Diabetic Complications Consortium

Application Title: Sexual dimorphisms and role of cGAS-STING Pathway in T2DN rats

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1. Project Accomplishments:

Diabetes and its renal complications are becoming an epidemic in the U.S. population. Diabetic kidney disease (DKD) is the leading cause of chronic renal pathology and, therefore, is the subject of major research efforts. DKD is one of the primary missions of the National Institute of Diabetes and Digestive and Kidney. Importantly, as described on the DiaComp website, diabetic complications manifest themselves differently between men and women. Understanding the molecular underpinnings of these manifestations is critical to designing tailored therapeutic approaches. This pilot proposal focused on sex differences in the progression of DKD. To explore the sexual dimorphisms in the development of DKD, we proposed to explore the development of DKD in type 2 diabetic nephropathy (T2DN) rats. The T2DN rat was previously created by the introgression of the mitochondria and some passenger loci from the Fawn Hooded Hypertensive rat into the background of the Goto-Kakizaki (GK; type 2 diabetic) rat. Our recent studies have demonstrated that T2DN rats develop renal and physiological abnormalities similar to clinical observations in human patients with DKD, including progressive glomerular and tubular damage and a significant decrease in renin-angiotensin-aldosterone system plasma levels, indicating these rats are an excellent model for studying the progression of renal damage in type 2 DKD (1). However, sexual dimorphisms have never been investigated in this model, and studies of sex differences are limited in DKD in general.

In this project we determined phenotypic characteristics of DKD progression related to the sex difference in T2DN rats and identified potential mechanisms responsible for renal and specifically glomerular injury. Our data revealed the functional divergence in DKD progression in male and female T2DN rats, similar to clinical observations in human patients (2). Furthermore, our studies have revealed that the cyclic GMP–AMP synthase (cGAS) / Stimulator of interferon genes (STING) signaling pathway is regulated differently between male and female T2DN rats (Khedr et al., in preparation).

References:

1. **Palygin O, Spires D, Levchenko V, Bohovyk R, Fedoriuk M, Klemens CA, Sykes O, Bukowy JD, Cowley AW, Jr., Lazar J, Ilatovskaya DV, and Staruschenko A**. Progression of diabetic kidney disease in T2DN rats. *Am J Physiol Renal Physiol* 317: F1450-F1461, 2019.

2. **Spires DR, Palygin O, Levchenko V, Isaeva E, Klemens CA, Khedr S, Nikolaienko O, Kriegel A, Cheng X, Yeo JY, Joe B, and Staruschenko A**. Sexual dimorphism in the progression of type 2 diabetic kidney disease in T2DN rats. *Physiological genomics* 53: 223-234,

2. Specific Aims:

A combination of *in vivo* and *ex vivo* studies was used to address the following Aims:

Specific Aim 1: To characterize the sexual dimorphisms in the progression of DKD in T2DN rats.

The goal of this Aim was to define the progression of DKD in both sexes in the setting of T2DM. T2DN rats, developed at the Medical College of Wisconsin, were used to study sexual dimorphisms in the setting of DKD. Despite the high degree of genetic similarity between T2DN and GK rats (97% at 681 loci), diabetes ensues earlier and progresses more severely in T2DN rats compared to the GK strain. Unlike other models, the natural course of renal disease in T2DN rats more closely parallels human DN. Renal structural abnormalities such as glomerular and tubular hypertrophy are observed at early ages (12 weeks) and precede the development of proteinuria. By six months of age, T2DN rats exhibited proteinuria, accompanied by renal histologic abnormalities such as focal segmental glomerulosclerosis (FSGS), mesangial matrix expansion, and thickening of basement membranes. **Figure 1** shows the progression of diabetes and some parameters of kidney injury in T2DN, GK, and Wistar rats (Wistar rats were selected as non-diabetic control since GK strain was initially developed by repeated inbreeding of Wistar rat).

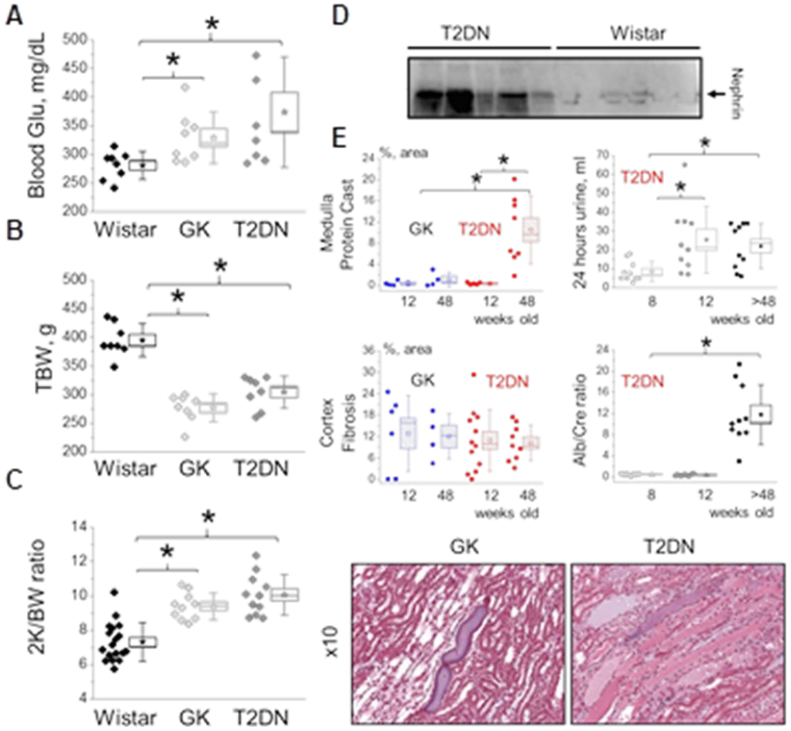

Figure 1. Progression of diabetes and DKD in T2DN male rats. Physiological parameters of DKD development and renal injury in aged-matched Wistar, Goto-Kakizaki (GK), and type 2 diabetic nephropathy (T2DN) rats (12 weeks for A-C, and 12 or 48 weeks for D and E). Shown are blood glucose levels (**A**), total body weight (**B**), ratio of kidney to body weight (**C**), urinary nephrin levels of T2DN and Wistar rats (**D**) and summary of kidney damage (**E**; % of protein casts, fibrosis, diuresis, albumin/creatinine ratios and histological examples of medullar protein cast in GK and T2DN rats).

Sex differences in T2DN rats: We described the development of advanced forms of DN and glomerular damage in T2DN rats (**Fig. 1**). Since the progression of renal disease in T2DN rats closely parallels that of human DKD, we questioned whether this strain also displays sexual dimorphisms comparable to the human population. For the characterization of T2DN rats, we used both male and female rats of the same age. We have found striking differences in the progression of DKD and kidney injury between sexes. Male and female T2DN rats at >48 weeks of age were used to evaluate hyperglycemia, kidney injury, and renal function during the advanced stage of DN. We found that there were significant differences in fasting blood glucose levels in males versus females (156 ± 8 vs. 124 ± 4 mg/dL, respectively). Glucose tolerance tests revealed a higher prevalence of glucose intolerance in males (378 ± 19 vs. 183 ± 48 mg/dL for males vs. females). The development of albuminuria and glomerular damage was also significantly different between sexes (11.8 ± 5.7 vs. 0.98 ± 0.64 Alb/Cre ratio, and 28 vs. 4 %

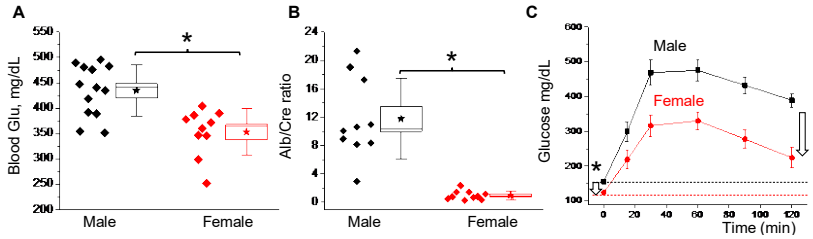

Figure 2. Sex difference in T2DN rats. Blood glucose levels (**A**), albuminuria (**B**), and glucose tolerance test (**C**) in male and female T2DN

with the highest damage score, male vs. female). These changes were accompanied by a significant increase in urinary nephrin shedding in males, suggesting pathological changes to the glomerular filtration barrier. Detailed analyses of plasma samples also indicated striking differences in cholesterol (261 ± 47 vs. 87 ± 3 mg/dL), blood urea nitrogen (22 ± 2 vs. 17 ± 1 mg/dL), albumin homeostasis, and alkaline phosphatase levels in males compared to females. Echocardiography analyses of the cardiac output, left ventricle wall thickness, fractional shortening, and heart rate for the majority did not differ between strains, suggesting that the DN-related effects on renal and metabolic phenotypes in the T2DN rats do not induce additional cardiac pathology. Shown in **Fig. 2** is a summary of blood glucose levels, albuminuria, and glucose tolerance in male and female T2DN rats. Kidney injury and expression of podocin and nephrin, which demonstrate glomerular damage, are shown in **Fig. 3**.

<u>Specific Aim 2:</u> To define the contribution of the cGAS-STING signaling pathway in the progression of DKD and its contribution to sex difference.

To define the molecular mechanisms involved in the progression of DKD and sex differences in T2DN rats, we applied RNA-Seq analysis to identify changes in the expression of genes involved in disease progression in young and old (12 and >48 weeks, respectively) T2DN male and female rats. One of the key observations from the RNA-Seq analysis was a significant change in genes participating in the cGAS-STING inflammatory pathway. Plotting of the gene expression values (RPKM) for 23 genes that participate in the cGAS-STING pathway for the four different animal cohorts is shown in **Fig. 4A**.

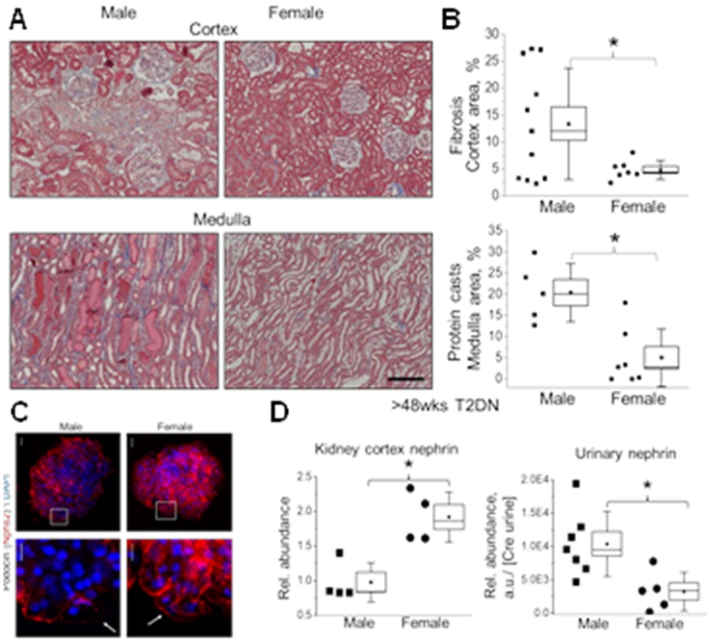

Figure 3. Sexual dimorphism in the progression of kidney and glomeruli injury in T2DN rats. ***A***, Representative images of cortical fibrosis/tubular injury and medullary protein cast formation in the kidneys of male and female T2DN rats (Masson's trichrome staining; >48 weeks of age). Scale bar = 100 µm. ***B***, Summary graphs indicating differences in cortical fibrosis and medullary protein cast formation in male and female kidneys of T2DN rats (>48 weeks of age). ***C***, Representative images of isolated glomeruli labeled with the podocyte marker podocin (Nphs2; red). Top: 3D structure projected into 2D image showing total podocin in the glomerulus. Bottom: single section of a z-stack indicating a focal region with low podocin expression in male and female T2DN glomerulus. ***D***, Summary graph for Western blotting analyses of nephrin, the major component in formation and maintenance of the podocyte slit diaphragm, in renal cortex and urine (nephrin shedding) in male and female T2DN rats at >48 weeks of age.

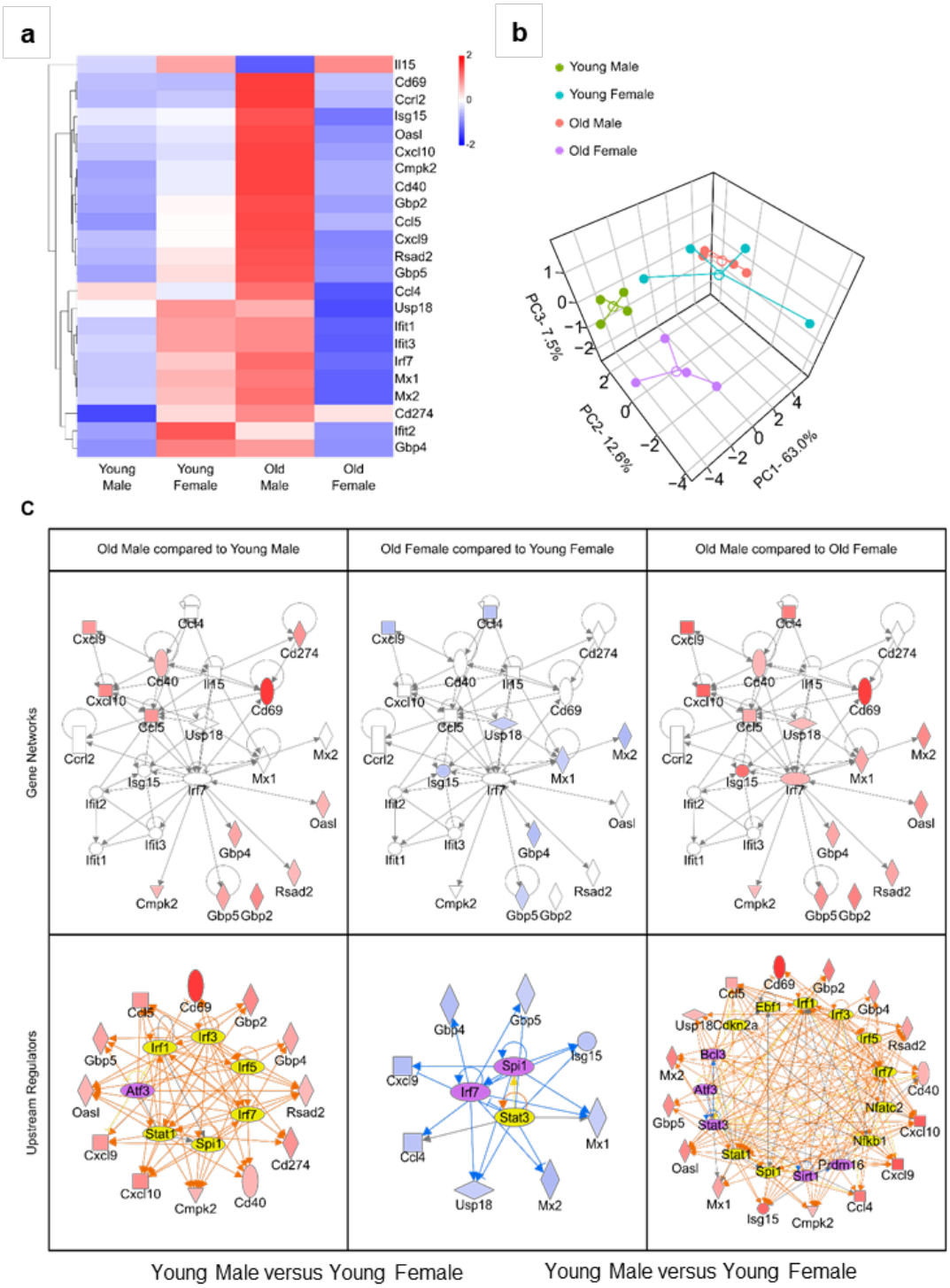

Figure 4. RNA-Seq analysis of cGAS-STING specific genes. A, RPKM expression level of genes normalized to the z-scale. **B,** PCA plot with centroids (open circles) and different animal groups (filled circles) using RPKM expression values of genes. **C,** Gene networks and upstream regulatory analysis of cGAS-STING specific genes for different animal groups. Gene networks: significant gene fold changes (fold change ≥ |2| and FDR < 0.05) with respect to controls are represented in red (upregulation) and blue (downregulation) nodes while the nonsignificant genes have white nodes. Upstream regulatory analysis: color scheme of nodes is same as in gene networks. Yellow and purple nodes represent predicted activated or inhibited transcription factors, respectively. Orange lines indicate predicted activation, blue lines indicate predicted inhibition, yellow lines indicate that the predicted relationship is inconsistent with gene expression while gray lines indicate no predicted effect.

The analysis revealed that the old males (>48 weeks) displayed the highest level of gene expression than all other groups indicating strong activation of genes in this pathway. Thus, concerning the sex and age differences, the older males had a higher expression of genes in this pathway than older females and younger males. We also performed principal component analysis (PCA) using RPKM expression values of the four different cohorts to assess the differences in gene expression patterns between the groups (**Fig. 4B**). PCA of these animals indicated that PC1 explains 63% of the data variance while PC2 and PC3 explain 12.6 and 7.5%, respectively. We found that the older males clustered the farthest from the older females and the younger males with a euclidean distance of 9.25 and 7.51, respectively. The younger females appear to cluster closer to the older males with a euclidean distance of about 5.13. Subsequently, we performed gene network and upstream regulatory analysis using the semantic-based algorithm IPA (**Fig. 4C**). Consistent with gene expression data, we observed that genes in the cGAS-STING network were highly expressed in the older male cohort compared to younger males and older females (**Fig. 4C**, left and right panels). Interestingly, some of these genes in the older female cohort were downregulated compared to younger females (**Fig. 4C**, center panel), indicating that in females, there is an attenuation of the cGAS-STING pathway with age. The upstream regulatory analysis revealed predicted activation of transcription factors involved in interferon responses (Irf1, Irf3, Irf5, Irf7) and other immune pathways (Stat1, Spi1, Nfatc2) in older males compared to females and younger males. We also observed downregulation of specific immune response transcription factors such as Atf3, Bcl3, and Stat3. Thus, data from this analysis indicates that the cGAS-STING pathway is highly active in older males as compared to the other groups, which is consistent with our previously reported progression of DKD in this strain.

3. Publications:

1. Kravtsova O., Bohovyk R., Levchenko V., Palygin O., Klemens C.A., Rieg T., **STARUSCHENKO A**. (2022) SGLT2 inhibition effect on salt-induced hypertension, RAAS, and sodium transport in Dahl SS rats. American Journal of Physiology: Renal Physiology 322(6):F692-F707. PMID: 35466690.
2. Spires D.R.*, Palygin O.*, Levchenko V., Isaeva E., Klemens C.A., Khedr S., Nikolaienko O., Kriegel A., Cheng X., Yeo J.-Y., Joe B., **STARUSCHENKO A**. (2021) Sexual dimorphism in the progression of type 2 diabetic kidney disease. *Physiological Genomics* 53(6):223-234. PMID: 33870721. # – APS*select.*
3. Khedr S., Alsheikh A.J., Spires D.R., Dissanayake L., Zietara A.P., Anne R., Kerketta R., Mathison A., Urrutia R., Palygin O., **STARUSCHENKO A**. Role of cGAS-STING pathway in aging and sexual dimorphism in diabetic kidney disease [In preparation].