Supporting Information

Ford et al. 10.1073/pnas.0809516106

Materials and Methods

Cell Lysis and Western Blot Analysis. Cells were directly lysed in 1X Laemmli buffer with 1 mM DTT. Cell lysates were separated according to size on 8–12% SDS-polyacrylamide gels and subsequently electrically transferred to PVDF membranes. Membranes were blocked for 1 hours at room temperature in TBS-Tween (0.01%) with 5% milk. Membranes were incubated with primary antibodies overnight at 4 °C in TBS-Tween (0.01%) with 3% milk, then washed three times for 10 minutes in TBS-Tween (0.01%). Visualization of proteins was performed via the addition of a secondary antibody conjugated to horseradish peroxidase to the membrane, which was then incubated for 1 hour at room temperature in TBS-Tween (0.01%) with 3% milk. Membranes were washed three times for 10 minutes in TBS-Tween (0.01%) and then incubated in ECL and developed with hyperfilm. Scanning was performed using a Bio-Rad (Hercules CA) GS-800 densitometer with Quantity One software.

Antibodies. Antibodies were used at the following dilutions: estrogen receptor α: HC-20 (Santa Cruz Biotechnology) 1:1,000, Wnt-5a: antibody developed in our laboratory against a Wnt-5a sequence with 100% homology between human and mouse 1:1,000 (1), progesterone receptor: 6A1, detects both A and B isoforms (Cell Signaling Technology) 1:1,000, caspase 3: (Cell Signaling Technology) 1:1,000, cleaved caspase 3: Asp-175 (Cell Signaling Technology) 1:1,000, Phospho-ERα (Ser 118): 16J4 (Cell Signaling Technology) 1:1,000, Tubulin: DM1A (Santa Cruz Biotechnology) 1:10,000. All secondary antibodies were from Dako Chemicals and were used at the following dilutions: goat anti-rabbit 1:10,000, goat anti-mouse 1:7,500, rabbit anti-goat 1:7,500.

RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction. RNA extraction was performed in a designated clean RNA area with the addition of 500 μl TRIzol to each sample. A 100-μl quantity of chloroform was then added and samples centrifuged at 4 °C for 10 minutes at 250 g. A 250-μl quantity of isopropanol was added to the clear upper phase and samples centrifuged at 4 °C for 15 minutes at 16,000 g. The supernatant was removed and the pellet was washed in 75% ethanol and resuspended in diethylenepyrocarbonate-treated water. RNA was treated with DNase 1 (Sigma) at 37 °C. The RNA concentration was measured using a Nanodrop Spectrophotometer ND-1000 (Bio-Rad). cDNA was synthesized from 1 μg of total RNA using M-MuLV reverse transcriptase (Fermentas) in a MJ Mini Personal Thermal Cycler (Bio-Rad, Hercules CA). All reverse transcriptase-polymerase chain reactions (RT-PCR) were performed in a designated clean PCR hood. RT-PCR was performed using a master mix containing 5 μl of 10x buffer, 5 μl of 25 mM MgCl$_2$, 1 μl 10 mM dNTP, 1 μl forward primer, 1 μl reverse primer, and 0.2 μl of Taq polymerase (Fermentas) per sample. Primer sequences were as follows. ERα forward: 5′ CAC CCT GAA GTC TCT GGA AG 3′, ERα reverse: 5′ GGC TAA AGT GGT GCA TGA TG 3′, cathepsin D forward: 5′ GTA CAT GAT CCC CTG TGA GAA GGT 3′, cathepsin D reverse: 5′ GGG ACA GCT TGT AGC CTT TC 3′, EBAG9 forward: 5′ GAT GCA CCC ACC AGT GTA AAG A 3′, EBAG9 reverse: 5′ AAT CAG GTT CCA TTG TTC CAA AG 3′, β-actin forward: 5′ TTC AAC ACC CCA GCC ATG TA 3′, β-actin reverse: 5′ TTG CCA ATG GTG ATG ACC TG 3′, Wnt-5a forward: GGA TTG TTA AAC TCA CTC TTC 3′, Wnt-5a reverse: 5′ ACA CCT CTT TCC AAA CAG GCC 3′, PR

forward: 5′ TCA TTA CCT CAG AAG ATT TAT TTA ATC 3′, PR reverse: 5′ ATT GAA CTT TTT AAA TTT TCG ACC TC 3′, murine ERα forward: 5′ CAA GGA GGG AGT GCG TCT GG 3′, murine ERα reverse: 5′ CAT CTA GGA CCA GGT CCT CAG C 3′, pS2 forward: 5′ TTC TAT CCT AAT ACC ATC CAC G 3′, pS2 reverse 5′ TTT GAG TAG TCA AAG TCA GAG C 3′, ERα promoter outer forward 5′ TTC TCC AAA TAA TAA AAC ACC TAC TAA 3′, ERα promoter outer reverse 5′ GTT TTT TTT GGG TTA TTT TTA GTA GAT TTT 3′, ERα promoter inner forward 5′ AGT GGG CCC CTA TTA AAT AAA AAA AAA CCC CCC AAA CC 3′, ERα promoter inner reverse 5′ AGT GGG CCC GTT AAT GTT AGG GTA AGG TAA TAG TTT TTG G 3′. All RT-PCRs were performed at least three times, and controls lacking reverse transcriptase were routinely included to rule out DNA contamination.

DNA Extraction and Bisulfite Treatment. DNA was extracted from cells with digestion buffer (150 mM NaCl, 15 mM Tris-HCl, 1 mM ethylenediamine tetraacetic acid (EDTA), and 0.1% sodium dodecyl sulfate) and 5 μl of proteinase K (20 mg/ml), incubated at 55 °C for 3 hours, and then at 95 °C for 10 minutes to inactivate the proteinase K. Phenol-chloroform extraction was performed, the DNA precipitated in ethanol, and the supernatant removed. Pellets were dried and resuspended in 20 μl of 10 mM Tris (pH 8)-1 mM EDTA buffer containing RNase, and the DNA concentration determined using a Nanodrop. A 1-μg quantity of DNA was then bisulfite treated using the EpiTect Bisulfite Kit according to manufacturer's instructions (Qiagen).

Methylation-Specific PCR. Bisulfite-treated DNA was subjected to methylation-specific PCR (MSP) for the internal control gene, MYOD1, and two regions in the ERα promoter that contain large CpG islands. The MYOD1 gene contains no regions of possible methylation and therefore serves as an internal control. Reactions were prepared in a clean PCR hood with reagents as described above. Primers for MSP were as follows. MYOD forward: 5′ CCAACTCCAAATCCCCTCTCTAT 3′, MYOD reverse: 5′ TGATTAATTTAGATTGGGTTTAGAGAAGGA 3′, MSP1 unmethylated (ER) forward: 5′ TTTTGGGATTG-TATTTGTTTTTGTTG 3′, MSP1 unmethylated (ER) reverse: 5′AACAAAATACAAACCATATCCCCA 3′, MSP1 methylated (ER) forward: 5′ TTTTGGGATTGTATTTGTTT-TCGTC 3′, MSP1 methylated (ER) reverse: 5′ AACAAAATA-CAAACCGTATCCCCG 3′, MSP2 unmethylated (ER) forward: 5′ ATGAGTTGGAGTTTTTGAATTGTTT 3′, MSP2 unmethylated (ER) reverse 5′ ATAAACCTACACAT-TAACAACAACCA 3′, MSP2 methylated (ER) forward 5′ CGAGTTGGAGTTTTTGAATCGTTC 3′, MSP2 methylated (ER) reverse 5′ CTACGCGTTAACGACGACCG 3′.

Fulvestrant Treatment. MDA-MB-231 cells were grown in normal media and treated with the antiestrogen Fulvestrant (Sigma) at a concentration of 10 μM. Cells were then stimulated, or not, with either recombinant Wnt-5a or Foxy-5 peptide for 120 hours. At the end of the experiment, lysates were taken as described in Cell Lysis and Western Blot Analysis, and Western blots were performed for PgR and ERα expression.

Luciferase Assay. MDA-MB-231 cells were transfected at 90% confluency in 10-cm plates using Lipofectamine 2000 (Invitrogen) with 3 μg of cytomegalovirus controlled *Renilla* reporter gene (2) and 17 μg of pGudLuc7ERE luciferase reporter

plasmid, which contains four EREs controlling the MMTV promoter and the luciferase gene (kindly provided by Michael Denison, University of California, Davis (3). Cells were allowed to rest overnight, then split into 24-well plates. Three hours later cells were stimulated with either Wnt-5a or Foxy-5 for 48 hours. Cells were then lysed in triplicate wells and analyzed with a dual luciferase reporter assay (Promega). Luciferase activity was normalized against Renilla activity, and readouts were averaged and adjusted to nonstimulated MDA-MB-231 controls. The experiment was performed three times. * $P < 0.05$.

Caspase 3 Activity Assay. MDA-MB-231 cells were grown in normal media and stimulated with recombinant Wnt-5a protein (0.6 μg/ml) or Foxy-5 peptide (100 μM) for 24 or 48 hours. Cells were then treated with Tamoxifen (Sigma) at a concentration of 1 μM for 20 hours. Caspase 3 activity was performed as previously via fluorescent spectrometry using the fluorogenic peptide DEVD-amc (Upstate Biotech) as substrate (4). Floating and adherent cells were lysed in caspase lysis buffer (10 mM Tris-HCl, 10 mM NaH_2PO_4/Na_2HPO_4, 130 mM NaCl, 1%

Triton-X-100, 10 mM NaPPi), and 50-μl triplicates added to the reaction wells with 200 μl HEPES buffer and 3 μl of DEVD-amc. Reactions were incubated at 37 °C for 1 hours and analyzed on a FLUOstar plate reader (BMG Lab Technologies). The total protein content of each lysates was measured using the Coomassie Plus Protein Assay, and readouts were averaged and adjusted accordingly. The experiment was performed six times and results averaged.

Nuclear Staining for Analysis of Apoptotic Cells. MDA-MB-231 cells were plated on cover slips and allowed to adhere. Wnt-5a (0.6 μg/ml) or Foxy-5 (100 μM) were added for 24 or 48 hours. Cells were then treated with tamoxifen (5 μM) for the last 20 hours. MCF-7 cells were used as a positive control. The cells were fixed for 15 minutes in ice-cold paraformaldehyde (4%), washed, and incubated in the dark with 10 μg/ml Hoechst 33342 stain (Invitrogen) for 10 minutes. The cells were washed with phosphate-buffered saline and mounted with Dako Cytomation fluorescent mounting medium. The morphology was analyzed with Nikon E800 Eclipse Microscope with 60× objective.

1. Jönsson M, Dejmek J, Bendahl PO, Andersson T (2002) Loss of Wnt-5a protein is associated with early relapse in invasive ductal breast carcinomas. *Cancer Res* 62:409–416.
2. Dejmek J, Säfholm A, Kamp Nielsen C, Andersson T, Leandersson K (2006) Wnt-5a/Ca2+-induced NFAT activity is counteracted by Wnt-5a/Yes-Cdc42-casein kinase 1alpha signaling in human mammary epithelial cells. *Mol Cell Biol* 26:6024–6036.
3. Rogers JM, Denison MS (2000) Recombinant cell bioassays for endocrine disruptors: Development of a stably transfected human ovarian cell line for the detection of estrogenic and anti-estrogenic chemicals. *In Vitro Mol Toxicol* 13:67–82.
4. Säfholm A, et al. (2008) A Wnt-5a-derived hexapeptide Foxy-5 inhibits breast cancer metastasis in vivo by targeting cell motility. *Clin Cancer Res* 14:6556–6563.
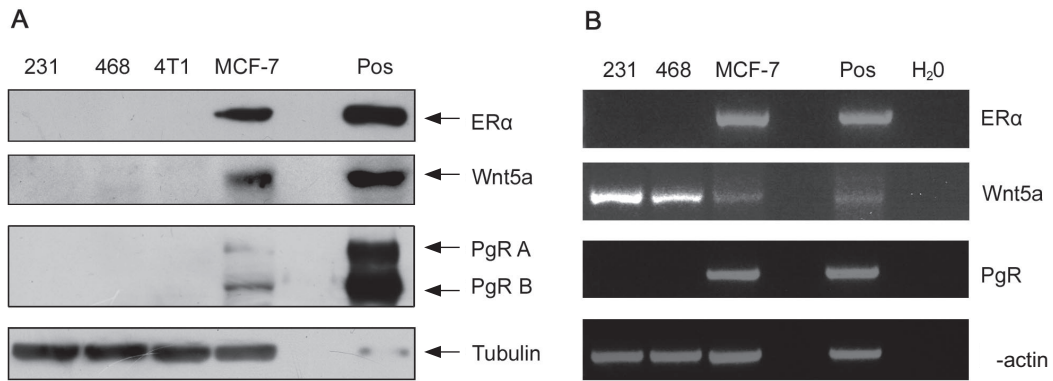
Fig. S1. Basal expression of ERα, PgR, and Wnt-5a in experimental cell lines. (*A*) Protein lysates from cells grown in culture were analyzed via SDS/polyacrylamide gel electrophoresis (PAGE) and Western blotting for proteins of interest. Tubulin expression was used as a loading control. (*B*) RNA was extracted from cell lines and subjected to cDNA synthesis and RT-PCR for our genes of interest. β-Actin expression was used as a housekeeping gene. The negative controls represent water controls. The T47D human breast cancer cell line was used as a positive control for both protein and mRNA analysis, as it is known to express all of the genes of interest for our study. Representative blots from at least four separate experiments are shown.
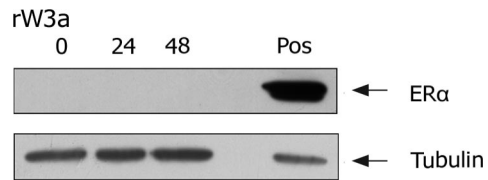
Fig. S2. Not even a high concentration of recombinant Wnt-3a up-regulates ERα. Breast cancer cells were grown in normal media and stimulated with 0.6 μg/ml recombinant Wnt-3a protein (rW3a). After treatment, cells were lysed and subjected to SDS/PAGE, transferred to PVDF membranes, and blotted for ERα expression. Tubulin expression was used as a loading control. T47-D cells expressing ERα were included as a positive control (Pos) to confirm the correct band size for ERα. This control experiment was performed twice, and a representative blot is shown.
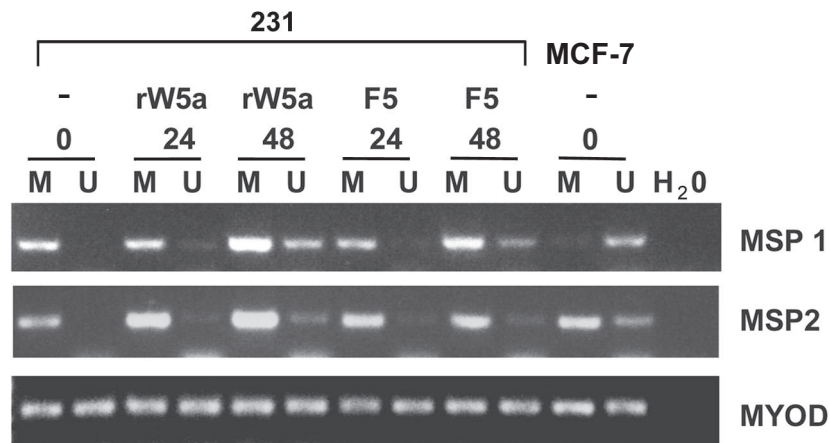
Fig. S3. Recombinant Wnt-5a and Foxy-5 reduce methylation of the ERα promoter. MDA-MB-231 cells were grown in normal media and stimulated with 0.6 µg/ml recombinant Wnt-5a protein (rW5a) or 100 µM Wnt-5a derived Foxy-5 peptide (F5), for 24 or 48 hours. After treatment, DNA was extracted from each sample and subjected to bisulfite modification. Bisulfite-treated DNA was then subjected to methylation-specific PCR using two set of primers to detect methylated (M) and unmethylated (U) ERα products. PCR for the MYOD1 gene lacking CpG islands served as an internal control. MCF7 cells, reported to be mostly unmethylated in these regions, served as a positive control. Representative agarose gels from four separate experiments are shown.
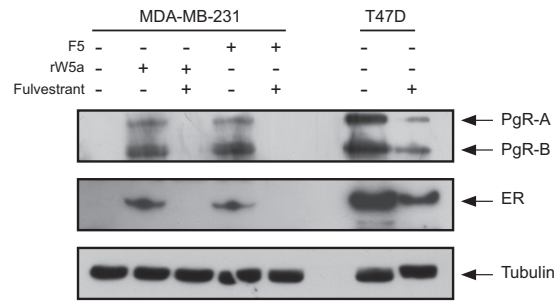
Fig. S4. Recombinant Wnt-5a and Foxy-5 reduce methylation at specific sites. Excel (Microsoft Corporation) file of bisulfite genomic sequencing (BGS) results. Numbers represent percentage methylation at a given cytosine. M, methylated. UM, unmethylated. DM, demethylated.

Fig. S5. Recombinant Wnt-5a and Foxy-5 do not directly up-regulate PgR. MDA-MB-231 cells were grown in normal media and either left untreated or treated with the antiestrogen Fulvestrant at a concentration of 10 μM. Cells were then stimulated, or were not stimulated, with either recombinant Wnt-5a (0.6 μg/ml) or Foxy-5 peptide (100 μM) for 120 hours. The PgR- and ERα-positive T47D human breast cancer cell line was used as a positive control. At the end of the experiment, lysates were analyzed via SDS/PAGE for PgR, ERα, and tubulin expression. Representative blots from three separate experiments are shown.
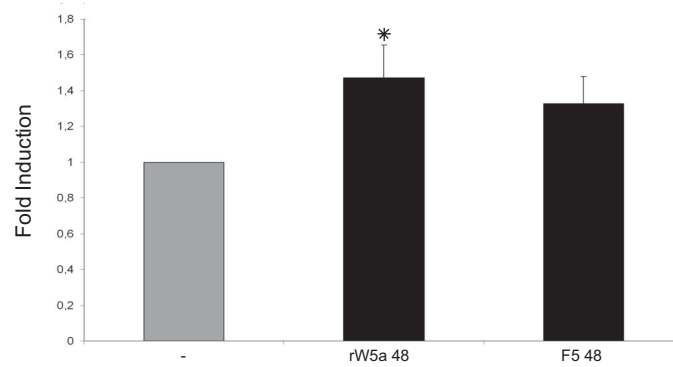
Fig. S6. Recombinant Wnt-5a and Foxy-5 activate ERα signaling as measured via luciferase assay. MDA-MB-231 cells were transiently transfected at 90% confluency in 10 cm plates with a pGudLuc7ERE luciferase reporter construct and a cytomegalovirus-controlled Renilla reporter gene, and then stimulated with either recombinant Wnt-5a (0.6 μg/ml) or Foxy-5 (100 μM) for 48 hours. Cells were lysed and analyzed with a dual luciferase reporter assay. Luciferase activity was normalized against Renilla activity, and readouts were averaged and adjusted to nonstimulated MDA-MB-231 controls. Values represent the mean of three separate experiments, with error bars representing standard deviation. * $P < 0.05$.
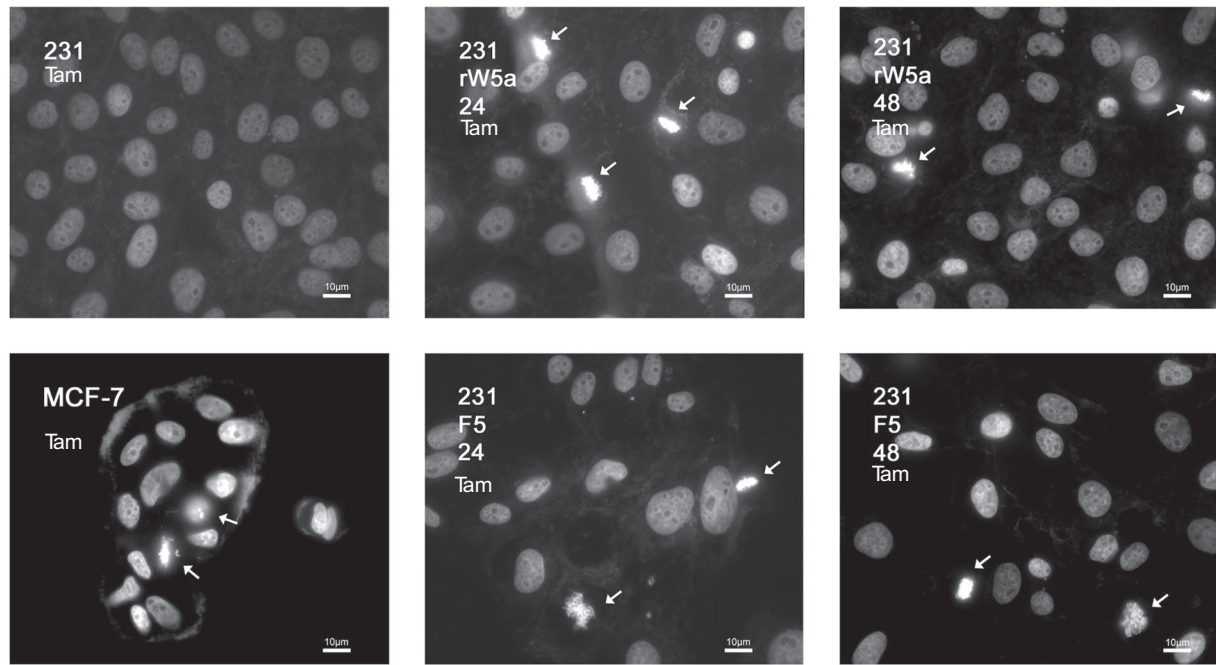
Fig. S7. Recombinant Wnt-5a and Foxy-5 increase the number of apoptotic cells after treatment with tamoxifen. MDA-MB-231 cells were grown in six-well plates and stimulated with recombinant Wnt-5a protein (rW5a, 0.6 μg/ml) or the Wnt-5a-derived Foxy-5 peptide (F5, 100 μM), for 24 or 48 hours. Cells were treated with Tamoxifen (5 μM) for the final 20 hours. Treated cells were stained with Hoechst to visually assess apoptotic cells displaying altered nuclear morphology per treatment. Arrows highlight apoptotic cells. Bars represent 10 μM. Pictures are representative of six separate experiments.
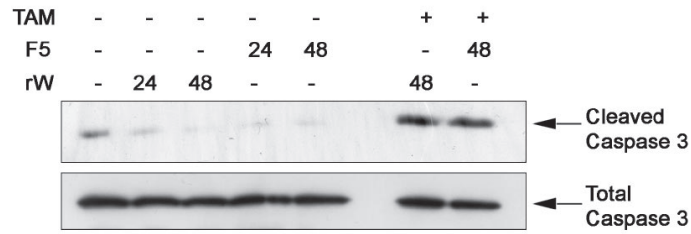
Fig. S8. Recombinant Wnt-5a or Foxy-5 alone do not increase the level of cleaved caspase-3. MDA-MB-231 cells were grown in normal media and stimulated with recombinant Wnt-5a protein (rW5a, 0.6 μg/ml) or the Wnt-5a derived Foxy-5 peptide (F5, 100 μM), for 24 or 48 hours. After treatment, cells were lysed and subjected to SDS/PAGE, transferred to PVDF membranes, and blotted for cleaved caspase-3 and total caspase-3 expression. MDA-MB-231 cells stimulated with recombinant Wnt-5a or Foxy-5 and then treated with tamoxifen (1 μM) for 20 hours were used as positive controls. Blots are representative of three separate experiments.
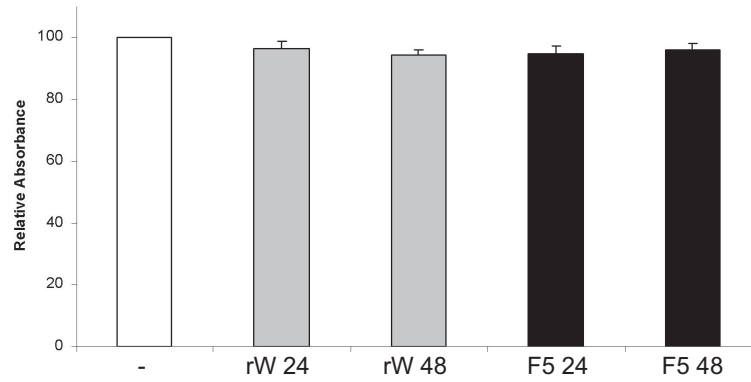
Fig. S9. Recombinant Wnt-5a and Foxy-5 do not inhibit cell proliferation alone. MDA-MB-231 cells were grown in normal media and stimulated with recombinant Wnt-5a protein (rW5a, 0.6 μg/ml) or the Wnt-5a-derived Foxy-5 peptide (F5, 100 μM), for 24 or 48 hours. MTT assays were then performed on cells to assess cell growth inhibition. MCF7 cells were treated with tamoxifen (5 μM) as a positive control. Graphs represent the average of three experiments, with error bars representing standard deviation.