Animal Models of Diabetic Complications Consortium
(U01 DK060905-10)

Annual Report
(2010)

"Role and Mechanisms of Epithelial Injury in Diabetic Nephropathy"

Mount Sinai School of Medicine
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Part A: Principal Investigator's Summary

1. Project Accomplishments

Role of Mpv17 family members in DN (Bottinger lab)

Role of mitochondrial Mpv17/Htra2 complexes in mitochondrial function and oxidative stress

- Analysis of wt and Htra2-/- MEFs shows increased mitochondrial superoxide production (MitoSox), and increased oxidative damage to mitochondrial DNA (mtDNA) in Mpv17/Htra2-deficient Htra2-/- MEFs.
- Conditionally-immortalized murine podocyte cell lines were established from mice carrying the Immorto transgene with the following Mpv17 backgrounds: +/+, +/-, -/-.

- PLANS
    - Analyze mitochondrial function, oxidative stress responses, mtDNA, metabolism, apoptosis, cell behaviors, etc. in conditionally-immortalized Mpv17 Podocytes under normal and diabetic culture conditions

Conditional deletion of Mpv17 in mice

- Gene targeting vector for insertion of loxP sites flanking exon 3 of murine Mpv17 was generated, sequence-verified, and sent to JAX lab (Rachel Wallace)
- AMDCC approved request to perform gene targeting in ES cells and production of chimeric mice by JAX laboratory. This project is in progress at JAX.

- PLANS

    - Receive chimeras from JAX lab
    - Establish germline transmission and validate Cre recombination at Mpv17 locus
    - Establish NPHS2-Cre / Mpv17$^{fl/fl}$ matings for podocyte-selective excision of Mpv17
    - Analyze phenotype of non-diabetic podocyte-selective Mpv17-/-
    - Establish and analyze STZ-induced T1D model in PodMpv17+/- and PodMpv17-/- mice (anticipated result: acceleration of DN lesions)

Conditional deletion of Mpv17l in mice

- We were unable to achieve germline transmission of targeted floxed Mpv17l allele in Agouti offspring despite exhaustive mating efforts and attempts at germline cell Cre recombination

- PLANS
    - Attempt to achieve Cre recombination to excise selectively Neo cassette by transfection of ES cell clones using Cre recombinase expression vectors

Genetics of glomerular disease susceptibility in muring diabetes models (Bottinger lab)

Diabetes-induced podocyte loss in DBA/2J

- Glomerular transcriptome analysis reveals association of loss of expression of genes functioning in oxidative phosphorylation and mitochondrial function with podocyte loss in DN-susceptible DBA/2J mice with diabetes-induced podocyte loss (DIPL), but not in resistant C57BL/6J mice without DIPL.
- Functional in vivo studies nonphoshorylating (uncoupled) respiration is reduced in glomeruli from DBA/2J, but not C57BL/6J mice, with early onset STZ-induced type I diabetes, consistent with loss of expression of respiratory chain complexes I – V.

- PLANS
    - Examine the effects of systemic antioxidant treatments on podocyte loss, mitochondrial function and DNA oxidation in diabetic DBA/2J mice (anticipated results: improvement of mitochondrial function and prevention of podocyte depletion)
    - Obtain mice with floxed alleles for complex I genes on C57BL/6J background, generate podocyte-selective Complex I heterozygous and homozygous knockout in control and STZ-induced T1D (anticipated results: het deletion of Complex I genes makes resistant C57BL/6J mice sensitive to DIPL and DN)

Identification of genetic factors associated with DIPL in BXD RI strains.

- DIPL is a heritable trait
- DIPL is correlated with increase in glomerular surface area
- DIPL loci map to distal chromosome 13 and 17 (Dipl13 and Dipl17).

- PLANS
    - Fine map and identify causative polymorphism(s)/gene(s) on Chr 13 and 17

Role of Cd36 in tubule-interstitial injury in DN (Susztak lab)

Successful generation and validation of inducible, tubular-specific CD36 transgenic mouse lines

- Tet-O-CD36 transgenic lines were generated and mated with tubular epithelial specific Pax8-rtTA mice to generate bitransgenic Pax8-rtTA/tet-O-CD36 transgenic mie
- Strong human CD36 transgene specific expression (mRNA and protein) was induced by doxycycline administration in mouse chow

- PLANS
    - Phenotype analysis of control and diabetic bitransgenic mice

Analysis of T1D and T2D models on inbred FVB/NJ genetic background

- Robust nephropathy was observed in Ove26 animals, while Akita, STZ, and db/db did not induce significant albuminuria on FVB/N background

- PLANS
    - Publish results

2. Collaborations

Project 1: Mpv17 (Bottinger Lab)
Collaboration with JAX Labs to generate Mpv17 loxP alleles in mice.

Project 2: DN-susceptibility genetics – diabetes-induced podocyte loss (Bottinger Lab)
Collabroation with Matthew Breyer group at Eli Lilly & Co.
Collaboration with Robert Williams group (Genenetworks) at University of Tennessee.

Projec 3: Cd36 (Susztak Lab)
No current collaborations

3. Address previous EAC comments

<u>Bottinger/Susztak October 2009 EAC comments</u>

- Bottinger continues his systems biology approach. He has picked reasonable intermediate phenotypes and appears to be on track. Future plans not in presentation. Susztak builds on her observation that CD36 is upregulated in diabetic tubules and is using renal tubule-specific upregulation of CD36 on diabetes permissive backgrounds to see if tubulointerstitial disease develops. No concerns with either project.

- The in vitro work on the Mpv17 proteins and their role in protection against oxidation induced apoptosis is progressing well. Whether the conditional knockout strategy to generate diabetic nephropathy models from these genes will work seems uncertain at this moment. Does the failure of the Mpv17 KO to develop hyperglycemia also extend to a type two model? Nevertheless, the efforts to generate condition Mpv17 KOs should have high priority compared to identifying substrates for Htra2. In terms of model development wouldn't it be more interesting to identify pathways leading to the low Mpv17 observed in DN?

Bottinger Response:

1. We have not pursued studies using Mpv17-/- in type 2 models because of genetic background issues. Mpv17-/- mice are on a complex mixed background and the db/db model is highly background dependent. Thus, extensive backcrossing would be required just to derived suitable Mpv17-deficient type 2 models.
2. We have prioritized the conditional Mpv17 ko approach (see collaboration with JAX Lab) and have not pursued identification of mitochondrial Htra2 protease substrates, as suggested by the EAC.

3. We agree that the identification of pathways leading to diabetes-induced loss of Mpv17 expression could provide important mechanistic insights in mitochondrial dysfunction, oxidative stress, and podocyte injury in DN. We plan to pursue these studies in the future once we will have ascertained in vivo validation of the mitoprotective role of Mpv17 in diabetes. In vivo validation studies with inducible, podocyte-specific transgenic overexpression of Mpv17 protects against podocyte-injury, podocyte-loss, and/or DN in diabetic models are in progress.

- The observation of podocyte loss in diabetic DBA2 is interesting and the approach of mapping contributing loci using the RI set is reasonable.

Bottinger Response

1. We have made considerable efforts to pursue these studies vigorously and have achieved excellent progress (see Project 2 progress report).

- For the CD36 project things seem to be on track after some setback due to colony problems. The new TA transgenic line may be very useful in the future. The analysis of the FvB background strains also provides useful data.

4. Publications

Original published manuscripts:

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*, 2009.

Original manuscripts under review:

Stefanie Krick, Gabriella Casalena, Ilse Daehn, Liping Yu, Wenjun Ju , Shaolin Shi, Su-yi Tsai, Steven Dikman, Bernd Schröppel and Erwin P Bottinger. Activation of HtrA2/Omi serine protease by Mpv17 in mitochondria protects podocytes against mitochondrial dysfunction and apoptosis. 2010, submitted

Published Abstracts

Poster TH-PO789, 2009 Annual Scientific Meeting of the ASN, San Diego
Activation of interferon and innate immunity pathways, followed by mitochondrial dysfunction and oxidative stress response are characteristic glomerular trancriptome profiles in inbred diabetic DBA/2J mice susceptible to diabetic nephropathy, compared with DN-resistant diabetic C57BL/6J mice
Haiying QI, Shaolin SHI, Yezhou SUN, Weijia ZHANG, Xiaoping LI, Jin CHEN, Taoran ZHANG, Erwin BOTTINGER

Submitted Abstracts

Abstract # 6115 submitted for ASN 2010 Denver
Glomerular mitochondrial dysfunction and oxidative mtDNA damage are hallmarks of susceptibility for podocyte depletion and diabetic nephropathy.
Gabriella Casalena, Ilse Daehn, Haiying Qi, Erwin Bottinger.

Manuscripts in preparation

Qi Haiying et al. Diabetes-induced podocyte loss is associated with impaired mitochondrial respiration, increased mitochondrial superoxide production, and increased oxidation of mitochondrial DNA.

Qi Haiying et al. Identification and characterization of quantitative trait loci for diabetes-induced podocyte loss on chromosome 13 and 17.

Part B: Individual Project Reports by Responsible Investigator

Project 1: "Role Mpv17-family members in DN"
Responsible Investigator: Bottinger, Erwin

Project 2: "Genetics of glomerular disease susceptibility in murine diabetes models "
Responsible Investigator: Bottinger, Erwin

Project 3: "Role of Cd36 in tubulo-interstitial damage of DN"
Responsible Investigator: Susztak, Katalin

Project 1: "Role Mpv17-family members in DN"

Responsible Investigator: Bottinger, Erwin

1. **Project Accomplishments:**

In vitro studies of Mpv17/Htra2 complexes in mitochondrial function and oxidative stress

Conditionally-immortalized murine podocyte cell lines from Mpv17+/+, +/-, -/- backgrounds
We have successfully established these multiple clones from immorto transgenic mice with each of the three Mpv17 genotypes to enable detailed mechanistic and interventional studies to define the functional roles, molecular targets and regulation of mitochondrial Mpv17 in podocytes under control and diabetic conditions. Podocyte lineage of all clones was established and confirmed by WT-1 staining of cultured cells (not shown).

Mpv17/Htra2 complexes protect against mitochondrial superoxide production
Loss of Htra2 sensitizes to increased mitochondrial superoxide production in Htra2-/- MEFs compared with wildtype control MEFs challenged with inhibitors of mitochondrial respiratory chain complexes (Figure 1).

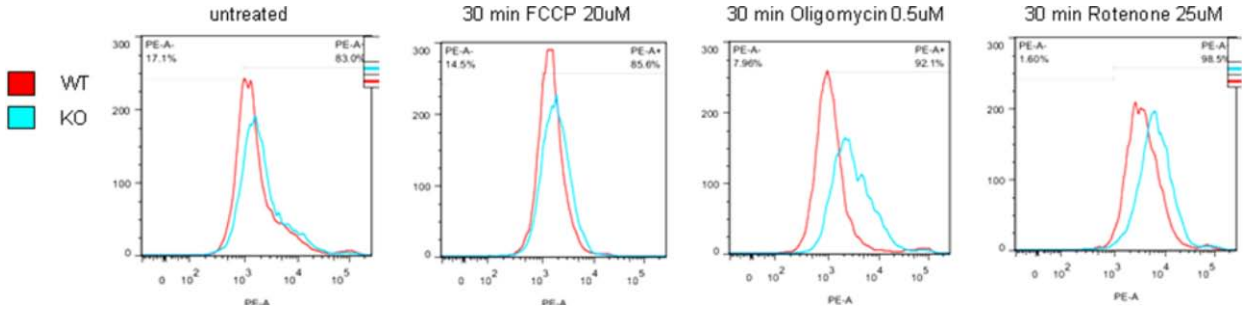


Figure 1

Plans for the upcoming year:

- Analyze mitochondrial function, oxidative stress responses, mtDNA, metabolism, apoptosis, cell behaviors, etc. in conditionally-immortalized Mpv17 Podocytes under normal and diabetic culture conditions

Generation of inducible transgenic and knockout models of Mpv17 in mice

Mpv17 floxed allele

To overcome the previous problem of failure to induce diabetes in conventional Mpv17-/- mice, we are generating mice carrying targeted Mpv17 floxed alleles in ongoing collaboration with JAX lab gene targeting group supported by AMDCC.

Research Design: We have designed the targeting strategy as shown in Figure 2. Five exons encoding ~ 74% of amino acids of Mpv17 protein (131 of 176 aa) which includes all four transmembrane domains will be floxed by loxP sequences and deleted in podocytes in presence of podocyte-specific Cre transgene (NPHS2-Cre). We have successfully made the targeting vector. In May, we have received approval from AMDCC SC and NIDDK to utilize JAX lab gene targeting resources to generate this model. The targeting vector was sent to JAX Lab at the beginning of June to perform gene targeting of the Mpv17 locus in ES cells and to produce chimeric mice using selected ES cell clones with the targeted allele.

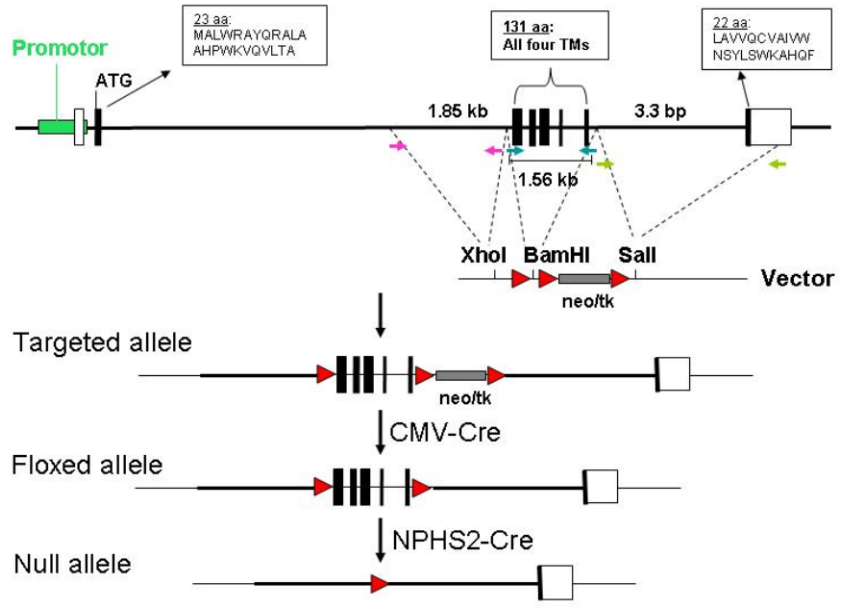


Figure 2.

Plans for the upcoming year:

- Receive chimeras from JAX lab
- Establish germline transmission and validate Cre recombination at Mpv17 locus
- Establish NPHS2-Cre / Mpv17$^{fl/fl}$ matings for podocyte-selective excision of Mpv17
- Analyze phenotype of non-diabetic podocyte-selective Mpv17-/-
- Establish and analyze STZ-induced T1D model in PodMpv17+/- and PodMpv17-/- mice (anticipated result: acceleration of DN lesions)

Mpv17l floxed exon 3 allele

Progress: We had previously generated seven chimeric mice with >50% agouti coat color from three ES-cell lines with targeted insertion of heterozygous loxP-flanked exon3 allele. However, even after six generations of offspring from these four chimeras, we could consistently obtain agouti-coat color offspring, but all carried exclusively wildtype Mpv17l allele. Recent preliminary experiments indicate that the floxed Mpv17l allele may induce toxicity in haploid germ cells. We have then intercrossing germline-active Cre mice with chimeras to attempt excision of loxP-flanked gene regions and/or the Neo-cassette in germ cells. However, this approach also failed to

produce offspring carrying floxed Mpv17l alleles, i.e. all offspring from 10 matings were wildtype at the Mpv17l locus. We are currently as a last-resort approach electroporating Mpv17l floxed ES cells in culture with Cre expression plasmids to eliminate the Neo cassette in vitro. If we succeed, we will use these ES cell clones for repeat blastocyst injections.

Plans for the upcoming year:

- Attempt to achieve Cre recombination to excise selectively Neo cassette by transfection of ES cell clones using Cre recombinase expression vectors

2. Collaboration:

Collaboration with JAX Labs to generate Mpv17 loxP alleles in mice.

Project 2: "Genetics of glomerular disease susceptibility in murine diabetes models "

Responsible Investigator: Erwin Bottinger

1. Project Accomplishments:

The onset of diabetes triggers a significant loss of podocytes in type I and type II diabetic patients [J Clin Invest 99:342; Kidney Int 59:2104], and type I and type II murine models of diabetes [Diabetes, 55:225]. Importanly, the number of podocytes is the strongest predictor of progression of diabetic nephropathy in a cohort of type II diabetic PIMA Indians [Diabetologia 42:1341]. Loss of podocytes in diabetic mice is associated with podocyte apoptosis and can be prevented by anti-oxidant scavengers [Diabetes 55:225]. Similar to differential genetic susceptibility in human diabetic nephropathy, diabetic inbred DBA2/J (D2) mice are more prone to albuminuria and mesangial expansion compared with diabetic C57BL/6J (B6) [Diabetes 54:2628].

During the previous project year, we demonstrated that inbred DBA2/J mice are susceptible and C57BL/6J mice are resistant to diabetes-induced loss of podocytes, suggesting that podocyte loss characteristic of diabetic nephropathy is a quantitative trait controlled by genetic factors.

In the current annual report, we describe new findings suggesting that

- Impairment of mitochondrial respiratory chain complexes and increased mitochondrial superoxide production are linked with DIPL in diabetic D2 mice
- DIPL is a heritable quantitative trait mapping to loci on chromosomes 13 and 17.

Impairment of mitochondrial respiratory chain complexes and increased mitochondrial superoxide production are linked with DIPL in diabetic D2 mice

We used microarrays to characterize glomerular transcriptome profiles associated with differential susceptibility for glomerular manifestations of diabetic nephropathy (DN) in diabetic D2 mice in comparison with DN resistant diabetic B6 mice. Diabetes was induced in 8-week-old D2 and B6 mice by multiple low-dose STZ protocol (AMDCC protocol). Six experimental groups with 9 animals per group included: B6C and D2C (B6 and D2 controls), B6D and D2D (diabetic B6 and D2), B6DI and D2DI (insulin-treated diabetic B6 and D2), respectively.

Time of onset and levels of hyperglycemia (>400mg/dl) were verified by blood glucose measurements. Animals were iron oxide perfused prior to sacrifice after 3 and 6 wks of diabetes, respectively, to enable magnetic separation of glomerular and tubular fractions. Genome-wide gene expression in glomerular fractions was examined using Affymetrix GeneChip platform. Pathway analysis of microarray results from non-diabetic control B6 and D2 demonstrated lower expression of oxidative phosphorylation and mitochondrial dysfunction pathway-related genes in glomeruli at baseline. Highly significant abnormalities in interferon signaling, antigen presentation, innate immunity, and complement system after 3 wks, and mitochondrial dysfunction, oxidative phosphorylation, ubiquinone biosynthesis, and oxidative stress response after 6 wks of diabetes were characteristic pathways in DN susceptible diabetic D2 mice, but not in diabetic B6 mice (Table 1).

Ingenuity Canonical Pathways	***Ratio (reg. genes/total pathway (%))***	
	Down	**Up**
BASELINE STRAIN DIFFERENCES (C57BL/6J : DBA/2J)		
1 Oxidative Phosphorylation	38/166 (23%)	6/166 (4%)
2 NRF2-mediated Oxidative Stress Response	23/185 (12%)	23/185 (12%)
3 Starch and Sucrose Metabolism	12/198 (6%)	13/198 (7%)
4 Fatty Acid Metabolism	12/192 (6%)	20/192 (10%)
5 Mitochondrial Dysfunction	26/172 (15%)	7/172 (4%)
3 WEEKS OF DIABETES (DBA/2J CONTROL : DBA/2J STZ)		
1 Antigen Presentation Pathway	0/39 (0%)	3/39 (8%)
2 Crosstalk between Dendritic Cells and Natural Killer Cells	0/98 (0%)	4/98 (4%)
3 LPS/IL-1 Mediated Inhibition of RXR Function	0/205 (0%)	6/205 (3%)
4 IL-8 Signaling	1/187 (1%)	4/187 (2%)
5 Hepatic Fibrosis / Hepatic Stellate Cell Activation	0/135 (0%)	4/135 (3%)
6 Complement System	0/36 (0%)	2/36 (6%)
7 IL-6 Signaling	0/95 (0%)	3/95 (3%)
8 MIF Regulation of Innate Immunity	0/48 (0%)	2/48 (4%)
9 Dendritic Cell Maturation	0/173 (0%)	4/173 (2%)
10 Myc Mediated Apoptosis Signaling	0/60 (0%)	2/60 (3%)
6 WEEKS OF DIABETES (DBA/2J CONTROL : DBA/2J STZ)		
1 Oxidative Phosphorylation	33/166 (20%)	1/166 (1%)
2 Mitochondrial Dysfunction	29/172 (17%)	3/172 (2%)
3 Nitrogen Metabolism	10/134 (7%)	1/134 (1%)
4 LPS/IL-1 Mediated Inhibition of RXR Function	15/205 (7%)	8/205 (4%)
5 Sulfur Metabolism	6/61 (10%)	0/61 (0%)
6 Xenobiotic Metabolism Signaling	19/294 (6%)	7/294 (2%)
7 Crosstalk between Dendritic Cells and Natural Killer Cells	2/98 (2%)	9/98 (9%)
8 Activation of IRF by Cytosolic Pattern Recognition Receptors	1/73 (1%)	8/73 (11%)
9 Antigen Presentation Pathway	0/39 (0%)	6/39 (15%)
10 Dendritic Cell Maturation	3/173 (2%)	12/173 (7%)

Our findings suggest that activation of interferon and immune pathways, and subsequent mitochondrial dysfunction and oxidative stress response are characteristic glomerular transcriptome signatures in DN susceptible diabetic DBA/2J mice.

Selected mitochondrial pathway genes were independently analyzed using qPCR (see Figure 3).

These included:
anti-oxidant genes

- catalase (Cat),
- superoxide dismutase 2 (Sod2),
- apoptosis-inducing factor, mitochondrion-associated, 1 (Aifm1)

and genes encoding respiratory chain complex proteins, including
Complex I

- NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7 (Ndufb7),
- NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4 (Ndufa4),
- NADH dehydrogenase (ubiquinone) Fe-S protein 3 (Ndufs3),
- NADH dehydrogenase (ubiquinone) flavoprotein 1 (Ndufv1),

Complex III

- ubiquinol-cytochrome c reductase (Uqcr)

Complex IV

- cytochrome c oxidase subunit VIIIA (Cox8a), and

Complex V

- ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit (Atp50).

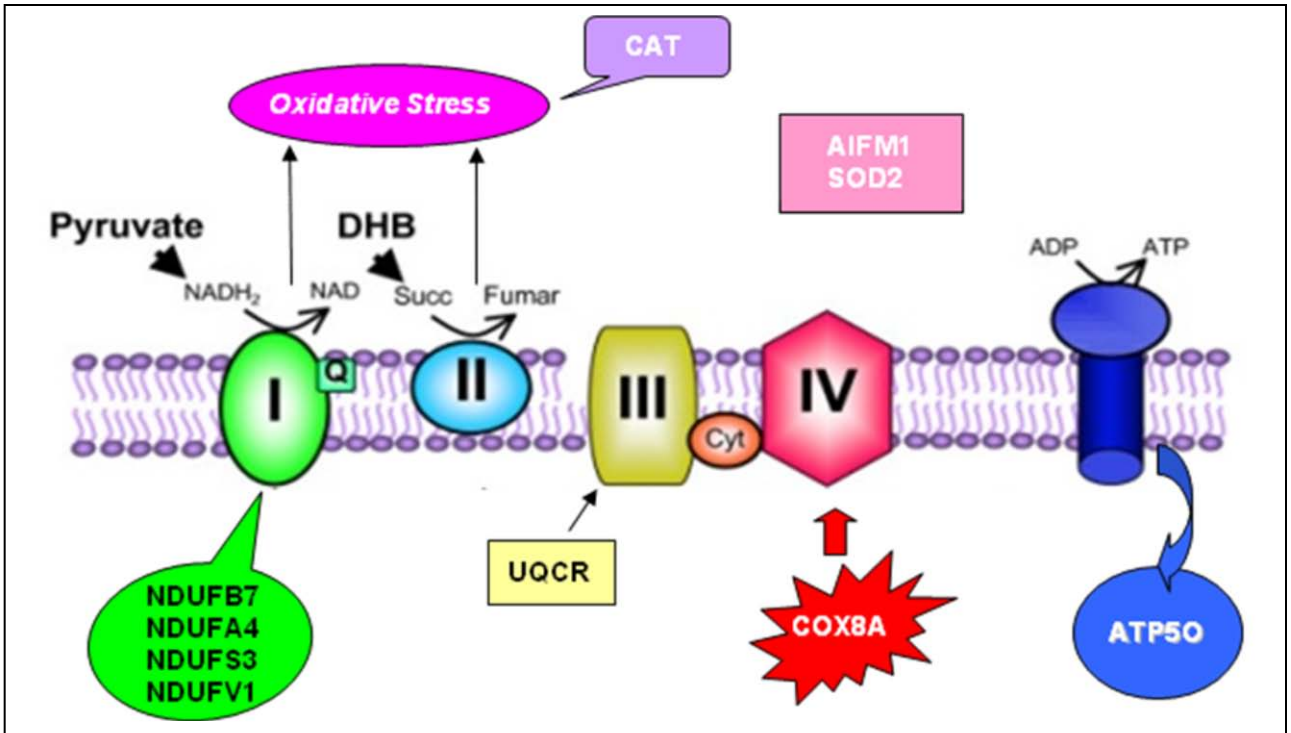


Figure 3

We found decreased levels of all tested gene expression profiles in diabetic D2, but not in diabetic B6 glomeruli exposed to 6 wks of T1D. Importantly, decreased expression was normalized in diabetic D2 STZ mice maintained normoglycemic with continuous insulin pump.

Nonphoshorylating (uncoupled) respiration is reduced in glomeruli from DBA/2J, but not C57BL/6J mice, with early onset STZ-induced type I diabetes.

Gene expression profiling revealed a striking downregulation of transcripts encoding protein complexes of the mitochondrial respiratory chain in DBA/2J but not in C57BL/6J mice (Table 1). Based on these findings, we hypothesized that onset of diabetes induces a reduction of nonphosphorylating (uncoupled) respiration in glomeruli of diabetic DBA/2J mice, whereas uncoupled respiration should not be inhibited by diabetes in C57BL/6J mice. To test his hypothesis, we induced T1D in B6 and D2 mice using the AMDCC low-dose Streptozotocin (STZ) protocol. Glomeruli were isolated by Fe++ bead perfusion protocol from tubular fractions and subjected to analysis of respiratory chain function five days after completion of STZ-treatment (prediabetic state), or seven days after onset of hyperglycemia (early T1D diabetic state). Blood glucose levels were elevated (>400 mg/dl) and comparable between B6 STZ (N=4) and D2 STZ (N=4).

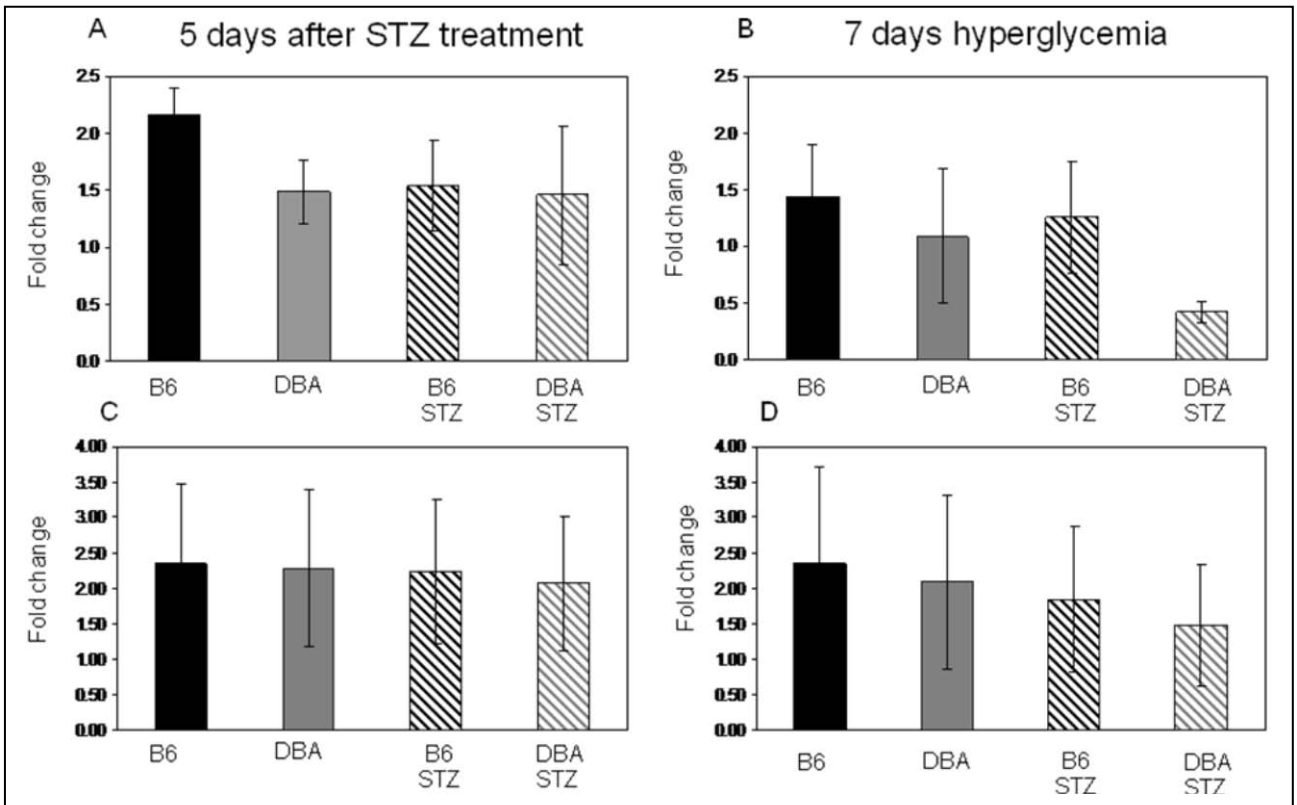


Figure 4. Nonphosphorylating respiration is severely reduced in glomerular preparations from DBA/2J mice after 7 days of STZ-induced hyperglycemia/diabetes. A, C) Average ratio of uncoupled (FCCP) over basal respiration in glomerular (A) and tubular preparations (C) from control B6 and DBA/2J mice, and B6 and DBA/2J mice (B6 STZ, DBA STZ) five days after completion of low dose STZ protocol (post-STZ, prediabetic state). B, D) Average ratio of uncoupled (FCCP) over basal respiration in glomerular (B) and tubular (D) preparation from control B6 and DBA/2J mice, and B6 and DBA/2J mice (B6 STZ, DBA STZ) 7 days after onset of hyperglcycemia/diabetes (~ 12 days after completion of low dose STZ).

Uncoupled respiration (respiratory chain complex function) was not significantly different in glomerular or tubular preparations from B6 STZ, D2 STZ, and control mice immediately prior to onset of hyperglycemia/diabetes (five days after STZ treatment) (Figure 4). After seven days of diabetes, uncoupled respiration was significantly reduced by 58% in D2 STZ compared to non-diabetic D2 controls, whereas respiration was not significantly different in diabetic B6 STZ mice compared with non-diabetic B6 controls (Figure 4B). Interestingly, there was no significant inhibition of uncoupled respiration in tubular fractions from diabetic D2 STZ compared to non-diabetic D2 mice (Figure 4D). We conclude that mitochondrial respiration is dramatically reduced after onset of diabetes in DN-susceptible D2 mice, but not in DN-resistant B6 mice. Furthermore, mitochondrial respiration is not inhibited in tubular fractions of both, B6 and D2 mice, consistent with the absence of tubular histopathology in both parental strains.

PLANS

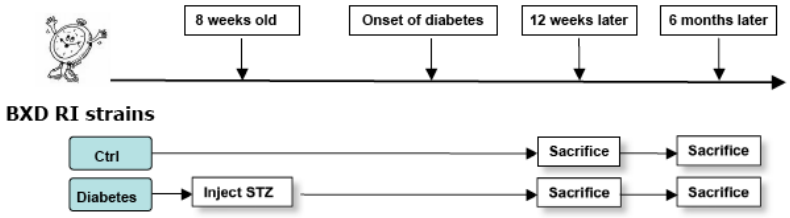
- Examine the effects of systemic antioxidant treatments on podocyte loss, mitochondrial function and DNA oxidation in diabetic DBA/2J mice (anticipated results: improvement of mitochondrial function and prevention of podocyte depletion)
- Obtain mice with floxed alleles for complex I genes on C57BL/6J background, generate podocyte-selective Complex I heterozygous and homozygous knockout in control and STZ-induced T1D (anticipated results: het deletion of Complex I genes makes resistant C57BL/6J mice sensitive to DIPL and DN)

DIPL is a heritable quantitative trait mapping to loci on chromosomes 13 and 17

Since D2 mice are susceptible to diabetes-induced podocyte loss while B6 mice are resistant as shown by our preliminary studies, these two strains as well as the BXD panel are the ideal materials for us to map the QTL of the susceptibility of podocyte loss in diabetes. We have established a collaboration with Matthew Breyer from Eli Lily & Co, and Robert Williams from University of Tennessee Memphis to accomplish this.

For each BXD strain, 7 diabetic mice were generated by STZ treatment and were sacrificed after 6 months of diabetes. Hyperglycemia was monitored by BG measurement and was comparable across strains. The Breyer group at Eli Lily measured UAE and other renal parameter. Our group analyzed glomerular cell phenotypes, including changes in podocyte count, WT1-negative glomerular cells, total glomerular cell count per glomerular section, as well as glomerular section area.

A summary of the experimental protocol is provided here:



Over 40 BXD RI strains were included with 7 diabetic mice/strain (STZ), and 4-5 control mice/strain (no STZ). Eight-week old BXD male mice received low-dose streptozotocin (50 mg/kg) by intraperitoneal injection for five consecutive days according to AMDCC protocol at Oak Ridge Laboratories. Vascular perfusion was performed through left ventricle with 4% paraformaldehyde(PFA) for 3 minutes and 18% sucrose for 5 minutes according to AMDCC protocol. The experimental phase of this work was performed under contract with Eli Lily & Co at Oak Ridge National Labs in collaboration with Dr. Matthew Breyer's group.

Our group received frozen and frozen kidney blocks from all BXD mice (a total of 38 strains completed the protocol) to apply our immunofluorescence based method of glomerular phenotyping, which includes triple-fluorescence staining with anti-WT1, anti-synaptopodin antibodies, and nuclear DAPI staining. Cell count data were uploaded to the GeneNetwork database and applications toolbox for statistical analysis and QTL mapping.

A phenotype correlation matrix comparing multiple quantitative phenotype traits WT1-negative cell count, podocyte count, total glomerular cell count, and glomerular surface area (μm^2) in 38 BXD RI strains and two parental strains exposed to 6 months of T1D was established by Spearman rank correlation and Pearson correlation statistics (Table 2). WT1-negative cell count and total glomerular cell count were highly correlated with each other and with glomerular surface area (GSA), but not with podocyte count (Table 2). Podocyte count was negatively correlated with glomerular surface area (Table 2). These results demonstrate that podocyte count after longterm T1D in BXD strains is an independent quantitative trait, while WT1-negative cell count (mesangial cells and endothelial cells) and total glomerular cell count determines the increase in glomerular volume associated with longterm T1D in some BXD strains. An important implication of these findings is that normalization cell counts for glomerular section area is not permissible.

Table 2

Spearman rank correlation rho

Pearson R

	WT1-negative cells	podocytes	Glomerular cells	Glom surface area (GSA)
WT1-negative cells	1	-0.377	0.895	0.743
Podocytes	-0.499	1	-0.003	-0.641
Glomerular cells	0.908	-0.088	1	0.53
Glom surface area (GSA)	0.773	-0.74	0.529	1

The highly significant inverse correlation between podocyte count and glomerular section area (r = -0.740; P = 7.39E-09) is shown in Figure 5. These results suggest that those BXD RI strains with susceptibility to diabetes-induced increase in glomerular volume are also susceptible to loss of podocytes, similar to parental DBA/2J.

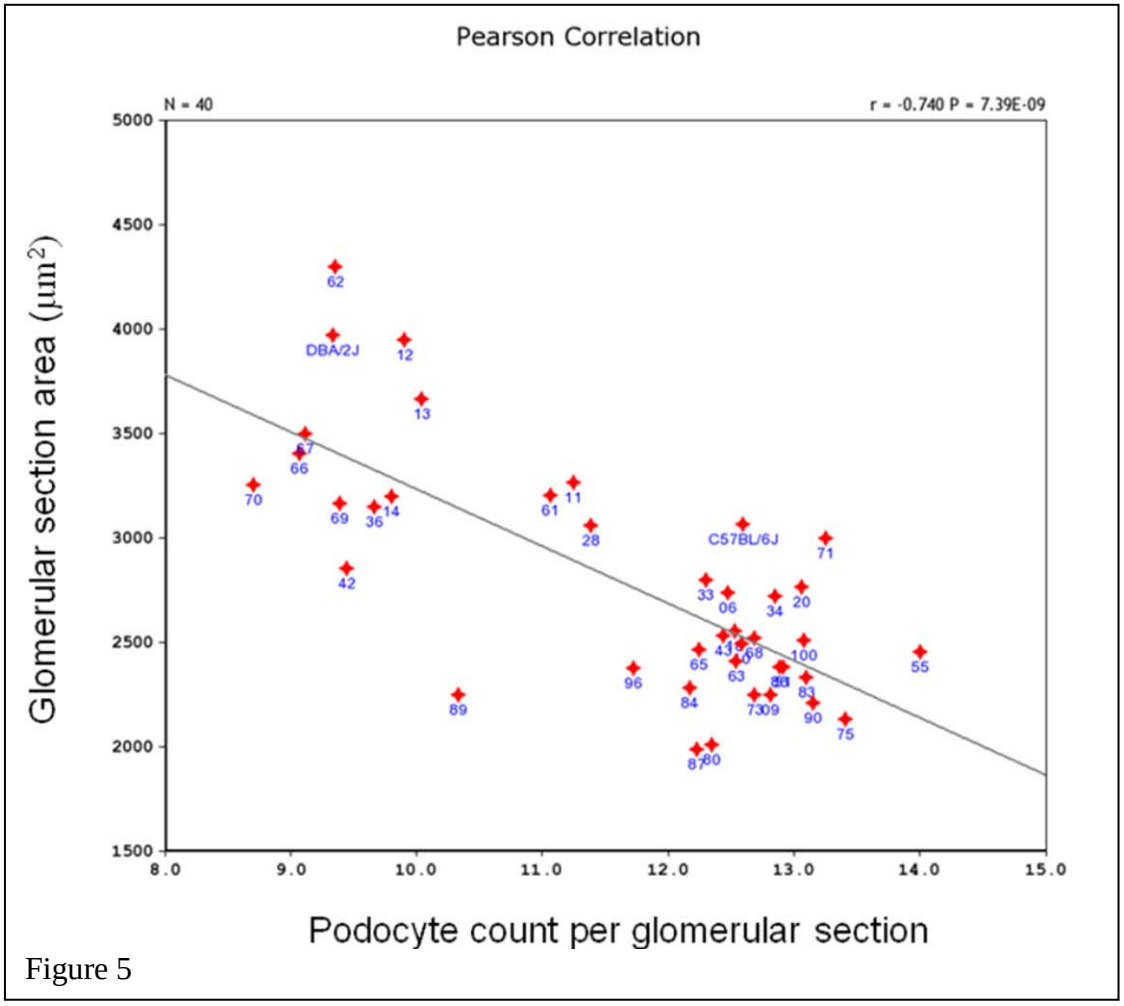


Figure 5

Figure 6 shows that podocyte number after 6 months of T1D in BXD strains can largely be categorized into two groups, resembling either B6 parental mice without diabetes-induced loss of podocytes or D2 parental mice with diabetes-induced loss of podocytes. Furthermore, podocyte count variability within individual BXD strains was much smaller compared with between BXD strain variability, suggesting considerable degree of heritability of diabetes-induced podocyte loss (DIPL).

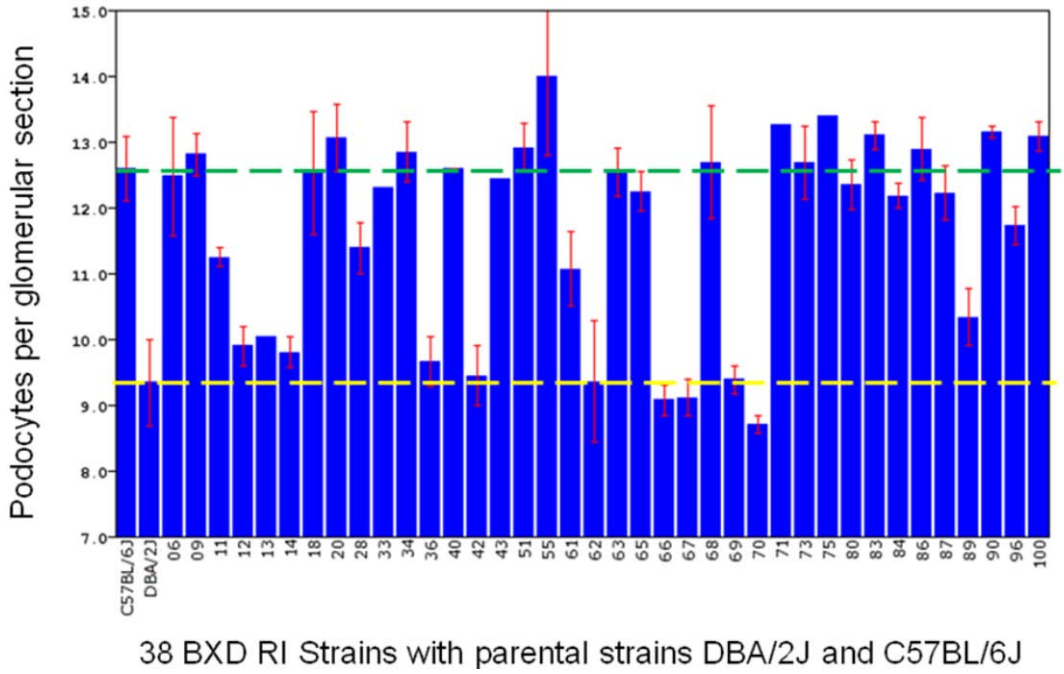


Figure 6

QTL mapping was carried out using WebQTL (www.genenetworks.org). Linkage was calculated across the whole genome using simple interval mapping. Linkage statistics are expressed as a likelihood ratio score (LRS). We estimated genome-wide significance of QTLs by calculating significant (p = 0.05) and suggestive thresholds by permutation analysis. The suggestive threshold represents the approximate LRS that corresponds to a genome-wide p-value of 0.63 and an average of one false positive per genome scan. This threshold is useful because it calls attention to regions of the genome in which additional analysis may be warranted. They are particularly helpful in trait meta-analysis when two different crosses pick up the same suggestive locus.

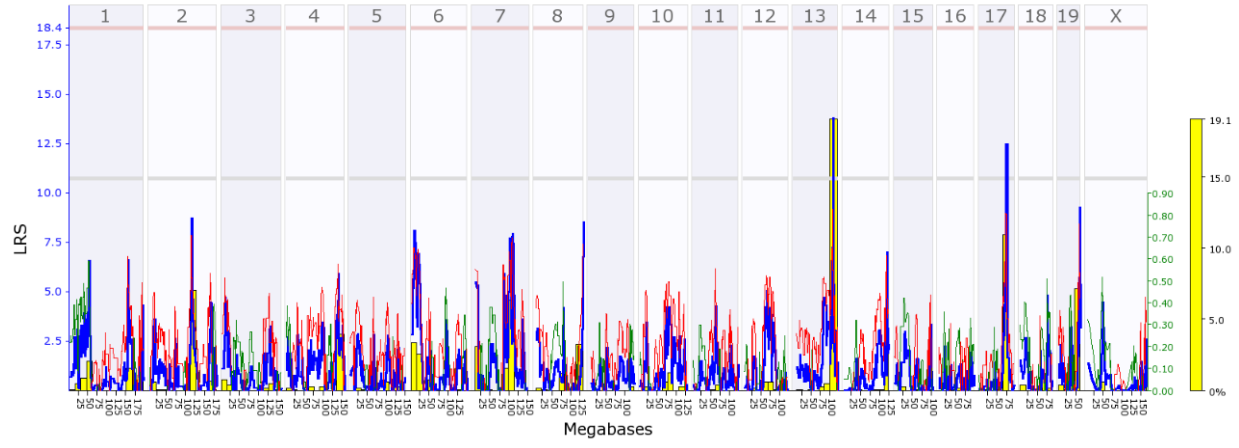


Figure 7

Confidence limits of QTL locations were calculated by mapping bootstrap re-samples of each dataset. In order to perform the bootstrap analysis, we randomly sampled the original data set 2000 times. In a single bootstrap sample, some strains were by chance represented one or more times, while others were not represented at all. We used WebQTL to generate 2000 bootstraps, map each data set, and store the location of the loci with the highest LRS scores. These loci were used to produce the yellow histograms plotted on the QTL maps (Figue 7). If the position of a QTL is robust, the particular composition of the sample will not change the position of the QTL peaks and the histogram of QTLs with high LRS will have a sharp peak.

This analysis shows two suggestive loci for DIPL, including the distal arm of chromosome 13 (Dipl13) and of chromosome 17 (Dipl17) (Figure 7). Detailed analysis of genes and polymorphisms between B6 and D2 genomic sequence localized to these intervals is in progress.

PLANS

- Fine map and identify causative polymorphism(s)/gene(s) on Chr 13 and 17

2. Collaborations

With other AMDCC Members: Matthew Breyer, Eli Lilly & Co
With other non-AMDCC PIs: Robert Williams, University of Tennessee, Memphis

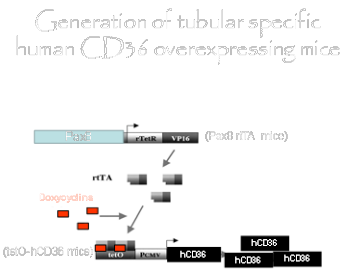
Project 3: "Role of Cd36 in tubulo-interstitial of DN"

Responsible Investigator: Katalin Susztak

1. Project Accomplishments:

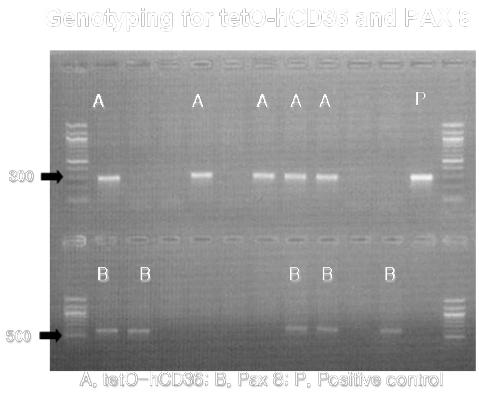
B. Rationale and Relevance

Our preliminary studies showed that as opposed to human diabetic nephropathy the currently examined murine diabetic animal models (db/db and STZ induced diabetes of C57B6J 129SvJ mice) do not develop significant tubulointerstitial fibrosis. We found that expression of a scavenger receptor; CD36 coincided with proximal tubular epithelial cell apoptosis and tubulointerstitial fibrosis in human DNP. CD36 expression was necessary and sufficient to mediate proximal tubular apoptosis induced by glycated albumins and free fatty acid palmitate through sequential activation of src kinase, and proapoptotic p38 MAPK and caspase3. In contrast, paucity of expression of Cd36 in PTEC in diabetic mice with diabetic glomerulopathy was associated with absence of tubular apoptosis and normal tubular epithelium. Mouse PTEC lacked Cd36 were resistant to glycated albumin induced apoptosis. Recombinant expression of CD36 in mouse proximal tubular epithelial cells conferred susceptibility to glycated albumin induced apoptosis. Our findings suggest that CD36 is as essential mediator of proximal tubular apoptosis in human DNP.



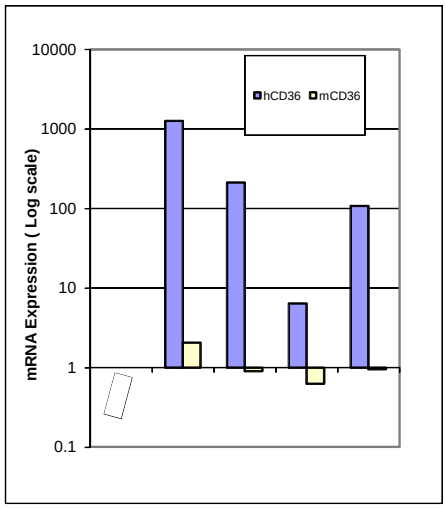
C. Summary of accomplishments

Since the initially proposed sglt2CD36 transgenic model did not result in tubular transgene expression, and after out setback with MPV virus infection of our animal colony, we are now on track with a new transgenic approach to enable inducible tubular expression of Cd36. For example, we generated a new transgenic line where CD36 is under the tetracycline responsive promoter, using the tet-O-CD36 transgenic construct. We crossed these animals with the Pax8-rtTA animals.



We generated tet-O-CD36 trangenic animals via pronuclear injection. The transgenic construct is shown on figure1. We obtained 64 founder lines. Four lines showed positive tail PCR and thereby successful integration of the transgene. All 4 positive lines were mated with Pax8-rtTA animals. A recent publication from the group of Robert Koesters (Nature Medicine Sept 2008) indicates a very strong and widespread expression of rtTA in the renal tubules of these animals. The Pax8rtTA animals were purchased from the Jackson Laboratories.

Double positive transgenic animals were identified by tail PCR, weaned at 3 weeks of age and started on doxycycline containing food at 4 weeks of age. Animals were sacrificed at 7 weeks of age (3 weeks after the initiation of the doxycycline food). Out of four transgene positive lines, three showed increased human CD36 (transgene) mRNA expression and two of them showed positive transgenic protein expression (Figure).
These results indicate the successful generation of tubule specific inducible Cd36 expressing animals.



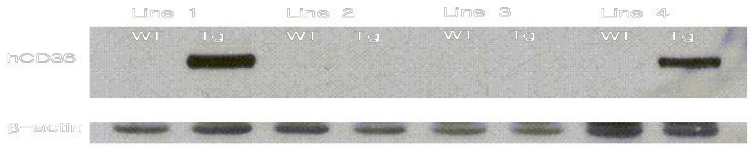
Phenotype analysis of these transgenic animals are in progress

A, Double transgenic animals are placed on doxycyline food at 3 weeks of age, at present they are 16 weeks of age, we observed no obvious phenotype or increased lethality in these animals.

B, In order to analyze the role of CD36 in diabetic nephropathy, animals were made diabetic by multiple low dose streptozotocin injection at 5 weeks of age. For these experiments all animals control (i.e. single transgenic) and double transgenic animals were placed on doxycyline containing food at 4 weeks of age and diabetes was induced at 5 weeks of age. We have 7 animals in each groups i.e. control, control diabetic, double transgenic, double transgenic diabetic. Currently they are about 15 weeks old and we plan to sacrifice them at 24 weeks of age.

C, We are performing in vivo and in vitro studies determining the mechanism of CD36 mediated tubular degeneration are ongoing. We already successfully generated primary tubular cells from wild type mice and from double transgenic animals. We are in the process of determining the expression level of key metabolic enzyme and glucose and lipid metabolite levels.

D, **Additional experiments**



Western Blot analysis of whole kidney lysates with an antibody specific for human Cd36 and beta actin. WT and Tg (pax8rtTA/tetOCd36) animals were sacrificed after 2 weeks course of doxycycline feeding (both wild type and double transgenic mice). Transgenic line1 and 4 showed positive expression of the transgene (cd36)

The CD36 transgenic animals are on FvB genetic background. To analyze the role of CD36 in diabetic nephropathy development we will need to make them diabetic. There are many different diabetic animal models are available on FvB background. Thus we are in the process of performing head to head characterization of the different diabetic animal models on the FvB background. We have analyzed and sacrificed STZ induced diabetic FvB mice, FvB db/db mice, Ove26 transgenic mice on FVB background and we also back-crossed the Akita mutation into the FvB background. Our preliminary results indicate only minor albuminuria and glomerular injury in the FvBAkita and STZ induced FvB mice, db/db mice on FvB background show significantly increased albuminuria similar to the BKlS dbdb mice. We observed the most robust nephropathy in the Ove26 animals. These animals developed high grade albuminuria and significant glomerulosclerosis.

Plans for the coming year

1, First priority for the coming year is the completion of the characterization of the control and diabetic Pax8 rtTA/tet-O-CD36 animals.

2. Collaboration:

No active collaboration