Supplemental Materials

Supplemental Figure Legends

Figure S1. PRKO mice display differences in glycoproteins and proteoglycans, Related to Figure 1

(A) Histological sections from uteri of vehicle **(a,b)** and hormonally stimulated **(c,d)** control (wild-type) and PRKO mice. Scale, 100μm **(B)** qPCR analysis of extracellular matrix proteins from control and PRKO mice treated with vehicle or hormones. n = 4. In all panels, error bars represent +/- SEM and data was analyzed using an unpaired two-tailed Student-T test. ***p<0.001; ****p<0.0001.

Figure S2. PR LacZ knock-in mice report expression of both PR isoforms, Related to Figure 2

(A) The LacZ reporter gene encoding β-gal was inserted by homologous recombination into exon 1 (E1) of the endogenous murine PR locus directly downstream of the ATG start sites for both PR isoforms, PR-B and PR-A. Dotted lines at the 5' and 3' flanks indicate areas of homology between the genomic locus and the LacZ reporter construct. A floxed neo cassette was used as a selection marker and excised through Cre-mediated recombination. Black arrows delineate the two distinct sets of forward and reverse primers (WT and LacZ) used for genotyping. **(B)** Both WT primers and LacZ primers reveal a 300bp band by PCR. **(C)** Heterozygous ($PR^{LacZ+/-}$) and homozygous ($PR^{LacZ+/+}$) mice are viable and survive into adulthood in a frequency identical to wild-type mice ($PR^{LacZ+/+}$). $PR^{LacZ+/+}$ mice are infertile. Animals depicted are 3 months of age. **(D,E,F)** β-gal positivity (blue) of transverse uterine (D), oviduct (E) and mammary gland

(F) sections (5μm) from $PR^{LacZ+/-}$ and $PR^{LacZ+/+}$ mice following different hormonal treatments as indicated. * indicates areas of enlarged glands after PMSG/HCG injection. Lumen, L; myometrium, M; Scale, 150μm. **(G)** β-gal positivity in the ovary vasculature. β-gal positive endothelial cells (arrowheads); β-gal positive smooth muscle cells (arrows); artery, A; vein, V; lymphatic, L. Scale, 50μm. **(H)** Histological sections of veins and arteries from X-gal stained non-reproductive tissues. Nuclei are stained with nuclear fast red. Thymic epithelial cell, TEC. Scale, 50μm.

Figure S3. Ectopic expression of PR in lung endothelial cells leads to enhanced permeability, Related to Figure 3

(A) Generation of PR^{ECKO} mice was accomplished by mating PRCE mice to constitutive VE-Cadherin Cre mice. **(B)** Presence of VE-Cadherin Cre was determined by a 100bp band using general EIIa Cre primers. A 300bp control band confirmed PCR efficiency. **(C)** VE-cadherin Cre expression (β-gal, blue) in the uterus and ovary. Scale bar, 50 μm. **(D)** Tiled images comparing control and PR^{ECKO} vascular density in the uterus. Vessels were stained for PECAM-1 (green). DAPI (blue) visualized nuclei. Scale bar, 0.5 mm. **(E)** FACS plot demonstrating the percentage of endothelial cells (PECAM+, VE-Cadherin+) isolated from the uterus for examination of Cre recombination. **(F)** PCR of PR expression in uterine endothelial cells from mice expressing VE-Cadherin Cre. PECAM was used as a marker of endothelial purity. **(G)** Transgenic mice containing the 950bp tie1 minimal promoter fragment directs PR expression to the endothelium. **(H)** Transgenic PR was detected in PRTg total lung lysates by Western blot analysis. **(I)** Comparison of transgenic (human) and endogenous (murine) PR in lung and uterus respectively. **(J)** Equal levels of total protein lysate from indicated organs were

evaluated for the presence of the human PR transgene. IgG indicates the level of antibody used for immunoprecipitation. **(K-M)** Quantification of vascular permeability in wild-type (WT) and PRTg mice normalized to respective organ weight. vehicle treated, V; P4 treated, V. n = 6. (**N)** *Ricinus communis agglutinin I* staining (arrows) in wild-type and PRTg vessels of the lungs. Scale, 150μm. In all panels, error bars represent +/- SEM and data was analyzed using an unpaired two-tailed Student-T test. ****p<0.001.

Figure S4. Inhibition of classical permeability mediators does not prevent progesterone mediated permeability, Related to Figure 4

(A) HUVECs infected with a PR lentivirus (green) were treated for 4, 8, and 24 hours with P4. PECAM-1 and VE-cadherin (white) were used to visualize junctions. DAPI (blue) shows nuclei. Arrows indicate areas of junctional disruption. Scale, 50μm. **(B)** P4 treatment (24h) of HUVECs infected with a GFP control construct (green). PECAM and β-catenin (white) were used to visualize junctions. Nuclei are stained with DAPI (blue). Scale, 50μm. **(C)** HUVECS grown on ECIS electrodes visualized in the presence or absence of P4 for 24h. Arrows denote areas of junctional breakdown represented by β-catenin staining (green). PR positive nuclei are stained in blue. **(D-H)** Inhibition of permeability mediators including: Src kinase (SU-6656, 10μM) **(D)**, PI3K (PI-103, 500nM) **(E)**, ROCK (Y27632, 10 μM) **(G)**, and VEGFR2 (SU4312, 10 μM) **(H)** and inhibition of microtubule reorganization (taxol, 100nM) **(F).**

Figure S5. Cell-matrix adhesion proteins are not affected by PR activation, Related to Figure 5.

(A) Western blot of FAK and β1-integrin from HUVECs treated with P4 for times indicated.

Loss of protein upon transcriptional repression of cell-cell adhesion molecules is similar to the kinetics of protein reduction upon P4 treatment, Related to Figure 5.

(B) Western blot depicting the kinetics of endothelial cell-cell junctional protein loss following cyclohexamide treatment.

Figure S6. NR4A2 is not involved in progesterone mediated permeability, Related to Figure 6.

(A) qPCR of NR4A2 following P4 treatment of PR infected HUVECs. Graph represents the average of three independent experiments. **(B)** PR binding peak upstream of the NR4A2 gene following HUVEC treatment with P4. **(C)** ChIP-qPCR of PR binding at the NR4A1 promoter. **(D)** Knockdown of NR4A2 by three independent siRNA constructs. **(E)** HUVEC monolayer resistance following knockdown of NR4A2 and treatment with P4 as indicated. In all panels, error bars represent +/- SEM and data was analyzed using an unpaired two-tailed Student-T test. **p>0.01, ***p>0.001, ****p>0.0001.

Figure S7. Effect of NR4A1 on endothelial cell-cell adhesion molecules and permeability, Related to Figure 7.

(A-C) Densitometry of VE-Cadherin **(A),** Claudin-5 **(B)** and PECAM-1 **(C)** from Westerns blots of HUVEC lysate infected with non-targeting (NT) or NR4A1 siRNA and treated with P4 for indicated times. **(D)** HUVEC monolayer resistance after infection with a NR4A1 adenovirus (MOI 10 and 20). **(E)** Western blot of PECAM, VE-Cadherin, and claudin-5 from HUVECs infected with a NR4A1 adenovirus. GAPDH = loading control. In all panels error bars represent +/- SEM.

Table S1. Mouse models used in this study, Related to Figure 1, 2, 3 and 7. Transgenic lines, genotypes and selected references are indicated,

Mouse Model	Genotype	References
PRCE	$PR^{loxP/loxP}$	Hashimoto-Partyka et al. 2006
PR^{ECKO}	VE-Cadherin Cre; $PR^{loxP/loxP}$	This work
PRKO	EIIa Cre; $PR^{loxP/loxP}$	Hashimoto-Partyka et al. 2006
PR^{LacZ}	PR-LacZ	This work
Tie1-PR	Tg (Tie1-PR)	This work
VE-Cadherin/ROSA	VE-Cadherin Cre; R26R	Alva et al. 2006; Soriano, 1999
NR4A1KO	PGK-Neo Exon 2	Lee et al. 1995
NR4A1-GFP	NR4A1-EGFP/Cre	Moran et al. 2011

Table S2. Gene Ontology terms from the analysis of genes directly repressed by PR, Related to Figure 6.

Term	**Count**	***p*-value**
Regulation of cell proliferation	33	2.15E-09
Response to organic substance	30	1.75E-08
Positive regulation of smooth muscle cell proliferation	8	8.22E-08
Regulation of smooth muscle proliferation	9	8.36E-08
Positive regulation of cell proliferation	21	2.08E-07
Blood vessel development	16	3.96E-07
Vasculature development	16	5.39E-07
Regulation of locomotion	14	7.84E-07
Enzyme linked receptor protein signaling pathway	18	1.22E-06
Angiogenesis	12	2.30 E-06

Extended Experimental Procedures

Mouse Models

PRLacZ mice were generated by insertion of the LacZ gene into exon 1 by homologous recombination directly downstream of the PR-B initiation site on chromosome 9. To facilitate insertion, the PRLacZ reporter construct was flanked by a 1.25 kb (short arm) segment and 6.6 kb segment (long arm) of the endogenous PR locus. The construct also included a floxed neomycin gene for selection. The neomycin gene was later removed by Cre excision through mating PRLacZ mice to EIIaCre transgenic mice (Lakso et al., 1996). Incorporation of the construct by homologous recombination was verified using PCR.

For generation of tie1-progesterone receptor (PRTg) mouse, the transgenic construct consisted of a 950bp tie1 minimal promoter fragment linked to human PR DNA. To distinguish between mouse endogenous PR and the human transgenic PR, a myc-tag was inserted preceding the stop codon of the PR construct. Mice were genotyped by Southern via digestion of total genomic DNA with *Ava*II and probing with an eGFP fragment of tie1-PR construct that distinguishes it from the endogenous PR locus.

Additional mouse lines and their respective genotyping including: VE-cadherin Cre (Alva et al., 2006), floxed PR (Hashimoto-Partyka et al., 2006), PRKO (Hashimoto-Partyka et al., 2006), NR4A1KO (Lee et al., 1995), NR4A1-EGFP/Cre (Moran et al., 2011) and R26R LacZ (Soriano, 1999) have previously been described. For determination of Cre recombination efficiency in PR^{ECKO} mice, endothelial cells were isolated from uterine endometrial tissue. Tissue sheets of endometrium were digested

with collagenase (50ug/ml) (Sigma, St. Louis, MO) and dispase (10ug/ml) (Sigma, St. Louis, MO) for 15min at 37C under agitation. The cell suspension was filtered using a 70μm then 40μm pore size and subjected to PECAM and VE-Cadherin staining (BD Bioscience, San Jose, CA). PCR was used to determine the presence of PR. Primers used included: PR/Cre (Hashimoto-Partyka 2006) and PECAM (5'TGCGATGGTGTATAACGTCACCTC, 5'TGCACCTTCACCTCGTACTCAATC).

Hormone Priming

Days 1-3 mice were injected subcutaneously each day with 100ng 17 β–estradiol (Sigma, St. Louis, MO) in 0.1ml sesame oil; Days 4 and 5 no treatment; Day 6-8, 1μg progesterone (Sigma, St. Louis, MO) and 6.7ng 17 β–estradiol. Inhibitors were concurrently administered with hormonal or vehicle treatment at the following concentrations: icabant (Sigma, St. Louis, MO) was injected ip at 500ug/kg, Sunitinib (Sigma, St. Louis, MO) was given orally (gavage) at 40mg/kg, and mifepristone 100mg/kg subcutaneously (RU486; Sigma, St. Louis, MO). For implantation experiments, pregnant mice (gestational day 3, 7 and 12) received two injections, either RU486, Sunitinib and vehicle, one day apart beginning on the gestational day being studied.

Morphometric analysis of uteri following hormone treatment

Microscopic images from the Zeiss LSM 510 META multiphoton microscope were imported into Image Pro (Media Cybernetics, Bethesda, MD) image analysis software. Cell density and vessel number were calculated by measuring boxed areas of endometrium and counting the number of nuclei and vessels, respectively, in the area of interest.

β-galactosidase staining

Briefly, vibratomed sections (300-400μm) were permeabilized with detergent, rinsed, and incubated with X-gal overnight. 5μm sections were stained with nuclear fast red for nuclei visualization and mounted on slides using Permount (Fischer Scientific, NJ). Bright field images were obtained using an Olympus BX40 microscope (Olympus, PA) with an Olympus F1H033971 camera (Olympus, PA). Images were taken at room temperature and objectives included: 4x UplanFl 0.13, 10xUPlanF1 0.3, 20x UPlanApo 0.8 oil, 40x UPlanApo 1.0 oil, and 100xUPlan Apo 1.35 oil iris Ph3. Images were analyzed using Magnafire software.

Permeability Assays

Mice were injected i.v. with *Lycopersicon esculentum* (Tomato) or *Ricinus communis agglutinin I* (RCA I) (Vector Laboratories, Burlingame, CA) to label the entire vasculature uniformly or mark sites of vascular permeability, respectively. Tissues were fixed by vascular perfusion of 1% paraformaldehyde, sectioned (300μm) on a Vibratome, mounted using 90% glycerol in PBS, and imaged using a Zeiss LSM 510 META multiphoton microscope. Z stack images were analyzed using Zen software (Zeiss, Germany). For albumin extraction, isolated endometrial tissue was incubated in HBSS containing protease inhibitors (Pierce, Rockford, IL) for 30 min. at 4C. Cells were removed by centrifugation at 14,000g for 20 min. and supernatant was run on a mouse albumin ELISA kit according to manufacturers instructions (GenWay Biotech, San Diego, CA).

Immunoprecipitation

Both organs and cells were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycolate, 1 mM EDTA, 1 mM sodium vanadate, 10 mM β-glycerophosphate, and protease inhibitors [1 mM phenylmethanesulfonylfluoride (PMSF), 20 µg/ml leupeptin, and 20 µg/ml aprotinin]). Immunoprecipitation of human PR from PRTg mice was performed using equal amounts of whole tissue extracts, as determined by the DC protein assay reagent (Bio-Rad Laboratories, Hercules, CA), followed by incubation with anti-human monoclonal PR antibody (clone PgR 1294, DAKO; Carpenteria, CA) and proteinA-sepharose beads.

ECIS Reagents

Inhibitors included PI-103 (100nM; EMD Millipore, Billerica, MA), paclitaxel (100nM; Sigma, St. Louis, MO), Y27632 (10 µM; Sigma, St. Louis, MO), SU6656 (10µM; EMD Millipore, Billerica, MA), and SU4312 (10µM; Sigma, St. Louis, MO). The PR lentiviral construct used for HUVEC overexpression of PR was described in detail previously (Goddard et al., 2013). HUVECs were pretreated with inhibitors for 2h before P4 treatment. For Nur77/TR3 functional experiments, a Nur family dominant negative adenovirus was a generous gifts from Dr. Peter Tontonoz and has been described previously (Pei et al., 2006). The NR4A1 overexpression was done using a construct designed by the Tontonoz lab and viral amplification done by ViraQuest (North Liberty, IA). Cells were incubated in adenovirus for 1h in the absence of serum and examined by ECIS 48 h after infection.

Immunocytochemistry of cultured endothelial cells

For immunocytochemistry, HUVECS were seeded onto Lab-Tek II 8-well slides (Thermo Scientific, Rochester, NY) and stimulated with 100nM P4. Cells were fixed for 20

minutes with 4% PFA, permeabilized with 0.1% Triton-X100, and blocked for 1h with 10% donkey serum. Primary antibodies were incubated overnight in 1% serum and included β-catenin (1:350, Sigma, St. Louis, MO), PECAM-1 (1:400, M-20; Santa Cruz Biotechnology, Santa Cruz, CA), VE-cadherin (1:200, C-20; Santa Cruz Biotechnology, Santa Cruz, CA), and ZO-1 (1:500, Clone 1A12; Invitrogen; Grand Island, NY). Alexa Fluor secondary antibodies were incubated for 1h at RT (1:300, Invitrogen, Grand Island, NY). Nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI, 1:1000; Invitrogen, Grand Island, NY). Images were acquired using a Zeiss LSM 520 multiphoton microscope (Zeiss, Germany).

siRNA transfection of HUVECs

Confluent HUVEC monolayers were transfected with a Stealth single siRNA (Invitrogen, Grand Island, NY) to NR4A1 and NR4A2 using siPORT reagent (Ambion, Life Technologies, Grand Island, NY). Briefly, siPORT was incubated for 30 min. at RT with Opti-MEM before addition and 20 min. RT incubation with the siRNA. Cells were washed and incubated in antibiotic free DMEM with 1% FBS followed by the addition of the siRNA mixture. Cells were incubated for 4 hours with the siRNA then washed and replaced with MCDB-131 with 10% charcoal stripped FBS. The procedure was repeated again 48 hours later and cells were used for experiments 48-72 hours after the final transfection. Knockdown efficiency was assessed using qPCR. Negative Control Hi GC siRNA was used as a control (Invitrogen, Grand Island, NY). The NR4A1 siRNA sequence is CACAUGUGCGGACACCAUAAUGCUG.

Chromatin Immunoprecipitation

Cells were then crosslinked with 1% formaldehyde, resuspended in 400 μl of lysis buffer (1% SDS, 20 mM EDTA and 50 mM Tris-HCl (pH 8.0)) containing protease inhibitors (Roche, Indianapolis, IN), and sonicated using Misonix cup-horn sonicator to achieve, on average, 200bp fragments for ChIP-seq and 500bp fragments for ChIP-qPCR. The lysate was diluted with ChIP dilution buffer containing 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA and 16.7 mM Tris-HCl (pH 8.1) and immunoprecipitated with 3 ug of anti-PR or IgG antibody overnight at 4 degrees. The complexes were captured using protein A Dynabeads (Invitrogen, Grand Island, NY) and washed twice with the following buffers: low-salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1); high-salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1) and 500 mM NaCl); LiCl wash buffer (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA and 10 mM Tris-HCl (pH 8.1)) and TE (10 mM Tris-HCl and 1 mM EDTA (pH 8.0)). After elution with 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1% SDS, crosslinks were reversed by overnight incubation at 65°C. Samples were then treated with RNase A for 30 min at 37°C and proteinase K for 2 h at 56°C. DNA was subsequently purified using Qiagen MinElute Columns according to manufacturers instructions. DNA concentration was measured using a Qubit (Invitrogen, Grand Island, NY). Primers for ChIP-qPCR included: hNR4A1 peak 1 (5’ CAGACTTTCCCCATCTCAGC, AGGAGGCGACAATGTAGCAG), hNR4A1 peak 2 (5’ TTGTTCTGTGCTGTGCTGTG, 5’ AGAGCAGGGGAAGGAAGAAA), and control region (5’ TCCCACTTCCAGAGAACCTG, 5’ ACAGACGCGGAGAACTCCTA).

ChIP-seq analysis

Debarcoding of the multiplex runs was performed using Fastx toolkit

(http://hannonlab.cshl.edu/fastx_toolkit). Tags were mapped to the human genome (hg19) using bowtie v0.12.7 (Langmead et al., 2009) excluding non-unique mappings (-m 1). 12-22 million uniquely mapped reads were obtained for each sample. Wig files were created using Homer (Heinz et al., 2010) and visualized on UCSC (Kent et al., 2002) genome browser as custom tracks. Peak identification was performed with MACS v1.3.7.1 (Zhang et al., 2008). Peaks for PR and PR+P conditions were called using either input, negative control (non-infected cells) or IgG control as a reference and only peaks that were present in all three comparisons were included in the final list of PR binding sites. To identify genes that are potentially regulated by PR, peaks were mapped to nearby genes within 50kb range from the transcriptional start site using Genomic Regions Enrichment of Annotations Tool (GREAT) (McLean et al., 2010). Peak intersections and overlaps with differentially expressed genes were performed using Galaxy (Blankenberg et al., 2010) and in house shell scripts.

RNA-seq analysis

Debarcoding of the multiplex runs was performed using in house shell script. Reads were then processed and aligned to the human genome (hg19) using TopHat v2.0.4 (Trapnell et al., 2009) with default parameters. Approximately 50 million and 42 million mapped reads where obtained for PR and PR+P samples, respectively. The aligned read files were further processed with Cufflinks v2.0.1 (Trapnell et al., 2010). Assemblies for PR and PR+P endothelial cells were merged using CuffMerge and differential expression was determined using Cuffdiff. Genes with a p-value smaller than 0.01 where considered as differentially expressed. For the generation of heatmaps for each gene log2 ratio of a given sample rpkm was divided with the average of the two

samples (PR and PR+P4) rpkm's and visualized using treeview (de Hoon et al., 2004).

qPCR primers

Primer sets for qPCR include: hVE-Cadherin (Forward: 5' CACCACCAGCTACGATGTGT, Reverse: 5' TCGTAGCCGTAGATGTGCAG); hClaudin-5 (Forward: 5' GAGGCGTGCTCTACCTGTTT, Reverse: 5' GTACTTCACGGGGAAGCTGA); hNR4A1 (Forward: 5' CTTCTCAAGGTCCCTGCACA, Reverse: 5' TCTTGTCAATGATGGGTGGA); hNR4A2 (Forward: 5' GGCGAACCCTGACTATCAAA, Reverse: 5' CTGGGTTGGACCTGTATGCT; mPR (Forward: 5' GGTGGGCCTTCCTAACGAG, Reverse: 5' GACCACATCAGGCTCAATGCT); mNR4A1 (Forward: 5' TTCTGCTCAGGCCTGGTACT, Reverse: 5' AATGCGATTCTGCAGCTCTT); mfibronectin (Forward: 5' ACAGAAATGACCATTGAAGG, Reverse: 5' TGTCTGGAGAAAGGTTGATT), mSyndecan1 (Forward: 5' ACTCTGACAACTTCTCTGGCTCTG, Reverse: 5' CTTCTTCTTCATCCGGTACAGCAT); mSyndecan4 (Forward: 5' CCAAGGAACTGGAAGAG, Reverse: 5' CCACGATCAGAGCTGCC); mKeratin (Forward: 5' TGCCTCCTCTCTCAGCCATGATCG, Reverse: 5' TCGCTTGACACCACCAGCAATAGC)

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