



OPEN

SUBJECT AREAS:

MECHANISMS OF
DISEASEEXTRACELLULAR SIGNALLING
MOLECULES

Elevated serum 1,25(OH)₂-vitamin D₃ level attenuates renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction in *kl/kl* mice

Received
30 May 2014Accepted
12 September 2014Published
9 October 2014Correspondence and
requests for materials
should be addressed to
Y.M. (ymuragak@
wakayama-med.ac.jp)* Current address:
Department of
Nephrology and
Hypertension,
Inselspital, Bern 3010,
Switzerland.Yujing Sun^{1,2}, Gengyin Zhou², Ting Gui^{1*}, Aiko Shimokado¹, Masako Nakanishi¹, Kosuke Oikawa¹, Fuyuki Sato¹ & Yasuteru Muragaki¹¹First Department of Pathology, Wakayama Medical University School of Medicine, 811-1 Kimiidera, Wakayama 641-0012, Japan, ²Department of Pathology, School of Medicine, Shandong University, Jinan Wen Hua Xi Road 44, Jinan 250012, PR China.

Previous studies have suggested that *Klotho* provides reno-protection against unilateral ureteral obstruction (UUO)-induced renal tubulointerstitial fibrosis (RTF). Because the existing studies are mainly performed using heterozygous *Klotho* mutant (HT) mice, we focused on the effect of UUO on homozygous *Klotho* mutant (*kl/kl*) mice. UUO kidneys from HT mice showed a significantly higher level of RTF and TGF- β /Smad3 signaling than wild-type (WT) mice, whereas both were greatly suppressed in *kl/kl* mice. Primary proximal tubular epithelial culture cells isolated from *kl/kl* mice showed no suppression in TGF- β 1-induced epithelial mesenchymal transition (EMT) compared to those from HT mice. In the renal epithelial cell line NRK52E, a large amount of inorganic phosphate (Pi), FGF23, or calcitriol was added to the medium to mimic the *in vivo* homeostasis of *kl/kl* mice. Neither Pi nor FGF23 antagonized TGF- β 1-induced EMT. In contrast, calcitriol ameliorated TGF- β 1-induced EMT in a dose dependent manner. A vitamin D₃-deficient diet normalized the serum 1,25 (OH)₂ vitamin D₃ level in *kl/kl* mice and enhanced UUO-induced RTF and TGF- β /Smad3 signaling. In conclusion, the alleviation of UUO-induced RTF in *kl/kl* mice was due to the TGF- β 1 signaling suppression caused by an elevated serum 1, 25(OH)₂ vitamin D₃.

Klotho was first identified in close association with a premature onset of aging phenotype in mice. Homozygous *Klotho* mutant mice (*kl/kl*) exhibit a shortened life span, infertility, ectopic calcification, skin atrophy, pulmonary emphysema, and osteoporosis, which resemble an aged state in humans¹. Mouse *Klotho* is predominantly expressed in the kidneys and slightly in the brain². Two forms of *Klotho* have been identified: membrane *Klotho* and secreted *Klotho*. Membrane *Klotho* functions as a co-receptor of the FGF23 receptor^{3,4}. FGF23, a member of the fibroblast growth factor (FGF) family, is a bone-derived hormone that exerts its function in the kidneys, to help maintain homeostasis in phosphate and vitamin D metabolism by regulating the sodium phosphate co-transporter and the key enzymes for vitamin D metabolism CYP27B1 and CYP24A1⁵⁻⁸. Similar to FGF23-deficient mice, *kl/kl* mice also exhibit hyperphosphatemia and an increased serum 1,25 (OH)₂ vitamin D₃ level¹, which are suspected to be the primary cause of premature aging because restricting vitamin D intake improves the *kl/kl* phenotype^{8,9}. Secreted *Klotho* is formed via either alternative splicing of the gene or shedding of the extracellular domain into the extracellular space and subsequently into the circulation^{2,10}.

Renal tubulointerstitial fibrosis (RTF) is the final common pathological condition of chronic kidney disease (CKD) regardless of the underlying cause¹¹. RTF is characterized by an excess accumulation of extracellular matrix (ECM), fibroblast activation, and a loss of functioning nephrons¹². Recent studies have suggested that *Klotho* plays an important role in RTF. Kidney injury, such as ischemia-reperfusion, peritoneal cisplatin injection, or angiotensin II administration, induce *Klotho* deficiency, whereas exogenous *Klotho* protein attenuates kidney injury¹³⁻¹⁷. In a mouse model of CKD, unilateral ureteral obstruction (UUO)-induced RTF is exaggerated in heterozygous *Klotho* mutant mice (HT) and is ameliorated in *Klotho* over-expressing mice. Secreted *Klotho* has been proven to interfere with TGF- β ¹⁸ and Wnt/ β -catenin¹⁹ signaling to suppress UUO-induced RTF.

kl/kl mice exhibit phenotypes that are very different from wild-type or HT mice. Hyperphosphatemia and high 1, 25(OH)₂ vitamin D₃ and/or FGF23 are the most noticeable serologic features. ECM deposition is increased in *kl/kl* mice even without UUO and is thought to be due to *Klotho* deficiency²⁰. However, an activated form of

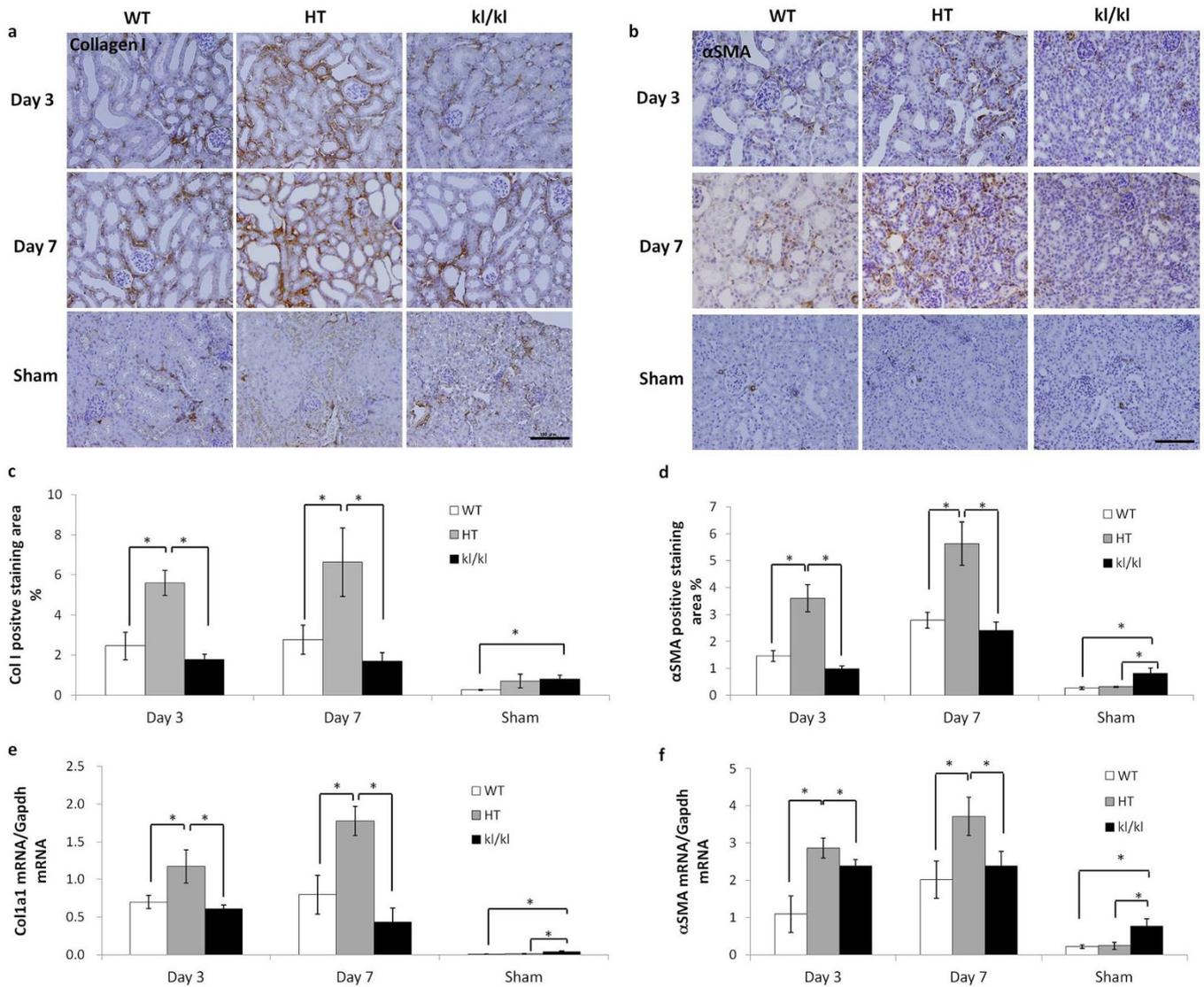


Figure 1 | *Kl/kl* kidneys showed the mildest UUO-induced renal tubulointerstitial fibrosis. (a, b): Representative pictures for immunohistochemistry of kidney sections from WT, HT, and *kl/kl* mice for collagen I (a) and α SMA (b). Scale bar: 100 μ m (c, d) Quantification of positive staining areas for collagen I (c) and α SMA (d). (e, f) Real-time PCR for Coll1a1 (e) and α SMA (f). Note that UUO-induced collagen I deposition and fibroblast activation were the most severe in HT mice at the protein and mRNA levels and were largely abolished in *kl/kl* mice. Data are presented as the means \pm S.D. * $P < 0.05$ ($n = 5$).

vitamin D and its analogues have been shown to protect the kidneys from fibrosis due to various kidney injuries^{21,22}. Also, 1, 25(OH)₂ vitamin D₃ reduces gene expression related to TGF- β -induced fibrosis in human uterine leiomyoma cells²³. An active form of the vitamin D₃ analogue maxacalcitol recruits a PPM1A/VDR complex to phosphorylated Smad3 to accelerate its dephosphorylation²⁴. Thus, the outcome of UUO-induced RTF becomes uncertain because of the opposing effect of an elevated vitamin D and Klotho deficiency. Because all of the existing studies on the effect of Klotho on UUO-induced RTF were performed using HT mice, whether the disrupted homeostasis in *kl/kl* mice has an effect is not known.

In this study, we compared the degree of UUO-induced RTF among wild-type, HT, and *kl/kl* mice and analyzed the underlying mechanisms.

Results

UUO-induced RTF is the mildest in *kl/kl* mice. Six week-old wild-type (WT), heterozygous Klotho mutant (HT), and *kl/kl* mice were subjected to UUO for 3 days or 1 week. The expression of RTF

markers collagen I and α SMA was assessed by immunohistochemistry and quantitative real-time PCR. RTF developed after the UUO operation in a time dependent manner in WT and HT mice compared to the sham-operated control groups. The expression of RTF markers was much higher in HT mice than WT mice, which is corroborated by previous reports^{18,20}. In the sham-operated groups, the expression of RTF markers was comparable between the WT and HT mice, but was increased more than 2-fold in the *kl/kl* mice. Conversely, *kl/kl* mice showed only a slight increase in the expression of fibrosis markers after UUO (Fig. 1).

TGF- β signaling is suppressed in *kl/kl* mice. Previous studies showed that Klotho protein interferes with TGF- β 1 binding to its receptor, resulting in suppressed TGF- β signaling¹⁸. As a result, UUO-induced RTF is exaggerated in HT mice²⁰. To examine whether the suppressed expression of RTF markers in *kl/kl* mice is a result of inhibited TGF- β signaling, the phosphorylation of Smad3 and TGF- β 1 expression was assayed. In parallel with RTF markers, after UUO, phosphorylated Smad3 (pSmad3) and TGF- β 1 expression were the highest in HT mice and were significantly suppressed

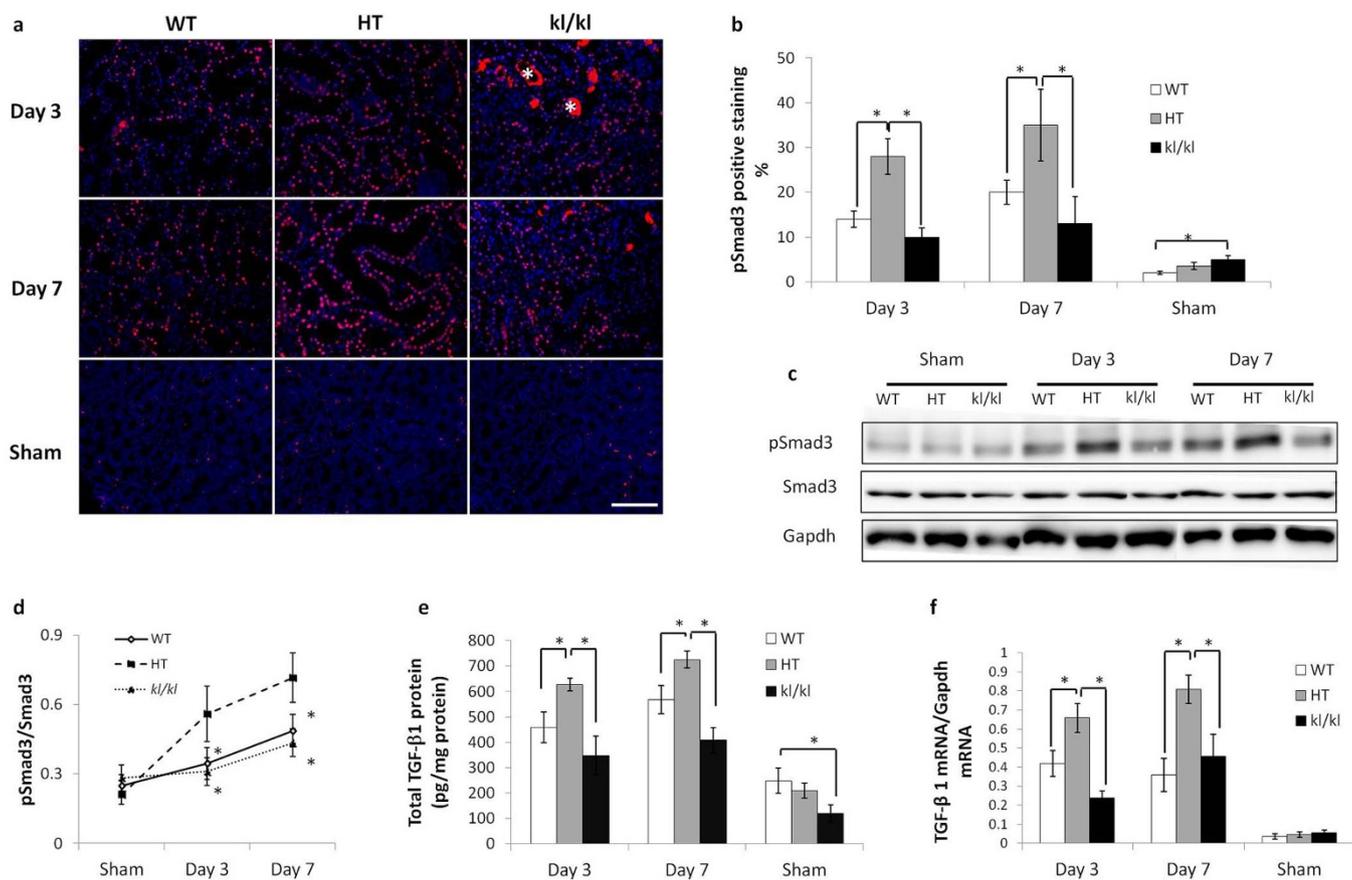


Figure 2 | UUU-induced TGF- β 1/Smad3 signaling is ameliorated in *kl/kl* mice. (a) Immunofluorescence for phosphorylated (p)-Smad3. Scale bar: 100 μ m (b) Comparison of the number of pSmad3-positive nuclei among WT, HT, and *kl/kl* kidneys. (c) Western blot for pSmad3 in UUU-treated kidneys. Full-length blots are included in the supplementary information. (d) Comparison by quantification of pSmad3 in western blot among WT, HT, and *kl/kl* kidneys. (e) The amount of TGF- β 1 protein in WT, HT, and *kl/kl* kidneys as measured by ELISA. (f) TGF- β 1 mRNA level as measured by real-time PCR. Note that UUU-induced TGF- β signaling was enhanced in HT kidneys compared to WT kidneys and was ameliorated in *kl/kl* kidneys. Data are presented as the means \pm SD. * $P < 0.05$ ($n = 5$).

in *kl/kl* mice. Interestingly, in the sham-operated groups, the *kl/kl* mice had a higher percentage of pSmad3 positive staining cells than the other two groups, whereas this group had the lowest TGF- β 1 content, suggesting that Smad3 phosphorylation in the sham-operated *kl/kl* mice was not a result of increased TGF- β 1 (Fig. 2).

No suppression of the TGF- β 1-induced epithelial-mesenchymal transition is observed in primary cultured proximal tubular epithelial culture cells isolated from *kl/kl* mice. Because *kl/kl* mice have disrupted phosphate and calcium (Pi/Ca) homeostasis, determining whether the suppressed TGF- β /Smad3 signaling is a result of complete depletion of Klotho or disrupted Pi/Ca homeostasis, most noticeably high phosphate, high FGF23, and high 1, 25(OH) $_2$ vitamin D $_3$, is difficult. To address the underlying molecular mechanism, we compared the expression of molecules involved in epithelial-mesenchymal transition (EMT) using primary cultured proximal tubular epithelial cells (PTECs) from WT, HT, and *kl/kl* mice. TGF- β 1-induced EMT was enhanced in HT and *kl/kl* cells compared to WT cells. The EMT markers showed higher α SMA and lower E-cadherin mRNA and protein expression at the indicated time point (Fig. 3). Smad3 phosphorylation was also enhanced in HT and *kl/kl* cells. Unlike UUU kidneys, *kl/kl* cells showed no suppression of TGF- β 1-induced EMT and Smad3 phosphorylation compared to HT cells. These results suggest that the suppressed TGF- β /Smad3 signaling observed in UUU-induced RTF in *kl/kl* kidneys was a result of disrupted Pi/Ca homeostasis and not complete Klotho depletion (Fig. 3).

TGF- β 1-induced EMT is suppressed not by high phosphate or FGF23 but by 1,25(OH) $_2$ vitamin D $_3$ in a dose-dependent manner. Previous studies showed that in *kl/kl* mice, serum phosphate and 1,25(OH) $_2$ vitamin D $_3$ increase sharply¹ and serum FGF23 concentration increased 2,000-fold compared to WT mice⁴. To identify an individual factor(s) that contribute to suppressed UUU-induced RTF expression in *kl/kl* mice, we used the normal kidney epithelial cell line NRK52E, and these cells were cultured in the presence of high phosphate (2 or 4 mM), FGF23 (0.2 or 2 μ g/ml), or 1,25(OH) $_2$ vitamin D $_3$ (0.1 or 1 μ g/ml) to evaluate their effect on TGF- β 1-induced EMT. In the cells treated with TGF- β 1 together with phosphate or FGF23 for 12 or 24 h, TGF- β 1-induced EMT was not affected at the mRNA or protein level compared to cells treated with TGF- β 1 alone. In contrast, 1,25(OH) $_2$ vitamin D $_3$ ameliorated TGF- β 1-induced Smad3 phosphorylation, α SMA expression, and E-cadherin suppression in a dose dependent manner at the mRNA and protein levels. This suppressive effect of 1,25(OH) $_2$ vitamin D $_3$ on TGF- β -induced EMT lasted for 72 h after TGF- β treatment (supplementary Fig. 1).

Interestingly, the cells cultured with high phosphate (4 mM) for 48 h had more Smad3 phosphorylation and higher α SMA expression compared to cells cultured in normal DMEM, although TGF- β 1 was absent. These results may explain the increased fibrosis markers observed in sham-operated *kl/kl* mice (Figs. 4 and 5).

Vitamin D-free diet normalizes serum 1,25(OH) $_2$ vitamin D $_3$ level and enhances the expression of UUU-induced RTF markers in

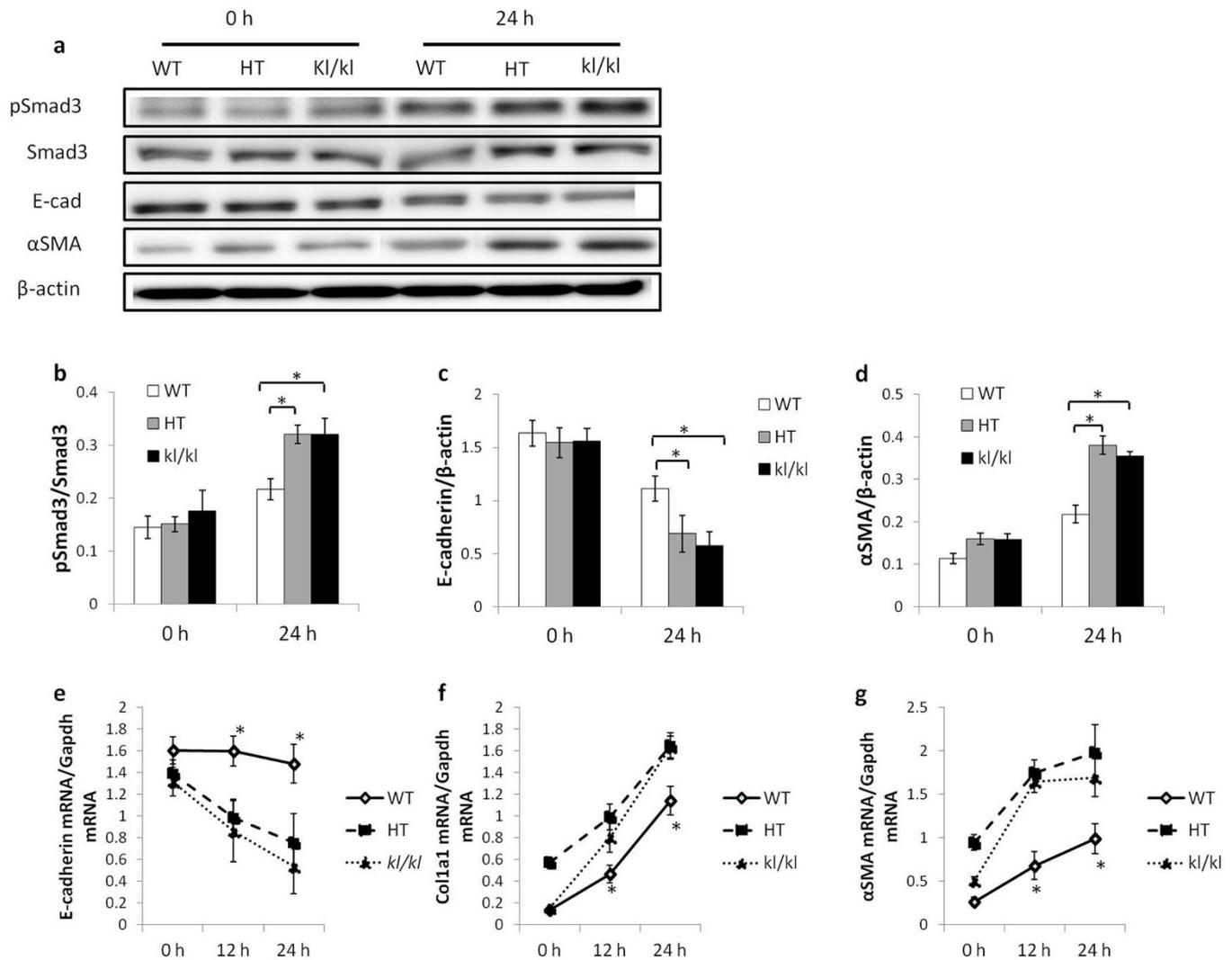


Figure 3 | TGF- β 1-induced EMT is enhanced in proximal tubular cells isolated from HT and *kl/kl* kidneys compared to WT kidneys. (a) Representative western blots for pSmad3, Smad3, E-cadherin (E-cad), α SMA, and β -actin. Full-length blots are included in the supplementary information. (b–d) Comparison by quantification of western blots for pSmad3 (b), E-cadherin (c), and α SMA (d). (e–g) Real-time PCR for E-cadherin (e), Col1a1 (f), and α SMA (g). Note that unlike in vivo, no difference in EMT markers was detected between HT and *kl/kl* cultured proximal tubular cells. Data are presented as the means \pm SD. * $P < 0.05$ compared to the HT group at the same time point. Experiments were repeated in triplicate.

***kl/kl* mice.** A vitamin D-free diet (D-diet) has been reported to maintain phosphate homeostasis and improve the *kl/kl* phenotype^{8,9}. In this study, we fed *kl/kl* mice on a D-diet to determine whether the decreased vitamin D level could enhance UO-induced RTF. In parallel with previous studies, the D-diet normalized serum 1,25(OH)₂ vitamin D₃ and increased the body weight of the *kl/kl* mice, such that they were indistinguishable from their WT littermates (Fig. 6).

After the UO operation, based on immunohistochemistry, expression of collagen I and α SMA was enhanced by the D-diet, with 3- and 4-fold increases, respectively, compared to *kl/kl* mice fed a standard diet, and mRNA expression showed a similar trend (Fig. 7).

In addition, the D-diet enhanced TGF- β /Smad3 signaling in UO-induced RTF in *kl/kl* mice. The percentage of pSmad3-positive cells increased about 2.5-fold as a result of vitamin D withdrawal. ELISA was performed to measure the TGF- β 1 content in kidney lysates. Protein and mRNA expression of UO-induced TGF- β 1 increased about 2-fold using the D-diet. Interestingly, in the sham-operated *kl/kl* mice fed the standard diet, TGF- β 1 protein expression was lower than the *kl/kl* mice fed the D-diet, while there were more pSmad3 positive cells in *kl/kl* mice fed the standard diet (Fig. 8).

Note that in sham-operated *kl/kl* mice fed a standard diet, the expression of RTF markers and activation of TGF- β /Smad3 signaling are slightly but significantly higher than *kl/kl* mice fed a D-diet. The results suggest that increased RTF marker expression in sham-operated *kl/kl* mice was not a result of Klotho depletion but the disrupted Pi/Ca homeostasis (Figs. 7 and 8).

Discussion

In this study, we demonstrated that UO-induced RTF was greatly suppressed in *kl/kl* mice compared to HT mice. Conversely, slightly but significantly more severe renal fibrosis was observed in sham-operated *kl/kl* kidneys than WT and HT kidneys. Because *kl/kl* mice exhibit a variety of abnormalities, among which phosphate retention and high serum 1, 25(OH)₂ vitamin D₃ are the most prominent changes, we hypothesized that disrupted Pi/Ca homeostasis in *kl/kl* mice may play an important role in the mild renal fibrosis in sham-operated mice and the suppression of RTF in UO-operated mice.

We observed that HT mice exhibit more severe UO-induced RTF compared to the WT group, which is consistent with previous reports²⁰. Klotho has been proven to protect the kidneys from injury-induced fibrosis by counteracting TGF- β /Smad3 and Wnt signal-

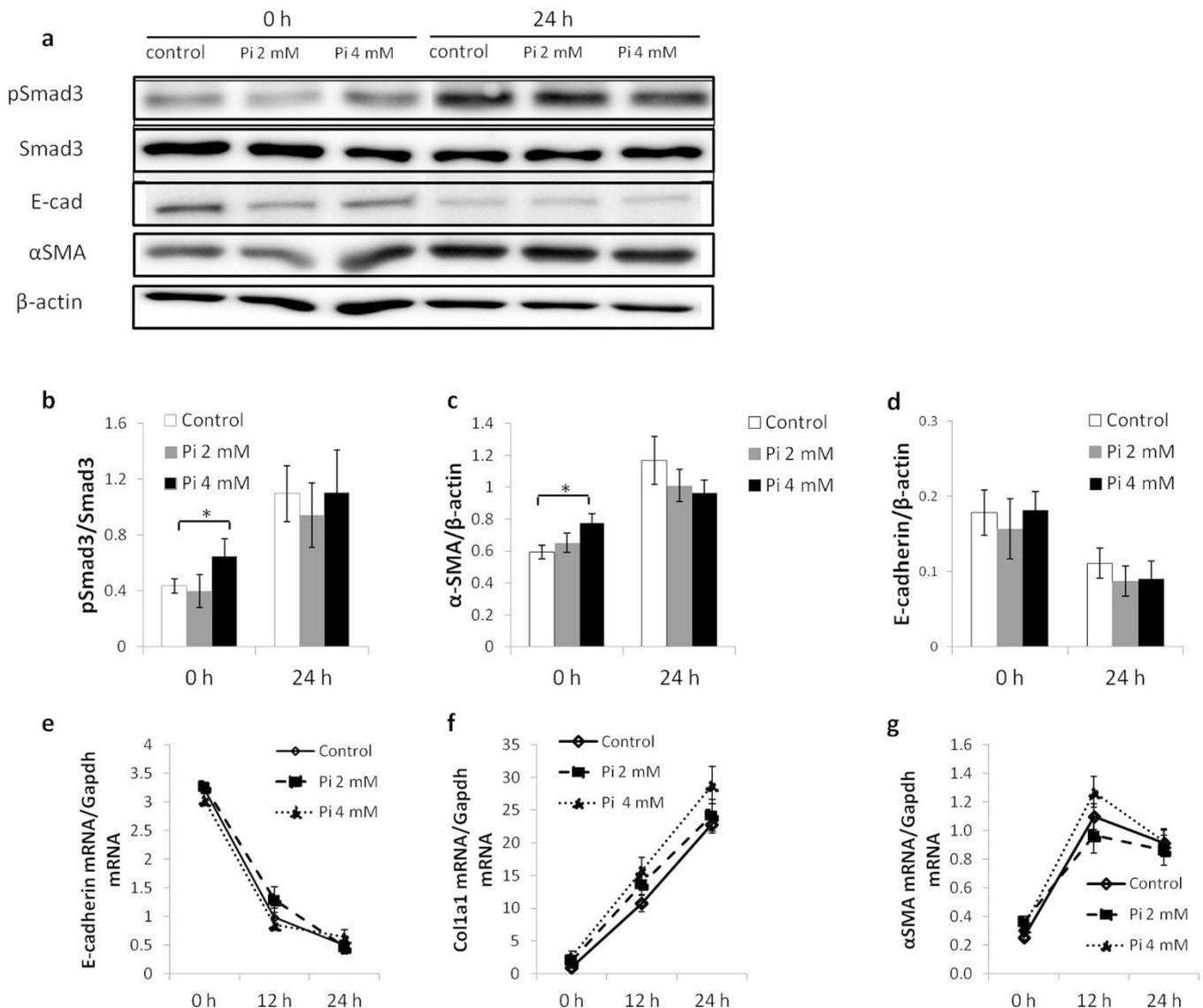


Figure 4 | TGF- β 1-induced EMT in NRK52E cells is not influenced by a high concentration of phosphate. (a) Representative western blots for pSmad3, Smad3, E-cadherin (E-cad), α SMA, and β -actin. Full-length blots are included in the supplementary information. (b–d) Comparison by quantification of western blots for pSmad3 (b), E-cadherin (c) and α SMA (d). (e–g) Real-time PCR for E-cadherin (e), Col1a1 (f), and α SMA (g). A high concentration of phosphate does not enhance TGF- β 1-induced EMT. Note that basal Smad3 phosphorylation and α SMA protein expression are enhanced in the presence of a high phosphate level. Data are presented as the means \pm SD. * $P < 0.05$. Experiments were repeated in triplicate.

ing^{18,19}. Klotho deficiency renders the kidneys more susceptible to acute insults, while exogenous Klotho expression attenuates renal fibrosis due to various causes^{13,14,18,20,25}. In rodent models, renal damage caused by several different kinds of stress, such as acute inflammatory stress and sustained circulatory stress, could suppress Klotho expression^{26,27}. In the human kidneys, decreased Klotho expression occurs as early as CKD stage 2²⁸. However, unexpectedly, UO-induced RTF in *kl/kl* mice was greatly attenuated. Because TGF- β /Smad3 signaling is believed to be the main driving force toward post-UO RTF²⁹, we examined TGF- β /Smad3 signaling, which turned out to be significantly suppressed in *kl/kl* kidneys. To identify the cause of this suppression, we examined TGF- β -induced EMT using primary cultured PTECs to exclude the humoral effects. Interestingly, unlike *in vivo* experiments, TGF- β 1-induced EMT was not suppressed in cultured *kl/kl* PTECs, and there was an increase in Smad3 phosphorylation. These results suggest that the suppression of UO-induced RTF in *kl/kl* mice might result from a disturbance in Pi/Ca homeostasis due to deficiency of Klotho. We have not investigated ERK/MAPK activation in cultured *kl/kl* PTECs because

the increase in Smad3 phosphorylation was significant, but it might be important to examine non-canonical TGF- β signaling, which also plays an important role in EMT.

Next, because phosphate retention, high serum FGF23, and high serum 1, 25(OH)₂ vitamin D₃ are the most prominent changes in *kl/kl* mice³⁰, we examined these factors individually using *in vitro* cultures. NRK52E cells were challenged with TGF- β 1 in the presence of high phosphate, FGF23, or calcitriol (1, 25(OH)₂ vitamin D₃). TGF- β 1-induced EMT was not affected by the presence of high phosphate or FGF23, but was inhibited by calcitriol in a dose dependent manner. These results suggest that a high level of 1, 25(OH)₂ vitamin D₃ but not phosphate or FGF23 may be the cause of the suppressed UO-induced RTF in *kl/kl* mice.

To confirm this mechanism, we sought to eliminate the effect of vitamin D in *kl/kl* mice by feeding a D-diet and determining the degree of UO-induced RTF. The D-diet normalized 1, 25(OH)₂ vitamin D₃ and phosphate levels in the blood of *kl/kl* mice and completely rescued the *kl/kl* phenotype, which is in agreement with previous reports⁸. As we predicted, the D-diet enhanced UO-

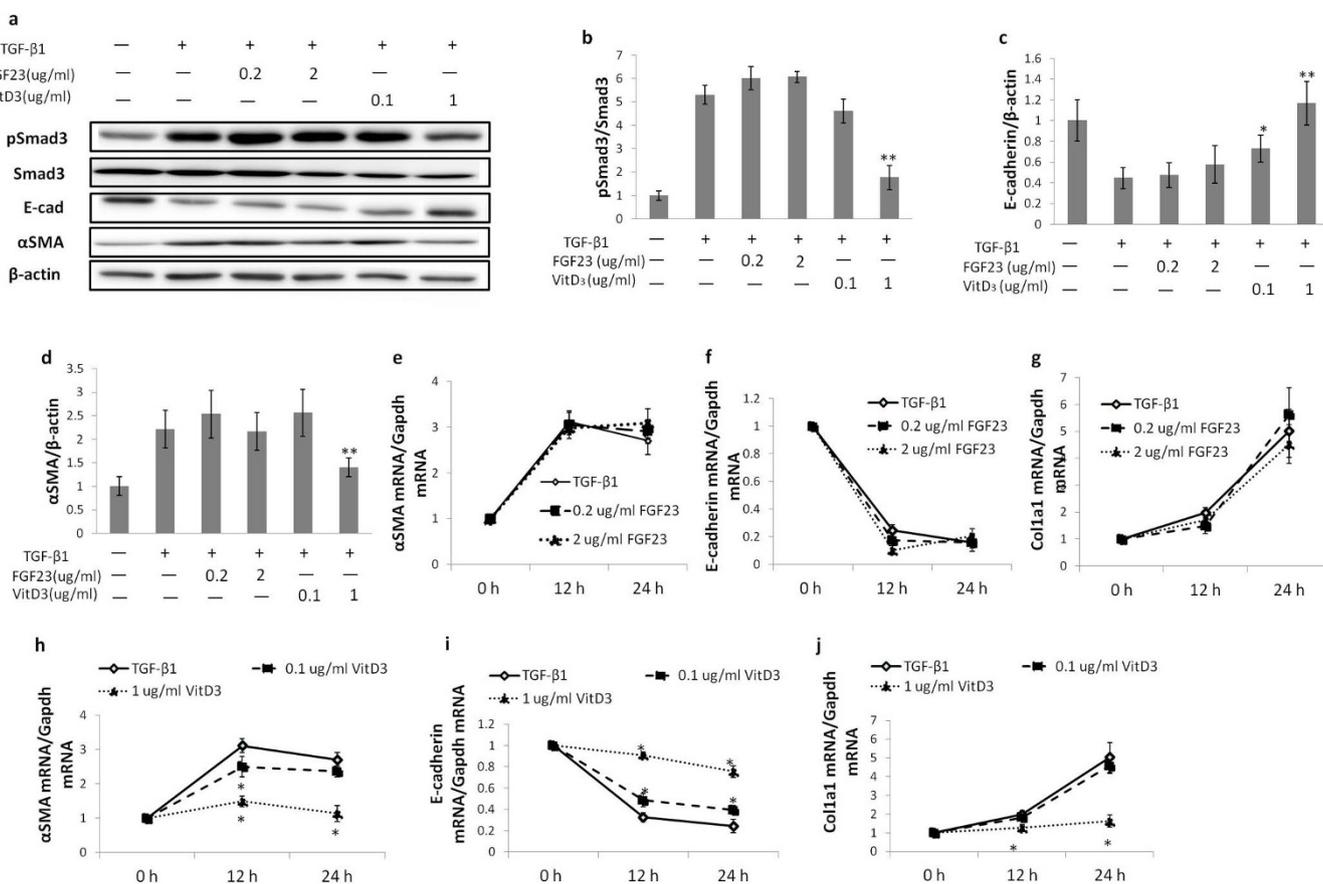


Figure 5 | TGF- β 1-induced EMT in NRK52E cells is suppressed by calcitriol. (a) Representative western blots for pSmad3, Smad3, E-cadherin, α SMA, and β -actin. Full-length blots are included in the supplementary information. (b–d) Comparison by quantification of western blots for pSmad3 (b), E-cadherin (c), and α SMA (d). (e–j) Real-time PCR results for E-cadherin (e, h), Col1a1 (f, i), and α SMA (g, j) in the presence of either FGF23 (e, f, g) or calcitriol (h, i, j). FGF23 have no effect on TGF- β 1-induced EMT at neither low nor high concentration. Note that calcitriol ameliorates TGF- β 1 induced EMT in a dose-dependent manner. Data are presented as the means \pm SD. * P < 0.05 versus cells treated with TGF- β 1 only. Experiments were repeated in triplicate.

induced RTF in *kl/kl* mice to the same levels as HT mice. In addition, TGF- β signaling was enhanced by eliminating vitamin D. Paricalcitol, a synthetic vitamin D analogue, has been reported to attenuate renal interstitial fibrosis in obstructive nephropathy via

repressing expression of TGF- β 1 and its type I receptor³¹. Moreover, vitamin D analogs are reported to ameliorate proteinuria and kidney injury by blocking Wnt/ β -catenin signaling³². We therefore conclude that an elevated level of 1, 25(OH)₂ vitamin D₃ in *kl/kl*

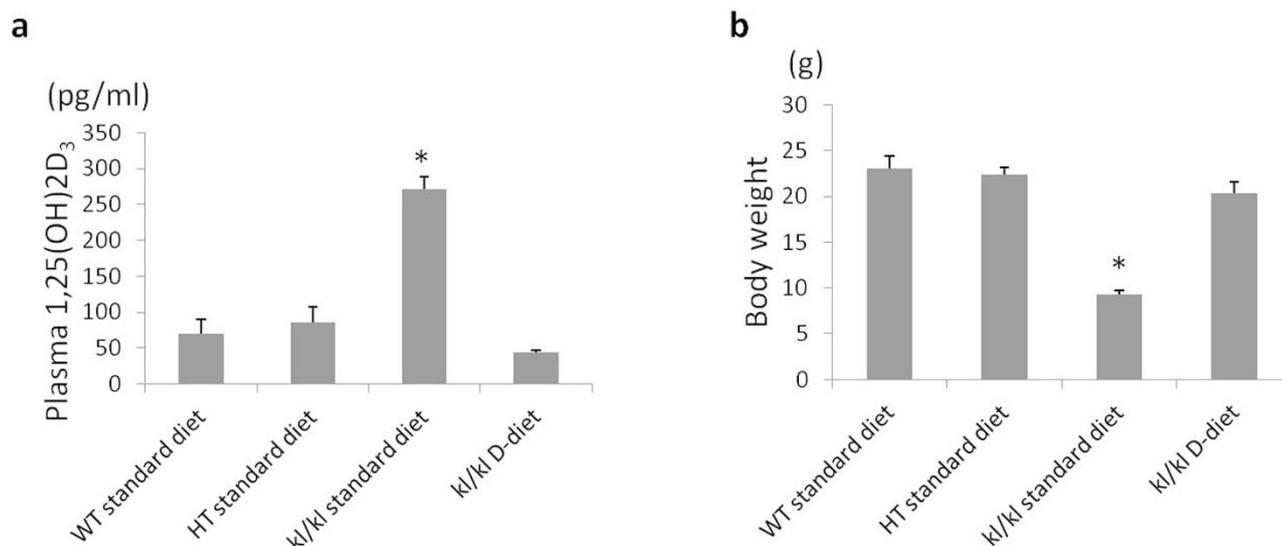


Figure 6 | A 1,25(OH)₂-vitamin D₃-deficient diet (D-diet) normalizes serum active vitamin D levels in *kl/kl* mice and rescues the *kl/kl* phenotype. (a) Serum level of active vitamin D. (b) Body weight. Data are presented as the means \pm SD. * P < 0.05 vs. WT (n = 4).

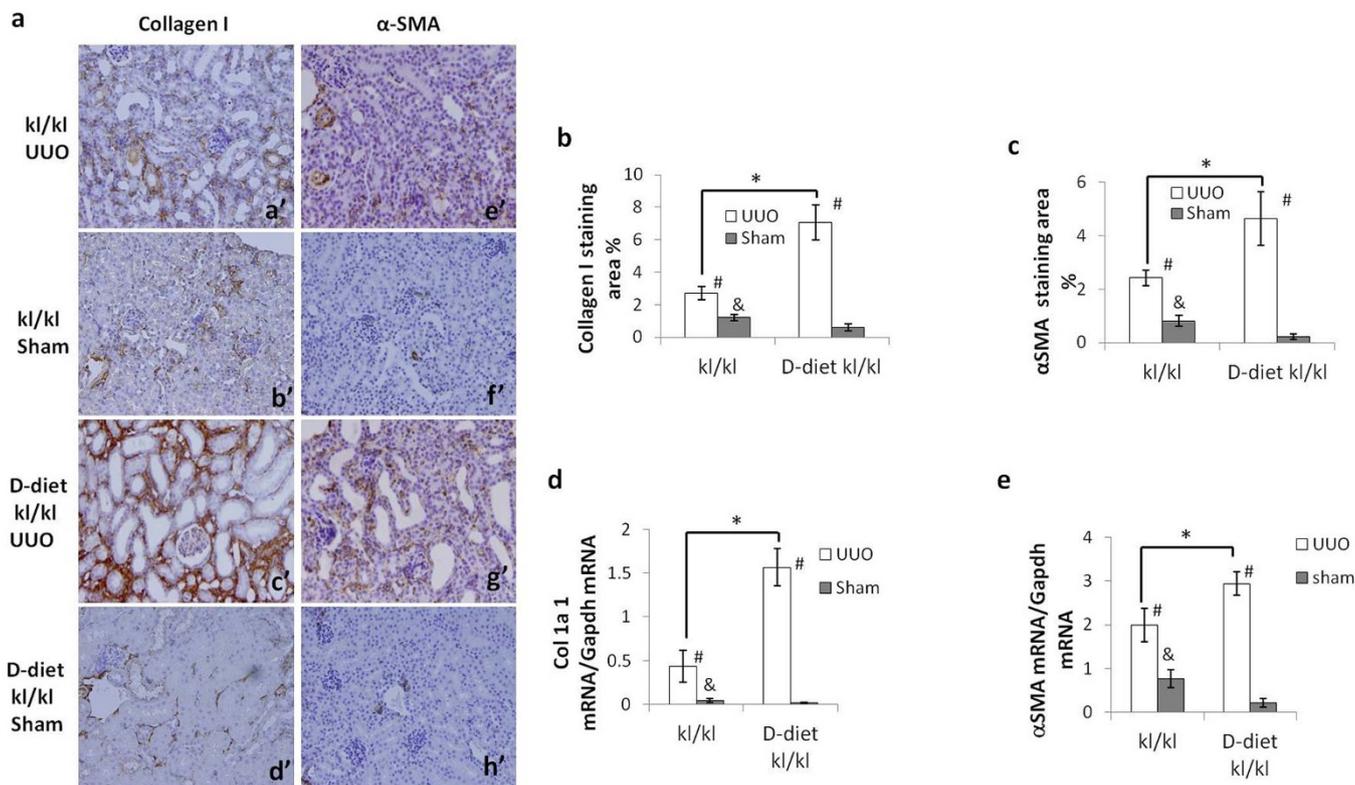


Figure 7 | A vitamin D₃-free diet enhances UO-induced RTF in *kl/kl* mice to the same level as HT mice. (a) Representative immunohistochemistry of kidney sections for collagen I (a–d) and α SMA (e–h) (b, c) Comparison by quantifying the positive staining area for collagen I (b) and α SMA (c). (d, e) Real-time PCR for Col1a1 (d) and α SMA (e). A vitamin D₃ free diet significantly enhances UO-induced protein and mRNA expression of collagen I and α SMA in *kl/kl* mice. Data are presented as the means \pm SD. * $P < 0.05$, # $p < 0.05$ vs. sham-treated kidneys, & $p < 0.05$ vs. D-diet *kl/kl* sham-treated kidneys (n = 5).

mice suppresses TGF- β /Smad3 signaling, resulting in amelioration of UO-induced RTF.

In sham-operated mice, there was no significant difference in the expression of RTF markers between WT and HT kidneys, whereas expression was significantly albeit mildly increased in *kl/kl* kidneys compared to WT and HT kidneys. Sugiura, H et al²⁰ suggested that this result may be due to failed TGF- β /Smad3 inhibition related to the absence of Klotho. However, our results did not support this hypothesis because the *kl/kl* PTECs expressed the same levels of EMT markers as WT and HT PTECs treated with TGF- β 1. In addition, *kl/kl* mice fed a D-diet did not have any RTF onset in the sham-operated group. Interestingly, NRK52E cells cultured for 48 h in the medium containing a high concentration of phosphate (4 mM) showed Smad3 phosphorylation and increased α SMA expression compared to the control medium (Pi 1 mM), suggesting that an increased expression of RTF markers in sham-operated *kl/kl* mice may be a result of a disrupted Pi/Ca homeostasis, that is, a high concentration of phosphate and not a result of failed TGF- β /Smad3 inhibition by Klotho. However, at this time, we cannot explain how a high concentration of phosphate phosphorylates Smad3.

In conclusion, we are the first to report that UO-induced TGF- β /Smad3 signaling is suppressed in *kl/kl* mice by an increased level of 1, 25(OH)₂ vitamin D₃, resulting in amelioration of UO-induced RTF. We identified 1, 25(OH)₂ vitamin D₃ to be a powerful suppressor of TGF- β /Smad3 signaling and protect the kidneys from RTF.

Methods

Animals, diets, UO animal models, and tissue preparation. All experimental protocols were approved by the Animal Studies Committee of Wakayama Medical University. The methods were carried out in accordance with the approved guideline.

This study used *klotho*/Jcl mice, which were purchased from CLEA Japan and described elsewhere¹. Mice were raised in a standard facility with free access to food and water.

Animals were fed a standard diet or vitamin D₃ free diet (D-diet). In the D-diet groups, heterozygous *klotho* female mice were maintained on a vitamin D₃ free diet (PMI nutrition international, Inc.) after pregnancy, in which the composition was identical to the standard diet except for missing vitamin D₃. The offspring of D-diet heterozygous *klotho* females were also fed a D-diet throughout the experiments.

Six- to eight-week-old male wild-type (WT), heterozygous (HT), and *kl/kl* mice from the standard diet and D-diet groups were used to perform UO. UO surgery was performed as described elsewhere^{33,34}. In short, male mice from each group (n = 5) were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine (5 mg/kg body weight). A left-flank incision was made to expose the left ureter, and then ligation was done with a 5-0 silk suture. The sham groups (n = 3) received the same procedure except for ureteral ligation.

At the time of sacrifice, the ligated kidneys (or sham kidneys) were removed and cut transversely. Tissues were fixed with 4% paraformaldehyde for histopathological examination or lysed by denaturing lysis buffer (50 mM Tris-HCl, pH 7.4, 1% triton X, 150 mM NaCl, 1 mM EDTA) with addition of a phosphatase inhibitor (2.5 mM sodium orthovanadate, 10 mM NaF, and 10 mM β -glycerol phosphate) and proteinase inhibitor (Roche). Lysed samples were stored in -80°C until use for western blot analysis or ELISA.

Immunohistochemistry and immunofluorescent staining. Immunohistochemistry was performed on paraffin sections using a microwave-based antigen retrieval technique. The antibodies used in this study include collagen I (Southern Biotechnology, Birmingham, AL), pSmad3 (Cell Signaling, Danvers, MA), α SMA (Sigma) and Fsp1 (Abcam). After incubation with the primary antibodies, sections were treated with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. For immunofluorescent staining, a Cy3-anti-rabbit IgG (Sigma Aldrich) was used as a secondary antibody.

Quantitative real-time polymerase chain reaction (qRT-PCR). SYBR Green PCR Master Mix (Takara Bio, Inc., Japan) was used to analyze mRNAs. Each sample was measured in duplicate, and gene expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (Gapdh). Sequences of the primers used for qRT-PCR are as follows: mouse-Col1a1 forward 5'-CAA CCT GGA CGC CAT CAA G-3' and reverse 5'-CAG ACG GCT GAG TAG GGA ACA-3'; rat-Col1a1 forward 5'-AGT GCA TTC AAC CTT ACC AA-3' and reverse 5'-TCA AGC AAG AGG ACC AAG

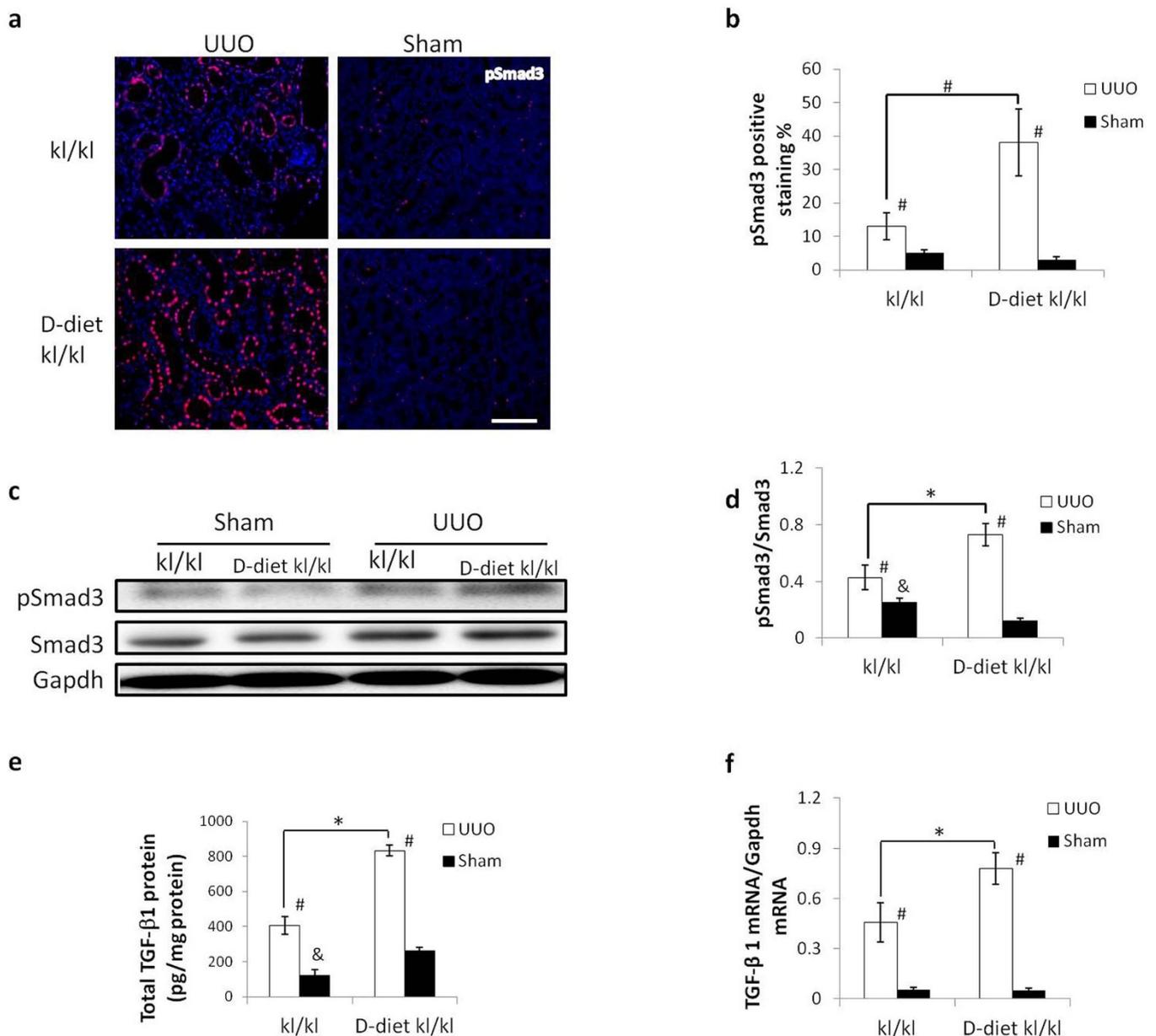


Figure 8 | TGF- β 1 signaling in *kl/kl* mice is enhanced by a vitamin D3 free diet. (a) Representative immunofluorescence for phosphorylated (p)-Smad3. (b) Western blot for pSmad3 in UUO- or sham-treated kidneys. Full-length blots are included in the supplementary information. (c) Comparison of the number of pSmad3-positive nuclei. (d) Comparison of pSmad3 to total Smad3 ratio in western blots. (e) TGF- β 1 protein expression in the kidneys as measured by ELISA. (f) TGF- β 1 mRNA expression as measured by real-time PCR. Amelioration of UUO-induced activation of TGF- β 1 signaling in *kl/kl* mice was abolished by a vitamin D3 free diet. pSmad3 positive nuclei and TGF- β 1 expression were increased at the protein and mRNA levels. Data are presented as the means \pm SD. * $P < 0.05$ ($n = 5$), # $P < 0.05$ vs. sham treated kidneys. & $P < 0.05$ vs. D-diet *kl/kl* mice.

C-3'; mouse and rat-Fsp1 forward 5'-GAA GTG AAG ACT CCT CAG ATG-3' and reverse 5'-CAC TAT GCT CAC AGC CAA C-3'; mouse-TGF- β 1 forward 5'-AAC TAT TGC TTC AGC TCC AGA GAG A-3' and reverse 5'-AGT TGG ATG GTA GCC CTT G-3'; rat-TGF- β 1 forward 5'-TGG AGC AAC ACG TAG AAC TCT-3' and reverse 5'-AGT TGG ATG GTA GCC CTT G-3'; mouse- α SMA forward 5'-AGA TCA CAG CCC TCG CA-3' and reverse 5'-AGA GTA CTT GCG TTC TGG AG-3'; rat- α SMA forward 5'-AGA TCA CAG CCC TCG CT-3' and reverse 5'-AGA ATA TTT GCG TTC TGG AG-3'; rat- α SMA forward 5'-ATG ATG AAG AGG GAG GTG G-3' and reverse 5'-CAT CAT TTC GAA TCA CTT CC-3'; mouse-Gapdh forward 5'-CCC ATG TTT GTG ATG GGT GT-3' and reverse 5'-GTG ATG GCA TGG ACT GTG GT-3'³⁵.

Western Blot Analysis. Tissue or cell lysate (20 μ g) was separated by SDS-PAGE and blotted on polyvinylidene difluoride membranes (Millipore, Billerica, MA). The primary antibodies were rabbit anti-pSmad3 (Acris Antibodies, Herford, Germany), rat anti-E-cadherin (Sigma Aldrich), mouse anti- α SMA (Sigma Aldrich), rabbit anti-Smad3 (Abcam), mouse anti- β -actin (Sigma Aldrich), and goat anti-GAPDH (Santa

Cruz). The signals were developed with the ECL Plus detection system (Amersham Bioscience, Buckinghamshire, United Kingdom).

TGF- β 1 ELISA assay. Kidney cortices were dissected and homogenized in denaturing lysis buffer containing 100 mM NaCl, 1% Triton X-100, and 0.05 M Tris-HCl, pH7.4. Protein concentrations were measured using a BCA method according to the manufacturer's instruction. Total TGF- β 1 was quantified using a TGF- β 1 ELISA kit (Invitrogen). Values were expressed as pg/mg protein for the protein extracts.

Measurement of active serum 1, 25(OH) $_2$ vitamin D $_3$ in mice. Six-week-old male mice were anesthetized with ketamine and xylazine; blood was collected from the inferior vena cava, and the serum was separated by centrifugation at 7,500 g for 10 min at 4°C ($n = 4$). The serum level of 1, 25(OH) $_2$ vitamin D $_3$ was measured by RIA (SRL Inc., Tokyo, Japan).

Cell culture. Primary proximal tubular epithelial cells were generated from the kidneys of WT, HT, and *kl/kl* mice using a method described previously³³ with minor



modification. Briefly, kidney cortices (6 wk male mice) were dissected, sliced, minced, and digested in 300 U/ml type II collagenase (Worthington Bio Corp) in FBS free DMEM in a shaking incubator at 37°C for 30 min. Collagenase was neutralized with 10% FBS/DMEM containing 100 U/ml penicillin and 0.1 mg/ml streptomycin. The suspension was triturated by pipetting and was passed through a 70 µm cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ). The samples were centrifuged (500 rpm, 5 minutes) to pellet the tubules, washed with 10 ml FBS free-DMEM, centrifuged, and washed twice more. The final pellet, consisting mostly of renal tubules, was resuspended in culture medium (REBM bullet kit, Clonetics) and incubated at 37°C in a 5% CO₂ incubator with medium changes every 2 days until approximately 80% confluent.

NRK52E cells were obtained from ATCC and cultured in DMEM with 5% FBS (Gibco) without antibiotic supplementation.

For TGF-β1 stimulation, cells underwent 24 h serum starvation and then were exposed to 5 ng/ml TGF-β1 (Cell signaling) for 12 or 24 h in the presence or absence of 0.2 or 2 µg/ml murine FGF23 (Peprotech) or 0.1 or 1 µg/ml Calcitriol (Cayman).

To make high phosphate growth medium, an equal volume of 100 mM NaH₂PO₄ and 100 mM Na₂HPO₄ were mixed to make 100 mM Pi stocks; 2 mM and 4 mM Pi medium were made by dilution of Pi stocks with DMEM (Pi concentration in DMEM is approximately 1 mM). NRK52E cells were grown in normal DMEM with 5% FBS or high Pi DMEM with 5% FBS until 80% confluent. TGF-β1 stimulation was performed with serum free normal DMEM or high Pi DMEM.

Quantitative Analysis. To quantify activated Smad3, the positive stained nuclei were counted in 20 consecutive high power fields (400×) and divided by the total number of nuclei. The number of collagen, and αSMA positive cells in the cortical interstitium was performed as described³⁶. Band intensity in the western blots was measured using Image J software.

Statistical Analysis. The data were analyzed with Student's *t*-test and one-way ANOVA with a Student–Newman–Keuls test (SPSS, 13.0) and expressed as means ± SD. For the data without Gaussian distribution, Kruskal–Wallis with post-hoc Mann–Whitney was used. *P* < 0.05 was considered to be statistically significant.

- Kuro-o, M. *et al.* Mutation of the mouse *klotho* gene leads to a syndrome resembling ageing. *Nature* **390**, 45–51 (1997).
- Shiraki-Iida, T. *et al.* Structure of the mouse *klotho* gene and its two transcripts encoding membrane and secreted protein. *FEBS Lett.* **424**, 6–10 (1998).
- Kurosu, H. *et al.* Regulation of fibroblast growth factor-23 signaling by *klotho*. *J. Biol. Chem.* **281**, 6120–6123 (2006).
- Urakawa, I. *et al.* *Klotho* converts canonical FGF receptor into a specific receptor for FGF23. *Nature* **444**, 770–774 (2006).
- Riminucci, M. *et al.* FGF-23 in fibrous dysplasia of bone and its relationship to renal phosphate wasting. *J. Clin. Invest.* **112**, 683–692 (2003).
- Murer, H., Hernando, N., Forster, I. & Biber, J. Proximal tubular phosphate reabsorption: molecular mechanisms. *Physiol. Rev.* **80**, 1373–1409 (2000).
- Wikvall, K. Cytochrome P450 enzymes in the bioactivation of vitamin D to its hormonal form (review). *Int. J. Mol. Med.* **7**, 201–209 (2001).
- Tsujikawa, H. *Klotho*, a Gene Related to a Syndrome Resembling Human Premature Aging, Functions in a Negative Regulatory Circuit of Vitamin D Endocrine System. *Mol. Endocrinol.* **17**, 2393–2403 (2003).
- Anour, R., Andrukhova, O., Ritter, E., Zeitz, U. & Erben, R. G. *Klotho* lacks a vitamin D independent physiological role in glucose homeostasis, bone turnover, and steady-state PTH secretion in vivo. *PLoS One* **7**, e31376 (2012).
- Chen, C. D., Podvin, S., Gillespie, E., Leeman, S. E. & Abraham, C. R. Insulin stimulates the cleavage and release of the extracellular domain of *Klotho* by ADAM10 and ADAM17. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 19796–19801 (2007).
- Harris, R. C. & Neilson, E. G. Toward a unified theory of renal progression. *Annu. Rev. Med.* **57**, 365–380 (2006).
- Zeisberg, M., Strutz, F. & Muller, G. A. Renal fibrosis: an update. *Curr. Opin. Nephrol. Hypertens.* **10**, 315–320 (2001).
- Haruna, Y. *et al.* Amelioration of progressive renal injury by genetic manipulation of *Klotho* gene. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 2331–2336 (2007).
- Shroff, R. C. *et al.* Chronic mineral dysregulation promotes vascular smooth muscle cell adaptation and extracellular matrix calcification. *J Am Soc Nephrol* **21**, 103–112 (2010).
- Mitani, H. In Vivo *klotho* Gene Transfer Ameliorates Angiotensin II-Induced Renal Damage. *Hypertension* **39**, 838–843 (2002).
- Panesso, M. C. *et al.* *Klotho* has dual protective effects on cisplatin-induced acute kidney injury. *Kidney Int.* **85**, 855–870 (2014).
- Sugiura, H. *et al.* *Klotho* reduces apoptosis in experimental ischaemic acute renal failure. *Nephrol. Dial. Transplant.* **20**, 2636–2645 (2005).

- Doi, S. *et al.* *Klotho* inhibits transforming growth factor-beta1 (TGF-beta1) signaling and suppresses renal fibrosis and cancer metastasis in mice. *J. Biol. Chem.* **286**, 8655–8665 (2011).
- Satoh, M. *et al.* *Klotho* protects against mouse renal fibrosis by inhibiting Wnt signaling. *Am J Physiol Renal Physiol* **303**, 51 (2012).
- Sugiura, H. *et al.* Reduced *Klotho* expression level in kidney aggravates renal interstitial fibrosis. *Am J Physiol Renal Physiol* **302**, F1252–F1264 (2012).
- Zhang, Z. *et al.* Renoprotective role of the vitamin D receptor in diabetic nephropathy. *Kidney Int.* **73**, 163–171 (2008).
- Tan, X., Li, Y. & Liu, Y. Paricalcitol attenuates renal interstitial fibrosis in obstructive nephropathy. *J. Am. Soc. Nephrol.* **17**, 3382–3393 (2006).
- Halder, S., Goodwin, J. & Al-Hendy, A. 1,25-Dihydroxyvitamin D3 reduces TGF-beta3-induced fibrosis-related gene expression in human uterine leiomyoma cells. *The Journal of clinical endocrinology and metabolism* **96**, 62 (2011).
- Inoue, K. *et al.* Maxacalcitol ameliorates tubulointerstitial fibrosis in obstructed kidneys by recruiting PPM1A/VDR complex to pSmad3. *Lab. Invest.* **92**, 1686–1697 (2012).
- Wang, Y. & Sun, Z. *Klotho* gene delivery prevents the progression of spontaneous hypertension and renal damage. *Hypertension* **54**, 810–817 (2009).
- Aizawa, H. *et al.* Downregulation of the *Klotho* gene in the kidney under sustained circulatory stress in rats. *Biochem. Biophys. Res. Commun.* **249**, 865–871 (1998).
- Ohyama, Y. *et al.* Molecular cloning of rat *klotho* cDNA: markedly decreased expression of *klotho* by acute inflammatory stress. *Biochem. Biophys. Res. Commun.* **251**, 920–925 (1998).
- John, G. B., Cheng, C. Y. & Kuro-o, M. Role of *Klotho* in aging, phosphate metabolism, and CKD. *Am. J. Kidney Dis.* **58**, 127–134 (2011).
- Schnaper, H. W. *et al.* TGF-beta signal transduction in chronic kidney disease. *Front Biosci (Landmark Ed)* **14**, 2448–2465 (2009).
- Kuro-o, M. *Klotho* and β*Klotho*. *Adv. Exp. Med. Biol.* **728**, 25–40 (2012).
- Tan, X., Li, Y. & Liu, Y. Paricalcitol attenuates renal interstitial fibrosis in obstructive nephropathy. *Journal of the American Society of Nephrology: JASN* **17**, 3382–3393 (2006).
- He, W., Kang, Y. S., Dai, C. & Liu, Y. Blockade of Wnt/beta-catenin signaling by paricalcitol ameliorates proteinuria and kidney injury. *J. Am. Soc. Nephrol.* **22**, 90–103 (2011).
- Gai, Z. *et al.* Trps1 haploinsufficiency promotes renal fibrosis by increasing Arkadia expression. *J. Am. Soc. Nephrol.* **21**, 1468–1476 (2010).
- Sato, M., Muragaki, Y., Saika, S., Roberts, A. B. & Ooshima, A. Targeted disruption of TGF-beta1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. *J. Clin. Invest.* **112**, 1486–1494 (2003).
- Choi, D. E. *et al.* Aliskiren ameliorates renal inflammation and fibrosis induced by unilateral ureteral obstruction in mice. *J. Urol.* **186**, 694–701 (2011).
- Cochrane, A. L. *et al.* Renal structural and functional repair in a mouse model of reversal of ureteral obstruction. *J. Am. Soc. Nephrol.* **16**, 3623–3630 (2005).

Acknowledgments

We thank Ms. Mayumi Akira for her technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research (24590488) from the Ministry of Education, Science, Sports, and Culture of Japan (to Y.M.).

Author contributions

Conceived and designed the experiments: Y.M., Y.S. Performed the experiments: Y.S., T.G., A.S. Analyzed the data: Y.S., G.Z., K.O., M.N., F.S., Y.M. Wrote the paper: Y.S., Y.M.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Sun, Y. *et al.* Elevated serum 1,25(OH)₂-vitamin D₃ level attenuates renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction in *kl/kl* mice. *Sci. Rep.* **4**, 6563; DOI:10.1038/srep06563 (2014).



This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder in order to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>