Diabetic Complications Consortium

Application Title: Defining Podocyte Injury in Human Diabetic Nephropathy by Single Nucleus RNA-Sequencing

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1. Project Accomplishments:

During this funding period, we successfully generated single nucleus RNA-sequencing (snRNA-seq) datasets from two healthy and two early diabetic human kidney samples. We have analyzed the first two samples and find striking differences in podocyte cell states in healthy vs. diabetic samples, but no major changes in other kidney states. We have identified differentially expressed genes and novel ligand-receptor signaling in the diabetic glomerulus. These studies will open the way to comprehensive analysis of biobanked human diabetic nephropathy samples using snRNA-seq.

2. Specific Aims:

Specific Aim 1: Generate single nucleus transcriptomes from a healthy kidney vs. kidney with known diabetic nephropathy. We will use material from the Boston Nephrectomy Cohort Biobank led by Co-Investigator Sushrut Waiker MD, MPH with whom we have ongoing collaborations. The healthy and diabetic tissues are already identified. We will generate a suspension of single nuclei using a validated protocol in our lab, FACS purify to remove all debris and sort individual nuclei into wells of a 96-well plate. We will generate barcoded single cell libraries using the SMART-Seq protocol and sequence the transcriptomes of 400 nuclei from each kidney to a depth of 200,000 reads per nucleus.

Results: We made outstanding process on this aim. We developed a protocol for snRNA-seq using the 10X Chromium platform which allowed us to generate 10-fold more snRNA-seq transcriptomes than we had originally planned. Moreover, these were from frozen kidney samples that had a low RNA Integrity Number (RIN) of between 2.0 – 3.0, which indicates degraded RNA. Despite this fact, we could detect a minimum of 3,000 unique genes per nucleus which allowed excellent clustering (Figure 1). The primary conclusion from our tSNE of the first two samples we processed (the other two have been sequenced but not analyzed yet) is very exciting. What we see is that all kidney cell type clusters co-project on the tSNE with one exception – podocytes. This means that in these samples with mild diabetic nephropathy, that podocytes are the primary cell type affected. This has given us a unique molecular window into early podocyte cell state changes in diabetic nephropathy (Figure 1). In aim 2, we analyzed in much greaeter detail the differences between healthy and diabetic podocytes.

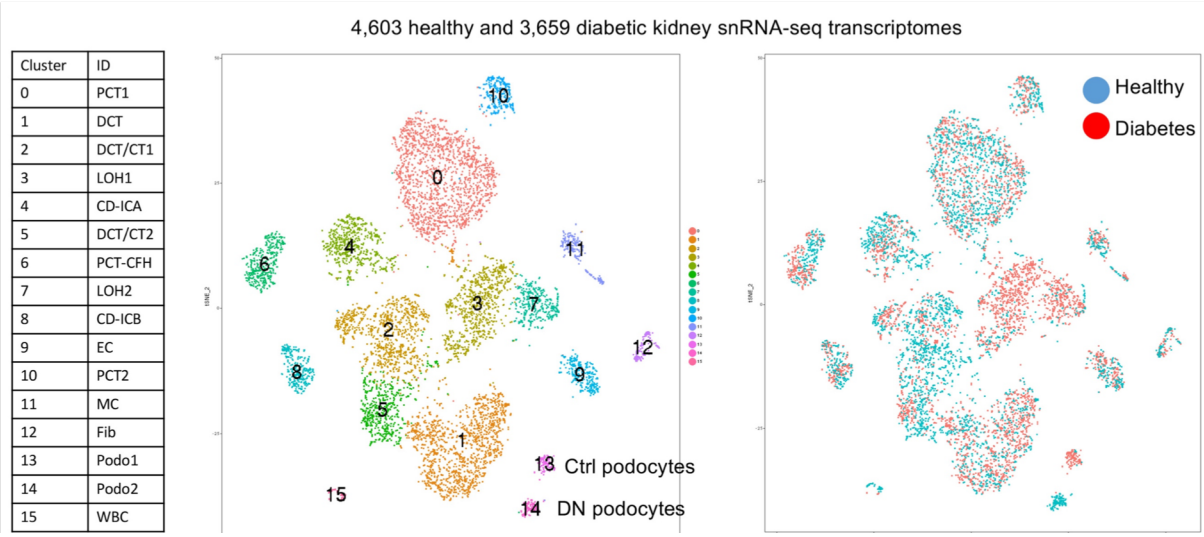
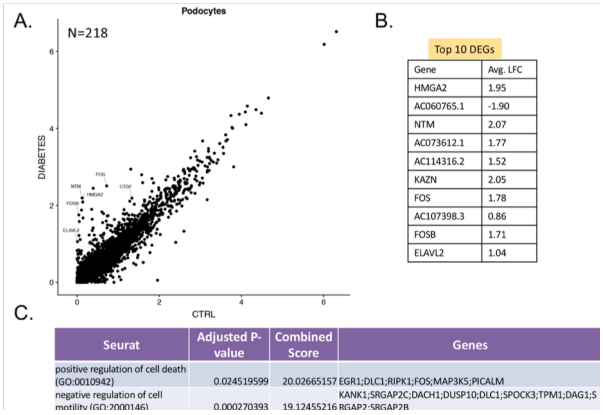


Figure 1. Healthy vs. diabetic kidney snRNAseq. We successfully generated 8,262 snRNA-seq transcriptomes from healthy or diabetic cryopreserved kidneys. When coprojecgted by tSNE, we observe 15 separate cell types. Notably, only podocytes cluster differently between healthy and diabetic samples (clusters 13 and 14), indicating that diabetic podocytes are fundamentally different from healthy ones – but other kidney cell types have no major changes by this analysis. Note also that we can detect a WBC cluster in the diabetic but not the healthy kidney sample.

Specific Aim 2: Perform comprehensive bioinformatic analysis on the sNuc-seq data using methods in use within the Humphreys Lab. We will batch-correct using ComBat, reduce dimensionality by tSNE and project onto 2d space. Cells will be clustered by SNN-cliq, Infomap or Louvain-Jaccard algorithms. We will use known markers to define cell types, and compare gene expression between healthy and diabetic cell types, focusing primarily on podocytes, proximal tubule and fibroblasts. We will perform gene ontology between cell types to infer disease processes in diabetic nephropathy. We will create a cell specific diabetic nephropathy gene expression atlas. Depending on dataset robustness, we will attempt to pseudotemporally order cells using healthy kidney as the start point (Monocle). We will validate marker gene expression by immunofluorescence and immunohistochemistry.

Results: We have focused our detailed bioinformatic analysis on healthy vs. diabetic podocytes, since we saw the greatest changes in these groups. The analysis revealed a number of differentially expressed genes (Figure 2 A,B). GO analysis suggests that cell death and cell motility are two major pathways activated by podocytes in response

Figure 2. A. Differentially expressed genes (DEG) between healthy and diabetic podocytes. **B.** Top 10 DEGs, healthy vs. diabetic podocytes. **C.** Gene Ontology analysis reveals that diabetic podocytes are characterized by "positive regulation of cell death" and "negative regulation of cell motility."



to the diabetic mileu. This is in keeping with both the podocyte depletion hypothesis (cell death signature) and foot process effacement (cell migration) that have been hypothesized to occur in diabetic nephropathy.

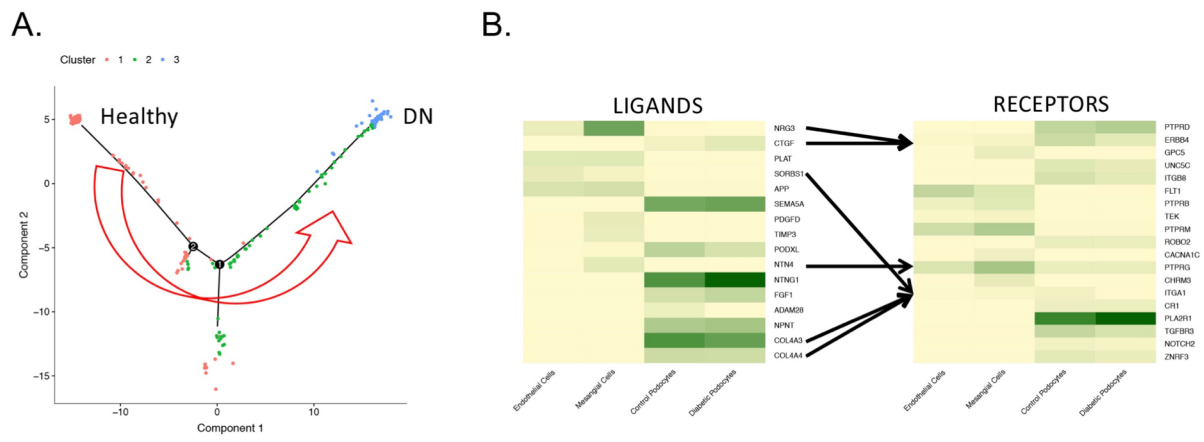


Figure 3. Cell trajectory and ligand-receptor analysis in diabetic podocytes. A. Pseudotemporal ordering of healthy to diabetic podocytes reveals a continuum of transcriptional changes. **B.** Receptor-ligand analysis of glomerular cell types reveals several novel intercellular communication pathways in diabetic glomerulopathy. In particular, mesangial cell upregulated neuregulin-3 signals to the ERBB4 receptor on podocytes in early DN.

We also performed a cell trajectory analysis using Monocle (Figure 3A). This revealed a smooth transition from healthy podocytes (upper left) to diabetic podocytes (upper right). This demonstrates the potential power of single nucleus analysis to reveal subtle state changes in early diabetic nephropathy. Finally, we performed a receptor-ligand analysis of upregulated ligands and receptors in glomerular cell types. This revealed very intriguing upregulation of known (CTGF) and novel (NRG3) signaling pathways. In particular, mesangial cell expressed Neuregulin-3 binds to its receptor ERBB4 present on podocytes. By contrast, diabetic podocytes upregulate CTGF and bind to ERBB4 in an autocrine signaling loop.

In conclusion, we consider this to have been a highly successful pilot study that sets the stage for generation of comprehensive cell atlases of human kidney diseases using cryopreserved specimens.

3. Publications:

1. H. Wu, Y. Kirita, E.L. Donnelly and **B.D. Humphreys**. Advantages of single nucleus over single cell RNA-sequencing of adult kidney: Rare cell types and novel cell states revealed in fibrosis. J Am Soc Neph, *Rapid Communication*, *in press*.

2. H. Wu, A.F. Malone, E. Donnelly, Y. Kirita, K. Uchimura, S.M. Ramakrishnan, J. Gaut and **B.D. Humphreys**. Single cell transcriptomics of a human kidney allograft biopsy defines a diverse inflammatory response. *J Am Soc Neph*, 29(8):2069-2080, 2018. ***Cover art**

3. A.F. Malone, H. Wu and **B.D. Humphreys**. Bringing Renal Biopsy Interpretation into the Molecular Age with Single Cell RNA-Sequencing. Semin. Nephrol. 2018; 38(1):31-39.

4. P.C. Wilson and **B.D. Humphreys**. Year in Review: Single cell genomics and gene editing: implications for nephrology. Nature Reviews Nephrology, *in press*.