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A MicroRNA196a2* and TP63 Circuit Regulated by Estrogen Receptor- α and ERK2 that Controls Breast Cancer Proliferation and Invasiveness Properties

Kyuri Kim[†], Zeynep Madak-Erdogan[†], Rosa Ventrella, and Benita S. Katzenellenbogen
Department of Molecular and Integrative Physiology, University of Illinois and College of Medicine at Urbana-Champaign, Urbana, IL, 61801

Abstract

Estrogen receptor α (ER α) is present in about 70% of human breast cancers and, working in conjunction with extracellular signal-regulated kinase 2 (ERK2), this nuclear hormone receptor regulates the expression of many protein-encoding genes. Given the crucial roles of miRNAs in cancer biology, we investigated the regulation of miRNAs by estradiol (E2) through ER α and ERK2, and their impact on target gene expression and phenotypic properties of breast cancer cells. We identified miRNA-encoding genes harboring overlapping ER α and ERK chromatin binding sites in ER α -positive MCF-7 cells and showed ER α and ERK2 to bind to these sites and to be required for transcriptional induction of these miRNAs by E2. Hsa-miR-196a2*, the most highly estrogen up-regulated miRNA, markedly down-regulated tumor protein p63 (*TP63*), a member of the p53 family. In ER α -positive and ER α -negative breast cancer cells, proliferative and invasiveness properties were suppressed by hsa-miR-196a2* expression and enhanced by hsa-miR-196a2* antagonism or TP63 target protector oligonucleotides. Hsa-miR-196a2* and TP63 were inversely correlated in breast cancer cell lines and in a large cohort of human breast tumors, implying clinical relevance. The findings reveal a tumor suppressive role of hsa-miR-196a2* through regulation of TP63 by ER α and/or ERK2 signaling. Manipulating the hsa-miR-196a2*-TP63 axis might provide a potential tumor-suppressive strategy to alleviate the aggressive behavior and poor prognosis of some ER α -positive as well as many ER α -negative breast cancers.

Keywords

estrogen receptor; breast cancer; ERK2; microRNA; proliferation; invasion

INTRODUCTION

The nuclear hormone receptor, Estrogen Receptor α (ER α), is a key regulator of the proliferation, differentiation and phenotypic properties of about 70% of human breast cancers, and extranuclear-activated protein kinase pathways are now known to collaborate with ER α in its actions in breast cancer cells [1,2]. Recently, we documented that ER α and ERK2, a downstream effector in the mitogen-activated protein kinase (MAPK) pathway, colocalized at ER α chromatin binding sites across the genome of breast cancer cells and that this protein kinase cooperated with ER α in regulating gene expression and proliferation

*Address correspondence to: Dr. Benita S. Katzenellenbogen, Department of Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, Urbana, IL, 61801, katzenel@illinois.edu, 217-333-9769.

[†]KK and ZME should be considered equal first authors

CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

programs [3]. MAPK signaling is often up-regulated in breast cancers and is believed to play a central role in breast cancer development and progression, and in eliciting changes in breast cancers that engender resistance to endocrine therapies and other treatment therapies [3–6]. Our previous work examined ER α and ERK2 collaboration in the regulation of protein-encoding genes [3]. However, in view of the broad influence of miRNAs in cancer biology, we have herein examined the impact of ER α and ERK2 in the regulation of non-coding miRNA genes.

MicroRNAs (miRNAs) are small, ca. 22 nucleotide non-coding RNAs that modulate gene expression by post-transcriptional repression [7–9]. Bioinformatics predictions suggest that mammalian miRNAs might regulate more than 30% of all protein-coding genes [8,10]. miRNAs can affect both the translation and stability of mRNAs by their sequence complementarity to the 3' UTR of the target genes [8,11]. However, additional functions of miRNAs are possible; for example, they could regulate pre-mRNA processing in the nucleus or act as chaperones modifying mRNA structure or modulating mRNA-protein interactions [12].

miRNAs are thought to function as both tumor suppressors and oncogenes [13,14], and they are of great interest in cancer because of their regulatory functions in proliferation, differentiation and apoptosis [15–17]. High-throughput miRNA expression profiling in breast cancer cell lines and tissues has identified a large set of miRNAs expressed at different levels in breast cancers as compared to the normal breast [18–20]. miRNA signatures predicting the expression levels of the estrogen, progesterone and HER2/*neu* receptors, which characterize different breast cancer subtypes, have also been examined to elucidate the role of these miRNAs in disease classification of breast cancer and as prognostic biomarkers [21–23]. Increasing evidence is also accumulating for an association between miRNAs, ER α signaling, and endocrine resistance in breast cancer [24–26].

Tumor Protein 63 (TP63) is a member of the p53 family that has oncogenic and tumor suppressive cell context-dependent activities in human cancer [27–30], with roles in tumor growth, apoptosis and metastasis [27,30,31]. The translational products of the *TP63* gene are crucial for the maintenance of a stem cell population in the human epithelium [32] and are necessary for the normal development of all epithelial tissues [33] including the mammary gland [32], and are present in the myoepithelial cells of adult breast tissue [34,35]. TP63 is found to be overexpressed in a subset of highly aggressive ER-negative breast cancers that represent a basal and myoepithelial phenotype and have a poor clinical outcome [36,37]. Among six isoforms of TP63, Δ Np63 α , which lacks the transactivating N-terminal region, is the predominant form expressed in many carcinomas and although it lacks the N-terminal transactivation region, Δ Np63 α via a C-terminal transactivation domain can regulate the expression of genes distinct from those regulated by the N-terminal transactivation domain [38–41].

In this study, we examine estrogen regulation of miRNA genes and the involvement of the nuclear receptor ER α and the protein kinase ERK2. We report on the marked up-regulation of hsa-miR-196a2* by estradiol, mediated by these two proteins in ER α -positive breast cancer cells, and their control of TP63 by hsa-miR-196a2* action. Our studies highlight a novel role of this hsa-miR-196a2*-TP63 circuit in the hormone regulation of ER α -positive breast cancer cells and show that this hsa-miR-196a2*-TP63 axis also operates in ER α -negative breast cancer cells to control proliferative and invasiveness properties.

METHODS

Cell Culture, RNA Extraction, and Real-Time PCR Analysis

MCF-7 and MDA-MB-231 breast cancer cells were routinely maintained in Minimal Essential Medium (Sigma-Aldrich, St. Louis, MO) supplemented with 5% calf serum (HyClone, Logan, UT) or in L-15 (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), respectively [3,42]. Four days before E2 treatment, cells were switched to phenol red-free MEM containing 5% charcoal-dextran-treated calf serum or phenol red-free L-15 containing 10% charcoal-dextran-treated fetal bovine serum, respectively. Medium was changed on day 2 and 4 of culture, and then cells were transfected with 20 nM of siGENOME Ctrl, siER α or siERK2 as described previously [3] using Dharmafect (Dharmacon Inc, Lafayette, CO). After 48 h of transfection, cells were treated for 24h with 0.1% ethanol or 10 nM E2. Total RNA was isolated, reverse-transcribed and analyzed by real-time PCR as described [43]. For the analysis of miRNA expression, total RNA was isolated, reverse transcribed using primers specific for each miRNA (Applied Biosystems, Foster, CA) and analyzed by real-time PCR using Taqman chemistry and primers from Applied Biosystems.

MicroRNA Array

Total RNA was isolated using Trizol reagent. Then miRNA was enriched using RT² qPCR-grade miRNA isolation kit according to the manufacturer's instructions. Two hundred nanograms of enriched small RNA were converted into cDNA using RT² miRNA First strand kit. The cDNAs were mixed with 2 $\times$ RT2 SYBR Green PCR Master Mix (SABiosciences, Frederick, MD) and dispersed into 384-well Human Genome miRNA PCR Array (MAH-3200E, SABiosciences) with 10 μ l/well reaction volume. The PCR array contained a panel of primer sets for 376 most abundantly expressed and best characterized human miRNAs, four small RNAs as the internal controls and four quality controls. The real-time qRT-PCR was performed on a ABI 7900 real-time PCR system (Applied Biosystems) with the cycling parameters: 95 $^{\circ}$ C for 10 min, then 40 cycles of 95 $^{\circ}$ C for 15 s, 60 $^{\circ}$ C for 30 s and 72 $^{\circ}$ C for 30 s. SYBR green fluorescence was recorded from every well during the annealing step of each cycle.

Anti-miR, pre-miR, siTP63 or TP63 miScript Target Protector Transfection

MCF-7 or MDA-MB-231 breast cancer cells were transfected with 20 nM Anti-miR-196a* (Applied Biosystems), pre-miR-196a* (Applied Biosystems) or negative controls (Anti-miR or pre-miR) using Dharmafect, or in other experiments with 20nM of siGENOME Ctrl or TP63. After 48 h of transfection, cells were treated with control 0.1% ethanol vehicle or 10 nM E2 for the times indicated. It is important to note that miR-196a and miR-196a* represent different mature miRNAs of the miR-196a2 stem loop and that they have different sequence and were each monitored using miRNA-specific primers in quantitative real-time PCR as described [44]. For the target protector studies, cells were transfected with 100 nM TP63 miScript target protector (Qiagen, Alameda, CA) or negative control protector for 48 h prior to treatment with 0.1 % ethanol or 10 nM E2 for 24 h, and then RNA isolation for gene expression analysis.

Proliferation, Soft Agar Colony Formation and Invasion Assays

Cell proliferation was assessed using WST-1 reagent (Roche Applied Science, Indianapolis, IN) as described [45]. Invasion assays used BDBioCoat Matrigel invasion chambers (BD Biosciences, San Jose, CA) with 10% fetal bovine serum as chemoattractant in the lower chamber as described [5,45]. Soft agar colony formation assays were performed as described [45].

Chromatin Immunoprecipitation (ChIP) Assays and Western Blot Analysis

ChIP assays were performed as described before [3,46]. Antibodies used were: ER α (HC-20) and ERK2 (D-2) from Santa Cruz Biotechnology, Santa Cruz, CA. Whole cell extracts were prepared in lysis buffer as described [3], and Western blot analysis used specific antibodies for ER α (HC-20, Santa Cruz); ERK2 (D-2, Santa Cruz); TP63 (ab53039, Abcam), and TP63 (4A4, Santa Cruz) and TP63 (4892S, Cell Signaling) for comparison; and β -actin (AC-15, Sigma).

Gene Expression Data from Human Breast Tumors

mRNA and miRNA expression datasets from 31 ER α -positive, 23 HER2-positive, and 78 basal breast tumors and 21 normal breast tissue samples were obtained from a previous study [47]. The mRNA and miRNA data were combined and analyzed using Cluster.3 software and visualized using Tree View Java.

RESULTS

Identification of miRNA genes harboring overlapping ER α and ERK2 binding sites in MCF-7 cells

To investigate possible collaboration between ER α and ERK2 in miRNA regulation, we examined ER α and ERK2 binding sites across the genome using ChIP-on-chip microarray analysis [3] and mapped ER α and ERK2 binding sites to regions that contain miRNA genes after E2 treatment of ER α -positive MCF-7 breast cancer cells. From this genome-wide analysis of ER α and ERK2 binding sites, using a 50 kb window around the transcription start site (TSS) of annotated miRNAs in the human genome, we identified nine miRNA genes (hsa-miR-196a2, hsa-miR-135a2, miR944, miR-101, hsa-miR-938, hsa-miR-615-3p, hsa-miR-190b, hsa-miR-21, and hsa-miR-190) harboring both ER α and ERK2 binding sites after E2 treatment, with eight of these nine miRNA-encoding genes (all except hsa-miR-190b) having overlapping ER α and ERK2 binding sites. The overlapping binding sites for ER α and ERK2 in four of these genes are shown in Figure 1A. We also conducted miRNA microarray analyses to evaluate miRNA expression profiles and identified miRNAs that were up-regulated (miR-196a2, hsa-miR-135a, hsa-miR-944, hsa-miR-101, hsa-miR-938 and hsa-miR-615-3p) by E2 after 6h of hormone exposure (Figure 1B). hsa-miR-21 was not changed at 6 h after E2 but was down-regulated by E2 at later times (12 and 24 h, not shown). ER α or ERK2 knock-down reduced expression of these miRNAs and prevented E2 regulation of these miRNAs that harbor both ER α and ERK2 binding sites (Figure 1B).

We verified, by ChIP assay, recruitment of ER α and ERK2 to the chromatin regions identified for the hsa-miR-196a2, hsa-miR-135a2, hsa-miR-944 and hsa-miR-101-1 genes. Both ER α and ERK2 were recruited to chromatin by 45 min of E2 treatment (Figure 2A, B). Moreover, we observed a marked increase in the occupancy by activated RNA Pol II (pSer5 RNA Pol II) of the TSS of all four miRNA genes upon E2 treatment (Figure 2C). For further investigation, we chose to focus in detail on the E2-stimulated miRNA, hsa-miR-196a2*, because it was highly up-regulated by E2 and harbored overlapping ER α and ERK2 binding sites close to (within 10 kb of) the TSS (Figure 1A and 1B), suggesting it might likely be a direct target of these two proteins.

Regulation of the E2 mediated increase in hsa-miR-196a2* levels by ER α and ERK2; miRNA target gene prediction; and characterization of ER α and ERK2 impact on target gene expression

Hsa-miR-196a2 and hsa-miR-196a2* are each transcribed from and are the mature miRNA products of the Pre-hsa-miR-196a2 stem loop. These two miRNAs have different sequences

and are predicted to target different genes. When expression levels of these two miRNAs were compared in MCF-7 cells using miRNA specific primers to make the cDNA and then using Taqman detection, we found that hsa-miR-196a2 was 2.5 times more highly expressed compared to hsa-miR-196a2* (Figure 3A). Next, we monitored the effect of estradiol (E2) on the levels of these two miRNAs over time. As a consequence of the recruitment of ER α and ERK2 to chromatin regulatory sites and the recruitment of active RNA Pol II to the TSS of the miR-196a2 gene as shown in Figure 2, hsa-miR-196a2* levels were up-regulated by 6 h of E2 treatment, and they continued to increase, reaching a 26-fold increase by 24 h after hormone treatment (Figure 3B). By contrast, when we monitored hsa-miR-196a2, we observed only a small, ca. 3-fold increase in its expression level (Figure 3B) so that by 24 h after E2, hsa-miR-196a2* would be the far more predominant form. As seen in Figure 3C, the up-regulation of hsa-miR-196a2* in response to hormone was completely abrogated by knock-down of either ER α or ERK2, indicating critical roles for ER α and ERK2 in this stimulation of hsa-miR-196a2*.

The microRNA databases and target prediction tools MicroCosm Targets (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>), PicTar (<http://pictar.mdc-berlin.de/>) and TargetScan (<http://www.targetscan.org/index.html>) were used to identify potential hsa-miR-196a2* targets. Based on the observation that hsa-miR-196a2* expression was up-regulated by E2 and this was eliminated by ER α or ERK2 knock-down, we utilized our microarray gene expression data after E2 treatment without or with ER α or ERK2 knock-down [3] to then narrow down the potential miRNA target genes to focus on in this study. These presumed target genes for hsa-miR-196a2* are shown in Figure 3D.

Hsa-miR-196a2* target genes, TP63, SPRY1, and TFAP2A mRNA levels were verified to be down-regulated after 24 h of E2 treatment and this regulation was lost upon knock-down of ER α or ERK2 (Figure 3E). Western blot analysis revealed that only the Δ Np63 α isoform was present in MCF-7 cells, and this was observed using 3 different TP63 antibodies listed in Methods. The protein level of the Δ Np63 α isoform of TP63 was reduced by E2 treatment of cells, with this down regulation of Δ Np63 α by E2 being prevented by ER α and ERK2 knockdown (Figure 3F).

Inhibition of hsa-miR-196a2* increases TP63 expression and MCF-7 cell proliferation

To verify the effect of hsa-miR-196a2* on regulation of target gene expression, antisense inhibition of hsa-miR-196a2* expression was conducted using Anti-miR oligonucleotides (Figure 4A). Treatment of MCF-7 cells with anti-miR-196a* increased the basal expression of the miR target gene, *TP63* (Figure 4B). TP63 mRNA expression was downregulated by E2 as shown in Figure 4B. We therefore next investigated the effect of hsa-miR-196a2* antagonism on breast cancer cell growth. Inhibition of hsa-miR-196a2* was found to enhance the proliferation of MCF-7 cells without and with E2 treatment (Figure 4C). We also performed the same experiment in another ER α -positive breast cancer cell line, BT474, and observed similar findings (Figure 4D).

TP63 is a direct target of hsa-miR-196a2* and hsa-miR-196a2* controls TP63 expression and growth of MCF-7 cells

To confirm the impact of miR up-regulation on target transcripts, we overexpressed hsa-miR-196a2* using pre-miR-196a* oligonucleotides (Figure 5A). The biological activity of miRNAs is primarily mediated by interaction with matching recognition sequences, usually in the 3' UTRs of target genes, and by translational repression. To determine whether TP63 is a direct target of hsa-miR-196a2*, we also utilized TP63 miScript target protector oligonucleotide sequence which selectively recognizes hsa-miR-196a2* target sequences

only on the TP63 3' UTR, thereby blocking the interaction between hsa-miR-196a2* and TP63 target mRNA.

Expression of pre-miR-196a* greatly suppressed TP63 expression (Figure 5A). Transfection of TP63 protector oligonucleotides increased basal TP63 mRNA levels, as expected, and reversed this down-regulated TP63 expression caused by pre-miR-196a* (Figure 5A). We observed decreased cell proliferation with pre-miR-196a* expression (Figure 5B), and the application of TP63 target protector resulted in enhanced proliferation of control MCF-7 cells. This target protector also reversed the inhibition of proliferation observed with pre-miR-196a* transfection (Figure 5B). To monitor the direct impact of down-regulated TP63, we knocked down TP63 with siRNA and showed that this reduction in TP63 (Figure 5C) was accompanied by decreased proliferation of control and E2 treated cells (Figure 5D).

Relationships between ER α , hsa-miR-196a2, and TP63 in human breast tumors and breast cancer cell lines; and impact of miR-196a on growth and invasion of ER α -negative breast cancer cells

To test whether these relationships were clinically relevant and to address how the expression levels and isoforms of TP63 and the level of hsa-miR-196a2* might be associated with ER α and MAPK pathway activity in human breast tumors, we examined the expression of TP63 and hsa-miR-196a2* in a large human breast tumor dataset that contained information on both mRNA and miRNA expression levels [47]. As shown in Fig 6A, tumors were clustered into ER and PR positive (top left); ER, PR and Her2 positive; Her2 positive (top middle); or triple negative (top right). We observed that the levels of hsa-miR-196 (a-1, a-2 and a-2*) miRNA were high (shown in red) in many of the ER α -positive tumors and in a subgroup of ER α -negative/HER2-positive tumors (Figure 6A). Of note also, a small portion of basal like tumors were also positive for hsa-miR-196, but overall levels were lower compared to ER α -positive or HER2-positive tumors. Most interestingly, when we monitored expression of hsa-miR-196a2* targets (Figure 6A middle), we observed an inverse correlation between levels of this miRNA and its targets (TP63, TRAP2A, SPRY1, NEUROD1, MGAT4A, IGF1) across the human breast tumors (Figure 6A).

We also compared levels of TP63 and hsa-miR-196-a2* in MCF-7 cells which are ER α -positive, in MDA-MB-231 cells which are ER α -negative, and in an MDA-MB-231 ER α -positive cell line in which we stably introduced ER α [42,48]. In the cell lines, we also observed an inverse relationship between miR-196a2* and the Δ Np63 α isoform of TP63, which was the only form of TP63 detected (Figure 6B), even when we monitored protein expression using three different antibodies. The level of the Δ Np63 α (ca. 64 kDa) protein was very high in the ER α -negative MDA-MB-231 cell line compared to MCF-7 cells and MDA-MB-231 ER+ cells. Moreover, introduction of ER α into MDA-MB-231 cells reduced expression of this protein (Figure 6B). As expected, when we monitored hsa-miR-196a2*, we observed the highest level in MCF-7 cells. MDA-MB-231 cells had extremely low hsa-miR-196a2* and introduction of ER α increased expression of this miRNA ca.10-fold, but to a level much below that present in MCF-7 cells (Figure 6C).

We overexpressed hsa-miR-196a2* using pre-miR-196a* oligonucleotides to examine the impact of down-regulated TP63 in ER-negative MDA-MB-231 cells (Figure 6D). We also utilized TP63 miScript target protector sequences along with hsa-miR-196a2* overexpression to confirm the direct targeting of TP63 by hsa-miR-196a2* in this cell line. In cell proliferation studies (Figure 6D), incubation with TP63 protector alone increased cell proliferation whereas overexpression of pre-miR-196a* reduced cell proliferation, which was reversed by TP63 protector (Figure 6D).

We next investigated the effect of pre-miR-196a* on invasive properties of these cells. We observed that pre-miR-196a* expression reduced *in vitro* invasion of MDA-MB-231 cells, monitored using Matrigel invasion chambers (Figure 6E). The effects of these treatments on the cellular level of the protein Δ Np63 α are also shown (Figure 6E, Western blot). To further examine the impact of pre-miR-196a* on tumor growth, soft agar colony formation assays were performed with and without pre-miR-196a* overexpression in MDA-MB-231 cells. After 8 days of incubation, colony numbers and colony size were greatly reduced with pre-miR-196a* transfection compared to control (Figure 6F). Similar suppressive effects of pre-miR-196a* on cell proliferation, invasion and colony formation and reversal of the suppression by target protector were observed in three other ER-negative breast cancer cell lines, MDA-MB-453, MDA-MB-468 and SKBR3 (Figure 7), supporting a tumor suppressive role of hsa-miR-196a2*.

DISCUSSION

Estradiol and ER α control hsa-miR-196a2* and TP63 expression, and there is an inverse relationship between miR-196a2* and TP63 in human breast cancer cells and tumors

In this report, we show that TP63 expression is controlled by the presence or absence of ER α and extracellular regulated kinase 2 (ERK2) in breast cancer cells through up-regulation of hsa-miR-196a2*, greatly impacting on cell phenotypic properties (Figure 8). Our observations that the E2-liganded ER α up-regulated hsa-miR-196a2* and thereby decreased expression of TP63 would indicate a molecular mechanism by which control of TP63 regulates tumor cell growth. Our experiments, using target protector oligonucleotide sequences that selectively block hsa-miR-196a2* interaction with its binding sequence on TP63, support the model shown in Figure 8 in which estradiol and ER α control of TP63 via hsa-miR-196a2* governs a hormone-regulated change in cell proliferation. Several reports suggest that prognosis, disease-free survival and chemotherapeutic response in a number of human cancers are worse when levels of the oncogenic Δ Np63 isoforms are elevated or when tumors specifically lose expression of the transcriptionally active TA form of p63 [49,50]. In head and neck squamous cell carcinomas and in other epithelial cancers [51], as we have observed here in ER α -positive and ER α -negative breast cancer cells, Δ Np63 α was the only form of TP63 observed, and this TP63 isoform regulates gene programs affecting cell proliferation and the malignant phenotype. This amino-terminally truncated Δ N isoform of p63 lacks the amino-terminal transactivation domain but maintains the DNA binding domain and therefore can bind to p53 response elements in chromatin and act as a dominant negative inhibitor of p53 and full length transcriptionally active TAp63 with which it can form hetero- and homo- oligomers [39,52]. Moreover, the inverse relationship between miR-196a2* and TP63 observed in breast cancer cell lines and also in human breast tumors implies that this circuit is likely operative in human breast tumors.

Regulation of proliferation by estrogens in breast cancer cells no doubt involves multiple pathways and cellular components. In previous studies, we and others have documented that the widespread gene regulatory effects of estrogens generally result in up-regulation of pro-proliferative and anti-apoptotic genes and down-regulation of anti-proliferative and apoptotic genes [53–55]. Because estrogens are generally pro-proliferative in ER α -positive breast cancer cells, it is noteworthy that we have identified in this study an estrogen-regulated pathway that proceeds via up-regulation of hsa-miR196a2* and consequent down-regulation of TP63, and has a net anti-proliferative effect. Thus, our findings highlight the fact that even in an estrogen-stimulated process that has a net pro-proliferative outcome, certain anti-proliferative circuits might also be active.

Further, we show that regulation of TP63 by hsa-miR196a2* also operates in ER α -negative breast cancer cells, but this hsa-miR-196a2*-TP63 circuit is obviously not estrogen

regulated in these cells. If this antiproliferative axis could be up-regulated independent of estrogen stimulation, for example by introduction of hsa-miR196a2*, it might potentially be useful as a tumor-suppressive strategy. While there are still many aspects in need of optimizing, progress is being made in the development of miRNA directed therapies [56–58] which hold promise for further investigation of these approaches.

ER α and ERK2 chromatin binding and regulation of miRNAs and the hsa-miR-196a2*-TP63 circuit

The involvement of miRNAs in human cancer is of increasing interest as a critical layer in the gene expression regulatory system at the post-transcriptional level, by destabilizing target mRNAs using an RNA interference mechanism and at the translational level by repressing the translation process [8,10,59]. High-throughput miRNA expression profiling in breast cancer cell lines and tumors has identified a large set of miRNAs expressed at different levels in breast cancer compared to the normal breast [18–20]. In this study, we have focused on elucidating the regulatory role of ER α and ERK2 in miRNA expression and understanding its impact on breast cancer target gene regulation. Using a genome-wide analysis of ER α and ERK2 binding sites upon E2 treatment in MCF-7 cells, we identified nine miRNAs that harbor both ER α and ERK2 binding sites within a 50 kb window around the TSS of annotated non-coding RNAs in the human genome. Eight of these miRNAs had overlapping ER α and ERK2 binding sites, implying a possible collaborative action between ER α and ERK2 in miRNA regulation.

For the most highly estrogen-stimulated miRNA, hsa-miR-196a2*, which we studied in detail, we showed that ER α and ERK2 directly bind to the overlapping chromatin binding sites near the miRNA gene and were required for transcriptional stimulation by E2. Loss of miRNA expression by depletion of ER α or ERK2 induced an increase in expression of the miRNA target gene, TP63, highlighting that ER α and ERK2 regulate expression of not only primary protein-encoding target genes but also expression of miRNAs to coordinate key functional outcomes mediated through miRNA-targeted genes.

hsa-miR-196a2* significantly reduced TP63 expression and cell proliferation, implying a tumor suppressor-like role for hsa-miR-196a2*. Δ Np63 α , the predominantly expressed TP63 isoform in cancer cells and in the breast cancer cell lines we investigated, is amino-terminally truncated and can oppose the transactivation capabilities of the full-length protein [27,30]. The ability of Δ Np63 α to enhance proliferation, colony formation and invasiveness properties of breast cancer cells we observed, supports findings by others for a role of TP63 in tumorigenesis and breast cancer progression [38,40,60].

We showed that hsa-miR-196a2* is involved in the control of breast cancer proliferation since the inhibition of hsa-miR-196a2* with specific anti-miR enhanced breast cancer cell growth. The importance of hsa-miR-196a2* in tumor cell proliferation was further supported by the observation that proliferation of ER α -positive and ER α -negative breast cancer cells was greatly reduced by overexpression of this miRNA. Our studies also revealed that *in vitro* invasion and colony formation were inhibited by enforced hsa-miR-196a2* expression and that this was reversed by TP63 target protector, indicating an important role played by TP63 in these cellular effects.

Of note, we found that the expression of TP63 was inversely correlated with hsa-miR-196a2* levels in a large cohort [47] of human breast tumors encompassing ER α -positive, ER α -negative/HER2-positive and basal tumor subtypes, suggesting clinical relevance. Relative levels of hsa-miR-196a2* were dependent on ER α expression, as well as Her2 expression, implying that both ER α and MAPK activity may control levels of this miRNA in tumors. Interestingly, levels of hsa-miR-196a2* correlated inversely, almost

perfectly, with the levels of its target genes, further supporting the functionality of the ER α -HER2/hsa-miR-196a2*/TP63 circuitry in breast tumors. It is of interest also that a single nucleotide polymorphism in hsa-miR-196a2* has been shown to be significantly associated with breast cancer risk [44].

Understanding the altered regulation of miRNAs by factors acting through ER α and ERK2 in breast cancer cells is of considerable significance, since these signaling pathways are heavily involved in the development and progression of breast tumors, as well as in the responsiveness of breast cancer patients to endocrine therapies [22–26,61,62]. In this study, we show that the regulation of hsa-miR-196a2* by ER α and/or ERK2 signaling in breast cancer may contribute to the divergent physiological properties and clinical outcomes of different subtypes of breast cancers, those that are ER α -positive and those that are ER α -negative, mediated through differential TP63 expression affecting cell growth and invasiveness properties. Understanding potential mechanisms of miRNA-modulated cellular responses driven by ER α and/or protein kinase signaling could offer new strategies in breast cancer therapy for different subtypes of breast tumors in accordance with their miRNA signatures. In particular, increasing hsa-miR-196a2* expression in both ER α -positive and ER α -negative breast cancers might prove potentially to have effectiveness as a tumor-suppressive strategy.

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Abbreviations

ChIP	chromatin immunoprecipitation
ERα	estrogen receptor-alpha
E2	estradiol
ERK2	extracellular signal-regulated kinase 2
miR	microRNA
TP63	tumor protein 63
TSS	transcription start site

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Figure 1. Association of ERα and ERK2 binding sites with miRNA gene promoters and impact of ERα or ERK2 knockdown on miRNA regulation by estradiol (E2)

(A) ERα and ERK2 binding sites are shown near four E2 and ERK2 regulated miRNA genes. Shown are the binding sites at the hsa-miR-196a2, hsa-miR-135a2, hsa-miR-944 and hsa-miR-101-1 locus. (B) Impact of ERα or ERK2 knockdown on expression of these miRNAs that harbor overlapping ERα and ERK2 binding sites. MCF-7 cells were transfected with siCtrl, siERα or siERK2 and treated with 0.1% ethanol (veh) or 10 nM E2 for 6 h and expression levels of each miRNA were determined from our miRNA microarray analysis. miRs are arranged from left to right based on magnitude of regulation by E2.

Figure 2. Characterization of ERα, ERK2, and pSer5 RNA Pol II recruitment to the regulatory regions of miRNA genes

ERα (panel A) and ERK2 (panel B) recruitment to the overlapping binding sites of hsa-miR-196a2, hsa-miR-135a2, hsa-miR-944, and hsa-miR-101-1 genes and recruitment of phospho-serine5 RNA Polymerase II to the transcription start site (TSS) of the respective miRNA genes (panel C) after cell treatment with 10 nM E2 for 0, 45, or 120 min.

Figure 3. Relative levels of hsa-miR-196a2* and hsa-miR-196a2 in MCF-7 cells; time course of the E2 stimulated increase in hsa-miR-196a2* and the requirement for ERα and ERK2; prediction of miR-196a2* target genes; and impact of ERα and ERK2 knockdown on target gene expression

(A) Relative levels of hsa-miR-196a2* and hsa-miR-196a2 in control MCF-7 cells. (B) Time course monitoring changes in the level of hsa-miR-196a2* and hsa-miR-196a2 after treatment with 10 nM E2 for the times indicated. (C) Impact of ERα or ERK2 knockdown on the response of hsa-miR-196a2* to E2 treatment. MCF-7 cells were transfected with siCtrl, siERα or siERK2 for 48 h and then treated with 0.1% ethanol (veh) or 10 nM E2 for the indicated times. Total RNA was harvested and the expression level of hsa-miR-196a2* was determined by quantitative RT-PCR. (D) List of E2 down-regulated genes that are potential targets of hsa-miR-196a2*. (E) Effect of ERα or ERK2 knockdown on expression of three potential target mRNAs of hsa-miR-196a2*, TP63, SPRY1, and TFAP2. MCF-7 cells were transfected with siCtrl, siERα or siERK2 for 48 h and then treated with 0.1% ethanol (veh) or 10 nM E2 for 24 h. (F) Effect of siERα or siERK2 treatment on the level of TP63 (ΔNp63α) protein, and on ERα and ERK2 and pERK1/2 protein levels monitored by western blot analysis. Cells were treated as described in panel E. β-actin was used as a loading control.

Figure 4. Anti-miR-196a* reduces expression of hsa-miR-196a2* and increases expression of TP63 and proliferation of MCF-7 and BT474 cells

(A) MCF-7 cells were transfected with Anti-miR-196a* or negative control (Ctrl) and treated with 0.1% ethanol (veh) or 10 nM E2 for 24 h. Total RNA was harvested and expression level of hsa-miR-196a2* was determined using Taqman probe based quantitative PCR. (B) Impact of anti-miR-196a* on the expression of TP63 in MCF-7 cells treated with 0.1% ethanol (veh) or 10 nM E2 for 24 h. * p -value < 0.05. (C) Impact of anti-miR-196a* on MCF-7 cell proliferation. Cells were transfected with 20 nM anti-miR-196a* and then were treated with control 0.1% ethanol (veh) or 10 nM E2 for 4 days. Cell proliferation was assessed using WST-1 reagent. ***, p -value < 0.001; ****, p -value < 0.0001. (D) Impact of anti-miR-196a* on BT474 cell proliferation. Cells were transfected with 20 nM anti-miR-196a* and then were treated with control 0.1% ethanol (veh) or 10 nM E2 for 4 days.

Cell proliferation was assessed using WST-1 reagent. *, p-value <0.1; ***, p-value < 0.001; ****, p-value < 0.0001.

Figure 5. Expression of pre-miR-196a* greatly reduces TP63 expression and MCF-7 cell proliferation and these are reversed by TP63 target protector
 (A) Impact of TP63 target protector and pre-miR-196a* on TP63 mRNA. MCF-7 cells were transfected with 100 nM TP63 miScript target protector or negative control protector (Ctrl) with or without 10 nM pre-miR-196a*. After 48 h, cells were treated with 0.1% ethanol (veh) or 10 nM E2 for 24 h and RNA was then isolated for gene expression analysis. ** p-value < 0.01. (B) Impact of TP63 target protector and pre-miR-196a* on MCF-7 cell proliferation. Cells were transfected with 100 nM TP63 miScript target protector or negative control protector (Ctrl) with or without 10 nM pre-miR-196a*. Cells were then grown for 4 days and proliferation was assessed using WST-1 reagent. ** p-value < 0.01. (C) Effect of siTP63 treatment on TP63 mRNA level in cells treated with 0.1% ethanol (veh) or 10 nM E2

for 24 h. (D) Impact of TP63 knockdown on MCF-7 cell proliferation. Cells were transfected with 20 nM siGENOME Ctrl (siCtrl) or siTP63 and then were treated with 0.1% ethanol or 10 nM E2 for 4 days and cell proliferation was monitored. ** p -value < 0.01.

Figure 6. TP63, hsa-miR-196a, and ERα expression in human breast tumors, and TP63-mediated regulation of cell growth and invasion by hsa-miR-196a2* overexpression and effect of TP63 target protector in MDA-MB-231 cells

(A) Expression of the miRNAs, hsa-miR-196a1 and a2 (representing the same miR sequence but from a gene on a different chromosome) and hsa-miR-196a2* (complementary to the a2) and TP63 mRNA expression in human breast tumors. Analyses were performed by us on datasets of miRNA and mRNA sequencing in 132 human breast tumors from [47] as described in Methods. Tumor data for ERα, PGR, HER2, and potential hsa-miR-196a2* target genes (TFAP2A, SPRY1, TP63, NEUROD1, MGAT4A, and IGF1) are also shown in this Tree View Java hierarchical clustering analysis. (B) Relative levels of TP63 ΔNp63α protein in MCF-7 cells, MDA-MB-231 cells and MDA-MB-231 cells stably containing ERα. (C) Relative levels of hsa-miR-196a2* in MCF-7 cells, MDA-MB-231 cells, and MDA-MB-231 ERα-positive cells. (D) Impact of pre-miR-196a* overexpression and TP63 protector on MDA-MB-231 cell proliferation. Cells were transfected with 100 nM TP63 miScript target protector or negative control protector (Ctrl) with or without 10 nM pre-miR-196a*, or cells were transfected with pre-miR-196a* alone, and then were grown for 4 days. Cell proliferation was assessed using WST-1 reagent. ** p -value < 0.01. (E) MDA-MB-231 cells were transfected with pre-miR-196a* (or negative control, Ctrl), or with target protector oligonucleotides or siTP63 alone or together as indicated, and were then analyzed in *in vitro* invasion assays. ** p -value < 0.01. Western blots shown at right indicate effects of these treatments on ΔNp63α protein level. Total ERK2 was monitored as the internal reference loading protein. (F) MDA-MB-231 cells were transfected with negative control pre-miR (Ctrl), pre-miR-196a* or pre-miR-196a* and TP63 target protector and then analyzed for soft agar colony formation. Images are at 4x magnification. Colony numbers

per field were determined. Colony size was measured using ImageJ software. * p -value < 0.1; **, p -value < 0.01.

Figure 7. Regulation of cell proliferation, colony formation, and invasion by hsa-miR-196a2* and effect of TP63 target protector in three ER-negative breast cancer cell lines (MDA-MB-453, MDA-MB-468 and SKBR3)

(A) Impact of pre-miR-196a* overexpression and TP63 target protector on cell proliferation. Cells were transfected with negative control pre-miR (Ctrl), 10 nM pre-miR-196a* alone or 10 nM pre-miR-196a* with 100 nM TP63 miScript target protector, and then were grown for 4 days. Cell proliferation was assessed using WST-1 reagent. **** p -value < 0.0001. (B) Cells were transfected with negative control pre-miR (Ctrl), 10 nM pre-miR-196a* alone or 10 nM pre-miR-196a* with 100 nM TP63 miScript target protector as indicated, and were then analyzed in *in vitro* invasion assays. *, p -value < 0.1; **, p -value < 0.01; ***, p -value < 0.001. (C) Cells were transfected with negative control pre-miR (Ctrl), 10 nM pre-miR-196a* alone or 10 nM pre-miR-196a* with 100 nM TP63 miScript target protector and then analyzed for soft agar colony formation. Images are at 4x magnification. Colony numbers per field were determined. Colony size was measured using ImageJ software. *, p -value < 0.1; **, p -value < 0.01; ***, p -value < 0.001.

Figure 8. Model schematically depicting the effect of estradiol (E2), ERα, and ERK2 on hsa-miR-196a2* and TP63 regulation and their impact on breast cancer cell phenotypic properties ERα and ERK2 up-regulate hsa-miR-196a2* upon cell treatment with E2, resulting in the down-regulation of TP63, with consequent decrease in cell proliferation. Although E2-ERα, in collaboration with ERK2, is overall pro-proliferative in ERα-positive breast cancer cells, this model, based upon our findings, reveals that E2-ERα-ERK2 does regulate a pathway that has a net antiproliferative and tumor-suppressive effect. Potential modulation of this hsa-miR-196a2*-TP63 circuit might be capable of reducing the growth stimulatory actions of estrogens in ERα-positive breast cancer. Our findings also suggest that enhancement of this axis in ERα-negative breast cancers by alteration in miR-196a2* expression might also suppress cancer cell invasiveness and the aggressiveness of these breast cancers in a manner independent of ERα.