Overexpression of Circulating Soluble Nogo-B Improves Diabetic Kidney Disease by Protecting the Vasculature

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Damage to the vasculature is the primary mechanism driving chronic diabetic microvascular complications such as diabetic nephropathy, which manifests as albuminuria. Therefore, treatments that protect the diabetic vasculature have significant therapeutic potential. Soluble neurite outgrowth inhibitor-B (sNogo-B) is a circulating N-terminus isoform of full-length Nogo-B, which plays a key role in vascular remodeling following injury. However, there is currently no information on the role of sNogo-B in the context of diabetic nephropathy. We demonstrate that overexpression of sNogo-B in the circulation ameliorates diabetic kidney disease by reducing albuminuria, hyperfiltration, and abnormal angiogenesis and protecting glomerular capillary structure. Systemic sNogo-B overexpression in diabetic mice also associates with dampening vascular endothelial growth factor-A signaling and reducing endothelial nitric oxide synthase, AKT, and GSK3β phosphorylation. Furthermore, sNogo-B prevented the impairment of tube formation, which occurred when human endothelial cells were exposed to sera from patients with diabetic kidney disease. Collectively, these studies provide the first evidence that sNogo-B protects the vasculature in diabetes and may represent a novel therapeutic target for diabetic vascular complications.

Diabetic nephropathy (DN), the leading cause of end-stage renal disease in the Western world, is characterized by structural changes in the kidney glomerular filtration barrier (1,2). This leads to enhanced glomerular permeability manifested as albuminuria, representing a common mechanism for renal and extrarenal diabetic vascular complications (3).

A complex network of vascular growth factors regulates the permeability and structure of the glomerular capillary filtration barrier (4). Glomerular levels of vascular endothelial growth factor-A (VEGF-A) and angiopoietin-2 (Angpt2) are upregulated in the early stages of DN, while Angpt1 is downregulated (5–7), a milieu associated with vascular remodeling, endothelial proliferation, and increased capillary permeability (1,4). Blockade of VEGF-A signaling (8) or restoration of Angpt1 levels in podocytes (7) ameliorates albuminuria and glomerular damage in rodent models of early DN. The effects of vascular growth factors on endothelial permeability in DN are partly mediated by nitric oxide (NO) signaling through modulation of endothelial NO phosphorylation ($eNOS^{Ser1177}$), which acts in an AKT-dependent manner (9). In diabetes, reduction in NO availability due to eNOS uncoupling (10) has been implicated in the pathophysiology of DN. Podocyte-specific overexpression of *Angpt1* activates eNOS (7) in diabetic mice, whereas the beneficial effect of VEGF-A blockade on albuminuria in DN is prevented in *eNOS* knockout mice (11).

Another pathway involved in vascular remodeling is the neurite outgrowth inhibitor (Nogo) family, which is

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encoded by one gene with three major isoforms: Nogo-A, -B, and -C (12), mainly expressed in the endoplasmic reticulum (13). Nogo-A and -C are found in the central nervous system and muscle tissue, respectively, while Nogo-B localizes to endothelial and smooth muscle cells within the vasculature (12). In physiology, loss of Nogo-B upregulates eNOS-NO and flow-mediated vasodilation, leading to hypotension (14). Mice lacking both Nogo-A and -B are viable and do not have major apparent vascular defects (15). However, vascular lesions are enhanced in Nogo A/B-deficient mice following injury, which can be prevented by gene delivery of full-length Nogo-B (15,16).

The full-length Nogo-B protein of 49 kDa can be cleaved into a shorter ~150-amino acid N-terminus fragment (17), which can then be secreted into the circulation as soluble Nogo-B (sNogo-B) (18). This Nogo-B N-terminus (identical in circulating sNogo-B and full-length Nogo-B within the cells) binds to its receptor NgBR, expressed in endothelial cells (ECs) on the cell plasma membrane and in the endoplasmic reticulum, leading to EC proliferation/vascular remodeling (15,19), angiogenesis during development, vascular repair, and cytoskeletal organization (12,20–23). Given the role of this N-terminal fragment of Nogo-B in vascular remodeling, we hypothesized that overexpression of sNogo-B in the circulation could have a protective role in the setting of diabetic kidney disease.

RESEARCH DESIGN AND METHODS

Materials and chemicals were purchased from Sigma-Aldrich (Gillingham, U.K.) and STARLAB (Milton Keynes, U.K.) unless otherwise stated.

Experimental Animal Model of Diabetes

To induce diabetes, 8- to 12-week-old (~20 g in weight) male DBA2J mice were administered with streptozotocin (low-dose multiple-injection protocol) (7,8). Mice were considered diabetic with a fed glycemia >22 mmol/L. Control nondiabetic littermates were injected with vehicle only (citrate buffer). Two weeks later, some diabetic and nondiabetic mice were administered an adeno-associated viral vector expressing 6xHis-Tag/sNogo-B (AAV–sNogo-B) (Supplementary Fig. 1*A*). The used vector, AAV/DJ, has a specific tropism for the liver and maintains a sustained expression of transgene for 15–17 weeks under the CMV promoter (24). The construct also contains a secretory alkaline phosphatase peptide, which drives the release of the 6xHis-Tag/sNogo-B protein in the circulation. To control for infection, other diabetic and nondiabetic mice were injected with AAV/DJ, driving the expression of GFP under the same promoter (AAV-GFP).

All mice were maintained for 12–14 weeks after induction of diabetes before sacrifice and tissues analysis. Prior to sacrifice, blood pressure was assessed with tail cuff methodology, and 24-h urine collection was conducted with mice kept in metabolic cages for creatinine, sNogo-B, and albuminuria determination (7). At sacrifice, full blood and plasma was collected (to assess for HbA_{1c}, creatinine by high-performance liquid chromatography, and sNogo-B levels). Kidney tissue was harvested for histology, electron microscopy (EM), and lysates of kidney cortex or isolated glomeruli frozen for further analysis (7).

Immunofluorescence

Proliferating Glomerular EC Determination

Glomerular Ki67/CD31 and CD31-positive glomerular ECs (GECs) per glomerulus were visualized with a fluorescent microscope (7); an average of 30–40 glomeruli per animal were studied and the means values used in the analysis.

Endothelial Glycocalyx Determination

Lectin staining was assessed as indicator of thickness/integrity of the endothelial glycocalyx in frozen kidney sections as described (25). A total of 10–15 capillary loops were analyzed per mouse from five to seven glomeruli, and the mean value for each animal was used in the analysis.

Electron Microscopy and Glomerular Ultrastructure Analysis

Mesangial volume fraction, glomerular basement membrane (GBM) thickening, podocyte number, and glomerular volume were studied as described (7,8). For detailed methodology, see the Supplementary Material.

sNogo, VEGF-A, Angpt1/2, and VEGFR2^{Tyr1173} Phosphorylation ELISA

sNogo-B in plasma, urine, and cell culture media was assessed by ELISA (BioLegend, San Diego, CA). ELISA was also used for kidney cortex VEGF-A (R&D Systems, Abingdon, U.K.), Angpt1/2 (Biomatik, Wilmington, DE), and VEGFR2 phosphorylation (PathScan; Cell Signaling Technology, Leiden, the Netherlands) levels; results were normalized for micrograms of total kidney cortex protein lysates.

Immunoblotting

Immunoblotting was performed on cells and tissue lysates (mouse renal cortex and isolated glomeruli) as described (7). The following primary antibodies were used: Nogo-B (N-terminus; R&D Systems); NgBR (Abcam, Cambridge, U.K.); α-tubulin (Santa Cruz Biotechnology, Heidelberg, Germany); β-actin; pan-AKT, GSK3β, phospho-AKT (Ser^{473}), phospho-eNOS (Ser^{1177}), and phospho-GSK3β^{Ser9} (Cell Signaling Technology); eNOS (Santa Cruz Biotechnology); and total β-catenin (Proteintech, Manchester, U.K.).

Culture of Mouse Lung ECs, Human GECs, and Human Podocytes

Primary lung ECs were isolated from adult C57BL/6J mice and cultured, up to three passages, as described (14).

Human GECs and podocytes were cultured as described (26,27). To examine the effect of high glucose (HG) and VEGF-A in fully differentiated GECs, cells were starved for 12 h in 1% serum followed by incubation in normal glucose (NG; 5 mmol/L glucose plus 20 mmol/L mannitol) or HG (25 mmol/L glucose) for 72 h in the presence or absence of VEGF-A (50 ng/mL).

Immunoprecipitation and Proximity Ligation Assay Experiments

For immunoprecipitation (IP) experiments, GECs were transfected with 6xHis-Tag/sNogo-B adenovirus (ADV–sNogo-B, a transgene identical to the AAV–sNogo-B construct) at a multiplicity of infection of 100 for 4 h and studied after 24 h; whole protein lysates were obtained and incubated with anti-NgBR (Novus Biologicals, Oxford, U.K.), anti–6xHis-Tag antisera (Thermo Fisher Scientific, Oxford, U.K.), or vehicle IgG. Immunoblotting was conducted on immunoprecipitates obtained with anti–6xHis-Tag and -NgBR antisera.

For the proximity ligation assay (PLA) experiments, cell culture media enriched with 6xHis-Tag/sNogo-B was obtained by transfecting confluent human GECs with ADV–sNogo-B (multiplicity of infection of 100) in full media for 4 h; subsequently, cells were put in 1% serum media and supernatant (containing 6xHis-Tag/sNogo-B protein, ~6,000 pg/mL) collected after 48 h; collected media was stored at 4°C and used within 6–12 h. Human differentiated GECs were then incubated with 6xHis-Tag/sNogo-B–conditioned media for 1, 5, and 15 min, fixed in 4% paraformaldehyde for 10 min, and then incubated with primary antisera against NgBR (1:100 dilution, rabbit anti–N-terminus NgBR, gift from R.Q.M.) and anti–6xHis-Tag (1:100 dilution) mouse monoclonal antiserum (Thermo Fisher Scientific). The NgBR–sNogo-B interaction was visualized with immunofluorescence following the manufacturer's instructions (Duolink In Situ Red Starter Kit Mouse/Rabbit).

Nogo-B Immunogold Staining in Glomeruli

Nogo-B immunogold staining was conducted in mouse renal cortex tissue as previously described using a sheep polyclonal anti–Nogo-B (N-terminus) antibody (R&D Systems) (8).

Nogo-B Immunohistochemistry

Nogo-B immunohistochemistry was conducted in mice and humans (patients with DN or thin basement membrane nephropathy [TBMN]) (Supplementary Table 1) kidney tissue paraffin sections using the streptavidin-biotin complex method with specific sheep polyclonal anti–Nogo-B N-terminus antibody (R&D Systems).

In human tissue, Nogo-B expression was quantified by assessing the percentage glomerular area with positive staining. Ten glomeruli were analyzed for each human biopsy, and the calculated average Nogo-B staining/area of glomeruli was then used for analysis.

Role of sNogo-B Overexpression in Human Umbilical Vein Endothelial Cell Tube Formation Assay

To examine the continuous paracrine/autocrine effect of sNogo-B on angiogenesis, human umbilical vein ECs (HUVECs) were transfected with ADV–sNogo-B or identical vector lacking sNogo-B cDNA (control vector) (Supplementary Fig. 1*B*) for 4 h; cells were then equilibrated (24 h) in complete medium and then plated in 96-well plates (5,000 cells/well) in EGM basal media containing sera (4% v/v) obtained from patients with type 1 diabetes mellitus (T1DM) susceptible to (DN+) or protected toward (DN−) the progression of DN (Supplementary Table 2). Tube formation was performed on Matrigel (BD Biosciences, Berkshire, U.K.) (28) in duplicate and assessed after 24 h. Tube length and number were analyzed in a blinded fashion with the Wimasis WimTube image system (29).

Statistical Methods

Differences among groups were analyzed by two-tailed Student *t* test or ANOVA with post hoc least significant difference (LSD) pairwise comparisons test for normally distributed variables. Kruskal-Wallis and Mann-Whitney nonparametric tests were used for not normally distributed variables (albuminuria and sNogo-B). Data are expressed as means ± SD for normally distributed data or as median and interquartile range for not normally distributed data. Analysis was conducted with IBM SPSS-22 software (IBM, New York, NY), and statistical significance was accepted at $P \leq 0.05$.

RESULTS

sNogo-B Overexpression Ameliorates Diabetes-Mediated Albuminuria and Hyperfiltration

Nondiabetic (ND) and diabetic (D) mice injected with AAV–sNogo-B were characterized by a 12-fold increase in circulating sNogo-B when compared with those injected with control vector (AAV-GFP) (ND-GFP vs. ND–sNogo-B, $P = 0.00001$; D-GFP vs. D–sNogo-B, $P = 0.0001$) (Fig. 1*A*).

Diabetes led to a significant increase in HbA_{1c} levels (ND-GFP vs. D-GFP, $P = 0.00008$; ND–sNogo-B vs. D–sNogo-B, $P = 0.00004$), which was paralleled by a loss of body weight (ND-GFP vs. D-GFP, $P = 0.0001$; ND–sNogo-B vs. D–sNogo-B, $P = 0.00001$), increased kidney/body weight ratio (ND-GFP vs. D-GFP, $P = 0.006$; ND–sNogo-B vs. D–sNogo-B, $P = 0.0002$), and a modest decrease in systolic blood pressure (ND-GFP vs. D-GFP, $P = 0.01$; ND–sNogo-B vs. D–sNogo-B, $P = 0.003$) (Table 1).

Glomerular permeability, measured by albumin excretion rate over 24-h urine collection, was increased in D mice compared with ND mice (ND-GFP vs. D-GFP, $P = 0.0001$) (Fig. 1*B*). Overexpression of sNogo-B in the circulation of D mice led to a significant 40–50% reduction in albuminuria (D-GFP vs. D–sNogo-B, $P = 0.04$) (Fig. 1*B*). This effect was independent of changes in systemic blood pressure, kidney/body weight, and glycemic control, which were similar in D mice administered either AAV–sNogo-B or AAV-GFP (Table 1). Diabetes-mediated renal hyperfiltration was evidenced by raised creatinine clearance levels (Fig. 1*C*) (ND-GFP vs. D-GFP, $P = 0.02$), and AAV–sNogo-B overexpression attenuated this effect (D-GFP vs. D–sNogo-B, $P = 0.04$) (Fig. 1*C*).

Urinary sNogo-B was measured in a subset of animals. Diabetes was paralleled by an increase in 24-h urine sNogo-B in both mice administered AAV-GFP or AAV–sNogo-B (Fig. 1*D*) (ND-GFP vs. D-GFP and ND–sNogo-B vs. D–sNogo-B, $P \leq 0.002$); no differences were observed in urine sNogo-B between AAV-GFP or AAV–sNogo-B mice within the ND and D group.

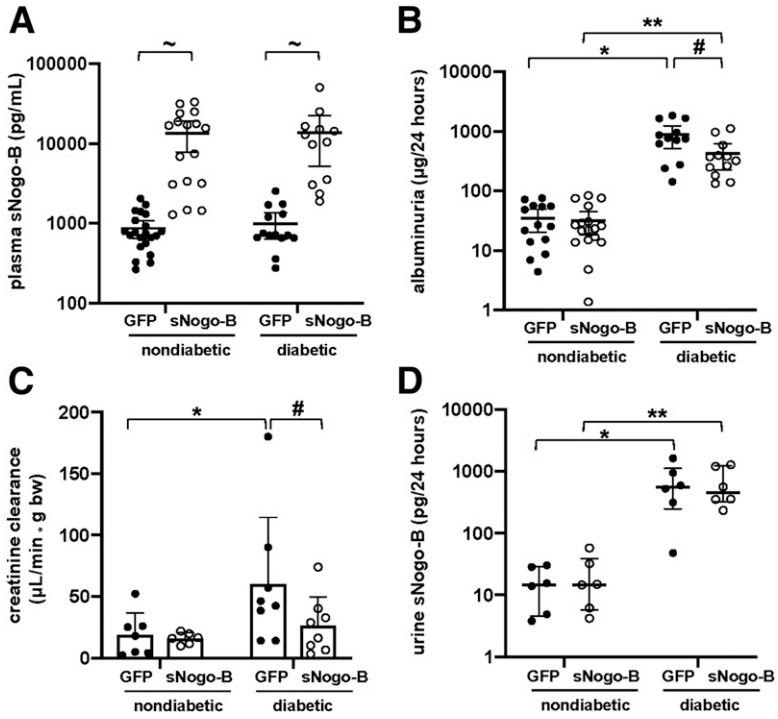

Figure 1—sNogo-B overexpression in the circulation ameliorates diabetic glomerulopathy. AAV–sNogo-B administration significantly increases plasma sNogo-B levels (*A*, n = 12–21/group) (measured at sacrifice, 12–14 weeks post–diabetes induction) ($\sim P \leq 0.0001$, ND-GFP vs. ND–sNogo-B and D-GFP vs. D–sNogo-B) and ameliorates diabetes-mediated albuminuria (*B*; n = 12–17/group), and hyperfiltration, measured as change in creatinine clearance (*C*; n = 6–8/group). Diabetes was paralleled by an increase in sNogo-B in the urine (*D*; n = 6/group); no differences were observed between mice with GFP or sNogo-B overexpression within the ND or D group (*$P \leq 0.02$, ND-GFP vs. D-GFP; **$P \leq 0.002$, ND–sNogo-B vs. D–sNogo-B; #$P \leq 0.04$, D-GFP vs. D–sNogo-B). *A*, *B*, and *D*: Kruskal-Wallis and Mann-Whitney test (median interquartile range). *C*: ANOVA with LSD post hoc test (mean ± SD). ●, AAV-GFP–treated mice; ○, AAV–sNogo-B–treated mice. bw, body weight.

Effects of sNogo-B Overexpression on GECs and Podocytes in Diabetes

The results above implicate sNogo-B as having a protective role in glomerular capillary permeability in diabetic kidneys. We therefore explored whether elevated circulating levels of sNogo-B might stabilize the glomerular vasculature and confer a healthier GEC and podocyte phenotype in diabetes.

Firstly, we examined GEC proliferation to assess the effect of sNogo-B on abnormal vascular remodeling that occurs in early diabetic glomerulopathy (1,7) (Fig. 2*A* and *B*). Diabetes increased the number of $CD31^+/Ki67^+$ cells per glomerulus by sevenfold, which was attenuated in mice with sNogo-B overexpression in the circulation (ND-GFP vs. D-GFP, P = 0.0001; D-GFP vs. D–sNogo-B, P = 0.005) (Fig. 2*B*). The total number of $CD31^+$ cells/glomeruli was not different between animals with increased sNogo-B in the circulation in either ND or D mice (mean ± SD $CD31^+$ cells/glomeruli: ND-GFP, 7.6 ± 1.37; ND–sNogo-B, 7.7 ± 1.8; D-GFP, 9.7 ± 2.4; D–sNogo-B, 9.5 ± 3.0; n = 7 animals/group, P = NS).

Table 1—Clinical and biochemical characteristics of DBA2J mice

	ND-GFP	ND–sNogo-B	D-GFP	D–sNogo-B
HbA_{1c}, % (mmol/mol) (n = 16–17/group)	4.5 (26) ± 1.7 (18)	4.3 (24) ± 1.5 (15)	7.7 (60) ± 2.2 (24)*	7.5 (59) ± 2.8 (30)**
BW (g) (n = 18–20/group)	28.6 ± 2.9	27.5 ± 3.56	23.6 ± 3.9*	21.6 ± 4.4**
SBP (mmHg) (n = 7–10/group)	112 ± 11	111 ± 11	98 ± 9*	93 ± 12**
Kidney weight/BW (mg/g) (n = 12–18/group)	8.7 ± 0.6	8.5 ± 0.6	9.9 ± 1.4*	10 ± 1.2**
Glomerular volume (μm^3) (n = 7–12/group, average 30 glomerular determinations per animal)	215,480 ± 45,972	231,523 ± 38,702	193,193 ± 22,518	239,640 ± 51,064

Data are expressed as mean ± SD. Clinical and biochemical characteristics in ND and D DBA2/J mice overexpressing sNogo-B in the circulation or control vector (GFP). BW, body weight; SBP, systolic blood pressure. ANOVA with post hoc LSD: *$P \leq 0.01$, ND-GFP vs. D-GFP; **$P \leq 0.003$, ND–sNogo-B vs. D–sNogo-B.

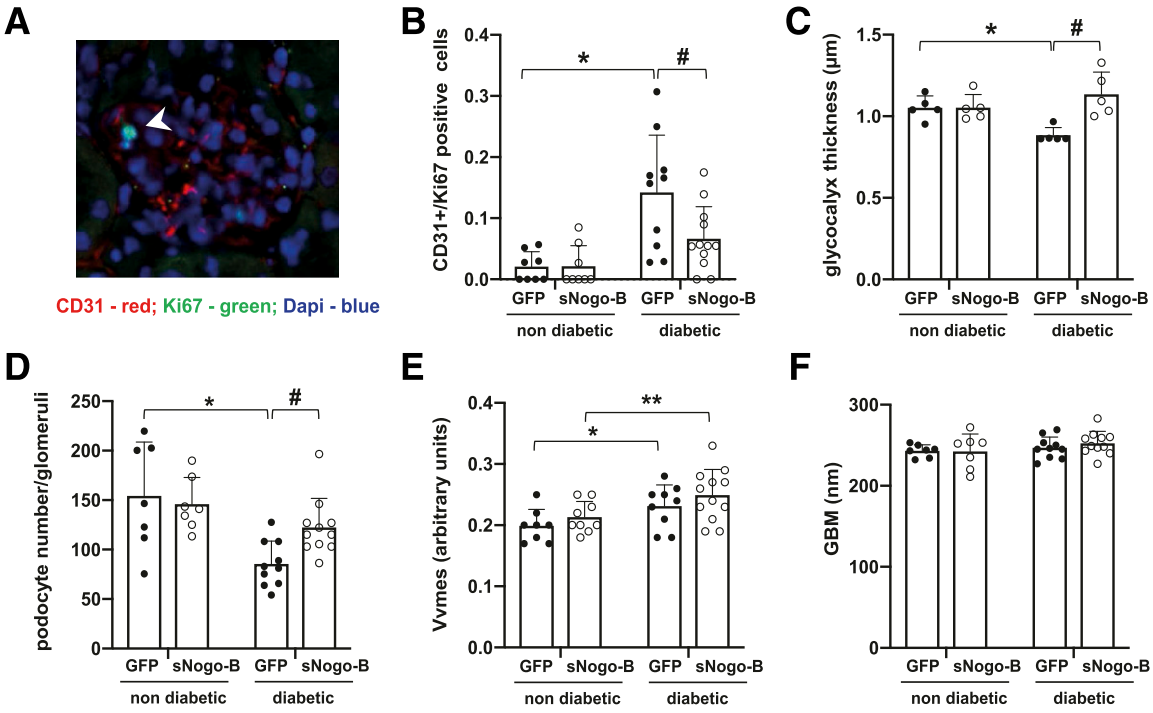

Figure 2—sNogo-B overexpression in the circulation ameliorates diabetes-mediated GEC proliferation, reduction in glycocalyx lectin content, and podocyte loss. *A*: GEC proliferation was detected by $CD31^+/Ki67^+$ cells (white arrowhead). Diabetes significantly increases the average number of glomerular $CD31^+/Ki67^+$ cells by sevenfold, which was reduced by sNogo-B overexpression in the circulation (*B*; *n* = 8–12/group, average of 30–40 glomeruli/animal). Diabetes led to a 20% reduction in the thickness of the glycocalyx (assessed as lectin content), which was prevented by sNogo-B overexpression in the circulation (*C*; *n* = 5/group, with 10–15 capillary loops studied per mouse). Similarly, diabetes-mediated glomerular podocytes loss was ameliorated by sNogo-B overexpression in the circulation (*D*; *n* = 7–11/group). Elevated sNogo-B circulating levels had no effect on the diabetes-mediated increase in mesangial volume fraction (Vvmes) (*E*; *n* = 9–12/group) or GBM thickness (*F*; *n* = 7–11/group) (*$P \leq 0.02$, ND-GFP vs. D-GFP; **$P = 0.02$, ND–sNogo-B vs. D–sNogo-B; #$P \leq 0.01$, D-GFP vs. D–sNogo-B). ANOVA with LSD post hoc test (mean ± SD) for all comparisons. ●, AAV-GFP–treated mice; ○, AAV–sNogo-B–treated mice.

We performed lectin staining as an indicator of thickness/integrity of the glomerular endothelial glycocalyx, which is known to contribute to vascular permeability (30). The estimated lectin thickness was significantly reduced in D mice but restored to baseline levels in mice administered AAV–sNogo-B (ND-GFP vs. D-GFP, $P = 0.009$; D-GFP vs. D–sNogo-B, $P = 0.0004$) (Fig. 2*C*).

Using EM (Supplementary Fig. 2), we found that diabetes was also paralleled by a significant decrease in the number of podocytes per glomerulus (Fig. 2*D*) (ND-GFP vs. D-GFP, $P = 0.0002$), increased the mesangial volume fraction (Fig. 2*E*) (ND-GFP vs. D-GFP, $P = 0.02$), but had no effect on GBM width (Fig. 2*F*) or glomerular volume (Table 1). The reduction in podocyte number observed in D mice was ameliorated by elevated circulating sNogo-B levels (D-GFP vs. D–sNogo-B, $P = 0.01$) (Fig. 2*D*). Overexpression of sNogo-B in the circulation did not alter diabetes-mesangial expansion (Fig. 2*E*), GBM width (Fig. 2*F*), or glomerular volume in diabetic mice (Table 1).

Effects of sNogo-B Overexpression in the Circulation on Vascular Growth Factors, eNOS, AKT, and GSK3β Signaling in the Kidney Cortex

To identify the molecular mechanisms by which sNogo-B might alter the endothelium in diabetes, we examined levels of the vascular growth factors VEGF-A, Angpt1, and Angpt2 in kidney cortex lysates. VEGF-A protein levels were significantly increased in D mice (Fig. 3*A*) (ND-GFP vs. D-GFP, $P = 0.009$) and were reduced when sNogo-B was overexpressed in the circulation (D-GFP vs. D–sNogo-B, $P = 0.03$) (Fig. 3*A*).

In accord with these findings, phosphorylation levels of the main VEGF-A receptor, VEGFR2, in the kidney cortex were significantly elevated in D mice, an effect that was dampened in mice with elevated circulating sNogo-B levels (ND-GFP vs. D-GFP, $P = 0.003$; D-GFP vs. D–sNogo-B, $P = 0.01$) (Fig. 3*B*). Angpt1/Angpt2 ratio was lower in D mice and was not altered by sNogo-B overexpression in the circulation (ND-GFP vs. D-GFP, $P = 0.03$; ND–sNogo-B vs. D–sNogo-B, $P = 0.001$) (Fig. 3*C*).

Given that upregulation of sNogo-B in the circulation was paralleled by altered VEGF-A signaling, for which the effects on endothelial permeability in DN are regulated by eNOS activation (9), we examined phosphorylation of eNOS. Diabetes led to a significant increase in the ratio of phosphorylated $eNOSs^{Ser1177}$/total eNOS (Fig. 3*D* and *E*) (ND-GFP vs. D-GFP, $P = 0.017$), which was prevented by sNogo-B overexpression (D-GFP vs. D–sNogo-B, $P = 0.05$) (Fig. 3*D* and *E*). As protein kinase B (AKT) activation is involved in EC proliferation and eNOS phosphorylation (9), we investigated the phosphorylation at the AKT^{Ser473} site. The ratio of AKT^{Ser473}

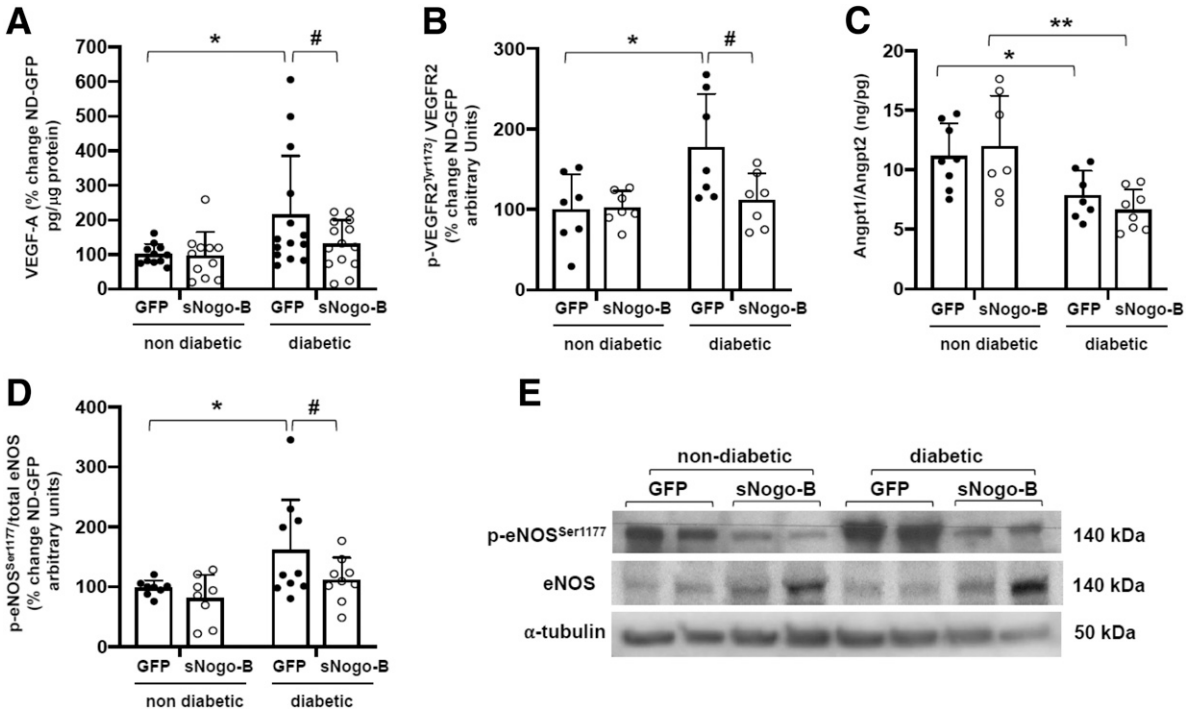

Figure 3—sNogo-B overexpression in the circulation ameliorates diabetes-mediated VEGF-A/VEGFR2 signaling and eNOS^{Ser1177} phosphorylation in kidney cortex lysate. Diabetes-induced kidney cortex VEGF-A expression (*A*; n = 11–14/group, in duplicate) and phosphorylated (p-)VEGFR2^{Tyr1173} (*B*; n = 7/group, in duplicate) was blunted in diabetic mice with sNogo-B overexpression in the circulation (*$P \leq$ 0.003, ND-GFP vs. D-GFP; #$P \leq$ 0.03, D-GFP vs. D–sNogo-B). Angpt1/Angpt2 ratio (*C*; n = 7 to 8/group, in duplicate) was reduced in diabetes (*,**$P \leq$ 0.03, ND-GFP vs. D-GFP and ND–sNogo-B vs. D–sNogo-B) but not altered by elevated sNogo-B circulating levels. The diabetes-mediated increase in ratio of p-eNOS^{Ser1177}/total eNOS was prevented by sNogo-B overexpression in the circulation (*D*, n = 8–10/group; *P = 0.017, ND-GFP vs. D-GFP; #P = 0.05, D-GFP vs. D–sNogo-B). *E*: Representative data showing Western blotting for p-eNOS^{Ser1177}, total eNOS, and α-tubulin as housekeeping gene. ANOVA with LSD post hoc test (mean ± SD) for all comparisons. ●, AAV-GFP–treated mice; ○, AAV–sNogo-B–treated mice.

phosphorylation/total AKT was upregulated in D mice, an effect that was significantly attenuated in mice injected with AAV–sNogo-B (ND-GFP vs. D-GFP, P = 0.0001; D-GFP vs. D–sNogo-B, P = 0.04) (Fig. 4*A*). The level of total AKT protein was downregulated by diabetes (ND-GFP vs. D-GFP, P = 0.002) (31), but elevated circulating levels of sNogo-B had no effect on total AKT protein expression in kidney cortex lysate.

We then investigated activation of GSK3β, a known substrate of AKT (32) that has been postulated as a therapeutic target for diabetic glomerulopathy (33). The ratio of phosphorylated GSK3β^{Ser9}/total GSK3β was upregulated in kidney cortex lysates of D mice when compared with ND mice (Fig. 4*B*) (ND-GFP vs. D-GFP, P = 0.006), consistent with previous reports (33). Notably, sNogo-B overexpression in the circulation was paralleled by a significant downregulation of GSK3β^{Ser9} phosphorylation/total GSK3β in D mice (D-GFP vs. D–sNogo-B, P = 0.04) (Fig. 4*B*). Total GSK3β protein expression was not altered in any of the experimental groups studied. As a known substrate of GSK3β, an important player in DN (34), we studied total β-catenin (35) protein levels in kidney cortex lysate. In line with GSK3β activation status, β-catenin was significantly upregulated in D mice when compared with ND mice (ND-GFP vs. D-GFP, P = 0.007) (Fig. 4*C*). sNogo-B overexpression in the circulation led to a significant downregulation of β-catenin in kidney cortex of D mice (Fig. 4*C*) (D-GFP vs. D–sNogo-B, P = 0.01), whereas β-catenin protein levels were unaffected in ND animals.

sNogo-B Binds to NgBR in Human GECs In Vitro

Next, we examined the physical interaction between sNogo-B and NgBR by IP and PLA experiments in human GECs (26). Firstly, we showed that GECs expressed NgBR in vitro. We then could detect an interaction between 6xHis-Tag/sNogo-B and NgBR with IP experiments (Supplementary Fig. 3*A*).

The sNogo-B/NgBR interaction was further confirmed with PLA experiments in which the sNogo-B/NgBR interaction was observed by identification of positive red/orange dots signals in GECs incubated with "conditioned media" containing sNogo-B protein as early as 1 min (Supplementary Fig. 3*B*).

Renal Cortical Levels of Full-Length Nogo-B Are Downregulated in Diabetic Mice and Restored by sNogo-B Overexpression in the Circulation

To further explore the renoprotective mechanisms of sNogo-B overexpression in the circulation, we investigated the expression of full-length Nogo-B in mouse and human kidney tissue in diabetes. With immunogold staining, combined

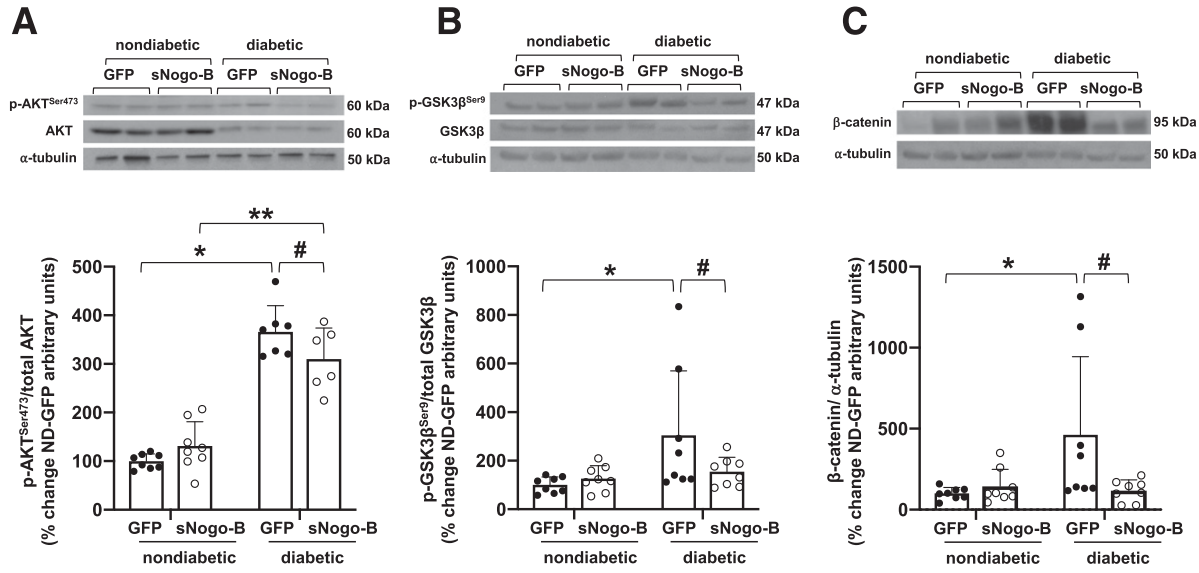

Figure 4—sNogo-B overexpression in the circulation modulates diabetes-mediated AKT^{Ser473} and GSK3β^{Ser9} phosphorylation, while preventing diabetes-mediated β-catenin upregulation. Diabetes was paralleled by a two- to threefold increase in the ratio of phosphorylated (p-)AKT^{Ser473}/total AKT (*A*; n = 6–8/group), a significant elevation of the ratio of p-GSK3β^{Ser9}/total GSK3β (*B*; n = 8/group) and increased total β-catenin levels (*C*; n = 8/group) in kidney cortex lysates. Diabetes-mediated AKT and GSK3β phosphorylation and upregulation β-catenin levels were partially or totally prevented by sNogo-B overexpression in the circulation (#$P \leq 0.04$, D-GFP vs. D–sNogo-B; *$P \leq 0.007$, ND-GFP vs. D-GFP; **$P = 0.0001$, ND–sNogo-B vs. D–sNogo-B). ANOVA with LSD post hoc test (mean ± SD) for all comparisons. ●, AAV-GFP–treated mice; ○, AAV–sNogo-B–treated mice.

with EM, we found that full-length Nogo-B protein was localized in both GECs and podocytes in ND male adult mice (Fig. 5*A*). Immunohistochemical staining confirmed the expression of full-length Nogo-B in the glomerular tuft and, as described, in cortical collecting duct (36) (Fig. 5*B*). Full-length Nogo-B protein, assessed by immunoblotting, in glomeruli enriched kidney cortex and isolated glomeruli lysates was two- to threefold downregulated in D mice when compared with ND controls (Fig. 5*C*) (ND-GFP vs. D-GFP, $P \leq 0.045$); sNogo-B overexpression in the circulation restored the diabetes-induced loss of full-length Nogo-B (D-GFP vs. D–sNogo-B, $P \leq 0.015$) (Fig. 5*C*). NgBR was expressed in isolated glomeruli but not modulated by diabetes or elevated sNogo-B circulating levels (Fig. 5*D*).

We also investigated the expression of full-length Nogo-B in human kidney biopsies obtained from two distinct glomerular diseases: DN and TBMN. DN represents a progressive proteinuric disease, while TBMN represents a benign nonproteinuric, nonprogressive disorder, featuring a uniformly thinned GBM (37) without any other significant glomerular pathology (Supplementary Table 1). Nogo-B expression was lower in glomeruli of kidney biopsies of patients with DN when compared with patients with TBMN (TBMN vs. DN, $P = 0.0001$) (Fig. 5*E*).

High Glucose and VEGF-A Promote Full-Length Nogo-B Downregulation and Increased sNogo-B Secretion in the Supernatant of GECs in Culture

Fully differentiated human GECs (26) incubated in NG or HG condition, with VEGF-A (50 ng/mL or vehicle) or a combination of HG and VEGF-A for 72 h, showed a 30–40% downregulation of full-length Nogo-B expression (NG vs. HG, NG vs. VEGF-A, and NG vs. HG plus VEGF-A, $P \leq 0.04$) (Fig. 6*A*). HG and/or VEGF-A–mediated full-length Nogo-B downregulation was paralleled by an upregulation of sNogo-B secretion in the supernatant (NG vs. HG, NG vs. VEGF-A, and NG vs. HG + VEGF-A, $P \leq 0.03$) (Fig. 6*B*).

sNogo-B Overexpression Corrects Altered Tube Formation Seen in HUVECs Cultured With T1DM/DN+ Serum

Finally, we explored the paracrine/autocrine effects of secreted sNogo-B overexpression on the ability of HUVECs, known to express NgBR (19), to differentiate in a tubelike structure (tube formation assay) (38) when incubated with the serum of patients with T1DM with (DN+) or without (DN−) DN (Supplementary Table 2).

sNogo-B overexpression led to a fivefold increase in sNogo-B released from cells into the supernatant when compared with ADV-control–transfected cells (ADV-control vs. ADV–sNogo-B within DN− and DN+, $P = 0.0001$) (Fig. 7*D*).

HUVECs incubated with serum of patients with T1DM/DN+ had decreased numbers and lengths of tubes when compared with cells incubated with serum of patients with T1DM/DN− (ADV-control, DN− vs. DN+, tube length, $P = 0.01$; tube number, $P = 0.04$) (Fig. 7*A–C*). Increased sNogo-B in the supernatant was paralleled by a correction of the impaired tube formation observed in cells incubated with DN+ serum (DN+, ADV-control vs. ADV–sNogo-B, tube length, $P = 0.02$; tube number, $P = 0.001$) (Fig. 7*A–C*).

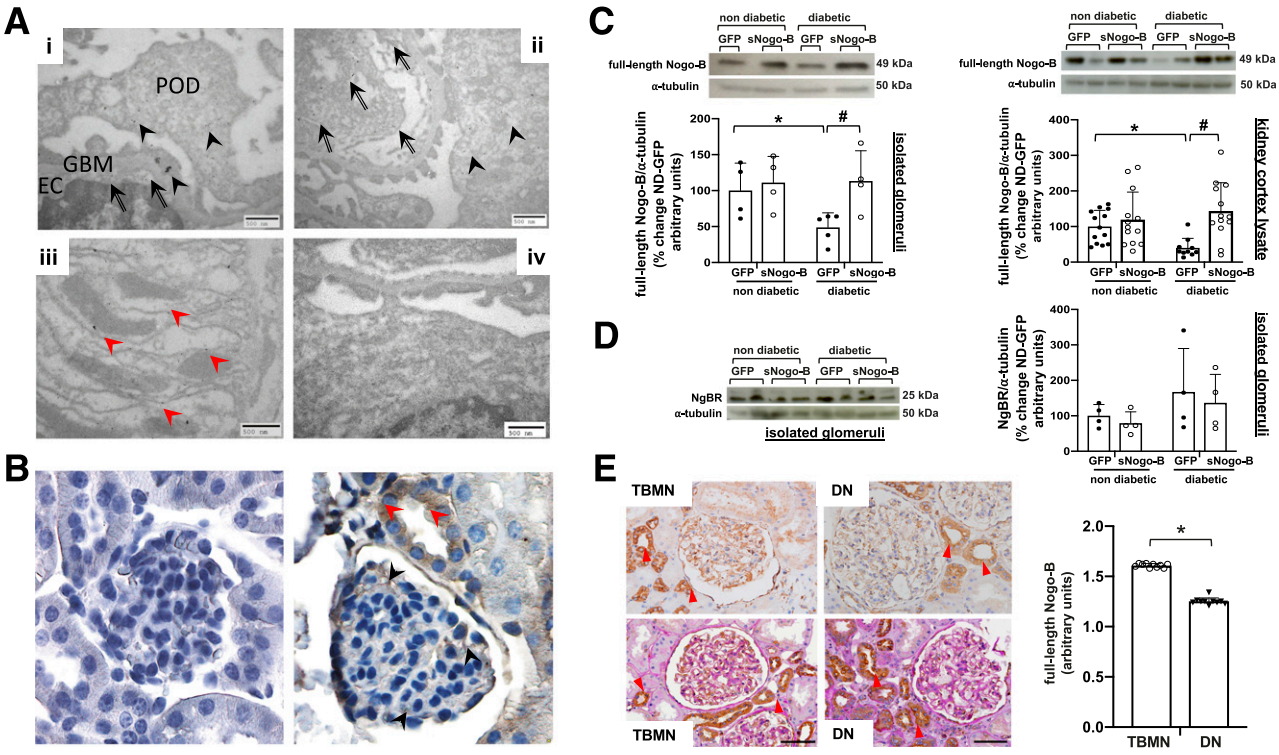

Figure 5— Full-length Nogo-B is expressed in glomeruli, and sNogo-B overexpression in the circulation prevents diabetes-mediated Nogo-B downregulation. *A*: EM showing the ultrastructure of the kidney glomerulus containing podocytes (POD), ECs, and the GBM. Immunogold labeling showed positive full-length Nogo-B expression in podocytes (black arrowheads) (*Ai*) and ECs (black arrows) (*Aii*). Collecting ducts (medullary section) (*Aiii*) are used as positive control, and negative control (omission of first antibody) is shown in *Aiv*. Scale bars, 500 nm. *B* (right): Positive signal for Nogo-B is observed by immunohistochemistry both in glomerular cells (black arrowheads) and in the cortical collecting duct (positive internal control, red arrowheads) (magnification ×40). *B* (left): Negative control-omission first anti–Nogo-B antiserum. *C*: Full-length Nogo-B protein expression was downregulated in kidney cortex cell lysate and isolated glomeruli of diabetic mice and was prevented by AAV–sNogo-B overexpression in the circulation (*C*; n = 10–13/group fort cortex lysate, n = 4 to 5 for isolated glomeruli; *$P \leq 0.045$, ND-GFP vs. D-GFP; #$P \leq 0.015$, D-GFP vs. D–sNogo-B). *D*: NgBR was detected in isolated glomeruli; no effect of diabetes or sNogo-B overexpression was noted. *E*: Nogo-B expression (brown staining) was detected by immunohistochemistry in kidney biopsies from patients with DN and TBMN. Positive staining in the cortical collecting ducts serves as positive internal control (red arrowheads). Scale bars, 100 μm. Quantitative data showing the area of the glomerular tuft containing positive Nogo-B staining (n = 10/group for both TBMN and DN with an average score from 10 glomeruli obtained for each biopsy; *$P = 0.0001$, TBMN vs. DN). *C* and *D*: ANOVA with LSD post hoc test (mean ± SD). *E*: Unpaired t test (mean ± SD). ●, AAV-GFP–treated mice; ○, AAV–sNogo-B–treated mice.

DISCUSSION

In this study, we demonstrated, in DBA2/J diabetic mice, that systemic overexpression of the N-terminal fragment sNogo-B, which binds to NgBR, improved diabetic glomerulopathy as evidenced by a reduction in albuminuria. This finding was associated with reduced GECs proliferation, restoration of glycocalyx thickness, maintenance of podocyte number, and dampening of VEGF-A signaling and eNOS, AKT, and GSK3β phosphorylation as assessed in glomeruli-enriched kidney cortex lysates. Finally, sNogo-B prevented the impairment of tube formation, which occurred when ECs were exposed to sera from patients with T1DM with DN.

The reduction in diabetes-mediated albuminuria seen in mice with sNogo-B overexpression in the circulation was possibly partly driven by a parallel fall in diabetes-mediated glomerular hyperfiltration; this was not accompanied by an increase in glomerular volume, which is in accord with other reports examining the early (up to 16 weeks of diabetes) phase of DN in DBA2J mice (39). Other potential confounders for the reduced albuminuria could be that sNogo-B overexpression in the circulation lowers blood pressure or be related to changes in glycemic control (2). However, we did not find any change in systemic blood pressure or glycemic control between diabetic mice with overexpression of sNogo-B in the circulation or control mice ruling out these possibilities. As previously described, the blood pressure observed in diabetic DBA2J mice was slightly lower than in nondiabetic ones (39).

Urinary sNogo-B was elevated in diabetes, likely due to the increased permeability of the filtration barrier. However, the excretion of sNogo-B was not increased in either nondiabetic or diabetic mice administered AAV-sNogoB, a finding most likely to be attributed to the large molecular weight of the 6xHis-Tag/sNogo-B (~80 kDa) (Supplementary Fig. 1*C*).

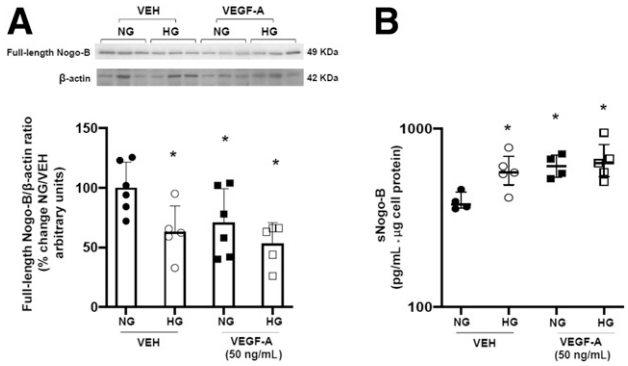

Figure 6—Incubation of GECs with HG and/or VEGF-A in vitro results in downregulation of full-length Nogo-B and parallel increase in sNogo-B secretion in the supernatant. Fully differentiated GECs were incubated with NG or HG for 72 h in the absence (vehicle [VEH]) or presence of VEGF-A (50 ng/mL). Full-length Nogo-B protein expression was significantly downregulated by HG and/or VEGF-A (*A*; n = 5 to 6, in duplicate). Conversely, HG and/or VEGF-A was paralleled with an increase in sNogo-B levels, expressed as pg/mL · μg of cell protein, in the supernatant (*B*; n = 4 to 5 in duplicate) (NG vs. HG, NG vs. VEGF-A, and NG vs. HG + VEGF-A, $^*P \leq 0.03$). *A*: ANOVA with LSD post hoc test (mean ± SD). *B*: Kruskal-Wallis and Mann-Whitney test (median and interquartile range). ●, NG + VEH; ○, HG + VEH; ■, NG + VEGF-A; □, HG + VEGF-A.

As albuminuria results from defects in the glomerular filtration barrier, we initially focused on the glomerular endothelium because the biological effects of sNogo-B and its receptor NgBR are primarily on the vasculature (14,19). Elevated circulating levels of sNogo-B prevented diabetes-mediated GEC proliferation as seen in the early stages of DN (1,7,40). sNogo-B overexpression in the circulation was also associated with amelioration of the diabetes-induced loss of lectin, an important component of the endothelial glycocalyx, known to contribute to vascular permeability (25,41). These changes were paralleled by a reduction in VEGF-A signaling. VEGF-A is a potent stimulator of EC proliferation (42), and dampening VEGF-A signaling ameliorates albuminuria and glomerular damage in DN (4). VEGF-A has also been implicated in the regulation of the thickness of the endothelial glycocalyx in diabetic mice with the antiangiogenic VEGF-A_{165b} isoform able to restore the diabetes-induced loss of the endothelial glycocalyx and albuminuria (41). There have been a few reports linking the sNogo-B/NgBR and VEGF-A/VEGFR2 systems, suggesting a concerted vasculoprotective role (20,43).

The effects on the endothelium may also be related to the changes seen in Wnt/β-catenin signaling. Activation of Wnt/β-catenin signaling (with cellular β-catenin accumulation) results in endothelial dysfunction, inflammation, and fibrosis and has been implicated in DN (34). Inactive GSK3β is also known to promote EC proliferation (44), with a phosphorylated (inactive) GSK3β favoring accumulation of cellular β-catenin (45), a promoter of angiogenesis and enhancer

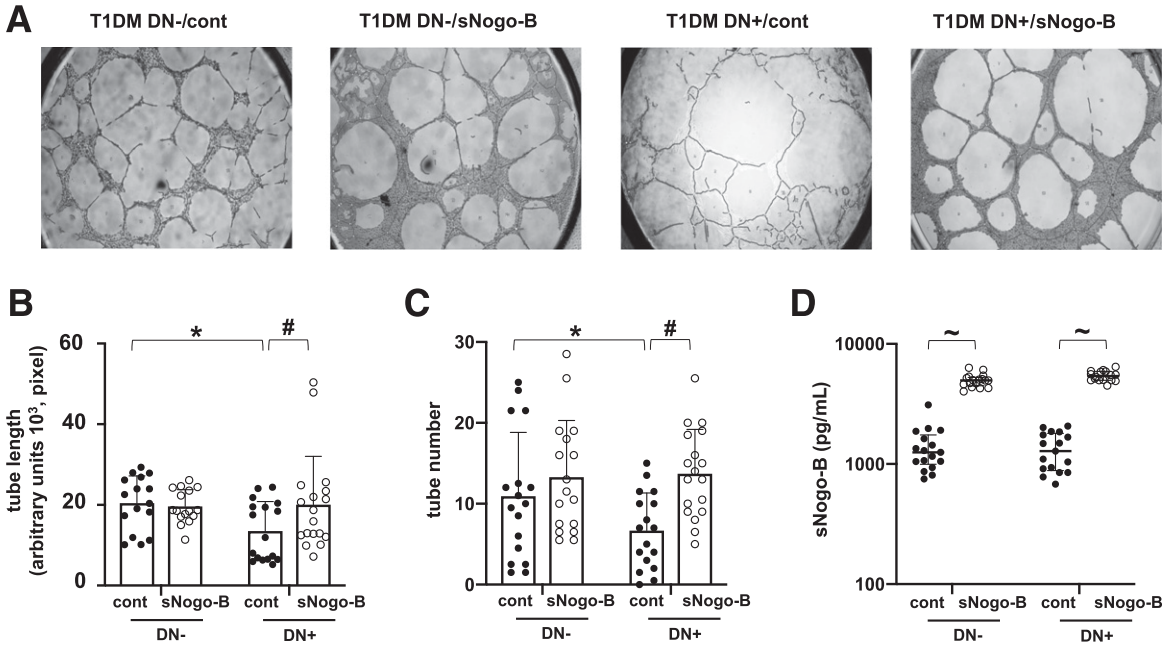

Figure 7—sNogo-B overexpression in the supernatant ameliorates impaired angiogenesis in HUVECs cultured with serum from patients with T1DM and DN. *A*: HUVECs transfected with either ADV-control (cont) or ADV–sNogo-B (sNogo-B) were seeded onto Matrigel for tube formation assay. HUVECs were then incubated with media containing (4% v/v) sera obtained from blood of patients with T1DM susceptible (DN+) or protected (DN−) toward the progression of DN (*A*; n = 16–18/group, in duplicate). An impairment in tube length (*B*) and number (*C*) was observed in HUVECs cultured with DN+ serum ($^*P \leq 0.04$, ADV-control, DN+ vs. DN−) was prevented by sNogo-B overexpression ($\#P \leq 0.02$, DN+, ADV-control vs. ADV–sNogo-B). *D*: sNogo-B levels in the supernatant of HUVECs transfected with ADV-control (cont) or ADV–sNogo-B (sNogo-B) vectors ($\sim P \leq 0.0001$, ADV-control vs. ADV–sNogo-B within DN− and DN+; n = 16 to 17/group, in duplicate). *B* and *C*: ANOVA with LSD post hoc test (mean ± SD). *D*: Kruskal-Wallis and Mann-Whitney test (median and interquartile range). ●, ADV-control–treated cells; ○, ADV–sNogo-B–treated cells.

of VEGF-A/VEGFR2 signaling (46). In our experiments, we showed that sNogo-B reduced GSK3β phosphorylation/β-catenin, which may contribute to preventing the angiogenesis response to diabetes-mediated vascular damage and dampen VEGF-A/VEGFR-2 signaling.

Increased eNOS phosphorylation and NO production have been implicated in hyperfiltration in the early phases of diabetic glomerulopathy (47,48), and the role of eNOS in oxidative stress (when in an "uncoupled state" [49]) is implicated in DN and GEC/podocyte damage (50). The reduction in diabetes-mediated eNOS phosphorylation, observed in kidney cortex lysates of diabetic mice with elevated sNogo-B circulating levels, would result in reduced eNOS-mediated NO production and oxidative stress, events that could contribute to a reduction in glomerular hyperfiltration (51) and in the prevention of diabetes-mediated loss of GECs glycocalyx and podocyte detachment. sNogo-B overexpression in the circulation associates with inhibition of diabetes-mediated modulation of VEGF-A/VEGFR2 and AKT/GSK3β/β-catenin system, signaling pathways that are implicated in podocyte injury/detachment and albuminuria (52–54). Finally, it might be possible that sNogo-B may have a direct effect on podocytes, as evidenced by our in vitro data showing expression of NgBR in human podocyte cells (Supplementary Fig. 4).

Full-length Nogo-B (assessed with a specific N-terminus antiserum) was found to be expressed in isolated glomeruli and glomeruli-enriched kidney cortex lysates and downregulated in diabetic mice. Full-length Nogo-B expression levels were also lower in glomeruli of proteinuric DN (a progressive chronic kidney disease) biopsy kidney samples when compared with TBMN, a benign glomerular disease without proteinuria, suggesting that the presence of proteinuria/albuminuria associates with reduced glomerular full-length Nogo-B levels. Whether low glomerular Nogo-B causes albuminuria is yet to be determined, but in experimental models of diabetic retinopathy, lack of full-length Nogo-B attenuated HG-induced cell migration and tube formation (EC differentiation) (55). In contrast to our findings, studies performed in humans' and rodents' renal biopsies (Nephroseq database, University of Michigan) showed no change in full-length Nogo-B mRNA expression in diabetic kidney tissue when compared with control tissue.

We also demonstrate that HG and/or VEGF-A determine, in GECs in culture, an increase in sNogo-B in the supernatant and a parallel reduction in cellular full-length Nogo-B (probed with specific N-terminus antiserum). These results support the cleavage of the full-length Nogo-B N-terminus as previously described (17), and future work will have to address the mechanisms behind this phenomenon.

We then explored the putative vasculoprotective role of sNogo-B in an angiogenesis experimental model in HUVECs cultured with sera of patients with T1DM susceptible to (DN+) or protected toward (DN−) the progression of DN (56). We demonstrate that circulating factors (sera from T1DM/DN+), other than glucose, negatively affect EC tube formation, and sNogo-B overexpression in the supernatant rescued this defect favoring EC differentiation toward a stable tube structure, an effect likely related to the patients' DN status rather than being driven by renal impairment, given that the average of renal function of the two groups of patients studied was 80–100 mL/min/1.73 m^2. Importantly, artificial upregulation of the N-terminus of Nogo-B (sNogo-B) in the supernatant is able, via a paracrine autocrine mechanism, to ameliorate the ability of ECs to migrate and differentiate into tubule-like structures (38), which translates into a more stable vasculature.

Collectively, our studies provide the first evidence that a primary increase of sNogo-B in the circulation protects the glomerular vasculature in diabetes. More studies will need to further dissect sNogo-B mechanisms of action and explore its potential benefit in diabetic animals with established kidney disease. sNogo-B could represent a novel targetable pathway for the treatment of diabetic chronic vascular complications.

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Author Contributions. I.H.-D., G.E.M., D.A.L., and L.G. led data discussion, interpretation, and manuscript writing with input from all other coauthors. I.H.-D., D.A.L., and L.G. contributed to experimental design and data collection together with J.P., C.A.R., X.B., J.K., K.E.W., M.F., G.E.F., F.A., A.E.H., F.F.H., G.E.M., and R.Q.M. D.A.L. and L.G. conceived the experiment. All authors approved the final manuscript. L.G. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Data and Resource Availability. The data sets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request. The viral vectors generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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