Animal Models of Diabetic Complications Consortium
(U01 HL70523)

Annual Report
(2004)

Duke/UNC/Stanford

Principal Investigator
Thomas M. Coffman, M.D.

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

		Page
Part A:	**Principal Investigator's Summary**	**4-7**
1.	**Project Accomplishments (2004)**	**4-5**
2.	**Collaboration within your group**	**5**
3.	**Collaboration with other AMDCC groups**	**5**
4.	**Pertinent non-AMDCC Collaboration**	**6**
5.	**Address previous EAC comments**	**6-7**
Part B:	**Project Reports by Responsible Investigators**	**8-19**
Component I:	**Model Development and Physiological Assessment**	
	PI's: Thomas M. Coffman, M.D. Nobuyo Maeda, Ph.D. Oliver Smithies, Ph.D	**9-14**
Component II:	**Production of Mice with Mutations in Candidate Susceptibility Genes for Diabetic Complications**	
	PI's: Nobuyo Maeda, Ph.D. Beverly H. Koller, Ph.D.	**15-17**
Component III:	**Generation of embryonic Stem Cell Lines from Diabetic Mouse Strains**	
	PI: Beverly H. Koller, Ph.D.	**18-19**

Animal Models of Diabetic Complications Consortium
(U01 HL70523)

Part A:

Principal Investigator's Summary

1. Program Accomplishments (2004):

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

- Continuing comparisons of susceptibility to renal complications of STZ-induced diabetes in common strains of laboratory mice (DBA/1 and Swiss Webster)
- Nephropathy screening in mice with candidate susceptibility mutations using the STZ model including *mPges1*$^{-/-}$, *Ace2*$^{-/-}$, and a renin-transgene
- Detailed phenotyping of C57BL/6*Ins2*$^{\text{Akita}}$ *Bdkr2*$^{-/-}$ mice
- Nephropathy screening in mice with candidate susceptibility mutations using a genetic model of type I diabetes (*Ins2*^{Akita} mouse)
- Vascular screening in mice with candidate susceptibility mutations crossed onto the *Apoe*$^{-/-}$ background

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

- 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

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

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

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 pathology studies 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 some of our lines 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. 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.

5. Responses to Previous EAC Comments:

Questions Regarding Kidney Models

"Encouraged to test models over a full time-course."

Experiments are currently in progress to test our most promising models over a longer time periods.

"Suggestion – try a simple nephrectomy to accelerate pathology."

Along with the extended testing periods, we are also evaluating the effects of nephrectomy on severity of renal pathology.

"The Akita/DBA/2 cross seems very promising but documentation of the more chronic course of at least 4 months and preferably 6 months is recommended."

As above, these studies are in progress.

"The DBA/2J strain may not be a good model for retinopathy because the mice develop increased intraocular pressure and other features of human glaucoma (see report from Jackson Lab). These abnormalities alter the retina and lead to apoptosis of ganglion cells. Interaction with Dr Kern is encouraged."

We have discussed this with Dr. Kern and will send some eyes to him for examination.

Questions Regarding Cardiovascular Models

"Should take the lead in standardizing a protocol for looking at calcification? This information should be included in CV Feldman tables. Follow Anne Marie Schmidt scoring system?"

In association with the Cardiovascular Committee, standardization of the CV protocols and reporting has been accomplished.

"The connective tissue growth factor studies seem promising and their cross breeding with apoE-/- may be of value."

This project continues to be a high priority for our group.

"Need to focus on 3-4 most promising animal models and start the phenotyping activities."

We agree with this suggestion and we have developed a list of 3-4 models for intensive phenotyping included in our most recent Feldman tables.

"Please report on extent (not only aortic root) and complexity (score)."

We will do this.

"Agree that Akita is a very promising model and should be pursued. Combine with high-fat/cholesterol diet and/or cross with apoEKO, with the potential of B2KO and aged animals."

Additional characterization of the Akita-B2KO model is in progress and work is in progress to cross with the ApoE knockout.

"The second component (candidate susceptibility genes for diabetic complications) is less compelling. While these two targets (PPAR P465L/+ and CTGF) are of potential interest, the lack of a unifying hypothesis limits enthusiasm for pursuing other random (albeit with some valid rationale) candidate genes. Since there are two models already produced, effort at this late stage may be better suited for crosses rather than generation of new models."

As suggested, we have primarily focused on models that have already been generated.

Animal Models of Diabetic Complications 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.

1. 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.

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.

As a mechanism to refine our phenotyping approaches and to establish a baseline for the susceptibility screens, we have devoted significant effort to physiological assessments in diabetic animals without superimposed mutations.

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 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). The first part of this work was reported in last year's report and has now been developed into a manuscript to be submitted in the next few weeks. We found that there was a

hierarchical response of blood glucose level to the STZ regimen among the strains (DBA/2>C57BL/6>MRL/Mp>129/SvEv>BALB/c). In all five strains, males demonstrated much more robust hyperglycemia with STZ than females. Accordingly, our analysis of nephropathy focused on males from each strain. 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. Renal pathological changes were limited to the development of mesangial expansion, and while there were some apparent differences among strains in susceptibility to renal pathological changes, there was a significant positive correlation between blood glucose and the degree of mesangial expansion suggesting that most of the variability in renal pathological abnormalities was due to differences in hyperglycemia.

We have now extended these studies to two additional strains: DBA/1 and Swiss Webster. The DBA/1 strain was studied for two reasons. First, there is a robust line of ES cells generated from this line that has been used to generate genetically modified mice. Second, this line is related to the DBA/2 line, which we have previously shown has enhanced susceptibility to STZ-induced diabetes and proteinuria. We evaluated the Swiss Webster line because of previous studies suggesting diabetes susceptibility. Sixteen weeks after STZ administration, the male Swiss Webster (588±7 mg/dl) and male DBA/1 (571±16 mg/dl) mice had marked hyperglycemia in the range that we had observed with the other highly sensitive strain, DBA/2 (588±7 mg/dl). Similar to our experience with STZ-induced diabetes in the other lines, females of both strains were relatively resistant to STZ. The significant levels of hyperglycemia were associated with the development of significant albuminuria compared to non-diabetic controls to levels of 119±26 µg/30gm/day in male, diabetic Swiss Webster mice and 245±56 µg/30 gm/day in the male, diabetic DBA/1 mice. This compared favorably with the level of proteinuria seen in the male, STZ-treated DBA/2 mice (106±39 µg/30 gm/day). Evaluation of kidney histology in these two strains is ongoing. Thus, in the low-dose STZ model, the DBA/1, DBA/2 and Swiss Webster strains are particularly susceptible to the development of hyperglycemia. While all 3 strains develop albuminuria after STZ, the level of albumin excretion was highest in the DBA/1 strain. Accordingly, at comparable levels of hyperglycemia in STZ diabetes, the DBA/1 strain seems to have the greatest susceptibility to renal injury.

2.A.i. 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 explored the *Ins2*^{Akita} mouse model of type I diabetes as an alternative to STZ. While the general character of renal involvement was similar between chemical and STZ diabetes, Akita mice on the C57BL/6 background developed more marked hyperglycemia, elevated blood pressures, and less variability in renal pathological responses compared to STZ-treated C57BL/6 mice. Based on this first set of studies, we concluded that the *Ins2*^{Akita} line could be a useful platform for model development.

In our studies of strain differences in the STZ model, we found that the C57BL/6 was relatively resistant to the development of hyperglycemia and nephropathy after STZ. By contrast, as discussed above, the DBA/2 line was found to be more susceptible. We were therefore interested in evaluating the effect of the DBA/2 background on the course of diabetes in the *Ins2*^{Akita} mouse model. To examine this issue, we generated and studied (DBA/2 x C57BL/6)F1 *Ins2*^{Akita} mice. These animals develop marked hyperglycemia that was sustained throughout the study period from 8 (451±16 mg/dl) through 24 weeks of age (580±29 mg/dl). This persistent hyperglycemia was associated with substantial albuminuria (283±53 µg/30

gm/day), which is significantly higher than level of albumin excretion in the STZ-treated DBA/2 animals (106±39 μg/30 gm/day). Despite their substantial levels of proteinuria, renal pathological findings at 6 months of age are limited to mesangial expansion. Nonetheless, of the wild-type mouse strains that we have examined, this one appears to have the most potential. As suggested by the EAC, we are carrying these animals for longer times and are performing uni-nephrectomies in some animals as maneuvers to attempt to accelerate disease. We are also carrying out back-crosses to generate *Ins2*^{Akita} mice on an inbred DBA/2 background.

In our studies of STZ-induced diabetes, the 129/SvEv strain responded only modestly in terms of both hyperglycemia and proteinuria. However, studies by our group and others have suggested that this strain may have enhanced susceptibility to renal injury. Based on these observations, we back-crossed the *Ins2*^{Akita} mutation onto the 129/SvEv background and assessed their propensity for developing hyperglycemia and proteinuria. 129/SvEv-*Ins2*^{Akita} mice developed robust hyperglycemia (495±31 mg/dl), compared to 219±25 mg/dl in 129/SvEv mice 16 weeks after STZ. The 129/SvEv-*Ins2*^{Akita} mice also developed generous levels of urinary albumin excretion (168±77 μg/30 gm/day) that were higher than those of C57BL/6-*Ins2*^{Akita} mice (45±3 μg/30 gm/day) or of 129/SvEv mice treated with STZ. Renal pathological evaluation of kidneys from the 129/SvEv-*Ins2*^{Akita} mice is in progress.

Thus, compared to STZ-induced diabetes, the *Ins2*^{Akita} model provides more significant hyperglycemia, increased blood pressures, more proteinuria, and more consistent mesangial expansion. Because of these characteristics, along with the simplicity of the genetic model (requires crossing in only a single mutant allele), we believe that the *Ins2*^{Akita} line offers significant advantages for model development. Moreover, we have now identified 2 genetic backgrounds, DBA/2 and 129/SvEv, that enhance proteinuria associated with the *Ins2*^{Akita} mutation. As many mouse lines with targeted genetic alterations are generated in ES cells from the 129 background, the 129/SvEv-*Ins2*^{Akita} line may be especially useful for developing models with accelerated nephropathy.

2.A.iii. Evaluation of differences in susceptibility to diabetes-associated atherosclerosis. One of our aims is to identify genetic variations that enhance atherosclerosis when animals become diabetic. Candidate genes include those affect oxidative stress, fibrosis and body fat composition. While some of the mutants such as Gulo-/- mice and eNOS deficient mice that we generated in the past have been extensively backcrossed to an C57BL genetic background, our newer mutants generated under the auspices of this program including the CTGF and PPARγ mutants were made using ES cells derived from 129/SvEv mice. Since backcrossing is laborious and time consuming and based on the enhanced susceptibility ot the 129 strain for diabetic albuminuria, we decided to examine the effects of diabetes on atherosclerosis in *Apoe*$^{-/-}$ mice on a pure 129/SvEv background. In order to determine the baseline effects of STZ-induced hyperglycemia on 129/SvEv-*Apoe*$^{-/-}$ mice, we treated 3-month old males with STZ (n=13). Blood glucose levels increased from 180±7 mg/dl to 421±16 mg/dl, and hyperglycemia was maintained for 5 months. Body weight decreased in diabetic mice from 26.8±0.7 gm to 24.5±1.0 gm, but increased to 27.4±0.5 gm in non-diabetic controls that did not receive STZ. Cholesterol increased slightly from 608±25 mg/dl to 735±21 mg/dl. Urinary excretion of albumin in diabetic *Apoe*$^{-/-}$ mice was 32±2 μg/day. Atherosclerosis measured at the aortic root was not different between diabetic (238±21 x10^6μm^2, n=13) and nondiabetic mice (268±51 x10^6μm^2, n=7). Plaque components were also similar. Plaques were all complex with fibrous caps, necrotic cores and cholesterol clefts (Complexity score =1). We have previously

observed that 129/SvEv *Apoe*$^{-/-}$ mice develop larger plaques in carotid artery and aortic arch compared to C57BL/6 *Apoe*$^{-/-}$ mice. We are currently evaluating whether STZ-induced hyperglycemia affects plaque development in these areas.

2.B. Screening of mouse lines with candidate susceptibility mutations for the development of nephropathy and vascular complications. We are continuing to screen mouse lines with targeted gene alterations that we hypothesized might accelerate the development of disease. The results for these studies are described below:

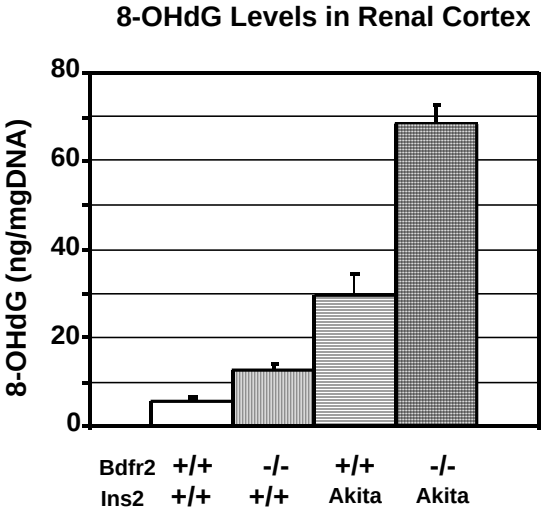
2.B.i. ACE2-deficient mice. Homologues of angiotensin converting enzyme (ACE) have been recently identified. One of these, ACE2, exhibits more than 40% identity at the protein level with the catalytic domain of ACE. Similar to ACE, ACE2 is expressed on the surface of certain endothelial cell populations. However, compared to the ubiquitous distribution of ACE, the expression pattern of ACE2 is more limited with most abundant expression seen in kidney followed by heart and testis. Their substrate specificities also differ; ACE2 hydrolyzes angiotensin II with high efficiency, but has much lower activity against angiotensin I. As a carboxymonopeptidase, ACE2 generated angiotensin 1-7 from angiotensin II. Because ACE2 is very highly expressed in the kidney and we have previously found that it is an important pathway for renal angiotensin II catabolism, we hypothesized that the absence of ACE2 might exaggerate the renal actions of angiotensin II in diabetes, thereby accelerating diabetic nephropathy. To test this possibility, we rendered male ACE2 knockout mice diabetic with STZ and tested them for the development of proteinuria and renal pathology. Blood glucoses were elevated in STZ-treated, male *Ace2*$^{-/y}$ mice (457±33 mg/dl) and these levels were similar to those of male wild-type littermates treated with STZ (408±27 mg/dl). Compared to non-diabetic controls, urinary albumin excretion was increased in the ACE2-deficient mice (117±32 µg albumin/mg creatinine), but this was not different from the level of albuminuria in diabetic, wild-type littermates (138±22 µg albumin/mg creatinine). Likewise, there was no difference in the extent of mesangial expansion observed in diabetic ACE2-deficient mice (0.67±0.18 vs 0.67±0.27). Thus, the absence of ACE2 does not affect the development of renal complications in STZ diabetes.

2.B.ii. mPGES1-deficient mice. Previous studies have suggested a role for prostaglandins in modulating the development of diabetic nephropathy. In particular, generation of vasodilator, anti-inflammatory prostanoids such as prostaglandin E2 have been suggested to have a beneficial effect in preventing end-organ injury. Synthesis of PGE2 is mediated by terminal synthases. The first PGE synthase to be characterized molecularly was a membrane-bound enzyme that was named mPGES1. Studies using *mPges1*$^{-/-}$ mice indicate that mPGES1 is responsible for the late phase of PGE_2 synthesis during inflammation via a pathway that depends on COX-2. Febrile responses to LPS are also mediated by mPGES1 and mPGES1 is the source of PGE_2 that facilitates acute inflammatory pain.

These data suggest that mPGES1 is a major source of PGE_2 synthesis in vivo. We hypothesized that specific inhibition of PGE_2 might exacerbate the renal injury in diabetes and therefore we carried out screening studies of mPGES1-deficient mice made diabetic by treatment with STZ. The line of mPGES1-deficient mice that we used for these studies was generated from ES cells derived from DBA/1 mice. Thus, the experiments were carried out using inbred DBA/1-*mPges1*$^{-/-}$ animals. As discussed above, the DBA/1 line is particularly susceptible to

STZ diabetes. By 16 weeks after STZ, the mPGES1-deficient mice developed marked hyperglycemia (525±41 mg/dl) that was similar to their wild-type DBA/1 controls (571±16 mg/dl). They also developed significant proteinuria (230±37 µg albumin/30 gm/day), but this was not significantly different from the STZ-treated, wild-type DBA/1 controls (203±39 µg/30 gm/day). PGE_2 excretion was reduced by approximately 50% in the knockouts, suggesting that mPGES1 is a significant (but not the only) source of urinary PGE_2 in this circumstance. However, we found no evidence for a major impact of this enzyme on diabetic renal disease.

2.B.iii. Further studies on the mechanism of enhanced diabetic renal injury in B2 bradykinin receptor-deficient mice. We have continued to characterize diabetic complications in *Ins2*^{Akita}*Bdkr2*$^{-/-}$ mice. 24-week-old *Ins2*^{Akita}*Bdkr2*$^{-/-}$ mice develop overt albuminuria, excreting the equivalent of >550mg albumin/day in humans, which contrasts with the microalbuminuria (equivalent to <150mg/day in humans) seen in their simply diabetic *Ins2*^{Akita}*Bdkr2*$^{+/+}$ littermates. The overt albuminuria is accompanied by marked kidney pathology, including mesangial expansion and interstitial fibrosis [Kakoki et al, 2005]. By age 48 weeks, genetically diabetic male mice lacking bradykinin B2 receptors develop marked kyphosis, alopecia, osteoporosis, skin atrophy, testicular atrophy and amyloid deposits. In contrast, their diabetic littermates with B2 receptors had only mild conditions. Significant increases in 8-hydroxy-2'-deoxyguanosine (see figure), point mutations and a large deletion in kidney mitochondrial DNA, a decrease in serum leptin levels, and significantly enhanced expressions of several cancer-suppressor genes including transforming growth factor β1, connective tissue growth factor, p53, a-synuclein and foxO1 were detected. The enhanced expression of these genes has been implicated in senescence. Thus our findings suggests that the accelerated aging is associated with diabetes in the *Ins2*^{Akita}*Bdkr2*$^{-/-}$ mice, and that bradykinin plays an important role in reducing diabetes-related senescence at least partly via the suppression of oxidative DNA damages and the subsequent alteration of the gene expression profile.



3. Plans for the coming year:

- Complete the phenotype screens that are in progress including: Constitutively expressed renin transgenic (*alb-Ren*-Tg), long term studies of F1(DBA/2 x C57BL/6*Ins2*^{Akita}) mice including a cohort that have undergone uninephrectomy, long term studies of 128/SvEv-*Ins2*^{Akita} mice including a cohort that have undergone uninephrectomy
- Continue back-crosses of *Ins2*^{Akita} on promising susceptibility backgrounds (DBA/1, DBA/2, 129/SvEv)
- Continued in-depth phenotyping of *Bdkr2*$^{-/-}$ C57BL/6*Ins2*^{Akita} animals

- Assess effect of hyperglycemia on vascular and kidney disease in the following mice: Vitamin C-deficient *Gulo*$^{-/-}$*Apoe*$^{-/-}$ mice compared to their Vitamin C- supplemented littermates, *Pparg*$^{P456L/+}$*Apoe*$^{-/-}$ mice compared to *Pparg*$^{+/+}$*Apoe*$^{-/-}$ mice, CTGF 1-, 2-, 3- and 4-copy mice, *Nos3*$^{-/-}$ mice compared to wild type mice on both chow and high fat diet.
- Examine effects of a high fat containing diet and genetic background (129 vs B6) on the STZ induced diabetes in *Apoe*$^{-/-}$ mice.

4. Most significant achievement.

Identifying genetic backgrounds that confer clear-cut susceptibility to proteinuria, independent of the level of blood glucose.

Publications:

1. Kakoki M, Takahashi N, Jeanette JC, Smithies O. Diabetic nephropathy is markedly enhanced in mice lacking the bradykinin B2 receptor. *Proc Nat Acad Sci* 101:13302-13305, 2004.
2. Gurley SB, Clare SE, Snow KP, Meyer TW, Coffman TM. Impact of genetic background on nephropathy in diabetic mice. To be submitted.

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. Generation and analysis of *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

We have introduced the P456L-PPARγ mutation onto the ApoE-deficient background. Preliminary assessment of the *Pparg*$^{P456L/+}$*Apoe*$^{-/-}$ mice showed that the moderate hypertension (8mmHg above normal), abnormal distribution of body fat but normal insulin resistance seen in *Pparg*$^{P456L/+}$ mice are retained on the *Apoe*$^{-/-}$ background. We are currently increasing the number of male mice to treat with STZ and these animals will be analyzed in the *Model development and physiological assessment* Component.

2.B. 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. In order to test whether genetically increased expression of connective tissue growth factor (CTGF) increases susceptibility to fibrosis, a hallmark of the end-stage diabetic nephropathy, we have been working to develop mouse models with increased CTGF expressions. Our earlier attempt to obtain mice with increased steady state levels of CTGF transcripts by replacing the endogenous 3'UTR sequence with that for bovine growth hormone was not successful. The high CTGF expression (about 5X normal) resulting from the increased stability of the message is apparently detrimental for normal development. We therefore applied a targeted duplication strategy in ES cells of 10 kb DNA that contains the *Ctgf* gene together with approximately 3.5 kb and 2 kb respectively of the 5' and 3' flanking sequences. Transmission of the duplicated endogenous *Ctgf* gene was successful. An average CTGF mRNA in the growing tails of pups heterozygous for the duplicated *Ctgf* locus (3-copy mice) was 160% that in the wild type littermates (2-copy mice). Homozygous mice (4-copy mice) have been born and appear normal. We are beginning to apply STZ treatments on 1-copy (mice heterozygous for the KO allele), 2-copy, 3-copy and 4-copy CTGF mice. We have also started crossing the *ApoE*$^{-/-}$ mice with 1-copy or with 3-copy CTGF mice in order to place the KO chromosome and the duplicated chromosome on an apoE-deficient background. These studies will be carried out in the *Model development and physiological assessment* Component.

C. Plans for the coming year

- We plan to treat the P465L-PPARγ *Apoe*$^{-/-}$ mice with STZ to induce diabetes and to assess its effects on diabetic complications.
- We will focus on generating mouse lines with the *Ctgf* gene duplication 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 achievement.

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. *J Clin Invest* 114:240-249, 2004.

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.

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 4 stable ES cell lines from NOD mice.

Publications

None.