The Effects of Global Knockdown of Cytochrome C Oxidase Assembly Protein (Sco2) in Diabetic Kidney Disease

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Abstract

The kidneys are highly metabolic, mitochondrial-rich organs that require vast amounts of ATP for their normal function. However, in diabetes, the ATP production decreases in the mitochondria. Type 2 diabetes, characterized by insulin resistance, has been the leading cause of Chronic Kidney Disease(CKD) with about 35% of diabetic people aged 20 years or older who acquire CKD.[1] Because the kidney requires large amounts of energy to function, the proper maintenance of mitochondria structure and function is crucial. Synthesis of Cytochrome C Oxidase (Sco2) has been identified as an essential protein for maintaining the function of mitochondria and for the assembly of Cytochrome C Oxidase, a key component of the mitochondrial respiratory chain. Although emerging evidence on the importance of Sco2 has been recently found, little is known about the role of Sco2 and the effects of the global knockdown of Sco2 in various kidney cells especially in a diabetic model. In this study, processed tissues were obtained and stained for different cell markers in various kidney cells (podocytes, endothelial cells, and tubular cells) by using immunofluorescence staining. Glomerular volume and mesangial expansion were qualitatively analyzed following Periodic-Acid Schiff's Staining. In this study, no significant difference was observed in tubular damage between the different genotype. Conversely, in the setting of diabetes and Sco2 mutation, podocyte injury seems to decrease and the Sco2 mutation seems to be a protective mutation. This is seen by podocyte marker staining and the albumin analysis. Finally, after staining for endothelial cell markers, there was a significant increase observed between wild type and the diabetic genotype and trend of an increase between the WT and the Sco2 knockdown genotype. Collectively, these data suggest that in a diabetic condition, the Sco2 mutation has no negative effect on tubules, a protective effect on podocytes, and a negative effect on endothelial cells.

Introduction

Diabetes, a chronic disorder that affects millions of people around the world, is becoming an epidemic.[2] In the United States, 30.3 million people have diabetes and by 2030, it is estimated that more than 54.9 million Americans will suffer from it.[3,4] More health care resources are estimated to be spent on diabetes than any other condition since it leads to damage to multiple organs. Diabetes is a lifelong chronic disease caused by a high glucose levels. The two most common forms of diabetes disorders are Type 1 and Type 2. The Type 1 diabetes is an autoimmune disease caused by the inability of the pancreas to produce sufficient amounts of insulin. Conversely, Type 2 diabetes is a metabolic disorder caused by insulin resistance, in which the cells are unable to use the insulin produced by the pancreas, and glucose is not converted into energy for cells.[5] According to the Center of Disease Control and Prevention, 90% of the diabetic population have Type 2 diabetes.[3] Globally, diabetes is the leading cause of chronic kidney disease with about 40% of patients who are diabetic eventually getting diabetic kidney disease (DKD).[6]

This is a problem because the kidneys are critical organs responsible for maintaining homeostasis by regulating water and electrolyte balance, regulation of calcium, filtering blood, and excretion of metabolic wastes.[7] The nephron, the key structural unit of the kidney, consists primarily of the glomerulus and the proximal tubules. The glomerulus is the filtering portion of the kidney which determines what portions of the blood will show up in the early urine. The tubules will reabsorb and secrete certain things which will ultimately determine what is in the urine.[7]

In a healthy patient, as seen in *Figure 1.A*, normal capillary loops are formed by endothelial cells. A relatively thin basement membrane and healthy podocytes with normal sized cell bodies with multiple foot processes coming off of them is seen. Podocytes are very important because they have a major role in the maintenance of the kidney filtration barrier.[8] Additionally, while there are some mesangial cells, they are not proliferating or putting down extracellular matrix. Lastly, the proximal tubule coming off the glomerulus with its neighboring cells with their brush borders attached can still be seen. Early diabetic changes are first seen in the glomerulus, whereas late presenting diabetic patients will progress to tubular damage and chronic kidney disease. In early diabetic nephropathy, which is shown in *Figure 1.B*, there is a marked increase in mesangial cell

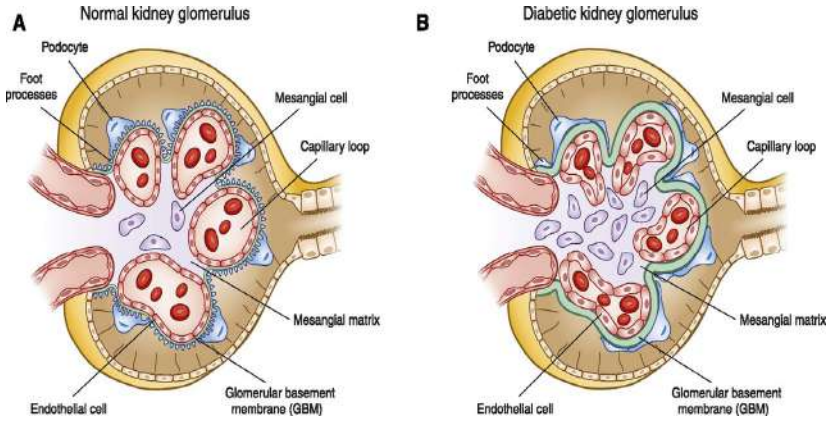

Figure 1:
(A) Morphology of Normal Kidney Glomeruli
(B) Morphology of Diabetic Kidney Glomeruli. Diabetic kidney disease leads to morphological and structural changes such as podocyte effacement thickening of the glomerular basement membrane, increase in mesangial cells, mesangial matrix expansion, and compression of endothelial cells.[8]

number as well as mesangial extracellular matrix. This starts to compress on the endothelial cells, causing the lumen to become smaller in size. Additionally, the basement membrane becomes thicker, podocyte foot process effacement starts to occur, followed by podocyte apoptosis, finally ending with a decrease in podocyte number. This is so detrimental because podocytes are post mitotic cells, meaning they are similar to neurons; once podocytes are lost, they cannot regenerate. The loss of the podocytes allow for proteins to be filtered through the glomerulus which can then finally start causing tubular damage as well.[9]

In addition to maintaining healthy kidneys, since the kidney is a highly metabolic organ, mitochondria are very important for the proper functioning of the kidney. Mitochondria are membrane-bound organelles that maintain a variety of cellular functions, such as the level of reactive oxygen species, making nucleotides, and regulating apoptosis. However, the most important function of the mitochondria is the production of ATP and supplying energy for basal cell function as well as cellular repair and regeneration.[10] A population of healthy and functional mitochondria is vital for these processes to be carried out. The mitochondria produce energy through the process of cellular respiration. Cellular respiration consists of three processes: glycolysis, Krebs cycle, and oxidative phosphorylation. Most of the ATP is formed through the oxidation and reduction of electrons in the electron transport chain (ETC) during oxidative phosphorylation. In the first step of cellular respiration, glucose is broken down into pyruvate during glycolysis. The pyruvate is converted into acetyl-CoA and enters the Krebs cycles which produces NADH and $FADH_2$ - electron carriers that transfer electrons to complex I and II respectively, in the ETC in the mitochondrial inner membrane (*Figure 2*). The electrons travel through Cytochrome C and then complex IV catalyzes the reduction of molecular oxygen forming H_2O.[11] In addition to the transfer of electrons, protons are actively pumped into the mitochondrial intermembrane space as seen in *Figure 2*. The protons than flow through the ATP synthase (complex V) and drives ATP phosphorylation by attaching a phosphate to ADP forming ATP.[10]

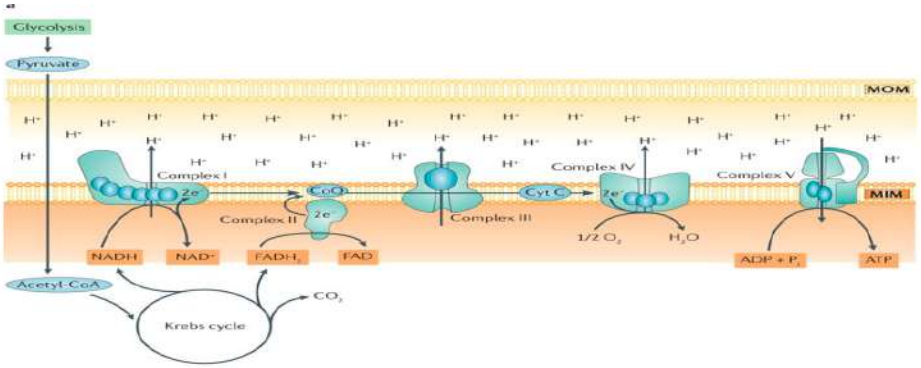

Figure 2: ATP production through ETC and Oxidative Phosphorylation [10]

The mitochondria's importance in the kidney has led to numerous publications discussing the mitochondria in diabetic kidney disease. It is still not known with accuracy if the mitochondria is important for initiating the disease process or if the mitochondria become damaged due to disease progression. Uncovering the role of mitochondria in this setting is important, but in order to do that, it is important to first understand mitochondrial processes. Firstly, it is important to recognize that mitochondria have their own genomes, which is seen as mtDNA in *Figure 3*. Mitochondria are dynamic organelles that are constantly going through fusion and fission. In healthy mitochondria, reactive oxygen species are formed at basal levels which cause some degree of damage to the mitochondria. When this occurs, MFN and OPA1 gene expression is induced and two mitochondria fuse together to become one large mitochondria and one set of mtDNA disappears. From there, DRP1 are expressed and the damaged portion is taken out of the mitochondria. Since the mitochondrial membrane potential will be disrupted, PINK1 and Parkin will be expressed in these mitochondria which allows for mitophagy to begin. This process allows for the controlled degradation of mitochondria without contents being spilled out into the cell. If mitophagy is unable to occur, Cytochrome C is released and the intrinsic apoptosis pathway is initiated. In order to maintain homeostasis, mitochondria number is tightly regulated and if there is a need to produce new mitochondria, there is an increase in PGC1*a* which leads to the duplication of mitochondrial DNA and increased mitochondria.[12]

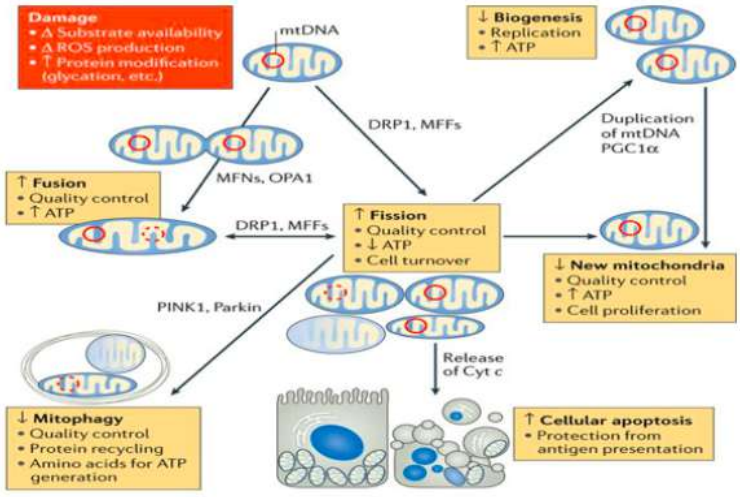

Figure 3: Alterations in mitochondrial dynamics in diabetic kidney disease [11]

In the setting of diabetes, there is a potential for mitochondrial damage due to a change in substrate availability, increase in reactive oxygen species, and an increase in protein modifications. This can lead to an increased amount of fusion in order to compensate for the increased damaged mitochondria. Unfortunately many portions of the mitochondria that could become damaged leading to an increased amount of fission that would occur. Despite this increase in fission, there is not enough autophagy

machinery to keep up with the fission which will lead to more apoptosis. Simultaneously, there will be a demand for new mitochondria, but not enough machinery to make new mitochondria because components were not saved through mitophagy. As a result there will be a decrease in biogenesis. This is problematic due to the importance of mitochondria in kidney function. If there is a decreased amount of mitochondria due to diabetes, then there will be less energy available thus negatively impacting kidney function.[12]

Another significant factor that can impact mitochondrial function and ATP production is the Synthesis of Cytochrome C Oxidase (Sco2) protein. Sco2 is an essential protein embedded in the inner mitochondrial membrane. It is a metallochaperone that is essential for the assembly of Cytochrome C Oxidase (Complex IV) because it is involved in the delivery of copper to subunit 1 and 2 to help with the formation of Complex IV.[13] However, when a mutation in Sco2 is present, patients may have fatal infantile cardioencephalomyopathy and severe complex IV deficiency in heart and skeletal muscle.[14] It has been found that within the first year of life, patients can develop severe cardiomyopathy, encephalopathy and myopathy and can die of cardiac failure.[15] The E140K missense allele specifically has been found in this patient population.[15]

In order to study the role of Sco2 within the mitochondria and the effects of the Sco2 mutation, previous researchers studied the E140K mutation which was present in most patients who had a Sco2 mutation. E140 residue forms an ion pair with K143, perhaps ensuring the integrity of the copper binding site of Sco2.[16] In this specific mutation, a point mutation occurs in which Glutamine-140 is converted to Lysine. When there is a mutation in one allele a knock-in mutation has occurred. As a result of this mutation, each patient carries at least one E140K missense allele. When a mutation occurs, copper binding does not occur. Although this mutation is specific to humans, researchers were able to apply this mutation in mice because mice have the E129 residue which is homologous to the human E140 residue.[16]

Previous research by Yang et al. has shown that a complete knockdown of both Sco2 alleles (KOKO) will result in instant death of normal, healthy mice. A single knockdown of the Sco2 allele (KIKI) showed biochemical, morphological, and functional defects in the kidney cells of healthy mice.[16] Additionally, prior research has also indicated that diabetes is one of the leading causes of chronic kidney disease and end stage renal disease.[6] However, minimal research has been conducted on the specific role of Sco2 and the effects of Sco2 mutation on a diabetic kidney.

The current study aims to bridge the gap by observing the Sco2 mutation in diabetic kidney tissue and investigating its effects on kidney function. The primary goal of this study was to determine the effects of a global knockdown of Sco2 in a diabetic injury mouse model on kidney function and to characterize the tubular, podocyte, endothelial damage to determine which cells are reliant on oxidative phosphorylation in the setting of diabetes.

To study the effects of this mutation, db/db and KI/KI db/db mice were obtained and various staining were performed on the kidney tissue obtained from these generated mice. The two types of staining that were conducted were immunofluorescent staining and Periodic-Acid Schiff (PAS) staining. Immunofluorescence staining was used to target podocyte cell markers, tubular cell markers, and endothelial cell markers to assess the damage within the tubules and glomeruli of the kidney. PAS staining allowed for both tubules and the glomeruli to be examined under the bright field microscope, so that mesangial expansion, glomerular volume, podocyte count, endothelial cell injury, and tubular injury can be assessed. The objective of this study was to observe the changes that occur between the wild type, db/db, and db/db KI/KI mice. It was hypothesized that the kidney, a highly metabolic organ with an abundance of mitochondria present, and a global knock-down of Sco2 will lead to cell damage in cells that need oxidative phosphorylation for survival in a diabetes model.

Materials and Methods

I. Obtaining Kidney Tissue Samples:

The C57BL/6 wild type(WT), diabetic(db/db), and diabetic Sco2 knock-in(KI/KI db/db) mice were grown until 24 week and perfused for another project. The mice were perfused with PBS and then the kidneys were fixed in 10% phosphate buffered formalin for 24 hours and switched to 70% ethanol prior to processing. After perfusion, the kidneys were taken out and were embedded in paraffin by histology and 3-um-thick sections were cut onto a slide and ready to be stained.

II. Genotyping:

In order to verify the genotype of each tissue used for this investigation, the mice tails were used to extract DNA and conduct genotyping. The tails were obtained and DNA extraction was performed by the mentor who followed standard ethical procedures and had an IACUC. 0.2 cm of the tail was cut off and placed in a 0.2 ml PCR tube. 30ul of extraction buffer was added to the tube. Samples were then heated to 95 °C for 30 minutes in the PCR under the DNA extract program. After 30 minutes, the samples were allowed to cool to room temperature and 30ul of Stabilization buffer was added.

After DNA extraction was completed, the PCR reaction for genotyping was and remainder of the procedure was performed by the author of this paper. To make the PCR master mix, 7.0 µl of H_20, 10 µl of blue choice, 1 µl of 10 µM of primer, and 2.0 µl of DNA per sample was used for both db/db and Sco2 KI/KI. For the db/db gene, db/+ , db/db, and WT were used as controls and for KI/KI db/db, the control were KO/+, KO/KI, KI/KI, and WT. Distilled H_2O was also used as a control to ensure that there was no contamination in the PCR reaction. PCR tubes were placed into the PCR to run under lepr db/db or Sco2 KI/KI.

After PCR was completed, restriction enzyme buffer was created. In new PCR tubes, 2.4 µl of buffer, 13.1 µl of H_2O, and 8µl of sample from PCR was added. Additionally, a restitutive enzyme specific to the gene was added: 0.5 µl of Rsal was added for db/db and 0.5 µl HindIII was added for Sco2 KI/KI. To allow the restriction enzymes to work, the tubes were placed into the 37 °C for 1 hour. Next, gel electrophoresis was performed to compare the genotypes. The samples were inserted into the wells of 3% agarose gel and the gel ran for 90-120 minutes for db/db and 120-150 minutes for Sco2 KI/KI db/db at 100 volts. Images were taken under UV light and the bands were compared with the control to verify the genotype of each sample.

III. Periodic Acid- Schiff's (PAS) Staining:

PAS staining helps in detecting PAS polysaccharides such as glycogen, and mucosubstances such as glycoproteins, glycolipids and mucins in tissues. Additionally, within the glomerulus of the kidney, PAS staining also allows us to determine the amount of mesangial expansion, glomerular volume, and tubular injury within the glomerulus.[5] After obtaining slides for WT, db/db, and KI/KI db/db genotypes, PAS staining was performed in order to determine the effects of the different genotypes on the kidney. The slides were baked for at least 1 hour at 37 °C and cooled at room temperature for 10 minutes. Once the slides were cooled, the hydration process was initiated. First, the slides were placed into Xylene bath for 5 minutes twice. Then, the slides were placed into 100% ethanol, 75% ethanol, and 25% ethanol for 3 minutes each. Due to the hydrophobic wax covering, the decreasing concentration of ethanol allowed for the cells to adapt to the deionized water and not burst on contact. After 3 washes in ethanol, the slides were placed in deionized water for 5 minutes. After the hydration process, the slides were immersed into periodic acid for 11 minutes. Next, the slides were placed in Schiff reagent for 15 minutes. Lastly, the slides were immersed in hematoxylin for 10 seconds per slide. After staining with hematoxylin, the slides were left in the sink and rinsed in distilled water for 5 minutes. After the completion of staining, the slides underwent the dehydration process which is simply just the reverse of the hydration process and then cover slipped. Once the slides dried, they tissue was observed under the bright field microscope and full scan images of the glomeruli and tubules were taken.

IV. Immunofluorescence staining for podocyte markers:

A. Immunofluorescence (IF) for Synaptopodin

Kidney sections from the 3 genotypes were stained for Synaptopodin, a podocyte marker. The immunofluorescent staining was a 2 day protocol.

On Day 1, the slides were baked in an oven at 65 °C for at least an hour and then the same hydration process used for PAS staining was applied to the slides. While the slides were immersed in deionized water,

200ml sodium citrate buffer was prepared at a 1:10 dilution. After 5 minutes, the slides were placed into the sodium citrate buffer and then placed in a pressure cooker at 120 °C for 10 minutes. Once the 10 minutes were complete, the slides were placed in the 4 °C and allowed to cool for 30 minutes. During this time, blocking buffer using 2% non-fat milk was prepared with a 3:2 dilution of tris - buffered saline(TBST) to milk. The slides were taken out of the 4 °C and washed in TBST (1:10) for 3 minutes. Then, the blocking buffer was added to each tissue and the slides were placed in the 37 °C to block for an hour. As the slides are being blocked, the Synaptopodin primary antibody (goat) was prepared with a 1:50 in milk. After an hour, tegh slides were washed in TBST and the primary antibody was placed onto the lisdes and then left to incubate overnight at 4 °C.

On Day 2, the slides were stained with secondary antibody R*a*G (1:300 dilution in milk). After washing with TBST, sections were incubated with fluorophore-linked tertiary antibody D*a*R 568 (1:300 in TBST). After staining, the slides were mounted using Prolong Gold antifade mounting media and then photographed using the microscope.

B. Immunofluorescence for WT1

Immunostaining was performed on the kidney using the same method for Day 1 IF staining for Synaptopodin (mentioned in the previous section) with the exception of WTI (mouse) primary antibody to target the WTI podocytes markers in the glomerulus.

On Day 2, the slides were washed in TBST and then fluorophore-linked secondary antibody D*a*M 647(1:300 in TBST). In order to quantify the WT1, dapi-positive WT1 was counted. Dapi stains the nucleus of the cell. In order to stain for Dapi, the tissue was stained with Hoechst stain (1:1000) and then washed in TBST. After staining, the slides were mounted using Prolong Gold antifade mounting media and then photographed using a microscope. To determine the amount of damage of podocytes within the glomeruli, Dapi + WT1 was counted for.

V. Immunofluorescence staining for tubule marker:

Immunostaining for the Lotus Lectin, a tubular marker for the brush borders of the proximal tubules, was conducted using the same method for Day 1 as the previous immunofluorescence staining with the exception of the antibody which was utilized for this staining. Lotus Lectin was prepared in TBST and placed onto the slides for incubation at room temperature for 10 minutes. After staining, the slides were mounted with the mounting media, images were taken on the microscope, and the percent area of Lotus Lectin was determined using Image J.

VI. Immunofluorescence staining for endothelial cells:

To analyze the differences between the three genotypes in endothelial cells, immunostaining for endothelial nitric oxide synthase(eNOS), an endothelial cell marker, was conducted. The same method was utilized for day 1 as previously stated, but this staining, eNOS primary mouse antibody was used instead. The primary antibody was prepared in non-fat milk at a dilution of 1:50. The slides were incubated overnight at 4 °C. On the second day, the slides were washed in TBST for 5 minutes and then a secondary antibody, D*a*M 647, was placed on the slides at a dilution of 1:300. The slides were incubated for 30 minutes at 37 °C. After 30 minutes, the slides were mounted with mounting media and images were taken under the microscope. Using Image J, the percent area stained within the glomerulus was determined.

VII. Albumin Assay:

An albumin assay was performed on the urine samples of the two genotypes, db/db and KIKI db/db, in order to determine the albumin concentration within the urine. The albumin assay consists of 5 major parts: coating, blocking, standards, HRP detection, and enzyme substrate reaction.

A master mix for coating was created using 1µl of capture antibody diluted with 100µl of coating per buffer. 100µl of master mix was pipetted into a 96 well plate. The plate was incubated for 1 hour at 4 °C and then the capture antibody was removed and plate was washed 3x with washing buffer. A plate plan was devised with duplicate wells for each standard and sample. Next, 200µl of blocking solution was added to each well and incubated for 30 minutes. After incubation, the blocking solution was removed and the wells were washed again with wash solution three times each. Using the standard dilution, 8 standards, B-I, were prepared. 100µl standards, samples of diluted urine, were transferred into the wells. The plate was sealed and was incubated for 60 minutes. The samples and standards were removed and wells were washed. 100µl of HRP Detection Antibody was added to each well at a dilution of 1:1000 with HRP Conjugate Diluent. The plate was incubated for an hour and the washed 5x. For the Enzyme Substrate reaction, 100µl of substrate solution(TMB) was transferred to each well and the plate was incubated for 15 minutes. The reaction is stopped by applying 100ul of stopping solution to each well. The plate was read at 450 nm for TMB and the albumin concentration was analyzed.

Results

I. Periodic Acid- Schiff's Staining:

In this project, a quantity of N=3 was used for each genotype for this staining. Representative images shown below were taken under bright field microscope after staining in order to qualitatively observe the trends occurring within the glomeruli and tubules of the three genotypes.

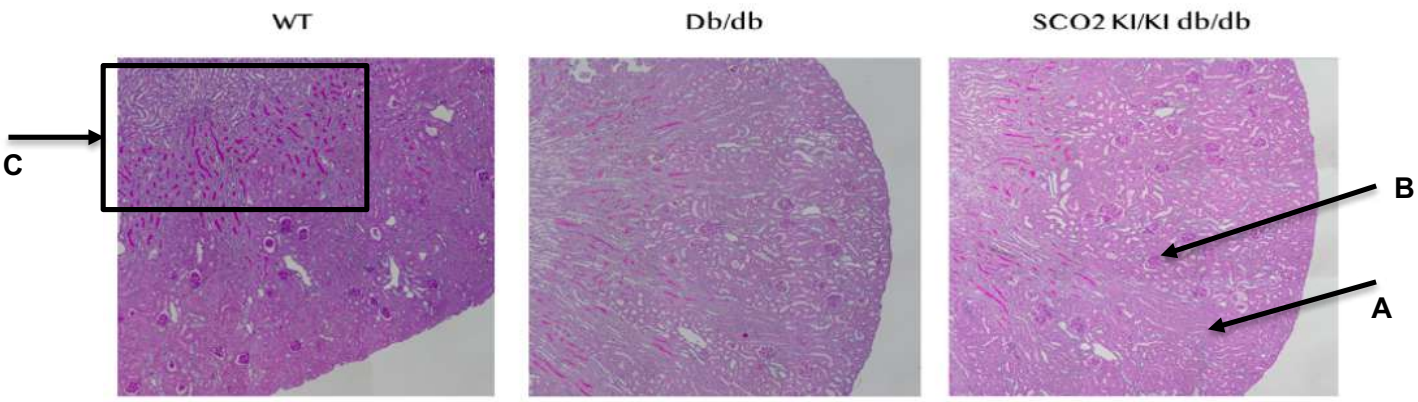

Figure 4: Periodic Acid -Schiff's staining was conducted for WT, db/db, Sco2 KI/KI db/db
Representative images taken by the author at a magnification of 10x under bright field microscope. (A) tubules (B) glomeruli (C) medullary region.

These images, shown in *Figure 4,* represent the general trends that occurred within the glomeruli and tubules in each staining. Changes that occurred in the tubules and glomeruli were qualitatively analyzed using these representative images. First, the tubules were analyzed. As shown in *Figure 4*, there was an apparent increase in white space in the tubules of both db/db and KIKI db/db tissues representing tubular injury. Next, the glomeruli was analyzed. The size of glomeruli seems to have increased in db/db and KIKI db/db indicating an increase in glomerular volume. Additionally, the pink area within the glomeruli was spreading which indicated increased mesangial expansion. Lastly, in the medullary region of the kidney, dark pink spot can be seen which represented the protein casts that have formed.

II. Immunofluorescence staining for podocyte markers:

A. Immunofluorescence for Synaptopodin

In order to assess the impact of Sco2 mutation in a db/db setting on podocytes, percent area stained of Synaptopodin was analyzed. Shown below is the representative images taken for all three genotypes (*Figure 5*).

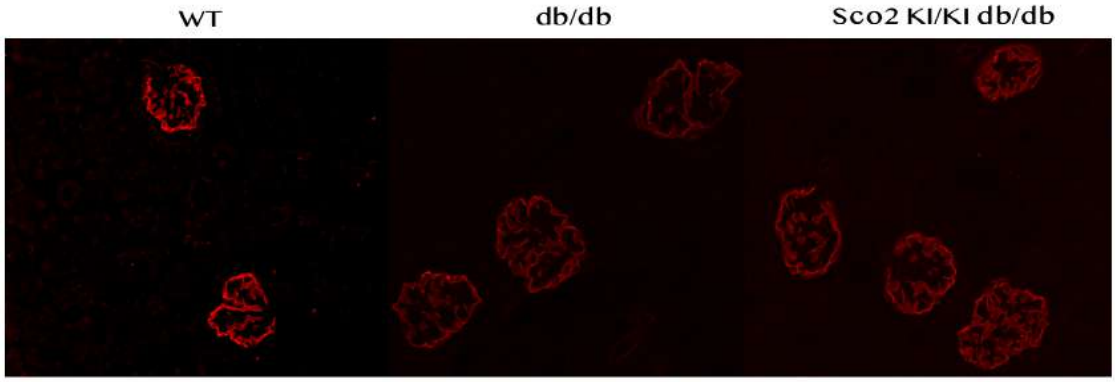

Figure 5: Immunofluorescence staining for Synaptopodin. Images were taken at 20x by the author.

Figure 5 shows that amount of red stained in WT and db/db is equal, while it looks like there is an increase in the amount of KI/KI db/db. Using Image J, percent area was measured per glomeruli. After quantifying the images, the data was plotted on Graphpad Prism and an ANOVA test was performed on the data (*Figure 6*). According to *Figure* 6, there was no significant change in percent area stained between the WT and db/db genotype. However, a trend of an increase in the percent area stained was observed in the KIKI db/db. This could indicate that Sco2 mutation may be beneficial in the setting of diabetes.

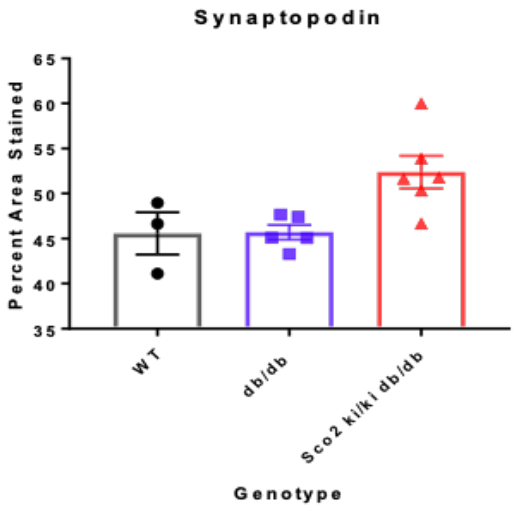

Figure 6: Synaptopodin quantification
Percent area was measured per glomeruli using Image J. This figure represents the percent area plotted using Graphpad prism.

B. Immunofluorescence for WT1

Images of WT1 and Dapi were taken both individually and merged. Representative images shown below were chosen to represent the general trend in podocyte damage using WT1 marker (*Figure 7*). Note than when Dapi and WT1 are merged, the bright pink dots represent podocytes. It can be seen in *Figure 7* that the number of podocytes is consistent in all three genotypes.

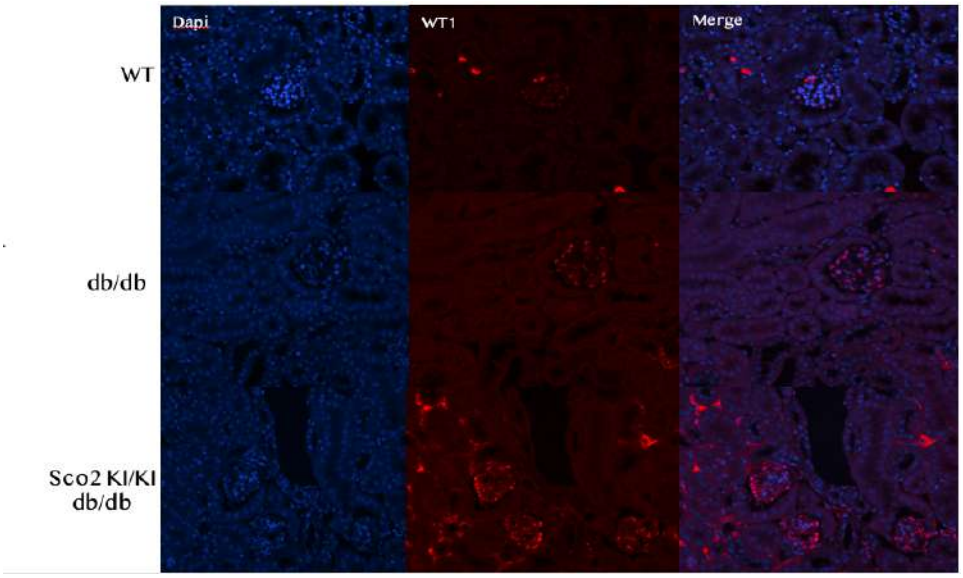

Figure 7: Images shown for dapi and WT1 staining. Images were taken by author on microscope at a magnification of 20x.

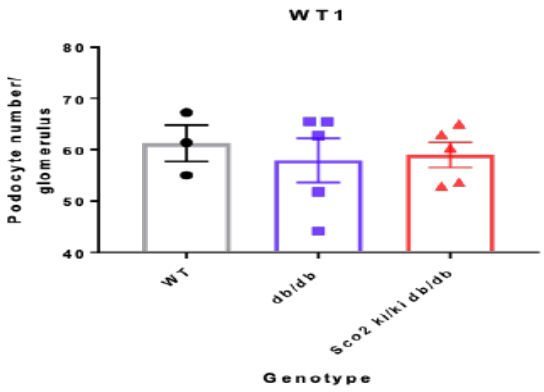

Figure 8: WT1 quantification
Podocyte number per glomeruli was counted Image J. This figure represents the chart of podocytes counted.

The amount of WT1 present in the glomeruli of each genotype was then quantified with Image J. WT1 was quantified in conjunction with dapi because WT1 is present in the nucleus of the podocytes. All dapi–positive WT1 were counted and the total podocyte count per glomerulus was determined. The data, shown on the left, was plotted on Graphpad Prism and analyzed using ANOVA test. *Figure 8* shows no significant difference between the podocyte count/glomerulus between any of the genotypes.

III. Immunofluorescence staining for tubule marker:

Lotus Lectin was analyzed in order to determine the percent of tubules present. After staining, images were taken under the microscope and representative images were chosen. *Figure* 9, down below, shows a relatively even amounts of Lotus Lectin in all three genotypes.

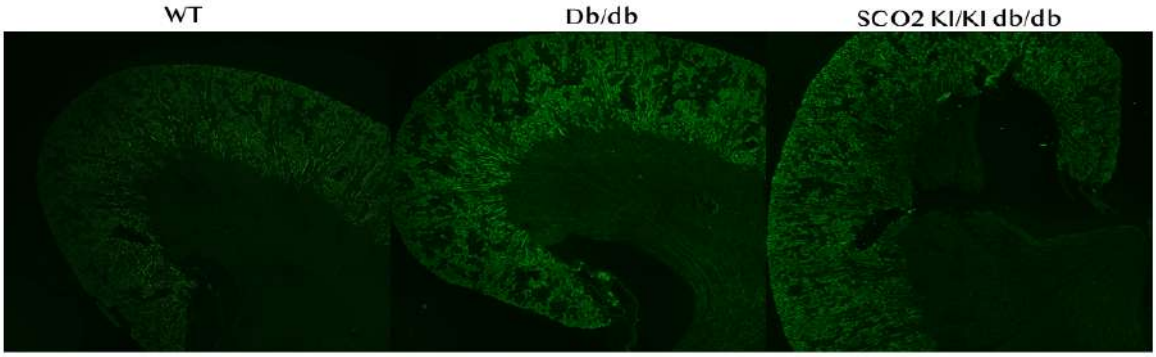

Figure 9: Images of Lotus Lectin staining taken by author of this paper at a magnification of 10x taken by author of this paper

Image J was used to quantify the Lotus Lectin and determine the percent area stained. The data was gathered on Graphpad Prism and an ANOVA test was conducted to determine the significance of the data. After graphing, it can be seen though *Figure 10*, that there is no significant difference in the percent of tubules stained. This suggests that there may not be as much tubular damage as previously thought.

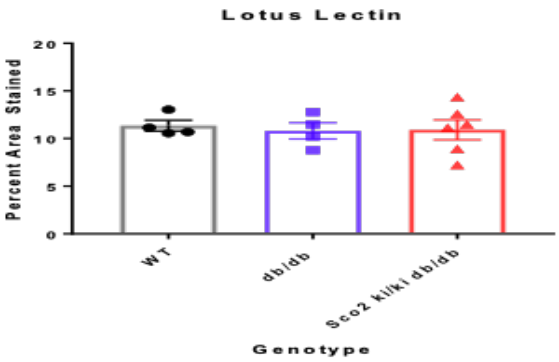

Figure 9: Lotus Lectin quantification
Percent area was measured using Image J. This figure shows the percent area of lotus lectin stained.

IV. Immunofluorescence staining for endothelial cells:

The three genotypes were stained for eNOS and then images focusing on the glomeruli were taken under the microscope. *Figure* 11 is the representative images chosen for eNOS so the difference in eNOS expression can be qualitatively analyzed. From *Figure 11*, it can be observed that there is an increase in eNOS visible in the glomerulus in db/db compared to the WT and then there seems to be less in the KI/KI.

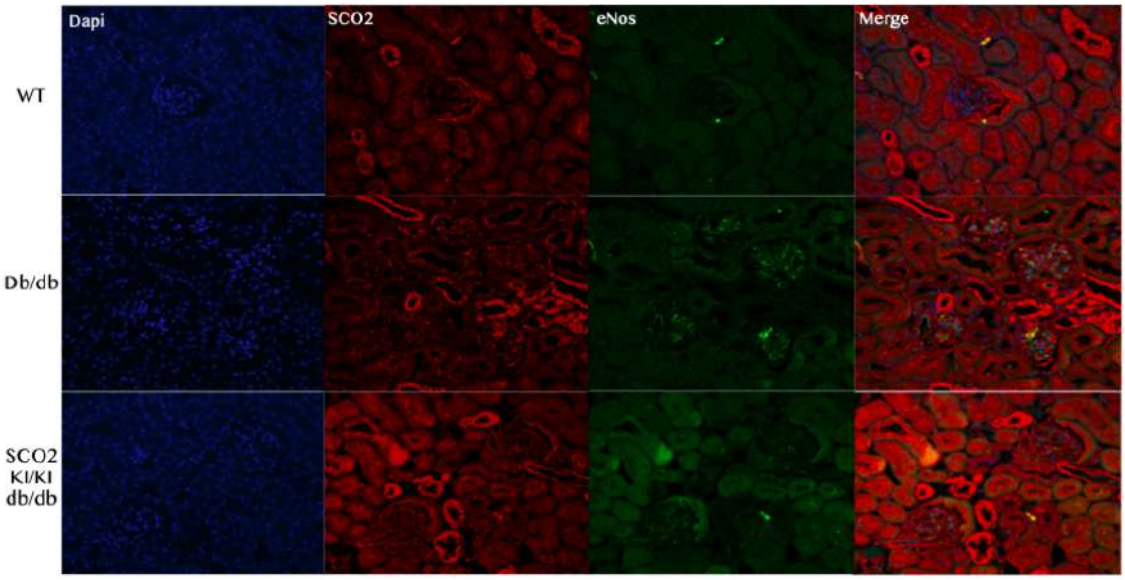

Figure 11: Representative images of eNOS taken at a magnification of 20x by the author

The percent area of eNOS stained was determined using Image J. The data was inputted into Graphpad Prism and ANOVA test was performed on this data set. From *Figure 12*, it can be determined that there was a significant increase in percent area stained between WT and db/db. There was 0% of eNOS stained in WT, while in db/db there was around 12%. Although not significant, there is also a trend of an increase in KI/KI db/db compared to WT however, there is a decreasing trend in eNOS between db/db and KI/KI db/db. This portrays the glomerular eNOS expression is probably represented due extensive injury.

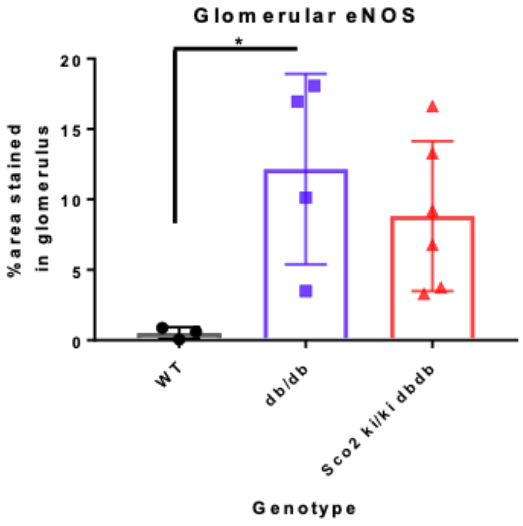

Figure 12: Graph of percent area of eNOS stained in the glomerulus for each genotype

V. Albumin Assay:

Urine was collected by the mentor from the db/db and KI/KI db/db mice over a 24 hour period. The albumin was measured using the Albumin ELISA Assay. Once the albumin concentration was calculated, the albumin concentration in mg/24 hr was graphed. As seen by *Figure 12* down below, there was a significant decrease albumin between db/db and KI/KI db/db. This suggests that the amount of protein filtered with the urine decreased indicating that the podocytes are functioning correctly.

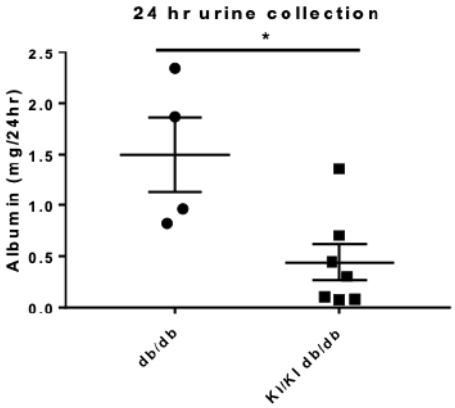

Figure 13: Albumin concentration in urine after 24 hour urine collection. Graph and urine collection done by mentor

Discussion

The novel findings of this study provide major contributions to the research on diabetic nephropathy by demonstrating the effects of a global knockdown of Sco2 in a diabetic injury model on kidney function. Additionally, it offers insight into which cells are impacted by the mutation in the setting of diabetes and how they are affected. This investigation found general morphological changes which were observed through the PAS staining. As seen in *Figure 4*, morphological changes were prominent in both db/db and KI/KI db/db genotypes. The damage seen in db/db genotype supports the previous research that states that diabetes has a negative effect on the kidney and leads to loss of proximal tubules, shedding of the brush border, glomerular mesangial expansion, and increase in glomerular volume.[5]

Additionally, it was also determined that the Sco2 mutation, in conjunction with diabetes, produced tubular and glomerular damage compared to WT. Despite these changes, as shown in *Figure 4*, db/db has more injury in the glomerulus and tubules compared to the Sco2 KI/KI db/db. This suggests that the reduction of energy produced by the mitochondria does have a negative effect on the tubules and the glomerulus but may not cause any additional injury compared to just the diabetic genotype. The findings observed in this study mirror those of the previous studies that have examined the effect of rotenone(ROT) treatment in a type 1 diabetes setting.[17] As seen in *Figure 14. D*, the streptozocin (STZ) kidney had the most glomerular and tubular injury whereas STZ + ROT had injury but at a smaller scale. *Figure 14.E.* represents glomerular area which quantitatively supports the images in *Figure 14.D.* The STZ + ROT had a significant decrease compared to the STZ even though it was an increase from the Ctrl. Similarly to this study, when the Sco2 mutation was present, the glomerulus has less injury compared to db/db.

Significant findings from this study indicate that Sco2 mutation in a diabetic setting have a protective effect on the podocytes. The results obtained from the immunofluorescence staining of Synaptopodin, although not statistically significant, reveal an increasing trend in expression of Synaptopodin in the KI/KI db/db genotype as represented in *Figure 6*. This data was inversely related to the albumin assay results which indicated that there was a significant decrease in the amount of albumin in the urine between the db/db and the KI/KI db/db (*Figure 13*). the albumin concentration in the urine decreased and the Synaptopodin expression increased in KI/KI db/db, the podocyte injury caused by diabetes is regulated suggesting that the mutations could have a protective effect on the podocyte.

Conversely, the WT1 data may refute this conclusion since the data shown in *Figure 8* shows no significant difference in podocyte number per glomeruli. This may propose that the mutation in a diabetic setting may not have a negative impact on the podocytes. However, in a study done by Wu in year, the mRNA expression of nephrin and podocin, 2 podocyte markers, were determined.[17] As depicted in *Figure 14 A*. and *B*, podocin and nephrin expression increased in the STZ model with the ROT treatment. Conversely, *Figure 14.C.* shows that the albumin concentration significantly decreased in the STZ with ROT. Although this data supports Type 1, the data shows a negative correlation between podocyte markers and albumin excretion in urine which is also observed in this study with type 2 diabetes and Sco2. This validates the Synaptopodin results and supports that Sco2 may have a protective effect on podocytes with the podocytes having less injury.

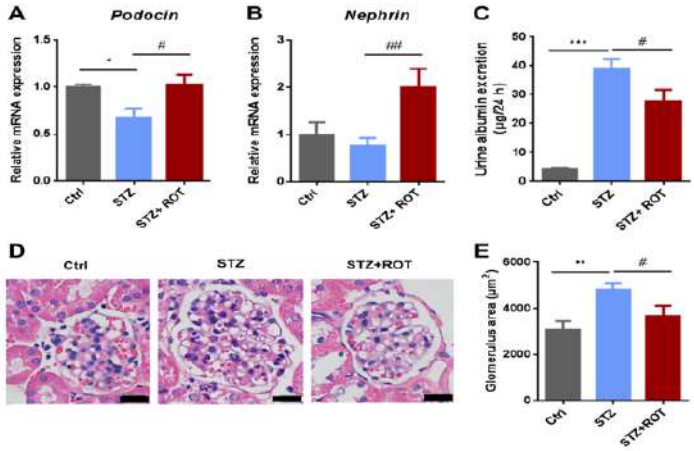

Figure 14: Type 1 diabetic mice were treated with treated with rotenone (ROT) which attenuated glomerular injury. (A) and (B) are quantitative analysis of podocyte-specific markers of podocin and nephrin. (C) albuminuria was measured after 24 hours of collecting urine. (D) is the representative images of PAS stained mouse kidney. (E) quantification of the glomerular area
N = 7 for control group, N = 9 for STZ group, and N = 9 for ROT group.[17]

Although the PAS staining showed that the mutation had seemingly negative ramifications, the results obtained through immunofluorescence staining for tubules produced contradictory results. As seen

in *Figure 9* and *Figure 10*, the percent stained of Lotus Lectin was approximately the same. Additionally, the one way ANOVA also reveals that there is no significant difference between the percent area stained in all three genotypes. This implies that there is a possibility that the Sco2 mutation in a diabetic condition has no negative effect on the tubules which is seemingly different from the results shown in the PAS stainings.

The analysis of immunofluorescence staining for eNOS showcases findings that suggest that the Sco2 mutation with the diabetic setting may be more injurious in endothelial cells. eNOS is expressed within the endothelial cells in order to maintain homeostasis. In a diabetic condition, the homeostasis of the endothelial cells is disrupted and results in an increase in expression of eNOS. Further statistical analysis revealed that there was a significant increase in the percent area of eNOS stained in the glomerulus which indicates that there was an increase in eNOS expression (*Figure 12*). The eNOS expression seems to have a correlation to the injury caused by diabetes. The results for the KI/KI db/db also show increasing trend in eNOS expression. However, *Figure 12* shows a decreasing trend in percent area of eNOS stained compared to db/db. This could signify an increase in endothelial damage when the mutation is present because too much damage could have an adverse effect on the expression of eNOS. Since there could be excessive damage to endothelial cells, eNOS is no longer able to manage the injury and maintain homeostasis, so it's expression decreases.

Limitations

There were identifiable limitations present in this study. The sample size used for many of the staining was useful in obtaining the current results, however, it limits the accuracy of the results. Due to the limited sample size, the changes in most staining was minimal and not significant. Further, the data for PAS staining was based off of qualitative data which provided visual representations of the injury, but determination of the difference between each genotype was significant or not was unable to be determined by simply just using the images.

Future Research

The data collected in this investigation provided significant insight on diabetic nephropathy in addition to Sco2 and its impacts on kidney function. Nevertheless, more research in the future is necessary to develop this project further and gain a stronger understanding of Sco2 and its effects on the kidney in DKD. In order to develop more accurate data which allows us to more precise changes between genotypes, an increased sample size needs to be obtained. Additionally, the IF stainings for all cell markers should be repeated in order to show the validity of the data especially since WT1 and Synaptopodin were contradictory as well as the PAS stains and the Lotus Lectin. Moreover, in further investigations, it would be important to use other markers, such as aquaporin-1, collagen1a1, nephrin, and isolectin, to obtain more results so

that stronger conclusions can be made. Lastly, the complex IV activity should be checked in order to exactly determine how much the Sco2 mutation influences the activity and affects ATP production.

Conclusion

The findings generated in this multifaceted investigation provided insight on the effects of the global knockdown of Sco2 in a diabetic injury model while also characterizing which cells in the kidney are affected and how they are affected.

- The knockdown of Sco2 in a diabetic model may not negatively impact the tubular cells as previously thought
- Synaptopodin and albumin are negatively correlated, which suggests that the Sco2 mutation in the setting of diabetes may have protective effects on the podocytes and podocytes would have less injury.
- eNOS expression increased in db/db but decreased in KI/KI db/db suggesting an increase in injury in the KI/KI db/db

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