have acquired phenotypic changes that mediate the spread of the cancer to distant tissues and organs. Cell migration is a highly regulated event and is controlled by the expression and localization of specific cell surface receptors, such as Integrins, Epithelial Growth Factor (EGF)- Receptor (EGFR), Insulin Growth Factor (IGF)- Receptor (IGFR), the reorganization of cytoskeletal elements, and the directed trafficking of molecules required for cell signaling and adhesion.^{8,110}

There are three distinctive types of morphologic events that are affiliated with early metastasis- epithelial mesenchymal transition (EMT)^{71,147}, collective invasion^{41,42} and amoeboid transcription^{85,115}. Most recently a fourth concept, inflammatory induced invasion, has been identified as another morphogenic episode in metastasis^{64,93,106}.

1.2.3.1. Epithelial Mesenchymal Transition

The most common of the morphogenic episodes associated with metastasis, EMT is a process where cells elongate, secrete ECM proteases which degrade the matrix and migrate out⁵³. Transforming Growth Factor β (GF β) induces EMT via RAS-GTPases signaling¹⁰² and facilitate changes to a more invasive phenotype in cancer cells in response to ligand growth factor stimuli, specifically IGF-1¹³³. Within the human body, tissue is comprised of two types of cells that aggregate and perform a specialized task; epithelial cells and mesenchymal cells. Epithelial cells are atypical in shape, adherent to other cells to form layers within the tissue; of which the latter is interspersed with structural carbohydrates and proteins that serve as the extracellular matrix. Mesenchymal cells are solitary cells that possess a strong migratory potential. Yet most cancers (at least 90%) are epithelial in origin¹²⁷.

Normal epithelial cells secrete E-cadherin, a regulatory protein that maintains adhesive (glue-like) structure between the cells and suppresses the release of pro-migratory growth molecules thereby ensuring that the cells remain in senescence and contact inhibition. With the advent of metastasis, E-cadherin function is lost and there is a gradual shift to N-cadherin expression; which is expressed in migrating cells.¹³³ After EMT, motile cells migrate mesenchymally where they extend projections called lamellipodia on the front of the cells while binding to specific surface cell surface receptors such as beta-integrins^{19,147}. This movement is largely regulated by several small GTPases like H-RAS, N-RAS, K-Ras, Rac1, Rho and Cdc42 GTPases activity¹³⁸.

Actin dependent contractile propulsion push the cell body forward while rear is released and integrin signaling engage the secretion of matrix metalloproteinases

(MMPs); degrading the ECM and generating gaps for cells to pass through. EMT cells are therefore self- directing and path-generating given the cells' proteolytic activity as they migrate.¹⁴²

The most unique aspect of EMT and tumor cell migration is the ability to survive without normal matrix components and the evasion of anoikis or adherence to foreign ECM as they move³⁴.

1.2.3.2. Amoeboid Transcription

Typically aggressive, motility in amoeboid transcription (AT) differs from EMT where protease- independent elongated cells acquire a spherical shape and then are deformed upon passage through interstices already present in the ECM¹¹⁵. Within AT cell migration and invasion is controlled by the Rho family of GTPases^{109,131}.

Unlike mesenchymal migration, suppression of Smurf1 influences RhoA activation, and initiates a signaling cascade that effect the generation of the spheroid structure and locomotion.¹³⁵ The high metastatic capacity of amoeboid transcription can be attributed to the tumor cells' competence in warping the collagen bed in the ECM which allows the cells to become invasive¹⁴⁵.

1.2.3.3. Collective Invasion

As the name implies, this type of invasion involve the movement of a group of cancer cells to neighboring tissues and organs. However, unlike EMT and amoeboid transcription, this type of invasion is somewhat controversial and believed to be less aggressive as it lacks key characteristics that facilitate metastasis.

On one hand, it is believed that during collective invasion, cells on the leading edges of the collective invasion zones may acquire partial EMT-like traits and drive the intravasation process²⁶. For example, cancer cells expressing high levels of basal epithelial markers such as cytokeratin 14 and p63 led invasion of the collective group of cells in different subtypes of breast cancer²⁶. Stromal fibroblasts via Rho activation also initiate collective invasion in cancer cells⁴⁷.

The other theory is that a unique mechanism distinct from EMT, may be utilized by tumor cells undergoing collective invasion³⁹.

1.2.3.4. Inflammation Induced Invasion

If EMT can be viewed as a simplistic feature of tumor cell invasion and amoeboid is the aggressive, complicated version, inflammation induced invasion can be the product of the aforementioned invasive processes. The crosstalk between the stroma and tumor cells in the microenvironment influence the invasive potential of tumor cells. In breast cancer, it was determined that cancer cells signaled the secretion of chemokines by mesenchymal cells located in the stromal bed, and these chemokines stimulate cancer cells to become aggressive and invasive⁶⁵.

Immune cells at tumor sites enable invasion by supplying MMPs. Tumor associated macrophages (TAMs) furnish cancer cells with growth factors and cancer cells reciprocate by providing colony stimulating factor 1 (csf1) thereby driving increasingly invasive behavior in tumor cells¹¹².

1.3 Growth Factor Driven Signaling in Tumor Metastasis

Metastatic factors such as HGF, EGF, TGF β and IGF are known to activate the endosomal pathway and the mechanisms can be analogous at key junctions thereby promoting a migratory response in tumor cells.

These Receptor Tyrosine Kinases (RTKs) can be internalized through two principal pathways: clathrin coated pits or caveolae. We will briefly discuss a few of these growth factors and their possible roles in early endocytic trafficking and tumor progression. A detailed review of most metastatic growth factors (whether mentioned here or not) with the exception of IGF-1 can be accessed from the following reference.⁶¹

1.3.1. Epithelial Growth Factor

Perhaps the most studied and understood receptor trafficking systems, EGFR signaling is a crucial aspect of breast cancer metastasis^{11,15,38,88,90}. Epithelial Growth Factor Receptor signaling is heavily regulated by endocytosis where EGF triggers the internalization of the EGFR complex via clathrin-mediated endocytosis^{57,123}, clathrin-independent mechanisms¹⁰⁴ and subsequent activation of the mitogen-activated protein kinases (MAPK) signaling pathway.

The Arf GAP with Rho GAP domain, ankyrin repeat, and PH domain 1 (ARAP1) protein controls EGFR trafficking via Rab5 activation and affect the rate of EGFR signal attenuation¹⁴⁹ and ensuing MAPK signaling. A recent study highlighted the role of clathrin-mediated endocytosis Epithelial Growth Factor directed chemotactic invasion of breast cancer⁹⁸. Rab5a is characterized as a significant molecule in ovarian cancer upon EGF stimulation.¹⁵⁵

On the other hand, Rab5c has been implicated in EGFR signaling by affecting integrin recycling and triggering cell invasion in some breast cancer cells.¹⁰³ Rab11a is known to modulate EGFR recycling and have a differential effect on proliferation and motility of MCF10A breast cells.¹⁰⁷ Inhibition of Rab35 fostered a shift from epithelial to mesenchymal status, and led to an increase in cell migration and invasion in tumor cells in response to EGFR activation.³

1.3.2. Hepatocyte Growth Factor

It has been previously reported that HGF signaling involves the activation of the Hepatocyte Growth Factor Receptor (MET) and the receptor undergoes rapid endocytosis via clathrin dependent and independent endocytic pathways.

Hepatocyte Growth Factor Receptor trafficking requires Rab5 and facilitates focal adhesion turnover, actin remodeling and sustained MAPK signaling thereby underscoring its role in tumorigenesis, cell migration and invasion.⁵² Further studies show that human growth hormone (HGH) exposure in cells expressing naturally occurring Met mutants boosted endocytic activity and led to tumor metastasis.⁶¹

1.3.3. Transforming Growth Factor β

The TGF β has an interesting dual role in cancer. As indicated earlier, metastasis necessitates the transformation from epithelial cell to mesenchymal cell phenotype. During this process, TGF β has been characterized in its role in epithelial mesenchymal transition (EMT) and tumorigenesis as having both a positive and negative influence in the promotion of cell migration and invasion.²⁹

The TGF β signaling involves the internalization of the Transforming Growth Factor β Receptor (TGF β R) through clathrin dependent and independent pathways and was found in EEA1 and caveolin positive vesicles, respectively.³¹ Furthermore, the intensity of TGF β was sustained through recycling of the receptor through ligand stimulation and clathrin internalization through Rab11 irrespective of ligand activation via Rab4 activity⁹¹.

It was also noted that lipid raft-caveolar induced rapid receptor turnover by the competitive binding of the internalized TGF β R with a small intracellular inhibitory protein, *mothers against decapentaplegic homolog 7* (Smad7) coupled with *SMAD specific E3 ubiquitin protein ligase 2* (Smurf2); of which this Smad7-Smurf2 complex tags the TGF β R for degradation³¹ through the proteasomal or lysosomal pathways.

While there is a dearth of information regarding the direct involvement of TGF β R endocytosis in cancer metastasis, it was recently reported that delayed endocytosis of the TGF- β RII was due to a gain-of-mutation of the receptor and enhanced TGF- β signaling. This resulted in a more invasive phenotype of human oral squamous carcinoma¹⁰⁸.

1.3.4. Insulin Growth Factor

The IGF system is a complex highly regulated network and its influence in cancer has been extensively reviewed by several groups^{6,14,17,77,82,116}. Here, the key features of IGF-1 and the downstream effectors in cancer biology is recapitulated. The IGF-1 system is comprised of the IGF ligands (IGF-1 and IGF-2), cell-surface receptors that mediate the biological effects of the IGFs, including the IGF-1 receptor (IGF-1R), the IGF-2 receptor (IGF-2R), the insulin receptor (IR), as well as a family of IGF-binding proteins (IGFBPs).

Most IGF-1 in circulation is produced by the liver, and IGF-1 synthesis and action is linked to the growth hormone (GH) system, typically referred to as the GH/IGF axis⁷⁰. IGF-1 is also synthesized in other organs and tissue where autocrine and paracrine mechanisms are essential. Insulin Growth Factor-1 activity is modulated in part by IGFBPs, where close to 99% is bound, leaving a small percentage to exert its influence on the body⁵¹.

Insulin Growth Factor-1 signaling may be both IGF-1R dependent or independent. Insulin Growth Factor signaling is initiated by the binding of the IGF-1 or IGF-2 ligand to the IGF-1R/ IGF-2R and the subsequent phosphorylation of tyrosine residues of specific cytosolic kinase domains^{35,82,111}.

The activated receptor transduces this phosphorylation activity to insulin receptor substrates (IRS) and Shc adaptor proteins; and starts a signaling cascade that involves the MAPK and phosphatidylinositol 3-kinase (PI3K) intracellular signaling pathways that are responsible for the diverse target actions of these growth factors, which include increases in cell division, cell size, protein synthesis, cell migration, and inhibition of apoptosis⁷⁸. Many of the signaling elements that are downstream from tyrosine kinase activation have been characterized, but several of the molecular events (such as the possible internalization of the receptor) that may occur before or concomitantly with kinase activation and whether these early events can influence the ultimate outcome of IGF-1-stimulated signal transduction have not been determined.

The IGF system is integral to a number of cellular processes ranging from normal growth and development of specific organs, such as in the nervous system, in which IGF signaling regulates neuronal proliferation, apoptosis, and cell survival^{82,111,116}.

Conversely, IGF also has a critical role in pathological situations, particularly tumorigenesis and a growing body of epidemiological studies has implicated free IGF-1 serum levels as a risk factor for the development and progression of breast, prostate, colon, and lung cancer^{35,46,111}. The adverse effect of IGF is unsurprising given the delicate balance that must be maintained, and the diverse and extensive regulatory function of IGF-1 in maintaining homeostasis.

Insulin Growth Factor-1 does not induce mutations but may promote cancer via the apoptotic pathway as it is a survival factor similar to PDGF, EGF and act via the IGF-1R to stimulate the phosphatidylinositol 3-kinase PI3K /Akt pathway^{49,78}. Kurmasheva et al. maintained that the IGF-1 mediates cell survival in adverse physiological conditions ranging from oxidative stress, low nutrient, cytotoxic or hypoxic environments.⁷⁴ Insulin Growth Factor-1 facilitates cell survival by regulating the expression of pro- and anti-apoptotic proteins. Specifically, IGF-1 binding to the IGF-1R ligand induces the activation of Akt which phosphorylates the pro-apoptotic protein Bad⁷⁸. This phosphorylation activity in turn, induces a conformational change in Bad and inhibited its interaction with the anti-apoptotic Bcl proteins and the subsequent activation of the caspase chain thereby allowing the transformed and pre-cancerous cells to escape apoptosis and survive.

Insulin Growth Factor-1 has been demonstrated to increase VEGF expression of tumor cells and inhibit apoptosis by binding to caspase 9 which is unable to cleave caspase 3 and halting the caspase cascade to apoptosis¹³². It is known that IGF-1 protect scells from anoikis which is a process that ensures cells undergo apoptosis when they are detached

and no longer able to adhere to the extracellular matrix and this protective action may be conferred to pre-metastatic tumor cells.

Insulin Growth Factor-1 levels increase in response to ECM degradation by matrix metalloproteinases (MMPs) and ensure the survival and hyperproliferative state of cancerous cells.¹²⁸ Insulin Growth Factor-1 increases the pro-metastatic property of cancer cells by reorganizing integrin receptors, and modulating the E-cadherin/ β -catenin complex which in turn, increase MMPs expression and the migratory potential of the cells¹³³ β -catenin as a result, accumulates in the nucleus (possible translocation from cytoplasm), and there is an increase cell proliferation by inactivation of glycogen synthase kinase 3B (GSK3B)³⁰.

Insulin Growth Factor-1 also facilitates the crosstalk between the PI3K and MAPK pathways via Ras (upstream effector of the p85 subunit of PI3K) which potentiates both the cell proliferation and cell survival cell response. Some interesting studies that proffered other links between IGF-1 and cancer where an inverse relationship exists between the tumor suppressor genes p53 and Wnt1 and low expression of the genes was dominant in highly aggressive breast cancer cells (estrogen receptor negative; ER-)³⁵.

It is now known that that circulating IGF-1 levels are higher in breast cancer patients compared with normal controls¹¹¹ while high IGF levels are associated with increased risk of breast cancer in pre-menopausal women.⁵⁴

There also exists an inherent relationship between IGF-1 and estrogen (E2). Estrogen has been shown to increase IGF-1 and IGF-1R expression and transcription, with the latter having a positive feedback and activating the IGF-1 signal pathways³⁵. Furthermore, it has been determined that the E2 antagonist, tamoxifen and other

antiestrogens suppress IGF-1 gene expression and MAPK and PI3K pathways.²⁷ Conversely in tamoxifen resistant cancer cells, IGF-1R expression as well as MAPK phosphorylation were activated in tamoxifen resistant cancer cells⁸⁷.

1.3.4.1. IGF-1R and Endocytosis

Despite the limited information regarding the function of endocytosis in IGF-1 driven tumorigenesis, recent evidence points to an increase in cell proliferation and inhibition of apoptosis upon IGF-1R stimulation and ensuing internalization and co-localization with clathrin and caveolin-1 in Ewing sarcoma cells.⁸⁶ Insulin Growth Factor-1 receptor is also localized in the nucleus in prostate, renal and breast tumor cells after translocation from the cell surface via clathrin mediated endocytosis.² Whether early endocytosis of IGF-1R is key in regulating tumorigenesis and cancer metastasis remains to be seen.

1.4. Endosomal Signaling and Intracellular Trafficking

Within a cell, homeostatic conditions and normal cell mechanisms and processes such as differentiation, cell migration, proliferation, gene transcription, vesicular trafficking, cytoskeleton modulation and organization and nuclear assembly are tightly regulated and rely heavily on the small GTPase activity.^{83,139} These monomeric GTPases are homologous to the heteromeric G-proteins where they hydrolyze the active bound form of GTP to the inactive bound form thereby acting as molecular switches⁶⁶ and cycling between the active GTP and inactive GDP bound forms via three classes of regulatory proteins: the GTPase activating proteins (GAPs), guanine nucleotide disassociation inhibitors (GDIs) and guanine exchange factors (GEFs)¹⁸.

One of the largest sub-families of the large Ras superfamily of small GTPases is the Rab proteins¹⁵³. They extend across more than 60 members in mammals and are highly

conserved from yeast to humans. The Rab family of proteins plays important regulatory roles in vesicle formation and transportation from plasma membrane to various organelles within the cell through the recruitment of small factors that include phosphatases, kinases and docking , adaptor proteins as well as actin filaments and microtubules in cell migration and mitosis.¹²⁶

In this way, given the myriad of functions that Rab proteins regulate during intracellular trafficking, it is unsurprising that there is a marked interest in Rab GTPase function and expression in tumor progression. The Rab proteins have been specifically linked to the deregulation of several signaling pathways in several cancers and also been implicated in the promotion of abnormal proliferation, migration and invasion of epithelial cells in tumor progression because of stimulation of membrane bound growth factor receptors²³.

Disruption of endocytosis confers growth factor and ECM self-sufficiency to cancer cells as discussed earlier, imparts growth factor and extracellular matrix autonomy to cancer cells by bypassing key endocytic processes such as lysosomal degradation and instead promote the recycling of growth factors as well as integrins. In response to this deviant endocytic pathway, cellular processes such as adhesion, migration, proliferation, polarity, asymmetrical division and overall survival are modified and represent emerging hallmarks of cancer⁹².

1.4.1. Differential Expression of Rab GTPases in Cancer Cells

Though the functional role of Rab GTPases have not been fully characterized in cancer cells, studies have shown that at the genomic levels, there is a deregulated expression of Rab in tumor cells which also differs within specific tissue.

The GTPase, Rab23, is over-expressed in bladder ⁵⁸, gastric ⁵⁹ and ovarian ²⁴ carcinoma whilst it is purported to be down-regulated in triple negative breast cancer^{21,22}. Similarly Rab25 have differing expression levels with increased expression in estrogen and progesterone receptor positive breast cancer ¹⁴⁸ and suppression in colon and head and neck tumors^{5,100}.

Down-regulation of Rab38 may be associated with metastasis in melanoma⁹⁵. In gastric cancer, other Rabs have been implicated such as Rab32, Rab34 and Rab40C of which hypermethylation of the Rab32 gene led to down-regulation in the tumor ¹²⁰ and conversely suppression of specific miRNAs; miR-9 and let-7a promoted the efflux of Rab34 and Rab40c expression⁸⁴.

Likewise, alterations in miRNA expression (specifically miRNA-451 and miRNA-101) led to non-small lung cancer via over-expression of Rab14 ¹³⁷ and upregulation of Rab5a in hepatocellular carcinoma, ¹¹⁹ respectively. Furthermore in highly metastatic lung cancer, post-translational modifications and an increase in gene expression mediated elevated Rab37 and Rab7 levels. ^{99,143} In liver cancer, Rab1b, Rab4b, Rab10, Rab22 and Rab24 are over-expressed ⁵⁵ whilst Rab1a is higher than normal in tongue cancer¹²¹.

It is also known that Rab2B levels are elevated in colon carcinoma¹⁰¹ while in thyroid associated adenomas, Rab5a and Rab7 are upregulated⁷⁵. Breast cancer metastasis and worse outcomes in patients have been attributed to high levels of Rab31 and Rab5a^{72,146}.

1.4.2. The Role of Rab GTPases in regulating Cell Migration

As mentioned earlier, one of the key characteristics of metastatic cells is the ability to migrate and invade through a compromised ECM and translocate to distal sites, thereby forming secondary tumors or metastases⁵³.

Rab35, a gtpase involved in Rho mediated actin cytoskeletal reorganization, is upregulated in migrating breast cancer cells in response to Wnt activation. Chau et al. demonstrated that actin dependent protrusions were generated by Rab35 acting on Rho GTPases²⁸. The silencing of Rab35 inhibited cell migration in MCF7 breast cancer cells.¹⁵⁷ Conversely, Rab35 mitigates cell migration in brain tumor cells³ which suggest that Rab GTPase modulation of cell migration may be tissue specific.

Similarly Rab25, a protein that plays a critical role in translocating and recycling integrin and other pro-migratory factors from endosomes to the plasma membrane have contrary actions on cell migration. In esophageal squamous cell carcinoma (ESCC), Rab 25 was characterized as a tumor suppressor as diminished Rab25 expression was seen as poor prognosis for esophageal cancer survival by enhancing migration and invasiveness of cancer cells.¹²⁹ Interestingly, it has also been shown that loss of Rab25 (function or expression) increased tumorigenesis in mammary epithelial cells²². However, Rab25 is also said to be an oncogene given its role in advanced stages of ovarian and breast cancers and is linked to an increase in malignancy and migration of the epithelial cells by modulating cell proliferation (both anchorage dependent and independent cells), trafficking of integrin and suppressing apoptosis²⁴. Specifically, Rab25 promotes cell survival by increasing PI3K signaling through the interaction with the Rab-coupling protein which in turn sustains the recycling of both EGFR and the fibronectin receptor (integrin $\alpha 5\beta 1$). It was determined that Rab25 accelerates cell migration by actively moderating the recycling of integrins via the chloride intracellular channel protein 3 where this process is required in the freeing of the cell's rear in cell migration and invasion³³.

Both Rab25 and Rab11 overexpression augmented the pace of cell migration by affecting small-scale fast recycling within the tips of the cell and basal long-distance transport from the rear end to the front of the migrating cell basal long-distance transport from the rear end to the front of the migrating cell respectively in HeLa and MDA-MB 231 cell lines⁶⁹.

A GTPase essential for early endosomal formation and trafficking, Rab5, is involved in lamellipodia formation and actin remodeling in response to growth factor stimulation^{105,124} and this may be attributed to tyrosine signaling and subsequent activation of Ras and Rab5 GAPs^{10,76}.

Rab5 is expressed differentially in cancer cells where Yu et al. identified Rab5a as a potential major factor in the transformation of tumor cells in human lung adenocarcinoma, to an aggressive phenotype where overexpression of Rab5a was associated with increasing metastatic capacity.¹⁵¹ The loss of Rab5a expression in HeLa and SiHa cells significantly suppressed or abrogated cancer cell motility and also down-regulated Rho A expression⁸⁰ whereas Rab5a overexpression elevated the proliferative activity of ovarian cancer cells¹⁵⁵.

Studies in flies (*Drosophila*) and mouse neurons have shown that cell migration can be mediated by endocytotic and trafficking mechanisms and dependent on Rab5 and Rab11 expression in epithelial cells^{68,118}. Migration of cells in hepatocellular carcinoma was also dependent on VEGF/PDGF (platelet derived growth factor) and EGFR mediated tyrosine kinase endocytosis via Rab5⁴⁴, hepatitis B induced Rab5 expression¹¹⁹ and Rab11^{62,63}. Actin remodeling and cell migration are dependent on Rac activity which is trafficked by Rab5 and affects the internalization of integrins during cell migration^{130,152}.

Lysosome trafficking is an important feature of the endocytic pathway particularly in the degradation growth factor receptors and / or ligands and is regulated by Rab7^{125,140}. Disruption of the endocytic pathway in EGF mediated signaling in cancer cells can be rescued by the activation of the autophagy and lysosomal pathway. A tumor suppressor and Bax family member, Bif1, led to the recruitment of Rab7 to the early endosome and facilitated the loss of cell migration of breast cancer cells by directing the degradation of the EGFR¹¹⁴.

Rabs	Cancer Type	Location	Cell Function	Effect on Cell Migration or Invasion	Refs
Rab4a (invasion)	melanoma	plasma membrane	protein transport	secrete procathepsin-L, translocates furin	9
Rab5a (invasion)	cervical, breast	Plasma membrane, Early endosome, Melanosomes	Fusion of PM to EE, protein transport	down-regulation of integrins, Rac1, CDC42, reduction in number of lamellipodia, transport of MT1-MMP and β 3 integrin	43,81
Rab5a (migration)	hepatocellular carcinoma, breast	plasma membrane, early endosome	Translocation of surface receptors to late endosome	lamellipodia formation and actin remodeling in response to growth factor stimulation	21,45,89,119,146
Rab7 (invasion)	liver, prostate	Late endosome, Lysosome, Melanosomes	Late endocytic transport	decrease protease secretion	125,140
Rab7 (migration)	breast, liver	late endosome, lysosome	exocytosis, late endosome		114,140

Rab8 (invasion)	lung adenocarcinoma	plasma membrane	vessicle trafficking	secretion and cleavage of (MT1-MMP	16
Rab11 (a,b,c) (invasion)	breast, colon, lung, ovarian, renal, endometrial, prostate, bladder and carcinoid types	recycling endosome	Long loop recycling	down regulates E-cadherin	150,156
Rab11 (a,b,c) (migration)	breast, colon, lung, ovarian, renal, endometrial, prostate, bladder and carcinoid types	recycling endosome	long loop recycling	stabilization of microtubules	20,48,69
Rab23 (invasion)	gastric cancer	Plasma membrane	vessicle trafficking		60
Rab25 (invasion)	colon, ovarian, head and neck	apical recycling compartment	integrin recycling	recycles integrins to psuedopodial tip of invasive cells, interact with CLIC3 (chloride intracellular channel protein 3) to recycle integrins from late compartment to the rear of migrating cells	5,24,73
Rab25 (migration)	breast, pancreatic , ovarian	apical endosomes	integrin recycling	sustained recycling of intergrin and growth factor receptors	22,24,33,69,129
Rab27a,b (invasion)	glioma, breast, liver	Plasma membrane, melanosomes	exocytosis	secrete IGF-2, MMP2 activation	32,56,94,136,144
Rab31 (invasion)	metastatic breast	Plasma membrane, transgoli network	anterograde transport	reduced adhesion to extracellular matrix proteins	50
Rab35 (migration)	breast, brain	sorting and recycling endosome tubules	recycling	activate Rho GTPases, actin dependent protrusions, increase recycling rate	3,28,157

Table 1: Papers demonstrating some of the Rab GTPases including their function in the promotion of cell invasion of tumor cells.

1.4.3. The Role of Rab GTPases in Cell Invasion

Cancer cells attain an array of invasive and malignant phenotypes which are tumor and/or site specific⁴⁰ and involve a variety of Rab proteins that have been implicated in the transition to metastasis where tumor cells become invasive and penetrate the basal cell membrane and the ECM. These tumor cells secrete an array of proteases such as MMPs, induce the compromised basement membrane to further release more proteases such as collagenase and growth factors, thereby allowing cancer cells to breach the basal membrane and migrate to distal sites. For example, it has been ascertained that Rab8 facilitates cell invasiveness in breast cancer cells at peripheral invasive structures¹⁶.

Further evidence of Rab7 as possible tumor and metastasis suppressor was illustrated by Steffan et al¹²⁵. Silencing of Rab7 expression has a diminutive effect on invasion and when Rab7 was present, lysosomes were directed away from cell surface, thereby decreasing protease secretion and metastatic capacity of prostate cancer cells¹²⁵.

Rab7 has also been associated with MT1-MMP secretion and the promotion of cell migration and invasion, where the dominant-negative mutant of Rab7 impairs both processes¹⁴⁰. On the other hand, early stage melanomas promoted tumor progression via Rab7 regulation of the lysosomal pathway, where high expression of Rab7 was an indicator of the high metastatic risk (increased proliferative and invasive outcome) in patients⁴. The contrary role of Rab7 may be attributed to cancer type, stage and specific growth factor stimulus.

Under hypoxic conditions within the tumor microenvironment, Rab11 maintained the stabilization of microtubules via the inactivation of GSK3-B and possible down

regulation of E-cadherin, which led to an increase of cell invasion of breast cancer cells^{150,156}.

Caswell and colleagues established that Rab25 interaction with $\alpha 5\beta 1$ integrin was essential in ovarian epithelial tumor cell invasion into a 3 dimensional (3D) fibronectin-enriched ECM and may additionally regulate MMP-dependent regulation^{16,20}. Rab coupling protein (RCP), an effector of Rab11 and Rab25, acts as an oncogene as it promotes breast cancer transformation¹⁵⁴. It is also purported that the invasive potential of cancer cells is augmented in RCP mediated recycling and signaling of the EGFR and integrin, downstream of a mutant form of p53⁹⁶.

Poor prognosis in invasive breast cancers are associated with Rab27 a and b isoforms, which are secretory GTPases that control vesicle exocytosis and secrete critical proinvasive growth regulators into the tumor microenvironment. Inhibition of Rab27 activity reduced tumor growth and dissemination of lung carcinoma in response to neutrophil mobilization that were in turn, dependent on Rab27 exosomal secretions¹³.

In primary hepatocellular carcinoma and lymph node metastasis, Rab27b is over-expressed³², and in ER+ breast cancer, Rab27b activates MMP2 through heat shock protein (Hsp) 90a secretion and stimulate invasion of breast cancer cells^{56,94}. Rab27a is involved in the exocytosis of endocrine cells and conferred an invasive and metastatic phenotype by inducing the secretion of IGF-2 in breast cancer cells¹³⁶.

The rate of IGF-2 secretion by Rab27 influenced the expression other growth factors such as VEGF into the tumor microenvironment, as well as MMPs, such as MMP-9, cathepsin D and the cyclin dependent kinases (CDK16)¹³⁶. It was also suggested that

Rab27 may act as an oncogene given its regulatory role of invasive growth and metastasis in breast cancer cells and association with poor prognosis in humans⁵⁶.

Like a few other Rab GTPases, such as Rab25, Rab27 have dual roles in tumorigenesis and can be said to be context dependent. While the studies outlined indicate a pro-tumorigenic and metastatic role of Rab27, a study by Li et al indicated that Rab27 may possess more anti-tumor characteristics and demonstrated in a mouse model *in vivo*, that immunization of exosomes derived from Rab27a-overexpressing cells suppressed tumor growth. Analysis of the mice spleens also revealed high levels of type 1 cytokines, interleukin-2 (IL2) and IFN- γ , in response to Rab27 generated exosomes. These cytokines are crucial in the regulation of anti-tumor immunity⁷⁹.

Rab23 also influences cell invasion in two distinct tumor types within gastric cancer, where silencing of Rab23 abrogated such activity⁶⁰. On the other hand, Rab31 overexpression engendered a shift from a highly invasive capacity to a less invasive and highly proliferative phenotype in an experimental mouse model⁵⁰.

Rab4 has also been implicated in the secretion of procathepsin-L, another significant protease required in the modulation of the tumor microenvironment where suppression of Rab4 expression led to a reduction of tumor mass.⁹ Growth factor stimulated breast cancer cells overexpressing Rab5a affected Rab4 and RABENOSYN-5-dependent endo/exocytic cycles in the ferrying of the matrix protease, MT1-MMP and $\beta 3$ integrin⁴³. This trafficking cycle produced a chemotactic dependent invasive and proteolytic mesenchymal response in breast carcinoma cells *in vitro* and *in vivo*⁴³. Under hypoxic conditions, increased cell invasion of tumor cells was also mediated by Rab4 dependent

recycling and translocation of furin, which interacts with the cytoskeletal protein filamin A at the cell surface.⁷

1.5. Conclusion

As the role of growth factor driven endocytosis in metastasis is slowly being characterized, part of the struggle that currently exists involves the correct identification of the crucial endocytic molecules that may be potential therapeutic targets in cancer treatment. These may include but not limited to Rab5, and its effectors the GEFs such as Rab interactor 1 (RIN1) and GTPase Activating Proteins (GAPs). As outlined lined above, there is a paucity of research specifically regarding the relationship between Rab GTPases and growth factors, particularly IGF-1, in cancer cell migration and invasion.

Whilst most Rabs are currently not considered to be oncogenic, there is growing evidence that may suggest otherwise. Endocytosis is an indispensable signaling mechanism in growth factor induced signaling, it is worth considering the early factors such as Rab5 that exert a great influence on intracellular trafficking and also as a coordinator in the crosstalk amongst signaling pathways in the propagating and promoting metastasis (Fig 2).

Further description of Rab function in cancer cell metastasis would be beneficial as these Rabs can be prospective biomarkers in determining cancer stage and outcome prognosis. This opens the doors to the development of new therapeutic methods to target these necessary proteins such as siRNA or peptide manipulations that can selectively affect Rab effectors.

1.6. Thesis Overview

This project aims to examine the trafficking and signaling pathways involved in the progression from dormant tumor cells to aggressive invading cells via Rab5 GTPase activity and interaction with IGF-1R, and subsequent downstream signal transduction pathways. The hypothesis is that **Rab5 GTPase activity is one of the key elements in proliferation, migration and invasion of cells in breast cancer** mediated by the IGF-1R. Therefore, specific aims were designed to address this hypothesis.

For the Aims, it was:

(I) to **determine whether small Rab GTPases mediate phenotypic changes in the promotion of proliferation, migration and invasion activities in breast cancer cells in the presence of IGF-1**

(II) to **elucidate the signaling mechanism induced by IGF-1 via Rab5 function in modulating malignancy of breast cancer cells**

(III) to **examine the role of Rab5-Guanine Exchange Factors (GEFs) in proliferation and migration of breast cancer cells induced by IGF-1.**

This thesis is divided into 5 chapters.

Chapter 1 is the INTRODUCTION and literature review. The content of this chapter has been submitted for publishing and is currently under-review.

Chapter 2 elucidates the importance of Rab5, early endocytic membrane protein, as a potential mediator of tumorigenesis and metastasis. The Rab5 activity was shown to be a modulator of cells invasion and migration of non-aggressive breast cancer cells, MCF7 in response IGF-1 stimulation. Further analysis revealed that the guanine exchange factor

(GEF) that interacts with Rab5, Rin1, also promotes cells migration and invasion of tumor cells.

Chapter 3 examines the suppression of the aggressive potential of the highly metastatic breast cancer cell line, MDA-MB231 in response to IGF-1 and normal growth media. Over-expression of the inactive mutant form of Rab5, Rab5:S34N, impaired the invasion and migratory activity of aggressive breast cancer cells. Rin1 over-expression also mediated a highly aggressive phenotype, while the mutant abrogated cell invasion and migration.

Chapter 4 reveals the effect of inhibition of the IGF-1 receptor and early endocytic pathway on cell migration and invasion in MCF7 cells. This study also demonstrates that these inhibitors affect downstream signaling proteins and genes: the metalloproteinase MMP9, Rab5a and MAPK and PI3K signaling pathways

Chapter 5 summarizes the relevance and significance of the above research with future possibilities resulting from this study.

1.7. Figures

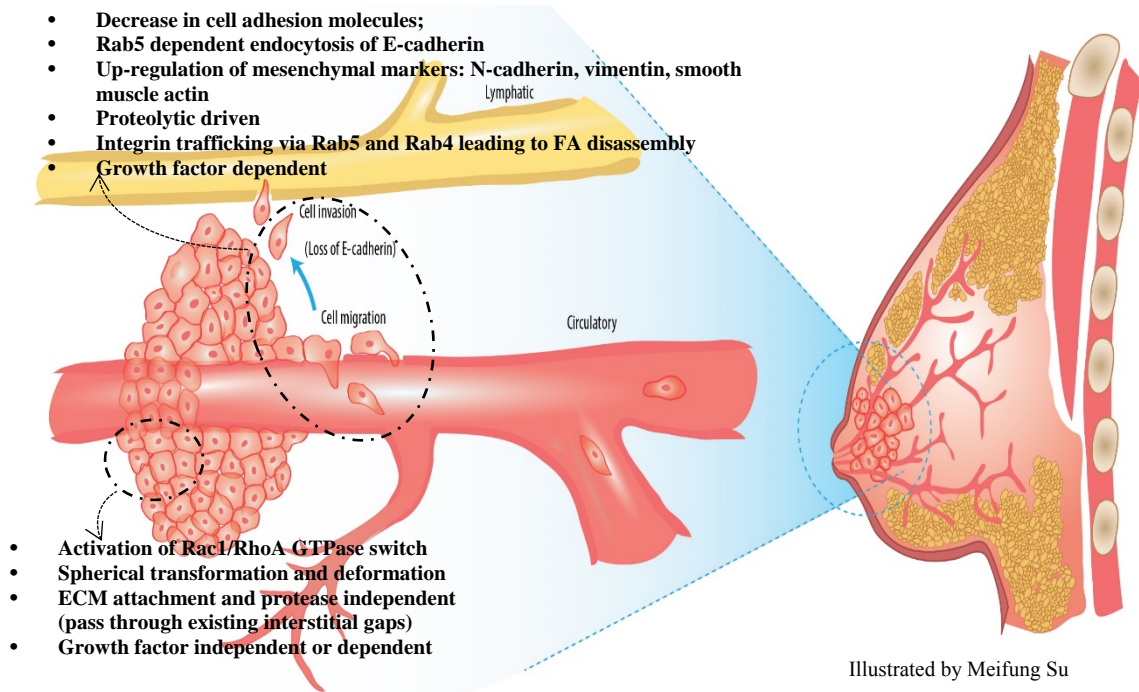


Figure 1. EMT-mediated invasion and amoeboid invasion in cancer metastasis. TAMs secrete cytokines and growth factors (EGF, TGF, HGF) and primary tumor cells respond to growth factor stimulation. These cells (dark in color) undergo EMT and invade and migrate into blood or lymphatic circulation. Some tumor cells may invade via AT by morphological conformation to spheroids and pass through pre-existing gaps in ECM. (EMT: epithelial-mesenchymal transition, MAT: mesenchymal-amoeboid transition, AT: amoeboid transition, TAMs: tumor activated macrophages)

IGF-1R as a possible target in tumor progression and metastasis

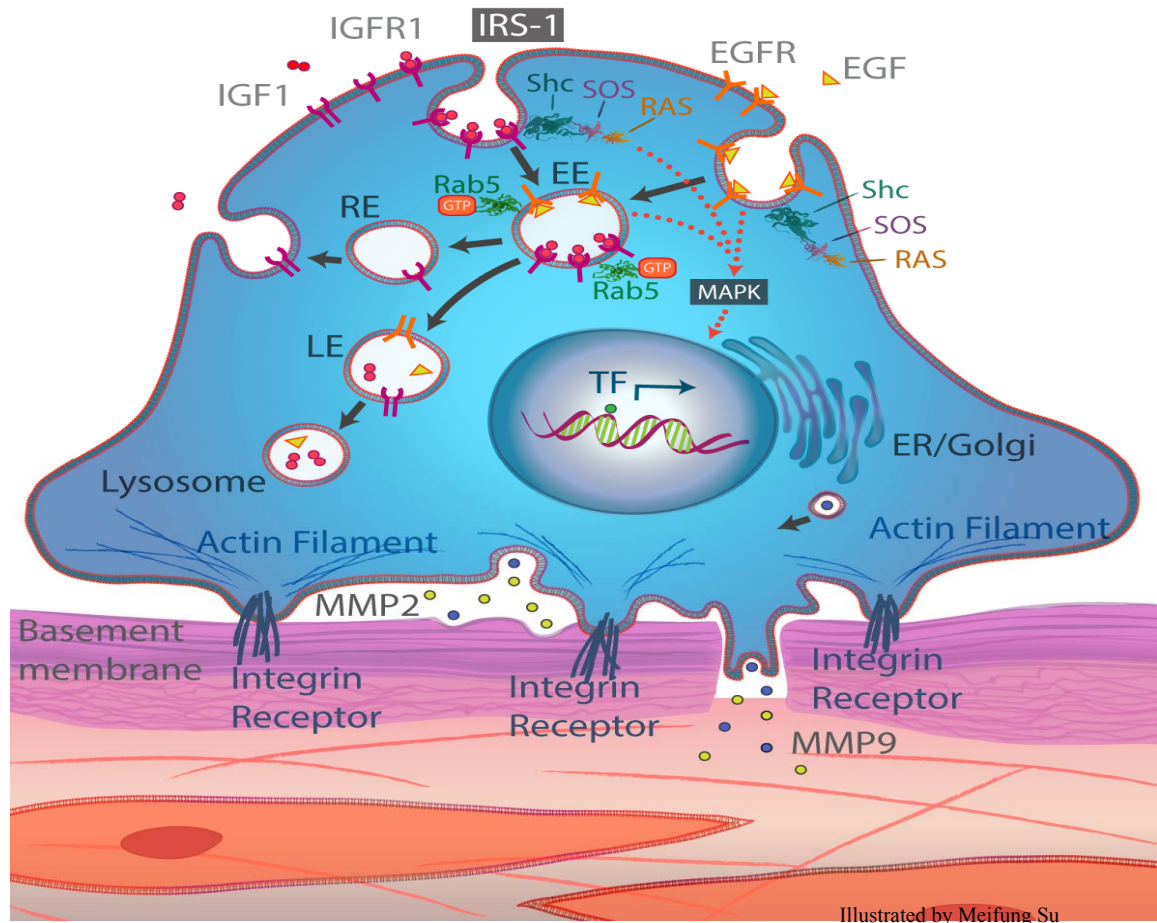


Figure 2. Illustration of the possible role IGF-1 plays in mediating metastasis in breast cancer cells. IGF-1 ligand binds to IGF-1 receptor. The activated receptor is internalized; Rab5 is recruited leading to the formation of the early endosome and subsequent launching of the downstream endocytic machinery and MAPK pathway. The IGF-1R is later shuttled back to the plasma membrane via the recycling endosome (RE), or to the lysosome through the late endosome (LE). It is known that IGF-1 can also lead to the transactivation of the EGFR¹¹³, internalization of the EGFR and subsequent degradation of the receptor. Activation of the MAPK pathway led to the transcription and secretion of metalloproteinases such as MMP2 and MMP9, degradation of the basement membrane, actin polymerization and reorganization of integrin receptors.

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CHAPTER 2.

Insulin-like Growth Factor-1 induces Migration and Invasion via Rab5 Activation

This chapter demonstrates the atypical non-invasive breast tumor cells (MCF7) acquire an aggressive phenotype, thereby becoming more proliferative, motile and invasive in response to IGF-1. Most importantly, early endocytosis of the IGF-1R regulated by Rab5 and its GEF RIN1 is implicated in this transition of breast cancer cells.

2.1 Abstract

Cell invasion, proliferation and migration are classic indicators of metastasis. Growth factors, while important for normal cellular processes, are also pro-metastatic. In this study, it was determined that invasion and migration in non-invasive MCF7 cells was regulated by growth factors via Rab GTPases. It was also indicated that the invasive and migratory properties of breast cancer MCF7 cells upon growth factor stimulation were abrogated in cell lines that only expressed the inactive (GDP-bound) form of Rab5 irrespective of growth factor stimulation. The invasive potential of breast cancer MCF7 cell lines expressing the Rab5WT and active (GTP-bound) form of Rab5 mutant (Rab5:Q79L) were noticeably greater when exposed to growth factors. In addition, we observed that Rab5 was activated in cells incubated with IGF-1 in a time and concentration dependent manner. Therefore, it can be concluded that Rab5 activation plays a crucial role in cell invasion, proliferation, migration particularly in response to growth factor stimulus.

2.2 Introduction

Cell migration and invasion are key components of metastasis where the atypical epithelial cells have acquired phenotypic changes that mediate the spread of the cancer to

distant tissues and organs. These processes are highly regulated and controlled by the expression and localization of specific cell surface receptors, such as integrin receptor, EGFR, IGFR; the reorganization of cytoskeletal elements; and the directed trafficking of molecules required for cell signaling and adhesion^{1,39,49}. Therefore it is unsurprising that cancer cells hijack and utilize these important cellular and biological functions ensuring the malignancy and spread of the disease to distal sites.

Trafficking of signaling molecules and ligand growth factors are regulated by the Rab family of proteins. These small monomeric GTPases play important regulatory roles in vesicle formation and transportation from plasma membrane to various organelles within the cell through the recruitment of small factors that include phosphatases, kinases and docking , adaptor proteins as well as actin filaments and microtubules in cell migration and mitosis⁵⁰.

Given the myriad of functions that Rab proteins regulate, it is unsurprising that there is a marked interest in Rab GTPase function and expression in tumor progression. The Rab proteins have been specifically linked to the deregulation of several signaling pathways in several cancers and also been implicated in the promotion of abnormal migratory and invasion of epithelial cells in tumor progression^{55,58,63}. For example, Rab25 is purported to be an oncogene given its role in advanced stages of ovarian and breast cancers and is linked to an increase in malignancy and migration of the epithelial cells by modulating cell proliferation (both anchorage dependent and independent cells), trafficking of integrin and suppressing apoptosis⁶. The silencing of Rab35 inhibited cell migration in

MCF7 breast cancer cells⁶⁷, while Rab11 and Rab27 facilitate an invasive phenotype and poor prognosis in breast carcinoma^{15,62,64}.

Rab5, a GTPase essential for early endosomal formation and trafficking, is involved in lamellipodia formation and actin remodeling. These physiological changes are in response to growth factor stimulation, the recruitment of Rac1 guanine-nucleotide-exchange factor (GEF) Tiam1 to early endosomes^{36,47} and these changes may be attributed to receptor tyrosine kinase (RTK) signaling and subsequent activation of Ras and Rab5 GAPs^{4,27}. Rab5a is expressed differentially in cancer cells where Yu et al. identified Rab5a as a potential major factor in the transformation of tumor cells in human lung adenocarcinoma, to an aggressive phenotype where overexpression of Rab5a was associated with increasing metastatic capacity⁶³.

Insulin Growth Factor -1 Receptor, a RTK, is involved in cell survival and proliferation of both normal and malignant phenotypes⁶⁰. Insulin Growth Factor signaling is initiated by the binding of the IGF-1 or IGF-2 ligand to the IGF-1R/ IGF-2R and the subsequent autophosphorylation of tyrosine residues of specific cytosolic kinase domains. The activated receptor transduces this phosphorylation activity to IRS and Shc adaptor proteins, and starts a signaling cascade that involves the MAPK and PI3K intracellular signaling pathways that regulate cell survival and proliferation. RTKs can be internalized through two principal pathways: clathrin coated pits or caveolae. The IGF-1R is internalized via both pathways⁷ and this may have a significant pathological effect in tumor progression.

The inherent relationship between Rab5 and IGF-1 in metastasis is more than implied despite the paucity of data establishing a clear link. As it currently stands, research have both alluded to IGF-1 involvement in endocytic pathway and the consequent activation of down signaling molecules ^{16,42}. The present study investigated Rab5 activity in response to IGFR activation in breast cancer.

Here, it has been determined that Rab5 expression was closely related with increased cell migration and invasion of MCF7 breast cancer cells. Furthermore, the guanine exchange factor, the Rab5 interfering 1 (RIN1), also induce breast metastasis through the enhanced endocytosis of the IGF-1R.

2.3 Materials and Methods

2.3.1 Cell Culture and Materials:

The MCF7 cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (w/v) fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml of streptomycin. The antibodies for EEA-1 and Rab5 were purchased from Cell Signaling Technology (Beverly, MA). RIN1 antibodies were from Abcam Inc. and secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). IGF-1 was purchased from Novus Biologicals (Littleton, CO). Other chemicals were obtained from Sigma unless otherwise stated.

2.3.2 Construction of recombinant pMX-puro retroviruses and cell lines

As previously described, the cDNAs of Rab5 and RIN1 were subcloned into the pMX-puro vector ³. Transfection of 60% confluent PhoA cells was performed using Fugene

(Roche) and the virus harvested after 48hrs post-transfection. MCF7 cell line was infected with the retrovirus tagged with green fluorescent protein (GFP) and Rab5, RIN1 and control cell lines were generated and selected after 72 hrs.

2.3.3 EEA1 pull-down assay

The MCF7 cells were lysed using a lysis buffer containing 20 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM DTT, 5 mM MgCl₂, 5% (v/v) glycerol and 1% (v/v) Triton-X-100 supplemented with 1 mM PMSF. Lysates (1 ml) were then incubated with 100 µl of glutathione beads containing ~10 µg of GST-EEA1 at 4°C while rocking for 1 hr. After incubation, the beads were washed three times using the lysis buffer. The pull-downs were subjected to SDS-PAGE and analyzed by immunoblotting using an anti-Rab5 antibody.

2.3.4 Cell Migration and Invasion Assays

Migration of MCF7 cell lines was measured using Transwell inserts (8 µm pores, Trevigen, MD). Cells suspended in serum-free and phenol free DMEM and applied to the upper chamber. Serum and phenol free media with or without IGF-1 added to the lower chamber and the plate incubated at 37°C for 24hrs. Non-migrated cells in the upper well were removed and the membranes were fixed and stained using 0.01% crystal violet. The membranes were either photographed or counted under a light microscope (×100) or the dye extracted and the absorbance reading obtained using a spectrophotometer.

For the Matrigel invasion assay, transwell inserts coated with 0.5 mg/ml Matrigel (BD Biosciences, MA) were used. Cells (10⁵ per well) suspended in serum-free and phenol

free DMEM were seeded onto the upper chambers of the precoated transwells. Serum and phenol free media with or without IGF-1 added to the lower chamber and the plate incubated at 37°C for 24hrs. Subsequently, the membranes were fixed, stained, and photographed as above or absorbance readings acquired.

2.3.5 Cell Proliferation Assay

The MCF7 cell lines expressing Rab5 WT, the inactive form of Rab5 (Rab5:S34N), the active form Rab5 (Rab5:Q79L), RIN1 and its isoforms, RIN2 and RIN3, and RIN1:R94A mutant or vector clone were seeded in 96-well plates at a density of 25×10^3 cells/well in 100 ml medium and incubated overnight. Cells were then serum starved for 24hrs followed by treatment serum and phenol red free DMEM with or without IGF-1 for 48 hrs. After, the MTT solution (5 mg/ml) was added to the plates and the cells incubated at 37 °C for 4 hrs. The formazan, derived from MTT by living cells, was dissolved in 10% SDS (150 ml per well), and the absorbance was measured at 595 nm. All MTT experiments were performed in triplicate and repeated at least 3 times.

2.3.6. Anchorage-Independent Growth Assay

Briefly, 1×10^3 cells of each cell line were suspended in 300µl of Dulbecco's Modified Eagle's medium containing 10% calf serum and 0.35% SeaPlaque low-melting temperature-agarose. These cells were plated over a 300µl layer of solidified Dulbecco's Modified Eagle's medium containing 10% calf serum and 0.6% agarose, and cells were allowed to settle to the interface between these layers at 37 °C. After 20 min, the plates were allowed to harden at room temperature for 30 min before returning to 37 °C. 100µl

of vehicle control or IGF-1 was added to wells and the plate incubated at 37 °C. The plates were fed every 2–3 days. After 12-14 days, colonies were photographed under low magnification (x10).

2.3.7 RNAi constructs and transfection

The RNAi directed against: mouse RIN1 [5'-UUAUACAUUUGCUUCACACCUAAGC-3'], was designed and synthesized by Ambion (Austin, TX). A scrambled RNAi was designed as a control [5'-CACCUGAAUCCGUGGUUCAA-3']. Prior to RNAi transfection, MCF7 cells were plated in growth medium without antibiotics at 30-50% confluency. RNAi (20 nM final concentration) transfection was performed using Lipofectamine-2000 (Invitrogen, Carlsbad, CA), as specified by Invitrogen. After transfection, cells were used either for cell invasion and migration assays.

2.3.8 Statistical Analysis

All samples in this study were analyzed in duplicate and each experiment was repeated three times. Values represent the mean \pm SEM of three independent experiments. To compare two groups, student's t test was utilized. A $p < 0.05$ was considered as statistically significant.

2.4 Results

2.4.1 IGF-1 stimulation triggers the endocytic pathway by upregulating Rab5 activity.

It is known that Rab5 is a key molecule in insulin and growth factor endocytosis and signaling^{3,5,16,22}. Furthermore, IGF-1 stimulation mediated the loss of surface IGF-1R and formation of Rab11 endosomes in glial cells⁴² indicating the role of the endocytic pathway in IGF-1 signaling. Given that Rab5 is required for early endocytic activity, the possible role of Rab5 in IGF-1 signaling was investigated in breast cancer cells, by preparing a stable cell line using a retroviral system in MCF7 cells. As described in the Materials and Methods, the cells were infected with a retrovirus encoding GFP-Rab5 constructs. Stable cell lines over-expressing Rab5 were stimulated with a physiological concentration of IGF-1. Rab5 activation upon IGF-1 stimulation was examined by precipitating the active or GTP-bound form of Rab5 with GST-EEA1 domain. After 5 mins of growth factor stimulation, the amount of GTP-bound form of Rab5 in cells expressing Rab5WT was 5 fold compared to control (Figure 3). The data show that IGF-1 stimulation promotes the interaction between EEA1 and Rab5 of which the latter must be in the active-GTP form. EEA1, a downstream effector of Rab5 is required to tether the active GTP-Rab5 to the membrane and facilitate the fusion of the early endosome in the trafficking of the activated receptor (Figure 3)^{5,31,46,51}.

2.4.2 Increase in the proliferative and invasive capacity of IGF-1 stimulated tumor cells is dependent on Rab5 activity.

Prior data have shown cell proliferation is dependent on Insulin receptor (IR) activation via Rab5 activation in liver cancer cells¹⁶. Rab GTPases act as regulatory

molecular switches where they hydrolyze the active bound form of GTP to the inactive bound form thereby acting as molecular switches²³ as they cycle between the active GTP and inactive GDP bound forms²⁴. Early endosome formation and receptor internalization and trafficking is dependent on Rab5 activation³⁸. Therefore, it was necessary to show that non-aggressive breast tumor cells overexpressing Rab5:Q79L would augment cell proliferation because of the rapid IGFR endocytosis. Hence, it is unsurprising a significant increase in cell proliferation was observed in GFP and Rab5WT in the presence of IGF-1 breast cancer cells compared to vehicle control (Figure 4a). The cells expressing the constitutively active mutant form of Rab5, Rab5:Q79L, had approximately 4.5 fold increase in cell proliferation when compared to GFP alone while the inactive form GDP bound form, Rab5:S34N, had the opposite effect and inhibited growth. Considering IGF-1 increased the proliferative capacity of the MCF7 breast cancer cells, another experiment was designed to examine whether IGF-1 does indeed play a key role via Rab5 activity in cell migration and invasion of tumor cells.

Within the tumor microenvironment, degradation of the extracellular matrix (ECM) releases growth factors including IGF, and they incur a bi-directional response to tumor cells growth factor secretion^{24,61}. Both the migratory and invasive action of MCF7 cells overexpressing Rab5WT (figures 4b and 4c respectively) was augmented in the presence of IGF-1.

In cells overexpressing the constitutive inactive GDP bound form of Rab5, Rab5:S34N, was significantly abrogated in both the vehicle control and IGF-1 stimulated cells. Interestingly when the GTP bound form of Rab5, Rab5:Q79L, was overexpressed,

migration and invasion of the MCF7 cells were greater than the rates of control cells in the absence and presence of IGF-1.

The invasive potential of Rab5WT was pronounced when compared to those in the migration assay (4 fold compared to 2.5), considering the cells must transverse a barrier, specifically the basement membrane. This can be attributed to the degradation of the ECM by secretion of proteolytic enzymes by the cancer cells and the subsequent release of pro- migratory and invasive growth factors¹⁰ from the matrigel, which in turn stimulate the MCF7 cells.

2.4.3. IGF-1 stimulates Rab5 activation in breast cancer cells overexpressing Rab5 specific GEFs

The switch from the inactive GDP to active GTP state of GTPases are enabled by small proteins called guanine exchange factors (GEFs)³⁵. The Ras interfering (RIN) family of proteins has been recognized as the GEFs for Rab5 and each one (RIN1, RIN2, RIN3) interacted preferentially to inactivated Rab5⁴⁴. RIN1 is known to interact with the EGFR²² and IR¹⁷. The role of RIN1 in tumor progression is contrary at best, suggesting that RIN1 expression in cancer progression can be growth factor and tissue specific^{2,9,14}. The MCF7 cells were transformed using a retrovirus system and selected for RIN1, RIN2 and RIN3. If RIN proteins were a requirement for the active Rab5 form, the pull-down assay was performed to verify active Rab5 presence in cells overexpressing RIN1, RIN2 and RIN3 in response to IGF-1. Cells were incubated with IGF-1 for 5mins and the amount of active Rab5 generated was quantified using SDS-PAGE after precipitating with GST-EEA1 protein. IGF-1 led to more Rab5 activation in tumor cells over

expressing RIN1 and RIN3 compared to the vehicle control. Though there was some Rab-GTP bound form present in RIN2 expressing cells, it was significantly less in cells overexpressing RIN1 and RIN3.

2.4.4. IGF-1 elicits a strong proliferative, invasive and migratory response in tumor cells overexpressing Rab5 GEFs

On the basis of our results above, it was deduced that expression of Rab5 GEFs may also induce cell proliferation and migration in breast cancer cells in response to IGF-1. This activity is attributed to the GEF activity in switching Rab5 to the GTP bound form and promoting proliferation in the breast cancer cells. Using the Boyden chamber as described in the Materials and Methods, a 2-fold and 4-fold increase in cell migration was observed in RIN1 and RIN3 cell lines respectively (Figure 6a). While RIN2 engendered an increase in migration in the presence of IGF-1, it was not much of an alteration when compared to the control cell line. Both RIN1 and RIN3 also garnered an augmentation of cell invasion in both cell lines (Figure 6b), with RIN3 having less invasion of cancer cells compared to RIN1 and the GFP control. Like the Rab5:Q79L mutant cell line, RIN1 cancer cells behaved more aggressively even when IGF-1 was absent. Whether or not this is significant still needs to be ascertained but initially suggests that Rab5 aberration may be key in metastatic progression.

To further establish the function of RIN1 in mediating pro-invasive response to IGF-1 in breast cancer cells, RNAi suppression of RIN1 expression was performed and the invasive response quantified (figure 9c). Temporal silencing of RIN1 gene expression in breast cancer cells significantly inhibited invasion of the cancer cells.

2.4.5. IGF-1 fostered in vitro tumor formation in cancer cell and is dependent on Rab5 activation.

Tumor formation occurs pre- and post-metastasis where the initial tumor may acquire anchorage independence and secondary tumor formation or re-colonization may be both anchorage dependent or independent¹². It is also important to note that as cancer cells continue to feed and increase in size, cells on the perimeter are typically the first cells to undergo changes and gain aggressive and highly motile tendencies. Most importantly, it only takes one cell for a secondary tumor to develop.

Here, it was necessary to show that IGF-1 influences colony formation in breast tumor cells, particularly through Rab5 activity. Cells overexpressing Rab5WT formed larger and more colonies compared to the vehicle control (Figure 10a.) In cells with RIN1 overexpression (Figure 10b), it was surprising to observe that colony formation, while greater in number and more pronounced in size in response to growth factor stimulation. Colonies were also numerous and larger in the absence of IGF-1 when compared to the control and even RIN3.

2.5 Discussion

Cancer cells become highly motile and invasive in response to small alterations in the tumor microenvironment and hijack important cellular functions such as cell migration¹². In light of this behavior, endosomal trafficking in cancer, particularly in the propagation of metastasis, is a major point of interest in cancer research. There is increasing evidence that the early endocytic molecule, Rab5 GTPase, is a foremost player in cell motility/migration of normal and cancer cells as reviewed by Stupack et al.⁵⁸, and also

engaged in lamellipodia formation and actin remodeling in response to growth factor stimulation^{37,48}.

While Rab5 is expressed differentially in cancer cells, when overexpressed, it was identified as a potential modulator of tumor cell transformation to aggressive phenotypes, also indicative of its possible role in metastasis^{63,66}. Additionally, Rab5 GTPases has been characterized as an essential signaling molecule in response to growth factor stimulation^{3,5,16,22}.

Here, it was demonstrated Rab5 had a critical role in the early phases of metastasis, specifically affecting the migratory and invasive capacity of breast cancer cells. Yet it is not fully determined that IGF-1 signaling is dependent on endocytic trafficking and may spur the activation and recruitment of the active form of Rab5 from the cytosol to the membrane.

Circulating IGF-1 levels are higher in breast cancer patients compared with normal controls⁴⁰ while high IGF levels are associated with increased risk of breast cancer in pre-menopausal women¹³. IGF-1 ensures the survival and hyper-proliferative state of cancerous cells. IGF-1 modulates cell proliferation by affecting changes in the apoptotic signaling pathways. IGF-1R is found in all breast cancer cell lines regardless of estrogen receptor status⁸.

Notably, IGF-1 is known to stimulate the estrogen receptor (ER) in the absence of estrogen itself, thereby allowing for more crosstalk between the IGF-1R and ER, phosphorylation of the ER and increased synthesis of IGF-1R and ER transcripts^{19,29,52}. IGF-1R activation mediate and conformational change in Bad, a pro-apoptotic protein,

which inhibited its interaction with the Bcl (anti-apoptotic) proteins and the subsequent activation of the caspase chain thereby allowing transformed and pre-cancerous cells to escape apoptosis and survive^{11,28,30}.

Curiously, Rab5 has been shown to be a key regulator in caspase mediated signal transduction and facilitates increased motility and survival of cancer cells^{56,57}. This suggests that in general, RTK internalization via Rab5, may also result in the increase of caspase expression and sequestration of integrins to early endocytic compartments suppressing apoptosis of cancer cells.

In the present study, the proliferative effect of IGF-1 on breast tumor cells was clearly evident in the presence of the growth factor and positively correlated to Rab5 expression (Figure 5a.). This may be attributed to modulation of apoptotic factors such as caspase-8^{26,53,55} or down-stream activation of adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1 (APPL-1) of which the latter has implications in growth factor dependent Rab5 activation in ovarian cancer⁶⁶. Studies have intimated that IGF-1 exerts a strong mitogenic and anti-apoptotic effect on cells and bolsters the pro-metastatic property of cancer cells by reorganizing integrin receptors and modulating the E-cadherin/ B-catenin complex which in turn increases the migratory potential of the cells^{54,59}.

These intracellular changes of the E-cadherin/ B-catenin complex increase MMPs expression, disintegration of the ECM and release of growth factors and push tumor cells to a mesenchymal phenotype⁵⁹. Rab5 function in the migration and invasion of cells due to IGF-1 induction of metastasis suggests that Rab5 mediates the internalization of the

activated IGF-1R, either through a clathrin dependent or independent mechanism. Specifically, cell migration was dependent on active Rab5 translocation to the plasma membrane and formation of early vesicles as the inactive GDP bound form, Rab5:S34N, suppressed the migratory aptitude of cancer cells. A similar pattern was established when the invasive capacity of cancer cells was also investigated in response to IGF-1 stimulation. It was also noted that there was a strong migratory and invasive capacity of tumor cells in the cells overexpressing the active Rab5, Rab5:Q79L, irrespective to IGF-1 stimulation. During metastasis, invading cancer cells acquire an autonomous trait where they are less reliant on external growth factors and move in response to internal signaling events and secretions. As acknowledged earlier, IGF-1 regulates the pro-survival transduction pathway⁶⁰, and it has been demonstrated that internal forces unleashed in cancer cells may also be due to caspases, in a non-apoptotic role. This can potentiate migration and invasion in tumor cells and is reliant on Rab5 activation⁵⁸.

Rab5 activity status seems to be critical in facilitating IGF-1 induced breast cancer cell migration and invasion, yet some studies imply that Rab5 may instead be a tumor suppressor. In part, this may be attributed to overexpression of a few of the GEFs for Rab5, the RIN family of proteins. Specifically it has been suggested that RIN1 may be a breast cancer tumor suppressor gene^{14,34}. However, this study suggests otherwise, that over-expression of RIN1 and RIN3 fostered an increase in migration and invasion of the cancer, with RIN1 eliciting a slightly larger rise in the metastatic potential of the cells compared to RIN3. This also implies that RIN1 expression instead, may be a key prognostic marker of cancer metastasis and progression^{9,45}.

RIN1 is recognized as a regulator of Rab5 as it possesses three features needed in early endocytic signaling; Src Homology 2 (SH2), Rab5-GEF and the Ras binding domains¹⁸. Though not shown, RIN1 interaction with the IGF-1R via the –SH2 domain is a possible precursor for the activation of ERK and PI3K/Akt signaling pathways.

Due to the highly homologous relationship between IGF-1 and insulin and their receptors, it is therefore likely that RIN1 would also regulate IGF-1R internalization and promote migration and invasion of breast cancer cells²⁰. Furthermore, IGF-1 can also trans-activate other growth factor receptors like the EGFR and of which the latter has been described in studies characterizing the EGFR relationship with RIN1^{2,25,43}.

Hence, it can be deduced that this is another mechanism that IGF-1 influences cell migration, proliferation and invasion of cancer cells. In Figure 9c, RIN1 as a principal switch in cancer cell metastasis was substantiated when depleted RIN1 levels negated the tumor cell invasion alluding to two possible roles of RIN1 in growth actor signaling in metastasis:

1. Action on Rab5 leading to its active form;
2. Thus, there is direct Rab5 activation and interaction with the cystolic tail of the IGF-1R ensuring the internalization of receptor and subsequent activation of the intracellular signaling pathways.

Jozic et al. showed that RIN1 facilitated the fusion of the insulin receptor to the early endosome and was Rab5 dependent²¹. Other Rab5 effectors such as Rabaptin are known to interact with the platelet-derived growth factor beta receptor (PDGFbetaR) and led to deleterious myeloproliferative phenotype in mice³². Rabaptin also functions

synergistically with another adaptor for Rabex5 GEF to activate Rab5 and promotes early endosomal fusion⁶⁵ and receptor internalization⁴¹. Whether these Rab5 effectors are also involved in IGFR trafficking in tumor progression has yet to be investigated.

In summary, this study clearly shows that Rab5 and well as its GEFs, RIN1 and RIN3, are central players in metastasis of cancer cells especially in the trafficking of the activated IGFR. How Rab5 and RIN1 specifically regulates invasion in response to IGF-1 must still be investigated. Mendoza et al. suggested that Rab5 modulated focal adhesion kinase activity and matrix metalloproteinases (MMPs) in cancer cells³³, yet little information is available as to IGF-1 role in facilitating this intracellular response. Here, it is shown that the change to an aggressive and invasive phenotype of non-invasive tumor occurred through Rab5 and RIN1 recruitment and activation in trafficking of the IGF-1R upon IGF-1R stimulation.

2.6 Figures

Figure 3.

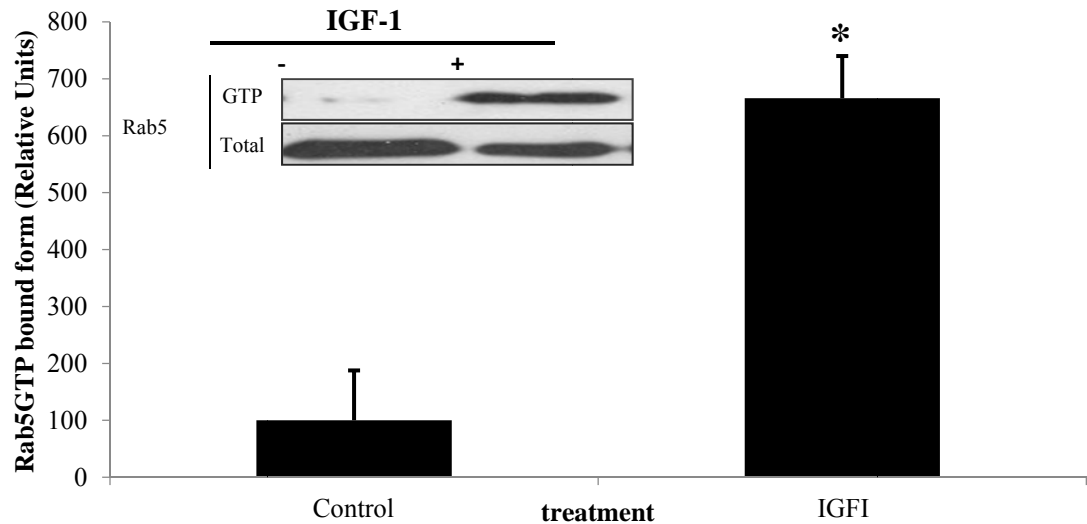
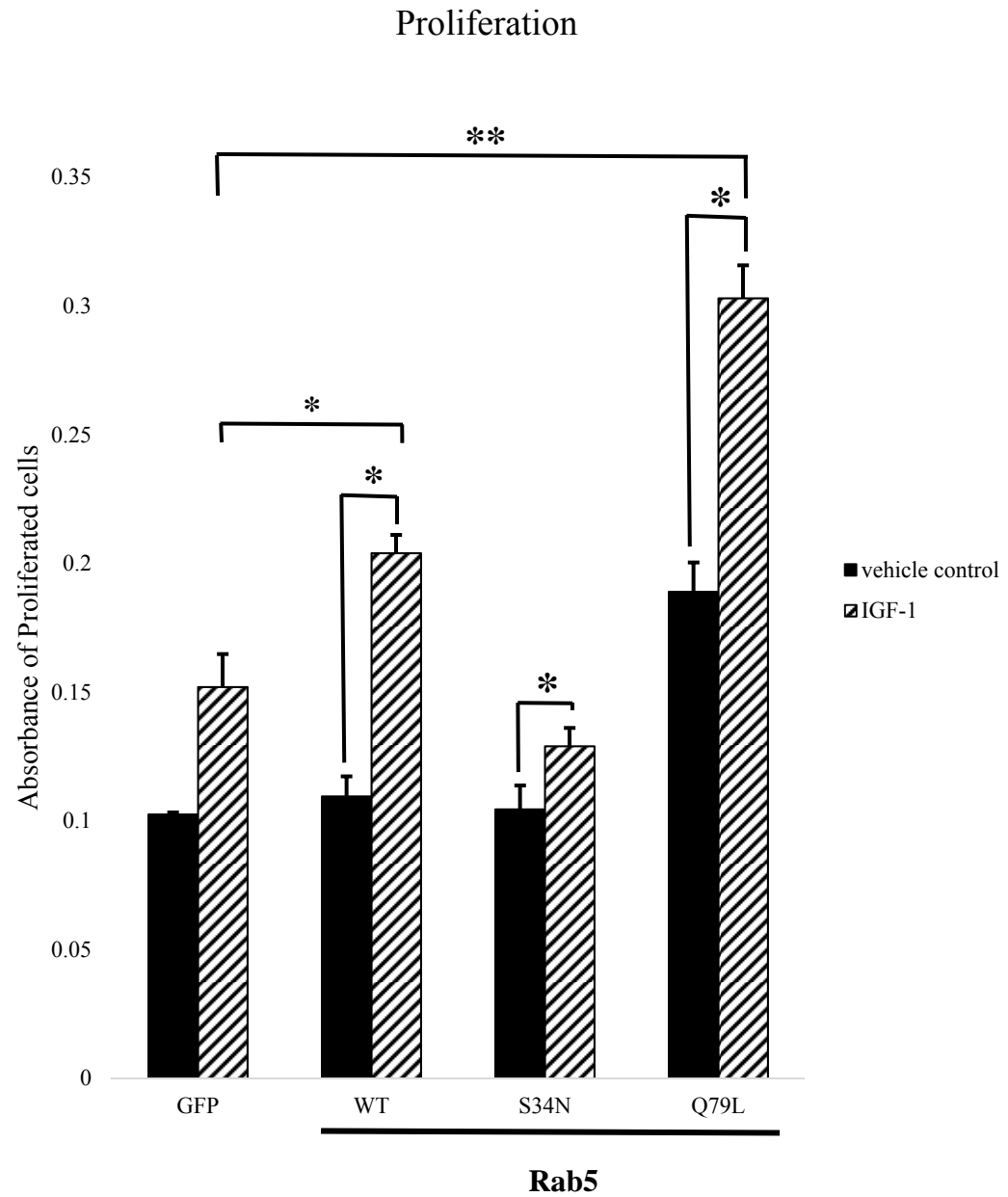


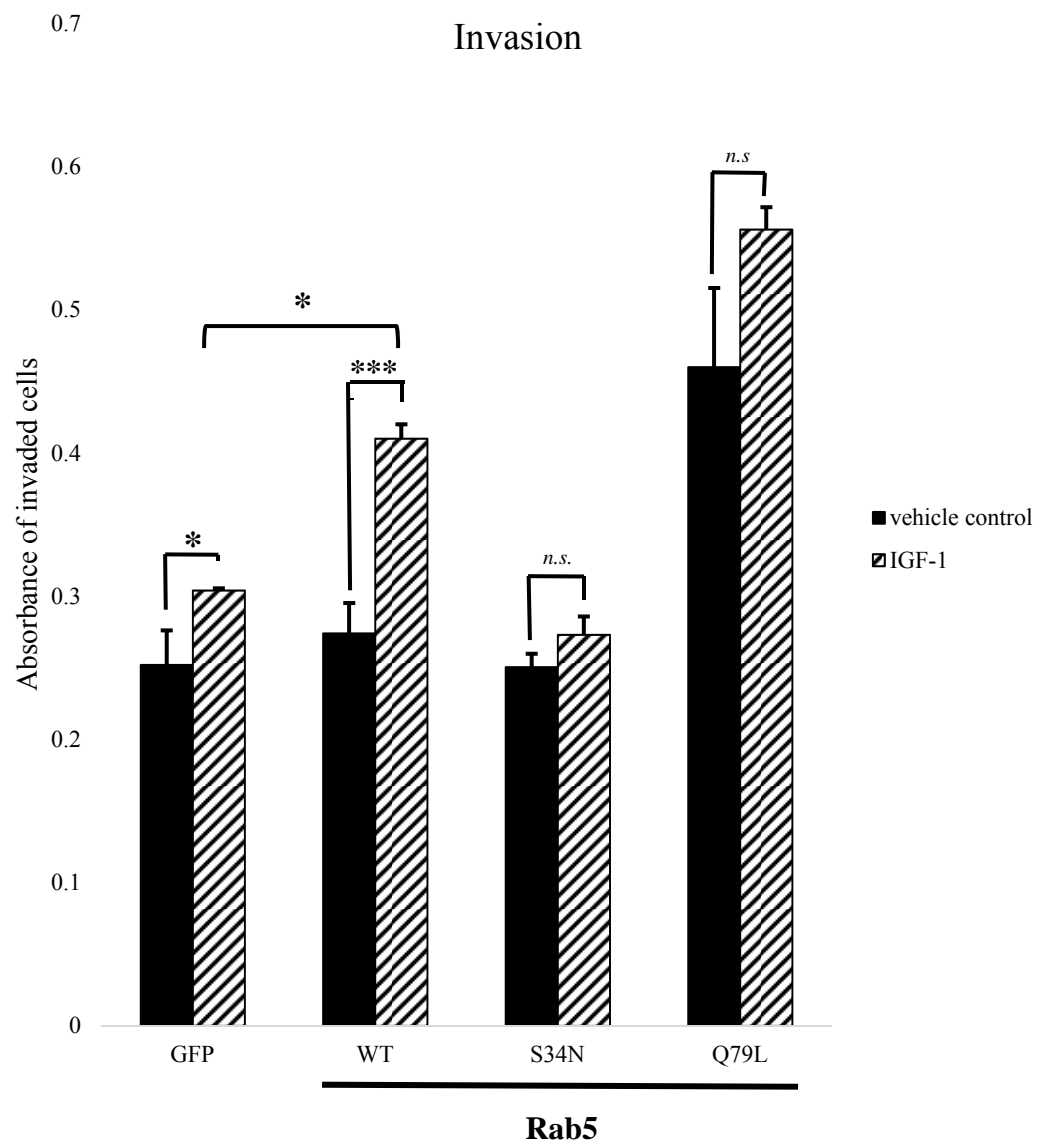
Figure 3. IGF-1 endocytosis is mediated by Rab5 protein expression in breast cancer cells. MCF7 cells expressing GFP-Rab5 was incubated in the absence or presence of IGF-1 at 37°C. After incubation, active or GTP-bound form of Rab5 was precipitated with GST-EEA1 domain at 4°C while rocking for 1 hr. After incubation, the beads were washed three times using the lysis buffer. The pull-downs were subjected to SDS-PAGE and analyzed by immunoblotting using an anti-Rab5 antibody. A representative immunoblot probed with anti-Rab5 or tubulin (loading control) is shown. Data represent the mean \pm SEM of three independent experiments. (*) represents statistically significant difference from control group ($P<0.05$).

Figure 4

(a)



(b)



(c)

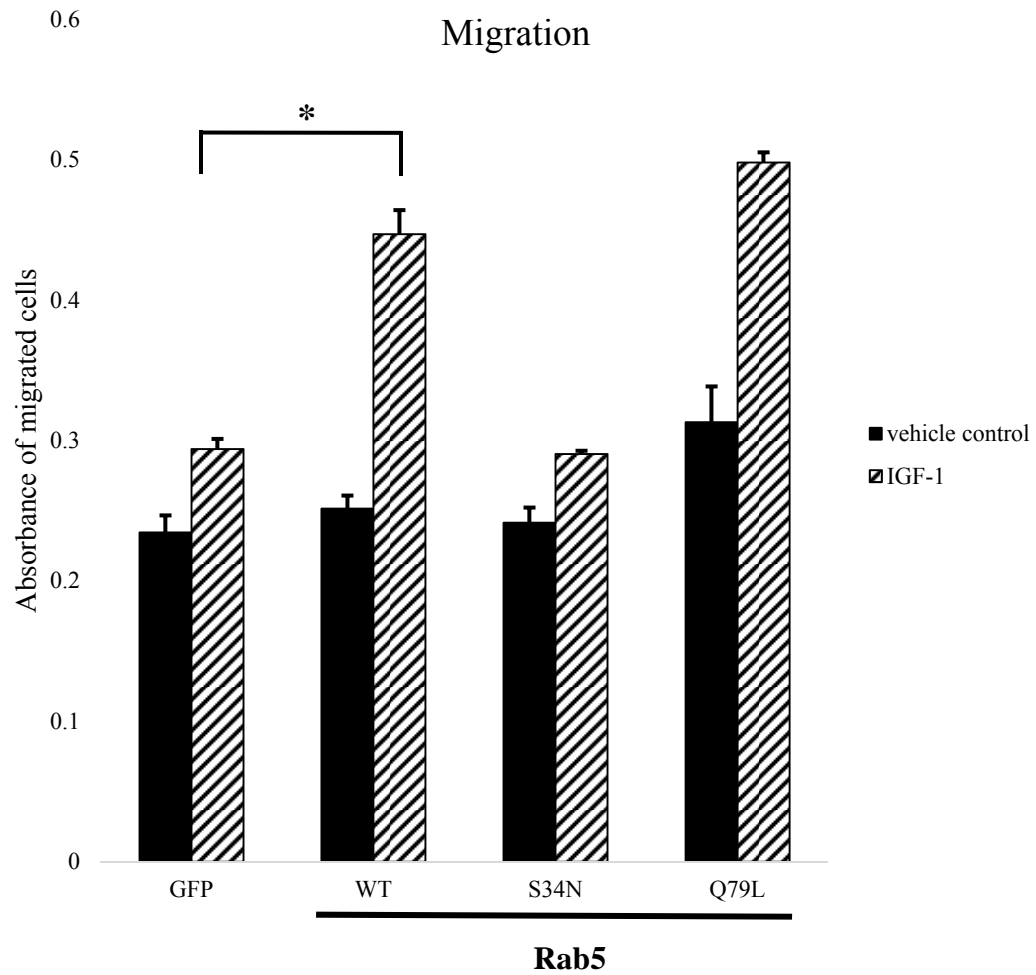


Figure 4. (a) IGF-1 enhances cell proliferation via Rab 5 activation. Cells expressing either GFP or GFP-Rab5:WT and its mutants were incubated in the absence or in the presence of IGF-1 in triplicate. Cell proliferation was assessed after 48hrs incubation

with vehicle or IGF-1 of cell culture, using the MTT proliferation assay (Sigma). Briefly after the incubation period, cells were added with 100 μ L of 5 mg/mL thiazolyl blue tetrazolium bromide (Sigma, St. Louis, MO) solution/well and were incubated further for 4hrs in a humidified atmosphere (37°C in 5% CO₂). The absorbance was measured at 560 nm in a microplate reader. Cell proliferation was expressed as the mean percentage absorbance compared to vehicle control \pm percentage SD for three independent experiments. In **Figures 4(b) and 4(c), IGF-1 mediates cell invasion and migration in MCF7 cells via Rab5 activation**. Cells were plated in a boyden chamber with or without basement extract membrane. Treatment groups were IGF-1 or vehicle control and cells were incubated at 37°C for 16h. Crystal violet staining was performed and the dye extracted and the OD measuring using a plate reader at 595nm. Cell migration and invasion absorbance reading were expressed as relative OD to control \pm SEM for three independent experiments where (*) denotes $p < 0.05$, (**) is $p < 0.01$ and (***) is $p < 0.005$.

Figure 5

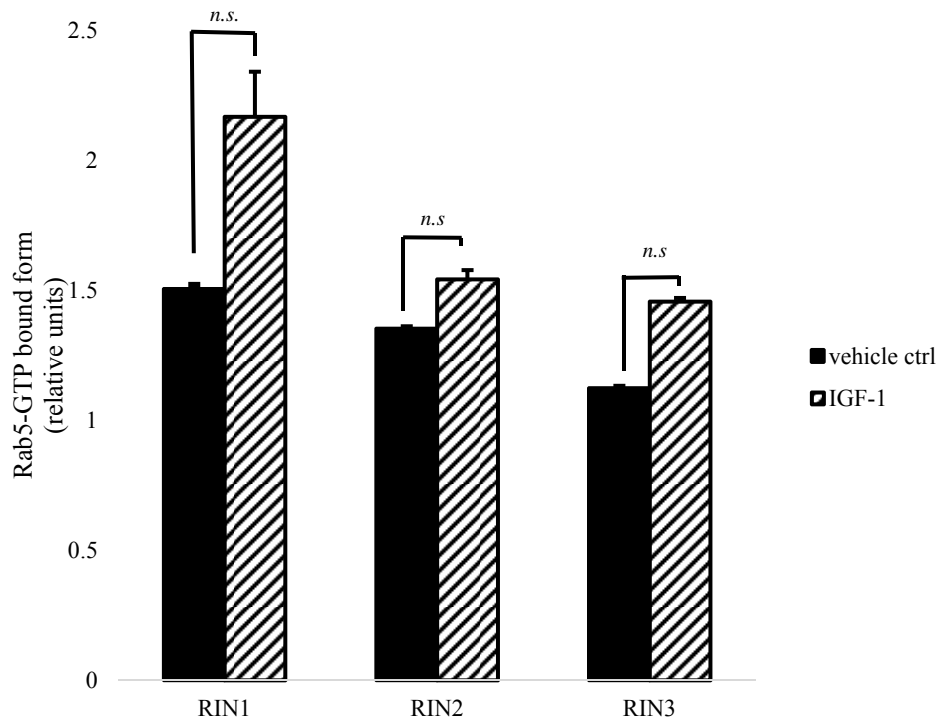
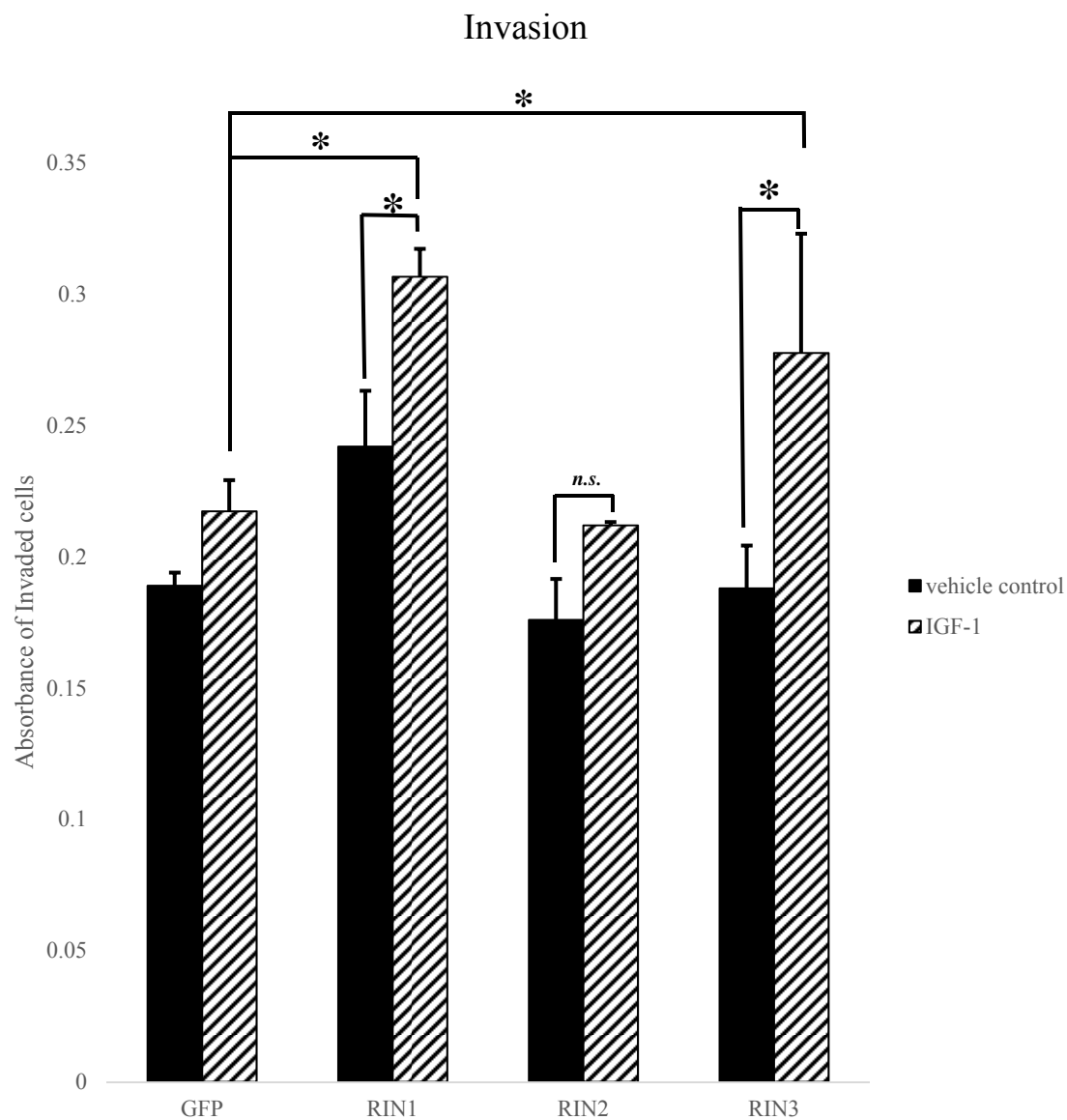


Figure 5. IGF-1 stimulates Rab5 activation in breast cancer cells overexpressing Rab5 specific GEFs MCF7 cells expressing the GEFs RIN1, RIN2, and RIN3 were stimulated with IGF-1 or vehicle control for 5 mins and assayed for active GTP-Rab5 as described in Materials and Methods. Data represent the mean \pm SEM of three

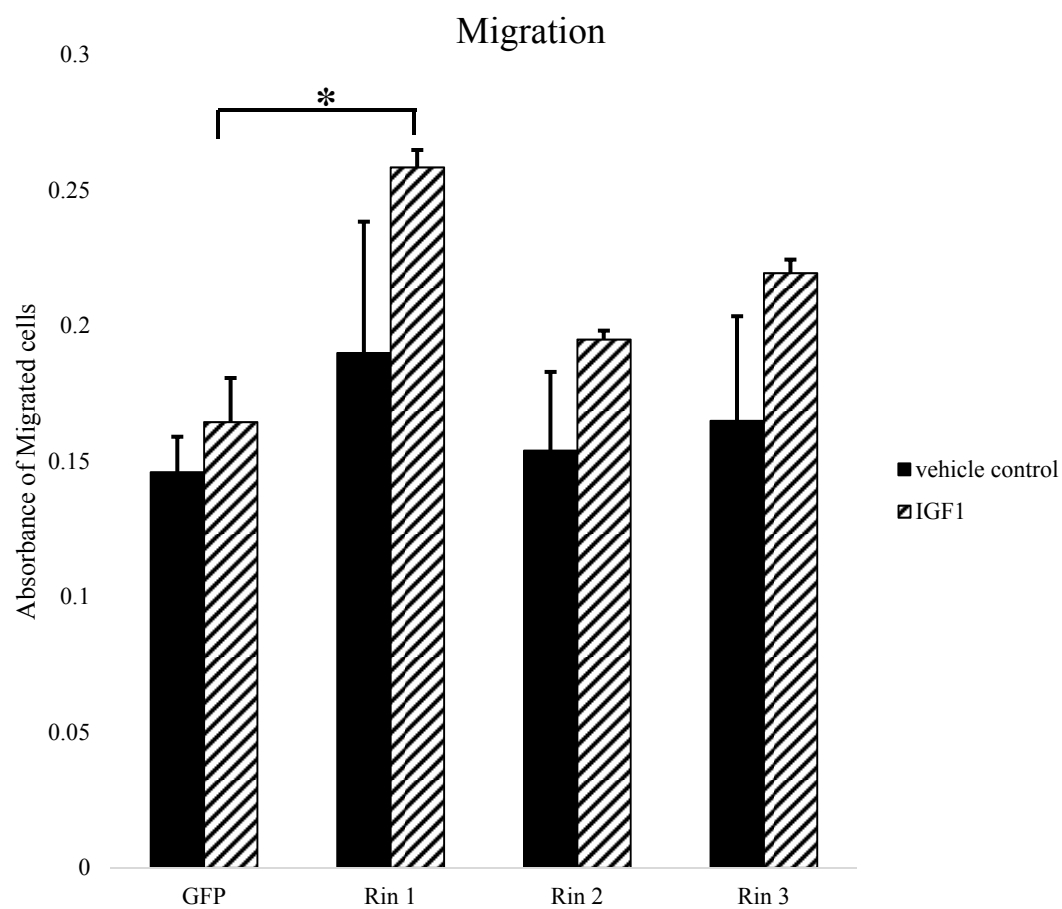
independent experiments. (*) represents statistically significant difference from control group ($P<0.05$); n.s. = no significance

Figure 6

(a)



(b)



(c)

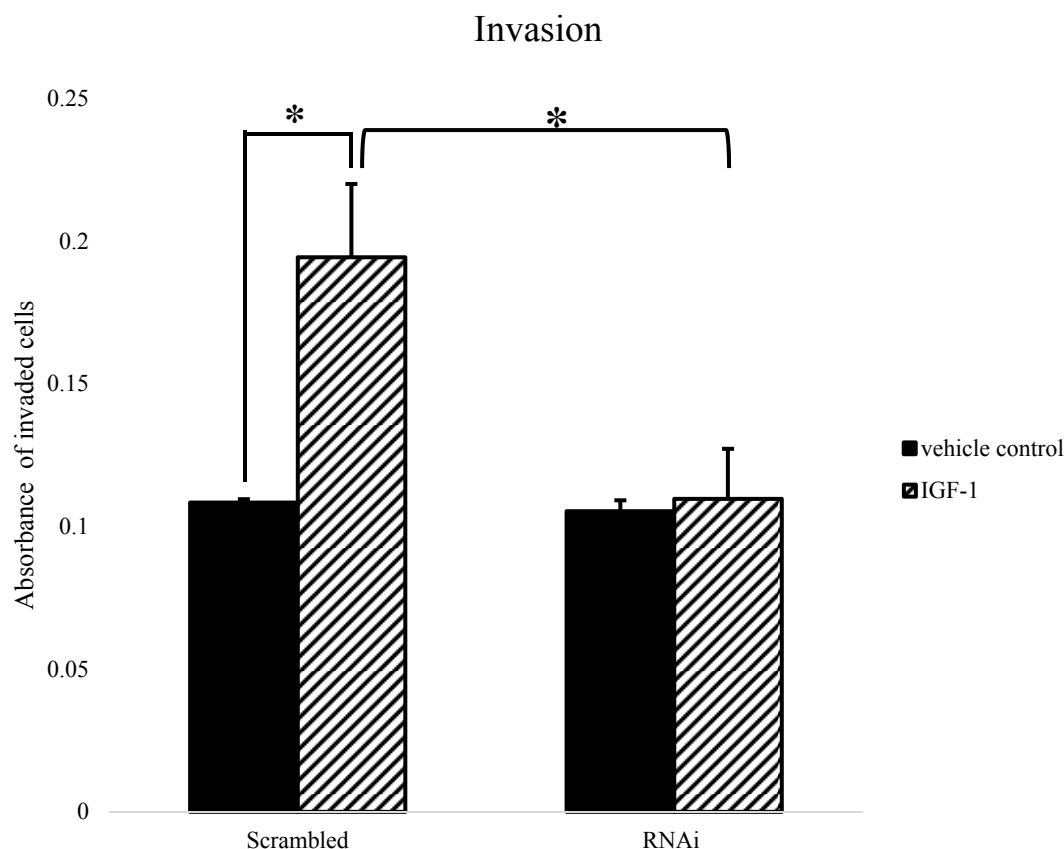
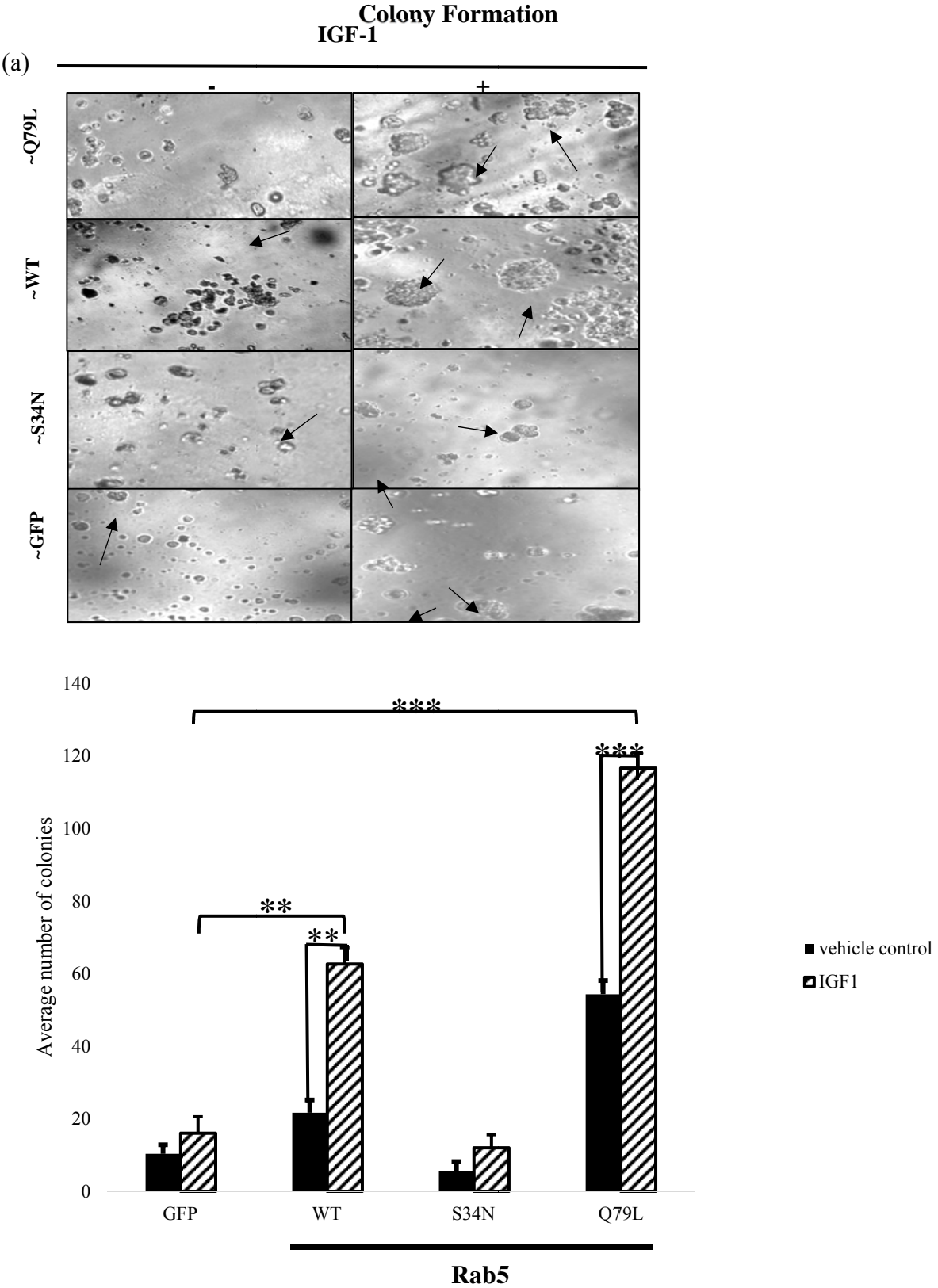


Figure 6. The Rab5 GEFs mediate cell invasion and migration in MCF7 cells via Rab5 activation. As previously outline in Figure 4, tumor cells expressing GFP and the Rab GEFs; RIN1 and RIN3. Cells were placed in a Boyden chamber with or without basement extract membrane (figures 6a and 6b respectively) Treatment groups were IGF-1 or vehicle control and cells were incubated ta 37°C for 16h. Crystal violet staining was performed and the dye extracted and the OD measuring using a plate reader at 595nm. Cell migration and invasion absorbance reading were expressed as relative OD to control \pm SEM for three independent experiments. (*) represents statistically significant difference from control group ($P < 0.05$)

Figure 7



(b)

IGF-1

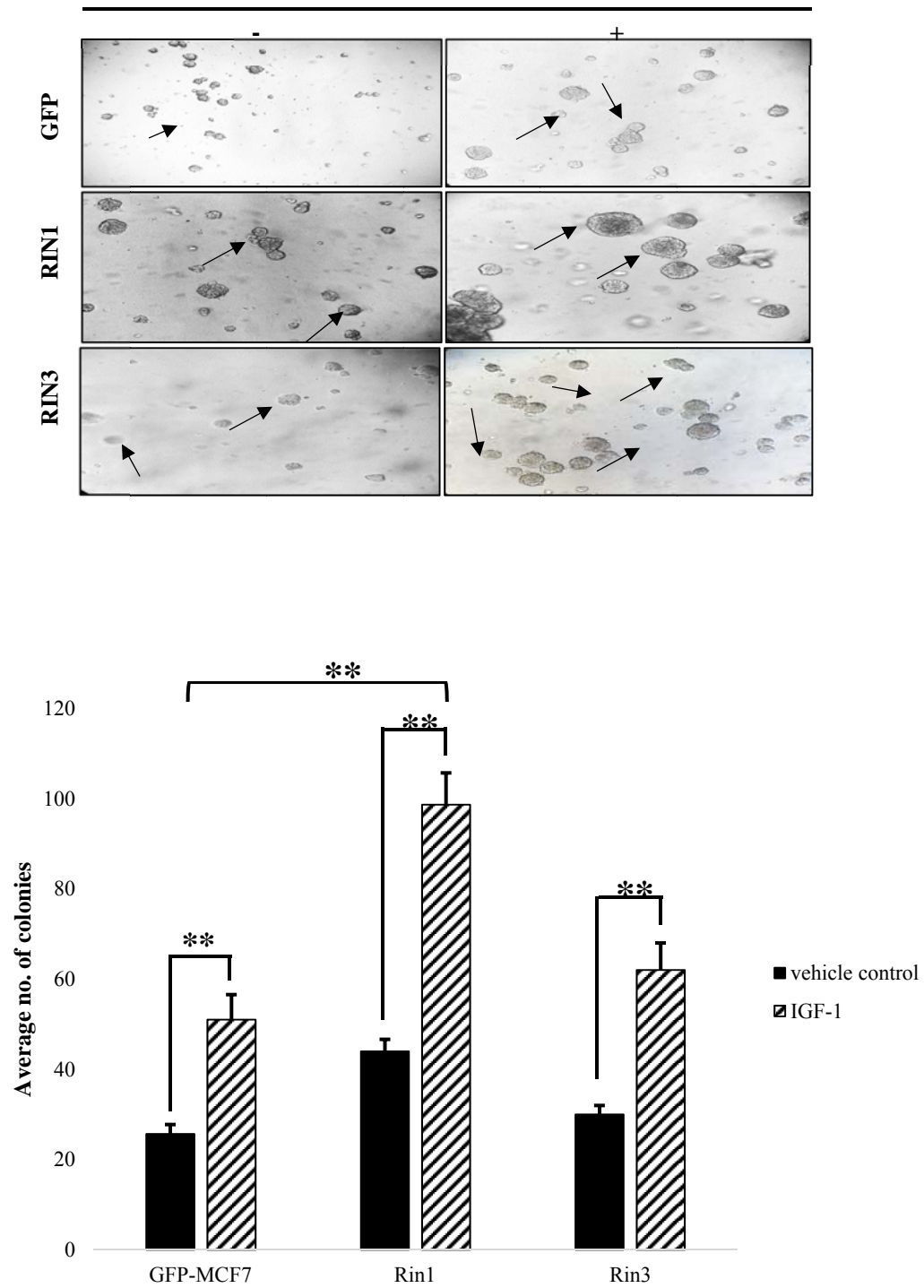


Figure 7. Rab5 and its GEFs promote colony formation in IGF1 stimulated breast cancer cells. Each cell line was plated over a 300ul layer of solidified Dulbecco's Modified Eagle's medium containing 10% calf serum and 0.6% agarose, and cells were allowed to settle to the interface between these layers at 37 °C. 100ul of vehicle control or IGF-1 was added to wells and the plate incubated at 37 °C. The plates were fed every 2–3 days. After 12-14 days, colonies were photographed under low magnification (x10). Each cell line was done in triplicate with data represent the mean \pm SEM of the three independent wells. (*) represents statistically significant difference from control group ($P < 0.05$); (**) is ($P < 0.01$) and (***) is ($P < 0.001$)

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CHAPTER 3.

Requirements of Rab5 Activity in Highly Invasive Breast Cancer Cell Lines.

This chapter sought to elucidate the functional role of Rab5 in facilitating cell migration and invasion in invasive breast cancer cells.

3.1 Abstract

Rab5 expression in cancer has been associated with diseases progression and prognosis. Growth factor-directed cell invasion and migration was dependent on Rab5 activation in non- invasive breast cancer cells. However, hardly any data are available regarding the role of Rab5 in invasive cells in the absence and presence of growth factors.

In this study, it is reported that the invasive and migratory properties of the triple negative highly invasive breast cancer cell line, MDA-MB-231, were abrogated in cells expressing the inactive (GDP-bound) form of Rab5 irrespective of growth factor stimulation, while the invasive potential of breast cancer cell lines expressing the Rab5:WT and the active form of Rab5 mutant (Rab5:Q79L) were noticeably greater. Rab:S34N significantly suppressed the migratory and invasive capacity of MDA-MB-231 cells. Interestingly, expression of RIN1, a Rab5 GEF, increases invasion and migration of MDA-MB-231 cells even in the absence of growth factors. In contrast, RIN1:R94A mutant partially mitigates both processes. Hence, it is reasonable to speculate that cell invasion and migration, a process that typically involves activation of IGF-1 receptor, can be intracellularly regulated without intervention or activation of growth factor receptors, but elicits a greater invasive response if growth factors are present.

3.2. Introduction

Over the last few years, Rab proteins have been specifically linked to the deregulation of several signaling pathways in a variety of cancers and also been implicated in the promotion of abnormal migratory and invasion of epithelial cells in tumor progression^{5,6,25}. Rab5, a member of this prolific Rab GTPase family, has generated a lot of interest in cancer development and progression given the myriad of cellular functions that the protein regulates. Rab5 is involved in vesicle formation, early endosome docking and fusion, translocation from plasma membrane to various organelles within the cell through the recruitment of small factors that include phosphatases, kinases, adaptor proteins as well as actin filaments and microtubules in cell migration and mitosis²⁹.

Endocytosis is an indispensable signaling mechanism in growth factor induced signaling, it is worth considering the early factors such as Rab5 that exert a great influence on intracellular trafficking and also as a coordinator in the crosstalk amongst signaling pathways in cancer pathogenesis. Within cancer biology, recent studies have pinpointed Rab proteins as a possible controller of the metastatic expression of cancer cells with influence on the degree of malignancy in lung and stomach cancer.

Based on the data derive from the first Aim, it was demonstrated that Rab5 activity drives the metastatic potential in non-invasive tumorigenic breast cancer cells. Whilst Rabs are currently not considered to be oncogenic, there is growing evidence that may suggest otherwise^{17,22,28,36,38,39}.

Insulin Growth Factor 1 (IGF-1) is pathologically critical in cancer development and progression. Epidemiological studies has implicated free IGF-1 serum levels as a risk factor for the development and progression of breast, prostate, colon, and lung cancer

^{11,23}. Davison et al. has shown that triple negative highly invasive breast cancer cells were highly sensitive to IGF-1 which promoted cell proliferation and survival ⁷. While there is a plethora of data regarding the IGF-1 and its receptor in cancer metastasis, there is a paucity of research specifically regarding the relationship between Rab GTPases and IGF-1R in cancer progression.

Interest has been building regarding the dynamic interplay between endocytosis of receptor tyrosine kinases (RTK) and signaling. During endocytosis, receptors are active within the endosomes¹⁶ thereby supporting the theory that membrane trafficking is a regulator in growth factor signal transduction. Metastatic factors such as hepatocyte growth factor (HGF), epithelial growth factor (EGF), transforming growth factor β (TGF β) and insulin growth factor 1 (IGF-1) are known to activate the endosomal pathway and the mechanisms can be analogous at key junctions thereby promoting a migratory response in tumor cells.

The role of clathrin-mediated endocytosis in EGF-directed chemotactic invasion of breast cancer²⁰ and internalization and co-localization of the IGF-1R with clathrin and caveolin-1 in Ewing sarcoma cells¹⁸ denoted the relationship of endocytosis and RTK trafficking in cancer. Other studies have established that IGF-1R is also localized in the nucleus in prostate, renal and breast tumor cells after translocation from the cell surface¹, and Rab5a is characterized as a significant molecule in ovarian cancer upon EGF stimulation⁴⁰.

On the other hand, Rab5c has been implicated in EGFR signaling by affecting integrin recycling and triggering cell invasion in some breast cancer cells²². Hepatocyte growth factor receptor (Met) trafficking requires Rab5 and facilitates focal adhesion turnover,

actin remodeling and sustained MAPK signaling thereby underscoring its role in tumorigenesis, cell migration and invasion¹².

Rab5-dependent IGF-1-signaling may a noteworthy aspect of cancer metastasis based on earlier results. Given that the potential role IGF-1R trafficking in invasive breast cancer is unknown, this study sought to explore and highlight the possible role of early endosomal trafficking in mediating phenotypic changes in the promotion of proliferation, migration and invasion activities of invasive breast cancer cells in the presence of IGF-1.

3.3. Materials and Methods

3.3.1. Media and Antibodies.

Trypsin and DMEM medium were purchased from Fisher Scientific. Basement extract membrane was obtained from Trevigen (Gaithersburg, CA). The following antibodies were used for Western blotting: Rab5, FAK, MMP-2, MMP-9, anti-ERK, cJun, p38, Akt, phospho-ERK, phospho-cJun, phospho-p38, phospho-Akt, GAPDH , (Cell Signaling Technology, Danvers,MA). Anti-mouse, and anti-rabbit IgG peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

3.3.2. Cell Culture.

The human breast cancer cell line, MDA-MB-231, was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in DMEM medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics (100 U/mL penicillin and 100 lg/mL streptomycin) at 37°C in a humidified atmosphere of 5% CO².

3.3.3. Construction of Recombinant pMX-puro retroviruses and cell lines

As previously described, the cDNAs of Rab5 and Rin1 and their mutants were subcloned into the pMX-puro vector ². Transfection of 60% confluent PhoA cells was performed using Fugene (Roche) and the virus harvested after 48 hours post-transfection. MDA-MB-231 cell line was infected with the retrovirus tagged with green fluorescent protein (GFP) and Rab5, Rin1 and control cell lines were generated and selected after 72 hours.

3.3.4. Rab5-GTP activation pull down assay

MDA-MB-231 cell lines were lysed using a buffer containing 20 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM DTT, 5 mM MgCl₂, 5% (v/v) glycerol and 1% (v/v) Triton-X-100 supplemented with 1 mM PMSF. Lysates (1.5ug) were then incubated with 1µl anti-active Rab5 monoclonal antibody (Neweast Biosciences, Mavern, PA) overnight at 4°C. The following day, cells were incubated with a protein A/G agarose bead slurry for 1 hr. After incubation, the beads were washed three times using the lysis buffer and resuspended in loading sample buffer. The pull-downs were subjected to SDS-PAGE and analyzed by immunoblotting using an anti-Rab5 antibody.

3.3.5. Cell Proliferation Assay

The viability of cells was determined by the MTT assay. MDA-MB-231 cell lines expressing Rab5 WT, the inactive form S34N, active form Q79L, Rin1, Rin2, Rin3, Rin1 mutant (R94A) or null vector were seeded in 96-well plates at a density of 25×10^3 cells/well and incubated overnight. Cells were then serum starved for 24 hours followed by treatment with serum and phenol red free DMEM with or without charcoal stripped 5% FCS for 48 h. The MTT solution (5 mg/ml) was added to the plates and the

cells incubated at 37 °C for 4 h. The formazan, derived from MTT by living cells, was dissolved in 10% SDS (150 µl per well), and the absorbance was measured at 595 nm on a plate reader. All MTT experiments were performed in triplicate and repeated at least 3 times.

3.3.6. Cell Migration/ Invasion assay

The ability of MDA-MB-231 cells passing through Matrigel coated or uncoated filters was measured by the Boyden chamber invasion assay using Transwell inserts (8 µm pores, Trevigen, MD). Cells suspended in serum-free and phenol free DMEM and applied to the upper chamber. Serum and phenol free media with or without FCS as the chemottractant added to the lower chamber and the plate incubated at 37°C for 24hrs. Non-migrated cells in the upper well were removed and the membranes were fixed and stained using 0.01% crystal violet. The membranes were either photographed or counted under a light microscope (×100) or the dye extracted and the absorbance reading obtained using a spectrophotometer.

For the Matrigel invasion assay, transwell inserts coated with 0.5 mg/ml Matrigel (BD Biosciences, MA) were used. Cells (10^5 per well) suspended in serum-free and phenol free DMEM were seeded onto the upper chambers of the precoated transwells. Serum and phenol free media with or without charcoal stripped FCS was added to the lower chamber and the plate incubated at 37°C for 24hrs. Subsequently, the membranes were fixed, stained, and photographed as above or absorbance readings acquired.

3.3.7. Wound-healing Assay

Cells were placed in inserts with a gap 500nm (\pm 50nm) (Ibidi, Verona, WI) and incubated at 37°C. After serum starvation for 24hrs, the inserts were removed, the wells

were washed with PBS and medium with or without 5% charcoal stripped FBS was added. Cell migration was determined under the inverted microscope and pictures were obtained at time = 0 and t= 16hrs.

3.3.8. Anchorage-Independent Growth Assay

Briefly, 1×10^3 cells of each cell line were suspended in 300ul of DMEM containing 10% calf serum and 0.35% SeaPlaque low-melting temperature-agarose. These cells were plated over a 300ul layer of solidified DMEM containing 10% calf serum and 0.6% agarose, and cells were allowed to settle to the interface between these layers at 37 °C. After 20 min, the plates were allowed to harden at room temperature for 30 min before returning to 37 °C. 100ul of vehicle control or IGF-1 was added to wells and the plate incubated at 37 °C. The plates were fed every 2–3 days. After 12-14 days, colonies were photographed under low magnification (x10).

3.3.9. Western Blot Analysis

MDA-MB-231 cells were lysed with RIPA buffer containing protease inhibitor. Equal amounts of protein (50 ug) were separated by SDS–PAGE gel electrophoresis and then were electrotransferred to nitrocellulose membrane. Subsequent to blocking the membrane with a solution containing 5% BSA- TBST buffer (20 mM Tris–HCl, pH 7.4, 150 mM qNaCl and 0.1% Tween 20) for 1hr, the membrane e was washed with TBST buffer and incubated with the primary antibodies. Membranes were then washed; probed with specific secondary antibodies and the proteins were detected by enhanced chemiluminescence.

3.3.10. Statistical Analysis

All samples in this study were analyzed in duplicate and each experiment was repeated three times. Values represent the mean \pm SEM of three independent experiments. To compare two groups, student's t test was utilized. $P < 0.05$ was considered as statistically significant.

3.4. Results

3.4.1. *IGF-1 endocytosis is mediated by Rab5 protein expression in breast cancer cells*

To study the effects of Rab5, GFP-Rab5 constructs were transduced into the MDA-MB-231 tumor cell line with a retrovirus system. Stable cell lines over-expressing Rab5 were stimulated with a physiological concentration of IGF-1. Rab5 activation upon IGF-1 stimulation was examined by precipitating the active or GTP-bound form of Rab5 antibody (see Materials and Methods). After 5 minutes of growth factor stimulation, the data shows that IGF-1 stimulation elicits a significant increase (a little over 3-fold) of active Rab5 (Figure 8), suggesting that IGF-1 signaling may be Rab5 dependent in MDA-MB-231 cells.

3.4.2. *IGF-1 stimulated Rab5 activation in breast cancer cell overexpressing Rab5 specific GEFs*

MDA-MB-231 cells were transformed using the aforementioned retrovirus system and selected for overexpression of RIN1, RIN2 and RIN3. As it was previously described, Rab5 activation can be described as molecular switch where GTPases cycle from an inactive to an active form. This switch is mediated by Rab5- specific GEFs and may also regulate RTK membrane trafficking.

To evaluate if cells expressing RIN1, RIN2 and RIN3 may be a requirement for Rab5 activation in IGF-1R trafficking, the pull-down assay was performed to verify active Rab5 presence in cells overexpressing RIN1, RIN2 and RIN3 in response to IGF-1. Cells were incubated with IGF-1 for 5mins and the amount of active Rab5 generated was quantified using SDS-PAGE after precipitating with active Rab5 monoclonal antibody.

3.4.3. Rab5 promotes cell migration and invasion in breast cancer cells

Cell migration and invasion were measured with the aid of a transwell system. Briefly, cells expressing the different Rab5 constructs were placed in the Boyden chamber in absence or presence of matrigel and incubated overnight. In Figures 10a and 10c, there was a marked increase in the migratory and invasive potential of cancer cells over expressing Rab5. As expected, cells expressing the constitutively inactive form of Rab5, Rab5:S34N, inhibited migration and invasion of the cancer cells compared to the control cells. While IGF-1 seems to have a profound impact on the migratory potential in the S34N cell line when compared to cell invasion, it was still less than both the Rab5WT and Rab5:Q79L active mutant.

These data also reveal that even in the absence of IGF-1, it seems that Rab5 activity encourages more migration and invasion in both WT and Q79L cell lines when compared to the GFP control. The migration abilities of the MDA-MB-231 breast cancer cell lines were also verified using the wound healing method after 18 hrs. Cells transduced with the Rab5-GDP bound form had a little migration, as the gap closure was similar to the 0hr timepoint and wider than GFP control. Both Rab5:WT and Rab5:Q79L cell lines demonstrated extensive gap closing which suggest that Rab5 activation may be key in modulating such activity.

3.4.4. The Ras-interfering family (RIN) increases cell proliferation, migration and invasion upon IGF-1 stimulation

Given the results above, it was speculated that expression of Rab5 GEFs may also induce cell proliferation, migration and invasion in breast cancer cells in response to IGF-1. In MDA-MB-231, IGF-1 has been purported to exert a strong proliferative effect ⁷ and it was necessary to verify if RIN expression may modulate this response in the breast cancer cells. Cells expressing RIN1, RIN2 and RIN3 were plated, incubated for 48hrs in the absence or presence of IGF-1 and the proliferative activity measured using the MTT assay as described in the Materials and Methods. Figure 11 depicts the promotion of cell proliferative by the RIN family of GEFs in response to IGF-1. To further validate the function of RIN1 in IGF-1 signaling in cancer progression, cell migration and invasion assays using the Boyden chamber as described in the Materials and Methods bolstered previous data that the invasive and migratory activity of cells were augmented in response to RIN1, RIN2 and RIn3 expression.

Figures 12a and 12b portrayed a strong increase in cell invasion and migration in RIN1 and RIN3 cell lines, even in the absence of IGF-1, suggesting that RIN activity be a driving force regardless of growth factor presence. RIN2 engendered an increase in migration in the presence of IGF-1, while invasion was not noticeable when compared to the control cell line.

3.4.5. Functional role RIN1 on the invasive and migratory potential in MDA-MB231 Cells

As described in Figures 13a and 13b, RIN1 appears to be a principal regulator in cancer cell metastasis. In Aim one, two scenarios arose with regard to RIN1 role in growth factor signaling:

1. Action on Rab5 leading to its active form;
2. Interaction with the cystolic tail of the IGF-1R ensuring the internalization of receptors and subsequent activation of the intracellular signaling pathways.

To further elucidate RIN1 function in growth factor signaling, stable cell lines expressing RIN1 and the RIN1 mutant, R94A were created and the proliferative, migratory and invasive capacities were studied. Jozic et al. showed that RIN1 facilitated the fusion of the insulin receptor to the early endosome and was Rab5 dependent¹⁵ via RIN1 activity. This study further supports the idea that RIN1 interacts with the IGF-1R via the -SH binding domain as the R94A mutant significantly diminished proliferation, migration and invasion in breast cancer cells. Interestingly during invasion, RIN1 seems to have a greater interaction in response to IGF-1R as the absorbance was greater in response to IGF-1 supplementation.

3.5. Discussion

Rab5 is an important factor in the regulation of intracellular vesicle transport and recently garnered interest in its role in the pathogenesis of certain diseases, specifically cancer. Earlier, it was shown that Rab5 may be an important protein in the onset of breast cancer metastasis as non -aggressive tumorigenic breast cancer cells acquired a highly

invasive and migratory phenotype when Rab5 was overexpressed. Furthermore, IGF-1, a potent mitogen seems to facilitate such changes as growth factors are integral in tumor progression^{4,9}.

Studies have shown that Rab5 is expressed differentially in different cancer subtypes^{38,40}. Expression of Rab5 in non-invasive and invasive breast cancer cell lines varies; where Rab5 distribution throughout the cells in different breast tumor grades were disparately localized³⁶.

As previously reported, an inherent relationship exists between RTK activation and internalization^{2,3,25} and has been attributed to Rab5 activation. Figures 11 and 12 showed that IGF-1 activated the IGF-1R resulting in sequestration of active Rab5 to early endosome and consequently the possible stimulation of the PI3K and MAPK pathways which are necessary in initiating cell migration and invasion. While this study is the first to show IGF-1 influence on the endocytic pathway on the migratory and invasive capacity of MDA-MB-231 cells, Yang et al provided possible answers that most if not all growth factors and their RTKs will have analogous behavior³⁶.

In MCF7 cells, EGF did not promote cell migration despite the associated increase of Rab5A expression. However, in MDA-MB-231 cells, the growth factor augmented the migratory potential in relation to Rab5 expression and may be due to the RTK levels in these cells. IGF-1R is found in all breast cancer cell lines regardless of estrogen receptor status⁷ and is predominant in both estrogen responsive MCF7 and estrogen unresponsive MDA-MB-231 cells. Although it is known that IGF-1 can stimulate the ER in the absence of estrogen, this study supplements data from the first aim that the IGF-1R

activation is necessary for migration and invasion of breast cancer cells and more than likely does not involve crosstalk between the ER.

In this study, the role of Rab5 in facilitating IGF-1 induced breast cancer metastasis is further clarified as overexpression of the Rab5WT and the active mutant form of Rab5 increased migration and proliferation. Conversely when inactive form of Rab5:S34N mutant, was expressed, proliferation and migration was stemmed. Taken together, it can deduced that the function of Rab5 is tightly linked to IGF-1 and IGF-1R signaling in breast cancer metastasis.

During migration and invasion of cancer cells, reorganization and reconstruction of the actin filament and microtubules are part of the morphological changes that these cells undergo^{26,37}. Cancer cells employ four key steps that facilitate this process: lamellipodia formation and extension at the forefront, translocation of integrins to the leading edge, contractile movement of the cell body and detachment of adhesion molecules at the rear edge²⁴. Actin remodeling and cell migration is dependent on Rho GTPase and Rac activity of which these proteins are trafficked by Rab13 and Rab5 respectively and affect the internalization of integrins during cell migration^{32,35,39}.

Rab5 depletion in HeLa and SiHa cells affected integrin trafficking, compromised filopodia and lamellipodia protrusion formation and reduced the cells' capacity to migrate and invade¹⁷, whereas in ovarian cancer, Rab5a overexpression elevated the proliferative activity of ovarian cancer cells⁴⁰. Migration of cells in hepatocellular carcinoma was dependent on VEGF/PDGF (platelet derived growth factor) and EGFR mediated tyrosine kinase endocytosis via Rab5¹⁰, hepatitis B induced Rab5 expression²⁸. Consequently, the data and studies involving growth factor/ RTK trafficking and Rab5

function collectively establish Rab5 as a regulator in IGF-1 mediated metastasis in breast cancer cells.

Though Rab5 activation may be a requirement in cancer cell proliferation, migration, invasion and survival, other factors may influence Rab5 activity and lead to the internalization of the phosphorylated receptor. The RIN family of proteins are regulators of Rab5 dependent early endocytosis via their GEF activity. They are homologous as they share an SH2 domain, an RH region, a Vps9 domain and an RA domain. Though controversial, RIN1 appear to exert its influence on Ras by inhibiting its action whilst binding to H-Ras via the RA domain thereby competing with RAF1^{33,34}. In Aim1, it was shown that both RIN1, RIN2 and RIN3 enabled MCF7 cells to garner a more hyper-proliferative and invasive characteristic and this is reflected in the triple negative highly invasive MDA-MB-231 cells. The conflicting role of RIN1 function can only be resolved by viewing RIN1 activity in RTK trafficking in a context-specific manner. On one hand, RIN1 may be a breast cancer tumor suppressor gene^{13,19} by suppression of cancer tumorigenesis and inhibition of transforming growth factor β (TGF β) signaling. The latter however has contrary roles in tumorigenesis and metastasis as typically in early non-metastatic tumor (epithelial to mesenchymal transition), TGF β levels are too low to facilitate such change, yet once cells are malignant, TGF β promote cancer progression and dissemination³¹. This may be the case for this study, as MDA-MB-231 are already quite aggressive and over expression of RIN1 has a paradoxical effect and instead augments proliferation and invasion of the cells in response to IGF-1. Previous studies have indeed highlighted RIN1 expression as a major indicator of cancer metastasis and progression^{8,27}.

IGF-1 has a significant effect on triple negative breast cancer cell lines by fostering proliferation and reducing apoptosis and attenuation of such response and when the IGF-1R expression was curbed⁷, cell proliferation and survival was abrogated. Modulation IGF-1R trafficking affected the proliferative, migratory and invasive potential of breast cancer cells. RIN1 beside possessing a Rab5 specific binding domain, also encodes a SH2 binding site that may interact with the insulin receptor substrate (IRS) on the phosphorylated tyrosine residues of the IGF-1R²¹. Here, mutation in the SH2 binding domain of RIN1 implied that RIN1 is crucial in IGF-1 signaling as cells expressing the RIN1 mutant, R94A, considerably moderated proliferation, migration and invasion of MDA-MB-231. Therefore it can be tentatively concluded that RIN1 is first recruited to the activated receptor, then engages Rab5 via its Vsp9 binding domain, with Rab5 subsequent activation. Comparatively, SH2 dependent interaction has been illustrated for RIN1 and other RTKs, specifically EGFR resulting in the recruitment and internalization of activated RTKs in endosomes^{14,30}.

Overall, the data presented shows that both Rab5 and RIN1 point to a novel relationship between IGF-1R trafficking and endocytosis in the regulation of migration and invasion of breast cancer cells. It remains to further elucidate if IGF-1R internalization may also be clathrin or caveolin dependent and the effect of IGF-1R trafficking on downstream signaling in IGF-1 driven breast cancer cell invasion and migration.

3.6. Figures

Figure 8

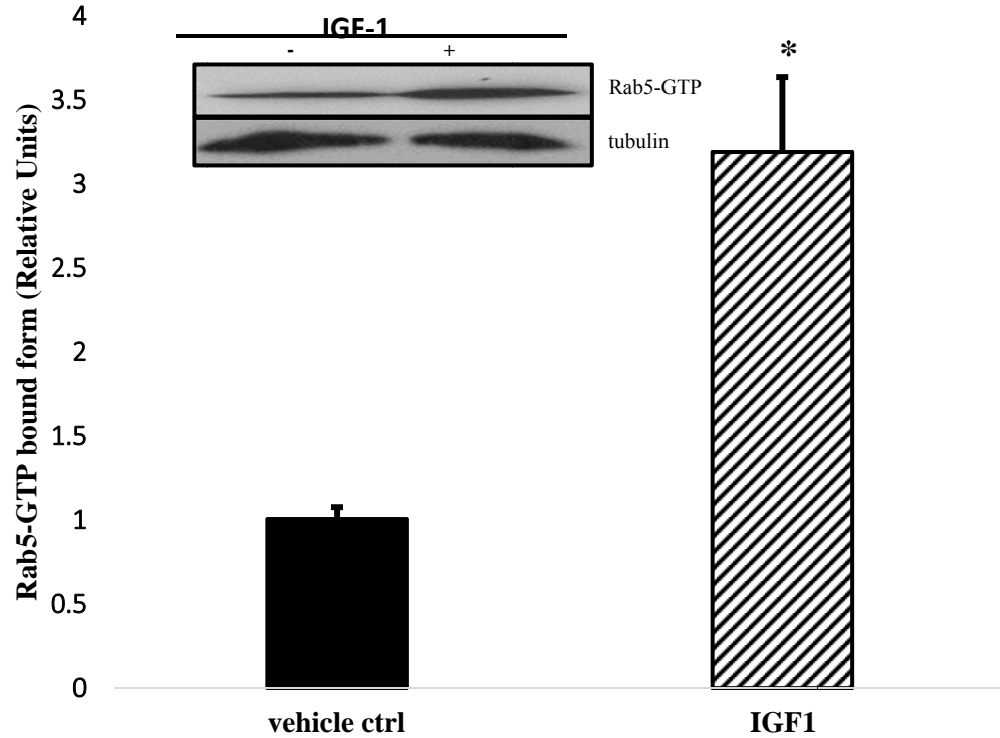


Figure 8. IGF-1 endocytosis is mediated by Rab5 protein expression in breast cancer cells. MDA-MB-231 cells expressing GFP-Rab5:WT were incubated in the absence or presence of IGF-1 at 37°C. After incubation, active or GTP-bound form of Rab5 was precipitated with GST-EEA1 domain at 4°C while rocking for 1hr. After incubation, the beads were washed three times using the lysis buffer. The pull-downs were subjected to SDS-PAGE and analyzed by immunoblotting using an anti-Rab5 antibody. A representative immunoblot probed with anti-Rab5 or tubulin (loading control) is shown. Data represent the mean \pm SEM of three independent experiments. (*) represents statistically significant difference from control group ($P < 0.05$).

Figure 9

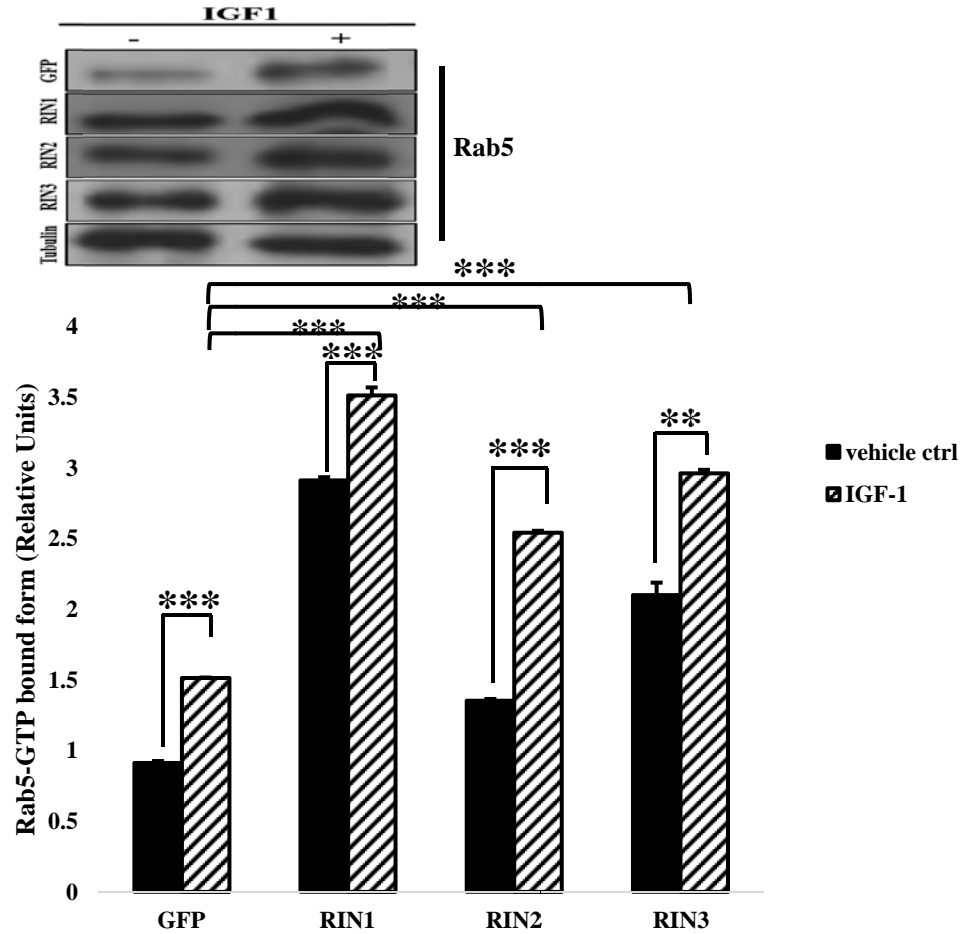
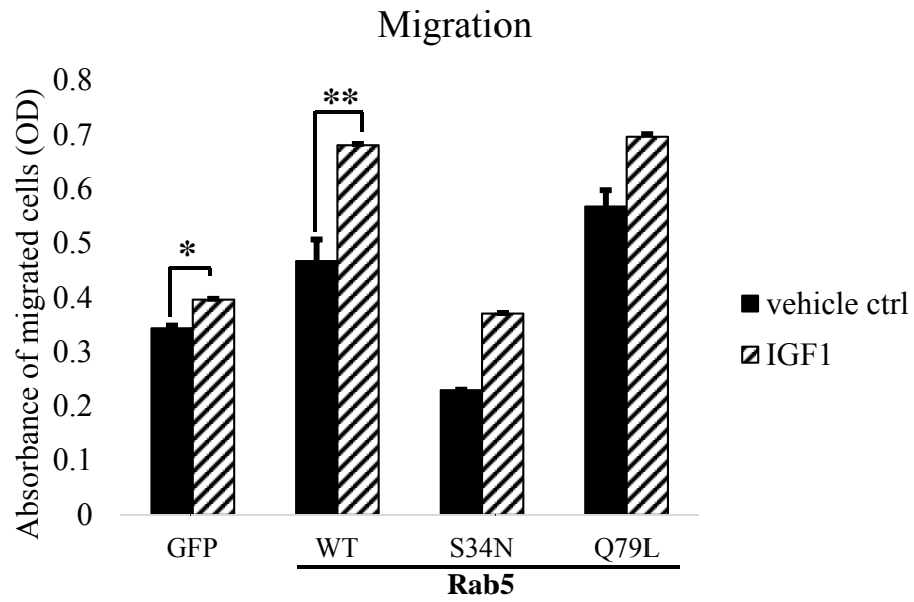


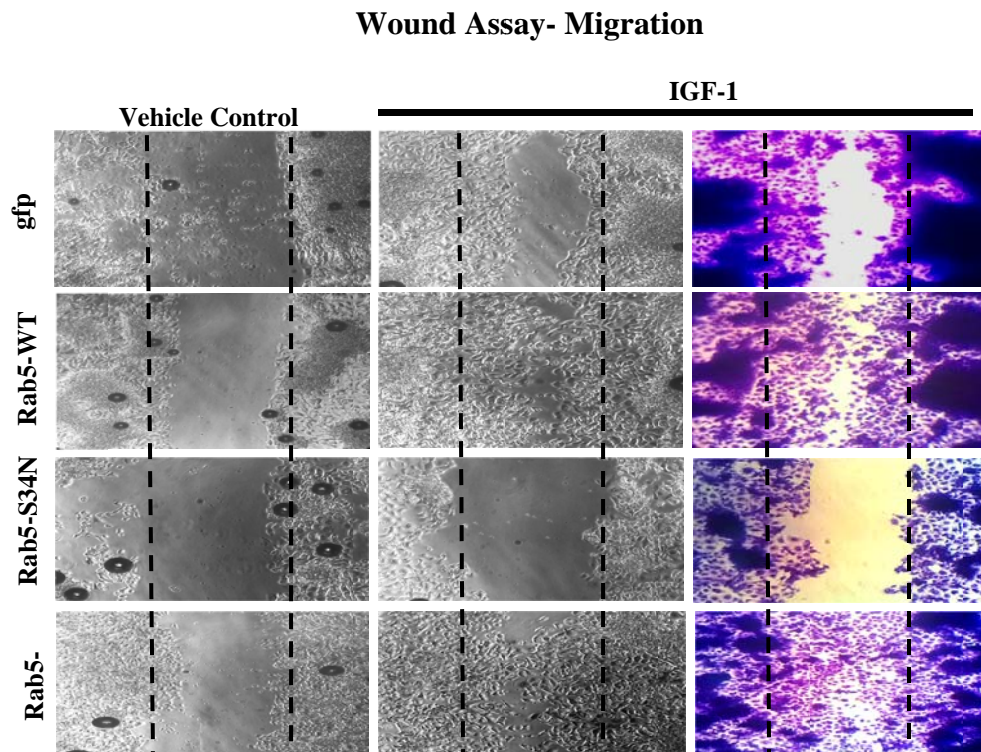
Figure 9. Rab5 GEFs facilitate Rab5 activation in response to IGF1 stimulation in invasive breast cancer cells. MDA-MB-231 cells expressing the GEFs Rin1, Rin2, and Rin3 were stimulated with IGF-1 or vehicle control for 5 minutes and assayed for active GTP-Rab5 as described in Materials and Methods. A representative immunoblot probed with anti-Rab5 or tubulin (loading control) is shown. Data represent the mean \pm SEM of three independent experiments. (*) represents statistically significant difference from control group ($P<0.05$), (**) is ($P<0.01$), (***) is ($P<0.001$)

Figure 10

(a)



(b)



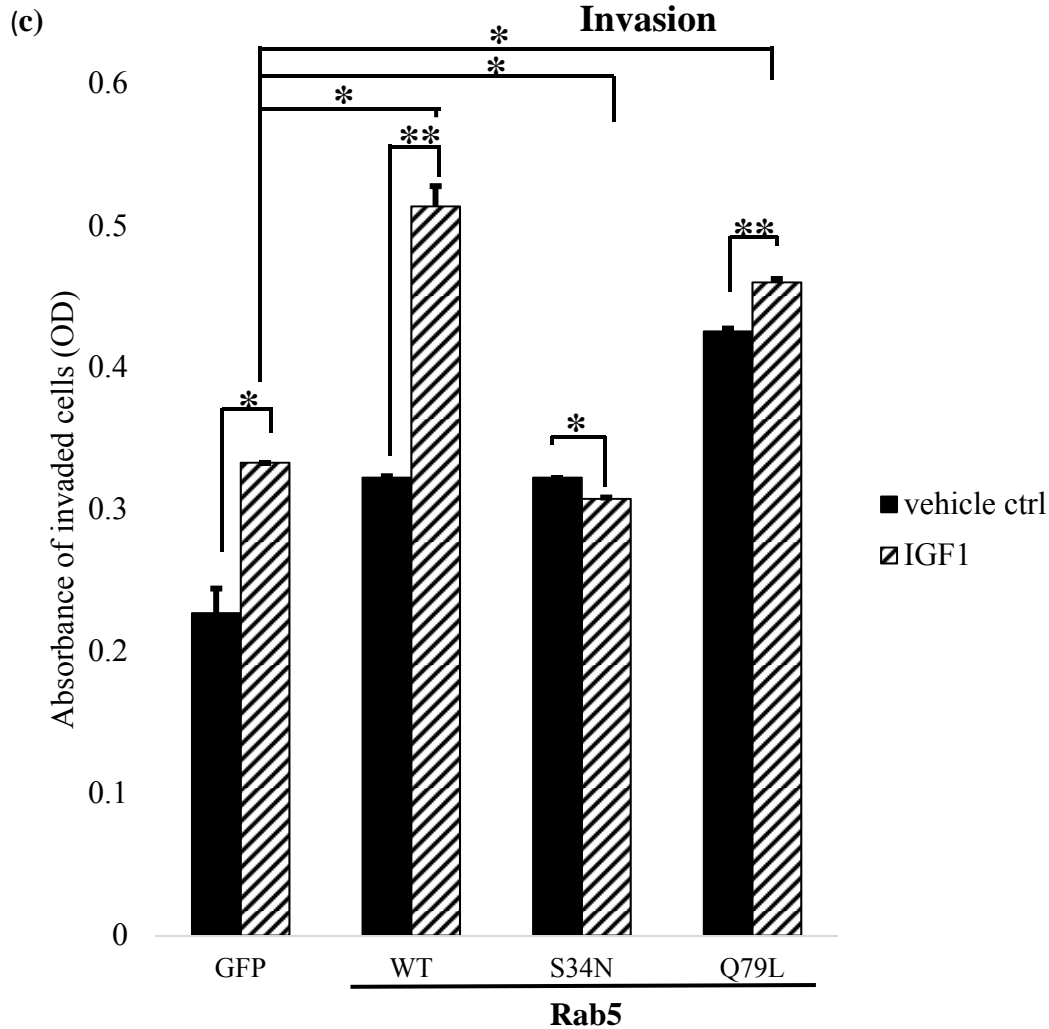


Figure 10. IGF-1 mediates cell invasion and migration in MDA-MB-231 cells via Rab5 activation. In Figures 10a and b, cells were plated in a Boyden chamber without or without basement extract membrane. Both kinase and caveolin inhibitors were added to cells for 1hr. Treatment groups were IGF-1 or vehicle control and cells were incubated at 37°C for 16hrs. Crystal violet staining was performed and the dye extracted and the OD measuring using a plate reader at 595nm. Cell migration and invasion absorbance reading were expressed as average \pm SEM for three independent experiments. For MDA-MB-231 cell migration (Fig. 10b), an IBIDI culture insert (IBIDI GmbH) was placed into wells of a 24 well plate and slightly pressed on the top to ensure tight adhesion. An equal number of cells were placed in each of the insert and the cells incubated for 16hrs. Gap closure was observed at a magnification of 10X and images recorded. Crystal violet staining was done for better imaging of the migration of the cells. Each cell line was done in triplicate with data represent the mean absorbance \pm SEM of the three independent wells. (*) represents statistically significant difference from control group ($P < 0.05$); (**) is ($P < 0.01$).

Figure 11.

Proliferation

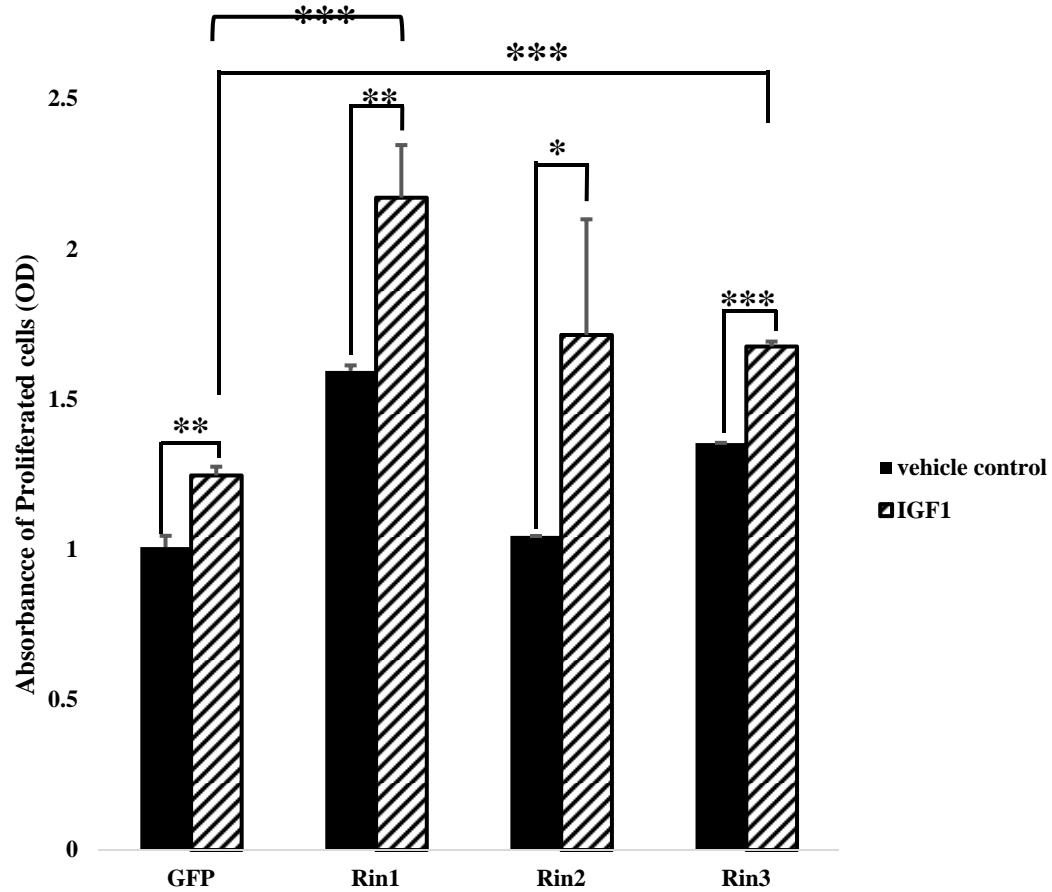
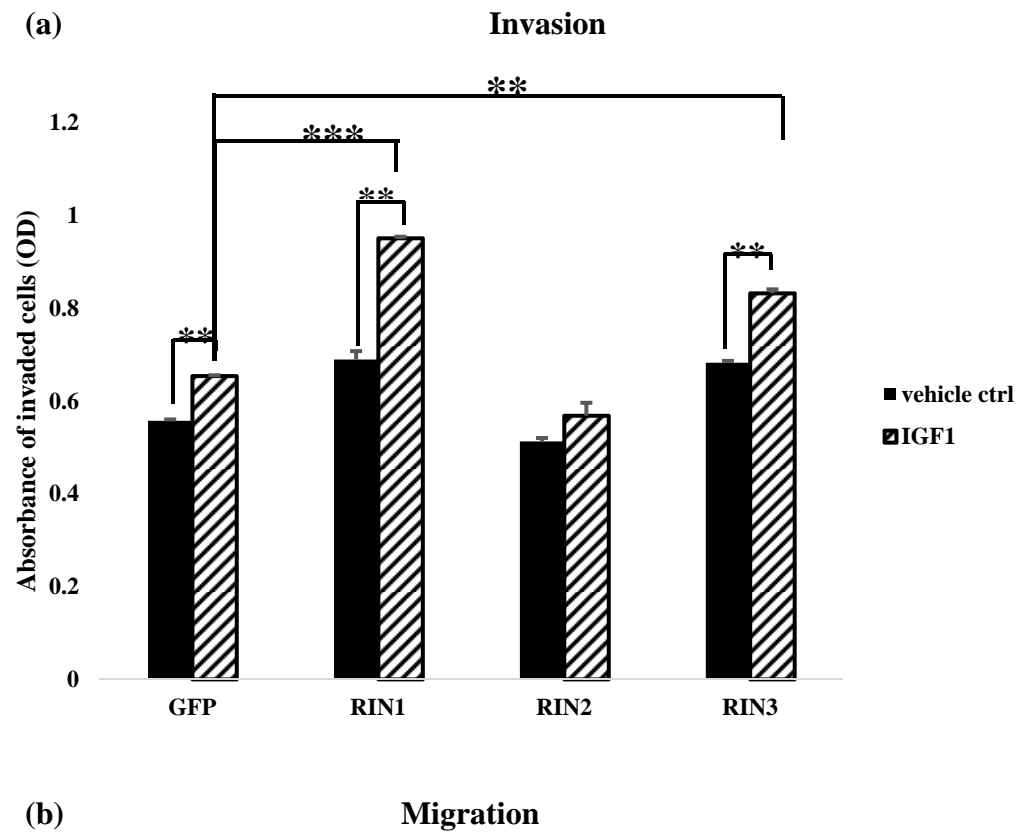


Figure 11. The Ras-interfering family (Rin) increases cell proliferation upon IGF-1 stimulation. Cells expressing either GFP, RIN1, RIN2 and RIN3 were incubated in the absence or in the presence of IGF-1 in triplicate. Cell proliferation was assessed after 48hr incubation with vehicle or IGF-1 of cell culture, using the MTT proliferation assay (Sigma). Briefly after the incubation period, cells were added with 100 μ L of 5 mg/mL thiazolyl blue tetrazolium bromide (Sigma, St. Louis, MO) solution/well and were incubated further for 4hrs in a humidified atmosphere (37°C in 5% CO₂). The absorbance was measured at 560nm in a microplate reader. Cell proliferation was expressed as the mean absorbance compared to vehicle control \pm SEM for three independent experiments. (*) represents statistically significant difference from control group ($P < 0.05$); (**) is ($P < 0.01$); (***) is ($P < 0.001$)

Figure 12



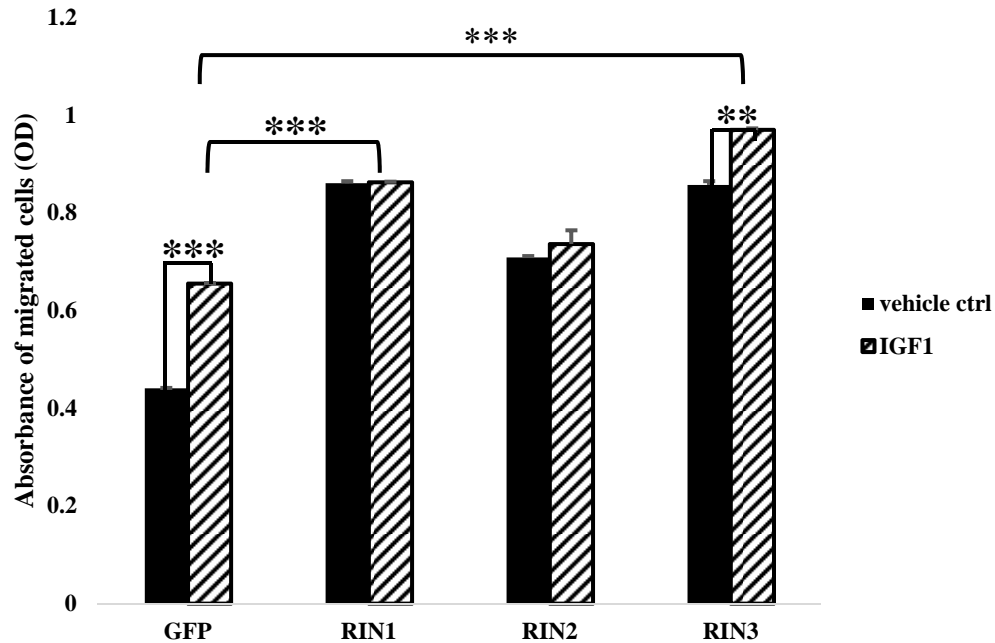


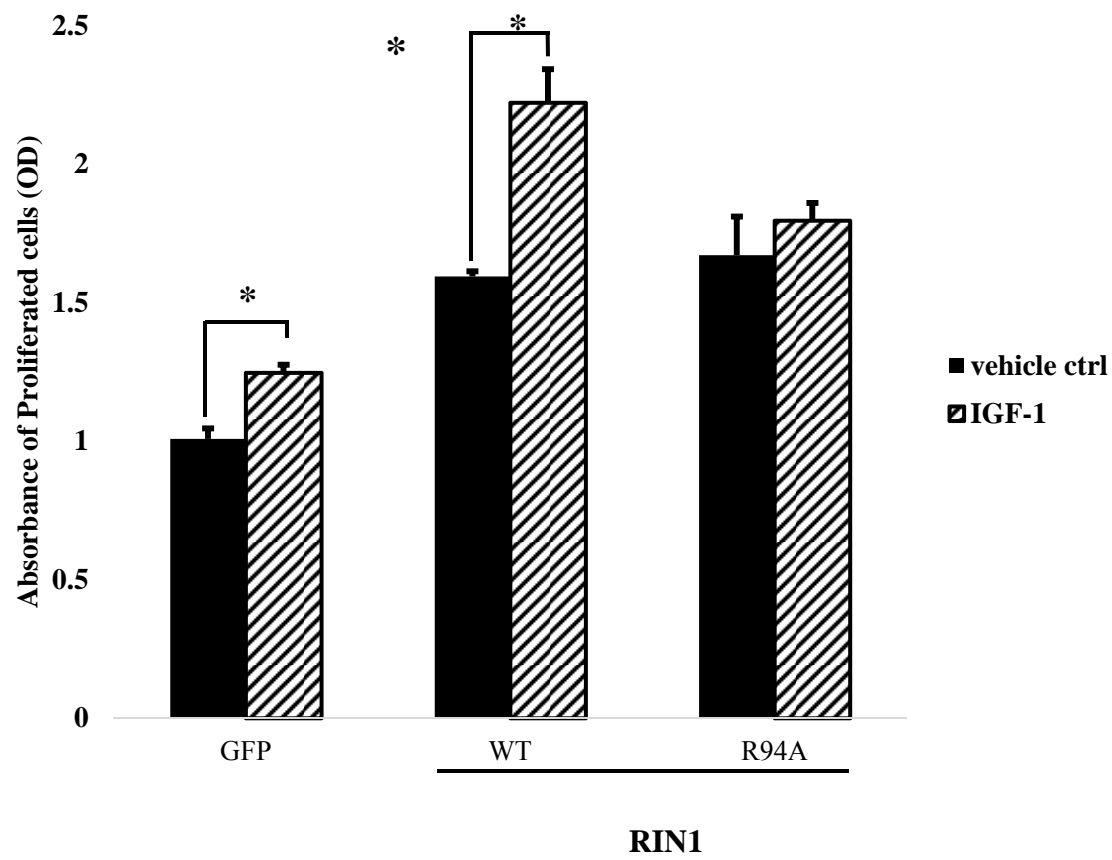
Figure 12. RIN1 and its family facilitate an increase in invasion and migration upon IGF-1 stimulation in cancer cells. Cells expressing either GFP, RIN1, RIN2 and RIN3 were incubated in the presence or absence basement extract membrane in a Boyden chamber in triplicate. Treatment groups were IGF-1 or vehicle control and cells were incubated to 37°C for 16hrs. Crystal violet staining was performed and the dye extracted and the OD measuring using a plate reader at 595nm. Cell invasion and migration was expressed as the mean absorbance compared to vehicle control \pm SEM for three independent experiments. (*) represents statistically significant difference from control group ($P<0.05$); (**) is ($P<0.01$); (***) is ($P<0.001$)

Figure 13

(a)

Proliferation





(b)

Migration

*

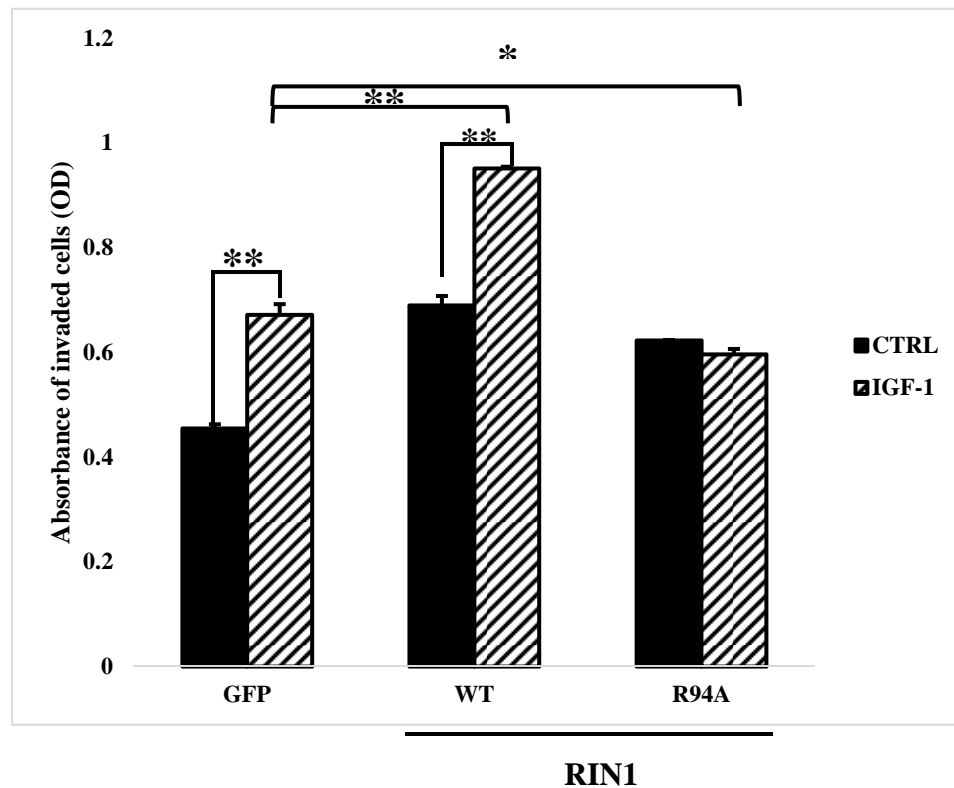
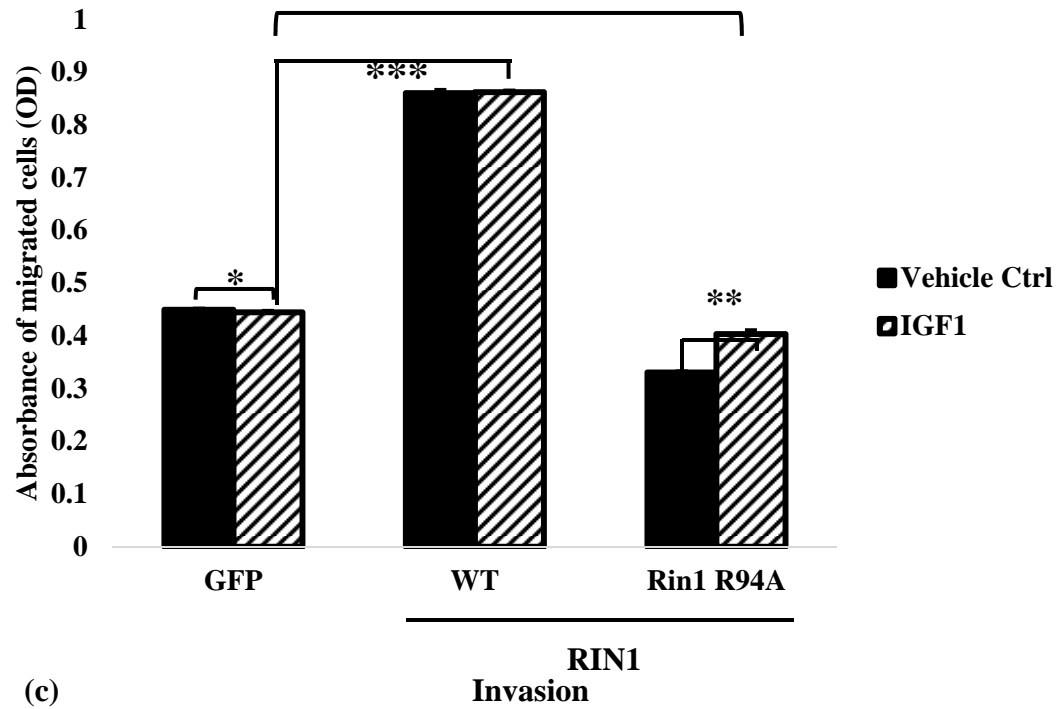


Figure 13a. IGF-1 enhances cell proliferation through GEF overexpression. Cells expressing either GFP , RIN1, and the RIN1 mutant R94A were incubated in the absence or in the presence of IGF-1 in triplicate. Cell proliferation was assessed after 48hr

incubation with vehicle or IGF-1 of cell culture, using the MTT proliferation assay (Sigma, St. Louis, MO). Briefly after the incubation period, cells were added with 100 μ L of 5 mg/mL thiazolyl blue tetrazolium bromide (Sigma, St. Louis, MO) solution/well and were incubated further for 4 h in a humidified atmosphere (37°C in 5% CO₂). The absorbance was measured at 595 nm in a microplate reader. Cell proliferation was expressed as the mean absorbance compared to vehicle control \pm SEM for three independent experiments. (*) denote p value < 0.05 for vehicle control. **Effect of Rin1 on the invasive and migratory potential in MDA-MB231 cells.** In Figure b, MDA-MB231 cells were infected with Rin1, Rin1 mutant R94A and the null vector GFP. Cells were placed in a Boyden chamber with or without basement extract membrane (Figures b. and c. respectively) Treatment groups were IGF-1 or vehicle control and cells were incubated to 37°C for 16hrs. Crystal violet staining was performed and the dye extracted and the OD measuring using a plate reader at 595nm. The average OD \pm SEM of the (b.) cell invasion and (c.) cell migration readings were obtained for three independent experiments. (*) denotes (P< 0.05), (**) is (P<0.01), (***) is (P<0.001).

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CHAPTER 4.

Caveolin dependent trafficking of IGF-1R is a key step in Cell Migration and Invasion of Breast Cancer Cells

Within this chapter, the functional roles of Rab5 and RIN1 in IGF-1R trafficking were further characterized. The effect on the gene expression of a key protein in cell invasion, MMPs was also investigated.

4.1. Abstract

IGF-1 signaling has been extensively studied in several developmental contexts and most recently within cancer pathogenesis. IGF-1 is known to increase cell proliferation and survival and may also be implicated in cell migration and cell invasion. However, there is a dearth of information regarding IGF-1R trafficking upon IGF-1 stimulation in breast cancer progression. It has been described that Rab5 and its GEF, RIN1, play key roles in RTK trafficking and influence the invasive and migratory potential of cells.

In this study, two non-invasive ER positive and highly invasive triple negative breast cancer cell lines were transfected with Rab5 and RIN1 constructs and the mechanisms surrounding IGF-1R endocytosis was investigated. Blocking of the IGF-1R revealed that IGF-1 signaling was contingent on receptor activation and recruited RIN1 and early endosome formation via a caveolae dependent mechanism. Furthermore, Rab5 and RIN1 overexpression increased MMP9 expression establishing a novel link among IGF-1R activation and signaling and MMP activation in migration and invasion of breast cancer cells.

4.2. Introduction

Like most if not all cancers, breast cancers commandeer highly regulated signaling pathways and alter these signaling pathways resulting in autonomous growth signal entities^{17,42}. They upset the delicate balance by promoting the binding of multiple growth factors to cell surface receptors, synthesize growth signals that can either bind to their own receptor, forming an autocrine loop or influence the secretion of more growth factors by normal cells into the stroma^{6,13}, which then binds again to the receptors on the cancer cells, creating a paracrine feedback loop.

In mammary epithelial cells, malignancy has been attributed to an increase in cell proliferation, survival and metastasis, specifically cell migration and invasion. IGF-1 has been strongly implicated in breast cancer progression as it facilitates cell proliferation and survival in breast cancer cells¹⁸. In fact, circulating IGF-1 levels have a strong positive correlation with worsening breast cancer prognosis, especially among menopausal women¹⁸, thereby indicating that breast cancer is not always hormonally dependent as previously believed.

While growth factors may induce transformations in signaling pathways in cancer pathogenesis, Rab proteins have also been specifically linked to the deregulation of several signaling pathways in several cancers¹². These small monomeric enzymes are implicated in the promotion of abnormal proliferation, migration and invasion of epithelial cells in tumor progression due to activation of membrane bound growth factor receptors. Rab5 is one such GTPase that is gaining notoriety in tumor progression and metastasis^{27,40}. As it was previously shown in the aforementioned Aims, Rab5 activity play a key role in cell migration and invasion in both estrogen positive and negative

breast cancer cell lines. Furthermore, it is believed that disruption of the endocytic pathway confers growth factor autonomy to cancer cells by passing key endocytic processes such as lysosomal degradation and instead promote the recycling of growth factors as well as integrins^{11,15,28}.

IGF-1 binds to a variety of insulin-like growth factor receptors (IGFRs) including the insulin receptor (IR). These receptor tyrosine kinases (RTKs), when activated, transduce signals through PI3K and MAPK-dependent mechanisms which are responsible for the diverse target actions of these growth factors. These include increases in cell division, cell size, protein synthesis, cell migration, and inhibition of apoptosis²¹. IGF-1 can also potentiate VEGF expression of tumor cells and inhibit apoptosis by binding to caspase 9, which is unable to cleave caspase 3 and halting the caspase cascade to apoptosis³⁶. The pro-survival effect of IGF-1 also allows detached pre-metastatic cells to escape anoikis²⁰, facilitate the secretion of more growth factors by cancer cells and induce ECM degradation by enabling the matrix metalloproteinases (MMPs) expression and secretion³⁹. IGF-1 also mediate cell migration of cancer cells by reorganizing integrin receptors, and modulating the E-cadherin/ β -catenin complex which in turn, augment MMPs expression and the migratory potential of the cells⁴¹.

The aforementioned studies (Aims 1 and 2) illustrated that IGF-1 signaling is mediated by Rab5 and RIN1 activities. Recently, it was acknowledged that IGF-1R is internalized via clathrin and caveolin dependent mechanisms in Ewing sarcoma³⁰ cells and is localized in the nucleus in prostate, renal and breast tumor cells after translocation from the cell surface via clathrin mediated endocytosis¹. Yet, during IGF-1 driven cell

invasion and migration of breast cancer cells, it remains unclear if IGF-1R trafficking is caveolin dependent or independent. In this study, IGF-1R trafficking utilized primarily caveolin dependent, and inhibition of IGF-1R activation and/ or internalization can alter cancer cell progression. Hence, there is a novel link between RIN1, Rab5, MMP9 expression and IGF-1R signaling in tumorigenesis and metastasis in both non-invasive and invasive cancer cells.

4.3. Materials and Methods

4.3.1. Media and Antibodies

Trypsin and DMEM were purchased from Fisher Scientific. Basement extract membrane was obtained from Trevigen (Gaithersburg, CA). The following antibodies were used for Western blotting: Rab5, MMP-9, anti-ERK, p38, Akt, phospho-ERK, phospho-cJun, phospho-p38, phospho-Akt, GAPDH, (Cell Signaling Technology, Danvers, MA). Anti-mouse, and anti-rabbit IgG peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Kinase inhibitors were obtained from Sigma (St. Louis, MO).

4.3.2. Cell Culture

The human breast cancer cell line, MDA-MB-231, was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in DMEM medium supplemented with 10% FCS, 2mM glutamine, and antibiotics (100 U/mL penicillin and 100µg/mL streptomycin) at 37°C in a humidified atmosphere of 5% CO₂.

4.3.3. Construction of recombinant pMX-puro retroviruses and cell lines

As previously described, the cDNAs of Rab5 and Rin1 and their mutants were subcloned into the pMX-puro vector⁵. Transfection of 60% confluent PhoA cells was

performed using Eugene (Roche) and the virus harvested after 48hrs post-transfection. MCF7 and MDA-MB-231 cell lines were infected with the retrovirus tagged with green fluorescent protein (GFP) and Rab5, Rin1 and control cell lines were generated and selected after 72hrs.

4.3.4. Cell Proliferation Assay

The viability of cells was determined by the MTT assay. MCF7 and MDA-MB-231 cell lines expressing Rab5 WT, Rin1, Rin1 mutant (R94A) or null vector were seeded in 96-well plates at a density of 25×10^3 cells/well and incubated overnight. Cells were then serum starved for 24hrs followed by treatment with serum and phenol red free DMEM with or without charcoal stripped 5% FCS for 48hrs. The MTT solution (5 mg/ml) was added to the plates and the cells incubated at 37 °C for 4hrs. The formazan, derived from MTT by living cells, was dissolved in 10% SDS (150µl per well), and the absorbance was measured at 595nm on a plate reader. All MTT experiments were performed in triplicate and repeated at least 3 times.

4.3.5. Cell Migration and Invasion Assays

The ability of MCF7 and MDA-MB-231 cells passing through Matrigel coated or uncoated filters was measured by the Boyden chamber invasion assay using Transwell inserts (8µm pores, Trevigen, MD). Cells were suspended in serum-free and phenol free DMEM and applied to the upper chamber. Serum and phenol free media with or without FCS as the chemotractant was added to the lower chamber and the plate incubated at 37°C for 24hrs. Non-migrated cells in the upper well were removed and the membranes were fixed and stained using 0.01% crystal violet. The membranes were either

photographed or counted under a light microscope ($\times 100$) or the dye extracted and the absorbance reading obtained using a spectrophotometer.

For the Matrigel invasion assay, transwell inserts coated with 0.5 μ g/ml Matrigel (BD Biosciences, MA) were used. Cells (10^5 per well) suspended in serum-free and phenol free DMEM were seeded onto the upper chambers of the precoated transwells. Serum and phenol free media with or without charcoal stripped FCS was added to the lower chamber and the plate incubated at 37°C for 24hrs. Subsequently, the membranes were fixed, stained, and photographed as above or absorbance readings acquired.

4.3.6. Wound-healing Assay

Cells were placed in inserts with a gap 500nm (\pm 50nm) (Ibidi, Verona, WI) and incubated at 37°C. After serum starvation for 24hrs, the inserts were removed, the wells were washed with PBS and medium with or without 5% charcoal stripped FBS was added. Cell migration was determined under the inverted microscope and pictures were obtained at time = 0 and 16hrs.

4.3.7. Anchorage-Independent Growth Assay

Briefly, 1×10^3 cells of each cell line were suspended in 300ul of Dulbecco's Modified Eagle's medium containing 10% calf serum and 0.35% SeaPlaque low-melting temperature-agarose. These cells were plated over a 300ul layer of solidified Dulbecco's Modified Eagle's medium containing 10% calf serum and 0.6% agarose, and cells were allowed to settle to the interface between these layers at 37 °C. After 20 min, the plates were allowed to harden at room temperature for 30 min before returning to 37 °C. 100ul of vehicle control or IGF-1 was added to wells and the plate incubated at 37 °C. The

plates were fed every 2–3 days. After 12-14 days, colonies were photographed under low magnification (x10).

4.3.8. RNA Isolation

After treatments of 15minutes, 1hr or 24 hrs, total RNA from MCF-7 cells was extracted using an RNAeasy mini kit (Qiagen) according to the manufacturer's instructions. The integrity and purity of the total RNA were checked using a Spectrophotometer (GE Healthcare).

4.3.9. Synthesis of Complementary DNA and Quantitative (real-time) PCR

Complementary DNA (cDNA) was created using qScript™ cDNA synthesis kit (Quanta BioSciences). Q-PCR was performed using the CFX96™ Real-Time PCR Detection System (Bio-Rad) in a two-step procedure using SSo Fast (Biorad). Amplification of 18s was performed to standardize the amount of sample cDNA. Primer were 18s, Rab5a, Rab5b, MMP9 (Life Technologies). All reactions were performed in a 96 well plate with the following cycling conditions: 40 cycles of 95°C for 15s, and 60°C for 30 s, 72°C 1 min. To report fold changes, $\Delta\Delta C_t$ values were obtained and fold change is derived as $2^{-\Delta\Delta C_t}$ with the value of each control sample was set at 1 and used to calculate the fold-change of target genes.

4.3.10. Statistical Analysis

All samples in this study were analyzed in duplicate and each experiment was repeated three times. Values represent the mean \pm SEM of three independent experiments. To compare two groups, student's t test was utilized. $P < 0.05$ was considered as statistically significant.

4.4. Results

Rab5 and RIN1 regulated IGF-1 signaling induced morphological changes in MCF7 cells consistent with Epithelial Mesenchymal Transition

Previous studies have shown that there is an intrinsic relationship between IGF-1 signaling and breast cancer^{34,41}. Here, the morphological changes that occurred in MCF7 breast cancer cells overexpressing Rab5 and RIN1, when treated with IGF-1, suggest this may be consistent with EMT.

In Figure 14a, cells were treated with IGF-1, and subsequently stained with phalloidin to visualize the actin filaments. In IGF-1 treated cells, more actin accumulated to the edge of the cells (Figure 14a. arrows), particularly in Rab5 and RIN1 overexpressing cell lines. Further examination also illustrated that some cells underwent a shift in epithelial morphology as they were more elongated and fibroblast-like, and similar in morphological characteristics like the invasive breast cancer cell line, MDA-MB-231²⁶.

4.4.2 Inhibition of IGF-1R trafficking modulated cell migration and invasion of MCF7 cells.

As it was ascertained in Aims 1 and 2, both Rab5 and RIN1 expression is required for IGF-1 signaling which drive cell migration and invasion. It is known that IGF-1 signaling is dependent on IGF-1R activation^{9,24}. IGF signaling is initiated by the binding of the IGF-1 ligand to the IGF-1R and the subsequent auto-phosphorylation of tyrosine residues of specific cytosolic kinase domains^{14,29,35} and the association specific adaptor molecules such as IRS, Shc²⁵ and RIN1²³. To determine if IGF-1 signaling was mediated through the IGF-1R in breast cancer cells, an IGFR inhibitor, AG1024³² was administered to the cells for 1hr prior to cell invasion and migration assays.

In Figures 14b and 14d, AG1024 abrogated migratory and invasive capacity of MCF7 cells expressing either Rab5 or RIN1, thereby suggesting that the IGF-1R is involved in IGF-1 signaling in breast cancer progression. Considering that Rab5 has been implicated in the endocytosis of RTKs, it was imperative that the actual mechanism of IGF-1R internalization was clarified. IGF-1R endocytosis has been said to be both clathrin and caveolin dependent³⁰. The latter, however has a stronger affinity in IGF-1 signaling^{7,37} and it was worth investigating if IGF-1 signaling in breast cancer was dependent on lipid raft formation. MCF7 cells were pre-treated for 1hr with the caveolin inhibitor i.e. Filipin, and the effect on migration and invasion observed using the Boyden chamber. Like the IGF-1R inhibitor, Filipin suppresses cell migration and invasion of MCF7 cells, particularly in RIN1 over-expressing cells. Cells overexpressing Rab5, while showing a decrease compared to the control, possessed a higher migratory and invasive potential like RIN1 expressing MCF7 cells, implying that there may be a possible link between RIN1 activity and caveolin invagination of the IGF-1R.

4.4.3 Colony formation requires caveolin dependent IGF-1R trafficking

During cancer progression there are two main phases of tumor formation, the onset of hyper-proliferative growth and primary colony formation during the pre-metastatic phase, and the secondary colony formation, post metastasis¹⁷. Based on previous results, it was illustrated that IGF-1 promotes aggressive tumor growth from single tumor cells in agarose suspension. Most importantly, it was dependent on Rab5 and RIN1 activity, suggesting that the IGF-1R may play an integral role in colony formation. In the presence of the AG1024 or Filipin inhibitors (Figure 14g), colony formation was significantly curbed, providing additional evidence that IGF-1R is involved in breast cancer

progression. Furthermore, this IGF-1R involvement requires trafficking to the early endosome via a caveolin dependent mechanism possibly through the invagination to the caveosome and docking on the early endosome.

4.4.4 AG1024 and Filipin modulates cell migration and invasion in MDA-MB-231 cells

Since cell migration and invasion was negated in MCF7 cells in the presence of inhibitors, it was interesting to see if in a highly invasive breast cancer cell line, the ability for cells to migrate and invade would also be affected. MDA-MB-231 cells were also transduced with GFP, Rab5 and RIN1 and pretreated with AG1024 or Filipin for 1hr. Migration and invasion was assayed using the Boyden chamber and after 18hrs, the absorbance of migrated and invaded cells was quantified.

As expected, there was a reduction in cell migration and invasion in the presence of the kinase or caveolin inhibitor (Figures 15 a , c, d and e). Unlike MCF7, Rab5WT-expressing MDA-MB-231 cells migrated and invaded less than RIN1 cells.

Cell migration and invasion was largely unaffected in RIN1:R94A cells compared to controls, implying that RIN1 is recruited upon IGF-1R stimulation prior to caveolin-dependent internalization of the receptor. Figure 15b, illustrated that inhibition of the IGF-1R significantly affected migration in both Rab5WT and RIN1 breast cancer cell lines supplementing the data obtained that IGF-1R must be involved in IGF-1 signaling in breast cancer progression.

4.4.5 RIN1 mediates IGF-1 induced expression consistent with IGF-1R trafficking in breast cancer cells

To determine if Rab5 expression is substantial upon IGF-1 stimulation in RIN1 expressing cancer cells, real-time PCR was performed with primers for Rab5a and Rab5b in both MCF7 and MDA-MB-231.

Figure 16a shows the role of Rab5a in cancer progression and the importance of the receptor in IGF-1 signaling during disease progression. In the presence of the kinase inhibitor, there was considerable down-regulation of Rab5a expression when compared to IGF-1 control (where the fold change >2 is considered significant) in cancer cells overexpressing RIN1. Addition of Filipin also induced marked suppression of Rab5a in the presence of IGF-1. This clearly denotes that Rab5a function in IGF-1 signaling in breast cancer cells may be dependent upon caveolin mediate internalization of the IGF-1R. In MDA-MB-231 cells, the outcome was similar to MCF7 with AG1024 repressing Rab5a gene activity (Figure 16d). To verify if other Rab5 isoforms may be involved in IGF-1R trafficking in breast cancer cells, Rab5b expression was observed. Surprisingly, Rab5b expression was comparable to Rab5a, where Rab5b was up-regulated significantly in the presence of IGF-1 and inhibited when AG1024 was introduced (Figures 16f). A key finding (Figures 16c and e) demonstrated that Rab5 gene expression was affected in cells expressing RIN1:R94A mutant, indicating that the ability of RIN1 to bind to the activated receptor is necessary for Rab5 function in early endocytosis of the receptor.

4.4.6 Endocytosis of the IGF-1R increased metalloproteinase expression in breast cancer cells

During cancer cell invasion, proteases are stimulated and secreted by growth factors including IGF-1^{17,41}. Given earlier data that illustrated acquisition of fibroblast like phenotype in MCF7 cells upon IGF-1 stimulation (Figure 14a), the next step was to ascertain if Rab5 and RIN1 trafficking of the IGF-1R facilitates protease expression. Using real time PCR with primers for MMP9 in both MCF7 and MDA-MB-231 cell lines, MMP9 gene expression was significantly enhanced in cells overexpressing Rab5 and RIN1. In MCF7 cell (Figure 17b), Rab5 expression elicited a 10-fold increase in MMP9 gene expression. RIN1 elicited lower but still significant levels of MMP9 mRNA, about 2.5 fold change in both MCF7 and MDA-MB-231 (Figure 17b and 17c respectively). Addition of AG1024 and Filipin to MCF7 cells (Figure 17c) reduced MMP9 expression with the fold changes at 1 or less, supplementing the cell invasion data that the inhibitors repress the invasive capacity of MCF7 in the presence of IGF-1. While one of the traits of MDA-MB-231 is a highly invasive phenotype, it is shown that MDA-MB-231 in response to IGF-1 can up-regulate MMP9 gene expression, via RIN1 activity (Figure 17d and e). Moreover, RIN1:R94A significantly suppressed MMP9 expression corroborating RIN1 and Rab5 roles in cell invasion.

4.5. Discussion

Proliferation, migration and invasion are regulated by a wide array of growth factors and cytokines in cancer metastasis⁸. Growth factor signaling are reliant on receptor phosphorylation and the utilization of specific inhibitors for RTKs are important in elucidating the role growth factors and their receptors in membrane trafficking and

signaling¹⁰ in cancer cell migration and invasion. In fact, RTK activation is pervasive during the endocytic process, from internalization to lysosomal and/or recycling processes.

Here (Figures 14b and 14d), it was demonstrated that the IGF-1 inhibitor, AG1024, suppressed the invasive and migratory activity of MCF7 breast cancer cells indicating that IGF-1 signaling was dependent on the IGF-1R. Rab5a expression was significantly diminished when the IGF-1R was blocked (Figure 16a), implying that the receptor must be activated for Rab5 mediated early endocytosis to occur. Confirmation that internalization of IGF-1R can be clathrin independent^{30,37} was validated by the addition of Filipin, indicating that IGF-1R endocytosis is caveolin-dependent and entailed Rab5 activation and early endocytic function (Figure 16b). Down-regulation of Rab5a through IGF-1R inhibition led to decreased cell invasion and migration, supporting previous reports from this study that growth factor driven cell migration and invasion was Rab5 dependent in breast cancer cells.

In MDA-MB-231 cells (Figures 15a and 15d), it appears that there was partial blocking of the IGF-1R in cells overexpressing RIN1, suggesting that IGF-1 signaling was also mediated by another mechanism independent of the IGF-1R. Within anchorage independent conditions, it was ascertained that IGF-1 interacts with the Integrin receptor ($\alpha\beta3$) and facilitates IGF-1 signaling regardless of IGF-1R inhibition¹⁶. Integrins are heavily involved in the cell migration process during cancer progression and Rab5 is known to regulate $\alpha\beta3$ endocytosis⁴⁰. Hence, it can be assumed that during cell migration of breast cancer cells, IGF-1 can also stimulate the $\alpha\beta3$ receptor, leading to the RIN1 interaction of Rab5 and sequestering of the active Rab5 complex to the cell

membrane and subsequent endocytosis of the $\alpha\text{v}\beta 3$ receptor. On the other hand, RIN1 binds to non-receptor ABL tyrosine kinases, where the latter arbitrates RTK phosphorylation and endocytosis, and directs actin remodeling in cell migration^{22,38}. RIN1 directed endocytosis of the RTK and the ultimate fate of the receptor involves a delicate balance between RIN1:Rab5 and RIN1:ABL interaction³. Specifically, ABL confers a protective influence on RTK activity, increase active Rab5 levels stabilized the receptor and shifted the mechanism to increased receptor internalization and recycling, and directed cell motility⁴.

In Figure 15a, despite the presence of Filipin, MDA-MB-231 cells overexpressing RIN1 may utilize ABL signaling via IGF-1R phosphorylation, bypassing caveolae receptor internalization. Taken together, it can be concluded during IGF-1-directed cell migration and invasion in breast cancer, IGF-1 signaling is both dependent and independent on IGF-1R.

IGF-1 has engendered morphological changes in breast cancer cells via TGF activation⁴¹ where atypical epithelial cells acquired an EMT configuration. This study revealed that in MCF7 cells overexpressing RIN1 and Rab5, IGF-1 stimulated breast cancer cells had modified morphological features as they were more elongated and fibroblast-like, typical of cells undergoing EMT¹⁷. During EMT, protease activity is integral, allowing cells to migrate and invade to distal tumor sites. Based on this, it was imperative to determine if MMP expression was correlated with IGF-1R trafficking in breast cancer cells.

MMPs are intergral family of endopeptidases involved in the metastatic potential of cancer. Walsh et al highlighted that IGF-1 significantly increased MMP expression and

subsequently the proteolytic and invasive response of MCF7 cells⁴¹. Few studies currently exist that illustrate the functional role of endocytosis in the regulation and trafficking of MMPs. In breast cancer cell lines expressing the estrogen receptor, Rab27b has been implicated in the regulation of MMP-2 activation through heat shock protein (Hsp) 90a secretion and stimulate invasion of breast cancer cells^{19,31}. Growth factor stimulated breast cancer cells overexpressing Rab5a affected Rab4 and Rabenosyn-5-dependent endo/exocytic cycles in the ferrying of the matrix protease, MMP-1 and β 3 integrin¹⁵. This trafficking cycle produced a chemotactic-dependent invasive and proteolytic mesenchymal response in breast carcinoma cells *in vitro* and *in vivo*¹⁵.

Here, it is shown that an inherent relationship may exist between endocytosis and MMP9 activity in the presence of IGF-1. In both ER positive and ER negative cell lines, MMP9 expression increased in response to IGF-1 stimulation (Figures 17a-e). Inhibition of the IGF-1R suppressed MMP9 levels showing persuasive the role of IGF-1 signaling in breast cancer cell invasion (Figures 17c and e). In the archetypal non-invasive MCF7 cells line, Rab5 over-expression garnered the most positive change in MMP9 gene expression (Figure 17b). This hints, not only of the potency of IGF-1 in accelerating the alteration to an aggressive breast cancer phenotype and behavior, but also the importance of Rab5 in mediating protease secretion and action during IGF-1R trafficking in breast cancer cell invasion.

As described above, IGF-1R internalization is caveolin dependent as Filipin hindered MMP9 gene expression in response (Figure 17c). Conversely, given the highly invasive nature of MDA-MB-231 cells, it was expected that MMP9 expression would be more dominant compared to MCF7². Yet, MMP9 gene expression, while significant, was

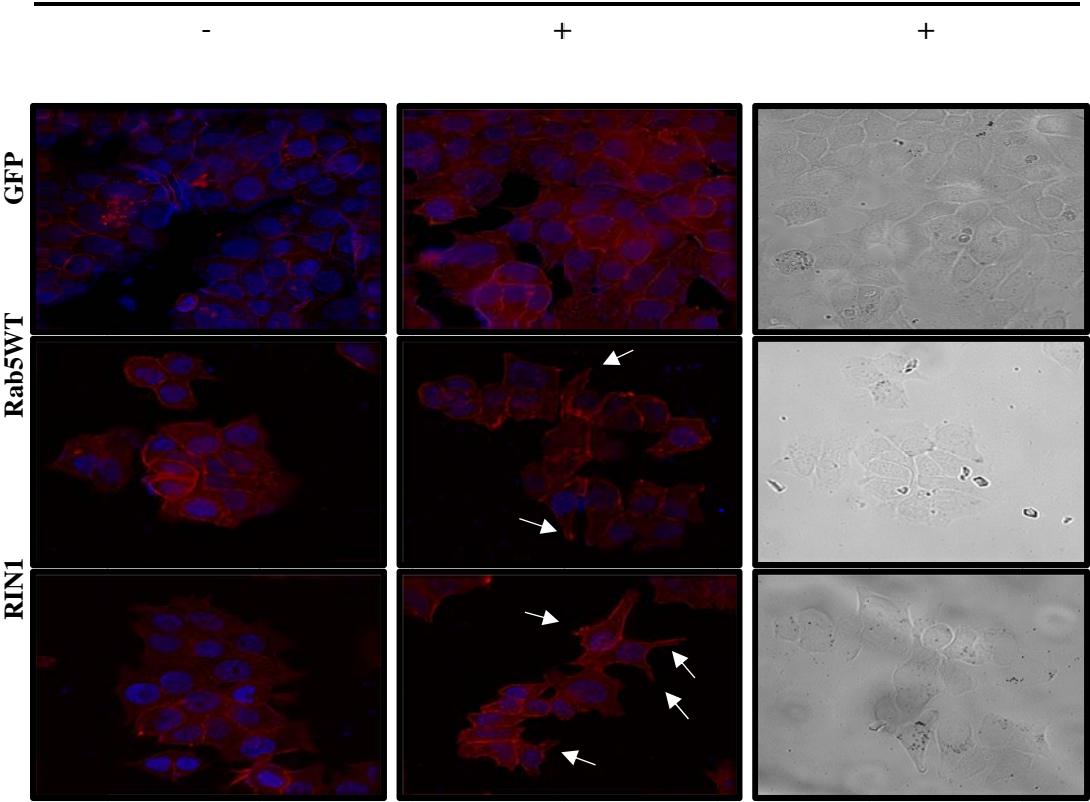
almost the same (about 2.5 fold) compared to protease expression in MCF7 cells. Perhaps this can be ascribed to the fact that as MDA-MB-231 is already highly invasive, the change in MMP9 expression in response to growth factor would not be as drastic compared to the MCF7 cells undergoing IGF-1-mediated transformation to an aggressive status. As expected, the RIN1:R94A cell line affected MMP9 expression. Therefore the inability for RIN1 to bind to the IGF-1R down-regulated MMP9 expression (Figures 17d).

In summation, this study illustrates a novel model in IGF-1 signaling in breast cancer progression. IGF-1 signaling relies heavily on the IGF-1R activation and caveolae-dependent trafficking through Rab5 and RIN1 activity. Additionally, this study show for the first time that there may be a link between caveolin dependent endocytosis and RIN1 activation in IGF-1 signaling. RIN1 overexpression increases endogenous caveolin-1 in early endosomes, and upon internalization, translocation and tethering of active caveosomes to the early endosomes is Rab5 dependent³³. The interplay between IGF-1R trafficking and MMP9 activation through RIN1 and Rab5 GTPases function elucidates one of the mechanisms of invading breast cancer cell. With the establishment of Rab5 and it GEF, RIN1 as crucial regulators in IGF-1 signaling in cancer cell migration and invasion, characterization of the RIN1- caveolin interaction as well as downstream signaling pathway need to be further studied.

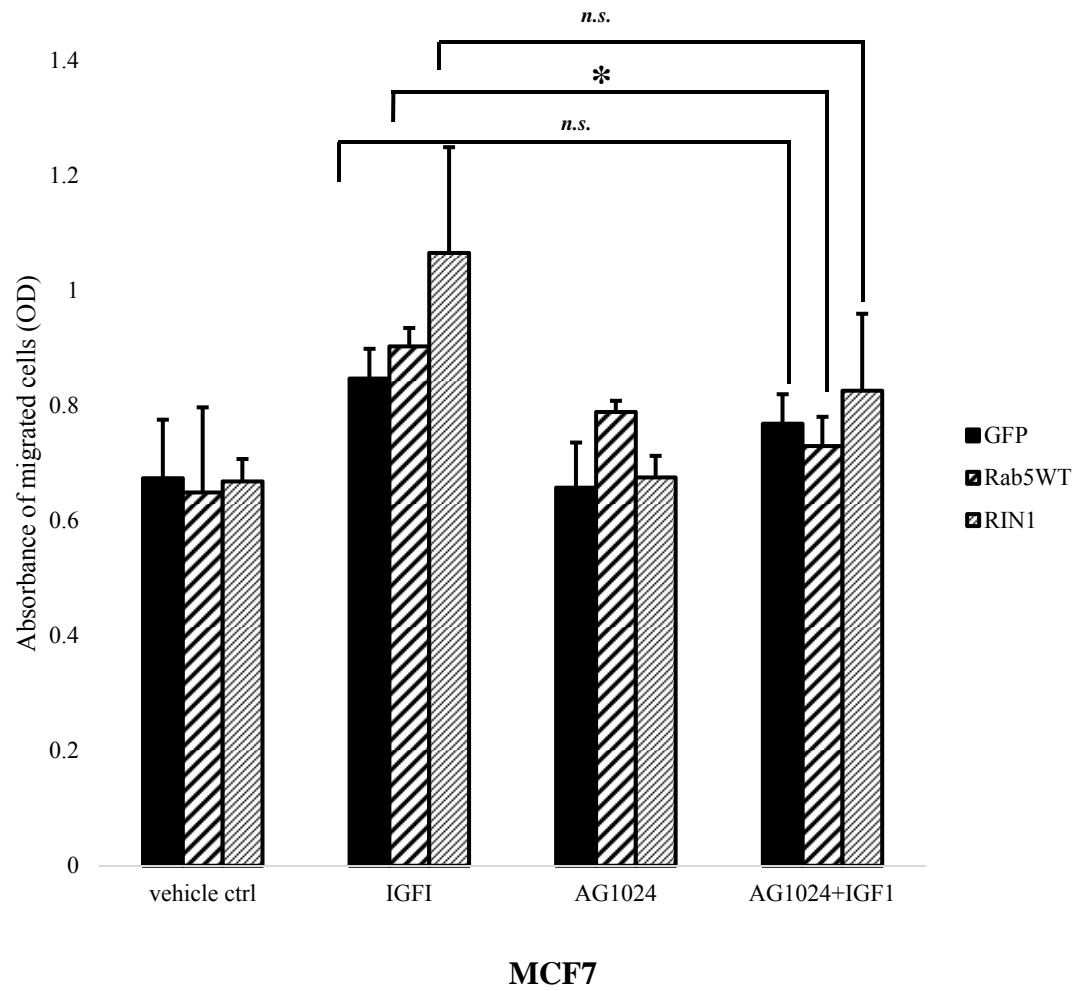
4.6 Figures

Figure 14a.

IGF-1

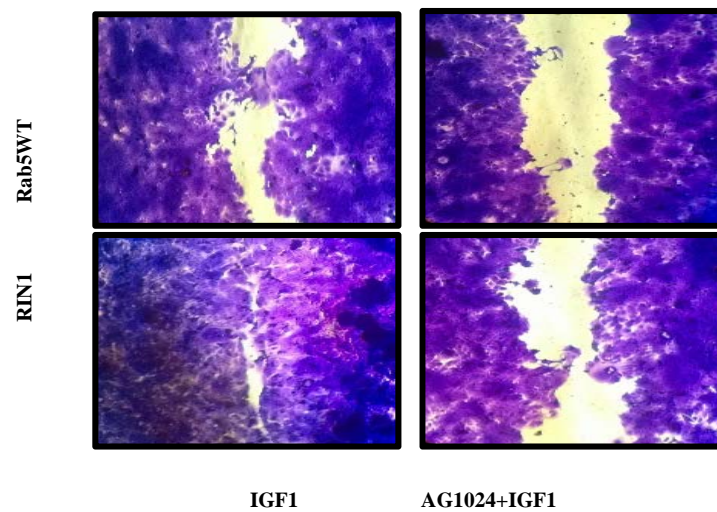


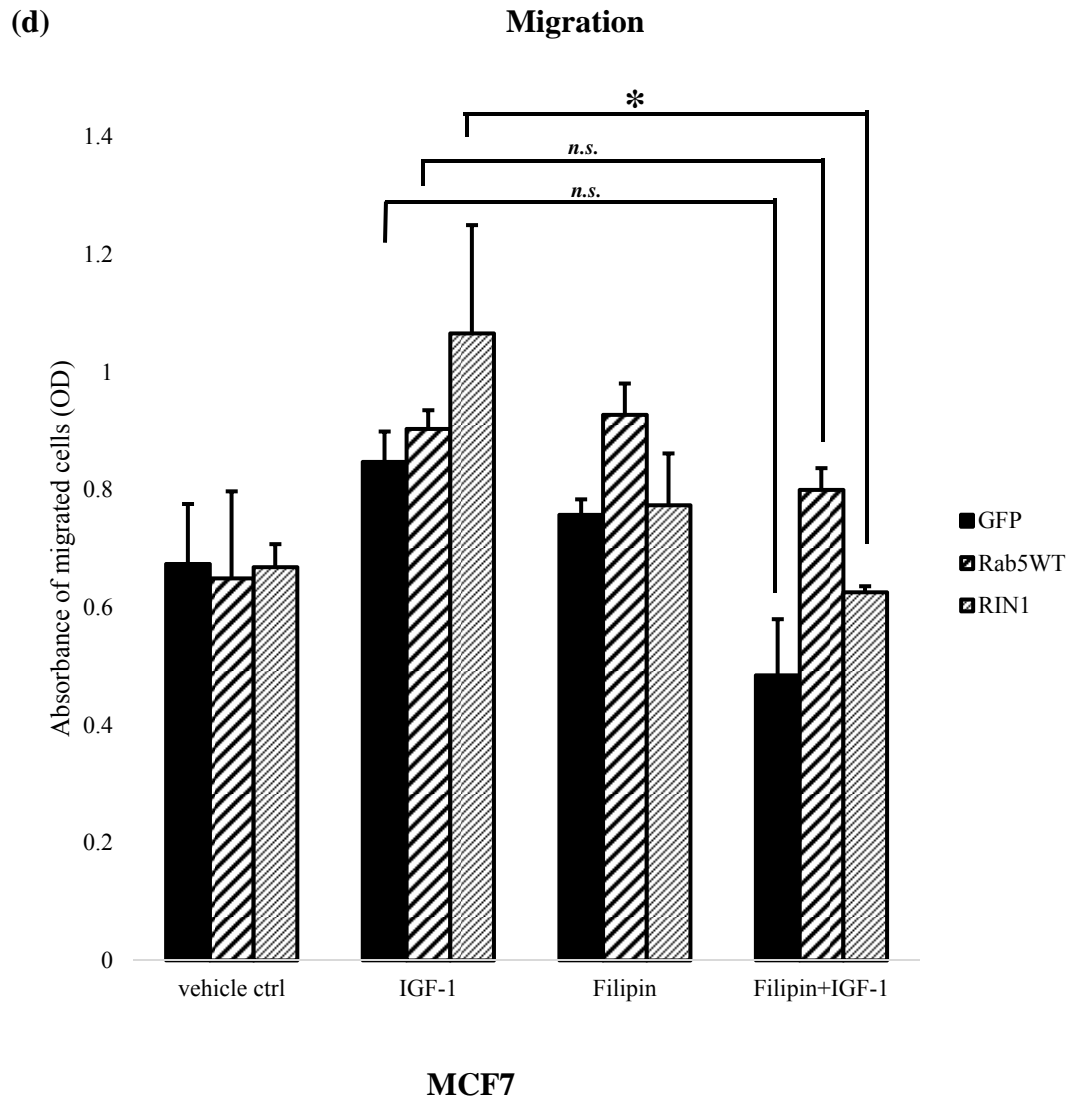
(b)



(c)

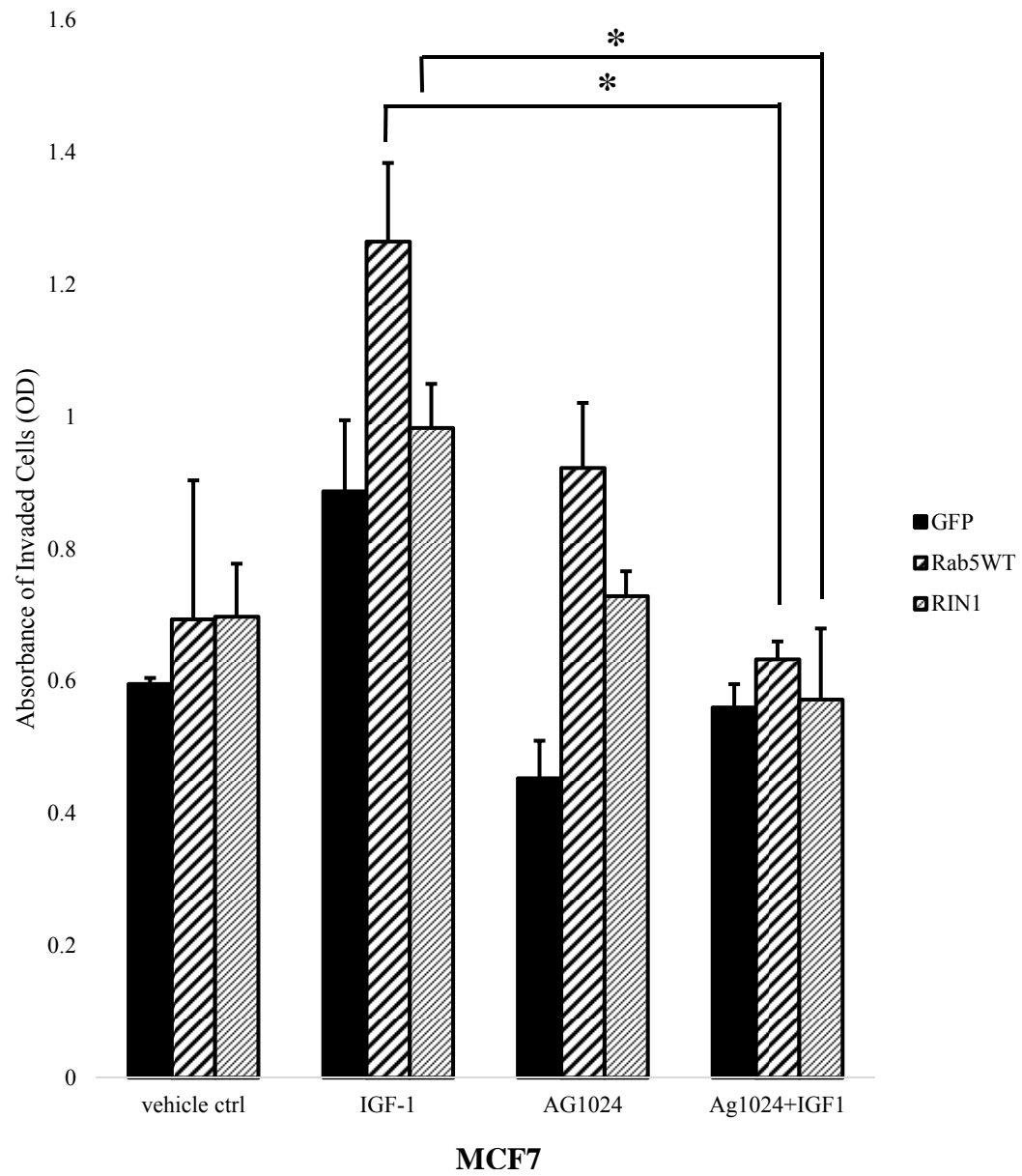
Cell Migration/ Wound Assay



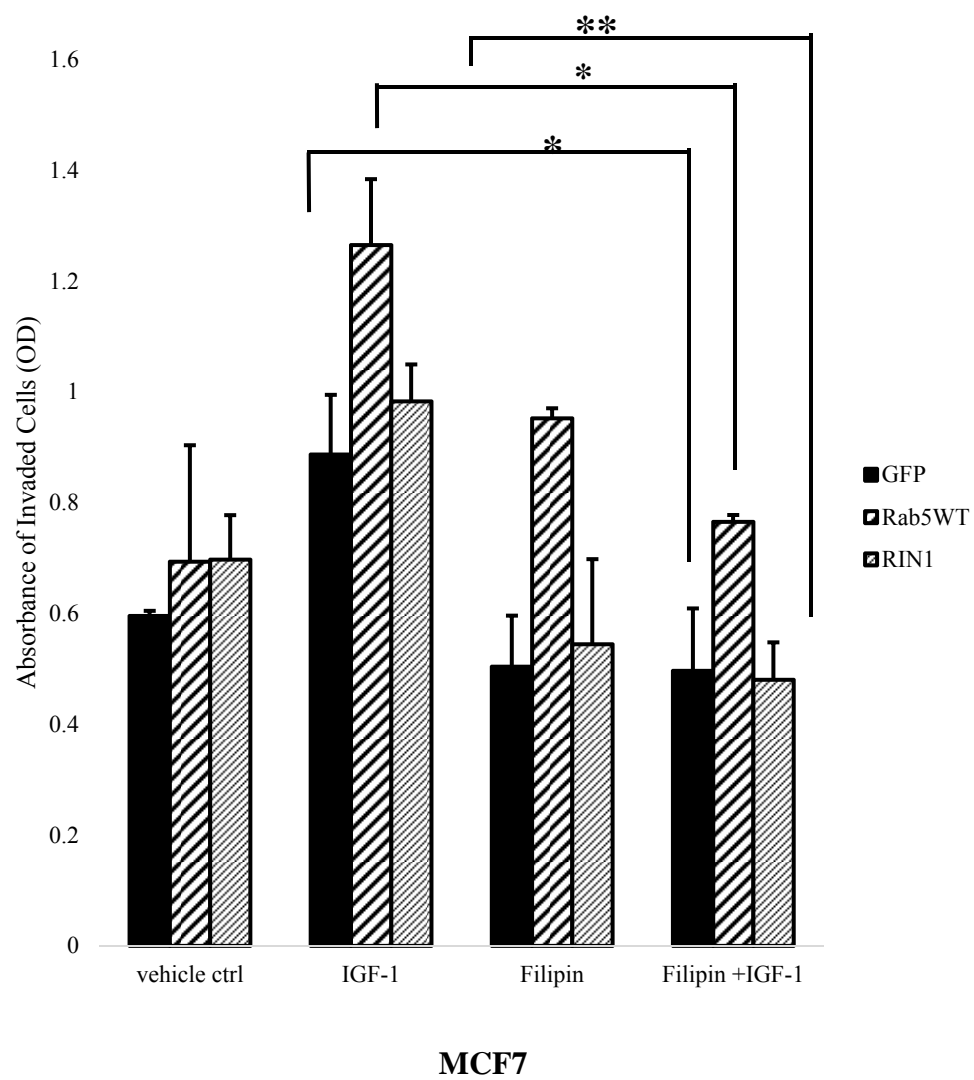


(e)

Invasion

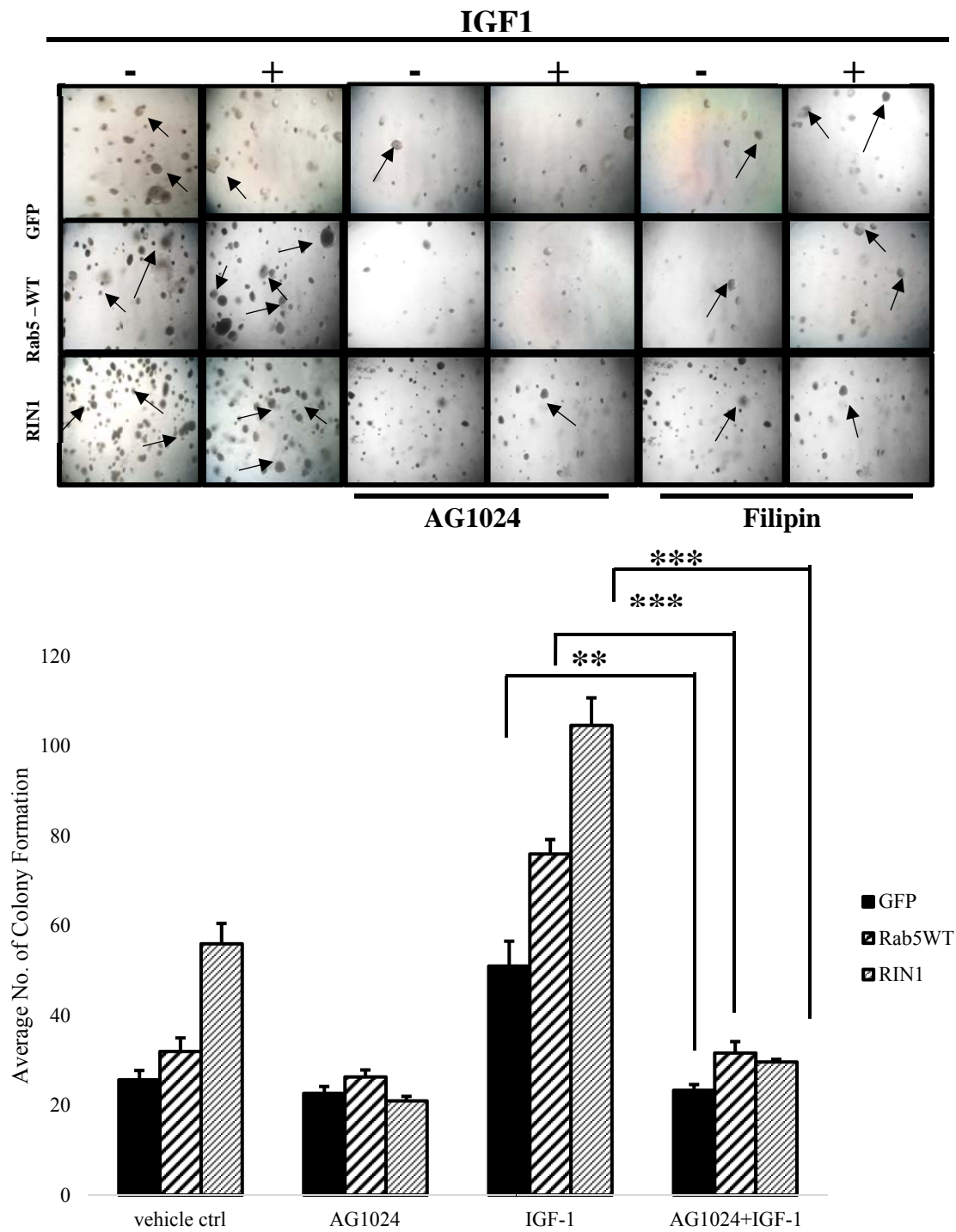


(f) **Invasion**



(g)

Colony Formation



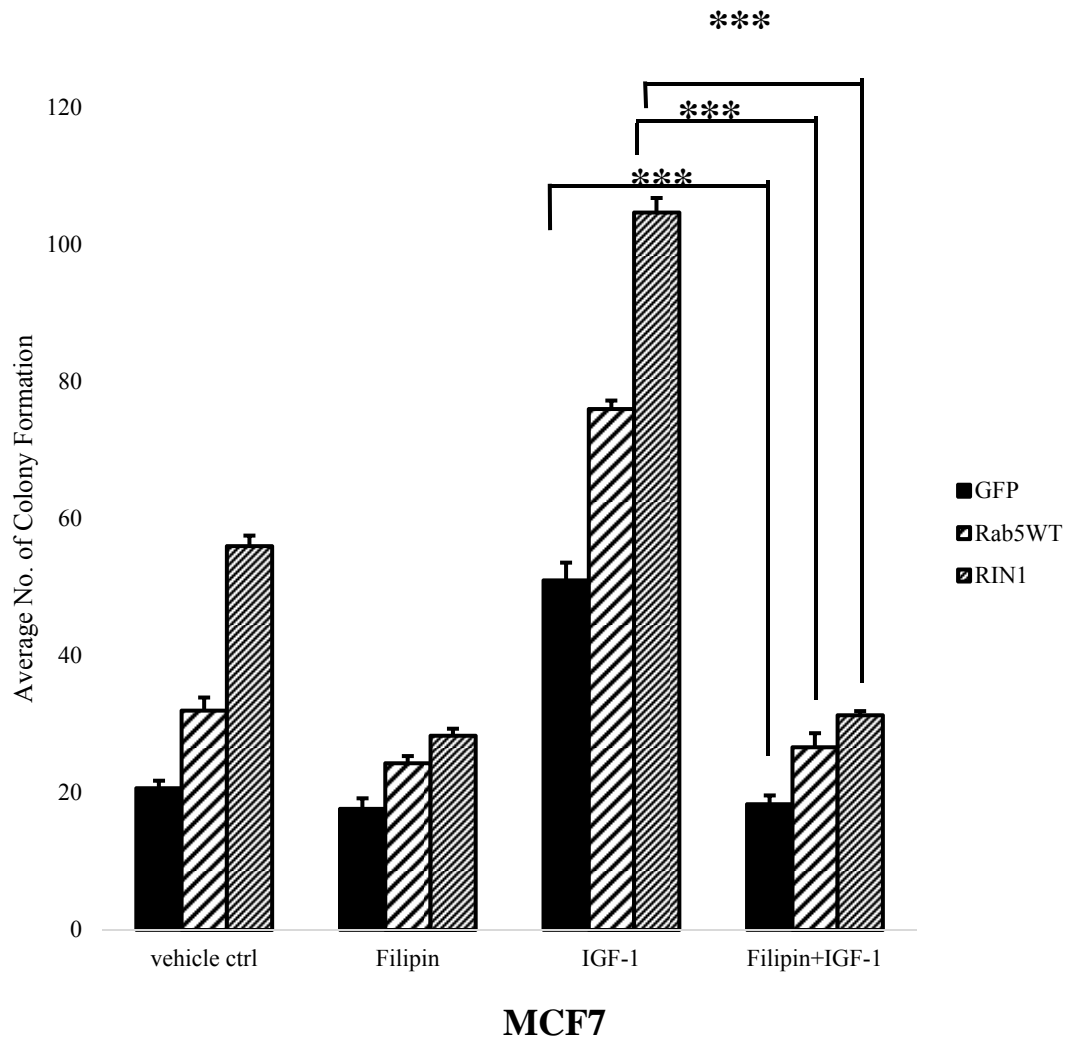
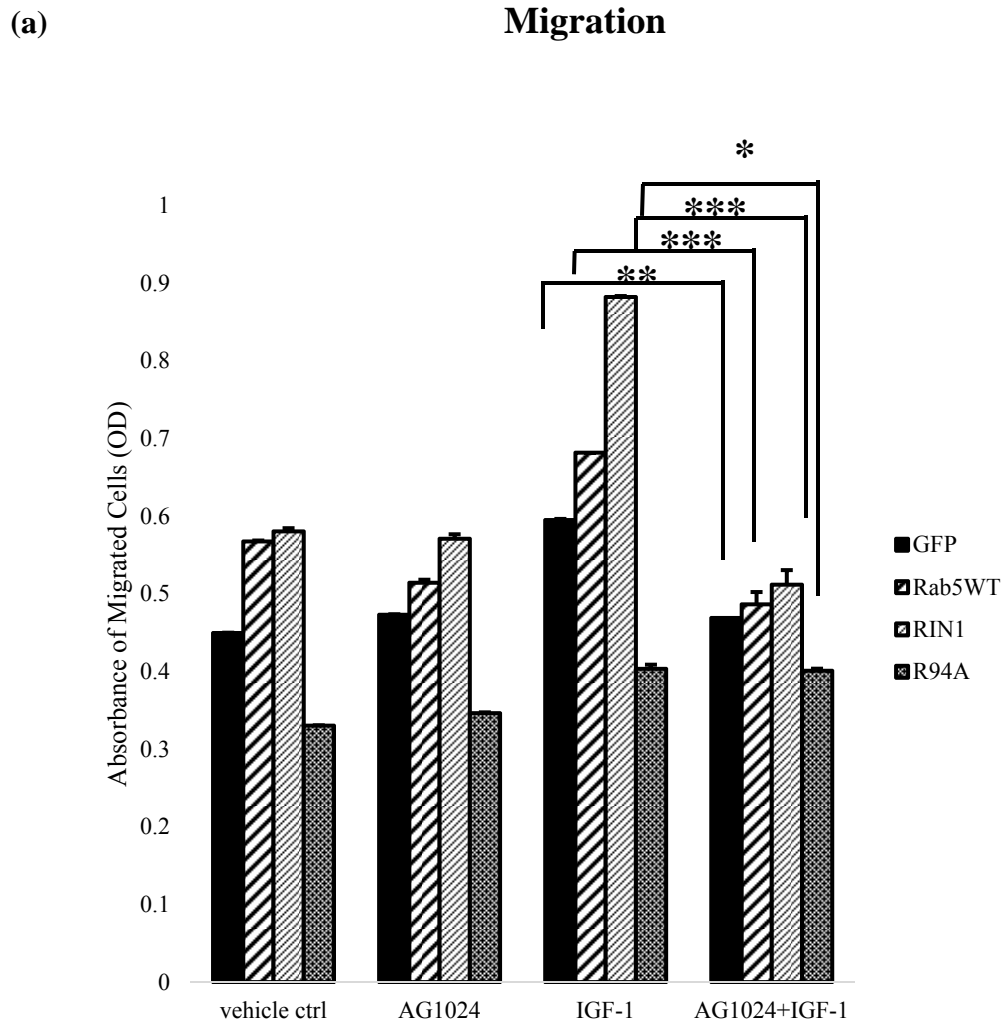


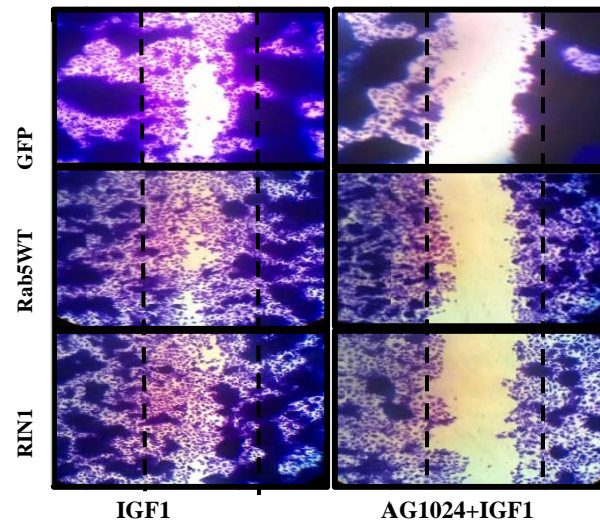
Figure 14a Rab5 and RIN1 influenced morphological changes in MCF7 cells when treated with IGF-1. Figure 14a illustrated the mobilization of actin fibers in MCF7 cells to the cell surface in response to IGF-1 with a strong response in Rab5 and RIN1 cell lines. Cells were seeded on coverslips, incubated for 18hrs in the absence or presence of IGF-1. After fixing and staining with actin phalloidin, the prepared slides were observed under a confocal microscope. Cytoskeleton was stained with phalloidin (red) and the nucleus with DAPI (blue) with more actin (red) was seen accumulating to the cell surface in IGF-1 treated cells. **In MCF7 cell lines, IGF-1 signaling requires IGF-1R activation and dependent on caveolin mediated internalization.** Figures 14b, c, e and f, migration and invasion of the tumor cells were affected by both the kinase and caveolin inhibitors. Cells were plated in a Boyden chamber without or without basement extract membrane. Both kinase and caveolin inhibitors were added to cells for 1hr. Treatment groups were

IGF-1 or vehicle control and cells were incubated at 37°C for 16hrs. Crystal violet staining was performed and the dye extracted and the OD measuring using a plate reader at 595nm. Cell migration and invasion absorbance readings were expressed as average \pm SEM for three independent experiments. Using an IBIDI gap closure system, Figure 14d is a representative picture depicting the effect of AG1024 on cell migration MCF7, where blocking of IGF-1R signaling down-regulated IGF-1R trafficking in Rab5 and RIN1 overexpressing MCF7 cells. In Figure 14g, cells were pretreated for 1hr with the inhibitors, followed by sparse seeding in agarose and cultured for 14 days with or without IGF-1. At the end, pictures of colony formation from single cells were taken and the average number of colonies were calculated for 3 random areas of each well. As shown, both Rab5 and RIN1 cells had more colonies in response to IGF-1. Inhibition of the IGF-1R and caveolin suppressed colony formation particularly in response to IGF-1. (*) denotes ($P < 0.05$), (**) is ($P < 0.01$), (***) is ($P < 0.001$)

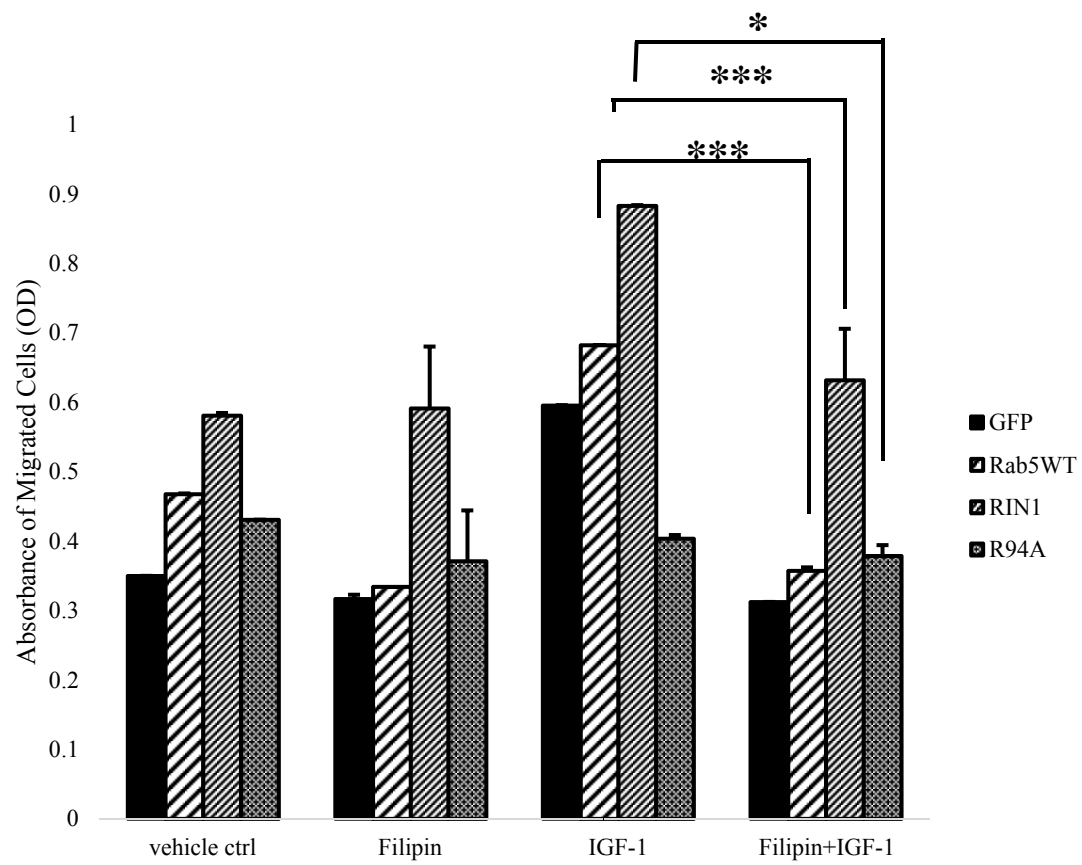
Figure 15



(b)

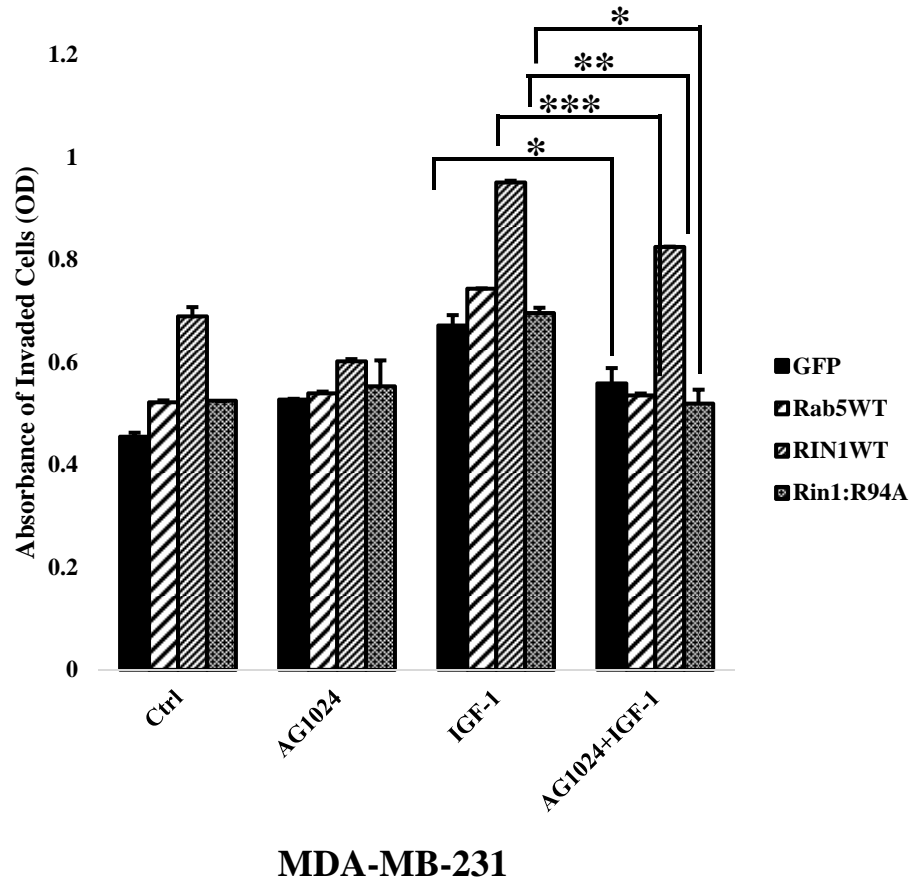


(c) Migration

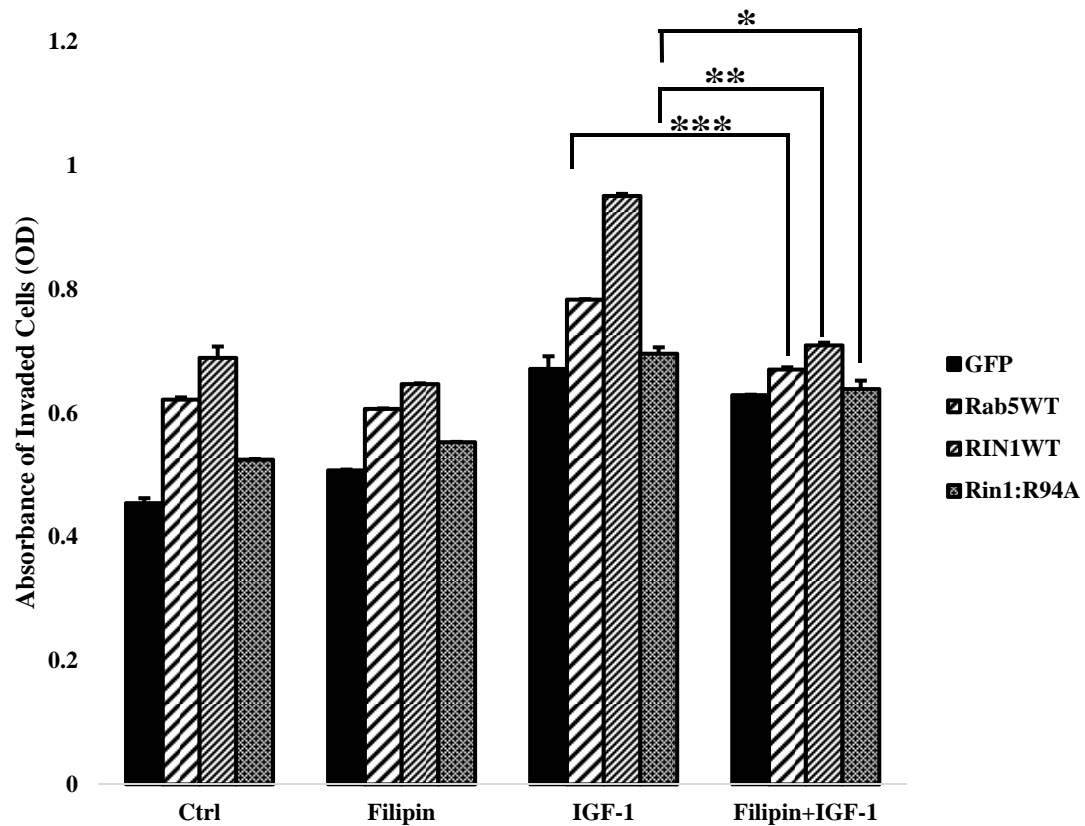


(d)

Invasion



(e) **Invasion**

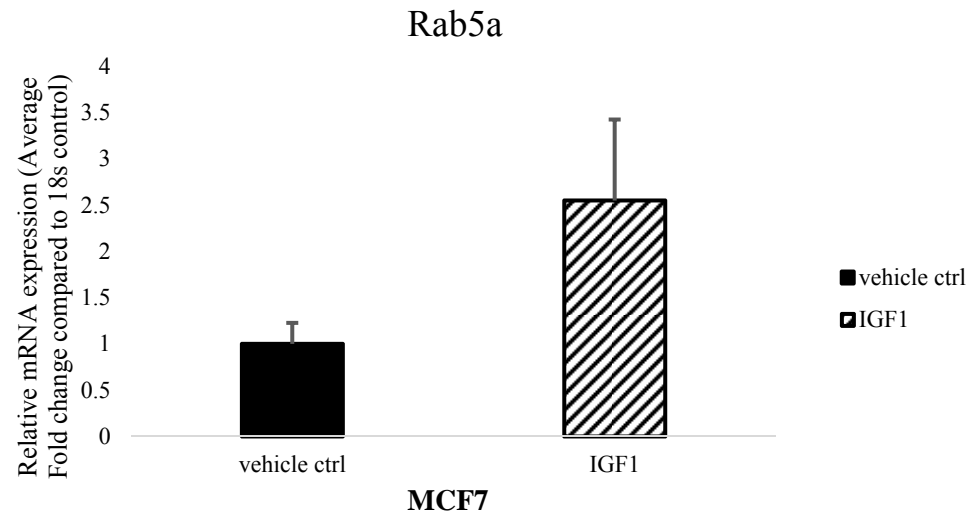


MDA-MB-231

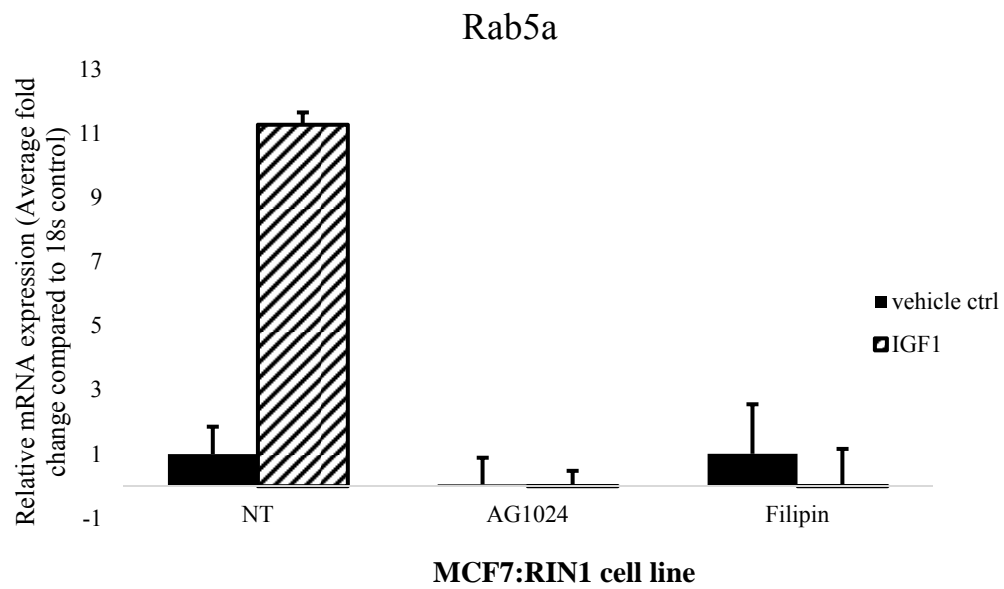
Filipin suppresses cell migration and invasion of MDA-MB-231 cells upon IGF-1 stimulation. (Figures 15a, c, d, e) Cells were plated in a Boyden chamber without or without basement extract membrane. Both kinase and caveolin inhibitors were added to cells for 1hr. Treatment groups were IGF-1 or vehicle control and cells were incubated at 37°C for 16h. Crystal violet staining was performed and the dye extracted and the OD measured using a plate reader at 595nm. Cell migration and invasion absorbance readings were expressed as average \pm SEM for three independent experiments. (*) denotes ($P < 0.05$), (**) is ($P < 0.01$), (***) is ($P < 0.001$). In Figure 15b, an IBIDI culture insert (IBIDI GmbH) was placed into wells of a 24 well plate and slightly pressed on the top to ensure tight adhesion. An equal number of cells were placed in each of the insert and the cells incubated for 16hrs. Gap closure was observed at a magnification of 10X and images recorded. Crystal violet staining was done for better imaging of the migration of the cells

Figure 16

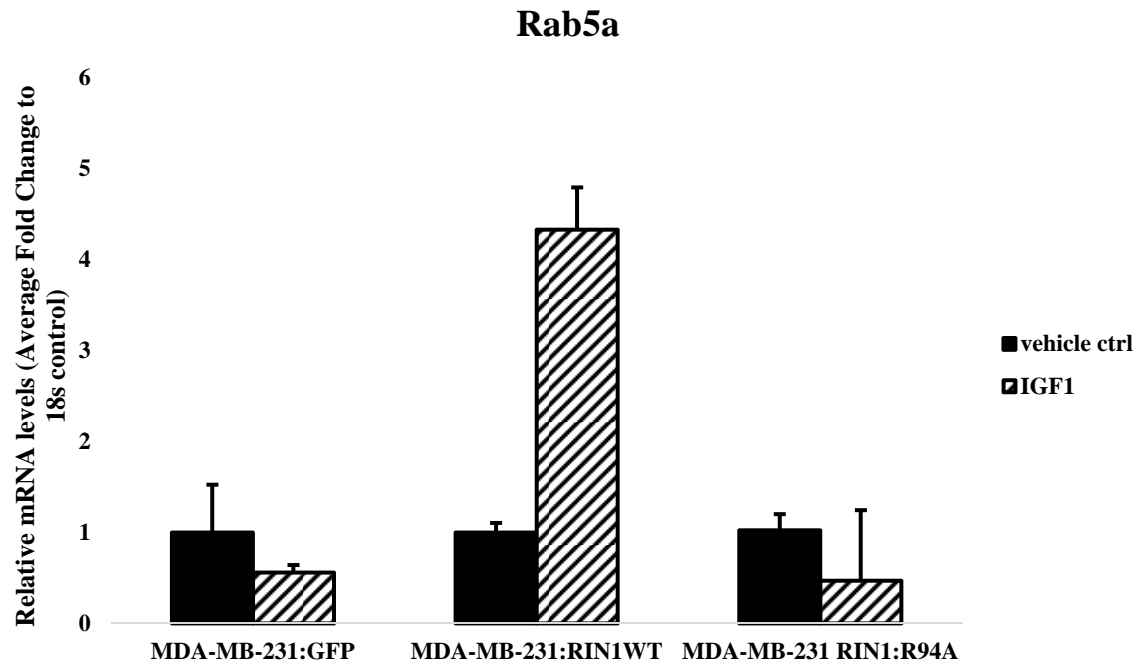
(a)



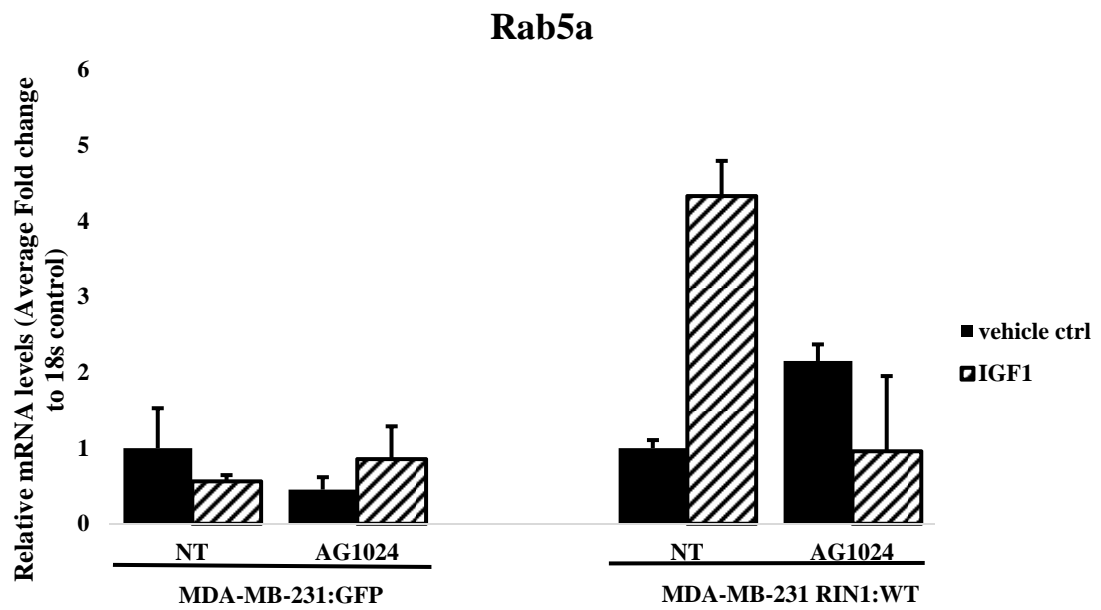
(b)



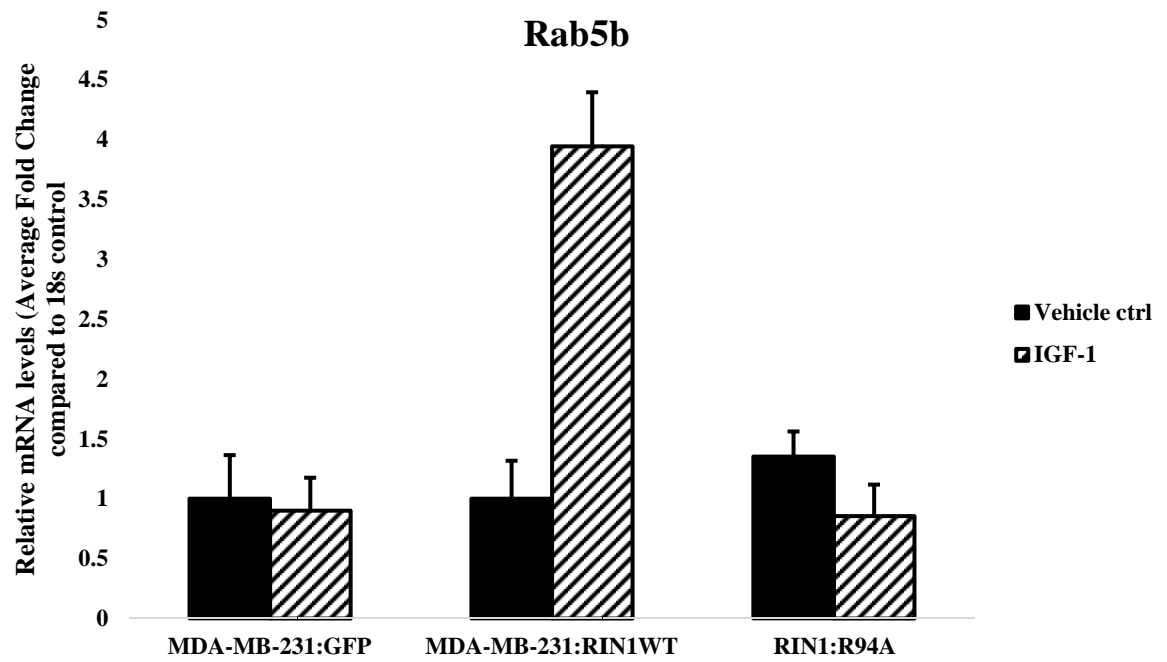
(c)



(d)



(e)



(f)

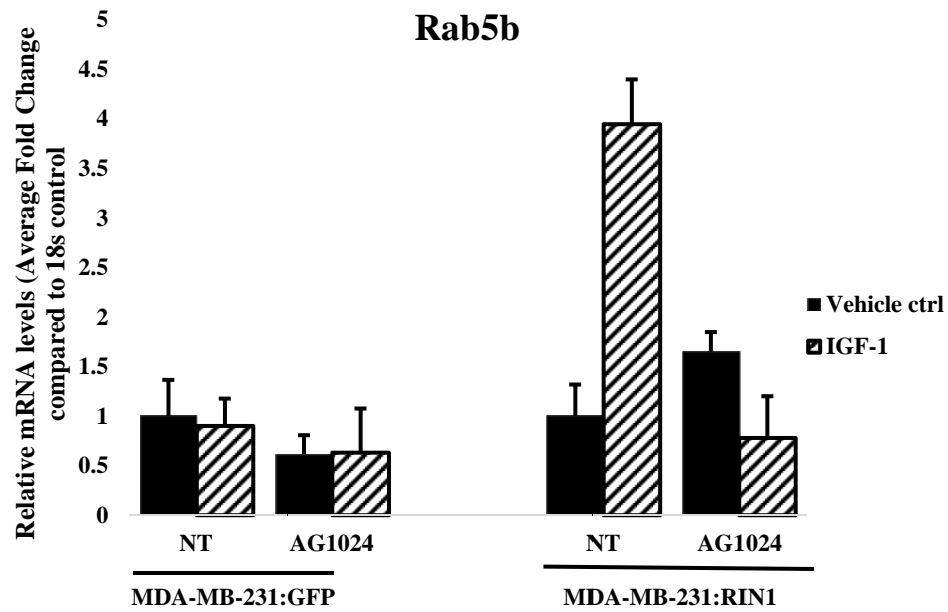
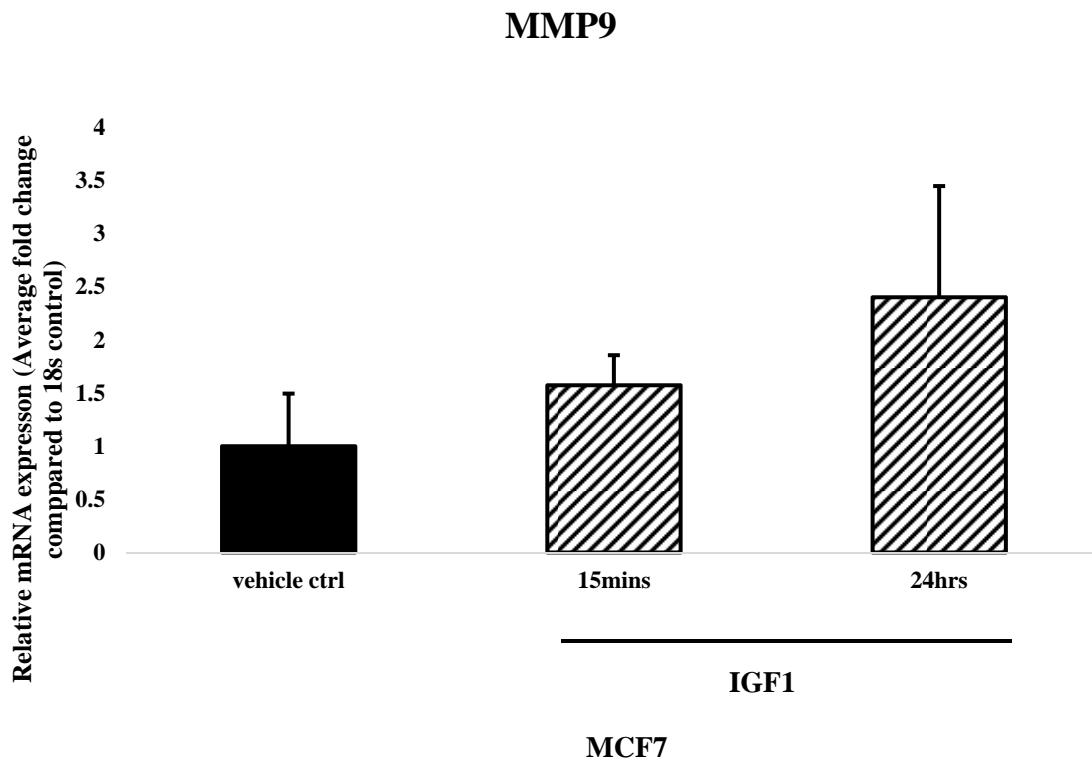


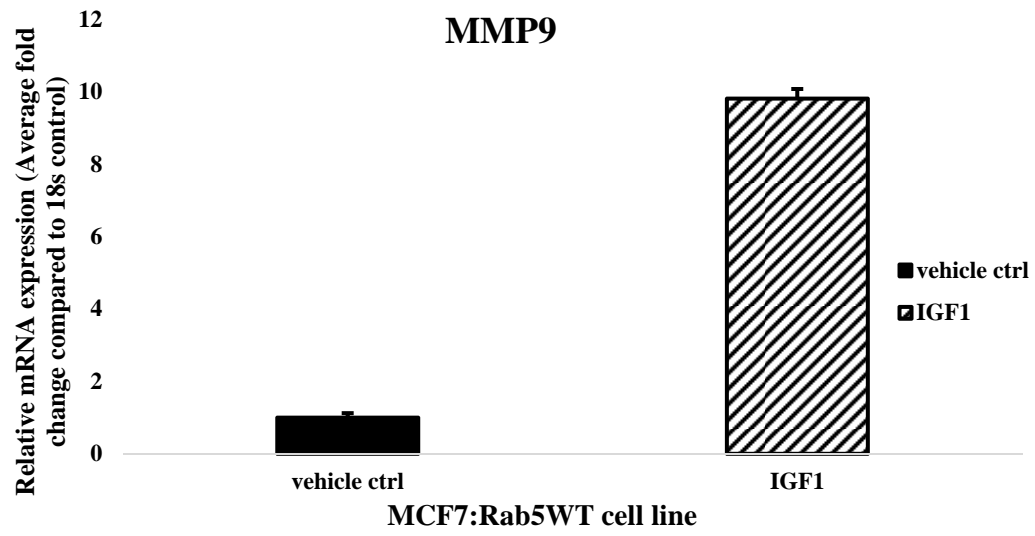
Figure 16. Real time PCR showed Rab5 expression and activation is dependent on IGF-1R phosphorylation and internalization via caveolae-mediated mechanism. Figure 16a shows Rab5a expression in wildtype MCF7 cells after IGF-1 stimulation. MCF7 and MDA-MB-231 cells overexpressing RIN1 were pre-treated with AG1024 or Filipin for 1hr and treated with IGF-1 or vehicle control. In Figure 16b, Rab5a levels were 11 fold compared with a sharp decrease in Rab5a expression in the presence of the inhibitors. Figures 16c and d complement the MCF7 data, showing Rab5a expression was also elevated upon IGF-1 stimulation and reduced in the presence of AG1024 and the RIN:R94A mutant. Rab5a had a 4-fold increase in gene expression with similarly inhibition of Rab5b gene levels in the presence of AG1024 in R94A cell lines.

Figure 17

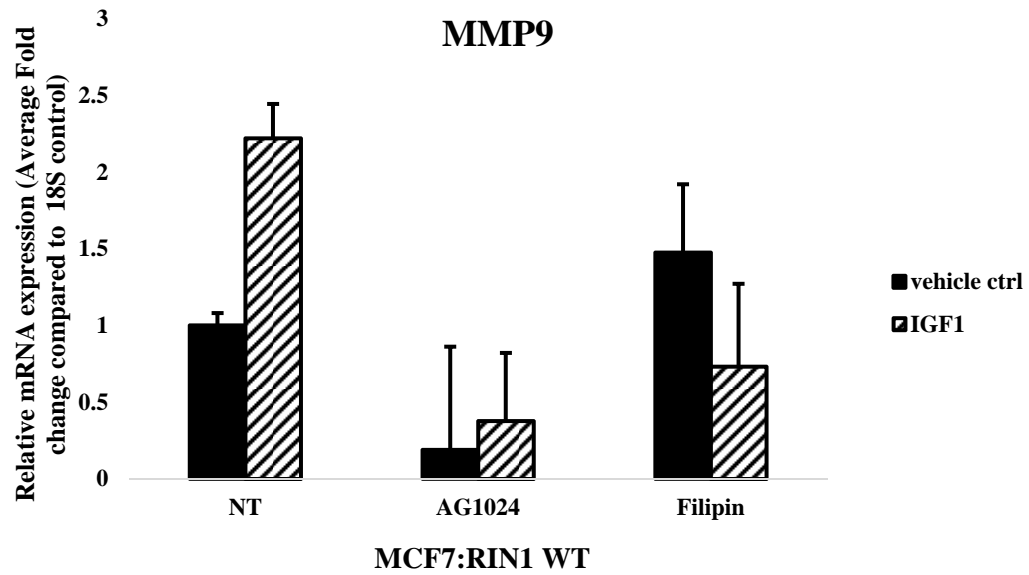
(a)



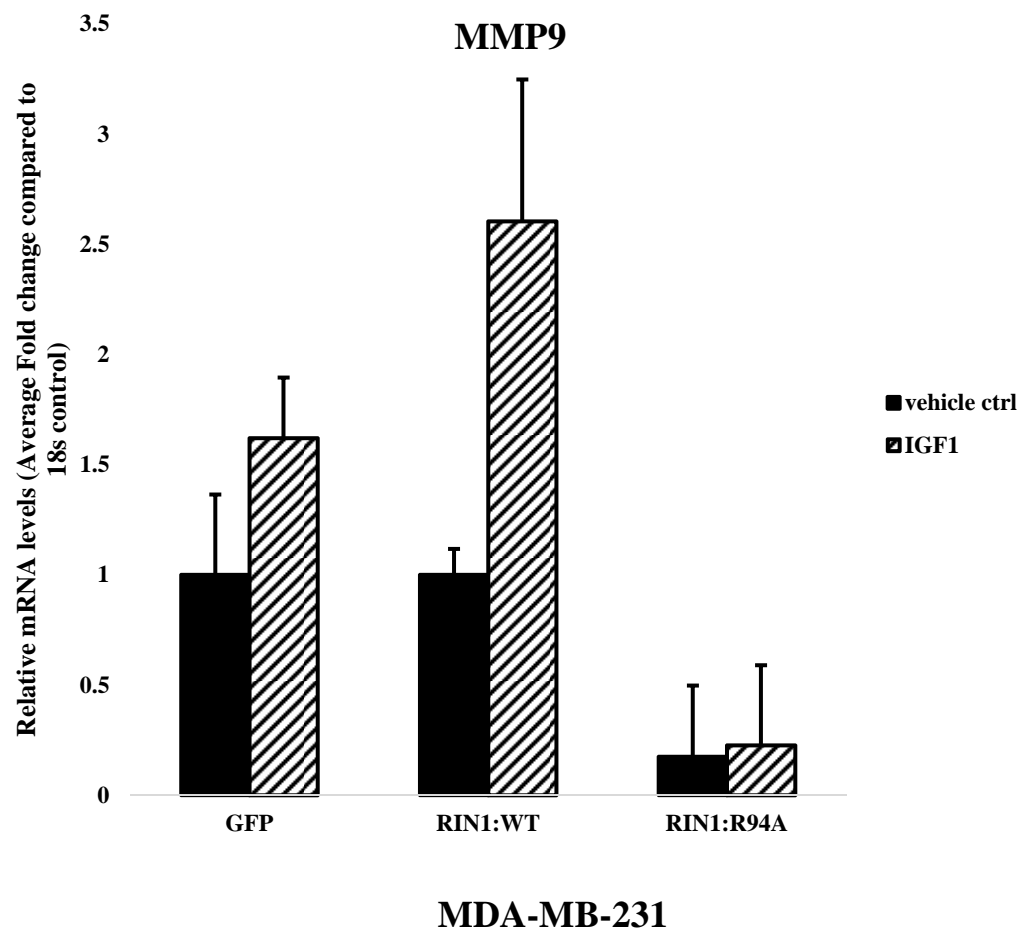
(b)



(c)



(d)



(e)

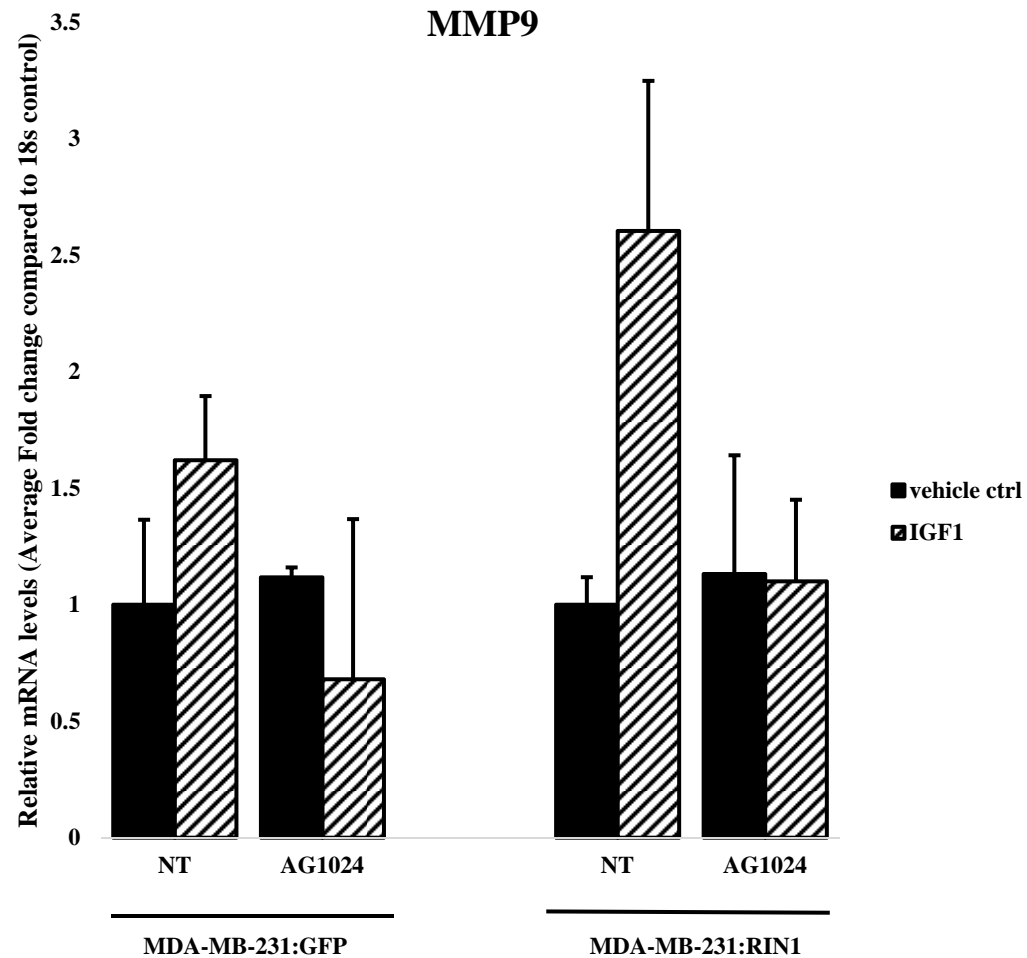


Figure 17. Real time PCR showed MMP9 expression and activation is dependent on IGF-1R phosphorylation and internalization via caveolae-mediated mechanism. MCF7 and MDA-MB-231 cells overexpressing Rab5a and RIN1 were pre-treated with AG1024 or Filipin for 1hr and treated with IGF-1 or vehicle control. Figure 17a. depicts MMP9 expression at an early and late time. In (b), MMP9 expression was notably elevated 11 fold compared to the vehicle control in 7 cells while in (d), disruption of the caveolae pathway also inhibited MMP9 expression while in RIN1:R94A mutant cells, MMP9 expression was also decreased. Figures (c) and (d) shows that the blocking of the IGF-1R affected MMP9 gene levels in both MCF7 and MDA-MB-231 cells overexpressing RIN1.

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Chapter 5

5.1. General Discussion

Endocytosis is a process where cells ingest macromolecules and particles via membrane bound substances. These events allow cells to digest and also recycle molecules to the exterior space. This process is called exocytosis and it functions opposite to endocytosis. These processes regulate several cellular mechanism from protein secretion, RTK endocytosis, recycling and degradation. Disruption of endocytosis within RTK trafficking has been linked to modifications in cellular processes such as adhesion, migration, proliferation, polarity, asymmetrical division and overall cell survival and represent the importance of Rab GTPases in cancer initiation and progression¹³¹.

Rab GTPases are considered to be master regulators of a multitude of trafficking pathways. Factors such as HGF, EGF, TGF β and IGF are known to activate the endosomal and exocytic pathways, thereby altering cell communication with the environment. The mechanisms can be comparable at key junctions where actin filaments and integrins are mobilized thereby promoting a migratory response in tumor cells. These RTKs can be internalized through two principal pathways: clathrin-coated pits or caveolae. In IGF-1 signaling, recent evidence highlighted an increase in cell proliferation and inhibition of apoptosis upon IGF-1R stimulation and ensuing internalization via co-localization with clathrin and caveolin-1¹²³. IGF-1R is also localized in the nucleus in

prostate, renal and breast tumor cells after translocation from the cell surface via clathrin mediated endocytosis.²

Recently, more attention has been directed to the mechanisms involving cancer progression and Rab GTPases function¹³¹. Cell migration and invasion constitute key hallmarks of cancer and specifically cancer metastasis⁶⁹. The transitions to a malignant and aggressive phenotype by normal cells are site specific⁵⁴. It also involves a variety of Rab proteins that have been implicated in the transition to metastasis where cells become invasive and penetrate the basal cell membrane and the ECM^{32,37}. These cells secrete an array of proteases such as MMPs; induce the compromised basement membrane to further release more proteases such as collagenase and growth factors, thereby allowing cancer cells to breach the basal membrane and migrate to distal sites.

As the role of growth factor-driven endocytosis in cancer cells is slowly being characterized, part of the struggle that currently exists involves the correct identification of the crucial endocytic molecules in growth factor signaling that may be potential therapeutic targets in cancer treatment. These may include but are not limited to Rab5, and its effectors^{49,172}. This report outlines an inherent relationship between Rab GTPases and IGF-1 in cancer cell migration and invasion. Whilst most Rabs are currently not considered to be oncogenic, this study otherwise provides evidence that some Rabs may be indeed so.

Here, it is reported for the first time that Rab5 and well as, RIN1, a specific Rab5 GEF, are central players in migration and invasion of MCF7 cells especially in the intracellular trafficking and signaling of the activated IGFR. The typically non-invasive MCF7 breast cancer cells were transfected with Rab5WT, Rab5 mutants (the GDP bound

form Rab5:S34N or GTP bound form Rab5:Q79L) or RIN1WT and the invasive and migratory potential were investigated. Interestingly, Rab5WT, Rab5:Q79L and RIN1 mediated significant migration and invasion of the tumor cells, with RIN1 and Rab5:Q79L also showing an induction (though not as significant) in the absence of IGF-1. The inactive form of Rab5, Rab5:S34N inhibited proliferation, migration and invasion of the cancer cells confirming Rab5 must be active and is necessary in IGFR endocytosis in IGF-1-dependent cancer cells. Further characterization of RIN1 importance in RTK trafficking was determined when transient silencing of RIN1 expression negated invasion of tumor cells. Other GEFs of Rab5 were also shown to influence the migratory and invasive potential of breast cancer cells with RIN3 demonstrating a more profound effect compared to RIN2.

Given that non-invasive tumor cells were directed to an aggressive status, it was logical to verify if in an invasive breast cancer cell line, MDA-MB-231, Rab5 and its GEFs are required in IGF-1 signaling. Using a stably transfected MDA-MB-231 cell line expressing Rab5:S34N, it was observed that the invasive and migratory properties increased irrespective of growth factor stimulations while the invasive potential of breast cancer cell lines expressing the wild type and active (GTP-bound) form of Rab5 were noticeably greater. Of interest, the expression of RIN1 increased invasion and migration of MDA-MB-231 cells even in the absence of growth factors. The results obtained warranted additional inquiry into the mechanism of RIN1 in enabling IGFR intracellular trafficking and signaling, i.e. through interaction with the receptor itself or only as a switch in Rab5 activation. The RIN1 mutant, RIN1:R94, possessed an aberration in the SH2 binding domain. This study clearly illustrated that RIN1 interacts with the cytosolic

tail of the IGFR as cancer cells expressing RIN1:R94A partially mitigated invasion and migration.

Thus far, IGF-1-induced migration and invasion of breast cancer cells necessitate active Rab5 sequestration via RIN1 activity. Consequently it was crucial to validate previous findings and ascertain if IGFR trafficking was caveolin dependent. IGF-1 treatment of MCF7 cells induced morphological changes consistent with EMT particularly in Rab5 and RIN overexpressing cells, i.e. more elongated and fibroblast-like. When kinase inhibitor for IGFR, AG1024 and caveolin inhibitor, Filipin, were administered to both MCF7 and MDA-MB-231 expressing, GFP, Rab5 or RIN1 constructs, the inhibitors impaired the migratory and invasive response of the breast cancer cell lines, suggesting that IGFR phosphorylation and internalization of the activated receptor was mediated by caveolin and caveosome formation upon IGF-1 stimulation. How does Rab5 and RIN1 fit into this schematic? Analysis of mRNA expression indicated that blocking of the IGFR or disruption of caveolin signaling in breast cancer cells expressing GFP or RIN1, suppressed Rab5 gene expression as well MMP9 secretion. The latter substantiates cell invasion data where the inhibitors averted the phosphorylation of the IGFR and its internalization despite RIN1 and Rab5 overexpression. Overexpression of Rab5 induced a significant increase in MMP9 expression and down-regulation in the presence of the inhibitors. In summation, this study illustrates a novel model in IGF-1 signaling in breast cancer progression. IGF-1 signaling is contingent on the IGF-1R phosphorylation and caveolae internalization via Rab5 and RIN1 function. Furthermore, it is reported that there may be a link between caveolin-dependent endocytosis and RIN1 activation in IGF-1 signaling. RIN1

overexpression has been shown to increase endogenous caveolin-1 in early endosomes, and upon internalization, translocation and tethering of active caveosomes to the early endosomes is Rab5 dependent¹⁵⁴. Therefore, the relationship between IGF-1R trafficking and MMP9 production is dependent on the activity of early endocytic trafficking machinery; thus RIN1 and Rab5 GTPases function within invading breast cancer cells.

5.2 Future Plans

Endocytosis is an indispensable molecular mechanism in growth factor induced signaling, and it is worth considering that early factors such as small GTPases and its GEFs exert a great influence on intracellular trafficking. Most importantly it is a coordinator in the crosstalk amongst signaling pathways in the propagating and promoting cancer cell survival. Further description of these small GTPases (e.g. Rab) function in cancer cell proliferation, migration and invasion, would be beneficial as these Rabs can be prospective biomarker in determining cancer stage and outcome prognosis. This idea opens the doors for the development of new therapeutic methods to target these necessary factors such as siRNA or peptide manipulations that can selectively affect Rab effectors.

While this study also demonstrates that Rab5 is a central player in the migration and invasion of cancer cells especially in response to IGF-1 stimulation, other questions abound including the role or activity of downstream effectors (PI3K and MAPK) in facilitating cell migration and invasion and as well as the mobilization of cytoskeletal

proteins such as focal adhesion kinase (FAK) and integrin receptor in IGF-1 mediate cancer progression. Even though it has been suggested that Rab5 modulates focal adhesion kinase activity in normal and cancer cells¹²⁷, yet little information is available as to how growth factors (i.e. IGF-1) regulate the molecular mechanisms of intracellular processes leading to survival in normal and cancer cells.

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