Fluid shear-stress induced TGF-β/ALK5 signaling in renal epithelial cells is modulated by MEK1/2

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**Supplementary figures**

**Fig. S1** Activation of SMAD2/3 signaling by fluid-flow in ciliated *Pkd1*^−/−^ PTECs.

a *Pkd1* gene knockout was confirmed by PCR on DNA of *Pkd1*^−/−^ PTECs, which only showed the deletion band, indicating deletion of exon 2-11 of the *Pkd1* gene. *Pkd1*^+/+^ cells only showed the lox band, indicating that the floxed region was still present. As control the *Pkd1*^del/lox^ mouse showed both
bands. Primers used LoxF: ACCCTCCCTGAGCCTCCAC; LoxR: CCACAGGGAAGCCATCATA; DelF: CACTGTGGTGCYGGGTTATC.

b-c Pkd1 mRNA expression (exon 1-3) is absent in Pkd1+/- PTECs, while Pkd2 expression is not reduced in Pkd1+-/PTECs. Expression of Pkd1 or Pkd2 was virtually not altered by fluid shear stress, as measured by quantitative PCR. Cone-plate induced fluid-flow in Pkd1+/- and Pkd1+-/PTECs at t = 6 (b) or 16 (c) hr; Hprt served as housekeeping gene to correct for cDNA input; data normalized to unstimulated PTECs; n=5 per condition; * indicates P < 0.05 using two-way ANOVA.

d Serum starvation induces cilia formation in Pkd1+/- PTECs. Cilia are visualized using anti-acetylated α-tubulin antibodies (red) and nuclei are stained with DAPI (blue).

e Relative expression of Ptgs2 (COX2) and Pai1 (plasminogen activator inhibitor 1; Serpine1), Fn1(EDA region; fibronectin) and Col1a1 (collagen, type I, alpha 1) is increased upon fluid-flow, as measured by quantitative PCR. Cone-plate induced fluid-flow in Pkd1+-/PTECs at t = 6 or 16 hr; Hprt served as housekeeping gene to correct for cDNA input; data normalized to unstimulated PTECs at 6 hr presented in Fig. 1; n=5 per condition; * indicates P < 0.05 using two-way ANOVA.

f SMAD3-SMAD4 (GACA12-Luciferase) transcriptional reporter activity was elevated, as measured upon 20 hr of fluid-flow stimulation. Data normalized to unstimulated PTECs; n=4 per condition; * indicates P < 0.05 using two-way ANOVA.

g Western blot analysis of p-SMAD2 and p-SMAD3 shows increased phosphorylation upon 6 hr and 16 hr fluid-flow stimulation. GAPDH served as loading control.

h Nuclear accumulation of p-SMAD2 (green; t = 6 hr, IF). Nuclei are visualized with DAPI (blue).

i Relative expression of Snai1 (Snail) and Vim (vimentin) is increased, while relative expression of Snai2 (Slug) and Cdh1 (E-cadherin) is reduced in Pkd1+/- PTECs stimulated with fluid-flow, as measured by quantitative PCR. Cone-plate induced fluid-flow in Pkd1+/- PTECs at t = 6 or 16 hr; Hprt served as housekeeping gene to correct for cDNA input; data normalized to unstimulated PTECs at 6 hr presented in Fig. 1; n=5 per condition; * indicates P < 0.05 using two-way ANOVA.

j Nuclear accumulation of Snail (green; t = 6 hr, IF). Nuclei are visualized with DAPI (blue).

k Relative expression of Ptgs2, Pai1, Fn1 and Col1a1 shown as fold change induction by fluid shear stress (cone-plate) compared to the no flow control (dashed line), as measured by quantitative PCR. Comparison between Pkd1+/- and Pkd1+-/PTECs at t = 6 or 16 hr showed stronger induction of target genes by fluid shear stress in Pkd1+-/PTECs; Hprt served as housekeeping gene to correct for cDNA input; data normalized to unstimulated PTECs (dashed line); n=5 per condition; * indicates P < 0.05 using two-tailed Student’s t-test.
Fig. S2 Expression of Wnt and Hedgehog targets in PTECs upon fluid-flow stimulation.

Relative expression of Wnt target genes (Ccn11, Axin2, Birc5, Lin7a, Ppard, Glis2, Insc) and hedgehog targets (Gli1, Gli2, Gli3) is virtually not altered by fluid shear stress. Cone-plate induced fluid-flow in Pkd1+/+ (a) and Pkd1−/− (b) PTECs at t = 6 or 16 h. Wnt target gene expression was measured by reverse transcriptase multiplex ligation-dependent probe amplification (RT-MLPA) as described previously (Leonhard et al. (2008) BMC Biotechnol., 8: 18). Briefly, cDNA was synthesized from total RNA and hybridized to probes (sequences available upon request) in a reaction containing MLPA probe mix and SALSA MLPA buffer (MRC-Holland) by incubation at 95°C for 1 min followed by 60°C for 4 h. Ligation of annealed oligonucleotides was performed at 54°C for 15 min followed by ligase inactivation at 98°C for 5 min. Products were amplified by PCR using SalsaTaq (MRC-Holland) and FAM or HEX-labeled primers. Amplified samples were mixed with Hi-Di formamide containing GeneScan-500 ROX size standard (Applied Biosystems), heated for 5 min at 95°C, and run on a 3730 DNA analyzer (Applied Biosystems). Data was analyzed using GeneScan 3.5 analysis software. Expression of housekeeping genes Ywhaz and Hprt served as reference for cDNA input. Peak ratios of target genes and housekeeping genes were calculated and results were normalized to unstimulated PTECs. n=3 per condition. Hedgehog targets were measured by quantitative PCR. Hprt served as housekeeping gene to correct for cDNA input; data normalized to unstimulated PTECs; n=5 per condition; * indicates P < 0.05 using one-way ANOVA.
Fig. S3 Expression of ligands and receptors in Pkd1-/- PTECs upon fluid-flow stimulation.

a Relative expression of Tgfb1, Tgfb2, Tgfb3, Inhba, Inhbb and b Tgfr1 (Alk5) and Acvr1b (Alk4) mRNA in Pkd1-/- PTECs upon fluid-flow. Cone-plate induced fluid-flow at t = 6 or 16 hr; qPCR, Hprt served as housekeeping gene to correct for cDNA input; data normalized to unstimulated PTECs at 6 hr presented in Fig. 3; n=5 per condition; * indicates P < 0.05 using two-way ANOVA, followed by post-hoc Fisher’s LSD multiple comparison.

c Levels of total and active TGF-β1 and TGF-β2 in the medium of Pkd1-/- PTECs collected after 6 or 16 hr fluid-flow. TGF-β3 levels in medium and TGF-β1, 2 and 3 levels in cell lysates were below the detection limit. Cone-plate induced fluid shear stress; TGF-β levels measured by ELISA; n=5 per condition; * indicates P < 0.05 using two-way ANOVA, followed by post-hoc Fisher’s LSD multiple comparison.
Fig. S4 Expression of SMAD2/3 targets upon TGF-β1 or activin B stimulation when using inhibitors.

a-c TGF-β1 induced expression of SMAD2/3 targets Pai1, Fn1, Col1a1 and Ptgs2, was decreased with 10 µM ALK4/5/7 inhibitor (a; n=3), 10 µM MEK inhibitor (b; n=6) or 10 µg/ml TGF-β neutralizing Ab (c; n=3).

d Activin B induced expression of SMAD2/3 targets Pai1, Fn1, Col1a1 and Ptgs2, was decreased with 5 µg/ml sActRIIB-Fc (n=3).

Relative mRNA expression measured by qPCR at t = 4 hr (a) or 16 hr (b-d). Hprt served as housekeeping gene to correct for cDNA input; data was normalized to unstimulated controls. * indicates $P < 0.05$ using two-way ANOVA, followed by post-hoc Fisher’s LSD multiple comparison. 

ALK inh = 10 µM ALK4/5/7 inhibitor (LY-364947); MEK inh = 10 µM MEK1/2 inhibitor (Trametinib, GSK1120212). TGF-β Ab = 10 µg/ml TGF-β neutralizing Ab (clone 2G7). sActRIIB = 5 µg/ml soluble activin receptor-IIB fusion protein
Fig. S5 MEK inhibition modulates fluid-flow or TGF-β1 induced expression of SMAD2/3 target genes.

a MEK inhibition (5 µM Trametinib; GSK1120212) reduces fluid-flow increased expression of *Fn1*, while fluid-flow increased expression of *Pai1, Col1a1, Ptgs2* and *Snai1* is further elevated. Parallel plate flow-chamber induced fluid-flow in PTECs at t = 16 hr; qPCR, *Hprt* served as housekeeping gene to correct for cDNA input; data normalized to unstimulated controls (fold change); n=3 per condition. * indicates *P* < 0.05 by two-way ANOVA, followed by post-hoc Fisher’s LSD multiple comparison.

b *Pai1* and *Fn1* expression was reduced by MEK inhibition (10 µM Trametinib; GSK1120212) upon low dose TGF-β1 (0.25-2 ng/ml) stimulation, as measured by quantitative PCR; *Hprt* served as housekeeping gene to correct for cDNA input; data normalized to unstimulated controls; n=2 per condition. * indicates significant difference compared to unstimulated control (0 ng/ml TGF-β1) or † significant difference upon MEK inhibition (*P* < 0.05 by two-way ANOVA, followed by post-hoc Fisher’s LSD multiple comparison).
### Supplementary tables

**Table S1** Primer sequences used for qPCR.

| Gene | Accession # | Forward primer | Reverse primer |
|------|-------------|----------------|----------------|
| Pai1 | NM_008871.2 | GCCAACAAGAGCCAATCAC | ACCCTTTCCCAGAGACCAG |
| Fn1  | NM_010233.2 | AATCCAGTCACAGCCAATCC | CCTGTCTTCTCTTCCGGTGTA |
| Col1a1 | NM_007742.4 | TGACTGGAAGAGGGAGAGT | AGACGGCTGAGTAGGGAACA |
| Ptgs2 | NM_011198.4 | ACTGGGCCATGGAGGGGA | ACCCTTTCCCAGAGACCAG |
| Snai1 | NM_011427.3 | CTTGTGCTCGACGACCTG | CAGTGGGAGCAGGGAATG |
| Snai2 | NM_011415.2 | GAACCTGACACACACAGGTTATT | TGCCGAGATGTCCTCAACAG |
| Cdh1 | NM_009864.3 | ATCCCTGCCCCTGCTGATT | ACCACCGTCTCTCCCCTGA |
| Vim  | NM_011701.4 | CCAACCTTTTCTCCCTGAA | TCGAGTGGTGGTCAACAGAG |
| Tgfb1 | NM_011577.2 | ACTATGGCTTCAGCTCCACAGA | AAGTTGCGATGAGCCCTT |
| Tgfb2 | NM_009367.3 | CAGGAGTGCTTCCTCCACAA | TCAACCTGACAAATCCTCCCT |
| Tgfb3 | NM_009368.3 | AGGATCCACCAACCAACACAC | CCAGTTGGCAAGGAAGCATAA |
| Inhba | NM_008380.1 | GACCTCGAGCATCACCCTT | TGCCCTCTCCGAAATCTCA |
| Inhibb | NM_008381.3 | CGAGATCATCAGCTTCCAG | CATAGGGGAGCAGTTCCAGG |
| Tgfb1 | NM_009370.3 | ACATCAGGCTCAGATCAAGGTT | CACCTGTCGCAATGCTTCTT |
| Avcr1b | NM_007395.3 | CGAAGATGCAATTCTGGGGAG | GAGTGGGCGTGGTAGGAG |
| Gli1  | NM_010296.2 | CGACCTGCAAACCGTATAC | AGAGATGGCAGGGGAAGGAG |
| Gli2  | NM_001081125.1 | TGTCGATGGAGATGAGGAGT | TTGGCTGAGTGGAAGGAG |
| Gli3  | NM_008130.2 | GCCATTCACGTCCAGGTC | TTGCCTGTCGATGAGGAG |
| Pkd1  | NM_013630.2 | GCCACCACGCTAGACCGCTG | TAGCACAACGCTCTTCTAATG |
| Pkd2  | NM_008861.3 | GGGAGCATCTCCAGTGGG | GATGCTGCAATGGAGTCC |