Animal Models of Diabetic Complications Consortium
(U01 HL087944)

Annual Report
(2010)

"Atherosclerosis and other complications in the hyperlipidemic BKS diabetic mouse"

University of California, Los Angeles

Principal Investigator
Richard C. Davis

Address: **UCLA Department of Medicine**
Cardiology Division
47-123 Center for Health Sciences
Los Angeles, CA 90095-1679

Phone: **(310) 206-4758**
FAX: **(310) 825-2450**

E-mail: **davisr@ucla.edu**

Table of Contents

Animal Models of Diabetic Complications Consortium
(U01 HL087944)

Part A:

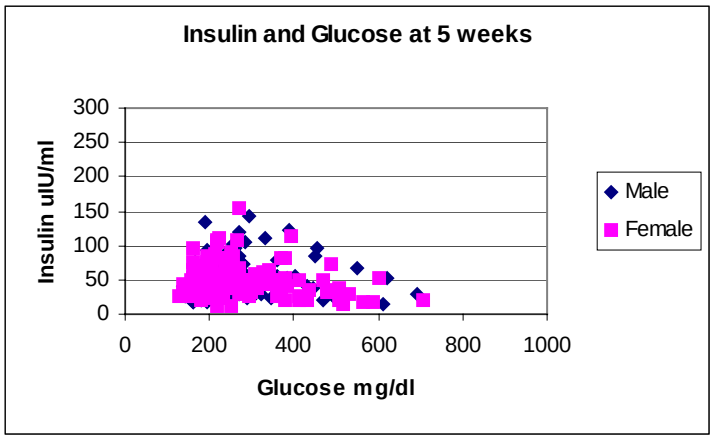
Principal Investigator's Summary

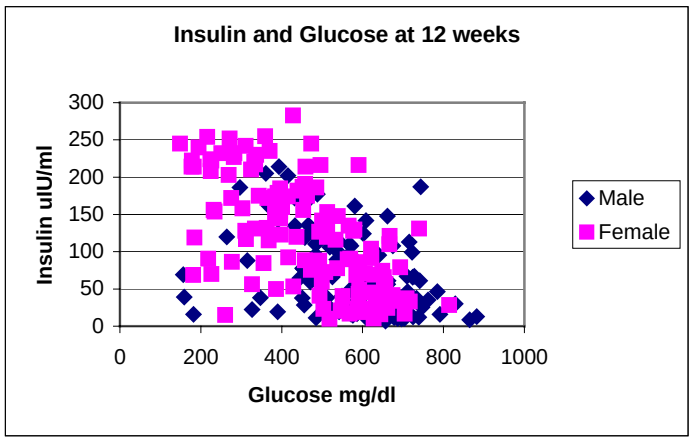
1. Program Accomplishments:

C57BLKS db/db mice have elevated insulin at 4-5 weeks of age and, by 6 months, have obvious depletion of pancreatic islets and hyperglycemia. By contrast, C57BL/6 db/db mice retain much better pancreatic function and glycemic control at the same age. C57BLKS mice are largely derived from C57BL/6 with about 70% of their genome apparently consisting of large blocks of DNA identical to B6 with the remainder coming from DBA/2 (~20%) and another uncharacterized and perhaps wild strain (>10%). Thus, the differential impact of the db/db mutation between C57BLKS and C57BL/6 must reside in the small fraction of the genome represented by DBA/2 and the unknown strain. Further, DBA and C57BLKS are similarly over-responsive to diabetogenetic stimuli, suggesting that the underlying loci in C57BLKS are predominantly located in the DBA/2-derived region of the genome.

In an effort to identify the genetic basis for the difference in diabetes susceptibility between C57BLKS and C57BL/6, we have examined obese F2 mice from a cross between C57BL/6 and DBA/2. Specifically, we wish to define loci important to these differences in insulin resistance, diabetic progression and lipid accumulation in the liver as driven by the db/db mutation. For this, we characterized two cohorts of mice; one at 5 weeks of age prior to anticipated islet depletion and another cohort at 12 weeks when C57BLKS db/db begin to show decreased insulin production but prior to the onset of significant diabetic complications.

Surprisingly, many of the F2 db/db animals at both 5 weeks and 12 weeks showed far more advanced diabetes in terms of plasma insulin and glucose levels than was anticipated from levels seen at these ages in C57BL/6 db/db and C57BLKS db/db animals. In particular, a significant number of animals had high glucose and low insulin suggestive of islet depletion seen in full diabetes. And, among animals that had so progressed to advanced diabetes, males were disproportionately represented, consistent with the increased diabetes susceptibility seen in males of C57BLKS and other strains.





While all animals in the cross were genetically obese, there was considerable heterogeneity among F2 animals in bodyweight and in percent body fat as determined by NMR. Thus, there are substantial genetic variations that impact body weight in this cross, even in the face of the extreme hyperphagia engendered by the db/db mutation. And, these genetic variations in body composition are strongly associated with differences in insulin

resistance and diabetes susceptibility. As evidenced in the correlation and p-value tables below, percent body fat among 5-week animals (shown in blue) is strongly and positively correlated with plasma glucose levels. Weaker correlation is also seen between plasma glucose and lipid accumulation in the liver.

p-Value Matrix

	Liver NMR Fat%	Body NMR Fat%	Plasma Glucose mg/dl	Insulin
Liver NMR Fat%	0	0.0415481	0.0087262	0.0276204
Body NMR Fat%	0.0888225	0	6.18E-10	0.0927811
Plasma Glucose mg/dl	2.60E-05	0.2665759	0	0.0919955
Insulin	7.74E-06	0.0346712	2.36E-24	0

W12 W5

Correlation Coefficient Matrix

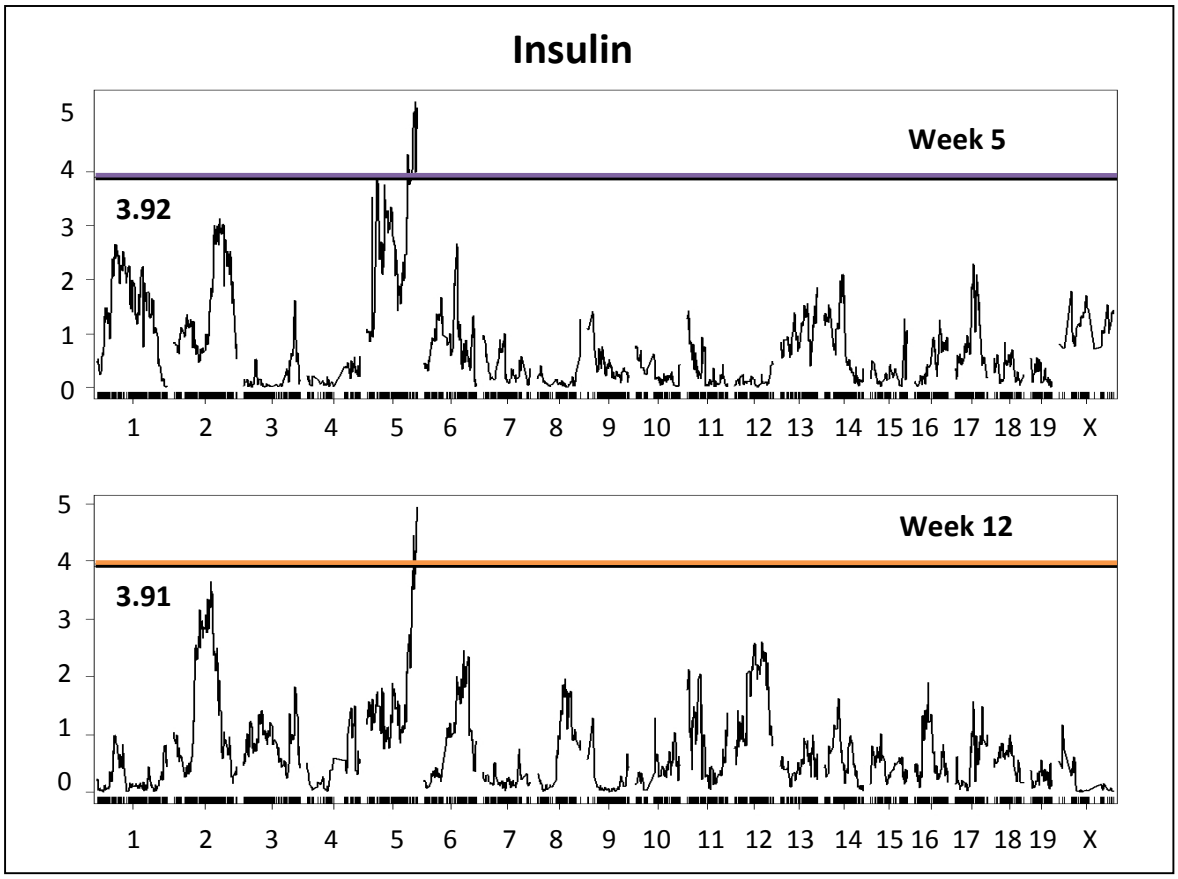
	Liver NMR Fat%	Body NMR Fat%	Plasma Glucose mg/dl	Insulin
Liver NMR Fat%	1	0.1300494	0.1668865	0.1404534
Body NMR Fat%	0.1087247	1	0.3813249	0.1074075
Plasma Glucose mg/dl	-0.265212	0.0711061	1	-0.107665
Insulin	0.2808103	-0.134466	-0.588863	1

W12 W5

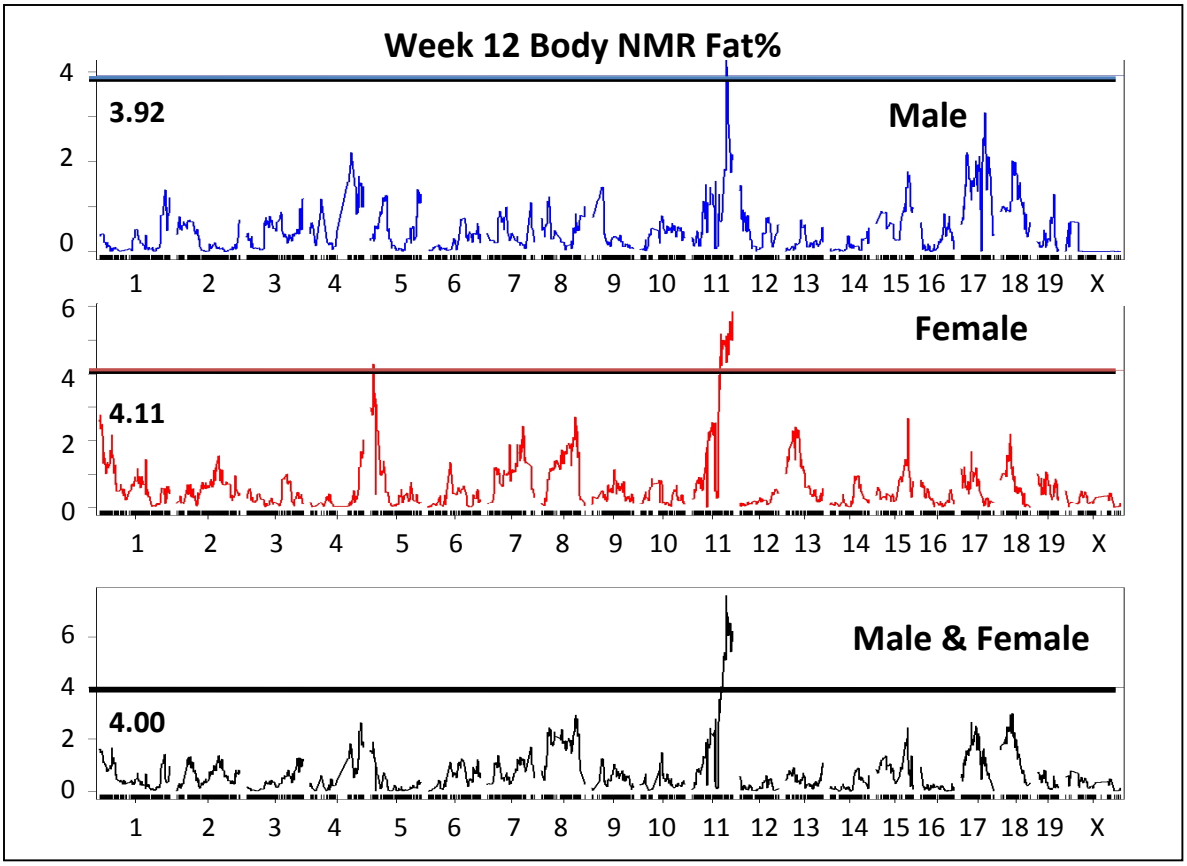
Interestingly, in 12 week animals (shown in red), the correlation of plasma glucose with percent body fat is largely lost, presumably reflecting the loss of bodyweight experienced in the more severely diabetic animals. However among these same 12 week animals, fat deposition in the liver shows a strong positive correlation with plasma insulin and equally significant negative correlation with plasma glucose levels. This is striking given the reverse situation in humans where fatty liver is positively associated with diabetes. However, this same inverse relationship is seen between C57BL/6 and C57BLKS where obese (db/db) animals with high fat content in the liver (C57BL/6) are more diabetes resistant than are animals with low liver fat (C57BLKS). A similar phenomenon is observed between diabetes susceptible BTBR-ob/ob animals and diabetes resistant C57BL/6-ob/ob mice. The mechanism for this inverse relationship is not understood.

As shown in the figures below, there is a single QTL for plasma insulin levels on distal chromosome 5 that reaches genome wide significance as determined by 1000 permutations of the data. A weaker QTL for plasma glucose levels is seen at the same location in 5 week animals but is not apparent in 12 week animals (data not shown).

Interestingly, while percent body fat shows a weak but significant correlation with insulin levels, the two traits map to separate genomic locations (Percent body fat by NMR maps to chromosome 11, see below.) Thus, while obesity is associated with diabetes-susceptibility, the fact that the traits map to separate locations is consistent with the idea that obesity does not directly drive insulin resistance.

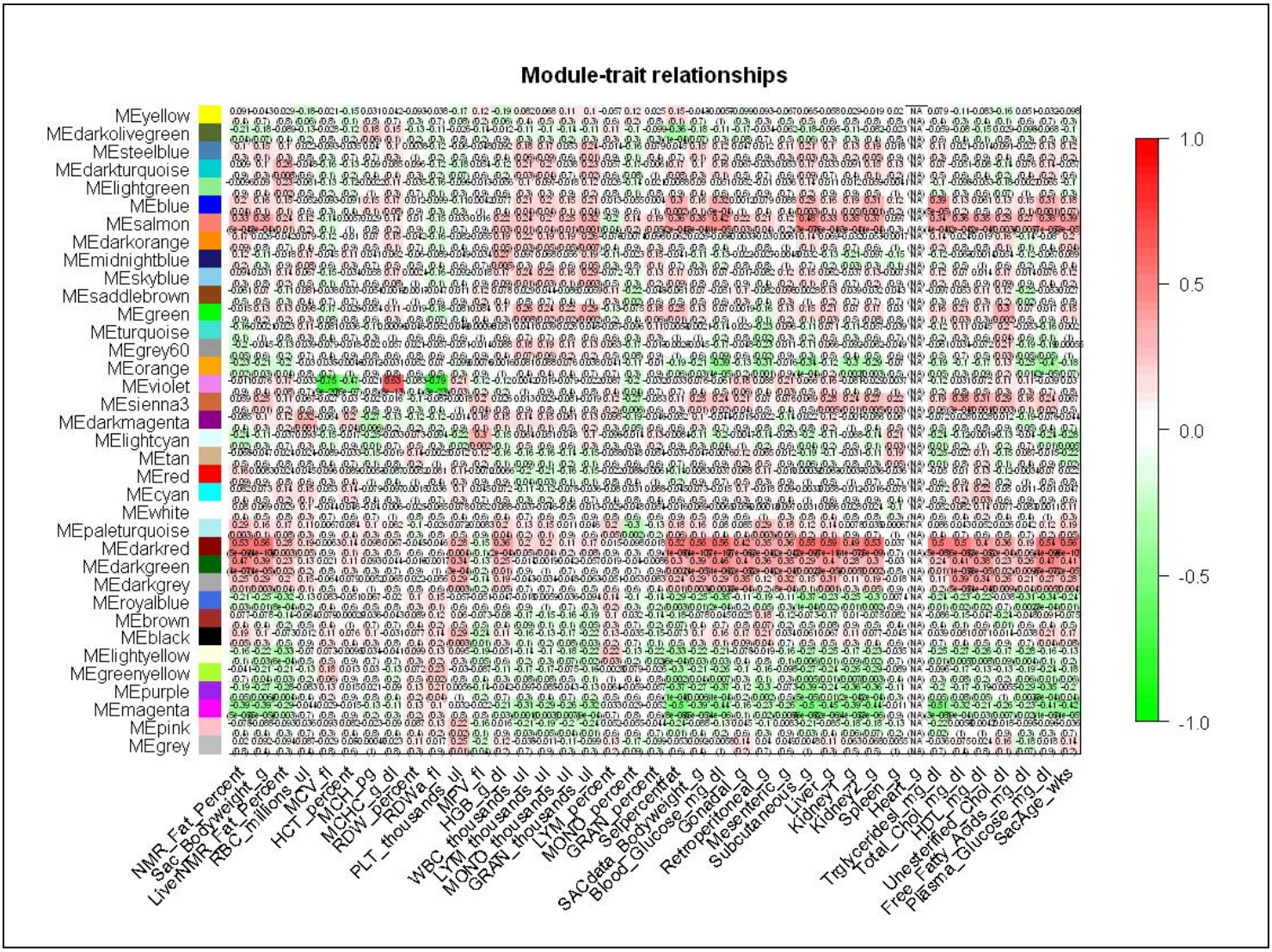


Insulin QTL on distal chromosome 5. The LOD score for genome wide significance (shown as a color bar at LOD ~3.9) was determined by analyzing the maximum LOD scores obtained for the insulin trait in repeated (1000X) permutations of the data.



Body fat QTL on distal chromosome 11. The LOD score for genome wide significance (shown as a color bar at LOD ~4) was determined by analyzing the maximum LOD scores obtained for the percent body fat in repeated (1000X) permutations of the data.

Gene expression analysis in the BXD F2 db/db cross. We are now analyzing gene expression data in several tissues from BXD F2 db/db animals using data from Affymetrics micro-arrays. After normalization of expression data and correction for background, we select the subset of genes that show significant variation in expression among the set of animals. In the example shown below for pancreatic islet-RNA, this constitutes a core set of about 7400 significantly varying genes. This expression data associated with these genes is used to carry out weighted gene co-expression network analysis (WGCNA) to identify sets of genes whose expression is highly correlated.

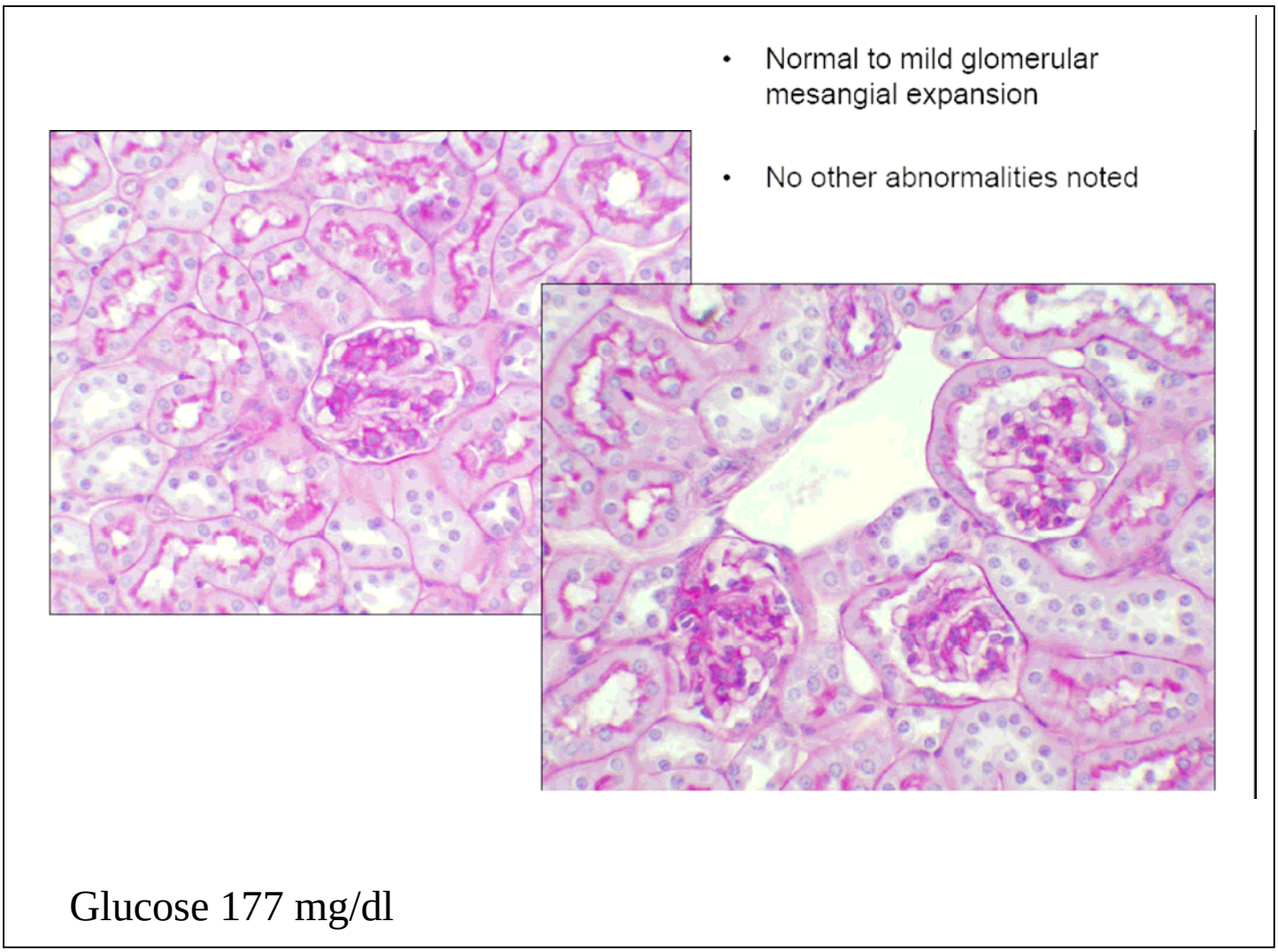


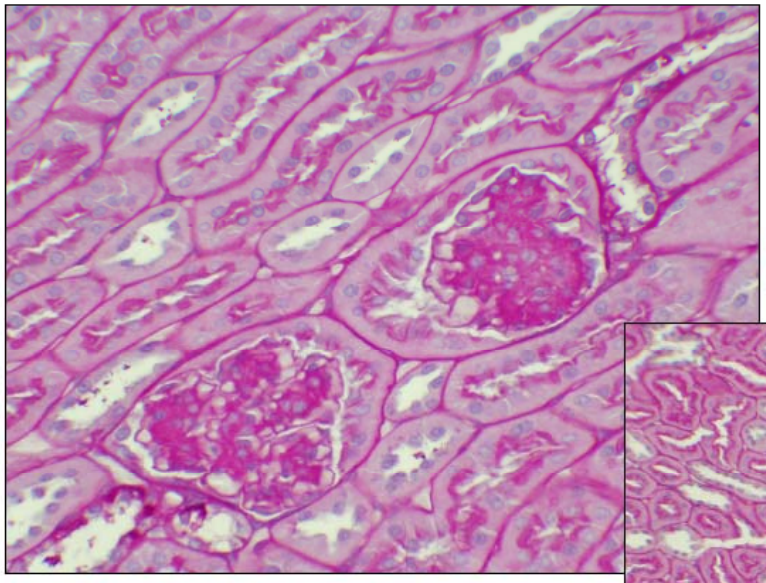
WGCNA results for pancreatic islet RNA from BXD F2 db/db animals. WGCNA identified 36 modules (sub-networks) in among the 7400 genes with significant variation in expression. The modules are identified by arbitrary color names and range in size from 21 genes to 1437 genes with a median size of 90 genes. In the table above, the red and green colorations indicate correlation between gene expression in the module and the phenotypic traits listed across the bottom. For instance the magenta module (173 genes near the bottom of the table) shows moderate negative correlation (medium dark green) with NMR-fat percent, Sac-bodyweight, Liver NMR fat percent as well blood glucose levels, liver and kidney weights and many of the plasma lipids. The darkred module shows moderately strong positive correlation with many of the same traits. In several cases, adjacent modules show similar but weaker correlations, as expected since adjacent modules have relatively high correlation in their expression profiles, whereas more distal modules are generally less

correlated. A similar analysis will be completed for each tissue where we have measured expression (Liver, adipose, islets, intestine) and we will use gene set enrichment analysis (GSEA) to identify specific metabolic pathways that are associated with the diabetes and complication related phenotypes observed in the BXD F2 db/db animals.

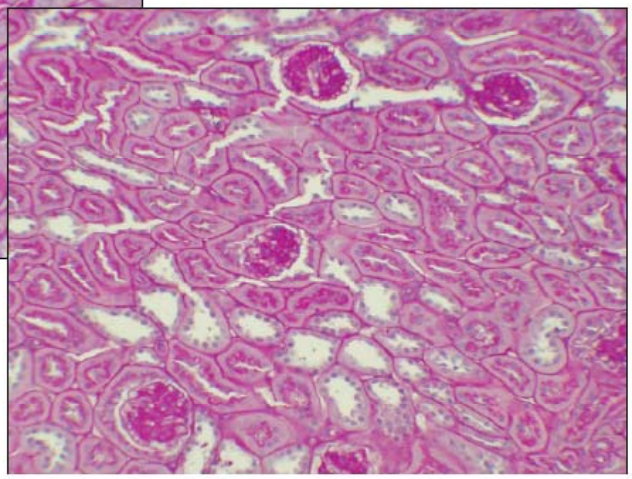
Diabetic Complications in the BXD F2 db/db cross: Under funding from an AMDCC pilot and feasibility grant we have forwarded heart and kidney tissues from a subset of these mice to Renee LeBoeuf at the University of Washington MMPC for histological analysis of traits related to diabetic cardiomyopathy and nephropathy. Our objective was to use the genotype data to identify QTLs for these complication-related phenotypes. Generally, the phenotyping effort was successful for the tissues analyzed. For example, BXD F2 db/db animals showed a wide range of nephropathy as shown in the figures below.

Nephropathy in BXD F2 db/db animals: Kidney histology in 12 week old BXD F2 db/db animals with increasing plasma glucose levels. Top: Plasma glucose 177 mg/dl. This animal with plasma glucose in the lower range of F2 animals showed little or no histological pathology. Middle: Plasma glucose 744 mg/dl. With plasma glucose in the higher ranges, increasing mesangial expansion was seen with some tubular damage. Bottom: Plasma Glucose 1307 mg/dl. In animals with the highest plasma glucose, extensive tubular damage was seen.



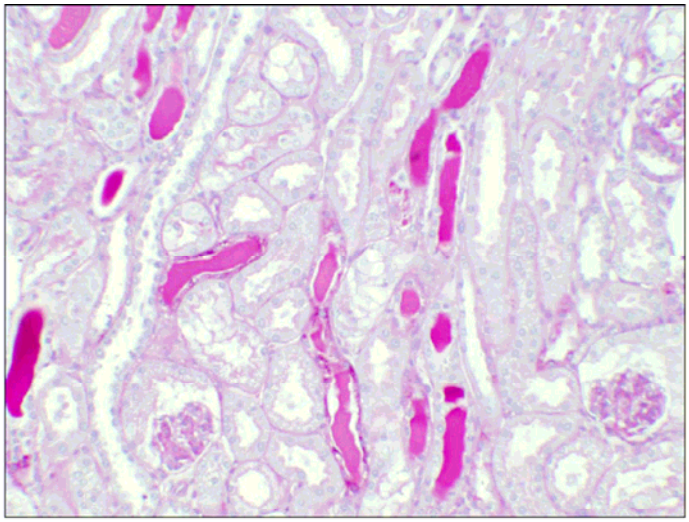


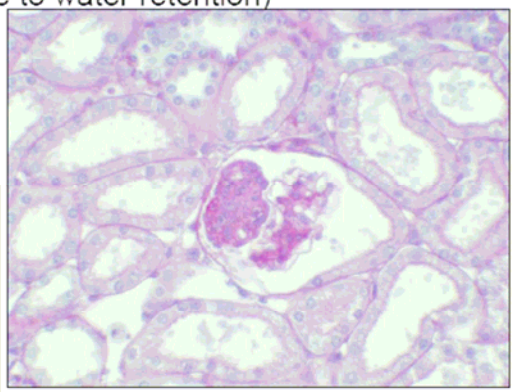
- Global, severe increase in glomerular mesangial matrix
- Areas of tubular damage

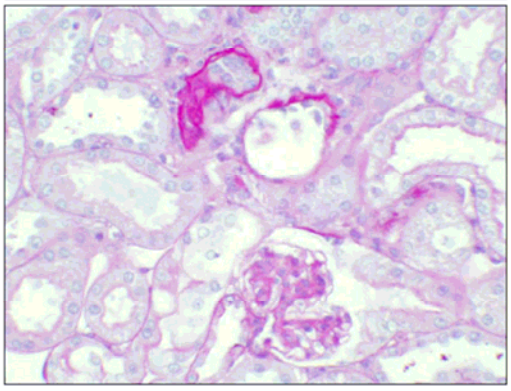


Glucose 744 mg/dl

- Moderate to severe mesangial matrix expansion
- Tubules appear somewhat dilated (possibly due to water retention)
- Prominent, PAS positive casts
- Focal thickened tubular basement membrane
- Focal tubular epithelial damage







Glucose 1307 mg/dl

Atherosclerosis was also measured in the proximal aorta of a set of BXD F2 db/db animals. Only early lesions were observed consistent with generally low plasma cholesterol in these mice. And, lesion area seemed relatively independent of plasma cholesterol but was more correlated with plasma glucose levels with no lesions present in normoglycemic animals.

BXDF2 Case	Plasma Glucose mg/dl	Total Cholesterol mg/dl	Leaflet (A) um^2 Lesion AREA	Leaftlet (B) um^2 Lesion AREA	Leaftlet (C) um^2 Lesion AREA
1223	177	213	0	2784.858	1891.24732
1171	183	196	0	0	0
1253	184	159	0	0	0
1191	368	174	3120.76	0	0
1160	661	192	4489	4974.088	3450.286
1177	744	200	3579.14226	1479.51	691.26504
1187	1307	376	0	0	0

We have now completed analysis of BUN and of glucose, albumin and creatinine in the urine. We are now using the genotype data to identify QTLs for these complication-related phenotypes.

2. Collaboration:

Many of the observations presented above derive from the ongoing work with the Seattle MMPC. In addition, we are developing collaborations here at UCLA to measure heart function through echocardiography (Dr. Yibin Wang, UCLA-Anesthesiology) and neuropathy as measured in footpad sensitivity (Dr. Nigel Maidment, Psychiatry and Behavioral Sciences). We will continue to work with Eva Feldman, as appropriate for histological assessment of neuropathy in footpads.

3. Address previous EAC comments:

Response to EAC comments:

- Dr. Davis' proposal has centered on identifying susceptibility genes and novel pathways involved in vascular complications of diabetes using 2 mouse studies: one is an intercross between B6/db and DBA/2, and the other is comparing BKS with B6. Some of this work was supported in part by an AMDCC P&F proposal which just ended. The current PI proposal follows up on this initial work using the above mouse models, and also studies the impact of 5-lipoxygenase on pancreatic function. Dr. Davis and colleagues are uniquely positioned to generate extremely important information about pathway identification involved in pathogenesis of vascular complications of Type 2 diabetes. These investigators are clearly among the world's leaders in their expertise and tools to accomplish these goals. In terms of progress, there appears to be minimal progress made on the 5LO-pancreatic project. During the last review, the committee asked them to repeat their findings, and to date, this has not been done. In terms of the genetic studies, preliminary data is beginning to emerge. However, one would have liked to have seen specifics of gene findings listed in the progress report, rather than only general comments

about ‘reduced expression of genes involved in TG synthesis’ (for example). Although Dr. Davis may wish to keep these findings somewhat confidential at this stage, it is important for the committee to know the numbers of genes that were changed, etc. One major issue with this project is the sheer volume of data generated and how to prioritize it…how many genes change and by how much do they change? What is considered to be ‘significant’ in terms of expression change? How do the investigators prioritize their findings? For example, if 100 genes related to kidney complications change, which genes and how many of the 100 get priority for subsequent study? And, if they find 100 genes changing for kidney and 100 for heart and 50 for lipids, which genes get studied first? Although we are sure this was most likely outlined in the original proposal, having an update in the progress report re: numbers of genes discovered and prioritization would be helpful. What appears to be missing in the progress report is a list of definitive findings for 2009 and the priorities that will occur from there.

RESPONSE: We agree that the sheer volume of data from micro-array profiling of gene expression in a large panel of genetically diverse animals necessitates a different approach to identifying significant disease-associated gene expression differences. When comparing two strains, one with diabetes and one without (for instance BKS db/db vs B6 db/db) we can ask about the changes in expression for specific genes in specific pathways. For example at the fall AMDCC meeting, we presented data for the reduced expression of several specific triglyceride-synthesis pathway genes. These expression differences were validated by quantitative PCR and were shown with error bars and p-values attesting to the degree and significance of the change in expression. So, it is clear that triglyceride synthesis pathway genes have reduced expression in livers of BKS db/db mice. And while this correlates with other phenotypic differences between the strains, there are at least three major limitations to this type of analysis. First, without further experimentation, it is difficult, perhaps impossible, to determine if the reduced expression of TG pathway genes is upstream (contributing to the diabetic phenotype) or downstream (resulting from the diabetes) or incidental. Secondly, there is little or no mapping information in the data. Every genotypic difference between the two inbred strains (and there are hundreds of thousands) correlates perfectly with every phenotypic difference so there is no way to assign the gene expression difference (or the phenotype differences) to a particular gene or even general chromosomal location. And third, the selection of genes for this type of analysis is necessarily based on some hypothesis. (In the case of TG synthesis genes, we were trying to account for the reduced fat accumulation seen in BKS db/db livers.) Thus, while hypotheses may be tested, no novel hypotheses are generated directly from the data.

Our approach to help solve these problems is to carry out expression analysis in a genetic cross or in a large panel of genetically diverse inbred strains that show varying degrees of diabetes and its complications. To varying degrees, this approach overcomes all the difficulties cited above in comparing two strains with differences in diabetes or its complications.
Mapping: There is mapping information for all phenotypes and expression differences that occur across the set of animals. (Whether this mapping information reaches statistical significance depends on the number of animals/strains examined, the strength of the variation among animals/strains, and the fraction of that variation that is genetic.)
Novel hypotheses: Because the comparison is carried out across many genetic backgrounds, the correlation of a diabetic phenotype with specific gene expression patterns identifies new genes and pathways associated with the trait.
Causality: There are increasingly powerful statistical methods to identify whether variation in a gene expression trait is upstream, downstream or incidental to the diabetes phenotype.

How many genes change and by how much do they change? There are many approaches to this question. Most analyses begin with some arbitrary selection cut-off. After background correction and normalization of expression values, some investigators pick all genes showing a specific fold change or greater. Or, pick the 5000 genes showing the greatest variation across the set of animals or strains. Or, pick all the genes whose

variation among mice or strains is statistically higher than the variation within strains. The objective of all these approaches is to select biologically significant differences in gene expression and to eliminate expression variation due to technical or environmental factors. None of these are perfect. However, the major analytical step is to identify clusters of genes whose expression is similarly regulated and to determine correlation of that group expression profile with the various diabetic or complication phenotypes. Within each expression cluster, there can be many differences in the magnitude of the change but the point is to link the expression pattern to the phenotypic pattern.

What is considered to be 'significant' in terms of expression change? Typically, we would not look for a specific magnitude of expression change to establish significance. Rather, the significance is in the tightness of the correlation between gene expression and a phenotype of interest or between gene-expression changes within a cluster of genes that are co-expressed.

How do the investigators prioritize their findings? Among the clusters or sub-networks of genes that correlate with complications, priority goes to those gene expression clusters that map to the same genetic location as is seen for the phenotype. This suggests that the same genetic variation is responsible for driving the observed differences in phenotype as well as the expression variation. And among the genes whose expression co-maps with the phenotype, special focus is given to genes whose physical location coincides with the QTL. This suggests that a sequence variation in that gene drives the observed variation in expression and underlies the phenotypic variation.

- Good progress with identification of QTLs. We believe Dr. Davis plans to identify specific genes by mapping eQTLs. Are any of the mouse DM QTLs ortholgous to "interesting/suggestive" loci in human DM association metaanalysis? The question being: is this approach informative for human disease

RESPONSE: As we presented at the Fall AMDCC meeting, many of the QTL overlap diabetes-related QTLs observed in humans or in other mouse crosses.

- Further characterization of the strain differences between C57BLKS and C57BL/6 mice. The B6 mice have increases in insulin over time compared to the BKS. They are using an Affy5k mouse SNP panel (with ~2500 informative SNPs between the two strains) to identify QTLs for phenotypic differences in F2 mice. There may be QTL for insulin on Ch5 and maybe 2. In addition, there may be SNPs in similar locations to explain the large variability of glucose levels. As previously mentioned, accurate assessment of phenotype is paramount for these correlations to be made. Thus, accuracy of phenotype evaluation must be ensured with repeated quality assurance measures taken.

RESPONSE: We agree and further note that mapping studies in the F2 cross are particularly vulnerable to this criticism because each animal is genetically unique. QTL mapping using the HMDP has both higher mapping resolution and the advantage that adequate numbers of animals for each strain may be bred to carry out multiple assessments of insulin resistance with an appropriate n for each assessment.

- The breeding of 5-LO Tg and KO with apoE(-/-) should provide interesting insight into diabetic vascular disease especially given the recent evidence that 5-LO activity is enhanced in diabetes.

RESPONSE: Breeding difficulties in our attempts to combine 5-LO, ApoE and db/db have caused us to hold up on this particular approach. We plan to evaluate 5-LO expression and its association with atherosclerosis in the Akita F1 panel. Depending on results, we may further assess atherosclerosis in Akita 5-LO tg animals.

- Data regarding the differential gene expression in the liver between BLKS and BL/6 mice are interesting and could contribute to the diabetes predisposition. Euglycemic clamp studies are described that intimate that hepatic insulin resistance precedes frank diabetes. Studies related to skeletal muscle will potentially be more informative as this tissue is the major contributor to whole-body insulin sensitivity.

RESPONSE: We agree that muscle studies are very interesting and the clamp studies shed some light on this issue. We have collected muscle tissue from the BXD db/db cross and are seeking funding for gene expression profiling and network analysis in this tissue.

- The collaborations with the U. of Michigan as well as other investigators are added benefits to this effort. It will be interesting to hear of any updates from these projects next year.

RESPONSE: In the context of screening large numbers of animals such as those collected for the BXD db/db cross or in the panel of Akita F1 animals, it has become apparent that we need to restrict our efforts to phenotypes that we can complete quickly within our laboratory or those that may be assessed in tissues that may be quickly stabilized for later analysis either by us or by collaborating laboratories. In the particular case of neuropathy in footpads, it appears that we were over ambitious in collecting tissues from the BXD db/db cross; footpad-histology requires a more complex fixation process than other tissues with which we are familiar and, the histological assessment should be carried out in a relatively narrow time-frame following fixation. We may still obtain histological insights from these animals but we have modified our approach for the Akita F1 animals. For B6 Akita diabetic mice, we have measured increases in times for foot withdrawal from a calibrated heat source and we are assessing our ability to carry out this measure in the full panel of mice in the week preceding euthanasia. One of the major advantages of the HMDP is that replicate mice may be generated for more complex phenotyping such as nerve fiber lengths in footpads. Our current efforts are focused on nephropathy and cardiovasucular phenotypes with only the simplest assays for other complications, e.g. bladder weight for uropathy.

- Dr. Davis' P&F proposal has centered on identifying susceptibility genes and novel pathways involved in vascular complications of diabetes using 2 mouse studies: one is an intercross between B6/db and DBA/2, and the other is comparing BKS with B6. It appears that the bulk of the proposed work has been completed by the investigator; however, final data analysis is in progress. The SNP analysis has been performed and the kidney analysis (with regard to phenotype analysis) has been performed. There is also a technical issue with the histology of the heart sections from the mice; these are still underway. The links between the two in terms of identifying genes and pathways important in kidney or heart vascular complications have not been completed. However, Dr. Davis is also a PI in the AMDCC, so this work is continuing as part of this new PI proposal. In sum, this was an ambitious undertaking that has led to the generation of new data related to vascular complications of diabetes. Data analysis is still ongoing, and will be continued through the PI's other AMDCC award.

RESPONSE: Correct

- Reasonable progress has been made on the P&F. Genotyping of F2 crosses down and several QTLs identified. Genome-wide expression analysis for eQTLs is process. Kidney histology shows glomerulosclerosis but minimal tubulointerstitial disease. No vascular leasions shown. The investigators have accomplished their goal to generate a F2 line between diabetic B6 and DBA/2 strains with a range of phenotypes. Use of eQTLs to identify candidate complication genes should be interesting. Renal histology similar to other rodent models for DN.

RESPONSE: Several groups have attempted to profile whole kidney RNA expression in mouse crosses with less than satisfactory results, perhaps because of the cellular heterogeneity of the kidney. Glomerular specific expression, in particular, has been difficult to detect in whole kidney RNA preparations. For this reason, we have redirected our expression profiling and network analysis to glomerular RNA from Akita F1 mice with parallel kidney histology being carried out at the Seattle MMPC.

- The P&F focuses on gene network-pathway-candidate genes for susceptibility for diabetes. It appears considerable progress has been made in terms of gene expression and network analysis. As mentioned for the overall project, the phenotyping of the traits is extremely important and should be documented with great precision. In particular, there is some concern regarding the lack of cardiac phenotyping available. Please give more detail as to the initial problems with the phenotyping effort and what plans are being made, with and without the Seattle MMPC to derive useful data beyond gene expression.

RESPONSE: As with other areas of phenotyping, we want to develop cardiac phenotypes that may be used in screening large numbers of animals such as those in a genetic cross or in a comprehensive strain survey. For these initial cardiac phenotype screens, we have been focusing on the following: For atherosclerosis we have been measuring early lesions in the proximal aorta and plasma levels of cholesterol and glucose. In the BXD db/db cross and in Akita F1 hybrids, we do not generally see large elevations in plasma cholesterol. And in the case of the BXD db/db cross, lesion areas, while low, seem more correlated with plasma glucose than with plasma cholesterol. This gives us hope for better mapping power in the Akita F1 project where hyperglycemia among strains is relatively consistent. Thus, we expect that, when the full F1 panel is complete, some QTL for lesions will correlate with diabetes-induced changes in plasma cholesterol while others will be driven by pathways independent of factors determining cholesterol levels, for instance alternative inflammatory pathways could be associated. For cardiomyopathy, we have several basic measures. In both the BXD db/db cross and Akita F1 panels, we have heart weight and tibia-length as measures of hypertrophy. While recently published results for the B6 Akita mouse show no hypertrophy, we anticipate that it may develop in some genetic backgrounds. With ultrasound, while we see the same diastolic dysfunction as published for B6 Akita males, we do not currently plan ultrasound as a feasible high-throughput screening tool. Instead, we have been focusing on lipid accumulation in the heart and levels of B-type naturitic peptide, both of which show strong and significant elevation in the B6 Akita. Both these assays are being carried out collaboratively with the MMPC.

4. Publications:

Davis, R. C., Castellani, L. W., Hosseini, M., Ben Zeev, O., Mao, H. Z., Weinstein, M. M., Jung, D. Y., Jun, J. Y., Kim, J. K., Lusis, A. J., and Peterfy, M. (2010) Early hepatic insulin resistance precedes the onset of diabetes in obese C57BLKS-db/db mice. Diabetes PMID: 20393148

Richard C. Davis, Yi Zhao, Zhiqiang Zhou, Pingzi Wen, Suzanne Yu, Hongxiu Qi, Melenie Rosales, Eric E. Schadt, Miklos Peterfy, Aldons J. Lusis (2010) Genetics of Obesity-Induced Diabetes in a Cross Between C57BL/6 and DBA/2. Submitted