Kidney fibrosis is the final common pathway of progressive kidney diseases, the underlying mechanisms of which is not fully understood. The purpose of the current study is to investigate a role of Piezo1, a mechanosensitive nonselective cation channel, in kidney fibrosis. In human fibrotic kidneys, Piezo1 protein expression was markedly upregulated. The abundance of Piezo1 protein in kidneys of mice with UUO or with folic-acid treatment was significantly increased. Inhibition of Piezo1 with GsMTx4 markedly ameliorated UUO or folic acid-induced kidney fibrosis. Mechanical stretch, compression or stiffness induced Piezo1 activation and pro-fibrotic responses in human HK2 cells and primary cultured mouse proximal tubular cells (mPTCs), which were greatly prevented by inhibition or silence of Piezo1. TGFβ-1 induced increased Piezo1 expression and pro-fibrotic phenotypic alterations in HK2 cells and mPTCs, which was again markedly prevented by inhibition of Piezo1. Activation of Piezo1 by Yoda1, a Piezo1 agonist, caused calcium influx and profibrotic responses in HK2 cells and induced calpain2 activation, followed by talin1 cleavage and upregulation of integrinβ1. Also, Yoda1 promoted the link between ECM and integrinβ1. In conclusion, Piezo1 is involved in the progression of kidney fibrosis and pro-fibrotic alterations in renal proximal tubular cells, likely through activating calcium-calpain2-integrinβ1 pathway.
Mechanosensitive Piezo1 channels mediate renal fibrosis

Xiaoduo Zhao¹,², Yonglun Kong¹,², Baien Liang¹,², Jinhai Xu³, Yu Lin⁴, Nan Zhou⁵, Jing Li⁶, Bin Jiang³, Jianding Cheng⁵*, Chunling Li²,³*, Weidong Wang¹,²,⁷*

¹ Department of Pathophysiology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, 510080, China; ² Institute of Hypertension, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, 510080, China; ³ Department of Physiology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, 510080, China; ⁴ Department of Pathology, Zhuhai Hospital, Southern Medical University, Guangzhou, 510282, China; ⁵ Department of Forensic Medicine, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, 510080, China; ⁶ The First Affiliated Hospital, Guangzhou University of Chinese Medicine, Guangzhou, 510405, China; ⁷ Department of Nephrology, The Seventh Affiliated Hospital, Sun Yat-sen University, Shenzhen, 518107, China

Running Title: Piezo1 and renal fibrosis

Please address inquiries to

Weidong Wang, MD, Ph.D
Department of Pathophysiology
Institute of Hypertension
Zhongshan School of Medicine
Sun Yat-sen University
74# Zhongshan Er Road
Guangzhou 510080
China
Phone: 86-20-87334840
Fax: 86-20-87334840
E-mail: wangwd6@mail.sysu.edu.cn

Please address inquiries also to

Chunling Li, MD, Ph.D E-mail: lichl3@mail.sysu.edu.cn
Jianding Cheng, MD, Ph.D E-mail: chengjd@mail.sysu.edu.cn

*co-correspondence
ABSTRACT

Kidney fibrosis is the final common pathway of progressive kidney diseases, the underlying mechanisms of which is not fully understood. The purpose of the current study is to investigate a role of Piezo1, a mechanosensitive nonselective cation channel, in kidney fibrosis. In human fibrotic kidneys, Piezo1 protein expression was markedly upregulated. The abundance of Piezo1 protein in kidneys of mice with UUO or with folic-acid treatment was significantly increased. Inhibition of Piezo1 with GsMTx4 markedly ameliorated UUO or folic acid-induced kidney fibrosis. Mechanical stretch, compression or stiffness induced Piezo1 activation and pro-fibrotic responses in human HK2 cells and primary cultured mouse proximal tubular cells (mPTCs), which were greatly prevented by inhibition or silence of Piezo1. TGFβ-1 induced increased Piezo1 expression and pro-fibrotic phenotypic alterations in HK2 cells and mPTCs, which was again markedly prevented by inhibition of Piezo1. Activation of Piezo1 by Yoda1, a Piezo1 agonist, caused calcium influx and profibrotic responses in HK2 cells and induced calpain2 activation, followed by talin1 cleavage and upregulation of integrinβ1. Also, Yoda1 promoted the link between ECM and integrinβ1. In conclusion, Piezo1 is involved in the progression of kidney fibrosis and pro-fibrotic alterations in renal proximal tubular cells, likely through activating calcium-calpain2-integrinβ1 pathway.

KEY WORDS: Piezo1, Fibrosis, Kidney
INTRODUCTION

Kidney fibrosis is the final common outcome of chronic kidney diseases, leading to the end stage of kidney diseases (1). The pathophysiological events during renal tubulointerstitial fibrogenesis are complex and involve several independent and overlapping cellular and molecular signaling pathways. Renal fibrogenesis is a dynamic and converging process, in which several types of cells, mainly, tubular epithelial cells and interstitial fibroblasts, play important roles in tubulointerstitial fibrosis after an insult to the renal parenchyma (2).

Kidney fibrosis is characterized by increased synthesis and inadequate degradation of extracellular matrix (ECM) in tubulointerstitial areas, which makes fibrotic regions are much stiffer than normal kidney tissue (1). Tubular epithelia in the kidney are exposed to different mechanical forces, from extracellular matrix on the basal side and from fluid shear stress in the luminal side. Tubular epithelium is able to detect tiny changes in matrix stiffness and in response, to adjust expression of genes and proteins which are important in cell proliferation, differentiation and apoptosis (3, 4). After severe or repeated injuries, tubular epithelial cells may exhibit a series of characteristic alterations and acquire mesenchymal traits, leading to pro-inflammatory and pro-fibrotic phenotypic changes (5, 6). An epithelial-mesenchymal transition (EMT) phenomenon was found in vitro after stimulation by transforming growth factor-β1 (TGF-β1), a well-known pro-fibrotic factor, but multiple lineage tracing studies don’t support the contribution of in vivo EMT to kidney fibrosis (7, 8). It is possible that EMT may represent tubular injuries, epithelial plasticity, or pro-fibrotic phenotypic changes.
TGF-β1 may directly induce tubular injuries and the production of ECM in the kidney through TGF-β1/Smad signaling, leading to renal fibrosis (9).

It has been known that epithelium may sense the microenvironments and transduce outside mechanical signals to intracellular chemical and electronic signals through mechanoreceptors, which include ion channels, integrins, G-protein coupled receptors, glycocalyx, etc (10-12). Piezo1 is a newly found mechanosensitive nonselective cation channel which extensively distributes in multiple cell types and tissues (13). Piezo1 is gated and tuned by cellular membrane bilayer tension directly (14). Physical forces such as shear stress, compression, stretch, and osmotic stress, induce a change in membrane tension and open Piezo1 channel to allow permeation of cations (K⁺, Na⁺, Ca²⁺ and Mg²⁺) with a slight preference for calcium (15). Piezo1 plays an important role in numerous physiological processes, such as determinants of vascular structure (16), urine osmoregulation (17) and blood pressure homeostasis (18). In the kidney, Piezo1 protein expression was found in the renal corpuscle, proximal and distal convoluted tubule, and the cortical and medullary collecting duct (17, 19).

Sensing and responding to mechanical signals are critical for the proper functioning of the nephrons and collecting ducts. Renal epithelial cells respond to changes in tubular fluid flow, intraluminal pressure and wall tension (20). In fibrotic lesion, kidney is gradually becoming stiffening, which is usually driven by the replacement of compliant cells with rigid matrix (such as collagen remodeling and the consequent activation in ECM signaling) and is further increased by crosslinking of these matrix fibrils (21). Normal stiffness of kidney cortex is about 4KPa, stiffness of cortex may increase to 35
KPa in human kidney with fibrotic pathological changes (22, 23). A previous study showed that Piezo1 sensed microenvironmental stiffness and transduces the mechanical cues into electronic and chemical signals in cells (24). Piezo1 was also shown to be involved in myofibroblast-fibroblast cross talk in fibrosis expansion (25). Mechanical stretch-induced EMT in alveolar epithelia (26) or in cholangiocarcinoma cells has been demonstrated via Piezo1 activation (27). It is recognized that mechanical stimulus is closely associated with kidney fibrosis, whether Piezo1 as a candidate mechanosensor is involved in progression of renal fibrosis is still unknown.

Integrins are also mechanoreceptors which are transmembrane protein complexes formed by α and β subunits. The extracellular domain of integrin interacts with ECM proteins and cytoplasmic domain interacts with cytoskeleton (28). When integrin binds to ECM proteins, outside environmental mechanical signals could be transmitted into cells, activating integrin-dependent intracellular kinase signaling to regulate cellular physiological processes. On the other hand, intracellular signals, especially calcium signals, activate intracellular domain of integrin, followed by clustering of integrins and the affinity of the integrins to the ECM ligand. Emerging evidence has shown an association between activation of Piezo1 and integrin-related signaling (29, 30).

In the current study, we hypothesized that activation of Piezo1 in renal tubular epithelia may contribute to the development of kidney interstitial fibrosis. We demonstrated that inhibition of Piezo1 at least partially prevented unilateral ureter obstruction (UUO) or folic acid-induced renal fibrosis. In vitro study showed that stimulation of Piezo1 induced pro-fibrotic phenotypic changes in tubular cells, likely through activating
calcium-calpain-integrin signaling. Targeting the Piezo1 pathway may offer a novel therapeutic strategy for ameliorating renal fibrosis.
RESULTS

Renal fibrosis was associated with increased Piezo1 protein expression

In order to examine a potential association between expression abundance of Piezo1 protein and fibrosis, Piezo1 labeling was performed on kidney specimens obtained from autopsy of decedents. The human kidney specimens from decedents died from traffic accidents with minimal pathological changes were used as controls. Water channel AQP1 is specially located in the proximal tubular cells and AQP2 is located in the collecting duct principal cells in the kidneys. Immunofluorescence showed that Piezo1 was generally expressed in human kidney tubules, including proximal tubular cells (co-localized with AQP1) and collecting duct principal cells (co-localized with AQP2) (Figure 1A). Masson’s trichrome staining showed more fibrotic lesions in specimens obtained from decedents with renal cysts, polycystic kidney, nephrotic syndrome or diabetic nephropathy than those from controls. Interestingly, in fibrotic kidney specimens more tubular labeling of Piezo1 particularly in proximal tubules was found than those in controls by immunohistochemistry (Figure 1B).

UUO not only causes elevation of tubular intraluminal pressure, persistent and non-relieved obstruction also leads to kidney interstitial fibrosis. The protein expression of Piezo1 in the kidney of mice started increasing since 30 minutes and significantly increased at the 3rd hour after UUO (Figure 2A and B), likely due to increased intraluminal pressure. At the 3rd and 7th day after UUO, the protein abundance of Piezo1 was markedly increased to 4 and 7.3-folds in the obstructed kidneys, compared
to sham mice, respectively (Figure 2C and D). Consistent with this, mRNA levels of Piezo1 were also increased approximately 4.5 and 10-folds compared to sham levels (Figure 2E), respectively. In contrast, mRNA levels of transient receptor potential canonical 1 (TRPC1) and transient receptor potential vanilloid 4 (TRPV4) capable of responding to membrane stretching were decreased or unchanged in the obstructed kidneys (Figure 2E). While TRPC6 mRNA expression levels were increased 1.7 and 4-folds at the 3rd and 7th day after UUO, respectively (Figure 2E). Immunofluorescence showed a few labeling of Piezo1 in tubular epithelia in sham mouse kidneys (Figure 2F-a and F-k), consistent with a previous study (31), however, the labeling intensity of Piezo1 was markedly stronger in tubular segments of the kidney in mice with 7UUO (Figure 2F-f and F-p) than that in sham, especially in the proximal tubular epithelial cells where AQP1 was located (Figure 2F-e and j). These data indicated that renal fibrosis was associated with upregulated expression of Piezo1.

**Inhibition of Piezo1 prevented UUO-induced kidney fibrosis**

Next, we investigated whether inhibition of Piezo1 improved UUO-induced renal fibrosis. Semiquantitative immunoblotting demonstrated that the protein abundance of fibrotic markers fibronectin and collagen I was dramatically increased in the kidney of mice after 3 days and 7 days UUO, which was markedly reversed by Piezo1 non-specific inhibitor GsMTx4 treatment (Figure 3A and B). Piezo1 protein expression was markedly increased in the kidneys of 3UUO and 7UUO mice and GsMTx4 could partially decrease Piezo1 expression in 3UUO, but not in 7UUO mice (Figure S1A-D). In canonical TGF-β1 signaling, Smad2 and Smad3 are two key downstream mediators
that are highly activated in the fibrotic kidney (32). In line with this, inhibition of Piezo1 by GsMTx4 also markedly decreased the protein expression of phosphorylated Smad2 at Ser465/467, phosphorylated Smad3 at Ser423/425 and total Smad2/3 in the kidney of mice with 3UUO and 7UUO (Figure 3A and C). Amelioration of fibrosis by GsMTx4 was confirmed by Masson's trichrome staining and immunohistochemistry for collagen I. Compared with sham mice, 7UUO caused more fibrotic lesions in tubulointerstitial areas and more extensive and stronger staining of collagen I, which was greatly prevented by GsMTx4 (Figure 3D). Consistent with this, increased mRNA expression levels of TGF-β1, α-SMA and fibronectin in the kidney of mice with 3UUO and 7UUO was partially suppressed by inhibition of Piezo1 (Figure 3E). These results suggested that inhibition of Piezo1 by GsMTx4 at least partially prevented UUO-induced tubulointerstitial fibrosis in the obstructed kidneys.

**Inhibition of Piezo1 prevented folic acid-induced kidney fibrosis**

To further investigate whether inhibition of Peizo1 also attenuate fibrosis in a non-obstructive kidney disease, a folic acid-induced nephropathy (FAN) mouse model was set up to exclude the possibility that upregulation of Piezo1 expression is only due to increased intraluminal pressure after UUO. Compared with controls, the protein abundance of Piezo1 in the kidney of mice with FAN was greatly increased, which clearly prevented by GsMTx4 (Figure 4A and B). Western blotting demonstrated that the protein abundance of fibronectin and collagen I was increased in the kidney of mice with FAN, which was again reversed by GsMTx4 (Figure 4A and B). Consistence with this, Masson's trichrome staining showed more fibrotic lesions in renal tubulointerstitial
areas of mice with FAN, which was also at least partially prevented by GsMTx4 (Figure 4C). mRNA levels of TGF-β1, α-SMA, and fibronectin were significantly increased in the kidney of mice with FAN, GsMTx4 reduced their expression levels (Figure 4D). Taken together, these findings indicated that inhibition of Piezo1 at least partially attenuated tubulointerstitial fibrosis in the kidney.

**Activation of Piezo1 by Yoda1 was associated with increased expression of pro-fibrotic factors**

As GsMTx4 is not a specific inhibitor of Piezo1, the role of Piezo1 in renal fibrosis was further verified by using a Piezo1 specific agonist Yoda1. Compared with controls, the protein abundance of fibronectin and TGF-β1 in the kidney was significantly increased in mice treated with Yoda1, although Piezo1 abundance was unchanged (Figure S2A and B). These data supported a role of Piezo1 activation in renal fibrosis.

**Piezo1 mediated mechanical stretch or compression-induced pro-fibrotic responses in HK2 cells**

UUO elevates membrane tension of tubular epithelium, due to the accumulation of tubular fluid and the deposition of ECM in interstitial spaces, if longer time. Increased tubular stretch is an inducer of pro-fibrotic phenotypic changes in tubular epithelial cells (6). As a mechanosensitive channel, whether Piezo1 mediated stretch-induced pro-fibrotic effects was investigated in human proximal tubule HK2 cells. First, by quantitative reverse transcription-PCR, we found mRNA expression level of Piezo1 in HK2 cells was high (Figure S3A). A cyclic stretch sustained for 24 hours at 20%
elongation increased Piezo1 mRNA levels about 1.5-folds in HK2 cells, which was suppressed by GsMTx4 at 5μM (Figure 5A). Consistent with this, a cyclic stretch induced a 3-fold increase in abundance of Piezo1 protein expression, which was markedly suppressed by GsMTx4 at 1 and 5μM (Figure 5B and C). Inhibition of Piezo1 by siRNA (Figure 4D) or GsMTx4 (Figure S3B) completely suppressed the elevated intracellular calcium levels ([Ca^{2+}]) invoked by mechanical stretch, indicating that Piezo1 channels were able to be activated by mechanical stretch in HK2 cells. Interestingly, mechanical stretch caused increased protein abundance of fibronectin and α-SMA, accompanied with decreased protein expression of an epithelial marker E-cadherin in HK2 cells, both of which were partially prevented by Piezo1 inhibitor GsMTx4 (Figure 5E and F), while GsMTx4 treatment didn’t cause any changes in expression of these proteins in controls (Figure 5E and F). To further examine the role of Piezo1 in stretch-induced pro-fibrotic alterations in HK2 cells, Piezo1 small interfering RNAs (siRNA) was used (Figure S3C and D). Piezo1 knockdown by siRNA completely prevented cyclic stretch-induced increased protein abundance of fibronectin and α-SMA and reversed protein expression of E-cadherin to control levels (Figure 5G and H). In UUO or FAN model, tubular epithelia were also subjected to compression from increased intraluminal pressure or accumulated ECM. HK2 cells were subjected to mechanical compression (15mmHg, at a frequency of 1HZ) using Flexcell-5000C Compression Plus System. Mechanical compression was associated with increased expression of Piezo1, fibronectin and α-SMA in HK2 cells, while GsMTx4 could partially prevent these pro-fibrotic alterations (Figure 5I and J). These
findings likely suggested that Piezo1 was involved in mechanical stretch or compression-induced pro-fibrotic alterations in HK2 cells.

*Matrix stiffness was associated with activation of Piezo1 in proximal tubular cells*

Renal fibrosis is characterized by increased tissue stiffness due to excessive ECM deposition in interstitial areas, leading to increased tubular membrane tension. Next, we aimed to investigate whether increased tissue stiffness induced by ECM deposition activated Piezo1 and the potential interaction between Piezo1 and ECM production. Polyacrylamide (PA) hydrogels can simulate the in vivo environment and could be a promising scaffolds material, mimicking the natural ECM (33, 34). HK2 cells were cultured on PA hydrogels with the modulus of 4 KPa to 35 KPa, which encompass the stiffness range from normal to fibrotic stiffness of kidney cortex. An Edu assay showed that proliferation of HK2 cells was greatly increased with stiffness of PA hydrogels (Figure 6A and B). With increases in stiffness, both protein and mRNA expressions of Piezo1 were significantly increased in HK2 cells cultured on PA hydrogels. Stiffness modulus of 8, 20, or 35KPa caused about 2-fold increases of Piezo1 mRNA expression (Figure 6C). The protein abundance of Piezo1 was increased about 3-folds under 8KPa, and approximate 5-fold increases were observed under 20 and 35KPa in HK2 cells (Figure 6D and E). With increases in stiffness, the protein abundance of fibronectin and α-SMA was significantly increased in HK2 cells (Figure 6D and E). At 20KPa modulus, increased fibronectin and α-SMA was inhibited by GsMTx4 (Figure 6F and G). The upregulation of Piezo1, fibronectin and α-SMA induced by stiffness
was also observed in primary cultured mouse proximal tubular cells (mPTCs) (Figure 6H and I), again, GsMTx4 prevented such a pro-fibrotic response (Figure 6J and K). These data suggested that Piezo1 was capable of sensing matrix stiffness, and increased matrix stiffness-induced pro-fibrotic alterations in tubular cells were, at least partially, depending on Piezo1 activation.

Piezo1 mediated TGF-β1-induced pro-fibrotic responses in proximal tubular cells

TGF-β1 induces tubular EMT and excessive production and deposition of ECM in tubulointerstitium (6), contributing to the early development and progression of renal interstitial fibrosis. We next examined the role of Piezo1 in TGF-β1-induced tubular pro-fibrotic alterations in HK2 cells. TGF-β1 treatment caused morphologic changes in HK2 cells, which was clearly prevented by GsMTx4 (Figure 7A). Consistent with this, western blots revealed that GsMTx4 prevented upregulation in protein expression of fibronectin and α-SMA, and reversed decreased protein abundance of E-cadherin in HK2 cells treated with TGF-β1 (Figure 7B and C). TGF-β1 induced about 3-fold increase in Piezo1 protein expression, which was partially prevented by GsMTx4 (p=0.06) (Figure 7B and C). Piezo1 silence with siRNA markedly prevented increases in protein expression of fibronectin and α-SMA, and a decrease in E-cadherin in HK2 cells treated with TGF-β1 (Figure 7D and E). In mPTCs, TGF-β1 clearly increased protein expression of Piezo1, fibronectin and α-SMA, which was inhibited by GsMTx4 (Figure 7F and G). Taken together, these data suggested an involvement of Piezo1 in TGF-β1 induced pro-fibrotic effects.
Recent data suggest a close association between TGF-β1 and stiff environment in development of fibrosis (35). Interestingly, with activation of Piezo1, HK2 cells seemed much sensitive to TGF-β1 treatment when cultured on stiffer PA hydrogels. After TGF-β1 treatment, the protein abundance of fibronectin was increased more in HK2 cells cultured on 20KPa PA hydrogels than those on 4KPa PA hydrogels (Figure S4A and B). It suggests a positive synergy between matrix stiffening and TGF-β1 in inducing Piezo1 activation in fibrosis.

Piezo1 activation by Yoda1 induced pro-fibrotic responses in proximal tubular cells

Mechanical stretch, compression, increased matrix stiffness, or TGF-β1, by activating Piezo1, leads to pro-fibrotic alterations in HK2 cells and mPTCs, we then ask whether a direct stimulation by a Piezo1 specific agonist, Yoda1, causes pro-fibrotic responses. First, the activity of Piezo1 was examined by the whole cell recording and cationic current was recorded. Yoda1 could induce marked cationic currents under different potential as shown in the current-voltage relationship (I-V) (Figure 8A). Under a high holding potential, puffing 10μM Yoda1 caused a significant current in HK2 cells (Figure 8B). These data confirmed expression of Piezo1 in HK2 cells and it functioned well under Yoda1 stimulation.

In a time-dependent manner, Yoda1 gradually caused morphologic changes in HK2 cells. HK2 cells in controls showed the typical cobblestone morphology of epithelial cells, while HK2 cells treated with Yoda1 exhibited elongation and hypertrophy after
24h treatment (Figure 8C). After 24-hour treatment with Yoda1, immunofluorescence showed a marked increase in fibronectin labeling intensity (Figure 8D). Consistent with morphological alterations, western blot demonstrated a time-dependent increase in the protein abundance of fibronectin and α-SMA, as well as a decrease in the protein expression of E-cadherin in HK2 cells treated with Yoda1 (Figure 8E and F), whereas Piezo1 silence with siRNA markedly prevented Yoda1-induced pro-fibrotic response (Figure 8G and H). Interestingly, Yoda1 stimulation caused a 2-fold increase in mRNA expression levels of TGF-β1 in HK2 cells (Figure 8I) and a 1.7-fold increase in protein abundance of TGF-β1 in the kidney of mice (Figure S2A and B), indicating that Piezo1 activation may induce TGF-β1 synthesis and secretion. The pro-fibrotic alterations caused by activation of Piezo1 were also seen in mPTCs treated with Yoda1 (Figure 8J and K). Taken together, these results suggested that activation of Piezo1 by Yoda1 directly induced pro-fibrotic phenotype changes in tubular cells and that activation of Piezo1 may be an important step in the development of fibrosis.

**Activation of Piezo1 induced calcium influx and enhanced interactions among calpain2, integrinβ1 and fibronectin**

Next we investigated the potential molecular mechanisms by which Piezo1 mediates renal fibrosis. Activation of Piezo1 by Yoda1 induced markedly increased calcium influx in HK2 cells (Figure 9A and B), consistent with previous studies. Calpain2, a calcium-dependent protease, is a member of the calpain families which can be activated by increased intracellular calcium. Inhibition of Piezo1 by Piezo-siRNA or GsMTx4 could reduce calpain activity in HepG2 cells or PC12 cells (36, 37). Calcium-calpain2
pathway was thus examined in our study. As shown in Figure 9, Yoda1 increased protein abundance of calpain2 in HK2 cells (Figure 9C-F). Piezo1 inhibition by siRNA attenuated Yoda1-induced upregulation of calpain2 (Figure 9E and F), indicating that calpain2 was an important downstream of Piezo1 (36-38). Piezo1 mediated calcium response seems largely dependent on extracellular calcium levels. When extracellular calcium was chelated by EGTA, increased calpain2 protein expression and pro-fibrotic changes induced by Yoda1 was significantly attenuated (Figure 9C and D), indicating that increased calcium influx mediated by Piezo1 may activate calpain2, which was involved in Yoda1-induced pro-fibrotic effects. To further investigate the role of calpain2 in Piezo1 activation-induced pro-fibrotic alterations in HK2 cells, calpain2 was knockdown by using CRISPR/Cas9-guided genome editing (Figure 9G and H). Compared with wildtype HK2 cells (WT HK2 cells), the protein abundance of fibronectin and α-SMA was greatly decreased in calpain2 knockdown HK2 cells (CAPN2-KD HK2 cells) after Yoda1 treatment, while E-cadherin protein expression was markedly upregulated (Figure 9G and H), indicating a suppression of pro-fibrotic response caused by calpain2 knockdown.

Talin1 regulates integrin affinity and provides a link between integrins and the cytoskeleton (39), and can be cleaved into active form by calpain2, mediating inside-out integrinβ1 activation and affinity (40). Western blot showed that the abundance of cleaved Talin1 protein (190kDa) was markedly increased in response to Yoda1 treatment, in association with a persistent increase of calpain2 protein abundance (Figure 10A and B). In CAPN2-KD HK2 cells, Yoda1 treatment failed to increase protein
expression of cleaved-Talin1 (Figure 10C and D).

As cleavage of Talin1 by calpain2 causes clustering of integrin on the plasma membrane and increased affinity of integrinβ1 (41), we next examined potential associations among activated Piezo1, calpain2 and integrinβ1 expression. Activation of Piezo1 by Yoda1 induced a significant increase in protein abundance of integrinβ1 in WT HK2 cells, which could be prevent by Piezo1 siRNA (Figure 10E and F). In CAPN2-KD HK2 cells, the abundance of integrinβ1 was lower than that in WT HK2 cells and Yoda1 treatment failed to induce integrinβ1 protein expression (Figure 10G and H), indicating a potential interaction between calpain2 and integrinβ1 when Piezo1 was stimulated. In addition, increased matrix stiffness also induced increase expression of calpain2 and integrinβ1 in HK2 cells and mPTCs, which could be attenuated by GsMTx4 (Figure S5A-D). In mice treated with Yoda1, the protein expression of integrinβ1 was also increased (Figure S2A and B). These results indicated that Piezo1 activation was probably an upstream event in integrinβ1 activation and following pro-fibrotic alterations. Co-IP assay showed that Yoda1 treatment induced a close binding of fibronectin to integrinβ1 (Figure 11A), which was confirmed by immunofluorescence demonstrating marked co-localization of fibronectin and integrinβ1 in WT HK2 cells treated with Yoda1 (Figure 11B), however, such a co-localization was not found in in CAPN2-KD HK2 cells treated with Yoda1 (Figure 11B). Immunofluorescence also demonstrated a prominent co-localization of Piezo1 and integrinβ1 in HK2 cells (Figure 11C). Focal adhesion kinase (FAK) is a downstream of integrin signaling and autophosphorylation of FAK plays an important role in integrin-
mediated signal transductions (42). Recent studies showed that integrin-FAK pathway was involved in the progression of fibrosis (43, 44). In our study, Yoda1 increased protein expression of phosphorylated FAK (p-FAK) (Tyr397) in HK2 cells, which was prevented by Piezo1 siRNA inhibition (Figure 10E and F). In CAPN2-KD HK2 cells, Yoda1 could not induce increased expression of p-FAK (Tyr397) (Figure 10G and H). The protein abundance of integrinβ1 and p-FAK (Tyr397) was also greatly increased in 7UOO mice, which was markedly reversed by Piezo1 inhibitor GsMTx4 treatment (Figure S6A and B).

Taken together, these results suggested that stimulation of Piezo1 induced activation of calcium-calpain2-Talin1 signaling and integrinβ1-FAK pathway, which may promote pro-fibrotic responses, presumably leading to kidney interstitial fibrosis.
DISCUSSION

The main finding of the current study is an association between Piezo1 activation and fibrosis in the kidney. In the fibrotic kidneys, tubular Piezo1 protein abundance was markedly increased, while inhibition of Piezo1 by a blocker GsTMx4 attenuated tubulointerstitial fibrosis induced by UUO or folic acid. We also demonstrated that Piezo1 stimulation by either mechanical stretch, compression, stiffness or an agonist Yoda1 induced pro-fibrotic alterations in HK2 cells, while TGF-β1-induced pro-fibrotic response was partially prevented by inhibition or silence of Piezo1. The involvement of Piezo1 in fibrosis is likely attributed to the activation of calcium-calpain2-integrinβ1 signaling.

Kidney fibrosis is associated with increased protein expression of Piezo1

Piezo1 is essential for transducing externally and internally applied forces at the plasma membrane, by which renal epithelial cells respond to both changes in fluid flow, intraluminal pressure, and wall tension (19, 20). In the kidney, piezo1 mRNA and protein expression was reported in segments of the rodent nephron and collecting duct (31). Piezo1 activity or Piezo1 transcript has been shown in immortalized proximal convoluted tubule cells (19) and the proximal tubule of mouse kidney and HK2 cells (45), respectively. Tubulointerstitial fibrosis is characterized by expansion of the space between tubular basement membrane and peritubular capillaries through ECM deposition (46), due to increased synthesis and inadequate degradation. ECM deposition inevitably leads to increase in peritubular compressional and tensional
forces. Excessive ECM accumulation also makes fibrotic regions (cortex, for example) much stiffer (35KPa) (22) than normal kidney tissue (4KPa) (23), which may cause an aberrant mechanical, microenvironmental and a stretch/compression stimulus to tubular epithelial cells. All these can be sensed by mechanosensors located on basolateral plasma membrane, among which is Piezo1 (19). In the present study, we found that in both human and mouse fibrotic kidneys, Piezo1 protein expression was much increased when compared with controls, particularly in proximal tubular epithelial cells. It is probably reasonable to believe that Piezo1 may be activated and upregulated when tubular epithelial cells respond to mechanical stress, e.g. fibrosis. Therefore, the observed upregulation of Piezo1 protein expression in the cortex may depend on the physiological or pathophysiological state of the kidney.

Piezo1 activation and upregulation in early time after UUO is likely attributing to tubular mechanical stretch caused by retrograde pressure shifts and urine pooling; while in sustained obstruction, the volume and continued pooling of urine provide major tubular stretch stimuli (47). Therefore, during UUO, tubular epithelial cells, at least partially, through Piezo1, sense stretch- or swelling forces in apical plasma membrane and stiff microenvironments in basolateral sides after fibrosis development. Besides, TRP ion channels located in epithelial cells also play important roles in sensing mechanical stimuli. After UUO, mRNA levels of TRPC1 and TRPV4 were slightly decreased or unchanged, whereas TRPC6 mRNA expression levels were shown 1.7- and 4-fold increase at the 3rd and 7th day, respectively. These findings likely indicated that in UUO Piezo1 responded to mechanical stimuli, together with other TRP channels. Activation
of Piezo1 in UUO may be largely attributed to tubular pressure, while in folic acid-induced kidney fibrosis, stretch and compression by deposited ECM, interstitial stiffness and/or certain pro-fibrotic factors (e.g. TGF-β1) may play important roles. Interestingly, inhibition of Piezo1 by GsMTx4 markedly attenuated UUO or folic acid-induced fibrosis in mice, strongly indicating the role of Piezo1 in the development of kidney fibrosis.

It should be noted that GsMTx4 is a specific inhibitor of cationic mechanosensitive channels, meaning that GsMTx4 not only inhibits Piezo1, but also has other targets (for example, TRP ion family) (48, 49). The stretch-evoked increase in intracellular Ca\(^{2+}\) concentration in urothelial cells was significantly attenuated by GsMTx4 to the level of Piezo1-knockdown cells (50), suggesting that GsMTx4 be able to inhibit Piezo1 effectively. In order to verify the role of Piezo1, Piezo1 specific agonist Yoda1 (no effect on Piezo2 or TRP ion family) was also used in animal study. Piezo1 stimulation by Yoda1 induced fibrotic alterations in the kidney of mice, supporting the involvement of Piezo1 in fibrosis. However, Yoda1 did not affect Piezo1 protein expression. The detailed molecular and biophysical mechanism is still unknown, but probably due to its direct interaction with Piezo1 or long-range membrane-delimited effects (through a change in membrane tension or curvature of the membrane) (51). Nevertheless, inhibition of Piezo1 by GsMTx4, at least, partially, contributes to attenuation of kidney fibrosis and activation of Piezo1 was involved in the development of renal fibrosis.

*Activation of Piezo1 by mechanical (stretch, compression, rigidity) or chemical (TGF-β1 or Yoda1) stimulation induced pro-fibrotic responses in tubular cells*
Mechanical stretch in tubular epithelial cells (and other types of cells) (26, 27) is a well-established model mimicking the pathogenic effects of tubular distention, which can provide a mechanism to stimulate an expression of fibrosis-related proteins (47, 52). In our study, stretch or compression markedly increased the protein expression of Piezo1 in HK2 cells and stretch induced increased intracellular calcium levels. Cyclic stretch or compression on HK2 or mPTCs caused significant increases of fibronectin and α-SMA, which were almost completely abolished by Piezo1 inhibition or silence, indicating that Piezo1 activation is essential for initiation of an early response to mechanical stimulation in renal tubular epithelial cells. During progression of fibrosis, kidney is becoming stiff due to the replacement of compliant cells with rigid matrix which is further increased by crosslinking of these matrix fibrils (21). Stiffness may provide a favored mechanical microenvironment to activate Piezo1. HK2 cells or mPTCs cultured on PA hydrogels with various levels of stiffness exhibited increased both the mRNA and protein levels of Piezo1, which was, again, in association with increased protein expression of fibronectin and α-SMA. Therefore, stiffness may enhance the mechanosensory and mechanotransduction capacity of tubular cells, leading to a pro-fibrotic alteration in HK2 cells or mPTCs, which was prevented when inhibiting activation of Piezo1.

TGF-β1 has been long considered as a key mediator of renal fibrosis, which promotes the accumulation of the ECM by enhanced synthesis of extracellular matrix proteins and by inhibiting their degradation (1, 32). Interestingly, Piezo1 inhibition by pretreatment with GsTMx4 or Piezo1 silence by siRNA dramatically prevented TGFβ1-
induced increases in fibronectin and α-SMA, and a decrease in E-cadherin in HK2 cells and mPTCs, suggesting a critical role of Piezo1 in initiation and persistence of fibrosis. This conception was further supported by the finding that specific Piezo1 agonist Yoda1 stimulated expression of fibrosis-related protein in HK2 cells and mPTCs.

**Piezo1 activation induced pro-fibrotic response through calcium-calpain-integrin pathway**

Mechanical signals propagate from the ECM and converge on cell surface adhesion receptors integrins, which connect intracellularly to the cytoskeleton within focal adhesion units (47). These stretch-activated cation channels are of fundamental importance in sensing and transducing external mechanical stresses (47) by a Ca\(^{2+}\)-dependent manner. Piezo1 stimulation by either stretch or Yoda1 induced Ca\(^{2+}\) influx-dependent increases in the [Ca\(^{2+}\)]. Piezo1 evoked instantaneous influx of calcium in tubular epithelial cells is probably a direct and immediate mechanotransduction response, and, likely, the first step leading to various intracellular events that may be involved in development of fibrosis, indeed, increases in [Ca\(^{2+}\)] have been found to play important roles in the development of renal interstitial fibrosis (53).

Calpain system was integrated as a downstream signals of Piezo1 (30) and calpains were shown to play critical roles in adhesion disassembly and in focal adhesion turnover (54). Calpain cleaves Talin1, which activates integrinβ1 clustering on the membrane and induces phosphorylation of FAK, a critical step in inducing pro-fibrotic phenotype changes in HK2 cells. Both in vivo (55) and in vitro (52) studies revealed
an important role of integrinβ1 in inducing tubulointerstitial fibrosis and tubular pro-fibrotic injuries. In the current study, either silencing of Piezo1 with siRNA or calpain2 knockdown significantly attenuated Yoda1-induced pro-fibrotic alterations, prevented cleavage of Talin1 and upregulation of integrinβ1 protein as well as phosphorylation of FAK in HK2 cells. Our data also showed that co-expression of integrinβ1 and fibronectin protein was markedly strengthened by stimulation of Piezo1 with Yoda1, indicating a closed binding of integrinβ1 and ECM once Piezo1 was activated. Therefore, our data likely suggested that Piezo1-mediated Ca\(^{2+}\) influx and corresponding activation of the calpain-Talin1-integrinβ1 pathway may be involved in pro-fibrotic phenotype alterations in HK2 cells when Piezo1 was activated (Figure 12).

**A reciprocal Piezo1-dependent feedforward mechanism in kidney fibrosis**

The interplay between physical forces and biochemical signaling pathways may regulate kidney fibrosis initiation and progression. In the UUO and FAN animal models, early inhibition of Piezo1 by GsMTx4 revealed a significant prevention of kidney fibrosis, likely indicating a possibility that Piezo1 activation is involved in initiation of fibrosis. Our unpublished data obtained from renal ischemia-reperfusion (I/R) injury model showed increased mRNA expression levels of Piezo1 in 24h I/R injury and in I/R-induced chronic kidney diseases rat models (unpublished data), supporting a role of Piezo1 in early stage of renal injury. Irrespective of the initial causes, it could be speculated that multiple insults to the renal parenchyma may stimulate Piezo1 in tubular epithelia, which cause Ca\(^{2+}\) influx and corresponding activation of calpain-Talin1-integrinβ1 signaling and ECM accumulation (an inside-out signaling), initiating
early events of fibrosis. During fibrosis progression, kidney tissue stiffening provides a favorable mechanical microenvironment to activate Piez1, the activity of which in calcium-dependent manner promotes the assembly of focal adhesions and activates integrin-FAK signaling (an outside-in signaling). Piezo1 signaling regulates the expression of genes and protein involved in ECM remodeling, which can further modulate tissue stiffness. In turn, the stiffer environment upregulates Piezo1 expression to increase the mechanosensory and mechanotransduction capacity of renal epithelial cells. Interestingly, TGF-β1 promotes expression and activation of Piezo1, inducing pro-fibrotic changes in both HK2 and mPTCs; on the other hand, Yoda1 stimulation was associated with increased expression of TGF-β1. These data likely indicated a reciprocal Piezo1-dependent feedforward mechanism between TGF-β1 and tubular mechanotransduction. Taken together, our data demonstrated a reciprocal, fibrosis-aggravating feedforward circuit between Piezo1-dependent mechanotransduction and aberrant tissue mechanics in tubular epithelial cells (Figure 12).

Our findings provide molecular insights into the integration between mechanical stimulation and biochemical signaling pathways in development of kidney fibrosis. We found increased expression of Piezo1 in kidney fibrosis and amelioration of fibrosis by Piezo1 inhibition. Piezo1 stimulation by crucial mechanical cues e.g. stretch, compression and matrix rigidity, TGF-β1 or a chemical agonist Yoda1, promoted pro-fibrotic responses in HK2 cells and mPTCs, through intracellular Ca²⁺-calpain-talin signaling that regulates concerted activation of integrin-associated FAK. Our findings
highlight the importance of external mechanical stress, mechanical sensors (Piezo1), and intracellular biological signaling pathways in the development of renal fibrosis, suggesting that blockade of the Piezo1 is probably a therapeutic option to delay the progress of kidney fibrosis.
MATERIALS AND METHODS

Animal models

Ten-week-old male C57BL/6J mice enrolled in these experiments were obtained from GemPharmatech (Nanjing, China). All mice were housed in an animal facility with a 12h light-dark cycle, water and chow ad libitum.

Unilateral ureteric obstruction (UUO) surgery was performed as previous described (56). To investigate whether Piezo1 was activated in the obstructed kidney with increased intraluminal pressure, mice were randomly divided into four groups: sham group, UUO for 30-minute group, UUO for 1-hour group and UUO for 3-hour group. To investigate whether Piezo1 was involved in UUO-induced kidney fibrosis, mice were randomly divided into six groups: sham group (3days), UUO for 3 days (3UUO) group and 3UUO + GsMTx4 group; sham group (7days), UUO for 7 day group (7UUO) and 7UUO + GsMTx4 group. Mice were subcutaneously injected on the back with 10mg/kg GsMTx4 dissolved in ddH2O (vehicle) every other day, starting on the first day after the surgery. The sham and UUO groups were given identical volumes of vehicle. The mice were sacrificed on day 3 and day 7 post-surgery and the obstructed kidneys were harvested for analysis.

To investigate whether Piezo1 was involved in folic acid-induced kidney fibrosis, mice were randomly divided into three groups: control group, folic acid group and folic acid + GsMTx4 group. In folic acid group, mice were treated with 250mg/kg folic acid
(MedChemExpress, China) dissolved in 0.3M sodium bicarbonate (vehicle) via intraperitoneal injection for 7 days. In folic acid + GsMTx4 group, mice were subcutaneously injected on the back with 10mg/kg GsMTx4 every other day, starting on the first day before folic acid treatment. Mice in control group received identical volumes of vehicle via intraperitoneal injection. The mice were sacrificed after 4 weeks and kidneys were harvested for analysis.

*Human kidney autopsy samples*

Human kidney autopsy specimens were obtained from the Center of Medicolegal Expertise, Sun Yat-sen University. Kidney specimens from decedents, dying of car accidents and without pathological changes, were used as normal control. Basic characteristics of decedents were listed in supplementary materials (Supplementary data I, Table 1).

*Cell culture and treatment*

Human proximal tubular cells (HK2 cells) were obtained from (ATCC, USA) and grown in DMEM/F12 (Corning, USA) containing 10% FBS (Quacell Biotechnology, USA) and 1% penicillin & streptomycin (Corning, USA) and maintained at 37°C in 5% CO₂ atmosphere. Mouse kidney proximal tubular cells (mPTCs) were prepared as previously described (57). Briefly, mPTCs were isolated from 10-week-old C57BL/6J mice and cultured in DMEM/F12 (Corning, USA) containing 10% FBS (Quacell Biotechnology, USA) at 37°C in 5% CO₂ atmosphere.

The HK2 cells and mPTCs were seeded on 6-well plate (Thermo Fisher Scientific, USA)
at a density of $2 \times 10^5$ cell/well for 24 hours and were serum-starved for 12 hours, the media was changed to fresh serum-free DMEM/F12 before treatment. For morphological assessments, the cells were fixed with 4% paraformaldehyde and photographed using a microscope (Leica) connected to a digital camera with a macro conversion lens. For pro-fibrotic response experiments, HK2 cells and mPTCs were treated with cyclic stretch for 24 hours or TGF-β1 (5ng/ml, R&D system, USA) for 48 hours, accompanied with either GsMTx4 (1μM or 5μM, TAIJIA biotech, China) or Piezo1 siRNA (RIBOBIO, China) pretreatments. Yoda1 (0.2μM, MedChemExpress, China) was used to stimulate HK2 cells for 1 to 24 hours, with or without Piezo1 siRNA. 1μM Yoda1 was used to stimulate mPTCs for 24 hours. All the cells were then collected for the protein or RNA analyses.

*Histologic analysis and immunofluorescence*

For histology, kidney tissues were fixed with 4% paraformaldehyde for paraffin embedding. 4μm tissue slices of kidney were prepared and stained via Masson’s trichrome, immunohistochemistry (IHC) and immunofluorescence. Paraffin-embedded kidney sections used for IHC studies were dewaxed, rehydrated, and incubated with Piezo1 (15939-1-AP, Proteintech, USA) or collagen I (BA0325, Boster, China) primary antibodies overnight at 4°C. The sections were subsequently incubated with goat anti-rabbit secondary antibodies (Thermo Fisher Scientific, USA), treated with diaminobenzidine, counterstained with hematoxylin and images were captured by the digital scanning microscope (Leica) with 20x and 40x magnification. Masson’s trichrome staining was used to further evaluate renal fibrosis.
Immunofluorescence staining was implemented as described in a previous study (56). The frozen sections of kidney tissue (8μm) or HK2 cells cultured on coverslips (20mm) from different groups were rinsed with PBS (Corning, USA), fixed with 4% paraformaldehyde, and incubated with primary antibodies against Piezo1 (15939-1-AP, Proteintech, USA), AQP1 (sc-25287, Santa Cruz, USA), AQP2 (sc-515770, Santa Cruz, USA), Fibronectin (BA1772, Boster, China) or Integrinβ1 (sc-9970, Santa Cruz, USA) overnight at 4°C. After the cells or sections were washed three times and incubated secondary antibodies conjugated with Alexa Fluor 488 and 555 (Thermo Fisher Scientific, USA), nuclei were stained with DAPI (4,6-diamidino-2-phenylindole, Thermo Fisher Scientific, USA). The samples were imaged using a fluorescence microscope (Leica).

Electrophysiology

HK2 cells were recorded under the voltage-clamp mode at room temperature using an integrated patch amplifier (Sutter Instrument, USA). HK2 cells were recorded in the ACSF composed of the following (in mM): 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 1 MgCl₂, 2 CaCl₂, 26 NaHCO₃, and 10 dextrose, bubbled with 95%O₂/5% CO₂. Patch pipettes (2–4 MΩ) were filled with the internal solution composed of the following (in mM): 140 CsCl, 10 HEPES, 0.2 CaCl₂, 5 MgATP, and 0.5 Na₃GTP, pH 7.2–7.3. The osmolarity of the internal solution was 275-290mOsm. Puff pressure was used for application of Yoda1. The tip diameter of puff micropipettes is about 2-5μm and puff pressure is between 1-5 psi: The horizontal distance from the tip of the puff micropipette to the recorded cell is about 20-40 μm and the angle between the puff micropipette and cell
surface is about 45°C. The tip of the puff micropipette is vertically 20-40µm above the recorded cell (z-axis). Cationic currents were recorded by puffing Yoda1 (10µM) for 350ms to the patched cells at holding potential of -90 (n=22), -45 (n=19), 0 (n=23), 45 (n=21) and 90mV (n=15). Only cells with series resistances <20 MΩ and input resistances >100 MΩ were included in the analysis. Cells were excluded if their input resistances changed >15% or if their series resistances changed >10% over the experiment. Recordings were filtered at 1 kHz and digitized at 10 kHz.

Cell mechanical stretch stress model

HK2 cells were seeded onto BioFlex culture plates (type I collagen-coated, Flexcell International Corporation, USA) at a density of 2×10⁵ cell/well for 24 hours. After being serum-starved for 12 hours, culture medium was replaced with fresh serum-free DMEM/F12. The BioFlex culture plates were placed on vacuum-based loading docks of the Flexcell FX-5000T apparatus (Flexcell International Corporation, USA) in the incubator and subjected to pulsatile mechanical stretch (20% of equibiaxial elongation) at a frequency of 0.3 Hz for 24 hours. Nonstretched cells (control group) were exposed to identical experimental conditions but without mechanical stretch.

Three-dimensional cultures and application of mechanical compression

The Flexcell-5000C Compression Plus System (Flexcell International Corporation, USA) was used to apply mechanical compression to HK2 cells. Cells were cultured in 1.5mg/ml type I collagen at a density of 2 × 10⁵ cell/well for 24hours. Cells were subjected to mechanical compression under the condition of 15mmHg at a frequency
of 1Hz for 24 hours (58), treated with or without 5μM GsMTx4. Cells cultured under the same condition with no stress were used as control.

**Plasmid and siRNA transfection**

Plasmid transfection CAPN2 knockdown plasmid was constructed. Briefly, knockdown of CAPN2 in HK2 cells was performed by CRISPR/Cas9-guided genome editing. Nucleotide sgRNA sequences: sgRNA 5’- CTTCTGACGAATCGCGCCA-3’ (223759291-223759349) were designed using sgRNA CRISPR design tool online (http://crispr.mit.edu) and cloned into the pSpCas9(BB)-2A-puro (PX459) plasmid (Addgene, catalog no.62988). After cloning, plasmids were purified and verified by sequencing. HK2 cells were seeded into six-well plates at 30-40% confluence and transfected with PX459 plasmid encoding a target specific sgRNA using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer’s instructions. Puromycin was then added to select the transfected cells after 24hr transfection. Knockdown efficiency for CAPN2 was assessed by western blot.

HK2 cells were transfected at 60% confluence in DMEM/F12 without FBS using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer’s instructions. The final Piezo1-siRNA (RIBOBIO, China) concentration was 100nM. Piezo1 suppression was confirmed by western blot and qPCR. The sequence of Piezo1 siRNA is 5’- GGCAGCGCATGAACCTTTCT-3’.

**Western blotting and coimmunoprecipitation (CO-IP) studies**

HK2 cells or kidney samples were lysed in protein lysis buffer for 15minutes on ice
before protein was extracted. Western blotting was performed by electrophoresis and incubation with primary antibodies against Fibronectin (BA1772, Boster, China), α-SMA (ab5694, Abcam, USA; BM0002, Boster, China; 14395-1-AP, Proteintech, USA), collagen I (BA0325, Boster, China), Calpain2 (11472-1-AP, Proteintech, USA), E-cadherin (20874-1-AP, Proteintech, USA), Integrinβ1 (12594-1-AP, Proteintech, USA), Piezo1 (15939-1-AP, Proteintech, USA; 28511-1-AP, Proteintech, USA), Smad2/3 (5678s, Cell Signaling Technology, USA), p-Smad2/3 (8828, Cell Signaling Technology, USA), p-FAK Tyr397 (sc-81593, Santa Cruz, USA), Talin1 (14168-1-AP, Proteintech, USA), GAPDH (60004-1-lg, Proteintech, USA), β-actin (66009-1-lg, Proteintech, USA) followed by the addition of horseradish peroxidase-labelled secondary antibodies. The blots were visualized in ECL detection systems and densitometric analysis was performed using AlphaEase Software.

The samples subjected to IP assay (Thermo Fisher Scientific, USA) were incubated with an anti-Integrinβ1 (sc-9970, Santacruz, USA) antibody in IP buffer overnight at 4°C. Protein A-sepharose beads were added to the samples, which incubated for another 12 hours. The sample were then washed and resuspended, and Western blotting was performed as described previously (56).

**Quantitative RT-PCR**

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) and then reverse transcribed with Reverse Transcriptase (AG bio, China). Resulting cDNAs were quantified by real-time PCR using SYBR green master mix (AG bio, China) on the Step
One Plus system (Applied Biosystems, USA). The sequences of the primers used were listed in supplementary materials (Supplementary data I, Table 2 and 3).

**Measurement of intracellular calcium levels**

To determine the intracellular calcium level, cells were incubated with 10nM Fluo-4 AM probe (Beyotime, China) for 30 mins at 37°C before subjected to Flow Cytometry analysis (Beckman coulter, USA) or fluorescence microscope (Leica).

**Polyacrylamide hydrogel**

Cell culture on polyacrylamide (PA) hydrogels of different stiffness was performed as described (33). The HK2 cells and mPTCs were seeded on PA hydrogels and grown in DMEM/F12 with 10% FBS and 1% penicillin & streptomycin at different stiffness conditions (4KPa, 8KPa, 20KPa and 35KPa), cultured for 5 days (HK2 cells) or 7 days (mPTCs) and then collected for protein and mRNA analysis. To determine whether Piezo1 mediated higher matrix stiffness induced pro-fibrotic effects in HK2 cells and mPTCs, HK2 cells were cultured on PA hydrogels for 1 day and mPTCs for 2 days, followed by 5μM GsMTx4 for another 4 days or 5 days. The proliferation of HK2 cells on PA hydrogels was detected by using an Edu labeling kit (RIBOBIO, China) according to the manufacturer’s recommendations. 5-ethynyl-2’-deoxyuridine (Edu) assay was used to detect the cell proliferative activity. Fluorescent images were obtained by fluorescence microscopy (Leica). The proportion of Edu-staining positive cells (red) to the total cells labeled by hochest (blue) indicated the proliferative rate.

**Statistical analysis**
Results are presented as the means ± SEM. Data were analyzed by one-way ANOVA and Student-Newman-Keuls tests for multiple comparisons. Statistical significance was accepted at the P < 0.05 level. The data analysis showed that the variance was homogeneous and accorded with normal distribution.

Study approval

All animal procedures were approved by the Animal Care and Use Committee of Sun Yat-sen University (Ethics Committee of ZSSOM on Laboratory Animal Care SYXK 2019-0209; Guangzhou, China). The study related to Human kidney autopsy specimens was conducted in accordance with the Declaration of Helsinki (2013).
AUTHOR CONTRIBUTIONS

X.Z., Y.K., B.L. and J.X. performed the experiments. X.Z., C.L. and W.W. designed the study, analyzed and interpreted the results, wrote and edited the manuscript. X.Z., Y.K., B.L. and J.X. assisted with main experiments. N.Z., J.L., B.J., J.C., C.L. and W.W. provided essential reagents and techniques for this study and reviewed the manuscript. X.Z., Y.K., B.L., J.X., N.Z., J.L., B.J., J.C., C.L. and W.W. approved the final version of manuscript. C.L. and W.W. conceived and supervised the study.
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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.
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LEGENDS

Figure 1. Increased expression of Piezo1 protein in fibrotic kidney diseases. (A) Co-localization of Piezo1 (red) and AQP1 (green) or AQP2 (green) in the kidney specimens from decedents died of traffic accidents, detected by immunofluorescence. Scale bar: 500μm. (B) Representative photomicrographs of Masson’s staining (Magnification 200×) and immunohistochemistry (Magnification 400×) of Piezo1 in the kidney specimens from decedents with different kidney diseases. AQP1, aquaporin-1; AQP2, aquaporin-2.

Figure 2. Increased expression of Piezo1 protein and mRNA in UUO mice. (A) and (B) Representative immunoblots and corresponding densitometry analysis of Piezo1 protein abundance in the kidney of Sham and UUO mice at the 30th minutes, the 1st hour and the 3rd hours after surgery. Data are shown as mean ± SEM (n=6 in each group). (C) and (D) Representative immunoblots and corresponding densitometry analysis of Piezo1 protein abundance in the kidney of Sham, 3UUO and 7UUO mice. Data are shown as mean ± SEM (n=4 in each group). (E) mRNA levels of Piezo1, TRPC1, 6 and TRPV4 in the kidney of Sham, 3UUO and 7UUO mice. Data are shown as mean ± SEM (n=4 in each group). (F) Co-localization of Piezo1 (red) and AQP1 (green) or AQP2 (green) in the proximal tubules or inner medullary collecting ducts of Sham and 7UUO mice, detected by immunofluorescence. Scale bar: 500μm.

* P<0.05 when compared with sham mice by one-way ANOVA with Student-Newman-Keuls test. UUO, unilateral ureteral obstruction; 3UUO, UUO for 3 days; 7UUO, UUO for 7days. TRPC, transient receptor potential canonical; TRPV, transient receptor potential vanilloid.
potential vanilloid; AQP1, aquaporin-1; AQP2, aquaporin-2.

**Figure 3. Inhibition of Piezo1 prevented UUO-induced kidney fibrosis.** (A-C) Representative immunoblots and corresponding densitometry analysis of collagen I, fibronectin, p-Smad2/3, Smad2/3 protein abundance in the kidney of Sham, 3UUO, 3UUO+GsMTx4, 7UUO and 7UUO+GsMTx4 mice. Data are shown as mean ± SEM (n=4 in each group). (D) Photomicrographs of Masson’s staining and immunohistochemistry of collagen I in the kidney of Sham, 7UUO, 7UUO+GsMTx4 mice (Magnification 400×). (E) mRNA levels of TGF-β1, α-SMA and fibronectin in the kidney of Sham, 3UUO, 3UUO+GsMTx4, 7UUO, 7UUO+GsMTx4 mice. Data are shown as mean ± SEM (n=4 in each group); * P<0.05 when compared with sham mice and # P<0.05 when compared with 3UUO or 7UUO mice by one-way ANOVA with Student-Newman-Keuls test. UUO, unilateral ureteral obstruction; 3UUO, UUO for 3 days; 7UUO, UUO for 7 days; TGF-β1, transforming growth factor-β1; SMA, smooth muscle actin.

**Figure 4. Inhibition of Piezo1 prevented folic acid-induced kidney fibrosis.** (A) and (B) Representative immunoblots and corresponding densitometry analysis of Piezo1, fibronectin and collagen I protein abundance in the kidney of CTL, FAN, FAN+GsMTx4 mice. Data are shown as mean ± SEM (n=5 in CTL group, n=6 in FAN and FAN+GsMTx4 group). (C) Photomicrographs of Masson’s staining in the kidney of CTL, FAN, FAN+GsMTx4 mice (Magnification 400×). (D) mRNA levels of TGF-β1, α-SMA and fibronectin in the kidney of CTL, FAN, FAN+GsMTx4 mice. Data are shown as mean ± SEM (n=5 in CTL group, n=6 in FAN and FAN+GsMTx4 group). * P<0.05
when compared with CTL mice and # P<0.05 when compared with FAN mice by one-way ANOVA with Student-Newman-Keuls test. CTL, control; FAN, folic acid nephropathy; TGF-β1, transforming growth factor-β1; SMA, smooth muscle actin.

**Figure 5.** Piezo1 mediated mechanical stretch or compression-induced profibrotic responses in HK2 cells. (A) mRNA level of Piezo1 in HK2 cells pretreated with 5μM GsMTx4 followed by cyclic stretch. Data are shown as mean ± SEM (n=6 in each group). (B) and (C) Representative immunoblots and corresponding densitometry analysis of Piezo1 protein abundance in HK2 cells pretreated with 1μM or 5μM GsMTx4 followed by cyclic stretch. Data are shown as mean ± SEM (n=4 in each group). (D) Fluo-4 AM was added to HK2 cells transfected with Piezo1 siRNA followed by cyclic stretch, and the fluorescence signals of calcium were detected by flow cytometry analysis. (E) and (F) Representative immunoblots and corresponding densitometry analysis of fibronectin, α-SMA and E-cadherin protein abundance in HK2 cells pretreated with 5μM GsMTx4 followed by cyclic stretch. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with Static and # P<0.05 when compared with Stretch by one-way ANOVA with Student-Newman-Keuls test. (G) and (H) Representative immunoblots and corresponding densitometry analysis of fibronectin, α-SMA and E-cadherin protein abundance in HK2 cells transfected with Piezo1 siRNA followed by cyclic stretch. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with scramble siRNA static and # P<0.05 when compared with scramble siRNA stretch by one-way ANOVA with Student-Newman-Keuls test. (I) and (J) Representative immunoblots and corresponding densitometry
analysis of Piezo1, fibronectin and α-SMA protein abundance in HK2 cells pretreated with 5μM GsMTx4 followed by 15mmHg compression for 24 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with CTL and # P<0.05 when compared with compression by one-way ANOVA with Student-Newman-Keuls test. SMA, smooth muscle actin.

Figure 6. Piezo1 sensed matrix stiffness and was involved in increased matrix stiffness-induced pro-fibrotic responses in HK2 cells and mPTCs. (A) and (B) Cell proliferation of HK2 cells cultured on polyacrylamide hydrogels with different stiffness, assessed by Edu. Scale bar: 500μm. Data are shown as mean ± SEM (n=4 in 4KPa group, n=5 in 8KPa group, n=8 in 20KPa group, n=9 in 35KPa group). (C) Piezo1 mRNA level of HK2 cells cultured on polyacrylamide hydrogels with different stiffness. Data are shown as mean ± SEM (n=4 in each group). (D) and (E) Representative immunoblots and corresponding densitometry analysis of Piezo1, fibronectin and α-SMA protein abundance in HK2 cells cultured on polyacrylamide hydrogels with different stiffness. Data are shown as mean ± SEM (n=4 in each group). (F) and (G) Representative immunoblots and corresponding densitometry analysis of fibronectin and α-SMA protein abundance in HK2 cells cultured on 4KPa and 20KPa polyacrylamide hydrogels, followed by treatment with GsMTx4. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with 4KPa and # P<0.05 when compared with 20KPa by one-way ANOVA with Student-Newman-Keuls test. (H) and (I) Representative immunoblots and corresponding densitometry analysis of Piezo1, fibronectin and α-SMA protein abundance in primary mPTCs cultured on
polyacrylamide hydrogels with different stiffness. Data are shown as mean ± SEM (n=4 in each group). (J) and (K) Representative immunoblots and corresponding densitometry analysis of fibronectin and α-SMA protein abundance in primary mPTCs cultured on polyacrylamide hydrogels with 4KPa and 35KPa stiffness, followed by treatment with GsMTx4. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with 4KPa and # P<0.05 when compared with 35KPa by one-way ANOVA with Student-Newman-Keuls test. mPTCs, mouse proximal tubular cells; Edu, 5-ethynyl-2′-deoxyuridine; SMA, smooth muscle actin.

Figure 7. Piezo1 was involved in TGF-β1-induced pro-fibrotic responses in HK2 cells and mPTCs. (A) Morphologic changes of HK2 cells pretreated with GsMTx4 followed by TGF-β1 treatment for 48 hours. Scale bar: 100μm. (B) and (C) Representative immunoblots and corresponding densitometry analysis of Piezo1, fibronectin, α-SMA and E-cadherin protein abundance in HK2 cells pretreated with GsMTx4 followed by TGF-β1 treatment for 48 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with CTL and # P<0.05 when compared with TGF-β1 by one-way ANOVA with Student-Newman-Keuls test. (D) and (E) Representative immunoblots and corresponding densitometry analysis of fibronectin, α-SMA and E-cadherin protein abundance in HK2 cells transfected with Piezo1 siRNA followed by TGF-β1 treatment for 48 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with scramble siRNA CTL and # P<0.05 when compared with scramble siRNA TGF-β1 by one-way ANOVA with Student-Newman-Keuls test. (F) and (G) Representative immunoblots and corresponding densitometry
analysis of Piezo1, fibronectin and α-SMA protein abundance in primary cultured mPTCs, pretreated with GsMTx4 followed by TGF-β1 treatment for 48 hours. Data are shown as mean ± SEM (n=6 in each group). * P<0.05 when compared with CTL and # P<0.05 when compared with TGF-β1 by one-way ANOVA with Student-Newman-Keuls test. CTL, control; TGF-β1, transforming growth factor-β1; SMA, smooth muscle actin; mPTCs: mouse proximal tubular cells.

**Figure 8. Activation of Piezo1 by an agonist Yoda1 induced pro-fibrotic responses in HK2 cells and mPTCs.** (A) and (B) Yoda1 induced the cationic currents in HK2 cells, (A) I-V curve exhibited the currents activated by 10μM Yoda1 recorded at different membrane potential at voltage-clamp mode. (B) Representative traces: recorded at -90 and +90mV by puffing a 10μM Yoda1 (short black arrow) for 350ms to HK2 cells. (C) Morphologic changes of HK2 cells treated with Yoda1 for 3 hours, 6 hours, 12 hours and 24 hours. Scale bar: 100μm. (D) Immunofluorescence of fibronectin in HK2 cells treated with Yoda1 for 24 hours. Scaler: 50μm. (E) and (F) Representative immunoblots and corresponding densitometry analysis of fibronectin, α-SMA and E-cadherin protein abundance in HK2 cells treated with Yoda1 for 3 hours, 6 hours, 12 hours and 24 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with CTL by one-way ANOVA with Student-Newman-Keuls test. (G) and (H) Representative immunoblots and corresponding densitometry analysis of fibronectin, α-SMA and E-cadherin protein abundance in HK2 cells transfected with Piezo1 siRNA followed by Yoda1 treatment for 24 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with scramble
siRNA CTL and # P<0.05 when compared with scramble siRNA Yoda1 by one-way ANOVA with Student-Newman-Keuls test. (I) mRNA expression of TGF-β1 in HK2 cells treated with Yoda1 for 24 hours. Data are shown as mean ± SEM (n=6 in each group). * P<0.05 when compared with CTL by unpaired student’s t test. (J) and (K) Representative immunoblots and corresponding densitometry analysis of fibronectin and α-SMA protein abundance in primary cultured mPTCs treated with Yoda1 for 24 hours. Data are shown as mean ± SEM (n=6 in each group). * P<0.05 when compared with CTL by unpaired student’s t test. CTL, control; SMA, smooth muscle actin; TGF-β1, transforming growth factor-β1; mPTCs: mouse proximal tubular cells.

Figure 9. Yoda1 induced calpain2 activation promoted pro-fibrotic responses in HK2 cells. (A) and (B) Fluo-4 AM was added to HK2 cells treated with Yoda1 for 1 hour, 3 hours, 6 hours and 24 hours, and the fluorescence signals of Ca²⁺ were detected by flow cytometry analysis and fluorescence microscope. Scale bar: 200µm. (C) and (D) Representative immunoblots and corresponding densitometry analysis of calpain2, fibronectin, α-SMA and E-cadherin protein abundance in HK2 cells pretreated with 2mM EGTA followed by Yoda1 treatment for 24 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with CTL and # P<0.05 when compared with Yoda1 by one-way ANOVA with Student-Newman-Keuls test. (E) and (F) Representative immunoblots and corresponding densitometry analysis of calpain2 protein abundance in HK2 cells transfected with Piezo1 siRNA followed by Yoda1 treatment for 24 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with scramble siRNA CTL and # P<0.05 when compared with
scramble siRNA Yoda1 by one-way ANOVA with Student-Newman-Keuls test. (G) and (H) Representative immunoblots and corresponding densitometry analysis of calpain2, fibronectin, α-SMA and E-cadherin protein abundance in WT HK2 cells and CAPN2-KD HK2 cells treated with Yoda1 for 24 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with CTL by one-way ANOVA with Student-Newman-Keuls test. CTL, control; WT, wildtype; CAPN2-KD, calpain2 knockdown; SMA, smooth muscle actin.

**Figure 10. Yoda1 activated calpain2-talin1-integrinβ1 pathway in HK2 cells.** (A) and (B) Representative immunoblots and corresponding densitometry analysis of calpain2 and Talin1 protein abundance in HK2 cells treated with Yoda1 for 3 hours, 6 hours, 12 hours and 24 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with CTL by one-way ANOVA with Student-Newman-Keuls test. (C) and (D) Representative immunoblots and corresponding densitometry analysis of Talin1 protein abundance in WT HK2 cells and CAPN2-KD HK2 cells treated with Yoda1 for 24 hours. Data are shown as mean ± SEM (n=6 in each group). * P<0.05 when compared with CTL by one-way ANOVA with Student-Newman-Keuls test. (E) and (F) Representative immunoblots and corresponding densitometry analysis of integrinβ1 and p-FAK (Tyr 397) protein abundance in HK2 cells transfected with Piezo1 siRNA followed by Yoda1 treatment for 24 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with scramble siRNA CTL and # P<0.05 when compared with scramble siRNA Yoda1 by one-way ANOVA with Student-Newman-Keuls test. (G) and (H) Representative immunoblots and
corresponding densitometry analysis of integrin β1 and p-FAK (Tyr 397) protein abundance in WT HK2 cells and CAPN2-KD HK2 cells treated with Yoda1 for 24 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with CTL by one-way ANOVA with Student-Newman-Keuls test. CTL, control; WT, wildtype; CAPN2-KD, calpain2 knockdown; FAK, focal adhesion kinase.

**Figure 1. Yoda1 enhanced the interaction between fibronectin and integrin β1 in HK2 cells.** (A) Immunoprecipitation assay showing the interaction between fibronectin and integrin β1 in WT HK2 cells with or without Yoda1 treatment. (B) Immunofluorescence of co-localization of fibronectin and integrin β1 in WT HK2 cells and CAPN2-KD HK2 cells treated with or without Yoda1. Scale bar: 50μm. (C) Immunofluorescence of co-localization of Piezo1 and integrin β1 in WT HK2 cells. Scale bar: 50μm. CTL, control; WT, wildtype; CAPN2-KD, calpain2 knockdown; FAK, focal adhesion kinase.

**Figure 2. A potential reciprocal Piezo1-dependent feedforward mechanism in kidney fibrosis.** During the progression of kidney fibrosis, the membrane tension of tubular epithelium (e.g. stretch or compression) is increased due to increased tissue stiffness induced by excessive deposition of extracellular matrix in tubulointerstitial areas and elevated intraluminal pressure from probably fluid accumulation in tubules, leading to activation of Piezo1 and influx of calcium, by this way, mechanical signals are transduced into intracellular chemical signals. Intracellular calcium activates Calpain2 that cleaved Talin1 into active form, which subsequently caused activation and clustering of integrin β1 on the basolateral membrane, promoting the deposition of
ECM (inside-out signal). Activation of Piezo1 also induces expression and synthesis of TGF-β1, further promoting ECM deposition. On the other hand, excessive deposition of ECM promotes tissue stiffening, which in turn increases the mechanosensory and mechanotransduction capacity of renal epithelial cells by activated Piezo1, aggravating the progression of renal fibrosis (outside-in signal).
Figure 1. Increased expression of Piezo1 protein in fibrotic kidney diseases. (A) Co-localization of Piezo1 (red) and AQP1 (green) or AQP2 (green) in the kidney specimens from decedents died of traffic accidents, detected by immunofluorescence. Scale bar: 500µm. (B) Representative photomicrographs of Masson’s staining (Magnification 200×) and immunohistochemistry (Magnification 400×) of Piezo1 in the kidney specimens from decedents with different kidney diseases. AQP1, aquaporin-1; AQP2, aquaporin-2.
Figure 2. Increased expression of Piezo1 protein and mRNA in UUO mice. (A) and (B) Representative immunoblots and corresponding densitometry analysis of Piezo1 protein abundance in the kidney of Sham and UUO mice at the 30th minutes, the 1st hour and the 3rd hour after surgery. Data are shown as mean ± SEM (n=6 in each group). (C) and (D) Representative immunoblots and corresponding densitometry analysis of Piezo1 protein abundance in the kidney of Sham, 3UUO and 7UUO mice. Data are shown as mean ± SEM (n=4 in each group). (E) mRNA levels of Piezo1, TRPC1, 6 and TRPV4 in the kidney of Sham, 3UUO and 7UUO mice. Data are shown as mean ± SEM (n=4 in each group). (F) Co-localization of Piezo1 (red) and AQP1 (green) or AQP2 (green) in the proximal tubules or inner medullary collecting ducts of Sham and 7UUO mice, detected by immunofluorescence. Scale bar: 500μm. * P<0.05 when compared with sham mice by one-way ANOVA with Student-Newman-Keuls test. UUO, unilateral ureteral obstruction; 3UUO, UUO for 3 days; 7UUO, UUO for 7 days; TRPC, transient receptor potential canonical; TRPV, transient receptor potential vanilloid; AQP1, aquaporin-1; AQP2, aquaporin-2.
Figure 3. Inhibition of Piezo1 prevented UUO-induced kidney fibrosis. (A-C) Representative immunoblots and corresponding densitometry analysis of collagen I, fibronectin, p-Smad2/3, Smad2/3 protein abundance in the kidney of Sham, 3UUO, 3UUO+GsMTx4, 7UUO and 7UUO+GsMTx4 mice. Data are shown as mean ± SEM (n=4 in each group). (D) Photomicrographs of Masson’s staining and immunohistochemistry of collagen I in the kidney of Sham, 7UUO, 7UUO+GsMTx4 mice (Magnification 400×). (E) mRNA levels of TGF-β1, α-SMA and fibronectin in the kidney of Sham, 3UUO, 3UUO+GsMTx4, 7UUO, 7UUO+GsMTx4 mice. Data are shown as mean ± SEM (n=4 in each group); * P<0.05 when compared with sham mice and # P<0.05 when compared with 3UUO or 7UUO mice by one-way ANOVA with Student-Newman-Keuls test. UUO, unilateral ureteral obstruction; 3UUO, UUO for 3 days; 7UUO, UUO for 7 days; TGF-β1, transforming growth factor-β1; SMA, smooth muscle actin.
Figure 4. Inhibition of Piezo1 prevented folic acid-induced kidney fibrosis. (A) and (B) Representative immunoblots and corresponding densitometry analysis of Piezo1, fibronectin and collagen I protein abundance in the kidney of CTL, FAN, FAN+GsMTx4 mice. Data are shown as mean ± SEM (n=5 in CTL group, n=6 in FAN and FAN+GsMTx4 group). (C) Photomicrographs of Masson’s staining in the kidney of CTL, FAN, FAN+GsMTx4 mice (Magnification 400×). (D) mRNA levels of TGF-β1, α-SMA and fibronectin in the kidney of CTL, FAN, FAN+GsMTx4 mice. Data are shown as mean ± SEM (n=5 in CTL group, n=6 in FAN and FAN+GsMTx4 group). * P<0.05 when compared with CTL mice and # P<0.05 when compared with FAN mice by one-way ANOVA with Student-Newman-Keuls test. CTL, control; FAN, folic acid nephropathy; TGF-β1, transforming growth factor-β1; SMA, smooth muscle actin.
Figure 5. Piezo1 mediated mechanical stretch or compression-induced pro-fibrotic responses in HK2 cells. (A) mRNA level of Piezo1 in HK2 cells pretreated with 5 μM GsMTx4 followed by cyclic stretch. Data are shown as mean ± SEM (n=6 in each group). (B) and (C) Representative immunoblots and corresponding densitometry analysis of Piezo1 protein abundance in HK2 cells pretreated with 1 μM or 5 μM GsMTx4 followed by cyclic stretch. Data are shown as mean ± SEM (n=4 in each group). (D) Flu-4 AM was added to HK2 cells transfected with Piezo1 siRNA followed by cyclic stretch, and the fluorescence signals of calcium were detected by flow cytometry analysis. (E) and (F) Representative immunoblots and corresponding densitometry analysis of fibronectin, α-SMA and E-cadherin protein abundance in HK2 cells transfected with 5 μM GsMTx4 followed by cyclic stretch. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with Static and # P<0.05 when compared with Stretch by one-way ANOVA with Student-Newman-Keuls test. (G) and (H) Representative immunoblots and corresponding densitometry analysis of fibronectin, α-SMA and E-cadherin protein abundance in HK2 cells transfected with Piezo1 siRNA followed by cyclic stretch. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with Scramble siRNA static and # P<0.05 when compared with Scramble siRNA stretch by one-way ANOVA with Student-Newman-Keuls test. (I) and (J) Representative immunoblots and corresponding densitometry analysis of Piezo1, fibronectin and α-SMA protein abundance in HK2 cells pretreated with 5 μM GsMTx4 followed by 15 mmHg compression for 24 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with CTL and # P<0.05 when compared with compression by one-way ANOVA with Student-Newman-Keuls test. SMA, smooth muscle actin.
Figure 6. Piezo1 sensed matrix stiffness and was involved in increased matrix stiffness-induced pro-fibrotic responses in HK2 cells and mPTCs. (A) and (B) Cell proliferation of HK2 cells cultured on polyacrylamide hydrogels with different stiffness, assessed by Edu. Scale bar: 500μm. Data are shown as mean ± SEM (n=4 in 4KPa group, n=5 in 8KPa group, n=8 in 20KPa group, n=9 in 35KPa group). (C) Piezo1 mRNA level of HK2 cells cultured on polyacrylamide hydrogels with different stiffness. Data are shown as mean ± SEM (n=4 in each group). (D) and (E) Representative immunoblots and corresponding densitometry analysis of Piezo1, fibronectin and α-SMA protein abundance in HK2 cells cultured on polyacrylamide hydrogels with different stiffness. Data are shown as mean ± SEM (n=4 in each group). (F) and (G) Representative immunoblots and corresponding densitometry analysis of fibronectin and α-SMA protein abundance in HK2 cells cultured on 4KPa and 20KPa polyacrylamide hydrogels, followed by treatment with GsMTX4. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with 4KPa and # P<0.05 when compared with 20KPa by one-way ANOVA with Student-Newman-Keuls test. (H) and (I) Representative immunoblots and corresponding densitometry analysis of Piezo1, fibronectin and α-SMA protein abundance in primary mPTCs cultured on polyacrylamide hydrogels with different stiffness. Data are shown as mean ± SEM (n=4 in each group). (J) and (K) Representative immunoblots and corresponding densitometry analysis of fibronectin and α-SMA protein abundance in primary mPTCs cultured on polyacrylamide hydrogels with 4KPa and 35KPa stiffness, followed by treatment with GsMTX4. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with 4KPa and # P<0.05 when compared with 35KPa by one-way ANOVA with Student-Newman-Keuls test. mPTCs, mouse proximal tubular cells; Edu, 5-ethynyl-2’-deoxyuridine; SMA, smooth muscle actin.
Figure 7. Piezo1 was involved in TGF-β1-induced pro-fibrotic responses in HK2 cells and mPTCs. (A) Morphologic changes of HK2 cells pretreated with GsMTx4 followed by TGF-β1 treatment for 48 hours. Scale bar: 100μm. (B) and (C) Representative immunoblots and corresponding densitometry analysis of Piezo1, fibronectin, α-SMA and E-cadherin protein abundance in HK2 cells pretreated with GsMTx4 followed by TGF-β1 treatment for 48 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with CTL and # P<0.05 when compared with TGF-β1 by one-way ANOVA with Student-Newman-Keuls test. (D) and (E) Representative immunoblots and corresponding densitometry analysis of fibronectin, α-SMA and E-cadherin protein abundance in HK2 cells transfected with Piezo1 siRNA followed by TGF-β1 treatment for 48 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with scramble siRNA CTL and # P<0.05 when compared with scramble siRNA TGF-β1 by one-way ANOVA with Student-Newman-Keuls test. (F) and (G) Representative immunoblots and corresponding densitometry analysis of Piezo1, fibronectin and α-SMA protein abundance in primary cultured mPTCs, pretreated with GsMTx4 followed by TGF-β1 treatment for 48 hours. Data are shown as mean ± SEM (n=6 in each group). * P<0.05 when compared with CTL and # P<0.05 when compared with TGF-β1 by one-way ANOVA with Student-Newman-Keuls test. CTL, control; TGF-β1, transforming growth factor-β1; SMA, smooth muscle actin; mPTCs: mouse proximal tubular cells.
Figure 8. Activation of Piezo1 by an agonist Yoda1 induced pro-fibrotic responses in HK2 cells and mPTCs. (A) and (B) Yoda1 induced the cationic currents in HK2 cells. (A) I-V curve exhibited the currents activated by 10μM Yoda1 recorded at different membrane potential at voltage-clamp mode. (B) Representative traces: recorded at -90 and +90mV by puffing a 10μM Yoda1 (short black arrow) for 350ms to HK2 cells. (C) Morphologic changes of HK2 cells treated with Yoda1 for 3 hours, 6 hours, 12 hours and 24 hours. Scale bar: 100μm. (D) Immunofluorescence of fibronectin in HK2 cells treated with Yoda1 for 24 hours. Scale: 50μm. (E) Representative immunoblot and corresponding densitometry analysis of fibronectin, α-SMA and E-cadherin protein abundance in HK2 cells treated with Yoda1 for 3 hours, 6 hours, 12 hours and 24 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with CTL by one-way ANOVA with Student-Newman-Keuls test. (G) and (H) Representative immunobLOTS and corresponding densitometry analysis of fibronectin, α-SMA and E-cadherin protein abundance in HK2 cells transfected with Piezo1 siRNA followed by Yoda1 treatment for 24 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with scramble siRNA CTL and # P<0.05 when compared with scramble siRNA Yoda1 by one-way ANOVA with Student-Newman-Keuls test. (I) mRNA expression of TGF-β1 in HK2 cells treated with Yoda1 for 24 hours. Data are shown as mean ± SEM (n=6 in each group). * P<0.05 when compared with CTL by unpaired student’s t test. (J) and (K) Representative immunoblots and corresponding densitometry analysis of fibronectin and α-SMA protein abundance in primary cultured mPTCs treated with Yoda1 for 24 hours. Data are shown as mean ± SEM (n=6 in each group). * P<0.05 when compared with CTL by unpaired student’s t test. CTL, control; SMA, smooth muscle actin; TGF-β1, transforming growth factor-β1; mPTCs: mouse proximal tubular cells.
Figure 9. Yoda1 induced calpain2 activation promoted pro-fibrotic responses in HK2 cells. (A) and (B) Fluo-4 AM was added to HK2 cells treated with Yoda1 for 1 hour, 3 hours, 6 hours and 24 hours, and the fluorescence signals of Ca2+ were detected by flow cytometry analysis and fluorescence microscope. Scale bar: 200µm. (C) and (D) Representative immunobots and corresponding densitometry analysis of calpain2, fibronectin, α-SMA and E-cadherin protein abundance in HK2 cells pretreated with 2mM EGTA followed by Yoda1 treatment for 24 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with CTL and # P<0.05 when compared with Yoda1 by one-way ANOVA with Student-Newman-Keuls test. (E) and (F) Representative immunobots and corresponding densitometry analysis of calpain2 protein abundance in HK2 cells transfected with Piezo1 siRNA followed by Yoda1 treatment for 24 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with scramble siRNA CTL and # P<0.05 when compared with scramble siRNA Yoda1 by one-way ANOVA with Student-Newman-Keuls test. (G) and (H) Representative immunobots and corresponding densitometry analysis of calpain2, fibronectin, α-SMA and E-cadherin protein abundance in WT HK2 cells and CAPN2-KD HK2 cells treated with Yoda1 for 24 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with CTL by one-way ANOVA with Student-Newman-Keuls test: CTL, control; WT, wildtype; CAPN2-KD, calpain2 knockdown; SMA, smooth muscle actin.
Figure 10. Yoda1 activated calpain2-talin1-integrinβ1 pathway in HK2 cells. (A) and (B) Representative immunoblots and corresponding densitometry analysis of calpain2 and Talin1 protein abundance in HK2 cells treated with Yoda1 for 3 hours, 6 hours, 12 hours and 24 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with CTL by one-way ANOVA with Student-Newman-Keuls test. (C) and (D) Representative immunoblots and corresponding densitometry analysis of Talin1 protein abundance in WT HK2 cells and CAPN2-KD HK2 cells treated with Yoda1 for 24 hours. Data are shown as mean ± SEM (n=6 in each group). * P<0.05 when compared with CTL by one-way ANOVA with Student-Newman-Keuls test. (E) and (F) Representative immunoblots and corresponding densitometry analysis of integrinβ1 and p-FAK (Tyr397) protein abundance in HK2 cells transfected with Piezo1 siRNA followed by Yoda1 treatment for 24 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with scramble siRNA CTL and # P<0.05 when compared with scramble siRNA Yoda1 by one-way ANOVA with Student-Newman-Keuls test. (G) and (H) Representative immunoblots and corresponding densitometry analysis of integrinβ1 and p-FAK (Tyr397) protein abundance in WT HK2 cells and CAPN2-KD HK2 cells treated with Yoda1 for 24 hours. Data are shown as mean ± SEM (n=4 in each group). * P<0.05 when compared with CTL by one-way ANOVA with Student-Newman-Keuls test. CTL, control; WT, wildtype; CAPN2-KD, calpain2 knockdown; FAK, focal adhesion kinase.
Figure 11. Yoda1 enhanced the interaction between fibronectin and integrinβ1 in HK2 cells. (A) Immunoprecipitation assay showing the interaction between fibronectin and integrinβ1 in WT HK2 cells with or without Yoda1 treatment. (B) Immunofluorescence of co-localization of fibronectin and integrinβ1 in WT HK2 cells and CAPN2-KD HK2 cells treated with or without Yoda1. Scale bar: 50μm. (C) Immunofluorescence of co-localization of Piezo1 and integrinβ1 in WT HK2 cells. Scale bar: 50μm. CTL, control; WT, wildtype; CAPN2-KD, calpain2 knockout; FAK, focal adhesion kinase.
Figure 12. A potential reciprocal Piezo1-dependent feedforward mechanism in kidney fibrosis. During the progression of kidney fibrosis, the membrane tension of tubular epithelium (e.g. stretch or compression) is increased due to increased tissue stiffness induced by excessive deposition of extracellular matrix in tubulointerstitial areas and elevated intraluminal pressure from probably fluid accumulation in tubules, leading to activation of Piezo1 and influx of calcium, by this way, mechanical signals are transduced into intracellular chemical signals. Intracellular calcium activates Calpain2 that cleaved Talin1 into active form, which subsequently caused activation and clustering of integrinβ1 on the basolateral membrane, promoting the deposition of ECM (inside-out signal). Activation of Piezo1 also induces expression and synthesis of TGF-β1, further promoting ECM deposition. On the other hand, excessive deposition of ECM promotes tissue stiffening, which in turn increases the mechanosensory and mechanotransduction capacity of renal epithelial cells by activated Piezo1, aggravating the progression of renal fibrosis (outside-in signal).