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Brms1 Coordinately Regulates Microrna To Suppress Breast

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BRMS1 COORDINATELY REGULATES MICRORNA TO SUPPRESS BREAST CANCER METASTASIS: THE EMERGENCE OF METASTAMIR

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ABSTRACT

The majority of cancer related mortality is attributed to complications associated with metastatic disease. Breast cancer metastasis suppressor 1 (BRMS1) suppresses metastasis of multiple cancer types in vivo and loss of nuclear BRMS1 is associated with ER-negative cancers and a high rate of proliferation. Many groups have shown BRMS1 to regulate the expression of multiple metastatic genes, yet until now no one has been able to account for how these many changes in gene expression occur.

In this work, we report that BRMS1 regulates a select set of genes called microRNA (miRNA), and these miRNA themselves can regulate metastasis. Using multiple human metastatic breast cancer cell lines, we compared BRMS1 and vector treated cell lines for miRNA populations using miRNA arrays. Remarkably, BRMS1 changed a rather small subset of miRNA and these miRNA have been implicated by our group and others to regulate metastasis. BRMS1 decreased the prometastatic miRNA 10b, 373, and 520c, while increasing the metastasis suppressing miRNA 146a, 146b, and 335. To determine if these changes in miRNA actually contribute toward BRMS1 metastasis suppression, we restored miR-10b expression in cells expressing BRMS1 and observed increased cellular migration and invasion. Furthermore, ectopic expression of miR-146 in metastatic breast carcinoma cells suppressed migration, invasion, and metastasis.

In addition to BRMS1 regulating individual miRNA, we observe changes in polycistronic miRNA. The polycistronic miRNA cluster miR-183, -96, -182 increases in cells expressing BRMS1. Thus far, groups have reported both metastasis suppressing and metastasis promoting phenotypes for members of this cluster. We have found that knockdown of this cluster in BRMS1 expressing cells results in increased invasion and migration in metastatic breast cancer cells while over expression inhibits. Taken together, these findings provide further mechanistic insight for how BRMS1 suppresses metastasis, and we provide new possible therapeutic targets for late stage metastatic disease.

Key words: Metastasis, mircoRNA, BRMS1, metastamiR

DEDICATION

This work is dedicated to my God and Savior who has blessed me with a wife, family, and education that I am eternally grateful for. To my raya, ahava, and dode: Britta, thank you for your never ceasing love, support, and encouragement. You've walked with me step in step these past 5 years, and I could not have done this without you. To my girls Delainey and Madeline, I love you dearly as you've put life into perspective for me and I pray I can be the father you both deserve. To my parents Tim and LaRae, thank you for teaching me hard work and perseverance. To my bride's parents Tom and Merrie, thank you for treating me like a son. To my brothers and sister, I love you dearly and have missed you these past 5 years. Finally, to my new brother and sister Brooke and Kent, I am truly thankful that we've been able to start our families together these past three years and love you both for blessing me with two beautiful nephews who will grow up and protect their cousins.

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INTRODUCTION

Breast Cancer Metastasis

Breast cancer has been recognized and treated by medicine dating back to 3000 B.C. in the ancient civilizations of Eygpt and Mesopotamia (1). Today, it is estimated that there will be over 1.5 million new cancer cases and nearly 600 thousand deaths from cancer in the United States alone for 2010 (2). The cancer most likely to be diagnosed in men and women are prostate and breast and each are the second leading cause of cancer related deaths. Treatments of this disease started with the application of topical ointments and evolved to Halstead's 1903A.D. publication, on the radical mastectomy (3). At the time, this surgical treatment option offered the most dramatic treatment and cure option in history with a reported survival increase of 85% (4). With the continued advances in surgical oncology, preventative detection, radiation, and chemotherapy, the most common cancers for men and women are today very curable if the tumor remains in the tissue of origin (98% Female Breast and 100% Prostate)(2). With that said, survival becomes very unlikely if the tumor spreads to distant tissues (23% Breast, 31% Prostate) and metastatic disease develops (2).

We define metastasis here, as the discontiguous spread of malignant cancer cells and the establishment of new foci. Unlike classification of the primary tumor, which dates back to ancient Greece (*oncos* –oncology), the term "metastasis" was termed only in the recent modern era by Jean Claude Recamier in 1829 (5). The process of metastasis can be broken down into several steps and begins with tumorigenesis. Vascularization is required for tumors to exceed a 1-2-mm diameter and therefore the secretion of angiogeneic agents play a crucial role in establishing a vascular system within the

adjacent host tissue (6). Next, local invasion of the stroma by tumor cells takes place by the remodeling and degrading the extracellular matrix (reviewed in 16). Tumor cells may either invade individually or in groups, but eventually breach the basement membrane and gain access to the thin-walled venules and/or lymphatic vessels. While in transit through the vasculature, tumor cells often form cellular aggregates with platelet cells which protect from the physical stresses of such travel as well as mask the tumor cell from immune cells and preventing death (8,9).

Eventually, the tumor cells arrest in a capillary bed, simply because of their physical size, and either extravasate into the new tissue, or proliferate within the vessel and burst it (10). Regardless the mechanism, proliferation of the tumor cells within the new tissue results in new microscopic, and eventually macroscopic, foci of tumor cells.

Every step in metastasis is rate limiting, in that should one step not succeed, metastasis is prevented. The process is rather inefficient as for every gram of tumor sheds 1-4 million cancer cells daily, and yet very few cells successfully metastasize (11). Further, using radio-labeled tumor cells, Fidler revealed that the vast majority of the millions of cells shed do not survive the physical stresses offered in circulation (12).

Metastasis suppressors

Given that so few tumor cells succeed to form new foci, many investigators correctly hypothesized that there were distinct genetic requirements for a tumor cell to metastasize, and those events were unique to those required of tumorigenesis. This hypothesis would indicate that there are genes which both suppress and promote metastasis. The task to identify the genes that promote metastasis has been rather

daunting as metastasis requires many functions for completion; however many have been identified, genes such as RhoC, and H-ras to increase metastatic potential (13,14,).

Given that every step in metastasis is rate limiting, there has been much more success in identifying the genes that suppress metastasis. To distinguish these genes from tumor suppressors (which do prevent metastasis from occurring), metastasis suppressors are genes that suppress metastasis, without blocking primary tumor formation. Metastasis suppressors have been found to be nuclear, cytosolic, membrane bound, and extracellular (reviewed in 15). Their functions include cell adhesion, cell cell communication, signaling, cell invasion, transcriptional and translational regulation (15).

The most common approach to identify these genes has been to compare for loss of heterozygosity (LOH) and any anomalies in karyotype in the different stages of human cancers. Once regions are identified, microcell-mediated chromosomal transfer (MMCT) can be used to re-introduce single wildtype chromosomes thought to encode the wildtype metastasis suppressor(s). Thus far, this method had identified genes on chromosomes 1, 2, 7, 8, 10-13, 16, 17, and 20 to suppress metastasis (15,16). As technology advanced, other methods such as subtractive hybridization, differential display, comparative genomic hybridization (CGH), real-time RT-PCR, microarray, deep-sequencing and proteomic approaches have been employed to identify additional metastasis suppressors.

Breast Cancer Metastasis Suppressor 1 (BRMS1)

In 1998, Welch and Wei cataloged the differential gene/protein expression and chromosomal abnormalities breast carcinoma acquire for metastatic potential (17). Their work revealed karyotypic changes that commonly occur in early-stage breast cancer (8p, 13q, 16q, 17p, 17q, and19p), whereas others typically occur later in breast cancer

progression (1p, 1q, 3p, 6q, 7q, 11p, and 11q). Among the most common changes in both familial or sporadic breast carcinoma were losses of genetic material on chromosome 11q. Many regions across the q-arm of chromosome 11 have been associated with breast cancer progression and the most common are amplifications and deletions involving regions near 11q13 (18). Data suggested that there are many critical genes in this region: genes that could act as oncogenes, tumor suppressors, metastasis promoting, and metastasis suppressing genes. Int-2, hst, bcl-1, glutathione S-transferase, CCND1, and EMS-1, all map to this region (11q13) and all have been demonstrated to be amplified in breast cancer (17).

Based on these observations and high-frequency deletions involving 11q13-q14 in late-stage, metastatic breast carcinomas, Welch and Weissman tested the hypothesis that chromosome 11q encodes a metastasis suppressor gene. Using MMCT of chromosome 11 and analysis of differentially expressed genes in MDA-MB- 435 metastatic breast carcinoma cells, the metastasis suppressor BRMS1 was discovered (17, 18,). In bladder cancer, BRMS1 has been correlated to metastatic potential (19). BRMS1 has been shown to suppress experimental metastasis of melanoma, non small cell lung carcinoma, and ovarian carcinomas (20, 21, 58). Clinical studies with BRMS1 have been difficult to interpret with regard to correlating survival and metastasis (22-26). These contradictions of function may be due to the fact that most studies measured mRNA expression and not protein. Unlike tumor suppressors, metastasis suppressors are not only deactivated by mutation, but by epigenetic silencing, mis-localization, and other events that go beyond simple cellular presence or absence of the protein (15). Frolova and colleagues recently demonstrated that BRMS1 was relatively equal in patient tumor samples; yet, the cellular

localization of BRMS1differred between low and high survival groups (27). Low five year survival correlated with cytosolic BRMS1, and nuclear BRMS1 with increased survival. One would expect such a finding, given BRMS1 has been found to be associated with SIN3:HDAC complexes, for which primarily nuclear functions are known (51).

Samant and colleagues initially characterized the mechanisms by which BRMS1 suppressed metastasic of breast carcinoma cells (28). In order to do so, they employed many *in vitro* surrogate assays of metastasis. Though metastasis can only be assessed *in vivo*, *in vitro* assays allow for determining possible step(s) a given gene may be acting in the metastatic cascade. To that end, BRMS1 decreased motility, the ability to grow in soft agar, and restored the ability to form functional homotypic gap junctions. Consistent differences were not observed for adhesion to extracellular matrix components (laminin, fibronectin, type IV collagen, type I collagen, Matrigel); growth rates *in vitro* or *in vivo*; expression of matrix metalloproteinases, or heparanase. Neither did BRMS1 expression up regulate expression of other metastasis suppressors. Others have studied the kinetics of BRMS1-expressing cells and found that BRMS1 causes fewer tumor cells to disseminate, and upon arriving at the secondary site they do not form macroscopic masses (29). Taken together, these data suggest that BRMS1 suppresses metastasis of human breast carcinoma by acting at multiple steps in the metastatic cascade and by some complex mechanisms.

In order to address these drastic changes in phenotype, many have sought to determine the molecular changes induced by BRMS1. As expected, re-expression of BRMS1 in metastatic breast carcinoma elicits the change of many genes/pathways

previously implicated in cancer progression. In summary, BRMS1 selectively down-regulates the phosphoinositide phosphatidylinositol (4,5) bisphosphate (PtdIns(4,5)P2) (30), downregulates osteopontin (31,32), inhibits NFκ B-mediated transcription (32), and inhibits EGFR signaling (33,51).

Additionally, several other groups, including ours, have performed both proteomic and transcriptomic analysis of BRMS re-expressing cells and have demonstrated hundreds of genes changes (34-36). Yet, despite these many changes in gene expression, the molecular mechanism(s) by which BRMS1 suppresses metastasis are still unknown.

Focus of Dissertation

The dissertation studies described here set out to determine if BRMS1 affects a set of genes called mircoRNA (miRNA). miRNA will be extensively discussed in the next chapter, but briefly, these non-coding, endogenous RNA molecules have been shown to regulate key processes and are often dysregulated in human cancer. miRNA were originally identified in *C.elegans* as a small non-coding-RNA (sncRNA let-7) which targeted lin-27 (54). For 15 years the gene that targeted lin-27 had been a critical missing piece in the *C.elegan* developmental timing pathway. The fact that let-7 was non-coding and anti-sense to its target was a novel discovery; it was thought to be an interesting molecular phenomenon unique to lower eukaryotes. That is until 2000, when three groups published three articles in the same issue of *Science* all demonstrating that sncRNA, now termed miRNA, was a conserved gene class in all eukaryotic organisms (55-57). Since

their “rediscovery,” there are now over 10,000 miRNA and they have been implicated in nearly every cellular process.

Just two years after their discovery in mammalian cells, miR-15a and miR-16-1 were the first miRNA described to facilitate cancer progression (37). Expression of these miRNA is inversely correlated to Bcl2 expression in CLL and both miRNA negatively regulate Bcl2 at a posttranscriptional level. Since then, hundreds of other miRNA have been shown to both promote and suppress tumorigenesis. Given the role of this new gene class in the development of the primary tumor, we hypothesized that BRMS1 changed mature miRNA expression, and that these miRNA changes were, in part, responsible for BRMS1 metastasis suppression.

This dissertation is important for two reasons. First, no one has shown metastasis suppressors to affect miRNA expression. Secondly, changes in miRNA may provide therapeutic targets for the treatment of metastatic breast cancer, for which few options exist.

LINKING METASTASIS SUPPRESSION WITH METASTAMIR REGULATION

by

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Abbreviations:

BRMS1, Breast Cancer Metastasis Suppressor1;

HDAC, histone deacetylase;

EGFR, epidermal growth factor receptor;

miRNA, microRNA;

miR, microRNA;

pri-miRNA, primary microRNA

pre-miRNA, premature microRNA

Abstract

Cancer metastasis requires the coordinate expression of multiple genes during every step of the metastatic cascade. Molecules that regulate these genetic programs have the potential to impact metastasis at multiple levels. BReast cancer Metastasis Suppressor 1 (BRMS1) suppresses metastasis by inhibiting multiple steps in the cascade through regulation of many protein-encoding, metastasis-associated genes as well as metastasis-regulatory microRNA, termed metastamiR. In this *Extra View*, we will highlight connections between BRMS1 biology and regulation of metastamiR.

Introduction

For cancer metastasis to develop, several processes must be completed, each requiring distinct, highly orchestrated genetic programs. Every step of metastasis is rate-limiting, meaning that when any step is blocked, a tumor cell cannot proceed to the next step. Metastasis suppressors are a group of gene products that, by definition, block metastasis but do not prevent primary tumor formation and may be targets for therapeutic intervention of the most deadly attribute of cancer cells [1-4]. The BRMS1 metastasis suppressor inhibits multiple steps of the metastatic cascade by regulating multiple metastasis-associated genes primarily through altered SIN3:histone deacetylase (HDAC) chromatin remodeling complexes [5-8]. Additionally, BRMS1 coordinately regulates multiple metastamiR (metastasis-associated microRNA), up-regulating metastasis-suppressing and down-regulating metastasis-promoting miRNA [9].

microRNA (miRNA) are an expanding class of non-coding RNA genes [10-12]. miRNA regulate gene expression, predominantly at a post-transcriptional level. miRNA genes are transcribed primarily by RNA polymerase II, and form a classic hairpin motif (pri-miRNA) while being transcribed. The hairpin is recognized and cleaved by the RNAse III Drosha in conjunction with the co-factor DiGeorge Syndrome critical region 8 (DGCR8) to generate the pre-miRNA that is exported to the cytoplasm. The enzyme Dicer and other ribonucleoproteins further process the hairpin into a mature ~22 nucleotide miRNA that is loaded onto RNA-induced silencing complexes (RISC) to target the 3' UTR of mRNA leading to regulation of translation [13, 14]. Given the small sequence of mature miRNA, a given miRNA can theoretically target hundreds of mRNA, thereby making them key regulators of a multitude of normal and pathological cellular

processes. Not long after their discovery in mammalian cells, miRNA dysregulation was reported in cancer. miR-15/16 were found to be frequently down-regulated, or deleted, in chronic lymphocytic leukemia [15]. Since then, miRNA have been shown to play both promoting and suppressing roles in most cancers (reviewed in [16]). More recently, several miRNA have been found to regulate the process of cancer metastasis independent of primary tumorigenesis [17].

Two recent publications prompted this *Extra View*. First, BRMS1 up-regulates the metastasis suppressive miR-146a/b expression and miR-146a/b alone can suppress breast cancer metastasis [18]. Second, BRMS1 coordinately regulates expression of multiple metastamiR [9]. In this *Extra View*, we will examine the known metastamiR targets of BRMS1 and how these actions coincide with the known biology of BRMS1.

BRMS1 regulates metastasis-associated genes

BRMS1 was discovered because microcell-mediated chromosomal transfer of chromosome 11 into metastatic breast cancer cells suppressed metastasis [19] . Following this observation differentially expressed genes were identified by differential display hybridization [20]. From those analyses, full-length BRMS1 was cloned. When BRMS1 was transfected into metastatic cells, orthotopic breast, melanoma, and non-small cell lung tumors were still able to grow but did not metastasize with the same efficiency [20-24]. With regard to biological mechanism, we and others have found that BRMS1 alters several components required for different steps in metastasis: restores gap junctional intercellular communication [25], promotes anoikis [22], decreases both growth in soft agar [20, 26] and migration/invasion [22, 23, 26]. At a molecular level, BRMS1 alters the transcriptome [27] and proteome [28, 29] when re-expressed. These changes include, but are not limited to:

epidermal growth factor receptor (EGFR; [8, 30]), osteopontin (OPN; [8, 31-33]), urokinase-type plasminogen activator (uPA; [34, 35]), C-X-C chemokine receptor 4 (CXCR4; [36]), and fascin [37]. These changes are believed to be primarily due to the interactions of BRMS1 with SIN3:HDAC chromatin remodeling complexes [5-8, 38]. Taken together, the working hypothesis for BRMS1 mechanism of metastasis suppression is as an epigenetic regulator of metastasis-associated gene expression.

MetastamiR – the roles of miRNA go beyond the primary

We hypothesized that in addition to metastasis-associated protein-coding genes being regulated by BRMS1, non-coding genes, including miRNA, would also be regulated by BRMS1. Using miRNA hybridization arrays, BRMS1 was found to regulate a subset of miRNA and several of these were already described metastamiR. Interestingly, those promoting metastasis were typically decreased while those that suppressed were generally increased [9].

Ma, Weinberg and colleagues were the first to discover and report the existence of a metastamiR, miR-10b [39]. miR-10b promoted tumor cell invasion *in vitro* and *in vivo* without affecting viability or proliferation. Importantly, metastasis was promoted *in vivo* (Note: Metastasis cannot be measured *in vitro*; only steps involved in metastasis can be modeled using *in vitro* assays.). Other metastasis-promoting metastamiRs have since been described with functional *in vivo* data including: miR-21, -143, -182, -373 and 520c (reviewed in [40]).

miR-143 and -182 promoted hepatocellular carcinoma and melanoma metastasis, respectively [41, 42]. miR-143 is up-regulated by NFκB and decreases cellular adhesion. miR-182 stimulates migration *in vitro* and is part of a cluster of microRNA located on

chromosome 7q31-q34 that includes miR-96 and miR-183 (miR-183-96-182). This region is frequently amplified in advanced human melanoma [43], further supporting the role of this cluster in aggressive behavior. Interestingly, this same cluster is associated with progressive hearing loss in humans [44, 45]. An important caveat to understanding the role(s) of miR-182 in controlling cancer cell behavior is that miRNA genes are often found in close proximity on chromosomes. miRNA clusters are genetically linked and frequently transcribed from a common promoter and generate polycistronic primary transcripts [46]. As a result, individual microRNA are expressed in a coordinated network, meaning that dissecting the roles of individual microRNA within a cluster is complicated by the co-expression of the others. So, while miR-182 has been individually examined, the miR-96 and -183 may also be involved in the cancers; however, to the best of our knowledge, no one has systematically studied individual and/or combinatorial functions for any of the clustered microRNA in a functional assay. We believe that such analyses will be critical to determining the role(s) of microRNA in cancer biology.

miR-21 promoted invasion and migration, while decreasing apoptosis. Transient knockdown of miR-21 with antagomir [47] significantly decreased experimental metastasis to lungs in breast and colorectal carcinoma cells [48]. miR-373 and -520c were identified via a high throughput screen of transduced non-metastatic MCF7 human breast cancer cells with a miRNA expression library. Transductants were screened using a transwell migration assay and both miR-373 and -520c emerged as promoters of *in vitro* migration and invasion [49]. Experimental metastasis assays further demonstrated that these were bona fide metastamiR as both increased metastases to multiple sites.

Another class of metastamiR are those that suppress metastasis. The metastasis suppressing metastamiR include: miR-31 [50], -146a/b [18], -206 [51], and -335 [51]. Many groups have shown a role for miR-146 in inflammation [52-55]. Both miR-146a and b inhibit invasion and migration of breast cancer cells, presumably via down-regulating NFκB by targeting IRAK1 and TRAF6 [53]. miR-146a/b further reduce the metastatic potential of cancer cells by decreasing expression of the invasion and metastasis promoting EGFR [18] and/or signaling molecule, ROCK1 [56]. These studies were extended *in vivo* to demonstrate that both miR-146a and b suppressed experimental metastasis in breast carcinoma cell lines [18]. miR-206 and -335 were the first suppressing metastamiR identified. Tavazoie *et al*. compared miRNA expression in metastatic variants derived from the human breast carcinoma cell line, MDA-MB-231 [51]. The expression of six specific miRNA were consistently low in metastatic variants. Three of them miR-335, 126, and -206 suppressed metastasis; but, miR-126 also inhibited cell proliferation and tumorigenesis. As a result, only miR-335 and -206 can be classified as metastasis suppressors. The key step at which miR-335 and -206 suppressed metastasis was inhibition of invasion and migration.

While a majority of metastmiRs seem to play key roles in tumor cell invasion and migration, thus far only one has been shown to have roles in multiple steps of the metastatic cascade. miR-31 was recently reported to inhibit cell invasion, promote anoikis, and suppress colonization of ectopic sites [50], leading to a 95% reduction in lung metastasis in an orthotopic model of breast cancer, while still allowing orthotopic growth. Using gene ontogeny analyses, miR-31 repressed frizzled 3 (Fzd3), integrin alpha-5

(ITGA5), myosin phosphatase-Rho-interacting protein (M-RIP), matrix metalloproteinase 16 (MMP16), radixin (RDX) and RhoA.

We acknowledge that expression of many more microRNA has been correlated with tumor aggressiveness, invasion, metastasis and survival. However, we have restricted this discussion solely to the microRNA for which functional *in vivo data* have been published showing selective regulation of metastasis. Undoubtedly, the number of metastamiR will continue to grow.

While multiple targets have been described for each metastamiR, the end result has been either an increase or decrease in metastasis. It is interesting that a known metastasis suppressor, BRMS1, increased the expression of metastasis-suppressing metastamiR and decreased the expression of metastasis-promoting metastamiR. Still unclear is how BRMS1 is coordinately regulating so many of the genetic programs associated with metastasis. It is likely that many of the metastamiR are linked by a finite number of signaling pathways and the BRMS1 is regulating expression by both direct and indirect mechanisms.

Key metastatic pathways regulated at multiple levels

Both metastamiR and metastasis suppressors act on selective steps of the metastatic cascade. Eleven miRNA have been shown to promote or block metastasis. This number is likely to grow because at least 20 more miRNA affect epithelial-mesenchymal transition (EMT), apoptosis, and angiogenesis, all steps important in metastasis. BRMS1 represents the first metastasis suppressor to regulate miRNA. It is likewise probable that some of the 25 other metastasis suppressors will directly or indirectly influence metastamiR expression.

Especially intriguing are the similarities between BRMS1 and miR-31. Both block multiple steps of the metastatic cascade; both block colonization of ectopic tissues; and both alter expression of several metastasis-associated proteins and signaling pathways.

Those findings, coupled with an explosion of papers describing miRNA and metastasis-associated steps compelled us to expand the focus of this mini-review to consider the state of the field. Furthermore, several clinical studies have identified correlations between miRNA expression and recurrence, development of metastases and/or survival (for a recent review, see [57]). Therefore, our goal is to focus on the evidence for metastamirs, the implications of their existence and some technical and theoretical considerations that emerge from their discovery.

miR-10b functions to positively regulate metastasis, ultimately by activating RhoC [39], the latter which is also decreased in BRMS1 cells [9]. The linkages between BRMS1, miR-10b and RhoC are intriguing; however, we remain cautious in our interpretation. The epithelial-mesenchymal-transition-inducing transcription factor, TWIST1, is a positive regulator of miR-10b. BRMS1-expressing cells down-regulate TWIST1 and miR-10b. Since the promoters for TWIST1 and miR-10b are still incompletely characterized, it is not yet possible to state definitively whether the BRMS1 effects are solely on TWIST1 or both TWIST1 and miR-10b.

Analogously, several of the molecules implicated in the miR-10b cascade are coincidentally regulated by BRMS1. For example, EGFR stimulates TWIST via Jak/stat signaling (but independent of Erk/MEK) to instigate the EMT [58]. BRMS1 down-regulates EGF signaling both directly (by decreasing receptor expression), and indirectly (by

attenuating phosphoinositide signaling) [30]. Decreased EGFR expression and the TWIST-miR-10b-RhoC axis could be the result of BRMS1 regulation of miR-146a/b, both of which target EGFR [18].

Our data, as well as the data from multiple other laboratories, clearly shows a heirearchical regulation of metastasis by BRMS1. Both direct and indirect effects on genes are operational within cells. BRMS1 can directly alter gene expression at the transcriptional level or via a transcriptional intermediate (i.e., by altering expression of a transcription factor, like TWIST). Concurrently, BRMS1 can regulate protein expression by manipulating transcription as described above, or by reducing translation by changing expression of miRNA.

Concluding remarks

It is in a multicellular organism's best interest that cells not migrate to other sites and integrate within another tissue. Such scenarios would create chaos and disrupt homeostasis. Thus, fail safe redundancies appear to be built into the circuitry that controls cancer metastasis. However, such redundancy would come at a cost, energetically inefficient. Even though the findings emerging from our studies on cancer metastasis begin to frame an intricate regulatory network for metastasis, our findings (along with those from many other laboratories) raise several additional questions:

- Why would a cell make multiple regulators to manipulate a single molecule? What types of cross-talk exist between the various mechanisms and molecules?
- Why would a cell make a single molecule (like a miRNA) that has so many targets? What confers specificity, if there is any? What other co-factors are involved, if any? Will the stoichiometry of miRNA expression be critical?

- Would metastamiR be useful clinical targets? If so, would the promiscuity of miRNA yield more or fewer off-target effects?
- Do all of the metastasis suppressors alter metastamiR expression? Is there overlap in the metastasis suppressor 'pathways?'
- Will exogenous miRNA have similar effects as endogenous miRNA? Are the biological consequences of re-introduction of individual miRNA artifactual when a miRNA exists within a cluster? Or should studies be done with pairs (or higher order) of miRNA?
- Are there functions of pri-miRNA and pre-miRNA in metastasis?
- What are the feedback mechanisms of miRNA (metastamiR) and metastasis suppressors and/or promoters?

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BREAST CANCER METASTASIS SUPPRESSOR 1 (BRMS1) COORDINATELY REGULATES METASTASIS-ASSOCIATED MICRORNA EXPRESSION

by

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Abbreviations:

BRMS1	Breast Cancer Metastasis Suppressor1
EMT	Epithelial-to-mesenchymal transition
miRNA	microRNA
miR	microRNA
Cy	Cyanine
hsa	*Homo sapiens*
EGFR	Epidermal growth factor receptor
MET	Mesenchymal-to-epithelial transition (reverse of EMT)
TTBS	Tween-20 containing Tris Buffered Saline
nt	nucleotide
DMEM	Dulbecco's modified Eagle'smedium
CMF-DPBS	calcium- and magnesium-free Dulbecco's phosphate buffered saline
RTQ	real-time quantitative PCR

ABSTRACT

Breast cancer Metastasis Suppressor1 (BRMS1) suppresses metastasis of multiple tumor types without blocking tumorigenesis. BRMS1 forms complexes with SIN3, histone deacetylases and selected transcription factors that modify metastasis-associated gene expression (e.g., EGFR, OPN, PI4P5K1A, PLAU). microRNA (miRNA) are a recently discovered class of regulatory, non-coding RNA, some of which are involved in neoplastic progression. Based upon these data, we hypothesized that BRMS1 may also exert some of its anti-metastatic affects by regulating miRNA expression. MicroRNA arrays were done comparing small RNA that were purified from metastatic MDA-MB-231 and MDA-MB-435 and their non-metastatic BRMS1-transfected counterparts. miRNA expression changed by BRMS1 were validated using SYBR Green RT-PCR. BRMS1 decreased metastasis-promoting (miR-10b, -373 and -520c) miRNA, with corresponding reduction of their downstream targets (e.g., RhoC which is downstream of miR-10b). Concurrently, BRMS1 increased expression of metastasis suppressing miRNA (miR-146a, -146b and -335). Collectively, these data show that BRMS1 coordinately regulates expression of multiple metastasis-associated miRNA and suggests that recruitment of BRMS1-containing SIN3:HDAC complexes to, as yet undefined, miRNA promoters might be involved in the regulation of cancer metastasis.

INTRODUCTION

Gene regulation by microRNA (miRNA) is a conserved mechanism in animals and plants (1). Endogenous, miRNA range from 15-28 nucleotides (nt) in *Homo sapiens* and, most commonly, negatively regulate gene expression, although some gene expression is positively regulated by miRNA. miRNA act as templates for RNA-induced silencing complexes (RISC) to target mRNA. Animal miRNA differ functionally from

plant miRNA in that they have imperfect base pairing with their target mRNA and more commonly inhibit protein translation than degrade mRNA (2). Imperfect or promiscuous base pairing allows animal miRNA to target multiple mRNA or even entire cellular pathways (3;4). To date, miRNA have been shown to regulate multiple cellular processes or pathways critical for neoplastic transformation and progression (5), including apoptosis (6;7), cell cycle regulation (8), differentiation (4;9;10), immune function (11) and metabolism (12;13). Up- and down-regulation of miRNA expression is correlated with development of multiple cancers (5;14), including breast carcinoma (15-17), and a growing number of miRNA also contribute to promotion and suppression of cancer invasion and metastasis (16;18-27).

Metastasis suppressors, defined by their ability to suppress metastasis without blocking orthotopic tumor growth, are an expanding family of >25 molecules (28;29). BRMS1 inhibits breast, melanoma, non-small cell lung and ovarian cancer metastasis in xenograft and syngeneic models (30-36). Expression of BRMS1 protein (30;37), but not necessarily mRNA (38;39), expression generally correlates inversely with survival and development of metastasis. BRMS1 associates with SIN3:histone deacetylase complexes (36;40;41) which are involved in chromatin structure and selective regulation of gene expression. Collectively, these factors lead to the hypothesis that BRMS1 suppresses metastasis by altering expression of metastasis-associated genes. Previous studies have identified selective regulation of the epidermal growth factor receptor (42), osteopontin (35;36;43;44), connexins (45) and urokinase plasminogen activator (46). We hypothesized that BRMS1 might also regulate recently discovered, metastasis-associated miRNA.

To determine whether BRMS1 regulates miRNA expression, we compared miRNA expression patterns in non-expressing and BRMS1 re-expressing breast carcinoma cells. miRNA expression was compared using microarrays imprinted with 328 known human miRNA probes and a selected common subset was further validated using quantitative real-time PCR (RTQ). In this report, we report that BRMS1 alters miRNA expression in metastatic breast carcinoma cells and notably down-regulated three of the four published metastasis-promoting miRNA (22;23;47) and up-regulated all three of the known metastasis-suppressing miRNA (16;24).

MATERIALS AND METHODS

Cell lines and cell culture.

MDA-MB-231 (231) and MDA-MB-435 (435) are human estrogen receptor- and progesterone receptor-negative cell lines derived from pleural effusions of metastatic infiltrating ductal breast carcinomas(48;49). Both cell lines form progressively growing tumors when injected into the mammary fat pads of immunocompromised mice. MDA-MB-435 cells develop macroscopic metastases in lungs and regional lymph nodes by 10-12 weeks post-inoculation, but infrequently metastasize after direct injection into the lateral tail vein or following subcutaneous injection. In contrast, MDA-MB-231 cells form macroscopic metastases when injected intravenously, but less commonly following injection into an orthotopic site. Both lines form osteolytic metastases following injection into the left ventricle of the heart (31;50-52). The origin of 435 has been questioned (53), however, this does not affect the interpretation of the results since BRMS1 suppresses metastasis of tumor cell lines from multiple tissue origins (33;34).

Parental cell lines were cultured in a mixture (1:1 *v:v*) of Dulbecco's modified Eagle's medium (DMEM) and Ham's-F12 medium (DMEM/F12; Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine, 0.02 mM non-essential amino acids and 5% fetal bovine serum (Invitrogen). All cultures were maintained without antibiotics or antimycotics on 100-mm tissue culture dishes (Corning, Corning, NY) at 37°C with 5 % CO_2 in a humidified atmosphere. When cultures reached 80-90% confluence they were passaged using a solution of 2 mM EDTA in Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate buffered saline (CMF-DPBS; Invitrogen). All cultures were regularly tested and confirmed negative for *Mycoplasma spp.* infection using a PCR-based test (TaKaRa, Shiga, Japan).

Constructs and transductions.

Cells (231 and 435) were transduced with GFP to facilitate tracking of cells *in vivo* (231^{GFP}/435^{GFP}) as previously described (50). Full-length BRMS1 cDNA was cloned into lentiviral constructs and transduced into 231^{GFP} and 435^{GFP} cells. Single cell clones (231^{BRMS1} and 435^{BRMS1}) were isolated and BRMS1 mRNA and protein expression was verified. Transduced cells were initially selected with puromycin (500 μg/mL) and maintained in puromycin (100 μg/mL) to ensure stable transduction. For routine culture, no antibiotic selection was used and expression has been verified to be stable for over two years.

MicroRNA arrays.

Cells were grown to 90% confluence, media aspirated, washed in ice-cold PBS, and lysed in acid phenol:chloroform (Ambion, Austin TX). Small RNA species (≤200nt) were isolated using *mir*Vana PARIS kit (Ambion) according to manufacturer's

instructions and immediately stored at -80°C. RNA quantification was performed with use of a DU 800 spectrophotometer (Beckman Coulter, Fullerton, CA). Three independently isolated samples were collected for every cell line.

miRNA array profiling was performed at the Vanderbilt Microarray Shared Resource according to the core's standard operating procedures (http://array.mc.vanderbilt.edu/microarray/expr/protocol.vmsr) as summarized below. RNA species (10-40 nt) were enriched using the flashPAGE fractionater (Ambion). The 3' ends were polyadenylated with modified amines; RNA from 231^{GFP} and 435^{GFP} were labeled with Cy5 while RNA from 231^{BRMS1} and 435^{BRMS1} cells were labeled with Cy3. The yields on the coupling reactions were typical of miRNA labeling reactions with no detectable CyDye, but a large increase in measurable RNA indicated that poly A polymerase was effective in adding the modified dNTPs to the miRNA (data not shown). RNA samples were suspended in 3X Hyb™ buffer (Ambion), heated for 95°C for 2 min, allowed to cool at room temperature for 1 min and then loaded onto mirVana miRNA bioarrays v2 (Ambion) with Maui DC cover slips (BioMicro Systems, Salt Lake City Utah). Hybridizations were performed using the Maui hybridization station at 42°C for 16 hr with Mix D settings in Maui A1-A3. Arrays washed once in Ambion SlideHyb™ low stringency buffer for 30 sec with mixing, followed by two Ambion SlideHyb™ high stringency buffer washes for 30 sec with mixing. Arrays were spun dry and scanned on the AXON 4000B scanner (Molecular Devices, Sunnyvale CA).

Raw GeneChip files from GeneChip Operating Software (GCOS, Affymetrix, CA) were uploaded and background was subtracted. Expression changes were normalized to the respective control cells (231^{GFP} or 435^{GFP}) in order to calculate the

intensity ratio/fold changes of the BRMS1-expressing counterparts (Cy5/Cy3). Data were sorted from greatest to least intensity. miRNA spots with foreground intensities less than 150 were disregarded as signals were too near background median. miRNA with values of -50 and -100 were also excluded as spot irregularity-affected fluorescence. Raw data from Axon were background-subtracted, normalized to the respective control cells (231^{GFP} or 435^{GFP}) in order to calculate the intensity ratio/fold changes of the BRMS1-expressing counterparts (Cy5/Cy3). miRNA spots with foreground intensities less than 150 were disregarded as signals were too near background median. miRNA with values of -50 and -100 were also excluded as spot irregularity-affected fluorescence.

Antibodies and immunoblotting.

BRMS1 monoclonal antibody clone 1a5.7 was used at 1:2500 and was previously described (36;54). Other antibodies used in this study were purchased and used at the titre indicated: anti-Twist1 (1:1000), anti-alpha tubulin (1:1000) and anti-EGFR (1:1000; Cell Signaling Technology, Danvers, MA), anti-HoxD10 E20 (1:200, Santa Cruz Biotechnology, Santa Cruz CA), anti-RhoC (1:1000: Abcam, Cambridge MA). Western blotting was performed as previously described (36;54).

Validation using real-time quantitative PCR.

miRNA expression was determined by collecting total RNA from 70–90% confluent cell cultures using Qiazol (Qiagen Inc, Valencia CA). RNA was purified using miRNeasy (Qiagen). MiScript primers (Qiagen Inc), designed to recognize specifically mature miRNA (i.e., not pre-miR or pri-miR), were: hsa-miR-10b_2 ,miR-96_1, hsa-miR-30e3p_1, hsa-miR-30e5p, hsa-miR-151, hsa-miR-320, hsa-miR-335_1, hsa-miR-

373_1, and hsa-miR-520c. All samples were normalized to small nuclear RNA U6 and fold changes were calculated as previously described (55).

RESULTS AND DISCUSSION

Small RNA (≤40 nt) were enriched from metastatic 231^{GFP}, 435^{GFP} and metastasis-suppressed BRMS1-transduced isogenic counterparts. BRMS1-associated mature miRNA expression changes were determined using miR-microarrays in three independent experiments. The top 25 consistently increased and decreased mature miRNA as a result of BRMS1 re-expression from both cell line comparisons are listed in **Table 1**. The arrays revealed changes in mature miRNA expression that could contribute to BRMS1 metastasis suppression. For example, the oncogenic miR-155 decreased in 435 cells and tumor suppressing let-7a increased in 231 cells; however, since the arrays were used as a screen for miRNA changes observed in both cell lines, many miRNA were excluded from further study in this report and are not included in **Table 1**. In general, the direction of miRNA expression change was consistent during the validations, but the magnitude of the changes was often under-appreciated on the arrays. The criteria for prioritizing candidates were: (*i*) technical replicates on the arrays were consistent; (*ii*) the direction of miRNA expression change was consistent in both $231/231^{BRMS1}$ and 435^{BRMS1} cell pairs; (*iii*) miRNA that target metastasis-associated mRNA (or proteins); and (*iv*) miRNA previously demonstrated to alter phenotypes associated with invasion and/or metastasis. Based on these criteria six miRNA were initially selected for further follow-up: miR-10b, -30e-3p, -30e-5p, -96, -151, -339 (**Fig. 1**).

Our original goals were to determine whether BRMS1 regulates miRNA and, if so, whether BRMS1-regulated miRNA are downstream mediators of BRMS1 metastasis

suppression. However, as these results were being collected, other laboratories reported on miRNA regulation of invasion and/or metastasis (16;22;23), compelling more detailed analysis of our data with regard to BRMS1 regulation of those miRNA (**Table 2**).

The first miRNA validated to promote metastasis was miR-10b (23). Knockdown of miR-10b in 231 cells decreased *in vitro* migration and invasion. Additionally, ectopic expression of miR-10b in HMEC and SUM149 cells promoted *in vitro* migration and invasion. In addition, there was increased dissemination to the lung and formation of macroscopic foci in the peritoneum by SUM159 cells (23). Correspondingly, miR-10b expression was suppressed in 231^{BRMS1} and 435^{BRMS1} cells by greater than 50%, which is consistent with prior data showing that BRMS1 suppresses invasion and metastasis. Furthermore, when miR-10b expression was examined in a panel of cells denoting breast cancer progression, BRMS1 reduced miR-10b levels similar to those found in non-metastatic cells (**Fig. 2**).

Ma et al. further showed that miR-10b down-regulated HoxD10 protein which led to an increased expression of RhoC, which is a positive regulator of metastasis (56-58). While RhoC mRNA expression decreased in both 231^{BRMS1} and 435^{BRMS1} cells (**Fig. 3**), HoxD10 mRNA increased in 435^{BRMS1} cells but decreased in 231^{BRMS1} cells. The differences observed when comparing cell lines suggest a non-linear pathway and/or other (i.e., non-HoxD10) mediators of miR-10b. Because we were unable to detect HoxD10 with the commercially available antibodies, we measured mRNA. The inconsistency of mRNA levels is consistent with the known roles of miRNA in regulating protein translation and with the findings reported by Ma et al.

Relatedly, Huang and colleagues (22) utilized a library of miR-transduced into human breast cancer cells in order to identify which miRNA promoted invasion *in vitro*. miR-373 and miR-520c stimulated migration and invasion *in vitro* and metastasis *in vivo*. Expression of miR-373 and -520c was decreased in 231^{BRMS1} and 435^{BRMS1} cells (**Fig. 4** and **Table 2**).

Tavazoie et al. (16) compared miRNA expression patterns in paired sets of 231 variants which showed preferential organ-selective metastasis patterns. miR-335, when over-expressed by 250-fold significantly suppressed lung and bone metastasis. Re-expression of BRMS1 suppressed metastasis to lungs and bone (31;34;36) and increased expression of the metastasis-suppressing miR-335 by 6-fold (**Fig. 4** and **Table 2**). In the same report, miR-126 was described as metastasis suppressing (16); however, it also suppressed orthotopic tumor growth, thereby excluding it as a metastasis suppressor.

Classically metastasis suppressor genes have encoded proteins; however, the demonstration that miR-335 and miR-146a/b suppress metastasis without blocking primary tumor growth show that the types of molecules considered to be regulating metastasis needs to be more inclusive. Thus far, few pathways have been linked between known metastasis suppressors. In this report, we show for the first time that BRMS1 coordinately regulates expression of multiple miRNA and their corresponding downstream targets (e.g., RhoC (this report) and epidermal growth factor receptor (42)). The findings are consistent with the emerging awareness that metastasis involves simultaneous control of multiple genes. Our observations beg the question as to whether other metastasis suppressors also mediate some or all of their affects through miRNA regulation. Prior studies relating miRNA and metastasis have focused attention primarily

toward identifying miRNA targets. Comparatively little is known about miRNA regulation (59;60). Ma et al. identified TWIST1, a major regulator of epithelial-mesenchymal transition (61), as a promoter of miR-10b expression (23). We discovered that BRMS1 decreases TWIST expression in whole cell lysates as well as nuclear fractions (**Fig. 3**). Moreover, BRMS1 mutants that do not suppress metastasis fail to down-regulate TWIST1 in 231 cells. Whether BRMS1 directly binds to the miR-10b promoter is still not known. But it is conceivable that BRMS1 might be part of a co-repressor complex(es) recruited to miR-10b since BRMS1 is a component of several SIN3:histone deacetylase complexes (36;41). Similarly, the mechanism(s) by which regulation of other miRNA occurs will be the object of intense future investigation. Given the decrease in such a prominent epithelial-to-mesenchymal transition (EMT) regulator (Twist1), one could speculate that BRMS1 suppresses metastasis by invoking the reverse process (mesenchymal-to-epithelial transition, MET) in metastatic cells; however, some published and unpublished data argue against this possibility. Several recent publications indicate that miR-200 family are associated with increased expression of E-cadherin (20;21;26;62;63), which is typically lost during EMT (64). Data presented in **Table 1** show decreases in miR-200 family members when BRMS1 is expressed. This is the opposite of what would be predicted. Moreover, changes in miR-200c are not consistent between 231 and 435 cells when BRMS1 is re-expressed. Also, we have previously reported that BRMS1 re-expression has no effect on E-cadherin mRNA or protein expression (65). Finally, BRMS1 consistently increases the vimentin expression, which is opposite of what one would expect if BRMS1 were regulating metastasis solely by regulating EMT.

Some miRNA expression changes observed in BRMS1-expressing 231 and 435 cells are large (miR-10b; 0.35/0.36, respectively), while others more subtle (miR-373; 0.69/0.53 and miR-520c; 0.65/0.59, respectively). Nonetheless, it is interesting that nearly all (6 out of 7; 86%) of the known metastasis-associated miRNA are regulated in the direction predicted for a metastasis suppressor. The exception was miR-21 which promoted metastasis in breast (47) and colorectal cancer (66) cells. BRMS1 increased miR-21 expression in 231 by ~2-fold, but did not change in 435. It is quite possible that in 231 cells, the combined effects changing expression of the other metastasis-associated miRNA may overwhelm the miR-21 up-regulation or the miR-21 expression change may be an attempt by cells to compensate for other changes.

Among the many miRNA changes observed in BRMS1-expressing cells, miR-146a and -146b expression were increased. Coupled with our prior reports that BRMS1 inhibits NFκB activity (46) and EGFR expression (42), NFκB regulation by miR-146a/b (25;67) and demonstration that EGFR is a target sequence for miR-146a/b, we tested the hypothesis that miR-146 was a downstream mediator of BRMS1 metastasis suppression. Transfection of miR-146a or miR-146b into 231 cells resulted in a significant suppression of lung metastases in experimental xenograft models (24). We emphasize that, unlike prior studies testing the role of miRNA in the processes of invasion and metastasis, the data reported here are based upon near physiologic expression levels of BRMS1 and the corresponding downstream miRNA. This point is key because off-target effects are more likely if experiments involve gross over-expression.

In summary, we demonstrate that BRMS1 regulates virtually all of the known miRNA regulating invasion and/or metastasis. Given that a single metastasis suppressor

regulates several key metastasis-associated miRNA, future studies should focus on which step(s) of the metastatic cascade are dysregulated in human disease and the specific miRNA expression regulatory elements that are regulated by BRMS1 directly.

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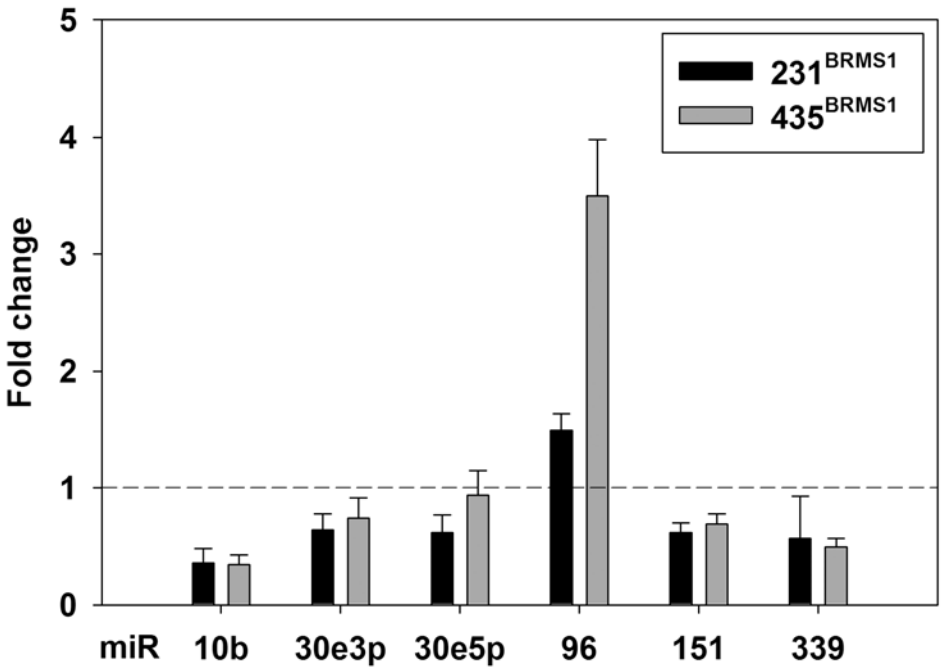

Fig. 1. BRMS1 re-expression changes miR levels in metastasis suppressed breast cancer cells. Total RNA was extracted from 231/231^{BRMS1} and 435/435^{BRMS1} cell lines, reverse transcribed and miR assessed using SYBR-green real-time RT-PCR.

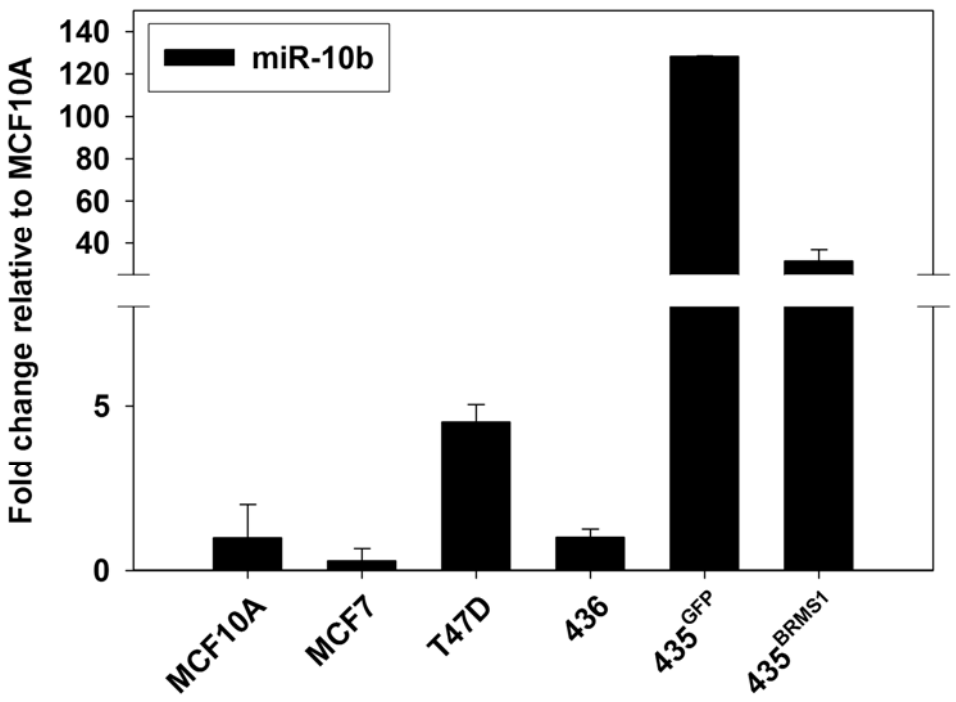

Fig. 2. BRMS1 restores miR-10b levels to those found in non-metastatic cells. A panel of breast cancer cell lines was evaluated for miR-10b expression. Total RNA was extracted, reverse transcribed and miR assessed using SYBR-green real-time RT-PCR.

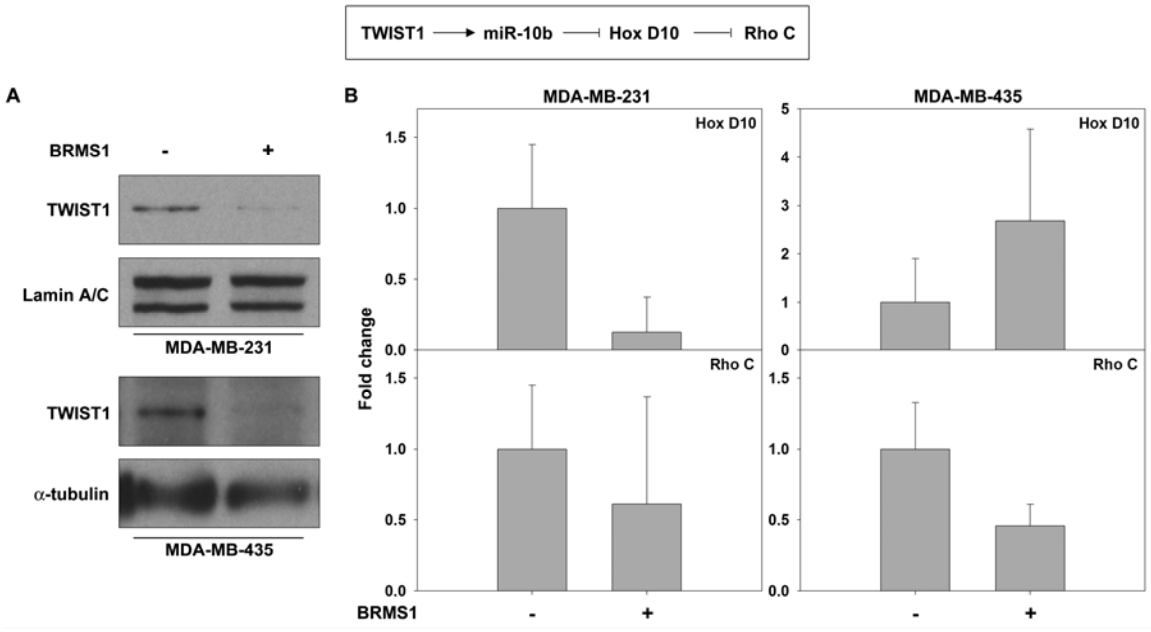

Fig. 3. BRMS1 decreases a pro-metastatic pathway. (*A*) Whole cell (435) and nuclear (231) fractions were obtained and probed for TWIST1. α-tubulin and Lamin A/C were used as loading controls for the whole cell and nuclear fractions, respectively. (*B*) Total RNA was collected from $231/231^{BRMS1}$ and $435/435^{BRMS1}$ cell lines. RNA (3 μL) was reverse transcribed and messages detected using Taqman primer probes for RhoC and HoxD10. Results are given as fold change relative to non-BRMS1 expressing 231 and 435 cells ± S.E.M. Ribosomal S9 was used as a normalization control for equal loading.

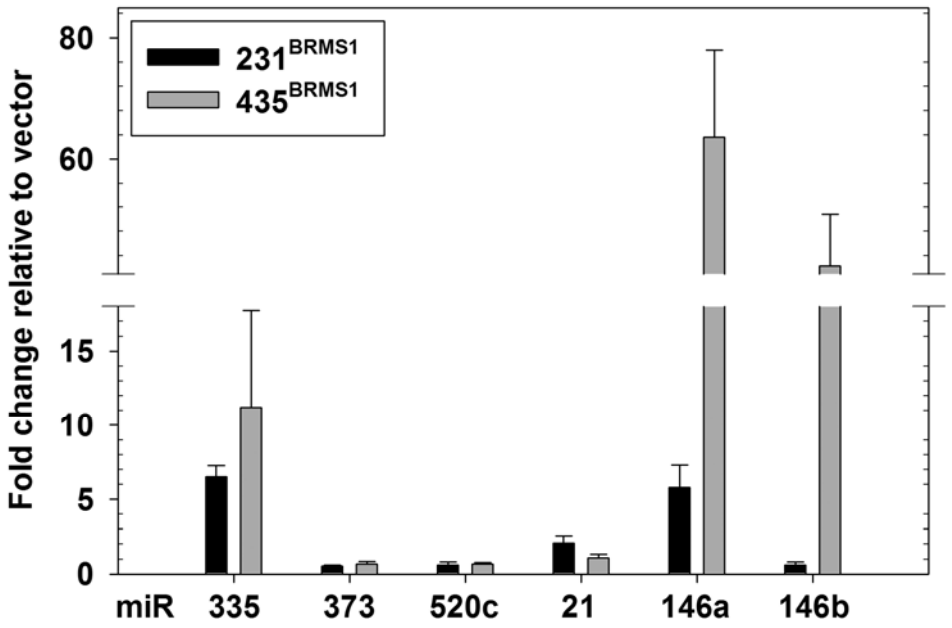

Fig. 4. BRMS1 decreases pro-metastatic and increases metastasis-suppressing miR in breast cancer cells. RNA was collected using Qiazol and the miRNeasy kits. Purified RNA (6 μL) was reverse transcribed and then amplified usin miR specific primer probes. Relative amounts were calculated using the comparative cycle threshold (C_T) method. The miR amounts were normalized to small nuclear RNA-U6. Results are given as fold change relative to non-BRMS1 expressing 231 and 435 cells ± S.E.M.

Table 1: Top 25 most commonly regulated miR following re-expression of BRMS1

Cell Line	Direction change	microRNA	Cy5:Cy3 Ratio	Locus	Accession No. Sanger database
MDA-MB-231	**DOWN**	hsa_miR_200a_AS	62.190	1p36	MIMAT0001620
		hsa_miR_200b	16.213	1p36	MIMAT0000318
		hsa_miR_380_3p	12.441	14q32	MIMAT0000735
		hsa_miR_326	8.200	11q13	MIMAT0000756
		hsa_miR_483	6.864	11p15	MIMAT0002173
		hsa_miR_210	3.964	11p15	MIMAT0000267
		hsa_miR_99b	3.580	19q13	MIMAT0000689
		hsa_miR_330	2.945	19q13	MIMAT0000751
		hsa_miR_324_5p	2.860	17p13	MIMAT0000761
		hsa_miR_324_3p	2.769	17p13	MIMAT0000762
		hsa_miR_320	2.684	8p21	MIMAT0000510
		hsa_miR_520h	2.621	19q13	MIMAT0002867
		hsa_miR_149	2.614	2q37	MIMAT0000450
		hsa_miR_423	2.553	17q11	MIMAT0001340
		hsa_miR_425	2.552	3p21	MIMAT0001343
		hsa_miR_501	2.478	Xp11	MIMAT0002872
		hsa_miR_452	2.430	Xq28	MIMAT0001635
		hsa_miR_511	2.414	10p12	MIMAT0002808
		hsa_miR_339	2.336	7p22	MIMAT0000764
		hsa_miR_151	2.299	8q24	MIMAT0000757
		hsa_miR_491	2.126	9p21	MIMAT0002807
		hsa_miR_362	2.064	Xp11	MIMAT0000705
		hsa_miR_17_3p	2.031	13q31	MIMAT0000071
		hsa_miR_18a_AS	1.969	13q31	MIMAT0002891
MDA-MB-231	**UP**	hsa_let_7a	0.295	22q13	MIMAT0000062
		hsa_let_7f	0.328	9q22	MIMAT0000067
		hsa_miR_128b	0.358	3p22	MIMAT0000676
		hsa_miR_30c	0.370	6q13	MIMAT0000244
		hsa_let_7g	0.384	3p21	MIMAT0000414
		hsa_miR_30a_3p	0.385	6q13	MIMAT0000088
		hsa_miR_21	0.386	17q22	MIMAT0000076
		hsa_miR_10a	0.396	17q21	MIMAT0000253
		hsa_miR_128a	0.408	2q21	MIMAT0000424
		hsa_miR_146b	0.429	10q21	MIMAT0002809
		hsa_miR_181a	0.434	1q31	MIMAT0000256
		hsa_miR_182	0.434	7q32	MIMAT0000259
		hsa_miR_96	0.435	7q32	MIMAT0000095
		hsa_miR_20a	0.436	13q31	MIMAT0000075
		hsa_miR_200c	0.436	12p13	MIMAT0000617
		hsa_miR_26a	0.463	3p22	MIMAT0000082
		hsa_let_7i	0.470	12q14	MIMAT0000415
		hsa_miR_15b	0.504	3q25	MIMAT0000417
		hsa_miR_148a	0.510	7p15	MIMAT0000243
		hsa_miR_30a_5p	0.515	6q13	MIMAT0000087
		hsa_miR_15a	0.518	13q14	MIMAT0000068
		hsa_miR_27a	0.520	19p13	MIMAT0000084
		hsa_miR_30e_5p	0.527	1p34	MIMAT0000692
		hsa_miR_16	0.540	13q14	MIMAT0000069
		hsa_miR_142_5p	0.541	17q22	MIMAT0000433

MDA-MB-435	**DOWN**	hsa_miR_34a	102.208	1p36	MIMAT0000255
		hsa_miR_126	99.692	9q34	MIMAT0000445
		hsa_miR_224	53.970	Xq28	MIMAT0000281
		hsa_let_7b	51.088	22q13	MIMAT0000063
		hsa_miR_155	50.785	21q21	MIMAT0000646
		hsa_miR_139	31.486	11q13	MIMAT0000250
		hsa_miR_199a	28.600	19p13	MIMAT0000231
		hsa_miR_452	25.500	Xq28	MIMAT0001635
		hsa_miR_148a	24.778	7p15	MIMAT0000243
		hsa_miR_200b	20.446	1p36	MIMAT0000318
		hsa_miR_222	16.220	Xp11	MIMAT0000279
		hsa_miR_200c	12.161	12p13	MIMAT0000617
		hsa_miR_380_3p	11.759	14q32	MIMAT0000735
		hsa_miR_503	7.627	Xq26	MIMAT0002874
		hsa_miR_138	7.141	3p21	MIMAT0000430
		hsa_miR_485_5p	6.773	14q32	MIMAT0002175
		hsa_let_7f	6.644	9q22	MIMAT0000067
		hsa_miR_21	6.037	17q22	MIMAT0000076
		hsa_miR_152	5.913	17q21	MIMAT0000438
		hsa_miR_125b	5.000	11q24	MIMAT0000423
		hsa_miR_100	4.076	11q24	MIMAT0000098
		hsa_miR_210	4.040	11p15	MIMAT0000267
		hsa_miR_520h	4.031	19q13	MIMAT0002867
		hsa_miR_221	3.980	Xp11	MIMAT0000278
MDA-MB-435	**UP**	hsa_miR_146a	0.001	5q33	MIMAT0000449
		hsa_miR_363	0.001	Xq26	MIMAT0000707
		hsa_miR_506	0.002	Xq27	MIMAT0002878
		hsa_miR_508	0.003	Xq27	MIMAT0002880
		hsa_miR_509	0.003	Xq27	MIMAT0002881
		hsa_miR_513	0.003	Xq27	MIMAT0002877
		hsa_miR_31	0.004	9p21	MIMAT0000089
		hsa_miR_105	0.006	Xq25	MIMAT0000102
		hsa_miR_211	0.009	15q13	MIMAT0000268
		hsa_miR_510	0.013	Xq27	MIMAT0002882
		hsa_miR_20b	0.016	Xq26	MIMAT0001413
		hsa_miR_146b	0.025	10q24	MIMAT0002809
		hsa_miR_9_AS	0.028	1q22	MIMAT0000442
		hsa_miR_204	0.032	9q21	MIMAT0000265
		hsa_miR_501	0.034	Xp11	MIMAT0002872
		hsa_miR_500	0.038	Xp11	MIMAT0002871
		hsa_miR_502	0.042	Xp11	MIMAT0002873
		hsa_miR_362	0.045	Xp11	MIMAT0000705
		hsa_miR_188	0.061	Xp11	MIMAT0000457
		hsa_miR_185	0.070	22q11	MIMAT0000455
		hsa_miR_497	0.079	17p13	MIMAT0002820
		hsa_miR_196a	0.087	17q21	MIMAT0000226
		hsa_miR_195	0.089	17p13	MIMAT0000461
		hsa_miR_96	0.182	7q32	MIMAT0000095

Small RNA were isolated from cell lines not expressing (MDA-MB-231 or MDA-MB-435) or expressing BRMS1, labeled with Cy5 or Cy3 and hybridized to arrays containing 328 human microRNA. Arrays were performed three times in independent

experiments. Only consistent changes are shown in this table.

Sanger database v12: http://microrna.sanger.ac.uk/sequences/

Subsequent to the evaluation of miR expression on the microarrays, homologs of some miR have been submitted to the Sanger databases. The chromosomal locations provided in this table correspond to the first miR deposited into the database.

Table 2: Summary of metastasis-associated miR changes following BRMS1 re-expression

miR	Accession No.	Change		Reported role(s) of miR in tumor progression	Reference(s)
Decrease		**231:231^{BRMS1}**	**435:435^{BRMS1}**		
10b	MI0000267	0.36	0.35	increased migration, invasion, metastasis	(23)
373	MI0000781	0.53	0.69	increased migration, invasion, metastasis	(22)
520c	MI0007801	0.59	0.65	increased migration, invasion, metastasis	(22)
Increase					
146a	MI00004	6	64	decreased invasion, metastasis	(24, 25)
146b	MI0003129	0.6	42	decreased invasion, metastasis	(24, 25)
335	MI0000816	6	11	decreased migration, metastasis	(16)
21	MI0000077	2.1	1.1	decreased migration, metastasis	(46, 62)

BREAST CANCER METASTASIS SUPPRESSOR 1 UP-REGULATES MIR-146, WHICH SUPPRESSES BREAST CANCER METASTASIS

by

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ABSTRACT

Breast cancer metastasis suppressor 1 (BRMS1) is a predominantly nuclear protein that differentially regulates expression of multiple genes, leading to suppression of metastasis without blocking orthotopic tumor growth in multiple human and murine cancer cells of diverse origins. We hypothesized that miR-146 may be involved in the ability of BRMS1 to suppress metastasis because miR-146 expression is altered by BRMS1, and because BRMS1 and miR-146 are both associated with decreased signaling through the NFκB pathway. BRMS1 significantly up-regulates miR-146a by 6-fold to 60-fold in metastatic MDA-MB-231 and MDA-MB-435 cells, respectively and miR-146b by 40-fold in MDA-MB-435 as measured by real-time quantitative RT-PCR (RTQ). Transduction of miR-146a or -146b into MDA-MB-231 down-regulated expression of epidermal growth factor receptor (EGFR), inhibited invasion and migration *in vitro*, and suppressed experimental lung metastasis by 69% and 84%, respectively (mean ± SEM: empty vector = 39 ± 6, miR-146a = 12 ± 1, miR-146b = 6 ± 1). These results further support the recent notion that modulating the levels of miR-146a or -146b could have a therapeutic potential to suppress breast cancer metastasis.

INTRODUCTION

Metastasis suppressors regulate diverse pathways to block metastasis without preventing orthotopic tumor growth (1). Breast cancer metastasis suppressor 1 (BRMS1) affects multiple steps in the metastatic cascade leading to ~90% metastasis suppression in xenograft models of breast carcinoma, melanoma, and ovarian carcinoma (2)(3). Mechanistically, BRMS1 regulates expression of multiple genes linked to metastasis, including osteopontin (OPN), urokinase-type plasminogen activator (uPA), epidermal

growth factor receptor (EGFR), fascin, and connexins (reviewed in (1)). Transcriptional regulation by BRMS1 has been proposed to occur through interaction with large SIN3:HDAC chromatin remodeling complexes based upon multiple protein-protein interaction studies (4)(3). A second, though not mutually exclusive, mechanism involves the negative regulation of nuclear factor κ-B (NFκB) through direct interaction with RelA/p65 and inhibition of IκBα phosphorylation (5)(6). Regardless of the specific mechanism by which BRMS1 regulates transcription, multiple changes in gene and protein expression have been verified by microarray and proteomic analyses when BRMS1 was re-expressed in cells (7)(8)(9).

We had hypothesized that BRMS1 could exert its anti-metastatic action by differentially regulating expression of microRNA (miRNA or miR). These are transcribed genes processed to single-stranded regulatory RNA of approximately 22 nucleotides (10). Mature miR repress protein expression primarily through base-pairing of a seed region with the 3' untranslated region (UTR) of the target mRNA leading to inhibition of translation and/or mRNA degradation. The hypothesis is supported by microarrays showing multiple changes in miR expression upon ectopic expression of BRMS1 (M.D. Edmonds and D.R. Welch in preparation).

Because miR-146 plays a role in regulating NFκB (11)(12) and because miR-146 is more abundantly expressed in BRMS1-expressing cells, we chose this miR to directly test whether miR-146 could be a downstream mediator of metastasis suppression by BRMS1. miR-146a and -146b are distinct genes encoded on chromosomes 5q33 and 10q24, respectively. The mature products have similar targets since they differ only by two nucleotides near the 3' end. Recently, miR-146a and -146b were shown to inhibit

both migration and invasion suggesting they play a role in metastasis (12). In this report, we show that BRMS1 up-regulates miR-146a in two breast cancer cell lines, MDA-MB-231 and -435, and miR-146b in MDA-MB-435, but not -231. miR-146a and -146b both reduce EGFR expression and suppress metastasis. These results add to the growing list of miR genes that regulate metastasis and provide a potential mechanism for how BRMS1 suppresses metastasis by altering the expression profile of metastasis-associated miR.

MATERIALS AND METHODS

Cell lines and cell culture

MDA-MB-231, -435, -436, T47D cell lines were cultured in a mixture (1:1, v/v) of Dulbecco's modified Eagle medium (DMEM) and Ham's F12 medium (Invitrogen; Carlsbad, CA), supplemented with 2 mM L-glutamine (Invitrogen), 0.02 mM non-essential amino acids (Mediatech, Herndon, VA), and 5% fetal bovine serum (Atlanta Biologicals, Norcross, GA). The immortalized, non-tumorigenic breast epithelial cell line MCF10A was cultured in a mixture (1:1, v/v) of DMEM and Ham's F12 medium supplemented with 5% horse serum, 2 mM L-glutamine, 0.02 mM non-essential amino acids, 10 ng/mL EGF, 0.5 μg/mL hydrocortisone, 0.1 μg/mL cholera toxin, and 10 μg/mL insulin (Sigma). MCF7 was cultured in minimal essential medium (MEM) with L-glutamine and Earle's salts (Invitrogen) supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acids, 10 μg/mL insulin, and 1 mM sodium pyruvate. Neither antibiotics nor antimycotics were used. All cell lines were confirmed negative for Mycoplasma spp. infection using a PCR-based test (TaKaRa, Shiga, Japan). Transduction of cells with BRMS1 or miR-146 was performed as previously described (12)(2)(3).

Quantitative Real-time RT-PCR

miRNA expression was determined by collecting total RNA from 70–90% confluent cell cultures. Media was aspirated, and cells were rinsed in ice cold CMF-DPBS. Cells were lysed with Qiazol (Qiagen, Valencia CA) and RNA separated using the miRNeasy system for purifying total RNA (Qiagen). MiScript primers specific for mature miRNA were hs-miR-146a_1 and hs-miR-146b_1 (Qiagen). All samples were normalized to small nuclear RNA U6 and fold changes were calculated as previously described (13).

Western Blotting

Total protein lysates were prepared as previously described (12) and probed for EGFR expression (rabbit polyclonal sc 03; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Relative expression level was determined by densitometry normalized to the expression of β-actin (mouse monoclonal sc 47778; Santa Cruz).

Metastasis Assays

Cells at 80 90% confluence were detached using 0.2 mM EDTA in Ca+2 , Mg+2 and NaHC03 free Hank's balanced salt solution (HBSS). Viable cells were counted using a hemacytometer and resuspended at a final concentration of 2.5 x 10^6 cells/mL in ice cold HBSS. Female athymic mice (3-4 wk; Harlan Sprague Dawley, Indianapolis, IN) were injected with 0.2 mL cell suspension into the lateral tail vein. Mice were necropsied 10 wk post inoculation following anesthesia with Ketamine:Xylazine and euthanasia by cervical dislocation. All organs were observed for presence of macroscopic metastases. Lungs were removed and fixed in a mixture of Bouin’s fixative and neutral buffered formalin (1:5 v/v). Mice were maintained under the guidelines of the National Institutes

of Health and the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Food and water were provided ad libitum.

Statistical Analyses

The number of lung metastases was compared for miR-146a or -146b transduced cell lines to the vector-only transduced line. A Kruskal-Wallis ANOVA of ranks procedure was used with Dunn's post hoc test. Calculations were performed using SigmaStat statistical analysis software (SPSS Inc., Chicago, IL). Statistical significance was defined as a probability $P \geq 0.05$.

RESULTS

BRMS1 up-regulates miR-146. BRMS1 has already been shown to regulate the expression of multiple genes involved in metastasis (1). Microarrays comparing the expression profile of miR between breast cancer cell lines with and without BRMS1 (at near physiologic expression levels) showed alteration of the expression of multiple miR genes that already have been implicated in the process of invasion and metastasis (M.D. Edmonds and D.R. Welch in preparation). We chose to focus studies with miR-146 since it was reported to be involved with regulation of NFκB. miR-146a was significantly up-regulated by introduction of BRMS1 into the MDA-MB-231 and -435 breast cancer cell lines (Fig. 1a). Additionally, miR-146b was significantly up-regulated by BRMS1 in MDA-MB-435, but not -231 (Fig. 1a).

To assess whether miR146a or -146b levels were altered in other breast cancer cell lines, a panel of breast derived cell lines were analyzed to measure relative levels of miR-146. Basal expression levels generally decreased for both miR-146a and -146b in tumorigenic, but weakly/non-metastatic cell lines MCF7, T47D, and MDA-MB-436

compared to the immortalized, but not tumorigenic breast epithelial MCF10A and a further decrease was found in metastatic MDA-MB-435 (Fig. 1b).

To address whether the miR-146 changes were specific to BRMS1, cells were transfected with siRNA targeting BRMS1. With a 70-90% reduction in BRMS1 expression, miR-146a/b were reduced by 20-50% at 48 hr (data not shown). The magnitude of the changes varied between experiments, but the trends were comparable, perhaps explained by miR stability and shared processing machinery (10).

miR146a and -146b down-regulate EGFR. Recently, BRMS1 was demonstrated to directly down-regulate transcription of EGFR (13). However, it was recently discovered that EGFR is also a predicted target of miR-146a and -146b, in addition to nine other human miR, based upon predicted base-pairing using miRBase targets (Sanger database) (14). The predicted target sequence is in the 3' UTR at position 4944-4964 of EGFR (numbering based on NG_007726; Fig. 2a). Since miR-146 was significantly up-regulated by BRMS1 and since EGFR is a predicted target of miR-146, we tested whether ectopic expression of either miR-146a or -146b could alter EGFR protein expression. A ~50% reduction in protein was demonstrated by immunoblotting and densitometry in the MDA-MB-231 cell line upon transduction with miR-146a or -146b (Fig. 2b). It is important to note that there is undetectable basal BRMS1 expression in MDA-MB-231 by RTQ or immunoblot.

miR146a and -146b suppress metastasis. Recently, miR-146a and -146b were shown to inhibit migration and invasion *in vitro* (12). To determine whether either miR could also suppress metastasis, an experimental metastasis assay was performed. MDA-

MB-231 cells transduced with miR-146a or -146b suppressed pulmonary metastasis by 69% and 84%, respectively (Fig. 3).

DISCUSSION

There is a growing list of miR genes that play specific roles in suppressing or enhancing metastasis. We hypothesized that BRMS1, a metastasis suppressor that regulates the expression of multiple genes, might directly or indirectly regulate expression of miR genes. We found a decrease in several pro-metastatic miR and an increase in metastasis suppressive miR upon ectopic expression of BRMS1 (M.D. Edmonds and D.R. Welch in preparation). Of the many miR expression changes, we focused on miR-146 because it is involved with regulation of the NFκB signaling pathway with which BRMS1 was already implicated and because miR146a and -146b have recently been shown to inhibit migration and invasion (12).

Ectopic BRMS1 expression significantly increased miR-146 expression. Moreover, transduction of miR-146a or -146b into MDA-MB-231 cells resulted in suppression of metastasis by 69-84%. While there have already been multiple mechanisms associated with BRMS1 suppression of metastasis, results presented in this study, for the first time, suggest that up-regulation of miR-146, whether direct or indirect, contributes to the ability of BRMS1 to suppress metastasis.

Precisely how a BRMS1–miR-146 axis could regulate metastasis is more difficult to determine. However, several possibilities can be deduced from the literature, noting that miR-146a and -146b are distinct genes encoded on different chromosomes. The data presented here emphasize that, despite their similarities, both miR are independently regulated. Recently, a G/C polymorphism was identified in the pre-miR-146a sequence

that reduced mature miR-146a levels (15). In addition, the transcription factor PLZF down-regulated miR-146a (16). It is likely, then, that miR-146a and -146b are regulated by different mechanisms depending on cell type and the environment in which cells find themselves. In fact, the difference noted between the MDA-MB-231 and -435 cell lines is not completely surprising since the lines are unrelated. In addition, NFκB is constitutively active in MDA-MB-231 but not in -435 (17). Because NFκB plays a role in regulating miR-146a and because BRMS1 regulates NFκB (5, 6), there exists a potential connection that may relate to differences between the regulation of miR-146a and -146b in these different cell lines.

Several targets have been predicted and identified for miR-146a and -146b including IRAK1 (12)(11), TRAF6 (12)(11), IL-8 (12), IL-6 (12), CXCR4 (16) and MMP9 (12). EGFR is an additional predicted target (14) and we recently showed that BRMS1 re-expression reduced EGFR in MDA-MB-231 cells by ~50% (13). Although EGFR transcription is directly down-regulated by BRMS1, miR-146 could be a redundant mechanism and a working model is proposed (Fig. 4). This possibility is supported by complete obliteration of EGFR expression in BRMS1-expressing MDA-MB-435 cells (13)(3) in which both miR-146a and -146b were up-regulated.

The organotropism of metastasis is mediated, in part, by differential expression of chemokine receptors and ligands on tumor cells and in various tissues. For example, breast carcinoma cells frequently express high levels of CXCR4 and frequently colonize lung and bone, which express abundant stromal cell derived factor-1 (SDF-1), the preferred ligand for CXCR4 (18). Interfering with CXCR4:SDF-1 interactions reduces metastatic potential (19). Although regulation of CXCR4 via a BRMS1-miR146 axis is

not directly tested here, we have observed a decrease in CXCR4 expression by microarray analysis with MDA-MB-231 and -435 cells following BRMS1 re-expression (data not shown). This is consistent with recent reports showing decreased CXCR4 in cells expressing miR-146a (16) or BRMS1 (20).

The biological observations notwithstanding, our findings are paradoxical with regard to NFκB. Baltimore and colleagues report that NFκB induces miR-146a following induction by lipopolysaccharide, with a corresponding induction of TRAF6 and IRAK1, the latter two which form a negative feedback loop (11)(Fig. 4). However, prior reports show direct BRMS1-RelA binding leading to decreased NFκB signaling (6). Based upon the latter, BRMS1 should reduce miR-146 expression. We observe the opposite. The most likely explanations relate to the aforementioned cell type-specific regulatory mechanisms, including as-yet-unknown co-factors that influence transcription.

These data reinforce the growing consensus that metastasis requires coordinated expression of multiple genes. Suppression of metastasis by BRMS1 may be explained by

decreased EGFR, OPN, and/or CXCR4, but we now show that miR-146 also contributes to BRMS1-mediated metastasis suppression. Likewise, the myriad downstream targets of BRMS1 can also be explained because of relative promiscuity of miRNA in targeting gene expression (10).

We also emphasize that the experiments reported here did not use super-physiologic expression of miR to achieve an effect on metastasis in contrast to many previously published reports showing functions of microRNA in cancer cells. This point is key for several reasons. Primarily, off-target effects are more likely if the expression is too high. Likewise, if miR or miR mimetics are to be translated into clinical practice, the efficacy needs to be achievable at reasonable concentrations. miR-146a and -146b were elevated 10- to 30-fold in transduced MDA-MB-231 (12), which is consistent with the 6- to 60-fold induction observed in BRMS1-expressing cells. Based upon our findings, miR-146a or -146b are promising targets for anti-metastatic therapy based upon the findings presented here.

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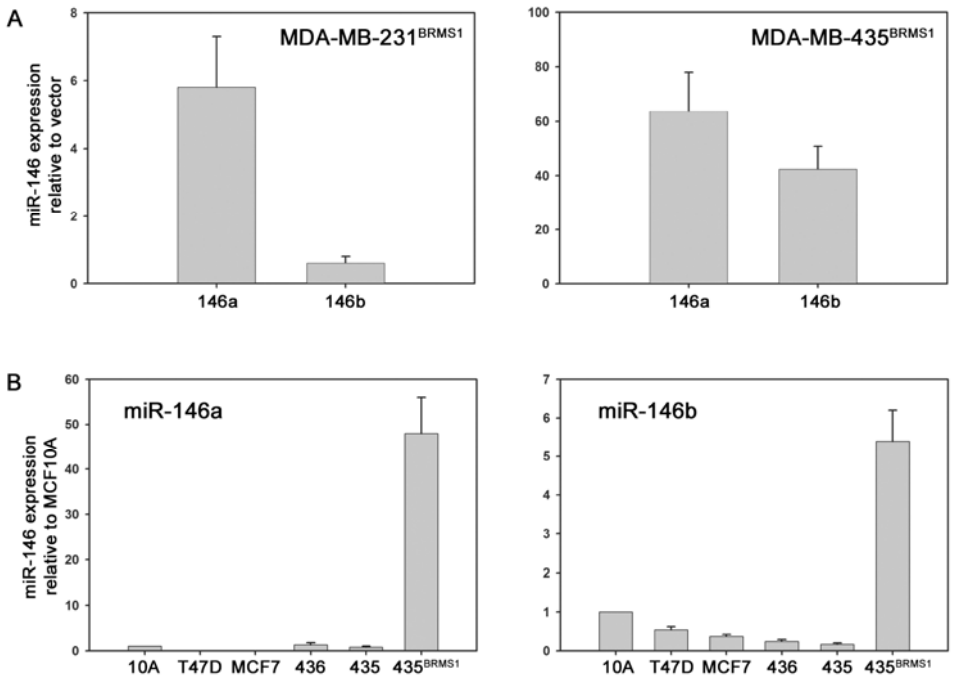

Figure 1. BRMS1 up-regulates miR-146. (*A*) Ectopic expression of BRMS1 in MDA-MB-231 or -435 breast cancer cell lines significantly enhanced the expression of miR-146a as demonstrated by real-time RT-PCR (~6 fold and ~60 fold, respectively). miR-146b expression was also increased in MDA-MB-435 (~40 fold). (*B*) Endogenous expression levels of miR-146a and -146b in multiple breast derived cell lines generally show decreased levels in tumorigenic but weakly/non-metastatic cell lines (MCF7, T47D, and MDA-MB-436) compared to the immortalized, non-tumorigenic breast epithelial cell line (MCF10A) and a further decrease was found in metastatic MDA-MB-435. Please note scale differences.

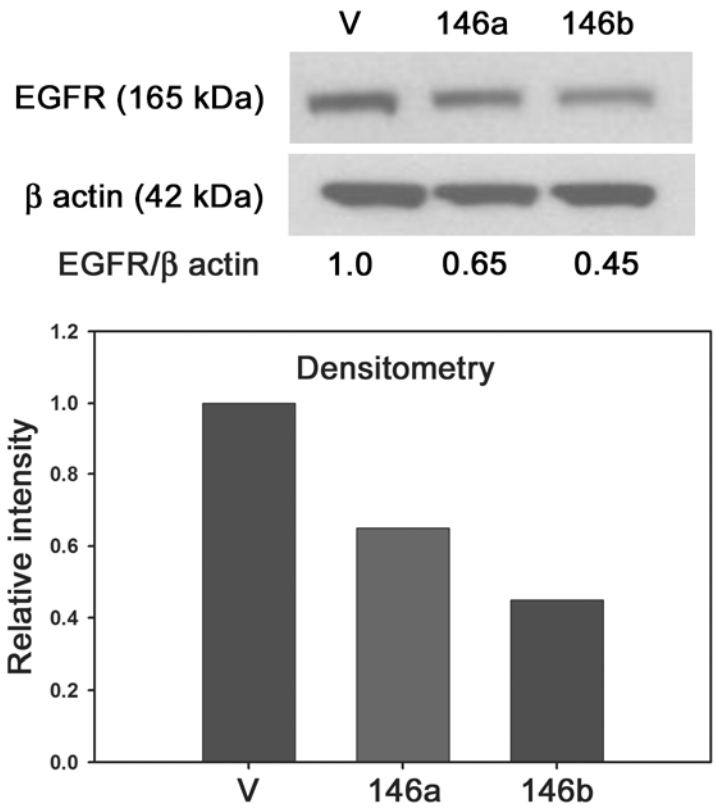

Figure 2. EGFR protein expression is decreased by miR-146. Ectopic expression of miR-146a or -146b in MDA-MB-231 resulted in a ~50% reduction in EGFR protein levels as measured by immunoblot and densitometry.

Lung Metastases			
MDA-MB-231 Variant	Incidence	Mean ± SEM	% Suppression
Vector	8/8	39 ± 6	0
miR-146a	9/9	12 ± 1	69
miR-146b	9/9	6 ± 1	84

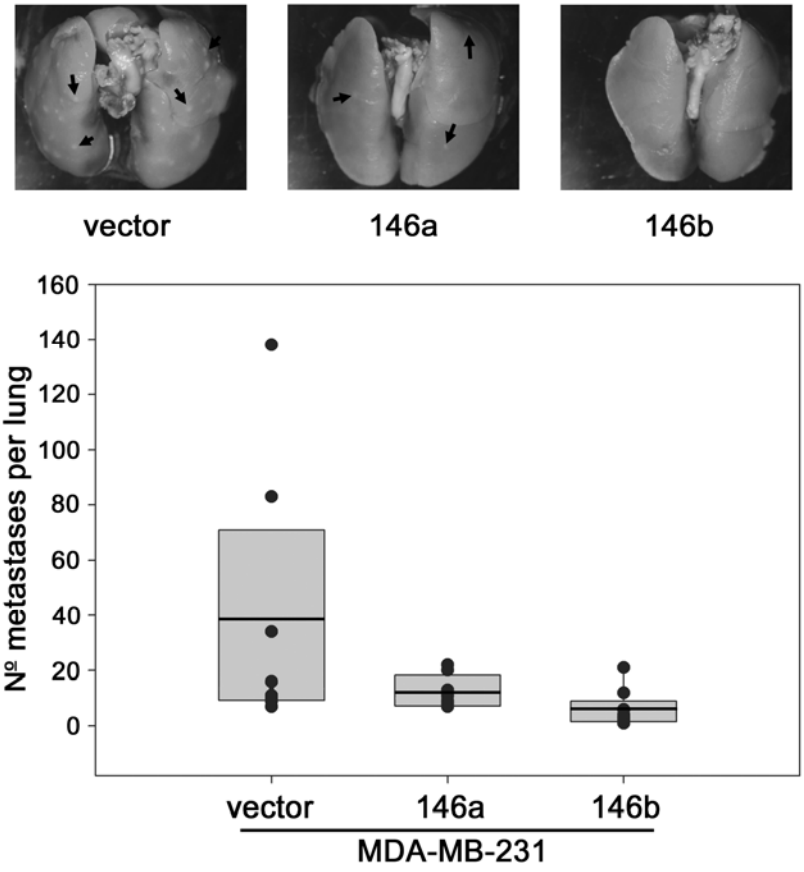

Figure 3. Metastasis is suppressed by miR-146. MDA-MB-231 cells expressing human miR-146a or -146b or vector-only were injected into the lateral tail vein of athymic mice and the lungs were analyzed for macroscopic metastases. The data is shown graphically with black dots representing the number of pulmonary metastases from each mouse; the box represents the 10^{th} and 90^{th} percentile; and the black line is the mean for each group. The table lists the incidence and the mean number of pulmonary metastases. Representative images for each group are pictured with arrows highlighting some of the individual lung metastases.

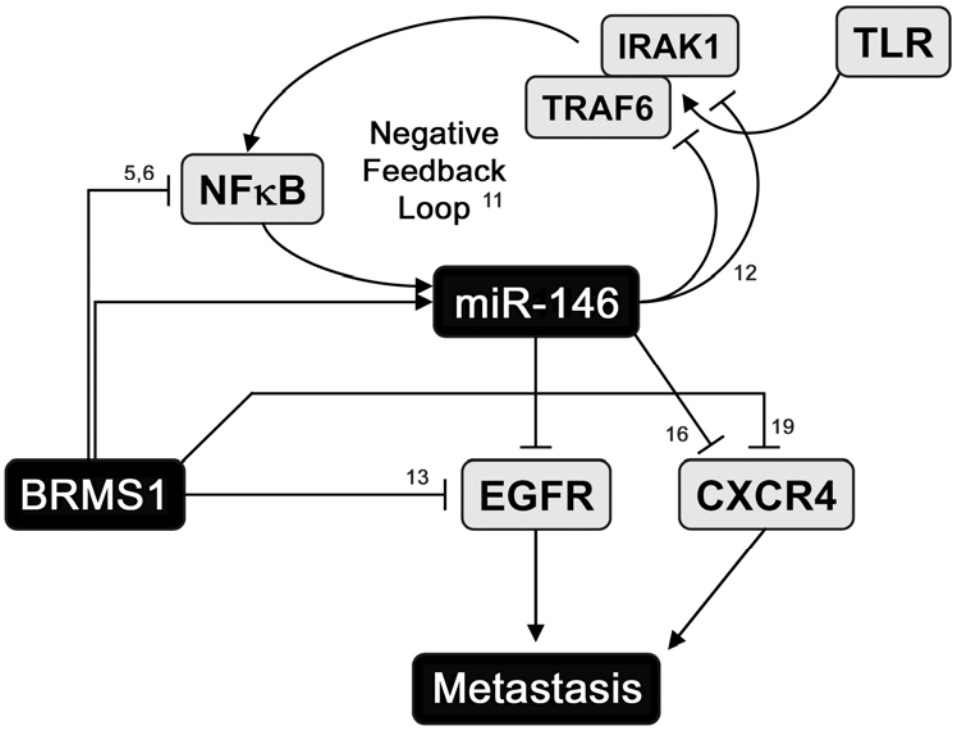

Figure 4. Working model for BRMS1-miR-146 axes in metastasis suppression. BRMS1 directly down-regulates transcription of EGFR (13), a predicted target of miR-146. Ectopic expression of miR-146a or -146b in cells that do not express BRMS1 have ~50% lower EGFR. BRMS1 may regulate CXCR4 directly or via miR-146. Both EGFR and CXCR4 correlate with increased metastasis. miR-146 upregulation by BRMS1 could be direct or indirect (by affecting NFκB). However, the connection through NFκB is parodoxical with a negative feedback loop described for NFκB and miR-146a in response to inflammatory stimulation (11).

MICRORNA CLUSTER 183 SUPPRESSES BREAST CANCER MIGRATION AND INVASION

by

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In preparation
Adapted for dissertation

ABBREVIATIONS:

BRMS1, Breast Cancer Metastasis Suppressor1

CTC, circulating tumor cells

EGFR, epidermal growth factor receptor

HDAC, histone deacetylase

EMT, epithelial to mesenchymal transition

miRNA, microRNA

miR, microRNA

pri-miRNA, primary microRNA

pre-miRNA, premature microRNA

183^{C}, miR-183, -96, -182

ABSTRACT

Metastasis is a multistep process requiring the coordinate regulation of numerous genes. MicroRNA are able to regulate hundreds of downstream target genes and have been shown to both promote and suppress metastasis. The Breast Cancer Metastasis Suppressor 1 coordinately regulates metastatic microRNA and increases miR-96 in metastasis suppressed breast carcinoma. We hypothesized that the entire miR-183,-96,-182 cluster (183^{C}) acts to decrease metastatic potential in breast carcinoma. Systematic knockdown of the miR-183^{C} resulted with increased invasion and migration in metastasis suppressed breast carcinoma cells. Further, over expression of these miRNA suppressed migration and invasion. Given these miRNA exist on a polycistronic RNA, we examined the consequences on the wildtype loci when exogenous miR-183^{C} components were expressed. Exogenous miR-96 and -182 increased the wildtype miR-183 cluster expression. These findings add to the growing list of miRNA that regulate metastatic potential and give further insight to the feedback mechanisms by which they are regulated.

INTRODUCTION

Metastasis is a multistep process in which many phenotypes must be exercised in order for a cancer cell to leave a primary tumor and colonize new tissues. The first step requires tumor genesis. After primary tumor formation, cells detach, invade through neighboring stroma and basement membranes, and eventually gain access to the vasculature (lymphatic or cardiovascular) (1). Once in circulation, cancer cells must survive the physical hazards of such transport and immune pressures brought forth by both innate and adaptive systems (2;3;4). Eventually circulating tumor cells (ctc) arrest

in capillary beds and either extravasate into the new tissue, or proliferate within the bed and eventually burst the vessel. The final outcome is tumor cells seeding a new tissue. The overall process of dissemination can be undergone in less than a few minutes.

Given the many steps mentioned above, it's logical to assume that an equal (or greater) amount of genetic and epigenetic events must occur for metastasis to take place. This rationale led many investigators to examine microRNA (miRNA) as modulators of metastatic potential. Individual miRNA are able to affect the expression of hundreds of genes, and could conceptually facilitate the vast and rapid changes in gene expression required for a cell to metastasize (5;6). Ma and colleagues first demonstrated this phenomenon and since then many miRNA have been shown to both promote and suppress metastasis, termed metastamiR (7-12). Several metastamiR have been linked to genes that have long been known to affect metastasis. For example, Twist1 was shown to promote expression of the prometastatic miR-10b (7). Likewise, the metastasis suppressor BRMS1 has been shown to coordinately influence the expression of multiple metatastamiR (13;14). BRMS1 increases the metastasis suppressing miRNA, while at the same time decreasing metastasis promoting miRNA. Array data also demonstrated that BRMS1 changed several other miRNA not previously associated with metastasis (13).

Here we examine the role of one of those miRNA, miR-96, which is increased in metastasis suppressed breast carcinoma cell lines. We find that miR-96, in addition to its other polycistronic cluster members miR-183 and 182, increase in metastasis suppressed breast carcinoma cells, and that these miR alone can suppress metastatic breast cancer migration and invasion. Additionally, the ectopic expression of cluster components caused an increase in expression of the wildtype miR-183^{C}.

MATERIALS AND METHODS

Cell lines and cell culture

MDA-MB-231 (231) and MDA-MB-435 (435) are human estrogen receptor- and progesterone receptor-negative cell lines derived from pleural effusions of metastatic infiltrating ductal breast carcinomas (15;16). Both cell lines form progressively growing tumors when injected into the mammary fat pads of immunocompromised mice. MDA-MB-435 cells develop macroscopic metastases in lungs and regional lymph nodes by 10-12 weeks post-inoculation, but infrequently metastasize after direct injection into the lateral tail vein or following subcutaneous injection. In contrast, MDA-MB-231 cells form macroscopic metastases when injected intravenously, but less commonly following injection into an orthotopic site. Both lines form osteolytic metastases following injection into the left ventricle of the heart (17-19). The origin of 435 has been questioned; however, recent publications have soundly reaffirmed the origins of the cell line and demonstrate the line to be of breast cancer in origin (20-22).

231 and 435 Parental and BRMS1 transduced counterparts were cultured in a mixture (1:1 *v:v*) of Dulbecco's modified Eagle's medium (DMEM) and Ham's-F12 medium (DMEM/F12, Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine, 0.02 mM non-essential amino acids and 5% fetal bovine serum (Invitrogen). Retropak cell line PT67 (Clontech, Mountain View, CA) was cultured in accordance to manufacturer's specifications. All cultures were maintained without antibiotics or antimycotics on 100-mm tissue culture dishes (Corning, Corning, NY) at 37°C with 5 % CO_2 in a humidified atmosphere. When cultures reached 80-90% confluence they were passaged using a solution of 2 mM EDTA in Ca^{2+}/Mg^{2+}-free Dulbeccos phosphate

buffered saline (CMF-DPBS; Invitrogen). All cultures were regularly tested and confirmed negative for *Mycoplasma spp.* infection using a PCR-based test (Agilent Technologies, USA).

Constructs and transductions

Cells (231 and 435) were transduced with full-length *BRMS1* cDNA that was cloned into lentiviral constructs. Transduced cells were initially selected with puromycin (500 μg/mL) and maintained in puromycin (100 μg/mL) to ensure stable transduction. For routine culture, no antibiotic selection was used and expression has been verified to be stable for over two years. miRNA were cloned into the pLXSN Retroviral Vector(Clontech Laboratories, Mountain View CA, USA). PCR primer sets used were:

hsa-miR-96 FWD: GGAATTCCCCTCGTCCAGTGTGTCCCC
hsa-miR-96 REV CGGGATCCCGCTCCAGAGACGGTAGCCCCC
hsa-miR-182 FWD GGAATTCCCCAGCAGGAAGGGGGACTG
hsa-miR-182 REV CCGCTCGAGCGGCTCCTCTTGGCAGCACCCC
hsa-miR-183 FWD CGGGATCCCGTGGGCTGCTGGTGTCTGG
hsa-miR-183 REV CGGGATCCCGCTCAAGGCAGAAGTGGGTAAGGT
hsa-miR-183/96 FWD CGGGATCCCGTGACCCACTCCCTCCCCAG
hsa-miR-183/96 REV CGGGATCCCGACTCCCCCACCTCTGCCCT
hsa-miR-182/96 FWD GGAATTCCCCTCGTCCAGTGTGTCCCC
hsa-miR-182/96 REV CCGCTCGAGCGGCTCCTCTTGGCAGCACCCC
hsa-miR-183^{C} FWD CCGCTCGAGCGGGCACTGTCCCTGTCTCCTTGAA
hsa-miR-183^{C} REV CCGCTCGAGCGGAGCTGACTTGAGGACCTGTGACC

The underlined section of primer denotes linker regions for restriction enzyme sites; miR-96 primers contain EcoR1 and BamH1 restriction sites; miR-182 primers contain EcoR1 and Xho1 restriction sites; miR-183 primers contain BamH1 restriction sites; miR-183/96 primers contain BamH1 restriction sites; miR-182/96 primers contain EcoR1 and Xho1 restrictions sites; miR-183^{C} primers contain Xho1 restriction sites. Genomic DNA from 231 and 435 cell lines were used as template. Platinum® Taq DNA Polymerase High Fidelity (Invitrogen CA, USA) was used to amplify miR-183^{C} (5300bp)

and GoTaq Polymerase (Promega WI USA) for the remaining sequences. Restriction enzymes (Promega WI USA) and DNA Ligase (Promega WI USA) were used in accordance to manufacturer's specifications.

Plasmids were heat shocked into MAX Efficiency Stbl2™ Competent Cells (Invitrogen CA, USA) according to manufacturers specifications. Colonies were screened and sequenced at the UAB Genetics Sequencing Core. Large scale plasmid collection was performed using the Qiagen Maxi Prep system (Qiagen Inc, Valencia CA).

Plasmids were transfected into retroviral packaging cell line PT67 (Clontech, Mountain View, CA) using Lipofectamine 2000 Transfection Reagent (Invitrogen CA, USA) and selected in accordance to the manufacturers specifications. Retrovirus secreted into PT67 media was filtered using 0.22μm low protein binding filter (Millipore, Carrington Ireland) to remove cellular debris. 200μL of filtrate was added to 70% confluent 231 and 435 cells and selected using 500 μg/mL of G418 for 3 weeks.

Real-time quantitative PCR

miRNA expression was determined by collecting total RNA from 70–90% confluent cell cultures using Qiazol (Qiagen Inc, Valencia CA). RNA was purified using miRNeasy (Qiagen Inc, Valencia CA). MiScript primers (Qiagen Inc, Valencia CA), designed to recognize specifically mature miRNA (i.e., not pre-miR or pri-miR), were: hsa-miR-96_1, hsa-miR-182, and hsa-miR-183. miScript Precursor Assays (Qiagen Inc, Valencia CA) were used for precursor miRNA detection. Total RNA was reverse transcribed using miScript Reverse Transcription Kit (Qiagen Inc, Valencia CA), and targets polymerized using miScript SYBR Green PCR Kit (Qiagen Inc, Valencia CA) on an Applied Biosystems 7500 Real-Time PCR System according to the manufacturers

specifications. All samples were normalized to small nuclear RNA U6 and fold changes were calculated using ABI 7500 v2.0.1 software.

miRNA knockdown.

Qiagen miScript inhibitors were used to transiently knockdown miRNA (anti-hsa-miR-96, -182, and -183). AntimiR were transiently transfected using HiPerFect transfection reagent (Qiagen Inc, Valencia CA). We performed time point analysis for 24, 48, 72hours at 5nmole, 25nmoles, and 50nmoles of inhibitor and observed the most robust and consistent knockdown at 48hours and 50nmoles inhibitor.

Scratch Assay

Cells were cultured to confluence on 12-well plates (50,000cells/well, initial plating) and four equatorial, linear scrapes were made with a sterile P200 pipette tip. After scratches were made, detached cells from scrape were removed by washing wells with room temperature DPBS (Invitrogen, Carlsbad, CA). Phase micrographs of the wound cultures were taken at 0 and 18 h (doubling time~24 hours). Photographs were analyzed by measuring the distance from the wound edge of the cell sheet to the original wound site. Migration activity was calculated as the mean distance between edges in 4 fields per well. Given that the scratch can be highly variable, we chose the fields adjacent the 4 areas of intersection. Both 0hr and 18hr pictures were taken in the same location. Each test group was assayed in quadruplicate, and results were expressed relative to parental and BRMS1 migration.

Motility and Invasion Assays

BD BioCoat (BD, Franklin Lakes, NJ USA) control 8.0 μm PET membrane inserts were used in accordance with manufacturers specifications in 24 well plates for

motility assay. BD BioCoat Matrigel Invasion Chambers, 8.0 μm PET Membrane 24-well cell culture inserts were used for invasion assays. Briefly, lower chambers were filled with 0.75 mL of room temperature 5% FBS-supplemented DMEM-F12 (Invitrogen, Carlsbad, CA). The inserts were rehydrated by adding 0.5 mL of room temperature DMEM-F12(no serum) and incubated at 37°C in 5% CO_2 for 2hrs and then media aspirated. Next, 0.5mL of DMEM-F12 containing $1x10^6$ cells were added to inserts and incubated at 37°C in 5% CO2 humidified atmosphere for 20hr. Inserts were removed and a cotton swab was applied to remove non-migrating cells. A second, pre-hydrated, cotton swab was again used to removing any remaining non-migrating cells. Inserts were fixed and stained using the Diff-Quick stain kit (Dade Behring, Inc. Newark, DE). Inserts were incubated in each solution for 2 minutes (Fixative, Solution1, Solution2), and rinsed with water between stains. Using a microscope to count migrating/invading cells, four fields per insert were scored (magnification 200×). Each group was done in quadruplicate and results were expressed as cell/field.

Metastasis Assays

Cells at 80-90% confluence were detached using 0.2 mM EDTA in Ca+2 , Mg+2 and $NaHC0_3$ free Hank's balanced salt solution (HBSS). Viable cells were counted using a hemacytometer and resuspended at a final concentration of 2.5×10^6 cells/mL in ice cold HBSS. Female athymic mice (3-4 wk; Harlan Sprague Dawley, Indianapolis, IN) were injected with 0.2 mL cell suspension into the lateral tail vein. Mice were necropsied 6 wk post inoculation following anesthesia with Ketamine:Xylazine and euthanasia by cervical dislocation. All organs were observed for presence of macroscopic metastases. Lungs were removed and fixed in a mixture of Bouin's fixative and neutral buffered

formalin (1:5 v/v). Mice were maintained under the guidelines of the National Institutes of Health and the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Food and water were provided ad libitum.

Statistical Analyses

The number of lung metastases was compared for miR-96, -182, or -182/96 transduced cell lines to the vector-only transduced line. A Kruskal-Wallis ANOVA of ranks procedure was used with Dunn's post hoc test. Calculations were performed using SigmaStat statistical analysis software (SPSS Inc., Chicago, IL). Statistical significance was defined as a probability $P \geq 0.05$.

RESULTS AND DISCUSSION

Thus far, the reported biology for miR-183, -96, -182 (183^C) in cancer has been very diverse (23-27). Some groups have demonstrated members of the cluster to act as an oncogene in breast cancer, and a tumor suppressor in pancreatic cancer (28;29). For example: miR-96 and -182 target the tumor suppressor FOXOA1 and promote proliferation in breast carcinoma cells; yet miR-96 targets K-RAS and induces apoptosis in pancreatic cancer. Others have shown that the entire miR-183^C down regulated in breast cancer stem cell compartments and that the metastasis suppressor BRMS1 increases miR-183^C thereby implicating a role for the cluster in decreasing metastatic potential (30;13). We sought to determine if miR-183^C was a mediator for the metastasis suppressed phenotype and first tested whether knockdown of the cluster in metastasis suppressed cells resulted in increased migration *in vitro*. Toward that end, the first test employed was a monolayer wound healing assay and the second a two chamber chemotaxis assay.

For the wound layer assay MDA-MB-435 (435), 435^{BRMS1}, and 435^{BRMS1} with systematic miR-183 knockdown (anti-miR-183^{C}) were compared for their ability to migrate into the wound. In preparing to manipulate (knockdown in this instance) the miR-183^{C}, we took caution as the three miR share a canonical seed region and may bind similar target sequences. We hypothesized that the combinations of miR-183^{C} members may have dramatically different phenotypes from the individual miR themselves. This led us to examine the affect of manipulating all possible combinations of the cluster (7 total), not just the individual miRNA. As previously reported, 435^{BRMS1} cells migrate less when compared to parental 435 cells (Fig 1a). Knockdown of miR-96 and miR 183^{C} increased cells migrating into the scratch.

Next, using the Boyden chamber assay, we further tested the migration ability of MDA-MB-231^{BRMS1} and $231^{BRMS1/anti\text{-}miR\text{-}183C}$ cells. As seen with the 435^{BRMS1} cells in the scratch assay, miR-183^{C} knockdown resulted in ~2 fold increase in migration (Fig2a). Likewise, retroviral transduction of miR-96, -182, and -182/96 into 231 cells decreased migration ~50% (Fig 2b). We also observed significant morphological changes in the $231^{miR\text{-}183C}$ cells that were not seen in the vector or BRMS1 cells. The changes only occurred during the Boyden chamber experiments and were only seen in the retroviral expressed $231^{miR\text{-}183C}$ cells, not the knockdowns. Morphologically the cells are much more round and epithelial than the mesenchymal appearing parental and vector cells (Fig 2c). These morphological changes, though only seen during the assay above, are consistent with the logic that an epithelial to mesenchymal transition (EMT) increases a cells metastatic potential and the reverse of such process (MET), possibly seen here, would lead to the phenotypes witnessed *in vitro*.

The above results led us to examine another key step in metastasis: invasion. These experiments were nearly identical to the migration chamber assay above, except these chambers contained a matrigel overlay. The matrigel contains matricellular proteins and mimics the extracellular matrix found in many tissues. For the breast cancer cells to invade to the bottom of the chamber, the matrix must be degraded and manipulated. As shown in figure 3b, cellular invasion increases when miR-96 is knocked down in 231^{BRMS1} and 435^{BRMS1} cells. Likewise, when miR-96, -182, and -182/96 were over expressed in 231 cells, invasion was suppressed (Fig 3a).

The ability of the miR-183^{C} to suppress invasion and motility *in vitro* was quite remarkable; however, these assays still remain surrogates to the phenotype we have set forth to study, metastasis. Metastatic potential is really only assayed *in vivo*, and we chose to use an experimental assay where breast carcinoma cells are introduced via tail vain to the venous circulation directly. In this assay, 231 cells arrest in the capillaries of the lung and colonize the lung; whereas, other non-metastatic lines such as the MCF-7 breast carcinoma cell line do not. The assay does have its technical limitations as it skips several of the early steps in the metastatic cascade. That withstanding, the assay still captures one of the most clinically crucial steps of metastasis, colonization of an ectopic site.

Parental 231, 231^{vector}, $231^{miR\text{-}96}$, $231^{miR\text{-}182}$, and $231^{miR\text{-}182/96}$ were all assayed for their metastatic ability. Though mean metastasis decreased 10% in $231^{miR\text{-}96\ cells}$, these differences were not statically significant, and the amount of metastases actually increased in $231^{miR\text{-}182}$ cells. These miRNA may act at the early steps of metastasis. Given the *in vitro* data demonstrating suppression of both invasion and migration, an additional

system that allows for spontaneous metastasis and encompasses all the steps of the metastatic cascade would fully address the effect of these miR on metastatic potential. The 435 cell line allows for such a study and after orthotoptic implantation into the mammary gland of nude mice, form metastases several months later.

We have shown the miR-183^{C} to regulate metastatic potential in breast carcinoma cells and are now led to ask how the cluster is regulated. Just after the cluster was implicated in cancer progression, Xu S and colleagues nicely described a function for miR-183^{C} in sensory specific cells (31). The cluster is reported to regulate core circadian machinery and may be involved in a feedback loop. The concept of miRNA involved in both feedforward and backward circuits is not new (32-36). However, it is interesting that many of the miRNA are polycistronic, and we asked whether exogenous miR-183^{C} components could influence the expression of endogenous mature miR-183^{C} members. Real-time quantitative PCR (RTQ) of RNA from 231^{miR-96} cells revealed that endogenous mature miR-182, and miR-183 increase. Also, in 231$^{miR-182}$ cells, mature miR-96 and miR-183 increase. And finally, in 231$^{miR-182/96}$ cells, mature miR-183 increases (Fig 4).

The increase in mature miRNA could arise from either transcriptional and/or posttranscriptional events. Polycistronic messages often adopt significant secondary structure that influences processing and protein binding (37). The exogenous miR-183^{C} cells (231^{miR-96} , 231$^{miR-182}$,231$^{miR-182/96}$) express the wildtype hairpin sequence which could promote a pro-cleavage structure in the endogenous 183^{C}. RTQ revealed increases in the premature sequences for the endogenous miRNA (Fig.5). In the 231^{miR-96} cells, miR-96 precursor sequence goes up, which is expected given that these cells have increased miR-96 levels. However, pre-miR-182 and pre- miR-183 hairpins also increase

indicating that the elevated levels of endogenous miR-183^{C} is likely regulated at the level of transcription, and not post-transcription. To ensure that the response was specific to miR-183^{C} and not a total increase in miRNA, we examined the non-related, yet pro-metastatic miR-21 and did not observe changes in this miRNA when miR-183^{C} was over expressed (12).

These findings call for careful analysis of manipulating members of the miR-183^{C}. Many, including ourselves, have performed transient knockdown of the miR-183 cluster and have deduced the change in phenotype to be a result of the manipulated miRNA alone. Though the interpretations of such data may not necessarily change, it's clear that these miR influence the expression of one another. Clinically, this feedback mechanism poses promising features, as adding one of the members increases the remaining cluster components and a reduction in metastatic potential.

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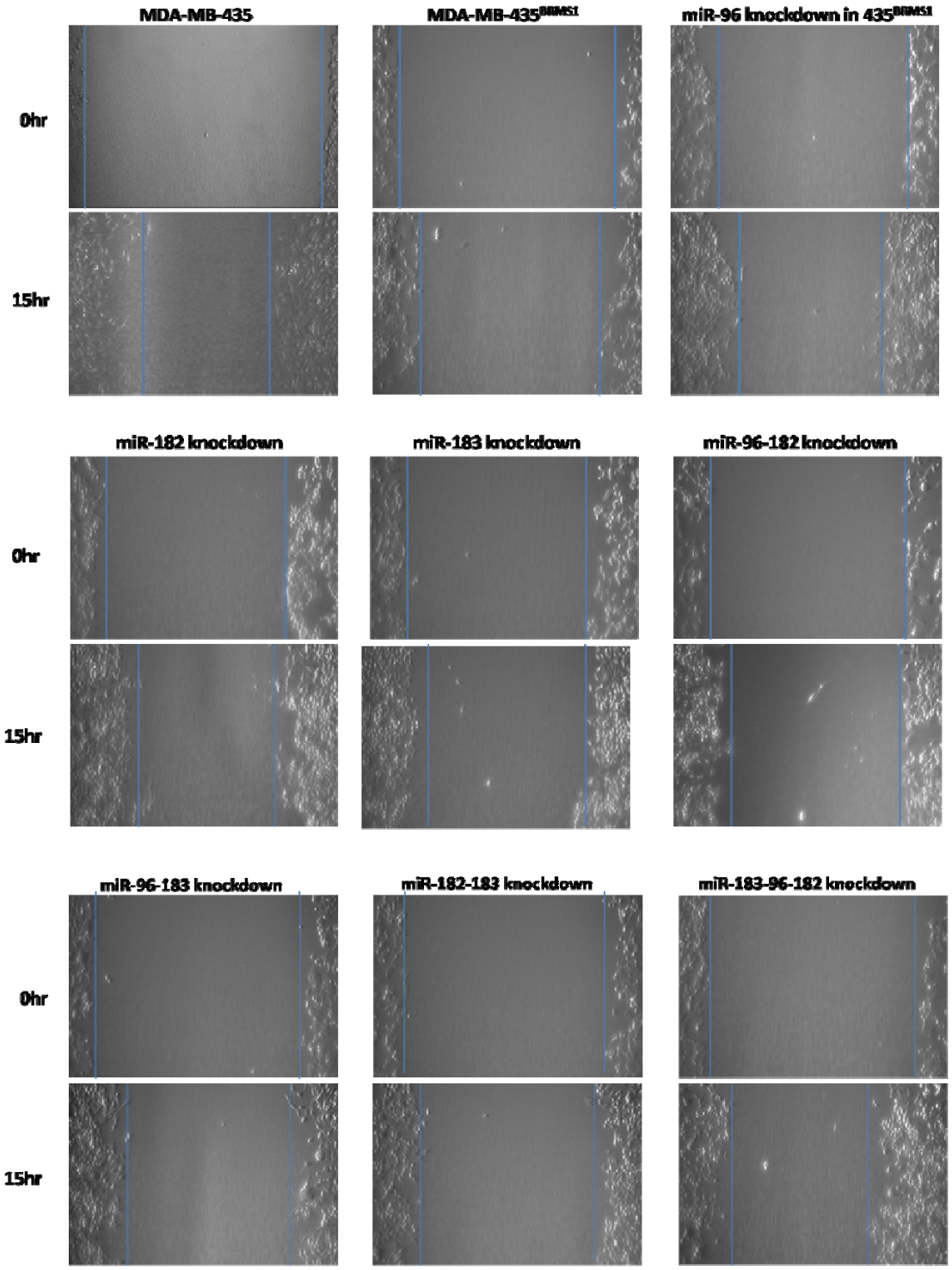

Fig. 1a. miR-96 and 183^{C} knockdown increases migration into an artificial wound. miR-183^{C} was transiently knocked down in MDA-MB-435^{BRMS1} cells and after 24hrs scratches were made in each experimental group. The pictures above were taken immediately after wounding, and then again after 15hrs.

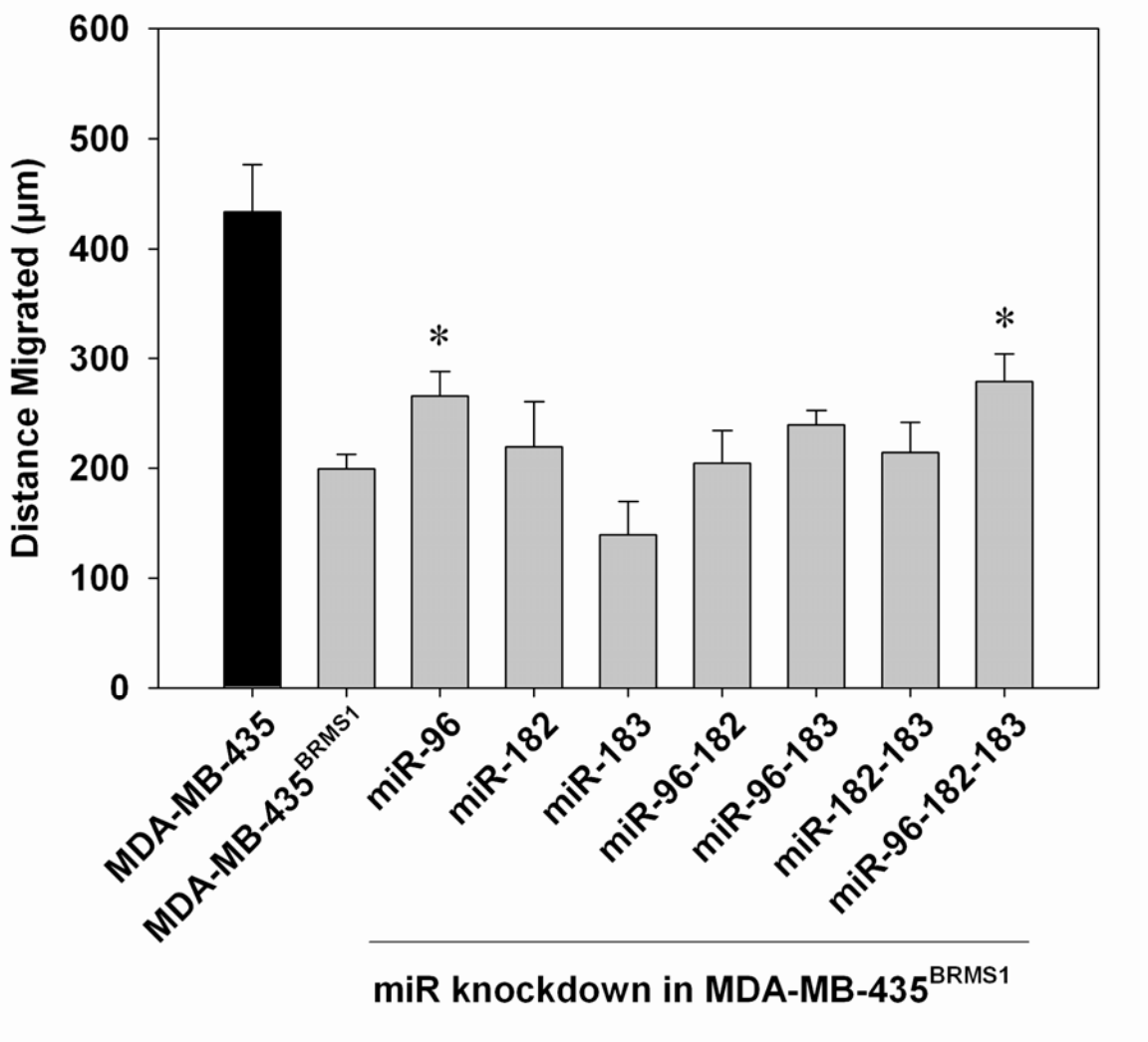

Fig. 1b. Distance migrated for wound heal assay. miR-96 and miR-183^{C} knockdown increased migration approximately 30% compared to 435^{BRMS1} cells. * $p \geq 0.05$.

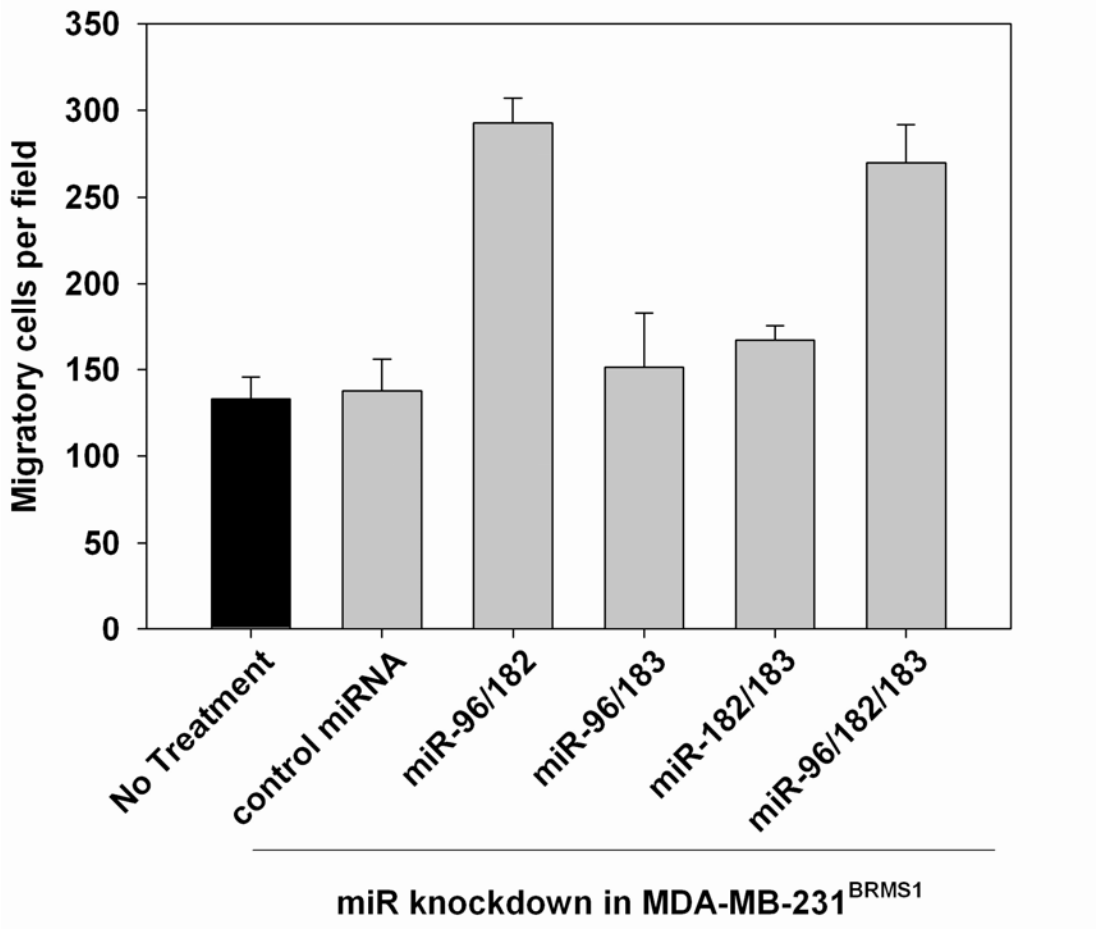

Fig. 2a. miR-183^{C} knockdown increases transwell migration in MDA-MB-231^{BRMS1} cells. Confluent (70-90%) 100mm plates were treated with anti miRNA for 24hrs and then cells were detached in 2mM EDTA. Cells ($1x10^5$) were added to the upper chamber of the transwell and allowed to migrate for 20hrs. Fields (4) were counted for each chamber and each group was performed in quadruplicate. Data is represented as average per group with SEM.

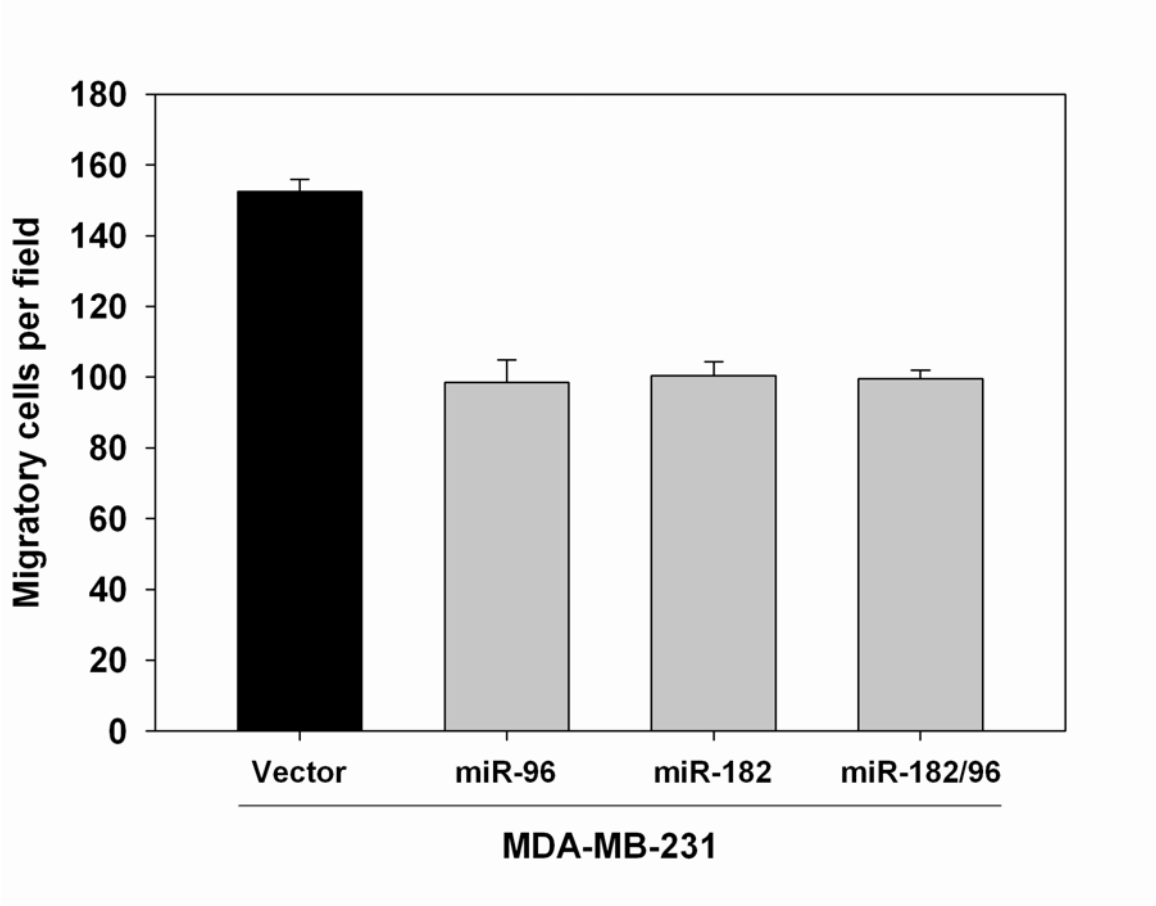

Fig. 2b. Ectopic expression of miR-96, 182, and 182/96 in MDA-MB-231 cells suppresses transwell migration. Retroviral transduced MDA-MB-231^{miR-96}, 231$^{miR-182}$, and 231$^{miR-182/96}$ cells ($1x10^5$) were added to the upper chamber of the transwell and allowed to migrate for 20hrs. Fields (4) were counted for each chamber and each group was performed in quadruplicate. Data are represented as average per group with SEM.

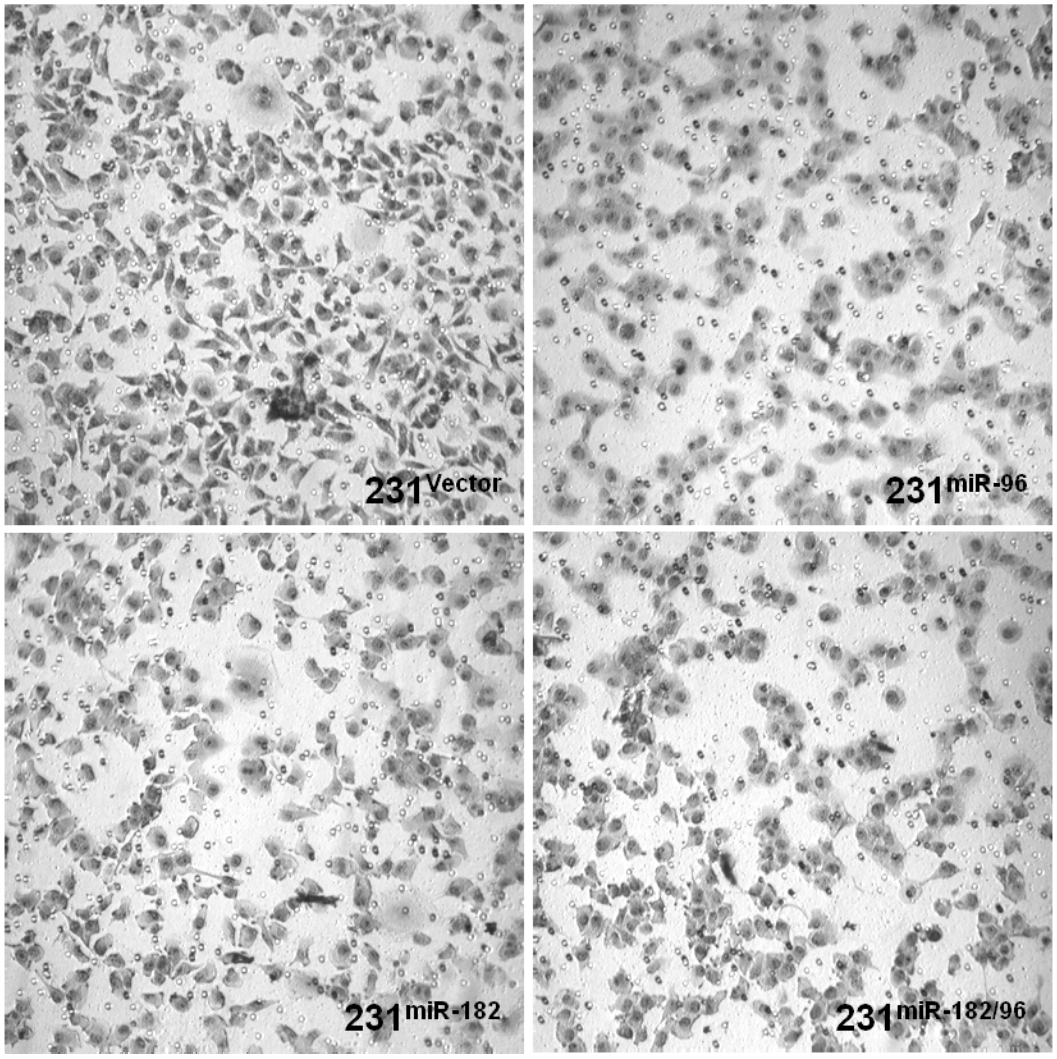

Fig. 2c. MDA-MB-231^{miR-96}, 231$^{miR-182}$ and 231$^{miR-182/96}$ expressing cells change morphology in transwell migration assay. In addition to decreased motility, MDA-MB-231^{miR-96}, 231$^{miR-182}$ and 231$^{miR-182/96}$ cells had dramatically different morphologies from 231^{Vector} and appear more round and epithelial in appearance.

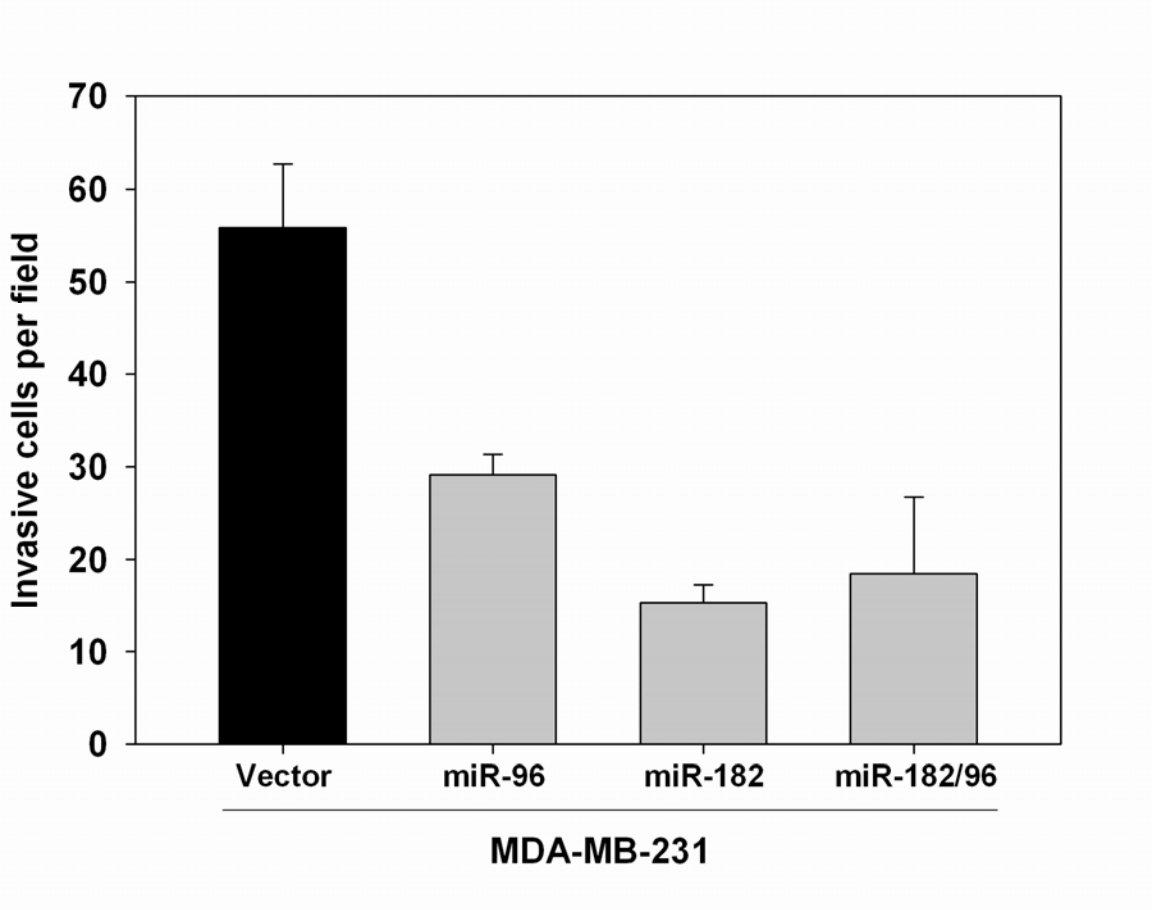

Fig. 3a. Ectopic expression of miR-96, 182, and 182/96 in MDA-MB-231 cells suppresses transwell invasion. Retroviral transduced MDA-MB-231^{miR-96}, 231$^{miR-182}$, and 231$^{miR-182/96}$ cells ($1x10^5$) were added to the upper chamber of the transwell and allowed to invade for 20hrs. Fields (4) were counted for each chamber and each group was performed in quadruplicate. Data are represented as average per group with SEM.

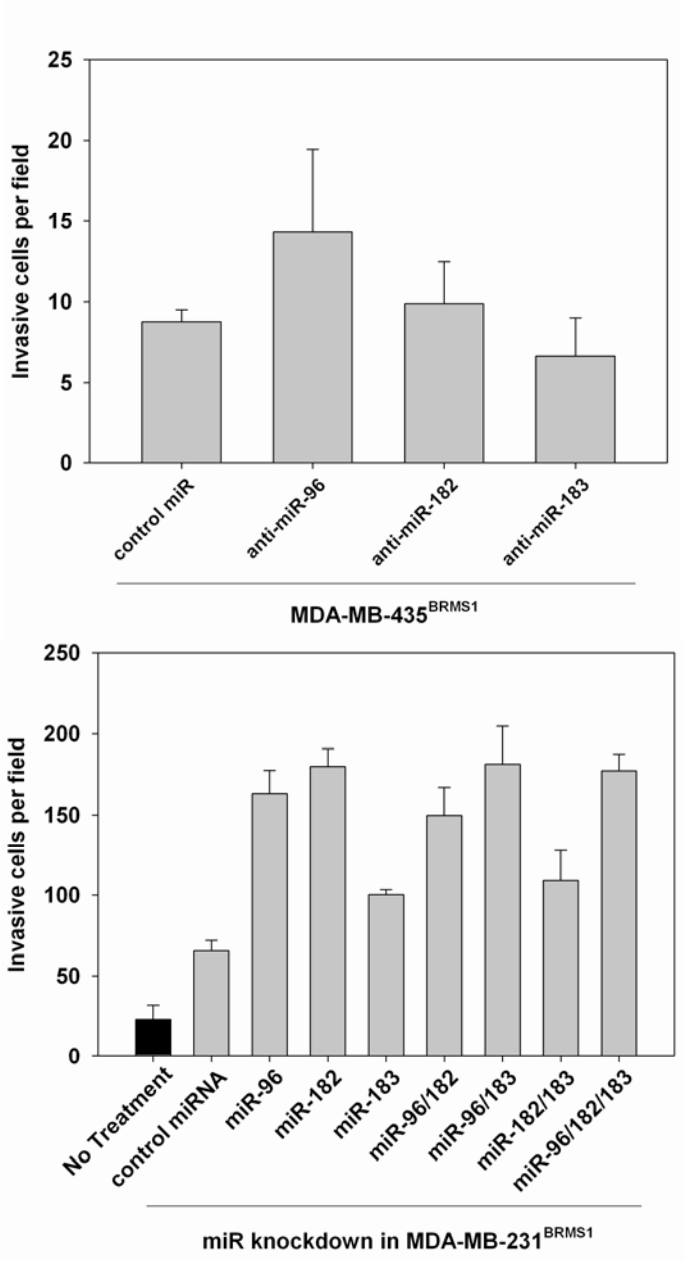

Fig. 3b. Knockdown of 183^{C} in BRMS1 cells increases transwell invasion. Confluent (70-90%) 100mm plates were treated with anti miRNA for 24hrs and then cells were detached in 2mM EDTA. Cells ($1x10^{5}$) were added to the upper chamber of the transwell and allowed to invade for 20hrs. Fields (4) were counted for each chamber and each group was performed in quadruplicate. Data is represented as average per group with SEM.

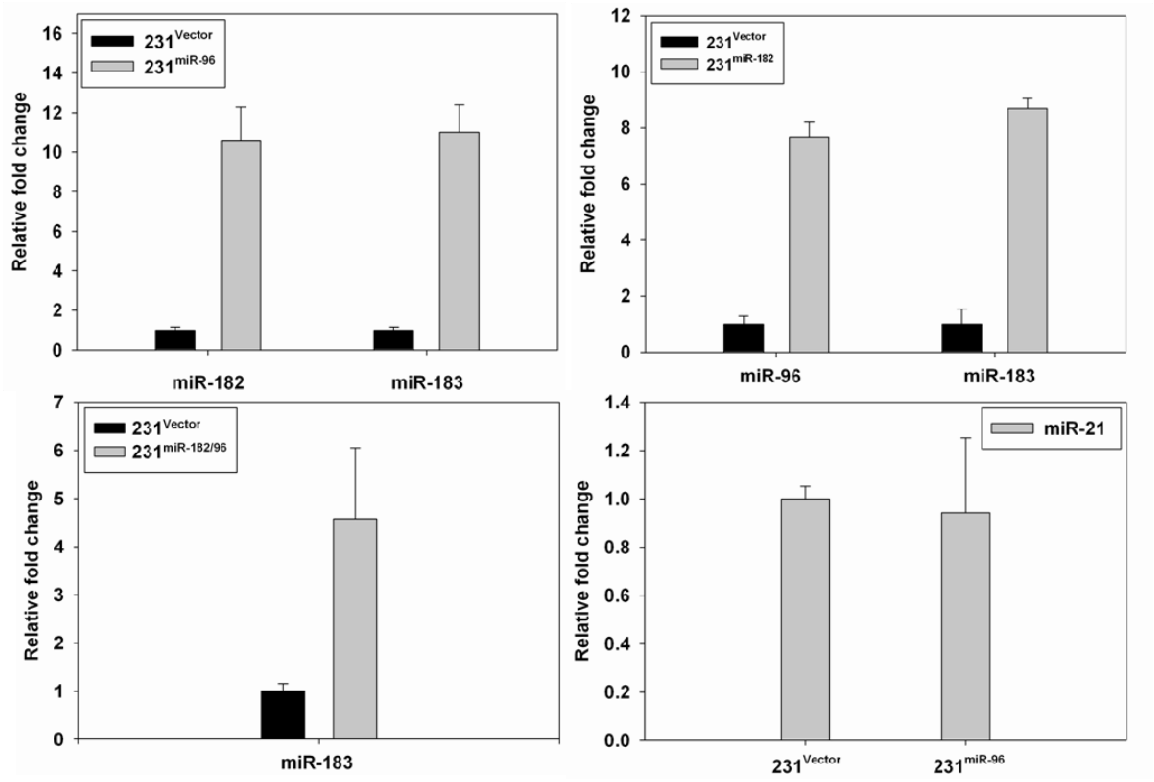

Fig 4. Exogenous miR-183^{C} components activate expression of the endogenous loci. Total RNA was extracted from MDA-MB-231^{Vector}, 231^{miR-96}, 231$^{miR-182}$ and 231$^{miR-182/96}$ cells, reverse transcribed and miR assessed using SYBR-green real-time RT-PCR.

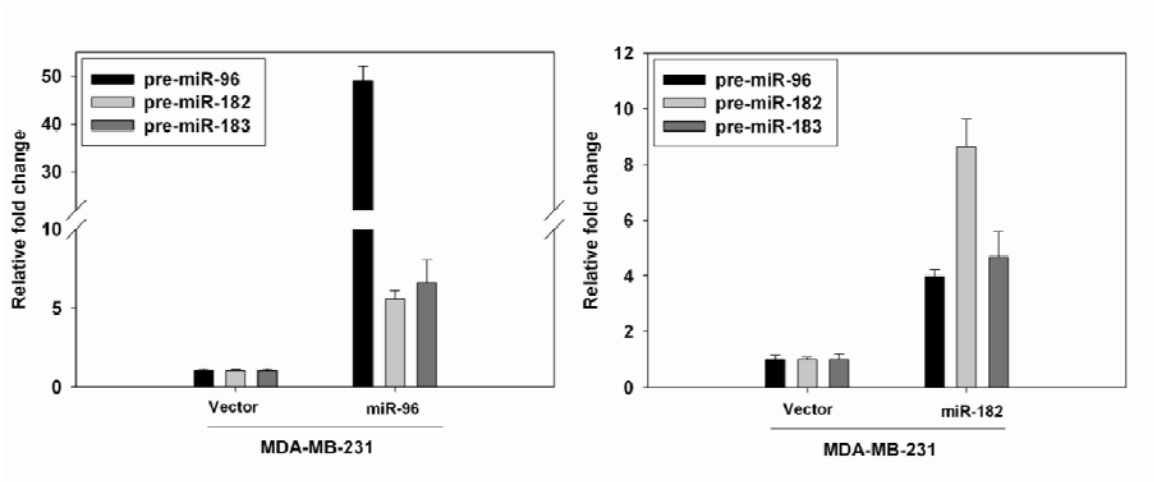

Fig 5. Exogenous miR-96 and -182 increase expression of miR-183^{C}. Total RNA was extracted from MDA-MB-231^{Vector}, 231^{miR-96}, and 231$^{miR-182}$ cells, reverse transcribed and pre-miR assessed using SYBR-green real-time RT-PCR.

Lung Metastasis

MDA-MB-231 variant	Incidence	Mean ± SEM
Parent	9/10	162 ± 22
Vector	8/10	144 ± 27
miR-96	10/10	130 ± 28
miR-182	10/10	154 ± 28
miR-182/96	10/10	157 ± 25

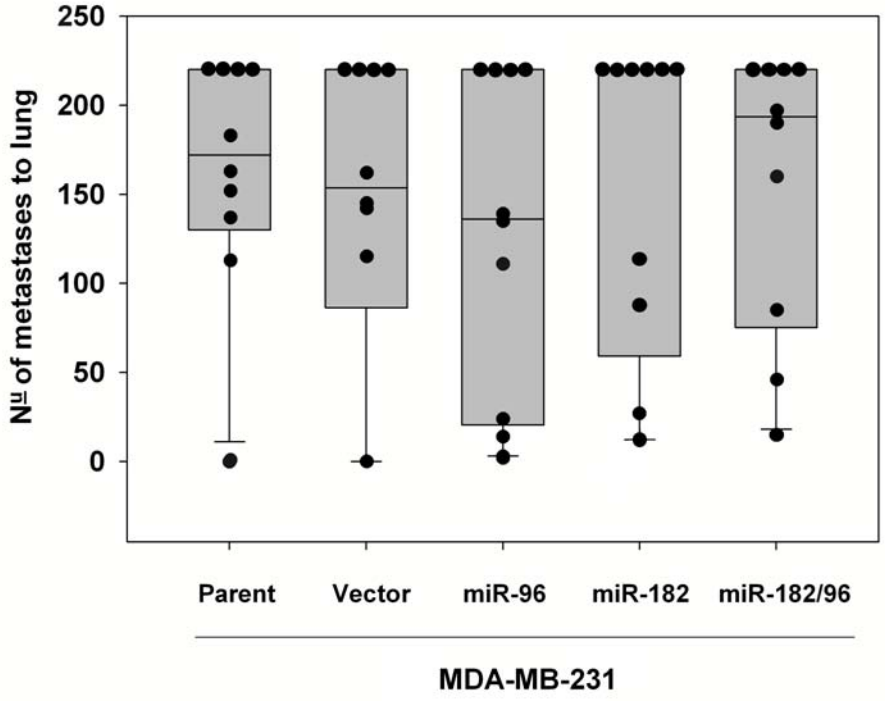

Figure 6. Experimental metastasis not suppressed by miR-96, 182, or 182/96. MDA-MB-231 cells expressing human miR-96, 182, 182/96 or vector-only, were injected into the lateral tail vein of athymic mice and the lungs were analyzed for macroscopic metastases. The data is shown graphically with black dots representing the number of pulmonary metastases from each mouse; the box represents the 10th and 90th percentile; and the black line is the mean for each group. The table lists the incidence and the mean number of pulmonary metastases. 220 metastases was the maximum amount of metastases that could be distinguished on a lung before the foci grew together and were undistinguishable.

CONCLUSIONS

In the past decade the scientific community has gone from learning the existence of a handful of small non-coding RNA in lower vertebrates to the now evident fact that miRNA are implicated in nearly every cellular process. As miRNA have been identified in human cancer, their discovery has not necessarily led toward new pathways and signaling cascades; rather a dovetailing of prior knowledge to the growing list of miRNA involved in cancer progression. For example, we now know that much of the oncogenic behavior of c-Myc stems from the microRNA cluster miR-17-92 (38). Dysregulated expression or function of c-Myc is one of the most common abnormalities in human malignancy and chromatin immunoprecipation experiments have shown that c-Myc binds directly to the miR-17-92 loci. E2F1, a transcription factor that regulates cell cycle progression, is a target of c-Myc and now we know it is negatively regulated by miR-17-5p and miR-20 (39).

Likewise, it has long been established that the tumor suppressor p53 is a transcriptional activator; yet, numerous reports indicated p53 could also repress the expression of specific genes either directly or indirectly, and at the transcriptional and post-transcriptional levels (reviewed in 40). These observations were made just as miRNA were found in mammalian cells and linking p53 to miRNA did not take long. The expression of miR-34a/b/c are all induced by DNA damage and oncogenic stress in a p53 dependent manner. Increased miR-34 leads to apoptosis and cellular senescence, whereas its reduction attenuates p53-mediated cell death (41-45).

When this dissertation work started, it had not yet been determined if miRNA would influence metastatic potential. We now know they do and like the classical

oncogene and tumor suppressor mentioned above, metastasis associated genes have also been found to have miRNA act in both up and downstream manners for their function.

These metastamiR both promote and suppress metastatic disease in nearly every cancer (46). BRMS1 suppresses metastasis in multiple tissue types, and elicits dramatic changes in gene expression. We've found that in metastasis suppressed breast carcinoma cells, BRMS1 is able to coordinately decrease pro-metastatic miRNA, while at the same time increase the metastasis suppressing miRNA. miRNA arrays led us to discover several metastamiR regulated by BRMS1 (10b, 373 520c, 335). Other miRNA such as 146a/b were linked to BRMS1 based on their overlapping functions. Both BRMS1 and miR-146a/b suppress *in vitro* migration and invasion in breast carcinoma cell lines. Further, both decrease epidermal growth factor receptor (EGFR) expression. Likewise, RTQ PCR revealed that miR-146a/b increase in both 231^{BRMS1} and 435^{BRMS1} cells. And finally, endogenous expression levels of miR-146a and -146b in multiple breast derived cell lines generally show decreased levels in tumorigenic but weakly/non-metastatic cell lines and BRMS1 restores expression of these miR. These observations led us to test whether 146a/b could decrease metastatic potential in an experimental metastasis model where 231 cells had 146a/b stably re-expressed. In short, 500,000 cells were injected into the lateral tail vein of athymic mice. 10 mice were used per group and the experiment duration was 10 weeks. The animals were euthanized and the lungs removed, fixed, and surface metastases were counted using a dissecting scope. cell lines expressing miR-146a/b suppressed metastasis, and this was the first time a metastasis suppressor was shown to regulate metastamiR.

Other metastamiR such as miR-10b decreased in BRMS1 cells and we hypothesized that metastatic potential would increase if it were added back to BRMS1 expressing cells. To do this, we transduced 231^{BRMS1} cells with human miR-10b under the control of a viral 5 LTR sequence, not the endogenous promoter as BRMS1 would likely prevent its expression. Figure 1 shows that over expression of miR-10b leads to a approximately 3 fold increase in migration and invasion. Expression of miR-10b was never greater than 2 fold, suggesting to us that the hairpin formation was less than ideal as several transductions were made. The increase in invasion is identical to that described by Li Ma et al. in 2007. RhoC, the downstream target and assumed effector molecule of 10b increased in $231^{BRMS1\text{-}miR\text{-}10b}$ cells (Fig 1). Interestingly the miR-10b target gene HoxD10 also decreased in the $231^{BRMS1\text{-}miR\text{-}10b}$ cells. Given the canonical method for miRNA function, this was not expected; yet, Kong et al. demonstrated in 2008 that miRNA function may differ based on the promoter by which its expression is derived (47). The miRNA targets do not change, yet the mode of action may. It appears that HoxD10 mRNA is degraded in the miR-10b cell line and regardless of the mechanism, HoxD10 is decreased.

As for the regulation of miR-10b, we reported in the previous chapter that Twist1 increases miR-10b expression. Twist1 can be activated via EGFR signaling. The metastamiR 146 targets EGFR, and we hypothesized that miR-146a/b expression could decrease miR-10b expression.Comparing miRNA from 231^{Vector}, $231^{miR\text{-}146a}$, and $231^{miR\text{-}146b}$ cells, we see that miR-10b is suppressed in the miR-146a/b cell lines (Fig. 2b). What was not expected was the finding that in $231^{BRMS\text{-}miR10b}$ cells, miR-146a/b decreases. There is no signaling yet reported that could explain these findings. Regardless, these

data link two very important metastamiR with having possibly opposing roles in breast cancer metastasis.

We next assayed the $231^{BRMS1/miR\text{-}10b}$ cells metastatic potential using an experimental metastasis model, identical to that used for the $231^{miR\text{-}146a/b}$ cells. As seen in figure 3, incidence of metastases increased in $231^{BRMS1/miR\text{-}10b}$ cells; however, metastasis did not. This leads us to conclude that the decrease of miR-10b in BRMS1 expressing cells may act to prevent metastases before the cancer cells enter circulation. These data also agree with the findings of Ma et al. who after identifying miR-10b as the first metastasis promoting miRNA, demonstrated that therapeutic knockdown of the miR in the primary tumor could prevent spontaneous metastasis (49). In their study they demonstrated successful pharmacologic knockdown of miR-10b in tumor tissues using a miR-10b antagomiR. Antagomir are chemically modified small nucleic acid sequences that bind miRNA of interest. The caveat to their therapeutic findings was that antagomir-10b treatment on tumor cells that have already disseminated did not prevent metastasis. To do this, they performed tail vein injection using mouse 4T1 cells. Despite reduction of miR-10b levels in mouse tissues (nearly 70%), both the PBS and antagomir mouse groups developed similar numbers of lung metastases. These data are in agreement with ours and indicate that manipulation of miR-10b does not affect the later stages of the metastatic process.

This dissertation work also sought to characterize the polycistronic cluster miR-183, -96, -182 for modulating metastatic potential. The first oncomiR was actually the polycistronic cluster miR-17-92 and we hypothesized that $miR\text{-}183^{C}$, which is increased in BRMS1 cells, could suppress metastasis. Though these experiments have already been

thoroughly described in the previous chapter, in summary, decreases of the cluster in the BRMS1 cells increased *in vitro* invasion and migration in both 231^{BRMS1} and 435^{BRMS1} cells. Experimental metastasis revealed the cluster not sufficient to suppress lung metastasis. Like miR-10b, we speculate miR-183^{C} may act at the early steps of the metastatic cascade and does not suppress metastasis once cancer cells disseminate. Another important possible explanation could be that miR-183^{C} may be necessary for BRMS1 metastasis suppression, but not sufficient. I make this statement based on the miR-183^{C} knockdown experiments in BRMS1 cells. Currently, the technology for the stable knockdown required for *in vivo* analysis does not yet exist and are thererore unable to fully test this hypothesis.

All three BRMS1 metastamiR groups (miR-10b, 146a/b, 183^{C}) tested *in vivo* highlight a key mechanism by which we believe BRMS1 exerts its ant-metastatic effects. As previously stated, it is the coordinate regulation of miRNA that leads to metastasis suppression in BRMS1 cells. Yes, miR-146a/b suppressed migration, invasion, and experimental metastasis, but not to the degree of BRMS1. For that matter, none of the three groups mimicked the BRMS1 phenotype entirely by themselves, and to explain this we employ the bacterial operon as a model to explain how BRMS1 suppresses metastasis. An operon gives a bacterium a specific phenotype and there are many different genes within that operon that operate or functino together to perform said phenotype. A classic example of this is the lac operon of E.*coli* (50). The operon is responsible for the transport and metabolism of lactose. Many processes take place to perform this function and each gene in the operon is responsible completing a specific part of said function.

Likewise, BRMS1 changes at least 9 known metastamiR, and by manipulating merely one or two of these miR, one should not expect to recapitulate the entire BRMS1 phenotype of metastasis suppression. These dissertation data demonstrate three key points. The first is that miRNA may exercise very strong effects on metastatic potential. The second, that metastamiR regulate one another as seen in the miR-10b-146 interplay, as well as the feed forward mechanism of miR-183^{C}. The final, that these miRNA can be coordinately regulated by a well known metastasis suppressor.

The strength by which miRNA can influence the outcome of metastatic disease is astonishing and many have sought to take advantage of these observations by manipulating miRNA to promote survival in metastastic disease. miRNA are stable in the circulation, very stable, and much more economical than peptides for synthesis purposes. This past decade has been one of discovery for miRNA research, and the next most likely one of application.

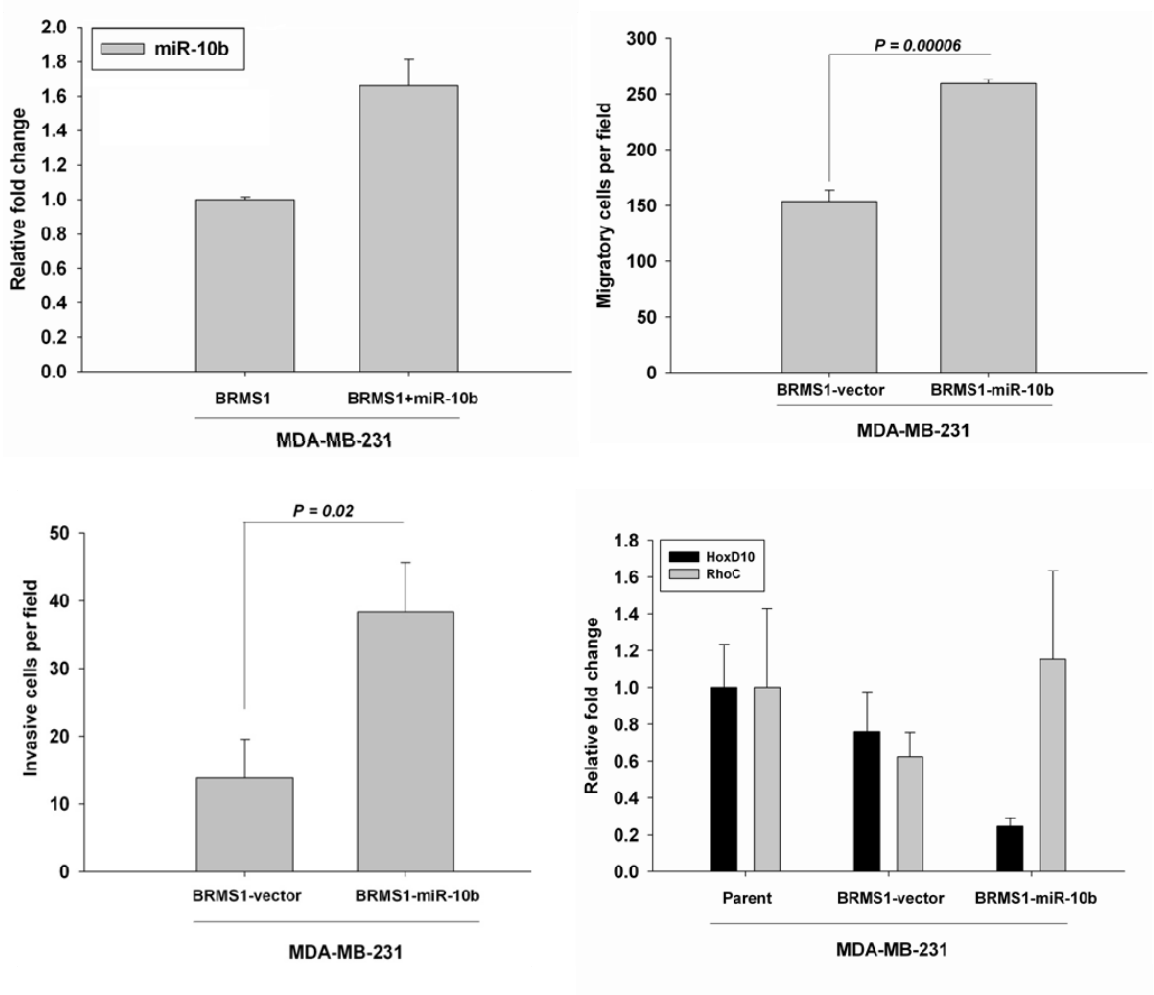

Figure 1. Re-expression of miR-10b in MDA-MB-231^{BRMS1} restores invasion and migration in metastastic breast carcinoma. Retroviral transduced MDA-MB-231$^{miR\text{-}10b}$ and 231^{vector} cells ($1x10^5$) were added to the upper chamber of the transwell and allowed to migrate/invade for 20hrs. Fields (4) were counted for each chamber and each group was performed in quadruplicate. Data is represented as average per group with SEM. Total RNA was extracted from MDA-MB-231, 231$^{BRMS1\text{-}vector}$ and 231$^{BRMS1\text{-}miR\text{-}10b}$ cell lines, reverse transcribed and miR assessed using SYBR-green real-time RT-PCR.

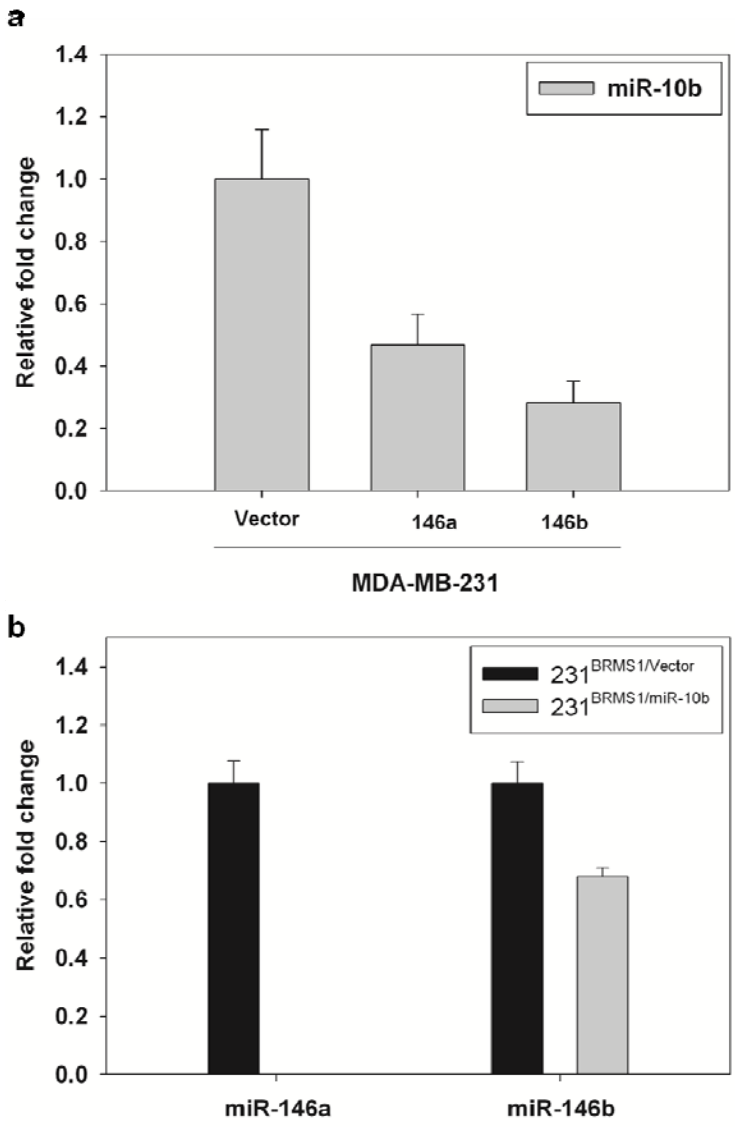

Figure 2. MicroRNA 10b and 146a/146b act to suppress one anothers expression. Ectopic expression of miR-146a/b in MDA-MB-231 decreases miR-10b expression. Likewise, re-expression of miR-10b in MDA-MB-231^{BRMS1} cells suppresses 146a/b expression. Total RNA was extracted from MDA-MB-231^{Vector}, 231$^{miR-146a}$, 231$^{miR-146b}$, 231$^{BRMS1/vector}$, and 231$^{BRMS1/miR-10b}$ cell lines, reverse transcribed and miRNA assessed using SYBR-green real-time RT-PCR.

Lung Metastasis

MDA-MB-231^{BRMS1} variant	Incidence	Mean ± SEM
Vector	7/10	6.8 ± 3.6
miR-10b	10/10	7.1 ± 2.3

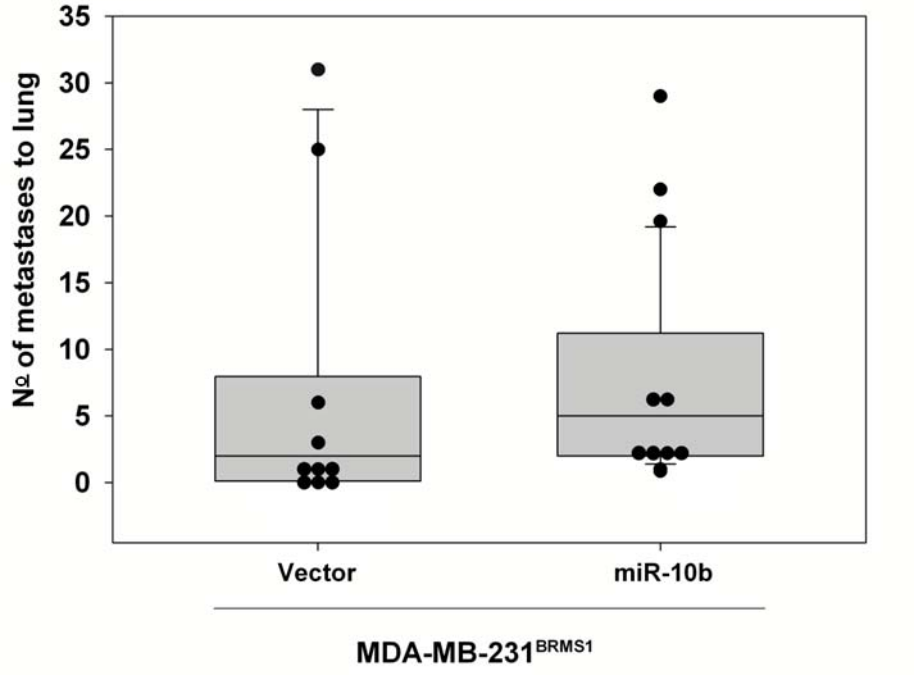

Figure 3. Experimental metastasis not restored by exogenous miR-10b in MDA-MB-231^{BRMS1} cells. MDA-MB-231 cells expressing human miR-10b or vector-only construct were injected into the lateral tail vein of athymic mice and the lungs were analyzed for macroscopic metastases. The data is shown graphically with black dots representing the number of pulmonary metastases from each mouse; the box represents the 10th and 90th percentile; and the black line is the mean for each group.

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APPENDIX

IACUC FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: June 11, 2010

TO: Welch, Danny
VH, G038 0019
934-2956

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: BRMS1 Regulation of miRNA to Suppress Metastasis (Mick Edmonds)
Sponsor: NIH
Animal Project Number: 1006S0368

On June 11, 2010, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
None	NA	0

Animal use is scheduled for review one year from June 2010. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 1006S0368 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
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