Profiling APOL1 Nephropathy Risk Variants in Genome-Edited Kidney Organoids with Single-Cell Transcriptomics

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ABSTRACT

Background
DNA variants in APOL1 associate with kidney disease, but the pathophysiological mechanisms remain incompletely understood. Model organisms lack the APOL1 gene, limiting the degree to which disease states can be recapitulated. Here we present single-cell RNA sequencing (scRNA-seq) of genome-edited human kidney organoids as a platform for profiling effects of APOL1 risk variants in diverse nephron cell types.

Methods
We performed footprint-free CRISPR-Cas9 genome editing of human induced pluripotent stem cells (iPSCs) to knock in APOL1 high-risk G1 variants at the native genomic locus. iPSCs were differentiated into kidney organoids, treated with vehicle, IFN-γ, or the combination of IFN-γ and tunicamycin, and analyzed with scRNA-seq to profile cell-specific changes in differential gene expression patterns, compared to isogenic G0 controls.

Results
Both G0 and G1 iPSCs differentiated into kidney organoids containing nephron-like structures with glomerular epithelial cells, proximal tubules, distal tubules, and endothelial cells. Organoids expressed detectable APOL1 only after exposure to IFN-γ. scRNA-seq revealed cell type-specific differences in G1 organoid response to APOL1 induction. Additional stress of tunicamycin exposure led to increased glomerular epithelial cell dedifferentiation in G1 organoids.

Conclusions
Single-cell transcriptomic profiling of human genome-edited kidney organoids expressing APOL1 risk variants provides a novel platform for studying the pathophysiology of APOL1-mediated kidney disease.
INTRODUCTION

Apolipoprotein L1 (APOL1)-mediated kidney disease accounts for a portion of the excess risk of chronic kidney disease (CKD) and end-stage kidney disease (ESKD) among African American patients\textsuperscript{1,2}. The APOL1 high-risk genotype, defined as the presence of two risk alleles (G1 or G2 coding variants), increases the risk of developing CKD, but not all individuals with the high-risk genotype develop disease\textsuperscript{3,4}. Much remains unknown regarding mechanisms and modifiers that render the disease incompletely penetrant, and complex interactions underlying these mechanisms are difficult to model outside APOL1’s native genomic locus. As such, current gaps in knowledge may not be fully addressed by induction of transgenic APOL1 expression in vivo or in vitro. Additionally, because APOL1 is widely expressed across different cell types, studying APOL1 risk variants solely within a specific type of cell (e.g., podocytes) may not fully capture how these variants affect the kidney.

Human kidney organoids derived from induced pluripotent stem cells (iPSCs) can be utilized to model genetic disease mechanisms in the native genomic context and cell-type heterogeneity within the kidney\textsuperscript{5–9}. Using CRISPR-Cas9 mediated genome editing, we engineered iPSCs homozygous for the G1 risk allele and differentiated these cells into three-dimensional kidney organoids. To evaluate cell-type specific effects of the APOL1 high-risk genotype, we also performed single-cell RNA-sequencing (scRNA-seq), which we and others have previously leveraged to uncover novel biology of how cell-specific phenotypes contribute to kidney development or disease in organoids and other models\textsuperscript{10–14}. Here we present the application of genome-edited iPSC-derived kidney organoids and single-cell transcriptomics to profile APOL1-mediated effects on kidney organoids relevant to disease processes.

METHODS

Induced Pluripotent Stem Cell (iPSC) Culture

iPSC lines previously derived from fibroblasts from a non-African ancestry donor (Harvard Stem Cell Institute, 1016SevA\textsuperscript{15–18}) and peripheral blood mononuclear cells from an African ancestry donor (WiCell, Penn134-61-26) were maintained in feeder-free culture on 10 cm dishes coated
with 0.5% Geltrex (Gibco) in Modified Tenneille’s Special Recipe 1 (mTeSR1, STEMCELL Technologies), supplemented with 1% Penicillin/Streptomycin (Gibco) and 0.02% Plasmocin (Invivogen). iPSCs were confirmed to be mycoplasma-free and below passage 48. They were passaged using 1:3 Accutase (STEMCELL Technologies).

**CRISPR Cas-9 Genome Editing**

*APOL1* G1 risk variants (rs73885319 and rs60910145) were introduced into the 1016SevA iPSC line through a genomic footprint-free approach (Figure 1A, Figure S1A)\(^\text{19,20}\). Briefly, the homology-directed repair (HDR) template containing the G1 variants was engineered using the MV-PGK-Puro-TK vector (Transposagen Bio), referred to as PMV vector, which houses a removable puromycin selection cassette flanked by two homology arms. The puromycin cassette is excisable by a piggyBac transposase, leaving only a “TTAA” sequence behind that can be seamlessly introduced into a coding sequence by carefully choosing sites where the change would be synonymous. The G1 variants were engineered by two-step PCR of G0 genomic DNA (gDNA) (Figure S1A, Table S1) to create the donor template for homology arm A, designed to flank the upstream portion of the puromycin selection cassette. Arm B, designed to flank the downstream end of the selection cassette, was amplified from G0 gDNA by traditional PCR. Both Arm A and Arm B underwent separate TOPO TA cloning reactions (Invitrogen) to insert into a stable vector for subsequent subcloning into the PMV vector. Stepwise sequential double restriction-enzyme digests and homology arm ligations were performed on the PMV vector with the following pairs of restriction enzymes: Not1-HF and Bbs1-HF, Nco1-HF, and Bsa1-HF (New England Biolabs). The ends of both homology arms bordering the cassette harbor the “TTAA” piggyBac transposase cut sequence, thus allowing for the transposase to excise the cassette from both ends and leave behind the “TTAA” sequence in a scarless fashion (Figure S1B). To make this genome editing event footprint-free, we selected a codon site that would allow the “TTAA” nucleotide sequence to be knocked in without altering the APOL1 amino acid sequence. We identified a leucine (an amino acid encoded by six different codons including “TTA”) flanked by an adenine to be the site of cassette entry and excision (Figure S1C). A guide RNA sequence with a suitable protospacer adjacent motif (PAM) was found nearby the excision
site (Figure 1A, Table S1) and cloned into gRNA_Cloning Vector (Addgene 41824). The donor template incorporates a point mutation at the PAM site to destroy it after HDR to prevent recutting. iPSCs were then electroporated with the guide vector, hCas9 (Addgene 41815), and the G1-PMV donor plasmid (control lines were electroporated with the guide vector only). 48 hours later, 10 µg/ml puromycin was added to iPSC culture to select for cells expressing the donor plasmid (Figure S1A). Seven days later, iPSC colonies were evaluated for insertion of HDR template by PCR and Sanger sequencing validation. After expansion of the successfully knocked-in colonies into separate lines, the piggyBac transposase expression vector (Transposagen Bio) was introduced by electroporation. Additional screening of genotype was performed to validate the puromycin cassette excision.

**iPSC-Derived Kidney Organoid Differentiation**

iPSCs were differentiated into kidney organoids following the previously published Freedman et al. protocol (Figure 1B). Briefly, iPSCs were dissociated with 1:3 Accutase and plated onto 24-well plates pre-coated with 0.5% GelTrex in mTeSR1 supplemented with 10uM Y-27632 ROCK Inhibitor (STEMCELL Technologies). 24 hours later another layer of GelTrex at 1.5% was added in mTeSR1 media. At the end of the fourth day the medium was replaced with Advanced RPMI (Gibco) supplemented with 12 µM CHIR-99021 and 10 ng/mL noggin (STEMCELL Technologies). Approximately 60 hours later, the medium was changed to Advanced RPMI with B27 (Gibco). Organoids were cultured in this medium until collection at day 25.

**Induction of APOL1 Expression and Endoplasmic Reticulum (ER) Stress**

Day 24 G0 and G1 kidney organoids in identically plated wells of a 24-well plate were treated with interferon-gamma (IFN-γ, 25ng/ml, PeproTech) for 24 hours to induce APOL1 expression. This dose approximates previous in vitro IFN-γ doses used for macrophage activation. ER stress was induced by adding 5 µM Tunicamycin (Tocris) for 24 hours.
Single-cell RNA Sequencing (scRNA-seq)

We performed scRNA-seq on G0 and G1 day 25 kidney organoids on the 1016SevA background. The organoids were treated with vehicle, IFN-γ, or both IFN-γ and tunicamycin for 24 hours. Organoids were dissociated from the well with TrypLE Express (Gibco) and processed into single-cell suspension by gentle intermittent pipetting while incubating in a ThermoMixer (Eppendorf) for up to 15 minutes. Single-cell libraries were prepared using the 10x Genomics Chromium droplet-based platform and the Single Cell 5’ Library Construction Kit (10x Genomics), which was chosen to increase read coverage over the 3’ chemistry. At least three technical replicates (different wells from the same experiment) were included in each prepared library, with targeted cell recovery of 4,000 cells per library. Each experimental condition was represented by at least two different libraries. These libraries were assessed for quality control, pooled, and sequenced on the NovaSeq 6000 (Illumina) through the University of Illinois Genomics Core. The libraries were processed for 150-base pair (bp) paired-end reads, at an average sequencing depth of 114,000 reads per cell.

Immunofluorescence

Organoids were fixed in 4% paraformaldehyde for 15 minutes at room temperature. After fixing, samples were washed in PBS, blocked in 5% donkey serum (Millipore)/0.3% Triton-X-100/PBS for one hour at room temperature, incubated overnight in 3% bovine serum albumin (Millipore)/PBS with primary antibodies, washed, incubated with Alexa-Fluor secondary antibodies (Invitrogen) and DAPI, and washed into PBS for storage. Primary antibodies included PODXL (AF1658; R&D; 1:500) and ECAD (ab11512; Abcam; 1:500). Stains included fluorescein-labeled LTL (FL-1321; Vector Labs; 1:500). Fluorescence images were captured using an inverted Nikon epifluorescence Eclipse Ti or A1R confocal microscope.

Real-Time Quantitative Polymerase Chain Reaction (RT q-PCR)

RNA was isolated from day 25 kidney organoids using the PureLink Kit (Invitrogen). RNA was then reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-qPCR reactions for APOL1 (Thermo Fisher, Hs01066280_m1) and ACTB
(Hs01060665_g1) were run in duplicate on the QuantStudio 5 Real-Time PCR System (Applied Biosystems). Relative APOL1 expression was calculated using the $2^{-\Delta\Delta CT}$ method. Statistical significance was tested using two-way ANOVA in Graphpad (Prism).

**Immunoblot**

Organoids were washed with PBS and lysed in RIPA buffer (Thermo Scientific) with protease inhibitor (Thermo Scientific) and benzonase nuclease (Thermo Scientific). Cell lysates were cleared by centrifugation for 10 minutes at 14,000 x g at 4º C. Proteins (20µg) were heated at 95ºC with β-mercaptoethanol and then separated by SDS PAGE (Invitrogen). Nitrocellulose membranes were blocked in 5% nonfat milk in PBST for 1 hr and then incubated in primary antibody overnight at 4ºC, followed by incubation with HRP-conjugated secondary anti-Ig (Abcam; 1:5000). The primary antibodies used in this study were anti-APOL1 (HPA018885; Sigma; 1:1000), anti-β-actin (#4970; Cell Signaling Technologies; 1:1000). Immunoblot signals were developed under chemiluminescence (Thermo Fisher) and digitally imaged (BioRad).

**Bioinformatic Analyses**

Approach to bioinformatic analyses of scRNA-seq data are presented in Supplemental Material.

**Data Availability**

All scRNA-seq data have been deposited in Gene Expression Omnibus under accession number GSE135663.

**RESULTS**

**Generation of Genome-Edited Risk-Variant Kidney Organoids**

Using CRISPR-Cas9 genome editing, we engineered an iPSC line homozygous for the APOL1 G1 risk alleles, with the G0 control on an isogenic background from the 1016SevA line. Unlike previous cell lines used to study risk-variant APOL1 through transgenic expression$^{24-27}$, this engineered line houses the G1 variant at its native genomic locus under the control of APOL1’s endogenous regulatory elements. To increase HDR efficiency, the donor template contained a
puromycin selection cassette flanked by 500 bp homology arms, one of which housed the G1 variants rs73885319 and rs60910145 (Figure 1A). Successful G1-variant knock-in and cassette removal were confirmed by Sanger sequencing (Figure 1A, Figure S1A-C). Because CRISPR-mediated genome editing can occasionally induce chromosomal changes28, we verified that our genome-edited iPSCs maintained a normal karyotype (Figure S1D-E).

To model the G1 variants within a kidney context, we differentiated the G0 and G1 1016SevA lines into kidney organoids using an adherent culture protocol we have previously established (Figure 1B)5–7,13,14. Both G0 and G1 1016SevA iPSC lines differentiated into kidney organoids without major structural differences, expressing markers of nephron structure including PODXL in glomerular epithelial cells, LTL in the proximal tubule, and E-cadherin in the distal tubule, in appropriately patterned and contiguous segments (Figure 1C).

**Single-Cell Transcriptomics of G0 and G1 Kidney Organoids**

To discover if risk-variant APOL1 exerts cell-specific effects in terms of APOL1 expression and function, we performed scRNA-seq on all cells collected from whole wells of G0 and G1 organoids differentiated from the 1016SevA line from three separate experiments. A total of 67,337 cells across genotype and experimental conditions passed quality-control metrics (see Supplemental Methods) and were analyzed using the integrated analysis workflow of Seurat v329 (Figure 2A-B), which was chosen to be the primary analysis pipeline to improve normalization for batch effects and evaluate differential expression across clusters.

To further confirm our findings, we also analyzed the data separately with an unsupervised deep embedding algorithm for single-cell clustering (DESC)30. Primary output of unsupervised clustering by Seurat v3 yielded 14 clusters (Figure 2B, S3A), seven of which were determined to consist of mesenchymal cells, while the others included glomerular epithelial cells at early and more mature stages, proximal and distal tubule, cycling cells, neurons, and endothelial cells (Figure 2C-D, Figure S3A) as determined by marker genes previously used in our scRNA-seq studies to identify differentiated cell types within human kidney organoids, based on direct
comparison of known markers to tissue samples (Figure 2C)\textsuperscript{13,14}. The abundance of mesenchyme versus epithelial lineages in these whole-well differentiations is relatively high but within the expected range for patient-derived iPSC lines, which vary substantially in their efficiency of differentiation from one individual to the next\textsuperscript{7,14}. These clusters were present in both G0 and G1 organoids (Figure S3B). Similar unsupervised clustering was also obtained using DESC (Figure S4A-B).

**IFN-γ Induces APOL1 Expression in G0 and G1 Kidney Organoids**

We next evaluated whether the organoids expressed \textit{APOL1}. RT-qPCR on RNA from whole-well organoids differentiated from G0 and G1 1016SevA lines and the G0 Penn134-61-26 line revealed that organoids expressed little detectable \textit{APOL1} under standard culture conditions (Figure 3A-B, Figure S2), consistent with one study showing low levels in human kidneys in vivo\textsuperscript{31}. IFN-γ has been implicated as a factor that may induce APOL1 expression in transgenic mice, although whether this occurs in humans remains unclear\textsuperscript{32}. Robust \textit{APOL1} expression (mean >4,000-fold over \textit{ACTB}, $P = 0.004$ by 2-way ANOVA) was induced in both G0 and G1 organoids when exposed to 25 ng/mL IFN-γ for 24 hours (Figure 3A). APOL1 protein expression was confirmed on immunoblot of whole-well organoids, with the appearance of a ~40 kDa band, the expected size for APOL1, only in the IFN-γ samples (Figure 3B, Figure S2).

To identify which organoid cell types express \textit{APOL1}, and underlying effects of \textit{APOL1} risk variants on gene expression, scRNA-seq was performed as described above. As with the RT-qPCR and immunoblot assays, scRNA-seq revealed that untreated organoids express little detectable \textit{APOL1}. However, exposure to IFN-γ for 24 hours induced \textit{APOL1} expression across multiple cell types, including glomerular epithelial cells, endothelial cells, and tubular cells (Figure 3C-D, Figure S5).

**Gene Expression Signatures of G1 Kidney Organoids Are Specific to Cell Type**

In contrast to an inducible \textit{APOL1} risk-variant overexpression cell model\textsuperscript{25}, G1 kidney organoids did not undergo appreciable cell death when \textit{APOL1} was expressed (Figure S3B). To determine
whether risk-variant APOL1 expression causes transcriptome-wide changes, we performed
differential expression analysis using Seurat v3. Comparing G0 and G1 organoids exposed to
IFN-γ, we found greater variance in gene expression patterns when examining each cell type
separately than when examining the whole organoid (Figure 4A). In IFN-γ stimulated organoids,
very few genes differentially expressed between G0 and G1 glomerular epithelial cells
overlapped with genes differentially expressed between G0 and G1 tubular cells (Figure 4B).
Indeed, the genes differentially expressed between G0 and G1 glomerular epithelial cells
stimulated with IFN-γ were enriched for biological processes related to metabolic function,
whereas tubular cells stimulated with IFN-γ exhibited differential (between G0 and G1)
expression of genes related to inflammation and protein targeting and processing (Figure 4C).

To visualize these cell-type specific gene expression patterns induced by IFN-γ, we generated
normalized transcript abundance on violin plots for each cluster (Figure 4D-E). We first plotted
genes previously identified by other studies to be potential modifiers of APOL1-mediated
kidney disease. A large linkage disequilibrium block on chromosome 6 containing UBD and
PPP1R18 may house disease-modulating genes33,34, with visible differences between APOL1
genotypes in UBD and PPP1R18 expression in the tubule and endothelial clusters (Figure 4D).
CXCL11 was previously found to be upregulated in the glomeruli of patients with APOL1-
mediated kidney disease24,34; it appears mildly more abundant in G1 endothelial cells (Figure
4D). Novel genes such as ASS1 are specifically upregulated in interferon-stimulated G1
glomerular epithelial cells, while CD74 and JAG1 are markedly downregulated in G1 glomerular
epithelial cells compared to G0 cells (Figure 4E).

**ER Stress Induces Differential Expression of Stress Response Genes in G1 Kidney Organoids**

Having identified these cell-type specific differences in G1 organoid gene expression, we next
evaluated whether introducing an additional stressor alters G1 cell phenotypes. Because
APOL1-mediated kidney disease is incompletely penetrant, others have proposed a “second-hit
hypothesis” that individuals carrying two APOL1 risk alleles develop disease after exposure to
environmental or genetic modifiers35,36. We tested whether ER stress could provide a stimulus
to alter stress response and cellular phenotypes in the high-risk genotype. ER stress was chosen because ubiquitin D, encoded by *UBD*, is involved in the ER stress response, and risk-variant *APOL1* appears to localize to the ER membrane\(^37\). Furthermore, ER stress is becoming an increasingly recognized driver of complex diseases, including CKD\(^38\)–\(^41\). To induce ER stress, we treated organoids with 5 \(\mu\)M tunicamycin for 24 hours and observed increased expression of unfolded protein response genes *EDEM1* and *HSPA5* by RT-qPCR (Figure 5A). Likewise, select UPR and apoptosis genes exhibit a trend of differences in average cell-type cluster expression, as seen on a heatmap of G0 and G1 GEC clusters (Figure 5B-C), although these genes did not reach the statistical significance threshold established by the integrated workflow of Seurat v3.

**ER Stress Induces Greater Dedifferentiation of G1 Glomerular Epithelial Cells**

In organoids treated with IFN-\(\gamma\) and tunicamycin, heatmap and violin plots of the podocyte markers in organoids treated with IFN-\(\gamma\) and tunicamycin revealed relatively decreased expression of *PODXL*, *NPHS1*, and *POSTN* in the G1 glomerular epithelial cell cluster, suggesting the G1 organoid may acquire a less differentiated state under stress (Figure 6A-B). We also discovered that G1 organoids treated with both IFN-\(\gamma\) and tunicamycin demonstrated less distinct cluster topology seen in partition-based graph abstraction (PAGA)\(^42\). More specifically, G1 glomerular epithelial cells became closer to the other cell types in spatial relationship, whereas G0 glomerular epithelial cells still remained more distinct from the other cell clusters (Figure 6C). Trajectory inference of G0 and G1 organoid glomerular epithelial cells (Figure 6D) revealed that G1 organoids, compared to G0 organoids, subjected to ER stress have more cells scattered along trajectory paths between clusters, as well as a smaller proportion of mature glomerular epithelial cells compared to early glomerular epithelial cells (Figure 6E-F). Collectively, these findings were consistent with potential dedifferentiation of G1 glomerular epithelial cells during stress.

**DISCUSSION**

We have conducted profiling of genome-edited iPSC-derived kidney organoids to detect the potential effect of *APOL1* risk variants on cellular phenotype and stress. With our novel
platform of integrating genome-editing, 3D kidney organoid culture, and single-cell transcriptomics, our model system recapitulates IFN- induced \textit{APOL1} expression and increased cellular stress in risk-variant organoids subjected to a “second hit” of ER stress. This system also demonstrated that gene expression signatures in risk-variant kidney organoids differ among cell types.

By expressing the G1 variants in their native genomic context, our approach circumvents challenges associated with studying \textit{APOL1} in model organisms and transgenic cell lines, for instance, transgenic overexpression that may introduce toxic non-physiological doses of \textit{APOL1} protein. Because \textit{APOL1}-mediated kidney disease demonstrates incomplete penetrance\textsuperscript{43,44}, the ideal model system would allow for interrogation of native genomic regulators, including distal enhancer elements and cell-specific long non-coding RNAs\textsuperscript{45}. Use of genome-editing rather than transgenesis preserves these regulatory interactions, including the response to IFN-\textgamma, and thus would facilitate validation of molecular modifiers of risk-variant \textit{APOL1} expression and function.

Another advantage afforded by our platform is the use of a G0 control on an isogenic background, made possible through footprint-free CRISPR-Cas9 genome editing. Our footprint-free method leaves no alterations to the amino acid sequence of \textit{APOL1} protein and also does not leave behind any additional DNA (such as loxP sites) that would potentially interfere with native non-coding regulation. Isogenic backgrounds, where the only difference is the knock-in or correction of the disease-associated DNA variants, eliminates other variants as variables. Thus, this approach reduces the potential for genetic and epigenetic heterogeneity, that would be expected among risk-variant patient-derived iPSCs and age- and gender-matched G0 patient-derived iPSCs, as potential confounding modifiers of \textit{APOL1} expression and function.

Because this platform utilizes 3D kidney organoids, we employed scRNA-seq to detect differences in molecular signatures among cell types in risk-variant organoids. Single-cell transcriptomics revealed cell-type specific gene expression differences between G0 and G1
organoids when \textit{APOL1} is induced, with little overlap between GECs and tubular cells, suggesting that risk-variant \textit{APOL1} could potentially alter transcriptional programs in a cell-type specific fashion. This result is concordant with findings that human genetic variation exerts cell-type specific effects in CKD\textsuperscript{11} and with a prior finding that glomerular and tubular compartments of \textit{APOL1}-mediated focal segmental glomerulosclerosis biopsies exhibit distinct gene expression patterns\textsuperscript{34}.

Furthermore, we demonstrated that additional stressors can lead to more pronounced dedifferentiation of glomerular epithelial cells in G1 organoids. Unlike prior models\textsuperscript{25,46}, our model system does not lead to much cell death in the G1 kidney organoids at 24 hours, nor does it activate many pro-apoptotic genes, consistent with the concept that another stressor is needed to induce a phenotype. We chose ER stress as an experimental condition because previous studies have indicated that risk-variant \textit{APOL1} may be regulated by \textit{UBD33}, a gene involved in the ER stress response, and that risk-variant \textit{APOL1} localizes to the ER rather than to lipid droplets\textsuperscript{37}. Expression of risk-variant \textit{APOL1} alone does not activate a significant UPR response (as seen in Figure 5B), but some differences can be seen for \textit{DDIT3}, \textit{EDEM1}, \textit{ERN1}, \textit{GSK3A}, and \textit{ERP44} upon tunicamycin stimulation, consistent with a “second-hit” hypothesis. With these changes in gene expression, accurate identification of cells attributed to the glomerular epithelial cell cluster among experimental conditions is essential. In this analysis, the glomerular epithelial cells were identified using the integration workflow of Seurat v3, which identifies conserved markers in clusters that are present in both control and stimulation conditions, decreasing the probability of cell type misclassification in the IFN-\gamma and / or tunicamycin treated organoids. With the cells appropriately assigned to the main glomerular epithelial cell cluster and early glomerular epithelial cell cluster across experimental conditions, we assessed dedifferentiation by the relative ratio of "early“ glomerular epithelial cells compared to both early and more mature cells in total. Because the differentiation and relative abundance of cell types did not differ between G0 and G1 organoids at baseline, the difference in this ratio between stressed G0 and G1 organoids most likely represented a dedifferentiation process induced by injury reproduced in data spanning technical replicates in experiments that
were repeated. Although defining the exact mechanisms of this “second-hit” in APOL1-mediated kidney disease is beyond the scope of this study, we demonstrate that our model system is capable of capturing the subtler phenotype of cellular dedifferentiation.

Our study also has limitations worth consideration. Our study did not include G2 variant organoids, although genome-editing to create an isogenic G2 line is underway and will be important in future studies. Also, our proof-of-concept single-cell transcriptomics used iPSCs from one parent line (1016 SevA) from a healthy non-African donor, so our work will require further validation from additional risk-variant APOL1 iPSC lines, preferably from patients with APOL1-mediated kidney disease, with matching isogenic genome-edited controls. In addition, the 1016 SevA iPSC line yields relatively immature glomerular epithelial cells, which limits the conclusions that can be drawn in terms of APOL1-mediated podocyte biology. Although our data reveals a cell-type specific molecular signature of G1 kidney organoids, further scRNA-seq and protein-level validation in organoids derived from other iPSC lines that yield mature glomerular epithelial cells would be needed to highlight disease-relevant cellular mechanisms. Another potential limitation of our model system is the lack of detected APOL1 expression at baseline without IFN-γ stimulation, a result that is consistent with one prior study but differs from a more recent study by Ma et al. suggesting baseline expression on immunohistochemistry. This perceived difference in expression could be due to the altered physiological or maturation state of the kidney organoids in vitro compared to tissue in vivo, to differential sensitivities of the various methodologies applied, or to variation of expression among different patients. It is nevertheless interesting and significant that APOL1 mRNA levels increase upon IFN-γ stimulation in organoids. Finally, the organoids generated in this study did not have vascular flow, so APOL1 expression and function in vasculature would not be fully captured despite the presence of endothelial-like cells. Likewise, while we have previously reported that organoid tubules are capable of selective solute transport, similar to proximal tubules, the organoids generated in this study lack a blood vessel conduit for continuous reabsorptive flux, and may not recapitulate the full range of filtrate reabsorption or specialized
ion exchange, a limitation that may also affect transcriptional profiles and phenotype of the tubular cells in our experiments.

Despite these limitations, our model system, which combines the power of footprint-free genome editing with organoid culture, provides a human-relevant platform to which future studies can be executed and provides new insight into the potential mechanisms of APOL1-mediated kidney disease in diverse kidney cell types. With the power of genome editing, evolving science, and scRNA-seq, this platform could launch larger-scale mechanistic and screening studies for APOL1-mediated kidney disease, enabling the identification of pathways that could be targeted therapeutically in high-risk populations to reduce the incidence of glomerular disease.

DISCLOSURES
J Himmelfarb reports personal fees from Maze Therapeutics, personal fees from Renalytix AI, and personal fees from Gilead outside the submitted work. B Freedman is an inventor on patent applications related to kidney organoid differentiation and disease modeling and an advisor for Chinook Therapeutics. The authors have nothing further to disclose.

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AUTHOR CONTRIBUTIONS
Esther Liu: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Supervision; Validation; Writing - original draft; Writing - review and editing
Behram Radmanesh: Conceptualization; Formal analysis; Investigation; Resources; Supervision; Visualization; Writing - original draft; Writing - review and editing
Byungha Chung: Data curation; Investigation; Methodology; Software; Visualization; Writing - review and editing
Michael Donnan: Funding acquisition; Resources; Writing - review and editing
Dan Yi: Investigation; Resources; Writing - review and editing
Amal Dadi: Data curation; Investigation; Visualization; Writing - review and editing
Kelly Smith: Data curation; Formal analysis; Investigation; Resources; Validation; Writing - original draft; Writing - review and editing
Jonathan Himmelfarb: Formal analysis; Funding acquisition; Investigation; Resources; Visualization; Writing - original draft; Writing - review and editing
Benjamin Freedman: Conceptualization; Investigation; Resources; Supervision; Writing - original draft; Writing - review and editing
Jennie Lin: Conceptualization; Data curation; Funding acquisition; Investigation; Supervision; Writing - original draft; Writing - review and editing

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FIGURES

**Figure 1.** Engineering APOL1-G1 kidney organoids from iPSCs. **A.** Schematic summarizing the CRISPR-Cas9 approach to knocking in the G1 risk variants rs73885319 and rs60910145 into the 1016SevA line, including the chosen protospacer, HDR donor template design leveraging the piggyBac transposon system, and Sanger sequencing validation of successful variant knock-in and selection cassette excision. **B.** Overview of the Freedman kidney organoid differentiation protocol, with light microscopy of the 1016SevA line forming tubular organoids. **C.** Confocal immunofluorescence images of nephron markers in representative G0 and G1 organoids on day 21 of differentiation.
(A) APOL1 gRNA Protoscaler and PAM site. gRNA Protoscaler: GAGAAGCTCGAGGAGAGCTTTGAAGC. PAM site: TTAA.

Puromycin Selection

piggyBac transposase

Footprint-free excision

G1 Variants

HDR Donor Template

TTAA TTAA TTAA TTAA

rs73885319 Ser342Gly

rs60910145 Ile384Met

(B) mTeSR1 Advanced RPMI

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Single Cells Cavitated Spheroids Mesenchyme Tubular Organoids

Day 0 1 2 4.5 6 14

(C) DNA PODXL LTL E-Cad Merge

G0 Day 21 100 µm

G1 Day 21 100 µm
Figure 2. Overview of single-cell transcriptomics of G0 and G1 APOL1 organoids. A and B. UMAP visualization of all whole-well G0 and G1 organoid cells profiled by scCRNA-seq, integrated using Seurat v3. C. Violin plot of marker genes used for cluster identification of cell types, color-coded according to labeling of nano-dissected UMAP in D, which separates out the main cell types seen in the organoid away from the extra mesenchyme captured in whole-well sequencing. EC = endothelial cells, GEC = glomerular epithelial cells, N = neuron, PT = proximal tubule, DT = distal tubule, Cyc = cycling, M = mesenchyme.
Figure 3. IFN-γ induces APOL1 expression in iPSC-derived kidney organoids. A. RT-qPCR of RNA isolated from whole-well G0 and G1 organoids reveal low endogenous APOL1 expression but a greater than 4,000-fold induction of APOL1 mRNA relative to ACTB after 24 hours of 25 ng/mL IFN-γ treatment (P = 0.004 by 2-way ANOVA). B. Immunoblot of APOL1 expression in G0 and G1 organoids + 25 ng/mL IFN-γ for 24 hours. C. Dot plot of APOL1 expression in G0 and G1 kidney organoids, with and without IFN-γ treatment. Brighter red dots indicate stronger expression level across cells, and dot size reflects percentage of cells in a cluster expressing APOL1. D. UMAP feature plots of nano-dissected clusters show APOL1 expression (red dots) at baseline or when treated with 25 ng/mL IFN-γ for 24 hours.
Differential expression of APOL1 under IFN-γ treatment.

**A**
- Graph showing relative APOL1 mRNA expression over β-Actin control.
  - Control vs. IFN-γ treated samples.
  - Significant difference indicated by $P = 0.004$.

**B**
- Western blot analysis showing IFN-γ, APOL1, and β-Actin expression levels.
  - lanes for G0 and G1 genotypes.
  - (+) indicates positive expression.

**C**
- Table summarizing average expression across different conditions.
  - Genotypes: G0, G1.
  - Conditions: M, Early GEC, PT, DT, GEC, EC.
  - Color scale for percent expressed.

**D**
- Heatmaps depicting APOL1 expression levels.
  - Genotypes: G0, G1.
  - Conditions: with or without IFN-γ treatment.
  - Color scale indicating expression levels.
Figure 4. scRNA-seq reveals cell-type specific differential expression patterns between G0 and G1 organoids treated with IFN-γ. A. Scatter plots of G0 vs. G1 organoid gene expression values across all cell types (left), among tubular cells (middle), and among glomerular epithelial cells (right). Greater variance is seen in the cell-type specific plots. B. Venn diagrams show little overlap of expression patterns across cell types when comparing which genes are upregulated in G1 IFN-γ stimulation over G0 IFN-γ stimulation. Similarly low overlap across cell types for genes downregulated in G1 IFN-γ stimulation. C. Gene ontology of the differentially expressed genes within each cell type, with -log(p-value) plotted for each biological process. Metabolic (green), stress (pink), cell and organelle function (purple), collagen / matrix (grey). D. Violin plots visualizing cell type-specific expression patterns of putative regulators^{24,33,34} of APOL1 abundance. E. Violin plots visualizing cell type-specific expression patterns of novel genes.
A Whole Organoid

B Genes Downregulated in G1
- 84
- 70

Genes Upregulated in G1
- 7
- 111

C Gene Ontology of Cell Type Specific Differential Expression

Tubule
- Protein targeting to ER
- Viral transcription
- RNA catabolism
- Proteasomal catabolism
- Developmental process
- ECM organization
- Wound healing
- Cell adhesion

Glomerular Epithelial Cells
- ATP metabolic process
- Coupled electron transport
- Protein targeting to ER
- Cristae formation
- ECM organization
- Cell activation
- Regulated exocytosis
- Immune effector process

D UBD
- PPP1R18
- CXCL11

E ASS1
- CD74
- JAG1
**Figure 5.** ER stress increases stress response in G1 kidney organoids. **A.** RT-qPCR of RNA isolated from whole-well G0 and G1 organoids reveals increased unfolded protein response (UPR) gene expression (*EDEM1, HSPA5*) in G1 organoids exposed to both IFN-γ and tunicamycin (*P* = 0.01 by 2-way ANOVA). **B.** Heatmap depicts relative average expression of UPR-relevant genes for G0 and G1 glomerular epithelial cells subjected to either IFN-γ alone or both IFN-γ and tunicamycin. **C.** Heatmap depicts relative average expression of apoptosis-relevant genes for G0 and G1 glomerular epithelial cells subjected to both IFN-γ and tunicamycin.
**Figure 6.** ER stress dedifferentiation of glomerular epithelial cells (GECs) in G1 kidney organoids. 

**A.** Heatmap depicts relative average expression of podocyte markers for G0 and G1 GECs and early GECs treated with either IFN-γ alone or both IFN-γ and tunicamycin. 

**B.** Violin plots of *PODXL* expression in the GEC clusters of G0 and G1 organoids subjected to either IFN-γ alone or both IFN-γ and tunicamycin. 

**C.** Partition-based graph abstraction (PAGA) visualizes trajectory inference of all organoid single cells. The more mature GECs are connected to non-mesenchyme cell clusters in the topology of the control organoids. For G0 organoids subjected to both IFN-γ and tunicamycin, the more mature glomerular cells are still more distant from the mesenchyme. PAGA visualization of G1 organoids subjected to both IFN-γ and tunicamycin reveals less distinct separation of GECs from early GECs and mesenchyme. 

**D.** Trajectory inference UMAP of all sequenced organoids was created using Monocle3. The trajectory of the relationship among mesenchyme, early GECs, and more mature GECs is circled and taken forward to **E,** where G1 organoids treated with both IFN-γ and tunicamycin (red) have a relatively smaller proportion of GECs to early GECs compared to G0 organoids subjected to the same stressors (orange). 

**F.** The relative number of GECs (black) to early GECs (white) is represented by the bar charts of G0 and G1 control organoids (left), G0 organoids treated with IFN-γ and tunicamycin (middle), and G1 organoids treated with IFN-γ and tunicamycin (right).