AMDCC Annual Report (2011)

PI: HARRIS, RAYMOND
Project Title: Generating Mouse Mutants with Diabetic Nephropathy
Grant Number: U01 DK061018

Abstract: The goal of the AMDCC is to develop animal models of diabetic complications that faithfully reproduce diabetic complications observed in humans. This proposal will provide a model of mouse model of diabetic nephropathy (DN) focusing on two key protective endothelial pathways: eNOS and prostacyclin synthase (PGIS). These pathways are not only co-localized within the endothelial cells but their activity is also biochemically interrelated through cellular levels of peroxynitrate, and both that have been implicated in human diabetic nephropathy, neuropathy, retinopathy and macrovascular disease. In the previous funding cycle, the Vanderbilt AMDCC site investigated the genetic underpinnings of diabetic nephropathy (DN) of mice. Those studies identified systemic eNOS deletion as a critical genetic modifier that converts C57BL/6 mice from a resistant strain to one that is susceptible to DN. Genetic disruption of endothelial nitric oxide synthase (eNOS or NOSIII), but not ApoE or LDLR was associated with a marked acceleration of DN in C57BL/6, not only characterized by a robust albuminuria, but also by dramatic mesangiolysis and expansion with decrease renal function (GFR). The involvement of eNOS as a clinically relevant modifier for risk of human diabetic nephropathy is bolstered by clinical studies showing that diabetics with an eNOS Glu298Asp polymorphism not only exhibit decreased eNOS activity but also an accelerated risk of renal failure {Noiri, 2002 #6975; Shin Shin, 2004 #8934}. Accumulating evidence implicates endothelial dysfunction in the pathogenesis of diabetic complications, particularly nephropathy and macrovascular disease {Schalkwijk, 2005 #9302}. Similarly polymorphisms have been identified in prostacyclin synthase (PGIS), although their specific role in the progression of diabetic nephropathy has not been established, The present proposal has two specific aims: Aim 1 will determine the role of endothelial eNOS in the progression of diabetic nephropathy; while To determine the role of Endothelial prostacyclin synthase in the progression of diabetic nephropathy. To achieve this we will generate conditionally targeted (floxed) eNOS and PGIS alleles, and cross these mice with a Tie2mERCre mouse, allowing temporally controlled deletion of these alleles specifically from the endothelium. These studies should allow the dissection of the role of these biochemical pathways in the progression of diabetic nephropathy in mice.

1. Program Accomplishments:

Hypothesis #1 Generating a mouse with targeted deletion of eNOS will provide a unique model for the study of diabetic microvascular complications.

Progress toward stated aims: Nitric oxide (NO) generated by eNOS enzyme plays a major role in vascular homeostasis. Further, recent studies of eNOS knockout mice have shown that abnormalities in eNOS expression and activity play a central role in the pathogenesis of diabetic nephropathy (DN). However, there remain uncertainties about the precise role of eNOS in DN. First, eNOS is present in a variety of non-endothelial cell types, including hematopoietic cells, cardiac cells, and renal tubular cells. Second, eNOS knockout mouse have been shown to exhibit congenital vascular and renal anomalies with high frequency, including septal defects, abnormal aortic valve development, and focal renal scars. Thus, a conditional knockout approach to target endothelial eNOS in adult diabetic mice is required to determine the role of endothelial eNOS in DN. To this end, we designed and generated a conditional allele (flox allele) in which the exons 9-12, encoding the sites essential to eNOS function, are excised by Cre-loxP recombination. Correct targeting events were confirmed by a series of Southern blot analysis and genomic PCR. Mice homozygous for floxed eNOS allele (flox/flox mouse) are healthy with morphologically normal kidneys, and their blood pressure and tissue eNOS protein levels do not differ from those in wild-type littermates. To evaluate the exons-deleted eNOS allele (lox allele), we also generated mice homozygous for lox allele (lox/lox mouse) by crossing with Sox2-Cre mice. The correct Cre-loxP recombination was confirmed by Southern blot analysis and genomic PCR. Compared with wild-type littermates, the lox/lox mice showed higher blood pressure levels that are comparable to eNOS -/- mice, and Western blot analysis exhibited deletion of the targeted eNOS protein sequences in tissues. These findings demonstrate that conditional targeting of eNOS gene can be achieved in our floxed eNOS mouse. This model should provide a useful reagent for elucidating cell or tissue-specific eNOS function. Endothelial specific eNOS targeting study is currently underway.

Plans for the year and completion of the project: The eNOS floxed mice are currently being backcrossed to the DBA/2 and 129/sve strains and will then be crossed with Akita+/- mice. At that time the role of endothelial specific eNOS will be assessed using the Sox-2-Cre as described above. In further studies, we will also determine the role of eNOS in kidney thick ascending limb by crossing with a THP-Cre that is available to us.

Hypothesis #2 The eNOS/db/db mouse serves as a valid mouse model of type II diabetic nephropathy and can serve as a platform to test potential novel therapeutic interventions.

Progress toward stated aims: We previously reported that deletion of eNOS in *db/db* mice (eNOS-/- db/db) leads to significant and early onset albuminuria, arteriolar hyalinosis, mesangiolysis and focal segmental and nodular glomerulosclerosis, similar to human diabetic nephropathy. Blood pressure was higher in eNOS-/- db/db mice than db/db mice (146 ± 6 vs. 120 ± 3 mmHg). eNOS-/- db/db mice (8 wks old, albuminuria 837 ± 79 μg/mg Alb/Cr) were randomly divided into 3 groups: vehicle, treatment with the ACEI, captopril or "triple therapy"

(hydralazine, resperine, HCTZ), and the animals were sacrificed after treatment for 12 wks. Captopril treatment led to significant decreases in blood pressure (102 ±5 mmHg), albuminuria (432 ± 101 vs. 2574 ± 974 μg/mg Alb/Cr of vehicle), and glomerulosclerosis index (0.37 ± 0.03 vs. 1.26 ± 0.29 of vehicle), along with less macrophage infiltration and decreased expression of nitrotyrosylated proteins (a marker of oxidative stress), Kim-1 (a marker of renal injury) and CTGF. Triple therapy reduced blood pressure to similar levels in captopril-treated mice (106 ± 4 mmHg). However, triple therapy was less effective in reducing albuminuria (1204 ± 180 μg/mg Alb/Cr) and glomerulosclerosis index (0.58 ± 0.07, P<0.01 vs. captopril group), along with less reduction of macrophage infiltration, nitrotyrosylated proteins, Kim-1 and CTGF. In vehicle treated eNOS-/- db/db, there was increased expression of $p22^{phox}$, a component of NADPH oxidase. Captopril significantly reduced $p22^{phox}$ expression, but triple therapy did not. Therefore, ACEI is more effective than triple therapy in reducing the progression of DN in eNOS-/- db/db mice, indicating an additional role for RAS inhibition in addition to blood pressure control.

Plans for the year and completion of the project*:* These studies indicate that therapeutic interventions known to affect progression of diabetic nephropathy in humans are also effective in this mouse model. We have therefore begun to use this model to test more novel interventions. As one example, we have begun a study of the use of EGF receptor tyrosine kinase inhibitors (erlotinib and gefiitinib). Our preliminary results indicate a potential role to decrease proteinuria in this model. At initiation of treatment (age 12 wks) urinary albumin/creatinine was 2105±437 μg/mg (n=4). After 4 wk treatment, it had decreased to 461±59 μg/mg. We will further assess this effect and determine whether renal histology and markers of injury are altered during progression of disease.

Hypothesis #3 Alterations in superoxide dismutase may be involved in either development or progression of diabetic nephropathy.

Progress toward stated aims: Superoxide dismutase (SOD) is a major defender against superoxide. We have reported that renal SOD1 (cytosolic CuZn-SOD) and SOD3 (extracellular CuZn-SOD) isoenzymes are remarkably down-regulated in KK/Ta-*Ins2*^{Akita} diabetic mice, which exhibit progressive diabetic nephropathy (DN), but not in DN-resistant C57BL/6- *Ins2*^{Akita} (B6-Akita) diabetic mice (JASN 20: 1303, 2009). To determine the role of SOD1 and SOD3 in DN, we generated SOD1, SOD3, and SOD1/3 double knockout B6-Akita mice and investigated their renal phenotype up to the age of 20 weeks. Increased glomerular superoxide levels were observed in $SOD1^{-/-}$ $SOD3^{+/+}$ and $SOD1^{-/-}$ $SOD3^{-/-}$ B6-Akita mice but not in $SOD1^{+/+}$ $SOD3^{-/-}$ B6-Akita mice. In parallel with glomerular superoxide excess, $SOD1^{-/-}$ $SOD3^{+/+}$ and $SOD1^{-/-}$ $SOD3^{-/-}$ B6-Akita mice exhibited increased urinary albumin levels and histopathologically progressive mesangial expansion, yet the severity of DN did not differ in these two groups. Significant differences were not observed in blood glucose, blood pressure, body weight, kidney weight or GFR among $SOD1^{+/+}$ $SOD3^{+/+}$, $SOD1^{-/-}$ $SOD3^{+/+}$, $SOD1^{+/+}$ $SOD3^{-/-}$, and $SOD1^{-/-}$ $SOD3^{-/-}$ B6-Akita mice. Interestingly, glomerular endothelial nitric oxide (NO) levels were markedly reduced in $SOD1^{-/-}$ $SOD3^{+/+}$ and $SOD1^{-/-}$ $SOD3^{-/-}$ B6-Akita mice, indicating that excessive superoxide scavenged NO. In conclusion, the present study demonstrates that deficiency of SOD1 rather

than SOD3 predominantly reduces renal defense capacity against superoxide and accelerates diabetic renal injury, possibly via endothelial dysfunction in DN-resistant mice.

Plans for the year and completion of the project: We will develop transgenic mice specifically overexpressing SOD1 in podocytes or endothelial cells to determine whether amelioration of ROS production in either cell type will slow progression if diabetic nephropathy.

2. Collaborations:

With AMDCC members:

- We have collaborated with Frank Brosius and Mathias Kretzler by isolating glomeruli and generating RNA from the eNOS/db/db mice.
- We have provided eNOS/db/db mice to Frank Brosius to develop a breeding colony at U. Michigan
- We have collaborated with Moshe Levi to test the effect of FXR-TGR5 dual agonist in wild type, db/db and eNOSdb/db mice. Further studies are planned

With Jax:

- We have collaborated closely with JAX in the development of the eNOS floxed mice.

With the MMPCs:

- We utilize the Vanderbilt MMPC for measurement of blood pressure by telemetry and indwelling catheters.

With other non-AMDCC PIs:

- We have collaborated with Paul Voyzen and Billy Hudson at Vanderbilt in proteomic analysis of glomeruli isolated from eNOSdb/db mice.
- We have collaborated with John Iacomini at Tufts by isolating glomeruli and generating RNA from eNOS db/db, db/db and wild type mice at different ages for studies of miRNA expression.
- We have collaborated with Andrew Avani at U. of Toronto by providing him STZ-eNOS and eNOSdb/db mice for study of early podocyte injury in diabetic nephropathy.
- We have collaborated with Francis Farrell at Centicor to test the potential efficacy of antibody therapy against proinflammatory cytokines in the eNOSdb/db model.

3. Address previous EAC comments:

- **Have you uploaded all of your published data on the eNOS models?**

It is in the process of being uploaded.

- ***Where does the CD1 P&F story stand? Was this ever published?***
  We have not yet published it, although we plan to submit an abstract for the upcoming ASN meeting.

- **Significant progress over last year. Clear plan for upcoming year. We anticipate that experimental goals will be achieved**. As indicated in the write-up above, we continue to make progress toward the stated goals of the grant.

- T***issue-specific deletion of eNOS is under way to allow studies of diabetes in the upcoming year. Work with the db/db mouse shows that eNOS is decreased and COX2 is increased; a COX2 inhibitor worsened diabetes. Not surprisingly, captopril ameliorated nephropathy in db/db eNOS KO mice. Work with Akita SOD1 KO, Akita SOD3 KO and Akito double KO support a role for SOD1 in protection. In the upcoming year older mice will be studied, as well as db/db SOD1 KO mice. The Harris group is making excellent progress, with a strong publication record. We wonder if increased expression of SOD1 in appropriate locations, perhaps via a transgenic approach, might ameliorate nephropathy?*** As noted in plans for hypothesis "3, we are considering developing transgenic mice with either selective podocyte or selective endothelial overexpression in order to determine the tissue specific role of reactive oxygen species in development of diabetic nephropathy.

- ***Below is a list of your AMDCC publications from the website. Should any publications be added or subtracted? Has all of the relevant data from these publications been uploaded to the website? Please work with Dr. Rick McIndoe to ensure that the website and database are up-to-date and complete***. We have one manuscript that has been just been accepted in JASN on the interactions of RAS, COX-2 and the prorenin receptor in mouse models of diabetic nephropathy. In addition, we currently have three additional manuscripts under review (on the role of RAS inhibition in the eNOS db/db model, the role of SOD1 vs SOD3 in diabetic nephropathy and the early podocyte injury in diabetic models with eNOS deficiency). We will submit these to the AMDCC website.

1. Mouse Models of Diabetic Nephropathy:A Midstream Analysis from the Animal Models of Diabetic Complications Consortium
   Frank C. Brosius IIIa, Charles E. Alpersb, Erwin P. Bottingerc, Matthew D. Breyerd, ThomasM. Coffmane, Susan B. Gurleye, Raymond C. Harrisf, Masao Kakokig, Matthias Kretzler, Edward H. Leiterh, Moshe Levii, Richard A. McIndoej, Kumar Sharmak, Oliver Smithiesg, Katalin Susztakl, Nobuyuki Takahashig, Takamune Takahashif
   Journal of the American Society of Nephrology : JASN, 2009 (20(12)), 2503 - 2512
2. Reduction of renal superoxide dismutase in progressive diabetic nephropathy.
   Fujita H, Fujishima H, Chida S, Takahashi K, Qi Z, Kanetsuna Y, Breyer MD, Harris RC,

Yamada Y, Takahashi T
Journal of the American Society of Nephrology : JASN, 2009 (20(6)), 1303 – 1313

3. Distinct roles for basal and induced COX-2 in podocyte injury.
   Cheng, H, Fan, X, Guan, Y, Moeckel, GW, Zent, R and Harris, RC
   JASN 20:1953-62, 2009
4. S6 kinase 1 knockout inhibits uninephrectomy- or diabetes-induced renal hypertrophy.
   Chen, J-K, Chen, J, Thomas, G., Kozma, SC and Harris, RC
   Am. J. Physiol (Renal)297:F585-93, 2009
5. Single amino acid substitution in aquaporin 11 causes renal failure.
   Tchekneva EE, Khuchua Z, Davis LS, Kadkina V, Dunn SR, Bachman S, Ishibashi K, Rinchik EM, Harris RC, Dikov MM, Breyer MD
   Journal of the American Society of Nephrology : JASN, 2008 (19(10)), 1955 - 1964
6. Insight into the genetics of diabetic nephropathy through the study of mice.
   Breyer MD, Qi Z, Tchekneva EE, Harris RC
   Current opinion in nephrology and hypertension, 2008 (17(1)), 82 - 86
7. Examining diabetic nephropathy through the lens of mouse genetics.
   Breyer MD, Tchekneva E, Qi Z, Takahashi T, Fogo AB, Harris RC
   Current diabetes reports, 2007 (7(6)), 459 - 466
8. Deficiency of endothelial nitric-oxide synthase confers susceptibility to diabetic nephropathy in nephropathy-resistant inbred mice.
   Kanetsuna Y, Takahashi K, Nagata M, Gannon MA, Breyer MD, Harris RC, Takahashi T
   The American journal of pathology, 2007 (170(5)), 1473 - 1484
9. Genetics of diabetic nephropathy: lessons from mice.
   Breyer MD, Tchekneva E, Qi Z, Takahashi T, Fogo AB, Zhao HJ, Harris RC
   Seminars in nephrology, 2007 (27(2)), 237 - 247
10. Nitric oxide stimulates cyclooxygenase-2 in cultured cTAL cells through a p38-dependent pathway.
    Cheng HF, Zhang MZ, Harris RC
    American journal of physiology. Renal physiology, 2006 (290(6)), F1391 - F1397
11. Expression of mediators of renal injury in the remnant kidney of ROP mice is attenuated by cyclooxygenase-2 inhibition.
    Cheng H, Zhang M, Moeckel GW, Zhao Y, Wang S, Qi Z, Breyer MD, Harris RC
    Nephron. Experimental nephrology, 2005 (101(3)), e75 - e85
12. Diabetic nephropathy: of mice and men.
    Breyer MD, Böttinger E, Brosius FC, Coffman TM, Fogo A, Harris RC, Heilig CW, Sharma K
    Advances in chronic kidney disease, 2005 (12(2)), 128 - 145
13. Mouse Models of Diabetic Nephropathy
    MATTHEW D. BREYER, ERWIN BÖTTINGER, FRANK C. BROSIUS, III, THOMAS M. COFFMAN, RAYMOND C. HARRIS, CHARLES W. HEILIG, AND KUMAR SHARMA (FOR THE AMDCC)
    Journal of the American Society of Nephrology : JASN, 2005 (16), 27 – 45