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ORIGINAL ARTICLE

Klotho recovery by genistein via promoter histone acetylation and DNA demethylation mitigates renal fibrosis in mice

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Abstract

Renal fibrosis is a common histomorphological feature of renal aging and chronic kidney diseases of all etiologies, and its initiation and progression are substantially influenced by aberrant epigenetic modifications of fibrosis-susceptible genes, yet without effective therapy. "Epigenetic diets" exhibit tissue-protective and epigenetic-modulating properties; however, their anti-renal fibrosis functions and the underlying mechanisms are less understood. In this study, we show that genistein, a phytoestrogenic isoflavone enriched in dietary soy products, exhibits impressive anti-renal fibrosis activities by recovering epigenetic loss of Klotho, a kidney-enriched anti-aging and fibrosis-suppressing protein. Mouse fibrotic kidneys induced by UUO (unilateral ureteral occlusion) displayed severer Klotho suppression and adverse expression of renal fibrosis-associated proteins, but genistein administration markedly recovered the Klotho loss and attenuated renal fibrosis and the protein expression abnormalities. The examination of possible causes of the Klotho recovery revealed that genistein simultaneously inhibited histone 3 deacetylation of Klotho promoter and normalized the promoter DNA hypermethylation by suppressing elevated DNA methyltransferase DNMT1 and DNMT3a. More importantly, genistein's anti-renal fibrosis effects on the renal fibrotic lesions and the abnormal expressions of fibrosis-associated proteins were abrogated when Klotho is knockdown by RNA interferences in UUO mice. Thus, our results identify Klotho restoration via epigenetic histone acetylation and DNA demethylation as a critical mechanism of genistein's anti-fibrosis function and shed new lights on the potentials of epigenetic diets in preventing or treating aging or renal fibrosis-associated kidney diseases.

Key messages
- Genistein prevents renal fibrosis and the associated Klotho suppression in UUO mice.
- Genistein upregulates Klotho in part by reversing the promoter histone 3 hypoacetylation.
- Genistein also preserves Klotho via relieving Klotho promoter hypermethylation.
- Genistein demethylates Klotho promoter by inhibiting aberrant DNMT1/3a expression.
- Genistein restoration of Klotho is essential for its anti-renal fibrosis function.

Keywords Renal fibrosis · Klotho · Genistein · Epigenetics · DNA methylation · Protein acetylation

Introduction

Renal interstitial fibrosis (renal fibrosis) is a typical histomorphological feature of renal aging and chronic kidney diseases (CKD) and characterized by myofibroblast trans-differentiation (MTD) and excessive deposition of extracellular matrix (ECM) proteins in renal interstitium [1]. At the subcellular level, renal fibrogenesis is regulated by a complex interplay between pro- and anti-fibrotic signaling and regulators. Pro-fibrotic signaling pathways such as TGFβ/Smad and Wnt/β-catenin promote MTD and the production of ECMs [2], while fibrosis-suppressors like Klotho, BMP-7, or Smad6/7 can block or interfere with the pro-fibrotic signaling. In addition, CKD patients with identical underlying diseases often experience various susceptibility and severity of renal fibrosis [3]. Studies investigating renal samples of patients or animals of renal fibrotic diseases detect DNA methylation alterations and aberrant

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histone deacetylase (HDAC) activities at the promoters of a number of genes known to be related to renal fibrosis [4–7], suggesting that the initiation and progression of renal fibrosis are additionally controlled by epigenetic modifications.

Epigenetics studies gene expression alterations regulated by DNA methylation, protein/histone acetylation, and miRNA interference. DNA methylation catalyzed by three bioactive methyltransferases DNMT1, DNMT3a, and DNMT3b adds a methyl group to a cytosine of CpG islands located in gene promoter and silences gene transcription via recruiting transcriptional repressor and histone deacetylase complex. On the other hand, histone acetylation is regulated inversely by histone acetyltransferases (HAT) or histone deacetylases that either positively or negatively affect gene transcription. Accumulating studies indicate that specific demethylating or HDAC inhibiting agents attenuated renal fibrosis by modulating the expression of a number of fibrosis-related genes in animal studies [8–11], making the identification of effective epigenetic drugs of lower side effects and the key target genes with therapeutic potentials an active area of renal research.

Dietary plant-derived food provides an easy source of safe bioactive substances with epigenetic modulating capacities. For example, sulforaphane and curcumin from eatable plants exhibit renal-protective properties via epigenetic modulations of DNA methylation or protein acetylation in animal studies [12–14]. Genistein, an isoflavone (4,5,7-trihydroxy isoflavone) enriched in soy and soy products with epigenetic regulatory potentials [15, 16], exhibits multiple tissue-protective activities against carcinogenesis, obesity, osteoporosis, and fibrotic disorders with a very low level of toxicity in most animal species [17, 18]. Genistein also displays effective renoprotection against cisplatin-induced kidney injury and diabetic nephropathy [19, 20], which involve active pro-fibrotic activities. However, whether genistein possesses anti-renal fibrosis function by epigenetic modulation and its cellular targets remains unknown.

Klotho is a renal tube epithelium-enriched anti-aging and fibrosis-suppressive protein. Klotho-deficient mice exhibit various aging phenotypes in almost all organs and spontaneously develop renal fibrosis [21]. Klotho transgenic overexpression or exogenous supplementation prevents renal fibrosis in various models of renal fibrotic disorders [22, 23]. Klotho exists in both membrane-bound and soluble forms. The formal mainly acts as a permissive co-receptor for fibroblast growth factor 23 [24]; whereas the soluble Klotho, derived from either membrane shedding of membrane Klotho or a secreted form due to differential splicing Klotho mRNA, is found in blood, cerebral spinal fluid, and urine and regulates the functions of various cellular membrane receptors and ion transporters by physical interactions or via its intrinsic glycosidase activities [25]. Klotho inhibits renal fibrosis by binding to Wnt or TGFβ

(transforming growth factor-beta) receptor, thereby interrupting the pro-fibrotic Wnt/β-catenin and TGFβ/Smad signaling [22, 23]. Klotho declined early in CKD patients [26, 27] and is severely suppressed after renal injury in animal models of renal fibrosis, which is prohibited by DNA methyltransferase (DNMT) or HDAC inhibitions [10, 28, 29]. Thus, it is believed that Klotho is an ideal target of epigenetic intervention in anti-renal fibrosis therapy.

In this study, we investigated the anti-renal fibrosis functions of genistein and explored the underlying mechanisms. We found that genistein is an impressive upregulator of Klotho, through which it effectively prevented renal fibrosis in unilateral ureteral occlusion (UUO) mice. We further demonstrated that genistein recovered Klotho loss via simultaneously normalizing the promoter histone 3 hypoacetylation and DNA hypermethylation. Thus, our results discover that genistein possesses dual epigenetic modulation activities that preserve a key anti-fibrosis protein Klotho in mouse fibrotic kidney, which confers its anti-renal fibrosis function.

Methods

Animal UUO model and genistein administration

C57BL/6 mice were purchased from the Model Animal Research Center of Nanjing University, and the experimental procedures were in accordance with the animal use guidelines and approved by the Institutional Animal Care and Use Committee of Nanjing University School of Medicine (Nanjing, China). Mice of eight-week-old male housed in the animal facilities of the medical school of Nanjing University, weighing 20 ± 0.5 g, were maintained with a standard diet and water ad libitum under 22 °C temperature and regular lighting conditions (12-h light/dark cycles).

Mouse model of renal fibrosis was established by a UUO procedure as detailed before [10]. The mice were randomly divided into four groups (6 mice in each group): (1) sham (5% DMSO); (2) genistein (MCE, USA, 10 mg/kgb, kilogram bodyweight, in 5% DMSO; intraperitoneal injection daily administered 1 day before UUO operation [20]); (3) UUO (5% DMSO); and (4) UUO + genistein treatment. Seven days after the initiation of UUO operation, the mice were sacrificed and the kidneys harvested by surgical procedure and stored at − 80 °C.

Cell culture and treatment

Human kidney tubular HK2 and human embryo kidney HEK293 cells (ATCC, USA) were cultured in DMEM/F12 or DMEM media, respectively as before [30], supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco, USA). Cells were cultured in a humidified 5% CO_2

incubator at 37 °C. Genistein, TGFβ (BD Biosciences, USA), fulvestrant (MCE, USA), trichostatin A (TSA, Selleck, USA) were added as indicated.

Histology

Kidney section preparations and Masson's trichrome staining were performed essentially as before [10]. Photomicrographs were obtained by a DP74 Olympus microscope (Olympus Inc., Japan). Renal fibrosis was calculated as the ratio of collagen deposition (blue color area in Masson's trichrome-stained sections) over the whole field area based on ten randomly selected non-overlapping fields for each animal and analyzed by Image J software.

Serum biochemistry

The blood was collected by intracardiac puncture immediately after the mouse sacrifice. Mouse sera were collected and analyzed for blood urea nitrogen (BUN) and creatinine (Cre) using a Chemray 430 automatic analyzer (Rayto Life & Analytical Sciences Co., Ltd., China). All serum samples were analyzed within 24 h after collection.

Western blot

Western blot analyses of renal tissues and cells were carried out as before [31, 32] with the following primary and secondary antibodies (suppliers): anti-Klotho rat monoclonal antibody (TransGenic, Japan), DNMT1 (Cell Signaling Tech., USA), DNMT3a (GeneTex, USA), DNMT3b (Epigentex, USA), E-cadherin (B&D Biosciences, USA), α-SMA (Santa Cruz, USA), β-catenin (Invitrogen, USA), collagen-I (Bioss, China), acetyl-Histone H3 (Millipore, USA), Histone3 (ABclonal, China), β-actin, goat anti-rabbit IgG-HRP, and goat anti-mouse IgG-HRP (Yifeixue Biotech, Nanjing, China). The blots were developed using an ECL plus Western blotting detection system (Vazyme, USA). The protein expression levels were determined by Image J software.

Reverse-transcription PCR

RT-PCR (reverse-transcription PCR) detecting Klotho mRNA in renal tissue was performed essentially as before [33] using the following primers: *Klotho*-F GGCTTTCCTCCTTT ACCTGAAAA; *Klotho*-R CACATCCCACAGATAGACAT TCG. *Gapdh* served as an internal control, *Gapdh*-F GGCCCGGTGCTGAGTATGTC; GAPDH-R TGCCTGCT TCACCACCTTCT. The relative quantification of RT-PCR data is presented as the expression ratio of Klotho over GAPDH gene.

Chromatin immunoprecipitation

ChIP (chromatin immunoprecipitation) assay is performed with renal tissues or HK2 cells to detect the association of acetylated histone 3 with Klotho promoter under various conditions using a protocol established before [28]. The Immunoprecipitation was performed with anti-acetyl histone H3 antibody (Millipore, Billerica, MA) or an isoform-matched IgG as a control. The immunoprecipitated DNA was analyzed by PCR using primers specific for mouse or human Klotho promoter as following: mouse, mKLpF (5′-GCTGAGTTG TACCTTACTGAG-3′) and mKLpR (5′-CACCATATCCCGTTCATCAC-3′); human, hKLpF (5′-GCCACCATGTTGGTGAATTT-3′) and hKlpR (5′-TCAC ACCTGTAATCCCAGCA-3′). After amplification, the PCR products were separated in 1.5% agarose gels and visualized under UV light by staining with ethidium bromide. The Image J software was used for optical densitometry analysis.

Methylation-specific PCR and bisulfite-sequencing PCR

MSP (methylation-specific PCR) and BSP (bisulfite-sequencing PCR) for *Klotho* promoter methylation examination of mouse kidney tissues and human HK2 cells were performed essentially as before [33]. The prediction of CpG islands in mouse *Klotho* promoter and the primer designs for methylation-specific PCR (MSP) or bisulfite-sequencing PCR (BSP) were performed by online program MethPrimer (http://www.urogene.org/methprimer) and depicted in Fig. 4a. MSP primer sequences for mouse were as follows: ME-mKL-F GGTATCGCG GGTATTTTTA ATC and ME-mKL-R CGACATAATCCCT AAAA TAATCGAC; UM-mKL-F TTAAT GGTA TTGTGG GTATTTTTAATTG and UM-mKL-R CAACA TAATCCCTAAA ATAATCAAC; for input DNA control, Inp-mF TAGTTTTA GGAAGGTAAAGGGA GTG and Inp-mR AAATACCCAAAAAAAAACACA ACAAA; for human Klotho promoter, ME-hKL-F AAAGAGA ATGAATTTGAGCGTTTAC and ME-hKL-R ACTCCGCT AACAATAATACCTACG; UM-hKL-F AAGAGAATGAAT TTGAGTGTTTATGA and UM-hKL-R TCCACTAACAATAATTACCTACAAA; for input DNA control, Inp-hF C CAACTCCAAATCCCCTCTCTAT and Inp-hR TGATTAA TTTAGATTGGGTTT AGAGAAGGA. PCR-amplified products were analyzed by electrophoresis on a 2% agarose gel and quantified by Image J software.

Primers for bisulfite-sequencing PCR of mouse *Klotho* promoter were Bis-mKL-F TAGTTTTAGGAAGGTAAAGG GAGTG and Bis-mKL-R AACAATAATTATCCAA AACAAAC. The PCR products were purified and cloned into pGEM-T Easy Vector (Promega, USA). Five to ten clones from each mouse were randomly chosen for sequencing analysis.

Luciferase assay

A mouse Klotho promoter reporter plasmid (mKLp-Luc) was constructed and assayed previously [33]. For luciferase assay, HEK293 cells were transiently transfected with plasmid mKLp-Luc plus a *Renilla* luciferase control plasmid with Lipofectamine 2000 (Invitrogen, USA) reagents. The transfected cells were treated with TGFβ and/or genistein for 48 h before luciferase activities were assayed by a microplate illuminometer using a dual luciferase reporter assay kit (Promega, USA). Luciferase activities were normalized to *Renilla* luciferase levels and expressed as relative fold changes.

Klotho suppression by RNA interferences

Klotho knockdown in cells or in kidneys was performed with shRNA or siRNA strategy, respectively, as before [33]. For Klotho knockdown in HK2 cells, a small hairpin RNA plasmid specific for human Klotho (shRNA-Klotho) and a control plasmid containing a scrambled sequence (shRNA-control) were constructed. shRNA-Klotho plasmid contains oligo sequences CTTCCTTATTTCACTGA AGATCTCGAGATCT TCAGT GAAATAAGGAAG. HK2 cell was transfected with control or shRNA-Klotho for 24 h before the experiments. For in vivo *Klotho* knockdown, a small interfering RNA targeting mouse *Kloth*o gene 5′-GCGACTACCCAGAG AGTAT-3′ and a scrambled RNA control (10 nm in 200 μl of PBS for each mouse, respectively) were injected into mouse tail vein 1 day before UUO operation.

Statistical analysis

The statistical analyses were based on at least three independent experiments for cell experiments or at least six mice in each group for animal studies. Data are represented as mean ± standard deviation (SD). Statistical significance was assessed by Student's *t* test for the comparisons of two groups and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for comparisons of multiple groups. $P < 0.05$ and $P < 0.01$ were considered statistically significant or very significant.

Results

Genistein alleviates UUO-incurred renal fibrosis and Klotho repression

We first tested our idea that genistein possesses anti-renal fibrosis functions in a well-established mouse model of UUO-incurred renal fibrosis. Mouse kidney subjected to UUO for 7 days displayed severe renal interstitial fibrosis

evidenced by Masson-stained collagen depositions (3.7 ± 0.7 to $23 \pm 5.7\%$ of blue-colored areas in Fig. 1a and the quantification in Fig. 1b, $P < 0.01$) and the functional loss indicated by increased serum creatinine and blood urine nitrogen (BUN) (Fig. 1c). Consistently, the UUO kidneys showed aberrant expressions of renal fibrosis-related proteins including epithelial marker E-cadherin, myofibroblast marker α-SMA (alpha-smooth muscle actin), β-catenin, and extracellular matrix type I collagen (Fig. 1d, e). However, genistein pretreatment at a dose 10 mg/kgb [20] significantly mitigated the renal fibrotic lesions (from 23 ± 5.7 to $8.8 \pm 5.8\%$ of blue-colored areas, Fig.1a, b; $P < 0.05$), the abnormal serum levels of creatinine and BUN (Fig. 1c), and the aberrant expression of renal fibrosis-associated proteins (Fig. 1d, e). As expected, UUO kidney suffered from marked reductions of Klotho protein and mRNA as reported previously [33], which were effectively corrected by genistein treatment (Fig. 1d, e). Since the restoration of endogenous Klotho reportedly protected the kidney from renal fibrosis in mice [10], these results suggest that genistein restoration of Klotho may contribute to its anti-renal fibrosis actions in UUO kidney.

Genistein attenuates TGFβ-induced pro-fibrotic protein expression and Klotho repression in kidney cells

To confirm the anti-renal fibrosis effects associated with Klotho restoration by genistein, we tested the genistein regulation of Klotho and the expressions of renal fibrosis-related proteins in renal tubule cells. Transforming growth factor beta (TGFβ) is considered the primary pathological factor that causes Klotho suppression and promotes renal fibrosis [33, 34]. Indeed, TGFβ treatment of renal tube HK2 cells led to reduced Klotho, E-cadherin, and increased α-SMA, β-catenin, and collagen I, which were reversed by genistein treatment (Fig. 2a, b). Further, TGFβ inhibited the transcriptional activity of a luciferase reporter driven by the mouse Klotho promoter, but the inhibitory effects were significantly relieved by genistein (Fig. 2c). Genistein possesses estrogenic activities [35, 36]. Administration of fulvestrant, an estrogen receptor (ER) antagonist, largely abolished the Klotho recovery by genistein (Fig. 2d), suggesting that genistein preserved Klotho at least in a significant part via its activation of estrogen receptor (ER).

Genistein anti-deacetylation properties contribute to the Klotho restoration

Previous studies reported that genistein exhibits HDAC inhibitory property in tumor cell lines [37] and Klotho downregulation in the fibrotic kidney can be relieved by HDAC inhibition [28, 38], suggesting that genistein inhibition of HDAC activities might contribute to the Klotho restoration. To test
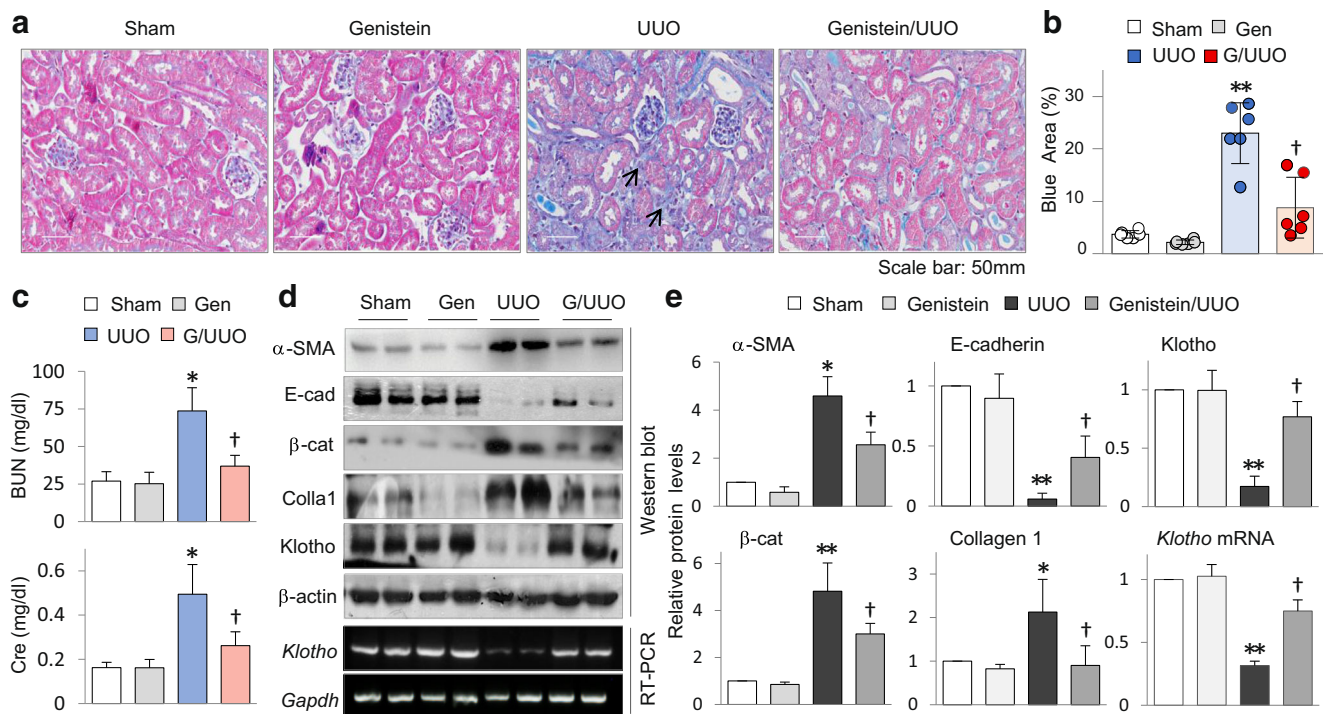
(TSA, Fig. 3a). Further, UUO kidney and TGFβ-treated HK2 cells displayed reduced histone 3 acetylation, possibly due to aberrant HDAC expressions. Genistein treatment marginally increased the histone 3 acetylation in the kidney of normal mice and untreated HK2 cells, but significantly reversed the histone 3 hypoacetylation in both UUO kidney and TGFβ-treated renal cells (Fig. 3b, c). In addition, this effect was partially inhibited by fulvestrant treatment (Fig. 3c, right panel), suggesting that genistein inhibition of HDAC activity relies in part on its estrogenic effect. To directly test whether genistein-induced histone 3 acetylation regulates Klotho, we examined the amounts of acetylated histone 3 that were associated with Klotho promoter by chromatin immunoprecipitation (ChIP) assay. The results showed that genistein slightly increased, but UUO and TGFβ significantly reduced, the amount of acetylated histone 3 associated with Klotho promoter in both mouse kidney and HK2 cells; however, genistein effectively inhibited the association reduction (Fig. 3d, e). Taken together, these results clearly demonstrate that genistein inhibition of HDAC activities contributes at least in part to the

Klotho restoration by genistein in both UUO kidney and TGFβ-treated renal cells.

Genistein attenuates pathological Klotho promoter hypermethylation in vitro and in vivo

Both human and mouse Klotho promoters are characterized by heavy CpG islands as depicted in Fig. 4a for mouse Klotho promoter. Klotho promoter hypermethylation has been reported in patients and in animal models of renal fibrotic diseases by others' and our previous studies [10, 33, 39], which accounts for the Klotho suppression. Again, we confirmed by methylation-specific PCR (MSP) that Klotho promoter methylation levels significantly increased in both mouse UUO kidney (Fig. 4b, c, from 34.9 ± 1.6 to $63.2 \pm 7.9\%$, $P < 0.05$) and TGFβ-treated HK2 cells (Fig. 4d, e, from 36.9 ± 4.3 to $54.6 \pm 11.5\%$, $P < 0.05$). However, genistein treatment brought the levels back to approximate $45.5 \pm 4.3\%$ and $37.2 \pm 6.3\%$ ($P < 0.05$), respectively. To confirm these results we further performed bisulfite-sequencing PCR (BSP), the golden
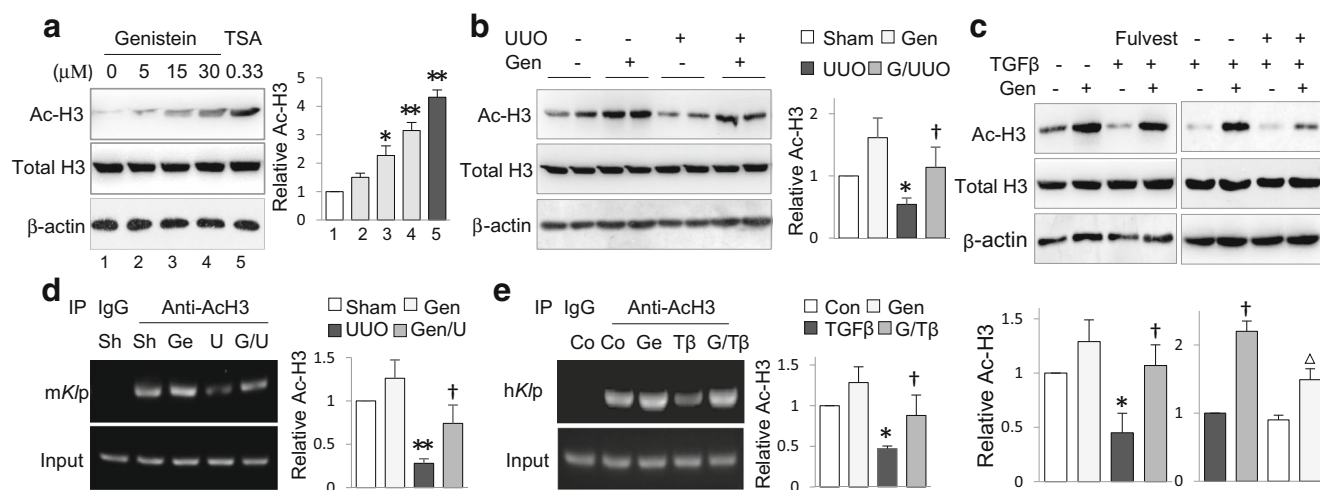
Fig. 3 Genistein anti-deacetylation property contributes to the Klotho restoration. **a** Genistein anti-deacetylation assay. HK2 cells were treated with increasing doses of genistein (Gen, 5, 15, and 30 μM) and the positive control TSA (0.33 μM) for 24 h, and then cell lysates were tested for acetylated (Ac-H3) and total histone 3 (T-H3) by Western blot. The quantifications are on the right side. **b** Renal histone 3 acetylation assay. Mouse kidney lysates from sham, genistein, UUO, and genistein-treated UUO mice (7 days, $n = 6$) were assayed for acetylated histone 3 with a pan-acetylated histone 3 antibody by Western blot. Two randomly selected samples from each group were presented. The levels of β-actin served as loading control. The quantifications were based on all experimental mice and are presented on the right side. **c** Acetylated histone 3 assay of the renal cell. HK-2 cells treated with genistein (15 μM) and/or TGFβ (5 ng/ml) for 24 h were assayed with anti-acetylated and anti-total histone 3 antibodies by Western blot. In a separate assay, cell lysates treated with TGFβ and genistein in the presence or absence of fulvestrant (Fulvest, 1 μM) under the same condition were also assayed. The quantifications are presented underneath the blots based on three independent experiments. **d** Mouse kidney ChIP assay. Antibodies against acetylated histone

H3 (AcH3) and an isoform-matched control (IgG) were used in ChIP assay to isolate acetylated chromatin fragments from renal cell lysates of sham, genistein (Gen), UUO, and genistein-treated UUO mice, respectively. The precipitated DNA fragments were further PCR-amplified with primer sets specific for mouse Klotho promoter (mKLpro). PCR products using DNAs prior immunoprecipitation served as input (Input) control. The PCR products were analyzed on a 1.5% agarose gel and visualized under UC light. The representative results are shown. The quantifications based on all experimental animals are presented on the right side. **e** Renal cell ChIP assay. HK2 cells treated with genistein (15 μM), TGFβ (5 ng/ml), or genistein plus TGFβ for 24 h were processed with ChIP assay similar to the abovementioned renal tissue ChIP, except a PCR primer set specific for human Klotho promoter was employed. The PCR products were analyzed on a 1.5% agarose gel and visualized under UC light. The quantifications were based on three independent experiments and are presented on the right side. Data are presented as mean ± SD. *$P < 0.05$, **$P < 0.01$ vs. sham or control; †$P < 0.05$ vs. UUO or TGFβ treatment; △$P < 0.05$ vs. TGFβ/genistein treatment
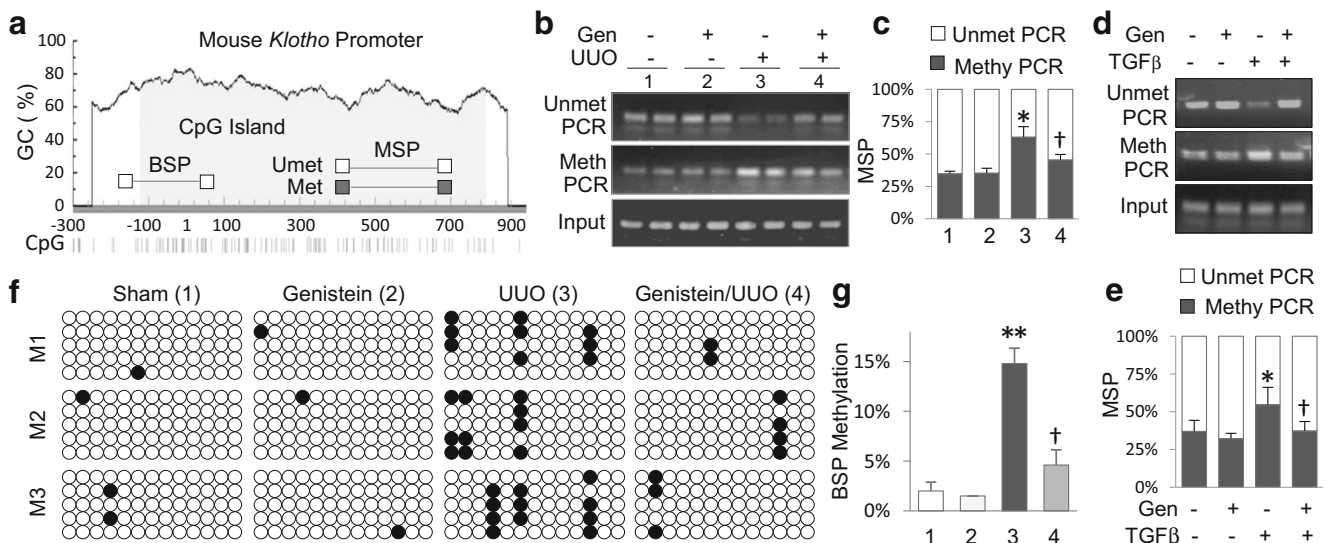
Fig. 4 Genistein attenuates pathological Klotho promoter hypermethylation in vitro and in vivo. **a** Schematic diagram of mouse Klotho promoter (-300 to 900 base pair related to the transcription start site). The guanine/cytosine contents are indicated on the Y-axis. The CpG islands are in gray. The approximate locations of methylated or unmethylated specific PCR (MSP) primers as well as primers for BSP are indicated. **b** MSP analysis of mouse kidney DNAs. Mouse kidney tissues from sham, genistein, UUO, and genistein-treated UUO mice (7 days, $n = 6$ as in Fig. 1) were subjected to MSP analysis using primer sets that were specific for mouse Klotho promoter as shown in **a**. PCR products were analyzed on 1.5% agarose gel and visualized under UV light. Two randomly selected samples from each group are shown. **c** Quantifications of **b** based on all experimental mice. **d** MSP analysis of

human Klotho promoter. HK2 cells were treated with genistein (15 μM) in the presence or absence of TGFβ (5 ng/ml) for 24 h, and then cellular DNAs were subjected to MSP analysis using a primer set specific for human Klotho promoter. **e** Quantifications of **d** based on three independent experiments. **f** BSP assay of experimental mice. Renal tissues from the abovementioned experimental mice were analyzed by BSP analysis. The black circle represents methylated cytosine and the white circle the unmethylated. Results of three mice (M1–M3) from each group and five clones from each mouse are shown. **g** Quantification of **f**. The statistical analysis was based on all experimental mice unless otherwise indicated, and the only representative results are shown. Data are presented as mean ± SD. *$P < 0.05$ vs. control cells or sham mice; †$P < 0.05$ vs. TGFβ treatment or UUO mice

standard for DNA methylation determination, on a different region of Klotho promoter (-234 to $+14$ related to transcription starting site). Similarly, UUO kidney showed a significant increase of Klotho promoter methylation from 2 ± 0.89 to $14.8 \pm 1.5\%$ ($P < 0.01$), but genistein treatment reduced the level to $4.6 \pm 0.15\%$ ($P < 0.05$, Fig. 4f, g). Since Klotho promoter hypermethylation is a driving force for Klotho suppression in UUO kidney, these results suggest that genistein demethylation of Klotho promoter substantially prevents the renal fibrotic Klotho loss.

Genistein normalizes aberrant expressions of DNMT1 and DNMT3a

In mammals, DNA methylations are catalyzed by the enzymatic activities of three bioactive DNA methyltransferases, including DNMT1, the key maintenance methyltransferase, and DNMT3a/DNMT3b, for de novo methylation. We found that TGFβ treatment and UUO incurred a dramatic increase of DNMT1 and DNMT3a in HK2 cells (Fig. 5a, b) and mouse kidney (Fig. 5c, d), respectively, but did not noticeably alter DNMT3b level. However, genistein treatment significantly inhibited the abnormal expressions of DNMT1 and DNMT3a in both TGFβ-treated HK2 cells (Fig. 5a, b) and

UUO kidney (Fig. 5c, d). Further, genistein inhibition of DNMT1 and DNMT3a was also sensitive to its estrogenic activity since the ER antagonist fulvestrant substantially prevented the inhibitory effects (Fig. 5e, f). Taken together, these results suggest that genistein possesses demethylating capacity by inhibiting DNMT1 and DNMT3a under renal fibrotic conditions, which explains its alleviations of Klotho promoter hypermethylation and Klotho depression.

Klotho is crucial for the normalization of fibrotic protein expression by genistein in renal cells

A critical question we wanted to address is whether Klotho is an essential cellular target that mediates genistein's anti-renal fibrosis functions. If genistein preservation of Klotho is crucial for its anti-fibrotic actions, then lack of Klotho would reduce or abolish the beneficial effects. Based on this assumption, we first investigated the effects of Klotho knockdown on genesis' anti-fibrotic activities in renal cells. We employed a control plasmid expressing a scrambled RNA sequence and a siRNA-interfering plasmid expressing a pre-miRNA hairpin (shRNA-Klotho) that specifically targeted and downregulated Klotho when transfected into HK2 cells (Fig. 6a (top panel), b). We then compared the effects of Klotho knockdown on
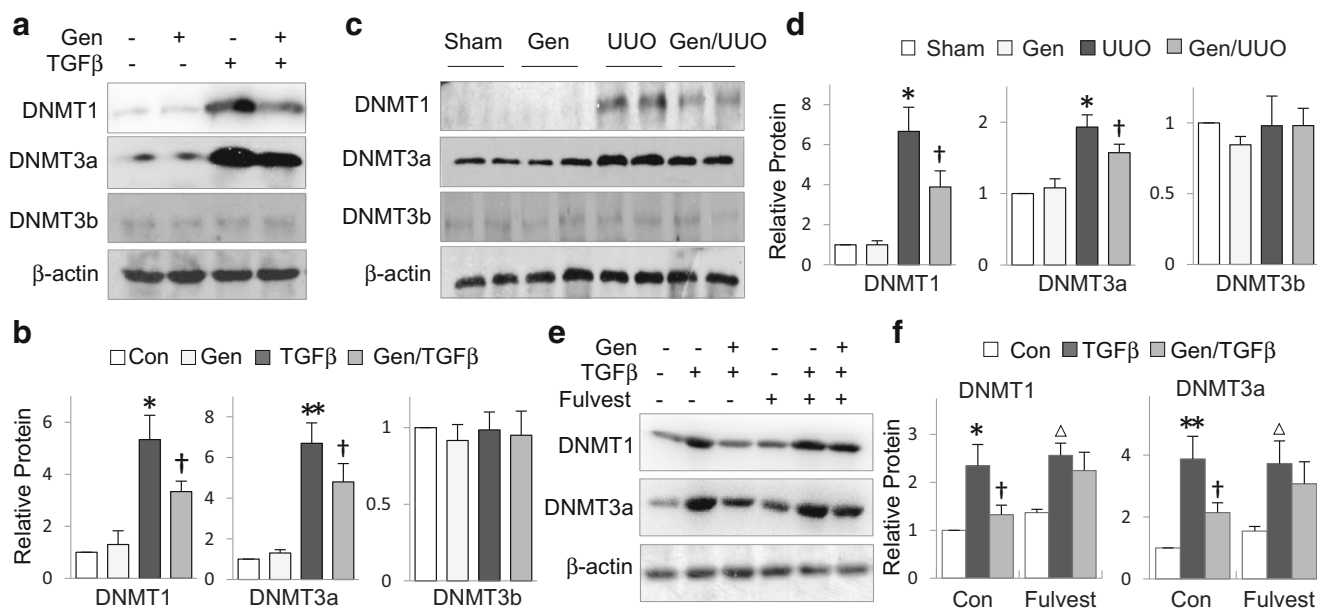
Fig. 5 Genistein mitigates the aberrant expressions of DNMT1 and DNMT3a in vitro and in vivo. **a** Renal cell DNMT assay. HK2 cells were treated with genistein (15 μM) in the presence or absence of TGFβ (5 ng/ml) for 24 h, and then the cell lysates were assayed for DNMT1, DNMT3a, and DNMT3b by Western blot. **b** Quantification of **a**. **c** Kidney tissue DNMT assay. The expression of DNMT1, DNMT3a, and DNMT3b from sham, genistein, UUO, and UUO mice ($n = 6$) were assayed by Western blot (two randomly selected samples from each group are shown). **d** Quantification of **c**, which was based on all animals tested. **e** Fulvestrant assay. HK2 cells were treated with or without genistein (15 μM) and TGFβ (5 ng/ml) in the presence or absence of fulvestrant (Fulvest, 1 μM) for 24 h, and then Klotho protein levels were assayed by Western blot. **f** Quantifications of **e**, which were based on three independent experiments. Data are presented as mean ± SD. *$P < 0.05$, **$P < 0.01$ vs. control cells or sham mice; †$P < 0.05$ vs. UUO mice or TGFβ treatment; ᐃ$P < 0.05$ vs. control in fulvestrant treatment

genistein improvements of the key fibrosis-related protein expressions after TGFβ treatment. The results showed that Klotho knockdown potentiated basal, but exacerbated TGFβ-induced expression of α-SMA, E-cadherin, β-catenin, and collagen I comparing to that in control cells (Fig. 6a, b, lane 1 vs. lane 5), suggesting that Klotho is an upstream and master regulator of the pro-fibrotic protein expressions. Again, genistein treatment significantly improved the TGFβ-induced abnormal protein expressions in control cells, but these inhibitory effects were only marginal in Klotho knockdown cells (Fig. 6a, b), supporting that Klotho is crucial for genistein's anti-fibrosis activity in renal cells.

Klotho is essential for the anti-renal fibrosis function of genistein in UUO mice

Further, we performed the mouse study to explore the critical roles of Klotho in genistein's anti-renal fibrosis functions in vivo. We adopted a small interference RNA (siRNA) strategy that effectively knocked down Klotho in mouse kidney (Fig. 7d, top panel). Similar to cell assay, Klotho knockdown not only prompted basal renal fibrotic histological changes (4 ± 0.6% vs. 7.6 ± 4.5% of blue-colored area) and the abnormal expressions of renal fibrosis-associated protein E-cadherin, α-SMA, β-catenin, and collagen I (Fig. 7d, land 1/2 vs. 7/8), but also aggravated UUO-incurred renal fibrotic lesions (31 ±

7.1% vs. 49 ± 11%, $P < 0.05$, Fig. 7a, b), serum BUN and creatinine productions (Fig. 7c), and the expressions of the renal fibrosis-associated proteins (Fig. 7d, lane 3/4 vs. 9/10). Genistein treatment effectively reduced the renal fibrosis severity (14 ± 6.5%, $P < 0.05$) and the levels of serum BUN and creatinine (Fig.7c), and significantly corrected the abnormal protein expressions in control siRNA-injected mouse kidney (Fig. 7d, left panel). Moreover, the above beneficial anti-renal fibrotic effects were largely abolished when Klotho was knocked down (Fig. 7a–e). Collectively, these results clearly indicate that genistein preservation of Klotho is a critical mechanism that confers genistein's anti-renal fibrosis functions in vivo.

Discussion

It is well recognized that many epigenetic drugs of small chemical compounds are cytotoxic and their off-target and the long-term effects of genome-wide hyperacetylation or demethylation raise safety concerns. "Epigenetic diets" are emerging as relatively safe bioactive supplementations for the prevention and treatment of epigenetic disorders [40]. In this study, we have revealed a prominent mode of anti-renal fibrosis action by genistein and identified Klotho as a key gene that mediated the beneficial effects. In mouse fibrotic
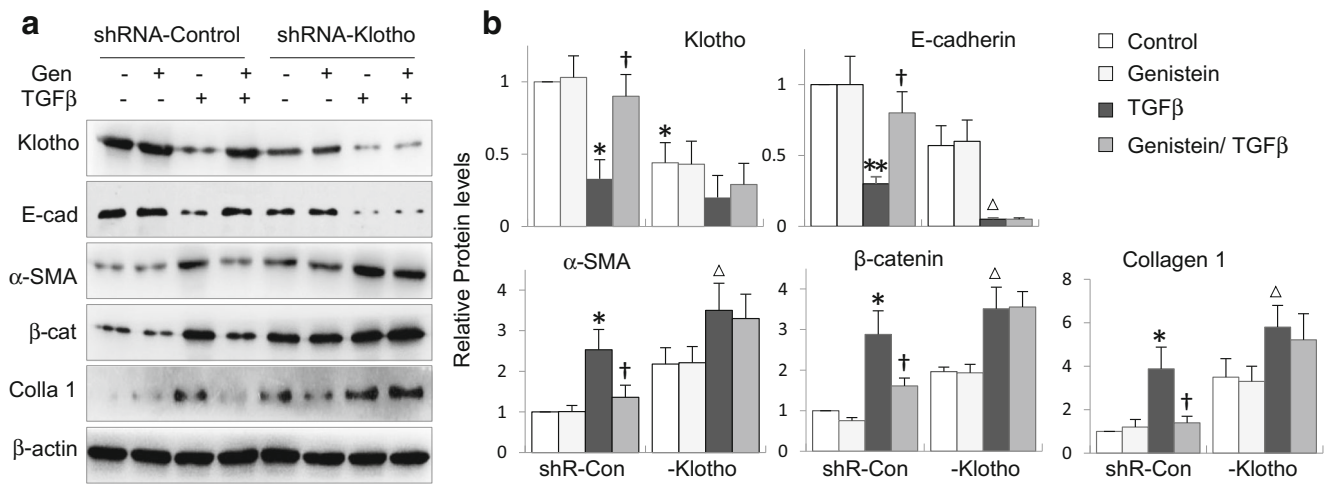
Fig. 6 Klotho is crucial for the normalization of fibrotic protein expression by genistein in renal cells. **a** HK2 cells were transfected with control or Klotho specific shRNA plasmid, and then treated with genistein (15 μM) in the presence or absence of TGFβ (5 ng/ml) for 48 h. The expressions of E-cadherin, α-SMA, β-catenin, collagen I, and Klotho were assayed by Western blotting. **b** Quantifications of protein levels in **a**. Cell assays were repeated three times and representative data are shown. Results are presented as mean ± SD of fold changes. *$P < 0.05$, **$P < 0.01$ vs. shRNA-control cells; †$P < 0.05$ vs. TGFβ-treated cells; △$P < 0.05$ vs. shRNA-Klotho-transfected control cells
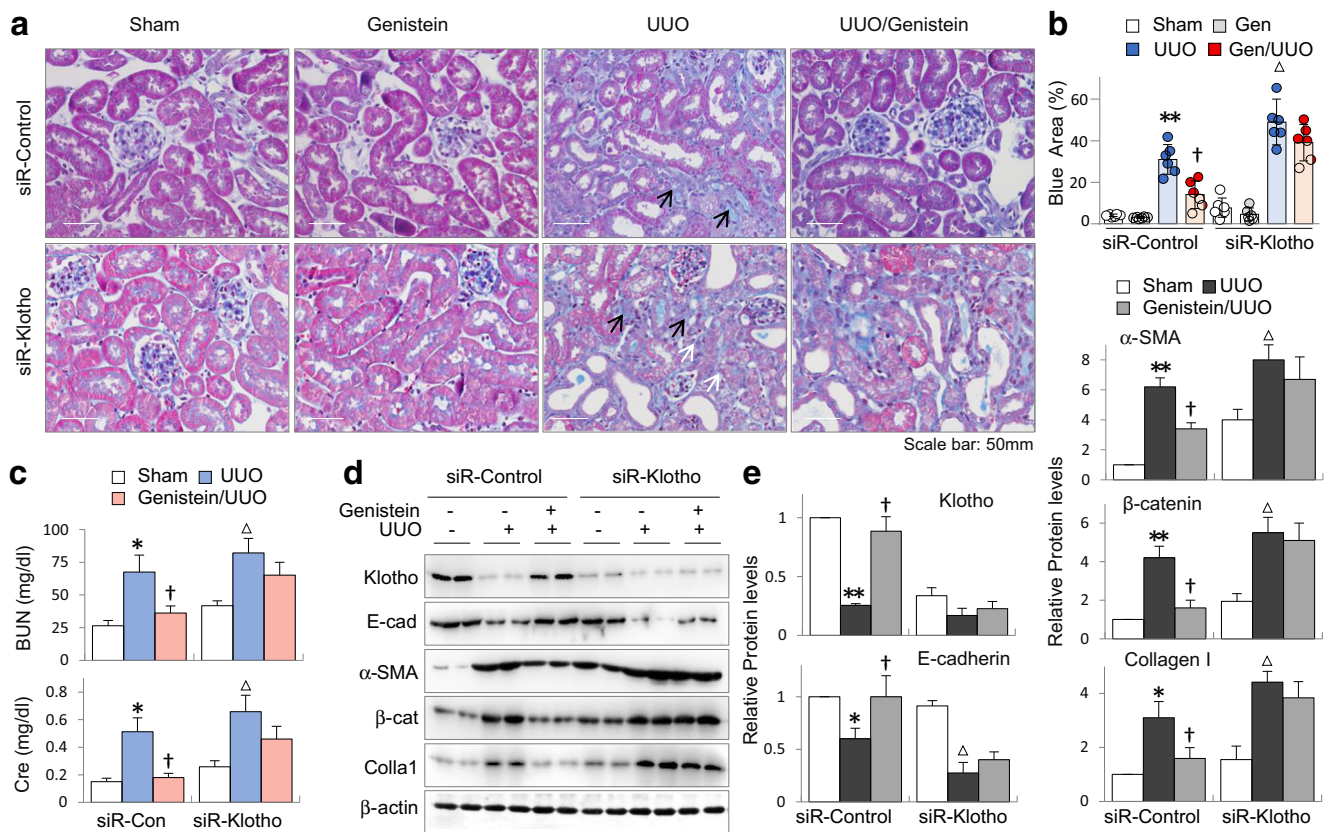

Fig. 7 Klotho is essential for the anti-renal fibrosis function of genistein in UUO mice. **a** Representative Masson's trichrome-stained kidney sections from siRNA-control- or siRNA-Klotho-injected sham, genistein (Gen), UUO, or genistein-treated UUO mice ($n = 6$ in each group, 7 days). Renal fibrosis stained in blue is indicated by arrows. **b** Quantification of mouse kidney fibrosis from all mice as in **a**. **c** Average concentrations of serum creatinine (Cre) and blood urine nitrogen (BUN) from experimental mice as above. **d** Renal lysates from the abovementioned animals were analyzed for the expression of Klotho, E-cadherin (E-cad), α-SMA, β-catenin (β-cat), and collagen I (Col-I) by Western blot. Two randomly selected samples from each group are shown. **e** Quantifications of average protein levels in **d** based on all mice tested. Results are presented as mean ± SD. *$P < 0.05$, **$P < 0.01$ vs. siRNA-control-treated sham mice; †$P < 0.05$ vs. siRNA-control-treated UUO mice; △$P < 0.05$ vs. siRNA-Klotho-treated sham mice

kidney incurred by UUO, Klotho is severely depressed mainly due to the histone deacetylation and DNA hypermethylation on Klotho promoter incurred by aberrant HDAC and DNMT1/DNMT3a induction. Genistein effectively corrected these epigenetic alterations and reversed the Klotho suppression. Moreover, genistein alleviated renal fibrosis in a Klotho restoration-dependent manner demonstrated by in vivo siRNA-mediated Klotho silencing assay. Therefore, our results clearly demonstrated that genistein exerts impressive anti-renal fibrosis functions essentially through Klotho restoration by a dual epigenetic regulatory mechanism.

Epigenetic alterations mediated by DNA methylation and protein acetylation affect gene transcription via different mechanisms but are mechanistically connected. Methylated CpGs on gene promoter are recognized and bound by methyl-binding proteins complexed with transcription co-repressors and HDACs. HDACs remove the acetyl groups from lysine on the core histone tails and restore the positive charge of lysine, resulting in chromatin compaction and gene transcription silencing [41]. Indeed, we detected the DNA hypermethylation that is associated with deacetylation of histone 3 on Klotho promoter in the fibrotic kidney. We further showed that simultaneous inhibition of the hypermethylation and histone deacetylation on Klotho promoter by genistein, even with a weaker acetylation capacity comparing to TSA (Fig. 3a), effectively recovered Klotho-deficit and reduced renal fibrosis lesions. These results provide the first evidence that Klotho restoration by a combination of DNA demethylation and histone acetylation strategies can restore Klotho more efficiently and suggest that many dietary bioactive substances with epigenetic regulatory capacities might prevent Klotho loss and renal fibrosis in a similar mode of actions, which are worthy of future exploration.

As we demonstrated that genistein prevented the Klotho loss via epigenetic modulations, the direct link between genistein and its simultaneous modifications of the abnormal protein hypoacetylation and DNA hypermethylation is still missing. Genistein as a phytoestrogen can bind to the estrogen receptor (ER) and exert its biological effects through either ER- or non-ER-mediated signaling pathways [42]. Estrogen receptor activations have been shown to promote DNA demethylation [43] and core histone acetylation [16] via unclarified mechanisms. Many endogenous and exogenous ER ligands such as 17β-estradiol and tamoxifen can either positively or negatively regulate the ER activities [43, 44]. One study reported that treatment of MDA-MB-231 cells with a combination of green tea polyphenols and sulforaphane led to Klotho reactivation through active chromatin modifications, which was partially dependent on ERα reactivation [45]. Our results further demonstrate that genistein inhibitions of aberrant DNMA1/3a elevation, histone 3 acetylation and Klotho depression are partially blocked by ER inhibitor (Figs. 2d, 3c, 5e), strongly suggesting that genistein's estrogenic effects contribute, at least in a significant part, to its Klotho restoration and anti-renal fibrosis functions, although the additional ER-independent pathways through which genistein exerts its epigenetic modulation functions are intriguing but currently unclear.

Klotho has been considered a sensitive biomarker of renal injury, and its levels closely and inversely correlate with the severity of renal fibrosis in patients and animals of CKD [46–48]. Previous and our current studies indicated that Klotho suppression in the fibrotic kidney is inhibited by either demethylating or HDAC inhibiting agents, suggesting that the aberrant expressions of DNMT1 and DNMT3a, and HDAC activities contribute to the Klotho suppression [10, 33, 38, 49]. Although the information regarding Klotho levels before patients developed renal diseases is limited, decreased plasma soluble Klotho is detected in aged populations in a study of 2496 participants, which correlated with the poor renal functions and incident CKDs [50]. This important observation implies that other than various renal injuries that suppress Klotho, people with prior epigenetic influences due to the life history of drug use, hazardous food consumption, illness, or unfavorable environmental exposures might lower their Klotho levels via epigenetic modifications, which would predispose them to renal fibrosis or exacerbate the renal fibrosis progression. Klotho is present in serum and urine and easily detectable. Future study of its levels in the susceptible populations might establish whether Klotho is a biomarker of pre-renal fibrotic conditions.

In conclusion, our study has uncovered an important feature of genistein restoration of Klotho that contributes significantly to its anti-renal fibrosis function, suggesting that dietary bioactive substances are potential sources of epigenetic drugs in clinical anti-renal fibrosis therapy. Our results also have other medical implications. Because Klotho is an anti-aging protein critically involved in aging and aging-related disorders such as tumor, cardiovascular, and neurodegenerative diseases [51], Klotho preservation by genistein or other dietary epigenetic regulators might retard or beneficially affect these pathological processes.

Compliance with ethical standards

References

1. Liu YH (2006) Renal fibrosis: new insights into the pathogenesis and therapeutics. Kidney Int 69:213–217

2. Woroniecki R, Gaikwad AB, Susztak K (2011) Fetal environment, epigenetics, and pediatric renal disease. Pediatr Nephrol 26:705–711

3. Wing MR, Ramezani A, Gill HS, Devaney JM, Raj DS (2013) Epigenetics of progression of chronic kidney disease: fact or fantasy? Semin Nephrol 33:363–374

4. Ko Y-A, Mohtat D, Suzuki M, Park ASD, Izquierdo MC, Han SY, Kang HM, Si H, Hostetter T, Pullman JM, Fazzari M, Verma A, Zheng D, Greally JM, Susztak K (2013) Cytosine methylation changes in enhancer regions of core pro-fibrotic genes characterize kidney fibrosis development. Genome Biol 14:R108

5. Smyth LJ, McKay GJ, Maxwell AP, McKnight AJ (2014) DNA hypermethylation and DNA hypomethylation is present at different loci in chronic kidney disease. Epigenetics 9:366–376

6. Nguyễn-Thanh TKD, Lee S, Kim W, Park SK, Kang KP (2018) Inhibition of histone deacetylase 1 ameliorates renal tubulointerstitial fibrosis via modulation of inflammation and extracellular matrix gene transcription in mice. Int J Mol Med 41:95–106

7. Kumar PGV, Periasamy R, Raghavaraju G, Subramanian U, Pandey KN (2017) Inhibition of HDAC enhances STAT acetylation, blocks NF-κB, and suppresses the renal inflammation and fibrosis in Npr1 haplotype male mice. Am J Physiol Renal Physiol 313:F781–F795

8. Reddy MA, Natarajan R (2015) Recent developments in epigenetics of acute and chronic kidney diseases. Kidney Int 88:250–261

9. Marumo T, Hishikawa K, Yoshikawa M, Fujita T (2008) Epigenetic regulation of BMP7 in the regenerative response to ischemia. J Am Soc Nephrol 19:1311–1320

10. Zhang Q, Yin S, Liu L, Liu Z, Cao W (2016) Rhein reversal of DNA hypermethylation-associated Klotho suppression ameliorates renal fibrosis in mice. Sci Rep 6. https://doi.org/10.1038/srep34597

11. Tampe B, Tampe D, Muller CA, Sugimoto H, LeBleu V, Xu X, Muller GA, Zeisberg EM, Kalluri R, Zeisberg M (2014) Tet3-mediated hydroxymethylation of epigenetically silenced genes contributes to bone morphogenic protein 7-induced reversal of kidney fibrosis. J Am Soc Nephrol 25:905–912

12. Vahid FZH, Nosrat-Mirshekarlou E, Najafi R, Hekmatdoost A (2015) The role dietary of bioactive compounds on the regulation of histone acetylases and deacetylases: a review. Gene 562:8–15

13. Senanayake GVBA, Wu L, Lee P, Juurlink BH (2012) The dietary phase 2 protein inducer sulforaphane can normalize the kidney epigenome and improve blood pressure in hypertensive rats. Am J Hypertens 25:229–235

14. Hu YML, Yang F, Tu H, Lin W (2016) Curcumin attenuates cyclosporine A-induced renal fibrosis by inhibiting hypermethylation of the Klotho promoter. Mol Med Rep 14:3229–3236

15. Day JK, Bauer AM, desBordes C, Zhuang Y, Kim BE, Newton LG, Nehra V, Forsee KM, MacDonald RS, Besch-Williford C et al (2002) Genistein alters methylation patterns in mice. J Nutr 132:2419S–2423S

16. Hong TNT, Pan W, Kim MY, Kraus WL, Ikehara T, Yasui K, Aihara H, Takebe M, Muramatsu M, Ito T (2004) Isoflavones stimulate estrogen receptor-mediated core histone acetylation. Biochem Biophys Res Commun 317:259–264

17. Ganai AAFH (2015) Bioactivity of genistein: a review of in vitro and in vivo studies. Biomed Pharmacother 76:30–38

18. Messina MJPV, Setchell KD, Barnes S (1994) Soy intake and cancer risk: a review of the in vitro and in vivo data. Nutr Cancer 21:113–131

19. Sung MJ, Kim DH, Jung YJ, Kang KP, Lee AS, Lee S, Kim W, Davaatseren M, Hwang J-T, Kim H-J, Kim MS, Kwon DY, Park SK (2008) Genistein protects the kidney from cisplatin-induced injury. Kidney Int 74:1538–1547

20. Elmarakby AA, Ibrahim AS, Faulkner J, Mozaffari MS, Liou GI, Abdelsayed R (2011) Tyrosine kinase inhibitor, genistein, reduces renal inflammation and injury in streptozotocin-induced diabetic mice. Vasc Pharmacol 55:149–156

21. Kuroo M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, Ohyama Y, Kurabayashi M, Kaname T, Kume E et al (1997) Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature 390:45–51

22. Doi S, Zou Y, Togao O, Pastor JV, John GB, Wang L, Shiizaki K, Gotschall R, Schiavi S, Yorioka N et al (2011) Klotho inhibits transforming growth factor-beta1 (TGF-beta1) signaling and suppresses renal fibrosis and cancer metastasis in mice. J Biol Chem 286:8655–8665

23. Satoh M, Nagasu H, Morita Y, Yamaguchi TP, Kanwar YS, Kashihara N (2012) Klotho protects against mouse renal fibrosis by inhibiting Wnt signaling. Am J Physiol Renal Physiol 303:F1641–F1651

24. Urakawa I, Yamazaki Y, Shimada T, Iijima K, Hasegawa H, Okawa K, Fujita T, Fukumoto S, Yamashita T (2006) Klotho converts canonical FGF receptor into a specific receptor for FGF23. Nature 444:770–774

25. Sanchez-Nino MD, Sanz AB, Ortiz A (2013) Klotho to treat kidney fibrosis. J Am Soc Nephrol 24:687–689

26. Akimoto T, Yoshizawa H, Watanabe Y, Numata A, Yamazaki T, Takeshima E, Iwazu K, Komada T, Otani N, Morishita Y, Ito C, Shiizaki K, Ando Y, Muto S, Kuro-o M, Kusano E (2012) Characteristics of urinary and serum soluble Klotho protein in patients with different degrees of chronic kidney disease. BMC Nephrol 13:155

27. Kitagawa M, Sugiyama H, Morinaga H, Inoue T, Takiue K, Ogawa A, Yamanari T, Kikumoto Y, Uchida HA, Kitamura S, Maeshima Y, Nakamura K, Ito H, Makino H (2013) A decreased level of serum soluble Klotho is an independent biomarker associated with arterial stiffness in patients with chronic kidney disease. PLoS One 8:e56695

28. Lin W, Zhang Q, Liu L, Yin S, Liu Z, Cao W (2017) Klotho restoration via acetylation of peroxisome proliferation–activated receptor γ reduces the progression of chronic kidney disease. Kidney Int 92:669–679

29. Sun CY, Chang SC, Wu MS (2012) Suppression of Klotho expression by protein-bound uremic toxins is associated with increased DNA methyltransferase expression and DNA hypermethylation. Kidney Int 81:640–650

30. Liu L, Lin W, Zhang Q, Cao W, Liu Z (2016) TGF-beta induces miR-30d down-regulation and podocyte injury through Smad2/3 and HDAC3-associated transcriptional repression. J Mol Med 94:291–300

31. Yang J, Yin S, Bi F, Liu L, Qin T, Wang H, Cao W (2016) TIMAP repression by TGFβ and HDAC3-associated Smad signaling regulates macrophage M2 phenotypic phagocytosis. J Mol Med 95:273–285

32. Bi F, Chen F, Li Y, Wei A, Cao W (2018) Klotho preservation by Rhein promotes toll-like receptor 4 proteolysis and attenuates lipopolysaccharide-induced acute kidney injury. J Mol Med 96:915–927

33. Yin S, Zhang Q, Yang J, Lin W, Li Y, Chen F, Cao W (2017) TGFβ-incurred epigenetic aberrations of miRNA and DNA methyltransferase suppress Klotho and potentiate renal fibrosis. Biochim Biophys Acta Mol Cell Res 1864:1207–1216

34. Bottinger EP, Bitzer M (2002) TGF-beta signaling in renal disease. J Am Soc Nephrol 13:2600–2610

35. Rahman Mazumder MA, Hongsprabhas P (2016) Genistein as antioxidant and antibrowning agents in in vivo and in vitro: a review. Biomed Pharmacother 82:379–392

36. Amiri Gheshlaghi S, Mohammad Jafari R, Algazo M, Rahimi N, Alshaib H, Dehpour AR (2017) Genistein modulation of seizure: involvement of estrogen and serotonin receptors. J Nat Med 71: 537–544

37. Groh IAM, Chen C, Lüske C, Cartus AT, Esselen M (2013) Plant polyphenols and oxidative metabolites of the herbal Alkenylbenzene Methyleugenol suppress histone deacetylase activity in human colon carcinoma cells. J Nutr Metab 2013:1–10

38. Lin W, Li Y, chen F, Yin S, Liu Z, Cao W (2017) Klotho preservation via histone deacetylase inhibition attenuates chronic kidney disease-associated bone injury in mice. Sci Rep 7:46195. https://doi.org/10.1038/srep46195

39. Zhang Q, Liu L, Lin W, Yin S, Duan A, Liu Z, Cao W (2017) Rhein reverses Klotho repression via promoter demethylation and protects against kidney and bone injuries in mice with chronic kidney disease. Kidney Int 91:144–156

40. Tollefsbol KALTO (2017) The influence of an epigenetics diet on the cancer epigenome. Epigenomics 9:1153–1155

41. Stancheva TCI (2008) Methyl-CpG binding proteins: specialized transcriptional repressors or structural components of chromatin? Cell Mol Life Sci 65:1509–1522

42. Setchell KDR, Cassidy A (1999) Dietary isoflavones: biological effects and relevance to human health. J Nutr 129:758S–767S

43. Yang-Ming Yang DS, Kandhi S, Froogh G, Zhuge J, Huang W, Hammock BD, An H (2018) Estrogen-dependent epigenetic regulation of soluble epoxide hydrolase via DNA methylation. Proc Natl Acad Sci U S A 115:613–618

44. Kim D, Lee AS, Jung YJ, Yang KH, Lee S, Park SK, Kim W, Kang KP (2014) Tamoxifen ameliorates renal tubulointerstitial fibrosis by modulation of estrogen receptor α-mediated transforming growth factor-β1/Smad signaling pathway. Nephrol Dial Transplant 29: 2043–2053

45. Sinha S, Shukla S, Khan S, Tollefsbol TO, Meeran SM (2015) Epigenetic reactivation of p21(CIP1/WAF1) and KLOTHO by a combination of bioactive dietary supplements is partially ER alpha-dependent in ER alpha-negative human breast cancer cells. Mol Cell Endocrinol 406:102–114

46. Chen J, Zhang X, Zhang H, Lin J, Zhang C, Wu Q, Ding X (2013) Elevated Klotho promoter methylation is associated with severity of chronic kidney disease. PLoS One 8:e79856

47. Shimamura Y, Hamada K, Inoue K, Ogata K, Ishihara M, Kagawa T, Inoue M, Fujimoto S, Ikebe M, Yuasa K et al (2012) Serum levels of soluble secreted alpha-Klotho are decreased in the early stages of chronic kidney disease, making it a probable novel biomarker for early diagnosis. Clin Exp Nephrol 16:722–729

48. Hu MC, Shi M, Zhang J, Quinones H, Kuro-o M, Moe OW (2010) Klotho deficiency is an early biomarker of renal ischemia-reperfusion injury and its replacement is protective. Kidney Int 78:1240–1251

49. Yang HC, Deleuze S, Zuo Y, Potthoff SA, Ma LJ, Fogo AB (2009) The PPARg agonist pioglitazone ameliorates aging-related progressive renal injury. J Am Soc Nephrol 20:2380–2388

50. Drew DAKR, Kritchevsky S, Ix J, Shlipak M, Gutiérrez OM, Newman A, Hoofnagle A, Fried L, Semba RD, Sarnak M (2017) Association between soluble Klotho and change in kidney function: the health aging and body composition study. J Am Soc Nephrol 28:1859–1866

51. Kuro-o M (2009) Klotho and aging. Biochim Biophys Acta Gen Subj 1790:1049–1058