

miR-139-5p is a regulator of metastatic pathways in breast cancer

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ABSTRACT

Metastasis is a complex, multistep process involved in the progression of cancer from a localized primary tissue to distant sites, often characteristic of the more aggressive forms of this disease. Despite being studied in great detail in recent years, the mechanisms that govern this process remain poorly understood. In this study, we identify a novel role for miR-139-5p in the inhibition of breast cancer progression. We highlight its clinical relevance by reviewing miR-139-5p expression across a wide variety of breast cancer subtypes using in-house generated and online data sets to show that it is most frequently lost in invasive tumors. A biotin pull-down approach was then used to identify the mRNA targets of miR-139-5p in the breast cancer cell line MCF7. Functional enrichment analysis of the pulled-down targets showed significant enrichment of genes in pathways previously implicated in breast cancer metastasis ($P < 0.05$). Further bioinformatic analysis revealed a predicted disruption to the TGF β , Wnt, Rho, and MAPK/PI3K signaling cascades, implying a potential role for miR-139-5p in regulating the ability of cells to invade and migrate. To corroborate this finding, using the MDA-MB-231 breast cancer cell line, we show that overexpression of miR-139-5p results in suppression of these cellular phenotypes. Furthermore, we validate the interaction between miR-139-5p and predicted targets involved in these pathways. Collectively, these results suggest a significant functional role for miR-139-5p in breast cancer cell motility and invasion and its potential to be used as a prognostic marker for the aggressive forms of breast cancer.

Keywords: biomarker; breast cancer; miRNA

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer in women and a leading cause of cancer mortality. One of the major determinants of breast cancer mortality is the stage of disease at diagnosis; patients who present with metastatic disease have a 5-year survival rate of 21% (Cardoso and Castiglione 2009). The progression of primary tumors to metastatic disease is known to involve (1) invasion of extracellular matrix and stromal layers by the tumor cells, (2) intravasation into the bloodstream to travel to a distant or-

gan, (3) extravasation into the parenchyma of distant tissues, and (4) colonization and outgrowth of tumors in the distant site (for review, see Fidler 2003). The molecular mechanisms underlying metastatic migration and invasion are only partially understood, despite several signaling pathways being implicated (Blanco and Kang 2011). Interaction between carcinoma cells and their neighboring stroma has also been shown to play a critical role (Bhowmick et al. 2004).

Recently, miRNAs have also been found to play a key role in metastases (Ma et al. 2007; Valastyan et al. 2009). miRNAs are short noncoding RNAs that suppress target gene activity predominantly through binding to target mRNAs and inhibiting their translation. miRNAs have also been shown to promote target gene degradation (for review, see Fabian et al. 2010; Huntzinger and Izaurralde 2011). Several miRNAs have

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causative or correlative links with metastasis. In the case of breast cancer, miR-21 overexpression has been shown to promote metastasis (Asangani et al. 2008; Zhu et al. 2008). miR-31 regulates the expression of the key metastatic genes integrin- $\alpha 5$, radixin, and RhoA (Valastyan et al. 2009). miR-200 has also been shown to increase metastatic potential of mammary carcinoma cell lines (Korpál et al. 2011), while other studies have observed an inverse correlation between miR-200 expression and tumor invasion and metastasis (Gregory et al. 2008; Korpál et al. 2008). Overexpression of miR-139-5p was recently shown to correlate with reduced metastatic activity in hepatocellular carcinoma and gastric cancer cells (Bao et al. 2011; Wong et al. 2011; Li et al. 2013) and down-regulated in glioblastoma (Li et al. 2013). Furthermore, in patients with invasive squamous cell carcinoma, loss of miR-139-5p expression is associated with increased metastatic disease (Mascaux et al. 2009).

Although a strong association between miR-139-5p and metastasis exists, there is little knowledge of the mechanisms by which it contributes to this process or of the gene networks it regulates; the specific pathways that are disrupted are still poorly understood. Only five targets of miR-139-5p have been identified and validated so far: FoxO1 (Hasseine et al. 2009), Rho-kinase2 (Wong et al. 2011), CXCR4 (Bao et al. 2011), RAP1B (Guo et al. 2012), and Type I Insulin-like GF (Shen et al. 2012). However, its role in breast cancer has not been studied so far.

miRNAs have the potential to be oncogenes or tumor suppressors in a given cellular context. Depending on the specific tissues or cancer type they are expressed in, miRNAs achieve functional specificity by targeting a core network of genes that belong to the same pathway. This interaction is highly dependent on the relative abundance of multiple mRNA targets. For instance, miR-17-5p is oncogenic in hepatocellular and colorectal carcinomas (Ma et al. 2012; Shan et al. 2013) and, in contrast, has been shown to have tumor-suppressive properties in cervical cancer cells (Wei et al. 2012). Similarly, miR-182-5p was shown to have oncogenic properties in bladder, ovarian, and breast cancers (Hirata et al. 2012; Liu et al. 2012; Krishnan et al. 2013), whereas it acts as a tumor suppressor in lung cancer (Sun et al. 2010). Given this molecular and context specificity of miRNAs, we wished to explore whether miR-139-5p was a potential oncomir of breast cancer and what the output of its functional repression was, and identify the network of genes possibly regulating its functions in the context of breast cancer.

RESULTS

miR-139-5p is de-regulated in human triple negative breast cancer samples

To assess the clinical relevance of miR-139-5p in human breast cancer, we measured its expression in a cohort of breast cancer patient samples ($n = 40$) that included the following

molecular subtypes of invasive ductal carcinomas—no special type (IDC-NST): triple negative ($n = 18$), Her2+ ($n = 4$), ER+/PR+ ($n = 9$); invasive lobular carcinomas (ILC) ($n = 3$); and normal breast tissue ($n = 6$) (Supplemental Table 1). The expression levels of miR-139-5p were assayed by qRT-PCR relative to an endogenous control RNU6B (Fig. 1A). We observe an increase in the levels of miR-139-5p in normal mammary tissue and several subtypes, but the triple negative subtype showed a marked variable pattern where 38% of the samples had lower expression compared to the normal controls. Since this subtype is heterogeneous at clinical, morphological, and molecular levels, it is possible that the low miR-139-5p expressing subgroup is one with a very different prognosis (Cheang et al. 2008), and further studies are warranted to try to validate this. Although the difference in the population average did not reach statistical significance, the loss of miR-139-5p expression may help to identify a new molecular subtype important for the biological understanding of disease and for clinical management within this invasive subgroup of breast cancer.

miR-139-5p is frequently down-regulated in invasive breast carcinoma

Next, we reviewed miR-139-5p expression in previously published data using TaqMan Low-Density Arrays to analyze 29 breast tumors and 21 normal adjacent controls (Romero-Cordoba et al. 2012). This sample cohort included invasive ductal carcinomas ($n = 26$), invasive lobular carcinomas ($n = 1$), invasive mucinous carcinomas (IMC) ($n = 1$), and ductal carcinoma in situ (DCIS) ($n = 1$). Of the IDCs, only five samples were triple negative. As shown in Figure 1B, miR-139-5p is significantly (P value < 0.0001) down-regulated in the tumor cohort compared to normal controls. To strengthen the validity of this expression profile, we also looked for changes in expression of known metastasis-associated miRNAs in breast cancer. Importantly, miR-139-5p expression positively correlates with miR-31 ($r = 0.44$) and miR-200b ($r = 0.36$), which are well-characterized anti-metastatic miRNAs in breast cancer (Korpál et al. 2008; Valastyan et al. 2009). This result suggested that miR-139-5p could be another marker for metastatic breast cancer besides the association with triple negative tumors.

To further investigate the expression of miR-139-5p across a larger cohort of patient samples, we chose to analyze a miRNA-seq data set (Farazi et al. 2011) consisting of normal breast tissue ($n = 16$) and various types of breast cancer including: adenoid cystic carcinoma ($n = 2$), apocrine carcinoma ($n = 4$), atypical medullary carcinoma ($n = 9$), metaplastic carcinoma ($n = 11$), mucinous carcinoma ($n = 1$), ductal carcinoma in situ ($n = 21$), and invasive ductal carcinoma ($n = 174$). Although the adenoid cystic carcinoma, a proportion of apocrine carcinomas, atypical medullary, and metaplastic carcinomas can be classified as basal-like molecular subtypes, they differ in their morphology, aggressiveness, and prognosis

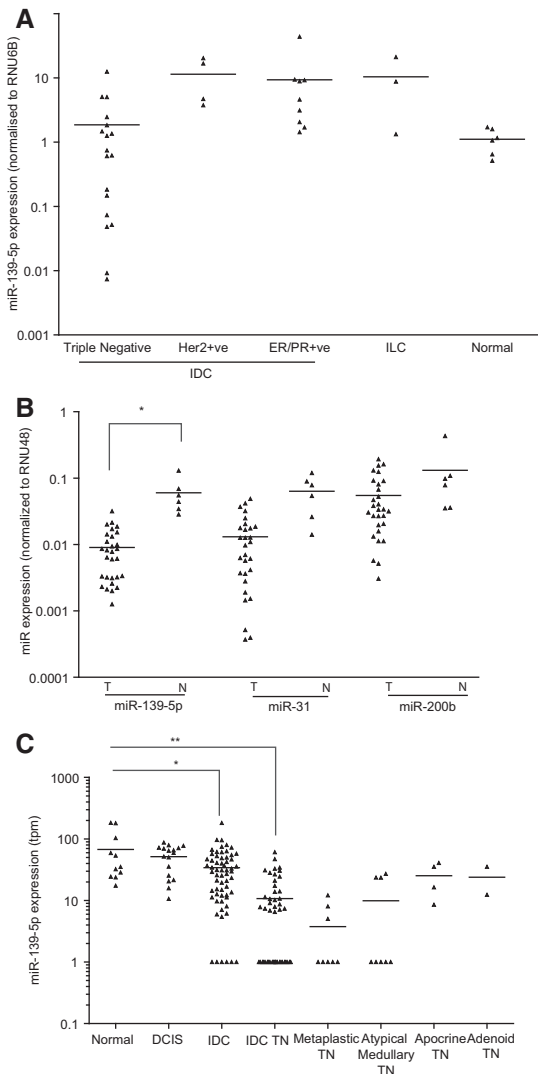


FIGURE 1. Expression analysis of miR-139-5p across tumor subtypes and normal tissue from human breast cancer patient samples. (A) Lines represent the mean of miR-139-5p expression (normalized to RNU6B) across sample subtypes: triple negative ($n = 18$), Her2+ ($n = 4$), ER+/PR+ ($n = 9$), invasive lobular carcinomas ($n = 3$), normal breast tissue ($n = 6$). Triangles represent the expression levels for individual patients. (B) Expression of miRNAs in breast cancer as assessed by TaqMan Low Density Arrays (Romero-Cordoba et al. 2012). Lines represent the mean expression of miR-139-5p, alongside well-characterized anti-metastatic miRNAs like miR-31 and miR-200b (normalized to RNU6B) across the sample cohort (comparing tumor versus normal along the x-axis). Triangles represent the expression levels for individual patients. Asterisks indicate significant difference between the expression of miR-139-5p in the tumour compared to the adjacent normal tissue ($[*] P < 0.0001$, Student's t -test). (C) miR-139-5p expression as assessed using miRNA-sequencing from Farazi et al. (2011) across normal breast tissue ($n = 16$) and a panel of breast cancer patient samples of various subtypes, including adenoid cystic carcinoma ($n = 2$), apocrine carcinoma ($n = 4$), atypical medullary carcinoma ($n = 9$), metaplastic carcinoma ($n = 11$), mucinous carcinoma ($n = 1$), ductal carcinoma in situ (DCIS) ($n = 21$), and invasive ductal carcinoma (IDC) ($n = 174$). Lines and scatter plot represent the mean across a subtype and the transcripts per million (tpm) of miR-139-5p in each sequenced sample, respectively. Asterisks indicate significant difference between the expression of miR-139-5p in the specific tumor subtype compared to the normal tissue. (**) $P < 0.0001$, (*) $P \sim 0.007$, Student's t -test.

(Yerushalmi et al. 2009; Marchio et al. 2010; Park et al. 2010). Metaplastic carcinomas are a heterogeneous group of tumors characterized by the presence of epithelial cells (spindle, squamous) with or without metaplastic elements such as bone, cartilage, and muscle. They are aggressive tumors with a poor prognosis (Park et al. 2010). Medullary carcinoma is a controversial entity defined by Ridolfi and, despite the high grade, is said to have a good prognosis. In contrast, tumors that do not fulfill the criteria for typical medullary carcinomas have been designated as atypical medullary carcinomas (Ridolfi et al. 1977). These are more aggressive and have morphological features and prognosis that overlap with high grade IDC (Ridolfi et al. 1977). Adenoid cystic carcinoma, despite falling into the basal-like category, is an indolent tumor in the breast compared to its salivary gland counterpart. Carcinomas with apocrine differentiation are heterogeneous, and the behavior is dependent on grade and stage (Yerushalmi et al. 2009; Marchio et al. 2010). Interestingly, as shown in Figure 1C, the subtypes that have the worst prognosis and the highest propensity to form distant metastases (metaplastic carcinomas, atypical medullary, and the high grade TN IDCs) exhibit the lowest levels of miR-139-5p expression. Within the IDCs, those patients with a triple negative phenotype show a more significant ($P < 0.001$) down-regulation compared to those that express either ER/PR or Her2 ($P \sim 0.007$). However, not all triple negative patient samples show a down-regulation of miR-139-5p expression as seen in Figure 1A and in some subtypes in Figure 1C, but the common feature among these results is that miR-139-5p is frequently lost in the most aggressive subtypes of breast cancer, suggesting that it may play a key role in the metastatic cascade of breast cancer.

Identification of direct targets of miR-139-5p using a biotinylated miRNA duplex

It is well established that the function of microRNAs depends on the expression of their targets (Sood et al. 2006) and that the differing expression of these targets in different cellular states can lead to opposing phenotypic outcomes—for example, overexpression of miR-17-5p acts as either an oncogene or a tumor suppressor depending on what tissue it is expressed in (Cloonan et al. 2008). Therefore, it is critical to robustly identify the direct targets of miR-139-5p in breast cancer cells. Given that target prediction programs are noisy and have high false-positive rates (Bentwich 2005; Sood et al. 2006), we resorted to an experimental identification of targets. We used the recently reported miRNA pull-down approach (Cloonan et al. 2011) that involves transfecting synthetic biotinylated miR-139-5p duplexes (Fig. 2A) into cells (in our case, MCF7 cells) and surveying the captured target mRNAs using microarrays. Figure 2B shows sample clustering of cells transfected with biotinylated miR-139-5p and the total lysates where total distance between samples indicates similarity, highlighting the reproducibility of the pull-

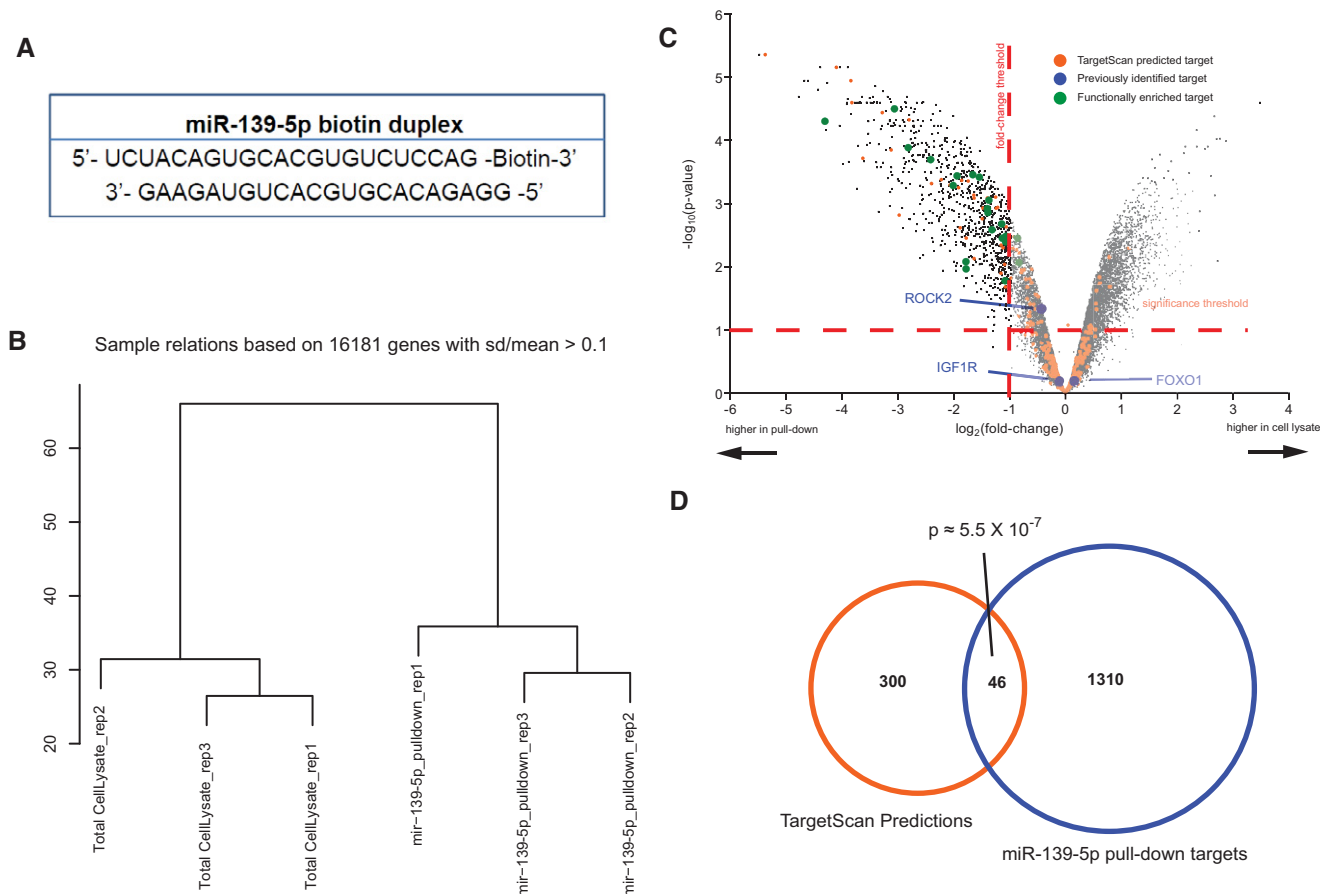


FIGURE 2. Identifying targets of miR-139-5p via biotin pull-down. (A) Sequence and position of biotin in the molecule used to transfect MCF7 cells for the pull-down approach used to identify biologically relevant targets of miR-139-5p. (B) Hierarchical clustering of microarray data was performed using the plotSampleRelations function in the *lumi* package. Total vertical distance between samples indicates similarity. (C) A “volcano plot” showing the \log_2 -transformed fold-change versus the \log_{10} -transformed P -value of that fold-change for every gene detected above background in the microarray. Genes highlighted in blue are targets validated by previous studies. Genes highlighted in orange are predicted by TargetScan to be targets of miR-139-5p, showing an enrichment of the targets in the pull-downs compared to the controls. Genes highlighted in green have known association with pathways frequently involved in metastasis. (D) Venn diagram showing the overlap of genes between TargetScan predicted targets of miR-139-5p (also expressed above background in HEK293Ts) and biotinylated miR-139-5p pull-down predicted targets. This overlap is significantly more than expected by chance.

down approach between biological replicates. We identified mRNAs significantly enriched in the biotin fraction compared to the input RNA at a 5% false discovery rate (FDR) threshold, with a fold-change > 2 (Fig. 2C). Using this approach, 884 probes (targeting 879 genes) were found to be significantly enriched in the pull-down fractions (Supplemental Table 2).

Next, we compared the miR-139-5p pull-down targets to TargetScan predicted targets and saw an overlap between the experimentally determined and the predicted targets which was significantly more than expected by chance ($P \approx 5.5 \times 10^{-7}$) (Fig. 2D), supporting the notion that we are enriching for biologically relevant targets of miR-139-5p. As expected, some targets identified in other pathologies are either not expressed in MCF7 cells (2/5) or do not meet our stringent threshold for target identification (Fig. 2C). Figure 2C highlights (in green) the significantly enriched genes that

are associated with the metastatic cascade, specifically in the context of breast cancer. miRNAs regulate cellular processes by concomitant suppression of a network of genes, so we included genes which do not pass our stringent threshold cutoff for target identification, since they belong to closely connected pathways associated with the invasive ability of cancer cells. Together, these results demonstrate that the pull-down has enriched for miR-139-5p’s biological targets.

miR-139-5p targets genes involved in pathways associated with metastasis

A gene set enrichment analysis (GSEA) was performed using ingenuity pathway analysis (IPA) on the miR-139-5p targets significantly enriched in the biotin pull-down. The list of pull-down-enriched 879 genes (Supplemental Table 2) was compared to 10 other random gene lists of the same size to

confirm specificity. A functional ontology was only considered significant if its $-\log(P\text{-value})$ was at least four standard deviations away from the mean $-\log(P\text{-value})$ of the 10 random gene lists. Supplemental Table 3 lists all canonical pathways (IPA) showing significant enrichment ($P\text{-value} < 0.05$) for miR-139-5p pull-down targets. As predicted, several significantly enriched pathways have been previously implicated in metastatic biology (Table 1), with confirmed roles for all but one in breast cancer.

Functional enrichment analysis identified several canonical pathways previously implicated in the metastatic cascade, but the specific mechanism by which miR-139-5p modulates key signaling pathways underlying these processes is still unknown. To explore this, we analyzed the list of significantly enriched targets from the pull-down approach and explored the literature for their known role specifically in cellular processes underlying metastasis, including cell proliferation, migration, and invasion. Shown in Figure 3 are key signaling cascades underlying these processes, with targets of miR-139-5p highlighted in dark gray ($\log_2 \text{FC} > 1$) and light gray ($\log_2 \text{FC} > 0.5$) and targets previously validated in other studies. The signaling molecules identified from this analysis were found to be part of three major pathways: (1) Wnt signaling which consists of canonical and noncanonical arms, depending on the ligand; canonical Wnt signaling occurs upon recruitment of β -catenin to the nucleus (Clevers and Nusse 2012), whereas the noncanonical streams work through downstream activation of molecules such as PRKC and RHO (Schlessinger et al. 2009), both of which are known players in breast cancer progression (Scheel et al. 2011); (2) receptor tyrosine kinases (RTK) signal through several pathways including RAS–MAPK and PI3 kinase, activating several downstream signal transducers, such as RAF1 and RAC1, and transcription factors such as MAPK3/1 (ERK1/2), NFKB, CEBPB, and TWIST1, all of which play important roles in breast cancer progression (Chaudhary et al. 2000; Bundy and Sealy 2003; Buchholz et al. 2005; Kim et al. 2008; Hong et al. 2011); (3) TGF β signaling which plays an important role in breast cancer (Buck and Knabbe 2006), epithelial

to mesenchymal transition (EMT) (Zavadil and Bottinger 2005), and breast cancer metastasis (Padua et al. 2008). Interestingly, TGF β also cross-talks with several other pathways, including estrogen receptor signaling (Matsuda et al. 2001), which have also been identified as miR-139-5p targets from our analyses. Also interestingly, proteins underlying this pathway were identified to be potentially regulated by miR-139-5p using target prediction software (Lee et al. 2013).

This analysis outlines the complex set of targets that appear to be regulated by miR-139-5p, a major proportion of them being involved in tumor progression and metastasis. It suggests possible mechanistic targets of miR-139-5p in the regulation of metastasis, warranting further functional characterization of its role in suppressing these functional phenotypes.

miR-139-5p does not alter proliferation or DNA profile in MDA-MB-231 cells

The pathways analysis suggested a role for miR-139-5p in promoting breast cancer metastasis. To confirm its relevance in processes underlying malignancy of tumor cells, we undertook a series of cell-based assays. Previous studies have reported a prognostic role for cell proliferation in the metastatic ability of tumors (Maeda et al. 1996; Panizo-Santos et al. 2000). Moreover, cell proliferation has been reported to be altered by miR-139-5p in human colorectal carcinoma through regulating the expression of RAP1B (Guo et al. 2012).

To test whether this phenotype was relevant in breast cancer, we generated four stable cell lines with inducible expression of miR-139-5p in MDA-MB-231 cells, a well-characterized invasive breast cancer cell line. Doxycycline-induced expression of miR-139-5p for each cell line was confirmed using TaqMan real time PCR (Fig. 4A). The four stable cell lines show between 20- and 200-fold higher expression of miR-139-5p in the presence of 1000 ng/mL doxycycline, which is also comparable to the difference in expression seen between normal breast tissues relative to tumor patient samples (Fig. 1C). Sequencing the small RNA population of the MDA-MB-231

TABLE 1. miR-139-5p target enriched canonical pathways and their association with tumor progression

Pathway	<i>P</i> -value	Associated with metastasis?	Cancer type	References
Protein ubiquitination pathway	1.00×10^{-5}	Yes	Breast	Kim et al. (2011)
ERK5 signaling	2.95×10^{-5}	Yes	Breast, prostate	Cronan et al. (2011); Ramsay et al. (2011)
Aminoacyl-tRNA biosynthesis	1.95×10^{-4}	Maybe (tumorigenesis)	Lung, colon	Kim et al. (2011)
N-Glycan biosynthesis	2.90×10^{-4}	Yes	Breast	Lau and Dennis (2008)
Glucocorticoid receptor signaling	3.55×10^{-4}	Maybe (tumorigenesis)	Breast	Moutsatsou and Papavassiliou (2008)
NGF signaling	3.90×10^{-4}	Yes	Breast	Adriaenssens et al. (2008)
Xenobiotic metabolism signaling	5.80×10^{-4}	Maybe (tumorigenesis)	Breast	Aust et al. (2005); Naushad et al. (2011)
HGF signaling	6.50×10^{-4}	Yes	Breast, melanoma	Maroni et al. (2007); Previdi et al. (2010)
PI3K/AKT signaling	1.15×10^{-3}	Yes	Breast, lung, thyroid	Smirnova et al. (2012); Xue et al. (2012)

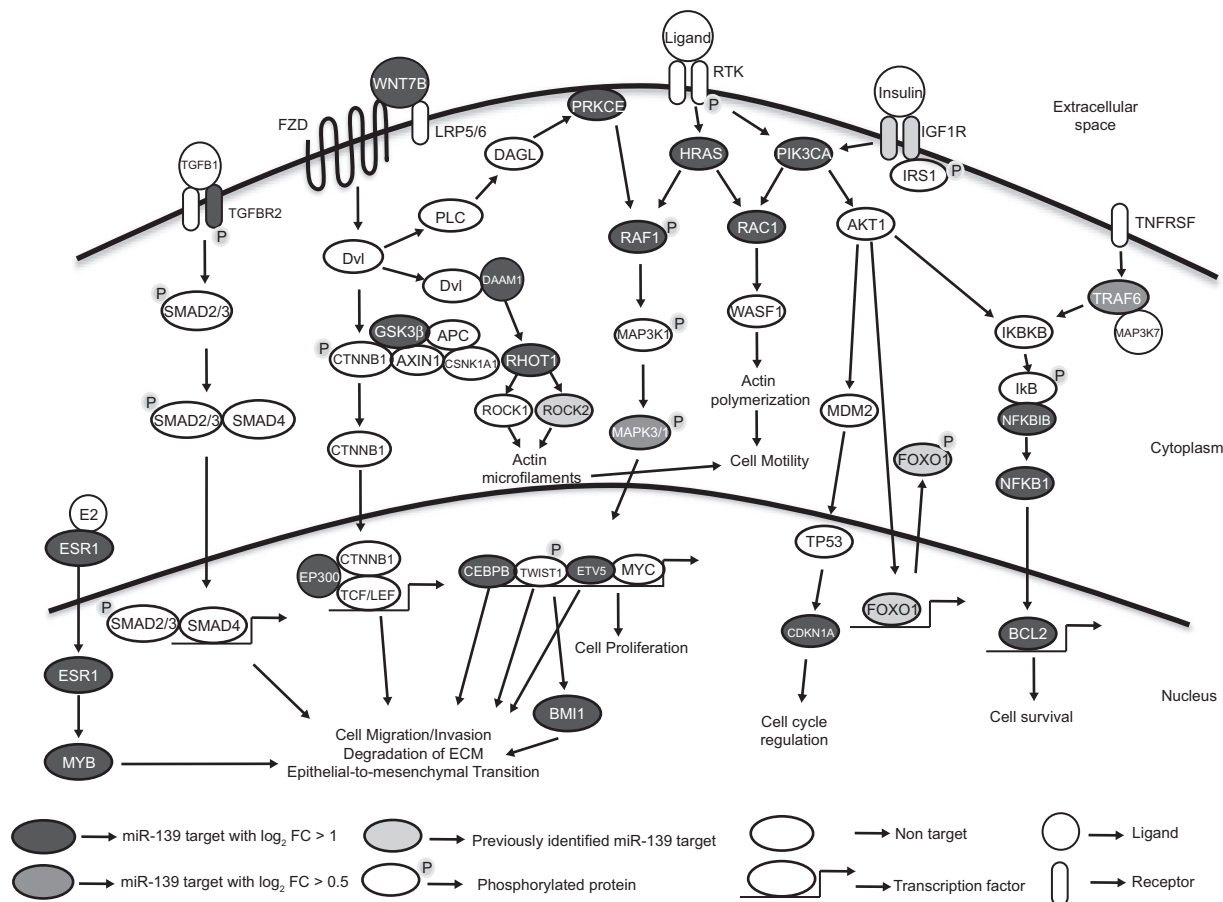


FIGURE 3. Overview of biotinylated miR-139-5p pull-down predicted targets involved in breast cancer invasion and metastasis. Illustration of major pathways being altered by miR-139-5p and the specific components that are targeted. These major pathways, TGF β , Wnt, and RTK-induced MAPK and PI3K all play a major role in the process of metastasis. Several components within each of these pathways are targeted by miR-139-5p in our pull-downs with \log_2 FC > 1 (dark gray) or \log_2 FC > 0.5 (light gray), or in previously reported studies leading to distinct downstream phenotypes that contribute to the metastatic properties of the cells.

cells (Supplemental Table 4) shows miR-139-5p to be expressed at ~ 300 transcripts per million (tpm), where the top 10% of miRNAs are expressed (miRNAs with <100 tpm excluded) with >1000 tpm. The highest expressed miRNA in the cell line had $\sim 74,000$ tpm (a difference of ~ 240 -fold between miR-139-5p and the highest expressed miRNA). This suggested our stable overexpression of miR-139-5p was within physiologically relevant levels. Additionally, an analysis of the data downloaded from The Cancer Genome Atlas (TCGA) that contains data from 893 breast invasive carcinomas and normal patient samples show expression of miRNAs in normal breast tissue to be between 100 tpm and 300,000 tpm with the top 10% of miRNAs with >10,000 tpm. These findings suggest our levels of expression are within the realms of normal copy numbers per cell. At this low level of overexpression, we did not observe any gross morphological changes or changes in cellular integrity.

All four cell lines were used in the subsequent cell-based assays. We performed MTT assays to measure the rate of proliferation over a time course of four days. Figure 4B shows no

significant difference in the proliferation rates of the parental (uninduced) cell lines versus the induced cell lines overexpressing miR-139-5p. We also performed FACS analysis of the PI-stained cells to measure any change in the DNA profile of MDA-MB-231 cells in response to miR-139-5p expression. As shown in Figure 4C, there was no statistically significant change in DNA profile. Together, these results suggest that overexpression of miR-139-5p has no effect on cell proliferation or progression through the cell cycle in MDA-MB-231 cells. This result also suggests that miR-139-5p may target different gene networks in other cancer types where alterations in proliferation rates have been shown to result from miR-139-5p overexpression.

miR-139-5p suppresses both invasion and migration in MDA-MB-231 cells

Cellular migration and invasion are key processes underlying metastasis. To test the hypothesis that miR-139-5p overexpression can suppress these phenotypes in breast

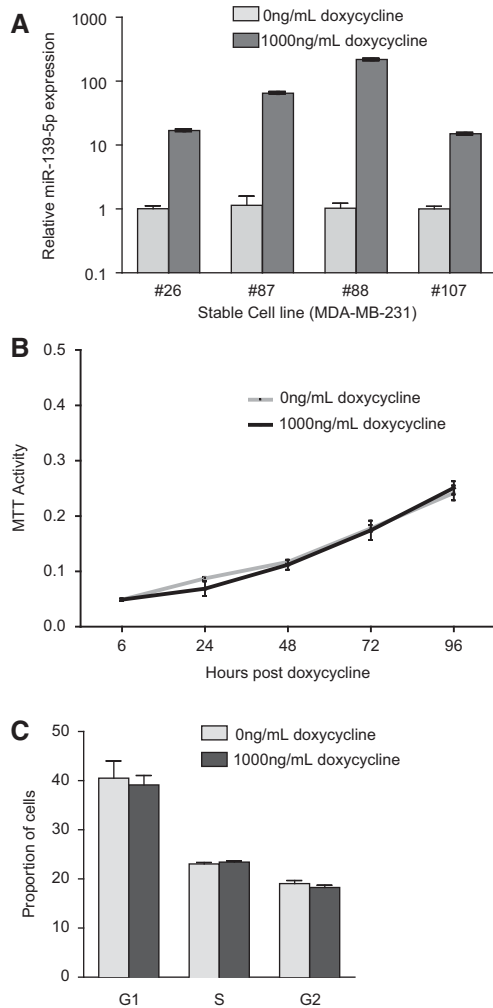


FIGURE 4. Overexpression of miR-139-5p does not induce a proliferative defect. (A) Expression of miR-139-5p as assessed by qRT-PCR in MDA-MB-231 cells (with low endogenous expression of miR-139-5p) stably transfected with miR-139-5p whose expression is induced in response to doxycycline. Four independent cell lines grown in the presence of 0 or 1000 ng/mL of doxycycline for 48 h are shown. RNU6B was used as an endogenous control for normalization of expression. (B) MTT cell proliferation assays of MDA-MB-231 cells stably expressing miR-139-5p. The graph plots the mean and SEM of the stable cell lines grown with either 0 or 1000 ng/mL doxycycline. The induction of miR-139-5p does not affect the proliferation rates of MDA-MB-231 cells. (C) DNA profile analysis of MDA-MB-231 cells stably expressing miR-139-5p. Graph shows the mean and SEM of the percentage of cells in different cell cycle phases, as assessed by FACS. There was no significant difference between MDA-MB-231 cells expressing or not expressing miR-139-5p.

cancer cells, we used the stable cell lines with inducible expression of miR-139-5p in MDA-MB-231 cells. The migratory ability of the stable cell lines was tested by quantifying their ability to traverse a transwell membrane in the presence (1000 ng/mL) or absence of doxycycline. The parent cell line stably expressing an empty vector without the miR-139-5p construct was included in the assay to identify potential side effects of the doxycycline treatment. Cells were plated

on the top of a transwell insert containing 8- μ M pores in basal medium and placed in a well containing medium enriched with EGF. After 6 h, cells that migrated to the bottom of the insert were stained with crystal violet and counted. Induction of miR-139-5p expression, upon addition of doxycycline, significantly decreased the migratory ability of the MDA-MB-231 cells to between 40% and 70% ($P < 0.05$) (Fig. 5A,B) of their original levels in the four stable cell lines tested.

The invasive ability of the cells was tested using a similar procedure as outlined above using transwells that contained a layer of matrigel to mimic the basement membrane. Cells were plated on top of the matrigel and invading cells were stained and counted 24 h after plating. Overexpression of miR-139-5p decreased the ability of the MDA-MB-231 cells to invade to 40%–70% (Fig. 5C,D) of the original levels in all four stable cell lines (reached significance in two of them [$P < 0.05$]). These assays reveal a novel role for miR-139-5p in the inhibition of properties that account for the metastatic potential of breast cancer cells. Similar effects of anti-invasive and anti-migratory roles for miR-139-5p have also been shown in other cancer types, like human hepatocellular carcinoma (Wong et al. 2011; Fan et al. 2012) and colorectal cancer cells (Shen et al. 2012).

Validation of miR-139-5p target-binding sites and change in protein expression in MDA-MB-231 cells

The initial pull-down analysis was performed in MCF7 cells, which is a breast cancer cell line, albeit less invasive than the MDA-MB-231 cells. Since these cells were used in all functional assays, we sought to validate the interaction between miR-139-5p and some of the genes enriched in the biotin pull-down using MDA-MB-231 breast cancer cells. The predicted binding sites (and ~60 nt of surrounding sequence) were cloned into the 3' UTR of the pMIR-REPORT Luciferase construct and transiently transfected into cells. Luciferase activity, indicative of translation from the plasmid, was measured in the presence of a miR-139-5p mimic or negative control mimic and normalized using β -galactosidase activity. Using this approach, we were able to validate five of seven genes selected (Fig. 6A), including *HRAS*, *NFKB1*, *PIK3CA*, *RAF*, and *RHOT1*. These genes are key modulators of the pathways previously discussed (Fig. 3). These data further strengthen our hypothesis that miR-139-5p targets a network of genes underlying cellular processes involved in metastasis. The validation of target binding in the MDA-MB-231 cells also suggests that these targets are possibly the mediators of miR-139-5p's influence on invasion and migration (Fig. 5). The high rate of validation is further evidence of the ability of the biotin pull-down approach to enrich for biologically relevant targets of the miRNA.

We further tested if regulation of these target genes by miR-139-5p could result in observable changes in their protein expression. Western blotting carried out with lysates

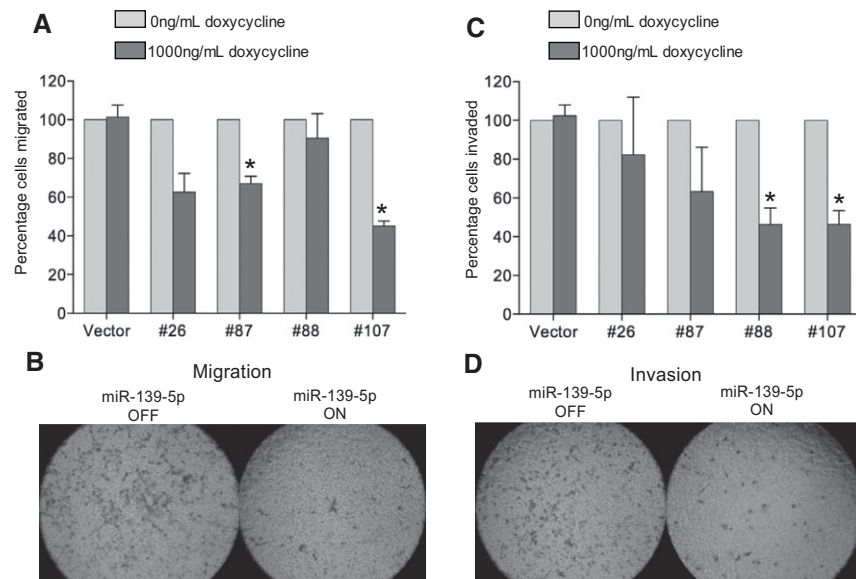


FIGURE 5. The effect of miR-139-5p overexpression on migration and invasion in MDA-MB-231 cells. (A–D) MDA-MB-231 cells stably expressing miR-139-5p and empty vector constructs were subjected to Boyden chamber transwell migration and invasion assays as described. Upon doxycycline-inducible (1000 ng/mL) expression of miR-139-5p. (A) The ability of the cells to migrate decreased to 40%–70% of the original levels in the stable cell lines. (B) Representative images of migrated cells not expressing miR-139-5p (left) and overexpressing miR-139-5p (right). (C) The ability of the cells to invade through a matrigel basement membrane-like matrix decreased to 40%–70% of original levels in the stable cell lines. (D) Representative images of invaded cells not expressing miR-139-5p (left) and overexpressing miR-139-5p (right). The cell lines stably expressing vector controls did not show significant changes in the invasion or migration assays. These experiments were performed with the vector and four different stable cell lines in triplicate. Data plotted are mean and SEM of three independent biological replicates with at least two technical replicates each. Asterisks indicate $P < 0.05$ in a Student's t -test.

from stably transfected MDA-MB-231 cells in the presence or absence of doxycycline showed reduction in the protein levels of NFKB1/p50 and PIK3CA and a dramatic loss in the levels of HRAS when miR-139-5p is overexpressed (Fig. 6B,C). Quantification of the levels of protein expression showed statistical significance in the reduction of HRAS and PIK3CA levels across all four stable cell lines, whereas the average 65% reduction of NFKB1/p50 was not statistically significant when including the increase in protein observed in one cell line. This difference could be attributed to the inherent differences in expression of miR-139-5p across the different stable cell lines and the rate of transcription, which might alter protein levels at different time points. These observations further substantiate our initial findings, showing that miR-139-5p does have a substantial effect on the levels of target genes that are relevant to tumorigenesis, cell migration, and invasion.

DISCUSSION

In prior studies, miR-139-5p has been shown to have anti-invasive, anti-migratory, and in some cases, anti-proliferative effects on cancer cells. However, studies focusing on its target cohort have been limited to FoxO1 (Hassaine et al. 2009),

Rho-kinase2 (Wong et al. 2011), and c-Fos (Fan et al. 2012) in hepatocellular carcinoma, CXCR4 (Bao et al. 2011) in gastric cancer cells, RAP1B (Guo et al. 2012), and Type I insulin-like growth factor (Shen et al. 2012) in colorectal cancer. In this study, we show miR-139-5p to be associated with human breast cancer, where its expression is frequently down-regulated in the more aggressive subtypes. Using the biotin pull-down method, followed by GSEA, we experimentally identified a large cohort of miR-139-5p targets in breast cancer cells, with known functions in pathways underlying the cellular migration and invasion processes. Overexpressing miR-139-5p in MDA-MB-231 cells reduced their invasive and migratory abilities. Together, these findings support the hypothesis that miR-139-5p is a potential anti-metastatic oncomir of solid tumors.

There are certain caveats to be considered when interpreting our results. For instance, although we did not observe gross morphological changes or changes in the cell cycle (Fig. 4B,C), it is possible that the introduction of an exogenous molecule could lead to changes in mRNA expression affecting our ability to detect miRNA targets. Reduced or absence of

target expression in the cell line being studied could lead to false negatives, and thus the targets identified in this study should not be considered to be a definitive list of all possible targets in all possible cell types. Similarly, overexpression of an exogenous miRNA (even at the low levels used in this study) could also lead to competition for gene targets with endogenous miRNAs, leading to false positives in our assay. Although we do not observe major disruption to the transcriptional landscape either here or in previous studies (Cloonan et al. 2011; Krishnan et al. 2013), this potential requires that studies of individual miRNA targets will need to be individually validated (Fig. 6). However, the inclusion of a small proportion of false positives is unlikely to affect the functional enrichment analysis performed here (Supplemental Table 3). This style of analyses has shown reliable results with a 0.25 FDR (Subramanian et al. 2005), and for strong biological signals, even a false-positive rate of 50% can yield accurate results (Cloonan et al. 2008). The accuracy of the analyses in this study is highlighted by our recapitulation of known biological functions for miR-139-5p (Wong et al. 2011; Fan et al. 2012; Shen et al. 2012).

miRNAs achieve specific regulation of cellular processes by concomitant suppression of a network of genes underlying the same function and/or pathways (Cloonan et al. 2008,

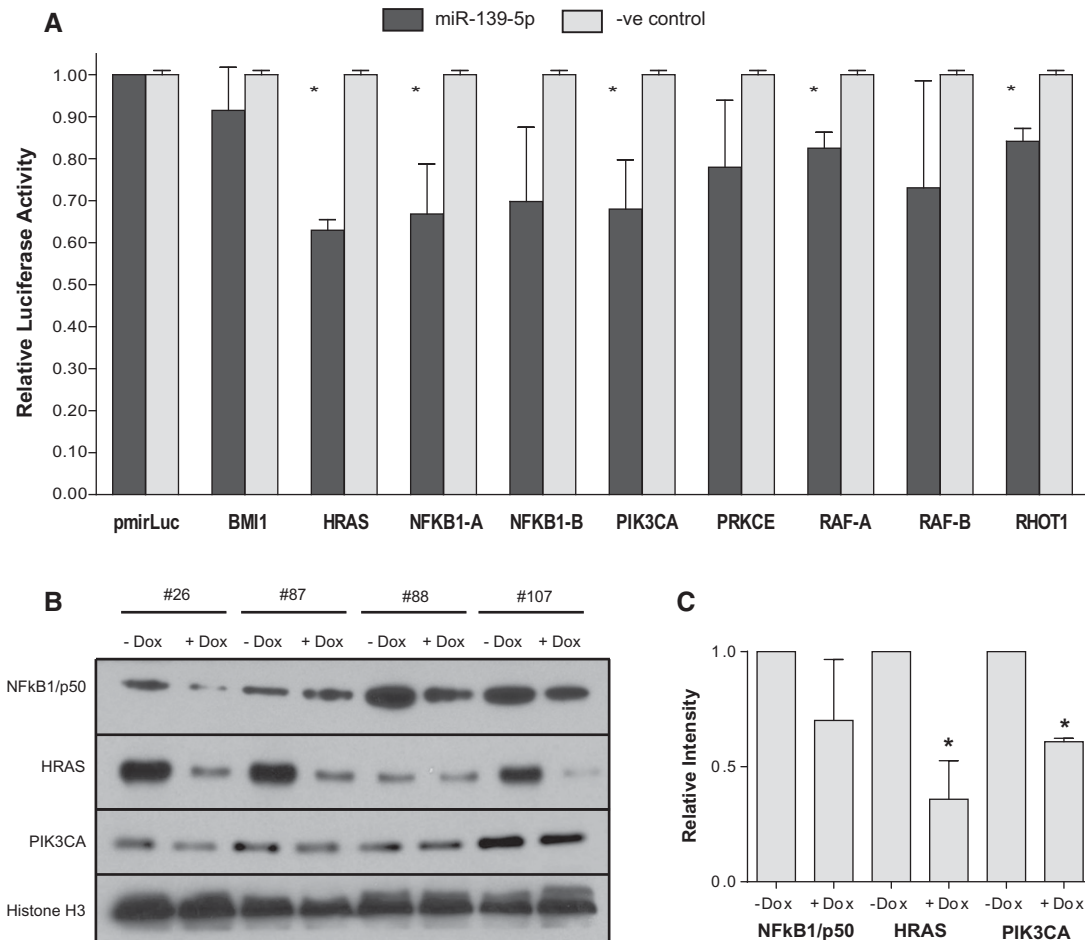


FIGURE 6. Validation of miR-139-5p targets using a luciferase assay and Western blots. (A) MDA-MB-231 cells were transiently cotransfected with 20 nM miR-139-5p or a control mimic with a pMIR-REPORT Luciferase construct containing the predicted binding site from the indicated target gene. Luciferase activity was normalized to β -galactosidase activity (asterisk indicates $P < 0.05$ as indicated in a Student's *t*-test). Data plotted are mean and SEM of at least two independent biological replicates with three technical replicates ($n \geq 2$). (B) Immunoblotting showing the effects on protein expression of NFKB1/p50, HRAS, and PIK3CA upon induction of miR-139-5p in stably transfected MDA-MB-231 cells. (C) Quantitation of the blots was carried out after normalization to loading control, showing a statistically significant reduction in levels of HRAS and PIK3CA across all stable cell lines. A substantial reduction in NFKB1/p50 was observed in three of four cell lines.

2011; Shirdel et al. 2011; Gennarino et al. 2012). In this study, we have shown miR-139-5p to be able to target several pathways underlying the invasive and migratory phenotypes of cancer cells. The Wnt signaling pathway activation has been shown in several cancers and more specifically in breast cancer (Schlange et al. 2007; Khalil et al. 2012). Schepeler et al. have shown that disruption of Wnt signaling leads to an increase in the levels of miR-139-5p, among other miRNAs, in colorectal carcinoma cells (Schepeler et al. 2012). In our study, we observe components of the Wnt pathway being targets of miR-139-5p. If disruption of Wnt in breast cancer cells also leads to increase in levels of miR-139-5p, it is possible that this miRNA could be part of a regulatory feedback loop that could keep the Wnt pathway in check in normal cells. The invasive and migratory capacity of cells can also be attributed to MAPK signaling, which can occur downstream from PRKC signaling through the Wnt-calcium pathway (Sheldahl et al.

1999) or through RTK-induced downstream Ras and PI3K signaling (Huang et al. 2004; Du et al. 2010).

Through luciferase assays and Western blotting, we were able to confirm Ras and PI3K members to be directly regulated by miR-139-5p, suggesting that the miRNA is likely inhibiting the invasive phenotypes through regulation of RTK-mediated downstream signaling. Additionally, NFKB, which can also be regulated by PI3K (Romashkova and Makarov 1999), has been shown to be a direct target of miR-139-5p, which is capable of conferring anti-apoptotic properties on metastatic cancer cells (Buchholz et al. 2005). Ras activates the canonical MAPK pathway (RAF \rightarrow MEK \rightarrow ERK), through which they regulate Rho GTPases, which are key players in cell migration and invasion (Vega and Ridley 2008; Makrodouli et al. 2011). Interaction of PI3K with Rho GTPase members Rac1 and Cdc42 has also been shown to regulate downstream actin reorganization (Tolias et al. 1995)

and cancer cell migration (Barber and Welch 2006), suggesting that both validated targets of miR-139-5p could be mediating anti-migratory and anti-invasive properties through the same effectors, the Rho GTPases. These properties are classical hallmarks of aggressive breast tumors, suggesting miR-139-5p to be anti-metastatic.

In this study, we show loss of miR-139-5p across the invasive subtypes in three different data sets. However, this loss does not always correlate with other molecular markers usually employed to classify breast cancers, e.g., the triple negative phenotype has been routinely used to classify aggressive breast cancers. More recently, the heterogeneity underlying this subgroup has been noted, warranting more detailed prognostic tools (Metzger-Filho et al. 2012). Consistent with this view, we observe a wide distribution in the expression of miR-139-5p in the triple negative breast cancers analyzed (Fig. 1A). Moreover, analysis of this and the Farazi et al. data sets (Fig. 1A,C) suggests that loss of miR-139-5p could help identify an aggressive subgroup of triple negative cancers. However, a comprehensive screen of a large number of patient samples, including those from relatively rare subtypes, is required to further understand the potential of miR-139-5p as a biomarker for aggressive breast cancer. Experimental validations as performed in this study across

other breast cancer cell lines and subtypes would help to identify its potential as a prognostic marker for the invasive property.

Being widely implicated in the progression of various cancers would indicate a common mode of action by miR-139-5p, presumably through the inhibition of signaling pathways underlying the metastatic traits of a wide range of carcinomas (Fig. 7). Metastatic properties of different carcinoma cells have been attributed to the pathways described in this study, among others, suggesting that inhibition of these pathways would be the initial step in reversing the aggressive nature of late stage tumors. Our study now shows experimental evidence for regulation of these pathways by a single miRNA. Future studies in the characterization of these downstream signaling pathways and the mode of miR-139-5p silencing in human cancers would provide further information for its potential use as a therapeutic in the reversal of the metastatic phenotype of breast and other cancers.

MATERIALS AND METHODS

Cell culture

MDA-MB-231 cells were maintained in DMEM (Life Technologies Australia) with 10% FBS and 1% Pen-strep and grown in a 5% CO₂ atmosphere at 37°C. MCF7 cells were maintained in similar conditions with the exception of 10 µg/mL bovine insulin (SigmaAldrich) added to the growth media. MDA-MB-231 and MCF7 cell lines were purchased from Cell Bank Australia.

Clinical samples, RNA purification, and qRT-PCR analyses

Human breast tumors were derived from the Brisbane Breast Bank, collected from consenting patients and with ethical approval from the research ethics committees of The Royal Brisbane & Women's Hospital and The University of Queensland. Histological type, tumor grade, tumor size, lymph node status, and ER, PR, and HER2 status were obtained from the pathology reports. ER, PR, and HER2 biomarkers were used to infer molecular subtype as luminal, HER2, or triple negative. Total RNA from human tumor samples was extracted using tumor homogenization followed by TRIzol extraction (Invitrogen).

Total RNA was purified from cell lines using the miRNeasy Mini Kit (Qiagen), and RNA integrity was assessed using an Agilent Bioanalyzer 2100. For mature miRNA, cDNA (5–10 ng total RNA) was synthesized using a TaqMan MicroRNA RT Kit (Applied Biosystems), and qRT-PCR was performed using a miR-139-5p MicroRNA TaqMan Assay (Applied Biosystems). All RT-PCR was performed on an Applied Biosystems 7000 SequenceDetection System. For small RNA expression analysis, RNU6B was used as an endogenous control to normalize the data.

Biotin pull-downs, microarray hybridizations

Pull-downs of miR-139-5p targets were carried out as previously described (Cloonan et al. 2011), using biotin-labeled oligonucleotides

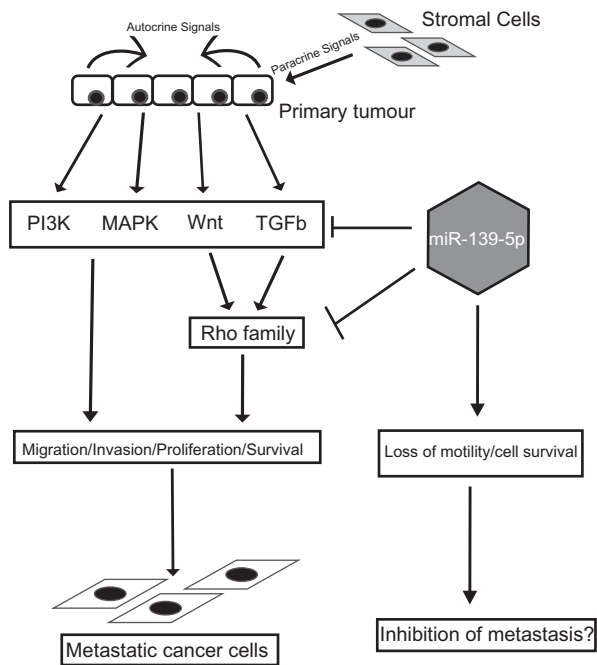


FIGURE 7. Model showing effect of miR-139-5p on tumor progression. Primary tumors, upon receiving autocrine and paracrine signals, respond by downstream activation of key signaling pathways such as TGF- β , Wnt, MAPK, and PI3K that are responsible for promoting their migratory, invasive, proliferative, and anti-apoptotic properties. Combined acquisition of these phenotypes enables the cells to become metastatic and seed distant organs. Shown in the figure is a potential model where miR-139-5p-mediated inhibition of these pathways can abrogate the onset of metastatic traits, potentially making them more susceptible for currently employed chemotherapeutic regimes.

specific for miR-139-5p (Fig. 2A). Briefly, the 50 pmol of biotin-labeled oligos (IDT) were transiently transfected into MCF7 cells, cultured for 24 h, followed by cell lysis and binding of 50 μ L myOne C1 Streptavidin Dynabeads (Invitrogen) to the RNA fraction for enrichment. Fifty nanograms of captured mRNA fractions (three independent biological replicates) were amplified and labeled using the Illumina Total Prep RNA Amplification Kit (Ambion) as per the manufacturer's instructions. Samples were profiled on Illumina Human HT-12 chips along with input total RNA from the same cells as negative controls. Microarray data have been deposited to the Gene Expression Omnibus and can be accessed with accession number GSE40411.

Bioinformatic analysis of pulldown microarray data

Microarray data were normalized using the *lumi* package (Du et al. 2008) by applying background adjustment, variance-stabilizing transformation (Lin et al. 2008), and robust spline normalization (Workman et al. 2002) successively. The *lmFit* and *eBayes* functions in the *limma* package (Smyth 2004) were used to test differential expression between the pull-down samples and the controls (Cloonan et al. 2011). The false discovery rate was calculated to account for multiple testing (Benjamini and Hochberg 1995). Probes that met the 5% FDR threshold (for one-sided tests) with a fold-change > 2 were considered significantly enriched in the pull-down. The transcripts (ENSEMBL V62) to which they matched exactly were considered putative targets of that miRNA. The targets enriched using the biotin pull-downs were analyzed using ingenuity pathway analysis as previously described (Cloonan et al. 2008).

Stable cell line generation

MDA-MB-231 cells stably expressing miR-139-5p were generated using the Mir-X Inducible miRNA Systems (Clontech). Briefly, MDA-MB-231 cells were transfected with the pTet-on Advanced Vector using Lipofectamine 2000 (Life Technologies Australia, Invitrogen Division), and cells stably expressing the plasmid were selected using 800 μ g/mL G418 (Life Technologies Australia) (maintenance concentration: 400 μ g/mL G418). Primers (Supplemental Table 5) were used to amplify the miR-139-5p hairpin from human genomic DNA and cloned into the pmRI-ZsGreen vector plasmid supplied and subsequently transfected into the 231-pTet-on parent line. Cells stably expressing the pmRI-ZsGreen-miR-139-5p were selected using 1 μ g/mL puromycin and further maintained in 0.5 μ g/mL puromycin. Stable expression of miR-139-5p was confirmed using TaqMan MicroRNA Assay (Applied Biosystems) specific for miR-139-5p (Fig. 4A).

MTT proliferation assays

Stable pmRI-MDA-MB-231 cell lines overexpressing miR-139-5p (1000 ng/mL dox) and parent stables (with no doxycycline) were plated at 1×10^4 cells per well. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) activity was assayed using a Cell Growth Determination Kit (SigmaAldrich) according to the manufacturer's instructions and detected on a PowerWave XS spectrophotometer (BioTek).

Flow cytometry for cell-cycle analysis

MDA-MB-231 cells stably expressing miR-139-5p were harvested and fixed in 70% ethanol at -20°C overnight. DNA was stained using 10 μ g/mL propidium iodide (SigmaAldrich), and RNA was removed using 200 μ g/mL RNase A (SigmaAldrich). Cells were filtered through 35- μ m cell strainer mesh (Becton Dickinson) and analyzed on a Becton Dickinson LSR II flow cytometer fitted with a 488-nm laser. Cell data were gated and analyzed using FlowJo 7.2.2 (Tree Star).

Cell invasion assay

MDA-MB-231 cells stably overexpressing miR-139-5p and parent stables (in the presence or absence of doxycycline) were counted and resuspended in serum-free media. Cells were plated at a density of 2×10^5 cells/well in the upper chamber of a matrigel (200 μ g)-coated transwell filter (8.0- μ m pore) from Corning. To the reservoir, 650 μ L of serum-free media with 0.1% BSA, 1% tet-FCS, and 10 ng/mL EGF was added. At 24 h, noninvaded cells on the upper side of the matrigel were removed carefully with a cotton swab. The cells bound to the lower side of the filter were washed twice with PBS and fixed with 5% glutaraldehyde at room temperature for 10 min. Fixed cells were washed twice with PBS and stained using 1% crystal violet. Excess stain was removed by washing with water, and the filters were dried overnight. Migrated cells were counted in six random fields and images obtained using light microscopy and camera.

Cell migration assay

MDA-MB-231 cells stably overexpressing miR-139-5p and parent stables (in the presence or absence of doxycycline) were counted and resuspended in serum-free media. Cells were plated at a density of 2×10^5 cells/well in the upper chamber of a transwell filter (8.0- μ m pore) from Corning. To the reservoir, 650 μ L of serum-free media with 0.1% BSA, 1% tet-FCS, and 10 ng/mL EGF was added. At 6 h, nonmigrated cells on the upper side of the filter were removed carefully with a cotton swab. The cells bound to the lower side of the filter were washed twice with PBS and fixed with 5% glutaraldehyde at room temperature for 10 min. Fixed cells were washed twice with PBS and stained using 1% crystal violet. Excess stain was removed by washing with water, and the filters were dried overnight. Migrated cells were counted in six random fields and images obtained using light microscopy and camera.

Luciferase assay to validate predicted binding sites

Predicted target sites of miR-139-5p were cloned into the HindIII and SpeI sites of the pMIR-REPORT Luciferase vector. Synthetic oligos (Supplemental Table 5) corresponding to 60 nt surrounding the target sequence were annealed before ligation into the pMIR-REPORT Luciferase vector. All constructs were verified by sequencing. MDA-MB-231 cells were cotransfected with 50 ng of a pMIR-REPORT Luciferase construct and 50 ng of pMIR-REPORT β -galactosidase Reporter Control Vector (Ambion) along with miR-139-5p or a negative mimic (Ambion) to a final concentration of 20 nM. Post-transfection, cells were incubated for 48 h prior to assaying. Luciferase activity was assayed using the Luciferase Assay System (Promega Corporation) and detected on a Wallac

1420 luminometer (PerkinElmer). β -galactosidase activity was determined using the β -Galactosidase Enzyme Assay System (Promega) and detected on a PowerWave XS spectrophotometer (BioTek). Luciferase activity was normalized to β -galactosidase activity in each well. Assays were conducted in triplicate and independently repeated at least twice.

Western blotting

MDA-MB-231 cells stably overexpressing miR-139-5p (in the presence or absence of 1000 ng/mL doxycycline) grown in 10-cm dishes for 24 h were washed gently in 5 mL of ice cold PBS and then lysed using 300 μ L of ice-cold aqueous lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, pH 8.0, 0.2% sodium azide, 50 mM NaF, 0.5% NP40) containing protease (Cat.# P8340, SigmaAldrich) and phosphatase inhibitors (Cat.# P5726 and P0044, SigmaAldrich). Lysates were spun at 12,000g for 30 min and the supernatants collected and stored at -80°C . Estimation of protein concentration was performed using Bradford reagent (Cat.# 500-0001, Biorad) using a standard curve created with known concentrations of BSA. Optical density measurements were carried out on an Ultrospec 6300 pro (Amersham Biosciences). Thirty micrograms of protein containing lysate were loaded onto each well of a NuPAGE SDS-PAGE gel (Invitrogen) and run for 1.5 h at 130V. Protein from the gel was transferred onto PVDF membrane (Millipore) using NuPAGE transfer buffer at 20V for 2 h. Following transfer, membranes were blocked in TBS-T containing 5% skim milk powder for 1 h at room temperature, after which they were incubated with primary antibody (1:500)-containing solution (in 5% BSA) overnight rocking at 4°C . Primary antibodies used were: rabbit anti-p50/p105 antibody (Cat.# 3035P, Cell Signaling), rabbit anti-HRAS (Cat.# SC520, Santa Cruz Biotechnology), rabbit anti-PIK3CA p110 antibody (Cat.# 4249S, Cell Signaling), and rabbit anti-histone H3 (Cat.# 9715, Cell Signaling). Membranes were then washed in TBS-T three times (30 min each), followed by incubation with anti-rabbit HRP secondary antibody (Cat.# 7074, Cell Signaling) at 1:5000 in TBS-T containing 5% skim milk powder for 1 h at room temperature. Membranes were then washed in TBS-T and developed using the SuperSignal West Dura Chemiluminescent Substrate (Cat.# 34076, ThermoScientific) on a Konica Minolta film processor (SRX 201A, Konica Minolta).

DATA DEPOSITION

Microarray data have been deposited to the Gene Expression Omnibus under accession number GSE40411.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

ACKNOWLEDGMENTS

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