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:

An important recent contribution of our group to understanding the pathophysiology of diabetic cardiomyopathy has been the demonstration of role that mitochondrial dysfunction plays in the pathogenesis of diabetic cardiomyopathy. Our earlier work focused on mitochondrial function, where we showed that in type 1 and type 2 diabetes, mitochondrial oxidative capacity was reduced. It models of obesity and type 2 diabetes, mitochondrial function was further limited by fatty acid-induced mitochondrial uncoupling. During the past year we focused further on understanding the mechanisms involved in mitochondrial dysfunction by performing proteomic analyses in CIRKO mice, in Akita mice and in CIRKO mice that were rendered hyperglycemic using STZ. The Data from the CIRKO and Akita mice were published in Circulation and Diabetes earlier this year (1,2). In brief we found that loss of insulin signaling was associated with coordinate reduction of fatty acid oxidation and TCA cycle proteins. Proteins of oxidative phosphorylation were not globally reduced but there were important changes in stoichiometry so that subunits of complex I were increased, whereas subunits of complex III were decreased. We believe that this change in stoichiometry might contribute to the increase in ROS generation that we also observed (1). The Akita study incorporated elements of the EAC's recommendations to phenotype across complications. Therefore we conducted a proteomic survey of cardiac, kidney brain and liver mitochondria, and coupled this with a detailed analysis of mitochondrial function and morphology. Of interest, we only observed mitochondrial dysfunction and altered mitochondrial structure in cardiac mitochondria but not in mitochondria from kidney, liver or brain (2). These observations underscore the important role of mitochondrial dysfunction in diabetic cardiomyopathy and suggest that mitochondrial dysfunction might be less important in the pathogenesis of nephropathy. In terms of the proteome, we observed tissue specific differences in the proteomic response. In diabetic Akita mice, fatty acid oxidation (FAO) proteins were less abundant in liver mitochondria, whereas FAO protein content was induced in mitochondria from all other tissues including the heart. Kidney mitochondria showed coordinate induction of tricarboxylic acid (TCA) cycle enzymes, whereas TCA cycle proteins were repressed in cardiac mitochondria. Levels of OXPHOS subunits were coordinately increased in liver mitochondria, whereas in mitochondria of other tissues OXPHOS proteins were largely unaffected. These data underscore differential degrees of remodeling of the mitochondrial proteome in tissues involved in diabetes complications, and represent an important benchmark for future studies that seek to examine mitochondrial proteomic changes in organs or tissues that are affected by diabetic complications.

The data from the STZ- CIRKO studies are not yet published but will be submitted soon for publication. By directly comparing STZ, CIRKO and CIRKO+STZ models we were able to dissect the relative contributions of loss if insulin signaling versus hyperglycemia to diabetic cardiac phenotypes and determine which pathophysiological changes were synergistic. Left ventricular

hemodynamic measurements revealed that left ventricular developed pressure and dP/dt measurements were impaired to similar extents in CIRKO and STZ mice. Only developed pressure but not dP/dt measurements showed additional impairment in CIRKO-STZ compared to CIRKO mice. With pyruvate, ADP-stimulated mitochondrial oxygen consumption and ATP synthesis rates were impaired to similar extents in saponin-permeabilized fibers of CIRKO and STZ mice, with only slight additional impairment in CIRKO-STZ mice. With palmitoyl-carnitine, respiration rates were not different among groups, and mitochondrial cristae density was decreased to similar extents in all groups. Comparative mitochondrial proteomics revealed downregulation of fatty acid oxidation proteins in CIRKO mitochondria but upregulation in STZ and CIRKO-STZ mitochondria, however TCA cycle and OXPHOS proteins were reduced in similar directions in CIRKO and STZ models. Thus, mitochondrial and contractile dysfunction in CIRKO-STZ hearts are largely recapitulated by impaired insulin signaling. Although proteomic profiles in STZ- and CIRKO-STZ hearts were similar in terms of FAO proteins (increased), mitochondrial dysfunction was similar to CIRKO hearts (where FAO proteins were reduced). Thus the induction of FAO proteins in STZ and CIRKO-STZ likely account for increased rates of FAO in diabetic hearts but additional shared mechanisms such as reduced TCA cycle and OXPHOS content which are present in STZ and CIRKO hearts likely contribute to the mitochondrial dysfunction. Cardiac steatosis was in increased in STZ and CIRKO STZ hearts but not in CIRKO hearts. Taken together these data suggest that at the level of the cardiomyocyte, impaired insulin action might be the major contributor to cardiac and mitochondrial dysfunction in diabetic cardiomyopathy. However the increased lipid delivery that occurs in diabetes contributes to changing the patterns of substrate utilization and lipid accumulation. Of interest, STZ diabetes was not associated with mitochondrial ROS overproduction or uncoupling of mitochondria. These findings were similar to observations made in Akita mouse hearts that were published by us in Diabetes last year. However, in CIRKO-STZ mice there was a significant increase in myocardial oxygen consumption and mitochondrial uncoupling. Taken together these data indicate that insulin resistance is a major contributor to mitochondrial uncoupling in the heart and provides a critical mechanism for the mitochondrial uncoupling and diminished cardiac efficiency that we previously reported in the hearts of *ob/ob* and *db/db* mice, which are models of severe insulin resistance, obesity and type 2 diabetes.

MHC-ACS: Studies in the MHC-ACS mice have a revealed a novel mechanism by which lipotoxicity leads to mitochondrial dysfunction in the heart. Specifically, a 2-fold increase in FA uptake in the heart so of these mice led to fragmentation of the mitochondrial network, which morphologically manifested as a 40% increase in mitochondrial number and volume density, that was associated with a dramatic reduction in mitochondrial size. This remodeling of the mitochondrial network impaired the access of long-chain FA substrates to mitochondria, which limited that ability of the heart to generate ATP from long chain fatty acids. In an in vitro model we have shown that exposure of cultured muscle cells to palmitate impairs mitochondrial fusion, which results in fragmentation of the mitochondrial network. Interestingly this process is reversible, when cells are returned to low-palmitate conditions, raising the possibility that lipotoxic disruption of mitochondrial networks might be reversible.

CIRKO-ACS: ACS mice were not insulin resistant, which was unexpected given the increased concentrations of ceramide and diacyl glycerol in the hearts of these animals. Thus generation of CIRKO-ACS mice would indeed introduce the additional component of insulin resistance. Mitochondria from CIRKO-ACS mice generated increased ROS relative to CIRKO mice. Interestingly, there was relatively little additional impairment in cardiac function in CIRKO-ACS mice, relative to CIRKO mice. Thus impaired insulin action appears to be dominant in terms of impairing cardiac function and that increased lipid uptake does not impair cardiac function further. However, the fact that ROS is increased in CIRKO –ACS mouse hearts leads us to believe that introduction of sod2 haplo-insufficiency could potentially lead to accelerated cardiac dysfunction by amplifying oxidative stress.

In preliminary studies we have begun to examine the contribution of autophagy to diabetic cardiomyopathy. Our studies so far suggest that rates of autophagy are increased in CIRKO and STZ models. We do not yet know if the increase in autophagy represents compensatory responses

to remove damaged mitochondria or is a maladaptive response that contributes to cardiac dysfunction.

Model 2:

This model represents the approved AMDCC model that is being generated at the Jackson Laboratories. Heterozyous *ROSA26* $^{netrin1/lacZ}$ mice were transferred to the Jackson Laboratories. Backcross to the C57BL6 background is almost completed and crossing to the Akita strain to generate *ROSA26* $^{netrin1/lacZ}$*.ins2*$^{+/C96Y}$ will get underway shortly. Once these mice are generated, they will be crossed with a tamoxifen-inducible cre that is driven by the beta-actin promoter.

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. Continue to characterize the role of autophagy in diabetic cardiomyopathy.
4. Backcross the ***ROSA26*** $^{netrin1/lacZ}$ to the Akita background and introduce the inducible cre transgene.

2. Collaboration:

Within AMDCC

1. We continue to collaborate with the Goldberg laboratory to determine substrate metabolic fluxes in his mouse models of lipotoxicity.

2. We have conducted detailed cardiac phenotyping of the double bradykinin receptor (b1b2R) KO on the Akita background from the Smithies' laboratory. In contrast, to the synergistic effect of b1b2 receptort 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.

With Jax

ROSA26 $^{netrin1/lacZ}$ mice have 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).

CIRKO mice have been sent to Dr. Barbara Huisamen at Stellenbosch University in South Africa where she is examining the impact of naturally occurring antioxidants on mitochondrial and cardiac function in animal models of diabetic cardiomyopathy.

We collaborated with Michael Lisanti's group at Jefferson University to characterize cardiac function and metabolism in the hearts of mice lacking caveolin 1 and 3 respectively.

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:

It would be interesting to further characterize MHC-ACS, and CIRKO-ACS hearts either invasively (PV Loops) and/or with Langendorff preps.

Isolated heart analyses have been done in ACS and CIRKO-ACS hearts. MHC-ACS hearts have mild contractile dysfunction, which is seen in hearts perfused in the presence of fatty acids. We believe that this is a consequence of the mitochondrial dysfunction that develops in these hearts. Because we did not see significant LV dysfunction in vivo, we subjected MHC-ACS hearts to pressure overload (transverse aortic constriction). Interestingly we did not see any additional acceleration in LV dysfunction relative to WT mice that were subjected to TAC. This contrasts with accelerated contractile and mitochondrial dysfunction, which we previously observed in banded CIRKO hearts. Thus the mitochondrial impact of lipotoxicity might be relatively mild relative to impact of impaired insulin signaling.

In CIRKO-ACS mice, mitochondrial morphology and function were similarly impacted. As noted by the investigators, this may be a model of lipotoxicity in the heart, although the true value of the model is still questionable as there is not a dramatic change in cardiac function.

I agree with this assessment. I think that impaired insulin action might be more important.

What is the role of netrin in the diabetic complications of reduced angiogenesis or neuropathy? Is endogenous netrin activity reduced in diabetes or is exogenous netrin supplementing other mechanisms?

We are not sure as if netrin deficiency per se contributes to the impaired angiogenic phenotypes of diabetes. Our netrin mouse was generated primarily as a platform that would enable us to increase angiogenesis in the face of complications in which impaired angiogenesis is believed to play an important role. However, we agree that this is an important question that we will address when we receive the netrin-Akita mice from JAX.

4. Publications:

Original Reports

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Reviews

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