

let-7 MicroRNAs Induce Tamoxifen Sensitivity by Downregulation of Estrogen Receptor α Signaling in Breast Cancer

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MicroRNAs (miRNAs) play an important regulatory role in breast tumorigenesis. Previously, we found that *let-7* miRNAs were downregulated significantly in formalin-fixed paraffin-embedded (FFPE) breast cancer tissues. In this study, we further found that endogenous levels of *let-7b* and *let-7i* miRNAs are inversely correlated with levels of estrogen receptor (ER)- α 36, a new variant of ER- α 66, in the FFPE tissue set. Bioinformatic analysis suggested that ER- α 36 may be another target of *let-7* miRNAs. To test this hypothesis, cotransfection of *let-7* mimics or inhibitors together with full-length or a fragment of ER- α 36 3'UTR luciferase construct was performed, and we found that *let-7b* and *let-7i* mimics suppressed the activity of reporter gene significantly, which was enhanced remarkably by *let-7b* and *let-7i* inhibitors. Both mRNA and protein expression of ER- α 36 were inhibited by *let-7* mimics and enhanced by *let-7* inhibitors. Furthermore, ER- α 36 mediated nongenomic MAPK and Akt pathways were weakened by *let-7b* and *let-7i* mimics in triple negative breast cancer cell line MDA-MB-231. The reverse correlation between *let-7* miRNAs and ER- α 36 also exists in Tamoxifen (Tam)-resistant MCF7 cell line. Transfection of *let-7* mimics to Tam-resistant MCF7 cells downregulated ER- α 36 expression and enhanced the sensitivity of MCF7 cells to Tam in estrogen-free medium, which could be restored by overexpression of ER- α 36 constructs without 3'UTR. Our results suggested a novel regulatory mechanism of *let-7* miRNAs on ER- α 36 mediated nongenomic estrogen signal pathways and Tam resistance.

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INTRODUCTION

The involvement of estrogen in mammary carcinogenesis has been known for more than 100 years (1). For a long time, the estrogen signaling was thought to be mediated by two major estrogen receptors (ER), ER- α and ER- β (2), which share a common structural architecture. ER- α is a 66 kD protein that functions as a transcription factor and regulates the transcription of estrogen-responsive genes. ER- α is comprised of six domains, A–F (3). The A/B region contains a ligand-independent transactivation domain (AF-1). Regions C and E

are responsible for DNA and ligand binding, respectively. A ligand-inducible transcription-activating function (AF-2) is present in the ligand-binding domain D/E/F (4). Recent research revealed the existence of a truncated form of ER- α with a molecular weight of 46 kD, which lacks the first 173 aa (AF-1 domain) of ER- α and is designated as ER- α 46 (5). The full-length ER- α therefore is recognized as ER- α 66. ER- α 46 functions to inhibit the transcriptional activity mediated by the AF-1 domain of ER- α 66 (5) and to signal a membrane-initiated estrogen pathway (6).

Later, a 36-kD novel isoform of ER- α 66, ER- α 36, was identified and cloned (7). The full-length cDNA accession number is BX640939. This cDNA is localized in the human genomic DNA sequence of clone RP1-130E4 on chromosome 6q24.2–25.3 (GenBank Accession Number AL078582) (7). ER- α 36 is transcribed from a promoter located in the first intron of the ER- α 66 gene and lacks both transcriptional activation domains (AF-1 and AF-2), but retains the DNA-binding, dimerization and partial ligand-binding domains. Additionally, it possesses an extra, unique 27-aa domain to replace the last 138 aa of the ER- α 66. ER- α 36 is localized predominantly on the plasma membranes and mediates membrane-initiated estrogen signal pathway (8) such as activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling pathway (9).

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ER- α 36 expression was detected in both ER- α 66-positive and-negative breast cancer tumors (10,11). High levels of ER- α 36 expression also are associated with tamoxifen resistance; breast cancer patients with tumors highly expressing ER- α 36 benefit less from tamoxifen treatment (12).

Dysregulated miRNA expression is associated frequently with the development of many types of human tumors. It was reported that almost half of the known mature human miRNAs are located in cancer-associated genomic regions, or fragile sites (13). The *let-7* family is considered as a tumor suppressor to inhibit malignant growth of many types of cancer cells by targeting RAS (14), HMGA2 (15,16) and c-Myc (17). Reduced expression of *let-7* miRNA has been reported in cancers of colon (18), lung (19), ovary (20) and breast (21).

In this study, we found a novel regulatory relationship between *let-7* miRNAs and ER- α 36. We demonstrated that there is an inverse correlation between ER- α 36 and *let-7* miRNAs in breast tissue and in the tamoxifen-resistant cell line. *let-7* miRNAs target ER- α 36 and negatively regulate its expression and function. In addition, *let-7* miRNAs (b and i) enhanced tamoxifen sensitivity of tamoxifen-resistant breast cancer cells by targeting ER- α 36 expression.

MATERIAL AND METHODS

Cell Culture

All breast cancer cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The normal breast cell line 184A1 was cultured in 10% Fetal Bovine Serum (FBS; Gibco, Carlsbad, CA, USA) and MEGM (MEBM plus SingleQuots; Clonetics, Walkersville, MD, USA). ER-positive breast cancer cell lines, MCF7, ZR-75-1, T47D and HB3396 were maintained in phenol-red free IMEM (Cellgro) plus 10% FBS, 1% nonessential amino acid, 10 mmol/L HEPES and 2 μ g/mL insulin. MDA-MB-231, MDA-MB-436, MDA-MB-468 and SK-BR-3 were cultured in DMEM

(Cellgro) plus 10% FBS. Tamoxifen-resistant MCF7 cell line (MCF7-Tam) was established by culturing ER-positive breast cancer MCF7 cells in 1 μ mol/L tamoxifen over 6 months and maintaining them in IMEM plus 1 μ mol/L tamoxifen (22). All cells were incubated at 37°C with 5% CO₂ atmosphere.

Quantitative Real-Time PCR

To analyze mature *let-7* miRNAs with real-time polymerase chain reaction (PCR), total RNA was isolated from the cell lines using mirVana miRNA isolation kit (Ambion Inc., Austin, TX, USA) or from FFPE tissues using the miRNeasy FFPE Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. We collected FFPE breast tissues from 13 benign, 16 ductal carcinomas *in situ* (DCIS) and 15 invasive ductal carcinomas (IDC), all of which were prepared as 50- μ m-thick sheets. The benign breast tissues were from surgical resections of abnormal breast lesions with non-neoplastic phenotypes such as calcification, fibrocystic changes or stromal fibrosis. cDNA was synthesized using 100 ng total RNA in a reaction volume of 15 μ L and following the protocol of Taqman microRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). After 15 \times dilution, 9 μ L cDNA was used for real-time PCR in 20 μ L reaction volume, including 10 μ L of TaqMan universal PCR master mix (no ampErase UNG) and 1 μ L primer mixture and Taqman probe (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed on 7900HT real-time PCR instrument (Applied Biosystems). PCR was initiated by two holds of 52°C 2 min and 94°C 10 min each, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The level of each miRNA was expressed following the 2^{- $\Delta\Delta$ Ct} or - Δ Ct method using the small nuclear RNA RNU48 as the internal reference.

To assess the expression levels of ER- α 66 and ER- α 36 with real-time PCR, total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). One microgram RNA was used for cDNA synthesis with the SuperScript III first-

strand synthesis system (Invitrogen). After 10 \times dilution, 4 μ L cDNA was used in 20 μ L reaction volume, which includes 4 μ L of 1.5 μ mol/L primers, 2 μ L of 2 μ mol/L probes, and 10 μ L TaqMan universal PCR master mix with ampErase UNG (Applied Biosystems). The reaction condition was the same as miRNA real-time PCR. β -Actin was the internal control (Applied Biosystems). The primers for ER- α 66 are: 5'-GCGGC CACGGACCAT-3' (forward); 5'-TTCCC TTGGATCTGATGCAGTA-3' (reverse); 5'-FAM-CCATGACCCTCCACACCAAA GCATC-TAMRA-3' (probe). The primers for ER- α 36 are: 5'-CAAGTGGTTTCCTC GTGTCTAAAG-3' (forward); 5'-ACGTC CACACACGGATTGA-3' (reverse); 5'-FAM-TGGTCATAAGGCCTCACAGT ATCCTGCA-TAMRA-3' (probe).

Transient miRNA Transfection for Western Blot and Growth Analysis

let-7 miRNA or negative control #1 miRNA of 60 nmol/L, inhibitor or inhibitor negative control #1 of 200 nmol/L (*let-7b*, Cat# PM11050; *let-7i*, Cat# PM10211; negative #1 AM17110; *let-7b* inhibitor, AM11050; *let-7i* inhibitor, AM10211; inhibitor negative control #1, AM17010) (Ambion) was transfected into cells with the siPORT NeoFX reagent (Ambion) following the manufacturer's protocol.

Western Blot Analysis

Cells were lysed with NP40 cell lysis buffer (250 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.4, 5 mmol/L EDTA, 50 mmol/L NaF, 1 mmol/L Na₃VO₄, 1% Nonidet P40, 1 mmol/L PMSF, proteinase and phosphoproteinase inhibitor cocktails). For protein extraction from FFPE tissues, FFPE sections (50 μ m thick) were transferred into a microcentrifuge tube and treated with xylene, followed by vortexing vigorously for 10 s. After centrifugation and removal of supernatant, the sections were washed with xylene for two more times, followed by one more wash with 1 mL of 100% ethanol. The resultant pellets were vacuum dried, then resuspended in 300 μ L of 20 mmol/L Tris-HCl

(pH 8.0) and 2% SDS and homogenized. The samples were then boiled at 100°C for 20 min and incubated at 60°C for 1 h. After centrifugation at 10,000g for 15 min at 4°C, the supernatant protein was saved for immunoblot analysis.

The quantified protein of 50 µg was mixed with gel-loading buffer and boiled for 5 min before loading onto 10% SDS-PAGE. After electrophoresis, the gel was blotted onto a PVDF membrane (Millipore, Billerica, MA, USA). The blots were probed with specific first antibodies and appropriate second antibodies. The signals were visualized with enhanced chemiluminescence (ECL) reagents (Pierce, Minneapolis, MN, USA) and documented with a FluorChem FC2 (Alpha Innotech, Santa Clara, CA, USA) image system. Gel density was quantified with Quantity One and expressed as a relative value against β-actin. The sources of antibodies are as follows: anti-ERK1/2 (#9102), anti-pERK1/2 (#9106), anti-Akt (#9272), anti-pAkt (Ser473, #9271), and anti-Her2 (#2242) are from Cell Signaling Technology (Danvers, MA, USA); anti-ER-α66 (RB-9016) was from ThermoScientific (Rockford, IL, USA); anti-ER-α36 antibody was an affinity-purified rabbit polyclonal anti-ER-36 antibody generated as a custom service from Pacific Immunology Corp (Ramona, CA, USA). The ER-α36 antibody was raised against a synthetic peptide antigen corresponding to the unique C-terminal 20 aa of ER-α36.

Plasmid Construction

QIAGEN Long Range 2 Step RT-PCR Kit (Qiagen, Cat# 205920) was used to amplify ER-α36 3'UTR. The PCR primers are as follows: CATGTAGCACTAGTCATCAAATCCGTGTGTGGAC (forward); CATGTAGCACGCGTTATCCCCACAGCATTCCCTC (reverse). The PCR product was applied to agarose gel electrophoresis, gel-purified with QIAquick Gel Extraction Kit (Qiagen, Cat# 28704), digested with *SpeI* and *MluI* (Promega, Fitchburg, WI, USA), and purified with QIAquick PCR Purification Kit (Qiagen, Cat# 28104). The pMIR-Report plasmid (Applied Biosystems) also was digested

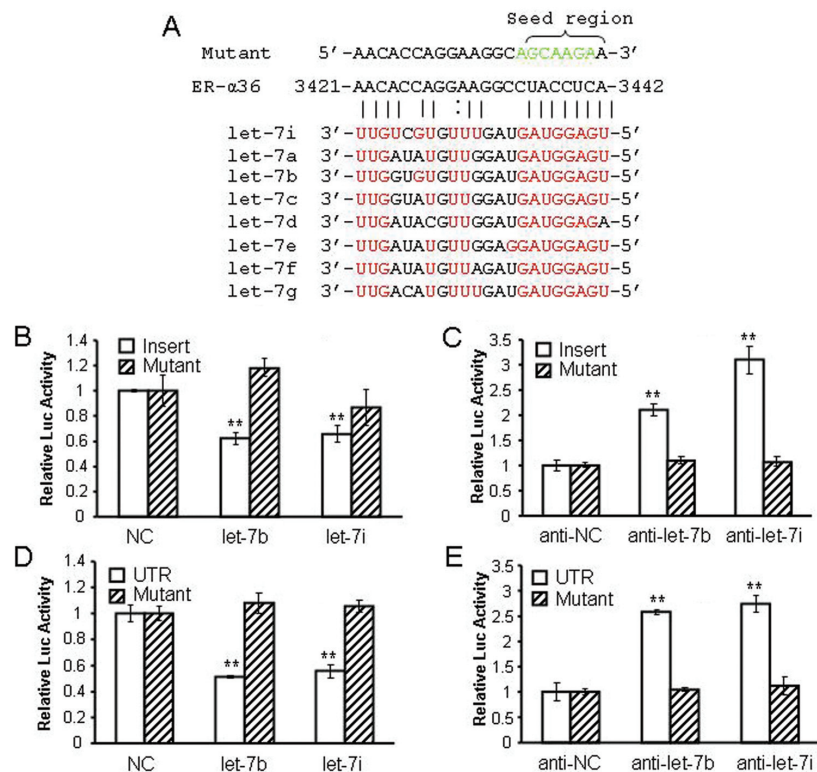


Figure 1. *let-7* miRNAs target ER-α36. (A) *let-7* target prediction: The pairing between *let-7* target miRNAs and 3'UTR of ER-α36 mRNAs. The paired nucleotides are highlighted in red. The mutated nucleotides in luciferase assays are highlighted in green. (B) *let-7b* and *let-7i* significantly inhibited the luciferase activity when the ER-α36 binding site was in the 3'UTR of the luciferase gene in MCF7 cells; they did not show inhibition when a mutated sequence was inserted into the 3'UTR of luciferase in MCF7 cells. (C) Anti-let7b and anti-let7i strongly induced luciferase activity when the putative binding site was inserted into the 3'UTR of luciferase in 184A1 cells; they did not show boost in luciferase activity when the binding site was mutated. (D) The same as in (B) except the full-length ER-α36 3'UTR was attached to luciferase gene. (E) The same as in (C) except the full-length ER-α36 3'UTR was attached to luciferase gene. **, $P < 0.01$.

with *SpeI* and *MluI*, and gel-purified. The ligation was performed with T4 ligase (Promega) in 10 µL volume. One µL of ligation product was transformed into DH5α Competent *E. coli* cells (Invitrogen, Cat# 18265-017). Positive clones were picked, amplified in LB broth with 100 µg/mL ampicillin. Reconstructed plasmids were extracted with EndoFree Plasmid Maxi Kit (Qiagen, Cat# 12362). The correct insert was verified by sequencing.

The full-length ER-α36 construct with mutated binding site for *let-7* miRNAs was generated with QuikChange Site-Directed Mutagenesis Kit (Cat# 200519; Agilent Technologies, Santa Clara, CA, USA). The sense primer

was CTAGAAACACCAGGAAGGCCA GCAAGAAAATAGCAACAGAG AAACC; the antisense primer was GGTTT CTCTGTTGCTATTTTCTTGCTGCCT TCCTGGTGTCTTAG.

A fragment of the 3'UTR of human ER-α36 gene, which harbors the putative *let-7* target sites, was synthesized (IDT, Coralville, IA, USA). With the *SpeI* and *HindIII* restriction sites at the ends, respectively, the sense strand is 5'-CTAG TGTTCCCTAGAAACACCAGGAAGGCC CTACCTCAAATAGCAACAA-3' and the antisense strand is 5'-AGCT TTGTT GCTATTTGAGGTAGGCCTTCTGTT GTTTCTAGGGAACA-3'. The corresponding mutant sequences were synthe-

sized as shown in Figure 1A. The sense and antisense oligonucleotides were annealed and ligated into the *SpeI*/*HindIII* sites of the pMIR-Report luciferase vector. The sequences were verified by DNA sequencing.

Luciferase Assay

Transfection was performed in 24-well plate using Lipofectamine 2000 (Invitrogen) in triplicate (23). *let-7* mimics, anti-*let-7* inhibitors, or negative controls were transfected with 0.25 µg luciferase construct and 0.25 µg β-galactosidase plasmid (Ambion) to check transfection efficiency. Twenty-four h after transfection, the luciferase activity was measured using Dual-Glo Luciferase Assay System (Promega) in Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA, USA).

MTT Cell Proliferation Assay

MCF7-Tam cells were transfected with *let-7* mimics or negative control in normal growth medium for 1 d, or in medium with 2.5% charcoal-stripped FBS for 2 d to exhaust endogenous estrogen. Ten thousand cells in the volume of 100 µL were seeded in a 96-well plate for 12 h and assay lasted for another 4 d. For tamoxifen treatment, 1 µmol/L tamoxifen was added into the growth medium. Each treatment had 12 repeats on the plate. Medium volume was increased to 200 µL after the assay began. At certain time points, the cells were incubated in 0.83 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) in growth medium at 37°C for 4 h and lysed in 100 µL of dimethyl sulfoxide at room temperature for 15 min. The absorbance in each well was measured at 490 nmol/L with an ELx800 Absorbance Microplate Reader (Biotek, Winooski, VT, USA). Cell growth was expressed as a fraction of absorbance at d 0.

Statistical Analysis

The correlation between *let-7* miRNA expression and ER-α36 was analyzed in SPSS 16.0 (SPSS Inc., Chicago, IL, USA). For comparison among means of different treatments, one-way analysis of vari-

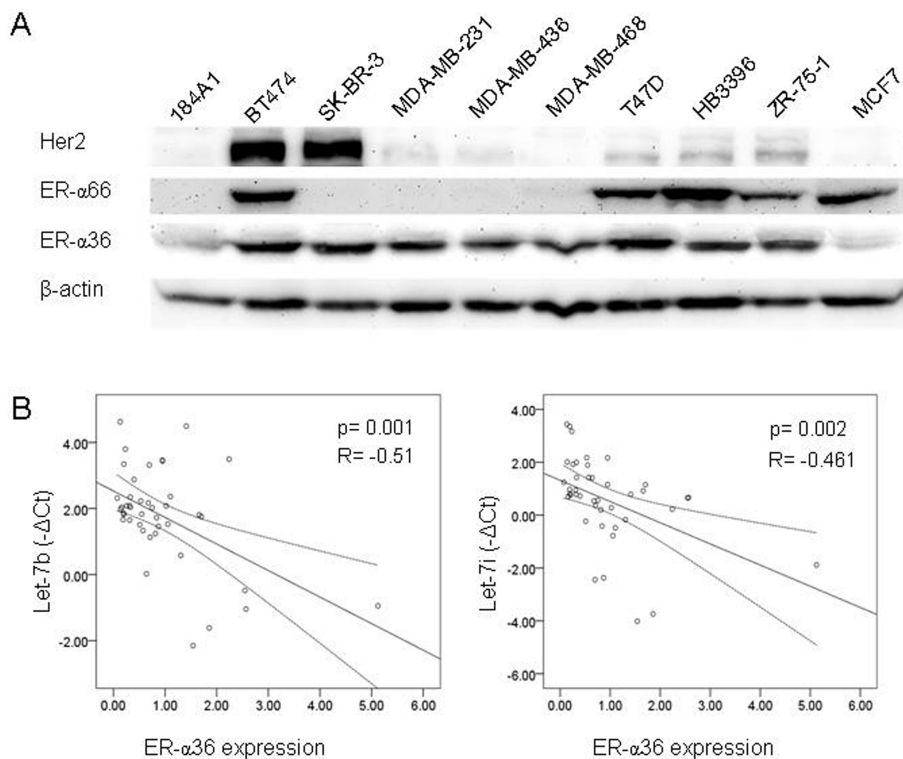


Figure 2. (A) The expression of HER2, ER-α66 and ER-α36 in commonly used breast cell lines. (B) The inverse correlation between the expression of *let-7b*, *let-7i* and the expression of ER-α36 in our collected FFPE breast tissues. Confidence Interval (95% CI) on Pearson's Correlation is shown in each chart.

ance (ANOVA) with the Tukey's adjustment was used for multiple comparisons in SPSS 16.0. * represents $P < 0.05$; ** represents $P < 0.01$.

All supplementary materials are available online at www.molmed.org.

RESULTS

ER-α36 Is Widely Expressed in Breast Cancer Cell Lines

After the cloning and identification of ER-α36 in 2005 (7), its important function has just begun to be recognized. We first checked the ER-α36 expression in some established breast cancer cell lines (Figure 2A) and found that ER-α36 was expressed in both ER-α66 positive and negative cell lines. ER-α36 is highly expressed in ER-α66 negative cell lines: SK-BR-3, MDA-MB-231, MDA-MB-436 and MDA-MB-468. It also was highly expressed in ER-α66 positive cell lines: T47D, HB3396

and ZR-75-1. However, there was only trace expression in the classical ER-α66 positive cell line MCF7 and in normal mammary epithelial cell line 184A1. Figure 2A shows the expression of Her2, ER-α66 and ER-α36 in breast cancer cell lines. Although a similar study was published previously (8), we had an expanded collection of cell lines and checked the expression of Her2 and ER-α66 simultaneously. In addition, the result of T47D is different from that in the previous study.

***let-7* miRNAs Target ER-α36**

Recently, we reported that the *let-7* family miRNAs are involved in regulation of ER-α66 expression (23). To examine if *let-7* miRNAs also regulate ER-α36 expression, we extracted proteins and total RNAs from 45 cases of FFPE breast cancer samples and assessed the expression levels of ER-α36 and *let-7* miRNAs with Western blot and real-time PCR analysis, respectively. We found an in-

verse correlation between ER- α 36 expression and two members of *let-7* family miRNAs (*let-7b* and *let-7i*) (Figure 2B).

A strong complementary match was found between *let-7* miRNAs and 3'UTR of ER- α 36 gene (see Figure 1A); *let-7* miRNAs match the 3,421 to 3,442 region located in the 3'UTR of ER- α 36 gene. Since ER- α 36 is not included in the databases used by the available softwares that predict potential miRNA-mRNA interaction, the complete sequence of ER- α 36 mRNA is shown in Supplementary Table 1, in which the coding sequence, 3'UTR, and *let-7* miRNA binding site are highlighted. The ΔG of the RNA duplex is -15.8 kcal/mole between the 3'UTR of ER- α 36 and *let-7i* (<http://mobyule.pasteur.fr/cgi-bin/portal.py?form=mfold>).

To determine whether ER- α 36 expression is subjected to regulation of *let-7* miRNAs, we employed two strategies for luciferase assay. At first, the putative binding and flanking sequences in the 3'UTR of ER- α 36 was cloned into the 3'UTR of luciferase in the pMIR-REPORT plasmid. Cotransfection assays with 60 nmol/L *let-7* miRNA mimics in MCF7 cells showed that both *let-7b* and *let-7i* potently decreased the luciferase activity, to about 60% of the activity in the control cells transfected with nonfunctional negative control miRNA (Figure 1B). Mutations in the putative *let-7* miRNAs binding sequences in the 3'UTR of ER- α 36 abrogated the inhibitory effects of these miRNAs (see Figure 1B). The effect of *let-7* inhibitors also was tested in cotransfection assays in the normal mammary epithelial 184A1 cell line that expresses high levels of *let-7* miRNAs (24). We found that *let-7b* and *let-7i* inhibitors strongly increased the activity (2- to 3-fold) of the luciferase reporter gene carrying the putative binding site from the 3'UTR of ER- α 36 gene (Figure 1C). Again, when the binding site was mutated, *let-7b* or *let-7i* inhibitor could no longer boost the expression of luciferase (see Figure 1C).

With the concern that secondary structure might interfere with the interaction between *let-7* miRNA and ER- α 36 3'UTR (25), we later cloned the entire ER- α 36

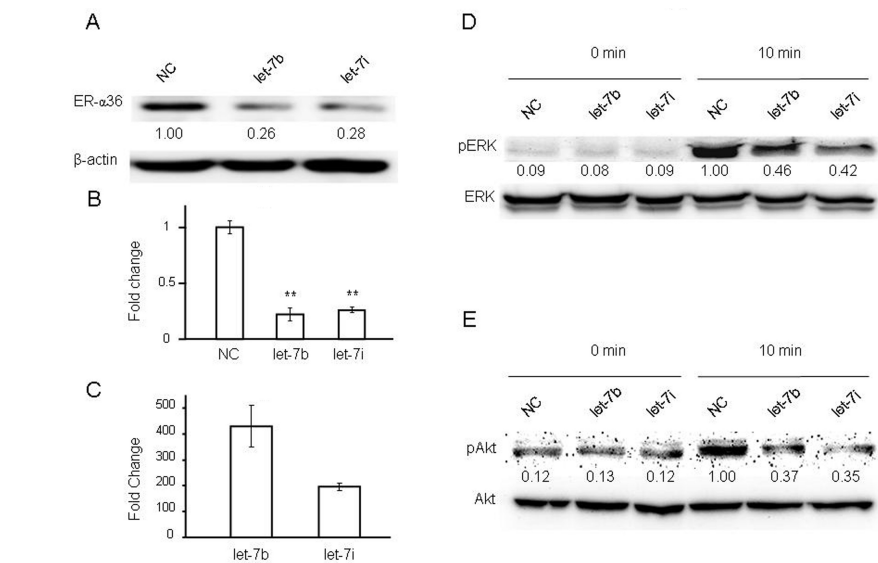


Figure 3. Effect of *let-7* miRNAs transfection on ER- α 36 expression and on nongenomic estrogen signal pathway in MDA-MB-231 cells. The protein level (A) and mRNA level (B) of ER- α 36 were inhibited 2 d after transfection of *let-7b* and *let-7i*. (C) Quantification of *let-7b* and *let-7i* compared with negative control transfections after 2 d using real-time PCR. (D) The nongenomic MAPK pathway was significantly inhibited after transfection of *let-7b* and *let-7i* miRNAs. (E) The nongenomic Akt pathway was inhibited significantly after transfection of *let-7b* and *let-7i* miRNAs. After transfection, MDA-MB-231 cells were cultured in IMEM with 2.5% charcoal-stripped FBS for 2 d and in IMEM with 1 mg/mL BSA for 1 d before 10 nmol/L or vehicle stimulation. **, $P < 0.01$.

3'UTR of about 4 kb into pMIR-REPORT plasmid. The primer regions are highlighted in Supplementary Table 1. Similar results were generated with the entire 3'UTR of ER- α 36 (Figures 1D, E). In MCF7 cells, *let-7* miRNAs could inhibit the luciferase activity (about 50% of control). However, when the binding site was mutated (see Figure 1A), there was no more inhibition by *let-7* miRNAs (see Figure 1D). In 184A1 cells, *let-7b* or *let-7i* inhibitors could boost the luciferase activity about 2.5-fold. However, when the binding site was mutated, there was no more increase in luciferase activity when *let-7* inhibitors were added (see Figure 1E).

Effect of *let-7* MiRNAs on ER- α 36 Expression and Nongenomic Signal Pathways

To further determine the inhibitory effects of *let-7b* and *let-7i* on expression of endogenous ER- α 36, we transfected their miRNA mimics separately into ER- α 36 negative breast cancer MDA-MB-231 cells

that express high levels of ER- α 36. We found that transfection of both *let-7b* and *let-7i* strongly inhibited ER- α 36 at the protein level compared with cells transfected with negative control miRNA (Figure 3A). At the mRNA level, real-time PCR analysis indicated that the two miRNA mimics had similar inhibitory effect on ER- α 36 mRNA expression (30% of the controls transfected with the negative control miRNA) (Figure 3B), indicating that *let-7* miRNAs inhibited ER- α 36 expression mainly through destabilizing ER- α 36 mRNA, which is in agreement with the previous study (26). The expression levels of *let-7b* and *let-7i* after 2 d of miRNA mimic transfection was shown in Figure 3C. *let-7b* achieved ~400-fold increase and *let-7i* reached ~200-fold increase compared with the negative control cells.

ER- α 36 is mainly localized on the plasma membrane and mediates the nongenomic estrogen pathways (7,8). We reasoned that the inhibition on ER- α 36 expression by *let-7* family miRNAs might

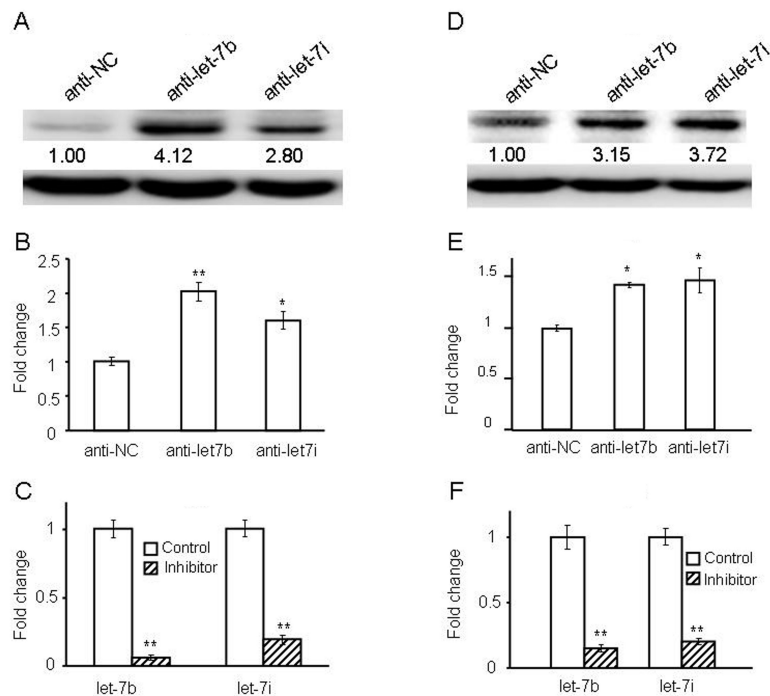


Figure 4. Effect of *let-7* inhibitors on ER- α 36 expression in MCF7 and 184A1 cells. The protein level (A) and mRNA level (B) of ER- α 36 were promoted 2 d after transfection with 200 nmol/L of anti-*let-7b* or anti-*let-7i* in MCF7 cell line. (C) Successful knockdown of *let-7b* and *let-7i* after transfection of anti-*let-7b* and anti-*let-7i* in MCF7 cells for 2 d. (D,E,F) The same as (A,B,C), respectively, except the experiment was performed in 184A1 cells.

dampen the nongenomic estrogen pathway. To test this hypothesis, we examined the activation of the MAPK/ERK and PI3K/Akt signaling pathways by estrogen in cells transfected with *let-7* miRNAs. The MDA-MB-231 cells transfected with *let-7b* and *let-7i* mimics or negative control were cultured in 2.5% charcoal-stripped FBS for 48 h and starved in serum-free medium for another 24 h. The transfected cells then were treated with 10 nmol/L of estradiol (E2) and the activation of ERK1/2 and Akt was examined with Western blot analysis using phos-ERK and phos-Akt specific antibodies. Transfection of *let-7b* and *let-7i* significantly inhibited estrogen-induced activation of the ERK1/2 (Figure 3D) and Akt (Figure 3E), presumably through downregulation of ER- α 36 expression. This result indicates that *let-7* miRNAs is involved in regulation of the nongenomic estrogen pathway.

To confirm the result, MCF7 and normal mammary epithelial 184A1 cells were transfected with anti-*let-7b*, anti-*let-7i*

inhibitor or negative inhibitor control. Figure 4 shows that both *let-7* inhibitors significantly enhanced the levels of ER- α 36 protein and mRNA expression (Figures 4A, B, D, E) while they reduced the levels of *let-7b* and *let-7i* miRNAs (see Figures 4C, F) in both MCF7 and 184A1 cell lines.

***let-7* MiRNA and ER- α 36 Expression in the MCF7-Tam Cell Line**

Antiestrogen resistance is a major obstacle in clinical breast cancer treatment. However, the exact mechanism is still unknown. In a tamoxifen-resistant MCF7 cell line, MCF7-Tam, we found that ER- α 36 protein level was greatly increased, whereas ER- α 66 protein was greatly reduced to trace level (Figure 5A). The mRNA level of ER- α 66 in MCF7-Tam was ~30% of parental cell line (Figure 5B). ER- α 36 mRNA expression in MCF7-Tam increased about 14-fold compared with tamoxifen-sensitive parental MCF7 cells (Figure 5C). Meanwhile, *let-7* miRNA lev-

els in MCF7-Tam cells were reduced significantly to less than half of the levels in parental MCF7 cells (Figure 5D). Thus, there is an inverse correlation between ER- α 36 and *let-7* miRNA expression.

We then examined whether transfection of *let-7* miRNAs could decrease the ER- α 36 expression in MCF7-Tam cells. We found that transfection of the *let-7* miRNA mimics significantly decreased both protein and mRNA levels of ER- α 36 (Figures 6A, C). This inhibition lasted for 6 d (Figures 6B, C). Transfection of *let-7b* and *let-7i* mimics achieved ~600- and ~300-fold increase of levels of *let-7b* and *let-7i* 2 d after transfection, and 100-fold 6 d after transfection compared with negative control (Figure 6D).

Recently, it was reported that the enhanced expression of ER- α 36 is involved in tamoxifen resistance (12). We then examined the tamoxifen sensitivity in tamoxifen-resistant MCF7-Tam cells transfected with *let-7* miRNAs or negative control miRNA. When grown in complete medium, which contained estrogen, no difference was detected between *let-7* transfection and negative control transfection (Figure 6E) with tamoxifen in the medium. However, when grown in E2-free medium, those cells transfected with *let-7b* or *let-7i* grew significantly slower than those transfected with negative control miRNA when tamoxifen was added in the medium (Figure 6F), which demonstrates that *let-7* miRNAs increase tamoxifen sensitivity by decreasing ER- α 36 expression. This hypothesis was validated further by the coectopic expression of ER- α 36 construct, which lacks 3'UTR, so that *let-7* miRNAs can no longer suppress its expression, and *let-7* miRNAs (see Figure 6F). When either *let-7b* or *let-7i* was co-transfected together with ER- α 36 expression vector, the sensitivity toward tamoxifen caused by high expression of *let-7* miRNAs was largely nullified by high ectopic expression of ER- α 36 protein (see Figure 6F). Therefore, the tamoxifen resistance in the MCF7-tam cell line is mainly due to high expression of ER- α 36, which is at least partially caused by low expression of ER- α 36 suppressor *let-7* miRNAs.

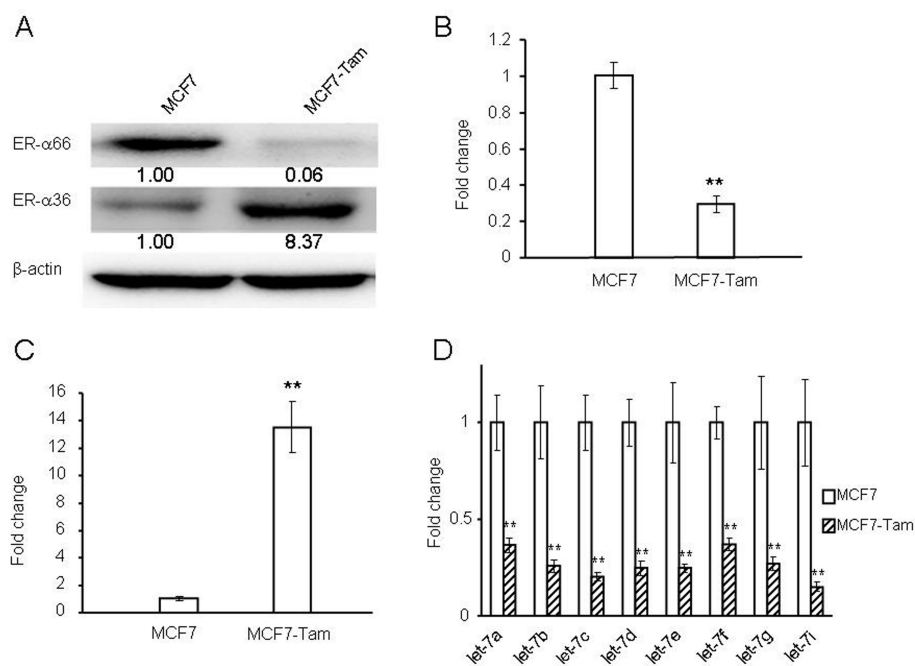


Figure 5. (A) ER- α 66 protein expression is inhibited and ER- α 36 protein expression is enhanced in MCF7 tamoxifen resistant (MCF7-Tam) cell line compared with parental MCF7. (B) mRNA of ER- α 66 is reduced in MCF7-Tam cell line. (C) mRNA of ER- α 36 is enhanced in MCF7-Tam cell line. (D) All *let-7* miRNAs are downregulated in MCF-7 Tam cell line. **, $P < 0.01$.

DISCUSSION

ER- α 36 is the product of a transcript initiated from the promoter located in the first intron of ER- α 66 gene. ER- α 36 is expressed in tumor tissues from ER- α 66 positive and negative breast cancer patients (12). Thus, breast cancer patients who are clinically characterized as ER-negative (lack of the nuclear ER- α 66 expression) may still express ER- α 36 (12,8). ER- α 36 inhibits the transcription regulatory function of ER- α 66 and ER- β (8) while ER- α 66 downregulates the promoter activity of ER- α 36 (27). We also found that ER- α 36 was expressed in both ER- α 66 positive and negative cell lines (see Figure 2A), indicating its important function in breast cancer.

let-7 family miRNAs are the first miRNAs discovered in human (28), and play important roles in maintaining stemness (21). Dysregulated *let-7* miRNA expression is involved in carcinogenesis (29). The expression of *let-7* miRNAs increases with differentiation and cancer development (21). The low level of *let-7* miRNAs in

stem cells is caused by uridylation mediated by Lin28 in concert with TUT4 (30,31). Because cancer cells have similar stemness as stem cells, *let-7* miRNAs are downregulated in many types of cancers, including breast cancer (18–21,32–33). Some targets of the *let-7* family have been found, including Ras, HMGA2 and c-Myc (14–16). Previously, we found that the *let-7* family miRNAs target ER- α 66 and downregulate its expression (24), suggesting that *let-7* miRNAs play an important role in the development of ER- α 66 positive breast cancer. In this study, we present strong evidence to demonstrate that *let-7* miRNAs also regulate a variant of ER- α 66, ER- α 36. The luciferase assay with the reporter gene harboring 3'UTR of ER- α 36 indicated that *let-7* miRNAs directly regulate ER- α 36 expression (see Figure 1). Although overexpression of *let-7* miRNAs could achieve a high level (over 300-fold, see Figure 3C), the inhibition on luciferase activity was less than 50% (see Figures 1B, D) while miRNA inhibitor could boost the luciferase activity 2~3-fold (see Figures 1C, E)

and only inhibited the corresponding miRNA 5-fold (see Figures 4C, F). We offer the following explanations. First, a specific miRNA inhibitor can inhibit a family of miRNAs. We checked the effect of anti-*let-7a*, anti-*let-7b* and anti-*let-7i* on the expression of *let-7a*, *let-7b* and *let-7i* in 184A1 cells (Supplementary Figure 1). Anti-*let-7a* not only inhibited the expression of *let-7a*, but also the level of *let-7b* and *let-7i*. Similar results were seen in anti-*let-7b* and anti-*let-7i*. We can reasonably deduce that the inhibitor for one family member of *let-7* can inhibit the levels of other family members, although the inhibitory effect may vary. In addition, the suppression of luciferase was done in the MCF7 cell line because it has a low expression of *let-7*, and the stimulation of luciferase was performed in the 184A1 cell line because it has a relatively high expression of *let-7*.

The findings of inverse correlation between the expression of *let-7* and ER- α 36 (see Figure 2B), and the downregulation of ER- α 36 by *let-7* miRNA transfection (see Figures 3A, B) demonstrated that *let-7* family miRNAs regulate ER- α 36 expression. Although there are previous studies that ER- α 36 inhibits the transcription regulatory function of ER- α 66 (8) while ER- α 66 downregulates the promoter activity of ER- α 36 (27), we do not observe any correlation between the expression of ER- α 66 and ER- α 36 in the FFPE sample set (data not shown), consistent with other studies (10,12). The results that *let-7* miRNA transfection impeded the nongenomic estrogen pathway (see Figures 3D, E) and enhances tamoxifen sensitivity in MCF7-Tam cells (see Figure 6) further indicates that *let-7* family miRNAs also regulate estrogen and antiestrogen signaling, presumably through regulation of ER- α 36 expression. Previous study shows that the knockdown of GPR30 does not affect nongenomic estrogen pathway, but knockdown of ER- α 36 significantly affects nongenomic estrogen pathway (34). The compound G1, which was regarded as the ligand of GPR30 actually binds to ER- α 36. In addition, ER- α 36 binds to estrogen *in vitro* (34). Our experiment strengthens the concept that ER- α 36 was the membrane es-

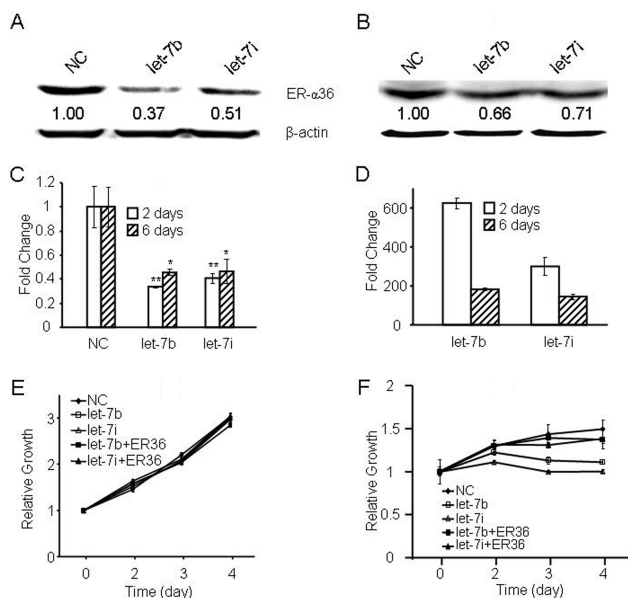


Figure 6. Knock down of ER-α36 by *let-7b* and *let-7i* increased tamoxifen sensitivity in MCF7-Tam cells. The protein level of ER-α36 after 2 d (A) and 6 d (B) post transfection of *let-7b* and *let-7i*. (C) The mRNA level of ER-α36 after transfection of *let-7b* and *let-7i*. (D) The forced overexpression of *let-7b* and *let-7i* compared with negative control transfection detected by real-time PCR. (E) The transfection of *let-7b* and *let-7i* does not affect tamoxifen sensitivity of MCF7-Tam in normal growth medium with 10% FBS. (F) Transfection of *let-7b* and *let-7i* significantly enhanced tamoxifen sensitivity of MCF7-Tam in growth medium with 2.5% charcoal-stripped FBS. The resistance to Tam could be restored by simultaneous cotransfection with ER-α36 construct without 3'UTR. *, $P < 0.05$; **, $P < 0.01$.

trogen receptor which mediates nongenomic estrogen pathway, because knock-down of ER-α36 by *let-7* significantly decreases the phosphorylation of ERK and Akt, which are critical components of MAPK and PI3K pathways (see Figure 3).

Tamoxifen is the most successful adjuvant therapy drug for ER-positive breast cancer patients. It is well known that tamoxifen acts as both agonist and antagonist of estrogen signaling. The acquired and *de novo* tamoxifen resistance in breast cancer patients is a significant clinical problem. Our current study revealed that MCF7-Tam cells exhibits enhanced expression of ER-α36 and decreased expression of ER-α66 (see Figures 5A–C), suggesting that ER-α36 is involved in tamoxifen resistance. Previous study showed that tamoxifen acts as an agonist in ER-α36-expressing cells by activation of the MAPK/ERK signaling pathway (8), and patients who have expression of both ER-α66 and ER-α36 will benefit less

from tamoxifen treatment (12). Here, we found that *let-7* family miRNAs are downregulated in MCF7-Tam cells that

have an enhanced expression of ER-α36 (see Figure 5D). The transfection of *let-7b* and *let-7i* miRNA not only inhibited the expression of ER-α36, but also enhanced the tamoxifen sensitivity in MCF7-Tam cells (see Figure 6F). The tamoxifen resistance can be restored by simultaneous overexpression of ER-α36 construct, which lacks 3'UTR (see Figure 6F). Therefore, our study indicates that downregulation of *let-7* miRNAs causes upregulation of ER-α36, which causes tamoxifen resistance. ER-α36 is an important predictive marker for tamoxifen therapy in ER-α66 positive breast cancer patients. In fact, tamoxifen can cause more endurable ERK phosphorylation than estrogen in ER-α36 expressing cells (8). In our previous study, we conclude that there is an inverse correlation between the expression of ER-α66 and *let-7* miRNAs when we compared benign tissues and ER-α66 positive breast cancer tissues (24). We did not check whether this inverse correlation exists between ER-α66 positive and negative breast cancer tissues because we did not have enough ER-α66 negative breast tissues (24). In this study, we observe a direct relationship between ER-α66 and *let-7* miRNAs between MCF7 and MCF7-Tam cells (see Figure 5A, B, D). This is not surprising because *let-7* miRNAs may

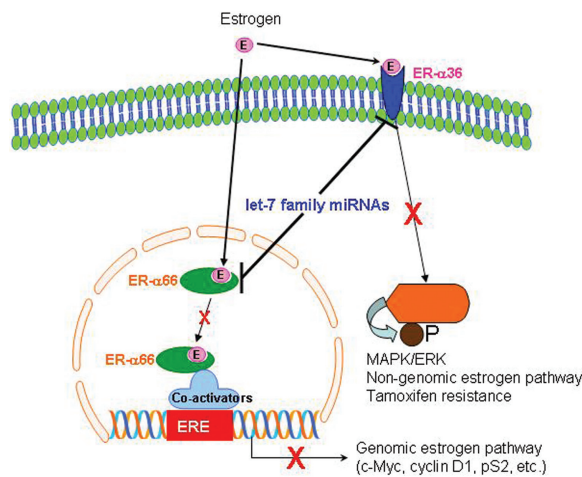


Figure 7. A schematic diagram about the regulatory role of *let-7* miRNAs on ER-α66 mediated genomic estrogen signaling and ER-α36 mediated nongenomic estrogen signaling in breast cancer.

help to upregulate the expression of ER- α 66 during the transition of normal cells into breast cancer cells, but may not be decisive in the downregulation of ER- α 66 during the process of ER- α 66 positive breast cancer into ER- α 66 negative breast cancer due to the fine tuning nature of the regulatory function of miRNAs (35,36). The mechanisms for downregulation of the expression of ER- α 66 in this process are unknown.

Combined with our previous study (24), this study demonstrates *let-7* miRNAs can regulate the expression of both ER- α 66 and ER- α 36 in breast cancer. The downregulation of *let-7* miRNAs in breast cancer causes the upregulation of ER- α 66 and ER- α 36. ER- α 66 mediates the genomic estrogen pathway, which includes the downstream transcription of c-Myc, cyclin D1 and pS2, etc. ER- α 36 mediates the nongenomic estrogen pathway, which includes phosphorylation of ERK and Akt, tamoxifen resistance, etc. Overexpression of *let-7* miRNAs can effectively inhibit these two pathways (see Figure 7).

CONCLUSION

let-7 miRNAs regulate the expression of ER- α 36, resulting in enhanced sensitivity to tamoxifen treatment in breast cancer. *let-7* could be therapeutic target for breast cancer treatment.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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