Protein S Protects against Podocyte Injury in Diabetic Nephropathy

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ABSTRACT

Background Diabetic nephropathy (DN) is a leading cause of ESRD in the United States, but the molecular mechanisms mediating the early stages of DN are unclear.

Methods To assess global changes that occur in early diabetic kidneys and to identify proteins potentially involved in pathogenic pathways in DN progression, we performed proteomic analysis of diabetic and non-diabetic rat glomeruli. Protein S (PS) among the highly upregulated proteins in the diabetic glomeruli. PS exerts multiple biologic effects through the Tyro3, Axl, and Mer (TAM) receptors. Because increased activation of Axl by the PS homolog Gas6 has been implicated in DN progression, we further examined the role of PS in DN.

Results In human kidneys, glomerular PS expression was elevated in early DN but suppressed in advanced DN. However, plasma PS concentrations did not differ between patients with DN and healthy controls. A prominent increase of PS expression also colocalized with the expression of podocyte markers in early diabetic kidneys. In cultured podocytes, high-glucose treatment elevated PS expression, and PS knockdown further enhanced the high-glucose–induced apoptosis. Conversely, PS overexpression in cultured podocytes dampened the high-glucose– and TNF-α–induced expression of proinflammatory mediators. Tyro3 receptor was upregulated in response to high glucose and mediated the anti-inflammatory response of PS. Podocyte-specific PS loss resulted in accelerated DN in streptozotocin-induced diabetic mice, whereas the transient induction of PS expression in glomerular cells *in vivo* attenuated albuminuria and podocyte loss in diabetic OVE26 mice.

Conclusions Our results support a protective role of PS against glomerular injury in DN progression.

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Diabetic nephropathy (DN) is a leading cause of ESRD in the United States.[1] Although the current management of tight glycemic control and renin

Significance Statement

Elucidating the mechanisms that mediate injury in early diabetic nephropathy is necessary for development of novel preventive and therapeutic measures. Aided by the proteomic analysis of an experimental rodent model of DN, the authors have identified protein S as a highly expressed protein in early DN, but not in late DN. Protein S is a well studied plasma glycoprotein that serves as an essential cofactor in pathways that regulate coagulation. The study demonstrates that this temporal increase in protein S signal transduction through Tyro3 receptor thwarts DN progression and that the eventual loss of protein S results in worsened DN outcome. The findings reveal a previously unrecognized role of protein S in kidney cells and suggest that approaches to elevate protein S signaling may have therapeutic benefit against DN progression.

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angiotensin system blockade slows the progression of DN, many patients continue to progress toward ESRD.[1] Thus, elucidating the mechanisms that mediate the early stages of diabetic kidney injury may help identify novel preventive and therapeutic measures for DN. Toward this goal, we performed proteomic analysis of glomeruli isolated from diabetic and nondiabetic control rats. We identified protein S (PS) to be among the highly upregulated proteins, which was reversed with insulin treatment. Interestingly, the increase in glomerular PS levels in diabetic rats did not correlate with the serum concentrations of PS, suggestive of kidney cell–specific upregulation of PS in the diabetic kidney.

PS is a plasma glycoprotein whose function is well established as an essential cofactor for activated protein C–dependent inhibition of coagulation factors FVa and FVIIIa.[2] In diabetic kidneys, thrombomodulin-dependent activated protein C formation has been shown to mediate cytoprotection by inhibiting glomerular endothelial cell and podocyte apoptosis,[3] suggesting that PS may have a renoprotective function. In addition to its anticoagulant function, PS also activates a family of protein tyrosine kinase receptors, Tyro-3, Axl, and Mer (TAM) receptors, that have multiple biologic functions.[4] Although PS and its structural homolog GAS6 are both ligands of TAM receptors, due to their differential binding affinities for individual TAM receptors,[5,6] PS and GAS6 have divergent functions.[7] GAS6 binds predominantly to Axl, with some affinity to Tyro3, but little affinity to Mer. In contrast, PS binds predominantly to Tyro3 and Mer with little affinity to Axl.[4,8] In the context of kidney injury, GAS6 has been shown to induce Axl-mediated mesangial cell proliferation and glomerular hypertrophy in early DN[9,10] and to promote inflammation in GN.[11,12] Although the role of PS in kidney cells has not been explored, in other cell types PS has been shown to be a negative regulator of immune and inflammatory responses, and involved in the clearance of apoptotic cells[13,14] and in the inhibition of VEGF-A–mediated angiogenesis.[15] Given these contrasting effects of PS in comparison to GAS6, we posited that the increased PS expression in early diabetic kidney injury might be renoprotective. Our results now show that PS is significantly increased in the glomerular cells including the podocytes during the early stages of DN, and that the loss of PS specifically in podocytes aggravated proteinuria, inflammation, and podocyte injury and loss in streptozotocin (STZ)-induced diabetic kidneys. Conversely, the induction of PS overexpression in glomerular cells attenuated albuminuria and podocyte loss in OVE26 diabetic mice, confirming the renoprotective role of PS in diabetic kidneys.

METHODS

Study Approval

All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee at Icahn School of Medicine at Mount Sinai.

Statistical Analyses

Data are expressed as mean±SEM. Unpaired *t* test was used to analyze data between two groups. ANOVA with Bonferroni *post hoc* test was used when more than two groups were present. All experiments were repeated at least three times, and representative experiments are shown. Statistical significance was achieved when $P<0.05$.

A complete, detailed description of methods can be found in the Supplemental Material.

RESULTS

Identification of PS from Proteomic Analysis of Diabetic Rat Glomeruli

Proteomic analysis was performed on isolated glomeruli of rats injected with either low-dose STZ or with citrate buffer vehicle. Rats were euthanized at either 6 or 12 weeks after the onset of diabetes in the experimental group (n=5 for each time point).

Table 1. Top ten identified proteins with most significant fold changes in expression levels at 12 wk after induction of diabetes

Protein Name	Gene	Cont 6 wk	Cont 12 wk	STZ 6 wk	STZ 12 wk	STZ+Ins 12 wk	*P* Value	Adjusted Q Value
C4b-binding protein α chain	C4bpa	1.0	1.0	2.1	2.4	1.6	1.055e−07	1.787e−05
Vitamin K–dependent PS	Pros1	1.0	1.0	2.1	2.4	1.7	1.055e−07	8.933e−06
Brain acid soluble protein 1	Basp1	1.0	1.0	0.5	0.4	0.7	2.266e−07	1.278e−05
DnaJ homolog subfamily C member 2	Dnajc2	1.0	1.0	0.9	0.4	0.4	2.266e−07	9.586e−06
Solute carrier family 6, member 18	Slc6a18	1.0	1.0	0.5	0.4	0.6	2.266e−07	7.669e−06
Apo A-I	Apoa1	1.0	1.0	1.5	2.3	1.3	4.028e−07	1.136e−05
Complement C4	C4	1.0	1.0	2.1	2.2	1.7	1.53e−06	3.699e−05
C4b-binding protein β chain	C4bpb	1.0	1.0	2.3	1.9	1.0	7.856e−05	0.0017
Solute carrier family 43, member 3	Slc43a3	1.0	1.0	1.4	1.9	1.1	7.856e−05	0.0015
Alanyl aminopeptidase	Anpep	1.0	1.0	0.6	0.5	0.7	<0.001	0.0018

Protein expression levels at 6 and 12 wk of diabetes (STZ 6wk, STZ 12wk) are indicated as fold changes relative to age-matched nondiabetic vehicle-injected controls. We used a Z-test to test for significance of fold changes for individual proteins, and the Benjamini–Hochberg procedure to adjust for multiple hypothesis testing. Cont, control vehicle-injected; STZ, STZ-injected; Ins, treated with insulin.

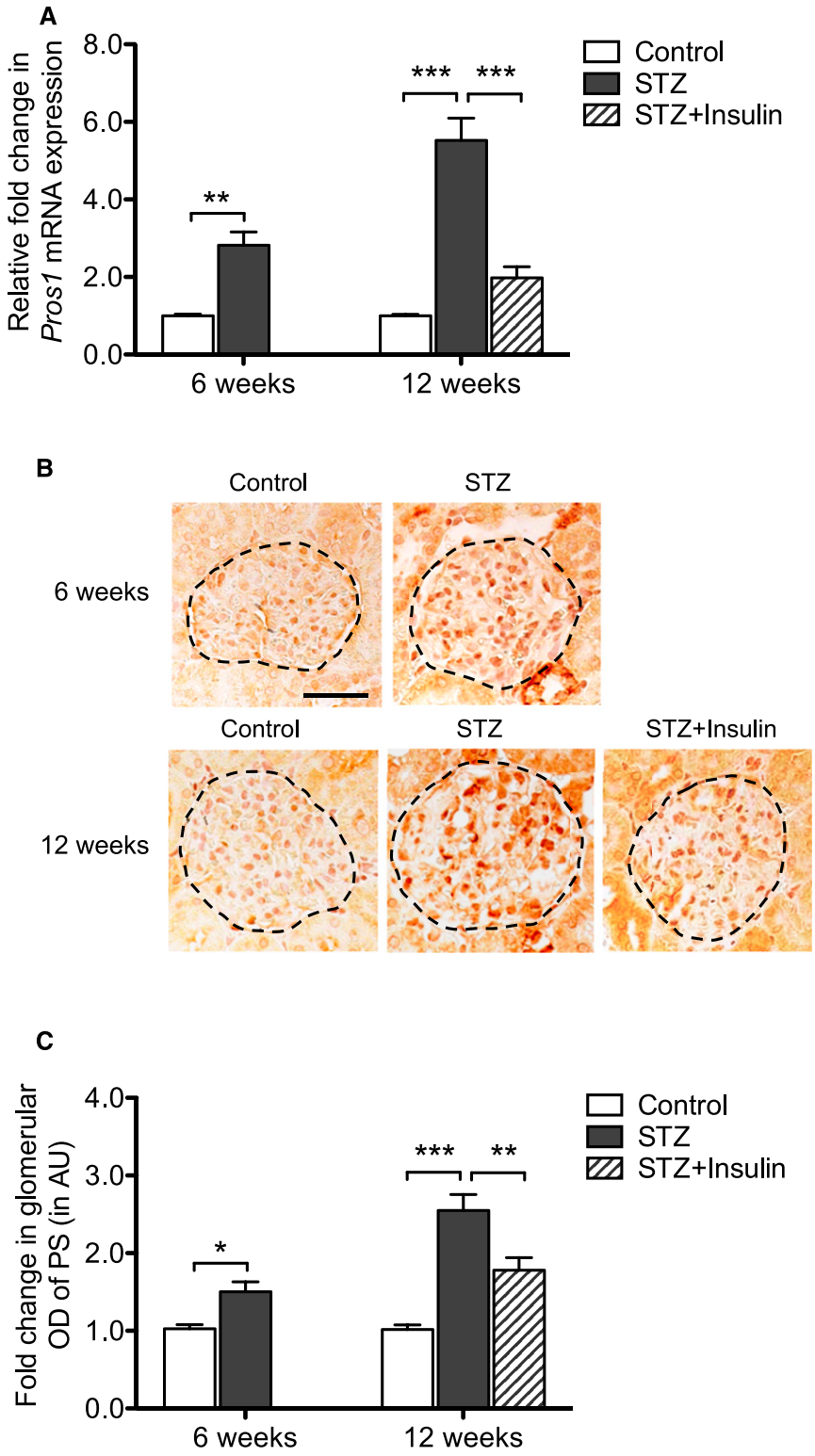


Figure 1. Expression of PS is increased in diabetic rat glomeruli. (A) Real-time PCR was performed to measure *Pros1* mRNA expression in glomeruli isolated from diabetic and control rats euthanized at either 6 or 12 weeks after the induction of diabetes (DM). Pros1 mRNA expression was also measured in diabetic rats treated with insulin between 6 and 12 weeks (DM+Insulin). (B) Representative images of immunostaining for PS are shown. Scale bar, 50 μm. (C) Glomerular region was selected, and OD was measured and quantified as a relative fold change to nondiabetic control mice (n=5; *P<0.05, **P<0.01, and ***P<0.001 when compared between indicated groups). AU, arbitrary units.

An additional group of diabetic rats were treated with insulin to maintain tight glycemic control between weeks 6 and 12 and euthanized at 12 weeks after the onset of diabetes (n=5). Sex- and age-matched nondiabetic control rats were used as controls (n=5 for each time point). Body weight, blood glucose, kidney-to–body weight ratio, and urine albumin-to-creatinine measurements are shown in Supplemental Table 1. The top ten of the proteins whose expression was significantly changed in diabetic rats, but was reversed by insulin treatment, are listed in Table 1. Among them, PS had nearly two- to three-fold increase in diabetic glomeruli at both 6 and 12 weeks of diabetes, but its level was significantly decreased in diabetic rats treated with insulin (Table 1). Because PS is a homolog of GAS6, which was shown to be involved in early diabetic glomerular hypertrophy and DN pathogenesis,[10] we selected PS for further study.

PS Expression Is Increased in Diabetic Glomeruli

Real-time PCR analysis and immunostaining showed that both mRNA and protein levels of PS were increased in the diabetic rat glomeruli compared with nondiabetic glomeruli and that their levels were partially reversed by insulin treatment (Figure 1, A–C). We did not detect any significant changes in serum levels of PS in all groups (Supplemental Figure 1A), but urinary PS levels increased significantly in diabetic rats at both 6 and 12 weeks (Supplemental Figure 1B).

Phenotypes of most rodent models of DN, including the STZ-induced DN, are considered to be representative of the early stage of DN. Recent studies suggest disparate or even opposite patterns of renal gene expression profiles between diabetic animals and patients,[16] which may be in part due to the differences in early (rodent models) versus late stages (human

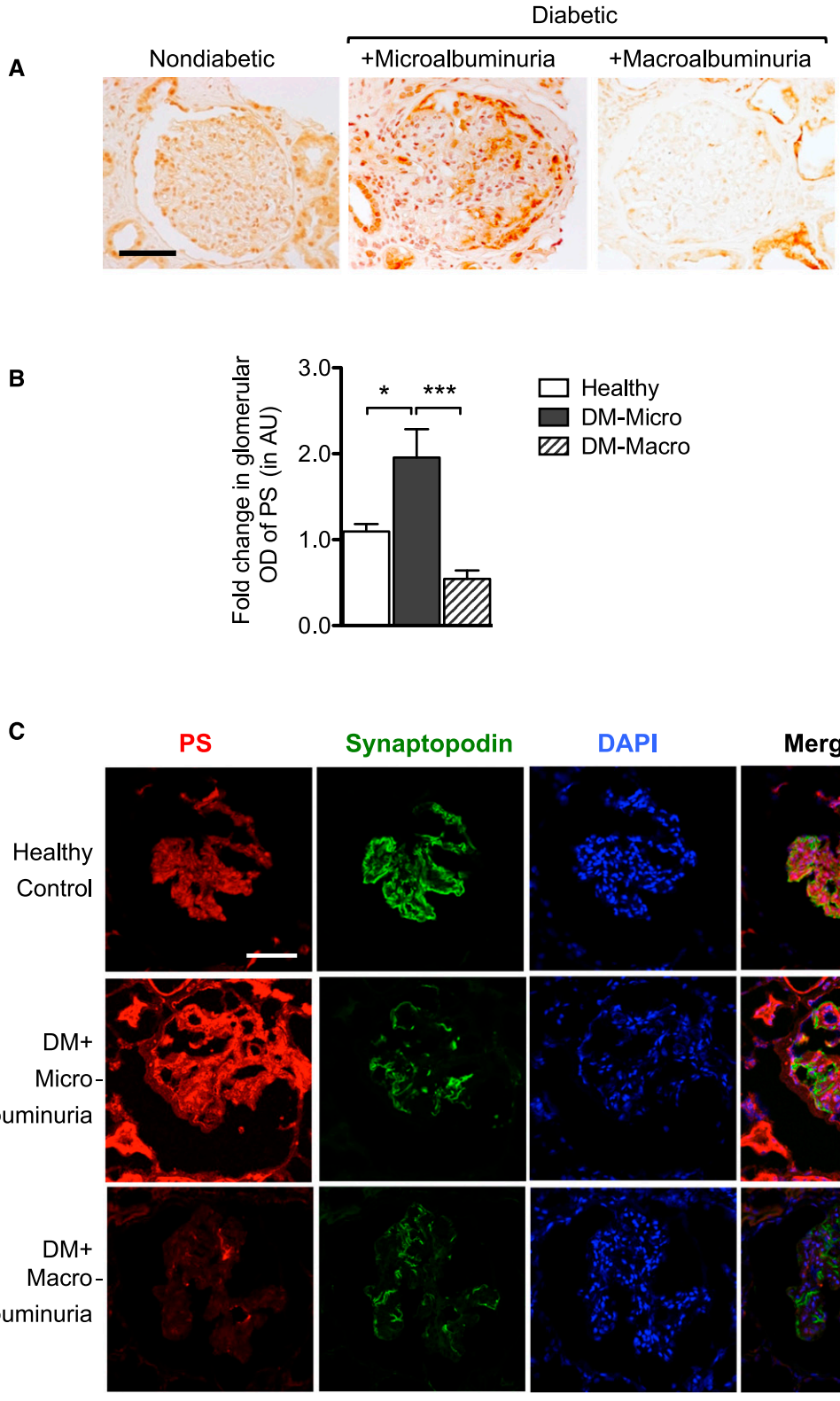


Figure 2. PS expression is altered in human DN. Immunostaining for PS was performed on healthy donor nephrectomy specimens and on kidney biopsy samples of patients with diabetes with microalbuminuria (UACR<300 mg/g) and with macroalbuminuria (UACR>300 mg/g). (A) Representative images of six subjects in each group are shown (400× original magnification; scale bar, 50 μm). (B) Glomerular area was selected, and OD was measured and quantified as a relative fold change to healthy donor specimens (n=6; *P<0.05 and ***P<0.001 when compared between indicated groups). (C) Representative images of diabetic human kidneys

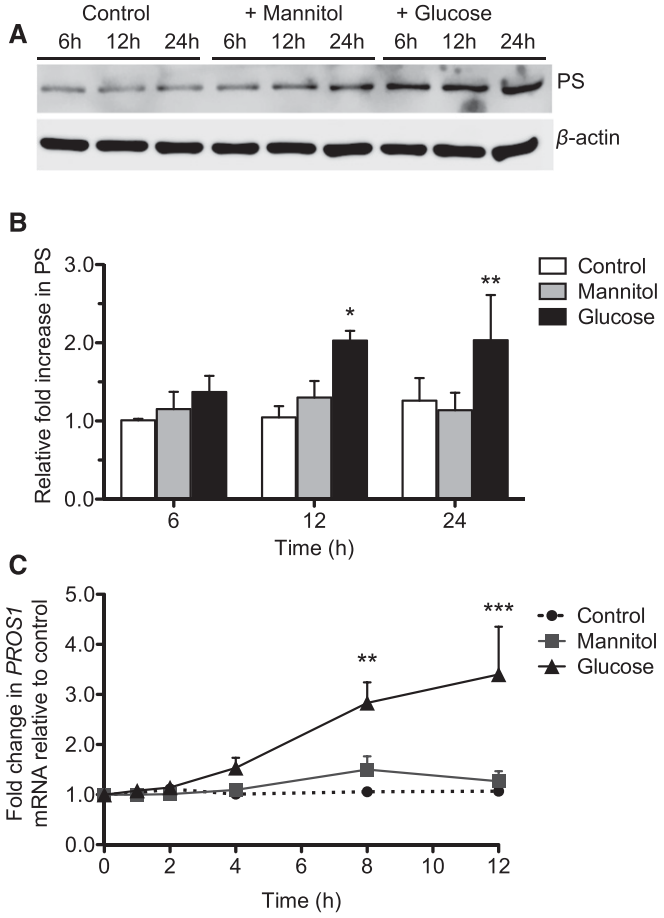


Figure 3. High glucose increases PS expression in cultured human podocytes. Human podocytes were incubated with normal glucose (5 mM), high mannitol (30 mM mannitol), or high glucose (30 mM) for 24 hours by adding the indicated concentrations of glucose and mannitol in the glucose-free medium. Western blotting and real-time PCR were performed to assess PS expression levels. (A) Representative western blot of three independent experiments is shown. (B) Densitometry analyses were performed for western blots. (C) *PROS1* mRNA expression was measured by real-time PCR. (n=3; *P<0.05, **P<0.01, ***P<0.001 versus all other groups.)

samples) of DN. Therefore, we next examined whether PS expression was altered in human diabetic kidneys with early and advanced DN (clinical information of the patients is summarized in Supplemental Table 2). Immunohistochemical analysis showed a marked increase of PS in glomerular cells of kidneys of patients with diabetes with microalbuminuria, but it was barely detectable in those with macro albuminuria (Figure 2, A and B). We further verified that the increased glomerular PS expression partially colocalized with podocyte marker synaptopodin (Figure 2C). Consistent with the above data in diabetic rats, there were no significant changes in plasma levels of free PS between control patients and patients with diabetes with early or late DN (Supplemental Figure 1, B and C). Our results suggest that there is a temporal increase of PS in kidneys of early DN, but not late DN, likely due to the local production of PS in glomerular cells.

High Glucose Increases PS Expression in Podocytes

Because PS expression was localized more prominently in podocytes of diabetic kidneys, we sought to determine the regulation of PS expression in cultured human podocytes. Incubation of podocytes with high glucose induced both mRNA and protein levels of PS in comparison to cells incubated in control media with normal glucose or with high mannitol (Figure 3, A–C), suggesting that the induction of PS in hyperglycemic milieu may account in part for the observed increase of PS in early DN kidneys. To determine whether insulin had a direct effect on PS regulation, we treated the podocytes with either high glucose or insulin and found that insulin alone did not induce PS (Supplemental Figure 2). The mechanism of reduced PS expression in kidney with advanced DN remains to be determined.

PS Regulates Glucose-Induced Apoptosis and NF-κB–Mediated Inflammation in Podocytes

Hyperglycemia-induced podocyte loss is an early event in DN.[17] Because PS is known to exert an antiapoptotic effect through the binding to and signaling through its cognate TAM receptors,[18] we next examined whether the reduced expression of PS might affect podocyte survival under high-glucose conditions. shRNA-mediated silencing of *PROS1* in podocytes incubated with high glucose led to a further increase in BAX expression and suppression of BCL-2 expression (Figure 4, A and B) and to increased Caspase-3 activity (Figure 4C), indicating that reduced PS expression exacerbated the high-glucose–induced podocyte apoptosis. These observations were further validated by using a second set of PS-specific shRNA to rule out any nonspecific effects (Supplemental Figure 3).

Because PS is a negative regulator of inflammatory responses,[19] and TNF-α–induced NF-κB signaling is a driver of DN pathogenesis,[20,21] we examined whether the overexpression of PS can mitigate the high-glucose– or TNF-α-induced inflammatory responses. Podocytes were transfected with either PS overexpressing vector (PS^{OE}) or control vector expressing green fluorescent protein (GFP^{OE}) together with NF-κB luciferase reporter and renilla luciferase plasmids. Forty-eight hours post-transfection, cells were exposed to

immunostained for both PS and synaptopodin (×400 original magnification). DAPI was used to visualize the nuclear staining. AU, arbitrary units; DAPI, 4′,6-diamidino-2-phenylindole; DM, diabetes mellitus; Macro, macroalbuminuria; Micro, microalbuminuria; PS, protein S; UACR, urinary albumin-to-creatinine ratio.

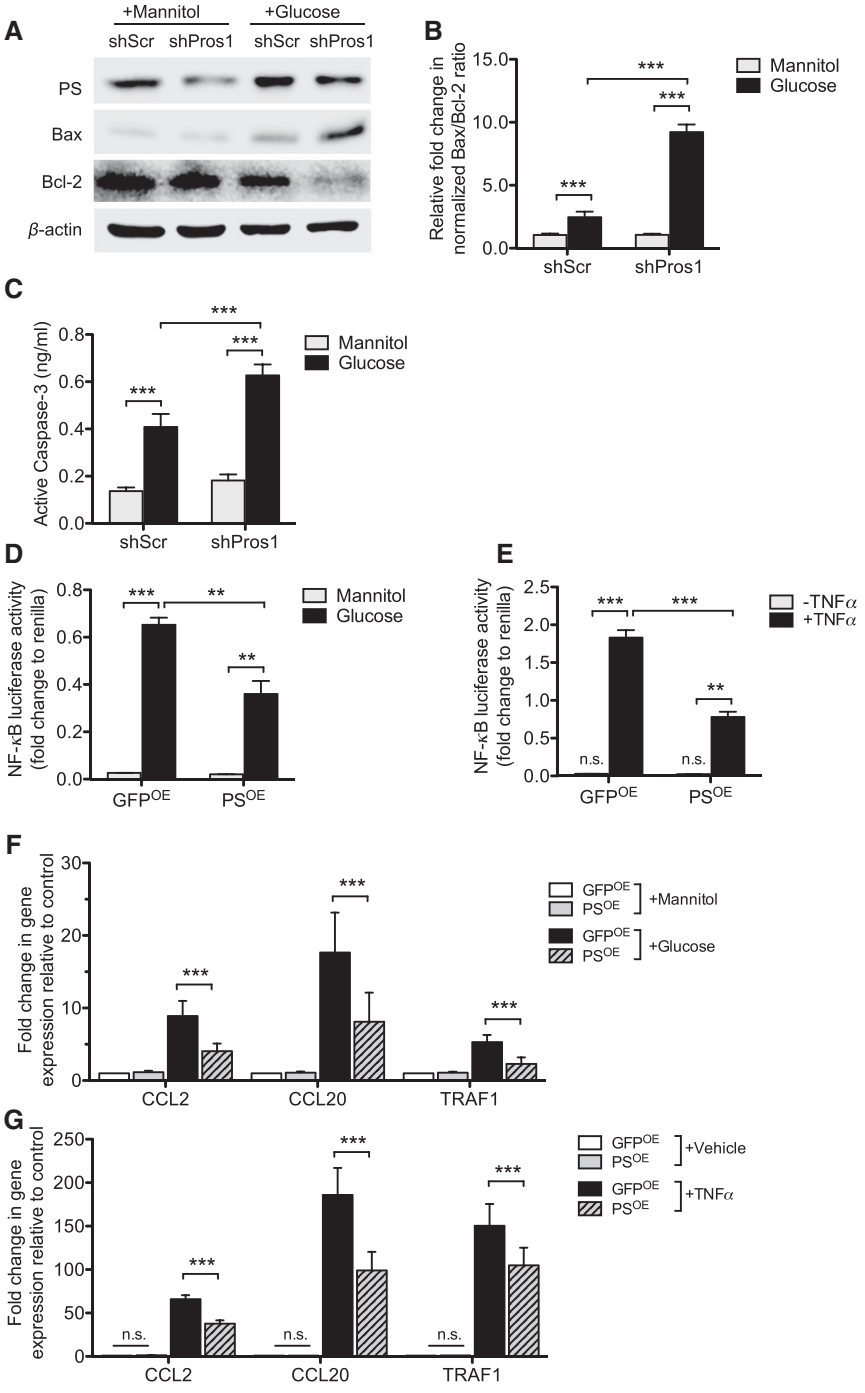


Figure 4. PS prevents hyperglycemia-induced apoptosis and dampens TNF-α-induced inflammation. (A–C) Podocytes stably transduced with lentivirus expressing either scrambled shRNA (shScr) or Pros1 shRNA (shPros1) were incubated with high glucose (30 mM) or high mannitol (5 mM glucose±25 mM mannitol) for 24 hours. (A) Western blots were performed for PS, BAX, and BCL2. Representative blots of three independent experiments are shown. Densitometry analyses were performed for western blots of Bax and Bcl-2 after normalization with β-actin. (B) Active Caspase-3 concentration was measured by ELISA. (n=3; *P<0.05, **P<0.01, and ***P<0.001 when compared between indicated groups.) (D-G) Podocytes were transiently transfected with PROS1 overexpressing vector (PS^{OE}) or control GFP vector (GFP^{OE}). NF-κB luciferase reporter assay (D) with high mannitol (25 mM) or high glucose (30 mM) for 48 hours or (E) with TNF-α (10 ng) for 24 hours. NF-κB luciferase reporter activity is shown as fold change to renilla luciferase activity. (F-G) Real-time PCR analysis of NF-κB–targeted gene expression (F) in high glucose condition or (G) with TNF-α treatment is shown. (n=3; *P<0.05, **P<0.01, and ***P<0.001 when compared between indicated groups.)

either high glucose or TNF-α (10 ng/ml) for an additional 24 hours. We found that the overexpression of PS suppressed both high-glucose– or TNF-α-induced NF-κB luciferase reporter activity (Figure 4, D and E), as well as the expression of several NF-κB–mediated proinflammatory genes, by real-time PCR (Figure 4, F and G).

To determine whether the antiapoptotic and anti-inflammatory effects of PS observed above are mediated by the activation of TAM receptors, we first examined their expression in podocytes. All three TAM receptors were expressed in podocytes, but only Tyro3 expression was upregulated in both protein and mRNA levels under high-glucose conditions (Figure 5, A–C, Supplemental Figure 4). To ascertain the contribution of each receptor in PS-mediated anti-inflammatory effects, we next silenced the individual TAM receptors by using lentiviral vectors expressing specific shRNAs to each receptor (Supplemental Figure 5, A and B). Indeed, the silencing of Tyro3, but not of Mer or Axl, led to a significant mitigation of the anti-inflammatory effects of PS in podocytes (Figure 5, D and E, Supplemental Figure 5, C and D), suggesting that Tyro3 specifically mediates the cytoprotective effects of PS in podocytes.

Diabetic Glomerulopathy Is Aggravated in Podocyte-Specific *Pros1*-Null Mice

Because global *Pros1* knockout mice are not viable,[13] and significant upregulation of PS was found in podocytes in early diabetic kidneys, we interrogated the effects of podocyte-specific loss of PS in DN. *Pros1* floxed mice (from Dr. G. Lemke) were crossed with *podocin*-Cre (*Pod-Cre*) transgenic mice (Jackson Laboratory, ME). Resulting $Pros1^{fl/fl}$;*Pod-Cre* (PS-KO) mice were viable, fertile, and indistinguishable from the $Pros1^{fl/fl}$ wildtype (WT) littermates. We confirmed the podocyte-specific

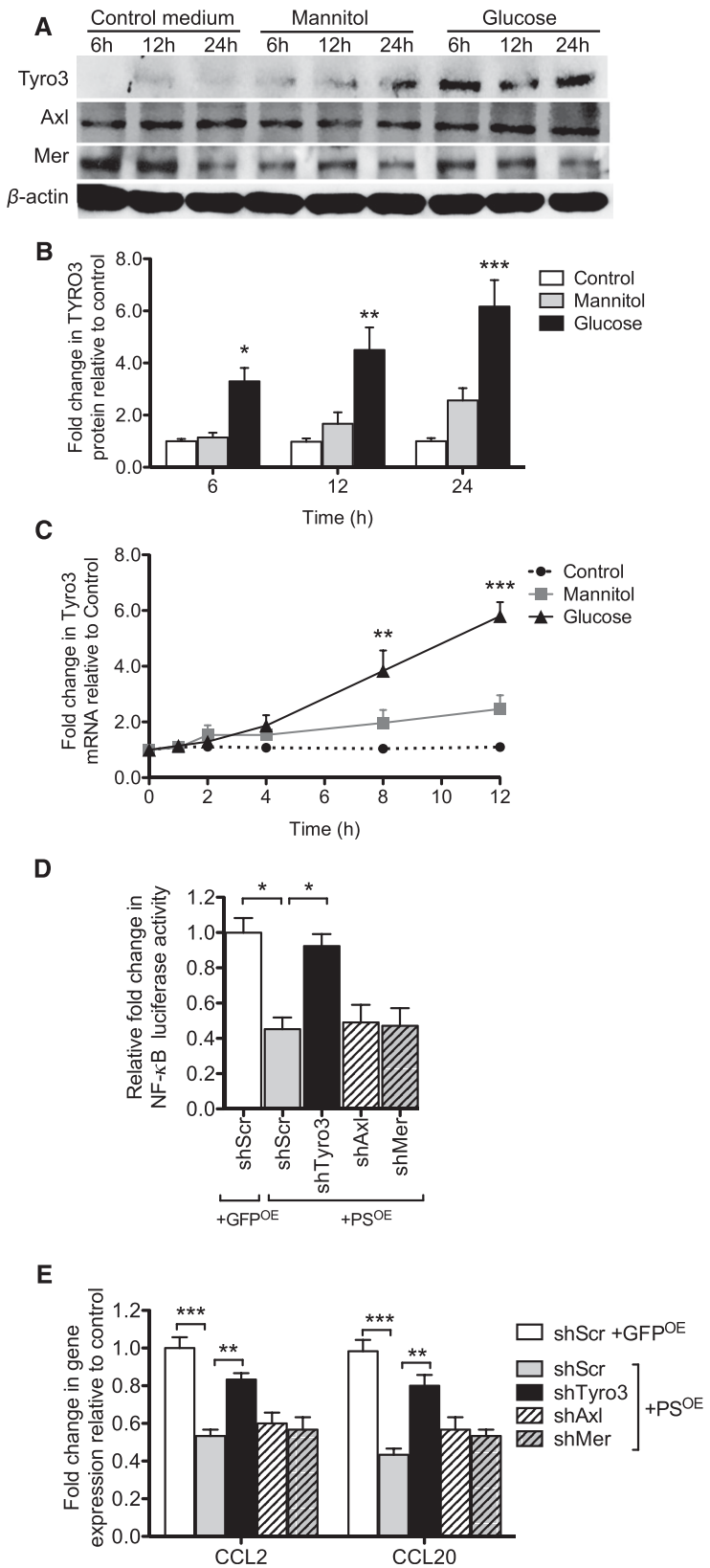
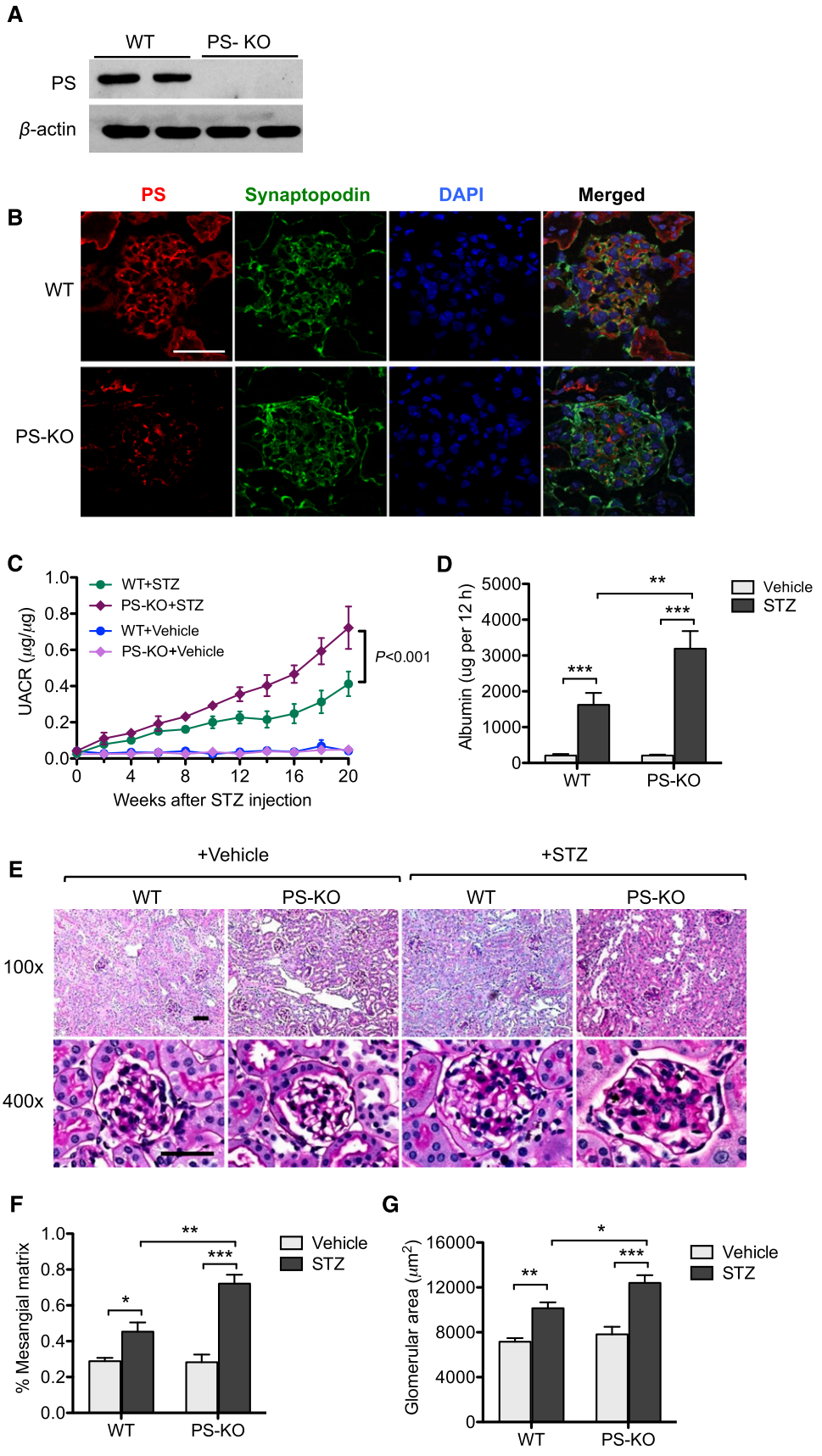


Figure 5. Tyro3 mediates the effects of PS in podocytes. (A–C) Podocytes were incubated with normal glucose, high mannitol, or high glucose for 24 hours. Western blots were performed for each TAM receptor. (A) Representative blots of three independent experiments are shown. Densitometry analysis of Tyro3 is shown (densitometric analysis of Axl and Mer are included in Supplemental Figure 2). (C) *PROS1* mRNA expression was measured by real-time PCR (mRNA expression of Axl and Mer are included in Supplemental Figure 2). (n=3; *P<0.05, **P<0.01, and ***P<0.001 versus Control.) (D and E) Podocytes stably transduced with lentivirus expressing either scrambled shRNA (shScr), Tyro3 shRNA (shTyro3), Axl shRNA (shAxl), or Mer ShRNA (ShMer) were transfected with NF-κB luciferase reporter and renilla luciferase, together with either control GFP overexpression vector (GFP^{OE}) or PS overexpression vector (PS^{OE}). (D) NF-κB luciferase reporter activity is shown as fold change to renilla luciferase activity. (E) Real-time PCR analysis of selected NF-κB–targeted gene expression was performed in these cells. (n=3; *P<0.05, **P<0.01, and ***P<0.001 when compared between indicated groups.)

ablation of *Pros1* by western blot analysis of primary podocytes isolated from PS-KO and WT mice (Figure 6A). Immunostaining of PS with synaptopodin further confirmed the loss of PS specifically in podocytes (Figure 6B). At baseline, PS-KO mice did not develop proteinuria and kidney injury when they were euthanized at 6–12 months of age (data not shown).

Diabetes was induced in 8-week-old WT and PS-KO mice with low-dose STZ injections, and all mice were euthanized 20 weeks postinjection. Blood glucose levels were monitored every 2 weeks, which were comparable between STZ-injected WT and PS-KO mice (Supplemental Figure 6A). Both WT and PS-KO diabetic mice displayed similar extents of weight loss (Supplemental Figure 6B) and kidney hypertrophy, as measured by kidney-to–body weight ratio (Supplemental Figure 6C), when compared with nondiabetic controls. However, we observed a significant increase in albuminuria in diabetic PS-KO mice in comparison to diabetic WT controls when examined by spot urine collection (Figure 6C) and by 12-hour urine collection at 20 weeks (Figure 6D). Histologic analysis of mouse kidneys also showed marked increase in mesangial matrix and in glomerular volume in


A
WT
PS- KO
PS
β-actin
B
PS
Synaptopodin
DAPI
Merged
WT
PS-KO
C
WT+STZ
PS-KO+STZ
WT+Vehicle
PS-KO+Vehicle
UACR (μg/μg)
P<0.001
Weeks after STZ injection
D
Albumin (ug per 12 h)
Vehicle
STZ
WT
PS-KO
E
+Vehicle
+STZ
WT
PS-KO
100x
400x
F
% Mesangial matrix
Vehicle
STZ
G
Glomerular area (μm2)
Vehicle
STZ

diabetic PS-KO mice as compared with the diabetic WT controls (Figure 6, E–G).

Consistent with worsened diabetic glomerulopathy in diabetic PS-KO mice, greater podocyte injury and loss were also observed in diabetic PS-KO mice (Figure 7). Electron microscopy revealed exacerbated podocyte foot process effacement in the diabetic PS-KO mice as compared with diabetic WT mice (Figure 7, A and B). In addition, there was a greater podocyte loss in diabetic PS-KO kidneys, as quantified by Wilms' tumor-1 (WT-1)–positive cells (Figure 7, C–E). Real-time PCR analysis confirmed the decreased expression of podocyte markers (synaptopodin, nephrin, synaptopodin, and podocin) in diabetic PS-KO glomeruli as compared with diabetic WT glomeruli (Figure 7F), consistent with increased podocyte injury and loss. Furthermore, increased cell death in diabetic PS-KO glomeruli was confirmed by TUNEL staining (Supplemental Figure 7). Moreover, real-time PCR analysis showed increased expression of inflammatory mediators in diabetic PS-KO glomeruli in comparison to diabetic WT glomeruli (Figure 7G). Taken together, our data demonstrate a protective effect of PS against diabetic podocyte injury and loss *in vivo*.

Induction of PS Expression in Diabetic Kidney Attenuated Albuminuria and Podocyte Injury

We next determined whether increased PS expression was sufficient to attenuate diabetic kidney injury *in vivo*. Because STZ-induced diabetes results in mild kidney disease, to detect the improvement of DN injury, we employed a diabetic mouse model with more severe DN, namely OVE26 mice in the FVB/N background.[22,23] We induced a transient overexpression of PS in the kidney of OVE26 mice through intrarenal arterial injection of recombinant adeno-associated virus (rAAV9) expressing PS, as described in the Methods. We first confirmed the efficacy of the method by injection of enhanced green fluorescent protein–expressing rAAV9 (rAAV9-eGFP) into the renal artery of the left kidney. Examination of the kidneys 3 weeks postinjection showed a robust expression of eGFP in the glomeruli of the left kidney, but not in those of the right kidney (Figure 8, A and B). Glomerular expression of eGFP was confirmed by real-time PCR analysis of eGFP mRNA levels in the isolated glomeruli and in kidney cortices (Supplemental Figure 8A). Importantly, we observed that significant eGFP expression colocalized with synaptopodin expression, as shown by the quantification of eGFP and synaptopodin double-positive fluorescence areas in the glomeruli (Figure 8, A and B), suggesting a high infection rate of podocytes by rAAV9. We next injected the *Pros1*-expressing rAAV9 (rAAV9-PS) into the left kidneys of OVE26 mice at age 10 weeks when they developed a significant amount of proteinuria.[22,23] Notably, 10-week-old OVE26 mice showed decreased PS expression compared with age-matched nondiabetic littermates (Supplemental Figure 8, B and C), consistent with the above observation that the severity of DN is associated with decreased PS expression. There was a significant increase in PS expression at 5 weeks postinjection in the rAAV9-injected (left) kidneys as compared with the contralateral (right) kidneys, which also showed a significant overlap with podocyte marker synaptopodin (Figure 8C), which was further confirmed by real-time PCR of isolated glomeruli (Figure 8D). Importantly, PS overexpression led to a significant reduction in mesangial expansion (Figure 8, E and F) and in podocyte loss (Figure 8, G and H) in the rAAv9-PS–injected left kidneys as compared with the uninjected right kidneys of OVE26 mice. To ascertain the effects on renal function by this transient overexpression of PS, we injected the rAAV9-PS or rAAV9-eGFP into both kidneys of diabetic OVE26 and nondiabetic control (WT) mice at 10 weeks of age, and the mice were similarly euthanized at 5 weeks postinjection. Real-time PCR analysis of isolated glomeruli confirmed a significant expression of PS in WT and OVE26 mice that received rAAV9-PS in comparison to those that received rAAV9-EGFP injection (Figure 8I). Urinary albumin-to-creatinine ratio showed a marked reduction of proteinuria in rAAV9-PS–injected OVE26 mice as compared with rAAV9-eGFP–injected OVE26 mice (Figure 8J). These data strongly suggest that PS could be a potential therapeutic target for DN.

DISCUSSION

We performed a proteomic analysis of diabetic rat glomeruli in order to identify proteins that may be involved in disease pathogenesis in early DN. We identified several proteins with significant changes in their expression in diabetic glomeruli at both 6 and 12 weeks post STZ injection and whose expression

Figure 6. Diabetic glomerulopathy is aggravated in PS-KO mice. (A) Western blot analysis of PS in the lysates of primary podocytes isolated from PS-KO and control WT mice. (B) Immunostaining of PS and synaptopodin in the kidney shows loss of PS specifically in podocytes in PS-KO mice. (C) Urinary albumin-to-creatinine ratio is shown over the course of 20 weeks post–diabetes onset. (D) The 12-hour urinary albumin excretion rate was determined at 20 weeks post–diabetes onset. (E) Representative images of periodic acid–Schiff–stained kidneys of vehicle- or STZ-injected WT and PS-KO at both low (×200) and high (×400) magnification. (F and G) Quantification of (F) glomerular area and (G) percentage of mesangial matrix area are shown (60 glomeruli per group; $n=6$; *$P<0.05$, **$P<0.01$, and ***$P<0.001$ when compared between indicated groups). DAPI, 4′,6-diamidino-2-phenylindole; PS-KO+Vehicle, PS-KO injected vehicle control; WT+Vehicle, WT injected with vehicle control; PS-KO+STZ, PS-KO injected with STZ; WT+STZ, WT injected with STZ; UACR, urinary albumin-to-creatinine ratio.

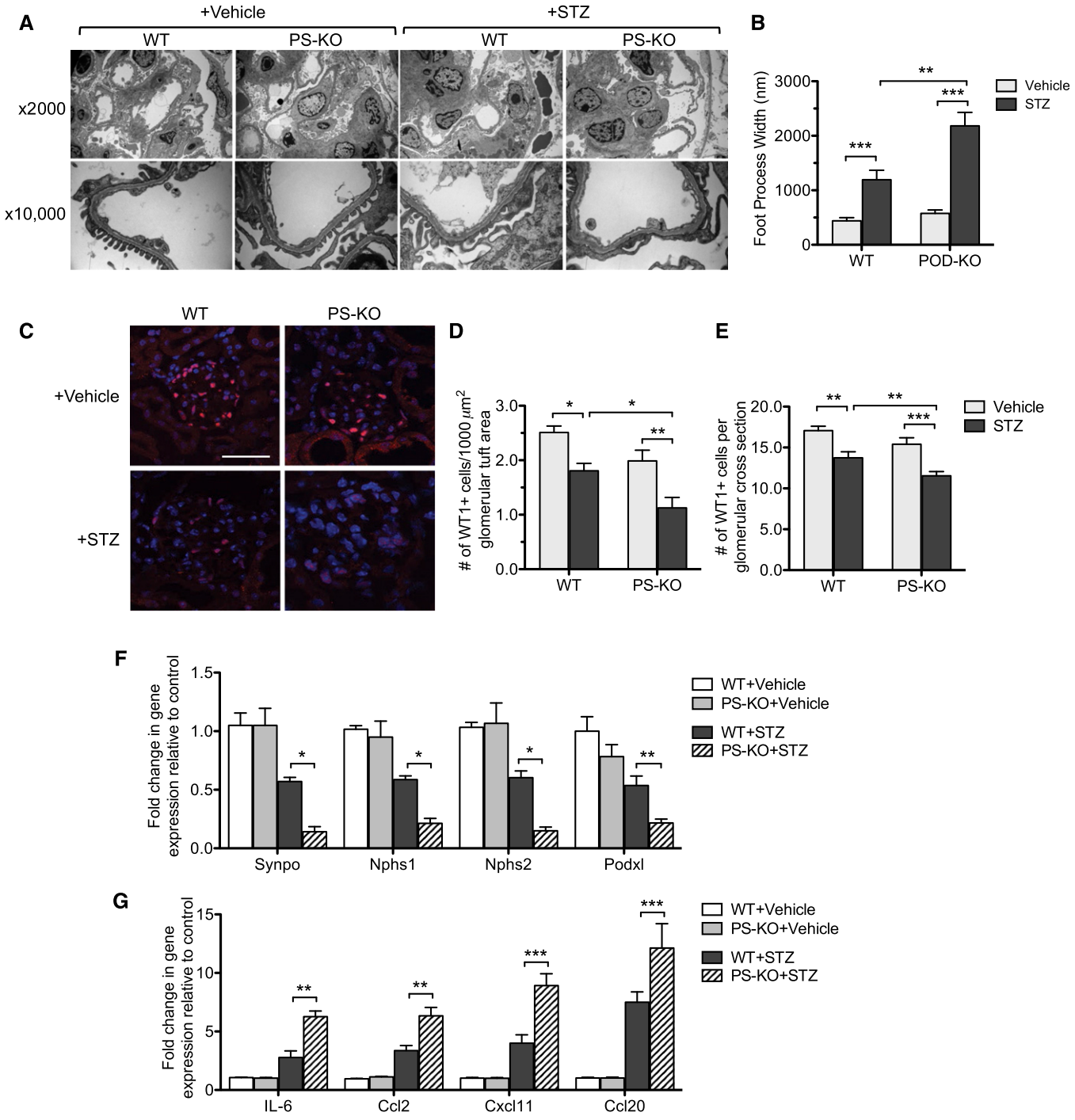


Figure 7. Podocyte injury and loss are worsened in diabetic PS-KO mice. (A) Representative transmission electron microscopy images of vehicle- or STZ-injected WT and PS-KO kidneys are shown at low (×2000) and high (×10,000) magnifications. (B) Quantification of foot process effacement is shown. (C) Representative images of WT-1 immunostained glomeruli are shown. (D and E) Quantification of WT-1+ cells are shown as (D) WT-1+ cell number per glomerular cross section and as (E) WT-1+ cell number per 1000 μm^2 glomerular tuft area. (F) mRNA levels of WT-1, nephrin, synaptopodin, and podocin are shown as a relative fold change to vehicle control. (G) mRNA levels of inflammatory markers from isolated glomeruli are shown as a relative fold change to vehicle control. (60 glomeruli per group; $n=6$; $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ when compared between indicated groups.)

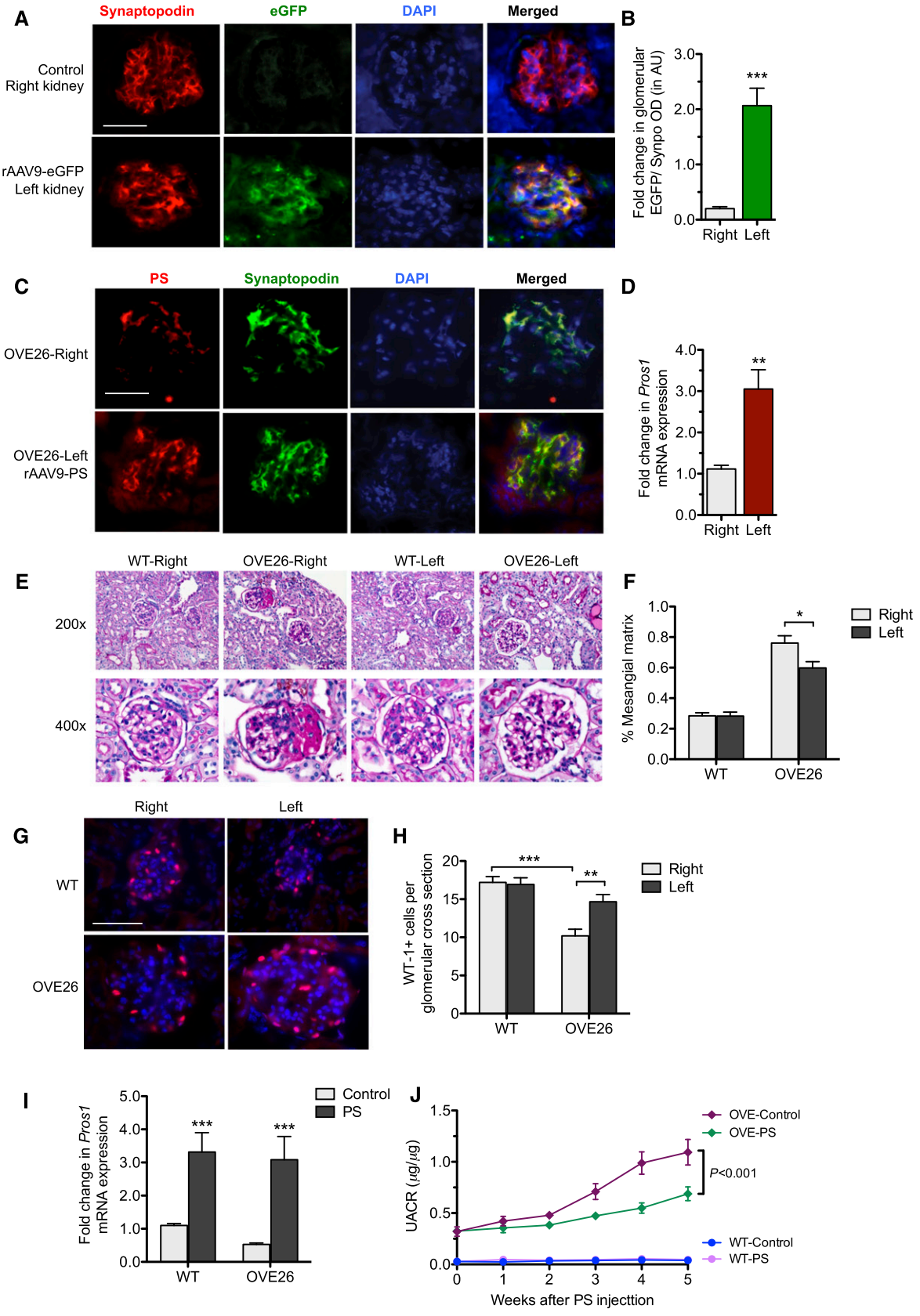


Figure 8. Induction of PS expression in glomeruli attenuates podocyte injury and albuminuria in OVE26 mice. Four mice received injection of GFP-expressing rAAV9 into the left kidney for 3 weeks while the right kidneys were used as controls. (A) Representative

was reversed when treated with insulin, suggesting that their expressions are likely to be regulated by hyperglycemia and/or insulin. Among the proteins identified, we chose to further interrogate the role of PS in early DN, because PS has previously been shown to have anti-inflammatory and anti-apoptotic effects through binding to TAM receptors,[18,24] and apoptosis and inflammation of kidney cells are key components of DN.

Although PS is present abundantly in the plasma and is made mostly in the liver, the plasma levels of PS did not change between diabetic and nondiabetic rats and in human patients with diabetes with or without DN, demonstrating that the observed changes in our proteomic analysis of isolated glomeruli are likely due to changes to local production of PS by glomerular cells, which was also confirmed by immunostaining. In support of these observations, it has been shown that PS is also synthesized in cells and tissues other than the liver, including endothelial cells and smooth muscle cells.[13] In contrast to our findings, the early studies suggest that the plasma PS levels increase in patients with diabetes.[25,26] However, a more recent study shows that the plasma PS level did not change between normal controls, patients with diabetes with or without DN.[27] The discrepancy among these studies could be due to the use of different assays and/or the heterogeneity of patient populations. Further studies with more standardized protocols and larger numbers of patient populations are required to reconcile the discrepant findings.

The regulation of PS expression is not well understood, because only a few studies have explored its mechanism. It was shown that miR-155 and miR-494 mediate the regulation of *PROS1*[28,29] and that *PROS1* 3′-UTR sequences contain three putative miR-494 binding sites. Interestingly, an increased expression of miR-155 was observed in the patients with DN and in the experimental DN animal models, which contributes to inflammation-mediated glomerular cell injury,[30,31] and elevated urinary miR-494 predicts the progression of kidney disease.[32] These studies suggest a potential role of miR-155 and miR-494 in the regulation of *PROS1* in DN. IL-6 has also been shown to induce PS expression through the activation of Stat3 pathway,[33] and Stat3 is known to be activated in diabetic kidneys.[34] In addition, AMP kinase has been shown to mediate flow-induced PS expression in endothelial cells.[35] We found that PS is expressed highly in podocytes and that high glucose, but not insulin, upregulates PS expression in cultured podocytes, suggesting that the effect of insulin in reducing PS expression *in vivo* is likely through the reduction in blood glucose. In human diabetic kidneys, we observed that PS expression is upregulated during the early stages, but not during the late stages of DN, suggesting that the upregulation of some factors induced by high glucose at early DN may be protective against the disease progression. We speculate that this protective mechanism is lost in patients with progressive DN and may contribute to its pathogenesis. Future studies are required to determine how *PROS1* is regulated in diabetic kidneys during the early and late stages of DN.

In this study, we chose to focus on the role of PS in podocytes because we observed its prominent expression in podocytes of human diabetic kidneys. Podocyte injury is considered to be an important early event in DN pathogenesis,[36] such that the reduction in podocyte density is the strongest predictor of progressive DN.[37,38] We found that PS protects against podocyte loss *in vivo* and reduces high-glucose– and TNF-α-induced inflammatory response in cultured podocytes. Nevertheless, we cannot rule out the expression of PS and its potential contribution against DN injury in other glomerular cells, such as glomerular endothelial cells. Given that the mice with global endothelial cell–specific knockout of PS show development of thrombosis,[13] the model may not be suitable for studying the effect of PS in DN; and glomerular endothelial cell–specific Cre mice are not currently available. Future studies are required to dissect the role of PS in other glomerular cells in DN.

Both ligands of TAM receptors, PS and Gas6, are constitutively γ-carboxylated on glutamic acid residues in their N-terminal domains by a vitamin K–dependent carboxylase during secretion from cells,[39] and γ-carboxylation of PS and Gas6 appears to be required for full activation of specific cognate TAM receptors.[6,40] It is not clear at present whether the upregulated PS specifically in glomerular cells including podocytes during diabetic injury is fully γ-carboxylated.

images of immunofluorescence costaining between eGFP and synaptopodin are shown to compare the eGFP expression and localization between right kidney (control) and left kidney (rAAV9-eGFP injected). (B) Quantification of fluorescence intensity of eGFP/synaptopodin ratio between right and left kidneys from these mice (n=4; ***P<0.001 compared with the right kidneys). (C) Diabetic OVE26 mice at age 10 weeks were injected with the rAAV9-PS in the left kidneys and were euthanized 5 weeks postinjection. Representative image of immunostaining for PS and synaptopodin shows extensive overlap. (D) *Pros1* mRNA levels were compared between left kidney (rAAV9-PS) and right kidney (control) by real-time PCR (n=4; **P<0.01 compared with right kidneys). (E) Representative images of PAS-stained kidneys. (F) Quantification of mesangial fraction (n=4; *P<0.05 compared with right kidneys). (G and H) Representative image of (G) WT-1 immunostaining and (H) quantification are shown (n=4; **P<0.01 and ***P<0.001 when compared between indicated groups). (I and J) In another set of experiments, OVE26 mice at age 10 weeks received either rAAV9-PS or rAAV9-eGFP in both kidneys. (I) Real-time PCR analysis confirmed an increase of PROS1 expression in the kidneys of mice injected with rAAV9-PS as compared with those injected with rAAV9-eGFP. (J) Urinary albumin-to-creatinine ratio (UACR) was determined weekly in these mice for 5 weeks (n=4; ***P<0.001 compared with between indicated groups). AU, arbitrary units; DAPI, 4′,6-diamidino-2-phenylindole; eGFP, enhanced green fluorescent protein; OVE-Control, OVE26 injected with rAAV9-eGFP; OVE-PS, OVE26 mice injected with rAAV-PS; WT-Control, wildtype injected with rAAV-eGFP; WT-PS, wildtype injected with rAAV9-PS.

Further studies are required to assess this important aspect of PS signal transduction. Nevertheless, our data strongly suggest that the effects of PS in podocytes are mediated through Tyro3, consistent with previous reports of preferential binding of PS to Tyro 3.[6,8] Moreover, our data further indicate that PS/Tyro3 signaling pathway confers protection against high-glucose–mediated cellular injury in cultured podocytes, and that the podocyte-specific loss of PS aggravates early diabetic kidney injury *in vivo*. We speculate that the loss of PS/Tyro3 upregulation removes this protection and contributes to progressive DN. Future studies are required to confirm whether the loss of Tyro3 specifically in podocytes similarly aggravates DN. Importantly, to assess whether the increased PS expression would confer therapeutic benefit, we examined the *in vivo* effect of transient induction of PS expression in OVE26 mice, a type 1 diabetic mouse model with severe DN.[22,23,41] We induced the PS expression in mice that had already developed significant proteinuria in order to detect a therapeutic effect. Indeed, the induction of PS expression in the glomerular cells by injection of a *Pros1*-overexpressing rAAV9, which showed a high prominent expression in podocytes, led to a significant improvement of DN, indicating that PS could be a potential therapeutic target for DN.

In conclusion, our data demonstrate a hyperglycemia-regulated renoprotective pathway mediated by PS in early DN. Future studies are needed to determine whether the therapeutic approaches to modulate this protective pathway specifically in the kidneys can prevent or halt the progression of DN without affecting the global coagulation activity.

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DISCLOSURES

None.

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