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Basic Research

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Genetic characterization of early renal changes in a novel mouse model of diabetic kidney disease

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Genetic factors influence susceptibility to diabetic kidney disease (DKD). Here we mapped genes mediating renal hypertrophic changes in response to diabetes. A survey of 15 mouse strains identified variation in diabetic kidney hypertrophy. Strains with greater (FVB/N(FVB)) and lesser (C57BL/6 (B6)) responses were crossed and diabetic F2 progeny were characterized. Kidney weights of diabetic F2 mice were broadly distributed. Quantitative trait locus analyses revealed diabetic mice with kidney weights in the upper quartile shared alleles on chromosomes (chr) 6 and 12; these loci were designated as *Diabetic kidney hypertrophy (Dkh)*-1 and -2. To confirm these loci, reciprocal congenic mice were generated with defined FVB chromosome segments on the B6 strain background (B6.*Dkh1/2f*) or vice versa (FVB.*Dkh1/2b*). Diabetic mice of the B6.*Dkh1/2f* congenic strain

developed diabetic kidney hypertrophy, while the reciprocal FVB.*Dkh1/2b* congenic strain was protected. The chr6 locus contained the candidate gene; Ark1b3, coding aldose reductase; the FVB allele has a missense mutation in this gene. Microarray analysis identified differentially expressed genes between diabetic B6 and FVB mice. Thus, since the two loci identified by quantitative trait locus mapping are syntenic with regions identified for human DKD, the congenic strains we describe provide a valuable new resource to study DKD and test agents that may prevent it.

Keywords: alloxan; congenic mice; diabetes; diabetic kidney disease; mouse model; quantative trait loci

Translational Statement

In this study, we have identified genetic loci and candidate genes that mediate DKD. Other studies have identified specific genes that contribute to early renal damage; however, there has not been any mouse model that showed a reciprocal effect. Our work provides not only a murine-susceptible DKD model with susceptibility loci identified in humans, but also a protected one. Genes within the loci can now be used as targets to identify the mechanism underlying early changes in DKD. The defined genes may also enable personalized care for patients and the development of targeted treatments for DKD to the human orthologs of the loci we identified.

DKD is the most common cause of end-stage renal disease in Western countries and occurs in about a third of subjects with either type 1 diabetes or type 2 diabetes (see the review by Alsahli and Gerich[1]). Early changes in the diabetic kidney include renal hypertrophy, with a disproportionate increase in glomerular matrix and mesangial cell volume,[2,3] thickening of the basement membrane and occlusion of the glomerular capillary lumen,[3-5] an elevated glomerular filtration rate,[6,7] and microalbuminuria.[8,9]

Disturbances in glucose homeostasis drive early DKD and can often be alleviated by strict glycemic control.[10,11] However, despite this strong relationship between hyperglycemia and renal disease, not all individuals with poorly controlled diabetes proceed to renal failure. Indeed, some individuals with well-controlled diabetes develop progressive kidney disease.[12] Thus, individual genetic factors likely play a role in determining the susceptibility to renal disease in diabetes that is supported by family studies.[13,14] Several genome wide association studies (GWAS) and meta-analyses have been conducted on various human populations to identify novel susceptibility loci for DKD.[15,16] However, these studies' findings remain inconsistent, demonstrating that it is difficult to identify the genetic mechanism involved in the complex disease of DKD (reviewed by Regele *et al*[17]), suggesting that a new approach could be useful.

Mouse models have been successfully used to define genetic susceptibility to complex diseases such as type 1 diabetes,[18-20] cancer,[21] schizophrenia,[22] atherosclerosis,[23] and Alzheimer's disease.[24] Hence mapping rodent susceptibility genes could help identify genes that influence DKD in humans. This approach was successfully applied to identify the genetic locus, *Rf1*, which confers increased susceptibility to end-stage renal disease, in rodent models[25] and in African American individuals.[26] Further evidence that there is genetic variability in kidney disease within rodent strains includes the description of an NZW-derived gene that increases the risk for lupus-induced glomerulonephritis,[27] the susceptibility of BioBreeding (BB) diabetes-resistant but not BB diabetes-susceptible rats to renal lesions,[28] and development of renal disease with obesity and type 2 diabetes in *db/db*, KK, and New Zealand White (NZO) mice.[25,29]

Hyperglycemia commonly induces rapid changes in kidney function and structure. This is evidenced from studies using glucose clamps in human volunteers where acute exposure to hyperglycemia even over the course of an hour increases glomerular filtration rate.[30] Furthermore, glucose perfusion increases glomerular filtration rate in kidneys isolated from normal rats.[31] Glomerular hyperfiltration is often observed in individuals with newly diagnosed type 1 diabetes.[32] Significantly, the presence of hyperfiltration in early diabetes has been linked with a greater risk of the subsequent development of DKD.[12,33] Therefore, identification of genes that regulate the early kidney responses to hyperglycemia in the mouse could provide relevant therapeutic targets for human DKD and perhaps other complications of diabetes.

Results

Changes in kidney weight and histopathology in response to diabetes

A survey of 15 mouse strains was conducted. Seven days after the induction of diabetes, body weight and kidney weight were recorded for both male and female (diabetic) mice. Age-matched nondiabetic controls were also characterized. The average weight of both kidneys represented the wet kidney weight (mg) relative to the body weight (g); this was calculated for each of the 15 strains (Figure 1). The FVB strain was chosen for further work because it had the greatest increase in kidney weight with diabetes (Figure 1). Although many strains exhibited a low kidney to body weight score, the B6 mouse was chosen as the other progenitor as it is one of the most commonly used inbred mouse strains and has well-characterized genetics. Kidneys from the FVB and B6 mice were taken for histologic analysis; the samples were blinded and scored for renal changes in response to diabetes.[34] Compared with the B6 diabetic controls (scored 1.89 ± 0.04) (Figure 2a), the FVB diabetic kidneys (21 days) showed a marked increase in glomerulosclerosis (2.59 ± 0.13; $P < 0.0001$) (Figure 2b). We used this strain to characterize the genetic basis of this trait, and

to map the genes that were responsible.

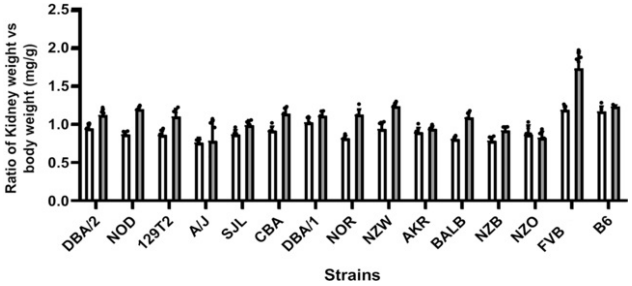

Figure 1 Murine strain survey of renal hypertrophy in diabetes. Kidney weight was measured and expressed as a ratio to body weight in both nondiabetic (white) and diabetic (gray) mice. *$P < 0.05$.

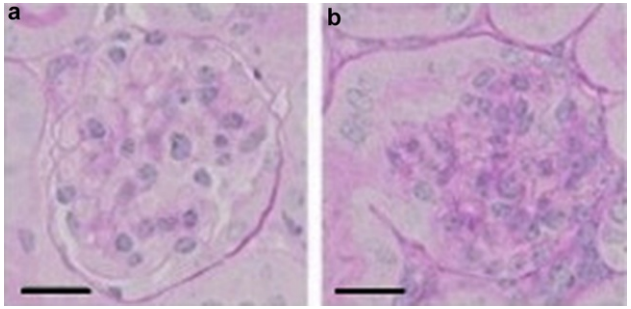

Figure 2 Renal histology of diabetic B6 and FVB mice. FVB diabetic kidneys (**b**) show a marked increase in glomerulosclerosis compared with the B6 (**a**). Black scale bar represents 20 μm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Characterization of (FVB × B6) F2 generation of mice

The kidney weight to body weight ratio (KW:BW) in the (FVB × B6) F2 mice displayed a broad distribution exceeding the range of both parental strains (Figure 3). A low-density (approximately 10 cM) genome-wide scan was performed by genotyping F2 mice (n = 88) in the highest and lowest quartiles for KW:BW at 95 markers spread across the 19 autosomal chromosomes. Logarithm of the odds (LOD) scores was calculated as a measure of linkage at each marker (Figure 4a). From this scan, we identified 2 major loci, one on chr6 and another on chr12 (Figure 4a).

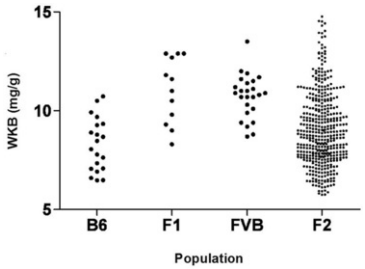

Figure 3 The distribution of wet kidney/body weight (WKB) amongst progenitor, F1, and F2 murine populations. B6 (n = 20), (FVB × B6) F1 (n = 12), FVB (n = 25), and (FVB × B6) F2 mice (n = 434). The *Y* axis represents the wet kidney weight (mg) normalized to body weight (g), WKB (mg/g). Except for F1 (male only), male and female mice are included, but not distinguished in this figure (no significant differences were seen between sexes).

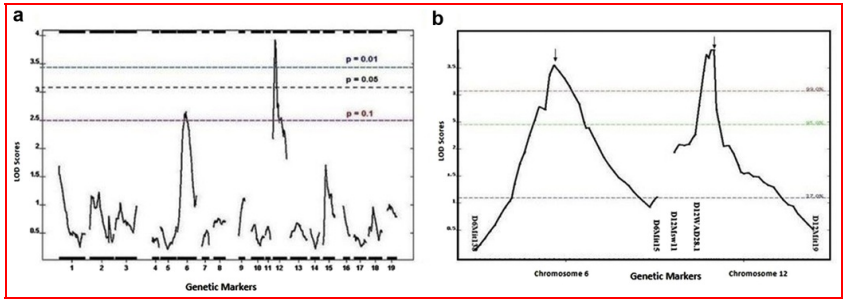

Figure 4 Main effect QTLs for the kidney weight to body weight ratio. (**a**) Logarithm of odds (LOD) scores are shown at markers for all autosomes, where the black line shows the $P = 0.05$ threshold for significant linkage, the red line represents the $P = 0.1$ threshold for suggestive linkage, and the blue line represents the $P = 0.01$ threshold. (**b**) Fine mapping of significant QTL marker positions is shown for chromosomes 6 and 12 with marker imputation at 1 Mb intervals. The Y axis represents the LOD score, the measure of genetic linkage. Significance threshold denoted by the 95th percentile ($P < 0.05$) (green line). Arrows indicate peak QTLs in chromosome 6 (*D6Mit188*, LOD Score 3.551) and chromosome 12 (*D12WAD39.8*, LOD Score 3.834). Loci were deemed significant if they exceeded the LOD threshold of 3.5. Permutation testing N = 1000.

To refine these regions, markers within the peak intervals were tested (Supplementary Table S1), and QTL analysis was performed (Figure 4b). The most significant markers were identified on chr6 at a position of 86 Mbp (LOD = 3.55) and on chr12 (LOD = 3.88) at 39.3 Mbp.

Analysis of the genotypes on chr6 for mice with renal hypertrophy (i.e., high KW:BW) in response to diabetes showed an absence of B6 homozygotes at this locus, suggesting that the FVB allele dominantly conferred susceptibility to diabetes-induced renal changes (Figure 5a). In contrast, mice that exhibited a low KW:BW ratio had greater B6 homozygosity. The opposite results were demonstrated on chr12, with none of the high KW:BW responders being FVB homozygous and the low KW:BW mice showing FVB homozygosity (Figure 5b). These results suggest that the FVB allele at this locus was protective (because all the protected mice were FVB homozygous). These loci are here after referred to as *diabetic kidney hypertrophy 1* (*Dkh1*) and *Dkh2*, respectively.

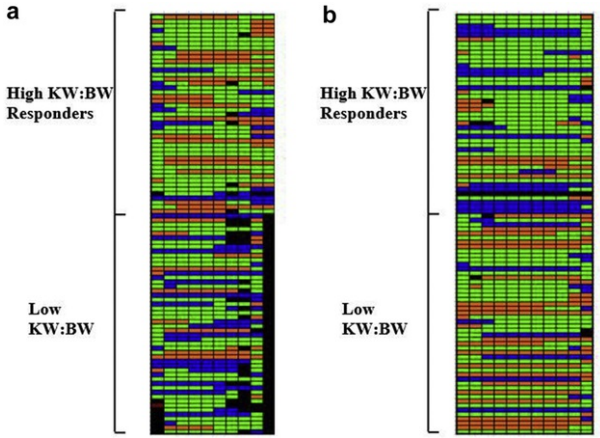

Figure 5 Genotype data for chromosomes 6 (a) and 12 (b). Each column represents individual markers. Each row represents a different mouse sample. Genotypes are shown as colored squares; B6 homozygous (blue), heterozygous (green); FVB homozygous (red); missing data (black). KW:BW, kidney weight to body weight ratio.

Test for gene-gene interaction (epistasis)

A 2-QTL scan was performed to determine whether the QTLs interacted additively or epistatically (Figure 6). The *Dkh1* locus on chr6 interacted additively with *Dkh2* on chr12, with LOD scores over 7. An epistatic interaction was observed using the method of Carlborg and Haley[35] between markers on chr6 and another locus on chr12, with LOD scores around 3. The new locus was associated with the marker D12Mit19 and the end of chr 12. These results indicate that there are 3 major loci, with *Dkh1* and *Dkh2* having additive effects and *Dkh3* interacting epistatically with *Dkh1*. This demonstrates the genetic complexity controlling diabetes-induced renal changes in these mice.

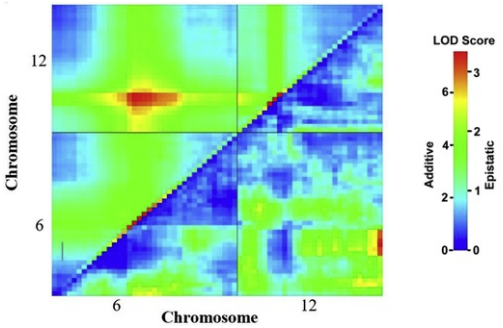

Figure 6 Interactive QTL analyses of mouse chromosomes 6 and 12. Additive interactions are shown above the diagonal. Epistatic interactions are represented below the diagonal. Chromosomes are shown on the horizontal and vertical axes. Significant thresholds are indicated in red on the heat map (scale at the right). Additive and epistatic logarithm of odds (LOD) scores were significant if they exceeded the values of 7 and 3, respectively.

Candidate genes showing differential gene expression

There are approximately 200 genes within the QTL intervals identified on chr6 and 12. We used the Sentix BeadChip Array MouseRef8 to examine global gene expression patterns between B6 and FVB diabetic and nondiabetic kidneys. Genes were identified that were differentially expressed between B6 and FVB kidneys at 21-day diabetic, using Illumina DiffScore.

Within the QTL regions, 16 genes on chr6 and 8 on chr12 were differentially expressed between the FVB and B6 diabetic mice at day 21 (Table 1). A total of 113 genes were differentially expressed between FVB nondiabetic and FVB diabetic mice (Supplementary Table S2). There were no significant changes in gene expression between B6 diabetic and nondiabetic controls. This suggests that changes in the expression of genes in the regions that we identified in kidneys from diabetic FVB mice were associated with kidney hypertrophy.

Table 1 Genes within the candidate regions differentially expressed between B6 and FVB diabetic and nondiabetic mice at 21 days after induction of diabetes

Gene name	Chromosome	log2-fold change	*P* value	Accession number	Gene function
MGST1	6	4.1525	0.0013	NM_019946.4	Microsomal glutathione S-transferase
INMT	6	3.1824	0.0005	NM_009349.1	N-methylation of endogenous and xenobiotic compounds
SSPN	6	2.8882	0.0019	NM_010656.2	A link between the F-actin cytoskeleton and the extracellular matrix
COL1A2	6	2.2643	0.0130	NM_007743.2	Forms pro-alpha2 chain of type I collagen
FGFR1OP2	6	1.9604	0.0028	NM_026218.2	Protein homodimerization activity
H2AFJ	6	1.4490	0.0382	NM_177688.2	Involved in the nucleosome structure of the chromosomal fiber
AKR1B3	6	1.2663	0.0105	NM_009658.2	aldo/keto reductase superfamily
ABCC9	6	1.2420	0.0132	NM_021041.2	Sulfonylurea receptor 2
PARP12	6	1.1947	0.0282	NM_172893.2	NAD+ ADP-ribosyltransferase activity
ART4	6	1.0005	0.0240	NM_026639.2	Post-translational modification
AKR1B8	6	0.9823	0.0268	NM_008012.1	aldo/keto reductase superfamily
TACSTD2	6	0.7366	0.0557	NM_020047.3	Encodes a carcinoma-associated antigen
KRAS	6	−1.2616	0.0118	NM_021284.4	Proto-oncogene

ANXA4	6	−1.3881	0.0159	NM_013471.1	Membrane-related events along the exocytotic and endocytotic pathways
SLC13A4	6	−2.0417	0.0033	NM_172892.1	Transporter activity and sodium:sulfate symporter activity
BCAT1	6	−2.0759	0.0165	NM_007532.2	Catalyzes the reversible transamination
MAP4K5	12	1.3232	0.0109	NM_024275.2	Encodes a member of the serine/threonine protein kinase family
1700047I17RIK1	12	1.2245	0.0276	NM_028527.1	Uncharacterized
MBOAT2	12	1.1832	0.0165	NM_001083341.1	1-Acylglycerol-3-phosphate O-acyltransferase activity
TTC15	12	−1.1545	0.0248	NM_178811.3	Affiliated with noncoding RNA
BC022687	12	−1.2319	0.0211	NM_145450.3	Clathrin binding box of aftiphilin containing 1
GREB1	12	−1.3871	0.0094	NM_015764.1	Important role in hormone-responsive tissues and cancer
SERPINA3H	12	−1.4869	0.0056	NM_001034870.2	Uncharacterized
SERPINA6	12	−1.8823	0.0035	NM_007618.2	Major transport protein for glucorticoids and progestins in the blood

Of interest are several genes on chr 6 linked to DKD: microsomal glutathione S-transferase 1 (*Mgst1*), a candidate for DKD;[36] collagen type I alpha 2 chain (*Col1a2*), a key feature of DKD;[37] aldo-keto reductase family 1, member B8 (*Akr1b8*) functions in biochemical processes including the polyol pathway that is linked to DKD;[38] and aldo-keto reductase family 1, member B3 (*Akr1b3*), which has been implicated in the amelioration of key endpoints in early diabetic nephropathy. Only 1 gene is known to be linked to DKD on chr 12, that is, growth regulation by estrogen in breast cancer 1-like (*Greb1l*), a gene reported as a target of retinoic acid signaling.[39]

Production and characterization of congenic mice

Reciprocal congenic strains were produced by repeated backcrossing, selecting breeders at each generation carrying introgressed genomic intervals identified by QTL mapping. These strains are denoted as FVB.*Dkh1/2*^{f} and B6.*Dkh1/2*^{b}. Where the peak QTL on chr 6 was genotyped between markers D6Mit15-D6Mit138 and the peak at D6Mit188, where the 99th percentile was between 77 and 89 Mbp. The corresponding QTL on chr 12 was genotyped between markers D12Mit19-D12Msw11 and the peak at D12WAD39.8, where the 99th percentile was between 37.3 and 42 Mbp. The QTL on chr 12 contains both *Dkh2* and *Dkh3*, where *Dkh3* is defined by marker D12Mit19 and is encompassed in the congenic strains. After induction of diabetes, differences in KW:BW were evident between these congenic strains and their relevant founder strain controls (Figure 7). The B6 regions intorgressed to the FVB strain protected against diabetes-induced increased kidney weight, whereas the FVB regions bred onto the B6 background conferred susceptibility to this trait on the resistant B6 background. These results confirmed our mapping of the *Dkh* QTLs in response to diabetes.

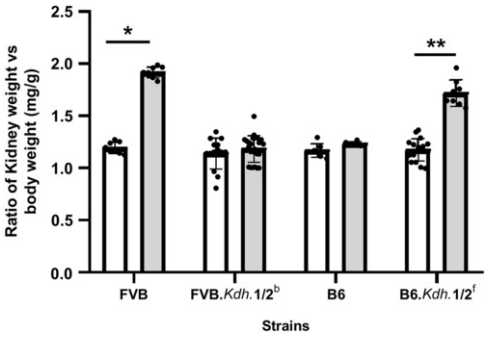

Figure 7 Characterization of renal response in congenic strains. Kidney weight measured by the kidney to body weight ratio, showing the difference between nondiabetic kidneys (white) and diabetic (gray). The introgressed B6 genes on the FVB background protect against increased kidney weight, whereas those of FVB on the B6 background resulted in an increase in kidney weight. *$P < 0.001$; **$P < 0.01$.

Glomerulosclerosis index

Tissue sections of nondiabetic and diabetic kidneys (at 21 days) were scored blind[40] and plotted. When compared with control counterparts, the diabetic FVB mice had evidence of glomerulosclerosis ($P < 0.001$) (Figure 8). The B6.*Dkh1/2*^{f} diabetic kidneys also had a significant increase in glomerulosclerosis compared with B6 diabetic controls ($P < 0.01$) (Figure 8). This congenic strain showed a degree of scarring in the glomeruli in response to diabetes, mediated by the introduction of genes from the FVB strain. By contrast, the FVB.*Dkh1/2*^{b} strain showed limited evidence of glomerulosclerosis indicating protection mediated by the genes introduced from the B6 strain.

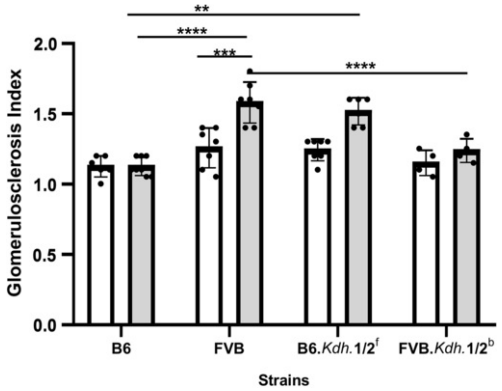

Figure 8 Glomerulosclerotic index in nondiabetic (white) and diabetic (gray) mice. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by 1-way analysis of variance and Tukey's multiple comparisons test, n = 4–9 per group.

Discussion

We aimed to identify genes responsible for the early renal changes seen in response to diabetes using a genetic approach. A strain survey determined that the FVB strain had the greatest renal hypertrophy (KW:BW) in response to diabetes, whereas the B6 strain did not. A whole genome scan of F2 progeny clearly identified 2 major loci on chromosomes 6 and 12. Both regions are orthologous to regions identified in the human GoKinD study,[41] in association with DKD. The QTL located on murine chromosome 6 has an orthologous region on human chromosome 7q. Two linkage studies in humans have also identified DKD-associated QTLs in this same region (maximum LOD = 3.1).[42,43] The second region on murine chromosome 12 corresponds to a region on human chromosome 7p. A linkage study of DKD in humans mapped a DKD QTL into this region (LOD score = 3.59).[44]

Within the QTL regions on chromosomes 6 and 12, there are approximately 200 genes. Of particular interest are candidate genes that lie within the QTL on chromosome 6. These include *Akr1* members B3, B8, B7, and D1. Of these 4 genes, members B3, B8, and D1 have known human orthologs. The most interesting of these genes is *Akr1b3,* which encodes aldose reductase and is homologous to human gene *AKR1B1*. Aldose reductase functions in the polyol pathway, catalyzing a reduced NAD phosphate–dependent reduction of sugar aldehydes to corresponding sugar polyols (discussed by Brownlee[45]). Flux of glucose through the polyol pathway is increased in cells exposed to hyperglycemia (reviewed by Brownlee[45]), and expression of aldose reductase is increased in DKD and other diabetic complications.[46,47] Studies such as these have led to the development of the hypothesis that the degree of aldose reductase gene expression may modulate risk for DKD. Thus, the congenic mice we developed in the present study will support further delineation of the contribution of *AKR1B1/Akr1b3* to risk for DKD.

Glomerulosclerosis is a feature of DKD as it progresses to end-stage kidney disease.[48] The congenic introduction of FVB susceptibility alleles into the less susceptible B6 genetic background induced diabetic kidney changes including glomerulosclerosis, similar to that of the parental FVB mouse, confirming that this DNA region was responsible for the renal changes identified. By contrast, the converse congenic strain, with B6 protective alleles introduced to the high-risk FVB strain, had protected against signs of DKD. These congenic strains provide confirmation that these genomic regions harbor genes that are differentially expressed and mediate these early renal changes in DKD. Further interrogation of these mouse lines should provide a better understanding of the underlying molecular basis of DKD and direct future studies to achieve the ultimate goal of identifying methods to prevent or better treat DKD.

Research design and methods

Mice

Inbred mouse strains (DBA/2, 129T2, SJL, CBA, DBA/1, NOR, NZW, AKR, NZB, NZO, BALB/c) were obtained from the specific pathogen-free colonies at the Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). Strains (NOD,

A/J, BALB/c, NZO, FVB, and B6) were obtained from the Animal Resources Centre (Perth, Australia). Both male and female mice were used between 8 and 12 weeks of age. Mice consumed an *ad libitum* diet of standard laboratory chow containing (w/w) 6% fat, 20% protein, 74% carbohydrate (Barastoc, New South Wales, Australia), and water. Artificial lighting was maintained to a 12-hour day/night cycle and the room temperature kept constant at 22 °C.

FVB/N (FVB) and C57BL/6 (B6) mice were mated and F1 mice were sibling-mated to generate F2 progeny (FVB × B6); these were then backcrossed to B6 mice to generate a backcross denoted as (B6.*Dkh1/2*^{f}) or backcrossed to FVB mice to generate a backcross denoted as (FVB.*Dkh1/2*^{b}). Each backcross was selectively bred to maximize the B6 genes in an FVB background and FVB genes in a B6 background at defined locations on chromosomes 6 and 12. Ethics approval was obtained from the Animal Ethics Committee of the University of Western Australia, and all experimental procedures were carried out in accordance with guidelines of the guide for the care and use of laboratory animals, Eighth edition (2011).

Induction for diabetes and kidney collection

At 6–8 weeks of age, all test mice were treated with Alloxan 90 mg/kg (Sigma-Aldrich, Australia), except the strains NOR and SJL, which were treated with 80 mg/kg alloxan, by tail vein injection. Each control mouse, matched for age and sex, received a saline injection. Mice were monitored daily by testing for urinary glucose and weekly for blood glucose and followed for 7 or 21 days after induction of diabetes (10–20 mice per group). Mice were considered diabetic if their urine glucose concentration was higher than 55 mmol/l (Keto-Diabur-Test 5000, Germany) and their blood glucose higher than 30 mmol/l (Accu-Check Performa Roche, Germany). At the experimental endpoints of day 7 or 21, animals were killed and renal hypertrophy was defined as the average kidney to body weight ratio of both kidneys. Whole kidneys were weighted and then placed in RNALater (Ambion) for gene expression studies or 10% formalin for histology.

Genotyping

DNA was extracted from the mouse liver or from tail snips as described previously.[48] The response of (FVB × B6) F2 progeny mice (n = 534) to alloxan was bimodal, where the majority (81%) developed diabetes. A subset of F2 samples was selected from the 434 diabetic F2 mice. This subset consisted of the upper 90%–100% (2.41–2.69 mg/g [male vs. female]) (n = 44) and lower 0%–10% (1.75–2.03 mg/g [male vs. female]) (n = 44) 10th percentiles as defined by the change in renal hypertrophy amongst diabetic F2 mice. Equal numbers of female and male mice were included in each group. Genotyping was conducted on samples from this subset. The introgressed strains were genotyped for 20 generations using flanking markers on chromosomes 6 (77–86 Mbp) and 12 (37.3–42 Mbp) (Supplementary Table S1).

The mice were genotyped by amplification of simple sequence length polymorphisms. The markers used in the polymerase chain reaction assays were selected from the JAX website (http://www.informatics.jax.org) or designed in-house and named as chromosome number, Western Australian Diabetes followed by the chromosome position (Supplementary Table S1). Each of the markers was tested for polymorphism between FVB and B6, using the following conditions: 96 °C for 2 minutes and then 35 cycles of 96 °C for 20 seconds, 55 °C for 20 seconds, and 72 °C for 20 seconds, followed by a final cycle of 72 °C for 2 minutes. Additional primers, amplifying single nucleotide polymorphisms , were designed for chromosome regions where there were no polymorphic microsatellite markers.

QTL analyses

All genotypic data were analyzed using J/Qtl.[49] The phenotype investigated was the natural log of wet KW (average of both left and right kidney) normalized to BW. QTL scans for main-effect and interacting QTL in inbred mice were performed as described.[50] LOD scores were subsequently computed at 2 cM intervals across mouse chromosomes 6 and 12 that contained significant linkage peaks. Loci were deemed significant if they exceeded the LOD threshold of 3.5. Significance was determined by permutation testing (N = 1000).[51]

Microarray analysis

RNA was extracted from diabetic (21 days) and nondiabetic kidneys homogenized in Trizol (Invitrogen) with the addition of β-mercaptoethanol, using a Precellys BeadBeader instrument. RNA was quantified using a Nanodrop spectrophotometer and quality checked with the Total RNA Nano 6000 Assay Kit (Agilent) and the Agilent Bioanalyser. All RNA integrity values were 8 or higher.

RNA (250 ng) was reversed transcribed using the Illumina TotalPrep RNA amplification Kit (Illumina). To generate the first strand cDNA, RNA was mixed at room temperature with the First Strand cDNA Mix (Illumina) and incubated at 42 °C for 2 hours. First-strand cDNA synthesis was primed with a T7 Oligo (dT) primer. Post incubation, the Second Strand cDNA was made according to manufactures instructions (Illumina).

In vitro transcription technology Master Mix (Illumina) was added to each cDNA sample. Samples were incubated at 37 ºC for 16 hours. cRNA purification was performed. Biotin-labeled antisense cRNA was quantified using a Nanodrop Spectrophotometer. cRNA was deemed appropriate for further use if the concentration was 200 ng/µl or higher.

A total of 750 ng cRNA in 5 µl nuclease free water was prepared after cRNA quantification and hybridized to Sentix Bead Chip Array MouseRef-8 (Illumina) according to the manufacturer's instructions. Chip scanning was conducted using the Bead Array Scanner (Illumina). Microarray expression data have been deposited in the Gene Expression Omnibus under Accession Number GSE123677.

Data analysis and statistics

Microarray data were analyzed using Illumina Bead Studio. The data were first filtered according to the detection P value. Illumina probes were analyzed for noise and nonspecific binding. Detection P values were computer based on the rank of the Z value of a probe relative to the Z values of the negative controls (Illumina) (with P value < 0.01).

Data were rank-invariant normalized to minimize the effects of variation arising from nonbiological factors. FVB diabetic kidney samples were normalized to B6 diabetic samples, and in a separate analysis, FVB nondiabetic kidney samples were normalized to FVB diabetic samples. DiffScore was generated to determine the variable expression of genes. DiffScores of greater than 13 or less than −13 were associated with $P < 0.05$. A DiffScore of greater than 30 or less than −30 was associated with $P < 0.01$.

Gene locations were determined using MGI Batch Query (http://www.informatics.jax.org) and Entrez Gene function from the NCBI website (http://ncbi.nlm.nih.gov).

Morphological changes in the diabetic kidney

One kidney, at 7 or 21 days of diabetes, was transversally cut into 4 equal pieces, and 2 alternating pieces were taken for processing and embedding into glycolmethacrylate or paraffin. Blocks were sectioned at 3 μm and stained with periodic acid-Schiff. Glomerulosclerotic index was assessed using a semiquantitative method[52] in 20 glomeruli from the 2 alternating pieces at a magnification of ×400. Glomeruli were graded according to the severity of damage, assessed on the following parameters: mesangial matrix expansion and/or hyalinosis of focal adhesions, true glomerular tuft occlusion, sclerosis, and capillary dilation. Specifically, grade 1 indicates <25% glomerular injury; grade 2, 26%–50%; grade 3, 51%–75%; and grade 4, >75%.

Glomerulosclerotic index was calculated using the following formula: Glomerulosclerotic index = $[(1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)]/n_1 + n_2 + n_3 + n_4$, where n_x is the number of glomeruli scored within each grade of glomerulosclerosis.

Disclosure

All the authors declared no competing interests.

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Author Contributions

LAB wrote the manuscript, bred, genotyped, and performed the congenic experiments; RW and CR genotyped the F2 mice and defined the QTLs; KAJ-D reviewed and edited the manuscript; LAG performed the histology and glomerulosclerotic index (GSI) analysis; ZC and YC conducted the strain survey and conducted experiments on the F2 mice; KLR involved in array analysis and documentation; JMF analyzed GSI results and reviewed and edited the manuscript; and GM planned the project, completed the gene mapping, and reviewed/edited the manuscript.

Supplementary Material

Table S1. Oligonucleotide primer sequences for Mit and WAD markers used to genotype (FVB × B6) F2 mice. Markers on chromosomes 6 and 12 are represented, and the primers that are associated with the congenic intervals are shown in red. The outermost primers determine the congenic intervals.

Table S2. Differential expression of genes between B6 and FVB diabetic and control kidneys. Genes differentially expressed on chromosome 6 between diabetic and control FVB kidneys: (i) genes differentially expressed on chromosome 12 between diabetic and control FVB kidneys; (ii) genes are listed in terms of the log-fold change, along with the P value, chromosome number, and accession number.

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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Supplementary Material

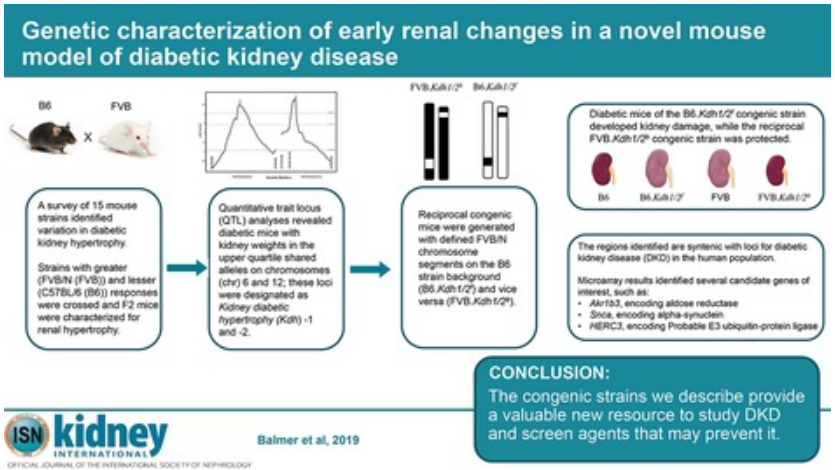
Multimedia Component 1

Table S1 Oligonucleotide primer sequences for Mit and WAD markers used to genotype (FVB × B6) F2 mice. Markers on chromosomes 6 and 12 are represented, and the primers that are associated with the congenic intervals are shown in red. The outermost primers determine the congenic intervals.

Multimedia Component 2

Table S2 Differential expression of genes between B6 and FVB diabetic and control kidneys. Genes differentially expressed on chromosome 6 between diabetic and control FVB kidneys: (i) genes differentially expressed on chromosome 12 between diabetic and control FVB kidneys; (ii) genes are listed in terms of the log-fold change, along with the *P* value, chromosome number, and accession number.

Graphical abstract



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