

# Altered Gene Expression Related to Glomerulogenesis and Podocyte Structure in Early Diabetic Nephropathy of *db/db* Mice and Its Restoration by Pioglitazone

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Glomerular injury plays a pivotal role in the development of diabetic nephropathy. To elucidate molecular mechanisms underlying diabetic glomerulopathy, we compared glomerular gene expression profiles of *db/db* mice with those of *db/m* control mice at a normoalbuminuric stage characterized by hyperglycemia and at an early stage of diabetic nephropathy with elevated albuminuria, using cDNA microarray. In *db/db* mice at the normoalbuminuric stage, hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), ephrin B2, glomerular epithelial protein 1, and Pod-1, which play key roles in glomerulogenesis, were already upregulated in parallel with an alteration of genes related to glucose metabolism, lipid metabolism, and oxidative stress. Podocyte structure-related genes, actinin 4 $\alpha$  and dystroglycan 1 (DG1), were also significantly upregulated at an early stage. The alteration in the expression of these genes was confirmed by quantitative RT-PCR. Through pioglitazone treatment, gene expression of ephrin B2, Pod-1, actinin 4 $\alpha$ , and DG1, as well as that of oxidative stress and lipid metabolism, was restored concomitant with attenuation of albuminuria. In addition, HIF-1 $\alpha$  protein expression was partially attenuated by pioglitazone. These results suggest that not only metabolic alteration and oxidative stress, but also the alteration of gene expression related to glomerulogenesis and podocyte structure, may be involved in the pathogenesis of early diabetic glomerulopathy in type 2 diabetes. *Diabetes* 55:2747–2756, 2006

**D**iabetic nephropathy is the leading cause of end-stage renal disease in the U.S., Japan, and most of Europe (1). Clinical features of diabetic nephropathy are development of albuminuria followed by persistent proteinuria and, later, reduction of glomerular filtration rate (2). Increased thickness of glomerular basement membrane and augmentation of glomer-

ular extracellular matrix are recognized as pathological hallmarks of diabetic nephropathy (2). Thus, glomerular injury is apparently critical for the initiation and progression of the disease. Several pathways are postulated as potential mechanisms of diabetic nephropathy, including renal hemodynamic changes, accretion of advanced glycation end products, intracellular accumulation of sorbitol, oxidation of glycoproteins by reactive oxygen species, and activation of protein kinase C (2,3). Recently, much attention has been paid to the role of podocyte injury in glomerular diseases, including diabetic nephropathy (3–6). However, the precise molecular mechanisms underlying diabetic glomerulopathy still remain unclear.

Microarray is a novel tool by which whole-genome analysis can identify new genes and pathways that are important for the pathophysiology of diabetic nephropathy (7). Although several laboratories recently performed cDNA microarray analyses of diabetic kidney (8–12), most of them examined gene expression of whole kidney, despite the importance of glomerular injury in diabetic nephropathy. In addition, analysis of whole kidney often makes it difficult to select genes associated with diabetic glomerulopathy because glomeruli occupy only a small part of the kidney. Only one of these reports showed the gene expression profile of glomeruli (12). However, because the report analyzed glomeruli from advanced diabetic nephropathy patients with apparent histological changes, it did not provide much information about the mechanism of early diabetic glomerulopathy.

In this study, we performed microarray analysis using isolated glomeruli from diabetic mice at a normoalbuminuric stage and an early stage of diabetic nephropathy with no apparent histological change in order to find the genes that are strongly associated with diabetic glomerular injury. This approach also enabled us to avoid the modification of gene expression profiles by cell component alteration. We analyzed *db/db* mice, a genetic model of type 2 diabetes with obesity and insulin resistance (13), because they exhibited histological changes resembling those in human diabetic nephropathy (13,14). Because accumulating evidence indicates that insulin resistance participates in the pathogenesis of diabetic nephropathy in type 2 diabetes (15), we also examined the effects of pioglitazone, one of the insulin sensitizers that improves insulin sensitivity, on the gene expression profile of *db/db* mice.

## RESEARCH DESIGN AND METHODS

Male diabetic *db/db* mice and their nondiabetic *db/m* littermates were used for this study. All mice were purchased from CLEA Japan (Tokyo). These *db/db*

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DG1, dystroglycan 1; GLEPP1, glomerular epithelial protein 1; HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ ; VEGF, vascular endothelial growth factor.

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TABLE 1  
Characteristics of experimental animals

	5 weeks	7 weeks
Body weight (g)		
<i>db/m</i>	21.5 ± 0.4	25.4 ± 0.3
<i>db/db</i>	22.8 ± 0.7	37.1 ± 0.3*
Blood glucose levels (mg/dl)		
<i>db/m</i>	182 ± 6	128 ± 12
<i>db/db</i>	238 ± 17†	575 ± 25*
Urinary albumin excretion (μg/16 h)		
<i>db/m</i>	8.9 ± 1.5	5.2 ± 1.1
<i>db/db</i>	12.2 ± 1.3	32.5 ± 6.3*
Urinary albumin excretion (μg/mg creatine)		
<i>db/m</i>	0.29 ± 0.06	0.16 ± 0.02
<i>db/db</i>	0.19 ± 0.07	0.49 ± 0.13*

Data are the means ± SE. Each group has  $n = 12$ . \* $P < 0.01$ , † $P < 0.05$  vs. *db/m*.

mice began to show hyperglycemia at 5 weeks of age and a significant increase in urinary albumin excretion at 7 weeks of age (Table 1). Mice were killed under pentobarbital anesthesia at 5 and 7 weeks of age to obtain kidney samples for isolation of glomeruli and immunohistochemistry.

To study the role of insulin resistance in the development of diabetic nephropathy, we administered pioglitazone (Takeda Pharmaceutical, Osaka, Japan), a peroxisome proliferator-activated receptor- $\gamma$  agonist, to two other groups of 5-week-old *db/db* mice for 2 weeks ( $n = 12$  in each). Pioglitazone was mixed with normal mouse chow and administered at a dose of 3 or 15 mg · kg body wt<sup>-1</sup> · day<sup>-1</sup> because 15 mg/kg of pioglitazone was reported to improve insulin sensitivity in *db/db* mice (16).

We obtained 16-h urine specimens from all mice at 5 and 7 weeks of age for the measurement of albumin excretion (17). Urinary albumin excretion was determined by enzyme-linked immunosorbent assay (Albuwell; Exocell, Philadelphia, PA) (17). Urinary creatinine levels were measured by enzymatic method (SRL, Tokyo) (17). For the insulin tolerance test, mice were fasted for 6 h and given 1.25 unit/kg i.p. human regular insulin (Novo Nordisk, Bagsvaerd, Denmark) (18).

**Isolation of glomeruli.** We prepared two isolated glomerular samples from each group. An isolated glomerular sample was obtained from the kidneys of six mice by differential sieving method, using mesh diameters of 45, 75, and 150 μm (19). The purity of each sample was confirmed by microscopy. The glomerular samples were ~80% pure on average, and there was no difference in purity among the samples.

**Microarray gene expression.** Total RNA was extracted from glomerular samples by the acid guanidinium-phenol-chloroform method, using Trizol reagent (Life Technologies) (20). We essentially followed the procedures described in detail in the GeneChip expression analysis manual (Affymetrix, Santa Clara, CA). In brief, 10 μg of total RNA was used for cDNA synthesis (Superscript II kit; Life Technologies, Rockville, MD). Biotin-labeled cRNA was produced through in vitro transcription of cDNA, using an ENZO BioArray high-yield RNA transcript labeling kit (Affymetrix). Fragmented cRNA (15 μg) was hybridized to an Affymetrix Murine Genome U74Av2 GeneChip at 45°C for 16 h. The samples were stained and washed according to the manufacturer's protocol on a Fluidics Station 400 (Affymetrix) and scanned on a GeneArray scanner (Affymetrix) (8,21).

Primary data extraction was performed with Microarray Suite 5.0 (Affymetrix) because analysis by Microarray Suite 5.0 is more reliable than other methods (22). Microarray Suite 5.0 software normalized the data of each microarray and compared the expression between the two different arrays. Moreover, the software could determine statistically whether each gene was present (reliably detected) or absent (not detected) in one array and whether each gene increased or decreased between two different arrays. Signal normalization across samples was carried out, using all probe sets, with a mean expression value of 500 (8,21). To allow comparisons between any two experiments, pairwise comparisons were made between *db/m* and *db/db* mice by Microarray Suite 5.0. Because two arrays were used for each group (*db/m* 1, *db/m* 2, *db/db* 1, and *db/db* 2), we performed four comparison analyses (i.e., *db/m* 1 vs. *db/db* 1, *db/m* 1 vs. *db/db* 2, *db/m* 2 vs. *db/db* 1, and *db/m* 2 vs. *db/db* 2). Genes showing an increased or decreased call in at least three of four comparisons were defined as genes showing a significant change. As an internal control, we chose GAPDH and confirmed that there was no difference in GAPDH expression level between each sample in microarray analysis.

**Podocyte culture.** Cultivation of conditionally immortalized mouse podocytes (a gift from Dr. Peter Mundel, Albert Einstein College of Medicine, Bronx, NY) was performed as reported previously (23). Briefly, cells were grown on a type 1 collagen-coated dish (IPC-03; Koken, Tokyo) at 33°C in the presence of 10 units/ml murine  $\gamma$ -interferon (Life Technologies, Gaithersburg, MD) in RPMI 1640 medium (Nihonseiyaku, Tokyo) supplemented with 10% FCS (Cansera International, Etobicoke, ON, Canada) and antibiotics. To induce differentiation, podocytes were maintained at 37°C without interferon. Before the experiment, cells were differentiated for 2 weeks without passage, followed by culture in RPMI 1640 containing 1% FCS supplemented with 5.6 mmol/l glucose (normal glucose) or 25 mmol/l glucose (high glucose) for 14 days.

**Quantitative RT-PCR.** We reverse transcribed 2.5 μg of total RNA using Ready-To-Go (Amersham Pharmacia Biotech, Piscataway, NJ) (20). TaqMan real-time quantitative PCR was performed and analyzed according to the manufacturer's instructions (Applied Biosystems, Foster City, CA) (24). Primers and probe sequences were selected by using Primer Express (Applied Biosystems). GAPDH was used for internal control because its expression level did not show a significant difference between *db/m* and *db/db* in our microarray analysis.

**Immunohistochemistry.** Kidneys were dissected immediately and fixed in 10% formalin, embedded in paraffin, and sectioned at 4 μm. Hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) was identified with monoclonal IgG HIF-1 $\alpha$  antibody 67 (Novus Biological, Littleton, CO) at a 1:2000 dilution, using the Tyramide signal amplification system (PerkinElmer Life Sciences, Boston, MA) (25). The number of HIF-1 $\alpha$ -positive cells was counted in 30 randomly selected glomeruli in the outer cortex.

**Statistical analyses.** Data are the means ± SE. Statistical analyses were performed using ANOVA followed by Scheffe's test.  $P < 0.05$  was considered statistically significant.

## RESULTS

**Characteristics of *db/db* mice.** At 5 weeks of age, *db/db* mice already showed significant hyperglycemia, whereas urinary albumin excretion did not increase compared with *db/m* mice (Table 1). There was no difference in renal histology between *db/m* and *db/db* mice at 5 weeks of age under light microscopic observation. At 7 weeks of age, *db/db* mice showed significant elevation of urinary albumin excretion (Table 1). However, no apparent histological difference was observed between *db/db* and *db/m* mice (data not shown). Thus, 5-week-old *db/db* mice exhibited features similar to the human normoalbuminuric stage, and 7-week-old mice showed features similar to the human early stage of diabetic nephropathy.

**Comparison analysis of gene expression profiles between *db/db* and *db/m* mice.** Table 2 shows 134 genes with an absolute relative log ratio >0.5 and showing significant change by Microarray Suite 5.0 analysis at 5 and/or 7 weeks of age. At 5 weeks of age, 105 genes were differentially expressed (65 increased, 40 decreased) between *db/db* and *db/m* mouse glomeruli. Among them, there were genes related to oxidative stress, glucose metabolism, lipid metabolism, cell growth, fibrosis, apoptosis, vasoactive mediators, calcium-binding proteins, coagulation, cell structure, and extracellular matrix components. We also observed significant differences in the expression of development-related genes, including several genes related to kidney development. At 7 weeks of age, 116 genes expressed differentially (72 increased, 44 decreased) between *db/db* and *db/m* mouse glomeruli. In addition to the genes showing differential expression at 5 weeks of age, genes related to cell structure, particularly podocyte structure, and solute carrier family were expressed differentially between *db/db* and *db/m*.

We next confirmed the differential expression of genes by quantitative real-time RT-PCR. Because glomerular response to injury is accompanied by activation of the development-related genes (26), we measured mRNA levels of kidney development-related genes, i.e., ephrin B2

TABLE 2  
Genes up- or downregulated in *db/db* mice at 5 and 7 weeks of age

Gene	ID	Fold change of genes up- or downregulated in <i>db/db</i> mice compared with <i>db/m</i>		Fold change of genes up- or downregulated in pioglitazone-treated <i>db/db</i> mice	
		5 weeks	7 weeks	Pioglitazone 3 mg/kg	Pioglitazone 15 mg/kg
<b>Oxidative stress related</b>					
Upregulated					
Glutathione-S-transferase $\alpha 2$	J03958	7.77*	9.49*	10.70	0.95†
Cytochrome P450 4a14	Y11638	6.52*	15.26*	9.76	0.76†
Cytochrome c oxidase subunit VIa	U08439	2.17*	14.90*	9.68	2.99
Glutathione S transferase $\omega 1$	AI843119	1.87*	1.73*	2.16	0.81†
Glutathione S transferase $\omega 1$	AI843119	1.87*	1.73*	2.16	0.81†
Glutathione S transferase $\theta 1$	X98055:	1.73*	1.52*	1.90	1.02
Metallothionein 1	V00835	1.61*	2.79*	4.7	1.14†
Calcipressin	AI846152	1.61	2.92*	2.16	1.26†
Cytochrome P450 4a10	AB018421	1.57*	2.34*	1.73	0.82†
Antioxidant enzyme AOE372	U96746	1.31	1.91*	1.58	0.90†
Downregulated					
Cysteine sulfonic acid decarboxylase	AW120896	0.31*	0.56*	0.48	1.06†
Cytochrome P450 2e1	X01026	0.43*	0.37	0.43	0.91†
Cytochrome P450 2a4	M19319	0.45*	0.32*	0.22	0.87†
Malic enzyme supernatant	J02652	0.48*	0.41*	0.51	1.11†
Glutamate cysteine ligase	U95053	0.50*	0.53*	0.34	0.96†
Extracellular superoxide dismutase	U38261	0.60*	0.70*	0.70	1.50†
Peroxisomal acyl-CoA oxidase	AF006688	0.74	0.56*	0.72	1.02†
<b>Lipid metabolism related</b>					
Upregulated					
Apolipoprotein E	D00466	3.00*	3.32*	4.98	1.16†
Stearoyl-coenzyme A desaturase 2	M26270	2.34*	2.06*	3.09	1.26†
Thioredoxin interacting protein	AI839138	1.96*	1.3	1.43	1.17
Oxysterol-binding protein like 5	AW121299	1.78*	1.41	2.15	1.09
Phosphatidic acid phosphatase 2b	AI847054	1.65*	0.95	2.93	2.10
Acetyl coenzyme A acyltransferase 2	AI849271	1.28*	1.82*	1.91	0.89†
Sterol-C4 methyl oxidase like	AI848668	1.13	2.06*	2.39	2.06
Acetyl-coenzyme A synthetase 2	AW125884	1.19	1.65*	2.16	0.99
Downregulated					
Lipoprotein lipase	M63335	0.13*	0.17*	0.09	0.93†
Alcohol dehydrogenase class I gene	M22679	0.22*	0.23*	0.21	1.08†
Degenerative spermatocyte homolog 2	AI852933	0.27*	0.25*	0.24	0.97†
$\alpha$ -Methylacyl-CoA racemase	U89906	0.36*	0.28*	0.37	0.98†
Diphosphate $\delta$ isomerase	AA716963	0.36*	0.35*	0.27	0.91†
Enoyl-coenzyme A hydratase	AJ011864	0.46*	0.43*	0.22	1.23†
Coenzyme A synthase	AI837229	0.53*	0.36*	0.29	0.88†
Fatty acid transporter protein 2	AF072757	0.68	0.54*	0.49	0.89†
Lysophospholipase 1	AA840463	0.72	0.61*	0.29	0.81
<b>Cell growth related</b>					
Upregulated					
Dual-specificity phosphatase 1	X61940	2.30*	0.90	1.31	0.86
Cysteine-rich protein 1	D88793	2.06*	1.91*	2.90	1.64
Prothymosin $\beta 4$	U38967	2.01*	1.57*	1.60	1.16
Calpactin I heavy chain (p36)	M14044	1.96*	1.31	2.04	1.18
Cyclin-dependent kinase inhibitor 1C	U22399	1.96*	1.25	1.95	1.39
Nuclear protein 1	AI852641	1.75*	2.11*	3.84	1.56
Minopontin	X13986	1.73*	2.93*	3.25	0.94†
Annexin A1	:AV003419	1.73*	1.15	2.62	1.15
Biglycan	X53928	1.65*	1.45	2.64	1.38
Cyclin-dependent protein kinase	AI849556	1.69	2.01*	2.23	1.17†
CDC28 protein kinase regulatory subunit 2	AA681998	1.19	1.96*	1.55	1.06†
Downregulated					
Sin3-associated protein (sap 30)	AF075136	0.48*	0.36*	0.25	0.76†
Ornithine decarboxylase	M12330	0.56*	0.37*	0.43	1.16†
FK506BP-rapamycin-associated protein 1	AI853977	0.58*	0.45*	0.70†	1.11†

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TABLE 2  
Continued

Gene	ID	Fold change of genes up- or downregulated in <i>db/db</i> mice compared with <i>db/m</i>		Fold change of genes up- or downregulated in pioglitazone-treated <i>db/db</i> mice	
		5 weeks	7 weeks	Pioglitazone 3 mg/kg	Pioglitazone 15 mg/kg
<b>Development related</b>					
Upregulated					
Ephrin B2	U30244	2.11*	1.63*	1.63	1.20†
Glomerular epithelial protein 1 (PtrpO)	U37465	1.73*	1.16	2.70	1.55
Secreted frizzled related protein sFRP-2	U88567	1.69*	0.90	2.47	2.20
Cytoplasmic protein Ndr1	U60593	1.61*	2.23*	3.23	2.11
Transcriptional factor 21 (Pod1)	AF035717	1.50*	0.61*	1.16†	0.93†
HIF-1 $\alpha$	Y09085	1.25	1.53*	1.83	1.13
Downregulated					
BTEB-1 (kif 9)	Y14296	0.46*	0.62*	0.70	1.01†
Iroquois homeobox protein 3	Y15001	0.61*	0.47*	0.34	1.64†
Cartilage-associated protein	AJ006469	0.62*	0.61*	0.80	1.35†
<b>Glucose metabolism related</b>					
Upregulated					
Phosphoglycerate mutase	AF029843	6.20*	5.47*	14.49	3.82
Transketolase	U05809	1.82*	1.91*	1.72	0.99†
Pyruvate kinase 3	X97047	1.78*	2.17*	2.23	1.32†
Glucose-6-phosphatase	U00445	1.61*	1.25	1.13	1.01
Phosphoenolpyruvate carboxykinase	AF009605	1.60*	1.64*	1.48	1.21
Pyruvate dehydrogenase kinase 3	AI853226	1.60*	2.12*	3.07	1.63
<i>N</i> -acetylneuraminidase	AA710564	1.38	1.96*	3.15	1.08†
Downregulated					
Pyruvate dehydrogenase kinase 3	AI842259	0.48*	0.49*	0.47	1.26†
$\beta$ -Galactosidase	M57734	0.57*	0.52*	0.63	1.16†
<b>Vasoactive mediator related</b>					
Upregulated					
Kallikrein	V00829	3.76*	4.26*	6.86	1.15†
Potential kallikrein gene	M13500	3.40*	3.86*	4.94	0.93
Epidermal growth factor-binding protein A	M1797	3.29*	3.96*	5.19	1.15†
Kallikrein 5	Y00500	3.24*	3.24*	3.66	0.81†
Mouse renin	M32352	2.93*	2.47*	3.31	1.43†
Glandular kallikrein	J00389	2.72*	2.65*	3.55	1.03†
Adrenomedullin	U77630	1.96*	2.52*	3.15	1.13
Serpin	M25529	1.69*	0.80	0.51	0.66
Carboxypeptidase N	AI182588	1.40*	2.11*	1.94	1.16†
<b>Coagulation, fibrinolysis</b>					
Upregulated					
Coagulation factor II receptor	AW123850	2.23*	1.91*	5.30	1.41
Protein S	L27439	1.61	1.86*	2.45	1.59
Tissue factor pathway inhibitor 2	D50586	1.38	2.17*	2.49	1.02†
Downregulated					
$\alpha$ 2-ntiplasmin	Z36774	0.30*	0.34*	0.27	1.38†
Anticoagulant protein C	AF034569	0.46*	0.40*	0.46	0.84†
<b>Cell structure</b>					
Upregulated					
Tubulin $\beta$ 2	M28739	1.96*	2.79*	2.34	1.30
Calponin 3	AW125626	1.65*	1.57*	1.82	1.05
Tubulin $\alpha$ 1	M28729	1.65*	1.32	1.78	1.13
(Podocyte structure)					
Dystroglycan 1	AV244370	1.38	1.69*	3.49	1.13†
Actinin 4 $\alpha$	AI836968	1.22	1.60*	4.33	1.52
<b>Apoptosis related</b>					
Upregulated					
Clusterin	D14077	3.69*	4.37*	8.34	1.13†
Gelsolin	J04953	2.40*	1.69*	2.16	1.32
Downregulated					
Midkine	M34094	0.34*	0.56*	0.50	0.87
B-cell leukemia/lymphoma 6	U41465	0.44*	0.54*	1.11†	1.32†

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TABLE 2  
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Gene	ID	Fold change of genes up- or downregulated in <i>db/db</i> mice compared with <i>db/m</i>		Fold change of genes up- or downregulated in pioglitazone-treated <i>db/db</i> mice	
		5 weeks	7 weeks	Pioglitazone 3 mg/kg	Pioglitazone 15 mg/kg
Calcium-binding protein					
Upregulated					
Calbindin-28K	D26352	6.52*	9.97*	13.76	1.19†
Calcium-binding protein D-9k	AF028071	5.70*	7.21*	11.31	1.15†
Calcyclin	X66449	1.34	1.86*	3.29	1.24
Calcium-binding protein S100A1	AF087687	1.69*	1.57*	1.60	0.77
Steroid related					
Upregulated					
Hydroxysteroid 11- $\beta$ -dehydrogenase 2	X90647	2.52*	3.86*	5.17	2.24
Glucocorticoid-regulated kinase	AW046181	2.12*	1.91*	3.07	1.28
Progesterone receptor membrane component 1	AF042491	1.34*	1.69*	2.51	0.98†
Downregulated					
Hydroxysteroid 17- $\beta$ -dehydrogenase 11	AA822174	0.22*	0.20*	0.23	0.99†
Extracellular matrix component					
Upregulated					
Nephronectin	AA592182	1.82*	2.58*	4.69	1.91
Procollagen type IV ( $\alpha$ 1)	M15832	1.49	2.12*	3.58	1.65
Procollagen type XVIII ( $\alpha$ 1)	U03715	1.22	1.91*	1.72	1.72
Fibrosis related					
Upregulated					
Endoglin	X77952	2.34*	1.34	3.45	1.63
Connective tissue growth factor	M70642	1.87*	1.78*	4.05	1.87
Chaperone					
Downregulated					
Nucleoplasmin 3	U64450	0.45	0.46*	0.32	0.65
Co-chaperone mt-GrpE#2	AF041060	0.80	0.54*	0.66	0.93†
Solute carrier family					
Upregulated					
Solute carrier family 8	AF004666	4.48*	7.89*	16.66	1.10†
Solute carrier family 3	AW122706	1.22	1.82*	2.71	1.40
Complement related					
Upregulated					
CD59 antigen	U60473	1.82*	1.61*	2.46	1.32
Downregulated					
C1q- and tumor necrosis factor-related protein 3	AI315647	0.48*	0.29*	0.42	1.30†
Others					
Upregulated					
WSB-1	AF033186	2.59*	6.69*	4.68	1.40†
Zinc finger protein 36	M58566	2.11*	1.35	2.28	1.55
Proline dehydrogenase 2	AA675075	1.96*	1.96*	1.90	0.69†
Smad 6	AF010133	2.11*	1.65	2.52	1.57
Cytotoxic T cell-associated protein 2	X15591	1.73*	0.89	1.93	1.20
Aldehyde dehydrogenase II	M74570	1.69*	2.11*	1.48	0.87†
Hephaestin	AF082567	1.68	2.86*	5.89	1.52†
$\alpha$ -Mannosidase II	X61172	1.49*	1.69*	2.23	1.03†
Carbonic anhydrase II	M25944	1.45*	1.92*	2.13	1.06†
Hexosaminidase A	U05837	1.38	1.69*	2.11	1.13
Protein tyrosine phosphatase receptor type D	D13903	1.25	1.87*	0.80	0.75†
Prominin-like 1	AF039663	1.13	2.12*	3.01	1.63
ROMK-2	AF012834	0.88	2.11*	1.11	1.03†
Downregulated					
CNDP dipeptidase 2	AI854839	0.08*	0.11*	0.08	0.85†
ATPase class VI 11a	AA690863	0.22*	0.22*	0.24	1.62†
UDP-glucuronosyltransferase 8	U48896	0.23*	0.21*	0.13	0.99†
Chemokine-like factor superfamily 6	AW125031	0.33*	0.34*	0.39	1.38†
Hepatic nuclear factor-1 $\beta$	AB008174	0.42*	0.88	1.05	1.18

Continued on following page

TABLE 2  
Continued

Gene	ID	Fold change of genes up- or downregulated in <i>db/db</i> mice compared with <i>db/m</i>		Fold change of genes up- or downregulated in pioglitazone-treated <i>db/db</i> mice	
		5 weeks	7 weeks	Pioglitazone 3 mg/kg	Pioglitazone 15 mg/kg
Interferon regulatory protein 6	U73029	0.46*	0.88	1.07	1.18
Suppressor of cytokine signaling-2	U88327	0.46*	0.49	0.57	1.14
Uromodulin	L33406	0.47*	0.43*	0.72	1.29†
Carbonic anhydrase IV	U37091	0.49*	0.54*	0.45	1.14†
Connexin 26	M81445	0.49*	0.65	0.97	1.52†
Growth hormone receptor	U15012	0.52*	0.56*	0.48	0.97†
Aldehyde dehydrogenase 4	U14390	0.59*	0.53*	0.68	1.24†
Tripartite motif protein 47	AW048347	0.70	0.59*	0.54	1.02†
Carboxypeptidase H	X61232	0.45	0.17*	0.42†	1.78†
Makorin	AA656621	0.69	0.58*	0.45	0.95
Nitrlase 1	AF069988	0.74	0.59*	0.76	1.07†
Cyclophilin C	M74227	0.77	0.55*	0.64	0.95†

Gene names were ordered according to the absolute value of the relative log ratio. \*Significant change compared with *db/m*, †significant change compared with untreated *db/db*, in comparison analysis by Microarray Suite 5.0.

(27), Pod-1 (28), glomerular epithelial protein 1 (GLEPP1) (29), and HIF-1 $\alpha$  (30), in isolated glomeruli. These four genes are reported to play important roles in glomerulogenesis (27–30). Real-time RT-PCR confirmed significant mRNA elevation of ephrin B2 at 5 and 7 weeks of age (2.2- and 2.9-fold of control at 5 and 7 weeks of age, respectively) (Fig. 1A). Although the upregulation of HIF-1 $\alpha$  was not significant by cDNA microarray analysis at 5 weeks of age, real-time RT-PCR revealed significant upregulation of HIF-1 $\alpha$  mRNA at both 5 and 7 weeks of age (2.1- and 2.9-fold of control at 5 and 7 weeks of age, respectively) (Fig. 1A). Because HIF-1 $\alpha$  is a transcription factor and it is more important to evaluate the expression of HIF-1 $\alpha$  protein, we also examined HIF-1 $\alpha$  protein expression by immunohistochemistry. HIF-1 $\alpha$  protein expression was significantly increased in glomeruli at 7 weeks of age (Fig. 4A, B, and D). GLEPP1 and Pod-1 were significantly upregulated only at 5 weeks of age, and Pod-1 was significantly downregulated at 7 weeks (GLEPP1: 2.3- and 1.1-fold of control at 5 and 7 weeks of age; Pod-1: 4.0- and 0.5-fold, respectively) (Fig. 1A).

We also confirm the differential expression of podocyte

structure-related genes, actinin 4 $\alpha$  (31) and dystroglycan 1 (DG1) (32), because morphologic changes of podocytes and podocyte injury play key roles in the development of diabetic nephropathy (3–6). Quantitative RT-PCR revealed significant upregulation of actinin 4 $\alpha$  and DG1 in isolated glomeruli of 7- but not of 5-week-old *db/db* mice compared with those of control (actinin 4 $\alpha$ : 3.4-fold; DG1: 2.9-fold) (Fig. 1B).

**mRNA expression in cultured podocytes under high-glucose conditions.** To examine whether the differential expression of kidney development-related genes and podocyte structure-related genes in isolated glomeruli of *db/db* mice could reflect the alteration in gene expression of podocytes, we next examined the expression of these genes in cultured podocytes by quantitative RT-PCR. Although Pod-1 mRNA expression was not detectable, ephrin B2, HIF-1 $\alpha$ , GLEPP1, actinin 4 $\alpha$ , and DG1 mRNA were detectable in cultured podocytes under normal glucose conditions. Ephrin B2 and HIF-1 $\alpha$  mRNA expression was significantly upregulated under high-glucose conditions (5.7- and 2.3-fold of control, respectively) (Fig. 2A). GLEPP1 mRNA expression also tended to increase (1.6-fold of control) (Fig. 2B). By contrast, actinin 4 $\alpha$  and DG1 mRNA did not show a significant change (Fig. 2B).

**Comparison analysis of gene expression profile between *db/db* and pioglitazone-treated *db/db* mice.** Insulin resistance is one of the important pathogenic factors for the diabetic nephropathy in type 2 diabetes. Indeed, improvement of insulin resistance by thiazolidinediones resulted in the reduction of albuminuria in diabetic nephropathy (33). Therefore, we examined the effects of pioglitazone on the gene expression profiles in *db/db* mice. Although administration of pioglitazone at a dose of 3 mg/kg did not affect hyperglycemia, insulin sensitivity, or albuminuria, pioglitazone at a dose of 15 mg/kg significantly reduced but did not normalize the blood glucose level, improved insulin sensitivity, and completely normalized urinary albumin excretion (Tables 3 and 4).

We first examined the effect of pioglitazone using microarray analysis. Table 2 shows genes in *db/db* mice whose differential expression was restored by pioglitazone

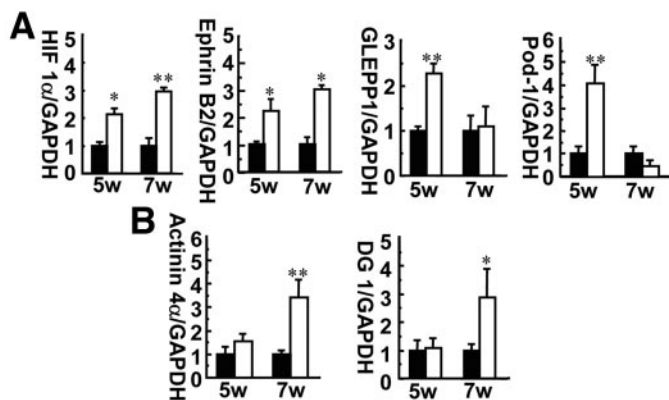


FIG. 1. mRNA expression of HIF-1 $\alpha$ , ephrin B2, GLEPP1, and Pod-1 (A) and actinin 4 $\alpha$  and DG1 (B) in isolated glomeruli from *db/m* and *db/db* mice at 5 and 7 weeks (w) of age by TaqMan real-time quantitative PCR. Values are the means  $\pm$  SE,  $n = 4$  for each group. \* $P < 0.05$ , \*\* $P < 0.01$  vs. *db/m* mice. ■, *db/m* mice; □, *db/db* mice.

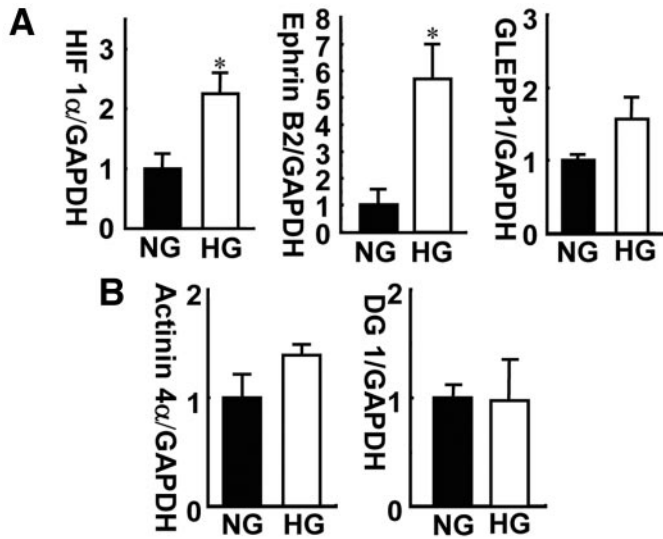


FIG. 2. mRNA expression of HIF-1 $\alpha$ , ephrin B2, and GLEPP1 (A) and actinin 4 $\alpha$  and DG1 (B) in cultured podocytes under high-glucose (HG) conditions by TaqMan real-time quantitative PCR. Values are the means  $\pm$  SE. ■, normal glucose (NG;  $n = 3$ ); □, high glucose ( $n = 3$ ). \* $P < 0.05$  vs. normal glucose. w, weeks.

treatment. Although the lower dose of pioglitazone (3 mg/kg) restored only a small number of genes (4 of 116), pioglitazone at the higher dose restored the alteration in more than two-thirds of the genes (81 of 116) in *db/db* mice. Pioglitazone restored most of the genes related to oxidative stress (16 of 17), lipid metabolism (11 of 14), glucose metabolism (4 of 8), development (5 of 7), cell growth (6 of 9), vasoactive mediator (6 of 8), and coagulation (3 of 5). Among these genes, the recovery of oxidative stress-, glucose metabolism-, and lipid metabolism-related gene expression by pioglitazone was compatible with previous reports (34,35). By contrast, only a small number of the genes related to fibrosis (0 of 2), apoptosis (1 of 3), calcium-binding protein (2 of 4), cell structure (1 of 5), and extracellular matrix component (0 of 3) were restored by pioglitazone.

We also examined mRNA expression of kidney development- and podocyte structure-related genes by quantitative RT-PCR. Upregulation of ephrin B2 and downregulation of Pod-1 were blunted by pioglitazone treatment (Fig. 3A). Although suppression of HIF-1 $\alpha$  mRNA expression was not observed in microarray analysis and RT-PCR, HIF-1 $\alpha$  protein expression was partially attenuated by pioglitazone (Fig. 4). Upregulation of actinin 4 $\alpha$  and DG1 genes was significantly attenuated by pioglitazone treatment (Fig. 3B).

## DISCUSSION

Although glomerular injury plays a central role in the development of diabetic nephropathy, most reports using microarray analysis have focused on the gene expression

TABLE 3  
Blood glucose levels of insulin tolerance test in *db/db* mice

	0 min	20 min	60 min	100 min	160 min
Untreated <i>db/db</i>	157 $\pm$ 56	135 $\pm$ 75	63 $\pm$ 12	91 $\pm$ 10	120 $\pm$ 13
<i>db/db</i> + 3 mg/kg pioglitazone	148 $\pm$ 30	172 $\pm$ 13	67 $\pm$ 9	94 $\pm$ 4	131 $\pm$ 4
<i>db/db</i> + 15 mg/kg pioglitazone	117 $\pm$ 9	87 $\pm$ 6	58 $\pm$ 7	38 $\pm$ 78*	53 $\pm$ 15*

Data are the means  $\pm$  SE. Each group has  $n = 4$ . \* $P < 0.01$  vs. untreated *db/db*.

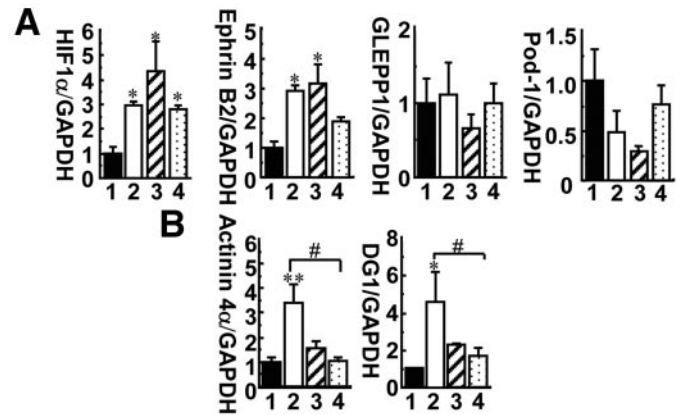
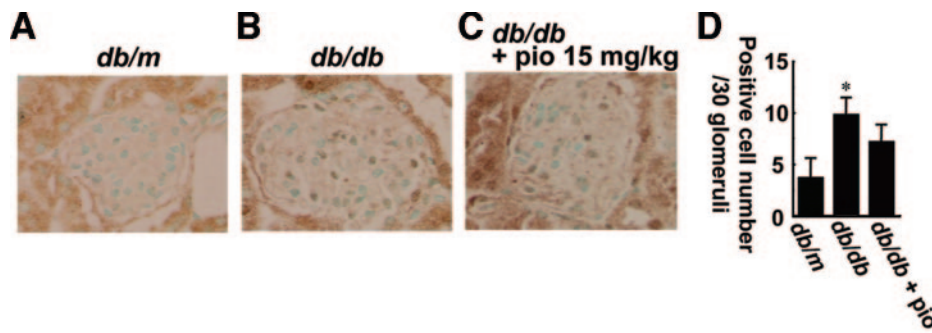


FIG. 3. mRNA expression of HIF-1 $\alpha$ , ephrin B2, GLEPP1, and Pod-1 (A) and actinin 4 $\alpha$  and DG1 (B) in isolated glomeruli from pioglitazone-treated *db/db* mice by TaqMan real-time quantitative PCR. 1, *db/m* mice; 2, untreated *db/db* mice; 3, *db/db* mice treated with 3 mg/kg pioglitazone; 4, *db/db* mice treated with 15 mg/kg pioglitazone. Values are the means  $\pm$  SE,  $n = 4$  for each group. \* $P < 0.05$ , \*\* $P < 0.01$  vs. *db/m* mice; # $P < 0.05$ .

profile of the whole kidney in diabetic animals with nephropathy. In the current study, we examined a gene expression profile of isolated glomeruli from *db/db* mice, a well-known type 2 diabetes model. To the best of our knowledge, this is the first report on the glomerular gene expression profile in type 2 diabetes models. Our microarray data showed differential expression of genes related to glucose and lipid metabolism, oxidative stress, vasoactive mediators, cell growth, and coagulation in isolated glomeruli between *db/db* and *db/m* mice at the normoalbuminuric stage. These results are compatible with previous reports (1–3).

Our first new finding is that the kidney development-related genes were already differentially expressed at the normoalbuminuric stage in the glomeruli of *db/db* mice. Although the pathophysiological significance of the kidney development-related genes we examined is not fully clarified in diabetic nephropathy, ephrin B2, HIF-1 $\alpha$ , Pod-1, and GLEPP1 participate in various stages and aspects of glomerulogenesis and are relevant to some types of glomerular injury, as previously suggested (26). Ephrin B2 is a transmembrane ligand of the ephrin B2 receptor (Eph) and its signaling pathway is required for vascular morphogenesis (36,37) and glomerular microvascular assembly (27). HIF-1 $\alpha$  is critical for renal vasculogenesis and glomerulogenesis (30), and nuclear localization of HIF-1 $\alpha$  increases in murine adriamycin nephrosis (38). Nyengaard and Rasch (39) reported an increase in glomerular capillary size and number in diabetic nephropathy, suggesting that angiogenesis is associated with glomerular injury. Actually, vascular endothelial growth factor (VEGF) plays a key role in the development of proteinuria and glomerular sclerosis in diabetic nephropathy (14). Although VEGF mRNA were not elevated at the normoalbuminuric



**FIG. 4.** Immunohistochemical analysis for HIF-1 $\alpha$  expression in the glomeruli of *db/db* mice at 7 weeks of age. **A:** *db/m* mice. **B:** *db/db* mice. **C:** *db/db* mice plus 15 mg/kg pioglitazone (pio). **D:** HIF-1 $\alpha$ -positive cell number in 30 glomeruli. Values are the means  $\pm$  SE,  $n = 4$  for each group. \* $P < 0.05$  vs. *db/m*.

stage in this study (data not shown), ephrin B2 and HIF-1 $\alpha$  mRNA were already upregulated at the normoalbuminuric stage and remained elevated at an early stage of diabetic nephropathy in isolated glomeruli of diabetic mice (Fig. 1). Because ephrin B2 and HIF-1 $\alpha$  relate to angiogenesis, and because HIF-1 $\alpha$  induces VEGF (40), elevation of ephrin B2 and HIF-1 $\alpha$  may be an important early step for glomerular angiogenic change in diabetic nephropathy. GLEPP1 is related to podocyte differentiation (29), and its expression decreases in dedifferentiated podocytes (41). Pod-1 is one of the transcriptional factors important for glomerulogenesis and podocyte differentiation (24,28). Both ephrin B2 and HIF-1 $\alpha$  are abundantly expressed in glomerular podocytes in the developing kidney (27,30). Taken together, podocyte injury may play a pivotal role in diabetic glomerulopathy, including glomerular angiogenic change. Other kidney development-related molecules (e.g., gremlin and transforming growth factor- $\beta$ ) were also suggested to contribute to the pathogenesis of diabetic nephropathy (42,43). Thus, the current study raises the possibility that the alteration of the kidney development-related molecules, particularly glomerulogenesis-related molecules (ephrin B2, HIF-1 $\alpha$ , GLEPP1, and Pod-1), is a key mediator for diabetic glomerulopathy. This possibility is strengthened by our finding that high glucose induced a similar pattern of changes in glomerulogenesis-related gene expression in cultured murine podocytes because hyperglycemia is a well-known determinant of diabetic nephropathy.

Another new finding in the current study is that extracellular matrix and cell structure-related genes were differentially expressed at an early stage of diabetic nephropathy. This is consistent with the development of mesangial expansion several weeks later in this model. Among these genes, we focused on genes playing important roles in podocyte structure, i.e., actinin 4 $\alpha$  and DG1. Actinin 4 $\alpha$  is an actin-cross-linking protein, and mice with mutant actinin 4 $\alpha$  revealed foot process fusion and podocyte vacuolization (31). DG1, a heavily glycosylated pe-

ripheral membrane protein located in podocytes, is thought to keep foot process shape, and it decreases in proteinuric renal diseases (32,44). Thus, the current study suggests that podocyte structure and function may already alter at an early stage of nephropathy. In contrast to previous reports, actinin 4 $\alpha$  and DG1 mRNA expression increased in this study. Induction of these genes might reflect the glomerular repairing process, as reported in a puromycin aminonucleoside nephrosis model (45).

Insulin resistance is a major feature of type 2 diabetes, and it precedes the onset of microalbuminuria. Greater degrees of insulin resistance are evident when urinary albumin excretion is elevated in type 2 diabetes (15), and hyperinsulinemia in the pre-diabetic state may contribute to microalbuminuria in type 2 diabetes (1). In our microarray analysis, alteration in most of the development-related gene expression was restored by pioglitazone treatment with amelioration of albuminuria and hyperglycemia. Among them, the restoration of ephrin B2 and Pod-1 were confirmed by RT-PCR, and nuclear localization of HIF-1 $\alpha$  was attenuated by pioglitazone. Although we could not evaluate the effect of pioglitazone on GLEPP1 gene expression because of its transient upregulation in this study, these results suggest that insulin resistance might be important in inducing the alteration in the expression of kidney development-related genes, including glomerulogenesis-related genes at early stages of nephropathy. Similarly, insulin resistance might also induce phenotype alteration of podocytes at an early stage of nephropathy because the upregulation of DG1 and actinin 4 $\alpha$  genes was attenuated by pioglitazone treatment. We could not rule out the possibility that hyperglycemia per se directly altered glomerulogenesis-related gene expression because high glucose stimulated expression of glomerulogenesis-related genes in cultured podocytes and because administration of pioglitazone improved insulin resistance as well as hyperglycemia.

In conclusion, we demonstrated that the differential expression of glomerulogenesis-related genes already took

**TABLE 4**  
Effects of pioglitazone treatment on body weight, blood glucose levels, and urinary albumin excretion

	Body weight (g)	Blood glucose levels (mg/dl)	Urinary albumin excretion (mg/16 h)	Urinary albumin excretion ( $\mu$ g/mg creatine)
Untreated <i>db/db</i>	37.1 $\pm$ 0.3	575 $\pm$ 25	32.5 $\pm$ 6.3	0.49 $\pm$ 0.13
<i>db/db</i> + 3 mg/kg pioglitazone	38.2 $\pm$ 0.2	521 $\pm$ 28	26.3 $\pm$ 4.0	0.42 $\pm$ 0.03
<i>db/db</i> + 15 mg/kg pioglitazone	41.7 $\pm$ 0.9*	295 $\pm$ 51*	12.6 $\pm$ 1.9*	0.21 $\pm$ 0.03*
<i>db/m</i>	25.4 $\pm$ 0.3	128 $\pm$ 12	5.2 $\pm$ 1.1	0.16 $\pm$ 0.02

Data are the means  $\pm$  SE. Each group has  $n = 12$ . \* $P < 0.01$  vs. untreated *db/db*.

place at the normoalbuminuric stage in the isolated glomeruli from *db/db* mice, whereas the expression of podocyte structure-related genes were altered at an early nephropathy stage with the elevation of microalbuminuria. We also showed that pioglitazone treatment restored most of the differential expression of glomerulogenesis- and podocyte structure-related genes. These findings suggest that the alteration of these genes might be relevant to the pathogenesis of diabetic glomerulopathy in type 2 diabetes with insulin resistance. Pioglitazone treatment even at the normoalbuminuric stage might be useful for the prevention of diabetic nephropathy.

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