ONLINE SUPPLEMENT

ENDOTHELIAL CELL TGF-β RECEPTOR ACTIVATION CAUSES TACROLIMUS-
INDUCED RENAL ARTERIOLAR HYALINOSIS

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MATERIALS AND METHODS

Animals
Wild-type male C57Bl/6J mice (The Jackson Laboratory) were treated with daily i.p. injections of either low-dose tacrolimus (TAC, LC Laboratories; 1 mg/kg/day), high-dose TAC (10 mg/kg/day), or vehicle (DMSO, <1% final concentration) for 1 week. FK12EC KO mice were generated by crossing Tie2-Cre mice (The Jackson Laboratory, Bar Harbor, ME) with mice containing lox P sites flanking either side of FKBP12 (generously provided by Dr. Susan Hamilton, Baylor College of Medicine). 1 Wild-type male C57Bl/6J mice served as controls and all animals were studied at 10-12 weeks of age. Mice were maintained on a 12:12 light/dark cycle and had access to standard chow ad libitum. All procedures were approved by the Texas A&M Health Science Center/Scott & White Memorial Hospital Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Confirmation of FK12EC KO
Cardiac endothelial cells were isolated from control and FK12EC KO mice, purified using PECAM antibodies conjugated to magnetic beads, then cultured for 2 passages. Protein levels of FKBP12 and FKBP12.6 were measured using a Li-COR Odyssey (see below) and an anti-FKBP12/12.6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) with β-actin serving as a loading control.

ELISAs
Blood was collected via cardiac puncture prior to euthanization and placed into EDTA Vacutainer tubes. Plasma was obtained and 50 µL was used in the ELISAs for both TGF-β1 and angiotensin II per the manufacturer’s protocol (Phoenix Pharmaceuticals, Inc., Burlingame, CA).

Histology
Control and FK12EC KO mice were anesthetized by isoflurane and euthanized by exsanguination. Right and left kidneys were isolated, decapsulated, and placed in 10% formalin. Renal tissue was processed routinely and embedded in paraffin. Sections (3-5 µm thick) were obtained on a microtome, deparaffinized, and stained with either hematoxylin and eosin or Masson’s trichrome. Histologic slides were reviewed and photographed at 400x final magnification using a Nikon Optiphot-2 microscope and Olympus digital camera. Scoring for hyalinosis was performed as described previously. 2 In brief, a blinded reviewer scored 25 fields per section for both the left and right kidneys of each mouse using a 1-4 scale (1 = hyalinosis evident in <10% of the field, 2 = 10-25%, 3 = 25-50%, and 4 = >50%) and the scores were averaged.

qRT-PCR
Quantitative real-time PCR was performed on a Stratagene Mx3005P to analyze mRNA expression in endothelium-intact aortas from control and FK12EC KO mice. Gene expression was measured using a Mouse Endothelial Cell Biology RT² Profiler™ PCR Array and was performed according to the manufacturer’s protocol (SABiosciences, Frederick, MD). Results are expressed as fold change compared to control with an increase or decrease of 2-fold considered statistically significant.
Immunoblotting
Endothelium-intact and endothelium-denuded aortas were processed and imaged as described previously. Some aortas isolated from control mice were denuded of endothelial cells by repeatedly injecting air through the aorta, treated with calcineurin autoinhibitory peptide (CAIP; 10 µM, 30 minutes; Calbiochem), or treated with the TGF-β receptor inhibitor SB-505124 (10 µM, 30 minutes; Sigma) followed by treatment with TAC (1 or 10 µM, 24 hours). Western blot analyses were performed using the following primary antibodies: TGF-β1 1:1000 (Abcam, Cambridge, MA), phospho-SMAD2/3 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA), SMAD2/3 1:1000 (Cell Signaling), collagen type I 1:1000 (BD Biosciences Transduction Labs; Franklin Lakes, NJ), fibronectin 1:1000 (Millipore, Billerica, MA), calcineurin 1:1000 (Cell Signaling), and β-actin 1:5000 (Sigma; St. Louis, MO). Secondary antibodies consisted of anti-mouse, anti-rabbit, or anti-goat IgGs conjugated to either Alexa-Fluor 680 or IR800Dye (LI-COR Biosciences; Lincoln, NE).

Calcineurin Activity Assay
Isolated aortas from FK12EC KO and control mice as well as isolated control aortas treated with TAC (1 µM or 10 µM) or CAIP (10 µM; Calbiochem) were homogenized in the presence of protease and phosphatase inhibitors. Vascular homogenates (250 µg of protein) were used to measure calcineurin activity which was performed using a Calcineurin Assay Kit according to the manufacturer’s protocol (Calbiochem). In brief, this colorimetric assay utilizes the RII phosphopeptide substrate and Malachite Green for the detection of free phosphate released by calcineurin. Values obtained were determined from a standard curve and analyzed.

Statistical Analyses
Results are presented as mean ± SEM. For serum TGF-β1 and angiotensin II levels the values obtained from the ELISA were compared to the mean of the controls in each trial and expressed as a % of controls. For multiple comparisons between TAC-treated and control mice, an analysis of variance was used followed by the Student’s-Newman-Keuls post hoc test. The two-tailed Student’s t-test was used to compare variables between FK12EC KO and controls. The significance level was set at 0.05. All analyses were performed using SigmaStat 3.5 software.

REFERENCES
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Supplemental Figure 1. Tacrolimus (TAC) treatment increased TGF-β receptor activation and vascular matrix protein production. Mice treated with low-dose TAC (LD; 1 mg/kg/day) exhibited increased aortic SMAD2/3 phosphorylation and increased protein levels of collagen and fibronectin. Representative images from 3 independent experiments (n=6-11 mice in each group).
Supplemental Figure 2. Mice lacking FKBP12 in endothelial cells do not exhibit changes in serum TGF-β or angiotensin II. (A) FK12EC KO mice were produced by breeding FKBP12<sup>flox/−</sup> mice (Dr. Susan Hamilton, Baylor College of Medicine) with Tie2-Cre mice. Deficient FKBP12 protein levels in cardiac endothelial cells isolated from FK12EC KO mice vs. control mice but FKBP12.6 is still present. FK12EC KO mice exhibited no differences in serum (B) TGF-β1 or (C) angiotensin II compared to control mice (n=3-6 in each group). Densitometry of FKBP12/12.6 to actin as a % of control; *p<0.05 vs. control.
Supplemental Figure 3. No differences in vascular calcineurin expression or activity in FK12EC KO mice. (A) Calcineurin protein expression was not changed in aortas from FKBP12EC KO mice vs. control mice. Densitometry for calcineurin to β-actin expressed as % of control. Representative images from 3 independent experiments (n=3-4 mice in each group). (B) Calcineurin activity assay results showed no difference between aortas from FK12EC KO and control mice (n=4 mice in each group).
Supplemental Figure 4. Similar inhibition of aortic calcineurin activity between tacrolimus (TAC) and the calcineurin autoinhibitory peptide (CAIP). Both TAC and CAIP significantly inhibited calcineurin activity to a similar extent. Vessels were treated for 24 hrs. Results are from 3 independent experiments (n=3-4 aortas in each group). *p<0.05 vs. control.