Supplementary Data

Supplementary Materials and Methods

Cell Culture

Cells were grown under standard conditions. Cell lines were tested for Mycoplasma via PCR using the Universal Mycoplasma Detection Kit (ATCC). MDA-MB-231, MDA-MB-468, BT549 and Hs578T were cultured in DMEM containing 10% FBS. MCF7-Vec and MCF7-HER2 cells were cultured in DMEM containing 10% FBS with insulin. T47D, HCC1954, BT474 and ZR-75-1 were cultured in RPMI containing 10% FBS. MCF7- WT and MCF7-TAMR cells were cultured in phenol red free low glucose DMEM supplemented with 10% FBS, non-essential amino acids and insulin. For estrogen deprivation studies, MCF7 cells were cultured in phenol red free DMEMF12 supplemented with insulin and 10% charcoal stripped FBS. T47D cells were cultured in phenol red free RPMI and 10% charcoal stripped FBS. All cells were cultured at 37°C in a humidified incubator containing 5% CO2.

Real-time PCR analysis

RNA samples were prepared by using Trizol according to the standard protocol. Real-time RT-PCR was carried out using RT2 Fast SYBR® Green/ROXTM qPCR Master Mix (SABiosciences). Data analysis was performed using the 2– ΔCT method for relative quantification, and all sample values were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression value (as the internal reference control).

Western blotting analysis

Protein samples were prepared by using M-PER protein extraction buffer (Cat # 78501, Thermo Scientific) added with 10% protease inhibitor, 2% Sodium Orthovanadate (1mM), 1% Sodium Floride (10mM). Immunoblotting was carried out as previously described (4).). All of the antibodies below were purchased from Cell Signaling. p-STAT3 (Tyr705, Cat. #9145); p-p65 (Ser536, Cat. #3033); p-ERK (Thr202/Tyr204, Cat. #4370); p-AKT (Ser473, Cat. #4058); p-IkB (Ser32, Cat. #2859); p-HER2 (Tyr1221/1222, Cat, #2243); STAT3 (Cat. #4904); p65 (Cat. ##8242); ERK (Cat. #4695); AKT (Cat. #4691); IkB (Cat. #4814); HER2 (Cat. #4290); Src (Cat. #2109) and β-actin (Cat. #3700). GAPDH (Cat. #SC-25778) was purchased from Santa Cruz biotechnology.

Flow Cytometry.

CD24 and CD44 antibodies (BD Biosciences) were used to examine cancer stem cell population. After the treatment cells were harvested and washed with PBS. Cells were then resuspended in 1X PBS+2% FBS solution and counted using 112 Biorad cell counter. 200K cells from each sample were transferred to fresh tube, centrifuged and resuspended in 100uls of 1X PBS+2% FBS. Cells were incubated with CD24-PE and CD44-FITC antibodies for 30min with mixing every 10 minutes. Cells were washed with PBS at the end of incubation, resuspended in 1X PBS+2% FBS and read in BD Accuri C6 flow cytometer.

Immunofluorescence

After indicated treatment, cells were washed with 1X PBS and fixed using 4% paraformaldehyde for 15minutes. Cells were washed thrice with 1X PBS and blocked using 1% Rat Serum, 0.01% triton X-100 and 1X PBS for 1 hour at room temperature. Cells were then incubated overnight with ERα antibody (Santacruz biotechnology) at 4 C. Next day, cells were

washed with PBS thrice and incubated with Alexa fluor 594 goat anti-rabbit IgG H&L (Thermo scientific) for 1 hour at room temperature. After 3 washes with PBS, cells were counterstained with DAPI for 15 minutes and mounted on slide. Cells were then imaged using confocal microscope using cy3 and DAPI channel.

Mammosphere Assay.

Cells were trypsinized and mechanically separated and passed through 40-μm filters to obtain single cell suspensions. Cells were plated at 10,000 cells per plate density in super–low-attachment plates in mammosphere media and cultured for 6-8 days. Quantification of mammosphere numbers was accomplished by transferring in 96 well plate.

Estrogen and progesterone treatment

To examine effect of estrogen and progesterone on gene expression and proliferation cells were first cultured in estrogen deprivation condition for 3-6 days. For estrogen deprivation, MCF7 cells were cultured in phenol red free DMEMF12 supplemented with insulin and 10% charcoal stripped FBS. T47D cells were cultured in phenol red free RPMI and 10% charcoal stripped FBS. Cells were then trypsinzed and seeded in desired cell culture dish and treated with estrogen and/or progesterone for indicated amount of time. To examine effect of miR-489 on estrogen dependent proliferation, estrogen deprived cells were treated with control siRNA, miR-489 mimic or miR-489 inhibitor for 6days followed by MTT assay to examine proliferation and colony formation assays to examine effect on progenitor/cancer stem-like cell populaiton.

Microarray analysis

T47D cells were seeded in 6-well culture dish, treated with 28nM scramble miRNA or miR-489 mimic for 72hrs. RNA was extracted with Trizol reagent, followed by clean-up and DNase I treatment with QIAGEN RNeasy mini kit in accordance with the prescribed protocol

provided with the kit. Quality control was performed with Agilent Bioanalyser before performing microarray. The data were normalized using the default quantile normalization with R/bioconductor package lumi version 3.2.2. The microarray data in this manuscript is available on the GEO database (GSE99728). A subset of identified genes was validated by q-PCR.

Supplementary Figure Legends

Fig. S1. Both MCF7-Vec and HER2 are sensitive to miR-489 mimic. A. Colony formation assay. Cells were treated with indicated microRNA mimics or inhibitors for 72 hours followed by colony formation assay for 7-10 days. **B.** Western Blot analysis of ERα signaling pathways.

Fig. S2: Schematic diagram showing process of miR-489 KO cell line generation.