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 previously reported, 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* in 2009.

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. As previously described, we opted for 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 did all the targeting at the University of Michigan but then sent the mice to the MGHC at JAX for backcrossing onto pure 129S6 background as well continued backcrossing of the NPHS2 (podocin) Cre mouse onto the same background. With the researchers at JAX, especially Racheal Wallace, we both mouse lines have been established on a pure 129S6/SvEvTac background. 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 produced Ins2 $^{Akita/+}$ JAK2$^{loxP/loxP}$ mice which are now being crossed to the NPHS2 Cre mice to produce triple heterozygote Ins2 $^{Akita/+}$ JAK2$^{loxP/+}$ NPHS2Cre/+ as well as double heterozygote JAK2$^{loxP/+}$ NPHS2Cre/+ mice for use in our experiments to test the effects of podocyte specific JAK2 overexpression in type 1 diabetes.

Proximal tubule specific cre mice, Tg(PEPCK-cre) have been crossed with stop-flox JAK2$^{loxP/loxP}$ mice, to produce proximal tubule cell-specific JAK2 transgenic mice. Male mice were made diabetic by the AMDCC low dose STZ protocol and were euthanized at around 28-30 weeks of diabetes. Despite initial results to the contrary in nondiabetic female mice (which could not be used for the low dose STZ experiments) the proximal tubule specific JAK2 transgenic male mice had disappointingly small increases (~30-40%) in JAK2 protein expression in kidney cortex regions on immunoblotting. To induce promoter activity of the PEPCK Cre construct, we placed the mice on acidic drinking water (thanks to the suggestion of Dr. Susztak, AMDCC investigator) halfway through the study. Unfortunately, this maneuver did not result in a substantial increase in JAK2 expression. Diabetes, body weight and albuminuria, as expressed by albumin/creatinine ratios, were altered in the diabetic groups but not by the presence of the JAK2 transgene (Figs. 1-3). These findings were not unexpected given that the site of enhanced JAK2 expression was restricted to the proximal tubular. However, initial assessment of the tubulointerstitial area, where some changes might have been expected, also did not reveal obvious differences between the JAK2 diabetic and wild-type diabetic animals.

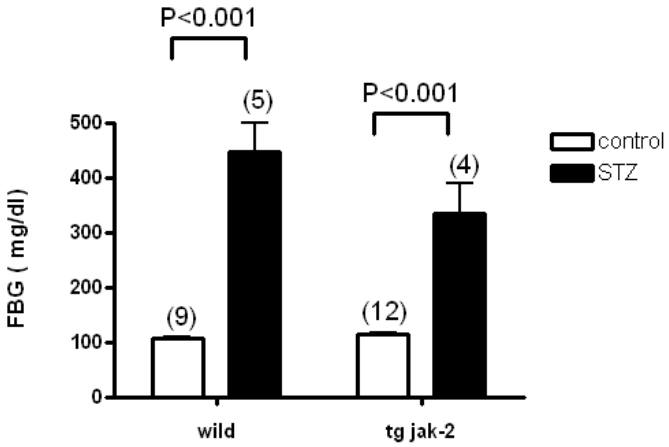

Fig. 1. Fasting blood glucose levels (FBG) in control and low dose streptozotocin-diabetic (STZ) proximal tubule-specific JAK2 transgenic (Tg jak-2) and wild-type male mice.

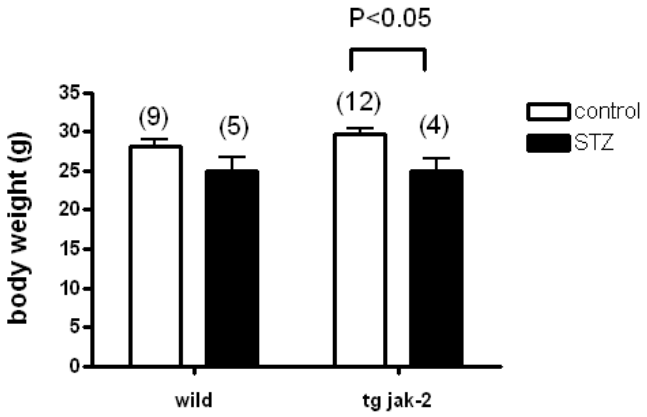

Fig. 2. Body weight in in control and low dose streptozotocin-diabetic (STZ) proximal tubule-specific JAK2 transgenic (Tg jak-2) and wild-type male mice. The diabetic transgenic mice were lighter than their non-diabetic littermates but were no different from the other groups.

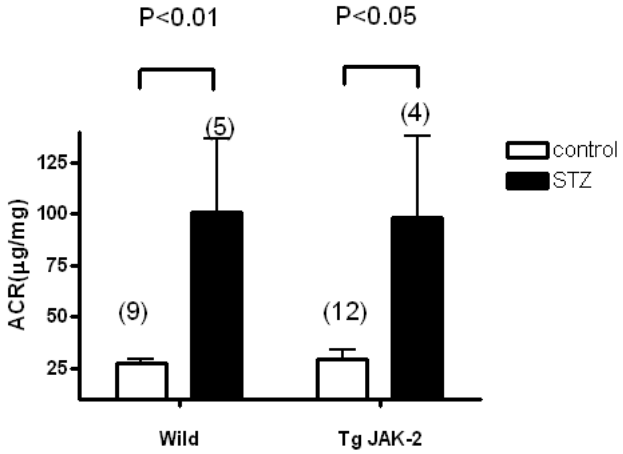

Fig. 3. Albumin/creatinine ratios in control and low dose streptozotocin-diabetic (STZ) proximal tubule-specific JAK2 transgenic (Tg jak-2) and wild-type male mice.

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

Previously, we 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 C57BLKS (BKS) db/db mice retards DN. 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 BKS db/+ background. We recently published the results of our characterization of these lines (epub ahead of print) of these lines. Immunoblots of glomerular lysates showed that transgenic mice had a 3.5-fold (line 1) and 2.1-fold (line 2) increase in GLUT1 content compared to wild-type mice. Both lines showed similar increases in fasting blood glucose and body weights at 24 wk of age compared to wild-type mice. Mesangial index (percent PAS positive material in the mesangial tuft) increased 88% (line 1) and 75% (line 2) in the wild-type diabetic mice but only 48% (line 1) and 39% (line 2) in the diabetic transgenic mice ($p<0.05$, transgenic vs. wild-type mice). This reduction in mesangial expansion was accompanied by a reduction in fibronectin accumulation, and vascular endothelial growth factor (VEGF) levels increased only half as much in the transgenic diabetic mice as in wild-type diabetic mice. Levels of nephrin, neph1, CD2AP, podocin, and GLUT4 were not significantly different in transgenic compared to wild-type mice. Taken together, increased podocyte GLUT1 expression in diabetic mice does not contribute to early diabetic nephropathy; surprisingly, it protects against mesangial expansion and fibronectin accumulation possibly by blunting podocyte VEGF increases. This manuscript was just published in *Am J Physiol*.

4. Transciptomic-metabolomic analysis of streptozotocin-diabetic mouse kidneys.

As a converse to our main hypothesis, changes in gene expression in mouse models of diabetic nephropathy and humans with diabetic nephropathy are likely to overlap significantly. Such changes are more likely to represent alterations that may produce the pathologic conditions found in both humans and mouse models.

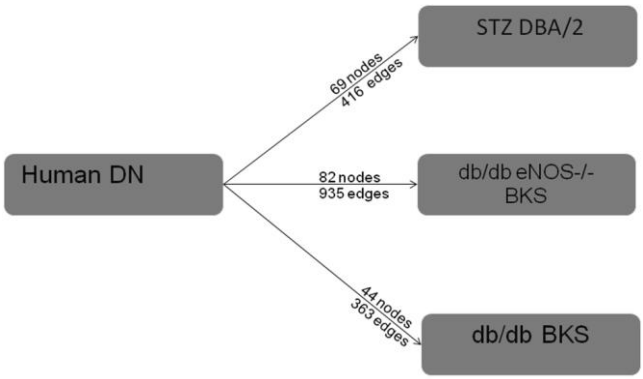

Fig. 4. Overlay of data from humans with type 2 diabetic glomerulopathy and each of the 3 murine models of diabetic nephropathy. The number of nodes (genes) and edges (interactions) for each comparison are noted.

We have finalized the general transcriptomic evaluation of glomeruli from db/db BKS mice, db/db eNOS -/- BKS mice, and streptozotocin-diabetic DBA/2 mice compared to that of humans with early type 2 diabetic nephropathy (Pima Indian cohort). These analyses showed substantial overlap between human and paralogous mouse gene expression. Specifically, the db/db BKS mice showed similar directional expression changes in 789 genes paralogous with those of the type 2 diabetic humans, when compared to nondiabetic control mice and humans, respectively. Glomeruli from eNOS -/- db/db BKS mice showed an overlap with human diabetic expression in 692 genes, and the STZ DBA/2 mice, for which only a limited data set was available, showed 352 overlapping genes with those in human diabetic glomeruli (Fig. 4). More comprehensive pathway comparisons have indicated that each model has 44-92 pathway nodes that are shared with humans with diabetic nephropathy (Fig. 5). When characterized in terms of enriched functional networks the eNOS -/- db/db mice showed extensive inflammation/stress gene activation with moderate amounts of angiogenesis gene activation, whereas

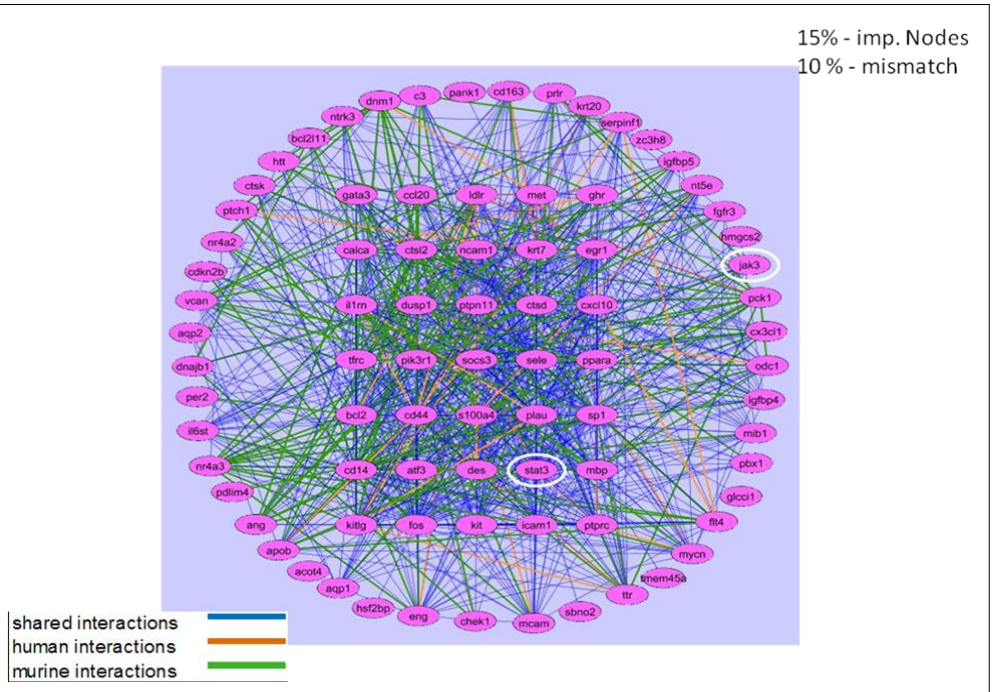

Figure 5. TALE analysis of human type 2 diabetic and db/db eNOS -/- mouse diabetic glomerular gene expression changes showing shared and species specific interactions between nodes in identified pathways altered in both species with diabetic glomerulopathy. The TALE overlay was allowed to have 10% mismatch between nodes and 15% imputed nodes in inter-species comparisons. STAT3, as a central node, and JAK3, as a more peripheral node, are circled as they lend further credence to the participation of the JAK/STAT pathway in progressive diabetic nephropathy in both species. The human-mouse comparisons for the other 2 murine models are not shown.

The top Biological processes enriched in the shared network .(Zscore>4.0)

BKS db/db eNOS -/-	BKS db/db	STZ DBA/2
Hum-eNOS -/-	**Hum-Db/db**	**Hum-STZ**
response to external stimulus	blood vessel development	blood vessel development
positive regulation of MAP kinase activity	vasculature development	vasculature development
cytokine-mediated signaling pathway	transmembrane receptor protein tyrosine kinase signaling pathway	enzyme linked receptor protein signaling pathway
response to wounding	angiogenesis	transmembrane receptor protein tyrosine kinase signaling pathway
response to stress	positive regulation of cellular metabolic process	angiogenesis
inflammatory response	cell surface receptor linked signal transduction	
transmembrane receptor protein tyrosine kinase signaling pathway	inflammatory response	
regulation of MAP kinase activity		

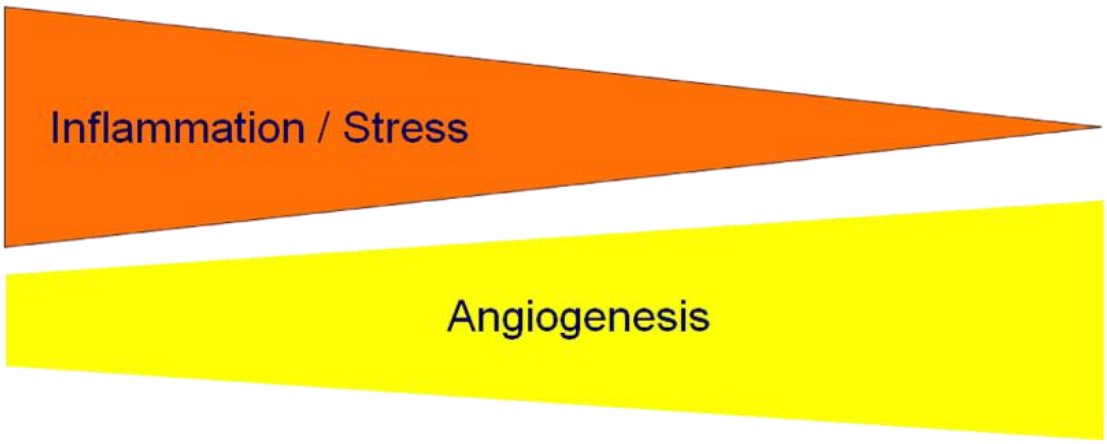

Fig. 6. Functional analysis of enriched networks in the 3 mouse model-human comparisons. Enriched human-mouse networks for each human-mouse model pair were grouped into biological processes and those processes with the highest scores were listed for each of the comparisons under the murine model. The shared human-mouse networks for the STZ DBA/2-human were enriched for those involved in angiogenesis, whereas the shared networks for the BKS db/db eNOS-/- mouse-human comparison were highly enriched in inflammation stress pathways while retaining some angiogenesis pathways. The shared networks between the BKS db/db mouse and humans were intermediate between the others.

the STZ DBA/2 mice showed substantial angiogenic gene activation with little activation of inflammation/stress genes, and the db/db mice were intermediate between the two (Fig. 6).

Plans for the Upcoming Year:

1. **Complete analysis of proximal tubular specific JAK2 trangsenic mice podocyte specific JAK2 transgenic mice**. The proximal tubule-specific and podocyte-specificJAK2 transgenic 129S6/SvEvTac diabetic animals will be fully phenotyped. We will also perform a more careful morphometric analysis of the tubulointerstitial compartment in both models. The JAK2 stop-flox transgene has been crossed onto the Akita/+ background obviating the need for STZ or other manipulations. Again, both models will be on a 129S6 genetic background. Prior to beginning the podocyte-specific trial assessment of JAK2 expression will be performed to ensure at least a 50% increase in glomerular JAK2 expression. If enhancement is <50% we will generate homozygous stop-flox JAK2 animals to boost JAK2 expression. At some point, we would like to combine the podocyte and proximal tubular JAK2 transgenic animals, but this will not be possible during the tenure of this grant.

As long as the at least one of the models shows significant enhancement of diabetic nephropathy, we will perform assessments of urine and plasma metabolites that differ between that model and controls at the end of the study, as we have previously published (1). We will also add in

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, if we have identified an improved model of nephropathy, we will perform an appropriate transcriptomic comparison with human expression data as noted in the preceding section.

2. JAK2 overexpression in HK2 (tubular) cells and mouse mesangial (glomerular) cells. We have established high transfection efficiency systems in both cell systems. We will perform transcriptomic analysis of transient JAK2 transfections of these cell lines to identify the prominent changes of expression in both normal and high glucose media. Networks analysis will be compared to those for glomeruli and/or cortex from the appropriate JAK2 transgenic mice from the previous experiments, as well as from the Pilot and Feasibility study noted below.

3. Continue analysis of human mouse model comparison. Networks which show consistent and highly significant patterns in expression will be systematically evaluated. We will select one to three networks for validation with RT-PCR analysis in one model (probably BKS db/db eNOS-/- mice) as foundations for future studies beyond the tenure of this grant.

2. Collaboration:

With other AMDCC PIs: Drs. Brosius and Kretzler 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 three mouse models, as noted above. Drs. Kretzler and Brosius worked closely with Dr. Ray Harris on the AMDCC project on the transcriptomic analysis of the eNOS -/- db/db mouse glomeruli, reported above, and nerve samples (see separate pilot project progress report). We are also collaborating with Drs. Thomas Coffman and Susan Gurley and the MCHC in the generation of the 129S6 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. Susztak was very helpful in our issues with the PEPCK Cre mouse.

Our AMDCC studies helped launch another collaborative effort to utilize mouse models for systems biological approaches to diabetic complications. Drs. Kretzler, Feldman and myself, along with Dr. Hosagrahar Jagadish, in Electrical Engineering and Computer Science, and Dr. Sub Pennathur, in Internal Medicine, have joined forces (as multiple principal investigators) in a NIDDK sponsored R24 grant entitled, “Integrated Systems Biology Approach to Diabetic Microvascular Complications.” This grant was just funded and will allow us to follow-up on some of the transcriptomic analyses described above to include proteomic and metabolomic validation of pathways involved in progressive diabetic complications and in response to therapy.

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 was published in Dec., 2009 in *J Am Soc Nephrol*.

With other non-AMDCC PIs: See above for the R24 grant collaborations. We work also closely with Dr. Christin Carter-Su (University of Michigan) and members of her laboratory on JAK/STAT signaling aspects, and with Dr. Sub Pennathur (University of Michigan) on oxidative markers, metabolomics and proteomics in diabetic complications. Dr. Brosius has continued close collaboration with Dr. Charles Heilig (University of Florida) on GLUT1 overexpression models of diabetic nephropathy which has resulted in 2 publications in Am J Physiol in 2010. Dr. Kretzler continues collaborations on diabetic nephropathy with numerous investigators internationally.

3. Address previous EAC comments:

2009 comments:

- Presented work to prove that overexpression of Jak2 is critical to DN phenotypes. Reasonable progress in generating Jak2 overexpressing mouse lines.

- Significant progress has been made on all aims of the P&F. The work is ongoing and the functional meaning of the pathway mapping needs to be pursued. Plans for prioritization of follow-up studies are not provided.

- The P&F seems very useful in the longer run but it is very early. The correct or best samples for comparison are an essential problem for this approach. For example, are the Pimas representative enough and which mouse models are best to compare? This is complex stuff and potentially useful but careful consideration of which mouse models to compare with which human samples is crucial.

We thank the committee for their comments and for the support of our general lines of investigation. In terms of prioritization of follow-up studies, we will utilize the TALE analysis to identify novel pathways or networks that are enriched most robustly in the mouse human comparisons, especially those that have not been explored previously, are biologically relevant and technically feasible. Obviously, only a small amount of such follow-up analysis can be done within the tenure of the current grant.

We believe the Pima samples represent a good representation of diabetic nephropathy as this population is relatively untrammeled by the effects of hypertension and vascular disease. Thus, gene expression changes in the kidney are likely to result purely result from diabetes and not cardiorenal disease. We believe that the full analysis of the 3 model comparison should shed some light on which model(s) may be most helpful in future comparisons and in future studies.

4. Publications:

1. 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, Feb;58(2):469-77. (*corresponding author).

2. Brosius FC 3rd, Alpers CE, Bottinger EP, Breyer MD, Coffman TM, Gurley SB, Harris RC, Kakoki M, Kretzler M, Leiter EH, Levi M, McIndoe RA, Sharma K, Smithies O, Susztak K, Takahashi N, Takahashi T; Mouse models of diabetic nephropathy J Am Soc Nephrol. 2009;20:2503-12.

3. Pop-Busui R, Roberts L, Pennathur S, Kretzler M, Brosius FC, Feldman, EL. The Management of diabetic Neuropathy in CKD. Am J Kidney Dis. 2010 Feb;55:365-85

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5. Zhang H, Schin M, Saha J, Burke K, Holzman LB, Filipiak W, Saunders T, Heilig C, Brosius FC III. Podocyte Specific Overexpression of GLUT1 Surprisingly Reduces Mesangial Matrix Expansion in Diabetic Nephropathy in Mice. Am J Physiol Renal Physiol. 2010 Apr 7; [Epub ahead of print].

6. Wang Y, Heilig K, Chen S, Saunders T, Minto A, Deb DK, Schlimme M, Chang A, Xiang M,Quigg R, Brosius F, Heilig CW. Transgenic Overexpression of GLUT1 Mouse Glomeruli Produces Renal Disease Resembling Diabetic Glomerulosclerosis, Am J Physiol RenalPhysiol. 2010 Apr 7; [Epub ahead of print]

5. References

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2. Berthier CC, Zhang H, Schin M, Henger A, Nelson RG, Yee B, Boucherot A, Neusser MA, Cohen CD, Carter-Su C, Argetsinger LS, Rastaldi MP, Brosius FC, Kretzler M: Enhanced expression of Janus kinase-signal transducer and activator of transcription pathway members in human diabetic nephropathy. *Diabetes* 58:469-477, 2009