Animal Models of Diabetic Complications Consortium
(U01 DK076133)

Annual Report
(2008)

"Adiponectin and Nox4 in Diabetic Kidney Disease"

University of California, San Diego
Thomas Jefferson University

Principal Investigator
Kumar Sharma

Address: 9500 Gilman Drive, Stein Building Suite 405-7
Phone: 858-822-0870
Fax: 858-822-7483
E-mail: kusharma@ucsd.edu

Table of Contents

		Page
Part A:	**Principal Investigator's Summary**	**3**
1.	**Project Accomplishments (2008)**	**4-5**
2.	**Collaboration**	**5-6**
3.	**Address previous EAC comments**	**6-7**
4.	**Publications**	**6-7**
Part B:	**Individual Project Reports by Responsible Investigator (if applicable)**	**N/A**
Project 1 (if applicable): "Title" Responsible Investigator: Name		**X**
Project 2 (if applicable): "Title" Responsible Investigator: Name		**X**
Project 3 (if applicable): "Title" Responsible Investigator: Name		**X**

Animal Models of Diabetic Complications Consortium
(U01 DK076133)

Part A:

Principal Investigator's Summary

1. Program Accomplishments:

We have made significant progress in our AMDCC unit despite a cross country move of our group from Thomas Jefferson University in Philadelphia to UCSD in California. Our original proposal sought to determine the role for adiponectin and Nox4 in diabetic kidney disease. **Our hypothesis was that adiponectin and Nox4 are key modifier genes for diabetic nephropathy and diabetic cardiovascular complications.** As described in the last annual report, the two new mouse models of Diabetic Nephropathy and Vascular Complications that we proposed were the:

a. diabetic adiponectin KO mouse
b. an inducible tissue specific Nox4 transgenic diabetic mouse

Recent Progress and Major Accomplishments

Project a. We have completed the characterization of renal function in the adiponectin KO (*Apn* KO) mouse with and without early diabetes. We found that the *Apn* KO mouse has an elevated level of albumin/creatinine as early as 4 weeks of age. In association with albuminuria there is foot process fusion and increased Nox4 in the podocytes of the *Apn* KO mouse. Treatment with exogenous adiponectin reversed the changes in the *Apn* KO mouse. The major signaling pathway that appeared to mediate the protective effect of adiponectin is the AMPK pathway which is stimulated via the Adipo R1 receptor in podocytes. **This data is of critical importance in the understanding of how early changes of kidney disease are regulated by adipokines in states of insulin resistance and obesity.** Furthermore, with development of diabetes with the low dose streptozotocin protocol, the *Apn* KO mice develop a marked increase in albuminuria and increased urine hydrogen peroxide levels. The marked increase in albuminuria occurs within 4 months of diabetes and is much greater than what is seen in the wild type diabetic mouse. That the marked albuminuria occurs in the C57Bl6J *Apn* KO mouse suggests that the loss of adiponectin plays a major role in podocyte response to hyperglycemia, even in the disease resistant C57Bl6 strain. This data has now been published in the ***Journal of Clinical Investigation***.

Ongoing studies will characterize the effect of chronic diabetes (6-12 months) on diabetic kidney disease in the *Apn* KO mouse. This data will be presented at the Steering Committee Meeting in June of 2008. Of major interest is the degree of glomerular volume increase that is present in the Apn KO mouse with and without diabetes. As glomerular volume increase is characteristic of obesity related kidney disease and diabetic kidney disease, we have formed a collaboration with Roland Blantz and Volker Vallon at UCSD to study the hemodynamic profile of these mice with and without diabetes. We are also studying the effect of diabetes using crossing of the *Apn* KO mouse with Akita in both C57Bl6 and the newly available Akita in the DBA2 background. Several groups have expressed interest in working with the *Apn* KO mouse and we have coordinated the transfer of the mice to the Jackson Labs with the support of Larry Chan of Baylor University, who created the mice, and Chris Ketchum. Of note, there have been several clinical studies that have linked adiponectin levels with varying stages of kidney disease in relation to obesity, hypertension, and diabetes. Furthermore, there has been linkage of *Apn* SNPs with human diabetic nephropathy in patients with type 1 diabetes. Thus there is likely to be a

very close translational connection of our studies identifying the mechanism of adiponectin's effect on glomerular podocytes with human disease. Indeed, we found that African Americans with obesity, who are at high risk of kidney disease, have a strong negative correlation between albuminuria and adiponectin levels. Additional translational studies will be pursued with various collaborators both within and external to the AMDCC.

Project b. The Nox4 transgenic mouse was originally proposed to be vascular smooth muscle specific based on our findings that Nox4 was increased in the vascular smooth muscle cells of the diabetic kidney in rats. However in the decorin KO diabetic mice, there was marked upregulation of Nox4 in mesangial cells and podocytes. In addition, podocyte Nox4 was increased in the *Apn* KO mouse and may thus play a major role in the early podocyte dysfunction associated with obesity and diabetes. Support for this concept come from the studies by Bottinger's group showing that albuminuria can be decreased with apocynin (Diabetes 2006), an NADPH oxidase inhibitor. If podocyte Nox4 is a major source of free oxygen radicals in podocytes then it would be appropriate to study a podocyte specific Nox4 transgenic mouse. We have recently begun a collaboration with Dr. Tom Leto of the NIH who had developed a murine construct for a tet regulated Nox4 transgene. We received this construct but had difficulty demonstrating robust regulation of the expression of Nox4 in 293 cells. We have therefore initiated the construction of a new construct in our lab to generate a tagged human Nox4 in a tet-inducible system using the new tight vector from Clontech. We have tested this construct in stably transfected 293 cells expressing the CMV-P-rtTA plasmid (Figure 1). Our preliminary data demonstrate that doxycycline stimulation stimulates Nox4 expression and there is functional activity of HA-Nox4 in response to doxycycline (Figure 2 and 3, respectively).

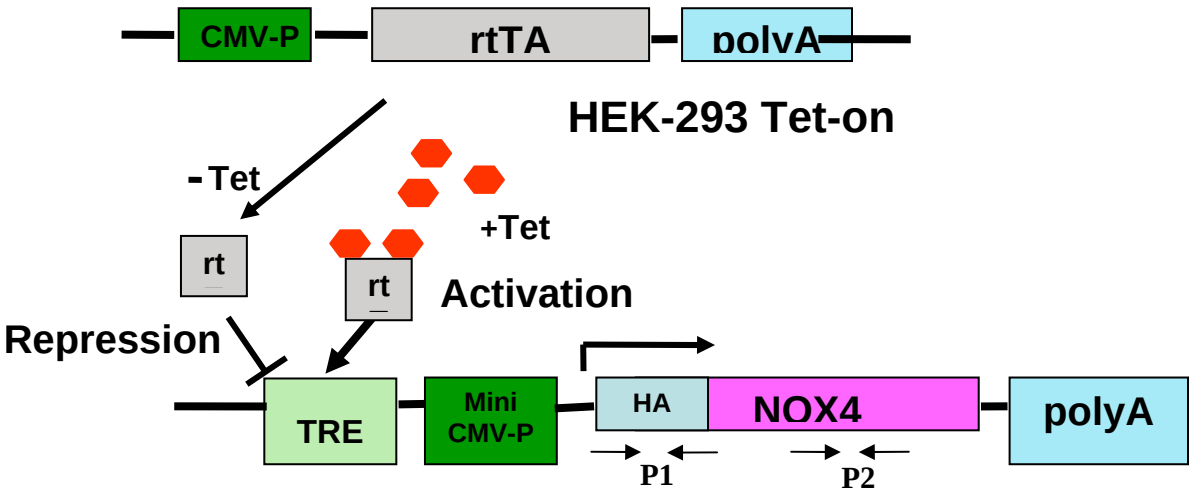

Figure 1. Generation of NOX4 in Tet inducible cell system. Construction of HA-tagged human Nox4 cDNA into Tet-on inducible pTRE system.

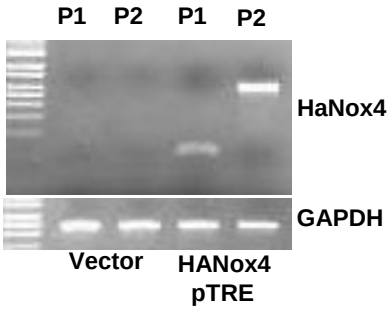

Figure 2. RT-PCR demonstrating expression of HA-tagged Nox4 in 293 cells. In cells transfected with empty vector there is no expression of the HA-tagged Nox4 (primers P1 for HA-Nox4) or endogenous Nox4 (P2, internal primers). In cells transfected with HA-Nox4 there is the appropriate sized band for HA-Nox4 with either set of primers.

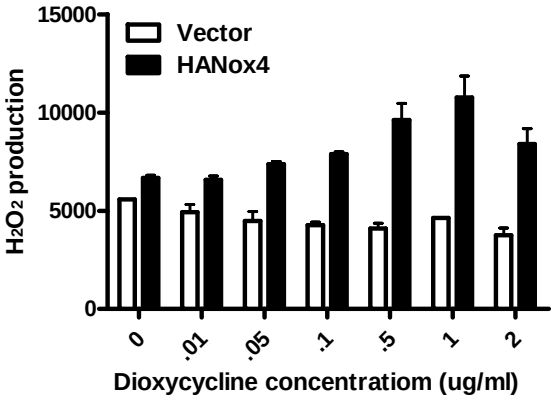

Figure 3. HA-tagged Nox4 is functional in 293 cells. In cells transfected with HANox4 there is a dose-response relationship with doxycycline for hydrogen peroxide production as an index of Nox4 activity. The peak concentration is at 1 ug/ml of doxycycline. There is no dose-response relationship in cells transfected with empty vector. Hydrogen peroxide measured with Amplex Red assay.

Thus, the results of our new studies demonstrate that our new construct is able to be regulated and is functional. The availability of anti-HA antibodies will allow us to track the transgene in vivo and localize cell specific expression. We are completing construction of a Flag-Nox4 construct as well and will test which of the two constructs have the best expression pattern in response to doxycycline. We are thus in position to submit this construct to Jackson Labs and will await the generation of the transgenic mouse. The overall project has been difficult due to the lack of cell survival with marked overexpression of Nox4. Overexpression of Nox4 may induce senescence and/or apoptosis. This may be of critical importance and relevance to podocytes in diabetic kidney disease. We recently demonstrated that podocytes express Nox4 (Figure 4) and Nox4 is stimulated by high glucose in podocytes (Figure 5):

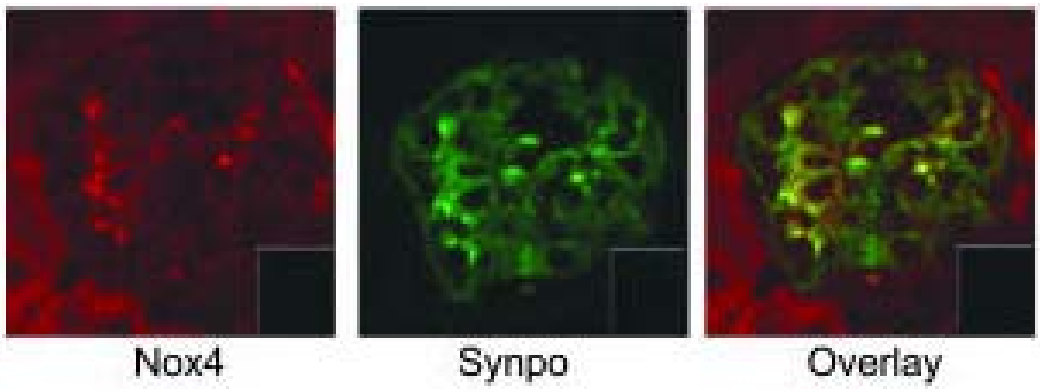

Figure 4. Nox4 expressed in podocytes of normal mouse glomeruli. Double staining with antibody to Nox4 (left panel) and Synaptopodin (center panel) demonstrated co-localization (right panel) in frozen tissue of normal mouse glomeruli. Insets in bottom right of each panel are negative controls.

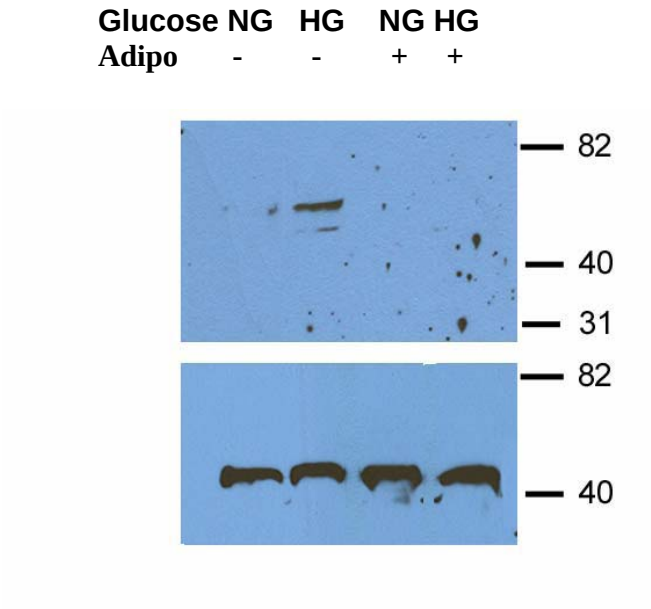

Figure 5. High glucose stimulated Nox4 in podocytes. Transferred proteins were immunoblotted with antibody to Nox4 (upper panel, band between 40 and 82 kDa) and β-actin (lower panel). Adiponectin is able to suppress Nox4 levels in podocytes.

Based on these results and our prior published data demonstrating stimulation of Nox4 protein in diabetic glomeruli (**Am J Pathol**. 2007 Nov; 171(5):1441-50), we have proposed a mouse model where we will specifically stimulate Nox4 in podocytes after the onset of diabetes using the tet-on inducible mouse crossed with the podocin rtTA mouse. By administering doxycycline to the mouse after the onset of diabetes, we will be able to determine if podocyte Nox4 is sufficient to result in podocyte dysfunction and dropout. Furthermore, the use of the inducible Nox4 mouse can be utilized by other members of the consortium to determine the role of Nox4 in specific cell types involved in diabetic retinopathy, neuropathy and cardiomyopathy and cardiovascular disease.

Plans for the Upcoming Year

We will submit the tagged inducble Nox4 construct to Jax by end of the present year and expect to have the transgenic mouse by end of year 3. Subsequent studies will study the effect of temporal upregulation of Nox4 in podocytes before and after onset of diabetes. By performing these studies in the C57Bl6 mouse we will be able to determine if selective alteration of podocyte function in the C57Bl6 mouse is sufficient to induce progressive nephropathy in this background.

Using the tagged Nox4 inducible constructs we will also examine the post-translational regulation of Nox4 in 293 cells, mesangial cells, and podocytes in response to high glucose and growth factors (TGF-b, TNF-a).

We will also characterize the Akita diabetic *Apn* KO mouse. Thus far there has been severe abdominal pathology in the Akita/Apn +/- mice. This observation will be confirmed in subsequent litters to determine if severe diabetes with relative adiponectin deficiency is a good model for diabetic gastropathy. The kidney disease has not as yet been evaluated in the Akita *Apn* KO diabetic mouse. The preliminary studies suggest that relative adiponectin deficiency (heterozygous Apn mice) is sufficient to develop severe diabetic end-organ complications when

crossed with Akita. These studies will likely have immediate clinical relevance as adiponectin deficiency is correlated with development of cardiovascular complications in type 1 diabetic patients as well as in patients with chronic kidney disease.

We have begun to establish new phenotyping expertise in several areas that will be of relevance to the AMDCC after the transfer to UCSD. In collaboration with Dr. Robert Naviaux, who is a leading member of the Mitochondrial Medicine Society, we have developed methodology for the accurate and quantitative characterization of mitochondrial functional and mtDNA damage indices. We have also begun a collaboration with Dr. Laura Dugan to comprehensively evaluate ROS formation in vivo using DHE labeling and paramagnetic spin resonance techniques. As these methods are optimized they will be made available to the AMDCC.

Preliminary Milestones for 2009 and Beyond
By 2009 we expect that the podocyte specific Nox4 inducible mouse will be generated. We will study the regulation of Nox4 in cell culture in relation to high glucose using our new constructs. The Akita x Apn KO mouse will be established and phenotyping of the diabetic complications will be inititated. By 2010 we will have completed the first round of phenotyping of the diabetic inducible Nox4 mice and the Akita Apn KO diabetic mice. In 2011 and 2012 the Apn and Nox4 transgenic mice will be studied in the DBA background and renal and cardiovascular complications will be investigated. The mice will be available for additional studies that will likely include retinopathy and neuropathy. Additional studies with fat feeding will determine the role of adiponectin and Nox4 in development of obesity related complications.

2. Collaboration:

With other AMDCC PIs
We have begun a collaboration with Dr. Oliver Smithies. Based on our recent studies we have identified that there are mitochondrial functional and mtDNA changes in diabetic kidneys. To determine if mtDNA damage may result in enhanced diabetic renal pathology we are studying the mitochondrial polγ KO mice. These mice will be made diabetic with Akita and/or STZ and phenotyped in collaboration with the Smithies lab.

We have also begun a pilot proposal with Dale Abel and the Smithies group to submit a proposal to characterize mtDNA damage and biomarkers in diabetic mice. Mitochondrial DNA damage will be characterized by measuring kidney and urine exosomal fractions by real time PCR with primers specific for mitochondria. Urine and kidney biomarkers for TGF-b1, CTGF, MCP-1, fetuin A and hemopexin will be studied as recent human studies have suggested these are potential useful biomarkers. As diabetic mice are well characterized, samples of urine and kidney tissue will be a valuable source to determine if levels of these candidate markers will provide useful and relevant information for human disease.

During the past year we are completing a sub-contract with Dr. Bottinger and the Mt. Sinai group. The double KO decorin/LDL receptor mouse has been studied with diabetes. Surprisingly this mouse did not develop a severe renal phenotype. High fat feeding in these mice led to an increase in albuminuria. The data is being finalized and will be submitted for publication in

2008. We hope to initiate studies with Dr. Moshe Levi with respect to fat feeding studies in future studies, possibly with the adiponectin KO mouse.

With Jax We have arranged for the transfer of the Adipo KO mice (with Larry Chan) and the podocin-rtTA mice (with Jeff Kopp) to Jackson Labs. As we are now in the final stages of the tagged inducbile Nox4 construct we will submit to Jackson Labs and concurrently develop the transgenic mouse at UCSD.

With the MMPCs At this point we have not yet sent samples to the MMPCs. We understand that the Washington group will be doing phenotyping of kidney histology and we plan to use these facilities to validate our scoring methods. We have developed our own scoring system in collaboration with a pathologist at UCSD, Dr. Andrew Mizisin. We look forward to collaborations with MMPC to formalize criteria for pathology scoring.

With other non-AMDCC PIs Drs. Barry Goldstein and Dr. Kevin Williams of Thomas Jefferson University have been co-investigators in our proposal. Dr. Goldstein recently announced his resignation from TJU and Dr. Williams will be taking over his role in his grants. As Dr. Williams has been a long-time collaborator with Dr. Sharma there should be no overall change in the collaboration. Dr. Williams will work with Dr. Sharma to help study the effect of diabetes on cardiovascular phenotype in the AdKO mice.

3. Address previous EAC comments:

EAC comments 9/2007

a. Progress has been slowed by Dr. Sharma's change in insititutions, although he has remained productive. A new model (tet-inducible podocyte deletion of *Nox4*) has been proposed. This model is inherently less interesting across complications. Will this be done first on B6? And then DBA? Get Nox 2 linkage stock from JAX?

b. Your decorin and adiponectin k/o's should be reposited with JAX by Spring 2008.

Response:

a. Our progress was delayed due to the move to UCSD and the necessity of decreasing the size of our mouse colonies. However we are now fully operational and all our mouse lines have been expanded (Apn KO, Akita). We have trained new personnel in our phenotyping methods and established the HPLC-creatinine protocol at UCSD. We feel that the new model that we proposed (tet-inducible Nox4 upregulation) is of much greater benefit to the consortium and the scientific community than the prior model that we had proposed (SM22a-Nox4 transgenic). The new model will allow temporal regulation of Nox4 in a cell-targeted manner. We have now established the construct and have shown doxycycline regulated gene expression and functional activity. The construct is also tagged to allow for discriminate identification of the transgene in tissues. We chose to initially study podocyte specific regulation of Nox4 due to our exciting data demonstrating an increase in Nox4 in podocytes stimulated with high glucose and Nox4 expression in mouse glomerular podocytes (**J Clin Invest.** 2008 May;118(5):1645-56, **Am J Pathol**. 2007 Nov; 171(5):1441-50.). Additionally, in coordination with data from other members of the consortium it is clear that podocyte function is critical in the development of

diabetic nephropathy and may also contribute to the strain differences of diabetic nephropathy between C57Bl6 and DBA mice. Finally, the availability of new cell specific rtTA mice for other target tissues will make this mouse a very valuable addition to the AMDCC. This will facilitate the study of Nox4 in relation to cardiac and retinal complications as specific promoters already exist to express rtTA in cardiac and retinal cells.

b. The adiponectin KO mice were generated by Larry Chan at Baylor University. Dr. Chan has agreed to reposit the mice to Jackson Labs and this is underway. The transfer has been coordinated by Chris Ketchum. The decorin KO mice were generated by Dr. Renato Iozzo at Thomas Jefferson University prior to the first funding period of the AMDCC. Although I had requested Dr. Iozzo to reposit the mice to Jackson Labs the final decision rests with Dr. Iozzo. This information has been transmitted to Chris Ketchum. We have also made arrangements with Dr. Kopp of the NIDDK to reposit the podocin-rtTA mice to Jackson Labs.

4. Publications 2007-8:

1. Tchekneva, E., Rinchik, E., Polosukhina, D., Kadkina, V., Dunn, S., **Sharma, K.,** Qi, Z., Fogo, A., Breyer, M.. Generation of dominant ENU-induced mutations that predispose mice to diabetic nephropathy. ***Journal of the American Society of Nephrology***, 18:103-12, 2007

2. Zhu, Y., Usui, H., **Sharma. K**. Regulation of Transforming Growth Factor-beta in Diabetic Nephropathy: Implications for Treatment in Diabetic Nephropathy. ***Seminars in Nephrology***, invited review Mar;27(2):153-60, 2007

3. Scalia R, Gong Y, Berzins B, Zhao LJ, **Sharma K**. Hyperglycemia Is a Major Determinant of Albumin Permeability In The Diabetic Microcirculation: The Role of {micro}-Calpain. ***Diabetes***, 2007 Apr 19; [Epub ahead of print]

4. Zhu, Y., Usui, H., **Sharma. K**. Regulation of Transforming Growth Factor-beta in Diabetic Nephropathy: Implications for Treatment in Diabetic Nephropathy. ***Seminars in Nephrology***, invited review Mar;27(2):153-60, 2007

5. Saltzman HE, **Sharma K,** Mather PJ, Rubin S, Adams S, Whellan DJ. Renal dysfunction in heart failure patients: what is the evidence? **Heart Fail Rev.** 2007 Mar;12(1):37-47.

6. Kalantar-Zadeh K, Kopple JD, Regidor DL, Jing J, Shinaberger CS, Aronovitz J, McAllister CJ, Whellan D, **Sharma K**. A1C and survival in maintenance hemodialysis patients. **Diabetes Care**. 2007 May;30(5):1049-55.

7. Ramachandra Rao SP, Wassell R, Shaw MA, **Sharma K**. Profiling of human mesangial cell subproteomes reveals a role for calmodulin in glucose uptake. Am J Physiol Renal Physiol. 2007 Apr;292(4):F1182-9.

8. Williams, K., Qiu, G., Zhu, Y., Dunn, S., McCue, P., Bottinger, E., Iozzo, R**., Sharma, K.** Decorin deficiency enhances progressive nephropathy in diabetic

mice. **Am J Pathol**. 2007 Nov; 171(5):1441-50.

9. **Sharma, K**, Rao, S., Qiu, G, Zhu, Y., Rao, S., Kataoke, H., Dunn, S., McCue, P., Chan, L., Falkner, B., Goldstein, B . Adiponectin regulates albuminuria and podocyte function in mice. **J Clin Invest.** 2008 May;118(5):1645-56.

Animal Models of Diabetic Complications Consortium
(U01 XX#####)

Part B:

Update by Individual Project Leaders
(not applicable)

Project 1 (if applicable): "Title"

Responsible Investigator: Name

1. Project Accomplishments:

Hypothesis

Recent Progress and Major Accomplishments

Plans for the Upcoming Year

Preliminary Milestones for 2009 and Beyond

2. Collaboration:

With other AMDCC PIs

With Jax

With the MMPCs

With other non-AMDCC PIs

3. Publications:

Please list

Project 2 (if applicable): "Title"

Responsible Investigator: Name

1. Project Accomplishments:

Hypothesis

Recent Progress and Major Accomplishments

Plans for the Upcoming Year

Preliminary Milestones for 2009 and Beyond

2. Collaboration:

With other AMDCC PIs

With Jax

With the MMPCs

With other non-AMDCC PIs

3. Publications:

Please list

Project 3 (if applicable): "Title"

Responsible Investigator: Name

1. Project Accomplishments:

Hypothesis

Recent Progress and Major Accomplishments

Plans for the Upcoming Year

Preliminary Milestones for 2009 and Beyond

2. Collaboration:

With other AMDCC PIs

With Jax

With the MMPCs

With other non-AMDCC PIs

3. Publications:

Please list