Materials and Methods

Animals

All animal–related procedures described were approved by the Tufts Medical Center Institutional Animal Care and Use Committee. Female (C57Bl/6 background, 10 week old) mice were used in this study. The generation of DPM and ERαKO(st) mice have been described previously (1, 2). Littermate transgene-negative wild type (WT) mice were used as controls.

Cell lines and culture methods

Carotid artery smooth muscle cells were cultured from carotid artery explants from WT, DPM and ERαKO(st) mice, as previously described (3), and grown in phenol red-free DMEM with 10% charcoal-stripped bovine growth serum (sBGS) in a humidified atmosphere of 95% air and 5 % CO2 at 37°C. VSMC from passage 7 to 10 were used for the experiments.

For VSMC proliferation assays, cells were plated in 96-well plates at a density of 2500 cells / well with DMEM containing 10% sBGS. Four hours after plating, the media was replaced by DMEM with either 3% sBGS or 5ng/ml PDGF (Life Technologies, Grand Island, NY), and treated with 100 nmol/L 17β-estradiol (E2, Sigma-Aldrich, St. Louis, MO) or EtOH vehicle. In a subset of experiments, okadaic acid (50nmol/L) was added to the culture medium 15 minutes prior to E2 treatment, then PDGF (5 ng/ml) was added 30min after the E2 treatment. The VSMC proliferation was assessed at the specified time points by the Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI) according to the manufacture's instructions. Subculture was

performed in WT and DPM in parallel, and these assays were repeated in three subclones in each genotype.

For migration assays, VSMC (2×10^5) were plated in 6 well plates with DMEM containing 10% sBGS. Twenty four hours after plating, the media was replaced with DMEM with 0.5 % sBGS, and 18 hr after starvation, a single scratch was made with a plastic p200 pipette tip. Cells were rinsed once with PBS to remove non-adhered cells, and incubated in DMEM with 0.5% sBGS containing E2, EtOH vehicle, PDGF or okadaic acid. At 24 hr after the scratch was made, bright-field images of 4 different positions for each condition were captured, and cell migration was measured as the number of cells that had entered the scratch. Each experiment was repeated 4 times.

To assess the effect of E2 on phosphorylation levels of kinases, VSMC (2×10^5) were plated in 6 well plates with DMEM containing 10% sBGS. Twenty four hours after plating, the media was replaced with DMEM without sBGS, and 6 hr after starvation, cells were treated with okadaic acid, 5-100 nmol/L E2 and PDGF as described above. Cells were washed with cold PBS 15 min after PDGF stimulation and then frozen immediately at -80 degrees.

For apoptosis assays, VSMC (2×10^4) were plated with DMEM containing 10% sBGS in 6 well plates where gelatin-coated coverslips were put on the bottom of each well. Twenty four hours after plating, culture media was replaced with DMEM with 0.5% sBGS, and 16 hr after starvation, cells were subjected to treatment with E2 or EtOH followed by stimulation with PDGF (5ng/ml) or sBGS (3%). At 24 hr after stimulation, cells were fixed by 4% paraformaldehyde and subjected to TUNEL staining using a

commercially available kit (In Situ Cell Death Detection Kit; Roche, Basel, Switzerland) as directed by manufacturer. DAPI staining was used for detection of nuclei.

qRT-PCR

To assess the effect of E2 on mRNA expression level of Proliferating Cell Nuclear Antigen (PCNA) in VSMC, cells were plated in 6 well plates with DMEM containing 10% sBGS for 24 hr. Then cells were switched to media containing 0.5% sBGS for 18 hr, treated with 100 nmol/L E2 or EtOH vehicle for 30 min and then with 5ng/ml PDGF, 100 mmol/L acetic acid with 0.1% BSA as vehicle control, or 3% sBGS for 24 hr before RNA was extracted using the RNeasy plus kit (Qiagen, Valencia, CA). cDNA was prepared using the Super Script VILO kit (Life Technologies), and qRT-PCR was performed using Quanti Tect SYBR Green (Qiagen) and the primers: *PCNA:* forward CTAGCCATGGGCGTGAAC, reverse GAATACTAGTGCTAAGGTGTCTGCAT, and *GAPDH*: forward CACTGAAGGGCATCTTGG, reverse CATTGTCATACCAGGAAATGAG.

Gene transfer

To test the role of ERα in VSMC on E2-mediated anti-proliferative effect, the adenovirus encoding both ERα and green fluorescent protein (GFP) (adeno-GFP-ERα) was introduced to VSMC derived from ERα KO mice. Adenovirus encoding GFP only (adeno-GFP) was used as a control. The generation of the adeno-GFP-ERα and -GFP was described previously (4). VSMC (2×10^5) were plated in 6 well plates with DMEM containing 10% sBGS and cultured for 24 hr, and then infected by adeno-GFP-ERα or -

GFP. Sixteen hours later, the media was replaced by DMEM containing 10% sBGS to wash out the adenovirus, and cells were then cultured for 8 hours. The cells were then re-plated in 96 well plates as described above for the VSMC proliferation assay. The efficacy of infection was assessed by counting cells expressing GFP fluorescent. Approximately 60-80 % of cells expressed GFP 24 hr after adeno adeno-GFP-ERα or -GFP infection (data not shown).

To examine the transcriptional transactivation potential of the E2 receptors, VSMC were plated in 12-well plates and cultured in DMEM with 10% sBGS overnight and were transfected with the reporter plasmid ERE-Luc (containing an estrogen response element (ERE) driving expression of the luciferase gene) and β-galactosidase by using Fugene HD (Roche). Following transfection, cells were treated with E2-containing media 24 h prior to harvest for determination of luciferase activity. Cells were lysed in passive lysis buffer (Promega), and luciferase assays (luciferase assay system, Promega) and β-galactosidase assays (Tropix, Bedford, MA) were performed according to the manufacturer's guidelines.

To test the role of protein phosphatase 2 (PP2) Ac in E2-induced inhibition of phosphorylation of Akt, VSMC (1×10^5) were plated in 6-well plates and cultured in DMEM with 10% sBGS, and siRNA (30 pmol) targeting PP2Ac and negative control RNA (Dharmacon, Chicago, IL) were transfected into VSMC by using Lipofectamine RNAiMAX (Life Technologies) according to the manufacture's instructions. Forty-eight hours after transfection, cells were treated with E2 followed by PDGF stimulation as described above, and then cells were harvested 15 min after PDGF stimulation.

Western blotting assay

Western blotting was performed as described previously (4). VSMC were lysed in RIPA buffer or IP buffer (Thermo Scientific, Lafayette, CO) containing protease inhibitor and phosphatase inhibitor cocktail (EMD, LaGrange, IL). Lysates were subjected to SDS-PAGE and then transferred onto PVDF membranes (Thermo Scientific). The membranes were probed using a primary antibody against, phospho-Akt, phospho-ERK, phospho-GSK3α/β, PTEN (Cell signaling, Danvers, MA), α-tubulin (EMD), ERK (Life Technology), GAPDH, ERα (MC20), Akt, PP2Ac, (Santa Cruz Biotechnologies, Santa Cruz, CA), MKP-1, and striatin (BD bioscience, San Jose, CA). The ECL-plus system (GE healthcare, Pittsburgh, PA) was used for detection.

PP2A activity assay

PP2A activity was measured by using PP2A immunoprecipitation phosphatase assay kit (Upstate Biotechnology, Lake Placid, NY). Cells were lysed by M-PER lysis buffer (Thermo Scientific) containing protease inhibitor cocktail. Phosphatase inhibitor was not added into the samples. Each sample (150 μg) was subjected to immunoprecipitation with PP2Ac antibody. Beads containing precipitated PP2Ac were added to a phosphatase reaction with threonine phosphopeptide in a shaking incubator. Samples were then aliquoted into two wells of a 96-well plate, into which malachite green detection solution was added. Plates were incubated for 15 min at room temperature and then read at 650 nm on an automated plate reader. Absorbance data was calculated by using the standard curve which ranges between 0-2000 pmol/L.

Statistical analysis

All data are shown as mean±SEM. Comparison between two groups was analyzed by the two-tailed Student's t-test. Multiple group comparison was performed by ANOVA followed by the Tukey procedure for comparison of means. Values of $P<0.05$ were considered statistically significant.

1. Moens SJB, Schnitzler GR, Nickerson M, Guo H, Ueda K, Lu Q, Aronovitz MJ, Nickerson H, Baur WE, Hansen U, Iyer LK, and Karas RH. Rapid estrogen receptor signaling is essential for the protective effects of estrogen against vascular injury. *Circulation.* 2012; 126: 1993-2004.

2. Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M. Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development*. 2000; 127:4277–4291.

3. Karas RH, van Eickels M, Lydon JP, Roddy S, Kwoun M, Aronovitz M, Baur WE, Conneely O, O'Malley BW, Mendelsohn ME. A complex role for the progesterone receptor in the response to vascular injury. *J. Clin. Invest.*. 2001; 108:611–618.

4. Lu Q, Surks HK, Ebling H, Baur WE, Brown D, Pallas DC, Karas RH. Regulation of estrogen receptor alpha-mediated transcription by a direct interaction with protein phosphatase 2A. *J. Biol. Chem.* 2003; 278:4639–4645.