ANIMAL MODELS OF DIABETIC COMPLICATIONS CONSORTIUM

(U01 DK61018)

Annual Report (2005)

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PART A:

PRINCIPAL INVESTIGATOR'S SUMMARY

Program Accomplishments:

The major theme of the AMDCC project at Vanderbilt is the identification and characterization of genetic modifiers causing diabetic nephropathy. Within this theme three related topics of research are: 1) characterization of candidate gene mutations exacerbating nephropathy in mice; 2) characterization of ENU mutants exhibiting diabetic nephropathy, and 3) Establishment of Phenotyping capability for renal function in diverse strains of mice.

Major accomplishments include:

1) Determination that eNOS-/- db/db mice exhibit more severe diabetic nephropathy than ApoE, LDLR or Cyp4a14 knockout mice. This model is the most robust model of DN we have characterized thus far, exhibiting both decreased GFR and robust albuminuria.
2) Establishment of two lines of C57BL/6 mice exhibiting novel dominant ENU induced mutations conferring excess albuminuria and reduced GFR in the setting of diabetes as compared with other mice of the same strain.
3 We are determining whether glucose control (reflected by fasting blood sugar, HbA1C, or glycated Hb) correlates with albuminuria in several strains of mice.
4) We demonstrated that different mouse strains exhibit more severe albuminuria and histopathological changes than C57BL/6J, especially KK and DBA. We are determining whether these lines will be suitable for QTL mapping.

Collaboration within the group

Project 1: Project 1 – " Characterization of Candidate genes predisposing to diabetic nephropathy.

This project is focused primarily on type II diabetes and defining the role of dyslipidemia and hypertension in exacerbating diabetic nephropathy. The project is now focusing on eNOS mutant mice. Project 2 characterizes type I diabetes in mice resistant to nephropathy. Both are utilizing phenotyping techniques developed by project 3, to measure glucose control, GFR, albuminuria, and glomerular histopathology. Dr. Takahashi and Dr. Harris are the lead investigators of this project and are working together with Dr. Breyer to measure GFR and albuminuria in these models.

Project 2

In collaboration with Dr. Gene Rinchik a member of our consortium at University of Tennessee, in a project directed by Dr. Breyer, we have generated over 375 diabetic ENU mutants and has generated two mutants lines increased albuminuria and reduced GFR in diabetic mice. Drs Rinchik and Breyer, will continue to collaborate as we move forward with mapping the mutations. Drs. Breyer, and Fogo have collaborated in characterization of renal pathological changes occurring in diabetic strains.

Project 3
Project 3 involves close work with projects 1 and 2 and has evolved a major goal of phenotyping diabetes and diabetic nephropathy in different strains of inbred mice. This past year we published a manuscript in Diabetes characterizing nephropathy in different inbred strains. This year, Dr. Zhonghua Qi in Dr. Breyer's group will characterize glucose control in wild-type and diabetic mice of different inbred line. This is important since in humans, the one factor that reliably reduces diabetic complications is improvied glucose control. In contrast, there is little information relating the level of glucose control to diabetic complications in mice. Dr. Qi will examine the relationship between fasting blood glucose and continuous blood glucose values measured with the Mini-med subcutaneous glucometer.

Collaboration with other AMDCC groups:
Our group continues to collaborate will other members of the nephropathy group within the AMDCC try and develop a "meta-analysis" relating glucose contol to albuminuria aind HbA1C in different strains of mice. While we had shipped eyes from diabetic mice from KK, DBA, C57BL/6 and FVB to Tim Kern for characterization of the severity of their retinopathy, we . Dr. Kern is also measuring glycated Hb with us to correlate these values with HbA1C values and serum glucose

Dr. Breyer's group has continued to collaborate with Dr. Kumar Sharma and Erwin Bottinger in the AMDCC program at Jefferson and Einstein to evaluate serum creatinine as an endogenous marker for determining GFR in mice. We've also collaborated with Tim Kern to validate which glycated hemoglobin determination is best for use in mice. Finally we are expecting to send ENU mutant mice up to the university of Michigan for cross phenotyping of neuropathy, and having the bladders sent to Dr. Daneshgari and eyes sent to Dr. Kern for phenotyping of uropathy and nephropathy respectively.

Pertinent non-AMDCC Collaborations
The VU AMDCC is collaborating with the VU-MMPC to determine the appropriate measurement for glycated Hbs in mice and comparing these to blood glucose. We are undertaking a pilot project looking at 24 hour continuous glucose monitoring in mice instrumented with a subcutaneous glucose sensor (MiniMed, Germany).

Dr. Charles Epstein's colony of the FVBOVE26 model of type I diabetes mellitus, at Univ of Louisville became contaminated with Murine Hepatitis virus and had to be rederived by Jackon labs. The colony is just now becoming available. Dr. Mary Loeken, (at Harvard) apparently lost her entire colony of FVB/N Akita mouse and is having to re-backcross the Akita mutation onto FVB. We continue to characterize the the Balb/C-Akita mouse developed by Dr. Ambra Pozzi at Vanderbilt. We have also have continued our collaboration with Dr. Rob Williams at Univ Tennessee, Memphis to plan the mapping of Quantitative trait loci that could contribute to DN in the prone DBA/2J mouse vs. the resistant C57BL/6 mouse.

Response to previous EAC comments:

Studying differences between male and female mice in the models we are using is problematic given the huge difference between glucose values in male and female C57BL/6 $Ins2^{Akita}$ mice. Female C57BL/6 $Ins2^{Akita}$ exhibit only borderline increased glucose while male C57BL/6 $Ins2^{Akita}$ fasting glucose is ~600mg/dl. For this reason all studies on the type I Akita mice have been restricted to male mice.

Regarding issues of cross-phenotyping, we are scheduled to send ENU76 mice to the university of Michigan on February 27th. Once delivered to the UofM the mice will be phenotyped for neuropathy and uropathy. Tissues will be processed for retinopathy.

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PART B:

UPDATE BY PROJECT LEADERS

Responsible Investigators: **Raymond C. Harris, M.D. & Takamune Takahashi**

Project Number and Title: **Project 1 – " Characterization of Candidate genes predisposing to diabetic nephropathy"**

A. Rationale and Relevance:

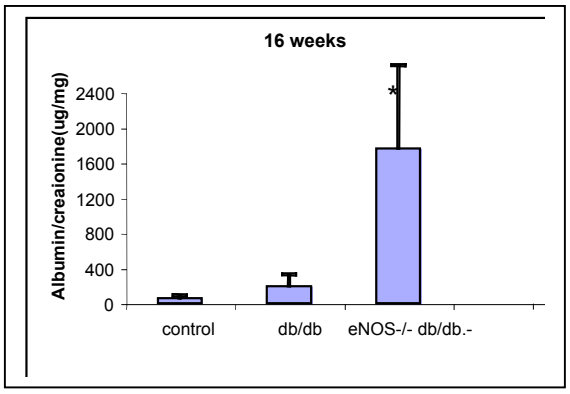
The goal of Project 1 has been to examine candidate modifier genes in either development or progression of diabetic nephropathy. We have tested candidate genes that were originally suggested from population studies indicating predisposition to development and/or progression of diabetic nephropathy in type I or type II diabetes. We have specifically concentrated genes related to potential alterations of endothelial function or alterations in regulation of lipoproteins as exacerbating factors in both type I and type II diabetes. Polymorphisms in eNOS and ApoE3 genes were associated with diabetic nephropathy. We therefore examined the effects of deletion of these genes on the development of diabetic nephropathy in mice.

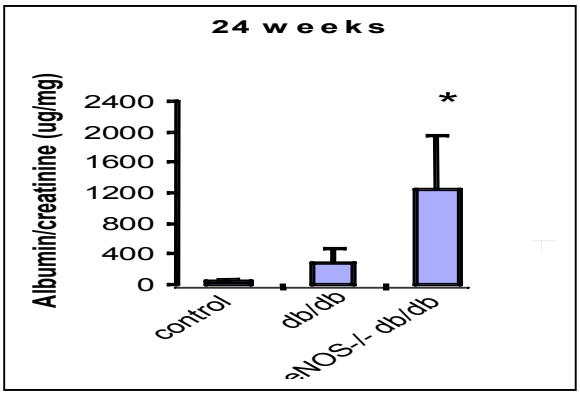
We utilized the streptozotocin model primarily as a model of insulinopenia and db/db mice as model of insulin resistance. Mice carrying the *db* mutation of the leptin receptor (i.e. $LepR^{db}$) do not respond to leptin over eat and homozygous *db/db* mice become obese by 3 to 4 weeks of age. Plasma insulin is elevated by 10 to 14 days and blood sugar is elevated by 4 to 8 weeks. In dib/db mice, the course and severity of the disease are influenced by genetic background of the mice. On the C57Bl/6J background, islet ß-cells undergo compensatory hyperplasia, and the mice display continued hyperinsulinemia throughout an 18-to 20-month life span.

The *db/db* mice on the C57BL/6J background develop minimal nephropathy. In contrast, when *db/db* is expressed on the C57BLKs background, the diabetes is more severe due to an insulinitis that results in progressive depletion of the insulin-producing ß-cells of the pancreatic islets, and death by 10 months of age. During this granting period, we have completed the backcross at the backcross of several modifier genes including ApoE-/-, LDLR-/- and eNOS-/- from C57BL/6 to C57BLKS at the 10^{th} generation (N10).

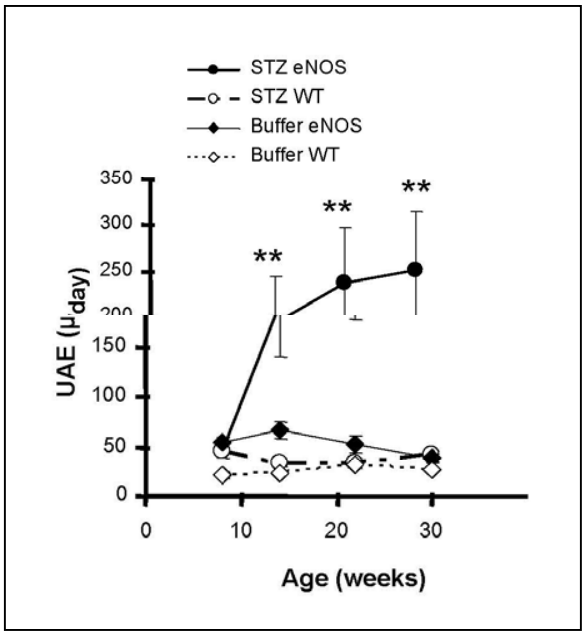
B. Summary of accomplishments

In 16-week-old non-obese C57BLKS mice (control), albumin to creatinine ratio (ACR) was 61 ±14 µg/mg (n=6). In db/db mice it was increased to 196±55, while in the eNOS-/- db/db mice it was increased to 1763±389. Similarly in 24 week old control mice albumin to creatinine ratio was 38 ±10 while db/db was 282 ±73 and eNOS-/- db/db mice were 1243±291 (Figure 1). These results indicated that proteinuria was





significantly increased in the presence of deletion of the eNOS gene. Similar increases in proteinuria were also seen in mice on the C57v/6 background with either streptozotocin-induced diabetes (Figure 2) or with type II **Figure 1 Albuminuria in db/db mice on the BKS background with or without genetic deletion of eNOS (eNOS-/-) at 16 and 24 weeks.diabetes (db/db)**.



In contrast there were only moderate increases in albuminuria in mice with deletions in either LDLR or apoE genes (350 and 377 µg/mg respectively at 26 weeks).

GFR was measured by the method of FITC-inulin clearance. AT 24 weeks, control mice on the BKS background had GFRs of 331±79 µ/min, db/db mice were 366±102 and db/db mice with eNOS deletion had GFRs of 164±76 (Figure 3A). A time course of GFR in eNOS-/- db/db mice indicated that at 8 wks of age, GFR was 390±120 µl/min and fell to 160±50 and 164±76 and 16 and 24 weeks, in respectively (Figure 3B).

Figure 2 Albumine excretion in C57B/6 Mice with streptozotocin-induce diabetes

Histologic changes were most striking in the eNOS db/db mice on the BKS background, with evidence of nodular glomerulosclerosis and arteriolar hyelinosis (Figure 4A), and with increased glomerular matrix content (indicated by fibronectin staining in Figure 4B). However, there were significant glomerular histopathology seen in the C57B/6 mice made insulinopenic with streptozotocin, as indicated by the glomerular basement membrane thickening and foot process effacement (Figure 4C) and increased podocyte VEGF expression (Figure 4D).

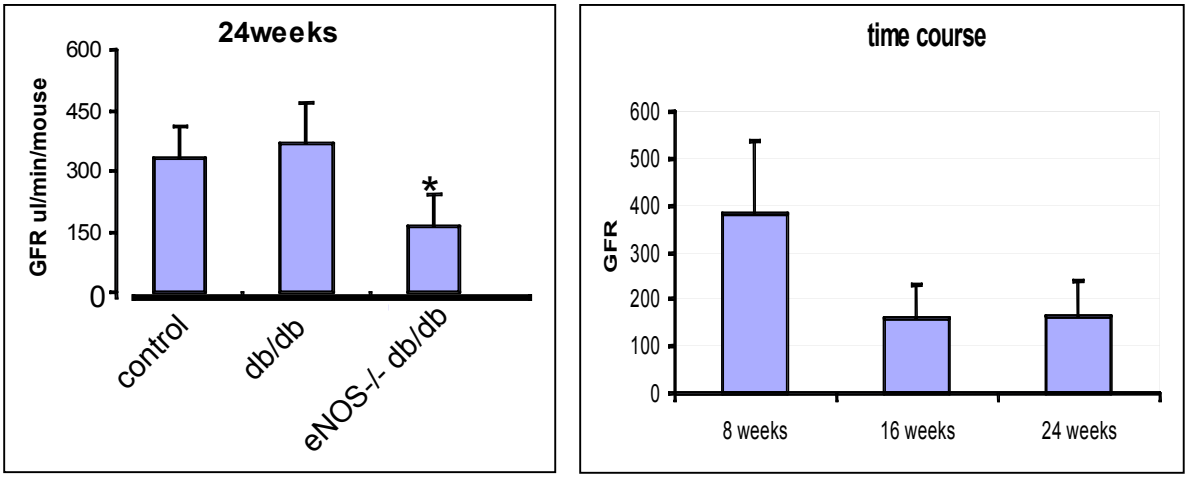
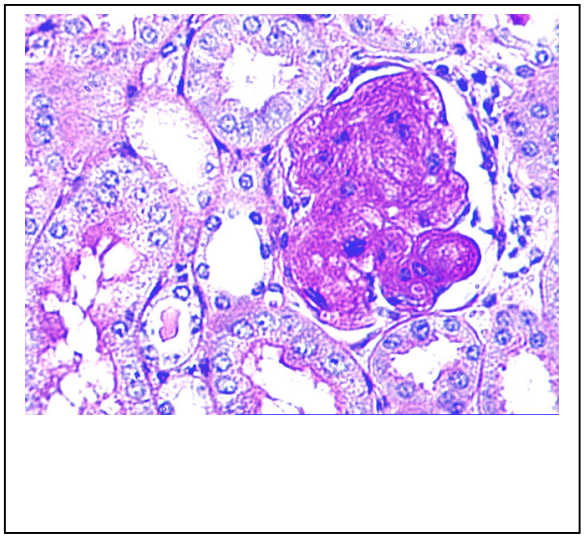
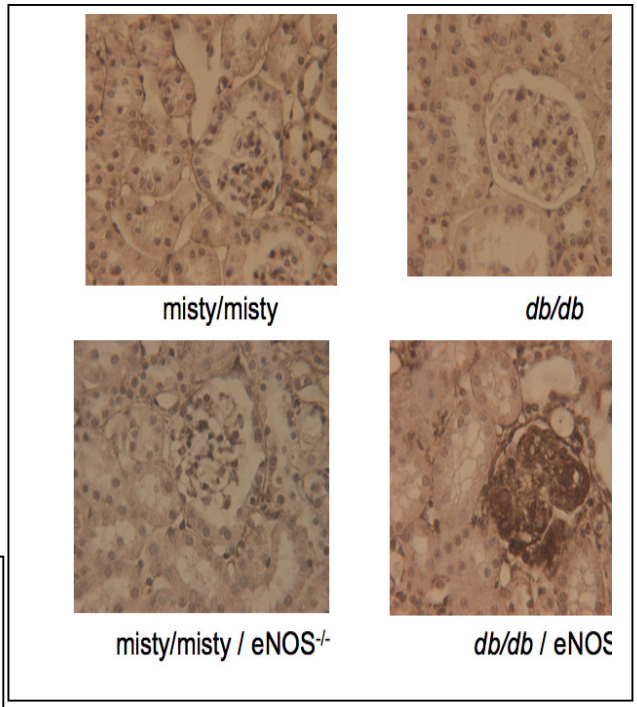
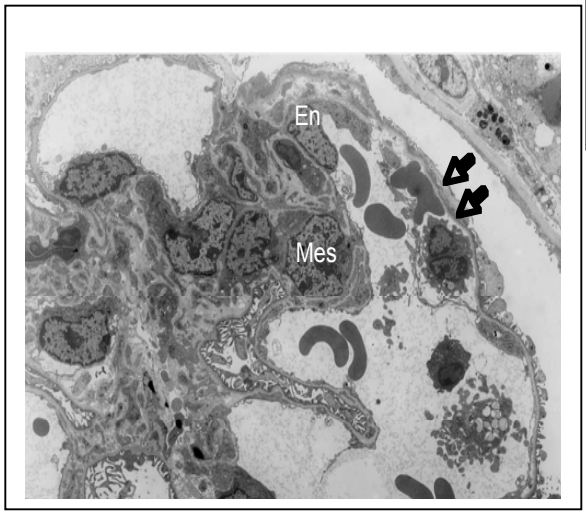


Figure 3 Glomerular filtration rate in mice on BKS background A) 24 wks. B time cours of GFR in eNOS-/- db/db mice

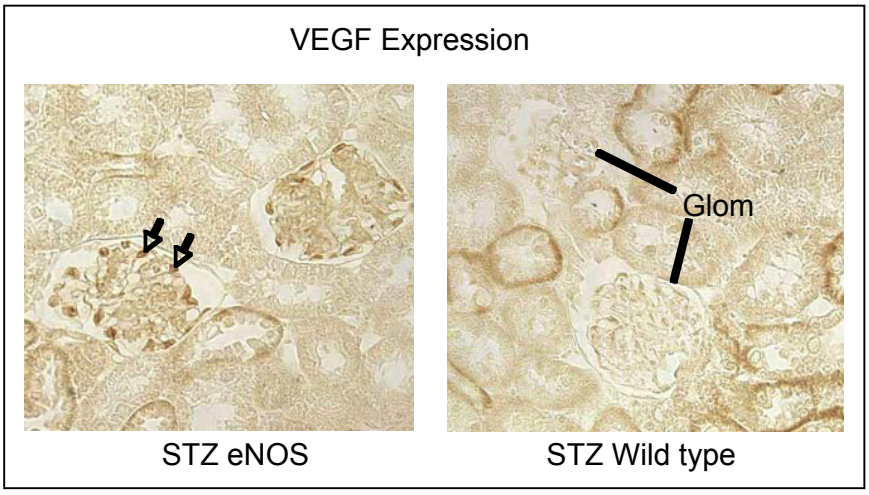
Figure 4 A) Histology of eNOS-/- db/db BKS background glomeruli showing nodular







nephrosclerosis B) fibronectin staining C) electron microscopy indicating foot process effacement and GBM thickening



C. Plans for the coming year

We will continue to characterize the development of nephropathy in heterozygous eNOS-/+ diabetic mice.

Figure 5 VEGF immunohistochemistry in glomeruli from diabetic mice with or without genetic deletion of eNOS. Note podocyte staining in eNOS-/-

D. Significant achievement and its importance

Endothelial nitric oxide synthase (eNOS) is a key enzyme in maintaining blood pressure and normal endothelial function. A polymorphism in eNOS is associated with increased risk of diabetic nephropathy as well as increased rate of development of diabetic nephropathy in people(1-3). These studies suggest that eNOS dysfunction might also predispose to diabetic nephropathy in mice.

Publications

ENOS null x db/db paper in preparation.

Cheol Whee Park, Hyeong Wook Kim, Seung Hyun Ko, Hyun Wha Chung, Sun Woo Lim, Chul Woo Yang, Yoon Sik Chang, Akira Sugawara, Youfei Guan, and Matthew D. Breyer. Accelerated diabetic nephropathy in mice lacking the Peroxisome proliferator activated receptor α. Diabetes (*in press*).

Responsible Investigators: **Matthew D. Breyer, M.D.**

Project Number and Title: **Project 2 – " A screen for dominant ENU mutants developing diabetic nephropathy"**

A. Rationale and Relevance:

As in man, genetic modifiers are critical determinants of the severity of renal injury in mice (4, 5). The extent of renal injury developing in insulin dependent diabetes (following streptozotocin treatment) in inbred mice is dramatically influenced by the genetic background (6, 7). Those studies recently published by our group and separately by the Duke AMDCC group, showed that while the DBA2/J is relatively sensitive to renal disease from diabetic nephropathy, C57BL/6 mice are resistant to renal disease. Mapping the precise genetic quantitative trait loci (QTLs) or SNPs responsible for these differences provides one approach to resolving the basis of these differences. However this approach might be complicated by epistatic interaction between multiple alleles may be necessary to develop nephropathy.

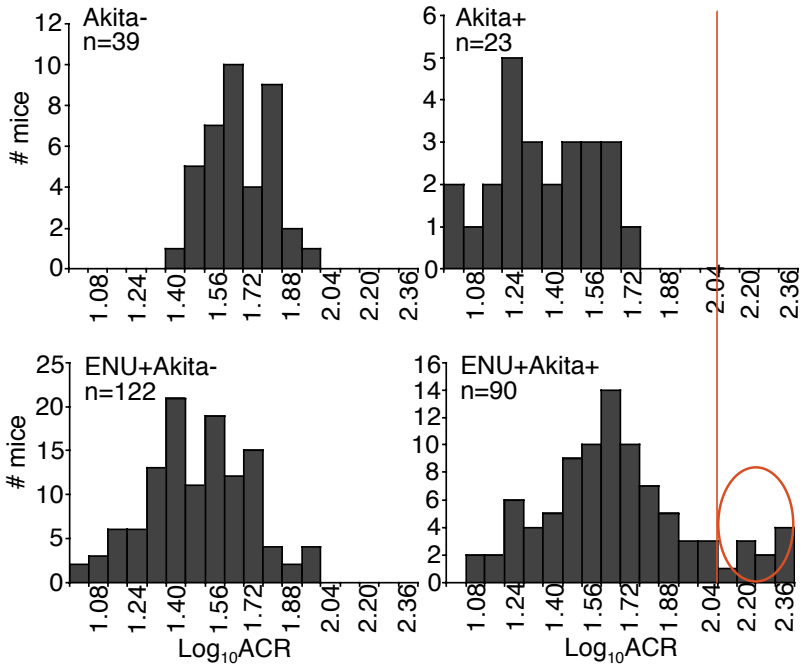
Mutagenesis offers advantages over QTL mapping in the analysis of complex traits (8). Chemical mutagenesis induces a high frequency of mutations affecting potentially every gene that might contribute to a given trait. In this project we utilized *N*-ethyl-*N*-nitrosourea (ENU) to perform a "sensitized screen" for mutants that induce renal dysfunction with diabetes mellitus being the "sensitizing" condition. ENU is a potent point mutagen that acts by transferring its ethyl group to oxygen or nitrogen radicals in DNA, which, if not corrected, results in mis-pairing with resulting A/T to T/A transversions and A/T to G/C transitions (9). ENU mutagenesis provides an unbiased approach for identifying novel and unpredicted genes that may contribute to the development of DN throughout the genome.

B. Summary of Accomplishments

<u>GENERATING novel Mouse mutants with diabetic nephropathy:</u>

We have now generated two lines of ENU induced mutant mice whereby type I diabetic Akita C57BL/6J progeny exhibited increased albuminuria and reduced GFR. These mutants exhibiting increased albuminuria with a Ualb:Creat ratio averaging 340.2±50 µg/mg. This value is more than 3 standard deviations more than the typical levels of albuminuria in C57BL/6J^{ins2akita} mice, defining them as prime candidates for founders carrying novel mutations conferring susceptibility to diabetic nephropathy.

Two out of 375 diabetic G1 founders were found to exhibit albumin excretion rates (AER) persistently 10 fold greater than AERs in non-mutagenized *Ins2*^{Akita} controls. This albuminuria trait was heritable and transmitted to ~50% of *Ins2*^{Akita} G2 and G3 progeny, consistent with a simple, dominantly inherited trait, but was never observed in non-diabetic offspring. Over the course of a year, albuminuric *Ins2*^{Akita} G2 and G3 progeny <u>developed reduced inulin clearance, with elevated BUN and plasma creatinine</u>. Glomerular histology revealed mesangial expansion, and glomerular basement membrane thickening determined by electron microscopy was enhanced in diabetic



mutant kidneys. Hereditary albuminuric ENU-induced mutants were re-designated as *Nphrp1* (nephropathy1) and *Nphrp2* (nephropathy2) mice for two generated lines. These novel mutants provide new robust mouse models of DN, and should help to elucidate the genetic basis underlying predisposition to diabetic nephropathy.

Fig 6) Identification of ACR outliers in diabetic G1 progeny. C57BL/6 (*Akita-)* and control $Ins2^{Akita}$ mice (*Akita+)* (upper panels) and G1 $Ins2^{+/+}$ mice (*ENU+Akita-*) (lower left panel) follow a normal distribution in Log_{10} ACR values. This is in contrast to the bimodal distribution observed in G1 $Ins2^{Akita}$ heterozygotes inheriting ENU mutation (*ENU+Akita+*) (lower right panel). Among ten identified outliers during primary screening (circled bars), six phenotypic variants showed persistent ACR over one year.

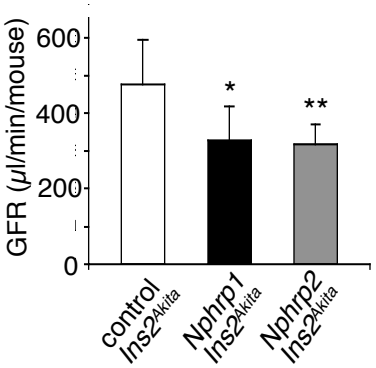


Fig 7) GFR in progeny from *Nphrp1* and *Nphrp2* founders. GFR was measured by FITC-inulin clearance in conscious mice. Control $Ins2^{Akita}$ mice, open bar, n=8; *Nphrp1/*$Ins2^{Akita}$ mutants, black bar, n=10; *Nphrp2/*$Ins2^{Akita}$ mutants, gray bar, n=11. *P < 0.05, **P=0.005 for *Nphrp1/*$Ins2^{Akita}$ and *Nphrp2/*$Ins2^{Akita}$ mice vs. control $Ins2^{Akita}$ mice. Values are mean ± SEM.

C. Plans for the coming year

The top priority for the upcoming year will be to generate N2 backcrosses of mutants from C57BL/6 onto Balb/C. These mice will allow us to map the location of the *nphrp1* and *nphrp2* mutations and ultimately identify the responsible gene. At present we have

D. Most significant achievement.

The propagation of these novel dominant diabetic nephropathy mutations and the establishment of these two new lines should allow us to identify potentially novel genes that cause the development of diabetic nephropathy in C57BL/6 mice, a strain that is otherwise resistant to DN.

Publications

Elena E. Tchekneva, Eugene M. Rinchik, Dina Polosukhina, Linda Davis, Veronika Kadkina, Steve R. Dunn, Kumar Sharma, Zhonghua Qi, Agnes B. Fogo, Matthew D. Breyer. A sensitized screen of ENU mutagenized mice identifies dominant mutants predisposed to diabetic nephropathy. (Submitted).

Responsible Investigators: **Matthew Breyer M.D. Agnes Fogo M.D.,**

Project Number and Title: **Project 3 – " Phenotypic Screens for diabetic nephropathy in inbred strains of mice"**

A. Rationale and relevance

1. *Assessment of Glucose control in mice:*

In human diabetes, the extent of glucose control critically determines the severity and incidence of complications including nephropathy (10, 11). A similar relationship between glucose control and diabetic complications has not been demonstrated in murine diabetes. Indeed, at present there have not been studies assessing the accuracy of measures of glucose control in mice. To address these deficiencies we have undertaken studies to determine whether HbA1C correlates with plasma glucose in mice and also to determine whether HbA1c or blood sugar control.

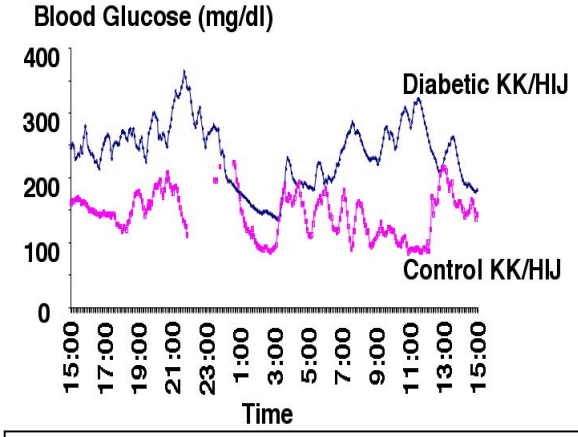


Figure 8: continuous monitoring of interstitial glucose concentration in diabetic and control KK mice

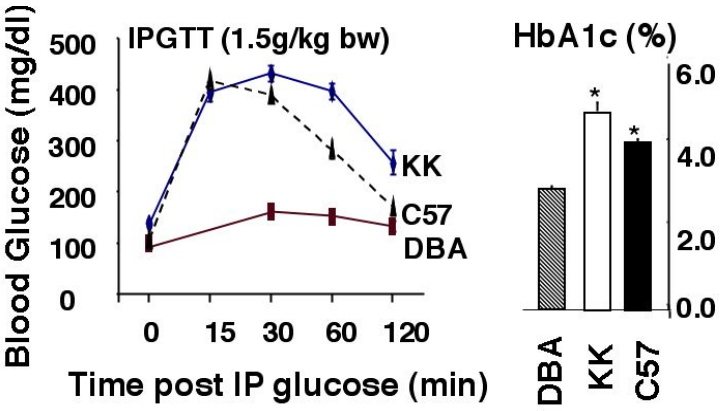


Figure 9: different glucose tolerance and HbA1c in different strains of mice.

B. Summary of Accomplishments

We have developed a technique for continuous 24 hour monitoring of interstitial glucose concentrations in mice (figure 8) (12). Integrated daily blood glusoe will be correlated with HbA1C, glycated hemoglobin values and fasting blood glucose levels. We have also determined that non-diabetic mice exhibit dramatically different glucose tolerance in different strains and that this correlates with different prevailing HbA1C levels (figure 9).

C. Plans for the coming year

We will correlate prevailing blood glucose in KK, C57 and DBA2 mice including control mice, STZ diabetic mice and STZ diabetic mice treated with insulin. We will then correlate this with albuminuria to see if glucose control impacts the severity of nephropathy in mice.

D. Significant Achievement

In the past year we established that the severity of diabetic nephropathy is dramatically affected by strain including effects on albuminuria and histopathological changes (7). We will now examine whether the severity of the disease within each of the strains is further affected by the level of hyperglycemia. **Publications:**

Zhonghua Qi, Hiroki Fujita, Jianping Jin, Linda S. Davis, Yihan Wang, Agnes B. Fogo, Matthew D. Breyer Characterization of Susceptibility of Inbred Mouse Strains to Diabetic Nephropathy. *Diabetes*. 2005 Sep;54(9):2628-37.

Yukiko Kanetsuna[1], Keita Hirano[1], Michio Nagata[2], Maureen A Gannon[1], Raymond C. Harris[1], Matthew D. Breyer[1], and Takamune Takahashi[1*]Characterization of Diabetic Nephropathy in a Transgenic Model of Nonobese Diabetes *American Journal of Physiology (In Press).*

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