MYDGF attenuates podocyte injury and proteinuria by activating Akt/BAD signal pathway in mice with diabetic kidney disease

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Abstract

Aims/hypothesis Myeloid-derived growth factor (MYDGF), mainly secreted by bone marrow-derived cells, has been known to promote glucagon-like peptide-1 production and improve glucose/lipid metabolism in mouse models of diabetes, but little is known about the functions of MYDGF in diabetic kidney disease (DKD). Here, we investigated whether MYDGF can prevent the progression of DKD.

Methods In vivo experiments, both loss- and gain-of-function strategies were used to evaluate the effect of MYDGF on albuminuria and pathological glomerular lesions. We used streptozotocin-treated *Mydgf* knockout and wild-type mice on high fat diets to induce a model of DKD. Then, albuminuria, glomerular lesions and podocyte injury were evaluated in *Mydgf* knockout and wild-type DKD mice treated with adeno-associated virus-mediated *Mydgf* gene transfer. In vitro and ex vivo experiments, the expression of slit diaphragm protein nephrin and podocyte apoptosis were evaluated in conditionally immortalised mouse podocytes and isolated glomeruli from non-diabetic wild-type mice treated with recombinant MYDGF.

Results MYDGF deficiency caused more severe podocyte injury in DKD mice, including the disruption of slit diaphragm proteins (nephrin and podocin) and an increase in desmin expression and podocyte apoptosis, and subsequently caused more severe glomerular injury and increased albuminuria by 39.6% compared with those of wild-type DKD mice ($p < 0.01$). Inversely, MYDGF replenishment attenuated podocyte and glomerular injury in both wild-type and *Mydgf* knockout DKD mice and then decreased albuminuria by 36.7% in wild-type DKD mice ($p < 0.01$) and 34.9% in *Mydgf* knockout DKD mice ($p < 0.01$). Moreover, recombinant MYDGF preserved nephrin expression and inhibited podocyte apoptosis in vitro and ex vivo. Mechanistically, the renoprotection of MYDGF was attributed to the activation of the Akt/Bcl-2-associated death promoter (BAD) pathway.

Conclusions/interpretation The study demonstrates that MYDGF protects podocytes from injury and prevents the progression of DKD, providing a novel strategy for the treatment of DKD.

Keywords Diabetic kidney disease · Myeloid-derived growth factor · Podocyte injury

Abbreviations

AAV Adeno-associated virus
BAD Bcl-2-associated death promoter

Bcl-2 B-cell lymphoma 2
BMDC Bone marrow-derived cell
BMSC Bone marrow mesenchymal stem cell
BMMNC Bone marrow mononuclear cell
BMC Bone marrow cell
BUN Blood urea nitrogen
DKD Diabetic kidney disease
GBM Glomerular basement membrane
GLP-1 Glucagon-like peptide-1
HFD High-fat diet
HG High glucose
KO Knockout
MA Mannitol

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Research in context

What is already known about this subject?

- Myeloid-derived growth factor (MYDGF) is a paracrine-acting protein produced by bone marrow-derived monocytes and macrophages
- A previous study has indicated that MYDGF promotes glucagon-like peptide-1 production and improves glucose/lipid metabolism in a mouse model of diabetes

What is the key question?

- Does MYDGF affect the progression of diabetic kidney disease?

What are the new findings?

- MYDGF ameliorates albuminuria and glomerular injury in a mouse model of diabetic kidney disease
- MYDGF preserves nephrin expression and protects against podocyte apoptosis both in vivo and in vitro
- The molecular mechanisms underlying these beneficial effects of MYDGF involve the activation of the Akt/BAD signalling pathway

How might this impact on clinical practice in the foreseeable future?

- The study suggests a critical role for MYDGF in preventing the development of diabetic kidney disease by protecting podocytes from injury and improving podocyte function through the activation of the Akt/BAD pathway, opening up a potential new therapeutic strategy for treating diabetic kidney disease

MYDGF	Myeloid-derived growth factor
NG	Normal glucose
PAS	Periodic acid–Schiff
PKA	Protein kinase A
rMYDGF	Recombinant MYDGF
RSK	p90 ribosomal S6 kinase
siRNA	Small interfering RNA
STZ	Streptozotocin
S6K	p70 ribosomal S6 kinase
TEM	Transmission electron microscopy
UACR	Urinary albumin/creatinine ratio
WT	Wild-type
WT-1	Wilms' tumour 1

Introduction

Diabetic kidney disease is one of the most common complications of diabetes and has become the leading cause of end-stage renal disease [1]. As the earliest appearing symptom, proteinuria is the hallmark of diabetic kidney disease (DKD) and is caused by disruption to the glomerular filtration barrier [2]. Podocytes present on the outer surface of the glomerular basement membrane (GBM), which contributes to maintaining the structure and function of the glomerular filtration barrier. Podocyte injury, such as alterations in the structures of podocytes or loss of podocytes, affects the integrity of the filtration barrier, resulting in proteinuria and glomerulosclerosis [3, 4]. Moreover, apoptosis is one of the

main reasons for podocyte loss, since podocytes are highly specialised, terminally differentiated and unable to be replaced or regenerated [5–7]. Therefore, protecting podocytes from injury is a promising therapeutic target for DKD treatment.

Bone marrow-derived cells (BMDCs) are a pool of progenitor and pluripotent stem cells including endothelial progenitor cells, haematopoietic stem cells and mesenchymal stromal cells that secrete a variety of cytokines, growth factors, exosomes and microvesicles [8]. Evidence in recent years has shown that BMDCs, such as bone marrow mesenchymal stem cells (BMSCs) and bone marrow mononuclear cells (BMMNCs), inhibit the progression of DKD [9, 10]. In rodent models, intravenous injection of BMSCs can preserve renal structure and ameliorate the development of diabetic nephropathy [11], and BMMNC treatment also improves glomerular function in early diabetic nephropathy [12]. In clinical trials, allogeneic BMSC transplantation can improve the glomerular filtration rate [13], and autologous BMMNC infusion also ameliorates albuminuria in diabetic nephropathy patients [14]. Moreover, increasing numbers of studies have demonstrated that the paracrine effect, as an alternative mechanism by which BMDCs secrete biologically active molecules, is a promising strategy for the treatment of DKD [8, 15, 16]. Myeloid-derived growth factor (MYDGF, also known as C19orf10) is also a paracrine-acting protein produced by bone marrow-derived monocytes and macrophages [17]. Our previous study has found that MYDGF can promote intestinal glucagon-like peptide-1 (GLP-1) production and improve glucose/lipid metabolism in a mouse model of type 2 diabetes

[18]. However, whether MYDGF also participates in the development of DKD remains unknown. Therefore, in this study, we investigated the effects of MYDGF on DKD and explored the possible mechanisms involved.

Methods

Human study

A total of 60 male type 2 diabetic patients with DKD admitted to the Department of Endocrinology, General Hospital of Central Theater Command from October 2018 to May 2019 were enrolled in this study. During the same period, 20 age-matched healthy male participants were selected as a control group. All the participants enrolled in this study signed informed consent. The study was approved by the ethics committee of the General Hospital of Central Theater Command. The eligibility criteria are provided in electronic supplementary material (ESM) Methods.

Clinical examination BMI and blood pressure were measured. See ESM [Methods](#) for further details.

Blood biochemical analysis HbA_{1c}, serum total cholesterol, triacylglycerol, HDL-cholesterol, LDL-cholesterol, creatinine and blood urea nitrogen (BUN) were measured, and eGFR was estimated. Plasma MYDGF level was tested in duplicate. See ESM [Methods](#).

Urine biochemical analysis All participants underwent a 24 h urinary albumin excretion test. See ESM [Methods](#).

Animals and treatments

Mydgf knockout mice *Mydgf* knockout (KO) and wild-type (WT) littermate mice were produced by mating *Mydgf*^{+/-} breeders of C57BL/6J background purchased from Shanghai Southern Model Biotechnology Company, China.

DKD mice Streptozotocin (STZ; Sigma-Aldrich, St Louis, MO, USA)-treated mice fed with high-fat diet (HFD) (41% energy from fat, 42% carbohydrate and 17% protein) were used to produce DKD models following previous studies [19, 20]. The WT and KO male mice aged 4 weeks were both fed an HFD for 4 weeks and then i.p. injected with STZ (50 mg/kg per day) for 5 consecutive days to generate a mouse model of type 2 diabetes [18]. Nine days later, the mice were considered diabetic when their nonfasting blood glucose levels exceeded 13.9 mmol/l for 2 consecutive days [21]. After the diabetic model was produced, the mice were maintained for an additional 6 weeks of continued HFD feeding.

Adeno-associated-virus-treated mice The *Mydgf* (GenBank accession number NM_080837.2) cDNA was synthesised and adeno-associated virus (AAV)-MYDGF was constructed as previously [18, 22]. The mice received a single injection of AAV-MYDGF or AAV-GFP at a dose of 1×10^{12} viral genomes through the tail vein. For small interfering RNA (siRNA) treatment, the *Akt*-specific siRNA (GenBank accession number NM_009652.3) and the scrambled control siRNA oligonucleotides were obtained and AAV-siRNA/*Akt* (si*Akt*) and AAV-siRNA/control (siCON) were produced as previously [18, 23]. Both kidneys of the mice were treated with si*Akt* or siCON at a dose of 1.6×10^{11} viral genomes using orthotopic injection [24, 25].

Bone marrow chimeric mice Bone marrow cells (BMCs) were flushed from the femurs and tibias of the WT and KO mice and transplanted into lethally irradiated (7.5 Gy) recipient WT and KO mice through the tail vein (1×10^6 cells per mouse) as previously described [17].

All the experiments followed the National Institutes of Health Guidelines for the Use of Laboratory Animals. All the animal procedures were approved by the animal ethics committee of the General Hospital of Central Theater Command. See ESM [Methods](#).

Metabolic and biochemical analyses

Glucose and insulin tolerance tests, blood pressure, blood biochemical analysis and urine biochemical analysis are detailed in ESM [Methods](#).

Histology

For the morphometric assessment, kidney sections were stained with a periodic acid–Schiff (PAS) kit (Sigma-Aldrich). For immunofluorescence staining, MYDGF, nephrin and podocin antibodies were used. To identify podocyte apoptosis, Wilms' tumour 1 (WT-1) and TUNEL double staining and nephrin and cleaved caspase-3 double staining were performed. The analyses are detailed in ESM [Methods](#). The antibodies are detailed in ESM Table 1.

Transmission electron microscopy

Several 1 mm cubes from the renal cortex were cut, fixed, stained, embedded for transmission electron microscopy (TEM) and quantified, as described previously [26, 27]. See ESM [Methods](#).

Glomeruli isolation and culture

Briefly, mice were perfused with Dynabeads M-450 (Invitrogen, Carlsbad, CA, USA). After perfusion, the kidney

tissue was digested, filtered and transferred to a magnetic particle concentrator, which gathered the glomeruli that contained Dynabeads. After isolation, the glomeruli were incubated in DMEM under various experimental conditions. See ESM [Methods](#).

Cell culture and treatments

Conditionally immortalised mouse podocytes (MPC5) were purchased from the Cell Bank of the BeNa Culture Collection of Beijing (BNCC337685) and cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% FBS (Invitrogen) and 5.5 mmol/l glucose. Podocytes were authenticated by morphology examination and nephrin expression. No mycoplasma contamination was detected.

Apoptosis assays

Apoptosis was determined using caspase-3 activity (detailed in ESM [Methods](#)) and flow cytometry (Beckman-Coulter, Indianapolis, IN, USA) after double staining with annexin V-FITC and propidium iodide (eBioscience, San Diego, CA, USA).

RT-PCR and western blot

RT-PCR was performed as previously [28]. The primer sequences are listed in ESM Table 2. Western blot was performed on isolated glomeruli or cell extracts as described previously [29]. The antibodies are listed in ESM Table 1.

Statistical analysis

All the quantified data are expressed as the means $\pm$ SEM. Data between groups were compared by the unpaired Student's *t* test or one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. A *p* value <0.05 was considered significant.

Results

MYDGF levels decrease in human participants and mice with DKD

Our previous report found that the plasma levels of MYDGF decreased in diabetic patients and mice [18]. Thus, we further explored MYDGF expression in DKD. Sixty male participants with type 2 diabetes and 20 age-matched healthy male control participants were enrolled for measurement of their plasma MYDGF levels (participant characteristics are detailed in ESM Table 3). According to the degree of albuminuria, patients were classified into a normoalbuminuric group, a microalbuminuric group and a macroalbuminuric group

Fig. 1 MYDGF expression declines in patients and mice with DKD, and MYDGF deficiency accelerates albuminuria and glomerular injury in DKD mice. **(a)** MYDGF levels in normoalbuminuric, microalbuminuric and macroalbuminuric diabetic and healthy participants ($n = 20$ per group). **(b, c)** Plasma MYDGF content **(b)** and bone marrow *Mydgf* mRNA expression **(c)** in DKD mice and control mice ($n = 10$ per group). The mRNA expression of the DKD mice is relative to that of the control mice. **(d)** Experiment schedule of the HFD/STZ-induced DKD mouse model study in *Mydgf* KO and WT mice ($n = 8$ mice per group). **(e)** Urinary albumin levels were measured at the termination of the study ($n = 6$ mice per group). **(f)** Representative PAS staining and **(g, h)** quantitative analyses of mesangial matrix expansion and glomerular volume in glomeruli ($n = 4$ mice per group). Scale bar, 20 μ m; original magnification $\times 400$. **(i)** Western blots and quantification analyses of fibronectin in glomerular lysates ($n = 3$ mice per group). **(j–l)** The mRNA levels of fibronectin, *Tgfb- β* (also known as *Tgfb1*) and collagen IV in glomerular lysates were determined using RT-PCR ($n = 6$ mice per group). The mRNA expression of the mice is relative to that of the WT-CON mice. Data are shown as the mean $\pm$ SEM. Student's *t* test was employed for comparisons between two groups. One-way ANOVA followed by Tukey's post hoc test for multiple comparisons was used for groups of three or more. * $p < 0.05$, ** $p < 0.01$ vs WT-CON group (except as otherwise shown in **a**); †† $p < 0.01$ vs KO-CON group; ‡ $p < 0.05$, ‡‡ $p < 0.01$ vs WT-DKD group. CON, control; FN, fibronectin; Macro., macroalbuminuric; Micro., microalbuminuric; MYDGF, myeloid-derived growth factor; Normo., normoalbuminuric; Vol., volume

(ESM Table 3). The results showed that the plasma MYDGF levels in diabetic participants were lower than those of control participants (Fig. 1a). Among the three diabetic groups, the plasma MYDGF levels decreased in the microalbuminuric group and further declined in the macroalbuminuric group compared with the normoalbuminuric group (Fig. 1a). For the mice, the plasma MYDGF levels and *Mydgf* expression in bone marrow decreased in HFD/STZ-induced DKD mice compared with control mice (Fig. 1b, c). These results indicate that MYDGF may be associated with the development of DKD.

MYDGF deficiency aggravates albuminuria, glomerular injury and metabolic characteristics in DKD mice

As mentioned above, some relationships were found between plasma MYDGF and DKD, and these results encouraged us to explore further the effect of MYDGF deficiency on DKD. We first generated *Mydgf* global KO mice, and MYDGF expression in the plasma and bone marrow were tested, which confirmed the absence of MYDGF in the KO mice (ESM Fig. 1a, b).

HFD/STZ-induced DKD models in WT and KO mice were then generated (Fig. 1d). In the DKD groups, the urinary albumin/creatinine ratio (UACR) levels increased, being 39.6% higher in the KO-DKD mice than the WT-DKD mice ($p < 0.01$) (Fig. 1e), with no significant changes in BUN or serum creatinine between the two groups (ESM Table 4). Moreover, PAS staining showed that the glomerular volume

and mesangium expansion increased in the KO-DKD mice compared with the WT-DKD mice (Fig. 1f–h). We also examined the glomerular expression for profibrotic molecules (fibronectin, TGF- β and collagen IV). Western blot and RT-PCR analyses showed that the levels of the profibrotic molecules were elevated in the KO-DKD mice compared with the WT-DKD mice (Fig. 1i–l). These results illustrate that loss of MYDGF may accelerate albuminuria, glomerulomegaly and glomerulosclerosis in DKD.

Considering that metabolic disturbance is the primary characteristic of DKD [30], we further investigated metabolic indicators. First, the DKD mice developed glucose intolerance and insulin resistance, and the KO-DKD mice displayed more severe impairment in glucose tolerance and insulin sensitivity than the WT-DKD mice (ESM Fig. 2a–d). Second, the DKD groups had dyslipidaemia, and lack of MYDGF resulted in a worse lipid profile (ESM Table 4). Third, MYDGF deficiency led to greater weight gain in KO-DKD mice than in WT-DKD

Fig. 2 MYDGF deficiency exacerbates podocyte injury and apoptosis in DKD mice. **(a)** Representative immunofluorescence images of nephrin in glomeruli. Scale bar, 20 μ m; original magnification $\times 400$. **(b)** Representative immunofluorescence images of podocin in glomeruli. Scale bar, 20 μ m; original magnification $\times 400$. **(c)** Representative TEM images of glomeruli. Scale bar, 1 μ m; original magnification $\times 5000$. **(d)** Representative images showing WT-1 (green) and TUNEL (red) double staining. Scale bar, 20 μ m; original magnification $\times 400$. **(e, f)** Quantification analyses of mean GBM thickness and mean foot process width in **(c)** ($n = 5$ mice per group). **(g)** Absolute count of the numbers of glomerular cells positive for both WT-1 and TUNEL (white arrows) as the means of 20 glomeruli ($n = 4$ mice per group). **(h, i)** Western blots and quantification analyses of nephrin and desmin in glomerular lysates ($n = 3$ mice per group). Data are shown as the mean $\pm$ SEM. One-way ANOVA followed by Tukey's post hoc test for multiple comparisons was used for groups of three or more. $*p < 0.05$, $**p < 0.01$ vs WT-CON group; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ vs KO-CON group; $^{++}p < 0.01$ vs WT-DKD group. CON, control

mice, without a difference in food intake between the two groups (ESM Table 4). These data show that loss of MYDGF may aggravate glucose and lipid metabolism in DKD.

MYDGF deficiency promotes podocyte injury in DKD mice

In view of the important role of podocytes on DKD [31], we next examined the effect of MYDGF deficiency on podocytes.

The results showed that nephrin and podocin expression in the glomeruli decreased in the DKD mice, and the expression further declined in KO-DKD mice compared with WT-DKD mice (Fig. 2a, b). Furthermore, TEM showed podocyte injuries in the DKD groups, as evidenced by GBM thickening and degradation or fusion of the podocyte foot process, and the injuries were aggravated in KO-DKD mice compared with WT-DKD mice (Fig. 2c, e, f). TUNEL staining in WT-1-positive cells revealed that the apoptotic podocytes increased in the DKD groups compared with the control groups, and

loss of MYDGF accelerated podocyte apoptosis in DKD mice (Fig. 2d, g), which was reconfirmed by cleaved caspase-3 staining in nephrin-positive cells (ESM Fig. 3). Western blot analyses showed that MYDGF deficiency decreased nephrin expression and increased desmin expression in DKD mice (Fig. 2h, i). Taken together, these results show that loss of MYDGF may exacerbate podocyte injury in DKD.

MYDGF restoration ameliorates albuminuria, glomerular injury and metabolic characteristics in vivo

Our animal study showed that MYDGF deficiency aggravated podocyte injury and promoted albuminuria in DKD mice. Thus, we further explored whether MYDGF restoration had a favourable impact on DKD. We first constructed an AAV vector to overexpress MYDGF (ESM Fig. 4a). For AAV treatment, the WT and KO mice received a single injection of AAV-MYDGF or AAV-GFP via the tail vein (Fig. 3a). Six weeks later, the AAV-MYDGF mice had higher plasma MYDGF and liver MYDGF expression than the AAV-GFP mice (ESM Fig. 4b–e). At the end of the six week period, MYDGF replenishment decreased albuminuria by 36.7% in the WT mice ($p < 0.01$) and 34.9% in the KO mice ($p < 0.01$) (Fig. 3b). PAS staining showed that the glomerular volume and mesangium expansion decreased in the mice injected with AAV-MYDGF compared with the mice injected with AAV-GFP (Fig. 3c–e). Consistently, western blot and RT-PCR analyses showed that the profibrotic molecule expression in the glomeruli declined in the WT-MYDGF and KO-MYDGF mice, compared with the respective AAV-GFP mice (Fig. 3f–i). Taken together, these results confirm that MYDGF ameliorates albuminuria, glomerulomegaly and glomerulosclerosis in DKD.

Accordingly, we assessed whether MYDGF restoration affected metabolism. After the 6 week intervention, MYDGF treatment improved hyperglycaemia and dyslipidaemia and decreased body weight gain (ESM Fig. 5e–k, ESM Table 5). These data demonstrate that MYDGF ameliorates glucose and lipid metabolism in DKD.

MYDGF restoration prevents podocyte injury in vivo

Next, we explored whether MYDGF restoration has a positive effect on podocytes in DKD mice. The results showed that nephrin and podocin expression was preserved in the glomeruli of both WT and KO mice injected with AAV-MYDGF compared with mice injected with AAV-GFP (Fig. 4a, b). TEM also showed that podocyte injuries were attenuated in both WT-MYDGF and KO-MYDGF mice compared with their respective AAV-GFP groups (Fig. 4c, e, f). TUNEL staining revealed that podocyte apoptosis decreased in the WT-MYDGF and KO-MYDGF mice compared with the WT-GFP and KO-GFP mice (Fig. 4d, g), reconfirmed by

cleaved caspase-3 staining (ESM Fig. 6), which indicated that MYDGF inhibited podocyte apoptosis. Additionally, western blot analyses showed that MYDGF treatment increased nephrin expression and decreased desmin expression in DKD mice (Fig. 4h). These results illustrate that MYDGF attenuates podocyte injury in DKD.

MYDGF prevents podocyte injury in vitro and ex vivo

To further corroborate the direct benefits of MYDGF on podocytes, we used podocytes from non-diabetic WT mice as an in vitro system. Podocytes pretreated with 25 mmol/l glucose (high glucose [HG]) for 24 h significantly induced cell apoptosis, which was chosen in the following study (ESM Fig. 7a, b). Additionally, exposure of the HG-induced podocytes to recombinant MYDGF (rMYDGF) prevented cell apoptosis in a concentration- and time-dependent manner (ESM Fig. 7c, d). Accordingly, rMYDGF treatment at 100 ng/ml for 24 h was used subsequently.

As shown in Fig. 5a, b, nephrin expression decreased under HG conditions, and rMYDGF treatment attenuated this effect. Moreover, flow cytometry analysis showed that more apoptotic podocytes were observed in the HG group than in the normal glucose (NG) group or the mannitol (MA) group, and rMYDGF treatment remitted this effect (Fig. 5e, f). Consistently, the expression of the antiapoptotic protein B-cell lymphoma 2 (Bcl-2) increased and the expression of the proapoptotic Bax and cleaved caspase-3 decreased in the HG-induced podocytes treated with rMYDGF (Fig. 5a, c, d).

In addition, glomeruli isolated from non-diabetic WT mice were used as an ex vivo system. Pretreatment of mouse glomeruli with 25 mmol/l HG for 96 h showed impaired nephrin expression and induced cell apoptosis, which was used subsequently (ESM Fig. 8a, b). Based on the rMYDGF treatment in vitro, the HG-induced glomeruli were subsequently treated with or without 100 ng/ml rMYDGF for 24 h. In parallel, the nephrin expression in glomeruli significantly decreased under HG conditions, and rMYDGF abated the effect (Fig. 5g, h). Consistently, rMYDGF treatment increased Bcl-2 expression and decreased the Bax and cleaved caspase-3 expression in HG groups (Fig. 5g, i, j). Taken together, these data further confirm that MYDGF attenuates HG-induced podocyte injury.

Akt/BAD signal is required for the benefits of MYDGF in DKD mice

We next explored the possible mechanisms through which MYDGF exerts protective effects on podocytes. Because our data showed that podocyte apoptosis increased in both DKD mice and HG-induced mouse podocytes, and MYDGF treatment attenuated this effect, we further measured the level of apoptosis-related Bcl-2-associated death promoter (BAD).

Fig. 3 MYDGF restoration ameliorates albuminuria and glomerular injury in WT and KO mice with DKD. **(a)** Schematic representation of the AAV treatment protocol for WT and KO mice ($n = 8$ mice per group). **(b)** Urinary albumin levels were measured weekly after AAV-MYDGF treatment ($n = 6$ mice per group). **(c)** Representative PAS staining and **(d, e)** quantitative analyses of mesangial matrix expansion and glomerular volume in glomeruli ($n = 4$ mice per group). Scale bar, 20 μm ; original magnification $\times 400$. **(f)** Western blots and quantification analyses of fibronectin in glomerular lysates ($n = 3$ mice per group). **(g–i)** mRNA levels of fibronectin, *Tgf- β* and collagen IV in glomerular lysates were determined using RT-PCR ($n = 6$ mice per group). The mRNA expression of the mice is relative to that of the control mice. Data are shown as the mean $\pm$ SEM. One-way ANOVA followed by Tukey's post hoc test for multiple comparisons was used for groups of three or more. $*p < 0.05$, $**p < 0.01$ vs WT-GFP group; $\dagger\dagger p < 0.01$ vs KO-GFP group. FN, fibronectin; Vol., volume

Expressed as a member of the *Bcl2* gene family, BAD had been shown to inhibit apoptosis in its phosphorylated form [32]. Consistent with previous studies [33, 34], in our in vitro study, podocytes treated with HG exhibited a decrease in p-BAD, and rMYDGF treatment partially reversed this defect (Fig. 6a, b). It is well known that p-BAD can be regulated by several kinases, such as Akt, protein kinase A (PKA), p90 ribosomal S6 kinase (RSK) and p70 ribosomal S6 kinase (S6K) [35–37].

Our study found that rMYDGF treatment obviously increased p-Akt but did not alter the expression of p-PKA and p-RSK (Fig. 6a, c–f). S6K is also a downstream target of Akt and can phosphorylate BAD [38]; p-S6K declined in HG groups, but rMYDGF treatment did not change the decline (Fig. 6a, g). Consistently, our in vivo experiment showed that p-Akt and p-BAD declined in the DKD groups, and AAV-MYDGF treatment improved the decline (Fig. 6h–k).

Fig. 4 MYDGF restoration attenuates podocyte injury and apoptosis in WT and KO mice with DKD. **(a)** Representative immunofluorescence images of nephrin in glomeruli. Scale bar, 20 μ m; original magnification $\times 400$. **(b)** Representative immunofluorescence images of podocin in glomeruli. Scale bar, 20 μ m; original magnification $\times 400$. **(c)** Representative TEM images of glomeruli. Scale bar, 1 μ m; original magnification $\times 5000$. **(d)** Representative images showing WT-1 (green) and TUNEL (red) double staining. Scale bar, 20 μ m; original magnification $\times 400$. **(e, f)** Quantification analyses of mean GBM thickness and mean foot process width in **(c)** ($n = 5$ mice per group). **(g)** Absolute count of the numbers of glomerular cells positive for both WT-1 and TUNEL (white arrows) as the means of 20 glomeruli in **(d)** ($n = 4$ mice per group). **(h)** Western blots and quantification analyses of nephrin and desmin in glomerular lysates ($n = 3$ mice per group). Data are shown as the mean $\pm$ SEM. One-way ANOVA followed by Tukey's post hoc test for multiple comparisons was used for groups of three or more. * $p < 0.05$, ** $p < 0.01$ vs WT-GFP group; †† $p < 0.01$ vs KO-GFP group

We then confirmed the involvement of the Akt/BAD pathway in the benefits of MYDGF by RNA interference-mediated gene silencing *in vivo* and *in vitro*. For siRNA treatment, the renal cortex in both kidneys of mice were injected with siAkt or siCON (Fig. 7a). After 7 days, we evaluated the silencing efficiency; as expected, a robust reduction in Akt expression was found in the kidneys, but did not occur in the liver (ESM Fig. 9a–d). After the siAkt treatment, p-BAD/BAD in glomeruli decreased (Fig. 7b, c), and the UACR level and glomerular injury increased (Fig. 7d–j). In cultured podocytes *in vitro*, siAkt effectively silenced Akt expression in podocytes (ESM Fig. 9e–g). As a result, Akt knockdown

partially abated the rMYDGF-mediated protection of podocytes (Fig. 5a–f). Taken together, these data substantiate that MYDGF promotes podocyte survival partially through the Akt/BAD-dependent pathway.

Bone marrow-derived MYDGF prevents albuminuria, podocyte injury and glucose metabolism in DKD mice

Based on the fact that MYDGF is a paracrine-acting protein derived from BMDCs [17], bone marrow chimeric mice were generated to specifically address the importance of BMC-derived MYDGF for DKD (Fig. 8a). Six weeks after BMC

Fig. 5 MYDGF improves HG-induced podocyte injury and apoptosis in vitro and ex vivo. (**a–f**) Conditionally immortalised mouse podocytes (MPC5) treated with NG (5.5 mmol/l glucose), HG (25 mmol/l glucose) or mannitol (5.5 mmol/l glucose +19.5 mmol/l D-mannitol) for 24 h. Transfected with siAkt or not, HG-treated podocytes were exposed to 100 ng/ml rMYDGF for another 24 h. In addition, the NG group, MA group and HG group without rMYDGF treatment were processed in parallel without changing culture medium. (**a–d**) Representative western blots and quantification analyses of nephrin, cleaved caspase-3, Bax and Bcl-2 proteins in podocytes after incubation. (**e, f**) Apoptosis rates of

podocytes were measured by annexin V–propidium iodide staining and flow cytometry analysis. (**g–j**) Isolated glomeruli were incubated with NG (5.5 mmol/l) or HG (25 mmol/l) for 96 h and then exposed to 100 ng/ml rMYDGF or not for another 24 h without changing culture medium. Representative western blots and quantification analyses of nephrin, cleaved caspase-3, Bax and Bcl-2 proteins in glomeruli after incubation. Data are represented as the mean $\pm$ SEM of three independent experiments. One-way ANOVA followed by Tukey's post hoc test for multiple comparisons was used for groups of three or more. * $p < 0.05$, ** $p < 0.01$ vs NG group; † $p < 0.05$, †† $p < 0.01$ vs HG+rMYDGF group

transplantation, the plasma MYDGF levels were measured to determine chimerism. No MYDGF was detectable in the plasma of the KO recipient mice transplanted with KO-BMCs, and the plasma MYDGF level increased in the KO recipient mice transplanted with WT-BMCs (Fig. 8b). However, the plasma level of MYDGF was obviously lower in the WT recipient mice receiving KO-BMCs than the WT recipient mice receiving WT-BMCs (Fig. 8b). After HFD/STZ

induction, transplantation of WT-BMCs into KO recipient mice markedly ameliorated albuminuria and glomerular injury concomitant with improvement in glucose metabolism (Fig. 8c–i, ESM Fig. 10). In contrast, transplantation of KO-BMCs into WT recipient mice worsened albuminuria and glomerular injury accompanied by impaired glucose metabolism (Fig. 8c–i, ESM Fig. 10). These results reveal that MYDGF produced by BMCs is sufficient and required for renoprotection in DKD mice.

Fig. 6 MYDGF activates Akt/BAD signalling in vivo and in vitro. **(a)** Representative western blot analyses for p-BAD (S136), BAD, p-Akt (S473), p-Akt (T308), Akt, p-PKA (T197), PKA, p-RSK (S381), RSK, p-S6K (T389) and S6K in podocytes. **(b–g)** Bar graphs show the average ratios of phosphorylated protein signal vs total protein signal. Data are shown as the mean $\pm$ SEM of three independent experiments. One-way ANOVA followed by Tukey's post hoc test for multiple comparisons was used for groups of three or more. ** $p < 0.01$ vs NG group; †† $p < 0.01$ vs HG group. **(h)** Representative western blots of p-BAD (S136), BAD, p-Akt (S473), p-Akt (T308) and Akt in glomerular lysates. **(i–k)** Quantitative analysis of **(h)** ($n = 3$ mice per group). Data are shown as the mean $\pm$ SEM. One-way ANOVA followed by Tukey's post hoc test for multiple comparisons was used for groups of three or more. ** $p < 0.01$ vs WT-GFP group; †† $p < 0.01$ vs KO-GFP group. NS, not significant

Discussion

The major findings of this study are that: (1) MYDGF ameliorates albuminuria and glomerular injury in DKD; (2) MYDGF preserves slit diaphragm protein expression and protects against podocyte apoptosis both in vivo and in vitro; and (3) the molecular mechanisms underlying these beneficial effects of MYDGF involve the activation of the Akt/BAD signalling pathway. To the best of our knowledge, our data show for the first time that MYDGF reverses damage to podocytes from diabetes and prevents the progression of DKD.

The main pathological features of DKD include glomerular hypertrophy, glomerular and tubular cell injury, mesangial matrix expansion and GBM thickening, contributing to

glomerulosclerosis, tubulointerstitial degeneration and glomerular capillary dysfunction, which results in increasing albuminuria and a gradual decrease in kidney function [39, 40]. In the present study, both loss- and gain-of-function strategies indicated that MYDGF significantly attenuated the development of DKD. Under the STZ/HFD induction, WT mice developed severe albuminuria and presented pathologic changes characteristic of DKD, such as glomerular hypertrophy, mesangial matrix expansion and GBM thickening. Moreover, these lesions were more severe in KO-DKD mice than WT-DKD mice. Conversely, overexpression of exogenous *Myd* gene remarkably ameliorated these lesions in both WT-DKD and KO-DKD mice. Overall, these data indicate an unequivocal role for MYDGF in preventing albuminuria and glomerular injury in DKD.

Fig. 7 *Akt*-specific siRNA vitates the positive effects of MYDGF on podocytes in vivo. **(a)** The experiment schedule of the HFD/STZ-induced WT-DKD mice receiving an injection of si*Akt* or siCON ($n = 10$ mice per group). **(b)** Western blots and **(c)** quantification analyses of p-BAD and BAD in glomerular lysates at the end of study ($n = 3$ mice per group). **(d)** Urinary albumin levels were measured at the termination of the study ($n = 6$ mice each group). **(e–g)** mRNA levels of fibronectin, *Tgf-β* and collagen IV in glomerular lysates were determined using RT-PCR ($n = 6$

mice each group). The mRNA expression of the si*Akt* mice is relative to that of the siCON mice. **(h)** Representative PAS staining and immunofluorescence images of nephrin and podocin in glomeruli. Scale bar, 20 μm; original magnification $\times 400$. **(i, j)** Quantitative analyses of mesangial matrix expansion and glomerular volume in glomeruli ($n = 6$ mice per group). Data are shown as the mean $\pm$ SEM. Student's *t* test was employed for comparisons between two groups. ** $p < 0.01$ vs siCON.; Vol. volume

Podocytes play a crucial role in the regulation of the glomerular filtration barrier. Nephrin and podocin are key components of the slit diaphragm that function as the major size- and charge-selective barrier to protein leakage [41]. Therefore, podocyte injury, including podocyte loss and the disruption of slit diaphragm proteins, plays an important role in the pathogenesis of DKD [42, 43]. In our in vivo experiment, podocin and nephrin expression decreased and desmin expression increased in the DKD mice, accompanied by increased podocyte apoptosis. *Mydgf* deletion significantly sharpened the decrease in slit diaphragm proteins and the increase in desmin expression and podocyte apoptosis. In contrast, overexpression of MYDGF markedly enhanced nephrin and podocin expression and decreased desmin expression and podocyte apoptosis in both WT-DKD and KO-DKD mice. Consistently, in both in vitro and ex vivo experiments, exposure of podocytes and glomeruli to HG led to the disruption of slit diaphragm protein and podocyte apoptosis, and rMYDGF treatment significantly ameliorated these effects.

Taken together, our findings establish that MYDGF treatment prevents podocyte injury by preserving slit diaphragm protein expression and decreasing podocyte apoptosis in DKD.

Next, we explored the signalling pathways that may explain the renoprotection of MYDGF in DKD. It is well known that the phosphoinositide 3-kinase (PI3K)/Akt signalling pathway plays an important role in protecting against podocyte apoptosis and improving proteinuria in DKD [44, 45]. Specifically, the activation of the PI3K/Akt signalling pathway enhances the phosphorylation of BAD, thereby upregulating downstream Bcl-2 and Bcl-xl and decreasing Bax and cleaved caspase-3, followed by an increase in the expression of the podocyte function protein nephrin [34, 46]. In the present study, we observed significant decreases in the phosphorylation levels of Akt and BAD in both DKD mice and HG-induced podocytes. Moreover, this inactivation of the Akt/BAD signalling pathway was partially abrogated after MYDGF treatment. Importantly, the protective effect of MYDGF is abolished by a blockade of Akt activation.

Fig. 8 Bone marrow-derived MYDGF prevents albuminuria and podocyte injury in DKD mice. **(a)** The experiment schedule of WT and KO mice receiving BMCs from WT or KO mice ($n = 8$ mice per group). **(b)** Six weeks after bone marrow transplantation, plasma MYDGF levels were measured ($n = 8$ mice per group). **(c)** Urinary albumin levels were measured at the termination of the study ($n = 8$ mice each group). **(d–f)** mRNA levels of fibronectin, *Tgf- β* and collagen IV in glomerular lysates were analysed by RT-PCR ($n = 6$ mice per group). The mRNA expression of the mice is relative to that of the WT $\rightarrow$ WT mice. **(g)** Representative PAS staining and immunofluorescence images of nephrin and podocin in glomeruli. Scale bar, 20 μ m; original magnification $\times 400$. **(h, i)** Quantitative analyses of mesangial matrix expansion and glomerular volume in glomeruli ($n = 3$ mice per group). Data are shown as the mean $\pm$ SEM. One-way ANOVA followed by Tukey's post hoc test for multiple comparisons was used for groups of three or more. $**p < 0.01$ vs WT $\rightarrow$ WT group; $^{\dagger\dagger}p < 0.01$ vs WT $\rightarrow$ KO group. Vol., volume

Cumulatively, we conclude that the activation of the Akt/BAD cascade is involved in the MYDGF-mediated beneficial effects.

Herein, we also find that MYDGF improves glucose and lipid metabolism in DKD, and these positive effects may be of partial benefit for the improvement of DKD. Of note, evidence in animals and humans has shown that GLP-1 can attenuate DKD [47, 48]. Our previous study has revealed that MYDGF stimulates GLP-1 production from intestinal L-cells in diabetic mice [18]. The increased GLP-1 may partially contribute to the improvement in DKD in the present study.

This study has some limitations. First, MYDGF is a secreted protein [49], but we have not identified the receptor(s)

mediating its effects. Second, we found that MYDGF treatment improves metabolism in DKD mice, and the mechanisms need to be further investigated in other tissues, such as adipose, pancreas and liver tissues. Third, many other renal cells, such as glomerular mesangial cells, glomerular endothelial cells and proximal tubule epithelial cells, are known to be involved in the progression of DKD. However, we did not further explore the effect of MYDGF on the other renal cells. Thus, some additional studies are needed.

In summary, the data suggest a critical role for MYDGF in preventing the development of DKD by protecting podocytes from injury and improving podocyte function through the activation of the Akt/BAD pathway. The identification of

MYDGF opens up a potential new therapeutic strategy for treating DKD and stimulates further research into the role of MYDGF in other disease states.

Data availability All data generated or analysed during this study are included in this published article (and its supplementary information files).

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Contribution statement MJH, YXL, LW, BG, LX, JD, JJZ and ML were responsible for data acquisition and interpretation. GDX, WM, BZ, YD, BYM and LMZ designed the study and drafted the article. LWX contributed to the conception and design of the study and analysis of data. All authors contributed to its revision and approved the manuscript for submission. GDX is the guarantor and, as such, is responsible for final approval of the version to be published.

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