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Kunnen, S.J.; Malas, T.B.; Formica, C.; Leonhard, W.N.; Hoen, P.A.C. 't; Peters, D.J.M.

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Comparative transcriptomics of shear stress treated *Pkd1*$^{-/-}$ cells and pre-cystic kidneys reveals pathways involved in early polycystic kidney disease

Steven J. Kunnen, Tareq B. Malas, Chiara Formica, Wouter N. Leonhard, Peter A.C. 't Hoen, Dorien J.M. Peters*

Department of Human Genetics, Leiden University Medical Center, 2300 RC Leiden, the Netherlands

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ABSTRACT

Mutations in the *PKD1* or *PKD2* genes are the cause of autosomal dominant polycystic kidney disease (ADPKD). The encoded proteins localize within the cell membrane and primary cilia and are proposed to be involved in mechanotransduction. Therefore, we evaluate shear stress dependent signaling in renal epithelial cells and the relevance for ADPKD. Using RNA sequencing and pathway analysis, we compared gene expression of *in vitro* shear stress treated *Pkd1*$^{-/-}$ renal epithelial cells and *in vivo* pre-cystic *Pkd1**del*** models. We show that shear stress alters the same signaling pathways in *Pkd1*$^{-/-}$ renal epithelial cells and *Pkd1*^{wt} controls. However, expression of a number of genes was slightly more induced by shear stress in *Pkd1*$^{-/-}$ cells, suggesting that *Pkd1* has the function to restrain shear regulated signaling instead of being a mechano-sensing activator. We also compared altered gene expression in *Pkd1*$^{-/-}$ cells during shear with *in vivo* transcriptome data of kidneys from *Pkd1*^{del} mice at three early pre-cystic time-points. This revealed overlap of a limited number of differentially expressed genes. However, the overlap between cells and mice is much higher when looking at pathways and molecular processes, largely due to altered expression of paralogous genes. Several of the altered pathways in the *in vitro* and *in vivo Pkd1*^{del} models are known to be implicated in ADPKD pathways, including PI3K-AKT, MAPK, Hippo, calcium, Wnt, and TGF-β signaling. We hypothesize that increased activation of selected genes in renal epithelial cells early upon *Pkd1* gene disruption may disturb the balance in signaling and may contribute to cyst formation.

1. Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by formation of many fluid-filled cysts and renal fibrosis, leading to deterioration or loss of renal function in adulthood [1,2]. ADPKD is caused by germline mutations in the *PKD1* or *PKD2* genes, encoding polycystin-1 (PC-1) and polycystin-2 (PC-2), respectively [3–5]. Somatic mutations in the unaffected allele of *PKD1* or *PKD2* can initiate cyst formation, the so called "second hit", but stochastic fluctuations in gene expression can also lower PC-1 or PC-2 below critical levels [6,7].

The PC-1 and PC-2 proteins co-localize throughout the cell membrane of renal epithelial cells, at the cell-cell contacts, extracellular matrix (ECM) and primary cilia, where PC-2 functions as a non-selective cation channel [8–10]. Primary cilia are central in organizing signaling systems that sense environmental cues, triggered by fluid flow and growth factor stimulation. Lack of the PC1-PC2 complex in cilia is proposed to play a role in cyst formation [11,12]. Moreover, mutations or deletions of other ciliary proteins can cause renal cysts in mouse models and patients, indicating the role of cilia during cystogenesis [13,14]. Several signaling pathways are modulated by cilium dependent and independent shear stress responses of renal epithelial cells, including Wnt, mTOR, STAT6/p100, TGF-β/ALK5 and MAPK signaling, as well as Ca^{2+} influx and Na^{+} and HCO_3^{-} reabsorption [9,15–25]. In addition, receptors of various signaling pathways have been identified in the primary cilium, including TGF-β, epidermal growth factor receptor (EGFR), Wnt and hedgehog signaling, suggesting that different signaling cascades are being regulated by this organelle [19,26–28].

Abbreviations: ADPKD, autosomal dominant polycystic kidney disease; cKO, conditional knock-out; CPM, counts per million; DEG, differentially expressed genes; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; FDR, false discovery rate; MAPK, mitogen-activated protein kinase; mTOR, mechanistic target of rapamycin; NGS, next generation sequencing; PC, polycystin; PI3K, phosphoinositide 3-kinase; PKD1, polycystic kidney disease 1 gene; PKD2, polycystic kidney disease 2 gene; PTEC, proximal tubular epithelial cell; TGF-β, transforming growth factor β

* Corresponding author.
E-mail address: D.J.M.Peters@lumc.nl (D.J.M. Peters).

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Cellular physiology and gene expression are determined by integration and interaction of the different signaling pathways, triggered by fluid shear stress and by growth factor or cytokine stimulation.

Luminal fluid shear and growth factors related signaling are essential for normal cell function, cell viability, tissue development and maintenance of organs [29–32]. In the kidneys, urinary volume, diuretics, and diet will expose the renal epithelial cells to variations in hydrodynamic forces including fluid shear stress, circumferential stretch, and drag/torque on apical cilia and probably also on microvilli [33]. For example, the kidney has the capacity to increase glomerular filtration rate in response to physiological stimuli. In addition, strong variations in hydrodynamic forces and shear stress are common in kidney diseases due to tubular dilation, obstruction and hyperfiltration, which occur in functional nephrons to compensate for lost glomeruli and tubules [34]. Renal shear stress is increased after unilateral nephrectomy [35,36], which accelerates cyst formation in *Ift88*$^{-/-}$ and *Pkd1*$^{-/-}$ mouse models [37,38]. Therefore, we hypothesize that shear stress induced alterations in cellular signaling may contribute to (early) cystogenesis.

To study shear stress dependent signaling in ADPKD, we examined the effect of fluid flow in proximal tubular epithelial cells (PTECs) without *Pkd1* expression and compared this with *Pkd1*^{wt} controls using RNA sequencing. In addition, we compared differential gene expression in *Pkd1*$^{-/-}$ PTECs during shear with *in vivo* transcriptome analysis of pre-cystic kidneys in *Pkd1*^{del} mice, in which fluid flow is still present. Functional enrichment analysis revealed that several *in vitro* disturbed signaling pathways in *Pkd1*$^{-/-}$ PTECs were also altered in pre-cystic kidneys of *Pkd1*^{del} mice.

2. Material and methods

2.1. Cell culture

SV40 large T-antigen immortalized murine proximal tubular epithelial cells (PTEC; *Pkd1*$^{-/-}$ and *Pkd1*^{wt}), derived from a *Pkd1*^{lox,lox} mouse, were generated and cultured as described previously [19,39]. Briefly, cells were maintained at 37 °C and 5% CO_2 in DMEM/F-12 with GlutaMAX (Thermo Fisher Scientific; #31331-093) supplemented with 100 U/mL Penicillin-Streptomycin (Thermo Fisher Scientific; #15140-122), 2% Ultroser G (Pall Corporation; #15950-017), 1x Insulin-Transferrin-Selenium-Ethanolamine (Thermo Fisher Scientific; #51500-056), 25 ng/L Prostaglandin E1 (Sigma-Aldrich; #P7527) and 30 ng/L Hydrocortisone (Sigma-Aldrich; #H0135). Cell culture was monthly tested without mycoplasma contamination using MycoAlert Mycoplasma Detection Kit (Lonza; LT07-318). New ampules were started after 15 passages.

2.2. Fluid shear stress stimulation and RNA sequencing

PTECs were exposed to laminar fluid shear stress (1.9 dyn/cm^2) in a cone-plate device as described previously [19]. Briefly, the cone-plate device, adapted from Malek et al. [40], was designed for 3.5 cm cell culture dishes (Greiner Bio-One). Cells were grown on collagen-I (Advanced BioMatrix; #5005) coated dishes until confluence, followed by 24 h serum starvation, before the start of the treatment to exclude effects of serum-derived growth-factors and to synchronize cells and cilia formation. Culture dishes were placed in the cone-plate flow system and incubated at 37 °C and 5% CO_2. The confluent cell monolayer of 9.6 cm^2 was subjected to constant laminar (Re = 0.3) fluid shear stress, using 2 ml serum-free DMEM/F-12 medium containing penicillin-streptomycin, with viscosity (μ) of 0.0078 dyn s/cm^2 [41], a cone with an angle (α) of 2° and a velocity (ω) of 80 rpm, generating a fluid shear stress ($\tau = \mu\omega/\alpha$) of 1.9 dyn/cm^2. Static control cells were incubated for the same time in equal amounts of serum-free DMEM/F12 medium containing penicillin-streptomycin at 37 °C and 5% CO_2. Cilia formation was checked on a parallel slide by immunofluorescence using anti-acetylated α-tubulin antibodies (Sigma Aldrich; #T6793) as previously described [19].

RNA sequencing was performed on isolated mRNA from fluid shear stress treated PTECs or static controls (n = 4 per condition) as previously described [20]. Briefly, next generation sequencing (NGS) was performed by ServiceXS (GenomeScan) using the Illumina® HiSeq 2500 platform. Illumina mRNA-Seq Sample Prep Kit was used to process the samples according to the manufacturer's protocol. Clustering and cDNA sequencing using the Illumina cBot and HiSeq 2500 was performed according manufacturer's protocols. A concentration of 5.8 pM of cDNA was used. All samples were run on Paired End mode and 125 bp long reads. HiSeq control software HCS v2.2.38 was used. Image analysis, base calling, and quality check was performed with the Illumina data analysis pipeline RTA v1.18.61 and/or OLB v1.9 and Bcl2fastq v1.8.4. At least 87.3% of bases had a Q-score ≥30. Reads were aligned to mouse genome build GRCm38 - Ensembl using TopHat2 version 2.0.10 [42]. Gene expression was quantified using HTSeq-Count version 0.6.1 [43], using default options (stranded = no, mode = union). Differential gene expression analysis was performed in R version 3.0.2 using DESeq (Version1.16.0). Differentially expressed genes were selected with an adjusted p-value (corrected for multiple hypotheses testing using Benjamini and Hochberg's False Discovery Rate method) of < 0.05. Counts per million (CPM) values were calculated by dividing the read counts of a gene by total read counts of all genes in a sample, which is a measure for the abundance of the transcript. Only genes with average CPM > 2 were considered. Raw RNA sequencing data was deposited online at http://www.ebi.ac.uk/arrayexpress/ (authors: S.J. Kunnen, D.J.M. Peters; year: 2018; ArrayExpress: E-MTAB-6640; ArrayExpress: E-MTAB-6641).

2.3. Experimental animals and RNA sequencing

Tamoxifen inducible kidney-specific *Pkd1*-deletion mouse model (tam-KspCad-CreER^{T2}; *Pkd1*$^{lox2\text{-}11;lox2\text{-}11}$, referred to as iKsp-*Pkd1*^{del}) and tamoxifen treatments were previously described [44–46]. Briefly, iKsp-*Pkd1*^{del} male mice were fed with tamoxifen (5 mg/day, 3 consecutive days) at adult age, *i.e.* between 13 to 14 weeks of age. Mice euthanized at determined time points (2, 3, and 6 weeks after tamoxifen treatment) had no visible signs of cystic disease (4 mice per time point). Adult male mice that did not receive tamoxifen treatment were used as *Pkd1*^{wt} controls (5 mice). RNA sequencing was performed on isolated mRNA from kidneys of iKsp-*Pkd1*^{del} and *Pkd1*^{wt} mice as previously described [46]. The local animal experimental committee of the Leiden University Medical Center and the Commission Biotechnology in Animals of the Dutch Ministry of Agriculture approved the experiments performed. Animal experiments have been carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

2.4. Pathway analysis

Functional enrichment analysis was performed with the online tool GeneTrail2 v1.5 (https://genetrail2.bioinf.uni-sb.de/) using standard settings of the over-representation analysis enrichment algorithm with correction for multiple hypotheses testing (FDR adjustment) according to Benjamini and Yekutieli [47]. From this source we included pathway databases (KEGG, BIOCARTA, REACTOME and WIKI). Up- and down-regulated genes by fluid shear stress were used as separate gene sets to discriminate between generally up- and down-regulated pathways. Terms with false discovery rate (FDR) < 0.01 were considered significantly enriched. Venn diagrams were made using Venny 2.1 (Oliveros, J.C. (2007–2015) Venny. An interactive tool for comparing lists with Venn's diagrams. http://bioinfogp.cnb.csic.es/tools/venny/index.html).

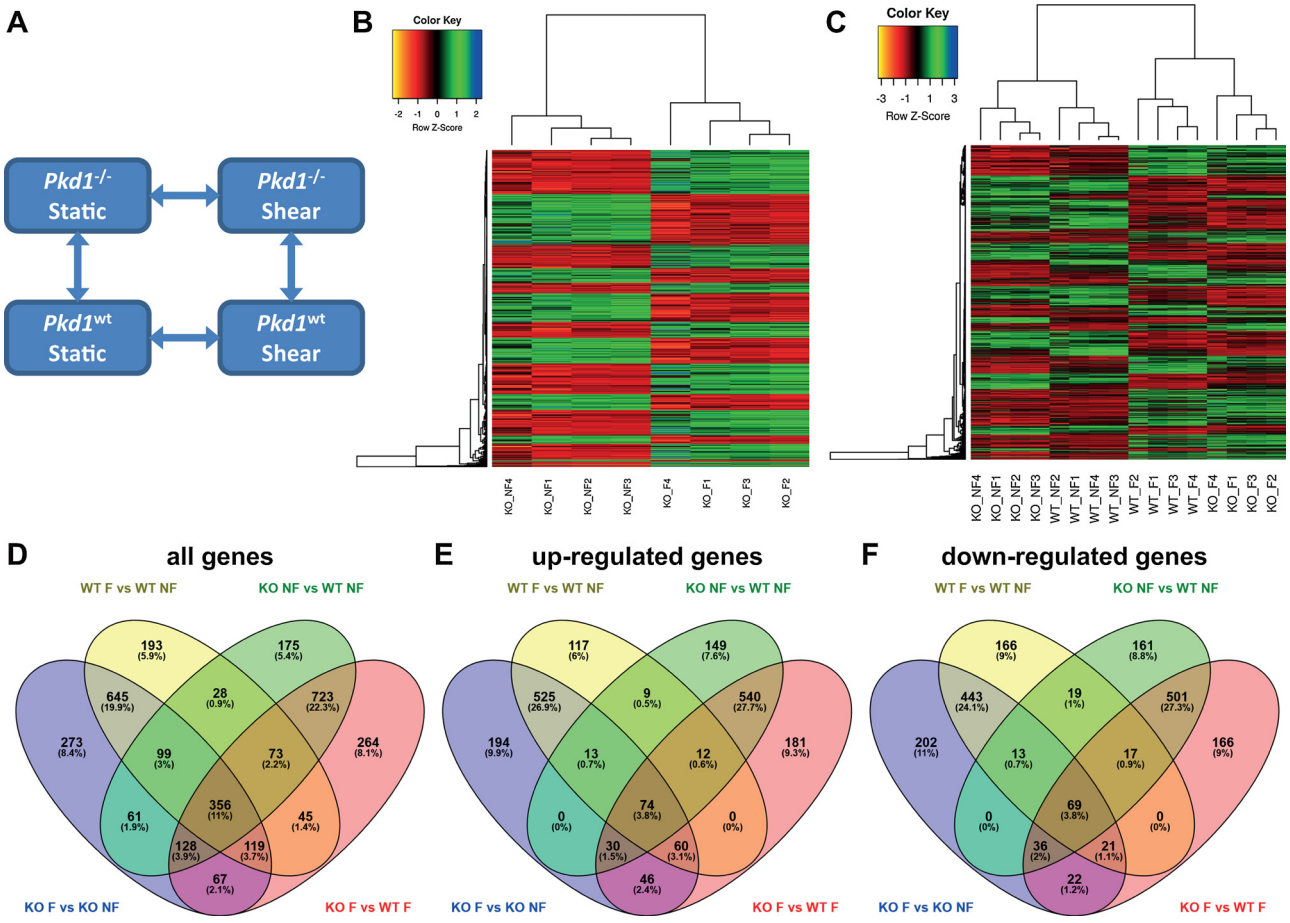

Fig. 1. Gene expression profiling in *Pkd1*$^{-/-}$ and *Pkd1*^{wt} PTECs shows a strong difference between fluid shear stress treated PTECs and static controls. (A) Groups used for the *in vitro* RNA-sequencing study and the comparisons that were made between shear stress treated *Pkd1*$^{-/-}$ or *Pkd1*^{wt} PTECs and static controls. (B) Heat map representing the differentially expressed genes ($p < 0.05$; CPM > 2) identified in 4 shear stress treated *Pkd1*$^{-/-}$ (KO) samples (F = Flow) and 4 static controls (NF = No Flow). (C) Heat map of differentially expressed genes ($p < 0.05$; CPM > 2) identified in *Pkd1*$^{-/-}$ (KO) and *Pkd1*^{wt} (WT) fluid shear stress treated samples and static controls. Unsupervised hierarchical clustering clearly distinguished fluid shear from static controls and grouped *Pkd1*^{wt} and *Pkd1*$^{-/-}$ within these clusters. (B, C) Expression values were normalized using the Voom function in *limma* R package. Hierarchical clustering was applied and values were scaled by row. (D–F) Venn diagrams of differentially expressed genes upon shear stress treatment or *Pkd1* gene disruption in PTECs. The overlap between shear stress treatment or *Pkd1* gene disruption is shown for all (D), up- (E) or down-regulated (F) genes. (%) means the percentage of genes of all (D), up-regulated (E) or down-regulated (F) genes.

3. Results

3.1. Fluid shear stress induced transcriptional changes in Pkd1$^{-/-}$ PTECs

To study shear stress altered gene expression, *Pkd1*$^{-/-}$ proximal tubular epithelial cells (PTEC) were exposed to fluid shear of 1.9 dyn/cm^2. Static controls were incubated without fluid flow treatment in equal amounts of medium. After 6 h fluid-flow or static exposure, total RNA was isolated and gene expression was analyzed using next generation sequencing (NGS) on the Illumina HiSeq 2500 platform. From the RNA sequencing data, the count per million (CPM) values were calculated by dividing the read counts of a gene by total read counts of all genes in a sample, which is a measure for the abundance of the transcript (Suppl. Table S1). Low expressed genes with an average CPM < 2 were excluded. In our previous study we presented the RNA sequencing results of shear stress treated *Pkd1*^{wt} PTECs [20], which we used as shear stress control in this study (Fig. 1A).

After normalization, a total of 1749 genes were differentially expressed ($p < 0.05$; CPM > 2) upon shear stress stimulation in *Pkd1*$^{-/-}$ PTECs (Table 1; Suppl. Table S2A). A heat map of *Pkd1*$^{-/-}$ PTEC samples shows a clear distinction between fluid shear stress treated samples and static controls (Fig. 1B). RNA sequencing results were compared to *Pkd1*^{wt} PTECs, again showing that shear stress samples clustered separately from the static controls, while the genotype has less influence on the hierarchical clustering (Fig. 1C). The comparison of *Pkd1*$^{-/-}$ and *Pkd1*^{wt} PTEC shows an overlap of 1220 differentially expressed genes (DEG) by shear stress (Table 1; Suppl. Table S2A and B). Furthermore, *Pkd1*$^{-/-}$ have 529 unique DEG compared to *Pkd1*^{wt}, while 339 genes were exclusively altered in *Pkd1*^{wt} cells in response to fluid shear stress (Fig. 1D–F; Table 1).

Table 1
Differentially expressed genes by shear stress in and *Pkd1*$^{-/-}$ and *Pkd1*^{wt} PTECs.

	Up	Down	Total
Pkd1$^{-/-}$	943	806	1749
Pkd1^{wt}	811	748	1559
Overlap	673	547	1220
Pkd1$^{-/-}$ only	270	259	529
Pkd1^{wt} only	138	201	339

Number of differentially expressed genes ($p < 0.05$; CPM > 2) of shear stress (flow) versus static (no flow) treated cultures in *Pkd1*$^{-/-}$ and *Pkd1*^{wt} PTECs. Low expressed genes were excluded with an enrichment filter of CPM (counts per million) < 2. *Pkd1*$^{-/-}$ only or *Pkd1*^{wt} only means genes differentially expressed upon shear, uniquely in *Pkd1*$^{-/-}$ or *Pkd1*^{wt} PTECs, respectively.

Table 2
Differentially expressed genes in *Pkd1*$^{-/-}$ *vs Pkd1*^{wt} PTECs.

	Up	Down	Total
No flow	828	816	1644
Flow	943	832	1775
Overlap	656	624	1280
No flow only	172	192	364
Flow only	287	208	495

Number of differentially expressed genes ($p < 0.05$; CPM > 2) of *Pkd1*$^{-/-}$ vs *Pkd1*^{wt} PTECs during static (no flow) or fluid shear stress (flow) conditions. Low expressed genes were excluded with an enrichment filter of CPM (counts per million) < 2. No flow only or flow only means genes uniquely differentially expressed in *Pkd1*$^{-/-}$ *vs Pkd1*^{wt} PTECs under static (no flow) or shear exposure (flow), respectively.

3.2. Pkd1 induced transcriptional changes in PTECs

To further investigate differences between the *Pkd1*$^{-/-}$ and *Pkd1*^{wt} cells, we compared the gene expression profiles (Fig. 1A). In static culture conditions there were 1644 genes differentially expressed between the *Pkd1*$^{-/-}$ and *Pkd1*^{wt} cells, while there were 1775 DEG during fluid flow (Table 2, Suppl. Table S2C and D). In total, 1280 genes were differentially expressed due to the *Pkd1* phenotype in both static and flow conditions, while smaller subsets of genes were uniquely differentially expressed in either of the two conditions (Fig. 1D–F). The lists of differentially expressed genes in PTEC cells were further analyzed using functional enrichment analysis.

3.3. Pathway analysis of altered gene expression upon fluid shear in *Pkd1*$^{-/-}$ and *Pkd1*^{wt} PTECs

We used GeneTrail2 v1.5 as tool for functional enrichment analysis [47] to identify biological pathways or processes associated with fluid-shear stress or the *Pkd1* phenotype in PTECs (*in vitro)*. The 4 lists of DEG (Suppl. Table S2A–D) were split into up- and down-regulated genes in order to get pathways that are generally up- or down-regulated. The up- or down-regulated biological annotations by fluid shear in *Pkd1*$^{-/-}$ and *Pkd1*^{wt} PTECs are presented in Supplementary Tables S3 and S4, respectively. We subdivided the biological annotations in core signal transduction, as well as cytokine/endocrine signaling, metabolism, cell-cell/matrix interaction, other cellular processes and diseases. Additionally, we used subgroup terms for the biological annotations similar to the subgroups used in the KEGG database. Several pathways or biological processes contain both up- or down-regulated genes, although in most cases there were more up-regulated genes in the enriched biological annotations. Pathway analysis of unique shear regulated genes in *Pkd1*$^{-/-}$ (270 up; 259 down) or *Pkd1*^{wt} (138 up; 201 down) PTECs are presented in Supplementary Table S5.

3.3.1. Core signaling pathways altered upon shear stress

The most prominently altered signaling pathways by fluid shear in *Pkd1*$^{-/-}$ PTEC are the mitogen-activated protein kinase (MAPK) and PI3K-AKT pathway (Table 3, Suppl. Table S3), which is similar to the shear response in *Pkd1*^{wt} PTEC (Suppl. Table S4). Both pathways have clearly more up-regulated than down-regulated genes, while one of the main inhibitors of the PI3K-AKT signaling, *i.e. Pten*, is down-regulated. MAPK and PI3K-AKT signaling show various interactions with other pathways that are altered by shear stress as well, both in *Pkd1*$^{-/-}$ and *Pkd1*^{wt} PTECs, including TGF-β, Wnt, p53 and JAK-STAT [19,20]. Of those pathways, TGF-β, Wnt, and p53 had clearly more up-regulated genes, suggesting that these pathways are up-regulated. In contrast, only JAK-STAT signaling contains more genes that were down-regulated, including receptors (*Ifngr1, Il6st, Lifr*) and signal transducers (*Stat1, Stat5a, Irf9*). Furthermore, our results indicate that Hippo, Rap1, Ras, TNF, FoxO, calcium, HIF-1, VEGF, mTOR and ErbB signaling are altered as well by fluid shear, both in *Pkd1*$^{-/-}$ and *Pkd1*^{wt} PTEC (Tables 3, S3 and S4). Furthermore, there are no pathways altered exclusively in *Pkd1*$^{-/-}$ or *Pkd1*^{wt} cells, but pathways are more or less active (Suppl. Table S5).

Altered Hippo signaling is probably attributed to interaction of YAP/TAZ with the TGF-β and Wnt signaling pathways, resulting in increased expression of Smad2/3 targets (*Serpine1, Ctgf, Smad7*) and TCF/LEF targets (*Myc, Cd44* and *Wisp1*), while transcriptional regulators Taz (*Wwtr1*), *Tead4*, *Tcf7l1* and *Axin2* are increased upon shear in *Pkd1*$^{-/-}$ PTECs only (Suppl. Table S5). Rap1 and Ras are core signal transducers of MAPK and PI3K-AKT signaling and show up-regulation of signaling related genes (*Rap1b, Rapgef5, Rras*), while several inhibitors of Rap1 and Ras (*Rap1gap, Rasa1, Rasal2, Rasa4, Syngap1, Sipa1l2*) and cytokines (*Angpt, Csf, Fgf, Igf, Pgf, Vegf*) were altered as well, contributing to the up-regulation of Rap1/Ras effectors (*Ets1, Ets2, Rassf1, Rassf5, Rin1*). In addition, activity of FoxO transcription factors can be modulated by MAPK, PI3K-AKT, JAK-STAT and insulin signaling, thereby modulating gene transcription upon interaction with Smad3/4 transcription factors. This FoxO-Smad interaction is revealed by shear induced up-regulation of genes involved in cell cycle (*Ccng2, Cdkn2b*, *Cdkn1a, Plk2, Plk3*) and DNA repair (*Gadd45a, Gadd45b, Gadd45g*), while other cell cycle genes were down-regulated (*Cdkn2d, Rbl2*), as well as autophagy genes (*Bnip3, Gabarapl1*). Altered TNF, FGF, PDGF, VEGF and ErbB signaling upon shear stress is attributed to altered gene expression of ligands (*Csf1, Csf3, Fgf1, Fgf9, Hbegf*, *Igf1, Pdgfa*, *Pdgfb*, *Pdgfc*, *Vegfa, Vegfb*) and receptors (*Egfr*, *Fgfr1*, *Fgfr2*, *Tnfrsf1b, Tnfrsf12a, Tnfrsf19, Tnfrsf21, Tnfrsf23, Relt*), while downstream components of MAPK and PI3K-AKT signaling are modulated as well.

3.3.2. Other biological processes altered upon shear stress

Expression of genes involved in various other cytokine and endocrine signaling pathways were also altered by shear in both *Pkd1*$^{-/-}$ and *Pkd1*^{wt} PTEC, including interleukin, insulin, B cell receptor, thyroid, estrogen, T cell receptor and chemokine signaling pathways. Altered expression include genes from aforementioned core signaling, as well as additional genes, including chemokines with C-C or C-X-C motifs (*Ccl2, Cx3cl1, Cxcl10, Cxcr4, Ccl27a, Cxcl15*). Regulation of cell-cell and extracellular matrix (ECM) interactions were altered by fluid shear in *Pkd1*$^{-/-}$ and *Pkd1*^{wt} PTEC, whereas much more genes involved in these interactions are up-regulated, including several actins, actinin, cadherins, β-catenin, cell adhesion molecules, collagens, fibronectin, integrins and laminins. The glycosaminoglycan biosynthesis pathway is induced by shear, which is involved in glycocalyx remodeling. Although several genes were differentially expressed in both *Pkd1*$^{-/-}$ and *Pkd1*^{wt} PTEC, a number of genes (*Hs2st1*, *Hs3st3b1*, *Sdc1* and *Sdc2)* were only induced by shear in Pkd1^{wt}, suggesting that this pathway is more controlled in *Pkd1*^{wt} cells (Suppl. Table S5). Genes involved in endocytosis were also increased by fluid shear in PTECs, which was reported in previous studies as well [48–50]. Genes involved in purine metabolism were altered by shear stress as well, which showed an equal number of up- and down-regulated genes. These include genes involved in cAMP processing (*Adcy1, Adcy7, Adcy9, Pde4d, Pde7a*), adenine nucleotide homeostasis (*Ak1, Ak2, Ak4, Ak5*), and RNA transcription (*Polr1b, Polr3d, Polr3e*). Genes involved in energy, carbohydrate, amino acid and cholesterol metabolism were mainly down-regulated by shear stress in *Pkd1*$^{-/-}$ and *Pkd1*^{wt} PTECs, while several other genes involved in energy metabolism and lysosomal degradation were exclusively down-regulated in *Pkd1*$^{-/-}$ cells (Suppl. Table S5). Other cellular processes altered by shear stress include the protein-protein interactions, Rho GTPase cycle and collagen metabolism. Only a small number of genes involved in apoptosis and cell cycle were altered by shear, indicating that these processes are not dramatically altered during shear exposure.

Table 3
Core signaling pathways affected by fluid shear stress in *Pkd1*$^{-/-}$ PTECs.

Pathway	Hits Up	FDR Up	Up-regulated genes	Hits Down	FDR Down	Down-regulated genes
MAPK	39/253	1.42E-24	CACNA1A; CACNA1G; CACNB3; DUSP1; DUSP4; DUSP6; DUSP7; EGFR; FGF1; FGF9; FGFR1; FLNA; FOS; GADD45A; GADD45B; GADD45G; HSPA2; HSPB1; MAP2K1; MAP2K3; MAP3K14; MAP4K4; MAPKAPK2; MYC; NFATC1; NFKB1; PAK1; PDGFA; PDGFB; PPP3CA; RAP1B; RASA1; RRAS; SRF; TGFB1; TGFB3; TGFBR1; TRP53; ZAK	13/253	7.78E-04	ARRB1; BDNF; CACNA1B; DUSP3; FGFR2; HSPA1A; MAP2K6; MAP3K1; MAP3K5; MAPT; MKNK2; RASGRP2; TGFB2
PI3K-AKT	44/347	2.35E-24	CDKN1A; COL1A1; COL27A1; COL4A3; COL4A4; COL5A1; CREB3L2; CSF1; CSF3; DDIT4; EGFR; EPHA2; F2R; FGF1; FGF9; FGFR1; FN1; IGF1; IL6RA; ITGA2; ITGA5; ITGAV; ITGB1; ITGB3; ITGB4; ITGB5; LAMB3; LAMC2; LPAR6; MAP2K1; MCL1; MYC; NFKB1; PDGFA; PDGFB; PDGFC; PHLPP1; PHLPP2; RHEB; SGK1; THBS1; TLR2; TRP53; YWHAH	19/347	1.10E-05	ANGPT1; ANGPT2; ANGPT4; COL3A1; COL4A5; FGFR2; ITGA1; ITGA6; OSMR; PIK3R1; PIK3R5; PKN3; PPP2R2C; PRKAA2; PTEN; RBL2; SGK2; VEGFA; VEGFB
Hippo	31/154	1.30E-22	ACTB; ACTG1; AXIN2; BMPR2; CRB2; CSNK1E; CTGF; CTNNB1; FGF1; FRMD6; FZD2; FZD6; FZD8; ID1; MYC; PARD6G; RASSF1; SERPINE1; SMAD3; SMAD7; TCF7; TCF7L1; TEAD4; TGFB1; TGFB3; TGFBR1; WNT7A; WNT7B; WNT9A; WWTR1; YWHAH	10/154	1.03E-03	APC2; BMP7; CDH1; ID2; PPP2R2C; RASSF6; TGFB2; WNT16; WNT6; WNT8B
Rap1	33/215	9.45E-21	ACTB; ACTG1; ADCY7; ARAP2; CSF1; CTNNB1; CTNND1; DOCK4; EGFR; EPHA2; F2R; FGF1; FGF9; FGFR1; ID1; IGF1; ITGB1; ITGB3; MAP2K1; MAP2K3; PARD6G; PDGFA; PDGFB; PDGFC; PLCE1; RAP1B; RAPGEF5; RASSF5; RRAS; SIPA1L2; THBS1; TLN2; VASP	16/215	3.36E-06	ADCY1; ADCY9; ANGPT1; ANGPT2; ANGPT4; CDH1; FGFR2; MAP2K6; PIK3R1; PIK3R5; PLCB1; RAP1GAP; RAPGEF3; RASGRP2; VEGFA; VEGFB
Wnt	21/143	8.71E-13	AXIN2; CSNK1E; CTNNB1; DAAM1; DAAM2; FZD2; FZD6; FZD8; MYC; NFATC1; NFATC2; NFATC4; PORCN; PPP3CA; SMAD3; TCF7; TCF7L1; TRP53; WNT7A; WNT7B; WNT9A	5/143	3.37E-01	APC2; PLCB1; WNT16; WNT6; WNT8B
Ras	25/227	1.12E-12	CSF1; EGFR; EPHA2; ETS1; ETS2; FGF1; FGF9; FGFR1; IGF1; MAP2K1; NFKB1; PAK1; PDGFA; PDGFB; PDGFC; PLA2G16; PLCE1; RAP1B; RAPGEF5; RASA1; RASAL2; RASSF1; RASSF5; RIN1; RRAS	11/227	4.09E-03	ANGPT1; ANGPT2; ANGPT4; FGFR2; PIK3R1; PIK3R5; RASA4; RASGRP2; SYNGAP1; VEGFA; VEGFB
TNF	19/109	1.12E-12	CCL2; CEBPB; CREB3L2; CSF1; CX3CL1; CXCL10; EDN1; FOS; IFI47; JUNB; LIF; MAP2K1; MAP2K3; MAP3K14; NFKB1; PTGS2; TNFAIP3; TNFRSF1B; VCAM1	4/109	5.05E-01	MAP2K6; MAP3K5; PIK3R1; PIK3R5
p53	14/68	1.99E-10	BID; CCNG2; CDKN1A; GADD45A; GADD45B; GADD45G; IGF1; IGFBP3; PERP; PMAIP1; SERPINE1; SFN; THBS1; TRP53	3/68	5.05E-01	CD82; PTEN; SESN1
FoxO	18/135	2.08E-10	CCNG2; CDKN1A; CDKN2B; CSNK1E; EGFR; FOXO1; GADD45A; GADD45B; GADD45G; IGF1; MAP2K1; PLK2; PLK3; SGK1; SMAD3; TGFB1; TGFB3; TGFBR1	12/135	1.86E-05	AGAP2; BNIP3; CDKN2D; FOXO6; GABARAPL1; PIK3R1; PIK3R5; PRKAA2; PTEN; RBL2; SGK2; TGFB2
TGF-β	13/82	2.69E-08	ACVR1; BMPR2; CDKN2B; ID1; INHBB; MYC; SMAD3; SMAD7; SMURF1; TGFB1; TGFB3; TGFBR1; THBS1	3/82	7.70E-01	BMP7; ID2; TGFB2
Calcium	15/182	2.44E-06	ADCY7; ATP2B4; CACNA1A; CACNA1G; EGFR; F2R; ITPKB; ITPR2; ITPR3; MYLK; PLCD3; PLCE1; PPP3CA; PTK2B; SPHK1	7/182	9.35E-02	ADCY1; ADCY9; ADRB1; ATP2B1; CACNA1B; PDE1C; PLCB1
HIF-1	10/112	1.48E-04	CDKN1A; EDN1; EGFR; HK2; IGF1; IL6RA; MAP2K1; NFKB1; SERPINE1; TFRC	12/112	4.10E-06	ANGPT1; ANGPT2; ANGPT4; ENO2; IFNGR1; MKNK2; PDK1; PFKL; PIK3R1; PIK3R5; SLC2A1; VEGFA
JAK-STAT	7/153	7.97E-02	CSF3; IL6RA; LIF; MYC; PIM1; SPRY1; SPRY4	12/153	5.04E-05	CBLB; IFNGR1; IFNLR1; IL6ST; IRF9; LIFR; OSMR; PIK3R1; PIK3R5; SOCS2; STAT1; STAT5A
VEGF	7/60	7.79E-04	HSPB1; MAP2K1; MAPKAPK2; NFATC2; PPP3CA; PTGS2; SPHK1	3/60	4.32E-01	PIK3R1; PIK3R5; VEGFA
Phosphatidylinositol	6/81	1.96E-02	DGKH; ITPKB; ITPR2; ITPR3; PLCD3; PLCE1	7/81	3.02E-03	INPP1; INPP5J; PI4KA; PIK3R1; PIK3R5; PLCB1; PTEN
mTOR	3/62	6.12E-01	DDIT4; IGF1; RHEB	6/62	4.77E-03	PIK3R1; PIK3R5; PRKAA2; PTEN; RRAGD; VEGFA
ErbB	7/87	4.91E-03	CDKN1A; EGFR; HBEGF; MAP2K1; MYC; NRG1; PAK1	4/87	2.74E-01	CBLB; PIK3R1; PIK3R5; STAT5A

Pathway analysis done on differentially expressed genes upon fluid shear stress in *Pkd1*$^{-/-}$ PTECs using GeneTrail2. The most significantly altered core signaling pathways of the KEGG database are shown and ordered by the lowest false discovery rate (FDR). Number of hits / total number of genes and the up- or down-regulated genes are presented. For the complete list of the pathway analysis of differentially expressed genes upon fluid shear stress, see Supplementary Table S3.

3.3.3. Cilia related gene expression upon shear stress

To investigate whether shear is affecting structural components of the cilium, we compared differential gene expression upon fluid flow with the SysCilia Goldstandard database for cilia related genes (http://www.syscilia.org/goldstandard.shtml). Only a minority of cilia genes was differentially expressed by shear and these genes were not involved in a specific cilia-assembly mechanism (Suppl. Table S6). Therefore, we conclude that shear stress does not result in major structural alterations in the experimental time-frame of 6 h.

From the pathway analysis we conclude that shear stress exposure in *Pkd1*$^{-/-}$ and *Pkd1*^{wt} PTEC modified expression of genes involved in the same signaling pathways and biological processes, suggesting that *Pkd1* is not directly involved in shear dependent activation of these pathways. These altered processes include several core signaling pathways, cytokine/endocrine pathways, cell-cell and ECM interactions, endocytosis, and various metabolic pathways. However, there were several genes involved in Hippo, Wnt and calcium signaling exclusively altered by shear in *Pkd1*$^{-/-}$ PTECs, while some important genes in the glycosaminoglycan biosynthesis were uniquely increased by shear in *Pkd1*^{wt} PTECs (Suppl. Table S5). Nevertheless, these pathways or molecular mechanisms are not uniquely altered in *Pkd1*$^{-/-}$ or *Pkd1*^{wt} cells, but our data show that the pathways are more or less active.

3.4. Differential activation of signaling in *Pkd1*$^{-/-}$ compared to *Pkd1*^{wt} PTECs

We compared the canonical signaling pathways that were altered by shear stress in *Pkd1*$^{-/-}$ *vs Pkd1*^{wt} PTECs. Scatter plots were constructed comparing the $\log_2$ fold change ($\log_2$ FC) values of differentially expressed genes by fluid shear in *Pkd1*$^{-/-}$ *vs Pkd1*^{wt} PTEC cultures normalized to their respective static controls (Suppl. Fig. S1). These plots show a substantial number of genes that have a higher $\log_2$ FC for *Pkd1*$^{-/-}$ cells for up-regulated (KEGG) pathways (MAPK, PI3K-AKT, Hippo, Rap1, Wnt, TNF, Ras and TGF-β), while this phenomenon was less evident for down-regulated pathways. These genes are more elevated in *Pkd1*$^{-/-}$ PTECs upon shear stress exposure compared to *Pkd1*^{wt} controls (Fig. 2), which we previously showed for TGF-β target genes [19]. Nevertheless, some of the pathways contain genes that are not differentially expressed between *Pkd1*$^{-/-}$ cells and *Pkd1*^{wt} controls. Differential gene expression between *Pkd1*$^{-/-}$ *vs Pkd1*^{wt} PTECs, presented in Suppl. Table S2C and D, was further assessed using functional enrichment analysis (Suppl. Tables S7 and S8).

3.4.1. Core signaling pathways altered upon in vitro Pkd1 gene disruption

Evaluating the absolute expression levels in *Pkd1*$^{-/-}$ compared to *Pkd1*^{wt} PTECs, revealed higher expression of genes involved in PI3K-AKT, Rap1, Hippo, MAPK, Ras, HIF-1, Wnt and TGF-β signaling pathways in shear-induced *Pkd1*$^{-/-}$ cells (Table 4, Suppl. Tables S7 and S8). Furthermore, FoxO, TNF, p53, calcium, hedgehog and JAK-STAT signaling were altered in *Pkd1*$^{-/-}$ PTECs as well. Most of the signaling pathways with altered expression in *Pkd1*$^{-/-}$ PTECs contain more up-regulated genes.

3.4.2. Other biological processes altered upon in vitro Pkd1 gene disruption

Other cytokine/endocrine signaling pathways are altered in *Pkd1*$^{-/-}$ PTEC as well, including GnRH, interleukin, insulin, thyroid and cytokine signaling. Biological processes that are increased in *Pkd1*$^{-/-}$ PTEC compared to *Pkd1*^{wt} PTEC include cholesterol biosynthesis, prostaglandin synthesis, carbohydrate and purine metabolism, while glycosaminoglycan metabolism is down-regulated. Several genes involved in cell-cell, extracellular matrix and semaphorin interactions were altered in *Pkd1*$^{-/-}$ cells, although there were both up- and down-regulated genes involved in these interactions. Interestingly, genes involved in endocytosis and circadian regulation were induced in *Pkd1*$^{-/-}$ cells compared to *Pkd1*^{wt}. Several cell cycle and apoptosis regulating genes were altered as well.

From the *in vitro* functional enrichment analysis, we conclude that several core signaling pathways and cellular processes were altered by both shear stress and *Pkd1* gene disruption, including PI3K-AKT, MAPK, Ras, Rap1, Hippo, Wnt and TGF-β signaling, as well as endocytosis and purine, cholesterol, carbohydrate and glycosaminoglycan metabolism. In addition, shear induced expression of several genes was stronger in *Pkd1*$^{-/-}$ PTECs.

3.5. Comparison of Pkd1 gene disruption in vitro and in vivo

Expression profiles of *in vitro Pkd1*$^{-/-}$ PTECs were compared with the RNA-sequencing data of the *in vivo* iKsp-*Pkd1*^{del} conditional knockout (cKO) mice (Suppl. Table S9). In the iKsp-*Pkd1*^{del} (adult) mouse model *Pkd1* gene disruption is specifically induced in renal epithelial cells, with the largest proportion in proximal tubular epithelial cells. In the early phase upon *in vivo Pkd1* disruption, there is still fluid flow, but no *Pkd1* expression in 40–50% of cells [36,38]. For this reason, the *in vitro Pkd1* phenotype during fluid shear exposure will be compared to the gene expression profile of early phase adult iKsp-*Pkd1*^{del} mice at 2, 3 or 6 weeks after gene disruption. At these pre-cystic time-points the kidneys did not show any sign of cyst formation and had 2KW/BW ratios comparable to *Pkd1*^{wt} mice (Fig. 3), while this iKsp-*Pkd1*^{del} model reaches end stage renal disease (ESRD) at 20 weeks after *Pkd1* gene disruption (Suppl. Fig. S2). The number of differentially expressed genes in iKsp-*Pkd1*^{del} mice (2, 3 or 6 weeks) *versus* wild-type controls was higher compared to flow-stimulated *Pkd1*$^{-/-}$ *versus Pkd1*^{wt} PTEC cells (Fig. 4A, Suppl. Table S2D, S9A–C). There was an overlap of 131 genes up-regulated and 48 genes down-regulated upon *Pkd1* disruption in *Pkd1*$^{-/-}$ PTEC cells and all three iKsp-*Pkd1*^{del} mice groups (Fig. 4B and C, Suppl. Table S10). The comparison between *in vitro* and *in vivo Pkd1*^{del} models was further assessed using functional enrichment analysis.

3.5.1. Core signaling pathways altered upon Pkd1 gene disruption

Altered gene expression caused by *Pkd1* disruption in both flow-stimulated *Pkd1*$^{-/-}$ PTEC cells and *Pkd1*^{del} mice was attributed to changes in several core signaling pathways, including PI3K-AKT, Rap1, Hippo, MAPK, Ras, FoxO, HIF-1, p53, calcium, Wnt, Hedgehog, JAK-STAT, TGF-β and TNF signaling (Fig. 5, Suppl. Figs. S3–S5, Suppl. Table S11). Most of the pathways had more up-regulated than down-regulated genes (Fig. 5), indicating that these signaling cascades are induced in *Pkd1*$^{-/-}$ PTEC cells and *Pkd1*^{del} mice compared to *Pkd1*^{wt} controls. A subset of genes in these pathways is identical but also paralogous genes were changed upon *Pkd1* disruption in cells and mice (Suppl. Fig. S3, Suppl. Table S11). For example, *Itgb6* in the PI3K/AKT pathway is up-regulated in *Pkd1*$^{-/-}$ PTEC cells and *Pkd1*^{del} mice, while several other integrin's were also up-regulated upon *Pkd1* gene disruption in the different models. Altered expression of identical genes in cells and mice is also observed for various signal transducers (*Adcy1, Ccng1, Egfr, Il6st, Itpkb*), transcription factor (*Creb3l2*), inhibitors (*Bcl2l1, Cdkn1a, Lats1, Nfkbia, Pten, Sipa1l*) and other genes (*Atp2a2, Camk2d, Pard6b, Plau*), as well as several paralogous genes, like collagens (*Col*), laminins (*Lam*), phospholipases (*Pla*), protein kinases (*Mapk, Prk, Jak, Pik3, Rapgef*), phosphatases (*Dusp, Ppp*), growth factors (*Bmp, Ctgf, Pdgf, Tgfb*), receptors (*Fgfr, Pdgfr*) and transcriptions factors (*Creb, Foxo, Smad, Tead*). These genes are active in several of the aforementioned pathways, suggesting that these genes might contribute to the *Pkd1* phenotype, since they are altered in both *in vitro* and *in vivo Pkd1*^{del} models. In contrast, altered expression of genes involved in Wnt signaling did not show a clear overlap between the *in vitro* and *in vivo* RNA sequencing data (Suppl. Table S11), but the overall effect in the two models indicates decreased non-canonical Wnt signaling. In *Pkd1*$^{-/-}$ PTEC cells, there is altered expression of several canonical Wnt ligands (Wnt proteins), receptors (Fz's), transcription factors (up: *Lef1, Tcf7l2;* down: *Tcf7l1, Tcf7*) and modulators (*up: Sfrp2;* down: *Frat2, Porcn, Sfrp1*), while non-canonical Wnt proteins (*Vangl1, 2, Prickle1*) and Ca^{2+}

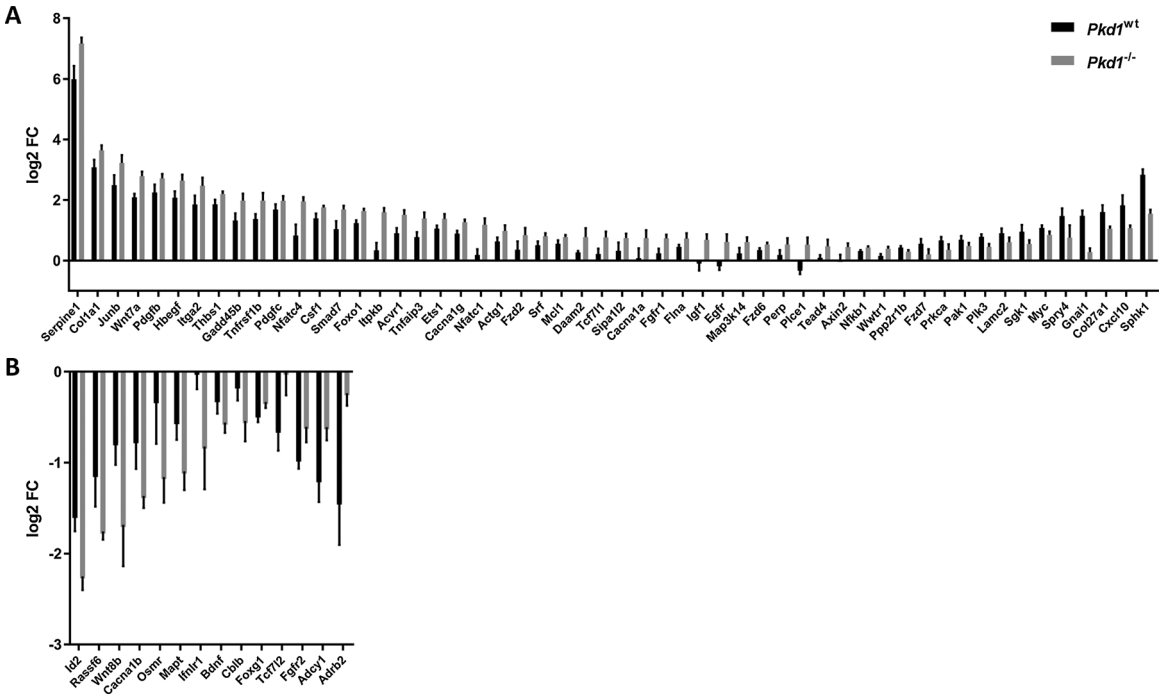

Fig. 2. Comparison of shear stress response in *Pkd1*$^{-/-}$ and *Pkd1*^{wt} PTECs.
Comparison of log_2 fold changes (log_2 FC) in *Pkd1*$^{-/-}$ PTECs and *Pkd1*^{wt} controls for differentially up-regulated (A) or down-regulated (B) genes upon shear stress treatment, normalized to their respective static controls. Several shear up-regulated genes show stronger shear induction in *Pkd1*$^{-/-}$ PTECs compared to *Pkd1*^{wt} controls. For shear down-regulated genes there were less genes differentially expressed between *Pkd1*$^{-/-}$ and *Pkd1*^{wt} PTECs. Only the genes are shown that were annotated to canonical signaling pathways (KEGG) using functional enrichment, as presented in Supplementary Tables S3–S4 and Fig. S1. All genes presented have a significantly different shear stress response between *Pkd1*$^{-/-}$ *vs Pkd1*^{wt} PTECs ($p < 0.05$ by a two-sample *t*-test with Welch's correction).

dependent NFAT (*Nfatc1,4)* transcription factors were decreased, which might suggest decreased non-canonical Wnt. In *Pkd1*^{del} mice there is decreased expression of several β-catenin inhibitors (*Chd8, Rb, Skp1a, Sox17*), while expression of β-catenin (*Ctnnb1*) itself is increased, as well as receptors (Fz's and *Lrp5,6*) and target genes (*Axin1, Ccnd1, Ccnd2, Ppard)*, suggesting increased canonical Wnt signaling. Down-regulation of *Dvl1* and Wnt proteins that activate non-canonical Wnt (*Wnt5b, Wnt7b, Wnt11*) may also suggest reduced non-canonical Wnt-signaling.

3.5.2. Other biological processes altered upon Pkd1 gene disruption

Other altered signaling pathways with mainly up-regulated genes in *Pkd1*$^{-/-}$ cells and mice include cytokine/endocrine signaling (GPCR, GnRH, interleukin, insulin, thyroid and estrogen signaling) as depicted in Supplementary Figure S4-5 and Table S11. Genes involved in protein interactions, ECM interactions, endocytosis, focal adhesion, cell adhesion, actin cytoskeleton, endoplasmic reticulum protein processing, adipogenesis, purine metabolism and circadian regulation were altered as well in both *Pkd1*$^{-/-}$ cells and *Pkd1*^{del} mice compared to *Pkd1*^{wt} controls. Cholesterol biosynthesis was up-regulated *in vitro*, but not *in vivo*, while lysosome related gene expression was only up-regulated *in vivo*. Genes involved in mRNA processing, protein translation, ribosomal proteins and electron transport were only down-regulated in iKsp-*Pkd1*^{del} mice (Suppl. Figure S5). This was attributed to down-regulation of various ATP synthases and transporters, NADH ubiquinone oxidoreductases, cytochrome C oxidases and reductases, eukaryotic translation initiation factor, RNA polymerases, general transcription factors, splicing factors and ribosomal proteins.

From the comparison between the *in vitro* and *in vivo* RNA sequencing data and pathway analysis we conclude that various identical and paralogous genes were altered upon *Pkd1* gene disruption in both model systems. These genes are already altered in the pre-cystic phase and are involved in several core signaling pathways, which suggest that these processes may contribute to *in vivo* cyst formation.

4. Discussion

In this study we present an overview of transcriptome alterations upon fluid shear stress exposure and *Pkd1* gene disruption in proximal tubular epithelial cells. We compared gene expression profiles of shear stress treated *Pkd1*$^{-/-}$ PTECs with *Pkd1*^{wt} controls and showed that 1219 genes were altered by fluid shear in both cell lines. Functional enrichment analysis revealed that shear regulated genes in *Pkd1*$^{-/-}$ and *Pkd1*^{wt} PTECs are involved in the same signaling pathways. Shear stress activated pathways include MAPK, PI3K-AKT, TGF-β, Wnt, p53, Hippo, FoxO, calcium and mTOR signaling, while only JAK-STAT signaling is down-regulated upon shear. Several of these pathways have been published previously [9,15–21,32]. Increased intracellular Ca^{2+} is one of the first responses of epithelial cells to the onset of shear, thereby modulating several signaling cascades, but it is currently under debate if the Ca^{2+} influx is cilium dependent [9,21,23–25,51]. In addition, the cilium is only involved in part of the shear stress response of PTECs, suggesting that other mechano-sensing complexes are involved as well [19,20].

Previously, we showed that inhibitors of TGF-β and MAPK/ERK signaling modulate a wide range of mechanosensitive genes, identifying these pathways as master regulators of shear-induced gene expression [19,20]. This is attributed to the many interactions of TGF-β and MAPK signaling with other pathways. One of these pathways is Hippo signaling, which controls organ size in animals and is modulated upon fluid shear. Interaction of the core components YAP and TAZ (also called *Wwtr1*) with TGF-β and Wnt signaling pathways has been described previously, thereby regulating Smad2/3 and TCF/LEF target gene expression, respectively [52–55]. In addition, activity of FoxO transcription factors can be modulated by MAPK, PI3K-AKT, JAK-STAT and insulin signaling, thereby modulating gene transcription upon

Table 4
Core signaling pathways altered in *Pkd1*$^{-/-}$ *vs Pkd1*^{wt} PTECs during shear stress.

Pathway	Hits Up	FDR Up	Up-regulated genes	Hits Down	FDR Down	Down-regulated genes
PI3K-AKT	42/347	7.74E-22	ANGPT4; BCL2L1; CDKN1A; COL11A1; COL4A2; COL4A3; COL4A4; CREB3; CREB3L2; CSF1R; CSF3; DDIT4; EGFR; EPHA2; FGF1; FGF9; FGFR2; FN1; G6PC2; GHR; GNG12; GNG5; IGF1; ITGA5; ITGA9; ITGB6; JAK2; LAMB3; LPAR1; LPAR3; MAP2K1; MCL1; MDM2; NGF; PDGFB; PHLPP2; PPP2R5A; PRKCA; PTEN; SGK1; TLR4; YWHAQ	26/347	6.82E-10	ANGPT1; COL27A1; COL3A1; COL5A1; COL6A1; COL6A2; COL6A3; EFNA1; EFNA5; FGF13; FGFR1; GNG11; IFNAR1; IFNAR2; IRS1; ITGB3; ITGB8; JAK3; LAMA5; OSMR; PDGFC; PDGFRB; PIK3CD; PIK3R5; PPP2R2C; PRKCZ
Rap1	29/215	1.98E-16	ADCY1; ADCY9; ANGPT4; ARAP2; CALM1; CSF1R; EGFR; EPHA2; FARP2; FGF1; FGF9; FGFR2; ID1; IGF1; LPAR1; LPAR3; MAP2K1; MAP2K6; NGF; PARD6B; PDGFB; PLCE1; PRKCA; RAP1GAP; RAPGEF3; RASGRP3; RASSF5; SIPA1L2; SIPA1L3	18/215	1.12E-07	ADCY3; ANGPT1; EFNA1; EFNA5; FGF13; FGFR1; GNAS; ITGB3; MAGI1; MAPK13; PDGFC; PDGFRB; PIK3CD; PIK3R5; PRKCZ; RALGDS; RAPGEF5; RASGRP2
Hippo	21/154	6.96E-12	AJUBA; BMP4; CRB2; CTGF; FGF1; ID1; LATS1; LEF1; LLGL2; PARD6B; SERPINE1; SMAD7; TCF7L2; TGFB2; TGFB3; WNT16; WNT7B; WNT8B; WNT9A; WWC1; YWHAQ	14/154	1.66E-06	FZD1; FZD5; FZD7; FZD8; GDF6; ID2; LLGL1; PPP2R2C; PRKCZ; SMAD1; TCF7; TCF7L1; TEAD3; WNT6
MAPK	23/253	7.11E-10	BDNF; CD14; DUSP3; DUSP6; DUSP8; ECSIT; EGFR; FGF1; FGF9; FGFR2; GADD45B; GADD45G; GNG12; MAP2K1; MAP2K6; MKNK2; NGF; PDGFB; PPP3CA; PRKCA; RASGRP3; TGFB2; TGFB3	17/253	2.80E-06	ARRB1; CACNG7; CDC25B; DUSP9; FAS; FGF13; FGFR1; HSPB1; IL1R1; MAP4K1; MAPK13; MECOM; NFATC1; PDGFRB; PLA2G4A; RASGRP2; RELB
Ras	20/227	2.82E-08	ANGPT4; BCL2L1; CALM1; CSF1R; EGFR; EPHA2; FGF1; FGF9; FGFR2; GAB2; GNG12; GNG5; IGF1; MAP2K1; NGF; PDGFB; PLCE1; PRKCA; RASGRP3; RASSF5	17/227	1.06E-06	ANGPT1; EFNA1; EFNA5; FGF13; FGFR1; GNG11; KSR1; PAK3; PDGFC; PDGFRB; PIK3CD; PIK3R5; PLA2G4A; RALGDS; RAPGEF5; RASGRP2; RGL2
FoxO	16/135	2.98E-08	AGAP2; CDKN1A; CDKN2B; EGFR; FOXO6; G6PC2; GADD45B; GADD45G; IGF1; IRS4; MAP2K1; MDM2; PTEN; SGK1; TGFB2; TGFB3	7/135	3.65E-02	CCNB1; IRS1; IRS2; MAPK13; PIK3CD; PIK3R5; SLC2A4
HIF-1	14/112	1.33E-07	ANGPT4; CAMK2D; CDKN1A; EDN1; EGFR; HK2; IGF1; MAP2K1; MKNK2; PFKFB3; PRKCA; SERPINE1; TFRC; TLR4	7/112	1.45E-02	ANGPT1; EGLN3; ENO2; LTBR; PFKFB1; PIK3CD; PIK3R5
Wnt	11/143	2.33E-04	CAMK2D; DAAM1; LEF1; PPP3CA; PRKCA; SFRP2; TCF7L2; WNT16; WNT7B; WNT8B; WNT9A	15/143	1.35E-07	FRAT2; FZD1; FZD5; FZD7; FZD8; NFATC1; NFATC4; PORCN; PRICKLE1; SFRP1; TCF7; TCF7L1; VANGL1; VANGL2; WNT6
TNF	7/109	1.78E-02	CREB3; CREB3L2; EDN1; MAP2K1; MAP2K6; NFKBIA; TRAF5	12/109	2.80E-06	BCL3; BIRC3; CEBPB; CX3CL1; CXCL10; FAS; LIF; MAPK13; PIK3CD; PIK3R5; PTGS2; TNFRSF1B
p53	10/68	4.40E-06	CCNG1; CDKN1A; GADD45B; GADD45G; IGF1; MDM2; PERP; PTEN; SERPINE1; SESN2	4/68	1.37E-01	BID; CCNB1; FAS; IGFBP3
Calcium	15/182	4.58E-06	ADCY1; ADCY9; ADRA1B; ADRB2; ATP2A2; CALM1; CAMK2D; EGFR; ITPKB; PLCE1; PPP3CA; PRKCA; PTK2B; SPHK1; TNNC1	5/182	7.38E-01	ADCY3; CD38; GNAL; GNAS; PDGFRB
Hedgehog	7/49	3.21E-04	BMP4; LRP2; PTCH1; WNT16; WNT7B; WNT8B; WNT9A	3/49	3.21E-01	GAS1; SMO; WNT6
JAK-STAT	11/153	3.99E-04	BCL2L1; CSF3; GHR; IL5RA; IL6ST; JAK2; PIM1; SOCS2; SPRY1; STAT5A; STAT5B	9/153	5.29E-03	IFNAR1; IFNAR2; IFNLR1; JAK3; LIF; LIFR; OSMR; PIK3CD; PIK3R5
TGF-β	8/82	7.97E-04	ACVR1; BMP4; CDKN2B; DCN; ID1; SMAD7; TGFB2; TGFB3	5/82	7.22E-02	CHRD; GDF6; ID2; PITX2; SMAD1
ErbB	8/87	9.67E-04	CAMK2D; CDKN1A; EGFR; HBEGF; MAP2K1; PRKCA; STAT5A; STAT5B	4/87	2.91E-01	PAK3; PIK3CD; PIK3R5; TGFA
NFkB	8/96	2.68E-03	BCL2L1; BLNK; CD14; GADD45B; NFKBIA; PLAU; TLR4; TRAF5	6/96	4.10E-02	BIRC3; CXCL12; IL1R1; LTBR; PTGS2; RELB
Phosphatidylinositol	7/81	4.05E-03	CALM1; DGKA; ITPK1; ITPKB; PLCE1; PRKCA; PTEN	5/81	7.04E-02	DGKH; PIK3C2B; PIK3CD; PIK3R5; SYNJ2
VEGF	4/60	1.30E-01	MAP2K1; PPP3CA; PRKCA; SPHK1	6/60	5.29E-03	HSPB1; MAPK13; PIK3CD; PIK3R5; PLA2G4A; PTGS2

Pathway analysis done on differentially expressed genes in *Pkd1*$^{-/-}$ *vs Pkd1*^{wt} PTECs during fluid shear exposure using GeneTrail2. The most significantly altered core signaling pathways of the KEGG database are shown and ordered by the lowest false discovery rate (FDR). Number of hits/total number of genes and the up- or down-regulated genes are presented. For the complete list of the pathway analysis of differentially expressed genes upon *Pkd1* disruption in PTECs, see Supplementary Table S7.

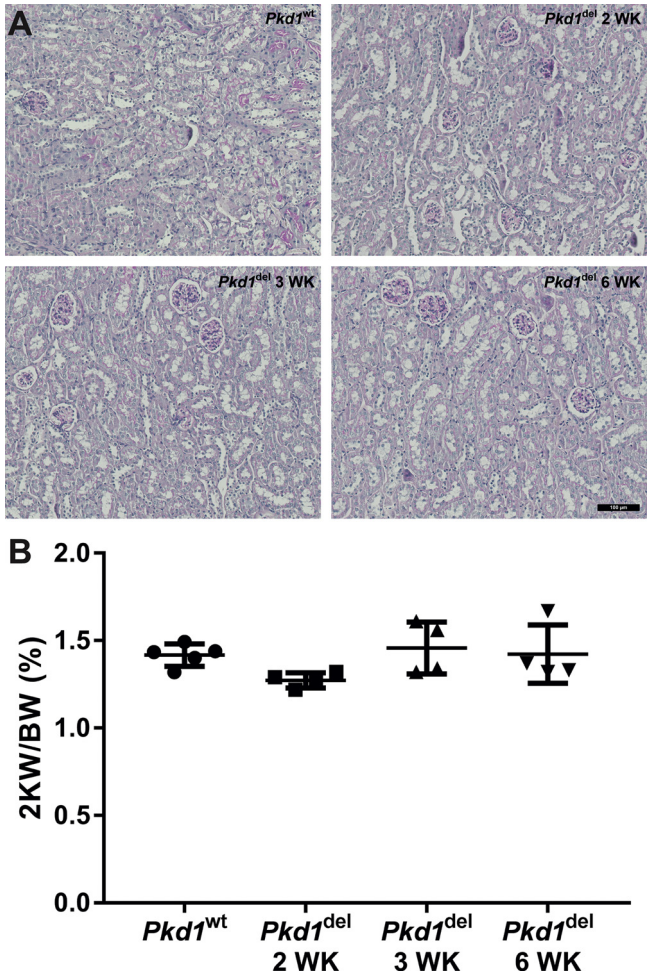

Fig. 3. Kidney morphology and kidney weight (KW/BW) of *Pkd1*^{wt} and pre-cystic iKsp-*Pkd1*^{del} mice.
(A) Representative images of periodic-acid Schiff (PAS) staining on formalin fixed, paraffin embedded kidney sections of *Pkd1*^{wt} (top left) and iKsp-*Pkd1*^{del} mice at 2 (top right), 3 (bottom left) or 6 (bottom right) weeks after gene disruption. No visual difference in kidney morphology between *Pkd1*^{wt} and pre-cystic iKsp-*Pkd1*^{del} mice; scale bar = 100 μm. (B) Similar kidney weight to body weight ratio's (2KW/BW%) in *Pkd1*^{wt} and iKsp-*Pkd1*^{del} mice at 2, 3 or 6 weeks after gene disruption.

interaction with Smad3/4 transcription factors [56].

Other shear regulated pathways include TNF, FGF, PDGF, VEGF and ErbB signaling, for which functional enrichment is largely attributed to altered gene expression of ligands, receptors and downstream components of MAPK and PI3K-AKT signaling. Important signal transducers of MAPK and PI3K-AKT signaling are Ras and Rap1, which shows again the interaction between cellular signaling pathways. Shear induced activation of several other cytokine or endocrine signaling pathways and shear altered expression of genes involved in cell-cell contacts, ECM, glycocalyx remodeling and endocytosis has been discussed in Kunnen et al. [20]. Remarkably, several genes involved in energy metabolism and autophagy (the process of lysosomal degradation) were exclusively down-regulated by shear stress in *Pkd1*$^{-/-}$ PTECs. These processes are also implicated in ADPKD, but how it affects cyst progression is currently unclear [57,58]. These findings suggest a complex interaction of processes and pathways to regulate the shear stress response in PTECs and to maintain cellular physiology.

Shear stress induced expression of a number of genes involved in MAPK, PI3K-AKT, Hippo, Rap1, Wnt, TNF, Ras and TGF-β was slightly more activated in *Pkd1*$^{-/-}$ PTECs compared to *Pkd1*^{wt} controls (Figs. 2 and S1), which we previously showed for TGF-β/ALK5 target genes [19]. Similarly, genes with differential expression in *Pkd1*$^{-/-}$ PTECs compared to *Pkd1*^{wt} cells, were indeed involved in PI3K-AKT, Rap1, Hippo, MAPK, Ras, Wnt and TGF-β signaling, which indicates that shear induced gene expression is further elevated due to *Pkd1* gene disruption. In addition, functional enrichment analysis of shear regulated genes that were exclusively induced in *Pkd1*$^{-/-}$ PTECs confirms that a subset of these genes is involved in Hippo, Wnt, MAPK and calcium signaling (Suppl. Table S5). These include several transcriptional regulators of the Hippo and Wnt signaling pathways. Overall, these data suggest that *Pkd1* has the function to restrain shear regulated signaling instead of being a mechano-sensing activator. Accordingly, *Ma et al.* previously proposed a role for *Pkd1* in restraining an unknown cilia-dependent signaling pathway involved in cyst formation [59]. Further research is required to investigate if and how *Pkd1* is inhibiting shear induced signaling. In addition, pathological shear stress can also elevate gene expression of aforementioned pathways compared to physiological levels of shear [20]. Moreover, strong variations in fluid shear stress are common in kidney diseases, including ADPKD, due to tubular dilation obstruction and hyperfiltration, which occur in functional nephrons, to compensate for lost glomeruli and tubules [34]. Increased or pathological shear stress after unilateral nephrectomy [35,36], can accelerate cyst formation in a *Pkd1*$^{-/-}$ mouse model [38]. This leads to the hypothesis that pathological shear stress and *Pkd1* gene disruption can both cause imbalanced cellular signaling, which may contribute to renal cyst formation and fibrosis.

To study shear stress dependent signaling in ADPKD, we compared changes in gene expression in *Pkd1*$^{-/-}$ PTECs during shear with *in vivo* transcriptome analysis of pre-cystic kidneys in mice. In the iKsp-*Pkd1*^{del} mouse model *Pkd1* gene disruption is specifically induced in around 40–50% of the renal epithelial cells, with the largest proportion in proximal tubular epithelial cells [36,38]. In the early phase upon *in vivo* *Pkd1* disruption, there is still fluid flow without signs of cyst formation. In contrast, in the late/end phase of PKD, there are many cysts and

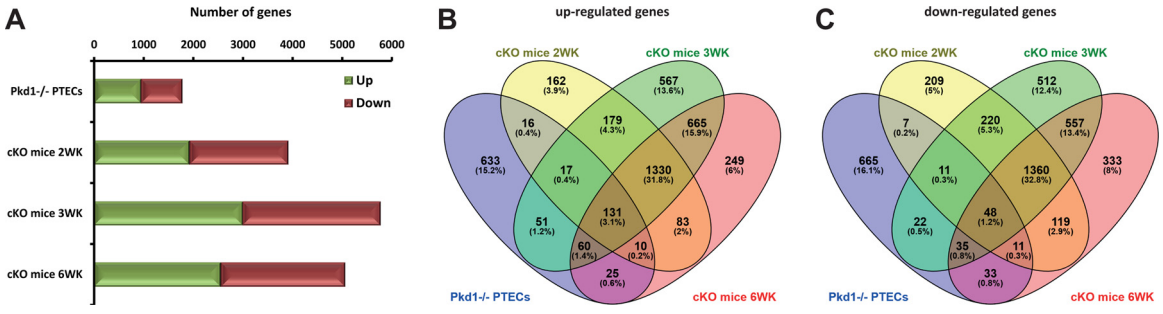

Fig. 4. Comparison of DEG upon *Pkd1* gene disruption in *Pkd1*$^{-/-}$ cells and iKsp-*Pkd1*^{del} mice.
(A) Number of differentially up- or down-regulated genes in *Pkd1*$^{-/-}$ PTECs or iKsp-*Pkd1*^{del} (cKO) mice at 2, 3 or 6 weeks (WK) after gene disruption. (B, C) Venn diagram of up-regulated (B) and down-regulated (C) genes showing the number of genes in overlap between *Pkd1*$^{-/-}$ PTEC cells and iKsp-*Pkd1*^{del} mice. (%) means the percentage of genes of all up-regulated or down-regulated genes.

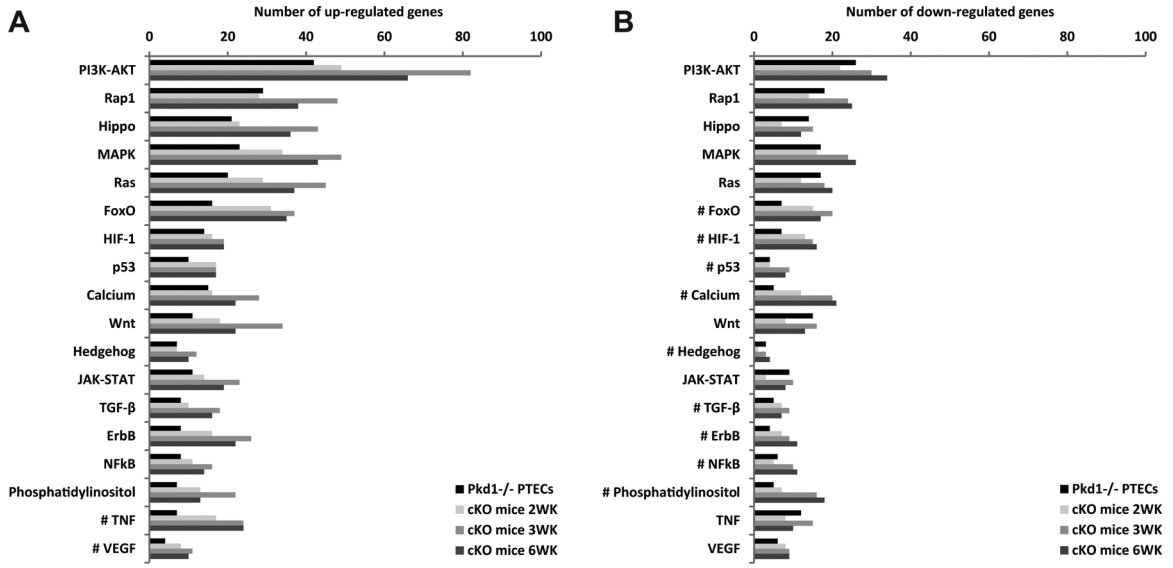

Fig. 5. Core signaling pathways altered upon *Pkd1* gene disruption in *Pkd1*$^{-/-}$ cells and iKsp-*Pkd1*^{del} mice.
Number of differentially expressed genes per condition, which were annotated to up-regulated (A) or down-regulated (B) core signaling pathways (KEGG) for *Pkd1*$^{-/-}$ PTECs or iKsp-*Pkd1*^{del} (cKO) mice at 2, 3 or 6 weeks (WK) after gene disruption. Pathways are ordered by lowest false discovery rate (FDR) of up-regulated pathways in *Pkd1*$^{-/-}$ PTECs. # = Not significantly enriched pathways in *Pkd1*$^{-/-}$ PTECs from functional enrichment analysis (FDR > = 0.01).

fibrotic tissue, causing disturbance or loss of fluid flow in numerous nephrons. At this stage, the remaining nephrons experience increased fluid shear to compensate for the cystic or fibrotic tissue. Therefore, altered signaling of biological pathways or processes in late phase PKD can be caused by several factors, like loss of fluid shear, increased shear, increased pressure, changes in tissue composition, fibrosis or inflammation [60]. For this reason, the *in vitro Pkd1* phenotype during fluid shear exposure in PTECs was compared with the gene expression profiles of pre-cystic adult iKsp-*Pkd1*^{del} mice at 2, 3 or 6 weeks after *Pkd1* gene disruption [46].

In the comparison between the *in vitro* and *in vivo* transcriptome analysis we identified 131 genes up-regulated and 48 genes down-regulated in *Pkd1*$^{-/-}$ PTECs and iKsp-*Pkd1*^{del} mice at all three time points (Suppl. Table S10). Therefore, we can conclude that these genes are already altered in the earliest phase upon *Pkd1* gene disruption. Looking at the pathways and molecular processes, we noticed extensive overlap between cells and mice, although this can largely be attributed to the expression of paralogous genes, rather than to the identical genes. Examples include collagens, integrins, kinases, phosphatases, growth factors, receptors, signal transducers, inhibitors and transcription factors. These genes are involved in several core signaling pathways (Suppl. Table S11) of which several are known to be implicated in ADPKD (*i.e.* PI3K-AKT, MAPK, Hippo, JAK-STAT and TGF-β signaling) [39,45,55,61–74], suggesting that these pathways are already modified at pre-cystic stage.

Interpretation of the Wnt signaling is complex since canonical (Wnt/β-catenin) or non-canonical (β-catenin-independent) Wnt signaling share components, but also may have reciprocal effects. In addition, non-canonical Wnt signaling can be subdivided into the Wnt/planar cell polarity (PCP) and Wnt/Ca^{2+} pathways. Genes involved in Wnt signaling do not show much overlap between *in vitro* and *in vivo* transcriptome data, however, the overall picture suggests decreased non-canonical Wnt signaling in *Pkd1*$^{-/-}$ cells and mice. Increased canonical Wnt might be involved in cyst formation, although aberrant PCP signaling at early stage has been suggested as well [67,75]. Recent data suggest that the polycystin-complex itself mediates Wnt-induced Ca^{2+} signaling, although independent of Fz-receptors [76,77].

Interestingly, several genes involved in endocytosis showed altered expression in *Pkd1*^{del} mice and PTEC cells, while this process was also increased by *in vitro* fluid shear exposure [48–50]. The involvement of endocytosis in several growth factor signaling cascades like TGF-β and MAPK [28,78], makes this finding more interesting, suggesting that altered endocytosis, upon shear stress or *Pkd1* gene disruption, might contribute to imbalanced signaling.

Besides the clear overlap in altered cellular signaling between *Pkd1*$^{-/-}$ cells and *Pkd1*^{del} mice, there were some differences. Genes involved in oxidative phosphorylation, mRNA processing, protein translation and ribosomal proteins were explicitly down-regulated in iKsp-*Pkd1*^{del} mice, while lysosome related gene expression was up-regulated. This might be caused by the different cell types that are present in the kidney, as well as variations in shear stress, and will depend on the overall biological context.

It has been hypothesized that critical Polycystin-1 levels are needed to restrain cellular and cilia related signaling [59,79]. Increased activation of selected genes in renal epithelial cells upon *Pkd1* gene disruption, as shown in this paper, may disturb the balance in signaling and might contribute to cyst formation. Of course, the signaling cascades that trigger cyst formation will depend on several factors, in addition to local and functional PKD protein levels, like renal injury, shear stress, inflammation, the metabolic status and more general, the biological context [60]. For example, a number of studies indicate that renal injury can accelerate cyst progression and fibrosis [67,80,81]. Numerous pre-clinical studies effectively inhibited implicated signaling pathways and reduced cyst formation and fibrosis. However, various clinical studies were unsuccessful to delay cyst growth in patients, while only Tolvaptan, a vasopressin receptor antagonist, has been approved as drug for ADPKD patients in a number of countries [82–86]. Therefore, effective therapies should target multiple signaling pathways, to re-establishing the balance in cellular signaling in renal epithelial cells and to maintain cellular homeostasis within physiological boundaries [60,87,88].

5. Conclusions

In conclusion, shear stress alters the same signaling pathways in *Pkd1*$^{-/-}$ PTECs and in *Pkd1*^{wt} controls *in vitro*. However, the expression of a substantial number of genes was slightly more elevated by shear in *Pkd1*$^{-/-}$ compared to *Pkd1*^{wt} cells, which are involved in Hippo, Wnt, MAPK, TGF-β and calcium signaling. Based on these results we hypothesize that *Pkd1* restrains shear stress induced signaling, rather than

being directly involved in shear dependent activation of these pathways.

A comparison of shear-induced changes in *Pkd1*$^{-/-}$ PTECs with *in vivo* transcriptome data of kidneys at three early pre-cystic time-points, revealed overlap in pathways and molecular processes, involving identical genes (approx. 180) as well as paralogous genes. These pathways include PI3K-AKT, MAPK, JAK-STAT, Hippo, p53, calcium, Wnt and TGF-β signaling, which are known to be implicated in the renal cyst formation as well. So, our results suggest that these processes are already altered at pre-cystic stage and may contribute to *in vivo* cyst formation caused by imbalanced signaling upon *Pkd1* gene disruption.

Compliance with ethical standards

Animal experiments have been carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

Author's contribution

SJK carried out experiments, pathways analysis and wrote the manuscript. TBM carried out bioinformatics gene expression analysis. CF and WNL carried out animal experiments.

PACH advised on the experimental setup and analysis. DJMP advised on the experimental setup, analysis and wrote the manuscript. All authors approved manuscript.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2018.07.178.

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