Impact of klotho on the expression of SRGAP2a in podocytes in diabetic nephropathy

Donghua Jin (✉ dhjin18@sina.com)

People's hospital of Suzhou new district <https://orcid.org/0000-0002-0273-9736>

Miao Jia

People's hospital of Suzhou new district

Yuxian Xie

People's hospital of Suzhou new district

Lihua Lin

People's hospital of Suzhou new district

Hong Qiu

People's hospital of Suzhou new district

Guoyuan Lu

The First Affiliated Hospital of Soochow university

Research Article

Keywords: diabetic nephropathy, podocyte, klotho, SRGAP2a

Posted Date: February 23rd, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1081413/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at BMC Nephrology on April 18th, 2022. See the published version at <https://doi.org/10.1186/s12882-022-02765-z>.

Abstract

Background Diabetic nephropathy (DN) is the major cause of renal failure, and glomerular podocytes play critical roles in the pathogenesis of DN by maintaining the glomerular structure and filtration barrier. Klotho and Slit-Robo GTP activating protein 2a (SRGAP2a) have been indicated to play protective roles in reducing renal injury, but whether there is an internal relationship between these two factors is unclear.

Methods In this study, we cultured differentiated rat podocytes in vitro and measured the SRGAP2a expressions by immunofluorescence staining, quantitative real-time PCR (qRT-PCR) and western blotting, after siRNA-mediated transforming growth factor β 1 (TGF- β 1) silencing, TGF- β 1 overexpression and in the presence of a reactive oxygen species (ROS) inhibitor. And we detected the expressions of SRGAP2a, small mother against decapentaplegic (Smad)2/3, phosphorylated-Smad2/3 (p-Smad2/3), Smad7, and NAD(P)H oxidase 4 (NOX4), ROS levels and podocyte cytoskeletal remodelling under high glucose (HG) and exogenous klotho conditions. In addition, we performed haematoxylin-eosin (HE) staining and immunohistochemistry with diabetic rat models to confirm the in vitro results.

Results The results indicated that SRGAP2a expression was significantly upregulated under siRNA-mediated TGF- β 1 silencing conditions or after adding a ROS inhibitor, but significantly downregulated with TGF- β 1 overexpression, in the presence of HG. The supplementation of exogenous klotho under HG conditions significantly increased the SRGAP2a expression, remodelled the actin cytoskeleton and altered the expressions of Smad2/3, p-Smad2/3, Smad7 and NOX4 and reduced the ROS generation in podocytes. Moreover, klotho administration protected renal injury in DN rats.

Conclusions This study indicated that klotho may modulate the expression of SRGAP2a by regulating the ROS and TGF- β 1 signalling pathways and provided theoretical support for klotho protein as a novel therapeutic strategy for treating DN patients.

Background

DN is the leading cause of chronic kidney disease (CKD), which has been recognized as a public health disease worldwide because it creates a high risk of cardiovascular disease and other complications [1–4]. Accumulating evidence has shown that glomerular podocytes, which are glomerular visceral epithelial cells residing on the urinary side of the glomerular basement membrane (GBM), play a critical role in the pathogenesis of DN by maintaining the glomerular structure and filtration barrier [5–8].

Klotho was initially identified as an anti-ageing gene [9–12]. There are three members in this family: α -, β -, and γ -klotho [13]. In this paper, the term klotho in the subsequent text refers to α -klotho. Klotho is a single transmembrane protein that consists of a small intracellular domain in the C terminus and a large extracellular domain in the N terminus [12, 14]. Membrane-bound klotho is suggested to be a coreceptor mainly for FGF23 signalling, regulating renal phosphate excretion [15–17]. The extracellular domain can be cleaved by a disintegrin and metalloproteinase (ADAM)-10 and ADAM-17 at the α cut site to become soluble klotho, which contains two functional repeats named KI1 and KI2 and are involved in the

circulation [12, 18, 19]. Generally, soluble klotho, which refers to this degradation fragment of membrane-bound klotho, is the functional form and is widely present in the blood, urine and cerebrospinal fluid [20–22]. Soluble klotho has been suggested to be an ideal biomarker for CKD based on findings from clinical and animal studies that klotho deficiency seems to be significantly associated with podocyte injury and renal fibrosis in human kidneys [23]. Moreover, extracellular supplementation is considered a novel therapeutic strategy for CKD to restore klotho levels and/or promote endogenous expression [24, 25]. Previous studies indicated that soluble klotho could inhibit TGF- β 1 activity to reduce renal fibrosis by directly binding to the TGF- β 1 receptor and preventing TGF- β 1 signalling [24, 26]. Klotho functions as a renoprotective factor by regulating multiple signalling pathways.

The normal function of podocytes depends on their actin cytoskeleton. Previous studies revealed that Rho family GTPases regulate podocyte actin dynamics, and abnormalities in Rho GTPase activity may result in podocyte mobility and induce proteinuria [7, 27–30]. SRGAP2a, a member of the Rho GTPase large family, has been shown to be primarily enriched in podocytes and is tightly correlated with the estimated glomerular filtration rate (eGFR) and proteinuria of DN patients [7]. The expression of SRGAP2a was shown to be decreased in DN patients and *db/db* mice, while increasing SRGAP2a levels in podocytes in *db/db* mice efficiently rescued the DN state [7]. Recent studies revealed that the expression of SRGAP2a decreased significantly in the presence of HG or TGF- β 1, and exogenous SRGAP2a largely rescued podocytes from HG- and TGF- β 1-induced damage [7, 31]. SRGAP2a has been shown to play protective roles against podocyte injury and proteinuria in DN [7, 31].

Therefore, there is a close relationship between the levels of klotho and SRGAP2a in CKD, including DN. However, whether klotho attenuates renal injury by regulating SRGAP2a in podocytes remains unclear. In this study, we cultured differentiated rat podocytes and measured the expression of SRGAP2a, the ROS levels, and key factors in TGF- β 1 and ROS signalling with or without exogenous klotho under HG conditions and analysed the relationship between klotho and SRGAP2a. And we confirmed the in vitro findings with diabetic rat models. The results provide theoretical support for klotho protein as a novel therapeutic strategy for treating DN patients.

Methods

Primary podocyte culture

We isolated two rat kidneys under aseptic conditions and dissected and gently ground the renal cortex. The tissues were rinsed by sequential passage through 180 μ m, 100 μ m and 75 μ m sieves at 4°C. The cells were harvested on 75 μ m sieves, resuspended in 2 ml of Dulbecco's modified Eagle's medium (DMEM), inoculated into three 75 cm² culture flasks and incubated at 37°C and 5% CO₂ for 7–8 days. The majority of cells observed with an electron microscope (Leica TCS SP8 STED, Germany) were primary podocytes.

Immunofluorescence staining

Primary podocytes were cultured for another 7 days after being trypsinized and subsequently passaged. Podocyte morphology was observed with an inverted phase contrast microscope (Leica TCS SP8 STED, Germany) when the cells covered the bottom of the dish. The cells were fixed with cold acetone and processed for immunofluorescence staining for the podocyte markers nephrin and synaptopodin.

Cell transfection

All siRNAs used in this study were synthesized by GenePharma (Suzhou). The siRNA-TGF- β 1 (TGF- β 1 siRNA-2) sequence consisted of a 21-nucleotide sense strand (5'-GACAAGUUCAAGCAGAGUACA-3') and an antisense strand (5'-UACUCUGCUUGAACU UGUCAU-3'). Podocytes were inoculated in 24-well plates and transfected with the designed siRNAs to mediate TGF- β 1 silencing with Lipofectamine™ 2000 transfection reagent according to the manufacturer's instructions (Thermo Fisher, US). Twenty-four hours after transfection, the podocytes were treated with HG and harvested for subsequent analysis such as SRGAP2a immunofluorescence staining, qRT-PCR and western blotting.

qRT-PCR

Podocyte samples were treated with mannitol (100 mM), glucose (100 mM), and glucose (100 mM) plus klotho (2000 pM) for 24 h, and samples without additional sugar treatment were used as negative controls. Then, the medium was removed from the 6-well cell culture plate, and 1× PBS solution (Sangon Shanghai) was added to wash the cells gently. Next, the 6-well plate was placed on ice, and 800 μ l of TRIzol reagent (Sigma, US) was added to each sample, which were repeatedly pipetted to dislodge all adherent cells, and the cells were transferred to 1.5 ml EP tubes. The total RNA of each sample was extracted according to the manufacturer's instructions and reverse transcribed with the PrimeScript™ RT reagent kit (TaKaRa, Japan). qRT-PCR was performed with SYBR Green detection mix (TaKaRa, Japan). The relative expression levels of genes in this study were normalized to actin expression, analysed by the $2^{-\Delta\Delta C_t}$ method, and summarized from three separately harvested podocyte samples. The primers used for qRT-PCR were showed in Table 1.

Table 1

Sequence information for primers used in qRT-PCR.

Gene	Forwad primer[5'-3']	Reverse primer[5'-3']
Actin	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTTCAG
SRGAP2a	TTCGGAGGAGCCGTGGTCCGCG	GTCTGCGCACACCGCCGCCTCAC
NOX4	GTATGTTTAGTTCCAATCGTCAG	ACATTATGGGTACTGAAGCAA
Smad2	GGATTGAGAGTCAAGATTGGGCA	ATTGGCTTCTCAAGATACCT
Smad7	ATCTGAAGGCGCTCACGCACT	AGATCCGGCCACCTGAACACTT
TGF- β 1	CTGGACACACAGTACAGCAAGGTCC	ATCATGTTGGACAACCTGCTCCACCT

Western blot analysis

Podocyte samples were treated as described above for qRT-PCR. Total protein was extracted from each sample to prepare cell lysates (Sangon, Shanghai, China) and stored at -20°C. The bicinchoninic acid (BCA) protein quantification method was used to ensure that the concentration of each sample was basically equal. Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes with electrophoresis systems (Tanon VE180 and Tanon VE186, Shanghai, China). The PVDF membranes were blocked with 5% (w/v) skimmed milk powder for 2 h and incubated at 4°C overnight with the following primary antibodies (1:1000): rabbit anti-Smad2/3, p-Smad2/3, Smad7 and NOX4 (Abcam, UK). After being washed with 1× PBS solution (Sangon, Shanghai, China) three times, the membranes were incubated with HRP-labelled goat anti-rabbit IgG secondary antibodies (1:5000) (Abcam, UK). Immunoreactivity was determined with enhanced chemiluminescence (ECL) reagent (Thermo Fisher, US). A gel imaging system (BIO-RAD Gel Doc XR+, US) and software (BIO-RAD Image Lab Software, Version 5.1 and SPSS 20.0) were used for imaging and statistical analysis. GAPDH was used as an internal control to ensure equal protein loading.

Fluorescein-conjugated phalloidin staining

The podocyte cytoskeletal remodelling with different treatments in this study were detected by fluorescein-conjugated phalloidin staining using test kits (APExBIO, Beijing, China) according to the manufacturer's instructions.

Animal experiment

The Wistar rats purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. were divided into two groups randomly. The control group rats were with free access to water and normal diet. The

modelled DN rats were supplied with high-fat-sugar diet (10% lard, 10% egg yolk powder, 5% sucrose), free access to water, and intraperitoneal injected with streptozotocin (70 mg/kg) six week later to induced DN. The DN rats were divided into two groups randomly. The klotho intervention rats were intraperitoneal injected with klotho proteins (0.02 mg/kg) for 7 d consecutively. The control and DN group were intraperitoneal injected with normal saline. The health status was monitored by a specific veterinarian. All animals were euthanized and sacrificed by cervical dislocation after treatment, and the kidney tissues were removed. All experiments with animals were approved by the Institutional Animal Care and Use Committee of Soochow University.

Immunohistochemistry

The kidney tissues were embedded in paraffin and cut into 4- μ m in thickness. After dewaxed and hydrated, the sections were washed with distilled water containing 3% hydrogen peroxidase to reduce endogenous oxidase activity. Then the tissue sections were incubated with anti-SRGAP2a antibody (Abcam, ab121977), anti-nephrin (Abcam, ab216692), anti-synaptopodin (Abcam, ab224491) for 2 hours at room temperature, and subsequently, incubated with goat-anti-rabbit antibody at room temperature for 40 minutes. The degree of staining was determined by developing with diaminobenzidine (DAB) chromogen (Bio-Rad, Inc., CA, USA).

Intracellular ROS generation analysis

We measured intracellular ROS generation with the fluorescent probe DCFH-DA, which is hydrolysed and generates nonfluorescent DCFH after passing through the cell membrane. The intracellular ROS oxidizes DCFH to produce fluorescent DCF. The level of DCF fluorescence intensity indicates the level of intracellular ROS.

Podocyte samples were treated as described above for qRT-PCR or western blotting, washed with cold 1 $\times$ PBS solution once, resuspended in serum-free DMEM and incubated with DCFH-DA (10 μ M)) for 30 min at 37°C. The samples were mixed every 3-5 min to ensure good contact between the probes and podocytes. After that, the podocytes were washed with serum-free DMEM three times and resuspended in 1 $\times$ PBS solution. Finally, the podocyte samples were analysed by flow cytometry (Life Attune NxT, US). FlowJo 10 software was used for data analysis.

Statistical analysis

Sigma Plot 12.0 and SPSS 20.0 were used for statistical analysis. All data are presented as the means $\pm$ SD. Independent group comparisons were performed using Student's *t*-test or one-way ANOVA with Bonferroni's post hoc test. A value of $P < 0.05$ was considered statistically significant.

Results

The culture of rat podocytes

Podocytes are important targets in various renal diseases. In this study, we performed all experiments in cultured differentiated rat podocytes. To harvest podocytes that were suitable for further study, we performed immunofluorescence labelling for nephrin (Abcam, UK) and synaptopodin (Abcam, UK), which are specific markers for differentiated podocytes (Fig. 1). The immunofluorescence results indicated that the cultured differentiated rat podocytes were pure enough for further study.

SRGAP2a involved in the TGF- β 1 and ROS signalling pathway

The expression of SRGAP2a is downregulated in cultured podocytes treated with TGF- β 1, and TGF- β 1 may induce the production of ROS by increasing the expression of NOX4 in human pulmonary artery smooth muscle cells [7,32]. Thus, we focused on the function of SRGAP2a in the TGF- β 1 and ROS signalling pathways. According to the results from qRT-PCR and western blotting, TGF- β 1 expressions were upregulated with HG treatment ($**p < 0.01$) (Supplemental Fig. 1). We performed siRNA-mediated silencing of TGF- β 1 and TGF- β 1 overexpression (Fig. 2; Supplemental Fig. 2). The results from immunofluorescence staining showed that SRGAP2a signals increased in TGF- β 1 siRNA group, but decreased in TGF- β 1-OE group, compared to the control without TGF- β 1 silencing under HG conditions (Fig. 2A). The results from qRT-PCR were in consistent with the immunofluorescence staining experiment that the mRNA expression of SRGAP2a showed significantly increased in TGF- β 1 siRNA group, and decreased significantly in TGF- β 1-OE group, compared to the controls ($**p < 0.01$) (Fig. 2B). The western blot analysis further verified the above results (Fig. 2C and D). The SRGAP2a signals from immunofluorescence staining in the presence of HG treatment was downregulated compared to the NC groups (Fig. 3A). When ROS inhibitors were added to the HG solutions, the SRGAP2a signals were obviously enhanced (Fig. 3A). The qRT-PCR and western blot results were consistent with the immunofluorescence staining findings (Fig. 3B-D). These results indicate that SRGAP2a is involved in TGF- β 1 and the ROS signalling pathway in rat podocytes.

Klotho increased SRGAP2a levels in HG-induced rat podocytes

To examine the effects of klotho on SRGAP2a, we performed immunofluorescence staining, qRT-PCR and western blot analysis of rat podocytes. The immunofluorescence staining results showed that the SRGAP2a signals decreased when the cells were treated with HG compared to the negative controls or mannitol groups (Fig. 4A) but increased obviously after the addition of soluble klotho protein to the HG solutions (Fig. 4A). The qRT-PCR results were in consistent with the immunofluorescence staining that the

soluble klotho addition promoted the mRNA expressions of SRGAP2a significantly ($**p < 0.01$) (Fig. 4B). Western blot analysis showed that the SRGAP2a protein level decreased significantly with HG treatment and increased significantly after klotho supplementation in HG solutions ($**p < 0.01$) (Fig. 4C and D), which confirmed the qRT-PCR results. Taken together, these results suggested that klotho could efficiently induce SRGAP2a expression under HG conditions in rat podocytes. In addition, we performed fluorescein-conjugated phalloidin staining to evaluate the number of F-actin stress fibers, the results from which showed that the mean actin per cell value was significantly decreased under HG conditions, but significantly upregulated by the klotho addition ($*p < 0.05$, $**p < 0.01$) (Fig. 4E and F).

Klotho regulated the expression of key factors in the TGF- β 1/Smad and ROS/NOX4 signalling pathways

To determine whether klotho prevents renal injury by regulating TGF- β 1 and ROS signalling in podocytes, we measured the expression of some key factors in the pathways. We quantified the expression of Smad2/3, Smad7 and NOX4 by qRT-PCR, which showed that the mRNA levels of Smad2/3 and NOX4 significantly increased in response to HG treatments compared to the NC groups but decreased significantly after the addition of klotho to the HG solutions ($*p < 0.05$, $**p < 0.01$) (Fig. 5A and C). Conversely, the transcript levels of Smad7 decreased significantly after HG treatment but slightly increased after klotho intervention ($*p < 0.05$) (Fig. 5B). The protein levels of Smad2/3, Smad7 and NOX4 measured by western blotting were consistent with the results from qRT-PCR ($*p < 0.05$, $**p < 0.01$) (Fig. 5D, E, G and H). The protein level of p-Smad2/3 was significantly increased with HG treatment but decreased by klotho intervention ($*p < 0.05$) (Fig. 5D and F). In addition, we measured ROS generation by flow cytometric analysis, which showed that ROS generation was extremely increased by HG treatment ($**p < 0.01$) (Fig. 6). However, the ROS levels significantly decreased after klotho intervention ($*p < 0.05$) (Fig. 6). These results demonstrated that klotho could regulate the expression of key factors in the TGF- β 1/Smad and ROS/NOX4 pathways in podocytes.

Klotho protects renal injury in diabetic rats

To figure out the effect of klotho in diabetic podocyte injury, we established the diabetic rats. The blood glucose, creatinine, urea level, total cholesterol level and triglyceride content were detected (Supplemental Table 1). The results from HE staining showed that rat kidney glomeruli were hypertrophic, mesangial matrix increased and erythrocyte stasis was visible in DN rats (Fig. 7A). Obvious vacuoles appeared in the cytoplasm of renal tubular epithelial cells (Fig. 7A). These pathological changes in DN kidney were rescued obviously by klotho supplement (Fig. 7A). Moreover, the SRGAP2a and podocyte makers of nephrin and synaptopodin protein expressions were decreased in DN rats but increased visibly after klotho administration from immunochemistry (Fig. 7B).

Discussion

DN is a common complication of diabetes mellitus, the major cause of renal failure and the leading cause of CKD [8, 33]. Podocyte injury, which is clinically indicated by progressive decline in the eGFR and increased proteinuria, plays a critical role in the pathogenesis of DN [5–8]. The specific function of podocytes is strongly dependent on the actin cytoskeletons of the interdigitating foot process between neighbouring podocytes [5, 7]. SRGAP2a was strongly suggested to protect podocyte function and structure [7]. In this study, we found that the expression of SRGAP2a was significantly decreased in cultured podocytes under HG conditions. And the DN rats also revealed downregulation of SRGAP2a expression. These results were consistent with the investigation showing that the SRGAP2a level was significantly decreased in DN patients [7]. These findings indicated that the specific expression of SRGAP2a was essential for proper podocyte function and structure.

TGF- β signalling regulates a series of biological processes and is indispensable in animal development [34]. Smad transcription factors occupy the core of this pathway [35]. According to this study, TGF- β 1 silencing stimulated SRGAP2a expression under HG conditions in podocytes. In addition, previous studies revealed that TGF- β 1 significantly reduced the podocyte level of SRGAP2a [7]. In summary, the molecular functions of SRGAP2a seemed to be tightly associated with TGF- β 1/Smad signalling. ROS generation was pharmacologically inhibited during HG + klotho treatment and resulted in the upregulation of podocyte SRGAP2a expression. Undoubtedly, SRGAP2a was involved in ROS signalling. Overall, we suggest that SRGAP2a plays protective roles against renal diseases via the TGF- β 1/Smad/ROS signalling axis. Increased ROS generation (oxidative stress) is consequently associated with the hyperglycaemia underlying DN [36–38]. Thus, we hypothesised that reducing oxidative stress might facilitate SRGAP2a expression to prevent podocyte injury in DN, since in diabetic mice, reducing oxidative stress efficiently improved kidney structure and function [39].

On the one hand, the serum klotho protein level is one of the early markers of renal diseases, as the level declines at the early stage of CKD and continuously decreases with the progression of CKD [24]. On the other hand, klotho protein is considered to be a novel therapeutic target [22, 24, 40]. In this study, we found that the expression of SRGAP2a significantly increased after the addition of klotho to HG solutions compared with the expression of SRGAP2a in HG-treated cultured podocytes. This might indicate that klotho protein actually stimulated SRGAP2a expression in podocytes to protect against injury. In addition, the expression of key factors in the TGF- β 1/Smad/ROS signalling pathway changed after klotho administration. The expression of NOX4 and Smad2/3 was greatly enhanced with HG treatment but significantly downregulated when klotho was added to the HG treatment. However, the expression of Smad7, an inhibitory Smad serving as a competitor for receptor-regulated Smad proteins such as Smad2/3, was contrary to the results of Smad2/3 and NOX4. The protein level of p-Smad2/3 showed the same trend as that of Smad2/3 and NOX4 in the presence of HG and exogenous klotho. These results suggest that klotho can regulate the expression of key factors in TGF- β 1/Smad/ROS signalling that are strongly associated with SRGAP2a in podocytes. NOX4 is the major source of ROS in the kidney [36–38]. NOX4-derived ROS mediate TGF- β 1/Smad signalling in many disease processes. In this study, ROS generation was analysed by flow cytometry and was enhanced under HG conditions but reduced significantly after klotho administration. In summary, exogenous klotho stimulates the expression of

SRGAP2a by substantially reducing ROS generation to prevent podocyte injury in DN. The TGF- β 1/Smad/ROS signalling pathway may regulate actin activity in kidney myofibroblast activation, human pulmonary artery smooth muscle cells and renal interstitial fibrosis [32, 41, 42]. In this study, klotho remodelled the actin cytoskeleton in DN rats. Thus, klotho may prevent podocyte dysfunction by regulating the expression of SRGAP2a and key factors of the associated regulatory pathway.

Conclusion

This study mimicked the medical treatment of DN patients with klotho protein. Our findings indicated that soluble klotho could interfere the expressions of SRGAP2a and key molecules in TGF- β 1/Smad/ROS signalling in cultured podocytes and maintaining the podocyte cytoskeleton under HG conditions, and protecting renal injury in DN rats. Further studies are necessary to precisely understand the role of klotho in protecting podocytes from DN. This research provides theoretical support for klotho protein as a novel treatment for DN patients.

Abbreviations

DN, Diabetic nephropathy; SRGAP2a, Slit-Robo GTP activating protein 2a; ROS, reactive oxygen species; Smad, small mother against decapentaplegic; p-Smad, phosphorylated-Smad; NOX4, NAD(P)H oxidase 4; HG, high glucose; HE, haematoxylin-eosin; CKD, chronic kidney disease; GBM, glomerular basement membrane; ADAM, a disintegrin and metalloproteinase; TGF- β 1, transforming growth factor β 1; eGFR, estimated glomerular filtration rate; DMEM, Dulbecco's modified Eagle's medium; BCA, bicinchoninic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DAB, diaminobenzidine.

Declarations

Ethics approval and consent to participate

The study has been approved by the ethnic committee of People's Hospital of Suzhou new district.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interest

The authors declare no relevant conflict of interest.

Funding

Author Donghua Jin is currently receiving a research grant from Collaborative fund of Hospital Management Foundation of Suzhou New District People's Hospital (no. SGY2021D01), a grant from Suzhou Citizens' Health Science and Technology Project“SYSD2020087”and a grant from Suzhou New District Science and Technology Bureau Key Project of Science Foundation (no. 2019Z005).

Authors' contributions

MJ and YX conceived and designed the study. LL analyzed the data. HQ conducted the experiments. DJ and GL wrote the manuscript. The final manuscript was read and approved by all the authors.

Acknowledgements

Not applicable.

References

1. Fineberg D, Jandeleit-Dahm KA, Cooper ME. Diabetic nephropathy: diagnosis and treatment. *Nat Rev Endocrinol*. 2013;9: 713-723.
2. Meza Letelier CE, San Martin Ojeda CA, Ruiz Provoste JJ, Frugone Zaror CJ. Pathophysiology of diabetic nephropathy: a literature review. *Medwave*. 2017; 17: e6839.
3. Reutens AT, Atkins RC. Epidemiology of diabetic nephropathy. *Contrib Nephrol*. 2011; 170: 1-7.
4. Wada J, Makino H. Inflammation and the pathogenesis of diabetic nephropathy. *Clin Sci (Lond)*. 2013; 124: 139-152.
5. Li JJ, Kwak SJ, Jung DS, Kim JJ, Yoo TH, Ryu DR, et al. Podocyte biology in diabetic nephropathy. *Kidney Int Suppl*. 2007; S36-42.
6. Ni L, Saleem M, Mathieson PW. Podocyte culture: tricks of the trade. *Nephrology (Carlton)*. 2012; 17: 525-531.
7. Pan Y, Jiang S, Hou Q, Qiu D, Shi J, Wang L, et al. Dissection of glomerular transcriptional profile in patients with diabetic nephropathy: SRGAP2a protects podocyte structure and function. *Diabetes*. 2018; 67: 717-730.
8. Tziomalos K, Athyros VG. Diabetic nephropathy: new risk factors and improvements in diagnosis. *Rev Diabet Stud*. 2015; 12: 110-118.
9. Hu MC, Shi M, Zhang J, Addo T, Cho HJ, Barker SL, et al. Renal Production, Uptake, and Handling of Circulating alphaKlotho. *J Am Soc Nephrol*. 2016; 27: 79-90.

10. Hu MC, Kuro-o M, Moe OW. Klotho and chronic kidney disease. *Contrib Nephrol.* 2013; 180: 47-63.
11. Lindberg K, Amin R, Moe OW, Hu MC, Erben RG, Ostman Wernerson A, et al. The kidney is the principal organ mediating klotho effects. *J Am Soc Nephrol.* 2014; 25: 2169-2175.
12. Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, et al. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature.* 1997; 390: 45-51.
13. Hu MC, Shiizaki K, Kuro-o M, Moe OW. Fibroblast growth factor 23 and Klotho: physiology and pathophysiology of an endocrine network of mineral metabolism. *Annu Rev Physiol.* 2013; 75: 503-533.
14. Matsumura Y, Aizawa H, Shiraki-Iida T, Nagai R, Kuro-o M, Nabeshima Y. Identification of the human klotho gene and its two transcripts encoding membrane and secreted klotho protein. *Biochem Biophys Res Commun.* 1998; 242: 626-630.
15. Ide N, Olauson H, Sato T, Densmore MJ, Wang H, Hanai JI, et al. In vivo evidence for a limited role of proximal tubular Klotho in renal phosphate handling. *Kidney Int.* 2016; 90: 348-362.
16. Kurosu H, Ogawa Y, Miyoshi M, Yamamoto M, Nandi A, Rosenblatt KP, et al. Regulation of fibroblast growth factor-23 signaling by klotho. *J Biol Chem.* 2006; 281: 6120-6123.
17. Urakawa I, Yamazaki Y, Shimada T, Iijima K, Hasegawa H, Okawa K, et al. Klotho converts canonical FGF receptor into a specific receptor for FGF23. *Nature.* 2006; 444: 770-774.
18. Chen CD, Podvin S, Gillespie E, Leeman SE, Abraham CR. Insulin stimulates the cleavage and release of the extracellular domain of klotho by ADAM10 and ADAM17. *Proc Natl Acad Sci U S A.* 2007; 104: 19796-19801.
19. Chen CD, Tung TY, Liang J, Zeldich E, Tucker Zhou TB, Turk BE, et al. Identification of cleavage sites leading to the shed form of the anti-aging protein klotho. *Biochemistry.* 2014; 53: 5579-5587.
20. Akimoto T, Yoshizawa H, Watanabe Y, Numata A, Yamazaki T, Takeshima E, et al. Characteristics of urinary and serum soluble klotho protein in patients with different degrees of chronic kidney disease. *BMC Nephrol.* 2012; 13: 155.
21. Imura A, Iwano A, Tohyama O, Tsuji Y, Nozaki K, Hashimoto N, et al. Secreted klotho protein in sera and CSF: implication for post-translational cleavage in release of klotho protein from cell membrane. *FEBS Lett.* 2004; 565: 143-147.
22. Kurosu H, Yamamoto M, Clark JD, Pastor JV, Nandi A, Gurnani P, et al. Suppression of aging in mice by the hormone Klotho. *Science.* 2005; 309: 1829-1833.
23. Cho NJ, Han DJ, Lee JH, Jang SH, Kang JS, Gil HW, et al. Soluble klotho as a marker of renal fibrosis and podocyte injuries in human kidneys. *PLoS One.* 2018; 13: e0194617.
24. Zou D, Wu W, He Y, Ma S, Gao J. The role of klotho in chronic kidney disease. *BMC Nephrol.* 2018; 19: 285.
25. Neyra JA, Hu MC. Potential application of klotho in human chronic kidney disease. *Bone.* 2017; 100: 41-49.

26. Sugiura H, Yoshida T, Shiohira S, Kohei J, Mitobe M, Kurosu H, et al. Reduced Klotho expression level in kidney aggravates renal interstitial fibrosis. *Am J Physiol Renal Physiol*. 2012; 302: F1252-1264.
27. Asanuma K, Yanagida-Asanuma E, Faul C, Tomino Y, Kim K, Mundel P. Synaptopodin orchestrates actin organization and cell motility via regulation of RhoA signalling. *Nat Cell Biol*. 2006; 8: 485-491.
28. Blattner SM, Hodgins JB, Nishio M, Wylie SA, Saha J, Soofi AA, et al. Divergent functions of the Rho GTPases Rac1 and Cdc42 in podocyte injury. *Kidney Int*. 2013; 84: 920-930.
29. Burridge K, Wennerberg K. Rho and Rac take center stage. *Cell*. 2004; 116: 167-179.
30. Gee HY, Saisawat P, Ashraf S, Hurd TW, Vega-Warner V, Fang H, et al. ARHGDI1 mutations cause nephrotic syndrome via defective RHO GTPase signaling. *J Clin Invest*. 2013; 123: 3243-3253.
31. Levi M, Myakala K, Wang X. SRGAP2a: a new player that modulates podocyte cytoskeleton and injury in diabetes. *Diabetes*. 2018; 67: 550-551.
32. Sturrock A, Cahill B, Norman K, Huecksteadt TP, Hill K, Sanders K, et al. Transforming growth factor-beta1 induces Nox4 NAD(P)H oxidase and reactive oxygen species-dependent proliferation in human pulmonary artery smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol*. 2006; 290: L661-L673.
33. Agardh CD. Diabetic nephropathy. *Nord Med*. 1992; 107: 215-216.
34. Shi Y, Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell*. 2003; 113: 685-700.
35. Joan Massagué, Seoane J, Wotton D. Smad transcription factors. *GENES & DEVELOPMENT*. 2020; 19: 2783-2810.
36. Avogaro A, de Kreutzenberg SV, Fadini GP. Oxidative stress and vascular disease in diabetes: is the dichotomization of insulin signaling still valid? *Free Radic Biol Med*. 2008; 44: 1209-1215.
37. Thallas-Bonke V, Thorpe SR, Coughlan MT, Fukami K, Yap FY, Sourris KC, et al. Inhibition of NADPH oxidase prevents advanced glycation end product-mediated damage in diabetic nephropathy through a protein kinase C-alpha-dependent pathway. *Diabetes*. 2008; 57: 460-469.
38. Brezniceanu ML, Liu F, Wei CC, Tran S, Sachetelli S, Zhang SL, et al. Catalase overexpression attenuates angiotensinogen expression and apoptosis in diabetic mice. *Kidney Int*. 2007; 71: 912-923.
39. Manickam N, Patel M, Griendling KK, Gorin Y, Barnes JL. RhoA/Rho kinase mediates TGF-beta1-induced kidney myofibroblast activation through Poldip2/Nox4-derived reactive oxygen species. *Am J Physiol Renal Physiol*. 2014; 307: F159-171.
40. Sopjani M, Rinnerthaler M, Kruja J, Dermaku-Sopjani M. Intracellular signaling of the aging suppressor protein klotho. *Curr Mol Med*. 2015; 15: 27-37.
41. Hotta Y, Uchiyama K, Takagi T, Kashiwagi S, Nakano T, Mukai R, et al. Transforming growth factor beta1-induced collagen production in myofibroblasts is mediated by reactive oxygen species derived from NADPH oxidase 4. *Biochem Biophys Res Commun*. 2018; 506: 557-562.
42. He T, Xiong J, Nie L, Yu Y, Guan X, Xu X, et al. Resveratrol inhibits renal interstitial fibrosis in diabetic nephropathy by regulating AMPK/NOX4/ROS pathway. *J Mol Med (Berl)*. 2016; 94: 1359-1371.

Figures

Figure 1

The immunofluorescence staining to label synaptopodin (A) and nephrin (B) of the cultured differentiated rat podocytes.

Figure 2

SRGAP2a is involved in the TGF- β 1 signalling pathways in rat podocytes. (A) The immunofluorescence staining of SRGAP2a in TGF- β 1 interference and TGF- β 1 overexpression conditions with HG treatments. (B) The qRT-PCR results showed that the expression of SRGAP2a in silencing TGF- β 1+ HG treatment and in TGF- β 1-OE + HG treatment. (C) The western blot results showed the SRGAP2a levels in silencing TGF-

$\beta 1$ + HG treatment and in TGF- $\beta 1$ -OE + HG treatment. (D) The quantitative analysis of the SRGAP2a protein levels in (C). All data were mean $\pm$ SD, (n = 6); * $p < 0.05$, ** $p < 0.01$. Bars = 50 μ m in (A).

Figure 3

SRGAP2a is involved in the ROS signalling pathways in rat podocytes. (A) The immunofluorescence staining of SRGAP2a after inhibiting ROS generation. (B) The qRT-PCR results showed that the expression

of *SRGAP2a* after inhibiting ROS generation. (C) The western blot results showed that the *SRGAP2a* level increased after inhibiting ROS generation. (D) The quantitative analysis of the *SRGAP2a* protein levels in (C). All data were mean $\pm$ SD, (n = 6); * $p < 0.05$, ** $p < 0.01$. Bars = 50 μ m in (A). NC, negative controls without additional treatment.

Figure 4

The SRGAP2a expressions and actin cytoskeleton of podocytes following HG and HG plus klotho treatment. (A) The immunofluorescence staining of SRGAP2a with HG and HG plus klotho treatment. (B) The qRT-PCR results showing SRGAP2a mRNA expressions with HG and HG plus klotho treatment. (C) The western blotting results showing SRGAP2a protein expressions with HG and HG plus klotho treatment. (D) The quantitative analysis of the SRGAP2a protein levels in (C). (E) The fluorescein-conjugated phalloidin staining of podocytes with HG and HG plus klotho treatment. (F) The quantitative analysis of the mean actin content per cell in (E). All data were mean $\pm$ SD, (n = 6); * p < 0.05, ** p < 0.01. Bars = 50 μ m in (A), 5 μ m in (E). NC, negative controls without additional treatment.

Figure 5

Klotho regulated the expression of Smad2/3, p-Smad2/3, Smad7, and NOX4. (A-C) The qRT-PCR results showed the expression of Smad2/3, Smad7, and NOX4 with HG and HG plus klotho treatments compared with the NC groups. (D) The western blot results showed that the protein levels of Smad2/3, p-Smad2/3, Smad7, and NOX4 changed with HG and HG plus klotho treatments compared with the NC groups. (E-H) The quantitative analysis of the results from (D). All data were mean $\pm$ SD, (n = 6); * p < 0.05, ** p < 0.01.

Figure 6

ROS generation with HG and HG plus klotho treatments. (A) Flow cytometry analysis showed that ROS generation in the HG and HG plus klotho treatments changed compared with that in the NC groups. (B) Statistical analysis of ROS generation, as determined by flow cytometry analysis. All data were mean $\pm$ SD, (n = 6); * $p < 0.05$, ** $p < 0.01$.

Figure 7

The HE staining (A) and Nephlin, Synaptopodin and SRGAP2a immunochemistry (B) of the kidneys from the modelled DN and DN plus klotho rats. Bars = 200 μm.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [NC3RsARRIVEGuidelinesChecklistfillable.pdf](#)

- [Additionalfile2.docx](#)
- [SupplementalFigure.docx](#)
- [SupplementalTable1.docx](#)