

Dual deletion of guanylyl cyclase-A and p38 mitogen-activated protein kinase in podocytes with aldosterone administration causes glomerular intra-capillary thrombi

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Natriuretic peptides exert not only blood-lowering but also kidney-protective effects through guanylyl cyclase-A (GC-A), a natriuretic peptide receptor. Signaling through GC-A has been shown to protect podocytes from aldosterone-induced glomerular injury, and a p38 mitogen-activated protein kinase (MAPK) inhibitor reduced glomerular injury in aldosterone-infused podocyte-specific GC-A knockout mice. To explore the role of p38 MAPK in podocytes, we constructed podocyte-specific p38 MAPK and GC-A double knockout mice (pod-double knockout mice). Unexpectedly, aldosterone-infused and high salt-fed (B-ALDO)-treated pod-double knockout mice resulted in elevated serum creatinine, massive albuminuria, macrophage infiltration, foot process effacement, nephrin and podocin reduction, and additionally, intra-capillary fibrin thrombi, indicating endothelial injury. Microarray analysis showed increased plasminogen activator inhibitor-1 (PAI-1) in glomeruli of B-ALDO-treated pod-double knockout mice. In B-ALDO-treated pod-double knockout mice, PAI-1 increased in podocytes, and treatment with PAI-1 neutralizing antibody ameliorated intra-capillary thrombus formation. *In vitro*, deletion of p38 MAPK by the CRISPR/Cas9 system and knockdown of GC-A in human cultured podocytes upregulated PAI-1 and transforming growth factor- β 1 (TGF- β 1). When p38 MAPK knockout podocytes, transfected with a small interfering RNA to suppress GC-A, were co-cultured with glomerular endothelial cells in a transwell system, the expression of TGF- β 1 was increased in glomerular endothelial cells. PAI-1 inhibition ameliorated both podocyte and endothelial injury in the transwell system signifying elevated PAI-1 in podocytes is a factor

disrupting normal podocyte-endothelial crosstalk. Thus, our results indicate that genetic dual deletion of p38 MAPK and GC-A in podocytes accelerates both podocyte and endothelial injuries, suggesting these two molecules play indispensable roles in podocyte function.

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KEYWORDS: albuminuria; aldosterone; endothelium; podocytes

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Translational Statement

The combination of an angiotensin receptor blocker and a neprilysin inhibitor is beneficial to patients with chronic heart failure and hypertension. However, it is not clear how natriuretic peptides/guanylyl cyclase-A (GC-A) signaling and their downstream target, p38 mitogen-activated protein kinase (MAPK) inhibition, are involved in podocyte homeostasis. In this study, we generated podocyte-specific p38 MAPK and GC-A double knockout mice, which exhibited kidney dysfunction, including both podocyte and endothelial damage by aldosterone and salt. This study elucidates the mechanism of podocyte and endothelium injury caused by a lack of these signals, providing prospective podocyte and endothelial protection targets.

Many clinical and experimental studies have shown that the renin-angiotensin-aldosterone system is involved in kidney diseases and hypertension.¹⁻³ In addition to the distal tubules and collecting ducts, recent studies have revealed that aldosterone acts on podocytes, mesangial cells, and endothelial cells, all of which are involved

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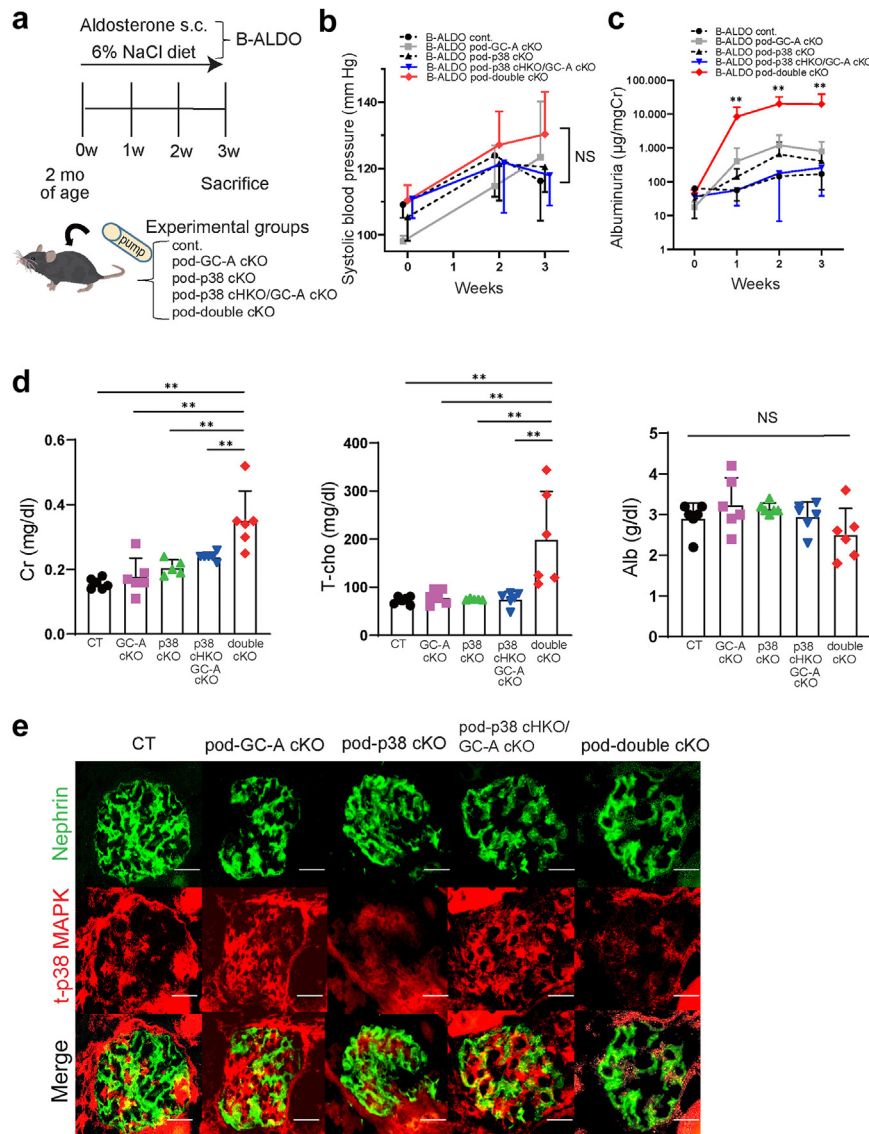


Figure 1 | Aldosterone-infused and high-salt diet-fed (B-ALDO)-treated podocyte-specific p38 mitogen-activated protein kinase (MAPK)/guanylyl cyclase-A (GC-A) double knockout (pod-double cKO) mice exhibited severe albuminuria and kidney injury. (a) Time course of the experiment. Aldosterone and high salt were administered to mice at 2 months of age for 3 weeks. Experimental groups were as follows: control (cont.; CT) mice, podocyte-specific GC-A knockout (pod-GC-A cKO) mice, podocyte-specific p38 MAPK knockout (pod-p38 cKO) mice, podocyte-specific p38 MAPK heterozygous/GC-A homozygous knockout (pod-p38 cHKO/GC-A cKO) mice, and pod-double cKO mice. **(b)** Time course of systolic blood pressure of B-ALDO-treated CT mice, B-ALDO-treated pod-GC-A cKO mice, B-ALDO-treated pod-p38 MAPK cKO mice, B-ALDO-treated pod-p38 cHKO/GC-A cKO mice, and B-ALDO-treated pod-double cKO mice. Systolic blood pressure at 3 weeks was 116.2 ± 4.9 , 123.3 ± 8.7 , 120.6 ± 2.8 , 120.8 ± 3.4 , and 134.8 ± 4.4 mm Hg, respectively. **(c)** Time course of albuminuria in these 5 groups. B-ALDO-treated pod-double cKO mice exhibited massive albuminuria. **(d)** Biochemical parameters (serum creatinine [Cr], serum total cholesterol [T-cho], and serum albumin [Alb]) in these 5 groups. Creatinine and total cholesterol levels of B-ALDO-treated pod-double cKO mice were higher than those of mice in other groups. Albumin of B-ALDO-treated pod-double cKO mice tended to be lower than that of mice in other groups. **(e)** The double-immunofluorescent staining with nephrin (green) and total p38 (t-p38) MAPK (red), indicating that t-p38 MAPK colocalizes with nephrin in control, pod-GC-A cKO mice, and pod-p38 cHKO/GC-A cKO mice, and that t-p38 MAPK did not colocalize with nephrin in pod-p38 cKO mice or pod-double cKO mice. Although the intensity of nephrin staining was reduced mostly in the glomeruli of B-ALDO-treated pod-double cKO mice, in this picture, a glomerulus with relatively preserved nephrin intensity was selected so that it was easier to see the merged image of t-p38 MAPK and nephrin. $n = 6$, each. Mean $\pm$ SD. $^{**}P < 0.01$. Bar = 20 μ m. NS, not significant. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

in glomerular injury caused by the activation of the aldosterone/mineralocorticoid receptor system.^{4–7} Natriuretic peptides have been shown to counteract renin-angiotensin-aldosterone system by inhibiting the secretion of renin, angiotensin-converting enzyme, and aldosterone and by

attenuating their signaling pathways.⁸ There are 3 types of natriuretic peptides: atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. Through guanylyl cyclase-A (GC-A), atrial natriuretic peptide and brain natriuretic peptide exert physiological effects, such as diuresis

Table 1 | Body and kidney weights and blood and urinary parameters in B-ALDO mice

Variable	B-ALDO CT	B-ALDO pod-GC-A cKO	B-ALDO pod-p38 cKO	B-ALDO pod-p38 cHKO/GC-A cKO	B-ALDO pod-double cKO
BW at 3 wk, g	26.6 ± 1.79	26.7 ± 1.24	26.4 ± 1.19	26.6 ± 1.14	26.9 ± 2.50
LKW at 3 wk, mg	245 ± 29.9	225 ± 9.6	228 ± 13.4	221 ± 35.3	225 ± 26.3
Serum TG, mg/dl	60.6 ± 22.8	54.2 ± 26.4	43.4 ± 13.2	47.8 ± 26.3	56.2 ± 9.41
Serum UN, mg/dl	25.6 ± 4.0	24.5 ± 5.8	21.5 ± 2.6	27.2 ± 4.6	45.1 ± 27.5
Serum sodium, mEq/L	159.0 ± 1.79	158.3 ± 2.35	157.4 ± 3.92	158.8 ± 2.99	163.8 ± 5.7
Serum potassium, mEq/L	5.08 ± 0.65	3.75 ± 0.70	3.90 ± 0.8	4.50 ± 1.11	4.44 ± 0.73
Urinary sodium, mEq/d	3.40 ± 1.65	1.31 ± 0.36	2.81 ± 0.93	2.47 ± 1.23	2.29 ± 1.05
Urinary potassium, mEq/d	0.72 ± 0.14	0.44 ± 0.23	0.72 ± 0.07	0.68 ± 0.08	0.67 ± 0.16

B-ALDO, aldosterone infused and high-salt diet fed; BW, body weight; CT, control; LKW, left kidney weight; pod-double cKO, podocyte-specific p38 mitogen-activated protein kinase/guanylyl cyclase-A double knockout; pod-GC-A cKO, podocyte-specific guanylyl cyclase-A knockout; pod-p38 cHKO/GC-A cKO, podocyte-specific p38 mitogen-activated protein kinase heterozygous/ guanylyl cyclase-A homozygous knockout; pod-p38 cKO, podocyte-specific p38 mitogen-activated protein kinase knockout; TG, triglyceride; UN, urea nitrogen.

Values are expressed as the mean ± SD. n = 6 for each group on BW, LTW, urinary sodium, and urinary potassium. n = 5 (CT), 6 (pod-GC-A cKO), 6 (pod-p38 cKO), 5 (pod-p38 cHKO/GC-A cKO), and 5 (pod-double cKO) on serum TG, serum UN, serum sodium, and serum potassium. There were no differences in these parameters among all B-ALDO-treated groups of mice. Comparisons were performed by 1-way analysis of variance with a Turkey *post hoc* test and the Dunnett test against B-ALDO CT group.

and vasodilation.⁹ We previously reported that uninephrectomized, aldosterone-infused, and high-salt-fed (ALDO) systemic GC-A knockout mice exhibited hypertension and massive albuminuria.¹⁰ To determine the site of GC-A action, we generated podocyte-specific GC-A conditional knockout (cKO) mice, demonstrating increased urinary albumin excretion, podocyte injury, and augmented phosphorylation of p38 mitogen-activated protein kinase (MAPK), thus indicating the importance of GC-A in podocytes.¹¹ Others have found that GC-A has a significant effect on podocytes in mice with mineralocorticoid deoxycorticosterone and excessive salt levels.¹²

The p38 MAPK, a serine/threonine protein kinase that plays important roles in cellular responses to external stress signals,¹³ is involved in cell differentiation, apoptosis, and autophagy,¹⁴ and it is activated by a series of cytokines and growth factors, in addition to stress and proinflammatory signals.¹⁵ The p38 MAPK is phosphorylated in experimental kidney injury models, according to several studies, and p38 MAPK inhibitors, such as FR167653, alleviate kidney disorders.^{11,16,17} In our previous report, the p38 MAPK inhibitor, FR167653, suppressed urinary albumin excretion in ALDO podocyte-specific GC-A cKO mice.¹¹

However, the effects of p38 MAPK genetic deletion in podocytes have yet to be elucidated. By generating podocyte-specific p38 MAPK and GC-A double-cKO mice, the study investigated the effects of p38 MAPK deletion in podocytes on glomerular injury with aldosterone and a high-salt diet in mice.

METHODS

Animal experiments

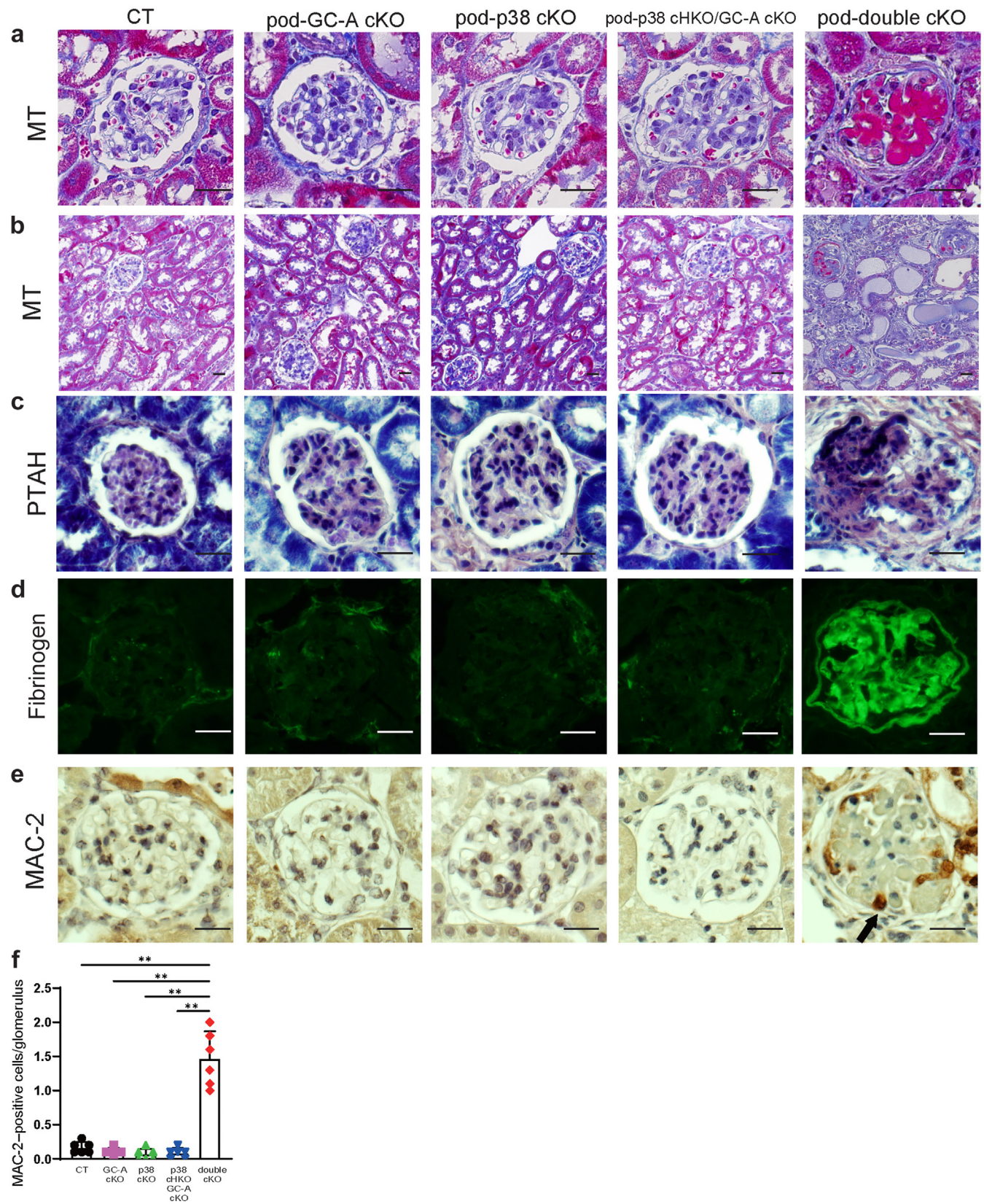
All animal experiments were performed in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions and were approved by the Animal Experimentation Committee of Kyoto University Graduate School of Medicine. We generated *Mapk14* floxed/floxed (fl/fl);neph1-Cre mice,^{18,19} *Npr1* (fl/fl);neph1-Cre mice,¹¹ *Mapk14* (fl/+);*Npr1* (fl/fl);neph1-Cre mice, and *Mapk14* (fl/fl);*Npr1* (fl/fl);neph1-Cre mice or *Mapk14* (fl/fl);*Npr1* (fl/fl) without neph1-Cre mice on a C57BL/6J background. *Mapk14* (fl/fl);*Npr1* (fl/fl) without neph1-Cre mice were used as a control. These male mice littermates were fed with a 6% high-salt diet and received aldosterone (0.2 µg/kg body weight per minute) or vehicle.¹⁰ We named these procedures bilateral kidney with aldosterone-administration (B-ALDO). Plasminogen activator inhibitor-1 (PAI-1) neutralizing antibody (HM2179; Hycult Biotech) or normal mouse

Table 2 | Body and kidney weights and blood and urinary parameters in VEH mice

Variable	VEH CT	VEH pod-GC-A cKO	VEH pod-p38 cKO	VEH pod-double cKO
BW at 3 wk, g	28.6 ± 0.59	27.3 ± 0.92	27.5 ± 0.22	28.1 ± 0.64
LKW at 3 wk, mg	215 ± 11.2	235 ± 16.6	225 ± 15.0	210 ± 15.8
Serum TG, mg/dl	49 ± 11.3	36.3 ± 9.0	33.0 ± 11.6	69 ± 47.4
Serum UN, mg/dl	37.4 ± 3.7	25.8 ± 5.6	32.0 ± 2.5	25.1 ± 2.7
Serum sodium, mEq/L	150.0 ± 4.03	153.3 ± 1.70	156.3 ± 4.03	152.3 ± 1.25
Serum potassium, mEq/L	5.77 ± 0.69	5.20 ± 1.14	6.00 ± 0.68	5.67 ± 0.21
Urinary sodium, mEq/d	2.39 ± 0.51	1.82 ± 0.59	1.25 ± 0.58	1.90 ± 1.18
Urinary potassium, mEq/d	0.24 ± 0.02	0.34 ± 0.08	0.26 ± 0.09	0.37 ± 0.10

BW, body weight; CT, control; LKW, left kidney weight; pod-double cKO, podocyte-specific p38 mitogen-activated protein kinase/guanylyl cyclase-A double knockout; pod-GC-A cKO, podocyte-specific guanylyl cyclase-A knockout; pod-p38 cKO, podocyte-specific p38 mitogen-activated protein kinase knockout; TG, triglyceride; UN, urea nitrogen; VEH, vehicle infused and high-salt diet fed.

Values are expressed as the mean ± SD. n = 4 for each group on BW, LTW, urinary sodium, and urinary potassium. n = 3 for each group on serum TG, serum UN, serum sodium, and serum potassium. There were no differences in these parameters among all VEH-treated groups of mice. Comparisons were performed by 1-way analysis of variance with a Turkey *post hoc* test and the Dunnett test against VEH CT group.



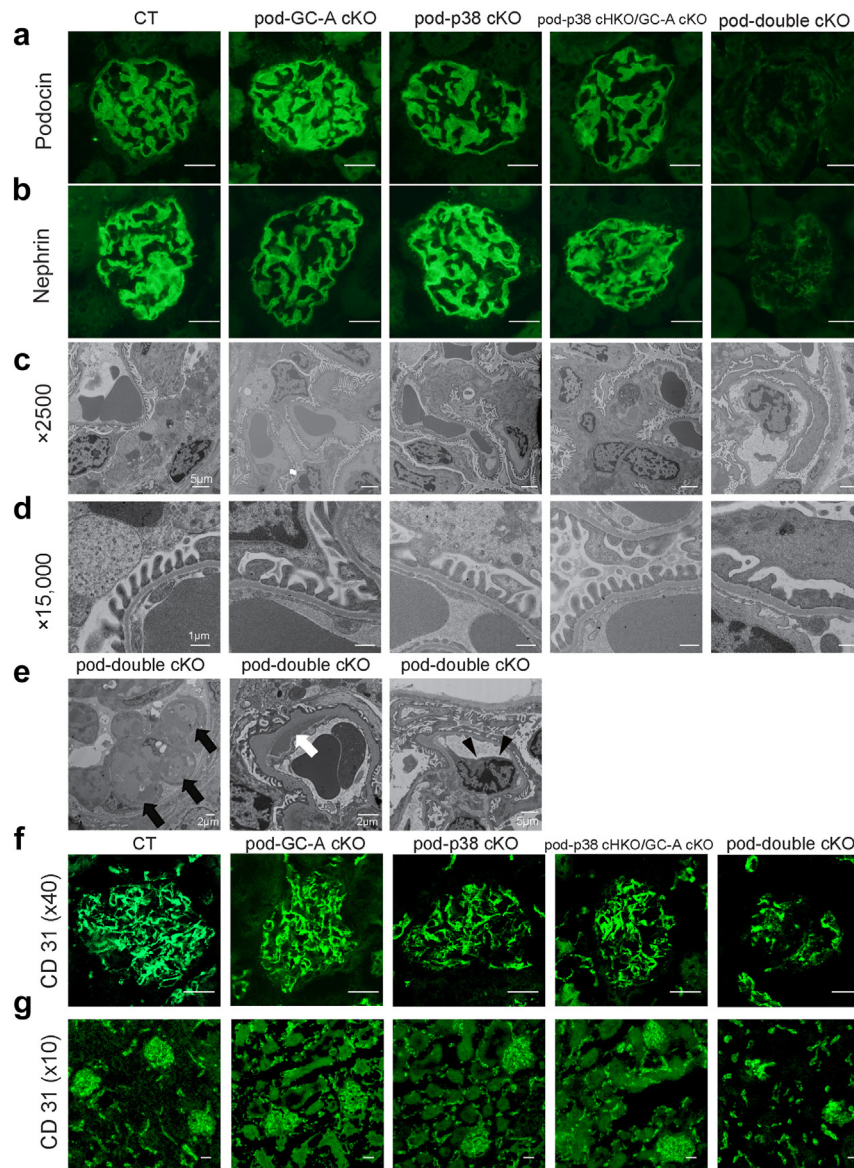


Figure 3 | Aldosterone-infused and high-salt diet-fed (B-ALDO)-treated podocyte-specific p38 mitogen-activated protein kinase (MAPK)/guanylyl cyclase-A (GC-A) double knockout (pod-double cKO) mice exhibited severe podocyte injury. (a,b) Representative images of podocin (a) and nephrin (b) by immunofluorescent study. The glomeruli of B-ALDO-treated pod-double cKO mice exhibited decreased staining intensity of podocin and nephrin. (c,d) Electron microscopic photographs of lower (c) and higher (d) magnification. B-ALDO-treated pod-double cKO exhibited severe foot process effacement and irregular thickening of the glomerular basement membrane. (e) B-ALDO-treated pod-double cKO mice exhibited intracapillary thrombi (black arrows), subendothelial exudative lesions (white arrow), and loss of fenestra and endothelial cell enlargement (black heads). (f,g) Representative images of CD31 in a high-power field (f; original magnification $\times 40$) and in a low-power field (g; original magnification $\times 10$) by immunofluorescent study. The glomerulus of B-ALDO-treated pod-double cKO mice showed weaker staining intensity of CD31, which indicated that B-ALDO-treated pod-double cKO mice exhibited endothelial injury. (a,b,f,g) Bar = 20 μ m. CT, control; pod-GC-A cKO, podocyte-specific GC-A knockout; pod-p38 cHKO/GC-A cKO, podocyte-specific p38 MAPK heterozygous/GC-A homozygous knockout; pod-p38 cKO, podocyte-specific p38 MAPK knockout. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Figure 2 | (continued) by Masson trichrome staining. In interstitial area, many protein casts were found in B-ALDO-treated pod-double cKO mice. (c) Histologic examination of glomeruli by phosphotungstic acid hematoxylin (PTAH) staining at 3 weeks. Periodic acid–Schiff–positive and red staining deposit by MT staining were stained violet in PTAH staining, indicating that intracapillary fibrin thrombi were formed in about 60% of glomeruli in B-ALDO-treated pod-double cKO mice. (d) Representative images of fibrinogen expression by immunofluorescent study. The formation of fibrin thrombi was remarkable in the glomerulus of B-ALDO-treated pod-double cKO mice. (e,f) Immunohistochemical study for MAC-2 (Galectin-3) and the number of MAC-2-positive cells in a glomerulus. B-ALDO-treated pod-double cKO mice exhibited increased number of MAC-2-positive cells. Mean $\pm$ SD. $^{**}P < 0.01$. Bar = 20 μ m. CT, control; pod-GC-A cKO, podocyte-specific GC-A knockout; pod-p38 cHKO/GC-A cKO, podocyte-specific p38 MAPK heterozygous/GC-A homozygous knockout; pod-p38 cKO, podocyte-specific p38 MAPK knockout. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

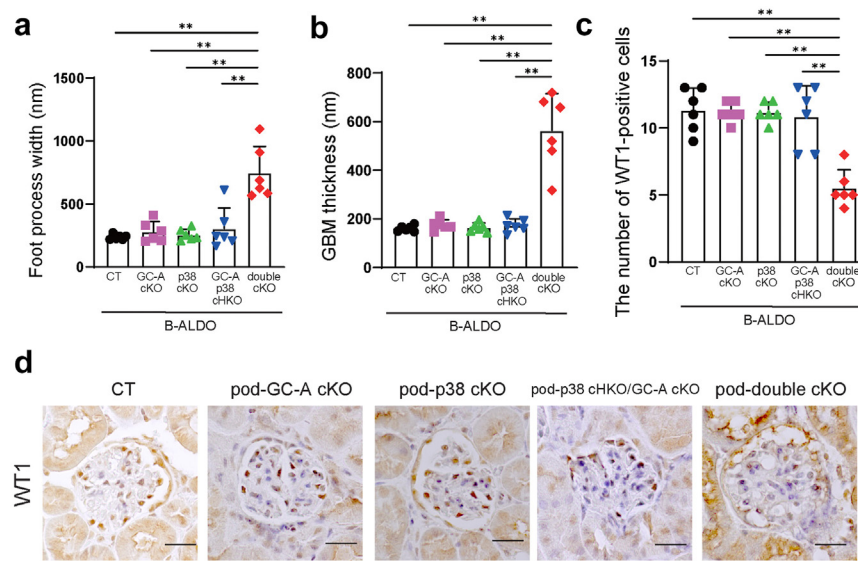


Figure 4 | Aldosterone-infused and high-salt diet-fed (B-ALDO)-treated podocyte-specific p38 mitogen-activated protein kinase (MAPK)/guanylyl cyclase-A (GC-A) double knockout (pod-double cKO) mice exhibited severe foot process effacement and irregular thickening of the glomerular basement membrane (GBM). (a) The width of foot processes in electron microscopic photographs. B-ALDO-treated pod-double cKO mice exhibited severe foot process effacement. (b) The width of GBM in electron microscopic photographs. B-ALDO-treated pod-double cKO mice exhibited irregular thickening of the glomerular basement membrane. (c) The number of Wilms tumor 1 (WT1)-positive cells in a glomerulus. (d) Immunohistochemical study for WT1 in the glomeruli. B-ALDO-treated pod-double cKO mice showed reduced number of WT1-positive cells. Mean $\pm$ SD. $**P < 0.01$. Bar = 20 μ m. CT, control; pod-GC-A cKO, podocyte-specific GC-A knockout; pod-p38 cKO/GC-A cKO, podocyte-specific p38 MAPK heterozygous/GC-A homozygous knockout; pod-p38 cKO, podocyte-specific p38 MAPK knockout. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

IgG (H1016; Hycult Biotech) was administered i.p. to B-ALDO-treated podocyte-p38 MAPK/GC-A conditional knockout (pod-double cKO) mice twice a week for 3 weeks.

Establishment of an MAPK14 knockout podocyte cell line

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology was employed to generate MAPK14 knockout human podocyte cell lines.

Cell culture and transfection

Differentiated wild human podocytes²⁰ and p38 knockout podocytes were transfected with *NPR1* small interfering RNA (Qiagen), as described previously,²¹ and then were stimulated with aldosterone. Transwells were used when human podocytes were cultured in the upper layer and glomerular endothelial cells (primary human glomerular microvascular endothelial cells; Cell Systems) or human umbilical vein endothelial cells (Lonza) were cultured in the lower layer. TM5441 (PAI-1 inhibitor; MedChemExpress) was administered to the podocytes.

Microarray analysis

Complementary RNA probes from RNA pool in B-ALDO-pod-double cKO and B-ALDO control mice were analyzed in an Affymetrix mouse genome 430 2.0 array at TAKARA Bio. The Gene Expression Omnibus accession number is GSE182344.

Statistical analysis

Values are expressed as the mean $\pm$ SD. Statistical analysis was performed with Graph Prism software (Version 9.00; GraphPad). An unpaired Student *t* test was used to compare differences between 2 groups, whereas comparisons of >2 groups were performed by

1-way analysis of variance with a Turkey *post hoc* test or the Dunnett test. The statistical significance level was set at $P < 0.05$.

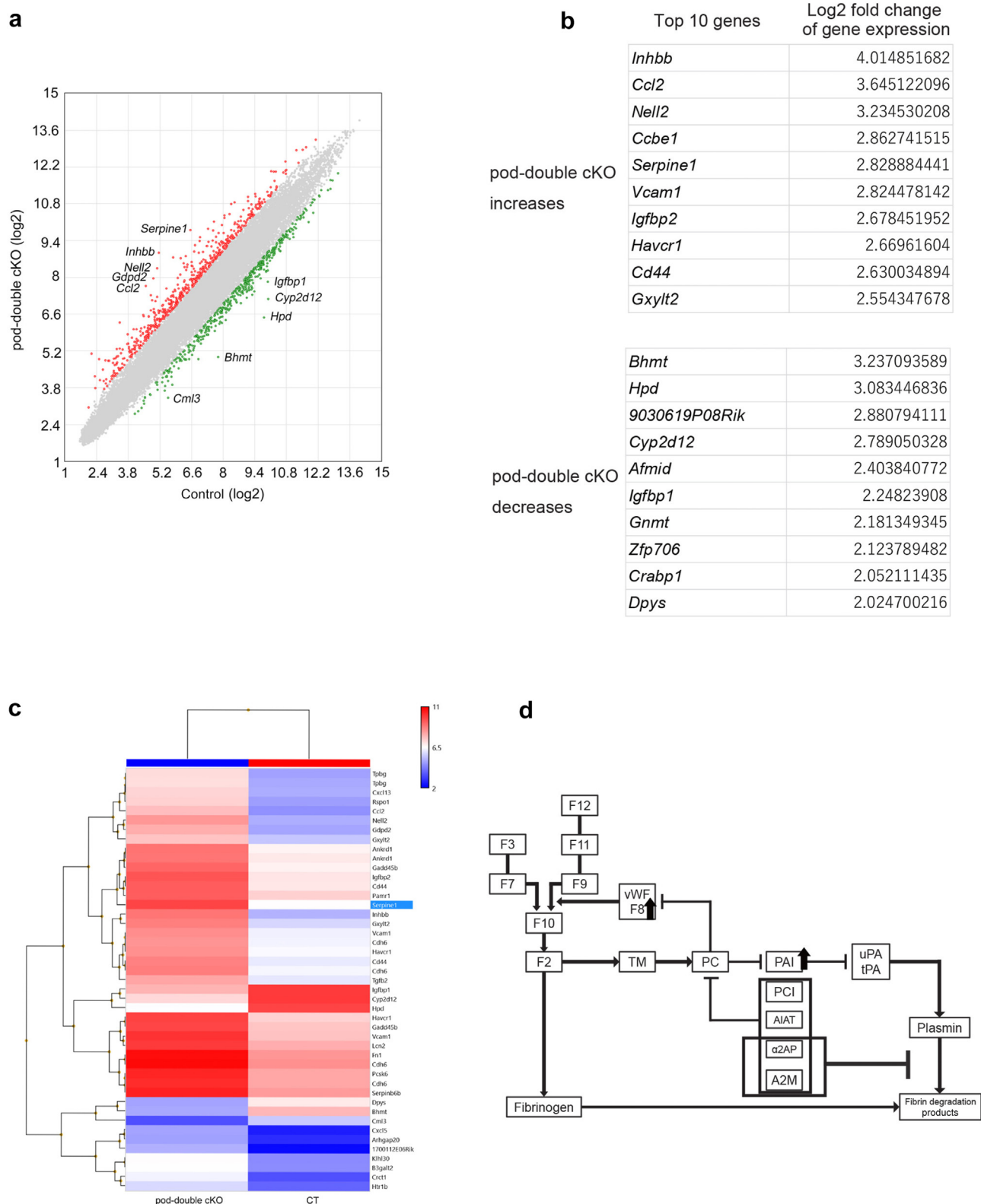
Detailed methods are described in the [Supplementary Methods](#) and [Supplementary Table S1](#).

RESULTS

Podocyte-specific deletion of p38 MAPK and GC-A in mice with aldosterone and high salt exacerbated glomerular injury with intracapillary fibrin thrombi

We generated male podocyte-specific GC-A knockout (pod-GC-A cKO) mice, podocyte-specific p38 MAPK knockout (pod-p38 cKO) mice, podocyte-specific p38 MAPK/GC-A double knockout (pod-double cKO) mice, podocyte-specific p38 MAPK heterozygous/GC-A homozygous knockout (pod-p38 cHKO/GC-A cKO) mice, and control (CT) mice. Blood pressure, albuminuria, and renal and cardiac tissue were normal in the 2-month-old healthy pod-double knockout mice ([Supplementary Figure S1A–C](#)). To induce glomerular injury, aldosterone and high salt were administered to mice at 2 months of age for 3 weeks without nephrectomy (B-ALDO). Vehicle (VEH)-treated mice were treated with a high-salt diet without aldosterone ([Figure 1a](#); [Supplementary Figure S2A](#)).

B-ALDO treatment increased systolic blood pressure ([Figure 1b](#)). Surprisingly, pod-double cKO mice treated with B-ALDO exhibited massive albuminuria ($20,540.9 \pm 8493.0$ μ g/mg creatinine) at 3 weeks ([Figure 1c](#)). Considering our previous findings,¹¹ we had speculated deletion of p38 MAPK in podocytes would ameliorate kidney damage; thus, this worsening albuminuria in pod-double cKO mice was unexpected. B-ALDO-treated CT mice showed slight albuminuria,



and B-ALDO-treated pod-GC-A cKO and pod-p38 cKO mice showed a mild increase of albuminuria (Figure 1c). The B-ALDO-treated pod-p38 cHKO/GC-A cKO mice tended to exhibit milder albuminuria than B-ALDO-treated pod-GC-A cKO mice, but this was not statistically significant. Consistent with these data, serum creatinine and total cholesterol levels of pod-double cKO mice with B-ALDO were high (Figure 1d). The serum albumin level of pod-double cKO mice with B-ALDO tended to be low (Figure 1d).

After 3 weeks of VEH administration, pod-double cKO mice exhibited hypertension and albuminuria but no elevation of serum creatinine (Supplementary Figure S2B–D). The body weight, blood test results, and urinary parameters of B-ALDO- and VEH-treated mice are presented in Tables 1 and 2, respectively.

Double-immunofluorescence staining of total p38 MAPK and nephrin, a podocyte marker, showed that total p38 MAPK colocalized with nephrin in control, pod-GC-A cKO mice, and pod-p38/GC-A cHKO mice, but not in pod-p38 cKO mice and pod-double cKO mice, indicating that p38 MAPK is deleted in podocytes of pod-p38 cKO mice or pod-double cKO mice (Figure 1e; Supplementary Figure S3A). Pod-double cKO mice with B-ALDO exhibited intracapillary massive deposits in some glomeruli, which stained red with Masson trichrome, violet with phosphotungstic acid hematoxylin, and pink with periodic acid–Schiff staining, indicating fibrin thrombi in capillaries (Figure 2a–c; Supplementary Figure S3B). Phosphorylation of p38 MAPK in glomeruli was increased in B-ALDO-treated pod-double cKO mice (Supplementary Figure S3C). In pod-double cKO mice with B-ALDO, many casts were found in the tubules (Figure 2b). VEH-treated mice of CT, pod-GC-A cKO, pod-p38 cKO, and pod-double cKO mice exhibited minor glomerular change (Supplementary Figure S4A–D). We performed immunofluorescent analyses for fibrinogen to ensure fibrin thrombi in the glomeruli (Figure 2d; Supplementary Figure S4E). The formation of fibrin thrombi was prominent in 60.7% of the glomeruli of B-ALDO-treated pod-double cKO mice (Figure 2d; Supplementary Figure S5A) but was not observed in other groups (Figure 2d; Supplementary Figures S4E and S5B). Macrophage infiltration is one of the characteristic features of glomerular injury. The number of MAC-2 (Galectin-3)-positive cells in B-ALDO-treated pod-double cKO mice was increased (Figure 2e and f). The number of MAC-2-positive cells in VEH-treated mice was low (Supplementary Figure S4H and I). The number of CD3-positive cells in a glomerulus in the B-ALDO-treated pod-double cKO mice increased (Supplementary Figure S6A and B). C3 deposition was not remarkable in all groups of mice, including B-ALDO-treated pod-double cKO mice (Supplementary Figure S6C).

These results indicate that deletion of GC-A and p38 MAPK in podocytes with B-ALDO stimulation presented with intracapillary thrombi in addition to massive albuminuria, kidney function deterioration, and glomerular macrophage infiltration.

Podocyte injury in B-ALDO-treated pod-double cKO mice

The intensity of podocin and nephrin staining was reduced in the glomeruli of B-ALDO-treated pod-double cKO mice (Figure 3a and b; Supplementary Figure S5C and E). On the other hand, strong podocin and nephrin staining patterns were detected in other groups, including VEH-treated mice (Figure 3a and b; Supplementary Figure S5D and F and Supplementary Figure S7A and B). Electron microscopic analysis revealed that pod-double cKO mice treated with B-ALDO had substantially more severe foot process effacement and irregular thickening of the glomerular basement membrane (Figure 3c and d and Figure 4a and b). In addition, B-ALDO-treated pod-double cKO mice exhibited an intracapillary thrombus (black arrows), sub-endothelial exudative lesions (white arrow), and loss of fenestra and endothelial cell enlargement (black heads; Figure 3e). VEH-treated pod-double cKO mice showed moderate foot process effacement as well as irregular thickening of the glomerular basement membrane (Supplementary Figure S7C–F). Mice in other groups showed only mild segmental foot process effacement (Figure 3c and d, Figure 4a; Supplementary Figure S7C–E). Next, we examined endothelial injury using CD31 and Tie-2 staining as an endothelial marker. The intensity of CD31 and Tie-2 staining was reduced only in the glomeruli of B-ALDO-treated pod-double cKO mice (Figure 3f and g; Supplementary Figure S5G and I, Supplementary Figure S8A and B), although it was well maintained in other groups, including VEH-treated mice (Figure 3f and g; Supplementary Figure S4F and G and Supplementary Figure S5G–J). These findings indicate that endothelial cells were severely injured in B-ALDO-treated pod-double cKO mice, and that B-ALDO pod-double cKO mice had both podocyte and endothelial injuries. When compared with the other groups of mice treated with B-ALDO, the number of Wilms tumor 1-positive cells in B-ALDO-treated pod-double cKO mice was reduced (Figure 4c and d). There is no significant difference in the number of Wilms tumor 1-positive cells in the VEH-treated groups (Supplementary Figure S7G and H).

These results indicate that deletion of GC-A and p38 MAPK in podocytes with B-ALDO stimulation causes podocyte loss and endothelial injuries in this model.

The glomerular gene expression in B-ALDO-treated pod-double cKO mice

A microarray experiment was conducted to investigate which genes are responsible for these severe kidney injuries

Figure 5 | (continued) showed particularly activated pathways. Factor VIII (F8) and plasminogen activator inhibitor-1 (PAI-1) were upregulated in B-ALDO-treated pod-double cKO mice. α 2AP, α -2 anti-plasmin; A2M, α -2 macroglobulin; AIAT, α -1 anti-trypsin; F2, factor II; F3, factor III; F7, factor VII; F9, factor IX; F10, factor X; F11, factor XI; F12, factor XII; PC, protein C; PCI, protein C inhibitor; TM, thrombomodulin; tPA, plasminogen activator, tissue type; uPA, plasminogen activator, urokinase; vWF, von Willebrand factor.

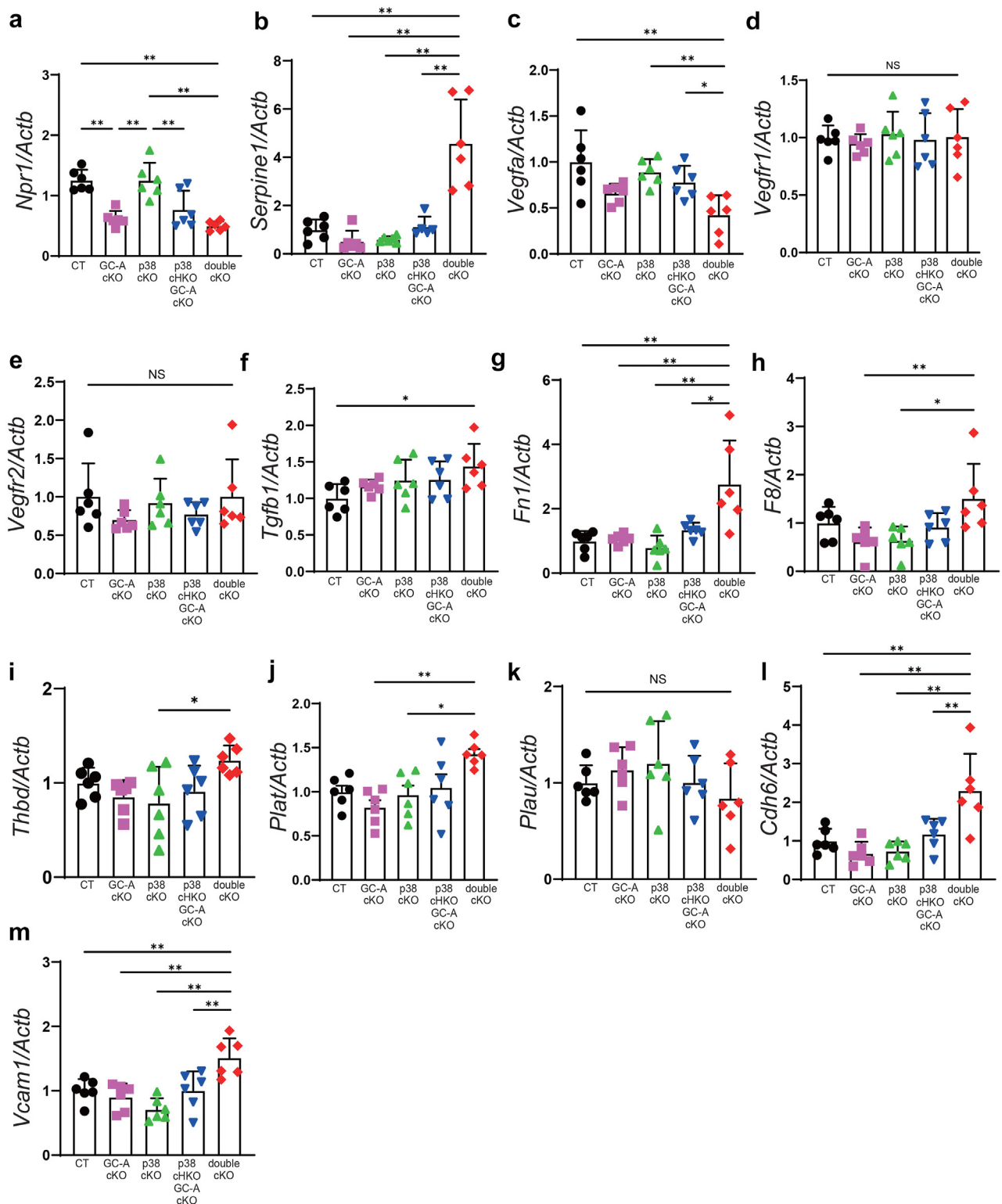


Figure 6 | Glomerular mRNA expression at 3 weeks after initiation of the experiment. Real-time reverse transcriptase–polymerase chain reaction analysis of *Npr1* (a; guanylyl cyclase-A [GC-A]), *Serpine1* (b; plasminogen activator inhibitor-1), *Vegfa* (c; vascular endothelial growth factor [VEGF]-A), *Vegfr1* (d; VEGF receptor 1 [VEGFR1]), *Vegfr2* (e; VEGFR2), *Tgfb1* (f; transforming growth factor- β 1), *Fn1* (g; fibronectin), *F8* (h; factor VIII), *Thbd* (i; thrombomodulin), *Plat* (j; plasminogen activator, tissue type), *Plau* (k; plasminogen activator, urokinase), *Cdh6* (l; cadherin 6), and *Vcam1* (m; vascular cell adhesion molecular 1). Mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$. CT, control; double cKO, podocyte-specific p38 MAPK/GC-A double knockout; GC-A cKO, podocyte-specific GC-A knockout; NS, not significant; p38 cHKO GC-A cKO, podocyte-specific p38 MAPK heterozygous/GC-A homozygous knockout; p38 cKO, podocyte-specific p38 MAPK knockout.

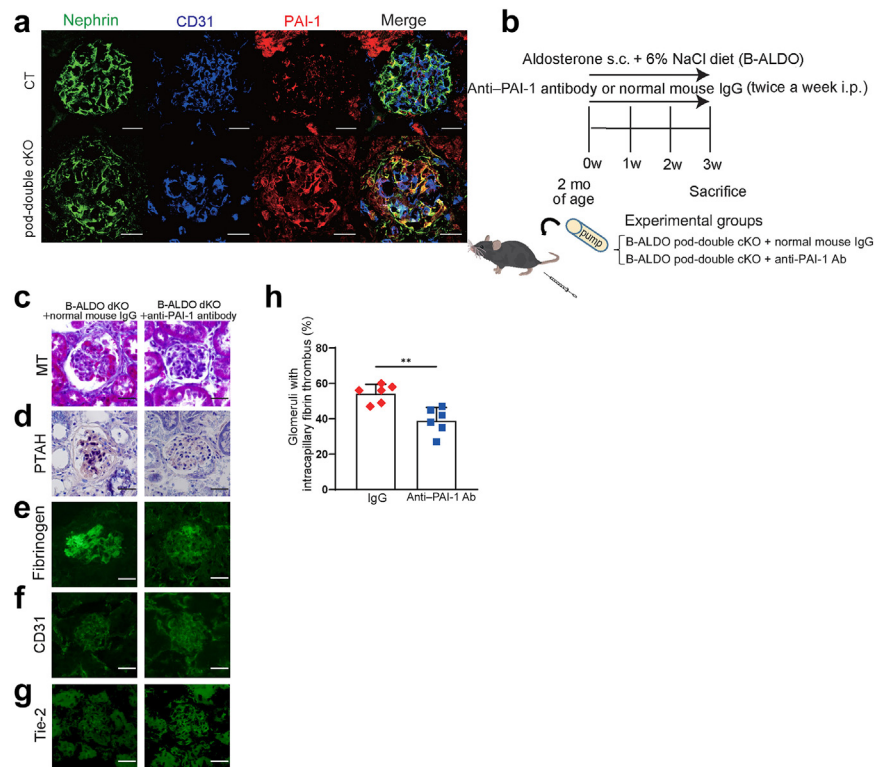


Figure 7 | Anti-plasminogen activator inhibitor-1 (PAI-1) antibody (Ab) ameliorated the kidney injury in aldosterone-infused and high-salt diet-fed (B-ALDO)-treated podocyte-specific p38 mitogen-activated protein kinase/guanylyl cyclase-A double knockout (pod-double cKO) mice. (a) Triple-immunofluorescent staining of nephrin (a podocyte marker; green), CD31 (an endothelial marker; blue), and PAI-1 (red). The staining intensity of PAI-1 was increased in B-ALDO-treated pod-double cKO mice and colocalized with nephrin. Staining intensity of nephrin and CD31 was mostly decreased in the glomeruli of B-ALDO-treated pod-double cKO mice. In this picture, a glomerulus with relatively preserved nephrin and CD31 intensities was selected so that the merged image of nephrin and PAI-1 staining could be easily seen. (b) Time course of the experiment. (c) Histologic examination of glomeruli by Masson trichrome (MT) staining. (d) Histologic examination of glomeruli by phosphotungstic acid hematoxylin (PTAH) staining. (e) Representative images of fibrinogen expression by immunofluorescent study. (f) Representative images of CD31 expression by immunofluorescent study. (g) Representative images of Tie-2 expression by immunofluorescent study. (h) The percentage of glomeruli with intracapillary fibrin thrombus. Mean $\pm$ SD. ** $P < 0.01$. Bar = 20 μ m. CT, control. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

in B-ALDO-treated pod-double cKO mice. The scatterplot with differences in glomerular mRNA expression between B-ALDO-treated pod-double cKO mice and B-ALDO-treated CT mice is presented (Figure 5a). The top 10 genes with difference in mRNA expression levels are shown in Figure 5b, and the heat map of top differentially expressed genes in pod-double cKO mice compared with control mice is shown (Figure 5c). The results of the Kyoto Encyclopedia of Genes and Genomes pathway analysis, especially the coagulation cascades, are shown in Figure 5d. PAI-1 (*Serpine1*) and factor VIII, indicated by black arrows, were particularly activated factors, and *Serpine1* was one of the candidate genes. We also confirmed that *Serpine1* and factor VIII were upregulated in B-ALDO-treated pod-double cKO mice (Figure 6b and h).

The glomerular expression of GC-A (*Npr1*) was decreased by $\approx 40\%$ in pod-GC-A cKO, pod-p38 cKO/GC-A cKO, and pod-double cKO mice compared with CT mice and pod-p38 cKO mice (Figure 6a; Supplementary Figure S9A). Next, we focused on the expression of the genes associated with thrombi from the results of the gene array (Figure 5a). The

glomerular expression of *Serpine1* in B-ALDO-treated pod-double cKO mice was increased (Figure 6b). Glomerular *Serpine1* expression in the B-ALDO-treated CT mice was high, but there were no significant differences between the VEH-treated groups (Supplementary Figure S9B). The glomerular expression of *Vegfa*, which was associated with podocyte-endothelial crosstalk, was downregulated in B-ALDO-treated pod-double cKO mice (Figure 6c). There was no significant difference in the expression of *Vegfa* mRNA expression between B-ALDO-treated CT mice and VEH-treated mice in all groups of mice (Supplementary Figure S9C). Glomerular *Vegfr1* (vascular endothelial growth factor receptor 1 [VEGFR]) and *Vegfr2* mRNA were not significantly different in B-ALDO-treated all groups of mice (Figure 6d and e). B-ALDO treatment increased glomerular *Vegfr1* and *Vegfr2* mRNA (Supplementary Figure S9D and E). VEGFR1 was not detected in the glomeruli by immunofluorescent staining, indicating its low protein level (Supplementary Figure S10A). Immunofluorescent staining revealed increased staining intensity of VEGFR2 in all B-ALDO-treated groups of mice compared with all

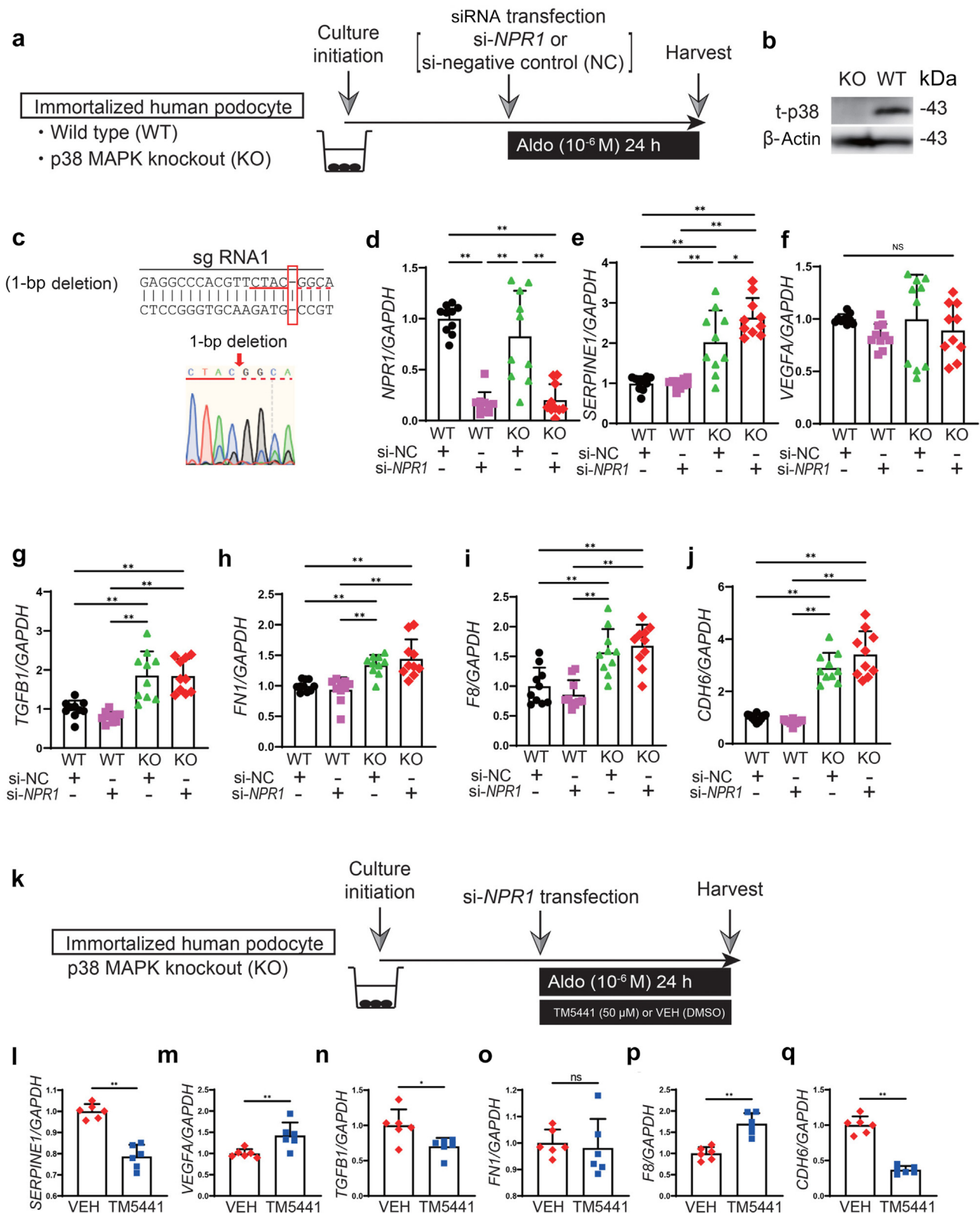


Figure 8 | Suppression of guanylyl cyclase-A (GC-A) and deletion of p38 mitogen-activated protein kinase (MAPK) in human induced podocyte injury *in vitro*. (a) The experimental protocol. We used immortalized wild-type human podocytes (WT) and p38 MAPK knockout podocytes (KO) using CRISPR/Cas9 system. We harvested podocytes 24 hours after transfecting small interfering (si)-NPR1 or control siRNA and administered aldosterone (10^{-6} M). (b) Western blotting of total p38 MAPK. p38 MAPK was completely deleted in p38 MAPK knockout podocytes. (c) Sanger sequencing analysis of cultured p38 MAPK knockout podocytes. (d–i) Analyses for mRNA expression of NPR1 (d; GC-A), SERPINE1 (e; plasminogen activator inhibitor-1 [PAI-1]), VEGFA (f; vascular endothelial growth factor-A), TGFB1 (continued)

VEH-treated groups of mice (Supplementary Figure S10B), suggesting activation of VEGFR2 pathway in all B-ALDO-treated groups of mice. The expression levels of *Tgfb1* and fibronectin (*Fn1*) in the glomeruli of B-ALDO-treated pod-double cKO mice were high (Figure 6f and g). Expression levels of *Tgfb1* and *Fn1* of the B-ALDO-treated CT mice were higher than those in VEH-treated mice, but there was no significant difference among the VEH-treated groups (Supplementary Figure S9F and G). Then, we next examined the expression of mRNAs of the genes associated with coagulation factor. The glomerular mRNA expression levels of coagulation factor VIII (*F8*), thrombospondin (*Thbd*), and tissue-type plasminogen activator (*Plat*) in B-ALDO-treated pod-double cKO mice were higher than those of B-ALDO pod-p38 cKO mice (Figure 6h–j). B-ALDO treatment increased *F8*, decreased *Plat*, and did not alter *Thbd* in glomerular mRNA expression (Supplementary Figure S9H–J). The glomerular expression level of F3 and urokinase-type plasminogen activator (*Plau*) was not significantly different in all B-ALDO-treated groups of mice, or in all VEH-treated groups of mice (Figure 6k; Supplementary Figure S9K and L and Supplementary Figure S11A). These findings suggest that activation of several molecules, including PAI-1, factor VIII, thrombospondin, and tissue-type plasminogen activator, may be involved in the formation of intracapillary thrombi.

Among many genes that were significantly increased in B-ALDO-treated pod-double cKO mice by gene microarray, we confirmed the increase of cadherin 6 (*Cdh6*) and *Vcam1* (Figure 6l and m). The glomerular mRNA expression of *Cdh6* and *Vcam1* of the B-ALDO CT mice was higher than that of VEH-treated mice (Supplementary Figure S9M and N). Neuropilin 1 and 2 (*Nrp1* and *Nrp2*) are shown to be coreceptors for VEGFRs. Glomerular *Nrp1* and *Nrp2* mRNA were not significantly different in all B-ALDO-treated groups of mice (Supplementary Figure S11B and C). B-ALDO treatment increased glomerular *Nrp1* and *Nrp2* mRNA (Supplementary Figure S9O and P). Immunofluorescent staining revealed increased staining intensity of neuropilin-1 in all B-ALDO-treated groups of mice (Supplementary Figure S10C), suggesting activation of neuropilin-1 pathway in these mice. Neuropilin-2 was not detected in the glomeruli by immunofluorescent staining, indicating its low protein level.

These results indicate that the glomerular mRNA expression of PAI-1 and extracellular matrix-related genes, including transforming growth factor beta 1 (TGF- β 1), was increased and mRNA expression of VEGF-A was decreased without the change of VEGFR signaling in B-ALDO-treated double cKO mice compared with B-ALDO-treated control mice.

PAI-1 neutralizing antibody ameliorated the glomerular injury in B-ALDO-treated pod-double cKO mice

Triple-immunofluorescent staining for PAI-1, nephrin (a podocyte marker), and CD31 (an endothelial marker) revealed increased staining intensity of PAI-1 and colocalization of PAI-1 and nephrin in B-ALDO-treated pod-double cKO mice (Figure 7a), indicating increased PAI-1 in podocytes. To investigate the significance of PAI-1 upregulation in podocytes, we administered PAI-1 neutralizing antibody or normal mouse IgG i.p. to B-ALDO-treated pod-double cKO mice twice a week for 3 weeks (Figure 7b). Masson trichrome, phosphotungstic acid hematoxylin, fibrinogen, CD31, and Tie-2 staining demonstrated that glomerular injury in B-ALDO-treated pod-double cKO mice was ameliorated by anti-PAI-1 antibody (Figure 7c–g). Although the urinary albuminuria between 2 groups was not statistically different (Supplementary Figure S12), the percentage of glomeruli in which intracapillary fibrin thrombi was observed in B-ALDO-treated pod-double cKO mice with anti-PAI-1 antibody was reduced compared with those treated with normal mouse IgG (Figure 7h). These results suggest that PAI-1 is one of the podocyte-derived endothelial injury factors that exhibit intracapillary thrombi in B-ALDO-treated pod-double cKO mice.

p38 MAPK inhibitor did not ameliorate kidney injury in B-ALDO-treated pod-double cKO mice

In this study, we found that in B-ALDO pod-GC-A cKO mice, deletion of p38 MAPK in podocytes aggravated kidney injury. We previously demonstrated that blocking p38 MAPK with a p38 MAPK inhibitor (FR167653) ameliorated glomerular injury in ALDO-treated pod-GC-A cKO mice.¹¹ We used FR167653 to investigate these differences in male B-ALDO-treated pod-double cKO mice (Supplementary Figure S13A). FR167653 in B-ALDO-treated pod-double cKO mice did not change the blood pressure (Supplementary Figure S13B), the urinary albumin excretion (Supplementary Figure S13C), serum creatinine, total cholesterol, and albumin (Supplementary Figure S13D), mesangial expansion, intracapillary fibrin thrombi, the number of MAC-2–positive cells (Supplementary Figure S13E–I), the reduced podocin and nephrin in glomeruli (Supplementary Figure S14A and B), the severe foot process effacement and glomerular basement membrane thickening (Supplementary Figure S14C–F), the number of Wilms tumor 1–positive cells (Supplementary Figure S14G and H), and mRNA expressions of *Tgfb1*, *Vegfa*, *Serpine1*, *Fn1*, *Cdh6*, and *Vcam1* (Supplementary Figure S15A–F). In this study, a p38 MAPK inhibitor did not ameliorate kidney injury in B-ALDO-treated pod-double

Figure 8 | (continued) (g; transforming growth factor [TGF]- β 1), *FN1* (h; fibronectin), *F8* (i; factor VIII), and *CDH6* (j; cadherin 6). (k) The experimental protocol. We cultured immortalized p38 MAPK knockout podocytes (KO) transfecting si-NPR1 and administered aldosterone (10^{-6} M) and TM5441 (PAI-1 inhibitor) or vehicle (VEH; dimethylsulfoxide [DMSO]) in the podocytes. (l–q) Analyses of podocyte mRNA expression of *SERPINE1* (l; PAI-1), *VEGFA* (m; VEGFA), *TGFB1* (n; TGF- β 1), *FN1* (o; fibronectin), *F8* (p; factor VIII), and *CDH6* (q; cadherin 6). Mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$. NC, negative control; sgRNA, single-guide RNA.

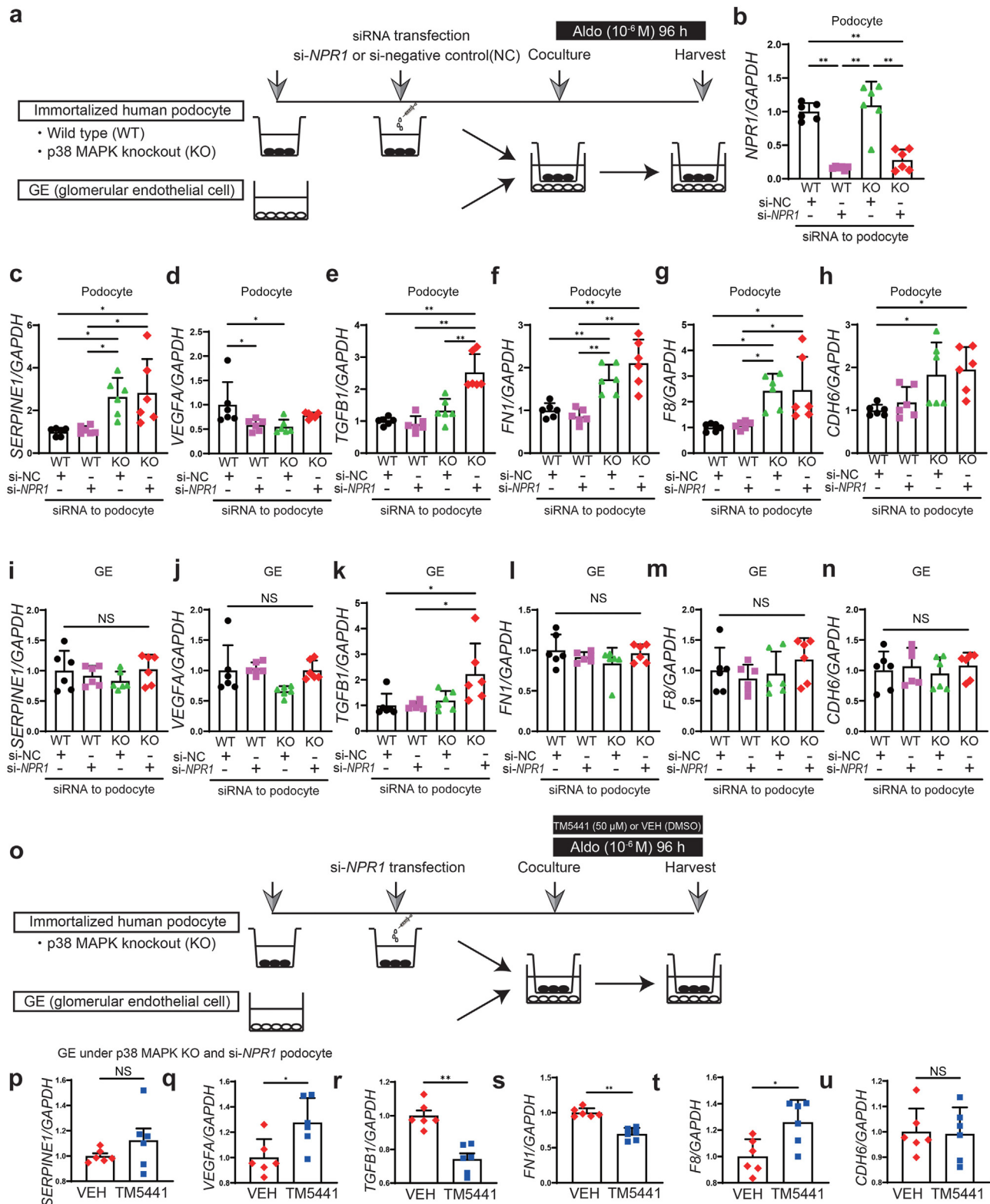


Figure 9 | Suppression of guanylyl cyclase-A (GC-A) and deletion of p38 mitogen-activated protein kinase (MAPK) in human podocytes induced not only podocyte damage but also glomerular endothelial cell damage in transwells. (a) The experimental protocol. We cultured immortalized wild-type human podocytes (WT) and p38 MAPK knockout podocytes (KO) in the upper layer and wild-type glomerular endothelial cells in the lower layer in the transwells. Transfection of small interfering RNA (siRNA) was performed only in podocytes. Glomerular endothelial cells (GEs) were cultured without transfection or gene deletion. We harvested podocytes and glomerular endothelial cells at 96 hours after transfecting small interfering (si)-NPR1 or control siRNA and administered aldosterone (10^{-6} M) in the podocytes. **(b–h)** Analyses of podocyte mRNA expression of NPR1 **(b)**; GC-A, SERPINE1 **(c)**; plasminogen activator inhibitor-1 [PAI-1], VEGFA **(d)**; vascular endothelial growth factor-A [VEGFA], TGFB1 **(e)**; transforming growth factor [TGF]- β 1, FN1 **(f)**; fibronectin, F8 **(continued)**

cKO mice, suggesting the importance of p38 MAPK in podocytes in this model.

Suppression of GC-A and deletion of p38 MAPK in human podocytes induced podocyte injury *in vitro*

The roles of p38 MAPK and GC-A in podocyte injury *in vitro* were then examined. Using the CRISPR/Cas9 method, we were able to delete p38 MAPK (MAPK14) in cultured human podocytes (Supplementary Figure S16A). Immortalized p38 MAPK knockout human podocytes were differentiated, then transfected with small interfering *NPR1* RNA, treated with aldosterone (10^{-6} M), and harvested 24 hours later (Figure 8a). MAPK14 was undetectable at the protein level in p38 MAPK knockout podocytes (Figure 8b). The Sanger sequencing analysis also revealed a 1-bp deletion in the target sequence, whereas it denied the possible off-target mutation of the candidate site (Figure 8c and Supplementary Figure S16B).

The expression level of *NPR1* was reduced to one-third by transfection (Figure 8d). Deletion of p38 MAPK and inhibition of GC-A upregulated *SERPINE1*, *TGFB1*, *FN1*, *F8*, and *CDH6* (Figure 8e and g–j) and tended to downregulate *VEGFA* (Figure 8f) in human cultured podocytes. In particular, *SERPINE1* expression was prominent (Figure 8e). Next, we administered TM5441 (PAI-1 inhibitor) or vehicle (dimethylsulfoxide) to p38 MAPK-null podocytes (Figure 8k). TM5441 decreased *SERPINE1*, *TGFB1*, and *CDH6* (Figure 8l, n, and q) and increased *VEGFA* and *F8* (Figure 8m and p) and did not alter *FN1* (Figure 8o), as mRNA in the treated podocytes, indicating that PAI-1 inhibitor ameliorated podocyte injury. These results are consistent with the *in vivo* results, showing that deletion of p38 MAPK and suppression of GC-A in podocytes induce podocyte injury partly through PAI-1 pathway.

In a coculture system, GC-A suppression and p38 MAPK deletion in human podocytes induced not only podocyte damage but also endothelium injury

In the next experiment, we used a transwell coculture system to culture immortalized wild human podocytes and p38 MAPK-null podocytes transfected with small interfering *NPR1* or control small interfering RNA in the upper layer and wild-type glomerular endothelial cells in the lower layer and harvested them 96 hours after aldosterone administration (10^{-6} M; Figure 9a). The expression level of *NPR1* was reduced to one-third of that of control podocytes by transfection (Figure 9b). Deletion of p38 MAPK and inhibition of GC-A upregulated *SERPINE1*, *TGFB1*, *FN1*, *F8*, and *CDH6* mRNA in human cultured podocytes and tended to downregulate *VEGFA* mRNA in human cultured podocytes

(Figure 9c–h). Deletion of p38 MAPK and inhibition of GC-A in podocytes upregulated *TGFB1* in the glomerular endothelial cells (Figure 9k). The expression of *SERPINE1*, *VEGFA*, *FN1*, *F8*, and *CDH6* in the glomerular endothelial cells did not exhibit any significant difference in each group (Figure 9i, j, and l–n). We obtained similar results using a transwell coculture system to treated podocytes in the upper layer and human umbilical vein endothelial cells in the lower layer instead of glomerular endothelial cells (Supplementary Figure S17). These results may indicate that some humoral factors, including *SERPINE1* and *TGFB1*, derived from podocytes, could work as cell-to-cell mediators, especially for glomerular endothelial cells. In the next experiment, we administered TM5441 (PAI-1 inhibitor) or vehicle (dimethylsulfoxide) to the previous transwell systems (Figure 9o). TM5441 treatment in the podocytes decreased *TGFB1* and *FN1* (Figure 9r and s) and increased *VEGFA* and *F8* (Figure 9q and t) and did not alter *SERPINE1* or *CDH6* (Figure 9p and u) in glomerular endothelial cells. These results suggest that inhibition of GC-A and p38 MAPK in podocytes causes glomerular endothelial injury, including upregulation of TGF- β 1 partly through the PAI-1 pathway.

DISCUSSION

Many reports have shown that inhibiting p38 MAPK has beneficial effects on inflammatory diseases.^{11,16,17} Consistent with these previous finding, we have already reported that inhibition of p38 MAPK by p38 MAPK inhibitor (FR167653) ameliorates glomerular injury in ALDO pod-GC-A cKO mice.¹¹ Analysis of podocyte-specific p38 MAPK knockout mice was novel, though Muller *et al.* have reported that inducible systemic p38 α MAPK knockout mice ameliorate tubular damage in anti-glomerular basement membrane nephritis.²² Unexpectedly, the present study demonstrated that kidney damage in B-ALDO pod-double cKO mice was greatly exacerbated. Some reports have shown that suppression of p38 MAPK exacerbates kidney lesions^{23–25} and that, in part, p38 MAPK has cytoprotective effects against kidney injury.²⁴ Our present study also demonstrated that genetic deletion of p38 MAPK in podocytes aggravated kidney injury, suggesting that the presence of an adequate level of p38 MAPK is cytoprotective against kidney injury.

We speculate that the discrepancy between these 2 studies is attributable to 2 factors: the degree to which p38 MAPK was inhibited and the site of action of the p38 MAPK inhibitor. First, in the previous study, we partially suppressed the effect of p38 MAPK by medication, but in this present study, we almost completely deleted p38 MAPK genetically. Second, in

Figure 9 | (continued) (g; factor VIII), and *CDH6* (h; cadherin 6). (i–n) Analyses of glomerular endothelial cell mRNA expression of *SERPINE1* (i; PAI-1), *VEGFA* (j; VEGFA), *TGFB1* (k; TGF- β 1), *FN1* (l; fibronectin), *F8* (m; factor VIII), and *CDH6* (n; cadherin 6). (o) The experimental protocol. We cultured immortalized p38 MAPK knockout podocytes (KO) transfected si-*NPR1* in the upper layer and wild-type glomerular endothelial cells in the lower layer in the transwells. We administered aldosterone (10^{-6} M) and TM5441 (PAI-1 inhibitor) or vehicle (VEH; dimethylsulfoxide [DMSO]) in the podocytes. (p–u) Analyses of the glomerular endothelial cell mRNA expression of *SERPINE1* (p; PAI-1), *VEGFA* (q; VEGFA), *TGFB1* (r; TGF- β 1), *FN1* (s; fibronectin), *F8* (t; factor VIII), and *CDH6* (u; cadherin 6). Mean $\pm$ SD. * P < 0.05, ** P < 0.01. NC, negative control.

the previous study, p38 inhibitor acts on the whole body, but in this present study, p38 MAPK is genetically deleted only in podocytes. By administering p38 MAPK inhibitor to B-ALDO-treated pod-double cKO mice, we found that the p38 MAPK inhibitor was effective in ALDO-treated pod-GC-A cKO mice, but that it is not effective on B-ALDO-treated pod-double cKO mice. These results indicate that p38 MAPK in podocytes is important in this model, but since this model also causes endothelial damage, we cannot conclude from these results alone that p38 MAPK is acting mainly on podocytes. We demonstrated that B-ALDO pod-double cKO mice exhibit severe foot process effacement and intracapillary thrombi, which resemble the glomeruli seen in the podocyte-specific VEGF knockout mice exhibiting thrombotic microangiopathy.^{26,27} Inhibition of VEGF in preeclampsia or suppression by anti-VEGF medications has been reported to cause thrombotic microangiopathy.^{27,28} Although the mechanism of thrombus formation in this study has not been fully investigated in detail, platelet thrombi are generally formed because of broad damage to endothelial cells, followed by fibrin deposition and fibrin thrombus formation. Tissue plasminogen activator produces plasmin and enhances the fibrinolytic system, and PAI-1 inhibits this system. The fact that PAI-1 was elevated in this study suggests that the fibrin thrombus formed by endothelial cell damage was not dissolved. Gregory *et al.* demonstrated that the fibrin-rich thrombus is shown by phosphotungstic acid hematoxylin staining.²⁹ In this study, B-ALDO-treated pod-double cKO mice had more deposits that stained violet in phosphotungstic acid hematoxylin staining, which is pathologically consistent with fibrin thrombi. Our results indicate that crosstalk is acting between podocytes and endothelial cells in B-ALDO-treated pod-double cKO mice, and that p38 MAPK in podocytes is necessary to protect not only epithelial cells but also endothelial cells from aldosterone-induced injury. We found PAI-1 elevation and VEGF downregulation in the glomeruli of B-ALDO pod-double cKO mice by microarray analysis. Many mediators of crosstalk between glomerular podocytes and endothelial cells have been reported.³⁰ Downregulation of VEGF-A in podocytes causes endothelial damage, and PAI-1 is involved in endothelial injury.^{31,32} We consider that one possible explanation for kidney injury in B-ALDO pod-double cKO mice is VEGF downregulation in podocytes, which causes endothelial damage, and that another is PAI-1 elevation in podocytes for endothelial injury in B-ALDO pod-double cKO mice.

Kitching *et al.* demonstrated that PAI-1 is an important factor in glomerular fibrin deposition in crescentic glomerulonephritis.³³ Furthermore, mice overexpressing PAI-1 form crescents with more extensive fibrin deposits.³³ It is becoming increasingly clear that PAI-1 plays a role in the interstitial recruitment of inflammatory macrophages and myofibroblast transition.³⁴ In B-ALDO-treated pod-double cKO mice, we demonstrated that PAI-1 increased in the podocytes and PAI-1 neutralizing antibody ameliorated the glomerular injury. Therefore, elevation of PAI-1 may be one of the important

mechanisms of kidney injury in B-ALDO pod-double cKO mice.

In vitro, deletion of p38 MAPK by the CRISPR/Cas9 system and suppression of GC-A by small interfering RNA upregulated PAI-1, TGF- β , fibronectin, factor VIII, and cadherin 6 in human cultured podocytes. Furthermore, using a coculture transwell system, podocytes and glomerular endothelial cells were cocultured, and the expression of TGF- β was upregulated in the glomerular endothelial cells under p38 MAPK-deleted and GC-A-downregulated podocytes. Moreover, we demonstrated that PAI-1 inhibitor ameliorated the podocyte injury and endothelial injury as well in the transwell system. Therefore, elevation of PAI-1 in podocytes by downregulation of GC-A and deletion of p38 MAPK may be associated with podocyte-endothelial crosstalk.

In conclusion, the genetical deletion of p38 MAPK in the podocyte aggravated podocyte injury in the setting of the absence of GC-A signaling, indicating that p38 MAPK in podocytes has an essential role in maintaining podocyte-endothelial interactions, especially under diseased conditions.

DISCLOSURE

HYo received research funding from Mitsubishi Tanabe Pharmaceutical Corporation; and honoraria from AstraZeneca and Mitsubishi Tanabe Pharmaceutical Corporation. MY received research grants from Mitsubishi Tanabe Pharmaceutical Corporation and Boehringer Ingelheim. All the other authors declared no competing interests.

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AUTHOR CONTRIBUTIONS

SS, HYa, Als, and HYo designed the study; SS, HYa, YK, and RY performed experiments; KPM, SO, TH, Aik, TI, KO, TT, TM, ARN, MY, and HYo performed the interpretation of results; SS and HYo wrote the article; and all authors approved the final version of the article.

SUPPLEMENTARY MATERIAL

[Supplementary File \(PDF\)](#)

Supplementary Methods.

Supplementary Figure S1. The renal and cardiac tissues, systolic blood pressure, and albuminuria of healthy (no intervention)

2-month-old pod-double cKO mice. (A) Histologic examination of glomeruli by periodic acid–Schiff staining. (B) Histologic examination of heart by hematoxylin–eosin staining. (C) Systolic blood pressure and albuminuria of 2-month-old healthy pod-double cKO mice. Mean $\pm$ SD. Bar = 20 μ m.

Supplementary Figure S2. VEH-treated pod-double cKO mice showed higher systolic blood pressure and more severe albuminuria than those of other VEH-treated groups, but all VEH-treated groups of mice did not show any significant differences in blood test results. (A) Time course of the experiment. (B) Time course of systolic blood pressure in VEH-treated CT mice, pod-GC-A cKO mice, pod-p38 MAPK cKO mice, and pod-double cKO mice (106.3 ± 3.3 , 103.0 ± 5.5 , 107.0 ± 5.6 , and 124.3 ± 1.5 mm Hg, respectively). The systolic blood pressure of VEH-treated pod-double cKO mice was higher than that of mice in other groups. (C) Time course of albuminuria in these 4 groups. VEH-treated CT, pod-GC-A cKO, pod-p38 cKO, and pod-double cKO mice exhibited slight albuminuria (45.3 ± 6.8 , 33.2 ± 9.0 , 50.5 ± 10.2 , and 386.5 ± 214.6 μ g/mg Cr, respectively). The albuminuria of VEH-treated pod-double cKO mice was more severe than that of mice in other groups. (D) Biochemical parameters (Cr, serum creatinine; T-cho, serum total cholesterol; Alb, serum albumin) in these 4 groups. Mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ versus other groups. $\times P < 0.05$ versus CT mice. $\dagger P < 0.05$ versus pod-GC-A cKO mice.

Supplementary Figure S3. B-ALDO-treated pod-double cKO mice exhibited deletion of total p38 MAPK in podocytes, periodic acid–Schiff (PAS)-positive deposits, and prominent phosphorylation of p38 MAPK. (A) The double-immunofluorescent staining with nephrin (green) and total p38 (t-p38) MAPK (red) in the higher magnification. (B) Histologic examination of glomeruli by periodic acid–Schiff staining at 3 weeks. B-ALDO-treated pod-double cKO mice exhibited PAS-positive deposits in glomeruli. (C) Histologic examination of p-p38 MAPK of glomeruli. Phosphorylation of p38 MAPK in glomeruli was increased in B-ALDO-treated pod-double cKO mice. Bar = 20 μ m.

Supplementary Figure S4. All VEH-treated groups of mice did not show any significant differences in renal histology. (A) Light microscopic analyses were performed at 3 weeks after vehicle administration, stained with PAS. (B) Histologic examination of glomeruli by Masson trichrome staining at 3 weeks. (C) Histologic examination of tubules and interstitial fibrosis by Masson trichrome staining at 3 weeks. (D) Histologic examination of glomeruli by phosphotungstic acid hematoxylin (PTAH) staining at 3 weeks. (E) Representative images of fibrinogen expression by immunofluorescent study. (F) Representative images of CD31 by immunofluorescent study. (G) Representative images of Tie-2 by immunofluorescent study. (H) Immunohistochemical study for MAC-2 in the glomeruli at 3 weeks. (I) The number of MAC-2-positive cells in a glomerulus. There were no differences in MAC-2-positive cells in these 4 groups. Mean $\pm$ SD. Bar = 20 μ m.

Supplementary Figure S5. The intensity of fibrinogen was increased, and the intensity of podocin, nephrin, CD31, and Tie2 immunostainings was reduced, in B-ALDO-treated pod-double cKO mice. The intensity of fibrinogen, podocin, nephrin, CD31, and Tie2 immunostainings was not statistically different in all VEH-treated types of mice. (A) The intensity of fibrinogen in B-ALDO-treated mice. (B) The intensity of fibrinogen in VEH-treated mice. (C) The intensity of podocin in B-ALDO-treated mice. (D) The intensity of podocin in VEH-treated mice. (E) The intensity of nephrin in B-ALDO-treated mice. (F) The intensity of nephrin in VEH-treated mice. (G) The intensity of CD31 in B-ALDO-treated mice. (H) The intensity of CD31 in VEH-treated mice. (I) The intensity of Tie2 in B-ALDO-treated mice. (J) The intensity of Tie2 in VEH-treated mice. Mean $\pm$ SD. ** $P < 0.01$.

Supplementary Figure S6. The number of CD3-positive cells in a glomerulus in B-ALDO-treated pod-double cKO mice increased, but C3 deposit was not found in all B-ALDO-treated types of mice. (A) Representative images of CD3 staining. (B) The number of CD3-positive cells in a glomerulus. The number of CD3-positive cells in a glomerulus increased in B-ALDO-treated pod-double cKO mice. (C) Representative images of C3 by immunofluorescent study. C3 deposit was not seen in all B-ALDO-treated types of mice. Mean $\pm$ SD. ** $P < 0.01$. Bar = 20 μ m.

Supplementary Figure S7. VEH-treated pod-double cKO mice did not exhibit reduction in podocin or nephrin staining or the number of Wilms tumor 1 (WT1)-positive cells but exhibited severe foot process effacement with irregular thickening of the glomerular basement membrane. (A,B) Representative images of podocin (A) and nephrin (B) expression by immunofluorescent study. All VEH-treated mice demonstrated strong staining of podocin and nephrin. (C,D) Electron microscopic photographs of lower (C) and higher (D) magnification. VEH-treated pod-double cKO mice exhibited severe foot process effacement with irregular thickening of the glomerular basement membrane. (E) The width of foot processes in electron microscopic photographs. (F) The width of glomerular basement membrane (GBM) in electron microscopic photographs. (G) Immunohistochemical study for WT1 in the glomeruli. (H) The number of WT1-positive cells in a glomerulus. There were no significant differences in the number of WT1-positive cells in these 4 groups. Mean $\pm$ SD. ** $P < 0.01$. Bar = 20 μ m (A,B,G).

Supplementary Figure S8. Representative images of Tie-2 by immunofluorescent study. Representative images of Tie-2 by immunofluorescent study in a high-power field (A; original magnification $\times 40$) and in a low-power field (B; original magnification $\times 10$). The glomerulus of B-ALDO-treated pod-double cKO mice showed weaker staining intensity of Tie-2, which indicated that B-ALDO-treated pod-double cKO mice exhibited endothelial injury. Bar = 20 μ m.

Supplementary Figure S9. Glomerular mRNA expression at 3 weeks in VEH- and B-ALDO-treated mice. Real-time RT-PCR analyses of *Npr1* (A; GC-A), *Serpine1* (B; PAI-1), *Vegfa* (C; VEGF-A), *Vegfr1* (D; Vegfr1), *Vegfr2* (E; Vegfr2), *Tgfb1* (F; TGF- β 1), *Fn1* (G; fibronectin), *F8* (H; factor VIII), *Thbd* (I; thrombomodulin), *Plat* (J; plasminogen activator, tissue type), *F3* (K; factor III), *Plau* (L; plasminogen activator, urokinase), *Cdh6* (M; cadherin 6), *Vcam1* (N; Vcam1), *Nrp1* (O; neuropilin 1), and *Nrp2* (P; neuropilin 2). Mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$.

Supplementary Figure S10. Immunofluorescent staining of VEGFR2 and neuropilin-1 revealed increased staining intensity of VEGFR2 and neuropilin-1 in all B-ALDO-treated groups of mice compared with those of all VEH-treated groups of mice. VEGFR1 staining was not detected in the glomeruli. (A) Representative images of VEGFR1 by immunofluorescent study. (B) Representative images of VEGFR2 by immunofluorescent study. (C) Representative images of neuropilin-1 by immunofluorescent study. Bar = 20 μ m.

Supplementary Figure S11. The glomerular mRNA expression level of F3 (tissue factor), *Nrp1*, and *Nrp2*. The glomerular mRNA expression level of F3 (A; tissue factor), *Nrp1* (B; neuropilin 1), and *Nrp2* (C; neuropilin 2) was not significantly different in all B-ALDO-treated groups of mice.

Supplementary Figure S12. The urinary albuminuria of B-ALDO-treated pod-double cKO mice with anti-PAI-1 antibody was not statistically different but tended to decrease compared with that of B-ALDO-treated pod-double cKO mice with normal mouse IgG at the end of the experiment.

Supplementary Figure S13. FR167653 (p38 MAPK inhibitor) did not ameliorate kidney injury in B-ALDO pod-double cKO mice. (A) Time course of the experiment. (B) Time course of systolic

blood pressure in B-ALDO-treated pod-double cKO mice with vehicle or FR167653. (C) Time course of albuminuria in B-ALDO-treated pod-double cKO mice with vehicle or FR167653. (D) Biochemical parameters (Cr, serum creatinine; T-cho, serum total cholesterol; Alb, serum albumin) in these 2 groups. (E) The Masson trichrome (MT) staining of these 2 groups. (F) The phosphotungstic acid hematoxylin (PTAH) staining of these 2 groups. (G) The fibrinogen immunostaining of these 2 groups. (H) The MAC-2 staining in these 2 groups. (I) The number of MAC-2-positive cells in a glomerulus is not statistically different in these 2 groups. Mean $\pm$ SD. Bar = 20 μ m.

Supplementary Figure S14. FR167653 (p38 MAPK inhibitor) did not ameliorate the kidney injury in B-ALDO-treated pod-double cKO mice. (A,B) Representative images of podocin (A) and nephrin (B) expression by immunofluorescent study. (C,D) Electron microscopic photographs of lower (C) and higher (D) magnification. (E) The width of foot process in electron microscopic photographs. (F) The width of glomerular basement membrane (GBM) in electron microscopic photographs. (G) Immunohistochemical study for Wilms tumor 1 (WT1) in a glomerulus. (H) The number of WT1-positive cells in a glomerulus. FR167653 did not ameliorate reduced number of WT1-positive cells. Mean $\pm$ SD. Bar = 20 μ m.

Supplementary Figure S15. p38 MAPK inhibitor did not ameliorate the kidney injury in B-ALDO-treated pod-double cKO mice. Glomerular mRNA expression at 3 weeks after aldosterone and FR167653 administration. Real-time RT-PCR analysis of *Tgfb1* (A; TGF- β 1), *Vegfa* (B; VEGF-A), *Serpine1* (C; PAI-1), *Fn1* (D; fibronectin), *Cdh6* (E; cadherin 6), and *Vcam1* (F; Vcam1). Mean $\pm$ SD.

Supplementary Figure S16. MAPK14 deletion of human podocyte by CRISPR/Cas9 system. (A) Designing sg-RNA for p38 MAPK knockout (KO) podocytes. (B) Sanger sequencing analysis of the off-target candidate site in cultured p38 MAPK KO podocytes.

Supplementary Figure S17. Suppression of GC-A and deletion of p38 MAPK in human podocytes induced not only podocyte damage but also human umbilical vein endothelial cell (HUVEC) damage in transwells. (A) The experimental protocol. We cultured immortalized wild human podocytes (WT) and p38 MAPK knockout podocytes (KO) in the upper layer and wild-type HUVECs in the lower layer in the transwells. Transfection of si-RNA was performed only in podocytes. HUVECs were cultured without transfection or gene deletion. We harvested podocytes and HUVECs at 72 hours after transfecting si-NPR1 or control small interfering RNA (siRNA) and administered aldosterone (10^{-6} M) in the podocytes. (B–H) Analyses of podocyte mRNA expression of *NPR1* (B; GC-A), *SERPINE1* (C; PAI-1), *VEGFA* (D; VEGFA), *TGFB1* (E; TGF- β 1), *FN1* (F; fibronectin), *F8* (G; factor VIII), and *CDH6* (H; cadherin 6). (I–N) Analyses of HUVEC mRNA expression of *SERPINE1* (I; PAI-1), *VEGFA* (J; VEGFA), *TGFB1* (K; TGF- β 1), *FN1* (L; fibronectin), *F8* (M; factor VIII), and *CDH6* (N; cadherin 6). Mean $\pm$ SD. * P < 0.05, ** P < 0.01.

Supplementary Table S1. TaqMan primer and probe sequences.

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