Macrophage Migration Inhibitory Factor Is a Possible Candidate for the Induction of Microalbuminuria in Diabetic *db/db* Mice

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Preventing the onset of microalbuminuria in diabetic nephropathy is a problem that needs urgent rectification. The use of a mouse model for diabetes is vital in this regard. For example, *db/db* mice exhibit defects in the leptin receptor Ob-Rb sub-type, while the *ob/ob* strain exhibits defects in the leptin ligand. These mouse strains demonstrate type 2 diabetes, either with or without microalbuminuria, respectively. The purpose of the present study was to use DNA microarray technology to screen for the gene responsible for the onset of diabetic microalbuminuria. Using Affymetrix Mouse Gene ST 1.0 arrays, microarray analysis was performed using total RNA from the kidneys of *ob* control, *ob/ob*, *db/m*, and *db/db* mice. Microarray and quantitative reverse transcription-polymerase chain reaction (RT-PCR) indicated that transcription of the macrophage migration inhibitory factor (MIF) gene was significantly enhanced in the kidneys of *db/db* mice. Western blotting showed that levels of MIF protein was enhanced in the kidneys of both diabetic *db/db* and *ob/ob* mice. On the other hand, elevation of urinary MIF excretion detected by enzyme-linked immunosorbent assay (ELISA) was only in *db/db* mice and preceded the onset of microalbuminuria. Immunofluorescence studies revealed that MIF was expressed in mouse kidney glomeruli. While MIF expression was enhanced in the diabetic kidneys of both mouse strains, the elevated secretion from *db/db* mouse kidneys may be responsible for initiating the onset of microalbuminuria in diabetic nephropathy.

Key words macrophage migration inhibitory factor; diabetic nephropathy; microalbuminuria; *db/db* mouse; *ob/ob* mouse

Diabetic nephropathy is the primary causative disease leading to dialysis in Japan.¹⁾ The cumulative incidence of nephropathy after 30 years of post-pubertal diabetes was reported to be significantly higher in type 2 diabetic patients (44.4%) than in type 1 diabetic patients (20.2%).²⁾ However, diabetic nephropathy does not occur in all patients with type 2 diabetes mellitus, implying that there might be an unknown underlying mechanism to prevent the onset of diabetic nephropathy. Establishing the identity of such a mechanism is of vital importance.

It is thought that the onset of microalbuminuria, considered to be a marker of early diabetic nephropathy, must be suppressed in order to inhibit the onset and aggravation of nephropathy in type 2 diabetes. Two murine strains are available as an animal model for type 2 diabetes: *db/db* mice in which microalbuminuria develops at 8 weeks of age³⁾; and *ob/ob* mice that do not develop microalbuminuria at all. It is thought that a gene cluster associated with the onset of microalbuminuria could therefore be identified by comparing differences in gene expression between these two mouse strains, without considering a specific disease-developing mechanism underlying microalbuminuria.

The purpose of this study was therefore to identify a gene associated with the onset of microalbuminuria in the type 2 diabetic model mouse using a DNA microarray approach. Macrophage migration inhibitory factor (MIF) was identified as a gene for which expression changed in *db/db* mice, but not in *ob/ob* mice. Since earlier reports indicate that MIF is associated with glomerulonephritis and proteinuria,⁴⁾ the present study also aimed to analyze the expression of MIF in the

kidneys of *db/db* and *ob/ob* mice.

MATERIALS AND METHODS

Animals Experiments involved male *db/db* and *ob/ob* mice and non-diabetic controls (*db/m* and *ob* control mice) that were 2 months of age. Urine was sampled using metabolic cages. Mice were anesthetized by intra-peritoneal injections of 50 mg/kg pentobarbital in order to obtain blood and kidney samples. Serum glucose was measured enzymatically using Glucose CII-test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Serum insulin and leptin was assayed using Mouse Insulin enzyme-linked immunosorbent assay (ELISA) kit (H-type) and Mouse Leptin ELISA kit, respectively (Shibayagi, Gunma, Japan). Urinary albumin was assayed using Albuwell M kit (Exocell Inc., Philadelphia, U.S.A.). Macrophage Migration Inhibitory Factor in plasma or urine was assayed by ELISA kit for mouse MIF (Uscn Life Science Inc., Houston, U.S.A.). Serum and urinary creatinine was assayed using HPLC as described previously.⁵⁾ All animal experiments were carried out in accordance with Teikyo University Guide for the Care and Use of Laboratory Animals.

Microarray Analysis Total RNA was extracted from the kidneys of *ob* control, *ob/ob*, *db/m*, and *db/db* mice and purified as described previously.⁶⁾ Microarray analysis was performed using Affymetrix Mouse Gene ST 1.0 arrays and analyzed using Expression Console (PharmaFrontier Co., Ltd., Kyoto, Japan).

Real-Time Polymerase Chain Reaction (PCR) cDNA was prepared from total RNA using a High-Capacity cDNA Reverse Transcription Kit. Real-time PCR analyses were subsequently conducted as described previously⁶⁾ using TaqMan

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probes corresponding to macrophage migration inhibitory factor (Mm01611157_gH), and 18s ribosomal RNA (rRNA) as an internal standard.

Western Blotting Kidneys were homogenized in a specific solution (20mM Tris, 3M Urea, 0.5% CHAPS, 0.1M NaCl, pH 7.4) containing protease inhibitors (Complete Mini, Roche Diagnostics Japan, Tokyo). Homogenates (5 μ g/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a Nitrocellulose membrane. After blocking, membranes were stained with anti-mouse MIF antibody (0.5 μ g/mL; FL-115 (sc-20121); Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) and horseradish peroxidase (HRP) conjugated anti-rabbit immunoglobulin G (IgG) antibody (1:10000, ZYM 65-6120, Life Technologies Japan Ltd., Tokyo). An HRP-conjugated anti- β actin antibody (1:40000, β actin C4, sc-47778, Santa Cruz Biotechnology, Inc.) was used to calibrate sample loading.

Products were detected with an ECL kit (GE Healthcare UK Ltd., Little Chalfont, U.K.).

Immunofluorescence Paraffin sections were prepared from the kidneys of *ob* control, *ob/ob*, *db/m*, and *db/db* mice which had been perfused with 4% paraformaldehyde in 30mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer. Deparaffinized sections were stained with anti-mouse MIF antibody (1:50, Santa Cruz Biotechnology, Inc.) and Cy5-conjugated anti-rabbit IgG (H+L) (1:100, A10523, Life Technologies Japan Ltd., Tokyo, Japan).⁷⁾

Statistical Analyses All data, except that arising from DNA microarrays, are expressed as mean $\pm$ standard error (S.E.M.). Differences between groups were analyzed statistically using the student's *t*-test. *p* values <0.05 were considered statistically significant.

Table 1. Comparison of Clinical State of Diabetes

	<i>dm/m</i>	<i>db/db</i>	<i>ob</i> control	<i>ob/ob</i>
Body weight (g)	24.2 $\pm$ 0.5	41.1 $\pm$ 0.9**	27.5 $\pm$ 0.4	45.9 $\pm$ 0.7**
Volume of urine (mL/d)	1.0 $\pm$ 0.1	11.9 $\pm$ 1.3**	1.3 $\pm$ 0.2	6.5 $\pm$ 0.8**
Serum glucose (mg/dL)	214 $\pm$ 11	615 $\pm$ 52**	308 $\pm$ 21	366 $\pm$ 28
Serum insulin (ng/mL)	2.3 $\pm$ 0.6	11.5 $\pm$ 3.3	0.9 $\pm$ 0.3	30.6 $\pm$ 6.3*
Creatinine clearance (mL/d/cm ²)	0.23 $\pm$ 0.04	0.53 $\pm$ 0.05**	0.26 $\pm$ 0.05	0.49 $\pm$ 0.04**
Urinary albumin (μ g/d)	45.5 $\pm$ 11.3	332.4 $\pm$ 79.9* ^{##}		31.5 $\pm$ 9.5
In-blood leptin (pg/mL)	49 $\pm$ 21	7828 $\pm$ 1232* ^{##}		n.d.

p* < 0.05, *p* < 0.01 vs. control, ^{##}*p* < 0.01 vs. *ob/ob* (*n* = 4–10, mean $\pm$ S.E.M.).

Fig. 1. Screening of Candidate Genes Underlying Microalbuminuria in Diabetic *db/db* Mice

(A) Microarray study and data analysis. STEP1: Genes for which the expression ratio of (*db/db*)/(*db/m*) was higher than the mean+2S.D. or lower than the mean-2S.D. were screened. Since the gene expression ratio of (*db/db*)/(*db/m*) was 1.020 $\pm$ 0.193 (mean $\pm$ S.D.) from a total of 25527 genes, 476 genes were induced by over 1.406-fold and 258 genes were suppressed below 0.634-fold. STEP 2: Genes for which the expression ratio of (*ob/ob*)/(*ob* control) was higher than the mean+1S.D. or lower than the mean-1S.D. were excluded as genes for which expression changed by diabetes. Since the expression ratio of (*ob/ob*)/(*ob* control) was 1.026 $\pm$ 0.168 from 25527 genes, 280 genes in 476 genes, and 53 genes in the 258 genes for which the expression ratio of (*ob/ob*)/(*ob* control) was between 0.853- and 1.194-fold were screened. STEP 3: Genes for which the expression ratio of (*ob* control)/(*db/m*) was higher than the mean+1S.D. or lower than the mean-1S.D. were excluded as genes for which expression changed by strain differences. The expression ratio for (*ob* control)/(*db/m*) was 1.010 $\pm$ 0.253 from 25527 genes. Consequently, 169 genes of the 280 genes, and 19 genes of the 53 genes, for which the expression ratio of (*ob/ob*)/(*ob* control) was between 0.757- and 1.263-fold were screened as candidates of microalbuminuria-related genes. (B) Messenger RNA levels of MIF determined by quantitative PCR. *ob* control vs. *ob/ob* and the *db/m* vs. *db/db* strains were compared. The amount of MIF transcription in the kidney was elevated in *db/db* mice (*n* = 4–6, mean $\pm$ S.E.M., **p* < 0.05).

RESULTS AND DISCUSSION

Serum glucose levels of *db/db* mice was significantly higher than those of *db/m* mice ($p=0.017$), while those of *ob/ob* mice tended to be higher than those of *ob* control mice ($p=0.056$). However, the urinary volume, serum insulin, and body weight of *db/db* and *ob/ob* mice were significantly higher than those of control mice. Therefore, type 2 diabetes-induced polyuria and obesity appears to have been demonstrated in both *db/db* and *ob/ob* mice. The obesity of *ob/ob* mice was caused by defective leptin, which normally suppresses over-eating by stimulating the satiety center in the hypothalamus. Obesity in *db/db* mice is known to be caused by defects in leptin signaling at the satiety center⁸⁾ (Table 1). Microalbuminuria were clearly observed in *db/db* mice ($332.4 \pm 79.9 \mu\text{g/d}$, $p < 0.01$ vs. *ob/ob* mice), however, neither *db/m* mice ($45.5 \pm 11.3 \mu\text{g/d}$) nor *ob/ob* mice ($31.5 \pm 9.5 \mu\text{g/d}$) were free from microalbuminuria (Table 1). This concurs with an earlier report³⁾ which reported that all *db/db* mice develop microalbuminuria at 8 weeks of age, while *ob/ob* mice did not develop microalbuminuria at all. Therefore, these two strains of diabetic mice are appropriate models for DNA microarray analysis in an attempt to identify microalbuminuria-related genes.

Creatinine clearance of *db/db* ($0.53 \pm 0.05 \text{ mL/d/cm}^2$) and *ob/ob* ($0.49 \pm 0.04 \text{ mL/d/cm}^2$) mice was significantly higher ($p=0.002$, 0.043) than those of *db/m* ($0.23 \pm 0.04 \text{ mL/d/cm}^2$) and *ob* control ($0.26 \pm 0.05 \text{ mL/d/cm}^2$) mice, respectively (Table 1). These results imply that microalbuminuria may not always be induced by glomerular hyper-filtration. Consequently, this indicates that there may be another candidate underlying the cause of microalbuminuria, other than glomerular hyper-filtration.

In order to screen candidates for microalbuminuria-related genes with *db/db* and *ob/ob* strains, we conducted three microarray data analysis steps (Fig. 1A). In STEP 1, up or down-regulated genes were determined as candidates for microalbuminuria-related genes. Genes for which the expression ratio of (*db/db*)/(*db/m*) was higher than (mean+2S.D.) or lower than (mean-2S.D.) were screened. In STEP 2, genes for which the expression ratio of (*ob/ob*)/(*ob* control) was higher than the mean+1S.D. or lower than the mean-1S.D. were excluded as genes for which expression changed not by microalbuminuria, but by diabetes. In STEP 3, genes for which the expression ratio of (*ob* control)/(*db/m*) was higher than the mean+1S.D. or lower than the mean-1S.D. were excluded as genes for which expression changed not by microalbuminuria, but by differences involving the genetic background of *db/db* and *ob/ob* strains. Of the 25527 genes expressed in the mouse kidney, the expression of 169 and 19 genes were enhanced or suppressed as candidates for microalbuminuria-related genes in *db/db* mouse kidney, respectively. Table 2 demonstrates the 169 up-regulated genes and the 19 down-regulated genes in the order of expression levels of *db/db* mouse kidney.

Microarray studies demonstrated that macrophage migration inhibitory factor gene (MIF) was the most expressed gene in the 169 up-regulated microalbuminuria-related genes. Using quantitative PCR, MIF mRNA levels in *db/db* mice were significantly higher than those in *db/m* mice (Fig. 1B). Moreover, the production of MIF is known to increase at the crescentic anti-glomerular basement membrane glomerulonephritis with nephrosis⁹⁾ while proteinuria is known to occur in glomerular

Table 2. DNA Microarray Data

Gene	Full name	Ratio
Up-regulated genes		
<i>Gm16379</i>	macrophage migration inhibitory factor-like	1.556
<i>Mif</i>	macrophage migration inhibitory factor	1.625
<i>Mif</i>	macrophage migration inhibitory factor	1.640
<i>Nr1d1</i>	nuclear receptor subfamily 1, gr. D, mbr. 1	1.558
<i>Aqp2</i>	aquaporin 2	1.626
<i>Prodh</i>	proline dehydrogenase	1.732
<i>Efh1</i>	EF hand domain containing 1	1.628
<i>Scnn1a</i>	sodium channel, nonvoltage-gated 1 alpha	1.447
<i>Vamp2</i>	vesicle-associated membrane protein 2	1.651
<i>Acot2</i>	acyl-CoA thioesterase 2	1.527
<i>Cyp2d9</i>	cytochrome P450, fam. 2d, polypeptide 9	1.469
<i>Hspa1a</i>	heat shock protein 1A	1.520
<i>Tspan7</i>	tetraspanin 7	1.464
<i>Ifi30</i>	interferon gamma inducible protein 30	1.642
<i>Pctk3</i>	PCTAIRE-motif protein kinase 3	1.478
<i>Cabc1</i>	chaperone, ABC1 activity of bc1 complex like	1.545
<i>Mfge8</i>	milk fat globule-EGF factor 8 protein, tr-var. 1	1.416
<i>Dmgdh</i>	dimethylglycine dehydrogenase precursor	1.523
<i>Cyp27a1</i>	cytochrome P450, fam. 27a, polypeptide 1	1.492
<i>Pagr5</i>	progesterone and adipoQ receptor family mbr. V	1.419
<i>Aqp3</i>	aquaporin 3	1.467
<i>Pdxk</i>	pyridoxal kinase	1.569
<i>Dnajb4</i>	DnaJ (Hsp40) homolog, subfam. B, mbr. 4	1.914
<i>Scnn1g</i>	sodium channel, nonvoltage-gated 1 gamma	1.421
<i>Sec14l1</i>	SEC14-like 1	1.517
<i>Hspa1a</i>	heat shock protein 1A	2.004
<i>Scnn1b</i>	sodium channel, nonvoltage-gated 1 beta	1.425
<i>Brd2</i>	bromodomain containing 2, var. 2	1.467
<i>Nicn1</i>	nicotin 1	1.408
<i>Gstt3</i>	glutathione S-transferase, theta 3	1.713
<i>Gys1</i>	glycogen synthase 1, muscle	1.578
<i>Hk1</i>	hexokinase 1, var. 2	1.517
<i>Abhd6</i>	abhydrolase domain containing 6	1.454
<i>Gsta4</i>	glutathione S-transferase, alpha 4	1.551
<i>Rnf186</i>	ring finger protein 186	1.835
<i>Angptl3</i>	angiopoietin-like 3	1.890
<i>Myof</i>	myoferlin	1.619
<i>Foxl1</i>	forkhead box 11	1.696
<i>Fam38a</i>	Fam38a protein gene	1.421
<i>Fmo4</i>	flavin containing monooxygenase 4	1.491
<i>Sfxn2</i>	sideroflexin 2	1.430
<i>Bag3</i>	BCL2-associated athanogene 3	1.490
<i>Slc14a1</i>	solute carrier fam. 14 (urea), mbr. 1	2.437
<i>Fbxo44</i>	F-box protein 44, tr-var. 1	1.442
<i>Deaf1</i>	deformed epidermal autoregulatory factor 1, tr-var. 2	1.426
<i>4930572J05Rik</i>	RIKEN cDNA	1.638
<i>Abp1</i>	amiloride binding protein 1, tr-var. 3	1.706
<i>Wsb1</i>	WD repeat & SOCS box-containing 1, tr-var. 1	1.928
<i>Gylt1b</i>	glycosyltransferase-like 1B	1.572
<i>Mpzl2</i>	myelin protein zero-like 2	1.605
<i>Sulf2</i>	sulfatase 2 (Sulf2)	1.422
<i>Dnajb1</i>	DnaJ (Hsp40) homolog, subfam. B, mbr. 1	1.413
<i>Fus</i>	fusion, from t(12;16) malignant liposarcoma	1.426
<i>Htra2</i>	HtrA serine peptidase 2	1.425
<i>Shmt1</i>	serine hydroxymethyltransferase 1	1.474
<i>Abtb2</i>	ankyrin repeat and BTB domain containing 2	1.488
<i>Syncn</i>	syncollin	1.440
<i>Slc45a3</i>	solute carrier family 45, mbr. 3	1.487
<i>2310081J21Rik</i>	adult male tongue cDNA, RIKEN	1.447

Table 2. DNA Microarray Data

<i>2310081J21Rik</i>	adult male tongue cDNA, RIKEN	1.447	<i>Cyp17a1</i>	cytochrome P450, fam. 17a, polypeptide 1	1.640
<i>2310081J21Rik</i>	adult male tongue cDNA, RIKEN	1.447	<i>Tbx18</i>	T-box18	1.610
<i>Cyp2s1</i>	cytochrome P450, fam. 2s, polypeptide 1	1.421	<i>Slc15a1</i>	solute carrier fam. 15 (oligopeptide), mbr. 1	1.676
<i>Aldoc</i>	aldolase C, fructose-bisphosphate	1.543	<i>Bex4</i>	brain expressed gene 4	1.427
<i>Mgll</i>	monoglyceride lipase	1.419	<i>Epha3</i>	Eph receptor A3	1.442
<i>Unc45a</i>	unc-45 homolog A	1.425	<i>Itgad</i>	integrin, alpha D	1.411
<i>Pfkip</i>	phosphofructokinase, platelet	1.503	<i>Rgn</i>	regucalcin	1.568
<i>Prkag3</i>	AMP kinase, gamma 3, non-catalytic subunit	1.790	<i>Olfr9</i>	olfactory receptor 9	1.477
<i>Shpk</i>	sedoheptulokinase	1.496	<i>Trpc7</i>	TRP cation channel, subfam.C, mbr. 7	1.490
<i>D430041B17Rik</i>	RIKEN cDNA	1.425	<i>Gm7039</i>	predicted gene similar to calmodulin.	1.435
<i>Fstl3</i>	folliculin-like 3	1.463	<i>Bicc1</i>	bicaudal C homolog 1	1.495
<i>Tubb2a</i>	tubulin, beta 2A	1.411	<i>Gpr101</i>	G protein-coupled receptor 101	1.450
<i>Bcl6</i>	B-cell CLL/lymphoma 6	1.522	<i>Olfr312</i>	olfactory receptor 312	1.558
<i>Upp2</i>	uridine phosphorylase 2	1.452	<i>Eda2r</i>	ectodysplasin A2 isoform receptor, tr-var. 1	1.419
<i>Pappa</i>	pregnancy-associated plasma protein A	1.532	<i>Spata18</i>	spermatogenesis associated 18	1.472
<i>Arndc4</i>	arrestin domain containing 4, tr-var. 1	1.503	<i>Gabrb1</i>	GABA-A receptor, subunit beta 1	1.643
<i>Ier5l</i>	immediate early response 5-like	1.452	<i>Vip</i>	vasoactive intestinal polypeptide	1.438
<i>Acot1</i>	acyl-CoA thioesterase 1	1.606	<i>V1ra3</i>	vomerionasal 1 receptor, A3	1.484
<i>Ppm1k</i>	protein phosphatase 1K	1.452	<i>Olfr1164</i>	olfactory receptor 1164	1.812
<i>2610301F02Rik</i>	MCG142258, isoform CRA	1.485	<i>Rai2</i>	retinoic acid induced 2, tr-var. 1	1.616
<i>AK220484</i>	cDNA sequence	1.600	<i>Lcn3</i>	lipocalin 3	1.429
<i>Atp2b4</i>	ATPase, Ca ²⁺ transporting, plasma mbr. 4	1.616	<i>Olfr1459</i>	olfactory receptor 1459	1.618
<i>Snrgp</i>	small nuclear ribonucleoprotein polypeptide G	1.450	<i>Spt1</i>	salivary protein 1	1.407
<i>Ddit4l</i>	DNA-damage-inducible transcript 4-like	1.507	<i>Olfr611</i>	olfactory receptor 611	1.588
<i>Sntal</i>	syntrophin, acidic 1	1.511	<i>A530021J07Rik</i>	putative uncharacterized protein	1.429
<i>Affl</i>	AF4/FMR2 fam., mbr. 1	1.407	<i>Olfr1166</i>	olfactory receptor 1166	1.482
<i>Cuedc1</i>	CUE domain containing 1	1.476	<i>Gm10667</i>	putative uncharacterized protein	1.661
<i>Ras11b</i>	RAS-like, fam. 11, mbr. B	1.487	<i>Hsd3b1</i>	hydroxy-Δ-5-steroid dehydrogenase, 3β-steroid-Δ-isomerase 1	1.714
<i>Gprc5b</i>	G protein-coupled receptor, fam. C, gr. 5, mbr. B	1.423	<i>Gm5793</i>	pseudogene	1.457
<i>Brms1</i>	breast cancer metastasis-suppressor 1	1.415	<i>Olfr1255</i>	olfactory receptor 1255	1.513
<i>Lypd6</i>	LY6/PLAUR domain containing 6	1.462	<i>Mup20</i>	predicted gene	1.764
<i>1810010H24Rik</i>	RIKEN cDNA	1.550	<i>Nlrp4c</i>	NLR family, pyrin domain containing 4C	1.557
<i>Hsd17b14</i>	hydroxysteroid (17-beta) dehydrogenase 14	1.432	<i>Svs3b</i>	seminal vesicle secretory protein 3B	1.641
<i>Tspan1</i>	tetraspanin 1	1.411	<i>5530400C23Rik</i>	putative uncharacterized protein	1.542
<i>Fbp2</i>	fructose bisphosphatase 2	1.488	<i>Olfr495</i>	olfactory receptor 495	1.494
<i>Th1l</i>	TH1-like homolog	1.465	<i>Cyp3a57</i>	cytochrome P450, fam. 3a, polypeptide 57	1.490
<i>Penk</i>	preproenkephalin	1.638	<i>Olfr1000</i>	olfactory receptor 1000	1.686
<i>Gpt</i>	glutamic pyruvic transaminase, soluble	1.527	<i>Ssx10</i>	synovial sarcoma, X member B, breakpoint 10	1.545
<i>Freq</i>	frequenin homolog	1.513	<i>Vmn2r78</i>	vomerionasal 2, receptor 78	1.742
<i>1110006O24Rik</i>	RIKEN cDNA	1.576	<i>V1rc21</i>	vomerionasal 1 receptor, C21	1.419
<i>Cck</i>	cholecystokinin	1.407	<i>Olfr516</i>	olfactory receptor 516	1.636
<i>Nes</i>	nestin	1.440	<i>Zfp811</i>	zinc finger protein 811	1.413
<i>Dph2</i>	DPH2 homolog	1.419	<i>Cyp2c68</i>	cytochrome P450, fam. 2c, polypeptide 68	1.549
<i>Aen</i>	apoptosis enhancing nuclease, tr-var. 1	1.557	<i>V1rd4</i>	vomerionasal 1 receptor, D4	1.517
<i>Krt34</i>	keratin 34	1.407	<i>LOC100045278</i>	predicted similar to ribosomal protein S25	1.513
<i>Fosb</i>	FBJ osteosarcoma oncogene B	1.599	<i>Wfdc6b</i>	WAP four-disulfide core domain 6B	1.467
<i>Cadps2</i>	Ca ²⁺ -dep. activator protein for secretion 2	1.482	<i>9830004L10Rik</i>	adult male bone cDNA	1.698
<i>Tmem54</i>	transmembrane protein 54	1.477	<i>Gm10848</i>	putative uncharacterized protein	1.469
<i>Nfil3</i>	nuclear factor, interleukin 3, regulated	1.466			
<i>Spag5</i>	sperm associated antigen 5	1.947			
<i>Gm9743</i>	predicted gene 9743	1.430			
<i>Chkb</i>	choline kinase beta	1.425			
<i>Grem2</i>	gremlin 2 homolog, cysteine knot superfamily	1.663			
<i>Bhlhe41</i>	basic helix-loop-helix family, mbr. e41	1.788			
<i>Zfp69</i>	zinc finger protein 69	1.569			
<i>Olfr373</i>	olfactory receptor 373	1.566			
<i>Zfp395</i>	zinc finger protein 395	1.409			
<i>Apoc1</i>	apolipoprotein C-I, tr-var. 1	1.424			
<i>Tspan2</i>	tetraspanin 2	1.462			
<i>1700047G07Rik</i>	putative uncharacterized protein	1.576			
<i>Chd7</i>	chromodomain helicase DNA binding protein 7	1.548			
<i>Gabarapl2</i>	GABA(A) receptor-associated protein like 2	1.593			

Table 2. DNA Microarray Data

Gene	Full name	Ratio
Down-regulated genes		
<i>Inmt</i>	indolethylamine <i>N</i> -methyltransferase	0.607
<i>Slco1a6</i>	solute carrier organic anion transporter fam., mbr. 1a6	0.581
<i>C730048C13Rik</i>	RIKEN cDNA	0.306
<i>Tmigd1</i>	transmembrane & immunoglobulin domain containing 1	0.631
<i>Etv1</i>	ets variant gene 1	0.588
<i>Stxbp5l</i>	syntaxin binding protein 5-like, tr-var.xb	0.633
<i>Aplnr</i>	apelin receptor	0.632
<i>Sp100</i>	nuclear antigen Sp100	0.615
<i>Asxl3</i>	isoform 2 of putative polycomb gr. protein	0.553
<i>Gbp6</i>	guanylate binding protein 6	0.629
<i>Gm4802</i>	predicted gene	0.524
<i>Vlrc26</i>	vomeronal 1 receptor, C26	0.615
<i>Gm11272</i>	predicted gene	0.633
<i>Prkar2b</i>	cAMP dependent protein kinase, type II beta	0.600
<i>Hmcn1</i>	hemicentin 1	0.567
<i>Lepr</i>	leptin receptor, tr-var. 3	0.613
<i>Vlrc10</i>	vomeronal 1 receptor, C10	0.539
<i>F730021E23Rik</i>	B6-derived CD11 +ve dendritic cells cDNA	0.619
<i>Gm9443</i>	predicted: similar to LNR42	0.611

Abbreviations: gr, group; mbr, member; fam, family; tr-var., transcription variant; dep, dependent.

epithelium-specific MIF transgenic mice.¹⁰) Thus, MIF was screened as a possible candidate for the induction of microalbuminuria in diabetic *db/db* mice by up-regulated transcription in the kidneys and by its own proteinuric properties.

Among the other genes in Table 2, muscle glycogen synthase 1 gene (*Gys1*) was reported that its polymorphism is related to hypertension and microalbuminuria.¹¹) Fructose-bisphosphate aldolase C gene (*Aldoc*) was reported that its expression was enhanced together with MIF gene in breast cancer.¹²) Integrin alpha D (*Itgad*) was reported to be related to macrophage migration.¹³) Although these genes may be revealed as further candidate genes for the induction of microalbuminuria, we decided to further investigate the protein profile of MIF in diabetic *db/db* and *ob/ob* mice.

Urinary excretion of MIF protein increased significantly only in *db/db* mice (Fig. 2A). Moreover, the increase of urinary secretion of MIF was observed significantly from 6-week-old (Fig. 2B). However, the urinary albumin was increased significantly from 8-week-old, as described in earlier report. Namely, the urinary secretion of MIF preceded microalbuminuria. Consequently, the urinary secretion of MIF is not induced by microalbuminuria, but a possible candidate for the inducer of microalbuminuria in diabetic *db/db* mice. Contrary to the MIF mRNA, MIF protein levels were significantly higher in the kidneys in both *db/db* and *ob/ob* mice

Fig. 2. Expression Profile of MIF Protein

Ob control vs. *ob/ob*, and the *db/m* vs. *db/db* strains were compared. Urinary excretion of MIF protein (A) and MIF protein levels in the kidney (C) ($n=4-6$, mean $\pm$ S.E.M., * $p<0.05$, ** $p<0.01$). The amount of MIF excretion in the urine was elevated in *db/db* mice. Weekly profile of urinary excretion of MIF (closed circle and continuous line) and albumin (open circle and dashed line) in *db/db* mice (B) ($n=3$, mean $\pm$ S.E.M., * $p<0.05$, ** $p<0.01$). The urinary excretion of MIF preceded the onset of microalbuminuria.

Fig. 3. Localization of MIF Protein in the Kidney

Expression of MIF protein was demonstrated in the tubules or interstitium (A,D) of the kidneys from control mice. MIF appeared to be localized to the Bowman's capsule in the kidneys of *ob/ob* mice (B,C) and *db/db* mice (E,F).

(Fig. 2C). MIF exists in cells in preformed pools and secreted in response to a variety of stimuli including inflammation and hypoxia.¹⁴ Consequently, MIF protein was secreted only from the kidneys of *db/db* mice, but was accumulated in the kidneys of both *db/db* and *ob/ob* mice. Another possible mechanism underlying the observed increase in urinary MIF protein is that MIF protein in the glomerular filtrate may not have been reabsorbed by the tubule and was thus excreted into the urine. However, MIF protein reabsorption failure at the tubule cannot explain the elevated levels of MIF mRNA in the kidneys of *db/db* mice. Consequently, it is possible that the observed increase in urinary MIF protein could be due to secretion of MIF protein from the kidneys of *db/db* mice.

The MIF receptor CD74 is increased in tubular cells and podocyte in human diabetic nephropathy. MIF engagement of CD74 activated mitogen activated protein (MAP) kinase and increased tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) expression in podocyte, which promote a modest increase in podocyte injury and cell death.¹⁵ Podocyte overexpression of MIF caused podocyte injury and proteinuria in mice without macrophage migration.¹⁰ Thus, we consider that the observed secretion of urinary MIF protein may be associated with the onset of microalbuminuria in diabetic *db/db* mice. Although it was suggested that in type 2 diabetes macroalbuminuria is the main predictor of mortality, independently of both eGFR and cardiovascular risk factors,¹⁶ the spectrum of diabetic nephropathy has recently expanded, as lack of significant albuminuria is present in 30% of dia-

betics with kidney function impairment.¹⁷ The MIF-induced TRAIL expression in tubular cell and podocyte also causes renal cell loss and impairment of kidney function. Thus, further study is necessary to clarify the mechanism of secretion of urinary MIF protein observed in diabetic *db/db* mice.

It has already been reported that MIF production in the kidneys of 8-month-old (32-week-old) *db/db* mice invaded by macrophages results in an increase in proinflammatory cytokines, MCP-1 and TNF- α .¹⁸ In the same report, the invasion of MIF-producing macrophages and an increase in proinflammatory cytokines were not observed in the kidneys of 2-month-old *db/db* mice. Our current study also did not result in the invasion of MIF-producing macrophages in the kidneys of 2-month (8-week)-old *db/db* mice. Rather, MIF was localized to the Bowman's capsule epithelium in the kidneys of both *db/db* and *ob/ob* mice (Fig. 3), in agreement with previous data concerning normal human kidneys.¹⁹ Furthermore, chemokines, which are normally induced by macrophages such as MCP-1 and TNF- α , were not elevated in our microarrays. In other words, there was no evidence of proinflammatory cytokine involvement in relation to microalbuminuria in the kidney of early 8-weeks-old *db/db* mice.

In conclusion, we suggest that the onset of microalbuminuria in early diabetic nephropathy may be associated with the urinary secretion of intra-renal MIF.

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