

Senescence marker protein-30/gluconolactonase deficiency exacerbates diabetic nephropathy through tubular injury in a mouse model of type 1 diabetes

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Keywords

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ABSTRACT

Aims/Introduction: Senescence marker protein-30 (SMP30) is abundantly expressed in renal proximal tubule cells, but its expression decreases with age. Previous studies have shown that reduced SMP30 expression could contribute to aging-associated deterioration of cellular function and tissue injury. In the present study, we investigated the effects of SMP30 deficiency on the pathogenesis of diabetic nephropathy.

Materials and Methods: Diabetes was induced using streptozotocin in male SMP30 knockout mice (KO) and wild-type mice at 7 weeks-of-age. Vitamin C was added to the drinking water to prevent vitamin C deficiency in KO mice. The mice were killed 12 weeks after the induction of diabetes.

Results: Urinary biomarkers for proximal tubule damage were significantly increased in non-diabetic KO mice compared with wild-type mice. Furthermore, diabetes-induced tubular damage was significantly exacerbated by SMP30 deletion. Morphological analysis showed a link between cortical tubulointerstitial fibrosis area and the degree of tubular damage. However, SMP30 deletion did not affect mesangial expansion. Tubular injury was associated with accumulation of hypoxia-inducible factor-1 α and increased hypoxia-inducible factor-1 α targeted gene expression. SMP30 deletion initiated oxidative stress; however, it did not exacerbate the oxidative stress seen in diabetic mice. In contrast, tubular inflammation was associated with SMP30 deletion only in diabetic mice.

Conclusions: Based on this evidence, we concluded that SMP30 deficiency exacerbates proximal tubule injury in diabetic mice. Decreased SMP30 could contribute to the increased incidence of various chronic kidney diseases, including diabetic nephropathy, with age.

INTRODUCTION

Diabetic nephropathy (DN) is the leading cause of end-stage renal failure, and an independent risk factor for cardiovascular mortality and morbidity. Risk factors, such as hyperglycemia, hypertension and aging, accelerate the progression of DN. DN is associated with the development of glomerular, vascular and

tubular lesions^{1,2}. Oxidative stress, inflammation, hypoxia and tubulointerstitial fibrosis are now recognized to play an important role in the development of diabetic proximal tubular injury, although the molecular mechanisms have not been completely elucidated^{3–5}.

Senescence marker protein-30 (SMP30) is a novel molecule whose expression decreases with age in a sex-independent manner^{6,7}. SMP30 transcripts have been detected in multiple tissues, and its amino acid alignment shows a highly conserved

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structure among humans, rats and mice⁸. We previously reported that SMP30 participates in Ca²⁺ efflux by activating the calmodulin-dependent Ca²⁺-pump in HepG2 cells and renal tubule cells, conferring on these cells a resistance to injury caused by high intracellular Ca²⁺ concentrations⁹. We further found that SMP30 functions as a glucolactonase (GNL) that is involved in L-ascorbic acid biosynthesis in mammals¹⁰. Studies using SMP30/GNL KO mice have suggested that reduced SMP30/GNL expression contributes to age-associated deterioration of cellular function and enhanced susceptibility to harmful stimuli in aged tissue^{11–17}; however, the physiological function of SMP30/GNL is still not entirely clear.

SMP30/GNL is abundantly expressed in proximal tubule epithelial cells. In addition, SMP30/GNL KO mice show clearly visible deposits of lipofuscin and senescence-associated β -galactosidase in tubular cells^{8,15}. However, the effects of decreased SMP30/GNL on renal injury have not been elucidated. Advanced age is a risk factor for the progression of DN leading to end-stage renal failure¹⁸, and it has been suggested that this is as a result of senescence of renal cells. In this context, decreased SMP30/GNL could contribute to the connection between the pathogenesis of DN and aging.

The present study investigated the effects of SMP30/GNL deficiency on the pathogenesis of DN. SMP30/GNL KO mice were used to investigate the question of whether decreased SMP30/GNL contributes to proximal renal tubular injury and accelerates the progression of DN.

MATERIALS AND METHODS

Animals and Experimental Protocol

All experimental procedures were approved by the Committee for Animal Research of the Kyoto Prefectural University of Medicine (Permit number: 231067). SMP30/GNL KO mice were generated as described earlier in the background strain C57BL/6¹¹. All studies were carried out on male mice using age-matched, wild-type (WT) male C57BL/6CrSlc mice (Shimizu Laboratory Supplies Co., Ltd., Kyoto, Japan) as controls. As we have previously reported, SMP30/GNL KO mice cannot synthesize vitamin C (VC) *in vivo*¹⁰. Therefore, to eliminate the confounding secondary effects of VC deficiency and to make this experimental model more relevant to human disease, the SMP30/GNL KO mice were maintained with water containing 1.5 g/L VC (a gift from DSM Nutrition Japan, Tokyo, Japan) in 10 μ mol/L ethylenediaminetetraacetic acid, pH 8.0. All mice had free access to VC-deficient chow (CL-2; CLEA Japan, Tokyo, Japan).

SMP30/GNL KO mice were maintained under VC (+) conditions after weaning. Diabetes (DM) was induced at 7 weeks-of-age by a single intraperitoneal injection of streptozocin (200 mg/kg in citrate buffer, pH 4.5; Sigma Aldrich, St. Louis, MO, USA). Mice serving as controls (Cont) were given the same volume of citrate buffer. A total of 7 days after streptozocin injection, mice with blood glucose levels over 300 mg/dL were selected. Mice were divided into four experimental groups

as follows: WT-Cont, KO-Cont, WT-DM and KO-DM. The mice were maintained for up to 12 weeks after induction of diabetes (20 weeks-of-age), and were killed under sodium aminobarbital anesthesia after an overnight fast.

Renal Histology and Morphometry

Kidneys were fixed in 10% (v/v) formalin and embedded in paraffin. Kidney sections (3 μ m) were stained with periodic acid-Schiff (PAS) for measurement of the mesangial area or assessment of tubular damage, and tubulointerstitial inflammation with Sirius Red (SR) for measurement of the fibrosis area. Stained sections were photographed and assessed using the color differentiation program Image J (National Institutes of Health, Bethesda, MD, USA). Glomerular tuft areas were estimated as the average of 50 randomly selected glomeruli, which were cut at the vascular pole in each animal and the relative mesangial area was expressed as PAS-positive area/glomerular tuft surface area. Determination of fibrosis by SR staining was estimated as the average of 10 randomly selected cortical tubulointerstitial areas in each animal, and expressed as the percentage of SR-positive area/total cortical area. Tubular damage and tubulointerstitial inflammation were quantified according to the method reported by Kiss *et al.*¹⁹ with minor modifications. Tubular damage was scored as 0 (non-existent), as 0.5 (thinning of the brush border with or without interstitial edema), 1 (thinning of the tubular epithelia with or without interstitial edema), 2 (denudation of the tubular basement membrane with or without interstitial edema) and 3 (tubular necrosis with or without interstitial edema)²⁰. Tubulointerstitial inflammation was scored as 0 (no mononuclear cells in the interstitium), 0.5 (focal mononuclear cell infiltration in the interstitium), 1 (focal mononuclear infiltration in the interstitium with tubulitis), 2 (diffuse mononuclear cell infiltration of the interstitium) or 3 (diffuse mononuclear cell infiltration of the interstitium with tubulitis). Tubulitis was defined as one or more mononuclear cells per tubular cross-section. Tubular damage and tubulointerstitial inflammation index was defined as the percentage of fields with the respective degree of injury in 10 randomly selected cortical tubulointerstitial areas in each animal. Tubular damage and tubulointerstitial inflammation score was calculated as the sum of all specific indices, whereby the index of fields with degree 0.5 was multiplied by 0.5, that of degree 1 \times 1, that of degree 2 \times 2 and that of degree 3 \times 3. Eight to ten mice in each group were included in the analysis. All classifications were carried out by an investigator who was blind to the experimental conditions.

Immunohistochemistry

Immunohistochemistry of kidney sections (3 μ m) was carried out using the following primary antibodies: anti-4-hydroxynonenal (4HNE) antibody (1:50 dilution; Japan Institute for the Control of Aging, Shizuoka, Japan) in Bouin's solution-fixed tissue and anti-hypoxia-inducible factor (HIF)-1 α antibody (1:100 dilution; NOVUS, Littleton, CO, USA) in 10% formalin-fixed

tissue. Target antigen retrieval entailed autoclaving for 10 min. Detection was carried out with diaminobenzidine as a substrate.

Positively stained tubulointerstitial areas were quantified as the average of 10 randomly selected cortical areas in each animal and expressed as the percentage of total cortical area at $\times 100$ magnification. Positively stained glomerular areas were quantified as the average of 50 randomly selected glomeruli in each animal and expressed as the percentage of total glomerular area at $\times 400$ magnification. Staining intensity was assessed using the color differentiation program Image J (National Institutes of Health).

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

Total ribonucleic acid (RNA) was isolated from frozen renal tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (0.5 μ g) was reverse-transcribed using Prime-Script RT Master Mix (TaKaRa Bio Inc., Shiga, Japan) for first-strand complementary deoxyribonucleic acid synthesis utilizing an oligonucleotide dT primer and random hexamer priming according to the manufacturer's recommendations.

Reverse transcription polymerase chain reaction was used to measure messenger RNA (mRNA) expression of HIF-1 α , p67-phox, SMP30, connective tissue growth factor (CTGF), monocyte chemotactic protein-1 (MCP-1) and peroxisome proliferator-activated receptor-gamma coactivator-1 α (PGC-1 α) using a Thermal Cycler Dice Real Time System II (TaKaRa Bio Inc.). Real-time SYBR[®] Premix Ex Taq II (TaKaRa Bio Inc.) was used along with the primers listed in Table 1. Relative expression levels of each targeted gene were normalized to beta-actin threshold cycle (CT) values and were quantified using the comparative threshold cycle $2^{-\Delta\Delta C_T}$ method as previously described²¹.

Biochemical Analysis

Non-fasted blood glucose levels were monitored every 2 weeks, and blood samples for measurement of fasting blood glucose, hemoglobin A1c and creatinine were taken at the end of the experiment after an overnight fast. Urine was collected at 20 weeks-of-age from mice housed in metabolic cages. Urinary concentrations of albumin (Albuwell; Exocell, Philadelphia, PA, USA), neutrophil gelatinase-associated lipocalin (NGAL; Mouse NGAL ELISA Kit; Bio Porto, Gentofte, Denmark) and kidney injury molecule 1 (KIM-1; Mouse TIM-1/KIM-1/HAVCR Immunoassay; R&D Systems, Minneapolis, MN, USA) were measured using a mouse-specific sandwich enzyme-linked immunosorbent assay system. N-acetyl- β -d-glucosaminidase (NAG) was measured by a colorimetric method. All urine measurements were expressed as total amount excreted in 24 h. Total VC levels in the liver were measured by high-performance liquid chromatography using an Atlantis dC18 5 μ m column (4.6 \times 150 mm; Nihon Waters, Tokyo, Japan) as previously reported²².

Statistical Analysis

Data are expressed as means \pm standard errors. Differences were analyzed by ANOVA followed by the Tukey–Kramer test. A $P < 0.05$ was considered statistically significant.

RESULTS

General Characteristics of Experimental Animals

General characteristics of experimental animals at the end of the experiment are shown in Table 2. VC supplementation increased total liver VC levels in SMP30/GNL KO mice to a level comparable with that in WT mice. Deletion of SMP30/GNL did not affect diabetes-induced renal enlargement. KO-DM and WT-DM mice showed a similar increase in blood glucose and hemoglobin A1c levels. It is noteworthy that the

Table 1 | Primer sequences for use in real time quantitative reverse transcription polymerase chain reaction

Gene name	Sequence	Size (bp)	GenBank accession no.	
<i>Beta-actin</i>	F	5'-CATCCGTAAAGACCTCTATGCCAAC-3'	171	NM007393
	R	5'-ATGGAGCCACCGATCCACA-3'		
<i>HIF-1α</i>	F	5'-AATCTGTTCCATTAGCAGGTGAAG-3'	151	NM010431
	R	5'-TGCCATGTACCAGAATCAAACCA-3'		
<i>P67-phox</i>	F	5'-ACTACTGCCTGACTCTGTGGTGTGA-3'	110	NM010877
	R	5'-CTGAGGCTCCGTAGTCTGCTTACTG-3'		
<i>SMP30/GNL</i>	F	5'-GAGGCAGCTGATGCTGGTAA-3'	92	NM009060
	R	5'-GAGCTGCAGTTCACCCCTGCATA-3'		
<i>CTGF</i>	F	5'-ACCCGAGTACCAATGACAATACC-3'	200	NM010217
	R	5'-CCGAGAACTTAGCCCTGTATG-3'		
<i>MCP-1</i>	F	5'-AGCAGCAGGTGTCCCAAAGA-3'	175	NM011333
	R	5'-GTGCTGAAGACCTTAGGCCAGA-3'		
<i>PGC-1α</i>	F	5'-GTACAACAATGAGCCTGCGAACA-3'	121	NM008904
	R	5'-TGAGGGCAATCCGTCTTCATC-3'		

senescence marker protein-30 (SMP30), CTGF, connective tissue growth factor; F, forward; GNL, glucolactonase; MCP-1, monocyte chemotactic protein-1; PGC-1 α , peroxisome proliferator-activated receptor-gamma coactivator-1 α ; R, reverse.

Table 2 | Characteristics of the experimental groups of mice at 20 weeks-of-age

	WT-Cont	KO-Cont	WT-DM	KO-DM
<i>n</i>	10	8	10	8
Bodyweight (g)	30.3 ± 0.5	26.5 ± 1.2	19.3 ± 0.9†‡	18.0 ± 1.0†‡
Blood glucose (mmol/L)	6.4 ± 0.2	7.7 ± 0.5	33.2 ± 1.3†‡	30.2 ± 1.6†‡
HbA1c (%)	5.0 ± 0.1	4.4 ± 0.2	11.6 ± 0.3†‡	9.9 ± 0.1†‡
Creatinine (μmol/L)	15.0 ± 1.8	13.3 ± 0.9	11.5 ± 0.9	10.6 ± 0.9
Kidney weight (μg/BWg)	6.0 ± 0.1	6.7 ± 0.2	9.9 ± 0.4†‡	10.8 ± 0.8†‡
UAE (μg/day)	7.6 ± 1.4	23.6 ± 1.4†	94.6 ± 21.3†‡	168.7 ± 24.6†‡§
NAG (mU/day)	30.9 ± 2.0	53.5 ± 15.7†	57.0 ± 9.8†	116 ± 30.8†‡§
NGAL (pg/day)	21.9 ± 2.2	61.5 ± 15.2†	302.8 ± 25.0†‡	1400.4 ± 385.6†‡§
KIM-1 (ng/day)	0.9 ± 0.07	2.6 ± 0.2†	3.7 ± 0.5†‡	15.3 ± 2.0†‡§
VC content (mg/g tissue)	140.2 ± 2.6	111.8 ± 0.5	132.6 ± 3.1	115.0 ± 3.1

† $P < 0.05$ vs wild-type (WT) control (Cont) mice, ‡ $P < 0.05$ vs knockout (KO)-Cont mice, § $P < 0.05$ vs WT diabetes (DM) mice. BW, bodyweight; HbA1c, hemoglobin A1c; KIM-1, kidney injury molecule 1; NAG, N-acetyl- β -D-glucosaminidase; NGAL, neutrophil gelatinase-associated lipocalin; UAE, urinary albumin excretion; VC, vitamin C.

urinary proximal tubule damage markers NAG, NGAL and KIM-1 were significantly increased in KO-Cont mice compared with WT-Cont mice ($P < 0.05$). Furthermore, SMP30/GNL deletion significantly increased expression of these tubular damage markers in diabetic mice (WT-DM vs KO-DM, $P < 0.05$). Urinary albumin excretion (UAE) levels showed a similar pattern to those of the tubular damage markers.

Renal Histology

Diabetic mice showed glomerular hypertrophy and an increase in mesangial areas. SMP30/GNL deletion did not affect these changes (Figure 1a,c,d). However, it is notable that the degree of tubulointerstitial fibrosis was significantly increased in KO-Cont mice compared with WT-Cont mice ($P < 0.05$). In addition, SMP30/GNL deletion significantly accelerated the diabetes-induced increase in tubulointerstitial fibrosis (WT-DM vs KO-DM, $P < 0.05$; Figure 1b,e).

Tubular damage and tubulointerstitial inflammation were evaluated after quantification (Table 3). Tubular damage was significantly increased in KO-Cont mice compared with WT-Cont mice ($P < 0.05$). Similar to the increase in tubulointerstitial fibrosis, diabetes-induced tubular damage was significantly exacerbated by SMP30/GNL deletion. However, a significant increase in tubulointerstitial inflammation was detected only in KO-DM mice (vs WT-DM, $P < 0.05$), suggesting that tubular inflammation is not induced by SMP30/GNL deletion alone.

HIF-1 α Expression

HIF-1 α is known to play an important role in the hypoxic response of tubular epithelial cells and tubulointerstitial fibrosis. HIF-1 α staining in the cortical tubulointerstitial area was significantly increased in KO-Cont mice compared with WT-Cont mice ($P < 0.05$; Figure 2a,c). Diabetes also increased HIF-1 α expression in the cortical tubulointerstitial area, and SMP30/GNL deletion significantly exacerbated the increase (WT-DM

vs KO-DM, $P < 0.05$; Figure 2a,c). The cytoplasmic presence of HIF-1 α could be caused by enhanced protein stabilization and accumulation before nuclear translocation²³. These changes were consistent with the degree of fibrosis. In the glomerular area, HIF-1 α expression was increased in diabetes, but SMP30/GNL deletion did not affect expression (Figure 2b,d). Analysis of HIF-1 α mRNA expression in whole kidneys showed a significant increase in KO-Cont mice compared with WT-Cont mice ($P < 0.05$; Figure 3). Among diabetic mice, there were no differences in HIF-1 α mRNA expression between KO and WT mice, suggesting that post-translational HIF-1 α stabilization could increase HIF-1 α protein content in KO-DM mice (Figure 3).

Oxidative Stress

The degree of oxidative stress was assessed by 4HNE immunostaining. In the tubulointerstitial area, the degree of oxidative stress was significantly increased in KO-Cont mice compared with WT-Cont mice ($P < 0.05$, Figure 4a,c). However, there were no differences between KO-DM and WT-DM mice, suggesting that hyperglycemia-induced oxidative stress might overwhelm the effects of SMP30/GNL deletion (Figure 4a,c). In the glomerular area, SMP30/GNL deletion did not affect the degree of oxidative stress (Figure 4b,d). Analysis of p67-phox mRNA expression in whole kidneys showed a similar pattern to that of 4HNE immunostaining in the cortical tubulointerstitial area, showing a significant increase in KO-Cont compared with WT-Cont mice ($P < 0.05$; Figure 3).

Expression of SMP30/GNL, CTGF, MCP-1 and PGC-1 α

CTGF is a typical HIF-1 α target gene, and has been shown to be essential for the epithelial to mesenchymal transition leading to tubulointerstitial fibrosis^{24,25}. MCP-1 is a pro-inflammatory factor, and contributes to tubulointerstitial inflammation

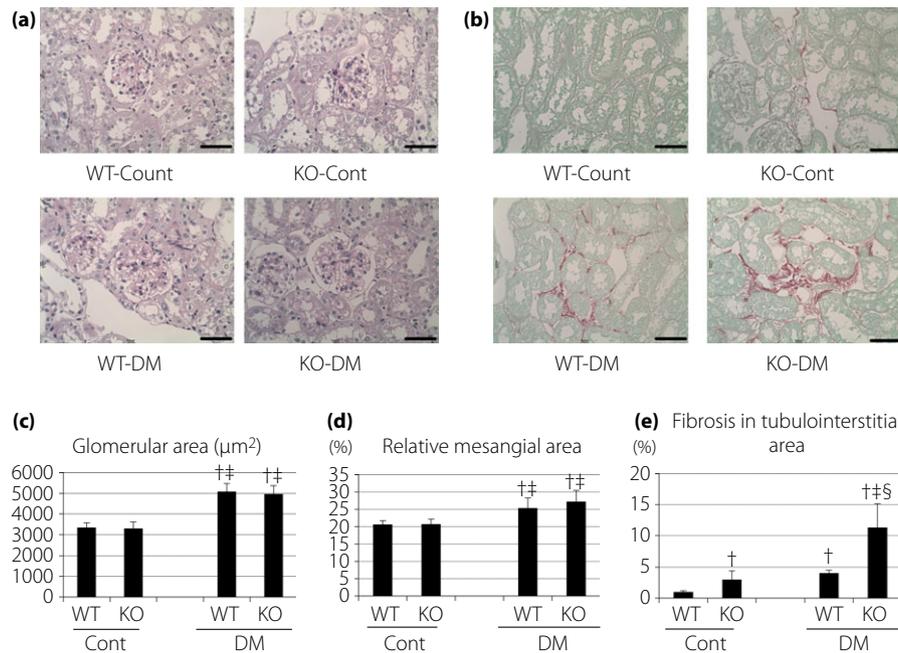


Figure 1 | Relative mesangial area and the degree of tubulointerstitial fibrosis. (a) PAS-stained kidney sections (magnification: ×400), (b) Sirius Red-stained kidney sections (magnification: ×400), (c) glomerular area and quantitative analysis of (d) relative mesangial area and (e) fibrosis at week 12 of the experimental period. Scale bar, 50 µm. Values are means ± standard error; †*P* < 0.05 vs wild-type (WT) control (Cont) mice, ‡*P* < 0.05 vs knockout (KO)-Cont mice, §*P* < 0.05 vs WT diabetes (DM) mice.

by attracting inflammatory cells to the interstitium²⁶. PGC-1α is a transcriptional co-activator, and regulates mitochondrial biogenesis and function, ultimately controlling cell survival²⁷.

Diabetes did not affect mRNA expression of SMP30/GNL in the kidneys of WT mice (Figure 3). mRNA expression of CTGF in whole kidneys showed a similar pattern to that of the cortical tubulointerstitial fibrosis and HIF-1α protein expression experiments, and was significantly increased in KO mice under both diabetic and non-diabetic conditions (WT-Cont vs KO-Cont, WT-DM vs KO-DM, *P* < 0.05; Figure 3). However, mRNA expression of MCP-1 and PGC-1α in KO mice was significantly upregulated only in diabetic mice (WT-DM vs KO-DM, *P* < 0.05; Figure 3).

DISCUSSION

The major finding of the current study was that SMP30/GNL deletion exacerbated diabetes-induced renal proximal tubule

damage with interstitial fibrosis. Urinary biomarkers of proximal tubule damage were associated with morphological changes. Furthermore, SMP30/GNL deletion caused tubulointerstitial changes even in non-diabetic mice. SMP30/GNL is abundantly expressed in renal proximal tubule cells, but its expression decreases with age. Hence, the present results suggest that reduced SMP30/GNL in renal proximal tubule cells could be a factor in the age-related decline in renal function and the progression of DN.

Morphological changes observed in the kidneys of non-diabetic SMP30/GNL KO mice were mild proximal tubule cell injury and tubulointerstitial fibrosis. Together with these findings, the lack of a significant increase in inflammation, as assessed by the inflammation score and expression of MCP-1 mRNA, indicates the presence of slow-progressing renal cell injury²⁸. The increase in oxidative stress in the tubular region is consistent with previous studies of SMP30/GNL KO mice^{8,13,14}.

Table 3 | Tubular damage and tubulointerstitial inflammation score

	WT-Cont	KO-Cont	WT-DM	KO-DM
<i>n</i>	10	8	10	8
Tubular damage	14.2 ± 2.5	21.3 ± 4.0 [†]	39.1 ± 12.8 ^{†‡}	58.7 ± 7.4 ^{†‡§}
Tubulointerstitial inflammation	20.9 ± 4.6	22.5 ± 0.9	38.2 ± 6.2 [†]	53.3 ± 10.8 ^{†‡§}

†*P* < 0.05 vs wild-type (WT) control (Cont) mice, ‡*P* < 0.05 vs knockout (KO)-Cont mice, §*P* < 0.05 vs WT diabetes (DM) mice.

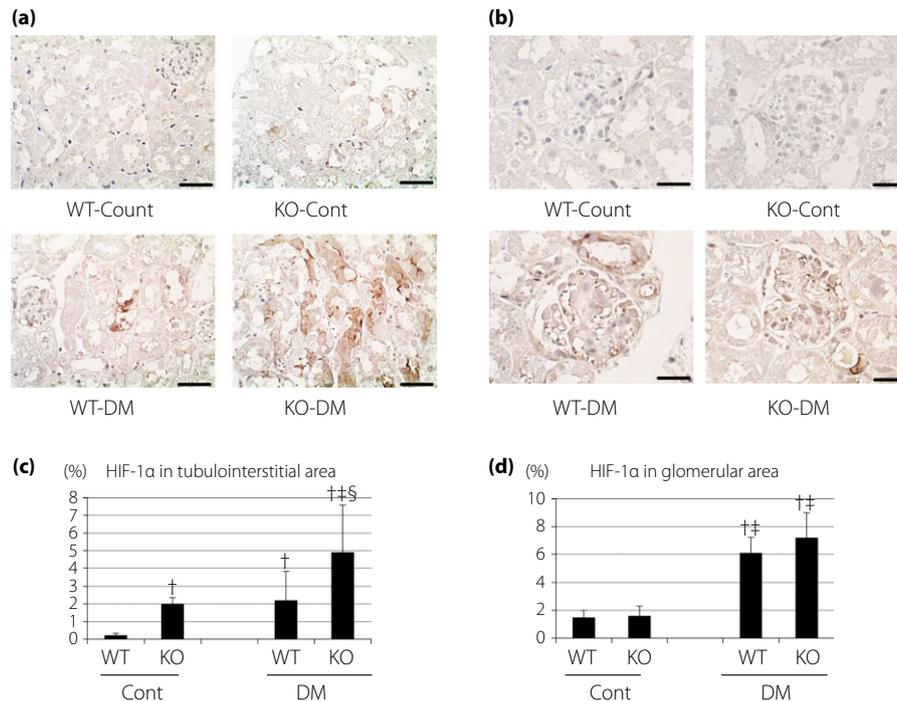


Figure 2 | Expression of hypoxia-inducible factor (HIF)-1 α . Immunohistochemistry of kidney sections using anti-HIF-1 α antibody, (a) cortical area and (b) glomerular area (magnification: $\times 400$), and (c, d) quantitative analysis of positively stained area at week 12 of the experimental period. Scale bar, 50 μ m. Values are means \pm standard error; $\dagger P < 0.05$ vs wild-type (WT) control (Cont) mice, $\#P < 0.05$ vs knockout (KO)-Cont mice, $\$P < 0.05$ vs WT diabetes (DM) mice.

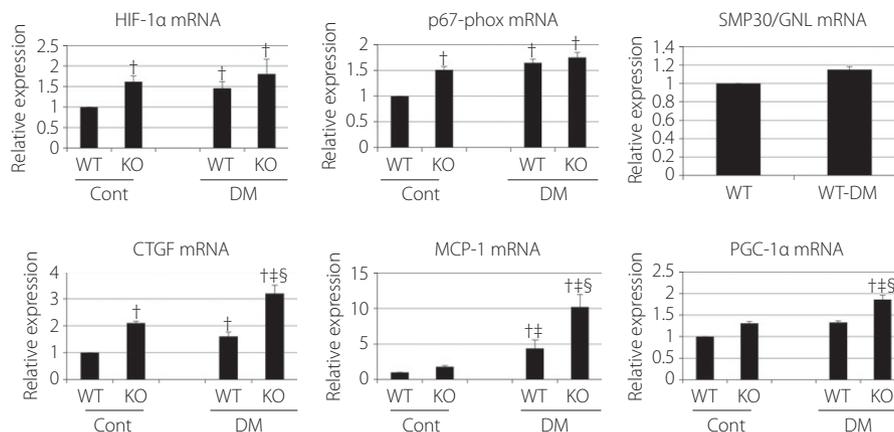


Figure 3 | Messenger ribonucleic acid (mRNA) expression of hypoxia-inducible factor (HIF)-1 α , p67-phox, senescence marker protein-30 (SMP30), connective tissue growth factor (CTGF), monocyte chemoattractant protein-1 (MCP-1) and peroxisome proliferator-activated receptor-gamma coactivator-1 α (PGC-1 α). Relative expression of HIF-1 α , p67-phox, SMP30, CTGF, MCP-1 and PGC-1 α in whole kidney were determined by reverse transcription polymerase chain reaction. mRNA content of wild-type (WT) control (Cont) mice was designated as 1.0. Values are means \pm standard error; $\dagger P < 0.05$ vs WT-Cont mice, $\#P < 0.05$ vs knockout (KO)-Cont mice, $\$P < 0.05$ vs WT diabetes (DM) mice.

Therefore, it is possible that increased intracellular reactive oxygen species might induce HIF-1 α expression and stabilization, resulting in the upregulation of CTGF, a direct target of

HIF-1 α . It has been suggested that prolonged activation of HIF-1 α in renal epithelial cells enhances maladaptive responses that lead to fibrosis and tissue destruction^{25,29}.

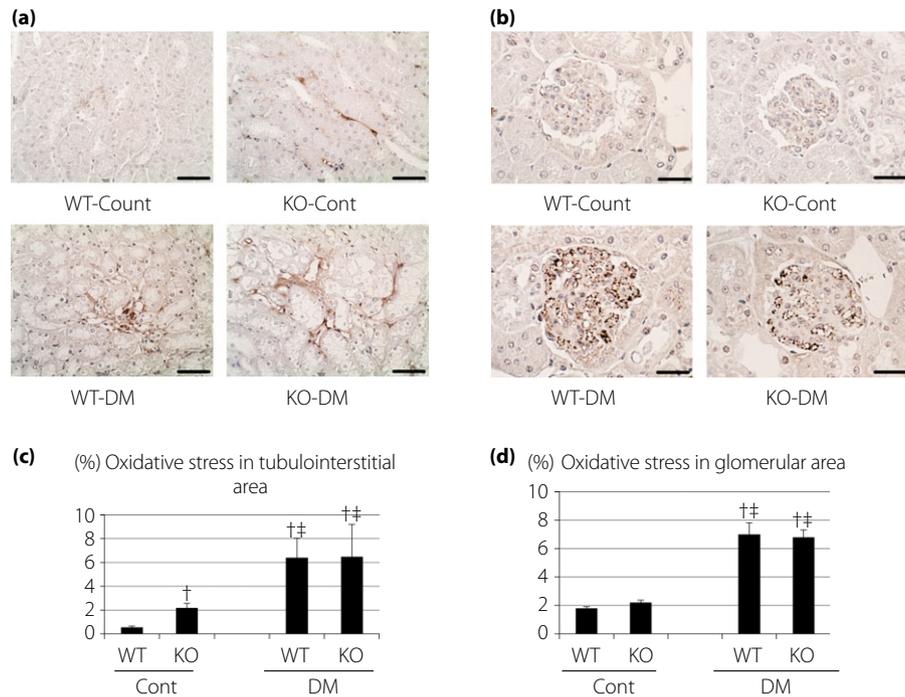


Figure 4 | Oxidative stress. Immunohistochemistry of kidney sections using anti-4-hydroxynonenal (4HNE) antibody, (a) cortical area and (b) glomerular area (magnification: $\times 400$), and (c, d) quantitative analysis of positively stained area at week 12 of the experimental period. Scale bar, 50 μm . Values are means \pm standard error; † $P < 0.05$ vs wild-type (WT) control (Cont) mice, †† $P < 0.05$ vs knockout (KO)-Cont mice. DM, diabetes.

Deletion of SMP30/GNL did not affect diabetes-induced morphological changes in glomeruli, as assessed by mesangial expansion and glomerular hypertrophy. These results are not unexpected when we consider the low expression of SMP30/GNL in glomeruli⁸. However, it should be noted that glomerular damage in SMP30/GNL KO mice is not completely excluded as a result of limitation of the experimental protocol. In contrast, as expected, SMP30/GNL deletion significantly enhanced diabetes-induced tubulointerstitial damage. Diabetic SMP30/GNL KO mice showed a marked increase in tubulointerstitial fibrosis and tubular damage, and increased HIF-1 α protein expression in the tubulointerstitial area and increased CTGF mRNA expression. Oxidative stress was markedly increased in both the tubulointerstitial and glomerular areas of diabetic mice; this increase could mask the mild oxidative stress induced by SMP30/GNL deletion. Therefore, together with the finding that HIF-1 α mRNA expression did not differ in WT-DM and KO-DM, this suggests that the increased HIF-1 α protein in the tubulointerstitial area might be a result of enhanced HIF-1 α stability. In this regard, although the precise mechanism remains to be elucidated, SMP30/GNL deletion in diabetes might inhibit HIF-hydroxylating enzymes. Furthermore, SMP30/GNL deletion in diabetic mice significantly increased inflammation, as assessed by inflammation scoring and MCP-1 mRNA expression. A number of studies have shown that hyperglycemia is central to the development of diabetic proximal tubule injury, which triggers oxidative stress,

inflammation, hypoxia and fibrosis⁴. SMP30/GNL deletion could affect the intricate interplay among these factors, resulting in considerable inflammation and fibrosis.

The precise mechanisms underlying renal tubule cell damage in SMP30/GNL KO mice remain to be defined. We have previously shown that SMP30/GNL participates in Ca²⁺ efflux by activating the calmodulin-dependent Ca²⁺-pump in HepG2 cells and renal tubular cells, conferring on these cells a resistance to injury caused by high intracellular Ca²⁺ concentrations.⁹ Therefore, high cellular Ca²⁺ concentrations would be expected in SMP30/GNL KO mice. High intracellular Ca²⁺ concentrations might promote mitochondrial Ca²⁺ influx and trigger opening of the mitochondrial permeability transition pore, resulting in mitochondrial swelling, increased generation of reactive oxygen species, mitochondrial membrane depolarization and decreased production of adenosine triphosphate³⁰⁻³². In fact, mitochondrial swelling has been shown in the livers and submandibular glands of SMP30/GNL KO mice^{8,33}. Taken together, these findings suggest that mitochondrial dysfunction induced by SMP30/GNL deletion contributes to renal tubule cell damage. However, the histological findings of the present study suggest that the cell damage induced by SMP30/GNL deletion is not sufficient to induce massive apoptosis and subsequent tissue degeneration. SMP30/GNL deletion did not significantly increase the number of terminal deoxynucleotidyl transferase dUTP nick-end labeling-positive cells in the renal proximal tubule either in diabetic or non-diabetic mice (data not shown).

The significantly increased PGC-1 α expression seen in KO-DM mice suggests that a compensatory pathway for mitochondrial dysfunction might be active in this system³⁴.

A recent study has shown that anti-aging gene, *KLOTHO*, deficiency exacerbated early diabetic nephropathy through glomerular injury in streptozocin-induced diabetic mice³⁵. Together with the present results of SMP30 deficiency, anti-aging genes could have a protective role in diabetic nephropathy. Further investigation into the targets and function of anti-aging genes, in addition to SMP30 and *KLOTHO*, will help develop new strategies for protection against diabetic nephropathy.

In conclusion, *SMP30/GNL* deficiency exacerbates proximal tubule injury in diabetic mice. In addition, *SMP30/GNL* deficiency results in tubule injury even in non-diabetic mice. These results suggest that decreased *SMP30/GNL* might contribute to the cross-talk between various chronic kidney diseases, including DN and aging. Understanding the molecular mechanisms of this tubular injury could lead to new treatment strategies and preventive measures for DN.

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