Online Supplement: Mouse Models of Diabetic Nephropathy

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Supplement Content:

Inbred strains that develop the most profound diabetic nephropathy

DBA2/J (JAX #671)

DBA/2 (dilute brown non-agouti) mice were among the first of the recognized inbred mouse strains. Male DBA/2 mice are substantially more sensitive to streptozotocin (STZ) than B6 mice; to avoid excess mortality they should only be administered a repetitive low dose STZ protocol, (40mg/kg i.p. every day for five days) to induce diabetes.[1-3] It should be noted that chronic diabetes induction by multiple low dose STZ appears to occur only in male mice in all genetic backgrounds that have been tested.

When made diabetic using this low dose STZ protocol, DBA/2 mice exhibit greater ACRs and more severe glomerulosclerosis than several other strains including diabetic B6, A/J, or BALB/C mice.[4, 5] Ongoing studies of diabetic DBA/2 mice suggest a decline in GFR following periods of diabetes of greater than 1 year (Breyer M, et al., unpublished). Thus, DBA/2 mice may represent a susceptible genetic background in which to study the development of diabetic nephropathy. For this reason AMDCC investigators recently back-crossed the dominant diabetogenic *Ins2*^{Akita} mutation[6] from B6 onto the DBA/2 background. This mutation is an autosomal dominant generally studied in heterozygous state (denoted as *Ins2*$^{Akita/+}$). This mutation impairs both beta cell function and survival because of extreme endoplasmic reticular stress exerted by severe misfolding of the insulin 2 gene product. Male DBA/2 *Ins2*$^{Akita/+}$ mice in

preliminary studies exhibit greater albuminuria than B6 male *Ins2*$^{Akita/+}$ mice or *Ins2*$^{Akita/+}$ mice on other genetic backgrounds (Figure 3). The DBA/2-*Ins2*$^{Akita/+}$ mice have less severe wasting than DBA/2 STZ-diabetic animals. The DBA/2-*Ins2*$^{Akita/+}$ mice are now commercially available from The Jackson Laboratory (D2.B6-*Ins2*$^{Akita/MatbJ}$ stock # 007562).

Several additional caveats should be noted regarding the care and use of these mice. Diabetic DBA/2 mice exhibit greater frailty and mortality and long-term studies require low doses of insulin to prevent cachexia and wasting, which typically precedes death. These mice frequently develop pyelonephritis, a potential contributor to their increased mortality, perhaps due in part to their profound glucosuria. In addition the polyuria seen in this strain, as well as many others, requires frequent bedding changes to reduce adverse effects.

C57BLKS

This C57BLKS (BKS) strain of mice apparently was derived from a genetic contamination of a colony of B6 mice that was originally recognized because of rapid rejection of B6 skin grafts due to MHC incompatibility. BKS shares approximately 88% genetic identity with B6, with many of the contaminating alleles apparently deriving from DBA/2. Like DBA/2 males, BKS males are more sensitive to multi-dose STZ induced diabetes. Fortuitously, these mice develop substantially higher blood sugars and greater degrees of albuminuria, mesangial expansion, and glomerular basement thickening than B6 mice when the type 2 diabetic *Lepr*$^{db/db}$ (db/db*)* mutation is studied on this background. Indeed, the BKS-db/db mouse is one of the best models of nephropathy associated with type 2 diabetes.[7] A recent study by members of the AMDCC demonstrates a wave of podocyte apoptosis in this model shortly after the onset of diabetes coincident with onset of albuminuria. Inhibition of NAPDH oxidase with apocynin

attenuates podocyte apoptosis, increases podocyte number and reduces albuminuria.[8] The renoprotective effects of exercise and uric acid inhibition have also been recently demonstrated in this model.[9, 10] Although BKS-db/db mice demonstrate mild tubulointerstitial damage (tubular cell ballooning),[10] typically they do not develop tubulointerstitial fibrosis.

129S6/SvEvTac

Previous studies in other experimental models of kidney disease suggest mice from the 129 strain are particularly susceptible to renal pathology and proteinuria.[11] Moreover, this strain is also salt-sensitive.[12] Accordingly, AMDCC investigators (Gurley S, et al., submitted) backcrossed *Ins2*$^{Akita/+}$ mice on to the 129S6/SvEvTac background. While full analysis of this strain has not yet been published, the 129S6/SvEvTac *Ins2*$^{Akita/+}$ mice are substantially more susceptible to development of albuminuria and mesangial expansion than B6-*Ins2*$^{Akita/+}$ animals. Although levels of proteinuria are less than in the DBA/2-*Ins2*$^{Akita/+}$ strain, 129S6/SvEvTac-*Ins2*$^{Akita/+}$ mice are more robust and appear less prone to renal infection. Since this model has only recently been reposited at The Jackson Laboratory, the extent of nephropathy has not yet been formally compared to the *Ins2*$^{Akita/+}$ mutation in the other strains shown in Figure 3. Importantly, it does not appear that all 129 substrains are equally susceptible to nephropathy. For example, there is no increased diabetic nephropathy in the 129X1 (formerly, 129/SvJ) substrain.[13]

FVB/N

The inbred FVB/N strain (FVB) is characterized by vigorous reproductive performance and has been a favorite for the generation of transgenic mice.[14] Recent metabolic analyses of

FVB mice indicate the highest counter-regulatory response to hypoglycemia of several strains tested.[15] Blood glucose levels (see Mouse Phenome Database: http://phenome.jax.org/pub-cgi/phenome/mpdgrcgi?rtn=views/measplot &brieflook=14302&userhilite=) are also higher and insulin levels relatively low in this strain compared to others.[15] AMDCC investigators have characterized renal complications in this strain under different diabetic conditions. Low dose STZ diabetic FVB mice develop a modest increase in 24-hr albumin excretion that is greater than found in STZ diabetic B6 mice, but substantially less than in STZ diabetic DBA/2 mice. Interestingly, there is no change in spot urine ACRs in the STZ diabetic FVB mice, perhaps due to an increase in creatinine excretion.[5] Minimal renal morphological changes are found in STZ diabetic FVB mice at 25 weeks of age, similar to STZ diabetic B6 mice. These changes are much milder than those that develop at the same time point of STZ diabetes in DBA mice.

The *Ins2*$^{Akita/+}$ mutation was also back-crossed onto the FVB/N background (FVB.B6-*Ins2*^{Akita}, JAX#6867).[16] Male FVB-*Ins2*$^{Akita/+}$ mice are severely hyperglycemic by the time of weaning (21 days of age). Similar to STZ diabetic FVB animals, FVB-*Ins2*$^{Akita/+}$ animals show no increase in urine ACRs but demonstrate a several-fold increase in 24 hr albuminuria when compared to non-diabetic controls. In addition, mesangial expansion and other glomerular changes are minimal in these animals. Most have normal glomeruli with little to no mesangial expansion, prominent juxtaglomerular expansion (renin accumulation) and granules in tubular epithelium. No other morphologic abnormalities were noted.

Chua *et al.* generated type 2 diabetic *Lepr*$^{db/db}$ (db/db*)* mice on a FVB background (FVB.BKS(D)-*Lepr*^{db}/ChuaJ, JAX#6654).[17] These mice are more insulin resistant and have more severe hyperinsulinemia and hyperglycemia than B6-db/db mice (JAX#697). FVB-db/db mice develop enlarged islets, but not islet destruction. Initial reports by *Wang et al.* indicate that

FVB-db/db mice develop relatively severe diabetic nephropathy[18] that is more robust than seen with STZ or the *Ins2*$^{Akita/+}$ mutation on this background. Glomerulosclerosis, tubulointerstitial fibrosis, increased expression of type IV collagen and fibronectin, and proteinuria are present. Electron microscopy studies indicate an increase in glomerular basement membrane thickness and podocyte foot process length. The authors also report a marked increase in neutral lipid deposits in glomeruli and tubules.

In general, diabetic FVB mice exhibit more polyuria than many other strains, with 24 hr urine volumes exceeding 10 ml. In addition, FVB mice develop significantly greater glomerular hyperfiltration after hyperglycemia than other diabetic strains.[5] Finally, one of the more advanced models of diabetic nephropathy so far reported, the OVE26 model (FVB(Cg)-Tg(*Ins2-CALM1*)26Ove Tg(Cryaa-TAg)1Ove/PneJ, JAX #5564) as discussed below, was generated on the FVB background.[19] In summary, severity of renal disease varies somewhat between the different FVB diabetic models. These differences might be related to studies under non-standardized conditions or environmental differences including severity of diabetes or differential exposure to microbial agents. For example, only mild renal changes are observed in cohorts of FVB-db/db mice of both sexes longitudinally profiled for the AMDCC at The Jackson Laboratory, even though their diabetic syndrome closely matches the published data.(14, 15) Given the ease of developing transgenic models on an FVB background and the vigorous reproductive capacity of these mice, further clarification of the susceptibility of this strain to diabetic nephropathy is needed.

Outbred Mice

To date, the AMDCC has focused on the use of inbred laboratory mice to study the

development of diabetic nephropathy. These studies clearly demonstrate the influence of genetic risk factors as contributors to diabetic nephropathy and at the same time simplify the problem by avoiding the genetic complexity of outbred human populations. Inbred mice are homozygous at all loci, whereas many loci in human populations are polymorphic. However, during the process of brother-sister mating used to derive inbred mouse strains, polymorphisms either decrease fecundity or are lethal when homozygous are lost. Consequently, if similar mutations contribute to human diabetic nephropathy, this variability would not be seen in inbred mouse models.[20]

High-dose STZ diabetes in an outbred mouse line, CD-1, reportedly results in severe nephropathy.[21] These mice develop ESRD associated with prominent tubulointerstitial nephritis and fibrosis within 3 months and die from diabetic complications by 6-7 months. The histopathologic lesions observed in these mice mimic human diabetic nephropathy, with glomerular hypertrophy, diffuse glomerulosclerosis, tubular atrophy, interstitial fibrosis and a progressive decrease in function.[21] However preliminary results by AMDCC investigators indicate significant diversity among individual CD-1 mice in the levels of albuminuria with low dose STZ diabetes. Such heterogeneity may more closely resemble the heterogeneity of human populations and thereby provide the opportunity to capture genes that contribute to increased albuminuria and diabetic nephropathy. Ongoing studies by the AMDCC will characterize this heterogeneity of diabetic nephropathy in CD-1 mice and initiate crosses for detecting heritable traits predisposing to development and/or progression of diabetic nephropathy.

Investigators in the AMDCC have also studied the effects of low-dose STZ diabetes in male outbred mouse line, Swiss Webster (SW) mice (Taconic #SW). The SW mice develop a robust hyperglycemic response with average blood glucose values of 588±12 mg/dl. Blood pressures are unaffected in diabetic SW mice but they develop relative tachycardia compared to

non-diabetic SW controls (751±16 vs. 617±33 bpm, p=0.003). After 4 months of diabetes, urinary albumin excretion increases by approximately 4-fold (119±16 versus 25±4 μg/d, p=0.002) (Gurley, SB et al., unpublished). Diabetic SW mice also develop modest mesangial expansion but the renal pathological assessment is complicated by the development of severe pyelonephritis in a number of animals. Significant glomerular sclerosis is absent. Thus, the severity of proteinuria and renal pathology observed after STZ treatment in the outbred Swiss Webster stock is within the range observed in several inbred mouse lines.[4, 22] As such, this particular outbred line offers little advantage for modeling nephropathy.

Negative AMDCC mouse models of diabetic nephropathy

Although a number of genetic modifications and alternate strains result in models of diabetic nephropathy that more closely resemble the human complication, there are a number of AMDCC models which do not result in worsening nephropathy over time. Some of these models have been surprises or have not always agreed with previously published models. The data for these models are available on the AMDCC website (www.amdcc.org). Some notable negative models are discussed in this section.

ACE-2 deficiency

A role for the renin-angiotensin-aldosterone system (RAAS) in the pathogenesis of diabetic nephropathy has been established through animal studies and clinical trials.[23] Thus RAS inhibitors reduce proteinuria and slow progression of kidney injury,[23] and mice expressing higher levels of angiotensin converting enzyme (ACE) develop more severe nephropathy when diabetic than mice with normal or reduced levels of ACE.[24] Accordingly, several studies have been

carried out by AMDCC investigators using mouse models with genetic modifications producing other differences in the activity of the RAAS. One of these models is a mouse with targeted disruption of the *Ace2* gene encoding angiotensin I converting enzyme (peptidyl-dipeptidase A)2, denoted ACE2.[25] ACE2 is a mono-carboxypeptidase homologue of ACE that metabolizes angiotensin II while also generating a peptide product (angiotensin 1-7) with putative physiological activities.[26, 27] Since ACE2-deficient mice have enhanced hypertensive responses to angiotensin II,[25] it was reasoned that ACE2-deficiency might promote the development of more severe nephropathy in diabetes. To test this hypothesis, diabetes was induced in ACE2-deficient mice and genetically matched wild-type controls on both B6 and 129S6/SvEvTac inbred backgrounds using the AMDCC low-dose STZ protocol (Gurley, SB, et al., unpublished). Similar levels of hyperglycemia develop in all groups. Blood pressures are elevated in the diabetic B6-ACE2-deficient mice compared to controls (130±2 vs. 125±7 mm Hg; p=0.03 Mann-Whitney), but are similar in diabetic wild-type and ACE2 null mice on the 129S6/SvEvTac background. Although there is a trend toward increased albumin excretion in diabetic ACE2 null mice, these differences do not reach statistical significance on either genetic background. Modest mesangial expansion is observed in all the groups and is unaffected by ACE2 genotype. Thus, ACE2-deficiency has no effect on the extent of diabetic renal disease. These results contrast with other reports indicating more substantial effects of ACE2 deficiency to exaggerate albuminuria and kidney pathology in STZ diabetic Sprague-Dawley rats,[28] db/db mice,[29] and *Ins2*^{Akita} mice.[30]

Agtr1a gene duplication

Kidney responses to diabetes were also examined in mice with a targeted duplication of

the *Agtr1a* gene encoding the major murine AT_1 angiotensin receptor isoform on an inbred 129/SvEv background.[31] As a consequence of this mutation, these mice have an approximately 40% increase in AT_1 receptor expression. After four months of diabetes induced by the low-dose STZ protocol, the extent of albumin excretion and glomerular pathology is indistinguishable between mice with increased AT_{1A} receptor levels and wild-type controls (Gurley, SB, et al., unpublished).

In this model and in the ACE2-deficient mouse, there is no acceleration of diabetic kidney injury. While RAAS activity is increased in both of these models, this enhancement is relatively modest and not as great as in the renin transgenic models, which demonstrate more overt activation of the RAAS and develop substantial proteinuria and glomerulosclerosis. Thus, there may be a threshold level of RAAS activation that is required to promote diabetic renal injury.

General and podocyte-specific SOD2 deficiency

Because of the overwhelming evidence that intracellular oxidative stress is critical to the cellular changes of progressive diabetic nephropathy,[32-41] some of the first models that were studied by AMDCC investigators for many complications were those in which oxidative stress was augmented. One such model is in mice with targeted deletion of the gene encoding superoxide dismutase (*Sod2*) also known as manganese or mitochondrial SOD. The murine *Sod2* gene had been targeted and placed on the B6 background by the time of the start of the AMDCC (JAX # 2973) but homozygotes for the deleted gene died within the first few weeks of life[42, 43] Therefore, B6-*Sod2*$^{+/-}$ heterozygous mice were studied initially. These mice have reduced glutathione levels and evidence of mitochondrial oxidative damage as well as reduced capacity

to recover from ischemia.[44-46]

The B6-*Sod2*$^{+/-}$ mice were made diabetic either by low-dose STZ or by breeding in the db/db mutation on a B6 background. The STZ diabetic B6-*Sod2*$^{+/-}$ mice were studied 24 weeks after STZ injections and the B6-db/db *Sod2*$^{+/-}$ mice were studied at 24 weeks of age (approximately 18 weeks of diabetes). Surprisingly, although there is a ~2-fold increase in total urinary albumin excretion in the STZ diabetic and a ~3-fold increase in the db/db mice compared to controls, there is no difference in albuminuria between the *Sod2*$^{+/+}$ mice and the *Sod2*$^{+/-}$ mice in either model, with or without diabetes (Brosius, FC, et al, unpublished). Similarly, although glomerular extracellular matrix (as assessed by PAS-positive staining) is increased significantly in both the STZ diabetic and db/db mice compared to controls, there is no difference between the *Sod2*$^{+/-}$ and the *Sod2*$^{+/+}$ groups. Interestingly, there is an increase in neuropathy in the B6-*Sod2*$^{+/-}$ db/db animals compared to *Sod2*$^{+/+}$ db/db mice, as reported by Vincent *et al.* for the AMDCC.[47] Therefore it appears that reduction in SOD2 levels contributes to diabetic neuropathy but not nephropathy, at least in this genetic background.

There is clear evidence that podocyte loss is a critical early step in the development of diabetic nephropathy,[48] and more recent data from AMDCC investigators and others confirm that this loss occurs in part through apoptosis.[49, 8] Moreover, at least a portion of this podocyte pathology is promoted by enhanced oxidative stress.[8] Therefore, AMDCC investigators studied the effect of total elimination of SOD2 expression in podocytes in B6 STZ diabetic and control mice. This was accomplished by breeding mice in which the *Sod2* gene had been targeted with two loxP sites allowing excision of the portions of the gene encoding exon 3[50] with mice expressing cre recombinase only in podocytes (JAX # 8205) driven by a podocin (*Nphs2*) promoter.[51] Again, there is no increase in albuminuria or mesangial expansion in mice with

podocyte-specific deletion of *Sod2* compared to wild-type littermate controls whether diabetic or not. Two caveats in interpreting these data are that it is not technically possible to document complete deficiency of SOD2 protein expression in podocytes *in vivo*, despite demonstrated efficacy of the podocin-cre recombinase in inactivating other genes,[52, 53, 51] nor are there sufficient glomerular samples to determine the effect of this manipulation on total reactive oxygen species or superoxide in these animals.

The lack of augmentation of nephropathy in the *Sod2* null models may be due to only partial deficiency in the heterozygotes, increased expression of other antioxidant genes in glomeruli in both models, or the general resistance of the B6 mouse to nephropathy as documented by AMDCC investigators[54, 5, 4] and others, and noted above. AMDCC investigators do not think that these negative data argue against the role of reactive oxygen species in the development of diabetic glomerulopathy, in general, or podocyte injury, specifically.

CD2AP deficiency

CD2-associated protein (CD2AP) is a widely-expressed cytoplasmic adaptor protein that has previously been implicated in podocyte slit-diaphragm protein complex formation.[55-57] CD2AP null mice develop progressive glomerulosclerosis early in life and CD2AP haploinsufficiency is associated with glomerular disease susceptibility in humans.[57, 55] CD2AP modulates TGFβ signaling in podocytes and tubular epithelial cells, whereby CD2AP interacts directly with ligand-activated TGFβ type I receptor (Tgfbr1), mediating direct activation of the PI3K/Akt survival pathway by Tgfbr1 kinase.[58] CD2AP promotes podocyte and tubular epithelial cell survival by functioning as a molecular switch that suppresses the Tgfbr1/SMAD-dependent apoptosis pathway and activates a Tgfbr1/PI3K-dependent survival pathway.[58]

AMDCC investigators (Suzstak K and Bottinger EP, unpublished) tested the hypothesis that podocyte survival is impaired in CD2AP-deficient diabetic mice, leading to accelerated podocyte depletion and glomerulosclerosis.[59] *CD2AP*$^{+/-}$ mice on a B6 background, made diabetic by low-dose STZ, do not develop increased albuminuria compared to diabetic *CD2AP*$^{+/+}$ mice after 12 weeks of diabetes. In contrast, *CD2AP* heterozygosity is associated with a significant 4-fold increase of albuminuria in *Ins2*$^{Akita/+}$ mice on a B6 background at 24 and 40 wks-of-age, respectively, compared to *CD2AP*$^{+/+}$ mice (ages combined: 253±282 versus 63±32, $P<0.05$) (Bottinger EP, unpublished). However, there is a large variation in albuminuria in B6-*CD2AP* heterozygous *Ins2*$^{Akita/+}$ mice, consistent with histopathological analysis of PAS-stained sections which showed severe glomerular lesions with tubular dilation in some B6-mice *CD2AP*$^{+/-}$ *Ins2*$^{Akita/+}$ mice, while glomerular lesions were not distinguishable from *Ins2*$^{Akita/+}$ mice in most *CD2AP*$^{+/-}$*Ins2*$^{Akita/+}$ mice.

Endothelial expression of RAGE

Mice transgenic for endothelial-specific expression of the receptor for advanced glycation endproducts (RAGE) driven by the vascular endothelial growth factor receptor-2 *(Flk1)* promoter were reported to develop glomerular lesions of human diabetic nephropathy and renal failure when subjected to experimental diabetes induced transgenically by islet cell expression of iNOS.[60] AMDCC investigators obtained *Flk1* promoter-RAGE transgenic mice from Dr. Yamamoto.[60] Of note, from the original report, these RAGE transgenic mice were generated on a mixed B6 × CBA/J F_1 background and subsequently mated for five generations with CD-1 outbred mice, prior to matings with the insulin promoter driven iNOS transgene on a CD-1 outbred background.[60] Thus, a precise definition of the genetic background of these RAGE

transgenic mice that were obtained by AMDCC investigators from the original investigators is not possible here.

AMDCC investigators (Bottinger, EP, et al.) have recently completed a screening experiment where *Flk1* promoter-RAGE transgenic and non-transgenic control littermates are subjected to type I diabetes by low-dose STZ model starting at 8 wks. Induction of hyperglycemia and albuminuria were monitored by standard AMDCC assays. Animals were sacrificed at 20 wks of age. After 12 weeks of STZ diabetes, there is no significant difference in albuminuria (~10x increase compared to non-diabetic controls) in 24 hr urine collection and serum creatinine between non-transgenic controls and *Flk1* promoter-driven flk-RAGE transgenic mice, with a comparable ~10x increase in albuminuria in both diabetic groups compared to non-diabetic controls after 12 weeks of STZ diabetes.

AMDCC investigators then intercrossed hemizygous *Flk1* promoter-RAGE monotransgenic mice with B6-*Ins2*$^{Akita/+}$ mice (Bottinger, EP, unpublished). Urinary albumin excretion and renal histopathology were analyzed at 24 and 48 wk of age. Similar to the negative findings obtained from the multiple low dose STZ protocol, endothelial expression of the RAGE transgene has no significant effect on the extent of urinary albumin excretion, mesangial expansion, or survival induced by diabetes after mating with B6-*Ins2*^{Akita}. Thus, AMDCC investigators were not able to confirm the accelerated lesions of diabetic nephropathy, originally reported in *Flk1* promoter-RAGE/Ins iNOS bitransgenic mice on a CD-1 background.[60] Several reasons could possibly account for the discrepancies between the two studies, including differences in genetic background (CD-1 in original report versus mixed B6 and CD-1 in AMDCC studies), or differences in the diabetes model (insulin iNOS transgene induced diabetes in the original report versus multiple low dose STZ and *Ins2*^{Akita}-induced

diabetes in the AMDCC study.

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