Animal Models of Diabetic Complications Consortium
(**U01** HL087947)

Annual Report
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Modeling Diabetic Cardiomyopathy and Microangiopathy in the Mouse

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Part A:

Principal Investigator's Summary

1. Program Accomplishments:

The University of Utah's participation in the Animal Models of Diabetes Complications Consortium proposed the generation of two mouse models. Model –1: *Modeling the role of insulin resistance, lipotoxicity and oxidative stress in the pathogenesis of diabetic cardiomyopathy* - ***CIRKO-ACS-sod2$^{+/-}$***
Model –2: *Modeling the role of impaired angiogenesis/arteriogenesis in the pathogenesis of microvascular complications of diabetes and to model the potential utility of increasing angiogenic potential as a strategy for preventing or reversing microvascular complications of diabetes.* – ***Inducible Netrin-Akita***

In addition we proposed hypothesis driven aims for both of these models.

MODEL 1: CIRKO-ACS-sod2$^{+/-}$.
The overall hypothesis that will be evaluated by this model is: *Diabetic cardiomyopathy is characterized by impaired myocardial insulin signaling, lipotoxicity and oxidative stress.* The proposed studies will test the following specific hypotheses:

1. The CIRKO-ACS-sod2$^{+/-}$ will meet the validation criteria for diabetic cardiomyopathy in terms of decreased contractile function, increased intramyocellular lipid and increased myocyte loss and fibrosis.
2. CIRKO-ACS-sod2$^{+/-}$ will exhibit increased rates of FA oxidation, decreased rates of glucose oxidation, increased MVO_2 and decreased cardiac efficiency.
3. The mechanism responsible for impaired myocardial function and substrate utilization in CIRKO-ACS-sod2$^{+/-}$ mice will be mitochondrial uncoupling on the basis of increased FA-mediated superoxide generation, leading to impaired mitochondrial energetics.
4. CIRKO-ACS-sod2$^{+/-}$ will develop rapid functional deterioration following hemodynamic stress such as pressure overload hypertrophy.

MODEL 2: Inducible-Netrin-Akita (*Tam-b-actinCRE.ROSA26* $^{netrin1/lacZ}$*.ins2*$^{+/C96Y}$).

The overall hypothesis that will be tested in this model is: *Impaired adaptive angiogenesis and arteriogenesis contributes to impaired myocardial remodeling following coronary ischemia, and to increased limb loss following femoral artery occlusion in diabetes.* These studies will utilize the inducible-netrin-akita mouse and take advantage of our ability to upregulate netrin expression in a temporal fashion by inducible activation of cre-recombinase following treatment of mice with tamoxifen. If inducible cardiomyocyte-restricted Cre-Netrin Akita mice are also developed, we can additionally determine if this approach will hold true in an organ-restricted manner as well. The studies proposed in this aim will initially determine the fidelity of the temporal (tamoxifen-inducible) gene expression system in inducible-netrin-akita mice. Based on preliminary data that we have obtained with the tamoxifen-regulated MHC Cre mouse (MCM-MHC) we are confident that we will be able to increase netrin expression in cardiomyocytes of netrin-Akita mice, and deem it likely that more widespread netrin activation will be obtained with the inducible beta-actin driven tamoxifen cre transgenic (*Tam-b-actinCRE.ROSA26* $^{netrin1/lacZ}$*.ins2*$^{+/C96Y}$).

The following hypotheses will be tested:

1. Tamoxifen treatment of *Tam-b-actinCRE.ROSA26* $^{netrin1/lacZ}$*.ins2*$^{+/C96Y}$ mice will increase netrin1 expression ubiquitously, including cardiomyocytes and skeletal muscle. Tamoxifen

treatment of *MCM-MHC.ROSA26* $^{netrin1/lacZ}$*.ins2*$^{+/C96Y}$ (if generated) will increase netrin expression in cardiomyocytes only.

2. Diabetic animals will exhibit accelerated myocardial remodeling following coronary artery occlusion and relative to control animals and the promotion of angiogenesis and arteriogenesis by netrin1 will reverse this phenotype
3. Diabetic animals will exhibit reduced recovery of hind-limb perfusion following femoral artery ligation relative to non-diabetic animals and the promotion of angiogenesis and arteriogenesis by netrin1 will reverse this phenotype

Recent Progress and Major Accomplishments

The **Inducible-Netrin-Akita (*Tam-b-actinCRE.ROSA26* $^{netrin1/lacZ}$*.ins2*$^{+/C96Y}$)**, was the model that the consortium chose to develop and initially characterize at the Jackson Laboratories. As this model was in an earlier stage of development we continued to characterize the ACS over-expressing mouse while awaiting production of the inducible Netrin –Akita. The first part of this report will summarize our findings to date in the **CIRKO-ACS-sod2**$^{+/-}$ project (Model 1) and the second part will summarize progress with the Netrin-Akita model (Model 2).

Model 1:

The **CIRKO-ACS-sod2**$^{+/-}$ originates from three component mice that will be used to generate a compound transgenic/gene targeted model. The respective components are (1) Cardiomyocyte Insulin receptor KO mice (CIRKO), (2) Cardiomyocyte-restricted low-level overexpression of Acyl CoA Synthase (MHC-ACS1), and (3) Germline heterozygous null mice for the mitochondrial superoxide dismutase (sod2$^{+/-}$). CIRKO mice were extensively characterized in the first round of the consortium and a number of publications have already been derived from this model (1-7). We have characterized MHC-ACS mice and the offspring of the CIRKO-ACS cross, which will be summarized in this report.

MHC-ACS: Palmitate uptake was determined in vivo by PET scanning and is increased in the hearts of these mice (**Figure 1**).

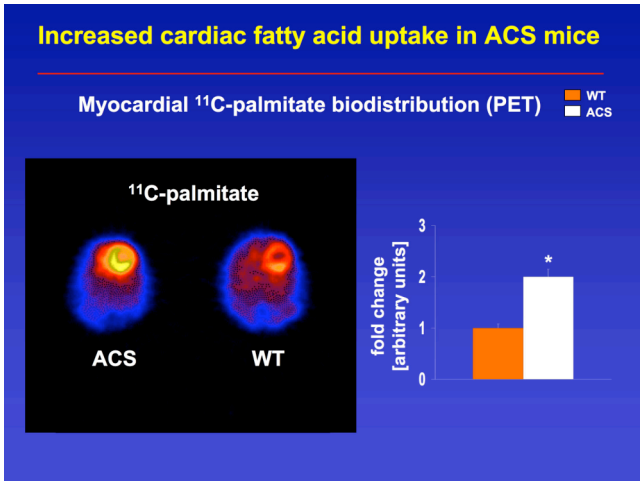


Figure 1: In vivo myocardial FA uptake in MHC-ACS mice as determined by ^{11}C-Palmitate PET scanning.

Unexpectedly, the increased lipid uptake was not directed towards increased FA oxidation. Instead, there was an accumulation of ceramide and diacylglycerol. These mice developed a striking change in mitochondrial morphology characterized by reduced mitochondrial size despite a clear biogenic response (**Figure 2**). The mitochondrial adaptations appear to be independent of changes in PPAR-alpha and PGC-1 alpha mediated signaling, which contrasts with observations in mouse models of type 1 and type 2 diabetes. Of interest mitochondrial dynamics are perturbed as evidenced by decreased mitochondrial localization of the regulator of mitochondrial fission Drp1. The altered mitochondria are associated with increased generation of superoxide, and there is an age-dependent decline in mitochondrial function as determined in permeabilized cardiac fibers. We also evaluated the mitochondrial cardiolipin pool. In control mice, the predominant FA species in cardiolipin are 18:2. In the MHC-ACS mice there is remodeling of the cardiolipin pool leading to an increase in 16:0 and 22:6 species. Despite these dramatic changes, cardiac function in vivo was only modestly impacted. Of relevance to the CIRKO-ACS project, insulin sensitivity was preserved in the hearts of MHC-ACS mice (**Figure 3**).

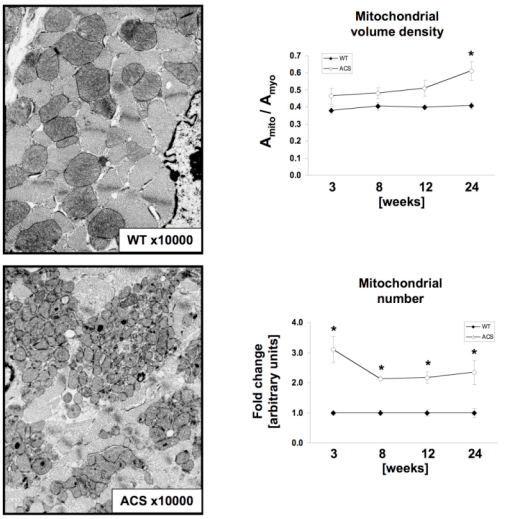


Figure 2: Mitochondrial morphology and volume density in MHC-ACS mice at ages as shown. * p<0.05 vs. controls.

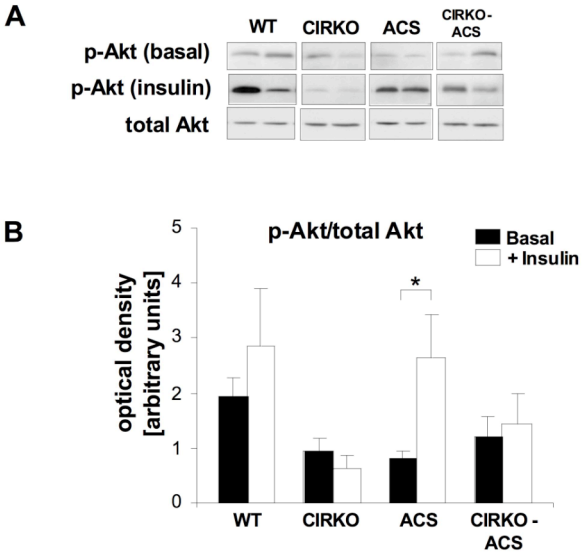


Figure 3: Basal and Insulin-stimulated Akt-phosphorylation in hearts from wildtype (WT), CIRKO, ACS and CIRKO-ACS mice. * p<0.05 versus basal.

CIRKO-ACS: We generated these mice initially on a mixed background and have backcrossed them 6-generations to C57BL6 and are currently on the 7th generation. In the interim we phenotyped mice on the mixed background. Loss of insulin signaling in MHC-ACS mice accelerated the age-dependent decline in mitochondrial dysfunction. Mitochondrial morphology and ROS overproduction were similar to findings observed in the MHC-ACS mice. Cardiac function was not substantially worsened relative to MHC-ACS. FAO gene expression was reduced, but there were deficiencies noted in the content of cytochrome C subunits. Taken together, the CIRKO-ACS mouse recapitulates mitochondrial dysfunction and oxidative stress but not increased myocardial FA utilization that seems to be driven primarily via activation of PPAR-alpha. We therefore hypothesize that increased lipid uptake via ACS1 directs lipid towards ceramide and DAG and leads to a remodeling of the mitochondrial membrane. We believe that these studies have identified one specific mechanism that may lead to lipotoxic cardiac dysfunction in diabetes. We would also predict that these mice would be sensitized to the effect of insulin-deficiency or high-fat feeding. We will be obtaining sod2$^{+/-}$ mice from the Cleveland Clinic group of the consortium, and will proceed to generate CIRKO-ACS-sod2$^{+/-}$ mice, which would be expected to demonstrate increased susceptibility to oxidative stress.

Model 2:

This model represents the approved AMDCC model to be generated at the Jackson Laboratories. We developed the targeting vector, successfully targeted embryonic stem cells and obtained chimeric mice. Germline transmission has now been verified. Heterozyous *ROSA26* $^{netrin1/lacZ}$ mice were recently transferred to the Jackson Laboratories where they will initially be backcrossed to the C57BL6 background and then to the Akita strain to generate *ROSA26* $^{netrin1/lacZ}$*.ins2*$^{+/C96Y}$. Once these mice are generated, they will be crossed with a tamoxifen-inducible cre that is driven by the beta-actin promoter. One of the goals of this model is to determine if inducible activation of netrin1 will reverse diabetes-associated impairment in angiogenesis following hind-limb ischemia and to determine if it will reverse of prevent diabetic neuropathy. We therefore conducted pilot experiments in which recombinant netrin1 was administered to the hindlimbs of outbred STZ-diabetic Swiss

Webster mice. These animals develop neuropathy after >4-months of diabetes as evidenced by decreased sural and sciatic nerve conduction velocities and increased hind-paw latencies **(Figure 4)**. Treatment of these mice with netrin, normalized hind-paw latencies **(Figure 5)**. These encouraging results suggest that inducible overexpression of netrin1 in the *ROSA26*$^{\text{netrin1/lacZ}}$*.ins2*$^{+/C96Y}$ mice should limit the progression of diabetic neuropathy.

Figure 4: Phenotyping of neuropathy in STZ-diabetic Swiss-Webster mice. Parameters measured were sural nerve, and sciatic nerve (SMNCV) conduction velocity; hind paw latency (HP) and tail flick (TF)

*p<0.05 vs. other groups

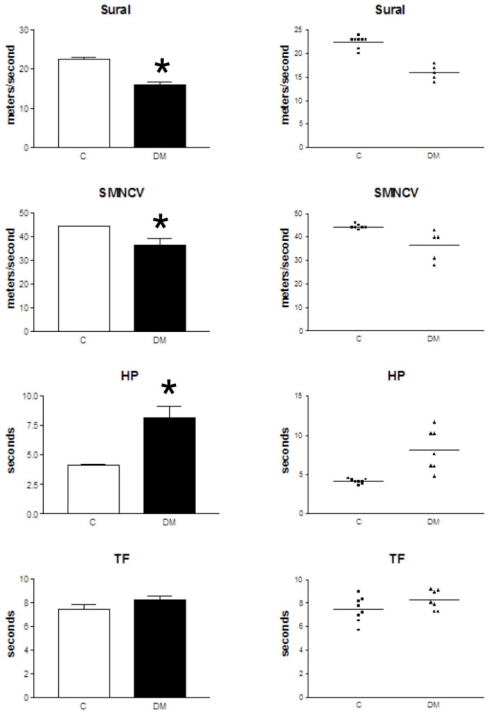
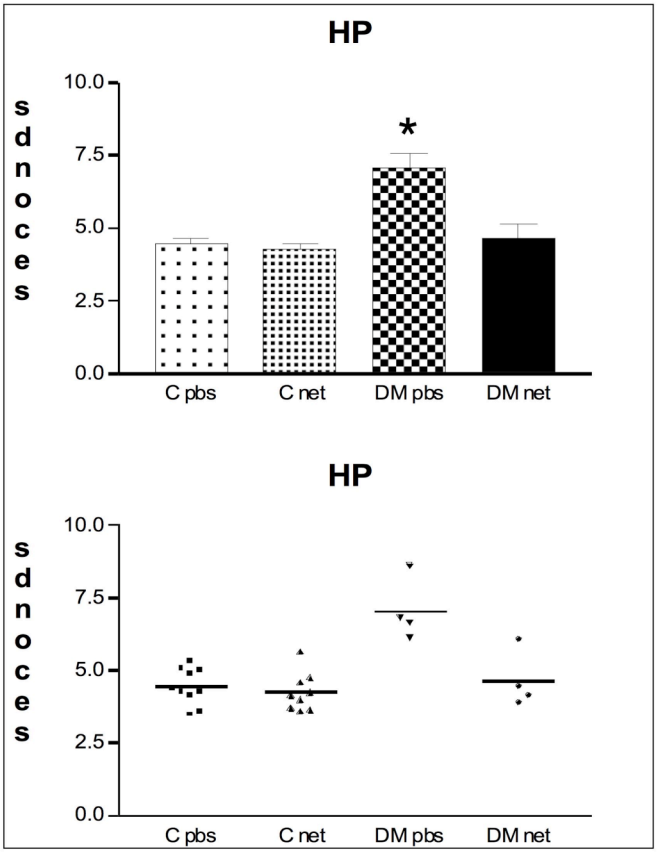


Figure 5: Normalization of hindpaw latency in netrin (net) versus vehicle (pbs) treated diabetic CD-1 mice.

*p<0.05 vs. other groups



Plans for the Upcoming Year

1. Complete the backcross MHC-ACS and CIRKO-ACS mice to the C57BL6 background.
2. Introduce sod2 heterozygous allele into the CIRKO-ACS mice on the C57BL6 background
3. Backcross the ***ROSA26*** $^{netrin1/lacZ}$ to the Akita background and introduce the inducible cre transgene.

Preliminary Milestones for 2009 and Beyond

1. Phenotypically characterize The CIRKO-ACS-sod2$^{+/-}$ as outlined in the specific aims.
2. Complete the generation of Tam-b-actinCRE.ROSA26 $^{netrin1/lacZ}$.ins2$^{+/C96Y}$ and phenotypically characterize as outlined in the specific aims.

2. Collaboration:

Within AMDCC

1. We have an active collaboration with Ira Goldberg, where we are characterizing substrate metabolism in the hearts of a mouse model of lipotoxic cardiomyopathy (mice with cardiomyocyte overexpression of a GPI-anchored lipoprotein lipase).

2. We transferred alpha- MHC Cre mice to Tom Coffman's Laboratory for the purpose of generating cardiomyocyte –restricted KO of type 1 angiotensin receptors.

3. We are collaborating with Eva Feldman's laboratory for neuropathy phenotyping of netrin-treated mice.

4. Isolated cardiomyocyte mRNA was sent to Moshe Levi for the determination of FXR gene expression.

With Jax

ROSA26 $^{netrin1/lacZ}$ mice have now been transferred to JAX and are being backcrossed to the C57BL6 and Akita strains.

With the MMPCs

We have been working with Craig Molloy at the UTSW MMPC to develop heart perfusions that use both ^{13}-C isotopomers, as well as ^{3}-H and ^{14}-C tracers to determine myocardial metabolism in isolated working mouse hearts. The goal of these studies is to compare tracer isotope and NMR – based methods, which provide complementary data on cardiac metabolic substrate utilization.

With other non-AMDCC PIs

Determining the role of insulin resistance in the potentially protective cardiac effects of isocaloric diets, which are rich in saturated fatty acids when given to mice with pressure overload hypertrophy. This hypothesis is being tested by William Stanley at the University of Maryland to whom we have sent mice with cardiomyocyte-restricted KO of insulin receptors (CIRKO).

Characterization of the response of PGC-1β deficient hearts to pressure overload hypertrophy (in collaboration with Antonio Vidal Puig at the University of Cambridge).

Determination of mitochondrial function in PGC-1α deficient hearts. (Collaboration with Daniel P Kelly Washington University School of Medicine)

Determination of the impact of loss of the small molecular weight heat shock chaperones (CRYAB and HSPB2) on cardiac mitochondrial function. (Collaboration with Ivor J. Benjamin, University of Utah School of Medicine).

3. **Address previous EAC comments:**

1. Abel

    a. *What is the current status of your model? When will it be shipped to JAX?*

As summarized above, we obtained germline transmission and heterozygous ROSA26 $^{netrin1/lacZ}$ mice have now been shipped to JAX.

    b. *Cardiomyopathy validation criteria appear to need some fine-tuning. The in vivo phenotyping is being performed by echo. The investigators appear to be getting all possible information from this modality (CO, EF, FS, HR, IVSd, LVDd, LVDs, LVPWd). While the parameters being assessed are appropriate, the parameters that are considered "dysfunctional" should be published so that direct (as opposed to relative) comparisons can be made (i.e., what is considered cardiac dysfunction and heart failure in a mouse). For the volume measurements, how many measurements are taken? Also, in vivo instrumentation should be considered as well in order to derive pressure measurements, dP/dt, and potentially oxygen content.*

We agree with this assessment and will discuss fine-tuning validation criteria at the steering committee meeting in Baltimore.

    c. *Interesting finding regarding the MHC-ACS mice (normal function in vivo but isolated perfused hearts indicated there is indeed contractile dysfunction). This in association with increased accumulation of TGs and age-related mitochondrial dysfunction. Mitochondria demonstrated increased ROS production, providing further rationale for limiting endogenous antioxidant systems (ACS w/ SOD2+/-). It will be important to determine the insulin sensitivity of these hearts.*

Remarkably, MHC-ACS mice are insulin sensitive, which provides additional justification for crossing with the CIRKO model.

    d. *The phenotyping data in the Akita highlights important potential differences between type1 and type2 DM.*

We agree. The revised manuscript describing these differences has been re-submitted to the journal Diabetes.

    e. *Development of the hind limb ischemia model is a good addition to the AMDCC phenotyping effort. Dr. Abel is encouraged to collaborate with Dr. Annex to compare/contrast models.*

We agree that this will be important, particularly as we move forward with the inducible Netrin-Akita model.

4. Publications:

Original Reports

1. Boudina S, Sena S, Theobald H, Sheng X, Wright JJ, Hu XX, Aziz S, Johnson JI, Bugger H, Zaha VG, **Abel ED**. Mitochondrial energetics in the heart in obesity related diabetes: Direct evidence for increased uncoupled respiration and activation of uncoupling proteins. 2007: *Diabetes* 56(10):2457-66.
2. Sena S, Rasmussen IR, Wende AR, McQueen AP, Theobald HA, Wilde N, Pereira RO, Litwin SE, Berger JP, **Abel ED** Cardiac Hypertrophy Caused by Peroxisome Proliferator Activated Receptor-Gamma Agonist Treatment Occurs Independently of Changes in Myocardial Insulin Signaling. 2007: *Endocrinology* 148:6047-53
3. Benjamin IJ, Guo Y, Srinivasan S, Boudina S, Taylor R, Rajasekaran NS, Gottlieb RA, Wawrousek E, **Abel ED,** Bolli R CRYAB and HSPB2 deficiency alters cardiac metabolism and paradoxically confers protection against myocardial ischemia in aging mice. *2007 Am J Physiol Heart Circ Physiol* 293(5): H3201-3209.
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5. Bray MS, Shaw CA, Moore MW, Garcia RA, Zanquetta MM, Durgan DJ, Jeong WJ, Tsai JY, Bugger H, Zhang D, Rohrwasser A, Rennison JH, Dyck JR, Litwin SE, Hardin PE, Chow CW, Chandler MP, **Abel ED**, Young ME. Disruption of the circadian clock within the cardiomyocyte influences myocardial contractile function, metabolism and gene expression. 2008: *Am J Physiol Heart Circ Physiol.* 294(2): H1036-47
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8. Lehman JJ, Boudina S, Banke NH, Sambandam N Phd, Han X, Young DM, Leone TC, Gross RW, Lewandowski ED, **Abel ED**, Kelly DP. The Transcriptional Coactivator PGC-1{alpha} is Essential for Maximal and Efficient Cardiac Mitochondrial Fatty Acid Oxidation and Lipid Homeostasis. 2008: *Am J Physiol Heart Circ Physiol. In Press.*

Reviews

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2. Abel ED, Litwin SE, Sweeney GD. Cardiac remodeling in obesity. *Physiological Reviews.* 2008: *88:389-419.*

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