Loss of Estrogen-Regulated microRNA Expression Increases HER2 Signaling and Is Prognostic of Poor Outcome in Luminal Breast Cancer

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Abstract

Among the genes regulated by estrogen receptor (ER) are miRNAs that play a role in breast cancer signaling pathways. To determine whether miRNAs are involved in ER-positive breast cancer progression to hormone independence, we profiled the expression of 800 miRNAs in the estrogen-dependent human breast cancer cell line MCF7 and its estrogen-independent derivative MCF7:2A (MCF7:2A) using NanoString. We found 78 miRNAs differentially expressed between the two cell lines, including a cluster comprising let-7c, miR99a, and miR125b, which is encoded in an intron of the long noncoding RNA LINC00478. These miRNAs are ER targets in MCF7 cells, and nearby ER binding and their expression are significantly decreased in MCF7:2A cells. The expression of these miRNAs was interrogated in patient samples profiled in The Cancer Genome Atlas (TCGA). Among luminal tumors, these miRNAs are expressed at higher levels in luminal A versus B tumors. Although their expression is uniformly low in luminal B tumors, they are lost only in a subset of luminal A patients. Interestingly, this subset with low expression of these miRNAs had worse overall survival compared with luminal A patients with high expression. We confirmed that miR125b directly targets HER2 and that let-7c also regulates HER2 protein expression. In addition, HER2 protein expression and activity are negatively correlated with let-7c expression in TCGA. In summary, we identified an ER-regulated miRNA cluster that regulates HER2, is lost with progression to estrogen independence, and may serve as a biomarker of poor outcome in ER+ luminal A breast cancer patients.

Introduction

The estrogen receptor (ER) is an estrogen-regulated transcription factor that controls the transcription of numerous coding and noncoding RNAs and is a key target for therapy in ER+ breast cancers (1, 2). In breast cancer, ER acts predominantly by binding to distal enhancer sites to mediate transcription (3). Downstream effectors of ER activity in breast cancer include genes with prooncogenic functions including survival and growth. It has been known for more than 40 years that a primary determinant of the response of breast cancers to endocrine therapy is the expression of ER, leading to the first stratification of breast cancer into ER+ and ER- subsets. More recently, refined subsets have been identified by gene expression profiles characteristic of clinical subtypes in which ER may play different roles (4–6).

miRNAs are small noncoding RNAs approximately 22 bp in length that regulate the expression of genes by targeting the 3′ untranslated regions (UTR) of mRNAs. These molecules have been demonstrated to play important roles in normal development and physiology as well as regulating a number of disease processes including breast cancer (7–9). miRNAs have been reported to be generally downregulated in cancers, and their loss leads to the increased expression of targeted genes, notably including oncogenes that lead to cancer progression. In breast cancer, a number of miRNAs have been reported to be abnormally regulated (10–13). ER has also been reported to regulate the expression of a number of miRNAs in response to its ligand estradiol (E2; refs. 14–17).

Here, we report the identification of miRNAs directly regulated by ER and differentially expressed in the estrogen-dependent ER+ breast cancer cell line MCF7 and its hormone-independent derivative MCF7:2A. The let-7c/miR99a/miR125b cluster is expressed in MCF7 cells where it is directly targeted by ER and both expression and ER binding are lost in MCF7:2A cells. Expression of this miRNA cluster is uniformly low in luminal B breast cancers, which have a worse outcome than luminal A. Within the luminal A subtype, low expression of the cluster predicts for poor patient outcome. We find that two members of the cluster, let-7c and miR-125b, inhibit HER2 protein expression and increased expression of the HER2 protein in luminal A tumors lacking expression of these miRNAs may mediate their poor outcome.

Materials and Methods

Cell culture and reagents

MCF7 cells were grown in high-glucose DMEM (Invitrogen) supplemented with 2 mmol/L L-glutamine, 10% (vol/vol) heat-inactivated FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) in a humidified incubator at 37°C and 5% CO2. The MCF7:2A, MCF7:5C, and MCF7:LTLT cell lines were
grown in phenol red-free high-glucose DMEM (Invitrogen) supplemented with 2 mmol/L L-glutamine, 5% (vol/vol) heat-inactivated FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). The MCF7:2A cells were also supplemented with 1 μmol/L letrozole. The MCF7:2A and MCF7:5C cell lines were obtained from V. Craig Jordan (Department of Oncology, Lombardi Cancer Center, Georgetown University Medical Center, Washington, DC) and the MCF7:LTLT cell line was obtained from Angela Brodie (Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, MD). The Dharmacon anti-miRs and miRNA mimics were obtained from ThermoFisher.

NanoString
A total of 2 x 10⁵ MCF7 and MCF7:2A cells growing in the exponential phase were seeded in 6-well plates and cultured for 2 days. The cells were then harvested for total RNA using the miRNeasy Kit (Qiagen). A total of 100 ng of total RNA was assayed using the Human miRNA NanoString Assay 2.0 Kit following the manufacturer’s instructions (NanoString). Differences in miRNA expression were analyzed using the NanoSTRIDE software program (18) with default settings. Clustering of the differentially expressed genes and heatmap generation was performed using the GenePattern Server (genepattern.broadinstitute.org). The volcano plot displaying the significance of the miRNA differences was produced using R version 3.0.2.

RT-PCR
For RT-PCR, total RNA was isolated using a combination of TRIzol (Sigma) and the RNeasy Mini Kit (Qiagen). First-strand cDNA was created using the Quantitect Reverse Transcription Kit (Qiagen) following the manufacturer’s protocol, was assayed using TaqMan miRNA assays (Life Technologies, Inc.), and the level of U6 RNA was used as a control. The expression of LINCO0478 was measured using the Power SYBR Green PCR Master Mix (Life Technologies, Inc.) with the following primers: 5'-GATCTGGAAACGCTTGTCC-3' (forward) and 5'-AGAGCTTCCCTCTCTCGG-3' (reverse). For the Ago1 experiments, the following primers were used: HER2: 5'-CTCTGTGAGTGGTAGAAA-3' (forward) and 5'-GGGACTTCTCCCTCTCTG-3' (reverse); Myc: 5'-CTCTGATTCATGCAGAACA-3' (forward) and 5'-GCTCTCTGAGAGAGTCTCC-3' (reverse); p21: 5'-AAAGACCATGTGGACCTGT-3' (forward) and 5'-CTGGTGCTC-3' (reverse).

Cell growth assays
To determine the rate of growth in the presence of miRNA mimics, 2.3 x 10⁵ MCF7:2A cells/mL were seeded into 6-well plates. The following day, the cells were transfected with 20 pmol of let-7c, miR99a, or miR125b miRIDIAN microRNA Mimics (ThermoFisher) or a negative control using the Lipofectamine RNAiMAX transfection reagent (Life Technologies, Inc.) following the manufacturer’s protocol. The cells were incubated at 37°C under 5% CO₂, passed into 96-well plates the following day (day 0), and allowed to proliferate. Triplicate wells were counted on days 1, 3, and 5 to determine the rate of growth.

Luciferase assays
A total of 3 x 10⁴ HEK 293 cells were seeded into 96-well plates. Twenty-four hours after plating, the cells were transfected with a psiCHECK2 vector encoding the entire 3' UTR of HER2 fused downstream of the Renilla luciferase gene and the firefly luciferase gene as a reporter with Lipofectamine 2000 following the manufacturer’s instructions. After incubation for 48 hours, the cells were lysed in 1 x Passive Lysis Buffer and assayed with the Dual-Luciferase Reporter Assay System (Promega) to measure the Renilla luciferase activity and that of firefly luciferase, which served as a transfection control.

Ago1 RNA immunoprecipitation
The Ago1 complex was immunoprecipitated as described in ref. 19. Briefly, A total of 2 x 10⁶ MCF7 and MCF7:2A cells in the growth phase were seeded in 10 cm plates. After 24 hours, the cells were harvested in 400 μL lysis buffer (100 mmol/L KCl, 5 mmol/L MgCl₂, 10 mmol/L HEPES, pH 7.0, 0.5% Nonidet P-40) supplemented with 100 μg/mL RNase Out (Invitrogen Cat# 10777-019) and Complete Protease Inhibitor Cocktail (Roche). The lysates were centrifuged, and 50 μL was set aside for input.

A total of 2 μg anti-Ago1 antibody (Abcam #ab5070) was prebound to protein A Dynabeads (Life Technologies). The antibody and lysate mixture was incubated overnight at 4°C. The next morning, the beads were collected by magnetic separation, and they were treated with DNaseI in NT2 buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1 mmol/L MgCl₂, and 0.05% Nonidet P-40) for 10 minutes at 37°C. The beads were then washed twice with NT2 buffer, treated with proteinase K to digest protein, and resuspended in 300 μL acid-phenol:chloroform (Ambion). The solution was centrifuged for 1 minute at 14,000 rpm at room temperature, the upper layer was collected, and the RNA was ethanol precipitated in the presence of GlycoBlue (Life Technologies, Inc.). The obtained RNA was resuspended in 30 μL water and used to generate cDNA and subsequent RT-PCR analysis.

Transfection and immunoblotting
MCF7 and MCF7:2A cells were transfected with 20 pmol of miRIDIAN miRNA anti-miRs or miRNA mimics as described above. Cells were incubated for 5 days, and whole-cell extracts were then harvested in RIPA buffer (TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide). Protein lysates were quantified using the BCA Protein Assay Kit (Pierce), and they were then separated in 4% to 12% NuPAGE Bis-Tris SDS/PAGE Protein Gels (Life Technologies) followed by transfer onto a polyvinylidene difluoride membrane. The membrane was blotted with anti-HER2 (2165; Cell Signaling Technologies) and β-actin (4967; Cell Signaling Technologies) antibodies followed by incubation with a secondary donkey anti-rabbit antibody (Pierce). The blots were developed using the Western Blotting Luminol Reagent (Santa Cruz Biotechnology).

Patient sample analysis
For patient sample analysis, data were extracted from the Breast Invasive Carcinoma provisional dataset in The Cancer Genome Atlas (TCGA) using the cBioPortal for Cancer Genomics CGDS-R version 1.1.19 package in R version 3.0.2. Kaplan–Meier analysis was performed using the Survival package version 2.37-7, and significance was determined using the log-rank test.

Results
miRNAs are differentially expressed in MCF7:2A versus MCF7 cells
To identify candidate miRNAs that may play a role in endocrine resistance, we compared miRNA expression between estrogen-
dependent MCF7 cells and the estrogen-independent derivative cell line MCF7:2A using the nCounter NanoString platform. Using RNA derived from MCF7 and MCF7:2A cells under standard culturing conditions, we found that a number of miRNAs are differentially expressed (Fig. 1A). Of the 800 miRNAs assayed by this method, 78 (9.8%) had significant differential expression ($P < 0.05$, 1.5-fold) in the two cell lines including 54 that were downregulated and 24 that were upregulated in MCF7:2A cells as compared with MCF7 cells (Table 1). Of these miRNAs, 57 are located within annotated sequences including coding and non-coding RNAs, and 21 are intergenic (Table 1). The top upregulated miRNA was miR148a (fold change: 10.6, $P$ value: 3.9 x $10^{-20}$), and the top downregulated miRNA was miR99a (fold change: –19.7, $P$ value: 5.1 x $10^{-25}$; Fig. 1B). We found that the miR17-92a cluster, previously been shown to be regulated by ER was upregulated, and that miR221/222, which was previously shown to regulate ER expression, was downregulated (16, 20) in MCF7:2A versus MCF7 cells. In addition, the clusters miR497/miR195, miR590-3p/miR590-5p, and miR30e/miR30e were significantly upregulated in MCF7:2A cells, whereas the let-7c/miR99a/miR125b cluster was downregulated (Supplementary Fig. S1).

The miR7c locus is downregulated in MCF7:2A cells

The most significantly underexpressed miRNA in MCF7:2A cells compared with parental MCF7 cells is miR99a. This miRNA is encoded in the intronic sequence of the long noncoding RNA (lncRNA) LINC00478 together with let-7c and miR125b (Fig. 2A), which are also downregulated in MCF7:2A cells (Fig. 1). Examination of ER binding near this miRNA cluster demonstrates that there is a loss of ER-binding activity at this locus in MCF7:2A versus MCF7 cells (Fig. 1). Interestingly, ER binding at the nearby NRIP1 gene is not lost.

All three miRNAs in this cluster are also downregulated in two additional estrogen-independent derivatives of MCF7 cells, MCF7:5C and MCF7:LTLT (Fig. 2B; refs. 22, 23). The

Figure 1. Differentially expressed miRNAs in MCF7:2A versus MCF7 cells. MCF7 and MCF7:2A cells were grown under standard culturing conditions, and small RNAs were extracted from each cell line. Each sample was then assayed for the expression of miRNA using nCounter NanoString assays. A, heatmap demonstrating the differentially expressed miRNAs found in the MCF7:2A and MCF7 cells including 54 upregulated and 24 downregulated miRNAs. B, volcano plot demonstrating the profile of the differentially expressed miRNAs in MCF7:2A versus MCF7 cells. This plot demonstrates the fold change ($x$-axis) and significance level expressed as the $-\log_{10}$ $P$ value ($y$-axis). The green circles represent the miRNAs downregulated in the MCF7:2A compared with MCF7 cells, and the red circles represent the miRNAs upregulated in the MCF7:2A compared with MCF7 cells. The blue circles indicate miRNAs that were not significantly expressed. Significance was determined with a $P$ value cutoff of 0.05 and a 1.5-fold change.
Table 1. miRNAs differentially regulated in MCF7:2A versus MCF7 cells

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survival than those expressing lower levels of miR99a, let-7c, and miR125b (Fig. 3C). Furthermore, the low-expressing luminal A subset has a similar outcome as luminal B patients (Supplementary Fig. S5). Because low expression of this cluster in patients with luminal A breast cancer indicates poor outcome and the luminal B subset is characterized by the low expression of this cluster and poor outcome (24), these data suggest that low let-7c/miR99a/miR125b expression is predictive of poor outcome for ER patients.

let-7c, miR99a, and miR125b inhibit MCF7:2A cell growth and target HER2

We next sought to determine whether the let-7c/miR99a/miR125b cluster has an effect on cell growth. MCF7:2A cells were transfected with each of the individual miRNAs, and the number of cells was counted every other day for 5 days. Although there was little to no difference in the growth rate of MCF7:2A cells transfected with a miRNA mimic control compared with untransfected cells, there was a significant decrease in the growth rate of cells transfected with miRNA mimics for let-7c, miR99a, and miR125b (Fig. 4A, top). In addition, when we transfected MCF7 cells with anti-miRs targeting each of the miRNAs, we found that anti-miRs directed against let-7c and miR125b significantly increased the growth of MCF7 cells, while the growth effects of anti-miR99a were insignificant. Together, these data suggest that loss of the let-7c/miR99a/miR125b cluster in MCF7:2A cells provides a growth advantage by permitting the expression of downstream miRNA targets.

We next sought to identify targets that may be responsible for the growth of these cells. A previous study reported that miR125b targets HER2 in an in vitro system (25). HER2 has also been shown to be responsible for the growth and activity of MCF7 cells that have been selected for estrogen-independent growth (22, 26) and is expressed at a higher level in MCF7:2A, MCF7:5C, and MCF7:LTLT cell lines. C and D, E2 regulates the expression of the cluster miRNAs and primary transcript. MCF7 cells were treated with E2 for 3 hours, and the level of let-7c, miR99a, miR125b, and LINC00478 expression was determined by RT-PCR. E and F, fulvestrant treatment leads to loss of the cluster miRNAs and LINC00478. MCF7 cells were treated with fulvestrant for 48 hours, and the level of let-7c, miR99a, miR125b, and LINC00478 expression was determined by RT-PCR. *, P < 0.01; **, P < 0.001; ***, P < 0.0001.
lLT cells compared with MCF7 cells. This expression pattern is in contrast with the level of ER protein expression, which is similar in the MCF7, MCF7:2A, and MCF7:5C cells and elevated in the MCF7:lLT cells (Fig. 4B). To determine whether HER2 protein expression is under miRNA control, we transfected MCF7:2A cells with miRNA mimics and measured the HER2 protein expression level in these cells after a period of 5 days. As expected, the miR125 mimic led to a decrease in HER2 protein expression as measured by Western blot analysis (Fig. 4C), whereas the miR99a mimic had little to no effect; however, let-7c also led to a decrease in HER2 protein expression (Fig. 4C). In addition, we found a significant decrease in the level of HER2 mRNA expression with let-7c overexpression (Supplementary Fig. S6). In contrast, no difference in HER2 mRNA level was found for miR125b overexpression as changes in mRNA level need not correlate with miRNA-mediated changes in protein expression. To further confirm that the HER2 protein is targeted by these miRNAs, we cloned the 3′-UTR of HER2, the gene that encodes the HER2 protein, downstream of Renilla luciferase, and determined changes in the level of luciferase activity in the presence of the mimics and anti-miRs of this miRNA cluster. Cotransfection of the HER2-UTR luciferase plasmid with let-7c led to a decrease in reporter expression that was similar to that for miR125b. In contrast, transfection with the mimic for miR99a had no effect (Fig. 4D). In addition, cotransfection of the HER2 3′-UTR luciferase reporter with anti-miRs confirmed that let-7c and miR125b act through the HER2 3′-UTR (Fig. 4E). These data suggest that let-7c and miR125b regulate HER2 at the protein level. In contrast with miR125b, which has been previously demonstrated to directly target the HER2 3′-UTR, let-7c is not predicted to target the HER2 3′-UTR. Thus, we attempted to determine the sequences targeted by let-7c in the HER2 3′-UTR by examining sites predicted by the Probability of Interaction by Target Accessibility (PITA) algorithm, which takes into account the free energy of base pair binding for potential sites (Supplementary Fig. S7A; ref. 27). However, mutation of these sites could not block the let-7c–mediated reduction in luciferase activity, suggesting that the effects on the HER2 3′-UTR mediated by let-7c may be indirect (Supplementary Fig. S7B). In examining targets previously reported to be regulated by let-7c that could mediate the effects of let-7c on HER2 expression, we found that there is strong downregulation of Dicer mediated by let-7c overexpression (Supplementary Fig. S7C). This observation suggests that the...
To further confirm that the HER2 gene is regulated by miRNAs in MCF7 cells, we examined its association with the Ago1 complex, which plays a role in translational silencing mediated by miRNA. We performed immunoprecipitation of the Ago1 complex in MCF7 and MCF7:2A cells and measured the level of associated HER2 mRNA (Fig. 4F). In contrast with the levels of the Myc or p21 mRNA in the Ago1 complex that are equivalent in MCF7 and MCF7:2A cells, the level of HER2 mRNA associated with the Ago1 complex is significantly reduced in MCF7:2A cells compared with MCF7 cells. These data support the conclusion that there is less miRNA-mediated regulation of HER2 expression in MCF7:2A cells compared with MCF7 cells, leading to greater HER2 protein expression in these cells.

HER2 protein expression and activity are negatively correlated with let-7c expression

To validate our cell model findings in actual patient samples, we examined whether there is a correlation between HER2 protein expression and activity and the expression of let-7c and miR125b miRNAs in patient samples using HER2 protein expression and phosphorylation data obtained from the TCGA cohort (Fig. 5 and Supplementary Fig. S7). We found that let-7c levels are significantly negatively correlated with HER2 protein expression (Fig. 5A; \( r = -0.28 \)) in the luminal A subset of patients. In addition, there was a similar negative correlation with the expression of the Tyr1248 phosphorylated form of HER2 (Fig. 5B; \( r = -0.16 \)).
sion in predicting which patients may benefit from treatment [1]. Insights into both breast cancer subtypes and increased precision in predicting outcome, but also more importantly may identify novel therapeutic strategies to overcome resistance. Expression profiling of mRNA genes has provided important insights into both breast cancer subtypes and increased precision in predicting which patients may benefit from endocrine therapy [4, 28]. More recently, miRNA expression levels have been explored both for predictive biomarker development and therapeutic target identification. Expression of miRNAs has been reported to be generally decreased during cancer progression [9]. By examining the miRNA expression profile of cell lines modeling estrogen-dependent and estrogen-independent ER\textsuperscript{+} cancers, we found that expression of the let-7c/miR99a/miR125b cluster is decreased during the progression to endocrine resistance. In data derived from a large cohort of primary breast cancers, this miRNA cluster was found to be uniformly reduced in luminal B tumors, a subset characterized by its aggressiveness, lower ER expression, and poorer survival in comparison with luminal A cancers (6, 29, 30). More significantly, luminal A tumors, which generally have more favorable outcome and a better response to endocrine therapy (31, 32), could be subdivided on the basis of the expression of this miRNA cluster. High cluster expression led to characteristically favorable outcome, whereas low cluster expression reflected patients with poor outcome.

Patient outcome could be directly related to the proteins targeted by the differentially expressed miRNAs; thus, we examined the expression of HER2, which was previously described as a miR125b target. Surprisingly, we found that let-7c also regulates HER2 expression. We found a negative correlation between let-7c miRNA expression and the expression of HER2 protein and phosphorylated HER2 in TCGA patient samples, but no correlation was found for miR125b. These data suggest that let-7c may be the most clinically relevant miRNA within the let-7c/miR99a/miR125b cluster. HER2 expression has been correlated with the expression of Lin28 and its homolog Lin28b [33]. These proteins bind the stem loop of let-7 family member precursors to directly inhibit the Drosha- and Dicer-mediated processing of their primary-miRNA precursors into mature let-7 miRNAs [34–38]. Moreover, Lin28 expression determines the expression of the let-7 family in tumors and cell lines [33, 39].

Previous studies have shown that the let-7 family controls the cell cycle, is associated with increased proliferation, and blocks tumorigenicity [40–42]. Moreover, Lin28 is transcriptionally regulated by Myc, which is an ER-regulated gene that is upregulated with progression to hormone independence [43, 44]. This protein is also targeted by let-7, suggesting a regulatory loop involving Lin28, let-7, and Myc [45–47]. As we found that let-7c could also target HER2, our data suggest that let-7 family members may be directly involved in the regulation of HER2 in Lin28-negative breast tumors.

Because many mRNAs are predicted to be targeted by the let-7c/miR99a/miR125b cluster, other targets of these miRNAs may also be significantly regulated in breast cancer. The mTOR protein, which is a downstream effector of the PI3K pathway [48], has been reported to be regulated by miR99a [49]; thus, it would be interesting to determine whether this miR99a targets the expression of mTOR, which has also been reported to play a role in endocrine resistance [50–52]. In addition, all three miRNAs are predicted to target insulin-like growth factor 1 receptor, which is a growth factor receptor that, like HER2, has been reported to be upregulated in estrogen-deprived breast cancer cells and is thought to be responsible for breast cancer cell signaling pathways. Thus, loss of expression of this miRNA cluster may play a role in the acquisition of endocrine resistance through the upregulation of multiple growth factor signaling pathways.

In summary, we have identified a number of miRNAs differentially expressed in estrogen-dependent versus estrogen-independent cells and have demonstrated that the let-7c/miR99a/miR125b cluster is group of miRNAs that regulate HER2 protein.
expression and when lost may lead to worse outcome for patients with luminal A tumors.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

**Authors’ Contributions**
Conception and design: S.T. Bailey, M. Brown
Development of methodology: S.T. Bailey
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.T. Bailey, T. Westerling
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.T. Bailey, M. Brown
Writing, review, and/or revision of the manuscript: S.T. Bailey, M. Brown
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**References**


Loss of Estrogen-Regulated microRNA Expression Increases HER2 Signaling and Is Prognostic of Poor Outcome in Luminal Breast Cancer

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