Estradiol downregulates miR-21 expression and increases miR-21 target gene expression in MCF-7 breast cancer cells

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ABSTRACT

Select changes in microRNA (miRNA) expression correlate with estrogen receptor α (ERα) expression in breast tumors. miR-21 is higher in ERα positive than negative tumors, but no one has examined how estradiol (E2) regulates miR-21 in breast cancer cells. Here we report that E2 inhibits miR-21 expression in MCF-7 human breast cancer cells. The E2-induced reduction in miR-21 was inhibited by 4-hydroxytamoxifen (4-OHT), ICI 182 780 (Faslodex), and siRNA ERα indicating that the suppression is ERα-mediated. ERα and ERβ agonists PPT and DPN inhibited and 4-OHT increased miR-21 expression. E2 increased luciferase activity from reporters containing the miR-21 recognition elements from the 3′-UTRs of miR-21 target genes, corroborating that E2 represses miR-21 expression resulting in a loss of target gene suppression. The E2-mediated decrease in miR-21 correlated with increased protein expression of endogenous miR-21-targets Pdcd4, PTEN and Bcl-2. siRNA knockdown of ERα blocked the E2-induced increase in Pdcd4, PTEN and Bcl-2. Transfection of MCF-7 cells with anti-sense (AS) to miR-21 mimicked the E2-induced increase in Pdcd4, PTEN and Bcl-2. These results are the first to demonstrate that E2 represses the expression of an oncogenic miRNA, miR-21, by activating estrogen receptor in MCF-7 cells.

INTRODUCTION

Although the precise sequence of events leading to breast tumors are not understood, lifetime exposure to estrogens is widely accepted as a major risk factor for the development of breast cancer. Estrogens promote cell replication by binding to the estrogen receptors α and β (ERα and ERβ). Ligand-activated ER acts genomically by binding directly to estrogen response elements (EREs) or by a ‘tethering mechanism’, e.g. by interacting with AP-1 (1) or Sp1 (2). These interactions recruit coregulators to initiate chromatin remodeling resulting in increased gene transcription (3). ER can also suppress target gene transcription, although the mechanisms involved are unresolved (4). In addition to its ER-mediated, genomic activity, E2 also has ‘non-genomic’ or ‘membrane-initiated’ effects, i.e. independent of ER-mediated transcription, that occur within minutes after estradiol (E2), or other ER ligand, administration (5,6).

Inhibition of estrogen action is used as the adjuvant therapy of choice to treat both pre- and post-menopausal women with breast cancer. The anti-estrogen/Selective ER Modulator (SERM) tamoxifen (TAM) is the ‘gold standard’ of treatment of women with ER positive tumors (7). TAM is a SERM because it has mixed agonist/antagonist activity in a cell- and gene-specific manner whereas Faslodex (Fulvestrant, ICI 182 780) has pure antiestrogen activity (8). Ablation of endogenous estrogen production using aromatase inhibitors (AIs, e.g. anastrozole, letrozole and exemestane) has an efficacy greater than TAM in preventing disease recurrence in post-menopausal breast cancer patients (9). Together, these data demonstrate the importance of endogenous estrogens in promoting breast cancer recurrence.

MicroRNAs (miRNAs) are a class of naturally occurring, small, non-coding RNA molecules distinct from small interfering RNAs (siRNAs) (10–12). miRNA genes are mostly transcribed by RNA polymerase II, processed by Drosha into short hairpin RNAs that are exported from the nucleus, and processed by Dicer to form mature 21–25 nucleotide miRNAs which are transferred to Argonaute proteins in RISC. miRNAs bind to the 3′-untranslated region (3′ UTR) of target mRNAs and either block the translation of the message or target the mRNA transcript to be degraded (13). miRNAs may also...
increase translation of select mRNAs in a cell cycle-dependent manner (14).

The human genome contains >700 miRNAs (15) and miRNAs are expressed in a tissue-specific manner (16). Each miRNA targets ~200 transcripts directly or indirectly (17). Aberrant patterns of miRNA expression have been reported in human breast cancer (16–40). A number of genes involved in breast cancer progression have been identified by in silico analysis to be targets of miRNAs that are deregulated in breast cancer (41) and some, e.g. AIB1 have been experimentally proven (42). We recently reported that miR-21 downregulates the translation of human PDCD4, a tumor suppressor in MCF-7 cells (43). Although miR-21 was identified as an ‘oncomiR’, was the most significantly up-regulated miRNA in breast tumor biopsies (37), and was significantly higher in ERzx+ than ERzx– breast tumors (40), no one has examined whether E2 or SERMs regulate miR-21 expression in human breast cancer cells.

In this study, we tested the hypothesis that miR-21, an ‘oncomiR’, is regulated by E2 in MCF-7 breast cancer cells. Although E2 increases proliferation of MCF-7 cells, we found that E2 inhibits miR-21 expression. Experiments were performed to test the effect of E2 on targets of miR-21. In silico analysis identified miR-21 seed elements in six target genes and these miRNA recognition elements (MREs) were cloned into the 3′UTR of a Renilla reporter for subsequent transcriptional evaluation and examination of the effect of antisense to miR-21 on Renilla luciferase. Antisense to miR-21 was used to confirm the importance of miR-21-MRE interaction in response to E2. Importantly, the E2-mediated decrease in miR-21 correlated with increased expression of miR-21-targets PDCD4, PTEN and Bcl-2 at the protein level. These results identify miR-21 as an E2-ER-regulated miRNA in MCF-7 cells.

MATERIALS AND METHODS

Cells and treatments

MCF-7 cells were purchased from ATCC and maintained as previously described (44). 17β-estradiol (E2), 4-hydroxytamoxifen (4-OHT), Actinomycin D (ActD, a transcriptional inhibitor) and cycloheximide (CHX, a protein synthesis inhibitor) were purchased from Sigma; ICI 182 780 (ICI), 4,4′,4′-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT, an ERz-selective agonist) and 2,3-his(4-hydroxyphenyl)-propionitrile (DPN, an ERβ-selective agonist) were purchased from Tocris. Prior to ligand treatment, the medium was replaced with phenol red-free IMEM supplemented with 5% dextran charcoal-stripped FBS (DCC-FBS) for 48 h (serum-starved). Where indicated, MCF-7 cells were pre-treated with 10 μg/ml ActD or 10 μg/ml CHX, for 1 h before ligand treatment. Cells were treated with ethanol (EtOH, the vehicle control) 0.01% final volume, 10 nM E2, 100 nM 4-OHT, 10 nM PPT, or 10 nM DPN, alone or in combination with 100 nM ICI for 6 h. For the indicated experiments, cells were pretreated with 100 nM ICI for 6 h prior to EtOH or E2 treatment.

miRNA microarray

RNA was isolated from MCF-7 cells treated with EtOH or 10 nM E2 for 6 h using the mirVana miRNA Isolation Kit from Ambion (Austin, TX) and was sent to LC Sciences (Houston, TX) (http://lcsiences.com/) where the RNA samples were labeled either with Cy3 or Cy5 and were hybridized with two identical, dual-color miRNA microarray chips (MRA-1001, LC Sciences). The array contains probes to detect mature miRNA sequences as well as pre-miRNAs in the Sanger miRNA registry (http://microrna.sanger.ac.uk/sequences/). Each human miRNA on the chip contains seven redundancies for each sequence to increase sensitivity. Microarray analysis was performed by LCS including background subtraction and data normalization to the statistical median of all detectable transcripts. Two lists of differentially expressed transcripts (based on a P-value < 0.01) from two chips were merged into one list and a statistical correlation between the two sets of data was calculated.

Constructs of miRNA-recognition elements (MREs)

For MRE sequences, synthetic DNA oligonucleotides (~35 bp) containing the MRE sequence (Supplementary Table 1) and ~5 bp adjacent sequences from each end were annealed and ligated into the NotI/XhoI sites located in the 3′UTR region of the pRL-TK Renilla luciferase reporter from Promega. Full-length (FL) 3′-UTRs of PDCD4 and RASA1 were amplified by PCR and inserted into the phRL-TK vector, similarly. All constructs were confirmed by DNA sequencing.

Quantitative real-time PCR (Q-PCR) analysis of miRNA and mRNA expression

miRNA-enriched total RNA was extracted from MCF-7 cells using the mirVana miRNA isolation kit (Ambion). Quantification of miRNAs was performed using TaqMan MicroRNA Assays (Applied Biosystems). U6 RNA was used for normalization of miRNA expression. For analysis of PTEN, PDCD4, BCL2 and TMEM49 mRNA expression, RNA was extracted using Trizol and quantitation was performed using TaqMan primers and probes from ABI using 18S for normalization. Analysis and fold change were determined using the comparative threshold cycle (Ct) method. The change in miRNA or mRNA expression was calculated as fold-change, i.e. relative to EtOH-treated (control).

Western blot

Cells were treated as indicated in individual figure and whole cell extracts (WCE) were prepared in modified RIPA buffer as described (22). Western analysis was performed and quantitated as described (19). Membranes were probed with ERα antibodies AER320 from NeoMarkers or HC-20 from Santa Cruz Biotechnology, ERβ antibody H150 (Santa Cruz Biotechnology), polyclonal PDCD4 antibody from Genetex, monoclonal PTEN antibody from Cell Signaling, or monoclonal Bcl-2 antibody from Assay Designs. Membranes were stripped and re-probed for β-actin (Sigma).
Transient transfection

MCF-7 cells were plated in 24-well plates at a density of 1.5 x 10⁴ cells/well in phenol red-free OPTI-MEM I reduced serum medium (GIBCO/Invitrogen) supplemented with 10% DCC-FBS. Transient transfection was performed using FuGene6 (Roche). For experiments in Figures 2 and 3A, each well received 10 ng of pGL3-pro-luciferase reporter (Promega) as a control and 10 ng of pRL-TK, Renilla luciferase reporter (Promega) containing the indicated MRE or 3′-UTR of miR-21 target genes. For some experiments, cells were also co-transfected with 2′-O-Me-anti-miR-21 [antisense (AS)-miR-21] and the control used was the negative control #1 from Ambion: a random-sequence 2′-O-Me modified RNA molecule that has been extensively tested in many human cell lines and tissues and validated to not produce any identifiable effect on known miRNA function (23). For Figure 3A, MCF-7 cells were transfected with 250 ng of pmir-21s-luc or pmir-21as-luc reporters described in (45) and 5 ng pRL-TK (control). Twenty-four hours after transfection, triplicate wells were treated with EtOH (vehicle control), E₂, 4-OHT- or 100 nM ICI 182 780 as indicated in the figure legend. The cells were harvested 30 h post-treatment using Promega’s Passive Lysis buffer. Luciferase and Renilla luciferase activities were determined using Promega’s Dual Luciferase assay. For Figure 2, Renilla luciferase was normalized by Firefly luciferase to correct for transfection efficiency. For Figure 3A, Firefly luciferase was normalized to Renilla luciferase. Fold induction was determined by dividing the averaged normalized values from each treatment by the EtOH value for each transfection condition within that experiment. Values were averaged from multiple experiments as indicated in the figure legends.

AS-control and AS-miR-21 transfection

MCF-7 cells were transfected with AS- duplexes and control-nonspecific siRNA obtained from Ambion using Lipofectamine RNAiMAX from Invitrogen according to the manufacturer’s protocol. Twenty-four hours post-transfection, the medium was replaced with phenol red-free IMEM with 5% DCC for 48 h and the cells were treated with ethanol (EtOH) vehicle control, 10 nM E₂, 10 nM PPT or 10 nM DPN for 24 h prior. Total RNA was isolated for Q-PCR analysis and WCEs were prepared and stored for 24 h at −80°C until western blot analysis. Each experiment was repeated for a total of three biological replicates. Western blots were quantified as above and the ratio of each protein/β-actin in the AS-control in EtOH-treated samples was set to 1 in each experiment.

ERα and ERβ knockdown by siRNA

MCF-7 cells were transfected with siRNA duplexes and control-nonspecific siRNA obtained from New England Biolabs (44). Forty-eight hours post-transfection, the cells were treated with 10 nM E₂, 10 nM PPT or 10 nM DPN for 6 h for mRNA analysis, or 24 h for protein analysis. Total RNA was isolated for Q-PCR analysis and WCEs were prepared and stored for 24 h at −80°C until western blot analysis.
ER modulator (SERM) agonist/antagonist activity. ICI reduced ERz protein by ∼30–50% in MCF-7 cells (Supplementary Figure 1), but had no effect on basal miR-21 expression (Figure 1). 4-OHT increased miR-21, indicating that 4-OHT opposes E2-induced miR-21 repression through ER binding. Since both 4-OHT and ICI relieved E2 suppression of miR-21, this reduction is ER-mediated.

Although ERz expression is higher than ERb in MCF-7 cells, both ER subtypes are expressed (44). To examine the contributions of ERz and ERb to the E2-induced reduction in miR-21, MCF-7 cells were treated with 10 nM PPT or 10 nM DPN, concentrations at which each is an ERz- or ERb-selective agonist, respectively (50). PPT and DPN, like E2, reduced miR-21 (Figure 1). E2 did not regulate miR-21 expression in ERz+/ERb+ T47D cells (Supplementary Figure 2), indicating cell-line-specific differences, similar to previous reports that E2 responses differ between MCF-7 and T47D cells (51–54). Together, these data indicate that both ERz and ERb contribute to miR-21 repression by E2.

Effect of E2 on miR-21 target gene reporter activity in MCF-7 cells
The biological activity of miRNAs is primarily mediated by interaction with matching recognition sequences in the 3′ UTRs of target genes and reducing translation. A ∼33-bp region from the 3′ UTR centering on the putative miR-21 miRNA regulatory element (miRNA recognition elements (MREs), also called a ‘seed element’, 5′-AT AAGCTA-3′), and minimally 4 bp flanking this sequence from the six genes listed in Supplementary Table 1 were cloned into the 3′ UTR of pRL-TK Renilla reporter plasmid. The pRL-TK-MRE or pRL-TK parental plasmids were transiently transfected into MCF-7 cells with pGL3-pro-luciferase as a control and cells were treated with EtOH or E2 (Figure 2A and B). If E2 reduces miR-21, we would expect an increase in the expression of Renilla but not Firefly luciferase activity since repression would be relieved. Figure 2C shows that E2 specifically increased the expression of the Renilla luciferase protein from the pRL-TK-MRE-bearing plasmid in MCF-7 cells with pGL3-pro-luciferase as a control. In contrast, E2 did not alter luciferase expression from the putative miR-21 MREs in Cerebral Cavernous Malformations 1 (CCM1) or a member of the RAS oncogene family (RAB6C). Thus, the E2-mediated decrease in miR-21 expression (Figure 1) resulted in lower amounts of miR-21 available to bind the MRE sequences from the TGFβ1, PDCD4, RASA1 and RASGRP1 genes, in turn reducing the targeting of these reporter transcripts for degradation/translational inhibition and thus increasing the amount of Renilla protein and luciferase activity. In contrast, the lack of change in Renilla activity from CCM1 and RAB6C indicates that the MREs in these genes do not appear to be targets of E2-induced reduction of miR-21 expression in MCF-7 cells under our assay conditions.

Effect of antisense to miR-21 target gene reporter activity in MCF-7 cells
If the E2-induced increase in Renilla luciferase from the MREs of the TGFβ1, PDCD4, RASA1 and RASGRP1 genes seen in Figure 2C is due to reduced levels of endogenous miR-21, then transfection of MCF-7 cells with antisense (AS)-miR-21 should have the same effect on luciferase activity. MCF-7 cells were transiently transfected with 2′-O-Me-anti-miR-21 (AS-miR-21) (Figure 2D). A 92% knockdown of miR-21 expression was achieved (Figure 5A). AS-miR-21 resulted in a significant increase in Renilla activity from pRL-TK reporters bearing the miR-21 MREs from the TGFβ1, PDCD4, RASA1 and RASGRP1 genes. In contrast, AS-miR-21 did not affect luciferase activity from the putative miR-21 MREs in CCM1 or RAB6C (Figure 2D). These data are in agreement with the E2 responses (Figure 2C), although E2 induced higher activity from the RASA1 reporter compared to the ASmiR-21. Overall, these data indicate that these MREs are bona fide targets of miR-21 regulation.

MRE and FL 3′-UTRs activities of PDCD4 and RASA1 in reporter assays in MCF-7 cells
Since sequences flanking the MRE affect miRNA binding and activity (55), it is important to compare the effect of E2 and AS-miR-21 in reporters bearing the MRE versus the FL 3′UTR of PDCD4 and RASA1 genes (Figure 2E). E2 induced greater luciferase activity from the FL than the PDCD4 MRE. AS-miR-21 increased reporter activity more from the MRE than the FL PDCD4. The AS-miR-21-induced increase in basal luciferase activity was comparable for the MRE and FL RASA1 reporters. AS-miR-21 transfection reduced the fold E2-induction for the MRE and FL PDCD4 and RASA1 reporters. The miR-21 knockdown data are consistent with E2-ER downregulation of miR-21 increasing reporter activity.

Regulation of primary (pri)-miR-21 promoter activity by E2, 4-OHT and ICI 182,780 in MCF-7 cells
miR-21 is located in the 10th intron of the TMEM49 gene (56). To test whether E2 regulates miR-21 gene expression through the ∼1 kb 5′flanking region previously reported to function as a promoter for miR-21 (45), transient transfection assays were performed using two constructs: pmiR-21s-luc and pmiR-21as-luc, corresponding to the sense (s) and antisense (as) orientations of this ∼1 kb region cloned in front of the Firefly luciferase gene (45) (Figure 3A). The activity from the pmiR-21as-luc reporter was ∼2% of that of the pmiR-21s-luc construct, indicating orientation-dependent promoter activity. If E2 represses miR-21 expression by an interaction of ER with the 5′ promoter, we should detect a decrease in luciferase reporter activity. E2 reduced luciferase activity ∼25% whereas 4-OHT increased pmiR-21 activity by ∼25% (Figure 3A). ICI abrogated the inhibition by E2, indicating that ER is responsible for reduction in reporter activity. E2 did not alter TMEM49 transcription (Figure 3B). To our
knowledge, this is the first examination of the effect of E2 on \textit{TMEM49} transcription. These data are consistent with the independent regulation of \textit{TMEM49} and miR-21 in HL-60 cells (56). Overall, these data agree with the direction, although not magnitude, of changes in endogenous miR-21 expression in response to E2, 4-OHT and ICI in MCF-7 cells (Figure 1) and indicate that the -1 kb promoter of miR-21 mediates in part, the observed reduction in miR-21 expression by E2.

**Actinomycin D (ActD) and cycloheximide (CHX) block E2-mediated miR-21 expression**

To determine whether the E2-mediated reduction in miR-21 expression is a direct effect of ER at the genomic level or requires synthesis of a secondary estrogen-responsive protein, MCF-7 cells were pretreated with the transcriptional inhibitor ActD or the protein synthesis inhibitor CHX prior to EtOH or E2.
Different from 10 nM E2, in Figure 2, E2 increased mRNA and protein expression in MCF-7 cells. MCF-7 cells were serum-starved for 48 h and then treated with EtOH, 10 nM E2, 10 nM PPT (ERα selective), or 10 nM DPN (ERβ selective) for 24 h prior to RNA isolation (A) or 24 h prior to WCE preparation (B) as described in 'Materials and methods' section. (A) Q-PCR was performed for the indicated genes and fold-expression determined compared to EtOH as described in 'Materials and Methods' section. Values are the average of four separate determinations ± SEM. (B) Western blot for the indicated proteins. The membrane was stripped and reprobed for β-actin for normalization as described in 'Materials and Methods' section. The blot shown is representative of three separate biological replicates. (C) Western data are presented as relative to non-treated (No TX) MCF-7 cells. The values in C are the mean ± SEM of three separate experiments. *Significantly different from the EtOH value for each protein, P < 0.01.

Effect of E2, PPT and DPN on endogenous miR21 target genes in MCF-7 cells

Since E2 reduced miR-21 expression in MCF-7 cells and increased the expression of miR-21 target reporter gene activity, the effect of E2 on the mRNA and protein levels of endogenous miR-21-target genes PDCD4, PTEN and BCL2 was examined by Q-PCR (Figure 4A) and Western blot (Figure 4B and C). To determine the relative contribution of the two ER subtypes to these effects, MCF-7 cells were treated with 10 nM PPT or 10 nM DPN, concentrations at which each is an ERα or ERβ-selective agonist, respectively (50). As expected based on the reporter assay data for PDCD4 in Figure 2, E2 increased...
mRNA (Figure 4A) and protein (Figure 4B and C) levels of PDCD4, results reflecting reduced miR-21 levels (Figure 1), thus increased transcript stability. Similar results were observed for BCL2. PPT also increased PDCD4 and BCL2 mRNA and protein levels, whereas DPN reduced PDCD4 and increased BCL2 mRNA levels (Figure 4A) while increasing protein amounts (Figure 4B). E2, PPT and DPN increased PTEN protein but not RNA levels (Figure 4A and C), suggesting translational inhibition. Overall, these data indicate roles for both ERα and ERβ in mediating the effects of E2 on miR-21 target gene expression, consistent with results shown in Figure 1.

**AS-miR-21 inhibits endogenous miR-21 target gene protein expression in MCF-7 cells**

To confirm the role of downregulation of miR-21 in the increase in protein expression of Pdcd4, PTEN and Bcl-2, MCF-7 cells were transfected with AS-control and AS-miR-21 plasmids followed by treatment with EtOH, E2, PPT and DPN for 24 h. If the ER-ligand-induced reduction in miR-21 causes an increase in target protein expression, then the AS-miR-21 should have the same effect. AS-miR-21 reduced miR-21 by 92% (Figure 5A). Specific knockdown of miR-21, and not miR-125a or miR-30b, was confirmed by Q-PCR (Figure 5A). AS-miR-21 significantly increased the basal Pdcd4, PTEN and Bcl-2 protein expression (Figure 5B and C). AS-control did not affect the observed increase in each protein in response to E2, PPT and DPN (compare Figures 4B, C and 5B, C). These data indicate that these genes are targets of repression by miR-21. No further increase in protein expression was detected with E2 or PPT treatment, but DPN significantly increased Pdcd4 and PTEN proteins (Figure 5C).

**Effect of ERα knock-down on E2-induced endogenous miR-21 target gene expression in MCF-7 cells**

To confirm the role of ERα in the observed decrease in miR-21 and increase in miR-21-target gene expression in response to E2 and PPT, MCF-7 cells were transfected with siRNA targeting ERα or control siRNA for 48 h and then treated with EtOH, 10 nM E2, PPT, or DPN for 6 h. Transfection of MCF-7 cells with siRNA for ERα reduced ERα mRNA expression by ~62% (Supplementary Figure 3) and ERα protein by 61%. In contrast, ERβ protein levels were unaffected (Figure 6A, see also Supplementary Figure 4). siERα blocked the E2-induced repression of miR-21 (Figure 6B). Concordantly, knockdown of ERα reduced the E2-stimulated expression of miR-21 target genes PDCD4, PTEN and BCL2 (Figure 6C). To confirm these findings at the protein level, western blots were performed using antibodies commercially available for Pdcd4, PTEN and Bcl-2 (Figure 6D). Results confirm that ERα knockdown reduced the E2- and PPT-induced protein expression of the miR-21 target genes PDCD4, PTEN and BCL2 to basal levels (Figure 6E). siERα also reduced DPN-stimulated expression of Pdcd4, PTEN and Bcl-2 proteins suggesting that at least part of the DPN response may be ERα-mediated.

**Effect of ERβ knock-down on miR-21 expression in MCF-7 cells**

To examine ERβ's role in mediating E2-suppression of miR-21 transcription, MCF-7 cells were transfected with siRNA targeting ERβ or control siRNA for 48 h and then treated with EtOH or 10 nM E2 for 6 h. siERβ reduced ERβ mRNA expression by ~70% and protein by 64% (Supplementary Figure 5A and B). Knockdown of ERβ reduced basal miR-21 by 73% and E2 treatment had no
Figure 6. ERα, but not ERβ, knockdown inhibits the E2-mediated decrease in miR-21 and thus reverses miR-21 target gene expression. (A) MCF-7 cells were not transfected (Not TF) or transfected with siControl RNA or siERα as described in ‘Materials and Methods’ section for 48 h and WCE were analyzed for ERα and ERβ by western blot as described in ‘Materials and Methods’ section. The same membrane was stripped and reprobed for β-actin for normalization. The% ERα knockdown was calculated relative to the Not TF control. (B) MCF-7 cells were transfected with siControl RNA or siERα for 48 h prior to treatment with EtOH or 10nM E2, PPT, or DPN for 6 h. RNA and protein were extracted and Q-PCR (B and C) or western blots (D and E) were performed for the indicated miR-21 targets as described in ‘Materials and Methods’ section. The blots shown are representative of three separate biological replicates. The values in (E) are the mean ± SEM of three to four separate experiments. (F) MCF-7 cells were transfected with siControl RNA or siERβ for 48 h prior to treatment with EtOH or 10nM E2 for 6 h. MiR-21 expression is the mean fold change ± SEM of four samples. Values are mean ± SEM. *Significantly different from the EtOH siControl for each protein, P < 0.05. **Significantly different from E2, PPT or DPN siControl value for that protein, P < 0.05.

Further effect (Figure 6F), siERβ resulted in a commensurate increase in basal PDCD4, PTEN and BCL2 mRNA and a loss of E2, DPN and PPT-stimulated PDCD4 and BCL2 transcription (Supplementary Figure 5C and Figure 4). With ERβ knockdown, PPT and DPN increased PTEN mRNA (Supplementary Figure 5C).

DISCUSSION

Since the oncomiR miR-21 was the most significantly up-regulated miRNA in breast tumor biopsies compared to normal breast tissue (37) and because estrogen stimulates breast tumorigenesis, the goal of this study was to determine if E2 regulates the expression of miR-21 in MCF-7 cells as an established human breast cancer model of estrogen responses. To our knowledge, this is the first report that E2 downregulates miR-21 and thus upregulates the protein expression of miR-21 target genes PDCD4, PTEN and BCL2 in MCF-7 human breast cancer cells. Furthermore, the ability of 4-OHT, ICI and siERα to block the E2 repression of miR-21 and the subsequent increase in Pdc4d, Pten and Bcl-2 proteins provide a mechanism for the E2 effect, i.e. through ERα activation. ERβ appears to regulate basal miR-21 expression in MCF-7 cells since knockdown of ERβ reduced miR-21 expression. ERβ represses/opposes ERα transcriptional activity and E2-induced cell proliferation (57–61). Stable transfection of MCF-7 cells with ERβ inhibited xenograft tumor growth, indicating that ERβ is a tumor suppressor (62). We observed that ERβ knock down reduced basal miR-21 and there was no further reduction in miR-21 expression with E2 treatment. These data appear to indicate a relief of repression of ERα’s inhibition of miR-21 transcription. Figure 7 shows a schematic model illustrating ER regulation of miR-21 and miR-21 regulation of its targets. Our results showing that E2 reduces miR-21 expression in MCF-7 are in agreement with recent reports that E2 down-regulated miR-21 in endometrial stromal cells (63) and in the uterus of ovariectomized mice (64).
Figure 7. ER regulates miR-21 expression and its downstream targets in a ligand-dependent manner. E2-ER (ERα and/or ERβ) inhibits miR-21 expression resulting in a loss of repression (indicated by the Xs) of Pdcdf4, PTEN and Bel-2 protein expression. E2-ERα directly increases BCL2 transcription (arrow, +). 4-OHT and ICI block ER-induced inhibition of miR-21 expression. E2-ER also regulates the expression of other miRNAs and mRNAs that, in turn, regulate other cellular pathways which impact the expression of PDCD4, PTEN and BCL2.

At the same time, given the established link between estrogen and breast carcinogenesis (65,66), one might expect E2 to upregulate miR-21 rather than inhibit miR-21 as shown here. Likewise, the increase in miR-21 expression by 4-OHT appears to contradict its anticipated anti-tumor role, but is consistent with 4-OHT's gene-specific SERM activity as indicated by its activity opposing E2's inhibition of miR-21 expression. For complex phenotypes including cell proliferation, genes and proteins are up- and down-regulated by a variety of interacting mechanisms that we are only beginning to understand and integrate. Our data are supported by a recent report showing that miR-21 expression was reduced in TAM-resistant MCF-7 cells (67), a finding likely reflecting the loss of ER-regulated responses in TAM-resistant cells. It is well-established that E2 and 4-OHT regulate transcription in a gene- and cell-specific manner (68–72) and the findings reported here add miR-21 to the list of ER-regulated genes. We conclude that our apparent 'contradictory data' of E2 down-regulating and 4-OHT increasing miR-21 expression add unexpected complexity to understanding of E2 action in breast tumorigenesis.

The reduction of miR-21 expression in response to E2 appears to be mediated, in part, by the -1kb promoter. However, because the reduction in transcription was only ~25% in the reporter assay compared to a ~80% reduction by Q-PCR analysis of miR-21 expression, it is possible that additional regions are also important in regulating miR-21 expression in response to E2. It has been established that E2 increases ERα binding to chromosome regions outside gene promoters (73,74). Analysis of the ERα promoter using TRANSFAC (http://www.gene-regulation.com/) identified a non-consensus ERE with a 2-bp spacer: 5′-AGCTGAgcTGACC-3′ located 883-bp upstream of the TATA-binding site. Previous studies showed no binding of ERα to an ERE with a 2-bp spacer in vitro (75). However, in addition to direct ERE binding, ERα regulates gene transcription by tethering to other transcription factors. Genes repressed by E2-ERα in MCF-7 cells lack EREs and instead have binding sites for Ikaros (IKZF1) and PAX homeobox factors, among others (76), that are also located in the miR-21 promoter. miR-21 is located in the 3′UTR of TMEM49 located at 17q23.1. Using data from Myles Brown’s online database of genomic E2-ERα-binding sites in MCF-7 cells from chromatin immunoprecipitation of ERα on-human genome tiled microarray data (ChIP-on-chip) for human chromosome 17 (73) http://research.dfci.harvard.edu/brownlab/datasets/index.php?dir=ER_MCF7_whole_human_genome/, we found that both E2-ERα and RNA polymerase II binding overlap with the 71-bp miR-21 gene (Supplementary Figure 6). AP-1 was shown to activate miR-21 transcription by direct interaction with three binding sites in the miR-21 promoter in response to PMA treatment of HL-60 cells (56). Although both ERα and ERβ interact with AP-1 to regulate gene expression, the direction of regulation (up or down) varies depending on the ligand, cell type, chromatin context and neighboring transcription factor-binding events (77,78). Here we showed that E2 did not alter TMEM49 transcription which supports previous results that TMEM49 and miR-21 are independently regulated (56). Further studies will be required to analyze the precise mechanisms mediating E2 repression of miR-21.

Both E2 and AS-miR-21 induced RASAI reporter activity; however, the magnitude of luciferase induction was higher with E2 than AS-miR-21. Although normalized relative luciferase between EtOH versus controlAS transfected cells is an unequal comparison, one possible explanation for this difference is that E2 alters the expression of other genes or pathways that selectively impact the RASAI reporter compared to the other reporters, e.g. TGFBI and PDCD4, that show similar luciferase activity.

Our data showing the downregulation of miR-21 by E2 correlated with upregulation of PDCD4 RNA and protein (Figure 4B and C) are in agreement with a report that blocking miR-21 using locked nucleic-acid-modified oligonucleotides increased PDCD4 mRNA and protein in MCF-7 cells (79). Furthermore, our results in the transient transfection assays indicate that miR-21 regulates PDCD4 by an MRE in the 3′UTR. The conclusion that E2 increases PDCD4 expression through inhibition of miR-21 expression in MCF-7 cells is further supported by data showing that AS-miR-21 inhibited E2-induced Renilla luciferase activity from the PDCD4 MRE and 3′-UTR in transfected MCF-7 (Figure 2B) and that AS-miR-21 mimics E2-induction of Pdcdf4 protein (Figure 5C). Our ERα knockdown experiments indicate that ERα is responsible for the E2-mediated inhibition of miR-21 expression and regulation of PDCD4 as well as other miR-21 target genes. The DNPinduced reduction in PDCD4 mRNA aligns with a report that DNPinhibited ERβ shows that the transcription of PPT-activated ERβ target genes in human breast cancer cells (57). The increase seen in Pdcdf4 protein after 24h of DNPtrtreatment may result from a secondary gene effect.

miR-21 functions as an oncogene and modulates tumorigenicity through regulation of Bcl-2 in MCF-7 cells (38). Inhibition of miR-21 expression by AS-miR-21 reduced Bcl-2 protein expression and increased apoptosis in MCF-7 cells in vitro and in tumor xenografts.
We found that E2, PPT and DPN increased PTEN protein examined miR-21 regulation of cells and tumors (35,84) but to our knowledge, no one has previous report showing no alteration of PTEN downregulation of miR-21. With ER this effect is mediated via ER knockdown. Our data contradict a previous report showing no alteration of PTEN expression in MCF-7 cells treated with 100 nM E2 for 24 h (85). This difference may be due to the lower, physiologically relevant E2 concentration and shorter treatment time used here.

In summary, we report for the first time that miR-21 is down-regulated in response to E2 in an ERα-dependent manner and that ERα regulates basal miR-21 expression. Furthermore, this inhibition correlates with up-regulation of miR-21 targets: PDCD4, PTEN and Bcl-2. The identification of miR-21 as a miRNA regulated by ER may open new avenues for potential therapeutic intervention in breast cancer treatment.

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