Received: 3 April 2018 | Revised: 28 June 2018 | Accepted: 2 July 2018

DOI: 10.1002/bit.26791

ARTICLE

WILEY BIOTECHNOLOGY & BIOENGINEERING

Dynamic microRNA activity identifies therapeutic targets in trastuzumab-resistant HER2$^+$ breast cancer

Joseph T. Decker[1] | Matthew S. Hall[1] | Rachel B. Blaisdell[1] | Kallen Schwark[1] | Jacqueline S. Jeruss[1,2] | Lonnie D. Shea[1]

[1]Department of Biomedical Engineering, University of Michigan, Ann Arbor, Michigan

[2]Department of Surgery, University of Michigan, Ann Arbor, Michigan

Correspondence
Lonnie D. Shea, Department of Biomedical Engineering, University of Michigan, 1119 Carl A. Gerstacker Building, 2200 Bonisteel Boulevard, Ann Arbor, MI 48109.
Email: ldshea@umich.edu

Funding information
National Cancer Institute, Grant/Award Number: 9R01CA214384

Abstract

MicroRNAs (miRNAs) are implicated in numerous physiologic and pathologic processes, such as the development of resistance to chemotherapy. Determining the role of miRNAs in these processes is often accomplished through measuring miRNA abundance by polymerase chain reaction, sequencing, or microarrays. We have developed a system for the large-scale monitoring of dynamic miRNA activity and have applied this system to identify the contribution miRNA activity to the development of trastuzumab resistance in a cell model of HER2$^+$ breast cancer. MiRNA activity measurements identified significantly different activity levels between BT474 cells (HER2$^+$ breast cancer) and BT474R cells (HER2$^+$ breast cancer cells selected for resistance to trastuzumab). We created a library of 32 miRNA reporter constructs, which were delivered by lentiviral transduction into cells, and miRNA activity was quantified by bioluminescence imaging. Upon treatment with the bioimmune therapy, trastuzumab, the activity of 11 miRNAs were significantly altered in parental BT474 cells, and 20 miRNAs had significantly altered activity in the therapy-resistant BT474R cell line. A combination of statistical, network and classification analysis was applied to the dynamic data, which identified miR-21 as a controlling factor in trastuzumab response. Our data suggested downregulation of miR-21 activity was associated with resistance, which was confirmed in an additional HER2$^+$ breast cancer cell line, SKBR3. Collectively, the dynamic miRNA activity measurements and analysis provided a system to identify new potential therapeutic targets in treatment-resistant cancers.

KEYWORDS
breast cancer, drug resistance, microRNA, trastuzumab

1 | INTRODUCTION

Targeted therapy for the treatment of breast cancer has significantly improved the expected outcomes for patients with specific disease subtypes. For example, the humanized HER2 antibody, trastuzumab, developed as a therapy for the treatment of HER2$^+$ breast cancers, has led to tremendous progress in the treatment of the 20% of breast cancer patients with amplified HER2 (Cobleigh et al., 1999; Romond et al., 2005; Slamon et al., 2001). Yet despite these advances, limitations remain, namely the development of resistance to targeted therapy (Nahta, Yu, Hung, Hortobagyi, & Esteva, 2006). Many mechanisms for resistance to trastuzumab have been suggested, including PTEN activation (Nagata et al., 2004), cyclin E overexpression (Scaltriti et al., 2011), and PI3K3CA pathway activation (Berns et al., 2007), among others (Gong et al., 2011; Gottesman, 2002; Valabrega, Montemurro, & Aglietta, 2007). While progress has been made as for new therapeutic strategies for

trastuzumab-resistant breast cancer, the precise mechanisms and systems responsible for treatment failure are active and critical areas of investigation.

One increasingly studied mechanism for resistance is altered expression of microRNA (miRNA) in the resistant cells. MiRNA is a class of small noncoding RNA that act as inhibitors of translation. MiRNAs recognize complementary sequences in the 3′ untranslated region (3′ UTR) and recruit Argonaut to the mRNA, causing degradation. MiRNA acts as a regulator for gene expression and can buffer against changes in endogenous promoter activity (Filipowicz, Bhattacharyya, & Sonenberg, 2008; Jonas & Izaurralde, 2015; Nilsen, 2007). MiRNA can act as an oncogene due to its regulatory role and have been associated with metastasis (Ma, Teruya-Feldstein, & Weinberg, 2007; Tavazoie et al., 2008; Yan et al., 2008) and epithelial–mesenchymal transition (Burk et al., 2008; Chang et al., 2011; Wellner et al., 2009). MiRNA abundance is often altered in cancer and has therefore been suggested as a prognostic indicator (Lu et al., 2005; Volinia et al., 2006). MiRNAs 21, 125, 145, and 155 were recently identified as significantly dysregulated in breast cancer and correlated with clinical outcome (Iorio et al., 2005). Additionally, several studies have linked dysregulation of the abundance of miRNAs such as miR-21 (Gong et al., 2011), miR-125 (Luo et al., 2017), miR-210 (Jung et al., 2012), or miR-375 (Ye et al., 2014) to differential sensitivity to trastuzumab therapy in $HER2^+$ cancer, indicating an essential role for miRNA activity in mediating the response to targeted therapy.

Currently, miRNAs associated with treatment resistance have been almost exclusively identified by their abundance relative to healthy tissues. However, tracking the abundance of miRNA may overlook critical mechanistic data as for the effects of the differential activity of these molecules. To this end, methods to track abundance of miRNA do not always correlate with miRNA activity (Mullokandov et al., 2012), indicating that abundance-based methods may not accurately delineate the impact of a particular miRNA on the physiology of the cancer cell. Furthermore, monitoring the dynamics of the miRNA may identify crucial mechanistic information or time points that is associated with miRNA function. New methods to dynamically track miRNA activity in treatment-resistant cancers could provide crucial insights currently lacking in the field of miRNA-mediated drug resistance.

In this report, we developed a system to monitor miRNA regulatory dynamics in trastuzumab-resistant $HER2^+$ breast cancer, to identify potential therapeutic targets and mechanisms that mediate resistance. The system for monitoring miRNA activity was based on adopting a technology termed TRACER (TRanscriptional ACtivity CEll aRray), which had been used to track the dynamic activity of transcription factors (Bernabé et al., 2016; Decker et al., 2017). TRACER utilizes a parallel reporter assay to probe the dynamics of transcription factor activity during treatment. These reporters consist of a luciferase gene whose expression is driven by a transcriptional response element. Luciferase measurements on living cells provide a dynamic measure of activity within the cells during treatment, which can be applied to a wide variety of culture systems or reporter constructs. We have previously used TRACER to identify transcriptional targets in PARP inhibitor-resistant BRCA-mutated breast cancer (Decker et al., 2017), as well as to determine transcriptional regulators of ErbB2-mediated (HER2-mediated) oncogenesis in breast cancer (Weiss et al., 2014). Herein, we developed a library of miRNA reporter constructs, which consists of constructs encoding for the luciferase gene with a miRNA recognition sequence in the 5′ region. Active miRNA can bind to the luciferase mRNA at the recognition site and lead to degradation of the luciferase mRNA, decreasing luminescence and providing a dynamic measurement of changes in miRNA activity. We characterized the miRNA activity in cells treated with trastuzumab to examine and identify the differential activity associated with resistance. A previously developed computational pipeline was used to determine key miRNA from the dynamic multivariate data, which were then validated experimentally.

2 | MATERIALS AND METHODS

2.1 | Cells and reagents

BT474 and SKBR3 cells were sourced from the American Type Cell Culture repository. BT474 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. SKBR3 cells were maintained in McCoy's 5A media supplemented with 10% fetal bovine serum. Media was replaced every 3 days and cells were passaged after they became 80% confluent. A trastuzumab-resistant cell line (BT474R) was established by continuous culture of the parental BT474 line in 10 μg/ml trastuzumab for greater than 6 months, as previously described (Scaltriti et al., 2011).

2.2 | MiRNA inhibitor assay

Cell viability was analyzed by MTS assay (Sigma, St. Louis, MO). Cells (1,500–3,000) were plated in each well of a 96-well tissue culture plate with 100 μl of medium. The next day, cells were transfected with either miR-21 Power Inhibitor (Exiqon, Vedbaek, Denmark) or random Power Inhibitor control inhibitor using JetPrime (PolyPlus, Illkirch, France). After 24 hr, the media was replaced with 100 μl of fresh media containing 10 μg/ml trastuzumab or vehicle control, as indicated, and the cells were grown for 3 days. Stock trastuzumab was prepared in deionized water and stock miRNA inhibitor was prepared in TE buffer. At the end of the treatment period, 10 μl of MTS solution was added to each well, the cells were incubated at 37°C for 1 hr, and absorbance was read at 490 nm. Data are presented as a percentage of the control cells cultivated under the same conditions or the absorbance of the wells. Two-way analysis of variance and Tukey's test for multiple comparisons was used to statistically evaluate differences between groups.

2.3 | Lentivirus

Lentivirus was produced by co-transfecting HEK-293T cells with previously described lentiviral packaging vectors (pMDL-GagPol,

pRSV-Rev, and pIVS-VSV-G; Dull et al., 1998) and lentiviral vectors using JetPrime (PolyPlus). After 48 hr, supernatants were collected and cell debris was spun down and removed. Viruses were concentrated using PEG-it (Systems Biosciences, Palo Alto, CA) and resuspended in phosphate buffered saline. Viral titer was measured using a qPCR Lentivirus Titration Kit (ABM, Richmond, BC, Canada).

2.4 | MiRNA activity arrays

MiRNA activity reporters consist of a single miRNA binding site cloned into the 3′ UTR of a phosphoglycerate kinase (PGK) promoter-driven firefly luciferase. Firefly luciferase was chosen as the reporter because of the linear relationship between luminescence and enzyme abundance over several orders of magnitude. Activity of the miRNA binding at the recognition site of a target mRNA leads to degradation and an overall reduction of signal from that reporter. MiRNA sequences were sourced from miRbase (Kozomara & Griffiths-Jones, 2013), with the exact complementary sequence to the miRNA used for the reporter. Reporter sequences were cloned between the *Nhe*I and *Xho*I recognition sites in the pmirGlo vector from Promega (Madison, WI). This reporter was excised from the original backbone using *Bgl*II and *Xho*I and cloned between the *Bam*HI and *Xho*I sites of the pCS-CG third generation lentiviral vector (Miyoshi, Blömer, Takahashi, Gage, & Verma, 1998).

Dynamic miRNA activity was measured for two cell lines. BT474 cells overexpress HER2 and are sensitive to trastuzumab. A resistant derivative of these cells (BT474R) was used as a model for acquired resistance to trastuzumab. Activity was measured for 32 different miRNAs during 48 hr of treatment with 10 μg/ml trastuzumab. Measurements acquired at several time points (0, 2, 4, 6, 8, 24, and 48 hr) allowed both initial differences between cell types as well as differences in response to trastuzumab treatment to be elucidated from the data.

All cell types were transduced with 10 MOI of each miRNA activity-reporting lentivirus. Transduced cells were then seeded on a 96-well plate with at least three technical replicates of each condition. All reporters were repeated with at least $n = 3$ for biological replicates. Two days after cell seeding, luciferase activity was measured using an IVIS Spectrum (Caliper Life Sciences, Hopkinton, MA). The 2-day period is sufficient time for ensuring lentiviral gene expression. After changing media, cells were treated with 10 μg/ml of trastuzumab, and the luciferase activity was measured for 48 hr. A blank reporter with no miRNA binding site was used as a control. Thus, all other miRNA activity was normalized with respect to blank activity and represented by miRNA/blank ratio. MiRNA reporters start at a high luminescence and are dimmer as the miRNA is more active and therefore degrading the luciferase mRNA. As such, less light emission correlates to higher miRNA activity.

2.5 | Statistical analysis

Activity levels for different miRNAs were normalized to a blank reporter with the corresponding treatment. We performed background subtraction and loss normalization to correct for systematic noise. All normalized miRNA activity levels were $\log_2$ transformed. Results of experiments are presented as the mean ± standard error unless otherwise indicated. Differences in means were evaluated by fitting an empirical hierarchical Bayesian linear model using the *limma* R package (Smyth, 2005). *p*-Values were adjusted using the false discovery rate correction (Benjamini & Hochberg, 1995). A *p*-value less than 0.05 was considered to be statistically significant.

2.6 | Classification analysis

Principal components analysis (PCA) and partial least-squared discriminant analysis (PLS-DA) were performed to identify multivariate combinations of factors and their time course that would separate the different conditions. Both analyses were performed using the mixOmics package in R (Rohart, Gautier, Singh, & Le Cao, 2017). Each individual 96-well plate included only a subset (10–16 of 32) of the measured miRNAs. Since both PCA and PLS-DA require the full complement of measured factors, we used randomly sampled individual biological replicates to generate 1,000 simulated experiments containing one biological replicate of every miRNA for PCA and PLS-DA. Data were normalized to blank reporter control and untreated control within an experiment before sampling, leaving three equivalent groups: Treated BT474 (HCP_BT474), treated BT474R (HCP_BT474R), and untreated BT474/BT474R (NT). Individual experiments were variance scaled to standardize all data before multivariate analysis.

2.7 | Network analysis

Network analysis of miRNA activity measurements was carried out using a modified version of NTRACER, which has been described previously (Bernabé et al., 2016). As with the classification analysis, biological replicate data was sampled to create 50 sets of complete measures for each run of the network analysis. A total of 500 runs was performed. Normalized activity measurements were variance scaled and an initial network topology inferred through several different techniques: Linear methods (PLSR [Mevik & Wehrens, 2007], similarity index [Siletz et al., 2013], linear ordinary differential equations based on TIGRESS [Haury, Mordelet, Vera-Licona, & Vert, 2012]), and nonlinear methods (ARACNE [Margolin et al., 2006], CLR [Faith et al., 2007], MRNET [Meyer, Kontos, Lafitte, & Bontempi, 2007]). Features were selected from the top 10% of edges for each inference method at each set of time points based on the relevant score for each inference technique. Possible connections between miRNAs were assigned a score of one if they were in the top 10% at least once in the set of time points. The results of each inference method summed between runs, and the final 10% of summed edges for the entire experiment were calculated after the conclusion of 500 runs of the inference code. Network edges were further pared by including only edges that were inferred through multiple inference methods to ensure high-quality connections in the final model. Networks were visualized and analyzed for eigenvector centrality using the R package *iGraph* (Csardi & Nepusz, 2006).

2.8 | Selection of relevant miRNA

We adapted a previously published method for identifying transcription factors that mediate drug resistance to this study of miRNA activity (Decker et al., 2017). Three different analysis methods were used to score miRNAs, with a total of four components leading to the score. These methods were selected to account for univariate differences between treatments, multivariate scoring and networked connections between factors. Statistical analysis using *limma* was used to compare treated BT474R cells to (a) untreated BT474R cells and (b) treated parental BT474 cells. Reporters were scored either a one (significant) or a zero (not significant) based on this analysis. Second, the network analysis from NTRACER was used to score the reporters based on their centrality to the network. Eigenvector centrality awarded scores between 0 and 1 based on how central the reporter was to the network. Finally, variable importance in projection (VIP) scores from the PLS-DA were used to measure the importance of each reporter within the multivariate context of these experiments. The maximum VIP score for each individual time point was used and scaled so 1 was the maximum value. The top scoring factor using these criteria was selected for further analysis.

3 | RESULTS

3.1 | Large-scale dynamic miRNA activity measurement

We initially investigated miRNA activity dynamics in two related cell lines that overexpress HER2 yet have different responses to trastuzumab: BT474 (parental) and BT474R (resistant). A total of 32 miRNAs were selected based on their previous association with drug sensitivity in cancer. These two cell lines had significantly different miRNA activity patterns at baseline, with a trend toward a higher number of active miRNAs in the treatment-sensitive cells. Two miRNAs were initially "active" in BT474R cells (miR-92 and miR-200a), which was observed as less luminescence compared with a blank control ($p < 0.05$), while 19 miRNAs were "active" in the parental BT474 cell line (Figure 1a,b). The most active of these miRNAs, in the BT474 cell line, were miR-21, miR-23b, and miR-32. Among the miRNAs that were significantly active in the BT474 cells, two were significantly different from BT474R activities at baseline, miR-20 and miR-32.

We next monitored dynamic miRNA activity in response to trastuzumab treatment in both cell lines. Significant alterations in miRNA activity were observed in both cell lines over 48 hr of treatment (Figure 2). A total of 11/32 (34%) miRNAs were significantly altered in BT474 cells ($p < 0.05$), with miR-100, miR-145, and miR-221 having the most substantial changes ($p < 0.01$). With respect to the resistant cell line (BT474R), a total of 20/32 (63%) miRNAs had significantly altered activity in response to trastuzumab. A total of 15 of these miRNAs were altered beyond the $p < 0.01$ threshold, with miR-21, miR-335, miR-200a, miR-32, miR-373, and miR-210 showing the most significant alterations ($p < 1e-7$). Hierarchical clustering of miRNA dynamics between the two cell lines demonstrated six broad

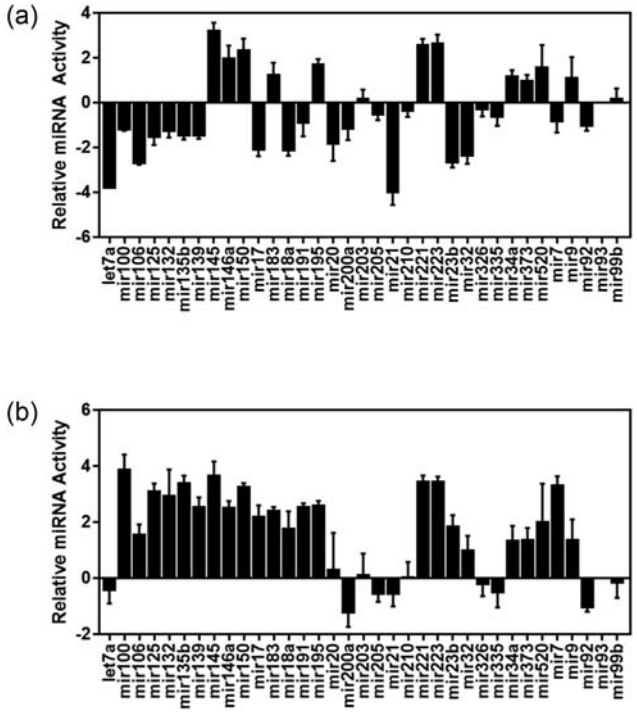

FIGURE 1 Static, initial microRNA (miRNA) activity measurements in (a) trastuzumab sensitive (BT474) and (b) resistant (BT474R) cells. Data is presented as log normalized difference between miRNA reporter and a blank control. Negative numbers indicated increased miRNA activity. Error bars represent standard error

clusters of miRNAs with similar activity between the two cell types. The two most unique clusters contained one factor (miR-21) and two factors (miR-7 and miR-18a).

Connections between miRNAs based on their activity were subsequently inferred through implementation of a compilation of network inference tools (Bernabé et al., 2016). Two networks were inferred, one for trastuzumab response in cells that respond to the drug (BT474; Figure 3a) and one for cells that were resistant to the drug (BT474R; Figure 3b). These networks utilized the aggregate connections inferred between miRNAs for each time point to produce connections and central hub nodes for each network. Hub nodes were identified as those whose eigenvector centrality score was in the top 10% of all nodes. Hub nodes were interpreted as important to the response to trastuzumab, and not necessarily as direct targets of trastuzumab treatment. Both networks had similar hub nodes at miR-20 and miR-21. Responsive cells had additional hubs at miR-92 and miR-183, while sensitive cells had hubs at miR-9 and miR-99b. These miRNAs represented hubs based on their influence at one or more times in the network, as inferred by one or more of the methods used to create the consensus network.

3.2 | Identification of controlling miRNA

We utilized both unsupervised (PCA) as well as supervised (PLS-DA) classification methods to determine a combination of variables that

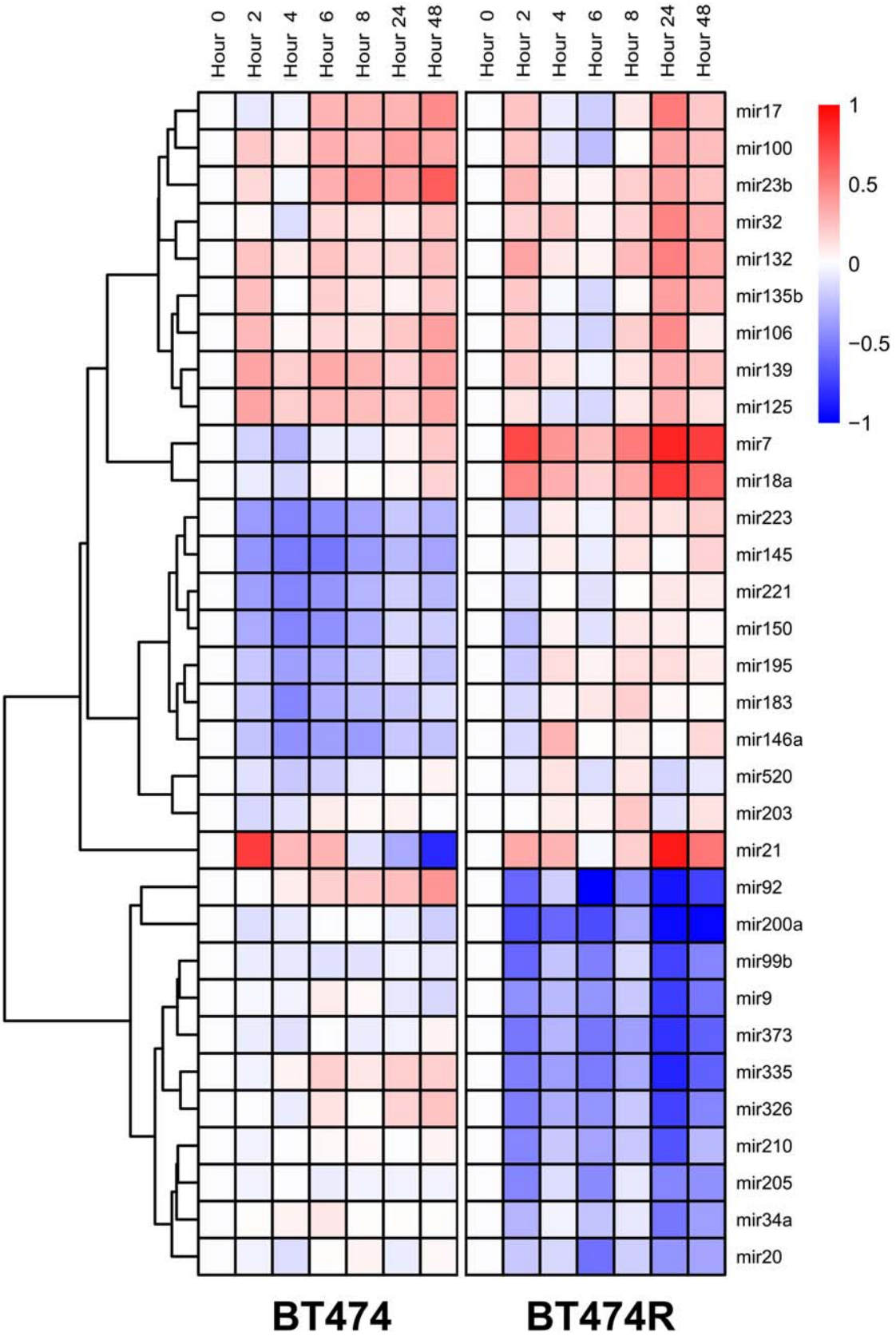

FIGURE 2 Dynamic microRNA (miRNA) activity in both BT474 (sensitive) and BT474R (resistant) cell lines during treatment with trastuzumab. Data is presented as log 2 normalized difference between miRNA reporter and a blank control. Brackets indicate groups identified through hierarchical clustering

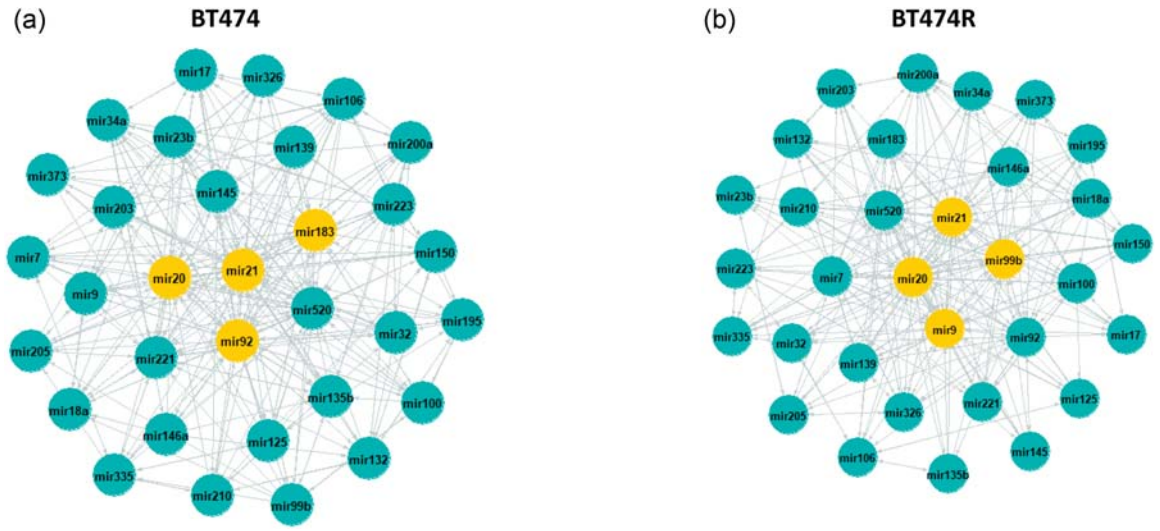

FIGURE 3 Network analysis for (a) BT474 and (b) BT474R cells over 48 hr treatment with trastuzumab. Yellow nodes are hubs identified by eigenvector centrality

separated BT474, BT474R and pooled normalized untreated controls. PCA was used to confirm the accuracy of separating the cells into three distinct types (Figure 4a). Two principal components were calculated, with PC1 explaining 20% of the sample variance and PC2 explaining 11% of the variance. Three broad groups of samples were found, which agreed with the three measurements that were used for the analysis (normalized untreated, treated BT474, treated BT474R). All conditions were normalized to the experimental control, and as such, both types of cells had similar values for the control and can be considered as one group for this analysis. A correlation circle of the top 10 variables (Figure 4b) had three clusters of factors and time points. miR-7, miR-18a, and miR-135b formed one cluster, while miRs 200a and 373 formed another along PC1. miRs 145, 146, 183, and 195 formed a cluster on PC2, with miR-23b forming solitary point as well. These variables were all-time point specific, which can be seen in the variable name (Figure 4b).

PLS-DA was used to identify a multivariate signature for the time-course changes in miRNA activity in the sensitive and resistant cell lines and also to identify how these cells lines differed from untreated control cells (Figure 5). We have previously demonstrated that this method provided superior classification for dynamic transcription factor activity data (Decker et al., 2017), and as such used this method to identify the linear combinations of miRNAs that best delineated treated and untreated sensitive and resistant cells. The time-course PLS-DA classified the cells into three groups (untreated aggregate, treated BT474, treated BT474R) with 98.9% accuracy from 10-fold cross validation using two components with 10 selected variables for each loading vector. Different factors were found to be highly loaded in LV1 (*X* axis) compared with LV2 (*Y* axis; Figure 5b,c). LV1 delineated resistant from sensitive and untreated cells. miR-21 after 48 hr was the highest loaded factor in LV1, with

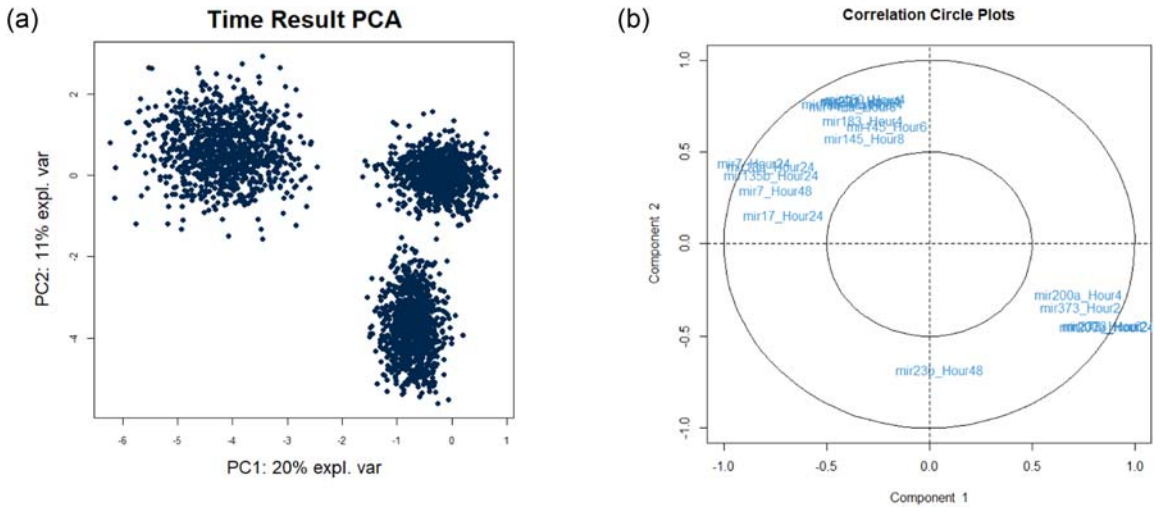

FIGURE 4 (a) Principal components analysis of time-course miRNA activity data. Data from separate partial experiments was randomly sampled to create 1,000 complete "experiments" for use analysis. (b) Correlation circle plot of selected activity measurements for the PCA model. miRNA: microRNA; PCA: principal component analysis

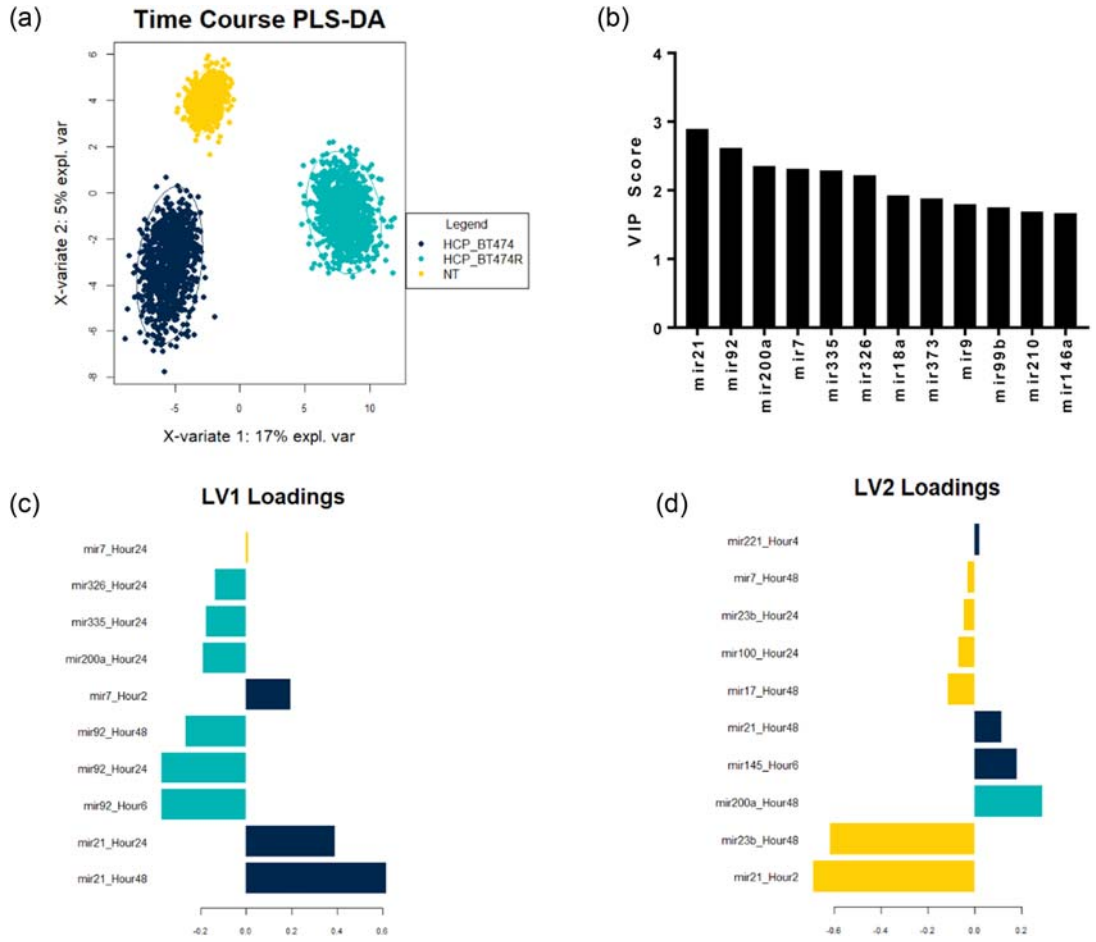

FIGURE 5 (a) PLS-DA plot separating bootstrapped samples from untreated, treated BT474 and BT474R cells. (b) VIP scores across for model. Similar scores were measured in both LVs. (c) Loadings in LV1. (d) Loadings in LV2. Colors indicate cell line/treatment with highest activity level. Ellipses in (a) represent 95% confidence intervals. PLS-DA: partial least-squared discriminant analysis; VIP: variable importance in projection

BT474 cells treated with trastuzumab having the highest level of activity. LV2 also delineated treated and untreated BT474 cells, with miR-150 after 4 hr as the highest loaded factor. VIP scores for the selected factors were also calculated (Figure 5d). These scores correspond to the relative estimated importance of each variable in the PLS-DA model. For the PLS-DA model associated with these experiments, the VIP scores were similar for both LV1 and LV2, with the highest score assigned to miR-21.

We subsequently tested the ability of our miRNA activity measurements to select relevant factors associated with trastuzumab resistance, using a method that combined statistical, classification, and network analyses (Decker et al., 2017). We scored our miRNAs based on their statistical significance between trastuzumab-resistant cells and untreated controls and treated responsive cells, centrality in the network analysis and importance in the PLS-DA model. This scoring system had a maximum possible value of 4, with the highest score factor selected for analysis; using these criteria, miR-21 was identified as the leading factor associated with trastuzumab resistance (Figure 6a). Initial activity measurements indicated increased miR-21 activity in BT474 cells relative to BT474R cells, with both lines having a decrease in activity in response to therapy. Based on the results with this model system, we hypothesized that miR-21 is a regulator of trastuzumab sensitivity, and specifically that inhibiting miR-21 activity will decrease the sensitivity to trastuzumab. This hypothesis was tested by transfection of a miR-21 inhibitor into SKBR3 cells, which served as a distinct model of $HER2^{+}$ breast cancer, unrelated to the study cells from which the hypothesis was derived. Transfection with a miR-21 inhibitor resulted in a 17% decrease in proliferation of SKBR3 cells relative to a random inhibitor control (Figure 6b). Both cells transfected with a miR-21 inhibitor, as well as those cells transfected with a random control, were sensitive to trastuzumab; however, the effect of the drug differed significantly between treatment groups (Figure 6c). The relative effect of adding trastuzumab to the system was significantly decreased in the presence of the miR-21 inhibitor compared to cells transfected with a random control inhibitor (17% decrease for miR-21 inhibitor condition compared with 40% in the control, $p < 0.001$). Collectively, treatment with trastuzumab resulted in decreased proliferation; however, inhibition of miR-21 reduced the impact of trastuzumab and thus contributed to the sensitivity to trastuzumab.

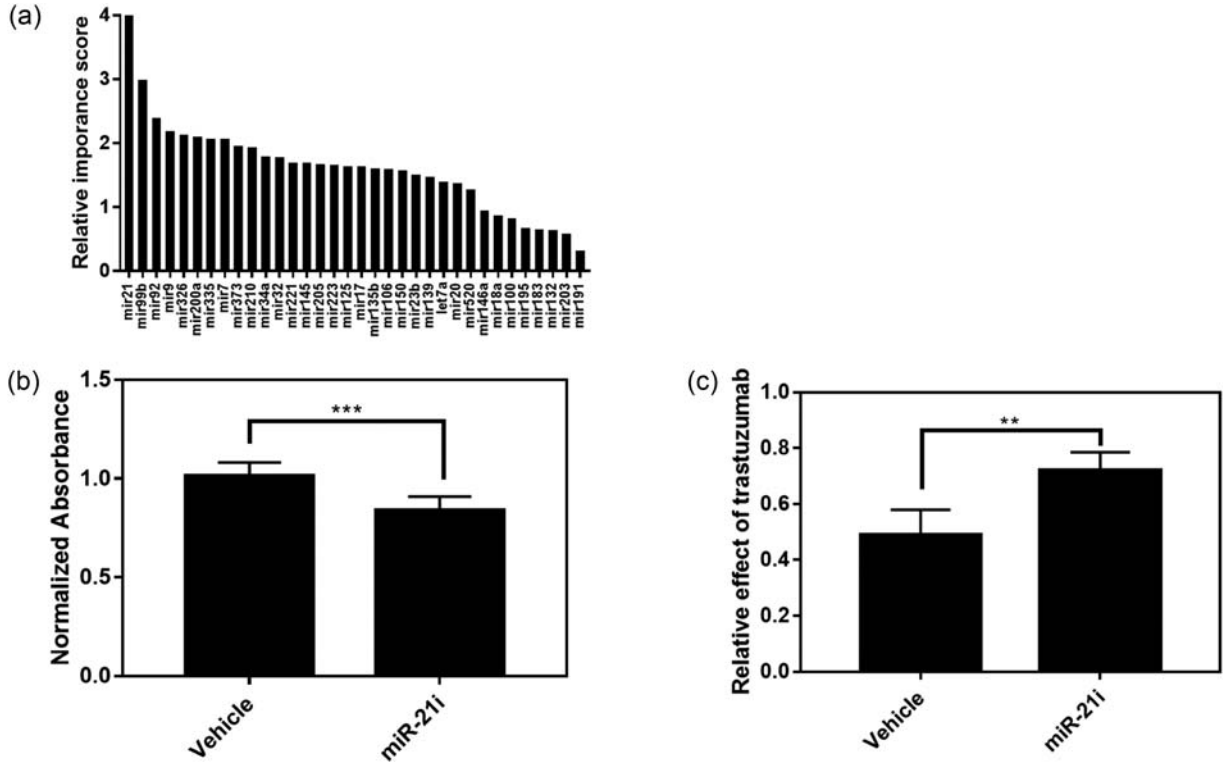

FIGURE 6 (a) Relative importance scores from statistical, network and classification identify miR-21 as important in trastuzumab resistance. (b, c) Proliferation assay for vehicle and trastuzumab-treated SKBR3 cells. (b) Effect of miR-21 on baseline proliferation of SKBR3 cells. (c) Effect of trastuzumab on proliferation in vehicle and inhibitor-treated cells. $^{**}p < 0.01$ and $^{***}p < 0.001$. miR-21: microRNA-21

4 | DISCUSSION

MiRNA has been the focus of intense study as both a biomarker and a therapeutic target (Pereira, Rodrigues, Borralho, & Rodrigues, 2013). In this report, we have used a new method, a miRNA activity array, to identify drug resistance mechanisms associated with HER2$^+$ breast cancer. We generated a library of miRNA reporters with 3′ UTRs that are exact matches to endogenous miRNAs from miRbase (Kozomara & Griffiths-Jones, 2013), which provides reporting for activity with maximum sensitivity and specificity. Our miRNA activity array expands upon our previous work developing a transcription factor activity array (Bellis et al., 2011; Bernabé et al., 2016; Decker et al., 2017) to measure the dynamic regulome in living cells. Both arrays utilize activity measurements of regulatory factors to provide a unique perspective on the regulome of the cell that can be missed through other techniques. Quantitative polymerase chain reaction and sequencing methods, the most commonly used methods to elucidate essential miRNAs in biological processes, are both expensive and often do not correlate well to the activity level of the miRNA (Mullokandov et al., 2012). Systems to report activity, therefore, have the potential to more effectively identify miRNAs that may serve as effective therapeutic targets. Our dynamic activity platform offered both a means to simultaneously quantify the activity of many relevant miRNAs, and implement the time-course of the dynamic activity to identify key factors controlling treatment response.

Our studies identified downregulation of miR-21 activity as associated with the intracellular response to trastuzumab and development of resistance. The identification of decreased miR-21 activity as contributing to trastuzumab-resistant HER2$^+$ breast cancer was performed using an updated analysis pipeline previously developed to recognize transcriptional targets of resistance to PARP inhibitors. This method combined statistical, classification and network analyses to identify factors that are consistently important in both the resistant phenotype, as well as the response to treatment in responsive and unresponsive cells. Time-course data provided a superior platform for both classification (Antonucci, De Rosa, Giusti, & Cuzzolin, 2015; Park, Koo, Kim, Sohn, & Lee, 2008; Robinson, Glonek, Koch, Thomas, & Davies, 2015) and network analysis, (Bar-Joseph, Gitter, & Simon, 2012; Bernabé et al., 2016) when compared with static data, which allowed the multiple techniques to converge on a single factor, miR-21.

Importantly, each method of analysis offered complementary information that was not available from a single technique. Statistical analysis provided a list of differential activities induced by the resistant phenotype, as well as treatment with trastuzumab. These measurements lack the multivariate context in which the different phenotypes can be distinguished. Network analysis provided a means to identify controlling hubs within the network and, when placed in the context of multivariate classification, to identify which factors that were essential in controlling treatment response. This report has updated the analysis pipeline to include a numerical score for each

factor, thus reducing bias that may be introduced in interpreting the results and providing a solid foundation for further experimentation. This particular study found miR-21 had the highest score. The next highest scoring factors, miR-99b and miR-92, have both been implicated in cancer aggressiveness and prognosis (Kang et al., 2012; Nilsson et al., 2012; Si et al., 2013; Wei et al., 2013). Specifically, miR-92a has been suggested as a partner to miR-21 as a biomarker for primary breast cancer (Si et al., 2013), supporting that this scoring method can identify relationships between factors associated with breast cancer, yet additional studies would be required to identify the precise mechanisms by which these factors may promote resistance to trastuzumab.

The relevance of miR-21 to HER2-targeted therapy was identified by its unique dynamics, centrality in the network, and ability to delineate sensitive and resistant cells in the multivariate analysis. The identification of miR-21 as a controlling factor for therapeutic response and resistance is supported by the literature for a number of different drugs and cancer types. MiR-21 activity has been implicated in the onset of drug resistance, and was reported to mediate resistance to treatments including cisplatin (Yang et al., 2013), gefitinib (Shen et al., 2014), and sorafenib (He et al., 2015). Specific to breast cancer, miR-21 abundance has been suggested as a biomarker for therapeutic response, (Müller et al., 2014; Si et al., 2013) and may confer resistance to trastuzumab (De Mattos-Arruda et al., 2015; Gong et al., 2011). We observed an initial decrease in miR-21 activity in both study cell types upon treatment with trastuzumab, followed by divergent activity patterns: Sensitive cells showed increased miR-21 activity while resistant cells maintained relatively low levels of activity (Figure 2). These results may be explained by the role of miR-21 in cell cycle regulation. Trastuzumab is considered to be cytostatic rather than cytotoxic to cells in vitro (Scaltriti et al., 2011; Vu & Claret, 2012), and as this therapy is antiproliferative (and is also why proliferation-based assays, such as the MTS used in this study, are appropriate). MiR-21 activity inhibition should increase expression of miR-21 target genes, such as the tumor suppressor PTEN, which would lead to a decrease in proliferation. We did indeed observe decreased proliferation in miR-21 inhibitor-treated SKBR3 cells, a second trastuzumab responsive cell line, compared with vehicle-treated cells, consistent with the proposed hypothesis. Furthermore, inhibiting miR-21 activity decreased the relative response to trastuzumab, which would be expected if trastuzumab acts in part through inhibition of miR-21 activity.

The regulome of the cell ultimately determines the cell fate, and disruption of the calibrated regulatory mechanisms of the cell can ultimately lead to oncogenic transformation. We advanced our previously described TRACER technology to encompass dynamic monitoring of miRNA activity and used this array to investigate the dynamics of miRNA activity in $HER2^+$ breast cancer to identify potential miRNA-mediated mechanisms for resistance. Both supervised and unsupervised classification could identify treated and untreated cells from both the resistant and responsive phenotypes, consistent with our previous observations using this method (Decker et al., 2017). Downregulation of miR-21 activity was identified through the multivariate analysis as a potential driving factor for the resistant phenotype in $HER2^+$ breast cancer cells. This hypothesis was confirmed by inhibiting the activity of miR-21, results that extended to an independent model of $HER2^+$ breast cancer. This tool for large-scale analysis of miRNA activity could be applied to numerous systems to identify the function of miRNA in cell fate determination, and these miRNAs may serve as either biomarkers or therapeutic targets.

ACKNOWLEDGMENTS

The authors would like to thank Lei Wan for her assistance in creating the trastuzumab-resistant cell line. This study was funded by NIH through grant number 9R01CA214384 (JSJ and LDS).

ORCID

Joseph T. Decker http://orcid.org/0000-0001-7914-4741
Lonnie D. Shea http://orcid.org/0000-0002-9296-9673

REFERENCES

Antonucci, A., De Rosa, R., Giusti, A., & Cuzzolin, F. (2015). Robust classification of multivariate time series by imprecise hidden Markov models. *International Journal of Approximate Reasoning*, *56*, 249–263.

Bar-Joseph, Z., Gitter, A., & Simon, I. (2012). Studying and modelling dynamic biological processes using time-series gene expression data. *Nature Reviews Genetics*, *13*(8), 552–564.

Bellis, A. D., Peňalver-Bernabé, B., Weiss, M. S., Yarrington, M. E., Barbolina, M. V., Pannier, A. K., … Shea, L. D. (2011). Cellular arrays for large-scale analysis of transcription factor activity. *Biotechnology and Bioengineering*, *108*(2), 395–403.

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B*, 289–300.

Berns, K., Horlings, H. M., Hennessy, B. T., Madiredjo, M., Hijmans, E. M., Beelen, K., … Bernards, R. (2007). A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. *Cancer Cell*, *12*(4), 395–402.

Burk, U., Schubert, J., Wellner, U., Schmalhofer, O., Vincan, E., Spaderna, S., & Brabletz, T. (2008). A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. *EMBO Reports*, *9*(6), 582–589.

Chang, C.-J., Chao, C.-H., Xia, W., Yang, J.-Y., Xiong, Y., Li, C.-W., … Hung, M. C. (2011). p53 regulates epithelial-mesenchymal transition (EMT) and stem cell properties through modulating miRNAs. *Nature Cell Biology*, *13*(3), 317–323.

Cobleigh, M. A., Vogel, C. L., Tripathy, D., Robert, N. J., Scholl, S., Fehrenbacher, L., … Slamon, D. J. (1999). Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *Journal of Clinical Oncology*, *17*(9), 2639–2639.

Csardi, G., & Nepusz, T. (2006). The igraph software package for complex network research. *InterJournal, Complex Systems*, *1695*(5), 1–9.

Decker, J. T., Hobson, E. C., Zhang, Y., Shin, S., Thomas, A. L., Jeruss, J. S., … Shea, L. D. (2017). Systems analysis of dynamic transcription factor activity identifies targets for treatment in Olaparib resistant cancer cells. *Biotechnology and Bioengineering*, *114*(9), 2085–2095.

Dull, T., Zufferey, R., Kelly, M., Mandel, R. J., Nguyen, M., Trono, D., & Naldini, L. (1998). A third-generation lentivirus vector with a conditional packaging system. *Journal of Virology*, *72*(11), 8463–8471.

Faith, J. J., Hayete, B., Thaden, J. T., Mogno, I., Wierzbowski, J., Cottarel, G., … Gardner, T. S. (2007). Large-scale mapping and validation of *Escherichia coli* transcriptional regulation from a compendium of expression profiles. *PLoS Biology*, *5*(1), e8.

Filipowicz, W., Bhattacharyya, S. N., & Sonenberg, N. (2008). Mechanisms of post-transcriptional regulation by microRNAs: Are the answers in sight? *Nature Reviews Genetics*, *9*(2), 102–114.

Gong, C., Yao, Y., Wang, Y., Liu, B., Wu, W., Chen, J., … Song, E. (2011). Up-regulation of miR-21 mediates resistance to trastuzumab therapy for breast cancer. *Journal of Biological Chemistry*, *286*(21), 19127–19137.

Gottesman, M. M. (2002). Mechanisms of cancer drug resistance. *Annual Review of Medicine*, *53*(1), 615–627.

Haury, A.-C., Mordelet, F., Vera-Licona, P., & Vert, J.-P. (2012). TIGRESS: Trustful inference of gene regulation using stability selection. *BMC Systems Biology*, *6*(1), 1.

He, C., Dong, X., Zhai, B., Jiang, X., Dong, D., Li, B., … Sun, X. (2015). MiR-21 mediates sorafenib resistance of hepatocellular carcinoma cells by inhibiting autophagy via the PTEN/Akt pathway. *Oncotarget*, *6*(30), 28867–28881.

Iorio, M. V., Ferracin, M., Liu, C.-G., Veronese, A., Spizzo, R., Sabbioni, S., … Croce, C. M. (2005). MicroRNA gene expression deregulation in human breast cancer. *Cancer Research*, *65*(16), 7065–7070.

Jonas, S., & Izaurralde, E. (2015). Towards a molecular understanding of microRNA-mediated gene silencing. *Nature Reviews Genetics*, *16*(7), 421–433.

Jung, E. J., Santarpia, L., Kim, J., Esteva, F. J., Moretti, E., Buzdar, A. U., … Calin, G. A. (2012). Plasma microRNA 210 levels correlate with sensitivity to trastuzumab and tumor presence in breast cancer patients. *Cancer*, *118*(10), 2603–2614.

Kang, J., Lee, S. Y., Lee, S. Y., Kim, Y. J., Park, J. Y., Kwon, S. J., … SON, J. W. (2012). MicroRNA-99b acts as a tumor suppressor in non-small cell lung cancer by directly targeting fibroblast growth factor receptor 3. *Experimental and Therapeutic Medicine*, *3*(1), 149–153.

Kozomara, A., & Griffiths-Jones, S. (2013). MiRBase: Annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Research*, *42*(D1), D68–D73.

Lu, J., Getz, G., Miska, E. A., Alvarez-Saavedra, E., Lamb, J., Peck, D., … Golub, T. R. (2005). MicroRNA expression profiles classify human cancers. *Nature*, *435*(7043), 834–838.

Luo, Y., Wang, X., Niu, W., Wang, H., Wen, Q., Fan, S., … Zhou, M. (2017). Elevated microRNA-125b levels predict a worse prognosis in HER2-positive breast cancer patients. *Oncology Letters*, *13*(2), 867–874.

Ma, L., Teruya-Feldstein, J., & Weinberg, R. A. (2007). Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature*, *449*(7163), 682–688.

Margolin, A. A., Nemenman, I., Basso, K., Wiggins, C., Stolovitzky, G., Favera, R., & Califano, A. (2006). ARACNE: An algorithm for the reconstruction of gene regulatory networks in a mammalian cellular context. *BMC Bioinformatics*, *7*(Suppl. 1), S7.

De Mattos-Arruda, L., Bottai, G., Nuciforo, P. G., Di Tommaso, L., Giovannetti, E., Peg, V., … Santarpia, L. (2015). MicroRNA-21 links epithelial-to-mesenchymal transition and inflammatory signals to confer resistance to neoadjuvant trastuzumab and chemotherapy in HER2-positive breast cancer patients. *Oncotarget*, *6*(35), 37269.

Mevik, B.-H., & Wehrens, R. (2007). The pls package: Principal component and partial least squares regression in R. *Journal of Statistical Software*, *18*(2), 1–24.

Meyer, P. E., Kontos, K., Lafitte, F., & Bontempi, G. (2007). Information-theoretic inference of large transcriptional regulatory networks. *EURASIP Journal on Bioinformatics and Systems Biology*, *2007*, 8–8.

Miyoshi, H., Blömer, U., Takahashi, M., Gage, F. H., & Verma, I. M. (1998). Development of a self-inactivating lentivirus vector. *Journal of Virology*, *72*(10), 8150–8157.

Müller, V., Gade, S., Steinbach, B., Loibl, S., von Minckwitz, G., Untch, M., … Schwarzenbach, H. (2014). Changes in serum levels of miR-21, miR-210, and miR-373 in HER2-positive breast cancer patients undergoing neoadjuvant therapy: A translational research project within the Geparquinto trial. *Breast Cancer Research and Treatment*, *147*(1), 61–68.

Mullokandov, G., Baccarini, A., Ruzo, A., Jayaprakash, A. D., Tung, N., Israelow, B., … Brown, B. D. (2012). High-throughput assessment of microRNA activity and function using microRNA sensor and decoy libraries. *Nature Methods*, *9*(8), 840–846.

Nagata, Y., Lan, K.-H., Zhou, X., Tan, M., Esteva, F. J., Sahin, A. A., … Yu, D. (2004). PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell*, *6*(2), 117–127.

Nahta, R., Yu, D., Hung, M.-C., Hortobagyi, G. N., & Esteva, F. J. (2006). Mechanisms of disease: Understanding resistance to HER2-targeted therapy in human breast cancer. *Nature Clinical Practice Oncology*, *3*(5), 269–280.

Nilsen, T. W. (2007). Mechanisms of microRNA-mediated gene regulation in animal cells. *Trends in Genetics*, *23*(5), 243–249.

Nilsson, S., Möller, C., Jirström, K., Lee, A., Busch, S., Lamb, R., & Landberg, G. (2012). Downregulation of miR-92a is associated with aggressive breast cancer features and increased tumour macrophage infiltration. *PLoS One*, *7*(4), e36051.

Park, C., Koo, J.-Y., Kim, S., Sohn, I., & Lee, J. W. (2008). Classification of gene functions using support vector machine for time-course gene expression data. *Computational Statistics & Data Analysis*, *52*(5), 2578–2587.

Peñalver bernabé, B., Shin, S., Rios, P. D., Broadbelt, L. J., Shea, L. D., & Seidlits, S. K. (2016). Dynamic transcription factor activity networks in response to independently altered mechanical and adhesive microenvironmental cues. *Integrative Biology*, *8*(8), 844–860.

Pereira, D. M., Rodrigues, P. M., Borralho, P. M., & Rodrigues, C. M. P. (2013). Delivering the promise of miRNA cancer therapeutics. *Drug Discovery Today*, *18*(5), 282–289.

Robinson, S., Glonek, G., Koch, I., Thomas, M., & Davies, C. (2015). Alignment of time course gene expression data and the classification of developmentally driven genes with hidden Markov models. *BMC Bioinformatics*, *16*(1), 196.

Rohart, F., Gautier, B., Singh, A., & Lê cao, K.-A. (2017). mixOmics: An R package for 'omics feature selection and multiple data integration. *PLoS Computational Biology*, *13*(11), e1005752.

Romond, E. H., Perez, E. A., Bryant, J., Suman, V. J., Geyer, C. E., Jr., Davidson, N. E., … Wolmark, N. (2005). Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *New England Journal of Medicine*, *353*(16), 1673–1684.

Scaltriti, M., Eichhorn, P. J., Cortés, J., Prudkin, L., Aura, C., Jiménez, J., … Baselga, J. (2011). Cyclin E amplification/overexpression is a mechanism of trastuzumab resistance in HER2+ breast cancer patients. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(9), 3761–3766.

Shen, H., Zhu, F., Liu, J., Xu, T., Pei, D., Wang, R., … Shu, Y. (2014). Alteration in Mir-21/PTEN expression modulates gefitinib resistance in non-small cell lung cancer. *PLoS One*, *9*(7), e103305.

Si, H., Sun, X., Chen, Y., Cao, Y., Chen, S., Wang, H., & Hu, C. (2013). Circulating microRNA-92a and microRNA-21 as novel minimally invasive biomarkers for primary breast cancer. *Journal of Cancer Research and Clinical Oncology*, *139*(2), 223–229.

Siletz, A., Schnabel, M., Kniazeva, E., Schumacher, A. J., Shin, S., Jeruss, J. S., & Shea, L. D. (2013). Dynamic transcription factor networks in epithelial-mesenchymal transition in breast cancer models. *PLoS One*, *8*(4), e57180.

Slamon, D. J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., … Norton, L. (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *New England Journal of Medicine*, *344*(11), 783–792.

Smyth, G. K. (2005). Limma: Linear models for microarray data, *Bioinformatics and computational biology solutions using R and Bioconductor* (397–420). Springer

Tavazoie, S. F., Alarcón, C., Oskarsson, T., Padua, D., Wang, Q., Bos, P. D., … Massagué, J. (2008). Endogenous human microRNAs that suppress breast cancer metastasis. *Nature*, *451*(7175), 147–152.

Valabrega, G., Montemurro, F., & Aglietta, M. (2007). Trastuzumab: Mechanism of action, resistance and future perspectives in HER2-overexpressing breast cancer. *Annals of Oncology*, *18*(6), 977–984.

Volinia, S., Calin, G. A., Liu, C.-G., Ambs, S., Cimmino, A., Petrocca, F., … Croce, C. M. (2006). A microRNA expression signature of human solid tumors defines cancer gene targets. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(7), 2257–2261.

Vu, T., & Claret, F. X. (2012). Trastuzumab: Updated mechanisms of action and resistance in breast cancer. *Frontiers in Oncology*, *2*, 62.

Wei, F., Liu, Y., Guo, Y., Xiang, A., Wang, G., Xue, X., & Lu, Z. (2013). MiR-99b-targeted mTOR induction contributes to irradiation resistance in pancreatic cancer. *Molecular cancer*, *12*(1), 81.

Weiss, M. S., Peñalver bernabé, B., Shin, S., Asztalos, S., Dubbury, S. J., Mui, M. D., … Shea, L. D. (2014). Dynamic transcription factor activity and networks during ErbB2 breast oncogenesis and targeted therapy. *Integrative Biology*, *6*(12), 1170–1182.

Wellner, U., Schubert, J., Burk, U. C., Schmalhofer, O., Zhu, F., Sonntag, A., … Brabletz, T. (2009). The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. *Nature Cell Biology*, *11*(12), 1487–1495.

Yan, L.-X., Huang, X.-F., Shao, Q., Huang, M.-Y., Deng, L., Wu, Q.-L., … Shao, J. Y. (2008). MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. *RNA*, *14*(11), 2348–2360.

Yang, S., Huang, C., Li, X., Yu, M., He, Y., & Li, J. (2013). MiR-21 confers cisplatin resistance in gastric cancer cells by regulating PTEN. *Toxicology*, *306*, 162–168.

Ye, X.-M., Zhu, H.-Y., Bai, W.-D., Wang, T., Wang, L., Chen, Y., … Jia, L. T. (2014). Epigenetic silencing of miR-375 induces trastuzumab resistance in HER2-positive breast cancer by targeting IGF1R. *BMC Cancer*, *14*(1), 134.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Decker JT, Hall MS, Blaisdell RB, Schwark K, Jeruss JS, Shea LD. Dynamic microRNA activity identifies therapeutic targets in trastuzumab-resistant HER2^{+} breast cancer. *Biotechnology and Bioengineering*. 2018;115:2613–2623. https://doi.org/10.1002/bit.26791