Genetics of Diabetic Nephropathy: Lessons From Mice

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Summary: Although diabetic nephropathy occurs only in a minority of diabetic patients (~30%), it is the major single cause of end-stage renal disease in the United States. Hyperglycemia and hypertension are important factors predisposing patients to nephropathy, however, accumulating evidence points to critical genetic factors that predispose only a subset of diabetic patients to nephropathy. Defining the genes responsible for nephropathy risk in human populations has proven challenging. Comparative genomics using the robust genetic reagents available in the laboratory mouse should provide a complementary approach to defining genes that may predispose to diabetic nephropathy in mice and human beings. In this article we review studies that have started to identify genetic risk factors for diabetic nephropathy in mice and the multiple approaches that may be used to elucidate the genetic pathogenesis of this disorder.

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Diabetic nephropathy (DN) is the major single cause of end-stage renal disease (ESRD) in the United States.[1] DN is a clinical syndrome composed of progressively worsening albuminuria, declining glomerular filtration rate (GFR), and increased risk for cardiovascular disease.[2] Importantly, DN is a late complication, occurring after approximately 20 years of diabetes in only a minority of all diabetic patients, making clinical studies of its evolution a daunting proposition.[2] Although hyperglycemia is clearly a prerequisite for the development of DN, alone it is insufficient for its development. Only 10% to 40% of all diabetic patients get DN, despite comparable levels of glucose control in those subjects developing DN versus those spared.[3] Sibling studies show a strong familial component for the risk of developing persistent proteinuria, suggesting a genetic basis for DN risk.[4-6] Together with studies supporting a familial predisposition to DN, a strong case for a genetic risk for DN has been made.[6-8] This realization has prompted the undertaking of several multimillion dollar clinical studies, yet to come to fruition, aimed at identifying these genetic risk factors.[4,9]

Given the recent completion of both mouse and human genomic DNA sequences, and the recognition of the high degree of homology between mouse and human genomes, comparative genomics provides a tractable alternative to expensive and laborious human genomic screens for identifying genetic risk factors predisposing to DN. Until recently, mouse models of DN remained poorly characterized with no reports of overt renal insufficiency. Even with recent studies characterizing the suitability of mouse models of DN,[10-14] the absence of reports of overt renal insufficiency in diabetic mice makes the interpretation of albuminuria and histopathologic changes problematic.[15] Microalbuminuria and histopathologic changes occur in human beings with diabetes, but without overt nephropathy,[16] so the significance of

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the reported levels of albuminuria or glomerulosclerosis seen in diabetic mice is unclear without knowledge of whether these changes presage renal failure. It may be that mouse studies simply have not been followed up for a long enough duration for renal failure to develop. However, without the knowledge that these models ultimately develop renal failure, the significance of preceding functional and histopathologic changes cannot be determined because the other alternative is that these changes do not ultimately evolve to nephropathy and the mice being studied are similar to those diabetic subjects who never develop nephropathy. If, as in human beings, genetic diversity is a key factor determining the risk for DN, then it is critical to test the evolution of DN in genetically diverse backgrounds in mice as well.

DIABETIC NEPHROPATHY IN INBRED MICE

Inbred mice represent a unique genetic resource, developed through repeated brother–sister mating for more than 20 generations (for definition see http://jaxmice.jax.org/info/inbred.html).[17,18] This mating strategy results in a progressive reduction of genetic complexity such that the surviving progeny ultimately become identical and homozygous at all loci. Thus, each individual mouse within an inbred strain is as genetically similar as are identical twins. From a genetic perspective, each mouse strain therefore can be viewed as an individual. As in human beings, in whom most individuals are resistant to DN, most inbred strains of diabetic mice do not appear to be susceptible to DN. This is based on results that showed that several inbred strains of diabetic mice showed only mild albuminuria, mild glomerulosclerosis, and no decrease in GFR.[19,20] Notably, the widely used C57BL/6J strain appears to be resistant to diabetic kidney disease and nephrosclerosis after five-sixths nephrectomy.[21,22] In contrast, other strains including C57BLKS, dilute brown nonagouti (DBA)2, and KK/HIJ develop substantially greater diabetic kidney disease including albuminuria and glomerulosclerosis.[19,20] These findings are consistent with the presence of specific modifier loci that confer susceptibility to DN among different strains.

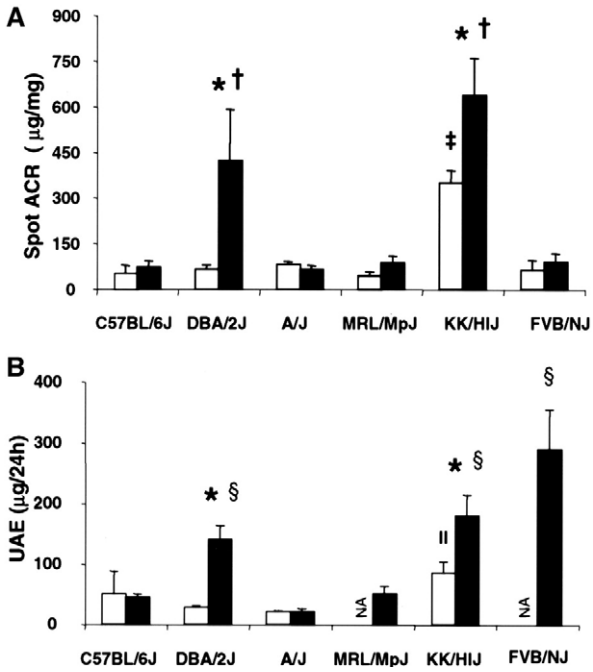

Figure 1. Development of albuminuria in STZ-induced diabetic inbred mice after 25 weeks of hyperglycemia. (A) Spot urine ACR in diabetic mice (■) and age-matched controls (□). *$P < .05$ (*t* test) versus respective age-matched controls; †$P < .05$ (analysis of variance [ANOVA]) versus diabetic C57BL/6J, A/J, MRL/MpJ, and FVB/NJ mice; ‡$P < .001$ (ANOVA) versus controls in other strains. (B) The 24-hour urine albumin excretion (UAE) in diabetic mice (■) and age-matched controls (□). Age-matched controls for MRL/MpJ and FVB/NJ strains were not available (NA) for studying. *$P < .05$ (*t* test) versus age-matched controls; §$P < .05$ (ANOVA) versus diabetic C57BL/6J, A/J, and MRL/MpJ strains; ‖$P < .05$ (ANOVA) versus controls in other studied strains. [20]

DBA2/J Mice

DBA mice were the first inbred mouse strain, originally selected for coat color by C.C. Little. Until recently, studies of diabetic kidney disease in this strain were limited. In the past 2 years, however, diabetic DBA2 mice produced by low-dose streptozotocin (STZ) were found to show a greater albumin-to-creatinine ratio (ACR) and more severe glomerulosclerosis than in diabetic C57BL/6 mice with diabetes of comparable duration (Fig. 1).[19,23] Ongoing studies

of this strain suggest that a decrease in GFR also may be observed after more prolonged periods of diabetes (unpublished observations), thus DBA2 mice may represent a susceptible genetic background in which to study the development of DN.

C57BLKS Mice

Studies of renal pathology and function suggest that DN is more severe in the C57BLKS than in the C57Bl/6J strain, using the *db/db* model of type 2 diabetes mellitus.[24,25] It seems likely that modifier genes directly predisposing to the development of nephropathy are involved in this disparity because it has been proposed that the C57BLKS strain represents a mixture of C57Bl/6J and DBA2/J genomes.[26] Genomic analysis showed that 84% of the alleles in the C57BLKS strain are shared with the C57BL/6J strain and 16% are shared with the DBA2/J strain, indicating genetic contamination early in the strain's history, but rather limited differences.[26] Published studies indicate the *db/db* mice do not develop glomerular/tubulointerstitial injury when expressed on the C57Bl/6J background, but have lesions consistent with DN (thickened basement membrane, mesangial hypercellularity, and expanded mesangial matrix) when on the C57BLKS background.[25,27,28] Mice expressing the db mutation in the leptin receptor ($LepR^{db}$) on C57BLKS background develop more severe hyperglycemia than the C57BL/6 strain because of an associated insulinitis that results in a combination of peripheral insulin resistance and insulin deficiency, whereas the C57BL/6J strain has only peripheral insulin resistance.[24,26,29] Therefore, it remains uncertain whether the modifiers in the *db/db* model simply make the diabetes more severe, or make the kidney more susceptible to glomerulosclerosis, or both.

FVB/N Mice

Accumulating evidence suggests that FVB/N mice also may be predisposed to renal fibrosis and DN, however, these studies are not definitive. FVB/N mice appear to be prone to nephrosclerosis after subtotal nephrectomy.[30] A recent study suggested the development of DN in FVB/NOVE26 mouse, a model of severe early onset type 1 diabetes secondary to the introduction of a calmodulin minigene driven by the rat insulin II promoter into FVB/NJ mice.[31] These mice develop diabetes within the first weeks of life and survive well over a year without insulin treatment. OVE26 diabetic mice show significant polyuria and significant albuminuria by 2 months of age (305 μg/24 h in OVE26 versus 20 μg/24 h in controls). The albumin excretion rate increases progressively with age and reportedly was greater than 15,000 μg/24 h at 9 months of age. There was accompanying glomerular and mesangial hypertrophy with diffuse and nodular expansion of mesangial matrix. Tubulointerstitial fibrosis also was observed. Glomerular basement membranes were thickened in OVE26 mice. Notably, inulin clearance, measured in anesthetized OVE26 mice, showed hyperfiltration at 2 to 3 months of age followed by a subsequent decrease at 5 to 9 months of age. The GFR in 9-month-old diabetic mice determined by inulin clearance was significantly lower than that of 9-month-old control mice. This latter feature is a potentially important finding because renal failure is an element of nephropathy missing from other mouse models of DN. Of note, OVE26 mice also reportedly showed hydronephrosis,[31] a feature not observed in STZ-induced diabetic FVB/N mice, so the contribution of hydronephrosis to the nephropathy phenotype remains to be determined.

The development of nephropathy in the OVE26 mice is in contrast to studies of FVB/NJ with STZ-induced diabetes, in which glomerulosclerosis and reduced GFR were not seen.[20] Also in those studies, unexpectedly the increased 24-hour albumin excretion rate was not accompanied by an increase in ACR,[20] raising questions regarding which value is valid in this extremely polyuric model. Interestingly, despite the apparent mild disease, in these latter studies the FVB strain was distinguished by dramatically greater glomerular hyperfiltration than other STZ-induced diabetic strains (Fig. 2). It may be that the lack of the observed reduction in GFR in the STZ-induced diabetes in FVB/N mice was because the studies were terminated earlier than those of the FVBOVE26

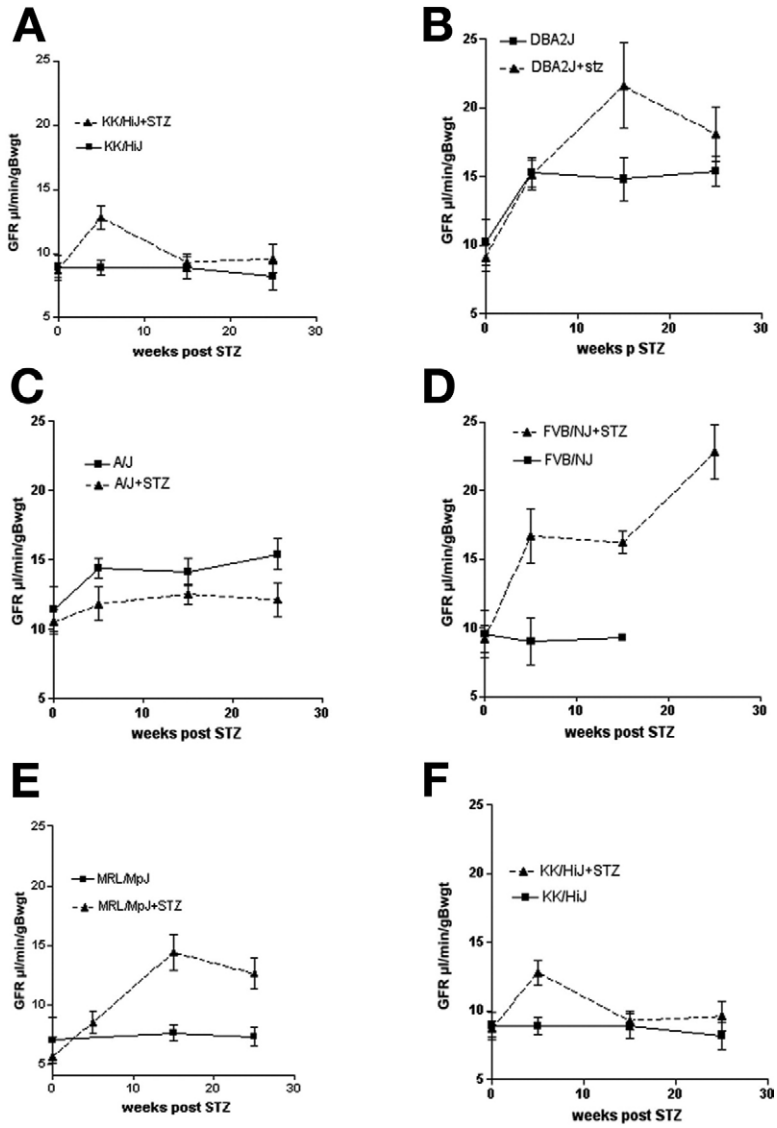

Figure 2. Time-dependent changes in GFR were determined by serial measurements of fluorescein isothiocyanate inulin clearance in groups of 6 different strains of inbred mice with or without low-dose STZ-induced diabetes mellitus. (A) ▲, KK/HiJ + STZ; ■, KK/HiJ; (B) ■, DBA2J; ▲, DBA2J + STZ; (C) ■, A/J; ▲, A/J + STZ; (D) ▲, FVB/NJ + STZ; ■, FVB/NJ; (E) ■, MRL/MpJ; ▲, MRL/MpJ + STZ; (F) ▲, KK/HiJ + STZ; ■, KK/HiJ.

mice (25 weeks of diabetic hyperglycemia in the STZ model versus 36 weeks of hyperglycemia in the OVE26 mice). More prolonged studies of STZ-treated FVB/N diabetic mice might therefore be informative.

129 Mice

Several substrains of 129 mice exist (for strain nomenclature see http://www.informatics.jax.org/mgihome/nomen/strain_129.shtml)[32] and it appears that at least some of these strains are more prone to nephrosclerosis than C57BL/6J mice after nephrectomy.[21,22] 129/Sv mice also are more prone to fibrosis than C57BL/6J mice after deoxycorticosterone acetate (DOCA)/salt treatment.[33] Studies of DN in this strain are limited, but in contrast to expectations based on the aforementioned studies, low-dose STZ treatment of 129/SvJ mice results in only mild albuminuria and moderate mesangial matrix expansion.[27,34] Similarly, STZ treatment of the 129S1/SvEv mice did not result in albuminuria or glomerulosclerosis.[19] Additional studies appear to be warranted given the appar-

ent predisposition of some 129 mice to renal fibrosis in other models.

BALB/c Mice

BALB/c mice have been useful in renal research because of their particular susceptibility to adriamycin-induced focal glomerulosclerosis.[35-38] In contrast, other mice, including C57BL/6J mice, are resistant.[35-38] There are few studies of DN in BALB/c mice, however, those studies that are available provide equivocal evidence regarding the susceptibility of this strain to DN.[19,39] Diabetes in BALB/c mice induced by low-dose STZ produced only mild glomerulosclerosis.[19,39] Although these same studies showed albuminuria was more robust than in other strains, their conclusion was that BALB/c mice were relatively resistant to nephropathy.[19] Care must be taken in interpreting these data because STZ itself can cause direct renal injury in a strain-dependent and dose-dependent manner, independent of hyperglycemia.[40]

KK/HIJ Mice

The KK strain was inbred in Japan by Kondo in 1944. KK/HIJ mice develop spontaneous diabetes mellitus associated with an insensitivity to insulin and an intolerance to glucose without hyperglycemia.[41] These insulin-resistant mice also develop significant glomerular lesions, and show significantly greater urine albumin excretion rates than other strains of inbred mice (Fig. 1). A recent study back-crossed KK mice to the albuminuria-resistant BALB/c strain, and mapped an albuminuria quantitative trait locus to chromosome 2 at 83.0 centimorgan.[42] The specific genes contributing to the development of albuminuria in KK mice remain to be identified. Although KK mice appear prone to renal fibrosis, whether they progress to renal insufficiency has not yet been determined.

KNOCKOUTS AND TRANSGENICS

By definition, specific gene targeting or transgenic mice do not allow gene discovery, but rather help to elucidate gene function and help to substantiate the role(s) of specific genes in the pathogenesis of DN. This approach has provided important insight into pathways leading to the development of DN. Some notable recent observations regarding transgenic mice and DN are described later.

Endothelial Nitric Oxide Synthase (eNOS) Knockout Mice

In human beings, a nonsynonymous single nucleotide polymorphism in eNOS (NOSIII) changing glutamine 298 to asparagine is associated with reduced NOS activity and also with accelerated DN.[43-45] Validation of an important role for eNOS activity in DN has been established recently through studies of $eNOS^{-/-}$ $LepR^{db/db}$ diabetic mice.[46] At 26 weeks, despite similar severity of hyperglycemia, $eNOS^{-/-}$ C57BLKS/J *db/db* mice showed much more dramatic albuminuria, histopathologic changes, and glomerular basement membrane thickening than $eNOS^{+/+}$ C57BLKS/J *db/db* mice. Even more notably, $eNOS^{-/-}$ C57BLKS *db/db* showed decreased GFRs to levels less than 50% of that in $eNOS^{+/+}$ C57BLKS *db/db*, as confirmed by increased serum creatinine and inulin clearance (Fig. 3). These mice provide one of the few diabetic mouse models documented to develop reduced GFR in addition to robust albuminuria and glomerulosclerosis.

Receptor for Advanced Glycosylation End-Products Transgenic Mice

Diabetic hyperglycemia is associated with nonenzymatic modification of proteins by glucose, or its downstream metabolic products, including triose phosphate intermediaries, nonenzymatically forming the early glycosylation products such as methylglyoxal.[47,48] Amine-catalyzed sugar fragmentation reactions modify lysine residues directly, forming N-(epsilon)-(carboxymethyl) lysine, a major product of oxidative modification of glycated proteins.[48,49] Alternatively, reaction of terminal amino groups (eg, on lysine) with glucose itself may form early glycation products (ie, Amadori products),[50,51] which rearrange to produce stable moieties that possess distinctive chemical cross-linking and biological properties, designated *advanced glycosylation end-products* (AGEs).[52] AGE-modified proteins activate a transmembrane receptor designated the *receptor for advanced glycosylation end-products*

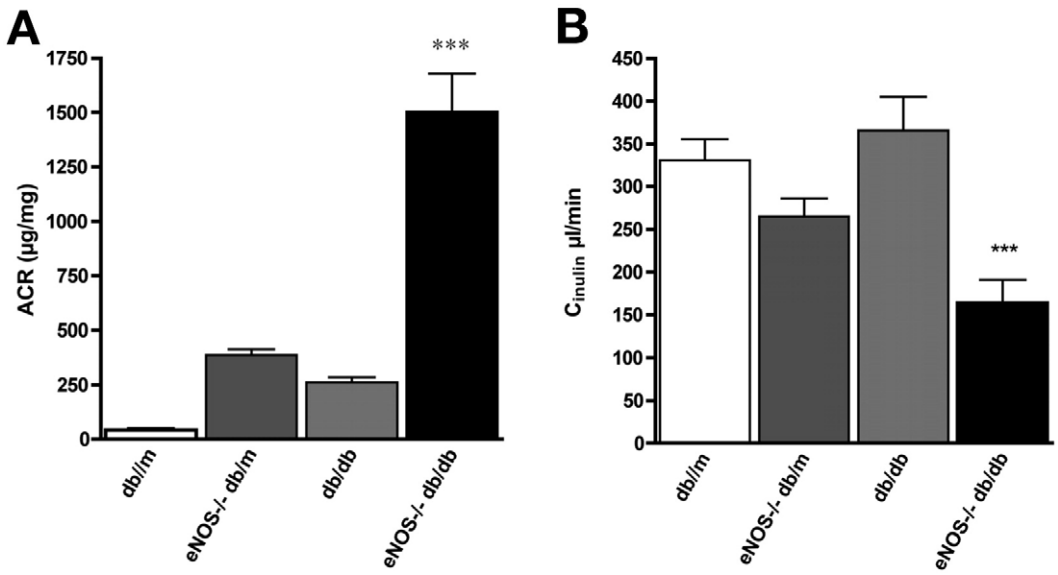

Figure 3. (A) Urinary ACR at 24 to 26 weeks of age. (B) GFR in nondiabetic and diabetic eNOS$^{-/-}$ and wild-type C57BLKS db/db. ***$P < .001$ ANOVA versus control, db/db, and eNOS$^{-/-}$. Bar height represents means ± SE for groups of at least 8 mice. Data from Zhao et al.[46]

(RAGE).[53] N-(epsilon)-(carboxymethyl) lysine adducts and endogenous ligands such as S100/calgranulin amyloid-β peptide and amphoterin proteins are major ligands for RAGE.[54,55] After ligation of the extracellular domain of RAGE, intracellular nuclear factor-κB–coupled events[54,55] as well as Cdc42-Rac-1-mitogen activated protein kinase (MAPK) p38 MAPK pathways[56,57] are activated. Binding of AGEs to RAGE activates a cell-signaling mechanism coupled to increased transforming growth factor-β and vascular endothelial growth factor expression, possibly contributing to the pathogenesis of diabetic complications.[53,58]

Transgenic mice overexpressing RAGE in endothelial cells or mesangial cells have provided important validation for a role for RAGE in the pathogenesis of DN. Recent studies of mice overexpressing RAGE driven by the megsin promoter, which is highly expressed in mesangial cells and back-crossed onto the outbred CD1 background, shows that these mice develop severe nodular glomerulosclerosis (Kimmelstiel-Wilson nodules) and dramatic albuminuria. The fact that the CD1 strain is outbred, and therefore not genetically homogenous, may play a role in the severity of the observed lesions. Although these mice also showed mildly increased serum creatinine and urea nitrogen, it is unclear what component of this was the result of volume depletion from diabetic polyuria.[10] Other studies of mice with RAGE driven by the endothelial selective flt1 promoter also are consistent with a role for RAGE in exacerbating DN.[59] However, these mice only developed a 4-fold increase in albuminuria over controls and true serum creatinine values likely were obscured by an artifact now recognized in mouse serum.[60-63]

Bradykinin Receptor Knockout Mice

Diabetic C57BL/6J Ins2^{Akita} mice were crossed with bradykinin type 2 receptor knockout mice (B2R$^{-/-}$). At 6 months of age, diabetic B2R$^{-/-}$ mice showed roughly 4 times greater albuminuria than B2R$^{+/+}$ mice and worse histopathology; a decreased GFR was not reported. As typifies many knockout studies, the common use of inbred C57BL/6J mice provides an experimental platform in which the unmanipulated diabetic mice show little albuminuria and only mild renal disease. This has the advantage of allowing only moderate increases in albuminuria to be sensitively detected above the low baseline levels in control diabetics. Conversely, a major disadvantage of using the C57BL/6J strain is that the overall severity of the renal

disease, on this genetic background, is not robust.

Although several studies have examined the impact of homozygous deletion of candidate genes (eg, bradykinin B2 receptor, apolipoprotein E) on the progression of DN in mice,[64] these studies do not report loss of GFR. Unbiased genome-wide approaches to discover novel genes predisposing to DN in mice have not been undertaken previously.

RANDOM MUTAGENESIS

Another advantage of mouse models is that they are amenable to large-scale random mutagenesis and phenotype-based screens to identify new genes that are associated with disease.[65-67] One approach is via N-ethyl-N-nitrosourea (ENU), a potent alkylating agent that causes random point mutations by alkylating genomic DNA, primarily inducing AT to TA point transversions and AT to GC transitions.[68] ENU is a supermutagen of spermatogonial stem cells that induces a mutation rate of approximately 10^{-3} per locus per gamete.[69] Thus, 1 in every 1,000 gametes from a mutagenized male might be expected to carry a mutation in a particular gene of interest. The use of phenotype-driven whole-genome mutagenesis to recover new ENU-induced heritable mutations in mice,[70-73] together with the extensive homology between the mouse and human genomes, makes mutagenesis an attractive approach to discover new genes predisposing to DN. Recent studies indicate up to 2% of ENU-induced progeny carry a heritable mutant phenotype.[65,74] The high frequency of ENU mutations potentially allows the generation of mice with a mutation of every gene that could contribute to any given phenotype. Mutagenesis offers distinct advantages for the analysis of the phenotypic traits over quantitative trait locus (QTL) analysis (see later), insofar as ENU-induced phenotypes arise from monogenic point mutations.[75] The consequence of this is that one can be confident that a phenotype observed in the mutagenized progeny is the result of a single gene mutation (either recessive or dominant, depending on the breeding scheme), rather than the consequence of epistatic interaction between multiple modifier genes, as can be the case with QTLs.[75]

Over the past 2 decades, ENU mutagenesis has been applied successfully to generate new mouse models of human disease and to discover novel genes involved in diverse phenotypes including behavioral, neurodegenerative, hearing, aging, seizures, hematologic, and metabolic disorders.[72] Some notable examples of ENU mutants include the *min* mouse, a mouse homolog with an ENU-induced mutation mapped to the mouse APC homolog, providing a murine model of familial adenomatous polyposis[76]; and discovery of the clock gene regulating circadian rhythm through an ENU mutation in this gene.[77] Roughly 70% of the 38,000 mutations identified in 1,500 genes responsible for human disorders are caused by point mutations and many of these act dominantly or semidominantly. It also is notable that there is evidence that dominant mutations may contribute to the genetic risk of DN in human populations.[78] Linkage analysis of 18 large Turkish families with type 2 diabetes mellitus and DN identified a QTL with a highly significant log odds ratio (LOD) of 6.1. Of particular relevance to this discussion is the fact that genetic transmission was fit best by a dominant mode of inheritance.[79] Based on these considerations, we recently executed a phenotype-driven screen to identify dominant ENU-induced mutants that converted a DN-resistant C57BL/6J strain to a DN-susceptible line.

We executed a screen of the progeny of a cross between male ENU-mutagenized C57BL/6J mice with female Akita diabetic mice. The *Akita* mutation in the insulin2 gene (*Ins2*^{Akita})[80,81] causes diabetes mellitus, allowing us to identify novel mutants predisposed to DM. In this case we screened progeny for mutants showing excess albuminuria. Two lines of C57BL/6J mice were identified that showed accelerated albuminuria. After a year of diabetes, albuminuric *Ins2*^{Akita} G2 and G3 progeny also showed reduced inulin clearance, with increased blood urea nitrogen and plasma creatinine levels.[82] Despite identical HbA_1C levels, diabetic ENU mutants showed greater mesangial expansion, increased glomerular basement membrane thickening, and greater kidney

weights than control Akita mice. These novel mutants should provide robust mouse models of DN. Further identification of the responsible mutant genes should help elucidate the genetic basis for individual predisposition to DN.

MAPPING QTLS

Inbred lines of mice also provide unique reagents for associating a region of a genome associated with traits that can be quantified (eg, serum creatinine level, serum cholesterol level, blood pressure) to identify genomic loci associated with these traits, known as QTLs. Classically, this is accomplished by examining F2 progeny of matings between affected versus unaffected individuals.[83] Comparative genomics has been proposed as an important adjunct approach to identifying QTLs in human beings. Implicit in this approach is that genes that naturally vary in one species to determine a particular trait also will be more likely to vary and impact that trait in human populations. Despite its promise, QTL mapping has been used successfully to discover only approximately 20 genes including SORCs, a novel gene contributing to obesity-induced type 2 diabetes mellitus[85]; lipoxygenase 12/15 (Alox15), shown to control bone density[86]; and Tnfsf4 (encoding OX40 ligand), which controls plasma lipid levels in mice and possibly human beings.[87] A list of cardiorenal disease QTLs concordant between rodents and human beings has been compiled (http://pga.jax.org/qtl/kidneyqtltable.htm). Concordance between QTLs contributing to several diseases in mice, human beings, and rats including hypertension,[88,89] proteinuria,[90] and renal disease[84] has been well established. Although yet to come to fruition, studies identifying genes predisposing to kidney disease and DN using QTL mapping are underway in human beings and rodents.

Another refinement facilitating the mapping of QTLs in rodents has been the generation of recombinant inbred lines (RI lines).[91-93] These lines represent heritable and stable admixtures of genomic contributions from 2 distinct genetic backgrounds (eg, C57BL/6J and DBA2 RI lines, also known as $B \times D$) and are homozygous at all loci for either B6 or D2 alleles.[92] There are approximately 80 unique lines for the B × D RI set. This allows repeated phenotyping of mice with known genetic contributions from each genetic background, reduction of the phenotypic experimental variability, and elimination of the need for genotyping each mouse (because each line has a known genetic contribution). A disadvantage of this approach, and the use of inbred lines in general, is that homozygous lethal mutations are lost from the genetic pool. Thus, if dominant mutations contribute to human DN and are homozygous lethal, their homologs will not be present in inbred mouse models. The use of outbred lines would be necessary to capture these genes.

CONCLUSIONS

Mice provide a highly flexible experimental platform on which to elucidate the genetic risk factors contributing to DN. These genetic approaches include the used of inbred strains, transgenic and knockout mice, and random ENU mutagenesis. Detailed and more prolonged studies of these murine models of diabetes mellitus have shown that many classic features of nephropathy, including nodular glomerulosclerosis, progressive albuminuria, and renal insufficiency, can be detected in selected mouse models. Identifying genes that predispose to DN in mice should provide insight into the pathogenesis of DN and inform genetic studies in human beings. Defining the genetic variants that predispose to versus those variants that protect against the development of nephropathy should help point the way to new therapeutic approaches to prevent kidney disease.

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