ANIMAL MODELS OF DIABETIC COMPLICATIONS CONSORTIUM
University of Michigan/Hopkins Group
(U01 DK60994)

UPDATE REPORT
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ANIMAL MODELS OF DIABETIC COMPLICATIONS CONSORTIUM (U01 DK60994)

PRINCIPAL INVESTIGATOR'S REPORT

Program Accomplishments:

Rodent models of diabetes fail to develop changes that closely resemble human diabetic nephropathy or neuropathy. For example, although several rat and mouse diabetic models develop early changes of human diabetic nephropathy (e.g., mesangial expansion, modest albuminuria), they do not develop nodular or diffuse glomerulosclerosis and progressive decline in renal function. Similarly, models of neuropathy show a limited form of typical diabetic neuropathy. While the reasons for the resistance of rodents to full-blown complications are likely multiple, they may include an increased resistance to oxidative stress or the absence of important genetic susceptibility genes. Our general strategic approach to this dilemma is to accelerate the injury of diabetes by predisposing critical cells in both the renal glomerulus and peripheral nerve to glucose-mediated oxidative injury by increasing glucose uptake via increased expression of facilitative glucose transporters or by reduction of enzymes that reduce oxidative stress.

This Progress Report presents a chronological account of our approach and resulting data. Because this approach, while involving the input of many investigators and several institutions, is not divided into separate projects, we have consolidated the report into a single narrative. The report is organized chronologically by years and, within each year, by model(see Table 1).

Table 1 **UM/Hopkins Mouse Strains**

Animal model	Background strain	current status	Phenotyping (begin-end)	Consortium collaborators
db/db	C57BL/6J	breeding; phenotyping complete	9-03; breeding stock	
db/db	C57B.KLS	breeding; begin neuro phenotyping	2-04 to 3-05; breeding stock	
SOD2 +/- STZ	C57BL/6J	Phenotyping complete; no increase in neuropathy or nephropathy	3-02 to 8-03	
SOD2 +/- db/db	C57BL/6J	Phenotyping complete; no increase in neuropathy or nephropathy	3-03 to 9 -03	
Nphs2 Cre//SOD2 loxP/loxP	C57BL/6J	Podocyte specific kos established	6-03 to 12-04	Vanderbilt
Nphs2 Cre//SOD2 loxP/loxP	C57BL/6J 129SvJ	Podocyte specific kos established	1-04 to 12-04	Vanderbilt
nestin Cre//SOD2 loxP/loxP	C57BL/6J	breeding currently	3-04 to 2-05	
synapsin Cre//SOD2 loxP/loxP	C57BL/6J	breeding currently	4-04 to 4-05	
AR tg//SOD2+/- STZ	C57BL/6	phenotyping ongoing; increased neuropathy in diabetics with double mutation	1-04 to 12-04	
AR tg//SOD2+/- db/db	C57BL/6	breeding	6-04 to 5-05	
GLUT4 -/- STZ	C57BL/6J	phenotyping ongoing; increased nephropathy in diabetics with mutation	11-01 to 5-04	Einstein
GLUT4 -/- db/db	C57BL/6J	on hold	~~11-03 to 10-04~~	Einstein

Nphs2 Cre //Glut4 loxP/loxP	C57BL/6J	Podocyte specific kos established; phenotyping ongoing	4-03 to 12-04	Utah
GCLC +/- STZ	C57BL/6J	on hold	~~6-03 to 5-04~~	
GCLC +/- db/db	C57BL/6J	phenotyping ongoing	7-03 to 6-04	
fyn -/-	C57BL/6J 129SvJ	phenotyping complete severe albuminuria in nondiabetics	6-03 to 8-03	
fyn -/- STZ	C57BL/6J 129SvJ	phenotyping completed: increased albuminuria in diabetics with mutation	6-03 to 8-03	
fyn -/-	129SvJ	rederivation completed; phenotyping ongoing. No albuminuria in nondiabetics	9-03 to 8-04	
fyn -/- and +/- STZ	129SvJ	phenotyping ongoing	9-03 to 9-04	
GLUT1 tg	C57BL/6J	phenotyping ongoing; increased glomerulosclerosis in nondiabetics	7-03 to 1-05	Hopkins
GLUT1 tg STZ	C57BL/6J	expanding lines	9-03 to 11-04	Hopkins
GLUT1 tg db/db	C57BL/6J	expanding lines for breeding	10-03 to 1-05	Hopkins
Nphs2 GLUT1 tg db/db	c57B.KLS	creating transgenics	7-04 to 10-05	Hopkins
Nphs2 cre //PPARγ loxP/loxP	C57BL/6J	breeding	4-04 to 8-05	UCLA
conditional Nphs2 cre	C57BL/6J	characterizing multiple lines	breeding stock	
synapsin cre	C57BL/6J	crossed with Rosa26	breeding stock	
nestin cre	C57BL/6J	crossed with Rosa26	breeding stock	

Year 1

SOD2 +/- STZ diabetic mice. Mitochondrial superoxide dismutase (SOD2) detoxifies highly reactive superoxide (O_2^-) by catalysis to hydrogen peroxide, which in turn is reduced in mitochondria by glutathione. The SOD2 -/- mice have a severe reduction in succinate dehydrogenase (complex II) and aconitase (a TCA cycle enzyme) activities suggesting that SOD2 maintains the integrity of mitochondrial enzymes susceptible to direct inactivation by superoxide. These abnormalities lead to cardiomyopathy and neurodegeneration with neonatal lethality. The SOD2 +/- animals have reduced enzyme activity in mitochondria from many tissues, show increased mitochondrial oxidative stress, and have increased mitochondrial swelling due to induction of the permeability transition pore but have no major pathophysiologic abnormalities and no neurodegeneration or nephropathy has been reported. Since the SOD2 -/- mice could not be used for long-term experiments, we reasoned that SOD2 +/- mice, which are viable, would still be susceptible to oxidative stress and diabetic complications. Two experiments were completed with essentially identical results. In the first experiment, there were 6 mice per group. In the second experiment, there were 10 mice per group. Data will be presented from the second larger experiment. Animals were made diabetic per the AMDCC protocol and monthly fasting tail blood glucoses confirmed diabetes. The 4 experimental groups were (C=control, D=streptozotocin diabetes): SOD2+/-D, SOD2+/+D, SOD2+/-C, SOD2+/+C. Diabetic animals remained hyperglycemic throughout the 6 month time period as evidenced by glycated hemoglobin measurements at 24 weeks: SOD2+/-D, 9.63 $\pm$ 0.7; SOD2+/+D, 11.2 $\pm$ 0.3; SOD2+/-C, 4.7 $\pm$ 0.1; SOD2+/+C, 4.9 $\pm$ 0.3.

The SOD2+/-D, SOD2+/+D both developed mild diabetic nephropathy. Urine microalbumin/creatinine ratios (uMa/Cr) were measured monthly starting 4 weeks after

induction of diabetes and 24 hour albumin excretion was determined at the end of the study (6 months of diabetes). Albuminuria (24 h) was increased roughly 2-fold in both diabetic groups compared to control animals: SOD2+/-D, 53.8 ± 11.2 μg; SOD2+/+D, 50.3 ± 12.0 μg; SOD2+/-C, 28.5 ± 10.2 μg; SOD2+/+C, 23.8 ± 3.5 μg. There was no significant difference between the SOD2+/-D and SOD2+/+D groups. Glomerular mesangial extracellular matrix expansion was assessed morphometrically by determining the relative area in the glomerular tuft that was positive for Periodic Acid Schiff (PAS) staining. At least 10 glomerular sections per animal were evaluated. Mild mesangial matrix expansion was detected in both diabetic groups. There was no difference between the SOD2+/-D and SOD2+/+D groups. The degree of nephropathy in both diabetic groups was quite mild. This relative resistance to nephropathy in the STZ C57BL/6J model has been confirmed by other AMDCC investigators.

To assess for the presence of diabetic neuropathy, tail flick analyses and nerve conductions studies were completed at 12, 16, 20 and 24 weeks on the 4 groups. The tail flick analysis constitutes a validated and quantitative assessment of sensory function. The "tail flick" consists of measurements of the time needed for an animal to withdraw his tail from a heat stimulus. There was no statistical difference in tail flick analyses or nerve conduction studies between diabetic and control animals. This absence of neuropathy was unlike what we had previously reported in the less inbred Swiss Webster mouse, and again suggested that the C57BL/6J mouse was relatively resistant to complications when diabetes was induced by streptozotocin.

Targeting the SOD2 gene and podocyte-specific deletion. In order to perform podocyte and neuron specific targeted deletion of the SOD2 gene, we cloned the murine SOD2 gene and created a targeting vector encoding a portion of the murine SOD2 gene in which exons 1 and 2 were flanked by loxP sites to allow for Cre recombinase directed excision of the area encompassed by the loxP sites. During this time the podocyte specific nphs2 Cre mouse was validated for specific and effective expression of Cre recombinase in podocytes. Nphs2 Cre mice were crossed with the Rosa26 mouse which expresses an interrupted "floxed" insert in the LacZ gene in all tissues. In the resultant progeny, Cre recombinase excises the "floxed" insert and thereby activates the LacZ gene. Cells in which Cre recombinase is expressed at sufficient levels to excise segments flanked by LoxP sites will be stained blue with X-gal in tissue sections. In the progeny of the Nphs2 Cre x Rosa26, all podocytes stained blue indicating uniform and sufficient expression of Cre recombinase in podocytes. Equally important, no other cells in the kidney, brain or other tissues showed evidence of Cre recombinase activity (Moeller, et al., Podocyte-specific expression of cre recombinase in transgenic mice. Genesis. 2003;35:39). During the first year, we developed plans to obtain previously characterized neuron specific Cre recombinase expressing mice, synapsin and nestin Cre mice.

GLUT4 -/- STZ diabetic mice. This model was studied because of the empirically observed predisposition of this model to glomerular injury noted when it was on a mixed 129SvJ/C57BL/6J background. It is a paradoxical model because glomerular mesangial cells and podocytes, both of which express GLUT4, should have reduced glucose uptake and thereby be protected from diabetic injury. However, we have previously shown that glomerular GLUT4 levels are reduced in experimental diabetes and that these changes accompany, and perhaps

precede, increases in expression of GLUT1. This reciprocal increase in GLUT1 may be the reason for the observed increased susceptibility to glomerular damage in the GLUT4 -/- animals. In any case, the initial observation, which was made by Dr. Maureen Charron and us, is the reason we have examined these animals, in collaboration with the Einstein/Jefferson group.

The baseline nephropathy found in the mixed background mice was no longer evident when the GLUT4 -/- mice were placed on a pure C57BL/6J background. We determined effects of STZ-diabetes on uMa/Cr in GLUT4 -/- and GLUT4 +/+ animals and found that at the end of the trial, GLUT4 -/- animals had substantially higher ratios than did controls but that these values were not significantly higher in diabetic animals. Similarly, PAS staining revealed little increase with STZ diabetes, but some increase in the GLUT4 -/- animals. Podocyte number was significantly decreased in the GLUT4 -/- mice (GLUT4 -/-, 15.4 ± 0.2 vs. Control, 18.2 ± 0.8 podocytes/glom) and decreased further with 33 weeks of STZ-diabetes.

Year 2

SOD2 and db/db transgenic mouse models. In an attempt to identify another diabetic model with a higher likelihood of developing microvascular complications a genetic model of type 2 diabetes, the db/db C57BL/6J mouse, was bred to the SOD2 heterozygotes (+/-). The db/db mouse with a leptin receptor mutation is obese with insulin resistance and develops hyperglycemia by 4 weeks of age. An experiment with 10 animals per group was conducted over a 24 week period. The groups were: SOD2+/- db/db, SOD2+/+ db/db, SOD2+/- db/+ and SOD2+/+ db/+. The fasting blood sugar profiles of these mice are presented in **Fig. 1**. After 16 weeks, fasting tail blood glucoses in the db/db mice began to normalize and by 24 weeks, had attained essentially normoglycemic levels. The 28 week glycated hemoglobin data parallel the fasting blood glucose levels but remained elevated at the end of the trial.

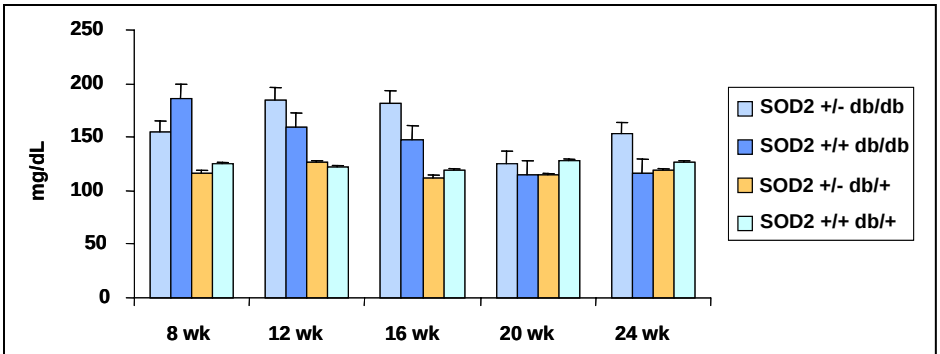

Fig. 1. Fasting blood sugars in C57BL/6J SOD2 +/- and +/+ db/db and db/+ mice

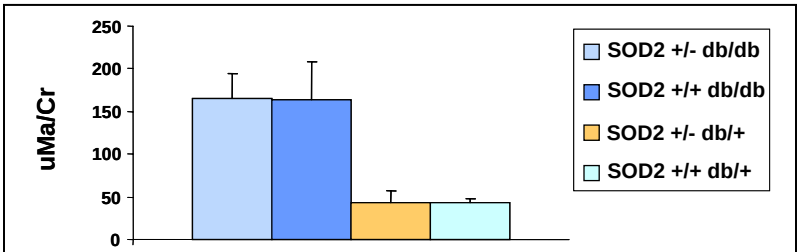

Fig. 2. Albuminuria (μg albumin/mg creatinine) in C57BL/6J SOD2 +/- and +/+ db/db and db/+ mice at 28 wk of age.

The extent of nephropathy was assessed as for the SOD2 STZ mice. The diabetic groups developed significant increases in uMA/Cr and 24 h albuminuria (**Fig. 2**). There was no

significant difference in albuminuria between the SOD2+/- db/db and SOD2+/+ db/db groups. Mesangial matrix expanded approximately 25% in both diabetic groups. There was no significant difference in matrix area between the SOD2+/- db/db and SOD2+/+ db/db groups.

In the first 12 weeks of diabetes, db/db mice, regardless of their SOD2 genotype, had prolonged tail flick times of greater than 10 sec, highly significantly different from db/+ mice.

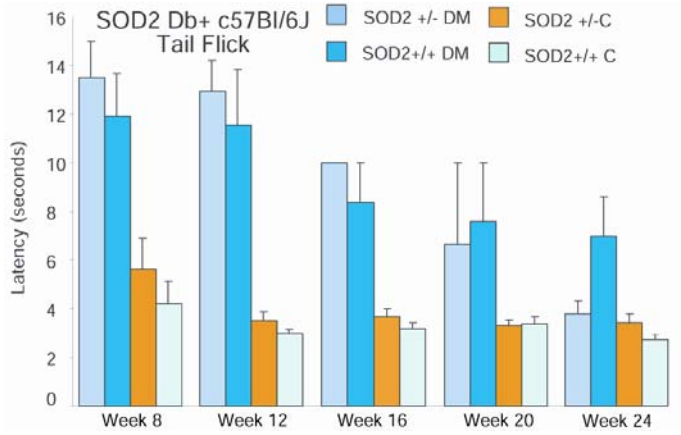

Fig. 3.

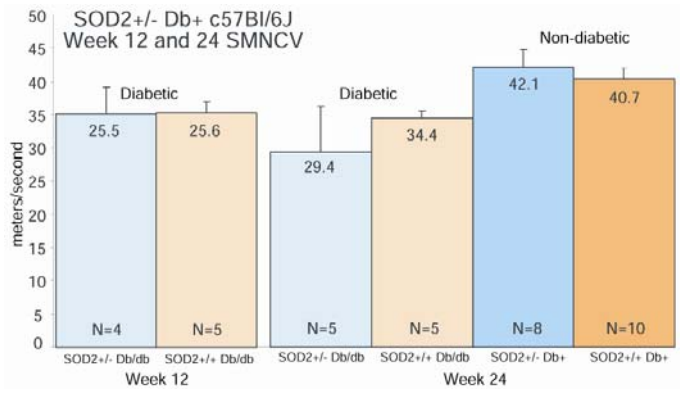

Fig. 4.

By week 16, as fasting blood sugars approached more normal levels, the tail flicks of the db/db mice were less than 10 msec, but still significantly longer than db/+ animals. This downward trend appears to continue at weeks 20 and 24, although the number of animals with measurable sensory loss by tail flick was small (**Fig. 3**). Like the tail flick analyses, the nerve conduction studies (tail motor distal latency, tail sensory and sciatic nerve conduction velocities) remained abnormal but not to the degree we have previously observed in the mutant leptin receptor rat model, the Zucker rat, or to the initial degree of abnormality observed when hyperglycemia was high (e.g., at 12 weeks) (**Fig. 4**). Collectively these data show impaired neural function due presumably to a combination of hyperglycemia and insulin resistance in the db/db animal, with a suggestion of a modest decrease in overall impairment as the animal becomes more normoglycemic. There was no statistically significant additive effect of the SOD2 +/- genotype.

Targeted SOD2 mice and podocyte specific SOD2 -/- mice. As we were preparing to initiate embryonic stem cell injections with our "floxed" SOD2 gene construct, we learned of the availability of a mouse line in which SOD2 had already been similarly targeted. In collaboration with AMDCC investigators at Vanderbilt University (Drs. Harris and Breyer) we obtained these mice to breed to the nphs2 Cre mice, and subsequently to neuronal-specific Cre mice. These animals were received in the latter half of year 2. The colony was expanded and a breeding strategy was established to secure populations of nphs2 Cre//SOD2 loxP/loxP and SOD2 loxP/loxP littermates. These models are currently being studied in year 3 (see below).

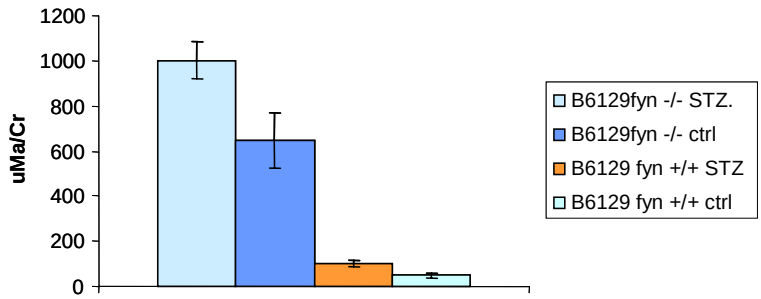

Fig. 5. Albuminuria (μg albumin/mg creatinine) in fyn -/- and +/+ on a mixed C57BL/6J and 129SvJ background 12 wk post-STZ injection.

Fyn -/- STZ diabetic mice on mixed 129SvJ/C57BL/6J background. The Src family member, fyn, directly associates with and phosphorylates membrane-associated Nephrin in podocytes (Verma, et al., Fyn binds to and phosphorylates the kidney slit diaphragm component Nephrin. J Biol Chem. 2003 ;278:20716). In that initial publication, fyn -/- mice had no significant increase in albuminuria compared with fyn +/+ mice, but there were significant changes in podocyte foot process morphology, suggesting that they could be susceptible to diabetic injury. Therefore, we initiated a trial of STZ diabetes in the fyn -/- and +/+ animals on the original mixed 129SvJ and C57BL/6J background. Interestingly, in our colony, nondiabetic fyn -/- mice developed massive albuminuria (**Fig. 5**) with a uMa/Cr of 649 ± 121. STZ diabetes however did increase uMa/Cr to 1002 ± 83. Because of those findings, we obtained embryonically derived fyn -/- mice on a pure 129SvJ background to determine if background effects could explain the massive albuminuria in nondiabetic animals. We also bred these animals to obtain fyn +/- 129SvJ mice to determine if gene dosage would alter albuminuria responses. These models are currently being studied in year 3 (see below).

Targeted GLUT4 mice and podocyte specific GLUT4 -/- mice. Because of the encouraging data suggesting that the STZ diabetic GLUT4 -/- mice developed enhanced nephropathy, we wished to assess whether podocyte specific ablation of the GLUT4 gene had a similar effect. Dr. Dale Abel, an AMDCC investigator at the University of Utah, had already developed a “floxed” GLUT4 mouse. We therefore sent him nphs2 Cre mice for breeding with the targeted GLUT4 mice. These mice are being studied in year 3 (see below).

GLUT1 transgenic mice. Based on our previous work showing that GLUT1 expression in rodent glomeruli increases in diabetes and that increased GLUT1 expression drives expression of extracellular matrix proteins, we developed a GLUT1 transgenic mouse to overexpress GLUT1 in glomerular cells as well as in nerve and other complication-prone tissues. The construct employs a modified beta actin promoter already utilized by Dr. Heilig for expression of GLUT1 antisense mice (Heilig et al., GLUT1-Deficient Mice Exhibit Impaired Development and Deformities Similar to Diabetic Embryopathy, Proc Natl Acad Sci, 2003;100:15613) that have a substantial reduction in glomerular GLUT1 levels. These mice are currently being studied in year 3 (see below).

Aldose reductase transgenic x SOD2 +/- mice. Although enhanced aldose reductase activity has been correlated to diabetic complications, especially neuropathy, diabetic aldose reductase overexpressing (AR tg) mice show only a mild increase in neuropathic findings when compared to control diabetic mice (Stevens MJ, et al., unpublished and Yagahashi, et al., Neuropathy in diabetic mice overexpressing human aldose reductase and effects of aldose reductase inhibitor. Brain. 2001;124:2448). Dr. Stevens has found that the diabetic AR tg mice

have a profound reduction in oxidative stress in the peripheral nerve when compared to diabetic control mice due to an increase in SOD activity. Therefore, in order to restore the full effect of aldose reductase activity on neuropathic changes, the AR tg mouse was crossed with the SOD2 +/- mice to generate AR tg//SOD2 +/- progeny. These mice are currently being studied in year 3 (see below).

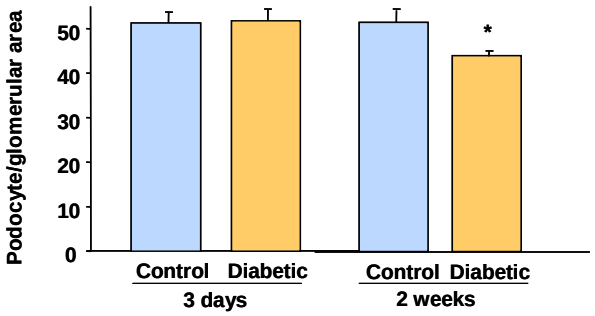

Fig. 6. Podocyte number in C57BL/6J mice 3 d or 2 wk post-STZ injection.

Podocyte loss in STZ diabetes in mice. We had previously determined that STZ diabetes leads to podocyte loss in rats, similar to the relatively early loss found in humans with very early diabetic nephropathy. In order to determine if such changes occur in mouse models, and importantly, to confirm that the AAMDC's accepted multiple low dose STZ protocol did not cause early toxic loss of podocytes, we examined podocyte number in C57BL/6J mice 3 days and 2 weeks after completion of STZ injections. There was no diminution of podocyte number after 3 days. However, at 2 weeks there was a small but significant decrease in podocyte number in the diabetic mice compared to controls (**Fig. 6**). These data confirm that podocyte loss is an early manifestation of diabetic nephropathy, but by itself does not presage substantial albuminuria and mesangial expansion, since C57BL/6J STZ mice develop very modest diabetic changes after 6 months. These findings also confirm that the AMDCC multiple low dose STZ protocol does not lead to toxic loss of podocytes.

Beginning of Year 3

Neuronal specific Cre mice. To determine efficacy and specificity of nestin Cre and synapsin Cre expression in these models, we crossed each of these mouse models with the Rosa 26 mouse. We characterized the expression of β-galactosidase in the progeny of these crosses at postnatal days 1, 7, 14, 21 and in adult mice for nestin Cre x Rosa26 mice and at days 1, 7, and 21 for synapsin Cre x Rosa26 mice. Three to 4 mice were examined per time point. In the nestin Cre x Rosa26 mice β-galactosidase was localized within neurons of the dorsal and ventral horns, large and small DRG neurons, and prevertebral sympathetic neurons. Not all neurons stained positive for β-galactosidase activity; morphometric analysis to determine the percentage of DRG neurons in which the reporter gene is activated is ongoing. In addition, β-galactosidase was detected in renal glomerular podocytes and especially in proximal tubular epithelial cells as well as other tubular cells. In the synapsin Cre x Rosa26 mice β-galactosidase was widely distributed in brain and spinal cord. Localization to dorsal root ganglia is being evaluated currently. Because nestin expression occurs in complications prone peripheral and autonomic neurons, we have bred the nestin-Cre mice with the floxed SOD2 mice and are waiting for sufficient numbers of mice to begin diabetes induction with streptozotocin.

GCLC+/- db/db mice. The γ-glutamate cysteine ligase (GCLC) heavy chain was targeted for disruption to produce a glutathione knockout mouse by two independent groups. This enzyme is an essential enzyme in glutathione synthesis. Homozygous disruption of the heavy subunit of GCLC is embryonically lethal. In contrast, the GCLC +/- mice are viable but show substantial decreases in GCLC protein and activity, and an approximately 20% decrease in glutathione levels. Thus, the GCLC +/- mouse should be a useful genetic model for mild endogenous oxidative stress, which could be substantially increased in diabetes. Given the resistance of normal C57Bl/6J mice to STZ diabetes, we bred these mice into the db/db C57BL/6J background resulting in 4 experimental groups: GCLC+/- db/db, GCLC+/+ db/db, GCLC+/- db/+ and GCLC+/+ db/+. This trial is ongoing. At 12 weeks, uMa/Cr levels are increased in both db/db groups. There was no significant difference between GCLC+/- db/db and GCLC+/+ db/db at 12 weeks. Smaller numbers have reached 16 weeks of age, but there is an suggestion that the uMa/Cr in GCLC+/- db/db mice is increasing at a faster rate than in the GCLC+/+ db/db mice. This impression will need to be confirmed as more animals reach this and later timepoints. Similarly, by 16 weeks, there is a substantial decline in both tail flick and nerve conduction velocities (**Fig. 7**) in the db/db mice. While the numbers of mice tested are small, there is a suggestion that the GCLC+/- db/db animals are more prone to neuropathy than are the GCLC+/+ db/db mice. Again, this will be tested as more animals reach this timepoint.

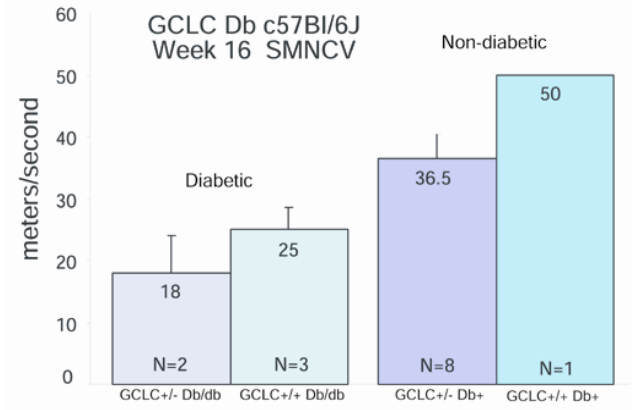

Fig. 7.

Effects of semisynthetic diet. The external advisory committee has expressed concern that lack of standardized, semisynthetic diets could result in skewed results due to variations in phytoestrogens or other hormones in regular rodent chow. Therefore, the GCLC db trial was performed with all animals on a semisynthetic diet (AIN - 76A Diet). The mice were given ad libidum access to the chow, but interestingly the db/db mice (whether GCLC +/+ or +/-) gained less weight than db/db mice on the regular Purina chow and developed fasting hyperglycemia that was even lower than levels in C57Bl/6J mice on a regular chow diet (**Fig. 8**).

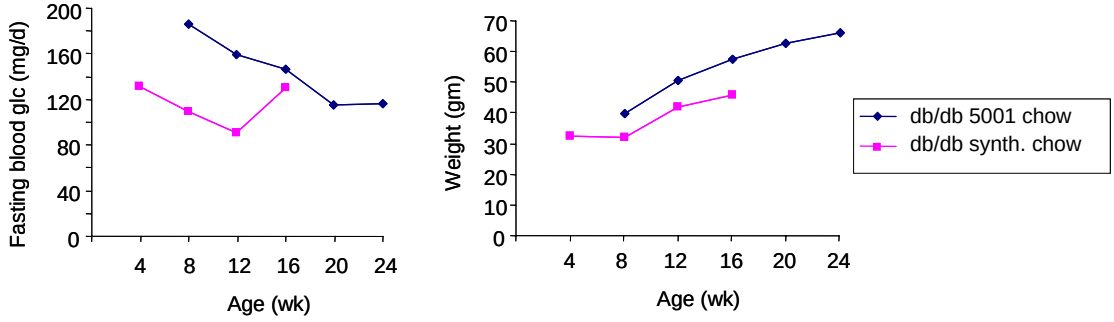

Fig. 8. Effect of semisynthetic chow (AIN - 76A Diet) on blood glucose and weight in C57BL/6J db/db mice

Podocyte specific SOD2 -/- mice. The breeding strategy has resulted in large numbers of nphs2 Cre//SOD2 loxP/loxP and SOD2 loxP/loxP mice for study. We are beginning immunohistochemical studies to confirm SOD2 deletion in the nphs2 Cre//SOD2 loxP/loxP podocytes. Initial groups have been injected with STZ.

Fyn -/- STZ diabetic mice on a pure 129SvJ background. Because of the high levels of albuminuria in the nondiabetic fyn -/- mice on the mixed 129SvJ/C57Bl/6J background, we obtained fyn +/-embryos that were on a pure 129SvJ background. The nondiabetic fyn -/- and +/- 129 SvJ mice have no significant increase in albuminuria up to 24 weeks of age. Therefore, the fyn +/+, +/- and -/- mice were subjected to STZ diabetes. A small number of STZ fyn +/- and +/+ mice are at 12 weeks post-STZ at this point. There is no significant albuminuria in either group at this timepoint.

Podocyte specific GLUT4 -/- mice. Dr. Abel's group at the University of Utah performed initial breeding and sent us animals for evaluation of diabetic nephropathy. At this point we are comparing the effects of STZ diabetes on nphs2 Cre//GLUT4 loxP/loxP and GLUT4 loxP/loxP mice. The first group should have Cre mediated disruption of both GLUT4 alleles in podocytes alone. We are utilizing immunohistochemistry to confirm this podocyte-specific knockout of GLUT4. Initial experiments suggest absence of podocyte GLUT4 from 12 week old nphs2 Cre//GLUT4 loxP/loxP mice and presence in GLUT4 loxP/loxP mice. We will extend these observations and assess total glomerular GLUT4 by immunoblots of pure glomerular lysates which we have been performing regularly. These mice are on a mixed FVB/C57BL/6J background. These mice have developed substantially greater fasting hyperglycemia in response to STZ than mice on pure C57BL/6J backgrounds with average fasting blood glucose of ca. 400 mg/dl at 8 weeks post-STZ injection. At this timepoint, nphs2 Cre//GLUT4 loxP/loxP and GLUT4 loxP/loxP mice still have similar and essentially normal uMa/Cr values (49.3 ± 24 vs. 38.5 ± 19.2 respectively).

GLUT1 transgenic mice. In order to obtain a generalized complications prone mouse model we developed a transgenic mouse in which human GLUT1 cDNA is driven by a 3kb portion of the human ß-actin gene 5'-flanking sequence plus 5' untranslated region and

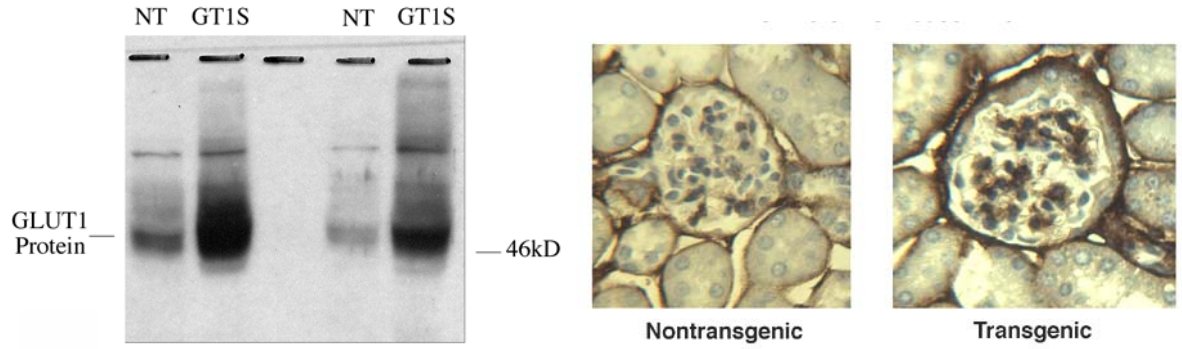

Fig. 9. Confirmation of GLUT1 overexpression in glomeruli in GLUT1 tg mice. Staining for GLUT1 appears to be extensive in the mesangium and perhaps enhanced in podocytes as well (right panel).

intervening sequence. This promoter has been successfully used by us to express a GLUT1 antisense mRNA in transgenic mice leading to substantial reduction in GLUT1 expression (Heilig, et al. GLUT1-Deficient Mice Exhibit Impaired Development and Deformities Similar to Diabetic Embryopathy, Proc Natl Acad Sci, 2003;100:15613). Early analysis of one founder line of the GLUT1 transgenic animals shows substantial GLUT1 glomerular overexpression (**Fig. 9**) and enhanced PAS positive extracellular matrix deposition in nondiabetic glomeruli. We are initiating trials to examine effects of STZ diabetes on nephropathy and neuropathy in this model within the next 2 months. We will also breed into the C57BL/6J db/+ background.

Podocyte specific GLUT1 overexpression in db/db C57BKS mice. Because the db/db model develops more substantial nephropathy and neuropathy in the C57BL/6J background than do STZ diabetic C57BL/6J mice, we would prefer to analyze our genetic manipulations in this model when possible. However, in many of the AMDCC centers, this model does not maintain fasting hyperglycemia and therefore, effects of diabetes may be limited. However, in a related strain, C57BKS (or BKS.Cg) the db/db mutation results in much more dramatic hyperglycemia that is sustained for at least 6 months. Therefore, this model would be ideal for genetic manipulations to study their effect on diabetic complications. However, since most genetic manipulations have been performed on 129SvJ and C57BL/6J backgrounds, bringing such genetic changes onto a pure BKS.Cg background would entail 2 years of breeding. If genetic models can be developed directly on this background, complications studies could be dramatically accelerated. Therefore, we have attempted to create a podocyte specific GLUT1 overexpressing transgenic mouse by microinjecting the DNA into BKS.Cg-m +/+ Lepr<db> (Jackson Stock# 000642) eggs. We have had initial success and have identified at least 7 potential db/m founder transgenic males. Progeny of these animals are being assessed for GLUT1 overexpression in podocytes and total GLUT1 expression in glomerular lysates. We are also setting up progeny for uMa/Cr determinations and neuropathy phenotyping.

Aldose reductase transgenic x SOD2 +/- STZ mice. These mice had NCV determinations 2 weeks after multiple low dose STZ injections. Motor nerve conduction velocities were reduced in the AR tg//SOD2 +/- mice (33 ± 4 m/sec) but not in the AR tg (46 ± 5 m/sec) or SOD2 +/- (41 ± 8 m/sec) animals. Many of the combined AR tg//SOD2 +/- animals died early from very severe hyperglycemia and were unavailable for nephropathy determinations. Although this model may be a difficult one to sustain over many months, we are hopeful that with small doses of insulin or even lower STZ administration, these mice will survive for 6 months of diabetes.

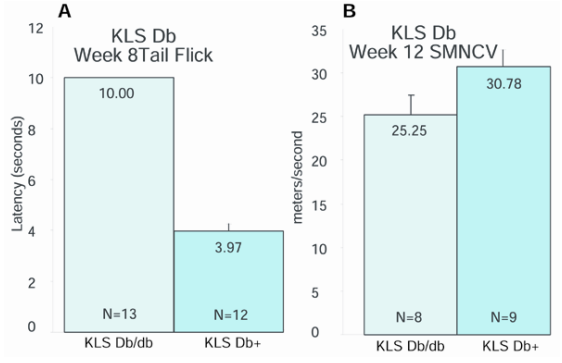

Fig. 10

db/db C57BKS mice. AMDCC data over the past 2 years have now amply demonstrated that C57Bl/6J mice are relatively resistant to developing nephropathy and neuropathy. Although db/db C7BL/6J mice are more susceptible to microvascular complications than the STZ C57Bl/6J animals, this model remained generally resistant to progressive nephropathy and neuropathy despite genetic modifications.

Moreover, as just noted, this model demonstrated a return to normoglycemia in several, though not all, of the AMDCC centers. In continued efforts to find the best genetically modified mouse for further neuropathy studies, we are completing the phenotyping of the KSLdb/db animals. Tail flick analysis (done at week 8) and sciatic nerve conduction studies (done at week 12) confirm significant neuropathy in these animals (**Fig. 10**), which will be further phenotyped when they reach 24 weeks.

Collaborations with other Groups:

Although many of our specific collaborations have been detailed in the progress report above, we have established the following intra-AMDCC collaborations:

1) Podocyte specific SOD2 knockout project is in collaboration with the Vanderbilt group.

2) Podocyte specific GLUT4 knockout project is in collaboration with the University of Utah group

3) GLUT4 knockout project is in collaboration with the Albert Einstein/Jefferson group.

4) Podocyte specific PPARg knockout project is in collaboration with the UCLA group.

5) All pertinent models from all the groups are to be sent to the University of Michigan for neuropathy phenotyping.

Pertinent non-AMDCC Collaborations:

Listed below are the major collaborative projects related to the consortium goals but independent of AMDCC:

1) JDRF Center of Excellence in the study of diabetic complications. This center encompasses a number of collaborative projects exploring the role of glucose transporters, oxidative stress, SH2B-β, growth hormone receptors in diabetic complications. It also includes several clinical projects testing antioxidants and other agents in the treatment of diabetic complications. The Center Director is Dr. Feldman. Drs. Brosius, Russell and Stevens have projects in this center. Drs. Christin Carter-Su, and Ram Menon are non-AMDCC collaborators in this center.

2) Dr. Feldman is PI for several collaborative NIH grants investigating the etiology, pathogenesis and treatment of diabetic polyneuropathy. Dr. Russell is a co-investigators on several of these grants.

3) Dr. Feldman is an investigator in neuropathy aspects of the multi-institutional Epidemiology of Diabetes Interventions and Complications (EDIC) study.

4) Dr. Brosius is PI on two NIH grants investigating the role of glucose transporters in vascular disease. Collaborators include several vascular biologists.

5) Drs. Feldman and Brosius are PIs on grant proposals with Dr. Michael Uhler (University of Michigan) in a collaborative informatics project attempting to define a common set of transcriptional events that occur early in diabetic neurons and podocytes.

6) Dr. Heilig is the PI on several funded and pending NIH proposals for the study of GLUT1 in diabetic and nondiabetic nephropathy and diabetic embryopathy as well as GLUT1 haplodeficiency syndromes. Dr. Brosius is a collaborator and consultant on these proposals.

7) Dr. Stevens is the PI on several collaborative grants investigating myocardial aspects of diabetic autonomic neuropathy.

8) Dr. Russell is the PI on a NIH project investigating the role of IGF-1 in oxidative stress and apoptosis in diabetic neuropathy; collaborators on this project include Drs. Feldman and Michael Brownlee (Albert Einstein College of Medicine). Dr. Russell is also the PI on a VA grant studying IGF-1 and Schwann Cells in neuropathy.

9) Dr. Holzman has collaborative projects and grants on glomerular podocyte cell biology and pathology with multiple glomerular disease investigators worldwide.

Summary of Accomplishments

The major accomplishments in the first 2½ years of the AMDCC by the Michigan/Hopkins group have been:

1) the development and validation of neuropathy phenotyping in mouse models and the neuropathy testing of several mouse models of diabetic neuropathy;

2) the development and initial evaluation of novel podocyte specific knockout animals;

3) the novel development of a transgenic model in BKS.Cg-m +/+ Lepr<db> mice allowing for direct testing of genetic manipulations on diabetic mouse background;

4) the establishment of a generalized GLUT1 overexpression model which should provide an enhanced model for all diabetic complications;

5) the development of the AR tg//SOD2 +/- mouse which develops profound diabetic neuropathy;

6) the demonstration that fyn -/- mice have substantial albuminuria that is augmented by diabetes and affected significantly by strain background.

Plans for the coming year

Plans for each of our extant models have been included under the Program Accomplishments section. We will also be testing effects of diabetic neuropathy on the IRS-1 knockout animals from the Joslin Clinic.

Most significant achievement.

The advances noted in the Summary of Accomplishments above are our most significant achievements; we will not attempt to further prioritize them.

Publications

Brosius FC III. Trophic Factors and Cytokines in Early Diabetic Glomerulopathy, Experimental Diab Res; 2003.

Siu BB, Saha J, Smoyer WE, Sullivan KA, Stevens MJ, Brosius FCIII. Podocyte Loss in Early Streptozotocin Diabetes: Prevention by Lipoic Acid Treatment, in revision, Kidney International.