Polycystin-2 is upregulated in fibrotic kidneys and inhibition of plycystin-2 by triptolide attenuates renal fibrosis

Ming Wu[1,2,3, *], Yanzhe Wang[1,2,3,*], Yin Jing[4,*], Junyan Lin[1,2,3], Dongping Chen[1,2,3], Di Huang[1,2,3], Chaoyang Ye[1,2,3]

[1]Department of Nephrology, Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine

[2]TCM Institute of Kidney Disease of Shanghai University of Traditional Chinese Medicine

[3]Key Laboratory of Liver and Kidney Diseases, Ministry of Education, Shanghai Key Laboratory of Traditional Chinese Clinical Medicine

[4]Department of Nephrology, The 960th Hospital of the PLA Joint Logistics Support Force

*These authors contributed equally to this paper.

Running head: PKD2 promotes renal fibrosis

Corresponding Author1: Prof. Chaoyang Ye

Corresponding Author 2: Dr. Ming Wu

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Abstract

Mutations in PKD2 gene, which encodes polycystin-2, cause autosomal polycystic kidney disease (ADPKD). Development of ADPKD is associated with progression of renal fibrosis. Whether renal fibrosis in ADPKD is a direct effect of polycystin-2 mutation or a consequence of cyst growth induced tubular obstruction is currently unknown. Polycystin-2 has been identified as a direct target of triptolide, and we used triptolide as a probe to study the role of polycystin-2 in renal fibrosis. To study the expression of polycystin-2, we established unilateral ureteral obstruction (UUO), unilateral ischemia-reperfusion injury (UIRI) and aristolochic acid nephropathy mouse models. Here we showed that polycystin-2 is up-regulated in these three mouse models and tightly correlated with the expression of collagen-I in a time dependent manner. Treatment with triptolide inhibited the expression of polycystin-2 and pro-fibrotic markers in UUO and UIRI models. Moreover, triptolide dose-dependently inhibited the expression of polycystin-2 and pro-fibrotic markers in rat renal fibroblasts or in TGF-β stimulated human renal epithelial (HK2) cells. Knockdown of PKD2 reduced the expression of pro-fibrotic markers in TGF-β stimulated or unstimulated HK2 cells. Finally, we showed that knockdown of PKD2 attenuated the inhibitory effect of triptolide on the expression of pro-fibrotic markers in TGF-β stimulated HK2 cells.

In conclusion, polycystin-2 is a pro-fibrotic protein suggesting that renal fibrosis in ADPKD kidneys is not a direct effect of PKD2 mutation.

Introduction

Autosomal polycystic kidney disease (ADPKD) is a leading cause of the end stage renal failure and it is caused by mutations in PKD1 or PKD2 gene, which encodes polycystin-1 or polycystin-2 respectively (1). Surprisingly, the expression of polycystin-1 and polycystin-2 were found to be up-regulated in human ADPKD kidneys, which is unexplainable (2). ADPKD is characterized by progressively enlargement of bilateral kidneys which is filled with numerous fluid-filled cysts and associated with massive deposition of interstitial extracellular matrix at the late stage of disease progression (1, 3). Hallmarks of renal fibrosis include overexpression of extracellular matrix fibrils (collagen-I, fibronectin, etc.), up-regulation of epithelial to mesenchymal transition (EMT) markers (a-SMA, vimentin, N-cadherin, etc.), and activation of TGFβ/Smad3 signaling pathway (3, 4). The mechanism of renal fibrosis in ADPKD is largely unexplored (1, 3). Several evidences indicate that defect in PKD genes directly induces the production of extracellular matrix proteins (3). However, renal fibrosis in ADPKD kidneys could also be the consequence of cyst growth induced tubular obstruction, which resembles the animal model of unilateral ureteral obstruction (UUO) (1). Indeed, heterozygous deletion of Pkd1 gene in mice exacerbated fibrosis in the kidney after renal ischemia reperfusion injury and numerous microcysts were observed in injured kidneys of Pkd1 mice (5).

Polycystin-2 has been identified as a direct target of triptolide, a natural compound extracted from a traditional Chinese herb Lei Gong Teng (6). Through polycystin-2-dependent calcium release, triptolide attenuated disease progression in a

mouse ADPKD model (6). We also showed the beneficial effect of triptolide in human ADPKD patients (7). Besides, triptolide ameliorated proteinuria, attenuated inflammation, and reduced renal fibrosis in several animal models of renal disease (8, 9). All these data imply that regulation of polycystin-2 activity by triptolide provides renal benefits. Here, we hypothesized that triptolide attenuated renal fibrosis through polycystin-2 in fibrotic kidneys.

In this study, we used triptolide as a probe to study the role of polycystin-2 in renal fibrosis.

Results

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

The time course of polycystin-2 expression was assessed in three different mouse models of renal fibrosis. Compared with sham kidneys, the expression of polycystin-2 was steadily increased in UUO kidneys or unilateral ischemia-reperfusion injury (UIRI) kidneys from day 3, day 7 to day 14, which was correlated with the expression of collagen-I by time (Figure 1A and 1B). Moreover, polycystin-2 was increased by time in aristolochic acid nephropathy (AAN) kidneys from day 3, day 7 to day 21 in a same pattern as the expression of collagen-I (Figure 1C).

Triptolide inhibits the expression of polycystin-2 and attenuates renal fibrosis in two mouse models

Massive interstitial collagen deposition was observed in UUO kidneys at 10 days after the operation as shown by Masson staining, and treatment with triptolide attenuated collagen deposition (Figure 2A). Increased pro-fibrotic markers like collagen-I, fibronectin, and a-SMA were observed in UUO kidneys by Western blotting and triptolide reduced the expression of these pro-fibrotic markers (Figure 2B). Interestingly, triptolide even inhibited the expression of polycystin-2, collagen-I and a-SMA in sham kidneys (Figure 2B).

In UIRI kidneys, triptolide attenuated the deposition of collagen and reduced the expression of pro-fibrotic markers (collagen-I, fibronectin, and a-SMA) (Figure 2C and 2D). In parallel, triptolide reduced the increased expression of polycystin-2 in

UIRI kidneys (Figure 2D).

Triptolide inhibits renal fibrosis through down-regulation of polycystin-2

The anti-fibrotic effect of triptolide was further confirmed *in vitro* using TGF-β stimulated human renal epithelial (HK2) cells or normal rat kidney fibroblast (NRK-49F) cells. Triptolide dose-dependently inhibited the expression of pro-fibrotic markers (fibronectin, N-cadherin, vimentin, and phosphorylated Smad3) in TGF-β stimulated HK2 cells from 10 nM to 1000 nM (Figure 3A). Similarly, the expression of pro-fibrotic markers (fibronectin, N-cadherin, and vimentin) were dose-dependently inhibited by triptolide in NRK-49F cells from 10 nM to 1000 nM (Figure 3B). The expression of polycystin-2 was also dose-dependently inhibited by triptolide in TGF-β stimulated HK2 cells or NRK-49F cells from 10 nM to 1000 nM (Figure 3A and 3B).

As shown by Figure 3C and 3D, PKD2 siRNA inhibited the expression of polycystin-2 in normal or TGF-β stimulated HK2 cells, which was correlated with down-regulation of pro-fibrotic markers (fibronectin, N-cadherin, vimentin, and phosphorylated Smad3). We further showed that triptolide inhibited the expression of polycystin-2 and pro-fibrotic markers (fibronectin, N-cadherin, vimentin, and phosphorylated Smad3) in PKD2 knockdown HK2 cells in a less extent than that in non-sense control (NC) siRNA transfected HK2 cells (Figure 3E).

Discussion

We first determined whether triptolide inhibits renal fibrosis through polycystin-2. Firstly, triptolide inhibited the expression of polycystin-2 in UUO and UIRI model of renal fibrosis, which was correlated with reduced interstitial renal fibrosis in injured kidneys. Surprisingly, we even observed reduced expression of polycysin-2 and pro-fibrotic markers by triptolide in sham kidneys. Secondly, triptolide dose-dependently reduced the expression of polycystin-2 and pro-fibrotic markers in two *in vitro* models of renal fibrosis. Thirdly, knockdown of PKD2 gene attenuated the inhibitory effect of triptolide on the expression of pro-fibrotic markers in TGF-β stimulated HK2 cells. Thus, we conclude that triptolide inhibits renal fibrosis through down-regulation of polycystin-2.

We next asked whether polycystin-2 is a pro-fibrotic protein in renal disease. A previous report showed that increased fibrosis was observed in Pkd2 heterozygous mouse kidneys after UIRI as compared to that in WT kidneys (10). However, the experiment was performed in Pkd2 mice of 4-6 months where the baseline tubular proliferation in heterozygous kidneys was significantly higher than that in WT kidneys, and normal Pkd2 heterozygous mice even developed cystic phenotype in kidneys at 9-12 months of age which were associated with increased interstitial fibrosis (10). Similarly, collagen overexpression was observed in *pkd1* or *pkd2* deficient zebrafish where pronephric cysts were also occurred (11). We found that polycystin-2 was up-regulated in three different mouse model of renal fibrosis in a time-dependent manner, which was tightly correlated with the expression of

collagen-I. Secondly, we have proved in this study that inhibition of polycystin-2 by triptolide attenuates renal fibrosis, and no renal cyst was observed in our experiments. Finally, knockdown of PKD2 reduced the expression of pro-fibrotic markers in TGF-β stimulated HK2 cells and even in unstimulated HK2 cells. Thus, we concluded that polycystin-2 is a pro-fibrotic protein in renal disease, which suggests that renal fibrosis in ADPKD kidneys is not the direct effect of PKD2 mutation.

Polycystin-2 is up-regulated in kidneys upon ischemic or toxic acute injury, which was thought to be a protective mechanism for injured kidneys (12). Interestingly, overexpression of renal polycystin-2 was also observed in non-orthologous rodent model of PKD (12). Surprisingly, up-regulation of polycystin-2 and even its partner protein polycystin-1 were observed in human ADPKD kidney samples by different labs over two decades ago (2). However, there is still no explanation for these observations. Our study suggests that the up-regulation of polycystin-2 in human ADPKD kidneys is a fibrotic response of cyst growth induced tubular obstruction.

In this study, we found that polycystin-2 is increased in fibrotic kidneys and promotes renal fibrosis. Our study not only revealed a novel biological function of polycystin-2 but also shed lights on the mechanism of renal fibrosis in ADPKD.

Materials and Methods

Animal studies

Wide type male C57BL/6 mice were used in this study. Animal experiments were approved by the ethic committee of Shanghai University of Traditional Chinese Medicine (PZSHUTCM18111601).

UUO operation was performed through twice ligation of the left ureter. Animals were sacrificed at day 3, 7 or 14 in a time-course experiment (n=5 for each time point). For interventional study, Mice were randomly divided into four groups (n= 6-7 mice per group). Sham or UUO mice were treated with vehicle or 0.25 mg/kg triptolide (Topscience, T2179, Shanghai, China) daily by intraperitoneal (i.p.) injection for 10 days starting from day 0. Mice were sacrificed at day 10.

For the UIRI model, left renal pedicles were clamped for 35 minutes. Animals were sacrificed at day 3, 7 or 14 in a time-course experiment (n=3 for each time point). For interventional study, mice were randomly divided into three groups (n= 6-7 mice per group) and were treated with vehicle or 0.25 mg/kg triptolide daily by i.p. injection starting from day 1 for 10 days. Mice were sacrificed at day 11.

To induce aristolochic acid nephropathy in mice, aristolochic acid I (AAI, A9451, Sigma; 5 mg/kg body weight) or normal saline (NS) was peritoneally injected once. Animals were sacrificed at day 3, 7 or 21 in a time-course experiment (n=5 for each time point).

Cell culture

To study the anti-fibrotic effect of triptolide, normal rat kidney 49F (NRK-49F) were exposed to different concentrations of triptolide (10 nM, 100 nM, and 1000 nM)

for 24h.

HK2 renal proximal tubular epithelial 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. 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

Mouse kidneys were fixed and embedded in paraffin. Four-μm-thick kidney section 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.

Western blotting analysis

Cell or kidney protein was extracted by using lysis buffer bought from Beyotime Biotech (Nantong, China), which was further subjected to SDS-PAGE gel electrophoresis and was electro-transferred to a polyvinylidene difluoride membrane. Protein targets were detected by antibodies against fibronectin (1:1000, ab23750, Abcam), pSmad3 (1:1000, ET1609-41, HUABIO), Collagen I (1:500, sc-293182, Santa Cruz),α-SMA (1:1000, ET1607-53, HUABIO), N-cadherin (1:1000, sc-59887,

Santa Cruz), vimentin (1:1000, R1308-6, HUABIO), GAPDH (1:5000, 60004-1-lg, Proteintech), orα-tubulin (1:1000, AF0001, Byotime).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

CY funded the project. MW, YJ, CY conceived and coordinated the study. MW wrote the paper. MW and YW, conducted the *in vitro* experiments. MW, YW, YJ, JL, DC 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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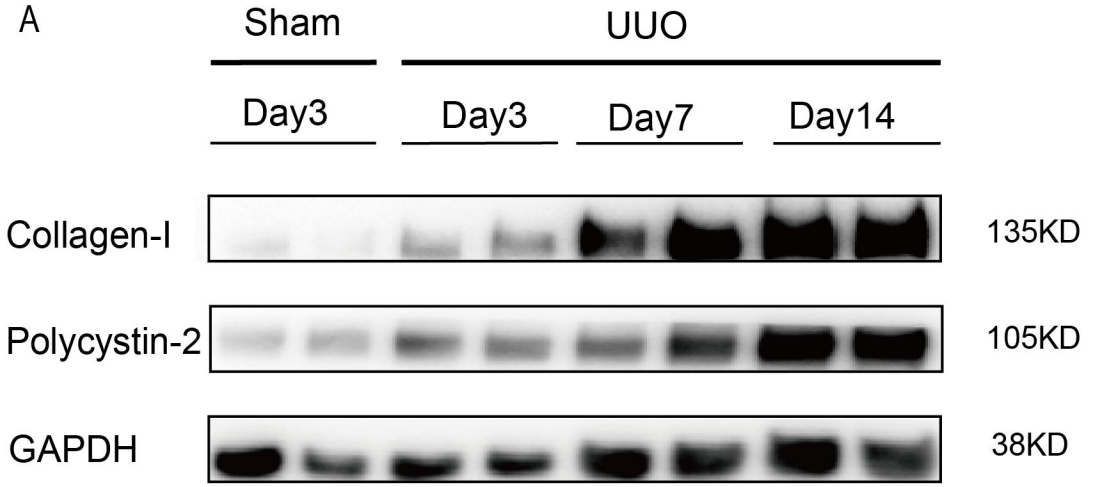
Figure legends

Fig. 1. Polycystin-2 is up-regulated in fibrotic kidneys.
The time course of polycystin-2 and collagen-I expression in (A) unilateral ureteral obstruction (UUO) kidneys, (B) unilateral ischemia-reperfusion injury (UIRI) kidneys and (B) aristolochic acid nephropathy (AAN) kidneys.

Fig. 2. Triptolide inhibits the expression of polycystin-2 and attenuates renal fibrosis in UUO and UIRI mouse kidneys.
Renal fibrosis was assessed by (A) Masson's trichrome staining and (B) the expression of collagen-I, fibronectin, a-SMA and polycystin-2 in UUO kidneys. (C) Masson's trichrome staining and (D) the expression of collagen-I, fibronectin, a-SMA and polycystin-2 in UIRI kidneys.

Fig. 3. Triptolide inhibit the expression of pro-fibrotic markers through polycystin-2 in fibrotic kidney cells.
(A) HK2 human renal epithelial cells were stimulated with TGF-β and treated with different concentration of triptolide for 48h. (B) normal rat renal fibroblasts were treated with different concentration of triptolide for 24h. (C) HK2 human renal epithelial cells were transfected with nonsense control (NC) or PKD2 siRNA (siPKD2) for 24h. (D) HK2 cells were transfected with NC or PKD2 siRNA, and then were stimulated with TGF-β for 48h. (E) HK2 cells were transfected with NC or PKD2 siRNA, and were stimulated with TGF-β and treated with triptolide for 48h. The expression of profibrotic markers were measured by Western blotting.

A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38KD

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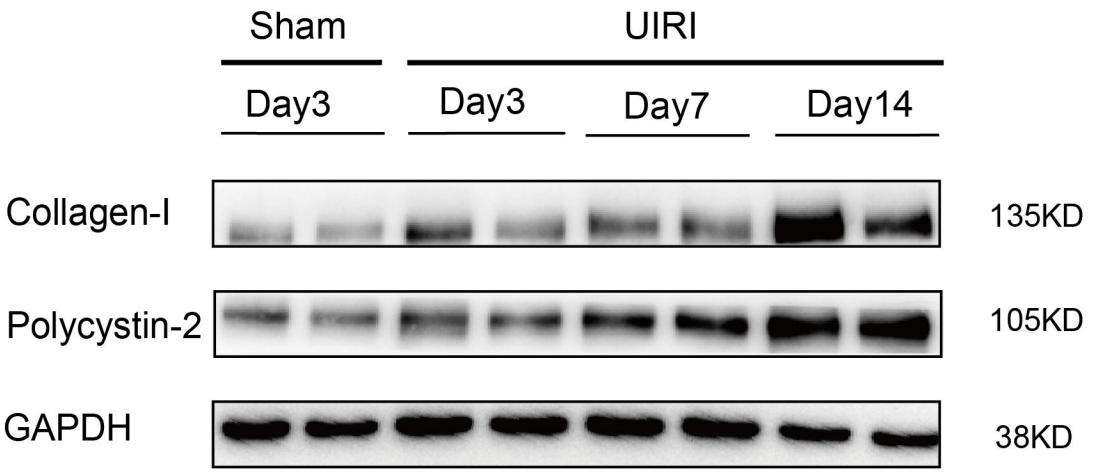
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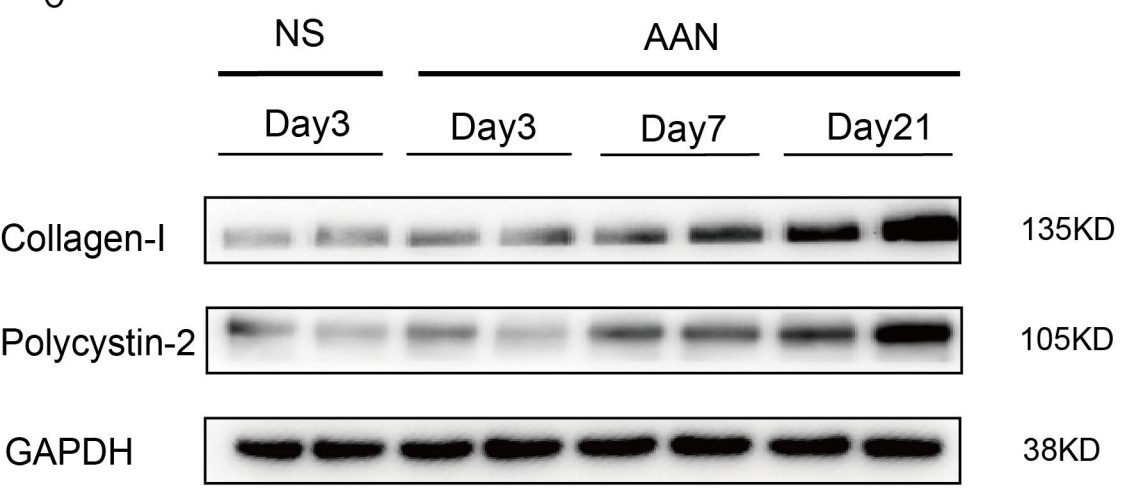
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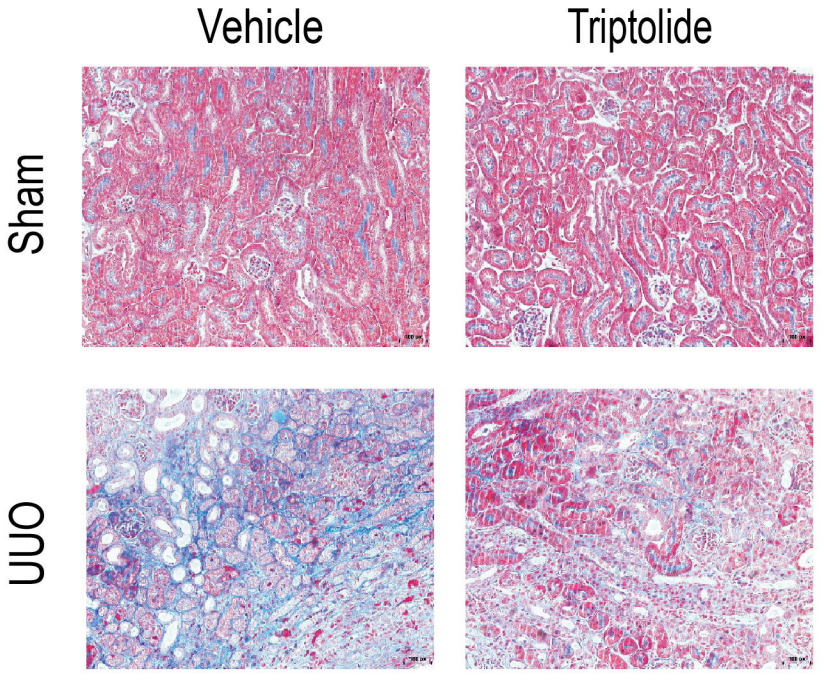
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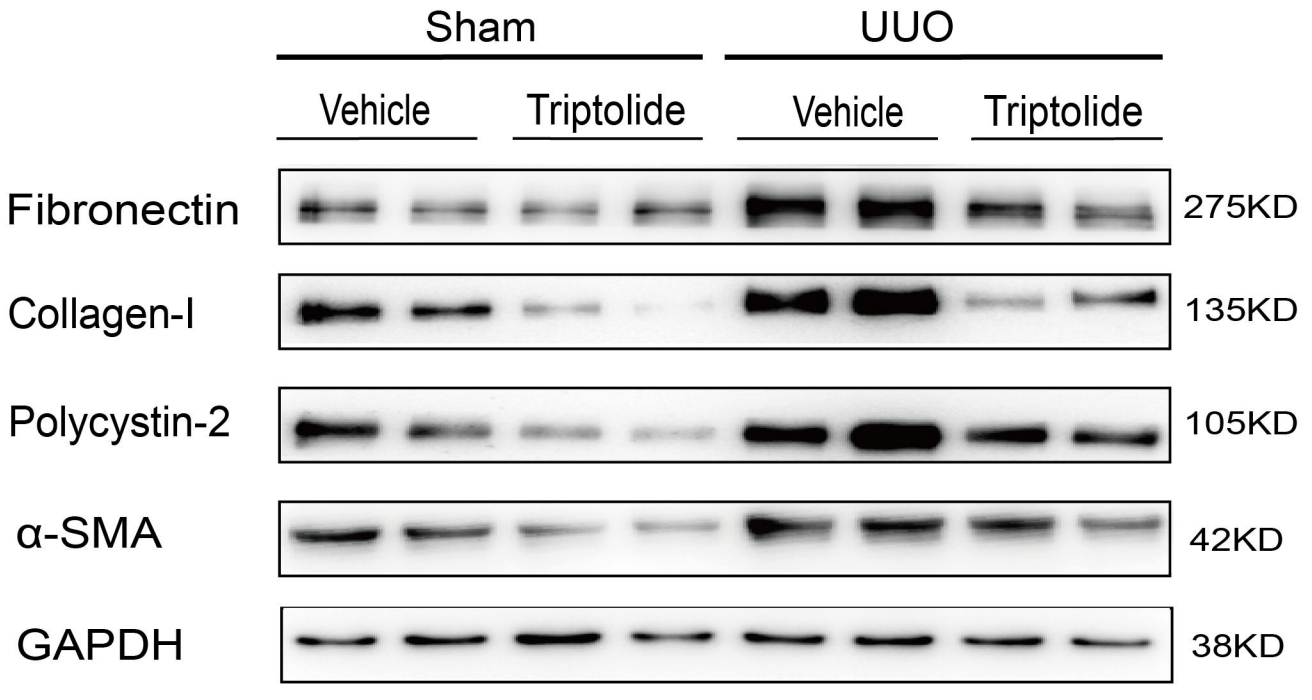
Triptolide
Sham
UUO

B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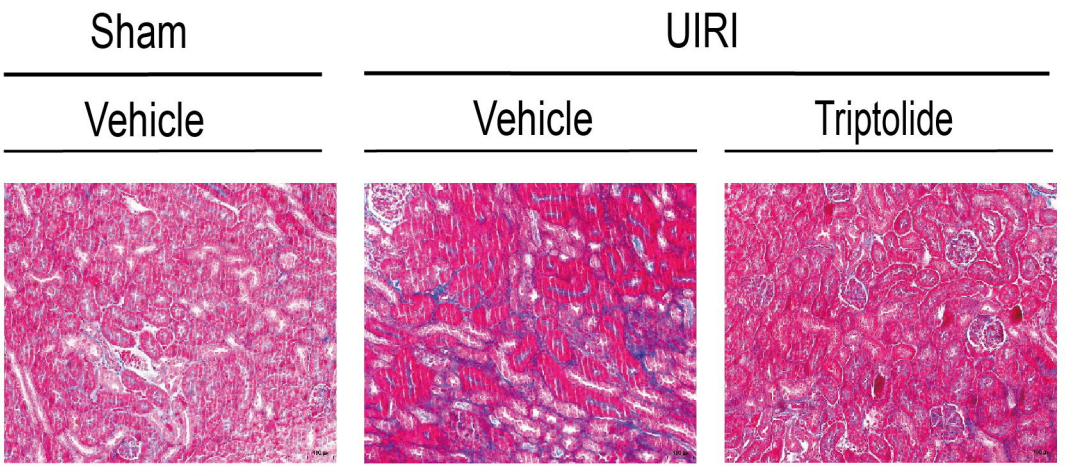
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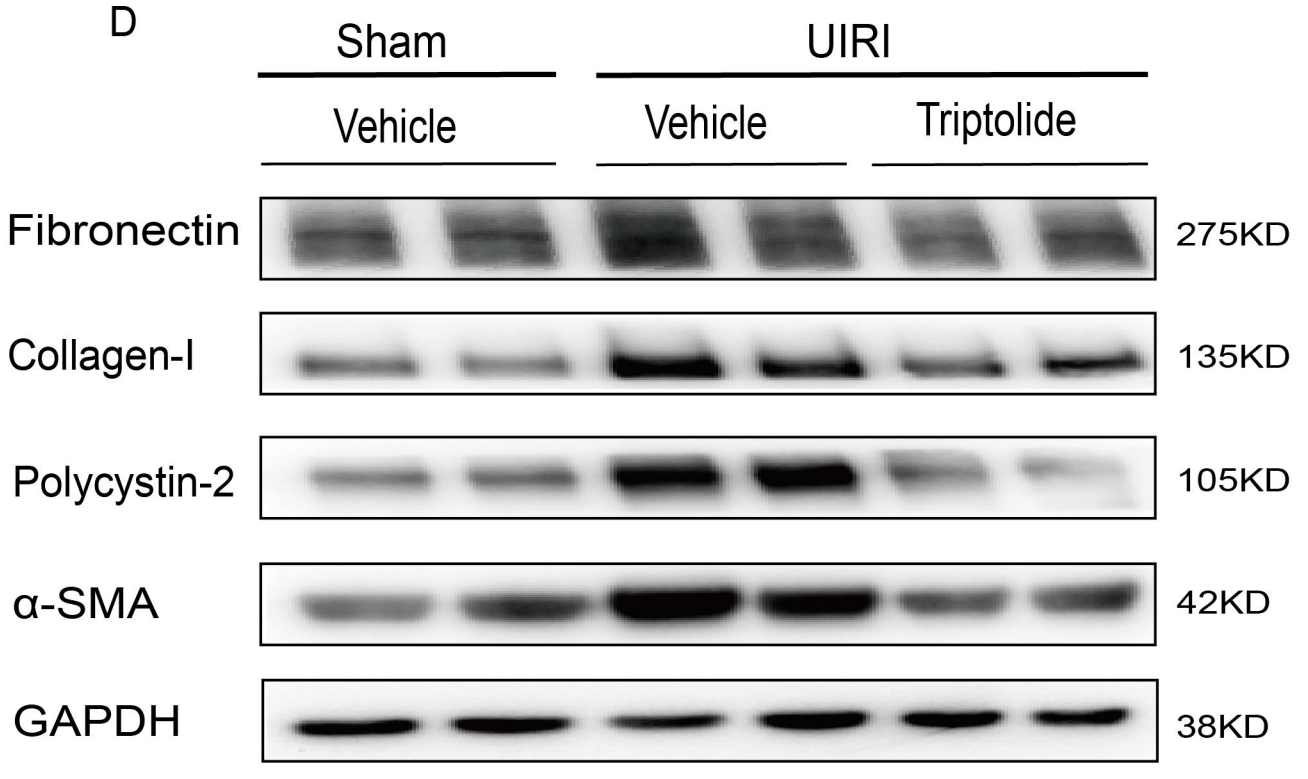
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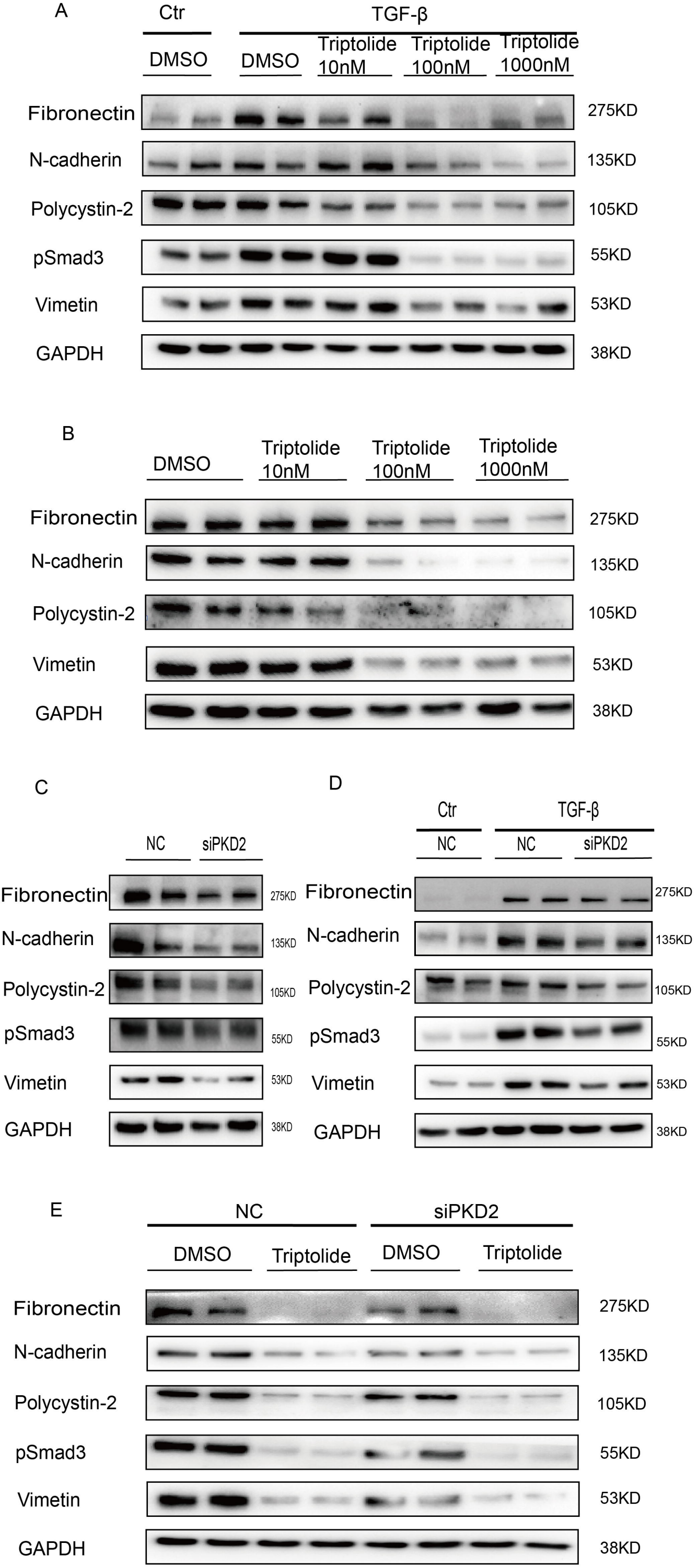
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