Animal Models of Diabetic Complications Consortium
(U01 HL70523)

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Duke/UNC/Stanford

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Part A:

Principal Investigator's Summary

1. Program Accomplishments:

The Duke-UNC-Stanford Unit of the Animal Models of Diabetic Complications Consortium (AMDCC) consists of a multi-disciplinary group of investigators with substantial experience in genetic engineering, in molecular and physiological phenotyping in mice, and in the study of rodent models of diabetes. Based on our cumulative expertise, we have been engaged in the study two diabetic complications: *nephropathy* and *vascular disease*. The major deficiency of current mouse models of these diabetic complications is their relatively mild severity that does not progress to reproduce the pathology seen in the later stages of human diabetes. Our efforts for model development have been based on the hypothesis that this lack of congruity with human disease is *not* due to a fundamental difference in the biology of mice and humans, but instead is due to the presence or absence in mice of genetic factors that modulate susceptibility to end-organ injury. Therefore, our work involves the introduction life-long genetic alterations into diabetic mice with the goal of exaggerating the severity of renal and vascular disease.

Our efforts can be divided into 3 areas: 1) *Model development and physiological assessment*, genetic alterations are introduced into diabetic mice with the goal of exaggerating the severity of renal and vascular disease, 2) *Generation of mice with mutations in candidate susceptibility genes for diabetic complications*, and 3) *Generation of embryonic stem cell lines from diabetic mouse strains* to facilitate genetic manipulation.

Major achievements have been:

A. Model development and physiological assessment

- Detailed phenotyping of C57BL/6*Ins2*^{Akita} *Bdkr2*$^{-/-}$ mice in older animals with more advanced disease, and identification of a potential mechanism for acceleration in disease caused by the absence of the bradykinin receptor
- Continuing comparisons of susceptibility to renal complications of diabetes in common strains of laboratory mice
- Determining the effects of lipoic acid supplements on the development of atherosclerosis on diabetic *Apoe*$^{-/-}$ mice

B. Production of mice with mutations in candidate susceptibility genes for diabetic complications

- Generation of mice lacking lipoic acid synthase

C. Generation of embryonic stem cell lines from diabetic mouse strains

- A number of ES cell lines have been generated from NOD mice but none have yet proven capable for germ-line transmission

2. Collaboration Within Our Group:

A. Model development and physiological assessment

This effort is primarily carried out by investigators at two sites: Duke (PI: Coffman) and UNC (PI: Maeda, Co-investigator: Smithies). The strain comparison studies and screening of a number of the candidate mutations have been carried out at the Duke site. Most of the studies involving vascular and nephropathy phenotyping of Apo E-deficient mice have been carried out at the UNC site. In addition, the generation and phenotyping of the B2 bradykinin receptor-deficient animals have been done at the UNC site. Investigators from the two sites meet regularly to discuss progress, to optimize phenotyping protocols, and to avoid overlap of effort. The histopathological analysis for the nephropathy screens are carried out by the Renal Histopathology and Morphometry Core at Stanford (PI: Meyer).

B. Production of mice with mutations in candidate susceptibility genes for diabetic complications

The production of new mouse lines with mutations of candidate gene loci is carried out at UNC (PI's: Maeda and Koller). These investigators have substantial experience in generation of mice with targeted genetic alterations, and these efforts take advantage of well-established facilities for ES cell manipulation and blastocyst injection at UNC. As these animals are generated and their basic phenotypes are characterized, they are passed directly to the groups involved in model development and physiological assessment.

C. Generation of embryonic stem cell lines from diabetic mouse strains

This activity is carried out at UNC (PI: Koller). This work also takes advantage of Dr. Koller's expertise in producing ES cell lines.

3. Collaboration with Other AMDCC Groups:

A. Core Facility for Evaluation of Renal Histopathology and Morphometry

This facility is located at the Stanford site (PI: Meyer) providing support for the *Model Development and Physiological Assessment* groups. Perfused and fixed kidney sections are shipped to Dr. Meyer from the sites where the animal work is done. They are subsequently sectioned, stained, and renal pathological abnormalities are evaluated and scored. This facility provides a mechanism for standardizing the renal pathological evaluations across the range of models that are being characterized by our program. Eyes from several of our lines, including the C57BL/6*Ins2*^{Akita} *Bdkr2*$^{-/-}$ line, have been shipped to Dr. Tim Kern in Cleveland for evaluation of retinopathy.

4. Pertinent Non-AMDCC Collaboration:

Dr. Robert Reddick at the Texas Health Center at San Antonio has served as a consultant for Dr. Maeda in the evaluation of atherosclerosis in mice in Project 1.

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Part B:

Update by Project Leaders

COMPONENT I: ***Model development and physiological assessment***

Responsible Investigators: **Thomas M. Coffman, M.D.**
Nobuyo Maeda, Ph.D.
Oliver Smithies, Ph.D.

1. Rationale and Relevance:

Based on our collective expertise, we have focused on the study of two diabetic complications: *nephropathy* and *vascular disease*. We recognize the deficiencies of current mouse models of these diabetic complications including their relatively mild severity and failure to recapitulate the pathology seen in the later stages of human diabetes**.** Our experimental approach has been based on the hypothesis that this lack of congruity with human disease is *not* due to a fundamental difference in the biology of mice and humans, but instead is due to the presence or absence of genetic factors that modulate susceptibility to end-organ injury. Therefore, in our approach to *model development,* we have introduced life-long genetic alterations (both positive and negative) into diabetic mice with the goal of exaggerating the severity of renal and vascular disease.

2. Summary of Accomplishments

Since the initiation of the grant, we have screened a number of mouse lines to determine their propensity for developing diabetic renal and vascular disease. As discussed below, we have used two diabetic models as platforms for these screening studies: STZ-induced diabetes and the *Ins2*^{Akita} mouse, a genetic model of type I diabetes. We have used the *Apoe*$^{-/-}$ mouse as a platform for developing models of vascular disease. Along with evaluations of the extent of atherosclerosis, we have also carried out evaluations of kidney phenotypes in diabetic *Apoe*$^{-/-}$ mice, reasoning that in addition to its effects to promote vascular disease, hyperlipidemia might also enhance the development of nephropathy.

2.A. Evaluation of differences in susceptibility for diabetic nephropathy between mouse strains. Our major experimental strategy is to superimpose planned genetic alterations onto models of diabetes as a means of accelerating and facilitating complications. However, we considered the possibility that there might be significant differences in susceptibility to diabetic nephropathy between strains of inbred mice, due to naturally occurring genetic variability. Moreover, identification of susceptible strains would have obvious utility for facilitating model development. Accordingly, we first initiated a side-by-side comparison of chemical and genetic diabetes in a series of common inbred laboratory mouse strains.

2.A.1. Strain differences in susceptibility to kidney pathology in STZ Diabetes. To first determine whether there were strain differences in susceptibility to renal consequences of diabetes, we compared the course of streptozotocin (STZ)-induced diabetes between five common inbred mouse strains: C57BL/6, MRL/Mp, BALB/c, DBA/2 and 129/SvEv (Gurley et al). We found a hierarchical response of blood glucose to a standardized STZ regimen among the strains (DBA/2>C57BL/6>MRL/Mp>129/SvEv>BALB/c). In all five strains, STZ caused much more robust hyperglycemia in males than females. STZ-induced diabetes was associated with modest levels of albuminuria in all of the strains, but was greatest in the DBA/2 strain, which also had the most marked hyperglycemia. However, as shown in **Figure 1**, renal structural changes on light microscopy were limited to the mesangium and consisted of minimal expansion of the mesangial matrix. While there were some apparent differences in the extent of

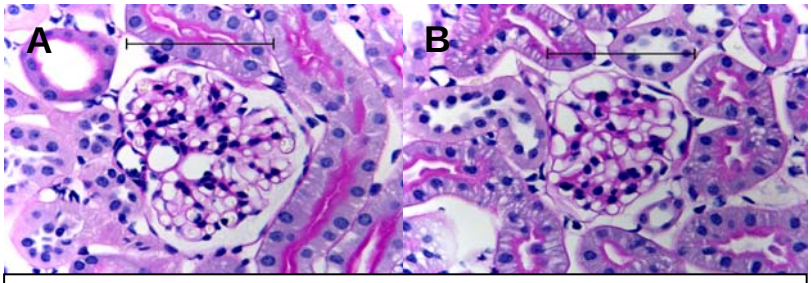

Figure 1: Representative glomerular changes in STZ-treated mice. (A) Normal glomerulus (B) Typical modest mesangial expansion.

mesangial changes between strains, there was a significant positive correlation across the groups between mesangial pathology scores and blood glucose suggesting that most of the variability in renal pathological abnormalities could be explained by differences in severity of hyperglycemia. These studies have recently been published (Gurley et al).

2.A.2. Strain differences in susceptibility to kidney pathology in the Ins2$^{+/C96Y}$ (Akita) model. Because of the mild pathological lesions and the variability in responses to STZ in mice, we considered the possibility that genetic models of diabetes might be better platforms for model development. Therefore, we next compared STZ-treated C57BL/6 animals to C57BL/6 mice heterozygous for the C96Y mutation of the insulin 2 (*Ins2*) gene (Gurley et al). This mutation causes abnormal processing of insulin resulting in islet cell dysfunction and apoptosis. Blood glucose values were significantly higher in C57BL/6- *Ins2*$^{+/C96Y}$ compared to C57BL/6 mice that received STZ (518±25 vs. 388±50 mg/dl; p=0.04). The C57BL/6- *Ins2*$^{+/C96Y}$ mice developed slightly more albuminuria (45.2±3.2 vs. 24.9±6.4 µg/30gm/24 hrs; p=0.02). Akita mice also developed significant renal hypertrophy with increased glomerular volumes, which were not observed in In contrast to the STZ-treated animals. However, the renal pathological changes were qualitatively similar to the STZ-treated animals, consisting of mild increases of matrix material in the mesangium. Because the C57BL/6 strain appears to be resistant to kidney damage in other models, we back-crossed the *Ins2*$^{+/C96Y}$ mutation onto a putatively more permissive background, 129/SvEv. The degree of hyperglycemia in the 129/SvEv- *Ins2*$^{+/C96Y}$ mice was virtually identical to the C57BL/6- *Ins2*$^{+/C96Y}$ group. However, on the 129 compared to the C57BL/6 background, Akita mice had more albuminuria (167.8±17 vs. 45.2±3.2 µg/30g body weight/24 hrs) with exaggerated mesangial pathology (**Figure 2**). These studies confirm the potent effects of genetic background to modulate the response to kidney injury in the Akita model, even with similar degrees of hyperglycemia.

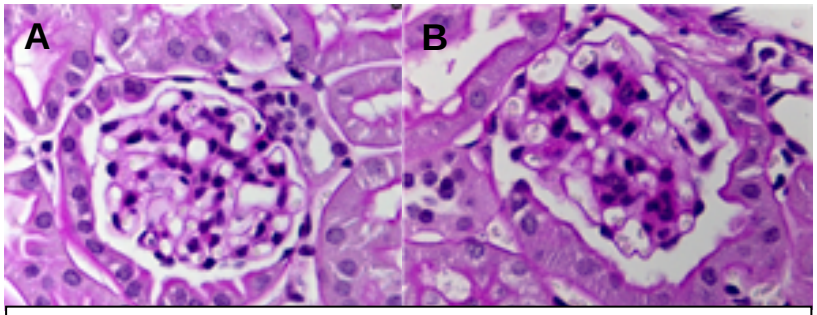

Figure 2: Representative glomerular pathology in 129/SvEv wild-type (A) and *Ins2*$^{+/C96Y}$ (B) mice.

2.A.3. Uni-nephrectomy to enhance the severity of proteinuria and renal pathology in diabetes. In various models of kidney disease, removal of one kidney (uni-nephrectomy) is a maneuver that has been used to enhance and accelerate the progression of renal injury. In preliminary studies, we used uni-nephrectomy as a possible approach for maximizing nephropthay in the (*Ins2*$^{+/C96Y}$) Akita model of diabetes. We first examined the effects of left nephrectomy carried out at 8 weeks of age, comparing wild-type (WT) and *Ins2*$^{+/C96Y}$ C57BL/6 mice (B6 Akita). As shown in Figure 3, nephrectomy produced a modest, but statistically significant increase in proteinuria in non-diabetic C57BL/6 mice (WT Nx). The level of

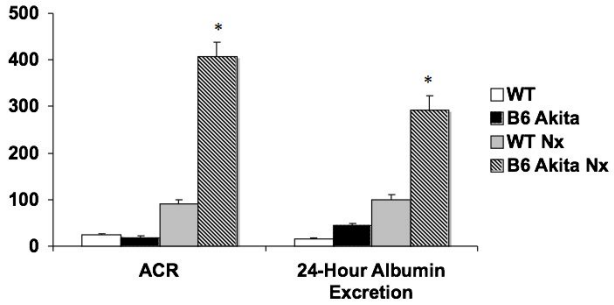

Figure 3. **Albuminuria is exaggerated in diabetic mice after uni-nephrectomy.** Urine albumin excretion is increased 6-fold in B6 Akita mice 16 weeks after left nephrectomy (*p<0.01 vs. B6 Akita with 2 kidneys).

proteinuria was further augmented by nephrectomy in the C57BL/6 *Ins2*$^{+/C96Y}$ mice (B6 Akita Nx), so that albumin excretion was increased by more than 6-fold compared to C57BL/6 *Ins2*$^{+/C96y}$ animals with two kidneys (B6 Akita). Thus, uni-nephrectomy significantly augmented albuminuria in C57BL/6 *Ins2*$^{+/C96Y}$ mice.

Thus, in two mouse models of type I diabetes, we found minimal renal pathological changes that were limited to modest mesangial expansion, corresponding to the earliest changes seen in humans with diabetes and micro-albuminuria. The extent of pathology could be substantially enhanced by provocative maneuvers such as uni-nephrectomy or super-imposing a more "permissive" genetic background. These maneuvers may be useful to incorporate as general approaches for model development.

2.B. Accelerated senescence in B2R-null Akita diabetic mice. Last year we reported that the lack of bradykinin B2 receptor accelerates diabetic nephropathy: increased urinary albumin excretion and mesangial expansion. We have recently found that the combination of diabetes with absence of B2R leads to an accelerated senescence clearly detectable by 12 months of age. Thus by 12 months of age, B2R null Akita diabetic mice show considerable alopecia, reduction in subcutaneous fat, marked kyphosis, which is often a manifestation of osteoporosis. These phenotypes are also present in Akita diabetic mice to a lesser degree. Thus, Fig. 4a shows the progressive decrease in femur mineral bone density in the order: wildtype > B2R null > Akita diabetic > Akita diabetic B2R null.

Other indicators of senescence, including accumulation

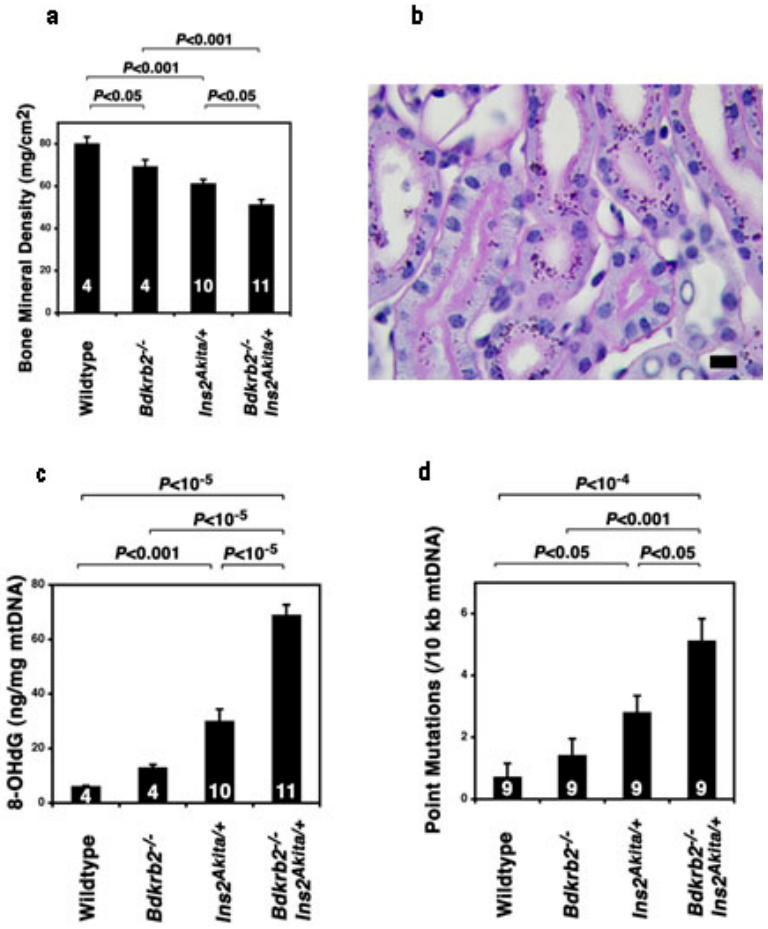

Figure 4. Senescence and mitochondrial DNA mutations of B2R-/- Akita diabetic mice. **a.** Bone mineral density of femurs of 48-week-old mice. **b.** Periodic Acid-Schiff stained kidney of a 48-week-old B2-/- Akita mouse (scale bar = 10 μm). Note prominent lipofuscin granules in proximal tubules. **c.** 8-hydroxy-2'-deoxyguanosine (8-OHdG) content in renal cortical mtDNA. **d.** Frequencies of point mutations in the cytochrome b gene in the mtDNA.

of lipofuscin granules in renal proximal tubule cells (Fig. 4b), increases progressively in mice that are wildtype < B2R null < Akita diabetic < Akita diabetic B2R null. Renal expression of genes implicated in senescence (TGF-β1, CTGF, p53, α-synuclein, FoxO1) [1-3] also increases in the same progression.

In kidney mitochondrial DNA, concomitant increases occur in 8-OHdG (Fig. 4c), point mutations (Fig. 4d) and deletions. Progressive increases likewise occur in thiobarbituric acid reactive substances (TBARS) in plasma, together with decreases in reduced glutathione in erythrocytes. Together these observations led us to the conclusion that absence of the B2R increases oxidative stress, mtDNA damage, and accelerates senescence in diabetic mice (Kakoki et al. *J Clin Invest*, In press).

2.C. Glomerular injury in STZ treated eNOS-/- mice. Since bradykinin and the B2 receptor protect mice from diabetic nephropathy and this action is at least partly mediated through NO, we have most recently made eNOS-/- mice diabetic to test whether lack of eNOS is sufficient to exacerbate diabetic complications. Absence of eNOS caused no difference in blood glucose levels, but as shown in Fig. 5A the eNOS-/- diabetic mice excreted significantly higher amount of albumin in their urine after three months of diabetes (closed bar), and the amount increased more after 6 months of diabetes (open bar) (our unpublished data). Histological assessment of kidney sections after 6 months revealed essentially normal glomeruli in the STZ treated eNOS wildtype mice. (NN in Fig. 6A) However, in the STZ-treated eNOS -/- mice (nn), glomerular sclerosis was apparent in >10% of glomeruli (Fig. 6B). The basement membrane was thickened, and microthrombi were present in some capillary loops (Fig. 6C), and some glomeruli were filled with PAS-positive collagenous material that obliterated the capillary lumens (Fig.6D).

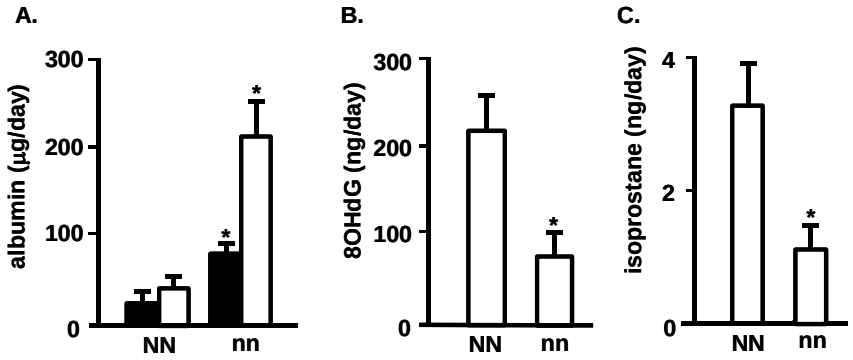

Figure 5. Diabetic nephropathy and oxidative stress of eNOS null diabetic mice. Daily excretion of albumin (A), 8OHdG (B), and isoprostane (C) in the urine of eNOS-/- (nn) and wildtype (NN) mice that were made diabetic by streptozotocin. Closed bars: 3 months of diabetes; open bars: 6 months of diabetes. *p<0.01 vs. wildtypes. (Li, F & Takahashi, N)

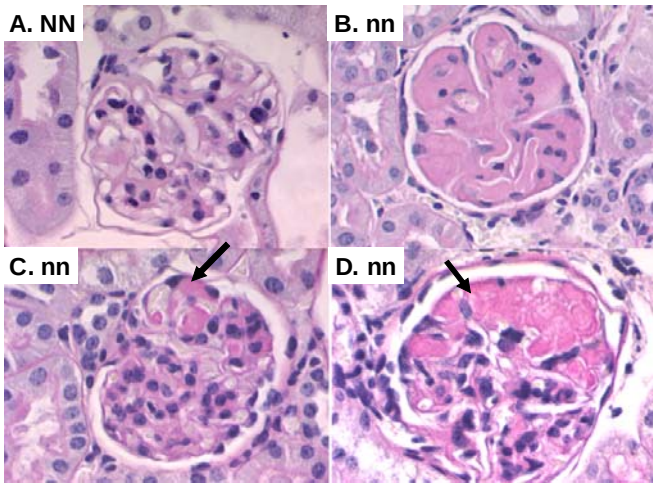

Figure 6. Histology of the kidneys from eNOS-/- (nn) and wildtype (NN) mice that have been diabetic for 6 months using streptozotocin. Arrows show microthrombi (C) and proteineous material (D).

Surprisingly, however, in the diabetic eNOS-/- mice urinary excretion of 8-OHdG and isoprostane, two markers of oxidative stress, was less than half of diabetic wild type mice (Figs. 5B, 5C). These studies consequently indicate that absence of

eNOS in STZ diabetic mice induces advanced diabetic glomerulopathy without increasing oxidative stress.

2.D. Lipoic acid decreases oxidative stress in STZ diabetic mice. Considerable evidence indicates that hyperglycemia increases oxidative stress and contributes to the increased incidence of atherosclerosis and cardiovascular complications in diabetic patients. To examine the effect of alpha-lipoic acid (LA), one of the most potent natural antioxidants, on atherosclerosis in diabetic mice, we made three-month-old apolipoprotein E-deficient (apoE-/-) mice diabetic by treating them with streptozotocin (STZ). At 4 weeks after starting the STZ treatment, a diet containing LA (1.65g/kg) was given to some of the mice. At 20 weeks, markers of oxidative stress were significantly lower in both the diabetic apoE-/- mice and their non-diabetic apoE-/- controls fed LA than in those without it. Remarkably, the LA completely prevented the increase in plasma total cholesterol, the increase in atherosclerotic lesions, and the general deterioration of health induced by the diabetes. These protective effects of LA were accompanied by a reduction of plasma glucose and an accelerated recovery of insulin producing cells in the pancreas, suggesting that part of its effects are due to protecting pancreatic beta cells from damage. Our results suggest that dietary LA is a promising protective agent for reducing the cardiovascular complications of diabetes.

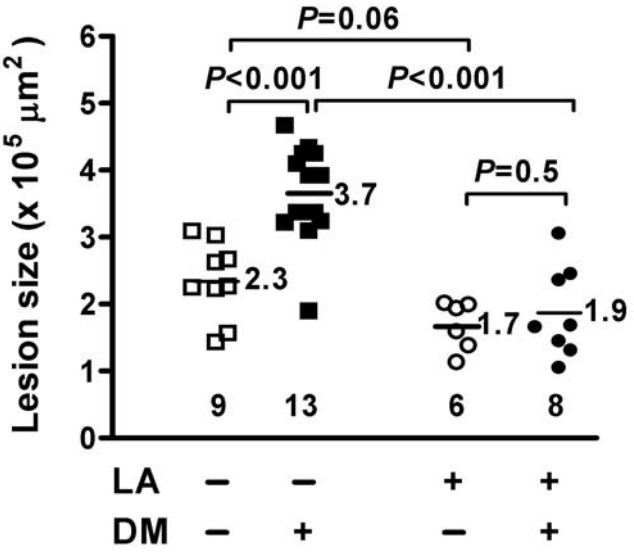

Figure 7. Aortic atherosclerotic lesion size of apoE-/- mice with or without LA in the diet. Each point represents mean lesion size in an individual mouse. Diabetic apoE-/- mice without LA (black squares), diabetic mice with LA (filled circles), non-diabetic apoE-/- mice without LA (open squares), and non-diabetic mice with LA (open circles). Horizontal bars indicate the average of each group with lesion size (μm^2) shown on the right. Numbers of animals in each group are indicated. P values are by Tukey-Kramer HSD. DM, diabetes mellitus consequent to STZ administration. Two-factor ANOVA analysis shows that STZ treatment (P<0.01) and dietary LA (P<0.0001) have highly significant effects, and interact strongly (P<0.01). (Yi, X & Maeda, N)

Publications:

Gurley SB, Clare SE, Snow, Snow KP, Hu A, Meyer TW, and Coffman TM. Impact of genetic background on nephropathy in diabetic mice. *Am J Physiol*; In press (2006).

Kakoki M, Kizer CM, Yi X, Takahashi N, Kim H-S, Bagnell CR, Edgell CS, Maeda N, Jennette JC, Smithies O. Senescence-associated phenotypes in Akita diabetic mice are enhanced by absence of bradykinin B2 receptors. *J Clin Invest*; In press (2006)

COMPONENT II: *Production of mice with mutations in candidate susceptibility genes for diabetic complications*

Responsible Investigators: **Nobuyo Maeda, Ph.D.**
Beverly Koller, Ph.D.

1. Rationale and Relevance: Using a number of mouse lines already available in our laboratories, we have embarked on screens of candidate susceptibility mutations that we have hypothesized would accelerate diabetic renal and vascular diseases (as described above). In addition, under the auspices of this part of our program, we are also generating new lines that we believe may be useful for model development. Furthermore, once susceptibility loci for diabetic complications are identified in humans, experimental tests for causality will be necessary. Mouse models have great utility for this type of experiment and our investigative group has considerable prior experience with such experiments. It is likely that genetic variants influencing susceptibility will not cause drastic loss of function mutations but instead will cause subtle changes (positive or negative) in expression or function of the affected gene. The physiological functions of candidate mutations can be directly tested by recapitulating the human polymorphisms in mice and determining their effects on the development of diabetic complications.

2. Summary of Accomplishments

2.A. Generating a model of endogenous deficiency of lipoic acid production. As discussed in section 2.D. of the description of Component I, we found that oxidative stress was, as expected, increased in the diabetic apoE-/- mice compared to the non-diabetic mice as judged by a decrease in erythrocyte glutathione (GSH) levels and an increase in plasma thiobarbituric acid reacting substances (TBARS). Feeding lipoic acid (LA) to the mice decreased the oxidative stress in diabetic apoE-/- mice to the similar levels in nondiabetic mice without supplement. Thus LA abolished the increase in oxidative stress due to diabetes. However, LA also increased the erythrocyte GSH and decreased plasma TBARS in non-diabetic mice, indicating the potential general utility of exogenous LA supplements for this purpose.

To examine the role of endogenously produced LA, we used the gene targeting to disrupt the *Lias* gene which codes for lipoic acid synthase. Homozygous embryos lacking Lias appear normal at the blastocyst stage, but their development is retarded globally by 7.5 days postcoitum (dpc), and all the null embryos die before 9.5 dpc (Figure 8). Supplementing the diet of heterozygous mothers with LA during pregnancy fails to prevent the prenatal deaths of the homozygous embryos. Thus, endogenous LA synthesis is essential for developmental survival, and cannot be replaced by LA in maternal tissues and blood (Yi and Maeda). This early embryonic death of Lias-/- mice shows that dietary LA cannot replace the lipoic acid moiety of the E2-containing enzyme complexes in the mitochondria of mice, which suggests that the protective effects of dietary LA on diabetic complications are not a result of its participation in enzyme reactions that involve E2. The Lias+/- heterozygotes appear normal, but have a somewhat reduced antioxidant capacity even when not stressed. We are currently carrying out experiments to determine whether these Lias+/- mice are more susceptible to diabetic complications.

Figure 8 **+/-** **-/-**

Publications:

None**.**

COMPONENT III: ***. Generation of embryonic stem cell lines from diabetic mouse strains***

Responsible Investigators: **Beverly Koller, Ph.D.**

A. Rationale and Relevance: Many of the approaches for model development that are being carried out within the Consortium require introducing complex genetic modifications onto a diabetic background. We propose to facilitate this process by generating embryonic stem cells from various diabetic mouse lines. The resulting special ES cell lines will be made available to other investigations to facilitate combining genetic modifications in the process of model development. Such ready availability of embryonic stem cells from various diabetic mouse lines would facilitate combining the genetic modifications that will be necessary for model development. Moreover, once we have developed better models, ES cells lines can be developed to facilitate genetic strategies for testing treatment interventions.

B. Summary of Accomplishments

To generate ES cell lines, 8-week-old NOD females are mated with NOD males and checked daily for the presence of a copulation plug. Pregnant females are killed 3.5 days after mating and the blastocysts flushed from the uterus. Blastocysts are placed individually into 35 mm plates seeded with embryonic feeders cells in media containing leukemia inhibitory factor (LIF). The growth of the blastocysts is monitored daily and when the size of the inner cell mass has increased four-fold, it is removed from the trophectoderm using a glass pipet, treated briefly with trypsin to disperse the cell mass into smaller aggregates, and plated on embryonic fibroblasts in LIF containing media. As these aggregates grow, those with ES cell morphology are again transferred to new dishes. When dishes contain more than 10 aggregates with ES cell morphology, we begin to transfer cells by trypsinization of the wells. Lines are expanded and typed as male or female by PCR and/or Southern analysis. As a control, lines are prepared from 129 mice at the same time. A table of our results to date are shown below:

Table 1: Generation of ES cell lines.

Strain	Cultured Blastocysts	Outgrowths	Lines Growing	Blastcoysts Injected	Germ-line Transmission
NOD	**212**	**30**	**4**	**64**	***12 pups born-0 chimeras***
129/SvEv	**10**	**8**	**1**	**-**	***Not tested***

Three ES lines with good morphology have been generated to date, using 3 different lots of serum. We were able to expand 2 of these, the other line eventually differentiated and was therefore unusable. One of these lines was karyotyped and injected into C57BL/6 blastocysts. A total of 64 blactocysts were injected and 12 pups were born from these injected blastocysts. However, so far, no chimeras have been generated.

Publications:

None.