Animal Models of Diabetic Complications Consortium
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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.

Generation of this model continues at JAX. The first part of this report will summarize new findings made in models related to 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$^{+/-}$** model has 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 mice for the mitochondrial superoxide dismutase (sod2$^{+/-}$). Additional updates related to these models are summarized below.

CIRKO: Our studies in the past year has focused on the findings that impaired insulin action in the cardiomyocyte leads to a significant increase in myocardial autophagy. The role of autophagy in the pathogenesis of diabetic cardiomyopathy is incompletely understood. On one hand, autophagy may play an important role in the removal of damaged organelles such as mitochondria. However, on the other hand excessive degrees of autophagy could contribute to cardiomyocyte death. Therefore a goal of our studies in the remaining year will be to understand the contribution of reduced insulin signaling to autophagy in the heart and the impact of this on cardiac structure and function in diabetic cardiomyopathy. We have also completed and submitted a manuscript describing the synergistic interactions between hyperglycemia and insulin resistance when hyperglycemia was induced in CIRKO mice. The key finding in this work was a dramatic increase in myocardial oxygen consumption, indicating an important role for insulin resistance in reducing cardiac efficiency.

MHC-ACS: We have completed studies that have characterized the consequences of a chronic and persistent increase in myocardial fatty acid uptake on cardiac mitochondria. As reported last year, we showed that chronic myocardial FA uptake led to fragmentation of the mitochondrial network. We have now conducted in vitro studies that have demonstrated that this is the result of palmitate-induced ceramide generation. We anticipate submitting this manuscript for publication in the next few months.

Oxidative Stress and Diabetic Cardiomyopathy: A goal of the CIRKO-ACS-sod2+/- model was to test the hypothesis that increased myocardial oxidative stress would accelerate cardiac dysfunction. We had initially planned to cross CIRKO-ACS mice with germline heterozygous KO mice for the sod2 allele. Because of the possibility of unexpected consequences of sod2 haploinsufficiency in the entire organism, we elected to use a heart-specific strategy by generating mice with cardiomyocyte specific heterozygous deletion of sod2. Not unexpectedly, cardiac-restricted sod2 overexpression resulted in early lethality from heart failure, but the heterozygous mice are viable. Thus, we will use these mice to determine if increased mitochondrial ROS generation will accelerate diabetic cardiomyopathy. We will initially use high-fat feeding and evaluate cardiac function, substrate metabolism and oxygen consumption rates and mitochondrial function. Based on our earlier studies in *ob/ob* and *db/db* mice, where we first reported the

importance of ROS-mediated mitochondrial uncoupling in the pathogenesis of diabetic cardiomyopathy, it is our expectation that sod2 haploinsufficiency in the heart will increase ROS-mediated mitochondrial uncoupling and accelerate cardiac dysfunction in response to high-fat feeding. While we remain committed to the goal of generating a mouse model with increased myocardial oxidative stress, we also chose to ask the opposite question, which is: Would increased ROS scavenging reverse cardiac dysfunction in diabetic cardiomyopathy? We have completing a project in which we administered a potent sod2 mimetic to two independent mouse models of type 2 diabetes; the UCP-DTA mouse and *db/db* mice. In both cases ROS scavenging improved mitochondrial function, providing strong evidence to support a role for pharmacological anti-oxidant strategies in improving cardiac function in models of type 2 diabetes.

Mechanisms of Insulin Resistance in the Heart: We completed a high-fat feeding study last year (Wright et al. Cardiovascular Research, 2009), which indicated that short-term high-fat feeding impaired insulin-mediated glucose uptake while preserving proximal insulin signaling to PI3 Kinase and Akt. The basis for diminished glucose uptake was a reduction in the levels of GLUT4 in the heart and a defect in GLUT4 translocation (1). A recent study in humans with diabetes in which cardiac and skeletal muscle biopsies were obtained also demonstrated that whereas proximal insulin signaling was impaired in skeletal muscle in diabetes, it was actually increased in the heart, despite reduced insulin-mediated glucose uptake (2). This leads to the interesting observation that myocardial insulin resistance exhibits spatial differences within the cell with defects at later steps, but normal or increased signaling at proximal steps in the signaling pathway. Thus in the face of hyperinsulinemia, increased proximal insulin could contribute to pathogenesis of diabetic cardiomyopathy via effects on activation of proximal insulin signaling. Support for this hypothesis was recently published in a collaborative study between my laboratory and the group at the University of Osaka, which suggest that increased insulin signaling indeed could worsen the cardiac function in a model of pressure overload cardiac hypertrophy (3).

Model 2:

This model represents the approved AMDCC model that is being generated at the Jackson Laboratories. Heterozyous *ROSA26* $^{\text{netrin1/lacZ}}$ mice were transferred to the Jackson Laboratories. Backcross to the C57BL6 background and crossing to the Akita strain to generate *ROSA26* $^{\text{netrin1/lacZ}}$*.ins2*$^{+/C96Y}$ is now completed. The final cross to introduce the tamoxifen-inducible cre that is driven by the beta-actin promoter has just started, so we anticipate having mice available in the next 2-3 months.

Plans for the Upcoming Year

1. Determine the impact of sod2 haploinsufficiency on cardiac function and metabolism and mitochondrial function in response to high-fat feeding.
2. Continue to characterize the role of autophagy in diabetic cardiomyopathy.
3. Perform initial characterization of the *Tam-b-actinCRE*.*ROSA26* $^{\text{netrin1/lacZ}}$*.ins2*$^{+/C96Y}$ mice. This will entail documenting tamoxifen-inducible netrin expression in the heart and hind limb, followed by studies to determine if netrin-1 overexpression will enhance recovery of hindlimb circulation following femoral artery ligation, and cardiac function following coronary artery ligation. The model will also be useful in determining if netrin-1 overexpression will retard the progression of diabetic neuropathy.

2. Collaboration:

Within AMDCC

1. We continue to collaborate with the Goldberg laboratory to determine substrate metabolic fluxes in his mouse models of lipotoxicity, and recently completed a collaborative study on the impact of deleted PPAR-α in the lipotoxic model of PPAR-γ overexpression. We determined cardiac substrate metabolism and mitochondrial function. This model indicated that increasing lipid storage as triglyceride in conjunction with increasing FA oxidation was able to ameliorate lipotoxic heart dysfunction despite increased myocardial lipid storage.

2. We completed an analysis of the impact of complete bradykinin receptor deficiency on diabetic cardiomyopathy by analyzing the b1b2R KO on the Akita background that were generated by the Smithies' laboratory. In contrast, to the synergistic effect of b1b2 receptor deficiency to accelerate nephropathy and neuropathy in Akita mice, loss of b1and b2 receptors do not worsen contractile or mitochondrial dysfunction in Akita hearts. These studies further underscore the importance of phenotyping across complications in multiple models, as underlying pathophysiological mechanisms are likely to be distinct. This work was recently published (4).

With Jax

ROSA26 $^{netrin1/lacZ}$ mice were transferred to JAX and were backcrossed to the C57BL6 and Akita strains as described above. The final cross with the tamoxifen-inducible Cre is now being conducted.

With the MMPCs

None.

With other non-AMDCC PIs

1. We have collaborated with E. Douglas Lewandowski at the University of Illinois in studies that determined that most of the fatty acid that is oxidized in the heart comes as a result of turnover of the endogenous myocardial triglyceride (TAG) pool. Interestingly the rate of turnover of this pool is increased in PPAR-α transgenic mice. The implication of this work for diabetic cardiomyopathy is that altered TAG pool dynamics could contribute to impaired substrate availability and contribute to left ventricular function. This manuscript is now in press at Circulation Research.
2. We have collaborated with Gary Sweeney at York University to determine the impact of adiponectin on myocardial fatty acid utilization and oxygen consumption. This manuscript is undergoing minor revisions for AJP.
3. We have collaborated with Issei Komuro at the University of Osaka to examine the interaction of IGF-1 and Insulin receptor signaling in physiological cardiac hypertrophy and the in studies of the role of excessive insulin signaling in the adverse left ventricular remodeling that accompanies pressure overload cardiac hypertrophy. This collaboration has yielded 2 publications (3,5).
4. We have collaborated with David Symons at the University of Utah in studies that have elucidated the mechanisms by which eNOS activity in the vasculature is diminished in response to high-fat feeding. Specifically we showed that the eNOS defect was independent of impaired insulin signaling and was most likely the result of a direct inhibition of eNOS function by fatty acids such as palmitate.

3. **Address previous EAC comments:** EAC comments are in italics.

Abel

- *Work in the Abel lab has continued to investigate the alterations in mitochondrial function and their role in diabetic complications in the heart. By electron microscopy, they showed preferential damage to myocardial mitochondrial morphology when compared to liver, kidney or brain in the Akita mouse. They have recently published that mitochondrial uncoupling and resultant oxidative stress may underlie myocardial insulin resistance. These studies highlight the potential differences in mechanisms of metabolic dysfunction in different tissues of importance in diabetes. They put forth the hypothesis that myocardial insulin resistance may be in part due to oxidative stress and mitochondrial uncoupling. To continue to investigate potential mechanisms, they have embarked upon proteomics studies of mitochondrial and found that insulin resistance is associated with a change in protein stoichiometry, which would lend itself to enhanced ROS production. A suggestion would be to try and separate different myocardial cells before mitochondrial isolation to determine effects on subsets of cells (endothelial cells, fibroblasts, myocytes). Also to investigate the possibility of separating matrix and membrane proteins. In general, significant progress on the stated aims has been made.*

Response: The suggestion to separate myocardial cells from other cells prior to mitochondrial isolation is a good one, but is technically challenging in terms of obtaining sufficient mitochondria from these populations with which to conduct proteomics analyses. However, we have recently obtained a Seahorse XF24 analyzer, which should allow us to examine cellular rates of oxygen consumption (a proxy for mitochondrial function) in various cell types from the heart. We have routinely separated mitochondrial matrix from membranes prior to proteomic analyses as shown in two recent publications from our laboratory (6,7).

- *A netrin transgenic mouse on the C57BL6 background is almost complete and crossing to the Akita strain is imminent.*

- *Dr. Abel's group has also collaborated with other Consortium members to investigate whether these mechanisms are universal in different diabetic complications. With the UNC group, they have found that the combination of the BR1/2 on Akita background had no synergistic effects on cardiac structure. Mitochondrial studies are being performed.*

4. **Publications:**

Original Reports

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9. Boudina S, **Abel ED**. Diabetic cardiomyopathy, causes and effects. *Rev Endocr Metabo Disord.* 2010. 11:31-9. PMC In progress.

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3. Shimizu, I., Minamino, T., Toko, H., Okada, S., Ikeda, H., Yasuda, N., Tateno, K., Moriya, J., Yokoyama, M., Nojima, A., Koh, G. Y., Akazawa, H., Shiojima, I., Kahn, C. R., Abel, E. D., and Komuro, I. (2010) *J Clin Invest* **120**(5), 1506-1514
4. Wende, A. R., Soto, J., Olsen, C. D., Pires, K. M., Schell, J. C., Larrieu-Lahargue, F., Litwin, S. E., Kakoki, M., Takahashi, N., Smithies, O., and Abel, E. D. (2010) *Endocrinology* In Press.
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