Supporting Information

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SI Text

Animal Studies. All animal work was performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Fox Chase Cancer Center. Female ovariectomized CB17-*SCID* mice, 6–8 weeks of age, were purchased from Taconic. One week prior to ELT3 cell injection, 17-beta estradiol or placebo pellets (2.5 mg, 90-day release, Innovative Research America) were implanted. For xenograft tumor establishment, 2×10^6 ELT3 cells were bilaterally injected into the rear flanks of the mice. For intravenous injections, 2×10^5 ELT3 or ELT3-Luc cells were injected into the lateral tail vein. Lung metastases were scored from 5-micron H&E-stained sections of each lobe by observers blinded to the experimental conditions. CI-1040 (PD184352) was obtained from Pfizer and was prepared in a vehicle of 10% Cremophore EL (Sigma), 10% ethanol, and 80% water. RAD001 was obtained from Novartis Pharma and was diluted in double-distilled water. CI-1040 (150 mg/kg day by gavage, twice per day) or RAD001 (4 mg/kg per day by gavage) was initiated 1 day post-cell inoculation.

Detection of ELT3 Cells by Real-Time PCR. Mouse blood (0.5 mL) was collected at indicated times by intraocular bleed, and red blood cells were lysed before DNA extraction. At death, the lungs were dissected and stored at -80°C for DNA extraction. Rat and mouse DNAs were quantified by using TaqMan-chemistry based real-time PCR assays. The assay for rat DNA was adapted from the method described by Walker *et al.* (1). The primers amplify a LINE repeat element (AC087102). The assay for mouse is for the gene Anf. The sequences (all 5′ to 3′) for the primers and probes are: Mouse Forward: GGCATCTTCTGCTGGCTCC; Reverse: GGCTA GAACCCTCCCCATTCT; Probe: 6FAM-CACTC-CATCGCTTTATCGCTGCAAGTG-BHQ1. Rat Forward: CAAGACGGATGATCAAAATGTG; Reverse: TCTCT-GTTTTAATCTTTGCCT CTCC; Probe: 6FAM-CCTGC-CAAGGGTATTCTTTTTCCTCATTTAAA-BHQ1. PCR master mix from Eurogentec was used for PCR. Primers and probe concentrations were 500 and 100 nM, respectively. Cycling conditions were 95°C, 15 minutes followed by 40 (2-steps) cycles (95°C, 15 sec; 60°C, 60 sec). Reactions were run using an ABI 7900 HT instrument. Each sample was analyzed using two different amounts of input DNA. Relative quantification was done using the $2^{-\Delta\Delta Ct}$ method (2).

Bioluminescent Reporter Imaging. ELT3 cells were transfected with 3 μg of pCMV-Luc (Invitrogen) using Nucleofection reagent (Amaxa) and selected with G418. Ten minutes prior to imaging, animals were injected with luciferin (Xenogen) (120 mg/kg, i.p.). Bioluminescent signals were recorded at indicated times post-cell injection using the Xenogen IVIS System (Xenogen). Total photon flux at the chest regions and from the dissected lungs was analyzed.

Cell Culture and Reagents. ELT-3 cells (Eker rat uterine leiomyoma-derived smooth muscle cells) were cultured in IIA complete medium supplemented with 15% FBS. Prior to the in vitro experiments, cells were maintained in media supplemented with 10% charcoal-stripped FBS for 3 days and then serum-starved for 24 hours in serum- and phenol red-free medium. 17 beta-estradiol (E_2, 10 nM, Sigma) or PD98059 (50 μM, Cell Signaling Technology) was added to the cells as indicated.

Immunoblotting and Antibodies. Cells were rinsed once in ice-cold PBS and lysed in PTY buffer (50mM Hepes, pH 7.5, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM $Na_4P_2O_7$, and 1% Triton) supplemented with phosphatase inhibitors. Lysates were resolved by SDS/PAGE electrophoresis and transferred onto Immobilon P membranes (Millipore). Cytoplasmic and nuclear fractions were separated using CellLytic Nuclear Extraction Kit (Sigma) before electrophoresis. The following antibodies were used for Western blot analysis: anti-Bim (Affinity BioReagents), anti-S6, anti-phospho-S6 (S235/236), anti-phospho-S6K (T389), anti-phospho-p42/44 MAPK (T202/Y204), anti-p42/44MAPK, anti-ELK1, anti-cleaved caspase 3 (all from Cell Signaling Technology), anti-Ki67 and antismooth muscle actin (Bio-Genex), anti-alpha-tubulin and anti-beta-actin (Sigma), anti-phospho-Raf-1 (S338) (Upstate Biotechnology), and anti-Raf-1 (Santa Cruz Biotechnology). Western blots were developed using horseradish peroxidase-conjugated secondary antibodies and ECL chemiluminescence (Amersham Biosciences).

Immunohistochemistry. Sections were deparaffinized, incubated overnight with primary antibodies at 4°C in a humidified chamber and then rinsed and incubated with biotinylated secondary antibodies for 30 minutes at room temperature. Slides were developed using the Broad Spectrum AEC Histostain-Plus (Invitrogen) or Histostain-Plus kit (Invitrogen), and they were counterstained with Gill's hematoxylin.

Anoikis Assay. ELT3 cells were cultured with or without 10 nM E_2 in serum free and phenol-red free medium supplemented with 10% charcoal-stripped FBS for 24 hours. Cells were harvested, plated onto 60 × 15 mm style Poly-hydroxyethyl methacrylate (PolyHEMA) culture dishes (Corning Incorporated) at a density of 1×10^6 cells/mL with or without E_2. Cell death as a function of DNA fragmentation was detected using Cell Death Detection ELISA kit (Roche Diagnostics).

Thymidine Incorporation. The surviving cells in suspension were plated in triplicate in 24-well plates and allowed to grow adherently for 24 hours. ^{3}H-thymidine (1 μCi) was added to the media and the cells were incubated at 37°C for 6 hours, washed with PBS, and lysed in 0.5 mL of 0.5 N NaOH plus 0.5% SDS. ^{3}H-thymidine incorporation was measured by scintillation counting.

Statistical Analyses. Statistical analyses were performed using Student's *t* test when comparing 2 groups. Results are presented as means + SD of experiments performed in triplicate. Differences were considered significant at $P < 0.05$.

1. Walker JA, *et al.* (2004) Quantitative PCR for DNA identification based on genome-specific interspersedrepetitive elements. *Genomics* 83:518–527.

2. Livak, KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 212-Delta Delta C(T)) method. *Methods* 25:402–408.