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Brief Communication

Complement Modulation of Anti-Aging Factor Klotho in Ischemia/Reperfusion Injury and Delayed Graft Function

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Klotho is an anti-aging factor mainly produced by renal tubular epithelial cells (TEC) with pleiotropic functions. Klotho is down-regulated in acute kidney injury in native kidney; however, the modulation of Klotho in kidney transplantation has not been investigated. In a swine model of ischemia/reperfusion injury (IRI), we observed a remarkable reduction of renal Klotho by 24 h from IRI. Complement inhibition by C1-inhibitor preserved Klotho expression *in vivo* by abrogating nuclear factor kappa B (NF-kB) signaling. In accordance, complement anaphylotoxin C5a led to a significant down-regulation of Klotho in TEC *in vitro* that was NF-kB mediated. Analysis of Klotho in kidneys from cadaveric donors demonstrated a significant expression of Klotho in pre-implantation biopsies; however, patients affected by delayed graft function (DGF) showed a profound down-regulation of Klotho compared with patients with early graft function. Quantification of serum Klotho after 2 years from transplantation demonstrated significant lower levels in DGF patients. Our data demonstrated that complement might be pivotal in the down-regulation of Klotho in IRI leading to a permanent deficiency after years from transplantation. Considering the anti-senescence and anti-fibrotic effects of Klotho at renal levels, we hypothesize that this acquired deficiency of Klotho might contribute to DGF-associated chronic allograft dysfunction.

Abbreviations: ANOVA, analysis of variance; CAPE, caffeic acid phenethyl ester; CKD, chronic kidney disease; CTR, control group; C1-INH, C1-inhibitor; DGF, delayed graft function; EGF, early graft function; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; IQR, interquartile range; IRI, ischemia reperfusion injury; NF-kB, nuclear factor kappa B; pNIK, phospho NF-kB inducing kinase; qRT-PCR, quantitative reverse-transcription PCR; SD, standard deviation; SEM, standard error of the mean; TEC, tubular epithelial cells; TNF, tumor necrosis factor

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Introduction

Delayed graft function (DGF) is a common complication after renal transplantation and negatively affects long-term graft function (1,2). DGF is characterized by a complex pathogenesis resulted from tubular injury, interstitial inflammation and altered microcirculation. Ischemia/reperfusion injury (IRI) plays a pivotal role in DGF (3–5). The activation of complement occurs in IRI leading to the release of pro-inflammatory mediators such as C5a and C5b-9 that exacerbate inflammatory response and subsequent renal tissue damage (3,6). Inhibition of complement activation significantly protects renal parenchyma by reducing infiltrating leucocytes, tubular damage and renal fibrosis (4,5,7). Klotho was originally identified in mice as anti-senescence gene; deficiency in Klotho led to a premature aging syndrome (8). Klotho is mainly expressed in renal tubular epithelial cells (TEC) in two forms such as the membrane-bound and the soluble Klotho (9). Membrane Klotho regulates mineral homeostasis while the soluble form released in blood, urine, and cerebrospinal fluid acts as endocrine factor regulating extra renal functions (10). Interestingly, Klotho is an early biomarker of acute kidney injury and is down regulated in IRI (11,12); few data are available on the mechanisms of modulation in

transplant patients (10,13). The aim of our study was to investigate the possible involvement of complement system in the modulation of Klotho in IRI and in patients affected by DGF.

Materials and Methods

Animal model

The animal model of renal IRI was developed in ten 4-month-old female large white pigs as previously described (14). Renal IRI was induced by clamping the left renal artery for 30 min followed by removing the clamp allowing reperfusion. Animals were randomized into two groups: control pigs (CTR) (group, n = 5) and treated pigs with C1 Inhibitor (C1-INH) (group, n = 5). Five minutes before the end of the ischemia time, recombinant C1-INH was diluted in saline solution and was injected in the ear vein (500 U/kg) (14); in the control group an equal volume of vehicle was infused at the same time point. Multiple biopsies were performed and animals were sacrificed 24 h after the surgical procedure. This study was conducted after the approval of the Ethical Committee of the Italian Ministry of Health.

Patients

In our study, we randomly selected seven consecutive adult first renal transplant recipients who developed DGF and seven patients that underwent renal biopsy within 15 days from transplantation for calcineurin inhibitors toxicity, used as control group. The presence of DGF was defined as the need for dialysis in the first week after transplantation. A wedge biopsy of donor kidney was performed before transplantation. A second graft biopsy was performed 7 to 15 days after transplantation in all patients with DGF. All patients included in the study were given 500 mg of methylprednisolone intra-operatively, followed by 250 mg of prednisone daily, with the dose tapered to 25 mg by day 8; 20 mg of a chimeric monoclonal anti-CD25 antibody (Simulect, Novartis) intravenously on day 0 and day 4; mycophenolate mofetil (Cell-Cept, Shanghai Roche Pharmaceuticals LTD, Shanghai, China; Roche Diagnostics, Milano, Italy) 1000 mg b.i.d and either cyclosporine A (Neoral, Novartis, C2 levels 800–1200 ng/mL) or tacrolimus (Prograf, Astellas, through levels 8–12 ng/mL). For evaluation of Klotho serum level, 16 patients who received renal cadaveric transplants 2 years before, with comparable and stable renal function were included in this retrospective analysis (eight DGF vs. eight early graft function [EGF] patients; EGF eGFR 73.03 ± 14.13 mL/min; DGF eGFR 63.29 ± 20.62 mL/min; $p > 0.05$). Serum samples of these patients were collected and stored at –80°C until use. The human studies were performed according to the principles of the Declaration of Helsinki and were approved by our local ethical committee (study number 4440) and conducted according to the guidelines of good clinical practice. Written informed consent was obtained from each patient before inclusion.

Cell culture

Immortalized Proximal TEC, HK-2, were grown to confluence in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mM L-glutamine (Sigma Aldrich, Milan, Italy), in 5% CO_2 at 37°C. Confluent cells were stimulated with C5a (Biovision, San Francisco, CA) at 10^{-7} M and/or CAPE (caffeic acid phenethyl ester, 20 ng/mL), a NF-kB inhibitor, for the indicated time period.

Klotho immunohistochemistry

Klotho staining was performed on human and swine paraffin-embedded renal sections with rabbit polyclonal anti-Klotho 1:200 antibody (Abcam, Cambridge, UK, for swine sections; Lifespan Biosciences, Seattle, WA, for human sections). The slides were deparaffinized, underwent epitope unmasking (by pressure-cooking in ethylenediaminetetraacetic acid-(EDTA) buffer for swine sections and by microwave and citrate buffer for human sections), then incubated with H_2O_2 (3%), tween-20 (0.1%), protein block solution (Dako, Glostrup, Denmark) and with the primary antibody. Primary antibody was detected by the Peroxidase/DAB Dako Real EnVision Detection System, according to manufacturer's instructions (Dako, Glostrup, Denmark). Sections were counterstained with Mayer haematoxylin (blue) and mounted with glycerol (DakoCytomation, Glostrup, Denmark). Negative controls were prepared with isotype control Ab. Digital images were obtained using Aperio ScanScope CS2 device (Aperio Technologies, Vista, CA) and further analyzed by ImageScope V12.1.0.5029 (Aperio Technologies, Vista, CA). Klotho staining was quantified using the algorithm for analysis of membrane signal (IHC Membrane Image Analysis algorithm, Aperio) and expressed as percentage of positive pixels in the analyzed area.

Confocal laser scanning microscopy

Paraffin-embedded renal sections were stained with primary antibodies anti-phospho nuclear factor kappa B inducing kinase (pNIK) ^{Thr559} (1:100 Santa Cruz Biotechnologies, Santa Cruz, CA) and anti-p65/RelA (1:50 Santa Cruz Biotechnologies) and the corresponding secondary antibodies (Alexa Flour 488 and 555 anti-rabbit, Molecular Probes, Eugene, OR). Negative controls were prepared by omitting the primary antibody. All sections were counterstained with TO-PRO-3 (Molecular Probes). Specific fluorescence was acquired using the confocal microscope Leica TCS SP2 (Leica, Wetzlar, Germany). Fluorescence signals were quantified using Adobe Photoshop software and expressed as area fraction (%).

RNA extraction and quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was isolated by Qiacube with RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and quantified by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). Its quality was assessed by electrophoresis on the agarose gel (1%). One-half microgram of total RNA was used in a reverse transcription (RT) reaction using the Quantitect reverse transcription Kit (Qiagen) according to the manufacturer's instructions. Human Klotho (sense 5′-CTTAAAAGCCATCAAGCTGGA-3′ and antisense 5′- GGCAACAA-CATCTTGTCCTG-3′), and glyceraldehyde-3 phosphate dehydrogenase (GAPDH), (sense 5′-GAAGGTGAAGGTCGGAGTCA-3′ and antisense 5′-GGGTGGAATCATATTGGAA-3′) specific primers were used for mRNA amplification and quantification. qRT-PCR were performed in triplicate on a Light Cycler 96 (Roche Diagnostics, Mannheim, Germany). Klotho expression levels were normalized with GAPDH expression using the 2ΔΔCt method.

Western blotting

Cell samples were homogenized in RIPA lysis buffer. Twenty micrograms of proteins from each cell lysate or 40 μg of proteins from tissue homogenate were separated on a pre-cast 4–15% polyacrylamide gel (BioRad, Hercules, CA) under reducing conditions and then electro transferred onto polyvinylidene diflouride membrane (Trans-Blot Turbo Midi PVDF, 0.2 μM; BioRad) by Trans-Blot Turbo transfer system (BioRad). Membranes were probed with rabbit anti-Klotho antibodies (1:1000, Lifespan Biosciences, Seattle, WA), and then incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit 1:7500 Santa Cruz). The same membranes were re-hybridized with mouse monoclonal anti-βactin antibody (1:20 000; Sigma, Saint Louis, MO). Immune complexes were detected by the ECL chemiluminescence system (Amersham Pharmacia, Little Chalfont, UK),

according to the manufacturer's instructions. The chemiluminescent blots were acquired by Chemidoc and analyzed using Image J software.

Immunophenotypic analysis

Klotho staining was performed with unconjugated rabbit polyclonal anti-Klotho antibody (LifeSpan Biosciences). At the end of the stimulation with C5a, cells were washed, fixed, permeabilized with Intraprep reagents (Instrumentation Laboratory, Milano, Italy) and incubated for 20 min at room temperature with the primary antibody. Then cells were washed and incubated with the secondary antibody AlexaFluor 488 (Molecular Probes) for 15 min. Data were obtained using a FC500 (Beckmann Coulter, Pasadena, CA) flow cytometer and analyzed by Kaluza software. Three independent experiments were performed. The area of positivity was determined using an isotype-matched mAb; a total of 10^4 events for each sample were acquired.

Klotho serum levels

Klotho level were quantified in patients serum by ELISA assay (IBL, Hamburg, Germany) according to manufacturer's instructions.

Microarray analysis

Raw data available under accession number GSE37838 (15) were downloaded from the Gene Expression Omnibus database and analyzed using the Agilent Gene Spring GX 12.5 software. Genes displaying differential expression between donor biopsies that developed DGF ($n = 12$) versus donor biopsies that developed EGF were detected by unpaired t-test with Storey and Tibshirani multiple testing correction (16), using a q-value cut-off of 0.05 and a fold-change cut-off of 1.4: only genes that were statistically significantly modulated were considered.

Statistical analysis

Experimental data were shown as median ± interquartile range (IQR) or as mean ± standard deviation (SD) or as mean ± standard error of the mean (SEM) as appropriated. Statistical analysis was performed using paired, unpaired Student t-test, Mann–Whitney test or ANOVA, as appropriate. A p-value <0.05 was considered statistically significant. All analyses were performed using GraphPad Prism 5.0 (San Diego, CA).

Results

Complement inhibition preserved tubular Klotho in IRI

We first investigated the modulation of Klotho in a swine model of renal IRI by immunohistochemical analysis. Before ischemia, Klotho protein was expressed in renal tubules but not at vascular and glomerular level, as expected (Figure 1A). After 24 h of reperfusion, we found a remarkable reduction of Klotho at tubular level (Figure 1B). On the contrary, inhibition of complement by C1-INH could preserve Klotho expression (Figure 1C; negative control 1D). Quantification of specific tubulo-interstitial staining showed that the down-regulation of Klotho in IRI compared to basal levels was statistically significant as well as the preservation by C-INH (Figure 1E, $p < 0.05$). These data were further confirmed by western blotting on renal protein lysate from tissue biopsies (Figure 1F, $p = 0.004$).

C5a down-regulated Klotho mRNA expression and protein production in renal TEC

C5a anaphylotoxin is a major mediator in complement-induced IRI (6,17). We then investigated whether C5a could regulate mRNA Klotho expression in proximal TEC. C5a induced a marked reduction of Klotho mRNA synthesis after 18 h and 24 h of stimulation (Figure 2A). By western blot analysis, we observed a significant decrease in protein synthesis of Klotho (Figure 2B) compared to basal condition at different time points.

Complement activation down-regulated Klotho expression in a NF-kB-dependent manner

Since Nuclear Factor kappa B (NF-kB) signaling is critical in regulating Klotho production, we next investigated its activation in our model by analyzing both the canonical and noncanonical pathways (18). IRI caused a strong activation of pNIK both at tubular and glomerular level, particularly at 30 min of reperfusion (Figure 3A). Complement inhibition abrogated NIK phosphorylation (Figure 3B); quantification of $pNIK^{Thr559}$ showed that the modulation of NIK was statistically significant (Figure 3C). We then performed an immunofluorescence staining for the p65/RelA subunit. As expected, we detected low level of p65/RelA in kidney biopsies before ischemia (Figure 3D). After 60 min of reperfusion, we observed an increase of p65/RelA signal in the cytoplasm (Figure 3E). Interestingly, after 24 h of reperfusion, p65/RelA transmigrated into the nucleus of tubular cells (Figure 3G; 3H arrows). C1-INH treated pigs showed impaired p65/RelA activation both at 60 min and 24 h from IRI (Figure 3F and I). We then exposed TEC to C5a in the presence of CAPE, a specific NF-kB inhibitor, *in vitro*. Interestingly, NF-kB inhibition abrogated C5a-induced Klotho reduction in TEC as indicated by cytofluorimetric analysis (Figure 3J). The observed bimodal expression of Klotho was related to apoptotic rates occurring in the different experimental conditions (Figure S2), since apoptosis inhibits Klotho expression (19).

Modulation of Klotho in transplant recipients with DGF

Finally, we investigated the modulation of Klotho in human allograft biopsies. Analysis of pre-implantation renal biopsies from cadaveric kidney donors showed that Klotho was significantly expressed at tubular level with a clear distribution along the basal membrane (Figure 4A and D). As control, renal biopsies performed by 7 days posttransplantation in patients with diagnosis of calcineurin-inhibitor tubular toxicity indicated a partial reduction in Klotho expression (Figure 4B and E). Interestingly, patients with DGF showed a dramatic reduction in Klotho that was barely detectable at tubular level (Figure 4C and F). When we compared tissue Klotho expression in DGF patients and in pre-implantation biopsies from the same donors, we found that the down-regulation was statistically significant (Figure 4G).

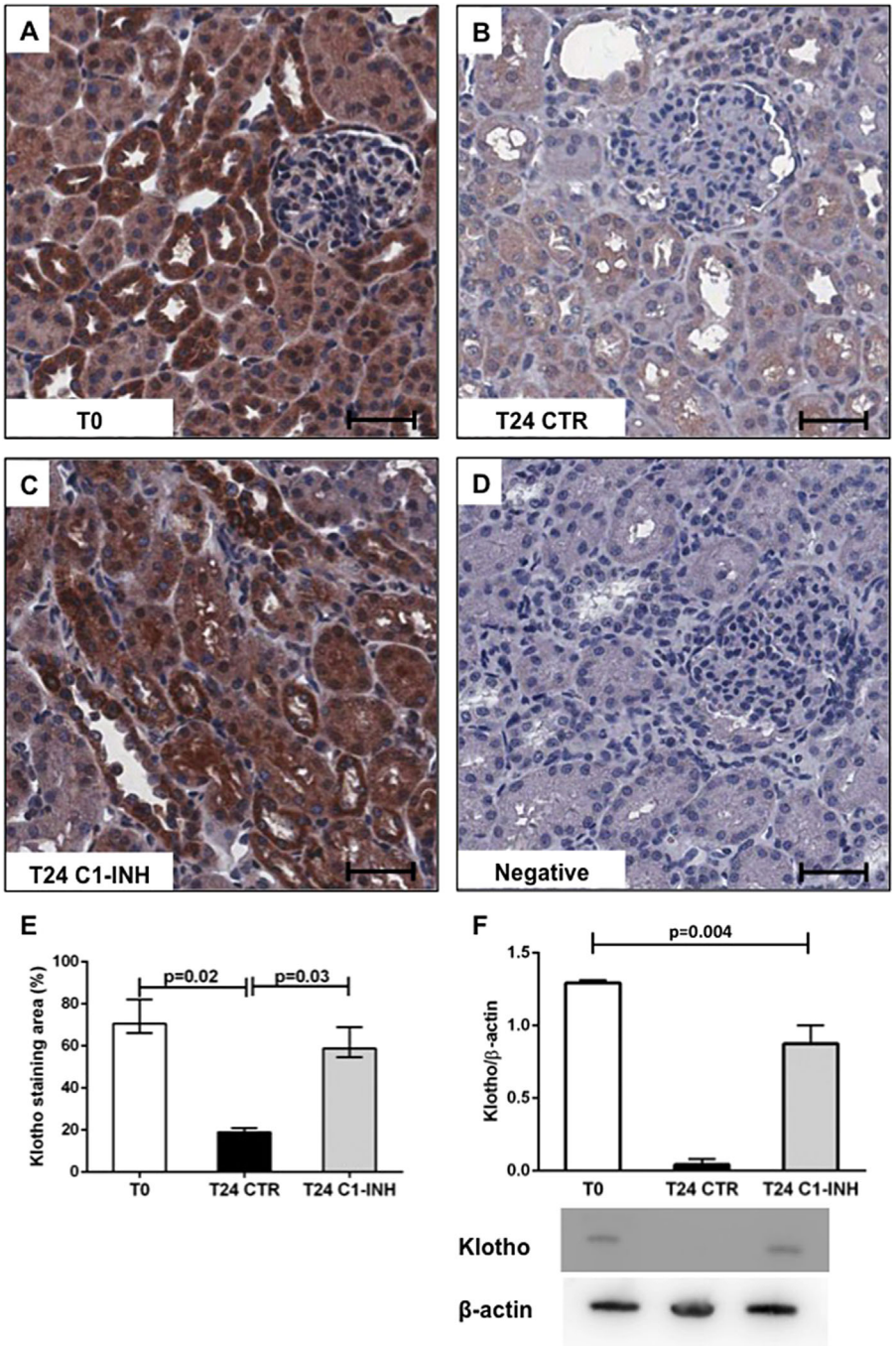

Figure 1: Complement inhibition preserved renal Klotho expression in a swine model of IRI. We performed an immunoperoxidase staining on renal paraffin-embedded section before ischemia (T0) (A) and at 24 h after reperfusion in control (T24 CTR n = 5) (B) and treated (T24 C1-INH n = 5) (C) pigs. Klotho immunohistochemistry antibody isotype negative control (D). Scale bar = 50 μm. Klotho staining was quantified as described in the Methods section and expressed as median ± IQR of at least five independent pigs for each group (E). Klotho protein expression was also evaluated by western blotting analysis on renal tissue homogenates from kidney biopsies. Results are expressed as median ± IQR of three independent pigs for each group; β-actin protein expression was used for normalization (F). Full-size western blot is shown in Figure S1. CTR, control group; C1-INH, C1-inhibitor; IHC, immunohistochemistry; QR, interquartile range; IRI, ischemia reperfusion injury.

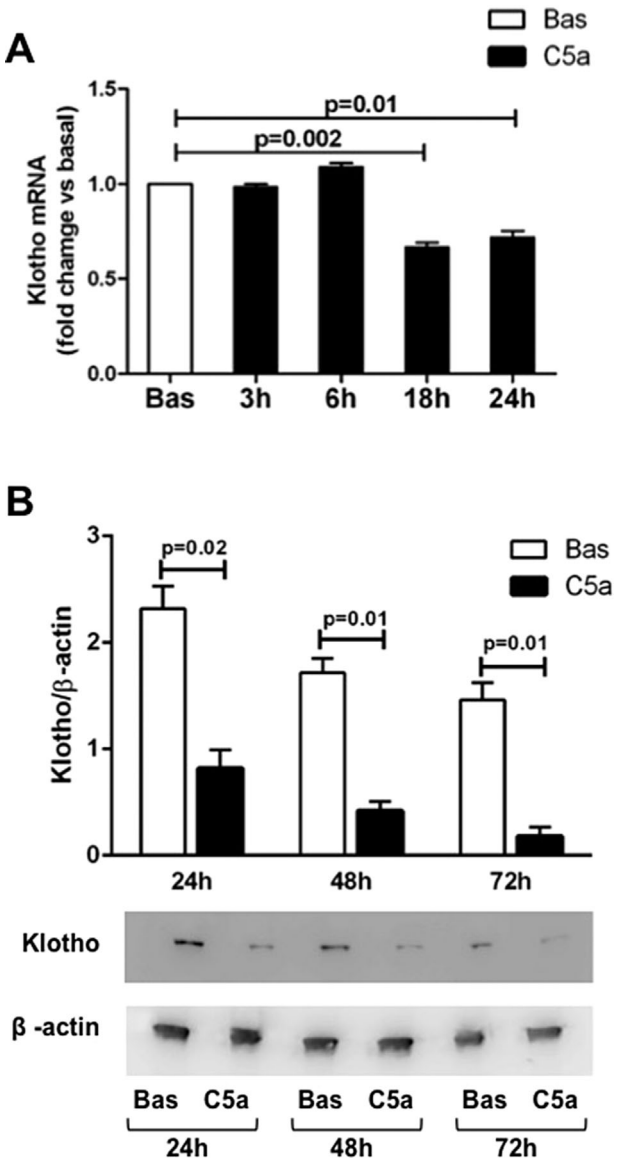

Figure 2: C5a reduced Klotho expression in TEC *in vitro*. (A) HK-2 cells were incubated with C5a at 10^{-7} M for 3, 6, 18, and 24 h. qRT-PCR revealed a significant decrease of Klotho mRNA after 18 and 24 h of incubation. Graph shows fold change in Klotho gene expression (normalized to GAPDH) between the basal and stimulated condition. Results are expressed as mean ± SEM and are representative of three independent experiments. (B) HK-2 cells were incubated with C5a at 10^{7} M for 24, 48, and 72 h. Western blot analysis showed a significant reduction of Klotho protein compared to basal condition at different time points; β-actin protein expression was used for normalization. Results are expressed as mean ± SD and are representative of three independent experiments. Full-size western blot is shown in Figure S1. GAPDH, glyceraldehyde-3 phosphate dehydrogenase; SEM, standard error of the mean; TEC, tubular epithelial cells.

Next, we searched microarray datasets in the literature that analyzed gene expression profiles of kidney tissue from transplant recipient collected in the early period posttransplantation (Gene Expression Omnibus, GEO database, http://www.ncbi.nlm.nih.gov/geo/). We identified microarray series GSE37838 (15) run on the U133Plus2.0 Affymetrix microarrays (Affymetrix, Santa Clara, CA) focusing on implantation allograft biopsies from patients that developed DGF (n = 12) versus patients with early graft function (EGF, n = 68). Interestingly, we found a down-regulation of Klotho gene expression with a Fold change $\geq$1.4 (false discovery rate $\leq$0.05) in patients that developed DGF comparing with EGF patients (Figure 4H). Finally, we investigated whether the down-regulation of Klotho synthesis was reversible after the resolution of DGF by measuring the soluble form of the protein. Interestingly, transplant patients who experienced DGF had significant low levels of soluble Klotho compared with patients with EGF after 2 years from transplantation (Figure 4I).

Discussion

In the present study, we demonstrated for the first time that the complement system is primarily involved in the down-regulation of Klotho in renal IRI. Inhibition of complement prevented the activation of NF-kB signaling in tubular epithelial cells resulting in preservation of Klotho production. Transplant patients with DGF presented a significant down-regulation of tubular Klotho expression resulting in a permanent deficiency of soluble Klotho after transplantation.

Klotho was originally identified as an anti-aging gene (8); Klotho deficiency in mice caused a syndrome resembling accelerated aging with a broad spectrum of organ abnormalities (8,20). Klotho is expressed in several organs such as parathyroid glands and choroid plexus but the kidney is the major site of production (10,20,21). Full-length Klotho is a transmembrane protein that acts as co-receptor for the fibroblast growth-factor-23 (10). A soluble form of Klotho was also identified and can be generated by cell membrane shedding or by alternative splicing (9). Recent studies showed that different forms of renal damage might lead to a down-regulation of Klotho such as IRI, ureteric obstruction or toxins like cisplatin (12). Following IRI in animal models, there was a remarkable reduction of Klotho in kidneys, followed by a reduced release in urines and blood (22,23). Interestingly, IRI led to Klotho reduction in plasma and urines as early as at 3 h after injury (22). However, the factors modulating Klotho expression in renal injury are not fully understood. Both systemic and local inflammation may decrease Klotho expression (12); inflammatory cytokines such as tumor necrosis factor (TNF)-alpha, and interferon-gamma reduced renal Klotho in rodent model and in TEC (11,24). Exogenous administration of TNF-like weak inducer of apoptosis, TWEAK, decreased expression of renal Klotho in mice (11). Since the

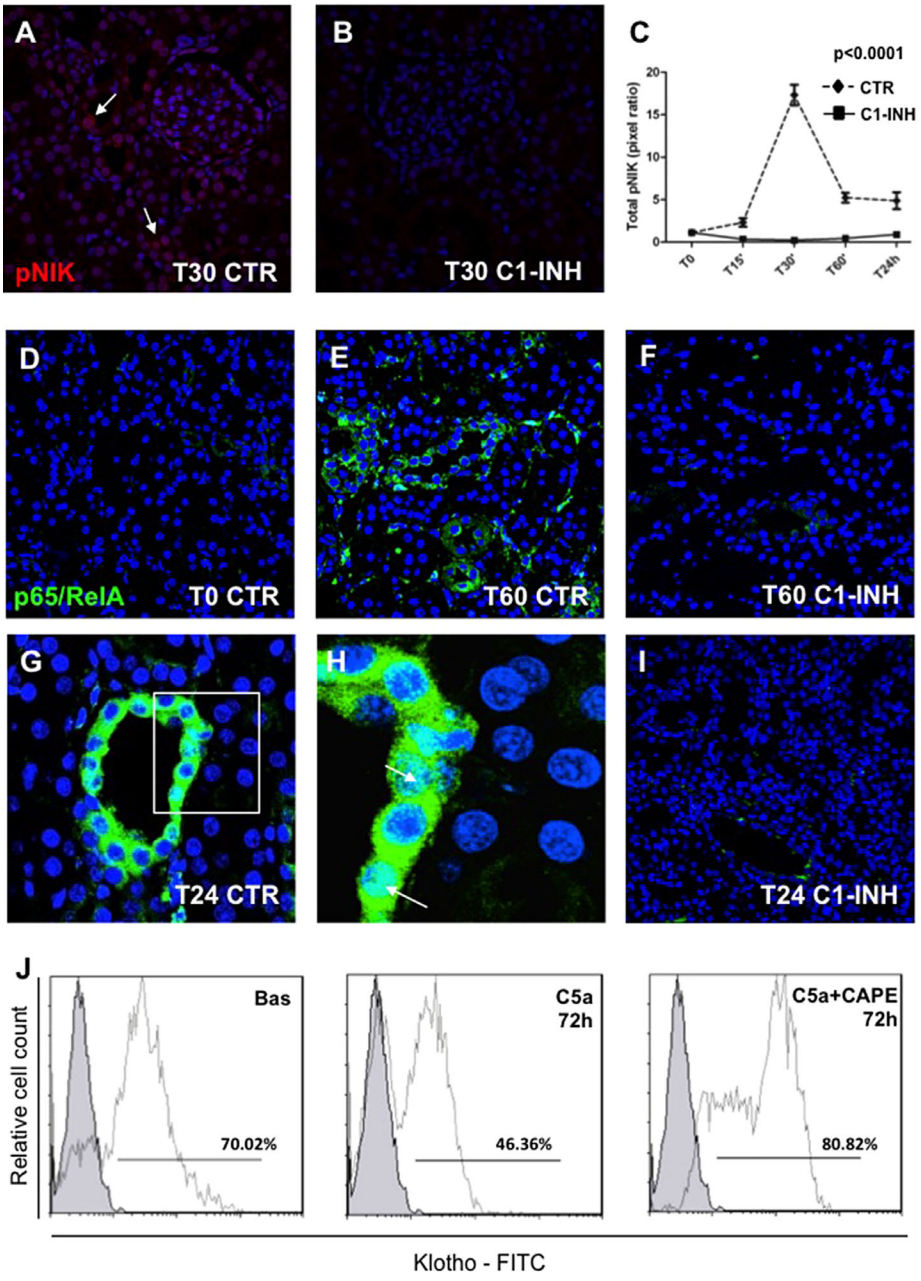

Figure 3: Complement activation down-regulated Klotho expression in a NF-kB-dependent manner. The activation of noncanonical NF-kB pathway was investigated by immunofluorescence staining for the phosphorylated form of NIK (red staining) on paraffin-embedded sections. Before ischemia, we observed a basal activation of NIK (data not shown). A striking activation of NIK at tubular and glomerular level was detected after 30 min (T30 CTR, arrows) from reperfusion (A). Complement inhibition by C1-INH led to a suppression of NIK phosphorylation (T30 C1-INH) (B). Total $pNIK^{Thr559}$ levels were quantified as described in the Methods section. Results are expressed as median $\pm$ IQR of at least five independent pigs for each group (C). Renal sections were stained for p65/RelA (green staining) to further analyze the activation of canonical NF-kB pathway. Before ischemia (T0), we detected low levels of p65/RelA (D). The cytoplasmic levels of these subunits increased after 60 min of reperfusion (T60 CTR) (E). After 24 h from reperfusion p65/RelA subunits transmigrated to the nucleus (T24 CTR) (G). Boxed area with zoom on p65/RelA nucleus transmigration was enlarged in H. The arrows indicate the merge (cyan) between green (p56/RelA) and blue (TO-PRO-3). In treated pigs, C1-INH reduced p65/RelA staining in the cytoplasm after 60 min (T60 C1-INH) (F) and 24 h from reperfusion (T24 C1-INH) (I). Magnification 630×. To-pro 3 was used to counterstain nuclei (blue). *In vitro*, the NF-kB inhibitor CAPE (20 ng/mL) abrogated C5a-induced Klotho reduction at 72 h in HK-2 cells as indicated by cytofluorimetric analysis. Results are representative of three independent experiments (J, $p < 0.05$). NF-kB, nuclear factor kappa B; NIK, NF-kB inducing kinase; CAPE, caffeic acid phenethyl ester; CTR, control group; C1-INH, C1-inhibitor.

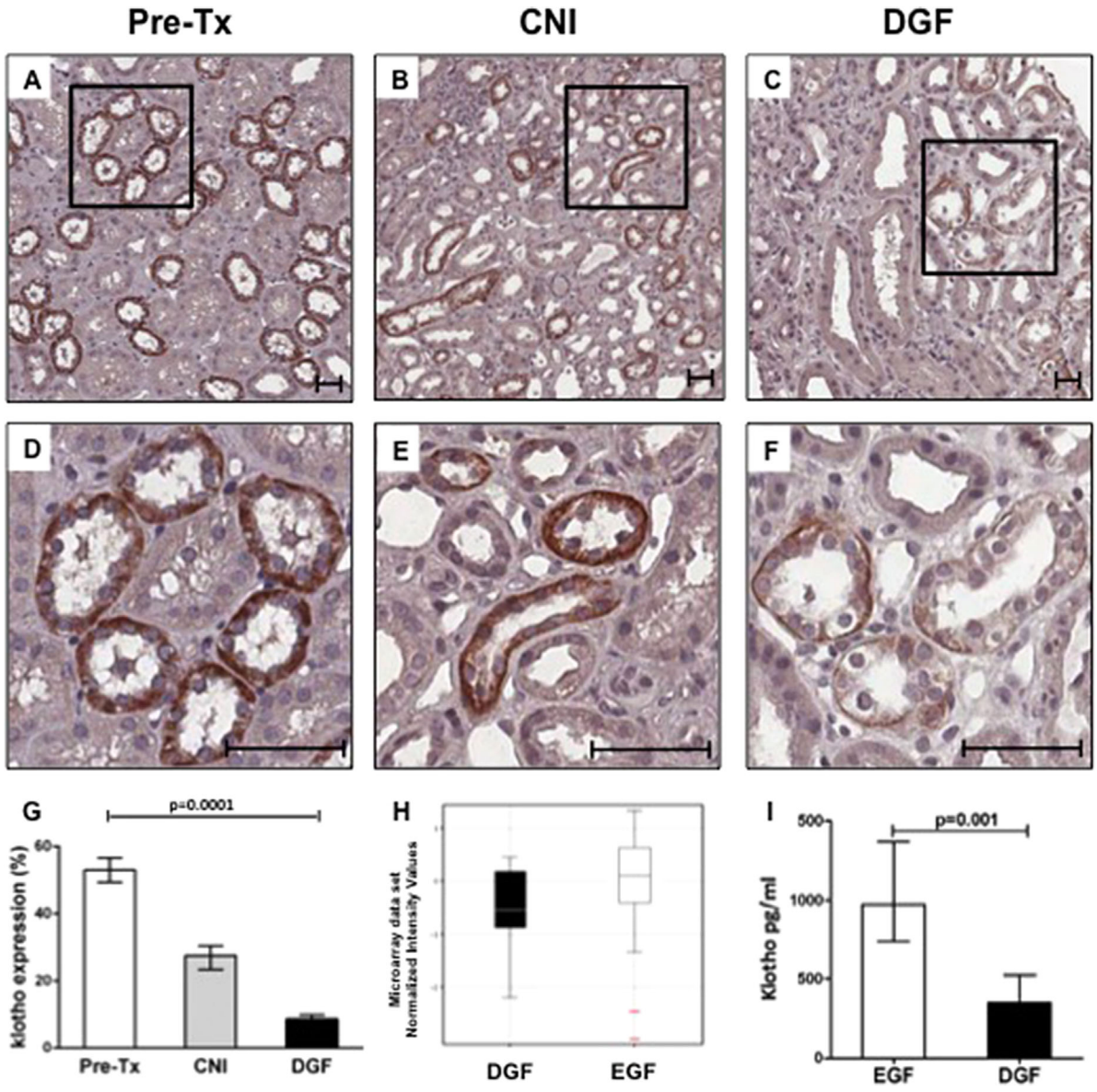

Figure 4: Klotho down-regulation in transplant recipients with DGF. Renal Klotho expression was evaluated in pre-transplant (Pre-Tx) graft biopsies, DGF patients and in transplant recipients with calcineurin inhibitors toxicity (CNI). Klotho was expressed in renal tubules with a clear distribution at basal membrane level in Pre-Tx biopsies (A, boxed area was enlarged in D). CNI group showed a lower tubular staining of Klotho (B, boxed area was enlarged in E). In DGF biopsies, Klotho expression dramatically decreased (C, boxed area was enlarged in F). Scale bar = 100 μm. Klotho staining was quantified as described in the section ''Methods'' and is expressed as median ± IQR of at least seven patients for each group (G). Box plot showing lower level of Klotho in implantation biopsies from patients that developed DGF compared to early graft function (H). Serum levels of Klotho were analyzed by enzyme-linked immunosorbent assay in patients with a history of DGF. Sera samples were collected 2 years after transplantation. Serum levels of Klotho were significantly reduced in the group of patients who had DGF respect to early graft function. Results are expressed as median ± IQR of at least eight patients for each group (I). CNI, calcineurin inhibitors toxicity; DGF, delayed graft function; ELISA, enzyme-linked immunosorbent assay; IQR, interquartile range.

complement system plays a pivotal role in inflammatory response during IRI (7,14,17), we examined the relationship between the complement system and Klotho expression in a swine model of IRI. Complement inhibition can effectively protect both tubular and endothelial renal cells in IRI by limiting tubular apoptosis (14) and endothelial dysfunction (5). In our animal model, we used C1-INH (25), with significant preservation of Klotho expression at tubular level. Between the different complement components, several evidences described C5a as the most effective anaphylotoxin in complement-mediated kidney injury (17). Moreover, TEC express specific

receptors for complement anaphylotoxins (6,17). Interestingly, C5a is capable to significantly down-regulate Klotho in TEC. We also found that C1-INH can suppress NF-kB activation *in vivo*; NF-kB inhibition could also abrogate C5a-induced Klotho reduction in TEC *in vitro*. Our data are in line with other animal models where Klotho was modulated in a NF-kB-dependent mechanism (11,12,18).

Few data are available on Klotho in renal transplant recipients. Our study showed a tissue down-regulation of Klotho in patients suffering for DGF, a particular form of acute kidney injury. In accordance, patients with acute kidney injury had lower levels of Klotho in urines (22). Both tissue and soluble Klotho were decreased in blood and urine in patients with chronic kidney disease (12,26); these results are comparable with our findings of reduced levels of Klotho in serum of DGF patients. DGF patients are indeed characterized by chronic allograft dysfunction that limits graft and even patient survival (1,2). In addition, decreased serum levels of renal-derived Klotho (20) might also have systemic and renal adverse consequences considering its pleiotropic functions including cytoprotection (27), preservation of endothelial function (28), inhibition of fibrosis (21,29). Klotho has also antioxidant, anti-inflammatory, and anti-senescence functions (12), that might support the regeneration of TEC maintaining nephron integrity (10,30). In conclusion, our data demonstrate for the first time a direct involvement of complement in Klotho down-regulation in IRI. Considering the central role of Klotho in preventing cellular senescence, we hypothesize that Klotho deficiency in DGF patients might play a central role in DGF-associated chronic allograft dysfunction.

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Disclosure

The authors of this manuscript have conflicts of interest to disclose as described by the *American Journal of Transplantation*. B. O. and E. v. A. are employed by Pharming NV, Leiden, the Netherlands.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1: **Uncropped western blots.**

Figure S2: **NF-kB inhibitor CAPE abrogated C5a-induced apoptosis in TEC *in vitro.*** CAPE, caffeic acid phenethyl ester; TEC, tubular epithelial cells.