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Estradiol-activated estrogen receptor α does not regulate mature microRNAs in T47D breast cancer cells

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ABSTRACT

Breast cancers are sensitive to hormones such as estrogen, which binds to and activates estrogen receptors (ER) leading to significant changes in gene expression. microRNAs (miRNA) have emerged as a major player in gene regulation, thus identification of miRNAs associated with normal or disrupted estrogen signaling is critical to enhancing our understanding of the diagnosis and prognosis of breast cancer. We have previously shown that 17β-estradiol (E2) induced activation of ERα in T47D cells results in significant changes in the expression of protein-coding genes involved in cell cycle, proliferation, and apoptosis. To identify miRNAs regulated by E2-activated ERα, we analysed their expression in T47D cells following E2-activation using both dual-color microarrays and TaqMan Low Density Arrays, and validations were carried out by real-time PCR. Although estrogen treatment results in altered expression of up to 900 protein-coding transcripts, no significant changes in mature miRNA expression levels could be confirmed. Whereas previous studies aiming to elucidate the role of miRNA in ER-positive breast cancers cell lines have yielded conflicting results, the work presented here represents a thorough investigation of and significant step forward in our understanding of ERα mediated miRNA regulation.

1. Introduction

Estrogen has a significant impact on the risk, development, maintenance, treatment and prognosis of breast cancer. The majority of breast tumors are dependent on estrogen for proliferation, hence therapeutic intervention typically consists of small molecule selective estrogen receptor modulators such as tamoxifen and raloxifene [1]. Estrogen exerts its function by binding to estrogen receptors (ER), which in turn bind to their cognate DNA sequences (EREs), leading to the recruitment of co-activators and chromatin remodeling complexes that destabilize chromatin structure by way of chromatin modifications, ultimately resulting in up- or downregulation of specific target genes. In addition to its genomic activity, estrogen has "nongenomic" or "membrane-initiated" effects (which are independent of the genomic effect) such as interaction with G-proteins, the p85 subunit of P13K, c-Src, and Cav-1 to initiate P13K/AKT and MAPK signaling cascades [2]. The estrogen receptor α (ERα) is essential for estrogen-dependent growth in breast cancer. Many genes associated with the control of cell cycle, proliferation, and apoptosis are regulated by ERα, which acts as the response determinant to endocrine therapy and prognosis in ERα-positive breast cancer [3,4].

microRNAs (miRNAs) are non-protein coding transcripts which have emerged as major gene regulators, controlling expression by blocking mRNA translation or targeting mRNA transcripts for degradation, by binding to the 3′-untranslated region (3′ UTR) of the target mRNAs located in the cytoplasm. miRNA encoding genes are transcribed by RNA polymerase II, processed by Drosha into short hairpin RNAs, which are then exported from the nucleus to the cytoplasm, and processed by Dicer to form mature 21–25 nucleotide miRNAs, which are finally transferred to Argonaute proteins to form RISC complex [5,6]. ER-positive breast cancers display a distinct expression profile featuring elevated expression of both let-7 and miR-21 miRNA family members compared to ER-negative breast cancers [7,8]. More targeted studies have reported that miR-221/222 can induce tamoxifen resistance through downregulation of ERα [9]. Thus, changes in miRNA expression correlate with diagnostic and prognostic markers used in breast cancer therapy.

Despite these findings, few studies have addressed the direct hormonal regulation of miRNA expression [2], and nearly all have used the ERα-positive MCF-7 breast cancer cell line as a model. Moreover, these have yielded conflicting results; one study

Abbreviations: miRNA, microRNA; E2-ERα, estrogen-activated ERα; TLDA, TaqMan Low-Density Array; E2, 17β-Estradiol; ERE, estrogen response elements.

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investigating the role of miR-21 in estrogen signaling found it to be downregulated in estradiol (E2)-activated ERα in MCF-7 breast cancer cells [5], while another showed the same miRNA being upregulated by hormone treatment in the same cell line [7]. These differences may be a consequence of differences in duration of estrogen treatment (6 h and 4 h, respectively), assay methods employed (TaqMan and SYBR Green Technology, respectively) or variations within the cell line (from culturing cells overtime).

Our group has previously performed a genome-wide study of the transcriptional effects of the estrogen receptors in T47D breast cancer cells [10]. We have shown that there is a significant change in transcript levels of a large number of genes following 24 h estrogen activation of ERα. Among the transcripts identified, genes involved in processes such as proliferation, apoptosis, cell signaling, development, and ion transport were overrepresented. miRNAs have been reported to be involved in similar biological processes [11], and we speculated that these function may be in part through ERα regulated miRNAs.

In this study, we performed a thorough analysis of mature miRNAs regulated by 24 h E2 activated ERα in T47D breast cancer cells. We used both dual-color microarray technique and TaqMan Low Density Arrays (TLDA). Microarray expression profiles were confirmed by real-time PCR using both SYBR green and TaqMan chemistry. Comparisons to previous work on regulated miRNAs were performed to validate results. Our results illustrate the inherent ambiguity and complexity of miRNA profiling, and provide valuable insights into the study area of miRNA regulation in these cells.

2. Materials and methods

2.1. Cell culture

T47D and MCF-7 cells were cultured in Dulbecco's modified Eagle's medium in proportion with F12 [DMEM/F12 (1:1)] supplemented with 5% FBS and 1% Penicillin Streptomycin (Invitrogen, Carlsbad, CA). The cells were synchronized by changing the medium to phenol red-free DMEM/F12 (1:1) medium supplemented with 5% dextran-coated charcoal-treated FBS (DCC) for 24 h. The serum was then reduced to 0.5% DCC for 48 h. The cultured cells were treated with either 10 nM of ER ligand 17β-estradiol (E2), vehicle (100% ethanol), or 10 nM ICI for 0–72 h. MCF10A cells were cultured in DMEM/F12 (1:1) supplemented with 5% FBS, 0.5 mg/ml Hydrocortisone, 20 ng/ml EGF, 10 g/ml Insulin, 2 nM D/L-Glutamine, and 1% Penicillin Streptomycin. MDA-MB-231 cells were cultured in DMEM/F12 (1:1) supplemented with 10% FBS, and 1% Penicillin Streptomycin. MCF10A, T47D, MCF-7, and MDA-MB-231 cells grown in complete FBS serum were used as positive control for miRNA expression levels. All cells were harvested in TRIzol (Invitrogen) for RNA extraction.

2.2. RNA extraction

RNA was extracted and purified using the miRNeasy Mini Kit (Qiagen, Valencia, CA) with DNase I DNA degradation according to manufacturer's protocol. RNA concentrations were measured using Nanodrop 1000 Spectrophotometer (Thermoscientific, Pittsburgh, PA) and RNA integrity was measured using Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA).

2.3. cDNA synthesis

For mRNA analysis: 1 μg of the extracted total RNA was used for each cDNA synthesis reaction. First-Strand cDNA was synthesized using SuperScript III and random hexamer primers (Invitrogen). A final concentration of 5 ng/μl of cDNA was prepared from stock for use in qPCR. For miRNA analysis: 1 μg of total RNA was used for each polyA tailing and First-Strand cDNA synthesis of miRNA using the NCode™ miRNA First-Strand cDNA Synthesis and qRT-PCR Kit (Invitrogen) according to manufacturer's protocol. For TaqMan miRNA assays: reverse transcription of 10 ng of total RNA was performed according to manufacturer's protocol using the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA).

2.4. miRNA microarray analysis

5 μg of isolated RNA from T47D cells treated with either control (EtOH) or 10 nM E2 for 24 h were sent to LC Sciences (Houston, TX) where microarray expression profiles were determined using human miRNA microarray dual color sample array by μParaflo® Microfluidic Biochip Technology (Sanger miRBase Release 14.0). The miRNA microarray contained 894 mature miRNA and 50 controls unique probes in quadruplets. Hybridizations were performed in duplicates with dye swap procedure. Differentially expressed miRNAs were considered significant when P-value < 0.10. Additional expression profiles for 381 miRNAs including U6 snRNA in quadruplets were additionally determined using TaqMan Low Density Array (TLDA) (Applied Biosystems, Foster City, CA) using TaqMan Human microRNA A cards v2.0 according to protocol using the 7900HT Fast Real-Time PCR system (Applied Biosystems). A starting total RNA amount of 700 ng was used, and this assay was performed in duplicate.

2.5. qPCR analysis of mRNA and miRNA expression

For mRNA expressions: 10 ng cDNA (from above), 1 pmol of each of the forward and reverse primers, and SYBR green PCR master mix in a 10 μl final reaction volume. ARHGDIA and 18S RNA were used for normalization of mRNA expression. For miRNA expressions: 16 ng of poly A cDNA (from above), 2 pmol of each of the specific forward primer and the universal primer, and SYBR green PCR master mix in a 10 μl final reaction volume. Expression of miRNA was additionally quantified using TaqMan microRNA assays, according to manufacturer's protocol (Applied Biosystems). U6 snRNA was used for normalization of miRNA expression for each method. snRNA U6 and miRNA basal expressions for each stage of serum starvation for T47D and MCF-7 cell lines, and snRNA U6 basal expression for each cell type are included in supplemental figures (Figs. S1–S3, respectively). All runs were performed using the 7500 Fast Real-Time PCR System (Applied Biosystems). Analysis and relative expressions were determined using comparative threshold cycle ($\Delta\Delta C_t$) method. The change in mRNA or miRNA expression was calculated as fold change relative to control (EtOH-treated). Melting curve analysis was performed for all SYBR green runs, confirming the amplification of one specific fragment. Student's t-test was used for statistical analysis, using two-tailed distribution and two-sample unequal variance parameters ($^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$).

2.6. Bioinformatics

For chromatin binding comparisons, the University of California Santa Cruz (UCSC) Genome Bioinformatics website, http://genome.ucsc.edu, was used to observe the relative distances between the possible miRNA promoter and ER binding sites, and miRNA and c-MYC binding sites using the coordinates from: ERα [12–14] and c-MYC [13] ChIP data. The following custom tracks were used: Carroll et al. (ERα: 10,599 tracks applied in hg17 format), Hua et al. (ERα: 1615 tracks applied in hg16 format, and c-MYC: 311 tracks applied in hg16 format), and Cicatiello et al. (ERα: 3561 tracks applied in hg18 format). miRNA coordinates were taken from miRBase

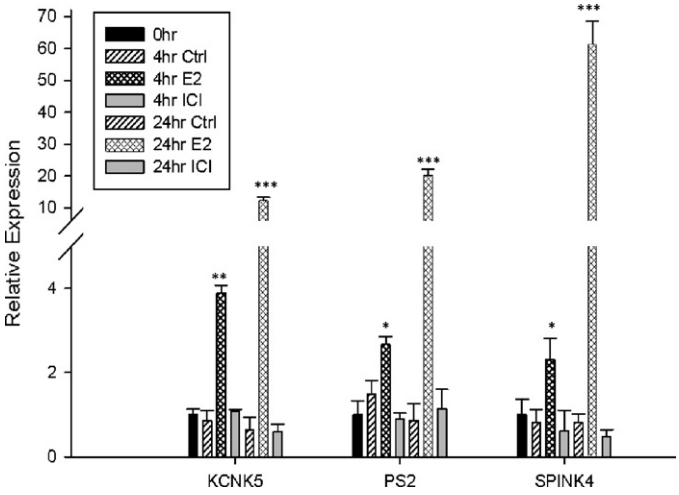

Fig. 1. Expression of ERα-regulated genes at varying time points. qPCR results showing E2-activated expression of KCNK5, PS2, and SPINK4. T47D parental cells were treated with EtOH, 10 nM E2, and 10 nM ICI for 4 h and 24 h. Expressions of these genes were normalized to the expression of ARHGDIA (reference gene). Student *t* test: $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$. Values are the average of three separate experiments ±SD.

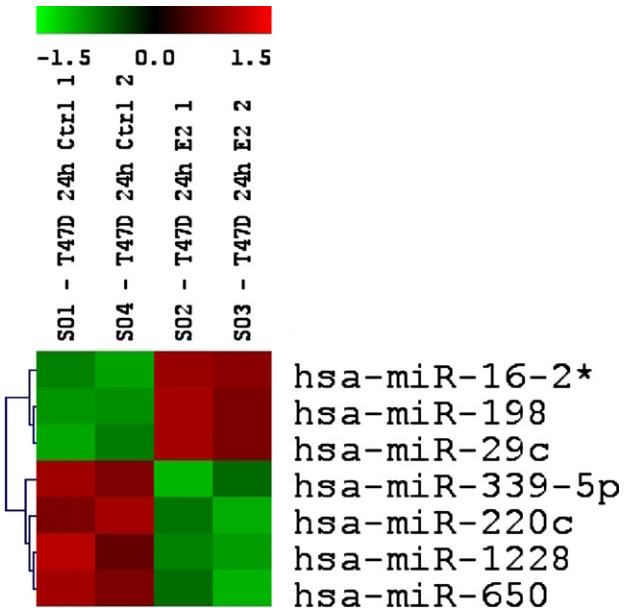

Fig. 2. miRNA profiling of E2-activated ERα in T47D cells. Microarray analysis of 24 h E2-activated ERα in T47D cells from LCSciences showing the seven most significantly regulated miRNA. All have *P*-value < 0.10. EtOH treated sample was used as a control. Analysis was done in duplicates.

(http://www.mirbase.org): the miRNA database in hg19 format and converted to the necessary usable format using UCSC LiftOver tool.

3. Results

3.1. Strong ERα mediated transcription of protein coding genes occurs at 24 h in T47D cells

We previously performed a gene expression analysis of 24 h E2-activated ERα (E2-ERα) in T47D cells and found significant changes in genes associated with proliferation, cell-cycle, and apoptosis [10]. In order to investigate the effect of various exposure times on ERα gene regulation, T47D cells were treated with a control (EtOH), 10 nM 17β-estradiol (E2) and 10 nM ICI for 0–24 h. ICI is a high-affinity ER ligand that antagonizes most estrogen-mediated action by ERα inhibition and degradation. The pS2 (TFF1), KCNK5, and SPINK4 genes, which have been shown to be regulated by ERα [10], were used to confirm ERα transcriptional activation by qPCR. These are involved in a range of cellular processes: PS2 is similar in function to some growth factors, KCNK5 is a potassium ion channel involved in breast cancer proliferation [15], and SPINK4 is a serine peptidase inhibitor. PS2 has a classical ERE in its promoter sequence, and KCNK5 has a binding site for ERα and is directly regulated through ERα DNA binding [15]. We expected that 24 h treatment would result in a marked response of these genes, and that regulation would occur solely through ERα. As shown in Fig. 1, significant differences were observed between time points. Whereas the KCNK5 gene had its maximum response at 1 h [15], all three genes showed a strong and significant response at 24 h; at this time point, genes were shown to be upregulated between 10- and 57-fold. ICI induced ERα depletion resulted in inhibition of expression, demonstrating the key role of this receptor in their regulation. Since the research objective was to measure mature miRNAs, a 24 h time point was chosen in order to ensure pre-miRNAs processing had taken place, and to ensure maximal transcriptional activation.

3.2. Profiling showed minimal ERα mediated regulation of miRNAs at 24 h

We have shown that at 24 h there is a massive transcriptional regulation in these cells by E2-activated ERα (E2-ERα), especially in processes such as proliferation and apoptosis. miRNAs have been reported to be involved in similar processes [11], and are transcribed as pre-miRNAs using RNA polymerase II in the same manner as protein coding genes. ERα positive tumors have been shown to express miRNAs differentially to ERα negative tumors. We therefore set out to test the hypothesis that, at 24 h, E2-ERα would be able to regulate pre-miRNA transcripts as substantial as the protein coding transcripts. miRNAs are not active until they are exported into the cytoplasm and processed into their mature form. In order to identify mature miRNAs that are regulated by E2-ERα, we compared miRNA expression of ethanol (control) and E2 treated T47D cells using miRNA microarrays. Only 7 miRNAs were indicated to be significantly regulated (Fig. 2) using a *P*-value of <0.1, whereas no regulated miRNA exhibited a *P*-value of <0.01. The remaining 801 miRNAs (Supplemental data) showed statistically insignificant regulation. This result indicated that miRNAs were only minimally regulated by ERα. To eliminate method bias, we performed an additional round of profiling using the TaqMan® Low Density Array (TLDA). This method indicated that 57 miRNAs were regulated (Supplemental data). Further analysis of these regulated miRNAs would be done using qPCR assays.

3.3. qPCR confirms non-significant ERα regulation of miRNAs

To validate array results, we performed qPCR on RNA from three separate treatments; the two replicated treatments used for the array and a third, additional treatment. First, we tested the 7 miRNAs identified by microarray using SYBR green technology, which detects mature miRNA alone. As seen in Fig. 3A, miR-29c and three more miRNAs showed a slight estrogen induced upregulation in all three treatments, in accordance with the array results, none of which were found to be statistically significant. The three

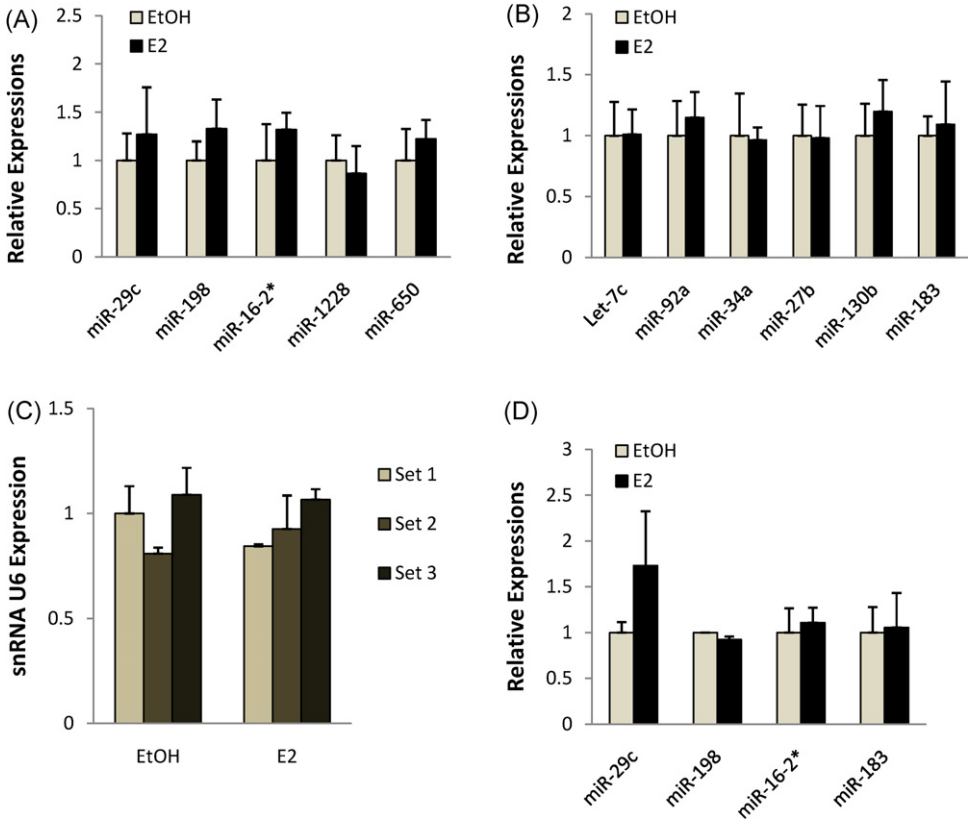

Fig. 3. Validations of miRNA microarray. (A) qPCR results to confirm top seven miRNA from LCSciences. Samples (T47D) were poly-A tailed and SYBR green technology was used to detect amplification. (B) qPCR results to confirm regulated miRNA from both LCSciences and TLDA lists. Samples (T47D) were poly-A tailed and SYBR green technology was used to detect amplification. (C) qPCR for snRNA U6 expression for each sample set of treatment (SYBR Green) (D) qPCR results to confirm regulated miRNAs from LCS and TLDA using TaqMan MicroRNA assays. Each miRNA was carried out in triplicate with each sample. All miRNA expressions were normalized to snRNA U6, except for 3D where relative levels of U6 were plotted against input RNA showing the level of variation between samples. Analysis and relative expressions were determined using comparative threshold cycle ($\Delta\Delta C_t$) method. All have $P > 0.10$. Values are the average of three separate experiments ±SD.

remaining miRNAs (one shown in Fig. 3A) showed no change. These results were obtained from an average of three biological sample sets, and were inconsistent with the array data that showed significant changes in expression levels. For example, miR-16-2* and miR-198 showed microarray-detected changes of 1.88 and 2.06-fold, respectively. We also tested miRNAs detected by the TLDA array alone, using the same SYBR green qPCR technology. We observed no significant changes in any of the miRNAs tested (examples shown in Fig. 3B). Also of note, these miRNAs were recorded as unchanged using microarray technology. U6 snRNA expression showed no variation between treatments and across the three biological samples (Fig. 3C). The data collected so far, lead to the reasonable conclusion that no miRNAs are significantly regulated by 24 h E2-activated ERα in T47D breast cancer cell lines.

To further confirm the accuracy of the validations and remove any bias, we used TaqMan miRNA technology to validate array results, and to verify that the qPCR results from SYBR were reproducible, given that TaqMan probes are more specific at detecting changes. As seen in Fig. 3D, miR-29c showed a 1.7-fold increase in expression, though not statistically significant. The remaining miRNAs tested (including miR-198, miR-16-2* and miR-183) showed no change. From these results, we conclude that miRNAs are not strongly regulated by 24 h E2-ERα in T47D cells. The miR-29c may be the only miRNA regulated, but variations between treatments reduced the significance of its expression. We also conclude that both microarray and TLDA technology generate false positives, but of these, the microarray results correlate better with qPCR results.

3.4. Previously reported estrogen-induced miRNA regulations could not be reproduced

In order to ensure comprehensive analysis of potentially regulated miRNAs, we tested a range of those previously reported in the literature, for which ERα induced regulation data remains largely inconclusive. Previous studies have reported that miR-125b, miR-107/103 [14], and miR-206 [2,4] are downregulated in the presence of E2 in MCF-7 cells. The miR-200 family has been reported to be upregulated by E2 [2] and miR-21 to be downregulated by ERα in MCF-7 cell lines [2,5] but not in T47D cell lines [5]. However, miR-21 has also been reported to be strongly upregulated by ERα in both MCF-7 and T47D breast cancer cell lines [7]. As seen in Fig. 4A, qPCR (SYBR green) revealed no significant changes in relative expression of these miRNAs, with the exception of a non-significant upregulation of miR-206. miR-206 and miR-21 were further analysed using TaqMan probes (Fig. 4B), and showed no significant changes in miR-21 expression (in agreement with our SYBR green qPCR results and Wickramasinghe et al. [5], but not with Bhat-Nakshatri et al. [7]). miR-206 showed different tendencies depending on the technique employed; SYBR green qPCR showed a non-significant upregulation, while TaqMan chemistry showed a non-significant downregulation of miR-206. Other miRNAs tested, including miR-98 and -101, showed no changes in expression. Previously

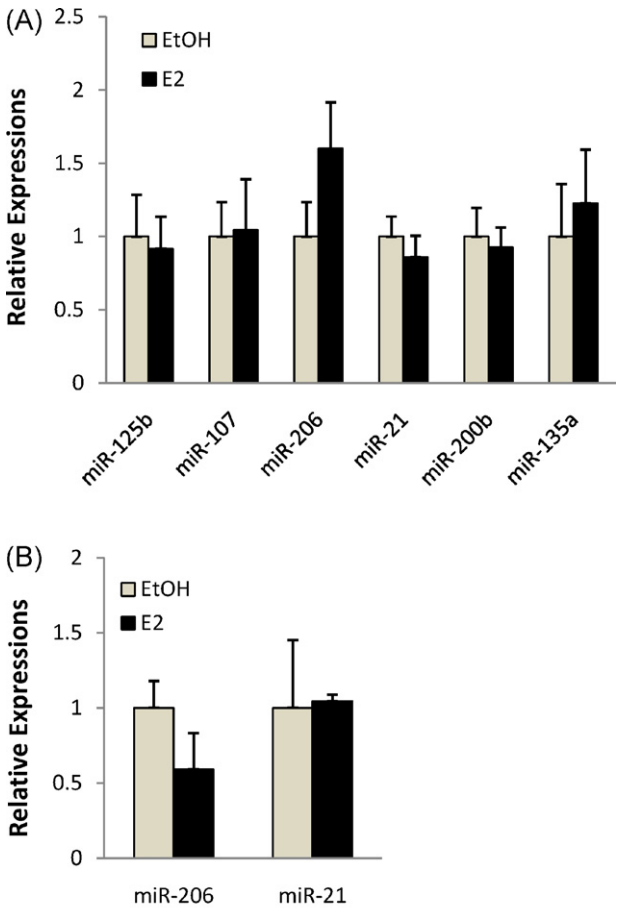

Fig. 4. Reproduction of previously reported miRNAs. qPCR results to confirm regulated miRNA from the literature. (A) Samples (T47D) were poly-A tailed and SYBR green technology was used to detect amplification. (B)TaqMan MicroRNA assays were used detect changes in expression. Each miRNA was carried out in triplicate with each triplicate sample. All miRNA expressions were normalized to snRNA U6. Analysis and relative expressions were determined using comparative threshold cycle ($\Delta\Delta C_t$) method. All have $P > 0.10$. Values are the average of three separate experiments ±SD.

reported results pertaining to ERα regulated miRNAs could not be reproduced. One possible explanation is that most previous studies have utilized MCF-7 cell lines, and may show patterns of miRNA regulation distinct from T47D.

In order to evaluate the importance of cell line specific miRNA regulation, we performed the miRNA studies in MCF-7 cells. MCF-7 cells were treated with control (EtOH) and E2 for 24 h, and the same protein coding genes tested to confirm treatment and response to E2. At 24 h E2 treatment, E2-responsive genes showed significant induction (Fig. 5A). We then performed qPCR analysis of miRNAs reported to be regulated by ERα in MCF-7 using SYBR green technology. As shown in Fig. 5B, no significant changes were observed in any of the miRNAs tested. However, previously reported downregulated miRNAs miR-21, -107, and -22 [2,8,14] showed some indications of downregulation following E2 treatment, although not significant ($P > 0.05$). Other miRNAs tested but not shown included miR-221, -222, -103, -200a, -125b, -18a, and -92a. To further investigate potential regulation we performed TaqMan miRNA analysis on miR-21 and -206 (Fig. 5C), which showed no significant changes, directly contradicting previous reports relating to these miRNAs [5,7]. U6 snRNA expression showed no variation between treatments and across the three biological samples (Fig. 5D). An important consideration was the fact that our data were collected 24 h after E2 exposure, whereas the literature largely reports 1, 4, 6, and 12 h time courses for exposure to E2, with some in between the 24 and 72 h range [14,16]. A time point assay was therefore conducted to address this variable.

3.5. Time-course study of miRNA reveals a slight variation in miRNA expression

Differences in miRNA regulation observed across multiple experiments may be a consequence of variable E2 exposure times. A time point assay on selected miRNAs was therefore carried out in order to verify the potential for miRNA expression changes different time points in the T47D cell line. Duplicate T47D cells treated with EtOH or E2 for 0, 1, 4, 8, 24, and 72 h were measured for miRNA expression changes, in particular miR-29c, -21, and -206. miR-29c showed variation in expression at different time points (Fig. 6A). Comparisons within time points between treated sample (EtOH vs. E2), showed a statistically insignificant upregulation of miR-29c at 24 h E2 treatment ($P > 0.05$), as detected in previous treatments (Fig. 3A). For miR-21 we noted a small statistically significant increase at 8 h E2 treatment only ($P < 0.05$) (Fig. 6B). At remaining time points, including the previously analysed 24 h (Fig. 3B), no significant changes were observed. miR-206 showed no significant estrogen regulation at any time point (Fig. 6C). miR-135a showed a significant upregulation at 72 h estrogen treatment ($P < 0.01$) (Fig. 6D). miR-183 showed no significant estrogen regulation at any time point (Fig. 6E). Overall the miRNAs varied slightly over the time-courses measured, yet, the T47D cell lines did not exhibit any major estrogen induced miRNA regulation at any time point.

3.6. miRNAs with an ERα chromatin-binding site nearby are not regulated in T47D cells

Estrogen activates ERα through binding to its ligand binding domain, which allows transcription by the receptors binding to EREs (gene specific) in the chromatin and subsequent induction of gene transcription. Potential miRNAs could be directly regulated by ERα, or indirectly via an ERα regulated transcription factor. The proto-oncogene c-MYC is upregulated by ERα, which in turn regulates its sets of gene targets, such as those involved in breast cancer progression [17,18]. Thus, it is plausible that miRNAs with either an ERα or a c-MYC binding site nearby might be up or downregulated following estrogen treatment within 24 h. Although we had not found any E2 induced miRNA regulations so far, we decided to perform an in-depth analysis of such miRNAs. Several studies have mapped ER binding regions using ChIP studies on MCF-7 breast cancer cells as a model of hormone-dependent breast cancer [12–14], similar ERα binding sites is functional in other breast epithelial cell lines such as T47D cells [12]. To study miRNAs with a potential ERα or c-MYC binding site nearby, we used the UCSC genome browser to search for ERα binding sites within 200 kb of the miRNA genomic location using the coordinates provided by previous studies on ERα [12–14] and c-MYC [13]. Of the miRNAs that had shown altered expression at any time point (miR-21, -206, -183, and -29c), miR-21 was shown to possess both ERα (two sites, 170 bases to 5 kb downstream) and c-MYC (134 kb upstream) binding sites located nearby (not shown). miR-196a, -301a, -652, and -342-3p were found to have ERα binding sites within 200 kb. We used qPCR to investigate their ERα regulation in both T47D and MCF-7 (Fig. 7A and B), and we observed no change in expression at 24 h. Others tested but not shown were miR-590-5p and miR-140-5p, and no changes were observed. Our results show that potential regulation of miRNAs through proximal or

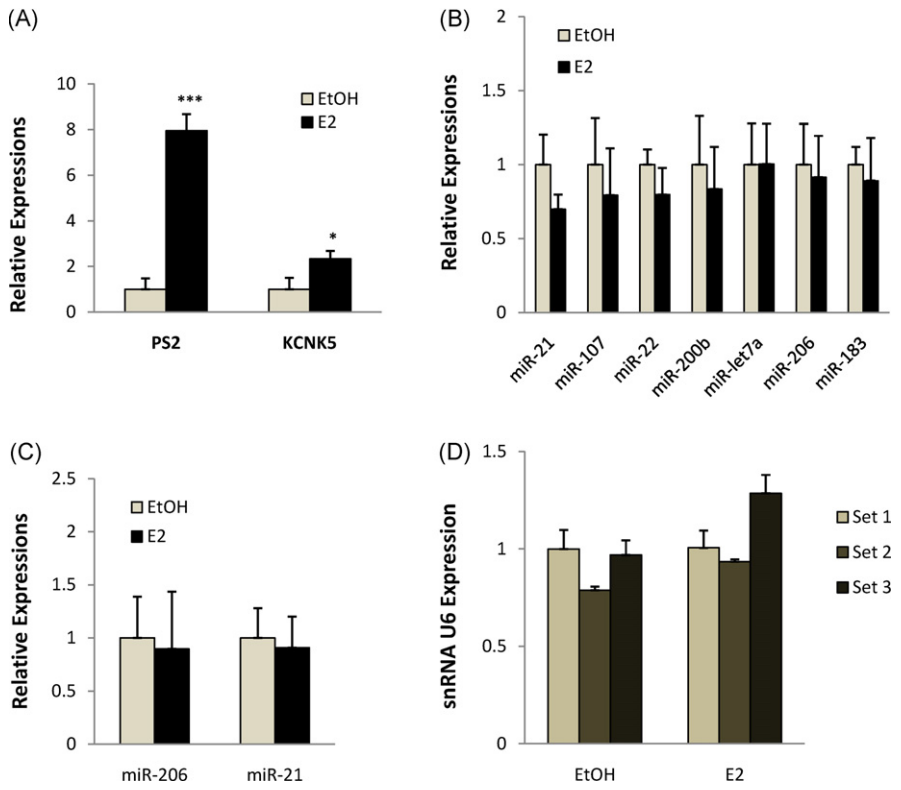

Fig. 5. miRNA regulation in other ERα-positive breast cancer cells. (A) qPCR results showing E2-activated expression of PS2 and KCNK5. MCF-7 parental cells were treated with EtOH or 10 nM E2 for 24 h. Expression of these genes was normalized to the expression of ARHGDIA (reference gene). Triplicate samples were used. Student *t* test: ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$. (B) qPCR results to confirm regulated miRNA from the literature. Samples were poly-A tailed and SYBR green technology was used to detect amplification (All have $P > 0.10$). (C) qPCR results using TaqMan MicroRNA assay method. (D) qPCR for snRNA U6 expression for each sample set of treatment. Each miRNA was carried out in triplicate with each triplicate sample (All have $P > 0.10$). All miRNA expressions were normalized to snRNA U6, except for 5D where relative levels of U6 were plotted against input RNA showing the level of variation between samples. All analysis and relative expressions were determined using comparative threshold cycle ($\Delta\Delta C_t$) method. Values are the average of three separate experiments ±SD.

distal ERα binding sites do not result in changes of mature miRNA expression.

3.7. miRNAs can differentiate between non-tumor and tumor cell lines

Although, T47D cells showed no major estrogen induced changes in miRNA expression, cell to cell variation is known to exist, and we have previously shown that basal miRNA levels can differ between different breast cancer cell lines [19]. In order to demonstrate and acknowledge differences in miRNA expression, we analysed changes in miRNA expression levels in non-tumor and triple-negative breast cancer cells as positive controls (Fig. 8). miR-200, -183, and -206 showed no variation among the two ER-positive breast cancer cells (T47D and MCF-7), but were reduced by approximately 50% compared to normal-like cell (MCF10A). miR-21 was greatly reduced in T47D cells and unchanged in MCF-7 cells. miR-200b, -135a, and -183 levels were also greatly reduced (levels close to zero) in triple-negative cells (MDA-MB-231) in relation to their expression in MCF10A. Thus, there is significant variation in miRNA expression between different cell lines, although these are not directly regulated by ERα, as shown in this study.

4. Discussion

The conflicting results for estrogen regulated miRNAs, such as miR-21, previously reported as both up-, and downregulated by ERα in MCF-7, and found to be regulated in neither MCF-7 nor T47D by the current study, may be due to several factors. The short length of mature miRNA, the heterogeneity in their GC content (melting temperature), and the fact that the target sequence is present in the primary transcript, makes miRNA expression profiling technically challenging [20]. Microarray-based methods are the most extensively used approach for miRNA identification and quantification, although methods such as cloning and northern blot are still used [8]. Previous studies have raised concern about the normalization procedure in miRNA microarray analysis. This is an important and critical step in miRNA microarray analysis [21], but because of the smaller population of miRNAs compared to mRNAs, the commonly used normalization method (e.g., whole-genome gene expression microarray) is not appropriate [22]. qPCR has some major advantages over microarrays in that it is fast, more sensitive, has a larger dynamic range and requires lower amount of starting material [20]. For miRNAs analysis, however, qPCR is challenging due to the short 22 nucleotide size, which requires additional steps to allow annealing of both forward and reverse primers.

Also, the effectiveness of the qPCR depends on several parameters including RNA extraction and integrity, cDNA synthesis, primer design, amplicon detection, and data normalization (reference gene). No reference miRNA had been identified to date, and instead small nucleolar U6 (snRNA U6) is commonly used for normalization of miRNA expression. Furthermore, the time of exposure to E2, cell variations, and method of detection are factors that could lead to variables in miRNA research. We also took into consideration the chemistry for detection of qPCR products. SYBR green detects mature miRNA but it cannot discriminate between different amplicons and binds to all double-stranded DNA, including non-specific products such as primer–dimers. We performed a melting point

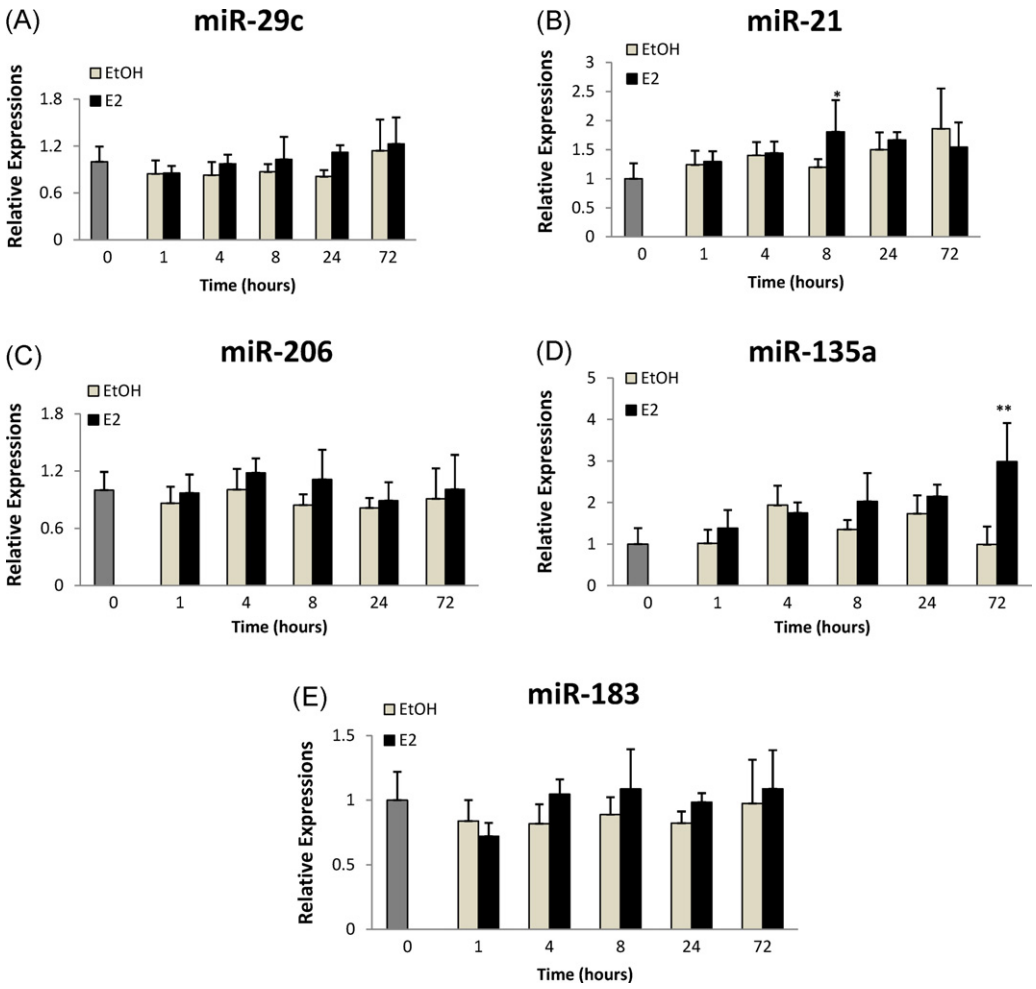

Fig. 6. Time assays of miRNAs suspected to be regulated. qPCR results for time point assay on: (A) miR-29C, (B) miR-21,(C) miR-206, (D) miR-135a, and (E) miR-183. Samples (T47D) were poly-A tailed and SYBR green technology was used to detect amplification. Each miRNA was done in triplicate with each duplicate sample. Expression levels were compared relative to the 0 h sample. All miRNA expressions were normalized to snRNA U6. Analysis and relative expressions were determined using comparative threshold cycle ($\Delta\Delta C_t$) method. All have $P < 0.955$. *P*-Student *t* test: $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$. Values are the average of two separate experiments ±SD.

analysis (dissociation curve) for each qPCR, which enabled us to monitor the homogeneity of the qPCR products. To eliminate the possibility of the detection method being the problem, we performed TaqMan miRNA assays using TaqMan probes as a more specific approach. Primer-dimers and other non-specific amplification products can still be formed, but they would not generate any fluorescent signal. All these differences may account for the variation in results on identifying miRNAs within the same cell type.

A previous study further validated the estrogen exposure time used in our study; Hua et al. carried out gene expression profiling in a time course study in an effort to map the ERα binding sites in MCF-7 cell line [13]. The highest number of responsive genes was seen at an E2 exposure time of 24 h. Also, it was reported that a few of these estrogen responsive genes found had ERα binding sites within 10–50 kb of the transcription start site [12,13]. Genes found to be out of that range may use transcription factors on a different chromosome, may be regulated from distal sites, may not be annotated, or may be independent of ER-binding [12,13]. Hua et al. further analysed their data by using distances within and greater than 200 kb to identify their ERα binding regions, validating our use of a distance within 200 kb for both proximal and distal interactions. It has been reported that E2-ERα interacts with the Drosha complex and reduces primary miRNA processing [23], and that E2-ERα also induces dicer mRNA [7]. However, according to our results, this does not result in changes of mature miRNA levels within 24 h of ERα activation. It has also been reported that processing of pre-miRNA to mature miRNA is delayed for at least 12 h after E2 stimulation, which was further confirmed by detecting an increase in the expression of mature miRNA from 24 h to 72 h after E2 stimulation [24]. Recently reported estrogen induced transcription of pri-miRNA has revealed that regulation of pri-miRNA occurs early (40 min–3 h) and those pri-miRNAs that undergo sustained and significant changes in expression are usually reflected as changes in the processed, mature miRNAs [25].

Differences in miRNA expression in ERα-positive and ERα-negative tumors have been observed in this and other studies. Although this is likely an effect of ERα expression, such as a higher level of differentiation in ERα expressing breast cancer, we suggest that it is not a direct effect of ERα transcription. We cannot exclude that ERα regulates transcription of pre-miRNA, but if so, this does not result in alterations in mature miRNA expression within 24 h. Also, E2-activated ERα is known to induce c-MYC expression [26,27], and c-MYC, in turn, regulates miRNA expression [24,28]. This suggests that there may be an indirect relationship between ERα signaling and miRNA expression. The exact relationship between miRNA and the ERα signaling in breast tumors remains unknown [29].

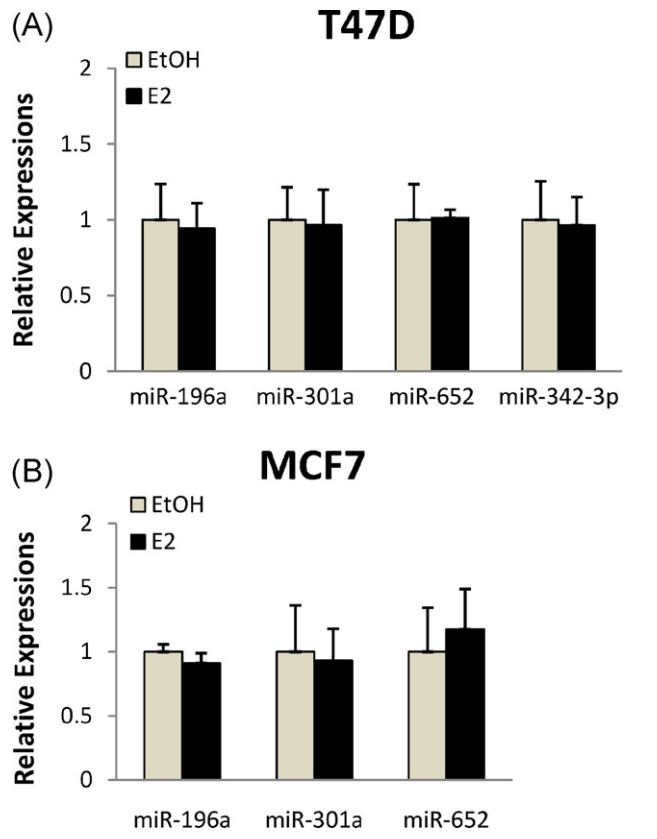

Fig. 7. Validation of miRNA with EREs within 200 kb of ERα-binding site. qPCR was used to validate miRNAs that have been reported to be regulated by ERα and have EREs < 200 kb from ERα-binding site. Samples (A) T47D and (B) MCF-7, were poly-A tailed and SYBR green technology was used to detect amplification. Each miRNA was done in triplicate with each triplicate sample (All have $P > 0.10$). All miRNA expressions were normalized to snRNA U6. All analysis and relative expressions were determined using comparative threshold cycle ($\Delta\Delta C_t$) method. Values are the average of three separate experiments ±SD.

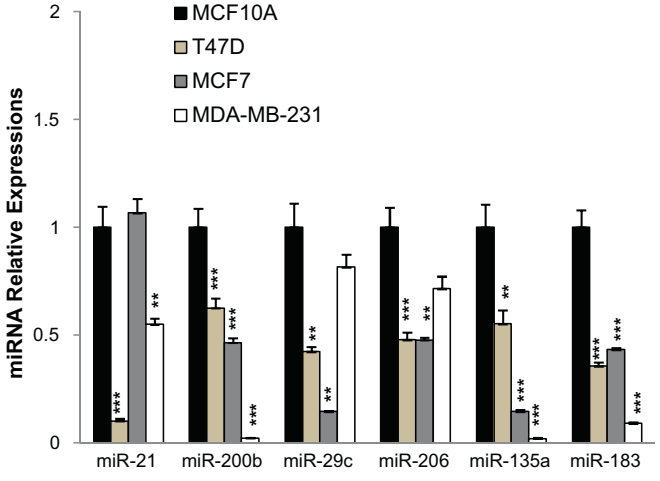

Fig. 8. Expression levels of miRNAs in different breast cancer sub-type and normal breast cell lines. qPCR (SYBR green) results showing selected miRNA expression levels in T47D, MCF-7, and MDA-MB-231, relative to their expression in MCF10A cells. All miRNA expressions were normalized to snRNA U6. Analysis and relative expressions were determined using comparative threshold cycle ($\Delta\Delta C_t$) method. Student t test: $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$. Values are the average of three separate experiments ±SD.

5. Conclusion

In conclusion, we have shown that although the T47D cell line demonstrates very strong estrogen-ERα mediated gene transcription, mature miRNAs are not directly regulated at 24 h. Understanding the complete mechanism of ERα is important in breast cancers, which would aid in better treatment and diagnosis of this disease. We believe that this study will contribute to narrowing the study area of ERα regulated miRNA in ERα positive breast cancer cells, and help to unravel the function of ERα in breast cancer cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2011.10.008.

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