ANIMAL MODELS OF DIABETIC COMPLICATIONS CONSORTIUM
(U01 HL70523)

UPDATE REPORT
(September 2001—January 2004)

Thomas M. Coffman, M.D.
Program Director
Duke/UNC/Stanford Unit

Thomas M. Coffman, M.D.
Duke University Medical Center
Department of Medicine
Division of Nephrology
Box 3014
Durham, NC 27710
Phone: (919) 286-6947 Fax: (919) 286-6879
Email: tcoffman@duke.edu

Table of Contents

PART A **Principal Investigator's Summary** **Page**

1. Program Accomplishments 4
2. Interrelationship of Projects 5
3. Collaborations with Other Groups 5
4. Pertinent non-AMDCC Collaborations 6

PART B **Progress Reports by Responsible Investigators**

Component I: Model Development and Physiological Assessment

PI's: Thomas M. Coffman, M.D. 8-15
Nobuyo Maeda, Ph.D.
Oliver Smithies, Ph.D

Component II: Production of Mice with Mutations in Candidate Susceptibility Genes for Diabetic Complications

PI's: Nobuyo Maeda, Ph.D. 16-18
Beverly H. Koller, Ph.D.

Component III: Generation of embryonic Stem Cell Lines from Diabetic Mouse Strains

PI: Beverly H. Koller, Ph.D. 19-20

ANIMAL MODELS OF DIABETIC COMPLICATION CONSORTIUM (U01 HL70523)

PART A:

PRINCIPAL INVESTIGATOR'S SUMMARY

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 accomplishments in these areas since the grant was initiated include:

1. Model development and physiological assessment

Comparison of susceptibility to renal complications of STZ-induced diabetes in common inbred strains of laboratory mice

Comparison of renal complications between STZ-induced diabetes and a genetic model of type I diabetes (the $Ins2^{Akita}$ mouse)

Nephropathy screening in mice with candidate susceptibility mutations using the STZ model including $Apoe^{-/-}$, $Bdkr2^{-/-}$, *Agtr1a* gene duplication

Nephropathy screening in mice with candidate susceptibility mutations using a genetic model of type I diabetes ($Ins2^{Akita}$ mouse)

Vascular screening in STZ-treated Apo E-deficient mice

Vascular screening in mice with candidate susceptibility mutations crossed onto the $Apoe^{-/-}$ background

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

Development of a mouse line with a P465L substitution in the PPARγ gene, analogous to a mutation in humans that is associated with severe insulin resistance and early onset of hypertension

Production of a series of mouse lines with altered expression of Connective Tissue Growth Factor (CTGF), a factor that acts downstream of TGF-β to promote tissue fibrosis

Generation of mice with enhanced expression of AT_{1A} angiotensin receptors using the strategy of altering the 3' untranslated region to promote mRNA stability

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

Two ES cell lines have been generated from NOD mice and their propensity for germ-line transmission is being tested

Interrelationships of sites:

1. 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 pathology studies for the nephropathy screens are carried out by the Renal Histopathology and Morphometry Core at Stanford (PI: Meyer).

2. 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.

3. 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.

Collaborations with other Groups (including Core Facilities):

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.

Pertinent non-AMDCC Collaborations:

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. Insulin clamp experiments on mice with PPARγ P465L mutation were carried out in collaboration with Dr. Jason Kim at the Mouse Metabolic Phenotyping Center at Yale University.

ANIMAL MODELS OF DIABETIC COMPLICATION CONSORTIUM (U01 HL70523)

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.

A. Rationale and Relevance:

Based on our cumulative 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.

B. 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.

As a mechanism to refine our phenotyping approaches and to establish a baseline for the susceptibility screens, we first carried out physiological assessments in diabetic animals without superimposed mutations.

1. 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 initiated a side-by-side comparison of STZ diabetes in a series of common inbred laboratory mouse strains: C57BL/6 (a strain highly susceptible to diet-induced obesity, type II

diabetes, and atherosclerosis), MRL/MpJ (which displays heightened wound healing and fibrosis), BALB/c (with a propensity to develop TH2-type immune responses), 129 (used as major source for most ES cells) and DBA/2 (a strain with low susceptibility to atherosclerosis). STZ was administered using the protocol adopted by the Steering Committee: 40 mg/kg/day IP for 5 days at 8 and 15 weeks of age. Blood glucose was monitored every 4-6 weeks from age 10 to 24 weeks. Blood pressures are measured and urine is collected for measurement of protein and creatinine at an early time point (12 weeks) and just prior to euthanasia (24 weeks). At the end of the study, kidney tissue is collected for pathological evaluation, immunohistochemistry, and frozen tissue is saved for isolation of RNA and gene expression analysis. We studied 14 animals from each strain (7 males and 7 females).

Across all strains, hyperglycemia was more marked in STZ-treated males compared to females. For example, in BALB/c mice at 16 weeks after STZ administration, blood glucoses were 249±44 mg/dL in males compared to 120±15 mg/dl in females ($p<0.001$). We also found substantial strain differences in sensitivity to STZ-associated hyperglycemia. For instance, hyperglycemia was more severe in male C57BL/6 males (387±26 mg/dl) than BALB/c males (249±44 mg/dl; $p<0.01$). The strain susceptibility to STZ-induced hyperglycemia was: DBA/2 > C57BL/6 > MRL > BALB/c > 129/SvEv. We also observed variable effects of diabetes on blood pressure across strains. Blood pressures tended to be lower in STZ-treated C57BL/6 male mice compared to controls (95±2 vs. 104±2 mm Hg; p=0.08); whereas STZ-treated male MRL animals had higher blood pressures than non-diabetic MRL male controls (117±2 vs. 111±2 mm Hg; p=0.03). Albumin excretion was also higher in diabetic MRL males than diabetic C57BL/6 males (96±65 vs. 25±16 µg/30 gm BW/day; p=0.03), despite less severe hyperglycemia in the MRL males (196±24 vs 387±26 mg/dl; $p<0.01$). Finally, there were also differences in development of pathological changes in the kidneys between strains, and this did not necessarily correlate with proteinuria or degree of hyperglycemia. For example, mesangial matrix expansion was significantly increased in diabetic compared to non-diabetic male C57BL/6 mice (0.78±0.15 vs 0.31±0.02; p=0.01), but there was no difference in the degree of mesangial expansion between diabetic and non-diabetic male MRL mice (0.52±0.2 vs 0.28±0.1; p=0.15). Taken together, these findings indicate that there are substantial differences in susceptibility to STZ-induced DM and to the physiological consequences of DM across genetically distinct inbred mouse strains. In this STZ model, the DBA/2 strain is particularly susceptible to the development of hyperglycemia, proteinuria, and mesangial matrix expansion compared the other strains that were tested. Thus, the DBA/2 strain may be a useful platform for developing nephropathy models. A manuscript is in preparation describing these studies (Gurley et al., *In preparation*).

1.A. Characterization of Renal Pathology of a Genetic Model of Type I Diabetes (the *Ins2*^{Akita} mouse). Because of problems related to variability and inconsistent duration of STZ-induced diabetes, we compared the character of renal abnormalities in C57BL/6 *Ins2*^{Akita} mice with C57BL/6$^{+/+}$ mice treated with STZ. Blood glucose was monitored every 4-6 weeks from age 10 to 24 weeks. Blood pressures are measured and urine is collected for measurement of protein and creatinine at an early time point (12 weeks) and just prior to euthanasia (24 weeks). At the end of the study,

kidney tissue was collected for pathological evaluation, immunohistochemistry, and frozen tissue is saved for isolation of RNA and gene expression analysis. We studied 7 male animals from each strain.

At the end of the study, blood glucose levels were significantly higher in the C57BL/6 *Ins2*^{Akita} mice (518±25 mg/dl) with C57BL/6$^{+/+}$ mice treated with STZ (332±36 mg/dl; p=0.0018). Along with enhanced hyperglycemia, blood pressures were elevated (112±4 vs. 95±2 mm Hg; p=0.002) and heart rates were reduced (545±13 vs. 666±8 beats/min; p=0.0006) in the C57BL/6 *Ins2*^{Akita} mice compared to controls. Proteinuria was also significantly increased in C57BL/6 *Ins2*^{Akita} mice (45.2 µg/30 gm/24 hrs) compared to STZ-treated C57BL/6$^{+/+}$ animals (24.9 µg/30 gm/24 hrs; p=0.0175). Consistent with the increase in proteinuria, mesangial volumes were modestly, but significantly increased in the C57BL/6 *Ins2*^{Akita} animals compared to genetically-matched, non-diabetic C57BL/6 mice (0.88±0.03 vs. 0.6±0.05; p=0.0004). Compared to STZ-treated C57BL/6 mice, there was no difference in mesangial volumes in the C57BL/6 *Ins2*^{Akita} mice (0.88±0.03 vs. 0.78±0.16; p=0.52). However, the degree of mesangial expansion was much more consistent within the group of C57BL/6 *Ins2*^{Akita} mice (SEM of 3% for C57BL/6 *Ins2*^{Akita} compared to 20% for the STZ-treated C57BL/6 animals).

Thus, compared to STZ-induced diabetes, the C57BL/6 *Ins2*^{Akita} model provides more significant hyperglycemia, increased blood pressures, more proteinuria, and more consistent mesangial expansion. Because of these advantages in the phenotype of renal disease, along with the simplicity of the genetic model (requires crossing in only a single mutant allele), we are using the C57BL/6 *Ins2*^{Akita} model to screen any models that appear promising with STZ. As discussed below, acceleration of diabetic proteinuria and renal pathology by B2 bradykinin receptor-deficiency was significantly amplified on the *Ins2*^{Akita} background, compared to STZ.

2. Screening of mouse lines with candidate susceptibility mutations for the development of nephropathy and vascular complications. As standard phenotyping procedures were being established and characterized in unmodified animals, we also began screens in mice with targeted gene alterations that we hypothesized might accelerate the development of disease. The results for these studies are described below:

2.A. Apolipoprotein E-deficient mice. Mice with targeted disruption of the apolipoprotein gene (*Apo E*) spontaneously develop hyperlipidemia and atherosclerosis. Because hyperlipidemia accompanies diabetic renal and vascular disease, we hypothesized that *Apoe*$^{-/-}$ mice might be more susceptible to diabetic renal and vascular disease. We are also using this line as our platform for vascular disease, so that most of the candidate mutations will be screened in combination with Apo E-deficiency. As an initial approach to determine the influence of hyperlipidemia on the development of diabetic kidney disease, we studied streptozotocin (STZ)-induced diabetes mellitus (DM) in wild-type, male C57/BL6 mice and Apo E knockout (*Apoe*$^{-/-}$) mice. DM was induced using the standard, low dose STZ protocol. Non-diabetic controls (Non-DM) were injected with citrate buffer and mice were studied for up to 6 months.

Diabetic *Apoe*$^{-/-}$ mice had increased albumin excretion rate after 3 months (65.8±7.8 vs 12.1±0.9 µg/d/30g, p<0.0001) and following 6 months of diabetes

(81.1±12.6 vs 12.3±1.4 μg/d/30g; $p < 0.0003$) compared to the diabetic *Apoe*$^{+/+}$ group. While there were no differences in body weights between the groups after 6 months of diabetes (30.3±2.5 vs 35.4±1.1g; p=0.13), enhanced albuminuria was associated with increased total kidney to body weight (18.6±1.6 vs 13.9±0.4 mg/g; p=0.02), doubling of urinary albumin/creatinine ratio (33.9±8.0 vs. 16.6±1.2 μg/mg; p=0.029), decreased hematocrit (40.6±0.9 vs. 44.1±0.9, p=0.029) and a non-significant trend towards elevation in plasma creatinine levels (0.43±0.05 vs. 0.30±0.04 mg/dL; p=0.127). Histologic analysis revealed large glomeruli, mesangial matrix expansion, mesangial cell debris (pycnosis), tubulo-interstitial fibrosis and tubular atrophy that was more severe in diabetic *Apoe*$^{-/-}$ mice compared to diabetic wild-type C57BL/6 mice. However, the overall severity of the changes, even in the Apo E-deficient mice, was relatively mild and did not reach the benchmarks defined by the Nephropathy Sub-Committee. Nonetheless, these data suggest that hyperlipidemia and/or lack of ApoE may enhance the development of diabetic kidney disease in mice.

We also evaluated the severity of atherosclerosis on the STZ-treated Apo E-deficient mice compared to non-diabetic *Apoe*$^{-/-}$ animals. After 16 weeks of diabetes, animals were sacrificed and the extent of atherosclerosis was determined using approaches that have been agreed upon by the CV Committee. The severity of atherosclerosis in the aortic root or in the aortic tree was not significantly different between diabetic and non-diabetic *Apoe*$^{-/-}$ mice. However, there were increased numbers of plaques in the diabetic animals (Arbitrary score of 3.0 vs 1.7; $p<0.007$).

2.B. Diabetic complications in "elderly" Apo E-deficient mice. There are some clinical reports suggesting that diabetic complications may be accelerated when the onset of diabetes occurs at an older age. Accordingly, we wished to examine whether mice rendered diabetic at an older age would develop more severe nephropathy and vascular disease. To this end, diabetes was induced in 6-month old (male) *Apoe*$^{-/-}$ mice and these animals were followed 4 months. At the end of this period, urinary albumin excretion was measured and the severity of renal and vascular pathology was determined. Compared to untreated controls, albuminuria increased by almost 10-fold in the diabetic group. Albumin:creatinine ratios were 202±12 and 24±5, respectively; $p<0.001$). The total kidney weight/body weight ratio was also increased (16.3±0.5 vs. 13.9±0.7; p=0.03). In addition, there was qualitiative evidence of glomerulomegaly, mesangial matrix expansion. Mesangial volumes were significantly increased in the diabetic animals (1.6±0.3) compared to controls (0.9±0.1).

Along with evidence of enhanced nephropathy, the older, diabetic Apoe-/- mice also had evidence of accentuated vascular disease. For example, the size of proximal aortic lesions were significantly increased in diabetics compared to the non-diebtic controls (% occlusion: 24.3±1.0 vs. 16.9±1.7; $p<0.01$). The overall lesion sizes in the aortic root were increased by approximately 40% ($p<0.01$) but there were no striking differences in the composition of complexity of plaques. Moreover, the extent of atherosclerosis in the aortic tree was not significantly enhanced by diabetes in this group of older Apr E-deficient mice.

2.C. Effects of hyperhomocysteinemia on diabetic complications. Because of the potential role of homocysteine to augment cardiovascular complications in humans,

we examined the impact of hyperhomocysteinemia on development of diabetic complications using a mouse line in which a targeted disruption of the Cystathione B Synthase (*Cbs*) gene was crossed onto the *Apoe*$^{-/-}$ background. *Cbs*$^{+/-}$*Apoe*$^{-/-}$ mice were treated with STZ and the severity of kidney and vascular disease were compared with STZ-treated *Cbs*$^{+/+}$*Apoe*$^{-/-}$ mice. After 16 weeks of diabetes, the renal and vascular findings in the *Cbs*$^{+/-}$*Apoe*$^{-/-}$ group were not significantly different from the *Cbs*$^{+/+}$*Apoe*$^{-/-}$ controls. Given that the homocysteine levels in the heterozygous are only mildly elevated, we repeated the experiments in mice that had been challenged with dietary L-methionine with and without folic acid supplementation (both administered in drinking water). Both supplements were introduced after 1 month of diabetes and maintained for a total of 3 months. Plasma homocysteine in both groups (L-methionine vs L-methionine plus folate) were similar at the initiation of dietary supplementation (7.9±1.9 vs 9.8±3.5 μmol/l). By the end of the study, supplementing L-methionine caused plasma homocysteine levels to increase to 82.9±25.7 μmol/l. Homocysteine levels increased to a similar level in the group receiving L-methionine + folate (74.4±15.8 μmol/l) indicating that the addition of folate had very little effect on elevated homocysteine levels in this circumstance. Despite the marked elevation in plasma homocysteine levels, levels of albuminuria (L-methionine: 107±26 μg/mg, n=8; L-methionine plus Folate: 162±24 μg/mg, n=6) were similar to diabetic *Cbs*$^{+/+}$*Apoe*$^{-/-}$ controls. Likewise, measangial volumes were similar between the two groups (1.0±0.1 vs. 0.9±0.1). Finally, the extent and character of aortic vascular lesions were not significantly different from those of the diabetic *Cbs*$^{+/+}$*Apoe*$^{-/-}$ controls. These studies therefore suggest that elevation of plasma homocysteine levels in mice does not accelerate the development of vascular or renal lesions in diabetes.

2.D. The absence of the B2 bradykinin receptor enhances diabetic nephropathy. As a part of the preliminary data described in our original application, we carried out studies to test whether mice with increased levels of the angiotensin-converting enzyme (ACE) gene would have enhanced susceptibility to diabetic nephropathy. The rationale for this experiment was based on reports linking a common polymorphism within intron 16 of the angiotensin converting enzyme (ACE) gene with the risk of nephropathy in patients with type I diabetes. This polymorphism accounts for much of the variance in plasma ACE levels in humans. Specifically, risk for nephropathy is attributed to the D allele, which is associated with higher plasma ACE levels. In collaboration with Dr. Alhenc-Gelas and his associates in Paris, studies were performed to test for a causal role of ACE gene function and increased plasma ACE levels in diabetic kidney disease. To this end, diabetes was induced by STZ in mice carrying one, two and three copies of functional ACE gene. The one-copy mouse is a heterozygote for the disrupted ACE allele, the two-copy mouse is a wild type mouse, and the three-copy mouse is heterozygous for the duplicated ACE gene. ACE mRNA levels and plasma ACE activities are directly correlated to the ACE gene copy number. In unperturbed animals, there is no difference in the blood pressure of the 1-, 2-, and 3-copy animals. However, after 4 weeks of diabetes, blood pressure increased significantly in the 3-copy animals ($p<0.05$) but remained at normal levels in the 1- and 2-copy mice. Furthermore, in the 3-copy animals, urinary albumin excretion significantly over the 12 weeks of study ($p<0.001$), but remained at basal levels in the 1- and 2-copy mice. The

mice with 3 copies of the ACE gene also developed significantly more mesangial expansion than 1- or 2-copy mice. These studies demonstrated that genetically determined levels of ACE gene expression affect the development of hypertension and proteinuria in the STZ diabetic mouse and they have now been published (Huang et al., *Proc Natl Acad Sci* 98:13330; 2001). While these studies clearly demonstrated the role of increased ACE levels in the development of kidney, the degree of augmentation of kidney disease was relatively mild. Moreover, the precise mechanism of this effect was not clear. Possibilities included an effect of enhanced ang II generation and/or augmented degradation of bradykinin or other related peptides. Based on our previous studies using computer modeling (Takahashi et al., *Endocrinology* 144:2184; 2003), we hypothesized that this effect was most likely due to inhibition of bradykinin actions.

To test this possibility, we evaluated the development of diabetic nephropathy in B2 bradykinin receptor knockout mice. In the first set of studies, the *Bdkr2*$^{-/-}$ mice were treated with STZ to induce diabetes. After 16 weeks, albuminuria tended to be higher in the *Bdkr2*$^{-/-}$ animals, but there was substantial variability in these measurements. Based on our observations (described above) that the degree of hyperglycemia and the magnitude of proteinuria and mesganial expansion were more marked and reproducible in the *Ins2*$^{\text{Akita}}$ animals, we crossed the C57BL/6 *Bdkr2*$^{-/-}$ mice with C57BL/6 *Ins2*$^{\text{Akita}}$ animals. The severity of diabetic renal disease was then evaluated in the C57BL/6*Ins2*$^{\text{Akita}}$ *Bdkr2*$^{-/-}$ mice. As shown in Figure 1, there was a dramatic increase in urinary albumin excretion of almost 10-fold in the male C57BL/6*Ins2*$^{\text{Akita}}$ *Bdkr2*$^{-/-}$ mice compared to male C57BL/6*Ins2*$^{\text{Akita}}$ *Bdkr*$^{+/+}$ mice, approaching the target specified by the Nephropathy Sub-Committee. While the quantitative scoring of the histopathology is in progress, the preliminary evaluation of the renal pathology shows impressive mesangial expansion that is substantially more marked than that seen in C57BL/6*Ins2*$^{\text{Akita}}$ *Bdkr*$^{+/+}$ animals. At 6 months of age, there is no evidence of glomerulosclerosis or interstitial fibrosis. Nonetheless, based on our qualitative assessment, this model has the most severe renal lesions of any that our group has tested to date. Along with the detailed pathological evaluation, we plan to evaluate these animals at later time points and to examine additional maneuvers that might accelerate disease including feeding a high-fat, western diet. In addition to representing a significant advance for model development, these results indicate a significant role for bradykinin, acting through its B2 receptor, to protect the kidney in diabetic nephropathy. They further suggest that potentiation of bradykinin levels may be an important mechanism of action of ACE inhibitors in diabetic nephropathy.

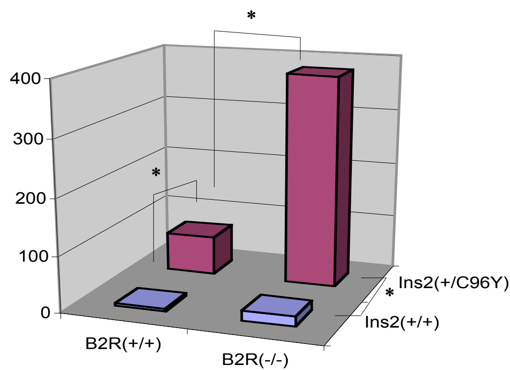

***Figure 1.* Proteinuria is enhanced in diabetic B2 bradykinin receptor knockout mice.** Urinary albumin:creatinine ratios are depicted in C57BL6 B2R$^{+/+}$ and $^{-/-}$ mice that are non-diabetic (*Ins2*$^{+/+}$) or heterozygous for the C96Y *Ins2* variant (*Ins2*$^{\text{Akita}}$).

2.E. *Agtr1a* gene duplication. The role of the renin-angiotensin system (RAS) in the pathogenesis of diabetic nephropathy has been long recognized. Thus, one of the strategies of our group has been to test genetic alterations that would be anticipated to augment the activity of the RAS as candidate susceptibility mutations for diabetic nephropathy. To this end, we carried out kidney phenotyping in mice with targeted duplication of the *Agtr1a* gene locus encoding AT_{1A} angiotensin receptor on an inbred 129/SvEv background. We compared animals homozygous for the targeted duplication with 4 copies of the *Agtr1a* locus to wild-type 129/SvEv mice. We have previously shown that levels of AT_{1A} receptor expression in the 4-copy mice are in the range of 150% of normal. Diabetes was induced with STZ using protocols adopted by the Steering Committee. At the end of 16 weeks, there were no differences in blood glucose, left kidney weight, urinary albumin excretion, urinary albumin:creatinine ratio, or blood pressure between diabetic male 4- and 2-copy animals. Thus, enhanced expression of AT1 receptors alone is not sufficient to augment proteinuria in diabetes. Pathological evaluation of the kidneys from these animals is in progress.

Table I

Groups	Blood Glucose (mg/dl)	Left Kidney Weight	Urinary Albumin Excretion (μg/24 hrs)	Urinary Albumin:Creatinine Ratio	Blood Pressure (mm Hg)
Agtr1a 4-copy	290±34	0.21±0.01	39.6±6.7	84.5±12.5	107±3
Agtr1a 2-copy	296±26	0.21±0.01	69.1±12.8	97.9±12.8	105±1

C. Plans for the coming year

Complete the phenotype screens that are in progress including: Thromboxane receptor (TP)-deficient line, tissue inhibitor of metalloproteinase 3 (TIMP3)-deficient line, constitutively expressed renin transgenic (*alb-Ren*-Tg), ACE2-deficient line, $Ppar^{P435L/+}$ line (see below), $Apoe^{-/-}$ 129/SvEv inbred line, F1(DBA/2 x C57BL/6$Ins2^{Akita}$) line, prostaglandin E synthase 1-deficient line
Generate crosses of promising susceptibility mutations with C57BL/6$Ins2^{Akita}$ and carry out phenotyping (including $Apoe^{-/-}$, *Alb-Ren*-Tg)
In-depth characterization of $Bdkr2^{-/-}$ C57BL/6$Ins2^{Akita}$ animals

D. Most significant achievement.

Our discovery that the absence of the B2 bradykinin receptor accelerates the development of diabetic nephropathy.

Publications

Kakoki M, Takahashi N, Jennette C, and Smithies). Nephropathy is profoundly enhanced in diabetic mice lacking the bradykinin B2 receptor. *In preparation* .

Gurley S, Hinkle S, Snow K, Coffman T. Effect of genetic background on susceptibility to renal injury in streptozotocin-induced diabetes in the mouse. *In preparation.*

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

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

A. 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.

B. Summary of Accomplishments

1. Generation of $\boldsymbol{Ppar^{P465L/+}}$ **mice.** Peroxisome proliferator-activated receptor gamma is a nuclear hormone receptor that promotes adipogenesis and macrophage differentiation. It enhances insulin-mediated glucose uptake and ligands to PPARγ are currently used as therapy for type II diabetes. PPARγ is also expressed in mesangial cells and its activation may directly attenuate diabetic glomerular disease, possibly by inhibiting mesangial growth, which occurs early in the process of diabetic nephropathy. Of many mutations found in the human PPARγ gene, P467L and V290M in the ligand-binding domain of PPARγ are of particular interest because Barroso et al recently reported that the subjects heterozygous with these mutations have severe insulin resistance. These patients also developed hypertension at an unusually early age. Although association between diabetic complications and these mutations in humans has not been studied, PPARγ gene is an important candidate gene.

Using homologous recombination in embryonic stem cells, we have generated mice that carry an amino acid substitution P465L equivalent to the P467L mutation that had been described in human patients. Mice homozygous for the mutation die at an early time point in embryonic development, but heterozygotes are apparently healthy with normal weight gain. It is these $Ppar^{P465L/+}$ animals that we have studied. Although these mice have total body fat mass that is similar to wild type mice, fat distribution is significantly altered. Subcutaneous (inguinal) fat is significantly increased whereas intraperitoneal (gonadal) fat is significantly reduced. While there is no indication of overt insulin resistance in these mice, insulin secretion from beta cells is significantly increased. Moreover, the $Ppar^{P465L/+}$ animals are hypertensive: their blood pressures are about 8 mmHg higher than wild type mice regardless of diet (fat, salt) or age ($P<0.001$). Kidney function is grossly normal in these mice and gene expression for

angiotensionogen in the liver, renin and angiotensin II receptor 1 in kidneys, and aldosterone synthase in adrenals are unaffected by the mutation. However, gene expression for renin-angiotensin system components is enhanced in fat depots suggesting that local activation of the RAS may contribute to the hypertension in the $Ppar^{P465L/+}$ heterozygotes. The initial phenotype screen of these mice is largely completed and they will soon be passed along to the *Model development and physiological assessment* Component.

2. Generation of mice with altered expression of Connective tissue growth factor (CTGF). A major manifestation of end-stage diabetic nephropathy in humans is renal fibrosis. However, the severity of fibrotic changes in current diabetic mouse models is minimal. CTGF, followed by induction by TGF-beta, is responsible for enhanced expression of collagen and extracellular matrix proteins in the injured tissues. Therefore, we have made targeted mutations in ES cells to (1) inactivate and (2) over-express the endogenous mouse *Ctgf* gene.

We found that the CTGF-deficient mice are born but die shortly after birth with apparent respiratory failure. Histologically, the lungs appear to be immature. A similar defect has been observed in mutants lacking TGF-beta3. A recent publication by others demonstrated that their $Ctgf^{-/-}$ mice have defective rib cage structure that prevents the proper inflation. We are planning to examine whether a 50% reduction of CTGF in heterozygote animals is sufficient to alter the development of fibrosis upon injury.

In a separate experiment, we have generated mice that should be prone to have enhanced expression of CTGF by replacing the endogenous 3'UTR sequence with that for bovine growth hormone to stabilize the CTGF mRNA without affecting the transcriptional regulation of the gene. Unexpectedly, we found that the chimeric mice that express these more stable CTGF transcripts exhibited dwarfism that is proportional to their extent of chimerism. Previously, overproduction of CTGF or TGF-beta in mice has been shown to cause similar defects in bone development. Furthermore, our chimeras failed to transmit the chromosome carrying the mutant gene, although weaker chimeras were able to transmit a normal 129 chromosome. This suggests that the level of gene expression, even in a heterozygous state, is sufficient to alter normal development. Therefore, in order to obtain a lesser but predictable increase in the *Ctgf* gene expression, we have duplicated the locus by homologous recombination in ES cells. The majority chimeras carrying the mutated ES cells that we have obtained to date have relatively poor contribution of modified cells judged by their coat color. This suggests that the likelihood of successful transmission of the mutant ES cell genome through the germ line is low. We are therefore currently in the process of generating additional chimeras.

C. Plans for the coming year

We plan to treat the P465L-PPARγ mice with STZ to induce diabetes and to assess its effects on diabetic complications.
We will introduce the mutation on *Apoe*$^{-/-}$ and on *Ins2*^{Akita} genetic backgrounds.
We will focus on generating mouse lines over-expressing the *Ctgf* gene as we believe that these will have the most relevance for generating better mouse models of diabetic complications.

D. Most significant achievement.

Our finding that generation of the *Pparγ*$^{P465L/+}$ mutation in mice replicates the hypertension and abnormal body fat distribution seen in human patients bearing this genetic variant is our most significant acheivement. These animals are mildly hyperinsulinemic but do not have overt insulin resistance. These observations may be useful in dissecting the etiology of human diabetic conditions. For example, while insulin resistance can cause hypertension in some cases, our experiments demonstrate that the PPAR mutation causes to hypertension that is independent of insulin resistance.

Publications

Tsai Y-S, Kim H-J, Takahashi N, Kim H-S, Hagaman JR, Kim JK, and Maeda N. Hypertension and abnormal fat distribution but not insulin resistance in mice with P465L-PPARγ. *Submitted*.

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	3	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.

C. Plans for the coming year

We will continue our attempts to produce germ-line competent ES cell lines from NOD.

D. Most significant achievement.

Development of 2 stable ES cell lines from NOD mice.

Publications

None.