Animal Models of Diabetic Complications Consortium
(U01 DK076139)

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

University of Michigan

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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:

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 (manuscript in revision, *Diabetes*). Our major emphasis has been on Jak2 as a factor potentially responsible for both glomerular and tubulointerstitial fibrosis in humans with DN. In support of this hypothesis, we have found that Jak2 overexpression in cultured mouse mesangial cells leads to enhanced Jak2 activity as determined by Stat3 phosphorylation (**Fig. 1**). In addition, using the same mesangial cells system we have found that Jak2 overexpression enhances oxidative stress in both normal and high glucose conditions, as determined by DCFDA fluorescence (**Fig. 2**).

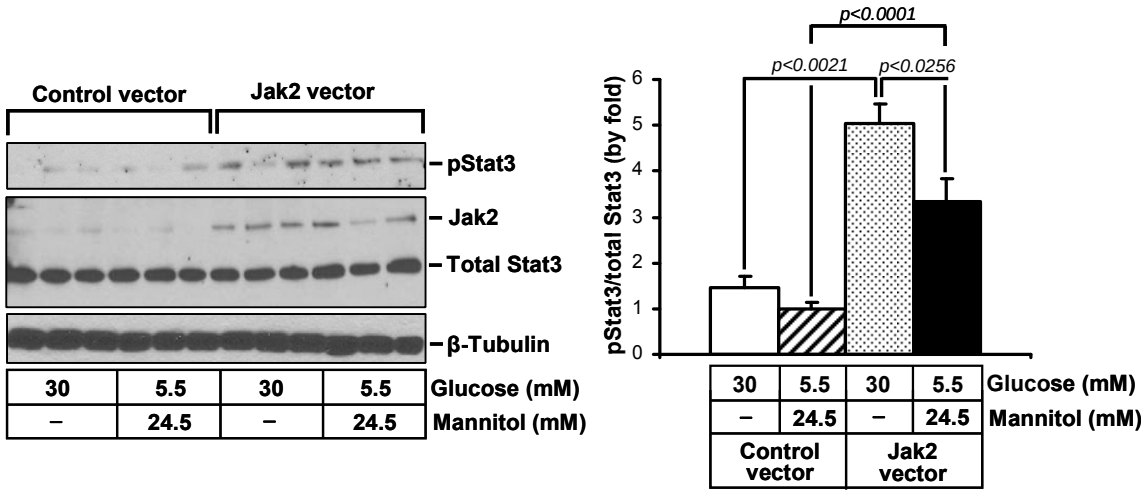

Figure 1. Effects of Jak2 overexpression and high glucose on Stat3 phosphorylation in Jak2 mesangial cells. Left panel: Jak2 (130 kDa), total Stat3 (79 kDa), phosphorylated Stat3 (79kDa) and β-tubulin (50 kDa) immunoblots of murine mesangial cells transfected with a Jak2 prk-5 plasmid or a control vector. Right panel: Phosphorylated Stat3 levels normalized to total Stat3 levels.

Although murine models do not develop the tubulointerstitial fibrosis and progressive decline in GFR that occur in humans with DN, the glomerular lesions in some murine DN models resemble those of early DN in humans. Although we found that murine models do not demonstrate increased mRNA and protein expression of Jak2 in glomerular and tubulointerstitial regions of diabetic mice, we determined that Jak activity, based on levels of Stat3 phosphorylation, was enhanced in glomeruli from STZ DBA/2J diabetic mice when compared to their non-diabetic congeners (**Fig. 3**), suggesting that inhibition of Jak2 activation in these mouse models could interrupt development of even the early stages of diabetic glomerulopathy.

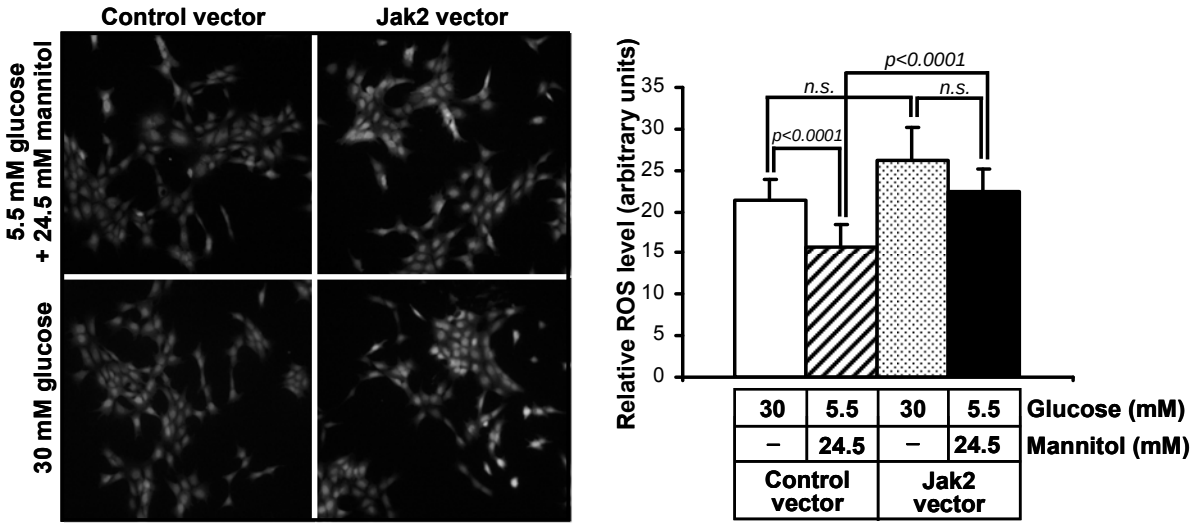

Figure 2. Effects of Jak2 overexpression and high glucose on ROS generation in mesangial cells. Left panel: DCF fluorescence in murine mesangial cells transfected with either Jak2 prk-5 or control vectors exposed to 5.5 mM D-glucose + 24.5 mM mannitol, or 30 mM D-glucose for 24h. Image exposure times were identical and are representative of those from 3 separate fields on 5 separate slides for each condition. Right panel**:** ROS quantitation from the DCF fluorescence experiments. Fluorescence intensity was determined using NIH image J. (n = 5 per group; 50 cells were assessed for each slide).

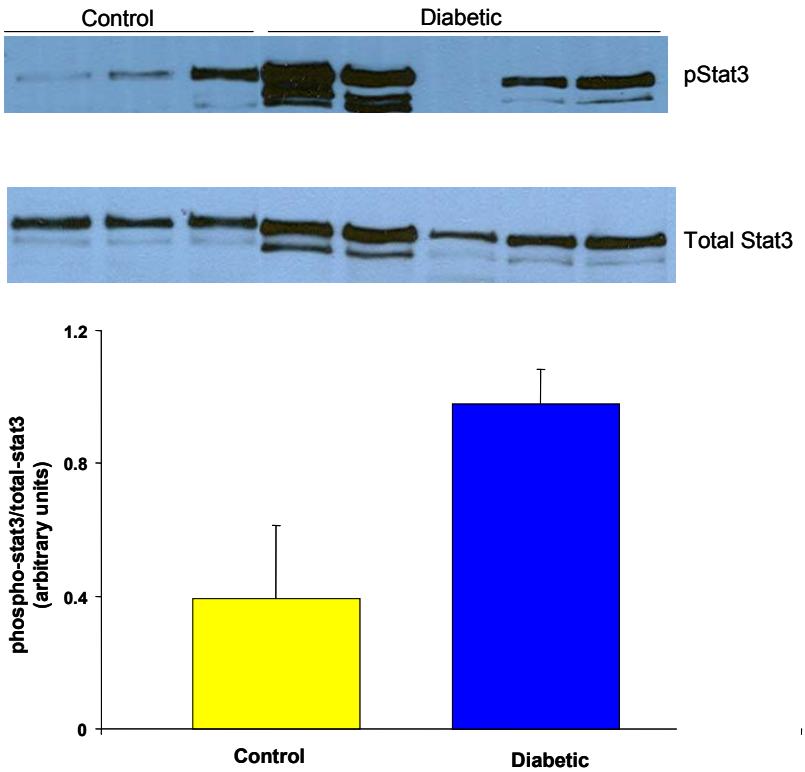

Figure 3. Stat3 phosphorylation (pStat3) and total Stat3 levels in glomeruli from DBA/2J mice either non-diabetic (control) or rendered diabetic by injection with STZ (low dose AMDCC protocol. pStat3 levels were significantly increased in the diabetic glomeruli ($p<0.05$; n =3 control, 4 diabetic).

To establish a more human-like model of DN we have therefore embarked on the development of a Jak2 transgenic mouse. In order to generate the most reliable model and one that would be of most use to other investigators, we have opted on an approach in which a stop-flox Jak2 construct has been "knocked-in" to the ROSA26 locus. This will allow generation 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 129SvEv strain to DN, we will breed our targeted mutation onto this background. At this point, we have developed 3 separate targeted ES cell lines that have been injected into blastocysts. We have over 50 chimeras generated many of which are > 95% Agouti. Mating of chimeras will begin at the end of May, 08 and hopefully germline transmission will be documented 6 weeks later. We are proceeding with this development through the Transgenic Core at the University of Michigan as

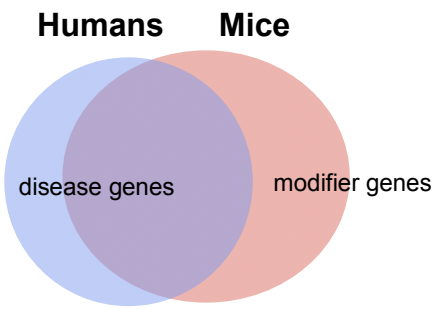

Figure 4. Venn diagram of the overlap of gene changes between diabetic complications in humans (blue circle) and mice (red circle). Important disease genes in humans will certainly overlap with those in mice (purple area), whereas some human disease gene changes are not represented in mice (area of blue without overlap). Mice have protective responses (modifier genes), not existing in humans that generally keep them from progressing to end-stage DN or DPN (red area without overlap).

the facility at JAX was processing too many other AMDCC models. The proximal tubule specific Cre mouse on the 129SvEv background, to be used to breed with our Stop/flox Jak2 mouse, has been developed and already utilized by Dr. Coffman's group as part of their AMDCC activities. We are currently backcrossing the nphs2 Cre transgene onto a 129SvEv background.

Based on our general hypothesis that diabetic complications in humans and mice converge in many respects but diverge in others because of a different mix of disease and modifier genes (**Fig. 4**), we have begin to explore where overlaps between the 2 species occur in both diabetic nephropathy and polyneuropathy, the latter in collaboration with AMDCC investigator, Dr. Eva Feldman, in order to identify important shared mechanisms. We have made such comparisons using transcriptomic profiles, and though most of the neuropathy work will be included in Dr. Feldman's progress report, we have included some of those collaborative findings below.

Following up Dr. Brosius' previous JDRF funded studies of thiazolidinediones (TZDs) in murine diabetic nephropathy, Dr. Kretzler has examined tissues from these mice to look at transcriptomic changes and we have found multiple similar responses both to diabetic injury and its prevention by TZDs in both mice and humans (**Fig. 5**). Similar studies performed with Dr. Feldman, have revealed significant overlap of gene networks between diabetic nephropathy in humans and diabetic nephropathy AND neuropathy in mice. A significant enrichment of eight gene ontology (GO) categories was defined that was shared among the 2 mouse and 1 human tissue, including the

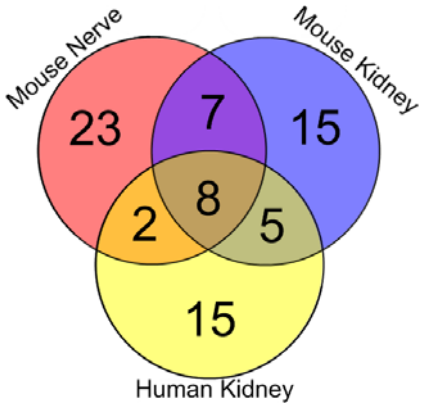

Figure 6. Gene ontology (GO) category overlap between murine diabetic nephropathy and neuropathy and and human diabetic nephropathy. Go classification of regulated mRNAs. Eight GO categories are shared between the 3 sources suggesting substantial overlap in mechanisms.

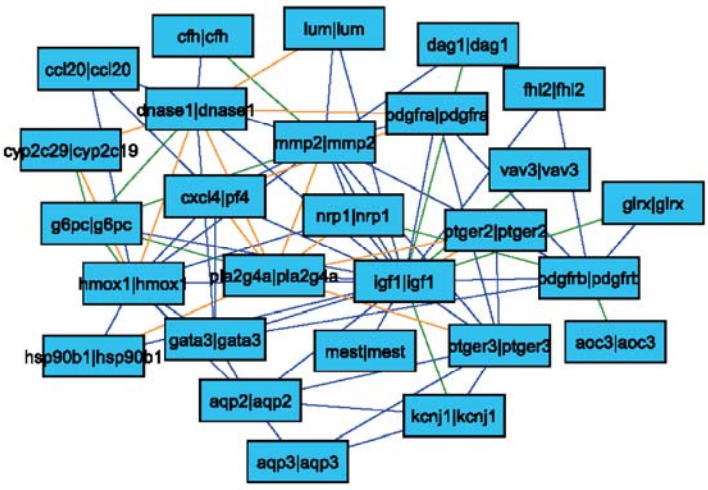

Fig 5. Cross species conserved transcriptional network. Transcriptional networks of genes regulated in human and murine diabetic nephropathy in response to TZDs were compared using the graphical matching tool TALE. Nodes indicate aligned mouse and human gene pairs (in the format of MouseGene|HumanGene), dark blue lines represent the conserved interactions in both species, green lines are the interactions only in human and orange lines are the interactions only in mouse.

categories mitochondrial enzymes, angiogenesis, extra-cellular matrix and collagen helix repeat proteins (**Fig. 6**).

In additional collaborative experiments with Dr. Feldman, we have confirmed and extended the AMDCC findings of Breyer's and Coffman's groups that the DBA/2J mice develop substantial albuminuria and anatomical evidence of renal injury at 12 wk of diabetes (**Table 1**). There is parallel evidence of diabetic polyneuropathy, as will be documented in Dr. Feldman's report and is summarized in Table 1. In these animals, rosiglitazone (3 mg/kg/day) starting 2 wk after completion of the low dose STZ protocol largely prevented both nephropathy and neuropathy and DPN with correction in thermal responses, NCVs and restoration of IENF density. There was no change in

Model	DM dura-tion (wk)	Behavior	NCV	Nerve Anatomy	Alb/Cr	Renal Anatomy	Ox. Stress Metabolites	Treatment
STZ DBA/2J	12	thermal hypo-algesia	↓sensory NCV by 30% ↓motor NCV by 20%	↓IENF by 30%	↑14-fold	↑mesangial matrix by 54% ↓podocyte no. by 40%	↑NT, ↑ HODE, ↑dityrosine	Rosiglitazone ↓↓DN, ↓↓DPN, ↓↓ox stress markers
Db/db BLKS	26 for DPN; 12 for DN	thermal hypo-algesia	↓sensory NCV by 50% ↓motor NCV by 50%	↓IENF by 50%, EM severe Mt abnormalities	↑4-fold	↑mesangial matrix by 50%	↑NT, ↑ HODE, ↑dityrosine	Resveratrol Prevented DN, ↓DPN

Table 1. **Mouse Models of diabetic nephropathy (DN) and neuropathy (DPN).** Ox. = oxidative; HODE = Hydroxyoctadecadienoic acids, EM = electron microscopy, IENF = intraepidermal nerve fiber, Mt = mitochondrial, NCV = nerve conduction velocity, NT = nitrotyrosine immunohistochemistry.

glycated Hb between treatment groups. We also completed a treatment trial of db/db BLKS mice using resveratrol (20 mg/kg/day) beginning at 8 wk of age with similar robust results with nephropathy and neuropathy and no change in glycated Hb.

Plans for the Upcoming Year:

1. Generation of podocyte and proximal tubular specific Jak2 trangsenic mice. As noted above, if things go well we could have germline transmission of our stop/flox Jak2 mouse on a 129Sv background in approximately 6 weeks. Once confirmed we will establish a breeding colony and send breeders to the core at JAX for distribution. At the same time we will begin breeding to the PEPCK Cre mouse on the 129SvEv background developed by Dr. Coffman's group as part of their AMDCC activities. This cross will generate the proximal tubule specific Jak2 overexpression and appropriate control mice. Similarly, as soon as the nphs2 Cre mouse has been backcrossed onto the 129SvEv background 8-10 generations (winter), we will begin generating nphs2 Cre/Jak2 mice for specific podocyte overexpression.

2. Determination of role of Jak2 in glomerulopathy of STZ DBA/2J mice. Because it appears that Stat3 phosphorylation is strongly induced in diabetic glomeruli from the susceptible strain, DBA/2J mice, we will determine the role of chronic Jak2 inhibition in the amelioration of nephropathy in this model. Our initial experiments will comprise initiation of diabetes with the AMDCC low dose streptozotocin protocol. Half of the diabetic animals would be implanted with pellets delivering AG-490 followed by phenotyping them with AMDCC methods for albuminuria, mesangial index, podocyte number, tubulointerstitial fibrosis.

3. Determination of Jak2 overexpression effects on evolution of diabetic nephropathy. We will make the proximal tubule specific Jak2 overexpressing and control littermate mice diabetic via the AMDCC low dose streptozotocin protocol, and phenotype them with AMDCC methods for albuminuria, mesangial index, podocyte number, tubulointerstitial fibrosis, creatinine clearance, and mRNA and protein expression in glomeruli and tubulointerstitial tissues for a number of markers such as Jak2, TGF-b, VEGF, nephrin, neph-1, fibronectin, GLUT2, etc. We will also analyze the tissues and urine for markers of oxidant injury (HODEs, dityrosine, etc.), and analyze the urine for changes in metabolites identified in our previous studies of DBA/2J mice (AJP-Renal in revision). Finally, transcriptomic analysis of glomeruli and tubulointerstitial compartments will be performed to compare to human progressive diabetic nephropathy databases.

4. Mechanistic cellular studies to examine effects of Jak2 overexpression. We will finalize our cultured cell line experiments documenting the effects of Jak2 overexpression on murine mesangial cells (ATCC CRL-1927) with STAT tyrosine phosphorylation, TGF-β and fibronectin gene expression, and reversal of such changes by incubation with AG-490, a JAK2/3 inhibitor. Transcriptomic changes will also be analyzed.

2. Collaboration:

With other AMDCC PIs: We continue to work in a highly interdependent manner with the laboratory of Dr. Eva Feldman. Our extensive collaborations were highlighted in the progress section above. We are collaborating with Dr. Coffman in our generation of the 129SvEv mouse lines as noted above. We are finalizing analysis of one model generated during the original AMDCC funding period with Dr. Abel. Dr. Kretzler continues to collaborate closely with Dr. Bottinger.

With Jax: see above under Jak2 transgenic model development.

With the MMPCs: none.

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). We have participated with Dr. Ron Koenig (University of Michigan) on analysis of a potential model of diabetic nephropathy. Brosius has continued close collaboration with Dr. Charles Heilig (University of Chicago) 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:

a. *Studies of Jak/Stat pathways in DN are progressing well. This is a very innovative approach that could also guide future studies within the consortium.*

We appreciate the EAC's support of this work and hope that current progress is also deemed acceptable.

b. *The Volker Haas PT cre (previously used by Coffman and Susztak) should be considered. NIH staff will work to get this animal reposited*.

We very much appreciate this recommendation and will now be using that mouse on the 129 SvEv background for our proximal tubule specific Jak2 transgenic mouse.

c. *We were particularly impressed with the work of Matthias Kretzler and think that this is a significant addition to the consortium*.

Thank you. Dr. Brosius couldn't agree more.

4. <u>Publications:</u>

1. Sullivan KA, Hayes JM, Wiggin TD, Backus C, Oh SS, Lentz SI, Brosius FC III, Feldman EL. Mouse models of diabetic neuropathy, Neurobiology of Disease, 2007;:276-85, 2007.

2. Qian Y, Feldman E, Pennathur S, Kretzler M, Brosius FC III. From Fibrosis to Sclerosis: Mechanisms of Glomerulosclerosis in Diabetic Nephropathy, in press, Diabetes, 2008.

Manuscripts submitted:

1. Berthier C, Zhang H, Schin ML, Henger A, Blattner S, Boucherot A, Carter-Su C, Rastaldi MP, Brosius FC, Kretzler M. Enhanced Expression of JAK-STAT Pathway Members in Human Diabetic Nephropathy.

2. Zhang H, Saha S, Schin ML, Byun[a] J, Kretzler M, Feldman EL, Weil DA, Pennathur S, Brosius FCIII. Rosiglitazone Prevents Renal and Plasma Markers of Oxidative Injury and Reverses Urinary Metabolite Abnormalities in the Amelioration of Diabetic Nephropathy.

3. Wiggin TD, Kretzler M, Pennathur S, Brosius FC, Feldman EL. Rosiglitizone Treatment Reduces Diabetic Neuropathy in Type 1 Diabetes.

4. Blauwkamp MN, Yu J, Schin MA, Burke KA, Berry MJ, Carlson BA, Brosius FC III, Koenig RJ. Podocyte specific knock out of selenoproteins does not enhance nephropathy in streptozotocin diabetic C57BL/6 mice.