Allelic depletion of grem1 attenuates diabetic kidney disease

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Objective Gremlin (grem1) is an antagonist of the bone morphogenetic protein family that plays a key role in limb bud development and kidney formation. There is a growing appreciation that altered grem1 expression may regulate the homeostatic constraints on damage responses in diseases such as diabetic nephropathy.

Research Design and Methods Here we have explored whether knockout mice heterozygous for grem1 gene deletion (grem1+/−) exhibit protection from the progression of diabetic kidney disease in a streptozotocin-induced model of type 1 diabetes.

Results A marked elevation in grem1 expression was detected in the kidneys of diabetic wild-type mice compared to littermate controls, particularly in kidney tubules. In contrast, diabetic grem1+/− mice displayed a significant attenuation in grem1 expression at 6 months of diabetes compared to age and sex-matched wild-type controls. Whereas the onset and induction of diabetes was similar between grem1+/− and wild-type mice, several indicators of diabetes-associated kidney damage such as increased glomerular basement membrane thickening and microalbuminuria were attenuated in grem1+/− mice compared to wild-type controls. Markers of renal damage such as fibronectin and connective tissue growth factor were elevated in diabetic wild-type, but not grem1+/− kidney. Levels of pSmad1/5/8 decreased in wild-type, but not grem1+/− diabetic kidneys, suggesting that BMP signalling may be maintained in the absence of grem1.

Conclusions These data therefore identify grem1 as a potential modifier of renal injury in the context of diabetic kidney disease.
Diabetic nephropathy (DN) represents the most common cause of end stage kidney disease worldwide, affecting approximately one third of diabetic patients (1). Extracellular signaling molecules such as transforming growth factor-beta (TGFβ), connective tissue growth factor (CTGF) and advanced glycation end-products (AGEs) are implicated as drivers of DN (2). Intracellular signalling events including Smad3 phosphorylation, PI3K→PKB/Akt pathway, and MAPK activation play a role in kidney cell damage during DN (3-5). However, the precise molecular mechanisms underlying the pathogenesis of DN remain incompletely understood, and thus additional research is needed to identify novel molecular targets that may be of potential therapeutic value.

Grem1 is a highly conserved, 24-26 kDa secreted glycoprotein member of the cysteine knot superfamily, with the ability to heterodimerise and antagonise bone morphogenetic proteins (BMPs), in particular BMP-2, 4 and 7 (6). Grem1 regulates outgrowth, chondrogenesis and apoptosis of the developing limb bud (6-8), as well as branching morphogenesis during kidney development (9). Mice homozygous for deletion of grem1 die shortly after birth due to complete renal agenesis, supporting a primary role for grem1 in the developing kidney (10; 11). Recent data have identified a role for grem1 in bone formation and bone mass (12; 13), as well as in pulmonary hypertension (14) and angiogenesis (15). A role for grem1 in DN was originally proposed by data from our group that identified grem1 upregulation in primary human mesangial cells treated with high glucose, and in kidneys of diabetic rats (16; 17). Grem1 was also upregulated in other in vitro models relevant to DN such as mesangial cells subjected to cyclical mechanical strain or TGF-β1, and, importantly, in biopsies from human DN patients (18-20). We recently demonstrated that grem1 upregulation is part of the transcriptomic response of tubular epithelial cells exposed to TGF-β1 (20). Increasing evidence suggests that the degree of grem1 expression correlates with disease severity in a variety of forms of renal fibrosis, including glomerular scarring in in vivo acute glomerulonephritis (21), tubular scarring in chronic allograft nephropathy and progressive membranous nephropathy (22). Levels of grem1 in the adult kidney are low, and disease-dependent upregulation of grem1 in DN may reflect a reactivation of quiescent gene expression in response to hyperglycemia and other stimuli (23). Together, these data point towards a contributory role for grem1 in diabetic microvascular complications.

This study addressed the hypothesis that reduced grem1 gene expression would provide protection in the diabetic kidney. Our data suggest that depletion of grem1 expression in mice attenuates early diabetic nephropathy-like changes in kidney.

**RESEARCH DESIGN AND METHODS**

**Experimental Animals:** All animal procedures were licensed by the Irish Department of Health and Children and approved by the local animal research ethics committee at University College Dublin. Grem1 heterozygote knockout mice (grem1+/-) were generated by Richard Harland, University of California at Berkeley, USA (10). Experimental animals were generated by crossing wild-type C57Bl/6J and grem1+/- mice, with male offspring used in the study. Mice were maintained in a conventional animal facility in standard caging, with free access to water and standard rodent chow. Genotyping was performed using DNA extracted from ear-punches as previously described (10).
**Induction of type 1 diabetes in mice:**

Seven to 10-week old male mice (both wild-type and grem1+/-), weighing approximately 19 g at the onset of the experimental protocol were genotyped and then randomly divided into two groups: A, treated with streptozotocin (STZ, Sigma) dissolved in 100 mM citrate buffer pH 4.5, or B, treated with citrate buffer alone (www.amdcc.org). STZ was dissolved in sterile citrate buffer and injected intraperitoneally (50 mg/kg) within ten minutes of preparation on 5 consecutive days. Fasting blood glucose levels were assayed after 6 h fast between 8 am and 2 pm with tail venepuncture at 2 pm. Diabetes mellitus was confirmed by two consecutive daily measurements of fasting blood glucose >15 mmol/L two weeks after STZ injection. To maintain weight and prevent ketoacidosis, all diabetic mice were commenced on long acting insulin (Insulotard®, Nova Nordisk) subcutaneously at a dose of one third IU three times weekly at week 18 of diabetes.

**Tissue pathology and urine collection:** Mice were housed individually in mouse metabolic cages (Technoplast®) for 24 h to collect urine 5 days pre-sacrifice. Urine volumes were recorded, and urinary glucose, ketone, and red blood cell levels were monitored semi-quantitatively with Multistix™ reagent strips (Bayer). Cardiac puncture was performed at the time of sacrifice. The left renal artery was clamped and the left kidney was removed, weighed, and dissected. The inferior renal pole was promptly processed for electron microscopy, and the superior renal pole was snap-frozen and stored at -80 °C for RNA and protein extraction. Renal perfusion was performed via cannulation of the left ventricle with an 18-gauge needle. Initial perfusion using gravity was performed with sterile normal saline (pH 7.4) for 5 min, followed by 4 % (w/v) paraformaldehyde (pH 7.4) for 5 min. The perfused right kidney was then removed and incubated in 4 % paraformaldehyde for 24 h at RT. Kidneys were processed and cut at 3 µm thickness on a rotary microtome and stained with hematoxylin/eosin, periodic acid Schiff, or picrosirius red. Stained sections were scored independently (single blinded) by pathologists using normal light microscopy. The amount of mesangial matrix was scored with PAS staining (scored 1-4) Collagen distribution in the cortex was scored with Sirius red (SR) staining using a normal light microscope (scored 1-5).

**Clinical biochemistry:** Urinary albumin was measured using the Albuwell M® kit, and urinary creatinine was measured using the Creatinine Companion® murine ELISA kit (Exocell, Philadelphia). Urine was collected in metabolic cages, urine volume recorded and 24 h urinary albumin excretion levels were assayed with the Albuwell M assay kit.

Plasma creatinine, serum lipids and whole blood HbA1c were measured at the Mouse Metabolic Phenotyping Core (MMPC), Vanderbilt Medical Centre, Nashville. Plasma creatinine was measured on a HPLC Zorbax SCX strong cation exchange column (Aglient, Wilmington, DE) as described (www.amdcc.org). HbA1c was measured using DCA 2000 analyser and cartridges (Bayer). Total plasma cholesterol and triglycerides were measured by standard enzymatic assays (Raichem®). HDL cholesterol was measured after precipitation of vLDL and LDL using dextran sulfate and magnesium.

**Estimation of creatinine clearance:**

Creatinine clearance was calculated utilising the equation:

\[
\text{Urine vol (µl) x urine creatinine (mg/dL) x 1440 (min)}
\]

and then corrected to total body weight at sacrifice (g) to give creatinine clearance in µL/min/gram body weight.
**Electron Microscopy:** The left inferior kidney pole was removed, diced into 1 mm cubes and fixed in 2.5 % (v/v) glutaraldehyde in 0.1 M cacodylate buffer. The samples were washed in 0.1 M cacodylate buffer and then postfixed in 1 % (w/v) aqueous osmium tetroxide. The tissues were washed, dehydrated through a graded series of ethanol and embedded in Spurr resin. Thick sections (0.5 μm) were cut, affixed to glass slides, stained with toluidine blue and viewed by light microscopy. Thin (100 nm) sections were cut from selected areas and viewed with an FEI CM-12 transmission electron microscope operated at 80 KeV. GBM thickness measurements were assessed as follows: 2 to 4 glomeruli were randomly selected in each slide and serial measurements were taken at intervals from the margins of the lamina rara interna to lamina rara externa. Images were taken and analyzed with an AMT XR41 Digital TEM Camera system. Up to 60 measurements were taken per kidney.

**Quantitative PCR:** Total RNA was isolated from snap-frozen renal poles by homogenisation in 1 ml Trizol® (GibcoBrl, Life technologies) using a Polytron® (Kinematica) and Qiagen RNeasy kit. Reverse transcription was performed using Superscript II™ (Invitrogen), followed by quantitative real time PCR on an ABI Prism 7700 sequence detection system. Mouse Grem1 (Mm00488615_S1), BMP-7 (Mm00432102_m1), fibronectin (Mm00482221), vimentin (Mm00432102_m1) and CTGF (Mm00515790_g1) real-time oligo probes were purchased from Applied Biosystems.

**Western Blotting:** Portions of renal pole were lysed in RIPA buffer containing 50 mM Tris-HCl pH 7.4, 1 % (v/v) NP-40, 0.25 % (v/v) sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, supplemented with fresh 1 mM phenylmethylsulfonyl fluoride, 1x protease inhibitor cocktail (Sigma), 1 mM NaF, 40 mM β-Glycerophosphate, 2 μM Microcystin, 1 mM sodium vanadate. Twenty five μg protein extract were then separated by 10 % (v/v) SDS-PAGE and blotted using phospho-Smad1/5/8 (Cell Signalling #95115), total Smad1/5/8 (Santa Cruz sc-6031R), BMP-7 (Abcam ab27569) or β-actin (Sigma) antibodies exactly as described (4).

**Statistical Analysis:** All data were plotted as mean +/- standard error of the mean (SEM). Student’s two-tailed t-tests or one-way ANOVA with Tukey Kramer multiple comparison post hoc tests were calculated using the Instat software package.

**RESULTS**

**Induction of type 1 diabetes in wild-type and grem1+/− mice:** Using the replacement β-galactosidase “knock-in” gene activity as a marker of grem1 promoter activity (10), grem1 gene expression was detected in heterozygous grem1+/− knockout cells (Fig. 1A). Importantly, homozygous grem1−/− knockout MEFs displayed approximately double the activity of β-galactosidase activity, suggesting that maximal grem1 expression required both copies of the grem1 gene. We therefore hypothesised that grem1 expression would be reduced in the kidneys of grem1+/− mice in response to hyperglycemia.

Type 1 diabetes was induced in grem1+/− and wild-type mice using streptozotocin injection, with time zero defined as the day of initial STZ injection. Mice were maintained in the diabetic state for 18 or 27 wk. Metabolic parameters, body weight, renal weight and serum glucose levels are presented in Table 1. Non-diabetic mice gained significant weight compared to diabetic mice, as assessed at study end (wild-type control mice body weight 32.0 g +/- 4.47, wild-type diabetic mice body weight 22.82 g +/- 3.22). No significant difference in total body weight between wild-type and grem1+/− mice, in both control and diabetic cohorts was detected (Table 1). Both diabetic groups
Diabetic nephropathy in grem1+/- mice

Diabetic mice developed marked fasting hyperglycemia that peaked at approximately 33 mmol/l and was maintained up to the 27 wk time-point (Fig. 1B). The onset and severity of hyperglycaemia was similar between wild-type and grem1+/- mice (Fig 1B). Marked and significant increases in HbA1c levels in diabetic mice were also detected at 18 and 27 wk of hyperglycemia (Fig. 1C), in both wild-type and grem1+/- groups. Thus, the onset, severity and progression of diabetes were similar in both wild-type and grem1+/- mice.

*Grem1 upregulation is reduced in grem1+/- mice kidneys:* We assessed the effects of type 1 diabetes on grem1 expression in kidneys from our experimental animals. Minimal grem1 mRNA was detected in both wild-type and grem1+/- control kidneys at both time points examined, but was dramatically increased in wild-type diabetic mice (Fig. 2A). Approximately 62-fold increased grem1 upregulation was detected at 18 wk, compared to 42-fold at 27 wk in wild-type diabetic mice (Fig. 2B). In contrast, grem1+/- mice manifested a 53-fold increase at 18 wk and an 8-fold increase at 27 wk in diabetic kidney (Fig. 2B). Importantly, the fold-change in grem1 mRNA upregulation at 27 wk in diabetic grem1+/- mice was significantly lower than that seen in diabetic wild-type mice at the same time-point (Fig. 2B). Increased grem1 protein was also detected in diabetic wild-type kidney sections compared to controls via Western blotting and immunohistochemistry (Fig. 2C, D and Suppl. Fig. 1). In contrast, the increase in grem1 protein was blunted in diabetic grem1+/- mice (Fig. 2C, D). These data suggest that deletion of one copy of the grem1 gene dramatically reduced grem1 induction in the diabetic mouse kidney.

**Early type 1 diabetes-induced structural changes are attenuated in grem1+/- kidney compared to wild-type mice:** Diabetic mice developed marked polyuria compared to age-matched controls, with no significant difference between wild-type and grem1+/- cohorts observed at 27 wk (wild-type control urine volume 3.35 ml +/- 2.79, wild-type diabetic 15.18 ml +/- 9.35, p<0.01; grem1+/- control 4.32 ml +/- 3.15, grem1+/- diabetic 12.31 +/- 10.6, p<0.05, Table 1). A significant increase in the ratio of left kidney: total body weight occurred in all diabetic animals compared to age matched control after 27 wk of diabetes, suggestive of renal hypertrophy (wild-type control vs. diabetic 5.409 +/- 0.19 vs. 9.077 +/- 0.38, p<0.001; grem1+/- control vs. diabetic 5.536 +/- 0.16 vs. 8.816 +/- 0.28, p<0.001, Table 1). The fold change in left kidney: total body weight was not significantly different between wild-type and grem1+/- mice (data not shown).

All diabetic mice developed a significant increase in glomerular basement membrane (GBM) thickness compared to non-diabetic age-matched controls at 27 wk of hyperglycemia (Fig. 3A, B). Interestingly, this increase was significantly greater in diabetic wild-type compared to diabetic grem1+/- mice. GBM thickness increased 47 % from baseline mean in age-matched control vs diabetic wild-type mice (Fig. 3B, open bars). In contrast, a more modest 14 % increase in GBM thickening was detected in control vs. diabetic grem1+/- mice at 27 wk (Fig. 3B, filled bars). The fold-change in GBM thickening was significantly lower in grem1+/- mice compared to wild-type (Fig. 3C), suggesting allelic depletion of grem1 prevented diabetes-induced early structural changes in the kidney.

Moderate increases in glomerular matrix secretion and interstitial collagen deposition were observed in diabetic wild-type and grem1+/- mice compared to control (Fig. 4A, C). No significant tubulointerstitial fibrosis was detected in either genotype up to 27 wk of diabetes (data not shown). Scoring of these sections revealed that the degree of increased staining of these markers of renal
damage were not significantly different between diabetic wild-type and grem1+/- mice (Fig. 4B, D). These data suggest that our model of type 1 diabetes in mice on a C57Bl/6J background manifest early DN-like changes in kidney, but do not develop more advanced renal disease.

**Diabetic grem1+/- mice exhibit attenuated changes in ACR and eGFR:** Urinary microalbumin was increased in diabetic wild-type mice compared to controls (control 34.02 µg +/- 4.53, diabetic 27 wk 129.32 µg +/- 16.05, p<0.001, Fig. 5A). In contrast, microalbuminuria was less severe in diabetic grem1+/- mice (control 33.09 µg +/- 3.00, diabetic 27 wk 71.47 µg +/- 17.54, p<0.05, Fig. 5A). Fold-change in microalbuminuria was significantly lower in grem1+/- diabetic mice compared to wild-types at 27 wk of diabetes (Fig. 5B). These data suggest that depletion of grem1 expression reduced microalbuminuria associated with early renal damage in DN.

Serum creatinine levels decreased significantly in wild-type diabetic mice compared to age-matched controls (Table 1). This decrease in serum creatinine did not occur in grem1+/- mice at either 18 or 27 wk of diabetes (Table 1). Albumin to creatinine ratios (ACR) increased in diabetic wild-type mice at both 18 and 27 wk of hyperglycemia (control 63.51 µg/mg +/- 15.25, diabetic 27 wk 594.97 µg/mg +/- 271.09, Fig. 5C). This increased ACR was greatly attenuated in diabetic grem1+/- mice (control 104.13 µg/mg +/- 13.68, diabetic 27 wk 287.10 µg/mg +/- 68.75, Fig. 5C). The smaller increase in grem1+/- mice was highlighted when fold-change was calculated, showing a significantly lower increase in grem1+/- mice ACR compared to wild-type at 27 wk (Fig. 5D).

Creatinine clearance increased in diabetic wild-type mice compared to control at 18 wk of diabetes, suggesting that glomerular hyperfiltration was occurring at this time point (Fig. 5E). In contrast, diabetic grem1+/- mice did not develop significant increases in creatinine clearance until the 27 wk time-point (Fig. 5E). A significantly higher fold-change in creatinine clearance was detected in grem1+/- mice compared to wild-type at 27 wk, suggesting that grem1 depletion delayed the onset of peak hyperfiltration in diabetic mice (Fig. 5F).

Serum lipids were elevated in diabetic wild-type mice, showing a significant increase in triglycerides and decrease in HDL typical of diabetic hyperlipidemia (Table 1). Diabetic grem1+/- mice also manifested a significant elevation in serum triglycerides but demonstrated a significantly lower HDL at baseline than wild-type controls, which failed to drop significantly in the setting of diabetes (HDL, wild-type control 77 g/dl +/- 3.69, grem1+/- control 58.3 g/dl +/- 2.9, Table 1).

**Grem1 mRNA correlates with indices of early indices of DN:** A significant correlation was detected between ACR and GBM thickening in both control and diabetic mice in our study (Fig. 6A). To assess whether grem1 mRNA correlated with indices of early diabetic kidney disease, grem1 levels were compared to changes in microalbuminuria and glomerular basement membrane thickening. The degree of grem1 expression correlated significantly with both ACR and GBM thickening in wild-type control and diabetic mice across the entire experiment (Fig. 6B, C). These data suggested that increased grem1 gene expression occurred in parallel with cellular damage during early diabetic kidney disease.

To explore the mechanism of partial protection from diabetes-induced damage in grem1+/- mice, we measured levels of genes implicated in kidney damage during diabetic nephropathy. Increased levels of fibronectin, vimentin and connective tissue growth factor (CTGF) were detected in diabetic wild-type kidney at 27 wk (Fig. 7). In contrast, no
significant increase was detected in grem1+/- diabetic kidney, suggesting that decreased grem1 levels reduces diabetes-mediated upregulation of genes involved in mediating glomerular and renal damage. Since grem1 is a negative regulator of bone morphogenetic protein-7 (BMP-7), a molecule that has been shown to mediate repair processes in the damaged kidney, we examined the effect of grem1 deletion on this protein. No significant changes in BMP-7 mRNA or protein levels were detected at either 18 or 27 wk of diabetes in either wild-type or grem1+/- mice (Suppl. Fig. 2 and data not shown). Similar to previous reports (24), levels of phospho-Smad1/5/8, a downstream target of BMP-7 signalling, were reduced in wild-type diabetic kidney at 27 wk (Fig. 8C, D). In contrast, no significant decrease in pSmad1/5/8 levels was detected in grem1+/- mice, potentially suggesting that BMP signalling is maintained in the diabetic kidney when levels of grem1 are reduced.

DISCUSSION

The BMP antagonist, grem1, regulates critical processes controlling limb-bud outgrowth and kidney development (6-8; 10; 11). Increased grem1 levels correlated with the pathogenesis of diabetic nephropathy and/or progressive renal fibrosis (19; 20). To address whether grem1 depletion would protect against diabetes-induced renal disease, we evaluated renal damage in type 1 diabetic mice lacking one copy of the grem1 gene (grem1+/-). We provide the first evidence that allelic depletion of grem1 causes a reduction in grem1 expression levels and protects against early diabetic nephropathy-like changes in the kidney.

Grem1+/- mice on a C57Bl/6J background were used in this study. Previous data compared the susceptibility of different genetic strains of mice, and showed that C57Bl/6J mice were “high responders” in terms of STZ-induced hyperglycemia (25). Consistent with these findings, severe and sustained hyperglycemia developed in both wild-type and grem1+/- mice (Fig. 1B). We demonstrate here that type 1 diabetes induced grem1 kidney mRNA and protein in wild-type mice. This reactivation of grem1 expression may be linked to a tissue repair mechanism in response to hyperglycemia and other insults that become maladaptive, leading to further kidney injury (23; 26). Factors such as transforming growth factor-beta (TGFβ), advanced glycation end-products (AGEs) and hemodynamic stress are features of diabetic nephropathy that have previously been shown to increase grem1 expression (17; 20). Since grem1+/- mice displayed a marked reduction in grem1 upregulation in diabetes (Fig. 2), we propose that the recapitulation of grem1 gene activation in the diabetic kidney involves both grem1 alleles.

A similar degree of renal hypertrophy was observed in both wild-type and grem1+/- diabetic mice (Table 1). These data suggested that reduction of grem1 expression prevented diabetes-induced increases in GBM thickness during early-stage DN. Staining for markers of more advanced kidney damage such as mesangial matrix secretion and interstitial collagen showed modest increases in diabetic mice (Fig. 3D-F). Previous data had indicated that C57Bl/6J mice were somewhat resistant to DN-like changes in kidney, manifesting modest increases in albuminuria and glomerular damage (25). Our study also detected modest increases in glomerular damage (Fig. 3), together with a significant increase in microalbuminuria (approx. 4.8 fold, Fig. 5A, B). Mice in our study developed severe hyperglycemia (~590 mg/dl) at 18 wk (Fig. 1B). In contrast, C57Bl/6 mice in the study of Gurley et al. manifest lower levels of hyperglycemia at 16 wk (388 mg/dl). Thus, the more robust hyperglycemia in our model may have been sufficient to induce microalbuminuria, but not gross structural changes in glomerulus or...
kidney tubules in our mice. We conclude based on the early structural changes evident that our diabetic mice develop mild but significant damage in the kidney, and that this early damage is attenuated when levels of \textit{grem1} are reduced by gene deletion. Microalbuminuria developed in wild-type diabetic mice compared to control (Fig. 5), but was attenuated in age-matched \textit{grem1}+/− mice (Fig. 5A, B). Diabetes-induced increases in albumin:creatinine ratio (ACR) were also reduced in \textit{grem1}+/− mice compared to wild-type (Fig. 5C). Values for creatinine clearance suggest that diabetes-induced hyperfiltration peaked in wild-type mice at 18 wk (Fig. 5E). In contrast, creatinine clearance values for \textit{grem1}+/− diabetic mice were lower at 18 wk compared to wild-type, and were higher at 27 wk, suggesting that the peak of hyperfiltration in diabetic \textit{grem1}+/− may occur at a later time compared to wild-type (Fig. 5E). Together with previous data identifying \textit{grem1} upregulation in high-glucose cell culture models and human DN biopsies, these data add to the growing body of evidence implicating \textit{grem1} in the pathogenesis of DN. Furthermore, our data provide the first evidence that a reduction in \textit{grem1} expression reduces early impairment of renal structure and function in the diabetic kidney. We are currently examining whether other vascular complications of diabetes are also altered in \textit{grem1}+/− mice. Preliminary evidence from our group suggests that \textit{grem1} deletion may also protect against aortic thickening in the diabetic state, potentially due to altered serum lipid profile (27).

Several markers of renal damage in diabetes have been identified, and levels of several of these genes were elevated in the wild-type, but not \textit{grem1}+/− diabetic kidney (Fig. 7). Thus, in the hyperglycaemic state, the triggers that increase the expression of genes contributing to glomerul sclerosis and tubular damage seem to be attenuated in the \textit{grem1}+/− kidney. \textit{Grem1} is an antagonist of BMP-2, 4 and 7, binding to these proteins and preventing their interaction with their cognate receptors (6; 8; 14). Of these, BMP-7 has attracted recent attention, as up to 90% loss of this protein has been reported in the kidneys of diabetic rats (28). Loss of BMP-7 was accompanied by an increase in \textit{grem1} expression, both changes likely driven by increased TGFβ1 in the diabetic kidney (28). Administration of recombinant BMP-7 or transgenic over-expression of BMP-7 in podocytes ameliorates renal injury in mouse models of diabetes (29) and lupus (30). Although significant changes in cellular BMP-7 mRNA or protein were not detected in our diabetic mice (Suppl. Fig. 2), our data shows that levels of phospho-Smad1/5/8, a downstream target of BMP signalling were reduced in wild-type, but not \textit{grem1}+/− diabetic kidney compared to controls (Fig. 8), suggesting that loss of \textit{grem1} expression facilitates sustained BMP-7 signalling in the diabetic kidney, extending the notion of a carefully coordinated balance between BMP signalling and \textit{grem1} in the disease state.

Previous reports have identified other genes such as TGFβ type II receptor, CTGF and p27Kip1 whose deletion confers protection against the sequelae of diabetic kidney disease. (31) (24) (32). (p27Kip1+/−) displayed an intermediate degree of protection compared to p27Kip1−/− mice, suggesting that p27Kip1 is haplo-insufficient in terms of its role in diabetic nephropathy (32). Our data suggest that \textit{grem1} may also be haplo-insufficient, as deletion of one allele of \textit{grem1} reduced \textit{grem1} expression and conferred a moderate degree of protection from structural renal damage induced by diabetes.

Since \textit{grem1}+/− mice developed less severe GBM thickening (Fig. 3), together with lower fold increases in ACR and eGFR (Fig. 5C-F) compared to wild-type controls, a reduction in \textit{grem1} levels using gene deletion may reduce both the onset and severity of renal disease in the diabetic kidney. Increased
grem1 levels correlated tightly with both GBM thickening and ACR (Fig. 6), suggesting that reactivation of grem1 in the diabetic kidney may occur in parallel with early pathological changes in renal structure and function. Since grem1+/- mice manifest less severe microalbuminuria and GBM thickening, these data suggest that grem1 upregulation contributes to glomerular damage in response to diabetes in the kidney.

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Statement of competing financial interests: The authors declare that there are no competing financial interests.
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Diabetic nephropathy in grem1+/- mice

Table 1. Metabolic and renal function parameters of control and diabetic mice. Data are expressed as mean +/- SEM with the number of animals per group shown in brackets. Data were compared using one-way ANOVA and Tukey Kramer post hoc analysis. *p<0.05, ** = p <0.01, *** p<0.001 compared to age-matched control of same genetic type.

<table>
<thead>
<tr>
<th>Group/ Parameter</th>
<th>Control +/- 18wk</th>
<th>Diabetic +/- 18wk</th>
<th>Diabetic +/- 27wk</th>
<th>Control grem1 +/-. 18wk</th>
<th>Diabetic grem1 +/- 18wk</th>
<th>Diabetic grem1 +/- 27wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, initial (g)</td>
<td>19.37+/-0.61 (10)</td>
<td>22.3+/-1.17 (6)</td>
<td>19.3 +/- 0.85 (10)</td>
<td>20.15 +/- 0.71 (11)</td>
<td>23.91 +/- 1.43(6)</td>
<td>20.02 +/- 0.47 (12)</td>
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<td>Body wt, final (g)</td>
<td>32.00 +/- 1.41(10)</td>
<td>20.64 +/- 1.24 (6)***</td>
<td>22.82 +/- -1.15 (10)***</td>
<td>30.95 +/- 0.66(11)</td>
<td>21.84 +/- -1.29(6)***</td>
<td>22.49 +/- -0.84 (12)***</td>
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<tr>
<td>FPG initial (mmol/L)</td>
<td>6.06+/-0.38(8)</td>
<td>6.32+/-0.18(6)</td>
<td>6.05 +/- 0.34(10)</td>
<td>5.4 +/- -0.27(11)</td>
<td>5.68 +/- -0.23(6)</td>
<td>6.79 +/- -0.27(12)</td>
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<tr>
<td>FPG final (mmol/L)</td>
<td>6.28+/-0.17(10)</td>
<td>&gt;33.3+/-0 (6)***</td>
<td>33.02+/-0.19(10)***</td>
<td>5.73+/- -0.21(11)</td>
<td>&gt;33.3+/-0 (6)***</td>
<td>32.64+/- -0.65 (12)***</td>
</tr>
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<td>HbA1c (%)</td>
<td>4.35+/-0.26(6)</td>
<td>13.06+/-0.79 (5)***</td>
<td>12.65 +/- -0.84(6)***</td>
<td>4.26+/- -0.08(9)</td>
<td>12.17 +/- -0.84 (7)***</td>
<td>12.51 +/- -0.53 (11)***</td>
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<tr>
<td>Urine volume pre sacrifice (ml)</td>
<td>3.35+/-0.93(9)</td>
<td>15.32+/-4.58(6)*</td>
<td>15.18+/- -2.96(10)**</td>
<td>4.32+/- -0.87(13)</td>
<td>18.59 +/- -5.95(6)**</td>
<td>12.31+/- -3.19(11)*</td>
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<td>Left kidney weight (g)</td>
<td>0.168+/-0.01(10)</td>
<td>0.185+/-0.01(6)</td>
<td>0.204 +/- -0.01(10)**</td>
<td>0.167 +/- -0.01(11)</td>
<td>0.202 +/- -0.01(6)*</td>
<td>0.199 +/- -0.01(11)*</td>
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<td>Left kidney: total body weight ratio (g/kg)</td>
<td>5.409 +/- -0.19(10)</td>
<td>9.074+/-0.27 (6)***</td>
<td>9.077 +/- -0.38 (10)***</td>
<td>5.536 +/- -0.16(11)</td>
<td>9.152 +/- -0.63 (6)***</td>
<td>8.816 +/- -0.28 (11)***</td>
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<td>Plasma creatinine (mg/dL)</td>
<td>0.072+/-0.01(12)</td>
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<td>0.042+/-0.01 (8)**</td>
<td>0.085+/- -0.01(11)</td>
<td>0.093+/- -0.01 (6)</td>
<td>0.071+/- -0.01 (9)</td>
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<td>Creatinine clearance (µl/min/g body wt)</td>
<td>13.72+/-1.35(8)</td>
<td>35.39+/-7.14(8)*</td>
<td>27.45+/--3.89 (8)</td>
<td>9.07+/- -0.71(10)</td>
<td>16.87+/- -3.44 (5)</td>
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<td>ACR</td>
<td>63.51+/-15.25 (9)</td>
<td>636.5+/-341.4 (3)</td>
<td>594.96+/- -664.04 (6)*</td>
<td>104.13+/- -13.7 (11)</td>
<td>284.76+/- -52.49 (3)</td>
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<td>UAER (24 h)</td>
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<td>129.59+/-34.07 (6)**</td>
<td>129.32 +/- -16.05 (8)***</td>
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<td>170.0+/- -9.60 (5)**</td>
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<td>HDL (g/dL)</td>
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<td>62.73+/-3.52 (6)*</td>
<td>59.4+/- -4.19(10)***</td>
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<td>Triglycerides (g/dL)</td>
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<td>124.5+/- -16.73 (6)*</td>
<td>130.71+/- -10.71(7)***</td>
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<td>LDL (g/dL)</td>
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<td>41.87+/- -6.79 (8)</td>
<td>85.5+/- -13.86 (4)</td>
<td>33.43+/- -6.80 (7)</td>
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**Figure Legends**

**Figure 1. Induction of type 1 diabetes in wild-type and grem1+/- mice.** A. Grem1 promoter activity was examined in embryonic fibroblasts from E13.5 mouse embryos. Lysates from wild-type (+/+), grem1+/- or grem1-/- cells were assayed for β-galactosidase activity as described. Results are representative of four experiments carried out in duplicate. B. Wild type and grem1+/- mice were injected intraperitoneally with either citrate buffer (control) or 50 mg/kg streptozotocin (STZ) for 5 consecutive days (week 0) according to established procedures (Methods). Fasting blood glucose levels were monitored biweekly for 27 wk using a glucometer and a drop of blood from the tail vein. Significant increases in blood glucose levels developed in both groups after 2 wk (p<0.001, n=10-12 mice per group) and were maintained over the 27 wk study time-course. Open square, wild-type control; filled circle, grem1+/- control; filled square, wild-type diabetic; open circle, grem1+/- diabetic. C. Whole blood was collected via cardiac puncture at time of sacrifice in both cohorts of mice. Percent HbA1c was assessed via ELISA as described in Methods. Significant increases in % HbA1c were detected in both cohorts at 18 and 27 wk of diabetes (mean +/- SEM, *** p< 0.001 using one-way ANOVA and Tukey-Kramer multiple comparison test, n=6-11 in each group).

**Figure 2. Diabetes-mediated induction of grem1 expression is attenuated in grem1+/- mice.** A. Total RNA was extracted from renal poles of control (“C”) and diabetic (“D”) wild-type (open bars) and grem1+/- (filled bars) mice at each time-point indicated. Quantitative TaqMan PCR was performed using mouse grem1 specific oligonucleotides as described. ∆∆Ct values were calculated by subtracting the Ct values for the 18s control from the corresponding grem1 value performed in the same tube, and altered mRNA levels were then calculated by setting the control in each age-group to 1. Data are plotted as mean +/-SEM. * p<0.05, Student’s unpaired t-test, n=4-6 for each group. B. Fold-change in grem1 mRNA was calculated by dividing the ∆∆Ct value for diabetic mice by the mean of the corresponding age-matched control group. Data are plotted as mean fold-change +/-SEM. *, p<0.05, Student’s unpaired t-test, n=4-6 per group. C. Protein extracts (20 µg) from control and diabetic wild-type and grem1+/- renal poles were probed by Western blot with grem1 antibody (R&D) and β-actin (Sigma). An approx 25 kDa band corresponding to grem1 was detected. D. Densitometry was performed using Scion image software and the intensity of grem1 expression was normalised to β-actin loading control. Data were then plotted as diabetic/control fold change for both wild-type and grem1+/- mice. ** p<0.01, Student’s t-test (n=3).

**Figure 3. Glomerular basement membrane thickening is attenuated in diabetic grem1+/- mice compared to wild-type.** Kidney pieces were processed as described in Methods. 100 nm sections were cut from the renal pole harvested from control and diabetic wild-type and grem1 +/- mice at 27 wk. Sections were viewed with an FEI CM-12 transmission electron microscope operated at 80 KeV. Glomeruli were randomly selected, viewed at 15,000 x magnification and serial measurements along the GBM were assessed. Arrows indicate the position of the glomerular basement membrane (A). Top left, non diabetic wild-type (+/+) control. Top right, diabetic wild-type (+/+). Bottom left, non-diabetic grem1+/- (+/-) control. Bottom right, diabetic grem1+/- Arrows indicate the thickness of the GBM. B. Quantitation of GBM thickness from all groups of mice. Up to 60 serial measurements were made from each individual glomerulus, and a mean value per mouse was calculated. Data are plotted as group mean +/- SEM. GBM thickness
was significantly higher in wild-type diabetic mice compared to non-diabetic controls (p<0.001 using one-way ANOVA and Tukey-Kramer multiple comparison test, n=7-11 per group). The increase observed in diabetic grem1+/- mice compared to controls did not reach significance (p=0.224). Fold change in GBM thickening. Mean GBM thickness values for each diabetic animal were divided by the mean thickness for control mice for both wild-type and grem1+/- groups. Mean fold change values were calculated for both wild-type and grem1+/- mice at 27 wk. *, p<0.05, Student’s two tailed t-test.

**Figure 4. Mild structural changes are evident in diabetic wild-type and grem1+/- mice by light microscopy.** Post mortem, mouse kidneys were fixed by perfusion fixation in situ using 4% (w/v) paraformaldehyde. 3 µm paraffin embedded sections were stained with (A) Sirius Red to detect interstitial collagen or (C) Periodic Acid Schiff to assess glomerular matrix secretion. Slides (n=5 for each group) were scored blindly by an independent renal pathologist on a scale of 0-4. Data are plotted as mean score +/- SEM for control (“C”) or diabetic (“D”) mice in wild-type (open bars) or grem1+/- (filled bars) mice. Observed increases in both glomerular matrix secretion (B) or collagen staining (D) did not reach significance using Student’s two-tailed t-test.

**Figure 5. Renal function impairment is attenuated in diabetic grem1+/- mice compared to wild-type.** A. Twenty four hour urine volumes were measured and levels of microalbumin measured using Albuwell ELISA assay as described. Values for control non-diabetic (27 wk), 18 wk and 27 wk diabetic groups of wild-type (open bars) and grem1+/- mice (filled bars) were plotted (n=6-11 per group except grem1+/- 18 wk, n=3). Data were analysed using one-way ANOVA with Tukey Kramer post hoc analysis (* p<0.05, ** p<0.01, *** p<0.001). B. Fold change in 24 h microalbuminuria was calculated by divided microalbumin values for individual diabetic wild-type and grem1+/- mice by the mean microalbumin value for the corresponding 18 or 27 wk control group. Mean fold-change values +/- SEM were plotted. *, p<0.05, using a two-tailed t-test. NS, non-significant. C. Albumin:creatinine ratio (ACR) was calculated by dividing urinary microalbumin by urinary creatinine, and plotted as µg/mg. Values from control (non-diabetic), 18 wk and 27 wk diabetic wild-type and grem1+/- mice were plotted (n=6-11; * p<0.05, one-way ANOVA with post hoc Tukey-Kramer multiple comparison test). D. Fold-change in ACR was calculated by dividing ACR values for individual diabetic wild-type and grem1+/- mice by the mean ACR value for the corresponding 18 or 27 wk control group. Mean fold-change values +/- SEM were plotted. NS, non-significant; *, p<0.05 using Student’s two-tailed t-test. E. Creatinine clearance was calculated as µl per minute per g body weight. Data from both control and diabetic wild-type and grem1+/- mice at time 0, 18 wk and 27 wk of diabetes were plotted (n=6-11; * p<0.05, *** p<0.001, NS, non-significant using one-way ANOVA with post hoc Tukey-Kramer multiple comparison test). F. Fold-change in creatinine clearance was calculated by dividing creatinine clearance values for individual diabetic wild-type and grem1+/- mice by the mean creatinine clearance value for the corresponding 18 or 27 wk control group. Mean fold-change values +/- SEM were plotted. NS, non-significant; *, p<0.05 using Student’s two-tailed t-test.

**Figure 6. Grem1 mRNA levels correlate with indices of renal damage.** A. Twenty four hour microalbumin values (µg) were plotted against ACR values (µg microalbumin/mg creatinine) for wild-type and grem1+/- control and diabetic mice over the course of the study (n=50). R² value of the trendline was calculated at 0.5846 (n=49), two-tailed p<0.0001 using Spearman’s rank
correlation analysis. B. *Grem1* mRNA levels were plotted against albumin creatinine ratio (ACR) values for wild-type and *grem1*+/- mice. The cohort of control and diabetic mice of both genotypes at 18 and 27 wk of diabetes were plotted (n=30). Spearman rank correlation analysis revealed an $R^2$ value of 0.5704 with a two-tailed $p$ value < 0.0001. (B). *Grem1* mRNA values were plotted against mean glomerular basement membrane (GBM) thickness for control and diabetic wild-type and *grem1*+/- mice at 27 wk of diabetes (n=17). $R^2$=0.533, two-tailed $p$-value<0.001, using Spearman’s rank correlation analysis.

**Figure 7. Upregulation of gene markers of diabetic nephropathy is attenuated in *grem1*+/- mice.** Total RNA was extracted from renal poles of control (“C”) and diabetic (“D”) wild-type (open bars) and *grem1*+/- (filled bars) mice at 27 wk of diabetes. Quantitative TaqMan PCR was performed using mouse fibronectin (A), vimentin (B) or connective tissue growth factor (CTGF, C) specific oligonucleotides as described. ∆∆Ct values were calculated by subtracting the Ct values for the 18s control from the corresponding test gene value performed in the same tube, and altered mRNA levels were then calculated by setting the control in each age-group to 1. Data are plotted as mean +/-SEM. * $p$<0.05, Student’s unpaired t-test, n=4-6 for each group. Increases in vimentin in wild-type diabetic kidney just failed to reach significance (p=0.079).

**Figure 8. Decreased pSmad1/5/8 phosphorylation is evident in wild-type, but not *grem1*+/- diabetic kidney.** A. Protein extracts from renal poles of control (“C”) and 27 wk diabetic (“D”) wild-type and *grem1*+/- mice were probed via Western blot using phospho-Smad1/5/8, total Smad1/5/8 and β-actin antibodies as described (n=3 mice per group). D. Densitometry was performed using Scion Image software. pSmad1/5/8 intensities were normalised to total Smad1/5/8 levels and plotted as relative intensity. * $p$<0.05 using Student’s unpaired t-test.
Diabetic nephropathy in grem1+/- mice

Figure 1.

A. 

B.

C.

Roxburgh et al., 2009
Figure 2.

A.

B.

Roxburgh et al., 2009
Figure 2.

C.  

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\[\text{grem1}\]

\[\beta\text{-actin}\]

D.  

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Roxburgh et al., 2009
Diabetic nephropathy in grem1+/− mice

Figure 3. A. Control Diabetic Roxburgh et al., 2009

Wild-type

Grem1+/−

B. C. Mean glomerular basement membrane thickness (nm)

Wild-type

Grem1+/−

C.

GBM thickening (diabetic/control)

Wild-type Grem1+/−
Diabetic nephropathy in grem1+/− mice

Figure 4.
A. Control Diabetic
   Wild-type grem1+/−
   Interstitial collagen

B. Interstitial collagen score (arbitrary units)
   wild-type grem1+/−
   C D C D

C. Control Diabetic
   Glomerular matrix

D. Glomerular matrix score (arbitrary units)
   wild-type grem1+/−
   C D C D

Roxburgh et al., 2009
Diabetic nephropathy in greml1+/- mice

Figure 5.

A.

![Graph A]

B.

![Graph B]

Roxburgh et al., 2009
Diabetic nephropathy in grem1+/- mice

Figure 5.

C.

**Albunin/Creatinine ratio (ug/mg)**

![Graph C showing Albunin/Creatinine ratio over time and conditions.

D.

**Albunin/Creatinine ratio (fold change)**

![Graph D showing Albunin/Creatinine ratio fold change over time and conditions.

E.

**Creatinine clearance (umol/min/g body weight)**

![Graph E showing Creatinine clearance over time and conditions.

F.

**Creatinine clearance (fold change)**

![Graph F showing Creatinine clearance fold change over time and conditions.

Roxburgh et al., 2009
Figure 6. A. 

Roxburgh et al., 2009

24 hr Microalbuminuria (μg)

ACR (μg microalbumin/mg creatinine)

$R^2 = 0.5826$

B.

Relative gremlin 1 mRNA levels (arbitrary units)

ACR (mg microalbumin/mg creatinine)

$R^2 = 0.5704$

C.

Relative gremlin 1 mRNA levels (arbitrary units)

Glomerular basement membrane thickness (nm)

$R^2 = 0.5330$
Figure 7.

A. Fibronectin

B. Vimentin

C. CTGF

Roxburgh et al., 2009
Figure 8.

A. 

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B. 

Graph showing pSmad1/5/8/total Smad intensity (arbitrary units) for wild-type and gremlin1+/- mice.

Roxburgh et al., 2009