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Tamoxifen differentially regulates miR-29b-1 and miR-29a expression depending on endocrine-sensitivity in breast cancer cells

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Abstract

Endocrine-resistance develops in ~ 40% of breast cancer patients after tamoxifen (TAM) therapy. Although microRNAs are dysregulated in breast cancer, their contribution to endocrine-resistance is not yet understood. Previous microarray analysis identified miR-29a and miR-29b-1 as repressed by TAM in MCF-7 endocrine-sensitive breast cancer cells but stimulated by TAM in LY2 endocrine-resistant breast cancer cells. Here we examined the mechanism for the differential regulation of these miRs by TAM in MCF-7 *versus* TAM-resistant LY2 and LCC9 breast cancer cells and the functional role of these microRNAs in these cells. Knockdown studies revealed that ER α is responsible for TAM regulation of miR-29b-1/a transcription. We also demonstrated that transient overexpression of miR-29b-1/a decreased MCF-7, LCC9, and LY2 proliferation and inhibited LY2 cell migration and colony formation but did not sensitize LCC9 or LY2 cells to TAM. Furthermore, TAM reduced DICER1 mRNA and protein in LY2 cells, a known target of miR-29. Supporting this observation, anti-miR-29b-1 or anti-miR-29a inhibited the suppression of DICER by 4-OHT. These results suggest miR-29b-1/a has tumor suppressor activity in TAM-resistant cells and does not appear to play a role in mediating TAM resistance.

Keywords

miRNA-29b-1; miRNA-29a; breast cancer; endocrine-resistance; estrogen receptor; tamoxifen

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1. INTRODUCTION

Breast cancer is the second leading cause of cancer-related deaths in women in the US [1]. Seventy percent of breast tumors express estrogen receptor α (ER α) [2, 3], making these patients eligible for endocrine therapies including aromatase inhibitors (AIs), *e.g.*, letrozole, and selective estrogen receptor modulators (SERMs), *e.g.*, tamoxifen (TAM). Unfortunately ~40% of patients initially responsive to endocrine therapies acquire resistance to TAM or AI therapy [4]. The selective estrogen receptor down regulator (SERD) fulvestrant (ICI 182 780) is used for the treatment of patients who relapse on AIs or TAM [5-8]. New clinical studies show that patients treated with AIs who have metastatic tumors with ER α ligand binding domain mutations show increased disease-free and overall survival with fulvestrant treatment [9]. These and other data implicate ER α as an important target in endocrine resistance. While still not completely understood, a variety of mechanisms play a role in endocrine resistance including increases in coactivators SRC-1 (NCOA1) and/or SRC-3 (NCOA3), activation of receptor tyrosine kinases (TRKs, *e.g.*, epidermal growth factor receptor (EGFR)) that crosstalk with ER α , and deregulation of apoptotic or cell survival signals [2, 10, 11].

MicroRNAs are 22 nucleotide (nt) noncoding RNA molecules that bind to the 3'-UTR region of target mRNAs to cause mRNA degradation or translational repression [12, 13]. microRNAs are dysregulated in breast tumors resulting in aberrant expression of target proteins involved in cellular processes including proliferation, apoptosis, and migration. A number of microRNAs play a role in mediating endocrine resistance (reviewed in [8, 11]); however, it is likely that additional microRNAs contribute to the emergence of endocrine-resistance. Previously we used microarray analysis to identify microRNAs that are differentially regulated between estradiol (E₂)- and TAM- sensitive MCF-7 and endocrine resistant LY2 human breast cells that were derived by long-term incubation of MCF-7 cells with a precursor to raloxifene and which are cross-resistant resistant to TAM and fulvestrant [14, 15]. Several microRNAs, including miR-29b-1 and miR-29a, were identified to be differentially regulated by 4-hydroxytamoxifen (4-OHT, the active metabolite of TAM) between MCF-7 and LY2 cells. However, the role of miR-29b-1/a in breast cancer remains unclear and the relationship between increased miR-29b-1 and miR-29a in 4-OHT-treated LY2 cells and TAM-resistance is unknown.

The miR-29 family includes 4 members encoded at two genomic sites. miR-29b-2 and miR-29c are encoded on chromosome 1q32.2 separated by 502 base pairs (bp) with miR-29b-2 upstream of miR-29c [16, 17]. miR-29b-1 and miR-29a are located on chromosome 7q32.3, separated by 652 bp, with miR-29b-1 upstream of miR-29a [18-20]. Each pair (miR-29b-2/c and miR-29b-1/a) is postulated to share a promoter and be transcribed as a single primary-microRNA, *i.e.*, pri-miR-29b-2/c and pri-miR-29b-1/a. All four miR-29s have the same seed sequence [17]. Interestingly, miR-29 family members have higher expression in Luminal A and B breast tumors compared with basal and HER2+ tumors [21].

Both oncogenic and tumor suppressive roles have been ascribed to miR-29 in breast cancer progression (summarized in Supplementary Table1) [22-24]. Forced overexpression of

miR-29b and miR-29a promoted an epithelial-to-mesenchymal transition (EMT) in MCF-7 and T47D cells while miR-29a repression reduced invasiveness of MDA-MB-231 triple negative breast cancer (TNBC) cells [25]. However, other studies indicate that miR-29a and miR-29b act as oncosuppressors in breast cancer cells by repressing cell proliferation, differentiation, and metastasis [21, 26, 27]. These conflicting studies underscore the need to further elucidate the role of miR-29b-1/a in breast cancer.

In the current study, we confirmed the opposing regulation of miR-29b-1 and miR-29a transcription by 4-OHT seen in the original microarray studies in MCF-7 and LY2 cells [14] using quantitative realtime PCR (qPCR). We examined the mechanism for this opposite regulation by 4-OHT in these cells and in additional TAM- and fulvestrant- resistant cell lines derived from MCF-7 cells: LCC2 and LCC9. Transient overexpression of miR-29b-1 and miR-29a decreased cell proliferation in all 3 cell lines and inhibited migration and colony formation of LY2 cells. Inhibition of miR-29b-1/a did not sensitize TAM-resistant LCC9 or LY2 cells to TAM. The increase in miR-29b-1/a in LY2 cells was associated with decreased protein expression of DICER, a *bone fide* target of miR-29b-1/a. Knockdown of miR-29b-1/a blocked 4-OHT mediated repression of DICER. Our results suggest that miR-29b-1/a do not appear to play a role in acquired tamoxifen-resistance in breast cancer cells.

2. MATERIALS AND METHODS

2.1. Cell culture/treatments

MCF-7 (purchased from American Type Culture Collection (ATTC, Manassas, VA, USA)), LCC2, LCC9 and LY2 (provided by Robert Clarke, Georgetown University) [15, 28, 29] cells were grown in phenol red IMEM supplemented with 5% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). These cells represent a model of progression to endocrine/TAM-resistance [30]. For the experiments described here, cells were grown in hormone depleted medium: phenol red-free IMEM supplemented with 5% dextran-coated charcoal-stripped fetal bovine serum (DCC-FBS, Atlanta Biologicals, Lawrenceville, GA, USA) for 48 h prior to experiments to reduce basal hormone-related activities [31]. Where indicated, cells were pretreated with 10 µg/ml actinomycin D (ACTD, a transcriptional inhibitor, Sigma, St. Louis, MO, USA) or 100 nM fulvestrant (ICI 182,780; Tocris, Ellisville, MO, USA) for 6 h prior to treatment. Treatments included vehicle control (ethanol (EtOH) or DMSO) or 100 nM 4-hydroxytamoxifen, (4-OHT; Sigma St Louis, MO, USA).

2.2. Transfection

MCF-7, LCC9, and LY2 cells were transiently transfected for 48 h with miR-29b-1/a inhibitor (Anti-miRTMs, Ambion, Austin, TX, USA), siERα (Silencer[®], Ambion), pre-miR-29b-1/a precursor (Pre-miRTMs, Ambion), using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) and Opti-MEM[®] Reduced Serum Medium (Invitrogen, Carlsbad, CA). Negative controls were Anti-miRTM negative control #1 (Ambion), Pre-miRTM negative control #1 (Ambion), or Negative Control #1 (Silencer[®], Ambion). Treatments were performed following transfections.

2.3. Quantitative real-time PCR (qRT-PCR)

RNA was isolated using the miRCURY RNA isolation kit (Exiqon, Vedbaek, Denmark) according to manufacturer's instructions. RNA concentration and quality was assessed using a NanoDrop spectrophotometer (Thermo Scientific, Rockford, IL, USA). The TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) and the High Capacity cDNA Reverse Transcription kit for RNA (PE Applied Biosystems, Carlsbad, CA, USA) were used to make cDNA for microRNA and mRNA respectively. Quantitative real-time PCR (qPCR) for pri-miR-29b-1/a, miR-29b-1/a, (Applied Biosystems) and *DICER1* [32] (IDT), was performed using TaqMan (Applied Biosystems/Life Technologies) or SYBR green (QIAGEN). Normalizers included RNU6B, RNU48 or RNU38, PPIA, 18S rRNA (Applied Biosystems) or GAPDH (SYBR green). qPCR was performed in the ABI Viia 7 (LifeTechnologies) with each reaction run in triplicate. Fold change was determined using the comparative threshold cycle (Ct) method relative to vehicle control [33].

2.4. Luciferase assay

To examine ER α regulation of miR-29b-1/a promoter, CHO-K1 cells were transiently transfected in triplicate in 24 well plates with pGL3 -1530/+165 miR-29b-1/miR-29a promoter fragment [18] and pGL4-hRluc-TK (*Renilla*, Promega, Madison, WI, USA) using FuGENE HD (Roche, Indianapolis, IN, USA) for 48 h as per the manufacturer's protocol. 18 h post transfection, cells were either not pre-treated or pre-treated for 6 h and then treated for 24 h prior to performing dual luciferase assay (Promega). Relative expression was determined by dividing averaged values from DMSO treated values.

2.5. Cell proliferation (BrdU), colony formation, and migration (wound healing) assays

MCF-7, LCC9 and LY2 cells were placed in hormone-depleted medium and transfected with precursor microRNA or anti-miR transcripts or 48 h and the following assays performed. For BrdU assays, cells were treated with 100 nM 4-OHT for 48 h and cell proliferation quantified using BrdU ELISA assay (Roche). For colony formation, following transfection, LY2 cells were counted and transferred to agarose plates and allowed to grow for 14 days to form colonies. Colonies were stained with crystal violet (0.005% w/v, Sigma) and counted using an inverted Nikon microscope ($\times 10$ objective). For cell migration assays, LY2 cells grown in hormonally depleted medium and transfected in six well plates for 24 h. Cells were wounded by scratching with a p20 pipette tip, and treated with 100 nM 4-OHT for up to 8 h. Images were captured at each time point at $4\times$ magnification using an EVOS microscope (Thermo Fisher Scientific Waltham, MA, USA). NIH Image J software (<http://imagej.nih.gov/ij/>) was used to analyze percent wound area.

2.6. Western blot

Whole cell extracts (WCE) were prepared in RIPA buffer (Sigma) with added phosphatase and complete protease inhibitors (Roche). Protein concentration was determined using the Bio-Rad DC protein assay (Bio-Rad). Where indicated, 10 or 15 μ g of protein was boiled for 5 min, electrophoresed on 10% SDS-PAGE gels and electroblotted on PVDF membranes (Bio-Rad) for western blotting with the following antibodies: ER α (G-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), DICER1, PTEN (Cell Signaling, Danvers, MA,

USA) and β -actin (loading control; Sigma). Bands were visualized using Carestream Image Station 4000R PRO with Carestream Molecular Imaging Software Version 5.0.2.30 (Carestream Health, Inc., New Haven, CT, USA) and quantified by UN-SCAN-IT Graph Digitizer Software 7.1 (Silk Scientific, Orem, UT, USA). The values were normalized to loading control and the normalized values for vehicle-treated and/or control-transfected cells were set to 1 for comparison within each cell line.

2.7. Statistical analysis

Statistical evaluations were performed using Student's t-test or one-way ANOVA followed by Tukey test in GraphPad Prism (La Jolla, CA, USA).

3. RESULTS

3.1. 4-OHT reduces expression of miR-29b-1/a in TAM-sensitive (TAM-S) MCF-7 cell and increases miR-29b-1/a expression in TAM-resistant (TAM-R) LCC2, LCC9 and LY2 human breast cancer cells

Our previous microarray analysis of microRNA expression revealed that 4-OHT reduced expression of miR-29b-1 and miR-29a in endocrine sensitive MCF-7 breast cancer cells and increased expression in endocrine resistant LY2 cells [14]. As a follow up on these initial observations, qPCR was performed to examine the effect of 4-OHT on miR-29b-1 and miR-29a expression in MCF-7 cells and a panel of MCF-7-derived cells lines showing progressive resistance to TAM and fulvestrant: LCC2, LCC9 and LY2 cells [15, 28]. For these experiments, cells were grown in hormone-depleted medium *i.e.*, incubated with phenol-red free IMEM + 5% DCC-stripped FBS + 1% P/S, for 48 h to reduce basal hormone-related activities [31] and then treated with 100 nM 4-OHT for 6 h. Confirming the microarray data [14], 4-OHT decreased the expression of miR-29b-1 and miR-29a in MCF-7 cells, but increased miR-29b-1 and miR-29a expression in the three endocrine-resistant cell lines: LCC2, LCC9 and LY2 cells (Fig. 1A). miR-29b-1/a-3p expression levels were higher than miR-29b-1/a-5p expression levels (Supplementary Fig. 1). Thus, we examined miR-29b-1-3p and miR-29a-3p in all subsequent experiments.

miR-29b-1 and miR-29a are transcribed from a common promoter as a single primary transcript [18]. Pri-miR-29b-1 and pri-miR-29a expression was reduced by 4-OHT in MCF-7 cells and increased by 4-OHT in LCC2 and LCC9 cells (Fig. 1B). These data suggest 4-OHT regulates pri-miR-29b-1/a transcription.

To examine if the 4-OHT regulation of miR-29b-1/a expression was mediated at the level of transcription, cells were preincubated with the transcriptional inhibitor actinomycin D (ACT D) (Fig. 1C and D). While ACT D inhibited basal expression of miR-29b-1 and miR-29a in all cells (data not shown), ACT D further inhibited miR-29b-1 and miR-29a expression in 4-OHT-treated MCF-7 cells. Conversely, ACT D inhibited the 4-OHT-induced miR-29b-1 (Fig. 1C) and miR-29a (Fig. 1D) expression in LCC9 and LY2 cells, suggesting a primary transcriptional response.

3.2. ER α mediates 4-OHT regulation of miR-29b-1/a transcription

The observed 4-OHT regulation of miR-29b-1/a transcription in these ER α + breast cancer cells suggests a possible role of ER α , and/or ER β , in mediating these effects. MCF-7 and LCC9 cells have higher ER α than ER β and higher ER α protein levels than LY2 [30]. To evaluate ER α 's role in mediating 4-OHT-regulation of miR-29b-1/a expression, MCF-7 and LCC9 cells were transfected with siER α or siControl. siER α reduced ER α protein levels by ~ 70% in MCF-7 and ~90% in LCC9 cells, respectively (Fig. 2A). *ESR1* mRNA levels were decreased by siER α in both cell lines (Fig. 2B). ER α knockdown had no significant effect on basal miR-29b-1/a expression in MCF-7 or LCC9 cells (Fig. 2C). ER α knockdown inhibited the decrease in pri-miR-29b-1/a (Fig. 2C), as well as the reduction in miR-29b-1 and miR-29a seen with 4-OHT treatment in MCF-7 cells (Fig. 2D).

Although ER α knockdown did not affect basal pri-miR-29b/1a expression in LCC9 cells (Fig. 2C), there was a significant increase in miR-29a (Fig. 2D). Knockdown of ER α blocked 4-OHT stimulation of pri-miR-29b-1/a (Fig. 2C) and mature miR-29b-1 and miR-29a expression (Fig. 2D). Comparable results were seen with ER α knockdown in LY2 cells (Supplementary Fig. 2). Together, these data suggest that ER α plays a role in mediating 4-OHT suppression of miR-29b-1/a expression in MCF-7 cells and 4-OHT stimulation of miR-29b-1/a expression in LCC9 and LY2 cells.

3.3. 4-OHT regulates miR-29b-1/a promoter activity

To examine the direct effect of 4-OHT on the 5' promoter of miR-29b-1/a, CHO-K1 cells were transiently transfected with an ER α expression vector [34] and a luciferase reporter containing -1530 to +165 of the human miR-29b-1/a promoter [18]. 4-OHT reduced luciferase activity and this was inhibited by fulvestrant (Supplementary Fig. 3). These data support the model that 4-OHT inhibits miR-29b-1/a promoter activity through ER α .

3.4. miR-29b-1/a inhibit cell proliferation but do not sensitize cells to 4-OHT inhibition

To examine the functional roles of miR-29b-1 and miR-29a in sensitivity to TAM, we evaluated how overexpression or inhibition of miR-29b-1 and miR-29a affected MCF-7, LCC9, and LY2 cell proliferation. Successful repression or overexpression of miR-29b-1 and miR-29a was confirmed by qPCR up to 6 d post transfection (Supplementary Fig. 4). Anti-miR-29b-1 or anti-miR-29a transfection had no effect on basal cell proliferation (Fig. 3A-C). However, inhibition of miR-29a blocked 4-OHT inhibition of MCF-7 proliferation (Fig. 3A). Further, the combined inhibition of miR-29b-1 and miR-29a resulted in 4-OHT stimulating MCF-7 cell proliferation, effectively reversing the normal antiestrogenic pharmacology of 4-OHT in MCF-7 cells (Fig. 3A). These data are commensurate with the inhibition of miR-29b-1/a expression by 4-OHT in MCF-7 cells (Fig. 1), suggesting a possible role for miR-29b-1 and miR-29a in 4-OHT's antiproliferative activity in MCF-7 cells.

Since 4-OHT stimulated miR-29b-1/a in TAM-resistant LCC9 and LY2, cells, we anticipated that inhibiting miR-29b-1 and/or miR-29a might sensitize these cells to 4-OHT inhibition. However, inhibition of miR-29b-1/a did not result in 4-OHT inhibiting cell proliferation (Fig. 3B, 3C). In contrast, inhibiting miR-29b-1 and/or miR-29a resulted in a

stimulation of LCC9 cell proliferation with 4-OHT treatment (Fig. 3B). There was no significant effect of anti-miR-29b-1 or miR-29a on 4-OHT-stimulated LY2 cell proliferation (Fig. 3C).

Transfection of pre-miR-29b-1 and/or pre-miR-29a inhibited basal MCF-7 and LCC9 cell proliferation (Fig. 3A, 3B). In LCC9 cells, the inhibition by pre-miR-29b-1/a transfection was reduced by 4-OHT, although the effect was not statistically significant for miR-29a (Fig. 3B). Transfection of pre-miR-29a, alone or in combination with pre-miR-29b-1, inhibited LY2 cell proliferation, but did not sensitize the cells to further inhibition by 4-OHT (Fig. 3C).

3.5. miR-29b-1/a inhibit LY2 cell migration and colony formation

Transfection of MDA-MB-231 triple negative breast cancer (TNBC) cells with pre-miR-29b increased invasion, whereas anti-miR-29b inhibited invasion [35]. To further elucidate the possible functional role of miR-29b-1/a in ER-positive breast cancer cells, cell migration and colony formation experiments were performed with LY2 cells. We chose LY2 cells because they are invasive whereas MCF-7 and LCC9 cells are not [36, 37]. In accordance with our observation for an antiproliferative role for miR-29b-1 and miR-29a in LY2 cells, transfection of LY2 cells with pre-miR-29b-1 and pre-miR-29a inhibited cell migration (Fig. 4A, 4B). 4-OHT inhibited LY2 cell migration by ~ 5% after 8 h; however, this inhibition was not detected in cells transfected with pre-miR-29b-1 or pre-miR-29a.

4-OHT inhibited LY2 colony formation. Transfection of LY2 cells with pre-miR-29b-1 and pre-miR-29a inhibited colony formation and these transfections enhanced 4-OHT inhibition of colony formation (Fig. 4C). Together, these data suggest that miR-29b-1 and miR-29a stimulate anti-migratory and anti-colony-forming activity in LY2 cells. It appears that overexpression of miR-29b-1 and miR-29a enhance LY2 cell responses to 4-OHT's antiestrogen activity on cellular migration and invasion.

3.6. miR-29b-1/a decrease DICER in LY2 cells

Next it was important to examine how 4-OHT's regulation of miR-29b-1/a transcription affected a *bona fide* target of miR-29b-1/a in these cells. To address the identity of such miR-29b-1/a targets, we searched online databases (mirdb.org[38]; microrna.org [39])) and identified DICER1 as one such candidate [40]. There are no previous reports of TAM-regulation of DICER1 transcription. Although DICER1 expression is upregulated in TAM-R MCF-7 cells [41], the mechanism was not examined. Transfection of LY2 cells with pre-miR-29b-1 or pre-miR-29a, individually or together, reduced DICER1 protein (Fig. 5A). Conversely, transfection with either anti-miR-29b-1 or anti-miR-29a, alone or in combination, increased DICER1 protein expression (Fig. 5A), suggesting that DICER1 is a target of miR-29b-1/a in LY2 cells. Similar results were observed for another *bona fide* target of miR-29b-1/a: tumor suppressor PTEN [42] (Supplementary Fig. 5). RNA seq data (GSE81620) show lower transcript levels of PTEN in LCC9 *versus* MCF-7 cells (Supplementary Fig. 6A). However, qPCR for PTEN reveal no difference in basal PTEN transcript levels in MCF-7 and LCC9 cells grown in hormonally depleted medium (5% DCC-FBS, phenol-red free IMEM) for 48 h and treated with DMSO (vehicle control) or 100

nM 4-OHT (Supplementary Fig. 6B). Significantly higher PTEN transcript levels were found in LY2 cells under these experimental conditions. We note that while 'serum starvation in 0.5% FBS had no effect on PTEN in MCF-7 cells, PTEN was increased in more aggressive adenocarcinoma cell lines including MDA-MB-468 TNBC cells [43]. Our data indicate that 4-OHT increased PTEN in MCF-7 cells but not in LCC9 or LY2 cells, commensurate with the TAM-resistant phenotype of these cells.

To determine whether 4-OHT would decrease DICER1 by increasing miR-29b-1/a in LY2 cells, cells were treated with 100 nM 4-OHT for 24 h following 48 h growth in hormone-depleted medium. Indeed, 4-OHT decreased *DICER1* mRNA (Fig. 5B) and protein (Fig. 5C). Next, we examined if anti-miR-29b-1 and anti-miR-29a would block 4-OHT downregulation of DICER1 expression. Anti-miR-29b-1/a transfection blocked 4-OHT downregulation of DICER1 protein (Fig. 5C) and transcript (Supplementary Fig. 7). We also show that 4-OHT downregulation of *DICER1* mRNA expression is mediated through ER α since knockdown of ER α blocked 4-OHT inhibition of DICER1 mRNA (Supplementary Fig. 8). These results suggest that the 4-OHT-stimulated increase in miR-29b-1/a in LY2 cells results in decreased DICER1 expression.

4. DISCUSSION

In this study, we demonstrated that ER α -mediates the 4-OHT-induced decrease in miR-29b-1 and miR-29a expression in endocrine-sensitive MCF-7 cells and the 4-OHT-induced increase in the transcription of these microRNAs in LCC2, LCC9 and LY2 cells. This supports our findings in a microarray analysis of 4-OHT-regulated microRNAs in MCF-7 and LY2 cells [14]. These 4-OHT-induced changes in mature miR-29b-1/a expression were initiated at the level of ER α -regulated transcription of pri-miR-29b-1/a. However, knockdown of ER α had no effect on basal pri-miR-29b-1/a transcription, or mature miR-29b-1 or miR-29a levels, suggesting the importance of the ligand 4-OHT in the ER α transcriptional response. A recent study identified a positive roles for Oct-4 (POU5F1 gene) and the E3 ubiquitin ligase SKP2 and a negative role for NKX3-1 in TAM-ER α stimulated gene transcription in MCF-7 cells [44]. This study did not examine regulation of miRNAs. The authors provided evidence for a 4-OHT-ER α /SKP2 complex and postulate this complex collaborates with Oct-4 to increase transcription of TAM-stimulated genes. To our knowledge, there are no reports of Oct-4 regulation of miR-29 family members. Oct-4 was increased and NKX3-1 decreased in TAM-R MCF-7 cells compared to parental MCF-7 cells [44]. We found no difference in Oct-4 (POU5F1) transcript levels between MCF-7 and LCC9 cells whereas SKP2 was increased and NKX3-1 decreased in LCC9 compared to MCF-7 cells (Supplementary Fig. 9). Future studies will be required to elucidate the role of these factors in 4-OHT-ER α stimulated miR-29b-1/a transcription in LCC9 cells.

We observed that transfection of both TAM-S MCF-7 and its TAM-R cell line derivatives with miR-29b-1 or miR-29a repressed cell proliferation. Transient overexpression of miR-29a also inhibited the growth of MDA-MB-453 TNBC cells [27], implying repression of cell growth by miR-29a is not dependent on ER α . If the 4-OHT-induced increase in miR-29b-1/a in TAM-R cells is involved in resistance, then we would expect that inhibiting

miR-29-b-1/a should enhance 4-OHT inhibition of cell proliferation. However, inhibition of miR-29b-1 or miR-29a did not enhance the sensitivity of the TAM-R cells to 4-OHT.

Likewise, transient overexpression of either miR-29b-1 or miR-29a in TAM-R LY2 cells decreased cell migration colony formation. Interestingly, overexpression of miR-29a alone or in combination with miR-29b-1 enhanced 4-OHT inhibition of LY2 colony formation. These data suggest that the 4-OHT-induced increase in miR-29b-1/a in LY2 may be involved in TAM-sensitivity, not resistance. Future studies will address if a combination of miR-29a and 4-OHT may lead to mesenchymal-to-epithelial transition (MET) in LY2 cells, although other gene expression changes are likely to contribute. The inhibitory effect of miR-29b-1 and miR-29a on LY2 cell migration and colony formation are the opposite of the effect of overexpression of these miRs in MDA-MB-231 TNBC cells. Overexpression of miR-29b enhanced the mesenchymal-like phenotype in MDA-MB-231 whereas transient transfection of antisense miR-29b decreased MDA-MB-231 cell migration [35]. The expression of ER α and other genes in LY2 cells may mediate the observed phenotypic difference in cellular responses to manipulation of miR-29b-1/a expression as TNBCs, by definition, lack ER α .

miR-29 family members contribute to resistance of chemotherapeutic agents [45-48]. For example, miR-29a contributes to resistance to Adriamycin and Docetaxel in breast cancer by repressing the tumor suppressor PTEN [45], fostering cell proliferation and inhibiting apoptosis. Transient overexpression of miR-29b increased MCF-7 cell migration and invasion by repressing the PTEN [35]. Upregulation of miR-29b was recently reported to enhance sensitivity of gastric cancer cells to cisplatin, while knockdown enhanced cisplatin resistance [46]. Likewise, loss of miR-29 inhibits cisplatin-induced cell death in ovarian cancer through upregulation of collagen type I alpha 1 (COL1A1) and inactivation of glycogen synthase kinase beta (GSK β) [47]. In pancreatic cancer, miR-29a contributes to resistance of gemcitabine through upregulation of the Wnt/ β -catenin signaling pathway [48]. These studies implicate the miR-29 family as mediators of drug resistance and sensitivity in several cancers through mechanisms that may be drug and cancer-type-specific. It is therefore essential to identify the deregulated pathways mediating responses to miR-29 in each disease type.

Although miR-29b-1 and miR-29a originate from the same primary transcript, sharing the same promoter and seed sequence [17, 18], they had similar but not identical functional outcomes in our study. This difference can be explained in part by nucleotide differences adjacent to the seed sequence which contribute to target specificity and posttranscriptional processing, including subcellular localization and stability [23, 49]. Specifically, a hexanucleotide terminal localization motif present in miR-29b-1 and not in miR-29a targets miR-29b-1 for nuclear enrichment, while miR-29a and other vertebrate microRNAs are predominantly cytoplasmic [49, 50]. The nuclear localization of miR-29b appears to depend on the cell type, suggesting cell-specific expression of the machinery involved in miR-29b nuclear import [51]. We speculate this difference in subcellular localization may cause differential gene regulation and hence, differences in the functional outcomes between miR-29b-1 and miR-29a. Further studies are needed to examine the subcellular distribution of miR-29a and miR-29b-1 in breast cancer cells.

miR-29b-1 and miR-29a downregulate DICER1 protein levels in LY2 breast cancer cells. Because of its role in microRNA processing from pre-miRs to mature microRNAs, DICER1 regulates cellular processes including cell differentiation, programmed cell death, senescence, DNA repair, and chromatin remodeling [52-56]. DICER1 expression was reported to be downregulated in breast cancer cells exhibiting mesenchymal phenotypes and in primary breast tumors of patients with lower disease free survival [57]. However, a larger study of primary breast tumors reported that DICER1 staining was associated with ER negativity, HER2+, high Ki67, and expression of basal-like biomarkers [58]. Our observation that 4-OHT reduced DICER1 by increasing miR-29b-1/a and the reported increase in DICER1 in ER α -negative breast tumors suggest a possible role for ER α in DICER1 regulation, although further experiments are needed to validate this suggestion. Further DICER1 expression was associated with reduced overall survival, except in HER2+ patients where moderate or strong DICER1 expression was associated with improved disease free survival [58] .

DICER1 mRNA expression was decreased in breast cancer tissues [59] and in invasive ductal breast carcinomas [52]. In ER α negative breast cancer cells, DICER1 is repressed by tumor promoting microRNAs including miR-103/107 [60], miR-222/221 [40], and miR-18a [61]. The differing reports on the role of DICER1 deregulation underscore the limited current knowledge of its role in cellular processes and mechanism of action. These discrepancies can, in part, be explained by heterogeneity and genetic diversity of cancers and cell types [62], target expression status, the cell type and the microenvironment of each cell [25].

We observed that TAM decreased DICER1 protein expression in the TAM-R breast cancer cells by upregulating miR-29b-1/a, suggesting a decrease in DICER1 may play a role in promoting TAM resistance. This is contrary to a previous report that stable DICER1-overexpression in MCF-7 breast cancer cells conferred TAM resistance by increasing breast cancer resistance protein (BCRP) [41]. However, miR-29b-1/a regulation of DICER1 was not examined. In prostate tumors, DICER1 mRNA levels were negatively correlated with miR-29b-1 [63]. Further studies are needed on the impact of DICER1 in TAM resistance. Increased expression of miR-29b-1 and miR-29a in 4-OHT-treated TAM-R cells also suggest that this microRNA family could target transcripts of tumor suppressor proteins. Indeed, we observed downregulation of the tumor suppressor PTEN, another *bona fide* target of miR-29 regulation [42]. Certainly additional miR-29 targets may contribute to the response of TAM-R cells to TAM.

5. CONCLUSION

Mechanisms for endocrine resistance in breast cancer are multifactorial (reviewed in [64]). Here we identified miR-29b-1/a as regulated in opposite direction by 4-OHT in TAM-S MCF-7 cells *versus* TAM-R LCC2, LCC9, and LY2 cells. We show that the TAM regulation of miR-29b-1/a is mediated by ER α in opposite direction, *i.e.*, stimulation in LCC9 and LY2 versus inhibition in MCF-7 cells. We found that inhibiting miR-29b-1/a in TAM-R LCC9 and LY2 cells did not increase their sensitivity to 4-OHT inhibition. Further studies to identify other targets of these microRNAs are needed. It is perhaps not surprising that

miR-29b1/a has a complicated set of effects, given that there are 632 predicted gene targets for has-miR-29a-3p and has-miR-29b-3p in the microRNA data base (<http://mirdb.org/miRDB/>). Examining the role of microRNAs in mediating endocrine resistance will provide new insights in elucidating defective cellular pathways and improving therapeutics for cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics 2016. *CA Cancer J Clin.* 2016; 66:7–30. [PubMed: 26742998]
2. Ring A, Dowsett M. Mechanisms of tamoxifen resistance. *Endocrine-related cancer.* 2004; 11:643–658. [PubMed: 15613444]
3. Johnston SR, Saccani-Jotti G, Smith IE, Salter J, Newby J, Coppen M, Ebbs SR, Dowsett M. Changes in estrogen receptor, progesterone receptor, and pS2 expression in tamoxifen-resistant human breast cancer. *Cancer research.* 1995; 55:3331–3338. [PubMed: 7614468]
4. Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet.* 1998; 351:1451–1467. [PubMed: 9605801]
5. Baum M, Brinkley DM, Dossett JA, McPherson K, Patterson JS, Rubens RD, Smiddy FG, Stoll BA, Wilson A, Lea JC, Richards D, Ellis SH. Improved survival among patients treated with adjuvant tamoxifen after mastectomy for early breast cancer. *Lancet.* 1983; 2:450.
6. Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids.* 2007; 72:7–25. [PubMed: 17169390]
7. Zhao M, Ramaswamy B. Mechanisms and therapeutic advances in the management of endocrine-resistant breast cancer. *World journal of clinical oncology.* 2014; 5:248–262. [PubMed: 25114842]
8. Egeland NG, Lunde S, Jonsdottir K, Lende TH, Cronin-Fenton D, Gilje B, Janssen EA, Soiland H. The Role of MicroRNAs as Predictors of Response to Tamoxifen Treatment in Breast Cancer Patients. *Int J Mol Sci.* 2015; 16:24243–24275. [PubMed: 26473850]
9. Fribbens C, O'Leary B, Kilburn L, Hrebien S, Garcia-Murillas I, Beaney M, Cristofanilli M, Andre F, Loi S, Loibl S, Jiang J, Bartlett CH, Koehler M, Dowsett M, Bliss JM, Johnston SR, Turner NC. Plasma ESR1 Mutations and the Treatment of Estrogen Receptor-Positive Advanced Breast Cancer. *J Clin Oncol.* 2016; 34:2961–2968. [PubMed: 27269946]
10. Nagaraj G, Ma C. Revisiting the estrogen receptor pathway and its role in endocrine therapy for postmenopausal women with estrogen receptor-positive metastatic breast cancer. *Breast cancer research and treatment.* 2015; 150:231–242. [PubMed: 25762475]
11. Muluhngwi P, Klinge CM. Roles for miRNAs in endocrine resistance in breast cancer. *Endocrine-related cancer.* 2015; 22:R279–300. [PubMed: 26346768]
12. Iorio MV, Croce CM. MicroRNAs in cancer: small molecules with a huge impact. *J Clin Oncol.* 2009; 27:5848–5856. [PubMed: 19884536]

13. Iorio MV, Croce CM. microRNA involvement in human cancer. *Carcinogenesis*. 2012; 33:1126–1133. [PubMed: 22491715]
14. Manavalan TT, Teng Y, Appana SN, Datta S, Kalbfleisch TS, Li Y, Klinge CM. Differential expression of microRNA expression in tamoxifen-sensitive MCF-7 versus tamoxifen-resistant LY2 human breast cancer cells. *Cancer Lett*. 2011; 313:26–43. [PubMed: 21955614]
15. Bronzert DA, Greene GL, Lippman ME. Selection and characterization of a breast cancer cell line resistant to the antiestrogen LY. *Endocrinology*. 1985; 117:1409–1417. [PubMed: 4029083]
16. Chang TC, Yu D, Lee YS, Wentzel EA, Arking DE, West KM, Dang CV, Thomas-Tikhonenko A, Mendell JT. Widespread microRNA repression by Myc contributes to tumorigenesis. *Nature genetics*. 2008; 40:43–50. [PubMed: 18066065]
17. Eyholzer M, Schmid S, Wilkens L, Mueller BU, Pabst T. The tumour-suppressive miR-29a/b1 cluster is regulated by CEBPA and blocked in human AML. *British journal of cancer*. 2010; 103:275–284. [PubMed: 20628397]
18. Mott JL, Kurita S, Cazanave S, Bronk SF, Werneburg NW, Fernandez-Zapico ME. Transcriptional Suppression of mir-29b-1/mir-29a Promoter by c-Myc, Hedgehog, and NF-kappaB. *Journal of cellular biochemistry*. 2010; 110:1155–1164. [PubMed: 20564213]
19. Kriegel AJ, Liu Y, Fang Y, Ding X, Liang M. The miR-29 family: genomics, cell biology, and relevance to renal and cardiovascular injury. *Physiol Genomics*. 2012; 44:237–244. [PubMed: 22214600]
20. Mishmar D, Rahat A, Scherer SW, Nyakatura G, Hinzmann B, Kohwi Y, Mandel-Gutfroind Y, Lee JR, Drescher B, Sas DE, Margalit H, Platzer M, Weiss A, Tsui LC, Rosenthal A, Kerem B. Molecular characterization of a common fragile site (FRA7H) on human chromosome 7 by the cloning of a simian virus 40 integration site. *Proc Natl Acad Sci U S A*. 1998; 95:8141–8146. [PubMed: 9653154]
21. Chou J, Lin JH, Brenot A, Kim JW, Provot S, Werb Z. GATA3 suppresses metastasis and modulates the tumour microenvironment by regulating microRNA-29b expression. *Nat Cell Biol*. 2013; 15:201–213. [PubMed: 23354167]
22. Wang Y, Zhang XW, Li H, Yu JP, Ren XB. The role of miRNA-29 family in cancer. *European Journal of Cell Biology*. 2013; 92:123–128. [PubMed: 23357522]
23. Schmitt MJ, Margue C, Behrmann I, Kreis S. MiRNA-29: a microRNA family with tumor-suppressing and immune-modulating properties. *Curr Mol Med*. 2013; 13:572–585. [PubMed: 22934851]
24. Jiang H, Zhang G, Wu JH, Jiang CP. Diverse roles of miR-29 in cancer (review). *Oncol Rep*. 2014; 31:1509–1516. [PubMed: 24573597]
25. Rostas JW 3rd, Pruitt HC, Metge BJ, Mitra A, Bailey SK, Bae S, Singh KP, Devine DJ, Dyess DL, Richards WO, Tucker JA, Shevde LA, Samant RS. microRNA-29 negatively regulates EMT regulator N-myc interactor in breast cancer. *Mol Cancer*. 2014; 13:200. [PubMed: 25174825]
26. Cittelly DM, Finlay-Schultz J, Howe EN, Spoelstra NS, Axlund SD, Hendricks P, Jacobsen BM, Sartorius CA, Richer JK. Progestin suppression of miR-29 potentiates dedifferentiation of breast cancer cells via KLF4. *Oncogene*. 2013; 32:2555–2564. [PubMed: 22751119]
27. Wu Z, Huang X, Huang X, Zou Q, Guo Y. The inhibitory role of Mir-29 in growth of breast cancer cells. *J Exp Clin Cancer Res*. 2013; 32:98. [PubMed: 24289849]
28. Brunner N, Boulay V, Fojo A, Freter CE, Lippman ME, Clarke R. Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. *Cancer Res*. 1993; 53:283–290. [PubMed: 8380254]
29. Brunner N, Boysen B, Jirus S, Skaar TC, Holst-Hansen C, Lippman J, Frandsen T, Spang-Thomsen M, Fuqua SA, Clarke R. MCF7/LCC9: an antiestrogen-resistant MCF-7 variant in which acquired resistance to the steroidal antiestrogen ICI 182,780 confers an early cross-resistance to the nonsteroidal antiestrogen tamoxifen. *Cancer research*. 1997; 57:3486–3493. [PubMed: 9270017]
30. Riggs KA, Wickramasinghe NS, Cochrum RK, Watts MB, Klinge CM. Decreased chicken ovalbumin upstream promoter transcription factor II expression in tamoxifen-resistant breast cancer cells. *Cancer research*. 2006; 66:10188–10198. [PubMed: 17047084]

31. Biswas R, Vonderhaar BK. Role of serum in the prolactin responsiveness of MCF-7 human breast cancer cells in long-term tissue culture. *Cancer Res.* 1987; 47:3509–3514. [PubMed: 3581086]
32. Kwon SY, Lee JH, Kim B, Park JW, Kwon TK, Kang SH, Kim S. Complexity in regulation of microRNA machinery components in invasive breast carcinoma. *Pathol Oncol Res.* 2014; 20:697–705. [PubMed: 24574065]
33. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc.* 2008; 3:1101–1108. [PubMed: 18546601]
34. Ivanova MM, Mazhawidza W, Dougherty SM, Minna JD, Klinge CM. Activity and intracellular location of estrogen receptors α and β in human bronchial epithelial cells. *Mol Cell Endocrinol.* 2009; 305:12–21. [PubMed: 19433257]
35. Wang C, Bian Z, Wei D, Zhang JG. miR-29b regulates migration of human breast cancer cells. *Mol Cell Biochem.* 2011
36. Clarke R, Liu MC, Bouker KB, Gu Z, Lee RY, Zhu Y, Skaar TC, Gomez B, O'Brien K, Wang Y, Hilakivi-Clarke LA. Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling. *Oncogene.* 2003; 22:7316–7339. [PubMed: 14576841]
37. Hoshiba T, Tanaka M. Breast cancer cell behaviors on staged tumorigenesis-mimicking matrices derived from tumor cells at various malignant stages. *Biochem Biophys Res Commun.* 2013; 439:291–296. [PubMed: 23978418]
38. Wong N, Wang X. miRDB: an online resource for microRNA target prediction and functional annotations. *Nucleic Acids Res.* 2015; 43:D146–152. [PubMed: 25378301]
39. Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. *Nucleic Acids Res.* 2008; 36:D149–153. [PubMed: 18158296]
40. Cochrane DR, Cittelly DM, Howe EN, Spoelstra NS, McKinsey EL, LaPara K, Elias A, Yee D, Richer JK. MicroRNAs link estrogen receptor alpha status and Dicer levels in breast cancer. *Horm Cancer.* 2010; 1:306–319. [PubMed: 21761362]
41. Selever J, Gu G, Lewis MT, Beyer A, Herynk MH, Covington KR, Tsimelzon A, Dontu G, Provost P, Di Pietro A, Boumendjel A, Albain K, Miele L, Weiss H, Barone I, Ando S, Fuqua SA. Dicer-mediated upregulation of BCRP confers tamoxifen resistance in human breast cancer cells. *Clin Cancer Res.* 2011; 17:6510–6521. [PubMed: 21878538]
42. Lin X, Zhou X, Liu D, Yun L, Zhang L, Chen X, Chai Q, Li L. MicroRNA-29 regulates high-glucose-induced apoptosis in human retinal pigment epithelial cells through PTEN. *In Vitro Cell Dev Biol Anim.* 2016; 52:419–426. [PubMed: 26822433]
43. Levin VA, Panchabhai SC, Shen L, Kornblau SM, Qiu Y, Baggerly KA. Different Changes in Protein and Phosphoprotein Levels Result from Serum Starvation of High-Grade Glioma and Adenocarcinoma Cell Lines. *Journal of Proteome Research.* 2010; 9:179–191. [PubMed: 19894763]
44. Bhatt S, Stender JD, Joshi S, Wu G, Katzenellenbogen BS. OCT-4: a novel estrogen receptor-[alpha] collaborator that promotes tamoxifen resistance in breast cancer cells. *Oncogene.* 2016; 35:5722–5734. [PubMed: 27065334]
45. Zhong SL, Li WJ, Chen ZY, Xu JJ, Zhao JH. miR-222 and miR-29a contribute to the drug-resistance of breast cancer cells. *Gene.* 2013; 531:8–14. [PubMed: 23994196]
46. Chen DD, Feng LC, Ye R, He YQ, Wang YD. [miR-29b Reduces Cisplatin Resistance of Gastric Cancer Cell by Targeting PI3K/Akt Pathway]. *Zhongguo yi xue ke xue yuan xue bao. Acta Academiae Medicinae Sinicae.* 2015; 37:514–519. [PubMed: 26564501]
47. Yu PN, Yan MD, Lai HC, Huang RL, Chou YC, Lin WC, Yeh LT, Lin YW. Downregulation of miR-29 contributes to cisplatin resistance of ovarian cancer cells. *International journal of cancer.* 2014; 134:542–551. [PubMed: 23904094]
48. Nagano H, Tomimaru Y, Eguchi H, Hama N, Wada H, Kawamoto K, Kobayashi S, Mori M, Doki Y. MicroRNA-29a induces resistance to gemcitabine through the Wnt/beta-catenin signaling pathway in pancreatic cancer cells. *International journal of oncology.* 2013; 43:1066–1072. [PubMed: 23900458]
49. Hwang HW, Wentzel EA, Mendell JT. A hexanucleotide element directs microRNA nuclear import. *Science (New York, NY).* 2007; 315:97–100.

50. Lee Y, Jeon K, Lee JT, Kim S, Kim VN. MicroRNA maturation: stepwise processing and subcellular localization. *The EMBO journal*. 2002; 21:4663–4670. [PubMed: 12198168]
51. Roberts TC. The MicroRNA Biology of the Mammalian Nucleus. *Mol Ther Nucleic Acids*. 2014; 3:e188. [PubMed: 25137140]
52. Poursadegh Zonouzi AA, Nejatizadeh A, Rahmati-Yamchi M, Fardmanesh H, Shakerizadeh S, Poursadegh Zonouzi A, Nejati-Koshki K, Shekari M. Dysregulated expression of Dicer in invasive ductal breast carcinoma. *Medical oncology (Northwood, London, England)*. 2015; 32:203.
53. Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. *Nature genetics*. 2007; 39:673–677. [PubMed: 17401365]
54. Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV, Hannon GJ. Dicer is essential for mouse development. *Nat Genet*. 2003; 35:215–217. [PubMed: 14528307]
55. Kanellopoulou C, Muljo SA, Kung AL, Ganesan S, Drapkin R, Jenuwein T, Livingston DM, Rajewsky K. Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. *Genes & development*. 2005; 19:489–501. [PubMed: 15713842]
56. Han L, Zhang A, Zhou X, Xu P, Wang GX, Pu PY, Kang CS. Downregulation of Dicer enhances tumor cell proliferation and invasion. *Int J Oncol*. 2010; 37:299–305. [PubMed: 20596657]
57. Hinkal GW, Grelier G, Puisieux A, Moyret-Lalle C. Complexity in the regulation of Dicer expression: Dicer variant proteins are differentially expressed in epithelial and mesenchymal breast cancer cells and decreased during EMT. *Br J Cancer*. 2011; 104:387–388. [PubMed: 21119658]
58. Caffrey E, Ingoldsby H, Wall D, Webber M, Dinneen K, Murillo LS, Inderhaug C, Newell J, Gupta S, Callagy G. Prognostic significance of deregulated dicer expression in breast cancer. *PLoS One*. 2013; 8:e83724. [PubMed: 24386264]
59. Yan M, Huang HY, Wang T, Wan Y, Cui SD, Liu ZZ, Fan QX. Dysregulated expression of dicer and drosha in breast cancer. *Pathol Oncol Res*. 2012; 18:343–348. [PubMed: 21898071]
60. Martello G, Rosato A, Ferrari F, Manfrin A, Cordenonsi M, Dupont S, Enzo E, Guzzardo V, Rondina M, Spruce T, Parenti AR, Daidone MG, Biciato S, Piccolo S. A MicroRNA targeting dicer for metastasis control. *Cell*. 2010; 141:1195–1207. [PubMed: 20603000]
61. Sha LY, Zhang Y, Wang W, Sui X, Liu SK, Wang T, Zhang H. MiR-18a upregulation decreases Dicer expression and confers paclitaxel resistance in triple negative breast cancer. *European review for medical and pharmacological sciences*. 2016; 20:2201–2208. [PubMed: 27338043]
62. Boyd LK, Mao X, Lu YJ. The complexity of prostate cancer: genomic alterations and heterogeneity, *Nature reviews. Urology*. 2012; 9:652–664. [PubMed: 23132303]
63. Bian X, Shen Y, Zhang G, Gu C, Cai Y, Wang C, Zhu Y, Zhu Y, Zhang H, Dai B, Ye D. Expression of dicer and its related miRNAs in the progression of prostate cancer. *PLoS One*. 2015; 10:e0120159. [PubMed: 25768283]
64. Clarke R, Tyson JJ, Dixon JM. Endocrine resistance in breast cancer--An overview and update. *Mol Cell Endocrinol*. 2015; 418(Pt 3):220–234. [PubMed: 26455641]

Abbreviations

miRNA	microRNA
DMSO	Dimethyl sulfoxide
EtOH	ethanol
4-OHT	4-hydroxytamoxifen
SERDs	selective ER downregulators
SERMs	selective ER modulators

TAM	tamoxifen
TAM-R	TAM resistant
TAM-S	TAM sensitive
TNBC	triple negative breast cancer
AI	aromatase inhibitors
E₂	estradiol
EMT	epithelial-to-mesenchymal transition
ER	estrogen receptor
ERα	estrogen receptor α
ERβ	estrogen receptor β
ERE	estrogen response element
PM	plasma membrane
qRT-PCR	quantitative real-time PCR
RAL	raloxifene

Highlights

- Tamoxifen activation of estrogen receptor α inhibits miR-29b-1 and miR-29a transcription in MCF-7 cells but stimulates their expression in endocrine-resistant LCC9 and LY2 cells
- Overexpression of miR-29b-1 and miR-29a inhibit MCF-7, LCC9, and LY2 cell proliferation and inhibit LY2 cell migration and colony formation
- Inhibition of miR-29b-1/a does not sensitize LCC9 and LY2 cells to tamoxifen
- Tamoxifen through ER α represses DICER1 by increasing miR-29b-1/a in LY2 cells
- Tamoxifen-induced miR-29b-1/a may be involved in endocrine sensitivity, not resistance

Figure 1. Differential regulation of miR-29b-1/a expression by 4-OHT in MCF-7, LCC2, LCC9, and LY2 breast cancer cells

Cells were grown in hormonally depleted medium for 48 h and treated with vehicle control (DMSO) or 100 nM 4-OHT for 6 h. A, C, and D) miR-29b-1 and miR-29a expression was normalized to RNU38 or RNU48. B) pri-miR-29b-1 and pri-miR-29a expression was normalized to GAPDH. C and D) Cells were pre-incubated with 10 µg/ml actinomycin D (ActD) for 1 h prior to 6 h treatment. Values are the average of 4-20 (A), 3-9 (B), and 3 (C,D) separate experiments $\pm$ SEM. * $p < 0.05$ versus control; # $p < 0.05$ versus 4-OHT in the same cell line.

Figure 2. ERα regulates miR-29b-1/a expression in MCF-7 and LCC9 cells

Cells were grown in hormonally depleted medium and transfected with siControl or siERα. 48 h post transfection, cells were treated for 6 h with EtOH or 100 nM 4-OHT. (A) ERα protein was examined in WCE and the blot was stripped and re-probed for β-actin for normalization. Values are the ERα/β-actin with siControl set to one for each cell line. Q-PCR for ESR1 (B); pri-miR-29b-1/a and pri-miR-29a (C); and miR-29b-1 and miR-29a (D). Values are the average of 4-6 separate experiments ± SEM. *p < 0.05 *versus* control-transfected cells. #p < 0.05 *versus* 4-OHT-treated Control-transfected cells.

Figure 3. Overexpression of miR-29b-1/a decreases cell proliferation

Cells were grown in hormonally depleted medium and transfected with anti-miR-control, anti-miR-29b-1/a, pre-miR control, or pre-miR-29b-1/a for 48 h prior to treatment with DMSO (vehicle control) or 100 nM 4-OHT for 48 h prior to BrdU assay. Values are the $\pm$ SEM of 3 separate experiments * $p < 0.05$ versus vehicle control-transfected cells; # $p < 0.05$ versus 4-OHT-treated control transfected, as indicated.

Figure 4. Overexpression of miR-29b-1/a decreases LY2 cell migration

LY2 cells were grown in hormonally depleted medium and transfected with pre-miR control or pre-miR-29b-1/a. (A-B) 24 h post transfection cells were treated with DMSO (vehicle) or 4-OHT for up to 8 h and images taken at indicated time points. (C) Two d post transfection, colony formation assay was performed for 14 d. (B) Values are the $\pm$ SEM of 3 separate measurements within an experiment. (C) Values are the $\pm$ SEM of 6 replicates. * $p < 0.05$ versus vehicle control-transfected cells; # $p < 0.05$ versus control transfected + 4-OHT-treated cells

Figure 5. miR-29b-1/a downregulate DICER in LY2 cells and 4-OHT decreases DICER1 mRNA and protein in LY2 cells

A) LY2 cells were transfected with Anti-miR-control, Anti-miR-29b-1, AS-miR-29a, or the combination for 48 h. B and C) LY2 cells were grown in hormonally depleted medium for 48 h and treated for 24 h with vehicle control (DMSO) or 100 nM 4-OHT. B) qPCR for DICER1 relative to 18S. Values are the average of triplicate determinations $\pm$ SEM in one experiment. For A and C, the blots were striped and reprobed for β -actin. Values are the ratio of DICER/ β -actin from the experiment shown. All experiments were repeated 3-4 times and representative data is shown. D) Model of suggested pathway for decreased DICER1 in 4-OHT-treated LY2 cells.