

High *Elmo1* expression aggravates and low *Elmo1* expression prevents diabetic nephropathy

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Contributed by Oliver Smithies, January 14, 2016 (sent for review December 29, 2015; reviewed by Roberto Ariel Gomez and Masaomi Nangaku)

Human genome-wide association studies have demonstrated that polymorphisms in the engulfment and cell motility protein 1 gene (*ELMO1*) are strongly associated with susceptibility to diabetic nephropathy. However, proof of causation is lacking. To test whether modest changes in its expression alter the severity of the renal phenotype in diabetic mice, we have generated mice that are type 1 diabetic because they have the *Ins2^{Akita}* gene, and also have genetically graded expression of *Elmo1* in all tissues ranging in five steps from ~30% to ~200% normal. We here show that the *Elmo1* hypermorphs have albuminuria, glomerulosclerosis, and changes in the ultrastructure of the glomerular basement membrane that increase in severity in parallel with the expression of *Elmo1*. Progressive changes in renal mRNA expression of transforming growth factor β 1 (TGF β 1), endothelin-1, and NAD(P)H oxidase 4 also occur in parallel with *Elmo1*, as do the plasma levels of cystatin C, lipid peroxides, and TGF β 1, and erythrocyte levels of reduced glutathione. In contrast, Akita type 1 diabetic mice with below-normal *Elmo1* expression have reduced expression of these various factors and less severe diabetic complications. Remarkably, the reduced *Elmo1* expression in the 30% hypomorphs almost abolishes the pathological features of diabetic nephropathy, although it does not affect the hyperglycemia caused by the Akita mutation. Thus, *ELMO1* plays an important role in the development of type 1 diabetic nephropathy, and its inhibition could be a promising option for slowing or preventing progression of the condition to end-stage renal disease.

reactive oxygen species | 3'-untranslated region | fibrosis

Diabetic nephropathy is the leading cause of end-stage renal diseases in developed countries (1). Although the control of blood glucose levels remains the mainstay to prevent diabetic nephropathy, cumulative epidemiological studies have also provided evidence that genetic factors partly account for the severity of the disease (2, 3), and human genome-wide association studies have reported several candidate genes conferring susceptibility or resistance to diabetic nephropathy.

The gene coding for engulfment and cell motility protein 1 (*ELMO1*), first discovered as a gene required for phagocytosis of apoptotic cells and cell motility (4), is a strong candidate, but proof of causation is lacking. In a genome-wide case control association study in Japan, over 80,000 single-nucleotide polymorphism (SNP) loci were tested and one SNP locus in the 18th intron of the *ELMO1* gene was found to be strongly associated with nephropathy due to type 2 diabetes ($\chi^2 = 19.9$; $P = 0.000008$; odds ratio, 2.7) (5). Later studies have demonstrated an association of SNPs in the *ELMO1* gene and the susceptibility to type 1 diabetic nephropathy in Caucasians (6, 7), Pima Indians (8), African Americans (9), and Chinese population (10), although some other studies have reported that the association is not significant (11, 12).

To determine whether modest genetic changes in the levels of *Elmo1* expression cause differences in the severity of diabetic nephropathy that occurs in type 1 diabetes, we have used our previously described method (13) to generate mice having five genetically graded levels of *Elmo1* mRNA expression ranging from ~30% to ~200% normal, and have studied their phenotypes after making them type 1 diabetic by crossbreeding them with

mice carrying the dominant Akita mutation in the insulin 2 gene (*Ins2^{Akita}*).

Here, we show that the severity of renal fibrosis and the amount of urinary albumin excretion in the Akita diabetic mice parallels the genetic levels of *Elmo1*. The indices of reactive oxygen species (ROS) also increase as the expression of *Elmo1* increases, although plasma glucose levels and blood pressure do not differ significantly among the mice with the five *Elmo1* genetic levels. These results indicate that *ELMO1* plays an important role in the development of diabetic nephropathy, probably by increasing oxidative stress.

Generation of Akita Diabetic Mice Having Five Genetically Different Levels of *Elmo1*

We first used our published method (13, 14) to generate mice having an *Elmo1* allele in which the 3'-untranslated region (3'-UTR) of the gene is replaced with that of the *cFos* gene (to form a low-expressing L allele) or with that of bovine growth hormone gene, *bGH* (to form a high-expressing H allele) (Fig. 1A). Comparison of the effects of these 3'-UTRs on gene expression shows that the stability of an mRNA having the natural *Elmo1* 3'-UTR is intermediate between that of an mRNA having the *Fos* 3'-UTR and that of an mRNA having the *bGH* 3'-UTR (Fig. 1B and C).

Mice having the five combinations of the low (L), WT (+), and high (H) alleles (*Elmo1^{L/L}*, *Elmo1^{L/+}*, *Elmo1^{+/+}*, *Elmo1^{H/+}*, and *Elmo1^{H/H}*) were bred and at age 40 wk have *Elmo1* mRNA expression graded in five steps from ~30% to ~200% of normal (Fig. 1D, Left).

Significance

About one-third of patients with type 1 diabetes mellitus develop nephropathy, which often progresses to end-stage renal diseases. The present study demonstrates that below-normal *Elmo1* expression in mice ameliorates the albuminuria and glomerular histological changes resulting from long-standing type 1 diabetes, whereas above-normal *Elmo1* expression makes both worse. Increasing *Elmo1* expression leads to aggravation of oxidative stress markers and enhances the expression of fibrogenic genes. Suppressing *Elmo1* action in human patients could be a promising option for treating/preventing the progressive deterioration of renal function in diabetes.

Author contributions: C.K.H., J.C.J., and M.K. designed research; C.K.H., A.S.C., R.G., H.-S.K., V.J.M., C.R.B., and M.K. performed research; H.-S.K. and M.K. analyzed data; and O.S. and M.K. wrote the paper.

Reviewers: R.A.G., University of Virginia; and M.N., University of Tokyo School of Medicine.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1600511113/-DCSupplemental.

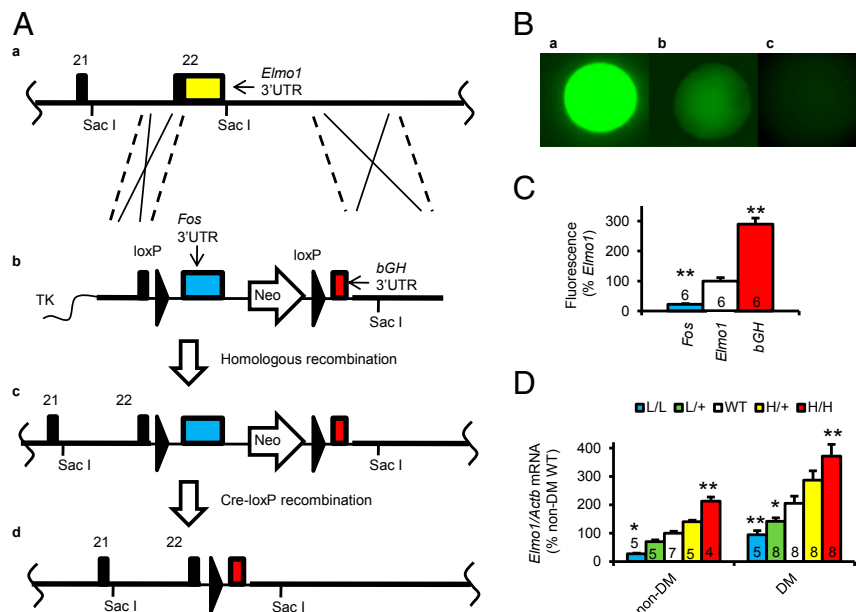


Fig. 1. Generation of Akita diabetic mice having five graded expression levels of *Elmo1*. (A) The gene-targeting strategy. (a) The target locus, *Elmo1*, into which an exogenous 3'-untranslated region (3'-UTR) is introduced by homologous recombination. Coding sequences and the endogenous 3'-UTR of the *Elmo1* gene are shown as black and yellow columns, respectively. (b) The targeting vector contains a loxP sequence, the *c-Fos* 3'-UTR (blue), a Neo gene with the MC1 promoter (pMC1), loxP, and the 3'-UTR of the bovine growth hormone gene (*bGH* 3'-UTR; red). TK, thymidine kinase gene. (c) The resulting locus after homologous recombination; the *Elmo1* gene is now in its low-expression form (*Elmo1^L*) because the stability of its mRNA is now controlled by the destabilizing 3'-UTR of *Fos*. (d) The resulting locus after Cre-loxP recombination; the *Elmo1* gene is now in its high-expression form (*Elmo1^H*) because the stability of its mRNA is now controlled by the stabilizing 3'-UTR of *bGH*. (B) In vitro comparison of the effects of 3'-UTRs and their G-rich elements (GREs) on expression of a green fluorescent protein gene. Fluorescence of colonies with the 3'-UTRs and GREs of *bGH*, *Elmo1*, and *Fos* is shown. (C) Mean fluorescence levels of the single cells with the 3'-UTR and GRE of *bGH*, *Elmo1*, and *Fos* from different colonies. * $P < 0.05$, ** $P < 0.01$ vs. *Elmo1*. (D) The mRNA levels of *Elmo1* in the kidney of the nondiabetic and Akita diabetic mice having five graded expression levels of *Elmo1* at age 40 wk. * $P < 0.05$, ** $P < 0.01$ vs. *Elmo1* WT genotype.

We then crossbred these mice with mice having the Akita mutation in insulin 2 gene (*Ins2^{Akita/+}*), to generate male Akita mice with the following genotypes—*Elmo1^{L/L}:Ins2^{Akita/+}* (L/L Akita), *Elmo1^{L/+}:Ins2^{Akita/+}* (L/+ Akita), *Elmo1^{+/+}:Ins2^{Akita/+}*

(Akita), *Elmo1^{H/+}:Ins2^{Akita/+}* (H/+ Akita), and *Elmo1^{H/H}:Ins2^{Akita/+}* (H/H Akita). Fig. 1D, Right, shows that these diabetic mice, like their nondiabetic counterparts, have *Elmo1* mRNA expression graded in five steps, still ranging from genetically increased levels of

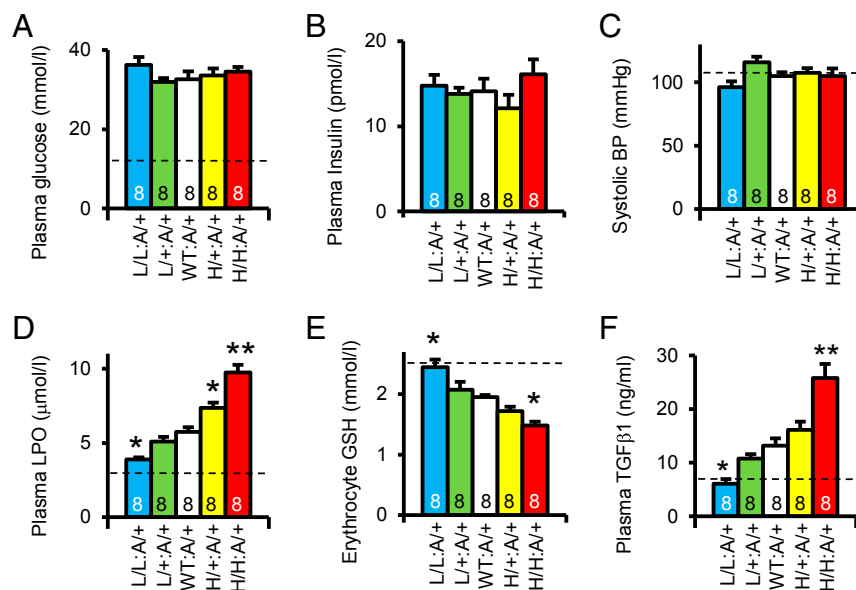


Fig. 2. Systemic parameters affecting diabetic nephropathy in Akita mice with five graded expressions of *Elmo1* at age 40 wk. Dotted lines indicate nondiabetic WT levels. * $P < 0.05$, ** $P < 0.01$ vs. *Elmo1* WT. (A) Plasma glucose levels. (B) Plasma insulin levels. (C) Systolic blood pressure (BP) determined with a tail-cuff method. (D) Plasma levels of lipid peroxide (LPO). (E) Erythrocyte levels of reduced glutathione (GSH). (F) Plasma levels of transforming growth factor β 1 (TGF β 1).

Elmo1 to ~200% relative to diabetic WT *Elmo1* mice but at an approximately two times higher level than nondiabetic WT *Elmo1* mice.

Systemic Parameters Affecting Diabetic Nephropathy in Akita Diabetic Mice with Five Graded Expressions of *Elmo1*

Plasma glucose and plasma insulin levels are not significantly different among the Akita diabetic mice with the five *Elmo1* genotypes (Fig. 2*A* and *B*), nor are their arterial pressures different (Fig. 2*C*). However, plasma lipid peroxides (a marker of

oxidative stress) are progressively increased (Fig. 2*D*), and the levels of reduced form of glutathione in erythrocytes is reduced (Fig. 2*E*) as the expression of *Elmo1* increases in the diabetic mice. Plasma levels of transforming growth factor β 1 (TGF β 1) also increase as the expression of *Elmo1* increases in the diabetic mice (Fig. 2*F*). These results demonstrate that increased expression of *Elmo1* does not affect plasma glucose levels and blood pressure in the diabetic mice, but that it progressively enhances oxidative stress and increases the plasma levels of the fibrogenic cytokine TGF β 1.

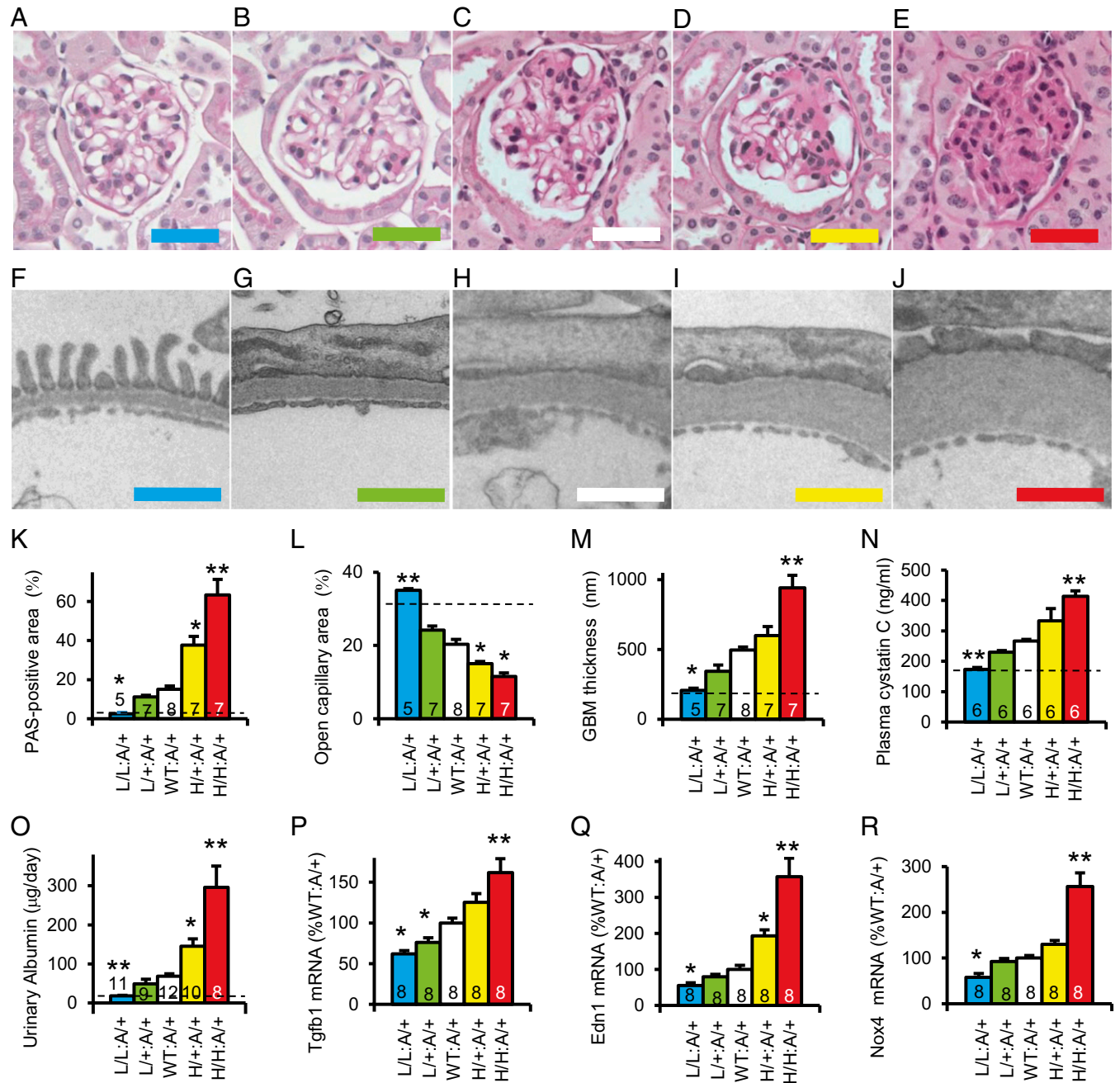


Fig. 3. Renal pathology in the Akita diabetic mice having five graded expression levels of *Elmo1* at 40 wk of age. Dotted lines indicate nondiabetic WT levels. * $P < 0.05$, ** $P < 0.01$ vs. *Elmo1* WT. (A–E) Periodic acid–Schiff (PAS) staining with hematoxylin of the glomerulus in 40-wk-old male mice. (Scale bar: 50 μ m.) (F–J) Transmission electron microscopy of the glomerular basement membrane in 40-wk-old male mice. (Scale bar: 1 μ m.) (A and F) L/L Akita mouse. (B and G) L/+ Akita mouse. (C and H) WT Akita mice. (D and I) H/+ Akita mouse. (E and J) H/H Akita mouse. (K) Quantification of PAS-positive mesangial material per total glomerular tuft cross-sectional area. (L) Percentage of open capillary area per glomerular tuft. (M) Thickness of glomerular basement membrane (GBM). (N) Plasma levels of cystatin C. (O) Urinary albumin excretion. (P) Renal expression of *Tgfb1*. (Q) Renal expression of *Edn1*. (R) Renal expression of *Nox4*.

Overexpression of *Elmo1* Increases and Underexpression of *Elmo1* Decreases Renal Histological Changes, Renal Excretory Function, Urinary Excretion of Albumin, and the Renal Expression of Fibrogenic Genes in Akita Diabetic Mice

Microscopic studies of the glomeruli of the diabetic mice (Fig. 3 A–E) showed that the diabetic mice with wild-type *Elmo1* alleles (WT:A/+) at age 40 wk had pathological changes in their glomeruli typical of diabetic nephropathy, including mesangial cell proliferation and accumulation of periodic acid–Schiff (PAS)-positive materials (Fig. 3C). These pathological changes were progressively exacerbated when expression of *Elmo1* increased above normal in the H/+A/+ and H/H:A/+ Akita diabetic mice (Fig. 3 D and E). In marked contrast, the pathological changes were completely absent in the Akita mice with the lowest expression of *Elmo1* (L/L:A/+; Fig. 3A), and were still reduced in the L/+A/+ mice (Fig. 3B) relative to those in the diabetic mice with wild-type *Elmo1* (Fig. 3C). We quantitated the renal pathology in the five *Elmo1* genotypes by measuring the fraction of PAS-positive area per glomerular tuft area, an indicator of mesangial expansion, and found that PAS-positive material increased more than 10-fold in the Akita diabetic animals as *Elmo1* expression increased (Fig. 3K). The open capillary area in the L/L:A/+ hypomorphs decreased from almost twice that in the WT:A/+ diabetic mice to less than one-half in the H/H:A/+ diabetic mice (Fig. 3L).

The pathology revealed by light microscopy was further evaluated by electron microscopy (Fig. 3 F–J). The results confirmed that, at age 40 wk, the Akita mice with wild-type *Elmo1* alleles (WT:A/+) had ultrastructural changes typical of advanced diabetic nephropathy, including a severalfold increase in the thickness of the glomerular basement membrane (GBM) together with marked podocyte effacement (Fig. 3H). The GBM thickening (quantitated in Fig. 3M) was progressively exacerbated in the H/+A/+ and H/H:A/+ mice with above-normal expression of *Elmo1* (Fig. 3 I and J). The ultrastructure of the glomeruli in the L/L:A/+ mice was indistinguishable from that in wild-type nondiabetic C57BL/6 mouse (Fig. 3 F and M). Podocyte effacement was present in the glomeruli of all of the diabetic mice except the L/L:A/+ mice with one-third normal *Elmo1* expression. We did not observe any obvious lesions in the renal vasculature other than in the glomeruli of the Akita mice. We conclude that the nephropathy observed in the mature Akita mice with type 1 diabetes is strongly affected by the expression of *Elmo1*, ranging from almost the same as in nondiabetic *Elmo1* WT mice to severe diabetic nephropathy.

We studied kidney function by measuring plasma cystatin C and found that the cystatin C levels increase as the expression of *Elmo1* is increased in Akita mice (Fig. 3N). Likewise, urinary excretion of albumin was progressively increased when expression of *Elmo1* increased (Fig. 3O).

Renal expression of genes encoding TGF β 1 (*Tgfb1*; Fig. 3P), endothelin-1 (*Edn1*; Fig. 3Q), and NAD(P)H oxidase 4 (*Nox4*; Fig. 3R), which have been demonstrated to be involved in fibrogenesis and epithelial–mesenchymal transition, are progressively increased as the expression of *Elmo1* is increased.

Thus, visual inspection of the light and electron microscope images of the glomeruli together with their quantitative evaluation show that below-normal *Elmo1* expression ameliorates the nephropathy caused by diabetes whereas above-normal expression exacerbates it.

Discussion

In the current study, we have shown that genetically increased levels of *Elmo1* in Akita diabetic mice lead to progressive increases in the concentrations of oxidative stress markers, in the severity of pathological features characteristic of diabetic nephropathy, including glomerular fibrotic changes and urinary

excretion of albumin and the renal expression of fibrogenic genes. In contrast, genetically decreased levels of *Elmo1* lead to less urinary output of albumin, less tissue fibrosis, and less expression of fibrogenic genes. Strikingly, the kidneys of the diabetic mice with ~30% *Elmo1* expression exhibited no pathological changes; their kidneys were the same as those of nondiabetic mice *Elmo1* WT mice. Remarkably the plasma level of lipid peroxides (a measure of oxidative stress) and the erythrocyte level of reduced glutathione in the 30% *Elmo1* diabetic mice are also indistinguishable from that in nondiabetic *Elmo1* WT mice. Plasma glucose levels and blood pressures of the diabetic mice were unaffected by differences in *Elmo1* expression. Thus, our results show that genetic increases in *Elmo1* expression causally aggravate the severity of diabetic nephropathy, whereas reducing expression of *Elmo1* to about one-third is sufficient to prevent any pathological effects of the diabetes.

Elmo1 was first discovered as a factor required for the phagocytosis of dying cells and cell migrations in *Caenorhabditis elegans* (4) that also acts as an upstream regulator of Rac1 in the Ras-related C3 botulinum toxin substrate (Rac) signaling pathway. *Elmo1* is part of the Rac–guanine nucleotide exchange factor (Rac-GEF), which converts inactive Rac-GDP into active Rac-GTP (15). Earlier investigations have shown that expression of *Elmo1* is increased in db/db type 2 diabetic mice relative to its expression in nondiabetic control mice (5). In our present study, we have found that *Elmo1* expression is also elevated in Akita type 1 diabetic mice.

A possible chain of events leading from *Elmo1* to an increase in ROS and to nephropathy is apparent. Thus, in KKA(y) mice, which develop obesity-related diabetic kidney disease, the activity of Rac1 is increased in mesangial cells, and treatment with a pan-Rac inhibitor (EHT1864) mitigates the renal pathology (16). Rac1, Rac2, and Rac3 increase the formation of superoxide anion via activation of NAD(P)H oxidases (17–20), which are encoded in *Nox* genes. Increased production of superoxide by the mitochondrial electron transport chain has been shown to be the causal link between elevated glucose and three major pathways of hyperglycemic damage (activation of protein kinase C, formation of advanced glycation end products, and activation of the polyol pathway) in cultured bovine endothelial cells (21), and there is growing evidence that NAD(P)H oxidase is a critical enzyme for producing ROS (22). Additionally, pharmacological inhibition of NAD(P)H oxidase with apocynin has been shown to reverse renal mesangial matrix expansion in streptozotocin-induced type 1 diabetic mice (23). Consequently, it is likely that increased expression of *Elmo1* enhances the development of diabetic nephropathy by activating Rac and increasing NAD(P)H oxidase, which in turn increases ROS.

A possible chain of events between increases in *Elmo1* expression and glomerulosclerosis is also apparent. Thus, pharmacological inhibition of different ROS sources, including NAD(P)H oxidase and the mitochondrial respiratory chain, decreases the transcription of *Tgfb1* via reduced activity of activated protein 1 (24), indicating that ROS-induced enhancement of the transcription of *Tgfb1* may at least in part account for the increase in *Tgfb1* expression in diabetes. In vitro overexpression of *Elmo1* in cultured COS cells increases the expression of *Tgfb1* and its downstream extracellular matrix genes, including fibronectin, collagen 1A1 (5), and integrin-linked kinase (5). Consequently, it is likely that the overexpression of *Elmo1* in diabetes increases *Tgfb1* expression and enhances the development of nephropathy via its effects on ROS formation.

In summary, we have studied the renal phenotypes of Akita type 1 diabetic male mice having five different genetically determined levels of *Elmo1* expression and find that *Elmo1* expression levels positively correlate with glomerular fibrosis and urinary excretion of albumin, although no significant differences in plasma glucose or blood pressure are seen among the five

genotypes. Most notable is our finding that the nephropathic effects of diabetes are completely prevented if *Elmo1* expression is genetically decreased to one-third, even though the diabetic hyperglycemia persists. Thus, *Elmo1* plays an important pathophysiological role in the development of albuminuria and the tissue fibrotic changes characteristic of diabetic nephropathy. The remarkable effects of modestly decreasing its levels genetically in mice suggest that pharmacologically suppressing *Elmo1* action in the kidney could be a promising option for preventing the progression of early stages of nephropathy to end-stage renal disease in patients with type 1 diabetes.

Materials and Methods

Animals. Mice having five graded expression levels of *Elmo1* were generated by targeted replacement of the 3'-UTR of *Elmo1* with unstable Fos 3'-UTR or with stable bGH 3'-UTR, as previously described (13).

To study the effects of *Elmo1* on the phenotype in diabetes, heterozygous and homozygous mice having hypomorphic (L) or hypermorphic (H) alleles for *Elmo1* generated on a C57BL/6 genetic background (Ingenious Targeting Laboratory) were crossbred with mice heterozygous for the Akita mutation in the insulin 2 gene on a C57BL/6 genetic background (The Jackson Laboratory).

All mice were kept under husbandry conditions conforming to the guidelines of the University of North Carolina (UNC) Institutional Animal Care and Use Committee.

Measurement of Biological Parameters. Plasma glucose levels were determined with the glucose oxidase method (Wako Chemical). Plasma insulin levels were determined with ELISA (Crystal Chem). Plasma TGF β 1 levels were determined with ELISA (Quantikine Mouse/Rat/Porcine/Canine TGF β 1 Immunoassay; R&D Systems). Plasma cystatin C levels were determined with ELISA (Quantikine Mouse/Rat Cystatin C Immunoassay; R&D Systems). Plasma

lipid peroxide was quantified as thiobarbiturate reactive substances (Cayman Chemical). The reduced form of glutathione (GSH) in erythrocytes was determined with a Glutathione assay kit (EMD Millipore). Metabolic balance studies were performed using metabolic cages (Solo Mouse Metabolic Cage; Tecniplast).

Histology. After cutting the inferior vena cava, the left ventricle was punctured with a 23-gauge needle and perfused with PBS for 3 min and with 4% (wt/vol) paraformaldehyde for 5 min. Thereafter, the tissues were dissected out and put in 4% (wt/vol) paraformaldehyde for at least 3 d. They were then paraffin embedded and sectioned. Stained sections were prepared by the Center for Gastrointestinal Biology and Diseases Histology Core at UNC and imaged on an Olympus BX61 microscope. For electron microscopy, grids were prepared by the Microscopy Services Laboratory at UNC and imaged on a Zeiss TEM 910 transmission electron microscope.

Blood Pressure and Pulse Rate Measurement. Blood pressure and pulse rate were measured with a tail-cuff method (25).

Quantitative Reverse Transcription-PCR. Total RNA was extracted from different tissues, and the mRNAs were assayed by quantitative reverse transcription-PCR as previously described (26). The primers and probes used to measure the mRNAs are shown in Table S1.

Statistical Analysis. Data are expressed as means \pm SEs. To compare groups, we used one-factor ANOVA. Post hoc pairwise comparisons were performed by Tukey-Kramer honestly significant difference test (JMP 8.0; SAS Institute).

ACKNOWLEDGMENTS. This work was supported by NIH Grants HL49277, HL70523, and HL71266, and by Career Development Award 2006-102 from Juvenile Diabetes Research Foundation.

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