Ciliary protein polycystin is required for renal tubulointerstitial fibrosis

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Running head: Polycystin promotes renal tubulointerstitial fibrosis

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Keywords: Polycystin; renal tubulointerstitial fibrosis; primary cilia; ADPKD; EZH2

Research highlights:

- Ciliary protein polycystin1 and polycystin-2 are up-regulated in fibrotic kidneys
- Inhibition or deletion of polycystins attenuates renal tubulointerstitial fibrosis
- Polycystins promote EMT in renal epithelial cells
- Epigenetic regulator EZH2 mediates polycystin-driven renal fibrosis.

Abstract

Polycystin-1 (encoded by *PKD1* gene) and polycystin-2 (encoded by *PKD2* gene) are located in the primary cilia, forming a transmembrane complex and function as a stress sensor; their mutation causes autosomal dominant polycystic kidney disease. Recent studies showed that increased expression of polycystins and other ciliary proteins contributes to organ fibrosis. However, the role of polycystins in renal tubulointerstitial fibrosis remains unclear. In this study, we demonstrated that polycystin-1 or polycystin-2 was highly expressed in the kidney of two different fibrotic mouse models and positively correlated with expression of collagen-I. Pharmaceutical inhibition of polycystin-2 with triptolide or genetic knockout of polycystin-2 reduced expression of epithelial-mesenchymal transition (EMT) markers and deposition of extracellular matrix proteins in fibrotic kidneys. Similarly, conditional knockout of *Pkd1* gene also attenuated renal fibrosis in mouse models. Moreover, we found that enhancer of Zeste homolog 2 (EZH2), a methyltransferase that promotes renal fibrosis, was positively correlated with expression of polycystins in fibrotic kidneys, and conditional deletion of the *Ezh2* diminished the anti-EMT and anti-fibrotic effect of *Pkd1* deletion. These data indicate that polycystins drive EMT and renal tubulointerstitial fibrosis through upregulation of EZH2. Targeting ciliary protein may provide a new strategy to treat patients with renal tubulointerstitial fibrosis.

Introduction

Chronic kidney disease (CKD) is a worldwide health concern affecting more than 10% of global population with a high mortality and morbidity (1). Renal fibrosis is the common pathway of various CKD progressing to end stage renal disease regardless of its original causes (1). Hallmarks of renal fibrosis include excessive deposition of extracellular matrix (ECM) proteins (such as collagen-I, and fibronectin), over-expression of epithelial-mesenchymal transition (EMT) markers (such as N-cadherin, α-SMA and vimentin), activation of pro-fibrotic signaling pathways (such as the TGFβ/Smad3 signaling pathway) (2). Recent studies showed epigenetic alterations during the progression of renal fibrosis (3). In particular, enhancer of Zeste homolog 2 (EZH2), a methyltransferase, has been identified as a critical mediator of renal fibrosis (4).

Polycystins were originally identified in autosomal dominant polycystic kidney disease (ADPKD), where mutations in *PKD1* or *PKD2* gene cause this disease (5). Polcystin-1 (encoded by *PKD1* gene) and polycystin-2 (encoded by *PKD2* gene) form a transmembrane complex and are located in the primary cilia functioning as a calcium channel, which can sense external stimuli such as growth factors, chemical or mechanic stress (6). Mutations in ciliary genes responsible for the assembly of primary cilia (such as kinesin family member 3A, KIF3A) can also induce cystogenesis in mouse kidneys (7). Accumulating evidence show that ciliary proteins are implicated in the development or disease condition of other organs, for example, bone development and blood pressure control (8-11). Recent studies showed that

ciliary proteins are also involved in organ fibrosis (6, 12). Although polycystins have been revealed to be up-regulated in kidneys following ischemic or toxic acute injury (13, 14), their roles in injury-induced renal tubulointerstitial fibrosis remains unclear.

In this study, we aimed to investigate the role of polycystins in renal tubulointerstitial fibrosis and their underlying mechanisms in models of renal fibrosis induced by unilateral ureteral obstruction (UUO) and aristolochic acid using genetic and pharmacological approaches. Polycystin-2 has been identified as a direct target of triptolide, a natural compound extracted from a traditional Chinese herb Lei Gong Teng (15), it was thus used to assess its efficacy in renal fibrosis. Our data demonstrate that either pharmacological inhibition of polycystin-2 or genetic deletion of polycystin-1 or -2 inhibited EMT and attenuated renal fibrosis through a mechanism associated with suppression of EZH2.

Results

The expression of polycystin-2 is increased in two mouse models of renal tubulointerstitial fibrosis

As the first step towards understanding the role of polycystins in renal fibrosis, we examined polycystin-2 expression in two mouse models of renal tubulointerstitial fibrosis in wild type (WT) induced by UUO and aristolochic acid I. Compared with sham kidneys, the expression of polycystin-2 was steadily increased from day 3 to 14 in the kidney following UUO, which was correlated with the expression of collagen-I over time (Figure 1A-1C). In the model of aristolochic acid nephropathy (AAN), polycystin-2 and collagen 1 began to increase at 3 days and further elevated at day 21 following aristolochic acid administration (Figure 1D-1F).

Inhibition of polycystin-2 attenuates renal tubulointerstitial fibrosis

Previous studies have shown that triptolide can bind to polycystin-2 and regulate its functions (15), we attempted to use triptolide as a molecular probe to examine the role of polycystin-2 in renal tubulointerstitial fibrosis in WT UUO mice. Masson staining showed that a massive deposition of interstitial collagens in UUO kidneys, and treatment with triptolide largely reduced extracellular matrix deposition (Figure 2A and 2B). Immunoblot analysis also indicated increased expression of ECM proteins (collagen-I) and EMT marker (α-SMA), and activated Smad3 (phosphorylated Smad3, pSmad3) in UUO kidneys and triptolide administration reduced expression of all those pro-fibrotic proteins (Figure 2C, and 2E-2G). Notably,

triptolide also inhibited expression of polycystin-2 in kidneys following UUO operation (Figure 2D and 2H). These data illustrate that triptolide protected kidneys against renal fibrosis and suggest that polycystin-2 upregulation is required for the development of renal fibrosis.

To verify the role of polycystin-2 in renal fibrosis, we examined the effect of Triptolide and polycystin-2 siRNA on the expression of EMT associated proteins in cultured human renal epithelial (HK2) cells. As shown in Figure 2I-2J, exposure of HK2 cells to TGF-β1 increased expression of fibronectin, N-cadherin and vimentin, and pSmad3, and presence of triptolide from 100 nM to 1000 nM reduced their expression (Figure 2I-2J), which is corresponding to its inhibition on polycystin-2 expression (Figure 2K and 2L). Similarly, transfection of PKD2 siRNA also reduced expression of these pro-fibrotic proteins, which was correlated with down-regulation of polycystin-2 (Figure 2M and 2P).

Since it has been reported that polycystin-2 is expressed in tissue fibroblasts (6), we also examined the effect of triptolide on the expression of proteins as described above in cultured normal rat kidney fibroblasts (NRK-49F). In line with our observations in HK2 cells, triptolide dose-dependently inhibited expression of these pro-fibrotic markers and polycystin-2 in this cell line (supplemental Figure 1A-1F).

Collectively, these data suggest that injury-induced upregulation of polycystin-2 may contribute to EMT, fibroblast activation and renal fibrosis.

Conditional knockout of polycystin-2 mitigates renal tubulointerstitial fibrosis in

mice

To confirm the pro-fibrotic role of polycystin-2, we generated *Pkd2* conditional knockout mice. As shown in Figure 3A and 3B, heterozygous (*Pkd2*$^{fl/+}$*;Cre/Esr1*$^{+}$) or homozygous (*Pkd2*$^{fl/fl}$*;Cre/Esr1*$^{+}$) conditional knockout mice of *Pkd2* exhibited reduced positive areas of Masson staining in UUO kidneys. Although the similar degree of dilated tubules was present in control (*Pkd2*$^{fl/+}$*;Cre/Esr1*$^{-}$) or heterozygous *Pkd2* knockout UUO kidneys without appearance of cysts, numerous small cysts (luminal diameter exceeding 50 μm) in addition of dilated tubules were observed in the kidney of homozygous *Pkd2* knockout mice (Figure 3A). Immunoblot analysis showed partial reduction of *Pkd2* expression in heterozygous and its absence in homozygous conditional knockout of *Pkd2*, which is corresponding to a different degree of pro-fibrotic markers (collagen-I, pSmad3, and α-SMA) (Figure 3C-3G).

The anti-fibrotic effect of polycystin-2 deletion was observed in AAN mouse models as well. As shown in Figure 3H-3N, mild renal interstitial fibrosis was seen in control (*Pkd2*$^{fl/+}$*;Cre/Esr1*$^{-}$) AAN kidneys, as indicated by Masson staining and immunoblot analysis of collagen-I, pSmad3, and α-SMA (Figure 3H-3N). Heterozygous deletion of *Pkd2* gene in *Pkd2*$^{fl/+}$*;Cre/Esr1*$^{+}$ mice showed reduced deposition of extracellular matrix and expression of pro-fibrotic markers in AAN kidneys, which was correlated with downregulation of polycystin-2 (Figure 3H-3N).

Together, all these data indicate that polycystin-2 promotes EMT and is pro-fibrotic in diseased kidneys.

Polycystin-1 is increased in fibrotic kidneys and conditional deletion of *Pkd1* gene attenuates renal tubulointerstitial fibrosis in mouse kidneys

Polycystin-2 intertwined with polycystin-1 in primary cilia mediates all the biological responses, we thus further examined the expression of polycystin-1 and other ciliary proteins in the mouse model with renal tubulointerstitial fibrosis. Compared with sham kidneys, the expression of polycystin-1 was increased from day 3 to day 14 in UUO kidneys (Figure 4A-4C). Similarly, the expression of KIF3A, another ciliary protein, was also up-regulated in UUO kidneys in a time dependent manner (supplemental Figure 2A-2C).

Using *Pkd1* conditional knockout mice, we found that heterozygous ($Pkd1^{fl/+}$;$Cre/Esr1^{+}$) or homozygous ($Pkd1^{fl/fl}$;$Cre/Esr1^{+}$) conditional knockout of *Pkd1* gene reduced the deposition of extracellular matrix in UUO kidneys as shown by Masson staining (Figure 4D-4E). Numerous small cysts were observed in homozygous *Pkd1* knockout mice (Figure 4D). Heterozygous or homozygous conditional knockout of *Pkd1* inhibited expression of polycystin-1 and pro-fibrotic markers (collagen-I, pSmad3, and α-SMA) in UUO kidneys as compared with control ($Pkd1^{fl/+}$;$Cre/Esr1^{-}$) UUO kidneys (Figure 4F-4J). Collectively, our data indicate that polycystin-1 promotes renal tubulointerstitial fibrosis as well.

Polycystins promote renal fibrosis through EZH2

EZH2 has been identified as an important epigenetic regulator in the development of renal tubulointerstitial fibrosis (4), we therefore hypothesized that

EZH2 would be involved in the pro-fibrotic effect of polycystins in injured kidneys. EZH2 was upregulated in mouse UUO kidneys as compared with sham-operated kidneys (Figure 5A, 5C, 5D and 5F). Heterozygous or homozygous conditional knockout of *Pkd2* or *Pkd1* gene dose-dependently inhibited expression of EZH2 in mouse UUO kidneys (Figure 5A, 5C, 5D and 5F). Upregulation of EZH2 was also observed in AAN kidneys in control mice, which was reduced by heterozygous deletion of *Pkd2* gene (Figure 5B and 5E). Conditional knockout of *Ezh2* reduced extracellular matrix deposition and the expression of pro-fibrotic markers (collagen-I, pSmad3, and α-SMA) in UUO kidneys (Figure 5G-5N). Inhibition of extracellular matrix deposition or the expression of pro-fibrotic markers (collagen-I, pSmad3, and α-SMA) by *Pkd1* deletion in *Ezh2* knockout kidneys ($Ezh2^{fl/fl}$;$Pkd1^{fl/fl}$;$Cre/Esr1^{+}$) was less than that in *Ezh2* control kidneys ($Ezh2^{+/+}$; $Pkd1^{fl/fl}$;$Cre/Esr1^{+}$). This suggests that polycystins promote renal fibrosis through a mechanism involved in the upregulation of EZH2.

Discussion

In the current study, we investigated the role of polycystins in renal tubulointerstitial fibrosis. We conclude that polycystins are pro-fibrotic in injured kidneys, which is supported by the following observations: (1) polycystin-1 and polycystin-2 were up-regulated in two different mouse models of renal fibrosis, which was positively correlated with the expression of ECM protein collagen-I; (2) inhibition of polycystin-2 by triptolide or siRNA reduced expression of pro-fibrotic markers in cultured renal epithelial cells; (3) pharmaceutical or genetic inhibition of polycystin-2 reduced partial EMT and renal fibrosis in UUO and AAN mouse models; (4) conditional knockout of *Pkd1* gene reduced pro-fibrotic marker expression and ECM accumulation in fibrotic kidneys. These data suggest that polycystins may hold the potential to be therapeutic targets for the treatment of chronic fibrotic kidney diseases.

Primary cilium is a no-motile, microtubule-based organelle protruding from cell membrane and acts as a senser transducing external signals into cells to control a variety of biological processes (16). Mutations in ciliary genes are involved in the pathology of many organs (17, 18). Recent studies showed that primary cilia play an important role in organ fibrosis (19). Conditional knockout of ciliary proteins such as IFT88 or polycystin-1 attenuates fibrosis in injured lungs or hearts (6, 12). In line with these studies, inhibition or conditional knockout of the functional ciliary protein prolycystin-1 or polycystin-2 in injured kidneys also attenuated renal tubulointerstitial fibrosis. Moreover, the expression of structural protein of primary cilia KIF3A is

positively correlated with the progression of renal fibrosis in fibrotic kidneys. All these data suggest that primary cilia are required for renal tubulointerstitial fibrosis. Targeting ciliary genes or primary cilium related signaling pathways may be a new strategy to prevent or treat renal tubulointerstitial fibrosis.

Previous studies showed that renal fibrosis is increased in *Pkd1* or *Pkd2* deleted kidneys after renal ischemia reperfusion injuries (14, 20). Nevertheless, the experimental design in these two studies was different from ours. In these two studies, *Pkd1* or *Pkd2* gene was globally knockout, which led to a different baseline, such as, an increased cell proliferation in the kidney of knockout mice as compared with that in wide type mice (14, 20). It has been shown that inhibition of proliferation associated signaling pathways such as the mTOR or MAPK pathway ameliorated renal tubulointerstitial fibrosis (21, 22), and thus the difference in baseline proliferation rate may lead to a different outcome in mouse model of renal fibrosis. To keep the same baseline condition, we only inactivated *Pkd* gene at one week before kidney injury in this study.

Partial EMT is an important mechanism underlying renal tubulointerstitial fibrosis (23). Under this pathological condition, renal tubular epithelial cells express profibrotic markers but do not migrate into interstitial areas (23). In this study, we showed that pharmacological inhibition or genetic deletion of polycystins reduced expression of EMT markers such as α-SMA, N-cadherin and vimentin in fibrotic kidneys and in TGF-β stimulated human renal epithelial cells, suggesting that polycystins are engaged in EMT. EMT also plays an important role in cancer

progression (24). It has been reported that the cancer risk in ADPKD recipient is lower than that in non-ADPKD recipient after renal transplantation, however the underlying biological mechanism is currently unknown (25). Thus, it would be interesting to study whether polycystin-mediated EMT is involved in the pro-tumor action of ADPKD genes.

EZH2 is an important epigenetic regulator during the development of renal fibrosis and has been intensively studied in recent years (3, 4). Studies showed that EZH2 promotes EMT in fibrotic kidneys and represses E-cadherin expression in renal cell carcinoma (26, 27). Thus, we hypothesized that polycystins promote renal fibrosis through EZH2-mediated EMT. Indeed, we found that polycystin is positively correlated with the expression of EZH2 and EMT markers, and genetic deletion of polycystins reduced expression of EZH2 and EMT markers in fibrotic kidneys. Importantly, genetic knockout of *Ezh2* diminished the anti-EMT and anti-fibrotic effect of *Pkd1* deletion in UUO kidneys. This suggests that polycystins play a role in regulating expression of EZH2 and that EZH2 acts downstream of polycystins to mediate EMT and renal tubulointerstitial fibrosis. It remains unclear how polycystins regulate expression of EZH2.

Activation of fibroblasts and subsequent production of a large amount of ECM proteins are hallmarks of renal tubulointerstitial fibrosis (28). In this study, we showed that inhibition of polycystin-2 by triptolide reduced expression of pro-fibrotic markers in renal fibroblasts, suggesting that polycystins may contribute to renal fibrogenesis through activating renal fibroblasts. In this context, it has been reported that primary

cilia are enriched in cardiac fibroblasts and deletion of polycystin-1 in fibroblasts attenuates cardiac fibrosis (6). As such, we speculate that there exist at least two mechanisms by which polycystin-2 drives renal fibroblast activation. On the one hand, polycystin-2 may induce partial EMT and G2/M arrest of renal tubular cells and then produces some profibrotic growth factors/cytokine that release to the interstitium to stimulate activation of renal fibroblasts. On the other hand, polycystin-2 may also directly induce activation of renal fibroblasts. Further study with specific deletion of polycystins in renal fibroblasts will address the latter issue.

Polycystins are the main focus of ADPKD research (29). The role of polycystins in CKD progression is rarely studied. Our study indicates that polycystins are pro-fibrotic and thus may play an important role in CKD progression. On this basis, polycystins hold the potential as therapeutic targets for CKD patients. Importantly, we demonstrated that treatment with triptolide, an inhibitor of polycystin-2 can effectively attenuate renal fibrosis in a murine model of renal fibrosis and triptolide-containing formulation is currently used in clinic in China to treat CKD patients (30). Nevertheless, more specific inhibitors of polycystin-2 should be designed and synthesized based on the structure of triptolide in the future and be tested in clinic to treat CKD patients with tubulointerstitial fibrosis.

In summary, our study revealed that polycystins promote EMT and renal tubulointerstitial fibrosis in kidney diseases through a mechanism involved in EZH2 upregulation, and suggests that targeting primary cilia associated signaling pathways may be a new therapeutic strategy to treat patients with renal fibrosis.

Materials and Methods

Animals

Only male mice were used in animal studies. Wide type (WT) C57BL/6 mice (SPF grade, 20-25g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. *Pkd1*$^{fl/fl}$ mice were obtained from Prof. Rudolf Wüthrich in University of Zurich, which were originally from the lab of Professor Gregory Germino in Johns Hopkins University School of Medicine (31). C57/BL6 *Pkd1*$^{fl/fl}$ mice were crossed with C57/BL6 tamoxifen-Cre (B6. Cg-Tg [Cre/Esr1]) 5Amc/J mice (stock 004682; Jackson Laboratories) as described previously (32). *Ezh2*$^{fl/fl}$ mice on a C57/B6 background were provided by Prof. Xi Wang from Capital Medical University and were further crossed with *Pkd1*$^{fl/fl}$*;Cre/Esr1*$^{+}$ mice (33). *Pkd2* conditional knockout mice with exon 3 deletion have cystic phenotype in the kidney (34). CRISPR/Cas9 technology was used to generate a mouse with loxP sites flanking exon 3 of the Pkd2 gene by Shanghai Model Organisms Center. *Pkd2*$^{fl/fl}$ were crossed with tamoxifen-Cre mice to generate *Pkd2*$^{fl/fl}$*;Cre/Esr1*$^{+}$ mice. Animals were housed in the animal facility of Shanghai University of Traditional Chinese Medicine according to local regulations and guidelines. Animal experiments were approved by the ethic committee of Shanghai University of Traditional Chinese Medicine (PZSHUTCM18102604).

Genotyping

Genomic DNA was prepared from mouse tail by using animal genomic DNA quick extraction kit (D0065M, Beyotime Biotech, Nantong, China) as described in the manufacturer's instructions. PCR genotyping was performed using the following primers: Pkd2-F, GTACACCTGTGCAGCACTCA; Pkd2-R,

GGAGCTCATCATGCCGTAGG; CRE-F, ATTGCTGTC ACTTGGTCGTGGC; CRE-R, GGAAAATGCTTCTGTCCGTTTGC; Pkd1-F, CCTGCCTTGCTCTACTTTCC; PKD1-R, AGGGCTTTTCTTGCTGGTCT; EZH2-F, CCCATGTTTAAGGGCATAGTGACATG; EZH2-R, ATGTGCAGGTCAGTCAGCAACTTCAG. Details regarding PCR conditions are available upon request.

Experimental Animal Protocol

UUO operation was performed through twice ligation of the left ureter with 4-0 nylon sutures. WT animals were sacrificed at day 3, 7 or 14 in a time-course experiment (n=5-6 for each time point). For interventional study, WT mice were randomly divided into four groups: 1) Sham/Vehicle (n=6), 2) Sham/triptolide (n=7), 3) UUO/Vehicle (n=7), and 4) UUO/triptolide (n=7) group. The stock of triptolide (Topscience, T2179, Shanghai, China) was dissolved in DMSO and further diluted in normal saline as working solution. Sham or UUO mice were treated with vehicle or 0.25 mg/kg triptolide daily by intraperitoneal (i.p.) injection for 10 days starting from day 0. Mice were sacrificed at day 10. Kidney samples were obtained for Western blotting or histological examinations. For conditional knockout mice (n= 7-9 mice per group), Cre recombinase activity was induced by intraperitoneally injection of tamoxifen (50 mg kg^{-1} day^{-1}, Apexbio, B5965) for three consecutive days at one week before UUO operations. Conditional knockout mice at 8 weeks of age were subjected to UUO operation and were sacrifice at day14.

To induce aristolochic acid nephropathy in mice, one dose of aristolochic acid I

(AAI, A9451, Sigma; 5 mg/kg body weight) or normal saline (NS) was peritoneally injected. WT animals were sacrificed at day 3, 7 or 21 in a time-course experiment (n=5-6 for each time point). Kidney tissues were collected. For conditional knockout mice (n= 6-8 mice per group), Cre recombinase activity was induced at 7 weeks of age by intraperitoneally injection of tamoxifen for three consecutive days at one week before the first AAI injection (5 mg/kg body weight), which was followed by a second AAI injection after one week (5 mg/kg body weight). Mice were sacrificed at 21 days after the second AAI injection.

Cell Culture

Normal rat kidney 49F (NRK-49F) cells, a rat kidney interstitial fibroblast cell line, were purchased from national infrastructure of cell line resource, Chinese academy of medical sciences. To study the anti-fibrotic effect of triptolide, NRK-49F cells were seeded in 6-well plates to 40–50% confluence and were starved with DMEM/F12 medium containing 0.5% fetal bovine serum (FBS) overnight before the experiment. The next day, cells were refreshed with DMEM/F12 medium with 0.5% FBS and then were exposed to different concentrations of triptolide (10 nM, 100 nM, and 1000 nM) for 24h. Protein was extracted from cell lysates for further analysis.

HK2 renal proximal tubular epithelial cells were obtained from the Cell Bank of Shanghai Institute of Biological Sciences (Chinese Academy of Science). HK2 cells were seeded in 6-well plate to 40-50% confluence, which were starved overnight with DMEM/F12 medium containing 0.5% fetal bovine serum. On the next day, fresh medium containing 0.5% fetal bovine serum was changed, and then cells were

exposed to 2.5 ng/ml TGF-β (Peprotech, Rocky Hill, NJ, USA) for 48h in the presence of various concentration of triptolide.

Nonsense control (NC) or human PKD2 siRNA were transfected by Lipofectamine 2000 (11668-027; Invitrogen) in HK2 cells using DMEM/F12 medium containing 10% fetal bovine serum according to the manufacturer's instruction. Protein was extracted from cell lysates at 48h after transfection to assess the efficacy of PKD2 siRNA (supplemental Figure 3). In another experiment, fresh medium containing 0.5% FBS was changed on the second day after transfection, and then cells were exposed to 2.5 ng/ml TGF-β (Peprotech, Rocky Hill, NJ, USA) for 48h with or without triptolide. The NC siRNA sequences were as follows: forward, 5′-UUCUCCGAACGUGUCACGUTT-3′; and reverse, 5′-ACGUGACACGUUCGGAGAATT-3′. The human PKD2 siRNA sequences were as follows: forward, 5′-GGACAUAGCUCCAGAAGGATT-3′; and reverse, 5′-UCCUUCUGGAGCUAUGUCCTT-3′.

Masson's Trichrome Staining

Mouse kidneys were fixed and embedded in paraffin. Four-μm-thick sections of paraffin-embedded kidney tissue was stained with hematoxylin, and then with ponceau red liquid dye acid complex, which was followed by incubation with phosphomolybdic acid solution. Finally, the tissue was stained with aniline blue liquid and acetic acid. Images were obtained with the use of a microscope (Nikon 80i, Tokyo, Japan).

Western Blotting

Cellular or kidney protein was extracted by using lysis buffer purchased from Beyotime Biotech (P0013, Nantong, China). The protein concentration was measured by using BCA Protein Assay Kit (P0012S, Beyotime Biotech, Nantong, China). Protein samples were dissolved in 5x SDS-PAGE loading buffer (P0015L, Beyotime Biotech, Nantong, China), which were further subjected to SDS-PAGE gel electrophoresis. After electrophoresis, proteins were electro-transferred to a polyvinylidene difluoride membrane (Merck Millipore, Darmstadt, Germany), which was incubated in the blocking buffer (5% non-fat milk, 20mM Tris-HCl, 150mMNaCl, PH=8.0, 0.01%Tween 20) for 1 hour at room temperature and was followed by incubation with anti-polycystin-1 (1:500, sc-130554, SantaCruz), anti-polycystin-2 (1:1000, 19126-1-AP, Proteintech), anti-polycystin-2 (1:1000, A3625, ABclonal), anti-KIF3A (1:500, SC-376680, SantaCruz), anti-EZH2 (1:1000, 5246S, Cell Signaling Technology) antibody, anti-fibronectin (1:1000, ab23750, Abcam), anti-pSmad3 (1:1000, ET1609-41, HUABIO), anti-Collagen I (1:1000, ab260043, Abcam), anti-α-SMA (1:1000, ET1607-53, HUABIO), anti-N-cadherin (1:1000, sc-59887, Santa Cruz), anti-vimentin (1:1000, R1308-6, HUABIO), anti-GAPDH (1:5000, 60004-1-lg, Proteintech), or anti-α-tubulin (1:1000, AF0001, Byotime) antibodies overnight at 4□. Binding of the primary antibody was detected by an enhanced chemiluminescence method (SuperSignal™ West Femto, 34094, Thermo Fisher Scientific) using horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG, 1:1000, A0208, Beyotime or goat anti-mouse IgG, 1:1000, A0216, Beyotime).

Statistical Analysis

Statistical analyses were performed by one-way ANOVA followed by a Dunnett's multiple comparisons test using GraphPad Prism version 5.0 (GraphPad, San Diego, CA). All data are expressed as means ± SD, and $P < 0.05$ was considered as statistically significant.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This work was supported by Key Disciplines Group Construction Project of Pudong Health Bureau of Shanghai (PWZxq2017-07); The Three Year Action Plan Project of Shanghai Accelerating Development of Traditional Chinese Medicine (ZY(2018-2020)-CCCX-2003-08); Shanghai Key Laboratory of Traditional Chinese Clinical Medicine (20DZ2272200), Shanghai, PR China; Scientific Research Foundation of Shanghai Municipal Commission of Health and Family Planning (201740193) to MW; National Natural Science Foundation of China (81873617; 82170747) to CY.

Author Contributions

CY funded the project. MW, SZ, JH, YJ, CY, DC conceived and coordinated the study. MW, SZ and JH wrote the paper. YW conducted the *in vitro* experiments. MW, YW, YJ, JL, DC, BT, MY and DH performed the animal experiments. YW performed and analyzed the Western blotting. All authors reviewed the results and approved the final version of the manuscript.

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Figure legends

Fig. 1. Polycystin-2 is up-regulated in fibrotic kidneys.

(A-C) The time course of polycystin-2 expression in sham or unilateral ureteral obstruction (UUO) kidneys from wide type (WT) mice. The expression of collagen-I and polycystin-2 was analyzed by Western blotting and quantified. One representative of at least three independent experiments is shown. (D-F) The time course of polycystin-2 expression in aristolochic acid nephropathy (AAN) kidneys from WT mice. The expression of collagen-I and polycystin-2 in normal saline (NS) or AAN kidneys was analyzed by Western blotting and quantified. N=5-6 in each experimental group and one representative of at least three independent experiments is shown. NS represents not significant. $^{**}p<0.01$. $^{***}p<0.001$.

Fig. 2. Pharmaceutical or genetic inhibition of polycystin-2 attenuates renal tubulointerstitial fibrosis.

WT mice received sham or UUO operation followed by treatment with vehicle or triptolide (TP) for 10 days and then were sacrificed at day 10. (A-B) Renal fibrosis was assessed by Masson's trichrome staining and quantified. Scale bar represents 100 μm. (C-H) The expression of collagen-I, phosphorylated Smad3 (pSmad3), α-SMA and polycystin-2 was analyzed by Western blotting and quantified. (I-L) HK2 human renal epithelial cells were stimulated with TGF-β and treated with different concentration of TP for 48 hours. Cell lysates were collected, and Western blot analysis was performed to determine expression of polycystin-2, fibronectin, N-cadherin, vimentin, and pSmad3, which was further quantified. (M-P) HK2 cells were transfected with nonsense control (NC) or PKD2 siRNA (siPKD2). On the second day, HK2 cells were stimulated with TGF-β for 48 hours. Cell lysates were collected, and Western blot analysis was performed to determine expression of polycystin-2, fibronectin, N-cadherin, vimentin, and pSmad3. N=7-9 in each experimental group and one representative of at least three independent experiments is shown. NS represents not significant. $^{*}p<0.05$. $^{**}p<0.01$. $^{***}p<0.001$.

Fig. 3. Conditional knockout of *Pkd2* attenuates renal tubulointerstitial fibrosis in mice.

Control (*Pkd2*$^{fl/+}$*;Cre/Esr1*$^{-}$), heterozygous (*Pkd2*$^{fl/+}$*;Cre/Esr1*$^{+}$) or homozygous (*Pkd2*$^{fl/fl}$*;Cre/Esr1*$^{+}$) *Pkd2* conditional knockout mice received sham or UUO operation and were sacrificed at day 14. (A-B) Renal fibrosis was assessed by Masson's trichrome staining. Scale bar represents 100 μm. (C-G) The expression of collagen-I, pSmad3, α-SMA and polycystin-2 was analyzed by Western blotting. To induce aristolochic acid nephropathy (AAN), control (*Pkd2*$^{fl/+}$*;Cre/Esr1*$^{-}$) or heterozygous (*Pkd2*$^{fl/+}$*;Cre/Esr1*$^{+}$) *Pkd2* conditional knockout mice were injected intraperitoneally with aristolochic acid I (AAI) or normal saline (NS) once a week for 2 weeks. *Pkd2* gene was inactivated at one week before AAI or NS injection. Mice were sacrificed at 3 weeks after the second AAI or NS injection. (H and J) Renal fibrosis was assessed by Masson's trichrome staining and quantified. Scale bar

represents 100 μm. (I, K, L, M) The expression of collagen-I, pSmad3, α-SMA and polycystin-2 was analyzed by Western blotting and further quantified. N=6-8 in each experimental group and one representative of at least three independent experiments is shown. *$p<0.05$. **$p<0.01$. ***$p<0.001$.

Fig. 4. Conditional knockout of *Pkd1* attenuates renal tubulointerstitial fibrosis in injured kidneys induced by UUO operation.

(A-C) The time course of polycystin-1 expression in sham or UUO kidneys from WT mice. The expression of collagen-I and polycystin-1 was analyzed by Western blotting and quantified. N=5-6 in each experimental group and one representative of at least three independent experiments is shown. Control (*Pkd1*$^{fl/+}$;*Cre/Esr1*$^{-}$), heterozygous (*Pkd1*$^{fl/+}$;*Cre/Esr1*$^{+}$) or homozygous (*Pkd1*$^{fl/fl}$;*Cre/Esr1*$^{+}$) *Pkd1* knockout mice received sham or UUO operation and were sacrificed at day 14. (D-E) Renal fibrosis was assessed by Masson's trichrome staining. Scale bar represents 100 μm. (F-J) The expression of collagen-I, pSmad3, α-SMA and polycystin-1 was analyzed by Western blotting and quantified. One representative of at least three independent experiments is shown. N=6-8 in each experimental group and one representative of at least three independent experiments is shown. *$p<0.05$. **$p<0.01$. ***$p<0.001$.

Fig. 5. Knockout of *Ezh2* abolished anti-fibrotic effect of *Pkd1* deletion in UUO kidneys.

(A-F) The expression of EZH2 was analyzed by Western blotting in *Pkd2* or *Pkd1* knockout mice after UUO operation or AAI injection and quantified. Control (*Ezh2*$^{+/+}$;*Pkd1* $^{fl/fl}$;*Cre/Esr1*$^{-}$), *Ezh2* (*Ezh2*$^{fl/fl}$;*Pkd1*$^{+/+}$;*Cre/Esr1*$^{+}$), *Pkd1* (*Ezh2*$^{+/+}$; *Pkd1*$^{fl/fl}$;*Cre/Esr1*$^{+}$) or *Pkd1/EZH2* (*Ezh2*$^{fl/fl}$;*Pkd1*$^{fl/fl}$;*Cre/Esr1*$^{+}$) knockout mice received UUO operation and were sacrificed at day 14. (G-H) Renal fibrosis was assessed by Masson's trichrome staining. Scale bar represents 100 μm. (I-N) The expression of collagen-I, pSmad3, α-SMA, EZH2 and polycystin-1 was analyzed by Western blotting. N=6-8 in each experimental group and one representative of at least three independent experiments is shown. NS represents not significant. *$p<0.05$. **$p<0.01$. ***$p<0.001$.

Supplemental Fig. 1. Triptolide reduces the expression of polycystin-2 and pro-fibrotic markers in renal fibroblasts.

Normal rat renal fibroblasts (NRF-49F) were treated with different concentrations of triptolide (TP) for 24 hours, and cell lysates were collected. (A-F) Western blot analysis was performed to determine the expression of polycystin-2, fibronectin, N-cadherin, and vimentin, which was further quantified. One representative of at least three independent experiments is shown. NS represents not significant. $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$.

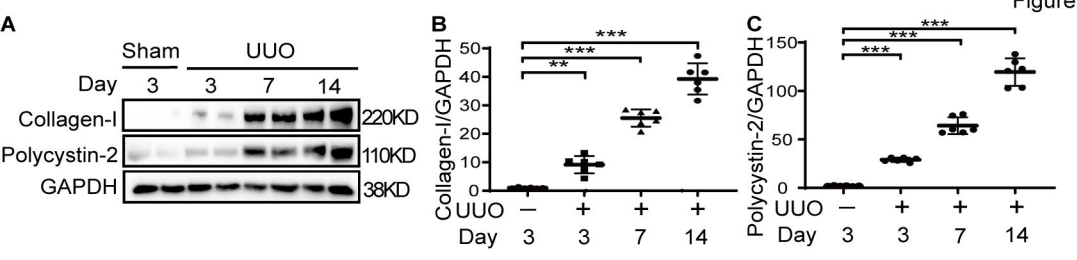
Supplemental Fig. 2. The expression of KIF3A in UUO kidneys.

The time course of KIF3A expression in sham or UUO kidneys from WT mice. (A-C) The expression of collagen-I and KIF3A was analyzed by Western blotting and quantified. N=5-6 in each experimental group and one representative of at least three independent experiments is shown. NS represents not significant. $^{**}p < 0.01$. $^{***}p < 0.001$.

Supplemental Fig. 3. Transfection efficiency of PKD2 siRNA in HK2 cells.

HK2 cells were transfected with NC or PKD2 siRNA. On the second day, HK2 cells were stimulated with TGF-β for 48 hours. Cell lysates were collected, and Western blot analysis and its quantification were performed to measure the expression of polycystin-2. One representative of at least three independent experiments is shown. $^{**}p < 0.01$.

Figure1


A
Sham
UUO
Day 3 3 7 14
Collagen-I
Polycystin-2
GAPDH
220KD
110KD
38KD
B
Collagen-I/GAPDH
UUO — + + +
Day 3 3 7 14
C
Polycystin-2/GAPDH
UUO — + + +
Day 3 3 7 14
D
NS
AAN
Day 3 3 7 21
Collagen-I
Polycystin-2
GAPDH
220KD
110KD
38KD
E
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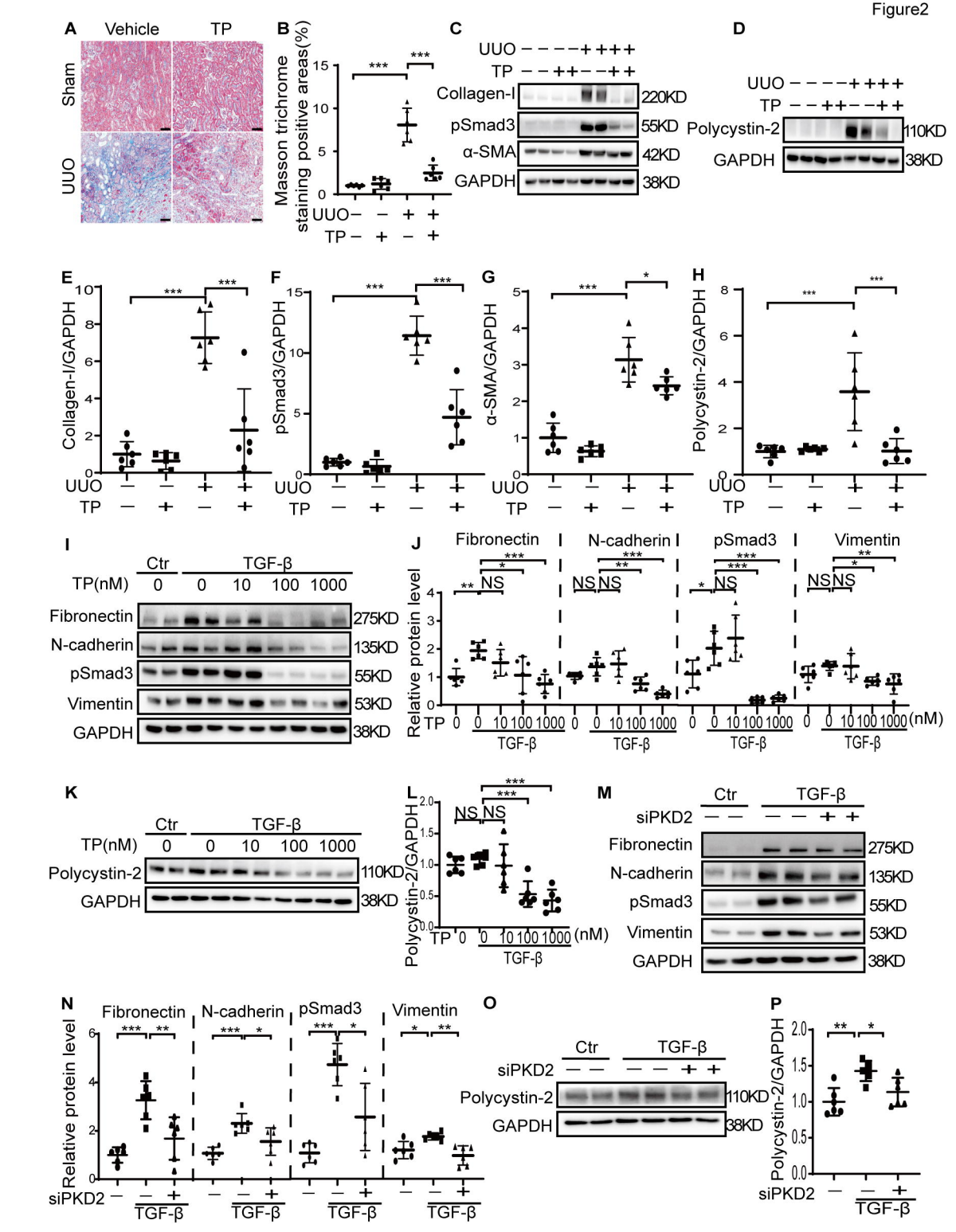
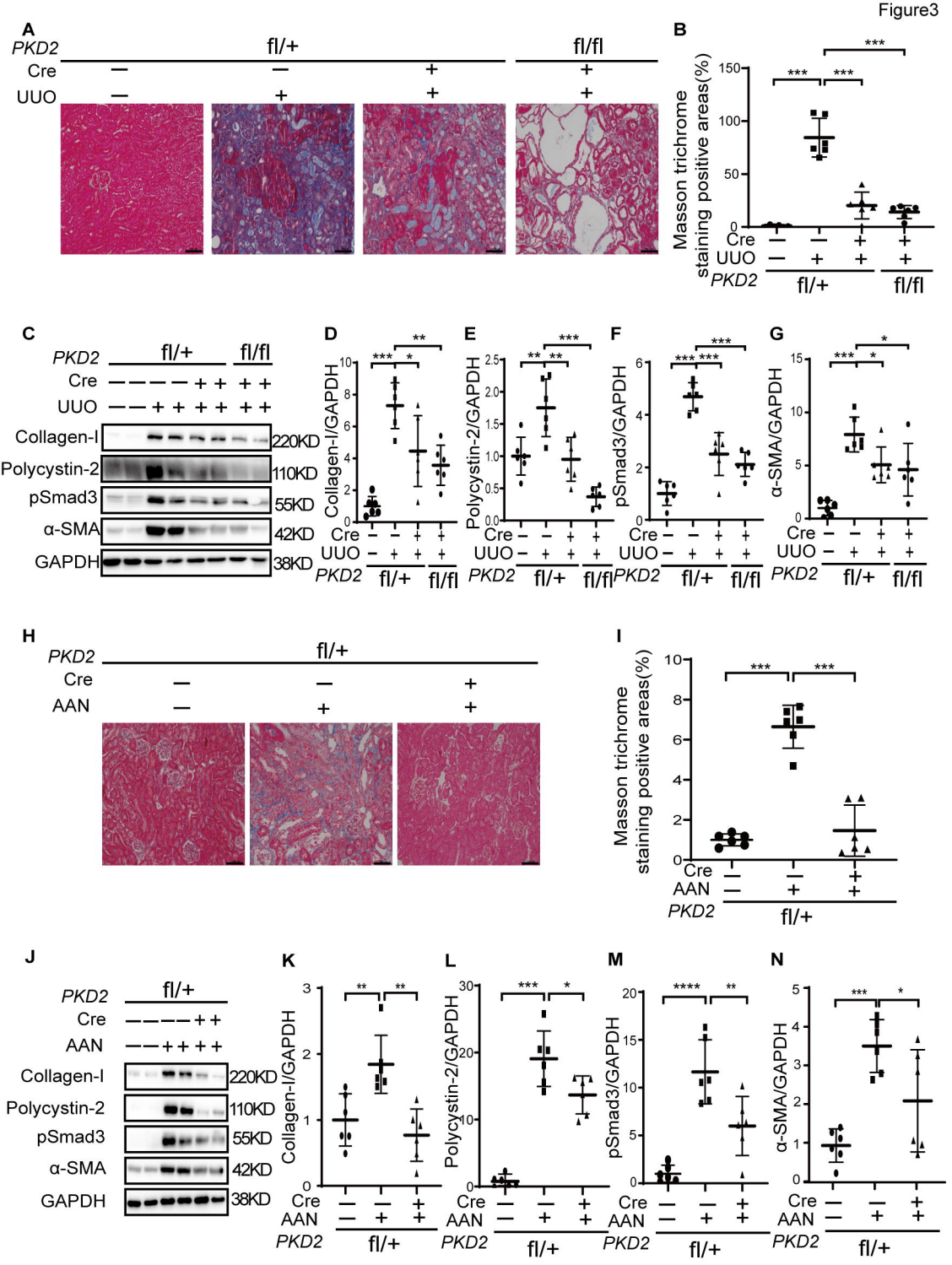
NS
AAN — + + +
Day 3 3 7 21
F
Polycystin-2/GAPDH
NS
AAN — + + +
Day 3 3 7 21


A
Vehicle
TP
Sham
UUO
B
Masson trichrome staining positive areas(%)
UUO
TP
C
UUO
TP
Collagen-I
pSmad3
α-SMA
GAPDH
220KD
55KD
42KD
38KD
D
UUO
TP
Polycystin-2
GAPDH
110KD
38KD
E
Collagen-I/GAPDH
F
pSmad3/GAPDH
G
α-SMA/GAPDH
H
Polycystin-2/GAPDH
I
Ctr
TGF-β
TP(nM)
0
0
10
100
1000
Fibronectin
N-cadherin
pSmad3
Vimentin
GAPDH
275KD
135KD
55KD
53KD
38KD
J
Fibronectin
N-cadherin
pSmad3
Vimentin
Relative protein level
TGF-β
(nM)
K
Ctr
TGF-β
TP(nM)
Polycystin-2
GAPDH
110KD
38KD
L
Polycystin-2/GAPDH
TGF-β
(nM)
M
Ctr
TGF-β
siPKD2
Fibronectin
N-cadherin
pSmad3
Vimentin
GAPDH
275KD
135KD
55KD
53KD
38KD
N
Fibronectin
N-cadherin
pSmad3
Vimentin
Relative protein level
siPKD2
TGF-β
O
Ctr
TGF-β
siPKD2
Polycystin-2
GAPDH
110KD
38KD
P
Polycystin-2/GAPDH
siPKD2
TGF-β


A
PKD2
fl/+
fl/fl
Cre
UUO
B
Masson trichrome staining positive areas(%)
C
Collagen-I
Polycystin-2
pSmad3
α-SMA
GAPDH
220KD
110KD
55KD
42KD
38KD
D
Collagen-I/GAPDH
E
Polycystin-2/GAPDH
F
pSmad3/GAPDH
G
α-SMA/GAPDH
H
AAN
I
J
K
L
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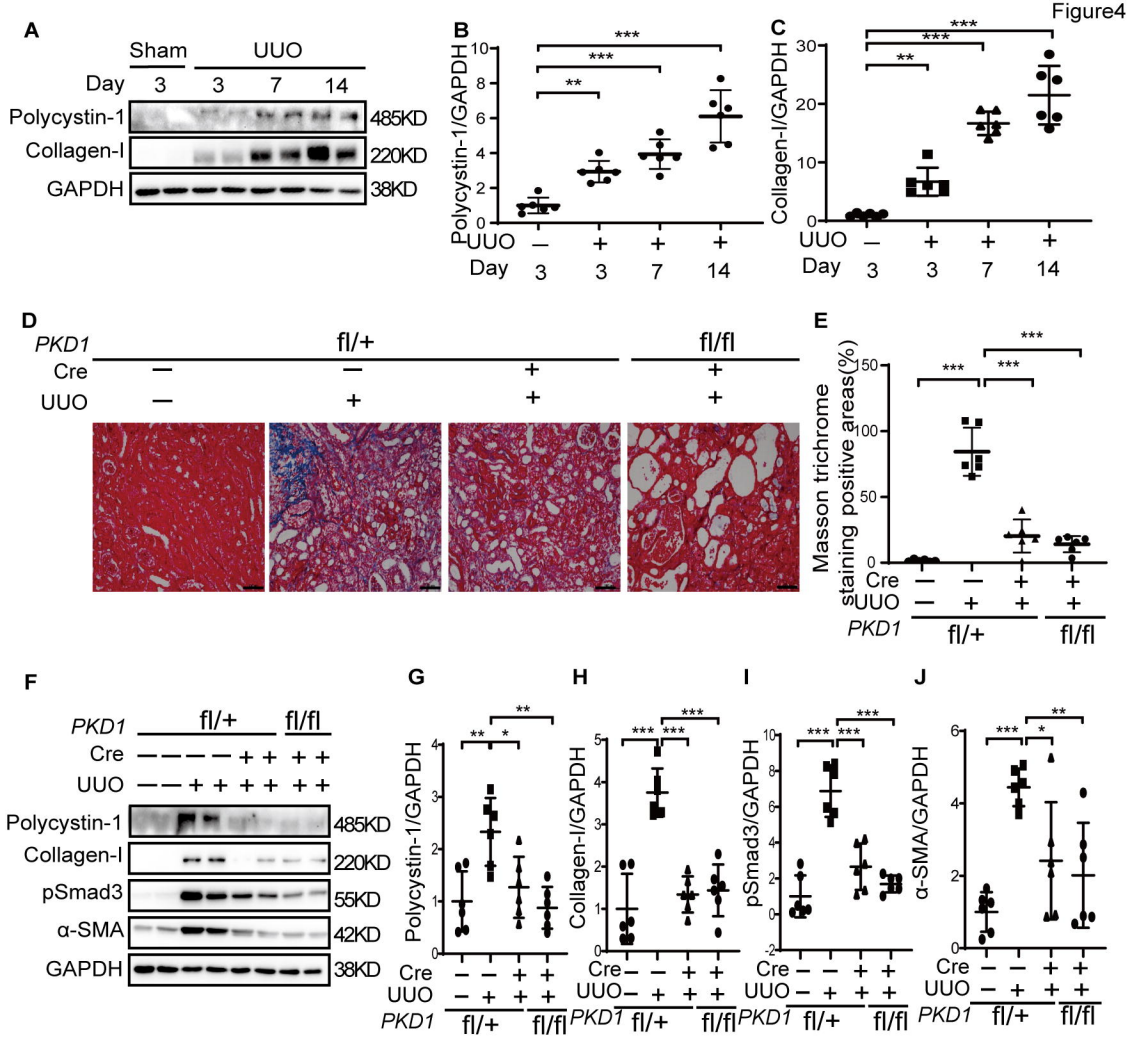
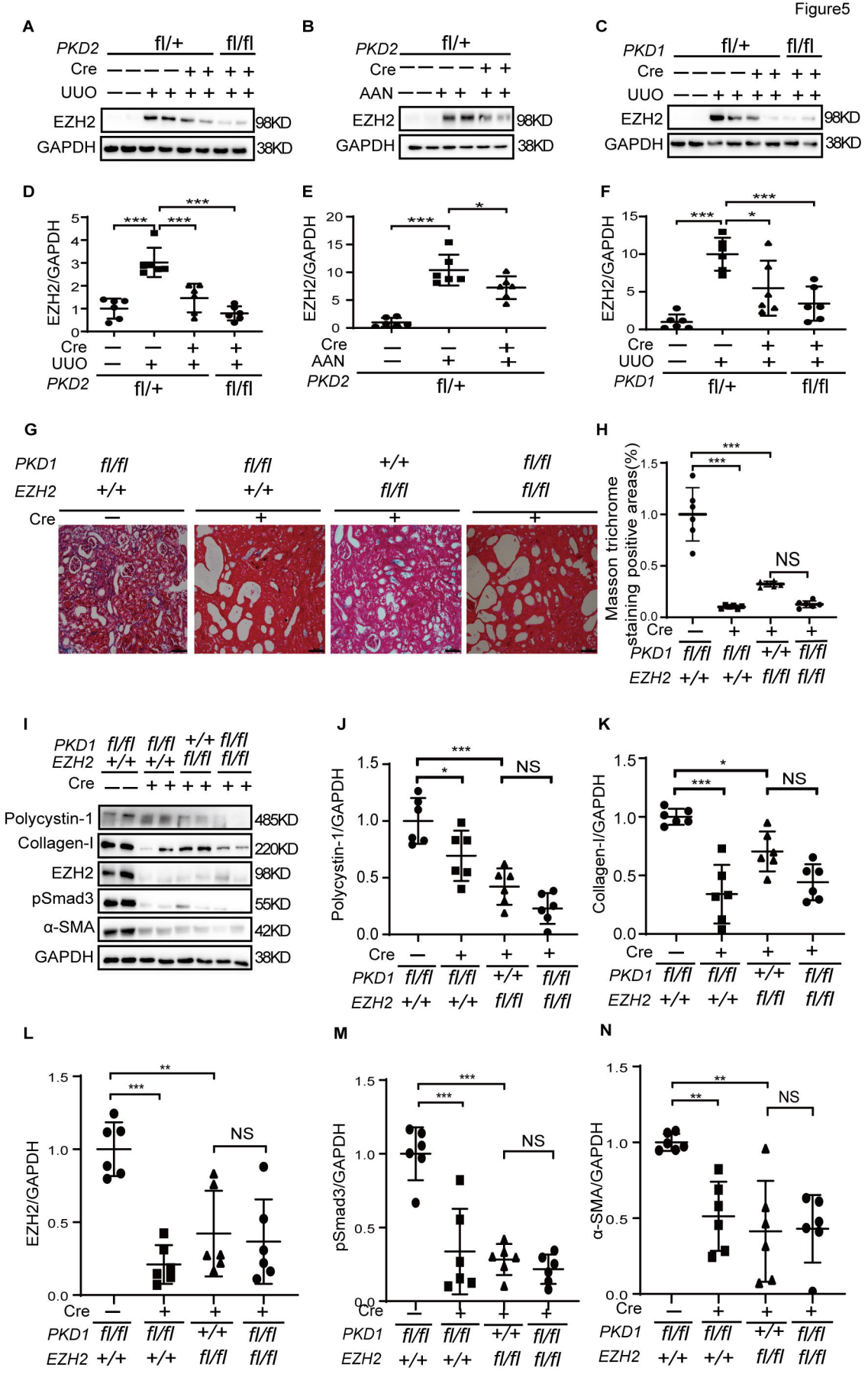


Figure4
A
Sham
UUO
Day 3 3 7 14
Polycystin-1
Collagen-I
GAPDH
485KD
220KD
38KD
B
Polycystin-1/GAPDH
UUO — + + +
Day 3 3 7 14
C
Collagen-I/GAPDH
UUO — + + +
Day 3 3 7 14
D
PKD1 fl/+ fl/fl
Cre — — + +
UUO — + + +
E
Masson trichrome staining positive areas(%)
Cre — — + +
UUO — + + +
PKD1 fl/+ fl/fl
F
PKD1 fl/+ fl/fl
Cre — — — — + + + +
UUO — — + + + + + +
Polycystin-1
Collagen-I
pSmad3
α-SMA
GAPDH
485KD
220KD
55KD
42KD
38KD
G
Polycystin-1/GAPDH
H
Collagen-I/GAPDH
I
pSmad3/GAPDH
J
α-SMA/GAPDH
Cre — — + +
UUO — + + +
PKD1 fl/+ fl/fl

Figure5


A
PKD2 fl/+ fl/fl
Cre
UUO
EZH2 98KD
GAPDH 38KD
B
PKD2 fl/+
Cre
AAN
EZH2 98KD
GAPDH 38KD
C
PKD1 fl/+ fl/fl
Cre
UUO
EZH2 98KD
GAPDH 38KD
D
EZH2/GAPDH
Cre
UUO
PKD2 fl/+ fl/fl
E
EZH2/GAPDH
Cre
AAN
PKD2 fl/+
F
EZH2/GAPDH
Cre
UUO
PKD1 fl/+ fl/fl
G
PKD1 fl/fl fl/fl +/+ fl/fl
EZH2 +/+ +/+ fl/fl fl/fl
Cre
H
Masson trichrome staining positive areas(%)
NS
Cre
PKD1 fl/fl fl/fl +/+ fl/fl
EZH2 +/+ +/+ fl/fl fl/fl
I
PKD1 fl/fl fl/fl +/+ fl/fl
EZH2 +/+ +/+ fl/fl fl/fl
Cre
Polycystin-1 485KD
Collagen-I 220KD
EZH2 98KD
pSmad3 55KD
α-SMA 42KD
GAPDH 38KD
J
Polycystin-1/GAPDH
NS
Cre
PKD1 fl/fl fl/fl +/+ fl/fl
EZH2 +/+ +/+ fl/fl fl/fl
K
Collagen-I/GAPDH
NS
Cre
PKD1 fl/fl fl/fl +/+ fl/fl
EZH2 +/+ +/+ fl/fl fl/fl
L
EZH2/GAPDH
NS
Cre
PKD1 fl/fl fl/fl +/+ fl/fl
EZH2 +/+ +/+ fl/fl fl/fl
M
pSmad3/GAPDH
NS
Cre
PKD1 fl/fl fl/fl +/+ fl/fl
EZH2 +/+ +/+ fl/fl fl/fl
N
α-SMA/GAPDH
NS
Cre
PKD1 fl/fl fl/fl +/+ fl/fl
EZH2 +/+ +/+ fl/fl fl/fl