Animal Models of Diabetic Complications Consortium (U01 DK076136)

Annual Report (2009)

"Angiogenic Signals in Diabetic Complications"

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Part A:

Principal Investigator's Summary

1. Program Accomplishments:

Hypothesis

In humans with diabetes, abnormal angiogenesis, defined as growth and proliferation of blood vessels from existing vascular structures, contributes to the development of end-organ damage. In this regard, "excessive" angiogenesis and increased activity of the vascular endothelial growth factor (VEGF) signaling pathway have been associated with diabetic complications such as retinopathy, and perhaps nephropathy. In contrast, inadequate angiogenesis with a reduced capacity to promote collateral blood vessel growth results in more severe manifestations of coronary and peripheral vascular disease in diabetes. However, the mechanisms responsible for the loss of control of angiogenesis in diabetes and how this dysregulation modulates tissue pathology are not clear. *We have hypothesized that abnormal signaling in VEGF-associated pathways is a critical factor in the pathogenesis of diabetic complications including nephropathy and peripheral artery occlusive disease (PAOD). Furthermore, we posited that distinct properties of key cellular targets in individual tissues determine the effects of diabetes on the local angiogenesis response, shaping the resulting pathology. We suggest for nephropathy the critical target cell is the podocyte and in PAOD it is skeletal muscle.*

Accordingly, to develop better models of diabetic nephropathy and PAOD, we will generate mouse lines with inducible alterations of angiogenic signaling pathways targeted to podocytes and skeletal muscle. Because both enhanced and diminished angiogenesis responses have independently been associated with diabetic complications, we will use models with up- or down-regulated angiogenic signaling. Some of these models have been generated and are ready to use; we propose others to be generated as a part of the consortium activities. The long-term goals of our studies are: (1) To understand how alterations in angiogenic factors contribute to the development of diabetic complications and (2) To develop mouse models of diabetic nephropathy and PAOD that more faithfully reproduce the respective human conditions.

Recent Progress and Major Accomplishments

SPECIFIC AIM I. To define the role of altered angiogenic signaling in podocytes on the development of albuminuria and nephropathy in diabetes. During the past year, our work in this specific aim has covered several areas. First, we have continued our previous work focused on understanding strain-specific genetic modifiers that affect susceptibility to diabetic kidney disease, and their relationship with expression of and signaling by angiogenic signaling pathways. In previous studies, we and other AMDCC investigators have carried out studies suggesting that mice bearing the *Ins2*$^{+/C96Y}$ mutation (Akita mice) may have significant advantages as a platform for developing models of diabetic nephropathy (DN). Since genetic factors play a key role in susceptibility to DN in humans, we investigated the role of genetic background on kidney injury in Akita mice. To this end, we back crossed the *Ins2*^{C96Y} mutation onto the 129/SvEv and DBA/2 backgrounds and compared the extent of renal disease with the C57BL/6-Akita line. While male mice from all three lines developed marked and equivalent hyperglycemia, there were significant differences in the level of albuminuria with DBA/2>129/SvEv>C57BL/6. Renal and glomerular hypertrophy were seen in all of the lines, but significant increases in mesangial matrix were observed only in the 129 and C57BL/6 backgrounds. F1(DBA/2 x C57BL/6) animals had levels of albumin excretion similar to the more

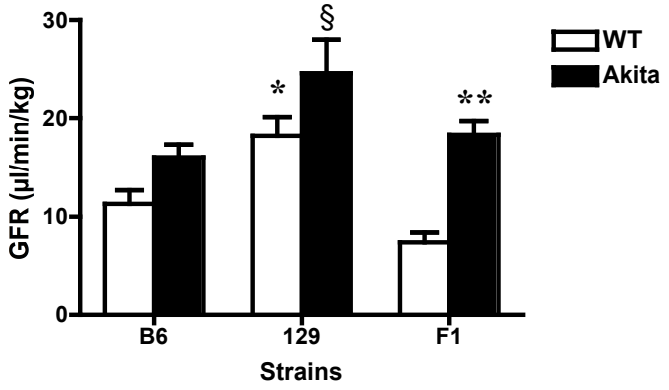

Figure 1. Glomerular filtration rate (GFR) in mice. Among the wild-type (WT) mice, GFR was highest in the 129/SvEv strain (*p<0.03 vs other WT). GFR tended to increase in diabetic mice on all strains, but the most marked increase was seen in the F1-Akita group (**p<0.008 vs F1-WT). (§p=0.026 vs B6 Akita)

susceptible DBA/2 parental strain, suggesting dominant pattern of inheritance for albumin susceptibility. However, the enhanced mesangial pathology in the F1 mice most closely resembled the C57BL/6 parental line, indicating genetic control of diabetic mesangial expansion may be distinct from that of albuminuria. These findings, which are described in a submitted manuscript (Gurley et al), indicate that genetic background has a powerful influence on determining the severity of renal injury in Akita mice. Based on the observed segregation of these traits in the F1 animals, mapping of susceptibility loci could be possible through F1 inter-crosses or backcrosses with the individual parental lines. We have now measured glomerular filtration rate (GFR) in wild-type and Akita mice on the C57BL/6, 129/SvEvTac, and F1(C57BL/6 x DBA/2) backgrounds. As depicted in Figure 1, there were significant baseline differences in GFR in non-diabetic mice from the different strains; GFR in wild-type 129/SvEv mice (18.2±1.9 µl/min/gm) was significantly higher than that of non-diabetic mice from the C57BL/6 (11.3±1.4 µl/min/gm; p=0.029) or F1(DBA/2 x C57BL/6) strains (7.4±1.0 µl/min/gm; p=0.0073). Comparing age-matched wild-type and Akita mice on each background, the presence of the *Ins2*^{C96Y} mutation and diabetes was associated with higher GFR. The extent of the increase in GFR was in the C57BL/6- and 129/SvEv-Akita mice was ≈35-40% and approached statistical significance (p=0.052 and 0.07, respectively). The proportional increase in GFR was much greater in the F1(C57BL/6 x DBA/2)-Akita animals (≈150%, p=0.011); this is also the group with the highest levels of albuminuria. These data indicate an association between genetic susceptibility to albuminuria and hyperfiltration, and suggest that GFR measurements should be included in future genetic screens. As discussed below, we have previously found that expression mRNAs for VEGF-A, VEGF-R1, soluble VEGF-R1, and VEGF-R2 were increased in Akita mice on the DBA/2 background.

In parallel with these studies, we have carried out studies to directly test whether local generation of VEGF by glomerular epithelial cells affects the development of proteinuria and kidney pathology in diabetes. To test this hypothesis, a genetic system allowing knock down of VEGF specifically in the glomeruli of diabetic mice was used consisting of an inducible *Cre-loxP* gene targeting system to excise the VEGF gene from podocytes of adult mice as described (Eremina et al. *NEJM* 358:1129, 2008). This work has been done in

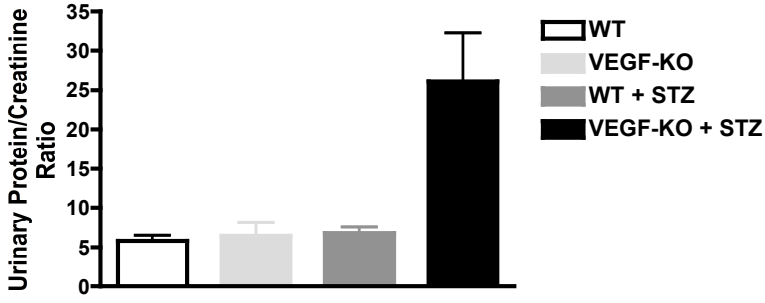

Figure 2. Urinary protein/creatinine ratio in mice.

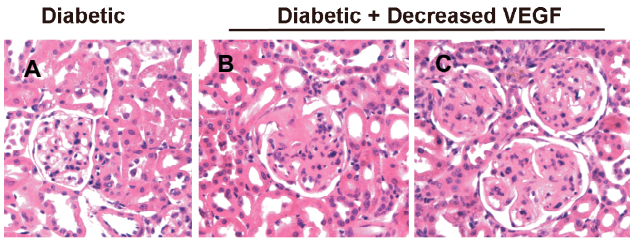

Figure 3. H & E stained sections from diabetic mice. (A) Wild-type mice treated with STZ (B & C) VEGF-KO mice with induced knock-down of VEGF production in podocytes treated with STZ.

Dr. Quaggin's laboratory in Toronto. Diabetes was induced by streptozotocin (STZ) injections beginning at 2.5 weeks of age. Mice were divided into four groups (n=80 total): wild-type (WT) non-diabetic, controls, VEGF-KO alone, VEGFKO + STZ, and WT + STZ. One week after the STZ injections, VEGFKO was induced by doxycycline (Dox) in drinking water, to eliminate the VEGF gene specifically in podocytes. Blood and urine were collected weekly to monitor blood glucose and urine protein concentrations for up to 10 weeks after STZ. As shown in Figure 2, significant proteinuria was only seen in the diabetic VEGF-KO mice. Moreover, 17% of the VEGF-KO + STZ mice died before the study end-point, while there was negligible mortality in the other groups. The representative photomicrographs depicted in Figure 3 show that the diabetic VEGFKO mice had significant glomerular pathology that was substantially worse than WT mice treated with STZ or non-diabetic VEGFKO mice (not shown). Thus, reduced production of VEGF within the glomerulus accelerates the progression of proteinuria and glomerular pathology in diabetic nephropathy. These results suggest that production of VEGF by podocytes may protect against glomerular injury in diabetes and that VEGF inhibitor therapy could be associated with a greater risk of renal toxicity in diabetics.

SPECIFIC AIM II. To define the role of altered angiogenic signaling in skeletal muscle in a model of peripheral artery occlusive disease. Deficient angiogenesis following ischemia may contribute to worse outcomes of peripheral arterial disease in patients with diabetes mellitus. Vascular endothelial growth factor and its receptors promote angiogenesis in PAD and, therefore, impaired activity of VEGF in diabetes might be one mechanism explaining the exaggerated severity of PAD in diabetic patients. Our primary hypothesis is that alterations in angiogenic signaling shape that manifestations of diabetic complications and the nature of these alterations may differ significantly between different organ systems. We previously documented changes in the vascular endothelial growth factor receptor-ligand family in mice fed a high fat diet. Specifically, we found that the soluble form of the VEGF Receptor-1 is down-regulated in the skeletal muscle of mice with type 2 DM. We proposed that down-regulation of this putative inhibitory receptor was a compensatory response to maintain critical down-stream signaling from the VEGF receptor which include the ability to phosphorylate (and thus activate) Akt and endothelial nitric oxide synthase. These data are consistent with other studies suggesting that VEGFR-1 antagonizes the actions of VEGFR-2, the major VEGF

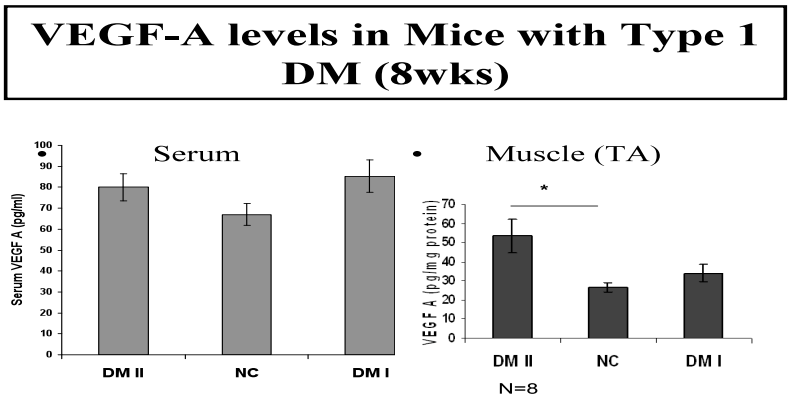

Figure 1. Mice with Type I DM have changes in levels of VEGF in skeletal muscle (right panel) that are very different from mice fed a high fat diet. Serum levels are not changed.

receptor for post-natal angiogenesis. We have now carried out studies to compare the effects of type 1 versus type 2 diabetes on these components of the VEGF system. As can be seen in **Figure 1**, VEGF levels are increased in skeletal muscle with type 2 but are unchanged in type 1 DM. The reductions in pAkt/Akt and pe-NOS/eNOS that we observed in mice fed a high fat diet were present, and indeed may be more pronounced in type 1 DM.

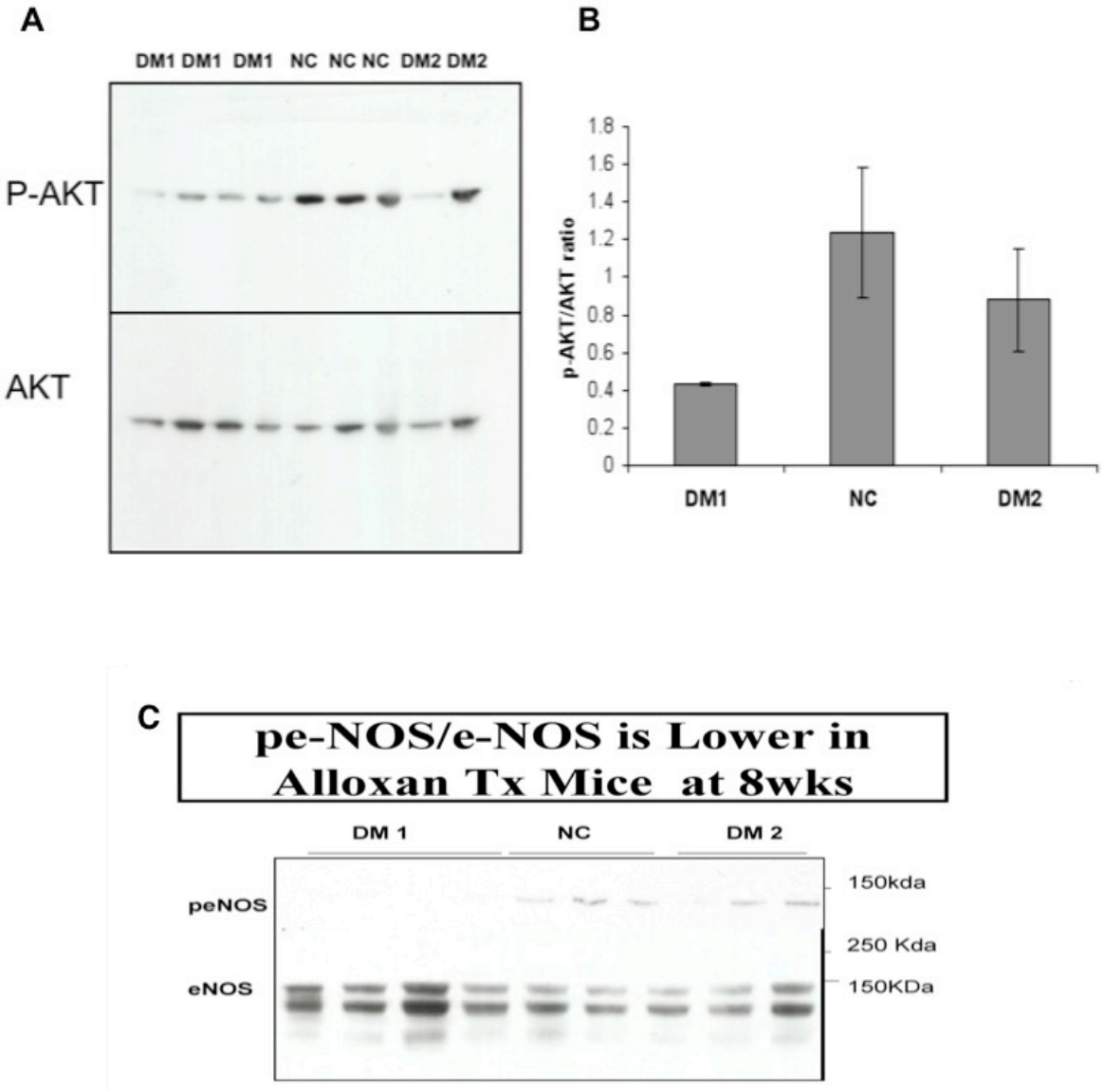

Figure 2. Mice with type I diabetes have changes in VEGF signaling in skeletal muscle (A and B) VEGF that are similar and perhaps worse then mice fed a high fat diet (type 2). © The ratio of phosphorylated (pe-NOS) to to total eNos (eNOS) is quite low in the animals with type I DM (DM1) compared normal chow (NC) or the high fat-fed mice (DM2).

In a previous study (Li et al. Diabetes 2007), we showed that both type 1 and type 2 DM models have impaired perfusion recovery following hind-limb ischemia. Having established that both models have similar changes in the VEGF ligand, we next sought to compare expression of VEGF receptors. We found that changes in the soluble VEGF receptor (sVEGFR1) were similar between the two models. However, as shown in the figure 3 below, expression of VEGFR2 was different at the protein (left panel) but not at the mRNA levels (right panel).

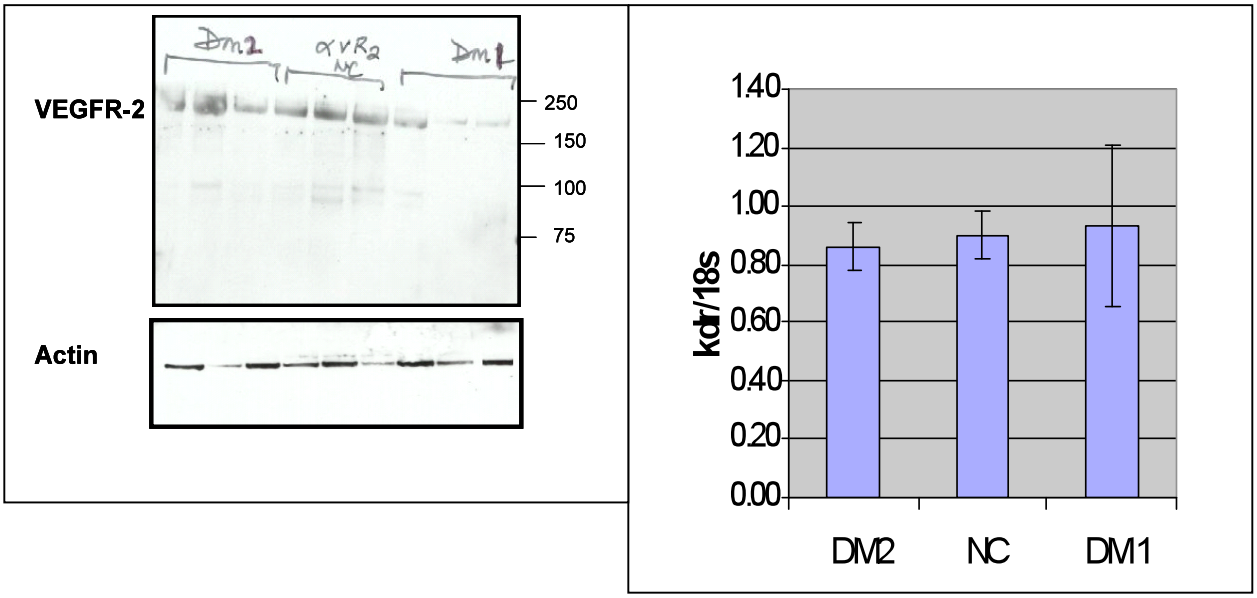

Figure 3. Protein and mRNA of VEGFR-2 in mice with Type 1 DM (DM1) compared to mice fed normal (NC) or high-fat (DM2) chow. DM1 mice show clear differences in the VEGFR2 protein levels without a difference in mRNA levels suggesting an effect on post-translational regulation.

From a clinical perspective, patients with peripheral arterial disease are treated similarly whether they have type 1 or type 2 DM. Thus, if confirmed these data may have therapeutic implications. Thus our ongoing work on this for the next year will be:

- Definitely establish the VEGF receptor ligand changes in muscle between the different models.
- Control for differences in the amount and extent of hyperglycemia.
- Examine the effects of glucose control.
- Examine apoptotic markers.
- Examine additional strains.
- Compare and contrast muscle and kidney from same mice.
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The hypothesis of the original application was that in humans with diabetes, abnormal angiogenesis contributes to the development of end-organ damage.
"Excess" angiogenesis = nephropathy
"Inadequate" angiogenesis = PAD

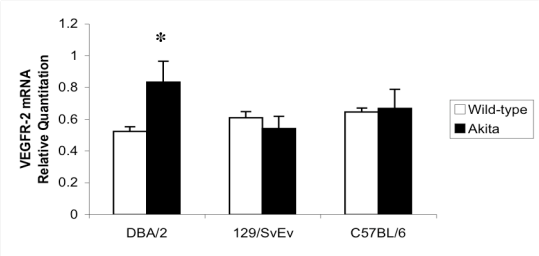
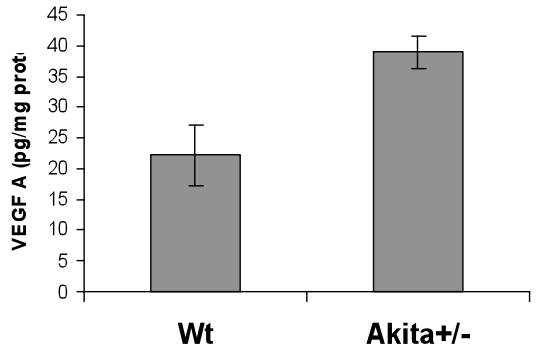


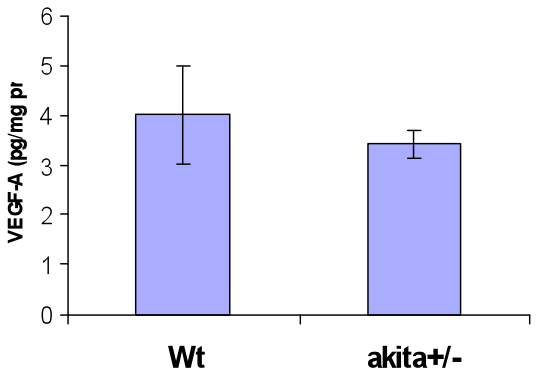
Figure 4. VEGFR-2 mRNA expression in renal cortex of wild-type and Akita mice.

To assess the association of VEGF levels with the extent of kidney disease, we compared mRNA levels for VEGF and its major angiogenic receptor (VEGFR2) in Akita mice and controls on three genetic backgounds, DBA/2, 129, and C57BL/6 which have a rank order of susceptibility to albuminuria of: DBA/2>129>C57BL/6. As shown in Figure 4, mRNA expression for VEGF and VGFR2 are increased in Akita mice on the most susceptible background (DBA/2). We have hypothesized that modulation of angiogenic signaling may differ in various target organs, including muscle and kidney. Accordingly, we compared levels of VEGF-A in kidneys and tibialis anterior muscle from Akita diabetic animals and genetically matched controls. As shown in Figure 5, VEGF-A levels were significantly higher in kidney cortex from Akita mice compared to non-diabetic controls. By contrast, there was no difference in VEGF levels in skeletal muscle. This is consistent with our hypothesis. The nature and extent of these differences will be explored during the next grant year.

Figure 5. VEGF-A levels in kidney and skeletal muscle. VEGF-A levels are increased in kidney cortex (left) of Akita mice vs. controls, but are unaffected in muscle (right)



p<0.05 Akita +/- vs. Wt



Plans for the Upcoming Year

During the next year, we will continue our ongoing work assessing the consequences of inducible elimination of VEGF expression from podocytes in adult mice on the course of STZ-induced diabetes. We will also carry out appropriate crosses so that similar studies can be done on the Akita background. Finally, we will continue our ongoing work characterizing the activity of VEGF-associated signaling pathways in our target tissues of interest, skeletal muscle and the glomerulus, to allow direct comparisons of the extent of angiogenic signal activation in these tissues and to understand how altered VEGF signaling may contribute to diabetic complications in these tissues.

Preliminary Milestones for 2009 and Beyond

1. ***Complete phenotypic characterization of diabetic mice with time- and cell-specific targeting of the VEGF gene in glomerular podocytes.*** These studies are in progress and will be completed during the next year.

2. ***Identifying the mechanism of attenuated VEGFR2 signaling in skeletal muscle during diabetes.*** This is a complex question, but we have made significant progress and our data now suggest that VEGFR1 may antagonize VEGFR2 signaling in skeletal muscle. We have recently obtained VEGFR1 knockout mice from Dr. Vickie Bautch from UNC-Chapel Hill to allow direct testing of this question.

3. ***Development of a mouse line with a capacity for cell-specific, inducible expression of HIF-1 alpha.*** We have floxed HIF-1 alpha mice in hand. These will be crossed with our lines allowing podocyte-specific deletion, and we are attempting to obtain skeletal muscel Cre lines to facilitate similar studies in skeletal muscle.

4. <u>Collaboration:</u>

We have interacted with the group at Jackson Laboratories over the past year. The 129/SvEvTac-*Ins2*^{Akita} line has now been successfully reposited.

5. <u>Address previous EAC comments:</u>

1. Coffman

a. The VEGF work continues to be interesting. The finding of more expression in susceptible strains and damage by VEGF inhibition seems on the surface contradictory. The notion of "rebound" is confusing.

We agree with the EAC that the idea of "rebound" in this setting might be confusing. In the development of our original hypothesis, we imagined that there might be a circumstance in which VEGF production might be impaired early in the course of diabetic nephropathy, and that this reduction could have pathophysiological significance. This might be followed by a compensatory increase in VEGF, which we called "rebound", and such a phenomenon has been seen in other settings. Of course, the consequences of an increase in VEGF generation will also depend on the availability of physiological receptors, as well as inhibitors and downstream signaling molecules. Our studies suggest that expression of these various components can also be altered with diabetes.

b. Could it be that there is just an optimal VEGF level? Is there a way to reconcile these findings with experiments?

Experiments carried out in the Quaggin laboratory over the past several years indeed suggest that there is an "optimal" level of VEGF in the glomerulus and deviation from this optimal range, either up or down, can have significant consequences. One approach to reconcile these findings is to test the consequences of enforced increases or decreases VEGF levels within the glomerulus in diabetes. Using

genetic models, this has been an area of focus for our project. So far, our studies indicate that any alteration of VEGF expression outside of the "optimal" range tends to augment glomerular injury in diabetes.

c. The studies of backgrounds' susceptibility are also interesting. Did females have the same pattern of albuminuria across strains?

We have focused on male mice in our studies since they develop more severe hyperglycemia and more marked kidney involvement in the models we have studied (STZ and Akita). In our hands, female mice develop only negligible albuminuria with these models.

6. Publications:

1. Kappas NC, Zeng G, Chappell JC, Kearney JB, Hazarika S, Kallianos KG, Patterson C, Annex BH, Bautch VL. The VEGF receptor Flt-1 spatially modulates Flk-1 signaling and blood vessel branching. *J Cell Biol* 2008; 181:847-858. PMID: 18504303

2. Xie D, Hazarika S, Andrich AJ, Padgett ME, Kontos CD, Donatucci CF, Annex BH. High cholesterol feeding in C57/Blc6 mice alters expression within the VEGF receptor-ligand family in corporal tissue. *J Sex Med* 2008; 5:1137-48. PMID: 18439153

3. Hazarika S, Angelo M, Li Y, Aldrich AJ, Odronic SI, Yan Z, Stamler JS, Annex BH. Myocyte-specific overexpression of myoglobin impairs angiogenesis after hind-limb ischemia. *Arterioscler Thromb Vasc Biol* 2008; 28:2144-50. PMID: 18818418

4. Facemire CS, Nixon AB, Griffiths R, Hurwitz H, Coffman TM. Vascular Endothelial Growth Factor Receptor 2 Controls Blood Pressure by Regulating Nitric Oxide Synthase Expression. *Hypertension* 2009; 54:652-8. PMID: 19652084

5. Gurley SB, Snow KP, Hu A, Meyer TW, and Coffman TM. Influence of Genetic Background on Albuminuria and Kidney Injury in *Ins2*$^{+/C96Y}$ (Akita) Mice. *Submitted*.

f. Project-generated Resources

None.