DISSECTING THE MECHANISM BY WHICH MICRORNA-200B INHIBITS BREAST CANCER METASTASIS

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Cellular and Molecular Biology-Doctor of Philosophy

2016

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MicroRNAs (miRNAs) are a large family of small non-coding RNAs that negatively regulate protein-coding gene expression post-transcriptionally via base pairing between the 5' seed region of a miRNA and the 3' untranslated region (3'UTR) of a target messenger RNA (mRNA). Recent studies have shown that miRNAs play a critical role in many diseases, including cancer. The microRNA-200 (miR-200) family consists of 5 members (miR-200a, -200b, -200c, -141, and -429) and has recently emerged as a prominent player in cancer initiation, progression, and metastasis. Studies also suggest that the miR-200 family could be a potential therapeutic for the treatment of cancer. Even though the role of the miR-200 family in cancer has recently been greatly studied, the role of this family on cancer metastasis continues to be controversial. Furthermore, many of these studies focus on the role of the entire family or an entire cluster of the miR-200 family in cancer metastasis and not the role of individual members of the family. Therefore to better understand the disease, this family, and to discover novel therapeutics, it is important to elucidate the role that each member of the miR-200 family plays in cancer metastasis.

Breast cancer is the most diagnosed cancer and second leading cause of cancer related death in women in the United States. Breast cancer can be classified into three main subtypes: luminal, Her2+, and triple negative. These subtypes are clinically defined by receptor status; luminal is defined by the presence of the estrogen receptor (ER), Her2+ by human epidermal growth factor receptor 2 (Her2) amplification, and triple negative by the absence of the estrogen receptor, progesterone receptor (PR), and Her2 amplification. Triple negative breast cancer

(TNBC) is a unique subtype of breast cancer that is often a highly invasive and metastatic form of breast cancer. TNBC has also been shown to have an overall poorer prognosis compared with other breast cancer subtypes. This is partly due to the inherent aggressiveness of TNBC and partly because it lacks effective targeted therapies. Therefore, chemotherapy is currently the only treatment option for metastatic TNBC and is only effective at the initial treatment stage. Consequently, there is an urgent need to better understand the underlying mechanism of TNBC aggressive behavior and identify novel targets for developing more efficient therapies for TNBC.

This study was performed to investigate the effect and mechanism of miR-200b on TNBC metastasis and identify targets for developing more efficient treatment for TNBC. We found that miR-200 expression was significantly reduced in the highly migratory and invasive, mesenchymal-like TNBC cells compared to other breast cancer subtypes. Expressing miR-200b in two of these highly migratory and invasive TNBC cells, MDA-MB-231 and SUM-159, dramatically reduced cell migration and lung metastasis in a mouse mammary xenograft tumor model. In this study we identified PKCα and ARHGAP18 as novel direct targets of miR-200b, and these proteins are inversely correlated with miR-200b expression in breast cancer cells. Furthermore, reduction of PKCα or ARHGAP18 protein expression significantly impaired the migratory capability of MDA-MB-231 and SUM-159 TNBC cells, and enforced expression of PKCα or ARHGAP18 impairs the inhibitory effect of miR-200b on cell migration and lung metastasis. Mechanistic studies revealed that miR-200b affects cell migration and lung metastasis by regulating key regulators of the actin cytoskeleton, Rac1 and RhoA. Overall, this study suggests that these proteins could serve as novel therapeutic options for the treatment of aggressive and metastatic TNBC.

ACKNOWLEDGMENTS

I would like to thank my mentor Dr. Chengfeng Yang for allowing me the opportunity to become his graduate student. I would like to thank him for giving his time, insight and help towards my project, patience with me, and constant support. He has helped guide me towards the scientist I am today, and I cannot thank him enough.

I would like to thank all the current and former members of the Yang laboratory that helped me and had scientific discussions with me during my project: Dr. Zhishan Wang, Dr. Yunfei Li, Ayda Alavi, Ryan Beyea, Theresa Fisher, Aaron Oom, and Amy Trinh.

I would like to thank my committee members for all their guidance and insight; Dr. Barbara Atshaves, Dr. Kathleen Gallo, Dr. R. William Henry, and Dr. Hua Xiao.

I would like to thank Dr. Suyun Huang (The University of Texas M.D. Anderson Cancer Center, Houston, TX) for providing MDA-MB-231 cells, Dr. Stephen Ethier (Wayne State University, Detroit, MI) for providing SUM-149 and SUM-159 cells, and Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY) for providing MDA-MB-231 LM2 4175 cells.

I would like to thank Dr. Sandra O'Reilly (Department of Physiology, Michigan State University, East Lansing, MI) for her expertise with the mouse xenograft and tail vein injections as well as IVIS imaging.

I would like to thank Amy Porter and Kathy Joseph (Investigative Histopathology Lab, Michigan State University, East Lansing, MI) for their expertise and help with histology sample preparation.

I would like to thank all the members of the Cellular and Molecular Biology Program at Michigan State University for providing me the opportunity to do research.

I would like to thank the members of the Cancer Research Network (CRN) for their help and guidance throughout my project.

I would like to thank my father, Kevin Humphries, my mother, Diana Humphries, my sister, Randielle Bilbia, and my brother-in-law, Kevin Bilbia, for all their support throughout my graduate school career as well as my life. The endless encouragement and love they have provided is what has motivated me to complete my PhD.

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KEY TO ABBREVIATIONS

3’UTR: 3’ untranslated region

4-OHT: 4-Hydroxytamoxifen

5’UTR: 5’ untranslated region

ABP: Actin-binding protein

ARHGAP18: Rho GTPase activating protein 18

ARHGAP19: Rho GTPase activating protein 19

ATP: Adenosine triphosphate

BPDE: Benzo(a)pyrene diolepoxide

BRCA1: Breast cancer 1

BrdU: 5-bromo-2’-deoxyuridine

Cdc42: Cell division cycle 42

CEA: Carcinoembryonic antigen

CLL: Chronic lymphocytic leukemia

CRISPR: Clustered regularly-interspaced short palindromic repeats

CTCs: Circulating tumor cells

DAPI: 4′,6-diamidino-2-phenylindole

DGCR8: DiGeorge syndrome critical region 8

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

ECM: Extracellular matrix

EMT: Epithelial-to-mesenchymal transition

ER: Estrogen receptor

F-actin: Filamentous actin

G-actin: Globular actin

GAP: GTPase activating protein

GDI: GDP dissociation inhibitor

GDP: Guanosine diphosphate

GEF: Guanosine nucleotide exchange factor

GFP: Green fluorescent protein

gRNA: Guide RNA

GST: Glutathione S-transferase

GTP: Guanosine triphosphate

GTPase: Guanosine triphosphatase

H&E staining: Hematoxylin and eosin

HBECs: Human bronchial epithelial cells

Her2: Human epidermal growth factor receptor 2

HUVECs: Human umbilical vein endothelial cells

MBC: Metastatic breast cancer

MET: Mesenchymal-to-epithelial transition

miRNA or miR: microRNA

miR-200: microRNA-200

miR-200b: microRNA-200b

mRNA: messenger RNA

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NMU: *N*-Nitroso-*N*-methylurea

NSCLC: Non-small cell lung cancer

OSCC: Oral squamous cell carcinoma

p53: Tumor protein p53

PBS: Phosphate buffered saline

PKCα: Protein kinase Cα

pre-miRNA: precursor miRNA

pri-miRNA: primary microRNA

qPCR: Quantitative polymerase chain reaction

Rac1: Ras-related C3 botulinum toxin substrate 1

RhoA: Ras homolog gene family, member A

RISC: RNA-induced silencing complex

RNA: Ribonucleic acid

SCID: Severe combined immunodeficiency

shRNA: short hairpin RNA

siRNA: small interfering RNA

TAMs: Tumor associated macrophages

TNBC: Triple negative breast cancer

Zinc finger E-box binding homeobox 1: ZEB1

Zinc finger E-box binding homeobox 2: ZEB2

µl: Microliter

µM: Micromolar

CHAPTER 1: INTRODUCTION

Part of this chapter represents a manuscript that was published in *Oncotarget* (2015) **6**: 6472-6498.

Authors who contributed towards this study were: Brock Humphries and Chengfeng Yang.

BREAST CANCER

The breast is a complex structure made up of fat, connective tissue, lobes, lobules, ducts, blood vessels and lymph nodes. Each breast has many lobules that branch out from the nipple, and each lobule consists of many alveoli. These alveoli are unique because they are lined with the milk-secreting cuboidal cells and surrounded by myoepithelial cells. During lactation, milk is carried from these lobules to the nipple by ducts. These structures are important because breast cancer most commonly originates from cells of these ducts and lobules.

Breast cancer is a breast tumor originating from breast tissues, and in its advance stages can metastasize to the bone, brain, liver, and the lung. Breast cancer is the most common form of cancer diagnosed (about 30% of all new female cancer cases each year) and second leading cause of cancer-related death (about 15%) in women in the United States (**Table 1.1.**). Furthermore, breast cancer is the most invasive type of cancer in women. Although breast cancer is 100 times more common in women, men can also get breast cancer. Risk factors that contribute to the development of breast cancer include sex, age, genetics, lack of childbearing or breastfeeding, increased levels of hormones, diet, and obesity.

Breast cancer can be classified into three main subtypes: Luminal, Her2+, and triple negative. Each of these subtypes is classified by receptor status of the primary tumor. Luminal breast cancer is defined by the presence of the estrogen receptor (ER), Her2+ by the presence of the human epidermal growth factor receptor 2 (Her2) amplification, and triple negative by the absence of the estrogen receptor, progesterone receptor, and Her2 amplification (**Table 1.2.**). Each of these subtypes of breast cancer is treated as a unique disease, each having their own regimen of treatment, and each has a varying prognosis. Breast cancer is typically treated with

Table 1.1. Estimated Cancer Rates and Deaths in Females for 2016

Estimated New Cancer Cases* **Female: 818,920**	**Estimated Cancer-Related Deaths*** **Female: 259,450**
Breast: **246,660 (30%)**	Lung & bronchus: **72,160 (28%)**
Lung & bronchus: **106,470 (13%)**	Breast: **40,450 (16%)**
Colon & rectum: **63,670 (8%)**	Colon & rectum: **23,170 (9%)**
Uterine corpus: **60,050 (7%)**	Pancreas: **20,330 (8%)**
Thyroid: **49,350 (6%)**	Ovary: **14,240 (5%)**

*Excludes basal and squamous cell skin cancers and in situ carcinoma except urinary bladder

Adapted from (1)

Table 1.2. Breast Cancer Subtypes

Subtype		**Molecular Profile***		**Prevalence**
Luminal A		ER+ and/or PR+ Her2-		30-70%
Luminal B		ER+ and/or PR+ Her2+		10-20%
Her2+		ER- PR- Her2+		5-15%
Triple Negative		ER- PR- Her2-		15-20%

* This is a general molecular profile for each tumor. Not all tumors in each subtype share the exact same molecular profile.

Adapted from (2-6)

surgery to remove the primary tumor, followed by endocrine or chemical therapeutics (whether targeted or general) and radiation.

TRIPLE NEGATIVE BREAST CANCER AND TREATMENT

Triple negative breast cancer (TNBC) is a unique histological subtype of breast cancer and highly heterogeneous disease that constitutes approximately 20% of all newly diagnosed breast cancer cases each year in the United States. TNBC is most common in women with African ancestry, women who have the BRCA1 mutation, and premenopausal women, however some recent studies have suggested that TNBC is also common in older, postmenopausal women as well. TNBC is often a highly invasive and metastatic form of breast cancer, and is associated with an overall poorer prognosis when compared to the other breast cancer subtypes (luminal A: 89%, luminal B: 83%, Her2: 77%, TNBC: 75% among the breast cancer subtypes in lymph node-negative breast cancer (7)). This is partly due to TNBC usually displaying more aggressive behavior and lacking effective targeted therapies (8,9). Chemotherapy is currently the only chemical treatment option for TNBC, and is only effective at initial treatment (10,11). This is because TNBC patients quickly develop therapeutic resistance to the drugs. Therefore, there is an unfulfilled need to understand the molecular mechanisms that underlie TNBC aggressiveness as well as identify novel therapeutic targets for this unique disease.

Although there is no targeted therapy treatment for TNBC, patients with TNBC still have treatment options. TNBC patients are usually treated locally, or treatment at the site of the cancer, as well as treated systemically, or treatment of the entire body. Local treatment refers to surgery to remove either the cancer only (lumpectomy) or part or all of the breast tissue (mastectomy), or to radiation treatment. On the other hand, systemic treatment refers to the use of chemical therapeutics to treat the cancer. These chemical therapeutics can either be given before or after treatment, and are usually anthracycline- or nonanthracycline-based regimens.

However, as with all chemical therapeutics the major problem with using these are the negative side effects.

EXTRACELLULAR MATRIX, ACTIN CYTOSKELETAL REMODELING, AND CANCER CELL MIGRATION

The extracellular matrix (ECM) is a complex network of polysaccharides and proteins (such as laminin, collagen, and fibronectin) which are secreted by cells. Two of the main functions of the ECM are to provide a structural element and to function as an adhesive substrate to the cell. In order to adhere to the ECM a cell needs to form cellular junctions by altering its actin cytoskeleton. One of the most important cell-to-ECM junctions is the focal adhesion, an anchorage site for actin filaments, because of the role it plays in cell migration and metastasis.

Actin is the most abundant protein in almost all eukaryotic cells. It is a highly conserved protein and has the ability to transition between monomeric G-actin and filamentous F-actin states. One of the most important roles that this transitioning plays is within the actin cytoskeleton. The actin cytoskeleton is a structure made entirely of actin that is involved in many aspects of cell biology, most importantly cell migration. The actin cytoskeleton is a highly dynamic structure, which is constantly being remodeled in response to the extracellular environment. These highly controlled dynamics are based on the constant assembly and disassembly of actin filaments that form the structures critical in cell migration. The three most important structures for actin-based cell motility are the lamellipodium, filopodium, and stress fiber. The lamellipodium is the sheet-like actin-based projection on the front, or leading edge, of the motile cell, the filopodium are the finger-like actin-based projections that extend from the lamellipodium, and stress fibers are contractile actomyosin bundles (12). Formation of these structures relies heavily on the polymerization of ATP-bound G-actin into actin filaments.

The first, and rate-limiting, step of actin polymerization is nucleation, in which a complex of three ATP-bound G-actin monomers forms (13-15). This complex can then be elongated

through binding of other ATP-bound G-actin monomers into an F-actin filament. These F-actin filaments are polarized with a fast-growing “barbed” end, in which the ATP-bound G-actin monomers preferentially bind, and a slower-growing “pointed” end. However, this is highly unstable because of the instability of the actin intermediates that make up the F-actin filaments. To overcome this, cells employ the use of actin-binding proteins (ABPs) that can modulate nucleation and the stability of the filament.

Actin-binding proteins are proteins that have the ability to specifically bind to actin, whether it is a monomer, polymer, or both. By binding to actin, these proteins can modulate processes such as actin nucleation and actin cytoskeleton organization (16). Actin-binding proteins such as Arp2/3, Spire, and Formin accelerate actin nucleation by bringing actin monomers together. Thymosin, profilin, and ENA/VASPs are actin-binding proteins that regulate actin filament assembly and elongation by bringing actin to the actin filament, and finally ABPs that regulate filament polymerization and depolymerization include cofilin, gelsolin, capping protein, and tropomyosin. Furthermore, there is evidence that suggests that these actin-binding protein families do not function individually, but that cross-talk between them occurs. It is this cooperative functionality that helps to shape the cells migratory capability.

Not only are ABPs regulated by cross-talk, but they are also regulated by small Rho GTPases. Rho GTPases are a family of small (~21 kDa) signaling G proteins that belong to the Ras superfamily of signaling proteins (17,18). The members of the Rho GTPase family have been shown to regulate many aspects of intracellular actin dynamics such as cell migration. Of the 20 members only three have been studied in detail: RhoA, Rac1, and Cdc42. RhoA has been shown to regulate the actin cytoskeleton to form stress fibers, Rac1 to form the lamellipodium, and Cdc42 to form the filopodium (17,18). These small Rho GTPases function as molecular

switches within the cell depending on whether GTP or GDP is bound. When loaded with GTP the Rho GTPases are in an “on” state, move to the cell membrane, and can interact with downstream effectors. However, the intrinsic phosphatase activity of the Rho GTPases can hydrolyze the GTP to GDP putting the GTPase in an “off” state and shuttle the GTPase to the cytoplasm.

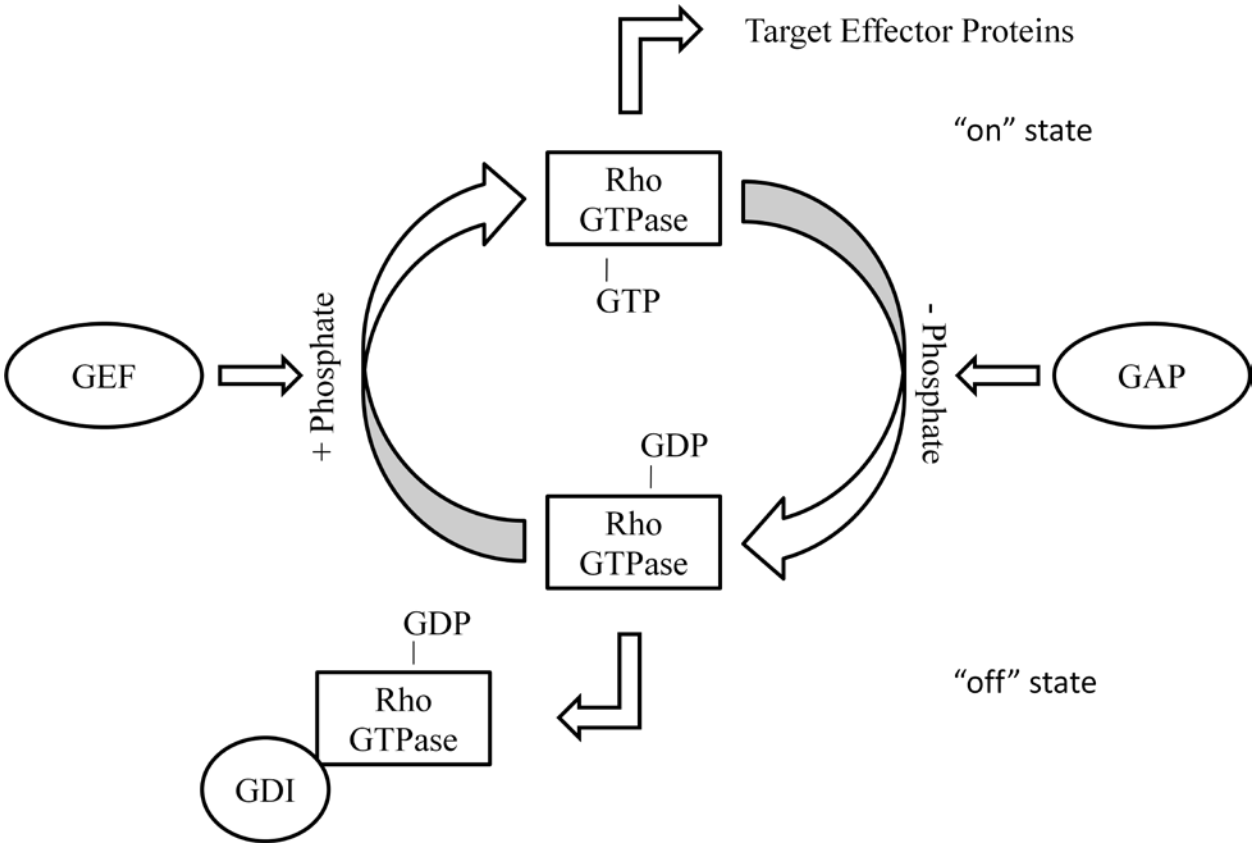
These “on” and “off” states of the Rho GTPases can be accelerated by interaction with certain regulators of G-protein signaling (**Figure 1.1.**). GTPase activating proteins (GAPs) accelerate the Rho GTPases intrinsic phosphatase capability, putting the GTPase into the “off” state (17-19). Conversely, guanine nucleotide exchange factors (GEFs) activate Rho GTPases by rapidly exchanging GDP with GTP (17-19). GDP dissociation factors (GDIs) also act to put the Rho GTPases into the “off” state by binding and sequestering GDP-bound Rho GTPases, not allowing these proteins to exchange GDP for GTP (17-19). Since the number of GAPs and GEFs outnumber the number of Rho GTPases by over 3 to 1, many of these GAPs, GEFs, and GDIs target the same Rho GTPase. However, some of these GAPs, GEFs, and GDIs have been shown to be specific for a single Rho GTPase over the others. Moreover, these regulators of G-protein signaling have been shown to be regulated by the small Rho GTPases themselves.

In order to migrate, a cell has to change its shape and stiffness to interact with the surrounding ECM and tissue. Therefore, the ECM acts as both a substrate as well as an obstacle for the cell to overcome in order to migrate. Cell migration is an essential process during development, for immune defense, wound healing, as well as for the progression of many diseases such as cancer. Cell migration consists of multiple steps (protrusion, adhesion, contraction, and tail retraction) that are heavily reliant on the constant remodeling of the actin cytoskeleton (22,23). In response to an external stimulus, a cell will form lamellipodium and filopodium as the main protrusive

structures. These actin-based structures will form at the leading edge of the cell, in the direction that the cell will be moving towards the stimulus. Consistent remodeling of the actin cytoskeleton during the formation of these structures is critical for generating the force needed to push the cell forward. Actin adhesions, or focal adhesions, assemble coordinately with the protrusions formed earlier in cell migration. As these new adhesions are formed the older, mature adhesions are disassembled and the actin and proteins involved are recycled. Although many other proteins are involved in the regulation of these processes, initial adhesion assembly is dependent on Rac activity, RhoA is required for the maturation of these newly formed adhesions, and focal adhesion kinase (FAK) is involved in adhesion turnover. Contraction moves the cytoplasmic mass forward through an actomyosin-based contractile force. This step requires RhoA-dependent stress fibers and focal adhesions to be formed throughout the cell and requires ATP consumption to accomplish. The final step is tail retraction in which the contractile tension generated by the actomyosin bundles causes the rear adhesions to break. The breaking of these rear adhesions ultimately leads to the cell moving forward, and the proteins involved in these adhesions recycle and form new adhesions at the leading edge of the cell.

The ability of a cell to migrate and invade allows it to change its position within and between tissues. Migration is not only important for normal cells during development and in processes such as wound healing, but this characteristic in cancer cells can help progression and promote metastasis. Therefore, more research is needed into understanding the molecular mechanism behind cancer cell motility to better and more efficiently target it in patients and improve prognosis.

Figure 1.1. GAPs, GEFs, and GDIs regulate small Rho GTPases. Small Rho GTPases are known as molecular switches inside the cell due to the fact that they cycle between "on" (GTP-bound) and "off" (GDP-bound) states. In the GTP-bound state, small Rho GTPases are able to regulate intracellular signal transduction by binding to effector molecules. This signaling can be terminated by the intrinsic GTPase capability of small Rho GTPases, and can be promoted by interaction with GTPase activating proteins (GAPs). Small Rho GTPases in the GDP-bound state can also interact with guanosine nucleotide dissociation inhibitors (GDIs), which sequester the small Rho GTPase and does not allow for GDP to be exchanged for GTP. In order for GDIs to release the small Rho GTPase, a release factor must be present (20,21). In the GDP-bound state, small Rho GTPases are unable to regulate downstream signaling, but can be reactivated by exchanging GDP for GTP. Interaction with a guanine nucleotide exchange factor (GEF) can promote the exchange of GDP for GTP activating the small Rho GTPase.



MICRORNAS

The first microRNA (miRNA) (lin-4) was discovered in 1993 (24,25), however the term "microRNA" wasn't introduced until 2001 (26-28). Within the next year it was reported for the first time that miRNAs are likely involved in cancer, by demonstrating that miR-15 and -16 were frequently deleted in chronic lymphocytic leukemia (CLL) (29). Since this discovery a focus has been put on identifying and determining the role of miRNAs involved in cancer development, progression, diagnosis and treatment. Through this effort, our understanding of how microRNAs function and the role they play in cancer has increased tremendously.

MiRNAs are a large family of small non-coding RNA molecules (over 2500 in humans: miRBase.org) that negatively regulate protein-coding gene expression post-transcriptionally. MiRNAs are initially transcribed mono- or polycistronically in the nucleus by RNA polymerase II into primary transcripts (termed primary miRNA, pri-miRNA) ranging from hundreds to thousands of nucleotides long, which are then polyadenylated and capped (30,31). These pri-miRNA transcripts are subjected to a microprocessing event carried out by a type III RNase Drosha and its binding partner DiGeorge Syndrome Critical Region 8 (DGCR8) to reduce the size of the transcript to ~70 nucleotides termed precursor- or pre-miRNA (32,33). The pre-miRNA is then exported to the cytosol by exportin-5, where it undergoes another processing event performed by another type III RNase Dicer resulting in a ~21–22 nucleotide miRNA duplex (34,35). After unwinding, one strand of the duplex is usually degraded, while the other strand, the mature miRNA, is associated with Argonaute and then incorporated into the RNA induced silencing complex (RISC), where miRNAs are then able to regulate the expression of their target genes.

Although it has been shown that miRNAs can bind in other places (for examples see (36,37)), miRNAs typically function by base pairing with the 3’ untranslated regions (3’-UTRs) of their target messenger RNAs (mRNAs) through the seed sequences of the miRNAs. The seed sequence of a miRNA is the second to the eighth nucleotide region at the 5' end of the mature miRNA that generates the specificity of each miRNA to its target mRNA. The base pairing between a miRNA and its target mRNAs can result in mRNA destabilization and degradation, translational inhibition, or mRNA direct cleavage (38-41). Down-regulation of mRNAs through miRNA-caused mRNA destabilization and degradation is common, which is usually mediated by the same imperfect miRNA:mRNA base pairing that leads to translational inhibition (42-44). Conversely, mRNA down-regulation through miRNA-caused mRNA direct cleavage occurs in rare cases, which usually requires more extensive base pairing (40,41). While a miRNA's seed sequence is usually the most prominent characteristic that determines the specificity of the miRNA:mRNA interaction, there are examples of miRNAs that have weak seed sequence binding but better overall complementarity which can direct the inhibition of gene expression (39,45,46).

MiRNAs are interesting because each can target multiple genes and can also share seed sequences with other miRNAs, therefore theoretically targeting the same genes as other miRNAs. It is for this reason that miRNAs have been thought to regulate upwards of two-thirds of all protein coding genes in humans (47). Furthermore, miRNAs have been shown to be involved in almost all aspects of cellular functions. Therefore it is probable that miRNAs play critical roles in cancer development and progression. Indeed there is a growing body of evidence suggesting that miRNAs may act as either oncogenes or tumor suppressors, and are involved in

the development, progression, and treatment of cancer (for reviews see (48,49)). These miRNAs that act as either oncogenes or tumor suppressors in a cell are often called oncomirs (50).

MICRORNAS, BREAST CANCER, AND METASTASIS

There is a growing body of evidence that has demonstrated that miRNAs play an essential role in disease development and progression, therefore understanding the role that miRNAs play in cancer development and progression is important for understanding the disease. There have been many studies that have shown that microRNAs are differentially expressed and are involved in breast cancer initiation, progression, diagnosis and prognosis. More precisely, Iorio and colleagues analyzed 76 breast cancer and 10 normal breast samples and found that 29 miRNAs of the probed miRNAs had significantly dysregulated expression in cancer versus normal breast tissues (51). A different study found that nine miRNAs were important for transition from normal breast to ductal carcinoma *in situ* and then to invasive ductal carcinoma (52). Furthermore, this group also found a separate signature consisting of five miRNAs that was associated with overall survival and time to metastasis. Lastly screening the serum of healthy and breast cancer patients, it was found that miR-222 is significantly increased in the serum of breast cancer patients compared to controls and therefore could be a valuable diagnostic marker for differentiating the two groups (53). This data, along with others, has demonstrated that miRNAs are promising tools that are important in breast cancer initiation, progression, diagnosis, and prognosis.

One of the major reasons for cancer-related death is metastasis. The earliest report of a miRNA contributing to metastasis showed that miR-10b promotes metastasis by reducing homeobox D10 (HOXD10) expression, resulting in an increase in the pro-metastatic gene, *RHOC* (54). Since then other miRNAs that have been implicated in promoting metastasis by targeting tumor suppressing genes including miR-9 (55), miR-21 (56-58), miR-373 (59), 520c (59), miR-29a (60), and miR-155 (61-64) among others. On the other hand, some of the miRNAs

that have been demonstrated to suppress metastasis by targeting pro-metastatic genes including miR-7 (65,66), miR-17/20 (67,68), and miR-145 (69-71) among others. These studies show the importance of studying miRNAs because they can be critical components within metastasis.

THE MICRORNA-200 FAMILY

The miRNA-200 (miR-200) family consists of five members, which form two clusters located in two different genomic regions. As shown in **Figure 1.2.**, the cluster I miR-200s in humans contains *miR-200b*, *-200a*, and *-429* (*miR-200b/200a/429*) located in an intergenic region of chromosome 1, and cluster II miR-200s contains *miR-200c* and *-141* (*miR-200c/141*) located on chromosome 12 (72,73). Alternatively, the miR-200 family members can also be divided into two functional groups based upon the similarities of their seed sequences (**Figure 1.3.**). MiR-200b, -200c, and -429 (Functional Group I) all share the same seed sequence and miR-200a and -141 (Functional Group II) both share the same seed sequence, with the two functional groups only differing in the seed sequence by one nucleotide (AAUACUG for miR-200b/200c/429 and AACACUG for miR-200a/141). The miR-200 family is highly conserved among vertebrate species and highly expressed within epithelial cells.

The expression of the miR-200 family can be regulated through interactions with, and modifications of their promoters. Recent studies suggest that modifications to the promoter regions of each of the miR-200 clusters can cause the loss of the expression of the miR-200 family in cancer. The promoter region of the *miR-200c/-141* cluster has been shown to be hypermethylated (74,75), whereas the *miR-200b/-200a/-429* cluster has been shown to be silenced primarily through polycomb group-mediated histone modifications (76) in cancer. Alternatively, the promoter regions of the miR-200 family can be bound by the transcription factors zinc finger e-box bind homeobox 1 (ZEB1) and 2 (ZEB2 also known as SIP1), specificity protein 1 (Sp1), and p53. When bound, ZEB1 and ZEB2 can inhibit the transcription of the entire miR-200 family, while Sp1 and p53 binding has been shown to lead to activation of transcription of the *miR-200b/200a/429* (77,78) and the *miR-200c/141* (78,79) clusters, respectively.

Figure 1.2. The miR-200 family two clusters are located on two different chromosomes. The miR-200 family consists of two clusters: Cluster I (*miR-200b*, *-200a*, and *-429* is located on chromosome 1) and Cluster II (*miR-200c* and *-141* is located on chromosome 12).

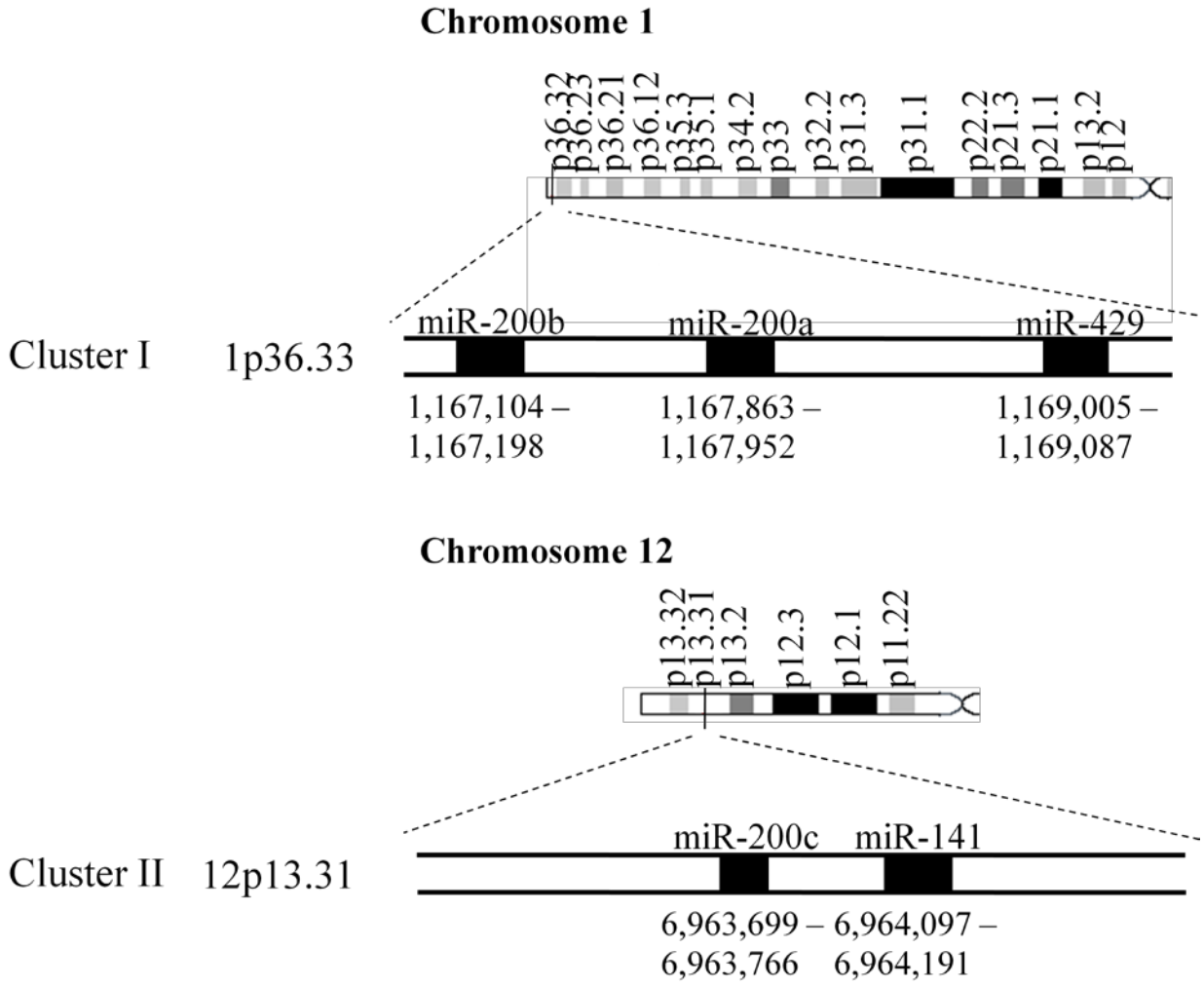
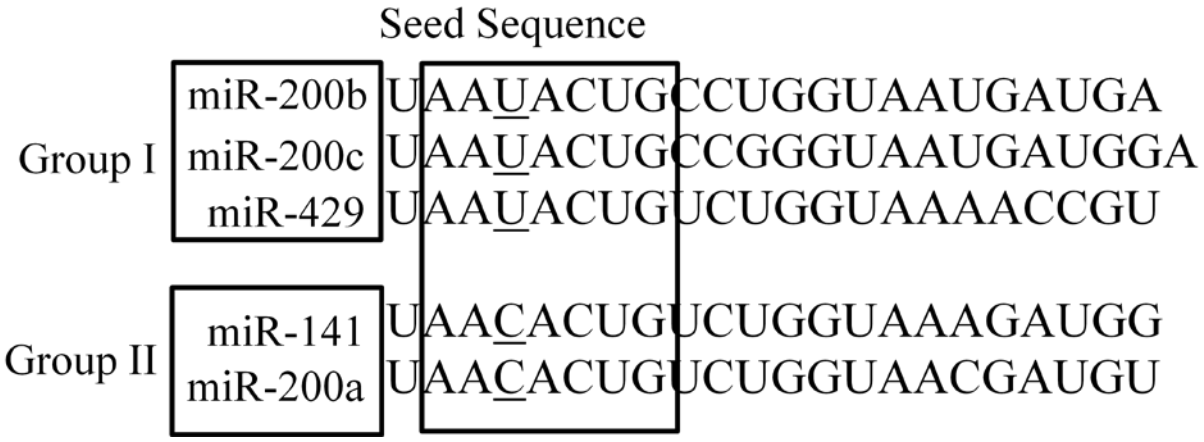


Figure. 1.3. The sequences of the mature miRNA-200 family members. The miR-200 family members can also be separated into two functional groups based upon their seed sequences. Functional Group I is composed of miR-200b, -200c, and -429 and Functional Group II consists of miR-141 and -200a. The seed sequences of these two functional groups only differs by one nucleotide: AAUACUG for group I and AACACUG for group II.



However, Kolesnikoff and colleagues also showed that Sp1-mediated activation of *miR-200b/200a/429* transcription can be disrupted by the expression and interaction of ZEB1/2 with its binding sites within the promoter (77).

THE MIR-200 FAMILY IN CELL TRANSFORMATION AND TUMORIGENESIS

Tumor initiation is a complex process by which normal cells are transformed into malignant tumor cells, which then produce a tumor. Throughout this process the molecular profile of the cell is changed in such a way as to allow these cells the ability to form a tumor. Recent research has suggested that the miR-200 family plays an important role in inhibiting cell malignant transformation and preventing tumor initiation.

Recent research done in our laboratory has been the first to show an important role of the miR-200 family in inhibiting and preventing cell malignant transformation by carcinogen exposure (80). Using immortalized human bronchial epithelial cells (HBECs) that either had normal p53 expression (HBECs) or p53 knocked down (p53lowHBECs), we were able to show that chronic exposure to a low concentration of arsenic caused epithelial to mesenchymal transition (EMT) and malignant transformation in p53lowHBECs, but not in p53 intact HBECs. Only thep53lowHBECs exposed to arsenic formed colonies in soft agar and formed tumors in a subcuteaneous injection of the cells into the nude mice. A miRNA microarray analysis showed that the expression levels of miR-200 family were drastically reduced in arsenic-transformed cells.

To determine whether down-regulation of the miR- 200 family plays a role in arsenic-induced cell malignant transformation, we first transiently re-expressed miR- 200b or -200c in arsenic-transformed cells and found that re-expression of miR-200b or -200c alone or together restored E-cadherin expression and the epithelial-like cellular morphology, and reduced the formation of colonies in soft agar. Then we generated miR-200b stable expression cells and found that stably re-expressing miR- 200b in arsenic-transformed cells abolished their ability to form colonies in soft agar, and tumors in nude mice when injected subcutaneously. Then we

overexpressed miR-200b in parental p53lowHBECs and found that forced expression of miR-200b prevented cellular transformation by chronic low dose arsenic exposure (80). Together, these findings suggest that loss of miR-200 expression plays a causal role in arsenic-induced cell malignant transformation and tumorigenesis.

A study investigating the mechanism of tobacco carcinogen-induced cell transformation showed that a 4 week treatment of p53 intact HBECs with genotoxic, but not cytotoxic, doses of *N*-Nitroso-*N*-methylurea (NMU) or Benzo(*a*)pyrene diolepoxide (BPDE) caused EMT and cell transformation as evidenced by occurrence of mesenchymal-like cellular morphology and increased soft agar colony formation (81). Further experiments revealed that expression of miR-200b and -200c were significantly reduced after 4 week carcinogen treatment. Transient or stable expression of these miRNAs in tobacco carcinogen-transformed HBECs restored the epithelial-like cellular morphology and reduced soft agar colony formation (81).

These two studies described above both looked at epigenetic silencing as a possible mechanism for the carcinogen-induced miR-200 expression loss seen in the HBECs. It was determined that the promoter regions of the miR-200 family were indeed highly methylated upon treatment with the carcinogen, and demethylation induced by DNA methyltransferase inhibitors or demethylation chemicals increased the expression of the miR-200 family. Therefore, arsenic or tobacco carcinogens may induce cell transformation by increasing the methylation of the promoter regions of, and subsequently leading to silencing of, the miR-200 family. Together, these studies suggest that loss of miR-200 expression may play an important role in the early stage of carcinogenesis.

THE MIR-200 FAMILY IN CANCER METASTASIS

Cancer metastasis is the result of a multi-step signaling cascade in which cancer cells move away from the primary tumor site to colonize distant organs and form secondary (or metastatic) tumors (82). Typically, cells that accomplish metastasis have undergone widespread genetic and epigenetic modifications that benefit the survival, growth, invasion and movement of the cell. Metastasis can be broken down into 6 steps (83): 1) growth and vascularization of the primary tumor, and nearby tissue invasion, 2) detachment of the cancer cells and migration away from the primary tumor, 3) intravasation into the blood stream and lymph nodes, 4) survival and circularization within the blood stream and lymph nodes, 5) attachment to the blood vessel wall and extravasation, and finally 6) colonization of the distant organs and growth of metastatic tumors. Each step symbolizes an important obstacle that the tumor cell must overcome to result in successful metastasis.

Current research on the miR-200 family has shown that the family can affect each step of the metastatic cascade. Therefore, the rest of this introduction will summarize these works in respect to the order of each metastatic step as described above.

EFFECT OF THE MIR-200 FAMILY ON TUMOR GROWTH, ANGIOGENESIS, AND NEARBY TISSUE INVASION

Once a cell undergoes transformation to a malignant cell, the checkpoints that limit its growth are bypassed resulting in uncontrolled growth. This uncontrolled growth leads to the formation of a primary tumor. Not only has the miR-200 family been shown to inhibit cellular malignant transformation, but studies have also shown that they are capable of suppressing tumor growth. For example, it was found that forced expression of miR-200a in meningioma cells (84), or expression of miR-429 in SW260 colorectal carcinoma cells (85), reduced xenograft tumor growth when injected into the flanks of SCID or nude mice, respectively. However, the reported effects of miR-200b on xenograft tumor growth are less consistent as some studies including ours have shown that expression of miR-200b decreased tumor growth (86,87) while others have shown that it has little or no effect on tumor growth (88,89). Therefore more work is needed to determine the role of the miR-200 family in this early step of metastasis.

Once a tumor reaches 1–2 mm^3 the cells at the center of the tumor are under hypoxic conditions and do not receive enough nutrients to grow (90). In order to combat this environment a tumor must initiate angiogenesis, which allows new blood vessels to form intricately within the tumor. Recent studies have shown that the miR-200 family can inhibit angiogenesis because the family targets multiple key players in this process.

In this regard, two separate studies have shown that miR-200b directly targets vascular endothelial growth factor A (VEGFA) (91,92), a ligand that is considered the master determinant for the activation of the angiogenic program. Furthermore, the miR-200 family has also been shown to target the VEGF receptors. For example, in A549 lung cancer cells, Choi and colleagues demonstrated that transient miR-200b expression reduced Flt1 (VEGF receptor 1) and

KDR (VEGF receptor 2) protein level, and a luciferase reporter assay confirmed the direct interaction between miR-200b and the 3′UTR of these proteins. Similarly, miR-200c has also been shown to directly target KDR (93). These findings were further confirmed by Roybal and colleagues showing that stable expression of cluster I miR-200s reduced protein and mRNA levels of the receptor tyrosine kinase Flt1 (VEGFR1) in lung adenocarcinoma 344SQ cells (94). Additionally, miR- 200a and miR-200b have also been shown to directly target the pro-angiogenic ligands interleukin 8 (IL-8) and chemokine (C-X-C motif) ligand 1 (CXCL1) to regulate angiogenesis in ovarian cancer (95). Taken together, these data suggest that the miR-200 family plays crucial roles in the metastatic cascade by down-regulating important players involved in angiogenesis.

Research in our lab has also shown an important role of miR-200b in inhibiting tumor angiogenesis. Ours and other studies showed that inoculation of arsenic-transformed cells produced invasive and metastatic xenograft tumors in nude mice (96-98), however, the underlying mechanism is not clear. To examine whether arsenic-transformed cells have a pro-angiogenic activity, we tested the effect of conditioned media from these cells on the tube forming ability of human umbilical vein epithelial cells (HUVECs). HUVECs cultured in conditioned media from arsenic-transformed cells formed extensive tubes compared to HUVECs cultured in conditioned media from non-transformed control cells, suggesting a pro-angiogenic capability of arsenic-transformed cells (99). To study the role of miR-200b in this process, we used conditioned media from arsenic-transformed cells that stably expressed miR-200b. It was found that HUVECs cultured in conditioned media from arsenic-transformed cells stably expressing miR- 200b formed significantly less tubes. Furthermore, the immunofluorescence staining of CD31, a marker of blood vessel endothelial cells, on xenograft tissues resulting from

the injection of arsenic-transformed cells stably expressing miR-200b showed a drastic reduction compared to CD31 staining on xenograft tumors resulting from the injection of arsenic-transformed vector control cells (99). Our additional mechanistic studies suggested that this reduction of angiogenesis by miR-200b is likely due to down-regulation of VEGF levels resulting from β-catenin sequestration at the plasma membrane by increased expression of E-cadherin. Together, these findings provide additional evidence supporting that miR- 200b is capable of inhibiting tumor angiogenesis.

Tumor nearby tissue invasion involves breakage of the boundaries of tissues where tumors originate from and the entry of cancer cells from the primary tumor into the surrounding stroma. Sossey-Alaoui and colleagues found that WAS protein family member 3 (WAVE3) was critical for the invasive properties of transformed cells (100). Further analysis revealed that miR-200b directly targets WAVE3 through interaction with its 3′UTR in MDA-MB-231 breast, LNCaP prostate, and HT29 colorectal cancer cells. To study the effects of WAVE3 on cell invasion, a Matrigel-invasion assay showed that cells treated with WAVE3 siRNA or that overexpression of miR-200b reduced the invasive capability, while using an anti-miR-200b oligonucleotide increased the number of invading cells. Expressing a WAVE3 mRNA that is resistant to miR-200b targeting also reversed the inhibitory effects of miR-200b on cell invasion, further suggesting a critical role for miR-200b in the inhibition of WAVE3- dependent cellular invasion.

Another study looked at the effects of miR-200 on targets that regulate the reorganization of the actin cytoskeleton to promote invasiveness (101). Transient transfection of a miR-200c mimic in MDA-MB-231 breast cancer cells showed a strong inhibitory effect on the invasive capabilities of these cells in Matrigel and when using a real-time cell analyzer. Furthermore, the

increase in the level of miR-200c was accompanied by a decrease in stress fiber formation that was not accompanied by a change in RhoA activity, suggesting that miR-200c likely acted downstream of RhoA. Transient transfection of miR-200c reduced both formin homology domain-containing protein 1 (FHOD1) and Mg2+/Mn2+-dependent protein phosphatase 1F (PPM1F) levels, and inhibition of the miR-200b/c/429 cluster in MCF7 breast cancer cells increased FHOD1 and PPM1F levels. A luciferase reporter assay confirmed that miR-200c directly targets the 3′UTR of these mRNAs in MDA-MB-231, MCF-7, and HEK- 293FT cells. Silencing of FHOD1 or PPM1F resulted in decreased invasion in MDA-MB-231 cells using the same assays as before, showing the importance of these targets in invasion.

Li and colleagues found that miR-200c expression was significantly lower in A549, H1299, and SPC-A-1sci non-small cell lung cancer (NSCLC) cells among other NSCLC cells (102). Transwell invasion assays showed that these cell lines that expressed lower levels of miR-200c had a higher invasive capability than other NSCLC cells. Furthermore when these highly invasive cells were transiently transfected with miR-200c mimics, their invasive abilities were significantly decreased compared to cells transfected with control oligos, which suggests a suppressive role for miR-200c on NSCLC cell invasion. Bioinformatic analysis suggested ubiquitin specific peptidase 25 (USP25) as a potential target for miR-200c and a luciferase reporter assay confirmed the direct binding of miR-200c to its 3′UTR. To further show the importance of this protein in cellular invasion, knockdown of USP25 significantly reduced cell invasion and its overexpression increased cell invasion as assayed by transwell invasion assays.

EFFECT OF THE MIR-200 FAMILY ON EPITHELIAL-TO-MESENCHYMAL TRANSITION AND TUMOR CELL MIGRATION

One of the most critical properties that a tumor cell must obtain in order to metastasize is the ability to move away from the primary tumor. One of the most widely studied cellular programs that tumors cells activate to gain this motility is known as the epithelial-to-mesenchymal transition (EMT). EMT is a process by which a normally polar, epithelial cell undergoes a change to a mesenchymal-like cell. By undergoing EMT, a cell is able to take on the characteristics of a mesenchymal cell and become more motile and invasive. Accompanying this morphological change is a shift in expressed proteins, and an increase in the production of transcription factors and extracellular matrix degrading enzymes within the cell. EMT occurs naturally during embryogenesis; however it is now thought to be a major contributor to the metastasis of epithelial-originated cancers.

Two of the major hallmarks of EMT include a loss of the epithelial markers such as E-cadherin, a cell-cell adhesion protein, and the increase in the expression of mesenchymal markers such as ZEB1, ZEB2 and other EMT-inducing transcription factors. These two hallmarks are mutually exclusive from the other within an epithelial cell. ZEB1 and ZEB2 act as E-cadherin repressors by directly binding to the E-boxes within the E-cadherin promoter (103-105), thus ZEB1 and ZEB2 are directly involved in the control of EMT by suppressing the expression of E-cadherin, and their expression promotes cell migration and invasion. Recent studies suggest that the miR-200 family is pivotal in regulating EMT by targeting ZEB1 and ZEB2 via direct interactions with their 3′UTRs (106-108). Through down-regulating ZEB1 and ZEB2 expression, the miR-200 family can effectively up-regulate cellular E-cadherin level and maintain a cell in a more epithelial-like state. However, ZEB1 and ZEB2 can also bind to the E-

box sites close to the transcription start site of each of the miR-200 clusters inhibiting their transcription, resulting in a negative feedback loop (109,110). Thus, ZEB1 and ZEB2 can keep a cell in a mesenchymal phenotype by repressing the transcription of both E-cadherin and the miR-200 family. Therefore, the interplay between the miR-200 family and ZEB1/ZEB2 plays an important role in driving the cell in to and out of EMT.

Cell migration is a critical step in the metastatic cascade where tumor cells move away from the primary tumor to enter the blood stream. Early studies on the miR-200 family have shown that the miR-200 family can suppress cell migration. Gregory *et al*. first reported the inhibitory effect of miR-200 on cell migration using a transwell migration assay (106). Using specific miR-200 inhibitors, this group found that the miR-200 inhibitors increased cell migration of Madin-Darby canine kidney epithelial cells, suggesting that the miR-200 family inhibits cell migration. Similarly, Park *et al*. found that expressing miR-200a/c in the highly metastatic MDA-MB-231 breast cancer cells significantly decreased their motility in a transwell migration assay (107). Both of these studies also showed that cells that have undergone EMT have an increased motility as well as an increase in the expression of the mesenchymal markers ZEB1, ZEB2 and vimentin. Furthermore, these and others have shown that the miR-200 family is a strong inhibitor of EMT, and that EMT resulting from the loss of the miR-200 family depends on ZEB1 and/or ZEB2 up-regulation. Therefore, it was concluded that the miR-200 family elicits this inhibitory effect on cell migration by targeting both ZEB1/2.

However, ours and other recent studies also suggest that the miR-200 family can inhibit cell migration independent of its effect on ZEB1/ZEB2. Li and colleagues found that mammary fat pad injection of the metastatic MDA-MB-231 LM2 breast cancer cells resulted in metastasis to the lung and bone, and this was greatly reduced by the stable expression of miR-200b or miR-

200c (88). This suppressive effect on metastasis was not seen when miR-141 was stably expressed in these cells suggesting that the functional group I miR-200s (miR-200b/-200c/-429) is able to repress metastasis in these cells. To determine whether miR-200 suppresses metastasis by targeting ZEB1, the ZEB1 expressing cDNA lacking the 3′UTR was engineered into the MDA-MB-231 LM2 cells that stably expressed miR-200b. It was found that miR-200b was still able to inhibit metastasis when ZEB1 was forcibly expressed, which implies that miR-200b can inhibit metastasis in a ZEB1-independent manner. Through invasion assays and luciferase reporter assays, it was determined that miR-200b regulates cell migration and metastasis by targeting moesin, and restoration of moesin prevents miR-200b from suppressing cell migration and metastasis.

Our recent studies have also implicated the miR- 200 family in the ZEB1-independent regulation of cell migration and metastasis. In the highly migratory arsenic-transformed cells and basal mesenchymal-like triple negative breast cancer cells, we found that PKCα expression levels are significantly higher, and re-expression of miR-200b reduced PKCα levels and inhibited cell migration as well as mammary tumor metastasis (87,111). Subsequent luciferase reporter assays revealed that miR-200b directly targets the 3′UTR of PKCα. Moreover, siRNA knockdown of PKCα significantly reduced cell migration. In contrast, enforced expression of PKCα reversed the inhibitory effect of miR-200b on cell migration and tumor metastasis with no significant effect on ZEB1 expression. These findings suggest that miR-200b suppresses cell migration and metastasis by targeting PKCα, which is independent of its effect on ZEB1.

By applying the Ago-HITS-CLIP technology for transcriptome-wide identification of direct miRNA targets in living cells, Bracken *et al* recently identified a good number of miR-200a and miR-200b targets (112). Further functional validation of the identified miR-200 targets

revealed that they constitute subnetworks that play crucial roles in enabling cancer cells to migrate and invade. The identified miR-200 targets are critically involved in Rho-ROCK signaling, invadopodia formation, matrix metalloproteinase activity, and focal adhesions. This work showed for the first time a global regulatory network directly regulated by miR- 200 family, which provided a novel mechanistic insight for miR-200 family maintaining the key features of the epithelial phenotype and preventing cell migration.

EFFECT OF THE MIR-200 FAMILY ON TUMOR CELL INTRAVASATION

Intravasation is a complex step that involves cancer cells entering the blood vessels or lymphatic system. It can be aided by gene changes that promote the ability of cancer cells to cross the basement and endothelial membranes that form the walls of the vessels. Notch signaling (113) and tumor-associated macrophages (TAMs) (114) are two example mechanisms that have been shown to positively modulate intravasation of cancer cells. In addition to these mechanisms, new blood vessels that have been formed by the primary tumor are leaky and therefore can also facilitate intravasation (115,116). It has been shown that stably expressing both miR-200 cluster members reduced the ability of cancer cells to enter the blood stream, and that E-cadherin overexpression can also decrease the number of cells in the blood stream (117). However, there has been little research done with respect to the mechanism by which miR-200 family reduces cancer cell intravasation. This is probably partly due to the difficulty in measuring intravasated cells in the blood stream, and that the miR-200 family has a strong suppressive effect on the earlier steps of the metastatic cascade. More research is needed to fully understand the intravasation process, and development of a system in which we can directly observe and measure intravasation is critical in doing that.

EFFECT OF THE MIR-200 FAMILY ON TUMOR CELL SURVIVAL IN CIRCULATION

After tumor cells have successfully intravasated into the blood stream, they can circulate throughout the body. These tumor cells that have entered the blood stream are known as circulating tumor cells (CTCs). Since the blood stream is such a harsh environment, these CTCs need to adopt a molecular profile that promotes survival in the blood stream. Therefore CTCs often reprogram certain cellular programs such as apoptosis and anoikis to survive. MiR-200 family members have been shown to regulate apoptosis and anoikis, and therefore may have an effect on tumor cell survival in circulation.

In the case of apoptosis, Uhlmann *et al*. found that in MDA-MB-231 breast cancer cells, overexpression of the miR-200b/c/429 cluster significantly reduced cell viability and increased apoptosis (118). It was further confirmed that PLCγ1 was a direct target of the miR-200b/c/429 cluster, and PLCγ1 knockdown resulted in reduced cell viability and increased caspase activity. Schickel *et al*. found that stably expressing miR- 200c in CAKI-1 kidney and HeyA8 ovarian or transiently expressing it in ACHN kidney cancer cells, caused these cells to be much more sensitive to CD95 (a death receptor)-mediated apoptosis when treated with a CD95 agonist (119). It was confirmed that miR-200c directly targets FAP-1, a known inhibitor of CD95-mediated apoptosis in these cells, and that the likely mechanism for the sensitivity seen was due to an increase of CD95 surface expression because of FAP-1 suppression. In another study, miR-200c was shown to target Noxa, a member of the Bcl-2 family, in MCF7 breast cancer cells (120). Through functional studies in these cells it was determined that miR-200c potentiates apoptosis to the clinically used proteasome inhibitor bortezomib by reducing Noxa levels. In addition, in meningiomas it was found that miR-200a directly targeted β-catenin to increase

apoptotic cell death (121). Therefore, downregulation of miR-200 family levels in tumor cells can help the cell survive within the bloodstream by reducing apoptosis.

Anoikis is a kind of apoptosis that is induced by inappropriate or inadequate cell-ECM attachment. Alteration of this complex signaling process can allow tumor cells to survive in the circulatory system that is normally unsuitable for these cells.

Howe *et al*. demonstrated that miR-200c levels were significantly reduced in BT549 and MDA-MB-231 triple negative breast cancer cells (122). Further work showed that stably expressing miR-200c induces anoikis in these cells by directly targeting TrkB, a neurotrophic tyrosine receptor kinase. Zhang and colleagues also studied the role of miR-200 in anoikis in breast cancer (123). Transfection of miR-200b in MDA-MB-231, BT549, and Hs578T TNBC cells increased the number of apoptotic cells in suspension-culture. Pin1, peptidylprolyl cis/trans isomerase, NIMA-interacting 1, was confirmed as a direct target of miR-200b, and simultaneously expressing miR-200b and an untargetable Pin1 resulted in a decreased number of cells undergoing anoikis in culture. However, in contrast to these studies, Yu *et al*. found that expression of miR-200a made breast cancer cells more resistant to anoikis (124). YAP1, yes-associated protein 1, was confirmed to be a direct target of miR-200a and by targeting this protein miR-200a allows cells to avoid anoikis. Given the contradictory results observed, the role of individual members of the miR-200 family in anoikis still needs to be further studied.

By regulating above cellular processes, cancer cells are able to survive in the harsh circulation system. In addition, immune system differentiation, activation, development, or response could also play critical roles in the survival of intravasated tumor cells. However, little research has been done specifically looking at the effect of the miRNA-200s on immune system

regulation. Therefore, more research is needed in order to further elucidate the role that these miRNAs play in this critical step of metastasis.

EFFECT OF THE MIR-200 FAMILY ON TUMOR CELL EXTRAVASATION AND METASTATIC COLONIZATION

The final steps of metastasis involve surviving CTCs coming to arrest in the blood vessels, extravasating from the blood vessels, and colonizing a distant organ. In order for extravasation to occur, CTCs must first come in contact the endothelium either by becoming lodged in smaller vessels or by specifically adhering to the endothelium. Once a circulating tumor cell extravasates from the blood vessels (likely facilitated by ZEB1 and N-cadherin expression (125)), these cells have a tendency to undergo mesenchymal-to-epithelial transition (MET) which is most likely due to the absence of the signals they received from the primary tumor to undergo EMT. MET facilitates the settling of a cancer cell at a distant organ by allowing these cancer cells to recover their epithelial properties. Once a cell settles in the distant organ, it begins to proliferate and colonize the organ. In striking contrast to the strong inhibitory effect of miR-200 family has on the early metastatic steps, studies have shown that miR-200 may promote metastatic colonization (117,126).

To study the role of miR-200 in the last step of the metastatic cascade, Korpal and colleagues profiled the levels of the miR-200 family in primary and metastatic samples and found that the miR-200 family was higher in metastatic secondary tumors (117). Moreover, profiling a group of mouse breast cancer cell lines (67NR, 168FARN, 4TO7, and 4T1) with different metastatic capabilities (67NR cells are unable to intravasate; 168FARN cells cannot extravasate efficiently; 4TO7 cells do not colonize distant organs well; and 4T1 cells are capable of completing all steps of metastasis) revealed that the most metastatic cells (4T1) had the highest level of miR-200 family expression. Similarly, Dykxhoorn *et al*. reported that the 4TO7 cells, lacking the capability of colonizing distant organs, had almost undetectable expression

levels of the miR-200b/c/429 cluster compared to the strongly metastatic 4T1 cells (126), suggesting a potential role for the miR-200b/c/429 cluster in colonization. To study the role of the miR-200s in metastatic colonization, Korpal *et al*. stably expressed cluster I (miR-200b/a/429: which will be referred to as C1), cluster II (miR-200c/141: C2) or clusters I and II (C1+C2) in the weakly metastatic 4TO7 cells. Importantly, it was found when C2 was stably expressed the other cluster (C1) also showed a significant increase in expression levels; however, C1 overexpression did not increase the expression levels of C2 miR-200 members. When 4T1 cells, the parental 4TO7 cells and modified 4TO7 cells were injected into the mammary fat pad of mice, it was found that all mice injected with 4T1 cells developed lung and liver metastases, while no mice injected with parental 4TO7 cells had any detectable metastases (126). In contrast, injection of the modified C2 or C1+C2 4TO7 cells (cell lines that both express high levels of all five miR-200 members) formed more lung metastases than the parental or C1 alone overexpressing 4TO7 cells (117). Furthermore, Dykxhoorn *et al*. also found that about 80% of mice injected with C2 4TO7 cells developed lung metastases, indicating that the 4TO7 cells that stably express miR-141/200c act similarly to the metastatic 4T1 cells (126). In addition, Korpal and colleagues also determined that this increase in metastasis by the stable expression of the miR-200s was not due to the increased E-cadherin expression because E-cadherin overexpression alone in these cells caused no increase in lung metastases that was seen in the C2 and C1+C2 cells (117). These data suggests that the miR-200 family targets pathways involved in inhibiting metastatic colonization.

To further determine the underlying mechanism behind miR-200 promotion of metastatic colonization, Korpal and colleagues used a tail vein injection model with the modified 4TO7 cells described above. Results from this experiment showed more lung metastases for all cell

lines with C2 and C1+C2 cells having the greatest effect. Knockdown of E-cadherin in C1+C2 cells did not affect their metastatic efficiency significantly (117). This again suggested that other targets of the miR-200 family are more important in colonization efficacy of a cancer cell. Through microarray and mass spectrometry analysis nine potential miR-200 targets were identified. Of these nine targets, three were confirmed as direct targets of miR- 200: cofilin 2 (Cfl2), low-density lipoprotein receptor-related protein 1 (Lrp1) and Sec23a, a key component of COPII vesicles. Functional analysis *in vitro* and *in vivo* revealed that miR-200 increased metastatic colonization by targeting Sec23a. Further analysis also revealed that Tinagl14 and Igfbp4, two secreted metastasis suppressors, are directly regulated by Sec23a (117). Interestingly, a recent study showed that the metastatic 4T1 cells, but not the poorly metastatic 4TO7 cells, can secrete miR-200s into extracellular vesicles (EVs) (127). Moreover, it was also found that the poorly metastatic 4TO7 cells can take up miR-200 from 4T1 EVs and become metastatic in a miR-200–dependent manner (127). This study provided novel evidence showing that metastatic capability can be transferred from metastatic to non-metastatic cancer cells through extracellular vesicles. In addition, this finding also suggests that circulating miRNAs are not only just cancer biomarkers; they are also functional being capable of promoting metastasis *in vivo*.

These studies suggest that even though the miR-200 reduces the number of cancer cells in the bloodstream, probably by strongly inhibiting the early metastatic steps, those cancer cells that have high expression levels of miR-200s and do manage to get through the extravasation step are more capable of colonizing a distant organ. Additionally since these studies have shown that it is the overexpression of the miR-200c/141 cluster that causes the increase in metastatic colonization, it is possible that the miR-200c/141 cluster may act as a suppressor for early steps

of metastasis, but facilitates post-extravasation events while the miR-200b/a/429 cluster suppresses metastasis at all steps. However, more research is needed to discern the function of each cluster as a whole and to elucidate the effect of each individual member of the miR- 200 family on the metastatic cascade.

THE MIR-200 FAMILY AS POTENTIAL DIAGNOSTIC AND PROGNOSTIC TOOLS

The search for biomarkers that can serve as an early detection method or as a predictive tool for prognosis is needed to increase the earlier diagnosis and thus the long term survival of cancer patients. The studies of miRNAs as potential diagnostic and prognostic tools mainly stem from reports showing that there are stable, cell-free miRNAs in the blood (termed circulating miRNAs) (128-131), and that circulating miRNAs have specific expression profiles for different cancers, although their origins are currently unclear. Further studies are needed to elucidate whether they come from tumor cell death, are secreted from tumor cells, or originate from blood cells associated with the tumor as well as elucidate their expression patterns in individual cancer types. It is because of this unknown that most studies only show a correlation between a certain cancer and miRNAs.

Studies showed potential in the use of members of the miR-200 family for cancer diagnosis. A genome-wide study by Madhavan *et al*. found that circulating miRNAs can act as diagnostic markers for circulating tumor cells (CTCs) in metastatic breast cancer (MBC) (132). Their analysis of CTC-positive versus CTC-negative MBC patients revealed distinct miRNA signatures for each group, and consequently 17 of these miRNAs were further studied. Of these 17 miRNAs, four of them were from the miR-200 family (miR-200b, -200a, -200c, and -141), and this group concluded that miR-200b was the best miRNA for determining CTC-positive MBC patients. Similarly, a study by Cheng *et al*. found that plasma miR-141 levels are increased in colorectal cancer, highly associated with stage IV colorectal cancer, and able to increase the detection of Stage IV colon cancer when combined with the commonly used colorectal cancer detection marker carcinoembryonic antigen (CEA) (133). In contrast, Park *et al*. reported that miR-200a was expressed at lower levels in the saliva of 38 patients with oral squamous cell

carcinoma (OSCC) compared to 38 healthy controls (134). These studies suggest that body fluid miR-200 family levels may have different diagnostic values for different types of cancers.

The use of the miR-200s as a prognostic marker also looks promising. Comparing the expression levels of the miR-200 family members in gastric cancer cell lines, Valladares-Ayerbes and colleagues found that miR-200c levels were much significantly higher in cancer cells compared to normal cells (135). Further analysis using patient samples showed an inverse correlation between miR-200c blood levels and prognosis, which suggests miR-200c as a potential prognostic biomarker for gastric cancer. Xu and colleagues were able to show that miR-200 family expression was significantly correlated with the status (benign, non-recurrent or recurrent primary, or metastatic) of a melanoma tumor, therefore expanding the potential role of the miR-200 family as a prognostic marker in this disease (136). In addition, Cheng *et al*. reported that circulating miR-141 levels were negatively associated with overall survival for the colon cancer patients (133). Furthermore, a high level of circulating miR-141 was found to be associated with high-risk (Gleason score ≥ 8) tumors (137), while a lower level of cluster I of the miR-200 family was correlated with relapse (138) in prostate cancer. High levels of miR-141 were also correlated strongly with decreased overall survival in the luminal subtypes of breast cancer (95). In contrast, it was found that low expression of cluster I miR-200s (miR-200b, -200a and -429) correlated with poorer overall survival in ovarian and endometrial cancer (139,140). Together, these findings suggest that miR-200 levels may have the potential to serve as indicators of cancer prognosis.

It is interesting to note that the majority of data regarding the miR-200 family in experimental model systems shows that the miR-200s suppress tumor development and progression. However, the clinical diagnostic and prognostic data summarized above are much

less consistent about the role of the miR-200s as a suppressor of tumor growth and metastasis. This inconsistence could be due to: (i) the levels of circulating miR-200s, not the levels of miR-200s in cancer tissues in most cases, were used for potential cancer diagnosis and prognosis prediction. However, the origin of circulating miRNA is currently unknown; (ii) it is likely that the role of miR-200s in cancer development and progression may be cancer type-dependent or even cancer subtype-dependent. Indeed, Pecot *et al*. recently reported that higher miR-200 levels in ovarian, lung, renal and basal-like breast adenocarcinomas are associated with improved clinical outcome. However, higher levels of miR-141 are significantly associated with worse clinical outcome of luminal subtypes breast cancer (95), suggesting that miR-200 may exhibit differential functions among different breast cancer subtypes.

THE POTENTIAL OF THE MIR-200 FAMILY IN CANCER THERAPY

The idea of miRNAs contributing to chemoresistance has been widely studied (141,142). The ability of a cell to avoid apoptosis (143) and to undergo EMT (144) have been shown to contribute to the chemoresistance of tumor cells. With the development of microarrays researchers have been able to determine the expression levels and patterns of miRNAs in chemoresistant cells, and this has allowed researchers to determine potential miRNAs involved in the process of apoptosis and chemoresistance.

In the case of apoptosis, Uhlmann *et al*. found that in MDA-MB-231 breast cancer cells, overexpression of the miR-200b/c/429 cluster significantly reduced cell viability and increased apoptosis (118). It was further confirmed that PLCγ1 was a direct target of the miR-200b/c/429 cluster, and PLCγ1 knockdown resulted in reduced cell viability and increased caspase activity.

Schickel *et al*. found that stably expressing miR-200c in CAKI-1 kidney and HeyA8 ovarian or transiently expressing it in ACHN kidney cancer cells, caused these cells to be much more sensitive to CD95 (a death receptor)-mediated apoptosis when treated with a CD95 agonist (119). It was confirmed that miR-200c directly targets FAP-1, a known inhibitor of CD95-mediated apoptosis in these cells, and that the likely mechanism for the sensitivity seen was due to an increase of CD95 surface expression because of FAP-1 suppression. In another study, miR-200c was shown to target Noxa, a member of the Bcl-2 family, in MCF7 breast cancer cells (119). Through functional studies in these cells it was determined that miR-200c potentiates apoptosis to the clinically used proteasome inhibitor bortezomib by reducing Noxa levels. In addition, in meningiomas it was found that miR-200a directly targeted β-catenin to increase apoptotic cell death (121). Therefore, downregulation of miR-200 family levels in tumor cells can help the cell survive within the bloodstream by reducing apoptosis.

Chemotherapeutic resistance of cancer cells is thought to be one of the primary causes of recurrence in cancer. Although inadequate delivery of the drug to the tumor can contribute to chemoresistance, cellular reprogramming also plays a major role in establishing this resistance. By turning off genes involved in chemosensitivity and turning on genes involved in chemoresistance, tumor cells can effectively evade the drug. Studies have shown that the miR-200 family plays a role in reducing chemoresistance by targeting these genes known to play a direct role in developing this resistance. Liu *et al*. found that the expression of miR-200c is decreased in melanoma tissues and cells, with a further decrease in metastatic primary melanoma tumors (145). In depth analysis revealed that miR-200c reduces the expression of ATP-binding cassette (ABC) transporters ABCG2, ABCG5 and MDR1 in WM115A melanoma cells. This is important because of their known involvement in the multidrug resistance seen in cancer (146). These findings suggest that downregulation of miR-200c may contribute to the development of chemoresistance in melanoma.

By generating a doxorubicin-resistant breast cancer cell line (BT474), Kopp *et al*. also showed that loss of miR-200c is important in developing chemoresistance (147). It was found that miR-200c was significantly downregulated in doxorubicin-resistant cells. When treated with a miR-200c inhibitor, these doxorubicin-resistant BT474 cells became even more resistant to doxorubicin treatment compared to control cells. In contrast, overexpressing miR-200c resensitized these doxorubicin-resistant breast cancer cells to doxorubicin treatment. Mechanistic studies from this group determined that miR-200c reduces drug resistance in these cells through targeting Neurotrophic Tyrosine Kinase, Receptor, Type 2 (TrkB) and BMI1 Polycomb Ring Finger Oncogene (BMI1) (147). In addition, another study revealed that loss of miR-200c expression is associated with poorly differentiated endometrial carcinoma; and restoration of

miR-200c in a papillary uterine cancer line significantly increased its chemosensitivity to the microtubule-targeting chemotherapeutics paclitaxel, vincristine, and epothilone B (148).

Using a miRNA microarray, Kovalchuk and colleagues found that the levels of miR-200a and miR-200c were significantly lower in MCF-7 breast cancer cells that were resistant to doxorubicin compared to the parental cells, suggesting that decreased expression of these miRNAs may contribute to doxorubicin resistance in breast cancer (149). Using the same techniques as Kovalchuk *et al*., Pogribny and colleagues found that in MCF-7 cells miR-200b and -200c expression were inversely correlated with resistance to cisplatin (150). However, in contrast to the findings from above studies, Hamano *et al*. found that miR-200c overexpression induces cisplatin resistance in esophageal cancer cells (TE8-R) (151), suggesting that the relationship between the miR-200c expression levels and chemoresistance may be cellular context and drug dependent.

A miRNA microarray done by Meng *et al*. in cholangiocyte cell lines showed that miR-200b and -141 are dysregulated in malignant cholangiocytes (152). By culturing cholangiocarcinoma cells (Mz-ChA-1) with gemcitabine in the presence or absence of miR-141 or miR-200b inhibitors, they were able to determine that the inhibition of miR-200b decreased gemcitabine-induced apoptosis. Separately, Rui and colleagues also found that decreased miR-200b levels are associated with resistance to docetaxel in a lung adenocarcinoma cell line (SPC-A1 and SPC-A1/docetaxel) (153). These findings suggest that decreased expression of miR-200b may play a critical role in chemoresistance.

A recent paper by Manavalan *et al*. has also shown a link between the miR-200 family and targeted therapy resistance in breast cancer cells (154). The expression levels of the miR-200 family members was determined by qPCR in MCF-7 cells that were either sensitive or resistant

(LY2) to endocrine treatment, and showed that the expression of miR-200b, -200a, and -200c was significantly decreased in the endocrine-resistant cell lines. To determine if these miRNAs affected sensitivity to endocrine treatment, the LY2 cells were transiently transfected with their precursor miRNA and cell viability was determined in the presence and absence of the antiestrogens 4-OHT (the active metabolite of tamoxifen) or fulvestrant (154). Results from this experiment showed that expression of miR-200b and miR-200c enhance the sensitivity of LY2 breast cancer cells to growth inhibition by both 4-OHT and fulvestrant. Therefore, the miR-200 family also plays a role in sensitivity to the specific targeted therapies available for breast cancer.

Though much of the research on the miR-200 family in cancer drug resistance has focused mostly on miR-200b and -200c, it is possible that the other members of the miR-200 family may also play similar roles in the process due to their similar seed sequences.

Although current studies on the miR-200 family have shown promising results, more work is needed to further understand the role this family plays in cancer. Future work on the miR-200 family can help with better understanding the mechanism by which miR-200s affect cancer initiation, metastasis, and relapse. Since much work has focused on the effect of whole clusters/groups on metastasis, more work is also needed to be done on individual members of the miR-200 family to elucidate their role in each step of the metastatic cascade. Moreover, most of the research done on individual miR-200 family members focuses on miR-200b or -200c, therefore more work is needed on miR-200a, -141 and -429 and their individual role in cancer. Since some of the data on the role of miR-200 family in cancer is controversial and cellular context dependent, it is important for future studies to tease apart which miR-200 family members act as a tumor suppressor and which may promote cancer progression. Furthermore, recent studies have shown that EMT is not necessary for cells to successfully metastasize to

distant organs, but is important for chemoresistance of cancer cells (155,156). Therefore, the other mechanisms by which miR-200 inhibits cancer metastasis needs to be fully elucidated. Completing these studies will lead to the discovery of more miR-200 targets and ultimately the development of novel and targeted therapeutic options for the treatment of cancer.

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CHAPTER 2: MICRORNA-200B TARGETS PROTEIN KINASE Cα AND SUPPRESSES TRIPLE NEGATIVE BREAST CANCER METASTASIS

This chapter represents a manuscript that was published in *Carcinogenesis* (2014) **35**: 2254-2263.

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ABSTRACT

Triple negative breast cancer (TNBC) is an aggressive subtype of breast cancer with poor prognosis and lacks effective targeted therapies. The microRNA-200 (miR-200) family is found to inhibit or promote breast cancer metastasis; however, the underlying mechanism is not well understood. This study was performed to investigate the effect and mechanism of miR-200b on TNBC metastasis and identify targets for developing more efficient treatment for TNBC. We found that miR-200 family expression levels are significantly lower in highly migratory TNBC cells and metastatic TNBC tumors than other types of breast cancer cells and tumors. Ectopically expressing a single member (miR-200b) of the miR-200 family drastically reduces TNBC cell migration and inhibits tumor metastasis in an orthotopic mouse mammary xenograft tumor model. We identified protein kinase Cα (PKCα) as a new direct target of miR-200b and found that PKCα protein levels are inversely correlated with miR-200b levels in 12 kinds of breast cancer cells. Inhibiting PKCα activity or knocking down PKCα levels significantly reduces TNBC cell migration. In contrast, forced expression of PKCα impairs the inhibitory effect of miR-200b on cell migration and tumor metastasis. Further mechanistic studies revealed that PKCα downregulation by miR-200b results in a significant decrease of Rac1 activation in TNBC cells. These results show that loss of miR-200b expression plays a crucial role in TNBC aggressiveness and that miR-200b suppresses TNBC cell migration and tumor metastasis by targeting PKCα. Our findings suggest that miR-200b and PKCα may serve as promising therapeutic targets for metastatic TNBC.

INTRODUCTION

Triple negative breast cancer (TNBC) is a unique subtype of breast cancer that is histologically defined by the absence of the estrogen receptor (ER), progesterone receptor (PR) and lack of human epidermal growth factor receptor 2 (Her2) overexpression (1,2). TNBC is often a highly invasive and metastatic form of breast cancer with an overall poorer prognosis compared with other breast cancer subtypes. This is partly due to TNBC usually displaying more aggressive behavior and lacking effective targeted therapies (3,4). Chemotherapy is currently the only treatment option for metastatic TNBC and is only effective at the initial treatment stage (5,6). There is an urgent need to better understand the underlying mechanism of TNBC aggressive behavior and identify novel targets for developing more efficient therapies for TNBC.

MicroRNAs (miRNAs) are a large class of small non-coding RNAs and regulate gene expression through binding to the 3′ untranslated region (3′UTR) of their target mRNAs, resulting in mRNA degradation or translation inhibition (7,8). miRNAs are found to be critically involved in many fundamental processes of cancer (8,9), although the underlying mechanisms have not been well understood for the majority of miRNAs. In breast cancer, miRNAs are shown to affect cancer cell survival, proliferation, differentiation, migration, invasion and metastasis (10–12). However, fewer studies on the role of miRNAs in TNBC have been done compared with other breast cancer subtypes. Further studying miRNA function in TNBC may lead to identification of novel therapeutic targets for TNBC.

Human miRNA-200 (miR-200) family consists of five members divided into two groups: the miR-200b/-200a/-429 group located on chromosome 1 and the miR-200c/-141 group located on chromosome 12 (13,14). Alternatively, the miR-200 family can be classified into two functional clusters based on the identities of their seed sequences: the miR-200b/-c/-429 cluster

and the miR-200a/-141 cluster. The miR-200 family members are among the first miRNAs reported to function as potent inhibitors of epithelial-to-mesenchymal transition (EMT) and as regulators of epithelial plasticity of cancer by directly targeting EMT inducing transcription factors zinc-finger E-box-binding homeobox factor 1 (ZEB1) and 2 (ZEB2; 15–21). Despite its well-established role in inhibiting EMT (15–19), a process thought to be important in cancer metastasis (22), the effect of miR-200 family on cancer metastasis has been shown to be controversial. Ectopic expression of either one group of miR-200 or the entire miR-200 family in cancer cells is able to suppress (23) or promote cancer metastasis (24,25). Moreover, relatively few studies have been done on the effect of a single member of miR-200 family on cancer metastasis. In addition, the mechanism of miR-200 function has not been well understood and only a limited number of miR-200 target genes that promote cell migration and cancer metastasis have been identified (26–28). It is essential to further investigate the effect of miR-200 family on cancer metastasis and identify their new targets that play crucial roles in cancer metastasis.

Protein kinase Cα (PKCα) is a member of PKC family of serine/ threonine kinases containing 10 isozymes, playing important roles in regulating cell migration and cancer metastasis (29,30). Particularly, recent studies revealed that PKCα functions as a central signaling node in breast cancer stem cells and has been proposed to be a valuable therapeutic target for certain breast cancer subtypes (31,32). Moreover, recent studies also showed that high PKCα levels were most commonly detected in high-grade TNBC tumors (32,33). However, little is known about the mechanism of PKCα dysregulation in breast cancer.

In this study, we identified PKCα as a new direct target of miR-200b and showed that miR-200b suppresses TNBC cell migration and metastasis by targeting PKCα, which in turn reduces Rac1 activation. The findings from this study not only provide mechanistic insights for

recent observations showing that metastatic TNBC tumors have high PKCα levels, but also suggest that miR-200b and PKCα may serve as promising therapeutic targets for metastatic TNBC.

MATERIALS AND METHODS

CELL LINES AND CELL CULTURE

MCF-7, T-47D, BT-474, MDA-MB-453, SKBR-3, MDA-MB-468, BT-20, Hs578T and BT-549 cell lines were purchased from and validated by American Type Culture Collection (ATCC, Manassas, VA). These cells were cultured following instructions from ATCC and used within 6 months of purchases. MDA-MB-231 cells, provided by Dr Suyun Huang (M.D. Anderson Cancer Center, Houston, TX) and authenticated by M.D. Anderson Cancer Center based on short tandem repeats, were cultured in Dulbecco's modified Eagle medium/F-12 supplemented with 5% fetal bovine serum and 1% Penicillin/Streptomycin (Pen/Strep) (Invitrogen, Carlsbad, CA). SUM-149 and SUM-159 cells, obtained from Dr Stephen Ethier (Wayne State University, Detroit, MI) who developed these cell lines, were cultured in F-12 supplemented with 5% fetal bovine serum, 1% Pen/Strep. All cells were cultured at 37°C in a humidified 5% CO2 atmosphere.

QUANTITATIVE PCR ANALYSIS

Cellular total RNAs were extracted using QIAGEN miRNeasy mini kit (Valencia, CA) for quantitative PCR (Q-PCR) analysis, which was carried out in ABI 7500 Fast Real-Time PCR System using TaqMan gene expression assays for the miR-200 family (Applied Biosystems, Foster City, CA). U6 snRNA was used to normalize relative miR-200 expression levels as described previously (34).

GENERATION OF MIR-200B STABLY EXPRESSING CELL LINES

Vector control (green fluorescent protein, GFP) and miR-200b stably expressing cells were generated by transducing cells with control (pMIRNA-GFP) or miR-200b precursor-expressing (pMIRNA-GFP-200b) lentiviral particles (System Biosciences, Mountain View, CA),

respectively, as described previously (34). The miR-200b stably expressing cell clones were selected by Q-PCR analysis of miR-200b levels in clones grown from series dilution culture of cells transduced with miR-200b precursor-expressing lentiviral particles.

GENERATION OF MIR-200B AND PKCα DOUBLE STABLY EXPRESSING CELLS

Human PKCα full-length complementary DNAs lacking the 3′UTR was purchased from OriGene Technologies (Rockville, MD) and cloned into pLenti6.3⁄V5-DEST™ vector (Invitrogen) following the manufacturer's instructions. Vector control (pLenti6.3) and PKCα-expressing (pLenti6.3-PKCα) lentiviral particles were packaged as described previously (35). To establish vector control, miR-200b and PKCα double stably expressing cells, miR-200b stably expressing cells were transduced with vector control (pLenti6.3) or PKCα-expressing (pLenti6.3-PKCα) lentiviral particles, respectively, and selected with Blasticidin.

GENERATION OF PKCα SHRNA STABLE KNOCKDOWN CELLS

Vector control and PKCα stable knockdown cells were generated by transducing cells with control (pLKO.1-puro) or PKCα short hairpin RNA (shRNA) expressing (pLKO.1-puro-PKCα-shRNA) lentiviral particles, respectively. The control and PKCα shRNA lentiviral constructs were purchased from Sigma (St Louis, MO) and the lentiviral particles were packaged as described previously (35). Cells were transduced with vector control or PKCα shRNA expressing lentiviral particles and selected with puromycin.

GENERATION OF PKCα 3′UTR LUCIFERASE REPORTER WILD-TYPE AND MUTANT-TYPE VECTORS AND DUAL LUCIFERASE REPORTER ASSAYS

A fragment of human PKCα 3′UTR containing nucleotide 1–1825 was synthesized by Blue Heron Biotech (Bothell, WA) and cloned into pMirTarget vector (OriGene Technologies), which served as the wild-type PKCα 3′UTR luciferase vector containing the miR-200b putative

binding site. To generate the mutant-type PKCα 3′UTR luciferase vector, the same fragment of PKCα 3′UTR was synthesized with the miR-200b putative binding site completely mutated. The mutated PKCα 3′UTR fragment was similarly cloned into pMir-Target, which served as the mutant-type PKCα 3′UTR luciferase vector. Dual luciferase reporter assays were performed as described previously (34). The relative luciferase reporter activity was calculated as the ratio of the wild-type or mutant-type PKCα 3′UTR firefly luciferase activity divided by the *Renilla* luciferase activity.

WOUND HEALING AND TRANSWELL CELL MIGRATION ASSAYS

Cell migration was determined by a wound healing assay and/or Transwell cell migration assay as described previously (36). A proliferation inhibitor mitomycin C (1 μg/ml) (Sigma) and GO6976 (1 μM) (Tocris Bioscience, Bristol) were added into the medium when the wound was created.

ORTHOTOPIC MOUSE MAMMARY XENOGRAFT TUMOR MODEL STUDIES

Six-week-old female nude mice (Nu/Nu, Charles River Laboratories) were used and maintained under regulated pathogen-free conditions. Animal protocols were reviewed and approved by the Michigan State University Institutional Animal Care and Use Committee. Mice were anesthetized before injections of 1 × 106 cells (MDA-MB-231-GFP, MDA-MB-231-GFP-200b, MDA-MB-231-GFP-200b-pLenti6.3 or MDA-MB-231-GFP-200b-pLenti6.3-PKCα) into the fourth mammary fat pad in 0.1 ml of 1:1 growth factor-reduced Matrigel (BD Biosciences). Animals injected with MDA-MB-231-GFP or MDA-MB-231-GFP-200b cells (eight mice in each group) were euthanized 8–10 weeks after injection (mice with tumors exceeding 1.0 cm limit were killed at week 8). Animals injected with MDA-MB-231-GFP-200b-pLenti6.3 or MDA-MB-231-GFP-200b-pLenti6.3-PKCα cells (five mice in each group) were killed 12 weeks

after injection. For determining cell proliferation in mammary tumor tissues, mice were injected [intraperitoneal (i.p.)] with 5-bromo-2′-deoxyuridine (70 mg/kg) 2 h before killing. Mammary tumors and lungs were harvested, fixed with 10% formalin solution, paraffin embedded for hematoxylin and eosin (H&E), immunohistochemistry and immunofluorescence staining.

IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE STAINING OF MOUSE MAMMARY TUMOR AND LUNG SECTIONS

Mouse mammary tumor and lung sections were prepared and subjected to H&E staining as described previously (37). The immunohistochemistry staining of 5-bromo-2′-deoxyuridine in mammary tumor sections was carried out using the ABC kit from Vector Laboratories (Burlingame, CA) as described previously (37). The presence of GFP in mouse lung sections was determined by performing GFP immunohistochemistry or immunofluorescence staining, or by staining sections with 4′,6-diamidino-2-phenylindole (DAPI) followed by directly viewing GFP fluorescence under a fluorescence microscope (Nikon Eclipse TE2000-U, Nikon, Melville, NY). The captured GFP fluorescent images were overlaid with the blue fluorescent images (nucleus DAPI staining) using MetaMorph software.

WESTERN BLOT ANALYSIS

Cells were lysed using Tris–sodium dodecyl sulfate and sodium dodecyl sulfate–polyacrylamide gel electorphoresis was used as described previously (34). These primary antibodies were used: anti-ZEB1, anti-E-cadherin, anti-PKCα (Cell Signaling Technology, Danvers, MA), anti-PKCβI (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Rac1 (Millipore, Temecula, CA) and anti-β-Actin (Sigma).

RAC1-GTP PULLDOWN ASSAY

Rac1-GTP pulldown assays were carried out to analyze active Rac1 levels following previously described protocol (38). Rac1-GTP levels were quantified using ImageJ software and the quantifications are presented as the relative Rac1-GTP levels (ratio of Rac1-GTP levels divided by the corresponding total Rac1 levels).

MTT ASSAY AND SOFT AGAR COLONY FORMATION ASSAY

The tetrazolium dye colorimetric test (MTT assay) was used to measure cell growth indirectly. Briefly, cells were cultured in 96-well plates (3–5 × 104 cells/well in 100 μl of complete culture medium) for 24, 48, or 72 h, respectively. At the end of culture, 50 μl of the MTT reagent (5 mg/ml) was added to each well and incubated for 4 h. Then, 200 μl of dimethyl sulfoxide was added to each well and incubated for another hour. The plate was read using a microplate reader (SpectraMAX Plus, Molecular Devices, Sunnyvale, CA) at a wavelength of 570 nm. Soft agar colony formation assay was performed as described previously (34). Colony formation in the agar was photographed and counted (if >100 μm) under a phase-contrast microscope after 4 week incubation.

STATISTICAL ANALYSIS

The statistical analyses for the significance of differences in numerical data (mean □} SD) were carried out by testing different treatment effects via analysis of variance (ANOVA) using a general linear model [Statistical Analysis System (SAS) version 9.1, SAS Institute, Cary, NC]. Differences between treatment groups were determined using a two sample *t*-test. A *P* value of <0.05 was considered statistically significant.

RESULTS

THE MIR-200 FAMILY LEVELS ARE EXTREMELY LOW IN BASAL MESENCHYMAL LIKE TNBC CELLS AND METASTATIC TNBC TUMORS AND ARE INVERSELY CORRELATED WITH TNBC CELL MIGRATORY ABILITIES

Although abnormal expression of miR-200 family has been observed in various types of cancers (26–28), their expression levels among different kinds of breast cancer cells and different subtypes of breast tumors are not well known. We first determined miR-200 family levels among 12 kinds of breast cancer cells. The basic features of these commonly used breast cancer cell lines were described previously (39). Compared with other breast cancer cells, the basal mesenchymal-like TNBC cells have extremely low levels of miR-200s (**Figure 2.1.A**). The only exception is that the Her2+ SKBR3 cells have very low levels of the miR-200b/-200a/-429 group (**Figure 2.1.A**). Moreover, we also compared miR-200 levels among different subtypes of breast cancers including ER+, Her2+, non-metastatic TNBC and metastatic TNBC tumors by analyzing a published breast cancer tissue miRNA microarray data set in the Gene Expression Omnibus (GEO). We found that metastatic TNBC tumors have the lowest levels of miR-200 family (**Figure 2.2.**). Basal epithelial-like TNBC cells display a differentiated and epithelial-like morphology and express high levels of E-cadherin (data not shown). In contrast, basal mesenchymal-like TNBC cells exhibit a fibroblast-like morphology and their E-cadherin expression is undetectable by western blot (data not shown). We examined and compared the migratory capabilities of different kinds of TNBC cells using a wound healing assay. As shown in **Figure 2.1.B**, while basal mesenchymal-like TNBC cells are able to fully close the wound within 24 h, basal epithelial-like TNBC cells close the wound marginally, indicating that basal mesenchymal-like TNBC cells migrate significantly faster than basal epithelial-like TNBC cells.

Figure 2.1. The miR-200 family levels in breast cancer cells are inversely correlated with their migratory capabilities. (**A**) Q-PCR analysis of miR-200 expression levels in breast cancer cells. The levels of miR-200 family are expressed relative to that of MCF-7 cells and are presented as mean ± SD (n = 3). * $p < 0.05$, compared with other types of breast cancer cells. (**B**) Comparison of TNBC cell migration by wound healing assay. Scale bar = 100 μm. Similar results were obtained in two repeated experiments.

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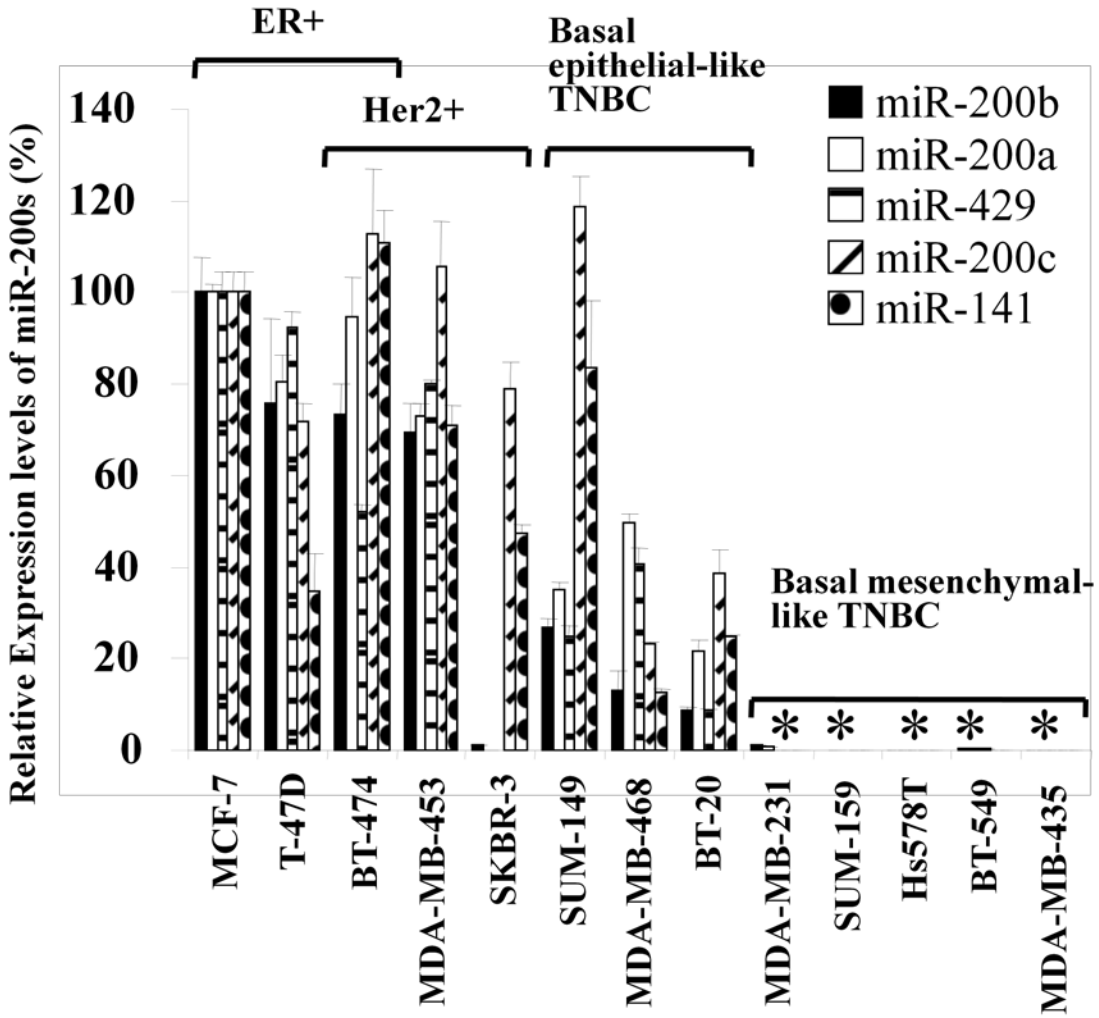

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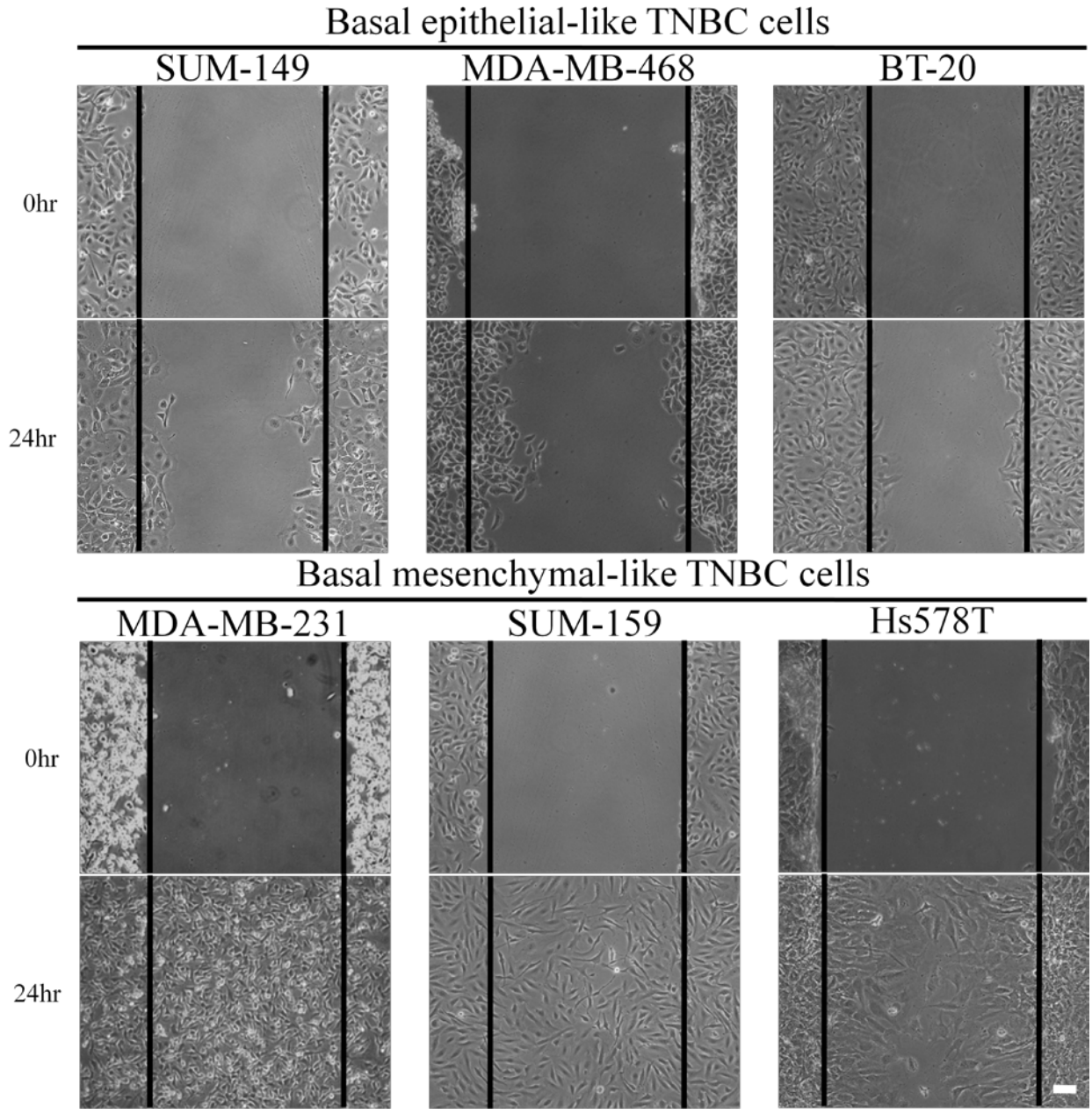
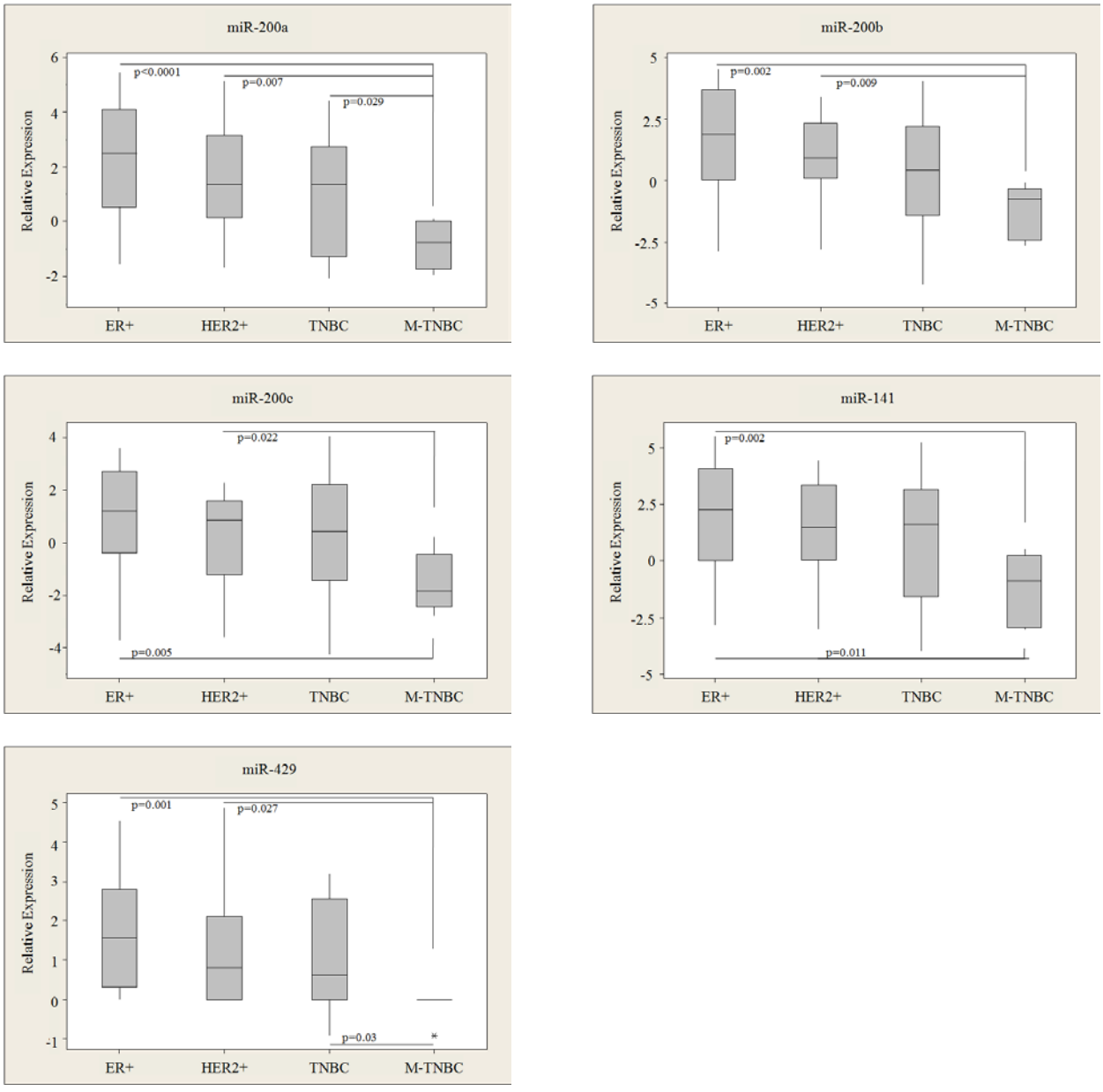


Figure 2.2. MiR-200 expression levels are significantly lower in metastatic TNBC (M-TNBC) tumors than other subtypes of breast cancer. The miR-200 expression values from a breast cancer tissue miRNA microarray data set in the Gene Expression Omnibus (accession number GSE39543) were log2 (Hy3/Hy5) transformed. A two sample ttest assuming unequal variance was applied to compare levels between different groups. A side-by-side boxplot was done to show the distribution of the log2 transformed relative expression values for different groups. ER+: n=14, HER2+: n=10, TNBC: n=10, M-TNBC: n=8.



Collectively, these results show that miR-200 levels in TNBC cells are inversely correlated with their migratory abilities.

STABLY RE-EXPRESSING MIR-200B IN BASAL MESENCHYMAL-LIKE TNBC CELLS SIGNIFICANTLY REDUCES THEIR MIGRATION AND SUPPRESSES MAMMARY TUMOR LUNG MICROMETASTASIS

To determine the effect of miR-200 on basal mesenchymal-like TNBC cell migratory behavior *in vitro* and metastatic capability *in vivo*, we chose to ectopically express a single member (miR-200b) of miR-200 family in two TNBC cells (MDA-MB-231 and SUM-159). This is due to the fact that limited studies have been done on the effect of a single member of miR-200 family, particularly miR-200b, on cancer metastasis. In contrast to GFP control cells that still exhibit mesenchymal-like morphology, miR-200b stably expressing cells display epithelial-like morphology as viewed under a bright light (**Figure 2.3.A**) or fluorescent microscope (**Figure 2.3.B**). Multiple miR-200b stably expressing clones were established, all having similar epithelial-like morphology and expressing high levels of E-cadherin, but low levels of ZEB1 (**Figure 2.3.C**). Q-PCR analysis showed that miR-200b levels in miR-200b stable expression cell clones are about 3-fold higher than MCF-7 cells, and about 1.7-fold higher than immortalized non-transformed human mammary epithelial cells (**Figure 2.3.D**). Four miR-200b stably expressing clones for each cell line were pooled together and used as miR-200b stably expressing cells for all subsequent experiments. Re-expressing miR-200b significantly reduces MDA-MB-231 and SUM-159 cell proliferation (**Figure 2.4.A**) as well as soft agar colony formation (**Figure 2.4.B**). Wound healing assays revealed that while GFP control cells are able to fully close the wound within 24 h, the miR-200b expressing cells only close the wound about 30% (**Figure 2.5.A**). These results show that re-expressing miR-200b is able to drastically

Figure 2.3. Effect of stably expressing miR-200b on TNBC cell morphology and the comparison of miR-200b expression levels among immortalized human mammary epithelial cell (HMLE), breast cancer MCF-7 cell, and miR-200b stably expressing cells. (**A**) Representative bright field images of GFP control and miR-200b stably expressing cells. (**B**) Representative overlaid bright field and fluorescent field images of GFP control and miR-200b stably expressing cells, showing all cells express GFP. (**C**) Western blot analysis of ZEB1 and E-Cadherin protein levels in GFP control and miR-200b stably expressing breast cancer cells. (**D**) Cellular levels of miR-200b were determined by Q-PCR and are expressed relative to that of HMLE cells. Data are presented as means ± standard deviations ($n = 3$). * $p < 0.05$, compared to HMLE cells; # $p < 0.05$, compared to MCF-7 cells.

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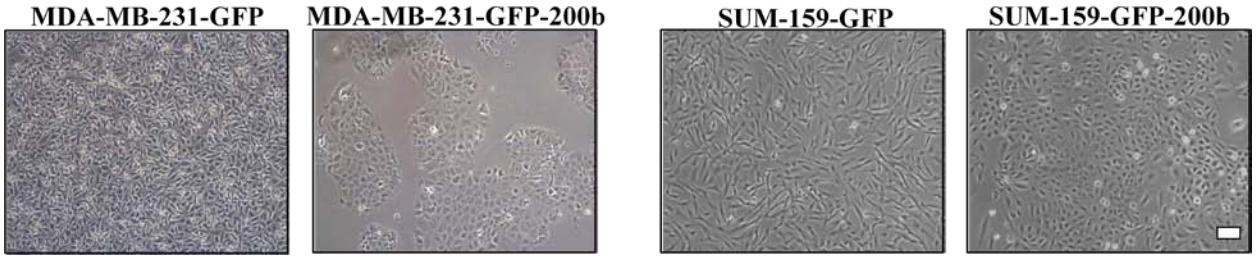

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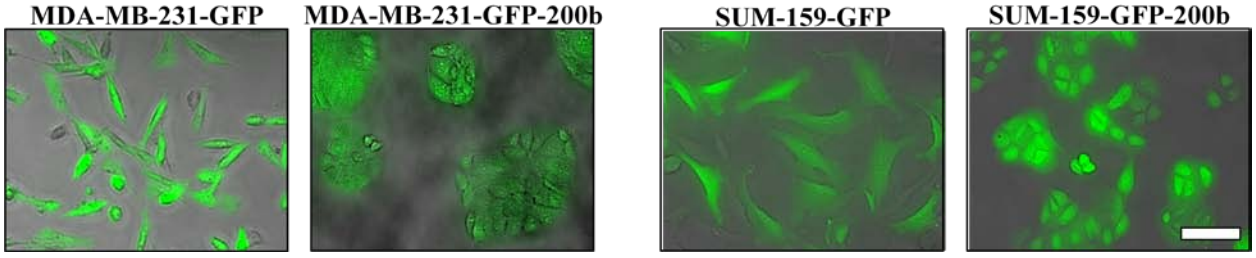

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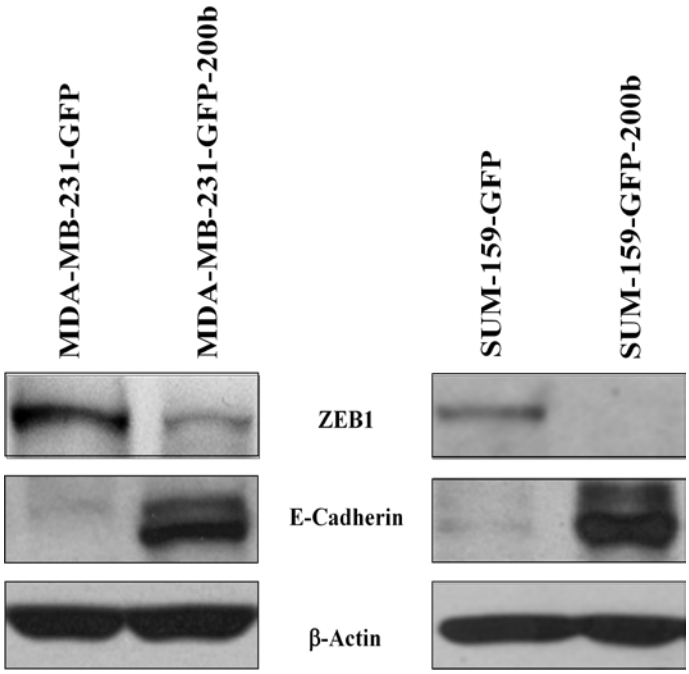

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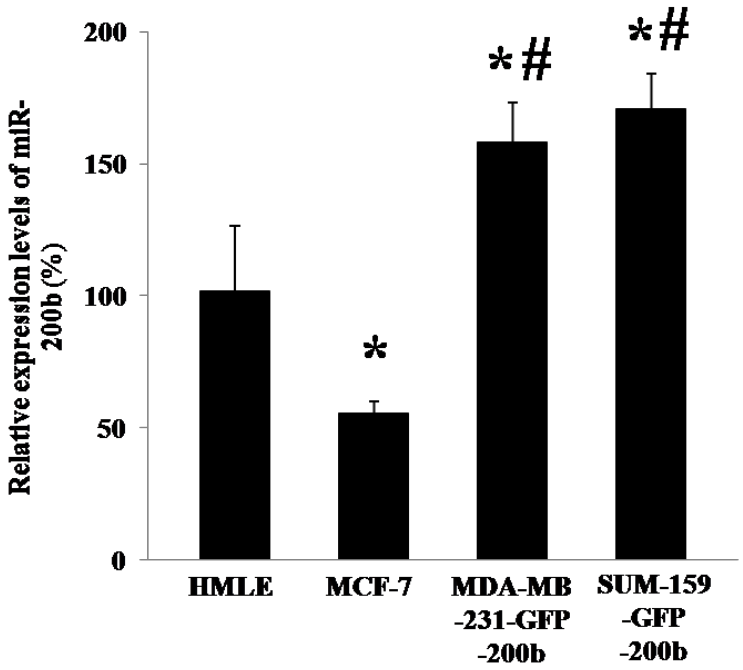

Figure 2.4. Stably expressing miR-200b reduces TNBC cell proliferation and colony formation in soft agar. (**A**) Three thousands cells were seeded into each well of 96-well plates for MTT assay to indirectly monitor cell proliferation up to 72 h. Data are presented as mean ± SD (n = 8). * $p < 0.05$, compared to the GFP control cells. (**B**) One thousand cells were used for soft agar colony formation assay as described in Materials and Methods. Data are presented as means ± standard deviations (n = 3). * $p < 0.05$, compared to the GFP control cells.

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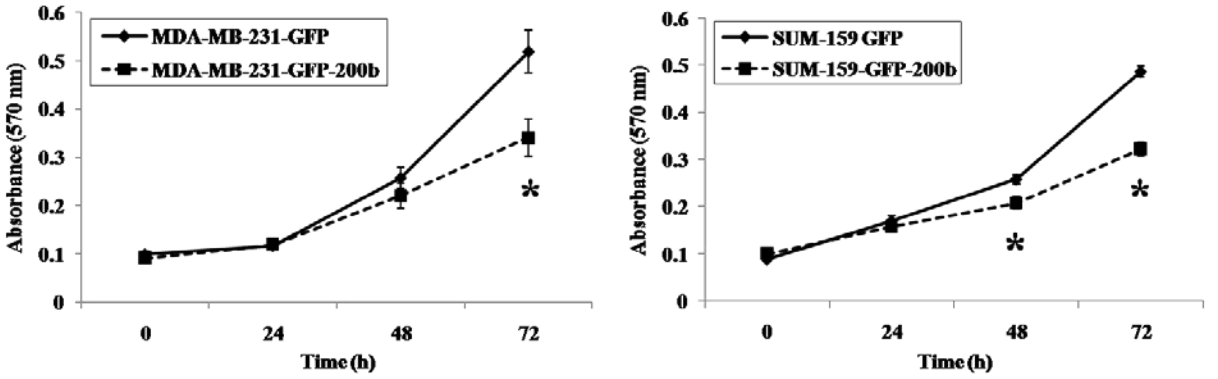

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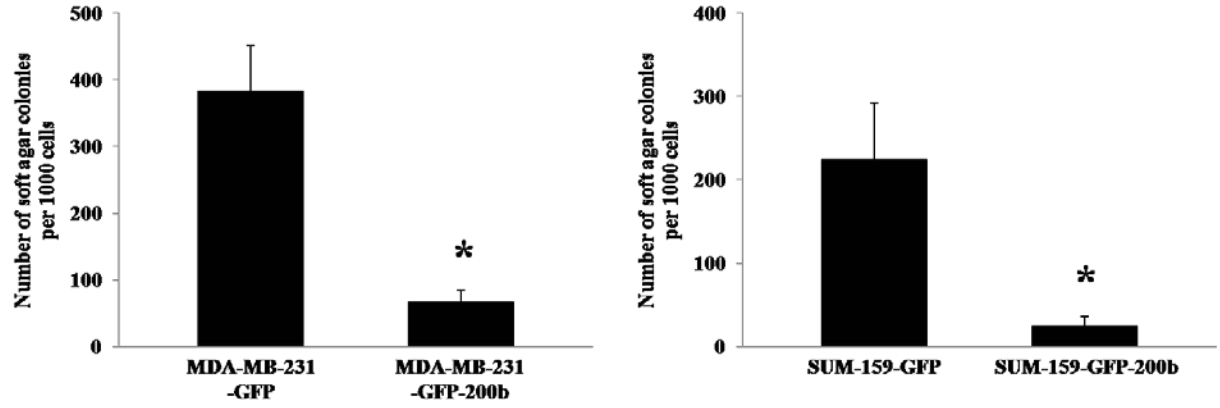

Figure 2.5. Stably expressing miR-200b in basal mesenchymal-like TNBC cells drastically reduces cell migration and inhibits mammary tumor metastasis. (**A**) Wound healing cell migration assay for GFP control and miR-200b stably expressing cells. (**B**) Representative images of GFP immunohistochemistry staining of lung sections from mice with mammary fat pad injection of MDA-MB-231-GFP or MDA-MB-231-GFP-200b cells as described in Materials and Methods. Brownish color inside the foci indicates GFP-positive staining. (**C**) Quantifications of GFP-positive immunohistochemistry staining foci in lung sections from mice with mammary fat pad injection of MDA-MB-231-GFP or MDA-MB-231-GFP-200b cells. (**D**) Representative overlaid images of GFP fluorescence (green color) and nuclear DAPI (blue color) staining in lung sections from mice with mammary fat pad injection of MDA-MB-231-GFP or MDA-MB-231-GFP-200b cells. Lung sections were first stained with DAPI, then viewed and photographed under a fluorescence microscope. Scale bar = 100 μm.

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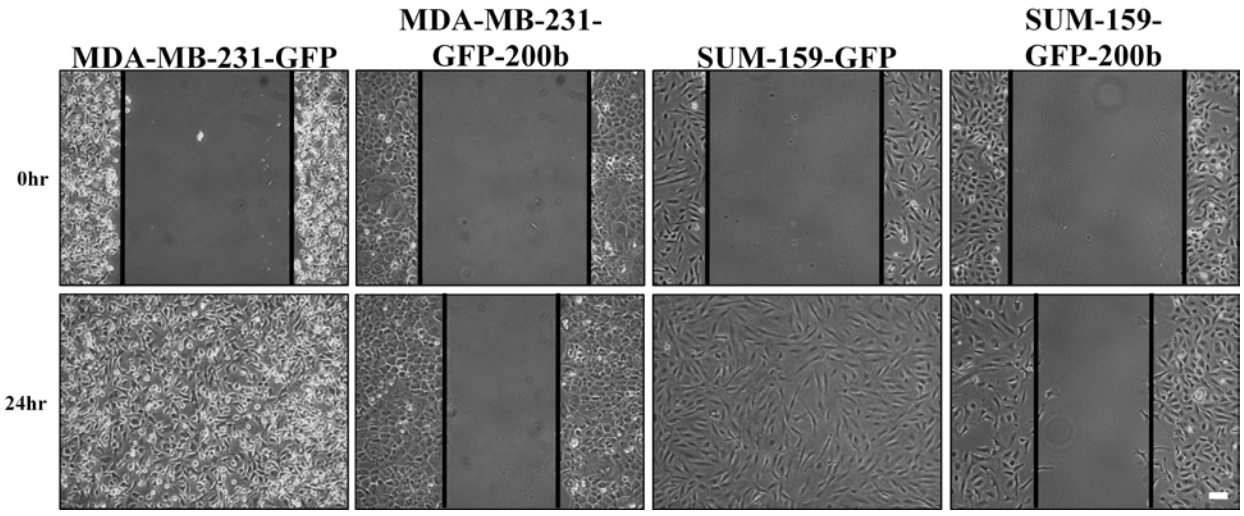

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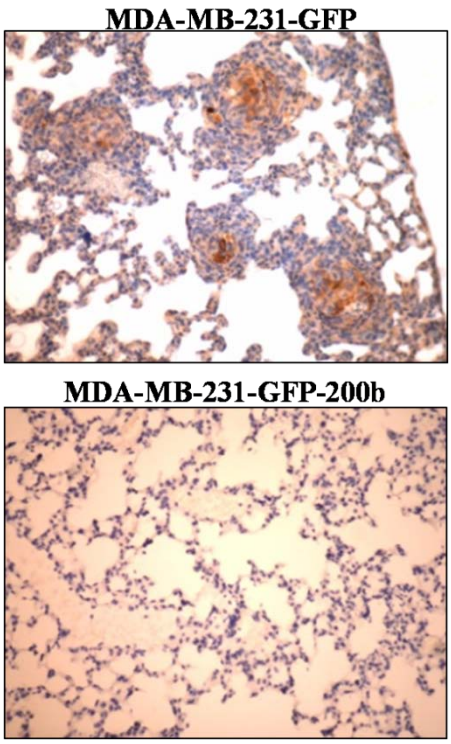

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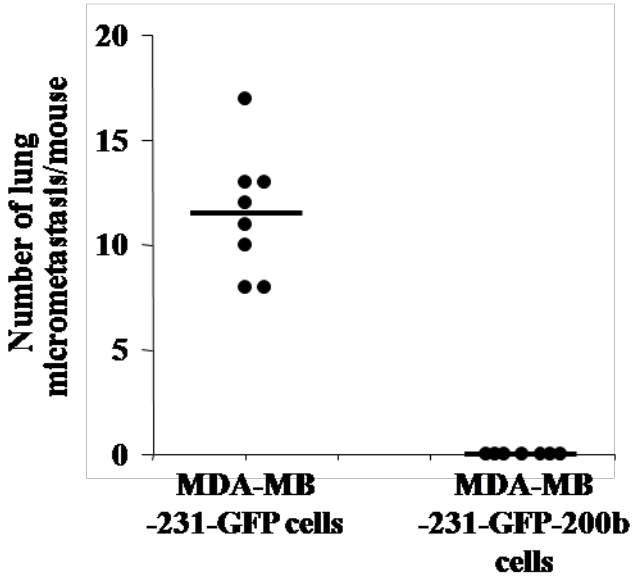

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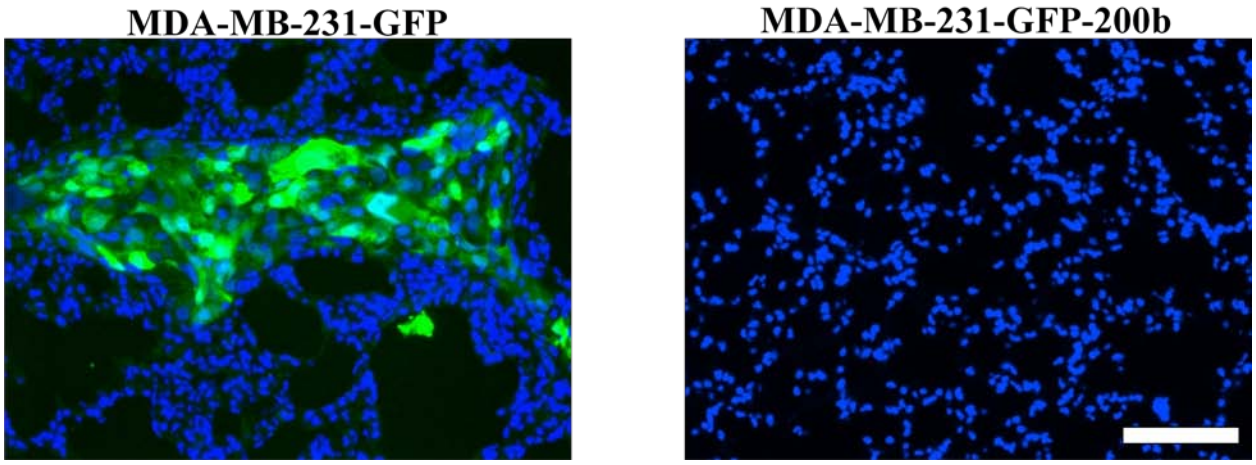
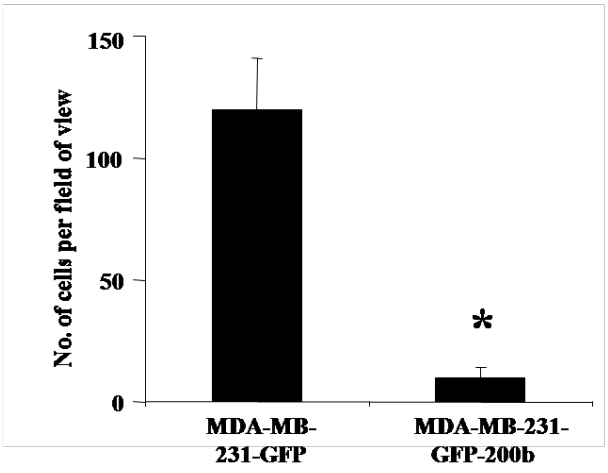


reduce TNBC cell migration, suggesting that loss of miR-200b expression plays an important role in the enhanced migratory behavior of basal mesenchymal-like TNBC cells. The inhibitory effect of stably expressing miR-200b on breast cancer cell migration is further confirmed by using another cell migration assay—Transwell cell migration assay (**Figure 2.6.**). To determine the effect of miR-200b on TNBC metastatic behavior *in vivo*, we performed a mouse orthotopic mammary xenograft tumor model study by injecting MDA-MB-231-GFP or MDA-MB-231-GFP-200b cells into the mammary fat pad. All eight mice injected with MDA-MB-231-GFP cells, and seven out of eight mice injected with MDA-MB-231-GFP-200b cells produced mammary tumors, which displayed a similar histology of poorly differentiated adenocarcinoma as revealed by H&E staining (**Figure 2.7.A**). Mammary tumors produced by MDA-MB-231-GFP-200b cells are significantly smaller than those of MDA-MB-231-GFP cells (**Figure 2.7.B**). Significantly less 5-bromo-2′-deoxyuridine positive staining is observed in mammary tumor tissues produced by miR-200b expressing cells (**Figure 2.7.C and D**), indicating that re-expressing miR-200b reduces tumor cell growth *in vivo*. Immunohistochemistry staining of GFP in lung sections showed that all mice injected with MDA-MB-231-GFP cells have GFP-positive staining foci, indicating the occurrence of lung micrometastasis (**Figure 2.5.B and C**). In striking contrast, no lung sections from mice injected with MDA-MB-231-GFP-200b cells have any detectable GFP-positive staining (**Figure 2.5.B and C**). Moreover, the presence of GFP in lung sections was further demonstrated by directly viewing GFP fluorescence under a fluorescence microscope (**Figure 2.5.D**). Together, these results indicate that stably expressing miR-200b is able to suppress mouse mammary xenograft tumor lung micrometastasis.

Figure 2.6. Stably expressing miR-200b significantly reduces MDA-MB-231 and SUM-159 breast cancer cell migration determined by Transwell cell migration assay. After 48 h culture, cells with 70-80% confluence were collected for Transwell cell migration assay as described in reference 36. Ten percent fetal bovine serum was used as the chemoattractant. The quantification of cell migration is presented as number of cells per field of view (means ± standard deviations, $n = 3$). * $p < 0.05$, compared to GFP Control cells.



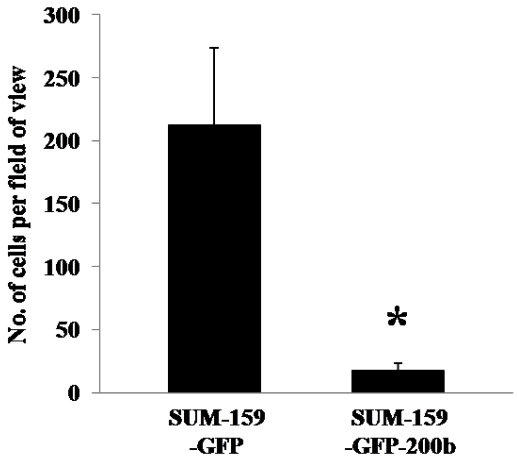


Figure 2.7. Effect of stably expressing miR-200b on mouse mammary xenograft tumor histology and growth. ***A.*** and ***B.*** Representative images of H&E staining (***A***) and the average volumes (***B***) of mouse mammary tumors resulting from injection of MDA-MB-231-GFP or MDA-MB-231-GFP-200b cells. ***C.*** and ***D.*** Representative images of BrdU immunohistochemistry staining (***C***) and the quantification of the staining (***D***) of mouse mammary tumor sections. Quantitative data are presented as means ± SD (n=7-8). * $p < 0.05$, compared to the GFP control cell tumor group. Scale bar = 100 μm.

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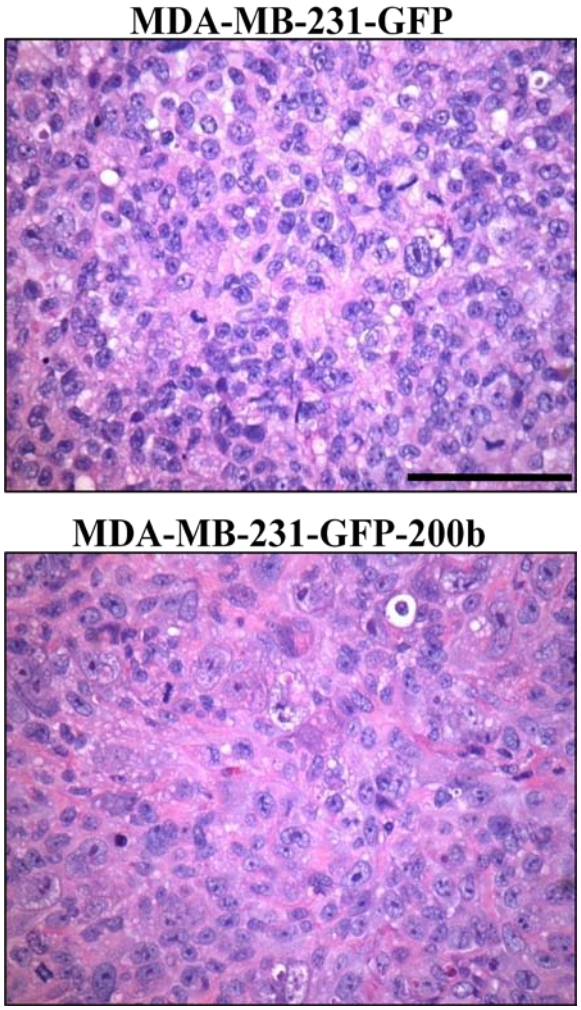

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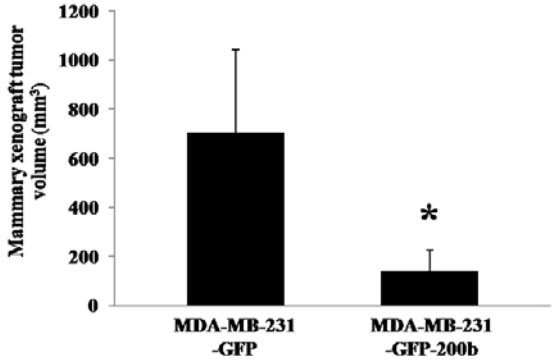

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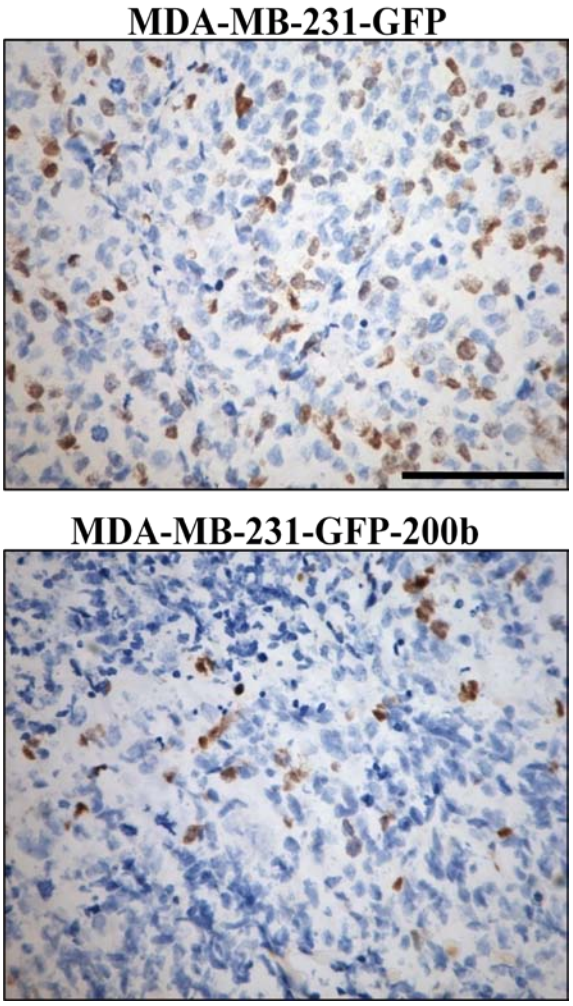

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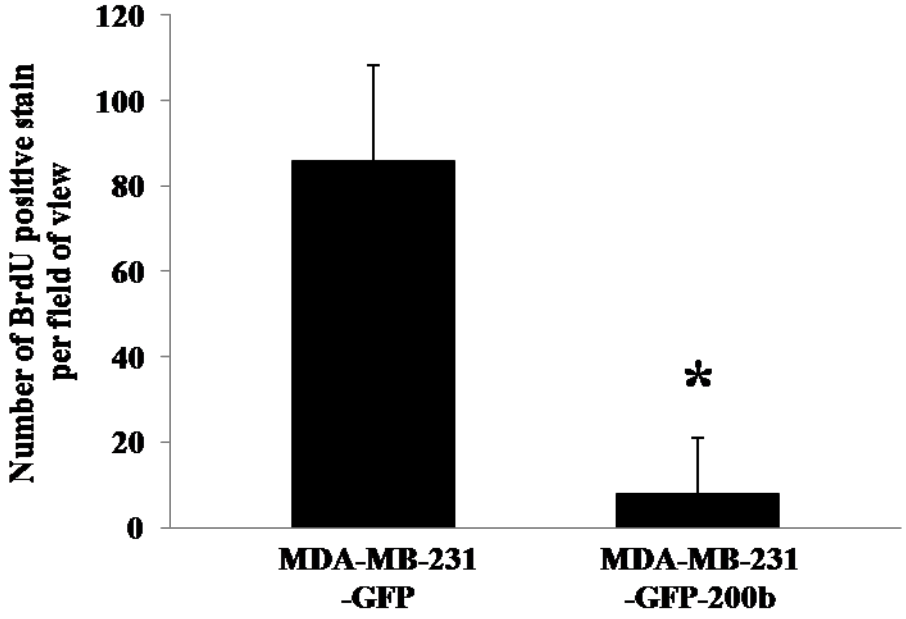

PKCα IS A DIRECT TARGET OF MIR-200B

We next wanted to investigate the underlying mechanism of miR-200b suppressing TNBC cell migration and tumor metastasis. Previous studies showed that EMT-inducing transcription factors ZEB1 and ZEB2 are direct targets of miR-200 family (15–19), implying that downregulation of ZEB1/2 by miR-200 may play an important role in its inhibitory effect on metastasis. However, a recent study reported that ZEB1 knockdown marginally reduces TNBC cell migration and that miR-200 could repress tumor metastasis through ZEB1-independent mechanisms (40). To identify miR-200b new targets that may play crucial roles in its inhibitory effect on TNBC cell migration and tumor metastasis, we performed a bioinformatic analysis. Among the predicted targets of miR-200b, we are particularly interested in PKCα based on recent studies showing its critical role in regulating breast cancer cell stemness, mouse mammary tumor metastasis and the association of its expression with high grade of TNBC (31–33). We first compared the expression levels of PKCα among 12 kinds of breast cancer cells. Western blot analysis showed that PKCα protein levels are remarkably higher in basal mesenchymal-like highly migratory TNBC cells than the weakly migratory ER+, Her2+ and basal epithelial-like TNBC cells (**Figure 2.8.A**), which is inversely correlated with miR-200b levels among these cells (**Figure 2.1.A**). Moreover, re-expressing miR-200b in basal mesenchymal-like TNBC cells drastically reduces the protein level of PKCα (**Figure 2.8.B**) but has no effect on the levels of other PKC isozymes (**Figure 2.9.**). A putative conserved binding site for miR-200b at nucleotide position 1319–1325 of human PKCα 3′UTR is predicted by two miRNA target-predicting software (TargetScan and DIANA-MICROT). We then generated the wild-type and mutant-type of PKCα 3′UTR luciferase reporter vectors. Dual luciferase reporter assays showed that re-expressing miR-200b in MDA-MB-231 and SUM-159 cells significantly reduces PKCα wild-

Figure. 2.8. PKCα is a direct target of miR-200b. (**A**) Western blot analysis of PKCα protein levels in 12 kinds of breast cancer cells. (**B**) Western blot analysis of PKCα protein levels in GFP control and miR-200b stably expressing cells. (**C**, **D**) Quantifications of PKCα 3′UTR wild-type and mutant-type vector luciferase reporter activity in GFP control and miR-200b stably expressing cells. The luciferase reporter activity (mean ± SD, $n = 3$) is expressed relative to control cells. * $p < 0.05$, compared with control cell group. Similar results were obtained in two repeated experiments.

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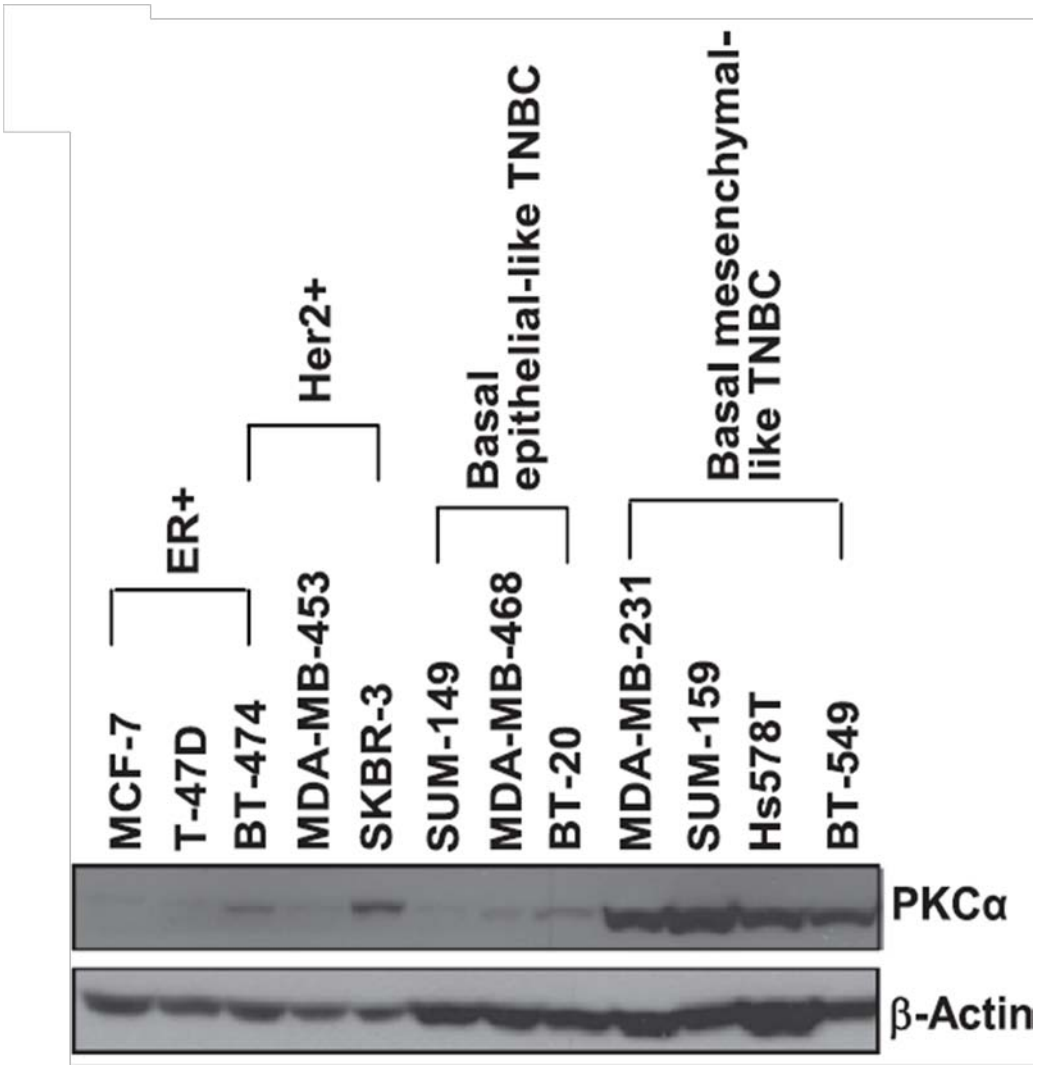

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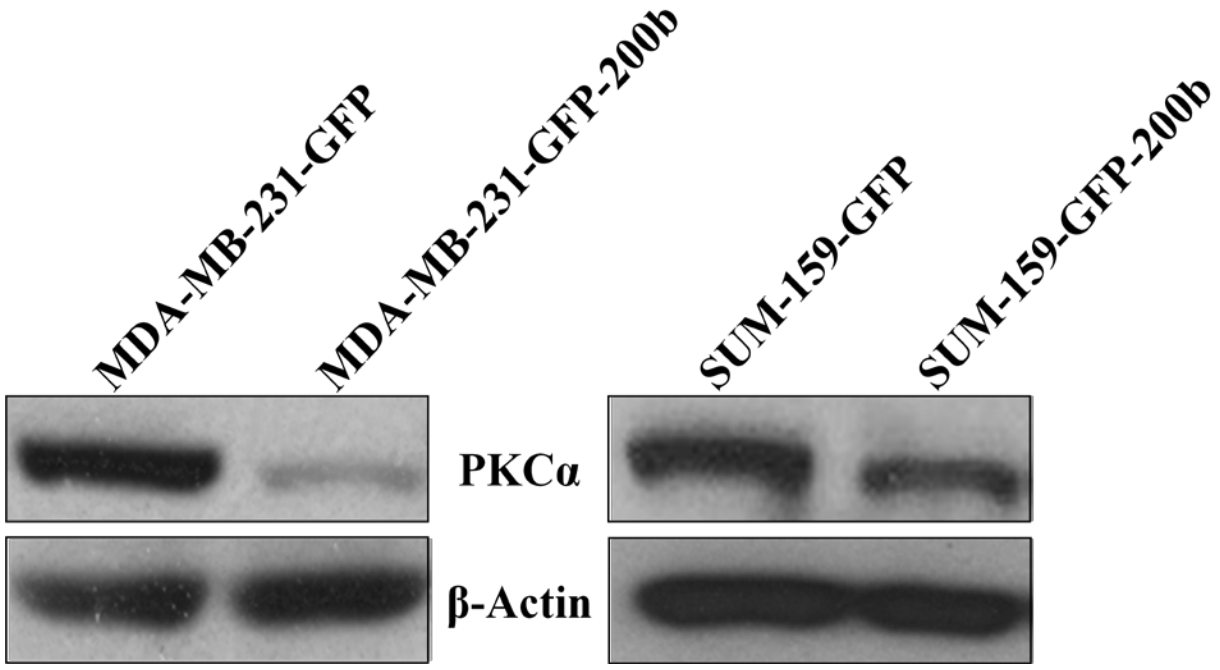

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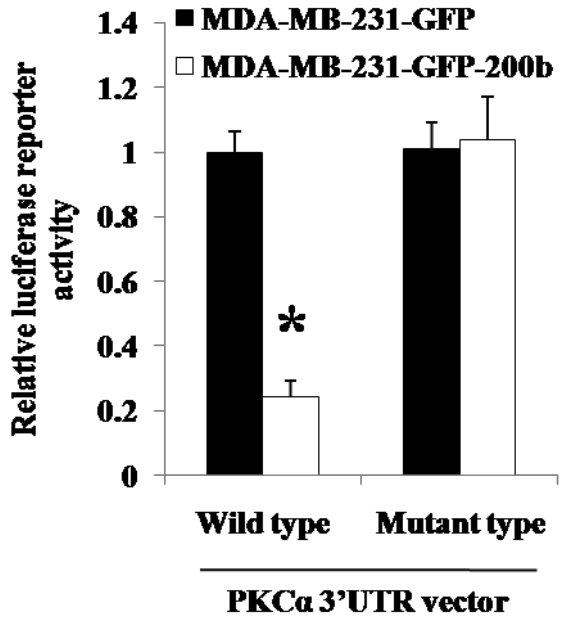

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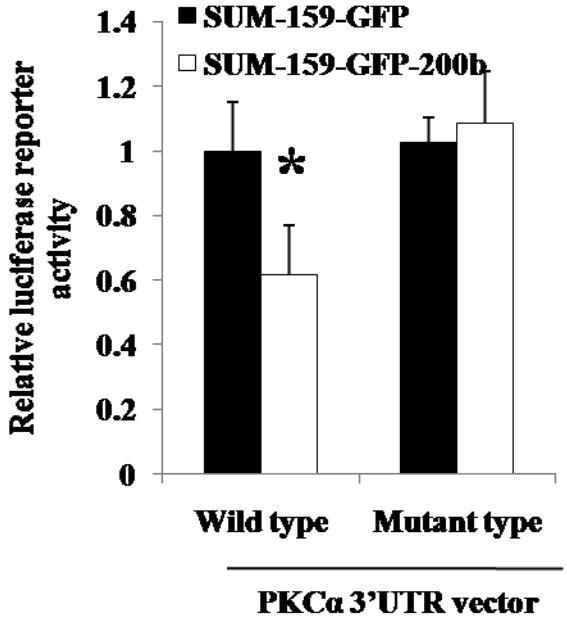
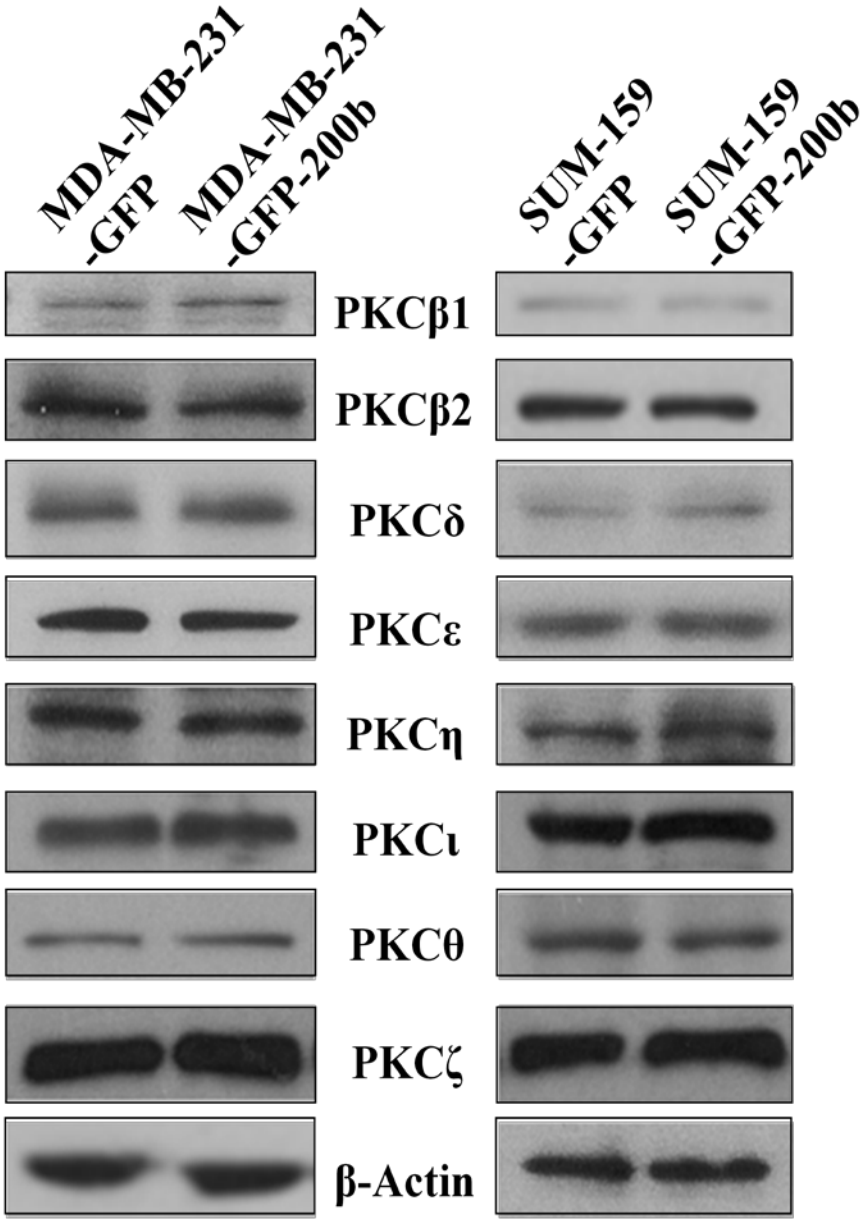


Figure 2.9. Stably expressing miR-200b has no significant effect on the protein levels of other PKC isozymes in MDA-MB-231 and SUM-159 breast cancer cells. After 48 h culture, cells with 70-80% confluence were harvested for Western blot analysis using specific primary antibodies for different PKC isozymes (Santa Cruz Biotechnology and BD Biosciences).



type 3′UTR luciferase reporter activity (**Figure 2.8.C**) but has no effect on the mutant-type 3′UTR luciferase.

INHIBITING OR KNOCKING DOWN PKCα REDUCESs TNBC CELL MIGRATION AND FORCED EXPRESSION OF PKCα IMPAIRS THE INHIBITORY EFFECT OF MIR-200B ON CELL MIGRATION AND TUMOR METASTASIS

We next wanted to determine whether downregulation of PKCα plays a role in the inhibitory effect of miR-200b on TNBC cell migration and tumor metastasis. We first used the inhibitor GO6976 that specifically inhibits PKCα and PKCβI activity. Wound healing assays showed that GO6976 treatment remarkably reduces MDA-MB-231 and SUM-159 cell migration (**Figure 2.10.A**), suggesting that PKCα may play an important role in TNBC cell migration. To further demonstrate this point, we generated PKCα shRNA stable knockdown cells. Western blot analysis revealed that PKCα level is drastically reduced, but PKCβI and ZEB1 levels are unchanged in PKCα stable knockdown cells (**Figure 2.10.B**). PKCα knockdown reduces cell proliferation by about 20% (**Figure 2.11.A**). However, wound healing assays showed that PKCα knockdown drastically reduces cell migration (**Figure 2.10.C**). Together, these results indicate that PKCα is essential for TNBC cell migration and downregulation of PKCα may play an important role in the inhibitory effect of miR-200b on TNBC cell migration. To further determine the role of PKCα in the inhibition of TNBC cell migration by miR-200b, we next overexpressed PKCα in miR-200b stable expression cells and generated PKCα-miR-200b double-stable expression cells. Forced expression of PKCα in MDA-MB-231-GFP-200b cells was confirmed by western blot (**Figure 2.12.A**). Forced expression of PKCα does not significantly change the levels of ZEB1, E-cadherin and miR-200b (**Figure 2.12.A and Figure 2.11.C**), and only increases cell proliferation by about 25% (**Figure 2.11.B**). However, forced

Figure 2.10. Inhibiting PKCα activity or knocking down PKCα expression reduces basal mesenchymal-like TNBC cell migration. (**A**) Effect of GO6976 (1 μM) treatment on basal mesenchymal-like TNBC cell migration determined by wound healing assay. (**B)** Western blot analysis of PKCα, PKCβI and ZEB1 protein level in control and PKCα shRNA knockdown cells. (**C**) Wound healing cell migration assay for control and PKCα shRNA knockdown cells. Scale bar = 100 μm. Similar results were obtained in two repeated experiments.

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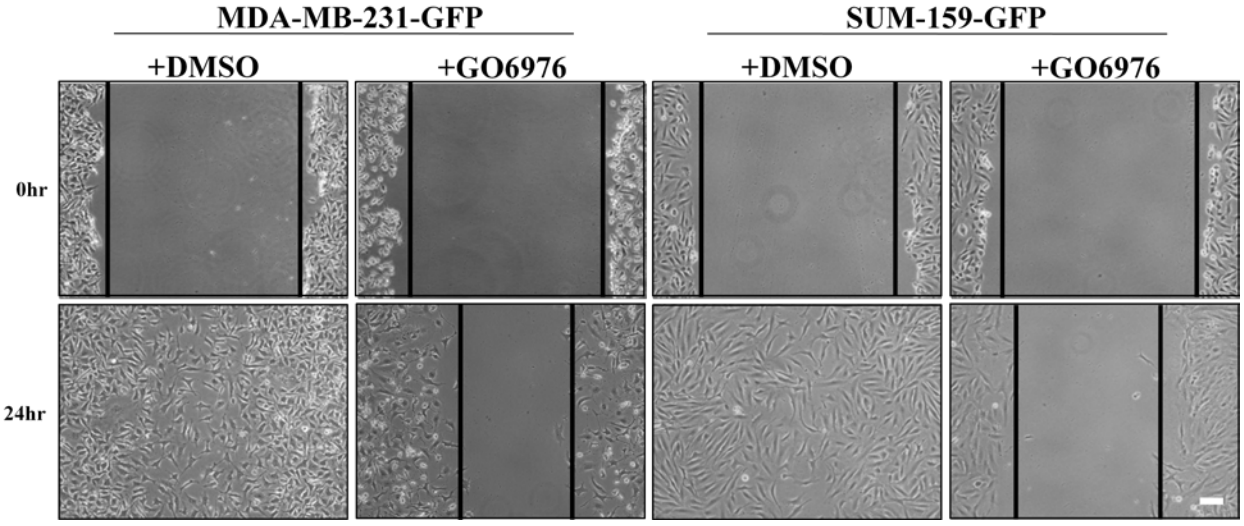

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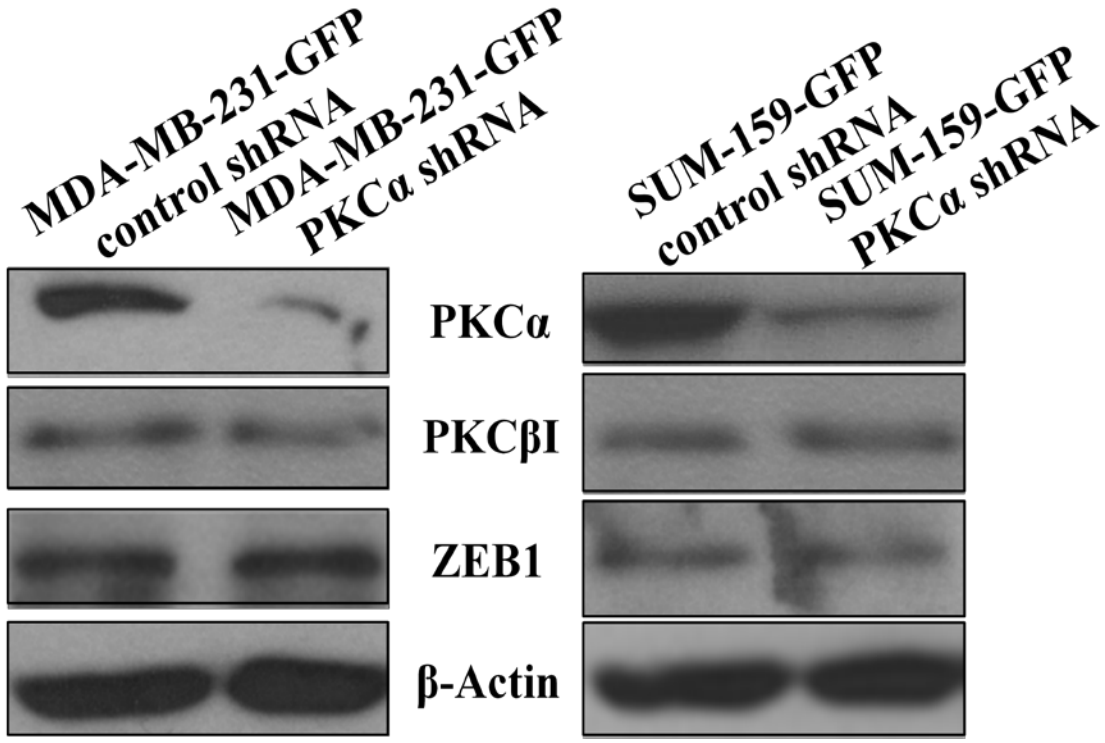

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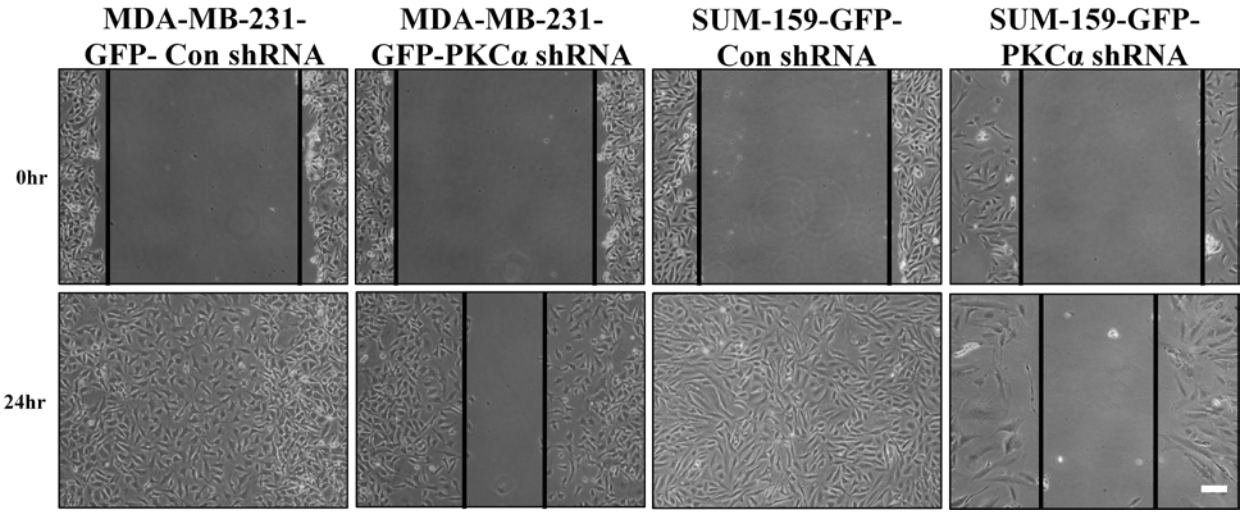

Figure 2.11. Effect of knocking down PKCα expression or forced expression of PKCα on TNBC cell proliferation determined by the MTT assay. Three thousands Control shRNA or PKCα shRNA cells (***A***), and MDA-MB-231-GFP-200b-pLenti6.3 or MDA-MB-231-GFP-200b-pLenti6.3-PKCα cells (***B***) were seeded into each well of 96-well plates for MTT assay to indirectly monitor cell proliferation up to 72 h. Data are presented as mean ± SD (n = 6-8). * p < 0.05, compared to the control shRNA cells or MDA-MB-231-GFP-200b pLenti6.3 cells. (**C**) Forced expression of PKCα has no significant effect on miR-200b expression level determined by Q-PCR using ABI TaqMan miR-200b Q-PCR assay. Data are presented as mean ± SD (n = 3).

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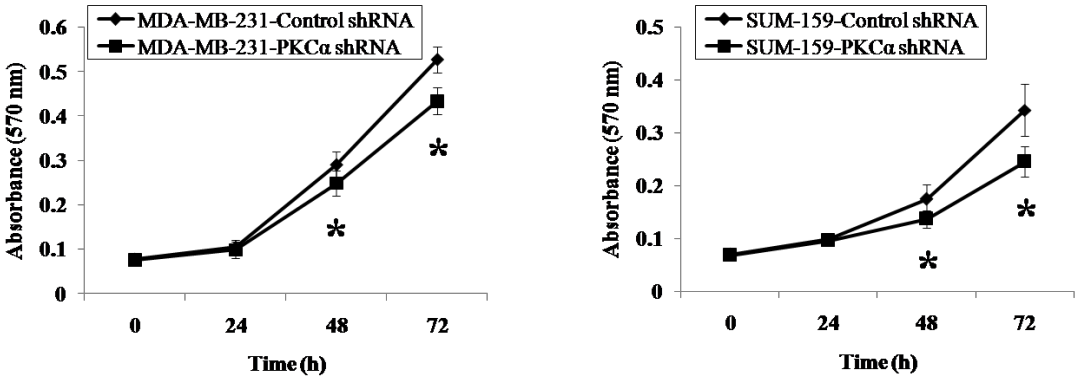

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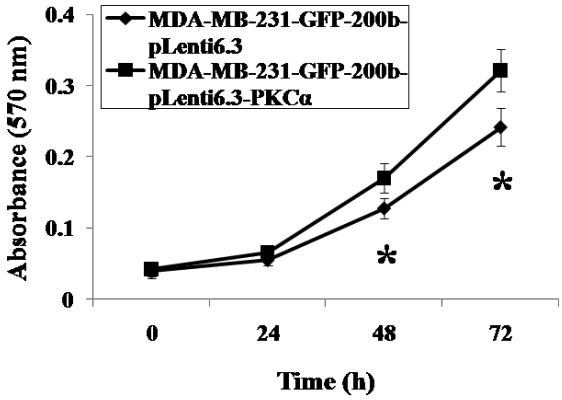

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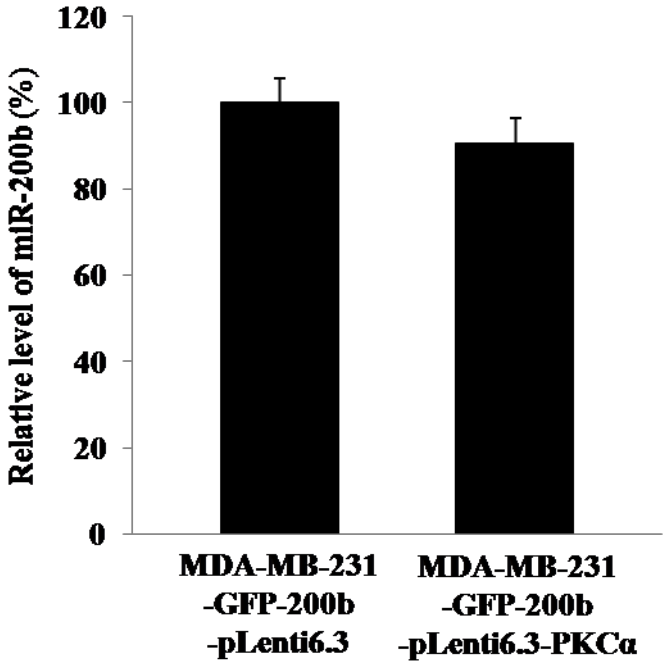
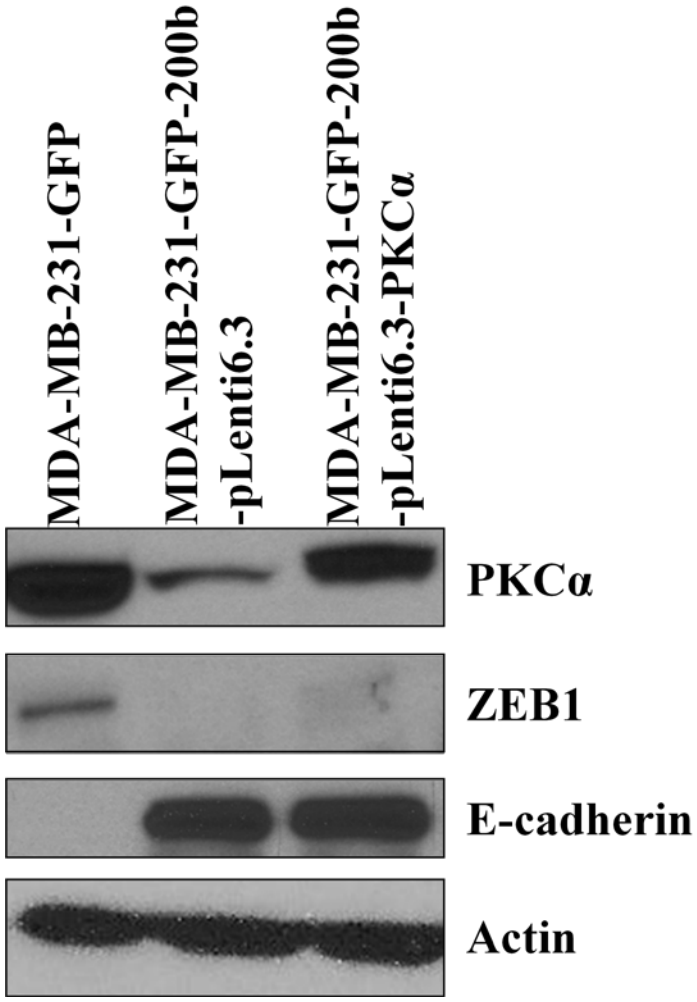


Figure 2.12. Forced expression of PKCα impairs the inhibitory effect of miR-200b on cell migration and tumor metastasis. (**A**) Western blot analysis of PKCα, ZEB1 and E-cadherin levels in MDA-MB-231-GFP, MDA-MB-231-GFP-200b-pLenti6.3 and MDA-MB-231-GFP-200b-pLenti6.3-PKCα cells. (**B**) Wound healing cell migration assay for MDA-MB-231-GFP-200b-pLenti6.3 and MDA-MB-231-GFP-200b-pLenti6.3-PKCα cells. Scale bar = 100 μm. (**C**) Representative images of H&E staining of lung sections from mice with mammary fat pad injection of MDA-MB-231-GFP-200b-pLenti6.3 or MDA-MB-231-GFP-200b-pLenti6.3-PKCα cells. Scale bar = 100 μm. (**D**) Representative overlaid images of immunofluorescence staining of GFP (red color) with nuclear DAPI staining (blue color) in lung sections from mice with mammary fat pad injection of MDA-MB-231-GFP-200b-pLenti6.3 or MDA-MB-231-GFP-200b-pLenti6.3-PKCα cells. Scale bar = 50 μm. (**E**) Representative overlaid images of GFP direct fluorescence (green color) with nuclear DAPI staining (blue color) in lung sections from mice with mammary fat pad injection of MDA-MB-231-GFP-200b-pLenti6.3 or MDA-MB-231-GFP-200b-pLenti6.3-PKCα cells. Scale bar = 50 μm.

A.



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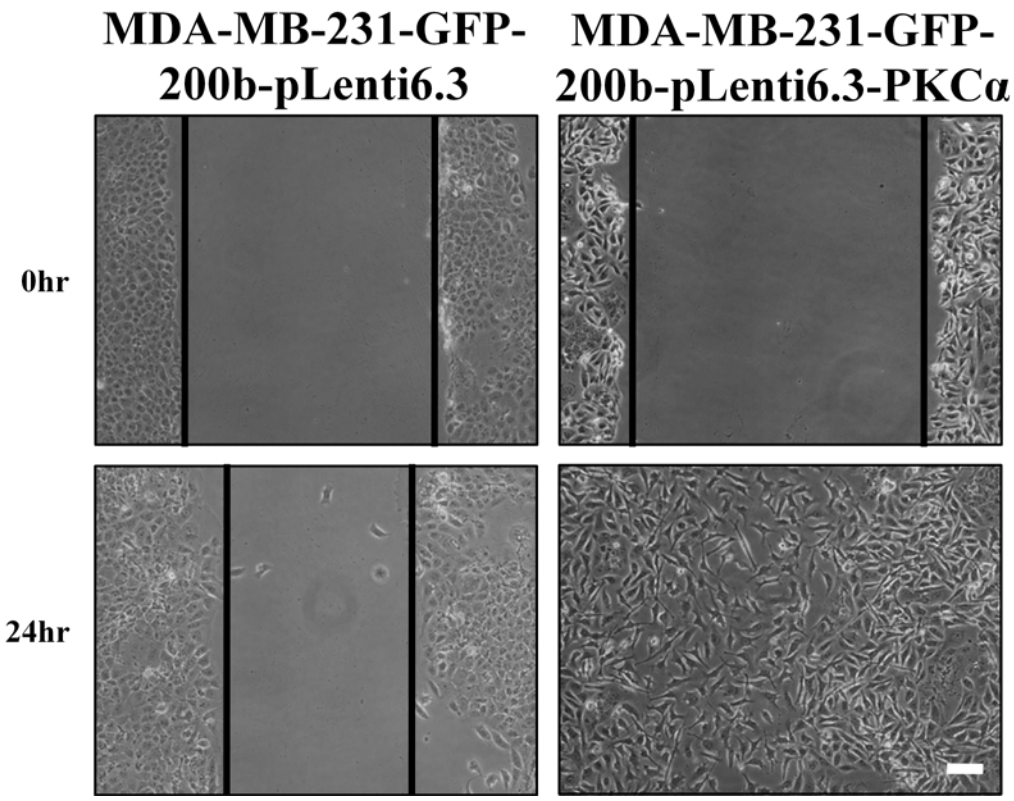
B.



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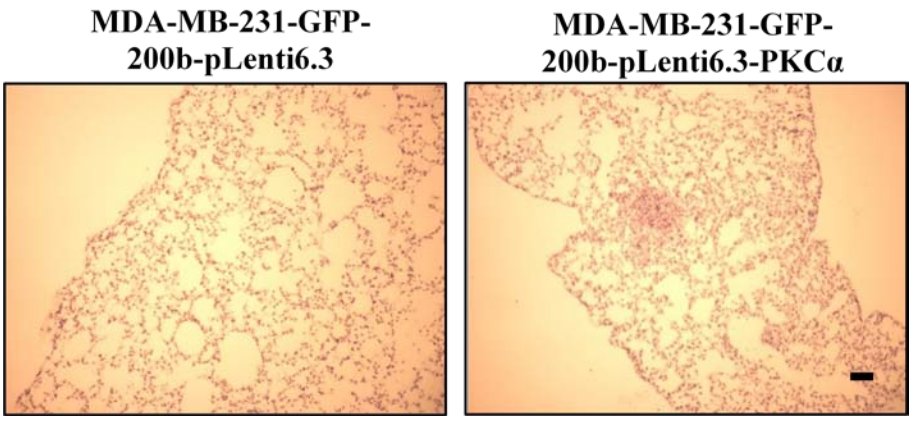

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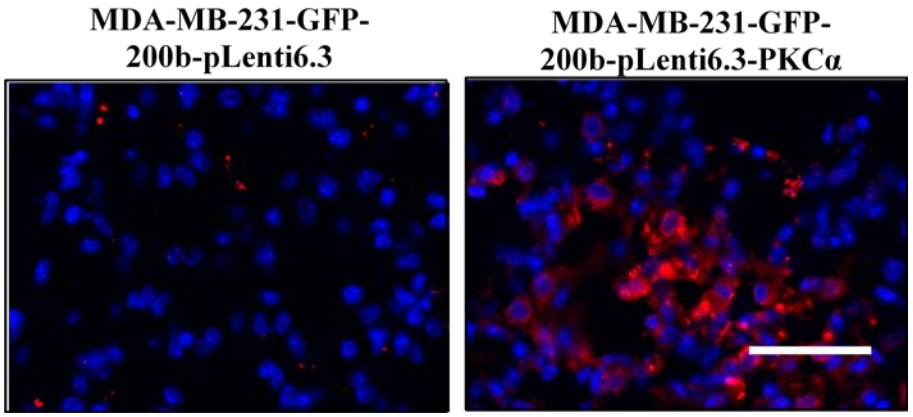
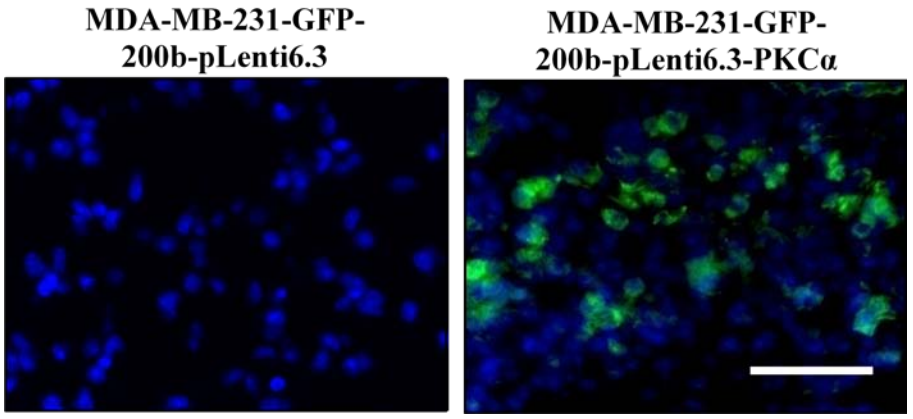


E.



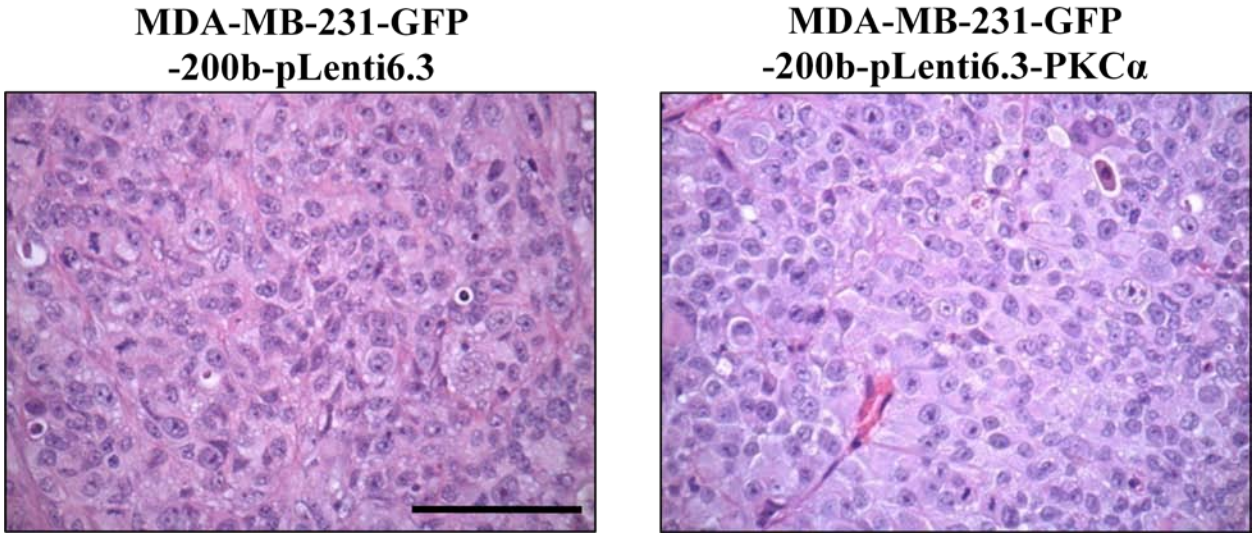
expression of PKCα in miR-200b expressing cells overcomes the inhibitory effect of miR-200b on cell migration as revealed by wound healing assays (**Figure 2.12.B**). Collectively, these results further indicate that downregulation of PKCα plays a key role in the inhibitory effect of miR-200b on TNBC cell migration. To investigate whether forced expression of PKCα is able to impair the inhibitory effect of miR-200b on tumor metastasis, vector control and PKCα-miR 200b double-stable expression cells were injected into mouse mammary fat pad. Similar histology of poorly differentiated adenocarcinoma is observed in mammary tumors produced by vector control (MDA-MB-231-GFP-200b-plenti6.3) and PKCα-miR-200b double-stable expression (MDA-MB-231-GFP-200b-plenti6.3-PKCα) cells (**Figure 2.13.A**). Moreover, forced expression of PKCα has no significant effect on mammary tumor growth (**Figure 2.13.B**). In line with results shown in **Figure 2.5.B–D**, no micrometastasis is observed in lung sections from mice injected with cells stably expressing miR-200b alone (**Figure 2.12.C–E and Figure 2.13.C**). In striking contrast, micrometastatic foci revealed by H&E staining (**Figure 2.12.C**), GFP immunofluorescence staining (**Figure 2.12.D**) and directly viewing GFP under a fluorescence microscope (**Figure 2.12.E**), are observed in lung sections from mice injected with PKCα-miR-200b double-stable expression cells. Quantifications of lung micrometastatic foci are shown in **Figure 2.13.C**. Together, these results indicate that forced expression of PKCα is able to impair the inhibitory effect of miR-200b on mammary tumor metastasis.

DOWNREGULATION OF PKCα BY MIR-200B REDUCES THE RHO GTPASE RAC1 ACTIVATION

Finally, we wanted to further determine the mechanism through which PKCα downregulation by miR-200b suppresses TNBC cell migration and tumor metastasis. One mechanism of PKCα promoting cell migration is via activating the Rho GTPases Rac and Cdc42,

Figure 2.13. Effect of forced expression of PKCα on mouse mammary xenograft tumor histology, growth and lung micrometastasis. (**A**) Representative images of H&E staining of mouse mammary tumors resulting from injection of MDA-MB-231-GFP pLenti6.3 or MDA-MB-231-GFP-200b-pLenti6.3-PKCα cells. Scale bar = 100 μm. ***B.*** and ***C***. The averages of mouse mammary xenograft tumor volumes (means ± standard deviations, *n* = 5) (**B**) and the quantifications of GFP immunofluorescence positive staining foci in lung tissue sections (**C**) from mice with mammary fat pad injection of MDA-MB-231-GFP-pLenti6.3 or MDA-MB-231-GFP-200b-pLenti6.3-PKCα cells.

A.



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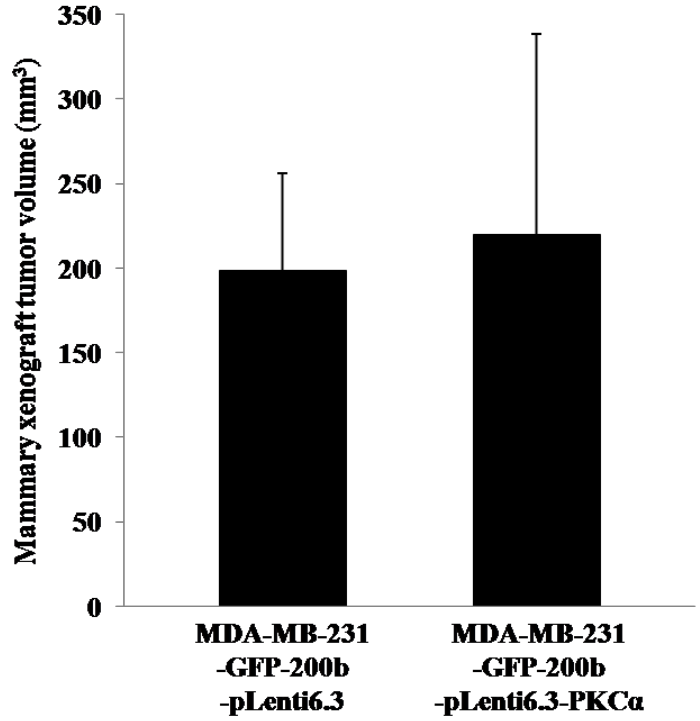
B.



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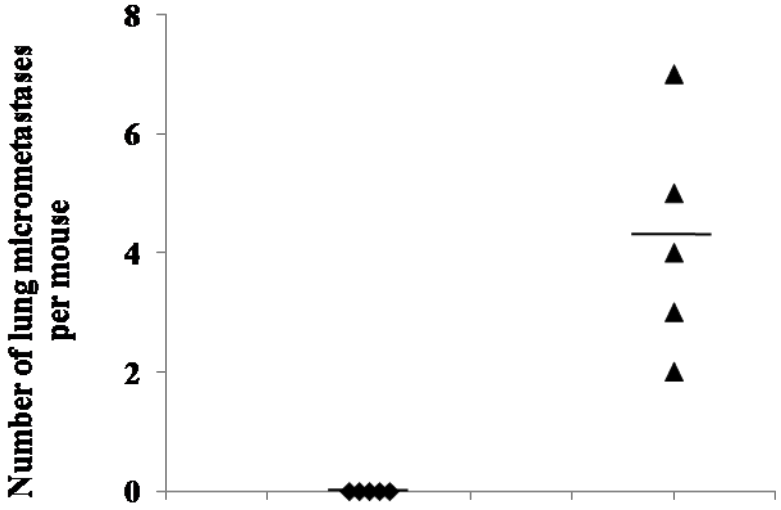
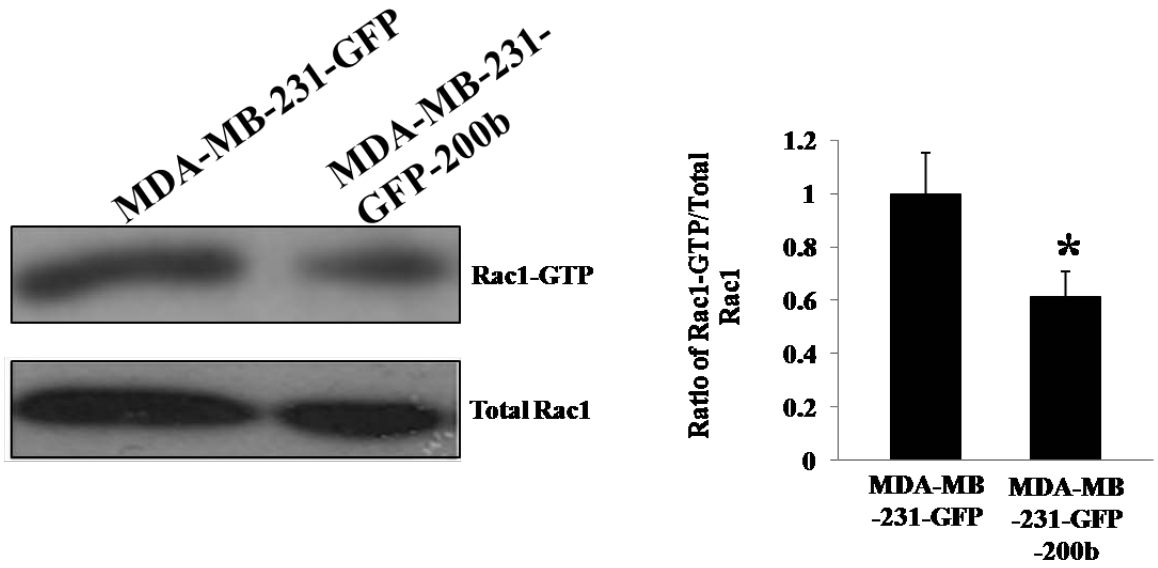


master regulators of cell migration (41,42). Studies showed that Rac is overexpressed and highly activated in invasive breast cancer and inhibiting Rac activity blocks the spread of breast cancer (43,44). We then determined whether re-expressing miR-200b has an effect on Rac1 activation in TNBC cells. Consistent with the reduced migration observed in miR-200b expressing cells, the active Rac1 (Rac1-GTP) levels are significantly lower in MDA-MB-231-GFP-200b and SUM-159-GFP-200b cells than their control counterparts (**Figure 2.14.A and B**), indicating that miR-200b is able to reduce Rac1 activation in TNBC cells. We next wanted to determine whether miR-200b suppresses Rac1 activation via downregulating PKCα. We found that inhibiting PKCα activity or knocking down PKCα expression both significantly reduce Rac1-GTP levels in MDA-MB-231 and SUM-159 cells (**Figure 2.14.C–E**). In sharp contrast, forced expression of PKCα in miR-200b stably expressing cells significantly increases Rac1-GTP levels (**Figure 2.14.F**). Collectively, these results indicate that miR-200b suppresses Rac1 activation by targeting PKCα.

Figure 2.14. Downregulation of PKCα by miR-200b reduces activation of the Rho GTPase Rac1. Rac1-GTP pulldown assay and quantifications for GFP control and miR-200b stably expressing cells (**A** and **B**), for GFP controls cells treated with dimethyl sulfoxide or GO6976 (1 μM) (**C** and **D**), for shRNA vector control and PKCα shRNA cells (**E**), and for miR-200b stably expressing alone and PKCα-miR-200b double stably expressing cells (**F**). Subconfluent cells were serum starved 24 h, and then incubated in fresh medium supplemented with 5% fetal bovine serum for 1 h and collected for Rac1-GTP pulldown assay. Rac1-GTP and total Rac1 levels were quantified using ImageJ software and the quantifications are presented as the ratio of Rac1-GTP levels divided by the corresponding total Rac1 levels (mean ± SD, $n = 3$) relative to that of control cells. * $p < 0.05$, compared with control cell or dimethyl sulfoxide-treated group.

A.



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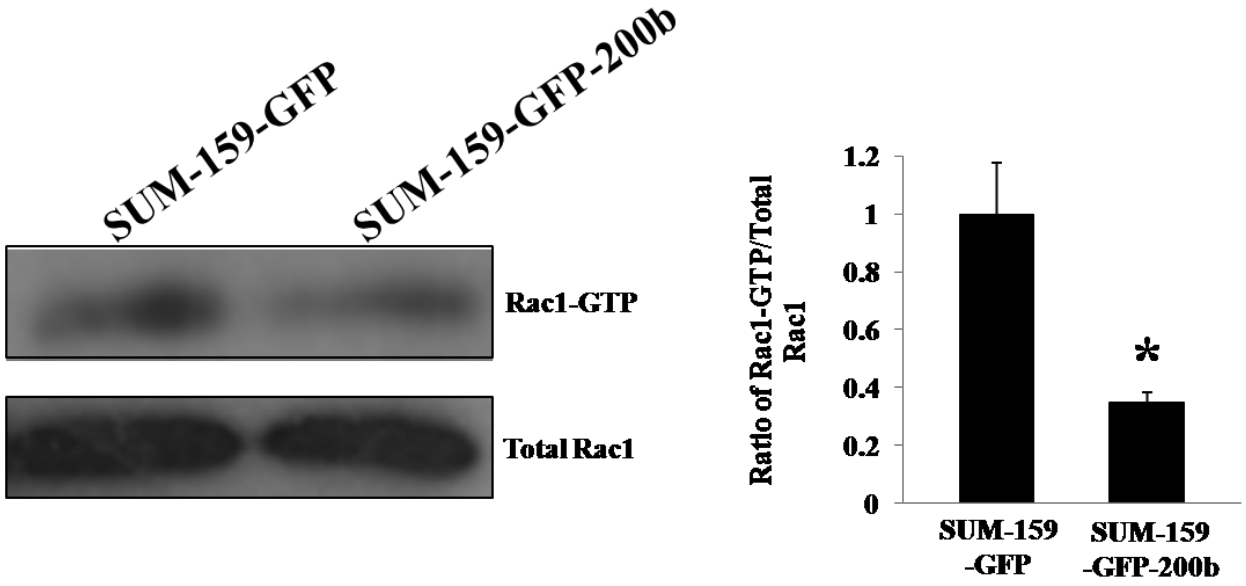
B.



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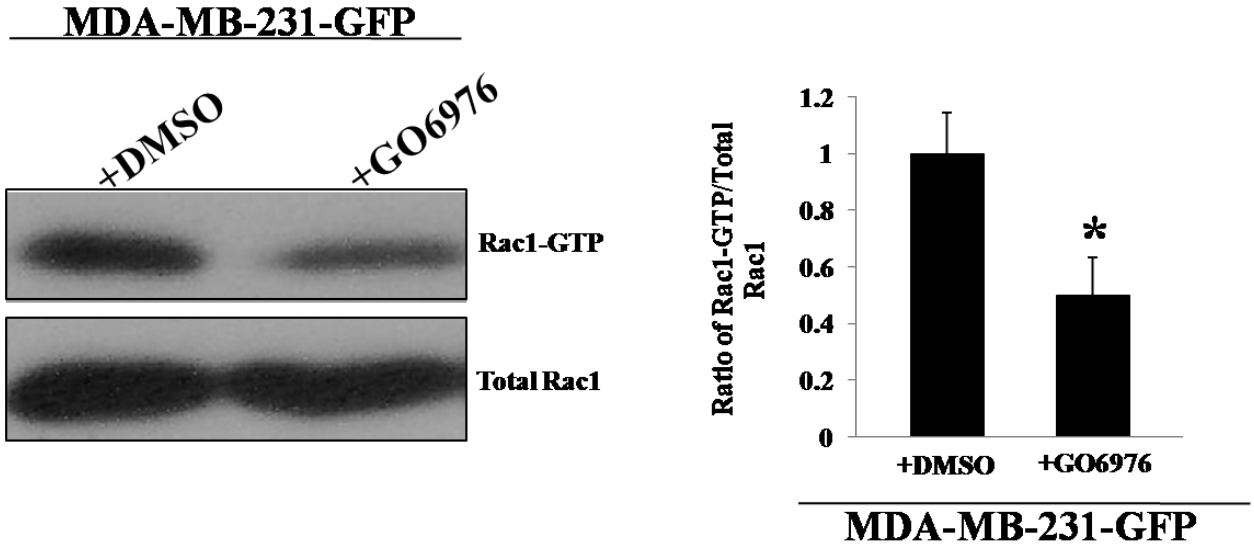

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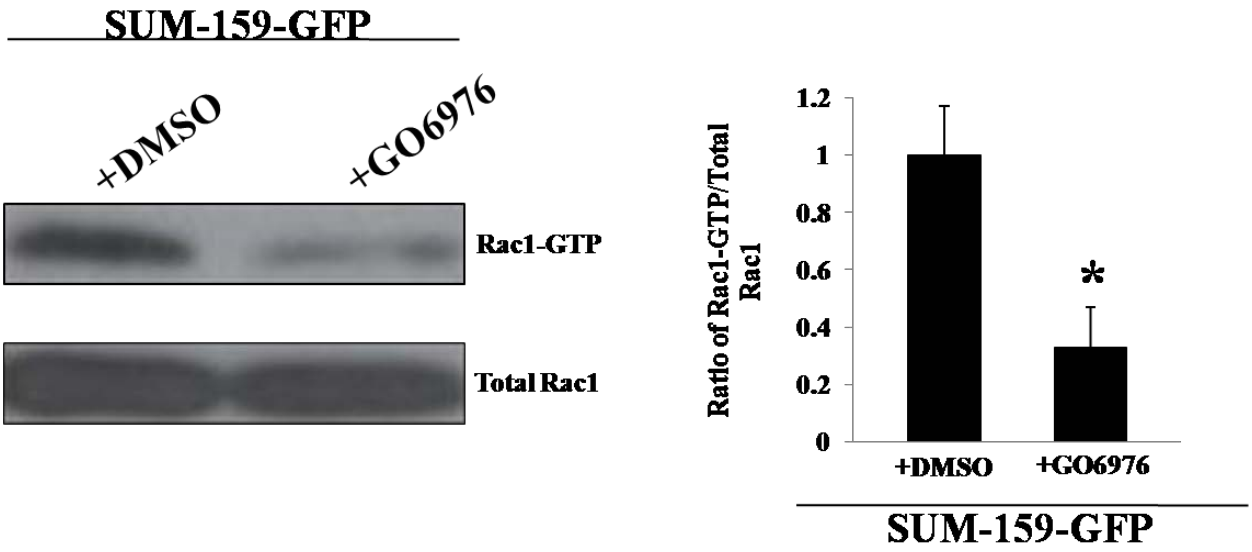
D.



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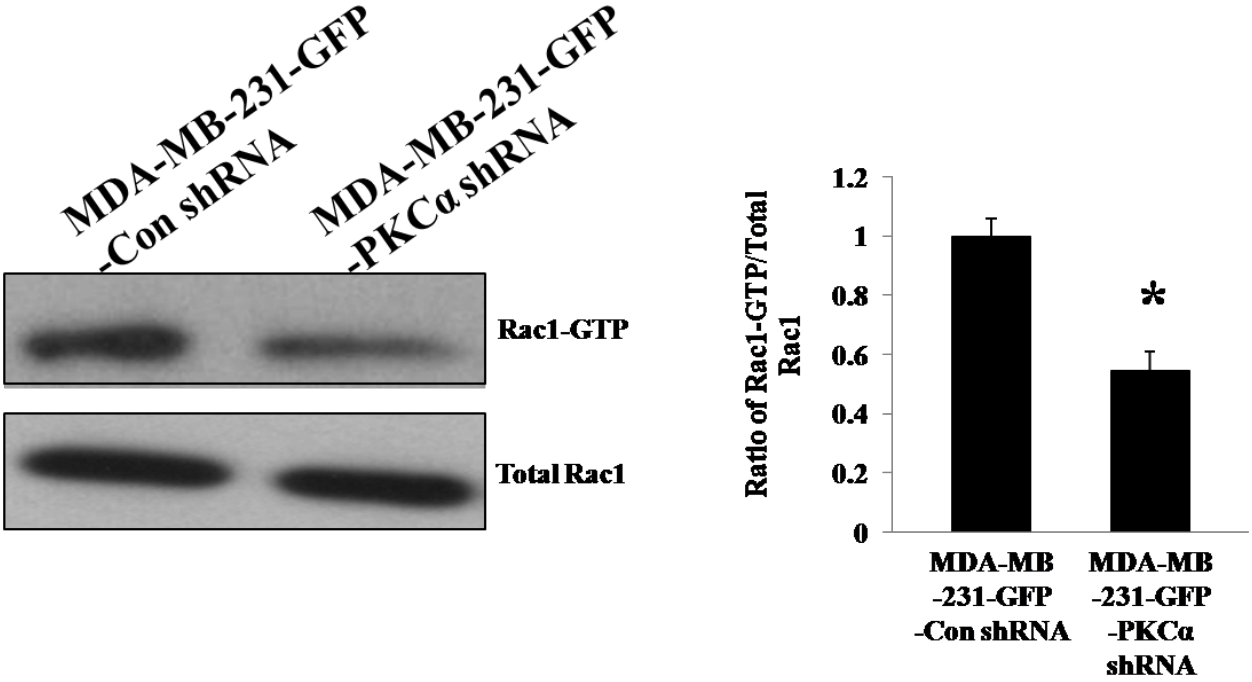

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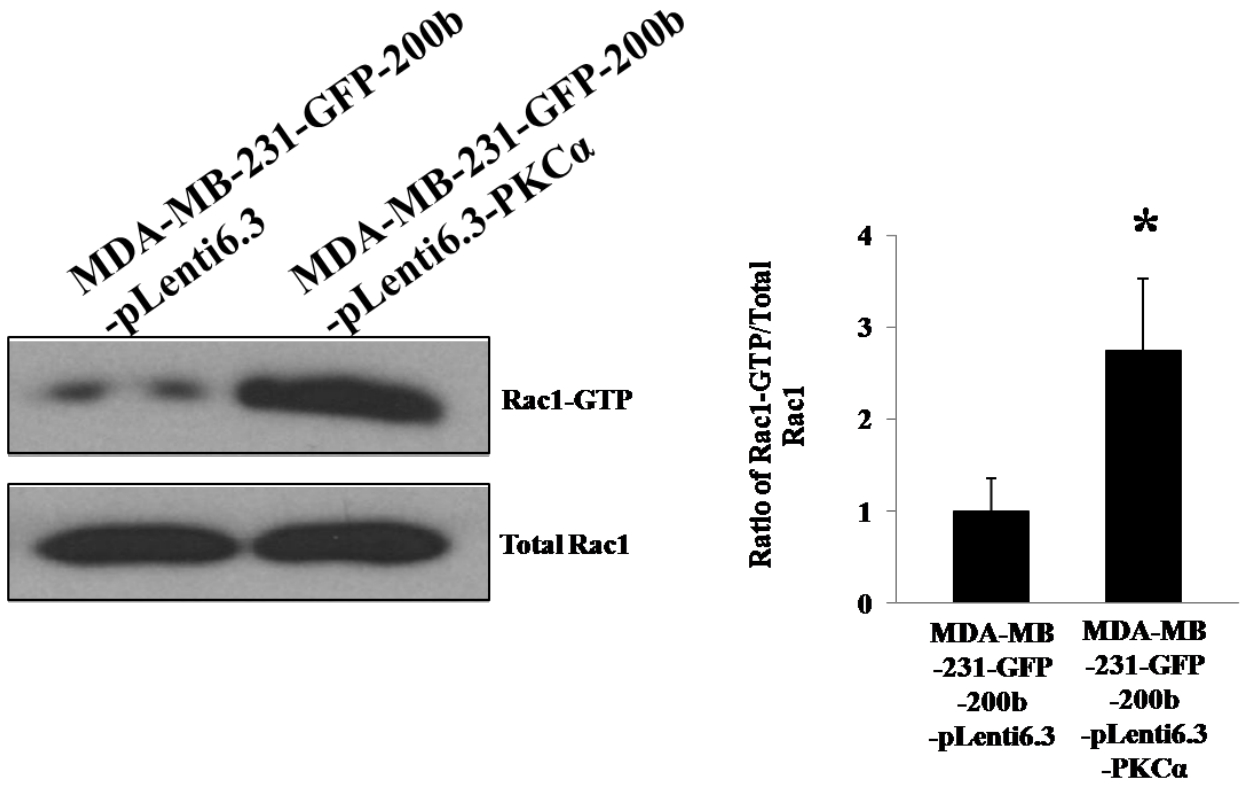
F.



DISCUSSION

Metastatic TNBC is a very aggressive subtype of breast cancer with poor prognosis and without efficient targeted therapies, underscoring the need for identifying novel targets to develop better treatment. In this study we found that miR-200 family levels are extremely low in highly migratory TNBC cells and metastatic TNBC tumors compared with other breast cancer subtypes. Moreover, we showed that ectopically expressing a single member (miR-200b) of the miR-200 family drastically reduces TNBC cell migration and suppresses mouse mammary tumor metastasis. We identified PKCα as a new direct target of miR-200b and found that PKCα levels are inversely correlated with the levels of miR-200b among 12 kinds of breast cancer cells. Inhibiting PKCα activity or knocking down PKCα expression in TNBC cells significantly reduces their migratory capabilities. In contrast, forced expression of PKCα in miR-200b stably expressing cells overcomes the inhibitory effect of miR-200b on TNBC cell migration and tumor metastasis, indicating a critical role of downregulation of PKCα in miR-200b inhibition of TNBC cell migration and tumor metastasis.

Despite the well-established role of miR-200 in inhibiting EMT, the reported effects of miR-200 on cancer metastasis are controversial. Our finding that ectopic expression of miR-200b inhibits TNBC cell migration and metastasis is consistent with a recent study showing that members of miR-200b/-c/-429 cluster repressed tumor metastasis (40), but contrasts with previous studies showing that miR-200 promoted metastasis (24,25). We think this inconsistency could be due to (i) differential targets and functions among individual members of, or between the two functional clusters of, the miR-200 family. Although there is only one nucleotide difference between the seed sequences of the two miR-200 functional clusters (miR-200b/-c/-429 and miR-200a/-141), these two clusters have been shown to have different targets and functions.

For example, Uhlmann *et al.* reported that the miR-200b/-c/-429 cluster, but not the miR-200a/-141 cluster, efficiently reduced phospholipase C γ1 expression; and the miR-200b/-c/-429 cluster exhibited a much stronger inhibitory effect on breast cancer cell invasion than the other cluster (45). Similarly, Li *et al.* found that ectopically expressing members from the miR-200b/-c/-429 cluster, but not from the miR-200a/-141 cluster, reduced breast cancer cell invasion and metastasis (40). The promoting effect of miR-200 on tumor metastasis was observed in mouse mammary tumor 4T07 cells overexpressing miR-200c/-141 group or miR-200c/-141 plus miR-200b/-a/-429 group (24,25). No metastatic promoting effect was observed in 4T07 cells overexpressing the miR-200b/-a/-429 group alone (25). Instead, Gibbons *et al.* (23) reported that overexpression of miR-200b/-a/-429 group inhibited tumor metastasis. Of note, forced expression of miR-200b/-a/-429 group did not significantly increase the level of miR-141 (23,25). Together, these findings clearly show differential effects of individual miR-200 family members or two functional clusters on tumor metastasis despite their high seed sequence homology. And (ii) cancer subtype-specific effect of the miR-200 family. In this study, we found that ER+, PR+ and Her2+ human breast cancer cells and tumors had significantly higher levels of miR-200 than aggressive TNBC cells and tumors, which is consistent with the findings from Korpal *et al*. showing that high miR-200 family level was associated with ER-positive status and correlated with poor distant relapse-free survival only in the ER-positive tumors but not in the ER-negative tumors (25).

Cancer metastasis is a multistep process. When cancer cells obtain migratory and invasive capabilities, they migrate away from the primary tumor site and initiate the metastatic process (46). Inhibition of cancer cell migration can therefore be crucial in reducing cancer metastasis. Cell migration is a dynamic process involving actin cytoskeleton reorganization

mediated by actin cytoskeleton-associated proteins and their regulators (42). In this study, we showed that PKCα is a new direct target of miR-200b and downregulation of PKCα plays a key role in the inhibitory effect of miR-200b on TNBC cell migration. Early studies showed that miR-200 may inhibit cancer cell migration by targeting certain actin cytoskeleton-associated proteins such as moesin and WAVE3 (Wiskott-Aldrich syndrome protein family member 3; 40,47,48). Our mechanistic study revealed that downregulation of PKCα by miR-200b inhibits TNBC cell migration probably through reducing the activation of Rac1, a key player in regulating actin cytoskeleton-associated proteins. Further studies are needed to determine the mechanism by which miR-200b reduces Rac1 activation. Our findings along with others indicate that miR-200 is capable of regulating actin cytoskeleton organization at multiple levels, which play critical roles in its inhibitory effect on cancer cell migration and metastasis.

The identification of PKCα as a new target of miR-200b is novel and important. In this study we found that (i) PKCα is a direct target of miR-200b; (ii) miR-200 expression is depleted in highly migratory TNBC cells and metastatic TNBC tumors; and (iii) the highest levels of PKCα are observed in highly migratory TNBC cells. These findings not only provide mechanistic insights for recent observations showing that high PKCα levels were most commonly detected in high-grade TNBC tumors (32,33), but also imply that our findings are potentially clinically relevant. PKCα was previously evaluated for breast cancer therapy, however only modest response to PKCα treatment was observed (49). We believe that the modest response to PKCα treatment may be largely due to lack of preselection of recruited patients for high expression of PKCα. Taken together, the findings from this study along with others strongly support the notion that PKCα could be a very promising target for treating metastatic TNBC, and PKCα should therefore be re-evaluated as a valid therapeutic target for

metastatic TNBC. In addition, due to the high homology of kinase domains among PKC isozymes, targeting a specific PKC isozyme by small molecule inhibitors has been shown to be a huge challenge (50). Our finding that miR-200b specifically targets PKCα but not other PKC isozymes provides an alternative approach for targeting PKCα. Moreover, previous studies revealed that EMT-inducing transcription factors ZEB1 and ZEB2 are direct targets of miR-200 family (15–19), implicating an important role of downregulation of ZEB1/2 by miR-200 in its inhibitory effect on cancer metastasis. In this study, we found that PKCα is a direct target of miR-200b and stably expressing PKCα impairs the inhibitory effect of miR-200b on breast cancer metastasis with no significant effects on ZEB1 and E-cadherin levels. These findings provided additional evidence supporting the idea that miR-200 could repress cancer metastasis through ZEB1-independent mechanisms (40).

In summary, we found that miR-200 level is extremely low in highly migratory TNBC cells and metastatic TNBC tumors. Stably expressing a single member (miR-200b) of miR-200 family greatly reduces TNBC cell migration and suppresses tumor metastasis. We identified PKCα as a new direct target of miR-200b and found that miR-200b suppresses TNBC cell migration and tumor metastasis by downregulating PKCα, which in turn reduces Rac1 activation. Our findings suggest that miR-200b and PKCα may represent promising targets for developing novel therapies for metastatic TNBC.

ACKNOWLEDGEMENTS

We thank Dr Suyun Huang (The University of Texas M.D. Anderson Cancer Center, Houston, TX) for providing MDA-MB-231 cells; Dr Stephen Ethier (Wayne State University, Detroit, MI) for providing SUM-149 and SUM-159 cells; and Dr Sandra O'Reilly (Department of Physiology, Michigan State University, East Lansing, MI) for excellent help with mouse injections.

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CHAPTER 3: DOWNREGULARION OF ARHGAP18 BY MIR-200B SUPRESSES TNBC MIGRATION AND METASTASIS BY ENHANCING RHOA ACTIVITY

Authors who contributed towards this study were: Brock Humphries, Yunfei Li, Zhishan Wang, Ayda Alavi, and Chengfeng Yang.

ABSTRACT

The constant assembly and disassembly of actin is not only important for normal cellular functions, but altered actin dynamics is also a key feature of metastasis. The Rho GTPase signaling pathway is one of the major pathways that regulate actin cytoskeleton reorganization, and research has suggested that these Rho GTPases promotes tumorigenesis and metastasis. Rho GTPases can be deactivated by Rho GTPase activating proteins (GAPs), which suggests a tumor suppressor role for these proteins. However, here we present data that suggests that RhoA acts as a tumor suppressor and ARHGAP18 displays a metastatic enhancer role, which challenges the current view of these proteins in cancer. Ectopic expression of miR-200b in highly migratory MDA-MB-231 and SUM-159 TNBC cells resulted in increased stress fiber and focal adhesion formation. Concurrent with this result, we also found that miR-200b expression increased RhoA activation and inhibited efficient focal adhesion turnover. Inhibition of RhoA signaling resulted in diminished stress fibers and promoted cell migration, which suggests a tumor suppressor role for RhoA. We identified Rho GTPase activating protein 18 (ARHGAP18) as a direct target of miR-200b that regulates RhoA activation in TNBC cells. Knockdown or CRISPR/Cas9 knockout of ARHGAP18 inhibited TNBC cell migration. CRISPR/Cas9 knockout of ARHGAP18 also greatly increased stress fiber and focal adhesion formation, as well as increased RhoA activation. In contrast, enforced expression of ARHGAP18 impaired the inhibitory effect of miR-200b on cell migration and lung metastasis. Therefore, results from this study show for the first time that ARHGAP18 plays an integral role in TNBC migration and metastasis, and may be a promising therapeutic for aggressive TNBC. Furthermore by showing a tumor suppressor role for RhoA and a metastatic enhancer role for ARHGAP18, our data disputes the current paradigm of Rho GTPase and Rho GAP activity in cancer.

INTRODUCTION

During cell migration a cell undergoes dramatic reorganization of the cell actin cytoskeleton, which is a highly dynamic structure capable of being constantly remodeled in response to changes in the extracellular environment. These highly controlled dynamics are based on the constant assembly and disassembly of actin filaments that form the structures critical in many cellular processes such as proliferation, differentiation, and migration (1,2). Not only is the actin cytoskeleton important for normal functions, but altered actin dynamics is a key feature of metastasis (3,4). Metastasis accounts for the majority of cancer-related deaths and is the result of the migration of cancer cells away from the primary tumor to distant organs. However, the process by which a cell successfully metastasizes depends on many different, and still unidentified, factors.

One of the major pathways involved in regulation of actin cytoskeleton organization is the Rho family GTPase signaling pathway. Rho GTPases are a family of small (~21kDa) signaling G proteins that belong to the Ras superfamily. These small Rho GTPases function as molecular switches within the cell depending on whether GTP or GDP is bound. When a GTPase is bound to GTP it is active and can interact with downstream effectors, and when bound to GDP the GTPase is inactive. Of the many Ras superfamily GTPases RhoA, Rac1, and Cdc42 are the three most well studied. These small Rho GTPases have been heavily studied in the aspect of metastasis and this research has suggested that the activation or overexpression of these GTPases strongly promotes tumorigenesis and metastasis (5,6).

The “on” and “off” states of the Rho GTPases can be accelerated by interaction with certain regulators of Rho GTPases. The GTPase activating proteins (GAPs) accelerate the Rho GTPases intrinsic phosphatase capability, putting the GTPase into the “off” state. Conversely,

the guanine nucleotide exchange factors (GEFs) activate Rho GTPases by rapidly exchanging GDP with GTP. The GDP dissociation inhibitors (GDIs) also act to put the Rho GTPases into the "off" state by binding and sequestering GDP-bound Rho GTPases, not allowing these proteins to exchange GDP for GTP. It is because of these functions that GEFs are thought to be oncogenic drivers of tumorigenesis and metastasis, and GAPs and GDIs as tumor suppressors (5). Currently, only a handful of these Rho GEFs, GAPs, and GDIs have been implicated to play a major role in cancer initiation and progression.

However, recent evidence has disputed this paradigm, suggesting that the aforementioned oncogenic effect of active RhoA may be tumor and cell-type specific (7,8). Indeed, other studies have also shown that the dominant RhoA mutation in angioimmunoblastic T-cell lymphoma (AITL) and peripheral T-cell lymphoma (PTCL) blocks the ability to interact with downstream effectors, suppress wild-type RhoA-mediated stress fiber formation, and promotes cell proliferation and invasion (9,10). Furthermore, another study found that RhoA mutations resulted in defective downstream signaling and promoted escape from anoikis in gastric cancer (11). Therefore, these recent studies suggest that wild-type RhoA has tumor suppressor functions, and mutated RhoA acts to promote tumorigenesis and metastasis.

This study aimed to build upon on our previous work of elucidating the mechanism behind how microRNA-200b (miR-200b) inhibits triple negative breast cancer (TNBC) metastasis. In this study we identified Rho GTPase activating protein 18 (ARHGAP18) as a novel direct target of miR-200b. Furthermore, we show that ARHGAP18 regulates RhoA activation and is critical for TNBC cell migration. We also show for the first time that ARHGAP18 acts as a metastatic enhancer by promoting metastasis, likely by decreasing RhoA activity, which further challenges the current paradigm of Rho GAPs as tumor suppressors.

Moreover, our study also supports the new paradigm which suggests a tumor suppressor role for RhoA. This study also further supports ours and others data suggesting a metastatic suppressive role for miR-200b in TNBC. Therefore, the findings from this study not only provide new mechanistic insights into TNBC metastasis, but also warrant more research into the role that the GTPase signaling pathway plays in cancer metastasis.

MATERIALS AND METHODS

CELL LINES AND CELL CULTURE

The source, authentication, and culture methods of MCF-7, T-47D, BT-474, MDA-MB-453, SKBR-3, MDA-MB-468, BT-20, BT-549, SUM-149, SUM-159, and MDA-MB-231 cells were described in our recent study (12). MDA-MB-231 LM2 4175 cells were provided by Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY) and cultured in the same media as MDA-MB-231 parental cells.

QUANTITATIVE PCR ANALYSIS

Cellular total RNAs were extracted using the QIAGEN miRNeasy mini kit (Valencia, CA) for quantitative PCR (Q-PCR) analysis, which was carried out in the ABI 7500 Fast Real-Time PCR System using TaqMan gene expression assays (Applied Biosystems, Foster City, CA) for ARHGAP18 and ARHGAP19. β-actin was used to normalize relative ARHGAP18 and ARHGAP19 expression levels as described previously (13).

GENERATION OF MIR-200B AND ARHGAP18 DOUBLE STABLE EXPRESSION CELLS

The miR-200b stable expressing cells were generated in our recent study (13). The miR-200b and ARHGAP18 double stably expressing cells were generated following previously described procedures (12). Briefly, human ARHGAP18 full-length complementary DNA lacking the 3'UTR was purchased from OriGene Technologies (Rockville, MD) and cloned into the pLenti6.3/V5-DEST^{TM} vector (Invitrogen) following the manufacturer's protocol. Vector control (pLenti6.3) and ARHGAP18-expressing (pLenti6.3-ARHGAP18) lentiviral particles were packaged as described previously (14). To establish a vector control, miR-200b stably expressing cells were transduced with pLenti6.3 lentiviral particles. To generate the double expressing cells,

miR-200b stably expressing cells were transduced with pLenti6.3-ARHGAP18 lentiviral particles. These cells were then selected using blasticidin. After the selection, the expression of ARHGAP18 was confirmed by Western blot.

GENERATION OF ARHGAP18 CRISPR KNOCKOUT CELLS

ARHGAP18-specific gRNA oligos were determined using the candidate gRNA targets provided by the Church lab (http://arep.med.harvard.edu/human_crispr/) (15), and cloned into the pX459 two-in one CRISPR targeting vector (Addgene) using the protocol provided by the Zhang lab (http://www.genome-engineering.org/crispr/?page_id=23) (16). The target sequence for ARHGAP18-1-1 is GAAATTGATCGATCAAATGGAGG and the target sequence for ARHGAP18-2 is GCTTCCGAAGGCTTACCTCGAGG. After successful cloning, the ARHGAP18 CRISPR targeting vector was transfected into MDA-MB-231 LM2 and SUM-159 cells and selected with puromycin for generating ARHGAP18 knockout cells.

GENERATION OF ARHGAP18 STABLY EXPRESSING CELLS IN ARHGAP18 CRISPR KNOCKOUT CELLS

ARHGAP18 CRISPR knockout cells were transduced with vector control (pLenti6.3) and ARHGAP18-expressing (pLenti6.3-ARHGAP18) lentiviral particles and selected with blasticidin for generating ARHGAP18 stably expressing cells in ARHGAP18 CRISPR knockout cells.

GENERATION OF ARHGAP18 3'UTR LUCIFERASE REPORTER WILD-TYPE AND MUTANT VECTORS AND DUAL LUCIFERASE REPORTER ASSAYS

A fragment of the human ARHGAP18 3′UTR containing nucleotides 1–822 was synthesized by Blue Heron Biotech (Bothell, WA) and cloned into pMirTarget vector (OriGene Technologies). This served as the wild-type ARHGAP18 3′UTR luciferase vector containing the

miR-200b putative binding site. Mutant-type ARHGAP18 3′UTR luciferase vector was generated by using the same fragment of ARHGAP18 3′UTR that was synthesized for the wild-type vector with the miR-200b putative binding site completely mutated. The mutated ARHGAP18 3′UTR fragment was then also cloned into pMir-Target vector. Dual luciferase reporter assays were performed as described previously (13). The relative luciferase reporter activity was calculated as the ratio of the wild-type or mutant-type ARHGAP18 3′UTR firefly luciferase activity over the *Renilla* luciferase activity.

WOUND HEALING CELL MIGRATION ASSAYS

Cell migration was determined by a wound healing assay as described previously (17). A proliferation inhibitor mitomycin C (1µg/mL) (Sigma Aldrich, St. Louis, MO) was added when the wound was created. When indicated, dimethyl sulfoxide (DMSO) or the RhoA inhibitor Y27632 (10µM) (Cayman Chemical, Ann Arbor, MI) was also added when the wound was created. The average percentage wound closure for each group (n=9) was determined by measuring and comparing the distance between the cells at the leading edge at 0hr and 24hr time points. The results are presented as ((1 – (remaining (24hr) wound area – initial (0hr) wound area)) *100).

ORTHOTOPIC MOUSE MAMMARY XENOGRAFT TUMOR MODEL STUDIES

Seven week old female nude mice (Nu/Nu, Charles River Laboratories) were used and maintained under regulated pathogen-free conditions. Animal protocols were reviewed and approved by the Michigan State University Institutional Animal Care and Use Committee. Mice were anesthetized before injections of 1×10^6 cells (MDA-MB-231-GFP-200b pLenti6.3 and MDA-MB-231-GFP-200b pLenti6.3-ARHGAP18) into the fourth mammary fat pad in 0.1mL of 1:1 growth factor-reduced Matrigel (BD Biosciences) to blank media. Animals injected with

MDA-MB-231-GFP-200b pLenti6.3 or MDA-MB-231-GFP-200b pLenti6.3-ARHGAP18 were euthanized 12 weeks post-injection. Mammary tumors, lungs, livers, and spleens were then harvested, fixed with 10% formalin, and paraffin embedded for hematoxylin and eosin (H&E) and immunofluorescence staining.

IMMUNOFLUORESCENT STAINING OF CULTURED CELLS AND MOUSE MAMMARY TUMOR AND LUNG SECTIONS

Mouse mammary tumor and lung sections were prepared and subjected to H&E staining as described previously (18). The presence of GFP in mouse lung sections was determined by GFP immunofluorescence staining. The captured GFP images were overlaid with the nuclear blue 4',6-diamidino-2-phenylindole (DAPI) fluorescent images using the MetaMorph software.

Cellular actin cytoskeleton organization and focal adhesion formation were revealed by vinculin and Rhodamine phalloidin immunofluorescence staining. Cells were cultured on cover slides in DMEM/F12 for MDA-MB-231 and F12 for SUM-159 cells for 48 hours. At 48 hrs cells were washed twice with PBS, fixed with 4% paraformeldehyde in PBS for 20 minutes, and then permeabilized using 0.15% Triton X-100 in PBS for 1-2 minutes. Cells were blocked with a 1:1 normal goat serum to PBS for 30 minutes, and incubated with vinculin (Sigma) in blocking solution overnight at 4°C. Cells were washed three times with PBS, stained with Rhodamine phalloidin (Molecular Probes) and anti-mouse IgG (H+L) CF633 (Sigma) for 1 hr, washed three times with PBS, and counterstained with 4',6'-diamidino-2-phenylindole (DAPI) for 5 minutes at RT. Cells were visualized and photographed using a Nikon Eclipse TE2000-U fluorescence microscope (Nikon Inc., Melville, NY). The red (Rhodamine phalloidin) and green (vinculin) fluorescent images were overlaid with blue (DAPI) fluorescent images using the MetaMorph software (Molecular Devices Corp., Dowingtown, PA).

WESTERN BLOT ANALYSIS

Cells were lysed by Tris-sodium dodecyl sulfate, and run using sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (13). Antibodies used were anti-ARHGAP18 (Thermo Scientific), anti-β-actin (Sigma-Aldrich), anti-Rac1 (Millipore, Temecula, CA), anti-RhoA, anti-ZEB1, anti-E-cadherin, and anti-PKCα (Cell Signaling Technology, Danvers, MA).

RHOA- AND RAC1-GTP PULLDOWN ASSAYS

RhoA- and Rac1-GTP pulldown assays were carried out as previously described (19). The active RhoA and Rac1 levels were quantified using ImageJ software (National Institute of Health, http://imagej.nih.gov/ij/) and quantifications are presented as relative RhoA- and Rac1-GTP levels (ratio of RhoA-GTP or Rac1-GTP levels divided by corresponding total RhoA or Rac1 levels).

MTT ASSAYS

Tetrazolium dye colorimetric test (MTT) was performed as previously described (12). Quantifications are presented as an average of at least 8 replicates.

STATISTICAL ANALYSIS

The statistical analyses for the significance in presented data (mean ± SD) were carried out by testing different treatment effects via analysis of variance (ANOVA) using a general linear model (Statistical Analysis System (SAS) version 9.1, SAS Institute, Cary, NC). A *t*-test was used to determine the differences between treatment groups. A *p*-value of <0.05 was considered statistically significant.

RESULTS

ENHANCED STRESS FIBER AND FOCAL ADHESION FORMATION, DEFECTIVE FOCAL ADHESION TURNOVER, AND STRONG RHOA ACTIVATION ARE DETECTED IN MIR-200B STABLE EXPRESSING TNBC CELLS

Since we have previously shown that miR-200b stable expression significantly reduces the activation of Rac1, a critical regulator of actin cytoskeleton reorganization (12), we decided to examine the effect of miR-200b expression on the actin cytoskeleton organization pattern in TNBC cells. Consistent with our previously observed inhibitory effect of miR-200b on Rac1 activity, the phalloidin staining revealed that lamellipodia formation is inhibited in miR-200b stably expressing MDA-MB-231 and SUM-159 TNBC cells (**Figure 3.1.A**). Moreover, strong stress fiber and focal adhesion formation were detected in miR-200b stably expressing cells (**Figure 3.1.A**). Since RhoA activation plays a key role in enhancing stress fiber and focal adhesion formation, we next determined RhoA activity level. RhoA-GTP pulldown assays showed that a significantly higher RhoA-GTP level is detected in miR-200b stably expressing cells (**Figure 3.1.B**), indicating that miR-200b expression causes high RhoA activation. Dynamic RhoA activitation and inactivation are critical for focal adhesion dynamic assembly and disassembly, which is vital for cell migration, therefore we next determined the effect of miR-200b expression on focal adhesion dynamic turnover. As shown in **Figure 3.1.C and Figure 3.2.**, the number of focal adhesions is decreased with the extension of serum incubation time in SUM-159-GFP and MDA-MB-231-GFP control cells, an indication of focal adhesion disassembly in these cells. In striking contrast, the number of focal adhesions does not decrease, but in fact increase as the serum incubation time increases in miR-200b stably expressing cells, an indication of defects in focal adhesion dynamic turnover. This suggests that miR-200b

Figure 3.1. miR-200b re-expression causes actin cytoskeleton reorganization and enhances focal adhesions by activating RhoA. (A) Representative overlaid images of immunofluorescence staining of phalloidin (red color) with vinculin (green) and nuclear DAPI staining (blue color) of MDA-MB-231- and SUM-159-GFP control and miR-200b stably expressing cells under normal culture conditions. Scale bar = 25µm. Images are taken at 600X. **(B)** RhoA-GTP pulldown assay and quantifications for MDA-MB-231 and SUM-159 control and miR-200b stably expressing TNBC cells. Subconfluent cells were collected for RhoA-GTP pulldown assay. RhoA-GTP and total RhoA levels were quantified using ImageJ software and the quantifications are presented as the ratio of RhoA-GTP levels divided by the corresponding total RhoA levels (mean ± SD, $n = 3$) relative to that of control cells. * $p < 0.05$, compared with the control group. **(C)** Representative overlaid immunofluorescent images of phalloidin (red color) with vinculin (green) and nuclear DAPI staining (blue color) of SUM-159-GFP control and miR-200b stably expressing cells. Cells were serum starved overnight and then serum activated for the indicated amount of time. Quantifications are presented as the number of focal adhesions (green dots) per cell (mean ± SD, $n \geq 40$). * $p < 0.05$, compared with GFP control cells. Scale bar = 25µm. Images are taken at 600X.

A.

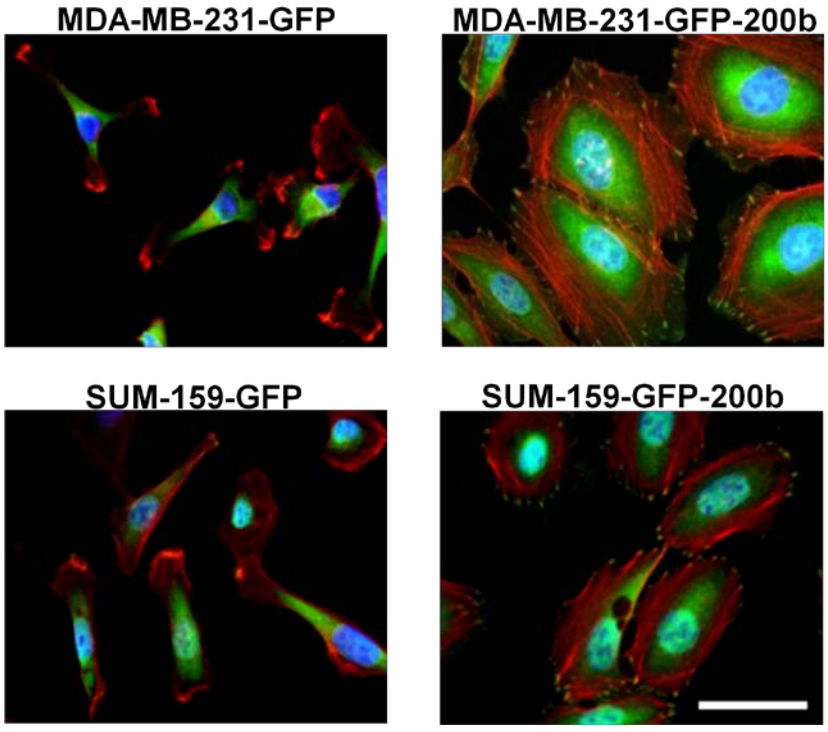


Figure 3.1. (cont'd)

B.

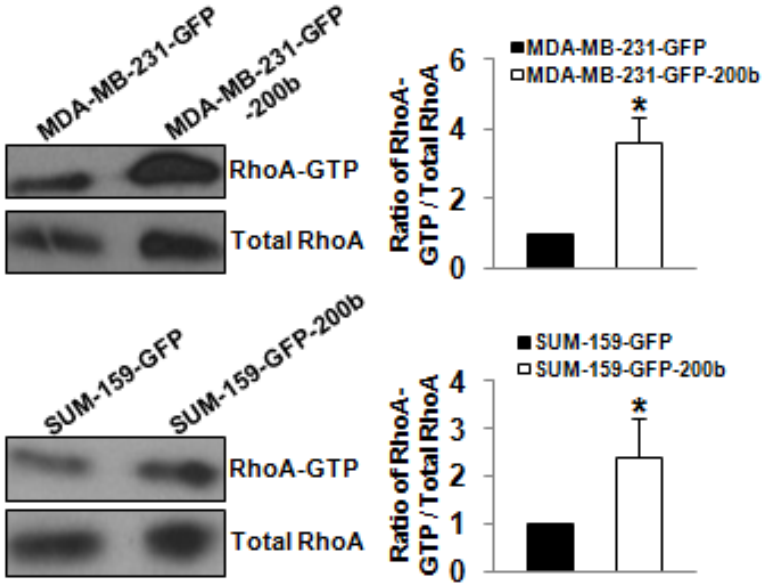


Figure 3.1. (cont'd)

C.

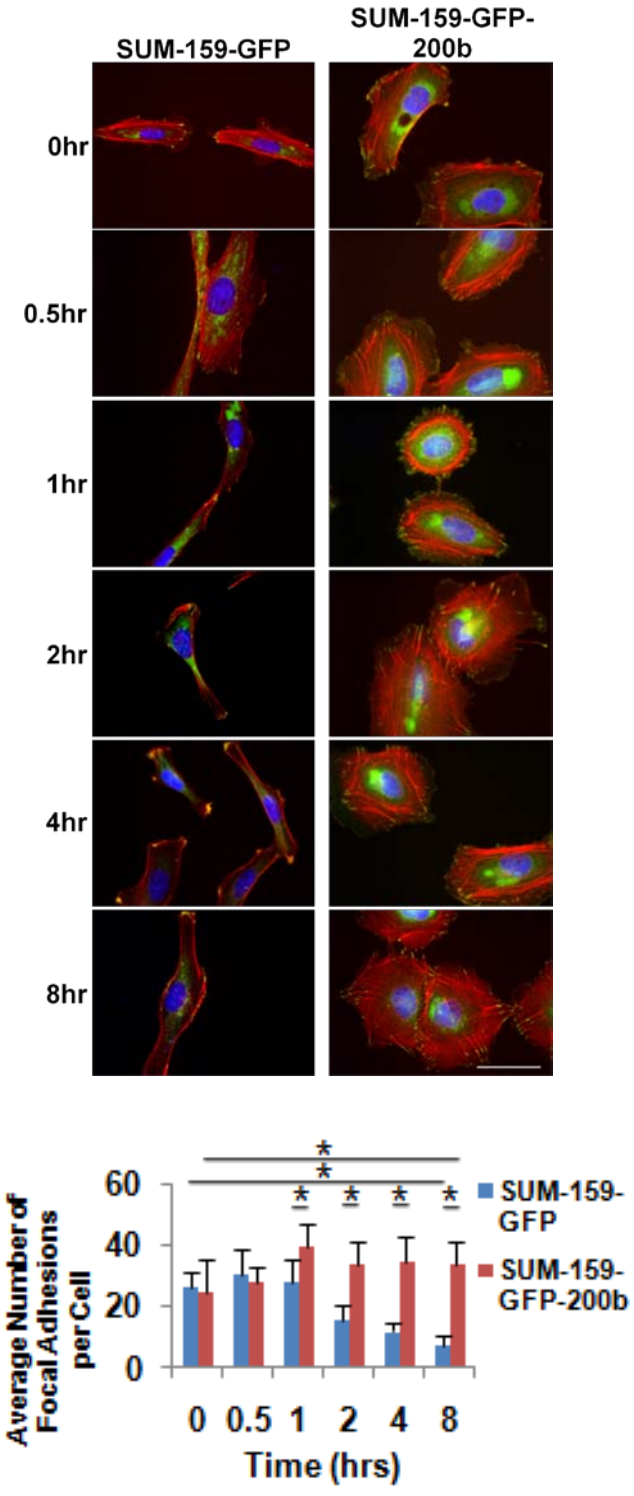
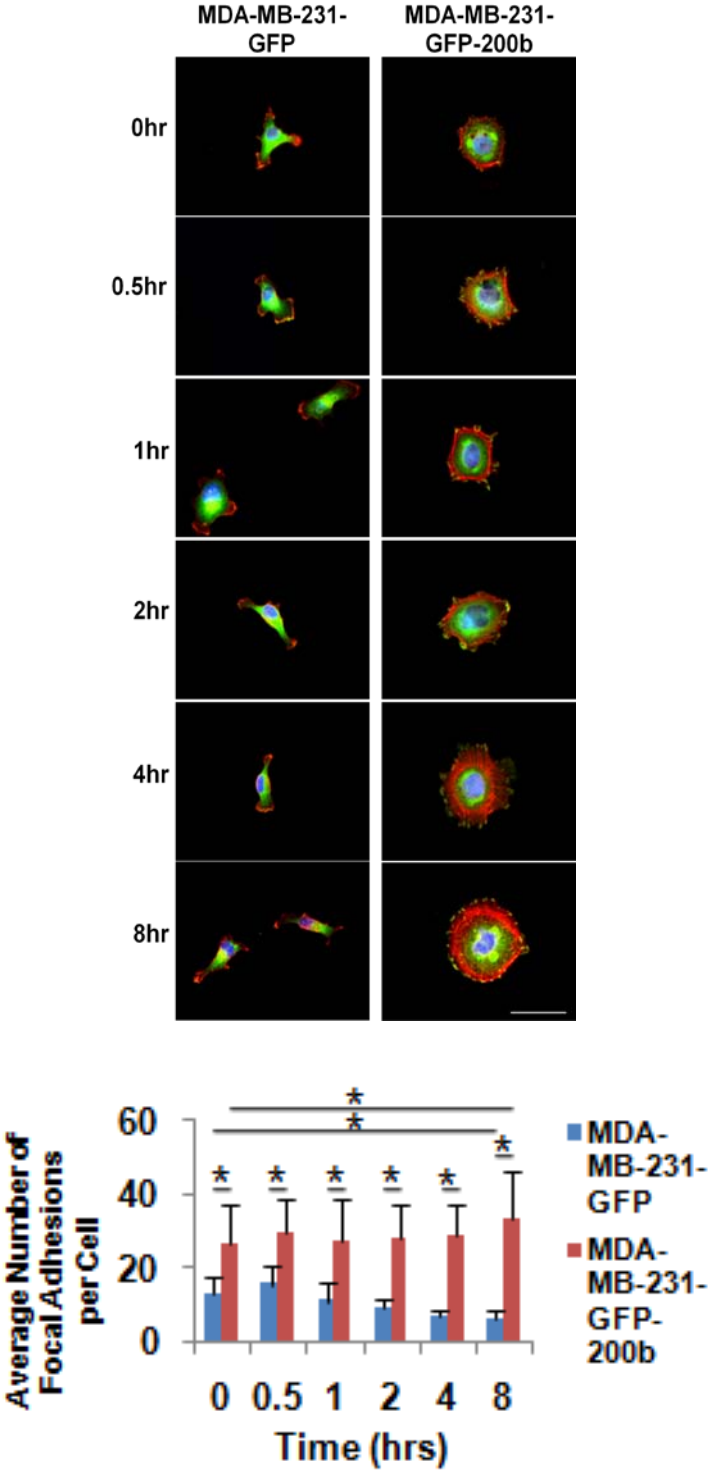


Figure 3.2. miR-200b stable expression increases focal adhesions and reduces focal adhesion turnover. Representative overlaid immunofluorescent images of phalloidin (red color) with vinculin (green) and nuclear DAPI staining (blue color) of MDA-MB-231 control and miR-200b stably expressing cells under serum activation for indicated amount of time. Quantifications are presented as the number of focal adhesions (green dots) per cell (mean ± SD, $n \geq 40$). * $p < 0.05$, compared with GFP control cells. Images are taken at 600X.



expression strongly increases RhoA activity, and this increased RhoA activity leads to enhanced stress fiber and focal adhesions formation and defected focal adhesion turnover.

INHIBITION OF RHOA SIGNALING REDUCES STRESS FIBER AND FOCAL ADHESION FORMATION, INCREASES RAC1 ACTIVATION, AND OVERCOMES THE INHIBITORY EFFECT OF MIR-200B ON TNBC CELL MIGRATION

To determine if increased RhoA activity is important for enhanced stress fiber and focal adhesion formation and the inhibitory effect of miR-200b on TNBC cell migration, we treated miR-200b stable expression cells with Y27632, a selective inhibitor of the key RhoA downstream effector ROCK. Inhibition of RhoA signaling by Y27632 significantly reduced stress fiber and focal adhesion formation in miR-200b stable expression cells (**Figure 3.3.A**). Moreover, inhibition of RhoA signaling also significantly increased lamellipodia formation (**Figure 3.3.A**), which is an indication of increased Rac1 activation. Indeed, Rac1-GTP pulldown assays further confirmed increased levels of Rac1-GTP in Y27632-treated miR-200b stably expressing cells (**Figure 3.3.B**). Wound healing cell migration assays were then carried out to determine the effect of inhibiting RhoA signaling on cell migration. Strikingly, it was found that inhibition of RhoA signaling overcomes the inhibitory effect of miR-200b on cell migration, as the wound is completely closed in Y27632-treated miR-200b stably expressing cells (**Figure 3.3.C**). This not only indicates that high RhoA activation plays a key role in the inhibitory effect of miR-200b on cell migration, but it also contrasts with the current view that RhoA activation promotes cell migration. Ultimately this data shows that increased RhoA activation is an important mechanism of miR-200b inhibition on TNBC cell migration.

Figure 3.3. Inhibition of Rho signaling overcomes the inhibitory effect of miR-200b on TNBC cell migration. (A) Representative overlaid immunofluorescent images of phalloidin (red color) with vinculin (green) and nuclear DAPI staining (blue color) of MDA-MB-231-GFP-200b and SUM-159-GFP-200b cells treated with or without the RhoA inhibitor Y27632 (10μM). Scale bar = 25μm. Images are taken at 600X. **(B)** Rac1-GTP pulldown assay and quantifications for MDA-MB-231-GFP-200b and SUM-159-GFP-200b cells treated with or without the RhoA inhibitor Y27632 (10μM). Subconfluent cells were collected for Rac1-GTP pulldown assay. Rac1-GTP and total Rac1 levels were quantified using ImageJ software and the quantifications are presented as the ratio of Rac1-GTP levels divided by the corresponding total Rac1 levels (mean ± SD, $n = 3$) relative to that of control DMSO treated cells. * $p < 0.05$, compared with control cell group. Inhibiting MDA-MB-231-GFP-200b and SUM-159-GFP-200b cells with or without the RhoA inhibitor Y27632 (10μM) significantly affects the wound healing capability **(C)**. Scale bar = 100 μm. Quantifications are presented as the percentage of wound closure after 24hrs (mean ± SD, $n = 3$). * $p < 0.05$, compared with the control DMSO treated group.

A.

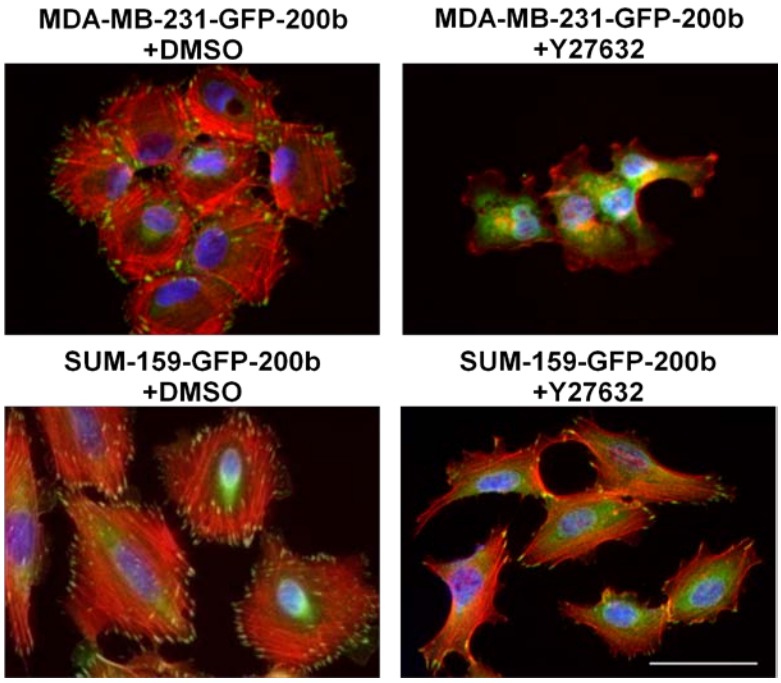
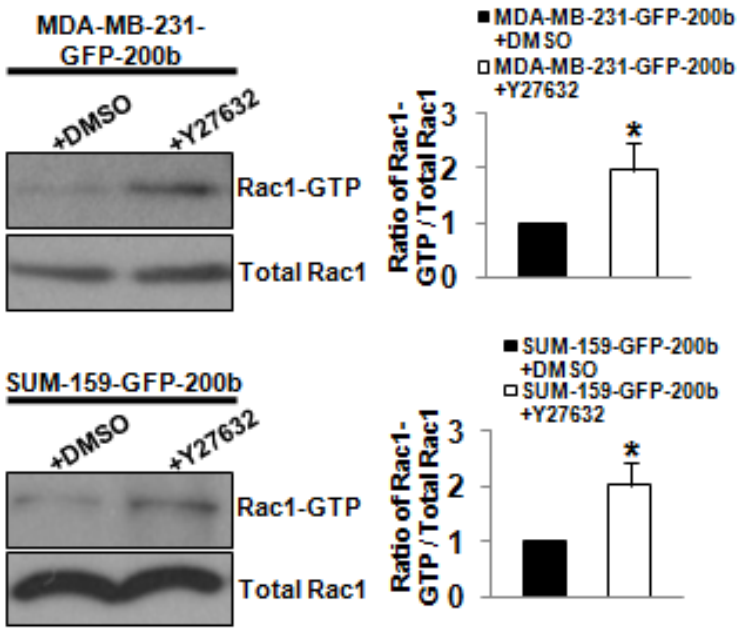
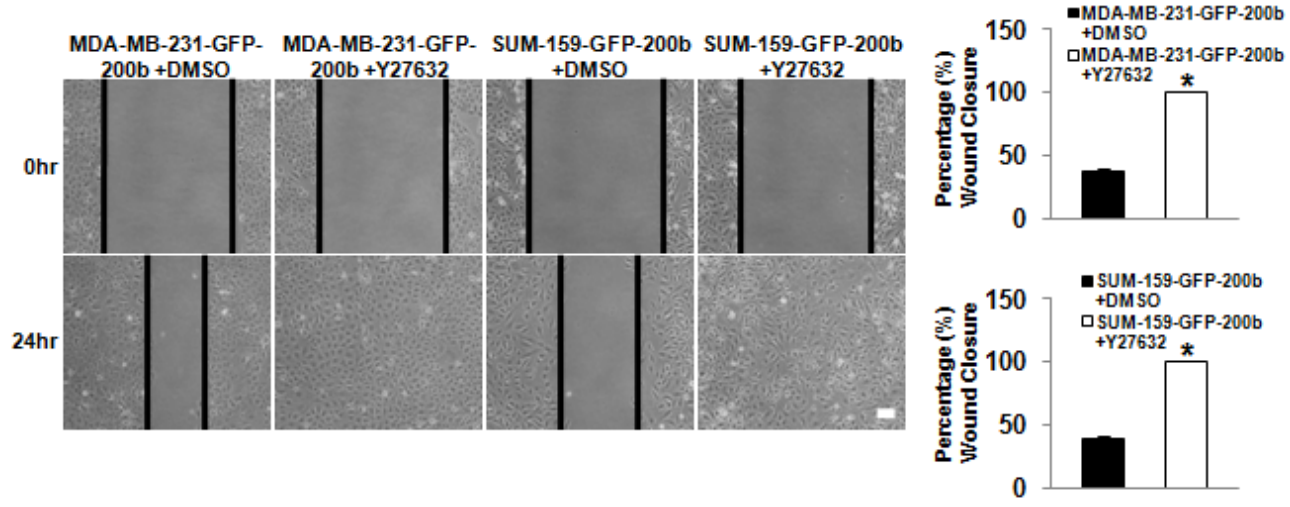


Figure 3.3. (cont'd)

B.



C.



THE EXPRESSION OF A RHOA-SPECIFIC GAP ARHGAP18 IS SIGNIFICANTLY HIGHER IN BASAL MESENCHYMAL-LIKE TNBC CELLS AND ARHGAP18 IS A DIRECT TARGET OF MIR-200B

We next wanted to investigate the molecular mechanism behind how miR-200b increases RhoA activation and inhibits TNBC cell migration and metastasis. Previous studies have shown that cross-talk exists between Rac1 and RhoA, and that Rac1 and RhoA can regulate each other through downstream effectors (20,21). Our previous work has shown that miR-200b reduces Rac1 activation by targeting PKCα and that inhibition of PKCα by GO6976 similarly reduces Rac1 activation in TNBC cells (12,22). Therefore, we first tested whether inhibition of Rac1 activity by inactivating PKCα with GO6976 treatment has any effect on RhoA activation in TNBC cells. We found that treatment of MDA-MB-231 and SUM-159 cells with GO6976 did not have any effect on RhoA activation (**Figure 3.4.**). Therefore, miR-200b increases RhoA activation through a mechanism other than reducing Rac1 activation by targeting PKCα. To identify other potential miR-200b targets that may play a crucial role in increased RhoA activity, we performed bioinformatic analysis. For this analysis, we are particularly interested in GTPase activating proteins (GAPs) because of their ability to inhibit Rho GTPase activation and are likely direct targets of miR-200b to increase RhoA activation. Using two different bioinformatic programs (TargetScan and microRNA.org), targets of miR-200b representing many different signaling pathways were identified. Among the potential targets, we particularly focused on ARHGAP18 and ARHGAP19 because of previous studies suggesting these GAPs are specific for RhoA. In order to determine if these RhoA GAPs play any role in regulating TNBC cell actin cytoskeleton organization, we performed a functional analysis by ectopically expressing GAP-specific siRNA. Knockdown of ARHGAP18 and ARHGAP19 were confirmed by western

Figure 3.4. Inhibition of Rac1 does not affect RhoA activation. RhoA-GTP pulldown in MDA-MB-231-GFP and SUM-159-GFP cells treated with DMSO or the Rac1 inhibitor GO6976. RhoA-GTP were quantified by ImageJ and are shown as RhoA-GTP levels divided by the corresponding total RhoA levels (mean ± SD, $n = 3$) relative to that of cells treated with DMSO. * $p < 0.05$, compared with the control group.

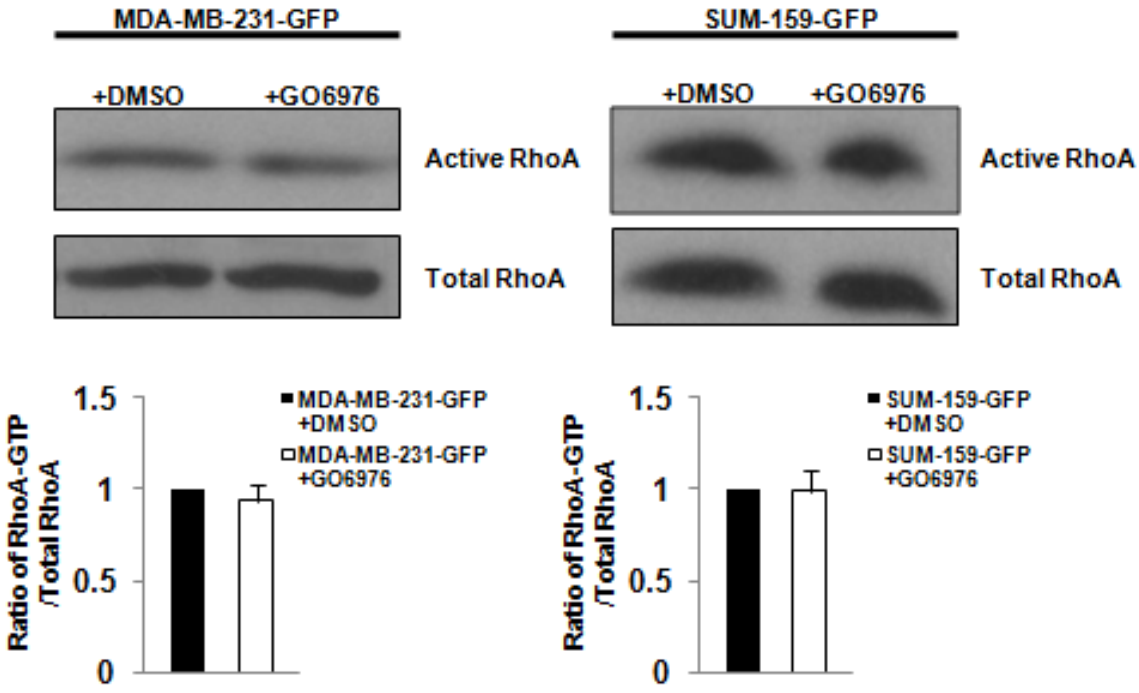
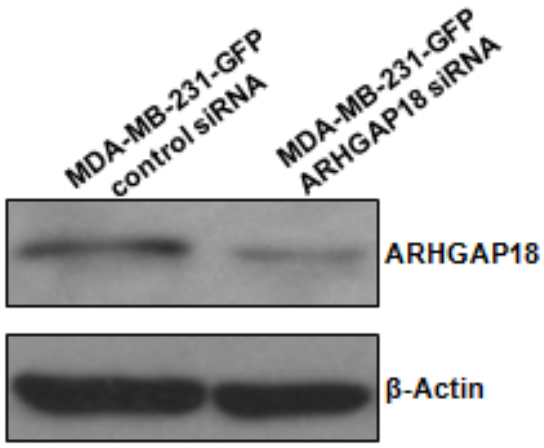


Figure 3.5. ARHGAP18 and ARHGAP19 siRNA significantly reduces protein and mRNA expression. (A) Western blot analysis of ARHGAP18 protein expression levels in MDA-MB-231-GFP cells treated with control or ARHGAP18 siRNA. (B) Q-PCR analysis of ARHGAP19 expression levels in MDA-MB-231-GFP cells. The levels of ARHGAP19 are expressed relative to that of control siRNA and are presented as mean ± SD (n=3). * $p < 0.05$ compared to control siRNA.

A.



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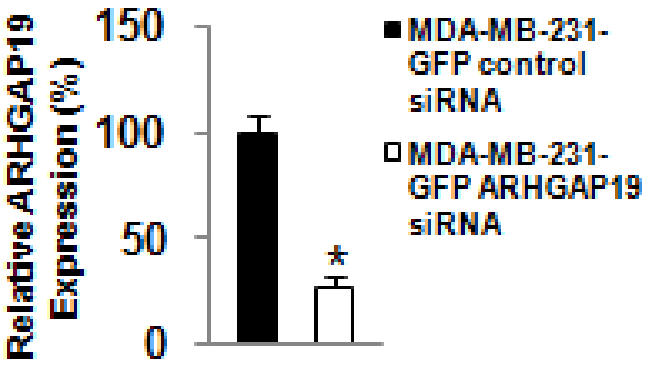


Figure 3.6. ARHGAP18 inhibition alters the actin cytoskeleton and activates RhoA. (A) Representative overlaid images of phalloidin (red), vinculin (green), and nuclear DAPI (blue) in MDA-MB-231-GFP and SUM-159-GFP TNBC cells treated with control or ARHGAP18 siRNA. Scale bar = 25µm. Images are taken at 600X. RhoA-GTP **(B)** and Rac1-GTP **(C)** pulldown assay and quantifications for MDA-MB-231-GFP and SUM-159-GFP control and ARHGAP18 siRNA treated cells. Rac1-GTP, RhoA-GTP, total Rac1, and total RhoA levels were quantified using ImageJ software and the quantifications are presented as the ratio of Rac1-GTP or RhoA-GTP levels divided by the corresponding total Rac1 or total RhoA levels (mean ± SD, $n = 3$) relative to that of control cells. * $p < 0.05$, compared with control siRNA group.

A.

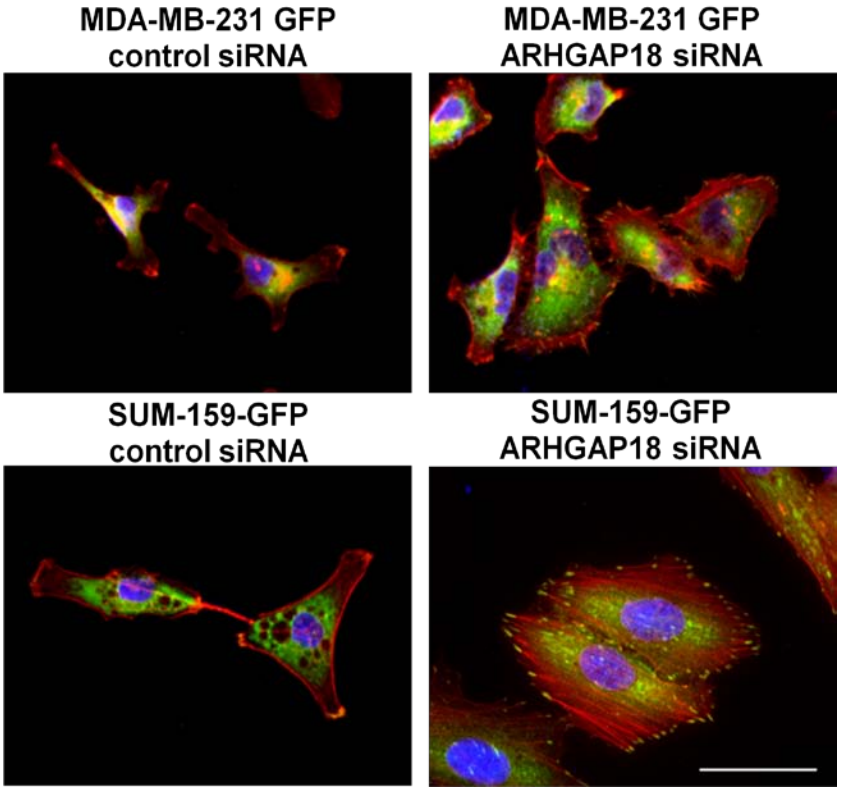
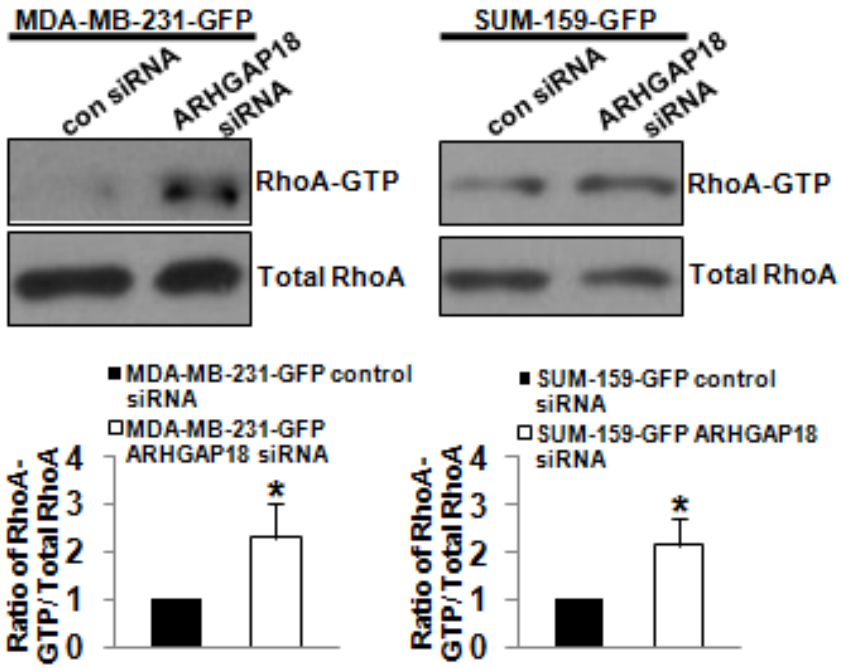
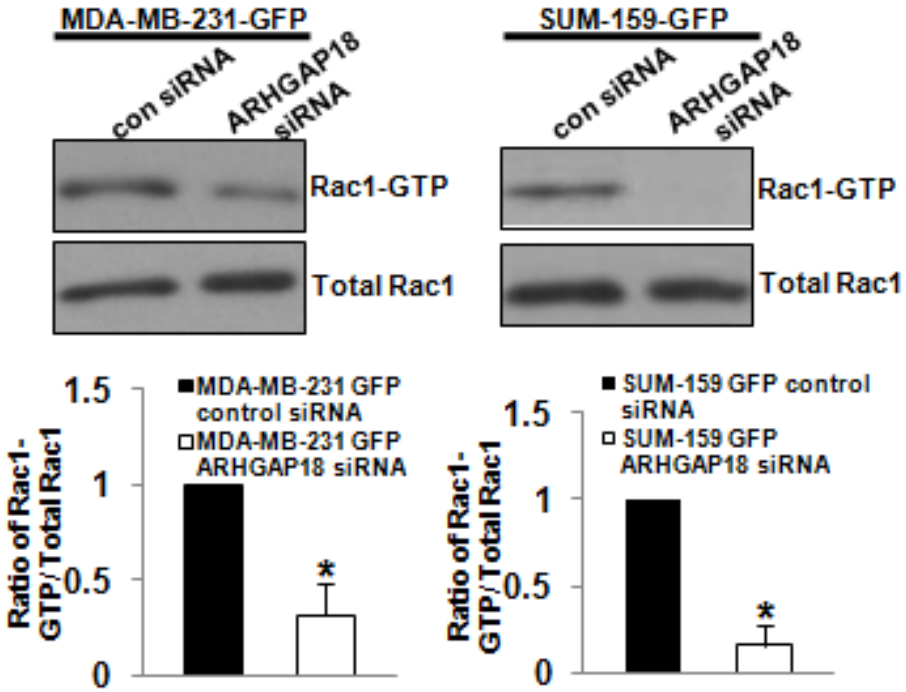


Figure 3.6. (cont'd)

B.



C.



blotting and Q-PCR, respectively (**Figure 3.5.A and 3.5.B**). Knockdown of ARHGAP18 showed a drastic decrease of lamellipodia formation and an increase in stress fiber and focal adhesion formation (**Figure 3.6.A**), while knockdown of ARHGAP19 had no dramatic effect on the actin cytoskeleton (**Figure 3.7.**). Moreover, ARHGAP18 knockdown also significantly increased RhoA (**Figure 3.6.B**) and decreased Rac1 (**Figure 3.6.C**) activation in TNBC cells, phenocopying the effect of miR-200b overexpression.

Since we have shown that ARHGAP18 knockdown has a significant impact on actin cytoskeleton organization, mimicking the effect of miR-200b overexpression, we next wanted to determine if ARHGAP18 is a direct target of miR-200b. First, we measured the expression levels of ARHGAP18 in MDA-MB-231 and SUM-159 TNBC parental and miR-200b stably expressing cells. Q-PCR analysis showed that in both of these TNBC cells mRNA levels were significantly reduced in cells that stably expressed miR-200b (**Figure 3.8.A**). Furthermore, protein expression levels were also reduced in cells that stably express miR-200b compared to control cells (**Figure 3.8.B**), suggesting that ARHGAP18 may be a target of miR-200b. To demonstrate that miR-200b is directly targeting ARHGAP18, we generated ARHGAP18 wild type and mutant type 3'UTR luciferase reporter vectors and performed a dual luciferase reporter assay. Results from the dual luciferase reporter assay show that stable expression of miR-200b in MDA-MB-231 and SUM-159 significantly reduces ARHGAP18 wild type luciferase reporter activity, but has no effect on mutant type luciferase reporter activity (**Figure 3.8.C**). Ultimately, this data shows that miR-200b directly targets ARHGAP18.

Consistent with our previous study showing that miR-200 expression levels are significantly lower in highly migratory TNBC cells and metastatic TNBC tumors than other subtypes of breast cancer cells and tumors, western blot analysis demonstrated that the

Figure 3.7. ARHGAP19 siRNA has no dramatic effect on the actin cytoskeleton of TNBC cell lines. Representative overlaid images of immunofluorescence staining of phalloidin (red color) with vinculin (green) and nuclear DAPI staining (blue color) of MDA-MB-231 and SUM-159 control cells treated with control or ARHGAP19 siRNA under normal culture conditions. Images are taken at 600X.

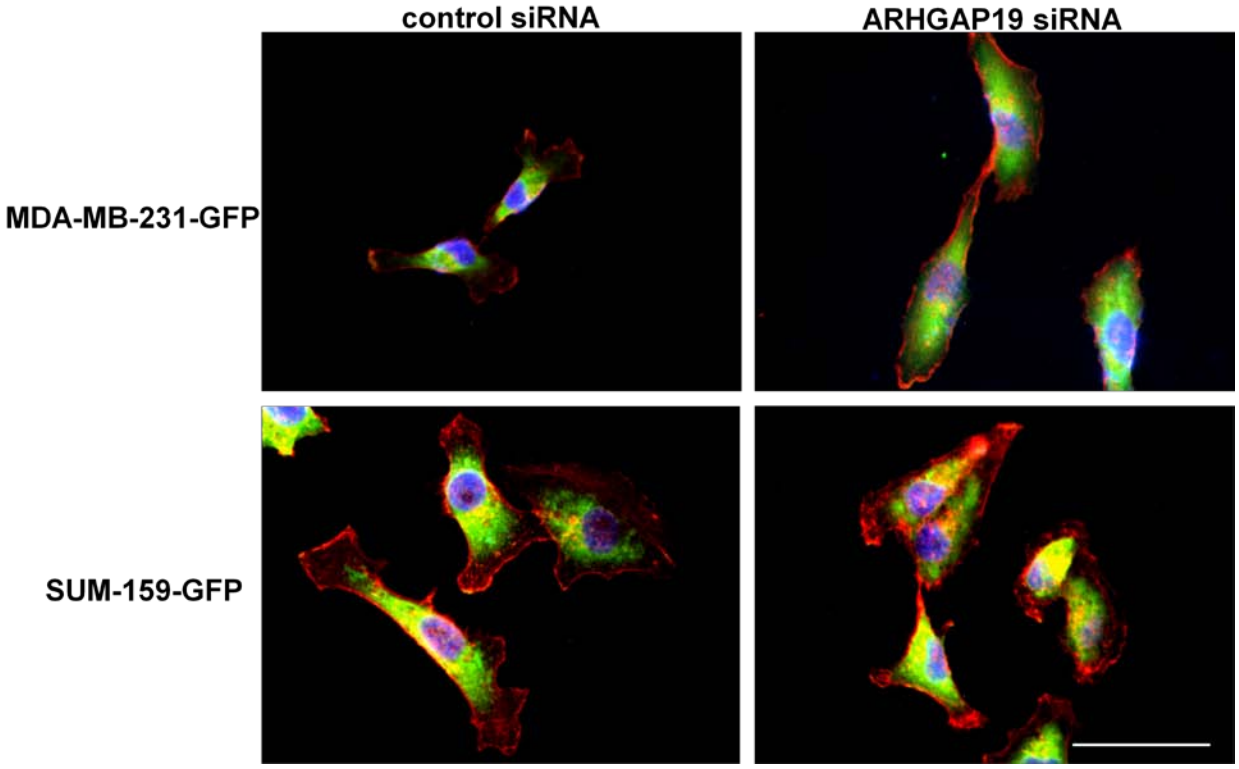
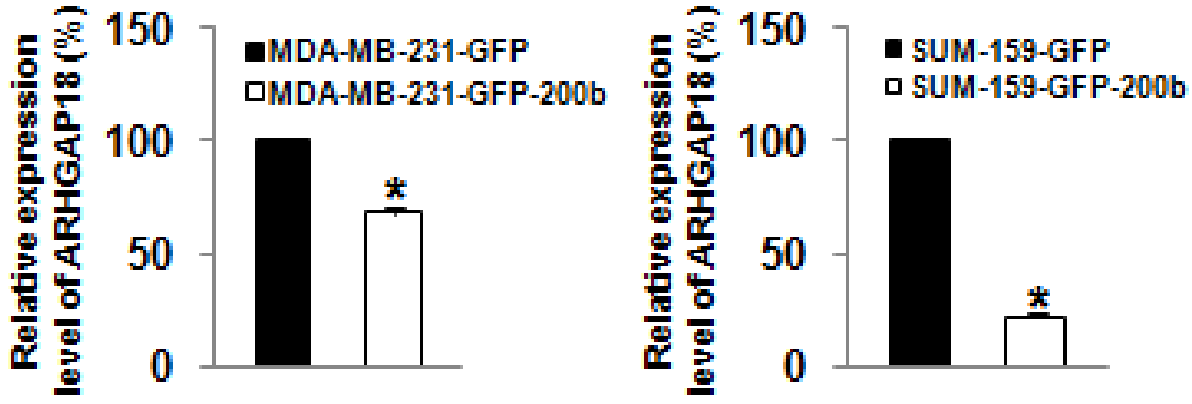


Figure 3.8. miR-200b directly targets ARHGAP18. mRNA **(A)** and protein **(B)** expression levels of ARHGAP18 in MDA-MB-231-GFP control and miR-200b stably expressing cells. **(C)** Quantifications of ARHGAP18 3′UTR wild-type and mutant-type vector luciferase reporter activity in GFP control and miR-200b stably expressing cells. The luciferase reporter activity (mean ± SD, $n = 3$) is expressed relative to GFP control cells.* $p < 0.05$, compared with the GFP control cell group.

A.



B.

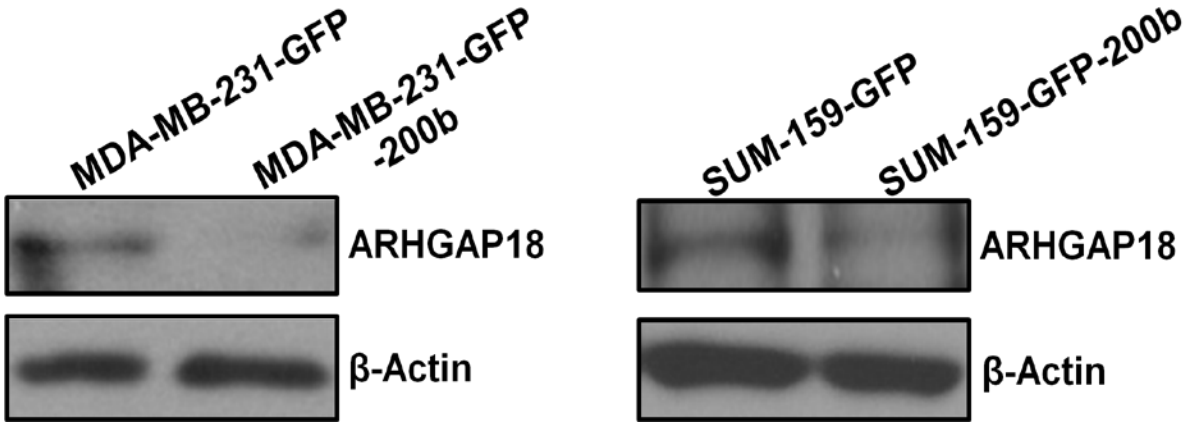


Figure 3.8. (cont'd)

C.

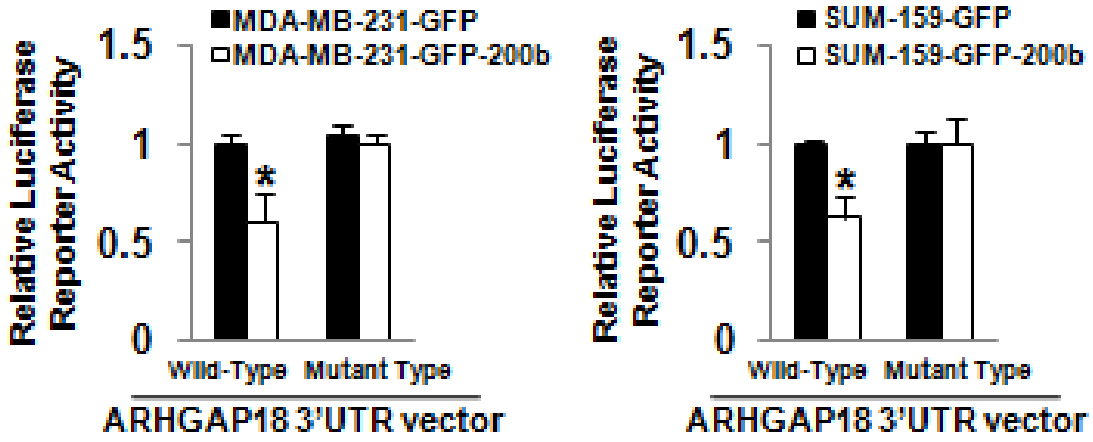


Figure 3.9. The expression of ARHGAP18 is high in TNBC cells and associated with worse DMFS and RFS in breast cancer patients. (A) Western blot analysis of ARHGAP18 protein levels across 12 kinds of breast cancer cells. Kaplan–Meier plots of distant metastasis free **(B)** and recurrence free **(C)** survival in breast cancer patients in high- and low-expression groups.

A.

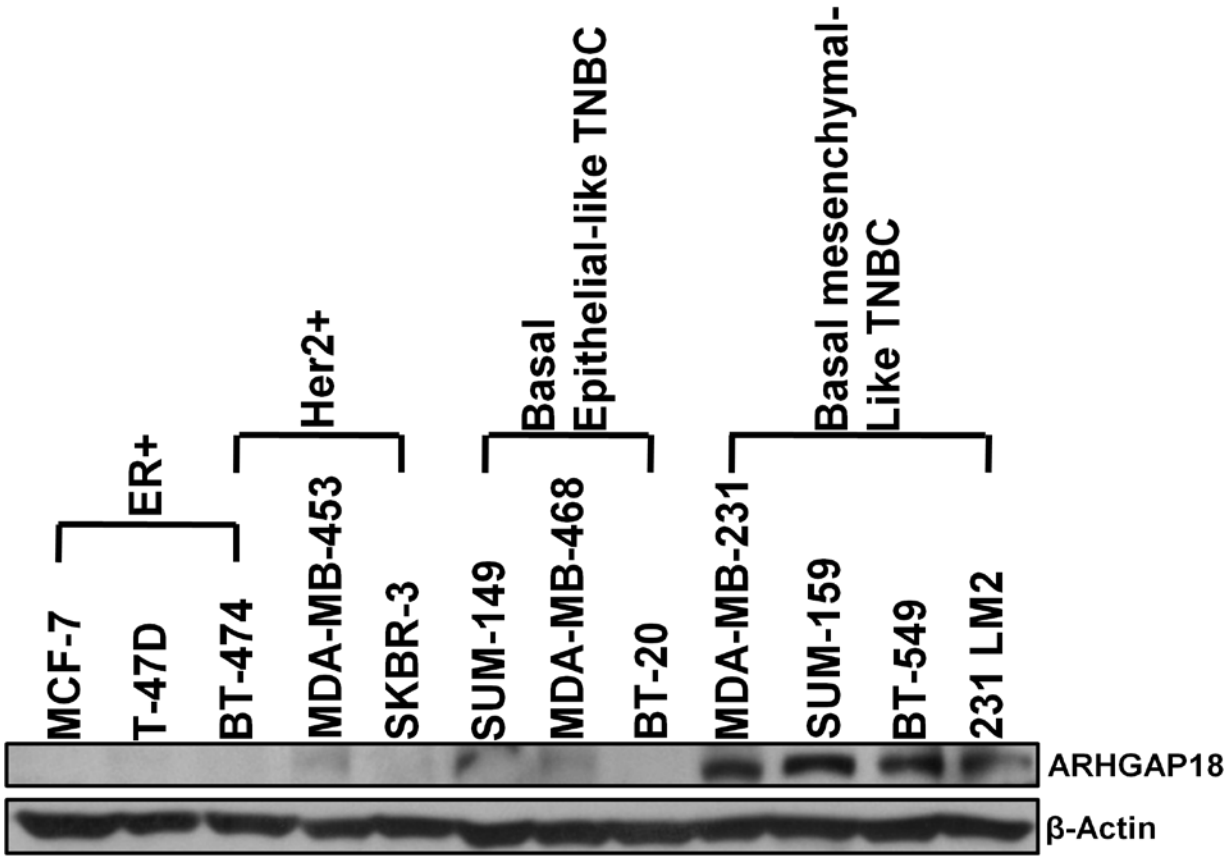
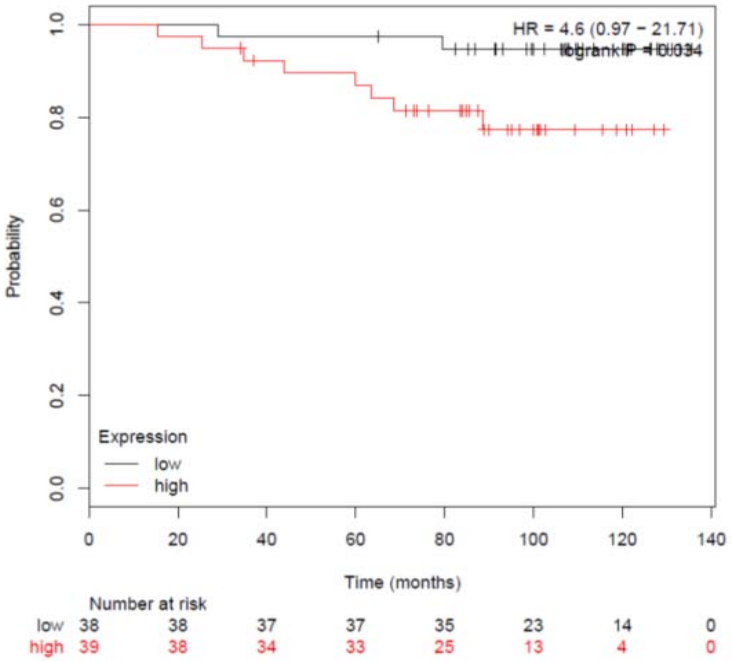
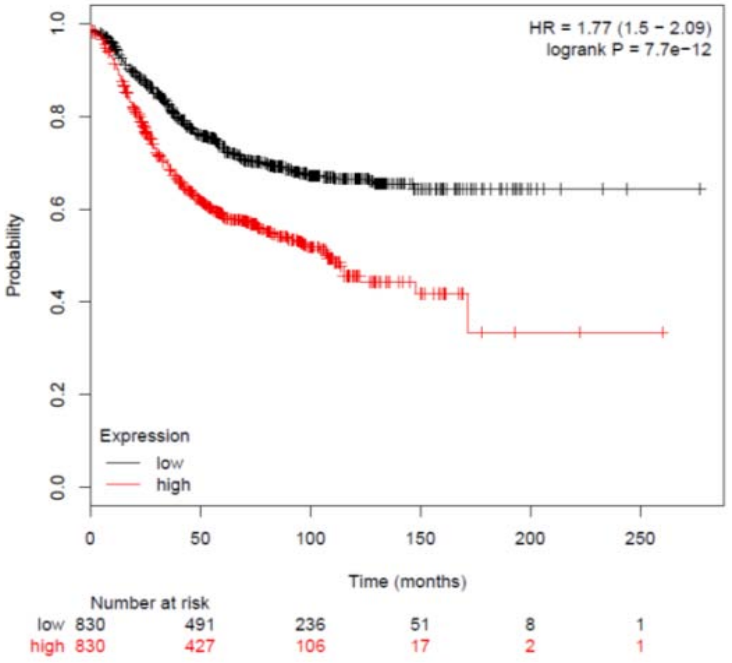


Figure 3.9. (cont'd)

B.



C.



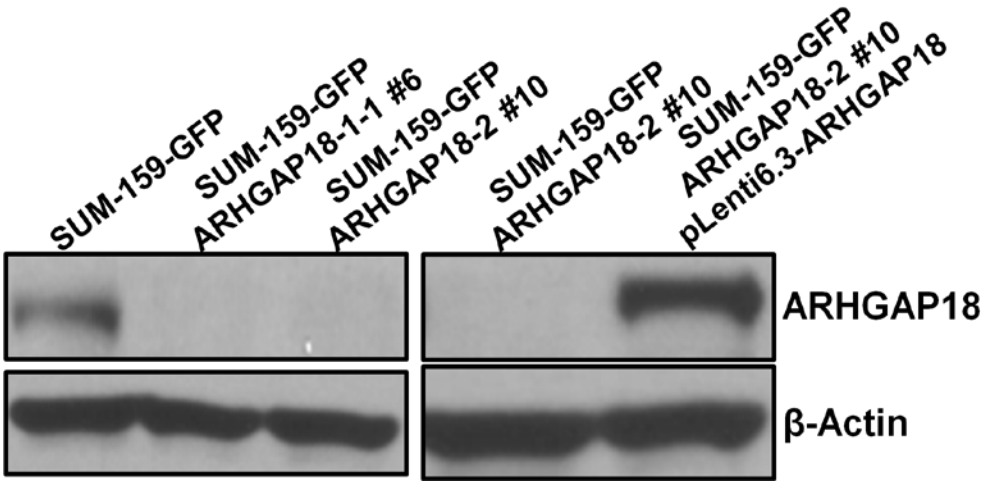
expression level of ARHGAP18 is dramatically higher in highly migratory TNBC cells than other types of breast cancer cells (**Figure 3.9.A**). To further determine whether ARHGAP18 plays a significant role in human breast cancer we performed bioinformatic analysis. Using a Kaplan-Meier plotter (kmplot.com), analysis on primary breast tumors revealed that high ARHGAP18 expression in breast cancer patients is correlated with significantly poorer distant metastasis free survival (**Figure 3.9.B**) and recurrence free survival (**Figure 3.9.C**). This data suggests that ARHGAP18 expression may play an important role in the triple negative breast cancer metastasis and recurrence.

CRISPR KNOCKOUT OF ARHGAP18 INCREASES RHOA ACTIVATION, BUT REDUCES TNBC CELL MIGRATION

Since high ARHGAP18 expression is strongly implicated in breast cancer cell migration and breast cancer metastasis and recurrence, we wanted to further explore the underlying mechanism. We knocked out ARHGAP18 expression using the CRISPR/Cas9 genome editing technique in the highly migratory and invasive SUM-159 (SUM-159-GFP ARHGAP18-1-1 #6 and SUM-159-GFP ARHGAP18-2 #10) and highly metastatic MDA-MB-231 LM2 4175 (MDA-MB-231-GFP LM2 ARHGAP18-1-1 #3 and MDA-MB-231-GFP LM2 ARHGAP18-2 #11) TNBC cell lines. ARHGAP18 knockout, and subsequent enforced expression of ARHGAP18 in knockout cells, was confirmed by Western blot (**Figure 3.10.A and 3.11.A**) and sequencing (**Figure 3.12.**). Control pLenti6.3 vector in MDA-MB-231 LM2, SUM-159-GFP, and ARHGAP18 CRISPR/Cas9 knockout cells has no significant effect on cell morphology and the actin cytoskeleton (data not shown). Moreover, knockout and/or enforced expression of ARHGAP18 had no significant effects on key EMT regulators ZEB1 and E-cadherin, as well as the previously identified miR-200b target PKCα (12) (**Figure 3.13.**). ARHGAP18 knockout

Figure 3.10. Knockout ARHGAP18 using CRISPR/Cas9 increases RhoA activation and reduces cell migration in SUM-159 cells. (A) Western blot analysis of ARHGAP18 protein expression levels in SUM-159-GFP and CRISPR/Cas9 knockout and ARHGAP18 overexpression cells. **(B)** RhoA-GTP pulldown in SUM-159-GFP parental and CRISPR/Cas9 knockout and ARHGAP18 overexpression cells. RhoA-GTP and total RhoA levels were quantified by ImageJ and are shown as RhoA-GTP levels divided by the corresponding total RhoA levels (mean ± SD, $n = 3$) relative to that of GFP parental cells. * $p < 0.05$, compared with parental group. **(C)** Representative overlaid images of phalloidin (red), vinculin (green), and nuclear DAPI (blue) of SUM-159-GFP parental and CRISPR/Cas9 knockout and ARHGAP18 overexpression cells. Scale bar = 25μm. Images are taken at 600X. **(D)** Wound healing assay of SUM-159-GFP parental and CRISPR/Cas9 knockout and ARHGAP18 overexpression cells. Scale bar = 100 μm.

A.



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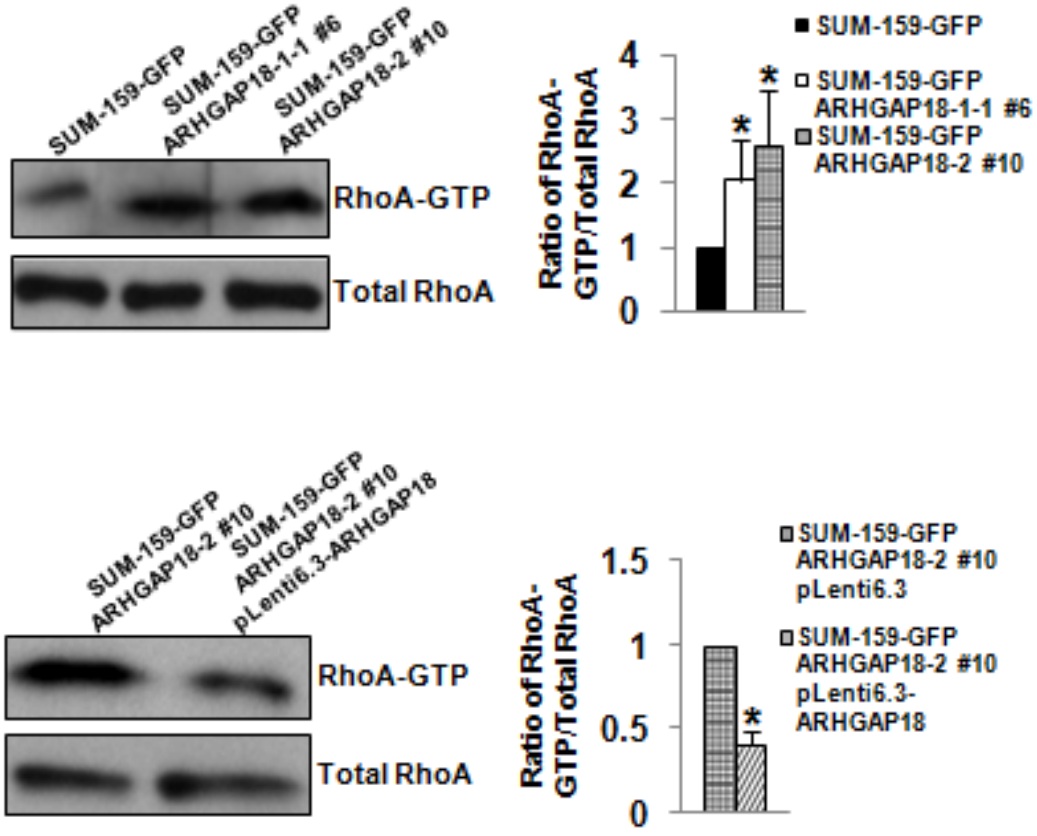

Figure 3.10. (cont'd)

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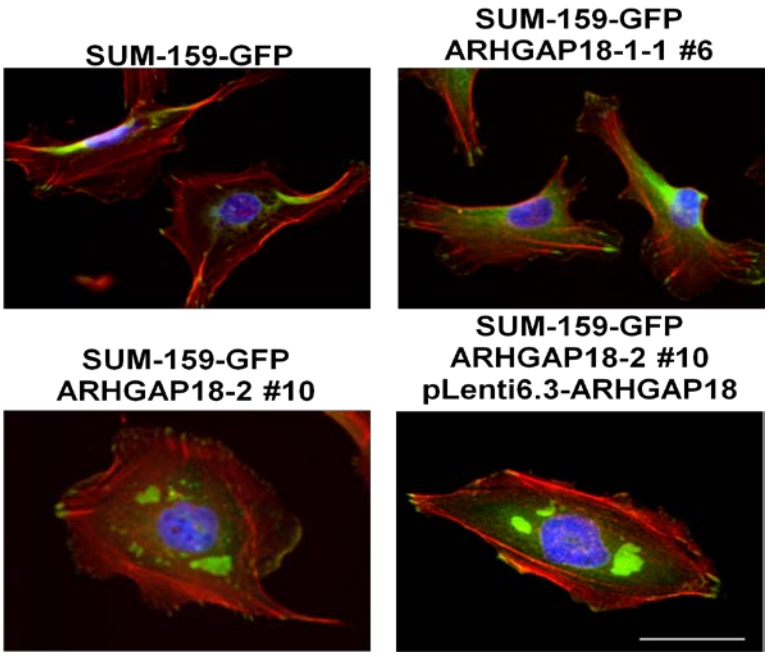

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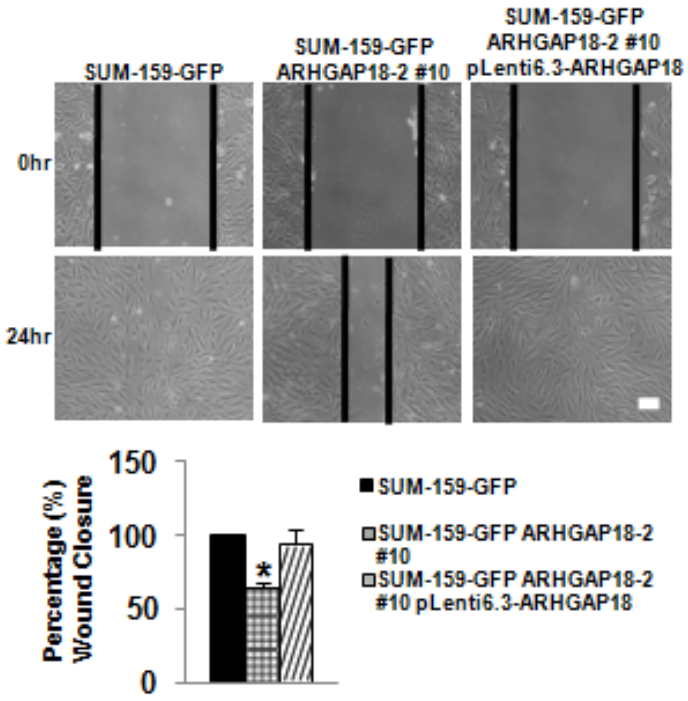
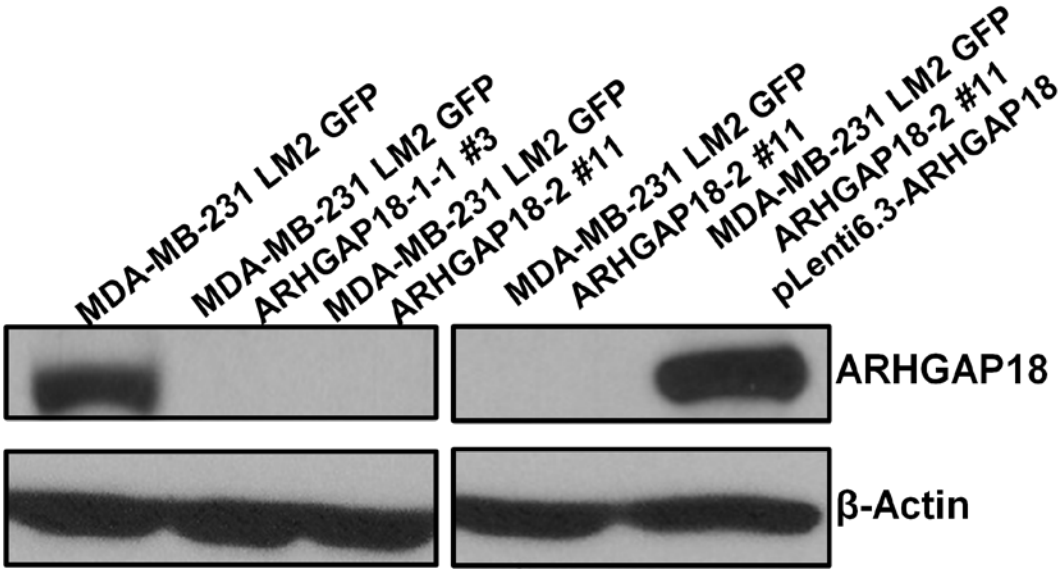


Figure 3.11. Knockout ARHGAP18 using CRISPR/Cas9 increases RhoA activation and reduces cell migration in MDA-MB-231 LM2 cells. (A) Western blot analysis of ARHGAP18 protein expression levels in MDA-MB-231 LM2 GFP and CRISPR/Cas9 knockout and ARHGAP18 overexpression cells. **(B)** RhoA-GTP pulldown in MDA-MB-231 LM2 GFP parental and CRISPR/Cas9 knockout and ARHGAP18 overexpression cells. RhoA-GTP and total RhoA levels were quantified by ImageJ and are shown as RhoA-GTP levels divided by the corresponding total RhoA levels (mean ± SD, $n = 3$) relative to that of GFP parental cells. * $p < 0.05$, compared with parental group. **(C)** Representative overlaid images of phalloidin (red), vinculin (green), and nuclear DAPI (blue) of MDA-MB-231 LM2 GFP parental and CRISPR/Cas9 knockout and ARHGAP18 overexpression cells. Scale bar = 25μm. Images are taken at 600X. **(D)** Wound healing assay of MDA-MB-231 LM2 GFP parental and CRISPR/Cas9 knockout and ARHGAP18 overexpression cells. Scale bar = 100 μm.

A.



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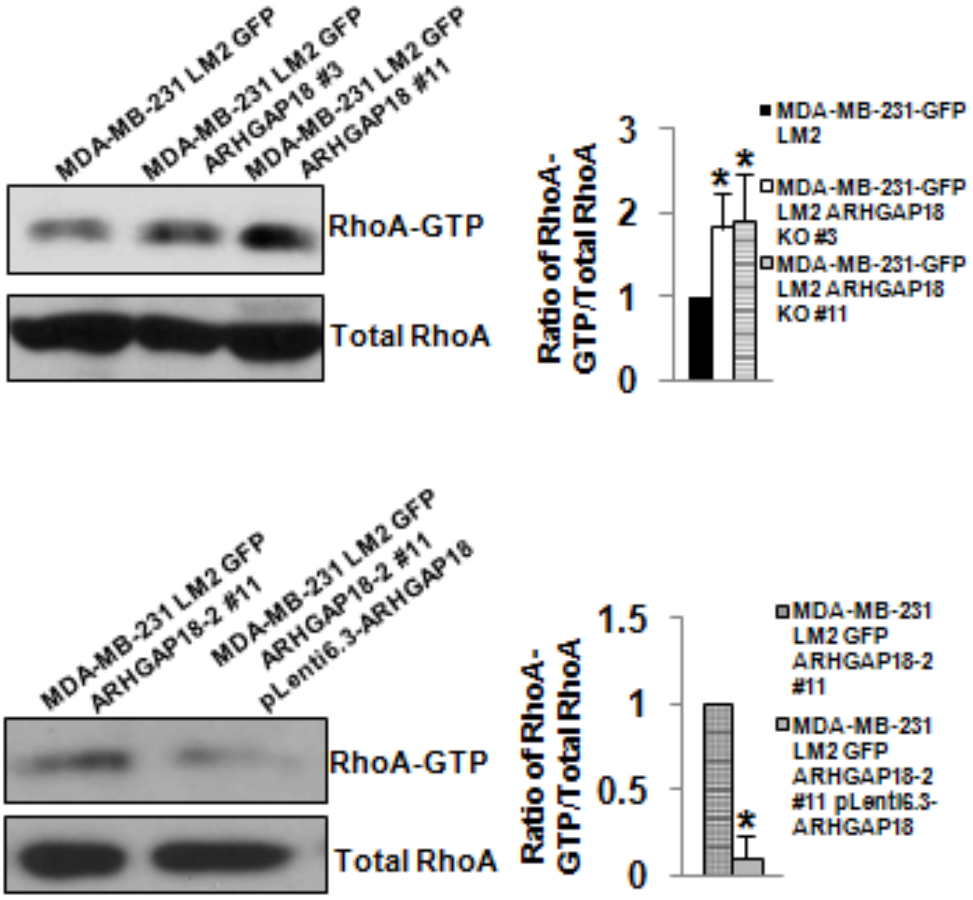

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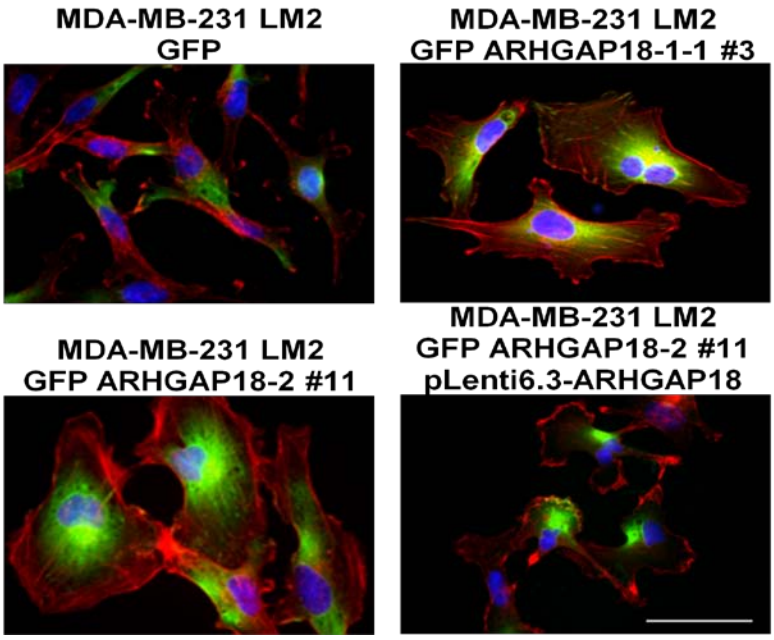

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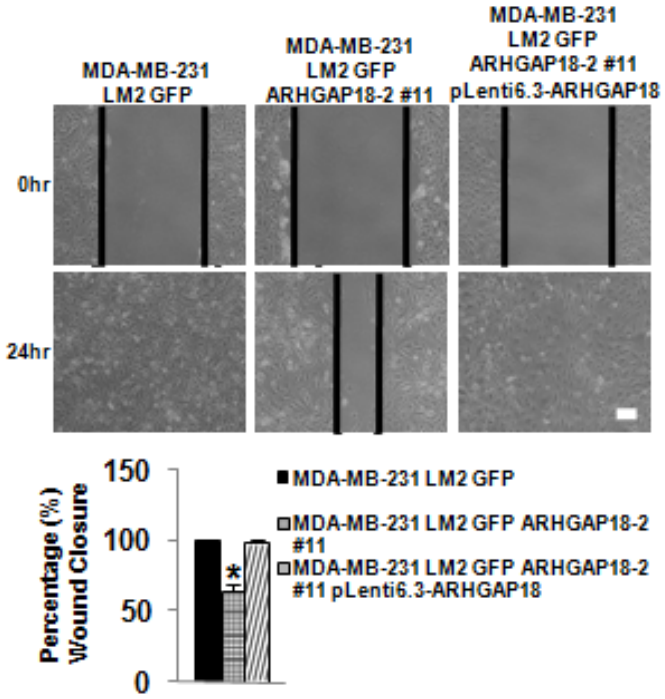


Figure 3.12. CRISPR/Cas9 results in insertions and deletions near the target PAM sequence. The sequences of the wild-type *ARHGAP18* region targeted by CRISPR/Cas9, and the resulting insertions/deletions detected in various clones used to test the function of ARHGAP18 in triple negative breast cancer.

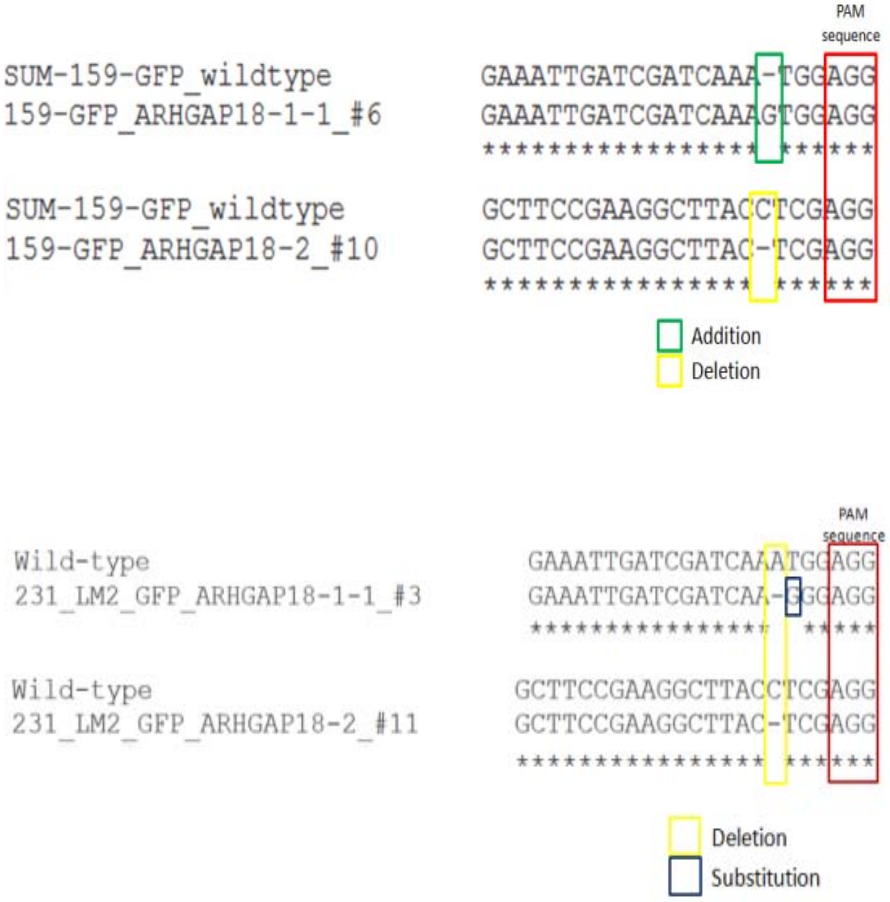


Figure 3.13. ARHGAP18 knockout by CRISPR/Cas9 and overexpression has no affect on EMT regulators and other known miR-200b targets. Western blot analysis of ZEB1, E-cadherin and PKCα protein expression levels in MDA-MB-231 LM2 GFP and SUM-159-GFP control and CRISPR/Cas9 knockout and overexpression cells.

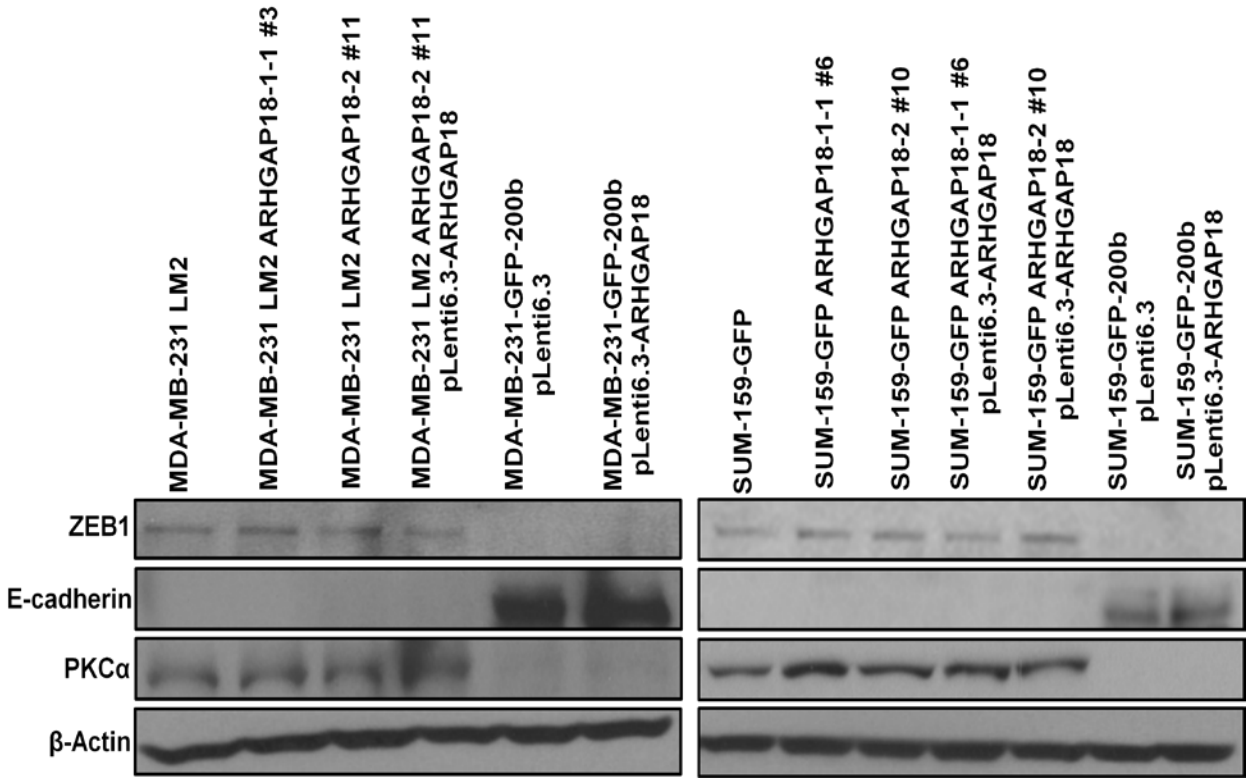
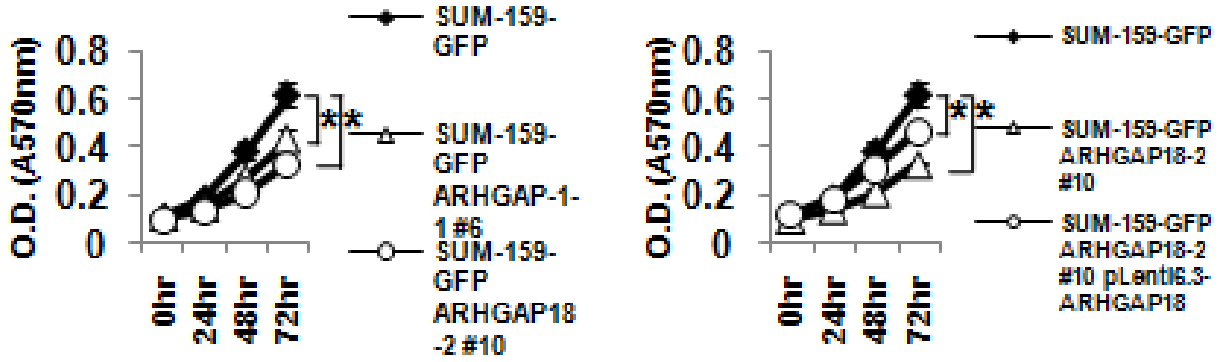


Figure 3.14. ARHGAP18 plays an important role in SUM-159 cell proliferation and migration. (A) Three thousand cells were seeded into each well of 96-well plates for MTT assay to indirectly monitor cell proliferation up to 72 h. Data are presented as mean ± SD (n=8). * $p < 0.05$, compared to the GFP control cells. (B) Knockout of ARHGAP18 significantly affects the wound healing capability of SUM-159-GFP cells. Scale bar = 100 μm.

A.



B.

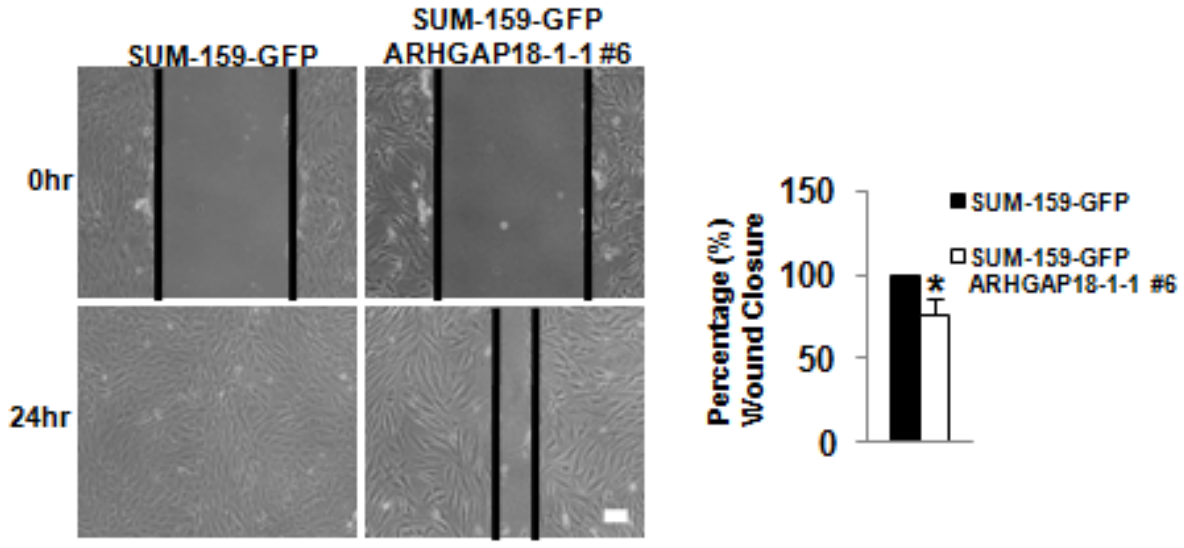
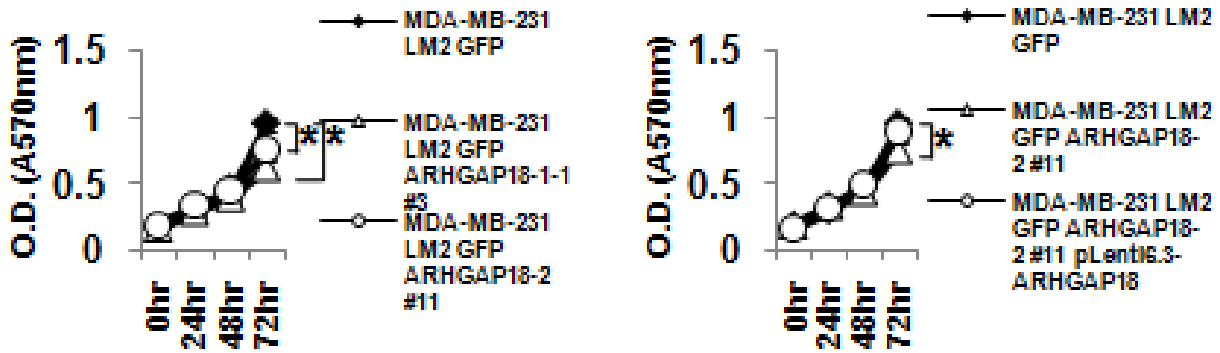
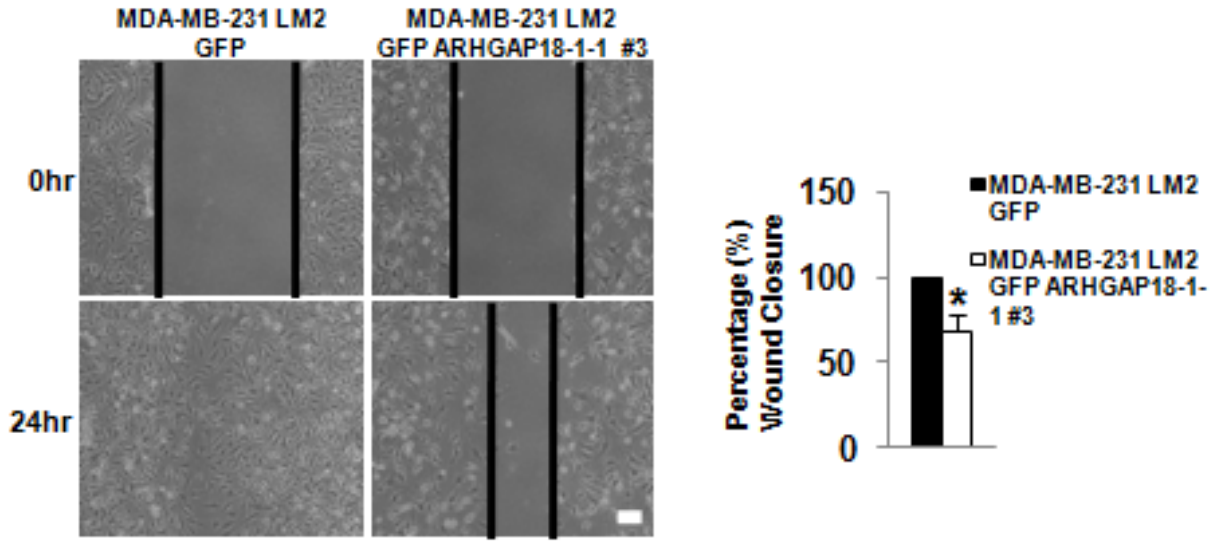


Figure 3.15. ARHGAP18 plays an important role in MDA-MB-231 LM2 cell proliferation and migration. (A) Five thousand cells were seeded into each well of 96-well plates for MTT assay to indirectly monitor cell proliferation up to 72 h. Data are presented as mean ± SD (n=8). * $p < 0.05$, compared to the GFP control cells. (B) Knockout of ARHGAP18 significantly affects the wound healing capability of MDA-MB-231 LM2 GFP cells. Scale bar = 100 μm.

A.



B.



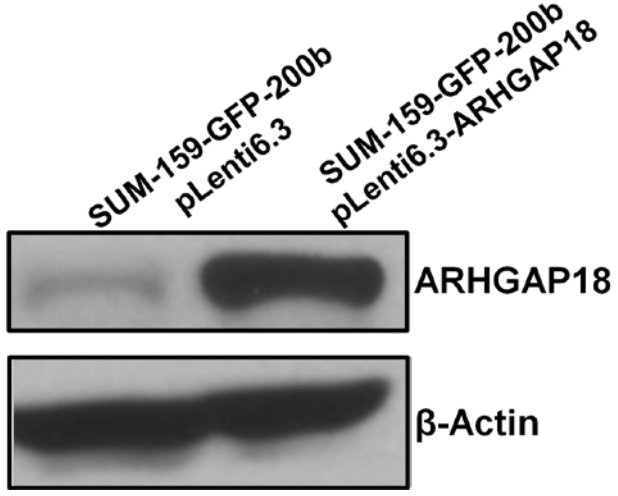
significantly decreased cell proliferation **(Figure 3.14.A and 3.15.A)**, and enforced expression of ARHGAP18 in knockout cells increases cell proliferation (**Figure 3.14.A and 3.15.A)** of both TNBC cell lines. As seen previously with ARHGAP18 knockdown using siRNA, CRISPR knockout of ARHGAP18 significantly increases RhoA activation, and enforced expression of ARHGAP18 in the knockout cells significantly reduces RhoA activation (**Figure 3.10.B and 3.11.B**). Furthermore, ARHGAP18 knockout decreases lamellipodia formation, but increases stress fiber and focal adhesion formation (**Figure 3.10.C and 3.11.C**). Concurrent with its effects on RhoA activity and actin cytoskeleton reorganization, ARHGAP18 knockout significantly reduces TNBC cell migration (**Figure 3.10.D and 3.11.D, Figure 3.14.B and 3.15.B)**; however, enforced expression of ARHGAP18 in the knockout cells reverses this phenotype (**Figure 3.10.D and 3.11.D**). This demonstrates that ARHGAP18 plays an important role in regulating actin cytoskeleton reorganization and promoting TNBC cell migration.

OVEREXPRESSION OF ARHGAP18 IN MIR-200B STABLY EXPRESSING CELLS OVERCOMES THE INHIBITORY EFFECT OF MIR-200B ON METASTASIS

We next wanted to determine whether expression of ARHGAP18 can overcome the inhibitory role of miR-200b on TNBC cell migration and tumor metastasis. We first enforced the expression of ARHGAP18 in miR-200b stably expressing cells and generated miR-200b-ARHGAP18 double-stably expressing cells. Enforced expression of ARHGAP18 in MDA-MB-231-GFP-200b and SUM-159-GFP-200b cells was confirmed by Western blot (**Figure 3.16.A and 3.17.A**), and the overexpression of ARHGAP18 does not affect the protein levels of E-cadherin, ZEB1, and PKCα (**Figure 3.13.**). ARHGAP18 overexpression does not significantly affect cellular proliferation of miR-200b stably expressing cells (**Figure 3.18.**). However, enforced ARHGAP18 expression significantly reduces RhoA activation (**Figure 3.16.B and**

Figure 3.16. Overexpression of ARHGAP18 in miR-200b stable expressing cells reduces RhoA activation and increases cell migration in SUM-159 cells. (A) Western blot analysis of ARHGAP18 expression levels in SUM-159-GFP-200b pLenti6.3 control and ARHGAP18 overexpression cells. **(B)** Representative immunofluorescent overlaid images of phalloidin (red), vinculin (green), and nuclear DAPI (blue) comparing SUM-159-GFP-200b pLenti6.3 control and ARHGAP18 overexpression cells. Scale bar = 25μm. Images were taken at 600X. **(C)** RhoA-GTP pulldown in SUM-159-GFP-200b pLenti6.3 control and ARHGAP18 overexpression cells. RhoA-GTP were quantified by ImageJ and are shown as RhoA-GTP levels divided by the corresponding total RhoA levels (mean ± SD, $n = 3$) relative to that of control cells. * $p < 0.05$, compared with parental group. **(D)** Wound healing assay of SUM-159-GFP-200b pLenti6.3 control and ARHGAP18 overexpression cells. Scale bar = 100 μm.

A.



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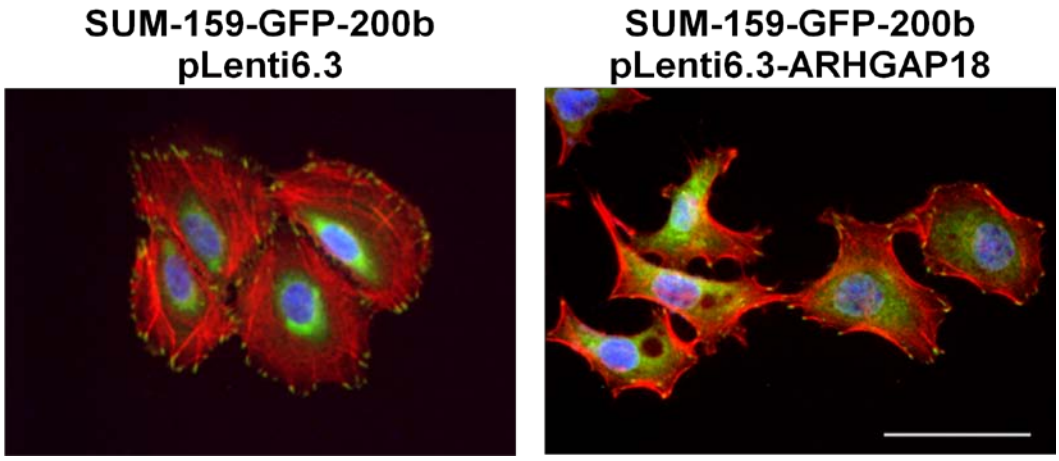
B.



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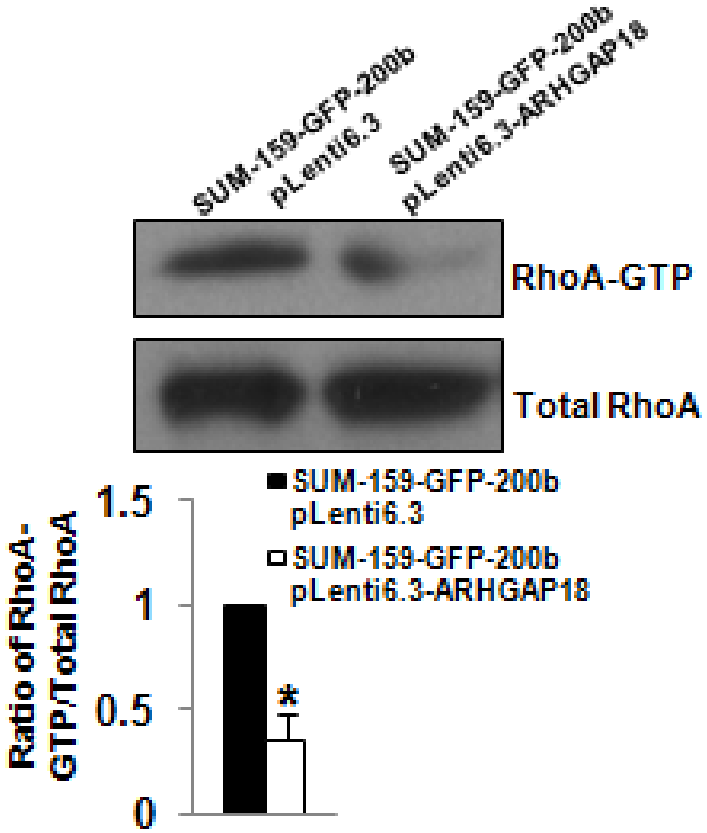

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D.

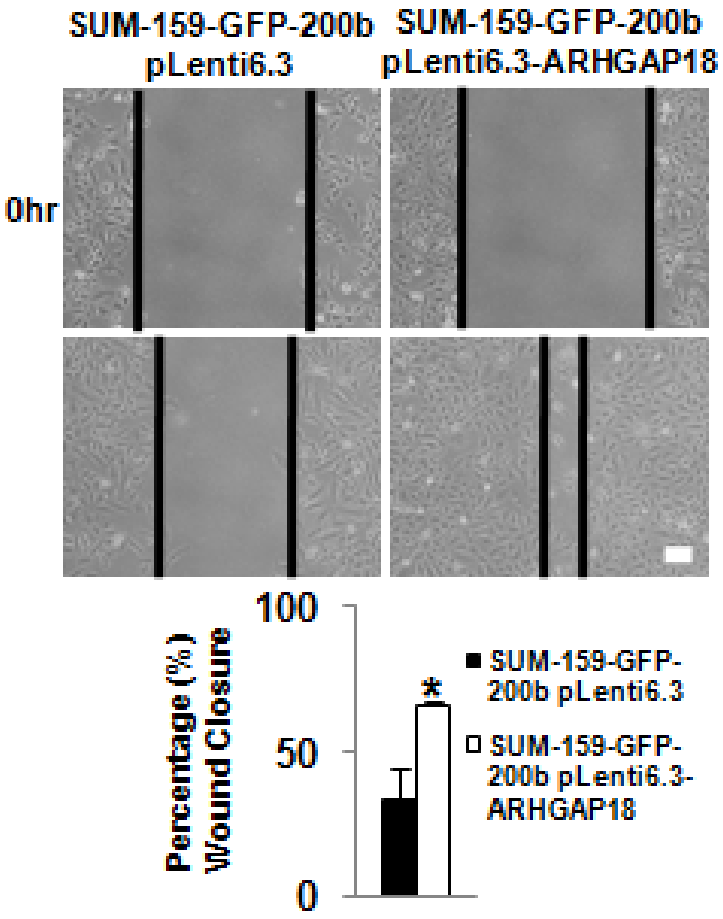


Figure 3.17. Overexpression of ARHGAP18 in miR-200b stable expressing cells reduces RhoA activation and increases cell migration in MDA-MB-231 cells. (A) Western blot analysis of ARHGAP18 expression levels in MDA-MB-231-GFP-200b pLenti6.3 control and ARHGAP18 overexpression cells. **(B)** Representative immunofluorescent overlaid images of phalloidin (red), vinculin (green), and nuclear DAPI (blue) comparing MDA-MB-231-GFP-200b pLenti6.3 control and ARHGAP18 overexpression cells. Scale bar = 25μm. Images were taken at 600X. **(C)** RhoA-GTP pulldown in MDA-MB-231-GFP-200b pLenti6.3 control and ARHGAP18 overexpression cells. RhoA-GTP were quantified by ImageJ and are shown as RhoA-GTP levels divided by the corresponding total RhoA levels (mean ± SD, $n = 3$) relative to that of control cells. * $p < 0.05$, compared with parental group. **(D)** Wound healing assay of MDA-MB-231-GFP-200b pLenti6.3 control and ARHGAP18 overexpression cells. Scale bar = 100 μm.

A.

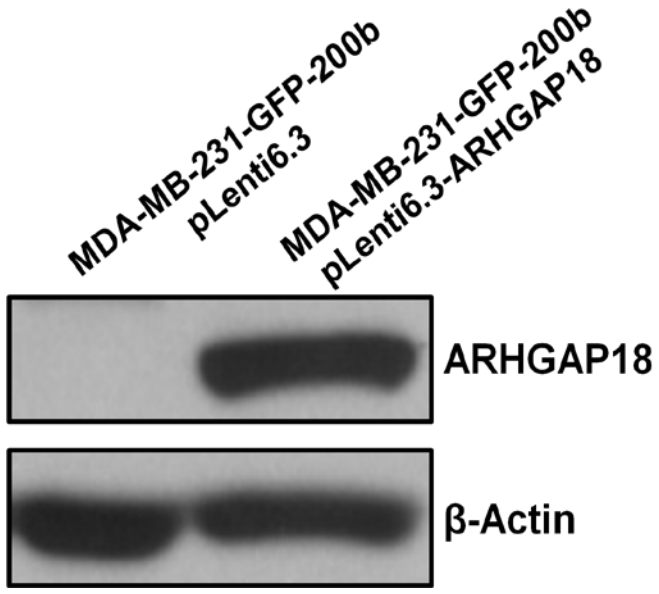
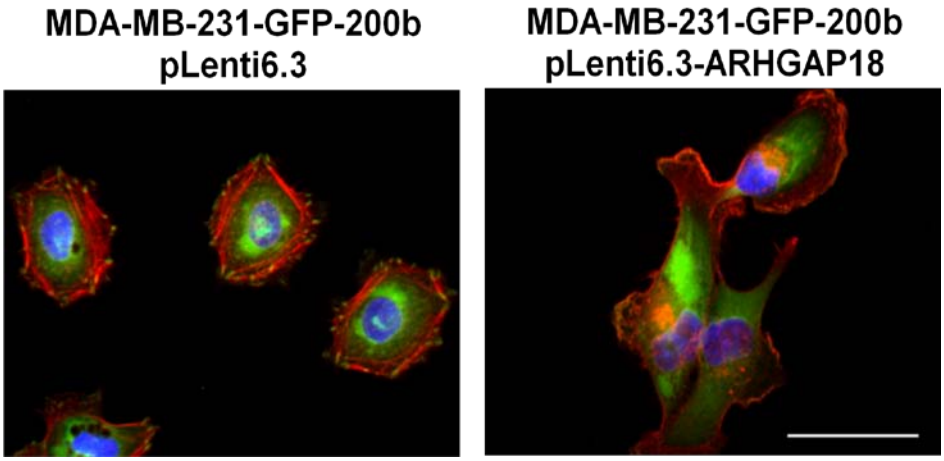


Figure 3.17. (cont'd)

B.



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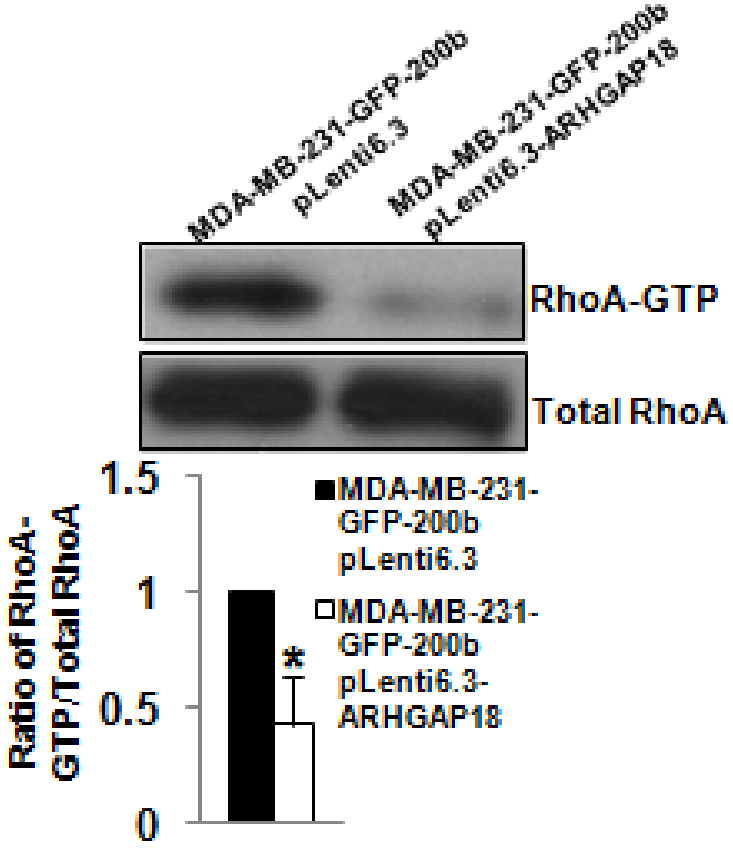

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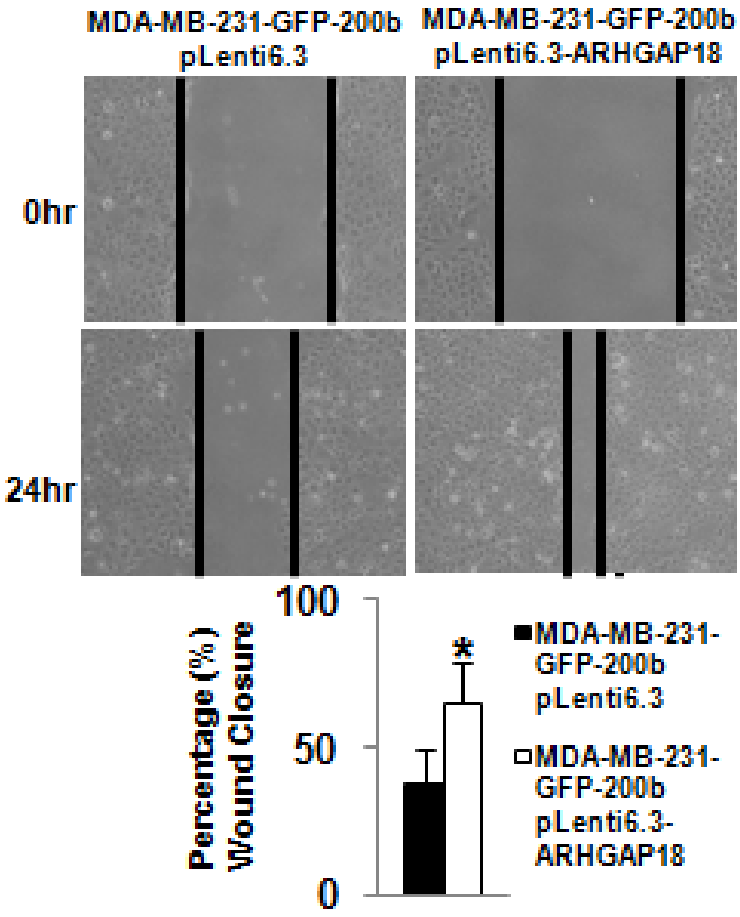
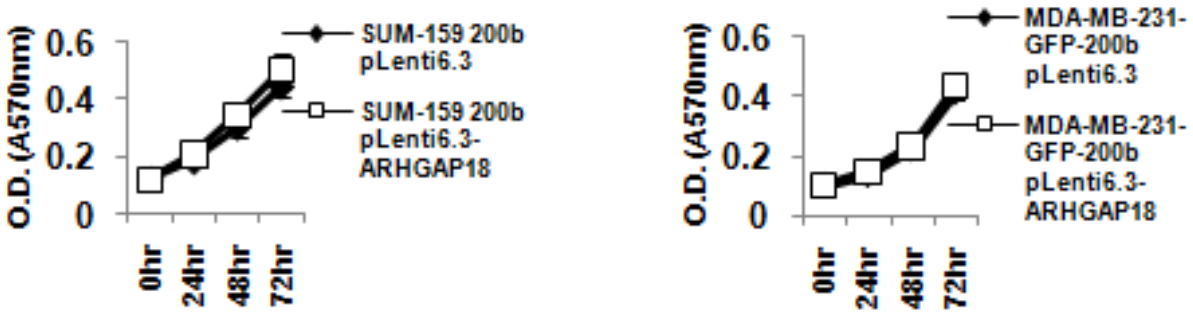


Figure 3.18. ARHGAP18 overexpression has no effect on miR-200b stable expression cell proliferation. (A) Three or Five thousand cells were seeded into each well of 96-well plates for MTT assay to indirectly monitor cell proliferation up to 72 h. Data are presented as mean ± SD (n=8).



3.17.B), stress fiber and focal adhesion formation (**Figure 8C and 9C**), and significantly increase cell migration in miR-200b stably expressing TNBC cells (**Figure 3.16.D and 3.17.D**).

Previous work in our lab has shown that stable expression of miR-200b in the metastatic MDA-MB-231-GFP cells is able to block lung metastasis in a mouse mammary xenograft tumor model (12). To investigate whether forced expression of ARHGAP18 is able to overcome the inhibitory effect of miR-200b on tumor metastasis, vector control and miR-200b-ARHGAP18 double-stably expressing cells were injected into the fourth mouse mammary fat pad. Overall, 4/5 mice injected with pLenti6.3 vector control and 6/6 mice injected with miR-200b-ARHGAP18 double-stably expressing cells had primary tumors. It was found that overexpression of ARHGAP18 has no significant effect on primary tumor growth. All mice injected with vector control cells had no observable micrometastases in the lungs, whereas all mice injected with miR-200b-ARHGAP18 double-stably expressing cells had micrometastatic foci as revealed by H&E staining and GFP immunofluorescence staining **(Figure 3.19.A and B)**. Therefore, these results show that enforced ARHGAP18 expression is able to overcome the inhibitory effect of miR-200b on TNBC lung metastasis.

Figure 3.19. Forced expression of ARHGAP18 impairs the inhibitory effect of miR-200b on tumor metastasis. (A) Representative images of H&E staining of lung sections from mice with mammary fat pad injection of MDA-MB-231-GFP-200b-pLenti6.3 or MDA-MB-231-GFP-200b-pLenti6.3-ARHGAP18 cells. Scale bar = 100 μm. (**B**) Representative overlaid images of immunofluorescence staining of GFP (red color) with nuclear DAPI staining (blue color) in lung sections from mice with mammary fat pad injection of MDA-MB-231-GFP-200b-pLenti6.3 or MDA-MB-231-GFP-200b-pLenti6.3-ARHGAP18 cells. Scale bar = 50 μm.

A.

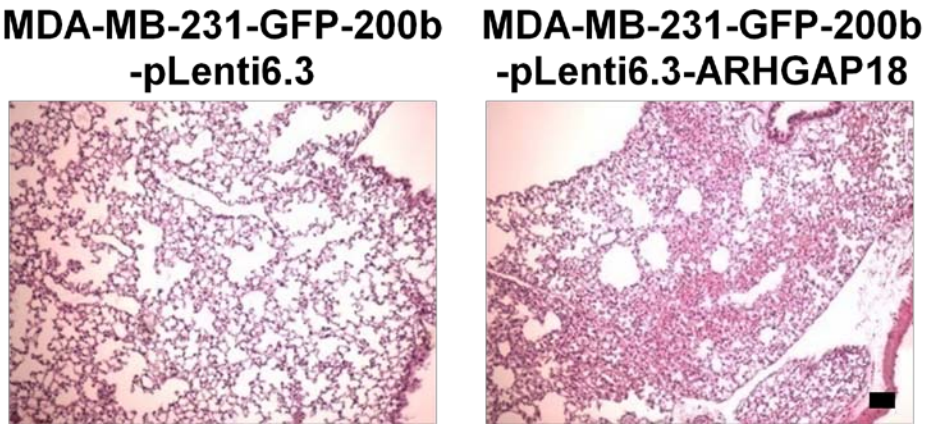
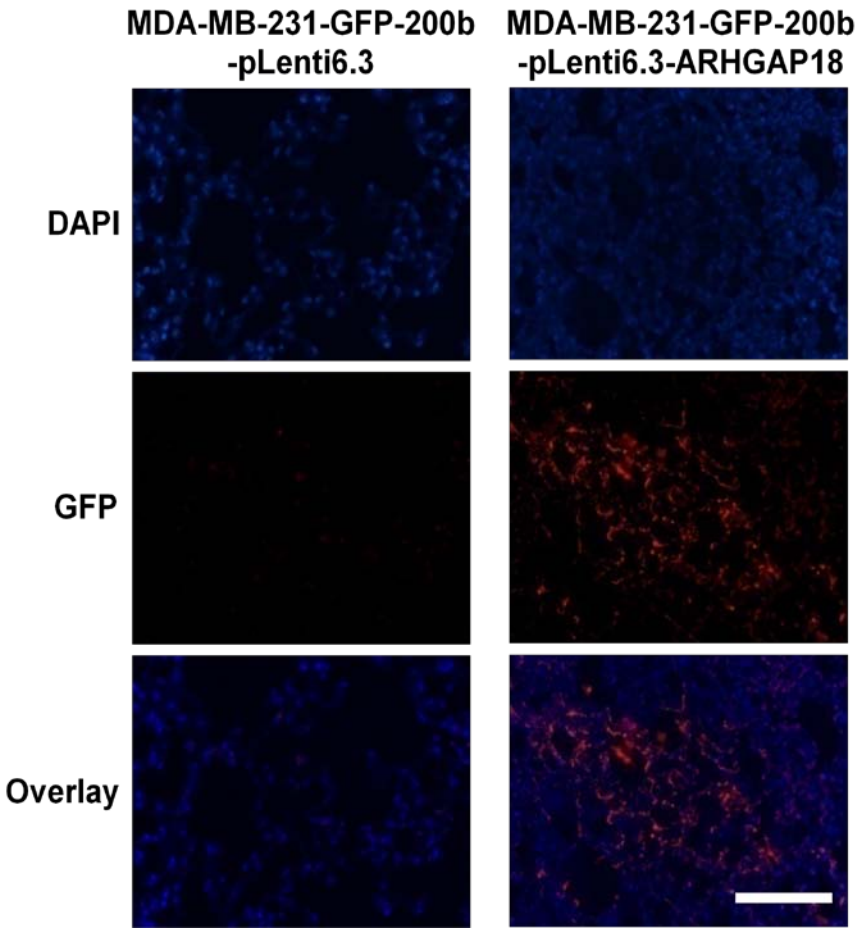


Figure 3.19. (cont'd)

B.



DISCUSSION

This study was performed to build upon our previous work that showed that miR-200b inhibits triple negative breast cancer (TNBC) metastasis. Our further studies into the mechanism behind how miR-200b inhibits TNBC metastasis revealed that an increase in RhoA activation is critical to the inhibitory effect of miR-200b. This increase in RhoA activation was shown to be because of the ability of miR-200b to directly target and decrease the expression of Rho GTPase activating protein 18 (ARHGAP18). The subsequent constitutive activation of RhoA also led to an increase of focal adhesions due to an inefficient focal adhesion turnover system, which greatly contributes to the reduced ability of these miR-200b cells to migrate. Therefore, RhoA in TNBC acts as a tumor suppressor by inhibiting cell migration, and consequently ARHGAP18 displays a metastatic enhancer role. These data are important because they challenge the current paradigms about the roles of Rho GTPases and Rho GAPs in metastasis.

Our previous studies have shown that miR-200b stable expression in TNBC cells significantly inhibits TNBC cell migration. Here we extend these results to show that miR-200b does this by increasing RhoA activation. This is in contrast to a recent study which shows that miR-200b expression decreases RhoA activation, but has no effect on Rac1 activation (23). This could be due to different cell lines used in the study. As shown in the recent study, miR-200b also targets multiple GEFs, Rho GTPase activators, in addition to several GAPs (23). The net effect of miR-200b on the activity of Rho GTPases may depend on its effect on differentially expressed GEFs and GAPs in different cell lines. Moreover, despite many reports suggesting crosstalk between Rac1 and RhoA (20,21), we did not find a bidirectional crosstalk. We found that increased RhoA activation reduces Rac1 activation, but reduced Rac1 activation has no effect on RhoA activation in TNBC cells. This unidirectionality has been reported previously in

fibroblast cells (24), but to our knowledge has not been seen in epithelial cancer cells. However, it has been shown that expressing a constitutively active mutant of either Rac1 or RhoA can increase Rho GTPase activity without altering the activity of the other Rho GTPase in glioblastoma cells (25), which suggests that it can occur in epithelial cancer cells. In our TNBC cells it is likely that mutations exist within the downstream effector proteins between Rac1 and RhoA in these cancer cells, or that in these TNBC cells Rac1 does not signal to RhoA. Further mechanistic studies revealed that the RhoA activation seen in miR-200b stably expressing cells is due to miR-200b directly targeting Rho GTPase activating protein 18 (ARHGAP18).

Rho GTPase activating protein 18 (ARHGAP18) is known as a RhoA-specific GAP (26,27), and previous studies have shown that ARHGAP18 expression is high in MDA-MB-231 TNBC cells (26). We have extended upon the results of this study to include all of the breast cancer subtypes, and found that high ARHGAP18 expression is specific to the highly migratory and invasive TNBC cells. Concurrent with previous studies (26,27), we also found that ARHGAP18 regulates RhoA activation. However, our mechanistic studies oppose the current model that Rho GAPs act as tumor suppressors in cancer. We demonstrated for the first time that knockdown or knockout of ARHGAP18 reduces TNBC cell migration and metastasis, and overexpression of ARHGAP18 back into these knockout cells rescues this phenotype. Moreover, our bioinformatic analysis also indicates that a higher level of ARHGAP18 expression is significantly associated with a much worse DMFS and recurrence of breast cancer. This suggests ARHGAP18 as a critical positive regulator of TNBC cell migration and tumor metastasis and highlights its potential as a therapeutic for aggressive TNBC.

Previous knowledge of the oncogenic role of RhoA typically came from using dominant-negative or constitutively active mutant expression approaches (8,28). However with recent

advances, evidence has shown that RhoA mutations are a major hallmark of angioimmunoblastic T-cell lymphoma (AITL) and peripheral T-cell lymphoma (PTCL) (9,10). Furthermore, it has recently been shown that gain-of-function mutations are highly recurrent in diffuse gastric cancer (29,30). These studies suggest that RhoA activity plays a tumor suppressive role in these diseases. In support of this, here we show that inhibition of RhoA signaling increased cell migration and metastasis of miR-200b stably expressing cells. This illustrates that miR-200b-dependent inhibition of TNBC migration and metastasis is dependent upon increased RhoA activation. One potential explanation for this may be the ability of miR-200b to act on focal adhesions through enhancing RhoA activation. Focal adhesion turnover is a critical part to cell migration, and is regulated by the dynamic activation and inactivation of RhoA (31-34). Previous studies have shown that RhoA inactivation is necessary for efficient focal adhesion turnover (35), however miR-200b stably expressing cells have constitutively active RhoA. Therefore, these cells have a high number of focal adhesions that cannot be efficiently turned over. This inability for the cells to turnover focal adhesions is largely due to miR-200b targeting ARHGAP18 causing RhoA constitutive activation. This highly activated RhoA displays a strong inhibitory effect on TNBC cell migration and tumor metastasis. Our data is supported with previous studies showing that a decrease in RhoA activation is critical for cancer cells to undergo epithelial-to-mesenchymal transition (EMT) and migrate away from the primary tumor (36,37).

Early studies suggested that Rho GTPases are oncogenic drivers of metastasis. Due to the ability of GAPs to promote GTPases to hydrolyze GTP to GDP and put them into an “off” state, GAPs are regarded as tumor suppressors (38). However, a recent study has shown that GAPs can instead function as oncogenes in cancer (39), and the data presented here further supports this study. Moreover, we also emphasize the tumor suppressive role that the miR-200 family plays in

TNBC by targeting the metastatic enhancer ARHGAP18. Furthermore contrary to the currently existing paradigm, we show that highly activated RhoA plays an important role in the inhibitory effect of miR-200b on TNBC cell migration and tumor metastasis likely by regulating focal adhesion turnover.

ACKNOWLEDGEMENTS

We thank Dr Suyun Huang (The University of Texas M.D. Anderson Cancer Center, Houston, TX) for providing MDA-MB-231 cells; Dr Stephen Ethier (Wayne State University, Detroit, MI) for providing SUM-149 and SUM-159 cells; Dr Sandra O'Reilly (Department of Physiology, Michigan State University, East Lansing, MI) for excellent help with mouse injections; and Amy Porter and Kathy Joseph (Division of Human Pathology, Michigan State University, East Lansing, MI) for excellent help with slide preparation.

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CHAPTER 4: SUMMARY AND CONCLUSIONS

Authors who contributed towards this were: Brock Humphries and Chengfeng Yang.

SPECIFIC AIMS AND RESULTS OF THE STUDY

The aim of this study was to investigate the role of the miR-200 family in triple negative breast cancer (TNBC) metastasis. It was hypothesized that the miR-200 family plays an inhibitory role on TNBC metastasis, and therefore by elucidating the molecular mechanism behind miR-200 inhibition of TNBC metastasis, novel therapeutic options will be discovered. We focused on the role of miR-200b because few studies have been done on the effect of individual miR-200 family members on cancer metastasis. A summary of the specific aims and the obtained results are shown below:

SPECIFIC AIM 1: To determine the expression of the miR-200 family in breast cancer and to elucidate the effect of miR-200b re-expression on cell migration and metastasis of triple negative breast cancer.

RESULTS:

1. miR-200 family expression was significantly reduced in the highly migratory, mesenchymal-like triple negative breast cancer cells (TNBC) and metastatic TNBC primary breast tumors.
2. miR-200 family expression was inversely correlated with TNBC cell migratory capability.
3. Stable expression of miR-200b in highly migratory TNBC cells inhibited cell migration.
4. Stable expression of miR-200b in highly migratory TNBC cells blocked lung metastasis.

SPECIFIC AIM 2: To determine the molecular mechanism behind miR-200b inhibition on cell migration and metastasis in triple negative breast cancer.

SUBAIM 1: To study the mechanism behind miR-200b inhibition of Rac1 activity.

RESULTS:

1. **1.** miR-200b stable expression in TNBC cells caused a decrease in lamellipodia and Rac1 activation.
2. **2.** PKCα expression was found to be higher in the highly migratory TNBC cells, inversely correlated with miR-200b expression, and identified as a direct target of miR-200b in TNBC cells.
3. **3.** Pharmacological inhibition of PKCα or PKCα knockdown inhibited TNBC cell migration and decreased Rac1 activation.
4. **4.** Enforced PKCα expression was able to overcome the inhibitory effect of miR-200b on cell migration and increased Rac1 activation.
5. **5.** Enforced PKCα in miR-200b stably expressing cells was able to overcome the inhibitory effect of miR-200b on TNBC lung metastasis.

SUBAIM 2: To study the molecular mechanism of RhoA activation by miR-200b.

RESULTS:

1. **1.** miR-200b stable expression in TNBC cells caused an increase in stress fibers, focal adhesion formation, and RhoA activation.
2. **2.** miR-200b stable expression inhibits TNBC focal adhesion turnover
3. **3.** Pharmacological inhibition of RhoA signaling in miR-200b stably expressing cells caused an increase in cell migration and an increase in Rac1 activation.
4. **4.** High ARHGAP18 expression is correlated with worse distant metastasis free and

recurrence free survival, and was found to be higher in the highly migratory TNBC cells.

5. ARHGAP18 expression was found to be inversely correlated with miR-200b expression and identified as a direct target of miR-200b in TNBC cells.

6. ARHGAP18 knockdown or CRISPR knockout increased stress fiber and focal adhesion formation, inhibited TNBC cell migration, and increased RhoA activation.

7. Enforced ARHGAP18 expression in CRISPR/Cas9 knockout cells rescues the ARHGAP18 knockout phenotype.

8. Enforced ARHGAP18 expression in miR-200b stably expressing cells reduced RhoA activation and can overcome the inhibitory effect of miR-200b on cell migration and lung metastasis.

LIMITATIONS OF THE STUDY

Although many positives can be taken from this study, there are some limitations to the study that must be considered:

1. Mouse mammary xenograft tumor models were used in this study using human cells. Since these studies were not done in humans, it does not fully recapitulate the human tumor microenvironment.

2. The CRISPR/Cas9 technology is becoming more mainstream in current research. However, there is no true control for CRISPR/Cas9. Re-expressing the knocked out protein back into the cells can serve as a control to show how important the protein is for the cellular function being studied. However, that does not account for potential off-target effects caused by using the CRISPR/Cas9 technology.

3. The selection process for CRISPR/Cas9 results in single cells and subsequently leads to clonal expansion. This means that the cell population that is used for the subsequent experiments comes from a single cell. Therefore, it is possible that the effects that are seen here are due to a single cell and are not representative of the population. To overcome this, multiple clones were screened and assayed. Since the clones all showed similar trends in many different cellular processes, we believe that a single clone can represent the whole population and the function of the protein being studied here.

4. The role that the miR-200 family plays in cancer metastasis remains controversial. This study suggests that the miR-200 family, in particular miR-200b, plays an inhibitory role on TNBC metastasis. However, the inhibitory role that miR-200b plays in breast cancer cannot be generalized to all cancers because other studies

have shown the dual role that the miR-200 family members play. Therefore, the role that miR-200b plays in cancer metastasis may be cell-type specific and more research is necessary to elucidate its role in each step of the metastatic cascade of different cancers.

STUDY OUTCOME

This dissertation investigated the role of miR-200b in triple negative breast cancer. Two individual studies in this project elucidated the molecular mechanism behind the inhibitory effect of miR-200b on triple negative breast cancer cell migration and metastasis.

Although further studies are needed to fully elucidate the molecular mechanism of miR-200b, we have preliminarily shown here that miR-200b expression plays an important role in blocking cancer cell migration and metastasis. It was found in these studies that miR-200b plays an inhibitory role in TNBC by regulating the actin cytoskeleton. Rac1 and RhoA activation were shown to be modulated upon stable expression of miR-200b in TNBC cells. Since Rac1 and RhoA are critical signaling nodes in all cancers, our findings could have a broader impact in terms of cancer treatment.

Early studies found that re-expression of miR-200b in TNBC cells resulted in decreased Rac1 activity. This decrease in Rac1 activity was concurrent with a dramatic reorganization of the actin cytoskeleton, with the formation of stress fibers and the loss of lamellipodia, and a decrease in cell migration. Further analysis revealed that PKCα is a direct target of miR-200b and is an upstream regulator of Rac1 activation. This is important because Rac1 expression has been shown to promote the metastatic process, and therefore has recently been of interest as a promising therapeutic target for the treatment of cancer angiogenesis and metastasis (1). However, targeting Rac1 has been shown to be difficult and have negative side effects. It is possible that by targeting the upstream effectors of Rac1, such as PKCα, it could overcome the difficulties of treating Rac1 directly and may be a viable treatment for cancer metastasis. Furthermore, other recent papers have suggested that PKCα is critical for breast cancer signaling

and breast cancer stem cells (2), and therefore PKCα treatment may be specific to breast cancer cells.

In addition to decreased Rac1 activity, miR-200b stable expression in TNBC cells also caused an increase in RhoA activation. Preliminary results showed that pharmacological inhibition of RhoA signaling increased cell migration in miR-200b stably expressing cells. This not only highlights the importance of RhoA signaling in TNBC cell migration, but also shows that miR-200b-dependent inhibition of TNBC metastasis is partly due to increased RhoA activation. It was also shown that pharmacological inhibition of RhoA signaling resulted in an increase in Rac1 activation and lamellipodia formation. Although previous studies have shown that crosstalk exists between Rac1 and RhoA, pharmacological inhibition of Rac1 did not result in an increase in RhoA activation. Possible explanations for this are that mutations exist within the crosstalk proteins between Rac1 and RhoA in these cancer cells, or that in these TNBC cells Rac1 does not signal to RhoA. ARHGAP18 was subsequently identified as a direct target of miR-200b, and as a RhoA-specific GTPase activating protein (GAP). ARHGAP18 knockout resulted in decreased cell migration and metastasis, and overexpression of ARHGAP18 in miR-200b stably expressing cells was able to overcome the inhibitory effect of miR-200b on TNBC metastasis.

Many studies have suggested that activation or overexpression of RhoA results in progression of cancer and leads to metastasis in different cancers (3-6). However, our results indicate that RhoA activation is important for miR-200b inhibition of TNBC metastasis. One potential explanation for this may be the ability of miR-200b to act on focal adhesions through enhancing RhoA activation. Focal adhesion turnover is crucial to cell migration, and is regulated by the dynamic activation and inactivation of RhoA (7-10). Previous studies have shown that

RhoA inactivation is necessary for efficient focal adhesion turnover (11). However, here we show that miR-200b stably expressing cells have constitutively active RhoA. Therefore, these cells have a high number of focal adhesions that cannot be efficiently turned over. This inability for the cells to turnover focal adhesions is largely due to miR-200b targeting ARHGAP18, which results in RhoA constitutive activation. This highly activated RhoA displays a strong inhibitory effect on TNBC cell migration and tumor metastasis. Our data is supported with previous studies showing that a decrease in RhoA activation is critical for cancer cells to undergo epithelial-to-mesenchymal transition (EMT) and migrate away from the primary tumor (12,13). Therefore, ARHGAP18 may be a potential therapeutic target in aggressive cancers that show decreased RhoA activation.

We also show here that pharmacological inhibition of RhoA signaling promotes cell migration. This can be explained by the crosstalk that exists between Rac1 and RhoA. Decreased RhoA signaling resulted in increased Rac1 activation, and increased Rac1 activation can lead to increased cell migration (14,15). RhoA inhibition, using Y27632, has been proposed to be a potential therapeutic target to control tumor cell invasion and metastasis (16), however we show here that this may promote metastasis by increasing cancer cell migration. Therefore, the therapeutic potential of Y27632 must be elucidated for different cancers, and may not prove useful for aggressive TNBC.

Therefore the data presented here suggest that both PKCα and ARHGAP18 altered TNBC cell migration by regulating the critical actin cytoskeleton regulators Rac1 and RhoA. This suggests that PKCα and ARHGAP18 may be promising therapeutics for the treatment of aggressive TNBC. Our data also challenges the current paradigm which suggests that Rho GTPase activation promotes metastasis, and Rho GAPs act as tumor suppressors.

FUTURE EXPERIMENTS

Some of the future experiments will need to further characterize the role of miR-200b in TNBC as well as other cancers. In particular, we show here that miR-200b plays a critical role as a negative regulator of TNBC proliferation, migration, invasion, and metastasis. However, we only show two of the critical regulators of these processes, PKCα and ARHGAP18. Individual microRNAs have each been hypothesized to target hundreds of different proteins within the cell (17), and as a result it is very likely that miR-200b targets more proteins that are involved in regulating these cellular processes in TNBC. Therefore, future studies will need to elucidate these other miR-200b-target proteins and their functions on TNBC. Moreover, the effect that miR-200b has on the later steps of metastasis has not yet been studied. Future studies will therefore need to confirm what is shown here and determine the specific role of miR-200b in each of the later steps, i.e. extravasation and colonization, of the metastatic cascade. In order to determine the effect on the later steps of the metastatic cascade, a tail vein injection can be performed.

The role that the miR-200 family plays in cancer metastasis remains to be controversial. Even though miR-200b was shown to be a potential tumor suppressor, other miR-200 family members, such as miR-200c, have been shown to promote cancer metastasis and be associated with poorer patient outcome in clinical patients (18-20). This highlights the potential dual role that this family plays in cancer. We showed here that miR-200b expression is significantly decreased in TNBC, but the clinical prognosis for these patients was not characterized. Furthermore, recently it was shown that miR-200b promotes cell proliferation, invasion, survival, and silencing miR-200b inhibits tumor growth *in vivo* in cancer (21-23). Therefore,

results shown here may be cell-type specific, and more research is needed into the role of miR-200b in other cancer types.

Many of the current studies on the miR-200 family focus specifically on cancer cell lines that express extremely high or low levels of the miR-200 family. These studies either knockout or re-express the miR-200 family back into these cells and study the effect the miR-200 family has on certain cellular functions. However, no study has determined the effect that the miR-200 knockout has on non-metastatic cells. Using miRZip lentivector-based anti-microRNA technology, individual miR-200 family members can be efficiently knocked out and then the effect on metastasis can be assayed in non-metastatic cells. This will further solidify the role that the members of the miR-200 family play in metastasis.

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