MicroRNA Sequencing of Long-Term Estrogen Deprived Breast Cancer Cells Reveals a Potential Role for miR-181a in Estrogen-Independent Growth

By

Erin L. Hayes

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Co-Chairperson: Joan Lewis-Wambi, Ph.D.

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Co-Chairperson: Lane Christenson, Ph.D.

_____

Jeremy Chien, Ph.D.

Date Defended: April 27, 2015

The Thesis Committee for Erin L. Hayes certifies that this is the approved version of the following thesis:

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Co-Chairperson: Joan Lewis-Wambi, Ph.D.

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Co-Chairperson: Lane Christenson, Ph.D.

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Abstract

Breast cancer is a genetically complex disease that affects over 230,000 people in the U.S. every year. Estrogen signaling has been shown to play a large role in the development and progression of breast tumors that express the estrogen receptor (ER). Thus, aromatase inhibitors (AIs), which prevent the synthesis of estrogen, have been increasingly valuable for the management of ER+ breast cancer. A frequent obstacle, however, is acquired resistance to AIs, characterized by heightened growth factor signaling and estrogen-independent growth. Another aspect of acquired AI resistance that is now being examined is the role of microRNA (miRNA). miRNAs are 18-22 base pair RNA molecules whose primary role is to inhibit expression of mRNA targets by inhibition of translation or by degradation of the mRNA. Here, we have conducted next-generation sequencing of the miRNAs from three breast cancer cell lines – one ER+ line that is estrogen-dependent (MCF-7), and two ER+ long-term estrogen deprived (LTED) models of estrogen-independent growth (MCF-7:5C and MCF-7:2A). We found that in the LTED MCF-7:5C and MCF-7:2A cells there were 338 and 241 miRNAs differentially expressed, respectively, compared to the MCF-7 cells. We also saw unique miRNA clusters expressed in each LTED cell line – on chromosome 14q32 in the MCF-7:5C cells and on chromosome 13q31 in the MCF-7:2A cells. Our initial *in vitro* work focused on the functional significance of miR-181a (overexpressed in both LTED cells). This work has shown that miR-181a is regulated by estrogen/ER signaling, that loss of miR-181a in the LTED MCF-7:5C cells has a significant effect on proliferation, and that overexpression of miR-181a increases the ability of MCF-7 cells to grow in an estrogen-deprived environment. Since our LTED MCF-7:5C cells are more sensitive to DNA damage and overexpress miR-181a, which targets BRCA1, it was surprising that these cells were not more sensitive to the PARP inhibitor olaparib, which may be explained by high basal expression of BRCA1. Overall, it is clear that the miRNA profile of estrogen-dependent breast cancer cells is unique from the estrogen-independent LTED cells, and miR-181a may have a functional role in the estrogen-independent phenotype of our LTED MCF-7:5C cells.

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Introduction

Breast cancer and the role of estrogen

Breast cancer is the second leading cause of cancer death and one in eight women will get breast cancer in her lifetime [1]. About 70% of all breast cancers express estrogen receptor alpha (ERα) and belong to the molecular subtypes luminal A or B [1, 2]. While the exact etiology of breast cancer is not known, there is strong evidence that estrogen plays a critical role in the development and progression of the disease. The earliest correlations of estrogen and breast cancer were reported in the late 19th century. In an 1896 issue of Lancet, Dr. George Beatson reported about a patient of his who presented with inoperable recurrent breast cancer, and because of his studies of lactation in sheep he predicted that removal of the ovaries would cause the breast cancer to regress [3]. Indeed, eight months after the oophorectomy, the woman showed no sign of the cancer [3]. In a similar report in 1900, Dr. Stanley Boyd concluded that oophorectomy can be used as adjuvant therapy for breast cancer patients who are older than 40 years of age but have not yet entered menopause [4]. These results are supported by a study showing that breast cancer xenografts in athymic, oophorectomized mice depend on the presence of estradiol, and another study that correlates breast cancer risk with levels of circulating estrogens, such as estradiol [5, 6].

Estrogen mediates its biological effects by binding to estrogen receptors ERα and ERβ, which are members of the nuclear receptor superfamily of ligand-inducible transcription factors [7, 8]. ERα is encoded by *ESR1*, a 473 kb gene located on chromosome 6, and has six functional domains, A-F, which include both ligand-binding and DNA-binding domains, as described by Kumar and co-workers (Fig. 1) [9]. There are two transactivation domains, AF-1 and AF-2, on ERα, which are binding sites for other transcription factors and coregulators of ERα. It is also important to note that there are multiple sites of phosphorylation on ERα (Fig. 1). For instance,

Ser118 phosphorylation by a MAPK (mitogen-activated protein kinase) or Ser167 phosphorylation by Akt (protein kinase B) can lead to activation of ERα, and when the expression of these kinases is aberrantly upregulated it can lead to ligand-independent phosphorylation and action of ERα [10, 11].

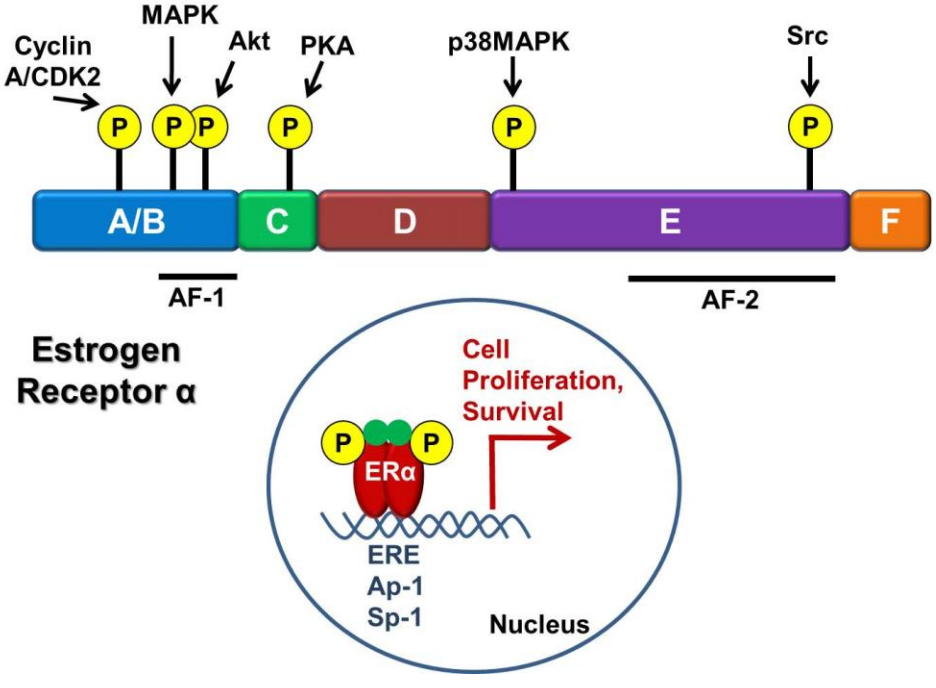


Figure 1. Functional domains and action of estrogen receptor alpha. The estrogen receptor alpha (ERα) protein has 6 functional domains (A-F) which allow it to perform its function as a transcription factor in the nucleus. This transcriptional regulation performed by ERα normally occurs when ERα is bound to its ligand – estrogen (green circles) – but can also occur in a ligand-independent manner. Both ligand-dependent and ligand-independent functions of ERα require phosphorylation and dimerization of the receptor and translocation to the nucleus where it performs its function as a transcriptional regulator of genes with promoter motifs such as estrogen receptor elements (ERE), or binding sites for the transcription factors Ap-1 and Sp-1. Many of the ERα target genes play a role in cell proliferation and survival.

In the classic model of estrogen signaling, estrogen diffuses across the cell membrane and binds ERα. The receptor dimerizes and translocates to the nucleus where it binds estrogen response elements (EREs) in the DNA, stimulating transcription of target genes involved in cell proliferation and survival (Fig. 1) [12]. Both ligand-bound and ligand-free ERα has been found bound to the promoter of ERα target genes, further confirming the existence of ligand-

independent ERα action [13]. ERα is also able to bind other transcription factors, such as Ap-1 and Sp-1, independent of EREs, where it may recruit co-activators to stimulate transcription of additional growth and survival genes [14]. Much of the molecular signaling mediated by ERα involves the MAPK and PI3K (phosphoinositide 3-kinase) pathways, the primary facilitators of cell growth and proliferation [15].

Endocrine therapies for breast cancer

Since ERα-positive breast cancers rely on estrogen signaling for proliferation, the most effective strategy to stop or slow the growth of these hormone-sensitive tumors is to block estrogen action in the tumor using endocrine therapy. Current endocrine therapies for ERα-positive breast cancer include: tamoxifen, the selective estrogen receptor modulator that antagonizes ERα function; fulvestrant, the pure anti-estrogen that degrades/downregulates ERα; and the more recent standard of care – aromatase inhibitors (AIs).

The aromatase enzyme (a cytochrome P450 heme-containing protein) is required for the synthesis of estrogen via aromatization of androgens such as testosterone [16]. Circulating levels of estrogen decrease as a woman enters menopause, since there is no longer production of estrogen by the ovaries. Thus, it is the local synthesis of estrogen by breast adipose tissue that plays a large role in the growth and survival of ERα-positive breast tumors [17]. Inhibition of aromatase activity in this adipose tissue is a rational treatment strategy to suppress estrogen production in the breast, thus inhibiting tumor growth.

Currently, there are three FDA-approved oral AIs in clinical use for the treatment of postmenopausal women with hormone receptor-positive breast cancer. These AIs can be divided into two categories: steroidal (exemestane) and non-steroidal (anastrozole, letrozole). There has been great interest in the development of AIs since it has been observed in clinical trials that they

can be more tolerable than tamoxifen, and are usually more effective or equivalently effective in clinical response rate and median time to progression [18]. Indeed, letrozole, anastrozole, and exemestane have all been shown to suppress circulating estrogen levels in post-menopausal breast cancer patients by more than 97% [19, 20].

Resistance to aromatase inhibitors

Unfortunately, the majority of patients treated with AIs are either intrinsically resistant or acquire resistance over time, leading to disease progression and death. Objective response rates for letrozole and anastrozole have been reported as 55% and 37%, respectively, indicating that nearly half of patients treated with non-steroidal AIs have innate resistance [21, 22]. In addition, nearly all breast cancers that show an initial response to AIs eventually develop an acquired resistance to the therapy [23]. There are several pathways that are implicated in the acquired AI-resistant phenotype. These include the MAPK [24], EGFR (epidermal growth factor receptor) [25], and PI3K pathways (Fig. 2) [26]. ERα has also been shown to play a role in AI resistance, in the form of a constitutively active ligand-independent mutant ERα [27], via different genome binding patterns [28], or simply by modified expression levels [29]. In fact, one study reveals that ligand-independent ERα activation is required for the development of an AI-resistant phenotype in the MCF-7aro (aromatase-overexpressing) cell line [30]. The phosphorylation of ERα by MAPK (Ser118) and Akt (Ser167) is often essential for the ligand-independent action of ERα, as discussed previously (Figs. 1, 2) [10, 11].

Since the mechanism by which AIs induce death of ERα-positive breast cancer cells often involves apoptosis, a disturbance in the balance of pro- and anti-apoptotic genes could also play a role in resistance to AI treatment (Fig. 2) [31]. Indeed, this imbalance has been shown in an aromatase-expressing MCF-7 cell line with a mutant ERα gene (K303R) [32]. The K303R

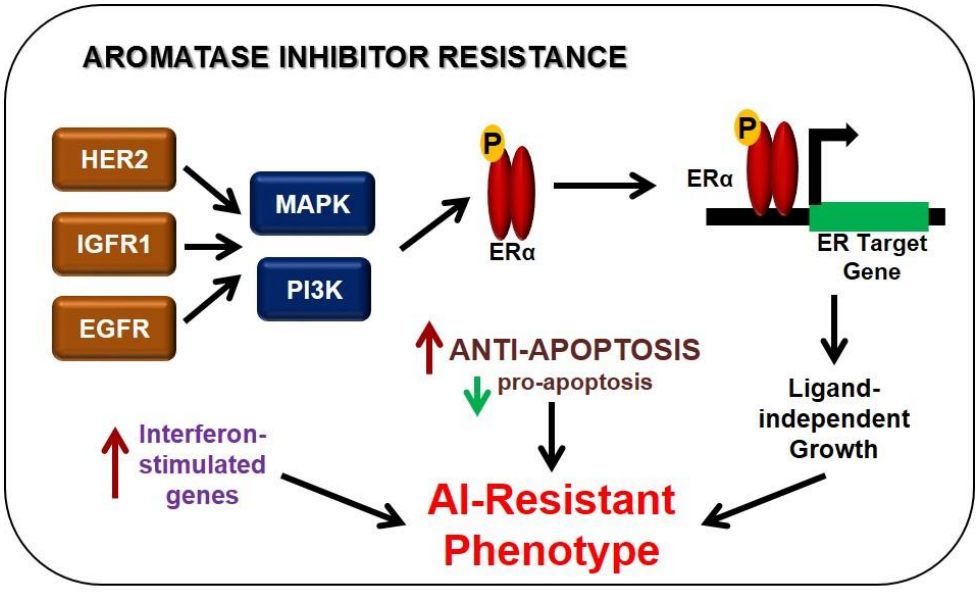


Figure 2. Mechanisms of aromatase inhibitor resistance in breast cancer cells. The mechanisms of AI resistance often involve growth factor pathway upregulation. The enhanced activity of growth factors such as MAPK can result in estrogen-independent phosphorylation and activation of ERα. In addition to growth factor signaling, interferon response genes and anti-apoptotic proteins have also been shown to have increased expression in AI-resistant cells.

mutation was shown to cause resistance to both tamoxifen and the AI anastrozole, and these K303R MCF-7/Aro cells have an increase in the Bcl-2 (anti-apoptotic)/Bax (pro-apoptotic) ratio, which is further exacerbated upon treatment with anastrozole [32].

The clonally selected LTED (long-term estrogen deprived) models MCF-7:5C and MCF-7:2A, used in our lab, have also revealed some of the genetic reprogramming that occurs when breast tumor cells are deprived of estrogen long term [33]. Both of these cell lines were derived from MCF-7 cells cultured in phenol red-free media (phenol red has estrogenic activity) containing estrogen-free serum for 8 months, followed by two rounds of limited dilution cloning [34, 35]. A summary of the characteristics that these cells display can be found in Table 1.

Jiang *et al.* first described the MCF-7:5C cells in 1992, indicating that they have the following characteristics: no significant alterations in ERα, loss of estrogen-stimulated PR expression, same ability as MCF-7 to bind ERE, less ERE reporter activity, and no response to

treatment with estrogen, fulvestrant, or tamoxifen [34]. Since then, it has been well-established that the MCF-7:5C cells undergo apoptosis following estrogen treatment when they are cultured in media containing charcoal-stripped fetal bovine serum instead of the original media which contained charcoal-stripped calf serum (Table 1) [36, 37].

Table 1. Characteristics of clonally-derived, long-term estrogen deprived MCF-7 cells – MCF-7:5C (5C) and MCF-7:2A (2A)

Characteristic	Cell Line(s)	Reference(s)	Comments
Cell line first described	5C	[34]	Publication in 1992
Cell line first described	2A	[35]	Publication in 1995
Express 77 kDa ERα	2A	[38-40]	Originally described as 80 kDa
Estrogen-induced apoptosis	5C	[33, 36, 37, 41-49]	This characteristic is unique to cells cultured in RPMI media containing 10% charcoal-stripped fetal bovine serum.
Differences in sensitivity to estrogen-induced apoptosis	5C and 2A	[33, 50]	The MCF-7:2A cells are less sensitive to estrogen-induced apoptosis than the MCF-7:5C cells.
BSO (Buthionine Sulfoximine) sensitization to estrogen-induced apoptosis	2A	[51, 52]	
Identification of proteins involved in estrogen-independent growth or increased aggression	5C	[53] [54] [55] [49]	PKCα PEDF cMYC via CDK9 IFITM1
Identification of proteins involved in estrogen-independent growth or increased aggression	5C and 2A	[56]	CEACAM6
Unique miRNA expression	2A	[57]	miR-125b

The MCF-7:2A cells were first described in 1995 by Pink *et al.* as an additional model of breast cancer cells that exhibit estrogen-independent growth [35]. In this first paper, MCF-7:2A cells were described as having the following characteristics: expression of an 80 kDa ERα in addition to wild type (Table 1), increased ERE reporter activity, and decreased growth upon treatment with fulvestrant and tamoxifen [35]. The larger ERα protein detected in these MCF-7:2A cells has since been described as a 77 kDa protein that does not play a functional role in the cells in terms of estrogen-mediated action, but may interfere with normal ERα activity [38-40].

In our lab, we have confirmed that the MCF-7:5C and MCF-7:2A cell lines have gained the ability to grow in an estrogen-independent manner, unlike their parental cell line, MCF-7, whose growth is estrogen-dependent (Fig. 3A). We have shown that our LTED cells also express

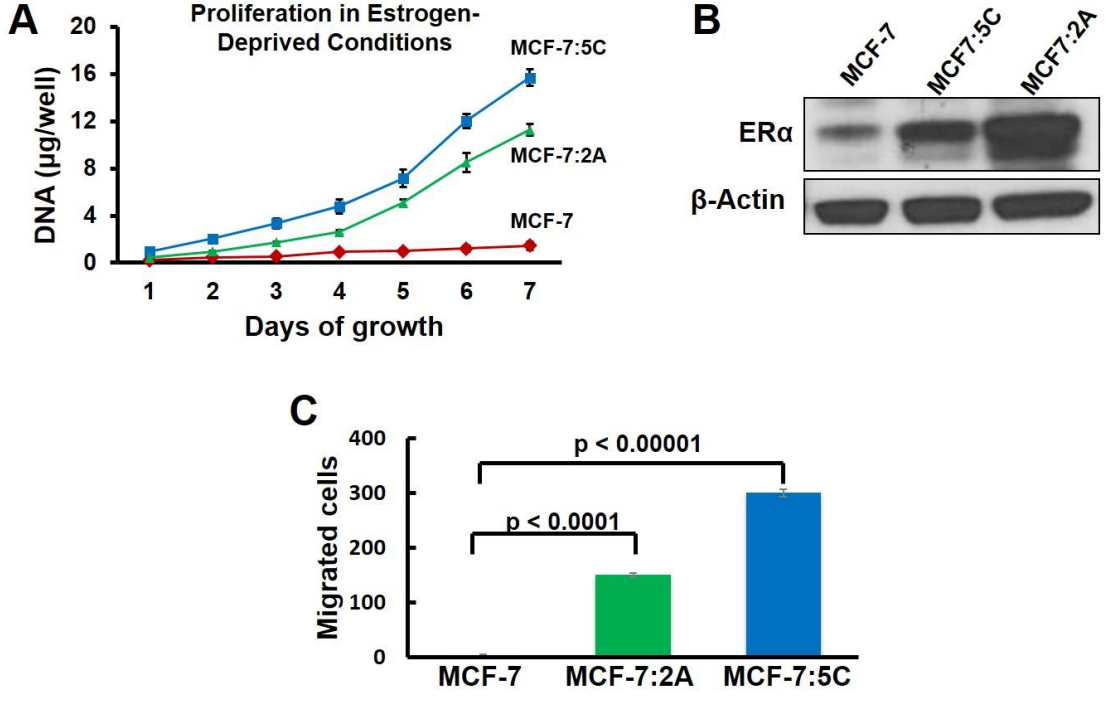


Figure 3. Phenotype of estrogen-dependent MCF-7 cells compared to estrogen-independent LTED MCF-7:5C and MCF-7:2A cells. (A) The MCF-7:5C and MCF-7:2A cells have gained the ability to proliferate in estrogen-deprived conditions, whereas the MCF-7 cells have significantly decreased proliferation in these conditions. **(B)** The basal expression of ERα is increased in the MCF-7:5C, and even more so in the MCF-7:2A cells compared to the MCF-7 cells. **(C)** The ability of the MCF-7:5C and MCF-7:2A cells to migrate through a transwell is significantly increased compared to MCF-7, indicating an increased ability to migrate.

ERα at higher levels than the MCF-7 cells (Fig. 3B), and are more aggressive – as indicated by their increased ability to migrate through a transwell (Fig. 3C).

Notably, recent findings from our lab have identified a critical role for interferon-stimulated genes in the estrogen-independent growth of the LTED MCF-7:5C cells (Fig. 2). Interferons are cytokines secreted by immune cells in response to viral infection or other pathogens [58]. One of these interferon-stimulated genes, IFITM1 (interferon induced transmembrane protein 1), has been shown to be markedly overexpressed (> 25-fold) in LTED MCF-7:5C breast cancer cells and AI-resistant human breast tumors (Table 1) [49]. Normally IFITM1 is important for inhibiting viral infection of cells, but it has also been associated with progression of cancers such as colorectal cancer and head and neck cancer [59-61]. Interestingly, we have found that overexpression of IFITM1 is associated with enhanced cell survival, proliferation, and invasiveness of LTED breast cancer cells, and knockdown of IFITM1 induces cell death and blocks the ability of the MCF-7:5C cells to migrate and invade [49].

microRNAs and breast cancer

microRNAs (miRNAs) are small 18-22 base pair RNA molecules that regulate the expression of target mRNAs by inhibiting translation or degrading the transcripts [62]. After miRNA genes are initially transcribed by RNA polymerase II, a process regulated by transcription factors and nuclear receptors, the transcripts undergo significant processing both in the nucleus and cytoplasm (Fig. 4) [63]. First, the pri-miRNA sequence is cleaved in the nucleus by Drosha, a class 2 RNase III enzyme that works in conjunction with DGCR8 (DiGeorge syndrome chromosomal region 8). The resulting pre-miRNA hairpin is exported from the nucleus to the cytoplasm via Exportin 5 where it is further cleaved by Dicer, another member of the RNase III family, resulting in a short double strand piece of RNA. The two strands of the

RNA then separate into the passenger strand, which often gets degraded, and the mature miRNA strand, which binds to argonaute (Ago) proteins to form the RISC (RNA-induced silencing complex) [62]. The miRNA of the RISC guides the complex to the 3'UTR of its target mRNAs. Perfect or near-perfect complementarity of the miRNA to the target mRNA stimulates Ago2 in

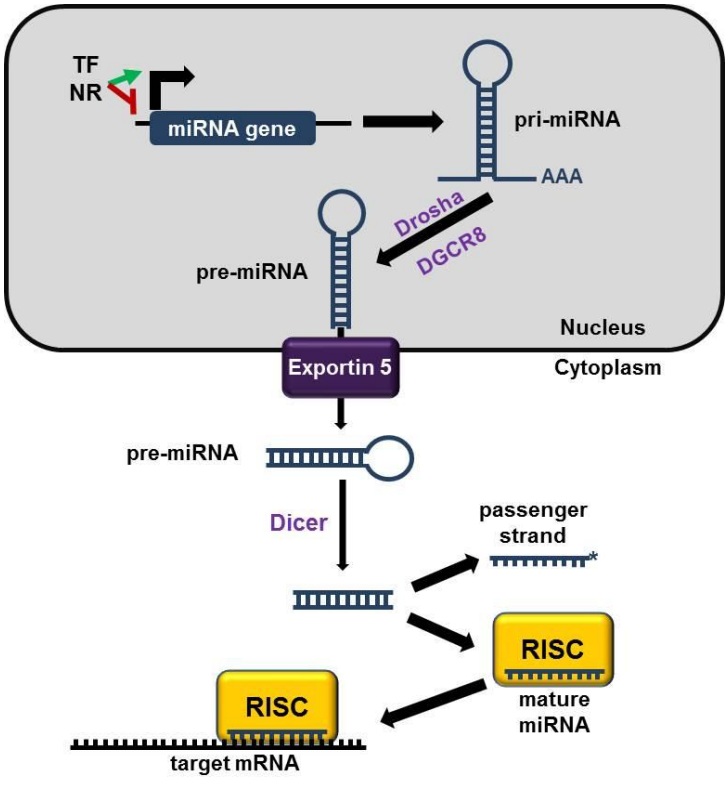


Figure 4. Standard pathway by which miRNAs are processed and loaded onto RISC to regulate gene expression. Regulation of miRNA expression is controlled at the miRNA promoter by transcription factors (TF) and nuclear receptors (NR). After transcription, the pri-miRNA is processed inside the nucleus by Drosha and DGCR8 to form pre-miRNA – a hairpin miRNA. Exportin 5 exports the pre-miRNA from the nucleus into the cytoplasm where it gets cleaved further by Dicer, resulting in a short double strand piece of RNA. These strands are separated into the passenger strand, which often gets degraded, and the mature strand, which is loaded onto the RISC (RNA-induced silencing complex) for action on target mRNA. If the mature miRNA sequence is a perfect or near-perfect match to its target mRNA 3'UTR, the transcript is degraded by Ago2. However, if there are enough mismatches between the miRNA and its target mRNA, the result is inhibition of translation.

the RISC to degrade the mRNA [64]. However, if there is only partial complementarity, the translation of the mRNA will be inhibited.

Expression profiles of miRNA in breast tumor samples have been correlated with biopathologic features such as hormone receptor status and proliferation index, and are used to distinguish between basal and luminal subtypes [65, 66]. For example, miRNAs overexpressed in basal, ERα-negative primary breast cancers include miR-150, which has been shown to promote breast cancer growth [67], and miR-135b, which correlates with early metastasis of

breast cancer cells [68]. miRNAs overexpressed in luminal, ERα positive breast cancers include miR-126 and miR10a, which are associated with an increase in patients' relapse-free time after tamoxifen treatment [69].

Beyond the use of expression profiles, specific miRNAs have been associated with regulation of genes involved in breast cancer. The let-7 miRNA family has been shown to regulate the self-renewal capacity of breast tumor-initiating cells derived from cell lines and primary patient tumors by inhibition of HRAS (Harvey rat sarcoma viral oncogene homolog) and HMGA2 (high mobility group AT-hook2), genes involved in self-renewal and differentiation, respectively [70]. miR-21, which targets many genes, including PTEN (phosphatase and tensin homolog), was identified to be upregulated in primary patient samples of invasive breast cancer compared to normal breast tissue by miRNA in-situ hybridization staining [71]. Two miRNAs, miR-373 and miR-520c, are considered metastasis-promoting miRNAs and are shown to be upregulated in lymph node metastases compared to primary tumor samples [72]. Promotion of tumor invasion and metastasis by miR-373 and miR-520c is likely achieved via suppression of the CD44 gene, which codes for a hyaluronan receptor and has been identified as a metastasis suppressor in breast cancer [73].

microRNAs and resistance to aromatase inhibitors

There has been little research done on the topic of miRNAs involved in AI resistance, but recent studies have begun to shed light on the field. Masri *et al.* conducted a miRNA expression analysis for four AI-resistant cell models, all derived from MCF-7aro (aromatase-overexpressing) cells [74]. MCF-7aro cells were treated with testosterone and each of three AIs until they acquired resistance. An LTED cell line (LTEDaro) was also derived from the MCF-7aro cells for comparison. When compared to LTEDaro cells and cells treated only with

testosterone, letrozole-resistant cells overexpressed miR-128a, suggesting it plays a role in AI resistance. miR-128a is associated with breast cancer aggressiveness, and is involved in regulation of TGFβR1 (transforming growth factor beta receptor 1) [75]. Inhibition of miR-128a in the letrozole-resistant cells led to re-sensitization to TGFβ growth-inhibitory effects [74]. It is also interesting to note that the miRNA expression profiles for the cells resistant to the steroidal AI (exemestane) and the non-steroidal AIs (letrozole, anastrozole) were distinct from one another, and the AI-resistant cells were distinct from the LTEDaro model of AI resistance, which supports the idea that multiple mechanisms of acquired resistance to AIs may exist [74].

In addition to the confirmed role of miR-128a, Masri *et al.* also briefly mentioned the downregulation of miR-125b in the letrozole-resistant, anastrozole-resistant, and exemestane-resistant cells compared to the LTEDaro cells [74]. A recent study in MCF-7:2A (LTED) cells by Bailey *et al.* confirms downregulation of miR-125b in AI-resistant cells and also indicates relevance of the other members of its miRNA cluster (a cluster is defined as miRNAs that are transcribed in one primary transcript and cleaved into individual hairpin loops in the nucleus by Drosha) [57]. The let-7c/miR-99a/miR-125b miRNA cluster encoded within the *LINC00487* gene was shown to be downregulated in MCF-7:2A cells compared to MCF-7 parental cells. A luciferase reporter assay confirmed that let-7c and miR-125b bind the HER2 (human epidermal growth factor receptor 2) 3'UTR, but it was revealed that while miR-125b directly regulates HER2 expression via the binding of HER2 3'UTR, let-7c regulation of HER2 is indirect via the inhibition of Dicer, one of the proteins involved in miRNA function [57]. Upon analysis of clinical data from The Cancer Genome Atlas, it was clear that let-7c and HER2 were inversely correlated in Luminal A breast tumors and that low expression of the let-7c/miR-99a/miR-125b cluster was associated with worse overall survival compared with patients who had high expression of this cluster [57].

Interestingly, the most recent study by Vilquin *et al.*, which also surveyed the miRNAs associated with AI resistance, contains results that conflict with the other two studies [57, 74, 76]. While Masri *et al.* and Bailey *et al.* found a downregulation of miR-125b in their AI-resistant models, Vilquin *et al.* showed an upregulation of miR-125b in AI-resistant models derived by long term treatment of MCF-7aro cells with letrozole (Res-Let) and anastrozole (Res-Ana) [76]. In fact, this study showed that miR-125b overexpression is sufficient to confer resistance to AIs in MCF-7aro cells and this is accomplished by activation of the Akt/mTOR pathway [76]. Analysis of primary breast tumors revealed that high levels miR-125b expression were associated with shorter relapse-free survival in luminal A breast cancers [76].

Another study of miRNA expression in MCF-7 cells revealed several miRNAs that were increased following letrozole treatment [77]. Perhaps the most interesting result was the upregulation of let-7f upon treatment with letrozole, as it inhibits the expression of the aromatase gene (*CYP19A1*) [77]. This study may reveal a mechanism of resistance similar to that of tamoxifen resistance – loss of the inhibitor's target. Just as tamoxifen resistance occurs in conjunction with absence of ERα, resistance to AIs may eventually develop due to pronounced loss of aromatase expression due to upregulation of certain miRNAs upon treatment.

In the past couple of decades, conducting genome- and transcriptome-wide expression analyses of cancer cells has become popular, and the development of microarrays and next-generation sequencing technologies have made these analyses possible. Next-generation sequencing has outpaced traditional sequencing as a faster and more accurate tool, and is used for many applications including DNA sequencing, transcriptome analysis, chromatin immnuoprecipitation analysis, and small RNA discovery and analysis. We have conducted next-generation sequencing of the miRNAs from three breast cancer cell lines – one ERα-positive line that is estrogen-dependent (MCF-7), and two ERα-positive LTED models of estrogen-

independent growth (MCF-7:5C and MCF-7:2A), which are considered models of the AI-resistant phenotype. Differential expression analysis revealed the differences in miRNA expression between the MCF-7 cells and the LTED MCF-7:5C and MCF-7:2A cells. Of the miRNAs that were determined to be upregulated in both MCF-7:5C and MCF-7:2A vs. MCF-7, we proceeded to conduct *in vitro* work to determine the potential role of miR-181a in estrogen-independent growth.

miR-181a has been described in multiple cancers, such as hepatocellular carcinoma [78-80], acute myeloid leukemia [81], cervical cancer [82], pancreatic cancer [83], and colorectal cancer [84, 85]. In addition, miR-181a plays a significant role in breast cancer aggressiveness [86], response to chemotherapy [87], metastasis [88], and formation of mammospheres [89]. The clinical significance of miR-181a in breast cancer has also been described by comparing miRNA expression in serum samples of 205 women who eventually developed breast cancer vs. 205 women who remained breast cancer free [90]. This study revealed an increase in the expression of 21 miRNAs in the samples from women who developed breast cancer, including miR-181a (15% overexpressed, $p = 0.05$) [90]. Since the serum samples were taken before the women developed breast cancer, miR-181a could potentially be used as a biomarker for early detection of breast cancer.

Transcription of the miR-181a-1/miR-181b-1 cluster on chromosome 1q32.1 has been shown to be regulated by MYCN (v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog), which binds directly to the miR-181a promoter [91]. The miR-181a promoter also contains multiple binding sites for the β-catenin/Tcf4 transcription factor complex, which has been correlated with enhanced expression of miR-181a, and Tcf4 was shown by chromatin immunoprecipitation to bind the miR-181a promoter [79]. In addition to regulation by MYCN and β-catenin/Tcf4, miR-181a expression has been shown to be upregulated by TGF-β, and

HER2 [79, 88, 92], and inhibited by NF-κB and estrogen signaling via ERα [89, 93]. In the microarray done by Masri *et al.*, miR-181a was shown to be overexpressed in the LTEDaro cells compared to parental MCF-7aro, which confirms a potential role of miR-181a in the estrogen-independent phenotype [74]. There are many targets of miR-181a, some of which play a role in regulating DNA damage repair, such as ATM (ataxia telangiectasia mutated) [86] and BRCA1 (breast cancer 1, early onset) [94]. Others, such as PTEN, PR (progesterone receptor), and Bim, are known to play roles in growth and apoptosis of cancer cells.

It is clear that there is a role for miRNAs in the development of resistance to AIs, as modeled by estrogen-independent and AI-resistant cells, but also clear is the need for more work to further characterize the miRNA expression profiles of AI-resistant cell models and tumors. Work in the field of miRNAs and AI resistance will contribute to the identification of biomarkers and novel therapeutic targets for AI-resistant breast tumors. In this study, we hypothesized that our estrogen-independent cell models of AI-resistance have unique miRNA profiles compared to parental estrogen-dependent cells, and that miR-181a has a specific role in the development of this estrogen-independent phenotype. This hypothesis was tested by the following specific aims:

I. To characterize the miRNA profiles of MCF-7 breast cancer cells and the long-term estrogen-deprived MCF-7:5C and MCF-7:2A cell models of aromatase inhibitor resistant breast cancer.

II. To determine the role of miR-181a in the estrogen-independent growth of the MCF-7:5C and MCF-7:2A long-term estrogen-deprived cell models of aromatase inhibitor resistant breast cancer.

Materials and Methods

Reagents

RPMI-1640, fetal bovine serum (FBS), antibiotic/antimycotic solution (containing 10,000 U/mL penicillin and 10 mg/mL streptomycin, 25 µg/mL of Fungizone®), non-essential amino acids (NEAA), L-glutamine, and TrypLE (containing trypsin and EDTA) were all obtained from Invitrogen (Grand Island, NY). Insulin (from bovine pancreas) and 17β-estradiol (E2) were obtained from Sigma-Aldrich (St. Louis, MO). E2 was dissolved in 100% ethanol at a stock concentration of 1 mM and stored at -20°C. Fulvestrant (ICI) was dissolved in 100% ethanol at a stock concentration of 1mM and stored at -20°C.

Primary antibodies were used against the following proteins: BRCA1 (1:200, Santa Cruz #642), ATM (1:200, Santa Cruz #23921), PTEN (1:1000, Cell Signaling #9559), ERα (1:200, Santa Cruz #544), and β-actin (1:15,000, Sigma-Aldrich #A5441). HRP-conjugated secondary antibodies were used against rabbit (Cell Signaling #7074) or mouse (Cell Signaling #7076).

Cell Culture Conditions

The ERα-positive hormone-dependent human breast cancer cell line MCF-7 was originally obtained from the American Type Culture Collection (Manassas, VA) and was maintained in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 µg/mL), Fungizone® (25 ng/mL), 1× NEAA (Invitrogen), and bovine insulin at 6 ng/mL (Sigma-Aldrich). The MCF-7:5C and MCF-7:2A cell lines were developed by long term estrogen deprivation of the MCF-7 parental cells followed by clonal selection [34, 35]. The MCF-7:5C and MCF-7:2A cells were maintained in phenol red-free RPMI-1640 supplemented with 10% dextran-coated charcoal-stripped FBS, 2 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 µg/mL), Fungizone® (25 ng/mL), 1× NEAA

(Invitrogen), and bovine insulin at 6 ng/mL (Sigma-Aldrich). Cells were cultured at 37°C in 5% CO_2 and were subcultured every three to four days.

RNA Isolation

Total RNA was extracted from breast cancer cell lines, in duplicate, for sample submission to the sequencing facility. Cells were plated on 60mm plates and grown 48 hours at 37°C, 5% CO_2. Cells were harvested immediately and lysed with Lysis/Binding Buffer (mirVana kit, Ambion). RNA extraction was carried out using the mirVana total RNA isolation protocol (Ambion). RNA concentration, 260/230 ratios, and 260/280 ratios were measured using a NanoDrop system immediately after isolation. Samples were kept in -80°C. Dilutions of these samples were prepared for submission to the sequencing core – 300ng/μL, 10μL total – and were analyzed on the Agilent 2100 Bioanalyzer for RNA integrity.

Library Preparation

Total RNA (1μg) was used to initiate the TruSeq Small RNA library preparation protocol (Illumina #RS200-0012 kit A). The small RNA fraction was ligated with 3' and 5' RNA adapters followed by a reverse transcription reaction and PCR amplification using 11 cycles of amplification. Size selection and purification of the cDNA library construct was conducted using 3% marker H gel cassettes on the Pippin Prep size fractionation system (Sage Science). The Agilent 2100 Bioanalyzer was used with the High Sensitivity DNA kit (Agilent #5067-4626) or the DNA1000 kit (Agilent #5067-1504) to validate the purified libraries. Libraries were quantified on the Illumina ECO Real Time PCR System using KAPA SYBR Universal Library Quant kit – Illumina (KAPA Biosystems KK4824). Following quantification, libraries were adjusted to a 2nM concentration and pooled for multiplexed sequencing.

Rapid Read Next-Generation Sequencing

miRNA sequencing was performed using the Illumina HiSeq2500 Sequencing System at the University of Kansas Medical Center – Genomics Core (Kansas City, KS). Libraries were denatured and diluted to the appropriate pM concentration (based on qPCR results) followed by clonal clustering onto the sequencing flow cell using the TruSeq Rapid Single Read (SR) Cluster Kit-HS (Illumina GD402-4001). The clonal clustering procedure was performed using the automated Illumina cBOT Cluster Station. The clustered flow cell was sequenced on the Illumina HiSeq 2500 Sequencing System in Rapid Read mode with a 1x50 cycle read and index read using the TruSeq Rapid SBS kit-HS (Illumina FC402-4002). Following collection, sequence data was converted from .bcl file format to FASTQ files and de-multiplexed into individual sequences for further downstream analysis.

Bioinformatic Analysis

To discover the miRNAs within the FASTQ files, the program miRDeep2 [95] was used. The reads were mapped to the hg19 human genome and, using a probabilistic model that scores reads based on structure and signature, the reads were identified using the microRNA database and novel miRNAs were predicted. Next, the Bioconductor package edgeR [96] was employed to normalize each miRNA read count to the total number of sequencing reads for each sample, and to determine the differential miRNA expression between the cell lines. The list of differentially expressed miRNAs was refined by using the following parameters: fold change greater than 1.5 and false discovery rate less than 0.05. Lists of genes targeted by the miRNAs were compiled by use of miRTarBase [97], a database of validated miRNA-target interactions.

miRNA Real-time quantitative PCR (qPCR)

For miRNA qPCR, total RNA was isolated from cultured cells using the mirVana RNA isolation kit (Ambion) according to manufacturer's protocol. cDNA was synthesized using the miRCURY LNA Universal cDNA synthesis kit II (Exiqon) using 10 ng of total RNA. qPCR was performed to evaluate the miRNA levels using the miRCURY ExiLENT SYBR® Green Master Mix (Exiqon), primer mix (Exiqon), and a 1:40 dilution of cDNA. The primer mixes were as follows: miR-103a-3p, #204063; miR-181a-5p, #204566; miR-221-3p, #204532; miR-222-3p, #204551; miR-127-3p, #204048.

Western Blot

Cells were harvested in cold 1X PBS and cell pellets were lysed in 100μL of lysis buffer (RIPA buffer, 1% protease inhibitor cocktail, and 1% phosphatase inhibitor). Lysates were sonicated and centrifuged to collect protein. The protein in each sample was quantified using the Bio-Rad protein assay (Bio-Rad) and samples were prepared for Western blot. 4-12% SDS-polyacrylamide gels were used to separate the proteins and the proteins were transferred to polyvinylidine difluoride membrane for 220 minutes at 30V on ice. The membranes were blocked in 5% non-fat milk prepared in Tris-buffered saline with Tween-20 (TBST). The membranes were then incubated in primary antibody, followed by three washes in TBST, and then incubated in the horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG. Bands of protein on the membrane were detected by addition of Amersham ECL Plus Western blotting detection reagents.

Drug and Radiation Treatments

For treatment with taxol, a 10 μM working stock was prepared in ethanol from a 10 mM stock and kept in -20°C. The 10 μM stock was diluted in media for treatments as needed. Dose

response curves required dilutions of 0.01, 0.1, 1.0, 10, and 100 nM. For treatment with olaparib (a gift from Dr. Jeremy Chien, University of Kansas Medical Center), a 50 µM stock was kept in -20°C and diluted in media for treatments as needed. For the treatment with multi-fraction dose ionizing radiation, cells were exposed to 2.5 Gy once per day for four consecutive days.

Proliferation Assays

The Cell Titer Blue® (Promega) assay was performed according to manufacturer's protocol to measure the viability of cells after treatment with chemotherapeutic drugs and radiation. Cells were seeded 5,000 per well in 96 well culture-treated plates and treatment was applied 24 hours later, after the cells had attached to the plate. Cell Titer Blue ® solution was diluted 1:5 in cell culture medium, added to cells, and cells were incubated at 37°C for 2 hours. Fluorescence was measured at 560/590 nm and graphed as a percentage of the untreated cells.

The CyQUANT DNA quantification assay was performed according to manufacturer's protocol to measure the proliferation rate of cells after transfection with the miR-181a inhibitor. Cells were seeded 5,000 per well in 96 well culture-treated plates and transfected 24 hours later, after the cells had attached to the plate. CyQUANT dye binding solution was prepared, added to cells, and cells were incubated at 37°C for 15 minutes. Fluorescence was measured at 485/530 nm and graphed as a percentage of the untreated cells.

Colony Formation Assays

Cells were plated in 12-well culture-treated plates at 500 cells per well and allowed to attach overnight. Transfection with miR-181a mimic or taxol treatments were done as indicated, and the cells were allowed to grow for 6 days uninterrupted. Medium was removed from each well and cells were rinsed with 1X PBS. Fixation and staining of colonies was done with a mixture of 0.5% crystal violet in 1:7 acetic acid:methanol for 2 minutes. A series of washes with 1X PBS was performed and images were taken and quantified using ImageJ software.

Transfections

For miR-181a knockdown experiments, cells were transfected with a mirVana miR-181a inhibitor or negative control inhibitor #1 (Ambion). Cells were transfected with 12.5nM of inhibitor using Lipofectamine 2000 according to the manufacturer's protocol in OPTI-MEM. Media was changed back to the normal complete media for each cell line 24 hours after transfection. Transfection efficiency was determined by qPCR for miR-181a expression.

For miR-181a mimic experiments, cells were transfected with a mirVana miR-181a mimic or negative control #1 (Ambion). Cells were transfected with 20nM of mimic using Lipofectamine 2000 according to manufacturer's protocol in OPTI-MEM. MCF-7 cells were plated for this experiment in estrogen-containing media and either kept in estrogen containing media (labeled E2-containing in figures), or switched to estrogen-deprived media (labeled E2-deprived in figures) 24 hours after transfection. Transfection efficiency was determined by qPCR for miR-181a expression.

For ERα knockdown experiments, cells were transfected with siRNA targeting ERα (siER) or a non-targeted siRNA (siCtrl) (Santa Cruz Biotechnology). Cells seeded in 6 well plates were transfected with 5nM of siRNA using Lipofectamine 2000 according to the manufacturer's protocol in OPTI-MEM. Transfection efficiency was determined by Western blot for ERα protein expression.

Statistical Analysis

All statistical comparisons of data were done using GraphPad Prism® software. Unpaired two-tailed t tests with 95% confidence intervals were performed for each set of data, and p-values were reported on the graphs.

Results

miRNAs differentially expressed in LTED versus MCF-7 cells

miRNA expression profiles were obtained for MCF-7 breast cancer cells, as well as the LTED, clonally-derived cell lines MCF-7:5C and MCF-7:2A by next-generation sequencing. The read counts were normalized by total reads and mapped to the hg19 reference genome using the program miRDeep2 [95]. miRNAs that were differentially expressed between the cell lines were discovered using the bioinformatic tool edgeR [96] (Fig. 5A). Fold change was analyzed

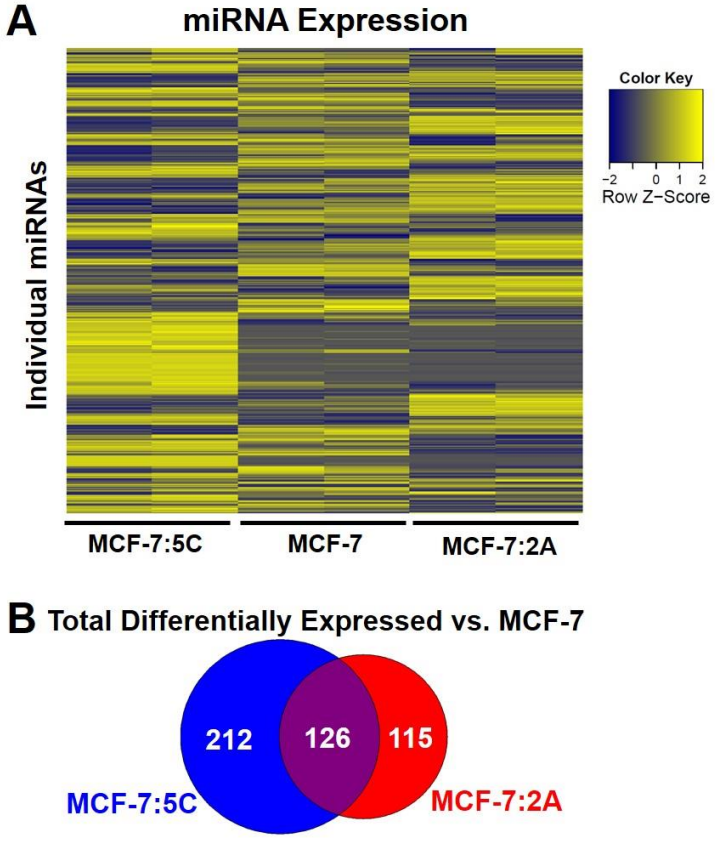


Figure 5. Next-generation sequencing of miRNAs reveals differences between estrogen-dependent and estrogen-independent cells. Rapid read 50-cycle next-generation sequencing of our estrogen-dependent MCF-7 cells and estrogen-independent MCF-7:5C and MCF-7:2A cells was conducted, and differential expression analysis was performed. **(A)** A partial heat map of miRNA expression in MCF-7 cells vs. the MCF-7:5C and MCF-7:2A cells. **(B)** 338 miRNAs are differentially expressed between MCF-7 and MCF-7:5C cells, while 241 miRNAs were differentially expressed between MCF-7 and MCF-7:2A cells.

for each of the following comparisons: MCF-7 vs. MCF-7:5C, MCF-7 vs. MCF-7:2A, MCF-7:5C vs. MCF-7:2A, and MCF-7 vs. [MCF-7:5C and MCF-7:2A]. When compared to MCF-7, MCF-7:5C had 338 differentially expressed miRNAs while MCF-7:2A had only 241 (Fig. 5B). Individual miRNA profiles were defined for each of the LTED cell lines, and there were 126 miRNAs that both LTED cell lines expressed in common, relative to MCF-7 (Table 2). The 126 miRNAs that were differentially expressed in both MCF-7:5C and MCF-7:2A include miRNAs that were also identified by the previous studies of miRNA expression in AI-resistant cells (Table 3), which indicates their relevance for further study [74, 98].

Table 2. microRNAs significantly differentially expressed in LTED cells vs. MCF-7

miRNA	Fold Change in MCF-7:5C vs. MCF-7	Fold Change in MCF-7:2A vs. MCF-7	Targets
miR-30a-5p	25.5	115	Eya2, AVEN, MTDH
miR-181a-5p	5.78	2.30	BRCA1, PTEN, ATM
miR-152-3p	4.26	3.25	DNMT1, IGF-IR, Rictor
miR-181b-5p	3.90	1.89	Bcl-2, ATM
miR-505-3p	2.99	2.13	SFRS1
miR-450b-5p	2.89	3.52	HER3, PAX9, ENOX2
miR-486-5p	2.82	3.50	PIM-1, FOXO1
miR-22-3p	2.18	2.35	ERα, TET2, Bmp7/6
miR-148a-3p	2.08	2.66	DNMT1, ROCK1
miR-424-5p	2.00	2.68	FGFR1, MAP2K1
miR-93-3p	-1.64	-1.59	TGFβR2, CDKN1A
miR-182-5p	-1.82	-2.40	RECK, MIM, FOXF2
miR-30d-5p	-2.07	-1.87	GRP78, MTDH
miR-27b-3p	-2.66	-1.66	PPARγ, ST14
miR-21-5p	-2.78	-2.27	TIMP3, PTEN, PDCD4
miR-342-3p	-4.96	-3.07	CCNB1, TP53
miR-10a-5p	-5.56	-8.36	Bcl-6, HOXD10
miR-221-3p	-13.4	-94.6	ERα, DDIT4, RB1
miR-222-3p	-17.3	-79.3	ERα, PUMA, TRPS1
miR-675-5p	-67.6	-231	RB, IGF1R

Table 3. miRNAs found to be upregulated in both LTED cell lines are also upregulated in the AI-resistant cell lines in other studies published on miRNAs and AI resistance

MCF-7:5C and MCF-7:2A	Masri *et al.* 2010	Bailey *et al.* 2015	Vilquin *et al.* 2015
miR-30a-3p/5p	EXE-R	MCF-7:2A	Res-Let, Res-Ana
miR-181a-5p	LTEDaro		
miR-152-3p			
miR-181b-5p	LTEDaro		
miR-505-3p		MCF-7:2A	
miR-450b-5p	EXE-R		
miR-486-5p			
miR-22-3p			
miR-148a-3p		MCF-7:2A	
miR-424-5p	EXE-R		

miRNAs differentially expressed in MCF-7:5C versus MCF-7:2A cells

Upon further bioinformatic analysis, it was apparent that the LTED cells had very unique expression profiles compared to one another, indicating that they have adapted to an estrogen-depleted environment through potentially different mechanisms (Fig. 6). In MCF-7:5C cells, which have lost expression of PR, a group of miRNAs on chromosome 14 is upregulated (Table 4). This upregulated group, which includes miR-127, miR-654, miR-889 and others, has been shown to be associated with Luminal B (ER+/PR-) breast cancer, consistent with the hormone receptor status of the MCF-7:5C cells [99]. The oncogenic, estrogen-responsive miR-17/92 cluster on chromosome 13 is upregulated in the MCF-7:2A cells (Table 5) and these miRNAs target proteins including PTEN, p21, and Bim. We chose one miRNA from the chromosome 14 region that was upregulated in the MCF-7:5C cells – miR-127 – and validated its expression by qPCR. Due to undetectable levels of miR-127 in the MCF-7 and MCF-7:2A cells, it was not possible to determine an exact relative expression value, but miR-127 was detected in the MCF-7:5C cells which confirms that miR-127 levels are highest in MCF-7:5C cells.

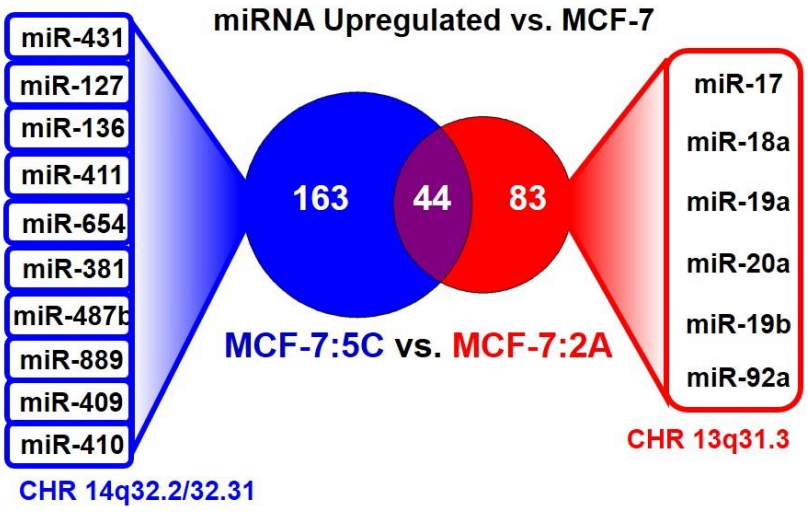


Figure 6. The miRNA expression of two LTED cell lines is unique. Analysis of the miRNAs upregulated only in MCF-7:5C or MCF-7:2A reveals miRNAs unique to each cell line. In the MCF-7:5C cells, miRNAs on chromosome 14q32 are upregulated, but these are not necessarily components of a single transcript. Whereas, the chromosome 13q31 cluster that is upregulated in MCF-7:2A cells encodes six miRNAs that are transcribed together and cleaved into individual pre-miRNA during processing of the RNA transcript in the nucleus.

Table 4. miRNAs upregulated in MCF-7:5C cells vs. MCF-7:2A cells include miRNAs from a cluster encoded on chromosome 14.

miRNA	Fold Change	CHR	Targets
miR-127-3p	1203	14q32.2	BCL6, BLIMP-1
miR-136-3p	622	14q32.2	PPP2R2A, E2F1
miR-431-5p	3341	14q32.2	Kremen1
miR-381-3p	475	14q32.31	MDR1, ID1, WEE1
miR-409-3p	1886	14q32.31	IFNγ, STAG2
miR-410-3p	557	14q32.31	MET
miR-411-5p	1687	14q32.31	FOXO1, YAF2
miR-487b-3p	1406	14q32.31	IRS1, BMI1, WNT5A
miR-654-3p	3246	14q32.31	CDKN1A
miR-889-3p	527	14q32.31	
miR-203a	1.60	14q32.33	c-JUN, p63, SNAI2
miR-181a-1-5p	2.52	1q32.1	BRCA1, PTEN, ATM
miR-181b-1-5p	2.06	1q32.1	Bcl-2, ATM
miR-205-5p	7.05	1q32.2	E2F1, LAMC1, PTEN
miR-29c-3p	1.63	1q32.2	DNMT3a, Akt3
miR-191-5p	1.66	3p21.31	CDK6, SATB1
miR-425-3p	1.75	3p21.31	CCND1, TACC3
miR-29a-3p	1.48	7q32.3	IFNAR1, NAV3

Table 5. miRNAs upregulated in MCF-7:2A cells vs. MCF-7:5C cells include miRNAs from a cluster encoded on chromosome 13, as well as many miRNAs that are upregulated by estrogen signaling.

miRNA	Fold Change	CHR	Estrogen-Upregulated?	Targets
miR-17-3p	1.86	13q31.3	YES	ICAM-1, TIMP3
miR-18a-3p	1.83	13q31.3	YES	CDC42, KRAS, HIF1α
miR-19a-3p	4.20	13q31.3	YES	PTEN, TNFα
miR-20a-3p	2.32	13q31.3	YES	LIMK1, BNIP2
miR-92a-1-3p	2.31	13q31.3	YES	VHL, PHLPP2
miR-200a-5p	1.75	1p36.33	YES	CTNNB1, ZEB2
miR-128-1-3p	1.78	2q21.3	YES	TGFBR1, BMI-1
miR-30a-3p	5.07	6q13	NO	LOX, CDK6
miR-550a-3p	2.72	7p14.3	NO	CPEB4
miR-196b-5p	2.62	7p15.2	YES	HOXA9, FAS
miR-25-5p	2.69	7q22.1	YES	DR4, SERCA2a
miR-489-3p	3.29	7q21.3	NO	PTPN11, SMAD3
miR-653-5p	3.12	7q21.3	YES	
miR-195-3p	1.85	17p13.1	YES	ARL2, IKKα, TAB3
miR-338-3p	3.36	17q25.3	YES	PREX2a, HIF1α
miR-424-3p	3.73	Xq26.3	YES	PDCD4, CCNE1

miR-181a is overexpressed in MCF-7:5C cells

In order to characterize the miRNAs expressed in these LTED cells, we found it ideal to begin with those that were differentially expressed in both MCF-7:5C and MCF-7:2A, compared to MCF-7. According to the sequencing data, a few of these shared miRNAs included miR-181a, miR-221, and miR-222. The qPCR levels of miR-181a were validated in the MCF-7:5C cells as very similar to the sequencing data (7-fold vs. 5.8-fold overexpressed, respectively), as were the levels of miR-221 (13.2-fold vs. 13.4-fold underexpressed) and miR-222 (18.4-fold vs. 17.3-fold underexpressed) (Fig. 7). While the MCF-7:2A cells didn't show as many similarities between qPCR levels and sequencing levels for miR-221 or miR-222, the levels of miR-181a were validated as similar to the sequencing expression (3.4-fold vs. 2.3-fold overexpressed) (Fig. 7).

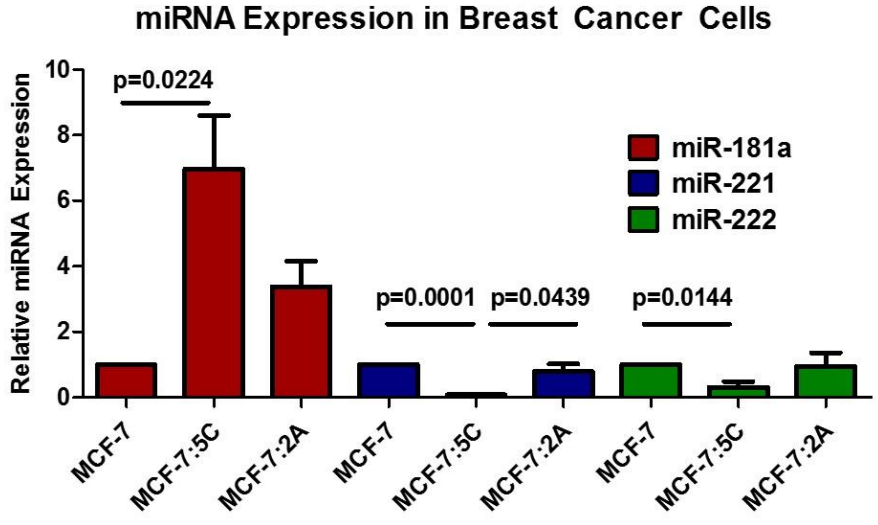


Figure 7. Validation of microRNA expression by qPCR. miR-181a expression was greater in the MCF-7:5C and MCF-7:2A cells, while miR-221/222 is decreased in MCF-7:5C compared to MCF-7. Not shown: miR-127 is highly expressed in MCF-7:5C, as measured by qPCR, but was not detectable in MCF-7 and MCF-7:2A.

Regulation of miR-181a by estrogen and estrogen receptor alpha

Estrogen has previously been shown to downregulate expression of miR-181a [93]. Thus, the overexpression of miR-181a in our MCF-7:5C cells cultured in the absence of estrogen, compared to MCF-7 cells cultured with estrogen, may simply be an artifact of media conditions. However, when miR-181a expression was measured in MCF-7 cells that were estrogen-deprived for 120 hrs (MCF-7Y), there was only a slight increase in expression, not nearly as high as the expression of miR-181a in the MCF-7:5C cells. To analyze this regulation of miR-181a expression further, the level of miR-181a was measured following treatment with 17β-estradiol, degradation of ERα by Fulvestrant (ICI), and knockdown of ERα by siRNA. Treatment of MCF-7Y cells with 17β-estradiol decreased miR-181a levels, and degradation of ERα by ICI blocked this regulation by estrogen – indicating the estrogen regulation of miR-181a expression is ERα-dependent (Fig. 8A). The estrogen-mediated downregulation of miR-181a expression in the MCF-7 cells at 48 hours post-treatment was further verified (Fig. 8B). Interestingly,

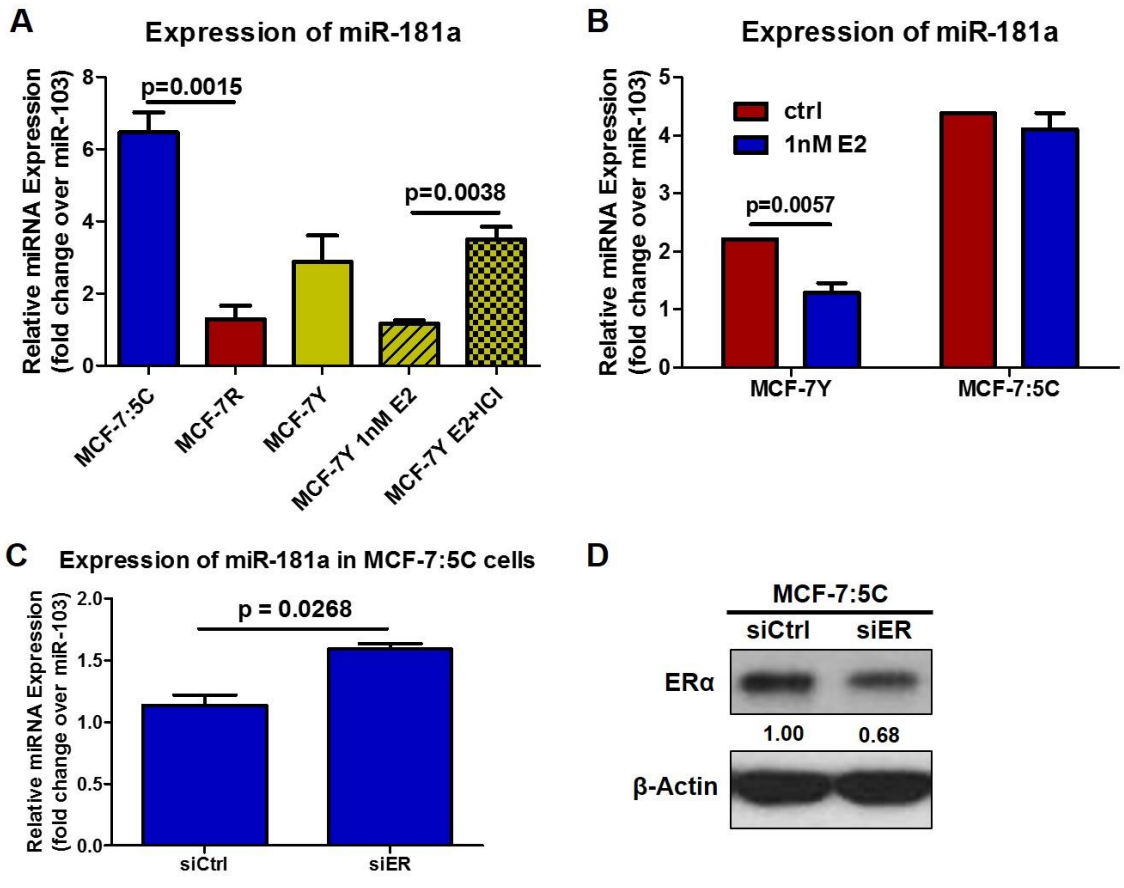


Figure 8. Estrogen downregulates the expression of miR-181a and this is dependent on action of ERα. (A) When MCF-7 cells, normally grown in red (estrogen-containing) media (MCF-7R), are deprived of estrogen for 120h (MCF-7Y), they still don't have as high a level of miR-181a as MCF-7:5C cells, and when these MCF-7Y cells are treated with 1nM 17β-estradiol (E2), expression of miR-181a decreases back to the same level as MCF-7R. This E2-downregulation of miR-181a is dependent upon ERα since 1μM ICI treatment, which causes degradation of ERα, blocks the E2-downregulation of mir-181a expression. **(B)** As already seen in **A**, the MCF-7Y cells are E2-responsive and decrease miR-181a expression upon E2 treatment (at 48 hrs). However, the MCF-7:5C cells respond to E2 in a different way, which may not include downregulation of miR-181a expression. **(C)** 48 hrs after knockdown of ERα (siER), miR-181a expression significantly increases in MCF-7:5C cells. **(D**) siER knockdown efficiency.

however, expression of miR-181a appears to be unaffected by 17β-estradiol treatment in the MCF-7:5C cells, indicating the difference in their response to estrogen compared to the estrogen-dependent MCF-7 cells (Fig. 8B). Even though estrogen doesn't affect the expression of miR-181a in MCF-7:5C cells, there could still be estrogen-independent action of ERα to regulate miR-181a. Knockdown of ERα by siRNA does in fact allow for an increase in the expression of miR-181a in MCF-7:5C cells 48 hours after ERα knockdown (Fig. 8C), indicating that there is

potential ligand-independent action of ERα in the MCF-7:5C cells that partially suppresses miR-181a expression.

Functional role of miR-181a in estrogen-independent growth

Since miR-181a expression was validated as being upregulated in both the MCF-7:5C and MCF-7:2A cells, we decided to analyze this miRNA for functional relevance in our cells. Loss-of-function studies using an inhibitor for miR-181a were used to identify the functional role of miR-181a in the MCF-7:5C and MCF-7:2A cells. The miR-181a inhibitor successfully decreased the levels of miR-181a in the cells compared to the negative inhibitor control (Fig. 9A), and the rate of proliferation was affected by this loss of miR-181a expression, as measured by CyQUANT DNA quantification. At 48 and 52 hours, the MCF-7:5C cells show a significant decrease in proliferation with the miR-181a inhibitor (Fig. 10B), while the MCF-7 and MCF-7:2A cells seem to be less affected by the loss of miR-181a (Fig. 10A, 10C).

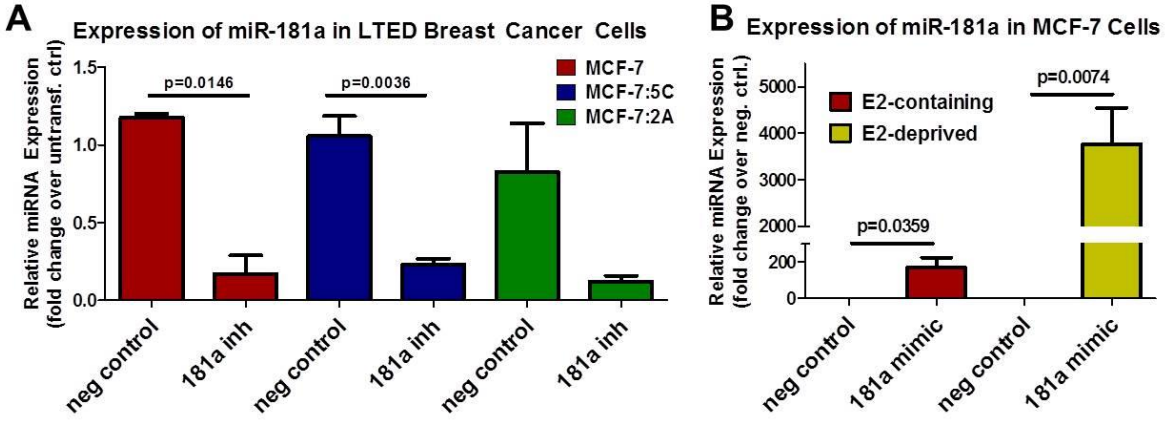


Figure 9. miR-181a knockdown and overexpression in breast cancer cells. A) Expression of miR-181a is decreased when cells are treated with the mirVana miR-181a inhibitor (181a inh). Neg control indicates transfection with the mirVana negative control inhibitor #1. **B)** Expression of miR-181a is increased when cells are treated with the mirVana miR-181a mimic (181a mimic). Neg control indicates transfection with the mirVana negative control #1.

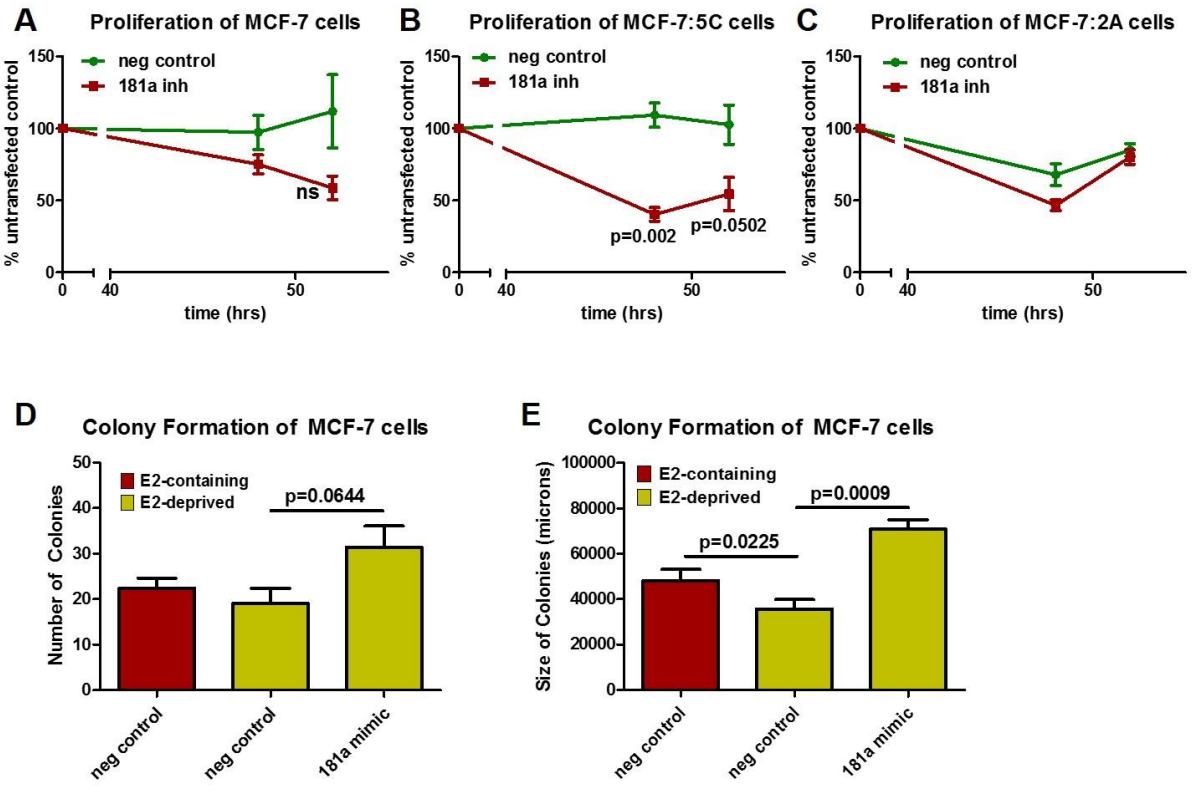


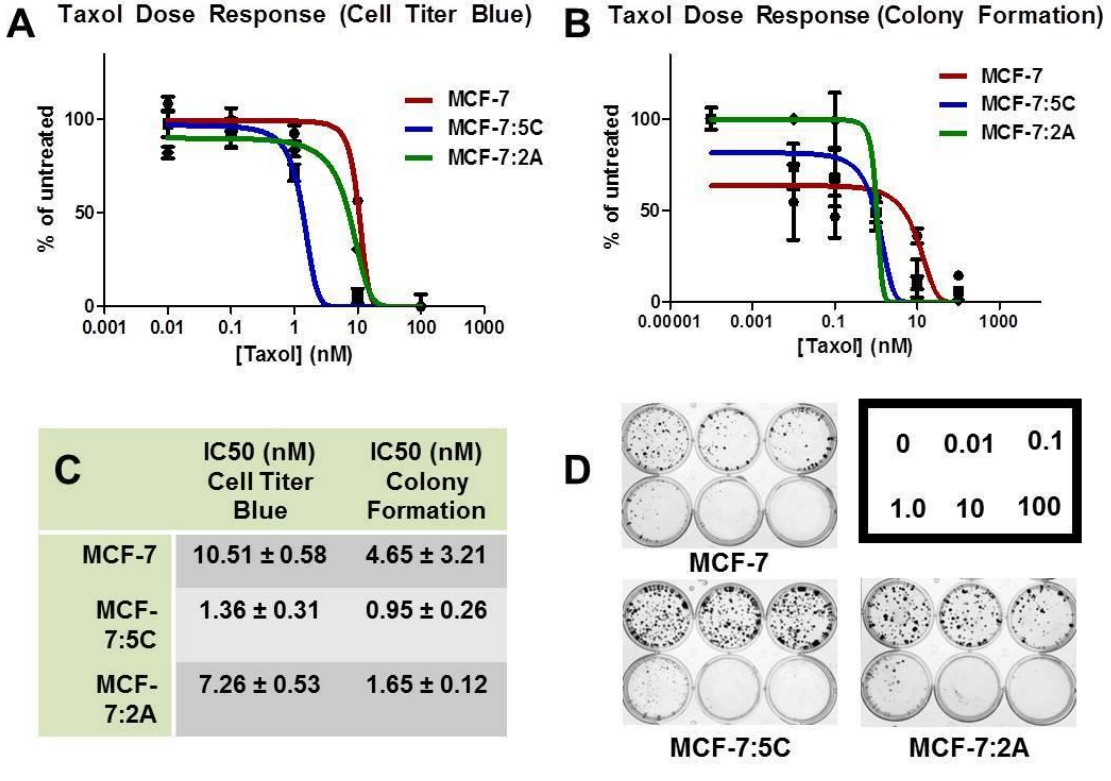
Figure 10. miR-181a affects ability of MCF-7 and MCF-7:5C cells to proliferate in E2-deprived conditions. Proliferation of MCF-7 cells **(A)** and MCF-7:2A cells **(C)**, as measured by CyQUANT DNA quantification, was not significantly affected by inhibition of miR-181a compared to negative control inhibitor-transfected cells. However, the proliferation of MCF-7:5C cells **(B)** was decreased at both 48 and 52 hours upon inhibition of miR-181a compared to the negative control inhibitor-transfected cells. **(D)** In addition, colony formation of MCF-7 cells decreases when the cells are deprived of E2 (neg control red vs. neg control yellow), and a miR-181a mimic was sufficient to rescue this decrease in number of colonies formed. While these differences in colony number were not significantly different, the size of the colonies **(E)** was significantly changed between the treatment conditions. The MCF-7 cells make smaller colonies when they are E2-deprived, and addition of miR-181a mimic results in colonies even larger than the MCF-7 cells in E2-containing media.

Conversely, a miR-181a mimic was transfected into estrogen-dependent MCF-7 cells, which was confirmed by qPCR (Fig. 9B). Two-dimensional colony formation assays of MCF-7 cells with and without miR-181a mimic reveal a decrease in the number of colonies that grow from estrogen-deprived MCF-7 cells compared to those cultured with estrogen, and this is rescued when MCF-7 cells are transfected with the miR-181a mimic (Fig. 10D). This trend is confirmed by the fact that the MCF-7 cells form smaller colonies in an estrogen-deprived environment when compared to estrogen-containing MCF-7, and this is rescued by the

transfection of the miR-181a mimic (Fig. 10E). These data support the hypothesis that miR-181a could have a functional role in the estrogen-independent proliferation of our cells.

miR-181a and sensitivity of estrogen-independent cells to DNA damage

miR-181a has been shown to play a role in regulating DNA damage repair proteins such as ATM [86] and BRCA1 [94]. Interestingly, the MCF-7:5C cells show a particular sensitivity to mitotic arrest and DNA damage by taxol (Fig. 11) and ionizing radiation (Fig. 12A). Thus, the idea that miR-181a is sensitizing these LTED MCF-7:5C cells to chemotherapy and DNA damaging agents was pursued.



C	IC50 (nM) Cell Titer Blue	IC50 (nM) Colony Formation
MCF-7	10.51 ± 0.58	4.65 ± 3.21
MCF-7:5C	1.36 ± 0.31	0.95 ± 0.26
MCF-7:2A	7.26 ± 0.53	1.65 ± 0.12

Figure 11. MCF-7:5C cells are more sensitive to taxol. Treatments with varying concentrations of taxol were used to create two separate dose response curves for each of our cell lines – one using the Cell Titer Blue viability assay **(A)** and the other by two-dimensional colony formation **(B).** The IC50 values were calculated according to each dose response curve and are listed in **(C). (D)** Representative images of colonies for each cell line after taxol treatment according to the upper right box of numbers (in nM).

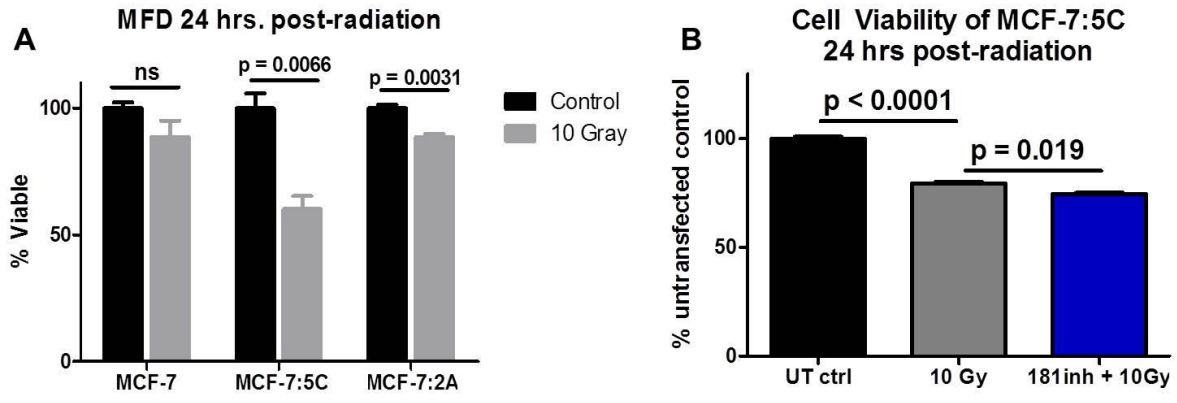


Figure 12. MCF-7:5C cells are more sensitive to DNA damage induced by ionizing radiation, especially when miR-181a is inhibited. (A) Multi-fraction dose (MFD) ionizing radiation treatments – 2.5 Gy every day for four consecutive days – significantly decreases the viability of MCF-7:5C and MCF-7:2A cells, with the most pronounced effect in the MCF-7:5C. **(B)** Inhibition of miR-181a further decreases the viability of MCF-7:5C cells in conjunction with MFD ionizing radiation.

First, we measured the protein levels of ATM, BRCA1, and PTEN (all miR-181a targets) in our cells following transfection with the miR-181a inhibitor or miR-181a mimic. Western blot analysis of ATM, BRCA1, and PTEN protein expression in MCF-7 and MCF-7:5C 48 hours after transfection with the miR-181a inhibitor revealed a consistent upregulation of all three proteins in the miR-181a-depleted MCF-7:5C cells (Fig. 13A). Treatment of MCF-7 cells with a

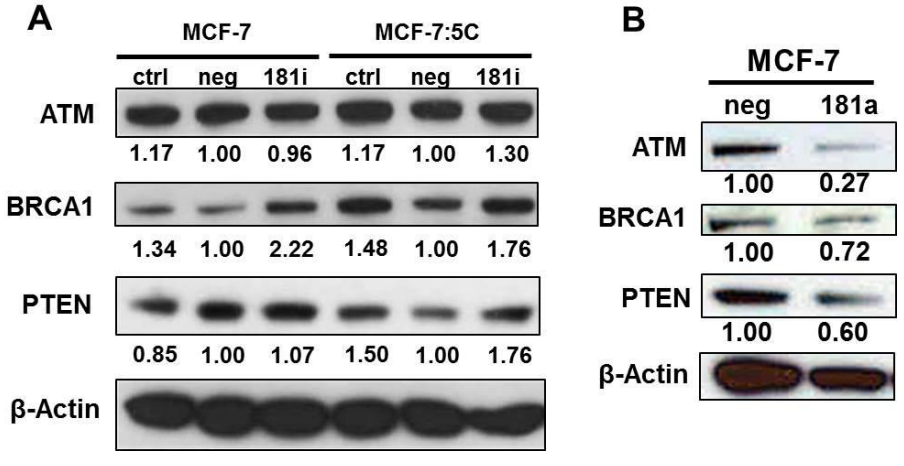
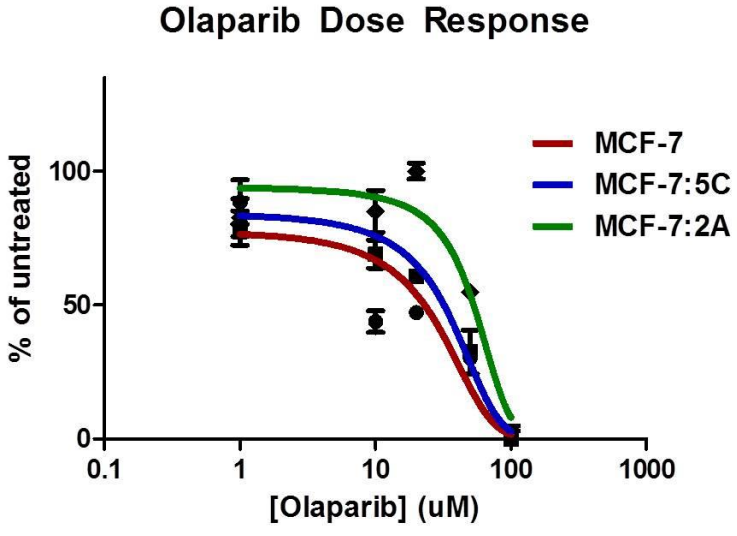


Figure 13. Expression of DNA damage repair proteins is affected by miR-181a. (A) The inhibition of miR-181a resulted in a slight increase of ATM, BRCA1, and PTEN expression in the MCF-7:5C cells at 48 hours after transfection, but this was not consistent in the MCF-7 cells. The overall expression of BRCA1 was higher in MCF-7:5C cells. Ctrl, untransfected control; neg, negative control inhibitor; 181i, miR-181a inhibitor. **(B)** The addition of miR-181a mimic (181a) to MCF-7 cells resulted in the decrease of its targets ATM, BRCA1, and PTEN compared to negative control-transfected cells (neg) at 48 hours after transfection.

miR-181a mimic for 48 hours resulted in downregulation of these same proteins (Fig. 13B), indicating that they are in fact targets of miR-181a in our cells.

Next, we wanted to understand the effect of a miR-181a inhibitor on the sensitivity of our MCF-7:5C cells to ionizing radiation. The cells were transfected with miR-181a inhibitor, were subject to ionizing radiation for four consecutive days (2.5 Gray per day), and cell viability was measured (Fig. 12B). The MCF-7:5C cell viability after this multi-fraction dose radiation was decreased further with inhibition of miR-181a (Fig. 12B). This was a peculiar finding given our initial hypothesis that miR-181a expression may be what is weakening the DNA damage response of our MCF-7:5C cells – we would expect knockdown of miR-181a to either increase or not affect the viability of these cells after radiation.

Finally, olaparib was used to test whether the inhibition of DNA damage repair proteins by miR-181a would make the LTED cells more sensitive to a PARP (poly-ADP ribose polymerase) inhibitor. MCF-7, MCF-7:5C, and MCF-7:2A cells were treated with olaparib, and the IC50 values were calculated from dose response curves (Fig. 14). The IC50 values reveal a trend that indicates the MCF-7 cells are most sensitive to the olaparib (IC50 = 23.06 ± 4.12), followed by the MCF-7:5C cells (IC50 = 32.08 ± 3.56), and the least sensitive cells MCF-7:2A (IC50 = 53.05 ± 5.16), though it should be noted that these results weren't statistically significant.



	IC50 (µM)
MCF-7	23.06 ± 4.109
MCF-7:5C	32.08 ± 3.564
MCF-7:2A	53.05 ± 5.158

Figure 14. Olaparib treatments reveal possible lack of sensitivity of MCF-7:5C and MCF-7:2A cells to PARP inhibitor. Treatment with olaparib revealed a trend that presents the MCF-7 cells as the most sensitive to PARP inhibition, while the MCF-7:5C and MCF-7:2A cells were less sensitive, as indicated by IC50 values below dose response curve. It should be noted, however, that none of the IC50 values were significantly different from the others.

Discussion

The role of miRNAs has been well established in multiple types of cancer, including breast cancer [100-102]. In addition to representing a specific molecular profile of certain pathological features in breast cancer [66], miRNAs are involved in development of resistance to endocrine therapies such as tamoxifen treatment [103-106]. More recently, studies have shown that the miRNA profile of breast cancer cells changes when the cells become resistant to AI therapy [74, 76, 98]. Our miRNA next-generation sequencing data from the LTED breast cancer cell lines MCF-7:5C and MCF-7:2A confirms the hypothesis that there are unique miRNA expression profiles in estrogen-independent vs. estrogen-dependent cells. There were 338 miRNAs differentially expressed in the MCF-7:5C cells and 241 miRNAs differentially expressed in the MCF-7:2A cells when compared to the parental MCF-7 cell line. Of those miRNAs, 126 were differentially expressed in both of the LTED cell lines vs. MCF-7. On the other hand, there were also unique miRNAs that were up- or down-regulated in one of the LTED cell lines versus the other.

A closer look at the miRNAs that were unique to each LTED cell line revealed an upregulation of specific chromosomal groups of miRNAs in MCF-7:5C (chromosome 14q32) and MCF-7:2A (chromosome 13q31). The miRNAs encoded on chromosome 14q32 are great in number, and the miRNAs we found to be upregulated in the MCF-7:5C cells represent a fraction of this group. Interestingly, many of the miRNAs we found upregulated in the MCF-7:5C chromosome 14 group, such as miR-127, miR-136, miR-411, and miR-487b, have been shown to be increased after estrogen deprivation (either naturally occurring or by ovariectomy) in mice and pigs [107, 108]. It has also been shown that the expression of miR-127 is upregulated by estrogen receptor-related gamma [109]. As seen in Table 4, many of these miRNAs target genes which are involved in cell proliferation, cell cycle, and immune response.

As mentioned earlier, the hormone receptor status of breast cancer cells can be predicted by some of these miRNAs – expression of certain chromosome 14q32 miRNAs is associated with ER+/PR- breast cancer [99]. This brings attention to a unique feature of the MCF-7:5C cells – lack of PR expression. While implications of this loss of PR are unexplored in the MCF-7:5C cells, there have been many studies that have tried to pinpoint the role of PR in the development of breast cancer. PR is important for normal breast development, especially of the mammary glands [110]. In studies of mice and human breast cancer cells, it seems that PR can act as an oncogene *in vivo*, and its *in vitro* role may be to prime breast cancer cells for growth, which is then sustained by action of additional proteins [111, 112]. A role for PR in breast cancer is further confirmed by the results from the Women's Health Initiative in 2002 where researchers found a greater increase of breast cancer risk in women who took hormone replacement therapy that included a combination of estrogen and progestin as compared to estrogen alone [113, 114].

The miRNA cluster on chromosome 13q31 which includes miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a, is upregulated in the MCF-7:2A cells. This cluster is part of an incredibly intricate pathway that includes dozens of transcription factors and a series of target proteins involved in various cellular processes from cell proliferation and death to heart and lung development to immune response [115]. This miR-17/92 cluster is also known as oncomiR-1, and has been shown to modulate tumor formation in B-cell lymphoma [116] and enhance cell proliferation of medulloblastomas [117].

Of the 126 miRNAs that were differentially expressed in both the MCF-7:5C and MCF-7:2A cells, we chose miR-181a as our first candidate for *in vitro* functional analysis. We have shown that miR-181a is overexpressed in both MCF-7:5C and MCF-7:2A cells vs. MCF-7, and the Masri *et al.* microarray also confirms overexpression of miR-181a in MCF-7aro LTED cells

[74]. miR-181a was interesting to our lab in particular since we have observed a greater sensitivity of the MCF-7:5C cells to DNA damage and mitotic arrest induced by ionizing radiation and taxol treatments compared to the MCF-7 cells, and miR-181a has been shown to target DNA damage repair proteins such as ATM [86] and BRCA1 [94]. Expression of miR-181a is associated with aggressive breast cancer, and inhibition of miR-181a sensitizes MDA-MB-231 breast cancer cells to the PARP inhibitor olaparib [86]. This sensitivity to olaparib is due to the decreased expression of DNA repair proteins by miR-181a, and another study has found that upregulation of miR-181a via TGF-β signaling induces a "BRCAness" phenotype in the MDA-MB-231 breast cancer cell line, referring to the synthetic lethality seen in BRCA-mutated breast cancers in response to PARP inhibition [94]. There is also evidence that AI-resistant breast cancer cells derived from long-term letrozole-treated xenograft tumors show increased reliance on the alternative nonhomologous end joining DNA repair pathway and are responsive to PARP inhibitors [118]. In addition, expression of miR-181a was shown to enhance drug sensitivity in mitoxantone-resistant MCF-7 cells through its regulation of BCRP [87]. Thus, it was our hypothesis that MCF-7:5C and MCF-7:2A cell proliferation may be affected by loss of miR-181a, but more importantly that these cell lines might be more sensitive to PARP inhibition due to the increased expression of miR-181a in these cell lines.

The proliferation of the MCF-7:5C cells was significantly affected by miR-181a knockdown at 48 and 52 hours when compared to treatment with the negative control inhibitor. This decrease in proliferation was not seen in the other LTED cell line MCF-7:2A, which further indicates the differences between these two cell lines. If these MCF-7:5C cells are relying on the expression of miR-181a in order to proliferate in an estrogen-deprived environment, then the next question is whether or not overexpression of miR-181a in estrogen-dependent MCF-7 cells allows them to grow in estrogen-deprived conditions. Indeed, there was an increase in the ability

of estrogen-deprived MCF-7 cells to proliferate and form large colonies when transfected with a miR-181a mimic. This indicates a potential functional relevance for miR-181a in maintaining estrogen-independent growth, though the mechanism by which this occurs is still unconfirmed.

When our cells were treated with olaparib, the MCF-7 cells were more sensitive to PARP inhibition, though it should be noted this was just a trend and the differences weren't significant. Given the overexpression of miR-181a in the LTED cells, their DNA repair proteins should be decreased and their sensitivity to PARP inhibition increased. Upon examining the protein expression of these DNA repair proteins we found that the basal expression of BRCA1 was highest in the MCF-7:5C cells, which may explain why it is not as sensitive to PARP inhibition as originally hypothesized. However, the question still remains as to why the MCF-7:5C cells are more sensitive to ionizing radiation and taxol, and this may be partially explained by examining the functionality of other DNA damage repair proteins in these cells.

The regulation of miR-181a is achieved by action of many different factors, one of which includes downregulation of miR-181a expression by estrogen signaling via ERα [93]. We are particularly interested in the effect of ERα on miR-181a expression since our LTED cells are deprived of estrogen and overexpress ERα compared to the estrogen-dependent MCF-7 cells. It is possible that the process of deriving the MCF-7:5C and MCF-7:2A cells using long-term estrogen deprivation allowed for significant upregulation of miR-181a. Estrogen treatment in the MCF-7:5C cells did not affect miR-181a expression, likely because these cells have a different response to estrogen than the parental MCF-7 cells. We did, however, confirm a downregulation of miR-181a in estrogen-deprived MCF-7 cells (MCF-7Y) 48 hours after estrogen treatment. If this estrogen regulation of miR-181a involves ERα, then loss of ERα should result in restored expression of miR-181a. This was in fact observed upon treatment of MCF-7Y cells with a combination of estrogen and ICI, which degrades ERα. Likewise, in our ERα knockdown

experiments, use of siRNA for ERα resulted in higher expression of miR-181a in the MCF-7:5C cells compared to the siCtrl-transfected cells. This observation in the MCF-7:5C cells indicates that ligand-independent ERα is capable of suppressing miR-181a levels to some extent.

The potential functional relevance of miR-181a in the LTED cells is encouraging, though not yet fully explained, and there are many miRNAs from our sequencing data that are yet to be explored. It will be important to pursue *in vitro* and *in vivo* analysis of miRNAs that are differentially expressed in both MCF-7:5C and MCF-7:2A, as well as those that were unique to each LTED cell line. Below, I will describe a series of experiments that would (1) fill in some mechanistic gaps involving miR-181a, and (2) utilize more information from the next-generation sequencing data to identify relevant miRNAs involved in estrogen-independent growth. While this list is not exhaustive, I believe it outlines the next steps relevant to the goal of this project:

1. Filling in the mechanistic gaps involving miR-181a
    A. Confirm a role for miR-181a in estrogen-independent growth
        i. Create an MCF-7 cell line that stably overexpresses miR-181a, and compare growth with normal MCF-7 cells after depriving these cells of estrogen and/or knocking down ERα.
        ii. Using an *in vivo* mouse model of breast cancer, overexpress miR-181a and then measure sensitivity to AI treatment as compared to mice that do not overexpress miR-181a.
        iii. Measure levels of miR-181a in serum samples from breast cancer patients who are AI-resistant and compare to patients who are AI-sensitive.
    B. Determine mechanism by which miR-181a controls estrogen-independent growth

i. Transfect miR-181a mimic into estrogen-deprived MCF-7 cells, isolate RNA from these cells and MCF-7 transfected with a negative control, then conduct next-generation RNA sequencing to identify a global profile of genes whose expression is downregulated with the miR-181a mimic.

ii. Use pathway analysis of genes altered with miR-181a mimic seen in RNA sequencing to identify key altered pathways, then conduct miR-181a target analysis – using both predicted and validated targets – for the altered genes seen the key pathways identified.

iii. To confirm direct miR-181a regulation of the candidate genes identified above, construct a luciferase reporter containing either the 3'UTR sequence of the candidate genes or a mutated 3'UTR sequence to see if miR-181a directly binds the 3'UTR in a sequence-specific manner.

C. Determine if miR-181a expression is directly regulated by ligand-independent ERα in the MCF-7:5C cells and/or ligand-dependent ERα in the MCF-7 cells

i. Analyze the promoter sequence of the miR-181a gene for ERE sites.

ii. If ERE sites are found in the miR-181a promoter, conduct a ChIP assay that immunoprecipitates ERα-bound DNA from MCF-7 and MCF-7:5C cells with and without estrogen, then use PCR primers specific to the miR-181a ERE sites to see if there is direct binding of ERα to those sites.

2. Utilize more information from the next-generation sequencing data

A. Explore the role of miR-30a in estrogen-independent growth.

i. All studies of miRNAs and AI resistance, including ours, show upregulation of miR-30a when cells are either AI-resistant (EXE-R, Res-Let, Res-Ana) [74, 76] or estrogen-independent (MCF-7:5C, MCF-7:2A) [57]. miR-30a has

recently been shown to suppress the growth, proliferation, migration, and invasion of breast cancer cells, which seems contrary to what we would expect from a miRNA that is upregulated in AI-resistant cells [119-121]. Thus, it would be worth conducting some preliminary functional experiments that could confirm how relevant miR-30a is in our estrogen-independent cells.

B. Explore the role of the miR-17/92 cluster on chromosome 13 in MCF-7:2A cells.

C. Explore the role of the group of miRNAs on chromosome 14 in MCF-7:5C cells.

Experiments that have been performed in this thesis regarding miR-181a, as well as those described in the additional experiments above, can also be done using miR-30a and miRNAs from the clusters on chromosomes 13 and 14 in order to understand their role in estrogen-independent growth. The information from these experiments may help to identify biomarker miRNAs that can predict the responsiveness or best alternative therapeutic option for AI-resistant breast tumors, or to identify novel therapeutic targets for the treatment of AI-resistant breast cancer.

With the use of innovative technologies, it is now possible to use miRNAs as biomarkers and compile biomarker panels for diagnosis and identification of therapeutic options for cancer, including AI-resistant breast cancer. Circulating miRNAs are ideal for clinical use, since they are highly stable and can be detected by a non-invasive manner in a blood sample. Circulating miRNA levels in breast cancer patients have been studied at diagnosis, in early stage tumors, after surgical resection, following chemotherapy/radiation treatments, and following metastatic relapse – all to understand the unique miRNA profiles throughout the progression of breast cancer [122-124]. Because of the low abundance of miRNAs in the blood, the use of powerful detection methods such as high-throughput sequencing will need to be employed in clinical settings.

Regarding therapeutic approaches, the miRNAs overexpressed in the LTED cells could be eliminated via RNA interference (RNAi), while those that are underexpressed in the LTED cells may be part of replacement therapies. There are over 20 RNAi-based therapies employing various methods of siRNA delivery currently in phase I clinical trials for the treatment of diseases such as viral infections, hereditary disorders, and cancer [125]. The only RNA replacement clinical trial to date began in April of 2013 as a strategy to deliver miR-34, a tumor suppressive miRNA that regulates expression of BCL-2 and MYC, to patients with liver-based cancers [126]. Though there are no published results on this MRX34 treatment yet, a recent update revealed that MRX34 has a manageable safety profile with only one incident of a dose-limiting toxicity [127].

Overall, we have described the miRNA profiles for two LTED breast cancer cell lines, and have explored the relevance of miR-181a as one functional player in the estrogen-independent growth associated with an AI-resistant phenotype. A more complete picture of the differentially expressed miRNAs in endocrine-resistant breast cancer, especially AI-resistant breast cancer, is crucial for the development of novel biomarker signatures and therapeutic strategies.

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