Supplemental Material

Detailed Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Post-PE human blood pressure study

The protocol was approved by the Partners Human Subjects Committee and all participants signed written informed consent. Women who were normotensive during all pregnancies (medical records either mention normotension or blood pressure values throughout pregnancy were <140 systolic and <90 diastolic mmHg) were recruited and included in the Prior Normotensive Pregnancy group (n=18). ACOG 2013 preeclampsia diagnosis standards[31] were used for inclusion of women in Prior Preeclampsia group n=10. Exclusion criteria were diabetes mellitus, renal or vascular disease and medications that affect blood pressure including hormonal contraceptives. Women had BP determined by automatic BP monitor (Dynamap) while in balance on high sodium (200 mEq/d) and low sodium (10 mEq/d) intake after 30 minutes supine at rest. The order of high salt and low salt was randomized so that some women had high salt first and some had low salt first. BP was measured every 5 minutes supine by automatic BP device over 30 minutes and BP results were averaged. Both groups had baseline SBP and DBP in the normotensive range in both high- and low-sodium balance. The number of years post-partum was missing for one woman. Only women who completed both low and high salt measurements were included in the analysis: Prior Normotensive Pregnancy group (n=13) and Prior Preeclampsia group (n=8) .

Animals

Model of PE: All animal studies were approved by Tufts University institutional review committee and conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Only female mice were used due to preeclampsia being a female-specific disease. Mice were singly housed during pregnancy in cages with corncob bedding with nestlet enrichment in rooms with 12hr light/12hr dark cycle. Female C57Bl/6 mice (RRID:MGI:2159769) were subjected to timed mating and gestation day 0 was denoted by the presence of a vaginal plug. Mice were randomized sequentially to alternate between control adenovirus (CMV-null vector= “control”) or sFlt1 adenovirus given by tail vein injected on gestation day 9. Exclusion criteria was based on sFlt1 adenovirus dose titrated to achieve a sFlt1 level > 60ng/mL, a level determined to be high enough to induce the PE phenotype without significant maternal mortality.[17,62] One cohort of mice was euthanized at GD 18 by terminal tissue harvest under isoflurane anesthesia to confirm PE and others were maintained in the post-partum period to study future susceptibility to hypertension.

SMC-specific mineralocorticoid receptor (MR) knockout mice: Six week old female MR fl/fl, SMA Cre-ER^{T2-} and Cre-ER^{T2+} mice on C57Bl/6 background were injected with 1mg tamoxifen (Sigma, dissolved in sunflower seed oil) for 5 consecutive days to induce specific SMC MR gene recombination as we have previously demonstrated.[32] At least 2

weeks later, mice underwent the same PE protocol used for the C57Bl/6 WT females, including timed mating and randomization to control or sFlt1 injection on gd9. No significant difference in any phenotype was found between Cre- or Cre+ mice injected with control (CMV-null) adenovirus, therefore control-injected SMC-MR-KO were combined with control-injected SMC-MR-WT for data analysis.

*Post partum hypertensive stimuli***:** Beginning 2 months post partum, mice were exposed to three consecutive hypertensive stimuli. Mice were fed 2% NaCl (Envigo TD.95078) to mimic moderate salt intake for 1 week, followed by standard laboratory diet (Envigo 2918) for 1 week. Then mice were fed 4% NaCl (Envigo TD.92034) to mimic high salt diet, and back to standard diet for a week. Finally, mice underwent implantation of osmotic pumps (Durect – Alzet Model 1002) to infuse angiotensin II (Sigma A9525) at 600ng/kg/min for 1 week to mimic increased renin-angiotensin-aldosterone system activation that occurs with aging.[24, 25]

Telemetric blood pressure monitoring: Singly housed mice were implanted with radiotelemetry catheters (TA11PA-C10, Data Sciences International) via the carotid artery into the aortic arch under isoflurane anesthesia as described[32] and allowed to recover for 7 days prior to measurement. Buprenorphine was used as post-operative analgesia (0.1mg/kg subcutaneous injection). In Figure 1, the cohort (n=13) was implanted prior to timed mating to measure blood pressure during pregnancy and 8 weeks post-partum. Systolic blood pressure was recorded for 30 seconds every hour and 24 hour averages are presented during pregnancy. In Figures 2 and 4, mice were implanted with devices 8 weeks post-partum and systolic pressure was measured at baseline and in response to hypertensive stimuli (C57 n=13, SMC MR colony n=30). During each hypertensive stimuli, systolic blood pressure was recorded 2 days prior ("baseline") and for 7 days during the stimuli. The mmHg change in systolic blood pressure for each day was calculated as ((Day X of stimuli- Baseline)/Baseline) and the area under this curve was calculated in Graph Pad Prism version 8 and graphed. This method normalizes each mouse to its own starting systolic blood pressure to assess the BP response to each intervention.

Humane endpoints: According with standard IACUC protocols, mice were euthanized if any of the following clinical signs were detected: dystocia, weight loss greater than 15% baseline weight, body condition score (BCS) < 2/5, inability to eat or drink, difficulty breathing, or poor ambulation.

Pulse wave velocity

Echocardiography was conducted using Doppler ultrasound (Vevo 2100, VisualSonics, Toronto, ON, Canada). In vivo pulse wave velocity (PWV) was measured in SMC-MR-WT and KO mice two months post-partum that had been implanted with radiotelemetry catheters (n=16). Mice were anesthetized with isoflurane and placed in a recumbent position with paws in contact with pad electrodes for ECG recording on a heated platform (37°C) and maintained with ~2.0% isoflurane during the procedure to maintain heart rate of 400-450 bpm. For PWV, the transit time between the proximal and distal abdominal aorta was determined by averaging distances between the foot of the flow waveform and

the R-wave of the ECG over 5 cardiac cycles at each location. PWV (mm/ms) was calculated by dividing the distance (mm) by the difference in transit times (ms) obtained at each location as previously described.[40]

Glomerular endotheliosis

Kidneys were harvested during pregnancy at GD17-18 (n=9), formalin fixed, paraffin embedded and sectioned. Slides with tissue sections of 5μm thickness were heated at 60-65°C, deparaffinized in three changes of xylene and rehydrated in decreasing alcohol concentrations. They were stained in Hematoxylin (Epredia 7211), differentiated with Clarifier 1 (Epredia 7401) and nuclei were enhanced with bluing solution (Avantik RS4368). Slides were briefly placed in 95% alcohol and counterstained with alcoholic eosin (Thermo Fisher 6766008), then dehydrated with increasing alcohol concentrations and cleared with xylene. Slides were mounted with a nonaqueous mounting media. A blinded renal pathologist evaluated multiple sections and glomeruli per kidney and assigned a score of 0, 0.5, 1, or 1.5 to each mouse kidney based on openness of capillary loops, size of glomerulus (swollen or shrunken) with a 0 score having no pathology, 0.5-1 having partial obstruction and 1.5 having severe evidence of glomerular endotheliosis.

Blood collection and assays

Plasma was collected in EDTA tubes (BD Microtainer 365974) via facial vein puncture at multiple timepoints during (gd17) and after pregnancy (2 months post-delivery). sFlt1 levels were measured using VEGFR1/Flt1 ELISA (R&D systems MVR100). Plasma aldosterone levels were measured using radioimmunoassay (Tecan MG13051) according to manufacturer instructions and as described in [33].

***Ex vivo* vascular function**

Wire myography: Third order mesenteric arteries were excised, dissected free of connective tissue, cut into 4 rings, mounted in wire myograph (Danish MyoTechnology) as in [33]. Smooth muscle and endothelial cell viability was confirmed by >1 milli-Newton (mN) constriction to 3μM phenylephrine and >50% dilation to 1μM acetylcholine. Vasoconstriction to four different concentrations of Angiotensin II was measured as the mN increase in force from baseline. Acetylcholine, phenylephrine, and angiotensin II (AngII, all sourced from Sigma) were dissolved in deionized water.

Pressure myography: Third-order mesenteric arteries were excised, dissected free of connective tissue, and both ends were cannulated on glass micropipettes, pressurized to 70mmHg and allowed to equilibrate. Endothelial and smooth muscle viability was confirmed via development of tone or constriction to 1μM phenylephrine and dilation to 1μM acetylcholine. Living Systems myograph and Danish MyoTechnology software was used to collect data. Solutions were made and myogenic tone was calculated as in [34].

Cell Culture

Mineralocorticoid receptor response element-Luciferase reporter assay: Pac1 Rat pulmonary artery SMC line (RRID: CVCL_U511) was grown in phenol red-free

Dulbecco's modified Eagle's medium (DMEM, Gibco) in 10% bovine growth serum. SMC biological replicates were treated with vehicle control or 50ng/mL sFlt (R&D Systems 7756-FL) for 24 hours. After sFlt1 treatment, the media was removed and replaced with fresh DMEM serum free medium. After 24 hours of sFlt1 washout, cells were transiently co-transfected with a luciferase reporter containing the mouse mammary tumor virus (MMTV) MR-responsive element (MRE)-luciferase reporter and a β-galactosidase expression plasmid as an internal control using Xfect Transfection Reagent (Takara Bio). The MRE luciferase reporter/beta gal plasmids and Xfect Polymer were diluted with Xfect Reaction Buffer. The plasmid to transfection reagent ratio was 0.3 µl of Xfect Polymer per 1 µg plasmid. To induce nanoparticle complex formation, the solution was incubated at room temperature in the dark for 15 min and then added dropwise to the cell culture medium. Six hours later, cells were stimulated with control or AngII (300nM) or aldosterone (10 nM) in serum free media for 24 hours. Luciferase was quantified by using Luciferin and β-galactosidase Detection Reagent (Promega). Luciferase activity was measured by Fisher Luminoskan Microplate Luminometer. Luciferase activity was divided by β-gal activity to normalize results for transfection efficiency. Each treatment was carried out in triplicate, and each experiment was performed a minimum of three times. sFlt1 (diluted in 0.1% BSA in PBS) and aldosterone (Aldo, 10 nM, Sigma) were dissolved in DMSO and appropriate control was added to each experiment. 24 hours after sFlt1 treatment, SMC replicates were treated with 10nM aldosterone or control for 24 hours and total RNA was isolated with the RNeasy mini kit (Qiagen 74106).

Western Blotting:
Pac1 SMC were treated with vehicle or sFlt1 (50ng/mL, R&D systems) for 24 hours and protein was harvested using TLB lysis buffer: 20 mM Tris-Cl, pH 7.5, 0.137 M NaCl, 2mm EDTA, pH 7.4, 1% Triton, 10% glycerol, 25 mM glycerol phosphate, phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Fisher). Lysate was run on 10% SDS PAGE gel, transferred to PVDF Transfer Membrane (Millipore), blocked with 5% nonfat milk and probed with appropriate primary antibodies. Antibodies used were mouse monoclonal anti-MR(1:500, Developmental Studies Hybridoma Bank RRID: AB_2722282) and rabbit anti-GAPDH (1:1000, Cell Signaling Technology). Secondary antibodies used were anti-mouse or anti-rabbit horseradish peroxidase secondary antibody (Cell Signaling Technology). Blots were imaged using ECL reagent (Fisher) and FluorChemE FE0504 Laboratory. Data quantification was performed using Fiji sofware.

VEGFA and VEGFR Quantification:
Pac1 and primary human aortic SMC (passage 4-5) were analyzed for expression of VEGFR using rabbit VEGFR1 (1:500), VEGFR2 (1:1000), GAPDH (1:2000) and secondary anti-rabbit (1:1000) from Cell Signaling Technologies. Human umbilical vein endothelial cell lysate (20% of the amount of SMC lysate, passage 4-5) was included as a positive control. Supernatant from Pac1 SMC grown in media with 10% bovine growth serum were analyzed using bovine VEGF ELISA (Invitrogen EB17RB) to determine presence of VEGF-A.

Mesenteric Vessel mRNA extraction and quantification

Mesenteric arcades were harvested, washed in saline and placed into RNA Later (Sigma R0901) for future dissection. Arteries and veins were dissected free of fat and combined from 2 mice for RNA extraction via Qiagen micro kit (Qiagen 74004). Only RNA with a 260:280 absorbance ratio greater or equal to 1.8 was reverse transcribed into cDNA.

Quantitative RT-PCR

RNA was reverse transcribed by using QuantiTect reverse transcription kit (Qiagen 205311) and rat gene-specific primers for Pac1 SMC (Table S2) or mouse specific primers for mesenteric vessels (Table S3). qRT-PCR was carried out by using an Applied Biosystems QuantStudio 3 using SsoFast EvaGreen SYBR-green supermix (Bio-Rad 1725201) per the manufacturer's instructions: initial enzyme activation (95°C for 30 seconds), followed by 40 cycles of denaturing (95°C for 5 seconds) and annealing and extension (55°C for 10 seconds). Ct values were obtained and analyzed by the $\Delta\Delta$Ct method to determine product fold change relative to housekeeping gene.

Supplemental Figures and Figure Legends

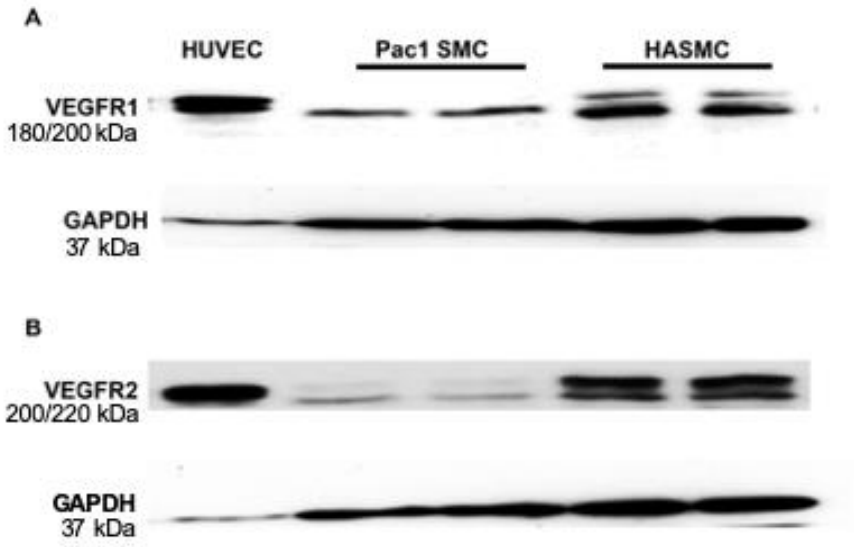

Figure S1: VEGFR1 and VEGFR2 are Expressed in Cultured SMC. Protein expression of: (**A)** vascular endothelial growth factor receptor (VEGFR) 1 and (**B)** VEGFR2 in cell lysate from proliferating human umbilical vein endothelial cells (loading 1/5 of SMC protein), Pac1 rat smooth muscle cells (SMC) and primary human aortic smooth muscle cells. GAPDH is included to indicate protein loading.

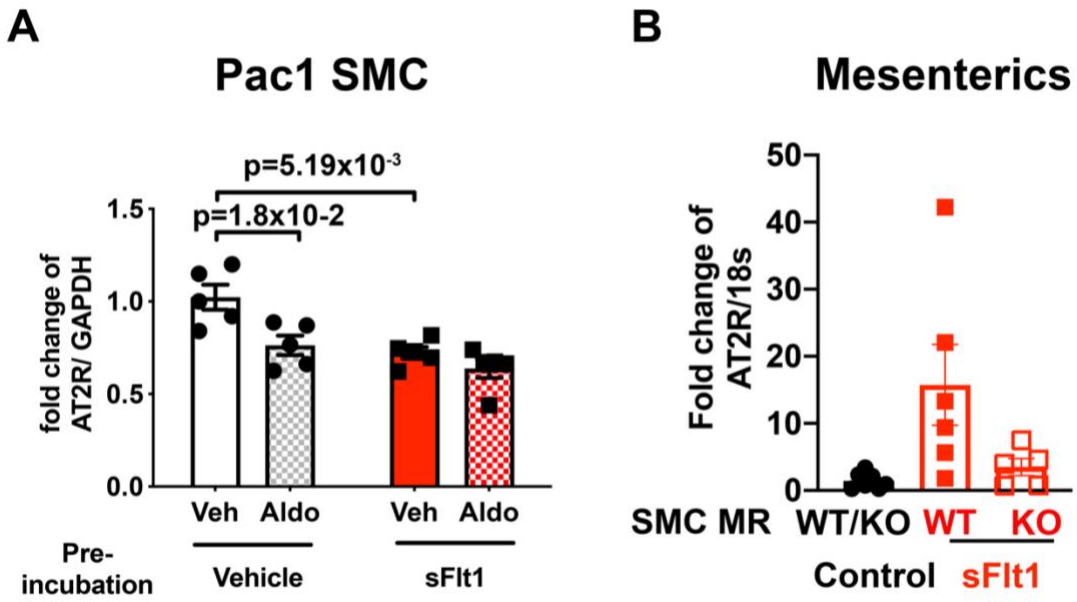

Figure S2. Impact of sFlt1 expression on angiotensin II type 2 receptor (AT2R) expression in SMC in vitro and mesenteric resistance vessels in vivo. (A) Pac1 SMC replicates were exposed to 50 ng/mL sFlt1 for 24 hours and then sFlt1 was removed for 24 hours and cells were stimulated with vehicle (Veh) or aldosterone (Aldo). RNA was extracted and analyzed for AT2R expression via qPCR. All groups n=5, two way ANOVA with Tukey post hoc test. **(B)** Mesenteric vessels from control pregnancy (Control), sFlt1 exposed SMC-MR-intact (WT sFlt1), and sFlt1 exposed SMC-MR-KO (KO sFlt1) littermates were harvested after exposure to post-partum repeated hypertensive stimuli and analyzed for AT2R expression via qPCR. Brown-Forsyth ANOVA test, Control n=7, WT sFlt n=6, KO sFlt n=5.

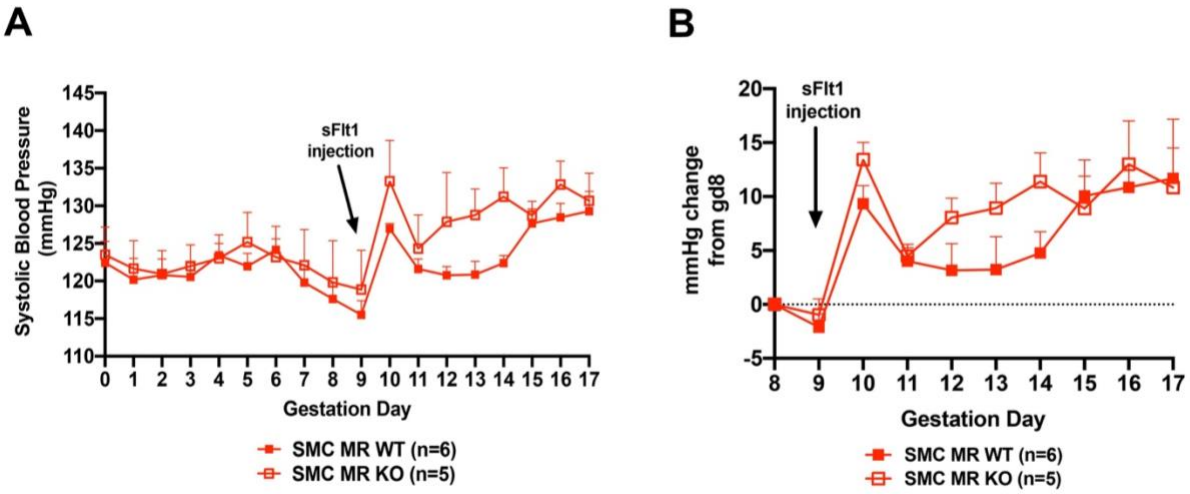

Figure S3: SMC MR KO does not impact systolic BP response to high sFlt1 in pregnancy. A) Systolic BP was measured using radiotelemetry during pregnancy in SMC MR WT (n=6) and KO mice (n=5). Experimental preeclampsia was induced in both groups via sFlt1 adenoviral injection on gestation day (gd) 9. BP significantly increased with gestation day ($p=1.0x10^{-4}$) whereas genotype did not impact BP in pregnancy (p=0.40). (**B)** Change in systolic blood pressure after sFlt1 injection compared to each mouse's pre-injection baseline (GD8). Graphs were analyzed using 2-way repeated measures ANOVA revealing no significant difference by genotype.

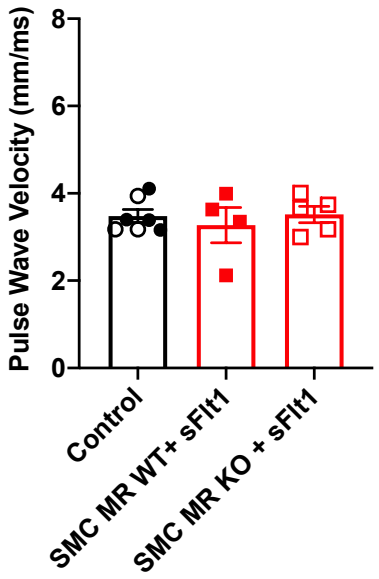

Figure S4. Aortic stiffness two months after prior sFlt1-induced preeclampsia. Aortic stiffness as measured by pulse wave velocity two months post-partum. PWV was not different between mineralocorticoid receptor (MR) intact (WT) or smooth muscle cell (SMC) MR knockout (KO) mice exposed to control or sFlt1-induced PE, prior to hypertensive stimuli. Control n=7, SMC-MR-WT sFlt1 n=4, SMC-MR-KO sFlt1 n=5.

Table S1: Population characteristics for human study. SD: Standard Deviation.

	Prior Normotensive Pregnancy (n=13)	Prior Preeclampsia (n=8)	P value
Age (SD), years	33.5 (6)	35.8 (4)	0.34
Years Post-Partum (SD)	4.3 (3)	3.2 (2.2)	0.30
BMI (SD), kg/m^2	24.1 (4.4)	26.9 (5.0)	0.22
Race			
White	8	5	
Asian	1	1	
Black	3	2	
Other	1	0	
Ethnicity			
Hispanic/Latina	5	0	
Non-Hispanic/Latina	8	8	
Hemoglobin A1C (SD), %	5.5 (0.3)	5.5 (0.3)	0.70
Systolic Blood Pressure (SD), mmHg	110 (9.4)	113 (12.4)	0.57
Diastolic Blood Pressure (SD), mmHg	70.7 (6.3)	75.9 (9.1)	0.19
Creatinine (SD), mg/L	0.74 (0.1)	0.75 (0.1)	0.85
Urine Sodium on High Salt (SD), mEq/L	210 (76)	212 (89)	0.97
Urine Sodium on Low Salt (SD), mEq/L	41 (29)	29 (19)	0.33

Table S2: Rat specific primers used for quantitative PCR.

Rat Primers	Amplicon size (bp)	Sequence
AT1R-Forward	130	GCCCTTCGGCAATCACCTAT
AT1R-Reverse		GCGAGACTTCATTGGGTGGA
MR-Forward	235	GCCCGGCAAATCTCAACAACTCAA
MR-Reverse		TTAGGGAAAGGAACGTCGTGAGCA
CTGF-Forward	150	CCTGGTCCAGACCACAGAGT
CTGF-Reverse		TTTTCCTCCAGGTCAGCTTC
GAPDH-Forward	164	GATGGTGAAGGTCGGTGTGA
GAPDH-Reverse		TGAACTTGCCGTGGGTAGAG

Table S3: Mouse specific primers used for quantitative PCR.

Mouse Primers	**Amplicon Size (bp)**	**Sequence**
AT1Rb-Forward	127	CAGCGTCAGTTTCAACCTCTACG
AT1Rb-Reverse		GGTGACTTTGGCGACCAGCATT
18s-Forward	64	CGCCGCTAGAGGTGAAATTC
18s-Reverse		TCTTGGCAAATGCTTTCGC