

## RESEARCH ARTICLE

# Klotho overexpression protects against renal aging along with suppression of transforming growth factor- $\beta$ 1 signaling pathways

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## Abstract

Klotho is an antiaging protein reported to suppress transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) signaling. Aging kidneys are characterized by interstitial fibrosis, accumulation of cell cycle-arrested cells, and increased levels of oxidative stress. TGF- $\beta$ 1 signaling is involved in these processes. In this study, we investigated whether klotho overexpression improves these features in the kidneys of aging mice and examined the inhibitory effect of klotho on signaling molecules related to transforming growth of TGF- $\beta$ 1. Klotho transgenic (KLTG) and wild-type (WT) mice were used, and 8-wk-old and 24-mo-old mice were defined as young and aging, respectively. We found that klotho expression was decreased in aging WT mice, but it was maintained in aging KLTG mice. Klotho overexpression improved the survival of 24-mo-old mice. Although the serum Ca<sup>2+</sup> level was significantly lower in aging KLTG mice than in aging WT mice, the serum phosphate level did not differ between these mice. Klotho overexpression attenuated the increases in blood pressure, serum blood urea nitrogen level, and serum creatinine level in aging mice. Interstitial fibrosis, accumulation of cell cycle-arrested cells, and oxidative stress did not differ between young KLTG and WT mice, but they were significantly suppressed in aging KLTG mice compared with aging WT mice. Furthermore, the expression of TGF- $\beta$ 1-related signaling molecules was increased in aging WT mice, whereas it was inhibited in aging KLTG mice. These data suggest that klotho overexpression protects against kidney aging along with suppression of TGF- $\beta$ 1 signaling pathways.

**NEW & NOTEWORTHY** Klotho is considered as an antiaging protein, and its overexpression may be a candidate therapy for protection against kidney damage with advanced aging. Although multiple factors are involved in the aging process, we showed that klotho overexpression inhibited interstitial fibrosis, accumulation of cell cycle-arrested cells, and increased levels of oxidative stress in the kidneys of aging mice, suppressing transforming growth factor- $\beta$ 1-related signaling pathways. The present data showed that klotho overexpression protects against age-associated kidney damage.

*aging; cell cycle arrest; interstitial fibrosis; klotho; oxidative stress*

## INTRODUCTION

Chronic kidney disease (CKD) is a major life-threatening health problem worldwide. In fact, according to the Global Burden of Disease Study 2019, CKD affected ~700 million people globally, which resulted in more than 1.3 million deaths in 1 yr (1). CKD progression is usually an irreversible change, and some patients eventually develop end-stage kidney disease and require renal replacement therapy. Clinically, renal functions decrease with aging (2), and major features of the aging kidney are characterized by interstitial fibrosis (3), accumulation of cell cycle-arrested cells (4), and increased levels of oxidative stress (5). However, considering these changes play an important role in maintaining physiological conditions, a therapeutic strategy against the aging process has not been established thus far.

More than two decades have passed since *Klotho* was first reported as an antiaging gene (genes are italicized and

proteins are not) (6). Indeed, *Klotho*-deficient mice exhibit phenotypes that resemble human aging as well as a short life span, whereas its overexpression extends life span (7). A clinical study has demonstrated an inverse relationship between human plasma klotho levels and age (8). Regarding the association between klotho and CKD, klotho expression decreases with the decline in renal functions (9). In rodent models of renal diseases, klotho deficiency intensifies renal damage, which suggests that age-mediated downregulation of klotho leads to the progression of renal dysfunction (10). Conversely, administration of klotho protein or overexpression of the *Klotho* gene exerted renoprotective effects (11–13). Thus, although klotho is currently considered to be a possible therapeutic target against CKD progression, the effects of klotho on renal aging remain unknown.

Klotho has three forms, and each form performs various functions. Briefly, membrane klotho functions as an obligate coreceptor for fibroblast growth factor-23 (FGF23) (14), which

promotes phosphate excretion into the urine. Secreted klotho inhibits growth factor signals from transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) (15), insulin-like growth factor-1 (IGF-1) (7), and Wnt (16). Moreover, secreted klotho promotes phosphate excretion into the urine independent of FGF23 (17) and  $\text{Ca}^{2+}$  reabsorption (18). Intracellular klotho recruits  $\text{Na}^+$ - $\text{K}^+$  pumps to the cell surface (19) and suppresses retinoic acid-inducible gene-1 and downstream inflammatory mediators (20). Among these, TGF- $\beta$ 1 reportedly participates in aging features, such as interstitial fibrosis (21), accumulation of cell cycle-arrested cells (22), and increased levels of oxidative stress (23). Therefore, the effects of klotho on renal senescence should be determined together with the expression of TGF- $\beta$ 1-related signaling molecules.

In this study, we used EFmKL46 klotho-overexpressing [klotho transgenic (KLTG)] mice and wild-type (WT) 129S1/SvImJ mice. We defined 8 wk of age as young and 24 mo of age as aging. We found that the 24-mo survival rate of KLTG mice was higher than that of WT mice. Next, we found that the renal age-related upregulation of senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) was suppressed in aging KLTG mice compared with aging WT mice, which was accompanied by increased body weight and decreased blood pressure, serum blood urea nitrogen (BUN), and serum creatinine. The serum  $\text{Ca}^{2+}$  level was significantly increased in aging KLTG mice, whereas the serum phosphate level did not differ between aging WT and KLTG mice. Furthermore, comparison of aging WT and KLTG mice revealed that klotho overexpression protected against renal aging characterized by interstitial fibrosis, accumulation of cell cycle-arrested cells, and increased levels of oxidative stress. Finally, we examined the expression levels of TGF- $\beta$ 1-related signaling molecules. Our data suggest that klotho overexpression exerts antiaging effects on mouse kidneys with improvement in aging-associated molecular changes.

## MATERIALS AND METHODS

### Animal Experiments

We used a transgenic mouse strain that overexpresses transmembrane klotho protein under the control of the ubiquitous human elongation factor-1- $\alpha$  promoter (EFmKL46), which was kindly provided by Dr. Hiroko Segawa and Dr. Kenichi Miyamoto (Tokushima University Graduate School, Tokushima, Japan). The expression levels of endogenous and exogenous *Klotho* genes have been described in a previous report (7). The background of the transgenic mice is 129S1/SvImJ (24), and WT mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). Mice were housed in a light- and temperature-controlled room and had free access to laboratory chow and tap water at the Institute of Laboratory Animal Science of Hiroshima University (Hiroshima, Japan). All animal experiments were performed in accordance with National Institutes of Health guidelines on the use of laboratory animals. The Institutional Animal Care and Use Committee of Hiroshima University approved all experimental protocols (Permit No. 28-104). All efforts were made to minimize the animals' pain and distress.

### Measurement of Biological Parameters

The blood pressure of 8-wk-old and 24-mo-old mice was measured using the tail-cuff method (Softron, Tokyo, Japan). Mice were euthanized at 8 wk or 24 mo of age under deep anesthesia. Body weight was measured before blood samples were collected by cardiac puncture. Analysis of blood samples was outsourced to SRL (Tokyo, Japan) to examine the serum concentrations of BUN by the urease-ultraviolet method (UN-S, Denka, Tokyo, Japan), creatinine by the Trinder reaction method (L-Type Creatinine M, Wako Pure Chemical, Osaka, Japan),  $\text{Ca}^{2+}$  by the Arsenazo III method (ACCURAS AUTO Ca II, Shino-Test, Tokyo, Japan), and inorganic phosphate by the molybdic acid direct method (Determiner L IP, Kyowa Medex, Tokyo, Japan) using a 7180 Clinical Analyzer (Hitachi High-Technologies, Tokyo, Japan). We also monitored death of aging mice before 24 mo to examine survival.

### Histology and Immunohistochemistry

Renal tissue samples were prepared as formalin-fixed, paraffin-embedded sections. Then, 2- $\mu\text{m}$ -thick sections were subjected to Masson's trichrome staining to assess the severity of tubulointerstitial fibrosis. The fibrotic area (stained blue) was quantified using Lumina Vision 2.20 (Mitani, Osaka, Japan) by examination of a predetermined field at  $\times 200$  magnification. Immunohistochemistry was performed on 4- $\mu\text{m}$ -thick sections with the following primary antibodies: rat monoclonal anti-klotho antibody (KO603, TransGenic, Fukuoka, Japan), mouse monoclonal anti- $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) antibody (A2547, Sigma-Aldrich, St. Louis, MO), rabbit polyclonal anti-collagen type I antibody (ab34710, Abcam, Cambridge, UK), rabbit monoclonal anti-vimentin antibody (ab92547, Abcam), rabbit polyclonal anti-p53 antibody (ab31333, Abcam), rabbit polyclonal anti-p16 antibody (ab54210, Abcam), rabbit polyclonal anti-p21 antibody (ab188224, Abcam), and rabbit polyclonal anti-8-hydroxy-deoxy-guanosine (8-OHdG) antibody (bs-1278R, Bioss, Boston, MA). Quantitative analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD).

### SA- $\beta$ -Gal Staining

Some kidney tissues were frozen in optical cutting temperature compound (Tissue-Tek OCT compound, Sakura Finetek Japan, Tokyo, Japan) to prepare cryosections. Then, 10- $\mu\text{m}$ -thick kidney cryosections were fixed in fixation solution (2% formaldehyde and 0.2% glutaraldehyde in PBS) at room temperature for 15 min. Sections were then rinsed with PBS and incubated overnight at 37°C with freshly prepared staining solution (pH 6.0).

### Quantitative RT-PCR

RNA extraction and quantitative RT-PCR were performed using the ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA). The specific oligonucleotide primers and probes for *Klotho* (assay ID: Mm00502002\_m1),  $\alpha$ -SMA (assay ID: Mm00725412\_s1), *collagen type I* (assay ID: Mm00801666\_g1), *vimentin* (assay ID: Mm01333430\_m1), and glyceraldehyde 3-phosphate

dehydrogenase (*GAPDH*; assay ID: Mm9999915\_g1) as an internal control were all from TaqMan Gene Expression Assays (Applied Biosystems).

### Western Blot Analysis

Renal tissues were lysed in Laemmli sample buffer (Sigma-Aldrich) or radioimmunoprecipitation sample buffer and sonicated using a Bioruptor II (Cosmo Bio, Tokyo, Japan). Lysates were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes, which were then blocked with 5% dry milk or Blocking One (No. 03953-95, Nacalai Tesque, Kyoto, Japan) in Tris-buffered saline with Tween 20, and then incubated overnight at 4°C with the following primary antibodies: rat monoclonal anti-klotho antibody, mouse monoclonal anti-GAPDH antibody (G8795, Sigma-Aldrich); mouse monoclonal anti- $\alpha$ -SMA antibody, rabbit polyclonal anti-collagen type I antibody, rabbit monoclonal anti-vimentin antibody (ab94527, Abcam); rabbit polyclonal anti-p53 antibody, rabbit polyclonal anti-p16 antibody (ab189034, Abcam); rabbit monoclonal anti-p21 antibody (ab109199, Abcam); mouse monoclonal anti-3-nitrotyrosine (3-NT) antibody (ab110282, Abcam); rabbit polyclonal anti-catalase antibody (PA5-29183, Invitrogen, Carlsbad, CA); mouse monoclonal anti-Mn-superoxide dismutase (Mn-SOD) antibody (MAB3419, R&D Systems); mouse monoclonal anti-TGF- $\beta$ 1 antibody (sc-130348, Santa Cruz Biotechnology, Dallas, TX); mouse monoclonal anti-Smad2 antibody (No. 3103, Cell Signaling Technology, Danvers, MA); rabbit monoclonal anti-phospho-Smad2 antibody (No. 3108, Cell Signaling Technology); rabbit monoclonal anti-Smad3 antibody (No. 9523, Cell Signaling Technology); rabbit monoclonal anti-phospho-Smad3 antibody (No. 9520, Cell Signaling Technology); rabbit monoclonal anti-Smad4 antibody (No. 38454, Cell Signaling Technology); rabbit monoclonal anti-connective tissue growth factor (CTGF) antibody (No. 86641S, Cell Signaling Technology); rabbit monoclonal anti-p44/42 MAPK (ERK) antibody (No. 4695, Cell Signaling Technology); rabbit monoclonal anti-phospho-ERK (Thr<sup>202</sup>/Tyr<sup>204</sup>) antibody (No. 4370, Cell Signaling Technology); rabbit polyclonal anti-JNK antibody (No. 9252, Cell Signaling Technology); rabbit polyclonal anti-phospho-JNK (Thr<sup>183</sup>/Thr<sup>185</sup>) antibody (No. 9251, Cell Signaling Technology); rabbit monoclonal anti-p38 antibody (No. 8690, Cell Signaling Technology); rabbit monoclonal antiphospho-p38 (Thr<sup>180</sup>/Tyr<sup>182</sup>) antibody (No. 4511, Cell Signaling Technology); rabbit polyclonal anti-protein kinase B (AKT) antibody (No. 9272, Cell Signaling Technology); rabbit monoclonal anti-phospho-AKT (Ser<sup>473</sup>) antibody (No. 4060, Cell Signaling Technology); and rabbit monoclonal anti-forkhead box protein O1 (FOXO1) antibody (No. 2880, Cell Signaling Technology). All gels were run at the same time. Quantitative analysis was performed using ImageJ software.

### Statistical Analysis

Results are expressed as means  $\pm$  SD. Statistical evaluation was performed using JMP 14 software. Statistical analysis was conducted using ANOVA followed by Tukey's post hoc test. The 24-mo survival rate was assessed by Kaplan-Meier analysis and the log-rank test.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Aging KLTG Mice Retain Klotho Expression Compared With Aging WT Mice

KLTG mice have a longer life span than WT mice (7). Therefore, we evaluated the klotho expression level in the kidneys as well as the life span of the mice. Western blot analysis (Fig. 1, A and B), quantitative RT-PCR (Fig. 1C), and immunohistochemistry (Fig. 1D) showed that klotho expression was decreased in aging WT mice but was maintained in aging KLTG mice. Next, we investigated the effect of klotho overexpression on life span. One mouse of 46 KLTG mice died within 24 mo, whereas 7 mice of 43 (16.3%) WT mice died during the observational period (Fig. 2A). SA- $\beta$ -gal is a hydrolase enzyme that catalyzes the hydrolysis of  $\beta$ -galactoside into monosaccharides, which is used as a senescence marker (25). SA- $\beta$ -gal staining was significantly increased in the kidneys of aging WT mice, but this increase was suppressed in the kidneys of aging KLTG mice (Fig. 2, B and C).

### Klotho Overexpression Improves Age-Associated Physiological Changes

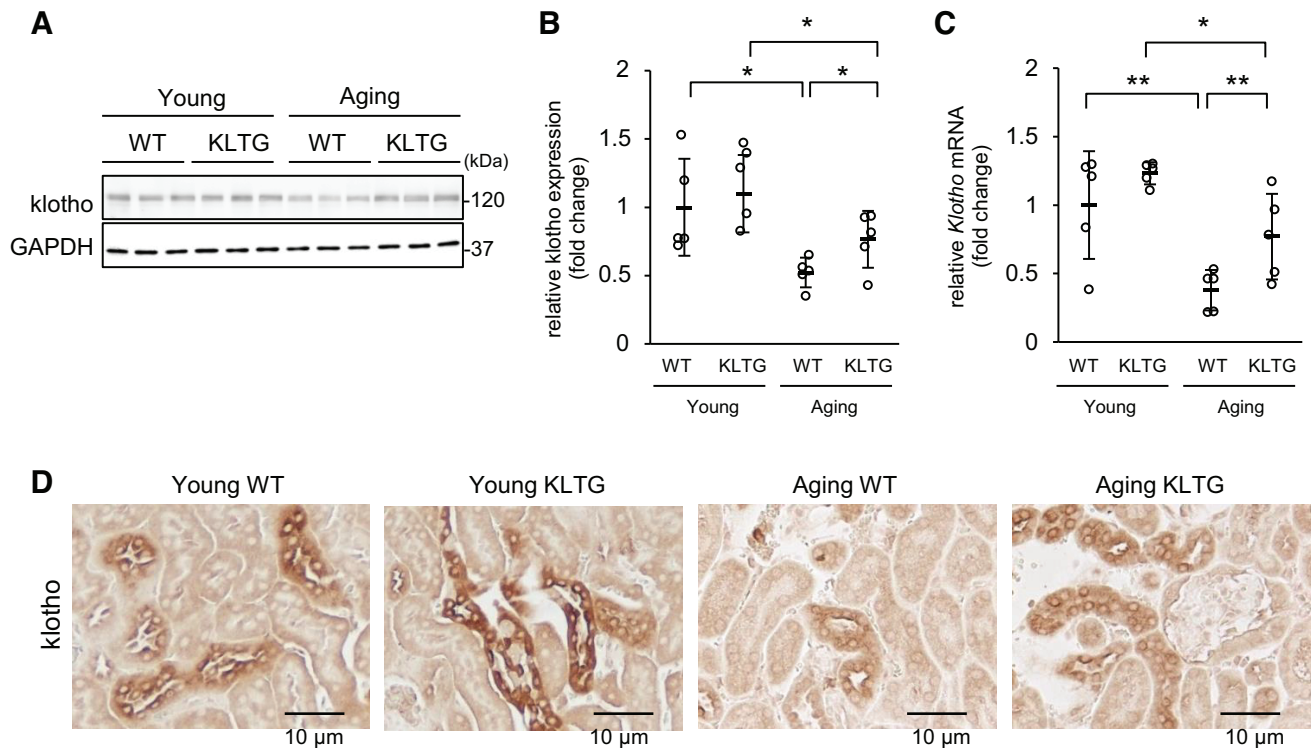
Body weight was measured immediately before euthanasia. The body weight of aging KLTG mice was higher than that of aging WT mice (Fig. 3A). Systolic blood pressure was increased in aging WT mice compared with young WT mice, but this increase was not observed in KLTG mice (Fig. 3B). Analysis of blood samples revealed that the serum levels of BUN and creatinine were significantly lower in aging KLTG mice than in aging WT mice (Fig. 3, C and D). Finally, the serum level of Ca<sup>2+</sup> was higher in aging WT mice than in aging KLTG mice (Fig. 3E), but we did not observe a difference in serum phosphate levels between aging WT and KLTG mice (Fig. 3F).

### Klotho Overexpression Protects the Kidneys From Age-Associated Fibrosis

Renal fibrosis is a pathological feature of the aging kidney (26, 27). Conversion of resident kidney cells to mesenchymal cells and the following production of extracellular matrix (ECM) proteins, which include collagens, play a pivotal role in this process (28). Masson's trichrome staining showed that deposition of ECM proteins was increased in the kidneys of aging WT mice compared with that in the kidneys of young WT mice, but this increase was attenuated in aging KLTG mice (Fig. 4, A and B). Next, Western blot analysis, immunohistochemistry, and quantitative RT-PCR revealed that the expression of mesenchymal markers  $\alpha$ -SMA and vimentin and ECM protein collagen type I did not differ between young WT and KLTG mice but was significantly lower in aging KLTG mice than in aging WT mice (Fig. 4, C–J).

### Klotho Overexpression Prevents Accumulation of Age-Associated Cell Cycle-Arrested Cells

Accumulation of cell cycle-arrested cells is a feature of renal senescence (4). Therefore, we evaluated the effect of klotho overexpression on the expression of cell cycle-related molecules in aging mice. Protein expression levels of cell cycle arrest markers p53, p16, and p21 were significantly



**Figure 1.** Aging klotho transgenic (KLTG) mice maintain klotho expression compared with aging wild-type (WT) mice. *A*: representative Western blot of klotho in the kidneys of WT and KLTG mice at 8 wk (Young) and 24 mo (Aging) of age. GAPDH was used as a loading control. *B*: quantitative analysis of the klotho expression level in *A* ( $n = 5$ ). *C*: quantification of relative *Klotho* mRNA levels in the kidneys determined by quantitative RT-PCR ( $n = 5$ ). *D*: representative images of immunohistochemical staining of klotho protein in the kidneys of KLTG and WT mice. Values are presented as means  $\pm$  SD. Data were analyzed using ANOVA followed by a Tukey's test. Statistical differences are indicated as  $*P < 0.05$  and  $**P < 0.01$ .

upregulated in aging WT mice, but this upregulation was not observed in aging KLTG mice (Fig. 5, *A–E*).

#### Klotho Overexpression Ameliorates Age-Associated Increases in Oxidative Stress

Oxidative stress plays a major role in the aging process (29). We, therefore, investigated the effects of klotho on the expression of oxidative stress-related molecules in aging kidneys. Western blot analysis revealed that 3-NT, a marker of oxidative nitric oxide damage (30), was significantly upregulated in aging WT mice compared with aging KLTG mice (Fig. 6*A*). In addition, expression of catalase and Mn-SOD, which are antioxidative stress molecules (31), was reduced in aging WT mice, but their expression was maintained in aging KLTG mice (Fig. 6, *A–D*). Furthermore, immunohistochemical staining revealed that expression of 8-OHdG, a marker of oxidative DNA damage, was significantly lower in aging KLTG mice compared with aging WT mice (Fig. 6, *E* and *F*).

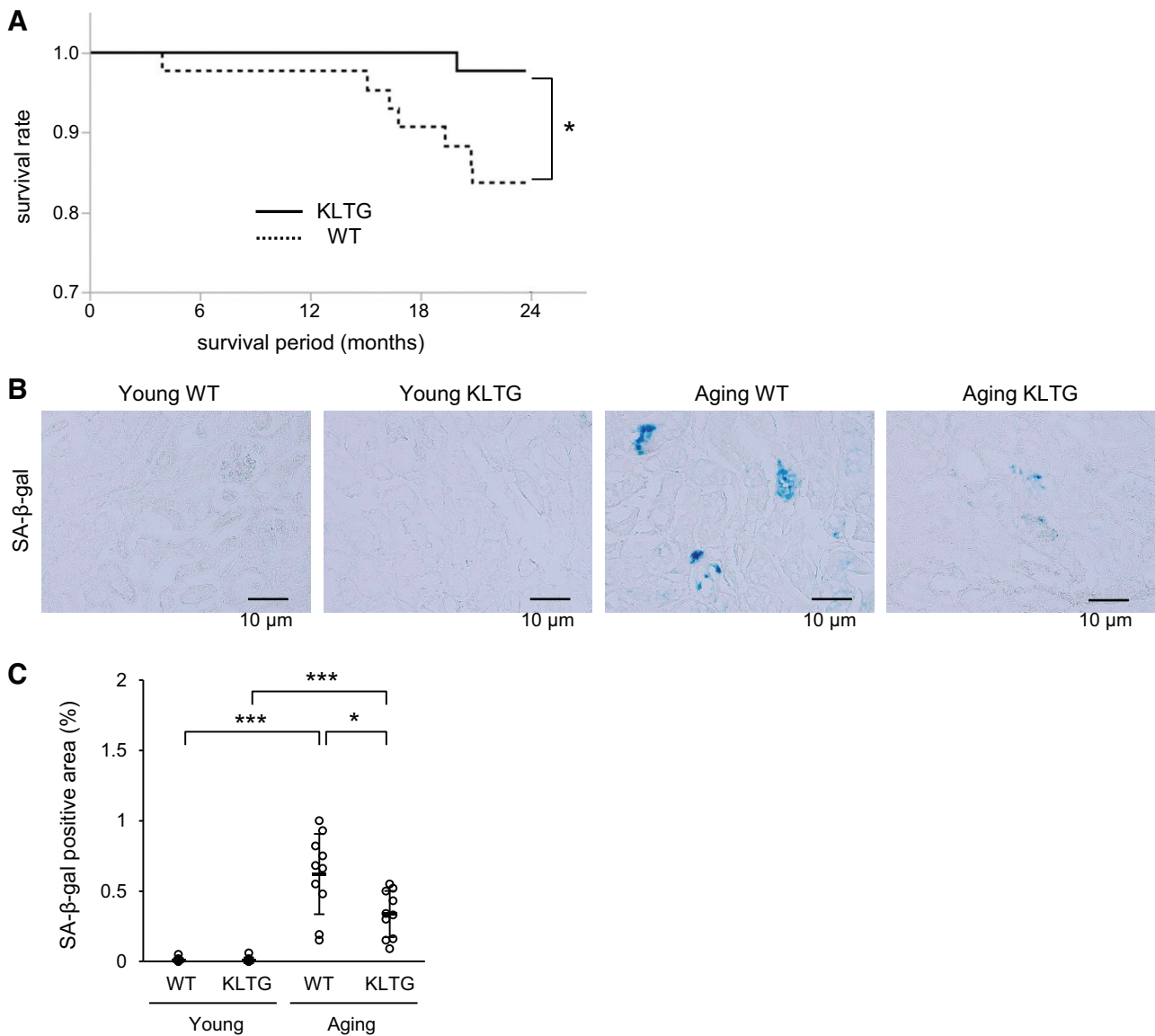
#### Klotho Overexpression Suppresses TGF- $\beta$ 1-Related Signaling

Klotho inhibits TGF- $\beta$ 1 signaling pathways (15), thereby contributing to suppression of renal fibrosis (21), accumulation of cell cycle-arrested cells (22), and oxidative stress (23). We examined the effects of klotho on the expression of molecules that are involved in TGF- $\beta$ 1 signaling pathways. Within TGF- $\beta$ 1-Smad signaling, expression levels of TGF- $\beta$ 1, Smad4, and CTGF, and phosphorylation levels of Smad2/3 were

significantly upregulated in aging WT mice, whereas this upregulation was suppressed in aging KLTG mice (Fig. 7, *B–F*). The MAPK and AKT cascades are non-Smad TGF- $\beta$ 1 signaling pathways (32). Western blot analysis revealed significant phosphorylation of ERK, JNK, p38, and AKT in the kidneys of aging WT mice. However, the phosphorylation level of these proteins was reduced in aging KLTG mice (Fig. 7, *G–J*). Finally, expression of FOXO1, which is negatively regulated by AKT (33), was higher in young and aging KLTG mice compared with WT mice (Fig. 7*K*).

## DISCUSSION

In this study, we found that klotho expression was decreased in aging WT mice but was maintained in aging KLTG mice. Klotho overexpression improved the 24-mo survival rate of mice and decreased the SA- $\beta$ -gal-positive area in the kidneys of aging mice. The body weight of aging KLTG mice was significantly higher than that of aging WT mice, and the age-associated rise in blood pressure was attenuated in aging KLTG mice. Although the serum  $\text{Ca}^{2+}$  level in aging KLTG mice was significantly lower than that in aging WT mice, the serum phosphate level did not differ between aging WT and KLTG mice. Klotho overexpression ameliorated the decline in renal functions in aging mice. Age-associated interstitial fibrosis, accumulation of cell cycle-arrested cells, and the increase in oxidative stress were significantly attenuated in aging KLTG mice. Moreover, the

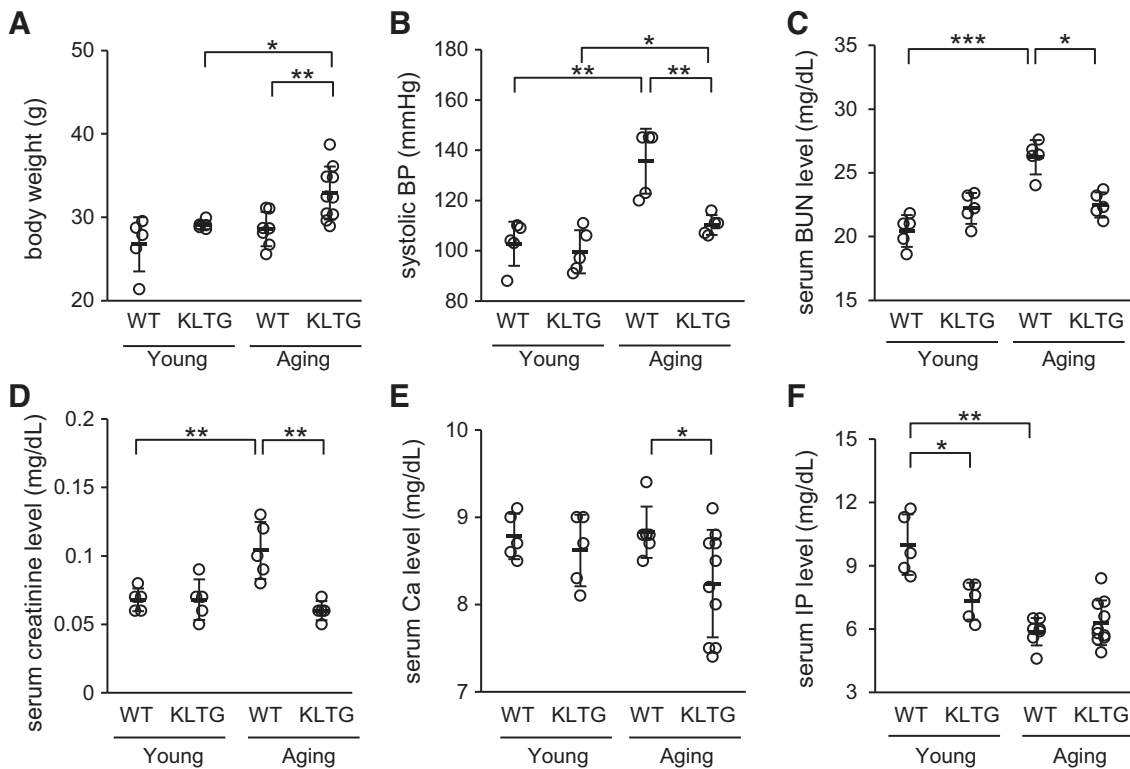


**Figure 2.** Klotho overexpression extends life span and protects kidneys against senescence. *A*: effect of klotho overexpression on life span. The 24-mo survival rate of wild-type (WT;  $n = 43$ ) and klotho transgenic (KLTG) mice ( $n = 46$ ) was assessed by Kaplan–Meier analysis and the log-rank test. *B*: typical images of immunohistochemical staining of senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) showing senescence changes in the kidneys of WT and KLTG mice at 8 wk (Young) and 24 mo (Aging) of age. *C*: quantification of the stained areas in *B* ( $n = 5$ ). Values are presented as means  $\pm$  SD. Data were analyzed using ANOVA followed by a Tukey’s test. Statistical differences are indicated as  $*P < 0.05$  and  $***P < 0.001$ .

expression of TGF- $\beta$ 1-related signaling molecules was upregulated in the kidneys of aging WT mice, but this upregulation was inhibited in aging KLTG mice. These findings suggest that klotho overexpression exerts antiaging effects on mouse kidneys and improves TGF- $\beta$ 1-related molecular changes.

Herein, klotho expression was higher in aging KLTG mice compared with aging WT mice, but it was not significantly different between young WT and KLTG mice. A previous study has reported that expression of a klotho transgene was weak compared with that of the endogenous klotho gene (7). Upregulation of the serum klotho level has also been observed in KLTG mice, which suggests that all forms of klotho increase in KLTG mice. Moreover, endogenous klotho expression was decreased in aging WT mice, and thus, the

expression level showed a significant difference between aging WT and KLTG mice. Although the precise mechanism of age-associated reduction in klotho expression remains unclear, several stimuli, such as oxidative stress, TGF- $\beta$ 1, and angiotensin II, contribute to the reduction in klotho expression (10, 34, 35). Among them, we have previously reported that oxidative stress and TGF- $\beta$ 1 induce epigenetic changes that are responsible for the reduction in klotho expression (36, 37). These findings suggest that, in addition to renal damage, klotho expression is negatively regulated by upregulation of several stimuli at the molecular level. We also found that KLTG mice had a lower mortality rate at 24 mo than WT mice and that klotho overexpression attenuated the age-associated upregulation of SA- $\beta$ -gal. The present data suggest that klotho extends life span and suppresses



**Figure 3.** Klotho overexpression improves age-associated physiological changes. *A–F*: quantification of body weight (*A*), systolic blood pressure (BP; *B*), and serum levels of blood urea nitrogen (BUN; *C*), creatinine (*D*), calcium (Ca; *E*), and inorganic phosphate (IP; *F*) in wild-type (WT) and klotho transgenic (KLTG) mice at 8 wk (Young) and 24 mo (Aging) of age ( $n = 5$ ). Blood samples were obtained at euthanasia and then analyzed. Values are presented as means  $\pm$  SD. Data were analyzed using ANOVA followed by a Tukey's test. Statistical differences are indicated as \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

renal senescence. A previous study has reported that *Klotho* mutant mice exhibit a smaller body than WT mice (6). In a previous study, the body weight of KLTG mice was similar to that of WT mice (7), indicating that klotho overexpression per se does not affect body weight in mice. However, in this study, the body weight of aging WT mice was lower than that of aging KLTG mice. Thus, although an effect of klotho overexpression on body weight was not observed in young KLTG mice, age-associated downregulation of klotho expression may explain the reduction in weight gain of aging WT mice.

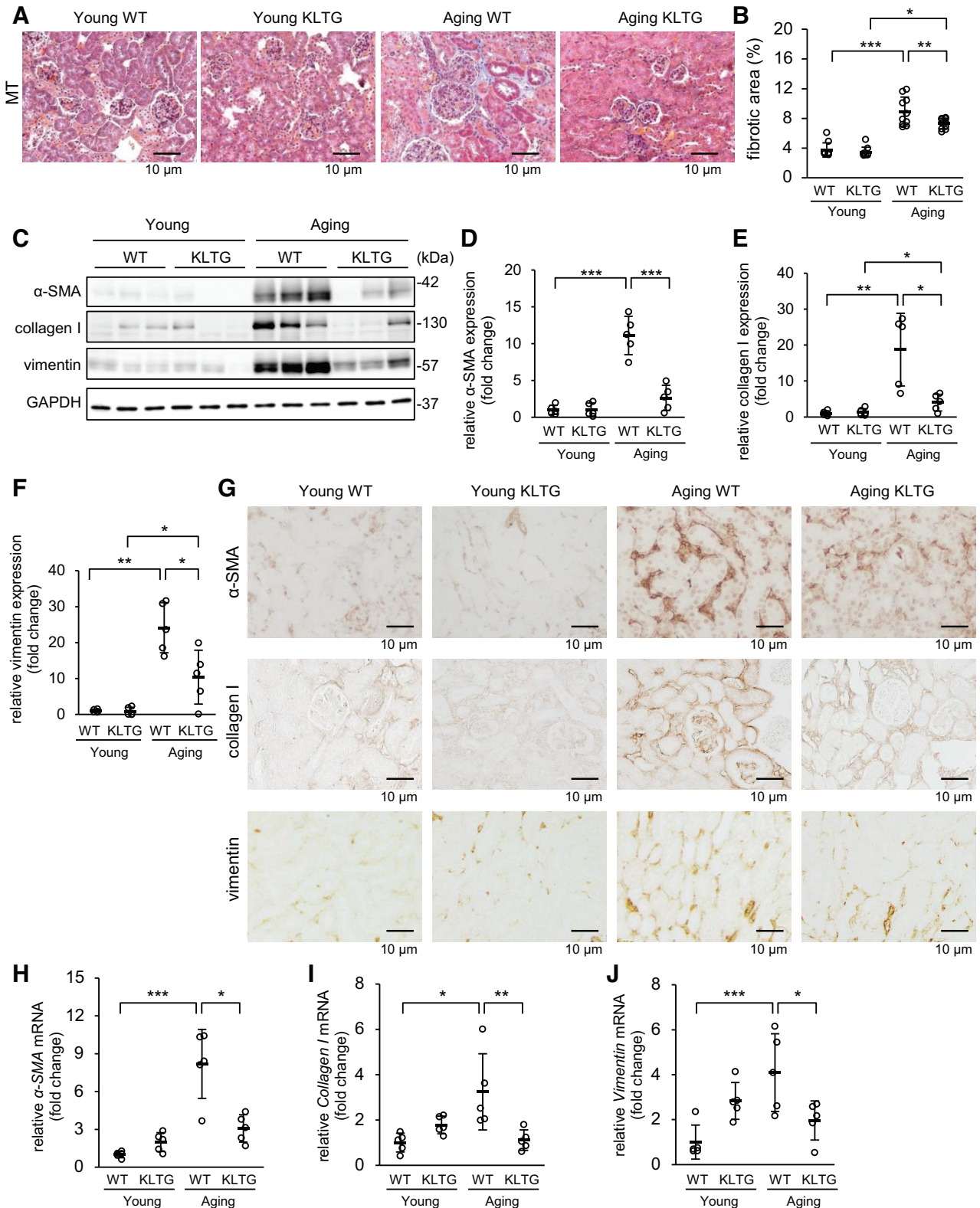
Klotho functions as a coreceptor of FGF23 and plays a pivotal role in phosphate excretion (14). Compared with young WT mice, the serum level of phosphate was significantly decreased in young KLTG mice, but it did not differ between aging WT and KLTG mice. Hence, the finding that aging WT mice exhibited lower body weight than aging KLTG mice suggests that despite the decrease in phosphate excretion due to the reduction in klotho expression, less dietary intake by aging WT mice resulted in the same level of phosphate as aging KLTG mice. Moreover, age-induced increases in the serum levels of BUN and creatinine were significantly suppressed in aging KLTG mice. The serum levels of BUN and creatinine reflect the glomerular filtration rate (GFR), which has clinically been shown to decrease yearly with aging (38). These data suggest that klotho overexpression ameliorates the age-associated reduction in GFR. In fact, although klotho is expressed in renal tubular cells but not in glomeruli, a previous study has reported that it suppresses glomerular

sclerosis in a mouse model of glomerular nephritis by functioning as a circulating hormone (39). In addition, even though we did not clarify whether klotho overexpression improves the decreased GFR or hypertension first, we showed that it ameliorated age-associated hypertension. Previous studies have also reported that klotho suppresses essential and salt-sensitive hypertension (40, 41). Thus, multiple mechanisms, including  $Ca^{2+}$ /phosphate metabolism, may be involved in the antihypertensive effect of klotho (42, 43). Pathologically, renal senescence is characterized by interstitial fibrosis (3), which is associated with GFR and predicts the development of end-stage kidney disease (44). In this study, renal fibrosis, which was enhanced in aging WT mice compared with young WT mice, was ameliorated by klotho overexpression. Taken together, klotho overexpression improves age-associated renal damage without reducing serum phosphate.

Renal fibrosis is a common pathological feature in the development of end-stage kidney disease (45), and TGF- $\beta$ 1-Smad signaling plays a central role in this process (46, 47). In this study, Smad2/3 phosphorylation levels and Smad4 expression were increased with aging, and these age-related changes were suppressed by klotho overexpression. These changes in Smad proteins in aging WT and KLTG mice are likely explained by the expression level of TGF- $\beta$ 1, suggesting that klotho overexpression suppresses renal fibrosis through downregulation of TGF- $\beta$ 1 expression. In addition, it has been shown that klotho protein binds directly to TGF- $\beta$

receptor type II, which inhibits TGF- $\beta$ 1-stimulated phosphorylation of Smad2/3 in a rat cell line of renal tubules as well as renal fibrosis in mice (15). Moreover, Smad4 forms a heteromeric complex with phosphorylated Smad2/3, which

plays a crucial role in mediating TGF- $\beta$ 1 signal transduction (46, 48). We have previously reported that Smad4 is upregulated in a mouse model of renal fibrosis and that inhibition of Smad4 expression suppresses renal fibrosis in mouse



kidneys and a cell line of renal fibroblasts (49). These findings suggest that both downregulation of TGF- $\beta$ 1 and inhibition of TGF- $\beta$ 1-Smad signaling participate in the antifibrotic effects in KLTG mice. According to previous studies, ERK, JNK, p38, and AKT are also responsible for the development of renal fibrosis (50–53). Consistently, our data demonstrated that klotho overexpression suppressed the MAPK and AKT pathways along with age-associated renal fibrosis. Although MAPK and AKT cascades are not specific pathways of TGF- $\beta$ 1 (54, 55), their inhibition may suppress renal fibrosis. Taking these findings together, aging KLTG mice exhibit a lower TGF- $\beta$ 1 level as well as an inhibitory effect on multiple TGF- $\beta$ 1-related signaling molecules, which may be implicated in the antifibrotic effect of klotho overexpression on age-induced renal fibrosis.

Expression of cell cycle arrest markers p53, p16, and p21 was increased in aging WT mice. Cell cycle arrest is induced by various stresses, such as reactive oxygen species, DNA damage, and oncogene activation, to protect tissues from proliferation of damaged cells (2). However, considering cell cycle-arrested cells exhibit a senescence-associated secretory phenotype, their accumulation contributes to chronic tissue injuries such as fibrosis and inflammation. In this study, klotho overexpression attenuated the accumulation of cell cycle-arrested cells along with inhibition of TGF- $\beta$ 1-related signaling molecules. Among the various cytokines, we have previously demonstrated that TGF- $\beta$ 1 directly induced upregulation of p53, p16, and p21 in a rat cell line of renal fibroblasts (56), which suggests that klotho overexpression ameliorates the accumulation of cell cycle-arrested cells through inhibition of TGF- $\beta$ 1 signaling. However, except for the evidence that expression of p21 is regulated by TGF- $\beta$ 1-Smad2/3 signaling (57), the signaling pathway through which TGF- $\beta$ 1 induces upregulation of p16 and p53 remains unknown. Regarding MAPKs, although ERK contributes to cell proliferation (58), a previous study has reported that activation of ERK leads to increased expression of p16 and p53 (59). Previous studies have also reported that JNK phosphorylation leads to increased p21 expression (60) and that p38 MAPK contributes to upregulation of p53, p16, and p21 (61). These findings suggest that inactivation of MAPKs may suppress the accumulation of cell cycle-arrested cells in aging KLTG mice. In addition to Smad and MAPKs, increased AKT phosphorylation reportedly increases the expression of p53 and p21 (60, 62). Thus, klotho overexpression may reduce the accumulation of cell cycle-arrested cells along with downregulation of multiple TGF- $\beta$ 1 pathways.

Oxidative stress is involved in the onset and progression of various diseases, and its level increases with the decline in renal functions (63). In this study, oxidative stress was increased in aging WT mice and this age-associated increase was suppressed in KLTG mice. Notably, klotho suppressed oxidative stress not only in tubular cells, but also in

glomeruli, even though klotho was not expressed in renal tubular cells (64). These findings suggest that secreted klotho participates in the beneficial effect against oxidative stress. According to previous studies, TGF- $\beta$ 1 is directly involved in the induction of oxidative stress during the development of fibrosis (23), and TGF- $\beta$ 1-induced oxidative stress contributes to the progression of fibrosis (65). Therefore, the inhibitory effect of klotho overexpression on both TGF- $\beta$ 1 expression and signaling pathways may contribute to the reduction in oxidative stress. In addition, MAPK cascade family proteins are activated under oxidative stress (66); however, inhibition of ERK, JNK, and p38 MAPK enhances oxidative stress resistance and prolongs life span (67). In this study, we showed that klotho overexpression attenuated the age-associated activation of these kinases in mice along with oxidative stress. Furthermore, klotho activates the FOXO transcription factor, which leads to the production of Mn-SOD (68). A previous study has reported that klotho suppresses the inhibition of IGF signaling, which plays an important role in the antioxidative stress effect of klotho (7). However, our data demonstrated that phosphorylation levels of MAPKs and AKT were reduced by 52–80% in aging KLTG mice compared with WT mice, despite evidence indicating that IGF-1 receptor mutation reduces the phosphorylation of p38 MAPK and AKT by 40–50% (67). Thus, the present study suggests that the inhibitory effect of klotho overexpression on TGF- $\beta$ 1 signaling, at least in part, contributes to the attenuation of age-associated oxidative stress in mice.

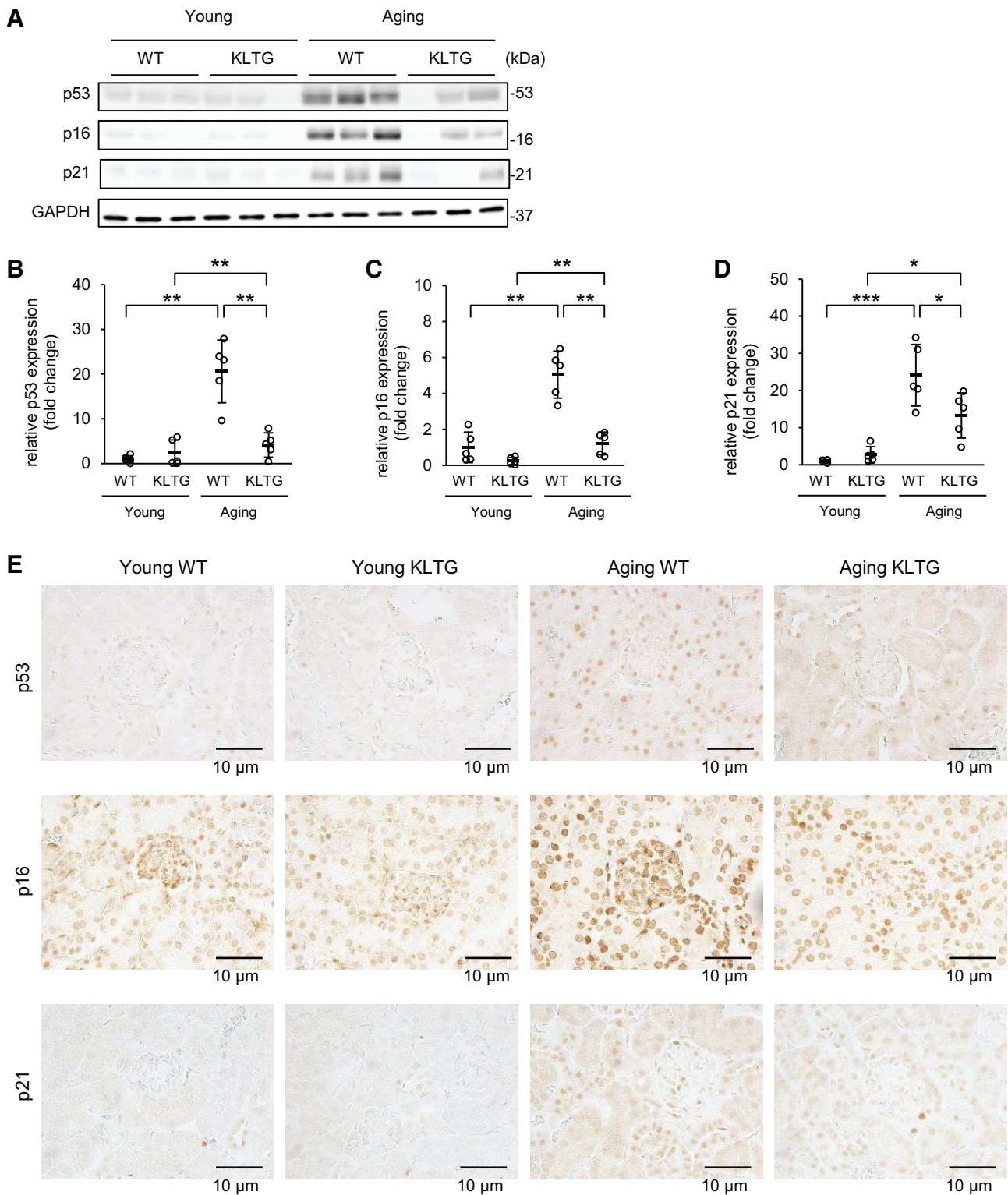
This is the first study, to our knowledge, to clearly show the effect of klotho overexpression on renal senescence. Klotho expression was decreased in aging WT mice compared with young WT mice. Klotho overexpression improved survival along with downregulation of age-induced SA- $\beta$ -gal expression in mouse kidneys. Body weight was higher in aging KLTG mice compared with aging WT mice. Blood pressure and serum levels of BUN and creatinine were higher in aging WT mice than in aging KLTG mice. The serum Ca<sup>2+</sup> level was significantly lower in aging KLTG mice than in aging WT mice, whereas the serum level of phosphate did not differ between them. We showed that klotho overexpression attenuated fibrosis progression, accumulation of cell cycle-arrested cells, and the increased level of oxidative stress. Our data also demonstrated that klotho overexpression suppressed the expression of TGF- $\beta$ 1-related signaling molecules in aging mice together with renal senescence. These data suggest that klotho exerts antiaging effects along with suppression of TGF- $\beta$ 1 signaling pathways.

### Perspectives and Significance

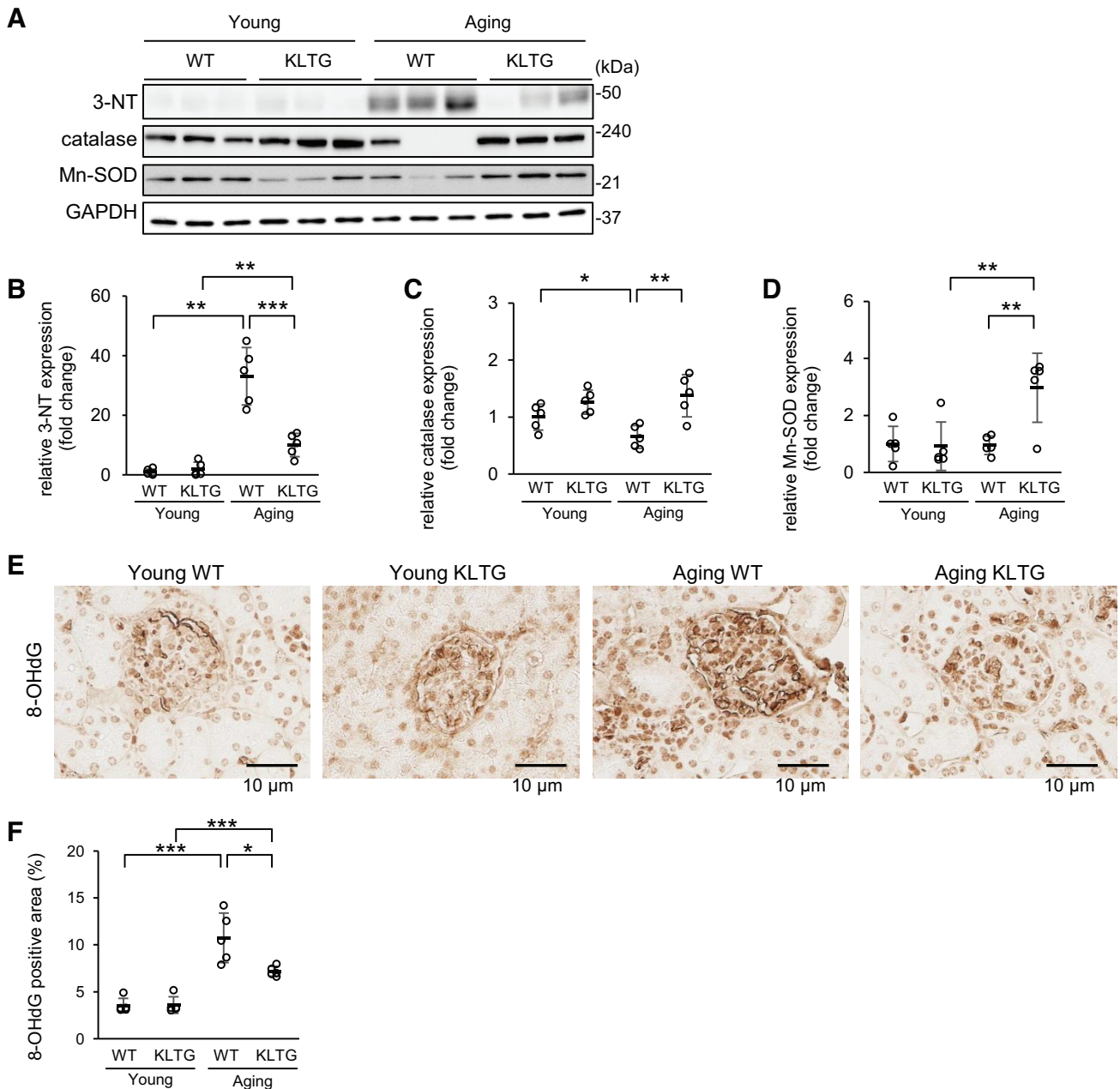
Life span is shortened in klotho-deficient mice but prolonged in KLTG mice. Therefore, klotho is currently

**Figure 4.** Klotho overexpression protects kidneys against age-associated fibrosis. *A*: representative images of Masson's trichrome (MT) staining showing fibrotic changes in renal tissues of wild-type (WT) and klotho transgenic (KLTG) mice at 8 wk (Young) and 24 mo (Aging) of age. *B*: quantification of the fibrotic areas in *A* ( $n = 5$ ). *C*: representative Western blot analysis demonstrating protein expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), collagen type I, and vimentin in the kidneys of each group. GAPDH was used as a loading control, and the blot is the same blot as shown in Fig. 1A. *D–F*: quantitative analysis of the fibrotic markers  $\alpha$ -SMA (*D*), collagen type I (*E*), and vimentin (*F*) in *C* ( $n = 5$ ). *G*: representative images of immunohistochemistry of  $\alpha$ -SMA (*top*), collagen type I (*middle*), and vimentin (*bottom*) in the kidneys of each group. *H–J*: quantification of relative  $\alpha$ -SMA (*H*), collagen type I (*I*), and vimentin (*J*) mRNA levels in the kidneys determined by quantitative RT-PCR ( $n = 5$ ). Values are presented as means  $\pm$  SD. Data were analyzed using ANOVA followed by a Tukey's test. Statistical differences are indicated as \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .





**Figure 5.** Klotho overexpression prevents age-associated accumulation of cell cycle-arrested cells. *A*: representative Western blot analysis of p53, p16, and p21 in the kidneys of wild-type (WT) and klotho transgenic (KLTG) mice at 8 wk (Young) and 24 mo (Aging) of age. GAPDH was used as a loading control, and the blot is the same blot as shown in Fig. 1*A*. *B–D*: quantitative analysis of the cell cycle arrest markers p53 (*B*), p16 (*C*), and p21 (*D*) in *A* ( $n = 5$ ). *E*: representative images of immunohistochemical staining of p53, p16, and p21 in the kidneys of each group. Values are presented as means  $\pm$  SD. Data were analyzed using ANOVA followed by a Tukey's test. Statistical differences are indicated as  $*P < 0.05$ ,  $**P < 0.01$ , and  $***P < 0.001$ .

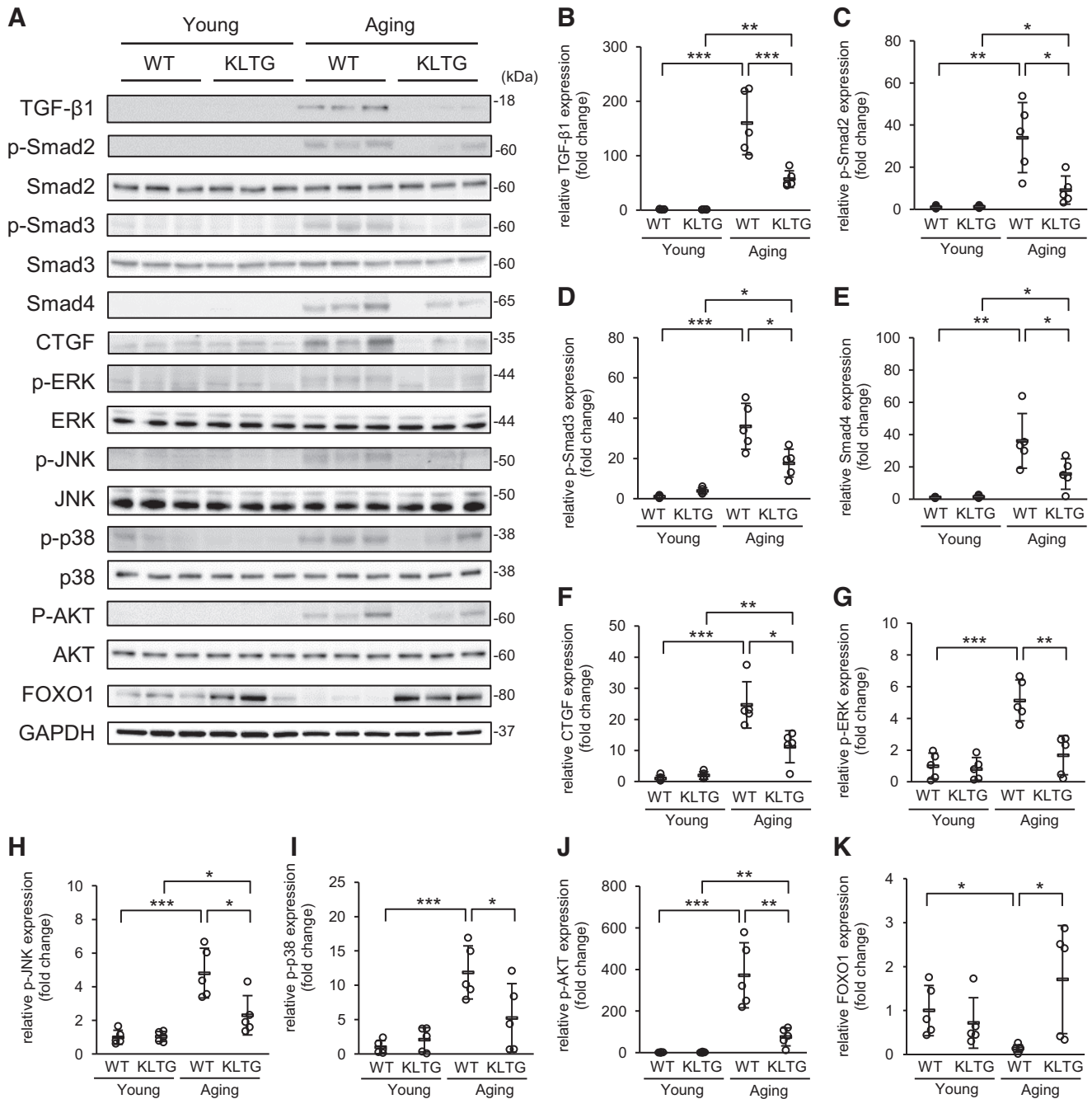


**Figure 6.** Klotho overexpression ameliorates the age-associated increase in oxidative stress. *A*: representative western blot analysis of 3-nitrotyrosine (3-NT), catalase, and manganese superoxide dismutase (Mn-SOD) in the kidneys of wild-type (WT) and klotho transgenic (KLTG) mice at 8 wk (Young) and 24 mo (Aging) of age. GAPDH was used as a loading control, and the blot is the same blot as shown in Fig. 1*A*. *B–D*: quantitative analysis of 3-NT (*B*), catalase (*C*), and Mn-SOD (*D*) in *A* ( $n = 5$ ). 3-NT and catalase/Mn-SOD were used as markers of oxidative nitric oxide damage and of antioxidative stress activity, respectively. *E*: representative images of immunohistochemical staining of 8-hydroxy-2 deoxy-guanosine (8-OHdG) showing oxidative DNA damage in the kidneys of each group. *F*: quantification of 8-OHdG-positive areas in *E* ( $n = 5$ ). Values are presented as means  $\pm$  SD. Data were analyzed using ANOVA followed by a Tukey's test. Statistical differences are indicated as  $*P < 0.05$ ,  $**P < 0.01$ , and  $***P < 0.001$ .

considered as an antiaging protein, and its overexpression may be a candidate therapy for protecting against kidney damage with advanced aging. Although multiple factors are involved in the aging process, we showed that klotho overexpression exerts an inhibitory effect on renal aging in mouse kidneys along with suppression of TGF- $\beta$ 1 signaling pathways. The present data showed that klotho provides a comprehensive approach for protecting against age-associated kidney damage.

## ACKNOWLEDGMENTS

We thank Dr. Kenichi Miyamoto and Dr. Hiroko Segawa (Department of Applied Nutrition, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Japan) for supplying klotho transgenic mouse strain EFmKL46 and its wild-type control. We also thank the Institute of Laboratory Animal Science at Hiroshima University for experimental mice and Mitchell Arico from Edanz (<https://jp.edanz.com/ac>) for editing a draft of this manuscript.



**Figure 7.** Klotho overexpression suppresses intracellular signals of multiple growth factors. **A:** representative Western blot analysis of intracellular signaling molecules in the kidneys of wild-type (WT) and klotho transgenic (KLTG) mice at 8 wk (Young) and 24 mo (Aging) of age. GAPDH was used as a loading control. **B–K:** quantitative analysis of transforming growth factor (TGF)-β1 (**B**), phosphorylated (p-)Smad2 (**C**), p-Smad3 (**D**), Smad4 (**E**), connective tissue growth factor (CTGF; **F**), p-ERK (**G**), p-JNK (**H**), p-p38 (**I**), p-AKT (**J**), and forkhead box protein O1 (FOXO1; **K**) in *A* (*n* = 5). Values are presented as means ± SD. Data were analyzed using ANOVA followed by a Tukey's test. Statistical differences are indicated as \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001.

## GRANTS

This work was supported by the Tsuchiya Memorial Medical Foundation.

## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

## AUTHOR CONTRIBUTIONS

S.D. conceived and designed research; H.O., T.I., and K.S. performed experiments; H.O., T.I., and K.S. analyzed data; H.O. and K.S. interpreted results of experiments; H.O. prepared figures; H.O. and Y.M. drafted manuscript; H.O. and Y.M. edited and revised manuscript; S.D., A.N., T.I., T.D., and T.M. approved final version of manuscript.

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