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# 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 ($E_2$) regulates miR-21 in breast cancer cells. Here we report that $E_2$ inhibits miR-21 expression in MCF-7 human breast cancer cells. The $E_2$-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. $E_2$ increased luciferase activity from reporters containing the miR-21 recognition elements from the 3′-UTRs of miR-21 target genes, corroborating that $E_2$ represses miR-21 expression resulting in a loss of target gene suppression. The $E_2$-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 $E_2$-induced increase in Pdcd4, PTEN and Bcl-2. Transfection of MCF-7 cells with antisense (AS) to miR-21 mimicked the $E_2$-induced increase in Pdcd4, PTEN and Bcl-2. These results are the first to demonstrate that $E_2$ 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, $E_2$ also has 'non-genomic' or 'membrane-initiated' effects, i.e. independent of ER-mediated transcription, that occur within minutes after estradiol ($E_2$), 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

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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 ERα+ than ERα– breast tumors (40), no one has examined whether $E_2$ 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 $E_2$ in MCF-7 breast cancer cells. Although $E_2$ increases proliferation of MCF-7 cells, we found that $E_2$ inhibits miR-21 expression. Experiments were performed to test the effect of $E_2$ 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 $E_2$. Importantly, the $E_2$-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 $E_2$-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 ($E_2$), 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 ERα-selective agonist) and 2,3-*bis*(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 $E_2$, 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 $E_2$ treatment.

### miRNA microarray

RNA was isolated from MCF-7 cells treated with EtOH or 10 nM $E_2$ for 6 h using the mirVana miRNA Isolation Kit from Ambion (Austin, TX) and was sent to LC Sciences (Houston, TX) (http://lcsciences.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 *Not*I/*Xho*I 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 *mir*Vana 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 $1.5 \times 10^4$ 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), $E_2$, 4-OHT or 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 $E_2$, 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 $E_2$, 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.

### Statistics

Statistical analyses were performed using Student's *t*-test or one-way ANOVA followed by Student–Newman–Keuls or Dunnett's post-hoc tests using GraphPad Prism (San Diego, CA).

## RESULTS

### $E_2$ regulates miR-21 expression in MCF-7 breast cancer cells

Estrogens promote breast tumor development by increasing transcription of protooncogenes and growth factors (46) and by negatively modulating the expression or functional activity of tumor suppressors (47). To determine the identity of primary $E_2$-regulated miRNAs in estrogen-responsive human breast cancer cells, ERα-positive MCF-7 human breast cancer cells were treated with 10 nM $E_2$ or EtOH (vehicle control) for 6 h. Among the $E_2$-down-regulated miRNAs, we selected miR-21 for further evaluation because miR-21 is an oncomiR and its expression is higher in ERα positive versus negative tumors (40). Furthermore, no one has examined if $E_2$ regulates miR-21 expression in breast cancer cells. Q-PCR using the TaqMan primer/probe sets from ABI indicated a ~60% reduction in mature miR-21 by $E_2$ (Figure 1). To determine the mechanism by which $E_2$ reduces miR-21, MCF-7 cells were pre-incubated with 100 nM ICI 182 780 (ICI, Faslodex), a pure antagonist of ER genomic action (48,49), or 100 nM 4-OHT, the active metabolite of the antiestrogen tamoxifen, and then treated with $E_2$. The effect of 4-OHT or ICI alone was also examined. If $E_2$ represses miR-21 expression by binding ER, then ICI should block the decrease. Because 4-OHT has mixed ER agonist/antagonist activity in a gene- and cell-specific manner, its effect on miR-21 expression could either mimic or oppose the $E_2$ effect, reflecting its selective

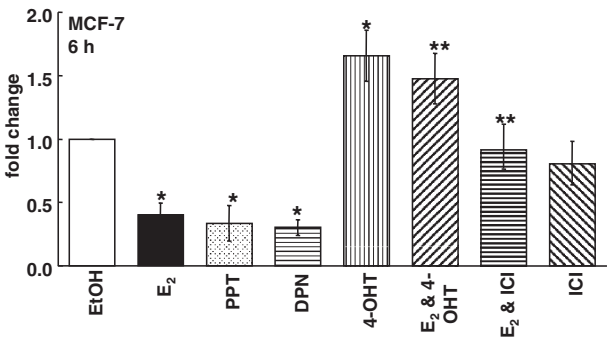


**Figure 1.** $E_2$ inhibits miR-21 expression. Summary of Q-PCR data on (mature) miR-21 expression. MCF-7 cells were treated with EtOH, 10 nM $E_2$, 10 nM PPT (ERα-selective), or 10 nM DPN (ERβ-selective) for 6 h. as indicated by the different fills. Where indicated MCF-7 cells were pretreated with 100 nM ICI 182 780 [ICI, an ER antagonist termed a 'selective ER disrupter' (SERD)] or 100 nM 4-OHT for 6 h and then ethanol or 10 nM $E_2$ was added for an additional 6 h. Values are fold increase compared to EtOH for each miRNA and were calculated as described in 'Materials and Methods' section. Values are the average of three to eight separate experiments ± SEM. *Significantly different from the EtOH control, $P < 0.05$. **Significantly different from $E_2$, $P < 0.05$.

ER modulator (SERM) agonist/antagonist activity. ICI reduced ERα 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 $E_2$-induced miR-21 repression through ER binding. Since both 4-OHT and ICI relieved $E_2$ suppression of miR-21, this reduction is ER-mediated.

Although ERα expression is higher than ERβ in MCF-7 cells, both ER subtypes are expressed (44). To examine the contributions of ERα and ERβ to the $E_2$-induced reduction in miR-21, 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). PPT and DPN, like $E_2$, reduced miR-21 (Figure 1). $E_2$ did not regulate miR-21 expression in ERα+/ERβ+ T47D cells (Supplementary Figure 2), indicating cell-line-specific differences, similar to previous reports that $E_2$ responses differ between MCF-7 and T47D cells (51–54). Together, these data indicate that both ERα and ERβ contribute to miR-21 repression by $E_2$.

### Effect of $E_2$ 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′-ATAAGCTA-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 $E_2$ (Figure 2A and B). If $E_2$ 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 $E_2$ specifically increased the expression of the *Renilla* luciferase protein from the pRL-TK- transforming growth factor β 1 *(TGFB1)*, Programmed Cell Death 4 (*PDCD4)*, RAS p21 Protein Activator 1 (*RASA1)* and RAS Guanyl Nucleotide-Releasing Protein 1 (*RASGRP1)* reporters in MCF-7 cells, data consistent with miR-21 downregulation by $E_2$. In contrast, $E_2$ 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 $E_2$-mediated decrease in miR-21 expression (Figure 1) resulted in lower amounts of miR-21 available to bind the MRE sequences from the *TGFB1*, *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 $E_2$-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 $E_2$-induced increase in *Renilla* luciferase from the MREs of the *TGFB1*, *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 *TGFB1*, *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 $E_2$ responses (Figure 2C), although $E_2$ induced higher activity from the *RASA1* reporter compared to the ASmiR-21. Overall, these data indicate that these MREs are bone 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 $E_2$ and AS-miR-21 in reporters bearing the MRE versus the FL 3′UTR of *PDCD4* and *RASA1* genes (Figure 2E). $E_2$ 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-mR-21 transfection reduced the fold $E_2$-induction for the MRE and FL *PDCD4* and *RASA1* reporters. The miR-21 knockdown data are consistent with $E_2$-ER downregulation of miR-21 increasing reporter activity.

### Regulation of primary (pri)-miR-21 promoter activity by $E_2$, 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 $E_2$ 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 $E_2$ represses miR-21 expression by an interaction of ER with the 5′ promoter, we should detect a decrease in luciferase reporter activity. $E_2$ reduced luciferase activity ~25% whereas 4-OHT increased pmiR-21 activity by ~25% (Figure 3A). ICI abrogated the inhibition by $E_2$, indicating that ER is responsible for reduction in reporter activity. $E_2$ did not alter *TMEM49* transcription (Figure 3B). To our

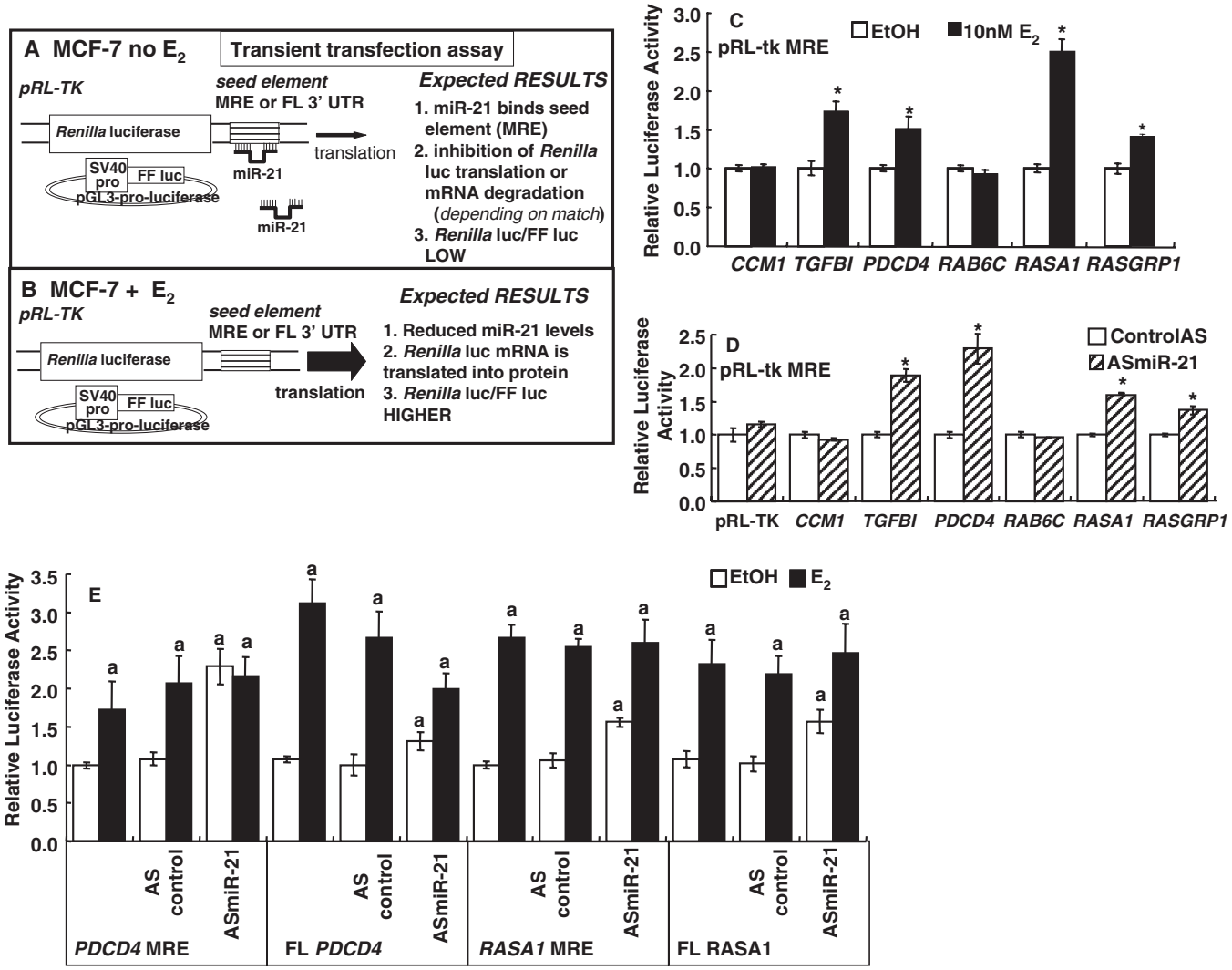


**Figure 2.** Luciferase reporter assay of putative miR-21 target genes and the effect of antisense (AS) to miR-21 on reporter activity. (**A**) Model of transient transfection assays in MCF-7 cells. MCF-7 cells were transiently transfected with pGL3-pro-luciferase and pRL-TK parental or pRL-TK containing putative miR-21 MREs from target genes (Supplementary Table 1) cloned in the 3′UTR as described in 'Materials and methods' section. Expected results are indicated without $E_2$ (**A**) and when cells are treated with $E_2$ (**B**). (**C**) MCF-7 cells were transfected as indicated and treated with EtOH or 10 nM $E_2$ for 24 h. *Renilla* luciferase was normalized by firefly luciferase to correct for transfection efficiency. Values are the average ± SEM of triplicate determinations. *Significantly different from EtOH control, $P < 0.01$. (**D**) MCF-7 cells were transfected with 2′-O-Me-antisense-miR-21 (ASmiR-21). *Renilla* luciferase reporter gene expression from the indicated gene MREs was determined and data analyzed as described in 'Materials and Methods' section. The control was a random-sequence 2′-O-Me modified RNA (control AS) as described in Materials and methods section. Values are the average ± SEM of triplicate determinations. *Significantly different from control AS, $P < 0.05$. (**E**) MCF-7 cells were transfected with the pRL-tk-MREs or FL 3′-UTRs as indicated. Indicated cells were co-transfected with ASmiR-21 or a control AS. Cells were treated with EtOH or 10 nM $E_2$ as indicated for 24 h. Dual luciferase reporter assays were performed and data quantitated as described in 'Materials and Methods' section. Values are the average ± SEM of triplicate determinations normalized to EtOH for each construct except that cells transfected with the ASmiR-21 were normalized against the control AS-EtOH value. [a]Significantly different from EtOH control, $P < 0.01$. [b]Significantly different from control AS transfected values, $P < 0.01$.

knowledge, this is the first examination of the effect of $E_2$ on *TMEM49* transcription. These data are consistent with the independent regulation of *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 $E_2$, 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 $E_2$.

### Actinomycin D (ActD) and cycloheximide (CHX) block $E_2$-mediated miR-21 expression

To determine whether the $E_2$-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 $E_2$

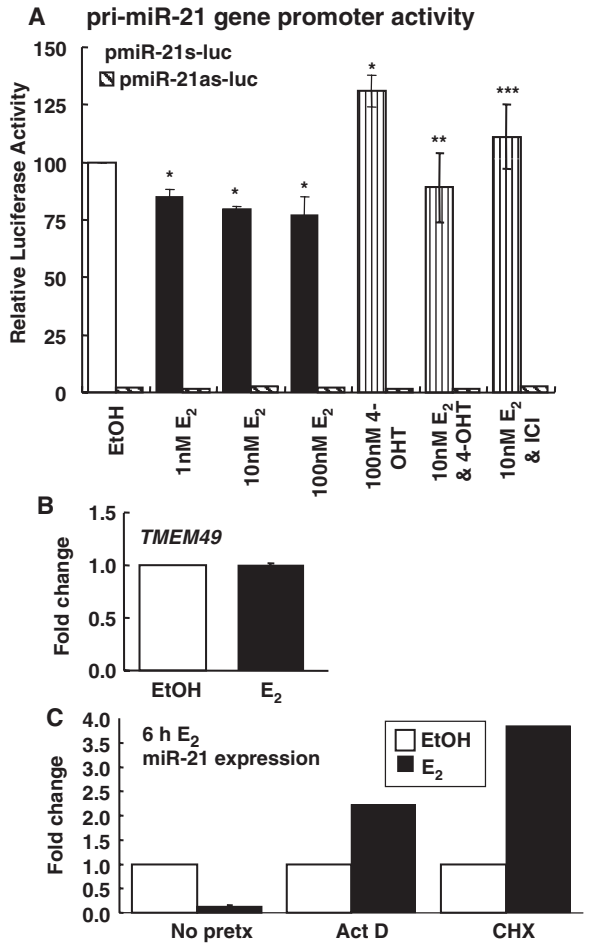


**Figure 3.** Regulation of miR-21 transcription in MCF-7 cells. (**A**) Effects of $E_2$, 4-OHT and ICI 182 780 (ICI) on the primary miR-21 (pri-miR-21) gene promoter in the sense (pmiR-21s-luc) or antisense (as) pmiR-21as-luc orientation. MCF-7 cells were transfected with pri-miR-21s-luc or pri-miR-21as-luc (hatched bars, values were very low) (45) and *Renilla* luciferase as an internal control. Cells were treated with the indicated concentrations of $E_2$, 4-OHT, or ICI for 24 h. Dual luciferase assays were performed and luciferase values were divided by *Renilla* values in the same sample. Values are the average ± SEM of triplicate determinations normalized to EtOH for the pmiR-21s-luc construct. *Significantly different from EtOH control, $P < 0.05$. **Significantly different from 4-OHT alone, $P < 0.05$. ***Significantly different from 10 nM $E_2$, $P < 0.05$. (**B**) $E_2$ does not affect *TMEM49* transcription in MCF-7 cells. miR-21 is encoded within the 10th intron of the *TMEM49* gene (56). MCF-7 cells were treated with EtOH or 10 nM $E_2$ for 6 h, total RNA was reverse transcribed and Q–PCR was performed. *TMEM49* was normalized to 18S. Values are the average ± SEM of triplicate determinations normalized to EtOH. (**C**) The $E_2$-induced decrease in miR-21 expression in MCF-7 cells is mediated in a primary transcriptional/genomic and secondary estrogen-target-dependent manner. MCF-7 cells were pre-treated with stripped medium or stripped medium containing 10 μg/ml ActD or CHX for 1 h before treatment with vehicle control (EtOH), or 10 nM $E_2$ for 6 h as described in 'Materials and Methods' section. miR-21 expression was determined using Q-PCR as described in 'Materials and Methods' section. The bar graph summarizes the fold change in miR-21 expression relative to no pretreatment (No pretx)-EtOH-treated cells.

treatment (Figure 3C). Pretreatment with ActD and CHX blocked $E_2$-mediated miR-21 repression, indicating that $E_2$-repression is mediated by both transcriptional (primary genomic) and secondary mechanisms.

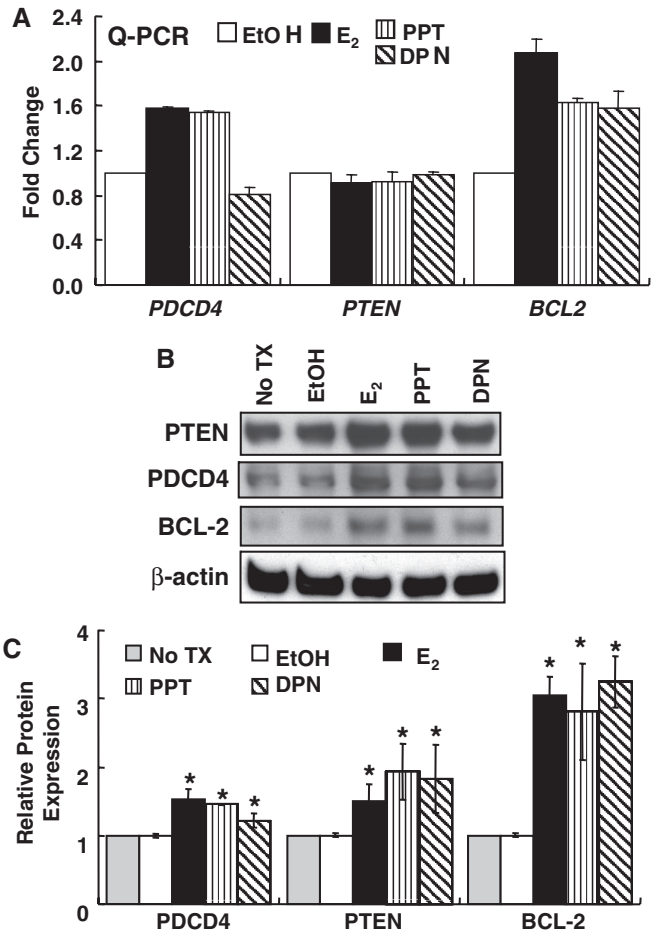


**Figure 4.** Effect of ER ligands on endogenous miR21 target gene mRNA and protein expression in MCF-7 cells. MCF-7 cells were serum-starved for 48 h and then treated with EtOH, 10 nM $E_2$, 10 nM PPT (ERα selective), or 10 nM DPN (ERβ selective) for 6 h prior to RNA isolation (A) or 24 h prior to WCE 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 $E_2$, PPT and DPN on endogenous miR-21 target genes in MCF-7 cells

Since $E_2$ reduced miR-21 expression in MCF-7 cells and increased the expression of miR-21 target reporter gene activity, the effect of $E_2$ 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, $E_2$ 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). $E_2$, 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 $E_2$ 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, $E_2$, 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 $E_2$, 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 $E_2$ or PPT treatment, but DPN significantly increased Pdcd4 and PTEN proteins (Figure 5C).

### Effect of ERα knock-down on $E_2$-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 $E_2$ and PPT, MCF-7 cells were transfected with siRNA targeting ERα or control siRNA for 48 h and then treated with EtOH, 10 nM $E_2$, 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 $E_2$-induced repression of miR-21 (Figure 6B). Concordantly, knockdown of ERα reduced the $E_2$-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 $E_2$- 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

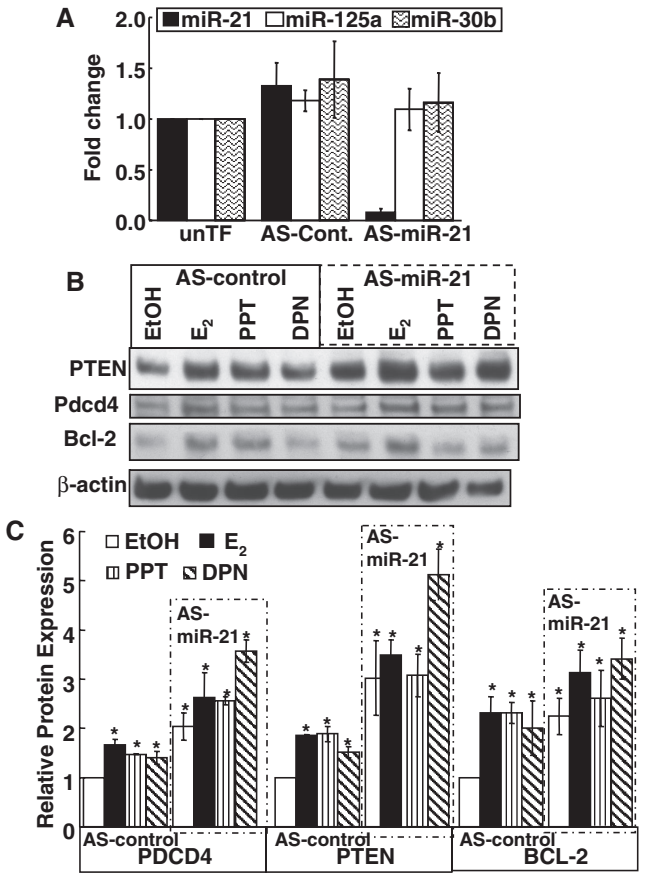


**Figure 5.** AS-miR-21 increases the expression of PTEN, PDCD4 and Bcl-2. (**A**) The specificity of AS-miR-21 to decrease miR-21was examined by Q-PCR in parallel with miR-125a and miR-30b as negative controls. MCF-7 cells were not transfected (unTF) or were transfected with AS-control or AS-miR-21 for 48 h prior to RNA harvest. Q-PCR was performed for the indicated miRs. The values are the average of three separate experiments, each run in triplicate, ± SD. (**B**) MCF-7 cells were transfected with AS-control or AS-miR-21 for 24 h prior to serum deprivation for 48 h and then 24 h treatment with EtOH, 10 nM $E_2$, PPT or DPN, as indicated. WCE were used for western blot for the indicated proteins as described in 'Materials and methods' section. 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**) The values graphed are the mean ± SEM of the normalized western data (each protein was normalized to β-actin input and then the ratio of each protein/β-actin in the AS-control in EtOH-treated samples was set to one in each experiment) in three separate experiments. *Significantly different from the EtOH AS-control for each protein, $P < 0.05$.

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 $E_2$-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 $E_2$ 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 $E_2$ treatment had no

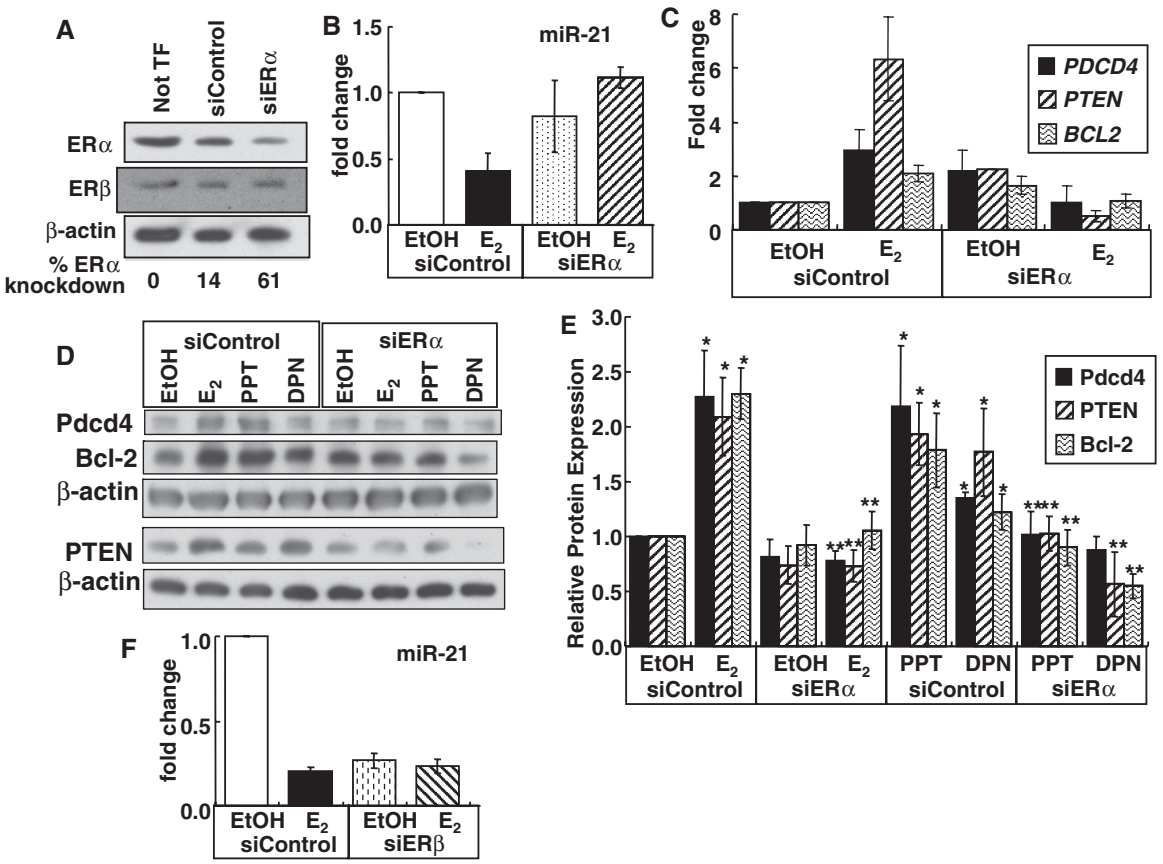


**Figure 6.** ERα, but not ERβ, knockdown inhibits the $E_2$-mediated decrease in miR-21 and thus reverses miR-21 target gene expression. (**A**) MCF-7 cells were not transfected (Not TF) or transfected with siControl RNA or siERα as described in 'Materials and Methods' section for 48 h and WCE were 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 10 nM $E_2$, 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 10 nM $E_2$ 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 $E_2$, 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 $E_2$, 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 $E_2$ 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 $E_2$ 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 $E_2$ repression of miR-21 and the subsequent increase in Pdcd4, Pten and Bcl-2 proteins provide a mechanism for the $E_2$ 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 $E_2$-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 $E_2$ 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 $E_2$ reduces miR-21 expression in MCF-7 are in agreement with recent reports that $E_2$ down-regulated miR-21 in endometrial stromal cells (63) and in the uterus of ovariectomized mice (64).

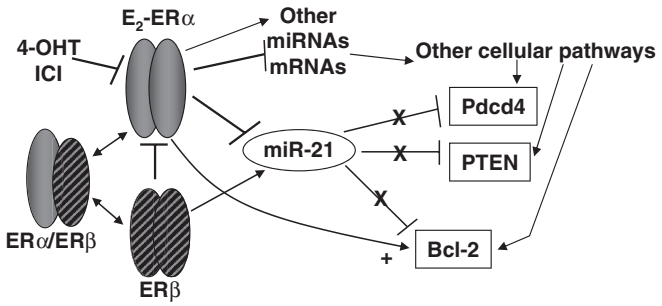


**Figure 7.** ER regulates miR-21 expression and its downstream targets in a ligand-dependent manner. $E_2$-ER (ERα and/or ERβ) inhibits miR-21 expression resulting in a loss of repression (indicated by the Xs) of Pdcd4, PTEN and Bcl-2 protein expression. $E_2$-ERα directly increases *BCL2* transcription (arrow, +). 4-OHT and ICI block ER-induced inhibition of miR-21 expression. $E_2$-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 $E_2$ 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 $E_2$'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 $E_2$ 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 $E_2$ down-regulating and 4-OHT increasing miR-21 expression add unexpected complexity to understanding of $E_2$ action in breast tumorigenesis.

The reduction of miR-21 expression in response to $E_2$ 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 $E_2$. It has been established that $E_2$ increases ERα binding to chromosome regions outside gene promoters (73,74). Analysis of the miR-21 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 $E_2$-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 $E_2$-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 $E_2$-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 $E_2$ 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 $E_2$ repression of miR-21.

Both $E_2$ and AS-miR-21 induced *RASA1* reporter activity; however, the magnitude of luciferase induction was higher with $E_2$ 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 $E_2$ alters the expression of other genes or pathways that selectively impact the *RASA1* reporter compared to the other reporters, e.g. *TGFB1* and *PDCD4*, that show similar luciferase activity.

Our data showing the downregulation of miR-21 by $E_2$ 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 $E_2$-increases *PDCD4* expression through inhibition of miR-21 expression in MCF-7 cells is further supported by data showing that AS-miR-21 inhibited $E_2$-induced *Renilla* luciferase activity from the *PDCD4* MRE and 3′-UTR in transfected MCF-7 (Figure 2B) and that AS-miR-21 mimics $E_2$-induction of Pdcd4 protein (Figure 5C). Our ERα knockdown experiments indicate that ERα is responsible for the $E_2$-mediated inhibition of miR-21 expression and regulation of *PDCD4* as well as other miR-21 target genes. The DPN- induced reduction in *PDCD4* mRNA aligns with a report that DPN-activated ERβ inhibits the transcription of PPT-activated ERα target genes in human breast cancer cells (57). The increase seen in Pdcd4 protein after 24 h of DPN treatment 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

in mice (38). Consistent with these findings, our data demonstrate that both $E_2$ and PPT decrease miR-21 and increase *BCL2* mRNA and protein expression in MCF-7 cells. *BCL2* expression has long been considered a good prognostic marker in breast cancer (80). DPN increased *BCL2* mRNA and protein expression; likely by ERα activation because $E_2$ regulates *BCL2* transcription in MCF-7 cells via ERα- Sp1 and AP1 interactions (81), we can not conclude that the increase in *BCL2* mRNA is due solely to $E_2$-mediated decreased miR-21. Further studies will be needed to dissect the relative contributions of multiple ERα-mediated pathways controlling *BCL2* gene expression.

*PTEN* is an important tumor suppressor (82) that has been identified as a breast cancer susceptibility gene (83). miR-21 regulates *PTEN* in human hepatocellular cancer cells and tumors (35,84) but to our knowledge, no one has examined miR-21 regulation of *PTEN* in breast cancer. We found that $E_2$, PPT and DPN increased PTEN protein levels without affecting PTEN transcript levels (Figure 4), indicating translational inhibition. Knockdown of ERα by siRNA blocked the $E_2$-mediated downregulation of miR-21 and the $E_2$-induced increase in *PTEN*, indicating that this effect is mediated via ERα, and commensurate with downregulation of miR-21. With ERβ knockdown, PPT and DPN increased *PTEN* mRNA; however, because $E_2$, PPT and DPN did not regulate *PTEN* mRNA in MCF-7 cells, it is likely that this increase is mediated by the loss of the expression of another *PTEN* transcriptional repressor with ERβ knockdown. Our data contradict a previous report showing no alteration of *PTEN* expression in MCF-7 cells treated with 100 nM $E_2$ for 24 h (85). This difference may be due to the lower, physiologically relevant $E_2$ concentration and shorter treatment time used here.

In summary, we report for the first time that miR-21 is down-regulated in response to $E_2$ in an ERα-dependent manner and that ERβ 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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*Conflict of interest statement*. None declared.

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