Animal Models of Diabetic Complications Consortium
(U01 DK076139)

Annual Report
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"Recapitulating Transcriptional Pathways of Human Diabetic Nephropathy in Mice"

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Part A:

Principal Investigator's Summary

1. Program Accomplishments:

Hypothesis: ***Current murine models fail to show human-like DN because they fail to replicate glomerular and tubulointerstitial gene expression changes that occur in humans with progressive DN. Replication of the critical transcriptomic profiles of patients with progressive DN should induce progressive DN in mice.***

Progress toward stated milestones:

1. Enhanced JAK/STAT pathway in human diabetic nephropathy but not in mouse models. As reported in last year's summary, our group identified transcriptomic profiles in humans with early and progressive DN that differed from those found in common murine models of this complication. We found particular increases in expression of several members of the JAK/STAT family in the glomeruli and tubulointerstitium of kidneys from patients with progressive DN which were generally not reproduced in 2 common murine models of DN, the streptozotocin DBA/2J and db/db C57BLKS mice. Increased expression of JAK2 in mesangial cells led to increased JAK2 activity as measured by enhanced STAT3 phophorylation. These findings were published in *Diabetes* this year (1), and therefore will not be further described.

2. Generation of proximal tubule and podocyte specific JAK2 transgenic mice. To establish a more human-like model of DN we have therefore developed a Jak2 transgenic mouse. In order to generate the most reliable model and one that would be of most use to other investigators, we opted on an approach in which a stop-flox Jak2 construct has been "knocked-in" to the ROSA26 locus. This allows generation of mice with cell-type specific overexpression of the Jak2 transgene by crossing the mouse with tissue specific Cre mice. Because of the enhanced sensitivity of the 129S6/SvEvTac strain to DN, we have bred our targeted mutation onto this background. We have demonstrated successful recombination and have established mouse lines of the stop-flox Jak2 mouse at both the University of Michigan and The Jackson Laboratory. Under the guidance of Racheal Wallace at The Jackson Laboratory, SNP typing has shown that the Jak2 conditional mice are on a pure 129S6/SvEvTac background. We have also worked with Ms. Wallace to establish the podocyte specific cre mouse, Tg(NPHS2-cre), on the 129S6/SvEvTac background, and these mice are now available for all AMDCC members and will be made freely available to the scientific community. The husbandry staff at The Jackson Lab are currently mating heterozygote (het) x het, het x 129S6, het x 129S6-Akita and het x Tg(NPHS2-cre) mice. The last of these described matings will produce podocyte-specific JAK2 transgenic mice. Proximal tubule specific cre mice, Tg(PEPCK-cre), which are on the 129S6/SvEvTac background were obtained from AMDCC investigators, Drs. Susan Gurley and Tom Coffman. These mice have been crossed with the stop-flox Jak2 mouse, to produce proximal tubule cell specific JAK2 transgenic mice. These mice have been made diabetic by the AMDCC low dose STZ protocol and are currently 12+ weeks through a 24 week trial of diabetes. Mice will be phenotyped using AMDCC criteria to determine whether JAK2 overexpression in the proximal tubule alone contributes to diabetic nephropathy. Similar trials will be initiated with the podocyte specific JAK2 transgenic mice.

3. <u>Characterization of the effect of podocyte-specific GLUT1-transgene on diabetic nephropathy in C57BLKS db/db mice</u>.

Previously, AMDCC investigators (Heilig C, Brosius F) have found that Increased expression of the facilitative glucose transporter, GLUT1, leads to glomerulopathy that resembles DN whereas prevention of enhanced GLUT1 expression in diabetic db/db C57BLKS mice retards DN (2). While many of the GLUT1-mediated effects are likely due to mesangial cell effects, we hypothesized that increased GLUT1 expression in podocytes also contributes to the progression of DN. Therefore, during the first 5 years of the AMDCC, we generated 2 podocyte specific GLUT1 transgenic mouse lines (driven by a podocin [Nphs2] promoter) on a C57BLKS db/+ background. We have now completed analysis of these lines.

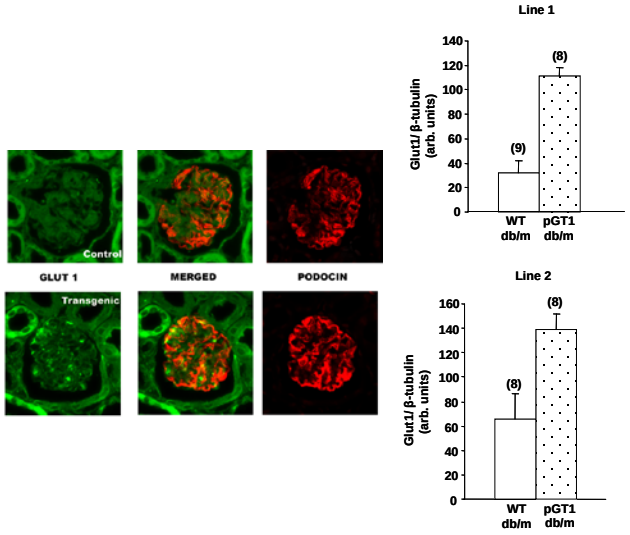

Fig. 1. Podocyte specific overexpression of GLUT1 in BLKS db/db mice. Two lines of mice were phenotyped with 2-3.5-fold overexpression of GLUT1 in glomerular extracts by immunoblotting. Immunohistochemistry for GLUT1 suggests that the increased expression is restricted to a subset of glomerular cells that are in podocyte distribution.

Progeny of the 2 founders were used to generate type 2 diabetic db/db and control db/+ littermate mice. Immunoblots of isolated glomerular lysates showed that tg mice had approximately 3.5-fold (line 1) and 2-fold (line 2) increase in GLUT1 content compared to wild-type (wt) mice (Fig. 1). Both lines showed similar increases in fasting blood glucose and similar rates of obesity with diabetes at 24 wk of age compared to wild-type mice. Similarly, and somewhat surprisingly, there was no difference in albuminuria or podocyte number between diabetic podocyte-specific GLUT1 animal and diabetic wild-type animals. However, the expansion in mesangial matrix in diabetic mice was reduced 52% (line 1; $p<0.05$) and 54% (line 2, $p<0.05$) (Fig. 1) in the transgenic diabetic mice which appeared to be accompanied by a reduction in fibronectin accumulation when compared to wild-type diabetic mice. There was no difference in mesangial matrix accumulation between nondiabetic wt and tg mice. Levels of nephrin, neph1, CD2AP, vascular endothelial growth factor (VEGF), podocin, GLUT4 were determined by immunoblotting of glomerular lysates from transgenic animals and controls. The increase in VEGF levels was significantly blunted in transgenic vs. wild-type diabetic mice (Fig. 3). There were no significant differences in the levels of the other measured proteins. Taken together, increased podocyte GLUT1 expression in

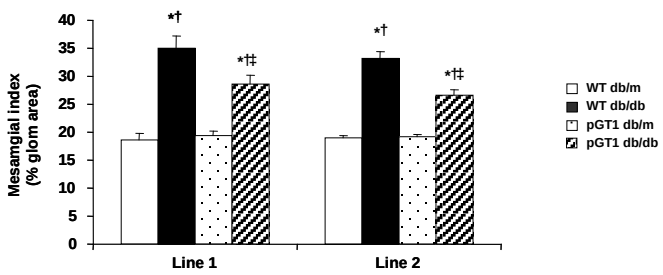

Fig. 2. Mesangial index (percent glomerular area occupied by extracellular matrix based on PAS staining). Mesangial expansion in diabetic db/db mice was reduced by presence of the podocyte-specific GLUT1 transgene. * $p<0.05$ compared to WT db/m mice, † $p < 0.05$ compared to pGT1 db/m mice. ‡ $p<0.05$ compared to pGT1 db/db mice.

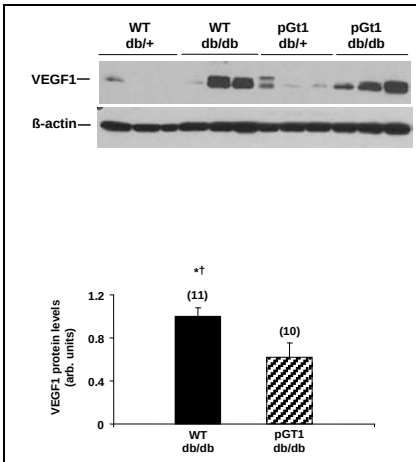

Fig. 3. VEGF1 immunoblots of glomerular samples showed a large increase in diabetic mice which was blunted by podocyte specific GLUT1 overexpression (* $p<0.05$).

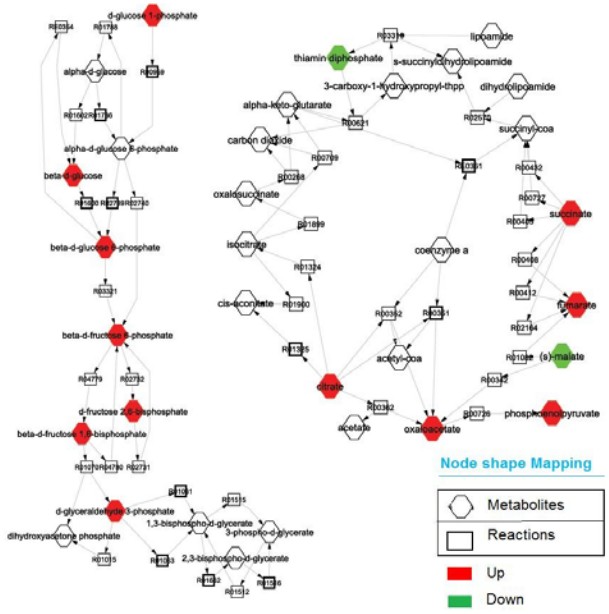

Fig. 4. Gene expression data were obtained from glomeruli of STZ diabetic and control DBA mice. Reactions potentially increased or decreased based on enzyme gene expression were visualized using Cytoscape. Predicted changes in glycolytic (left panel) and citric acid cycle pathway (right panel) metabolites are color coded based on direction of regulation (green down-regulated and red up-regulated).

diabetic mice does not enhance diabetic nephropathy; surprisingly, it protects against mesangial expansion and fibronectin accumulation possibly by blunting podocyte VEGF secretion. The manuscript reporting these results will be submitted within the next month.

4. <u>Transciptomic-metabolomic analysis of streptozotocin-diabetic mouse kidneys</u>. We performed transcriptomic analysis of glomeruli obtained from DBA/2 mice made diabetic with low-dose STZ for 24 weeks and non-diabetic DBA/2 mice. Mouse genes were translated to their corresponding human homologues and mapped to the Entrez Gene identification in *H. sapiens* Recon1. Mapped genes were assigned to one of three categories: up-regulated in diabetes, down-regulated in diabetes or not differentially expressed in diabetes. Based on these changes, predictions were made for metabolite accumulation. As part of this analysis, robust changes in many metabolites in the glycolytic pathway and the citric acid cycle were predicted (Fig. 4). We have now determined relative levels of metabolites in these pathways from renal cortices of db/db C57BLKS mice (a type 2 diabetes model). [We are also obtaining metabolite data from renal cortices of STZ DBA mice but these data are not yet extensive enough to analyze.] We analyzed renal cortical samples from 5 control, nondiabetic db/m mice and 5 diabetic db/db animals with diabetes and robust early diabetic nephropathy. Metabolites from homogenized mouse renal cortical tissue were separated by hydrophilic interaction chromatography (HILIC) using a Luna NH_2 column on an Agilent 1200 HPLC instrument. Quantitative analysis of glycolytic and citric acid cycle intermediates was then performed by electrospray ionization/tandem mass spectrometry (ESI/MS/MS) in the multiple reaction monitoring mode (MRM) utilizing an Agilent triple quadrapole mass spectrometer. Relative abundance of the metabolites was determined by comparing extracted ion chromatograms of the respective metabolites. Data were normalized for protein content of the starting material and comparing relative ion intensity of ^{13}C-succinate internal standard for extraction efficiency, and relative abundance was determined (Fig. 5). Consistent with the transcriptomic prediction, there was a marked elevation of glycolytic intermediates such as glucose 6-phosphate (G6P), glyceraldehyde 3-phosphate (G3P), 3-phosphoglycerate (3-PG), phosphoenolpyruvate (PEP) and pyruvate (* $p<0.05$). Metabolites in the citric acid cycle showed a similar trend with elevations in oxaloacetate (OA), citrate, isocitrate and fumarate (Fig 2). Only two metabolite levels (succinate and malate) were in variance

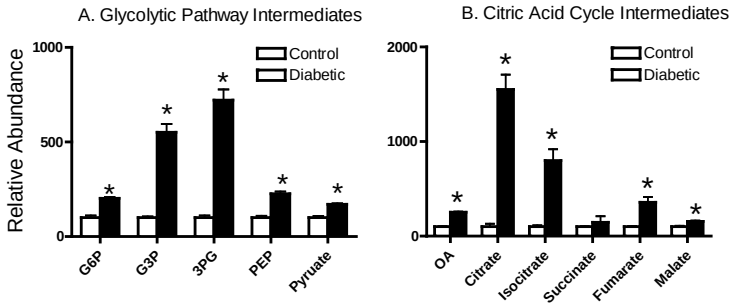

Fig. 5. Relative abundance of A) glycolytic and B) citric acid cycle Intermediates in control and diabetic renal cortex determined by ESI/MS/MS * $p < 0.05$.

with transcriptomic prediction. These data demonstrate the feasibility of a targeted metabolomic approach and serve as a proof-of-principle that transcriptome data can be utilized as a filter to direct targeted metabolomic studies.

Plans for the Upcoming Year:

1. **Continued analysis of proximal tubular specific JAK2 trangsenic mice and generation and analysis of podocyte specific JAK2 transgenic mice**. The proximal tubule specific JAK2 transgenic 129S6/SvEvTac diabetic animals will be fully phenotyped as for our previous models (see podocyte specific GLUT1 transgenic animal description, above). We will also perform careful morphometric analysis of the tubulointerstitial compartment in these animals. This will determine whether increased JAK2 expression in proximal tubule epithelia is sufficient to worsen nephropathy in diabetic mice. We will shortly begin breeding to establish the podocyte specific JAK2 trangenic 129 S6/SvEvTac mice. These animals will be made diabetic with the low-dose AMDCC STZ protocol and fully analyzed after 6 months of diabetes. Terminal studies may not occur till the end of the year or start of the following year.

We will also perform assessments of urine and plasma metabolites that differ between the two JAK2 transgenic mice models and controls at the end of the study, as we have previously published (3), to compare to what was found in STZ DBA2/J urines in the earlier study, as another way to help identify metabolites that may accurately predict worsening DN and/or response to therapy. We will also add in at least 2 measurements of tail-cuff blood pressure in each of the experimental and control models to determine whether JAK2 overexpression in podocytes or proximal tubule has an effect on blood pressure. Finally, we perform transcriptomic analysis of the glomeruli and tubulointerstitium (renal cortex) of the animals in these 2 studies, as we have previously reported (1). Such data have been extremely informative from previous mouse models, and such studies will help confirm that the molecular response has been altered in these tissues to a more human-like configuration, which will be critical ancillary (or rather, primary) data in support of the functional and histopathology phenotyping.

2. Final analysis and reporting of the effects of GLUT4 knockout and podocyte specific GLUT4 knockout on the development of diabetic nephropathy in C57BL/6 mice. These models were created and largely analyzed in the first 5 years of the AMDCC. However, final phenotyping will take place over the next 4 months and the manuscript on this model will be submitted.

2. Collaboration:

With other AMDCC PIs: We continue to work in a highly interactive manner with the laboratory of Dr. Eva Feldman. Our extensive collaborations include completion of an AMDCC pilot project in which transcriptomic data were derived from kidney and nerve tissue of BLKS db/db mice, DBA/2 STZ diabetic mice, and C57BLKS eNOS -/- db/db mice. Drs. Kretzler and Brosius worked closely with Dr. Ray Harris in the AMDCC pilot project on the transcriptomic analysis of the eNOS -/- db/db mouse glomeruli and nerve samples (see separate pilot project progress report). We are also collaborating with Dr. Thomas Coffman and the MCHC in the generation of the 129SvEv mouse lines as noted above. The project on the GLUT4 knockout mice continues work with Dr. Dale Abel as well. Dr. Kretzler continues to collaborate closely with Dr. Erwin Bottinger.

Dr. Brosius led the efforts to summarize AMDCC nephropathy accomplishments, including updates on validation criteria, phenotyping (including pathology phenotyping recommendations), the effect of genetic background on nephropathy, AMDCC nephropathy models (including negative models), and future goals. This paper is now in press in *J Am Soc Nephrol*.

With Jax: There have been extensive interactions in the generation of the various cre and Flox models on the 129S6/SvEvTac background an noted above under JAK2 transgenic model development.

With the MMPCs: Dr. Brosius worked closely with Drs. Levi, Alpers and Breyer to codify phenotyping recommendations and validation criteria for diabetic nephropathy in murine models. These are fully discussed in the review paper in *J Am Soc Nephrol*. Dr. Brosius served as the AMDCC representative at the MMPC meeting in Sept., 2008.

With other non-AMDCC PIs: We work closely with Dr. Christin Carter-Su (University of Michigan) and members of her laboratory on JAK/STAT signaling aspects, and with Dr. Sub Pennathur on oxidative markers, metabolomics and proteomics in diabetic complications (University of Michigan), who provided the metabolite correlations shown in Fig. 5. Dr. Brosius has continued close collaboration with Dr. Charles Heilig (University of Florida) on GLUT1 overexpression models of diabetic nephropathy and with Dr. Maureen Charron (Albert Einstein College of Medicine) on GLUT4 models. Dr. Kretzler continues collaborations on diabetic nephropathy with numerous investigators internationally.

3. Address previous EAC comments:

Previous comments: "The Brosius-Kretzler collaboration is quite interesting since the studies will test hypotheses generated from state-of-the-art analysis of kidney expression libraries generated from micro-dissected glomeruli and tubulointerstitium. Comparison of these expression profiles with profiles generated from diabetic mouse models suggests that mice fail to replicate human DN changes in JAK-STAT molecule expression. Studies are ongoing to conditionally overexpress Jak2 in podocytes and proximal tubules in diabetic mice. Progress is good.

"The Jak2 tissue specific overexpression seems reasonable based on the human data despite the lack of overexpression in the diabetic mice."

We thank the committee for their comments and have continued the work they suggested as noted above.

4. Publications:

1. Zhang H, Saha J, Byun J, Schin M, Lorenz M, Kennedy RT, Kretzler M., Feldman EL, Pennathur S, Brosius FC III. Rosiglitazone reduces renal and plasma markers of oxidative injury and reverses urinary metabolite abnormalities in the amelioration of diabetic nephropathy. Am J Physiol Renal Physiol, 2008; 295:F1070-81.

2. Wiggin TD, Kretzler M, Pennathur S, Sullivan, KA, Brosius FC, Feldman EL. Rosiglitazone treatment reduces diabetic neuropathy in streptozotocin-treated DBA/2J mice. Endocrinology, 2008;149:4928-37.

3. Qian Y, Feldman E, Pennathur S, Kretzler M, Brosius FC 3rd, From fibrosis to sclerosis: mechanisms of glomerulosclerosis in diabetic nephropathy, Diabetes. 2008;57:1439-45.

4. Buller C L, Loberg RD, Fan M-H, Zhu Q, Park JL, Vesely E, Inoki K, Guan K, Brosius FC III. A GSK-3, TSC2, mTOR pathway regulates glucose uptake and GLUT1 glucose transporter expression, Am J Physiol Cell Physiol, 2008;295:C836-43.

5. Brosius FC III. New insights into the mechanisms of fibrosis and sclerosis in diabetic nephropathy. Rev Endocr Metab Disord. 2008;9:245-54.

6. Berthier CC, Zhang H, Schin ML, Henger A, Nelson RG, Yee B, Boucherot A, Carter-Su C, Argetsinger LS, Rastaldi MP, Brosius FC, Kretzler, M. Enhanced Expression of JAK-STAT Pathway Members in Human Diabetic Nephropathy, Diabetes, 2009, 58:469-77.

7. Frank C. Brosius III, Charles E. Alpers, Erwin P. Bottinger, Matthew D. Breyer, Thomas M. Coffman, Susan B. Gurley, Raymond C. Harris, Masao Kakoki, Matthias Kretzler, Edward H. Leiter, Moshe Levi, Richard A. McIndoe, Kumar Sharma, Oliver Smithies, Katalin Susztak, Nobuyuki Takahashi, Takamune Takahashi for the Animal Models of Diabetic Complications Consortium, Mouse Models of Diabetic Nephropathy, J Am Soc Nephrol, in press, 2009.

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5. References

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2. Chen SL, Heilig KO, Brosius FC, Heilig CW: Diabetes increases glomerular GLUT1, and antisense-GLUT1 protects against diabetic glomerulosclerosis. In *36th Annual Meeting of the American-Society-of-Nephrology* San Diego, California, Lippincott Williams & Wilkins, 2003, p. 46A-46A
3. Zhang H, Saha J, Byun J, Schin M, Lorenz M, Kennedy RT, Kretzler M, Feldman EL, Pennathur S, Brosius FC, 3rd: Rosiglitazone reduces renal and plasma markers of oxidative injury and reverses urinary metabolite abnormalities in the amelioration of diabetic nephropathy. *Am J Physiol Renal Physiol* 295:F1071-1081, 2008