

Matrix metalloproteinase (MMP) -10 aggravates podocyte injury in glomerulonephritis

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Abstract

Background. Podocytes are integral to maintaining glomerular filtration barrier. Our previous research underscored the crucial role of podocyte guanylyl cyclase-A (GC-A) in the pathogenesis of severe albuminuria in both systemic and podocyte-specific GC-A knockout mice subjected to heminephrectomy, high salt and aldosterone (ALDO) treatment. This study investigates the role of matrix metalloproteinase-10 (MMP-10) on glomerular injury, which was found to be highly expressed in glomeruli of ALDO-treated GC-A knockout mice.

Methods. To investigate the role of MMP-10 in glomerulonephritis, we used MMP-10 knockout mice subjected to anti-glomerular basement membrane (GBM) nephritis. Additionally, we created systemic GC-A and MMP-10 double knockout mice, as well as podocyte-specific GC-A and systemic MMP-10 double knockout mice, to analyze glomerular injury. *In vitro*, changes in inflammatory mRNA are examined in MMP-10-overexpressing or -knockdown mouse podocytes following stimulation of tumor necrosis factor (TNF)- α .

Results. We demonstrate that MMP-10 is highly expressed in the kidneys of patients with glomerulonephritis. MMP-10 knockout mice showed less albuminuria and lower expression of pro-inflammatory and pro-fibrotic mRNAs compared to control mice in anti-GBM nephritis. Additionally, systemic or podocyte-specific GC-A and systemic MMP-10 knockout mice exhibited improved albuminuria, preserved nephrin expression, and reduced glomerular basement membrane thickening compared to systemic or podocyte-specific GC-A knockout mice with ALDO treatment. MMP-10 was co-localized with podocytes and endothelial cells. *In vitro* studies using mouse podocytes revealed that MMP-10 overexpression upregulated inflammatory mRNA changes induced by TNF- α , whereas MMP-10 knockdown mitigated inflammation. Co-culture of mouse podocytes with human endothelial cells showed reduced inflammation following MMP-10 reduction in endothelial cells. Moreover, activated MMP-10 cleaved nephrin *in vitro*, contributing to podocyte injury.

Conclusion. These findings suggest that GC-A ablation leads to upregulation of MMP-10, resulting in nephrin loss, and that systemic deletion of MMP-10 ameliorates GC-A-induced podocyte injury. (291 words)

Translational statement

Podocytes are crucial for maintaining the glomerular filtration barrier and preventing proteinuria. This study highlights the role of matrix metalloproteinase-10 (MMP-10) in podocyte injury. MMP-10 was highly expressed in guanylyl cyclase- A (GC-A) knockout mouse glomeruli and in patients with glomerulonephritis. Deletion of MMP-10 in mice led to improved albuminuria and reduced glomerular damage in anti-glomerular basement membrane nephropathy models, as well as in systemic or in podocyte-specific GC-A knockout mice treated with ALDO. These findings suggest that inhibition of MMP-10 could be a novel therapeutic target to mitigate podocyte injury and prolong renal function.

Key Learning Points

What was known:

- MMP-10 has been implicated in various inflammatory processes and tissue remodeling.
- Guanylyl cyclase-A (GC-A) plays a protective role in podocytes, and its deletion is linked to podocyte injury and albuminuria.
- The molecular mechanisms through which MMP-10 contributes to podocyte injury in the context of GC-A knockout models had not been thoroughly investigated.

This study adds:

- This study identifies MMP-10 as a key mediator of podocyte injury by cleaving nephrin, a critical protein for maintaining the glomerular filtration barrier.
- MMP-10 deletion significantly reduces albuminuria and glomerular damage in systemic and podocyte-specific GC-A knockout mice, suggesting its role as an active participant in kidney disease pathology.

Potential impact:

- Targeting MMP-10 could become a novel therapeutic strategy to protect podocytes, reduce proteinuria, and slow the progression of CKD.
- Understanding the MMP-10/nephrin interaction provides new insights into therapeutic targets, potentially influencing the development of policies related to CKD management.

Key Words: anti-GBM glomerulonephritis, endothelial cells, natriuretic peptides, aldosterone

Introduction

Chronic kidney disease (CKD) currently affects approximately 850 million people globally, with prevalence expected to rise due to aging populations and increased risk factors like diabetes and hypertension [1]. Proteinuria is a well-established risk factor not only for kidney failure but also for cardiovascular diseases [2-5]. Reducing proteinuria is therefore essential for improving renal outcomes [6]. Podocytes play a crucial role in maintaining the glomerular filtration barrier due to their unique architecture, which is essential for blood filtration and the prevention of proteinuria [7]. These cells are terminally differentiated with limited capacity for postnatal proliferation and regeneration, which makes podocyte loss a defining feature of the irreversibility of kidney failure [8]. Therefore, preventing podocyte injury is critical for improving outcomes in CKD.

Aldosterone has been reported to cause podocyte disorders, and inhibition of renin-angiotensin-aldosterone system has been shown to protect podocytes [9]. Aldosterone and natriuretic peptides work antagonistically to maintain homeostasis, such as regulating blood pressure and blood volume [10]. This opposite effect is also observed in podocyte injury. Guanylyl cyclase-A (GC-A), one of the natriuretic peptides (NP) receptors, is a membrane-bound enzyme that catalyzes the conversion of guanosine triphosphate to cyclic guanosine monophosphate (cGMP). We previously reported the protective effect of GC-A in podocytes against aldosterone infusion, using systemic GC-A knockout mice [11] and podocyte-specific GC-A knockout mice [12]. Both systemic and podocyte-specific GC-A knockout mice treated with heminephrectomy, aldosterone and high salt diet (ALDO) demonstrated massive albuminuria, mesangial expansion and podocyte injury. The NP-GC-A-cGMP signaling pathway has several protective effects on podocytes, including regulating the glomerular filtration rate by maintaining pressure in the glomerular capillaries [13], exerting anti-apoptotic and anti-fibrotic effects [14], and preserving cytoskeletal organization [15]. However, the precise molecular mechanism underlying the protective effect of the NP/GC-A pathway in podocytes has not been fully elucidated. Therefore, we investigated the molecular mechanisms in GC-A knockout mice to identify novel targets for podocyte protection in this study.

Materials and Methods

Human Biopsy Samples

This study was conducted according to the principles expressed in the Declaration of Helsinki. Patients with renal dysfunction or abnormal urine tests who underwent renal biopsy were enrolled in the study after obtaining informed consent. This study was approved by the Ethics Committee for Human Research of the Graduate School of Medicine, Kyoto University (Approval No. G562). The clinical features of the enrolled patients are detailed in Supplementary Table S1, and glomerular Periodic acid-Schiff staining images were shown in Supplementary Figure S1.

Aldosterone Infusion Model

Mice were anesthetized by intraperitoneal administration of medetomidine 0.75 mg/kg, butorphanol 5 mg/kg, and midazolam 4 mg/kg. Uninephrectomy was performed by removing left kidney and ligating the renal pedicles 2 weeks before subcutaneous implantation of an osmotic minipump (ALZET 2004, Cupertino, CA). All mice were fed a diet containing 6% NaCl after implantation. Blood pressure measurement and urine collection were performed biweekly.

Statistical Analysis

Data are expressed as mean $\pm$ standard deviation (SD). Variables were evaluated using a two-tailed unpaired Student's t-test or one-way analysis of variance with the Dunnett's test for comparison of multiple means. Pearson's correlation analysis was used to assess the relationship between two variables from Nephroseq Database. All statistical analysis was performed with JMP16 statistical software (SAS Institute). P-values < 0.05 were considered statistically significant.

For further details, please refer to the Supplementary Methods.

Results

MMP-10 as a Candidate Gene for Podocyte Damage in GC-A Knockout Mice and Its High Expression in Patients with Glomerulonephritis

We first conducted a comparative analysis of glomerular mRNA from uninephrectomized wild-type control and systemic GC-A knockout mice treated with ALDO using microarray analysis to identify novel genes involved in glomerular injury in GC-A knockout mice (Figure 1A). The scatterplot demonstrates differences in glomerular mRNA expression between control and systemic GC-A knockout mice (Figure 1B). Genes with expression changes of eight-fold or greater in systemic GC-A knockout mice compared to control mice are highlighted in Figure 1C. Among these, we focused on matrix metalloproteinase-10 (MMP-10) due to its role in extracellular matrix remodeling and controversial effects on inflammation and fibrosis. *Mmp10* mRNA expression was significantly higher in ALDO-treated GC-A knockout mice compared to ALDO-treated control mice (Figure 1D, the sequences of primers and probes are listed in Supplementary Table S2). To further explore MMP-10 expression in human glomerular diseases, we performed immunohistochemical staining of kidney biopsy specimens from patients with various glomerulonephritis. MMP-10 was rarely expressed in glomeruli from patients with no apparent glomerular disease (minor glomerular abnormality), whereas MMP-10 expression was markedly increased in proliferative lesions of glomerular diseases (Figure 1E). MMP-10 expression was particularly elevated in crescentic lesions in ANCA-associated vasculitis or proliferative lesions in lupus nephritis, suggesting that MMP-10 expression is enhanced by inflammation. Glomerular MMP-10-positive cells showed a significant increase of MMP-10 expression in patients with IgA nephropathy, diabetic nephropathy, lupus nephritis and ANCA-associated vasculitis, compared with minor glomerular abnormality (Fig. 1F). To investigate *MMP10* mRNA expression in human kidneys further, renal transcriptomic datasets from the Nephroseq repository were analyzed. *MMP10* mRNA expression was upregulated in kidneys from CKD patients compared to healthy controls (Figure 1G). Additionally, glomerular *MMP10* mRNA expression correlated well with serum creatinine levels of patients with vasculitis (Figure 1H). These findings suggest that glomerular expression of MMP-10 plays a significant role in the progression of renal diseases in both humans and mice.

MMP-10 Knockout Mice Show Reduced Glomerular Damage in Anti-Glomerular Basement Membrane (GBM) Nephritis

To further elucidate the role of MMP-10 in glomerulonephritis, we induced anti-GBM nephritis in MMP-10 knockout and wild-type control mice. Mice were administered anti-GBM serum at eight weeks of age and euthanized 14 days later (Figure 2A). Body weight changes in each group are shown in Supplementary Figure S2A. Both control and MMP-10 knockout mice displayed linear deposition of rabbit and mouse IgG along the basement membrane, indicating that MMP-10 deletion did not affect autologous antibody production (Supplementary Figure S2B). Induction of anti-GBM nephritis resulted in upregulation of *Mmp10* mRNA expression and enhanced MMP-10-positive area in glomerular lesions (Figure 2B and 2C). Western blot analysis revealed that MMP-10 expression was significantly upregulated in control mice with anti-GBM nephritis (Figure 2D). Histological examination revealed increased crescent formation in control mice with GBM nephritis at day 14, while MMP-10 knockout mice showed significantly fewer crescent formations (Figure 2E). Electron microscopy demonstrated severe basement membrane thickening in control mice with anti-GBM nephritis and only mild thickening in MMP-10 knockout mice (Figure 2F). Although there were no significant differences in urinary protein levels between vehicle-treated control and MMP-10 knockout mice, MMP-10 knockout mice with anti-GBM nephritis exhibited significantly reduced urinary protein levels compared to control mice with anti-GBM nephritis (Figure 2G). Control mice with nephritis showed reduced serum albumin and elevated total cholesterol due to massive proteinuria, but these changes were ameliorated in MMP-10 knockout mice (Figure 2G). Serum creatinine and blood urea nitrogen (BUN) levels were higher in control mice with anti-GBM nephritis compared to MMP-10 knockout mice, but body and kidney weights showed no significant differences between the groups (Figure 2H). Glomerular expression of pro-fibrotic genes (*Tgfb1*, *Ctgf*, *Fn1*), pro-inflammatory genes (*Ccl2*, *Emr1*), and other MMPs (*Mmp2*, *Mmp9*) were all increased in control mice with anti-GBM nephritis, and these changes were attenuated in MMP-10 knockout mice (Figure 3A-C). MAC-2 staining revealed the number of infiltrated macrophages in glomeruli was significantly lower in MMP-10 knockout mice with anti-GBM nephritis compared to control mice with anti-GBM nephritis (Supplementary

Figure S2C). Furthermore, double immunofluorescence staining revealed that MMP-10 co-localized with nephrin (a podocyte marker) and CD31 (an endothelial cell marker) in control mice with anti-GBM nephritis (Figure 3D and 3E). These findings indicate that MMP-10 deletion significantly reduces proteinuria and prevents structural deterioration of podocytes in anti-GBM nephritis.

Ablation of MMP-10 Reduces Glomerular Injury in ALDO-Treated GC-A Knockout Mice

To examine the effect of MMP-10 deletion in ALDO-treated GC-A knockout mice, we generated systemic GC-A and MMP-10 double knockout (DKO) mice by crossing GC-A knockout mice with MMP-10 knockout mice. These mice underwent uninephrectomy two weeks before the initiation of aldosterone administration, followed by a 6% salt diet and continued aldosterone and salt loading for four weeks (Figure 4A). *Mmp10* mRNA expression was significantly elevated in GC-A knockout mice (Figure 4B), whereas *Npr1* mRNA expression was slightly downregulated in MMP-10 knockout mice compared to control mice with no significant difference (Figure 4C). Western blot analysis revealed that MMP-10 expression was significantly upregulated in ALDO-treated GC-A knockout mice (Figure 4D). Immunofluorescence analysis confirmed that MMP-10 expression was upregulated in ALDO-treated GC-A knockout mice (Figure 4E). Heart weight was significantly higher in GC-A knockout mice, and this increase was reduced in DKO mice, while kidney weight and serum parameters remained unchanged between the GC-A knockout and DKO groups (Supplementary Figure S3A and S3B). Both ALDO-treated systemic GC-A knockout and DKO mice exhibited severe hypertension compared to ALDO wild-type mice, whereas ALDO-treated MMP-10 knockout mice had slightly elevated blood pressure comparable to wild-type mice (Figure 4F). Urinary albumin excretion and total urinary protein levels were significantly reduced in ALDO-treated DKO mice compared to ALDO-treated GC-A knockout mice at four weeks (Figure 4G). There were no serological changes between GKO and DKO groups despite the changes in urinary protein levels (Supplementary Figure S3C). Histological analysis revealed severe glomerular sclerosis and mesangial expansion in ALDO-treated GC-A knockout mice compared to ALDO-treated control and MMP-10 knockout mice, and these changes were attenuated in ALDO-treated DKO mice (Figure 4H). WT-1 staining indicated a loss of podocytes in ALDO-treated GC-A knockout mice and preservation of podocytes in ALDO-

treated MMP-10 knockout and DKO mice (Figure 4I). Electron microscopy showed thickening of GBM in ALDO-treated GC-A knockout mice, which was significantly improved in ALDO-treated DKO mice (Figure 4J). Expression levels of *Ctgf*, *Mmp2*, *Fnl*, and *Tgfb1* were elevated in ALDO-treated GC-A knockout mice and significantly ameliorated in DKO mice (Figure 5A). Furthermore, MMP-10 was also co-localized with nephrin and CD31 in ALDO-treated GC-A knockout mice consistent with the result of wild-type mice with anti-GBM nephritis (Figure 5B and 5C). These findings suggest that the exacerbation of podocyte injury by systemic GC-A deletion is mediated via the MMP-10 pathway.

Improvement of Podocyte Injury in Podocyte-Specific GC-A Knockout Mice Through MMP-10 Ablation

ALDO-treated MMP-10 and GC-A double knockout mice showed severe podocyte injury, but the importance of GC-A in podocytes was not fully investigated. Therefore, we generated systemic MMP-10 knockout and podocyte-specific GC-A knockout mice (p-DKO mice) by crossing MMP-10 knockout mice with *Npr1* floxed/floxed (fl/fl) nephrin-Cre mice. Control mice (*Npr1* fl/fl without Cre, Ctrl), MMP-10 knockout/*Npr1* fl/fl mice without Cre (MKO), podocyte-specific GC-A knockout mice (*Npr1* fl/fl nephrin-Cre, p-GKO), and p-DKO mice also underwent ALDO treatment (Figure 6A). *Mmp10* mRNA expression was upregulated in p-GKO mice compared to control mice, and *Npr1* mRNA expression was significantly reduced in p-GKO and pDKO mice (Figure 6B). Immunofluorescence staining also revealed the upregulation of MMP-10 in p-GKO mice (Figure 6C). Both p-GKO and p-DKO mice exhibit similarly elevated systolic blood pressure (Figure 6D) or no changes in heart or kidney weight (Supplementary Figure S4A) compared to control mice following ALDO treatment. However, urinary albumin excretion in ALDO-treated p-DKO mice was significantly reduced compared to ALDO-treated p-GKO mice (Figure 6E). Serum parameters such as aldosterone levels (Supplementary Figure S4B), creatinine and albumin (Supplementary Figure S4C), and pro-fibrotic mRNA expression (Supplementary Figure S4D) showed no significant differences between p-GKO and p-DKO mice. Histological analysis revealed mesangial expansion in ALDO-treated p-GKO mice compared to ALDO controls and MMP-10 knockout mice, with these changes ameliorated in ALDO-treated p-DKO mice (Figure 6F). WT-1 staining demonstrated loss of podocytes in ALDO-treated p-GKO mice and maintenance of podocytes in

ALDO-treated p-DKO mice (Figure 6G). ALDO-treated p-GKO mice showed thickening of GBM similar to systemic GC-A knockout mice, and these changes were significantly improved in ALDO-treated p-DKO mice (Figure 6H). These results reveal that ALDO-treated podocyte-specific GC-A knockout mice exhibited mild proteinuria relative to systemic GC-A knockout mice, without mRNA changes, and these minor injuries were also improved by MMP-10 deletion.

Overexpression of MMP-10 in Podocytes Exacerbates Inflammation; MMP-10 from Endothelial Cells Is Crucial for Podocyte Injury

Podocyte injury in MMP-10 knockout mice was ameliorated in both the ALDO model and the anti-GBM nephritis model *in vivo*, underscoring the importance of MMP-10 in nephritis progression. To further investigate this, we conducted *in vitro* studies focusing on the function of MMP-10 in glomerular cells. To assess the role of GC-A in podocytes, small interfering RNA (siRNA) targeting *Npr1* was transfected into Mouse Podocyte Clone-5 (MPC-5) cells (Figure 7A, the target sequences of siRNAs were listed in Supplementary Table S3). TNF- α stimulation upregulated *Mmp10* and *Ccl2* mRNA expression, while transfection with siGC-A effectively suppressed *Npr1* mRNA expression. Additionally, TNF- α -induced upregulation of *Mmp10* and *Ccl2* mRNA was significantly enhanced in GC-A-knockdown podocyte compared to control podocytes (Figure 7B). Next, MMP-10-expressing plasmids were transfected into MPC-5 cells to identify the effect of MMP-10 in podocytes (Figure 7C). MMP-10 expression following transfection was confirmed by immunofluorescence staining for MMP-10 (Figure 7D) and Western blot analysis for FLAG (DYKDDDDK)-tagged protein (Figure 7E). *Mmp10* mRNA expression was elevated by TNF- α stimulation, and *Il6* and *Ccl2* mRNA expression was upregulated in MMP-10-overexpressing podocytes compared to wild-type podocytes, while *Npr1* expression remained unchanged across all groups (Figure 7F). In contrast, MMP-10 knockdown via siRNA transfection into MPC-5 cells significantly suppressed the TNF- α -induced alterations in *Mmp10*, *Ccl2* and *Synpo* (synaptopodin) mRNA expression (Supplementary Figure S5A, S5B). Immunofluorescence staining in the anti-GBM nephritis model and ALDO model revealed the presence of MMP-10 in glomerular endothelial cells, prompting a focus on the role of MMP-10 in vascular endothelial cells. To explore this further, human glomerular endothelial (GE) cells

with or without MMP-10 knockdown were investigated (Supplementary Figure S5C). Although siRNA transfection significantly reduced *MMP10* mRNA expression, inflammatory changes induced by TNF- α were not changed by MMP-10 knockdown (Supplementary Figure S5C, S5D). To assess the interaction between endothelial-derived MMP-10 and podocytes, co-culture experiments were performed using mouse MPC-5 cells and human GE cells with or without mouse GC-A and human MMP-10 knockdown (Figure 7G). The mRNA expression of each cell type was examined using mouse- or human-specific probe sets. Human *MMP10* mRNA in GE cells (Figure 7H) and mouse *Npr1* mRNA in MPC-5 cells (Figure 7I) were successfully suppressed by siRNA transfection. While human *CCL2* mRNA expression remained unchanged in GE cells, *Ccl2* mRNA expression was upregulated in GC-A knockdown podocytes co-cultured with wild-type GE cells compared to wild-type podocytes with wild-type GE cells. However, this change was suppressed in GC-A knockdown podocytes co-cultured with MMP-10 knockdown GE cells. Although *Mmp10* mRNA expression in MPC-5 cells were upregulated by GC-A knockdown, co-culture with MMP-10-knockdown GE cells did not mitigate this effect. Additionally, mouse *Synpo* mRNA expression was downregulated in GC-A knockdown podocytes, and ameliorated in podocytes co-cultured with MMP-10 knockdown endothelial cells (Figure 7I). These results suggest that endothelial MMP-10 contribute to podocyte deterioration as well as MMP-10 in podocytes.

MMP-10 Knockout Mice Show Protection Against Podocyte Injury Through Nephrin Cleavage Inhibition

Given the observed attenuation of podocyte injury in MMP-10 knockout mice, we focused on the proteolytic activity of MMP-10. Western blot analysis of nephrin in anti-GBM nephropathy mice revealed nephrin loss in control mice, which was mitigated in MMP-10 knockout mice (Figure 8A). GC-A and MMP-10 knockout mice also showed less nephrin reduction compared to GC-A knockout mice (Figure 8B). Immunofluorescence staining also revealed the reduction of nephrin by anti-GBM nephritis and systemic or podocyte-specific GC-A knockout with ALDO treatment, and these changes were ameliorated by knockout of MMP-10 (Figure 8, C–E). Additionally, incubation of human nephrin with recombinant human MMP-10 resulted in nephrin fragmentation, similar to fibronectin, while adiponectin remained unaffected by MMP-10 incubation (Figure 8F). Moreover,

we transfected nephrin-expressing plasmid and MMP-10-expressing plasmid into COS-7 cells to investigate the nephrin cleavage activity *in vitro* (Figure 8G). The results revealed that overexpression of MMP-10 alone did not affect the expression of nephrin, while MMP-10 stimulated with TNF- α reduced the expression of nephrin (Figure 8H). These findings confirm the proteolytic activity of MMP-10 against nephrin and suggest that MMP-10 knockout protects nephrin integrity, contributing to the prevention of podocyte injury.

Discussion

This study provides compelling evidence for the critical role of matrix metalloproteinase-10 (MMP-10) in the progression of podocyte injury, with a specific focus on GC-A knockout mice. Our findings indicate that MMP-10 contributes significantly to podocyte injury by cleaving nephrin, a crucial component of the glomerular filtration barrier. Moreover, the deletion of MMP-10 in both systemic and podocyte-specific GC-A knockout models alleviated albuminuria and glomerular damage, underscoring its potential as a therapeutic target in CKD. The detrimental effects of MMP-10 on podocytes and the protective effects of its inhibition have garnered attention recently [16]. Microarray analysis identified MMP-10 as a candidate gene implicated in podocyte injury among several mRNAs elevated in the glomeruli from GC-A knockout mice treated with aldosterone and high salt diet. MMP-10, also known as stromelysin-2, is a member of the matrix metalloproteinase family containing a zinc ion in its catalytic domain [17]. Matrix metalloproteinases are well-known for their role in degrading extracellular substrates and activating bioactive molecules in various diseases [18], and MMP-10 has also been reported to be involved in wound healing [19, 20], malignant tumor invasion [21], and atherosclerosis [22]. In our study, MMP-10 was found to be strongly expressed in the glomeruli from various glomerulonephritis patients, which is consistent with earlier reports [16]. It was also shown that MMP-10 positive cells were more abundant in patients with lupus nephritis and ANCA-associated vasculitis than in patients with focal segmental glomerulosclerosis (FSGS) and membranous nephropathy. Since this is the first report to demonstrate that glomerular MMP-10 expression varies according to the primary disease, the number of patients included in this study is limited and further studies are needed to elucidate the details. Transcriptomic analysis using Nephroseq database also revealed correlations between glomerular *MMP10* expression and renal function in patients with glomerulonephritis. In the field of nephrology, it has also reported that circulating MMP-10 is correlated with progression of diabetic kidney disease [23], and that MMP-10 knockout mice attenuate podocyte injury induced by Adriamycin nephropathy [16]. MMP-10 has been implicated in a variety of diseases and these findings suggested MMP-10 was a common factor in podocyte disorders.

Our findings extend the understanding of MMP-10's role in glomerular injury. MMP-10 ablation protected against anti-GBM nephritis by suppressing pro-inflammatory and pro-fibrotic mRNA changes and

reducing the infiltration of MAC-2-positive cells. Given that macrophages are key players in glomerular crescent formation [24, 25], reducing their infiltration is a critical mechanism by which MMP-10 deletion improves glomerulonephritis. Our group has previously demonstrated the significance of MMP-10 in macrophage-mediated peritoneal inflammation and fibrosis [26]. This study supports the hypothesis that MMP-10 influences macrophage behavior, contributing to its effects on glomerular inflammation.

It was also demonstrated that both systemic and podocyte-specific GC-A knockout mice exhibit increased albuminuria, which is mitigated by MMP-10 knockout. GC-A, also known as natriuretic peptide receptor 1 (NPR1), expressed throughout the nephron [11] and regulates natriuresis by inhibition of salt reabsorption in proximal tubules, loop of Henle, and collecting ducts [27, 28]. However, prior research on podocyte-specific GC-A knockout mice revealed that the renoprotective effects of natriuretic peptides are directly mediated via GC-A in podocytes without influence of hemodynamic or neurohumoral responses to NP/GC-A signaling [29]. While the precise mechanisms of GC-A mediated protection against podocyte injury remain unclear, several studies have implicated downstream pathways such as transient receptor potential canonicals 6 (TRPC6) [29] or p38 mitogen-activated protein kinase (MAPK) [12, 30]. Inhibition of TRPC6 or p38 MAPK has been shown to improve podocyte damage in podocyte-specific GC-A knockout mice, suggesting that NP/GC-A signaling negatively regulates these pathways, thereby exerting protective effects. The gain-of-function mutation in TRPC6 was reported as a cause of FSGS [31] and the cGMP pathway negatively regulated TRPC6 [32], supporting the hypothesis that TRPC6 upregulation in podocyte-specific GC-A knockout mice exacerbates injury. Additionally, cGMP-dependent protein kinase type I (PKG1) inhibits TGF- β activated kinase 1 (TAB1)-p38 MAPK in cardiac myocytes [33] and phosphorylated p38 MAPK was enhanced in ALDO-treated GC-A knockout mice as we have previously reported [11]. Thus, p38 MAPK was thought to be also regulated negatively by cGMP.

This study also sheds light on the role of MMP-10 in glomerular cell interactions. Immunofluorescence studies demonstrated that MMP-10 is expressed in both podocytes and endothelial cells. The interaction between endothelial cells and podocytes is a burgeoning area of research, with vascular endothelial growth factor (VEGF), angiopoietins [34] and endothelin-1 [35] identified as potential mediators. Previous studies have

shown that MMP-10 is secreted by endothelial cells in response to VEGF [36] and thrombin [37] stimulation, although its role in glomerular injury was not fully elucidated. Our findings reveal MMP-10 from endothelial cells aggravates podocyte inflammation similar to MMP-10 overexpression in podocytes themselves, even though endothelial MMP-10 knockdown showed no effect against inflammatory mRNA change in endothelial cells. Previous reports from our group suggested the importance of podocyte-endothelial interaction in podocyte-specific p38 MAPK and GC-A knockout mice via plasminogen activator inhibitor-1-induced thrombotic pathway [30], further underscoring the significance of crosstalk between these cells. Podocyte-specific GC-A knockout mice showed upregulated albuminuria and slight changes in renal function and glomerular mRNA changes compared to control mice consistent with our previous report. [12] The reasons for the less pronounced elevation in pro-inflammatory mRNA or proteinuria in podocyte-specific GC-A knockout mice remains unclear, but the apparent increase in heart weight in systemic GC-A knockout mice suggests that heart failure may have exacerbated renal damage akin to cardiorenal syndrome like mechanism.

The protective effect of MMP-10 in podocytes has been noted previously, we revealed several novel findings about MMP-10. This study demonstrated that protective effect of GC-A knockdown is due to the suppression of MMP-10, and that MMP-10 is expressed in glomerular endothelial cells and its suppression ameliorates podocyte injury. MMP-10 is also expressed in the glomeruli of patients with glomerulonephritis, and its expression may differ depending on the types of glomerulonephritis. Moreover, this study also clarifies that MMP-10 directly cleaves nephrin, a finding that has important implications for understanding podocyte injury mechanisms. Fibronectin, gelatin and casein were already known as major MMP-10 substrates, [38] this is the first report about degradation of nephrin by MMP-10. Although MMP-10 has been previously reported to degrade zonula occludens-1 (ZO-1) but not nephrin or podocin [39], our data show that MMP-10 activated by APMA can degrade nephrin *in vitro*. Nephrin is crucial for maintaining the slit diaphragm's structure and function, and its loss leads to disrupted cell-cell adhesion and severe proteinuria [40]. While MMP-7 is already known to degrade nephrin [41], our study identifies MMP-10 as another protease capable of nephrin cleavage, reinforcing its role in podocyte injury, particularly in GC-A knockout mice subjected to aldosterone-induced injury or severe glomerulonephritis like anti-GBM nephritis.

This investigation has some limitations. The number of patients with glomerulonephritis included in this examination was limited, and the precise mechanisms underlying NP/GC-A/MMP-10 axis remain to be fully elucidated. Additionally, ALDO procedures affect the entire body, and potential harmful effects on other organs, such as heart or blood vessels, cannot be ruled out. ALDO treatment contains uninephrectomy, and it is atypical in the clinical setting. However, aldosterone-treated podocyte-specific GC-A knockout mice without unilateral nephrectomy excrete less albumin than ALDO-treated those mice [30], but have increased urinary albumin excretion, suggesting that uninephrectomy is considered an additional factor in podocyte injury. Although systemic MMP-10 knockout mice provided valuable insights, it was not possible to determine which cellular MMP-10 is most important in regulating podocyte integrity. Cell-specific MMP-10 knockout mice were not available and should be a focus of future research. In conclusion, the deletion of MMP-10 offers significant protection against podocyte injury and helps maintain nephrin integrity in GC-A knockout mice subjected to aldosterone and high-salt diet-induced stress, as well as in cases of anti-GBM nephritis. Our findings reveal that MMP-10 is expressed in both podocytes and endothelial cells within the glomeruli, where it contributes to podocyte injury through nephrin cleavage. These results suggest that MMP-10 plays a crucial role in the progression of glomerulonephritis and identifies it as a potential therapeutic target for podocytopathy.

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Author contributions

T. Ishimura, K. Osaki, H. Yokoi conceived and designed the study. T. Ishimura and K. Osaki conducted experiments. T. Ishimura, K. Osaki, S. Sugioka, A. Ishii, H. Yamada, N. Toda, S. Ohno, Y. Kato, T. Matsusaka and T. Tokudome interpreted the results and contributed to discussion. T. Ishimura, K. Osaki and H. Yokoi wrote the manuscript. H. Yokoi and M. Yanagita supervised this study. T. Ishimura and H. Yokoi are the guarantors of this work and had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors approved the final version of the manuscript.

Data Availability Statement

The datasets analyzed in this study are available from the Gene Expression Omnibus repository under the following accession numbers: GSE276626.

Conflict of Interest Statement

H. Yokoi has received Speakers Bureau from AstraZeneca, and research grants from Chugai Pharmaceutical, Otsuka Pharmaceutical, and Kyowa Kirin Co. Ltd. M. Yanagita has received research grants from Mitsubishi Tanabe Pharmaceutical Corporation and Boehringer Ingelheim. All the other authors declared no competing interests.

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Figure legend

Figure 1 | MMP-10 mRNA expression in human and mouse kidney revealed by microarray analysis. (A)

The protocol of ALDO model in control and guanylyl cyclase-A (GC-A) knockout mice for microarray analysis.

(B) The scatterplot with differences in glomerular mRNA expression between control and GC-A knockout mice.

Red dots represent upregulated genes with 2-fold or more in GC-A knockout mice and blue dots represent

downregulated genes with 0.5-fold or less in GC-A knockout mice compared to control mice. (C) The top 10

elevated genes in glomerular mRNA expression levels from GC-A knockout mice compared to control mice.

(D) Expression of MMP-10 mRNA in GC-A knockout and control mice by RT-qPCR. (n = 4, each) (E)

Immunohistochemical staining for MMP-10 in various glomerulonephritis patients' glomeruli. Patients' data

were shown in Supplementary Table S1. (F) The number of glomerular MMP-10 positive cells in patients with

various diseases. MGA (n = 3), IgAN (n = 13), MN (n = 3), FSGS (n = 3), DMN (n = 3), LN (n = 3), and AAV

(n = 5). (G) Renal expression of MMP-10 in healthy control and chronic kidney disease patients examined by

microarray analysis. The box shows 25-75 percentiles, and the vertical extending lines indicates adjacent values

(the most extreme values within 1.5 interquartile range of the 25 and 75 percentile of each group). Middle lines

indicate median values. (H) Correlation between glomerular expression of MMP-10 and serum creatinine in

vasculitis patients examined by microarray analysis. R^2 ; coefficient of determination, Scale bars represent 100

μm . Ctrl; control mice (wild-type littermate), GKO; GC-A knockout mice, MGA; minor glomerular abnormality,

IgAN; IgA nephropathy, MN; membranous nephropathy, FSGS; focal segmental glomerular sclerosis, DMN;

diabetic nephropathy, LN; lupus nephritis, AAV; ANCA associated vasculitis. Data in Figure 1G and 1H were

obtained from Nephroseq Database. Values are expressed as means $\pm$ SD. * $P < 0.05$, ** $P < 0.01$. \$; $P < 0.05$ vs

MGA, #; $P < 0.05$ vs MGA, FSGS, ##; $P < 0.05$ vs all other groups except for DMN, ###; $P < 0.05$ vs all other

groups.

Figure 2 | Anti-glomerular basement membrane (GBM) nephritis in MMP-10 knockout mice. (A) The

protocol of anti-GBM nephritis model in control and MMP-10 knockout mice. (B) Glomerular mRNA

expression of *Mmp10* in control mice treated with vehicle or anti-GBM antibody. (C) Western blot analysis for

MMP-10 in control mice treated with vehicle or anti-GBM antibody. (n = 4, each) (D) Immunohistochemical staining of MMP-10 in each group. Scale bars represent 50 μ m. (E) Representative images of periodic acid-Schiff staining of the glomeruli in each group. The graph shows ratio of glomeruli with crescent formation. Scale bars represent 50 μ m. (F) Representative images of electron microscopy of the glomeruli in each group. The graph shows GBM thickness (n = 4, each). Scale bars represent 1 μ m. (G) Time course of urinary protein excretion and serum albumin and total cholesterol. (H) Serum creatinine (Cr), blood urea nitrogen (BUN), body weight (BW) at sacrifice, and the ratio of total kidney weight (KW) and body weight. Ctrl/vehicle, n = 7; MKO/vehicle, n = 7; Ctrl/GBM, n = 8; MKO/vehicle, n = 7. Values are expressed as means $\pm$ SD. n.s.; not significant, $\dagger P < 0.05$ vs other groups, $*P < 0.05$, $**P < 0.01$.

Figure 3 | Inflammation and podocyte damage in MMP-10 knockout mice with anti-GBM nephritis. (A-C) Glomerular mRNA expression of pro-fibrotic (A), pro-inflammatory (B), other MMP (C) genes in control and MMP-10 knockout mice treated with vehicle or anti-GBM antibody. *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) was used as control. (D) Double immunofluorescent staining of nephrin and MMP-10 in control and MMP-10 knockout mice glomeruli treated with vehicle or anti-GBM antibody. (E) Double immunofluorescent staining of CD31 and MMP-10 in control and MMP-10 knockout mice glomeruli treated with vehicle or anti-GBM antibody. Square areas are enlarged and shown in high power field (HPF) images. White arrows indicate co-localized regions. Ctrl/vehicle, n = 7; MKO/vehicle, n = 7; Ctrl/GBM, n = 8; MKO/vehicle, n = 7. Scale bars represent 50 μ m. Values are expressed as means $\pm$ SD. $*P < 0.05$, $**P < 0.01$.

Figure 4 | The amelioration effect against aldosterone and high salt diet in GC-A/MMP-10 double knockout mice. (A) The protocol of ALDO model in control, MMP-10 knockout, GC-A knockout, and GC-A/MMP-10 double knockout mice. (B, C) mRNA expression of *Mmp10* and *Npr1* in glomeruli. (D) Western blot analysis for MMP-10 in control mice and GKO mice. (n = 4, each) (E) Representative images of immunofluorescence staining for MMP-10 in each group. Scale bars represent 50 μ m. (F) Systolic blood pressure (SBP) change in each group. (G) Time course of urinary protein excretion (UP) and albumin creatinine

ration (ACR). (H) Representative images of periodic acid-Schiff staining of the glomeruli in each group. The graph shows ratio of mesangial matrix fraction and total glomerular area. Scale bars represent 50 μm . (I) Representative images of immunohistochemical staining of WT-1 in each group (n = 5, each). The graph shows number of WT-1 positive cells in a glomerulus. Scale bars represent 50 μm . (J) Representative images of electron microscopy of the glomeruli in each group. The graph shows GBM thickness (n = 4, each). Ctrl, n = 7; MKO, n = 6; GKO, n = 5; DKO, n = 5. Scale bars represent 1 μm . Values are expressed as means $\pm$ SD. ND; not detected, n.s.; not significant, $\dagger P < 0.05$ vs other groups, $\#P < 0.05$ vs Ctrl group, $*P < 0.05$, $**P < 0.01$.

Figure 5 | The mRNA changes and localization of MMP-10 in GC-A knockout mice with or without MMP-10 deletion. (A) Glomerular mRNA expression of *Ctgf*, *Mmp2*, *Fnl1*, and *Tgfb1*. (B) Double immunofluorescence staining of nephrin and MMP-10 in glomeruli. (C) Double immunofluorescence staining of CD31 and MMP-10 in glomeruli. Square areas are enlarged and shown in high power field (HPF) images. White arrows indicate co-localized regions. Ctrl; control mice, MKO; MMP-10 knockout mice, GKO; Systemic GC-A knockout mice, DKO; Systemic GC-A and MMP-10 knockout mice. Ctrl, n = 7; MKO, n = 6; GKO, n = 5; DKO, n = 5. Values are expressed as means $\pm$ SD. Scale bars represent 50 μm . $*P < 0.05$, $**P < 0.01$.

Figure 6 | The protective effect against aldosterone and high salt diet in podocyte-specific GC-A and systemic MMP-10 double knockout mice. (A) The protocol of ALDO model in control, MMP-10 knockout, podocyte-specific GC-A knockout, and podocyte-specific GC-A/systemic MMP-10 double knockout mice. (B) mRNA expression of *Mmp10* and *Npr1* in glomeruli. (C) Representative images of immunofluorescence staining for MMP-10 in each group. Scale bars represent 50 μm . (D) Systolic blood pressure (SBP) change in each group. (E) Time course of albumin creatinine ratio (ACR) in each group. (F) Representative images of periodic acid-Schiff staining of the glomeruli in each group. The graph shows ratio of mesangial matrix fraction and total glomerular area. Scale bars represent 50 μm (n = 7, each). (G) Representative images of immunohistochemical staining of WT-1 in each group (n = 5, each). The graph shows number of WT-1 positive cells in a glomerulus. Scale bars represent 50 μm . (H) Representative images of electron microscopy of the

glomeruli in each group. The graph shows GBM thickness (n = 4, each). Scale bars represent 1 μ m. Values are expressed as means $\pm$ SD. ND; not detected, n.s.; not significant, †P < 0.05 vs other groups, #P < 0.05 vs Ctrl group, *P < 0.05, **P < 0.01.

Figure 7 | Aggravation of inflammatory changes in GC-A knockdown or MMP-10 overexpressing podocytes, and amelioration in podocytes co-cultured with MMP-10 knockdown endothelial cells. (A) The cell culture protocol of GC-A knockdown mouse podocytes (MPC-5 cells). (B) mRNA expression of *Npr1*, *Mmp10* and *Ccl2* in MPC-5 cells with or without GC-A knockdown. (C) The cell culture protocol of mouse MMP-10 expressing MPC-5 cells. (D) Representative images of MMP-10-overexpressing cells. Red color indicates MMP-10, and blue color indicates DAPI. (E) Western blot analyses of MPC-5 cells for FLAG and β -actin with or without MMP-10 overexpression. (F) mRNA expression of *Npr1*, *Mmp10*, *Ccl2* and *Il6* in MPC-5 cells with or without MMP-10. Only *Il6* mRNA expression is at 2 hours after TNF- α stimulation. (G) The co-culture protocol of MPC-5 cells and glomerular endothelial cells (GE cells). (H) mRNA expression of human *MMP10* and *CCL2* in GE cells. (I) mRNA expression of *Npr1*, *Ccl2*, *Mmp10* and *Synpo* in MPC-5 cells. Scale bars represent 50 μ m. Values are expressed as means $\pm$ SD. n.s.; not significant, *P < 0.05, **P < 0.01.

Figure 8 | Amelioration of nephrin loss in MMP-10 knockout mice and direct cleavage of nephrin by MMP-10. (A) Western blot analysis for nephrin and β -actin in control and MMP-10 knockout mice with or without anti-glomerular basement membrane nephropathy. (B) Western blot analysis for nephrin and β -actin in control, MMP-10 knockout, GC-A knockout, and GC-A/MMP-10 double knockout mice treated with ALDO treatment. (C) Immunofluorescence staining for nephrin in control and MMP-10 knockout mice with or without anti-glomerular basement membrane nephropathy. (D) Immunofluorescence staining for nephrin in control, MMP-10 knockout, GC-A knockout, and GC-A/MMP-10 double knockout mice treated with ALDO treatment. (E) Immunofluorescence staining for nephrin in control, MMP-10 knockout, podocyte-specific GC-A knockout, and podocyte-specific GC-A/systemic MMP-10 double knockout mice treated with ALDO treatment. (F) Representative silver staining images of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Activated

MMP-10 cleaved nephrin and fibronectin, but did not cleave adiponectin. (G) The cell culture protocol of nephrin and MMP-10 overexpressing COS-7 cells. OE; overexpression. (H) Western blot analysis for FLAG and β -actin in COS-7 cells. Scale bars represent 50 μ m. Values are expressed as means $\pm$ SD. *P < 0.05, **P < 0.01.

Supplementary Material

Supplementary File (PDF)

Supplementary Methods

Supplementary Reference

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Supplementary Figure S2. Body weight change, IgG deposition, and macrophage infiltration into glomeruli in wild-type and MMP-10 knockout mice with anti-GBM nephropathy model. (a) Body weight changes in wild-type and MMP-10 knockout mice with or without anti-GBM nephropathy. (b) Immunofluorescence staining for rabbit and mouse IgG. (c) Representative images of immunohistochemical staining for MAC-2 in the glomeruli from each group. The graph shows the number of MAC-2-positive cells in glomeruli. Ctrl; control mice, MKO; MMP-10 knockout mice. Values are expressed as means $\pm$ SD. Scale bars represent 50 μ m. n.s.; not significant, **P < 0.01.

Supplementary Figure S3. The organ weight and serum parameters in systemic GC-A knockout mice with or without MMP-10 knockout. (a) Body, heart and kidney weight. (b) Serum aldosterone levels. (c) Serum creatinine, urea nitrogen, total cholesterol and albumin. Ctrl; control mice, MKO; MMP-10 knockout mice, GKO; systemic GC-A knockout mice, DKO; MMP-10 and GC-A knockout mice. Values are expressed as means $\pm$ SD. n.s.; not significant, *P < 0.05, **P < 0.01.

Supplementary Figure S4. The organ weight, serum parameters and mRNA expression in podocyte-

specific GC-A knockout mice with or without MMP-10 knockout. (a) Body, heart and kidney weight. (b) Serum aldosterone levels. (c) Serum creatinine, urea nitrogen, total cholesterol and albumin. (d) Glomerular mRNA expression of *Ctgf*, *Mmp2*, *Fnl*, and *Tgfb1*. Ctrl; control mice, MKO; MMP-10 knockout mice, pGKO; podocyte-specific GC-A knockout mice, pDKO; podocyte-specific GC-A knockout and systemic MMP-10 knockout mice. Values are expressed as means $\pm$ SD. n.s.; not significant.

Supplementary Figure S5. The effect of MMP-10 knockdown in mouse podocyte clone-5 (MPC-5) cells and glomerular endothelial (GE) cells. (A) The protocol for MMP-10 knockdown in MPC-5 cells. (B) mRNA expression of *Mmp10*, *Ccl2*, and *Synpo*. (C) The protocol for MMP-10 knockdown in GE cells. (D) mRNA expression of *MMP10*, *CCL2*, and *IL1B*. Ctrl; control, KD; MMP-10 knockdown. Values are expressed as means $\pm$ SD. n.s.; not significant, * $P < 0.05$, ** $P < 0.01$.

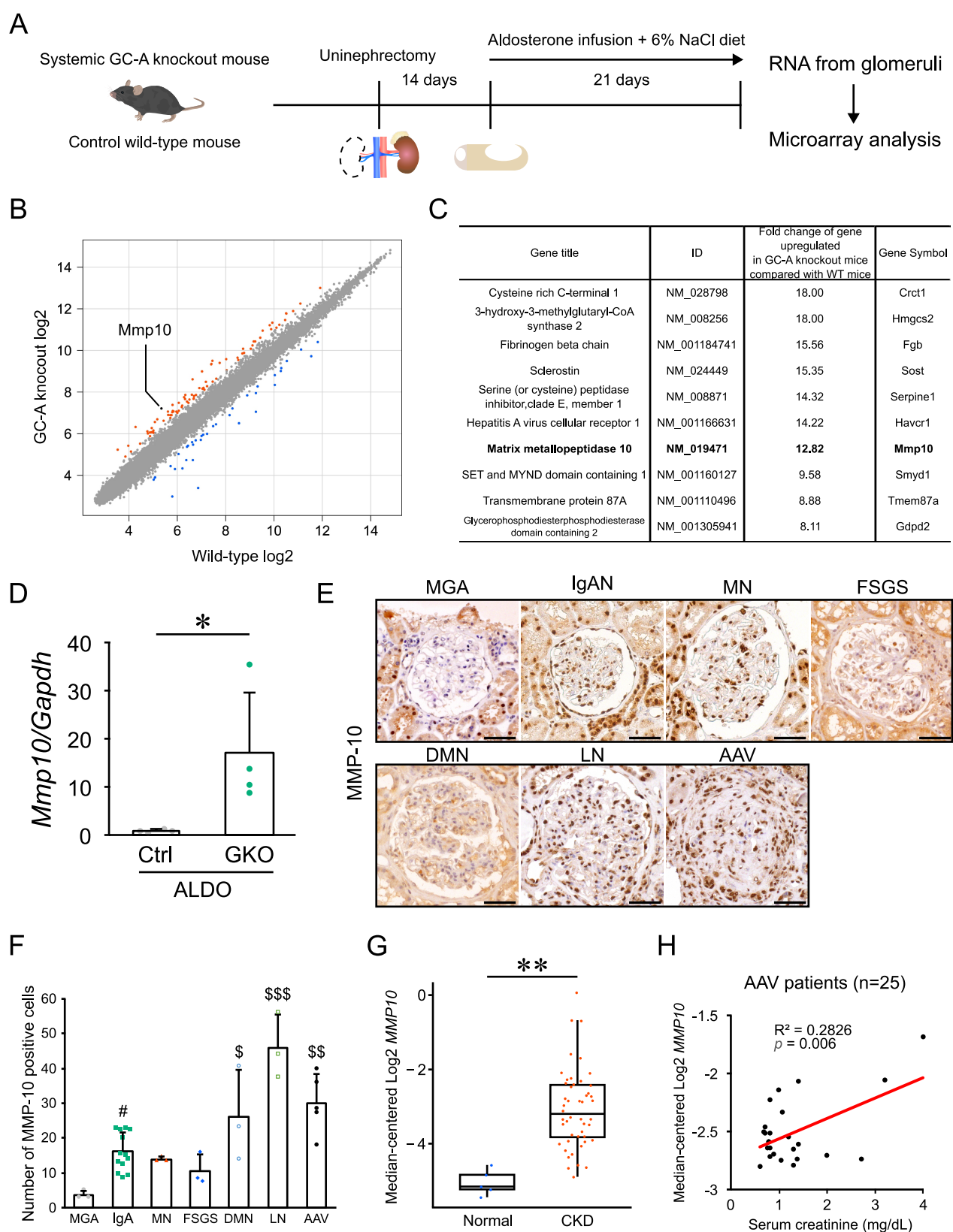


Figure 1. T. Ishimura et al.

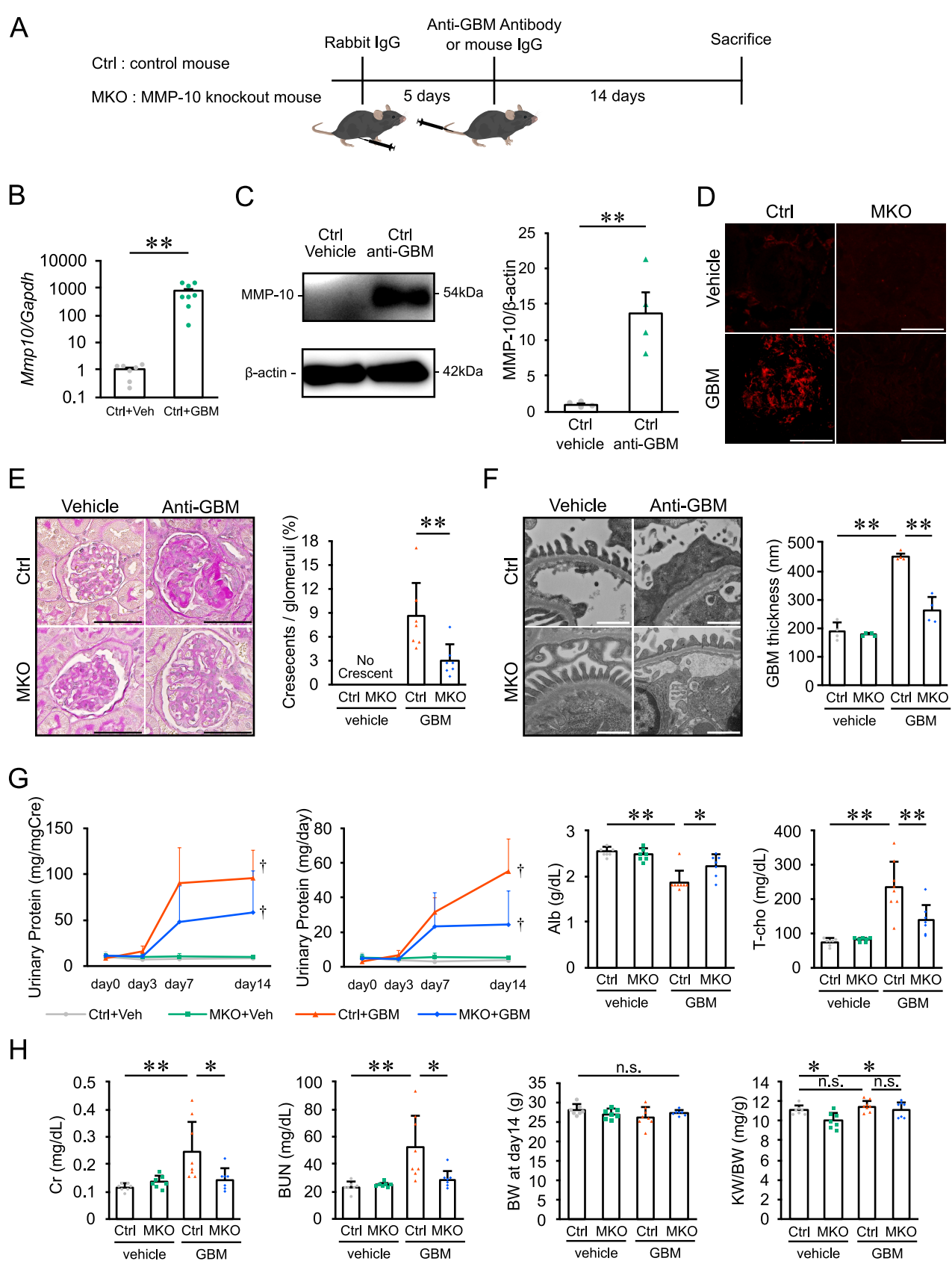


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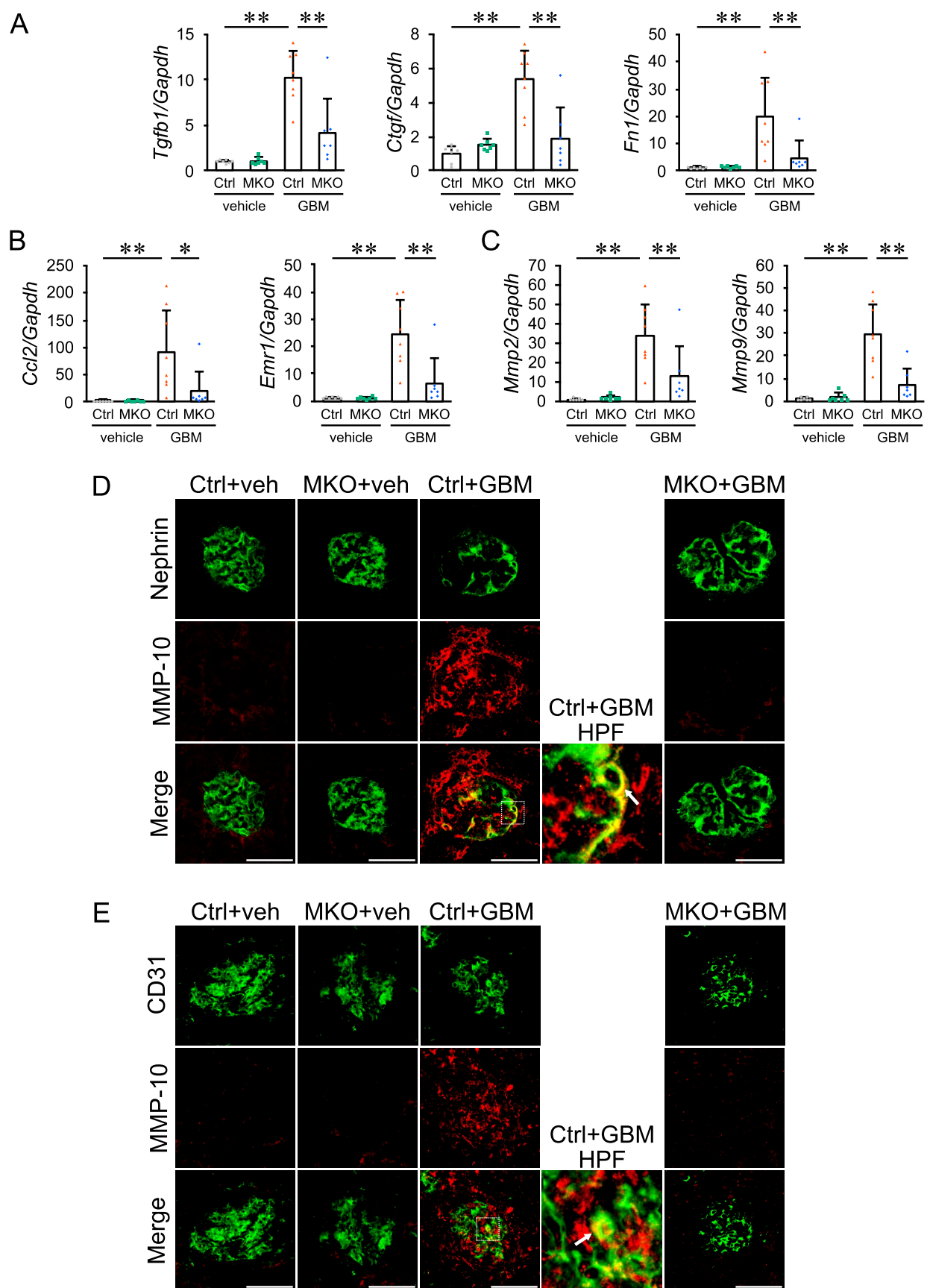


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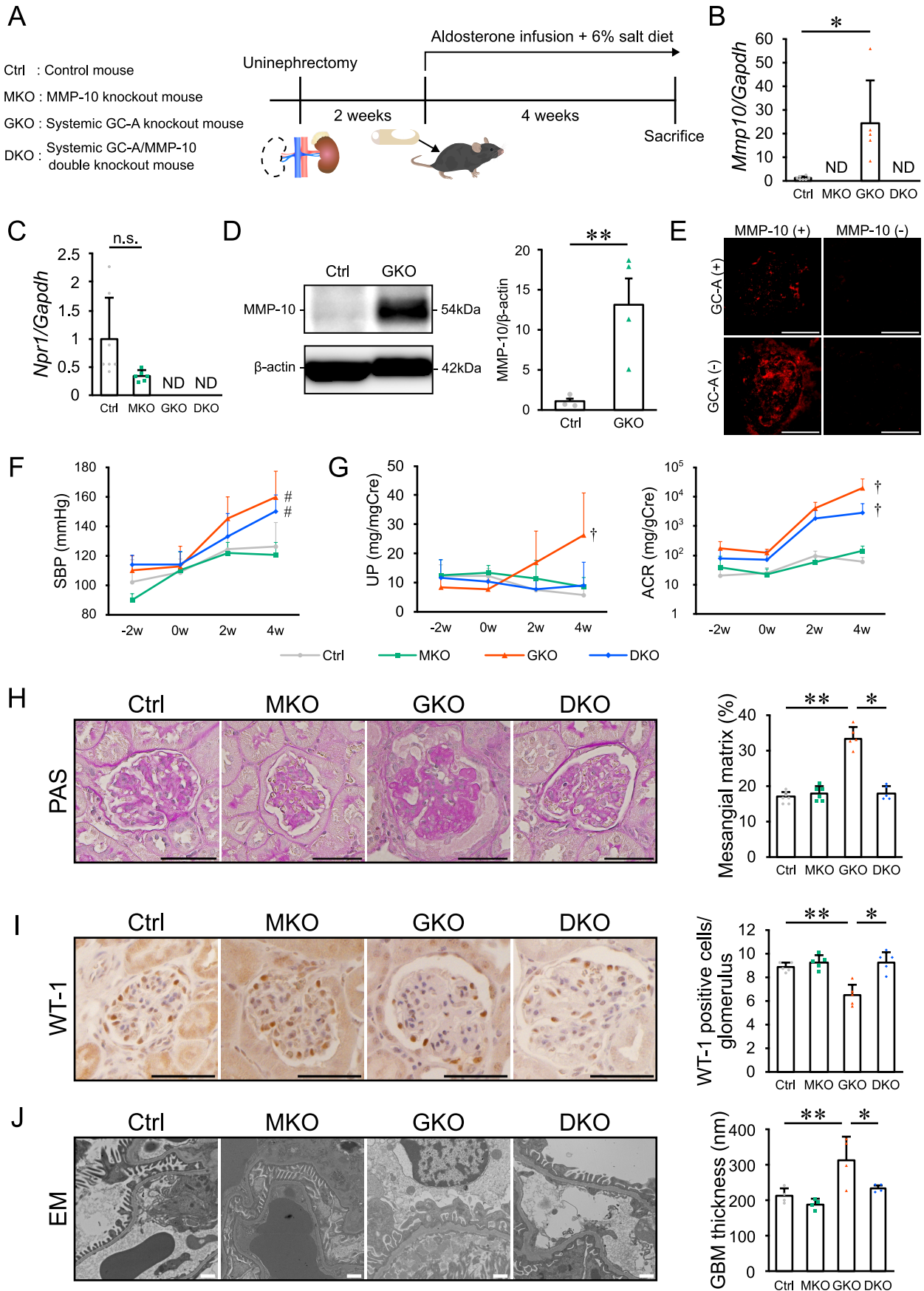


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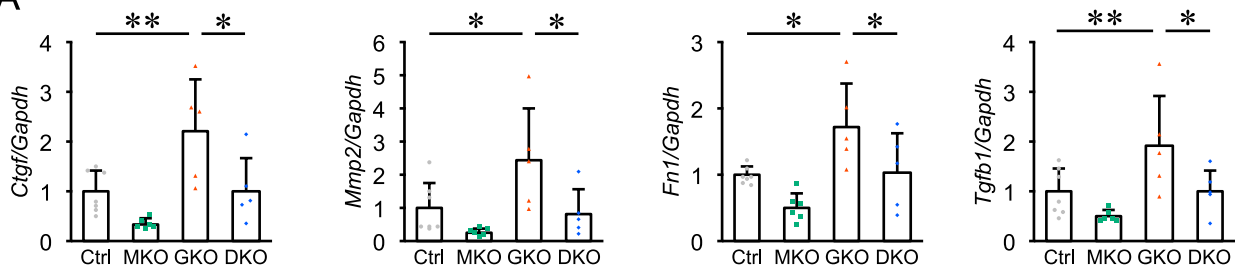
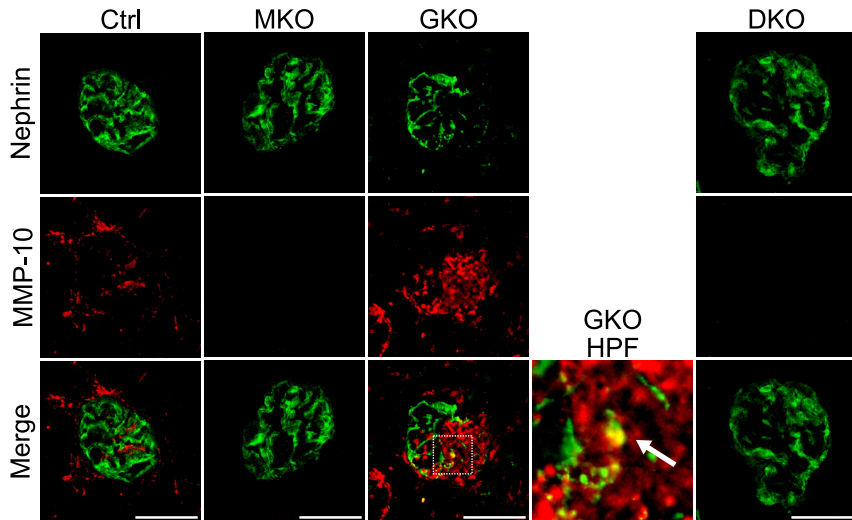
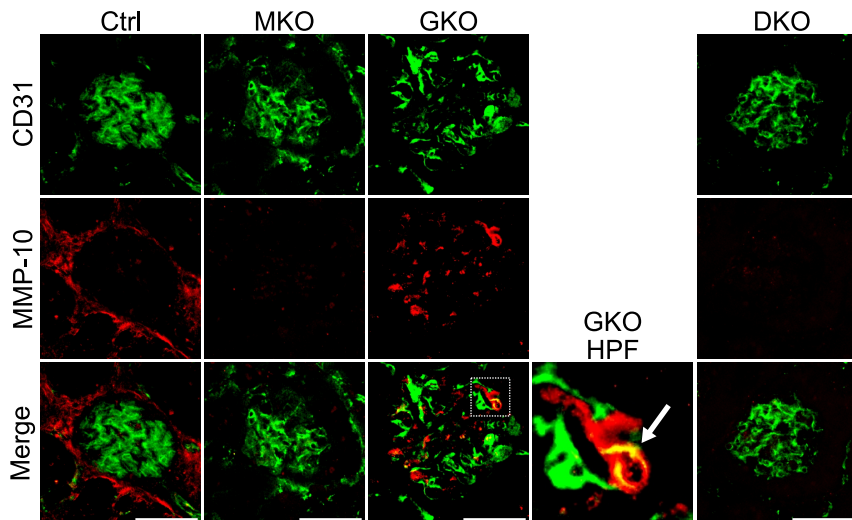
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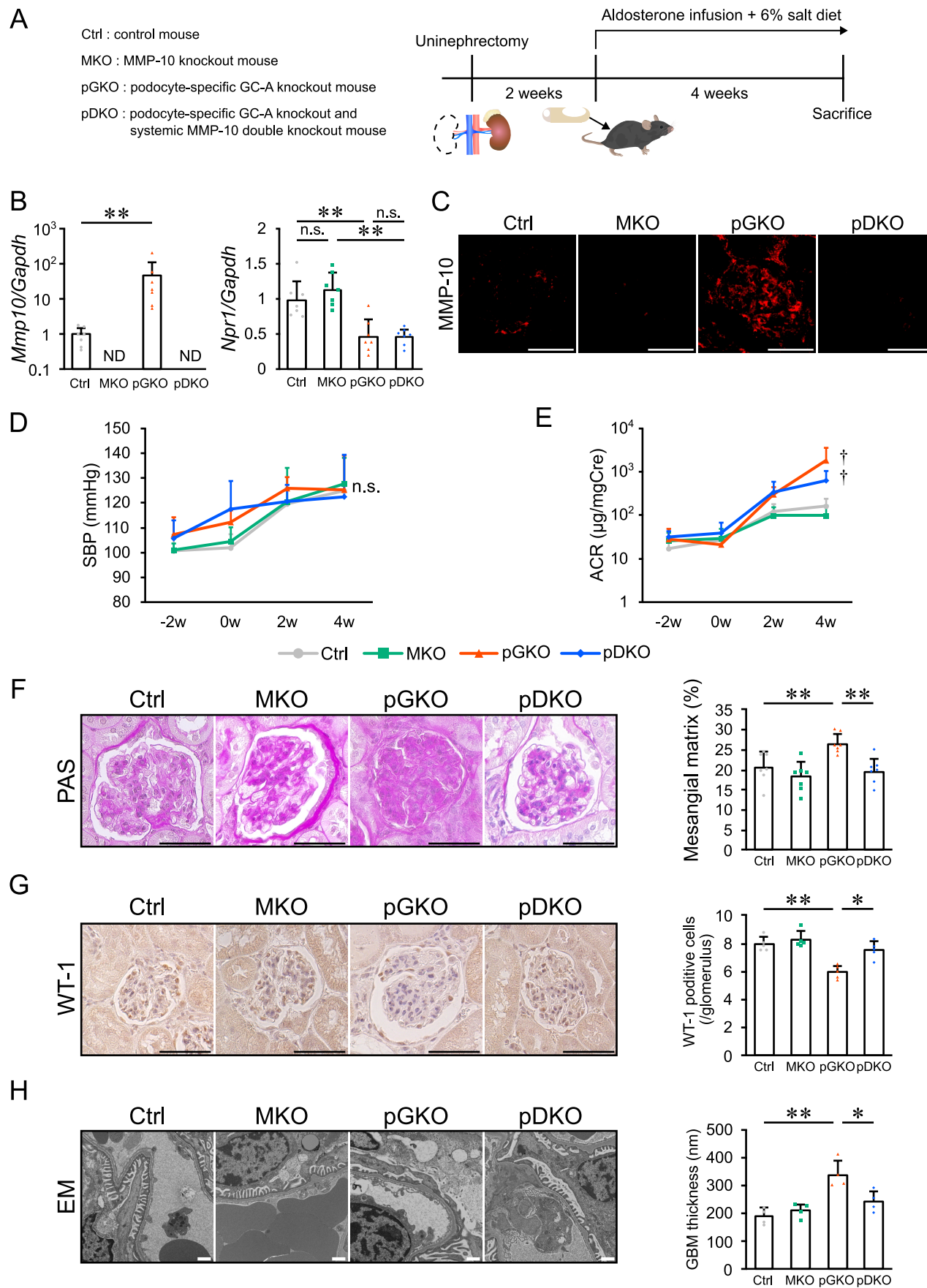


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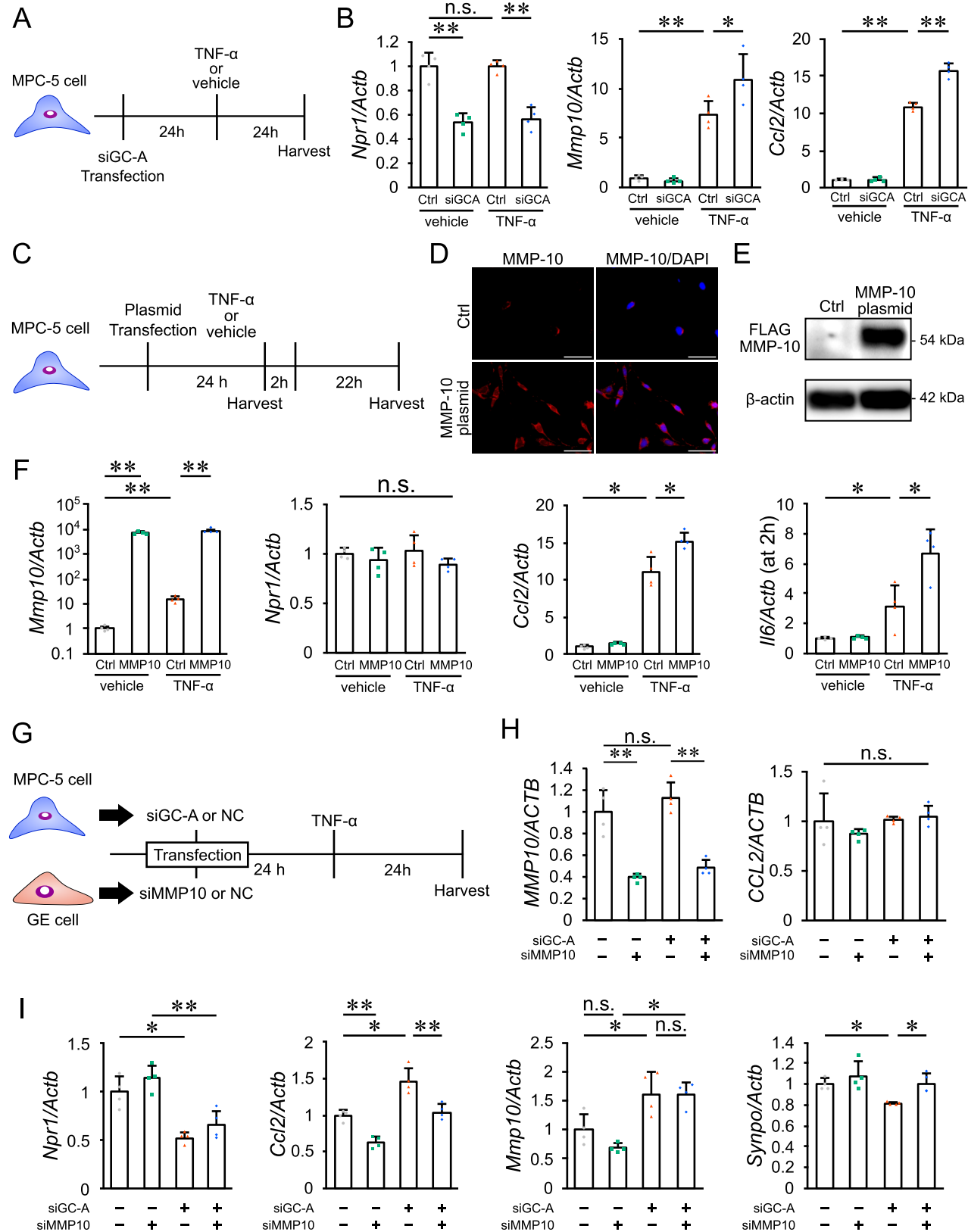


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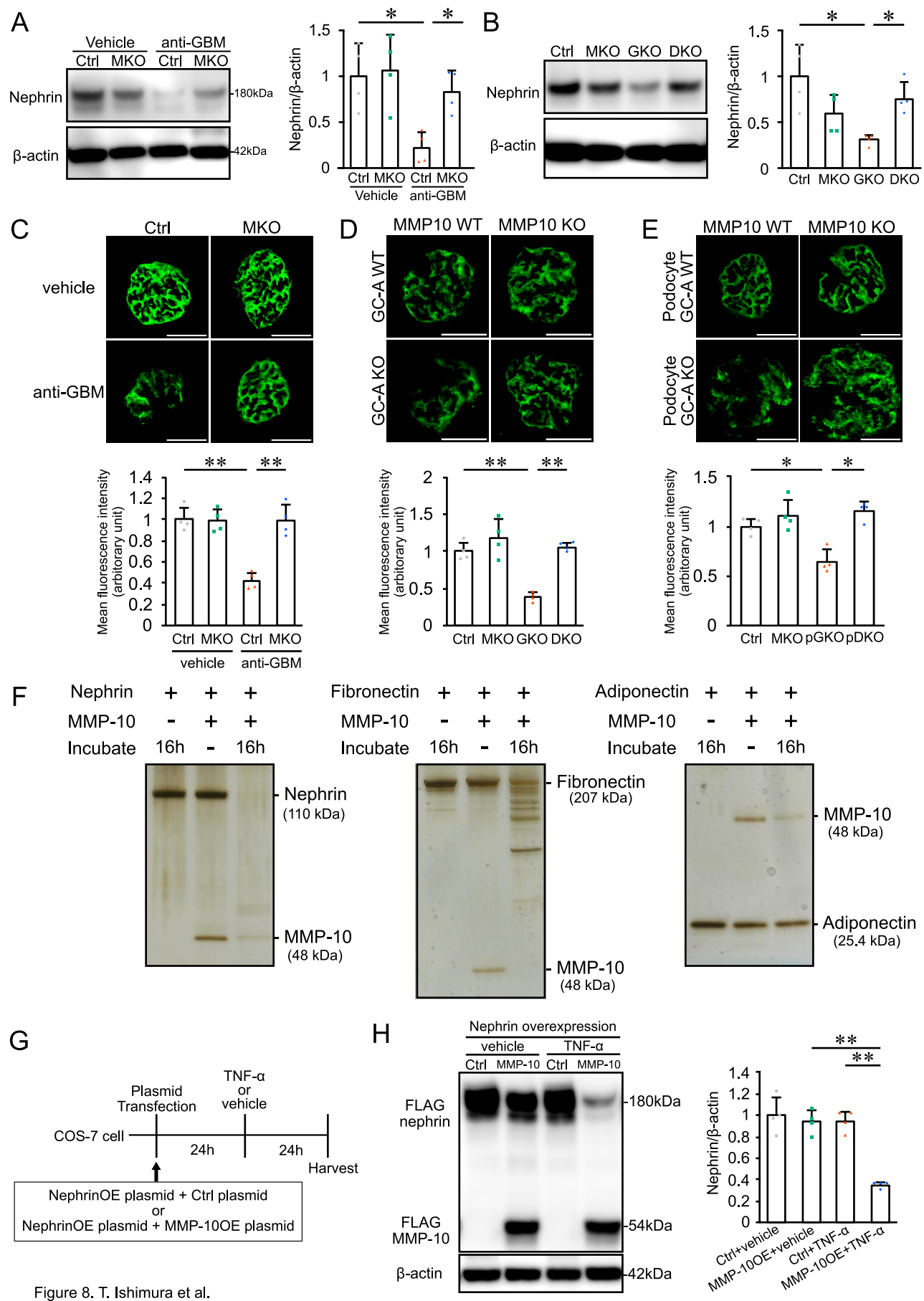


Figure 8. T. Ishimura et al.

Supplementary Material

Title

Matrix metalloproteinase (MMP) -10 aggravates podocyte injury in glomerulonephritis

Authors

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Supplementary Methods

Reagents and Antibodies

Primary antibodies used for immunohistochemical and immunofluorescence studies were anti-MMP-10 antibody (MAB910, R&D systems, Minneapolis, MN), anti-MAC-2 (CL8942F, Cedarlane, Ontario, Canada), anti-Wilms tumor 1 (WT1) antibody (sc-15421, Santa Cruz Biotechnology, Dallas, TX), anti-CD31 antibody (550274, BD Bioscience, Franklin Lakes, NJ), anti-nephrin antibody (AF3159, R&D Systems). Primary antibodies used for Western blot analysis were anti-DYKDDDDK (FLAG) tag antibody (014-22383, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and anti- β -actin antibody (A5441, Sigma-Aldrich, St Louis, MI). Antibodies were diluted 1:1000 for Western blotting and 1:100 for immunohistochemical and immunofluorescence staining. FLAG-tagged mouse MMP-10 expression vector was purchased from Origene Technologies (Rockville, MD). The primer set of *Gapdh* was purchased from ThermoFisher Scientific (Waltham, MA).

Transcriptomic Analysis Using Nephroseq Database

Data from the Nephroseq database (www.nephroseq.org, University of Michigan, Ann Arbor, MI, USA) were utilized to compare *MMP10* mRNA expression with clinical parameters. Expression data were derived from the “Nakagawa CKD Kidney” [1] dataset, which includes five healthy (normal) and 48 CKD human kidney biopsy samples, and the “Ju CKD Glom” [2] dataset, which comprises 23 micro-dissected glomerular samples from patients with vasculitis. The “Nakagawa CKD Kidney” samples were analyzed with the Agilent Whole Human Genome Microarray 4x44K and samples in “Ju CKD Glom” were analyzed with Affymetrix human genome U133A 2.0 microarray.

Animal Experiments

The study protocol was approved by the Kyoto University Animal Experiment Ethics Review Committee (Approval No. MedKyo23154). MMP-10 knockout mice (B6;129S5-Mmp10tm1Lex/Mmucd) were purchased from the Mutant Mouse Resource and Research Center (Davis, CA) and GC-A knockout mice were generated on 129/SVJ background as previously described [3]. Podocyte-specific GC-A knockout mice were generated by crossing Nephhrin-Cre mice with *Npr1* flox/flox mice [4]. MMP-10 knockout mice and GC-A knockout mice were backcrossed with C57BL/6J mice more than eight times and congenic. Male C57BL/6J mice were purchased from Clea Japan (Tokyo, Japan). Wild-type male littermates were used as control mice for MMP-10 knockout mice and GC-A knockout mice, and male *Npr1* flox/flox mice without Nephhrin-Cre were used as controls for podocyte-specific GC-A knockout mice. All mice were euthanized under deep sedation, and kidneys and hearts were collected for mRNA and protein analyses, as well as histological examination. All mice were maintained in a specific pathogen free room with a controlled light-dark cycle (12/12 hours) and temperature (20-22°C) at the Institute of Laboratory Animals of Graduate School of Medicine, Kyoto University. All mice used in this study were male littermates, and food and water were provided ad libitum. All animal experiments were performed in accordance with the Guidelines on the Use of Laboratory Animals of the National Institutes of Health.

Histology and Immunohistochemistry

Renal sections (1 μm thick) were obtained from paraffin-embedded tissue blocks and stained with Periodic Acid-Schiff as previously described [5]. The stained samples were examined by light microscopy (IX81, Evident, Tokyo, Japan), and electron microscopic examination was performed using an electron microscope (H-7600, Hitachi, Tokyo, Japan). Mesangial matrix areas in 5 glomeruli were evaluated using Metamorph Microscopy Automation & Image Analysis Software (Molecular Devices, San Jose, CA) and averaged. For immunohistochemical analysis of mouse WT-1 and MAC-2, sections were processed as previously described [6, 7]. Briefly, the sections were deparaffinized, followed by antigen retrieval by microwave heating or trypsinization, and then incubated with each primary antibody. The number of positive cells in 10 random glomeruli was measured and averaged for murine WT-1 and MAC-2, and the number of positive cells in all glomeruli was measured and averaged for human MMP-10.

Immunofluorescence Staining

Frozen sections (4 μm) fixed with cold acetone for 10 minutes. For nephrin or CD31 staining, sections were incubated with each antibody for 1 hour and then incubated with FITC-conjugated donkey anti-goat or anti-rat IgG. For MMP-10 staining, sections were incubated with Hilyte Fluor 555-labeled (Dojindo, Kumamoto, Japan) anti-MMP-10 antibody for 1 hour. The samples were then visualized using a fluorescence microscope (IX-81).

Western Blotting

Western blot analysis was performed as previously described, with some modifications [7]. Protein extracts were subjected to electrophoresis and transferred onto PVDF filters. Filters were incubated with each primary antibody, and immunoblots were then developed using horseradish peroxidase-linked secondary antibodies and a chemiluminescence kit. The obtained images were processed and analyzed using ImageJ software version 1.53 (<https://imagej.nih.gov/ij/>). β -actin was used as an internal control.

Induction of Anti-Glomerular Basement Membrane (GBM) Nephritis

An accelerated form of anti-GBM nephritis was induced in MMP-10 knockout mice or wild-type control mice. Anti-GBM antiserum was prepared in rabbits as described previously [8]. Mice were immunized via intraperitoneal injection of 0.25 mg/20 g body weight (BW) of normal rabbit IgG (MP Biomedicals, Santa Ana, CA) emulsified with complete Freund's adjuvant (Difco, Detroit, MI) at 8 weeks of age, and anti-GBM antibody or vehicle (normal rabbit serum) was injected into the tail vein 5 days later. Urinary protein levels were measured on days 0, 3, 7, and 14 after induction of anti-GBM nephritis. Mice were euthanized at 2 weeks after induction of anti-GBM nephritis under deep sedation, and blood and kidney samples were collected for biochemical and histological analyses.

Measuring Blood Pressure and Collecting Urine Samples.

Blood pressure was measured indirectly by the tail-cuff method (MK-2000ST; Muromachi Kikai, Tokyo, Japan) and urine samples were collected with metabolic cages (Shinano manufacturing, Tokyo, Japan) for 24 hours.

Blood and Urine Parameter Measurements.

Serum creatinine, BUN (blood urea nitrogen), serum total cholesterol, serum albumin, urinary total protein and urinary creatinine were measured by enzymatic method (SRL, Tokyo, Japan). Urinary albumin levels were measured with albumin ELISA kits (Exocell, Philadelphia, PA).

Collecting Glomeruli by Sieving Methods

Glomeruli were isolated by the graded sieving methods as described previously [9]. Briefly, we removed and discarded perirenal fat and medulla with scissors and sharp forceps and cut kidneys into small pieces. Three sieves (100 μ m, 75 μ m, 30 μ m) were piled up in order from top, and small pieces of kidney were placed top of the sieves and minced with plunger flange. Smaller segment went downside of the sieves and washed with phosphate buffered saline. The glomeruli remained on the top of 30 μ m sieve and collected by pipetting. Collected glomeruli were centrifuged at 15000 r.p.m. for 5 minutes at 4 °C and used for RNA and protein extraction.

RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction (PCR)

Total RNA was extracted using AllPrep DNA/RNA/Protein Mini Kit (Qiagen). ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) was used for the synthesis of first-strand complementary DNA from total RNA as described previously [10]. Applied Biosystems TaqMan gene expression assay probes were applied to measure the expression levels of mouse mRNAs. The primers and probes were designed to detect only murine or human cDNA, respectively, without cross-reactivity (each sequence was listed in Supplementary Table S2). Real-time quantitative PCR was performed using the QuantStudio 3 Realtime PCR system (Applied Biosystems, Waltham, MA) with Premix Ex Taq (TAKARA BIO, Shiga, Japan). The expression level of each mRNA was expressed relative to the GAPDH or β -actin mRNA level.

Affymetrix GeneChip Array

Systemic GC-A knockout and wild-type mice were performed uninephrectomy before aldosterone infusion and high salt diet for 3 weeks. Total glomerular RNA from these mice was subjected to microarray assay as previously reported [10]. Biotin-labeled cRNA was synthesized by in vitro transcription reaction using total RNA by GeneChip 3' IVT PLUS Reagent Kit (ThermoFisher Scientific). The obtained biotin-labeled cRNA was fragmented and hybridized to GeneChip Expression array (45 °C, 60 r.p.m. rotation speed, 16 hours). The arrays were stained and washed with GeneChip Hybridization, Wash and Stain Kit using an automatic staining and washing device, and images of the arrays were acquired using GeneChipTM Scanner 3000 7G. Data normalization, log transformation, statistical analysis, and pattern study were performed with the Expression ConsoleTM Software 1.4.1 (ThermoFisher Scientific). Microarray analysis of gene expression profiles is provided at the GEO data base (GSE276626).

Cell Culture, Transfection and Immunocytochemistry

Conditionally immortalized mouse podocytes, Mouse Podocyte Clone-5 (MPC-5), were kindly provided by Dr. Peter Mundel and cultured as described previously with some modification [11, 12]. Briefly, MPC-5 cells were cultured with

RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaille, France), interferon-gamma (IFN- γ ; PeproTech, Cranbury, NJ), insulin–transferrin–selenite supplement (Gibco), and penicillin G (100 U/mL), streptomycin (100 μ g/mL), and amphotericin B (250 ng/mL) for effective cell division. MPC-5 cells were differentiated with incubation in IFN- γ (–) media at 37 °C for 2 weeks before transfection. Primary human glomerular microvascular endothelial cells (GE cells, ACBRI 128) were purchased from Cell Systems (Kirkland, WA) and cultured with medium kit without serum and with cultureboost-RTM (4Z3-500R, Cell Systems). COS-7 cells (African green monkey kidney fibroblast-like cell line) were obtained from American Type Culture Collection and cultured with DMEM containing 10% FBS with antibiotics. For knockdown analysis, MPC-5 cells were transfected with NPR1 small interfering RNA or negative control RNA (QIAGEN), and GE cells were transfected with MMP-10 small interfering RNA (QIAGEN). For overexpressing analysis, Myc-DDK-tagged MMP-10 expressing plasmid (Origene Technologies) or pCMV-6 entry was transfected to MPC-5 cells. Myc-DDK-tagged mouse nephrin expressing plasmid was produced as previously [13] and transfected into COS-7 cells. siRNAs and plasmids were transfected by a pulse of electroporation with Nucleofector II (Lonza Group AG, Basel, Switzerland) and Ingenio Electroporation Kit (Mirus Bio, Madison, WI). Transfected cells were seeded to 6 well plates and maintained in normal serum medium for 24 hours, and stimulated with tumor necrosis factor-alpha (TNF- α , 10 ng/mL) in medium with 0.3% FBS for 2 or 24 hour. For immunocytochemistry, transfected MPC-5 cells were transferred into chamber slide and fixed with 4% paraformaldehyde for 15 minutes. Permeabilization of the cells was performed using 0.1% Triton X-100 in phosphate buffered saline for 10 minutes, and then incubated with Hilyte Fluor 555-labeled anti-MMP-10 antibody for 1 hour. Slides were mounted with VECTASHIELD Mounting Medium with DAPI (4', 6-diamidino-2-phenylindole) (Vector Laboratories, Newark, CA).

Co-culture Methods

MPC-5 cells and GE cells were co-cultured using direct co-culture method. Briefly, MPC-5 cells and GE cells were cultured in 10 cm dishes independently, and both cells were transfected with different siRNA. After transfection, cells were mixed in medium kit without serum with cultureboost-RTM to a 1:1 ratio and seeded to 6 well plates. Co-cultured cells were stimulated with 10 ng/mL TNF- α after 24 hours and collected after 48 hours. The RNA expression of MPC-5 cells was quantified by RT-qPCR using probes for human, and the RNA expression of GE cells was quantified using probes for mice.

Nephrin Cleavage Test by MMP-10

Human recombinant MMP-10 (R&D systems) was activated by incubation with 100 mM 4-aminophenylmercuric acetate (APMA) at 37 °C for 2 hours. Human recombinant full-length nephrin was incubated with activated MMP-10 at 37 °C for 16 hours. Recombinant fibronectin and recombinant adiponectin were also incubated with activated MMP-10 as positive control and negative control. Following electrophoresis, proteins were detected using Pierce Silver Stain kit (ThermoFisher Scientific).

Supplementary Reference

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Supplementary Table S1. Patients' data included in this study.

Clinical characteristic of patients presented in Figure 1E.

Number	Clinical Diagnosis	Age	Gender	Serum creatinine (mg/dL)	Urinary protein (g/gCr)
Patient 1	Minor glomerular abnormality	44	Female	0.6	0.044
Patient 2	IgA nephropathy	21	Female	0.7	0.98
Patient 3	Membranous nephropathy	67	Male	0.8	2.96
Patient 4	FSGS	24	Male	1.3	11.1
Patient 5	Diabetic nephropathy	62	Male	0.8	0.58
Patient 6	Lupus nephritis	31	Female	0.6	0.68
Patient 7	ANCA associated vasculitis	22	Male	3.3	4.39

Clinical characteristic and the number of glomerular MMP-10 positive cells in Figure 1F.

Clinical diagnosis	Age	Gender (M/F)	Cr (mg/dL)	Urinary Protein (g/gCr)	The number of MMP-10 positive cells
Minor glomerular abnormality	35.7±11.9	1/2	0.67±0.06	0.22±0.30	3.63±1.19
IgA nephropathy	29.9±18.7	8/5	0.81±0.21	0.77±0.69	16.04±5.44
Membranous nephropathy	59.0±17.4	3/0	0.8±0	2.17±1.79	13.89±0.67
FSGS	55.3±27.2	1/2	1.50±0.62	9.43±7.68	10.61±4.70
Diabetic nephropathy	63.0±1.0	2/1	1.53±1.10	4.63±4.72	26.00±13.59
Lupus nephritis	34.4±7.6	0/3	0.73±0.23	2.28±1.40	45.84±9.57
ANCA-associated vasculitis	52.8±17.8	4/1	2.54±1.13	1.63±1.67	29.92±8.52
Total	42.3±20.5	19/14	1.18±0.84	2.25±3.55	19.9±12.79

Supplementary Table S2. Primer and dual-labelled probe sequences for real-time PCR

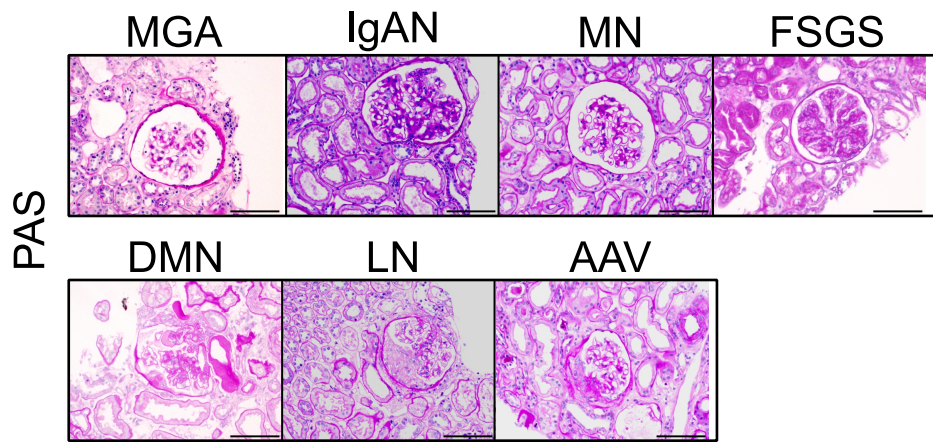
Target	Space	Forward primer	Reverse Primer	Probe
<i>Mmp10</i>	Mm	5'-ACCAATCTGCTCAGCGTATCC-3'	5'-ACTACTGTCCTTTCTTTGAAAATTTGT-3'	5'-6FAM-TGACACAAGGCCACCCAAGCATGGATC-TAMRA-3'
<i>Npr1</i>	Mm	5'-GGGGCACTGTCACTGATGG-3'	5'-GTGTCCCGATCTCCATTTCTATCA-3'	5'-6FAM-CATCACTCAGCGGATGTGGAACCGAAGC-TAMRA-3'
<i>Ccl2</i>	Mm	5'-CGGCTGGAGCATCCAGT-3'	5'-ATTGGGATCATCTTGCTGGTGAAT-3'	5'-6FAM-TCAGCCAGATGCAGTTAACGCCCCAC-TAMRA-3'
<i>Emr1</i>	Mm	5'-TGGTGGTCATAATCTCTGCTTCTG-3'	5'-AGACAGGCCCCAGGAAACTC-3'	5'-6FAM-CCCGTCTCTGTATTCAACCAGCAGCGATT-TAMRA-3'
<i>Tgfb1</i>	Mm	5'-GACGTCACTGGAGTTGTACGG-3'	5'-GCTGAATCGAAAGCCCTGT-3'	5'-6FAM-AGTGGCTGAACCAAGGAGACGGAA-TAMRA-3'
<i>Fn1</i>	Mm	5'-ATCATTTTCATGCCAACCAAGTT-3'	5'-TCGCACTGGTAGAAGTTCCA-3'	5'-6FAM-CCGACGAAGAGCCCTTACAGTTCCA-TAMRA-3'
<i>Ctgf</i>	Mm	5'-TTCCCCGAGAAGGGTCAAGCT-3'	5'-TCCTTGGGCTCGTCACACA-3'	5'-6FAM-CCTGGGAAATGTGCAAGGAGTGG-TAMRA-3'
<i>Mmp2</i>	Mm	5'-CCTGGTTTACCCTTTCTGCT-3'	5'-CGAGCGAAGGGCATAAAA-3'	5'-6FAM-CCCAGATACCTGCACCACCTTAAGTGTGC-TAMRA-3'
<i>Mmp9</i>	Mm	5'-CGTGTCTGGAGATTCGACTTGA-3'	5'-GCAGAAATAGGCTTTGCTTGGTA-3'	5'-6FAM-TTCCAGGGCACACCAGAGAACTCCTATCC-TAMRA-3'
<i>Il6</i>	Mm	5'-CCAGAAACCGCTATGAAGTTCCT-3'	5'-CACCAGCATCAGTCCCAAGA-3'	5'-6FAM-TCTGCAAGAGACTTCCATCCAGTTGCC-TAMRA-3'
<i>Tnfa</i>	Mm	5'-CCTCTTCTCAATCCTGCTTGTG-3'	5'-GCCATTGGGAACTTCTCATCC-3'	5'-6FAM-CCACCACGCTCTTGTCTACTGAACTTCG-TAMRA-3'
<i>Synpo</i>	Mm	5'-TTCCGAGTGGCATCCTTAAGTC-3'	5'-GCTGCTGCTTGGTAGGTTCA-3'	5'-6FAM-CGCAGGACTCCATTCTCTGGCAGATAGC-TAMRA-3'
<i>Actb</i>	Mm	5'-AGCTTCTTTCAGCTCCTTCG-3'	5'-GGGAGCATCGTcGCCCG-3'	5'-6FAM-CACACCCGCCACCAGTTCGCCATG-TAMRA-3'
<i>MMP10</i>	Hs	5'-CCCACTCTACAATCATTACAGA-3'	5'-GCATCTCAGATCCCGAAGGAAC-3'	5'-6FAM-CTCGCCAGTTCCGCCTTTCGCAA-TAMRA-3'
<i>CCL2</i>	Hs	5'-CTGTGCCTGCTGCTCATAGC-3	5'-CAGGTGACTGGGGCATTGATTGC-3	5'-6FAM-CTTCATTCCGCAAGGGCTCGCTCAGC-TAMRA-3'
<i>IL1B</i>	Hs	5'-CTGGATTCAATGAGGAGACTTGC-3'	5'-GGAAGTGGATCAGGACTTTGTAC-3'	5'-6FAM-ACAGCTCTGGCTTGTCTCTCACTACTCTCA-TAMRA-3'
<i>ACTB</i>	Hs	5'-CACAGAGCCTCGCCTTTGC-3'	5'-CATGCCGGAGCCGTTGTC-3'	5'-6FAM-CACCCGCCGCCAGCTCACCATG-TAMRA-3'

Mmp10; Matrix metalloproteinase 10, *Npr1*; natriuretic peptide receptor 1, *Ccl2*; C-C motif chemokine 2, *Emr1*; EGF-like module-containing mucin-like hormone receptor-like 1, *Tgfb1*; transforming growth factor-β1, *Fn1*; Fibronectin 1, *Ctgf*; Connective tissue growth factor, *Mmp2*; Matrix metalloproteinase 2, *Mmp9*; Matrix metalloproteinase 9, *Il6*; Interleukin-6, *Tnfa*; Tumor necrosis factor-α, *Synpo*; Synaptopodin, *IL1B*; Interleukin-1β, *Actb*; β-actin, Mm; Mus musculus, Hs; Homo sapiens

Supplementary Table S3. Target sequences of each small interfering RNA.

Target	Space	Target sequence
<i>Npr1</i>	Mm	CAGGGTTGTCCTGAACTTTAA
<i>Mmp10</i>	Mm	AGCATGGATCTTGCTCAGCAA
<i>MMP10</i>	Hs	TTGGCCCTCTCTTCCATCATA

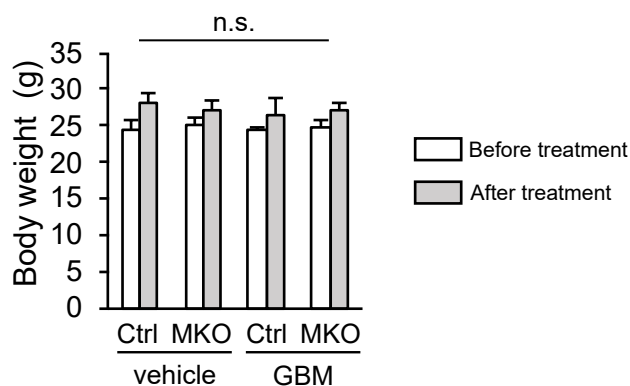
Mmp10; Matrix metalloproteinase 10, *Npr1*; natriuretic peptide receptor 1, Mm; Mus musculus, Hs; Homo sapiens



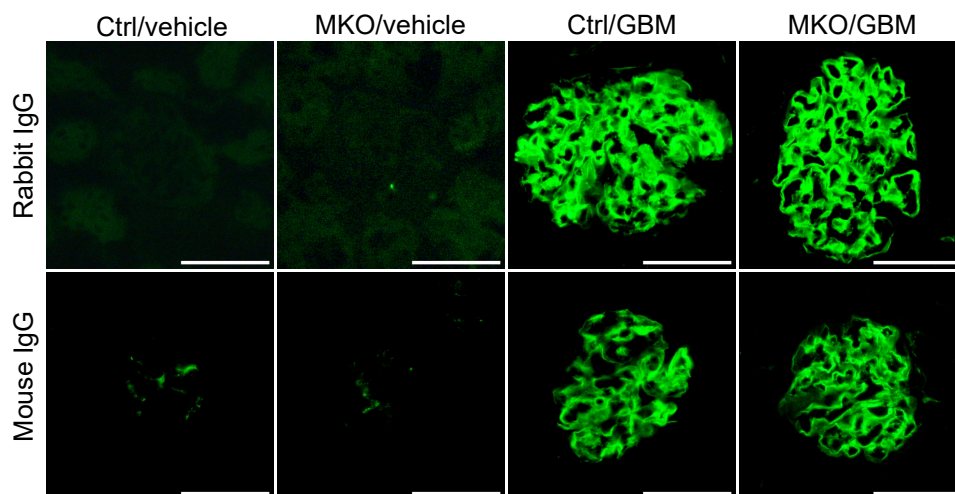
Supplementary Figure S1. Periodic acid Schiff staining of renal biopsy samples from patients with glomerulonephritis.

Scale bars represents 100 μ m. MGA; minor glomerular abnormality, IgAN; IgA nephropathy, MN; membranous nephropathy, FSGS; Focal segmental glomerulosclerosis, DMN; Diabetic nephropathy, LN; lupus nephritis, AAV; ANCA associated vasculitis.

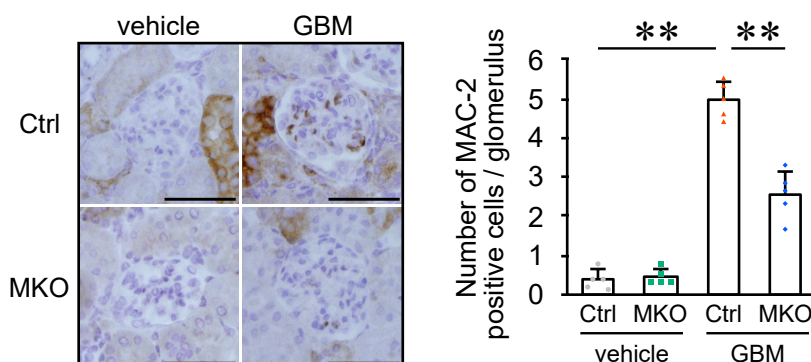
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B

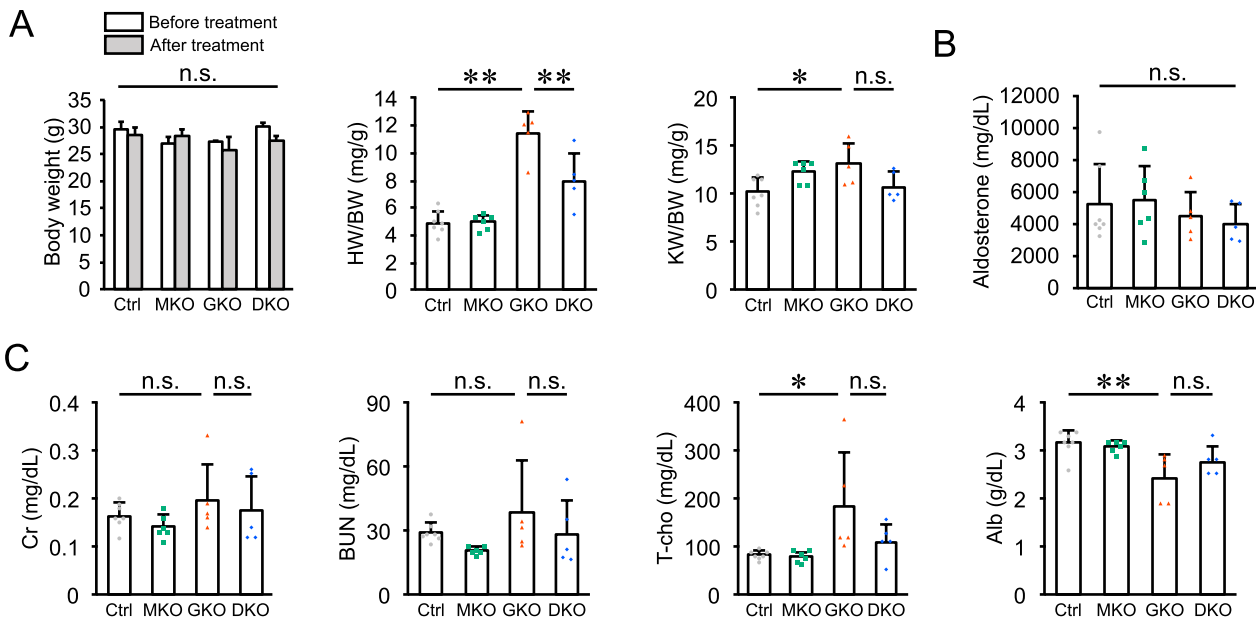


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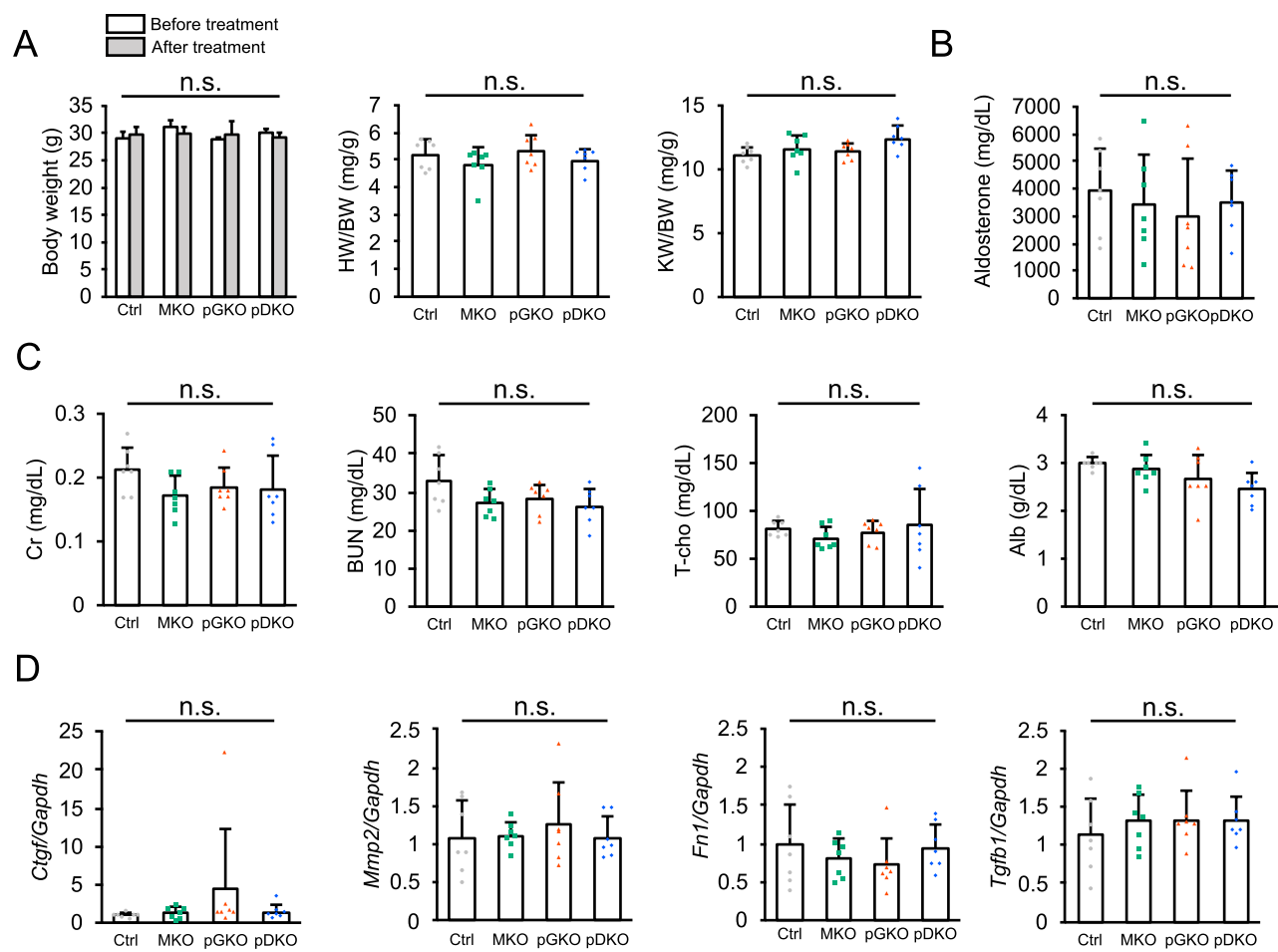


Supplementary Figure S2. Body weight change, IgG deposition, and macrophage infiltration into glomeruli in wild-type and MMP-10 knockout mice with anti-GBM nephropathy model.

(a) Body weight changes in wild-type and MMP-10 knockout mice with or without anti-GBM nephropathy. (b) Immunofluorescence staining for rabbit and mouse IgG. (c) Representative images of immunohistochemical staining for MAC-2 in the glomeruli from each group. The graph shows the number of MAC-2-positive cells in glomeruli. Ctrl; control mice, MKO; MMP-10 knockout mice. Values are expressed as means $\pm$ SD. Scale bars represent 50 μ m. n.s.; not significant, **P < 0.01.

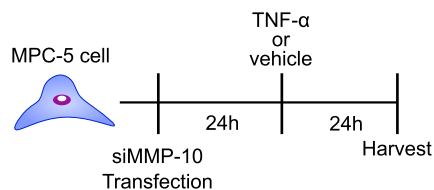


Supplementary Figure S3. The organ weight and serum parameters in systemic GC-A knockout mice with or without MMP-10 knockout. (a) Body, heart and kidney weight. (b) Serum aldosterone levels. (c) Serum creatinine, urea nitrogen, total cholesterol and albumin. Ctrl; control mice, MKO; MMP-10 knockout mice, GKO; systemic GC-A knockout mice, DKO; MMP-10 and GC-A knockout mice. Values are expressed as means $\pm$ SD. n.s.; not significant, *P < 0.05, **P < 0.01.

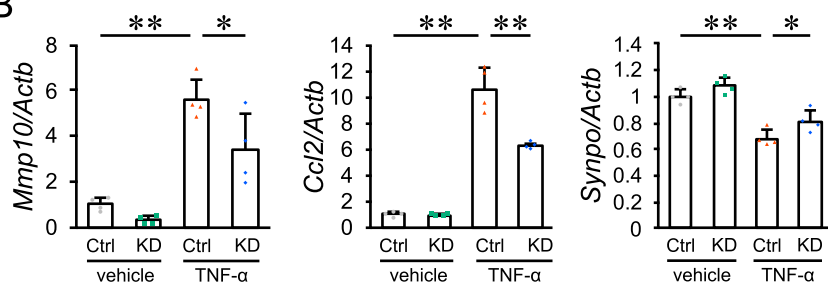


Supplementary Figure S4. The organ weight, serum parameters and mRNA expression in podocyte-specific GC-A knockout mice with or without MMP-10 knockout. (a) Body, heart and kidney weight. (b) Serum aldosterone levels. (c) Serum creatinine, urea nitrogen, total cholesterol and albumin. (d) Glomerular mRNA expression of *Ctgf*, *Mmp2*, *Fn1*, and *Tgfb1*. Ctrl; control mice, MKO; MMP-10 knockout mice, pGKO; podocyte-specific GC-A knockout mice, pDKO; podocyte-specific GC-A knockout and systemic MMP-10 knockout mice. Values are expressed as means $\pm$ SD. n.s.; not significant.

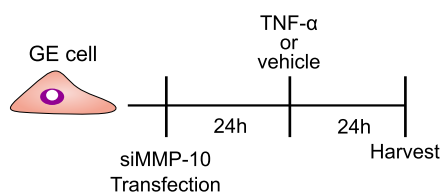
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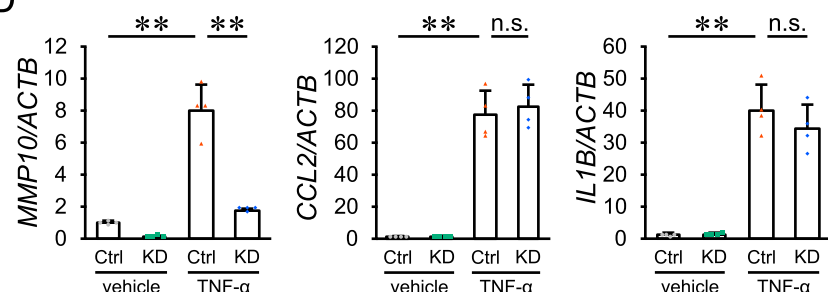
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D



Supplementary Figure S5. The effect of MMP-10 knockdown in mouse podocyte clone-5 (MPC-5) cells and glomerular endothelial (GE) cells. (A) The protocol for MMP-10 knockdown in MPC-5 cells. (B) mRNA expression of *Mmp10*, *Ccl2*, and *Synpo*. (C) The protocol for MMP-10 knockdown in GE cells. (D) mRNA expression of *MMP10*, *CCL2*, and *IL1B*. Ctrl; control, KD; MMP-10 knockdown. Values are expressed as means $\pm$ SD. n.s.; not significant, * $P < 0.05$, ** $P < 0.01$.