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MicroRNA ‘signature’ during estrogen-mediated mammary carcinogenesis and its reversal by ellagic acid intervention

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Abstract

Dysregulated miRNA expression has been associated with the development and progression of cancers, including breast cancer. The role of estrogen (E₂) in regulation of cell proliferation and breast carcinogenesis is well-known. Recent reports have associated several miRNAs with estrogen receptors in breast cancers. Investigation of the regulatory role of miRNAs is critical for understanding the effect of E₂ in human breast cancer, as well as for developing strategies for cancer chemoprevention. In the present study we used the well-established ACI rat model that develops mammary tumors upon E₂ exposure and identified a ‘signature’ of 33 significantly modulated miRNAs during the process of mammary tumorigenesis. Several of these miRNAs were altered as early as 3 weeks after initial E₂ treatment and their modulation persisted throughout the mammary carcinogenesis process, suggesting that these molecular changes are early events. Furthermore, ellagic acid, which inhibited E₂-induced mammary tumorigenesis in our previous study, reversed the dysregulation of miR-375, miR-206, miR-182, miR-122, miR-127 and miR-183 detected with E₂ treatment and modulated their target proteins (ER, cyclin D1, RASD1, FoxO3a, FoxO1, cyclin G1, Bcl-w and Bcl-2). This is the first systematic study examining the changes in miRNA expression associated with E₂ treatment in ACI rats as early as 3 week until tumor time point. The effect of a chemopreventive agent, ellagic acid in reversing miRNAs modulated during E₂-mediated mammary tumorigenesis was also established. These observations provide mechanistic insights into the new molecular events behind the chemoprevention action of ellagic acid in and treatment of breast cancer.

Keywords

Breast cancer; microRNAs; Ellagic acid; Estrogen; Carcinogenesis

1. Introduction

Breast cancer is the most commonly diagnosed cancer among women in the United States and is the second leading cause of cancer deaths among women [1]. The development of

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Competing interests

The authors declare that they have no competing interests.

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breast cancer has been associated with numerous risk factors including genetic, environmental, hormonal, and dietary influences [2; 3]. Despite all of the available data on breast cancer risk, the etiology of the majority of human breast cancers is still not readily identifiable. Accumulating data from epidemiological studies, experimental animal models, and cell culture studies have shown that reproductive hormones, particularly estrogens, play a critical role in breast cancer etiology. Nearly 70% of breast tumors express estrogen receptor (ER) [4]. Up-regulated ER during early stages of tumorigenesis has been identified as an important factor in stimulating proliferation of mammary cells leading to tumor development [5]. Other breast cancer risk factors, such as age at menarche, postmenopausal obesity, and hormone replacement therapy, are believed to increase risk by increasing estrogens.

MicroRNAs (miRNAs) are endogenous small noncoding RNAs of 20–25-nucleotide that are involved in post transcriptional control of gene expression. miRNAs regulate gene expression by binding to 3' untranslated region (UTR) of their target mRNAs causing degradation or translational silencing of targeted transcripts [6]. Recent studies documented aberrant miRNA expression profiles in breast cancer compared with normal breast tissues, with tumor suppressors miRNAs miR-10b, -125b and -145 down-regulated and oncogenic miRNAs miR-21 and miR-155 up-regulated [7]. Moreover, miRNA signatures were reported to reliably predict breast cancer biopathological features, such as ER, PR and HER2/neu receptor status [8]. Kovalchuk *et al* suggested deregulation of several cellular epigenetic processes such as alterations in DNA methylation, histone modifications, and aberrant miRNA expression play crucial role in the mechanism of E₂-induced mammary carcinogenesis in ACI rats, especially in the tumor initiation process [9].

Since the role of miRNAs in progression of cancer is evident, targeting miRNAs has been considered as a viable treatment option. Accumulating experimental evidence has shown that over-expression or knock-out of specific miRNAs could inhibit cancer cell proliferation and regression of tumors in animal models [10; 11; 12]. Recent reports have also indicated the ability of pharmacological agents and dietary components to modulate the alterations of miRNA expression induced by carcinogens [13; 14; 15]. Ellagic acid (EA) is a plant polyphenolic abundant in raspberries, pomegranates, and walnuts [16; 17]. In other experimental cancer models, EA was reported to decrease the incidence of chemically-induced lung [18], mammary [19], small intestinal [20], colon [21] and oral [22] tumors. In previous work we demonstrated the cancer preventive effects of dietary intervention using EA against E₂-mediated mammary tumors in the ACI rat model [23; 24; 25].

The goals of this study were to: (i) measure the changes in expression of miRNAs during the course of E₂ treatment in the rat mammary tissue; (ii) delineate canonical/functional pathways regulated by genes that are potential targets of the modulated miRNAs modulated; (iii) analyze effect of EA intervention on the E₂-modulated miRNAs and, (iv) determine the expression pattern of select targets at mRNA and protein expression levels. By using this approach, multiple genes and pathways involved in the E₂-mediated mammary carcinogenesis have been identified simultaneously leading to identification of potential therapeutic targets of EA.

2. Materials and Methods

2.1. Tissue samples

Female Augustus Copenhagen Irish (ACI) rats following an approved protocol from the Institutional Animal Care and Use Committee (IACUC). Non-tumor (distal normal mammary) and tumor tissues were obtained from two E₂-induced mammary tumorigenesis studies: Study 1 in which female ACI rats were treated without (control) or with continuous

low doses of E₂ and euthanized at 3, 12 (close to the appearance of first palpable mammary tumor) and 26 (when tumor incidence reaches nearly 90%) weeks. Study 2 [24] in which female ACI rats were treated without (control) or with continuous low doses of E₂ alone, EA alone or in combination with EA and euthanized after 8, 16 and 28 weeks. E₂ was administered via subcutaneous silastic implants (1.2 cm, 9 mg E₂), while EA was administered via the diet (400 ppm w/w). Tissues from these studies were stored at -80°C. For initial microarray studies, archived tissues from the Study 1 were utilized, while tissues from Study 2 were used to investigate the effect of EA intervention on miRNA modulation. Animal experimentation protocol was approved from the Institutional Animal Care and Use Committee (IACUC) at University of Louisville.

2.2. Isolation of RNA

mirVana miRNA Isolation kit (Applied Biosystems) was used to isolate total RNA for microarray analysis and mRNA expression studies. Small RNA was further enriched from total RNA for qPCR analysis of miRNAs according to the manufacturer's protocol. Trace genomic DNA in the crude total RNA samples was removed by incubation with 10 units of DNase I per 100 µg RNA (Ambion, Austin, TX) at 37 °C for 30 min. The concentration of the total and small RNA was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and RNA integrity was verified with a Bioanalyzer 2100 (Agilent, Palo Alto, CA).

2.3 miRNA expression profiling microarray

Total RNA containing low molecular weight RNA was hybridized to a GeneChip® miRNA Array (Affymetrix, Santa Clara, CA) at 48°C and 60 rpm for 16 hrs, then washed and stained on Fluidics Station 450 (Fluidics script FS450_0003) and finally scanned on a GeneChip® Scanner 3000 7G using Affymetrix Command Console 1.0 (Affymetrix, Santa Clara, CA). Microarray signals were analyzed and heat maps were generated using Partek Genomic Suite 6.5 RMA algorithm. ANOVA and Correlation analyses (Pearson's Pairwise Comparison and Spearman's rank correlation) were performed and FDR reports were generated using Partek Pro Software (Partek, St. Charles, MO). Following the identification of differentially expressed miRNAs, the predicted targets for these miRNAs were identified using TargetScan 6.2 data base. Functional profiling and canonical pathway analysis was carried out by uploading the data set of predicted targets for the significantly altered miRNAs to the Ingenuity Pathways Analysis (IPA).

2.4 qRT-PCR validation of miRNA microarray data

Molecules demonstrating consistent modulation at 2 or more time points in microarray observations were selected for qRT-PCR validation. For analysis of the 11 selected miRNAs (miR-122, -127, -142-5p, -182, -183, -205, -206, -25, -335, -375 and -429), the individual TaqMan human MicroRNA Assays were used. Briefly, 25 ng of total RNA was reverse-transcribed in a final volume of 20 µl with 12.5 nM of each RT primer using the TaqMan MicroRNA Reverse Transcription Kit. TaqMan miRNA PCR kit was used to perform PCR reactions on the ABI 7900 Real-Time PCR System (Applied Biosystems, Foster City, CA). The reactions were initiated in a 96-well optical plate at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative miRNA expression was assessed using the differences in normalized Ct (Δ Ct) method after normalization to 5s rRNA. Fold changes were calculated by $2^{-\Delta\Delta Ct}$.

2.5 qRT-PCR for target gene expression

One-Step SYBR green qRT-PCR Kit (Quanta Biosciences, Gaithersburg, MD) was used to perform cDNA synthesis and PCR amplification simultaneously from 100 ng of total RNA

according to the manufacturer's instructions. Reactions were run under the following conditions: hold at 50°C for 10 min, 95°C for 5 min, then 40 cycles at 95°C for 10 sec and 60°C for 30 sec. Relative gene expression was assessed using the differences in normalized Ct ($\Delta\Delta Ct$) method after normalization to 18s rRNA. Fold changes were calculated by $2^{-\Delta\Delta Ct}$.

2.6 Immunoblot analysis

The mammary tissue lysates (40 μg) were resolved on SDS-polyacrylamide gel and immunoblotted with various primary antibodies as previously described [26], ESR1 (Estrogen receptor α) (Proteintech, Chicago, IL), Bcl-2, CCNG1 (cyclin G1) (Santa Cruz Biotechnology, Santa Cruz CA), RASD1 (RAS, dexamethasone-induced 1), Bcl-w, FoxO3a (Forkhead box O3a), FoxO1 (Forkhead box O1), (Cell Signaling, Danvers, MA) and CCND1 (cyclin D1) (Abcam Cambridge, MA) were purchased. Appropriate secondary antibody was used and detection carried out using enhanced chemiluminescence (Thermo Scientific, Waltham, MA). Equal loading of the proteins was confirmed by staining the polyacrylamide gel with coomassie blue stain.

3. Results

3.1 miRNA expression profile during E₂-mediated mammary tumorigenesis

The effect of E₂ on miRNA expression in non-tumor and tumor tissues of ACI rats was analyzed using microarray for the expression levels of 351 rat specific miRNAs. Principal component analysis (PCA) was performed on 28 rat mammary samples (3 normal mammary samples each from 3 and 12 weeks untreated (control) and E₂-treated groups; 5 samples of non-tumor (distal normal mammary) and 6 tumors tissues from E₂-treated group and 5 from untreated group at the 26 week time point) and view the global changes of miRNA expression induced by E₂ treatment at different treatment time points. The normalized Ct values of 351 miRNAs were used for this analysis and the results for the first 3 principal components are shown in Figure 1a. The samples could be roughly divided into 2 groups. Untreated (control) samples at 3, 12 and 26 weeks are grouped together while E₂-treated samples including the non-tumor and tumor tissues are clustered together. Within the E₂-treated group, animals showed a much higher level of variation than the control, which may be caused by larger interindividual responses to the E₂ treatment. The PCA results suggest that the miRNA expression was globally altered by E₂ treatment at 3, 12 and 26 weeks. We performed statistical comparison between the groups of samples (combining a 1.5 fold change threshold and ANOVA $p < 0.05$). Unsupervised hierarchical clustering analysis of the positively detected (Ct < 35) miRNAs is shown in Supplementary Fig. S1. We identified 33 miRNAs that were significantly different between the untreated control and E₂-treated groups at two or more time points. Clustering of the samples was performed for a second time using the 33 significantly modulated miRNAs. The resulting dendrogram could be divided into two clearly separated sections: a first section containing the untreated control samples and a second containing the E₂-treated non-tumor and tumor samples (Fig 1b), emphasizing that the miRNA profile of the E₂-treated group was dramatically different from the untreated controls. Supplementary Table 1 summarizes the significant changes in the miRNAs detected in non-tumor and tumor samples from E₂-treated groups at different time points. The number of aberrantly expressed miRNAs increased with the duration of E₂ treatment. After 3 and 12 weeks of E₂ treatment, 15 and 31 miRNAs were down-regulated and 36 and 27 miRNAs were up-regulated, respectively; 26 weeks of E₂ treatment caused down-regulation of 26 and 65 miRNAs and up-regulation of 35 and 49 miRNAs in non-tumor and tumor tissues, respectively. A venn diagram (Fig 1c) depicts the number of overlapping miRNAs during the course of E₂ treatment. The fold change of 33 aberrant miRNAs in the non-tumor and tumor tissues of E₂-treated samples after 26 weeks compared to untreated control tissues is shown in Fig. 1d.

3.2 Verification of miRNA microarray results by qRT-PCR

To validate the findings of microarray analysis, we selected 6 up-regulated (miR-182, -375, -183, -34c, -196c and -429) and 5 down-regulated (miR-122, -127, -335, -205 and -206) of 33 differentially expressed miRNAs (2 fold). The qPCR and microarray results were compared by plotting the qPCR cycle threshold (Ct) value versus the log₂ of the array signal for each miRNA at 3, 12 and 26 week. An inverse correlation is expected between the two methods since increasing miRNA expression is associated with increasing log₂ signal in microarray and decreasing Ct values in qPCR. Thus, the plots are expected to show a linear correlation (R= -1) with a slope of -1. All of the miRNAs followed the same trend of expression as seen in the microarray experiment, thus validating the microarray platform (Supplementary Fig. S2a). The correlation between the two methods was 0.96-0.60 (Supplementary Fig. S2b).

3.3 Computational prediction of putative targets and pathway analysis

We used the computational algorithm TargetScan to identify potential target genes of the significantly modulated miRNAs during E₂ treatment. The miRNA target genes were input into Ingenuity Pathway Analysis (IPA) software and the related functions and pathways were identified. As expected, cancer was the highest scoring disease and disorder; gene expression was the highest molecular and cellular functions and molecular mechanism of cancer and axonal guidance signaling were the highest scoring canonical pathways in IPA analysis (Supplementary Fig. S3; Supplementary Table 2). An overlay of predicted targets from 26 week tumor tissue on E₂-mediated breast cancer pathways clearly suggested modulation of all the key molecules responsible in breast tumorigenesis (Supplementary Fig. S4).

3.4 Effect of EA intervention on E₂-modulated miRNAs

After identifying the 33 miRNAs that were modulated during E₂-mediated mammary tumorigenesis, we examined the effect of dietary EA intervention on the expression of selected miRNAs. Prior to analyzing the effect of EA intervention, we performed qPCR analysis of select miRNAs from the Study 1 and Study 2 to determine if the expression levels of miRNAs were in agreement between the two studies. We selected 9 miRNAs that showed consistent modulation at two or more time points during the course of E₂ treatment and for which literature reports supported an association of these miRNAs with breast cancer development or ER modulation [27; 28; 29; 30; 31; 32; 33; 34]. miRNA expression trends were in good agreement between the Study 1 and Study 2. Few miRNAs displayed fold change variations and were not time-dependent (miR-205, -206, -182 and -335), while other miRNAs (miR-183, -127, -122 and -375) showed time-dependent or consistent modulation between the 2 studies (Supplementary Fig. S5). Six of 9 miRNAs were altered significantly by EA: the oncogenic miRNAs, miR-183, -182 and -375 were repressed and the down-regulated tumor-suppressor miRNAs, miR-122, -127 and -206 levels were increased (Fig. 2). The effect of EA on miRNAs -205 and -335 did not attain statistical significance (Supplemental Fig. S6a). The raw Ct values of housekeeping gene 5S rRNA of untreated, the E₂ alone and E₂+EA at different time points suggested no significant influence of E₂ alone or in combination with EA on its expression (Supplementary Fig. S6b). EA alone-treated group showed similar miRNA expression trends as untreated control (Data not shown). Thus, using an E₂-mediated rat mammary carcinogenesis model, we demonstrate that the expressions of several aberrant miRNAs during mammary tumorigenesis were reversed by EA intervention.

3.5. Altered miRNAs are associated with estrogen/breast cancer signaling pathways

An unbiased computational approach was used to evaluate the potential functional consequences of six miRNAs reversed by EA intervention (Fig. 2) based on combinatorial effects of the predicted targets of the miRNAs using IPA. A total of 2034 targets were predicated for the 6 miRNAs (miR-122, -127, -182, -183, -206 and -375), of these 2016 could be mapped to signaling networks and 1697 predicated targets were found to be network-eligible. Cancer was the top bio-function of the predicted targets of the 6 miRNAs with 557 molecules (Supplementary Fig. S7a). Identification of this network function further supports the role of these predicated targets in breast carcinogenesis. Cellular and molecular functions significantly influenced by these network-eligible molecules, is shown in supplementary Fig. S7b. Representative predicted targets of the six miRNAs are depicted in Fig. 3a. These targets were further overlaid for their interaction with three networks, molecular mechanism of cancer, E₂-mediated breast cancer and ER signaling, that are presumed to play a key role in the breast tumorigenesis progression (Fig. 3b). These data suggest an important role for the miRNAs whose E₂-induced changes were reversed by EA during mammary tumorigenesis in ACI rats.

3.6 Favorable modulation of miRNA targets by ellagic acid

Selected predicted targets of the six miRNAs that showed reversal of E₂ alterations by EA (Fig. 2) were analyzed for mRNA expression levels at 8, 16 and 28 weeks by qPCR (Fig. 4). Twenty eight weeks (tumor time point) non-tumor and tumor tissues were analyzed for protein expression by western blot (Fig. 5). We observed modulation of the predicted targets ER (miR-206), CCND1 (miR-206), CCNG1 (miR-182, -122), FOXO1 (miR-182, -183), FOXO3a (miR-182), Bcl-2 (miR-127, -182 -206), Bcl-w/Bcl2l2 (miR-122), RASD1 (miR-375) with EA treatment. ER is overexpressed under the influence of E₂ leading to tumorigenesis in ACI rat mammary gland and is a target of a miR-206, miR-375 and -182 [27; 29; 35]. ER mRNA expression levels were significantly reduced with EA treatment at 16 and 28 weeks compared to E₂-alone treatment. CCND1, another target of miR-206 that was down-regulated by E₂ (Fig. 2), is also increased by E₂ and this increase was inhibited both at mRNA and protein levels by EA (Fig. 4 and Fig 5). CCNG1, a target of miR-122 and -127 mRNA expression levels, were significantly decreased at 16 and 28 weeks in non-tumor tissue compared to E₂ alone treatment. Although there was a trend for a decrease in tumor tissue with EA intervention, it did not attain statistical significance for mRNA levels, but protein expression of CCNG1 was significantly decreased (Fig. 5). This is commensurate with increased miR-122 and -127 inhibiting CCNG1 translation. Another target of miR-122, Bcl-w, was also decreased at the mRNA level in tumor tissues with EA treatment (Fig. 4). Protein expression trends were in agreement with the mRNA levels at 28 weeks (Fig. 5). FoxO1 and FoxO3a are targets of miR-182 and are repressed during breast cancer development [32]. Overexpression of miR-182 is known to cause FOXO1 and FOXO3a degradation/translational repression in several cancers [35; 36]. Expression levels of both FoxO1 and FoxO3a were increased with EA treatment, suggesting that the decrease in miR-182 by EA instigated this increase in FOXO protein levels. Antiproliferative factor RASD1 is a major target of miR-375 and has been associated with down-regulation of ER expression in breast cancer cells [27]. Decreased miR-375 expression by EA treatment was observed and corresponded with increased expression levels of RASD1 protein.

4. Discussion

The fundamental roles of miRNAs in tumor formation and progression have been identified [6]. One hallmark in most human breast cancers is increased expression of ER. ER-positive breast cancer cells are dependent on E₂ for their growth, survival and progression. E₂-dependent differential expression of miRNAs and their target genes has been reported [9;

34], but specific miRNA changes during premalignant and malignant stages remain elusive. Moreover, limited information is available about the potential of natural chemopreventive or chemotherapeutic agents to target the deregulated miRNAs in breast cancer. The ACI rat is an ideal model to study the influence of E₂ during mammary tumorigenesis as this model develops spontaneous tumors during E₂ treatment and these tumors share similar histology and pathology (e.g., ER +, PR+) as the majority of human breast cancers [37].

In this study, we first asked whether miRNAs are modulated during E₂-mediated carcinogenesis. To address this question we analyzed changes in miRNA expression during E₂-induced tumorigenesis in normal mammary and tumor tissues. Our results suggest that deregulation of miRNAs is an early event and the number of aberrant miRNAs increase with the duration of E₂ exposure. Several of these miRNAs remained modulated during tumor development, suggesting deregulation of molecular events during E₂-mediated mammary tumorigenesis. The miRNA 'signature' clearly differentiated E₂-treated non-tumor and tumor tissues from untreated rat normal mammary tissue. Several reports have previously identified miRNAs modulated by E₂ and dysregulated in breast and other human cancers [27; 28; 29; 30; 31; 32; 33; 34]. Among these, miR-182, -375, -183, -196, -34c, -205 and -429 were up-regulated, whereas, miR-122, -127, -335 and -206 were down-regulated during the carcinogenesis process under the influence of E₂ [38]. Our results are in agreement with a previous study of miRNA alterations in this rat model that reported an increase in number of modulated miRNAs with the duration of E₂ exposure [9]. Up-regulated miR-20a, -25, -375 and down-regulated miR-99a, -127 and -335 were common among the miRNAs identified in the previous report [9]. However, there were several miRNAs that were dissimilar between the study by Kovalchuk *et al* [9] and our study. The reason for these differences observed could be attributed to several factors: a) the miRNA microarray platform used b) the time points observed - notably, Kovalchuk *et al.*, examined miRNAs alterations at 6, 12 and 18 weeks, whereas in our study analyses were performed at 3, 12 and 26 weeks. c) dose of E₂ - single pellet containing 25 mg of 90-day release E₂ [9] versus less than 2-3 mg E₂ release by silastic implants in 180 days; and d) difference in cut-off parameters used to identify significant miRNAs.

A single miRNA can target hundreds of mRNA transcripts and since miRNAs alterations occur simultaneously and modulate their respective targets, thus the global effect is the sum of all effects coordinated by individual miRNAs [39]. In our study, computational target prediction of the aberrant miRNAs in E₂-induced mammary tumorigenesis provided insights into the global molecular networks regulated by these miRNAs. We focused on 6 aberrantly expressed miRNAs that were either down-regulated (miR-122, -127 and -206) or up-regulated (miR-182, -183 and -375) by E₂ and for which EA resulted in reversal of their expression in non-tumor and tumor tissues (Fig. 2). It is interesting to note that the predicted targets of these miRNAs are associated with cancer pathways such as molecular mechanism of cancer, ER signaling, and E₂-dependent breast cancer signaling (Fig. 3d). These findings are consistent with previous studies demonstrating that E₂ causes many physiological changes and subcellular effects in breast cells [5]. It is likely that E₂-induced dysregulation of miRNAs in the mammary gland is an early event in mammary carcinogenesis and may be involved in regulating biological pathways involved in tumorigenesis.

Compelling experimental evidence has suggested that miRNAs could be novel targets to induce drug sensitivity, inhibit proliferation, and suppress cancer cell invasion and metastasis [10; 11; 12]. Natural agents such as curcumin, EGCG (Epigallocatechin gallate), diindolylmethane, and isoflavones have been shown to alter the expression of specific miRNAs, leading to increased sensitivity of cancer cells to conventional therapeutic agents, and enhanced inhibition of tumor growth [14; 40; 41; 42; 43; 44]. These studies were, however, performed *in vitro*. Studies examining the role of natural agents on miRNAs *in*

vivo are limited. In one such study, modulation of vinyl carbamate-induced miRNAs in mouse lung tumors by dietary indole-3-carbinol was examined [15]. This study showed reduced levels of miR-21, -31, -130a, -146b and -377 with indole-3-carbinol compared to carcinogen alone treated mice. In another study, oral administration of chemopreventive agents such as N-acetylcysteine, oltipraz, indole-3-carbinol, 5,6-benzoflavone and phenethyl isothiocyanate (as single agents or in combination) in cigarette smoke-exposed rat lungs, was shown to affect the baseline miRNA expression, indicating protective effects during early carcinogenesis [13]. However, no study has been conducted to elucidate the role of dietary chemopreventive agents on miRNAs during mammary carcinogenesis.

This laboratory has extensively studied EA for its chemopreventive effects in E₂-mediated mammary tumorigenesis in the ACI rat model [23; 24; 25]. Earlier we reported a significant reduction in tumor volume (69%) and tumor multiplicity (49%) with EA intervention [24]. This study for the first time examined the role of EA on deregulated miRNAs during E₂-induced mammary tumorigenesis and supports our hypothesis that the inhibitory effects of EA in mammary carcinogenesis is, in part, mediated by regulation of miRNAs causing changes in the biological behavior of cells. EA decreased the expression of several oncogenic miRNAs and increased the expression of down-regulated tumor-suppressor miRNAs. miR-182 and -183 are up-regulated in breast cancer cells and in ductal carcinoma *in situ* compared with normal breast epithelium, supporting the idea that these two miRNAs may play important roles in the development of breast cancer [45]. miR-183, and -182 expressions were shown to correlate with ER and HER2/neu receptor expression [8]. In agreement with these literature reports, we observed increased expression of miR-182 and -183 by E₂ that could be associated with the up-regulation of ER in the ACI rat mammary glands. miR-182 negatively regulates tumor suppressors genes, involved in cell differentiation (FOXO1), invasion and metastasis (FOXO3a), and DNA damage repair (BRCA1 and FOXO3a) [35]. Increased expression of miR-182 in breast cancer cells has been linked with down-regulation of FOXO1, responsible for orchestrating genes involved in apoptotic response, cell cycle checkpoints, and cellular metabolism [32]. Here we demonstrate for the first time that EA reduced the expression of miR-182 and -183 and increased FOXO proteins in the EA-treated rat mammary glands compared to E₂ alone-treated rats.

Overexpression of miR-375 has been reported in lung [46] and breast cancers [27], while it was down-regulated in gastric [47] and liver [48] tumors. Here we observed that E₂ increased miR-375 during the progression of tumorigenesis in rat mammary glands. Increased expression of miR-375 is suggested to indirectly increase ER expression and thus cellular proliferation; this is an interesting observation as nearly 70% of breast cancers are ER -positive [4; 27]. Consistent with our observation, Kovalchuk *et al* reported increased expression of several miRNAs including miR -375 during the early stages of E₂-induced mammary carcinogenesis in ACI rats [9]. miR-375 was demonstrated to modulate ER by repression of an antiproliferative factor, RASD1. Further, this mechanism of action was confirmed by overexpression of RASD1 which resulted in down-regulation of ER mRNA levels [27]. In our study, EA decreased miR-375 and ER expression, and increased RASD1 levels, thus providing a mechanism by which EA may interfere with tumor cell proliferation through the down-regulation of ER .

EA effectively caused reversal in the expression trends of miR-206 and its targets, ER , CCND1 and Bcl₂, suggesting its benefits in ER positive breast cancers [29]. In addition to up-regulation of ER as noted above, CCND1 overexpression is a frequent event in breast cancer [49]. miR-206 decreases endogenous ER and CCND1 levels via two specific target sites within the 3'-UTR of the mRNA transcripts [31]. In our study, an inverse correlation was observed with lower miR-206 and overexpression of its targets ER and CCND1 during

the tumorigenesis process in ACI rat mammary glands. Similar expression trends of miR-206 and ER expression in human breast tumors have been reported [30].

Similar to tumor suppressor miR-206, E₂ repressed miR-122 and -127 expression, resulting in increased expression of their targets CCNG1 and Bcl-w. miR-122 has been associated with progression and poor clinical outcomes of breast cancer patients [50]. Although complete reversal of miRNA expression as with miR-206 was not observed, EA treatment improved the expression of repressed miR-122 and -127 and caused down-regulation of targets CCNG1, and Bcl-w. Moreover, unlike other miRNAs, the effect of EA on miR-122 expression during early time points was not evident, and remains a subject of further investigation.

5. Conclusion

In summary, the results presented here demonstrate that EA reversed the effects of E₂ on oncomiR and tumor suppressor miRNA expression in E₂-treated ACI rat mammary gland carcinogenesis. These results along with our earlier observations of EA's ability to efficiently decrease the tumor burden and multiplicity [24] provide new mechanistic insights into how EA inhibits E₂ carcinogenesis by reversal of aberrant miRNAs (miR-122, -127, -182, -183, -205 and -375) and their targets (ER, CCND1, RASD1, FoxO3a, FoxO1, CCNG1, Bcl-w and Bcl-2). The data presented here re-emphasize EA to be promising in the prevention and treatment of breast cancer. In view of the non-toxic characteristics of EA, and other natural agents, combining them with conventional chemotherapeutics could be considered as a promising strategy for clinical therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

E₂	17 -estradiol
miRNA	microRNA
ER	estrogen receptor
PR	progesterone receptor
ACI rat	August and Copenhagen–Irish rats
EA	Ellagic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PBS	phosphate-buffered saline
qRT-PCR	quantitative reverse transcription -polymerase chain reaction
cDNA	complementary deoxynucleic acid
IPA	Ingenuity pathway analysis
CCND1	cyclin D1
RASD1	RAS, dexamethasone-induced 1

FOXO	Forkhead box
CCNG1	cyclin G1
Bcl-2	B-cell lymphoma 2

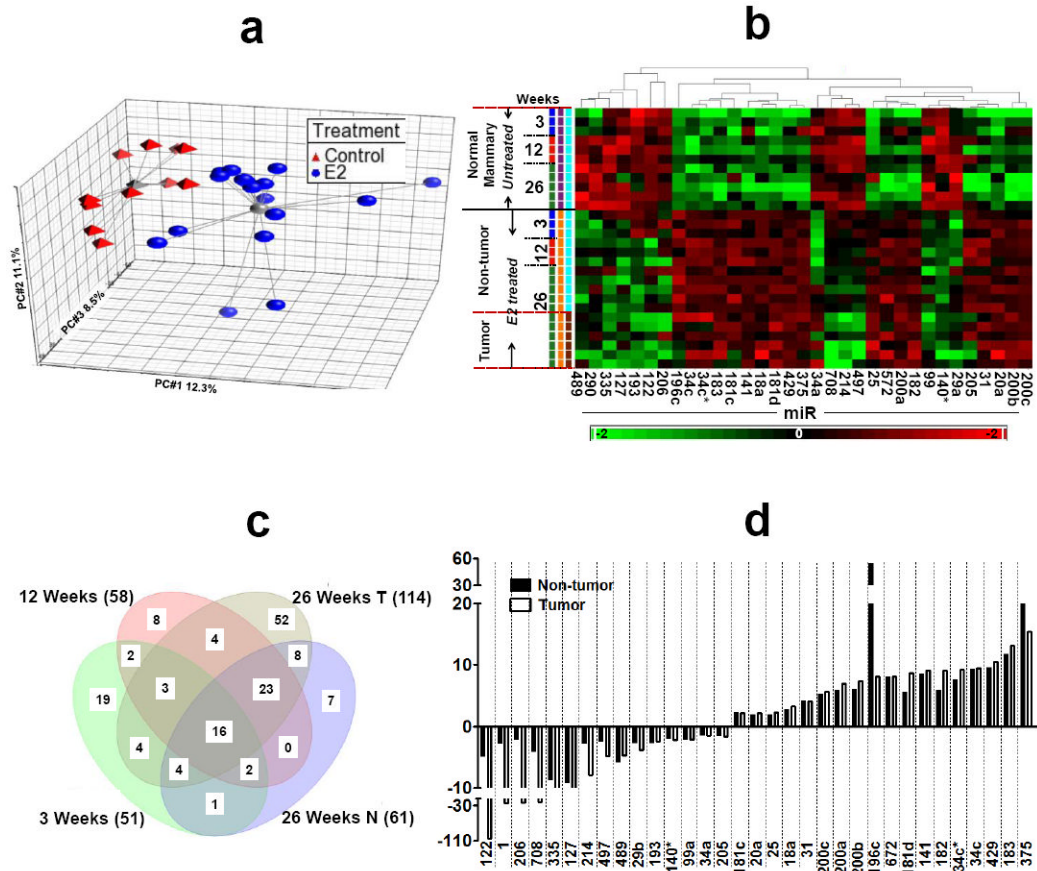


Figure 1. miRNA expression profile during E₂-mediated mammary tumorigenesis
a) Principal component analysis of ANOVA-selected miRNAs. Red triangles represent untreated control animals and blue circles represent E₂-treated animals. The grey lines connect to a centroid (grey triangle or grey ball). The PC#1, PC#2 and PC#3 represents the first three largest variable principle components accounting for the majority of the variance within the dataset; **b)** Hierarchical clustering of 28 samples and 33 differentially expressed miRNAs. Rows represent sample, and columns represent miRNA. The bar code at the bottom represents the color scales of the log₂ values; **c)** Venn diagram of miRNAs regulated at different time points of E₂ treatment. All miRNAs are significant at $p < 0.05$. MiRNAs at the intersections represents the overlapping/common miRNAs modulated by E₂ at different treatment while miRNAs that are located outside the overlaps can be considered E₂ independent miRNAs; **d)** the bar diagram represents fold change of the top 33 miRNAs in the non-tumor (distal normal mammary) and tumor tissues of ACI rat after 26 weeks of E₂ treatment.

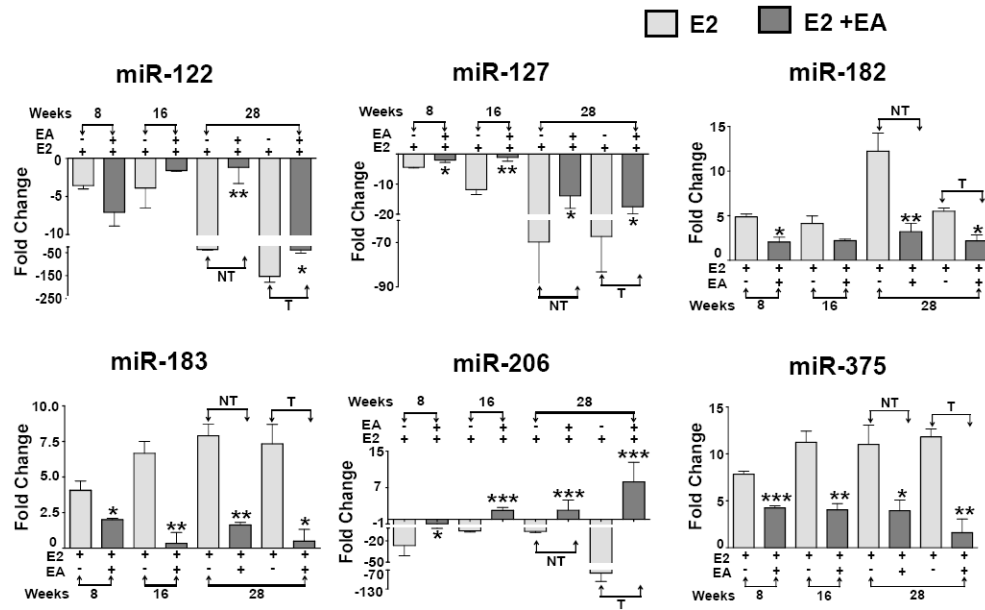


Figure 2. Effect of EA on aberrant miRNAs during E₂-mediated mammary tumorigenesis
 Select miRNAs were analyzed by qRT-PCR in mammary and tumor tissues of E₂-alone and EA-treated ACI rats. Data represents mean \pm SD of fold change in mRNA expression relative to the untreated control of five animals each. *p<0.05 **p<0.01, ***p<0.001. NT represents non-tumor (distal normal mammary) and T represents tumor tissue.

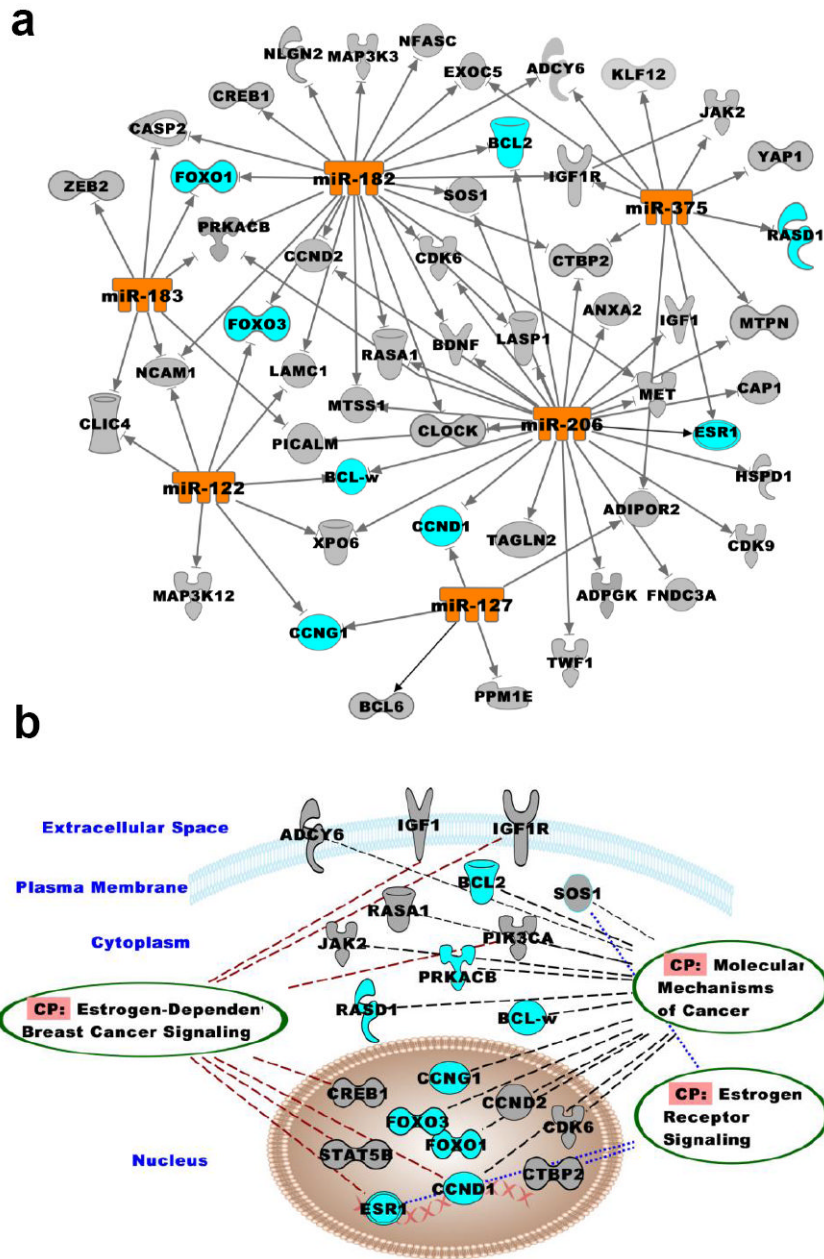


Figure 3. Effect of aberrant miRNAs on breast cancer signaling pathways
 Pathway analyses of the 6 selected miRNAs (miR-122, -127, -182, -183, -206 and -375) were performed using IPA. **c)** Interactions of the select miRNAs with their putative targets is shown; **d)** Canonical function overlays (ovals) indicate associated proteins important in breast cancer signaling pathway.

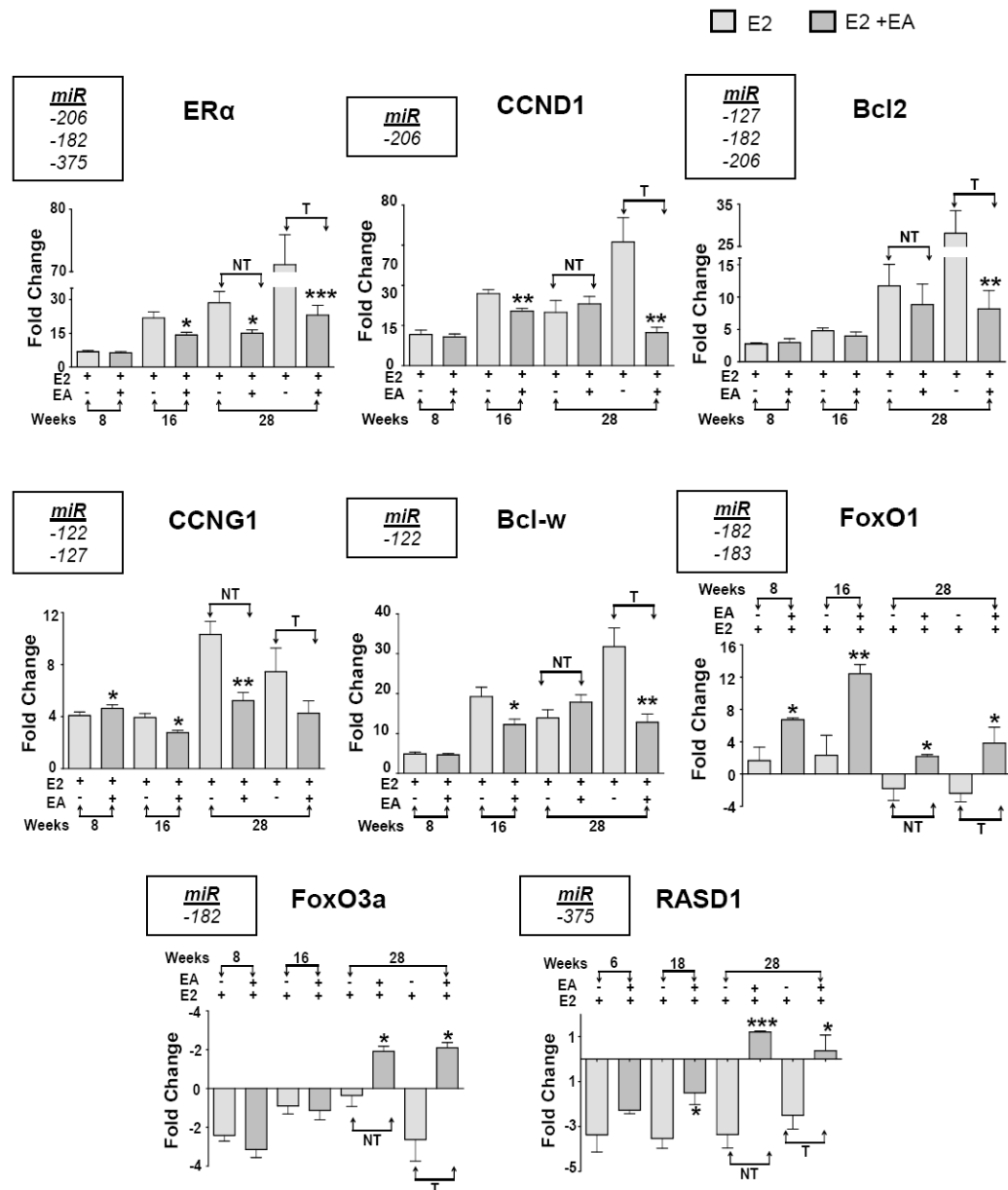


Figure 4. Effect of EA on potential targets of aberrant miRNAs

Potential mRNA targets of 6 miRNAs (listed in left corner rectangular box) - ER, CCND1, CCNG1, FOXO1, FOXO3a, Bcl-2, Bcl-w/Bcl2l2, RASD1 were analyzed by qRT-PCR.

Data represents mean \pm SD of fold change in mRNA expression relative to the untreated control in triplicate. * $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$. NT represents non-tumor (distal normal mammary) and T represents tumor tissue.

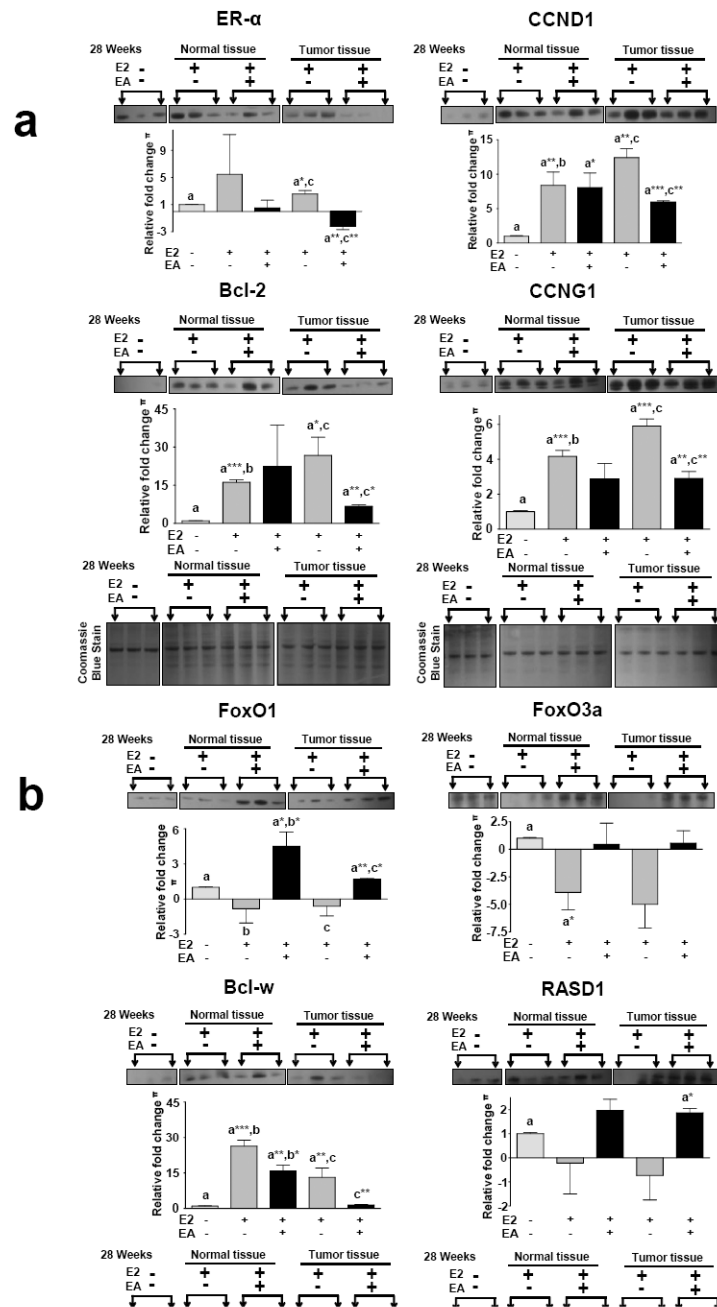


Figure 5. Effect of EA on protein expression levels

Western blot analysis was performed for the represented proteins. The top panel proteins (Fig 5a and 5b) were stripped and re-probed with different antibody (bottom panel). Equal Loading was confirmed by Coomassie blue staining. Densitometry was performed using imageJ software and the bar graph is given below each blot. Representative blots are shown.