Experimental Animals
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Original

# Progranulin deficiency attenuates tubulointerstitial injury in a mouse unilateral ureteral obstruction model

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**Abstract:** Progranulin (PGRN) may have two opposing effects—inflammation and anti-inflammation—in different diseases. Although previous studies have reported that PGRN is involved in liver fibrosis, its involvement in tubulointerstitial fibrosis remains to be fully elucidated. Herein, we investigated these issues using PGRN-knockout (KO) mice treated with unilateral ureteral obstruction (UUO). Eight-week-old male PGRN-KO and wild-type (WT) mice were euthanized 3 and 7 days following UUO, and their kidneys were harvested for histopathological analysis. The renal expression of PGRN was evaluated by immunohistochemical and/or western blot analyses. The renal mRNA levels of markers related to inflammation (*Il1b*, *Tnf*, *Il6*, *Ccl2,* and *Adgre1*) and fibrosis (*Tgfb1*, *Acta2*, *Fn1*, and *Col1a2*) were evaluated using quantitative PCR. Histological changes such as renal tubular atrophy, urinary casts, and tubulointerstitial fibrosis were significantly improved in UUO-KO mice compared with UUO-WT mice. Quantitative PCR revealed that the mRNA expression levels of all inflammation- and fibrosis-related markers were lower in UUO-KO mice than in UUO-WT mice at 3 and/or 7 days after UUO. Moreover, PGRN and GRN protein levels were higher in the kidneys of UUO-WT mice than in mice that did not undergo UUO. Elevated GRN levels associated with excess PGRN levels may be involved in the occurrence of renal inflammation and fibrosis in UUO mice.

**Key words:** progranulin, renal fibrosis, unilateral ureteral obstruction model

## Introduction

In patients with chronic kidney disease, inflammation promotes fibrosis, which leads to end-stage kidney disease [1, 2]. Progranulin (PGRN) is a 593-amino-acid glycoprotein; it is a growth factor that is involved in embryogenesis, tissue repair, tumorigenesis, and inflammation [3]. PGRN is encoded by *GRN* and is expressed in many cell types, including epithelial cells, immune cells, neurons, and adipocytes [4]. Although full-length PGRN exerts anti-inflammatory effects by inhibiting tumor necrosis factor (TNF)-α signaling [5], proteolytically processed PGRN—known as granulins (GRNs)—may stimulate the production of inflammatory cytokines [6].

Zhou *et al.* [7] reported that PGRN has renoprotective effects in a mouse model of acute renal failure. However, we have reported that serum PGRN levels are increased in patients with type 2 diabetes and renal function decline, and that elevated serum PGRN levels predict future glomerular filtration rate decline in patients with type 2 diabetes [8, 9]. These conflicting results might be attributed to the opposing actions of PGRN and GRN [10].

In the present study, we therefore investigated the effects of PGRN knockout (KO) on unilateral ureteral obstruction (UUO)-induced renal tubulointerstitial inflammation and fibrosis.

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## Materials and Methods

### Animals

*Grn* heterozygous KO mice, developed by Kayasuga *et al.*, were obtained from the RIKEN BioResource Center (RBRC02370, Ibaraki, Japan). The mice were backcrossed to C57BL/6JJmsSlc at least eight times. Genotyping of *Grn* homozygous KO mice was performed as previously described [11]. Wild-type (WT) mice (C57BL/6JJmsSlc) were used as controls. The mice were individually housed in plastic cages with free access to food and water throughout the experiment. All animal experiments were approved by the Ethics Review Committee for Animal Experimentation of the Juntendo University Faculty of Medicine (document no. 1450), and all animals were treated according to the guidelines for animal experimentation at Juntendo University in Tokyo, Japan.

### Experimental protocol

Six-week-old male mice were divided into the following six groups: sham-operated WT mice (Control-WT: 3 days after sham operation, n=6; 7 days after sham operation, n=8), UUO-operated WT mice (UUO-WT: 3 days after UUO, n=4; 7 days after UUO, n=10), and UUO-operated PGRN-KO mice (UUO-KO: 3 days after UUO, n=4; 7 days after UUO, n=9). The UUO surgery or a sham operation was performed on 8-week-old PGRN-KO and WT mice. UUO was performed according to standard procedures [12]. Briefly, mice were anesthetized, and a minor incision along the abdominal midline was performed in a sterile environment. The left lower ureter was tied with 3-0 silk sutures at two sites—one proximal to the kidney and the other distal—and ligated. Sham-operated mice underwent ureter exposure but not ligation. At 3 or 7 days after the operation, mice were euthanized with an intraperitoneal injection of pentobarbital (0.05 mg/g body weight) and their kidneys were harvested for subsequent analysis.

### Quantitative PCR (qPCR)

mRNAs were isolated from the kidney cortex and gene expression was analyzed using qPCR, as described previously [13]. For total RNA purification, the RNeasy Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions. For qPCR, TaqMan Gene Expression Master Mix and TaqMan probes (Applied Biosystems, Foster City, CA, USA) were used (Supplementary Table 1). The expression levels of *Grn, Il1b, Tnf, Il6, Ccl2, Tgfb1, Acta2, Fn1, Col1a2, Adgre1, Tnfr1, Tnfr2, Epha2, Sort1,* and *Lrp1* in the kidneys were measured using commercially available assays (Applied Biosystems). For each gene, the average threshold cycle (CT) was subtracted from the corresponding average CT for *Gapdh* for each sample to obtain ΔCT. Fold increases in other groups compared with the Control-WT group were then calculated using the $2^{-\Delta\Delta CT}$ method.

### Analysis of renal histology

For histological assessment, kidneys were fixed in 4% neutral buffered paraformaldehyde, embedded in paraffin, and sectioned into 4-$\mu$m-thick slices. Sections were then dewaxed using standard sequential techniques and stained with periodic acid-Schiff and Azan Mallory staining. We randomly captured at least five images at 200× magnification using the CellSens software (ver.1.12, Olympus, Tokyo, Japan). The percentage of cortical tubular necrosis was assigned using the following grades: grade 0, normal; 1, <20%; 2, 20–40%; 3, 40–60%; 4, 60–80%; and 5, >80%, as described previously [14]. To evaluate interstitial fibrosis in UUO, the intensity of positive Azan staining was quantified using KS400, a computer-aided manipulator (Carl Zeiss Vision, Munich, Germany), at the cortical interstitium in 10 randomly selected fields that contained at least two glomeruli at 200× magnification [15].

### Immunohistochemistry

Kidneys were embedded in optimal cutting temperature compound, frozen, cut into 4-$\mu$m sections using a cryostat, fixed in 4% fresh paraformaldehyde for 3 min, and blocked with 10% normal goat serum (Dako, Glostrup, Denmark) for 30 min. Immunohistochemical analyses were then performed using polyclonal sheep anti-PGRN antibodies (1:20 dilution in 1% bovine serum, AF2557; R&D Systems, Minneapolis, MN, USA) overnight at 4°C. Next, the sections were incubated with Alexa Fluor 488 anti-rabbit antibody (1:500 dilution; rabbit polyclonal, ab150181; Abcam, Cambridge, UK) for 40 min at room temperature. Nuclei were stained with 4′,6-diamidino-2-phenylindole.

### Western blot analysis

Proteins were extracted from the kidneys using radioimmunoprecipitation assay buffer with protease and phosphatase inhibitors before undergoing western blot analysis using a polyclonal antibody raised against PGRN, which also recognizes GRNs. Proteins were separated by NuPAGE Bis-Tris gradient gels (Thermo Fisher Scientific, Waltham, MA, USA), transferred to polyvinylidene fluoride membranes (ISEQ00010; Sigma-Aldrich, St Louis, MI, USA), and immunoblotted with specific primary antibody for PGRN (1:400 dilution, sheep polyclonal, AF2557; R&D Systems) in

Blocking One solution (Nacalai tesque, Inc., Kyoto, Japan) overnight at 4°C. After washing with Tris-buffered saline, membranes were incubated with peroxidase-conjugated secondary antibody (1:20,000 dilution, rabbit anti-sheep immunoglobulin/horseradish peroxidase, P0163; Dako) for 2 h at room temperature. Detection was performed using enhanced chemiluminescence (Thermo Fisher Scientific) before the membranes were photographed.

### Statistical analysis

Variables with a continuous distribution that followed a normal pattern are presented as the mean ± SD. Statistical analyses were conducted using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA). The unpaired *t*-test was used for comparisons between two groups. For comparisons between multiple groups, differences in between-group means were determined using one-way analysis of variance. Furthermore, Tukey's multiple comparisons test was performed for statistical comparisons, with $P$ values <0.05 considered significant.

## Results

### Effects of PGRN on UUO-induced tubular injury

UUO causes marked tubulointerstitial inflammation and injury. To investigate whether PGRN contributes to this pathogenesis, we performed UUO in the PGRN-KO mice. Tubulointerstitial inflammation was evaluated by periodic acid-Schiff staining of kidney sections (Fig. 1). There was no significant difference in tubular injury on day 3 after UUO between UUO-WT and UUO-KO mice. However, quantitative analysis of tubular injury on day 7 after UUO revealed significantly fewer tubular lesions in UUO-KO mice than in UUO-WT mice.

### UUO-induced tubulointerstitial fibrosis in WT and PGRN-KO mice

We evaluated UUO-induced tubulointerstitial fibrosis to investigate the role of PGRN in renal fibrosis (Fig. 2). Azan Mallory staining revealed no significant differences in renal fibrosis on day 3 after UUO between UUO-WT and UUO-KO mice. However, renal fibrosis on day 7 after UUO was significantly suppressed in UUO-KO mice compared with UUO-WT mice.

### mRNA expression levels of inflammation-related genes in UUO-operated kidneys

To investigate the effects of PGRN on the inflammatory response in more detail, the mRNA levels of inflammation-related genes (*Il1b, Tnf, Il6, Ccl2,* and *Adgre1*) in the kidneys were measured using qPCR. The mRNA levels of all inflammatory cytokines except *Adgre1* were significantly different between UUO-KO and UUO-WT mice on day 3 after UUO, but did not differ on day 7. By contrast, the mRNA levels of *Adgre1* were not significantly different between UUO-KO and UUO-WT mice on day 3, but significantly differed on day 7 after UUO (Fig. 3).

### mRNA expression levels of fibrosis-related genes after UUO

To further evaluate the effects of PGRN on UUO-induced fibrosis, we analyzed the mRNA levels of fibrosis-related genes (*Tgfb1, Acta2, Fn1,* and *Col1a2*) using qPCR. The qPCR analysis revealed that the mRNA

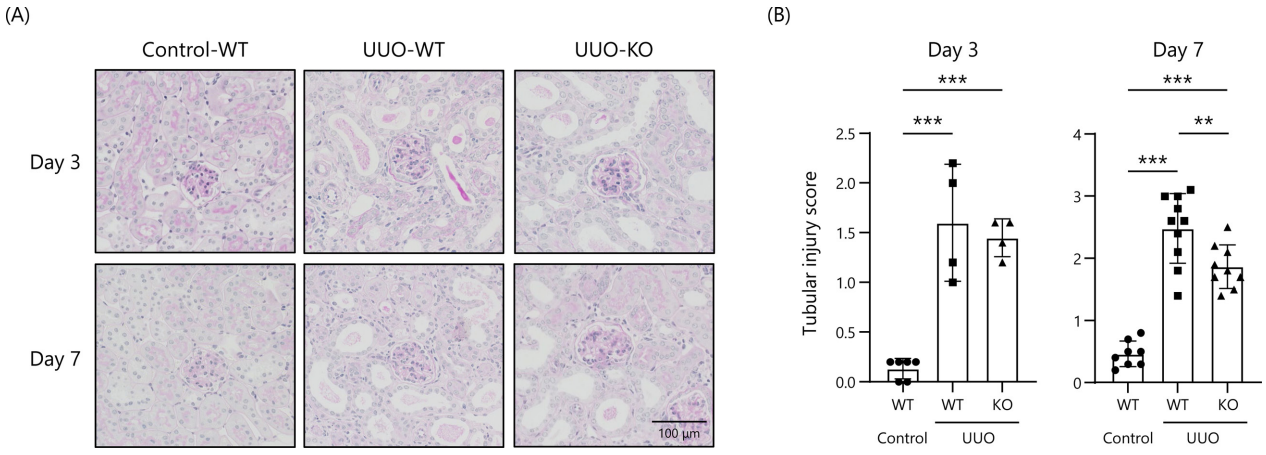


**Fig. 1.** Histological analyses of tubular injury with periodic acid-Schiff staining. Tubular injury scores were lower in unilateral ureteral obstruction (UUO)-knockout (KO) mice than in UUO-wild-type (WT) mice. (A) Representative images of periodic acid-Schiff staining in kidney sections at days 3 and 7 after UUO. (B) Quantitative analysis of tubular injury scores. Comparisons among groups were performed using one-way analysis of variance. Scale bar: 100 $\mu$m. **$P$<0.01. ***$P$<0.001.

levels of fibrosis-related genes in the kidney were lower in UUO-KO mice than in UUO-WT mice on both days 3 and 7 after UUO (Fig. 4).

## PGRN and GRN protein expression levels in UUO-operated kidneys

Although the mRNA levels of renal *Grn* were comparable between UUO-WT and Control-WT mice on day 3 after UUO, they were significantly higher in UUO-WT mice than in Control-WT mice on day 7 (Fig. 5A).

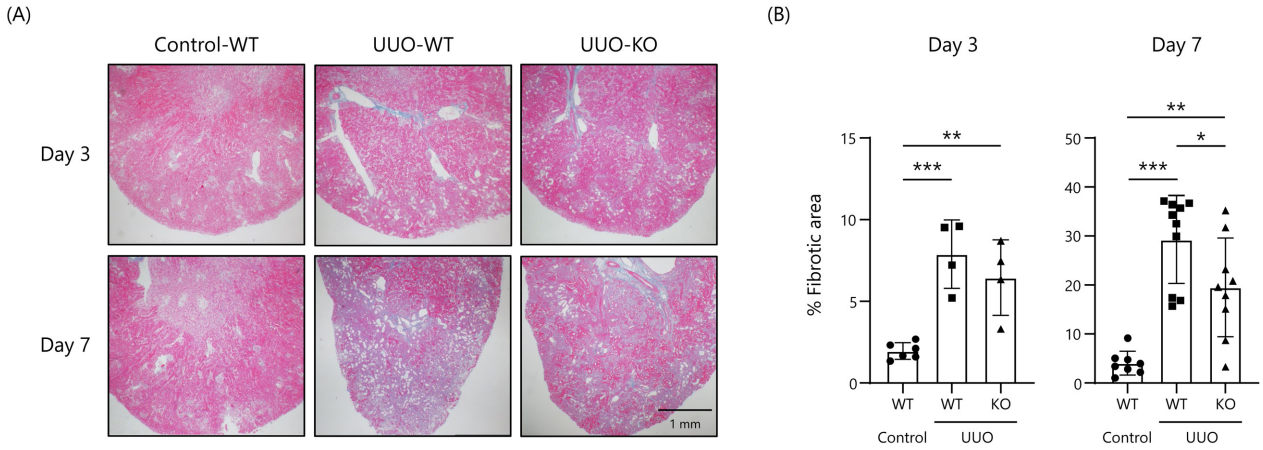


**Fig. 2.** Immunohistochemistry analysis with Azan Mallory staining. Renal fibrosis was lower in unilateral ureteral obstruction (UUO)-knockout (KO) mice than in UUO-wild-type (WT) mice. (A) Representative images of Azan Mallory staining in kidney sections at days 3 and 7 after UUO. (B) Quantitative analysis of fibrosis. Comparisons among groups were performed using one-way analysis of variance. Scale bar: 1 mm. **P*<0.05. ***P*<0.01. ****P*<0.001.

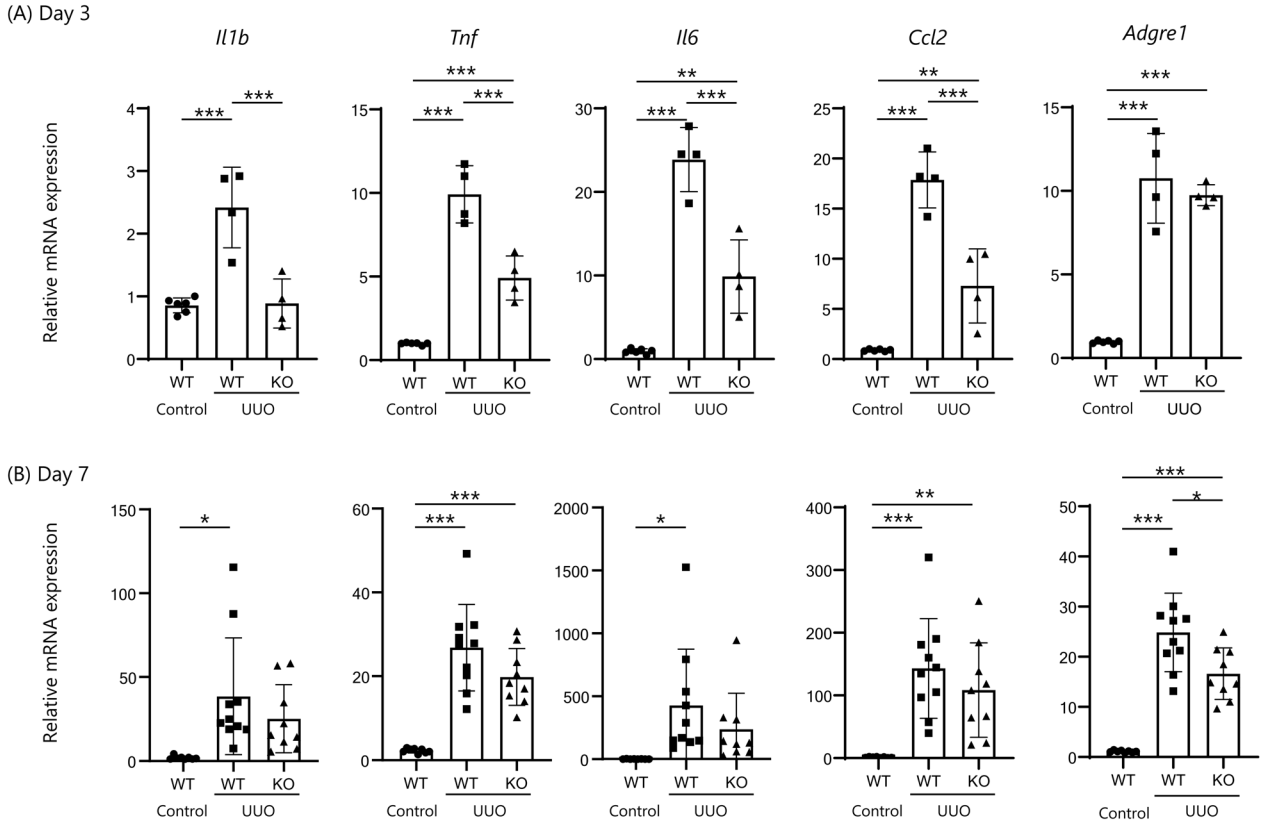


**Fig. 3.** mRNA expression levels of inflammation-related genes in the kidneys using real-time PCR analysis. The mRNA levels of inflammation-related genes were lower in unilateral ureteral obstruction (UUO)-knockout (KO) mice than in UUO-wild-type (WT) mice. Comparisons among groups were performed using one-way analysis of variance. **P*<0.05. ***P*<0.01. ****P*<0.001.

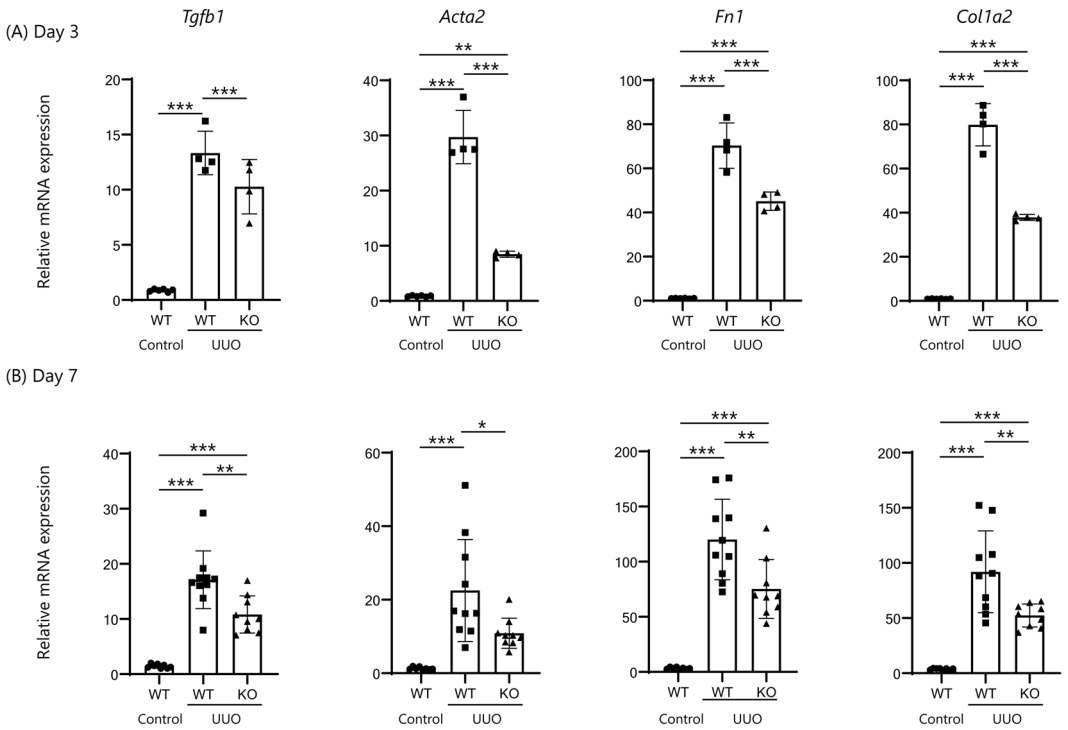


**Fig. 4.** mRNA expression levels of fibrosis-related genes in the kidneys using real-time PCR analysis. The mRNA levels of fibrosis-related genes were significantly lower in unilateral ureteral obstruction (UUO)-knockout (KO) mice than in UUO-wild-type (WT) mice on days 3 and 7 after UUO. Comparisons among groups were performed using one-way analysis of variance. **P*<0.05. ***P*<0.01. ****P*<0.001.

The absence of PGRN expression in PGRN-KO mice was confirmed using immunofluorescence staining. In UUO-WT and Control-WT mice, PGRN was mainly localized in the proximal tubules (Fig. 5B). Furthermore, PGRN protein levels in the proximal tubules appeared higher in UUO-WT mice than in Control-WT mice.

Next, we confirmed the protein expression levels of PGRN in kidneys using western blotting. The protein levels of PGRN were significantly different between Control-WT and UUO-WT mice on day 3 and 7 after UUO (Fig. 5C). The protein levels of GRN were significantly higher in the kidneys of UUO-WT mice on days 3 compared to those in Control-WT mice, but did not differ on day 7.

### PGRN receptor mRNA expression levels in UUO-operated kidneys

Given that PGRN influences multiple molecular pathways, we examined the mRNA levels of PGRN receptors on cell membranes. There were no significant differences in the mRNA levels of TNF receptors (*Tnfr1* and *Tnfr2*), *Epha2*, or *Lrp1* between UUO-KO and UUO-WT mouse kidneys. However, the mRNA levels of renal sortilin (*Sort1*) were significantly higher in UUO-WT mice than in UUO-KO mice (Fig. 6).

## Discussion

UUO is a widely used experimental model of renal interstitial fibrosis, which occurs primarily through an interstitial inflammatory response [16]. In the present study, we demonstrated that PGRN deficiency ameliorated the progression of UUO-induced renal inflammation and fibrosis. Our findings suggest that renal PGRN expression may play a pathogenic role in the progression of kidney injury. However, although PGRN is important in various physiological and pathological conditions, its role in the kidneys remains controversial. For example, studies have suggested that PGRN has anti-inflammatory effects in central nervous system disorders, arthritis, and acute ischemia–reperfusion renal injury [7, 17–19]. However, Matsubara *et al.* [20] reported that PGRN exerts pro-inflammatory effects through adipose tissue interleukin-6 production in mice with obesity and insulin-resistant diabetes. Furthermore, serum PGRN levels are reportedly inversely correlated with estimated glomerular filtration rate and positively correlated with albuminuria [8].

In the present study, the UUO-KO mice exhibited significant reductions in renal inflammation and fibrosis compared with the UUO-WT mice. However, experimental evidence indicates that PGRN deficiency exac-

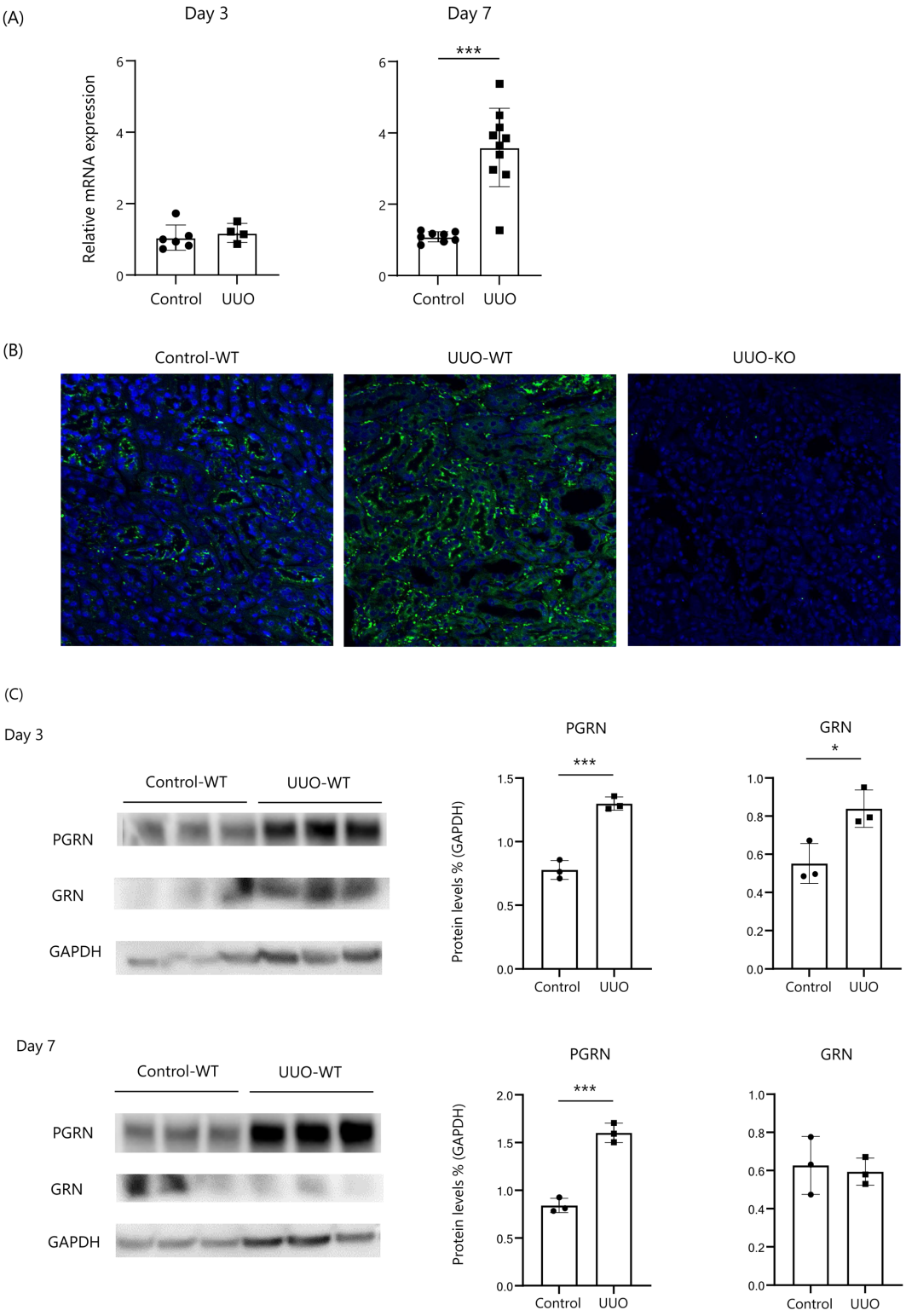


**Fig. 5.** Progranulin (PGRN) and granulin (GRN) expression levels in unilateral ureteral obstruction (UUO)-operated kidneys. (A) mRNA expression levels of *Grn* in the kidneys of UUO-wild-type (WT) and Control-WT mice at days 3 and 7 after UUO. PGRN mRNA levels in the kidneys were higher in UUO-WT mice than in Control-WT mice. Comparisons among groups were performed using an unpaired *t*-test. ***$P$<0.001. (B) Immunofluorescence staining for PGRN in kidney sections. PGRN expression was localized to the tubules. Blue and green indicate 4′,6-diamidino-2-phenylindole and PGRN, respectively. (C) PGRN and GRN protein levels in UUO-operated kidneys by western blotting. PGRN protein levels were significantly higher in the kidneys of UUO-WT mice on day 3 and 7 after UUO compared to those in Control-WT mice. Comparisons among groups were performed using an unpaired *t*-test. *$P$<0.05. ***$P$<0.001. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KO, knockout.

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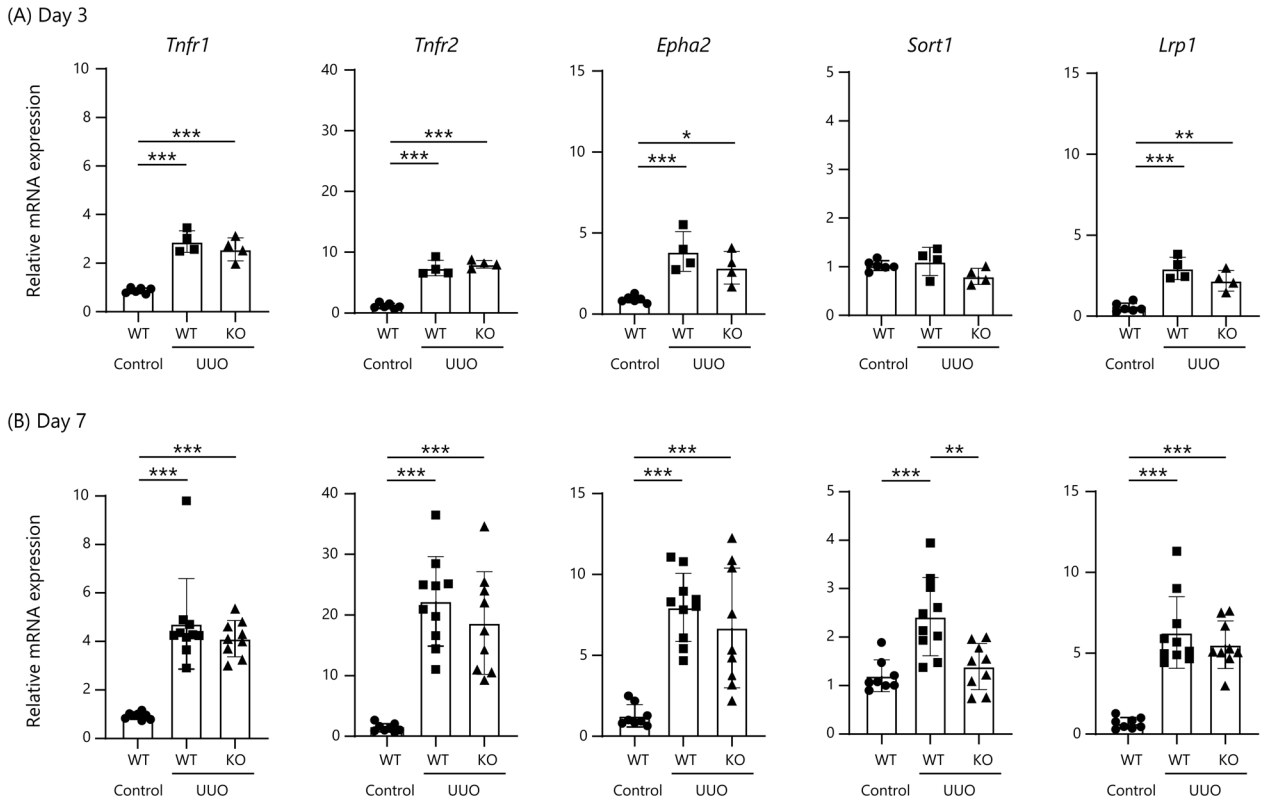


**Fig. 6.** Progranulin receptor mRNA expression levels in the kidneys using real-time PCR analysis. The mRNA levels of sortilin (*Sort1*) were significantly reduced in unilateral ureteral obstruction (UUO)-knockout (KO) mice compared with UUO-wild-type (WT) mice at day 7 after UUO. Comparisons among groups were performed using one-way analysis of variance. **P*<0.05. ***P*<0.01. ****P*<0.001.

erbates renal dysfunction and glomerular structural alterations, and the administration of recombinant human PGRN protects against podocyte injury under STZ-induced diabetic conditions [21]. One possible reason for these discrepancies is that PGRN has anti-inflammatory actions, whereas released GRNs have the opposite effect—they increase the production of pro-inflammatory cytokines [6]. In a study using a mouse model of cerebral infarction, the protease elastase was increased at the infarction site with PGRN overexpression and increased production of GRN. Furthermore, the administration of a neutrophil elastase inhibitor reduced inflammatory responses in this mouse model. Together, these results suggest that GRNs cleaved from overproduced PGRN may be involved in the inflammatory response after cerebral infarction [19]. In the present study, on day 3 after UUO, the renal gene expression levels of the macrophage marker F4/80 (*Adgre1*) did not differ between UUO-WT and UUO-KO mice, suggesting that the reduced inflammation in UUO-KO animals was not the result of suppressed macrophage infiltration. Nonetheless, the reduced renal inflammation in UUO-KO compared with UUO-WT mice may be caused by the absence of PGRN cleaved by macrophage-derived elastase (i.e., inflammatory GRNs). Several studies have reported that tissue inflammation is stronger in PGRN-deficient experimental animals than in WT mice. Most of these reports state that PGRN binds to TNF receptors and inhibits their downstream signaling pathways, thus suppressing inflammation [17]. However, some reports indicate that PGRN is not a direct regulator of TNF-α-dependent signaling [22]. In addition to TNF receptors, several other PGRN receptors have been identified, including ephrin type-A receptor 2, sortilin, and low-density lipoprotein receptor-related protein 1 [23]. In the current study, only sortilin mRNA expression levels were higher in UUO-WT mice than in UUO-KO mice. This finding suggests that the pleiotropic functions of PGRN may be mediated by sortilin, a regulator of pro-inflammatory cytokine secretion.

Markers of both inflammation and fibrosis were significantly elevated after the UUO procedure. However, although the levels of inflammatory markers exhibited a greater increase at 7 days after UUO than at 3 days, the levels of fibrosis markers remained unchanged between 3 and 7 days. Inflammation generally leads to fibrosis of the kidney. Sogawa *et al.* [24] examined the gene expression of inflammation and fibrosis markers in

UUO-operated mice at 3, 7, and 14 days after UUO. They reported that the expression of inflammation markers such as interleukin-18 peaked 7 days after UUO, but that the induction of fibrosis genes was delayed, and that fibrosis genes remained elevated 14 days after UUO. In the present study, fibrosis markers may thus have deteriorated further if they had been assessed after day 7.

We were unable to determine whether increased PGRN protein levels worsened renal impairment. In the present study, PGRN expression levels were higher in UUO mice than in control mice, and UUO-induced kidney inflammation was markedly reduced in PGRN-KO mice. These results suggest that PGRN might be involved in the progression of renal tubular damage caused by UUO. PGRN contains a secretory signal peptide and 7.5 GRN domains: para-GRN, GRN-1 (G), GRN-2 (F), GRN-3 (B), GRN-4 (A), GRN-5 (C), GRN-6 (D), and GRN-7 (E). During the inflammatory process, neutrophils and macrophages secrete matrix metalloproteinases, neutrophil elastase, and proteinase 3. These enzymes can degrade PGRN into a 6-kDa granulin peptide, which primarily has an inflammatory role [25]. Zhang *et al.* [26] reported that both GRN A and B are highly expressed in the kidney. Despite being derived from the same precursor, the varying levels of GRN peptides in different organs may be attributed to differences in PGRN processing and lysosomal degradation [27]. Rolinson *et al.* [25] reported transcript expression levels after incubation with individual GRN peptides. Pro-inflammatory functions were suggested for GRN A and B via protein kinase B/mitogen-activated protein kinase signaling, and for GRN C via the folate pathway. The other GRNs seemed to regulate mostly transcription factors, activators, or repressors. Together, these findings suggest that different GRN peptides have different effects. Although the present study did not examine which peptides caused increased GRN, GRN was increased in the kidneys of WT mice, in which inflammation was exacerbated by the UUO operation compared with kidneys in which PGRN was absent.

In conclusion, our results suggest that PGRN may be involved in tubulointerstitial inflammation and fibrosis in the pathogenesis of UUO in mice.

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