Supplemental Information

**FOXA2 suppresses endometrial carcinogenesis and epithelial-mesenchymal transition by regulating enhancer activity**

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Supplemental Methods

Endometrial cancer (EC) cell lines

a) Mouse EC cell line (FP) derivation and culture

A mouse EC cell line (FP) was established from a fresh endometrial tumor derived from a one-year-old BAC-Spr2f-Cre; Foxa2L/L, PtenL/L mouse. The tumor was washed with PBS, cut into 2-3 mm fragments with a sterile surgical scalpel and digested in Collagenase V/Dispase II enzymatic solution (0.4 mg/ml Collagenase V and 1.25 U/ml Dispase II: Sigma) in Advanced DMEM/F12 (Thermo Fisher Scientific) at 37°C for 1 hr with intermittent shaking. Tissue digestion was stopped by adding Advanced DMEM/F12 containing 10% FBS (Sigma). The tumor fragments were triturated 15-20 times and centrifuged at 800 rpm for 5 min. The isolated cells were washed twice in Advanced DMEM/F12 and cultured in Advanced DMEM/F12 medium containing 10% FBS and 1% penicillin-streptomycin (Thermo Fisher Scientific) at 37°C, 5% CO₂. The cells were passaged 3 times to obtain pure epithelial cells and the characteristic cobblestone epithelia cell morphology was confirmed by phase contrast microscopy.

b) Human EC cell lines

Human EC cell lines (Ishikawa, MFE-319, EN, AN3CA, HEC-1-A, HEC-1-B, E1, EJ, EFE-184, MFE-280, MFE-296, RL95-2, KLE) were cultured in standard media per ATCC recommendations supplemented with heat-inactivated FBS and antibiotics (50 U/mL penicillin and 50 mg/L streptomycin) in a humidified atmosphere at 37°C containing 5% CO₂. A normal human endometrial epithelial cell line (HEuEC, Lifeline Cell Technology #FC-0078) was maintained in ReproLife reproductive medium (Lifeline Cell Technology #LL-0068). Cell lines were confirmed to be Mycoplasma negative with the Mycosensor PCR assay kit (Agilent Genomics #302108).

c) Lentiviral transduction

To re-express FOXA2, Ishikawa, MFE-319 and FP cells were transduced with pLenti-C-Myc-DDK vector only (ISK-EV, MFE-319-EV, FP-EV) or vector containing human and mouse FOXA2 (ISK-
FOXA2, MFE-319-FOX2, FP-Foxa2) ORF clone (OriGene) following manufacturer’s instructions. Stable cell lines were obtained following puromycin selection. For Myc expression, mouse FP-Foxa2 cells were transduced with pLenti-C-Myc-DDK vector containing mouse full length Myc (FP-Foxa2-Myc) gene (G&P Bioscience) following manufacturer’s instructions. FOXA2 was knocked down in HEC-1-B cell line through a 29-mer FOXA2 specific shRNA (FOXA2<sup>KD</sup> HEC-1-B) and through a 29-mer scrambled shRNA in pGFP-C-shLenti vector (OriGene) with puromycin selection. To enforce E-cadherin protein expression, FOXA2<sup>KD</sup> HEC-1-B cells were transduced with pLenti-C-Myc-DDK vector only (FOXA2<sup>KD</sup> HEC-1-B-EV) or vector containing a human CDH1 (FOXA2<sup>KD</sup>/Lenti-CDH1 HEC-1-B) cDNA (OriGene) per the manufacturer’s instructions.

**Endometrial organoids**

**a) Culture and expansion**

Endometrial organoids were derived from 16-20 week old BAC-Spr2f-Cre; Foxxa2<sup>L/L</sup>, BAC-Spr2f-Cre; Pten<sup>L/L</sup> or BAC-Spr2f-Cre; Foxxa2<sup>L/L</sup>, Pten<sup>L/L</sup> mice. The uteri were washed in PBS, cut into small pieces (2-3 mm) using sterile scalpels and digested in Collagenase V/Dispase II enzymatic solution (0.4 mg/ml Collagenase V and 1.25 U/ml Dispase II: Sigma) in Advanced DMEM/F12 (Thermo Fisher Scientific) at 37°C for 1 hr with intermittent shaking. The digestion mixture was triturated several times to disrupt epithelial glands into single cells. Tissue digestion was stopped by adding Advanced DMEM/F12 containing 10% FBS. Isolated cells were pelleted, resuspended in 98 µl FACS buffer (HBSS + 3% FBS) + 2 µl of FCR blocking buffer (Miltenyi Biotec) and incubated at 4°C for 10 min. After blocking, cells were pelleted and incubated with EpCAM antibody (1:40 dilution, BD Biosciences) at 4°C for 1 hr. Cells were washed, stained with propidium iodide (0.5 µg/ml, BD Biosciences) and used for flow sorting of EpCAM<sup>+</sup>/PI<sup>-</sup> live epithelial cells. FACS sorted epithelial cells were resuspended in Matrigel (Corning) and 20 µl of Matrigel-cell suspension (500 cells in 20 µl Matrigel) was plated in individual wells of a 48-well
plate and incubated at 37°C for 5 min to solidify. The dome containing cells was overlaid with 250 µl of organoid expansion medium (Advanced DMEM/F12 supplemented with pen/strep [1%], Glutamax [2mM, Thermo Fisher Scientific], insulin-transferrin-selenium [1%, Thermo Fisher Scientific], nicotinamide [1mM, Sigma], N-2 supplement [1%, Thermo Fisher Scientific], B-27 supplement [2%, Thermo Fisher Scientific], R-Spondin1 [100 ng/ml, Peprotech], Noggin [100 ng/ml, Peprotech], FGF10 [100 ng/ml, R&D Systems], EGF [50 ng/ml, Peprotech] and Wnt3a [50 ng/ml, Peprotech]) to form organoids with media changed every 3 days.

\[ \text{b) Fixation and staining} \]

For immunostaining, the day 12 organoids were harvested from Matrigel by scraping with a pipette tip, washed in PBS, and fixed in 4% PFA (Electron Microscopy Sciences) for 20 min at room temperature (RT). Following fixation, organoids were gently pelleted and overlaid with 200 µl of low melting agarose (Lonza) at 42°C and permitted to solidify. The agarose plugs containing organoids were embedded in paraffin and 5 µm sections were prepared. The sections were deparaffinized in xylene followed by rehydration through a series of graded ethanol. Antigen retrieval was performed using sodium citrate buffer (pH 6.0, Vector Laboratories). Slides were washed with TBS containing 0.1% Tween 20 and incubated with cytokeratin, E-cadherin and GM130 antibodies (details in the resources table) overnight at 4°C. Sections were washed and incubated with Alexa Fluor secondary antibodies for 1 hr at RT and cover slipped using VECTASHIELD antifade mounting media with DAPI (Vector Laboratories). Images were taken on a Nikon ECLIPSE Ni fluorescence microscope. For 3D z-stack imaging, whole-mount organoids were fixed, permeabilized and stained with antibodies followed by imaging on a Zeiss LSM880 inverted confocal microscope.

\[ \text{c) Proliferation assays} \]

Organoids were harvested from Matrigel using cell recovery solution (Corning) and trypsinized in TrypLE express enzyme (Thermo Fisher Scientific). The cells were resuspended in a 15 µl
Matrigel drop containing 500 cells in the wells of a 96-well plate in triplicate, overlaid with 150 µl of organoid expansion medium and incubated at 37°C containing 5% CO₂. The luminescence signal was recorded with a Tecan Spark 10M microplate reader on day 3, 5, 7 and 10 with Cell Titer-Glo 3D reagent (Promega).

Tumor grafting

All procedures for tumor xenograft mice were approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee. Six- to eight-week-old female NOD scid gamma mice (Jackson Laboratory) were used. Ishikawa (ISK) or FP cells (1 X 10⁶) +/- transduced with FOXA2 lentiviral particles (OriGene, as described above) were injected subcutaneously into the flanks of the mice (n = 4/group) in 200 µL of sterile DPBS and Matrigel (1:1). Tumor dimensions were measured twice in a week by a digital caliper and volumes were calculated using the formula (length x width²)/2. Tumor weights were determined at the end of the experiment.

Immunohistochemistry and quantification

Human EC TMA slides (US Biomax) were deparaffinized in xylene followed by rehydration in graded alcohols and water. Antigen retrieval was performed using sodium citrate buffer (pH 6.0, Vector Laboratories). Slides were washed with TBS containing 0.1% Tween 20 and incubated with 3% hydrogen peroxide (H₂O₂) in water for 15 min to block endogenous peroxidase. Sections were blocked with 2% BSA in PBS containing 0.1% Triton X-100 for 1 hr at RT. After blocking, tissue sections were incubated with primary antibodies against FOXA2, ERα or PR (details in the resources table) at 4°C overnight. Slides were then washed and incubated with peroxidase-conjugated secondary antibodies (Vector Laboratories) and DAB substrate (Agilent Dako) to detect bound antibodies. Tissue sections were counterstained with hematoxylin. Images were captured using an Aperio AT2 slide scanner (Leica Biosystems) and analyzed using Aperio
ImageScope software. Immunohistochemistry intensity score (H-score) was calculated from pixel intensity values (the sum of 3 x % of pixels with strong staining + 2 x % of pixels with moderate staining + 1 x % pixels with weak staining).

**Immunostaining**

Ishikawa and HEC-1-B cells were cultured on glass slides, fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 20 min and processed for immunofluorescence. Cells were permeabilized in PBS containing 0.5% Triton X-100 for 10 min at 4\(^\circ\)C, rinsed in PBS/Glycine buffer (PBS containing 0.1 M Glycine), blocked in immunofluorescence buffer (130 mM NaCl, 7 mM Na\(_2\)HPO\(_4\), 3.5 mM NaH\(_2\)PO\(_4\), 0.1% BSA, 0.2% Triton X-100, 0.05% Tween 20) containing 10% goat serum and incubated with primary antibodies overnight at 4 \(^\circ\)C. Cells were then washed in TBS containing 0.1% Tween 20, incubated with Alexa Fluor secondary antibodies for 1 hr at RT and cover slipped using VECTASHIELD antifade mounting media with DAPI (Vector Laboratories). Images were captured on a Nikon ECLIPSE Ni fluorescence microscope using NIS-Elements BR software.

**Western blots**

EC cell lines were lysed in ice-cold RIPA buffer (Thermo Fisher Scientific) containing protease and phosphatase inhibitors (Sigma). Following lysis, the protein extracts were centrifuged at 12,000 rpm for 5 min at 4\(^\circ\)C and supernatant was collected. 20 \(\mu\)g of protein was mixed with 4X NuPAGE LDS Sample Buffer (Thermo Fisher Scientific), incubated at 95\(^\circ\)C for 5 min and resolved by 4-12\% NuPAGE gels (Thermo Fisher Scientific). Transfer to PVDF membrane (Sigma) was with the Trans-Blot Turbo Transfer System (Bio-Rad Labs). The membrane was blocked in TBS-T (0.1\% Tween 20 in TBS) containing 5\% skim milk (w/v) for 1 hr at RT and probed with primary antibody at 4\(^\circ\)C overnight. The membrane was washed and incubated with horseradish peroxidase (HRP) conjugated secondary antibody (Amersham) for 1
hr at RT and developed using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific) for detection of HRP and the protein bands were detected by chemiluminescence using a ChemiDoc Imaging System (Bio-Rad Labs). β-Actin or GAPDH were used as loading controls.

**Total RNA isolation, cDNA synthesis and qRT-PCR**

Total RNA was isolated from EC cell lines using the RNeasy Plus Mini Kit (Qiagen) following the manufacturer’s instructions. cDNA was synthesized from 1 µg of total RNA using SuperScript VILO master mix (Thermo Fisher Scientific). The cDNA was amplified using sequence specific primers (see resources table below). Quantitative real-time PCR (qRT-PCR) was performed using RT² SYBR Green ROX qPCR Mastermix (Qiagen) on a QuantStudio Real-Time PCR System (Applied Biosystems) through a pre-incubation step, and 40 amplification cycles. The comparative Ct ($2^{-\Delta\Delta C_{t}}$) method was used to compare the expression level of the target gene with the housekeeping gene (GAPDH) in different groups.

**Library preparation, RNA seq and data analysis**

Total RNA samples were quantified by Qubit fluorometer and submitted to the McDermott Next Generation Sequencing Core at UT Southwestern for library preparation. 1 µg of DNase treated high quality RNA (RIN score ≥ 8 per Agilent 2100 Bioanalyzer) was prepared with the TruSeq Stranded Total RNA LT Sample Prep Kit (Illumina). Total RNA was depleted of its rRNA and fragmented before strand-specific cDNA synthesis. cDNA was poly-A tailed and indexed adapters were ligated. After adapter ligation, samples were PCR amplified, purified with Ampure XP beads, and validated again on the Agilent 2100 Bioanalyzer. Samples were Qubit quantified before being normalized and run on the Illumina HiSeq 2500 using SBS v3 reagents with a read configuration of 75 bp, single end reads. 25-35 million reads were generated per sample.
The Fastq files were subjected to quality check using fastqc (version 0.11.2, http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and fastq_screen (version 0.4.4, http://www.bioinformatics.babraham.ac.uk/projects/ fastq_screen). Fastq files were mapped to Homo Sapiens (hg19, UCSC) and Mus musculus reference genomes (mm10, UCSC) using STAR (1). Gene quantification was performed using featureCounts (2) and the differential expression analysis was performed using edgeR (3).

Only genes with log2CPM >0 and log2FC >1 were considered. Different significance levels were used to define differentially expressed genes depending on the scenarios. Pathway analysis was performed using webgestalt (http://www.webgestalt.org/). The threshold for enriched biological processes in the gene ontology was set to FDR <0.05. The dot plots were generated using ggplot2 (https://ggplot2.tidyverse.org/reference/geom_dotplot.html).

Chromatin Immunoprecipitation (ChIP) and data analysis

The chromatin that was bound by acetylated Histone in ISK-EV and ISK-FOXA2 cells was analyzed. Crosslinking was carried out using 1% formaldehyde at RT. Formaldehyde was quenched with 1.25 M glycine. Cells were washed with PBS, lysed, sonicated, and the chromatin was used in a ChIP reaction using Histone H3 acetyl Lys27 antibody and HighCell# ChIP kit protein G (Diagenode) per the manufacturer’s recommendation (4). The ChIP DNA was reverse crosslinked, eluted and purified (5), and used to prepare next generation sequencing libraries (KAPA Hyper Prep, KAPA Biosystems, MA). The ChIP sequencing (ChIP-Seq) libraries, after appropriate size selection using AMPure XP Beads (Beckman Coulter Sciences, IN) were sequenced with a read-length of 75bp at the UTSW McDermott Sequencing Core. The quality of the raw data was checked using fastqc (v0.11.8) (6). Raw reads were mapped to hg19 using BWA (v 0.7.17-r1188) (7). Low quality and duplicated reads were removed using Picard (2.20.3, http://broadinstitute.github.io/picard). Peak calling was performed by MACS2 (v 2.1.0) (8) using
default parameters with input DNA as negative control. Deeptools (2.4.2) (9) was used to plot average coverage figures and heatmaps. The transcription start site (TSS) positions were obtained from UCSC table browser (https://genome.ucsc.edu/cgi-bin/hgTables). Integrated Genomics Viewer (IGV, v2.8.0) (10) was used to visualize the ChIP-seq peak signals.

**Cell cycle analysis**

Ishikawa (ISK) and FP cells (70-80% confluent) transfected with empty vector (ISK-EV and FP-EV) and transduced with FOXA2 lentiviral particles (ISK-FOXA2 and FP-Foxa2) were fixed in 2% paraformaldehyde solution for 10 min at RT. After fixation cells were washed in PBS and permeabilized in PBS containing 0.1% Triton X-100 for 15 min at RT. Lysates were passed through a 40 µm nylon cell strainer (Fisher Scientific) to remove cell debris, followed by incubated with 50 µg/mL of propidium iodide (PI, BD Biosciences) to stain DNA and 100 µg/mL of RNaseA to remove excess RNA. A total of 1 x 10^6 nuclei from the total population were analyzed on a FACS analyzer with 488 nm LASER line to detect PI-stained nuclei. The percentage of cells within the G0/G1, S and G2/M phases of the cell cycle were determined by analyzing the histogram output using FlowJo analysis software.

**IncuCyte cell proliferation and scratch assay**

The IncuCyte live-cell imaging and analysis system was used for cell proliferation and wound closure assays. FOXA2 +/- Ishikawa and FP cells (5,000 cells/each well of flat bottom 96-well plate) were grown to 90-100% confluency with images acquired every 6 hrs. Cell proliferation curves were determined with IncuCyte software by plotting normalized confluency (ratio of confluency at each 6 hr interval to confluency at time 0).

For wound closure assays, Ishikawa cells were cultured to reach 90-100% confluency and a single homogenous scratch per well of 96-well plate was made with the WoundMaker tool (Essen Bioscience). Cells were washed twice and replaced with complete medium. Images of migrating
cells near the wound were taken every 4 hrs and wound width (distance between leading edges of each cell front) was calculated with the IncuCyte software.

**Cell adhesion assays**

*FOXA2 +/- Ishikawa* cells (150,000) were seeded onto MicroMatrix ECM array slide (Advanced BioMatrix) and allowed to adhere for 16 hrs. Following cell attachment, the slide was washed in DPBS to remove unbound cells. The cells adhered to micromatrix spots after the wash were captured and counted per the manufacturer’s instructions.

**Transwell migration and invasion assays**

HEC-1-B cells (control and *FOXA2* knockdown) were grown till 70-80% confluency and serum starved for 4 hrs. For migration assays, 1 x 10^5 cells in serum-free medium were placed in the upper chamber of 24-well Transwell inserts (8-µm pore size, Fisher Scientific) and complete medium containing 10% FBS was added to the lower chamber. For invasion assays, inserts were coated with Matrigel and DPBS (20 µL Matrigel + 100 µL DPBS), incubated at 37°C for 2 hrs and then dried at RT overnight. Cells in serum-free medium were added to the upper chamber of the insert and medium containing 10% FBS was added to the lower chamber. After 16 hrs, migrated or invaded cells on the insert membrane were fixed in 70% alcohol and stained with crystal violet (0.5%) for 10 min. Cells in the inner chamber were removed with cotton swabs. Images of migrated or invaded cells attached to the membrane were quantified with ImageJ (NIH, USA).
| Antibodies                                    | Source                        | Identifier                        | Dilution                      |
|----------------------------------------------|-------------------------------|-----------------------------------|-------------------------------|
| Rabbit pAb anti-FOXA1                       | Millipore Sigma               | Cat #HPA050505; RRID: AB_2681148  | IHC: 1:500; WB: 1:1000        |
| Rabbit mAb anti-FOXA2 (EPR4466)              | Abcam                         | Cat #ab108422; RRID: AB_11157157  | IHC: 1:500; IF: 1:500; WB: 1:1000 |
| Rabbit pAb anti-H3K27ac                     | Active Motif                  | Cat #39134; RRID: AB_2722569      | 2 µg for 25 µg chromatin/ChIP |
| Rabbit mAb anti-Acetyl-Histone H3 (Lys27) (D5E4) | Cell Signaling Technology    | Cat #8173; RRID: AB_10949503      | WB: 1:500                     |
| Rabbit mAb anti-Histone H3 (D1H2)           | Cell Signaling Technology    | Cat #4499; RRID: AB_10544537      | WB: 1:1000                    |
| Rabbit mAb anti-Estrogen Receptor α (D6R2W) | Cell Signaling Technology    | Cat #13258; RRID: AB_2632959      | IHC: 1:500                    |
| Rabbit mAb anti-Progesterone Receptor A/B (D8O2J) | Cell Signaling Technology   | Cat #8757; RRID: AB_2797144       | IHC: 1:500                    |
| Rabbit mAb anti-c-Myc (Y69)                 | Abcam                         | Cat #ab32072; RRID: AB_731658     | WB: 1:1000                    |
| Rabbit mAb anti-E-Cadherin (24E10)          | Cell Signaling Technology    | Cat #3195; RRID: AB_2291471       | IF: 1:200; WB: 1:1000         |
| Mouse mAb anti-Vimentin (VI-10)             | Abcam                         | Cat #ab20346; RRID: AB_445527     | IF: 1:200; WB: 1:1000         |
| Rabbit mAb anti-β-Catenin (D10A8)           | Cell Signaling Technology    | Cat #8480; RRID: AB_11127855      | IF: 1:200; WB: 1:1000         |
| Rabbit pAb anti-Snail + Slug                | Abcam                         | Cat #ab180714; RRID: AB_2728773   | WB: 1:1000                    |
| Mouse mAb anti-SIP1/ZEB2 (E-11)             | Santa Cruz Biotechnology     | Cat #sc-271984; RRID: AB_10708399 | WB: 1:1000                    |
| Rabbit mAb anti-Cyclin A2 (E1DST)           | Cell Signaling Technology    | Cat #91500; RRID: N/A             | WB: 1:1000                    |
| Rabbit Ab anti-Cyclin B1                    | Cell Signaling Technology    | Cat #4138; RRID: AB_2072132       | WB: 1:1000                    |
| Rabbit mAb anti-Cyclin D1 (SP4)             | Abcam                         | Cat #ab16663; RRID: AB_443423     | WB: 1:1000                    |
| Rabbit mAb anti-Cyclin E1 (D7T3U)           | Cell Signaling Technology    | Cat #20808; RRID: AB_2783554      | WB: 1:1000                    |
| Rabbit mAb anti-GAPDH (14C10)               | Cell Signaling Technology    | Cat #2118; RRID: AB_561053        | WB: 1:4000                    |
| Mouse mAb anti-β-Actin (8H10D10)            | Cell Signaling Technology    | Cat #3700; RRID: AB_2242334       | WB: 1:4000                    |
| Mouse mAb anti-GM130 (Clone 35)             | BD Biosciences               | Cat #610822; RRID: AB_398141      | IF: 1:100                     |
| Rabbit pAb anti-Cytokeratin                 | Abcam                         | Cat #ab9377; RRID: AB_307222      | IF: 1:200                     |
| Rabbit mAb anti-PTEN (138G6)                | Cell Signaling Technology    | Cat #9559; RRID: AB_390810        | IF: 1:200                     |
| Phalloidin-TRITC                            | Millipore Sigma               | Cat #P1951; RRID: AB_2315148      | IF: 1:400                     |
| Phalloidin-FITC                             | Millipore Sigma               | Cat #P5282; RRID: N/A             | IF: 1:400                     |
| Mouse Ab anti-CD326 (Ep-CAM) (Clone G8.8)   | BD Biosciences               | Cat #563214; RRID: AB_2738073     | FACS: 1:40                    |
| Donkey anti-rabbit IgG, HRP linked Ab        | Amersham                      | Cat #NA934; RRID: AB_772206       | WB: 1:1000                    |
| Sheep anti-mouse IgG, HRP linked Ab          | Amersham                      | Cat #NA931; RRID: AB_772210       | WB: 1:1000                    |
| Alexa Fluor 555 goat anti-rabbit IgG (H+L)  | Thermo Fisher Scientific      | Cat #A-21429; RRID: AB_2535850    | IF: 1:250                     |
| Reagents                                      | Source               | Identifier               |
|----------------------------------------------|----------------------|--------------------------|
| Alexa Fluor 555 goat anti-mouse IgG (H+L)   | Thermo Fisher Scientific | Cat #A-21424; RRID: AB_141780 | IF: 1:250 |
| Alexa Fluor 488 goat anti-rabbit IgG (H+L)  | Thermo Fisher Scientific | Cat #A-11008; RRID: AB_143165 | IF: 1:250 |
| ImmPRESS HRP horse anti-rabbit IgG           | Vector Laboratories   | Cat #MP-7401; RRID: AB_2336529 | N/A     |
| Collagenase V                                | Millipore Sigma      | Cat #C9263; CAS #9001-12-1 |
| Dispase II                                   | Millipore Sigma      | Cat #D4693; CAS #42613-33-2 |
| FCR blocking buffer                          | Miltenyi Biotec      | Cat #130-092-575          |
| Propidium Iodide staining solution           | BD Biosciences       | Cat #556463               |
| Advanced DMEM/F12                            | Thermo Fisher Scientific | Cat #12634010             |
| HBSS                                         | Thermo Fisher Scientific | Cat #14170120             |
| Fetal Bovine Serum                           | Millipore Sigma      | Cat #F4135                |
| Penicillin-streptomycin                      | Thermo Fisher Scientific | Cat #15240062             |
| Glutamax                                     | Thermo Fisher Scientific | Cat #35050061             |
| Insulin-Transferrin-Selenium (ITS)           | Thermo Fisher Scientific | Cat #41400-045             |
| Nicotinamide                                 | Millipore Sigma      | Cat #N0636; CAS #98-92-0  |
| N-2 supplement                               | Thermo Fisher Scientific | Cat #17502048             |
| B-27 supplement                              | Thermo Fisher Scientific | Cat #12587010             |
| Recombinant murine R-Spondin-1               | Peprotech            | Cat #315-32               |
| Recombinant murine Noggin                    | Peprotech            | Cat #250-38               |
| Recombinant murine EGF                       | Peprotech            | Cat #315-09               |
| Recombinant murine Wnt-3a                    | Peprotech            | Cat #315-20               |
| Recombinant mouse FGF-10                     | R&D Systems          | Cat #6224                 |
| Matrigel                                     | Corning              | Cat #356231               |
| Cell recovery solution                       | Corning              | Cat #354253               |
| TrypLE Express Enzyme                        | Thermo Fisher Scientific | Cat #12604021             |
| Puromycin dihydrochloride                    | Thermo Fisher Scientific | Cat #A1113803             |
| Hexadimethrine bromide (Polybrene)           | Millipore Sigma      | Cat #H9268; CAS #28728-55-4 |
| Paraformaldehyde 16% solution EM grade       | Electron Microscopy Sciences | Cat #15710               |
| Sea Plaque low melting agarose               | Lonza                | Cat #50100                |
| Antigen unmasking solution, Citrate-based    | Vector Laboratories   | Cat #H3300                |
| Tween 20                                     | Millipore Sigma      | Cat #P1379; CAS #9005-64-5 |
| TBS buffer                                   | VWR Life Science     | Cat #97064-338            |
| Phalloidin, Fluorescein Isothiocyanate Labeled | Millipore Sigma   | Cat #P5282                |
| Vectashield Vibrance Antifade Mounting Media Medium with DAPI | Fisher Scientific | Cat #H-1800             |
| RIPA lysis and extraction buffer             | Thermo Fisher Scientific | Cat #89900               |
| Protease inhibitor cocktail                  | Millipore Sigma      | Cat #P8340                |
| Phosphatase inhibitor cocktail                | Millipore Sigma      | Cat #P5726                |
| NuPAGE LDS Sample Buffer                     | Thermo Fisher Scientific | Cat #NP0007               |
| NuPAGE gels                                  | Thermo Fisher Scientific | Cat #NP0321BOX             |
| PVDF membrane                                | Millipore Sigma      | Cat #IPVH00010            |
| Hydrogen peroxide                            | Fisher Scientific     | Cat #BP2633500; CAS #7722-84-1 |
| SuperScript VILO master mix                  | Thermo Fisher Scientific | Cat #11755050             |
| RT² SYBR green ROX qPCR master mix           | Qiagen               | Cat #330524               |
| Product Name                                                                 | Manufacturer                  | Catalog Number |
|----------------------------------------------------------------------------|-------------------------------|----------------|
| RNase A                                                                    | Thermo Fisher Scientific       | Cat #12091021  |
| Cell strainer snap cap, 5 ml                                               | Fisher Scientific             | Cat #0877123   |
| Cell culture inserts                                                       | Fisher Scientific             | Cat #0877121   |
| Endometrial cancer tissue array                                             | US Biomax                     | Cat #EMC1021   |
| ReproLife™ Reproductive Medium Complete Kit                                 | Lifeline Cell Technology       | Cat #LL-0068   |

### Cell lines

| **Homo sapiens**              | **Source**        | **Identifier or Reference** |
|-------------------------------|-------------------|-----------------------------|
| Ishikawa                      | Millipore Sigma   | Cat #99040201               |
| AN3CA                         | ATCC              | Cat #HTB-111                |
| HEC-1-A                       | ATCC              | Cat #HTB-112                |
| HEC-1-B                       | ATCC              | Cat #HTB-113                |
| RL95-2                        | ATCC              | Cat #CRL-1671               |
| KLE                           | ATCC              | Cat #CRL-1622               |
| EN                            | DSMZ              | Cat #ACC 564                |
| MFE-319                       | DSMZ              | Cat #ACC 423                |
| EFE-184                       | DSMZ              | Cat #ACC 230                |
| MFE-280                       | DSMZ              | Cat #ACC 410                |
| MFE-296                       | DSMZ              | Cat #ACC 419                |
| EL                            | Obtained from M. Takayama | PMID: 7669752 |
| EJ                            | Obtained from M. Takayama | PMID: 12889855 |
| Endometrial (Uterine) Epithelial Cells (HEuEC)                             | Lifeline Cell Technology      | Cat #FC-0078               |

| **Mus musculus**              | **Source**        | **Additional information**  |
|-------------------------------|-------------------|-----------------------------|
| FP                            | This paper        | See 'Mouse EC cell line (FP) derivation and culture' in Methods |

### Commercial Assays

| Assay Name                                   | **Source**   | **Identifier** |
|----------------------------------------------|--------------|----------------|
| RNeasy Plus Mini Kit                         | Qiagen       | Cat #74136      |
| Mycosensor PCR assay kit                     | Agilent Genomics | Cat #302108   |
| ECM Select Array Kit Ultra-36                | Advanced Biomatrix | Cat #5170    |
| Cell Titer-Glo 3D Cell Viability Assay       | Promega      | Cat #G9683      |
| Liquid DAB+, 2-component system, Immunohistochemistry visualization | Agilent Dako | Cat #K346811-2 |
| Super Signal West Dura Extended Duration Substrate | Thermo Fisher Scientific | Cat #34076     |
| HighCell# ChIP kit protein G                 | Diagenode    | Cat #C01010063  |

### Vectors

| **Genes**                                         | **Source** | **Identifier** |
|---------------------------------------------------|------------|----------------|
| FOXA2 shRNA (TCCATCAACAACCTCATGTCTCGGAGCA)        | OriGene    | Cat #TL304478  |
| Foxa2 (NM_010446) mouse tagged ORF clone lentiviral particle | OriGene | Cat #MR227354L3V |
| FOXA2 (NM_153675) human tagged ORF clone lentiviral particle | OriGene | Cat #RC211408L3V |
| E Cadherin (CDH1) (NM_004360) human tagged ORF clone lentiviral particle | OriGene | Cat #RC220731L1V |
| Mouse c-Myc/Myc Full-length Gene in Lentivector, pre-packaged lentiviral Particles | G&P Biosciences | Cat #LEV0011   |
| Human  | Forward (5' to 3') | Reverse (5' to 3') |
|--------|-------------------|-------------------|
| AGR2   | ATTGGCAGAGCAGTTTGTCC | GAGCTGTATCTGCAAGGTTCGT |
| CNTN1  | GCCCCATGACAAAGAAAGAC | CGACATGATCCCAGGTGATT |
| FOXA2  | GAGAAAGAAATCCATAACACCCCC | TTCTTTCCCGGTTCCTTCCTTAT |
| GAPDH  | GCCACATCGCTCACAGACCCCAT | GAAGGGGTCTTGGATGGCAA |
| PCDHA1 | CAACCCTGACTGCGTTACT | GGATACGTGGCGCCACTGCT |

| Mouse  | Forward (5' to 3') | Reverse (5' to 3') |
|--------|-------------------|-------------------|
| Agr2   | TGGACGAATGCCACCACAGT | AGTGATGTCTGCCCTACCC |
| Cdh1   | GTCTCTCATGGCCTTTCG | CTTTATATGGCGCAGGAC |
| Cdh2   | TGAAACGGGCGGATAAAGAG | GGTCCACATATCCTGGT |
| Cntn1  | CCTCATTCCGATTCCTGAAAC | TCCCTGAACGTGACCCAA |
| Ctnnb1 | ATGATTCCGAACCTTGCC | AGCTCCAGTACACCCCTTA |
| Foxa2  | AGCAGAGCCCCAACAAGA | AGAGAGATGGCGGATGGAG |
| Gapdh  | CAACTACATGGTCTACATGTT | CTGCCTCTGGAAAGATG |
| Snai1  | GTCTCTCTTCTGCTCCACC |GGGCTGGCACTGGTCTTC |
| Snai2  | TCCCCATTAGTGACGAAAGA | CCCACTGCTCAGTACATTC |
| Vim    | CAGGGTGAGGAGAAATTGC | CACCTTTCCGTTCAAGGTCAAG |
| Zeb1   | CCATACGAATGGCCGAACCT | ACAACCGCTTTGACCACA |
| Zeb2   | GAGCTTTGACACCAGACTC | TTGCGAGACTGCCCTTGAT |
Supplemental References

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Supplemental Figures and Legends

Supplemental Figure 1. FOXA2 re-expression suppresses MFE-319 cell proliferation.

(A) Western blot shows enforced FOXA2 expression following lentiviral transduction in MFE-319 cells (MFE-319-FOXA2). For negative controls, cells were subjected to lentiviral transduction with empty vector.

(B) Immunofluorescence of FOXA2 (red) and actin (green) in MFE-319-EV and MFE-319-FOXA2 cells confirms expected nuclear localization in the latter. Nuclei were stained with DAPI (blue). Scale bars, 50 µm.

(C) Cell proliferation assay by live cell imaging of MFE-319-EV and MFE-319-FOXA2 cells showing growth suppression by FOXA2. Percent confluency was normalized to time 0 (n=3). Data represents mean ± SEM; ****, P <0.0001, t-test.
A

Enriched GO pathways in up-regulated genes

B

Enriched GO pathways in down-regulated genes
Supplemental Figure 2. Enriched Gene Ontology (GO) pathways in ISK-FOXA2 cells.

(A and B) The dot plots show the identified GO biological pathways. GO analysis was performed on significantly up- and downregulated genes by RNA-seq (fold change > 2, \( P < 0.0001 \), FDR <0.005). The size of the dots represents the number of the genes associated with the GO term and the color of the dots represents the FDR values as shown in the inset.
Supplemental Figure 3. Heatmap of differentially expressed cell adhesion and EMT genes in FOXA2 re-expressing and knockdown cells.

(A) Heatmap shows differentially expressed cell adhesion genes after FOXA2 expression in Ishikawa (ISK) cells.

(B) Heatmap shows differentially expressed EMT genes in FOXA2 knockdown HEC-1-B cells.
Supplemental Figure 4. Confirmation of FOXA2 and PTEN loss along with ERα and PR-A/B in Foxa2/Pten mouse uteri.

IHC panel shows expression patterns of FOXA2, PTEN, ERα and PR-A/B in Foxa2/Pten mouse uteri at 2 and 12 months of age compared to wild-type (WT) mouse. Black dashed line shows luminal epithelium in WT mouse. Tumor cells are marked by an asterisk. le, luminal epithelium; ge, glandular epithelium; scale bars, 50 μm.
Supplemental Figure 5. Confocal z-stack images of FP and FP-Foxa2 organoids.

Brightfield images of day 5 FP-EV and FP-Foxa2 organoids. Organoids were stained with actin (red) and counterstained with DAPI (blue). Confocal z-stack images of 2μm thickness at indicated slices are shown. Scale bars, 50 μm.
Supplemental Figure 6. Foxa2 re-expression suppresses EMT.

(A) Heatmap showing top 50 differentially expressed genes between FP-EV and FP-Foxa2 cells.

(B and C) Enriched up- and downregulated GO pathways in FP-Foxa2 cells. GO analysis was performed with the significantly up- and downregulated genes by RNA-seq (fold change >2, \( P <0.0001\), Log2CPM >0).

(D) Relative fold-change in expression of different EMT markers in FP-Foxa2 vs FP-EV cells (n=3). Data represents mean ± SEM; **, \( P <0.01\); ***, \( P <0.001\); ****, \( P <0.0001\), multiple t-tests.

(E) Protein expression by western blot in FP-EV and FP-Foxa2 cells.

(F) Relative fold change expression of cell adhesion genes (Agr2 and Ctnn1) in FP-Foxa2 vs FP-EV cells (n=3). Data represents mean ± SEM; **, \( P <0.01\); ****, \( P <0.0001\), multiple t-tests.
Supplemental Figure 7. MYC is negatively regulated by FOXA2, with functional consequences.

(A) MYC, and MYC targeted cell cycle checkpoint protein expression in human (ISK-FOXA2) and mouse (FP-Foxa2) EC cell lines by western blot.

(B) Expression of Myc targeted cell cycle checkpoint proteins in FP-Foxa2 cells after lentiviral transduction of Myc.

(C) Growth comparison of FP-Foxa2 and FP-Foxa2-Myc cells by IncuCyte real-time live cell imaging (n=3). Error bars represent mean ± SEM; ****, P <0.0001, t-test.
Supplemental Movie 1 (see separate Supplemental Movie File). Movie shows confocal z-stack images of 2μm thick slices of FP-EV and FP-Foxa2 organoids. Organoids were stained with actin (red) and counterstained with DAPI (blue).

Supplemental Tables (see separate Supplemental Table File)

Supplemental Table 1. List of differentially expressed genes (P < 0.0001, FC > 2, Log2CPM > 0) in ISK-FOXA2 vs ISK cells. FC, fold change; CPM, counts per million reads mapped; LR, likelihood ratio, FDR, false discovery rate

Supplemental Table 2. List of component genes in up- and down-regulated gene ontology (GO) categories.

Supplemental Table 3. List of differentially expressed genes (FDR < 0.01, FC > 2, Log2CPM > 0) in FOXA2KD HEC1B vs HEC1B cells. FC, fold change; CPM, counts per million reads mapped; LR, likelihood ratio, FDR, false discovery rate

Supplemental Table 4. List of differentially expressed genes (P < 0.001, FC > 2, Log2CPM > 0) in FP-Foxa2 vs FP cells. FC, fold change; CPM, counts per million reads mapped; LR, likelihood ratio, FDR, false discovery rate