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Research Article

The Ca2+/calmodulin-dependent protein kinase IIα (Thr286Asp) transgenic mice: a novel mouse model of severe insulin-dependent diabetes

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Abstract

Diabetes mellitus is the leading cause of blindness and end-stage renal disease. To understand the pathogenesis of diabetic complications, suitable animal models for this disease have been needed. The activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in pancreatic β-cells has been thought to play a central role in Ca^{2+}-mediated insulin secretion. We generated transgenic mice over expressing the constitutively active-type CaMKIIα (Thr286Asp) in β-cells, which showed very high plasma glucose levels and exhibited the features of diabetic nephropathy and retinopathy. In cDNA microarray analysis osteopontin mRNA increased in CaMKIIα transgenic mice. In quantitative real-time RT-PCR analyses, not only M1 macrophage marker genes but also M2 macrophage marker genes were over expressed in renal cortex of CaMKIIα transgenic mice. The mice were crossed with conditional knockout mice in which platelet-derived growth factor receptor-β gene (*Pdgfr-β*) was deleted postnatal. The increased oxidative stress in the kidneys of the CaMKII α transgenic mice, which was shown by the increased urinary 8-hydroxydeoxyguanosine excretion and the increased expression of NAD (P) H oxidase 4, was decreased by *Pdgfr-β* deletion. The CaMKIIα (Thr286Asp) transgenic mice will be valuable as a novel model of severe insulin-dependent diabetes accompanied by an early progression of diabetic micro vascular complications.

Abbreviations

ATP: Adenosine triphosphate; cADPR: Cyclic adenosine diphosphate-ribose; CaM: Calmodulin; CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II; CCR2: CCmotif receptor 2; CHI3l3: Chitinase 3-like 3; DM: Diabetes mellitus; ERG: Electroretinography; IL-1β: Interleukin-1β; IP_3 : Inositol 1,4,5-trisphosphate; MCP-1: Monocyte chemotactic protein-1; MGL2: Macrophage galactose *N*-acetyl-galactosamine specific lectin 2; MRC1: Mannose receptor C-type1; NAD^+: Nicotinamide adenine dinucleotide; NOS2: Nitric oxide synthase 2; NOX4: NAD(P)H oxidase 4; 8-OHdG: 8-hydroxydeoxyguanosine; PDGF: Platelet-derived growth factor; PDGFRs: Platelet-derived growth factor receptors; RyR: Ryanodine receptor; TG: Transgenic; TNFα: Tumor necrosis factor α; WT: Wild type.

Introduction and Background

Diabetes mellitus (DM) is a disease characterized by hyperglycemia and is caused by absolute or relative insulin deficiency, sometimes associated with insulin resistance [1]. As a consequence of its micro vascular pathology, DM is the leading cause of blindness, end-stage renal disease, and a variety of neuropathies [2]. Approximately 30% of type 1 DM patients suffered from diabetic nephropathy eventually undergo renal dialysis or transplantation [3]. Nephropathy is thus a life-threatening complication of DM and is the leading cause of end-stage renal disease in developed countries. The features of diabetic nephropathy include persistent albuminuria, a progressive decline in renal function, and histopathologically mesangial expansion followed by glomerulosclerosis [4]. However, the molecular mechanisms leading to end-stage renal disease in DM have not been fully understood.

Analysis and Interpretation

Mechanism of Insulin Secretion

Cyclic ADP-Ribose in Insulin Secretion

Mobilization of Ca^{2+} from intracellular stores in the endoplasmic reticulum is required for insulin secretion from pancreatic β-cells. Inositol 1,4,5-trisphosphate (IP_3) is thought to be a second messenger for intracellular calcium mobilization, while in islet microsomes cyclic adenosine diphosphate-ribose (cADPR) induces mobilization of Ca^{2+}. In the process of glucose metabolism, adenosine triphosphate (ATP) is generated. ATP induces cADPR formation from nicotinamide adenine dinucleotide (NAD^+)by inhibiting the cADPR hydrolase activity of CD38.CD38 has enzymic activites of both cADPR synthesis from NAD^+(ADP-ribosyl cyclase activity) and cADPR hydrolysis to produce ADP-ribose (cADPR hydrolase activity) [5]. cADPR functions as a second messenger for Ca^{2+} mobilization from

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endoplasmic reticulum for glucose-induced insulin secretion from pancreatic β-cells [6].

cADPR Requires Calmodulin-Dependent Protein Kinase II For Intracellular Ca^{2+} Mobilization

In sea urchin eggs, calmodulin (CaM) directly interacts with the ryanodine receptor (RyR) to enhance the cADPR-mediated Ca^{2+} release [7]. In rat islets, CaM sensitized and activated the cADPR-mediated Ca^{2+} release from islet microsomes. It is reported that cADPR-mediated Ca^{2+} mobilization for insulin secretion is achieved by the activated Ca^{2+}/calmodulin-dependent protein kinaseII (CaMKII) not by the direct interaction of CaM and Ca^{2+} release [8].

Glucose stimulation to pancreatic β cell induces cADPR accumulation in islets by inhibiting cADPR hydrolase activity of CD38 by ATP. Glucose stimulation also activates CaMKII, and the activated kinase phosphorylates RyR to sensitize the Ca^{2+} channel for the cADPR signal.As shown in Figure 1, cADPR acts as a second messenger for intracellular Ca^{2+} mobilization via RyR, and induces insulin secretion [8-10]. Thus, CaMKII is suggested to be essential kinase for glucose–induced insulin secretion.

A Novel Mouse Model of Diabetic Nephropathy

Mouse Model of Diabetic Nephropathy

Spontaneously diabetic animals such as no obese diabetic mice developed only limited lesions of diabetic nephropathy such as mild mesangial sclerosis [11]. The same was the case with chemically induced diabetic rodents such as streptozotocin-induced diabetic model [12]. To understand the pathogenesis of diabetic nephropathy and to develop preventive and therapeutic methods against it, suitable animal models for this disease have been needed.

A Novel Model of Severe Insulin-Dependent Diabetes

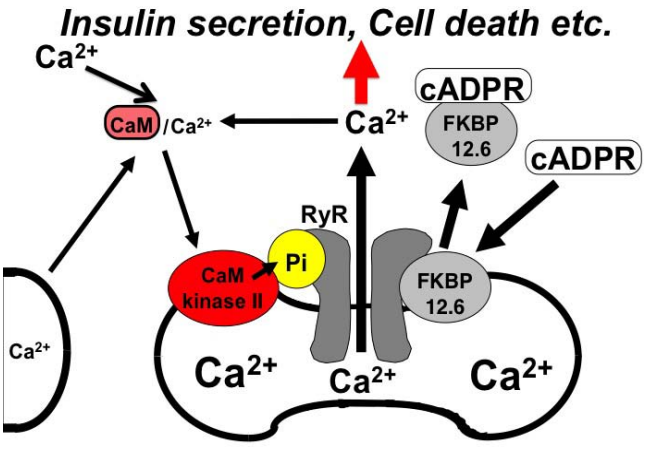

Figure 1: Possible involvement of CaMKII in cADPR mediated intracellular Ca^{2+} mobilization (adopted from [8]). cADPR binds to FK506 binding protein 12.6 (FKBP12.6) to release Ca^{2+} dissociating FKBP12.6 from RyR. CaMKII phosphorylates RyR to sensitize and activate the Ca^{2+} channel (Pi, phosphorylation of RyR by CaMKII). Ca^{2+}, released from intracellular stores and/or supplied from extracellular sources, further activates CaMKII and amplifies the process. In this way, Ca^{2+}-induced Ca^{2+} release can be explained. Increased Ca^{2+} activates many physiological/pathological events such as glucose-induced insulin secretion and cell death.

Recently we generated transgenic (TG) mice over expressing the mutant form (Thr286Asp) of CaMKIIα in pancreatic β-cells [13]. Western blot and immunehistochemical analyses showed that CaMKIIα protein was over expressed in pancreatic β-cells of the TG mice. Cell proliferation in the TG islets was severely impaired as assessed by *in vivo* BrdU labeling analysis. NF-κB accumulated in nuclei of the TGβ-cells at postnatal day (P) 21, which was associated with DNA laddering. The pancreatic insulin content was already significantly (p<0.01) lower in the TG mice than in the wild type (WT) mice at P7. The pancreatic insulin positive areas became negligible in the TG mice at P28 (Figures 2a-2c)). Serum insulin levels in the TG mice also decreased progressively along with aging, and were scarcely detectable at P28 (Figure 2d).

One hundred percent of TG mice developed severe hypoinsulinemic diabetes by P28. Blood glucose levels were significantly (p< 0.001) higher in the TG mice (44.4± 4.4mmol/l, n = 13) than in WT mice (8.9 ± 0.7mmol/l, n = 13) (Figure 3a).

Hemoglobin A1C levels were also significantly (p< 0.001) higher in the TG mice (9.4 ± 0.61%, n = 8) than in the WT mice (3.4 ± 0.08%, n = 13), suggesting that the TG mice will be valuable as a novel model of severe insulin-dependent DM.

Diabetic Nephropathy and Retinopathy in Cam Kinase Iiα TG Mice

The TG mice at P140-P168 developed severe renal and retinal lesions. Urinary albumin/ creatinine ratio (Figure 3b) as well as blood urea nitrogen (BUN) level were significantly elevated in the TG mice, indicating that the TG renal function is impaired. Histological analyses of kidneys revealed that body weight/kidney weight ratio, glomerular area, mesangial area, and mesangial/glomerular area ratio all increased in the TG mice (Figures 3c and 3d). The microscopic lesions noted by Periodic acid -Schiff (PAS) staining in the TG kidneys also consisted of glomerular hypertrophy, mesangial cell proliferation, mesangial expansion, and glomerulosclerosis (Figures 3e and 3f). Retinas of the TG mice demonstrated a dramatic loss of neuronal nuclei (NeuN)-positive nuclei, indicating a massive disruption of ganglion cells. Moreover, disorganization of the inner nuclear layer was evident in the TG retina. The corneal electro retinography (ERG) for maximal responses revealed waves each typical to a-waves, oscillatory potentials and b-waves for WT mice, but no discernible waves for the TG mice (Figures 3g and 3h).Thus, the TG mice were considered a good model of diabetic complications.

Diabetic Nephropathy and Inflammation

Macrophage infiltration has been reported to increase significantly in diabetic kidneys and thought to play significant roles in the progression of diabetic nephropathy [14,15]. There are at least two subtypes of resident macrophages in tissues [16]. One is referred to as M1 macrophages, which are classically activated by Th1 stimuli. M1 macrophages express high levels of proinflammatory cytokines and enhance tissue inflammatory response. Another is M2 macrophages, which are alternatively activated by Th2 stimuli. M2 macrophages express high levels of anti-inflammatory cytokines such as IL-10, and participate in the promotion of tissue repair, remodeling and vasculogenesis.

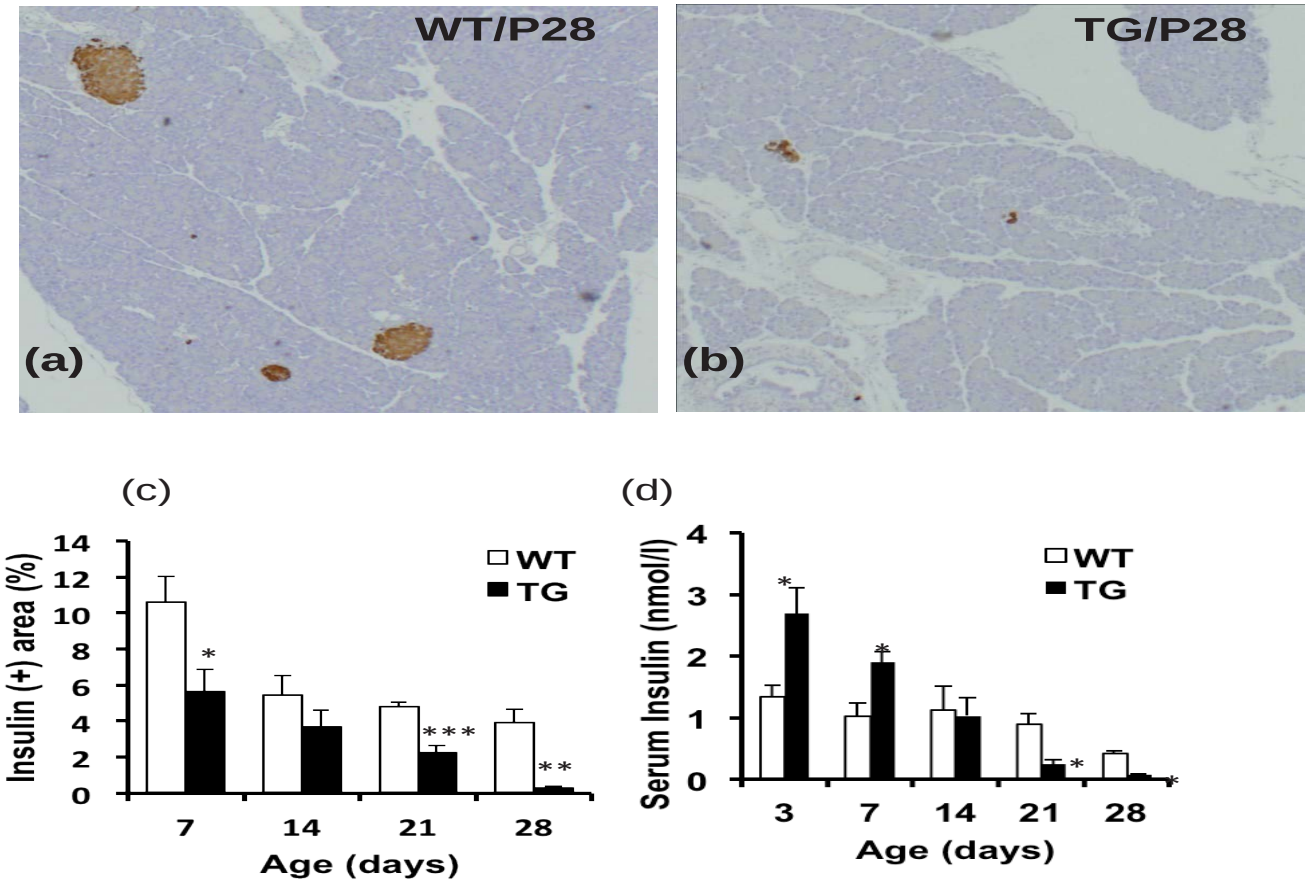

Figure 2: Immunohistochemical detection of insulin-producing pancreatic β cells in the representative sections of pancreas from WT mice (a) and the TG mice over expressing CaMKIIα (Thr286Asp) under the control of *Insulin II* promoter (b) at P28 (adopted from [13]). Percentages of insulin-positive area in whole pancreas section were determined at the indicated ages (c). Levels of insulin in serum in WT mice and the TG mice were determined at the indicated ages (d). The values are the means ± SE of at least four mice per group. *p< 0.05; **p< 0.01; ***p< 0.001 compared with age-matched WT mice by Student's *t*-test (adopted from [13]).

Diabetic Nephropathy and M1/M2 Macrophage

Expression of M1 macrophage markers such as CD11c, chemokine CCmotif receptor 2 (CCR2), tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), monocyte chemotactic protein-1 (MCP-1), and nitric oxide synthase 2 (NOS2) in kidney was then analyzed. Among them, the mRNA levels of TNFα (1.77-fold) and MCP-1 (4.77-fold) were significantly higher ($p < 0.05$ for both) in the TG mice compared to WT mice (Figure 4a). M2 macrophage markers such as mannose receptor C-type1 (MRC1), chitinase 3-like 3 (CHI3l3), macrophage galactose *N*-acetyl-galactosamine specific lectin 2 (MGL2), CD209a, and interleukin-10 (IL-10) were next analyzed. Among them, the mRNA level of CHI3l3 (5.72-fold) was significantly higher ($p < 0.001$) in the TG mice compared to WT mice (Figure 4b). These results indicate that not only M1 macrophages but also M2 macrophages participate in the formation of diabetic nephropathy in the TG mice.

Diabetic Nephropathy and Osteopontin

In order to identify genes whose expression levels were changed in the TG kidney, we carried out cDNA microarray analysis of mouse kidneys at P84. Genes with mean fold changes above 2.00 or less than 0.60 were listed (Table).

The fold changes are mean comparisons between the TG kidney and WT kidney.

Osteopontin mRNA increased 2.35 fold in the TG kidney. Osteopontin is a chemokine-like, extracellular matrix-associated protein with diverse functions [18]. The elevated expression of osteopontin in renal cortex has also been reported in murine models of diabetes such as streptozotocin-induced diabetic rats [19] and *db/db* mice [20]. We performed immunohistochemical detection of osteopontin in the kidneys of TG and WT mice at P140. The glomeruli and the tubular epithelial cells were stained much more strongly for osteopontin in the TG kidney as compared to the WT kidney (Figures 5a and 5b). Osteopontin may recruit macrophages to renal cortex and induce inflammatory immune response.

Platelet Derived Growth Factor in Diabetic Nephropathy

Platelet-derived growth factor (PDGF) is a potent mitogen that stimulates extracellular matrix accumulation in mesangial cells [21]. PDGF family members, PDGF-A, PDGF-B, PDGF-C and PDGF-D, are assembled as disulphide-linked homo- or heterodimers [22]. These signals are mediated by two types of PDGF receptors (PDGFRs),

Table 1: Genes up- or down-regulated in CaMKIIα (Thr286Asp) TG mouse kidney (adopted from [17]).

Gene Name	Function	Gene Bank#	Fold
Up-regulated cyclin D2	cell-cycle	NM_009829	6.70
sodium channel, nonvoltage-gated 1 beta	ion transport	NM_011325	4.40
nectin-lke 1	calcium ion binding	NM_053199	3.40
Notch-regulated ankyrin repeat protein	regulation of transcription	NM_025980	3.30
calcium channel, voltage-dependent, gamma subunit 3	calcium ion transport	NM_019430	3.21
pleckstrin homology domain containing, family B member 1	protein binding	NM_013746	3.18
RAS protein activator like 1	GTPase activator activity	NM_013832	3.12
receptor-interacting serine-threonine kinase 3	IκB kinase/ NFκB cascade	NM_019955	2.99
membrane-spanning 4-domains, subfamily A, member 6D	signal transduction	NM_026835	2.72
granzyme A	apoptosis	NM_010370	2.57
osteopontin	**immune response**	**NM_009263**	**2.35**
guanine nucleotide binding protein, alpha 11	G-protein signaling pathway	NM_010301	2.34
hydroxyacid oxidase 3	fatty acid metabolic process	NM_019545	2.23
lipopolysaccharide binding protein	lipid binding	NM_008489	2.15
interleukin 21 receptor	interleukin receptor activity	NM_021887	2.15
interleukin enhancer binding factor 3	protein amino acid methylation	NM_010561	2.06
catenin delta 2	cell adhesion	NM_008729	2.00
Down-regulated tumor rejection antigen gp96	molecular chaperone	NM_011631	0.59
solute carrier family 21, member 1	renal organic anion transport	NM_013797	0.58
carbonic anhydrase 4	renal bicarbonate reabsorption	NM_007607	0.56
testis specific gene A2	sperm mobility	NM_025290	0.55
pleckstrin	T-cell activation	NM_019549	0.53
hydroxysteroid 11-beta dehydrogenase 1	cortisol metabolism	NM_008288	0.51
lipoprotein lipase	lipid transporter activity	NM_008509	0.473
lymphocyte antigen 6 complex, locus E	GPI anchor binding	NM_008529	0.460
Mpv17 transgene, kidney disease mutant-like	molecular function	NM_033564	0.421
Mouse ornithine decarboxylase	polyamine biosynthesis	NM_013614	0.348
DNA segment, Chr 7, Roswell Park 2 complex, expressed	hydrolase activity	NM_033080	0.332
hemoglobin, beta adult minor chain	iron ion binding	NM_016956	0.205

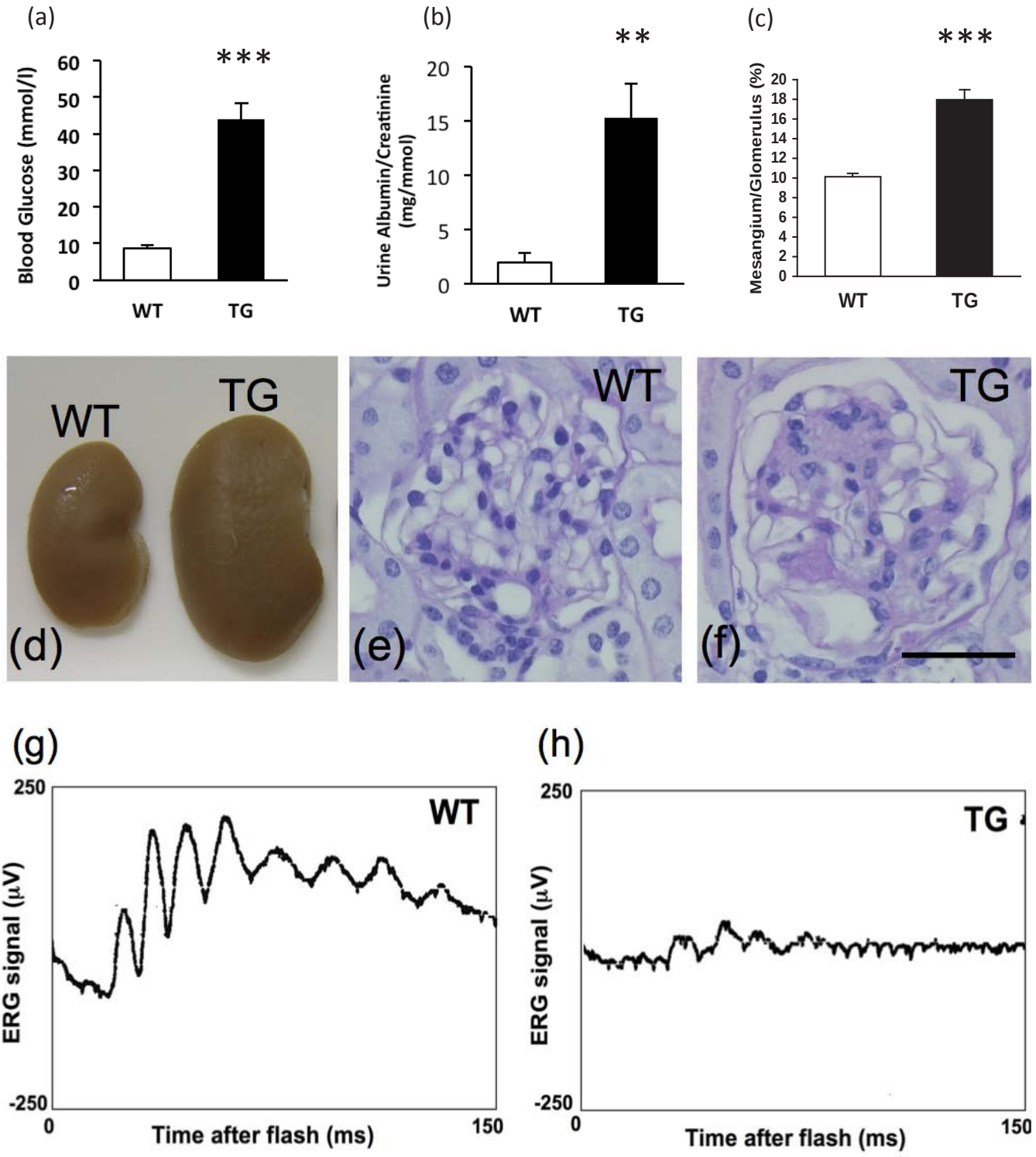

Figure 3: Phenotypic characterization of WT mice and the CaMKIIα (Thr286Asp) TG mice at P140 (adopted from [13, 17]). (a) Blood glucose levels. (b) Urine albumin/creatinine ratio. (c) Mesangial area/ glomerular area. White bars and black bars indicate means ± SE for WT mice and the TG mice, respectively. Statistical analyses were performed by Student's *t*-test. ***p*<0.01; ****p*<0.001 compared with the values for age-matched WT mice. Macroscopic finding of kidneys from WT and the TG mice at P140. (d) PAS-staining of WT kidney. (e) and the TG kidney (f). Scale bar = 50 μm. Representative ERGs for maximal responses in WT mice. (g) and the TG mice. (h) at P140. The ERG signals (μV) were recorded at 0-150 ms after the flash.

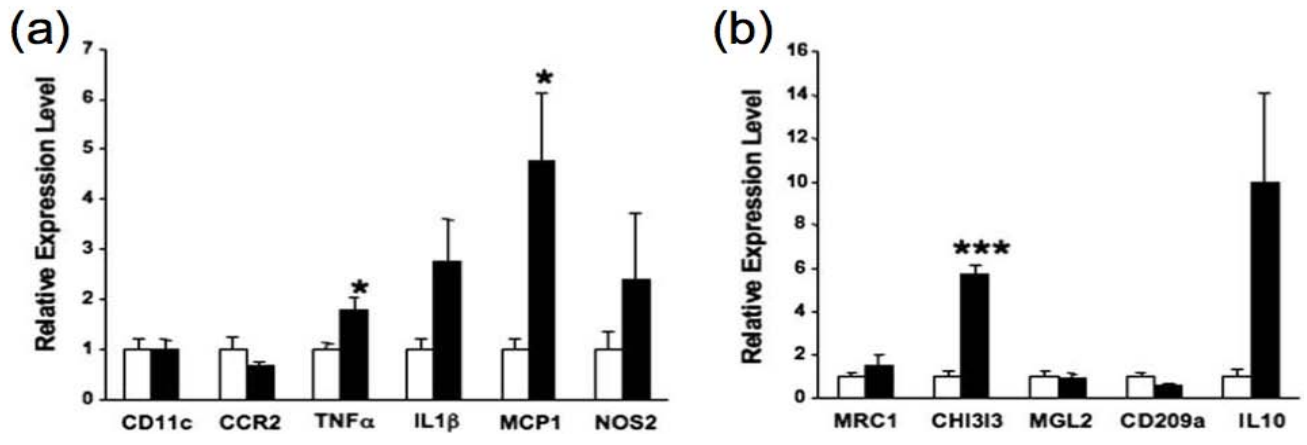

Figure 4: Quantitative real-time PCR analyses of macrophage marker mRNAs (a, b) (adopted from [17]). (a) mRNA levels of M1 macrophage markers (CD11c, CCR2, TNFα, IL1β, MCP1 and NOS2). (b) mRNA levels of M2 macrophage markers (MRC1, CHI3l3, MGL2, CD209a and IL10). In (a, b), the respective mRNA levels normalized to 18S ribosomal RNA (internal control) levels were shown. White bars and black bars indicate means ± SE for WT mice and TG mice, respectively. Statistical analyses were performed by Student's *t*-test. **p*<0.05; ****p*<0.001 compared with the values for age-matched WT mice.

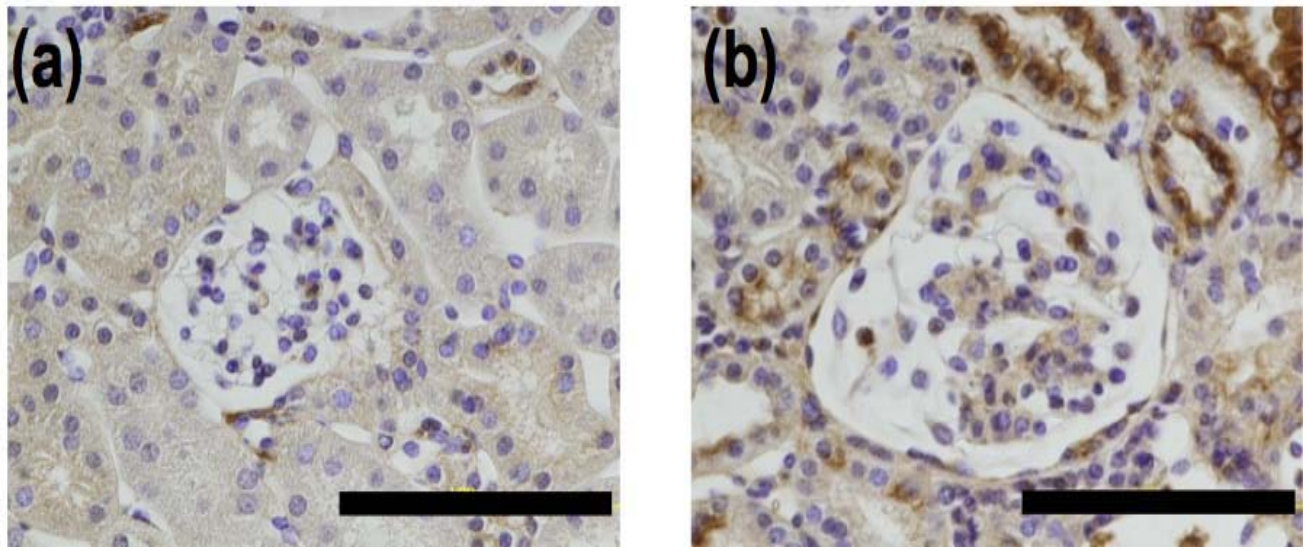

Figure 5: Immunohistochemical detection of osteopontin (a, b) in the kidneys of WT mice (a) and TG mice (b) (adopted from [17]). Images are representative from studies of n=2 mice for each genotype. Scale bars = 100 μm.

PDGFR-α and PDGFR-β.

In the diabetic kidney, up regulation of the PDGF pathway has been shown in experimental diabetic nephropathy [23-25] and in the kidneys of patients with diabetes [26]. Among them, the production of PDGF-B and PDGFR-β is specifically increased and correlates to the progress of glomerular lesions such as diabetic nephropathy [27,28]. The *in vitro* exposure to high glucose also induces PDGF-B production in human proximal tubular epithelial cells and mesangial cells, and PDGFR-β production in mesangial cells [29-31]. A number of specific interventions aimed at neutralizing PDGF-Bor -D or blocking PDGFR-β have been shown to reduce mesangial cell proliferation and matrix accumulation and to ameliorate renal dysfunction in experimentally induced glomerulonephritis [32-35]. In contrast, the effects of anti-PDGF therapy on diabetic nephropathy have not been well characterized so far [36]. To our best knowledge, intervention studies involving PDGF are limited to the work showing that tyrosine kinase inhibition with 4-(4-Methylpiperazin-1-ylmethyl)-*N*- [4-metthyl-3-(4-pyridin-3-ylpyrimidin-2-ylamino) phenyl] benzamide monomethanesulfonate (imatinib), a chemical tyrosine kinase inhibitor, retards the development of diabetic nephropathy in diabetic mice [37].

Camkiiα (Thr286Asp) Transgenic and *Pdgfr-B* Conditional Knockout Mice

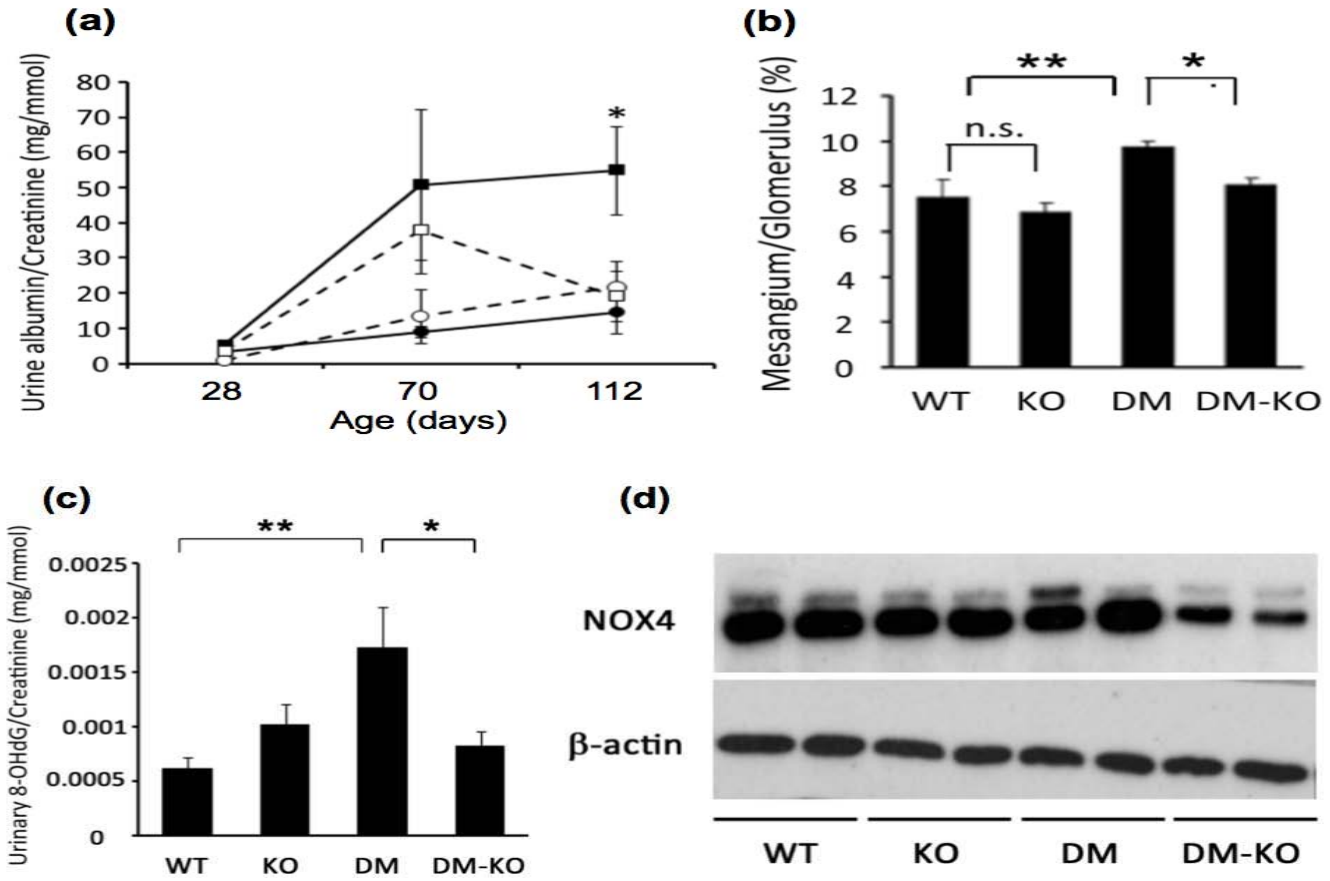

Figure 6: Deletion of *Pdgfr-β* improved diabetic nephropathy in DM mice [39]. (a) Urinary albumin/creatinine ratio at the indicated days of age. (b) Mesangial area/glomerular area ratio at P112. Data shown are the means±SE of at least six mice per group. *p<0.05; **p<0.01 (c) Urinary 8-OHdG levels at P112. Data shown are the means±SE of at least six mice per group. *p<0.05; **p<0.01 (d) Representative images of western blotting of renal cortex with anti-NOX4 and anti-β-action antibodies.*p<0.05; **p<0.01.

Conditional *Pdgfr-β* knockout mice (actin-Cre-ER$^{TM+/-}$-*Pdgfr-β* $^{flox/flox}$ mice), which express a fusion protein consisting of Cre recombinase and a mutated form of the mouse oestrogen receptor ligand-binding domain under the control of the actin promoter, were generated as described previously by Tokunaga et al [38]. By crossing the *CaMKIIα*$^{Tg/+}$ mice and the *actin-Cre-ER*$^{TM+/-}$- *Pdgfr-β* $^{flox/flox}$ mice, *CaMKIIα*$^{Tg/+}$- *actin-Cre-ER*$^{TM+/-}$ *Pdgfr-β* $^{flox/flox}$ mice were generated. We examined the following four mouse strains with a CD-1 background: *CaMKIIα*$^{+/+}$ *-actin -Cre-ER*$^{TM-/-}$ *-Pdgfr-β* $^{flox/flox}$ mice (WT); *CaMKIIα*$^{+/+}$ *-actin -Cre-ER*$^{TM+/-}$ *- Pdgfr-β* $^{flox/flox}$ mice (KO); *CaMKIIα*$^{Tg/+}$ *-actin-Cre-ER*$^{TM-/-}$ *-Pdgfr-β* $^{flox/flox}$ mice (DM); and *CaMKIIα*$^{Tg/+}$ *-actin -Cre-ER*$^{TM+/-}$ *-Pdgfr-β* $^{flox/flox}$ mice (DM-KO). All four strains of mice were similarly treated with tamoxifen at P28. The blood glucose levels of the DM and DM-KO mice increased markedly from P21, and reached a level higher than 44.4mmol/l thereafter. Deletion of *Pdgfr-β* did not affect body weight, blood glucose levels or blood pressure in either normoglycaemic control mice or diabetic CaMKIIα transgenic mice [39].

Diabetic Nephropathy in Camkiiα (Thr286Asp) Transgenic and *Pdgfr-B* Conditional Knockout Mice

The urinary albumin to creatinine ratio was higher in the DM mice than in the WT mice at P112. The deletion of Pdgfr-βdecreased the ratio significantly in diabetic CaMKIIα transgenic mice (Figure 6a). Deletion of Pdgfr-β improved the pathological changes in glomeruli observed in CaMKIIα transgenic mice. Microscopic examinations demonstrated that the glomeruli of DM mice were significantly larger than those in WT mice. They frequently showed the sclerotic changes of glomeruli, such as segmental changes at P112. The mesangial area to glomerular area ratio in DM-KO mice was significantly lower than in DM mice (Figure 6b).

The urinary 8-hydroxydeoxyguanosine (8-OHdG) level measured as oxidative stress marker was significantly higher in DM mice than in WT mice, and was significantly lower in DM-KO mice than in DM mice at P112 (Figure 6c). The production of NAD(P)H oxidase 4 (NOX4), a renal homologue of NAD(P)H oxidase, tended to be up regulated in DM mice, and significantly down regulated in DM-KO mice (Figure 6d).

It is reported that NOX4 is activated by PDGF-B followed by the generation of reactive oxygen species [40,41]. Inhibition of NOX4 ameliorates diabetic nephropathy with decreasing NAD (P) H-dependent reactive oxygen species generation [42]. These reports suggest that down regulation of NOX4 may be involved, at least partly,

in the improvement of diabetic nephropathy observed in DM-KO mice. Our present study suggests that enhanced PDGFR-β signaling plays important roles in the development of diabetic nephropathy *in vivo* and that increased oxidative stress is involved in this process.

Conclusion

The CaMKIIα (Thr286Asp) transgenic mice established here in would be valuable as a novel model of severe insulin-dependent diabetes accompanied by an early progression of diabetic micro vascular complications.

Acknowledgements

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