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Inhibition of TRPC6 channels ameliorates renal fibrosis and contributes to renal protection by soluble klotho

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Abstract

Fibrosis is an exaggerated form of tissue repair that occurs with serious damage or repetitive injury and ultimately leads to organ failure due to the excessive scarring. Increased calcium ion entry through the TRPC6 channel has been associated with the pathogenesis of heart and glomerular diseases, but its role in renal interstitial fibrosis is unknown. We studied this by deletion of *Trpc6* in mice and found it decrease unilateral ureteral obstruction-induced interstitial fibrosis, and blunted increased mRNA expression of fibrosis-related genes in the ureteral obstructed kidney relative to that in the kidney of wild-type mice. Administration of BTP2, a pyrazol derivative

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DISCLOSURE

These experiments were performed by Yueh-Lin Wu in partial fulfillment of the requirements of the Ph.D. degree at Taipei Medical University, Taipei, Taiwan. N.O. and N.X.B. are research scientists from Boehringer Ingelheim Pharmaceuticals, Inc. C.-L. H. received a sponsor research agreement fund from Boehringer Ingelheim Pharmaceuticals to study the role of klotho in a related project separate from the current work under consideration.

AUTHORS CONTRIBUTIONS

Y.-L.W. conducted animal and gene expression experiments with the assistance of J.X. S.A. conducted electrophysiological recordings. N.O. and N.X.B. conducted ISH experiments. M.-H.L. synthesized BTP2. L.B. provided Trpc3 and Trpc6 gene deleted mice. Y.-L.W. wrote the initial draft of manuscript. All authors contributed to discussions, and writing and editing of the paper. C.-L.H. supervised the entire project and wrote the final paper.

known to inhibit function of several TRPC channels, also ameliorated obstruction-induced renal fibrosis and gene expression in wild type mice. BTP2 inhibited carbachol-activated TRPC3 and TRPC6 channel activities in HEK293 cells. Ureteral obstruction caused over a 10-fold increase in mRNA expression for TRPC3 as well as TRPC6 in the kidneys of obstructed- relative to the sham-operated mice. The magnitude of protection against obstruction-induced fibrosis in *Trpc3* and *Trpc6* double knockout mice was not different from that in *Trpc6*-knockout mice. Klotho, a membrane and soluble protein predominantly produced in the kidney, is known to confer protection against renal fibrosis. Administration of soluble klotho significantly reduced obstruction-induced renal fibrosis in wild type mice, but not in *Trpc6*-knockout mice, indicating that klotho and TRPC6 inhibition act in the same pathway to protect against obstruction-induced renal fibrosis. Thus klotho and TRPC6 may be pharmacologic targets for treating renal fibrosis.

Keywords

Klotho; TRPC6; TRPC3; UUO; Fibrosis; BTP2

INTRODUCTION

Chronic kidney disease (CKD) is an increasingly growing global health burden. In United States and worldwide, CKD affects approximately 10% of the general population.[1] Fibrosis is a hallmark of progressive renal disease, regardless of the underlying cause.[2] After injury and nephron loss, the remaining nephrons hypertrophy to meet the increased demand. In severe cases, the process becomes maladaptive, causing injury to remaining nephrons and further nephron loss, thus leading to a vicious cycle that eventually results in renal failure. Scar formation is an important part of tissue repair, yet excess leads to fibrosis and organ failure. Clinically, CKD progression correlates best with renal interstitial fibrosis rather than with glomerular pathology.[3] Therefore, a better understanding of the pathways that cause renal fibrosis and promote disease progression is important for developing effective therapeutic options.

Cells have adopted calcium ion (Ca^{2+}) as an important signaling molecule.[4] Ca^{2+} signaling impacts many aspects of cell functions, for example, cell proliferation and differentiation.[5] The canonical transient receptor potential (TRPC) family channels are nonselective, Ca^{2+}-permeable cation channels expressed in the plasma membrane of many tissues.[6] Within this channel family, TRPC6 has been increasingly recognized to be involved in the pathogenesis of many diseases, such as familial focal segmental glomerulosclerosis.[7]

Proliferation of fibroblasts and its activation into myofibroblasts is a key step in renal tissue repair and fibrosis.[2,8] Myofibroblasts are fibroblast-like mesenchymal cells with contractile capability. They often express α-smooth muscle actin (αSMA),[8] synthesize extracellular matrix (ECM) macromolecules and remodel the ECM.[9] In the kidney, following injury or in response to cytokines and other factors, myofibroblasts can be generated by different activation, differentiation and transdifferentiation processes.[8] In skin and cardiac healing models, TRPC6 is necessary and sufficient for myofibroblast differentiation induced by angiotensin II (Ang II) or transforming growth factor-β (TGF-β).[10] The self-sustaining

abnormal Ca^{2+} entry and gene expression is responsible for disease pathogenesis of many gain-of-function TRPC6 channelopathies. TRPC3 and -6 genes contain nuclear-factor-of-activated-T cells (NFAT) promoter response elements. Increased Ca^{2+} entry via TRPC3/6 may further stimulate expression of TRPC3/6 channels by activating calcineurin/NFAT signaling, causing positive feed-forward amplification and unremitting gene expression.[11] A recent study implicates TRPC3 in the pathogenesis of renal fibrosis.[12] The role of TRPC6 in renal fibrosis is yet unknown.

Klotho is a single-pass type-1 transmembrane protein predominantly produced in the kidney that exerts some antiaging function.[13] Membranous klotho interacts with fibroblast growth factor receptor (FGFR) and functions as a coreceptor for the ligand fibroblast growth factor 23 (FGF23), a bone-derived endocrine factor that negatively regulates body phosphate homeostasis.[14,15] Soluble klotho, cleaved from the ectodomain of membrane klotho, is a protein with pleiotropic functions including regulation of ion transport and growth factor signaling by acting as a paracrine or endocrine factor.[16] Prior studies have shown that loss of klotho aggravates renal fibrosis while overexpression of klotho inhibits TGF-β1 signaling and suppresses renal fibrosis by antagonism of TGF-β1 and Wnt/β-catenin signaling.[17–19] Our recent data demonstrate heterozygous *klotho*-hypomorphic (*kl*/+) CKD mice have aggravated cardiac fibrosis relative to wild-type (WT) CKD mice, and that klotho protects against uremic cardiac pathology, at least partly, by downregulation of TRPC6-mediated abnormal Ca^{2+} signaling in the heart.[20,21]

In the present study, we test the hypothesis that TRPC6 is critical for myofibroblast generation in kidney fibrosis. Given multiple potential pathways for soluble klotho to protect organs, we further investigated the relationship of TRPC6 with klotho's protective action on renal fibrosis. We report that inhibition of TRPC6 achieved by *Trpc6* gene deletion or pharmacological means protects against renal fibrosis and that TRPC6 inhibition acts in the same pathway of protection by soluble klotho. We discuss the implications of our results in light of current literature.

RESULTS

UUO induces renal fibrosis, and *Trpc6* knockout in mice partially ameliorates UUO-induced fibrosis

Unilateral ureteral obstruction (UUO) is a common experimental model for the study of mechanisms of renal fibrosis. It has the advantage to induce accelerated renal fibrosis while recapitulating essential cellular processes in the progression of interstitial fibrosis, including cellular infiltration, myofibroblast generation and accumulation, increased ECM deposition, and tubular atrophy.[22] To elucidate the role of TRPC6 in the renal fibrosis, we asked if *Trpc6* knockout would alter fibrosis and expression of the genes involved in fibrogenic processes using a UUO experimental model. *Trpc6*$^{-/-}$ mice were previously described.[21] After 10 days, expression of multiple fibrosis-related gene markers was markedly increased in the obstructed kidney relative to contralateral sham kidney in WT and *Trpc6*$^{-/-}$ mice (Figure 1a–h). The increase in expression of mRNAs for collagen-1, connective tissue growth factor (CTGF), αSMA, matrix metalloproteases MMP-2, vimentin, TGF-β1, and MMP-9 in UUO kidneys of *Trpc6*-knockout mice was blunted relative to WT mice. Expression of snail-1, a

marker of mesenchymal-to-epithelial transitions (EMT) in kidney fibrosis,[23] was not different in UUO kidneys between *Trpc6*$^{-/-}$ and WT mice (Figure 1h). Basal level expression of these genes in sham kidneys was not different between *Trpc6*$^{-/-}$ and WT mice. Histological evidence of fibrosis was evaluated by staining collagen using Masson's trichrome staining. The increases in Masson's trichrome-positive areas in WT UUO versus sham kidneys indicate marked interstitial fibrosis induced by UUO (Figure 1i and j; see Supplementary Figure S1 for low-magnification view). Consistent with the finding of UUO-increased *Trpc6* gene expression, *Trpc6* deletion decreased fibrosis as shown by decreased trichrome-positive areas. Basal Masson's trichrome staining in sham kidneys of *Trpc6*$^{-/-}$ mice was comparable to staining in WT animals.

Pharmacological Inhibition of TRPC ameliorates renal fibrosis

BTP2 (4-methyl-4-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]-1,2,3-thiadiazole-5-carbox anilide) is a TRPC channel blocker without subtype selectivity among members of the TRPC family.[24] It was shown that administration of BTP2 attenuated cardiac hypertrophy presumably by inhibiting cardiac TRPC3 and TRPC6 channels.[24] We first tested the effects of BTP2 on recombinant TRPC3 6 and 7 channel activities in HEK293 cells. As shown, BTP2 inhibited the activity of TRPC3 and TRPC6 with similar efficacy (Figure 2a and b) at concentrations in agreement with a previous report.[24] BTP2 also inhibited TRPC7 activity at similar concentrations (Figure 2c). At 10-fold higher concentration (100 μM), BTP2 had no effect on an inward-rectifying K^+ channel ROMK or TRPV5, a member of the V (vanilloid)-type TRP channel group (Figure 2d and e), demonstrating specificity of BTP2 for TRPC channels.

Having demonstrated the specificity and efficacy of BTP2 on diacylglycerol (DAG)-sensitive TRPC3/6/7 channels, we next examined the effect of BTP2 on the development of renal fibrosis in UUO model. Daily intraperitoneal injection of BTP2 at 2mg/kg for 7 days significantly ameliorated the expression of collagen-1, CTGF, αSMA, MMP-2, vimentin, and TGF-β1, in the obstructed kidney (Figure 3a–f). BTP2 treatment also significantly reduced collagen deposition as shown by Masson's trichrome staining (Figure 3g and h). Similar to *Trpc6* gene deletion, inhibition of TRPC channels by BTP2 had little effect on sham kidneys (Figure 3g and h).

UUO induces upregulation of TRPC3 and TRPC6 expression

TRPC3 and TRPC6 form heteromultimers in some cell types.[25,26] Both TRPC channel genes contain NFAT-responsive elements in their promoters that may lead to eedforward amplification of abnormal Ca^{2+} signaling. Therefore, we examined the expression of TRPC3 and TRPC6 in UUO kidneys. As shown in Figure 4, UUO for 10 days induced a more than 20 -fold increase in mRNA expression for TRPC6 in UUO kidney compared to the contralateral sham kidney in WT mice. UUO also caused an increase of TRPC3 mRNA expression (Figure 4). Thus, both TRPC3 and TRPC6 are upregulated in UUO-induced renal fibrosis.

Trpc3/6 double knockout (DKO) mice are protected against UUO renal fibrosis, but the effect is not additive compared to single *Trpc6*- or *Trpc3*-knockout mice

Next, we asked whether *Trpc3* and *Trpc6* double deletion in mice confers more protection against renal fibrosis than deletion of *Trpc6* or *Trpc3* alone. To test this, we used *Trpc6* single knockout, *Trpc3* single knockout, *Trpc3* and *Trpc6* double knockout (*Trpc3*$^{-/-}$; *Trpc6*$^{-/-}$), and WT mice in 10-day UUO model. Histopathology revealed that renal fibrosis was attenuated in UUO kidney of *Trpc3/6*-DKO mice relative to WT mice, as evidenced by significantly less collagen deposition by Masson's trichrome stain (Figure 5a and b). UUO-induced fibrosis was similarly attenuated by *Trpc6*-KO. Quantitative analysis of fibrosis scores revealed that the protection against fibrosis by *Trpc6*-KO and DKO were not significantly different (Figure 5b, inset). Like the *Trpc6*-KO, compared to UUO kidney of WT mice, *Trpc3*-KO and DKO mice have significantly reduced profibrotic gene expression in UUO kidney for collagen-1, CTGF, αSMA, MMP-2, vimentin, and TGF-β1 (Figure 5c–h). Compared to *Trpc6*-KO mice, the reduction in mRNA expression in *Trpc3*-KO and in DKO mice was not significantly different.

Klotho administration attenuates renal fibrosis after UUO

Previously, we found that soluble Klotho present in the systemic circulation inhibits TRPC6 currents in cardiomyocytes by blocking phosphoinositide-3-kinase (PI3K)-dependent exocytosis of TRPC6 channels.[21] Given that inhibition of TRPC6 ameliorates renal fibrosis (Figure 1 and 2), we asked whether soluble klotho may protect against renal fibrosis via inhibition of TRPC6. To test this hypothesis, we first examined whether soluble klotho ameliorates UUO-induced fibrosis in our experimental setting. Recombinant soluble klotho consisting of the entire human klotho ectodomain (10μg/kg) was administered by intraperitoneal injection on day 0 after surgery and then every other day until Day 7 after UUO in WT mice. Soluble klotho treatment attenuated UUO-induced upregulation of collagen-1, CTGF, αSMA, MMP-2, and vimentin in UUO kidney (Figure 6a–e). Notably, klotho did not significantly reduce UUO-induced TGF-β1 mRNA expression (Figure 6f) while inhibition of TRPC6 did (Figure 1f and 3f). Consistent with the gene expression findings, histological analysis of renal sections stained with Masson's trichrome revealed that klotho treatment significantly decreased fibrotic area (Figure 6g and h).

Administration of soluble klotho has no additional protection against renal fibrosis in *Trpc6*$^{-/-}$ mice

Next, we investigated the relationship between TRPC6 and klotho pathways in protecting against renal fibrosis. To address this issue, we compared the effect of soluble klotho administered via intraperitoneal injection on WT and *Trpc6*$^{-/-}$ mice. We reasoned that, because complete deletion of *Trpc6* only confers partial amelioration of fibrosis, effect of soluble klotho treatment should be additive to *Trpc6* deletion if soluble klotho works independently of TRPC6. In vehicle treatment groups, *Trpc6* deletion attenuated UUO-induced upregulation (shown as fold increase in UUO kidney over sham kidney) of collagen-1, CTGF, αSMA, MMP-2, vimentin, and TGF-β1 mRNA expression relative to WT (Figure 7a–f). While klotho treatment also attenuated upregulation of collagen-1, CTGF, αSMA, MMP-2, and vimentin mRNA expression compared to vehicle-treated WT mice, it

did not further decrease gene expression in *Trpc6*$^{-/-}$ mice (Figure 7a–e). Consistent with results shown in Figure 6f, klotho treatment did not reduce UUO-induced TGF-β1 mRNA expression in WT mice (Figure 7f). Furthermore, klotho treatment did not decrease TGF-β1 mRNA expression in *Trpc6*$^{-/-}$ mice beyond the level of protection conferred by *Trpc6* deletion. Quantitative analysis of fibrosis scores revealed that the protection against fibrosis by *Trpc6*-KO and by klotho were not additive (Figure 7g). Thus, *Trpc6*-KO and klotho likely protect against UUO-induced fibrosis by affecting the same pathway. Supporting the notion that klotho's action is upstream of that for *Trpc6* deletion, we found that klotho administration reduced UUO-induced upregulation of *Trpc6* mRNA expression (Supplementary Figure S2A). For comparison, UUO did not increase the expression of TRPV5 Ca^{2+} channel normally expressed in the distal renal tubules, and klotho had no effect on TRPV5 expression (Supplementary Figure S2B).

Expression of TRPC6 in interstitial fibroblasts in UUO-induced fibrosis and the effects of BTP2 and Klotho

TRPC6 channels are widely expressed and in many different cell types.[6] In the kidney, TRPC6 expression is known in glomerular podocytes, mesangial cells, renal tubular epithelial cells, vascular endothelial and smooth muscle cells as well as interstitial fibroblasts. Since TRPC6 gain-of-function in podocytes is thought to be causal in FSGS,[7] increased function of TRPC6 channels in various cell types could directly or indirectly contribute to renal injury leading to fibrosis. Because it is recognized that Ca^{2+} entry through TRPC6 plays a necessary and sufficient role for myofibroblast generation in response to stimulation by injury factors such as Ang II and TGF-β,[10] we focused on interstitial cell expression of TRPC6 in the current study. Because TRPC6 expression in normal kidney tissue is fairly low, we validated our *in situ* hybridization (ISH) probe for *Trpc6* in UUO kidneys and found that the *Trpc6*-specific probe, but not a negative control probe, readily detected *Trpc6* mRNA in interstitial cells (Supplementary Figure S3A,B, S4). The *Trpc6* mRNA signals were detectable but quite low in sham kidneys, but strongly present in UUO kidneys (Supplementary Figure S3C,D). Higher magnification for better evaluation of cell shape and localization allowed identification of many positive interstitial cells as fibroblasts, and also showed that *Trpc6* mRNA positive cells include podocytes and vascular smooth muscle cells (Supplementary Figure S5). Next, we examined the effect of BTP2 and klotho on *Trpc6* mRNA expression in UUO kidneys. Compared to vehicle treatment, BTP2 treatment markedly reduced *Trpc6* mRNA expression in UUO kidneys (Figure 8). Similarly, klotho treatment attenuated *Trpc6* mRNA expression in UUO kidneys (Figure 9).

DISCUSSION

Fibrosis is a maladaptive tissue repair process where the excessive scarring disrupts tissue architecture and nutrient supply, thereby contributing to a relentless decline of organ function.[2] Because interstitial fibrosis is one of the best indicators of disease progression, studies to improve our knowledge of the underlying modulators and pathways seek to identify more effective therapeutic targets. Intracellular Ca^{2+} plays an essential role in regulating many vital cell functions such as signaling transduction, contraction, relaxation,

proliferation, and differentiation.[4,5] Abnormal increases in intracellular Ca^{2+} levels contribute to pathogenesis in many diseases, including cardiac hypertrophy,[26] arrhythmogenesis,[27] hypertension,[28] and familial focal segmental glomerulosclerosis (FSGS).[7] Increased Ca^{2+} flux through TRPC channels is frequently associated with disease pathogenesis. This is in part due to the fact that TRPC channels particularly TRPC3 and TRPC6 contain NFAT-responsive elements in their promoters and thus participate in a feedforward vicious cycle of sustained elevation of intracellular Ca^{2+} and unremitting gene activation. Here, we provide compelling evidence that TRPC6 channels are involved in the pathogenesis of kidney fibrosis. We show that *Trpc6* gene expression is upregulated in the injured kidney of UUO mice. Genetic deletion of *Trpc6* or pharmacological inhibition of TRPC6 channel function attenuates UUO-induced kidney fibrosis.

The TRPC family includes seven members, TRPC1 through 7, and is broadly divided into two groups based on structural and functional similarities: TRPC1/4/5, which are not sensitive to DAG, and DAG-sensitive TRPC3/6/7.[6] TRPC2 is a pseudogene in human. TRPC3, 6 and 7 share a high degree of amino acid identity (70–80%).[6] TRPC3 and TRPC6 channels are not only highly homologous in structure, but also share functional, regulatory and pharmacological similarities, as well as forming heteromultimers in some cell types.[25,26] A recent study using *Trpc3* knockout mice and pharmacological blockage of TRPC3 in rats demonstrates an important role of TRPC3 in the pathogenesis of tubulointerstitial damage and renal fibrosis.[12] In the present study, we show that both TRPC3 and TRPC6 expression are markedly increased in UUO kidney. Yet, double *Trpc3* and *Trpc6* knockout confers the same degree of protection against kidney fibrosis as single *Trpc6* knockout. These results suggest that TRPC3 and TRPC6 channels work in the same pathway, possibly in the context of formation of TRPC3/TRPC6 heteromultimers,[25,26] to contribute to the pathogenesis of fibrosis.

Recent studies have demonstrated that klotho is protective for many organs including the kidney. Klotho expression is markedly downregulated in CKD mouse models as well as in humans, providing an association between loss of klotho and CKD.[29] It has also been shown that klotho expression is reduced in the diseased kidney of UUO mice and that heterozygous klotho-deficient mice exhibit worse fibrosis relative to WT mice as evidenced by higher levels of fibrosis gene markers and more collagen deposition in trichrome staining.[18] In addition, UUO-induced interstitial fibrosis and tubular atrophy were significantly alleviated in klotho-overexpressing transgenic mice.[30] Multiple pathways have been proposed to mediate klotho protection of the kidney including antagonism of TGF-β1, antagonism of Wnt/β-catenin, and inhibition of renin-angiotensin- aldosterone system.[19,31,32] Supporting findings of previous studies,[19,33] we found that administration of recombinant soluble klotho protein suppresses UUO-induced renal fibrosis. Knowing that TRPC6 activation is involved in the pathogenesis of UUO-induced fibrosis and our prior finding that soluble klotho is cardioprotective by TRPC6 inhibition, we asked whether soluble klotho might protect the kidney via a TRPC6 pathway. We found that while soluble klotho ameliorates UUO-induced fibrosis in WT mice it confers no additional protection in *Trpc6*-knockout. These results support the conclusion that in our experimental setting, soluble klotho acts to inhibit the same pathway of TRPC6 function.

How do our findings and conclusion fit with the current literature? As a DAG-sensitive channel, TRPC6 activity is stimulated by phospholipase C (PLC)-activating hormones. Davis et al., recently demonstrated that activation of TRPC6 is critical for cardiac and dermal fibrosis and it is acting downstream of Ang-II as well as noncanonical TGFβ signaling cascade.[10] Further, Wnt signaling pathway modulates TRPC6 expression.[34] Conceivably, the protective effects of soluble klotho through Ang-II, TGFβ, Wnt/β-catenin may converge on or somehow interact with the TRPC6 pathway. In this scenario, inhibition of TRPC6 could underpin renal-protection by soluble klotho through Ang-II, TGFβ and Wnt/β-catenin as well. Certainly, multiple mechanisms may contribute to effect of anti-fibrosis by soluble klotho. Interestingly, we found that administration of soluble klotho does not affect the expression of TGFβ1 while yet decreasing the expression of collagen-1, CTGF, αSMA, MMP's, and vimentin. Consistent with our findings, Doi et al also reports that soluble Klotho does not reduce TGF-β1 expression, but directly binds the type-II TGF-β receptor to exert inhibition of TGF-β1 signaling.[19] In contrast, others have shown that klotho suppresses TGF-β1 expression and klotho deficiency leads to increased TGF-β1 expression.[18,3] Differences in the genetic background of mice and context of experimental settings and/or experimental models may explain the apparent discrepancy. Of note, our results show that *Trpc6* expression in interstitial fibroblasts is upregulated in UUO kidneys, and that BTP2 and klotho each markedly reduces expression in these cells, suggesting that they are targets of the protective action. Besides interstitial fibroblasts, TRPC6 is expressed in multiple cell types in the kidney. Klotho's action on TRPC6 in other cell types may also contribute to the protection against UUO-induced renal fibrosis. Future studies will investigate these questions.

In summary, we show that activation of TRPC6 contribute to the pathogenesis of UUO-induced renal fibrosis. Inhibition of TRPC6 function either by genetic deletion or pharmacological blockade ameliorates the fibrosis. Inhibition of TRPC6 may be a convergent point for renal protection by soluble klotho through multiple pathways.

METHODS

Animals

Animal protocol was approved by the UTSW Institutional Animal Care and Use Committee. *Trpc6*$^{-/-}$ and *Trpc3*$^{-/-}$ mice have been described.[21,35] Each mouse line was backcrossed to 129/SvJ mice for > eight generations to achieve congenic background. Wild-type littermates from backcrossing were used as controls. Age and weight matched male mice at 8–10 weeks age were used in experiments unless otherwise specified.

UUO model

Under general anesthesia, the left ureter was exposed through a midline abdominal incision and double-ligated with 4–0 silk. Sham operation was performed on the contralateral kidney. WT, *Trpc6*$^{-/-}$ and *Trpc3/6*-DKO mice were subjected to UUO for 10 days. After surgery, kidneys were harvested for histological analysis of fibrosis by trichrome staining and for measurement of mRNA expression by quantitative real-time PCR. In separate experiments, WT mice underwent UUO and received of BTP2 injection (2 mg/kg daily by i.p.) or vehicle

(polypropylene glycol) (Sigma-Aldrich, St Louis, MO) for 7 days. To test the efficacy of klotho in renal fibrosis after UUO injury, recombinant human klotho consisting of the entire klotho ectodomain (R&D Systems Inc. Minneapolis, MN; at 10 ug/kg in 0.1 ml of 10 mM phosphate buffered saline) was administered via intraperitoneal injection to mice immediately after ureteral ligation and then administered every other day until 7 days after surgery. PBS-treated animals were used as controls.

Renal tissue preparation and histopathology

Left kidneys were excised, decapsulated, and cut in half through a midsagittal plane. One-half was fixed with 4% paraformaldehyde, the other one-half stored in RNAlater® RNA Stabilization Solution (Life Technologies, Carlsbad, CA) for further RNA extractions. The paraformaldehyde-fixed tissue was embedded in paraffin, sectioned (in 4-mm thickness), and stained with Masson's trichrome. A blinded investigator randomly selected five fields on the coronal section in a high-power field (original magnification 200X). The researcher analyzed the fibrotic area with the ImageJ program by thresholding the collected images, then creating selections of the fibrotic areas (marked blue by Masson-trichrome) as previously described.[18,36] The severity of tubulointerstitial fibrosis was indicated as the ratio of the fibrotic area to the total area.

Real-time quantitative RT–PCR analysis of mRNA

Total RNA was extracted from kidney samples with Trizol (Invitrogen, Waltham, MA), and reverse-transcribed into cDNA using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). mRNA abundance was analyzed by CFX Connect™ Real-Time PCR Detection System and iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories). Nucleotide sequence for PCR primers are shown in the Supplementary Table S1. The expression was normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) in the same sample.

Electrophysiological recording

Transfection of HEK293 cells and ruptured whole-cell patch-clamp recording of TRPC3/6/7 channels,[21] ROMK channels,[37] and TRPV5 channels[38] were performed as previously described by us.

In situ hybridization

The paraformaldehyde-fixed, paraffin embedded kidney tissues were sectioned in 4-mm thickness. Sections were attached on SuperFrost Plus Slides (Thermo Fisher Scientific, Waltham, MA) and stored at −80°C until used for ISH. Pretreatment an d hybridization conditions were as described (RNAscope Sample Preparation Pretreatment Guide for FFPE Tissue; Advanced Cell Diagnostics, Inc.) using manual and automated RNAscope Probes for Trpc6 and a standard negative control, Dapb (a bacterial gene) as previously decribed.[21] Detection was by RNAscope 2.0 HD Detection Kit BROWN (catalog no. 320497) and hematoxylin counterstain provided structural landmarks. Automated scan of ISH slides used an Aperio ScanScope to capture digital images at magnification ×40.

Statistical analyses

Statistical comparison was made between control and experimental groups conducted during the same time period. Each experiment was performed at least twice at separate times with similar results. Data are presented as mean ± SEM. Statistical comparisons between two groups of data were made using the two-tailed unpaired Student's t-test. Multiple comparisons were determined using one-way ANOVA followed by Tukey's multiple comparison tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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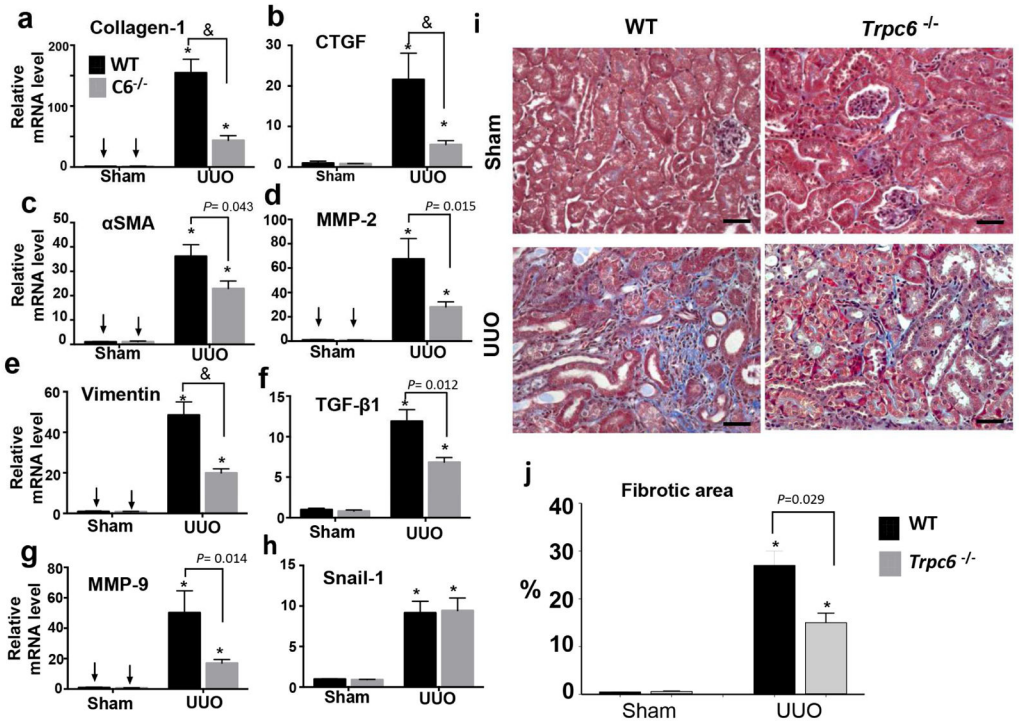

Figure 1. ***Trpc6*** **deletion ameliorates UUO-induced renal fibrosis**

(**a–h**) mRNA levels of collagen-1, CTGF, αSMA, MMP-2, vimentin, TGF-β1, Snail-1, and MMP-9 on day 10 after UUO determined by quantitative RT-PCR. Expression levels of mRNA were normalized to GAPDH and shown as fold change relative to WT sham kidney (WT sham was given value 1). Arrows indicate bar height not visible, where mean ± SEM values for relative mRNA levels for sham WT and sham Trpc6$^{-/-}$ were 1.00 ± 0.22 and 1.07 ± 0.25 for collagen-1, 1.00 ± 0.18 and 1.09 ± 0.23 for αSMA, 1.00 ± 0.13 and 1.36 ± 0.25 for MMP-2, 1.00 ± 0.21 and 0.94 ± 0.09 for vimentin, and 1.00 ± 0.29 and 0.80 ± 0.07 for MMP-9, respectively. (**i**) Representative Masson's trichrome staining of kidney sections at day 10. Scale bars, 10 μm. (**j**) Semiquantitative scores of interstitial fibrosis. All values are means ± SEM, $n = 6$ each. $^{\&}P < 0.01$ between indicated groups. $^{*}P < 0.01$ UUO vs Sham for respective WT or *Trpc6*$^{-/-}$ mice.

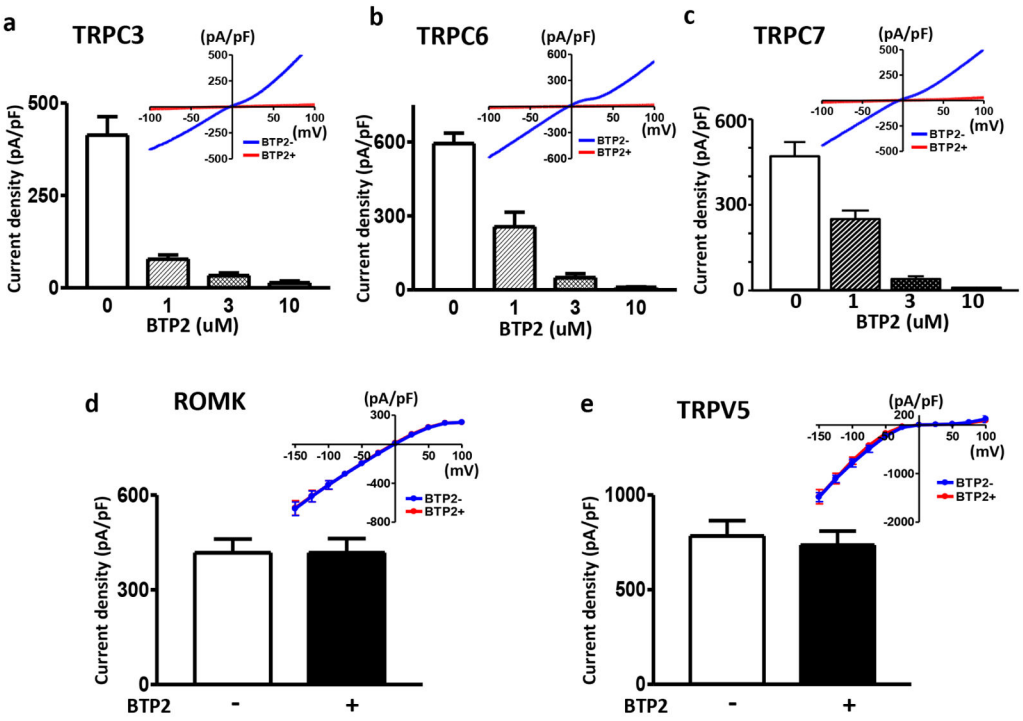

Figure 2. Selective inhibition of TRPC channels by BTP2

HEK293 cells were transiently transfected with plasmids encoding TRPC3 (**a**), TRPC6 (**b**), TRPC7 (**c**), ROMK (**d**), and TRPV5 (**e**) and activity of respective channels were recorded by ruptured whole-cell patch-clamping (as described in METHODS). Concentration of BTP2 used is 100 μM for ROMK and TRPV5. Data are means ± SEM (n = 6–10 each) of inward current density at −100 mV. In each condition, current density of mock-transfected cells is < 20 pA/pF. Current-voltage (I–V) relationship curves are characteristic for each type of channels studied.

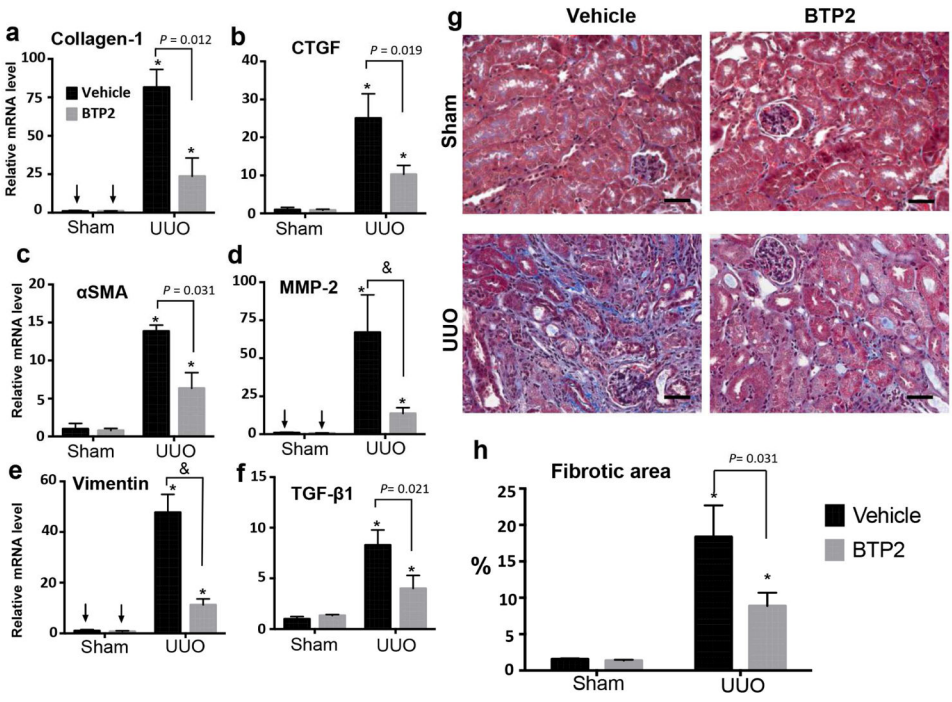

Figure 3. Inhibition of TRPC channels by BTP2 ameliorates UUO-induced renal fibrosis in WT mice

(**a**–**f**) mRNA levels of collagen-1, CTGF, αSMA, MMP-2, vimentin, and TGF-β1 on day 7 after UUO determined by quantitative RT-PCR. Expression levels of mRNA were normalized to GAPDH and shown as fold changes relative to sham kidney of vehicle treatment group (WT sham + vehicle was given value 1). Arrows indicate bar height not visible, where mean ± SEM values for relative mRNA levels for sham vehicle and BTP2-treated groups were 1.00 ± 0.36 and 1.01 ± 0.17 for collagen-1, 1.0 ± 0.26 and 0.63 ± 0.19 for MMP-2, and 1.00 ± 0.30 and 0.80 ± 0.15 for vimentin. (**g**) Representative Masson's trichrome staining of kidney sections at day 7. Scale bars, 10 μm. (**h**) Semiquantitative scores of interstitial fibrosis. All values are means ± SEM, $n = 6$ each. $^{\&}P < 0.01$ between indicated groups. $^{*}P < 0.01$ UUO vs Sham for respective vehicle or BTP2 treatment mice.

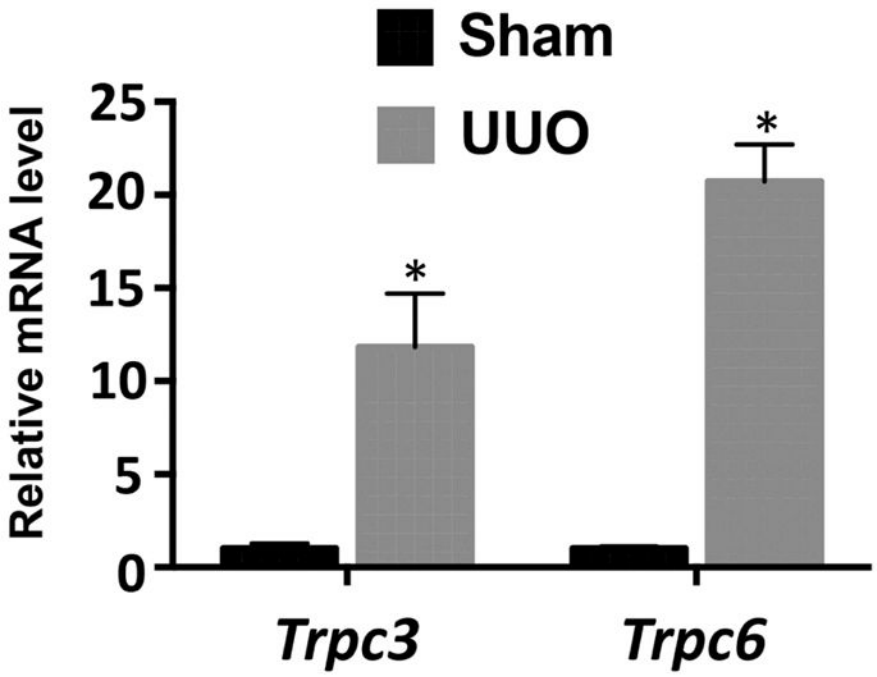

Figure 4. Upregulation of *Trpc3* and *Trpc6* expression in UUO kidney of WT mice

mRNA levels of TRPC3 and TRPC6 on day 10 after UUO determined by quantitative RT-PCR. Expression levels of mRNA were normalized to GAPDH level and shown as fold change relative to sham kidney (value = 1). All values are means ± SEM, n = 6 each. $^{*}P <$ 0.01 UUO vs Sham for respective TRPC3 and TRPC6 group.

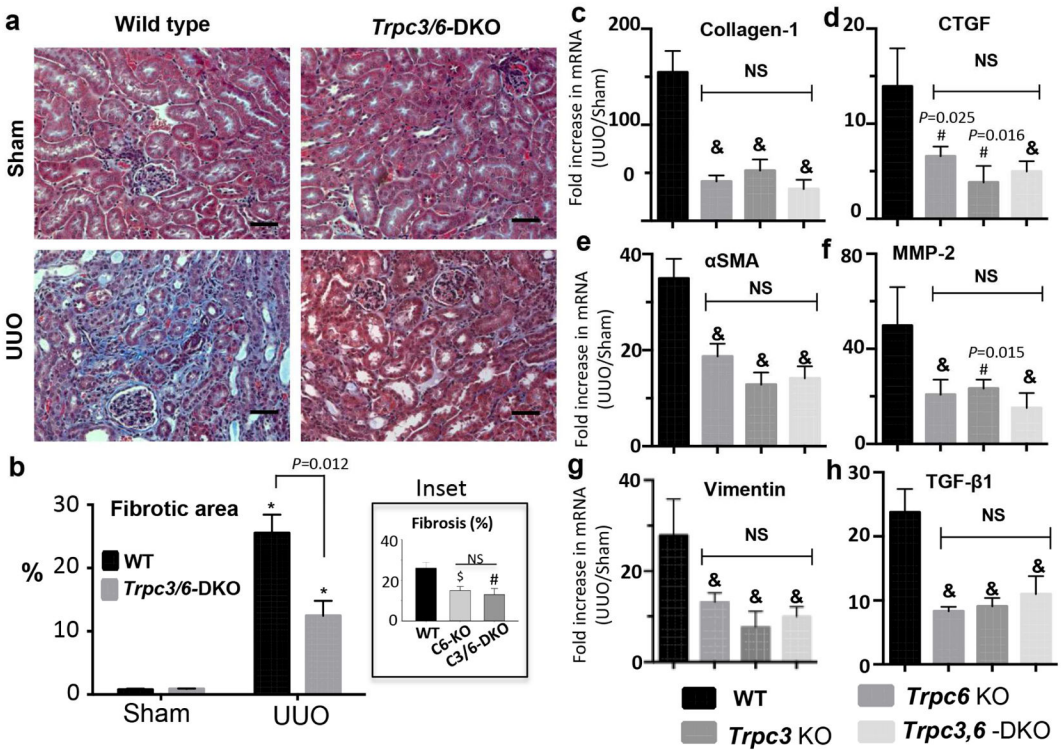

Figure 5. Effects of single and double deletion of *Trpc3* and *Trpc6* on UUO-induced renal fibrosis

(**a**) Representative Masson's trichrome staining of kidney cortex sections at day 10. Scale bars, 10 μm. (**b**) Semiquantitative scores of interstitial fibrosis (n = 5 each). All values are means ± SEM. $^{*}P < 0.01$ UUO vs Sham for respective WT or *Trpc3/6*-DKO mice. Inset shows UUO-induced fibrosis between WT, *Trpc6*-KO and *Trpc3/6*-DKO. Data for *Trpc6*-KO are from Figure 1c. $^{\$}P = 0.029$; $^{\#}P = 0.012$ versus WT mice. (**c**–**h**) mRNA levels determined by quantitative RT-PCR for collagen-1, CTGF, αSMA, MMP-2, vimentin, and TGF-β1 on day 10 after UUO. Expression levels of mRNA were normalized to GAPDH level and shown as fold change relative to respective sham kidney. n = 5–6 for each group. All values are means ± SEM. NS, not significant between indicated groups. $^{\#}P < 0.05$ (exact values given); $^{\&}P < 0.01$ vs WT mice.

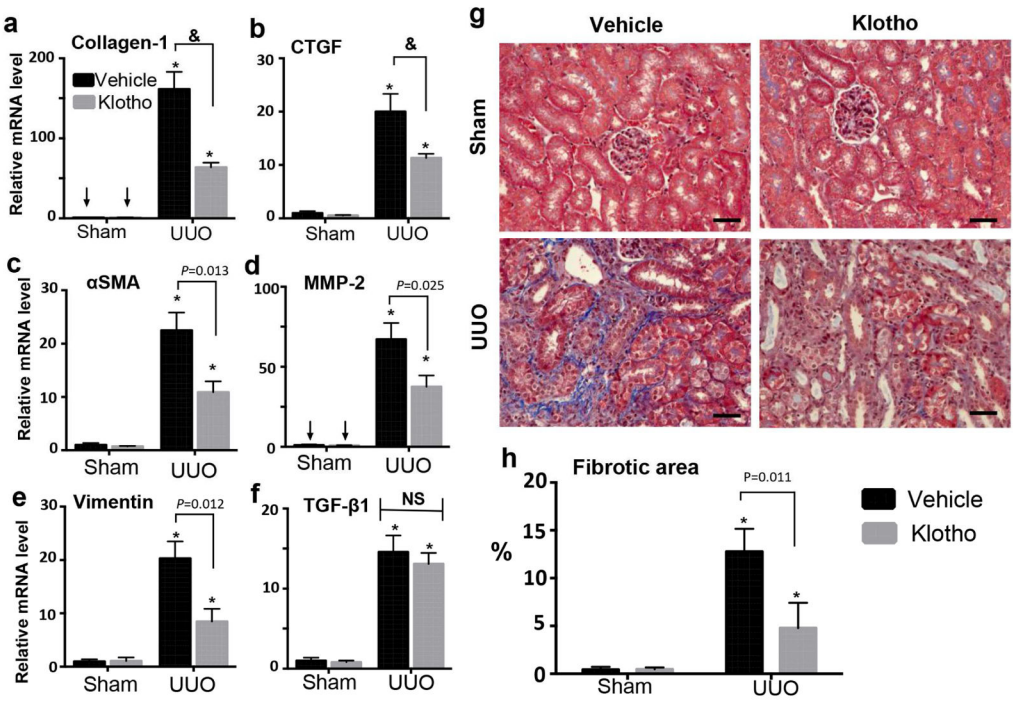

Figure 6. Recombinant soluble klotho suppresses renal fibrosis in WT UUO kidney

(**a**–**f**) mRNA levels of collagen-1, CTGF, αSMA, MMP-2, vimentin, and TGF-β1 on day 7 after UUO determined by quantitative RT-PCR. Expression levels of mRNA were normalized to GAPDH level and shown as fold changes relative to WT sham kidney of vehicle-treated group (value = 1). Arrows indicate bar height not visible, where mean ± SEM values for relative mRNA levels for sham vehicle and sham klotho-treated groups were 1.00 ± 0.20 and 0.99 ± 0.26 for collagen-1, and 1.00 ± 0.32 and 0.85 ± 0.17 for MMP-2. (**g**) Representative Masson's trichrome staining of kidney sections at day 7. Scale bars, 10 μm. (**h**) Semiquantitative scores of interstitial fibrosis. All values are means ± SEM, n = 6 each. $^{\&}P < 0.01$; NS, not significant between indicated groups. $^{*}P < 0.01$ UUO vs Sham for respective vehicle or klotho-treated mice.

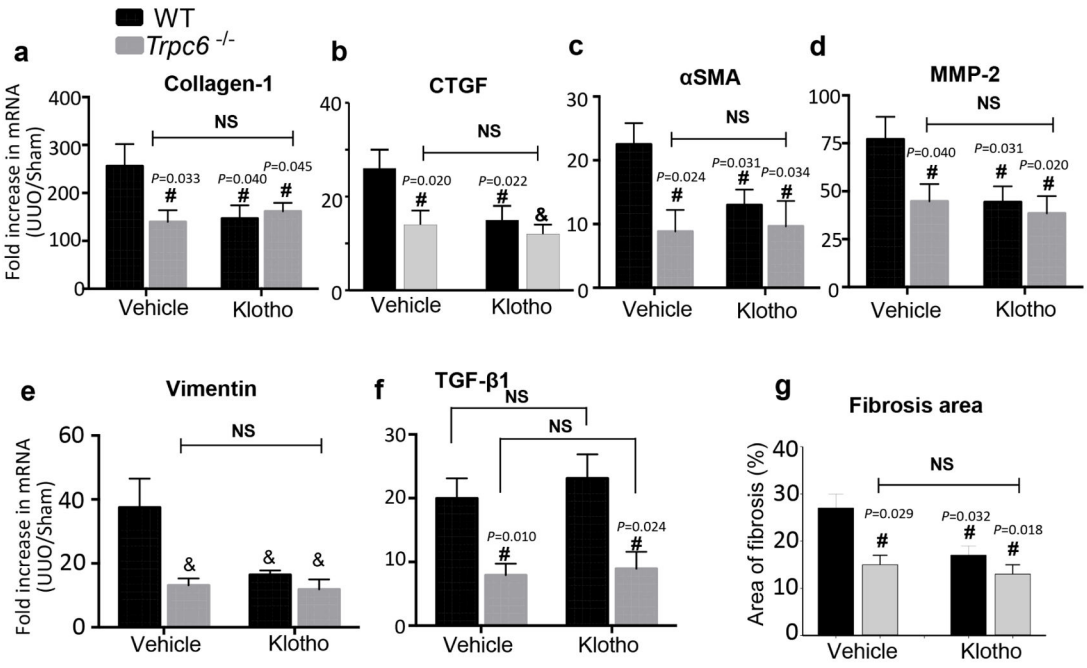

Figure 7. Effect of soluble klotho on UUO-induced fibrosis in WT and *Trpc6*$^{-/-}$ mice

(**a**–**f**) mRNA levels of collagen-1, CTGF, αSMA, MMP-2, vimentin, and TGF-β1 in WT and *Trpc6*$^{-/-}$ mice after 7 days UUO with or without klotho treatment. Expression levels of mRNA were normalized to GAPDH level and shown as fold changes relative to respective WT or *Trpc6*$^{-/-}$ sham kidney. n = 6 for each group. All values are means ± SEM. NS, not significant between indicated groups. $^{\&}P < 0.01$; $^{\#}P < 0.05$ (exact values shown) vs WT vehicle-treated mice. (**g**) Semiquantitative scores of interstitial fibrosis. All values are means ± SEM, $n = 6$ each. $^{\#}P < 0.05$ (exact values shown) vs WT vehicle-treated mice.

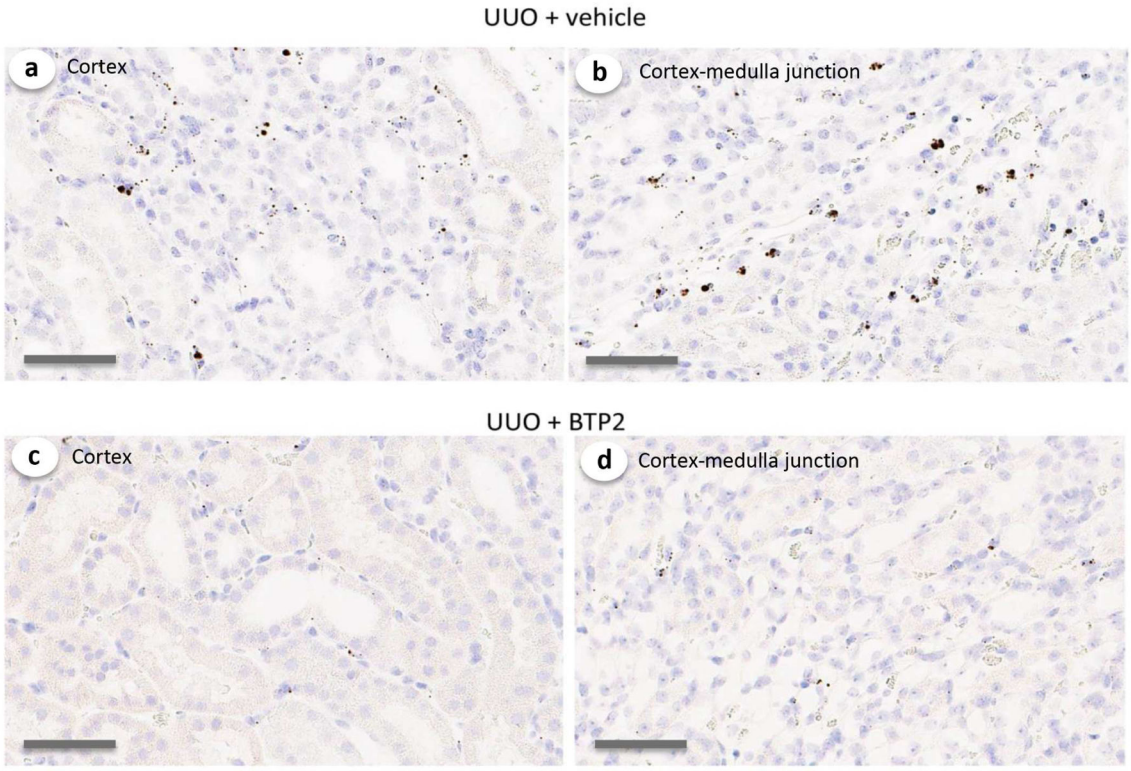

Figure 8. Effect of BTP2 on *Trpc6* mRNA expression in WT UUO kidneys

(**a**–**d**)Representative images of *Trpc6* mRNA expression examined by ISH in WT mice after 7 days UUO and pretreated with vehicle (**a**, **b**) or BTP2 (**c**, **d**). (**a**, **c**) Sections through cortical areas. (**b**, **d**) Sections through cortex-medulla junctional areas. Tissue sections were counterstained to provide structural landmarks. Cell nuclei, blue color; mRNA signal for *Trpc6*, dark-brownish color. See Supplementary Figure 3 for negative control. Scale bars, 60 μm.

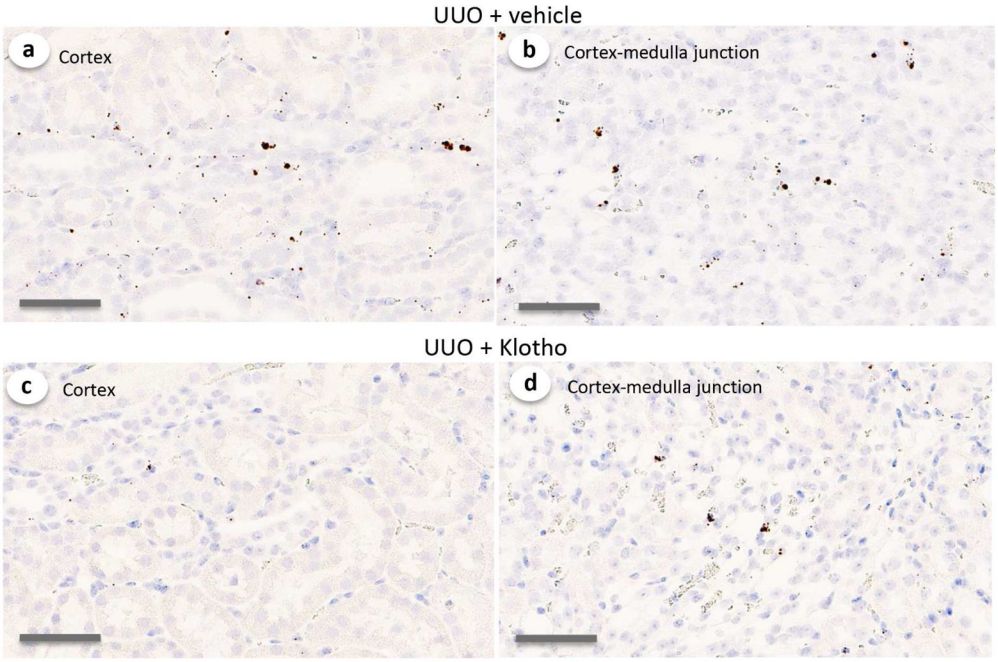

Figure 9. Effect of soluble Klotho on *Trpc6* mRNA expression in UUO kidneys

(**a**–**d**)Representative images of *Trpc6* mRNA expression examined by ISH in WT mice after 7 days UUO and pretreated with vehicle (**a**, **b**) or soluble Klotho (**c**, **d**). (**a**, **c**) Sections through cortical areas. (**b**, **d**) Sections through cortex-medulla junctional areas. Tissue sections were counterstained to provide structural landmarks. Cell nuclei, blue color; mRNA signal for *Trpc6*, dark-brownish color. See Supplementary Figure S3 for negative control. Scale bars, 60 μm.