Animal Models of Diabetic Complications Consortium
(U01 DK076139)

Annual Report
(2007)

"Recapitulating Transcriptional Pathways of Human Diabetic Nephropathy in Mice"

University of Michigan

Principal Investigator
Frank C. Brosius

Address: 5520 MSRB I, 1150 W. Medical Center Dr., Ann Arbor, MI 48109-0380
Phone: 734-936-5645
E-mail: fbrosius@umich.edu

Table of Contents

Animal Models of Diabetic Complications Consortium
(U01 DK076139)

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 tubulointerstital 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.***

Recent Progress and Major Accomplishments:

Human Studies

While glomerular mesangial expansion and podocyte loss are important early features of diabetic nephropathy, tubulointerstitial injury and fibrosis is critical for the progression of diabetic nephropathy to end stage renal disease. We have therefore analyzed the expression of genes in the tubulointerstitium as well as glomeruli of renal biopsies from diabetic nephropathy patients to identify pathways that may be activated in both regions. Renal biopsies were obtained from 22 patients with early type 2 diabetic nephropathy (microalbuminuria), 12 patients with progressive diabetic nephropathy with urine albumin/creatinine >300 mg/g and tubulointerstitial fibrosis and 12 healthy controls. Glomerular and tubulointerstitial mRNAs were individually microarrayed and underwent bioinformatic analysis. Changes in gene expression were confirmed by real-time RT-PCR. Finally, immunohistochemistry was performed on independent biopsy samples from patients with normal kidneys, progressive diabetic nephropathy, lupus nephritis and hypertensive nephrosclerosis. In this analysis we have found particular increases in expression of several members of the Jak/Stat family in the tubulointerstitium of kidneys with progressive diabetic nephropathy (**Fig. 1**). Jak1-3, and Stat 1

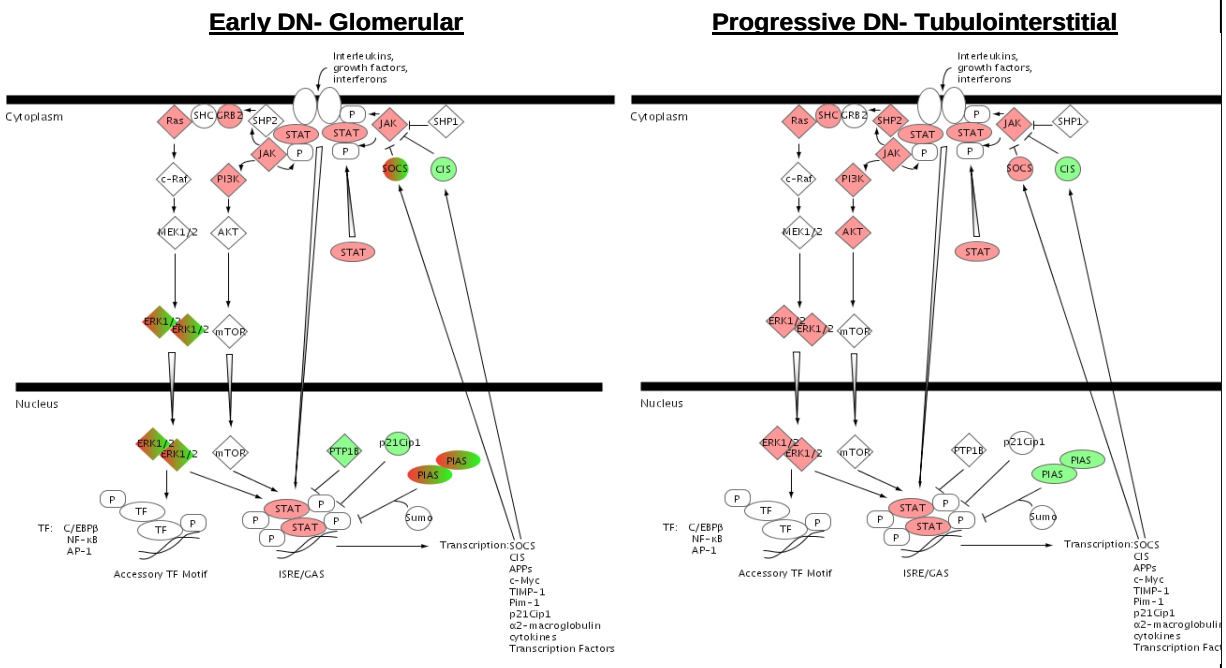

Fig. 1. Changes in mRNA expression for Jak/Stat family members in early and progressive human diabetic nephropathy (DN). Red indicates increased expression; green, decreased expression.

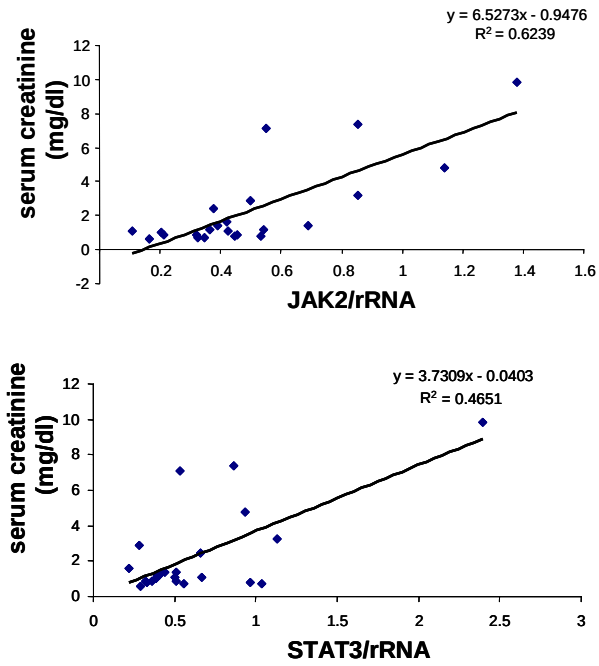

Fig. 2. Correlation of tubulointerstitial Jak2 or STAT3 expression with serum creatinine in patients with diabetic nephropathy.

and 3 were each expressed at significantly higher levels in glomeruli and/or tubulointerstitium of diabetic nephropathy patients. There was a further relative increase in Jak3 expression in the tubulointerstitium in patients with progressive diabetic nephropathy (19-fold). Serum creatinine of the patients with diabetic nephropathy strongly correlated with tubulointerstitial Jak1-3, Stat1 and Stat3 expression (R^2=0.3-0.75) (e.g., **Fig. 2**). Immunohistochemistry (see **Fig. 3**) found a robust increase in Jak3 and Jak2 expression in proximal tubular epithelia and glomerular cells in diabetic nephropathy compared to controls. These data suggest a critical role for the chronic activation of Jak2 and Jak3 signaling pathways in the pathogenesis of progressive diabetic nephropathy in humans.

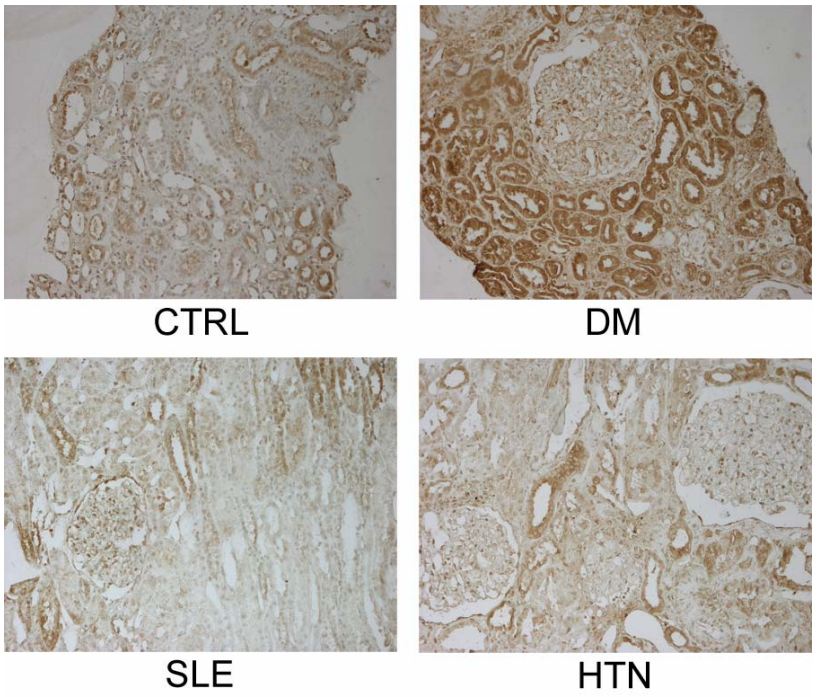

Fig 3. Representative Jak2 immunohistographs on kidney biopsies from patients with no kidney disease (CTRL), progressive diabetic nephropathy (DM), lupus nephritis (SLE), or hypertensive nephrosclerosis (HTN). Jak2 expression is substantially and significantly increased in the proximal tubular cells, glomeruli and interstitium of the patients with diabetic nephropathy and not other progressive kidney diseases.

Animal Studies.

We have now relatively fully characterized Jak/Stat expression in two major mouse models of diabetic nephropathy. In general, mRNA expression for Jak/Stat family members did not increase in glomerular or cortical samples from either db/db BLKS mice (type 2 model) or streptozotocin DBA/2J mice (type 1 model) (e.g., **Figs. 4 and 5**). The only exception to this was a significant increase in Jak3 expression in both cortex and glomeruli from DBA/2J animals (Fig. 6) and a roughly 2-fold increase in Stat1 mRNA expression in glomeruli of DBA/2J mice (not shown).

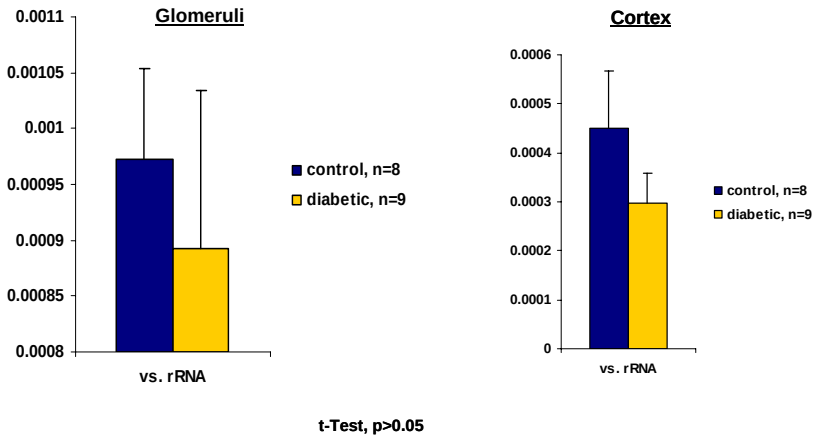

Fig. 4. Real time PCR assessment of Jak2 mRNA expression in DBA/2J glomeruli and cortex from animals 6 months after streptozotocin injection or age-matched non-diabetic controls.

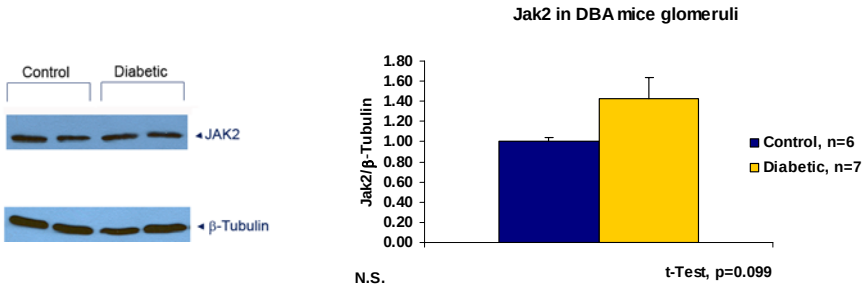

Fig. 5. Immunoblot assessment of Jak2 protein expression in DbA/2J glomeruli and cortex from animals 6 months after streptozotocin injection or age-matched non-diabetic controls.

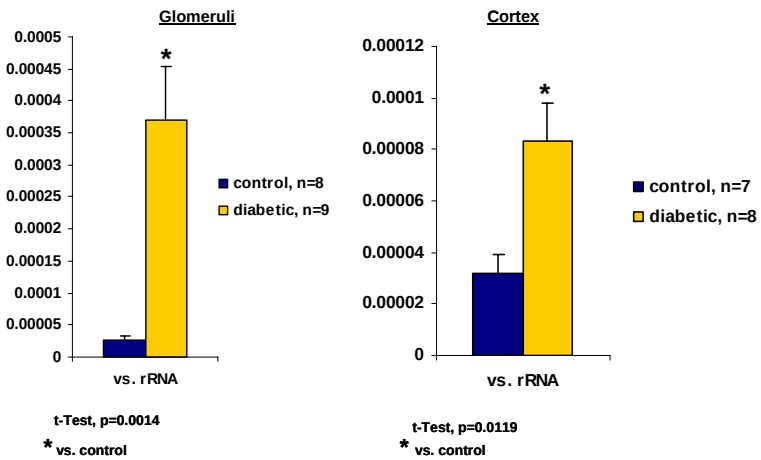

Fig. 6. Real time PCR assessment of Jak3 mRNA expression in DbA/2J glomeruli and cortex from animals 6 months after streptozotocin injection or age-matched non-diabetic controls.

Evidence that Jak2 expression alters cellular responses.

While it is clear that diabetic nephropathy is associated with enhanced expression of many Jak/Stat family members, it is not certain that such enhanced expression has effects on Jak/Stat signaling. As a first attempt to investigate this critical point, we have analyzed the effects of Jak2 overexpression on reactive oxygen species accumulation as determined by DC-DFDA fluorescence, with or without elevated extracellular glucose, in cultured murine mesangial cells. We found that enhanced Jak2 expression was associated with substantial increases in DC-DFDA fluorescence especially in the presence of high glucose in cultured mesangial cells (**Fig. 7**). These results suggest that enhanced Jak2 expression can lead to cellular effects that are likely consequences of enhanced Jak/Stat signaling.

Jak2 overexpression strategy.

At the first steering committee meeting of the new AMDCC, the model selected by the committee was the proximal tubular Jak2 transgenic mouse. To generate this mouse model, we initially generated a conventional trangene for injection utilizing the pRK-5/JAK2 murine cDNA provided by Dr. James Ihle (GenBank accession number 6680507) and the modified SGLT2-Cre construct we

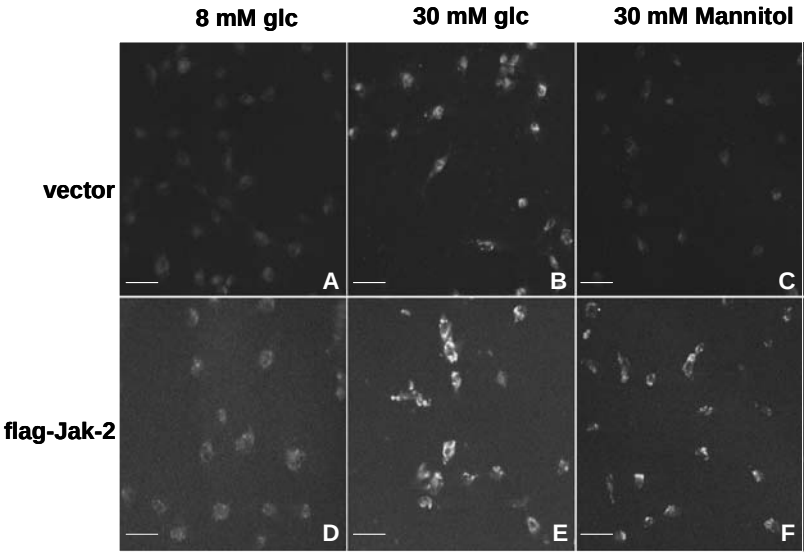

Fig 7. DC-DFDA fluorescence, reflecting accumulation of reactive oxygen species, in cultured murine mesangial cells transfected with a Flag-tagged Jak2 cDNA or with a control vector, and exposed to different levels of extracellular glucose or mannitol for 1 hr. Flourescence intensity was determined using NIH Image J. N = 3 per group; 50 cells were assessed on each slide.

have obtained from Rubera, et al (Specific Cre/Lox recombination in the mouse proximal tubule. *Journal of the American Society of Nephrology. 15):2050-6, 2004*). The construct was easily generated and the primers that we designed were able to detect a copy of single transgene. However, at this time we found that Dr. Katalin Susztak from the Mt. Sinai/Albert Einstein AMDCC group was unable to detect significant cre expression from the SGLT2 construct with the identical promoter sequence as we had utilized. Though she subsequently generated a conventional transgenic line that did show adequate proximal tubule specific expression especially in diabetic mice, we had already embarked on generating a knock-in of the Jak2 cDNA with a Floxed pA Stop cassette in front of the Jak2 sequence into the ROSA26 locus (**Fig. 8**). This will allow us to generate a Jak2 sequence that can be expressed in any tissue and will be regulated by crossing. with tissue specific Cre mice to allow excision of the 5' stop codon and expression of the transgene in the specific cell type of choice. This construct has now been generated, and after sequencing, will be sent to the Mouse Generation and Husbandry Core (MGHC) at The Jackson Laboratory for generation of the targeted line of mice on a FVB background.

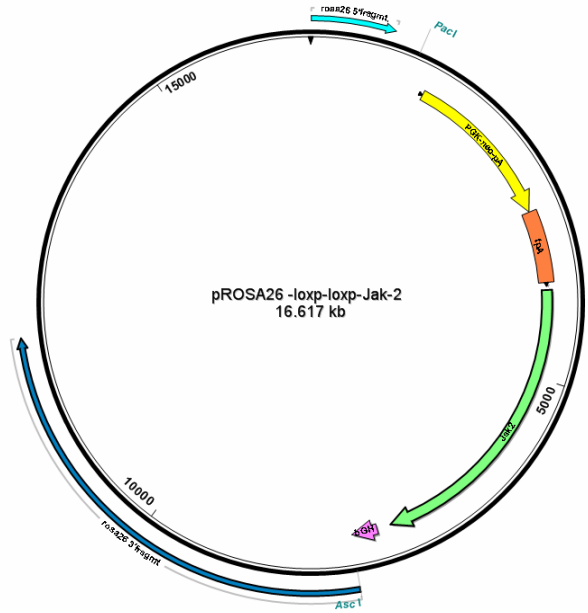

Fig 8. Representation of Jak2 targeting vector which will be used to knock-in the Jak2 cDNA into the Rosa26 locus to ensure robust expression. Tissue specific expression will be controlled by Cre recombinase excision of the upstream floxed TpA cassette containing an in frame stop codon upstream of the Jak 2 sequence.

Plans for the Upcoming Year:

1. **Generation of podocyte and proximal tubular specific Jak2 trangsenic mice**. The targeting vector will be sent shortly to the MGHC. Once a targeted line is established we will cross with a nphs2-Cre

mouse used successfully previously by our laboratory to generate podocyte specific Jak2 overexpressing mice. Similarly, we will cross with the best extant proximal tubule specific Cre mouse (as determined at that time by the Steering Committee) to generated proximal tubule specific Jak2 overexpressing mice. As soon as these animal lines are established, they will be evaluated in diabetic studies for progressive diabetic nephropathy.

2. Mechanistic studies to examine effects of Jak2 and Jak3 overexpression. Cultured cell lines, murine mesangial cells (ATCC CRL-1927) and HK-2 cells (proximal tubule cell line; ATCC CRL-2190) will be transfected or infected with Jak2 and Jak3 vectors to determine effects on downstream signaling as evidenced by Jak2-3 and STAT 1-5 tyrosine phosphorylation, TGF-β and fibronectin gene expression, and reversal of such changes by incubation with AG-490, a JAK2/3 inhibitor. Cells will be exposed to either 30 mM glucose or 6 mM glucose + 24 mM mannitol.

3. **Transcriptomic analyses**. If these initial studies show substantial activation of Jak/Stat pathway signaling, we will perform transcriptomic analyses of the overexpression system (specific Jak and specific cell line) that shows the major activation of downstream signaling. These transcriptomic studies, to be performed in the laboratory of the Co-PI, Dr. Kretzler, will help identify novel or unexplored pathways of signaling that could be important for Jak-Stat effects in progressive diabetic nephropathy.

Preliminary Milestones for 2009 and Beyond:

Likely milestones will be the generation of several tissue specific Jak2 overexpressing lines in several backgrounds that should help determine the role of Jak2/Stat signaling in the pathogenesis of progressive diabetic nephropathy. Similarly, establishment of specific Jak/Stat pathways that appear to be highly correlated with progression will be analyzed.

2. Collaboration:

With other AMDCC PIs: We continue to work in a highly interdependent manner with the laboratory of Dr. Eva Feldman. Models are shared, analyzed together and resources of the two laboratories are combined whenever possible. We are continuing to analyze one model generated during the original AMDCC funding period with Dr. Dale Abel at the University of Utah. We have provided animals, constructs and other advice to Dr. Firouz Daneshgari at the Cleveland Clinic. Dr. Kretzler continues to collaborate closely with Dr. Erwin Bottinger (Mt. Sinai School of Medicine).

With Jax: see above under Jak2 overexpression strategy

With the MMPCs: none so far.

With other non-AMDCC PIs: Dr. Brosius has continued close collaboration with Dr. Charles Heilig on GLUT1 overexpression models of diabetic nephropathy. Dr. Kretzler has collaborations on diabetic nephropathy with numerous investigators internationally.

3. Address previous EAC comments:

NOT APPLICABLE THIS YEAR

4. Publications:

Manuscript in preparation:

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 Jak2 and Jak3 in Human Diabetic Nephropathy.

Abstracts:

1. Anissa Boucherot, Anna Henger, Clemens D.Cohen, MaryLee Schin, Kathleen Burke, Ingrid Bayer, Holger Schmid, Maria P. Rastaldi, Detlef Schlondorff, Frank C.Brosius, Matthias Kretzler. Jak/Stat activation in diabetic nephropathy (DN) in humans but not mice: Transcriptomic analysis of human diabetic nephropathy, American Society of Nephrology, 2006.
2. Celine C. Berthier[1], Anissa Boucherot[1,2], Clemens C. Cohen[2], Anna Henger[1,2], Hongyu Zhang[1], MaryLee Schin[2], Kathleen Burke[2], Holger Schmid[2], Maria P. Rastaldi[3], Detlef Schlondorff[2], Frank C. Brosius[1], Matthias Kretzler[1,2] [for the ERCB consortium]. Jak/Stat regulation in humans with diabetic nephropathy (DN) but not in mice: Transcriptomic analysis of human diabetic nephropathy. *European Renal Cell Study Group (ERCSG), Paris, France, 2007.*