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Dysregulation of microRNA expression in acquired endocrine-resistant breast cancer.

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DYSREGULATION OF MICRORNA EXPRESSION IN ACQUIRED ENDOCRINE-RESISTANT BREAST CANCER

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DEDICATION

This dissertation is dedicated to my parents for their constant love, encouragement and support over the course of my life and my Ph.D. program. I would also like to dedicate this to my husband, Roshan James for his motivation that helped in the completion of my graduate studies.

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ABSTRACT

DYSREGULATION OF MICRORNA EXPRESSION IN ACQUIRED ENDOCRINE-RESISTANT BREAST CANCER

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MicroRNAs (miRNAs) regulate gene expression at the post-transcriptional level by repressing translation or stimulating mRNA degradation. In this study, I tested the hypothesis that miRNAs are differentially expressed in antiestrogen-sensitive MCF-7 versus -resistant LY2 human breast cancer cells. Microarray analyses identified 97 miRNAs that are differentially expressed between two estrogen receptor alpha (ERα) -positive human breast cancer cell lines: endocrine-sensitive MCF-7 versus -resistant LY2 cells under basal conditions. Opposite expression of miRs-10a, -21, -22, -125b, -181, -200a, -200b, -200c, -221, and -222 was confirmed between MCF-7 and LY2 cells. The ER antagonist ICI 182,780 (fulvestrant or Faslodex) generally blocked the effect of estradiol E_2 and 4-hydroxytamoxifen (4-OHT) regulated miRs, *i.e.*., miR-10a, miR-21, miR-22, miR-200a, miR-221, and miR-222, indicating that these responses in MCF-7 cells are ER-mediated. Time-dependent variation in basal (ethanol, the vehicle), E_2, and 4-OHT regulation of the top 8 miRNAs was detected in MCF-7 cells. Bioinformatic analyses to impute the biological significance of the identified miRNAs by identifying their computationally predicted target genes in the human genome using TargetScan, PicTar, and the Sanger

miRBase Targets databases was performed. Thirty six putative mRNA targets were identified. Agreement in the direction of anticipated regulation was detected for 12 putative targets. These miRNAs showing opposite expression between these two breast cancer cell lines may be involved in endocrine resistance.

MiR-200 family includes two clusters *i.e.* miR-200 a/200b/ 429 and miR-200c/141 encoded on chromosome 1 and chromosome 12, respectively. Lower miR-200a, miR-200 b and miR-200c expression was observed in estrogen-independent LCC1 and endocrine-resistant LCC2, LCC9, and LY2 compared to the parental, endocrine-sensitive MCF-7 human breast cancer cell line. ZEB1 protein was found to be expressed in endocrine-resistant LY2 cells but not in endocrine-sensitive MCF-7 cells. LY2 cells did not express E-cadherin, a ZEB1 target which is a marker for epithelial phenotype. This is the first demonstration that LY2 cells have undergone EMT as part of their endocrine-resistant phenotype. Concomitant with miR-200 decrease, there was an increase in ZEB1 mRNA expression in LY2 cells. Overexpression of miR-200b or miR-200c in LY2 cells changed the cellular morphology from a mesenchymal to an epithelial appearance and sensitized cells to inhibition by 4-OHT and fulvestrant. These studies indicate that reduced expression of miR-200 and a corresponding increase in ZEB1 protein is an indicator of endocrine-resistance in breast cancer cells.

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CHAPTER I: INTRODUCTION TO BREAST CANCER

BREAST CANCER AND ESTROGEN SIGNALING

Breast cancer is the leading non-cutaneous form of cancer that is diagnosed in women of the United States [1]. According to the American Cancer Society, it is estimated that in 2011 about 230,000 women will be diagnosed with invasive breast cancer and approximately 39,520 women are expected to die from breast cancer [2] (http://www.cancer.org/Research/CancerFactsFigures/BreastCancerFactsFigures/breast-cancer-facts-and-figures-2011-2012). Although the highest incidence of breast cancer is seen on non-hispanic white women, death due to this cancer is highest in African-American women because they have triple-negative breast cancer (TNBC). In TNBC, which is the most aggressive form of breast cancer, cells do not express estrogen receptor (ER), progesterone receptor (PR) or the epidermal growth factor receptor (HER2/neu/erbB2). In the last two decades, the incidence rates for breast cancer in women over 50 has declined by 2 % due to early detection and better therapy, including the use of the antiestrogen tamoxifen.

http://www.breastcancer.org/symptoms/understand_bc/statistics.jsp .

The breast tissue is composed of milk producing lobules and ducts that connect lobules to the nipple. The luminal epithelial cells that line the ducts of the breast are regulated by steroid hormones, such as estrogens and progesterone and peptide hormones *i.e.* Prolactin, Oxytocin. Estrogens are steroid hormones produced by the ovary. The three forms of estrogen produced in the ovary are Estrone (E_1),

Estradiol (E_2) and Estriol (E_3). E_1 is the major form of estrogen in postmenopausal women, E_2 is the predominant form of estrogen in pre-menopausal women and E_3 is primarily synthesized in pregnancy. Estrogens promote cell replication by binding to their receptors, estrogen receptors alpha (ERα) and beta (ERβ) that mediate the subsequent recruitment of coregulators, chromatin remodeling complexes, and RNA polymerase leading to transcription of estrogen responsive genes [3]. The two modes of signaling through the ER include genomic and non genomic.

Genomic ER signaling - Binding of E_2 to ER induces a conformation change to the ER leading to its activation and binding to DNA sequences called estrogen response elements (EREs). This is the classic or genomic mode of estrogen signaling. Alternatively, the E_2-ER complex may interact with DNA indirectly by a 'tethering' mechanism involving direct interaction of ER with transcription factors including AP-1, Sp1 or NF-κB [4]. Depending on the type of transcriptional coregulators associated with ER, the E_2-ER complex can have different effects [5,6]. For example, E_2-ER binds to the ERE of the NRF-1 gene in MCF-7 cells and recruits AP1 coactivator and RNA polymerase to increase the expression of TFAM mitochondrial gene [7]. Ligand independent ER signaling involves protein kinases that phosphorylate and activate ER.

Non-genomic ER signaling - In addition to genomic ER signaling a small percent of total cellular ERα that are localized in the plasma membrane (PM) can initiate rapid activation of intracellular phosphorylation cascades mediated by extracellular signal regulated kinases 1/2 (ERK1/2) and phosphoinositide 3-kinase (PI3K or AKT) [8,9]. This rapid E_2-initiated cascade is referred to as "membrane-initiated" or "non-genomic" ER signaling and is independent of gene transcription, although 'non-

genomic' estrogen activation of signaling pathways can stimulate gene transcription in breast cancer cells [10].

GPR30/ GPER: In addition to ERα, there is a plasma membrane-bound estrogen receptor that is a G-coupled protein receptor (GPR30/GPER) [11,12]. Filardo *et al.* have shown that E_2 can activate the MAPK signaling cascade via binding to GPR30 leading to transcription of cell proliferative genes [13] . ER antagonists ICI 182, 780 and 4-OHT were found to activate GPR30 and mediate MAPK signaling [14].

In addition, ligand activated membrane ERα can phosphorylate and activate key signaling molecules including Epidermal Growth Factor Receptor (EGFR), c- src, Shc and p85α regulatory subunit of MAPK [15-17]. Although this mechanism is independent of E_2-mediated transcription, the activation of signaling pathways may affect downstream molecules, subsequently leading to nuclear ER activity and also induce transcription of E_2-responsive genes [18]. Thus there is cross-talk between growth factor receptor pathways and genomic ER signaling [17].

ENDOCRINE THERAPIES FOR THE TREATMENT OF BREAST CANCER

Estrogens are natural ligands of ER. Blocking estrogen-mediated tumor growth has been the mode of breast cancer treatment for many years. Endocrine therapies either target ERα action or block estrogen synthesis. Antiestrogens (e.g., Tamoxifen (TAM) and Raloxifene (RAL)) function by binding to the ER and blocking its transcriptional activity. ICI 46, 474 (now known as TAM) was originally developed by Imperial Chemical Industries (ICI) Ltd. Pharmaceuticals Division [19]. It was developed by a group of scientists that included Arthur Walpole, Dora Richardson and Michael J.K Harper. Although initial studies focused on its role as a contraceptive drug, it was Walpole who later on suggested its use as an antiestrogen.

He identified that TAM acts as an estrogen in the mouse vagina while it is an antiestrogen in rat vaginal epithelium and uterus [20,21]. Walpole also suggested that TAM may inhibit tumor formation in the breast. After his death in 1977, significant studies on TAM's role as a SERM (Selective Estrogen Receptor Modulator) as well as an antitumor agent was investigated by Craig V. Jordan [22-25] .

It was in 1977 that TAM was approved by the FDA to be used clinically as an antiestrogen in the United States. Since then, TAM, a non-steroidal antiestrogen, has been widely used for the prevention and treatment of ERα-positive breast cancer [26,27]. TAM has cell-type-specific mixed agonist/antagonist activity, and is thereby classified as a SERM [28]. SERMs are classified according to its structure, which are more or less similar to E_2. TAM is a triphenylethylene which is metabolized in the liver to its active form 4-OHT by the cytochrome P450 enzyme CYP2D6. In the breast, 4-OHT acts as an ER antagonist and competes with E_2 for binding to the ER. For over 30 years, TAM has been the 'gold standard' for the treatment of breast cancer in pre-menopausal women [29]. One of the disadvantages of TAM is that women on TAM therapy have an increased risk of uterine cancer due to its agonist activity in the uterus [30].

RAL and arzoxifene are benzothiophenes. RAL avoids some of the side effects associated with TAM due to its antagonist activity in both breast and uterus. RAL was first approved in 1997 by the FDA to be used in the treatment of osteoporosis in post-menopausal women [31]. It was subsequently demonstrated to prevent recurrent disease and primary breast cancer by a number of clinical trials [32]. The RUTH (Raloxifene Use For The Heart) is a significant trial that demonstrated the use of RAL in the treatment of osteoporosis in post-menopausal women who had cardiovascular disease [33]. The trial showed that RAL is effective not only in the

treatment of osteoporosis but also for ER-positive breast cancer. Results of the STAR (Study of Tamoxifen and Raloxifene) trial that was published in 2006 further confirmed the role of RAL in the treatment of osteoporosis as well as invasive breast cancer in women [34].

Another class of antiestrogens used in the treatment of breast cancer is called Selective Estrogen Receptor Downregulators (SERD) with the prototype fulvestrant. It is a SERD because it binds the ligand binding domain (LBD) noncovalently, and targets ERα to the 26S proteasome for degradation [35].

Aromatase inhibitors (AIs) are a class of antiestrogens that block estrogen synthesis [36]. In post-menopausal women, estrogens are no longer synthesized by the ovaries. However estrogens are synthesized from either locally produced or adrenal androgenic precursors (e.g. DHEA or Dehydroepiandrosterone, DHEA-sulphate) in peripheral tissues including adipose tissue, brain, skin, bone, endometrium, and breast [37]. In these tissues, the enzyme aromatase (*CYP19A1*) catalyzes the conversion of androgens, (*i.e.*, testosterone and androstenedione) to estrogens (*i.e.*, E_1 and E_2, respectively). The three most commonly used AIs are letrozole, anastrozole and exemestane. AIs have been shown to be more effective than TAM in promoting disease-free survival in post-menopausal women [38]. Exemestane is a steroidal AI that bind to the substrate-binding site of aromatase enzyme and irreversibly inhibits its action [39]. Letrozole and Anastrozole are non-steroidal AIs that bind reversibly to the substrate binding site of the aromatase enzyme [4]. Unlike TAM, AIs do not increase the risk of endometriosis and thromboembolism [40]. However AIs increase osteoporosis and fractures along with side effects such as hot flashes and headaches. The use of AIs is limited by musculoskeletal pain which some breast cancer survivors find intolerable [41].

MECHANISMS OF ENDOCRINE/TAMOXIFEN-RESISTANCE

Although hundreds of thousands of women are alive today because TAM prevents breast cancer recurrence [42], the majority of tumors that initially respond to TAM develop resistance. It is estimated that 40% of the women who receive adjuvant hormonal therapy acquire endocrine resistance [43,44]. The mechanisms of acquired endocrine/TAM- resistance remain to be fully elucidated despite intense study by many labs over the past 25 years [45]

Loss of ERα and aberrant expression of coregulators contribute to endocrine-resistance- Acquired endocrine resistance, which occurs after a woman was initially successfully treated on TAM, was originally thought to be due to loss of ERα expression. Mechanisms involved in the loss of ERα expression include activation of EGFR and MAPK signaling [46,47], methylation of ERα promoter [48] and hypoxia [49]. However, ERα was found to be lost in only 15-20% of TAM-resistant cancers, suggesting other mechanisms for the development of endocrine/TAM-resistance [50]. Recent studies show that a variant of ERα called ERα36 is expressed in tumors that are not responsive to TAM [51]. Another variant of ERα called ERα46 was found to be decreased in TAM-resistant breast cancer [52].

Other factors that play a role in the development of endocrine/TAM-resistance include altered expression of coregulators [53-56]. For example, AIB1 (also known as SRC-3 or NCoA-3), a nuclear coactivator is overexpressed in breast tumors and is associated with increased ERα mediated transcription [56] and reduced TAM-responsiveness in patients [57]. On the other hand, reduced expression of Nuclear receptor co-repressor (NCoR) was observed in a mouse model of human breast cancer and correlated with TAM-resistance [58]. Low NCoR is associated with a shorter disease-free survival and is used as predictor of TAM-resistance in ERα-positive

breast cancer [59]. Increased expression of AP-1, Sp1 and NF-κB transcription factors have been associated with endocrine resistance [60-62]. TAM-liganded ERα acts as an antagonist and increases AP-1 and NF-kB transcriptional activities in MCF-7 breast cancer cells [63]. TAM-liganded ERβ was found to act as an agonist and induce AP-1 regulated gene transcription in MCF-7 and MDA-MB-453 breast cancer and Ishikawa endometrial cancer cells transfected with an ERβ expression plasmid [63]. This leads to enhanced cell proliferation and growth, resulting in TAM-resistance. Increased NF-κB transcriptional response was reported in TAM- resistant and ERBB2-positive cell lines, MCF-7/HER2 and BT474 [62]. The study showed that the increase in NFkB transcriptional response was reversed by the NF-kB inhibitor Parthenolide leading to better response to TAM. The study also showed that the increased TAM-responsiveness in these cells was due to enhanced recruitment of the corepressor NCoR to the TAM-ER complex upon treatment with the inhibitors.

Post-translational modifications of ERα contributes to endocrine resistance - Posttranslational modifications such as phosphorylation, sumoylation and acetylation can alter the conformation of ERα such that it affects its ligand binding properties and interaction with proteins such as coregulators [64-66]. Phosphorylation of ERα takes place mainly by activation of kinase pathways such as protein kinase A (PKA), MAPK and Src pathways [67-69]. Some of the phosphorylation sites within ERα that can alter the response of cells to TAM include Ser102/4/6, Ser 118 and Ser 305 [66]. The Ser 118 residue on ERα can be phoshorylated by pathways such as MAPK, PI3K, CDK7 and IKKα [70-72] . E_2 or EGF can induce the MAPK pathway and thus phosphorylate Ser118 leading to enhanced ERα activation [73]. Phosphorylation of ERα on Ser118 by MAPK was found to reduce its affinity for TAM [72]. ERα that is

phosphorylated at Ser-118 was also found to have decreased DNA binding capacity when bound to TAM. Another consequence of phosphorylation of ERα at Ser 118 is its altered interaction with coactivator AIB1 [72]. Phosphorylation of ERα at Ser305 by PKA has been shown to result in TAM resistance by altering the interaction of ERα with coactivator NCoA-1(also known as SRC-1), and promote transcription by TAM [74-76]. P21-activated protein kinase-1(Pak1) phosphorylates ERα at Ser305. This leads to transactivation of ERα independent of ligand binding and increases ERα-mediated transcriptional activity. As a result there is reduced responsiveness of tumors to TAM [77,78]. Hence expression of Pak1 is considered as a poor prognostic marker for TAM resistance.

Altered glycosylation is an early step in carcinogenic transformation [79] and yet is an understudied field of knowledge regarding modification of ERα and other proteins that can move between the plasma membrane and nuclear compartment. Glycosylation of ERα at the hydroxyl group of its Ser or Thr residues has been reported [80,81]. O-linked N-acetyl glucosamine (O-GlcNAC) residues were identified on ERα that was isolated from bovine calf uteri and insect cells expressing mouse ERα [80]. The Thr575 residue on the C-terminal fragment of ERα was identified as a major site of glycosylation site in these ERα. Additional glycosylation sites were later identified on Ser10 and Thr50 of mouse ERα [81]. However these modifications were not found to alter the DNA-binding properties of ERα.

Acetylation of transcription factors by histone acetyl transferases (HATs) such as p300, CBP and P/CAF makes the DNA accessible to nuclear coregulators [82]. Acetylation of ERα by p300 at Lys residues 266 and 268 in its hinge/ ligand binding domain was reported by Mi *et al.* [83]. Their study demonstrated that acetylation of

ERα did not modify its localization or ability to bind E_2 or coregulators. However, acetylation increased ERα's DNA binding properties and transactivation. Contrary to this observation, mutation of Lys302 and 303 residues of ERα to Arg enhanced its activation by E_2 [84]. They found that acetylation is specific to Lys302 and 303 residues in the hinge region of ERα. This indicates an inhibitory effect of acetylation on the sensitivity of ERα to ligands. Mutation of Lys residues could thus contribute to development of breast cancer. Interestingly, an ERα K303R mutation has been reported in breast cancer patients with atypical hyperplasia [85]

Sumoylation is a post- translational modification involving addition of SUMO (small ubiquitin-like modifier) moieties to proteins. SUMOylation is increased in carcinogenesis [86]. ERα is sumoylated by (SUMO)-1, at the Lys residues in the hinge region [87]. Sumoylation enhanced ERα transcriptional activity. Proteins PIAS1 and PIAS3 were found to stimulate sumoylation of ERα in the presence of ligands, *e.g.*, E_2 and TAM [87]. This would be expected to increase ERα turnover. Using mutation studies involving substitution of Lys residues in the hinge region with Arg, the authors observed that there was an inhibition of sumoylation and reduced ERα transcriptional activity. Thus posttranslational modifications of ERα alter its transcriptional activity as well as interaction with coregulators.

Aberrant growth factor receptors and cytoplasmic signaling contribute to endocrine resistance

Role of Receptor Tyrosine Kinases (RTK) - Enhanced RTK signaling mediated by growth factor overexpression or intrinsic activation has a role in endocrine resistance. Further, overexpression of EGFR, insulin like growth factor receptor (IGFR) and the mutant human epidermal growth factor receptor (HER-2/neu or ERBB2) contribute to

endocrine/TAM-resistance because they activate cell survival/proliferation responses independent of estrogen-ER activity [88-91].

Endocrine resistance also involves activation of the intracellular protein kinase pathways, (*e.g.* the MAPK and the PI3K) that are downstream of plasma membrane-initiated EGFR, IGFR and ERBB2 signaling [89,92]. Deregulation of these pathways could be due to genetic modifications such as mutation of PTEN, a tumor suppressor that inhibits PI3K, or due to amplification of ERBB2 [64,93,94]. The mechanism of ERBB2 overexpression in endocrine resistance is not fully understood. However it is proposed that the intrinsic activation of ERBB2 and subsequent activation of the MAPK pathway that leads to phosphorylation of ERα at Ser118 results in its ligand-independent activation [94-96]. This would lead to non-responsiveness of cells to TAM-mediated repression of ERα-mediated transcription. Another mechanism of antiestrogen-resistance in ERBB2 overexpressing cells is the impaired recruitment of transcriptional corepressors such as NCoR and SMRT by TAM-occupied ERα [94]. As a result there is reduced responsiveness of ERBB2-overexpressing cells to TAM. This was seen in ERBB2-expressing tumor samples.

Role of cell cycle modulators - Aberrant expression of cell cycle regulators that are targets of antiestrogens can lead to endocrine resistance [97]. Overexpression of MYC, cyclin E1 or cyclin D1, inactivation of the retinoblastoma (Rb) tumor suppressor, or the decreased expression of cyclin-dependent kinase (CDK) inhibitors p21 and p27 lead to loss of antiestrogen-responsiveness [98-102]. c-Myc, a nuclear transcription factor has mitogenic effects similar to E_2 and is, in fact, an immediate early gene product stimulated by E_2-ERα [103]. MYC promotes cell cycle progression by regulating cell cycle modulators (*e.g.* p21 and p27) [104]. MYC overexpression has been shown to promote TAM-resistance by suppressing the

expression of *CDKN1A* that encodes p21 which is a repressor of cell cycle regulators cyclin-cdk2 [105]. This relief of repression of cyclin E1 CDK2 complexes by p21 results in enhanced growth rate in response to TAM. Along with CDK2, Cyclin E1 promotes entry of cells into the S-phase of the cell cycle [97]. Cyclin D1 overexpression also leads to increased tumor growth in response to TAM [106]. This is due to enhanced cyclin D1 binding to ERα through STAT3, thus activating ERα. TAM was found to enhance binding of ERα to STAT3 in the presence of cyclin D1, thus promoting tumor growth. Overexpression of MYC and Cyclin D1 and Cyclin E1 has also been reported in patients with breast cancer [107].

Loss of Rb function has also been linked to endocrine resistance [102]. Rb is a critical regulator of cell growth [108]. Inactivation of Rb function due to phosphorylation has been reported in breast cancer [109]. This loss of function is associated with aberrant cyclin/CDK activity. Other factors that can result in loss of Rb function in breast cancer includes mutation and epigenetic silencing [110]. Aberrant Rb pathway function has also been reported in ER-negative breast cancer and is used as a predictor of poor response to TAM therapy [111].

CHAPTER II: SIGNIFICANCE OF MICRORNA EXPRESSION IN ENDOCRINE/TAMOXIFEN -RESISTANT BREAST CANCER

BIOGENESIS OF MICRORNA

Regulation of gene expression is critical for the normal development of an organism. MicroRNA (miRNA) are a class of short non-coding RNA that regulate gene expression at the post-transcriptional and/or translational level [112] . These RNAs, which are 21-22 nucleotides in length, regulate a number of cellular processes including growth, development, differentiation, apoptosis and cell cycle [113]. miRNAs were first described by Lee *et. al.* in *Caenorhabiditis elegans* [114]. Today there are over 9200 publications listed in PubMed on miRNAs in humans, reflecting the interest in how these RNAs post-transcriptionally regulate protein expression and cell function.

miRNAs are given a three lettered prefix depending on the species that they originate in *i.e.*, hsa for *homo sapiens*, mmu for mouse, and so on [115]. If miRNAs originate from different genomic loci, they are assigned a numerical suffix, *i.e.* hsa-miR-29b-1 and hsa-miR-29b-2. miRNAs that differ by a few bases are given a lettered suffix of the form miR-125a and miR-125b. Those miRNAs that originate from opposite arms of the hairpin precursor are assigned suffixes of the type miR-142-5p and miR-142-3p. miR-21 and miR-21* refers to miRNAs that arise from the same hairpin precursor. An asterix in the miRNA name indicates that it is a less predominant form of the miRNA[116]. miRNA cluster arises due to gene duplication, *e.g.*, the miR-200 cluster of miRNAs is located in two chromosomes, *i.e.*, miR-200a,

miR-200b and miR-429 are located on chromosome 1 and miR-200c and miR-141 are located on chromosome 12. Each cluster is transcribed into a common precursor RNA. A miRNA family refers to miRNAs that arise from a common ancestor and whose sequences are similar *e.g.* miR-221 and miR-222 family.

MiRNA are located mostly in the introns of protein coding genes [117]. In eukaryotes, most miRNAs are transcribed by RNA polymerase II into long transcripts called primary miRNA (pri-miRNA) [118]. A few miRNAs are transcribed by RNA pol III, *e.g.*, miR-515-1, miR-517a, miR-517c [119]. Primary miRNA is processed into ~70 nucleotide precursor miRNAs (pre-miRNA) by a ribonuclease III (RNase III) enzyme Drosha in association with an RNA binding protein DGCR8 (together called the Microprocessor complex). Recent studies show that some intronic miRNAs (mirtrons) are processed by splicing machinery instead of Drosha and DGCR8 [120]. Pre-miRNA is then exported from the nucleus to the cytoplasm by Exportin and Ran-GTP. In the cytoplasm, the pre-miRNA is further cleaved by RNase III Dicer along with RNA-binding proteins TRBP and PACT (in humans) to a mature double stranded miRNA. Mature miRNAs is incorporated into the RNA-induced silencing complex (RISC) consisting of Argonaute 2 (Ago2) and TRBP protein. The miRNA duplex unwinds and the RISC degrades one of the strands of the miRNA (passenger strand, *e.g.*, miR-21*) while the functional strand (guide strand, *e.g.*, miR-21) targets messenger RNA (mRNA) for degradation or translational repression. According to miRBase (version 18, November 2011), the human genome encodes more than 1500 human miRNAs.

MICRORNA FUNCTION

MiRNAs affect mRNA stability or repress translation. The 5'UTR of miRNA harbours a 2-8 nucleotide seed region that binds to the 3'UTR of mRNA in a

complementary fashion. Perfect base pairing between the miRNA and mRNA leads to mRNA degradation [121]. This type of perfect complementarity between miRNA and the target mRNA is seen in mostly in plants and rarely in vertebrates and viral miRNAs.

In most metazoans, there is mostly imperfect base pairing leading to translational repression. Many theories have been proposed for the repression of translation by miRNAs. One theory states that the AGO2 protein of the miRNA-ribonucleoprotein (miRNP) complex (consisting of mature miRNA, Ago2 and TRBP protein) competes with the eIF4E elongation factor from binding to the 5' cap of mRNA, thus repressing translation [122]. Another study shows that the elongation factor eIF6 is a major target of miRNPs. The authors of this study propose that AGO2 interacts with eIF6 and inhibits joining of the 60S ribosomal subunit to the 40S initiation complex [123]. It has also been proposed that repression of translation occurs at post-initiation stages of translation due to slow elongation of mRNA [124,125]. Repression of mRNAs by binding of miRNPs induce deadenylation mediated by a glycine-tryptophan protein called GW182 and poly (A) binding protein (PABP) which in turn recruits deadenylase CCR4 and CAF1, and subsequently results in decay of mRNAs [126,127]. An alternate mechanism of repression by miRNA is by sequestration of mRNA in P-bodies [128]. mRNAs that undergo translational repression by the RISC were found to accumulate in these P-bodies [129]. In eukaryotes, there are two modes of mRNA decay induced by miRNAs. The mRNA is degraded either by 3'→ 5' exosome activity or by removal of the 5' cap followed by 5'→3' degradation catalyzed by XRN1 [130].

Currently there are over 10, 000 miRNAs that have been reported in different organisms [131]. It is estimated that miRNAs regulate ~50% of protein-coding

genes in the human genome [132]. MiRNA target prediction software programs have shown that each miRNA can have more than one mRNA target. MiRNAs have been shown to fine-tune the expression of genes to allow optimal expression during different stages of development. In humans, miRNAs exhibit tissue specific expression and regulate cellular processes by targeting key proteins and signaling networks [133].

REGULATION OF MICRORNA EXPRESSION

Role of miRNA processing proteins - MiRNA expression is regulated at different levels. The ratio of the ribonuclease Drosha and its binding partner DGCR8 is tightly regulated to ensure proper pri-miRNA processing [134] . DGCR8 stabilizes Drosha. Drosha in turn regulates DGCR8 levels by cleaving and thus inactivating it. Thus a tight feedback loop maintains the cellular Drosha/DGCR8 ratio [135].

A number of co-activators and co-repressors can alter Drosha activity. Transforming growth factor beta (TGFβ) signaling and bone morphogenetic protein (BMP) and SMAD proteins stimulate, while the nuclear factor NF90-NF45 heterodimer suppresses Drosha activity [136,137]. ERα along with helicases p68 and p72, and Drosha have been shown to affect Drosha complex formation, thus repressing pri-miRNA processing[138].

Defects in Exportin 5 results in accumulation of pre-miRNAs in the nucleus [139]. Another ribonuclease that is a key point of regulation in the miRNA biogenesis pathway is Dicer. Altered Dicer expression can affect processing of pre-miRNA to mature miRNA. Dicer mutation has been reported in non-small cell lung cancer and prostate cancer [140,141]. It has been shown that Dicer cofactors, TRBP and PACT are critical in maintaining the stability of Dicer in cells [142,143].

Increased phosphorylation of TRBP by MAPK signaling was found to enhance Dicer activity and promote miRNA processing [144].

Role of Ago2 proteins – After cleavage of the pre-miRNA by the ribonuclease Dicer, one of the strands of the mature miRNA is incorporated into the RISC by Ago2 protein. Although the human genome encodes about 8 Ago proteins, Ago2 is the major protein involved in RNA cleavage and silencing. The level of Ago2 determines the amount of mature miRNA synthesized. Ago2 is subject to regulation at the transcriptional and post-transcriptional level. For example in MCF-7 breast cancer cells Ago2 expression is inhibited by E_2 and EGF-MAPK signaling [145].

Regulation of miRNA transcription - A number of transcription factors have been shown to regulate miRNA expression. For example, p53 has been shown to increase the expression of miR-34 and miR-107 families [146,147]. MYC stimulates the expression of miR-17-92 cluster of miRNAs in lymphoma cells [147]. In neuronal cells, the RE1 Silencing Transcription Factor (REST1) inhibits transcription of miR-124 by recruiting histone deacetylases (HDACs) and methyl CpG binding protein MeCP2 to the miR-124 gene promoter [148]. The transcription of primary miRNA transcripts have been shown to be regulated by platelet-derived epidermal growth factor (PDGF) and transforming- growth factor-beta (TGF-β) [149,150]

Steroid hormone regulation of miRNA expression- Steroid hormones and their receptors have been reported to regulate miRNA expression in a variety of cancer cell lines [151-153]. Because the focus of my dissertation is the role of miRNA expression in endocrine resistant breast cancer, I will focus on estrogen regulation of miRNA expression [154,155]. E_2 regulation of miRNA expression was studied by Cohen *et. al.* in adult zebrafish [156]. miR-196b and Let 7h were up-regulated, and miR-130c and miR-101a were downregulated by E_2 treatment in this study. Further,

Hoxb8a, a target of miR-196b was downregulated regulated by E_2 in zebrafish. In a model of mammary carcinogenesis in rats, E_2 was found to regulate the expression of miRs after 6, 12 and 18 weeks of treatment in female August Copenhagen Irish (ACI) rats [157]. Some of the miRNAs that were altered after E_2 treatment include miR-22*, miR-99a, miR-127, miR-499, miR-17-5p, miR-20a, and miR-92. Castellano *et. al.* have shown that E_2 upregulates the expression of miRNAs encoded by the miR-17-92 and miR-106a-363 paralogous cluster in MCF-7 breast cancer cells [158]. Microarrays identified 23 miRNAs to be downregulated by E_2 in MCF-7 cells [159]. Of these, the expression of 8 were confirmed by quantitative real time PCR (Q-PCR) in MCF-7, BT474, T47D and ZR-75-1 breast cancer cells. The expression of pri-miR-21 and pri-miR-181a were also found to be regulated by E_2 in MCF-7 cells. Another study by Cicatiello *et al.* identified miR-424 and miR-760 to be increased by E_2 while miR-107, miR-570 and miR-618 were found to be decreased by E_2 [160]. This study identified miRNA binding sites in the mRNA of E_2-regulated target genes by global mapping.

Work done in our laboratory has also shown that E_2 regulates miRNA expression in MCF-7 breast cancer cells [161,162]. E_2 was found to decrease miR-21 expression which in turn increased the expression of its target genes, PDCD4 and BCL2. Contrary to our results, Nakshatri *e. al.* reported an increase in miR-21 expression in MCF-7 cells after treatment with 10nM E_2 for 4 h [163]. However, others have likewise reported that E_2 reduces miR-21 expression [159,164-166] A summary of miRNAs that are regulated by E_2 was recently reviewed [167]

Epigenetic control of miRNA expression –DNA methylation and histone modifications regulate miRNA expression. These mechanisms have been attributed to the aberrant expression of miRNAs seen in diseases such as cancer. For example,

hypermethylation of miR-134, miR-34b/c, miR-137, miR-342 has been reported in different types of cancer [168,169]. Hypomethylation of let 7a-3 has been reported in lung adenocarcinoma [170]. The promoter of miRNA genes *e.g.* miR-1 and miR-27a are altered by histone modification [171]. Acetylation of miR-1 gene promoter results in its decreased expression in lung cancer. HDAC inhibitors reversed this effect.

MICRORNA AS ONCOGENES OR TUMOR SUPPRESSORS

Aberrant miRNA expression has been reported in a number of diseases including cancer [172]. MiRNAs act as oncogenes or tumor suppressors by regulating the expression of genes associated with key pathways. As such, their expression and the regulation of their expression is of keen interest in cancer research, both for use as clinical biomarkers and as targets for prevention or treatment.

MicroRNAs as tumor suppressors- miR-15a and miR-16 act as tumor suppressors by negatively regulating the anti-apoptotic gene BCL2. Downregulation of miR-15a and miR-16 has been observed in chronic lymphocytic leukemia (CML), resulting in increased expression of BCL2 and anti-apoptotic activity [173]. miR-34a is downregulated in colon, ovarian, lung and pancreatic cancers [174-176]. It targets the oncogene MYCN [177]. miR-34 indirectly controls p53 activation through SIRT1 [178] . The Let-7 family of miRNAs were found to be deleted in a number of cancers including lung, colon and lymphomas [179,180]. They act as tumor suppressors by negatively regulating cell cycle regulators, CDK6 and CCND [181]; and oncogenes such as RAS, MYC and HMGA2 by translational repression [182,183].

MicroRNAs as oncogenes- One of the most important miRNAs that acts as an oncogene is miR-21. It has been shown to repress the expression of tumor suppressors such as the programmed cell death protein (*PDCD4*), tropomyosin

(*TPM1*), phosphatase and tensin homologue (*PTEN*) and maspin[184-188]. miR-21 is overexpressed in lung, breast, pancreatic and prostate cancer [189-191].

In addition to miR-21, the miR-17-92 cluster is overexpressed in aggressive lung cancer and its putative targets include *PTEN* and *RB2* [132,192]. The expression of miR-155 is increased in most lymphomas, *e.g.*, miR-155 was found to be significantly decreased in Burkitt's lymphoma and Diffuse Large cell B-lymphoma (DLBCL)[193]. It has been suggested that this overexpression of miR-155 downregulates the expression of the transcription factor PU.1 that is important in B-cell differentiation [194].

MICRORNA EXPRESSION IN BREAST CANCER

MiRNAs are aberrantly expressed in breast cancer [195,196]. MiRNA expression is either upregulated or downregulated in breast cancer cells or tumors when compared to normal breast tissue. miRNAs have been shown to have a role in tumor progression by altering the expression of oncogenes and tumor suppressors [197]. miRNAs promote metastasis and invasive properties of breast cancer cells. Expression profiling of miRNAs in solid tumors and breast tissues have identified miRNAs that are associated with breast cancer subtypes [198]. A few examples of the miRNAs that are deregulated in breast cancer include tumor suppressors such as Let-7, miR-125, miR-200, and the oncogenic miRs such as miR-21 and miR-155 [199]. Downregulation of the tumor suppressor Let-7a alters the expression of its targets Ras and HMGA2 [200]. miR-125a and miR-125b have been shown to target tumor suppressors ERBB2 and ERBB3 [201]. Downregulation of Let-7a, miR-200 and miR-205 promotes epithelial-to-mesenchymal transition (EMT) in breast cancer [202-204]. The targets of miR-200 include nuclear transcription factors ZEB1, ZEB2; phospholipase C, gamma 1(PLCG1) and TGF-β2 [205-207]. Other metastatic

promoting miRNAs that promote migration and invasion of cancer cells include miR-10b, miR-520c and miR-373 [208,209]. miR-10b targets HOXD10 expression [210]. HOXD10 has been shown to target genes involved in angiogenesis and cell migration [211]. Loss of HOXD10 thus promotes an invasive phenotype in breast cancer.

The miRNAs that are oncogenic, and overexpressed in breast cancer, are miR-21 and miR-29, miR-34, miR-155, and miR-210 [199]. MiR-21 has been shown to promote tumor formation by targeting PDCD4, PTEN, TPM1, and BCL2 [187,212]. The miR-17/92 cluster has been shown to have a dual role as a tumor suppressor and an oncogenic miRNA. Hossain *et al.* reported its role as a tumor suppressor by reducing the expression of the ER coactivator AIB1 [213]. AIB1 was originally identified as an oncogene by Myles Brown [214] and further studies have confirmed AIB1's role in breast cancer [215-217]. Li *et al.* described miR-17-5p of the 17/92 cluster to be a metastasis promoting miRNA in breast cancer by suppressing HMG box-containing protein 1HBP1 expression [218]. HBP1 is a suppressor of Wnt/β-catenin signaling. Downregulation of HBP1 by miR-17-5p activates Wnt/β-catenin signaling and thus promotes migration and invasion of MCF-7 breast cancer cells.

Analysis of miRNA expression in breast tumors using tissue microarray identified a number of miRNAs that are differentially expressed in breast cancer tissue when compared to normal tissue, *e.g.,* miR-10b, miR-21, miR-145 were upregulated in breast cancer tissue [219]. There was also a correlation between miRNA expression, ER and PR status, lymph node status in these samples. In another study, miR-342 and miR-520g were found to be overexpressed in ER-positive and HER2 positive tumors when compared to normal tissue [220]. miRNA signatures associated with specific breast cancer cell type such as luminal and basal-like has been reported [198]. Let-7a, miR-21, miR-141 and miR-214 were expressed in

luminal cell type while miR-145 and miR-205 were associated with the myoepithelial cell type [221].

Circulating miRNAs have been detected in the blood, plasma and serum of breast cancer patients [222,223]. The level of plasma miRNAs was higher in breast cancer patients when compared to control groups [224,225]. For example miR-425*, Let 7c were found to be higher in the plasma of women with early stage breast cancer when compared to healthy controls. Higher expression of miR-10b and miR-34 was found in the serum of breast cancer patients correlated with metastasis [222]. Systemic miR-195 and Let-7a were higher in breast cancer patients when compared to normal subjects[226]. The expression of these miRNAs also correlated with ER status [227]. These reports indicate that miRNAs could be valuable diagnostic markers in the prognosis and treatment of breast cancer.

MICRORNAs REGULATE ERα ACTIVITY

MiRNAs regulate ERα expression and activity. MiR-206 downregulates ERα mRNA and protein level in MCF-7 human breast cancer cells [228]. This study showed that miR-206 expression is increased in ERα/ERBB2-negative tumors when compared to ERα-positive tumor specimens. Adams *et al.* reported a double negative feedback loop between miR-206 and ERα in MCF-7 cells [229]. Another study by the same group reported increased miR-206 expression in MCF-7 and T47D breast cancer cells [230]. This increased miR-206 expression was found to suppress estrogenic effects in cells by decreasing the expression of ERα and coactivators such as NCoA-1, AIB1 and GATA3 that are involved in ERα signaling. The authors propose that enhanced EGF signaling contributes to the loss of ERα by increasing miR-206 expression. This loss of ERα is one of the factors responsible for the

transformation of cells from luminal to a basal-type. Among the other miRNAs that target ERα are miR-22, miR-221 and miR-222. miR-22 degraded ERα mRNA [231]. The expression of this miRNA was lower in ERα-positive breast cancer cell lines such as MCF-7, T-47D and BT474 when compared to ERα-negative cell lines including MDA-MB-231 and SK-Br3 [232]. In contrast to miR-22, miR-221/222 suppressed ERα protein and not mRNA [233]. This study identified two binding sites for miR-221 and miR-222 in the 3'UTR of ERα. Further, a negative feedback loop between miR-221/222 cluster and ERα has been reported in breast cancer cells [234]. miR-221/222 represses ERα, which in turn represses the expression of miR-221/222. Thus miR-221/222 acts as a tumor suppressor in ERα-positive cells. However Rao *et al.* have shown that prolonged treatment of cells with E_2 or fulvestrant releases miR-221/222 from the negative feedback loop. This results in increased miR-221/222 expression leading to enhanced cell proliferation and endocrine-resistance [235].

Let-7 family of miRNAs target ER-α66. There is an inverse correlation between the expression of Let -7 family and its target ERα-66 in breast cancer samples [236]. Let-7 family also targets an isoform of ERα, ERα-36 that is located in the plasma membrane [237]. Loss of Let-7 family was found to confer TAM-resistance due to increased ER-α36 and enhanced non-genomic signaling. Among the other miRNAs that target ERα include miR-18a, miR-18b, miR-193b and miR-302c [238]. This study utilized a protein lysate microarray approach where a library of pre-miRs were transfected into MCF-7 and T47D cell lines, and the expression of ERα was analyzed in the protein lysates.

MICRORNA AND ENDOCRINE RESISTANCE

Computational analysis of microRNAs have identified miRNAs and their targets that have a role in fulvestrant resistance [239]. miR-221/222 has been reported to promote TAM-resistance by targeting ERα and the cell cycle regulator p27 (also known as Kip1) [233,240,241]. Miller *et al.* showed that miR-221/222 is overexpressed in ERα-negative cells lines and tumors, as well as HER2-positive tumors. Overexpression of miR-221/222 was associated with fulvestrant-resistance [235]. Prolonged fulvestrant treatment induced miR-221/222 expression in MCF-7 cells. Some of the signaling pathways that are involved in promoting the miR-221/222-mediated fulvestrant resistance and oncogenic activity include the β-catenin, TGF-β and p53 pathways [235]. miR-221/222 is also increased in $CD44^{+}CD24^{-/low}lineage^{-}$ human breast cancer stem cells, indicating a role for these stem cells in drug resistance [242]. A study by Lu *et al.* shows that anti-miR-221 suppressed the growth of TAM-resistant xenografts in mice [243]. miR-15a/16 was found to downregulate the anti-apoptotic gene BCL2 and promote TAM resistance in MCF-7 cells expressing the HER2delta16 mutant [244]. miR-451, a tumor suppressor miRNA was reported to be suppressed in MCF-7 derived cell lines that are TAM-resistant [245]. miR-451 targets the expression of 14-3-3ζ, an anti-apoptotic gene that is overexpressed in TAM-resistant tumors and is also associated with poor clinical outcome in breast cancer. Thus the loss of miR-451 in ER-positive breast cancer upregulates 14-3-3ζ and contributes to TAM-resistance.

A PubMed search for 'MicroRNA and endocrine resistance in breast cancer' generates 12 publications as of September 2012. Most recently, global miRNA expression profiling using Exiqon microarrays in 152 ERα + tumors in 52 patients who received adjuvant tamoxifen as mono-therapy identified 10 miRNAs that

discriminated between patient samples according to patient outcome[246] . Patients with recurrence in general had lower levels of miRs- 190b, 339-5p, 520c-3p, 520g, 520h, 139-3p, 204, 502-5p, 365, and 363. However, none of the data were statistically significant except that miR-7 increased with tumor grade. The authors concluded that "the miRNA profile does not seem to provide information with regard to the probability of recurrence following adjuvant tamoxifen-treatment in post-menopausal ER+ breast cancer patients." [246]. However, they also noted that tumor heterogeneity might be a factor in miRNA expression.

DISSERTATION SPECIFIC AIMS AND HYPOTHESES

As reviewed in Chapter I, breast cancer is one of the leading causes of death among women in the United States [1]. Although TAM prevents recurrence of breast cancer in women, about 40% of women who receive adjuvant hormonal therapy acquire resistance [53,248]. The mechanisms of acquired endocrine resistance remain to be fully elucidated [249]. This study was focused towards identifying miRNAs that are dysregulated in acquired endocrine/TAM- resistant breast cancer. By identifying miRNAs that are differentially expressed in antiestrogen-sensitive MCF-7 cells versus antiestrogen-resistant LY2 breast cancer cells, it would be possible to use them as prognostic markers of antiestrogen resistance in breast cancer.

Our laboratory identified miRNAs that are differentially regulated by TAM in endocrine-sensitive MCF-7 and endocrine-resistant LY2 human breast cancer cells [161,247]. Microarray expression profiling identified miRNAs that are regulated by E_2 and TAM. They were found to be regulated in the opposite direction in MCF-7 and LY2 cells. Q-PCR was preformed to confirm the expression 12 miRNAs that

showed significant opposite expression between the two cell lines. Bioinformatic prediction identified 36 putative mRNA targets of the 12 miRNAs whose expression was validated by Q-PCR. Some of the mRNA targets include *PDCD4*, *BCL2*, *CYP1B1*, *ERBB3*. *ZEB1*, a target of miR-200 family of miRNAs and a promoter of EMT, was found to be overexpressed in LY2 cells when compared to MCF-7 cells. This was a significant finding as it indicated that LY2 cells represent cells that have undergone EMT. This study identified miR-200 family of miRNAs to have a role in suppressing endocrine-resistance in breast cancer.

Identification of specific miRNAs and their gene targets will advance our understanding of mechanisms of antiestrogen/endocrine-resistant breast cancer. The overall hypothesis of this proposal is that **miRNAs are dysregulated in acquired endocrine-resistant breast cancer.**

The specific aims to test the hypothesis were:

SPECIFIC AIM 1: Identify miRNAs oppositely regulated by 4-OHT in antiestrogen-sensitive MCF-7 and antiestrogen-resistant LY2 human breast cancer cells.

I hypothesized that there is inverse regulation of miRNAs in tamoxifen-sensitive versus-resistant breast cancer cell lines, and that the regulation of these miRNAs that are altered in tamoxifen resistance is mediated through the ER. Microarray was used to identify miRNAs that are differentially expressed between MCF-7 and LY2 cells. Results of the microarray analyses were validated by quantitative real time polymerase chain reaction (Q-PCR).

SPECIFIC AIM 2: Identify the genes targeted by select miRNAs and correlate these miRNAs with changes in mRNA in MCF-7 and LY2 cells.

I hypothesized that changes in miRNA expression correlate with changes in target gene expression in MCF-7 and LY2 cells. miRNAs thus mediate dysregulated expression of target oncogenes or tumor suppressors and hence alter critical gene pathways in tamoxifen/endocrine-resistance. Bioinformatics was used to identify putative targets of select miRNAs that were identified and some were experimentally verified in Aim 1.

SPECIFIC AIM 3: Determine if knockdown of miRNAs upregulated and overexpression of miRNAs downregulated in endocrine-resistant breast cancer cells restore endocrine-sensitivity.

I hypothesized that changes in miRNA and mRNA expression correspond to changes in functional outcomes in cells. These changes can be reversed by knockdown or overexpression of those miRNAs that are up or downregulated respectively. Transfection using anti-miRNA or precursor miRNA oligonucleotides followed by functional assays were used to determine whether knockdown or overexpression of miR-200 restored endocrine-sensitivity/resistance and alter biological functions in cells.

CHAPTER III: DIFFERENTIAL EXPRESSION OF MICRORNAs IN TAMOXIFEN- SENSITIVE MCF-7 *VERSUS* TAMOXIFEN-RESISTANT LY2 BREAST CANCER CELLS

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1. INTRODUCTION

The ability of selective estrogen receptor modulators (SERMs, *e.g.*, tamoxifen and raloxifene) and aromatase inhibitors (AI) to prevent disease recurrence in patients whose initial breast tumors expressed estrogen receptor alpha (ERα) provides compelling data supporting the role of ERα in the pathogenesis of breast cancer [250]. Unfortunately, approximately 40% of patients relapse after tamoxifen (TAM) or other endocrine therapies [251]. The mechanisms for the acquired resistance to endocrine therapies is complex and, even in the presence of continued ERα expression, includes amplification of growth factor signaling pathways, *e.g.*, epidermal growth factor receptor (EGFR), MAPK, PI3K/AKT, JNK, and p38 MAPK [251-253], but the role of microRNAs in endocrine-resistance remains to be fully elucidated.

MicroRNAs are short, non-coding RNAs that regulate gene expression at the post-transcriptional level by direct binding to the 3'UTR of mRNA targets within the ribonucleoprotein RNA-induced silencing (RISC) complex, causing translational repression usually accompanied by mRNA decay [254,255]. miRNAs regulate diverse cellular processes including differentiation, replication, migration, and apoptosis [256]. Microarray technology has been used to generate miRNA profiles

and demonstrate aberrant miRNA expression in a variety of cancers including breast tumors and cell lines [257-262]. These miRNA expression profiles correlate with classification of tumor grade and patient prognosis [257,258,263]. Altered miRNA expression in cancer may result from chromosomal rearrangements, deletions or epigenetic modifications in DNA or chromatin structure [263]. Bioinformatic analyses are used to identify putative mRNA targets of miRNAs, thus linking miRNAs to the regulation of complex protein networks involved in a variety of cellular functions [264]. miRNAs in breast cancer cells function as tumor suppressors, *e.g.*, Let-7 family members, miR-125a and miR-125b, and miR-200; or as oncogenes, *i.e.*, 'oncomirs', *e.g.*, miR-21, miR-10b, miR-155, and the miR-17-92 cluster [262,265].

miRNAs are processed from longer transcripts called precursor (pre)-miRNAs by Dicer within the cytoplasm. Pre-miRNAs are, in turn, the products of the processing, within the nucleus by DROSHA, of the initial miRNA gene transcripts called primary (pri)-miRNAs [266]. Recent studies have identified miRNAs regulated by estradiol (E_2) in breast cancer cells and other cells and tissues (reviewed in [267]). For example, we and others reported that miR-21 and the Let-7 family of miRNAs are downregulated by E_2 in breast cancer cells [260,261,267,268]. Interestingly, E_2 upregulates transcription of miR-17-92 and its paralog miR-106a-363 clusters in MCF-7 human breast cancer cells, but appears to delay processing of the miR-17-92 gene product into its final miRNAs, including miR-18a and miR-20a [269], although the mechanism remains to be identified.

There are only a few studies of miRNA in TAM/endocrine-resistant breast cancer cells. Cell-based studies found that miRNA-221/222 are overexpressed in TAM-, fulvestrant-, and tumor necrosis factor (TNF)- resistant derivative of MCF-7

cells [270-272]. However, no one has examined the effect of TAM on the expression of miRNAs in TAM-sensitive *versus* resistant breast cancer cells.

To investigate whether antiestrogen-resistance correlates with changes in miRNA expression, we profiled miRNA expression in TAM- sensitive MCF-7 and TAM/endocrine-resistant LY2 human breast cancer cells. LY2 cells were derived from MCF-7 by serial passage in the antiestrogen LY 117018, a precursor to Raloxifene (RAL) [273], and express wild-type ERα mRNA levels similar to MCF-7 cells [274], but are resistant to TAM, RAL, and fulvestrant (ICI 182,780) [275]. I hypothesized that differences in miRNA expression with TAM treatment between the TAM-sensitive MCF-7 *versus* TAM-resistant LY2 cells would identify miRNAs and their mRNA gene targets contributing to antiestrogen-sensitivity and resistance, respectively. miRNA microarrays were used to identify TAM-regulated miRNAs in these two cell lines. This study identified 97 miRNAs that were differentially expressed between the two cell lines and focused on 12 miRNAs that showed the greatest difference in expression between the two cell lines. Quantitative real time polymerase chain reaction (Q-PCR) was used to confirm the results obtained by microarray. In addition to miRNAs differentially regulated in the two cell lines, eight endogenous controls, including 6 miRNAs, 5S rRNA, and SNORD38B, were identified from the microarray data and their expression confirmed by Q-PCR.

A search of the Sloan-Kettering Targets and Expression (http://www.microrna.org/microrna/getDownloads.do) dataset was used to identify 36 putative gene targets of these miRNAs from amongst those that were reported to be regulated by 4-OHT in MCF-7 cells [276]. Q-PCR was used to examine the expression of 8 miRNAs. Q-PCR and Western analyses were used to examine the expression of gene/protein targets of the miRs- 21, 125b, 200a, 200b, 200c, 221 and

222: *PDCD4*/Pdcd4, *BCL2*/Bcl-2, *CYP1B1*, *ERBB3*/ErbB3, *ESR1*/ERα, and ZEB-1. Results from this chapter show opposite regulation of select miRNAs and target proteins between the two cell lines, thus indicating a putative role of these miRNAs in TAM/endocrine resistance.

2. MATERIALS AND METHODS

2.1 Cells and treatments

MCF-7 human breast cancer cells were purchased from ATCC (Manassas, VA, USA) and maintained in IMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). LY2 tamoxifen/fulvestrant-resistant human breast cancer cells were provided by Dr. Robert Clarke, Georgetown University, and were used at P<16 from this source. LY2 cells were originally derived from MCF-7 cells by selection in increasing concentrations of LY 117018 [273]. LY2, selected for resistance to LY 117018, are cross-resistant to TAM, raloxifene, fulvestrant (ICI 182,780), and are ERα positive, although ERα protein expression is lower than MCF-7 cells [273,277]. LCC1, LCC2, LCC9 are also derivatives of MCF-7 cell lines that are E_2, tamoxifen, and multiple SERM-independent, respectively [278], and were provided by Dr. Robert Clarke, Georgetown University, and were, like LY2, used at P<16 from this source. MDA-MB-231 'triple negative' breast cancer cells were purchased from ATCC. E_2 and 4-OHT were purchased from Sigma (St. Louis, MO, USA). ICI 182,780 was from Tocris (Ellisville, MO, USA). Prior to treatment, the medium was replaced with phenol red-free IMEM supplemented with 5% dextran charcoal-stripped FBS (DCC-FBS) and 1% penicillin/streptomycin (stripped medium) for 48 h (referred to as 'serum-starving' or 'serum starved' cells). Cells were treated with ethanol (EtOH, the

vehicle control, 0.01% final volume), 10 nM E_2 or 100 nM 4-OHT for 6 h. For the microarray profiling, 4 separate experiments (biological replicates) were performed at different times over a 6 month period for each cell line. Note: Referrals in the text to ER and not specifically to ERα or ERβ indicated that either ERα or ERβ or both may be involved in the response tested.

2.2 MicroRNA microarray analyses

RNA was isolated from MCF-7 and LY2 cells, treated as above, using the mirVana miRNA Isolation Kit from Ambion (Austin, TX, USA) and sent to Exiqon (http://exiqon.com//) where the RNA samples were labeled with either Hy3 or Hy5 fluorescent labels and hybridized into the miRCURY LNA™ microarray (miRbase 11.0 human array). This microarray featured 1275 *bone fide* and putative human miRNAs plus additional controls. Four separate experiments (biological replicates) were performed. Data analysis was performed by Exiqon as follows: clustering of miRNAs was performed using log2 (Hy3/Hy5) ratios which passed the filtering criteria on variation across sample groups using a two tailed T-test p-value < 0.001. The Hy3 signals were normalized using the single color approach 'Quantile' followed by a background correction. The data were deposited in GEO as GSE28267 http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE28267. The subset of miRNAs showing the highest variation among the 1275 miRNAs were used for clustering which provided a subset of 50 miRNAs that showed maximum variation between the two cell lines. The heat map (Figure 1) shows the result of clustering of miRNAs. The miRNA clustering tree is shown on the left and top. Each column represents a treatment and each row a miRNA.

2.3 RNA isolation and quantitative Real-Time-PCR (Q-PCR) for miRNA expression

miRNA-enriched total RNA was extracted from MCF-7 and LY2 cells treated as above using the miRNA isolation kit (Exiqon). The quality and quantity of the isolated RNA was analyzed using a NanoDrop spectrophotometer and Agilent Bioanalyzer. cDNA was synthesized using the miRCURY LNA™ first strand cDNA synthesis kit (Exiqon) and Q-PCR was performed using the miRCURY LNA™ SYBR Green master mix (Exiqon) using the miRNA primer sets for miR-10a, -21, -22, -125b, -181a, -200a, -221 and -222 (Exiqon). SNORD38B and 5SRNA were used for normalization of miRNA expression. Analysis and fold change was determined using the comparative threshold cycle (Ct) method. The change in miRNA expression was calculated as fold-change, *i.e.*, relative to EtOH-treated (control).

2.4 RNA Isolation, RT-PCR and Q-PCR for mRNA expression

RNA was extracted from cells using Trizol (Invitrogen) or RNeasy (Qiagen). The High Capacity cDNA Reverse Transcription kit (PE Applied Biosystems) was used to reverse transcribe total RNA using random hexamers. Q-PCR for *BCL2*, *CYP1B1*, *ERBB3*, *ESR1*, *PDCD4*, and 18S using Taqman primers and probes as Assays-on-Demand was performed in the ABI PRISM 7900 SDS 2.1 (PE Applied Biosystems) using relative quantification. Analysis and fold differences were determined using the comparative CT method. Fold change was calculated from the ΔΔCT values with the formula $2^{-\Delta\Delta CT}$ and data are relative to EtOH-treated cells.

2.5 Whole cell and nuclear lysate preparation for western blotting

Whole cell lysates were prepared and western blots were performed as described [277]. Nuclear extracts (NE) were prepared using the NE-PER kit from Thermo Scientific (Rockford, IL, USA). Antibodies were purchased as follows: ERα (Santa Cruz Biotechnology, Santa Cruz, CA, USA), ERβ (H150, Santa Cruz, CA, USA), Argonaute 2 (Anti-Ago2, clone 9E8.2, #04-642, Millipore, Billerica, MA,

USA), Pdcd4 (GeneTex,Irvine, CA), Bcl-2 (Assay Designs, Plymouth Meeting, PA), E-cadherin (Cell Signaling, Danvers, MA, USA), α-tubulin (Thermo Scientific, Rockford, IL, USA), β-actin (Sigma, St. Louis, MO, USA). The ZEB-1 antibody was generously provided by Dr. Douglas Darling, University of Louisville. Chemiluminescent bands on the PVDF membranes were visualized on a Kodak Carestream Imager using Carestream Molecular Imaging software (New Haven, CT, USA).

2.6 Statistical analysis

Data preprocessing was performed on two sets of samples sent to Exiqon at different times (sample set 1 and 2 contained 6 and 14 cell treatments, respectively; different miRNA chips were utilized for the 2 sets of samples) separately before combining them for further analysis. Two-step filtering (1) excluding empty and blank spots and (2) keeping only those spots for which foreground intensities were greater than 1.1 x background intensities for 2 or more samples in the 6-sample group and 10 or more samples in the 14-sample group was done before normalization. For the remaining spots, background intensities were subtracted from the foreground intensities. Since even after the filtering step, some spots had backgrounds larger than foregrounds; we treated those as missing and imputed them using the *k*-nearest neighbor algorithm. Normalization within-arrays was performed using the loess method [279], while for between-arrays the quantile method was applied. The two sets of samples were then matched by their miRNA names and combined for further analysis.

In order to identify miRNAs which are expressed by MCF-7 and LY2 cells treated with EtOH and 4-OHT and by MCF-7 cells treated with E_2, the four technical replicates on each chip and the four arrays (biological replicates) corresponding to

each of the five treatment groups (n=20) were averaged. All expression values were represented as log2 ratios of Hy3 (experimental) *versus* Hy5 (universal reference). Differential expression of miRNAs between different TAM-sensitive and TAM-resistant cell lines treated with either 4-OHT or EtOH were determined by fitting a hierarchical linear model using the *limma* package [280] and testing the corresponding contrasts of interest, *e.g.*, MCF-7 *vs.* LY2 treated with 4-OHT, MCF-7 *vs.* LY2 treated with EtOH, and E_2 *vs.* 4-OHT treated MCF-7 cells, for each miRNA. Fold change, adjusted t-statistic, unadjusted and false discovery rate (FDR) adjusted p-values were calculated for each miRNA for each comparison. Of the 225 miRNAs that passed the filter for analysis, only those miRNAs with adjusted p-values below 0.10, *i.e.*, FDR of 10%, were considered as differentially expressed.

2.7 Gene pathway analysis

Functional and network analyses of differentially expressed miRNAs gene expression changes were performed using Ingenuity Pathways Analysis (IPA) 8.8 (Ingenuity® Systems, http://www.ingenuity.com). Networks were generated using 12 differentially expressed miRNAs (Figure 3) that were uploaded into IPA. Analysis considered all genes from the dataset that met the 2-fold (p-value < 0.05) change cut-off and that were associated with biological functions in the Ingenuity Pathways Knowledge Base. For all IPA analyses, Fisher's exact test was used to determine the probability that each biological function assigned to the genes within the data set was due to chance alone.

3. RESULTS AND DISCUSSION

Identification of miRNAs differentially expressed in MCF-7 and LY2 cells.

To identify miRNAs that might be involved in TAM- resistance, we compared the miRNA transcription profiles between MCF-7 TAM-sensitive and LY2 TAM-resistant cells in response to 4-OHT using ethanol as the vehicle control. The cells were treated for 6 h, a time point selected as that at which maximal primary ERα-gene target transcription occurs [281]. Since serum levels of 4-OHT in breast cancer patients on oral TAM-citrate are 8-18 nM and breast tumors concentrate 4-OHT to 74 nM – 1.5 μM [282], the 100 nM 4-OHT concentration used in our experiments is at the lower range of that found in women on TAM therapy. In addition, MCF-7 cells were treated with 10 nM E_2, as per previous investigations of miRNA transcriptional responses [260,261,268,269,283]. Four separate experiments were performed for each treatment group and cell line.

A total of 97 miRNAs exhibited differential expression between TAM-sensitive MCF-7 and TAM-resistant LY2 cells with either EtOH or 4-OHT treatment (Figure 1, Tables 1 and 2). Forty-seven miRNAs were exclusively differentially expressed between the two cell lines in the presence of EtOH and 21 miRNAs were exclusively differentially expressed between the two cell lines in the presence of 4-OHT. Twenty-nine miRNAs were commonly differentially expressed between the two cell lines both with treatment by EtOH or 4-OHT. A Venn diagram is provided to schematically represent these results (Figure 2, left hand side). We represent the same data by separating up-regulated and down-regulated miRNAs on the right hand side of Figure 2. For example, 53 miRNAs demonstrated enhanced and 23 miRNAs demonstrated reduced expression in MCF-7 cells when compared to LY2 cells treated with the vehicle control EtOH (Table 1). Twenty-nine miRNAs demonstrated

increased and 21 miRNAs demonstrated decreased expression in MCF-7 cells when compared to LY2 cells treated with 4-OHT (Table 2). Differentially expressed miRNAs for EtOH-treated MCF-7 *versus* LY2 are shown in Table 1, 4-OHT-treated MCF-7 *versus* LY2 are shown in Table 2, and E_2 *versus* 4-OHT-treated MCF-7 are shown in Table 3. Of the total 225 miRNAs analyzed, 128 miRNAs were not differentially expressed between MCF-7 and LY2 in cells treated with EtOH or 4-OHT (data not shown). One miRNA, miR-423-5p demonstrated higher and miR-181a, demonstrated lower expression in MCF-7 cells treated with E_2 compared to MCF-7 cells treated with 4-OHT (Table 3).

From that list of 76 miRNAs showing opposite direction of expression in MCF-7 *versus* LY2 cells (Figure 2, Tables 1 and 2), 12 miRNAs were selected for further study (Figure 3A and B). The microarray expression data show that miR-10a, miR-22, miR-29a, miR-125b, miR-181a, and miR-222 were lower in EtOH-treated MCF-7 than in LY2 cells. In contrast, miR-21, miR-93, and miR-200a, b, and c were lower in EtOH-treated LY2 than MCF-7. Of these miRNAs, only miR-21 and miR-181a were E_2 regulated, *i.e.*, inhibited by E_2, in MCF-7 cells. Of these miRNAs exhibiting opposite expression in MCF-7 and LY2 cells, miR-10a, miR-21, miR-22, miR-125b, miR-181a, miR-200a and miR-222 were selected for Q-PCR validation. In addition, we included miR-221 for analysis because of its reported role in TAM/endocrine resistance [270], although its expression was not significantly different between MCF-7 and LY2 cells in the microarray. A literature review of the relationship between these miRNAs and breast cancer is summarized in Appendix 1.

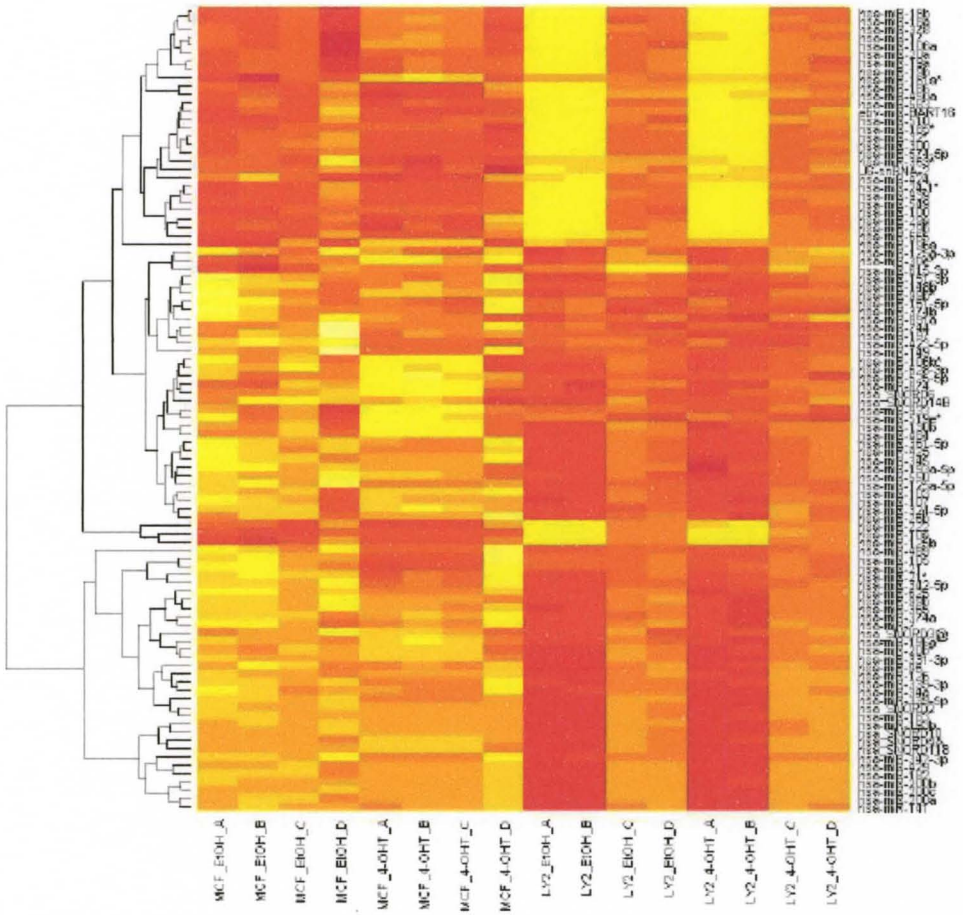


Figure 1: Heat map (hierarchical clusters) of significant differences in miRNA expression between MCF-7 and LY2 cells.

MCF-7 and LY2 cells were treated with EtOH, or 100 nM 4-OHT for 6 h and miRNA expression determined by microarray analyses of 4 separate experiments. The heatmap shows 97 miRNAs significantly differentially expressed (adjusted p-value < 0.10) for at least one of the two comparisons between cell lines (MCF-7/EtOH *versus* LY2/EtOH and MCF-7/4-OHT *vs.* LY2/4-OHT, see Tables 1 and 2, respectively). Each row of the heat map represents a gene, and each column represents a cell line/treatment group (as labeled at the bottom. Yellow indicates an increase in miRNA gene expression (relative to the other expression measurements in the same row) and orange/red indicates a decrease in expression.

Table 1: MicroRNAs Differentially Expressed in MCF-7/EtOH *vs.* LY2/EtOH in human breast cancer cells.

Gene expression from TAM-sensitive (MCF-7) and TAM-resistant (LY2) human breast cancer cells treated with EtOH were filtered and the average fold change for each gene was calculated for MCF-7 *vs.* LY2. Only those genes that demonstrated a statistically significant (adjusted $p < 0.10$) increase or decrease in expression for the MCF-7/EtOH *vs.* LY2/EtOH expression comparison are included in this table. Note that MCF-7/EtOH *vs.* LY2/EtOH means that expression for LY2 cells was utilized as reference group. Therefore, ratios above 1.0 indicate higher expression in MCF-7 than LY2 whereas ratios below 1.0 indicate lower expression in MCF-7 vs. LY2 cells

[1] empirical Bayes' t-statistic from the hierarchical linear model fitted by *limma*, see Materials and Methods for description

[2] p-Value corrected for the false-discovery rate (FDR)

MCF-7/EtOH > LY2/EtOH		Fold change^	t-Stat	p-Value	Adjusted p-value^
GeneIDs	miRNA gene family				
10928	hsa-miR-125a-5p	2.41	5.25	0.000	0.007
42576	hsa-miR-342-5p	7.15	4.36	0.000	0.026
14301	hsa-miR-361-5p	2.36	4.30	0.000	0.026
42914	hsa-miR-550	2.07	4.05	0.001	0.028
17608	hsa-miR-425	2.19	4.03	0.001	0.028
17878	hsa-miR-193a-5p	2.16	3.91	0.001	0.030
11078	hsa-miR-365	2.32	3.80	0.001	0.032
27565	hsa-miR-423-5p	1.54	3.78	0.001	0.032
17573	hsa-miR-625	3.03	3.68	0.002	0.037
42934	hsa-miR-345	1.83	3.62	0.002	0.038
42524	hsa-miR-21*	5.26	3.58	0.002	0.038
17463	hsa-miR-151-3p	1.76	3.48	0.003	0.040
11184	hsa-miR-99b	2.36	3.32	0.004	0.047
32884	hsa-miR-342-3p	11.25	3.31	0.004	0.047
42912	hsa-miR-339-3p	4.01	3.25	0.005	0.048
42794	hsa-miR-489	13.07	3.24	0.005	0.048
27378	hsa-miR-374a	2.33	3.24	0.005	0.048
42739	hsa-miR-339-5p	7.16	3.08	0.007	0.057
19007	hsa_SNORD38B	2.54	3.08	0.007	0.057
42538	hsa-miR-196a*	2.44	3.03	0.007	0.061
27217	hsa-miR-34a	3.54	2.90	0.010	0.069
10987	hsa-miR-193b	8.53	2.89	0.010	0.069
14302	hsa-miR-374b	1.62	2.88	0.010	0.069
42783	hsa-miR-197	1.57	2.87	0.010	0.069
19008	hsa_SNORD2	4.45	2.86	0.011	0.069
10977	hsa-miR-183	5.13	2.81	0.012	0.070
19011	hsa_SNORD10	11.15	2.80	0.012	0.070
11260	hsa-miR-151-5p	1.53	2.73	0.014	0.070
13147	hsa-miR-96	4.19	2.73	0.014	0.070
42887	hsa-miR-331-3p	2.78	2.73	0.014	0.070
19585	hsa-miR-148b	1.44	2.71	0.015	0.071
17427	hsa-miR-200c	13.02	2.69	0.015	0.071
19013	hsa_SNORD14B	1.49	2.67	0.016	0.071
4610	hsa-miR-126	3.19	2.66	0.016	0.071
10975	hsa-miR-182	8.02	2.63	0.017	0.074
10919	hsa-miR-103	1.62	2.62	0.018	0.074
19604	hsa_SNORD44A	7.87	2.62	0.018	0.074
42810	hsa-miR-149	2.26	2.60	0.018	0.074
10923	hsa-miR-107	1.90	2.60	0.018	0.074
3980	hsa-miR-7	2.58	2.59	0.019	0.074
42477	hsa-miR-324-5p	1.48	2.57	0.020	0.076
5740	hsa-miR-21	3.02	2.55	0.021	0.077
18900	hsa-miR-200b	10.83	2.52	0.022	0.077
11000	hsa-miR-200a	10.72	2.52	0.022	0.077
27559	hsa-miR-744	1.45	2.52	0.022	0.077
27961	hsa-miR-891a	1.37	2.47	0.024	0.084
17882	hsa-miR-20b*	2.62	2.45	0.025	0.085
17942	hsa-miR-125a-3p	1.42	2.38	0.029	0.091
10946	hsa-miR-141	11.11	2.38	0.029	0.091
5250	hsa-miR-105	2.60	2.37	0.029	0.091
13171	hsa-miR-429	5.25	2.34	0.031	0.096
42958	hsa-miR-628-3p	1.41	2.33	0.032	0.096
17898	hsa-miR-99b*	1.40	2.33	0.032	0.096
MCF-7/EtOH < LY2/EtOH					
13485	hsa-miR-10a	0.09	-5.59	0.000	0.007
30787	hsa-miR-125b	0.09	-4.00	0.001	0.028
42649	hsa-miR-20a	0.43	-3.53	0.002	0.040
42762	hsa-miR-665	0.30	-3.46	0.003	0.040
18847	hsa-miR-450a	0.43	-3.44	0.003	0.040
10998	hsa-miR-19b	0.39	-3.10	0.006	0.057
42529	hsa-miR-939	0.34	-3.10	0.006	0.057
42644	hsa-miR-18b	0.38	-2.93	0.009	0.069

MCF-7/EtOH > LY2/EtOH		Fold change[A]	t-Stat	p-Value	Adjusted p-value[B]
GeneIDs	miRNA gene family				
42650	hsa-miR-17	0.45	−2.88	0.010	0.069
11105	hsa-miR-378	0.46	−2.85	0.011	0.069
10997	hsa-miR-19a	0.40	−2.77	0.013	0.070
19581	hsa-miR-100	0.30	−2.77	0.013	0.070
42648	hsa-miR-106a	0.45	−2.77	0.013	0.070
42588	hsa-miR-18a	0.40	−2.75	0.013	0.070
17904	hsa-miR-185*	0.63	−2.73	0.014	0.070
27672	hsa-miR-615-3p	0.68	−2.70	0.015	0.071
42798	hsa-miR-549	0.36	−2.66	0.016	0.071
11040	hsa-miR-29b	0.35	−2.53	0.021	0.077
42575	hsa-miR-24-1*	0.40	−2.51	0.022	0.078
10990	hsa-miR-196a	0.26	−2.43	0.026	0.088
11013	hsa-miR-181a*	0.56	−2.39	0.028	0.091
17358	ebv-miR-BART16	0.65	−2.37	0.029	0.091
11142	hsa-miR-510	0.64	−2.31	0.033	0.098

Table 2: MicroRNAs Differentially Expressed in MCF-7/4-OHT *vs.* LY2/4-OHT in human breast cancer cells

Gene expression values from TAM-sensitive (MCF-7) and TAM-resistant (LY2) human breast cancer cells treated with 100 nM 4-OHT for 6 h were filtered and the average fold change for each gene was calculated for MCF-7 vs. LY2. Only those genes that demonstrated a statistically significant (adjusted $p < 0.10$) increase or decrease in expression for the MCF-7/4-OHT *vs.* LY2/4-OHT expression comparison, were included in this table. Note that MCF-7/4-OHT *vs.* LY2/4-OHT means that expression for LY2 cells was utilized as reference group. Therefore, ratios above 1.0 indicate higher expression in MCF-7 relative to LY2 and conversely, ratios below 1.0 indicate lower expression in MCF-7 compared to LY2 cells.

[1] empirical Bayes t-statistic from the hierarchical linear model fitted by *limma*, see Materials and Methods for description

[2] p-Value corrected for the false-discovery rate (FDR)

MCF-7/4-OHT > LY2/4-OHT		Fold change[A]	t-Stat	p-Value	Adjusted p-value[B]
GeneIDs	miRNA gene family				
10919	hsa-miR-103	2.07	3.97	0.001	0.053
27378	hsa-miR-374a	2.49	3.50	0.003	0.074
42538	hsa-miR-196a*	2.70	3.38	0.003	0.074
42539	hsa-miR-933	1.67	3.37	0.003	0.074
42698	hsa-miR-484	2.09	3.35	0.004	0.074
42808	hsa-miR-874	1.82	3.31	0.004	0.074
42887	hsa-miR-331-3p	3.33	3.21	0.005	0.077
42564	hsa-miR-26b	1.81	3.17	0.005	0.077
14301	hsa-miR-361-5p	1.86	3.11	0.006	0.077
10936	hsa-miR-130b	2.38	3.05	0.007	0.080
11175	hsa-miR-525-5p	1.45	3.01	0.008	0.083
19605	hsa_SNORD6	1.63	2.95	0.009	0.086
42934	hsa-miR-345	1.62	2.88	0.010	0.089
42477	hsa-miR-324-5p	1.55	2.86	0.011	0.089
19604	hsa_SNORD4A	8.83	2.76	0.013	0.089
11184	hsa-miR-99b	2.04	2.76	0.013	0.089
10923	hsa-miR-107	1.96	2.74	0.014	0.089
11078	hsa-miR-365	1.83	2.72	0.014	0.089
17608	hsa-miR-425	1.69	2.70	0.015	0.089
19585	hsa-miR-148b	1.44	2.70	0.015	0.089
17882	hsa-miR-20b*	2.86	2.67	0.016	0.089
13132	hsa-miR-519e*	1.57	2.64	0.017	0.089
4610	hsa-miR-126	3.15	2.63	0.017	0.089
42914	hsa-miR-550	1.60	2.61	0.018	0.092
11224	hsa-miR-30e*	1.62	2.56	0.020	0.097
19005	hsa_SNORD118	7.50	2.55	0.021	0.098
17854	hsa-miR-106b*	1.57	2.54	0.021	0.098
19011	hsa_SNORD10	8.85	2.53	0.021	0.098
10928	hsa-miR-125a-5p	1.52	2.52	0.022	0.098
MCF-7/4-OHT < LY2/4-OHT					
13485	hsa-miR-10a	0.10	-5.33	0.000	0.011
18847	hsa-miR-450a	0.33	-4.42	0.000	0.039
17358	ebv-miR-BART16	0.49	-3.99	0.001	0.053
11279	U6-snRNA-2	0.55	-3.69	0.002	0.074
42762	hsa-miR-665	0.29	-3.60	0.002	0.074
11023	hsa-miR-222	0.14	-3.42	0.003	0.074
27544	hsa-miR-363*	0.59	-3.24	0.005	0.077
30787	hsa-miR-125b	0.15	-3.12	0.006	0.077
42529	hsa-miR-939	0.34	-3.11	0.006	0.077
29575	hsa-miR-32*	0.49	-3.09	0.006	0.077
17904	hsa-miR-185*	0.61	-2.95	0.009	0.086
11040	hsa-miR-29b	0.31	-2.84	0.011	0.089
18739	hsa-miR-186	0.44	-2.81	0.012	0.089
17295	hsa-miR-583	0.58	-2.78	0.013	0.089
11039	hsa-miR-29a	0.34	-2.75	0.013	0.089
11142	hsa-miR-510	0.59	-2.73	0.014	0.089
42798	hsa-miR-549	0.35	-2.72	0.014	0.089
27740	hsa-miR-574-5p	0.46	-2.70	0.015	0.089
42513	hsa-miR-300	0.51	-2.68	0.016	0.089
42575	hsa-miR-24-1*	0.38	-2.64	0.017	0.089
42965	hsa-miR-424	0.44	-2.60	0.018	0.092

Table 3: MicroRNAs Differentially Expressed in MCF-7/E2 *vs.* MCF-7/4-OHT in human breast cancer cells.

Gene expression from TAM-sensitive (MCF-7) and TAM-resistant (LY2) human breast cancer cells were filtered and the average fold change for each gene was calculated for MCF-7 treated with E_2 vs. MCF-7 treated with 4-OHT. Only those genes that demonstrated a statistically significant (adjusted $p < 0.10$) increase or decrease in expression for the MCF-7/E_2 *vs.* MCF-7/4-OHT expression comparison were included in this table. Note that MCF-7/E_2 *vs.* MCF-7/4-OHT means that expression for MCF-7 cells treated with 4-OHT was utilized as reference group. Therefore, ratios above 1.0 indicate an increase E_2 relative to 4-OHT-treated MCF-7 cells and ratios below 1.0 indicate a decrease in expression in 4-OHT-treated cells relative to E_2-treated MCF-7 cells.

[1] empirical Bayes t-statistic from the hierarchical linear model fitted by *limma*, see Materials and Methods for description

[2] p-Value corrected for the false-discovery rate (FDR)

MCF-7/E_2 versus MCF-7/4-OHT		Fold change[A]	t-Stat	p-Value	Adjusted p-value[B]
GeneIDs	miRNA gene family				
27565	hsa-miR-423-5p	1.63	4.29	0.000	0.052
11013	hsa-miR-181a*	0.32	-4.73	0.000	0.040
42865	hsa-miR-181a	0.23	-3.84	0.001	0.094

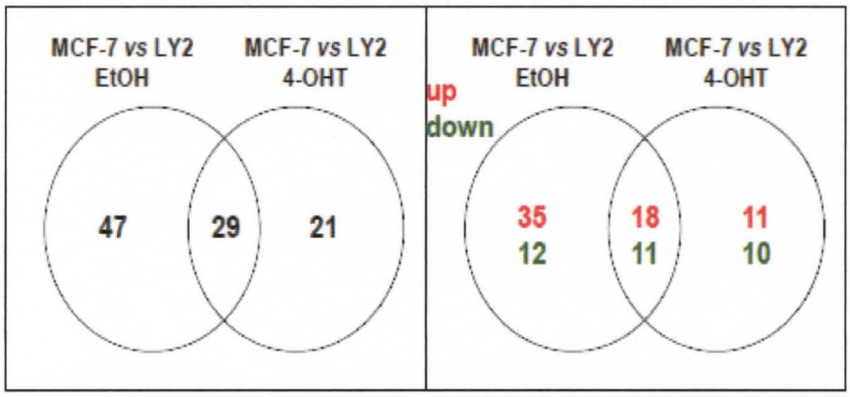
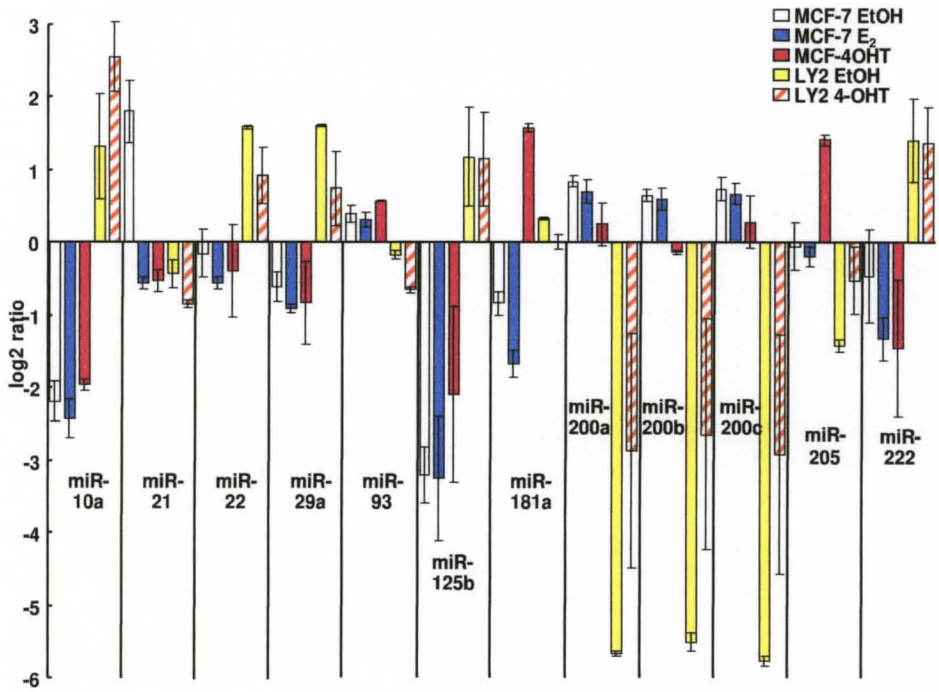


Figure 2: Venn diagrams summarizing differentially expressed (DE) miRNAs. A) Venn diagram of miRNAs differentially expressed between TAM-sensitive (MCF-7) and TAM-resistant (LY2) human breast cancer cells by the indicated treatments (EtOH and 4-OHT). Cells were treated as described in Figure 1. B) Venn diagram showing miRNAs either UP- (increased expression) or DOWN- (reduced expression) regulated between TAM-sensitive (MCF-7) and TAM-resistant (LY2) human breast cancer cells by treatment.

Figure 3: Select miRNAs that are differentially expressed in MCF-7 (TAM-S) and LY2 (TAM-R) breast cancer cells.
These 12 miRNAs were identified as differentially expressed in microarray analysis of miRNAs in EtOH, E_2 or 4-OHT treated cells. Values are log2(Hy3/Hy5) ratios in the sample *versus* the common reference pool. Each value is the avg. ± SEM of 4 separate experiments.



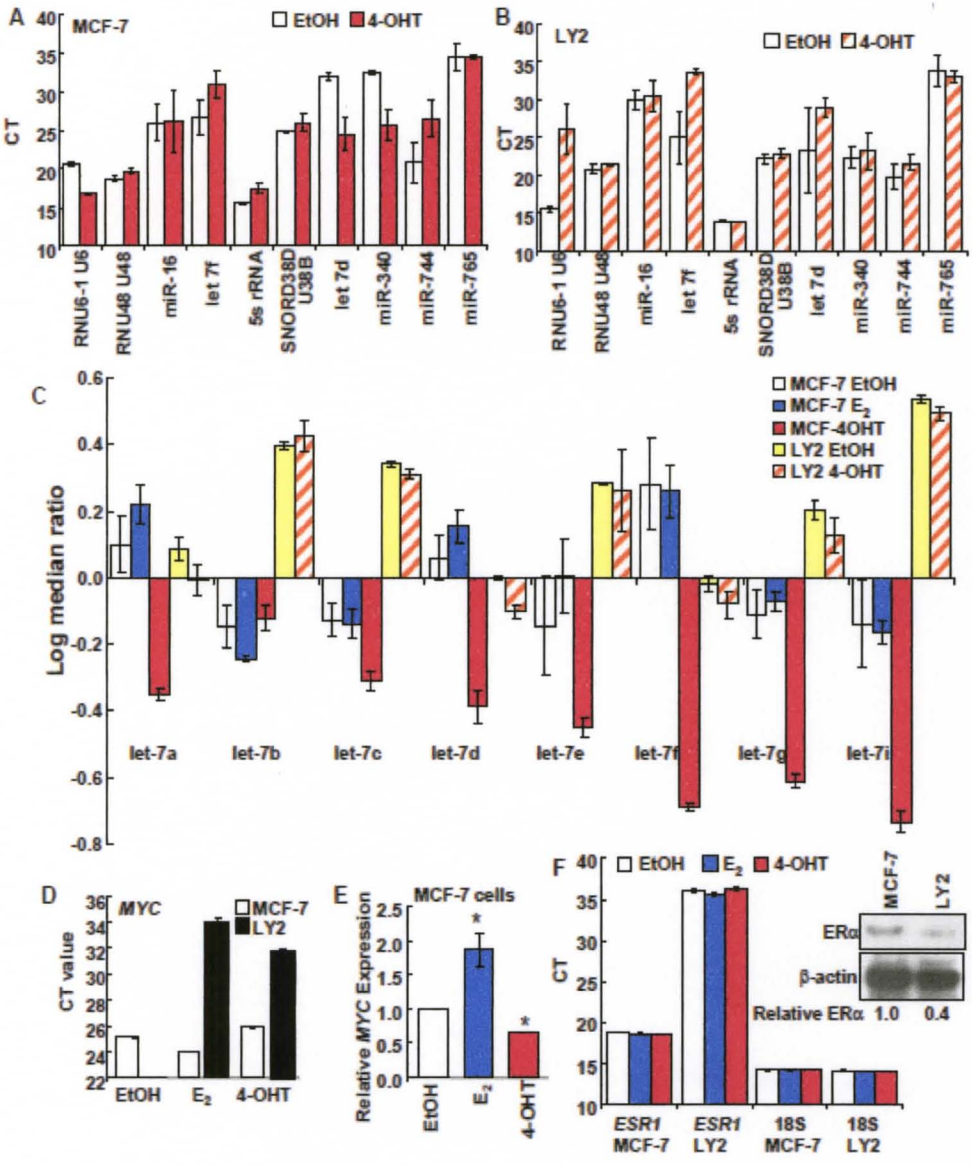
Selection of endogenous control genes for Q-PCR normalization and validation of select miRNAs by Q-PCR

Prior to performing Q-PCR to confirm the miRNA microarray data it was necessary to identify endogenous control genes (ECG) for normalization of miRNA transcript expression. First, we compared the expression of U6 (RNU6-1) and U48 (RNU48) RNA genes, traditionally used as controls for miRNA expression [284-286], in MCF-7 and LY2 cells after 6 h of 4-OHT or EtOH treatment (Figure 4A and 4B). U6 expression was increased by 4-OHT in MCF-7 and reduced by 4-OHT in LY2 cells. U48 expression was comparable between the two cell lines and unaffected by 4-OHT.

Eight additional candidate ECG were identified as showing low variation in expression in the miRNA microarray: high signal: miR-16, Let-7f, and 5SrRNA; medium signal: SNORD38D (U38B), Let-7d, and miR-340; low signal: miR-765, miR-744, miR-887, miR-92b. Eight of these ECG were screened for their expression in MCF-7 and LY2 cells after 6 h or vehicle (EtOH) or 100 nM 4-OHT treatment (Figure 4A and 4B). Two general conclusions can be made from these data: 1) ECG expression differs between the two cell lines; 2) 4-OHT affects ECG expression more in MCF-7 than LY2 cells. Expression of Let-7f was reduced by 4-OHT in both cell lines. miR-744 was reduced by 4-OHT whereas Let-7d and miR-340 were increased by 4-OHT in MCF-7 cells. Overall, the best ECG in MCF-7 and LY2 cells are U48, 5S rRNA, U38B, and miR-765 for high, medium, and low expression miRNAs, respectively. Because of the low expression of miR-765, we selected 5S rRNA, U48, and U38B to normalize miRNA expression in the rest of the studies in this chapter.

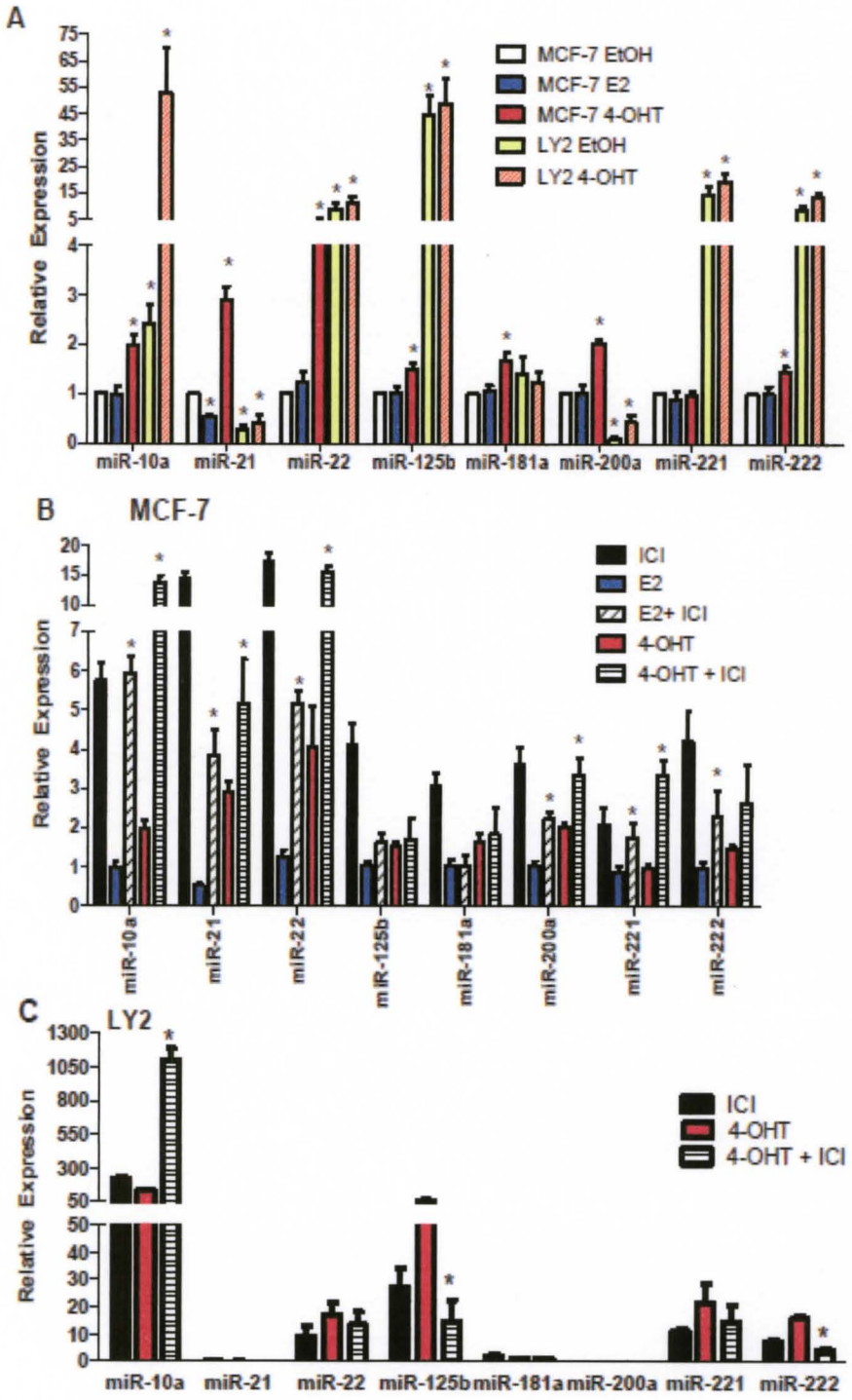
Figure 4: Selection of endogenous control genes for analysis of miRNA expression by Q-PCR

The expression of RNU6-1 (U6) and RNU48 (U48) RNA genes, traditionally used as controls for miRNA expression and eight candidate endogenous control genes (ECG) identified by miRNA microarray: high signal: miR-16, Let-7f, and 5SrRNA; medium signal: SNORD38D (U38B), Let-7d, and miR-340; low signal: miR-765 and miR-744 were examined in MCF-7 (A) and LY2 (B) cells treated for 6 h with EtOH (vehicle control) or 100 nM 4-OHT. (C) The expression of the Let-7 family members was determined by Exiqon Microarray analysis of miRNAs in EtOH, E_2 or 4-OHT treated cells, as indicated. Values are log2 (Hy3/Hy5) ratios in the sample versus the common reference pool. Each value is the avg. ± SEM of 4 separate experiments. D) The relative expression of MYC in MCF-7 and LY2 cells treated with EtOH, 10 nM E_2, or 100 nM 4-OHT for 6 h was determined by Q-PCR and CT values are the mean of 3 separate determinations ± SEM. E) Relative MYC expression in MCF-7 was normalized to 18S. * $p < 0.05$ versus EtOH control. F) The relative expression of ESR1 (ERα) in MCF-7 cells treated as in panel D. CT values are shown as avg. ± SEM of 3 replicates in one experiment. The inset shows a western blot of ERα protein. The blot was stripped and reprobed for β-actin. The ratio of ERα/β-actin in MCF-7 was set to 1 and the relative expression of ERα in MCF-7 was 0.4 = 60% lower ERα in LY2 compared to MCF-7 cells.


A
MCF-7
EtOH
4-OHT
CT
RNU6-1 U6
RNU48 U48
miR-16
let 7f
5s rRNA
SNORD38D U38B
let 7d
miR-340
miR-744
miR-765
B
LY2
C
MCF-7 EtOH
MCF-7 E2
MCF-4OHT
LY2 EtOH
LY2 4-OHT
Log median ratio
let-7a
let-7b
let-7c
let-7d
let-7e
let-7f
let-7g
let-7i
D
MYC
MCF-7
LY2
CT value
E
MCF-7 cells
Relative MYC Expression
F
ERα
β-actin
Relative ERα 1.0 0.4
ESR1 MCF-7
ESR1 LY2
18S MCF-7
18S LY2

Although Let-7a was reported to be an ECG for miRNA [285], Let-7a expression was increased by E_2 and reduced by 4-OHT in MCF-7 cells (Figure 4C). Let-7 family members are highly conserved in sequence and function across species [287]. Misregulation of Let-7 leads to a less differentiated cellular state and the development of cancer; hence, Let-7 family members are considered as tumor-suppressor miRNAs [288,289]. We observed that 4-OHT repressed the expression of all eight Let-7 family members in MCF-7 cells and none of the Let-7 family members in LY2, commensurate with a less differentiated cellular state. Let-7b, Let-7c, Let-7g, and Let-7i showed opposite expression between MCF-7 and LY2 cells. Since Let-7a [290] and Let-7g [291] downregulate Myc and high Myc expression results in a negative feedback loop inhibiting Let-7a expression [287], we examined Myc mRNA in MCF-7 and LY2 cells (Figure 4D). Based on the higher Let-7 expression in LY2, we expected lower Myc in LY2 and our data confirmed significantly lower Myc expression in LY2 compared with MCF-7 cells (Figure 4D). In agreement with an earlier report [292], E_2 increased and 4-OHT inhibited Myc transcription in MCF-7 cells (Figure 4E). Since transient overexpression of Let-7a, Let-7b, and Let-7i was reported to inhibit ERα expression in MCF-7 cells [293], we examined *ESR1* mRNA and protein levels in MCF-7 and LY2 cells. As expected, *ESR1* mRNA and ERα protein were lower in endocrine-resistant LY2 compared to endocrine-sensitive MCF-7 cells (Figure 4F). The reduced expression of ERα in LY2 also reflects higher expression of miR-221 and miR-222 that have been reported to suppress ERα expression [234,294,295].

Figure 5: Q-PCR analysis of the miRNA expression in MCF-7 and LY2 cells.
A) Cells were treated with EtOH, 10 nM E_2, or 100 nM 4-OHT for 6 h. Where indicated MCF-7 (B) and LY2 (C) cells were pretreated with 100 nM ICI 182,780 for 6 h. Values are the average of 3-8 separate experiments were normalized by U38 or 5S rRNA and are expressed as fold relative to EtOH-treated MCF-7 expression for each miRNA. In A: * Significantly different from EtOH in MCF-7. In B and C: * Significantly different from E_2 or 4-OHT in the absence of ICI.


A
Relative Expression
MCF-7 EtOH
MCF-7 E2
MCF-7 4-OHT
LY2 EtOH
LY2 4-OHT
miR-10a
miR-21
miR-22
miR-125b
miR-181a
miR-200a
miR-221
miR-222
B
MCF-7
Relative Expression
ICI
E2
E2+ ICI
4-OHT
4-OHT + ICI
miR-10a
miR-21
miR-22
miR-125b
miR-181a
miR-200a
miR-221
miR-222
C
LY2
Relative Expression
ICI
4-OHT
4-OHT + ICI
miR-10a
miR-21
miR-22
miR-125b
miR-181a
miR-200a
miR-221
miR-222

To validate the changes in miRNA expression detected in the miRNA microarrays, Q-PCR was performed on 7 of the 12 miRNAs in Figure 3: miR-10a, miR-21, miR-22, miR-125b. miR-181a, miR-200a and miR-222 in MCF-7 and LY2 cells treated with EtOH or 100 nM 4-OHT for 6 h, and MCF-7 cells treated with 10 nM E_2 for 6 h (Figure 5A). In addition, miR-221 was analyzed because it, along with miR-222, has been reported to be overexpressed and involved in endocrine-resistance in breast cancer cells [270,283,296].

For comparison between the two cells lines, miRNA expression was normalized to the value in EtOH treated MCF-7 cells. There was general agreement in the direction (up- or down- regulation) of miRNA expression in MCF-7 and LY2 cells between the Q-PCR and microarray data. The exception is that 4-OHT increased miR-200a in MCF-7 cells in Q-PCR. In agreement with other recent results examining E_2-regulation of miRNA expression in MCF-7 cells [260,261,268,269], the miRNA expression changes in response to E_2 or 4-OHT were less than five-fold. E_2 decreased miR-21 expression in MCF-7 cells, as observed in our earlier experiments [268] and as reported by others [261]. 4-OHT increased miR-21 expression in MCF-7 cells. LY2 cells had lower expression of miR-21 and miR-200a, in agreement with the data in the microarray (Figure 3). A recent analysis of miRNA expression in breast tumors by deep sequencing showed upregulation of miR-21 in ER+ breast tumors relative to normal breast tissue and triple negative breast tumors [297].

To determine if the effects of E_2 and 4-OHT on miRNA expression were ER-mediated, MCF-7 cells were pretreated with 100 nM ICI 182,780 (ICI, Fulvestrant) for 6 h, a time that reduces ERα protein and activity [298]. For each of the eight miRNAs, ICI increased expression relative to EtOH in MCF-7 cells (Figure 5B). These data suggest that unliganded ER may suppress transcription of these miRNAs

or that some component regulating miRNA expression or processing is inhibited by unliganded ER. Alternatively, since ICI is an agonist for GPER/GPR30 [13,299], it is possible that ICI activates intracellular signaling pathways, *e.g.*, MAPK, that increase miRNA expression. For example, MAPK increases miRNA expression by phosphorylating TRBP, a component of the Dicer complex that processes pre-miRNA into mature miRNA [300]. Testing GPER/GPR30 is beyond the scope of the current study. ICI ablated the inhibition of miR-21 by E_2 in MCF-7 cells. These data indicate that E_2-occupied ER suppresses the transcription of miR-21. The combination of ICI and 4-OHT did not further increase miRNA expression in MCF-7 cells with the exception of miR-10a, a result seen in both MCF-7 and LY2. While the mechanism involved for the increase in miR-10a appears to be, at least in part, ER-mediated, future studies are needed to address this mechanism in greater detail. There is only one report about miR-10a regulation [301]. That report found that miR-10a expression increased as mouse embryonic stem cells differentiated into smooth muscle cells [301]. Others reported that miR-10a associates with the 5' UTR of mRNAs encoding ribosomal proteins, enhances their translation, increases global protein synthesis, and thus contribute to oncogenesis [302]. For miR-21, miR-125b, and miR-181a, 4-OHT inhibited the stimulation over basal expression detected with ICI treatment in MCF-7 cells.

For LY2 cells, ICI had no significant effect on basal miRNA expression (Figure 5C). These data indicate that, unlike MCF-7 cells, the regulation of miRNA expression in LY2 cells is independent of ER. These data are in agreement with the estrogen-independent, endocrine-resistant phenotype of LY2 cells [273,303]. As discussed above, ICI and 4-OHT synergistically increased miR-10a transcription. ICI reduced 4-OHT-stimulated miR-125b and miR-222 expression (Figure 5C), a result

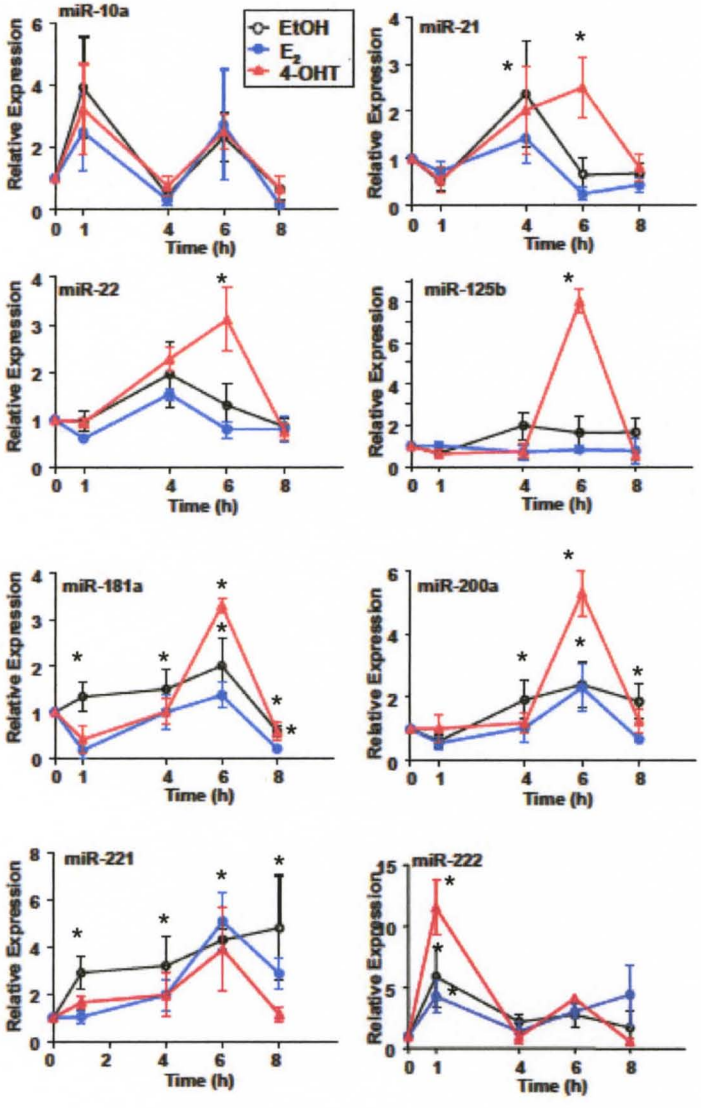
implicating ER involvement in 4-OHT-regulating the expression of these miRNAs, a result commensurate with higher miR-125b in ERα/PR-positive than ERα/PR-negative breast tumors [304]. The apparent synergy of ICI and 4-OHT in upregulating miR-10a transcription may be mediated by GPR30/GPER, for which both ICI and 4-OHT are agonists [12]. However, others have reported that an ERα variant called ERα36, and not GPR30, mediates non-genomic ER signaling, including ICI agonist activity [305]. ERα36 arises from a promoter in the first exon of ERα, but lacks both the N- and C terminal transcription activation domains, AF-1 and AF-2, respectively, of full-length wild type ERα66 [306,307]. Further studies would be required to examine ERα36 expression in LY2 cells. However, ERα36 was not detected using an antibody that recognizes epitopes conserved in ERα66 and ERα36 [307,308] (Appendix 4).

Time course of miRNA expression in MCF-7 cells

Time-dependent changes in the expression of 8 miRNAs were detected after 1, 4, 6, and 8 h treatment with EtOH, E_2, or 4-OHT (Figure 6). E_2 repressed the expression of miR-22, miR-125b, miR-181a, miR-200a (except at the 6 h time point), and miR-221 (except at the 6 h time point) relative to EtOH. 4-OHT increased expression of miR-21a, miR-22, miR-181a, and miR-200a relative to EtOH at the 6 h time point. 4-OHT inhibited miR-221 expression, although the difference was not statistically significant at the 6 h time point. To our knowledge, there are only two reports examining the effect of E_2 on miRNA at various times (0, 1, 3, 4, 6, and 12 h) of treatment in MCF-7 cells [260,269]. The time-course of miR-21 expression does not agree with a previous report showing E_2 increased miR-21 over time [260]. This difference is likely the result of differences in the MCF-7 cells used since Bhat-

Nakshatri *et al.* used MCF-7 cells stably transformed with a bicistronic vector control [260] whereas we used MCF-7 cells at passages less than 9 from ATCC.

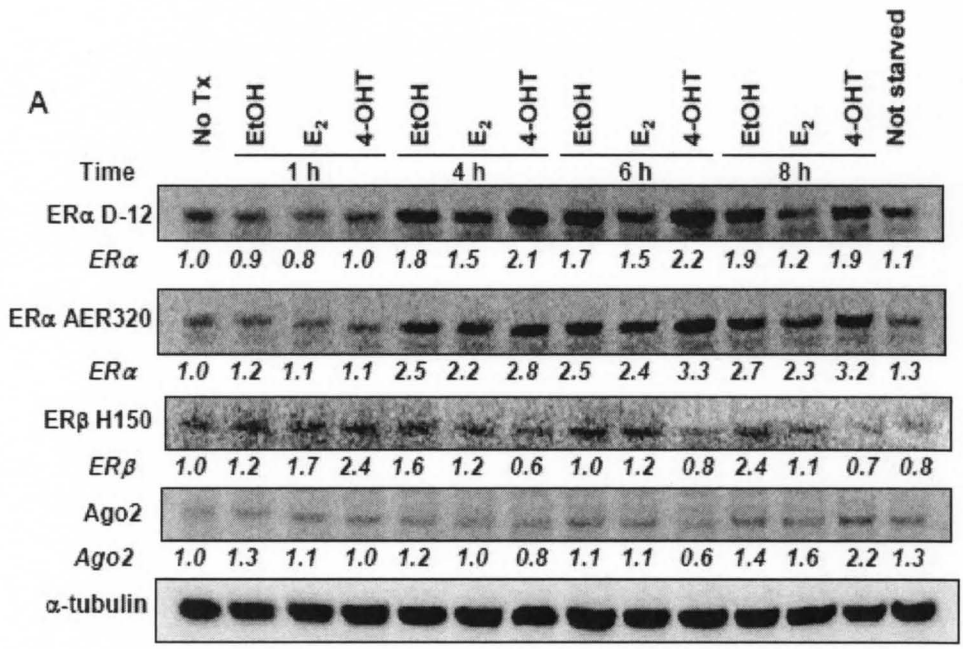
Figure 6: Time course analysis of miRNA expression. MCF-7 cells serum starved for 72 h and were treated for 1, 4, 6 and 8 h with EtOH, 10 nM E_2, or 100 nM 4-OHT. Values are the avg. ± SEM of 3-6 separate experiments in which each point was run in triplicate. Values were normalized by 5S rRNA and are expressed as fold relative to basal (time 0).

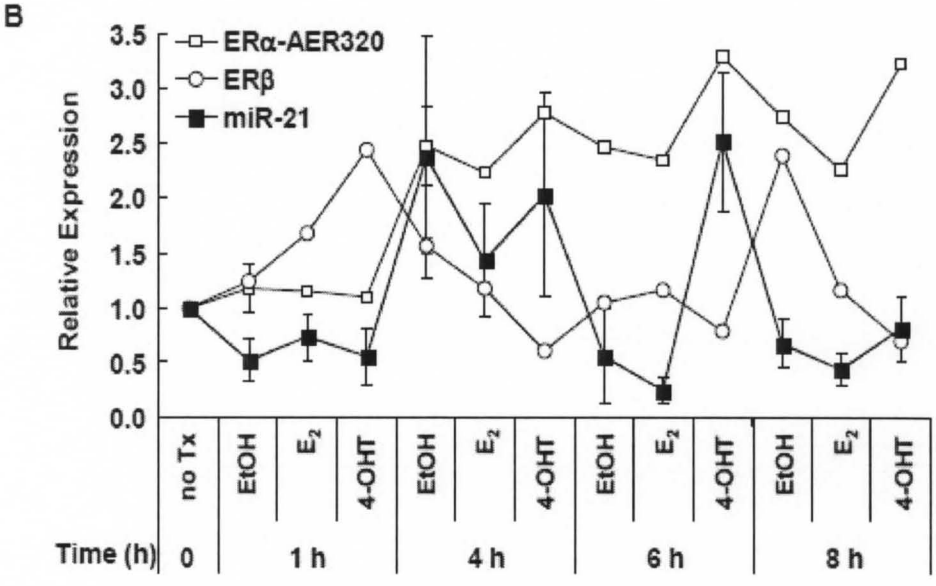


Time course of E_2 and 4-OHT regulation of ERα, ERβ, and Argonaute-2 (Ago2)

To determine if changes in miRNA expression with time reflect changes in ERα, ERβ, or Ago 2 expression, MCF-7 cells were treated with EtOH, E_2, or 4-OHT for 1, 4, 6, or 8 h prior to western blot for ERα, ERβ, and Ago2 protein expression (Figure 7A). These data show that ERα was increased after 4 h of treatment with EtOH and remained increased through the 8 h time course in MCF-7 cells. Consistent with previous investigations [309], E_2 reduced ERα and 4-OHT stablized ERα in MCF-7 cells. ERβ was increased with EtOH, E_2, and 4-OHT treatment for 1 h, but at 4 h, E_2 and 4-OHT reudced ERβ. At 6 h, only 4-OHT reduced ERβ. At 8 h, ERβ was increased and this increase was inhibited by E_2 and 4-OHT. Ago2 was unaffected by EtOH until 8 h when there was an increase in Ago2. Ago2 was decreased by E_2 and 4-OHT with time and the increase in Ago2 with EtOH at 8 h was further increased by 4-OHT. This is the first examination of the effect of E_2 or 4-OHT on Ago2 expression. The changes in miR-21 expression with time and treatment correspond to the expression of ERα protein and at some time points/treatments appear to inversely correspond to ERβ protein expression (Figure 7B). These data suggest that E_2-ERα regulates miR-21 expression.

Figure 7: Time-dependent changes in ERα, ERβ, and Ago2 expression in E2- or 4-OHT- treated MCF-7 cells. MCF-7 cells were serum-starved for 48 h and either untreated (No Tx) or treated for 1, 4, or 6 h with EtOH, 10 nM E_2, or 100 nM 4-OHT. A) WCE (30 μg protein) were separated on SDS-PAGE gels and western blotted with two different ERα antibodies (D-12 and AER320), ERβ antibody H150, or an antibody for Ago2. The blot was stripped and re-probed for α-tubulin as a loading control. The values below each blot are the ratio of the indicated protein/α-tubulin normalized to the No Tx control. The last lane shows MCF-7 cells that were not serum-starved or treated. B) The relative expression of miR-21 in MCF-7 cells treated as indicated are plotted with ERα (AER320) and ERβ protein expression. The miR-21 data are the same as in Figure 6.


A
No Tx
EtOH
E2
4-OHT
Not starved
Time
1 h
4 h
6 h
8 h
ERα D-12
ERα
1.0 0.9 0.8 1.0 1.8 1.5 2.1 1.7 1.5 2.2 1.9 1.2 1.9 1.1
ERα AER320
ERα
1.0 1.2 1.1 1.1 2.5 2.2 2.8 2.5 2.4 3.3 2.7 2.3 3.2 1.3
ERβ H150
ERβ
1.0 1.2 1.7 2.4 1.6 1.2 0.6 1.0 1.2 0.8 2.4 1.1 0.7 0.8
Ago2
Ago2
1.0 1.3 1.1 1.0 1.2 1.0 0.8 1.1 1.1 0.6 1.4 1.6 2.2 1.3
α-tubulin

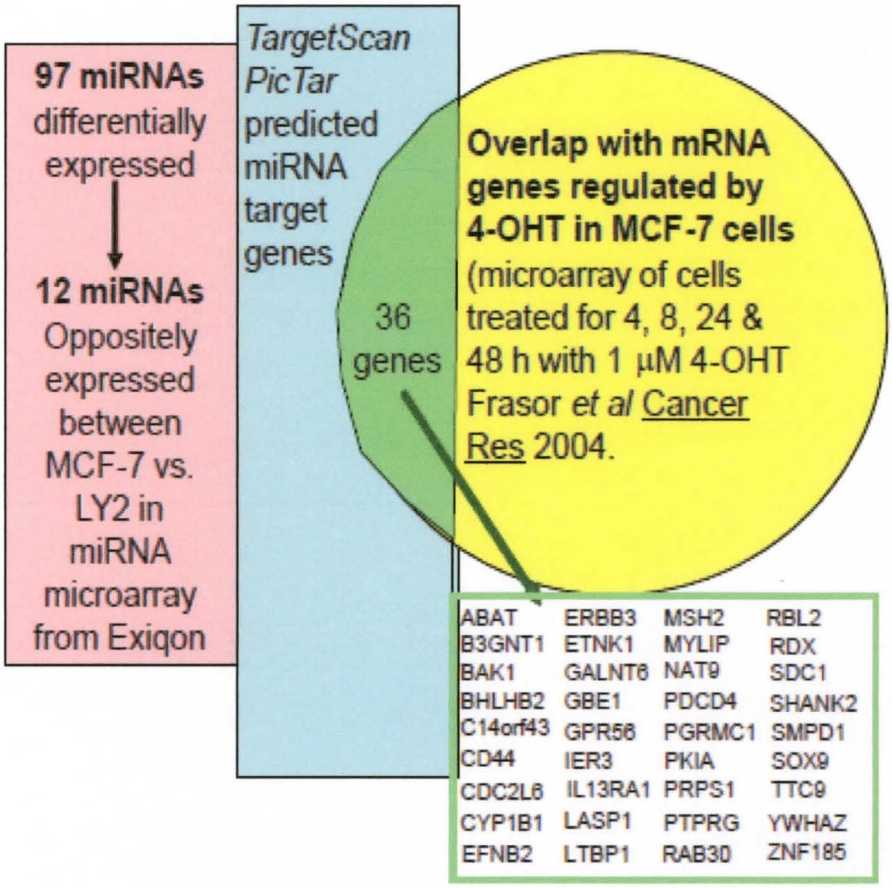

B
Relative Expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ERα-AER320
ERβ
miR-21
no Tx
EtOH
E2
4-OHT
Time (h)
0
1 h
4 h
6 h
8 h

Computational identification of miRNA target mRNA genes in 4-OHT-treated MCF-7 cells

mRNA targets of the 12 miRNAs that were differentially expressed between MCF-7 and LY2 cells (Figure 2) were identified *in silico* using target identification software:, Target Scan 5.1 (http://www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de/), miRanda (http://www.microrna.org/microrna/getGeneForm.do), and miR Base Release 15 (http://www.mirbase.org/). This data was integrated with mRNA targets regulated by 4-OHT after 4, 8, 24 and 48 h treatment of MCF-7 cells [276]. The gene symbols were transcribed from [276] and were searched against the miRanda predicted Human Target Site Predictions data contained in the file named human_predictions_aug2008.txt, downloaded from http://www.microrna.org/microrna/home.do. It was necessary to update several of the HUGO gene symbols presented in [276] in order to be consistent with the current HUGO identifiers for these transcripts in the human predictions dataset. Examples include updating C1orf24 to FAM129A, and RENT1 to UPF1. All genes listed in [276] were found the predicted target of at least one miRNA in the human_predictions_aug2008.txt dataset with the exception of SNCG, SLC16A5, RAP140, LOC441453, ELF3, LSS, CLIC3, EHD4,SERPINA1, EGFL5, SRD5A1, and KRT13. This analysis identified 36 genes that were regulated by 4-OHT in MCF-7 cells and which are putative targets of the 12 miRNAs identified in miRNA microarray analyses (Figure 8, Appendix 2). Appendix 2 also lists the putative miRNA target mRNA genes, their mRNA expression in 4-OHT treated cells at 8 and 48 h from [310], and whether these data agree with the data on miRNA expression in response to 4-OHT (Figure 5). In general, the predicated gene targets agree with the direction of miRNA expression in 4-OHT treated MCF-7 cells.

In order to identify gene networks involving 12 miRNAs that were differentially expressed in LY2 and MCF-7 cells (Figure 3), Ingenuity Pathway Analysis (IPA) was performed. Networks created by IPA are groups of proteins that interact directly or indirectly with genes or proteins in a dataset. As expected, IPA identified cancer as the top category followed by reproductive system disease and cellular development as significantly associated with the miRNAs in the data set (Appendix 6). Core analysis using IPA generated 2 networks containing the 12 differentially expressed miRNAs and key proteins involved in breast cancer (Appendix 7). Network 1 and 2 shows 35 and 9 molecules respectively (Appendix 7). The identity and cellular location of these molecules are provided in Appendix 3. Functional analysis with IPA tools identified 14 molecules in network 1 and 5 in network 2 as having roles in breast cancer (indicated by red lines in Appendix 7). Network 1 identified Myc as a central node, although none of the miRNAs directly connect to Myc. miR-10a mapped to a number of gene targets in Network 1 while miR-200b mapped to only one target, *VIM*. However, recent studies show that downregulation of the miR-200bc/429 cluster is associated with breast tumor progression through upregulation of phospholipase C gamma 1 (PLCG1) which, in turn, regulates cell mobility, proliferation, and viability [311]. *APC*, *MYC*, *CYRB*, *CASP3*, *CSF1*, *UNCX*, *NPTX1*, miR-10a, miR-22, miR-29a, miR-93, miR-200a, miR-205 and miR-222 are the genes associated with breast cancer in this network. Network 2 centers on estrogen receptor in breast cancer and supports our observation that the expression of miR-21 in MCF-7 cells is regulated by ER (Figure 4B).

Figure 8: Computational identification of mRNA gene targets of 12 miRNAs oppositely expressed in MCF-7 and LY2 cells. Target prediction software was used to identify mRNA targets of the miRNAs. Predicted genes were overlapped with microarray data of 4-OHT regulated genes by [310]. This identified 36 gene targets as indicated.



PDCD4, BCL2, CYP1B1, and ERBB3 are differentially expressed in MCF-7 and LY2 cells

Among the 36 putative gene targets in Appendix 2, we focused first on the *PDCD4* tumor suppressor because we had previously identified *PDCD4* tumor suppressor as a *bone fide* mRNA target downregulated by miR-21 in MCF-7 cells [268]. Since miR-21 expression was significantly lower in LY2 than MCF-7 cells, we anticipated that *PDCD4* mRNA and protein expression would be higher in LY2 than in MCF-7 cells. However, *PDCD4* mRNA was undetectable in EtOH-treated LY2 cells. With 4-OHT treatment, *PDCD4* mRNA was detected at low levels in LY2 cells (Figure 9A). Neither E_2 nor 4-OHT affected *GADPH* expression and *GADPH* CT values were similar in MCF-7 and LY2 cells, indicating that the quality of the RNA was not an issue in the lack of *PDCD4* expression in control (EtOH)-treated LY2 cells (Appendix 5). 4-OHT reduced *PDCD4* mRNA in MCF-7 cells (Figure 8A and 8B), consistent with the increase in miR-21 induced by 4-OHT. As reported previously, E_2 increased *PDCD4* mRNA (Figure 9A and 9B). We did not detect Pdcd4 protein expression in LY2, although Pdcd4 was expressed in MCF-7 (Figure 8C).

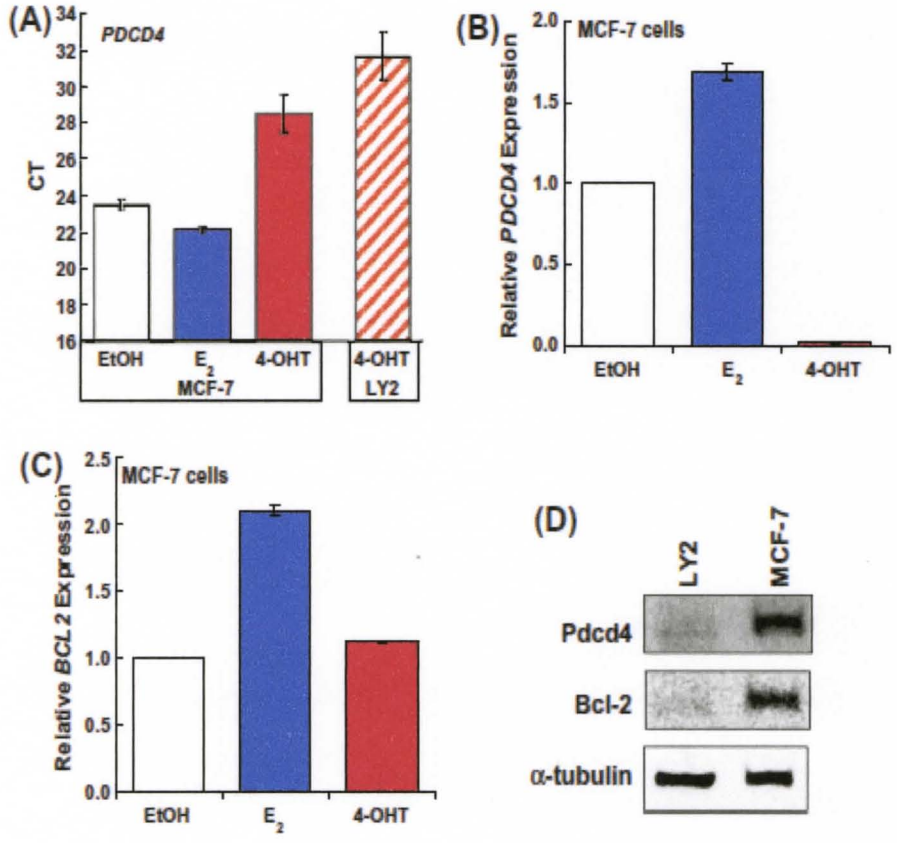
The anti-apoptotic, pro-survival *BCL2* is also a target of miR-21 [268]. Again, since miR-21 expression was lower in LY2, we expected higher *BCL2* expression in LY2 than MCF-7 cells. However, *BCL2* mRNA could not be detected in LY2 cells, whether EtOH or 4-OHT treated (data not shown). As expected based on our previous data and the work of others [268,312], E_2 increased *BCL2* mRNA in MCF-7 cells. 4-OHT had no significant effect on *BCL2* mRNA expression in MCF-7 cells. We did not detect Bcl-2 protein expression in LY2, although Bcl-2 was expressed in MCF-7 cells (Figure 9C). Others reported that 1 μM 4-OHT suppressed Bcl-2 expression in MCF-7 cells after 7 d of treatment [313].

CYP1B1 is a cytochrome P450 enzyme implicated in the metabolism of exogenous and endogenous substrates, including E_2, and *CYP1B1* polymorphisms are associated with breast cancer risk [314]. *CYP1B1* was stimulated by 8 h treatment with 1 μM 4-OHT in MCF-7 cells [310] and is a putative target of regulation by miR-200 family members that are reduced in LY2 compared to MCF-7 cells. Because 4-OHT increased miR-200a expression, we examined *CYP1B1* expression after 6 h treatment with 4-OHT or EtOH in MCF-7 and LY2 cells. *CYP1B1* mRNA expression was very low (CT ~ 39) in LY2 cells and 4-OHT did not affect *CYP1B1* expression (Figure 10A). These results are in contrast to a previous report showing 2-6-fold higher *CYP1B1* in TAM- and fulvestrant- resistant cell lines derived from of MCF-7 cells [315]. The reason for this difference in *CYP1B1* expression may be cell line- or cell culture- condition mediated. As another possible difference, we noticed that the endocrine resistant cell lines used in the previous report were supplemented with insulin [315], whereas we do not supplement our cell culture media with insulin. Although studies in diabetic rats indicate that insulin represses hepatic *CYP1B1* [316], the regulation of *CYP1B1* by insulin in breast cancer cells has not, to our knowledge, been examined. *CYP1B1* mRNA expression was higher in MCF-7 than LY2 cells, but was not significantly regulated by E_2 or 4-OHT with 6 h treatment in MCF-7 cells. These data reflect previous findings regarding detection of *CYP1B1* expression in MCF-7 cells [317]. We did not detect an increase in *CYP1B1* with 6 h of treatment, as reported for 12 h of E_2 treatment in MCF- 7 cells [318]. The reduction of miR-200b and miR-200c detected with 4-OHT treatment in MCF-7 cells in microarray (Figure 3) would be expected to increase targets of these miRNAs, including *CYP1B1*. Indeed, *CYP1B1* mRNA was increased in the microarray study with 8 h of

1 μM 4-OHT treatment (Appendix 2) [310]; but after 6 h of treatment, no reduction in *CYP1B1* was detected.

ERBB3 is an oncogene that is a member of the epidermal growth factor receptor (EGFR) family of transmembrane tyrosine kinases that is bound by heregulin and is involved in the pathogenesis and progression of breast cancer [319]. *ERBB3* is frequently over-expressed in breast cancer and increased in TAM resistance [319,320]. *ERBB3* is a putative target of miR-22, miR-125b, miR-221, miR-222, miR-93 according to our bioinformatic analyses and is a *bone fide* target of miR-125b [201]. Increased expression of these miRNAs in LY2 cells would be expected to reduce the expression of *ERBB3* in LY2 cells. In agreement with this idea, we did not detect *ERBB3* mRNA in LY2 cells, even when treated with 4-OHT (Figure 10B and data not shown). We did not detect regulation of *ERBB3* mRNA by E_2 or 4-OHT with 6 h treatment in MCF-7 cells. Others reported that *ERBB3* mRNA was inhibited by 48 h treatment of MCF-7 cells with 1 nM E_2 and this inhibition blocked by 1 μM TAM in MCF-7 cells [321]. The difference in time of treatment is likely responsible for differences in *ERBB3* regulation.

Figure 9: miR-21 target genes expression in MCF-7 and LY2 cells. MCF-7 and LY2 cells were serum-starved for 48 h and then treated with EtOH, 10 nM E_2, or 100 nM 4-OHT for 6 h prior to RNA isolation (A) or 24 h prior to WCE preparation (B) as described in Materials and Methods. (A) Q-PCR was performed for the indicated genes and fold-expression determined compared to EtOH as described in Materials and Methods. Values are the average of 3 separate determinations ± SEM. (B) Western blot for the indicated proteins. The membrane was stripped and reprobed for α-tubulin for normalization as described in Materials and Methods. The blot shown is representative of three separate biological replicates.



ESR1 and ERα protein expression is lower in LY2 than MCF-7 cells

MiR-221 and miR-222 expression was higher in LY2 compared to MCF-7 cells. miR-221 and miR-222 are overexpressed in ERα-negative and TAM-resistant breast cancer cell lines [270,295]. Knockdown of miR-221 and miR-222 in MDA-MB-468 breast cancer cells partially restored ERα expression and TAM-sensitivity [295]. *ESR1* mRNA expression is lower in LY2 than MCF-7 cells (Figure 10C). Western confirmed lower ERα protein expression in LY2 cells when compared to MCF-7 cells (Figure 4F). These data agree with reports that miR-221/222 is overexpressed in TAM-resistant breast cancer cell lines and suppresses ERα expression.

miR-200-regulated ZEB1 is reduced in LY2 cells

miR-200 family members suppress expression of the transcription factor ZEB1 that initiates epithelial to mesenchymal transition (EMT) by repressing transcription of E-cadherin and other genes regulating cell polarity [203,205,322-326]. Because all three miR-200 family members were expressed at significantly lower levels in LY2 than MCF-7 cells, we examined ZEB1 as a miR-200 target in MCF-7, LCC1, LCC2, LCC9, LY2, and MDA-MB-231 breast cancer cells by western blot (Figure 11A). LCC1 are estrogen-independent derivatives of MCF-7 cells and LCC2 and LCC9 are also endocrine-resistant derivatives of MCF-7 cells [278]. MDA-MB-231 serve as a positive control since ZEB1 expression is higher in MDA-MB-231, but not in MCF-7 [327]. As expected, ZEB1 was not expressed in MCF-7, but was expressed in MDA-MB-231 (Figure 11A). LY2 cells express ZEB1, indicating that this cell line has undergone EMT. However, LCC1, LCC2, and LCC9 cells do not express ZEB1, indicating that these estrogen-independent (LCC1) and tamoxifen/endocrine-resistant (LCC2 and LCC9) cell lines have not undergone EMT.

Because E-cadherin is inversely correlated with ZEB1 expression and inversely correlated with miR-200c [324], we examined E-cadherin in MCF-7, LY2, and MDA-MB-231 cells (Figure 11B). E-cadherin was not expressed in LY2 or MDA-MB-231 cells, indicating that LY2 cells have undergone EMT. This is, to our knowledge, the first demonstration of EMT in the LY2 endocrine-resistant breast cancer cell line.

Figure 10: CYP1B1, ERBB3, and ESR1 gene expression in MCF-7 and LY2 cells. MCF-7 and LY2 cells were serum-starved for 48 h and then treated with EtOH, 10 nM E_2, or 100 nM 4-OHT for 6 h prior to RNA isolation (A) or 24 h prior to WCE preparation (B) as described in Materials and Methods. Q-PCR was performed for the indicated genes and fold-expression determined compared to EtOH as described in Materials and Methods. Values are the average of 3 separate determinations ± SEM.

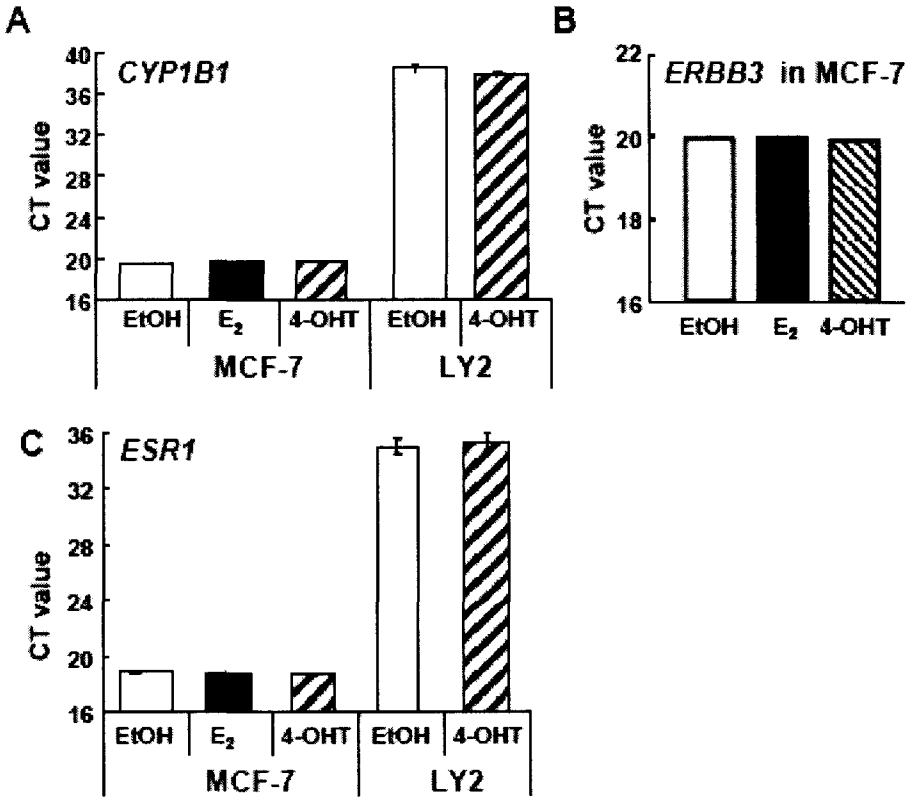
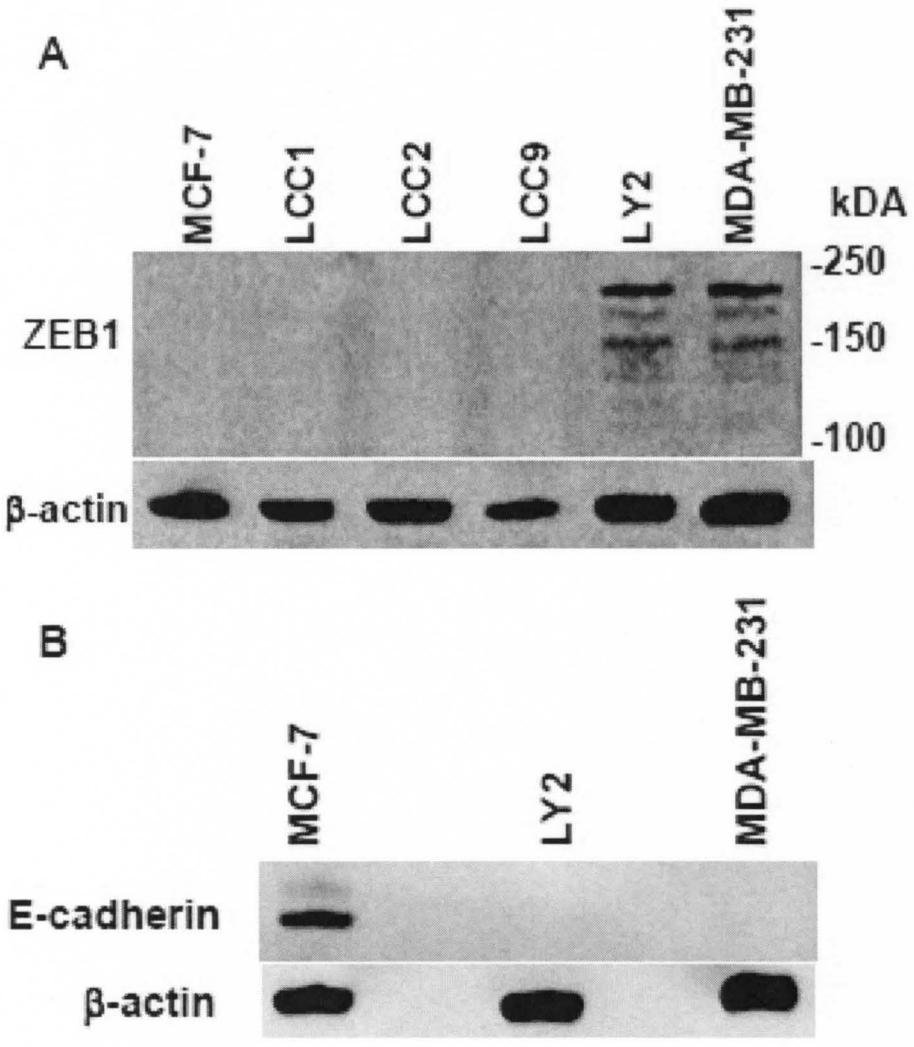


Figure 11: ZEB1 and E-cadherin expression. Whole cell lysates or nuclear extracts were prepared from the indicated breast cancer cell lines. Identical amounts (30 μg) of protein were immunoblotted for ZEB1 and E-cadherin as described in Materials and Methods. The membranes were stripped and reprobed for β-actin. These blots are representative of three separate experiments.


A
MCF-7
LCC1
LCC2
LCC9
LY2
MDA-MB-231
kDA
-250
-150
-100
ZEB1
β-actin
B
MCF-7
LY2
MDA-MB-231
E-cadherin
β-actin

CHAPTER IV: REDUCED EXPRESSION OF MIRNA-200 FAMILY IN LY2 CELLS CONFERS RESISTANCE TO TAMOXIFEN AND FULVESTRANT

1. INTRODUCTION

As previously discussed in earlier chapters, miRNAs regulate gene expression at the post-transcriptional level [328]. miRNAs are aberrantly expressed in different types of cancer including breast cancer [197].

EMT (epithelial-to-mesenchymal transition) is a hallmark of metastatic cancer [329]. EMT is induced by several signaling pathways such as TGF-β, Wnt and Notch [330,331]. It is characterized by loss of the epithelial marker E-cadherin due to gene methylation or repression by upregulation of transcription factors Zinc finger E-box binding homeobox domain proteins such as ZEB1 (also known as TCF1 or δEF1) or ZEB2 (also known as SIP1), Snail1/2 and TWIST that repress E-cadherin in epithelial cells [332-335]. As a result, cells acquire a mesenchymal phenotype characterized by the expression of markers such as vimentin and N-cadherin [336]. Reduced expression of miRNA-200 and miR-205; and increased expression miR-221/222 are implicated in EMT and metastasis [337]. Aberrant expression of these miRNAs has been reported to increase metastatic breast cancer [338,339]. The link between the development of endocrine resistance and EMT in breast cancer is still not clearly understood. Studies have shown that endocrine resistance confers metastatic properties to cells. For example, some endocrine-resistant cells and tumors show activation of the β-catenin pathway and induction of Snail and TWIST that contribute

to EMT by repressing E-cadherin transcription [340-342]. A few studies have shown that miRNAs have a role in conferring endocrine-resistance which subsequently led to induction of EMT and metastasis. For example, re-expression of miR-375 restored TAM sensitivity and reverted EMT in breast cancer cells [343]. Prolonged growth of MCF-7 breast cancer cells as mammospheres induced EMT and resistance to TAM [344]. Notably, these MCF-7 mammospheres exhibited higher expression of miR-221/222 and reduced expression of miR-200c, miR-203 and miR-205 compared to MCF-7 cells [344].

The miR-200 family of miRNAs are derived from two chromosomal locations: miR-200b, miR-200a, and miR-429 are located on chromosome 1p36; miR-200c and miR-141 are located on 12p13 [203]. The miR-200bc/429 cluster differs from the miR-200a/141 cluster by the fourth nucleotide (U to C) in the seed region and this means that they regulate different genes in breast cancer [206]. Reduced expression of miR-200 family of miRNAs has been observed in breast, ovarian, endometrial, lung and gastric cancers compared to normal tissue [322]. Many studies have identified an inverse relationship between the expression of miR-200 family and its targets ZEB1 in cells [205,345-347]. We recently reported increased expression of ZEB1 protein and loss of its target E-cadherin in an endocrine-resistant cell line LY2 compared to parental MCF-7 human breast cancer cells[247]. We observed that the LY2 cell line had undetectable levels of miR-200 family members compared to the parental MCF-7 cell line, suggesting a role for miR-200 in tamoxifen/endocrine-resistance and loss of ZEB1 repression.

Here I examined the expression of miR-200a, miR-200b, and miR-200c and their regulation by E_2 and 4-OHT, an active TAM metabolite in a panel of ERα positive breast cancer cell lines representing progression towards

endocrine/tamoxifen-resistance. Further, I tested how overexpression of miR-200b and miR-200c affected LY2 cell morphology, expression of *ZEB1* and vimentin, and cell proliferation. I also examined how knockdown of miR-200b and miR-200c affected the sensitivity of MCF-7 cells to TAM and fulvestrant. Lastly, I examined if epigenetic modification of miR-200b and miR-200c could be responsible for the lower expression of these miRNAs in LY2 cells compared to the parental MCF-7 cells.

2. MATERIALS AND METHODS

Cell culture

MCF-7 human breast cancer cells were purchased from ATCC (Manassas, VA, USA) and maintained in IMEM supplemented with 10% fetal bovine and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) serum [203]. LCC1, LCC2, LCC9, and LY2 are derivatives of MCF-7 cells that are E_2, tamoxifen, and multiple SERM-independent, respectively, and were graciously provided by Dr. Robert Clarke, Georgetown University. Prior to treatment, the medium was replaced with phenol red-free IMEM supplemented with 5% dextran charcoal-stripped FBS and 1% penicillin/streptomycin (stripped medium) or 48 h (referred to as 'serum-starving').

Chemicals

E_2 and 4-OHT were purchased from Sigma-Aldrich (St. Louis, MO). ICI 182,780 was from Tocris (Ellisville, MO). Cells were treated with ethanol (EtOH, the vehicle control, 0.01% final volume) 10 nM E_2 or 100 nM 4-OHT for 6 h as indicated. Where indicated LY2 cells were treated with 2.5 μM 5-aza-2'-deoxycytidine (5-aza-dC, Sigma-Aldrich, St. Louis, MO) alone or in combination with 100 ng/μl Trichostatin A (TSA, Sigma-Aldrich) for 72 h, with TSA added 16 h prior to RNA extraction.

RNA isolation and quantitative Real-Time-PCR (Q-PCR) for miRNA and mRNA expression

miRNA-enriched total RNA was extracted from MCF-7 and LY2 cells treated as above using the miRNA isolation kit (Exiqon, Woburn, MA). The quality and quantity of the isolated RNA was analyzed using a NanoDrop spectrophotometer. cDNA was synthesized using the miRCURY LNA^{TM} first strand cDNA synthesis kit (Exiqon) and Q-PCR was performed using the miRCURY LNA^{TM} SYBR Green master mix (Exiqon) using the miRNA primer sets for miR-200a, miR-200b or -miR-200c (Exiqon). SNORD38D, SNORD48 and 5SRNA were used for normalization of miRNA expression. Analysis and fold change was determined using the comparative threshold cycle (Ct) method. The change in miRNA expression was calculated as fold-change, *i.e.*, relative to EtOH-treated (control).

For mRNA expression, the High Capacity cDNA Reverse Transcription kit (PE Applied Biosystems) was used to reverse transcribe total RNA using random hexamers. Q-PCR for *ZEB1* was performed using SYBR green in the ABI PRISM 7900 SDS 2.1 (PE Applied Biosystems,Carlsbad, CA) using relative quantification. The sequence of ZEB1 primers is described in [205]. Analysis and fold differences were determined using the comparative CT method. Fold change was calculated from the ΔΔCT values with the formula $2^{-\Delta\Delta CT}$ and data are relative to EtOH-treated cells.

Transient transfection

MCF-7 or LY2 cells were transfected with either anti-miRNAs (antimiRs, Ambion, Life Technologies, Carslbad, CA) or precursor microRNA (pre-miRs, Ambion) respectively for miR-200b or miR-200c using Lipofectamine RNAimax (Invitrogen)

reagent. After 1 or 5 d, RNA was isolated (as described above) to confirm knockdown or overexpression of miR-200b or miR-200c.

MTT assay

MCF-7 or LY2 cells were grown in 96 well plates. Following transfection with anti-miRs or pre-miRs respectively for 24 h or 5 days, cells were treated with vehicle control EtOH, 10 nM E_2, 100 nM or 1 μM 4-OHT, 100 nM or 1 μM fulvestrant for 4 or 6 days. 20 μl of Cell Titer reagent (Promega, Madison, WI) was added to the wells and absorbance was read at 490 nm using a spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA).

Whole cell preparation for western blotting

Whole cell lysates were prepared and western blots were performed as described in [161]. Protein concentrations were determined by BioRad DCC protein assay (Hercules, CA).

Antibodies and reagents

Antibodies were purchased as follows: E-cadherin (Cell Signaling, Danvers, MA), vimentin (Santa Cruz Biotechnology, Santa Cruz, CA), β-actin (Sigma-Aldrich). Chemiluminescent bands on the PVDF membranes were visualized on a Kodak Carestream Imager using Carestream Molecular Imaging software (New Haven, CT).

Microscopy images

LY2 cells were untransfected or transfected with pre-miR-200b or pre-miR-200c or negative control for 48 h (described above). Images were captured using a digital microscope (EVOS, AMG, Bothell, WA) at a magnification of 20x and 100 μm scale.

Statistical analysis

Statistical evaluations were performed using GraphPad PRISM. Student's t-test was used to compare control and treatment values. P-values indicate statistical significance.

3. RESULTS

Expression of miR-200 family in MCF-7, LCC1, LCC2, LCC9 and LY2 human breast cancer cells

Microarray analysis of miRNA expression revealed low miR-200a, miR-200b and miR-200c expression in LY2 endocrine-resistant breast cancer cells compared to MCF-7 endocrine-sensitive breast cancer cells [161]. To follow up on this initial observation, the expression of miR-200a, miR-200b and miR-200c was measured by Q-PCR in a panel of human breast cancer cell lines, *i.e.*, MCF-7 cells and LCC1, LCC2 and LCC9 cells that were derived from the parental MCF-7 cell line by propagation in mice (LCC1), and then in long-term culture with tamoxifen (LCC2) or fulvestrant (LCC9) [348]. LY2 tamoxifen/fulvestrant-resistant human breast cancer cells were independently derived from MCF-7 cells by culture in a precursor to raloxifene: LY 117018 [349]. LY2 are cross-resistant to TAM, raloxifene, fulvestrant (Faslodex or ICI 182,780), and are ERα positive, although ERα protein expression is lower than MCF-7 cells [247]. These cells represent a model of the progression of breast cancer cells towards TAM/endocrine-resistance.

The effect of E_2 and 4-OHT on miR-200 expression was examined by Q-PCR in the cell lines described above (Figure 12). 10 nM E_2 and 100 nM 4-OHT significantly decreased miR-200a and miR-200b expression in MCF-7 cells. Similarly, E_2 significantly decreased the expression of miR-200a, miR-200b and miR-200c in estrogen-independent, but tamoxifen-sensitive LCC1 cells. However, there was no effect of E_2 and 4-OHT on the expression of miR-200 family in LCC2 and LY2 (Figure 13A-C). In LCC9, E_2 and 4-OHT decrease miR-200a and miR-200b expression and 4-OHT decreases miR-200c expression. LCC1 cells showed highest basal miR-200a expression. LY2 cells had undetectable levels of miR-200 family expression (Figure 12A-C). This is the first report of 4-OHT regulation of miR-200 family expression in LCC1, LCC2, LCC9 and LY2 cells. We and others previously reported that E_2 reduces miR-200 family expression in MCF-7 cells [159,161]

E_2 and 4-OHT regulate ZEB1 in MCF-7, LCC1, LCC2, LCC9 and LY2 human breast cancer cells

miR-200 family members repress ZEB1 [203,322,345]. Next we evaluated ZEB1 mRNA expression in each cell line. Basal expression of ZEB1 was lower in LCC1, LCC2, and LCC9 compared to MCF-7 (Figure 14A). As previously reported, ZEB1 expression was higher in LY2 compared to MCF-7 [247]. E_2 and 4-OHT decreased the expression of ZEB1 in MCF-7 cells (Figure 14A). There was no significant effect of either E_2 or 4-OHT on ZEB1 expression in LCC1, LCC2, or LCC9 cells (Figure 14B). In contrast, in LY2 cells, E_2 increased the expression of ZEB1 mRNA. Notably, there is an inverse relationship between the expression of miR-200 family and ZEB1 in LY2 cells (compare Figures. 12 and 14A).

Figure 12: Q-PCR analysis of the expression of miR-200 family in MCF-7, LCC1, LCC2, LCC9 and LY2 cells. Cells were treated with vehicle control EtOH, or 10 nM E_2 or 100 nM 4-OHT for 6 h. Values are the average of 3-4 experiments normalized to 5SrRNA or SNORD38D or SNORD48 and are expressed as fold relative to EtOH-treated MCF-7 expression. * $p<0.05$ *versus* MCF-7 EtOH treated.

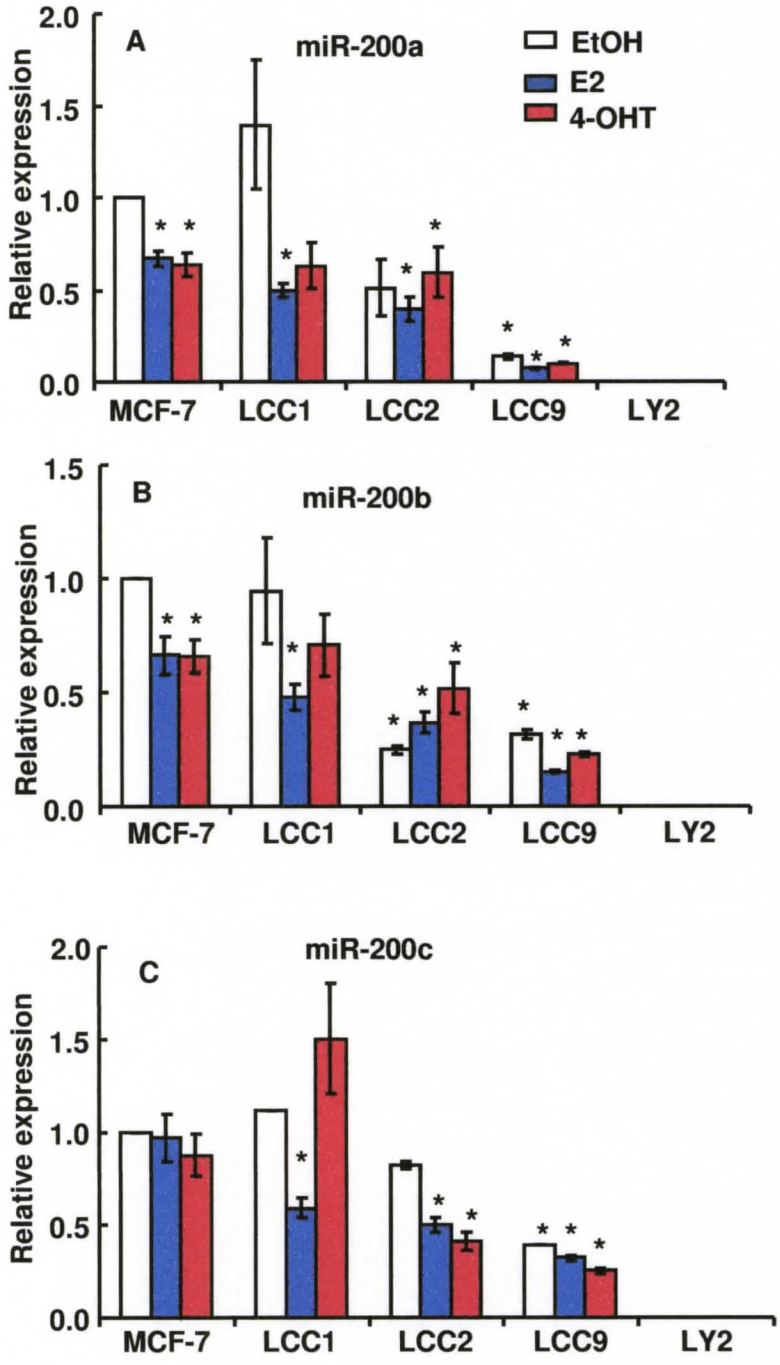


Figure 13: Q-PCR analysis of the expression of miR-200 family in MCF-7, LCC1, LCC2, LCC9 and LY2 cells. Cells were treated with vehicle control EtOH, or 10 nM E_2 or 100 nM 4-OHT for 6 h. Values are the average of 3-4 experiments normalized to 5SrRNA or SNORD38D or SNORD48 and are expressed as fold relative to EtOH-treated expression for each cell line. * $p<0.05$ *versus* EtOH treated for each cell line.

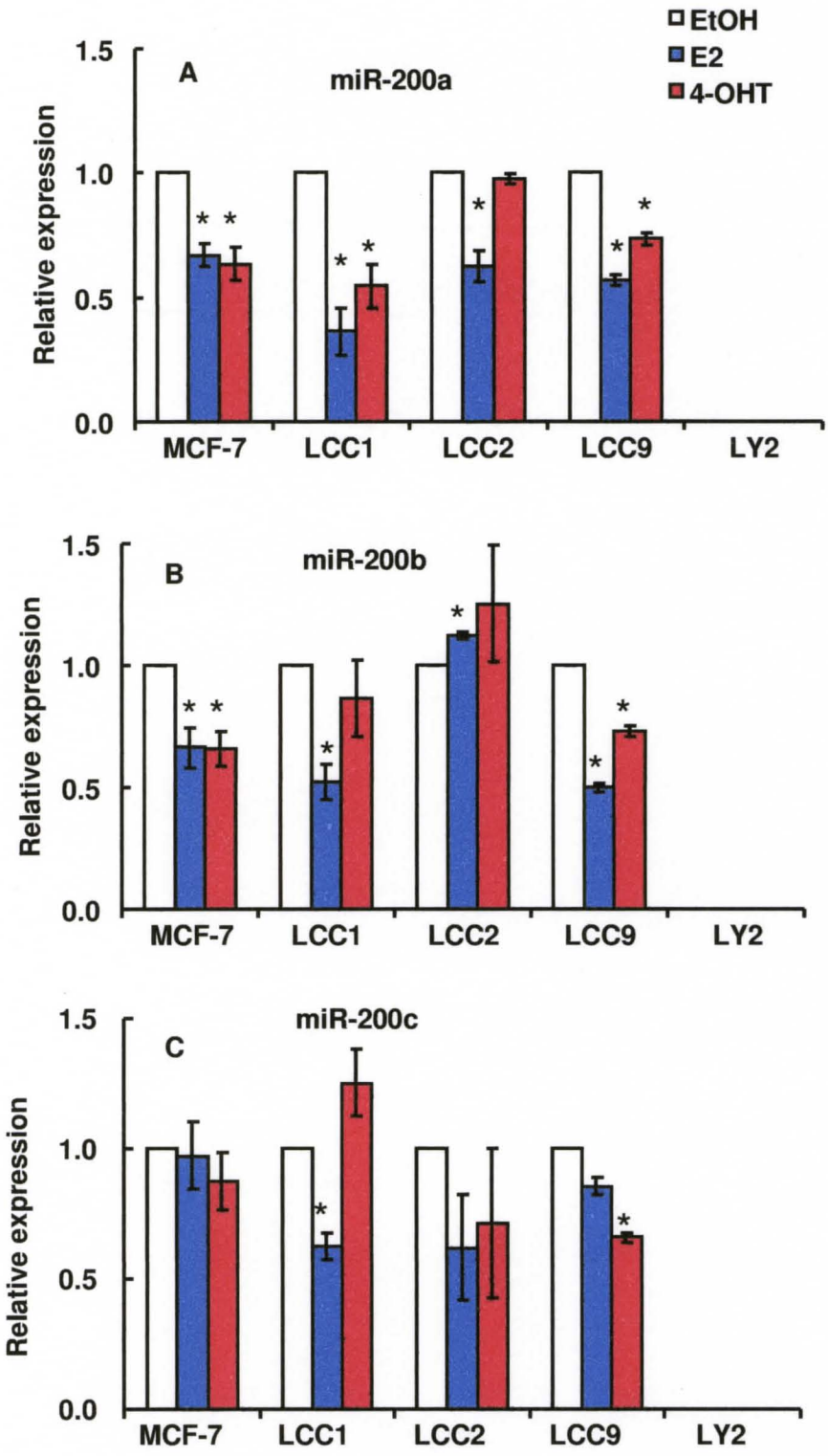
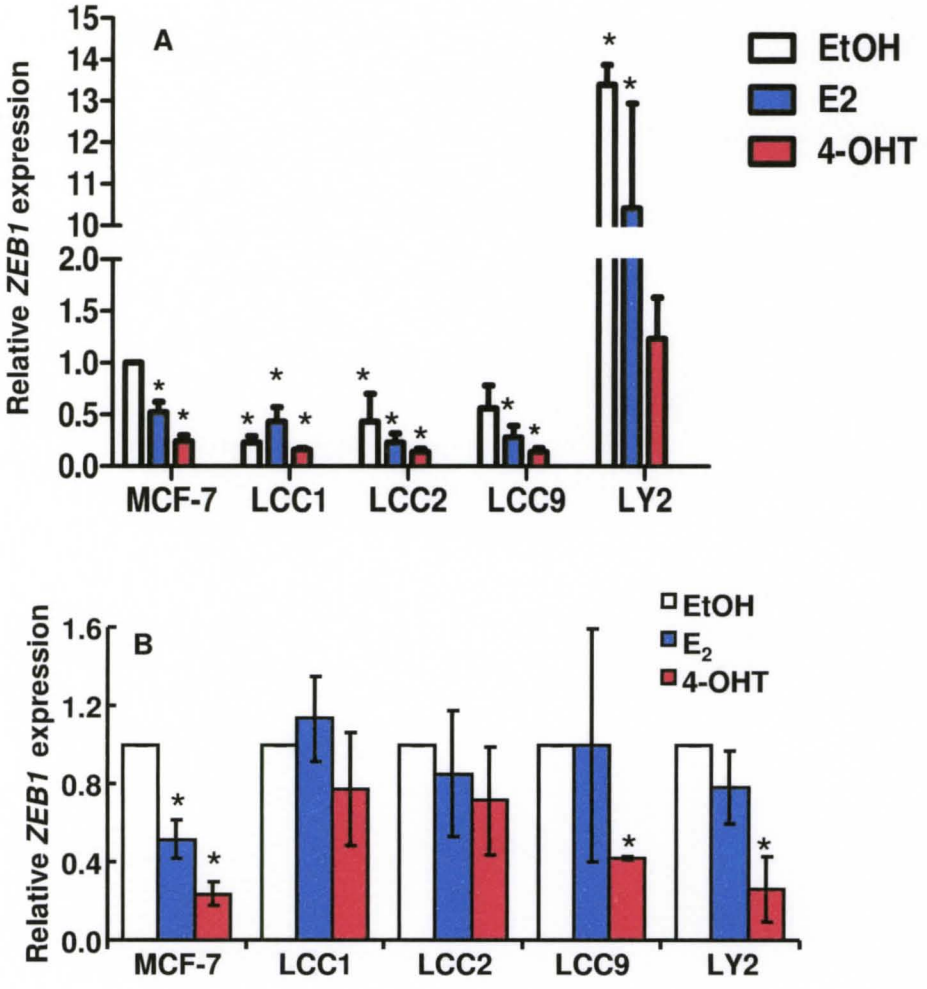

EtOH
E2
4-OHT
A
miR-200a
Relative expression
1.5
1.0
0.5
0.0
MCF-7
LCC1
LCC2
LCC9
LY2
B
miR-200b
C
miR-200c

Figure 14: Q-PCR analysis of the expression of ZEB1 mRNA in MCF-7, LCC1, LCC2, LCC9 and LY2 cells. Cells were treated with vehicle control EtOH, or 10 nM E_2 or 100 nM 4-OHT for 6 h. A. Values are the average of 3-4 experiments normalized to GAPDH and are expressed as fold relative to EtOH-treated MCF-7 expression for each cell line. *p<0.05 significantly different from MCF-7 EtOH treated. B. Values are the average of 3-4 experiments normalized to GAPDH and are expressed as fold relative to EtOH-treated for each cell line. *p<0.05 significantly different from EtOH treated for each cell line.



Overexpression of miR-200b or miR-200c in LY2 cells enhanced their sensitivity to 4-OHT or fulvestrant

To examine if expression of miR-200 family members affects sensitivity of endocrine-resistant LY2 cells to antiestrogens, cells were transiently transfected with precursors for miR-200a, miR-200b and miR-200c and MTT cell viability assays were performed in cells treated with vehicle control, 4-OHT, or fulvestrant for 6 days. Increased miR-200a, miR-200b and miR-200c expression was confirmed by Q-PCR even 7 days after transfection (Figure 15A). Treatment of nontransfected or control miRNA-transfected LY2 cells with 4-OHT or fulvestrant had no effect on cell viability (Figure 16A). LY2 cell viability was unaffected by overexpression of miR-200a regardless of treatment (Figure 16A). Overexpression of miR-200b increased LY2 cell sensitivity to inhibition by 4-OHT and fulvestrant. Overexpression of miR-200c reduced basal LY2 viability and fulvestrant, but not 4-OHT, further inhibited LY2 viability.

To determine if a shorter time of pre-miR-200b and pre-miR-200c transfection increases sensitivity of LY2 cells to inhibition by 4-OHT and fulvestrant, cells were transfected with pre-miR-200b or pre-miR-200c for 24 h and then treated with higher concentrations of 4-OHT and fulvestrant for 4 days. Q-PCR confirmed miR-200b and miR-200c overexpression in the transfected cells 5 d after transfection (Figure 15B). Notably the level of miR-200b was higher after 7 d than 5 d. Cell viability assays demonstrated lower basal level of proliferation in cells overexpressing miR-200b or miR-200c regardless of treatment (Figure 16B).

Figure 15: Overexpression of miR-200b or miR-200c in LY2 cells transfected with pre-miR-200b or pre-miR-200c or negative control. LY2 cells were transfected either with negative control or pre-miR-200a, pre-miR-200b or pre-miR-200c. RNA was harvested at 5 or 7 days and Q-PCR performed to confirm overexpression of miR-200a, miR-200b or miR-200c. Values are the mean ± SEM of triplicate determinations.

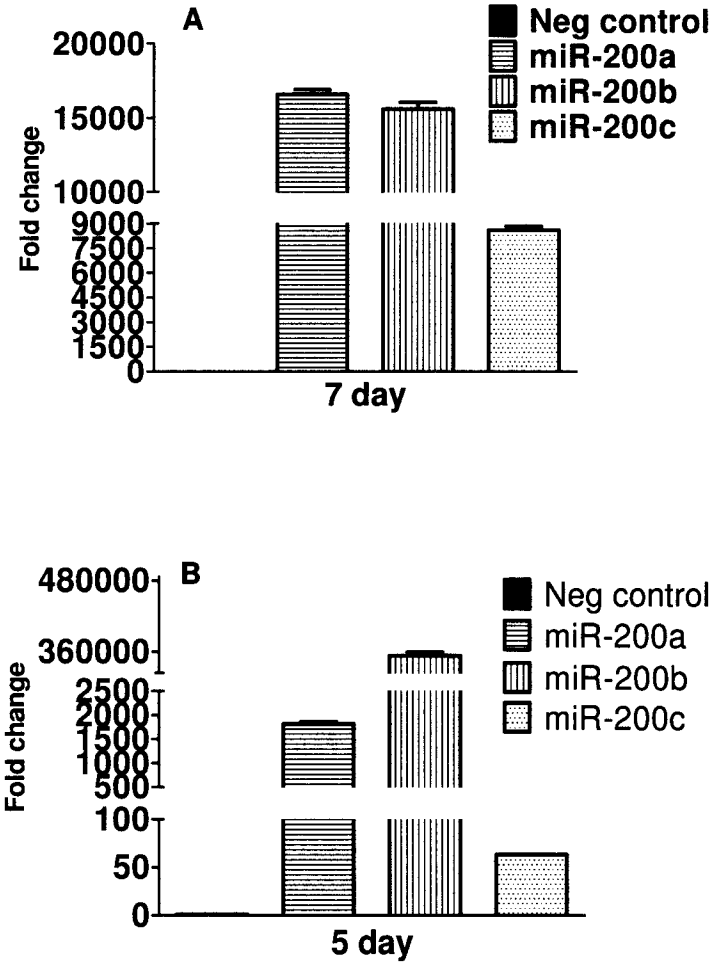
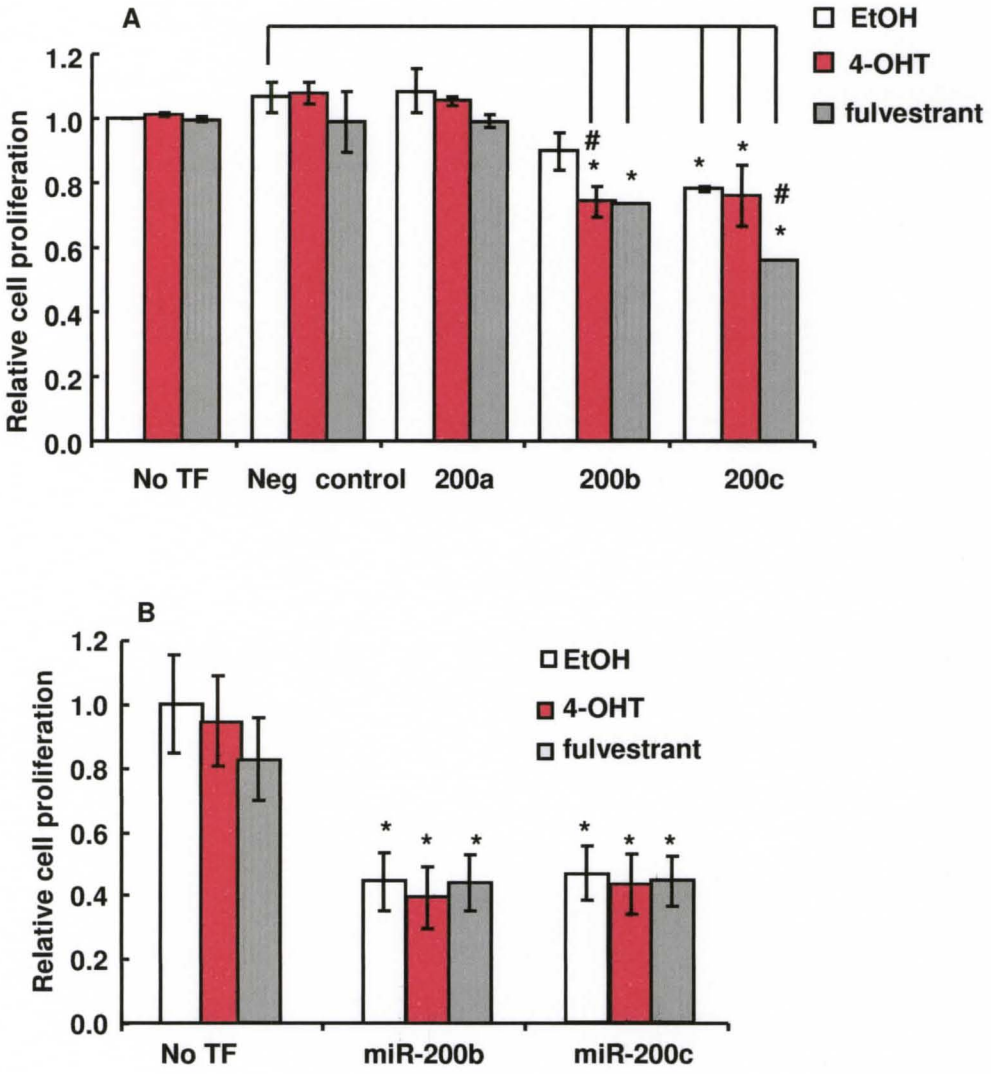


Figure 16: Overexpression of miR-200b or miR-200c restores sensitivity of LY2 cells to 4-OHT and fulvestrant. A. LY2 cells were either untransfected (No TF) transfected with negative control (Neg control) or pre-miR-200a, miR-200b or miR-200c. 5 days post-transfection, cells were starved for 24 h and treated with 100 nM 4-OHT or 100 nM fulvestrant for 6 days *p<0.05 *versus* LY2 EtOH treated negative control. # p<0.05 *versus* LY2 EtOH treated for each miRNA. B. LY2 cells were either untransfected (No TF) or transfected with pre-miR-200b or miR-200c. 1 day post-transfection, cells were treated with 1 µM 4-OHT or 1 µM fulvestrant for 4 days and MTT assays were performed. Values are the mean ±SEM of 3 experiments.*p<0.05 *versus* LY2 EtOH treated No TF.



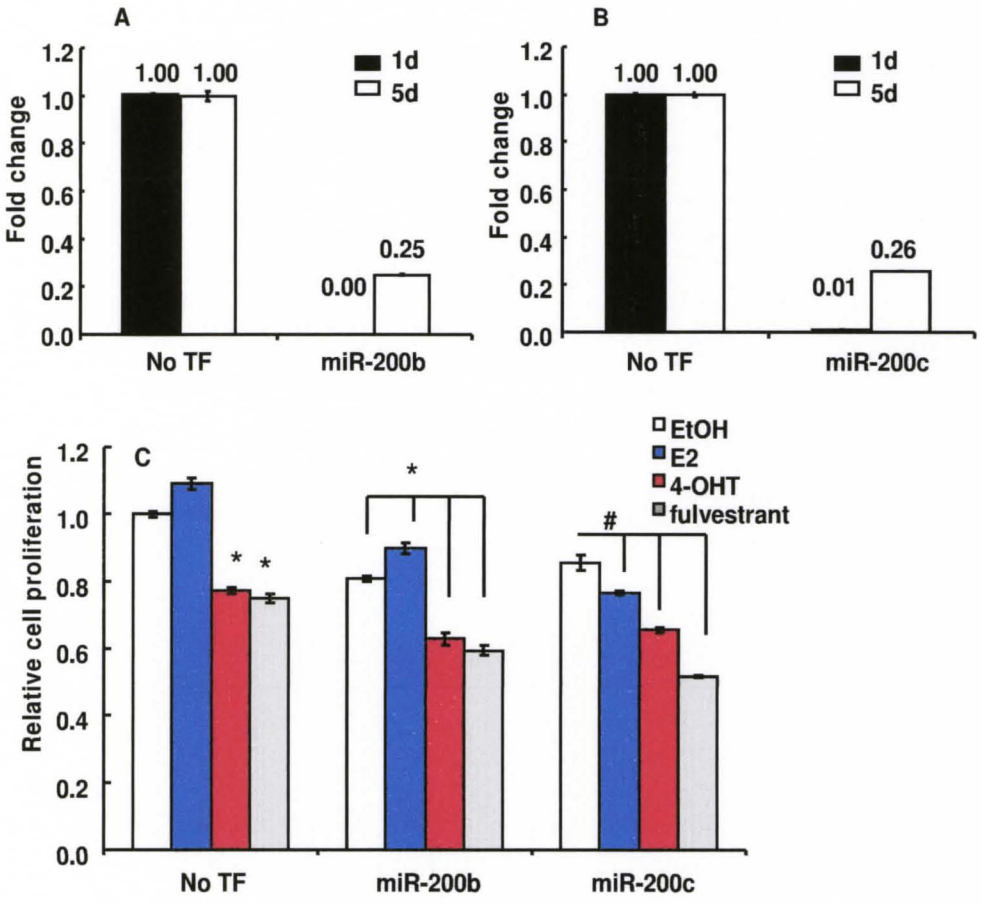
Converse experiments were performed using anti-miRNA to knockdown the expression of miR-200b or miR-200c in MCF-7 cells (Figure 17A and B). Surprisingly, knockdown of miR-200b and miR-200c reduced basal MCF-7 cell viability. However, there was no change in the sensitivity of cells to 100 nM 4-OHT or fulvestrant after knockdown of miR-200b or miR-200c (Figure 17C) in MCF-7 cells, indicating that other factors also contribute to the sensitivity of breast cancer cells to antiestrogens.

Overexpression of miR-200b or miR-200c changes morphology of LY2 cells to a 'cobblestone' shaped appearance

Overexpression of miR-200b and miR-200c (Appendix 8A) altered LY2 cell morphology (Figure 18). LY2 cells showed a change from an elongated to a more epithelial or 'cobble-stone' shaped appearance (Figure 18C and D). Overexpression of miR-200a had no effect on LY2 cell appearance, in agreement with the lack of effect of miR-200a on cell viability (Figure 18B). Overexpression of a control miRNA had no effect on cell appearance (Appendix 8B). Previous studies have reported the reversal of EMT in MDA-MB-231 breast cancer cells with miR-200c overexpression of [347]. These results are in agreement with my study and indicates that LY2 cells undergo change in morphology upon overexpression of miR-200b or miR-200c.

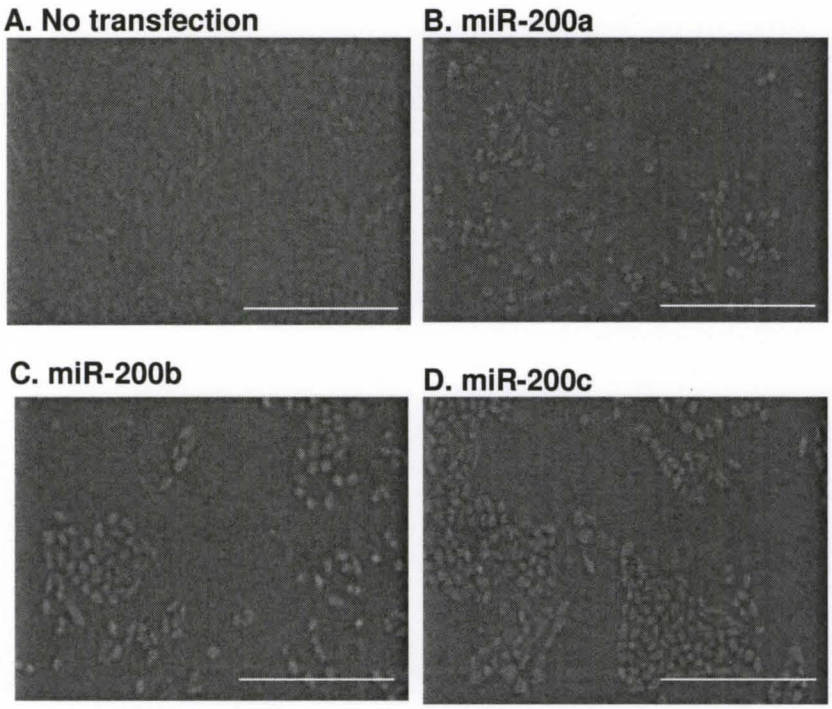
Figure 17: Knockdown of miR-200b or miR-200c does not promote resistance of MCF-7 to 4-OHT or fulvestrant.

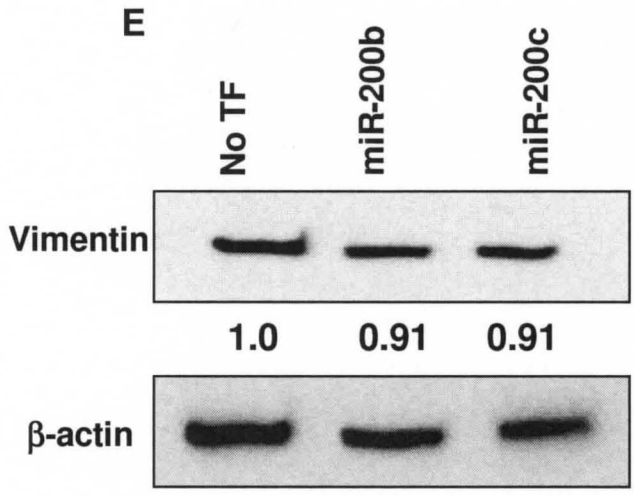
MCF-7 cells were either transfected with anti-miR -200b or anti-miR-200c. 1 day post-transfection, cells were treated with 100 nM 4-OHT or 100 nM fulvestrant for 4 days and MTT assays were performed. Values are the mean ±SEM of 3 experiments. *P<0.05 versus EtOH treated (miR-200b). # p<0.05 versus EtOH treated (miR-200c)

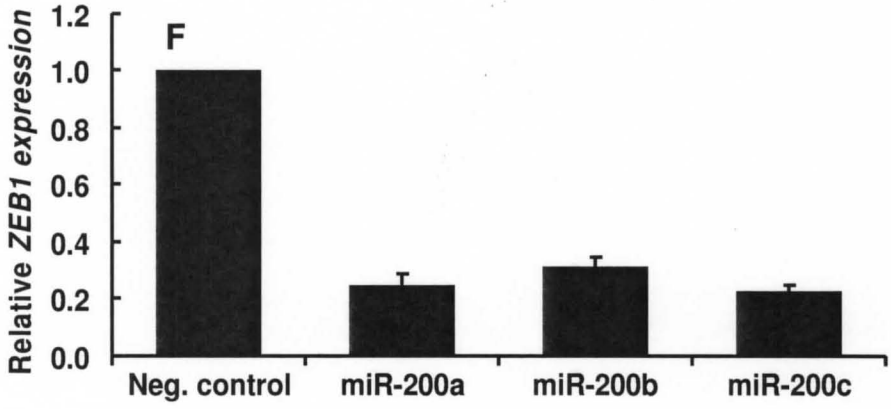


To confirm if the observed change in morphology of LY2 cells was due to reduced expression of mesenchymal markers, vimentin protein was examined in cells expressing transfected with miR-200b or miR-200c. Overexpression of miR-200b or miR-200c in LY2 cells did not induce MET (mesenchymal- to - epithelial transition) as indicated by unaltered vimentin or E-cadherin expression in cells transfected with miR-200b or miR-200c (Figure 18E). However, there was a decrease in ZEB1 mRNA in cells transiently overexpressiong miR-200a, miR-200b or miR-200c (Figure 18F). These results indicate that factors other than the reduction of miR-200b and miR-200c contribute to EMT in LY2 cells.

Figure 18: Overexpression of miR-200 family changes morphology of LY2 cells. LY2 cells were transfected with pre-miR-200a, or pre-miR-200b or pre-miR-200c for 3 days. A-D. Images of LY2 cells captured using a digital microscope (20x magnification, bar-100 μm scale). E. Expression of vimentin protein in cells expressing miR-200a, miR-200b or miR-200c. Images and Western blots are representative of 3 separate experiments. F. Expression of ZEB1 mRNA in LY2 cells expressing miR-200a, miR-200b or miR-200c or negative control. Results are the mean ±SEM of two separate experiments.



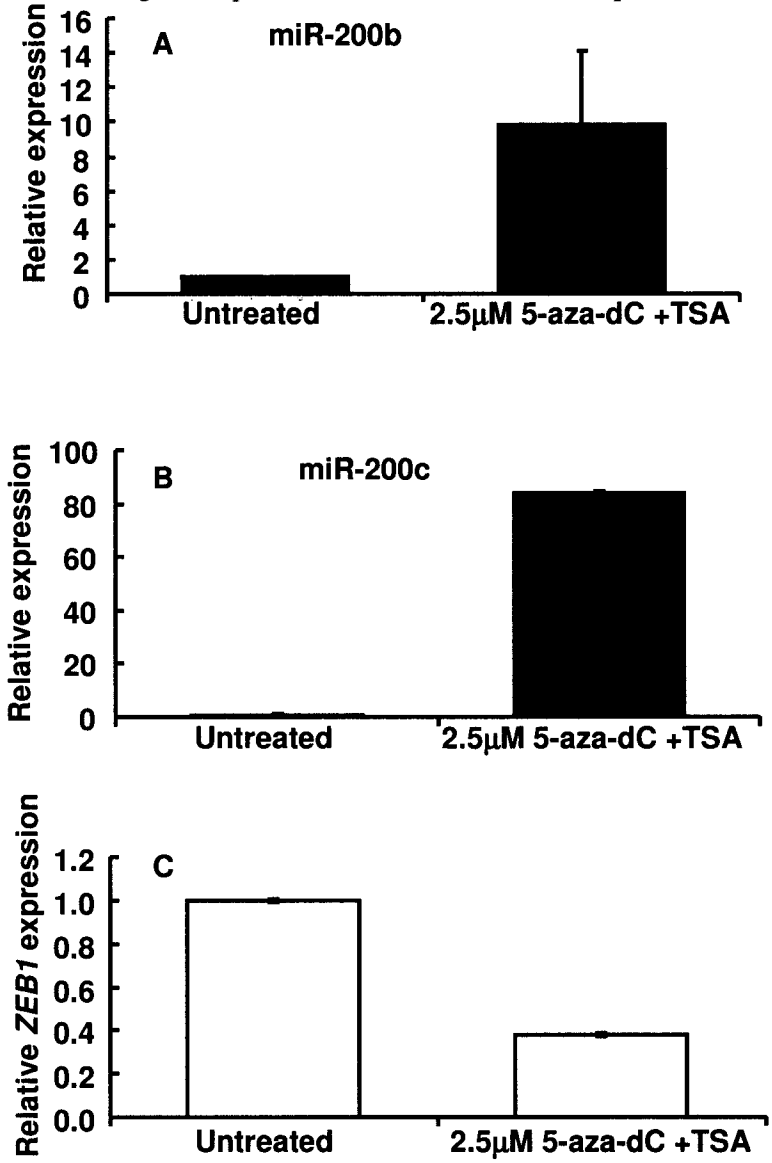

E
No TF
miR-200b
miR-200c
Vimentin
1.0
0.91
0.91
β-actin

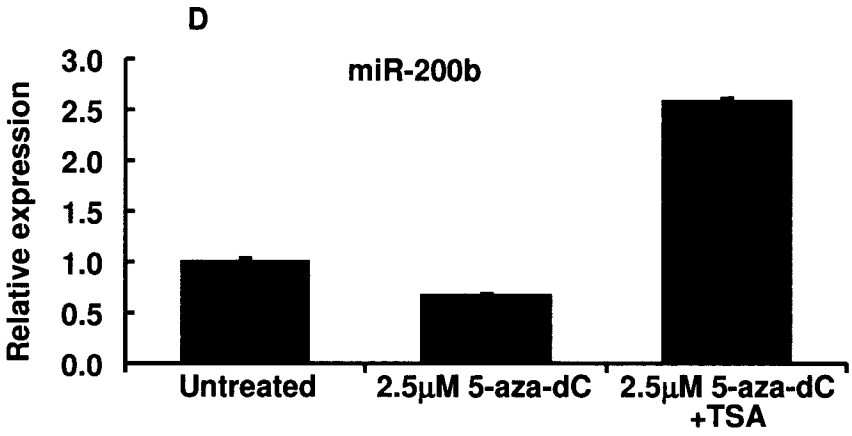

F
Relative ZEB1 expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Neg. control
miR-200a
miR-200b
miR-200c

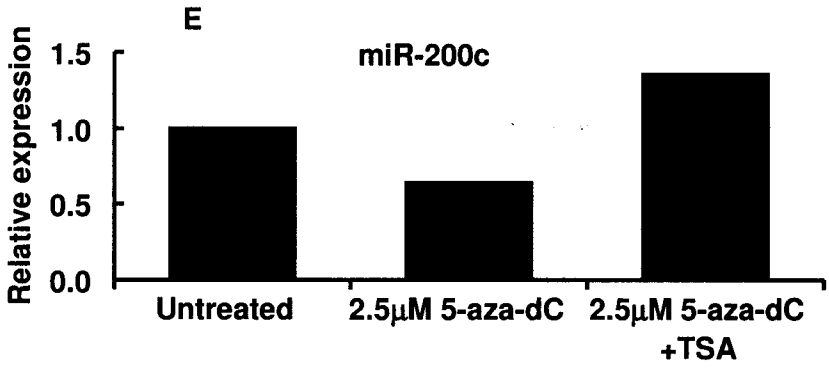
Inhibitors of deacetylation and methylation increase miR-200 family expression in LY2 cells

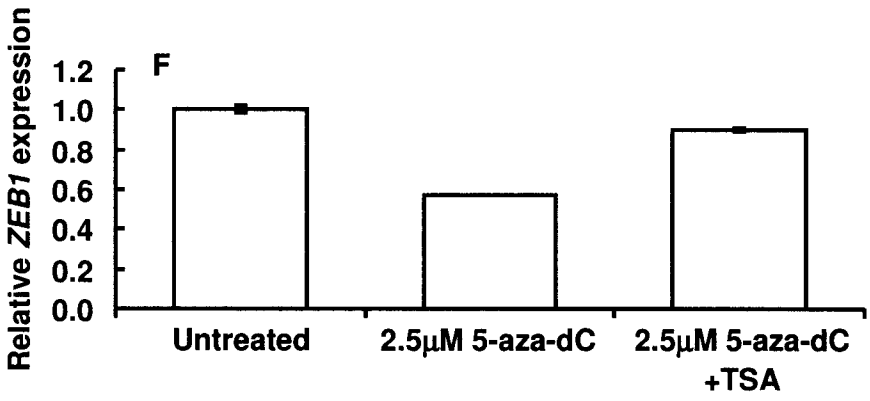
One possible mechanism responsible for reduced expression of miR-200 family members in LY2 cells could be DNA methylation or histone deacetylation. Previous studies have shown that the CpG island near the miR-200c/miR-141 transcription start site is methylated in fibroblasts and tumors cells that are miR-200c or miR-141-negative [350,351]. To determine if decreased expression of miR-200 family members in LY2 cells is due to epigenetic silencing, LY2 cells were treated with 2.5 μM 5-aza-dC alone in combination with 100 ng/μl trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor for 72 h. TSA was added in the last 16 h of the treatment period [352]. 2.5 μM 5-aza-dC and TSA increased the expression of miR-200b and miR-200c in LY2 cells (Figure 19A and B). Concomitant with the increased expression of miR-200b and miR-200c, there was a decrease in expression of its target, ZEB1 mRNA upon combined treatment with 2.5 μM 5-aza-dC and TSA (Figure 19C). To determine if the observed decrease in ZEB1 mRNA level is due to a direct effect of the inhibitors, MCF-7 cells were treated with 2.5 μM 5-aza-dC alone or in combination with 100 ng/μl TSA. Combined treatment with 5-aza-dC and TSA did not alter the expression of ZEB1 in MCF-7 cells (Figure 19F). However, it increased the expression of miR-200b and miR-200c in MCF-7 cells (Figure 19D and E).

Figure 19: 5-aza-dC and TSA co-administration results in an increase in miR-200b and miR-200c expression, and a concomitant decrease in ZEB1 mRNA in LY2 cells. A-C. LY2 cells were treated with 2.5 μM 5-aza-dCA in combination with 100 ng/μl TSA for 72 h. TSA was added only during the last 16 h of treatment. Q-PCR was used to determine the fold change in the expression of miR-200b, or miR-200c, or ZEB1 mRNA. A an C are the mean ± SEM of 2 separate experiments. B is the mean of triplicate determinations in one experiment. D-F. MCF-7 cells were treated with 2.5 μM 5-aza-dCA alone or in combination with 100 ng/μl TSA for 72 h. TSA was added only during the last 16 h of treatment. Q-PCR was used to determine the fold change in the expression of miR-200b, or miR-200c or ZEB1 mRNA. Values are the average of triplicate determinations in one experiment.




D
miR-200b
Relative expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Untreated
2.5μM 5-aza-dC
2.5μM 5-aza-dC +TSA


E
miR-200c
Relative expression
1.5
1.0
0.5
0.0
Untreated
2.5μM 5-aza-dC
2.5μM 5-aza-dC +TSA


F
Relative ZEB1 expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Untreated
2.5μM 5-aza-dC
2.5μM 5-aza-dC +TSA

4. DISCUSSION

In this study I identified a novel role for miR-200b and miR-200c in modulating the sensitivity of endocrine-resistant LY2 cells to 4-OHT and fulvestrant in addition to the previously reported ability of these miRNAs to stimulate EMT. A progressive decrease in the expression of miR-200a, miR-200b, and miR-200c was detected in an MCF-7-derived cell line model of tamoxifen/endocrine resistance. The parental estrogen- dependent, endocrine-sensitive MCF-7 cells express all three miR-200 family members. With the LCC2, LCC9, and LY2 cells derived for progressive tamoxifen and fulvestrant resistance, I observed a graded reduction in miR-200 family expression (Figure 12 A-C).

This is the first report demonstrating that overexpression of miR-200b and miR-200c enhanced the sensitivity of breast cancer cells to growth inhibition by antiestrogens 4-OHT and fulvestrant. Here I demonstrated that overexpression of miR-200b or miR-200c for 11 days increased sensitivity of LY2 cells to 100 nM 4-OHT and fulvestrant (Figure 16A). A shorter 5 day period of overexpression of miR-200a and miR-200b reduced basal LY2 cell viability independent of antiestrogen treatment (Figure 16B). One possible explanation for these different results is lower levels of miR-200b and miR-200c in cells after longer periods of transfection. Hence the decrease in basal cell viability in the control treated cells may be due to higher miR-200b and miR-200c levels at earlier times after transfection. This result may explain why no additional inhibitory effect of 4-OHT on cell viability was detected.

A role for miR-200 family in drug resistance was reported in ovarian cancer [323]. Overexpression of miR-200c increased the sensitivity of ovarian cancer cells to a microtubule targeting drug Paclitaxel [323]. This was mediated by miR-200c targeting of a microtubule associated protein TUBB3. Overexpression of miR-200c

reversed resistance of UMUC3 bladder cancer cells to anti-EGFR therapy [353]. Similarly gemcitabine-resistance in pancreatic cancer is associated with decreased miR-200 expression [354]. Resistance of pancreatic cancer cells to gemcitabine was reduced by treatment with natural compounds such curcumin, alone or in combination. This was accompanied by increased miR-200 expression [353,355-357]. CDF, an analogue of curcumin was found to increase the sensitivity of pancreatic cancer cells to gemcitabine through inactivation of NFkB and COX-2 pathways which led to increased expression of miR-200b and miR-200c [356]. These studies show that miR-200 has a dual role in drug resistance.

Our results show that there is an inverse relationship between the expression of miR-200 family (miR-200a, miR-200b and miR-200c) and ZEB1 mRNA in LY2 cells. These data are in agreement with other reports showing an inverse correlation between miR-200 family and ZEB1 expression in basal-like, TNBC cells such as MDA-MB-231 and BT-549 cell lines [203,205,345,347].

LY2 cells overexpressing miR-200b or miR-200c showed a change in morphology from a 'cobblestone' or mesenchymal phenotype to a spindle shaped or epithelial phenotype (Figure 18C and D). However cells expressing miR-200a did not show a change in morphology or change in sensitivity to antiestrogens (Figure 16A and 18B). miR-200b and miR-200c share the same seed sequence [206]. The similarity in effects of these miRNAs can be attributed to this identical seed sequence of miR-200b and miR-200c. These results indicate a novel role for miR-200b and miR-200c in cellular morphology and response to 4-OHT and fulvestrant. Other studies have observed a reversal of EMT in aggressive breast cancer cell lines transfected with miR-200c or miR-141 [203,205,346,347]. For example, overexpression of miR-200b and miR-200c reverted EMT in mesenchymal breast

cancer cell lines MDA-MB-231 and BT-549 by repressing the transcription and translation of transcription factors ZEB1 and ZEB2 [205,325]. Similar results were observed in miR-200b and miR-200b transfected MDCK- Paz (Madin-Darby canine kidney cells) [203]. Likewise, ectopic expression of miR-200c restored E-cadherin expression and reversed the mesenchymal phenotype in NMuMG (normal murine mammary epithelial cells) and 4TO7 breast carcinoma cell lines that had undergone EMT [346]. Our data showing a change in morphology of LY2 cells overexpressing miR-200b and miR-200c are in agreement with these observations, although we did not detect a reduction in vimentin or an increase in E-cadherin protein expression (data not shown).

One of the mechanisms for decreased miR-200 expression in LY2 cells could be due to epigenetic changes in the promoter, *e.g.*, DNA methylation and histone deacetylation. CpG island methylation of miR-200c/miR-141 promoter has been previously reported in breast and prostate cancer cells [350,351,358]. Treatment of MDA-MB-231 and BT549 breast and PC3 prostate cancer cells with 3 μM 5-aza-dC, an inhibitor of DNA methylation, increased miR-200c and miR-141 expression [350]. Analysis of histone modification by chromatin immunoprecipitation (ChIP) revealed dimethylation of Lysine 9 of histone H3 in miR-200c/miR-141-negative MDA-MB-231 breast cancer cells, indicating repression of the miR-200c/miR-141 cluster. The data provided in this chapter agrees with these reports of epigenetic silencing of the miR-200 family. I demonstrated that treatment of LY2 cells with 2.5 μM 5-aza-dC in combination with TSA increased miR-200b and miR-200c expression when compared to untreated cells (Figure 19). There was a concomitant decrease in the expression of ZEB1 mRNA.

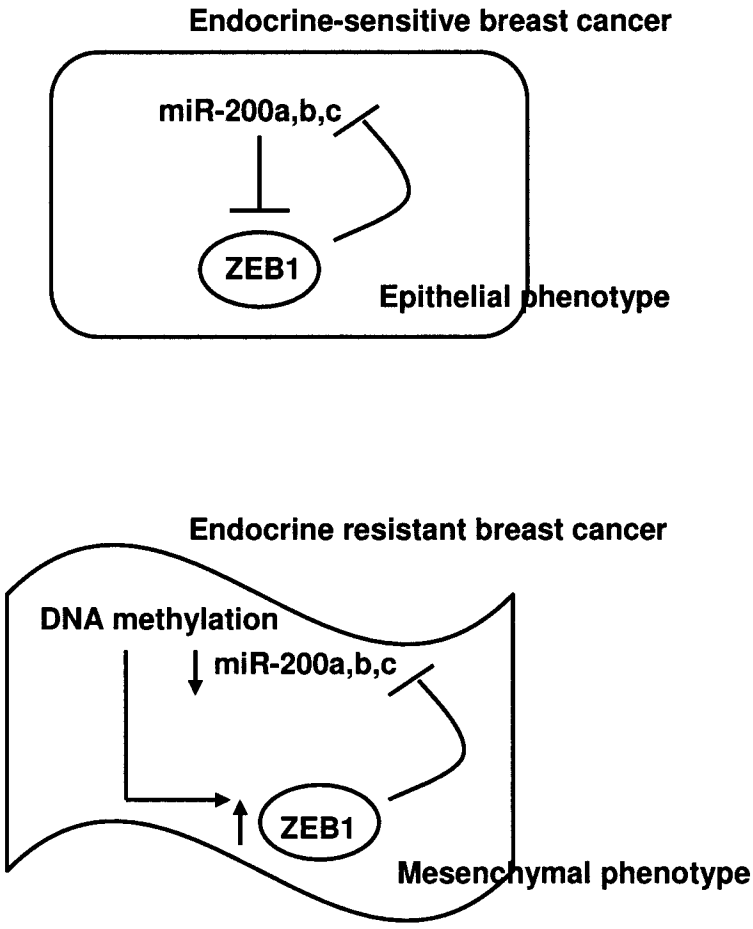
Previous studies have shown that endocrine resistance is accompanied by loss of cell-to-cell adhesion and EMT. Hiscox *et al.* showed that induction of EMT in MCF-7 breast cancer cells that are tamoxifen- resistant (TAM-R), is due to EGFR mediated phosphorylation and activation of the β-catenin pathway [340]. This promotes EMT and subsequently metastasis of cancer cells. Another study by the same group demonstrated that fulvestrant-resistant MCF-7 and T47D cells show enhanced migration and invasion due to overexpression of the c-Met receptor protein [359]. Further, elevated Src activity was also found to contribute to the invasive phenotype of TAM-R MCF-7 cells [360]. A recent study shows that prolonged mammosphere culture of MCF-7 cells makes them metastatic as well as resistant to tamoxifen [361]. Induction of Snail 1 transcription factor promotes EMT in MCF-7 TAM-R cells by downregulation of the epithelial marker E-cadherin [341]. This effect was seen upon overexpression of a peptidyl-prolyl isomerase Pin1 which, in turn, activates GSK-3β or NFκB. These reports indicate a link between aberrant activation of signaling pathways such as NFκB to EMT and endocrine resistance. However, there is only one report of miRNA regulation of both EMT and endocrine-resistance in breast cancer cells [343]. That study showed that overexpression of miR-375 increased sensitivity of TAM-R MCF-7 cells to treatment with TAM by decreasing the expression of Metadherin (MTDH). My identification of reduced miR-200 expression as a marker of endocrine resistance of breast cancer cells is a novel finding.

Although miR-200 is considered as a tumor suppressor miRNA, there are some reports of its role as an oncogene. There are many reports of miR-200 family expression as a marker of poor prognosis and chemoresistance in ovarian cancer [362-364]. Contrary to the expected decrease in miR-200 expression in metastatic cells,

Dykxhoorn *et al.* observed high levels of miR-200b and miR-200c in 4T1 metastatic mouse mammary tumor cells [365]. In concordance, 4T1 cells showed low ZEB1 and high E-cadherin expression. These results indicate that mR-200 has a dual pattern of expression, *i.e.*, it suppresses EMT while it promotes metastatic colonization after cells have invaded a distant site. Further, Korpal *et al.* reported pro-metastatic effects of miR-200 in a mouse model of breast cancer metastasis [366]. miR-200 was found to target Sec23a, a suppressor of metastasis that regulates the cell-secretome. These studies shed light on the dual role of miR-200 depending on the cell context.

In summary, the results presented in this chapter reveal a novel role for the loss of miR-200b and miR-200c in contributing to the loss of antiestrogen sensitivity in endocrine resistant breast cells. Of course, additional factors are also involved. Although most studies have identified a role for miR-200 as a suppressor of EMT, my studies provide new evidence (Fig.16A) to show miR-200 as a suppressor of endocrine resistance in breast cancer cells. Future experiments are needed to assess the dual role of miR-200b and miR-200c in cancer progression so that appropriate targets can be used for targeted therapy. This would enable the use of this miRNA as a marker of endocrine-resistant breast cancer.

Figure 20: Proposed model of miR-200 function in endocrine-resistant breast cancer. In endocrine-sensitive breast cancer, miR-200 suppresses ZEB1 expression and promotes an epithelial phenotype. Decreased expression of miR-200 family in endocrine-resistant breast cancer is a result of DNA methylation, and results in increased ZEB1 expression and a change in morphology of cells from an epithelial to a mesenchymal appearance.



CHAPTER V: RESEARCH IMPLICATIONS

Adjuvant endocrine therapy is widely used in the treatment of premenopausal and postmenopausal women with ER-positive breast cancer. It involves the use of SERMs, *e.g.*, TAM, Raloxifene, and pure antiestrogens, *e.g.*, fulvestrant, that interfere with estrogen receptor function [248,367]. However, about 40 % of women who receive adjuvant hormonal therapy acquire endocrine resistance [56]. Aberrant activation of growth factor signaling pathways, such as the EGFR, and the mutant EGFR (Her2), and altered expression of co-regulators are some of the mechanisms involved in endocrine resistance [368]. However, the complexity of endocrine resistance remains to be fully elucidated. My dissertation focused on identifying miRNAs that are aberrantly expressed in endocrine-resistant breast cancer. Deregulated expression of miRNAs has been reported in a number of cancers and these small RNAs are becoming increasingly important players affecting different signaling pathways [262]. By identifying miRNAs that are dysregulated in endocrine-resistant breast cancer, it is possible to test their use as markers of resistance.

One major finding of my study is that there is an opposite pattern of expression of miRNAs in antiestrogen-sensitive MCF-7 *vs.* -resistant LY2 cells. In Chapter III, I identified 97 miRNAs that are significantly differentially expressed in MCF-7 and LY2 cells. This is a significant finding as it shows that acquisition of endocrine resistance is associated with altered miRNA signature. Results from this chapter are important as this is one of the few studies that have profiled miRNAs in endocrine-resistant breast cancer cells.

My study identified reliable endogenous control genes (ECGs) for normalization of miRNA Q-PCR data. Identification of an ideal ECG for normalization of gene expression data has remained a challenge for years. Ideally, a good ECG expression is one whose expression is independent of treatment condition and cell types. I demonstrated that the most commonly used ECGs, *i.e.*, snoRNAs such as RNU6-1, vary in expression with 4-OHT treatment in MCF-7 and LY2 cells. SNORD38D, 5SrRNA and SNORD48 were identified as good ECGs as they showed the least variation with 4-OHT treatment. They also exhibited a consistent pattern of expression in MCF-7 and LY2 cells (Figure 4). My study shows the importance of the use of multiple ECGs to validate Q-PCR data. This is critical in miRNA gene analysis as the use of an inconsistent ECG leads to bias in results.

Regulation of miRNA expression by the ER has been studied by very few groups [158,159,162,163]. As indicated by results in Chapter III, ICI studies demonstrated that the expression of miR-10a, miR-21, miR-22, miR-200a, miR-221 and miR-222 are ER-mediated in MCF-7 cells (Figure 5B and C). ICI treatment alone relieved the basal expression of these miRNAs, indicating that ER normally suppresses their expression in MCF-7 cells. There are reports of ERα being a target of the miR-22 and miR-221/222 family [231,234]. Increased expression of miR-221/222 has also been reported in TAM- and fulvestrant- resistant breast cancer [283,369]. Higher expression of miR-221/222 family and lower expression of ERα in LY2 cells compared to MCF-7 cells agree with these reports. More studies are required to determine if ERα or ERβ are involved in the expression of the miRNAs identified in Chapter III.

One important finding in my study is that there is a variable pattern of miRNA expression at different time points. 4-OHT increased the expression of miRNAs after

6 h of treatment (Figure 6). There was a corresponding increase in ERα protein levels after 6 h of 4-OHT treatment, further implying that the expression of miRNAs could be ER-mediated.

Bioinformatic analysis identified 36 putative mRNA targets of the 12 miRNAs that were analyzed in Aim 1 (Figure 8). Expression of *PDCD4*, *BCL2*, *CYP1B1*, *ESR1* and *ERBB3* was validated at the mRNA and protein level. Although there was agreement in the direction of expression of specific miRNAs and their putative targets, discrepancy was seen in the expression of PDCD4 and BCL2 protein in LY2 cells. Contrary to the expected increase in PDCD4 and BCL2 protein with lower miR-21 in LY2 cells, there was undetectable PDCD4 and BCL2 protein in LY2 cells. It is likely that these genes are under regulation by other miRNAs or other mechanisms such as epigenetic modification. Additionally, there are reports of phosphorylation of PDCD4 by Akt and S6K1 leading to its proteasomal degradation in HEK293 cells [370,371].

In Chapter IV, I report lower expression of miR-200 family members as cells progress towards endocrine-resistance (Figure 12). The role of miR-200 in drug resistance in ovarian, bladder and pancreatic cancer has been studied [323,353,355]; however, there are no reports of its role in antiestrogen-resistant breast cancer. An interesting discovery in Chapter IV of my dissertation is that restoring miR-200 family members, miR-200b and miR-200c increased sensitivity of LY2 cells to inhibition by TAM and fulvestrant treatment (Figure 16A). These data imply that miR-200 contributes to endocrine-sensitivity of LY2 cells. However, the converse experiment (Figure 17) using antisense to miR-200b and miR-200c in MCF-7 cells did not make them resistant to TAM or fulvestrant. This implies that miR-200 is just

one of the players in the complex network of genes and pathways associated with endocrine resistance.

Overexpression of miR-200 changed morphology of LY2 cells from an elongated, mesenchymal to an epithelial appearance. This agrees with previous reports of the ability of miR-200 to reverse EMT in TNBC cells such as MDA-MB-231 and BT-549 [205,345,347]. It was intriguing to find that LY2 cells did not undergo MET when transfected with miR-200a, miR-200b or miR-200c as there was no decrease in expression of the mesenchymal marker, vimentin, nor an increase in epithelial marker, E-cadherin. Perhaps there are other factors in addition to miR-200 overexpression that are required to induce MET in LY2 cells. Previous studies have shown that ERα inhibits EMT by suppressing the expression of transcription factors such as SLUG and Snail 1 [372-375]. LY2 cells have lower ERα when compared to MCF-7 cells (Figure 4F). Future studies could determine if restoration of ERα induces MET and restore sensitivity of LY2 cells to treatment with 4-OHT and fulvestrant.

Although most studies have focused on the role of miR-200 as a tumor suppressor, there are a few reports of miR-200 as an oncogene promoting mestastasis in breast cancer. Pro-metastatic effects of miR-200 family have been reported in an animal model of lung metastasis [366]. This would be a major drawback in the use of miR-200 as therapy to restore endocrine-responsiveness in cells. The expression of miR-200 family in tumor samples of patients resistant to TAM or fulvestrant therapy remains to be tested. This would give a clear understanding of the pattern of expression of this miRNA and for its use in therapy.

Overall, this study contributes to better understanding of miRNAs that are dysregulated in endocrine-resistant breast cancer. By analyzing the miRNA signature

of breast cancer patients, it would be possible to determine if they are susceptible to developing resistance to these antiestrogens such as TAM and fulvestrant. This would be beneficial in a timely and effective treatment of patients with endocrine-resistant breast cancer. My study on miR-200 family provides a novel link between miRNA regulation of EMT and endocrine-resistant breast cancer. Finally, my study demonstrates that miR-200 is a significant contributor of sensitivity to antiestrogen therapy. By elucidating the exact mechanism involved in regulation of miR-200 expression, this could be an attractive target to restore TAM-responsiveness in cells.

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APPENDICES

APPENDIX 1: Differentially expressed miRNAs in MCF-7 estrogen/TAM-sensitive versus LY2 TAM/ endocrine resistant cells and their roles in breast and other cancers.

miRNA	Comments
miR-10a	miR-10a inhibits *hoxd4* gene expression in breast cancer cells [376]. miR-10a interacts with the 5' untranslated region of mRNAs encoding ribosomal proteins to enhance their translation and increases global protein expression [302]. In embryonic stem cells, RA induces binding of p65 to the miR-10a promoter, leading to an increased in miR-10a and enhanced miR-10a expression suppresses HDAC4 expression [301]. miR-10a is upregulated in bladder cancer and targets FGFR3 [377]. miR-10a expression is increased by retinoic acid-induced NFκB activation during smooth muscle cell (SMC) differentiation from mouse embryonic stem cells (ESCs) [301].
miR-21	miR-21 is an oncomiR that is significantly up-regulated in all types of breast tumors [378] and in breast cancer cell lines [257]. miR-21 is overexpressed in all solid tumors (lung, breast, stomach, prostate, colon, and pancreatic) [258]. The role of miR-21 in breast cancer was recently reviewed [262]. miR-21 was significantly higher in ERα+ *vs.* ERα-, ErbB2 - *vs.* ErbB2 +, and in PR+ *vs.* PR- breast tumors [304]. Hypoxia increasedmiR-21 expression in MCF-7 [379]. miR-21 in mammary gland was increased after 18 wks of E_2 treatment of female ACI rats [157]. Both E_2 and ICI decreased miR-21 in human endometrial stromal and glandular epithelial cells, but when combined, miR-21 expression returned to basal [380]. E_2 suppressed and ICI increased miR-21 in human myometrial smooth muscle cells [381]. E_2 inhibited the ICI-induced increase in miR-21 in these cells [381]. E_2 and Progesterone reduced miR-21 expression on the uterus of ovex mice [382]. Angiotensin II increased miR-21 expression, aldosterone secretion and proliferation in H295R human adrenocortical cells [383]. miR-21 expression was significantly reduced in tamoxifen-resistant MCF-7 cells [270]. Another group reported that E_2 (4 h) increased miR-21 in MCF-7 cells [260]. Knockdown of miR-21 in ER-negative/basal- like MDA-MB-231 breast cancer cells decreased cell migration *in vitro* and the formation of tumors in the lungs of female nude mice after tail vein injection of the si-miR-21 transfected MDA-MB-231 cells [187]. miR-21 was downregulated in MCF-7 cells with 48 h of treatment with 10 μg/ml Polyphenon-60 (green tea extract) [384]. miR-21 expression is induced by all-trans retinoic acid in ERα+ breast cancer cells [385].
miR-22	MiR-22 was increased in rat mammary gland by 6 and 12 wks. of E_2 treatment of female August Copenhagen Irish (ACI) rats [157]. MiR-22 was differentially expressed between patients with a short time to distant metastasis (TDM) (*i.e.*, tumor aggressiveness) versus those with a long TDM [386]. miR-22 represses ERα expression by directly targeting the ERα mRNA 3' UTR [231]. miR-22 is a tumor suppressor that represses the c-Myc-binding protein MYCBP, a positive regulator of c-Myc [387]. miR-22 repressed the c-Myc-binding protein MYCBP, a positive regulator of c-Myc, which resulted in inhibition of growth of MCF-7 breast cancer cells [387]. miR-22 is a tumor suppressor [387].
miR-29a	The miR-29 family is a tumor suppressor miRNA [388]. miR-29a expression was down-regulated in invasive lung and pancreatic cell lines and re-expression of miR-29a reduced the *in vitro* invasive ability of lung and pancreatic cancer cell lines [389].

	miR-29a was downregulated in prostate tumors [390]. miR-29 regulates CDK6 as well as oncogenes Tcl-141 and Mcl-1; cell growth and survival genes, YY1, p85, CDC42 and DNMT3; as well as natural killer and T-cell inhibitor B7-H3 [388]. miR-29a targets the 3'UTR of B7-H3, a surface immunomodulatory glycoprotein that inhibits natural killer cells and T cells, thus offering a mechanism by which loss of miR-29a plays a role in immune escape by solid tumors [391]. miR-29 is induced by Wnt signaling and in turn, the negative regulators of Wnt signaling, Dikkopf-1 (Dkk1), Kremen2, and secreted frizzled related protein 2 (sFRP2), are direct targets of miR-29a in osteoblasts [392]. miR-29a was downregulated in human prostate cancer tumors ~ 42% [390].
miR-93	miR-93 is in the tri-cistronic miR-106b cluster that expresses miR-106b, miR-93 and miR-25. microRNAs mir-17, mir-106a, mir-106b, mir-93, mir-20, and mir-18 are ancient paralogs [393]. expression of miR-93 was up-regulated in hypoxic trophoblasts [394]. miR-93 was among the miRNAs determined to be the most stable miRNA normalizers in normal human tissues [286]. miR-93 was upregulated in HTLV-1-transformed human T-cell lines and primary peripheral blood mononuclear cells from adult T-cell leukemia patients [395]. miR-93 inhibits p21 expression, thus reducing a 'brake' on cell cycle progression [396]. . miR-93 is upregulated in squamous cell carcinoma (SCC), a type of nonsmall cell lung carcinoma (NSCLC) [397]. miR-93 is upregulated in human hepatocellular carcinoma (HCC) compared to normal hepatic tissues [398].
miR-125b	miR-125b is a tumor suppressor miRNA in breast cancer [262]. miR-125b is consistently down-regulated in human breast cancer cell lines [257]. miR-125b was upregulated in Taxol-resistant breast cancer cell lines, 435TRa NA 435TRP compared to their parental MDA-MB-435 cell line [399]. Significantly higher in ErbB2- vs. ErbB2+ tumors [304]. Significantly higher in ERα+ than ERα- tumors [304]. Significantly higher in PR+ than PR- tumors [304]. miR-125b inhibits ERBB2 and ERBB3 translation [201] miR-125b expression was increased by hypoxia in MCF-7 cells [379]. miR-125b was downregulated in epithelial ovarian cancer [400] and in squamous cell carcinoma (SCC) of the tongue [401]. E_2 down-regulated miR-125b in mouse splenic lymphocytes [402]. miR-125b was identified as a suppressor of the pathway activator Smoothened in cerebellar neuronal progenitor and tumor cells [403]. miR-125b is reduced in human hepatocellular carcinoma (HCC) [398] and high miR-125b was correlated with good survival of HCC patients [404]. miR-125b was downregulated in prostate tumors [390]. miR-125b is located at chromosome 11q23-24, one of the regions most frequently deleted in breast, ovarian, and lung tumors [405]. miR-125b repressed C-Raf protein expression in MDA-MB-453 breast cancer cells. miR-125b is upregulated in endometriosis [406]. E_2 did not affect miR-125b expression in macrophages [407].

	miR-125b inhibits MUC1 protein expression in MCF-7 breast cancer cells [408]. miR-125b confers paclitaxel resistance in breast cancer cells by suppressing Bak1expression [399].
miR-181a	miR-181a expression was reduced by E_2 in MCF-7 cells [261]. miR-181a and miR-181b are down-regulated in human gliomas and act as tumor suppressors which triggered growth inhibition, induced apoptosis and inhibited invasion in glioma cells [409]. Regulates oncogene TCL1 in Chronic Lymphocytic Leukemia (CLL) [410] miR-181b was over-expressed in tumors compared to normal colorectal samples [411,412]. miR-181b was downregulated in during retinoic acid-induced differentiation of human acute promyelocytic leukemia [413]. miR181b-1, miR-181c were down-regulated in prostate cancer [414]. miR-181a was upregulated in 4-OHT-resistant MCF-7 cells [270]. Predicted targets [381]: hsa-miR181a 9q33.3 *ESR2, ABI1, ABI3BP, ADAM11, BMP3, BMPER, BMPR2, EGR1, EGR3, FGFR3, MMP14, MPP5, NCOA2, NEGR1, PAK4, PAK7, RUNX1, S100PBP, SMAD7, SOX5, SOX6, STC1, TGFBI, TGFBR1, TIMP3, TSC22D2 [381].* miR-181d was up-regulated greater than twofold in breast cancer compared with normal adjacent tissue [415]. miR-181 inhibits the translation of p27kip1 (p27), a cell cycle inhibitor and tumor suppressor, in myeloid cell differentiation [416]. Antisense-microRNA oligonucleotides (AMOs) against miR-181a inhibited the growth of A549 human lung adenocarcinoma cells [417]. Overexpression of miR-181a in A549 lung cancer cells sensitized the cells to the lethal action of cisplatin by stimulating Bax oligomerization and the activation of proapoptotic caspase [418]. miR-181a was reduced in human NSCLC tissues [419]. miR-181a inhibits the expression of a large number of zinc finger genes (ZNFs) by interacting with seed elements within the coding regions [420].
miR-200 family members miR-200a miR-200b miR-200c	miR-200a correlated with ERα status in human breast tumors [304]. miR-200a expression significantly up-regulated in all types of breast tumors compared to adjacent normal tissue [378]. Significantly > in ERα+ than ERα- human breast tumors (1) and PR+ than PR- breast tumors(1), miR-200a is expressed in MCF-7 and other epithelial breast cancer cell lines [203]. miR-200a was increased in colorectal cancer cell lines [421] and in epithelial ovarian cancer [400]. miR-200 expression was reduced in tamoxifen-resistant MCF-7 cells [270]. All five members of the microRNA-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) and miR-205 were markedly downregulated in cells that had undergone EMT in response to transforming growth factor (TGF)-beta [203] The miR-200 family inhibits expression of the related transcriptional repressors ZEB1/deltaEF1 and SIP1/ZEB2 in epithelial cells and play a major role in preventing these factors from triggering epithelial to mesenchymal transition (EMT) [203]. However, miR-200 family members differentially regulate EGF-driven invasion, viability, apoptosis and cell cycle progression of breast cancer cells, with the miR-200bc/429 cluster showing stronger effects than the miR-200a/141 cluster [422].

	miR-200a expression is increased in ovarian tumors compared to normal ovary [423]. miR-200a expression was reduced in endometriosis [406]. miR-200a is upregulated in squamous cell carcinoma (SCC), a type of nonsmall cell lung carcinoma (NSCLC) [397]. E_2 (4 h) increased miR-200a 2-fold in MCF-7 cells [260]. E_2 (48 h) reduced miR-200c expression in MCF-7 cells [261]. miR-200a regulated ZEB1 expression, thus regulating epithelial to mesenchymal transition (EMT) [424]. Likewise, there is a double negative feedback loop of miR-200ab and ZEB1, which regulates E-cadherin expression, in EMT [325]. miR-200c was highly expressed in MCF-7 cells with lower and more variable expression in MDA-MB-231 cells [425]. TCF8, also termed ZEB1, which is a key regulator of epithelial to mesenchymal transition (EMT) by repressing E-cadherin expression, was identified as the target of miR-200c in A549 lung cancer cells [324]. Recent studies have implicated a negative feedback loop of miR-200c and ZEB1 in cancer cells including breast [205,322,323,325]. miR-200b and miR-200c are reduced in lymph node metastases of primary breast tumors [209].
miR-205	Early studies reported that miR-205 expression was higher in ERα/PR-positive breast tumors and reduced in ErbB2-positive breast tumors [304]. Expression of miR-205 was restricted to the myoepithelial/basal cell compartment of normal mammary ducts and lobules and miR-205 expression was reduced or completely eliminated in matching tumor specimens [221]. miR-205 expression is reduced in breast cancer cells that had undergone EMT in response to transforming growth factor (TGF)-beta or to ectopic expression of the protein tyrosine phosphatase Pez [203]. miR-205 was downregulated in breast tumors and miR-205 directly targets HER3 and inhibits the activation of the downstream mediator Akt [426]. The reintroduction of miR-205 into SKBr3 breast cancer cells inhibited colony formation and increased inhibition by tyrosine-kinase inhibitors Gefitinib and Lapatinib, abrogating the HER3-mediated resistance and restoring a potent proapoptotic activity [426] MCF-7 and MDA-MB-231 express lower miR-205 than non-malignant MCF-10A cells [296]. Ectopic expression of miR-205 in MCF-7 cells significantly inhibited cell proliferation and overexpression of miR-205 inhibited MDA-MB-231 cell invasion and metastasis to lung when injected into nude mice [296]. ErbB3 and vascular endothelial growth factor A (VEGF-A) are direct targets for miR-205 [296].
miR-221	Upregulated in 4-OHT-resistant [270] and Fulvestrant-resistant [272] MCF-7 cells. Variably expressed in human breast tumors: highly expressed in some and completely absent in other specimens [221]. miR-221 is part of a gene cluster also expressing miR-222, a close homologue of miR-221 [427]. Both miRNAs share an identical seed sequence [427]. Reduction of the p27Kip1 tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation [427]. MiR-221 and miR-222 both directly target the 3' untranslated regions of p27 and p57 mRNAs [428]. MiR-221 and miR-222 are elevated in ERα-negative breast cancer cells and

	both directly target the 3' untranslated region of ESR1 (ERα) and reduced ERα expression [295]. ERα inhibits miR-221 expression [234]. miR-221 was upregulated in Taxol-resistant breast cancer cell lines, 435TRa NA 435TRP compared to their parental MDA-MB-435 cell line [399]. miR-221 is down-regulated in prostate cancer [414] and regulates tumor suppressor ARHI expression [429]. miR-221 inhibits expression of p53 upregulated modulator of apoptosis (PUMA) in breast and lung cancer cells [430].
miR-222	Upregulated in 4-OHT-resistant [270] and Fulvestrant-resistant [272] MCF-7 cells. Variably expressed in human breast tumors: highly expressed in some and completely absent in other specimens [221]. miR-222 was upregulated in Taxol-resistant breast cancer cell lines, 435TRa NA 435TRP compared to their parental MDA-MB-435 cell line [399]. Overexpression of miR-222 in hepatocellular cancer activates AKT signaling [431]. Overexpression of miR-221 and 222 increased proliferation in ERα+ MCF-7 breast cancer cells and reduced ERα protein levels [234]. ERα inhibits miR-222 expression [234]. miR-222 inhibits expression of p53 upregulated modulator of apoptosis (PUMA) in breast and lung cancer cells [430]. Overexpression of miR-222 activates AKT signaling in hepatocellular carcinoma by suppressing the protein phosphatase 2A subunit B (PPP2R2A) [431].

APPENDIX 2:
mRNA gene targets of the indicated miRNAs differentially expressed in MCF-7 versus LY2 cells that were identified in Frasor *et al.* Cancer Research 2004

					Frasor et al Cancer Res. 2004	
		microarray	**microarray**	**in MCF-7**	8 h 4-OHT*	48 h 4OHT*
mRNA target	**miRNA**	**MCF-7 + 4-OHT**	**LY2 + 4-OHT**	**expect mRNA**	4 h 4-OHT	24 h 4-OHT
SMPD1	hsa-miR-10a	-2	3	higher than LY2	0.62*	0.49*
EFNB2	hsa-miR-10a				1.2	2.7
GPR56	hsa-miR-10a				1.4	2.3
SDC1	hsa-miR-10a				1.2	2
LTBP1	hsa-miR-10a				0.7	2.8
B3GNT1	hsa-miR-125b	-2	1	higher than LY2	1.4	2
ERBB3	hsa-miR-125b				0.61*	0.53*
C14orf43	hsa-miR-125b				2	1.5
GGA2	hsa-miR-125b				0.9	2.5
NAT9	hsa-miR-125b				0.9	0.5
SDC1	hsa-miR-125b				1.2	2
BAK1	hsa-miR-125b				0.49*	0.27*
IL13RA1	hsa-miR-125b				1.1	2.6
ZNF185	hsa-miR-125b				1	2.3
PDCD4	hsa-miR-200a	0.3	-3	lower in MCF-7	0.7	0.5
ETNK1	hsa-miR-200a			than in LY2	0.9	2
RBL2	hsa-miR-200a				0.54*	0.91*
BHLHB2	hsa-miR-200a				1.6	3
PTPRG	hsa-miR-200a				1	4.3
CDC2L6	hsa-miR-200a				0.7	2.5
IL13RA1	hsa-miR-200a				1.1	2.6
ZNF185	hsa-miR-200a				1	2.3
PDCD4	hsa-miR-200b	-0.1	-3	lower in MCF-7	0.7	0.5
B3GNT1	hsa-miR-200b			than in LY2	1.4	2
RDX	hsa-miR-200b				1	3
EFNB2	hsa-miR-200b				1.2	2.7

C14orf43	hsa-miR-200b				2	1.5
ABAT	hsa-miR-200b				0.6	0.3
CYP1B1	hsa-miR-200b				24.01*	4.24*
GBE1	hsa-miR-200b				0.8	2.5
PKIA	hsa-miR-200b				0.9	3.7
PDCD4	hsa-miR-200c	0.3	-3	lower in MCF-7	0.7	0.5
B3GNT1	hsa-miR-200c			than in LY2	1.4	2
RDX	hsa-miR-200c				1	3
ETNK1	hsa-miR-200c				0.9	2
EFNB2	hsa-miR-200c				1.2	2.7
ABAT	hsa-miR-200c				0.6	0.3
CYP1B1	hsa-miR-200c				24.01*	4.24*
GBE1	hsa-miR-200c				0.8	2.5
PDCD4	hsa-miR-21	-0.5	-0.9	lower in MCF-7	0.7	0.5
B3GNT1	hsa-miR-21			than in LY2	1.4	2
RDX	hsa-miR-21				1	3
ETNK1	hsa-miR-21				0.9	2
ABAT	hsa-miR-21				0.6	0.3
LTBP1	hsa-miR-21				0.7	2.8
PTPRG	hsa-miR-21				1	4.3
SMPD1	hsa-miR-22	-0.4	1	higher in MCF-7	0.62*	0.49*
GALNT6	hsa-miR-22			than in LY2	0.7	0.4
ERBB3	hsa-miR-22				0.61*	0.53*
TTC9	hsa-miR-22				0.47*	0.31*
RBL2	hsa-miR-22				0.54*	0.91*
YWHAZ	hsa-miR-22				1.5	2
IL13RA1	hsa-miR-22				1.1	2.6
ERBB3	hsa-miR-222	-1.5	1.4	higher in MCF-7	0.61*	0.53*
EFNB2	hsa-miR-222			than in LY2	1.2	2.7

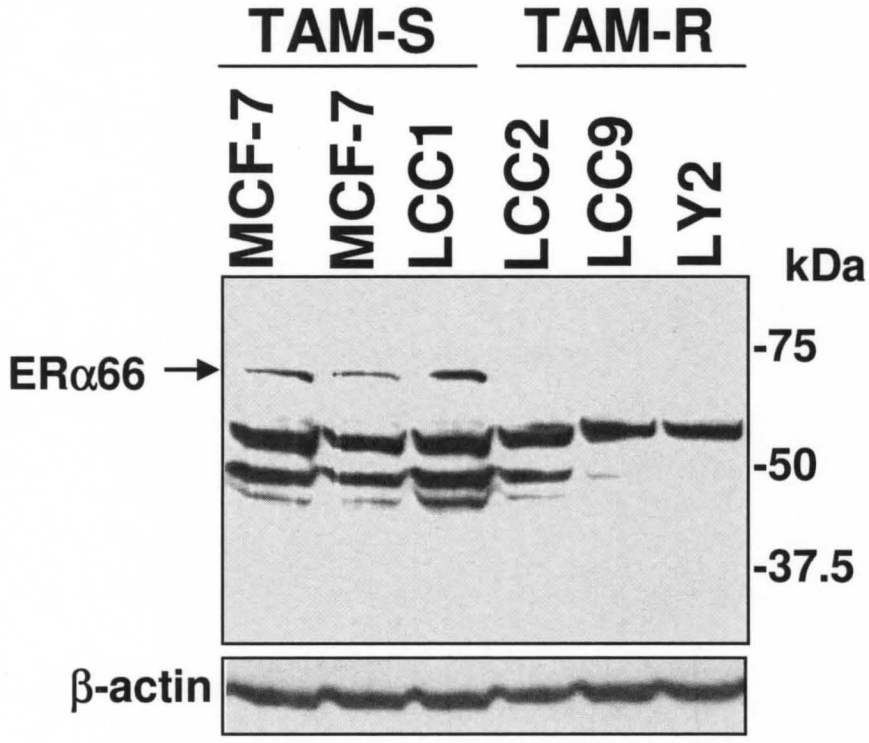
CYP1B1	hsa-miR-222				24.01*	4.24*
MYLIP	hsa-miR-222				0.7	0.4
CDC2L6	hsa-miR-222				0.7	2.5
PRPS1	hsa-miR-222				2.2	3
SMPD1	hsa-miR-29a	-0.8	0.7	higher in MCF-7	0.62*	0.49*
RAB30	hsa-miR-29a			than in LY2	1.3	2.2
C14orf43	hsa-miR-29a				2	1.5
CD44	hsa-miR-93	0.6	-0.7	lower in MCF-7	1	2
SHANK2	hsa-miR-93			than in LY2	0.8	2.5
RAB30	hsa-miR-93				1.3	2.2
RDX	hsa-miR-93				1	3
ERBB3	hsa-miR-93				0.61*	0.53*
EFNB2	hsa-miR-93				1.2	2.7
TTC9	hsa-miR-93				0.47*	0.31*
C14orf43	hsa-miR-93				2	1.5
RBL2	hsa-miR-93				0.54*	0.91*
LASP1	hsa-miR-93				1	2.5
MSH2	hsa-miR-93				0.8	2.1
IER3	hsa-miR-93				5	4.3
CDC2L6	hsa-miR-93				0.7	2.5
PKIA	hsa-miR-93				0.9	3.7
YWHAZ	hsa-miR-93				1.5	2
DNAJC12	has-miR-181a	1.6	0.01	lower in MCF-7	0.5	0.3
				than in LY2		

APPENDIX 3: Identity and location of genes shown in networks 1 and 2 (Appendix 7)

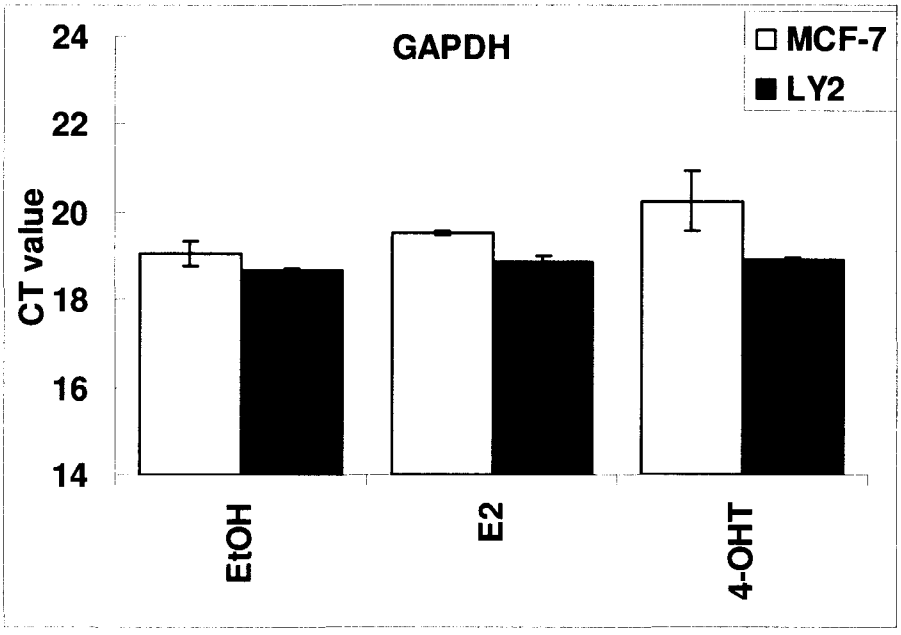
Symbol	Entrez Gene Name	Location
Network1		
APC	adenomatous polyposis coli	Nucleus
ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	Cytoplasm
BLZF1	basic leucine zipper nuclear factor 1	Cytoplasm
BPHL	biphenyl hydrolase-like (serine hydrolase)	Cytoplasm
CASP3	caspase 3, apoptosis-related cysteine peptidase	Cytoplasm
CCDC88B	coiled-coil domain containing 88B	Nucleus
CSF1	colony stimulating factor 1 (macrophage)	Extracellular Space
CYR61	cysteine-rich, angiogenic inducer, 61	Extracellular Space
DGAT1	diacylglycerol O-acyltransferase 1	Cytoplasm
DNAJB5	DnaJ (Hsp40) homolog, subfamily B, member 5	unknown
FBLN5	fibulin 5	Extracellular Space
FMNL3	formin-like 3	unknown
HOXA1	homeobox A1	Nucleus
LMNA	lamin A/C	Nucleus
MAP4	microtubule-associated protein 4	Cytoplasm
MIR10A (includes EG:406902)	--	unknown
MIR125B (human)	--	unknown
MIR200A (includes EG:406983)	--	unknown
MIR200B (includes EG:406984)	--	unknown
MIR205 (includes EG:406988)	--	unknown
MIR22 (includes EG:407004)	--	unknown
MIR222 (includes EG:407007)	--	unknown
MIR29A (includes EG:407021)	--	unknown
MIR93 (includes EG:407050)	--	unknown
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	Nucleus
MYT1L	myelin transcription factor 1-like	Nucleus
NPTX1	neuronal pentraxin I	Extracellular Space
PARP6	poly (ADP-ribose) polymerase family, member 6	unknown
PLK2	polo-like kinase 2	Nucleus
RAB10	RAB10, member RAS oncogene family	Cytoplasm
retinoic acid	--	unknown
RSF1	remodeling and spacing factor 1	Nucleus
TDG	--	Nucleus
UNCX	UNC homeobox	unknown
VIM	vimentin	Cytoplasm

Network 2		
beta-estradiol		unknown
Estrogen Receptor		unknown
IL6	interleukin 6 (interferon, beta 2)	Extracellular Space
MAP2	microtubule-associated protein 2	Cytoplasm
Mapk		unknown
MIR181A (human)		unknown
MIR200C (includes EG:406985)		unknown
MIR21 (includes EG:406991)		unknown
PGR	progesterone receptor	Nucleus

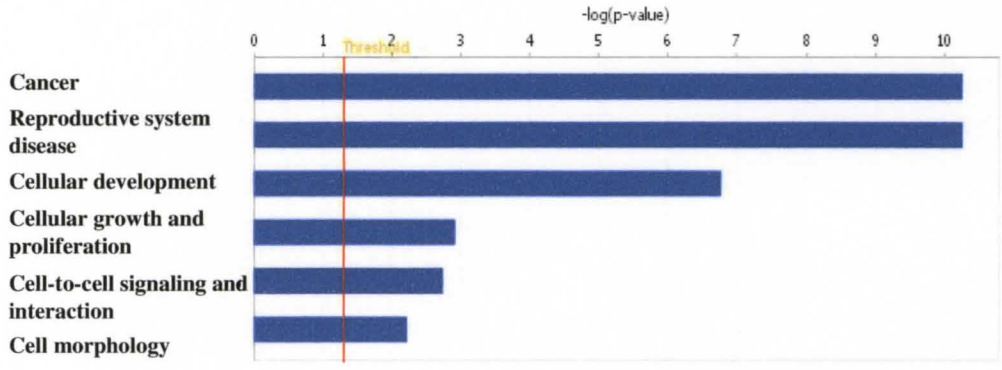
APPENDIX 4: ERα36 is not expressed in MCF-7 or LY2 cell lines. Whole cell extracts (30 μg) indicated breast cancer cell lines (tamoxifen (TAM)- sensitive (S) or TAM-resistant (TAM-R) were immunoblotted for ERα with G-20 (SantaCruz Biotechnology). The membrane was stripped and reprobed for β-actin. ERα36 lacks the A/B and F domains of ERα66, but contains the DNA binding domain (DBD, C region), hinge region (D region), and most of the ligand binding domain (LBD, E region). It also has an extra, unique 27aa domain instead of the last 138 aa encoded by exons 7 and 8 of the hERα66 gene (Wang *et al.* PNAS 103: 9063-8, 2006) . The G-20 antibody recognizes ERα aa 281-360 in the DBD and hinge region and thus, it should recognize ERα36, but we do not see any evidence of ERα36.



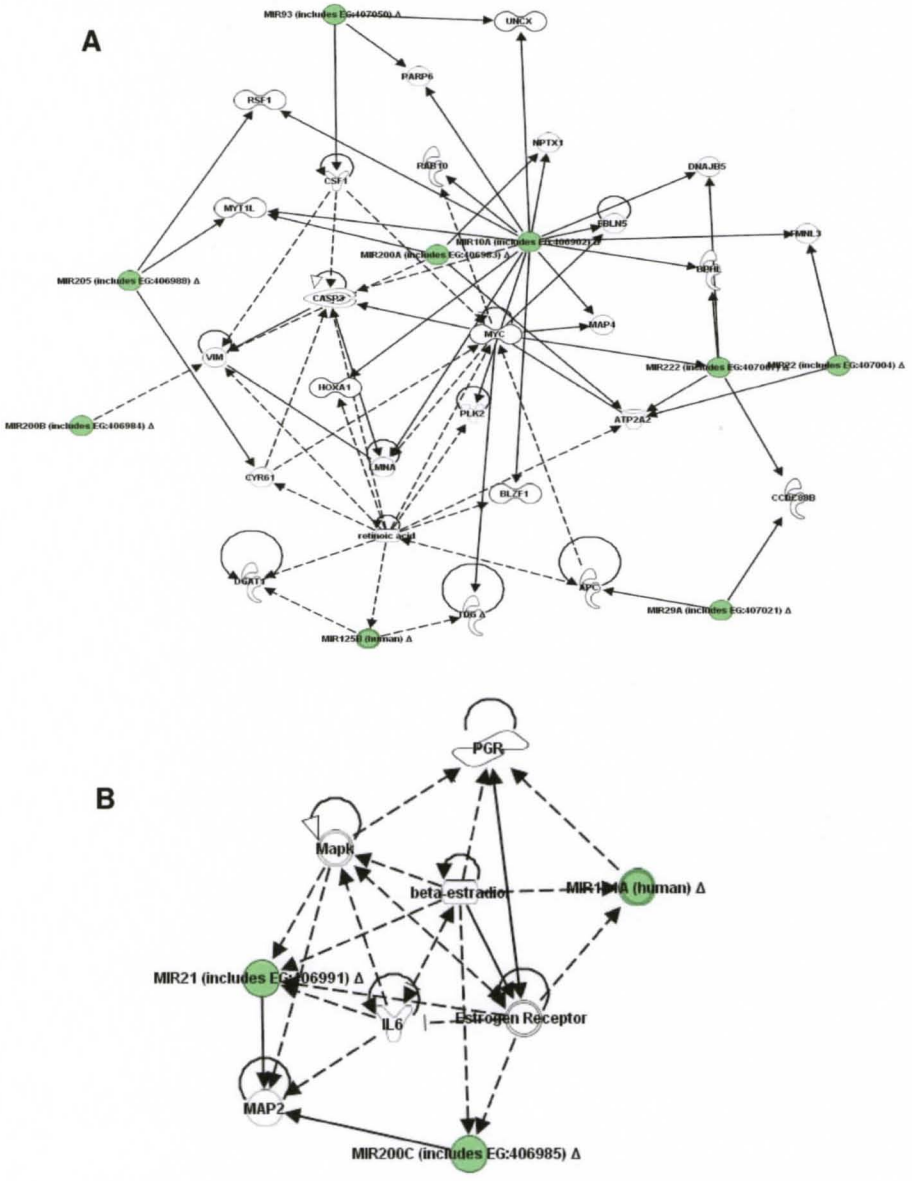
APPENDIX 5: GAPDH levels in MCF-7 and LY2 are comparable. MCF-7 and LY2 cells were treated with 10 nM estradiol (E_2) or 100 nM 4-hydroxytamoxifen (4-OHT) for 6 h. Quantitative real time PCR was employed to evaluate glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression because GAPDH is often used as a control gene for normalization of "test" mRNAs. Values are CT (cycle threshold) values and are the mean +/- SEM of triplicate determinations.



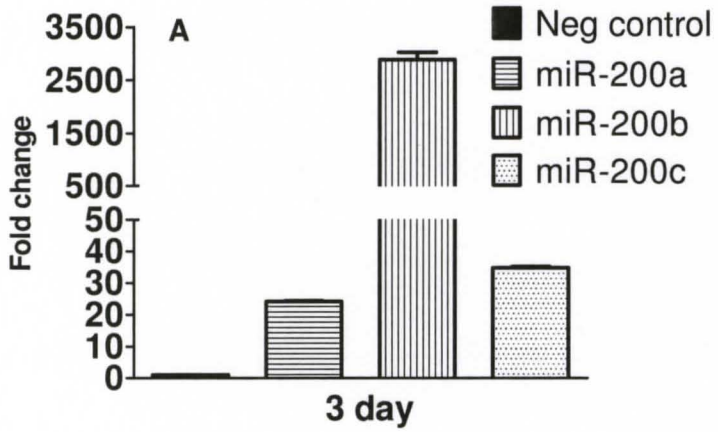
APPENDIX 6: Results of Ingenuity Pathway Analysis. The top biological function and disease categories enriched with miRNAs in our dataset (Figure 3) are displayed along the x-axis. The y-axis displays the -(log) significance. Taller bars are more significant than shorter bars. Functions are listed from most significant to least and the red vertical line denotes the cutoff for significance (p-value of 0.05).



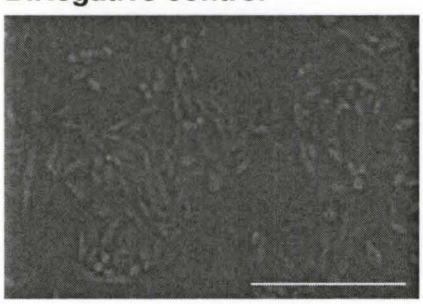
APPENDIX 7: Ingenuity Pathway Analysis (IPA) identified 2 networks of molecules that interact with the 12 miRNAs that are differentially expressed in MCF-7 versus LY2 breast cancer cells. Core analysis identified 2 separate networks that are associated with our miRNA dataset (Figure 3). Analysis identified a total of 44 molecules, 35 in Network 1 (A) and 9 in Network 2 (B).



Appendix 8: Overexpression of miR-200b or miR-200c in LY2 cells transfected with pre-miR-200b or pre-miR-200c or negative control. LY2 cells were transfected either with negative control or pre-miR-200a, pre-miR-200b or pre-miR-200c. A. RNA was harvested after 3 days and Q-PCR performed to confirm overexpression of miR-200a, miR-200b or miR-200c. Values are the mean ± SEM of triplicate determinations. B. Image of LY2 cells transfected with negative control, captured using a light microscope (20x magnification, 100 μm scale).



B.Negative control



Appendix 9

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EDUCATION

2012	Ph.D. in Biochemistry and Molecular Biology (dissertation defended 20.September 2012 with degree to be conferred at December 2012 graduation) University of Louisville School of Medicine, Louisville, KY
2011	M.S. in Biochemistry and Molecular Biology, University of Louisville School of Medicine, Louisville, KY
2007	M.Sc. in Biochemistry, Bangalore University, Karnataka, India
2005	B.Sc. in Microbiology Chemistry, Botany, Bangalore University, Karnataka, India

PUBLICATIONS

1. **Teng, Y*., Manavalan, T.T.***, Hu, C., Medjakovic, S., Jungbauer, A., and Klinge, C.M.. Endocrine Disruptors Fludioxonil and Fenhexamid Stimulate miR-21 Expression in Breast Cancer Cells. * equal contribution as first authors. Toxicological Sciences, *in press*, 2012, # TOXSCI-12-0551-R1.

2. **Manavalan, T.T.**, Teng, Y., Appana, S.N., Datta, S., Kalbfleisch, T.S., Li, Y., and Klinge, C.M. Differential expression of microRNA expression in tamoxifen-sensitive MCF-7 versus tamoxifen-resistant LY2 human breast cancer cells. Cancer Letters 313: 26-43, 2011. PMID:21955614

3. Wickramasinghe, N.S., **Manavalan, T.T.**, Dougherty, S.M., Riggs, K.A., Li, Y., and Klinge, C.M. Estradiol downregulates miR-21 expression and increases miR-21 target gene expression in MCF-7 breast cancer cells. Nucleic Acids Res. 37: 2584-2595, 2009. PMID:19264808

BOOK CHAPTERS

1. **Manavalan, T.T and Klinge, C.M**, Estrogen regulation of microRNA expression. Book chapter. Advances of Genomic science. *in press*. (Editor - Christian Neri)

HONORS AND AWARDS

2011 Second place in the Graduate Student poster competition (2nd of 127 posters) at the 10th Annual Brown Cancer Retreat, October 28, 2011. (**Manavalan, T.T.**, Teng, Y., Datta, S., Kalbfleisch, T.S., Li, Y. and Klinge, C.M. Differential expression of miRNAs in antiestrogen-sensitive MCF-7 versus antiestrogen-resistant LY2 human breast cancer cells. Abstract #84)

2011 Third place in the poster competition at the 13th annual Institute for Molecular Diversity and Drug Design (IMD^3) Symposium March 8, 2011. (**Manavalan, T.T.,** Teng, Y., Datta, S., Kalbfleisch, T.S., Li, Y. and Klinge, C.M. Differential expression of miRNAs in antiestrogen-sensitive MCF-7 versus antiestrogen-resistant LY2 breast cancer cells. Abstract #16)

2010 Second place in the poster competition at the 12th annual Institute for Molecular Diversity and Drug Design (IMD^3) Symposium March 9, 2010 (2nd of 32 posters). (**Manavalan, T.T.,** Wickramasinghe, N.S., Datta, S., Kalbfleisch, T., Li, Y.and Klinge, C.M. Differential expression of microRNAs in tamoxifen-sensitive versus - resistant human breast cancer cell lines. Abstract #18)

2009 Third place in the Graduate Student poster competition (3rd of 82 posters) 7th annual Brown Cancer Center Retreat, October 29, 2008. (**Manavalan, T.T.**, Wickramasinghe, N.S., Li, Y., and Klinge, C.M. Regulation of miRNA expression by 4-hydroxytamoxifen in MCF-7 breast cancer cells. Abstract #49)

2007- 2009 Integrated Program in Biomedical Sciences (IPIBS) Fellowship, University of Louisville School of Medicine, Louisville KY 40202

RESEARCH EXPERIENCE

Dissertation research: University of Louisville School of Medicine, 2007-present (Advisor: Dr. Carolyn M. Klinge)

- Identified miRNAs that are differentially expressed in antiestrogen-sensitive MCF-7 and anti-estrogen-resistant LY2 human breast cancer cells. Using bioinformatics, putative targets of the dysregulated miRNAs were identified.
- Correlated changes in miRNA expression with mRNA and protein changes of key tumor suppressors and oncogenes associated with breast cancer.
- Investigated the role of microRNA-200 family members in modulating resistance to antiestrogens, Tamoxifen and Fulvestrant.

TECHNICAL SKILLS

Molecular biology techniques: mammalian cell culture, miRNA analyses, QRT-PCR for mRNA and miRNA, Western blotting, transient transfection, immunocytochemistry

EDUCATIONAL ACTIVITIES

- Attended Trainee day at the 93rd annual meeting of the Endocrine Society, June 4, 2011
- Teaching assistant for Advanced Biochemistry II (BIOCHEM 647), Spring 2009
- Trained 2 undergraduate students (summer of 2009 and 2011)

POSTERS

Abstracts presented at National meetings:

1. **Manavalan, T.T** and Klinge, C.M. Loss of miR-200 family of microRNAs confers resistance to tamoxifen in human breast cancer cells. (Abstract #1096), Abstracts of the 103rd annual meeting presented at the American Association for Cancer Research (AACR),Chicago, IL March 31-April 4, 2012

2. **Manavalan, T.T,** Klinge, C.M., Datta, S., and Kalbfleisch, T.S. Differential Expression of MiRNAs In Antiestrogen-Sensitive MCF-7 versus Antiestrogen-Resistant LY2 Breast Cancer Cells. (Abstract P3-68) Abstracts of the 93rd annual meeting of the Endocrine Society, Boston, MA, June 4-7, 2011.

Abstracts presented at local meetings:

1. Barry, P., Riggs, K.A., **Manavalan, T.T.**, Patel, N.S., and Klinge, C.M. Role of estrogen receptor alpha isoform hERα46 in tamoxifen resistant breast cancer. (Abstract SMED 2) Research Louisville! University of Louisville School of Medicine, Louisville, KY, October 21, 2008.

2. **Manavalan, T.T.**, Wickramasinghe, N.S., Li, Y., and Klinge, C.M. Regulation of miRNA expression by 4-hydroxytamoxifen in MCF-7 breast cancer cells. (Abstract GRD-30) Research Louisville! University of Louisville School of Medicine, Louisville, KY, October 21, 2008.

3. Wickramasinghe, N.S., **Manavalan, T.T.**, Riggs, K.A., Li, Y., and Klinge, C.M. Estradiol regulates miR-21 and its targets via ERalpha in MCF-7 breast cancer cells. (Abstract RA-53) Research Louisville! University of Louisville School of Medicine, Louisville, KY, October 22, 2008. *This poster was awarded third place in the Postdoctoral Fellow/Research Associate poster competition (3rd of 70 posters).

4. **Manavalan, T.T.**, Wickramasinghe, N.S., Li, Y., and Klinge, C.M. Regulation of miRNA expression by 4-hydroxytamoxifen in MCF-7 breast cancer cells. (Abstract# 49) 7th annual Brown Cancer Center Retreat, October 29, 2008.

5. Wickramasinghe, N.S., **Manavalan, T.T.,** Riggs, K.A., Li, Y., and Klinge, C.M. Estradiol downregulates miR-21 expression and miR-21 gene targets in MCF-7 breast cancer cells. Abstract 3051 presented at the 31st annual San Antonio Breast Cancer Symposium, Dec. 10-14, 2008.

6. Wickramasinghe, N.S., **Manavalan T.T.**, Dougherty, S. M., Yong Li, and

Klinge, C.M. Estradiol regulates miR-21 and its targets via ER-alpha in MCF-7 breast cancer cells. (Abstract # 10) 11th annual Institute for Molecular Diversity and Drug Design (IMD3) Symposium, March 10, 2009. *This poster was awarded the first place in the poster competition.

7. Kapur, A., **Manavalan, T.T.**, Wickramasinghe, N.S., Klinge, C.M. Regulation of protein targets of microRNAs by 4-hydroxytamoxifen in breast cancer cells. SROP poster presentations, University of Louisville School of Medicine, Louisville, KY, August, 2009.

8. **Manavalan T.T.**, Wickramasinghe, N.S., Datta, S., Kalbfleisch, T., and Klinge, C.M. Differential expression of microRNAs in tamoxifen-resistant versus-sensitive human breast cancer cells. Abstract #13 presented as a poster at the 2009 Colloquium in Biochemistry and Molecular Biology (BMB), University of Louisville School of Medicine, August 21, 2009. (Honorable Mention (2nd of 17 posters selected for presentation by the BMB Research Committee).

9. **Manavalan, T.T.**, Wickramasinghe, N.S., Datta, S., Kalbfleisch, T., Li, Y. and Klinge, C.M. Differential expression of microRNAs in tamoxifen-sensitive versus - resistant human breast cancer cell lines. (Abstract GRD-54) Research Louisville! University of Louisville School of Medicine, Louisville, KY, October 15, 2009.

10. **Manavalan, T.T.**, Wickramasinghe, N.S., Datta, S., Kalbfleisch, T., Li, Y. and Klinge, C.M. Differential expression of microRNAs in tamoxifen-sensitive versus - resistant human breast cancer cell lines. (Abstract #54) 8th Annual Retreat, James Graham Brown Cancer Center, Louisville, KY, November 6, 2009.

11. **Manavalan, T.T.**, Wickramasinghe, N.S., Datta, S., Kalbfleisch, T., Li, Y. and Klinge, C.M. Differential expression of microRNAs in tamoxifen-sensitive versus - resistant human breast cancer cell lines. (Abstract #18) 12th annual Institute for Molecular Diversity and Drug Design (IMD3) Symposium, IMD3 Symposium, March 9, 2010.

12. **Manavalan, T.T.**, Teng, Y., Datta, S., Kalbfleisch, T.S., Li, Y. and Klinge, C.M. Differential expression of miRNAs in antiestrogen-sensitive MCF-7 versus antiestrogen-resistant LY2 breast cancer cells. (Abstract GRD-42) Research Louisville! University of Louisville School of Medicine, Louisville, KY, October 13, 2010.

13. **Manavalan, T.T.**, Teng, Y., Datta, S., Kalbfleisch, T.S., Li, Y. and Klinge, C.M. Differential expression of miRNAs in antiestrogen-sensitive MCF-7 versus antiestrogen-resistant LY2 breast cancer cells. (Abstract # 67) 9th annual Brown Cancer Center Retreat, Louisville, KY. November 5, 2010.

14. **Manavalan, T.T.**, Teng, Y., Datta, S., Kalbfleisch, T.S., Li, Y. and Klinge, C.M. Differential expression of miRNAs in antiestrogen-sensitive MCF-7 versus antiestrogen-resistant LY2 breast cancer cells. (Abstract #16) 13th

annual Institute for Molecular Diversity and Drug Design (IMD3) Symposium, March 8, 2011.

15. Bell, J.D., Teng, Y., **Manavalan, T.T.**, Kareparembil, S.A, Klinge, C.M. DHEA stimulates miR-21 expression in breast cancer cells. SROP poster presentation, University of Louisville School of Medicine, Louisville, KY, August 1, 2011.

16. **Manavalan, T.T.,** Teng, Y., Datta, S., Kalbfleisch, T.S., Li, Y. and Klinge, C.M. Differential expression of miRNAs in antiestrogen-sensitive MCF-7 versus antiestrogen-resistant LY2 breast cancer cells. Abstract #3 presented as a poster at the 2011 Colloquium in Biochemistry and Molecular Biology (BMB), University of Louisville, School of Medicine, August 26, 2011.

17. **Manavalan, T.T.**, Teng, Y., Datta, S., Kalbfleisch, T.S., Li, Y. and Klinge, C.M. Differential expression of miRNAs in antiestrogen-sensitive MCF-7 versus antiestrogen-resistant LY2 human breast cancer cells. (Abstract GRD-53) Research Louisville! University of Louisville School of Medicine, Louisville, KY, October 11, 2011.

18. **Manavalan, T.T.**, Teng, Y., Datta, S., Kalbfleisch, T.S., Li, Y. and Klinge, C.M. Differential expression of miRNAs in antiestrogen-sensitive MCF-7 versus antiestrogen-resistant LY2 human breast cancer cells. (Abstract #84) 10^{th} annual Brown Cancer Center Retreat, Louisville, KY. October 28, 2011.

19. **Manavalan, T.T** and Klinge, C.M. Loss of miR-200 family of microRNAs confers resistance to tamoxifen in human breast cancer cells. (Abstract #17), 14^{th} Annual IMD^3 (Institute for Molecular Diversity and Drug Design) Symposium, March 13th, 2012.