AMDCC Annual Report (2011)

PI: LEVI, MOSHE
Project Title: Novel Models of Diabetic Nephropathy
Grant Number: U01 DK076134

Abstract: Background: In a) OVE26 mice with type 1 diabetes, b) C57Bl/6 mice with diet induced obesity and insulin resistance, and c) db/db mice with type 2 diabetes mellitus, we have found increased renal expression of the transcriptional factors, i) the sterol regulatory element binding proteins 1 and 2 (SREBP-1 and SREBP-2), and ii) the carbohydrate response element binding protein (ChREBP), which result in increased synthesis and accumulation of triglyceride and cholesterol. The lipid accumulation is associated with development of robust glomerulosclerosis, tubulointerstitial fibrosis, and proteinuria. We have also found that the Farnesoid X Receptor (FXR) is highly expressed in the kidney and the expression of FXR and its target enzymes is decreased in the diabetic kidney. Furthermore we have determined that FXR is an important regulator of SREBP-1 and ChREBP expression as well as oxidative stress, advanced glycation end products (AGEs/RAGE), pro-inflammatory cytokines, and fibrosis inducing growth factors. **Hypothesis:** Based on these finding we propose that FXR plays an important role on the pathogenesis of diabetic nephropathy. We hypothesize that deletion of FXR will markedly enhance and overexpression of FXR will attenuate diabetic nephropathy in mouse models of type 1 (OVE26) and type 2 (db/db) diabetes. Mouse Model 1: A) We will generate FXR knockout mice, currently on the C57Bl/6 genetic background, on FVB, and if need be and DBA/2J genetic backgrounds, 2 genetic backgrounds that have been documented to have increased susceptibility to diabetic nephropathy. B) We will then cross breed FXR KO mice on FVB background with i) OVE26 mice (type 1 diabetes) on FVB background or ii) db/db mice (type 2 diabetes) on FVB background to determine if FXR deletion accentuates and accelerates diabetic nephropathy. C) We will generate renal podocyte specific FXR knockout mice on FVB background using the Lox-Cre approach (FXRf/f mice crossed with Nphs2 Cre mice). D) We will then crossbreed podocyte FXR KO mice with i) OVE26 mice or ii) db/db mice. Mouse Model 2: A) We will generate renal podocyte specific conditional and inducible FXR transgenic mice in the FVB Background. B) We will then crossbreed the podocyte specific FXR transgenic mice with OVE26 or db/db mice to determine if increased expression of FXR attenuates or prevents diabetic nephropathy. Phenotyping: In these mice we will determine a) the manifestation of diabetic nephropathy, including glomerular filtration rate, glomerulosclerosis, tubulointerstitial fibrosis, and proteinuria and b) the cellular and biochemical mechanisms that mediate diabetic renal injury including lipid and carbohydrate metabolism, inflammation, fibrosis, oxidative stress, and AGEs/RAGE.

1. Program Accomplishments:

Hypothesis:

Our hypothesis was that compared to FXR KO mice on the nephropathy resistant C57BL/6 strain, FXR KO mice on the FVB or DBA/2J strains would have increased nephropathy, especially when these mice were made diabetic with streptozotocin and/or crossed with the Akita mice.

Progress toward stated aims:

In collaboration with NIH AMDCC and Jackson Labs FXR KO mice have now been generated in all 3 strains and we have characterized the FXR KO mice and FXR KO x Akita mice on the FVB and DBA/2J genetic backgrounds and FXR KO x STZ mice on the C57Bl/6 genetic background.

FXR KO C57Bl/6 and FXR KO C57Bl/6 x STZ

These results have been published in the journal of DIABETES in 2010. Below is the summary of our finding. Although the C57Bl/6 strain is resistant to diabetic nephropathy we have found that in the absence of FXR diabetic nephropathy is accelerated in FXR KO x STZ mice in the C57Bl/6 genetic background.

Below we are presenting the data for the mice fed a low fat chow diet.

As presented in our Diabetes publication these effects are markedly accentuated and accelerated in mice fed a western diet.

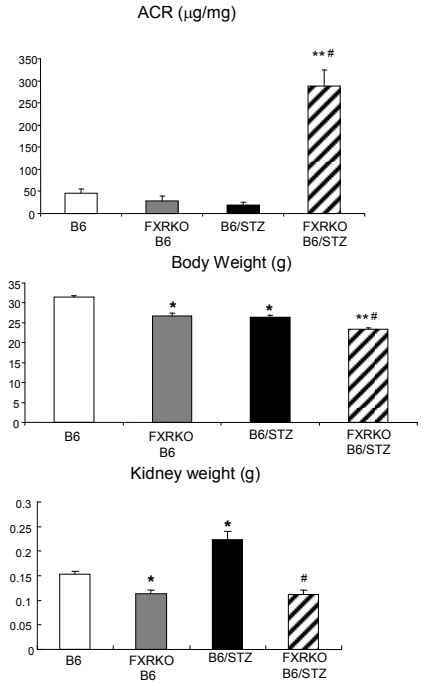

Figure 1: FXR KO C57Bl/6 x STZ mice develop marked proteinuria.

Figure 2: FXR KO C57Bl/6 x STZ mice have decreased body weight.

Figure 3: While C57Bl/6 x STZ mice have increased kidney weight, FXR KO C57Bl/6 x STZ mice do not.

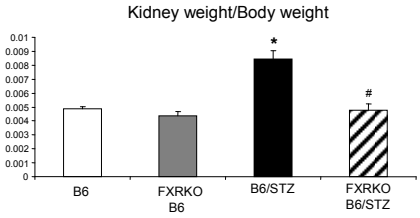

Figure 4: While C57Bl/6 x STZ mice have increased kidney weight/body weight ratio, FXR KO C57Bl/6 x STZ mice do not.

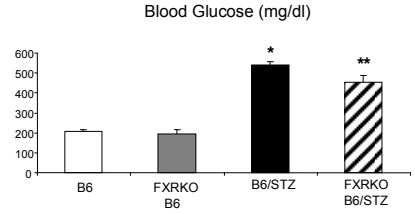

Figure 5: Blood glucose levels are similarly increased in C57B/6 x STZ and FXR KO C57Bl/6 x STZ mice.

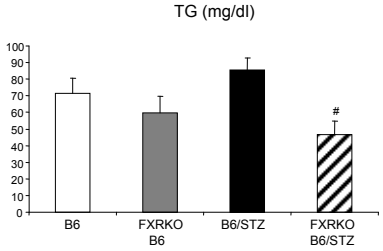

Figure 6: Serum triglyceride is not increased in FXR KO C57Bl/6 x STZ mice.

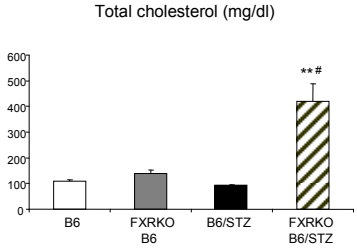

Figure 7: Serum total cholesterol is increased in FXR KO C57Bl/6 x STZ mice.

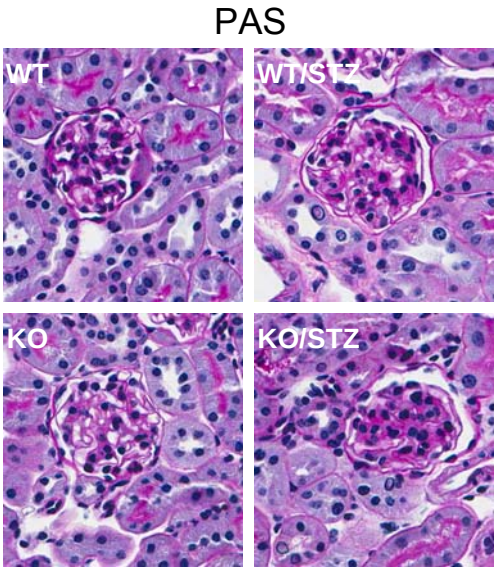

Figure 8: There is increased PAS positive matrix accumulation in FXR KO C57Bl/6 x STZ mice.

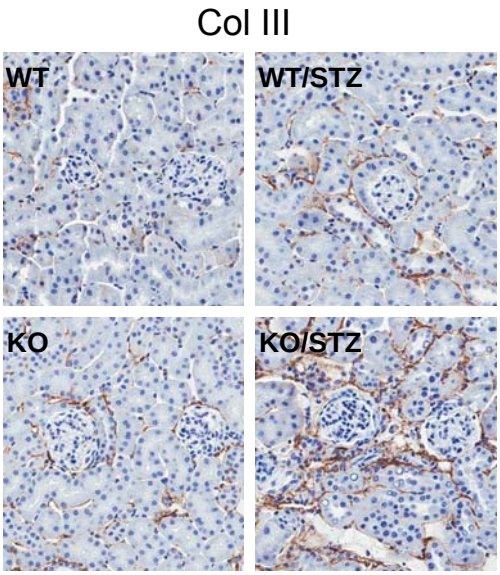

Figure 9: There is increased type III collagen expression and accumulation in FXR KO C57Bl/6 x STZ mice.

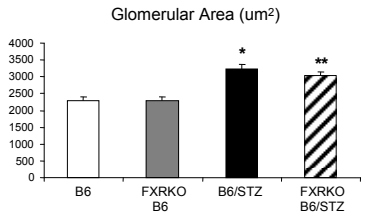

Figure 10: Both C57Bl/6 x STZ mice and FXR KO C57Bl/6 x STZ mice have increased glomerular area.

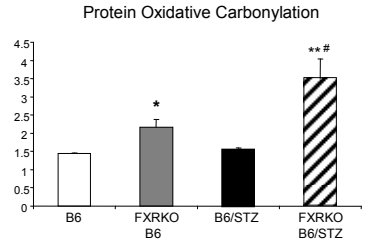

Figure 11: FXR KO mice have increased protein oxidative carbonylation which is further accentuated in FXR KO C57Bl/6 x STZ mice.

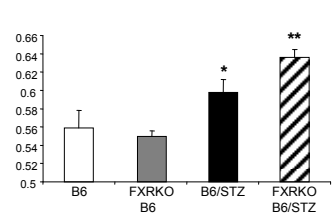

Figure 12: C57Bl/6 x STZ mice have increased NF-κB activation which is further accentuated in FXR KO C57Bl/6 x STZ mice.

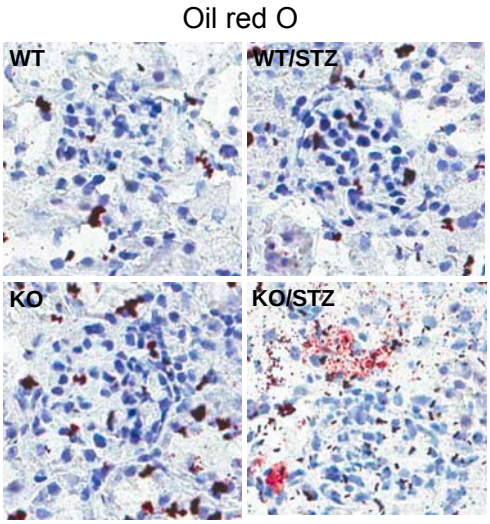

Figure 13: FXR KO C57Bl/6 x STZ mice have increased accumulation of oil red o positive lipids (triglycerides and cholesterol ester).

FXR KO FVB and FXR KO x Akita FVB

Akita mice on the FVB genetic background have moderate increase in urinary albumin (Figure 14) and urinary nephrin (Figure 15) excretion. However when crossed with the FXR KO mice on the same genetic background there are marked increases in both urinary albumin and urinary nephrin excretion. Studies in progress are determining the podocyte abundance for each condition.

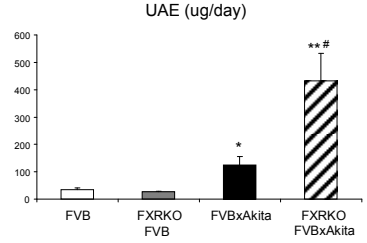

Figure 14: Akita mice on the FVB genetic background have moderate increase in urinary albumin which is further accentuated in FXR KO x Akita FVB mice.

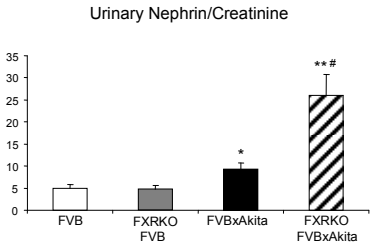

Figure 15: Akita mice on the FVB genetic background have moderate increase in urinary nephrin which is further accentuated in FXR KO x Akita FVB mice.

Interestingly the Akita x FXR KO mice develops systolic hypertension (Figure 16) which has also been observed at Jackson Labs. At this time the mechanisms for the increases in systolic BP remains to be determined.

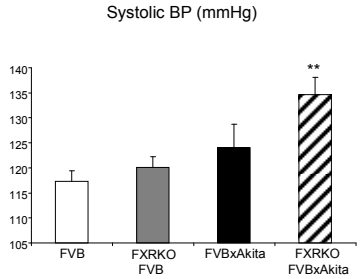

Figure 16: FXR KO x Akita FVB mice develop significant increase in systolic blood pressure.

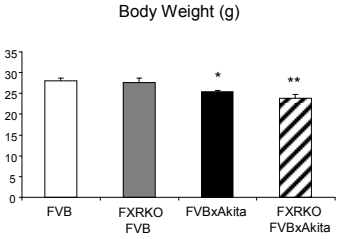

Figure 17: Akita FVB and FXR KO x Akita FVB mice decreases in body weight.

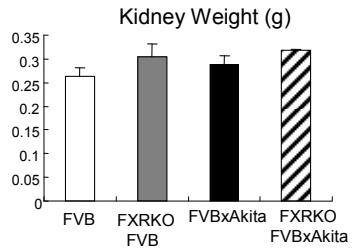

Figure 18: Akita FVB and FXR KO x Akita FVB mice no not have changes in kidney weight.

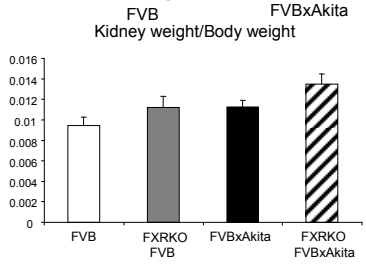

Figure 19: Akita FVB and FXR KO x Akita FVB mice no not have changes in kidney weight/body weight ratio.

Both the Akita and Akita x FXR KO mice are equally hyperglycemic (Figure 20). There are however significant differences in serum lipid composition (Figures 21-22).

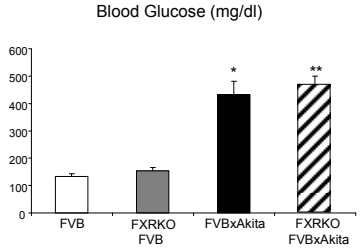

Figure 20: Akita FVB and FXR KO x Akita FVB mice develop similar degree of hyperglycemia.

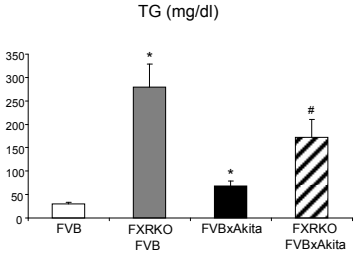

Figure 21: FXR KO FVB and FXR KO x Akita FVB mice have significant increases in serum triglyceride levels.

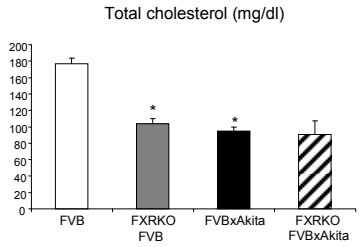

Figure 22: FXR KO FVB and FXR KO x Akita FVB mice have significant decreases in serum cholesterol levels.

PAS staining indicates that mesangial expansion and glomerular volume are increased in the Akita mice (Figures 23-24) and these increases are further amplified in the Akita x FXR KO mice. We are now performing quantitative histomorphometry and additional stains to further characterize the glomerular pathology.

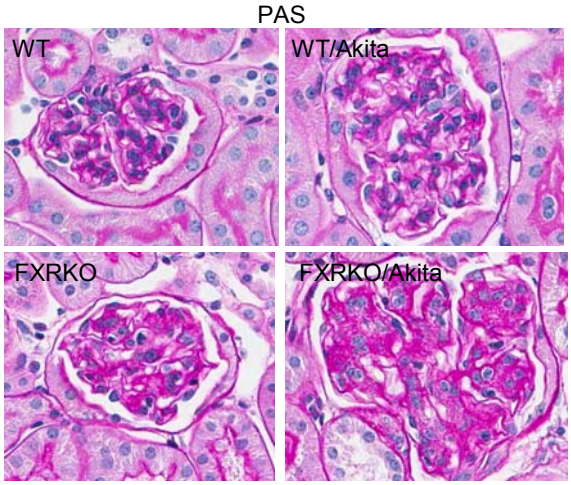

Figure 23: PAS staining indicates that mesangial expansion is increased in the Akita FVB mice and this increase is further amplified in the FXR KO x Akita FVB mice.

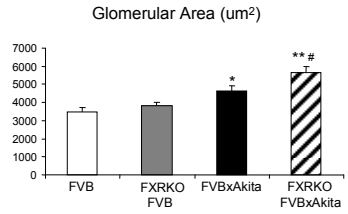

Figure 24: Glomerular area is increased in the Akita FVB mice and this increase is further amplified in the FXR KO x Akita FVB mice

Immunofluorescence microscopy further indicates that there is increased fibronectin accumulation in the glomeruli but not in the tubulointerstitium (Figures 25-26) of the Akita x FXR KO mice. We are now performing immunofluorescence microscopy and immunohistochemistry for additional extracellular matrix proteins.

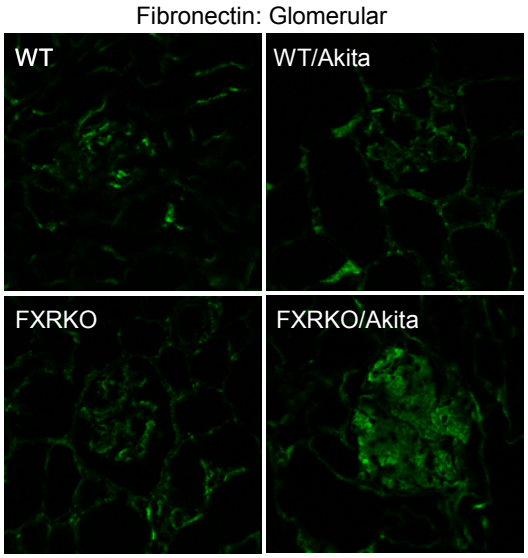

Figure 25: Immunofluorescence microscopy indicates that there is increased fibronectin accumulation in the glomeruli of the Akita x FXR KO FVB mice.

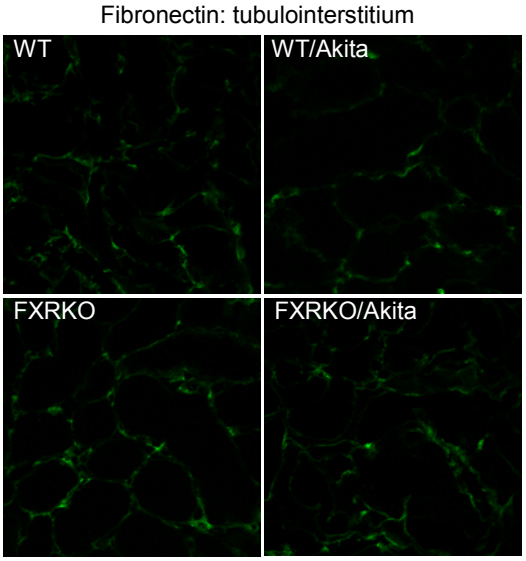

Figure 26: Immunofluorescence microscopy indicates that there is no increase in fibronectin accumulation in the tubulointerstitial cells of the Akita x FXR KO FVB mice.

Further analysis indicates that protein oxidation is increased in FXR KO mice but not in diabetic mice (Figure 27). We are now performing immunofluorescence microscopy and immunohistochemistry to determine expression of oxidized proteins and lipids in the kidney as well as determining the molecular mechanisms of alteration in oxidative stress.

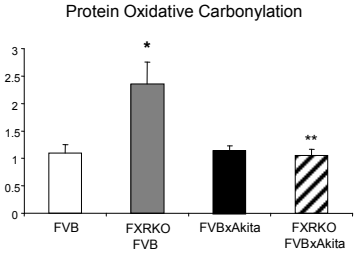

Figure 27: Protein oxidative carbonylation is increased in FXR KO mice but not in Akita X FXR KO FVB mice

Western blot analysis however indicates that there is increased inflammation in the Akita x FXR KO mice as assessed by NF-κB activity (Figure 28). Work in progress will determine the expression of proinflammatory cytokines in the kidney.

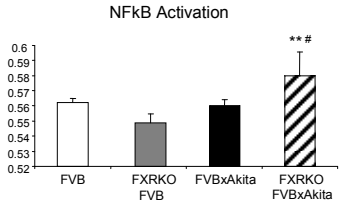

Figure 28: Akita x FXR KO FVB mice has increased NF-κB activation.

Oil red O staining (which stains for neutral lipids including cholesterol ester and triglycerides) indicates that while there is a mild increase in FXR KO mice there is marked increase in Akita x FXR KO mice (Figure 29). Work in progress will determine quantitative lipid composition analysis as well as the molecular mechanisms for the altered lipid composition.

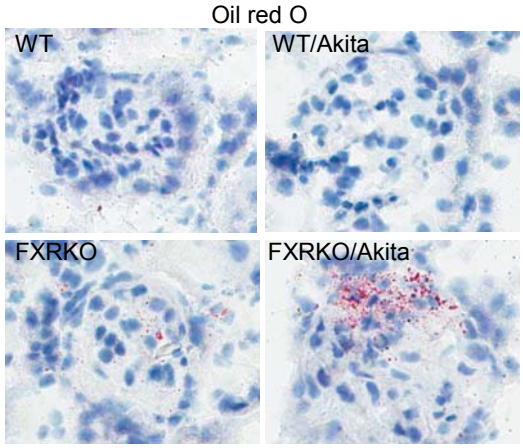

Figure 29: Oil red O staining (which stains for neutral lipids including cholesterol ester and triglycerides) indicates that while there is a mild increase in FXR KO mice there is marked increase in Akita x FXR KO FVB mice.

FXR KO DBA/2J and FXR KO x Akita DBA/2J

Akita mice on the DBA/2J background develop significant proteinuria (Figure 30), as also reported by Tom Coffman et al at Duke (AJP Renal Physiology 2010). FXR KO does not further amplify this effect.

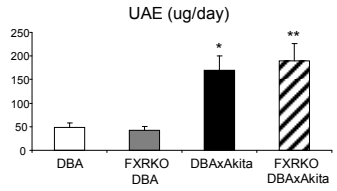

Figure 30: Akita DBA/2J and Akita x FXR KO DBA/2J mice develop significant proteinuria.

There are no significant differences in systolic blood pressure (Figure 31), as also reported by Tom Coffman et al at Duke (AJP Renal Physiology 2010).

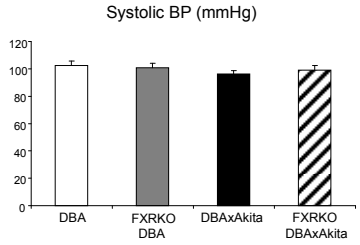

Figure 31: Akita DBA/2J and Akita x FXR KO DBA/2J mice do not have alterations in systolic blood pressure.

Kidney weight to body weight ratio is similarly increased in both Akita and Akita x FXR KO mice (Figures 32-34). The results in Akita mice are in agreement with the report by Tom Coffman et al at Duke (AJP Renal Physiology 2010).

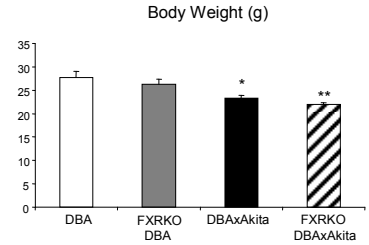

Figure 32: Akita DBA/2J and Akita x FXR KO DBA/2J mice have decreases in body weight.

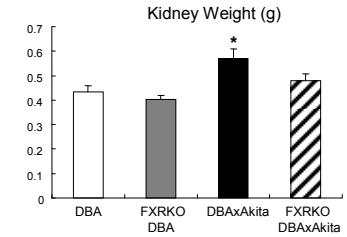

Figure 33: Akita DBA/2J but not Akita x FXR KO DBA/2J mice has increase in kidney weight.

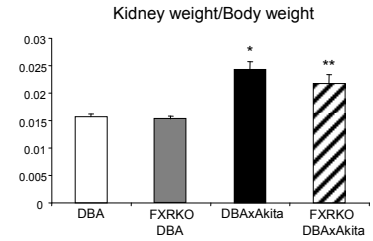

Figure 34: Akita DBA/2J and Akita x FXR KO DBA/2J mice have increases in kidney weight/body weight ratio.

Both Akita and Akita x FXR KO mice develop similar degree of hyperglycemia (Figure 35). However both serum triglyceride levels and serum total cholesterol levels are markedly increased in the Akita x FXR KO mice (Figures 36-38).

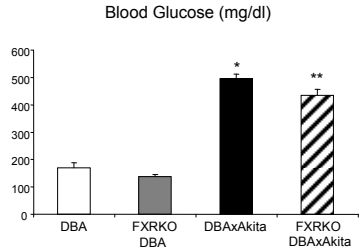

Figure 36: Akita DBA/2J and Akita x FXR KO DBA/2J mice develop similar degree of hyperglycemia.

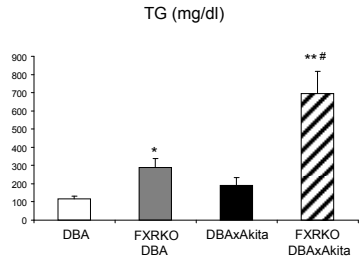

Figure 37: FXR KO DBA/2J mice has increase in serum triglycerides which is further amplified in Akita x FXR KO DBA/2J mice.

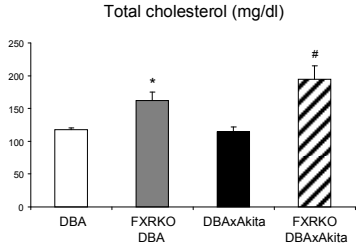

Figure 38: FXR KO DBA/2J and Akita x FXR KO DBA/2J mice have increases in serum cholesterol.

PAS staining indicates that there is no significant mesangial expansion and no increase in glomerular volume in Akita mice or Akita x FXR KO mice (Figures 39-40). The results in Akita mice are in agreement with the report by Tom Coffman et al at Duke (AJP Renal Physiology 2010).

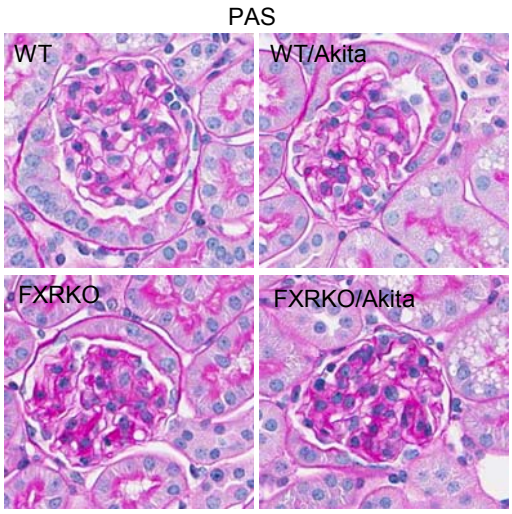

Figure 39: PAS staining indicates that there is no significant mesangial expansion in Akita mice, in FXR KO mice, or Akita x FXR KO DBA/2J mice.

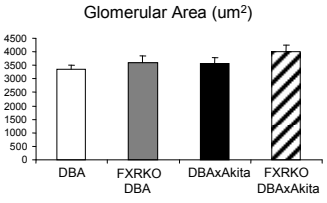

Figure 40: There is no significant change in glomerular area in Akita mice, in FXR KO mice, or Akita x FXR KO DBA/2J mice.

Immunofluorescence microscopy however indicates that there is increased fibronectin accumulation in the glomeruli (Figure 41) of the Akita x FXR KO mice. There is only a mild increase in the tubulointerstitial cells (Figure 42). We are now performing immunofluorescence microscopy and immunohistochemistry for additional extracellular matrix proteins.

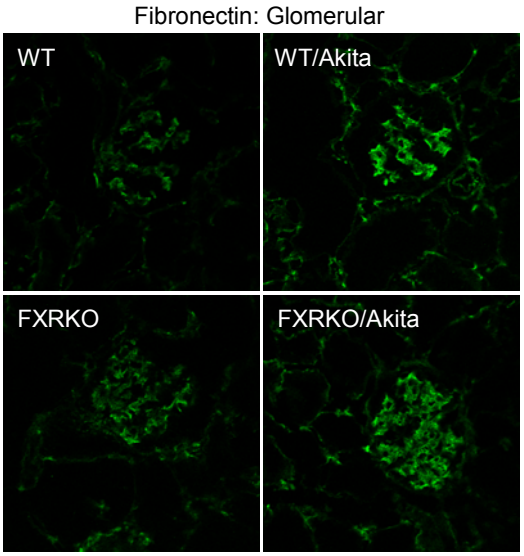

Figure 41: There is increased fibronectin accumulation in the glomeruli of Akita, FXR KO, and especially Akita x FXR KO DBA/2J mice.

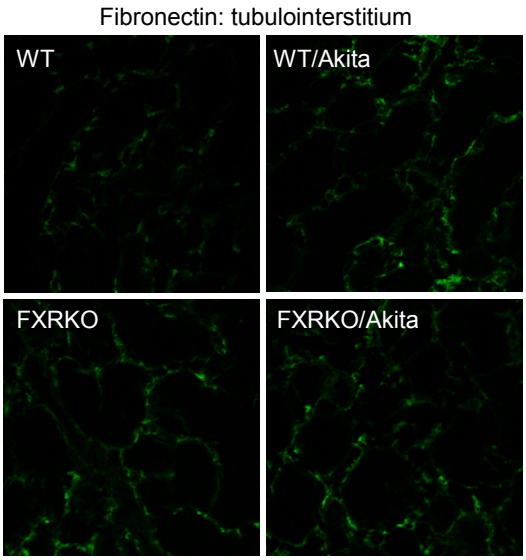

Figure 42: There is a mild increase in fibronectin accumulation in the tubulointerstitial cells of Akita, FXR KO, and Akita x FXR KO DBA/2J mice.

Further analysis indicates that protein oxidation is increased in FXR KO mice but not in diabetic mice (Figure 43). We are now performing immunofluorescence microscopy and immunohistochemistry to determine expression of oxidized proteins and lipids in the kidney as well as determining the molecular mechanisms of alteration in oxidative stress.

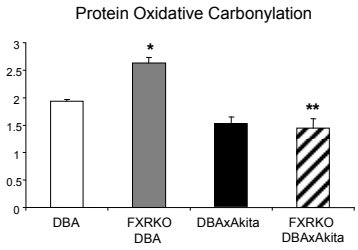

Figure 43: Protein oxidative carbonylation is increased in FXR KO mice but not in Akita X FXR KO DBA/2J mice

Western blot analysis also indicates that there is no increased inflammation in the Akita x FXR KO mice as assessed by NF-κB activity (Figure 44). Work in progress will determine the expression of proinflammatory cytokines in the kidney.

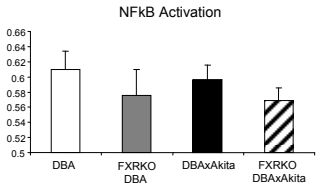

Figure 44: There is no increase in inflammation in the FXR KO, Akita, or Akita x FXR KO mice as assessed by NF-κB activity

Oil red O staining (which stains for neutral lipids including cholesterol ester and triglycerides) indicates that while there is no increase in FXR KO mice there is increased oil red o staining in Akita x FXR KO mice (Figure 45). Work in progress will determine quantitative lipid composition analysis as well as the molecular mechanisms for the altered lipid composition.

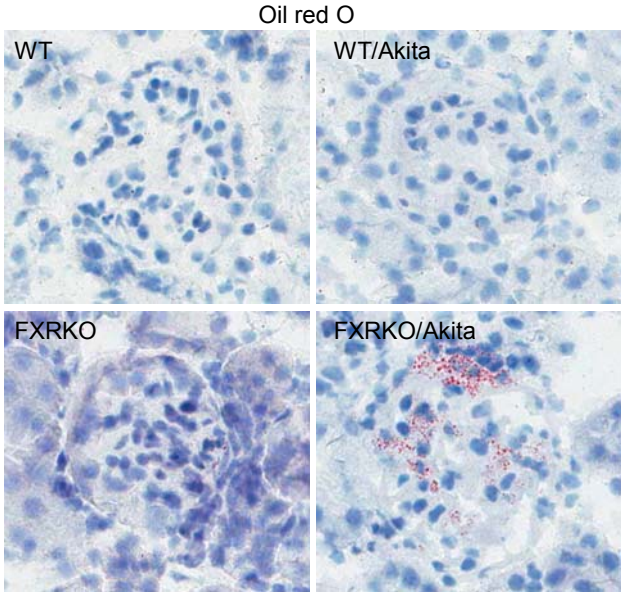

Figure 45: There is increase in oil red o staining (neutral lipids) in Akita x FXR KO mice

Plans for the year and completion of the project:

Our plans are to complete the above analyses so that we can publish our comparative results ASAP.

We are also determining the effects of western diet in the Akita DBA/2J and Akita x FXR KO DBA/2J strains as we have found that the DBA/2J strain is very susceptible to western diet induced alterations in lipid metabolism and renal disease especially in the presence of hyperglycemia.

In addition we are also now examining the 129/SvEv strain in our facility and we would like to complete analyses of all four strains.

2. **Collaborations:**

With other AMDCC PIs:

We have started a strong collaboration with Frank Brosius, Matthias Kretzler, and Subramanian Pennathur at the University of Michigan to perform comprehensive transcriptomic and metabolomic analyses of our kidney samples for identification of the regulation of critical and novel pathways in above models as well as diabetic mice treated with FXR ligands.

We also have a strong interaction and collaboration with Ira Goldberg at Columbia University because of our concurrent interest in atherosclerosis and also because of our belief of similarities between atherosclerosis and glomerulosclerosis.

With Jackson:

We have generated the strains above which we are characterizing.

With the MMPCs:

We have used the Seattle MMPC for our renal pathology.

With other non-AMDCC PIs:

We have collaborated with Frank Gonzalez at NIH/NCI to generate the FXR KO and FXR floxed mice.

We have an active ongoing collaboration with Jeffrey Kopp at NIH/NIDDK to generate FXR podocyte and FXR proximal tubular studies which will be used for our collaborative studies in diabetic as well as non-diabetic renal disease models.

3. **Address previous EAC comments:**

Please address each comment

- **The JAX data appears to indicate that FXR knockout buffers hyperglycemia (compared to Akita controls) and causes liver damage (based on GLDH levels). Have you seen similar effects?**

  We have not seen significant differences in blood glucose effects between Akita versus Akita x FXR knockout mice (Figure 20 and Figure 35). Actually based on some investigators' findings that FXR deficiency per se causes glucose intolerance we would have expected further increases in serum glucose levels, which we have not seen.

Since FXR plays an important role in regulation of liver bile acid metabolism, FXR deficiency could cause liver damage. In the FXR KO mice on the FVB genetic background we have seen an increase in serum ALT levels but not AST levels. We have just ordered the GLDH kit and we will be measuring GLDH levels as well.

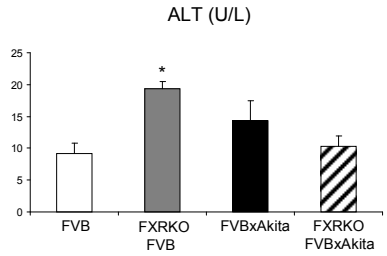

Figure 46: Serum ALT level is increased in FXR KO but not Akita x FXR KO mice.

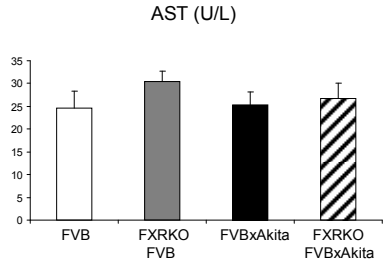

Figure 47: There are no changes in serum AST levels in FXR KO mice.

- **It would be interesting to know more about which kidney cell type(s) is relevant for FXR-dependent prevention of diabetic complications.**

We have used four complementary techniques to determine the renal localization of FXR. 1) We have isolated glomeruli and proximal tubules from the renal cortex using a) Microdissection, b) Dynabeads and Percoll gradient, and c) Laser Capture Microdissection microscopy: each technique indicated that FXR mRNA expression is much higher (at least 4-fold) in proximal tubules compared to glomeruli. 2) We have performed immunofluorescence microscopy and immunohistochemistry (IHC was performed by Dr. Jill Verlander at the University of Florida). Both techniques indicate that (as shown in the figure below) FXR is mainly expressed in the proximal tubule.

This has prompted us to develop mice with FXR overexpression in a) proximal tubules and b) podocytes to determine the in vivo role of each in models of diabetes and renal disease.

Meanwhile while FXR expression is much higher in the proximal tubules, our cell culture studies indicate that both in mesangial cells and also in podocytes FXR is protective against high glucose induced expression of inflammatory cytokines, oxidative stress, and fibrotic growth factors.

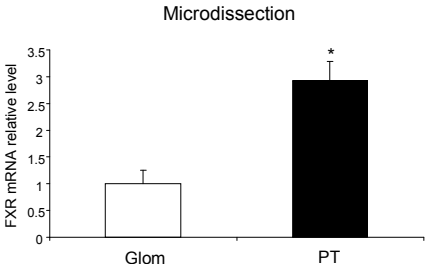

Figure 48: In glomeruli and proximal tubular cells isolated by manual Microdissection FXR mRNA abundance is 3-fold higher in proximal tubules.

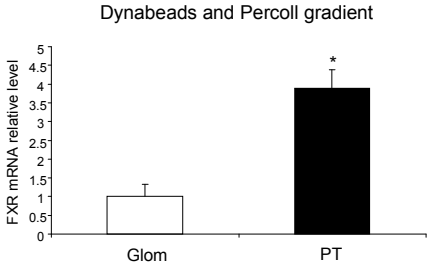

Figure 49: In glomeruli and proximal tubular cells isolated by Dynabeads and Percoll gradient FXR mRNA abundance is 3.5-fold higher in proximal tubules.

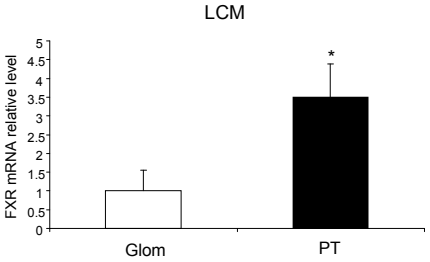

Figure 50: In glomeruli and proximal tubular cells isolated by LCM FXR mRNA abundance is 3-fold higher in proximal tubules.

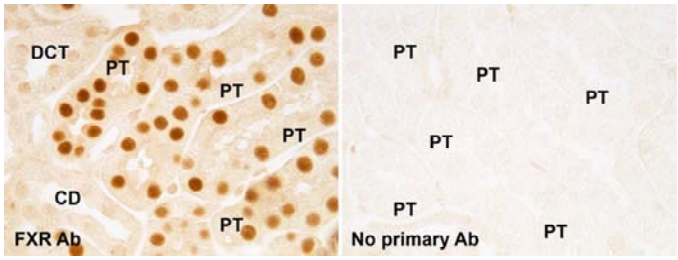

Figure 51: Immunohistochemistry indicates that FXR protein is strongly expressed in the proximal tubule and there is only faint staining in glomeruli.

- **The request to discuss discrepancies in JAX and Colorado mice is not addressed in the report.**

  It is not clear which discrepancies between Jackson Labs and Colorado mice are of concern. The EAC may be referring to the FXR KO mice on the FVB genetic background. If that is the case potential variables include i) high altitude which has known effects on diabetic nephropathy, ii) differences in diet, iii) differences in housing conditions, iv) differences in microbial

environment, and v) differences on the age of the mice at the time of the study.

- **Productivity is difficult to judge with mixture of relevant and irrelevant publications.**

  Our publication list includes only FXR relevant publications. In the case of vitamin D it is well known that there may be cross talk between the two related nuclear hormone receptors and in fact we have found that in vivo vitamin D receptor agonists induce increased expression of FXR (please see our most recent manuscript in AJP Renal) and FXR agonists induced increased expression of VDR (unpublished results).

- Productive over prior funding year. Goals for upcoming year continue to focus on FXR expression and ligands in diabetic kidney disease.

  Thank you. We continue our studies with FXR KO animals on FVB and DBA/2J genetic backgrounds. We have recently developed FXR pCALL2 mice that allow us to generate generalized FXR transgenic mice as well as proximal tubule or podocyte FXR transgenic mice. In addition we have been testing FXR, TGR5, and dual FXR-TGR5 ligands in models 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.

  This list has now been updated.

1. Diabetic Nephropathy is Accelerated by Farnesoid X Receptor Deficiency and Inhibited by Farnesoid X Receptor Activation in a Type 1 Diabetes Model
   Wang XX, Jiang T, Shen Y, Caldas Y, Miyazaki-Anzai S, Santamaria H, Scherzer P, Lewis L, Gonzalez FJ, Adorini L, Pruzanski M, Kopp JB, Verlander JW, Levi M
   Diabetes, 2010 (59), 2916-2927
2. Farnesoid X Receptor Activation Prevents the Development of Vascular Calcification in ApoE–/– Mice With Chronic Kidney Disease
   Shinobu Miyazaki-Anzai, Moshe Levi, Adelheid Kratzer, Tabitha C. Ting, Linda B. Lewis, and Makoto Miyazaki
   Circulation research, 2010 (106), 1807 - 1817
3. The farnesoid X receptor modulates renal lipid metabolism and diet-induced renal inflammation, fibrosis, and proteinuria.
   Wang XX, Jiang T, Shen Y, Adorini L, Pruzanski M, Gonzalez FJ, Scherzer P, Lewis L, Miyazaki-Anzai S, Levi M
   American journal of physiology. Renal physiology, 2009 (297(6)), F1587 - F1596

4. Mouse Models of Diabetic Nephropathy: A Midstream Analysis from the Animal Models of Diabetic Complications Consortium
Frank C. Brosius IIIa, 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
Journal of the American Society of Nephrology : JASN, 2009 (20(12)), 2503 – 2512
5. FXR Modulates Renal Lipid Metabolism and Fibrosis and Diabetic Nephropathy.
Jiang T, Wang XX, Scherzer P, Wilson P, Tallman J, Takahashi H, Li J, Iwahashi M, Sutherland E, Arend L, and Levi M:
Diabetes 2007 (56), 2485-2493
6. Nuclear hormone receptors in diabetic nephropathy
Wang XX, Jiang T, Levi M
Nature Review Nephrology 2010 (6), 342-351
7. Functional characterization of the semi-synthetic bile acid derivative INT-767, a dual FXR and TGR5 agonist
Rizzo G, Passeri D, De Franco F, Ciaccioli G, Donadio L, Rizzo G, Orlandi S, Sadeghpour B, Wang XX, Jiang T, Levi M, Pruzanski M, Adorini L:
Molecular Pharmacology 2010 (78), 617-630
8. The Vitamin D Receptor Agonist Doxercalciferol Modulates Dietary Fat Induced Renal Disease and Renal Lipid Metabolism
Wang XX, Jiang T, Shen Y, Santamaria H, Solis N, Arbeeny CM, Levi M
American Journal of Physiology Renal Physiology 2011(300), F801-F810