

SUPPLEMENTAL DATA AND METHODS

Title: A Pkd1-Fbn1 Genetic Interaction Implicates TGF- β -Signaling in the Pathogenesis of Vascular Complications in ADPKD

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Supplementary Figure 1. TGF- β Receptor Expression in *Pkd1*^{KO/+} VSMC and *Pkd1*^{KO/KO} mouse embryonic fibroblasts (MEFs).

Supplementary Figure 2. Activation of TGF- β -responsive Promoter in MDCK cells stably expressing PKD1.

Supplementary Figure 3. The Sm22 α Cre recombinase is efficient in vivo.

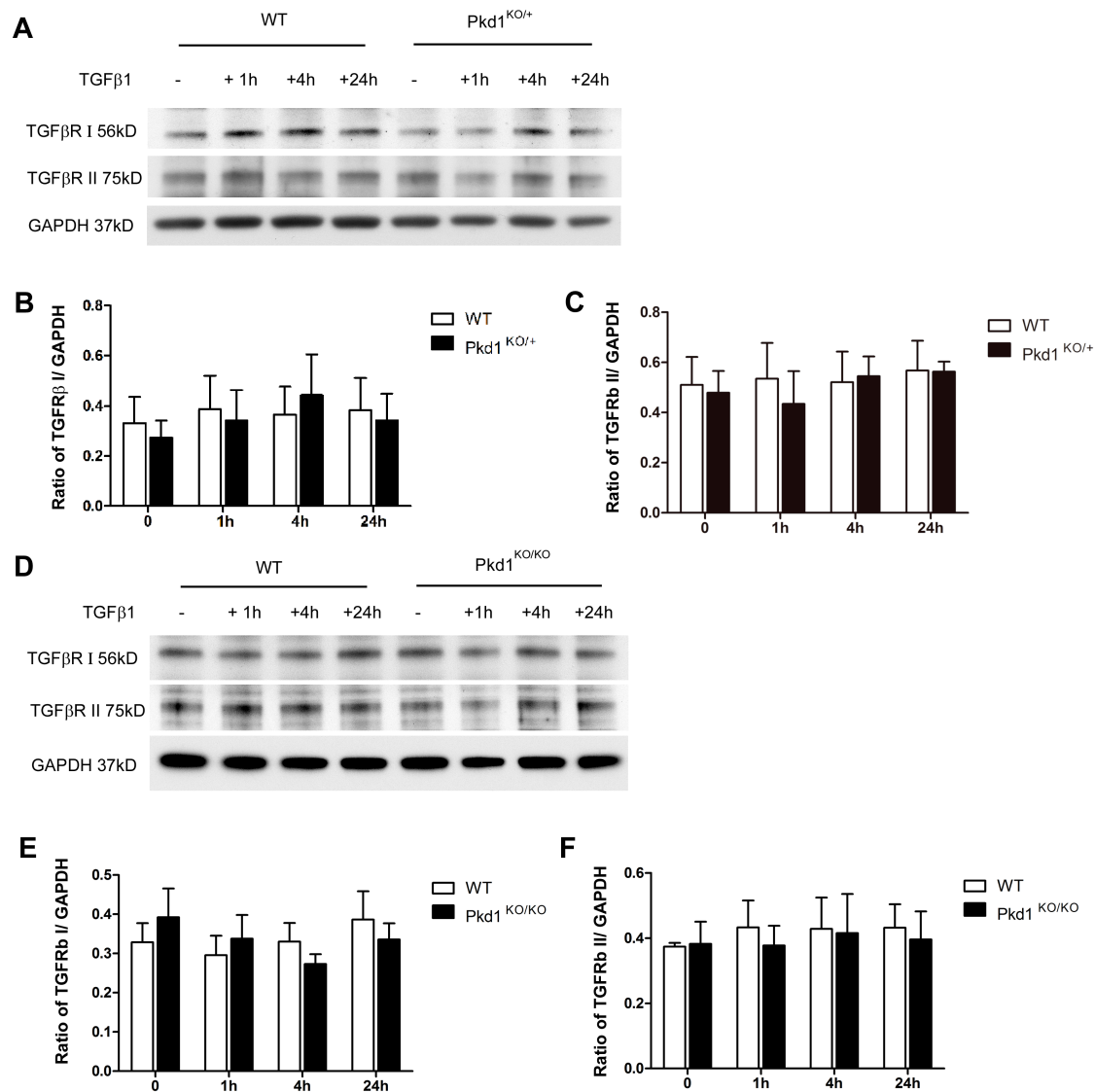
Supplementary Figure 4. Histopathology of Aortic Specimens.

Supplementary Table 1. Deletion of *Pkd1* in vascular smooth muscle cells

Supplementary Table 2. Aortic measurements in 6-month old animals

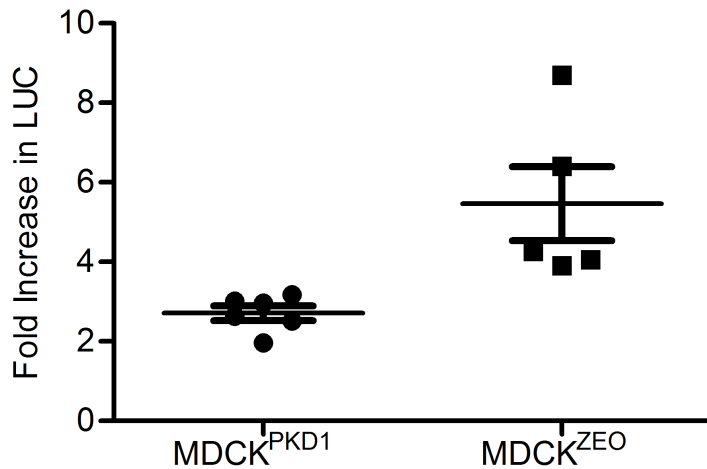
Supplementary Methods

Supplementary References

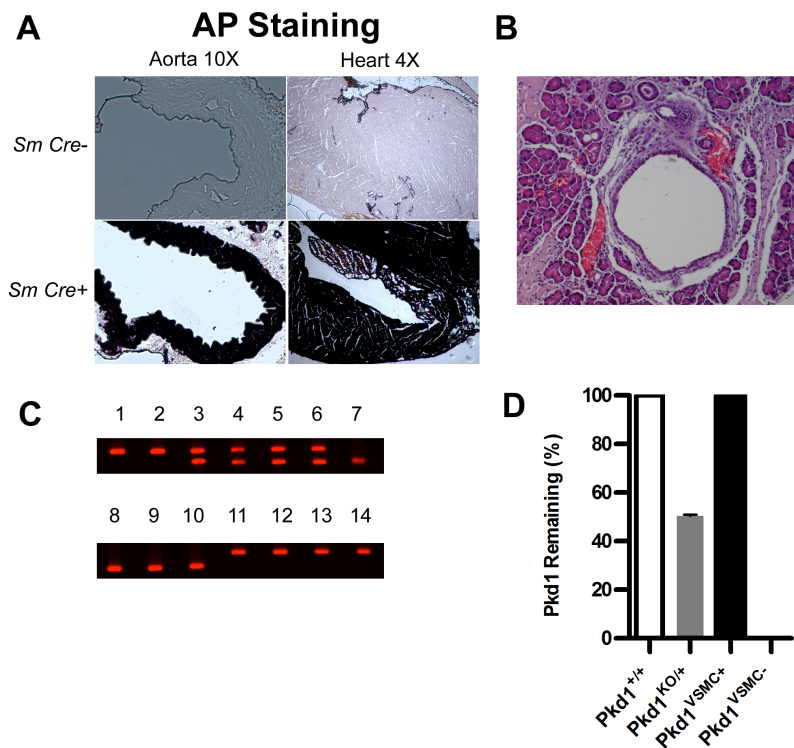


Supplementary Figure 1. TGF-β Receptor Expression in *Pkd1*^{KO/+} VSMC and *Pkd1*^{KO/KO} mouse embryonic fibroblasts (MEFs). (A, B, C) VSMC were isolated from *Pkd1*^{KO/+} and wild type control aortas (WT). (D, E, F) MEFs were derived from *Pkd1*^{KO/KO} or wild type littermate controls. Cells were serum starved over night and then incubated with 4μg/ml TGF-β1 for 1 hour, 4 hours or 24 hours as indicated. Cell lysates were used to prepare Western blots which were probed with antibodies to TGF-β Receptor I (Abcam) or to TGF-β Receptor II (Santa Cruz). Membranes were stripped and re-probed with anti-GAPDH as a loading control. (B, C, E, F) *Pkd1*^{KO/+} VSMC and

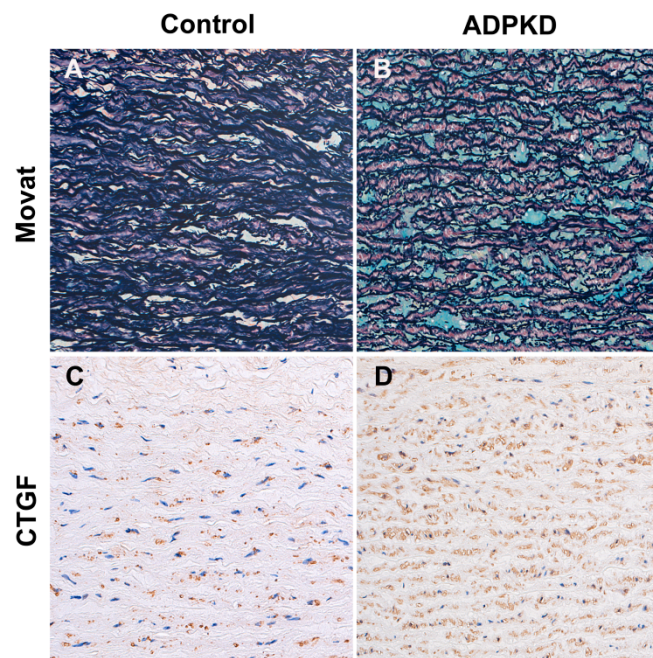
Pkd1^{KO/KO} MEFs did not show statistically significant changes in TGF- β 1 or TGF- β 2 receptor expression at baseline or after treatment with TGF- β 1. (N=3 for each experiment, P>0.05).



Supplementary Figure 2. Activation of TGF- β -responsive Promoter in MDCK cells stably expressing PKD1. MDCK cell lines that stably express human *PKD1* (MDCK^{PKD1}, N=3 independent clones) and vector controls (MDCK^{ZEO}, N=2 independent clones) were transfected with the p3TP-Lux TGF- β reporter and p-TK *renilla* luciferase. Following transfection, cells were incubated in the presence or absence of TGF- β 1 (4ng/ml) overnight and luciferase activity was assayed. Luciferase activity (LUC) was normalized to renilla activity for each plate. The fold change relative to untreated cells was averaged for all control lines (MDCK^{ZEO}) vs. PKD1 expressing stable cell lines (MDCK^{PKD1}). Upon TGF- β 1 treatment, MDCK^{ZEO} had a 5.46 fold increase in LUC activity vs 2.71 for the MDCK^{PKD1}, P=.01. Each data point represents the average of an experiment that was repeated in triplicate.



Supplementary Figure 3. The Sm22 α Cre recombinase is efficient in vivo. (A) Cre mediated recombination was visualized with the Z/AP reporter line. Alkaline phosphatase staining of heart and aorta from *Pkd1^{cond/+}; Z/AP* mice with and without the *SM22 α -Cre⁺* transgene reveals efficient recombination in vascular smooth muscle cells. (B) Large pancreatic cyst from a 1-month old *Pkd1^{cond/KO}; Sm22 α -Cre⁺* mouse. (C) SM22 α -Cre mediated recombination of the floxed *Pkd1* allele. A 3-primer PCR was designed to amplify a 180 base pair (bp) *Pkd1* wild type band (lane 7), a 250 bp deleted (null) band (lane 1, 2) and both bands equally in *Pkd1^{KO/+}* DNA samples (lanes 3-6). DNA was prepared from N=4 adult *Pkd1^{condKO}; Sm22 α -Cre⁺* aortas (lanes 11–14) and N=3 adults *Pkd1^{cond/+} Sm22 α -Cre⁻* aortas after removal of the endothelial cell layer and the adventitia. Only the 250 bp band is visualized in DNA from *Pkd1^{condKO}; Sm22 α -Cre⁺* aortas, indicating complete deletion of *Pkd1*. (D) Quantification of wild type *Pkd1* band remaining.



Supplementary Figure 4. Histopathology of Aortic Specimens. Aortic samples were collected from the ADPKD patient described in the text and a 19-year old control undergoing cardiac transplant surgery. (A, B) Movat's stain showing that the elastic fibers in the ADPKD aorta (A) are smaller in diameter and more fragmented with increased matrix deposition in comparison to control (B). These changes are typical of TGF- β related aortopathies. (C, D) Aortic sections were stained with antisera to CTGF, a prototypical TGF- β response gene. There is increased staining in the ADPKD aorta. All images acquired at a magnification of 64X.

Supplementary Table 1. Deletion of *Pkd1* in vascular smooth muscle cells

<i>Pkd1</i> ^{cond/cond} vs. <i>Pkd1</i> ^{KO/+} : <i>Sm22α-Cre</i> ⁺		
Offspring genotype	Expected Ratio, %	Obtained Ratio, %
<i>Pkd1</i> ^{cond/KO} : <i>Sm22α-Cre</i> ⁺	25	26.0(n=32)
<i>Pkd1</i> ^{cond/KO} : <i>Sm22α-Cre</i> ⁻	25	24.0(n=30)
<i>Pkd1</i> ^{cond/+} : <i>Sm22α-Cre</i> ⁺	25	30.0 (n=38)
<i>Pkd1</i> ^{cond/+} : <i>Sm22α-Cre</i> ⁻	25	20.0 (n=25)

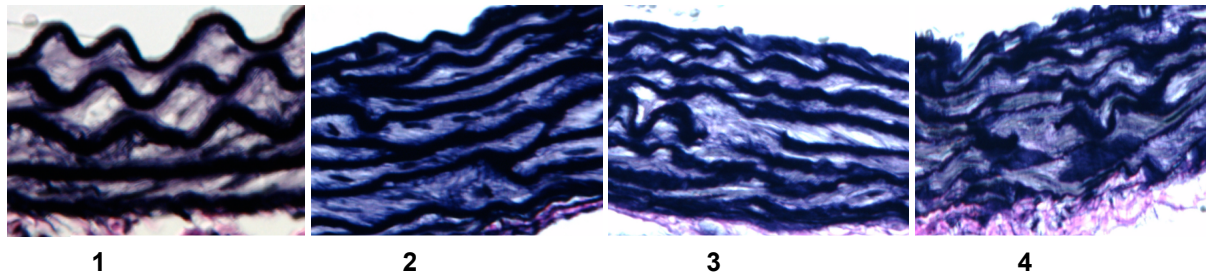
Supplementary Table 2. Aortic measurements in 6-month old animals

Diameter, mm	<i>Pkd1</i> ^{VSMC+}	<i>Pkd1</i> ^{VSMC-}
Aortic valve	1.33±0.13	1.40±0.14
Aortic root	1.70±0.08	2.02±0.22*
Ascending	1.33±0.05	1.43±0.05**

Data are the means ±SD from (n=4) animals, **P*=0.007, ***P*=0.006

Supplementary Methods

Analysis of Aortic Wall Architecture. Architectural damage and elastic fiber fragmentation were graded at 4 sites by a minimum of three blinded observers using a scale of 1 to 4. A score of “1” indicated smooth, uniform and unbroken elastic fibers with close apposition of elastic fibers. A score of “4” meant diffuse disruption of elastic fibers with an increased amount of matrix and loss of the smooth relationship between elastic fibers. Blinded observers were given the following examples to use as a reference for grading.



Features: Increase in elastic fragmentation, variable elastic thickness. Increasing extracellular matrix and loss of the smooth relationship between elastic layers.

Echocardiography. All echocardiograms were performed on awake, unsedated mice using the VisualSonics Vevo 660 V1.3.6 imaging system and a 40 or 60-MHz transducer (model RMV603, VisualSonics, Inc., Toronto). The aorta was imaged in the parasternal long axis view and 3 images were obtained at the level of the sinuses of Valsalva at each time point by a single echocardiographer who was blinded to the mouse genotype.

In Vivo Reporter Analysis. Cre mediated recombination was visualized with the Z/AP double reporter line¹ Frozen sections were prepared for alkaline phosphatase staining using the method of Lobe et al¹.

Analysis of Cre Deletion. Genomic DNA was prepared from the aortic media (largely

composed of VSMC) after removing the endothelial cell layer and the adventitia. A 3-primer PCR amplification for 25 cycles was performed with SYBR green Master Mix (Applied Biosystems) using primers and conditions previously described². Both *Pkd1* wild type (WT) and conditional alleles give a 180 bp band while the null or deleted (KO) allele yields a 250 bp band. The relative intensity of the two bands was determined using the Molecular Imager SystemH Pharos FxPlus™ (Bio-Rad) and used to calculate the relative *Pkd1* deletion (intensity KO band/intensity of WT band + KO band).

TGF- β Reporter Assays: MDCK cell lines that stably express human *PKD1* (MDCK^{PKD1zeo}, N=3 independent clones) and controls (vector expressing cell line with undetectable endogenous PC1 expression, N=2 independent clones) have been previously described³. Lipofectamine 2000 (Invitrogen) was used to co-transfect MDCK cell lines with the p3TP-Lux TGF- β reporter (gift of J. Massague and J. Wrana) and p-TK *renilla* luciferase (pRL-TK) (Promega) as an internal control for transfection efficiency⁴. Following transfection, cells were incubated in the presence or absence of TGF- β 1 (4ng/ml) overnight and luciferase activity was assayed. The luciferase activity was normalized to the *renilla* activity for each sample. The fold change for treated/untreated cells was averaged for all PKD1 expressing cell lines and compared with all control cell lines. Experiments for each cell line were repeated in triplicate at least two times.

Methods used for Quantification of Immunohistochemistry. In Figure 2 staining for pSmad2 and CTGF was quantified using the FRIDA analysis tool, which provides relative quantification of color intensity as described⁵. Within the aortic media region of interest (ROI), the amount of brown staining and its intensity were determined. These values were multiplied to create a single value reflecting pSMAD2 and CTGF staining.

In Figure 6, stained slides were digitized using a ScanScope CS (Aperio; Vista, CA) slide scanner. Immunohistochemical staining for pSmad2 and CTGF were then analyzed using the color deconvolution tool in the Aperio analysis toolkit ⁶. The color brown was deconvoluted from the image and both its intensity, binned into weak, medium and strong categories, and the percent of the media ROI contained in each bin was calculated. A scoring system, calculated as $1X(\%Weak) + 2X(\%Medium) + 3X(\%Strong)$ was used, which provided a score between 1-300⁷.

In Figure 8, the percentage of nuclei staining for pSMAD2 was determined for control, ADPKD and LDS aortae. Medium power images were evaluated for each subject in order to visualize the entire media. The image was opened in Image J (NIH) and a 6 x 8 grid was applied to each medium power image. We analyzed 48 fields for each sample that included 343, 550 and 401 nuclei for control, LDS and ADPKD aortas, respectively. The number of nuclei and brown-staining (pSMAD2⁺) nuclei were counted for each grid area and then summed. The percentage of brown nuclei was determined by dividing brown nuclei by total nuclei.

Supplementary References

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