Supplementary Material

Long Non-Coding RNA H19 Acts as an Estrogen Receptor Modulator That is Required for Endocrine Therapy Resistance in ER$^+$ Breast Cancer Cells

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Supplementary Figure S1. Decreasing *H19* expression >70% significantly decreases LCC9 cell viability.

(A) *H19* levels were decreased in LCC9 and LCC2 cells with sh*RNA* fragments using the lentiviral transduction (sh*H19*LCC9, sh*H19*LCC2). Scrambled sh*RNA* expressing cells were used as controls (Sc-LCC9 and Sc-LCC2). *H19* expression was examined in the transduced cells by qPCR normalized to the *GAPDH* transcript levels and the values are represented as fold changes. Average *H19* expression and standard deviation (SD) from 3 independent experiments are shown in the bar graphs.

(B) *H19* transcript levels in the LCC9^{H19low} cells [showing >70% decrease in *H19* levels] and the LCC9^{H19high} cells [showing <70% knockdown in *H19* expression (LCC9^{H19high})] were determined by qPCR and normalized to the *GAPDH* transcript levels and the values are represented as fold changes. Average expression and SD from at least 4 different experiments are shown in the bar graphs. *P<0.05, ***P<0.0005

(C) Effect of Fulvestrant (ICI) on cell viability on LCC9^{H19low} was compared to the LCC9^{H19high}. Vehicle control (ethanol) treated cell viability was set to 100% and average and SD from at least 5 independent experiments are shown in the bar graphs. ***P<0.0001, ****P<0.0005.

Supplementary Figure S2. c-MET signaling regulates *H19* expression in LCC9 cell.

(A) c-MET receptor expression was determined by flow cytometry in MCF-7, LCC9 and the LCC2 cells. A representative histogram is shown and mean fluorescence intensities and SD from 3 independent experiments are shown as bar graphs. (B) LCC9 cells were treated with various concentrations of Tivantinib (TIV) and *H19* expression was

determined after 24 hrs by qPCR. Average *H19* expression (relative to the *GAPDH* transcript levels) and SD from 3 independent experiments are plotted as bar graphs. **P*<0.05, ***P*<0.005, ****P*<0.0001.

Supplementary Figure S3. *NOTCH* regulates *H19* expression in the endocrine therapy-resistant cells but not in the therapy sensitive cells.

(A) LCC9 cells were treated with various doses of a Notch signaling inhibitor RO4929097 (RO) for 24 hrs. *HES1* and *H19* transcript levels were measured by qPCR and normalized to the *GAPDH* transcript levels. Average expression and SD from at least 3 independent experiments are shown in the bar graphs. (B) MCF-7, LCC2, and the LCC9 cells were treated with RO (250 μM) and *H19* expression was determined by qPCR and normalized to the *GAPDH* transcript level. Average expression and SD from 3 independent experiments are shown as bar graphs. (C) $T47D^{ICI\text{-}Res}$ and the $T47D^{Tam\text{-}Res}$ were treated with RO (250 μM), TIV (50 nM) and ICI (100 nM) or 4-OHTam (Tam, 100 nM) for 24 hrs and cell viability was measured. Vehicle control cell viability was set to 100%. Average cell viability and SD from 3 independent experiments are shown as bar graphs. **P*<0.05, ***P*<0.005, ****P*<0.0005, *****P*<0.0005.

Supplementary Figure S4. Significantly reduced *H19* expression reduces ERα expression.

(A) *H19* expression was decreased in the LCC2 cells with sh*RNA* fragments using lentiviral transduction and ERα (*ESR1*) protein expression was measured by flow cytometry. A representative histogram is shown and median fluorescence intensities and

SD from 3 independent experiments are depicted as bar graphs. (B) *ERα* (*ESR1*) transcript levels in the LCC9^{H19low} and the LCC9^{H19high} cells (from Fig. 5A) were determined by qPCR and normalized to the *GAPDH* transcript levels. Average expression and SD from at least 4 different experiments are shown as bar graphs. ***P<0.0005. (C) *ESR1* expression was decreased in the LCC9 cells with sh*RNA* fragments using lentiviral transduction. *ESR1* and *H19* transcript levels were determined by qPCR and normalized to the *GAPDH* transcript levels and the values are represented as fold changes. Average expression and SD from at least 4 different experiments are shown as bar graphs. ***P<0.0005.

Supplementary Figure S5. *H19, NOTCH4 and MET* expression correlates with poor overall survival in breast cancer patients with ER$^+$ tumours.

The survival differences between the high and low risk groups for combined (*H19, NOTCH4, and MET* genes) risk score was assessed by Kaplan-Meier curves and shown for TCGA (A) and METABRIC (B) data sets. The combined risk scores are significantly associated with the overall survival in both datasets representing independent cohorts of ER+ breast cancer patients.

Supplementary Materials and Methods

RT-PCR and quantitative PCR

RNA was obtained using the Trizol reagent (Invitrogen, Thermo Fisher Scientific, USA) and 1 µg of total RNA was reverse transcribed to cDNA using the i-Script cDNA synthesis kit (BioRad, Hercules, CA, USA). The cDNA was then used as a template for quantitative PCR (qPCR, CFX Connect 96, BioRad, Hercules, CA, USA) and transcript levels were determined using gene-specific primers. Relative transcript expression for each gene was normalized to the *GAPDH* transcript levels.

Analysis of Clinical data

Datasets involved in this study were from The Cancer Genome Atlas (TCGA)[1] and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC)[2]. ER+ cases were selected from the TCGA (798 out of 1,098 breast cancer cases) and METABRIC (1,508 out of 1,992 breast cancer cases) data sets and normalized transcript expression levels[3] for *H19*, *MET*, and *NOTCH4* genes were obtained. For METABRIC data, the normalized gene expression levels from the European Genome-Phenome Archive (https://ega-archive.org/dacs/EGAC00001000484) were obtained.

The association between *H19, NOTCH4 and MET* transcript expression and patient's overall survival was determined using the Cox's proportional hazards (COX-PH) model[4] and then the coefficients extracted from COX-PH models were used to generate a signature risk score by combining the expression information of the three genes in TCGA and METABRIC data separately [Risk score = coeff_1 * H19 gene expressions + coeff_2 * MET gene expressions + coeff_3 * NOTCH4 gene expressions, where coeff_1, coeff_2, coeff_3 are the coefficients of *H19, MET, NOTCH4* extracted from the COX-PH models.

The transcript expression level for each gene and the combined risk score were binarized into high risk and low risk groups using R package xtile function with a probability parameter set to 0.55. The survival differences between the high and low risk groups for each gene as well as their combined risk score were assessed by Kaplan-Meier (KM) curves[5].

Estrogen signaling in breast cancer cells

For estrogen deprived growth culture experiments, MCF7 cells were maintained in PRF-DMEM media supplemented with 5% charcoal-stripped serum (v/v) (2× charcoal/dextran-treated FBS) (estrogen-depleted growth media). The cells were maintained for 2 days and treated with vehicle control or 100nM ICI. RNA was extracted from the cells at (at the indicated time points).

Flow cytometric analysis

Single cell suspensions of MCF-7, LCC9 and LCC2 cells were pre-blocked with 2% FBS-containing HBSS supplemented with 10% human serum for 15mins and stained with anti MET antibody (Cell Signaling Technologies) and anti-Rabbit PE (Biolegend) secondary antibody. MET protein expression was then determined by the Guava8HT Flowcytometer (Millipore). Propidium Iodide dye was used to distinguish dead and live cells. FlowJo software was used to obtain Mean Fluorescence Intensities.

Generation of ICI- and Tam-resistant T47D cells

T47D cells (originally obtained from Dr. Edwards (Baylor College of Medicine, Houston [6]) have been maintained in the current laboratory for > 20 years. These cells were

115 authenticated recently (October, 2016) using STR analyses (Genetica Cell Line Testing,
116 Labcorp, Burlington, NC, USA). All experiments were carried out using cells growing
117 between passages 5-20. T-47D cells were regularly maintained in phenol red free
118 Roswell Park Memorial Institute ((RPMI)-1640, Sigma) medium supplemented with 10%
119 fetal bovine serum (FBS) (Gibco, ThermoFisher Scientific, USA) [7]. To generate
120 endocrine therapy resistant cells, T-47D cells were maintained in phenol red free RPMI
121 (Sigma, Missouri, USA) supplemented with 2% FBS and grown in presence of vehicle
122 control or 100nm ICI or 500nm 4-OHTam for 4 months. The cells were passaged once
123 70-75% confluence was reached and growth medium was replaced every 3 days. During
124 the first few weeks, cell growth was strongly affected by ICI and 4-OHTam treatment.
125 However, their growth rate eventually recovered leading to development of ICI- and
126 Tam- resistant T47D ($T47D^{ICI\text{-}Res}$, $T47D^{Tam\text{-}Res}$) cells. At this point cells were kept in
127 growth medium supplemented with ICI or 4-OHTam until used for experiments.

Supplementary References

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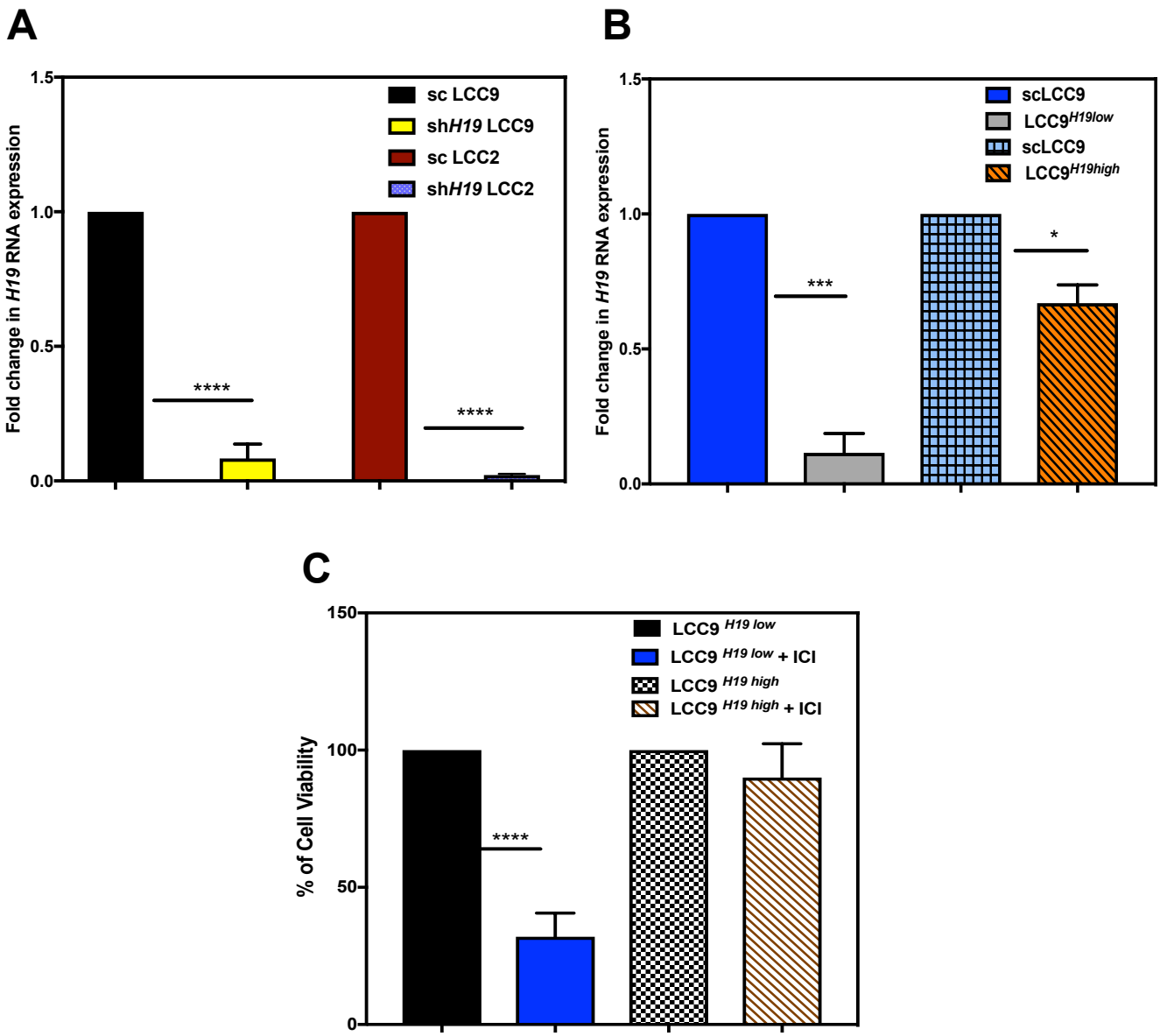
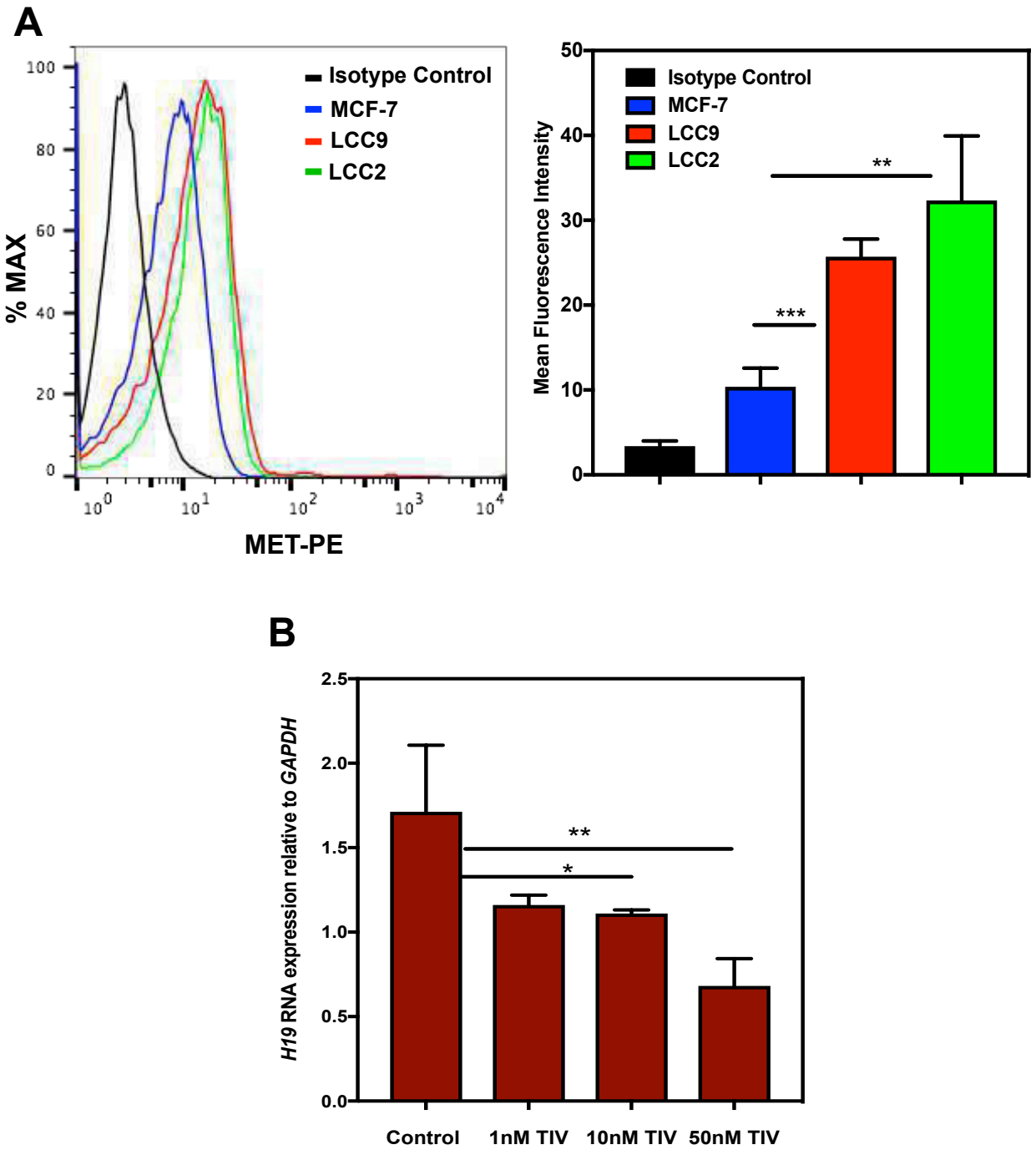
Supplementary Figure S1



Supplementary Figure S2



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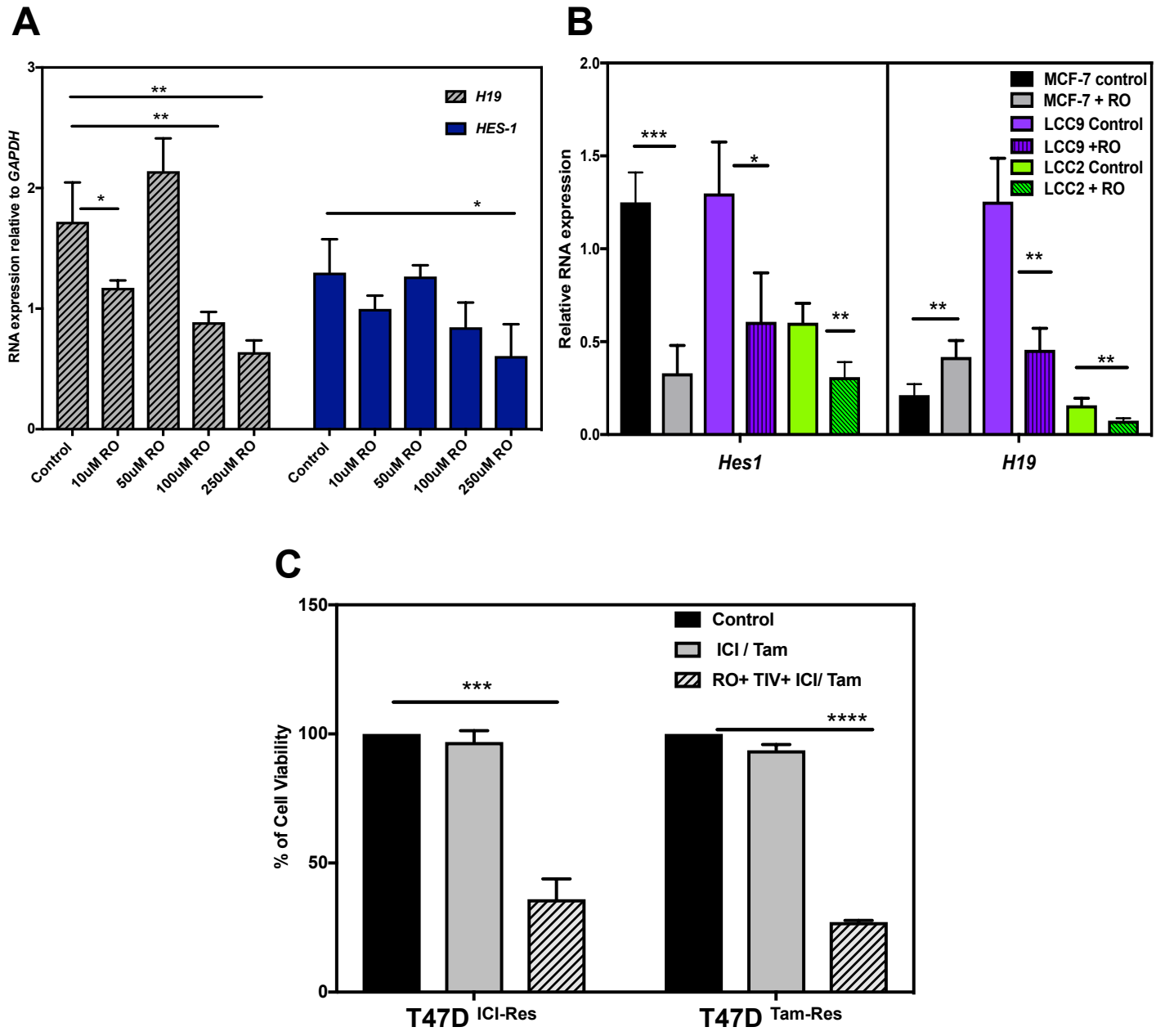
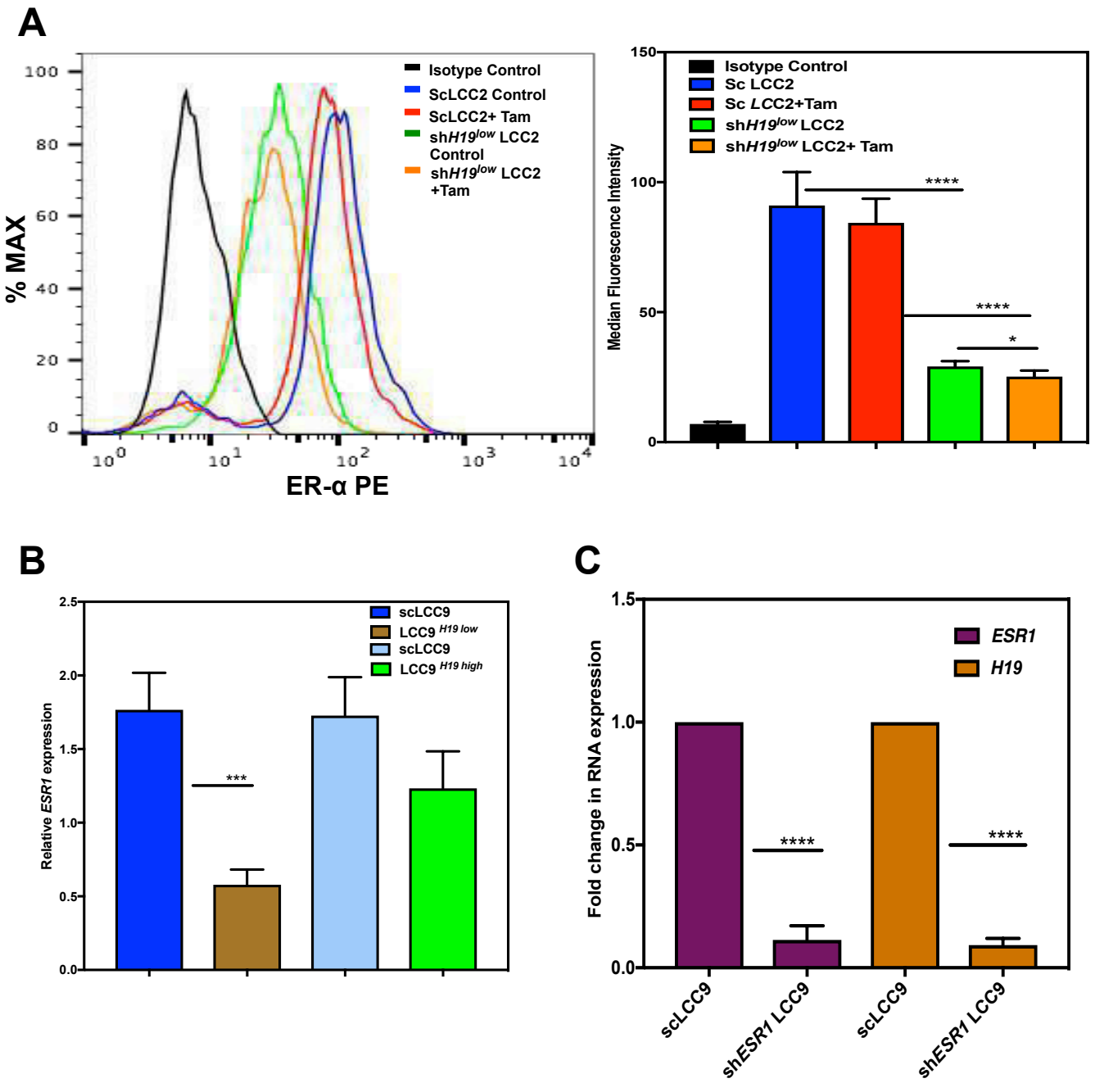


Supplementary Figure S4



Supplemental Figure S5