AMDCC Annual Report (2011)

PI: SMITHIES, OLIVER
Project Title: Bradykinin, NO and Mitochondrial DNA Damage in Diabetic Complications
Grant Number: U01 DK076131

Abstract: In our previous work we have demonstrated that genetic factors controlling the production of bradykinin (BK) and nitric oxide (NO) influence greatly the development of renal complications in mice made diabetic with streptozotocin (STZ) or by the Akita diabetogenic C86Y mutation in Ins 2. We also showed that diabetic nephropathy and several indicators of senescence increase progressively in the order wildtype < bradykinin receptor B2 null < Akita diabetic < B2 receptor null Akita diabetic. 8-OHdG content, point mutations and deletions in mitochondrial (mt) DNA increased in the same progression, as did indicators of oxidative stress. We now propose three specific aims and the generation of two new mouse models to determine the interplay between genetic factors that influence BK action, the production of NO, and diabetes-related increases in mutations in mtDNA. Specific aim 1 will determine the effect on diabetic complications of eliminating both BK receptors throughout the body, or in a tissue or cell specific manner; the effects of reducing oxidative stress in these mice will also be determined. Specific aim 2 will investigate the relationship between glomerular damage and mtDNA mutations in eNOS -/- diabetic mice in which we have found that oxidative stress is paradoxically less than in eNOS +/+ diabetic mice. Specific aim 3 will test the hypothesis that increasing the frequency of mtDNA mutations by introducing a proof reading defect into mitochondrial DNA polymerase gamma will exacerbate the complications in Akita diabetic mice even though oxidative stress is not further increased over that due to the diabetes alone.

Hypothesis.

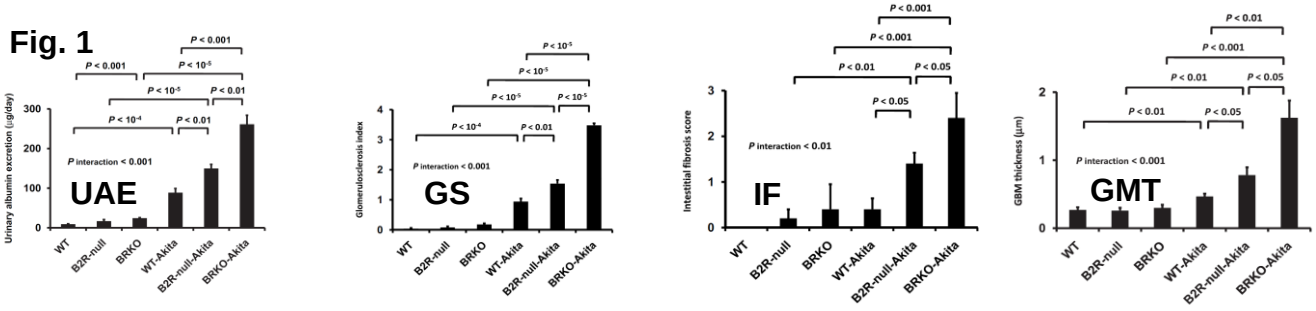
An association in humans between the development and progression of diabetic nephropathy and the D allele at the locus coding for the angiotensin converting enzyme (ACE), and work carried out during the first round of AMDCC studies (reviewed in Kakoki & Smithies, 2009) led us to hypothesize that that the major cause of diabetic complications is mitochondrial damage attributable to oxidative stress and mitochondrial mutations, and that the extent of these complications is strongly influenced by bradykinin and nitric oxide.

To test this hypothesis we proposed three specific aims. The first was to determine the effect on diabetic complications of eliminating both known bradykinin receptors (B1R and B2R) throughout the body or in a tissue specific or cell specific manner. The second was to explore the interaction between diabetic complications and oxidative stress using diabetic mice with or without reduced NO production because of a genetic absence of endothelial nitric oxide synthase (eNOS). The third was to determine whether a global or tissue specific increase in mitochondrial DNA mutations would increase diabetic complications, using Akita diabetic mice that have an editing defect in their mitochondrial DNA polymerase.

Progress towards stated aims, including plans for the year.

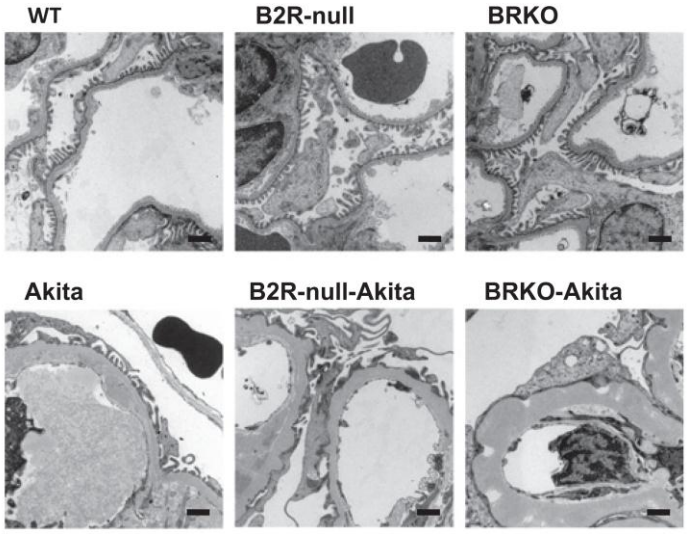
We have completed the work directed towards **the first part of specific aim 1** -- to determine the effect on diabetic complications of eliminating both known bradykinin receptors (B1R and B2R) throughout the body -- and three papers have been published (Kakoki & Smithies, 2009; Kakoki et al., 2010; and Wenge et al., 2010). The first is an invited review of the kallikrein-kinin system in health and in diseases of the kidney. The second (Kakoki et al., 2010) describes an analysis of the effects of absence of B2R or of both B1R and B2R throughout the body on the complications caused by type 1 diabetes induced by the Akita mutation in the *Ins2* gene. As expected from the hypothesis, all diabetic complications were worsened in mice with both bradykinin receptors knocked out (BRKO) compared to those lacking only B2R (B2R-null). For example, the urinary albumin excretion (UAE) of non- diabetic mice lacking B2R or B1R plus B2R was 17 and 30x normal, and there was a strong super-additive interaction between diabetes and absence of the receptors. All other indicators of nephropathy tested, including glomerulo-sclerosis (GS), interstitial fibrosis (IF), glomerular basement membrane thickening (GMT), were similarly progressively enhanced relative to wild type (WT) mice in the same order: WT<B2R-null<BRKO<WT-Akita<B2R-null-Akita<BRKO-Akita when lack of the receptors and diabetes were combined (Figure 1).

Fig. 1



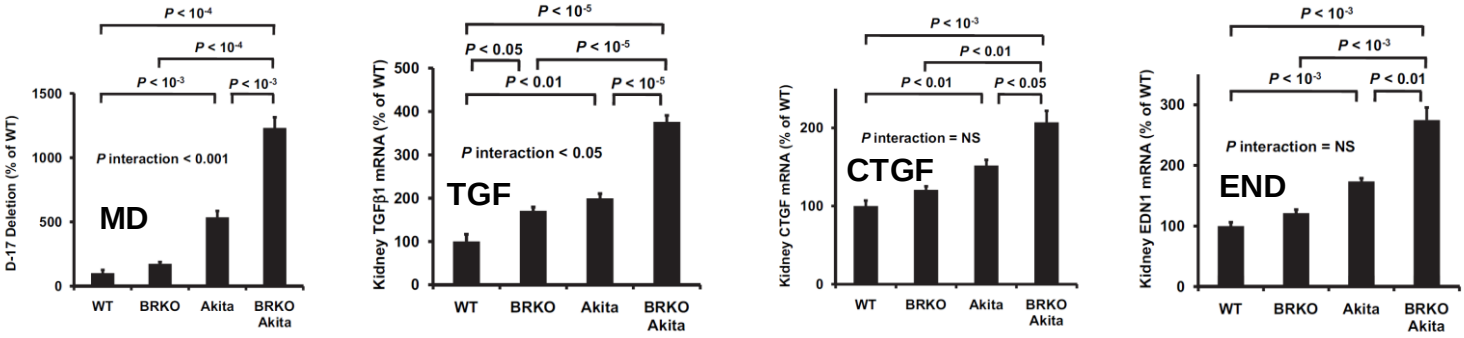
Absence of the receptors in non-diabetic mice had only small effects relative to those seen when the animals were also diabetic, as illustrated by electron micrographs of the renal glomerular capillaries of the mice that we studied. Note the marked increase in glomerular basement membrane thickness in the BRKO-Akita diabetic mice (Figure 2).

Fig. 2



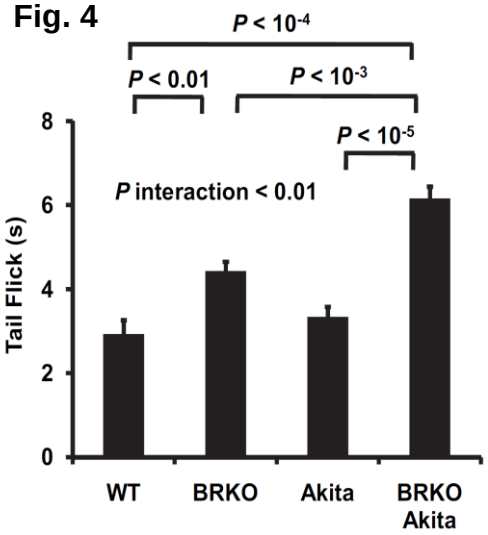
Absence of the receptors and diabetes combined to produce many other potentially pathogenic effects. Thus similar progressive increases in the order WT<BRKO<Akita<BRKO-Akita were observed (Figure 3) in deletions in kidney mitochondrial DNA (MD), in the renal expression of the fibrogenic factors, tissue growth factor β1 (TGF) and connective tissue growth factor (CTGF), and in the expression of endothelin-1 (END).

Fig. 3



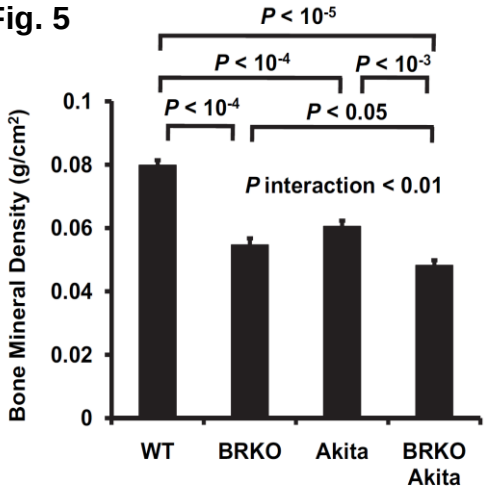
Dr. Eva L. Feldman (an AMDCC member) and her colleagues collaborated with us to study the neurological defects of the diabetic mice lacking both receptors. We found that their lack caused a highly significant increase in neuropathy, as exemplified by the time taken for the mice to respond to a painful stimulus (Figure 4). Lack of the receptors and diabetes again interacted super-additively.

Fig. 4



Likewise, Dr.Kunjie Hua in our department of nutrition collaborated with us to study the bone mineral densities of the mice. Lack of the both receptors markedly increased the osteoporotic effects of diabetes, again super-additively (Figure 5).

Fig. 5

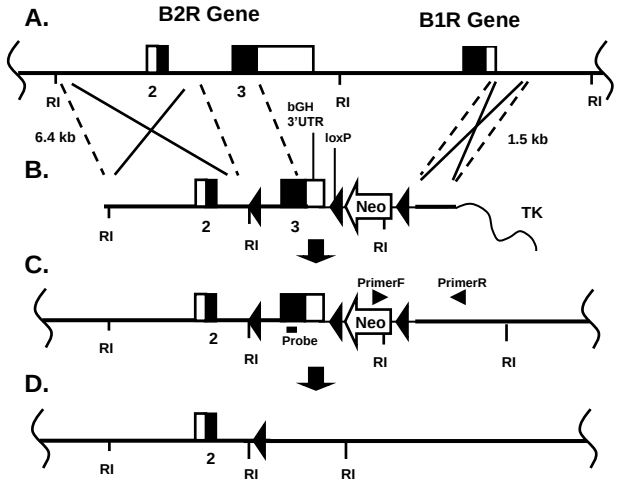


We concluded that lack of B1R and B2R exacerbates diabetic complications, and that the enhanced renal injury which occurs in diabetic mice lacking B1R and B2R may be mediated by a combination of increases in oxidative stress, mitochondrial DNA damage and over expression of fibrinogenic genes (Kakoki et al., 2010).

Our third paper (Wenge et al., 2010) resulted from a collaboration with Dr. E. D. Abel (another AMDCC member) to study the effects of lack of the two receptors on the cardiac function caused by diabetes. Surprisingly, we found that complete loss of bradykinin expression does not worsen cardiac function or increase myocardial fibrosis in diabetes. These findings underscore the need for studies of tissue specific effects in studying the pathogenesis of diabetic complications.

The **second part of specific aim 1** is directed towards this end: namely determining tissue and cell specific effects of lack of both bradykinin receptors. Figure 6(A) shows the shows the genes coding for B1R and B2R, and the targeting vector which we made (B) for use by Dr. Peter Reifsnyder (an AMDCC member in Jackson Laboratories) to modify C57BL/6 ES cells and generate mice having the targeted allele (C) which expresses B2R. We now have these mice in our colony, and are breeding them with mice carrying a Cre transgene appropriate for various target tissues. The locus that will result from the Cre-Lox recombination (D) is null for both receptors.

Fig. 6



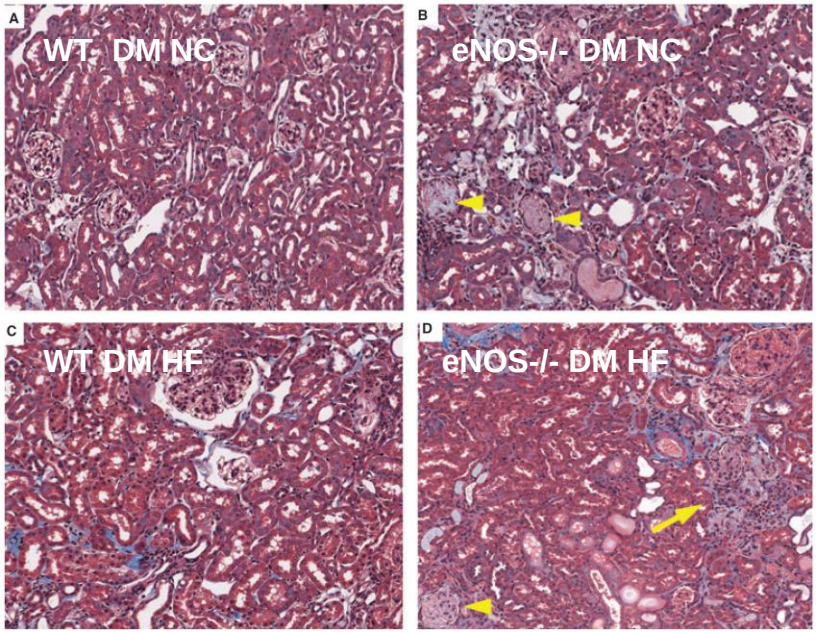
Our primary targets are endothelial cells, glomerular epithelial cells (podocytes), and proximal tubule cells. Mice carrying the requisite Cre transgenes are already in our colony at UNC or are ordered and pending shipment from JAX or the European Mutant Mouse Archive (EMMA). The mice with the Cre transgenes will be mated to the mice received from Jackson Laboratories to complete this part of our project.

The work planned for **Specific Aim 2** -- to explore the interaction between diabetic complications and oxidative stress using diabetic mice with or without reduced NO production because of a genetic absence of endothelial nitric oxide synthase (eNOS) -- has been completed, and two papers have been published (Li et al., 2010; Wang et al., 2011).

The first paper (Li et al.,2010) describes experiments in which $eNOS^{-/-}$ mice and $eNOS^{+/+}$ (wildtype) mice were made diabetic with streptozotocin and fed either normal chow(NC) or a high fat (HF) diet. As expected, non-diabetic mice lacking endothelial NOS ($eNOS^{-/-}$), on either diet, developed no remarkable renal pathology. However, the effects of absence of eNOS ($eNOS^{-/-}$) and of the HF diet were clearly demonstrable in the mice made diabetic (DM). Lifespan was reduced in the $eNOS^{-/-}$ DM NC mice, and was further shortened by the HF diet. Necropsy suggested that some of these mice had died of massive bleeding in the abdomen or thorax, while others had clots in the mesenteric artery accompanied with intestinal necrosis (see below).Urinary excretion of albumin was significantly increased about fourfold by the absence of eNOS without a significant change in creatinine clearance. The HF diet had minimal effects on urinary albumin.

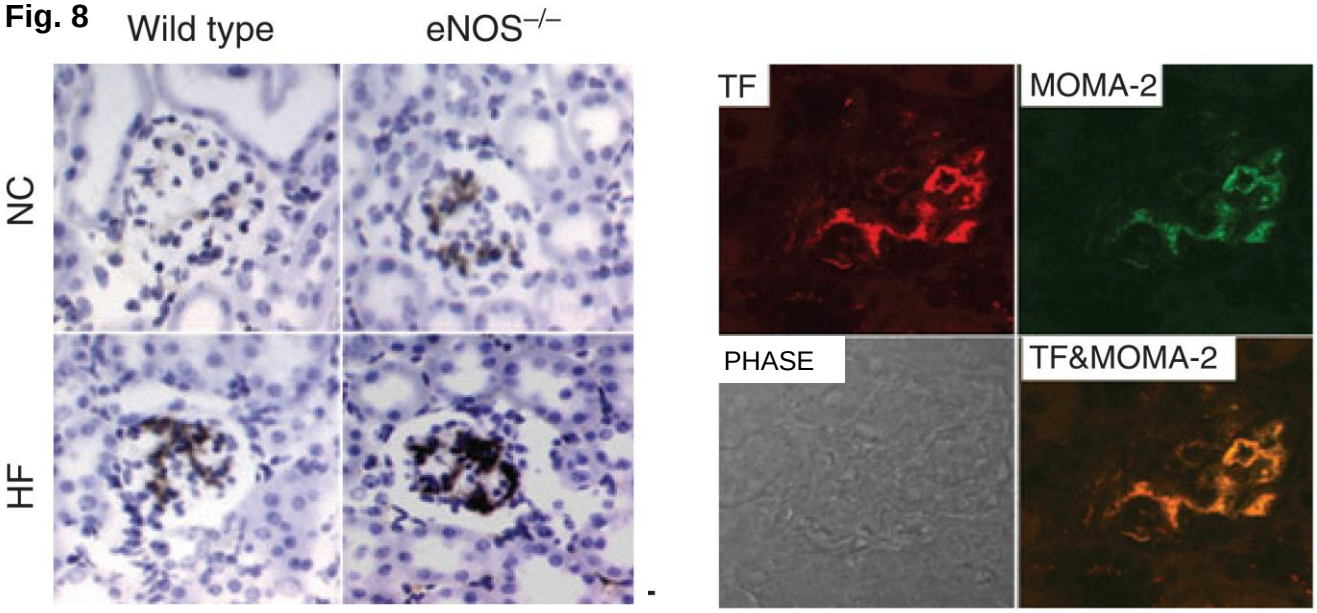
Fig. 7

Nevertheless (Figure 7), the diabetic mice (DM) lacking eNOS and fed normal chow (NC) had severe glomerulo-sclerosis (arrow heads) and tubulointerstitial fibrosis (arrows) together with thickening of the glomerular basement membrane (GBM). The HF diet more than doubled the number of glomeruli with sclerosis.



The bleeding and clotting observed in some of the eNOS -/- diabetic mice at necropsy prompted us to investigate the status of their coagulation system with emphasis on the kidney. We found that all the diabetic mice (but none of the non-diabetic mice) had fibrin deposits in their glomeruli (dark staining in left panel of Figure 8) which were increased by lack of eNOS and by the HF diet.

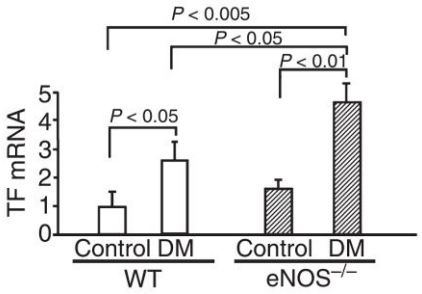
Fig. 8

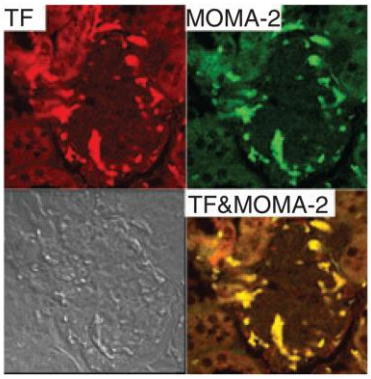


The presence of fibrin suggested a local activation of the clotting cascade, and TF was readily detected in the glomeruli of mice that were diabetic. Renal expression of a variety of inflammatory factors was likewise increased. This expression was stronger when the mice lacked eNOS. The TF positive cells were macrophages residing in the mesangial area (right panel of Figure 8).

Figure 9 shows that significant increases in renal TF mRNA and of TF in glomerular macrophages can be seen in mice that had been diabetic for only 5 weeks and have not yet developed overt renal pathology or significantly increased urinary albumin excretion. Thus increased TF activity precedes the nephropathy induced by diabetes and absence of eNOS.

Fig. 9





Oxidative stress plays an important role in diabetic nephropathy. Accordingly, we determined the plasma levels of carboxy-methyl lysine (CML) and the urinary excretion of 8OHdG, two well documented indicators of oxidative stress. Although both were substantially increased by diabetes, absence of eNOS had no detectable effect on their levels in the diabetic mice fed normal chow. Surprisingly however these indicators of oxidative stress were significantly decreased in the eNOS-/- diabetic mice fed the HF diet.

We concluded that our data indicate a causal link between TF and the exacerbation of diabetic nephropathy in mice lacking eNOS, with the condition being significantly worsened by enhanced inflammatory responses to a high fat diet (Li et al., 2010).

The second paper relevant to **specific aim 2** -- to explore the interaction between diabetic complications and oxidative stress using diabetic mice with or without reduced NO production because of a genetic absence of endothelial nitric oxide synthase (eNOS) -- describes a more comprehensive set of experiments which we then carried out using mice that were non-diabetic or had the genetic form of type 1 diabetes induced by the dominant Akita mutation (A) and were either wildtype at the eNOS locus (+/+), or heterozygous null (+/-) or homozygous null (-/-). To increase the vigor of the mice required for these experiments, we generated them using parents that were from two different inbred strains. Consequently the progeny that we used for our experiments are genetically as uniform as inbreds, but have the vigor of hybrids, in which many of the detrimental recessive genes present in any particular inbred strain of mice are covered by a wild type allele from the other strain unless they have a recent common ancestor. [They are the genetic equivalent in mice of human identical twins.]

The resulting six groups of mice showed multiple indicators of diabetic nephropathy - - albuminuria, changes in GFR, mesangial expansion, mesangiolysis, glomerulosclerosis, GBM thickening and tubulointerstitial fibrosis- -which were progressively enhanced in the order: eNOS$^{+/+}$ Akita < eNOS$^{+/-}$ Akita < eNOS$^{-/-}$ Akita. These data were used in preparing a document (appended to this report as 06-15-10 breeding scheme.pdf) published on the AMDCC website describing appropriate breeding schemes and selection of animals for use in studying the effects on diabetic complications of various genetic variations.

The beautiful gradation in their phenotypes is illustrated in the bar diagrams showing urinary albumin excretion (UAE) and GFR (Figure 10). +/+ is wild type, +/- is heterozygous eNOS null, -/- is homozygous eNOS null. Akita diabetes is indicated by A.

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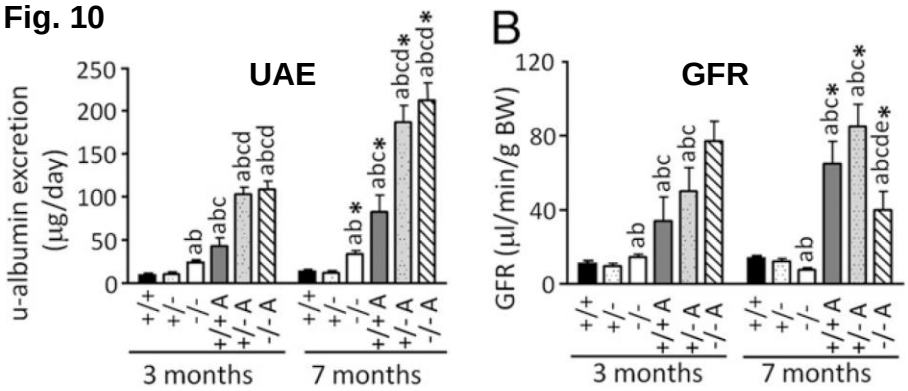
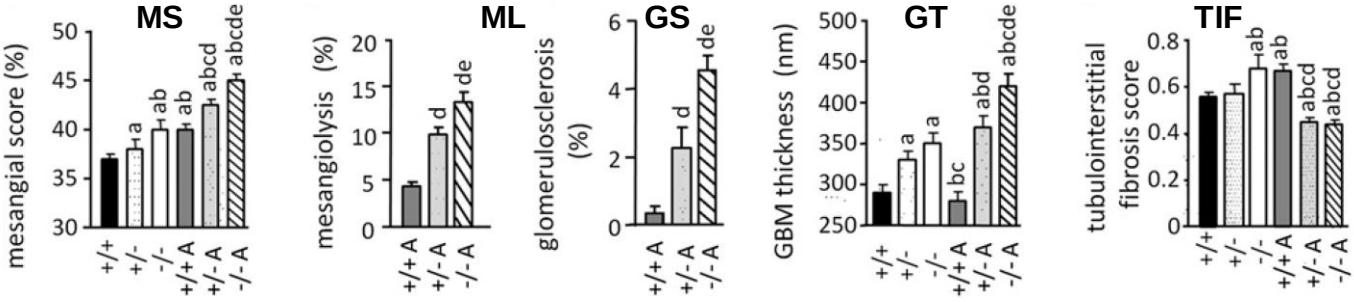
Fig. 10



Other important indicators of nephropathy were similarly graded, including mesangial score (MS), mesangiolysis (ML), glomerular sclerosis (GS), GMB thickness (GT), and tubulointerstitial fibrosis (TIF) (Figure 11).

Fig. 11



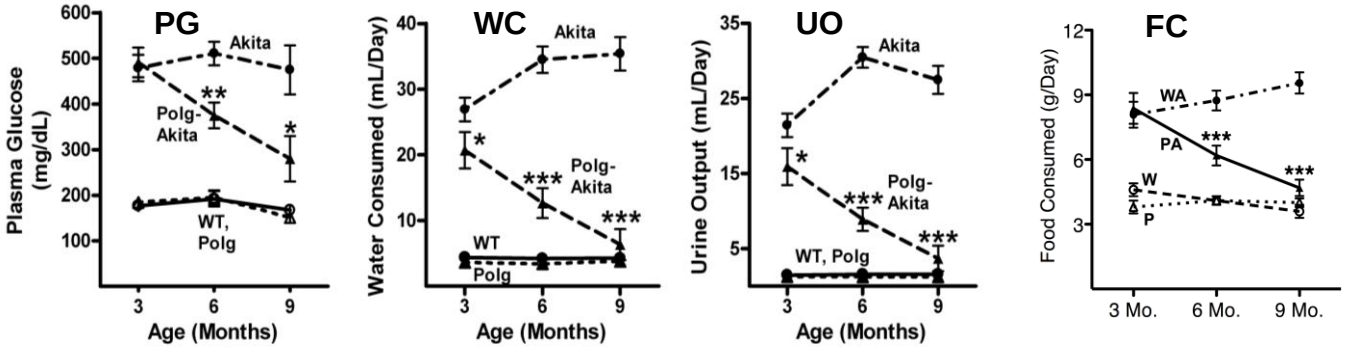
Particularly notable from the point of view of relevance to human conditions is the clear demonstration that the diabetic mice that were eNOS+/- heterozygotes (and still have about 25% normal eNOS expression) developed marked nephropathy. The level of eNOS expression in these heterozygotes is comparable to that (~30% normal) in the 5 to 9% of humans who have a G894T variation at the NOS3 locus (the human equivalent of eNOS). We consequently summarized our experiments with the statement that "by using genetically uniform and robust B6/129 hybrid F1 mice, we have shown that **a modest decrease in *eNOS*, comparable to that associated with polymorphisms in the human *NOS3* gene, markedly enhances the development of diabetic nephropathy.** Most of the damaging effects are independent of changes in blood pressure."

The results also confirmed our previous demonstration of the importance of tissue factor and inflammation in the development of the nephropathy. They also reinforced and extended our surprising findings related to oxidative stress, namely that the increased oxidative stress caused by diabetes in the $eNOS^{+/+}$ Akita mice is decreased by reduced expression of eNOS. Indeed, the oxidative stress of the diabetic mice with decreased levels or absence of eNOS is less than that of non-diabetic wild type mice. We therefore exclude increased oxidative stress as a factor in the exacerbation of diabetic nephropathy resulting from reduced expression of eNOS (Li et al., 2010; Wang et al., 2011).

Work is essentially complete towards **the first part of specific aim 3** -- to determine whether a global increase in mitochondrial DNA mutations increases diabetic complications using Akita diabetic mice that have an editing defect in their mitochondrial DNA polymerase. A paper describing this work is under review (Fox et al., 2011).

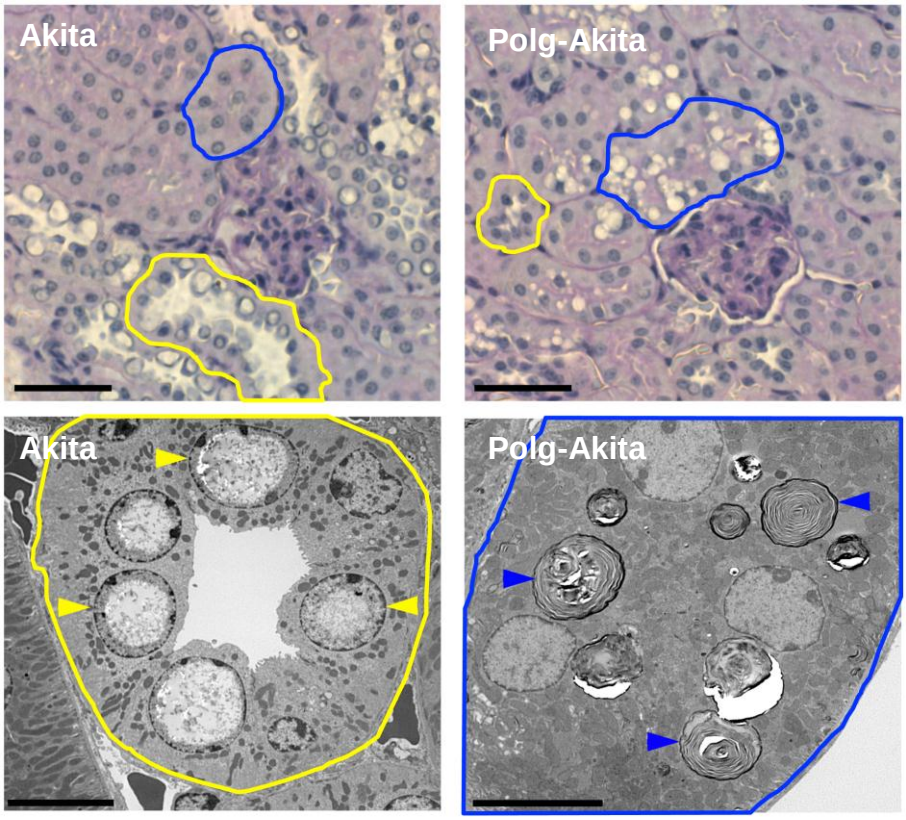
At 3 months of age, both the Akita and Polg-Akita male mice exhibited all the symptoms normally associated with type 1 diabetes, including high plasma glucose (PG) levels, water consumption (WC), urine output (UO) and food consumption (FC). Surprisingly however, as the Polg-Akita mice aged their diabetic symptoms progressively decreased, so that by 9 monhs age they had virtually disappeared (Figure 12).

Fig.12



Kidney function in the diabetic mice at 3, 6 and 9 months age, as judged by urinary albumin excretion (UAE), was 2 to 3 fold higher than in their non-diabetic mice siblings, although it never reached the level AMDCC considers to be overt proteinuria. The Polg mutation did not affect UAE. Nevertheless, despite the absence of effects of Polg on albumin excretion, light and electron microscopy revealed clear signs of nephropathy (Figure 13).

Fig. 13



Thus the proximal tubules (outlined in blue) of the 9 month old Akita mice (left panels) appeared normal, but the nuclei of their distal tubules (outlined in yellow) had glycogen inclusions (yellow arrow heads) due to the extreme hyperglycemia and high urinary glucose present in these untreated type 1 diabetic mice. In contrast, the proximal tubules of the Polg-Akita mice (right panels), which at 9 months were nearly euglycemic, no longer had nuclear glycogen inclusions in their distal tubules, but their proximal tubules (outlined in blue) had lamellated cytoplasmic inclusions (blue arrow heads) indicative of mitochondrial turnover at some time during the preceding months.

We then set out to determine the link between the Polg mutation and the near normal glucose of the 9 month Polg-Akita mice.

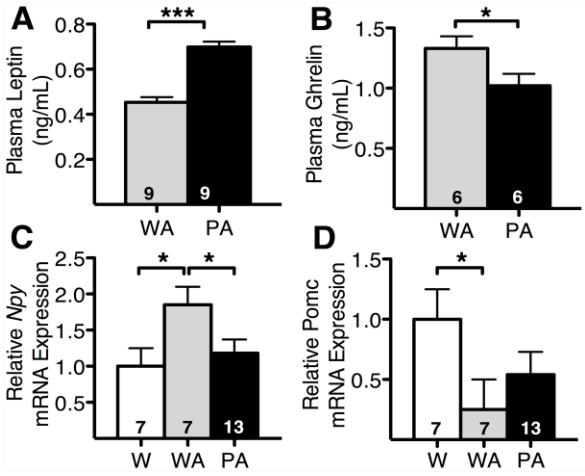
The Akita mutation, a cysteine to tyrosine substitution in insulin produced from the *Ins2* gene, induces an unfolded protein response in the beta cells of the pancreas accompanied with a progressive devastation of the secretory pathway organelles of the beta cells and an increase in apoptosis. The disappearance of hyperglycemia in the Polg-Akita mice suggested the possibility that these mice might have increased their production of insulin, or have become less impaired in their ability to handle glucose. However, our observations do not support this possibility. Thus, the Akita and Polg-Akita mice have very similar plasma insulin levels (approximately 20% of non-diabetic mice). Nor could we detect any differences in the morphology and number of pancreatic islet cells of the Akita and Polg-Akita mice. Additionally, their responses to an oral glucose load did not differ significantly. We concluded that the effect of the Polg mutation on plasma glucose was not via changes in beta cell function or in glucose handling.

We next considered the possibility that the marked decrease in plasma glucose in the Polg-Akita mice relative to the Polg mice could be due to an increase in the damage to intestinal epithelium already easily seen in non-diabetic Polg mice, or to intestinal transporters important for glucose uptake, or to pancreatic pro-enzymes. However, we found no differences in these factors in the mice sacrificed at 9 months age.

Accordingly, we considered the possibility that the decrease in food intake could be due to a change in the appetite of the Polg-Akita mice.

Remarkably, we found that the plasma levels of two known regulators of appetite were changed. Plasma leptin (a suppressor of appetite) was increased; plasma ghrelin (a stimulator of appetite) was decreased. Likewise the expression in the hypothalmus of Pomc (an anorexic propeptide) was decreased, while that of Npy (an orexic neuropeptide) was increased (Figure 14).

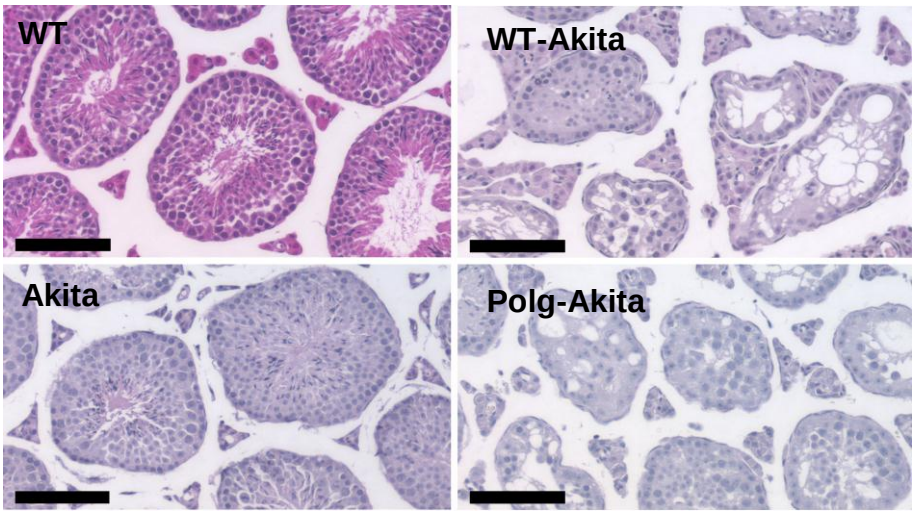
Fig.14



Testosterone is a known stimulator of appetite, and the testes of male mice with the mutant Polg become severely atrophic as they age. There was therefore a strong possibility that testicular damage in the Polg-Akita mice could decrease testosterone production sufficiently to impair their appetite.

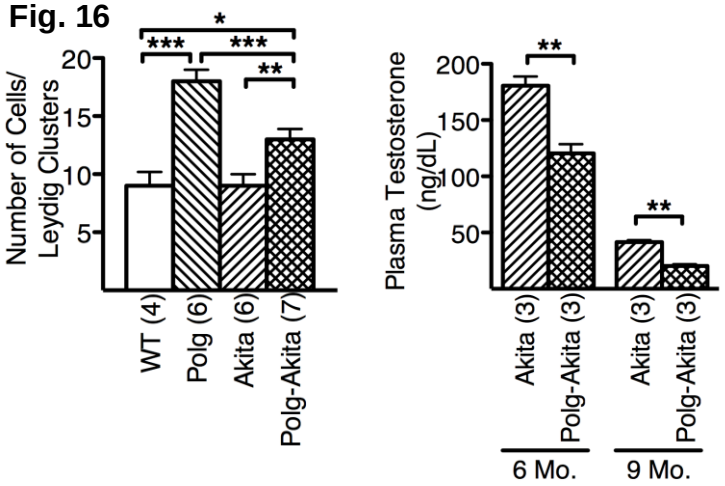
Histology of the testes of the WT, Akita, Polg and Polg-Akita mice (Figure 15) confirms the severe loss of spermatogenesis known to occur in mice having the mutant mtDNA polymerase, although spermatogenesis was not significantly affected by the Akita mutation. However, a morpho-metric analysis of the Leydig cell clusters in the testes of the mice, combined with assays of their plasma testosterone levels, shows that the two mutations do in fact interact and decrease the testosterone levels of the Akita diabetic mice.

Fig. 15



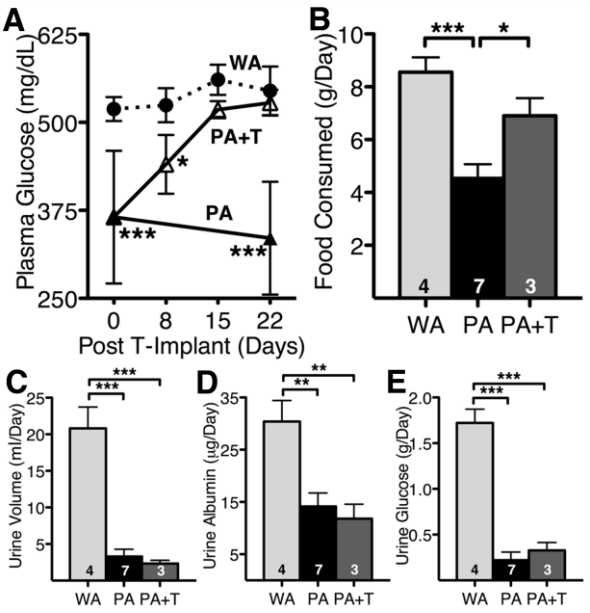
Thus, as illustrated in Figure16, although the Polg mutation increases the number of Leydig cells per cluster in both the WT non-diabetic mice, plasma testosterone levels in the diabetic mice are decreased by the Polg mutation. Furthermore, electron micrographs revealed an increase in electron dense lipofuscin in the Leydig cells of the Polg-Akita mice (an indicator of mitochondrial turnover) compared to their Akita siblings. We consequently concluded that the Polg mutation has a strong and direct negative impact on testicular function and testosterone levels of Akita diabetic mice.

Fig. 16



This result prompted us to test whether testosterone supplementation would restore the diabetic phenotype of the Polg-Akita mice to the level seen when they were young. We therefore implanted pellets to give a continuing physiological dose of testosterone to 6 month old Polg-Akita mice (Figure 17). Within less than a month the plasma glucose levels of the implanted mice returned to the high levels seen in Akita mice with wild type Polg, and the food consumption of the implanted mice increased to about midway between those of the Akita and untreated Polg-Akita mice. Urine volumes remained low, suggesting that the renal tubular glucose re-absorptive capacity of the implanted mice was sufficient to prevent the massive diuresis seen in Akita diabetic mice with normal Polg.

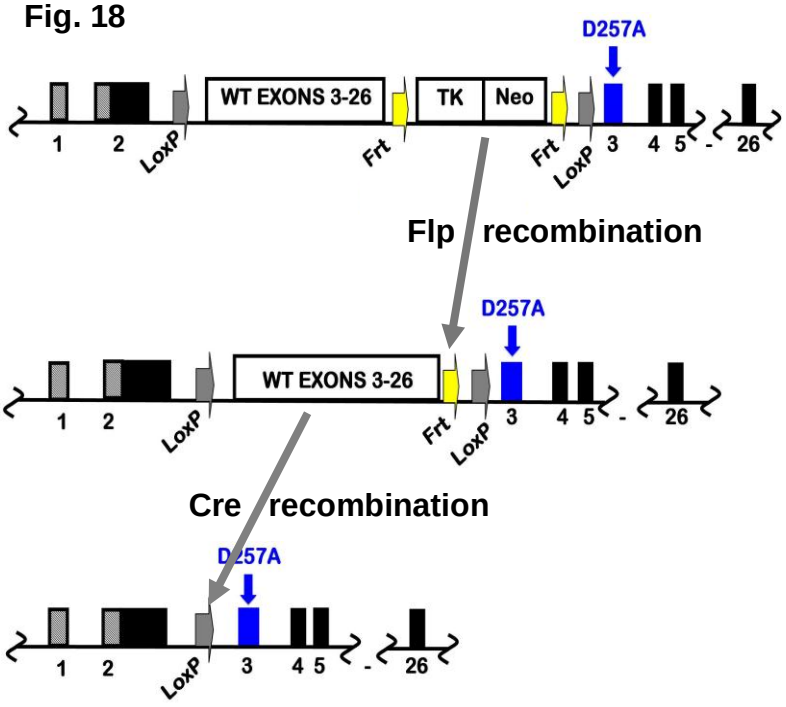
Fig. 17



We concluded that increased mtDNA mutations decreased the diabetic symptoms of aging mice as a result of appetite suppression triggered by decreased testosterone production associated with severe damage to the testis. Distal tubular damage in the kidney decreased as the hyperglycemia disappeared, but proximal tubular damage increased because of mitochondrial debris resulting from the mutant Polg (Fox et al., under review).

Towards t**he second part of specific aim 3** -- to determine whether a tissue-specific increase in mitochondrial DNA mutations will increase diabetic complications -- we constructed a targeting vector that will allow us to change a normal Polg gene to the non-editing mutant. The top line of Figure.18 shows the form of the Polg gene obtained after the targeting but before the action of either of the recombinases. We have obtained chimeras that are at this stage. To complete this part of our project we will mate these chimeras to mice carrying a ubiquitously expressed form of the Flp recombinase (available from JAX) to remove the selectable TK and Neo genes used for targeting . This will generate a form of the Polg gene (second line) that will produce normal Polg in all tissues. Homozygotes for this modified normal gene will then be exposed to a tissue specific Cre recombinase of our choice to produce the mutant form of Polg in selected target tissues. Our primary targets are the same as for Specific Aim 1, namely: endothelial cells, glomerular epithelial cells (podocytes), and proximal tubule cells, and we will use the same tissue-specific forms of Cre as for that aim. A paper describing these results is under review (Fox et al., 2011).

Fig. 18



Response to EAC's Comments.

Comment 1. Has all of your eNOS and B1R/B2R data been uploaded to the website?

Response. The answer is "Yes". A paper (Wang et al., 2011) describing the **eNOS** data was published on Feb.1, 2011 following electronic release on Jan 18, 2011. The relevant data have been uploaded to the website. Additionally, as described above, data from this paper were used to illustrate a document published on the AMDCC website titled "Breeding Scheme and Selection of Animals for AMDCC Experiments". We attach to the present report a PDF of this document ("06-15-10 breeding scheme.pdf"). A paper (Kakoki et al., 2010) describing the **B1R/B2R** work was published on Jun 1 2010 following electronic release on May 17, 2010. The data from this paper have been uploaded to the website.

Comment 2. The data for the tissue factor (TF) studies should be more explicitly presented.

Response. Our published paper (Wang et al., 2011) explicitly presents the data in our studies that implicate **TF** as critically important in determining the increased diabetic nephropathy resulting from reduced expression of eNOS (see for example Figures 8 and 9, above). We concluded that our data indicate a causal link between this increased nephropathy and TF.

Publications Since Last Report

Kakoki, M and Smithies, O. The kallikrein-kinin system in health and in diseases of the kidney. Kidney Int. 75(10):1019-1030 (2009) PMID: 19190676.

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Breeding Scheme and Selection of Animals for AMDCC Experiments

It is extremely important to measure quantitative parameters (e.g. plasma glucose, urinary albumin, etc.) in a way that minimizes the effects of any adventitious variables, such as seasonal changes, dietary differences, any differences in the degrees of backcrossing, or unplanned differences in genotypes. This means that, whenever possible, mice of the critical genotypes should be obtained from genotypically identical matings carried out at or close to the same time. Archival data are not appropriate.

The breeding scheme to generate the experimental animals can vary in complexity, depending on the fertility of the two genders, whether the *Testgene* is to be studied in homozygotes or in heterozygotes, and whether the diabetes is induced genetically or by STZ. However, it is important to remember that, when looking for the effects of the *Testgene* genotype on diabetic complications, the aim is to compare the *Testgene* $^{mutant/mutant}$ (or *Testgene* $^{mutant/wt}$) diabetic animals with their diabetic litter mates that are wildtype at the *Testgene* locus (*Testgene* $^{wt/wt}$).

When testing the effects of homozygosity for a mutant (such as a knockout)*Testgene* on diabetes induced by the dominant *Ins2*^{Akita} mutation, a conservative and usually trouble-free breeding scheme is:

Testgene $^{mutant/wt}$ & *Ins2* $^{wt/wt}$ female (Inbred1) x *Testgene* $^{mutant/wt}$ & *Ins2* $^{Akita/wt}$ male (Inbred2)

Males and females of six genotypes result -- the three *Testgene* genotypes, each with or without diabetes:

1.	*Testgene* $^{mutant/mutant}$	:	*Ins2* $^{Akitat/wt}$	(Inbred or F1)
2.	*Testgene* $^{mutant/wt}$	:	*Ins2* $^{Akita/wt}$	(Inbred or F1)
3.	*Testgene* $^{wt/wt}$	:	*Ins2* $^{Akita/wt}$	(Inbred or F1)
4.	*Testgene* $^{mutant/mutant}$	:	*Ins2* $^{wt/wt}$	(Inbred or F1)
5.	*Testgene* $^{mutant/wt}$	:	*Ins2* $^{wt/wt}$	(Inbred or F1)
6.	*Testgene* $^{wt/wt}$	:	*Ins2* $^{wt/wt}$	(Inbred or F1)

Note that both parents, although inbred, are heterozygotes for the mutant form of the *Testgene*, which are almost always healthier and have more offspring than homozygous mutants. The offspring can be F1 hybrids (genetically as uniform as inbreds but hardier), or can be kept inbred if this is preferred. Note also that this mating produces the essential genotypes (usually 1 & 3) as littermates. Studying all six genotypes is very informative, but not essential. However, if *Testgene* heterozygotes are included (1,2, & 3), the functional consequences of different levels of expression of the *Testgene* on diabetic complications can be determined. If wildtype and mutant *Testgene* mice are studied on both diabetic and non-diabetic backgrounds (1,3,4 & 6), additive, super-additive or sub-additive interactions between the *Testgene* and diabetes can be detected.

In the case of diabetes induced by the Akita mutation in heterozygous state, it has been the AMDCC experience (1) that a higher level of chronic hyperglycemia is seen in males, and (2) that the development of diabetic complications is age-dependent and largely male-limited. Thus, the investigator could cull females as soon as sex can be determined and wean only males, thereby reducing costs and saving colony space.

When testing the effects of transgenes, it is generally advisable to avoid homozygosity since undesirable artifacts (insertional mutagenesis leading to homozygous lethality or other major physiologic/metabolic anomalies) can result independent of the presence or absence of diabetes.

When homozygous transgenes are not needed, or when the homozygous knockout of a *Testgene* is lethal, a simpler breeding scheme can be used:

Testgene $^{mutant/wt}$ & *Ins2* $^{wt/wt}$ female (Inbred1) x *Testgene* $^{wt/wt}$ & *Ins2* $^{Akita/wt}$ male (Inbred2)

Males and females of four genotypes result:

7.	*Testgene* $^{mutant/wt}$	:	*Ins2* $^{Akita/wt}$	(Inbred or F1)
8.	*Testgene* $^{wt/wt}$	:	*Ins2* $^{Akita/wt}$	(Inbred or F1)
9.	*Testgene* $^{mutant/wt}$	:	*Ins2* $^{wt/wt}$	(Inbred or F1)
10.	*Testgene* $^{wt/wt}$	:	*Ins2* $^{wt/wt}$	(Inbred or F1)

We append recent results that Dr. Nobuyuki Takahashi has obtained in an experiment to determine the effects of *Nos3* genotype on diabetic nephropathy (Wang et al., 2011). He studied all six male genotypes resulting from *Nos3*$^{+/-}$ *Ins2* $^{wt/wt}$ female (129SvEv) x *Nos3*$^{+/-}$ *Ins2* $^{Akita/wt}$ male (C57BL/6) matings. The data obtained from the resulting F1 mice are internally very consistent, and the error bars are relatively small. Interestingly the phenotypes of all six genotypes are distinguishable and progressively more severe in going from 6 to 1.

Reference

Wang, C-H, Li, F, Hiller, S, Kim, H-S, Maeda, N, Smithies, O, and Takahashi, N. A modest decrease in endothelial NOS in mice comparable to that associated with human NOS3 variants exacerbates diabetic nephropathy. Proc. Natl. Acad. Sci. USA 108(5): 2070-2075 (2011) PMID: 21245338 PMCID: PMC3033253.

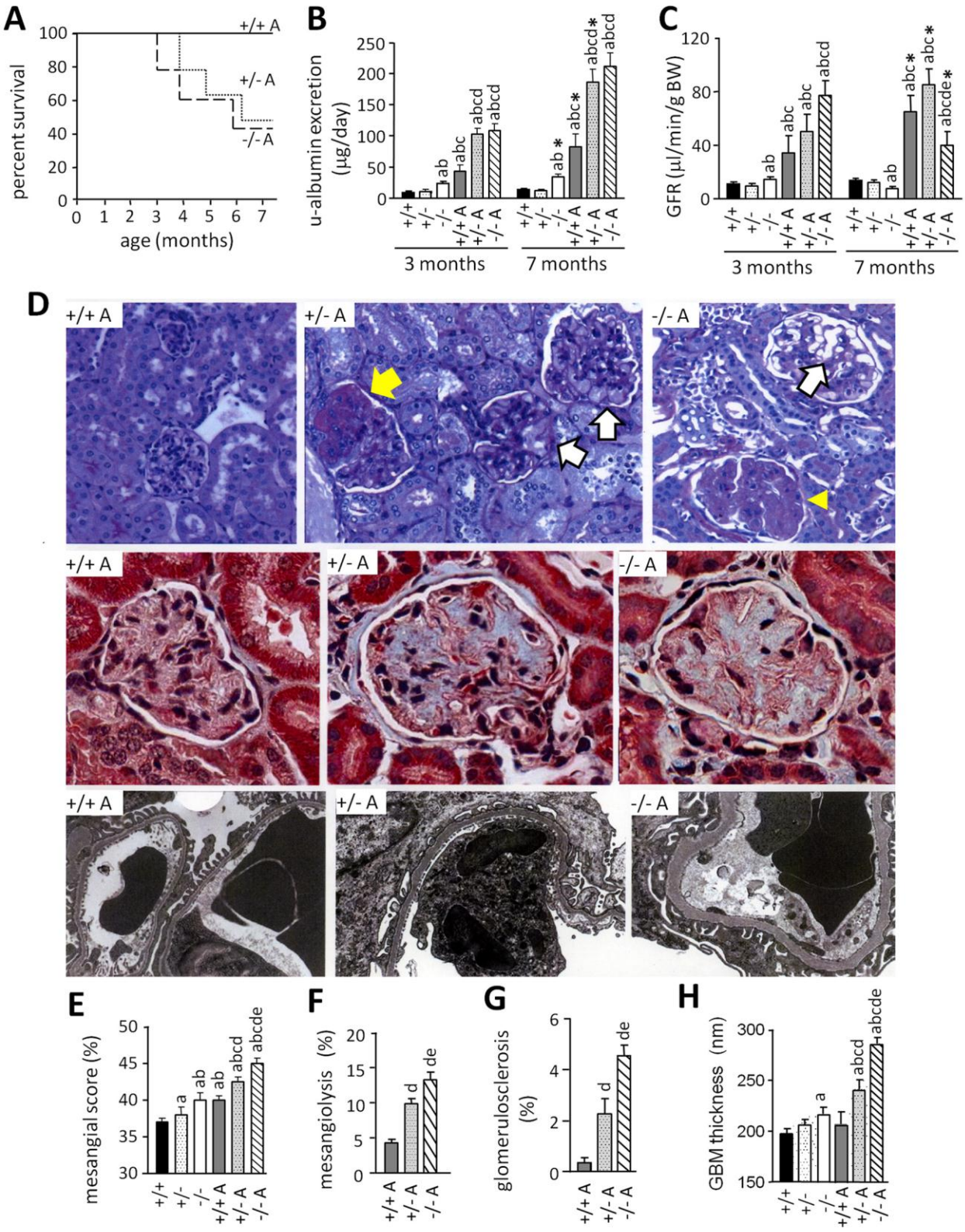

Figure 1. Survival rates, renal function, and histology of F1 (129SvEv x C57BL/6J) mice with different eNOS genotypes and Ins Akita mutation. A. Survival rates of three groups of diabetic mice with different eNOS genotypes. All non-diabetic mice regardless of the genotype of eNOS survived until the end of the experiment (not shown). B. Daily urinary albumin excretion when animals were at 3 months and 7 months old. C. GFR estimated by creatinine clearance when animals were at 3 months and 7 months old. D. Representative kidney morphology of 7 month-old diabetic mice with different eNOS genotypes. Top panels: PAS stain (original magnification x100). Yellow arrow: mesangial expansion; open arrow: mesangiolysis; yellow arrow head: glomerulosclerosis. Middle panels: glomeruli with Masson Trichrome stain (original magnification x200). Bottom panels: transmission electron micrographs with original magnification of x 12,500. E-H. Quantification of mesangial expansion (E), mesangiolysis (F), glomerulosclerosis (G), and GBM thickness (H) of 7 month-old mice. There was no glomerulosclerosis in any of non-diabetic mice regardless of eNOS genotypes. +/+, +/-, and -/- designate eNOS genotypes. A: Ins2Akita. Data are mean ± SEM. n≥8. a, b, c, d, e: $p<0.05$ vs. +/+, +/-, -/-, +/+ A, +/- A, respectively. *$p<0.05$ vs. values when the same mice were 3 months old.

Table 1 Characteristics of 3-month-old F1 (129SvEv x C57BL/6J) males having wild type and mutant *eNOS* and *Ins2* genes

Genotype	+/+	+/-	-/-	+/+ Akita	+/- Akita	-/- Akita	diabetes (D)	genotype (G)	DxG
BW (g)	29.4 ± 0.9	30.5 ± 0.8	28.0 ± 0.9	26.4 ± 0.9	28.0 ± 0.5	26.9 ± 0.7	0.002	N.S.	N.S.
BP(mmHg)	109.8 ± 2.3	127.6 ± 3.3^{a}	137.8 ± 4.0^{ab}	107.6 ± 3.6^{c}	126.8 ± 2.3^{acd}	142.8 ± 2.6^{abde}	N.S.	<0.0001	N.S.
glucose (mg/dl)	165 ± 19	199 ± 21	165 ± 20	422 ± 29^{abc}	467 ± 17^{abc}	515 ± 21^{abc}	<0.0001	N.S.	N.S.
food intake (g/day)	3.9 ± 0.5	4.6 ± 0.7	3.6 ± 0.7	5.1 ± 0.8	7.0 ± 0.5^{abcd}	8.4 ± 0.5^{abcd}	<0.0001	N.S.	0.05
water intake (ml/day)	3.0 ± 0.3	3.0 ± 0.2	3.0 ± 0.3	23.3 ± 3.6^{abc}	26.5 ± 2.0^{abc}	26.3 ± 2.6^{abc}	<0.0001	N.S.	N.S.
urine volume (ml/day)	1.5 ± 0.5	1.0 ± 0.2	1.3 ± 0.2	20.4 ± 1.6^{abc}	24.1 ± 2.5^{abcd}	21.1 ± 2.0^{abcd}	<0.0001	N.S.	N.S.

Data are mean ± SEM. n≥8. BW, body weight; BP: blood pressure; glucose: plasma glucose. GxD, designates *p* values of interaction between genotype and diabetes. N.S.: not significant. +/+, +/-, -/- designate eNOS genotypes. A: Akita. a,b,c,d,e: p<0.05 vs. +/+, +/-,-/-, +/+Akita, +/-Akita, respectively.

Table 2 Characteristics of 7-month-old F1 males having wild type and mutant *eNOS* and *Ins2* genes

Genotype	+/+	+/-	-/-	+/+ Akita	+/- Akita	-/- Akita	diabetes (D)	genotype (G)	DxG
BW (g)	33.2 ± 1.0	36.5 ± 1.3	37.8 ± 1.2	26.5 ± 1.2^{abc}	29.3 ± 1.2^{abcd}	33.3 ± 0.9^{cde}	0.002	<0.0001	N.S.
BP (mmHg)	117.0 ± 2.3*	127.0 ± 2.9^{a}	135.8 ± 2.9^{ab}	119.0 ± 2.9^{bc}*	134.0 ± 2.9^{abd}*	145.8 ± 2.7^{abcde}	<0.01	<0.0001	N.S.
glucose (mg/dl)	166 ± 16	203 ± 21	216 ± 20	465 ± 20^{abc}	500 ± 19^{abc}	514 ± 22^{abc}	<0.0001	N.S.	N.S.
food intake (g/day)	4.0 ± 0.4	3.8 ± 0.5	3.6 ± 0.5	9.5 ± 0.9^{abc}*	8.9 ± 0.8^{abc}*	10.8 ± 1.1^{abc}*	<0.0001	N.S.	<0.05
water intake (ml/day)	3.0 ± 0.2	2.7 ± 0.2	2.5 ± 0.2	25.5 ± 3.0^{abc}	31.9 ± 3.5^{abcd}	34.4 ± 2.7^{abcd}*	<0.0001	N.S.	N.S.
urine volume (ml/day)	1.0 ± 0.2	1.4 ± 0.2	1.2 ± 0.3	22.8 ± 3.0^{abc}	32.8 ± 3.5^{abcd}*	28.3 ± 2.5^{abcd}*	<0.0001	N.S.	N.S.
kidney weight (mg/g BW)	15 ± 2	14 ± 1	14 ± 1	19 ± 2^{abc}	20 ± 2^{abc}	21 ± 2^{abc}	<0.0001	N.S.	N.S.

Data are given as mean ± SEM. n≥8. BW, body weight. ; BP: blood pressure: glucose: plasma glucose. GxD, designates *p* values of interaction between genotype and diabetes. N.S.: not significant. +/+, +/-, -/- designate eNOS genotypes. A: Akita. a,b,c,d,e: p<0.05 vs. +/+, +/-, -/-,+/+Akita, +/-Akita, respectively.
*p<0.05 vs. values when the same mice were 3 months old.