Matrix metalloproteinase-9 deficiency attenuates diabetic nephropathy by modulation of podocyte functions and dedifferentiation

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Matrix metalloproteinase-9 deficiency attenuates diabetic nephropathy by modulation of podocyte functions and dedifferentiation

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Diabetic nephropathy is characterized by excessive deposition of extracellular matrix protein and disruption of the glomerular filtration barrier. Matrix metalloproteinases (MMPs) affect the breakdown and turnover of extracellular matrix protein, suggesting that altered expression of MMPs may contribute to diabetic nephropathy. Here we used an MMP-9 gene knockout mouse model, with in vitro experiments and clinical samples, to determine the possible role of MMP-9 in diabetic nephropathy. After 6 months of streptozotocin-induced diabetes, mice developed markedly increased albuminuria, glomerular and kidney hypertrophy, and thickening of the glomerular basement membrane. Gelatin zymographic analysis and western blotting showed that there was enhanced MMP-9 protein production and activity in the glomeruli. However, MMP-9 knockout in diabetic mice significantly attenuated these nephropathy changes. In cultured podocytes, various cytokines related to diabetic nephropathy including TGF-β1, TNF-α, and VEGF stimulated MMP-9 secretion. Overexpression of endogenous MMP-9 induced podocyte dedifferentiation. MMP-9 also interrupted podocyte cell integrity, promoted podocyte monolayer permeability to albumin, and extracellular matrix protein synthesis. In diabetic patients, the upregulation of urinary MMP-9 concentrations occurred earlier than the onset of microalbuminuria. Thus, MMP-9 seems to play a role in the development of diabetic nephropathy.

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease (ESRD), and it affects 10–40% of diabetic patients. Hyperglycemia, hypertension, and genetic predisposition are the main risk factors for the development of DN. However, glycemic control along with currently available pharmacotherapies may delay, but do not stop, the progression of DN toward ESRD. Therefore, identifying the key signaling culprits of DN in order to explore novel therapeutic agent demands immediate attention.

Glomerular basement membrane (GBM) thickening and glomerular extracellular matrix (ECM) accumulation-induced Kimmelstiel-Wilson nodules are pathological hallmarks of DN. Matrix metalloproteinases (MMPs) affect the breakdown and turnover of ECM, suggesting that altered MMP expression may contribute to DN. Among various MMPs, MMP-9 digests collagen IV of the basement membrane, and it has been documented as a central corpus in diabetic retinopathy and tissue remodeling. It is thus important to define whether and how MMP-9 could contribute to DN. It has long been recognized that tubulointerstitial lesions have an important role in the progression of DN and renal tubular cell dedifferentiation has been considered a critical
step in tubulointerstitial damage. Recent studies have indicated that podocytes also undergo dedifferentiation in DN, which causes foot process effacement, albuminuria, and ultimately results in glomerular sclerosis and kidney fibrosis. \(^{14-17}\). Furthermore, it is known that a complex network of molecular signals is involved in cell dedifferentiation, and MMP-9 is able to induce renal tubular cell dedifferentiation in \textit{vivo}. \(^{18,19}\) Accordingly, in this study, we showed the influence of the targeted deletion of the MMP-9 gene in an animal model of DN; we used podocyte culture to reveal the potential stimulators of MMP-9 and investigated the effects of MMP-9 on podocyte cell functions. Finally, we tested the hypothesis that DN patients have higher urinary MMP-9 concentrations than non-DN patients, and that the upregulation of MMP-9 occurs earlier than the onset of microalbuminuria in DN patients.

RESULTS

Upregulation of intraglomerular MMP-9 activity in a DN mouse model

To clarify the expression pattern of MMP-9 in DN, we created a DN mouse model. As shown in Figure 1, there was a greater MMP-9 production and activity in glomeruli in diabetic mice and MMP-9 is co-stained with nephrin, a podocyte marker. As expected, no MMP-9 protein production or activity could be detected in glomeruli in MMP-9\(^{-/-}\) mice. These findings indicated that induction of diabetes can stimulate MMP-9 activation and increase protein production in kidney glomeruli.

Deficiency of MMP-9 attenuates diabetic kidney injury

To determine the potential pathological role of MMP-9 in DN \textit{in vivo}, we examined the severity of kidney injury in MMP-9\(^{-/-}\) and MMP-9\(^{+/+}\) mice after the development of diabetes. The blood sugar and hemoglobin A1c levels were comparable between the two diabetic groups (Supplementary Figure online). There was no difference in the 24-h urine albumin levels between nondiabetic MMP-9\(^{-/-}\) and MMP-9\(^{+/+}\) mice throughout the 6 months of the study. Urinary albumin was significantly elevated in diabetic MMP-9\(^{+/+}\) mice starting from the second month of diabetes \((P<0.05; \text{Figure 2a})\), and the extent of urinary albumin progressively increased in diabetic mice through the fourth and sixth month \((P<0.01).\) However, as shown in Figure 2a, the diabetic MMP-9\(^{-/-}\) mice had significantly less 24-h urinary albumin than diabetic WT mice \((P<0.05).\) After killing the mice at the sixth month, kidney glomerular volume was calculated. As shown in Figure 2b and c, diabetic mice had increased glomerular volume compared with nondiabetic mice \((P<0.01, \text{separately})\). However, diabetic

![Figure 1](https://example.com/figure1.png)

**Figure 1** Increased glomerular matrix metalloproteinase-9 (MMP-9) protein expression and catalytic activity in diabetic mice. (a) Immunostaining of MMP-9 in control and diabetic kidneys. MMP-9 was expressed in glomeruli and upregulated in diabetic nephropathy; 4,6-diamidino-2-phenylindole (DAPI) is used for nuclear counterstaining. (b) Zymography and western blot analysis from the sieved glomeruli lysate showed that both MMP-9 activity and protein expression were upregulated in MMP-9\(^{+/+}\) mice after induction of diabetes \((15\,\text{μg} \text{ protein per well, } n = 12 \text{ each group, } P < 0.01).\) As expected, no MMP-9 enzymatic activity or protein expression was detected in MMP-9\(^{-/-}\) mice with or without diabetes. Intraglomerular MMP-9 is costained with podocyte marker nephrin in wild type (MMP-9\(^{+/+}\)) and MMP-9\(^{-/-}\) mice. DM, diabetes mellitus.
MMP-9–/– mice had significantly mitigated glomerular hypertrophy compared with diabetic MMP-9+/+ mice (P < 0.01). Moreover, progressively increasing glomerular filtration rate (GFR) was observed in diabetic mice, and ablation of MMP-9 significantly decreased GFR hyperfiltration in DN (P < 0.05; Figure 2d). Kidney weights were compared among the four groups. As shown in Figure 2e and f, less kidney hypertrophy was noted in diabetic MMP-9–/– mice compared with diabetic MMP-9+/+ mice (P < 0.05).

We further evaluated the thickness of GBM by electron microscopic examination in kidney tissues. As illustrated in Figure 3, induction of diabetes increased GBM thickness, and diabetic MMP-9–/– mice had significantly lower GBM thickening than diabetic MMP-9+/+ mice (P < 0.05). These data indicated that deletion of MMP-9 significantly ameliorated albuminuria and prevented structural alterations of the diabetic kidneys.

**Diabetes-related cytokines stimulate podocytes to secrete MMP-9**

MMPs are major physiological determinants of ECM degradation, and diverse cytokines may stimulate MMP activation in diabetes. High-glucose and advanced glycoprotein end products are well-known diabetes-specific mediators, and we first assessed the expression of other potential cytokines in mouse kidney tissue. As shown in Figure 4a, the western blotting showed that tumor necrosis factor-α (TNF-α), transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), and angiotensin II protein expression levels were upregulated in diabetic kidneys.

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**Figure 2 | Matrix metalloproteinase-9 (MMP-9)-deficient mice have attenuated functional and pathological changes in diabetic nephropathy.** (a) Twenty-four-h urine albumin was determined in nondiabetic and diabetic mice both in wild-type (WT) and MMP-9-deficient mice throughout the study of 6 months. Microalbuminuria occurred at the second month and progressed to the end of the study. The diabetic MMP-9–/– mice had less 24-h urine albumin than diabetic WT mice. (b, c) After the mice were killed at the sixth month, kidney glomerular volume was calculated. Six months of diabetes caused significant glomerular hypertrophy, and diabetic MMP-9–/– mice had mitigated glomerular hypertrophy compared with diabetic WT mice. (d) Progressively increased glomerular filtration rate (GFR) was observed in diabetic mice, and diabetic MMP-9–/– mice had reduced GFR hyperfiltration compared with diabetic WT mice during the whole study period. (e, f) Kidney weights were compared among the four groups. Kidney hypertrophy was observed in diabetic mice, and the diabetic MMP-9–/– mice had significantly decreased kidney weight, when compared with diabetic WT mice. (*P < 0.05 compared with nondiabetic WT mice; **P < 0.01 compared with nondiabetic WT mice; #P < 0.05 compared with diabetic WT mice, ##P < 0.01 compared with diabetic WT mice) (n = 12 each group for all animal experiments.)
We then evaluated MMP-9 and MMP-2 expression in response to various cytokines in cultured podocytes. Among various diabetes-related cytokines, TGF-β1, TNF-α, and VEGF, but not high glucose, angiotensin II, or advanced glycoprotein end-product bovine serum albumin, significantly stimulated MMP-9 activation in a time- and dose-dependent manner. In contrast, the activity of the other collagenase MMP-2 remained constant (Figure 4b). These
findings suggest that diabetes-related cytokines are able to stimulate MMP-9 secretion by podocytes.

Endogenous and exogenous MMP-9 interrupts podocyte junction integrity, induces cell dedifferentiation, and increases ECM production

Podocyte slit diaphragm, a modified adherens junction, is an essential component to maintain normal glomerular filtration barrier. As various cytokines stimulate MMP-9 in DN, to investigate the effects of enhanced glomerular MMP-9 on the slit diaphragm, we studied the effect of MMP-9 overexpression in cultured podocytes. MMP-9 overexpression significantly downregulated slit diaphragm protein nephrin and promoted the expression of mesenchymal markers fibroblast-specific protein-1 and fibronectin. In addition, integrin-linked kinase (ILK), a key intracellular mediator for cell dedifferentiation, was also significantly upregulated at mRNA and protein levels (Figure 5a and b). To further evaluate the effect of exogenous MMP-9, cultured podocytes were exposed to recombinant active MMP-9 (rMMP-9). To rule out a cytotoxic effect of rMMP-9, a cell viability assay using MTT reagent was performed. Podocytes incubated with low to high concentrations of rMMP-9 (0.1, 1, or 5 μg/ml) did not alter cell viability in 96 h (data not shown). Exogenous MMP-9 induced cultured podocyte dedifferentiation in a dose-dependent manner (Figure 5c). Zonula occludens-1, a cell tight junction-associated protein, is located at the slit diaphragm. As depicted in Figure 5d, immunofluorescence staining demonstrated abundant continuous zonula occludens-1 at the sites of cell-cell contacts. It is noteworthy that after incubation with rMMP-9, the linear zonula occludens-1 staining became interrupted.

We then determined the albumin permeability of podocytes using in vitro transwell assay. After the monolayer podocyte had achieved confluence and thermoshifted for 14 days, treatment with 1 μg/ml rMMP-9 caused the podocytes to permit increased leakage of albumin from the lower to the upper chamber (Figure 6a). Observation of cell ultrastructures

![Figure 5](image)

Figure 5 | Matrix metalloproteinase-9 (MMP-9) induces podocyte dedifferentiation. Cultured podocytes are exposed to endogenous and exogenous MMP-9. (a) MMP-9 overexpression was confirmed by zymography; the reverse transcription-polymerase chain reaction (RT-PCR) results showed that MMP-9 overexpression downregulates mRNAs of nephrin and upregulates mesenchymal proteins fibronectin and fibroblast-specific protein-1 (FSP-1). Integrin-linked kinase (ILK) was also upregulated. (n = 3) (b) The results of protein expression in western blot. (n = 3) (c) The semiquantitative PCR and western blots illustrate that exogenous MMP-9 downregulated slit diaphragm proteins and upregulated mesenchymal proteins in a dose-dependent manner. (d) Protein expression pattern of podocytes; note that the abundant linear distributed slit diaphragm protein zonula occludens-1 (ZO-1) and P-cadherin between cell-cell junctions became interrupted after exposure to rMMP-9. On the contrary, the ILK, FSP-1, and fibronectin were upregulated.
by electron microscopy showed that administration of rMMP-9 (0.5 μg/ml) for 1 week interrupted podocyte adherens junction integrity and stimulated podocytes to synthesize new basement membrane (Figure 6b). These results from cultured podocytes were consistent with evidence showing that elevated glomerular MMP-9 is able to induce podocyte dedifferentiation, interrupt podocyte adherens junction integrity, and stimulate GBM synthesis.

Podocytes secrete MMP-9 in patients with DN
We examined the MMP-9 expression in kidney tissues from individuals with and without DN. As shown in Figure 7a, MMP-9-positive glomerular cells could be detected in DN kidneys, and the MMP-9-positive area was colocalized with podocyte marker synaptopodin, but not with the endothelial cell marker CD 31. However, MMP-9 was negatively stained in non-diabetes mellitus (DM) kidney samples.

Increased urinary MMP-9 precedes albuminuria in diabetic patients
To determine the time course by which MMP-9 may affect the development of microalbuminuria, we examined the MMP-9 concentrations in urinary samples retrieved from freshly voided urine of 104 diabetic patients and 23 healthy subjects. The clinical characteristics of study subjects are listed in the supplement. As shown in Figure 7b, there was a significantly increased urinary MMP-9-to-creatinine ratio in DM patients without microalbuminuria, when compared with healthy controls (control vs. DM patients without microalbuminuria, 1.3 ± 3.0 vs. 18.4 ± 4.1 pg/mg, P<0.01). Furthermore, DM patients with microalbuminuria had higher urinary MMP-9 concentration compared with DM patients without microalbuminuria (DM patients without microalbuminuria vs. DM patients with microalbuminuria, 18.4 ± 4.1 vs. 26.1 ± 8.2 pg/ml, P<0.05). However, there was no difference in urinary MMP-9 in DM patients with microalbuminuria and macroalbuminuria. These findings suggest an earlier elevation of urinary MMP-9 before the onset of microalbuminuria in diabetic patients.

DISCUSSION
In this study, we created a DN mouse model and demonstrated that induction of diabetes upregulates MMP-9 expression and activity in kidney glomeruli. Deletion of MMP-9 in diabetic mice significantly attenuated albuminuria, reduced glomerular hyperfiltration, partly recovered kidney size, and diminished the thickness of GBM. In cultured podocytes, incubation with diabetes-related cytokines upregulated MMP-9 activity, and MMP-9 promoted podocyte dedifferentiation, interrupted podocyte cell-cell
integrity, and enhanced albumin leakage. Furthermore, we showed that MMP-9-positive glomerular cells could be detected in patients with clinical evidence of DN, but not in non-DN patients. DN patients had high urinary MMP-9 concentrations than healthy controls, and an earlier upregulation of MMP-9 was observed in diabetic patients before the onset of microalbuminuria. These findings provide clear evidence that MMP-9 has a pivotal role in the development of DN.

It is widely recognized that microalbuminuria is one of the earliest clinical markers of DN, and the appearance of albumin in urine suggests a compromised glomerular filtration barrier. Podocyte foot processes interdigitate with the counterparts of their neighboring cells to form slit diaphragm. This membrane-like structure constitutes the final barrier to prevent protein loss during convective fluid flow from the vascular to the urinary space. Any injury to podocytes that disrupts slit diaphragm structural and functional integrity would eventually lead to a defective glomerular filtration, thereby causing albuminuria, and thus the onset of albuminuria is most closely associated with podocytopathy. Emerging evidence indicates that podocytes’ phenotypic conversion after injury is one of the most important factors that lead to proteinuria and renal fibrosis. In the current study, we clearly illustrated that glomerular MMP-9 protein expression and catalytic activity are enhanced in DN. A previous study showed that TGF-β stimulation enhanced podocyte MMP-9 expression as a dedifferentiation marker, and our experiments further demonstrated that MMP-9 per se is able to induce podocyte dedifferentiation. In addition, we found that MMP-9 disrupts podocyte cell-cell junction integrity and increases albumin filtration fraction. We also discovered that not only TGF-β1 but also other various cytokines are able to stimulate podocyte MMP-9 secretion. These findings indicate that increased intraglomerular MMP-9 catalytic activity is a common pathway of albuminuria in DN and explain the reason why TGF-β-neutralizing antibody attenuated glomerular

Figure 7 | Matrix metalloproteinase-9 (MMP-9) expression in human podocytes and enhanced MMP-9 urinary concentrations in patients with diabetic nephropathy (DN). (a) MMP-9 was IHC-stained and immunofluorescence costained with epithelial marker CD 31 and podocyte marker synaptopodin in human kidney samples. MMP-9 was negatively stained in kidney samples from nondiabetic subjects. In DN samples, MMP-9 was positive stained in some glomerular cells. The MMP-9-positive cells localized outside the glomerular capillary loop and colocalized with podocytes. Arrowheads indicate yellow color representing colocalization of MMP-9 and synaptopodin. (b) DN patients had higher urinary MMP-9 concentrations, and the urinary MMP-9 concentration became elevated before the onset of microalbuminuria. IHC, immunohistochemistry.
sclerosis but not albuminuria in previous DN studies.\textsuperscript{23,24} Bai et al.\textsuperscript{25} reported that the cultured podocytes have an increased MMP-9 secretion when incubated in high-glucose medium for 2–3 days, and the catalytic activity returns to normal after 5 days of incubation. In our experiments, high-glucose culture condition tends to increase MMP-9 catalytic activity, but at a modest level, especially when compared with other DM-related cytokines. ILK has been proven to regulate cell adhesion and ECM accumulation.\textsuperscript{26} ILK has been shown to have a central role in integrin-mediated cell signaling and is involved in the cross talk between GBM and the specialized slit diaphragm.\textsuperscript{27} ILK expression is induced in glomeruli of various diseases with typical podocyte lesions of foot process effacement and GBM denudation, including DN.\textsuperscript{28,29} Under diseased conditions, ILK outside-in signaling senses the ECM microenvironment change, induces cell dedifferentiation, and activates an inside-out signaling to reduce podocyte matrix adhesion.\textsuperscript{20,21,30} In the current study, we observed that MMP-9 upregulates ILK and promotes podocyte dedifferentiation, suggesting that MMP-9 modulates podocyte dedifferentiation through the ILK pathway. The pathological hallmarks of DN are characterized by excessive amassing of ECM with thickening of GBM and increased amount of mesangial matrix. Numerous studies have documented hyperglycemia, advanced glycoprotein end-products, angiotensin II, TGF-\(\beta\), ILK, interleukins, and TNF-\(\alpha\) involvement in the DN, and most of these signal pathways ultimately activate transcription factors affecting glomerular ECM accumulation.\textsuperscript{31–34} It was initially thought that MMPs may be globally protective through antagonism of ECM accumulation, but growing evidence indicates that MMPs are also involved in inflammation and tissue fibrosis in kidney disease.\textsuperscript{35} For example, transgenic MMP-2 overexpression in renal tubular cells causes renal fibrosis in mice,\textsuperscript{36} although a study has also proven that MMP-9 can induce murine renal tubular dedifferentiation \textit{in vitro}.\textsuperscript{19} A study from clinical kidney biopsy samples also illustrated that increased MMP-9 expression is an independent risk factor for kidney fibrosis.\textsuperscript{37} Genetic MMP-9 ablation has been reported to have a beneficial effect on ischemia reperfusion nephropathy, nephritic serum nephritis, and obstructive nephropathy animal models.\textsuperscript{38–41} Although the pathological role of MMP-9 in DN has been widely speculated upon, to the best of our knowledge, we are the first group to use genetic knockout mice to investigate its regulation under experimental conditions, and MMP-9 is a highly inducible enzyme, which is regulated mostly at the transcription level. In current experiments, the six DM-related cytokines did not induce MMP-2 activation in cultured podocytes. Some,\textsuperscript{48} but not all,\textsuperscript{49} clinical studies indicate that DM patients have increased serum MMP-9 concentrations. Our study illustrated that DM patients have an elevated urinary MMP-9 concentration even earlier than the onset of microalbuminuria. The molecular weight of MMP-9 (92 kD) is much larger than albumin (66 kD), which means it is very unlikely that urinary MMP-9 is filtrated from serum. The tissue staining of DN samples also illustrated that kidney MMP-9 is produced mainly from podocytes.

There are several limitations of the current study. First, although renal function is normal at 9 months of age, C57BL/6J strain MMP-9\textsuperscript{−/−} mice have been reported with increased interstitial fibrosis and tubular dilation at 12 months of age.\textsuperscript{50} As C57BL/6J strains are relatively resistant to the development of DN,\textsuperscript{51} we used FVB strain MMP-9\textsuperscript{−/−} and MMP-9\textsuperscript{+/+} mice in the current study because they develop marked albuminuria and hyperfiltration after diabetes.\textsuperscript{51} When the mice were killed at 6 months of age, we found that the renal function and pathology were indistinguishable between the two nondiabetic groups; thus, this issue hardly affects the interpretation of the current study result. Although one may question the role of cell dedifferentiation in renal disease,\textsuperscript{52,53} it is clear that MMPs are a part of its regulation under experimental conditions, and MMP-9 has also been found to induce renal tubular cell dedifferentiation.\textsuperscript{18,19} We found that DM patients have elevated urinary MMP-9 before the onset of microalbuminuria, and there are two potential explanations of this finding. It might only be the relevant to the common early renal adaptation of hyperglycemia, or the elevated urinary MMP-9 is actually an early marker or even a mediator for DN. Further clinical
studies are needed to determine whether baseline urinary MMP-9 is an independent parameter of albuminuria and GFR decline. Another concern may be related to the source of intrarenal MMP-9. Kluger et al. recently reported that leukocyte-derived MMP-9 has a crucial role in macrophage recruitment in nephrotoxic serum nephritis. MMP-9-deficient mice were shown to have reduced macrophage infiltration and pathological change, and bone marrow transplantation with wild-type-derived cells restored the disease severity. As leukocytes are MMP-9-rich cells, it is not surprising that deficiency of MMP-9 may suppress leukocyte-induced proinflammatory response in acute nephrotoxic nephritis. In contrast to the acute toxic nephritis, DN is a chronic disease and has no necrotic tissue; the macrophage infiltration is also modest in DN. Our data also demonstrated that MMP-9 is mainly produced from podocytes in diabetic kidney (Figure 1). Despite these limitations, our study still showed that MMP-9 is involved in DN development. Uncontrolled hyperglycemia enhances the activation of several inflammatory cytokines in the kidney and induces abnormal ECM synthesis, whereas some of these cytokines further stimulate podocytes to secrete MMP-9 to balance ECM accumulation. However, chronic activation of MMP-9 disrupts slit diaphragm integrity, induces podocyte dedifferentiation, and reduces cell–matrix adhesion. These events eventually result in GBM thickening, glomerular hypertrophy, and albuminuria in DN (Figure 8).

Conclusions
Taken together, our findings indicate that intraglomerular MMP-9 activation has an important role with regard to albuminuria and subsequent kidney damage in DN. Selective MMP-9 inhibition attenuates DN in an animal model and may be an attractive treatment strategy to treat DN in clinical practice.

MATERIALS AND METHODS

DN animal model
To clarify the effects of MMP-9 on DN, we created type 1 diabetes in MMP-9−/− mice and their wild-type controls and evaluated the subsequent diabetic kidney damages. MMP-9−/− (Jax lab stock number 007084, FVB strain) and their FVB control MMP-9+/− mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Diabetes was induced by daily intraperitoneal injections of streptozotocin (50 mg/kg×5 days) in 8-week-old male mice. Induction of DM was confirmed with fasting blood sugar >300 mg/dl 2 weeks after streptozotocin injection. Diabetic mice received a small dose of insulin (0.1 U) injection every other day; this dose of insulin does not affect blood sugar significantly, but it is able to prevent ketoadiosis and body weight loss. The mice were divided into four groups: the MMP-9+/+ control group, MMP-9+/− DM group, MMP-9−/− control group, and MMP-9−/− DM group (n = 12 for each group). All mice were housed under standard conditions with normal food, and were killed 6 months after induction of diabetes. All experimental procedures involving animals were approved by the institutional animal care committee of National Yang-Ming University, and complied with the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

GFR and albuminuria measurement
Mouse GFR was assessed by fluorescein isothiocyanate-inulin clearance method, as previously described. For measurement of the urine albumin, mice were placed in metabolic cages. The 24-h urine was centrifuged and the albumin level was determined using a commercialized enzyme-linked immunosorbent assay (ELISA Kit, Albuwell M kit, Exocell, Philadelphia, PA).

Measurement of histopathological change
Mice were killed after 6 months of diabetes, and the kidneys were carefully removed and weighed. The severity of DN was evaluated by glomerular hypertrophy, mesangial expansion, and GBM thickness. Ten randomly selected glomeruli from each mouse were used to measure glomerular volume, which was calculated by serial sections, as described previously. The severity of these histological changes was assessed using a digital camera and the MetaMorph software (Molecular Devices, Sunnyvale, CA). A part of the kidney tissue was sieved in order to retrieve glomeruli for protein expression and catalytic activity analysis.

Podocyte culture and treatment
Conditional immortalized murine podocytes were kindly provided by Professor Peter Mundel. Podocytes were cultured as previously
described. Briefly, in permissive condition, growth medium consisting of RPMI 1640 containing 10% fetal bovine serum supplemented with 50 μg/ml interferon-γ in 33 °C supported cell proliferation. After switching the cells to a medium lacking INF-γ at a temperature of 37 °C, the cells exited the cell cycle and started differentiation. For enhancement of nephrin expression, vitamin-D (100 nmol/l) and all-trans retinoid acid (1 μmol/l, Sigma-Aldrich, St Louis, MO) were added 7 days after thermoshift in part of the experiments. For overexpression experiments, an MMP-9 plasmid (pCMV-Sport6.1) constructed by cloning a 2.2-kb MMP-9 complementary DNA fragment (cDNA clone MGC: 54599 IMAGE: 6309245) was transfected by Lipofectamine LTX under permissive conditions. All cell experiments were performed 14 days after thermoshift and triplicated.

Zymography
To examine the effects of various diabetic mediators on MMP-9 secretion, podocytes were treated with α-glucose, advanced glycation-end product bovine serum albumin (R&D Systems, Minneapolis, MN), angiotensin II (Sigma-Aldrich), VEGF (ProSpec, East Brunswick, NJ), TNF-α (R&D Systems), and TGF-β1 (R&D Systems), because these cytokines are proven to increase and to have an important role in DN. The conditioned media were collected to analyze the MMP-9 activity by gelatin zymography. A constant amount of conditioned medium was loaded onto 8% sodium dodecyl sulfate-polyacrylamide gel containing 0.1% gelatin. After electrophoresis, the gel was incubated at 37 °C for 18 h in a developing buffer containing 50 mmol/l Tris-HCl, 0.2 mol/l NaCl, 5 mmol/l CaCl₂, and 0.02% Brij 35. The gel was then stained with Coomassie blue. Proteinase activity was detected as unstained bands on a blue background representing areas of gelatin digestion.

Monolayer cell permeability assay
Podocyte monolayer cell permeability reflects albuminuria in vitro and was measured as described previously. Briefly, podocytes (2×10⁵ podocytes per well) were grown on type 1 collagen-coated transwell plates (0.4-μm pore; Corning, Tewksbury, MA) in nonpermissive conditions for 14 days. Cells were incubated with rMMP-9 (EMD Millipore, Billerica, MA) for 12 and 36 h. Cells were washed twice with phosphate-buffered saline supplemented with 1 mmol/l each of MgCl₂ and CaCl₂. The upper compartment was filled with 0.25 ml of RPMI 1640 alone, and the lower compartment was filled with 0.5 ml of RPMI supplemented with 40 mg/ml bovine serum albumin and incubated for 4 h at 37 °C. Total protein concentrations in the upper compartment were determined using a Bio-Rad protein assay (Hercules, CA).

Western blot analysis and reverse transcription-polymerase chain reaction
Proteins in cell and glomerular lysates were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and detected by western blot analysis according to the established protocols. The primary antibodies used were as follows: anti-nephrin (Santa Cruz Biotechnologies, Santa Cruz, CA), anti-MMP-9 (Chemicon, Billerica, MA), anti-TGF-β1 (Abcam, Cambridge, MA), anti-VEGF (Santa Cruz Biotechnologies), anti-angiotensin II (Genetex, Irvine, CA), anti-TNFSF15 (Abcam), anti-ILK (Abcam), anti-fibroblast-specific protein-1 (Dako, Hamburg, Germany), anti-P-cadherin (R&D Systems), anti-fibronectin (Santa Cruz Biotechnologies), anti-β-actin, and anti-tubulin. For reverse transcription-polymerase chain reaction analysis, total RNA was extracted with the TRizol Plus RNA purification kit (Invitrogen, Carlsbad, CA). First strand complementary DNA was synthesized from total RNA using oligo (dT) as primers. Real-time PCR and semiquantitative evaluation of target mRNA transcripts were performed using the following primers: fibronectin, nephrin, P-cadherin, ILK, fibroblast-specific protein-1, and actin. The primer pair sequence is listed in supplement.

Immunofluorescence staining
Podocytes grown on type I collagen-coated coverslips or kidney cryosections were fixed for 15 min at room temperature in 4% paraformaldehyde, followed by permeabilization with 0.4% Triton X-100 in phosphate-buffered saline for 10 min. After blocking with 5% bovine serum albumin in phosphate-buffered saline for 30 min, samples were incubated with primary antibodies against zona occludens-1 (Invitrogen), nephrin, fibronectin, P-cadherin, ILK, and fibroblast-specific protein-1. Nonimmune immunoglobulin G served as a negative control, and no staining was observed. The slides were viewed under an Olympus FV10i confocal microscope (Olympus, Tokyo, Japan).

Electron microscopy
Mouse kidney cortex and cultured podocytes growing on transwell membranes were fixed using 2.5% glutaraldehyde, followed by 0.5% OsO₄. They were then series dehydrated and embedded according to routine procedures. Thin sections were viewed and digitally recorded using a transmission electron microscope (JEM-1230, JEOL, Tokyo, Japan). The GBM thickness was determined by the orthogonal intercept method. In essence, this applies a correction factor for oblique sectioning to the harmonic mean of a large number of random orthogonal intercept measurements of the GBM of two or more glomeruli, as described by El-Aouni et al. In the current study, five glomeruli of each mouse were reviewed.

Human urinary and kidney biopsy samples
To identify the source of MMP-9, four formalin-fixed kidney-biopsied tissues from DN patients were costained with MMP-9 antibody (Chemicon AB19016), endothelial cell marker CD-31 (Dako), or podocyte marker synaptopodin (Acris, Herford, Germany), whereas normal kidney tissues from the opposite site to the tumor in two nephrectomy patients were used as control. To compare urinary MMP-9 concentrations, DM patients with preserved renal function (estimated GFR > 60 ml/min per 1.73 m²) and healthy controls were enrolled in a cross-sectional study. Urine samples were spun at 3000 r.p.m. for 10 min to remove debris; urine samples with white blood cell > 10 per high-power field were excluded and supernatants were stored at −80 °C until use. Study subjects were categorized into four groups: the control, the DM without microalbuminuria, the DM with microalbuminuria, and the DM with macroalbuminuria group. Urinary MMP-9 concentration was measured using the commercially available ELISA kit (Quantikine human total MMP-9 ELISA kit, R&D Systems) and corrected for creatinine concentration. Blood biochemistry data were measured with a Hitachi 7600 chemical auto-analyzer (Hitachi, Tokyo, Japan), whereas urinary albumin and creatinine were measured with an Olympus AU640 auto-analyzer (Olympus). Clinical serum and urinary biochemistry measurements were performed in a College of American Pathologists qualified central lab. The human study part was approved by Taipei Veterans General Hospital Institutional Review Board, and all enrolled subjects signed informed consent.
Statistical analysis
All values are expressed as mean and s.d. unless otherwise specified. Statistical analysis of the data was performed using the SPSS software (IBM Corporation, Armonk, NY). Comparison between groups was carried out by analysis of variance and Bonferroni’s correction. A level of \( P < 0.05 \) was considered statistically significant.

DISCLOSURE
All the authors declared no competing interests.

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S-YL initiated the study, contributed to experiments, and wrote the manuscript. A-HY contributed to the study design and electron microscopic photography. D-CT, C-CL, and W-CY contributed to the data collection and reviewed the manuscript. J-WC and GS-S contributed to data interpretation. P-HH and S-JL are the guarantors of this work and, as such, had full access to all the data in the study, and they take responsibility for the integrity of the data and the accuracy of the data analysis. This study was supported, in part, by the following research grants: the National Science Council, NSC 101-2314-B-075-038, NSC 98-2314-B-075-035, and UST-UCSD International Centre of Excellence in Advanced Bio-engineering NSC-100-2911-I-009-101-A2; VGH-V102B-016, VGH-V100E2-002, and VGH-V102E2-002 from Taipei Veterans General Hospital; and a grant from the Ministry of Education’s ‘ AIM for the Top University’ Plan. Funding agencies had no role in study design, data collection, analysis, decision to publish, or preparation of the manuscript.

SUPPLEMENTARY MATERIAL
Table S1. Body weight, BUN, kidney weight, and relative mesangial area of kidney tissues of study mice.
Figure S1. Diabetes mellitus induced by streptozotocin intraperitoneal injection.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/ki

REFERENCES


