PLOS ONE

RESEARCH ARTICLE

microRNA-200c regulates KLOTHO expression in human kidney cells under oxidative stress

Kenichi Morii[1], Satoshi Yamasaki[2], Shigehiro Doi[1]*, Taisuke Irifuku[1], Kensuke Sasaki[1], Toshiki Doi[1], Ayumu Nakashima[1,3], Koji Arihiro[4], Takao Masaki[1]*

1 Department of Nephrology, Hiroshima University Hospital, Hiroshima, Japan, **2** Center for Rheumatic Diseases, Kurume University Medical Center, Kurume, Japan, **3** Department of Stem Cell Biology and Medicine, Graduate School of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan, **4** Department of Anatomical Pathology, Hiroshima University Hospital, Hiroshima, Japan

* sdoi@hiroshima-u.ac.jp (SD); masakit@hiroshima-u.ac.jp (TM)

Citation: Morii K, Yamasaki S, Doi S, Irifuku T, Sasaki K, Doi T, et al. (2019) microRNA-200c regulates KLOTHO expression in human kidney cells under oxidative stress. PLoS ONE 14(6): e0218468. https://doi.org/10.1371/journal.pone.0218468

Editor: Partha Mukhopadhyay, National Institutes of Health, UNITED STATES

Received: January 10, 2019

Accepted: June 3, 2019

Published: June 14, 2019

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: This work was supported by a grant from Ryokufukai. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Abstract

KLOTHO deficiency is associated with the progression of kidney dysfunction, whereas its overexpression exerts renoprotective effects. Oxidative stress suppresses KLOTHO expression in renal epithelial cells but upregulates microRNA-200c (miR-200c) in human umbilical vein endothelial cells. In this study, we investigated whether oxidative stress-induced miR-200c is implicated in KLOTHO downregulation in human renal tubular epithelium (HK-2) cells. HK-2 cells were stimulated with hydrogen peroxide (H_2O_2) to examine the effect of oxidative stress. A luciferase reporter containing the *KLOTHO* 3′-UTR was used to investigate the effect of miR-200c on *KLOTHO* mRNA metabolism. The expressions of KLOTHO, oxidative stress markers, and miR-200c were determined in human kidney biopsy specimens. H_2O_2 suppressed KLOTHO expression without a reduction in *KLOTHO* mRNA levels but upregulated miR-200c expression. Similarly, transfection of a miR-200c mimic reduced KLOTHO levels and luciferase activity without a reduction in *KLOTHO* mRNA levels. In contrast, transfection of a miR-200c inhibitor maintained KLOTHO expression. Immunofluorescent assay revealed KLOTHO was present in the cytosol and nuclei of HK-2 cells. In human kidney biopsies, KLOTHO expression was inversely correlated with levels of oxidative stress markers (8-hydroxy-2′-deoxyguanosine: $\rho = -0.38$, $P = 0.026$; 4-hydroxy-2-hexenal: $\rho = -0.35$, $P = 0.038$) and miR-200c ($\rho = -0.34$, $P = 0.043$). Oxidative stress-induced miR-200c binds to the *KLOTHO* mRNA 3′-UTR, resulting in reduced KLOTHO expression.

Introduction

Chronic kidney disease (CKD) is recognized as a risk factor in the development of end-stage kidney disease [1], and all-cause mortality [2–5]. Consequently CKD has a substantial economic burden [6]. Currently, oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and anti-oxidant defenses [7]. Although past studies have reported that increased ROS levels play a pivotal role in the progression of CKD [8,9],

ROS are also involved in physiological processes, including cell signaling [10], gene expression [11], and cell growth [12]. Therefore, inhibition of ROS has not been established as a therapy for CKD [13]. In addition to ROS damage *per se*, recent studies have revealed that oxidative stress also participates in renal damage through the downregulation of renoprotective factors [14–16]. These findings indicate that oxidative stress-induced downregulation of such factors is a potential therapeutic strategy to prevent the progression of CKD.

KLOTHO is a single-pass transmembrane protein consisting of 1012 amino acids [17,18], and is strongly and weakly expressed in distal renal tubular epithelial cells and proximal renal tubular epithelial cells, respectively [19]. In addition to phosphate excretion, KLOTHO exhibits multiple functions, including the amelioration of oxidative stress [20,21], and inhibition of signaling pathways of insulin growth factor [22], Wnt/β-catenin [23], transforming growth factor-β1 [24], and mechanistic target of rapamycin signaling [25]. Overexpression of the *Klotho* gene or injection of KLOTHO protein shows beneficial effects in rodent models of various renal diseases [26]. These findings suggest that maintaining KLOTHO expression is a novel therapeutic strategy during the development of CKD. However, another study showed that hydrogen peroxide (H_2O_2), a ROS, contributed to the downregulation of KLOTHO expression in renal epithelial cells [14,15], causing renal damage [27]. Therefore, the underlying mechanism by which H_2O_2 decreases KLOTHO expression should be clarified to identify a therapeutic target.

Gene expression is regulated by epigenetic alterations, including histone modification, DNA methylation and microRNA (miRNA) expression [28–31]. Among these, miRNAs, which are small, endogenous, non-coding and single-stranded RNAs of 21–25 nucleotides, play a major role in repressing gene expression post-transcriptionally by binding to specific sites within the 3′-untranslated region (3′-UTR) of a target gene mRNA [32–34]. H_2O_2 upregulated microRNA-200c (miR-200c) in human umbilical vein endothelial cells [35], and, notably, there are two putative miR-200c binding sites in the 3′-UTR of the *KLOTHO* mRNA. These findings led us to the hypothesis that H_2O_2 suppresses KLOTHO expression through the induction of miR-200c. To test this, we investigated whether miR-200c regulates KLOTHO expression in kidney cells under oxidative stress.

In this study, we show that H_2O_2 suppresses KLOTHO expression without reducing levels of *KLOTHO* mRNA. We also show that H_2O_2-induced miR-200c downregulates KLOTHO expression by binding to the *KLOTHO* mRNA 3′-UTR. Last, KLOTHO expression is associated with markers of oxidative stress and miR-200c in renal biopsy samples from IgA nephropathy patients. These findings indicate that oxidative stress suppresses KLOTHO expression through the induction of miR-200c.

Materials and methods

Cell culture

Human renal proximal tubular epithelium (HK-2) cells were obtained from the American Type Culture Collection (CRL-2190, Lot No. 61218770, Manassas, VA). Mycoplasma was not detected during the experimental period. The cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS) (Nichirei Bio Science, Tokyo, Japan) and penicillin/streptomycin (Nacalai, Kyoto, Japan). For stimulations, HK-2 cells were treated with 100 μM H_2O_2 (Sigma-Aldrich, St. Louis, MO) for 6–24 hours (hrs) and 100–1000 μM paraquat (Sigma-Aldrich) for 24 hrs. ERK (#6560), JNK (#6232), p38 (#6564) and control (#6568) siRNAs were purchased from Cell Signaling Technology (Danvers, MA). Cells were transfected using Lipofectamine 2000 Reagent (Invitrogen, Waltham, MA) in accordance with the

manufacturer's protocol. After incubation with transfection complexes for 24 hrs, the medium was changed, and the cells were stimulated with 100 μM H_2O_2 for 24 hrs.

miRNA transfection

To examine the effect of miR-200c in HK-2 cells, hsa-miR-200c mimic (miRVana miRNA mimic, Applied Biosystems, Foster City, CA) or mimic control (miRVana miRNA mimic negative control, Applied Biosystems) were transfected into HK-2 cells using Lipofectamine RNAiMAX (Invitrogen) in accordance with the manufacturer's instructions. To evaluate the inhibitory effects of miR-200c and miR-21 on KLOTHO expression, hsa-miR-200c and hsa-miR-21 inhibitor (miRVana miRNA inhibitor, Applied Biosystems) or inhibitor control (miRVana miRNA inhibitor negative control, Applied Biosystems) were transfected into HK-2 cells using Lipofectamine RNAiMAX (Invitrogen). Mimic control or inhibitor control were used as negative controls.

Western blotting

Western blot analysis was performed as described previously [24,36,37]. Primary antibodies used were rat monoclonal anti-human KLOTHO antibody (KM2076, TransGenic, Kobe, Japan), mouse monoclonal anti-α-TUBULIN (TUBA) antibody (T9026, Sigma-Aldrich, St. Louis, MO), rabbit monoclonal anti-ERK1/2 antibody (#4696, Cell Signaling Technology), rabbit monoclonal anti-JNK antibody (#9252, Cell Signaling Technology) and rabbit monoclonal anti-p38 antibody (#8690, Cell Signaling Technology). The intensity of detected proteins was determined using ImageJ software (version 1.50i; National Institutes of Health, Bethesda, MD).

Gene expression

1) Quantitative PCR (q-PCR) for *KLOTHO*, *ACTIN B* (*ACTB*), pri-hsa-miR-200c and pri-hsa-miR-21. Total RNA was extracted from conditioned cells using an RNeasy Mini Kit (Qiagen, Venlo, Netherlands). For the synthesis of complementary DNA (cDNA), total RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). *KLOTHO* and *ACTB* mRNAs were quantified using TaqMan Gene Expression Assays (assay ID: Hs00183100_ml for *KLOTHO* and assay ID: Hs01060665_gl for *ACTB*) (Applied Biosystems) and a 7500 Fast Real-Time PCR (RT-PCR) System (Applied Biosystems). *ACTB* was used to verify equal sample loading. The expressions of pri-hsa-miR-200c and pri-hsa-miR-21 were quantified by TaqMan Pri-miRNA Assays (assay ID: Hs03303157_pri for pri-hsa-miR-200c, Hs03302625_pri for pri-hsa-miR-21) (Applied Biosystems) and a 7500 Fast RT-PCR System. The amplification of specific PCR products was confirmed by the $2^{(-\Delta\Delta CT)}$ method with dissociation curve analysis for each primer pair.

2) q-PCR for miRNA. For q-PCR analysis of hsa-miR-200c, hsa-miR-21 and U6 snRNA, RNA was extracted from conditioned cells using a miRNeasy Mini Kit (Qiagen). Five nanograms of RNA were converted to cDNA using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). q-PCR was performed using TaqMan MicroRNA Assays and a 7500 Fast RT-PCR. U6 snRNA was used as a reference gene.

KLOTHO 3′-UTR reporter assay

A *KLOTHO* 3′-UTR reporter clone in pMirTarget (pMirTarget-KL3′-UTR) was obtained from OriGene (SC217236, Rockville, MD). HK-2 cells were transfected with the plasmid for 4 hrs using Lipofectamine 2000 Reagent (Invitrogen) in accordance with the manufacturer′s

protocol. Has-miR-200c mimic, mimic control, has-miR-200c inhibitor or inhibitor control were simultaneously transfected with the reporter plasmid in some experiments. After changing the medium, HK-2 cells were cultured for a further 12 hrs before sampling. The cells were lysed using passive lysis buffer (Promega, Madison, WI) and expression from the luciferase reporter construct was quantified using the Luciferase Reporter Assay System (Promega) on an Infinite 200Pro plate reader (Tecan, Kanagawa, Japan). The luciferase activity was normalized against protein quantity.

Plasmid construction

Site-directed mutation of the miR-200c target sites in the pMirTarget-KL3′-UTR was generated using a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) in accordance with the manufacturer's instructions. The resulting plasmid was named pMirTarget-KL3′-UTR-MUT, which contained two 6 nucleotide substitutions at sites 568–573 and 1904–1909. Primer pairs used for construction were as follows; (forward, 5′-GAATGTTCCTTTCGAAAGCAATGCTTCTATCAAATACTCTGCGGAATTTATGTATCTGG TTAATGACATACTTGGAGAGCAA-3′; reverse, 5′-TTGCTCTCCAAGTATGTCATTAACCAG ATACATAAATTCCGCAGAGTATTTGATAGAAGCATTGCTTTCGAAAGGAACATTC-3′) and (forward, 5′-TCCTTGACTGTAAAGAGAAGTAATTTTGCTCCTTGATAACTGCGGATATTAA TAATAAATCTGCCTGCAACTTTTTGCCTTCTT-3′; reverse, 5′-AAGAAGGCAAAAAGTTGC AGGCAGATTTATTATTAATATCCGCAGTTATCAAGGAGCAAAATTACTTCTCTTTACAGTC AAGGA-3′).

Clinical sample collection and ethics statement

Kidney specimens were obtained by renal biopsy at Hiroshima University Hospital between April 2014 and December 2016 from 35 patients who were diagnosed with IgA nephropathy. The patients' demographic and clinical characteristics are shown in S1 Table. The Japanese glomerular filtration rate (GFR) equation based on serum creatinine (Cr) was used to estimate glomerular filtration rate (eGFR). eGFR (mL/min/1.73 m^2) = $194 \times Cr^{-1.094} \times Age^{-0.287}$ (× 0.739 if female). This study adhered to the declaration of Helsinki and was approved by the Ethics Committee of Hiroshima University (E-633-2). Informed consent was obtained in the form of opt-out on the web-site. The ethics committee waived the requirement for written informed consent because of the retrospective nature of the study.

Histology and immunohistochemistry of human kidney tissue

The following primary antibodies were used: rat monoclonal anti-human KLOTHO antibody (KM2076, TransGenic), mouse monoclonal anti-8-hydroxy-2'-deoxyguanosine (8-OHdG) antibody (MOG-020P, Japan Institute for the Control of Aging, Shizuoka, Japan) and mouse monoclonal anti-4-hydroxy-2-hexenal (4-HHE) antibody (MHH-030n, Japan Institute for the Control of Aging). Immunostaining of KLOTHO and 8-OHdG was performed as described previously [38]. 4-HHE was identified with the EnVision System (Dako, Santa Clara, CA). A positive area was quantified as the mean of five randomly selected fields using ImageJ software (National Institutes of Health).

Immunofluorescence assay

HK-2 cells were washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (Nacalai) and permeabilized with 0.5% Triton X-100 (Nacalai) at room temperature. After blocking with 5% Blocking One Histo (Nacalai) for 10 minutes (min), the cells were incubated

with rabbit polyclonal KLOTHO antibody (1:100, PA5-21078, ThermoFisher Scientific) at 37˚C for 30 min. After washing with PBS, the cells were incubated with Alexa Flour 488 goat anti-rabbit IgG (1:10,000, Invitrogen) at 37˚C for 30 min in the dark. The nucleus was labeled with 4′,6-diamidino-2-phenylindole (DAPI) (H-1200, Vector Laboratories). Images were captured using a Keyence BZ-9000 fluorescence microscope.

In situ hybridization

In situ hybridization (ISH) was performed on formalin-fixed paraffin-embedded human kidney biopsy specimens. Double digoxigenin (DIG)-labeled miRNA probes were designed by Exiqon (Venlo, Netherlands) to target has-miR-200c-3p. ISH was performed using a miRCURY LNA microRNA ISH Optimization Kit (Exiqon) in accordance with the manufacturer's instructions. Proteinase-K incubation was performed with 15 μg/mL for 25 min. The miRNA probe was used at 80 nM, and the U6 snRNA probe at 2 nM. The U6 snRNA and scrambled probes were used as a positive technical control and a negative control, respectively. The miR-200c positive area was quantified as the mean of five randomly selected fields using LuminaVision (version 4.2.1.2; Mitani, Tokyo, Japan).

Statistical analysis

Results are expressed as the mean ± standard deviation. Statistical analyses were performed using SPSS statistical software (version 25; IBM Corporation, Armonk, NY). Comparison between two groups was analyzed by the Mann-Whitney *U*-test. For multiple group comparison, the Mann-Whitney *U*-test with Bonferroni correction was applied. The correlation was calculated using Spearman's rank correlation coefficient. Values of $P < 0.05$ were considered statistically significant.

Results

KLOTHO expression is inhibited by H_2O_2

We carried out *in vitro* experiments to investigate the underlying mechanism by which oxidative stress regulates *KLOTHO* gene expression in HK-2 cells. First, we examined the mRNA and protein levels in HK-2 cells with or without H_2O_2 stimulation. Protein levels of KLOTHO decreased in HK-2 cells with H_2O_2 stimulation, whereas *KLOTHO* mRNA levels were induced at 6 hrs and 12 hrs compared with controls (Fig 1A and 1B).

Next, we used a luciferase reporter system to investigate the effect of H_2O_2 on the translation of *KLOTHO* mRNA in HK-2 cells. A reporter plasmid harboring the 3′-UTR of *KLOTHO* mRNA was used to analyze whether the expression of KLOTHO is mediated by its 3′-UTR. The activity of the luciferase reporter harboring the *KLOTHO* mRNA 3′-UTR was significantly reduced by H_2O_2 stimulation in HK-2 cells (Fig 1C).

miR-200c and miR-21 are upregulated by H_2O_2

We used the online prediction tool, microRNA.org [39], to assess the potential binding of miR-200c to the 3′-UTR of *KLOTHO* mRNA. There are two possible miR-200c binding sites in the *KLOTHO* 3′-UTR (Fig 2A and 2B). Base pairing between the *KLOTHO* 3′-UTR and miR-200c is shown (Fig 2B). Quantification of the expression of pri-miR-200c and miR-200c in HK-2 cells showed they were significantly induced by H_2O_2 stimulation (Fig 2C). We also found that microRNA-21 (miR-21), another miRNA with a predicted binding sequence in the 3′-UTR of *KLOTHO* mRNA, was upregulated by H_2O_2 stimulation (Part A in S1 Fig).

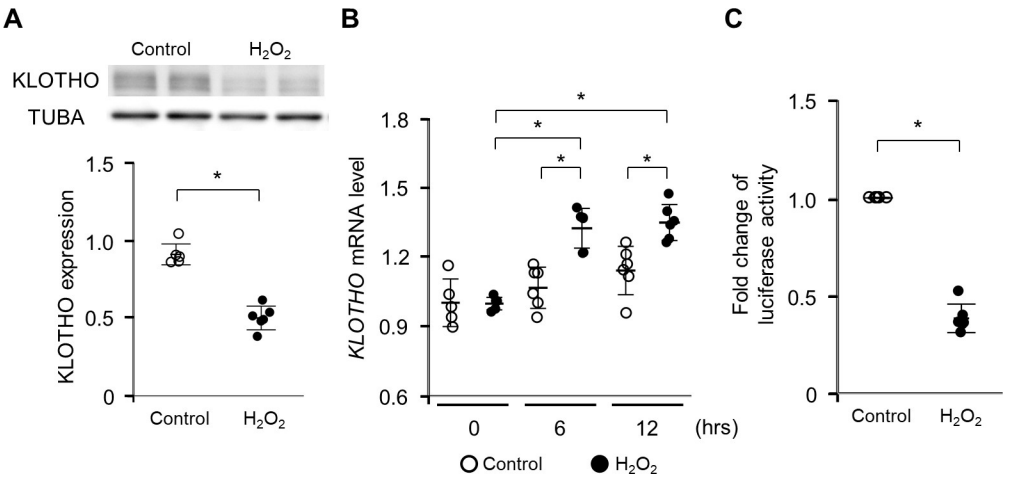

Fig 1. H_2O_2 suppresses KLOTHO expression in HK-2 cells at the level of translation. (A) Protein expression of KLOTHO in HK-2 cells treated with 100 μM H_2O_2 for 24 hours (hrs). Band intensities were analyzed and normalized against levels of α-TUBULIN (TUBA) using densitometry. (B) Expression of *KLOTHO* mRNA in HK-2 cells treated with 100 μM H_2O_2 for the indicated times. (C) Luciferase activities in HK-2 cells transfected with pMirTarget Vector harboring human *KLOTHO* 3′-UTR with or without 100 μM H_2O_2 treatment for 12 hrs. Luciferase activity was normalized against protein amount. Values represent individual measurements and the mean ± SD. Data were analyzed using the Mann-Whitney *U*-test or the Mann-Whitney *U*-test with Bonferroni correction. $^*P < 0.05$, n = 6.

https://doi.org/10.1371/journal.pone.0218468.g001

miR-200c inhibits KLOTHO expression

To examine the inhibitory effect of miR-200c on the metabolism of *KLOTHO* mRNA, we compared protein levels, luciferase activity and mRNA levels in HK-2 cells with or without the transfection of miR-200c. The transfection of miR-200c suppressed protein levels of KLOTHO in HK-2 cells (Fig 3A). Consistent with these results, co-transfection of miR-200c with the *KLOTHO* 3′-UTR reporter plasmid dampened luciferase activity (Fig 3B). Moreover, we examined the mRNA expression of *KLOTHO* in HK-2 cells to determine the effect of miR-200c on *KLOTHO* mRNA metabolism. The expression of *KLOTHO* mRNA was not significantly different between HK-2 cells transfected with miR-200c or control RNA (Fig 3C). Bioinformatics analysis indicated the presence of two potential binding sites of miR-200c in the *KLOTHO* 3′-UTR. To determine whether these sites were actual targets of miR-200c, we mutated both sites (Fig 3D) and examined plasmid luciferase activity. Mutations of these sites restored luciferase activity (Fig 3E). Immunofluorescent assay revealed that the miR-200c mimic reduced KLOTHO expression, and the expression of KLOTHO was observed in the cytosol and nuclei of HK-2 cells (Fig 3F).

We further examined the effect of miR-200c on KLOTHO expression in H_2O_2-stimulated HK-2 cells with or without transfection of the miR-200c inhibitor. As shown in Fig 4A, the miR-200c inhibitor upregulated the protein level of KLOTHO compared with control RNA. The expression level of *KLOTHO* mRNA was not significantly different between HK-2 cells transfected with miR-200c inhibitor or control RNA (Fig 4B), although *KLOTHO* mRNA was upregulated by H_2O_2 stimulation compared with control transfected HK-2 cells without exposure to H_2O_2 (S2 Fig). By immunofluorescent assay, miR-200c inhibitor was shown to retain KLOTHO expression (Fig 4C).

The H_2O_2-induced miR-21 also has a putative binding site in the *KLOTHO* mRNA 3′-UTR; however, the miR-21 inhibitor did not improve KLOTHO protein expression (Part B in S1 Fig). Paraquat, another oxidative stress inducer, reduced KLOTHO expression (S3 Fig).

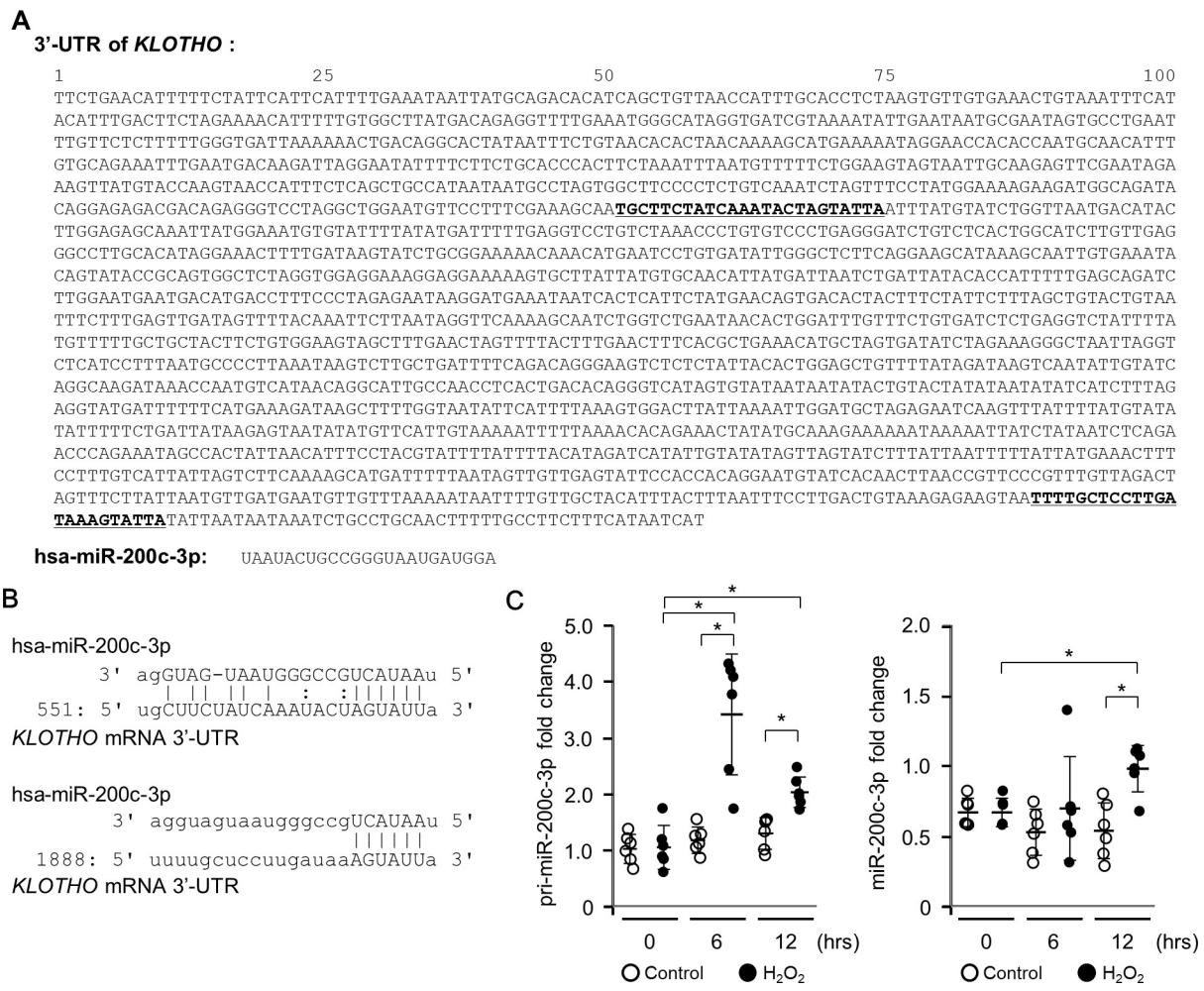

Fig 2. miR-200c is complementary to the human *KLOTHO* mRNA 3′-UTR and is upregulated by H_2O_2 exposure. (A) Putative miR-200c binding sites (underlined sequence) in the *KLOTHO* 3′-UTR sequence predicted by an online algorithm (www.microrna.org). (B) Predicted target sites of miR-200c in the *KLOTHO* mRNA 3′-UTR. There are two possible binding sites. (C) q-PCR analysis of pri-miR-200c and miR-200c expression in HK-2 cells cultured with or without 100 μM H_2O_2 at the indicated time points. U6 snRNA was used for normalization. Values represent individual measurements and the mean ± SD. Data were analyzed using the Mann-Whitney *U*-test with Bonferroni correction. $^{*}P < 0.05$, n = 6.

https://doi.org/10.1371/journal.pone.0218468.g002

KLOTHO expression is inversely correlated with levels of oxidative stress markers and miR-200c in human kidney specimens

We examined the degree of oxidative stress in renal biopsy samples obtained from patients with IgA nephropathy (n = 35) by immunohistochemical staining. Details of the clinical characteristics are shown in S1 Table. Consistent with a previous report [38], the expression of oxidative stress markers, 8-OHdG and 4-HHE, were clearly detected in all samples. To evaluate the link between oxidative stress and KLOTHO expression in human kidneys with IgA nephropathy, we performed immunohistochemical staining for KLOTHO. As shown in Fig 5A, KLOTHO was detected in distal renal tubules and was inversely correlated with levels of 8-OHdG ($\rho = -0.38$, $P = 0.026$) and 4-HHE ($\rho = -0.35$, $P = 0.038$) (Fig 5B).

We also examined miR-200c expression in the same series of specimens using ISH. miR-200c was detected in distal renal tubules (Fig 6A). Consistent with our *in vitro* examination, miR-200c expression was inversely correlated with KLOTHO expression ($\rho = -0.34$,

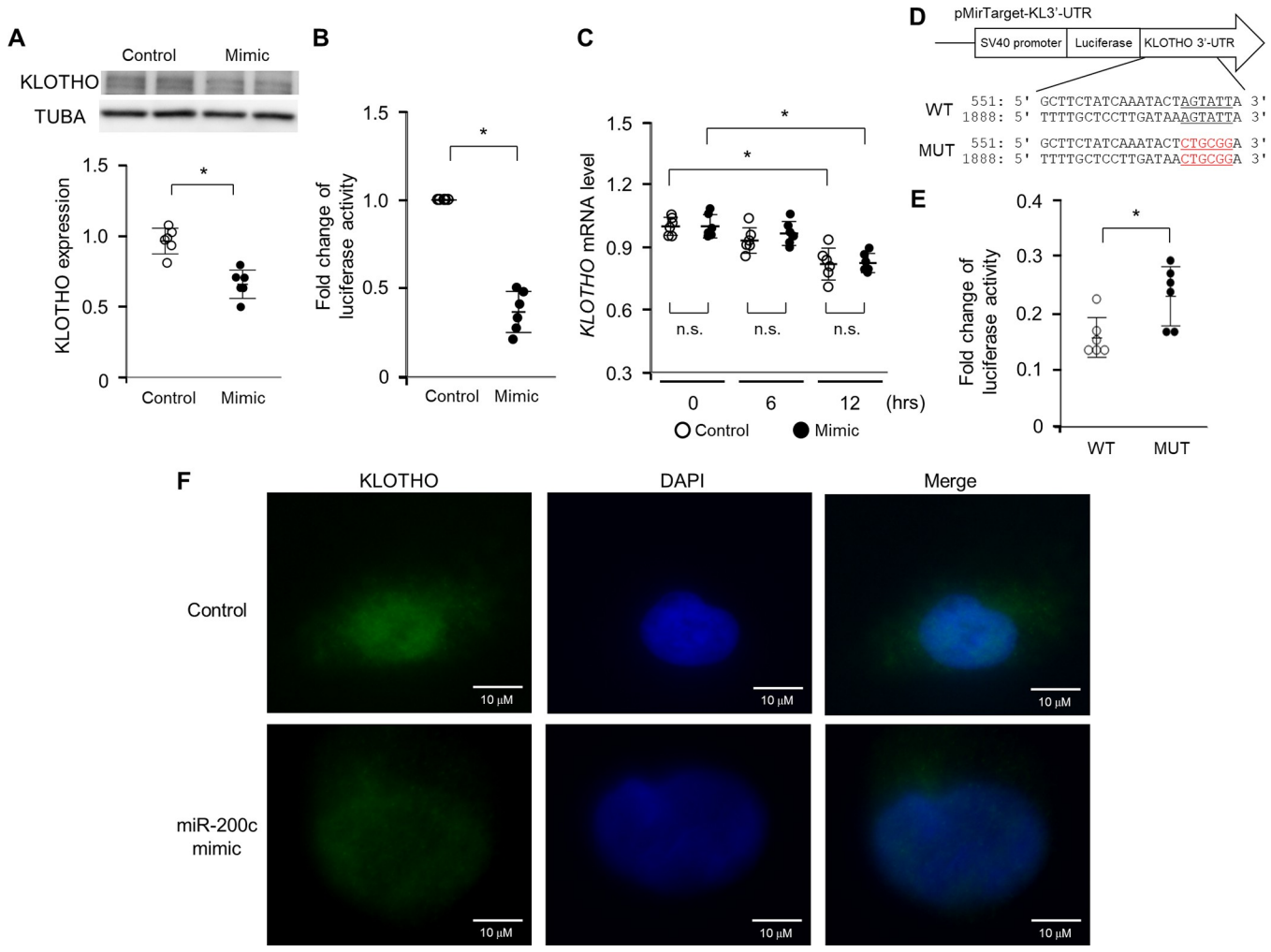

Fig 3. miR-200c decreases KLOTHO expression in HK-2 cells by translational repression. (A) KLOTHO protein expression in HK-2 cells 24 hrs after transfection with 25 nM mimic control or miR-200c mimic. Cells were cultured for another 48 hrs without mimic control or miR-200c mimic before sampling. Band intensities were analyzed and normalized against TUBA using densitometry. (B) A *KLOTHO* 3′-UTR reporter plasmid in combination with 50 nM mimic control or miR-200c mimic was transfected into HK-2 cells for 4 hrs. After a medium change, HK-2 cells were cultured for another 12 hrs before sampling. Luciferase activity was normalized against protein amount. (C) HK-2 cells were transfected with 50 nM mimic control or miR-200c mimic for 4 hrs and cultured for another 24 hrs before harvesting total RNA. *KLOTHO* mRNA levels were evaluated by q-PCR. (D) Mutations were introduced into the 3′-UTR of *KLOHO* mRNA as indicated. (E) The effect of 100 nM miR-200c mimic on the reporter activity of wild type (WT, pMirTarget-KL3′-UTR-WT) and mutant (MUT, pMirTarget-KL3′-UTR-MUT) plasmids in HK-2 cells was measured by luciferase assay. (F) HK-2 cells were stained with anti-KLOTHO antibody and Alexa Fluor 488-labeled goat anti-rabbit IgG. KLOTHO protein was evaluated under fluorescence microscopy. Scale bar = 10 μm. Values represent individual measurements and the mean ± SD. Data were analyzed using the Mann-Whitney *U*-test or the Mann-Whitney *U*-test with Bonferroni correction. $^{*}P < 0.05$, n = 6. n.s.; not significant.

https://doi.org/10.1371/journal.pone.0218468.g003

$P = 0.043$), whereas it was positively correlated with levels of 8-OHdG ($\rho = 0.39$, $P = 0.020$) and 4-HHE ($\rho = 0.53$, $P = 0.002$) (Fig 6B).

Discussion

In this study, we show that H_2O_2 suppressed KLOTHO expression in HK-2 cells. We also show that H_2O_2 induced the expression of miR-200c, which has two putative binding sites in the 3′-UTR of *KLOTHO* mRNA. Our *KLOTHO* 3′-UTR reporter assay indicated that miR-200c downregulates KLOTHO expression. In renal biopsy specimens of patients with IgA nephropathy, miR-200c was mainly detected by ISH in distal tubules, where KLOTHO is also

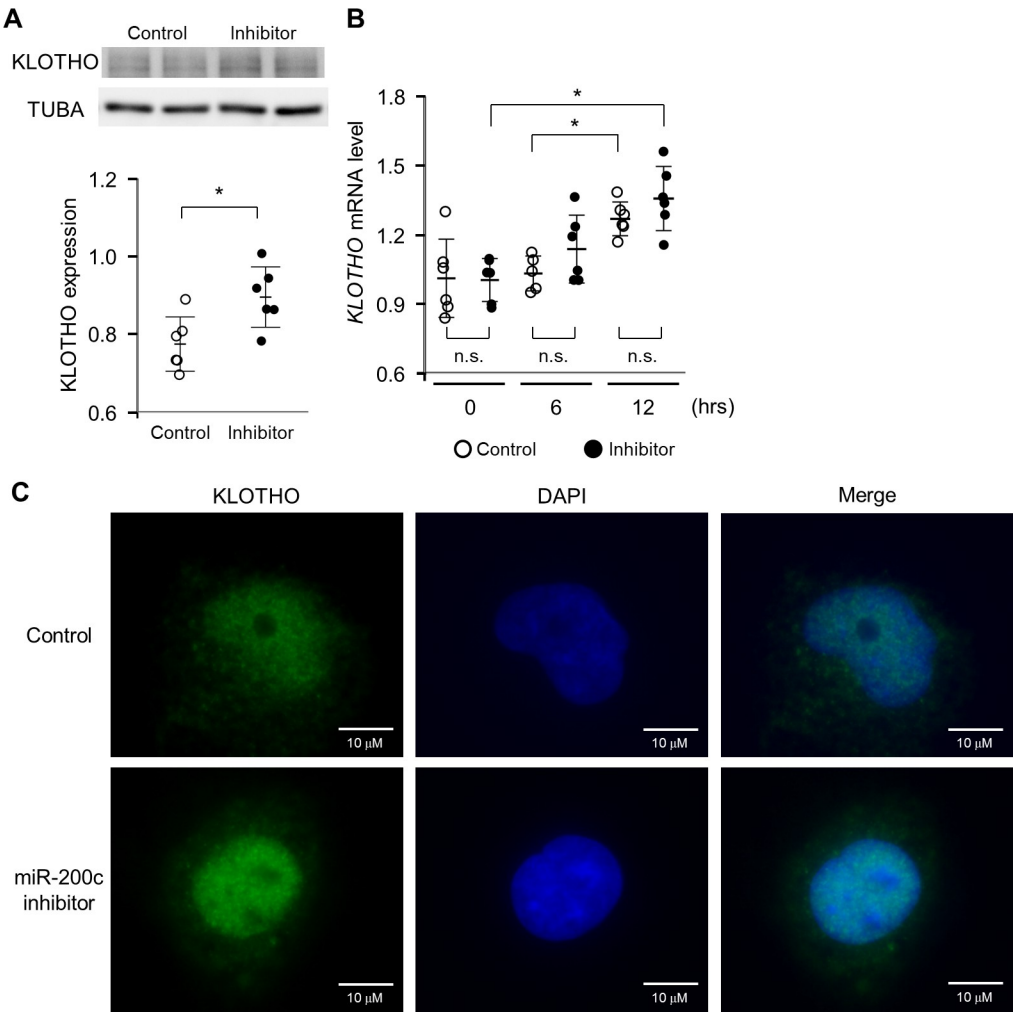

Fig 4. KLOTHO protein is preserved by inhibiting miR-200c in HK-2 cells. (A) The effect of a miR-200c inhibitor on KLOTHO protein expression in HK-2 cells treated with H_2O_2. KLOTHO protein expression in HK-2 cells treated with 100 μM H_2O_2 for 24 hrs after the transfection of inhibitor control (25 nM) or miR-200c inhibitor (25 nM) for 4 hrs. Band intensities were analyzed and normalized against TUBA using densitometry. $^*P < 0.05$, n = 6 (B) The effect of a miR-200c inhibitor on *KLOTHO* mRNA expression in HK-2 cells treated with H_2O_2. HK-2 cells were transfected with inhibitor control (25 nM) or miR-200c inhibitor (25 nM) and 12 hrs later were treated with 100 μM H_2O_2. *KLOTHO* mRNA was detected by q-PCR. (C) HK-2 cells were stained with anti-KLOTHO antibody and Alexa Fluor 488-labeled goat anti-rabbit IgG. KLOTHO protein was evaluated under fluorescence microscopy. Scale bar = 10 μm. Values represent individual measurements and the mean ± SD. Data were analyzed using the Mann-Whitney *U*-test or the Mann-Whitney *U*-test with Bonferroni correction. $^*P < 0.05$, n = 6. n.s.; not significant.

https://doi.org/10.1371/journal.pone.0218468.g004

expressed. Moreover, the KLOTHO immunostained area was inversely correlated with areas positive for oxidative stress markers and miR-200c. Importantly, another miRNA candidate that we expected to regulate KLOTHO expression, miR-21, did not affect KLOTHO expression. These data indicate that oxidative stress reduces KLOTHO expression through the induction of miR-200c.

miRNA binds to the 3′-UTR of a target mRNA to suppress target gene expression by inhibiting translation or mRNA degradation [33]. In the present study, H_2O_2 inhibited KLOTHO protein expression without reducing *KLOTHO* mRNA levels, indicating that miR-200c suppresses KLOTHO expression at the mRNA level, not at the transcriptional level. Indeed, endonucleolytic cleavage of mRNA occurs only when the sequence of the miRNA is completely

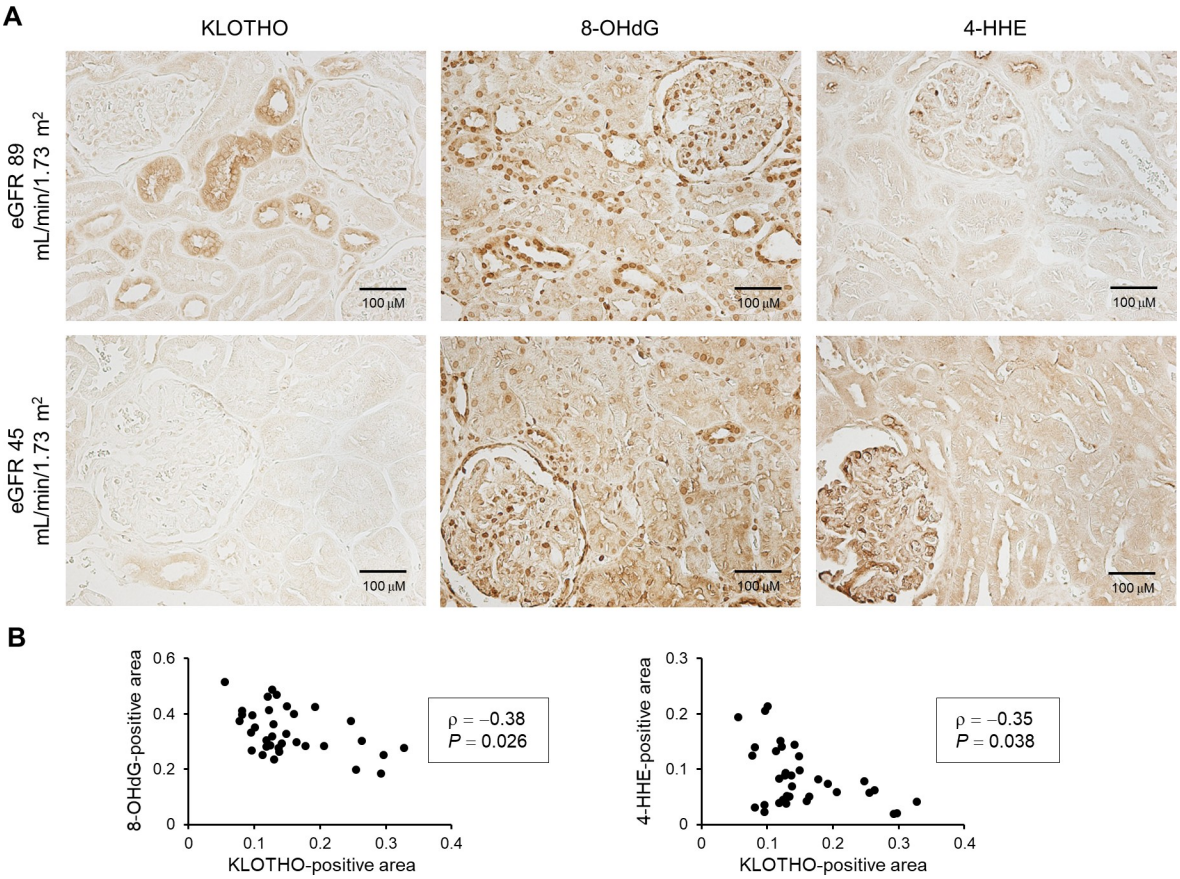

Fig 5. KLOTHO expression inversely correlates with oxidative stress markers in kidney biopsy specimens from IgA nephropathy patients. (A) Representative images of KLOTHO and oxidative stress markers (8-OHdG and 4-HHE) in patients with immunoglobulin A nephropathy. The levels of 8-OHdG and 4-HHE were higher, and those of KLOTHO were lower, in kidney specimens from patients with reduced eGFR compared with patients with conserved eGFR. Scale bar = 100 μm. (B) KLOTHO levels are inversely correlated with 8-OHdG ($\rho = -0.38$, $P = 0.026$) and 4-HHE ($\rho = -0.35$, $P = 0.038$) levels. Correlations were calculated using Spearman's rank correlation coefficient. n = 35.

https://doi.org/10.1371/journal.pone.0218468.g005

complementary with that of the target gene, and this is rare in mammals [40,41]. As shown in Fig 2B, the miR-200c sequence and the putative binding sites in the 3′-UTR of *KLOTHO* mRNA were not perfectly matched in humans. We also provide evidence that the transfection of a miR-200c mimic reduced KLOTHO expression, and that luciferase activity was decreased without any reduction in *KLOTHO* mRNA levels. These findings suggest that miR-200c inhibits KLOTHO expression through translational repression, but not by the degradation of *KLOTHO* mRNA.

A number of studies have described the involvement of oxidative stress in the development of various kidney diseases, such as diabetic kidney disease (DKD) [42], and acute kidney injury (AKI) [43,44]. However, in the BEACON trial (Bardoxolone Methyl Evaluation in Patients with Chronic Kidney Disease and Type 2 Diabetes Mellitus: the Occurrence of Renal Events), antioxidant therapy with bardoxolone methyl increased the risk for cardiovascular disease without a beneficial effect on the incidence of end-stage kidney disease in patients with DKD [45]. A possible explanation is that the oxidative response *in vivo* is not always detrimental and may be physiologically important. Therefore, the systemic inhibition of oxidative stress may lead to adverse effects. However, KLOTHO overexpression exhibited a protective effect in

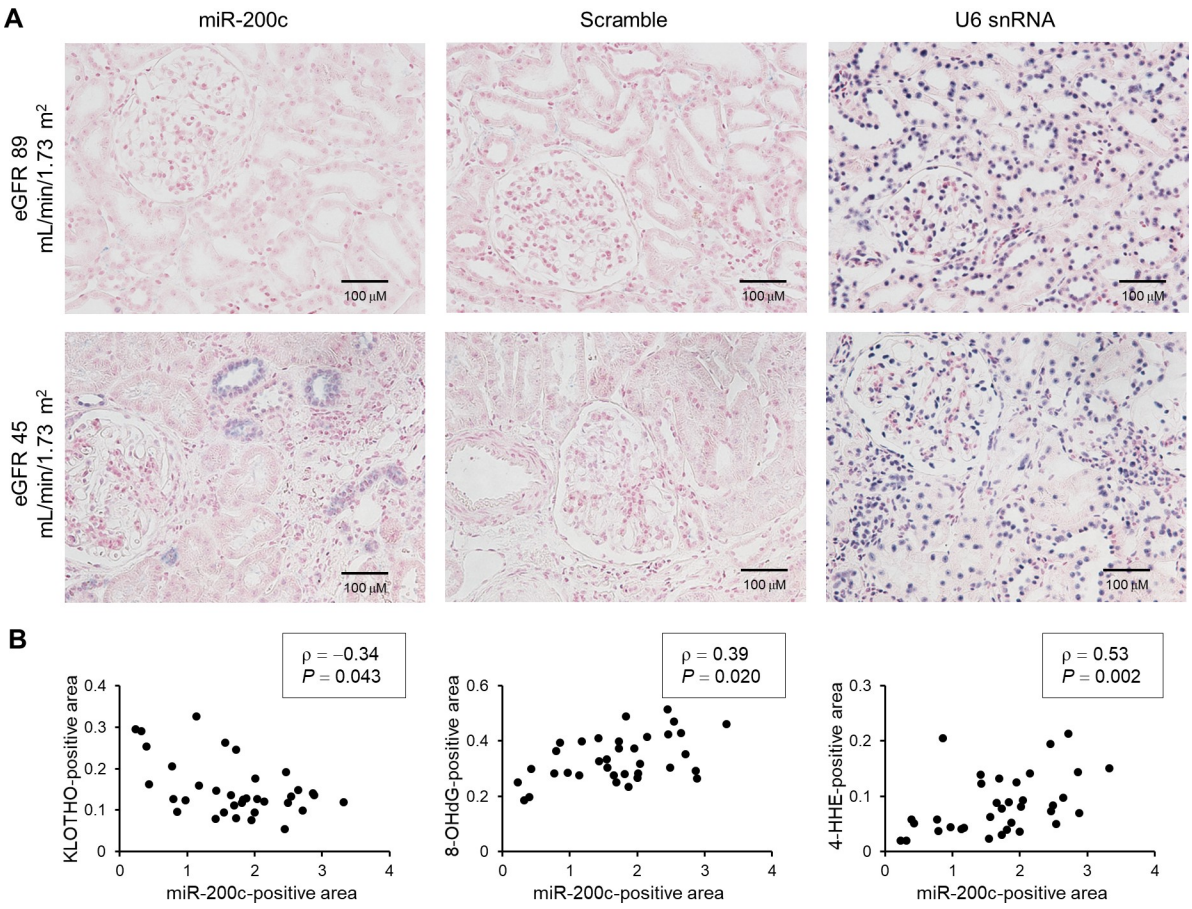

Fig 6. miR-200c expression inversely correlates with KLOTHO expression, and positively correlates with oxidative stress marker levels in kidney biopsy specimens from IgA nephropathy patients. (A) Representative images of miR-200c in the kidneys of patients with IgA nephropathy detected by *in situ* hybridization. Scrambled and U6 snRNA probes were used as negative and positive controls, respectively. Scale bar = 100 μm. (B) miR-200c levels are inversely correlated with KLOTHO levels (ρ = −0.34, *P* = 0.043), but positively correlated with 8-OHdG (ρ = 0.39, *P* = 0.020) and 4-HHE (ρ = 0.53, *P* = 0.002) levels. Correlations were calculated using Spearman's rank correlation coefficient. n = 35.

https://doi.org/10.1371/journal.pone.0218468.g006

various rodent models of renal diseases as well as heart diseases [23,46–48]. The current data suggest that KLOTHO downregulation induced by oxidative stress is an attractive therapeutic strategy.

Renal fibrosis is the most common pathological feature of CKD regardless of the underlying disease [49]. During the development of renal fibrosis, a major source of extracellular matrix (ECM) proteins results from the transformation of resident fibroblast cells into myofibroblasts, while epithelial-mesenchymal transition (EMT) accounts for 10% of ECM proteins in this process [50]. miR-200a prevents EMT, leading to protection from renal fibrosis [51,52], while miR-29 inhibits renal fibrosis through the prevention of ECM deposition [53–56]. These miRNAs can be regarded as beneficial for kidneys; however, several miRNAs might have detrimental effects on kidneys. For example, miR-21 [57,58], miR-192 [59–61], and miR-433 [62], exacerbated renal fibrosis in mice. Both miR-339 and miR-556 decreased the expression of KLOTHO *in vitro* [63], and in this study we show that oxidative stress-induced miR-200c was involved in repressing KLOTHO protein expression, because transfection of a miR-200c inhibitor maintained KLOTHO expression. KLOTHO deficiency caused renal fibrosis, whereas the overexpression or injection of KLOTHO ameliorated it [23,24,27]. Combined,

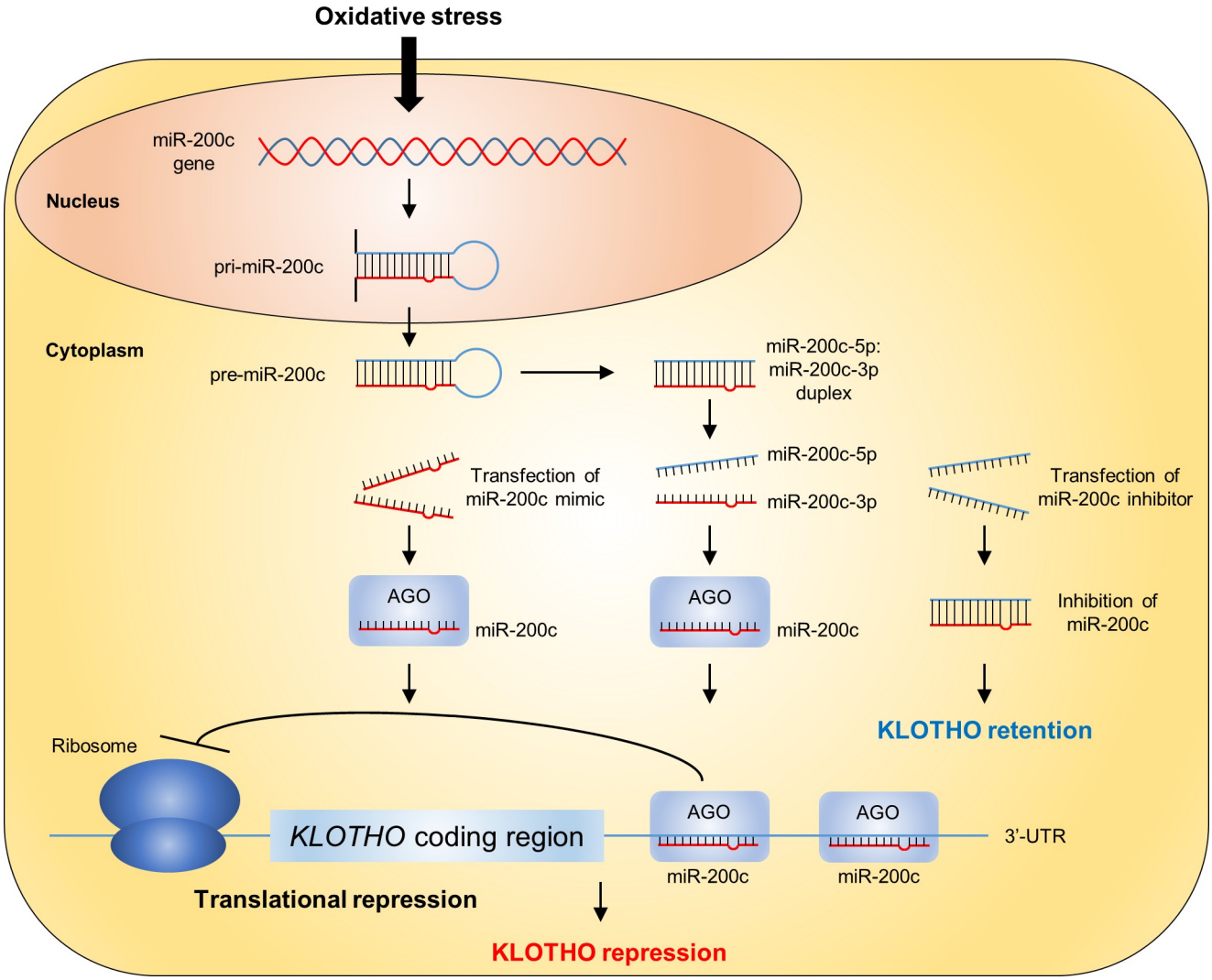

Fig 7. Summary of the results. In HK-2 cells, oxidative stress induced pri-miR-200c and miR-200c, a miRNA that is complementary with the 3′-UTR of *KLOTHO* mRNA at two different sites. miR-200c binds to the *KLOTHO* mRNA 3′-UTR with Argonaute protein (AGO) to suppress the expression of KLOTHO by inhibiting translation. Transfection of a miR-200c mimic reduced KLOTHO expression without a reduction in *KLOTHO* mRNA levels and transfection of a miR-200c inhibitor maintained KLOTHO expression.

https://doi.org/10.1371/journal.pone.0218468.g007

these findings indicate that the inhibition of miR-200c exhibits a beneficial effect in tissues that express KLOTHO protein.

The inhibition of miR-200c induced the expression of zinc finger E-box-binding homeobox (ZEB) 1 and ZEB2, resulting in the promotion of EMT through a reduction of E-cadherin in cells that do not express KLOTHO [64,65]. In contrast, KLOTHO protein confers the ability to prevent EMT by various mechanisms, such as PI3K/Akt.GSKβ3/Snail signaling [66], Wnt/β-catenin signaling [67], and TGF-β1 signaling [24]. Thus, the effect of miR-200c on renal fibrosis remains controversial despite our assumption that the inhibition of miR-200c may show beneficial effects against renal fibrosis. It should, therefore, be investigated in an animal model of renal fibrosis. However, the *KLOTHO* 3′-UTR sequence is different between humans and rodents, raising the possibility that another miRNA may influence KLOTHO expression in mice or rats. Moreover, although previous studies have reported that H_2O_2 induced the activation of mitogen-activated protein kinases [68,69], the inhibition of these

signaling pathways did not improve KLOTHO expression (S4 Fig). Major limitations of this study are that we did not assess the actual effect of miR-200c on KLOTHO expression *in vivo*, and that we could not identify the transcriptional factor responsible for miR-200c-mediated KLOTHO downregulation.

Although immunohistochemistry indicated that KLOTHO was expressed mainly in the cytoplasm of distal tubular cells and not the nucleus, immunofluorescent staining of HK-2 cells revealed that, in addition to the cytosol, KLOTHO was present in the nucleus. HK-2 cells are mainly derived from proximal tubular cells, suggesting that the cellular localization of KLOTHO might be different between cell types. Previous studies reported that KLOTHO exists as secreted, transmembrane and intracellular forms [18,70,71], and that KLOTHO expression is observed at the peripheral portion of the nucleus and the nucleolus in choroid plexus cells and cerebellar Purkinje cells [72]. These findings indicate that the localization of KLOTHO in HK-2 cells is similar to that in brain cells. However, miRNAs were reportedly localized at all major cellular organelles [73]. In this study, we obtained consistent data from our *in vitro* study and immunohistochemistry on human biopsy samples that oxidative stress decreased KLOTHO expression even though its localization was different in these experiments. The resulting data suggest that oxidative stress-induced miR-200c plays an important role in the downregulation of KLOTHO in proximal and distal tubular cells.

In summary, we show that H_2O_2 suppresses KLOTHO expression without a reduction in *KLOTHO* mRNA levels. The luciferase activity of a *KLOTHO* 3′-UTR reporter was decreased in response to H_2O_2 stimulation, indicating that an H_2O_2-induced miRNA regulates KLOTHO expression. A candidate miRNA is miR-200c, which has two possible binding sites in the *KLOTHO* 3′-UTR. Transfection of a miR-200c mimic decreased KLOTHO expression, whereas transfection of a miR-200c inhibitor maintained KLOTHO expression (Fig 7). In human renal biopsy samples, the levels of oxidative stress markers, such as 8-OHdG and 4HHE, were correlated with miR-200c and KLOTHO expression. These findings suggest that oxidative stress suppresses KLOTHO expression through the induction of miR-200c.

Supporting information

S1 Fig. miR-21 is upregulated by H_2O_2 exposure, but does not alter KLOTHO expression in HK-2 cells. (A) q-PCR analysis of pri-miR-21 and miR-21 expression in HK-2 cells cultured with or without 100 μM H_2O_2 at the indicated time points. U6 snRNA was used for normalization. (B) KLOTHO protein expression in HK-2 cells treated with 100 μM H_2O_2 for 24 hrs after the transfection of an inhibitor control (50 nM) or miR-21 inhibitor (50 nM) for 24 hrs. Band intensities were analyzed and normalized against TUBA using densitometry. $^{*}P < 0.05$, n = 6. Values represent individual measurements and the mean ± SD. Data were analyzed using the Mann-Whitney *U*-test or the Mann-Whitney *U*-test with Bonferroni correction. n.s.; not significant.
(TIF)

S2 Fig. Upregulation of *KLOTHO* mRNA levels by H_2O_2 stimulation is conserved in miR-200c inhibitor transfected HK-2 cells. The effect of H_2O_2 stimulation on *KLOTHO* mRNA expression in HK-2 cells transfected with miR-200c inhibitor was investigated. HK-2 cells were transfected with inhibitor control (25 nM) or miR-200c inhibitor (25 nM) and 12 hrs later they were treated with 100 μM H_2O_2. *KLOTHO* mRNA was detected by q-PCR. $^{*}P < 0.05$, n = 6. Values represent individual measurements and the mean ± SD. Data were analyzed using the Mann-Whitney *U*-test or the Mann-Whitney *U*-test with Bonferroni correction.
(TIF)

S3 Fig. Paraquat suppresses KLOTHO expression in HK-2 cells. KLOTHO protein expression in HK-2 cells treated with Paraquat for 24 hrs.
(TIF)

S4 Fig. Inhibition of the MAP kinase pathway does not restore KLOTHO suppression by H_2O_2 in HK-2 cells. (A, C, E) KLOTHO protein expression in HK-2 cells treated with 100 μM H_2O_2 for 24 hrs after the transfection of siRNAs (si-ERK, si-JNK and si-p38) or negative control siRNA (25 nM) for 24 hrs. Band intensities were analyzed and normalized against TUBA using densitometry. (B, D, E) MAP kinase expression in HK-2 cells treated with siRNAs. MAP; Mitogen-activated Protein, ERK; Extracellular Signal-regulated Kinase, JNK; c-Jun N-terminal Kinase.
(TIF)

S5 Fig. Full length western blots for Figs 1A, 3A and 4A.
(TIF)

S1 Table. Clinical characteristics related to renal function of IgA nephropathy patients.
(TIF)

Author Contributions

Conceptualization: Kenichi Morii, Satoshi Yamasaki, Shigehiro Doi.

Formal analysis: Taisuke Irifuku, Kensuke Sasaki, Toshiki Doi, Ayumu Nakashima.

Funding acquisition: Shigehiro Doi, Takao Masaki.

Investigation: Kenichi Morii.

Methodology: Satoshi Yamasaki, Shigehiro Doi.

Project administration: Shigehiro Doi, Takao Masaki.

Resources: Shigehiro Doi, Koji Arihiro.

Supervision: Shigehiro Doi.

Writing – original draft: Kenichi Morii.

Writing – review & editing: Satoshi Yamasaki, Shigehiro Doi.

References

1. Norris KC, Greene T, Kopple J, Lea J, Lewis J, Lipkowitz M, et al. Baseline predictors of renal disease progression in the African American study of hypertension and kidney disease. J Am Soc Nephrol. 2006; 17: 2928–2936. https://doi.org/10.1681/ASN.2005101101 PMID: 16959828
2. Webster AC, Nagler E V, Morton RL, Masson P. Chronic kidney disease. Lancet. 2017; 389: 1238–1252. https://doi.org/10.1016/S0140-6736(16)32064-5 PMID: 27887750
3. Tonelli M, Wiebe N, Culleton B, House A, Rabbat C, Fok M, et al. Chronic kidney disease and mortality risk: a systematic review. J Am Soc Nephrol. 2006; 17: 2034–2047. https://doi.org/10.1681/ASN.2005101085 PMID: 16738019
4. Mahmoodi BK, Matsushita K, Woodward M, Blankestijn PJ, Cirillo M, Ohkubo T, et al. Associations of kidney disease measures with mortality and end-stage renal disease in individuals with and without hypertension: a meta-analysis. Lancet. 2012; 380: 1649–1661. https://doi.org/10.1016/S0140-6736(12)61272-0 PMID: 23013600
5. Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. N Engl J Med. 2004; 351: 1296–1305. https://doi.org/10.1056/NEJMoa041031 PMID: 15385656

6. Honeycutt AA, Segel JE, Zhuo X, Hoerger TJ, Imai K, Williams D. Medical costs of CKD in the medicare population. J Am Soc Nephrol. 2013; 24: 1478–1483. https://doi.org/10.1681/ASN.2012040392 PMID: 23907508
7. Cachofeiro V, Goicochea M, de Vinuesa SG, Oubiña P, Lahera V, Luño J. Oxidative stress and inflammation, a link between chronic kidney disease and cardiovascular disease. Kidney Int Suppl. 2008; 74: S4–S9. https://doi.org/10.1038/ki.2008.516 PMID: 19034325
8. Krata N, Zagożdżon R, Foroncewicz B, Mucha K. Oxidative stress in kidney diseases: The cause or the consequence? Arch Immunol Ther Exp (Warsz). 2017; https://doi.org/10.1007/s00005-017-0496-0 PMID: 29214330
9. Dounousi E, Papavasiliou E, Makedou A, Ioannou K, Katopodis KP, Tselepis A, et al. Oxidative stress is progressively enhanced with advancing stages of CKD. Am J Kidney Dis. 2006; 48: 752–760. https://doi.org/10.1053/j.ajkd.2006.08.015 PMID: 17059994
10. Ha H, Lee HB. Reactive oxygen species as glucose signaling molecules in mesangial cells cultured under high glucose. Kidney Int Suppl. 2000; 77: S19–S25. http://www.ncbi.nlm.nih.gov/pubmed/10997686 PMID: 10997686
11. Sedeek M, Callera G, Montezano A, Gutsol A, Heitz F, Szyndralewiez C, et al. Critical role of Nox4-based NADPH oxidase in glucose-induced oxidative stress in the kidney: implications in type 2 diabetic nephropathy. Am J Physiol Renal Physiol. 2010; 299: F1348–F1358. https://doi.org/10.1152/ajprenal.00028.2010 PMID: 20630933
12. Buetler TM. Role of superoxide as a signaling molecule. News Physiol Sci. 2004; 19: 120–123. https://doi.org/10.1152/nips.01514.2003 PMID: 15143206
13. Sedeek M, Nasrallah R, Touyz RM, Hebert RL. NADPH oxidases, reactive oxygen species, and the kidney: friend and foe. J Am Soc Nephrol. 2013; 24: 1512–1518. https://doi.org/10.1681/ASN.2012111112 PMID: 23970124
14. Liu YN, Zhou J, Li T, Wu J, Xie SH, Liu H, et al. Sulodexide protects renal tubular epithelial cells from oxidative stress-induced injury via upregulating Klotho expression at an early stage of diabetic kidney disease. J Diabetes Res. 2017; 2017: 1–10. https://doi.org/10.1155/2017/4989847 PMID: 28929120
15. Mitobe M, Yoshida T, Sugiura H, Shirota S, Tsuchiya K, Nihei H. Oxidative stress decreases klotho expression in a mouse kidney cell line. Nephron Exp Nephrol. 2005; 101: e67–e74. https://doi.org/10.1159/000086500 PMID: 15976510
16. Calabrese V, Cornelius C, Leso V, Trovato-Salinaro A, Ventimiglia B, Cavallaro M, et al. Oxidative stress, glutathione status, sirtuin and cellular stress response in type 2 diabetes. Biochim Biophys Acta. 2012; 1822: 729–736. https://doi.org/10.1016/j.bbadis.2011.12.003 PMID: 22186191
17. Ohyama Y, Kurabayashi M, Masuda H, Nakamura T, Aihara Y, Kaname T, et al. Molecular cloning of rat klotho cDNA: markedly decreased expression of klotho by acute inflammatory stress. Biochem Biophys Res Commun. 1998; 251: 920–925. https://doi.org/10.1006/bbrc.1998.9576 PMID: 9791011
18. 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. https://doi.org/10.1038/36285 PMID: 9363890
19. Hu MC, Shi M, Zhang J, Pastor J, Nakatani T, Lanske B, et al. Klotho: a novel phosphaturic substance acting as an autocrine enzyme in the renal proximal tubule. FASEB J. 2010; 24: 3438–3450. https://doi.org/10.1096/fj.10-154765 PMID: 20466874
20. Qian Y, Guo X, Che L, Guan X, Wu B, Lu R, et al. Klotho reduces necroptosis by targeting oxidative stress involved in renal ischemic-reperfusion injury. Cell Physiol Biochem. 2018; 45: 2268–2282. https://doi.org/10.1159/000488172 PMID: 29550818
21. Yamamoto M, Clark JD, Pastor J V., Gurnani P, Nandi A, Kurosu H, et al. Regulation of oxidative stress by the anti-aging hormone klotho. J Biol Chem. 2005; 280: 38029–38034. https://doi.org/10.1074/jbc.M509039200 PMID: 16186101
22. Kurosu H, Yamamoto M, Clark JD, Pastor J V, Nandi A, Gurnani P, et al. Suppression of aging in mice by the hormone klotho. Science. 2005; 309: 1829–1833. https://doi.org/10.1126/science.1112766 PMID: 16123266
23. Satoh M, Nagasu H, Morita Y, Yamaguchi TP, Kanwar YS, Kashihara N. Klotho protects against mouse renal fibrosis by inhibiting Wnt signaling. Am J Physiol Renal Physiol. 2012; 303: F1641–F1651. https://doi.org/10.1152/ajprenal.00460.2012 PMID: 23034937
24. Doi S, Zou Y, Togao O, Pastor J V., John GB, Wang L, et al. Klotho inhibits transforming growth factor-β1 (TGF-β1) signaling and suppresses renal fibrosis and cancer metastasis in mice. J Biol Chem. 2011; 286: 8655–8665. https://doi.org/10.1074/jbc.M110.174037 PMID: 21209102
25. Lin Y, Kuro-o M, Sun Z. Genetic deficiency of anti-aging gene klotho exacerbates early nephropathy in STZ-induced diabetes in male mice. Endocrinology. 2013; 154: 3855–3863. https://doi.org/10.1210/en.2013-1053 PMID: 23928372

26. Mencke R, Olauson H, Hillebrands JL. Effects of klotho on fibrosis and cancer: A renal focus on mechanisms and therapeutic strategies. Adv Drug Deliv Rev. 2017; 121: 85–100. https://doi.org/10.1016/j.addr.2017.07.009 PMID: 28709936

27. 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–F1264. https://doi.org/10.1152/ajprenal.00294.2011 PMID: 22338084

28. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet. 2004; 5: 522–631. https://doi.org/10.1038/nrg1379 PMID: 15211354

29. Mimura I, Tanaka T, Nangaku M. New insights into molecular mechanisms of epigenetic regulation in kidney disease. Clin Exp Pharmacol Physiol. 2016; 43: 1159–1167. https://doi.org/10.1111/1440-1681.12663 PMID: 27560313

30. Reik W. Stability and flexibility of epigenetic gene regulation in mammalian development. Nature. 2007; 447: 425–432. https://doi.org/10.1038/nature05918 PMID: 17522676

31. Slotkin RK, Martienssen R. Transposable elements and the epigenetic regulation of the genome. Nat Rev Genet. 2007; 8: 272–285. https://doi.org/10.1038/nrg2072 PMID: 17363976

32. Stefani G, Slack FJ. Small non-coding RNAs in animal development. Nat Rev Mol Cell Biol. 2008; 9: 219–230. https://doi.org/10.1038/nrm2347 PMID: 18270516

33. Pasquinelli AE. MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. Nat Rev Genet. 2012; 13: 271–282. https://doi.org/10.1038/nrg3162 PMID: 22411466

34. Bartel DP. MicroRNAs: Target Recognition and Regulatory Functions. Cell. 2009; 136: 215–233. https://doi.org/10.1016/j.cell.2009.01.002 PMID: 19167326

35. Magenta A, Cencioni C, Fasanaro P, Zaccagnini G, Greco S, Sarra-Ferraris G, et al. MiR-200c is upregulated by oxidative stress and induces endothelial cell apoptosis and senescence via ZEB1 inhibition. Cell Death Differ. 2011; 18: 1628–1639. https://doi.org/10.1038/cdd.2011.42 PMID: 21527937

36. Irifuku T, Doi S, Sasaki K, Doi T, Nakashima A, Ueno T, et al. Inhibition of H3K9 histone methyltransferase G9a attenuates renal fibrosis and retains klotho expression. Kidney Int. 2016; 89: 147–157. https://doi.org/10.1038/ki.2015.291 PMID: 26444031

37. Sasaki K, Doi S, Nakashima A, Irifuku T, Yamada K, Kokoroishi K, et al. Inhibition of SET domain-containing lysine methyltransferase 7/9 ameliorates renal fibrosis. J Am Soc Nephrol. 2016; 27: 203–215. https://doi.org/10.1681/ASN.2014090850 PMID: 26045091

38. Yamada K, Doi S, Nakashima A, Kawaoka K, Ueno T, Doi T, et al. Expression of age-related factors during the development of renal damage in patients with IgA nephropathy. Clin Exp Nephrol. 2015; 19: 830–837. https://doi.org/10.1007/s10157-014-1070-2 PMID: 25504369

39. Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. Nucleic Acids Res. 2008; 36: D149–D153. https://doi.org/10.1093/nar/gkm995 PMID: 18158296

40. Llave C, Xie Z, Kasschau KD, Carrington JC. Cleavage of scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science. 2002; 297: 2053–2056. https://doi.org/10.1126/science.1076311 PMID: 12242443

41. Yekta S, Shih IH, Bartel DP. MicroRNA-directed cleavage of HOXB8 mRNA. Science. 2004; 304: 594–596. https://doi.org/10.1126/science.1097434 PMID: 15105502

42. Miyata T, Suzuki N, van Ypersele de Strihou C. Diabetic nephropathy: are there new and potentially promising therapies targeting oxygen biology? Kidney Int. 2013; 84: 693–702. https://doi.org/10.1038/ki.2013.74 PMID: 23486514

43. Pavlakou P, Liakopoulos V, Eleftheriadis T, Mitsis M, Dounousi E. Oxidative stress and acute kidney injury in critical illness: pathophysiologic mechanisms-biomarkers-interventions, and future perspectives. Oxid Med Cell Longev. 2017; 2017: 1–11. https://doi.org/10.1155/2017/6193694 PMID: 29104728

44. Billings FT 4th, Pretorius M, Schildcrout JS, Mercaldo ND, Byrne JG, Ikizler TA, et al. Obesity and oxidative stress predict AKI after cardiac surgery. J Am Soc Nephrol. 2012; 23: 1221–1228. https://doi.org/10.1681/ASN.2011090940 PMID: 22626819

45. de Zeeuw D, Akizawa T, Audhya P, Bakris GL, Chin M, Christ-Schmidt H, et al. Bardoxolone methyl in type 2 diabetes and stage 4 chronic kidney disease. N Engl J Med. 2013; 369: 2492–2503. https://doi.org/10.1056/NEJMoa1306033 PMID: 24206459

46. Xie J, Yoon J, An SW, Kuro-o M, Huang CL. Soluble klotho protects against uremic cardiomyopathy independently of fibroblast growth factor 23 and phosphate. J Am Soc Nephrol. 2015; 26: 1150–1160. https://doi.org/10.1681/ASN.2014040325 PMID: 25475745

47. Hu MC, Shi M, Gillings N, Flores B, Takahashi M, Kuro-o M, et al. Recombinant α-klotho may be prophylactic and therapeutic for acute to chronic kidney disease progression and uremic cardiomyopathy. Kidney Int. 2017; 91: 1104–1114. https://doi.org/10.1016/j.kint.2016.10.034 PMID: 28131398

48. Hu MC, Shi M, Cho HJ, Adams-Huet B, Paek J, Hill K, et al. Klotho and phosphate are modulators of pathologic uremic cardiac remodeling. J Am Soc Nephrol. 2015; 26: 1290–1302. https://doi.org/10.1681/ASN.2014050465 PMID: 25326585

49. Tampe D, Zeisberg M. Potential approaches to reverse or repair renal fibrosis. Nat Rev Nephrol. 2014; 10: 226–37. https://doi.org/10.1038/nrneph.2014.14 PMID: 24514753

50. LeBleu VS, Taduri G, O'Connell J, Teng Y, Cooke VG, Woda C, et al. Origin and function of myofibroblasts in kidney fibrosis. Nat Med. 2013; 19: 1047–1053. https://doi.org/10.1038/nm.3218 PMID: 23817022

51. Wang B, Koh P, Winbanks C, Coughlan MT, McClelland A, Watson A, et al. MiR-200a prevents renal fibrogenesis through repression of TGF-β2 expression. Diabetes. 2011; 60: 280–287. https://doi.org/10.2337/db10-0892 PMID: 20952520

52. Xiong M, Jiang L, Zhou Y, Qiu W, Fang L, Tan R, et al. The miR-200 family regulates TGF-β1-induced renal tubular epithelial to mesenchymal transition through Smad pathway by targeting ZEB1 and ZEB2 expression. Am J Physiol Renal Physiol. 2012; 302: F369–F379. https://doi.org/10.1152/ajprenal.00268.2011 PMID: 22012804

53. Du B, Ma LM, Huang MB, Zhou H, Huang HL, Shao P, et al. High glucose down-regulates miR-29a to increase collagen IV production in HK-2 cells. FEBS Lett. 2010; 584: 811–816. https://doi.org/10.1016/j.febslet.2009.12.053 PMID: 20067797

54. Qin W, Chung AC, Huang XR, Meng XM, Hui DS, Yu CM, et al. TGF-β/Smad3 signaling promotes renal fibrosis by inhibiting miR-29. J Am Soc Nephrol. 2011; 22: 1462–1474. https://doi.org/10.1681/ASN.2010121308 PMID: 21784902

55. Chen HY, Zhong X, Huang XR, Meng XM, You Y, Chung AC, et al. MicroRNA-29b inhibits diabetic nephropathy in db/db mice. Mol Ther. The American Society of Gene & Cell Therapy; 2014; 22: 842–853. https://doi.org/10.1038/mt.2013.235 PMID: 24445937

56. Lin CL, Lee PH, Hsu YC, Lei CC, Ko JY, Chuang PC, et al. MicroRNA-29a promotion of nephrin acetylation ameliorates hyperglycemia-induced podocyte dysfunction. J Am Soc Nephrol. 2014; 25: 1698–1709. https://doi.org/10.1681/ASN.2013050527 PMID: 24578127

57. Zarjou A, Yang S, Abraham E, Agarwal A, Liu G. Identification of a microRNA signature in renal fibrosis: role of miR-21. Am J Physiol Renal Physiol. 2011; 301: F793–F801. https://doi.org/10.1152/ajprenal.00273.2011 PMID: 21775484

58. Zhong X, Chung AC, Chen HY, Dong Y, Meng XM, Li R, et al. MiR-21 is a key therapeutic target for renal injury in a mouse model of type 2 diabetes. Diabetologia. 2013; 56: 663–674. https://doi.org/10.1007/s00125-012-2804-x PMID: 23292313

59. Kato M, Zhang J, Wang M, Lanting L, Yuan H, Rossi JJ, et al. MicroRNA-192 in diabetic kidney glomeruli and its function in TGF-beta-induced collagen expression via inhibition of E-box repressors. Proc Natl Acad Sci. 2007; 104: 3432–3437. https://doi.org/10.1073/pnas.0611192104 PMID: 17360662

60. Chung ACK, Huang XR, Meng X, Lan HY. MiR-192 mediates TGF-β/Smad3-driven renal fibrosis. J Am Soc Nephrol. 2010; 21: 1317–1325. https://doi.org/10.1681/ASN.2010020134 PMID: 20488955

61. Putta S, Lanting L, Sun G, Lawson G, Kato M, Natarajan R. Inhibiting microRNA-192 ameliorates renal fibrosis in diabetic nephropathy. J Am Soc Nephrol. 2012; 23: 458–469. https://doi.org/10.1681/ASN.2011050485 PMID: 22223877

62. Li R, Chung AC, Dong Y, Yang W, Zhong X, Lan HY. The microRNA miR-433 promotes renal fibrosis by amplifying the TGF-β/Smad3-Azin1 pathway. Kidney Int. 2013; 84: 1129–1144. https://doi.org/10.1038/ki.2013.272 PMID: 23868013

63. Mehi SJ, Maltare A, Abraham CR, King GD. MicroRNA-339 and microRNA-556 regulate klotho expression in vitro. Age (Dordr). 2014; 36: 141–149. https://doi.org/10.1007/s11357-013-9555-6 PMID: 23818104

64. Jiao A, Sui M, Zhang L, Sun P, Geng D, Zhang W, et al. MicroRNA-200c inhibits the metastasis of non-small cell lung cancer cells by targeting ZEB2, an epithelial-mesenchymal transition regulator. Mol Med Rep. 2016; 13: 3349–3355. https://doi.org/10.3892/mmr.2016.4901 PMID: 26935975

65. Park SM, Gaur AB, Lengyel E, Peter ME. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes Dev. 2008; 22: 894–907. https://doi.org/10.1101/gad.1640608 PMID: 18381893

66. Zhu Y, Xu L, Zhang J, Xu W, Liu Y, Yin H, et al. Klotho suppresses tumor progression via inhibiting PI3K/Akt/GSK3β/Snail signaling in renal cell carcinoma. Cancer Sci. 2013; 104: 663–671. https://doi.org/10.1111/cas.12134 PMID: 23433103

67. Chang B, Kim J, Jeong D, Jeong Y, Jeon S, Jung SI, et al. Klotho inhibits the capacity of cell migration and invasion in cervical cancer. Oncol Rep. 2012; 28: 1022–1028. https://doi.org/10.3892/or.2012.1865 PMID: 22710352

68. Ruffels J, Griffin M, Dickenson JM. Activation of ERK1/2, JNK and PKB by hydrogen peroxide in human SH-SY5Y neuroblastoma cells: role of ERK1/2 in H_2O_2-induced cell death. Eur J Pharmacol. 2004; 483: 163–173. https://doi.org/10.1016/j.ejphar.2003.10.032 PMID: 14729104

69. Dabrowski A, Boguslowicz C, Dabrowska M, Tribillo I, Gabryelewicz A. Reactive oxygen species activate mitogen-activated protein kinases in pancreatic acinar cells. Pancreas. 2000; 21: 376–384. https://doi.org/10.1097/00006676-200011000-00008 PMID: 11075992

70. Li S-A, Watanabe M, Yamada H, Nagai A, Kinuta M, Takei K. Immunohistochemical localization of Klotho protein in brain, kidney, and reproductive organs of mice. Cell Struct Funct. 2004; 29: 91–99. https://doi.org/10.1247/csf.29.91 PMID: 15665504

71. 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. https://doi.org/10.1016/j.febslet.2004.03.090 PMID: 15135068

72. German DC, Khobahy I, Pastor J, Kuro-o M, Liu X. Nuclear localization of Klotho in brain: an anti-aging protein. Neurobiol Aging. Elsevier Inc.; 2012; 33: 1483.e25–1483.e30. https://doi.org/10.1016/j.neurobiolaging.2011.12.018 PMID: 22245317

73. Makarova JA, Shkurnikov MU, Wicklein D, Lange T, Samatov TR, Turchinovich AA, et al. Intracellular and extracellular microRNA: An update on localization and biological role. Prog Histochem Cytochem. Elsevier B.V.; 2016; 51: 33–49. https://doi.org/10.1016/j.proghi.2016.06.001 PMID: 27396686