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PRINCIPAL INVESTIGATOR: Harikrishna Nakshatri, Ph.D.
Nikail R. Collins

CONTRACTING ORGANIZATION: Indiana University
Indianapolis, IN 46202-5012

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Harikrishna Nakshatri, Ph.D.
Nikail R. Collins

Email: hnakshat@iupui.edu

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14. ABSTRACT Estrogen receptor alpha (ERa) mediates transcriptional effects of estrogen. Estrogen inducible proteins c-Myc and E2F family are required for optimal ERa activity and secondary estrogen response, respectively. The purpose of this study was to investigate whether estrogen regulates it's target gene expression through microRNAs. We show that estrogen induces 21 and represses 7 microRNAs, which potentially control 420 estrogen-regulated and 757 non-estrogen regulated mRNAs at post-transcriptional level. Estrogen induced the expression of eight Let-7 family microRNAs, miR-98 and miR-21, which by reducing c-Myc and E2F2 proteins level, may attenuate estrogen response. Consistent with the role of Let-7 in differentiation of cancer stem cells, estrogen reduced ALDH1-positive breast cancer stem subpopulation of MCF-7 cells. The protein kinase AKT reduced estrogen-inducible expression of Let-7 microRNAs and may disrupt attenuation of estrogen response. Significance: Luminal subtype A breast cancers contain functional ERa, are well differentiated and display favorable prognosis. Estrogen:ERa-mediated differentiation pathway in these cancers is yet to be elucidated. We propose that estrogen-regulated Let-7 family microRNAs contribute to differentiated phenotype of ERa-positive breast cancers. The phenotype and the clinical course of ERa-positive breast cancers, particularly response to anti-estrogen therapy, may be dependent on the balance between estrogen-induced tumor suppressor (let-7 family) and oncogenic (mir-21) microRNAs. Our studies also reveal a negative regulatory loop controlling estrogen response through microRNAs and highlights differences in estrogen-induced transcriptome and proteome.

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estrogen, microRNA, gene expression

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Introduction:

Estrogen controls several biological processes by functioning as a ligand for nuclear receptors estrogen receptor alpha (ERα) and beta (ERβ) (1). ERs may participate in the genomic (transcriptional) and non-genomic actions of estrogen (1, 2). The genomic action involves binding of ERα to the regulatory regions of target genes either directly or through protein-protein interaction. DNA-bound ERα then recruits various co-regulatory molecules to induce chromatin modifications to either increase or decrease the levels of target gene transcripts. Several extracellular signal activated kinases phosphorylate and modulate ERα activity. The major kinases that modulate ERα activity include ERK1/2, AKT, RSK, PAK1, p38 kinase and SRC (2-4). These kinases may alter estrogen response and confer resistance to anti-estrogen therapy in breast cancer (2).

A significant number of studies so far have focused on estrogen:ERα-mediated transcriptome. The effect of estrogen on gene expression at post-transcriptional level, particularly by recently discovered microRNAs, is yet to be elucidated. MicroRNAs are a class of evolutionarily conserved non-coding RNAs that control gene expression at post-transcriptional level (5, 6). They regulate gene expression through direct cleavage of the target mRNAs or inhibiting translation through eIF6 upon binding to target sequences in the 3' untranslated region of transcripts (5, 7). However, control of gene expression at the level of translation by microRNA is cell cycle dependent (8). Each microRNA is predicted to target 90-300 mRNA species and together affect ~30% of the protein coding genes (9). Cellular stress and RNA binding proteins, which usually bind to sequences in between microRNA recognition sites on mRNAs, determine the target specificity of microRNAs (10, 11). The additional functions of microRNAs include transcriptional activation of genes with complementary promoter sequences (12) and chromatin modification (13, 14).

The majority of microRNAs are transcribed by the RNA pol II enzyme (a minority by pol III) to produce a primary-microRNA (15, 16). Approximately 50% of microRNAs are transcribed from introns of protein coding genes while the rest are intergenic with primary transcripts as long as 4 kb (16). The primary-microRNA form specific hairpin-shaped stem-loop structure, enter a microprocessor complex and then processed to 60-to-70-nt pre-miRNA. These pre-miRNAs are further processed to a short microRNA in the cytoplasm and incorporated into RNA-induced silencing complex (RISC) where they regulate translation or stability of their target mRNAs. The processing of the microRNAs is under additional control by RNA binding proteins such as LIN28 (17).

The miRNA genes usually appear in polycistronic clusters and more than 50% of miRNA genes are located in cancer associated genomic regions of fragile sites (5, 18). Thus, because of their enormous influence on expression of multiple genes, an alteration in the expression of a single microRNA can have profound effect on cellular physiology. Indeed, specific reduction or upregulation of microRNAs is observed in cancers. For example, *RAS* oncogene is frequently activated/mutated in cancers (19). Translation of RAS is controlled by Let-7 microRNA whose expression is often reduced in cancers (20, 21). In general, it is believed that Let-7/miR-98 family microRNAs prevent the re-expression of oncofetal genes in adult tissues and loss of their expression leads to re-expression of oncofetal genes in cancer (22, 23). In contrast, miR-17-92 cluster is overexpressed in lung cancer and these microRNAs induce tumor growth possibly by

targeting tumor suppressors PTEN and Rb2 (24). Glioblastomas show upregulation of miR-221 whereas testicular cancers overexpress miR-372 and miR-373 (24, 25).

Three recent reports describe microRNA expression pattern in breast cancer; two of these have evaluated the expression pattern in relation to ERα, ErbB2 and intrinsic subtypes (26-28). Overall, microRNA levels were lower in poorly differentiated tumors compared to well-differentiated tumors. MicroRNA profile rather than mRNA expression profile correlated more accurately with cell differentiation. Expression of Let-7 family microRNAs was lower in ErbB2-positive breast cancers compared to other cancer types. ErbB2-positive breast cancers overexpressed 43 microRNAs compared to ErbB2-negative breast cancers. Similarly, 43 microRNAs were expressed at higher level in ERα-positive breast cancers compared to ER-negative breast cancers (26). Blenkiron et al., described microRNA expression profile in relation to intrinsic subtypes of breast cancer (27). Breast cancers have been classified to five distinct subtypes (luminal subtypes A and B, Her-2+/ER-, basal-like, and normal-like subtypes) based on gene expression pattern with unique molecular characteristics and prognostic significance (29, 30). Luminal subtype A and B correspond to ERα-positive breast cancers with subtype A expressing higher levels of ERα and having a better prognosis than subtype B (30). ERα expression in breast cancer may be regulated by microRNAs as estrogen increases miR-206 expression, which in turn inhibits ERα translation by binding to 3' UTR of ERα mRNA (31). Luminal type A and basal type breast cancers displayed distinct pattern of microRNA expression (27). The ErbB2-positive tumors shared many of the microRNAs with basal tumors with the exception of miR-106a, miR-18a, miR-93, miR-155 and miR-135b, which were expressed at lower levels than in basal cancer types (27).

Despite enormous progress in understanding the expression pattern of microRNA in breast cancer, regulation of their expression in different breast cancer subtypes is not known. We focused on the effect of estrogen on microRNA expression in breast cancer cells with two goals. One was to determine whether any of the microRNAs expressed at higher levels in luminal type A/ ERα-positive breast cancer are regulated by estrogen. The second was to determine whether genes that are regulated by estrogen at transcription levels are subsequently controlled by estrogen-regulated microRNAs. We also examined whether extracellular signal activated kinases such as AKT modulate estrogen regulated microRNA expression. Results from this study revel a unique role for estrogen in regulating the expression of luminal type A-enriched microRNAs with tumor suppressor and oncogenic functions and the influence of AKT in altering the effects of estrogen.

Body:

Objectives:

1) Determine estrogen-regulated expression of microRNAs in MCF-7 cells using microRNA bioarrays and investigate their role in estrogen-mediated repression of genes and proliferation.

2) Investigate whether microRNAs induced by c-Myc are involved in estrogen-mediated repression of genes.

Progress on Objectives 1 and 2:

Estrogen regulates the expression of microRNAs

Our ongoing studies with parental MCF-7 cells with retrovirus vector alone (MCF-7pQXIP) and MCF-7 cells overexpressing constitutively active AKT (MCF-7/CA-AKT) revealed 1667 and 1908 genes, respectively, bound to ERα upon estrogen treatment (manuscript under revision). However, not all of the genes with ERα binding sites had an altered transcript level after estrogen treatment. Also, not all estrogen-regulated genes, as identified by microarray analysis of both cell types with or without estrogen treatment for four hours, contained ERα binding sites suggesting that there are other mechanisms by which estrogen regulates gene expression. In fact, among 833 estrogen-regulated genes in MCF-7pQXIP cells, only 299 genes contained ERα binding sites. Although secondary estrogen response through estrogen regulated transcription factors such as E2F1/2 can explain the lack of correlation between ERα binding and target gene expression (32), control of gene expression by estrogen-regulated microRNAs has not been investigated so far. To investigate this possibility, we determined microRNA expression pattern in MCF-7pQXIP and MCF-7CA-AKT cells with or without estrogen treatment for four hours as detailed below.

MicroRNA microarray consisted 248 human microRNAs. With a statistical cutoff p value <0.05 and fold change >1.5 or <0.7, we observed 21 estrogen-inducible and seven estrogen-repressible microRNAs in MCF-7pQXIP cells (Table 1). Estrogen increased the expression of eight members of Let-7 family (≥ 2.2 fold increase), which are abundantly expressed in luminal type A breast cancer (27). AKT completely changed the pattern of estrogen regulation of microRNA expression. In MCF-7CA-AKT cells, estrogen increased the expression of only one microRNA but reduced the expression of 20 microRNA species. Basal expression of 11 microRNAs was lower in MCF-7CA-AKT cells compared to MCF-7pQXIP cells whereas one microRNA displayed elevated expression in MCF-7CA-AKT cells. Seven of the microRNA that displayed lower basal expression in MCF-7CA-AKT cells were estrogen-inducible in MCF-7pQXIP cells. Only three microRNAs (miR-143, miR-506 and miR-98) showed similar estrogen-dependent regulation in both cell types.

We performed microRNA-qPCR that quantitatively measures only mature microRNAs to confirm estrogen-inducible expression of Let7f, miR-21 and miR-98 (Figure 1A). Consistent with results of microarray, estrogen increased the expression of these microRNAs in MCF-7pQXIP cells, which was substantially lower, yet estrogen-inducible, in MCF-7/CA-AKT cells. miR-98 was induced by 14-fold whereas Let-7f and miR-21 were induced by ~five-fold in MCF-7pQXIP cells by four hours and dropped to basal level by 24 hours. Estrogen inducible expression of Let-7f, miR-21 and miR-98 was also observed in two other ERα-positive cell lines T47-D and BT-474 (Figure 1B).

Kinetics of induction in T47-D, which is a luminal type A cell line, mirrored to that of MCF-7 cells. In contrast, estrogen-inducible expression of these microRNAs was observed at much earlier time point in BT-474 cells, which represents luminal type B (ERα+/HER2+.

Putative targets of estrogen-regulated microRNAs

Estrogen-inducible expression of Let-7 family and reduced basal and estrogen-inducible expression of this family members in MCF-7CA-AKT is interesting because reduced expression of this family is linked to loss of differentiation and increased self-renewal of progenitor cells (33, 34). Both Let-7 family and miR-98 have the same seed sequence and target same mRNAs. Ras family oncogenes and HMGA2 are the well-characterized targets of Let-7 (21, 35). By targeting Ras, Let-7 reduces self-renewal whereas by reducing HMGA2, it enhances differentiation without effecting rate of proliferation (36). c-Myc is also a target of Let-7 (37) and miR-21 (38). E2F1 and E2F2, two estrogen-inducible transcription factors involved in secondary estrogen response (32), contain non-conserved Let-7 interaction sites. NCOA3/AIB1 is a target of miR-17-5p (39), which in our studies is an estrogen-inducible microRNA (Table 1). EZH2 is a multifunctional protein that integrates Wnt and estrogen signaling in breast cancer cells and the abnormal function of this protein is linked to several diseases (40). EZH2 is subject to post-transcriptional regulation by miR-26a in myotubes (41). Based on TargetScan analysis, EZH2 is a likely target of mir-124a and mir-506, both of which are repressed by estrogen. By repressing the levels of these microRNAs, estrogen can cause an increase in EZH2 protein. The above genes thus constituted potential targets of estrogen-regulated microRNAs.

We confirmed estrogen-mediated repression of Ras protein in MCF-7pQXIP cells, which was not observed in MCF-7CA-AKT cells (Figure 2A). We observed repression of AIB1 and induction of EZH2 by estrogen in both cell types. Additionally, while E2F1 and E2F2 displayed delayed E2-inducible increase in protein levels in MCF-7pQXIP cells, basal levels of both of these proteins were elevated with further estrogen inducible increase in MCF-7CA-AKT cells. Previously, we reported little difference in the kinetics of estrogen-induced increase in c-Myc mRNA levels in parental and CA-AKT overexpressing MCF-7 cells (3). However, estrogen-induced increase in c-Myc proteins is advanced in MCF-7CA-AKT cells compared to MCF-7pQXIP cells (Figure 2A). None of the previous microarray profiling studies including our study observed an effect of estrogen on EZH2 and Ras mRNA levels and our ChIP-chip data did not identify ERα binding sites in these genes. We believe that estrogen changes levels of Ras, AIB1 and EZH2 through microRNA-mediated post-transcriptional mechanisms.

The effect of Let-7f/miR-98 and miR-21 knockdown on estrogen-induced levels of c-Myc, E2F1 and E2F2 proteins

We used locked nucleic acid (LNA) mediated knockdown of Let-7f/miR98 to determine whether reducing the levels of these microRNA leads to changes in the estrogen-inducible expression of c-Myc, E2F1, and E2F2 at protein level. Neither LNA against Let-7f/miR-98 nor LNA against miR-21 had any effect on estrogen-inducible expression of E2F1 (Figure 2B). Basal level of E2F2 was substantially higher in miR-21 LNA treated MCF-7pQXIP cells compared to control LNA-treated cells, which was

further increased by estrogen. LNA against Let-7f/miR-98 but not miR-21 increased the basal levels of c-Myc proteins. LNA against Let-7f or miR-98 individually was ineffective, possibly due to their redundant targets. Estrogen mediated reduction of AIB1 protein levels was unaffected by LNA against Let-7f/miR-98 or miR-21 (Figure 2B). We were unable to observe an effect of LNA against Let-7f/miR-98 on Ras protein levels possibly due to the effects of other estrogen-induced Let-7 family members on translation of Ras (data not shown). Also, due to undetectable levels of HMGA2 protein in MCF-7 cells, the effect of estrogen on this protein could not be evaluated (data not shown).

Bioinformatics analysis of potential targets of estrogen-regulated microRNAs identify additional estrogen-responsive genes

We next determined whether any of the estrogen-regulated genes are the targets of estrogen-regulated microRNAs. To facilitate this analysis, we first identified estrogen-inducible and estrogen-repressed genes in MCF-7pQXIP and MCF-7CA-AKT cells by microarray analysis of untreated and four-hour estrogen treated cells. These estrogen-regulated as well as non-estrogen regulated genes were then scanned for microRNA seed sequences. The 21 estrogen-induced microRNAs in MCF7pQXIP cells belong to 10 distinct microRNA families. These microRNAs may target 94 estrogen-inducible, 142 estrogen-repressible and 541 non-estrogen regulated genes in MCF-7pQXIP cells. Detailed statistical analysis is presented in Table 2.

Similar analysis as above was performed with microRNAs repressed by estrogen. Four estrogen-repressed microRNA families, which included 5 repressed microRNAs, potentially target 67 estrogen-inducible, 116 estrogen-repressible, and 407 non-estrogen target genes in MCF-7pQXIP cells (Table 2). Note that has-miR-302b* and has-miR-524* were excluded from this analysis due to the lack of prediction from TargetScan. All together, estrogen-regulated microRNAs target 1021 genes in MCF-7pQXIP cells.

Similar analysis in MCF-7CA-AKT cells showed that estrogen-inducible microRNAs may target 20 estrogen-inducible genes, 20 estrogen-repressed genes and 68 non-estrogen-regulated genes. MicroRNAs repressed by estrogen in these cells may target 148 estrogen-inducible genes, 111 estrogen-repressed genes and 554 non-estrogen target genes (Table 2). Together, estrogen-regulated microRNAs potentially control 850 genes in MCF-7CA-AKT cells.

Estrogen reduces number of breast cancer progenitor cells as defined by ALDH1-positivity.

We initially developed MCF-7 cells overexpressing Let-7f, miR-21 or miR-98. Although we were able to obtain cells overexpressing these microRNAs (10-2000 fold higher expression levels than control), these cells behaved similar to parental cells with respect to proliferation and some of the target gene expression. These results suggested that microRNA overexpression alone is not sufficient for reduction of target genes or to have a biological effect. Additional parameters such as proteins that target microRNAs for their targets may be essential to see the effect of overexpressed microRNAs. These negative results prompted to us conduct studies described below.

Alcohol dehydrogenase 1 (ALDH1)-positive but not the previously defined CD44+/CD24- phenotype has been suggested to identify tumorigenic progenitor cells of the breast (42). Also, ALDH1-positive cells from the normal mammary gland have been

shown to represent progenitor cells (34). These progenitor cells express very little Let-7 and the Let-7 level increases upon differentiation. Let-7 overexpression also promotes differentiation of these cells to ALDH1-negative phenotype. Another study described that breast cancer "tumor initiating cells", as defined by CD44+/CD24- phenotype, express lower level of Let-7 and forced expression of Let-7 leads to differentiation (36). MiR-21, although generally considered as oncogenic, induces differentiation of embryonic stem cells by reducing the levels of c-Myc, Oct-4, Nanog and Sox2 (38). These results suggest a potential role for estrogen-inducible microRNAs in differentiation of normal or cancerous progenitor cells.

We examined whether estrogen-induced Let-7 and miR-21 microRNAs reduces the number of ALDH1-positive subpopulation of MCF-7 cells. Consistent with this possibility, estrogen treatment reduced the number of ALDH1-positive MCF-7pQXIP and MCF-7CA-AKT cells (Figure 3). ALDH1-positive subpopulation of MCF-7 cells could only be detected when cells were maintained in dextran-charcoal treated serum containing media but not in regular serum containing media, which has sufficient estrogen to activate ERα. Despite lower level of estrogen-inducible expression of Let-7f, miR-98 and miR-21, estrogen still reduced ALDH1-positive subpopulation in MCF-7CA-AKT cells indicating that that residual estrogen-mediated induction of Let-7 or some other microRNAs compensate to induce differentiation of these cells. As we reported previously with parental MCF-7 cells (43), MCF-7pQXIP and MCF-7CA-AKT cells were of CD44-/CD24+ phenotype. Therefore, the effect of estrogen-induced Let-7s on differentiation of CD44+/CD24- cells could not be evaluated. In fact, none of ERα-positive cell lines that we have examined contain CD44+/CD24- subpopulation (data not shown). Our repeated attempts to delineate the role of Let-7f, miR-98 and miR-21 in estrogen-induced loss of ALDH1-positive subpopulation using LNA-mediated knockdown did not give interpretable results possibly due to redundant function of other estrogen-induced microRNAs in differentiation of ALDH1-positive cells.

MiR-21 regulatory region contain ERα binding site.

We used our ChIP-on-chip data set and other bioinformatics tools to investigate how estrogen regulates the expression of microRNAs. MicroRNAs can be transcribed as independent units or be a part of intronic sequences of a large transcribed gene (16). The microRNAs that reside in the intergenic sequence are transcribed as 3-5kb transcripts with clearly defined 5' and 3' boundaries. The promoter/enhancer regions of such microRNA genes are enriched for five transcription factors binding sites: MSX-1, TLX2 (Hox11L1), CDC5, SRF and ZNF238 (16). Table 3 provides summary of estrogen-regulated microRNAs that contain ERα binding sites in their regulatory region or located in the intronic region of known estrogen-regulated genes. We further evaluated ERα binding to miR-21 regulatory region by ChIP assay. Location of miR-21 transcribed region in relation to ERα binding site and the ChIP assay confirming ERα binding are shown in Figure 4. Unliganded ERα bound to three regions in MCF-7pQXIP cells (Figure 4A). Estrogen reduced the number of binding sites to one. ChIP assay was used to confirm ERα binding to the region II (central region). Unliganded ERα binding to this region was observed in MCF-7pQXIP cells (10-fold higher levels of PCR product compared to IgG control), which was reduced modestly upon estrogen treatment. However, ERα binding to this region was observed only in the presence of estrogen in

MCF-7CA-AKT cells and total ERα binding to this region with or without estrogen was substantially lower than in MCF-7pQXIP cells. Thus, extracellular signals as well as estrogen control ERα binding to the regulatory regions of miR-21. In summation, our studies reveal the mechanism by which estrogen changes expression pattern of microRNAs and consequences of these changes on the post-transcriptional control of gene expression.

Key Research Accomplishments:

- ☐ **Estrogen regulates the expression of 28 microRNAs; 21 are induced and seven are repressed.**
- ☐ **The serine/theronine kinase AKT abrogates estrogen-regulated expression of microRNAs**
- ☐ **Estrogen-regulated microRNAs reduce protein levels of estrogen inducible genes c-Myc and E2F2.**
- ☐ **Because c-Myc and E2F2 positively regulate ERα activity and secondary estrogen response, respectively, estrogen-regulated microRNAs may serve as negative regulatory loop in estrogen response.**

Reportable outcome:

1) Abstract and oral presentation in Era-of-Hope DOD meeting July 2008 (P63-2, page 422, please see the appendix).
2) Although a manuscript initially submitted to Cancer Cell was not accepted, a revised version is currently being prepared for submission to Journal of Biological Chemistry or Molecular and Cellular Biology.

Conclusions: Figure 5 shows our current model of how estrogen-regulated microRNAs may control breast cancer progression. We propose that signaling pathways that change the balance between estrogen-regulated tumor suppressor and oncogenic microRNAs dictate estrogen response, differentiation, and progression of the disease.

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P63-1: HORMONES OF PREGNANCY, AFP, AND REDUCTION OF BREAST CANCER RISK

Herbert Jacobson, Nicole Lemanski, Amithi Narendran, Anu Agarwal, James Bennett, and Thomas Andersen
Albany Medical College

Background: Parity profoundly reduces women's risk of acquiring breast cancer later in life. It has been reasoned that hormones (either estradiol E_2 or estriol E_3), progesterone (P), or human chorionic gonadotropin (hCG) in the serum of pregnant women might lead to that reduction in risk. These agents have been shown to reduce breast cancer incidence in carcinogen-treated virgin rats. We investigated the hypothesis that exogenously added E_2, E_3, P, or hCG are not the proximal effectors of risk reduction, but that they elicit α-fetoprotein (AFP) from the non-pregnant liver and that AFP is the proximal agent by which reduction of breast cancer risk is obtained.

Methods: Methylnitrosourea (MNU)-exposed animals were treated with saline, E_3, E_2 + P, E_3 + P, hCG, or were allowed to experience pregnancy, and AFP levels were measured in the serum and subsequent tumor incidence was recorded. Human HepG2 liver cells in culture were treated with E_3, E_2 + P, P, or hCG and elicited AFP was measured in the media. The HepG2 culture media containing elicited AFP was assessed for its ability to inhibit proliferation of T47D cells when applied to these human breast cancer cells in culture, and to inhibit the estrogen-induced phosphorylation of the estrogen receptor in T47D cells.

Results: For each condition in the prevention studies, hormone treatment reduced the incidence of breast cancer to an extent similar to that reported by the original studies. In each condition, AFP levels in serum were elevated over that in control animals. In culture, treatment of human liver cells with E_3, E_2 + P, or hCG, but not P alone, led to increased levels of AFP in the media. Media containing hCG-elicited AFP inhibited the estrogen-stimulated proliferation of T47D cells in culture, and inhibited phosphorylation of the estrogen receptor, whereas, estrogens and hCG did not inhibit the growth of these tumor cells in culture.

Conclusion: Since the hormones of pregnancy elicit AFP from the liver, and AFP but not the hormones of pregnancy has direct anti tumor properties, it is concluded that AFP is the proximal agent through which reduction in breast cancer incidence is realized from the experience of pregnancy.

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P63-2: CONTROL OF ESTROGEN-REGULATED MICRORNA EXPRESSION BY AKT

Harikrishna Nakshatri,[1] Nikhail R. Collins,[1] Yunlong Liu,[1] Michael J. Thomson,[1] Guohua Wang,[1] Poornima Bhat-Nakshatri,[1] Tim Geistlinger,[2] Jason S. Carroll,[1] Myles Brown,[2] and Scott Hammond[3]
[1]Indiana University-Purdue University, Indianapolis, [2]Dana-Farber Cancer Institute, and [3]University of North Carolina at Chapel Hill

Background and Objective: Estrogen induces or represses gene expression by acting as a ligand for estrogen receptor alpha (ERα) and ERbeta. To date, majority of mechanistic studies were focused on estrogen inducible gene expression and anti-estrogen based therapies were mostly designed and assessed for their ability to inhibit estrogen-induced gene expression. However, recent studies show that ~70% of genes regulated by estrogen are repressed in breast cancer cells suggesting that estrogen-repressible genes play a significant role in breast cancer. Such high percentage of repression is achievable if estrogen modulates a gene regulatory system other than classical transcription initiation machinery. The objective of this study was to investigate whether estrogen regulates gene expression through microRNAs and whether extracellular signal activated kinases such AKT modulate estrogen-regulated microRNA expression.

Methodologies: RNA from untreated and estrogen-treated (10-8 M, 3 hours) MCF-7 (pQXIP) and MCF-7 cells overexpressing constitutively active AKT (CA-AKT) were hybridized to miRNA-array comprising 248 human microRNAs. MicroRNA expression was further verified by qRT-PCR. The effect of estrogen-regulated microRNAs on target gene expression was determined by western blot analysis. Chromatin-immunoprecipitation coupled microarray was used to identify ERα binding sites in genomic region encoding microRNAs. Various bioinformatics tools were used to predict mechanisms of estrogen-regulated microRNA expression and to identify putative targets of microRNAs.

Results: Estrogen increased the expression of 21 microRNAs and decreased the expression of 7 microRNAs in pQXIP cells. AKT completely changed the pattern of microRNA expression as estrogen increased the expression of one and reduced the expression of 20 microRNAs in CA-AKT cells. Estrogen increased the expression of most of the Let-7 family members in pQXIP but not CA-AKT cells and consequently reduced the protein levels of Let-7 target Ras in pQXIP cells. Five of the estrogen-regulated microRNAs contain ERα binding sites while the remaining microRNAs appears to be regulated by transcription factors whose expression is under the control of estrogen and/or AKT. MicroRNA upregulated by estrogen may target ~486 (326+160) whereas microRNAs downregulated by estrogen may target ~551 (348+203) estrogen-regulated genes; however, multiple microRNAs may target the same gene thus overall number of genes may be lower than predicted.

Conclusions: Estrogen controls gene expression at post-transcriptional level through microRNA; Ras is one of the main target. By enhancing the expression of Let-7 family, estrogen may promote differentiation of luminal epithelial cells, which is abrogated by extracellular signal activated kinases such as AKT.

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P63-3: HISTONE DEACETYLASE 2 (HDAC2) MEDIATES THE POTENTIATION OF TAMOXIFEN BY HDAC INHIBITORS

Pamela N. Munster, Elona Bicaku, Douglas C. Marchion, and Morgen L. Schmitt
H. Lee Moffitt Cancer Center & Research Institute at University of South Florida

Background: Histone deacetylases (HDACs) play a central role in many biological functions and may be involved in the pathogenesis of breast cancer. HDAC inhibitors have been shown to modulate estrogen receptor (ER) expression and may restore tamoxifen sensitivity to hormone-insensitive tumors. The interaction of HDAC inhibitors with tamoxifen suggests an important role of HDACs in ER signaling; however, very little is known as to which HDAC enzyme interacts with ER and which HDAC enzyme(s) is a relevant target for antihormonal therapy. We evaluated the roles of specific HDAC isoenzymes and their inhibition on both ER and progesterone receptor (PR) signaling and their importance in response to endocrine therapy.

Method: The roles of individual HDAC isoenzymes on ER and PR expression and their functions were evaluated by depletion of select HDAC enzymes using siRNA or pharmacological inhibition.

Results: Co-treatment of breast cancer cell lines with HDAC inhibitors and the anti-estrogen, tamoxifen, resulted in synergistic antitumor activity with simultaneous depletion of both ER and PR. Selective inhibition of HDAC2, but not HDAC1 or HDAC6, was sufficient to potentiate tamoxifen-induced apoptosis in ER/PR-positive cells. Depletion of HDAC1 and HDAC6 was associated with downregulation of ER but not PR. Only the selective depletion of HDAC2 downregulated both ER and PR expression and was sufficient to potentiate tamoxifen.

Conclusions: Selective depletion of HDAC2 resulted in simultaneous depletion of ER and PR, and potentiated the effects of antihormonal therapy in ER-positive cells. A more effective pharmacological inhibition of HDAC2 and evaluation of HDAC2 and PR as therapeutic targets or as predictive markers in hormonal therapy may be considered when combining HDAC inhibitors and hormonal therapy. The effect of HDAC enzymes is currently being evaluated in clinical samples.

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P63-4: PROLACTIN ANTAGONIZES CHEMOTHERAPEUTIC-INDUCED CYTOTOXICITY IN BREAST CANCER CELLS

Nira Ben-Jonathan and Elizabeth W. Lapensee
University of Cincinnati

Background: Breast cancer patients are treated with various anti-cancer drugs, including the DNA damaging agent doxorubicin and the microtubule altering drugs taxol and vinblastine. However, resistance to chemotherapy is a major concern, with patients especially unresponsive to the DNA intercalating drug cisplatin. Prolactin (PRL) is a 23kd protein whose target is the breast, where it acts as a mitogen and survival factor. PRL is produced by both the pituitary and the breast, with expression of its receptors higher in tumors than in the normal breast. We previously reported that nude mice inoculated with PRL-overexpressing breast cancer cells developed faster growing tumors with increased expression of the anti-apoptotic protein Bcl-2.

Specific Aims: Given its action as a survival factor, we hypothesized that PRL antagonizes the cytotoxic effects of chemotherapeutic drugs in breast cancer. The specific aims were to: (1) characterize the sensitivity of several breast cancer cell lines to anti-cancer drugs and determine if PRL antagonizes their cytotoxicity, (2) examine if PRL opposes cisplatin-induced apoptosis, and (3) determine the mechanism by which PRL protects these cells from cisplatin cytotoxicity.

Results: Cisplatin, doxorubicin, vinblastine, and taxol induced dose-dependent decreases in cell viability that were completely or partially antagonized by low doses (25 ng/ml) of PRL. Such protection was observed using MDA-MB-468 (468) and MDA-MB-231 as well as T47D cells. The remainder of the experiments focused on the effects of cisplatin on 468 cells. To determine whether PRL antagonizes apoptosis, cells were labeled with Annexin-V/propodium iodide and analyzed by flow cytometry. Cisplatin-induced apoptosis was prevented upon pretreatment with PRL. As was also revealed by flow cytometry, cisplatin induced a G2/M cell cycle arrest. A combination of staining for phosphorylated histone H3 and Western blotting for cyclin B expression confirmed cell arrest at G2; this checkpoint was bypassed by PRL treatment. DNA damage was assessed by staining with γ-H2AX, which recognizes double-strand breaks.

Table 1: The effect of estrogen on microRNA expression in MCF-7pQXIP and MCF-7CA-AKT cells. Cells were treated with estrogen for four hours and microRNA expression array analysis was performed. Only those microRNA whose expression differences with a p value of <0.05 among untreated and estrogen treated cells are presented. miR-98 was induced by estrogen in both cell types (2.1 fold in MCF-7pQXIP cells, 1.4 fold in MCF-7CA-AKT cells) whereas miR-143 (to same level) and miR-506 (0.50 in MCF-7pQXIP cells and 0.3 in MCF-7CA-AKT cells) were repressed by estrogen in both cell types.

Estrogen-inducible microRNAs:

MCF-7pQXIP cells		**MCF-7CA-AKT cells**	
Name	***Fold change***	***Name***	***Fold change***
miR-Let-7f	**3.2**	**miR-520d**	**4.8**
miR-Let-7a	**2.9**		
miR-Let-7d	**2.7**		
miR-Let-7c	**2.6**		
mir-Let-7g	**2.6**		
miR-203	**2.4**		
miR-Let-7b	**2.3**		
miR-Let-7e	**2.2**		
miR-98	**2.1**		
miR-21	**2.0**		
miR-200a	**2.0**		
miR-103	**1.8**		
miR-200c	**1.8**		
miR-107	**1.7**		
miR-17-5p	**1.7**		
miR-23a	**1.6**		
miR-200b	**1.6**		
miR-30c	**1.6**		
miR-30b	**1.5**		
miR-424	**1.5**		
miR-let7i	**1.5**		

Estrogen-repressible microRNAs

MCF-7pQXIP cells		MCF-7CA-AKT cells	
Name	***Fold change***	***Name***	***Fold change***
miR-302b*	**0.4**	**miR-524**	**0.3**
miR-506	**0.5**	**miR-518d**	**0.3**
miR-524*	**0.5**	**miR-518e**	**0.3**
miR-27a	**0.6**	**miR-506**	**0.3**
miR-27b	**0.6**	**miR-409-5p**	**0.3**
miR-143	**0.6**	**miR-216**	**0.3**
miR-9	**0.7**	**miR-518c***	**0.5**
		miR-526b	**0.5**
		miR-34b	**0.5**
		miR-337	**0.5**
		miR-146	**0.5**
		miR-128b	**0.5**
		miR-124a	**0.5**
		miR-211	**0.6**
		miR-143	**0.6**
		miR-128a	**0.6**
		miR-126*	**0.6**
		miR-126	**0.6**
		miR-1	**0.6**
		miR-10b	**0.7**

MicroRNAs that display differences in basal expression between two cell types: MicroRNAs with fold change >1 are expressed at higher levels in MCF-7CA-AKT cells whereas fold change <1 represent microRNAs expressed at higher levels in MCF-7pQXIP cells

Name	Fold change	Name	Fold change
miR-520d	**5.1**	**miR-200a**	**0.6**
miR-let7g	**0.5**	**miR-182**	**0.6**
miR-337	**0.5**	**miR-17-5p**	**0.6**
miR-20b	**0.5**	**miR-1**	**0.6**
miR-Let-7i	**0.6**	**miR-203**	**0.6**
miR-98	**0.6**	**miR-200c**	**0.7**

Table 2: Number of genes potentially targeted by estrogen-regulated microRNAs in MCF-7pQXIP and MCF-7CA-AKT cells. FC= Fold change

	mRNAs induced by estrogen (FC > 1.5)	**mRNAs repressed by estrogen (FC≤ 1.5)**	**non-estrogen-regulated mRNAs (-1.5<FC<1.5)**
Estrogen up-regulated microRNA targets in MCF-7pQXIP cells	94	142	541
Estrogen down-regulated microRNA targets in MCF-7pQXIP cells	67	116	407
Estrogen up-regulated microRNA targets in MCF-7CA-AKT cells	20	20	68
Estrogen down-regulated microRNA targets in MCF-7CA-AKT cells	148	111	554

Table 3: Estrogen regulated microRNAs with ERα binding sites or located in the intragenic region of estrogen-regulated genes. miR-27b is located within aminopeptidase gene, which is an estrogen inducible and displayed 5 and 8 ERα binding sites in estrogen treated MCF-7pQXIP and MCF-7CA-AKT cells, respectively.

MicroRNAs with ERα binding sites		
MicroRNA	*Fold change in microRNA expression after E2 treatment in MCF-7pQXIP cells*	*ERα binding sites after estrogen treatment*
miR-21	1.98	1
miR-23a	1.62	4
miR27a	0.64	4
miR27b	0.60	5

MicroRNAs located in the intragenic regions of estrogen-regulated genes			
MicroRNA	*Fold change in microRNA levels after estrogen treatment of MCF-7pQXIP cells*	*Host gene*	*Estrogen effect on the host gene*
miR30c	1.58	NF-YC	-1.2
miR-9	0.654	C1orf61	1.16
Let-7c	2.6	C21orf34	-1.9
Let-7g	2.6	TMEM113	1.3

Figure Legend:

Figure 1: A) Estrogen-inducible expression of Let-7f, miR98 and miR-21 in MCF-7pQXIP and MCF-7CA-AKT cells. MCF-7pQXCIP and MCF-7CA-AKT cells were treated with estrogen (10^{-8}M) for indicated time and microRNA was subjected qRT-PCR. Normalization between samples was done using RNU66, small RNAs encoded in the intron of RPL5 gene (chr1:93,018,360-93,018,429), as an internal control. Data is from three independent experiments, which showed similar pattern of estrogen effects, although fold increase varied from experiment to experiment (10 to 35 fold in case of miR-98 for example). B) Estrogen-regulated expression of Let-7f, miR-21 and miR-98 in T47-D and BT-474 cells.

Figure 2: Putative targets of estrogen-induced microRNAs. A) MCF-7pQXIP and MCF-7CA-AKT cells were treated with estrogen for indicated time and Western blotting was performed. Note the failure of estrogen to reduce Ras expression in MCF-7CA-AKT cells, which is consistent with reduced estrogen-inducible expression of Let-7f and miR-98 in MCF-7CA-AKT cells compared to MCF-7pQXIP cells. B) LNA against Let-7f/miR-98 and miR-21 differentially affect basal and estrogen-inducible levels of c-Myc and E2F2 proteins in MCF-7pQXIP cells. Cells were treated with control or specific LNA and treated with estrogen for 24 hours. Western blot analysis was done with indicated antibodies. Average expression levels from three independent experiments, as measured by densitometric scanning, are indicated. Although the magnitude of LNA effect varied from experiment to experiment, trend was identical in all three experiments. Note the effects of LNA against Let-7f/miR98 and miR-21 on E2F2 and c-Myc but not E2F1 and AIB1 protein levels.

Figure 3: Estrogen reduces the number of breast cancer progenitor cells as defined by ALDH1-positivity. Cells were treated with estrogen for 24 hours and subjected to ALDEFLUOR (ALDE) staining with or without DEAB. DEAB staining allows separation of ALDH1-positive cells from background staining. Number of ALDH1-positive cells under different conditions in three experiments is also presented. Variation in number of ALDH1-positive cells between experiments is most likely due to differences in residual estrogen in charcoal-stripped serum.

Figure 4: MiR-21 regulatory region contains ERα binding site. A) ERα DNA binding patterns to the genomic region harboring miR-21 gene on chromosome 17. Colored bars represent ERα binding sites in two cell types observed under untreated and estrogen treated condition. B) ChIP analysis of ERα binding to the genomic region (central bar) shown in A. ChIP DNA obtained with ERα antibody was subjected to Q-PCR with specific primers and relative enrichment of ERα binding is shown after normalizing ERα binding in untreated MCF-7pQXIP cells to one unit.

Figure 5: A model depicting the possible effects of estrogen-regulated microRNAs on estrogen response and differentiated phenotype of ERα-positive breast cancer cells.

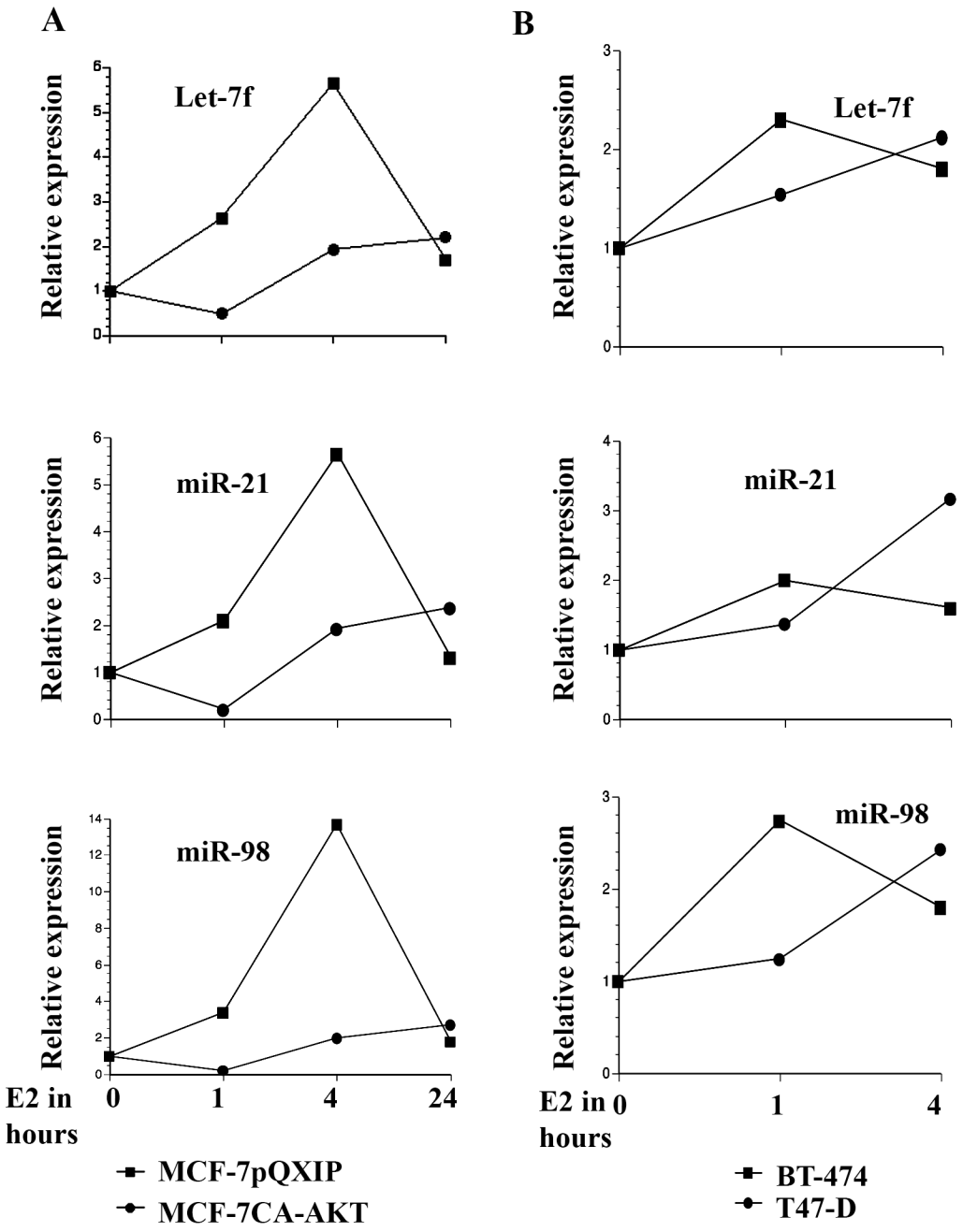

A
B
Let-7f
miR-21
miR-98
Relative expression
E2 in hours
0
1
4
24
MCF-7pQXIP
MCF-7CA-AKT
BT-474
T47-D

Figure 1

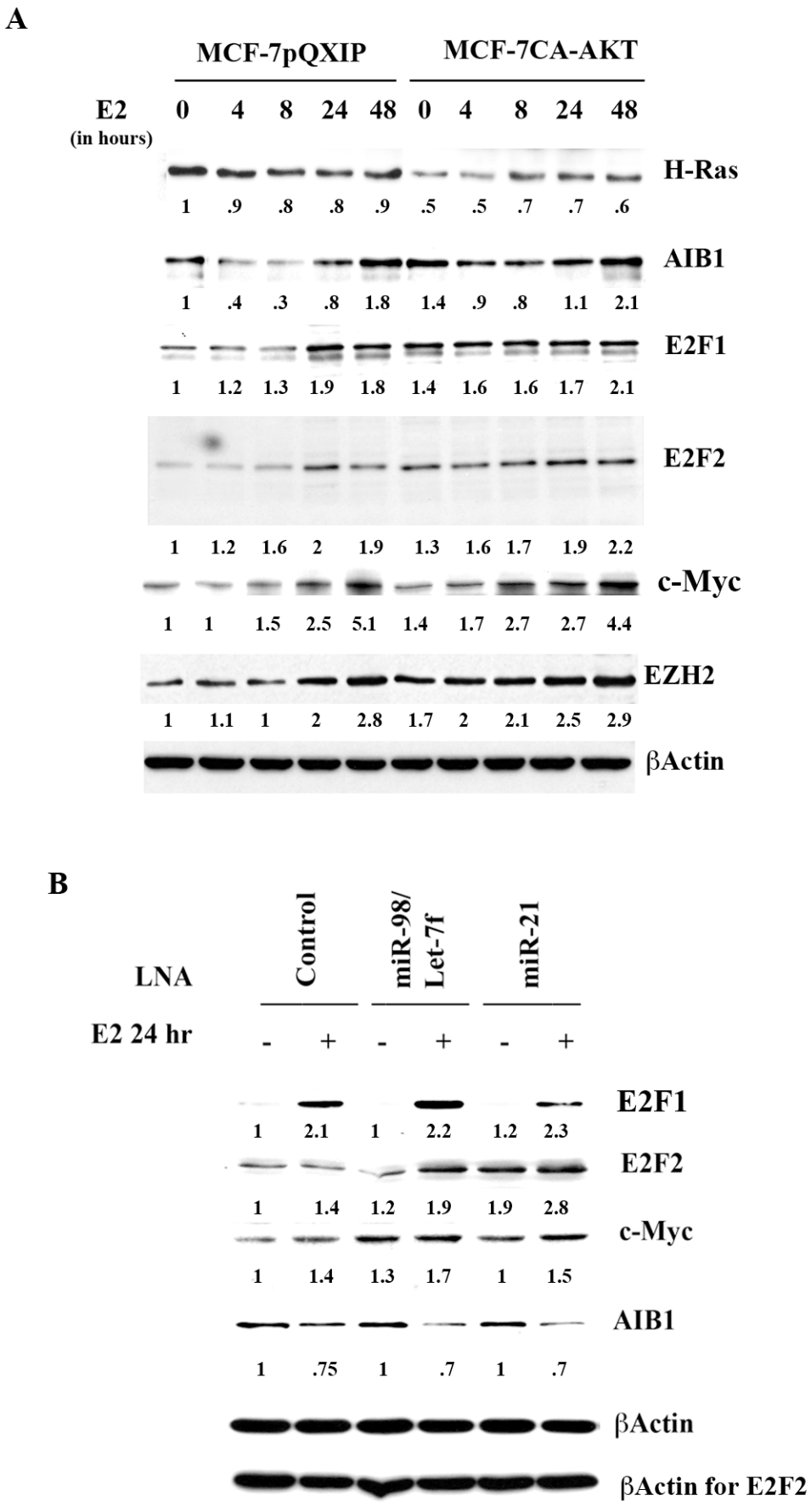
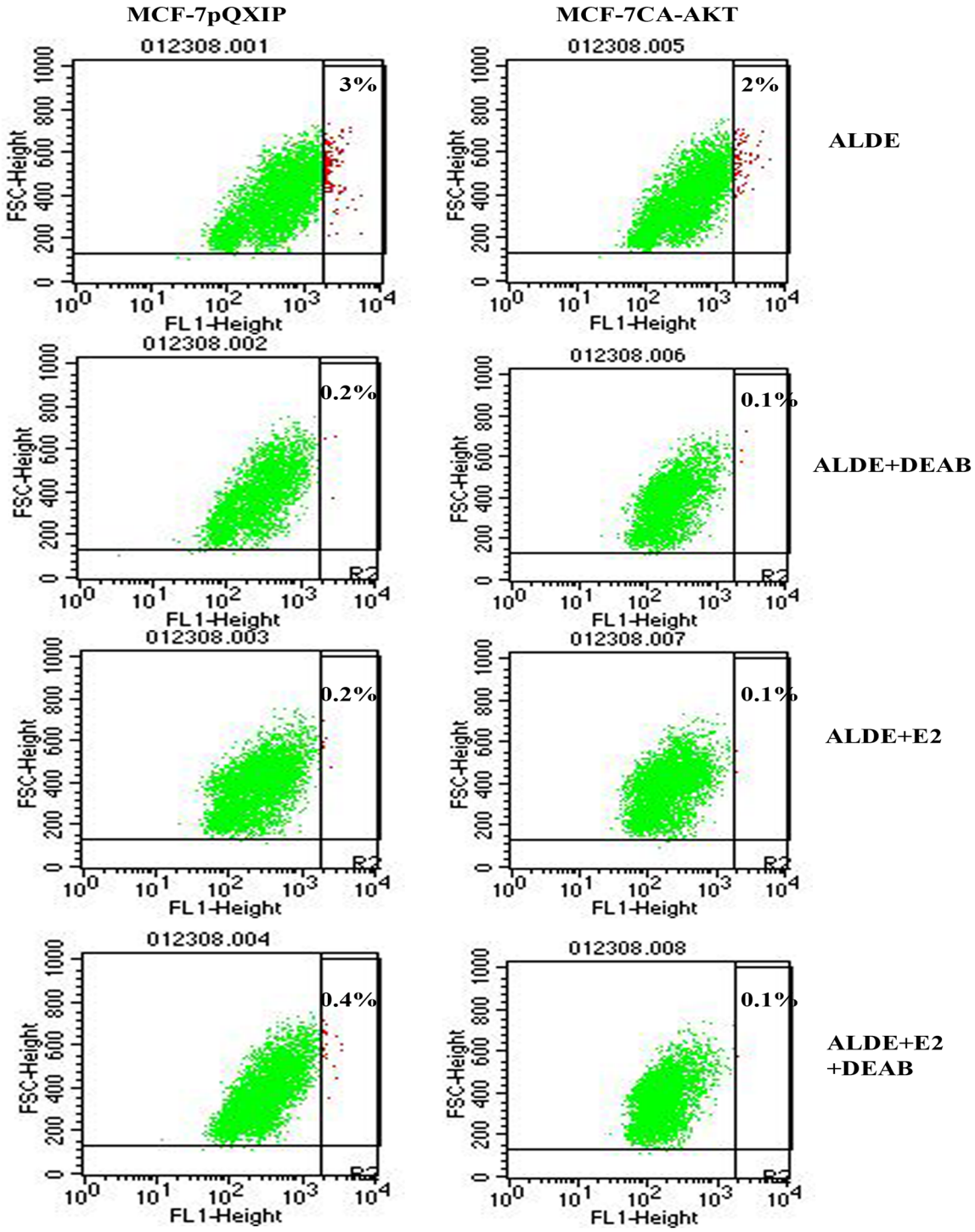

A
MCF-7pQXIP
MCF-7CA-AKT
E2 (in hours)
0 4 8 24 48 0 4 8 24 48
H-Ras
1 .9 .8 .8 .9 .5 .5 .7 .7 .6
AIB1
1 .4 .3 .8 1.8 1.4 .9 .8 1.1 2.1
E2F1
1 1.2 1.3 1.9 1.8 1.4 1.6 1.6 1.7 2.1
E2F2
1 1.2 1.6 2 1.9 1.3 1.6 1.7 1.9 2.2
c-Myc
1 1 1.5 2.5 5.1 1.4 1.7 2.7 2.7 4.4
EZH2
1 1.1 1 2 2.8 1.7 2 2.1 2.5 2.9
βActin
B
LNA
Control
miR-98/ Let-7f
miR-21
E2 24 hr
- + - + - +
E2F1
1 2.1 1 2.2 1.2 2.3
E2F2
1 1.4 1.2 1.9 1.9 2.8
c-Myc
1 1.4 1.3 1.7 1 1.5
AIB1
1 .75 1 .7 1 .7
βActin
βActin for E2F2

Fig.2



	MCF-7pQXIP		MCF-7CA-AKT	
	Ethanol	Estrogen	Ethanol	Estrogen
Experiment #1	3.0	0.2	2.0	0.1
Experiment #2	1.3	0.4	0.8	0.4
Experiment #3	2.8	2.2	1.8	1.1

Fig. 3

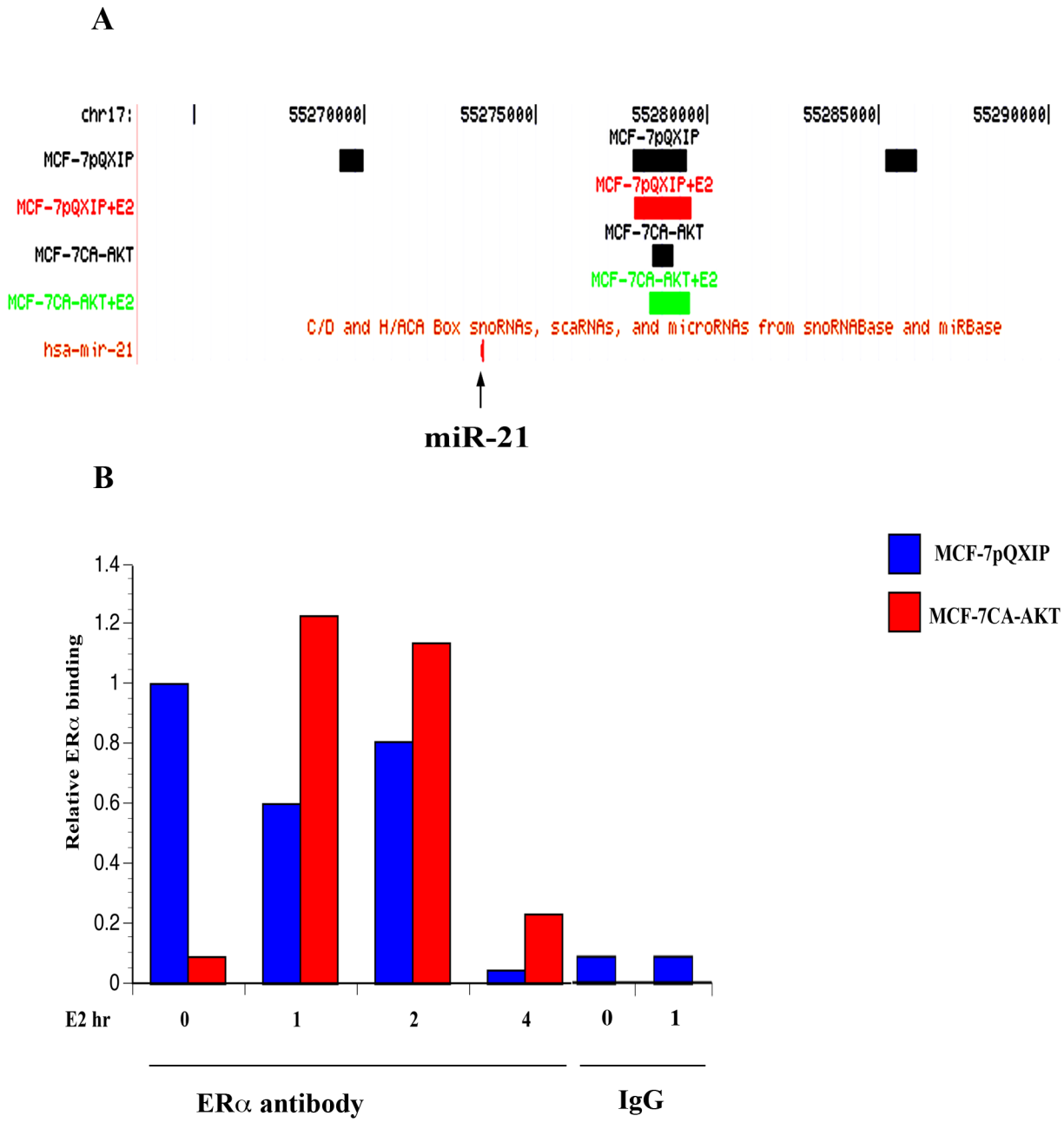

A
chr17:
55270000
55275000
55280000
55285000
55290000
MCF-7pQXIP
MCF-7pQXIP+E2
MCF-7CA-AKT
MCF-7CA-AKT+E2
C/D and H/ACA Box snoRNAs, scaRNAs, and microRNAs from snoRNABase and miRBase
hsa-mir-21
miR-21
B
MCF-7pQXIP
MCF-7CA-AKT
Relative ERα binding
1.4
1.2
1
0.8
0.6
0.4
0.2
0
E2 hr
0
1
2
4
0
1
ERα antibody
IgG

Fig. 4

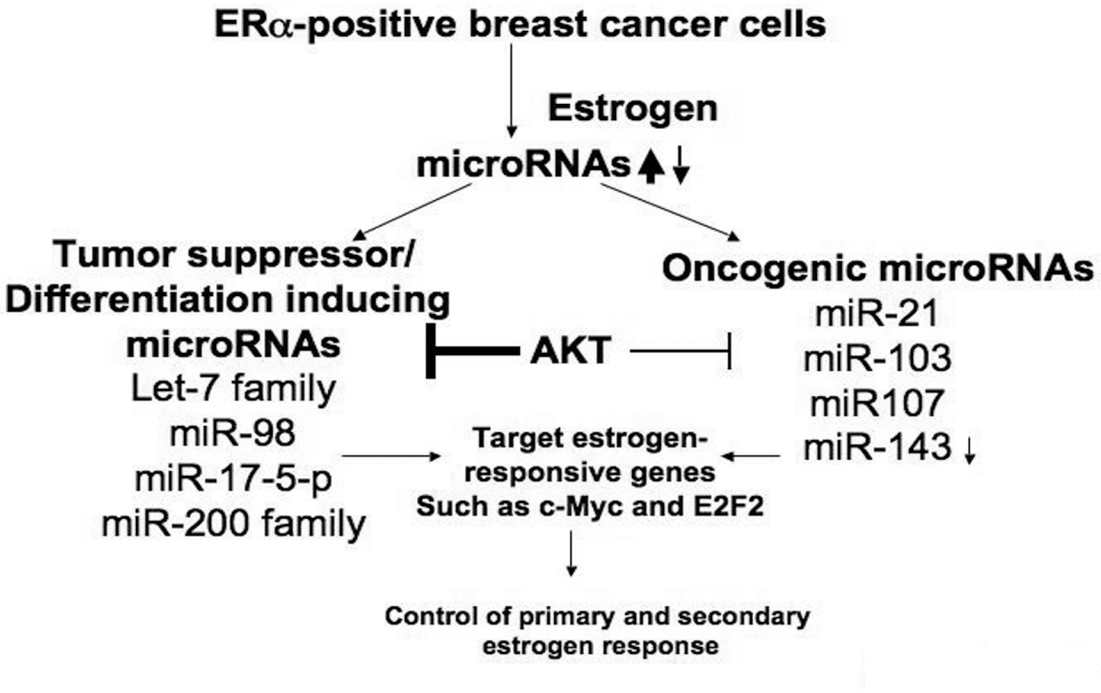

ERα-positive breast cancer cells
Estrogen
microRNAs
Tumor suppressor/
Differentiation inducing
microRNAs
Let-7 family
miR-98
miR-17-5-p
miR-200 family
AKT
Oncogenic microRNAs
miR-21
miR-103
miR107
miR-143
Target estrogen-
responsive genes
Such as c-Myc and E2F2
Control of primary and secondary
estrogen response

Figure 5