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TITLE: Intercellular Signaling and Biomechanics of Podocytes and Endothelial Cells in FSGS

PRINCIPAL INVESTIGATOR: Dr. Ilse Daehn

CONTRACTING ORGANIZATION: Icahn School of Medicine at Mount Sinai

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14. ABSTRACT The molecular mechanisms for glomerular cell crosstalk and regulatory inter-cellular feedback remain poorly understood. In experimental models of focal segmental glomerulosclerosis (FSGS), we described a novel hypothesis of glomerular endothelial cell and podocyte crosstalk where perturbations of microvascular homeostasis initiate mitochondrial stress and dysfunction of glomerular endothelial cells as well as loss of endothelial glycocalyx. This in turn results in subsequent podocyte depletion and irreversible sclerosis mediated by soluble endothelial cell secreted factors. We hypothesize that endothelial cell dysfunction mediates podocyte injury and loss through a mechanobiological pathway that can be therapeutically targeted. We will test this hypothesis with our microengineered 3-D glomerulus-on-chip platform in endothelial cells and podocytes derived from human induced pluripotent stem cell (hiPSC), and validate these findings in experimental mouse models of FSGS. Studies thus far show our ability to grow hiPSCs and to inhibit the production of stress secreted factors with RNAi intervention and novel small molecule antagonists.					
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1. INTRODUCTION:

Chronic kidney disease affects more than one in ten Americans and is a major public health problem, both in terms of disease burden and health expenditure as it can lead to kidney failure and eventual need for dialysis or kidney transplantation. Currently, there are no specific therapies for CKD that can prevent the progression into end stage renal disease and kidney failure. We have shown that communication between two different cell types in the kidney in response to injury is critical to trigger disease progression. There is very little known about this type of cell-to-cell communication; however, we have recently identified specific molecules that could be affecting the physical resilience of kidney cells. In this project, we propose to examine this new communication paradigm and validate these promising findings, using state-of-the-art nanotechnology and patient-derived stem cells. This approach will allow us to explore the specific biological function of each cell type and develop targeted therapeutics. The proposed studies will help validate the cause and timing of key events for each cell type during disease development and provide a better understanding of cell communication with the long-term objective to develop new and more effective therapies for this devastating disease.

2. KEYWORDS:

glomerular endothelial cells; glycocalyx; podocytes; induced pluripotent stem cell, glomerular disease

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: To define the effect of glomerular endothelial cell secreted stress factors on podocyte function.

- 1.1- Characterize the differentially affected signaling landscape in hiPSC-derived kidney podocytes stimulated by endothelial cell secreted factors in vitro
- 1.2- Quantify patient-specific factors released by hiPSC-derived endothelial cells under stress conditions
- 1.3- Determine 3-D spatiotemporal differential pathway activity of podocytes in a mouse model of endothelial injury in vivo
- 1.4- Use dynamical modeling to identify nodes and connections as druggable targets that can reverse the altered signaling landscape

Milestone #1: Co-author manuscript on BMPER specific effects on podocytes

Specific Aim 2: To reverse podocyte specific pathways that respond to endothelial cell secreted factors

- 2.1- To design structure-activity relationships to identify small molecular antagonists that can reverse the altered signaling landscape
- 2.2- Characterize cellular physiology and biomechanics of hiPSC-derived podocytes stimulated by endothelial cell secreted factors with and without small molecule correction
- 2.3- Establish the effects of endothelial cell secreted factors on cytoskeletal dynamics and focal adhesions of podocytes in vitro with and without small molecule correction
- 2.4- Determine the efficacy of top small molecular target in a mouse model of chronic endothelial injury in vivo

Milestone #2: Co-author manuscript on 3-D glomerular cell cocultures

Milestone #3: Co-author manuscript on glomerular cell crosstalk and inhibition of endothelial cell secreted molecules

What was accomplished under these goals?

Specific Aim 1: To define the effect of glomerular endothelial cell secreted stress factors on podocyte function.

Major activities. We proposed the endothelial dysfunction could mediate podocyte loss in FSGS and in other diseases where podocyte depletion occurs concomitantly with endothelial dysfunction such as in diabetic kidney disease (DKD). The data from Daehn lab (Initiating PI at the Icahn School of Medicine at Mount Sinai) suggests that perturbation of microvascular homeostasis in hyperglycemia initiate mitochondrial stress and dysfunction of glomerular endothelial cells, and this leads to endothelial dysfunction in mice with genetic susceptibility to DKD (in DBA/2J mice), not in resistant diabetic mice (C57BL/6J mice). We have demonstrated that endothelial dysfunction by oxidative stress mediates podocyte loss, and a selective mitochondrial-targeted reactive oxygen species (ROS) scavenger prevented mitochondrial oxidative stress and dysfunction selectively in endothelial cells, podocyte loss, albuminuria and glomerulosclerosis (PMID: 27899487, PMID: 32641054). In this past year, the Daehn lab have established a protocol for isolating stable glomerular endothelial cell line that allows for photo-activation of a subset of mitochondria causing a switch in fluorescence from green to red; mitoDendra2-GECs. MitoDendra2-GECs allows for live imaging of fluorescent mitochondria's distribution, fusion, and fission events without staining the cells (doi: 10.3791/64147; See Appendix). Figure 1 demonstrates the mitoDendra2-GECs before and after photobleaching showing the conversion of green mitochondria to red. Over time mitochondria matrix exchange suggest successful fusion and fission events with co-labeled yellow mitochondria. These are a great tool for studying the mitochondria responses to different stimuli and we are currently exploring the roles of the different stressors on glomerular endothelial mitochondrial dysfunction that result in the release of cytotoxic molecules that mediate podocyte injury and depletion. This cell line is instrumental for establishing stress responses in GECs and inform conditions for treatment of hiPSC-derived endothelial cell experiments. For achievement of the milestones under sub-aims 1.1 and 1.2, the Azeloglu lab and Daehn lab have now established iPSC stress responses to stress stimuli and confirmed the increased BMPER expression by treatment with ET-1 (Figure 2).

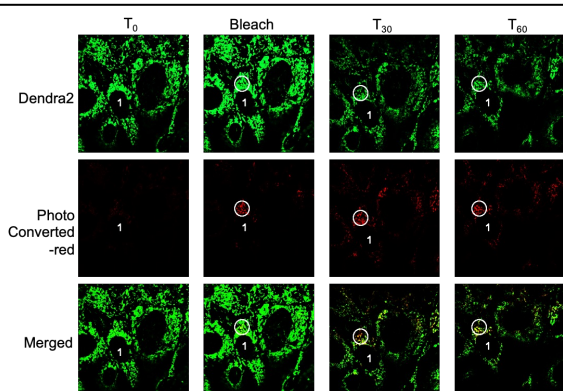


Figure 1. mitoDendra2-GECS before and after photoconversion of mitochondria from green to red. O= photo-converted region (ROI) at 0, 30 and 60 minute timepoints.

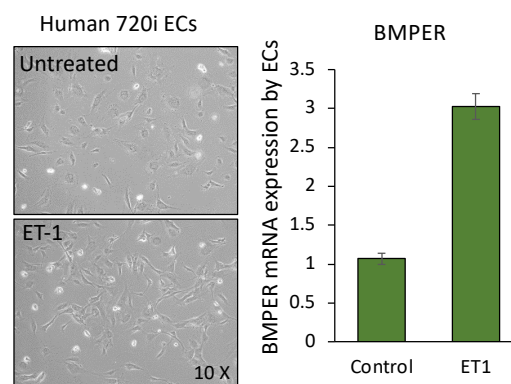


Figure 2. Representative image of untreated control and treated with Endothelin-1 (ET-1) 200 ng/ml 720i human endothelial cells (ECs) derived from iPSCs. mRNA expression of BMPER from control and ET-1 treated 720i human Ecs.

Following our efforts from last year, Azeloglu lab concluded the initial tests of a new robust microfluidic glomerulus-on-chip model system using both immortalized human cell lines and iPSC-derived endothelial cells and iPSC-derived podocytes. Our system comprised a synthetic membrane with small pore size (1 μm) that allowed vigorous filtration of both albumin and inulin through the system in its cell-free state mimicking the *in vivo* state. The system allows independent coating of extracellular matrix molecules, modification of mechanical forces and shear and rapid testing of the effect of secreted factors on filtration barrier integrity in a component-specific manner. Using only open-sourced system components (**Figure 3**), we were able to reliably quantify the circulating levels of inulin and albumin in both the endothelial and filtrate sides of the system and “visualize” filtration status in real-time.

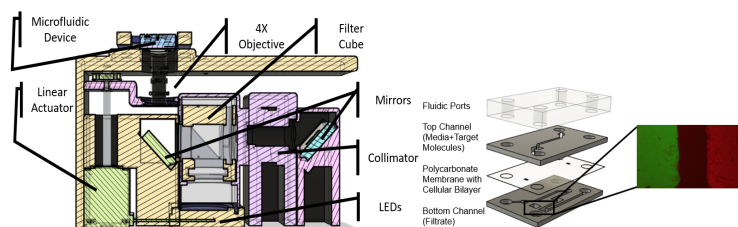


Figure 3. (A) Real-time optical sensor for tracking filtration barrier integrity using completely open-sourced materials and design using the FAIR principles. The system uses dual color fluorescent tracking of inulin and albumin in the endothelial and ultrafiltrate sides of the microfluidic device placed on the top left segment. (B) The microfluidic circulation is designed such that the protein concentrations in the endothelial and ultrafiltrate sides, which are separated by an optically-clear 1- μm pore-sized polycarbonate membrane, can be visualized simultaneously in real-time.

To begin to explore the potential BMPER targets in glomeruli, The Daehn and Azeloglu labs embarked on the collection of human glomeruli from patients for the discovery of BMPER binding targets and signaling *ex-vivo*. Since February 2022, twenty human partial or full nephrectomy samples have been collected and processed for the isolation of glomeruli. We have collected thousands of glomeruli for experiments in Aim 1.3, and have also developed a protocol for treatment of glomeruli with biotin bound BMPER, with the goal to isolate BMPER binding molecules by LC/MS-MS. These studies have yielded a lot of high-content data that we anticipate completing the analysis in the next couple of weeks. Our preliminary proteomic results show strong enrichment of podocyte specific cytoskeletal and slit diaphragm proteins (which are detected at exceptionally high coverage levels) being identified in the immunoprecipitated milieu, suggesting (1) successful precipitation of target protein complexes without disintegration, and (2) potential preferential uptake of BMPER locally within the secondary and tertiary foot processes of podocytes (Figure 4). In order to visually confirm the spatial integration of BMPER on the podocyte processes, we have also developed a protocol for treatment of glomeruli with FITC bound BMPER to visualize uptake (Figure 5). Temporal assessment of BMPER uptake by glomerular cells indicated was confirmed to occur around 15-30 minutes. Figure 5C depicts BMPER green puncta on glomerular cell membranes after 15 minutes treatment at RT.

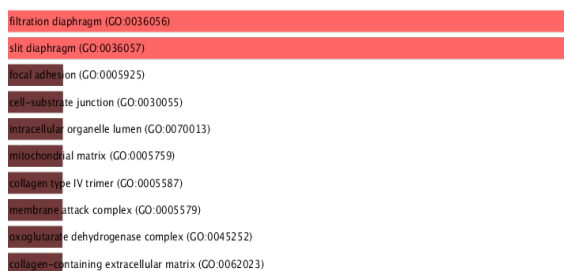


Figure 4. Gene ontological enrichment of top subcellular localization for LC-MS/MS identified proteins that are immunoprecipitated after BMPER treatment shows the strong enrichment for podocyte foot processes and slit diaphragm proteins.

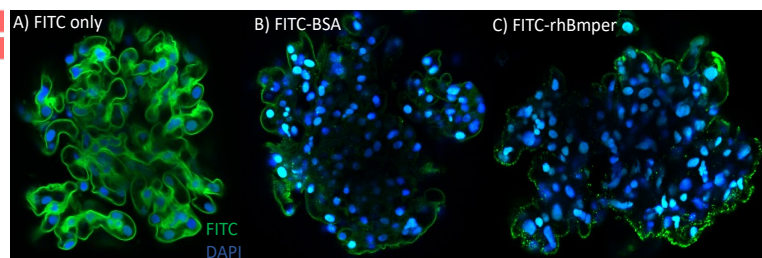


Figure 5. Representative image of A) 15 min FITC treated human glomerulus, B) conjugated FITC-BSA (100 nM) treated human glomerulus both showing diffused fluorescence, and C) 15 min FITC-BMPER (100 nM) treated human glomerulus showing green fluorescent puncta on cell membrane.

Specific Aim 2: To reverse podocyte specific pathways that respond to endothelial cell secreted factors

In our last report period, Dr. Das (Co-Investigator at Long Island University) began the design structure-activity relationships (SAR; study approach) to identify small molecular antagonists that can inhibit BMPER activity as part of Sub-Aim 2.1. Daehn lab tested all 8 small molecule compounds on GEC viability, proliferation and cytotoxicity and selected 4 molecules with the better safety profile: BT663, BT666, BT669, BT670. These were tested on Edn1 stressed GECs together with RNAi against BMPER as a control for inhibition of expression at the RNA level. All molecules tested had inhibitory activity at different concentrations. These results are very encouraging, and we continue to test the molecules on other endothelial functional tests. For Aim 2.4, we have begun pre-clinical testing of the molecules and performed pilot studies with mouse models of FSGS. First, we tested C57BL/6 mice with high dose Adriamycin (ADR; 25 mg/kg) mouse model known to develop glomerular sclerosis and albuminuria after 6 days post injection. Glomerular levels of BMPER we increases and increased BMPER expression was prevented by daily injections of BT663 (5mg/kg; Figure 6A,B). Importantly histopathology of glomeruli in ADR mice was also prevented by BT663 injections (Figure 6C,D) and improved the increased albuminuria (not shown). Next, albuminuria in a model of podocyte specific activation of TGFbRI signaling inducible by doxycycline (Dox) model; NephTgfbRI mice, was significantly ameliorated by BT663 treatment (Figure 6D). This was associated with decreased BMPER expression and improved glomerular histopathology (not shown). These data suggest that antagonizing BMPER with BT663 prevents early disease onset and preserved glomerular structures despite the podocyte specific insult; ADR or podocyte specific activation of TGFbRI signaling by Dox. We are working on improving the drug delivery by gavage or minipump for longer experiments.

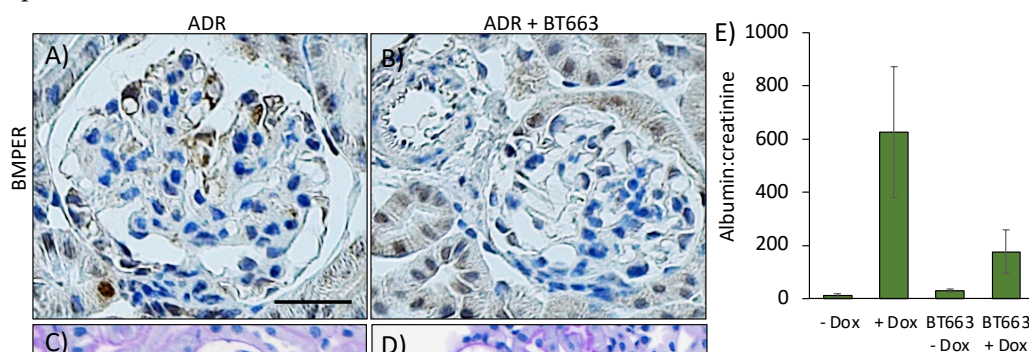


Figure 6. Representative image of IHC for BMPER expression on kidney cortex of A) day 6 adriamycin (ADR) treated mice without and with B) BT663 5mg/kg. Representative PAS staining of C) ADR treated mice without and D) with BT663 co-treatment. Scale bar = 50µm E) Urine albumin:creatinine of NephTgfbRI mice +/- Dox 4 days, +/- BT663.

What opportunities for training and professional development has the project provided?

One of the trainees in the Azeloglu Lab, Ms. Tess Fallon, received two separate awards at the Summer Bioengineering Meetings SB3C, including the “Best Undergraduate Research Presentation Award” for her work on the glomerulus-on-chip construction. She was able to attend the SB3C 2022 Meeting in-person on June 20-22, 2022. One of the four female electrical engineering majors at Columbia, Tess is currently working on preparing her work for peer-reviewed publication. Another doctoral student who has been working on developing the microfabricated glomerular culture system used in this project, Ms. Smiti Bhattacharya, was chosen as an ASN TREKS Fellow and attended the experimental training workshop on June 18-25, 2022. A prior NIH F31 fellow, Smiti’s success was featured by Columbia University on the Department of Mechanical Engineering website (<https://www.me.columbia.edu/smiti-bhattacharya-selected-participate-2022-kidney-treks-american-society-nephrology-asn-tutored>). We note that both promising postdoctoral fellows (one in each PI’s group), the summer engineering undergraduate student, and the doctoral student who has concluded the new design of the microfabricated chips are all women, who are highly underrepresented in engineering. These trainees, along with several others are continuing our “Kidney Tissue Engineering” journal club that meets every other Thursday to discuss recent literature and its implications on our project. In addition, Dr. Lassen in Daehn Lab (supported by this award) has an abstract accepted and will be attending and presenting at this year’s ASN Kidney week in November 2022.

How were the results disseminated to communities of interest?

We have presented our findings in the Summer Bioengineering meeting as noted above. In addition, our community engagement plans have resulted in strong recruitment success for our summer engineering program within our division, which has supplied one of the trainees that has contributed to this project. Last year, five of the six recruited engineering students were underrepresented minorities including one student from Jackson State, a Historically Black College and University.

What do you plan to do during the next reporting period to accomplish the goals?

Specific Aim 1. To define the effect of glomerular endothelial cell secreted stress factors on podocyte function.

Experiments with human induced pluripotent stem cell (hiPSC-) derived kidney endothelial cell by Azeloglu lab and Dr Daehn will continue to determine the stress responses and levels of secreted stress candidates; BMPER, FBN1 and AREG. Concomitant proteomics experiments using hiPSC-derived podocytes sourced from the same individual will enable mapping of cytoskeletal responses to endothelial cell secreted stress factors. By combining these with the transcriptomic experiments, we will begin dynamic modelling of the podocyte response to identify nodes and connections as druggable targets that can reverse the altered signaling landscape in podocytes. Multicellular microfluidic system will also be tested by the Azeloglu lab using multiple endothelial and podocyte lines from the same hiPSC sources for its ability to selectively filter inulin and retain albumin. These experiments will enable mapping of basal filtration barrier integrity in multiple kidneys-on-chip and allow determination of a quantitative range for the experiments under Specific Aim 2. Pilot studies to establish a model of experimental preeclampsia in mice, where disease is characterized by endothelial dysfunction, will be performed by the Daehn lab in this funding period. We will adopt the soluble VEGF receptor 1 administration (sFlt1 model) to induce glomerular injury and determine expression levels and localization of our candidate crosstalk molecules in an experimental model of preeclampsia with endotheliosis.

Specific Aim 2. To reverse podocyte specific pathways that respond to endothelial cell secreted stress factors.

We are encouraged by the preliminary results from the selected BT663 molecules described in the previous section. Ongoing studies by Deahn lab will confirm the safety of the molecules and will inform the next molecular design choices and to minimize potential toxic effects. We will conduct in vitro dose response assays (with and without the stress factors) and use MTT, annexin V and high-content cytoskeletal screening assays to characterize the pharmacodynamic response of the agents on hiPSC-derived podocytes treated with endothelial cell secreted stress factors BMPER. With the Azeloglu lab, we will also determine cytoskeletal dynamics and focal adhesions of podocytes in vitro with and without small molecule correction using an automated high-content imaging system allows for high-throughput treatment and image acquisition in a 96-well format. Podocyte morphometrics and biomechanics will also be characterized using an automated high-content fluorescent imaging system that is coupled with atomic force microscope elastography. We will create multidimensional correlation maps for intracellular organelle and microfilament distributions and the spatial subcellular elastic moduli. We will then harmonize the biomechanical maps and morphometric measurements for stress factor versus vehicle treated cells with and without small molecules. We will also perform bulk RNAseq at the IC10 and IC50 levels to map out the transcriptomic response.

Studies with animals will establish the pharmacodynamics of BT663 as well as longer termed injury models will be tested, and we will include diabetic kidney disease models.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

We have successfully generated stable hiPSC-derived endothelial cells from healthy volunteers of different race, sex and ethnicities.

Based on our earlier findings with replication-incompetent 3rd generation Lentivirus BMPER ShRNA, blocking BMPER at a transcription level was beneficial in prevented albuminuria without adverse consequences in a podocyte depletion mouse model. Our new preliminary studies using BMPER small molecule antagonists show effective inhibition at micro molar concentrations without impacting glomerular endothelial cell survival and are safe in animal models of glomerular disease. Hence suggesting that at least one of the candidate cytotoxic secreted stress factors by endothelial cells seems to be a druggable target in endothelial cells.

What was the impact on other disciplines?

Nothing to Report at this point.

What was the impact on technology transfer?

A technology disclosure was made by Dr. Azeloglu to Mount Sinai Innovation Partners office about the potential use of our biochip design on the quantitative evaluation of cellular motility. Dr. Daehn has begun discussions with Mount Sinai Innovation Partners office about the potential development of BT-663 for therapeutic evaluation.

What was the impact on society beyond science and technology?

Our curated diverse population of hiPSC-derived endothelial cells and podocytes were already instrumental in identifying potential sex-based differences in regulation of hypertensive diseases. While the results from our experiments are still in an early phase with these cell lines, we think that our technology and cell inventory will advance the discovery of markers that lead to sex-based differences in disease mechanisms.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

During this past funding period we had to slow our research efforts after lab closures for four months during COVID-19 pandemic and lockdown imposed by New York City in 2020. This has impacted both the cell culture studies which require constant management and all planned animal experiments. We were working at 50% capacity due to social distancing requirements until early 2021. At the same time, a substantial amount of our workforce had voluntarily reported to hospital posts to support the frontline response. In addition, we have collaborated with other clinical labs to support our division's research effort against COVID-19, which has a disproportionately heavy impact on the kidneys. We recognize that these were unprecedented events that inevitably affected us and much of the scientific community.

Changes that had a significant impact on expenditures

Dr. Das moved institution after the award notice and before the funding payments began in year 1. Hence indirect costs from his new institution impact the Direct Costs received by him/his lab.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals
Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

1. Bouchareb, R., Yu, L., Lassen, E., Daehn, I. S. Isolation of Conditionally Immortalized Mouse Glomerular Endothelial Cells with Fluorescent Mitochondria. *J. Vis. Exp.*(187), e64147, doi:10.3791/64147 (2022). Published. Acknowledgement of federal support: yes.
2. Daehn IS, Duffield JS. The glomerular filtration barrier: a structural target for novel kidney therapies. *Nat Rev Drug Discov.* 2021 Oct;20(10):770-788. Review. Published. Acknowledgement of federal support: yes.
3. Ebefors K, Lassén E, Anandakrishnan N, Azeloglu EU, Daehn IS. Modeling the Glomerular Filtration Barrier and Intercellular Crosstalk. *Front Physiol.* 2021 Jun 2;12:689083. Review. Published. Acknowledgement of federal support: yes.
4. Lassén E, Daehn IS. Molecular Mechanisms in Early Diabetic Kidney Disease: Glomerular Endothelial Cell Dysfunction. *Int J Mol Sci.* 2020 Dec 11;21(24):9456. Review. Published. Acknowledgement of federal support: yes.

Books or other non-periodical, one-time publications.

Editorial or Commentary

1. Daehn I.S. Merscher S.M. (2022). Molecular Mechanisms of Proteinuria, Volume II. *Frontiers in Medicine.* In press. Acknowledgement of federal support: yes.
2. E Lassen, IS Daehn. (2022) Hypertrophy and glomerular cell adaptation through crosstalk leads to glomerular injury after kidney transplantation." *Kidney Int* **101**(4): 673-676. Published. Acknowledgement of federal support: yes.
3. Das B., Daehn I.S. (2022) Tackling chronic kidney disease in diabetic patients with finerenone. *Trends Pharmacol Sci* **43**(9): 799-800. Published. Acknowledgement of federal support: yes.
4. Lassén, E., Bouchareb R., Daehn I.S. (2022). Podocyte as the link between sterile inflammation and diabetic kidney disease. *Kidney Int* **102**(4): 688-690. Published.
5. Anandakrishnan N, Azeloglu EU. Kidney tissue engineering for precision medicine. *Nat Rev Nephrol.* 2020 Nov;16(11):623-624. Published. Acknowledgement of federal support: yes.
6. E Lassen, IS Daehn. Clues to glomerular cell chatter in FSGS: via endothelin-1/ETAR *Kidney International Reports.* 2021 July 01,P1758-1760. Published. Acknowledgement of federal support: yes.

Other publications, conference papers and presentations.

Nothing to report

- **Website(s) or other Internet site(s)**

Nothing to Report.

- **Technologies or techniques**

Nothing to Report.

- **Inventions, patent applications, and/or licenses**

Nothing to Report.

- **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Ilse Daehn PhD
Project Role:	PI (Initiating)
Researcher Identifier (e.g. ORCID ID):	0000-0001-9915-5376
Nearest person month worked:	3
Contribution to Project:	Dr. Daehn has planned and performed cell and mice experiments. Dr. Daehn has also written manuscripts.
Funding Support:	DoD, NIH
Name:	Bhaskar Das PhD
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.6
Contribution to Project:	Dr. Das has developed the small molecule inhibitors
Funding Support:	NIH, DoD
Name:	Pratik Yadav PhD
Project Role:	Postdoc
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.2
Contribution to Project:	Dr. Yadav has performed experiments and written manuscripts.
Funding Support:	NIH, DoD
Name:	Emelie Lassen PhD
Project Role:	Postdoc
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Dr. Lassen has performed mouse experiments and written manuscripts.
Funding Support:	DoD
Name:	Liping Yu
Project Role:	Lab manager
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Ms. Yu has performed mouse experiments.
Funding Support:	DoD and NIH
Name:	Evren Azeloglu PhD
Project Role:	PI (Partnering)
Researcher Identifier (e.g. ORCID ID):	0000-0001-6137-109X
Nearest person month worked:	2
Contribution to Project:	Dr. Azeloglu has planned and performed bioengineering and cell culture experiments. He also has written manuscripts.
Funding Support:	DoD, NIH, Renalytix AI

Name:	Merve Zuvin PhD
Project Role:	Postdoc
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	7
Contribution to Project:	Dr. Zuvin has been the project lead performing most of the design work, bioengineering and cell culture experiments and manuscript preparation.
Funding Support:	DoD
Name:	Nanditha Anandakrishnan PhD
Project Role:	Postdoc
Researcher Identifier (e.g. ORCID ID):	0000-0003-2452-2527
Nearest person month worked:	1
Contribution to Project:	Dr. Anandikrishnan has assisted bioengineering and cell culture experiments and manuscript writing.
Funding Support:	DoD, NIH
Name:	Hillary Han
Project Role:	Computational Research Associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4
Contribution to Project:	Ms. Han has performed omics analytics.
Funding Support:	DoD
Name:	Stefanie DeFronzo
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Ms. DeFronzo has performed experiments evaluating chip performance.
Funding Support:	DoD, NIH
Name:	Smiti Bhattacharya
Project Role:	PhD Student
Researcher Identifier (e.g. ORCID ID):	0000-0002-5965-571X
Nearest person month worked:	1
Contribution to Project:	Ms. Bhattacharya has improved the design of the chip.
Funding Support:	DoD, NIH
Name:	Yi-bang Chen
Project Role:	Assistant Professor
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Dr. Chen oversaw omics experiments.
Funding Support:	DoD, NIH

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Newly funded active support for Dr. Daehn:

1 U54CA267776-01 (MPI: Benn, Campbell, Nestler, Richardson)

Dates: 09/24/2021 – 08/31/2026

Sponsor: NIH/NCI

Title: NIH FIRST Cohort Cluster Hiring Initiative at Icahn School of Medicine at Mount Sinai

Goals: The major goals are leadership, administration, communications, and integration across cores, recruitment of a diverse cohort and oversight of advancement/promotion and retention activities and tracking and evaluation.

Role: Co-Investigator

Newly funded active support for Dr. Azeloglu:

1R01DK131047-01 (MPI: Gusella, Azeloglu)

Dates: 09/24/2021 – 7/31/2025

Sponsor: NIH/NIDDK

Title: Biomechanical drivers of cystogenesis

Goals: The goal is to combine computational modeling and cell biology approaches to study the role of cell biomechanics that underlie polycystic kidney disease progression

Role: PI

AGR-23802 (MPI: Azeloglu, Campbell)

01/15/2022 – 01/14/2024

Sponsor: Aurinia Pharmaceuticals Inc

Title: Calcineurin inhibitors in kidney pathophysiology

Goals: The major goal of this grant is to determine the cytoskeletal interactions and processes associated with calcineurin inhibitors

Role: PI

GCO-22-0256 (PI: Azeloglu)

Dates: 04/01/2022 – 03/31/2023

Sponsor: FuzeHub, NYS

Title: Manufacturability Optimization of a Novel Vascular Access Device

Goals: The goal is to optimize the design for manufacture and bring an implantable vascular access device to the marketplace.

Role: PI

R37CA251733 (PI: Gallagher)

Dates: 08/01/2022 – 07/31/2026

Sponsor: NIH/NCI

Title: How elevated triglycerides promote triple negative breast cancer growth and metastasis

Goals: The aim of this grant is to the mechanisms through which elevated circulating triglycerides promote breast cancer development and progression

Role: Co-Investigator

U2CDK129502-01 (PI: Melamed, Site PI: Campbell)

Dates: 07/01/2022 – 06/30/2027

Sponsor: NIH/NIDDK

Title: New York Consortium for Interdisciplinary Training in Kidney, Urological and Hematological Research

Goals: Train a new generation of investigators who will recognize the value of interdisciplinary collaboration in KUH (Kidney, Urological and Hematological) research

Role: Co-Investigator

3R01DK127139-02W1 (PI: Nadkarni)

Dates: 06/01/2022 – 05/31/2023

Sponsor: NIH/NIDDK

Title: Elucidating Genetic and Environmental Second Hits in Racial and Ethnic Minorities with APOL1 High- Risk Genotypes (Supplement)

Goals: The overarching goal of this supplement to our existing R01 grant is to generate critical insights into the long-term effects of COVID-19 on kidney health in African Americans.

Role: Co-Investigator

What other organizations were involved as partners?

Organization Name:	Long Island University
Location of Organization:	Brooklyn Campus
Partner's contribution to the project:	Co-Investigator involved in the design and synthesis of novel small molecules as potential the BMPER, AREG and FBN1 inhibitors.
Funding Support:	NIH, DoD

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

Isolation of Conditionally Immortalized Mouse Glomerular Endothelial Cells with Fluorescent Mitochondria

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Abstract

Glomerular endothelial cell (GEC) dysfunction can initiate and contribute to glomerular filtration barrier breakdown. Increased mitochondrial oxidative stress has been suggested as a mechanism resulting in GEC dysfunction in the pathogenesis of some glomerular diseases. Historically the isolation of GECs from *in vivo* models has been notoriously challenging due to difficulties in isolating pure cultures from glomeruli. GECs have complex growth requirements *in vitro* and a very limited lifespan. Here, we describe the procedure for isolating and culturing conditionally immortalized GECs with fluorescent mitochondria, enabling the tracking of mitochondrial fission and fusion events. GECs were isolated from the kidneys of a double transgenic mouse expressing the thermolabile SV40 TAg (from the Immortomouse), conditionally promoting proliferation and suppressing cell differentiation, and a photo-convertible fluorescent protein (Dendra2) in all mitochondria (from the photo-activatable mitochondria [PhAM^{excised}] mouse). The stable cell line generated allows for cell differentiation after inactivation of the immortalizing SV40 TAg gene and photo-activation of a subset of mitochondria causing a switch in fluorescence from green to red. The use of mitoDendra2-GECs allows for live imaging of fluorescent mitochondria's distribution, fusion, and fission events without staining the cells.

Introduction

The glomerulus is critical for blood filtration by restricting the passage of large molecules through the glomerular filtration barrier^{1,2}. The glomerulus contains four cell types: parietal epithelial cells, podocytes (visceral epithelial cells), glomerular endothelial cells (GEC), and mesangial cells³. The glomerular endothelium is characterized by a unique vascular structure, as per the presence of fenestrae

required for large filtration volumes⁴. The apical surface of the glomerular endothelium is covered with a negatively charged glycocalyx layer and a coat called the endothelial surface layer that creates a space between the endothelium and blood. This structure provides high charge selectivity

restricting the passage of negatively charged molecules such as albumin and preventing leukocyte and platelet adhesion⁵.

GECs are very sensitive to metabolic changes, such as the hyperglycemia associated with the diabetic milieu. Indeed, diabetes leads to increased circulation of noxious substances, the saturation of glucose metabolism pathways, and disturbed cellular redox balance^{3,6}. Moreover, the increase in reactive oxygen species induces mitochondrial dysfunction, which affects endothelial function⁷.

The overall goal of the current protocol is to isolate immortalized glomerular endothelial cells with fluorescent mitochondrial features. Indeed, the cell culture of primary GECs has a limited proliferative cycle and early senescence⁸. In addition, the presence of fluorescent mitochondria helps examine fission and fusion events in response to hyperglycemia or any other treatment. As an alternative method, other labs used h-TERT to immortalize cells *in vitro*⁹.

The method described here allows for the isolation of conditionally immortalized mitoDendra2 glomerular endothelial cells from 4-6-week-old animals (**Figure 1**). This detailed protocol describes the use of transgenic mice (*H-2K^b-tsA58*) harboring the simian virus 40 large tumor antigen (SV40 TAG) gene^{10,11} for generating thermolabile conditionally immortalized cells. The tsA58 TAG gene product is functional at the permissive temperature of 33 °C under the control of the inducible 5' flanking promoter of the mouse *H-2K^b* gene, which is increased above basal levels upon exposure to interferon gamma (IFN γ), therefore maintaining the conditional proliferation phenotype¹². *H-2K^b* is rapidly degraded at the non-permissive temperature of 37 °C in the absence of IFN γ , removing the immortalizing function of the tsA58Tag in cells and allowing the cells to develop a more differentiated phenotype^{13,14,15}. Optional crossing of

H-2Kb-tsA58 transgenic mice with PhAM mice, which express a mitochondria-specific (subunit VIII of cytochrome c oxidase) Dendra2-green, allows the live detection of fluorescent mitochondria¹⁶. Dendra2 green fluorescence switches to red fluorescence after exposure to a 405 nm laser¹⁶. When mitochondria fuse after photo-switching, they form elongated shapes that appear yellow from the exchange of green and yellow material or appear red when they undergo fission^{7,17}. The mitoDendra2-GECs are a great tool for studying the cellular responses of GEC mitochondria to different stimuli.

Protocol

All animal procedures described here were approved by the IACUC at Icahn School of Medicine at Mount Sinai. We used three male mice (*H-2Kb-tsA58* transgenic mice with photo-activatable mitochondria [PhAM]) purchased from Jackson lab and kept on a normal chow diet.

1. Working conditions and preparations

1. Clean the working space inside the laminar flow hood using 70% ethanol and UV-C light.
2. Prepare the bead's washing buffers. Make buffer-1 using 0.1 M sodium phosphate at pH 7.4-8.0, buffer-2 using 1x phosphate-buffered saline (PBS) without calcium and magnesium supplemented with 0.1% bovine serum antigen (BSA), 3 mM EDTA, pH 7.4, and buffer-3 using 0.2 M Tris with 0.1% BSA, pH 8.5.

2. Magnetic particle concentrator

1. Preparation of magnetic beads
 1. In a 1.5 mL microcentrifuge tube, gently mix 200 μ L of magnetic beads (4×10^8 beads) with 200 μ L of buffer-1.

NOTE: Prepare 200 μL of magnetic beads per mouse 1 day before tissue harvesting. The beads will be used to harvest the glomeruli.

2. Place the tube in a magnetic particle concentrator for 1 min, then carefully aspirate the supernatant. Remove the tube from the magnet and resuspend the washed beads in 200 μL of buffer-1.
3. Place the tube containing the magnetic beads in the magnetic particle concentrator for 1 min and gently aspirate the supernatant.
4. Inactivate the free tosyl groups by washing the magnetic beads 2x, 5 min each, using 200 μL of buffer-2 by placing the tube in the magnetic particle concentrator and discarding the buffer.
5. Incubate the magnetic beads in 200 μL of buffer-3 for 24 h at 4 °C, or for 4 h at 37 °C, to inactivate the remaining free tosyl groups. Place the tube in the magnetic concentrator for 1 min, gently aspirate the supernatant, and discard it.
6. Wash the magnetic beads 1x with 200 μL of buffer-2 for 5 min at 4 °C. Place the tube back in the magnetic concentrator for 1 min and gently aspirate the supernatant. Remove the tube from the magnetic concentrator and resuspend the magnetic beads in 200 μL of buffer-2 to obtain 4×10^8 beads/mL.

NOTE: At this stage, it is possible to leave the beads at 4 °C for up to 15 days.

3. Isolation of mouse kidney glomerular cells

1. Prepare the perfusion material: 30 mL syringe, 25 G butterfly needle. Prepare the isolation material: sterile 10 cm dish, scalpel, magnetic particle concentrator, 40 μm cell strainer, 100 μm cell strainer.

2. Prepare 100 mL of 1x Hank's Balanced salt solution (HBSS) supplemented with 1% antibiotics and adjust the pH to 7.35. Filter the solution with a 0.2 μm filter and keep it under the hood to avoid contamination.
3. Mix 200 μL of magnetic beads from step 2.1.6 with 20 mL of 1x HBSS.

NOTE: For each mouse, 200 μL of beads diluted in 20 mL of HBSS 1x will be needed.

4. Prepare a 1 mL aliquot of collagenase type-II (125 CDU/mg) at 1 mg/mL in 1x HBSS solution, pH 7.35. Pre-warm the RPMI with 10% FCS medium in a water bath at 37 °C. Pre-warm the endothelial cell culture medium at 37 °C (see **Table of Materials** for details).

NOTE: The RPMI will be used to neutralize the collagenase.

5. Pre-coat two wells of a 6-well plate with 2 mL of 10 $\mu\text{g/mL}$ collagen IV (1 mL/well) for 45 min at room temperature. Wash the plate 3x with 1x PBS to remove the acetic acid used to dilute the collagen. Use the coated plates immediately or store them at 2-8 °C for up to 4 weeks.

NOTE: Sealing the plate(s) with a transparent film is preferred to prevent drying out. The collagen coating facilitates the attachment of cells to the plate.

6. Clean and sterilize all the surgical instruments in **Figure 2** by using 70% ethanol and subsequently autoclaving them for 30 min. Clean the surgical workspace with 70% ethanol.
7. Anesthetize the mice with an intraperitoneal injection of ketamine/xylazine (10 mg/mL ketamine and 1 mg/mL xylazine). To confirm that the animal is fully anesthetized, check for the toe pinch reflex.
8. Fix the paws of the mouse with 19 G needles to hold it in place on a dissection platform. Clean the chest and

the abdomen with 70% ethanol, and open the abdomen along the chest with scissors a (**Figure 2**).

9. Insert a butterfly needle into the left ventricle (**Figure 3A**) and inject about 100 μ L of the bead solution (from step 1.2) to expand the blood circulation.
10. Carefully break the inferior vena cava below the kidneys with a 19 G needle (**Figure 3A**). Continue the perfusion initiated in step 3.9.
NOTE: Alternatively, a mini pump can be used at speed: 18.0+, time: 5 min. Good perfusion is important to harvest a great number of glomeruli bearing magnetic beads.
11. Gently remove the fat tissue with tweezers and excise both kidneys with thin surgical scissors c (**Figure 2**) and put them in a plate with 1 mL of 1x HBSS on ice. Mince the kidney with scissors b (**Figure 2**) then with a sterile scalpel to 1 mm small pieces as shown in **Figure 3B**.
12. Next, incubate the minced kidney with 1 mL of collagenase type II from step 3.4 for 35 min at 37 °C with gentle tilting using a horizontal shaker. The tissue should be completely digested after this step, as shown in **Figure 3C**.
13. Add 4 mL of RPMI with 10% FBS (from step 3.4) to neutralize the collagenase. Filter the digested tissue through a sterile 100 μ m cell strainer into a 50 mL tube. Use the bottom of a sterile 1.5 mL microcentrifuge tube to stir the digested tissue against the strainer to allow the glomeruli to go through.
14. Rinse the cell strainer with 15 mL of 1x HBSS and then centrifuge the flow-through at 200 x g for 5 min at room temperature. At this point, the pellet contains three layers; the beads with glomeruli are visible in the middle layer of the tube, the upper layer consists of smaller

structures such as tubules, and the bottom layer is debris (**Figure 3D**).

15. Carefully aspirate the supernatant and the top white layer. The remaining pellet contains the beads bearing the glomeruli and debris (**Figure 3D**). Resuspend the pellet with 1.5 mL of 1x HBSS and transfer into a 2 mL tube.
16. Place the tube in the magnetic concentrator (**Figure 3E**), aspirate the supernatant, and then wash the beads 2x with 1 mL of 1x HBSS; at this stage, put 20 μ L of the cell suspension on a slide to verify the presence of glomeruli under the microscope (**Figure 3F**; a glomerulus [black arrow], and some beads [yellow arrows] in the suspension).
17. Carefully aspirate the supernatant, resuspend the pellet in endothelial cell growth medium (**Table of Materials**), and transfer it to a clean 2 mL tube. Add 2 μ L of IFN γ (100 U/mL) to the tube and plate the cells on to one well of a 6-well plate. Incubate the cells at 33 °C.
18. After 3 days of culture, carefully change the medium by pipetting out 1 mL of the medium and replacing it with 1 mL of fresh media. At this stage, the cells are heterogeneous with a mixture of glomerular cells (**Figure 4G**).
- NOTE:** In the outgrowing culture, there will be a mix of glomerular cells and some magnetic beads (**Figure 4G**). Notice that all the cells should show green, fluorescent mitochondria.
19. After 7 days of culture, completely aspirate the medium and replace it with a fresh 2 mL of endothelial cell growth medium supplemented with IFN γ (100 U/mL). At this stage, the cells start proliferating but are still heterogeneous (**Figure 4A**).

20. After 10 days of culture when the cells reach ~80% confluency, passage the cells using trypsin as described in step 5.1 and then transfer them into a T25 flask coated with collagen IV.
21. After 1 week (total ~21 days of culture), passage the cells to a T75 flask coated with collagen IV.

4. Preparation of beads for endothelial cell isolation

1. Start by coating the sheep anti-rat beads with a rat anti-mouse CD31 antibody^{3,18,19}. Use 10 μ L of beads for each T75 flask.
2. Wash 10 μ L of beads 3x with 200 μ L of 1x PBS supplemented with 0.1% BSA, 2 mM EDTA, 1% penicillin/streptomycin. Place the tube in the concentrator magnet to pipette the washing buffer each time.
3. Resuspend the beads in 200 μ L of PBS, 1% BSA, 2 mM EDTA, 1% penicillin/streptomycin. Add 4.8 μ L of rat anti-mouse CD31 antibody^{7,18,19}, place the tube on a horizontal shaker and gently mix for 1 h at room temperature, and then keep the tube at 4 °C overnight.

5. Isolating the glomerular endothelial cells with CD31-coated beads

1. Add 3 mL of 0.25% trypsin to a T75 flask and incubate the cells at 37 °C for 5 min. Neutralize the trypsin with 3 mL of endothelial growth medium, transfer the cells to a 15 mL tube, and then centrifuge at 200 x g for 5 min.
2. Aspirate the supernatant and wash the cells in 1 mL of PBS containing 1% BSA, 2 mM EDTA, 1% penicillin/streptomycin. Centrifuge at 200 x g for 5 min.
3. Resuspend the pellet in 200 μ L of coated beads prepared in step 4, and add 800 μ L of PBS containing 1% BSA, 2

mM EDTA, 1% penicillin/streptomycin. Incubate the cells for 45 min with continuous shaking at 37 °C, 5% CO₂.

4. Place the tube in the magnetic concentrator and wash the cells 4x with 1x PBS containing 1% BSA, 2 mM EDTA, 1% penicillin/streptomycin. Resuspend the cells in 3 mL of endothelial growth medium supplemented with 100 U/mL IFN γ .
 5. Plate 1.5 mL of cells/well in collagen IV-coated wells on a 6-well plate. Culture the cells under permissive conditions at 33 °C.
- NOTE:** The isolated cells express simian virus 40 (SV40) large T antigen tsA58, enabling the activation of continuous proliferation at 33 °C (permissive condition).
6. After 4 days in culture, remove 750 μ L of the medium and replace it with the same volume of fresh medium supplemented with 100 U/mL IFN γ .
 7. After 10 days to 14 days of culture, check for growing CD31 positive GECs colonies (**Figure 4C**) that express fluorescent mitochondria using an epifluorescence microscope with a 488 nm filter.
 8. After 21 days of culture, the cells may have reached 80%-90% confluency; transfer them into a T25 flask. After reaching confluency, cells can be maintained with RPMI growth medium and 10% FCS.

NOTE: Before reaching confluence, the cells may look heterogeneous (**Figure 4B**). However, once they are confluent, they acquire a cobble-stone morphology.

9. At this stage, the cells could be cryopreserved. Upon reaching 80% confluency, add 3 mL of 0.25% trypsin to the cells and incubate them at 37 °C for 5 min. Neutralize the trypsin with an equal volume of growth medium supplemented with 10% FBS.

10. Centrifuge at 200 x *g* for 5 min. Discard the supernatant and resuspend the cells in 3 mL of freezing medium (RPML, 20% FBS, 10% DMSO). Aliquot the cells in cryo-tubes and store them in liquid nitrogen vapor temperature.

6. Cultivating the mitoDendra2-GECs

1. Thaw the cells from the cryoaliquot, from step 5.10, in a water bath at 37 °C and transfer them into a 15 mL tube with 10 mL of pre-warmed endothelial growth medium.
2. Centrifuge the suspension at 200 x *g* for 5 min at room temperature. Remove the medium, and transfer the pellet to the collagen IV-coated T25 cm² cell culture flask with pre-warmed endothelial cell growth medium supplemented with 100 U/mL IFN γ and incubate at 33 °C.
3. Change the medium the next day. When the cells reach 70%-80% confluency, usually after 5 days, transfer them to a T75 cm² cell culture flask.
4. To differentiate the cells to endothelial phenotype, transfer the cells to non-permissive conditions at 37 °C in growth media without IFN γ .

7. Passaging of mitoDendra2-GECs

1. Remove the medium, wash the cells with PBS, and add 3 mL of warm 0.25% trypsin-EDTA solution (T75 cm² flask), incubate at 37 °C for 5 min.
2. When the cells are detached, add 5 mL of growth medium and pipette up and down. Centrifuge the suspension at 200 x *g* for 5 min at room temperature and transfer the cells to a new collagen IV-coated flask or plate.

NOTE: Passaging should be done once a week at a ratio of 1:4.

8. Characterization of mitoDendra2-GECs

1. Perform immunofluorescence staining for CD31 on differentiated cells^{7,18,19,20} (**Figure 4D**). Use other markers such as vWF and Isolectin-B4 as well²⁰.
2. To confirm mitochondrial specificity of Dendra2, label cells with mitochondrial-specific markers by incubating the cells in growth medium containing 100 nM of mitochondrial tracker for 30 min at 37 °C, 5% CO₂.
3. Remove the medium and wash the cells gently with pre-warmed medium without phenol red to perform live imaging.

NOTE: Alternatively, cells may be fixed using formaldehyde fixatives (4%) for 30 min followed by washing several times in PBS. Fixed cells can be stored at 4 °C in 1x PBS until use. A confirmation with flow cytometry is also possible: label cells with anti-CD31, anti-PECAM, anti-CD45. GECs should stain negative for podocyte markers (synaptopodin, Nphs1, Podxl), mesangial cell markers (PDGFR β , angiotensinogen), and tubular cell markers (Aquaporin, Aminopeptidase-N). These markers serve as negative controls. Due to the mitochondrial-specific Dendra2 expression of these cells, avoid using the FITC channel.

9. Live cell imaging of mitochondria structural changes in response to oxidative stress or high glucose

1. Plate 1 x 10⁵ GECs on a 35 mm glass-bottom dish coated with collagen IV using endothelial cell growth medium (**Table of Materials**). After 24 h, change to medium without phenol red. Cells have green, fluorescent mitochondria.

2. Set the environmental chamber at 37 °C, 5% humidity, and 5% CO₂ 1 h prior to the start of the experiment.
3. Under a confocal microscope, use a 40x/1.2 W objective to adjust the focus and select the region of interest. Incubate the cells with 2mL of 5 mM or 25 mM glucose for 60 min.
4. Expose the cells to a 405 nm laser (4% laser power) to photo-convert a subpopulation of mitochondria from green to red (**Figure 5A-B, D-E**).
5. Use the 561 nm laser to excite Dendra2 in the unconverted state to visualize the red fluorescence. See the detailed photo-switching protocol as previously described¹⁶. The fusion events appear as yellow fluorescence after several minutes because of green matrix and red matrix fusion (**Figure 4C, F**).

Representative Results

In this article, a detailed protocol for the isolation of conditionally immortalized glomerular endothelial cells with stable fluorescent mitochondria (mitoDendra2-GECs) is described (**Figure 1**). The use of young 6-10-week-old mice is essential to obtain a substantial number of healthy cells. After 3 days of culture, cells start growing slowly from the isolated glomeruli, as shown in **Figure 3G**. After 7 days, cells are heterogeneous, showing other glomerular cell types, such as podocytes, parietal epithelial cells, and mesangial cells (**Figure 4A**). After reaching 70% to 80% confluency, other glomerular cells were removed using a positive selection of

endothelial cells with magnetic CD31 labeled beads (**Figure 4B-C**). The mitoDendra2-GECs expressed CD31 (**Figure 4D**) and were negative for podocyte marker as shown by synaptopodin negative staining (**Figure 4E-F**).

The fluorescent mitochondrial feature in the mitoDendra2-GECs allows for studying mitochondria morphology under various conditions *in vitro*. Here, we tested the effect of high glucose (25mM) on the mitochondria structure of mitoDendra2-GECs (**Figure 5D-F**). Compared to the elongated mitochondria visible in cells under normal glucose (5mM; **Figure 5A-C**), high glucose-induced fragmentation or fission of mitochondria as observed by prominent spheroid-shaped mitochondria (**Figure 5E**). Furthermore, we used a 405 nm laser to photo-convert a selected subpopulation of mitochondria in a single live mitoDendra2-GEC (**Figure 5A,D**). In **Figure 5B** and **Figure 5E**, successful photo-switching of mitochondria from green (488nm) to red (561 nm) in the selected area is presented. Importantly, it allowed for witnessing the fusion events in mitoDendra2-GECs grown under normal glucose, as can be observed by the yellow merged green and red fluorescence as a result of mitochondria matrix fusion (**Figure 5C**). In contrast, in the high glucose-treated mitoDendra2-GEC, the mitochondria were fragmented and were mainly red (**Figure 5F**), suggesting that, in the time frame tested, the fission/fusion events were delayed or inhibited due to the damage caused by the high glucose treatment, and this is consistent with previous reports^{18,19}.

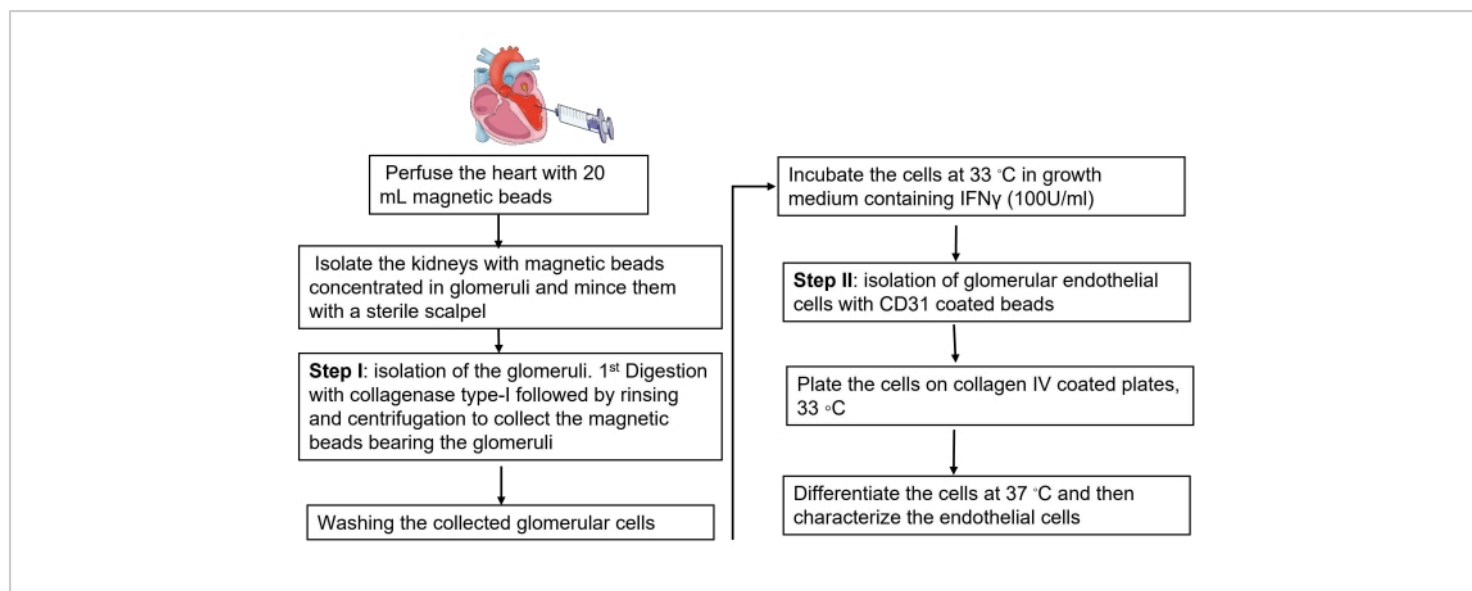


Figure 1: Representative description of murine GEC isolation. The diagram represents the significant steps of GEC isolation, starting from perfusing mice heart with magnetic beads to isolate the kidney glomeruli, the digestion of collagenase, the culture of glomerular cells, and finally, the purification of GEC using CD31-coated beads. [Please click here to view a larger version of this figure.](#)

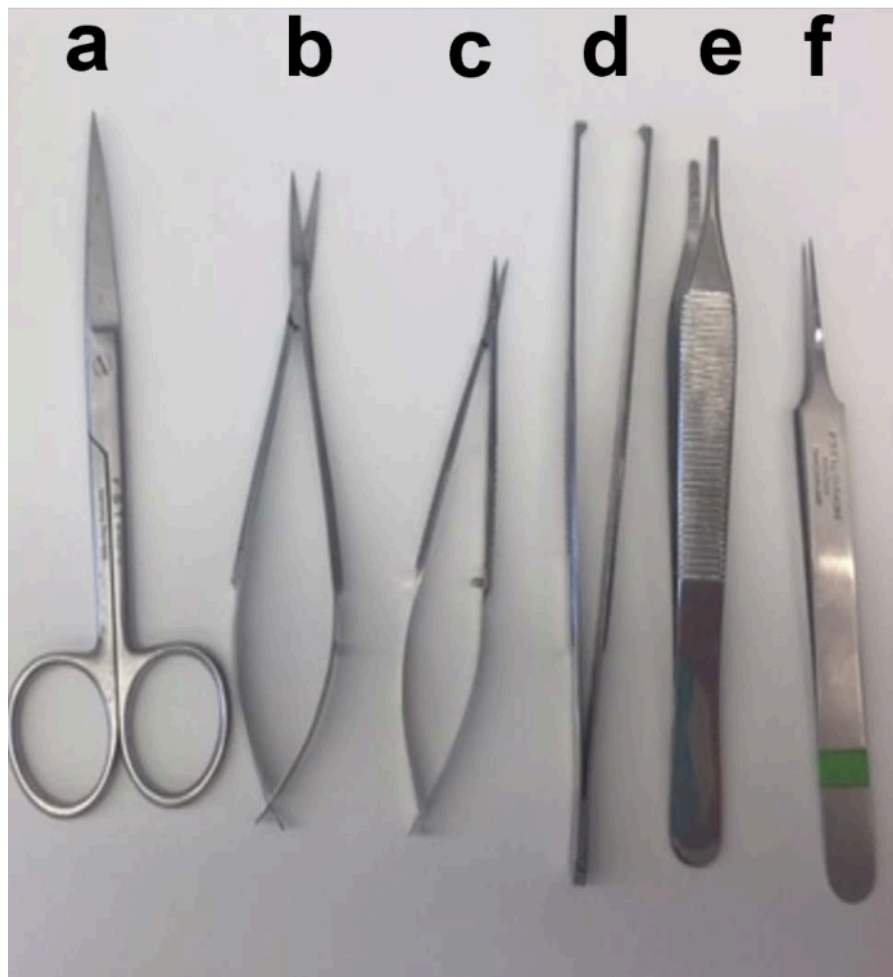


Figure 2: Description of kidney dissection. Representative image of all the sterile surgical instruments needed for the dissection. Scissors (a) are needed to open the skin of the mouse, and a separate pair of scissors (b,c) are needed to isolate the kidney. Tweezers (d) and (e) are needed to hold the skin. Tweezer (f) is needed to collect the dissected kidney. [Please click here to view a larger version of this figure.](#)

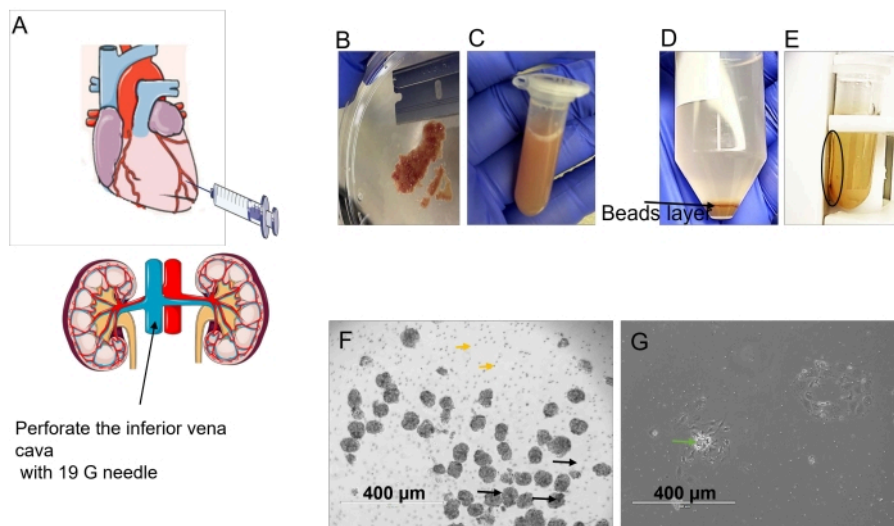


Figure 3: Description of glomeruli harvesting. (A) Perfuse the left ventricle with the HBSS-beads solution using a 20 mL syringe. Perforate the inferior vena cava with a 19 G needle. (B) After perfusion, remove the fat from the kidney and cut it into 1 mm pieces. (C) Digest the tissue with collagenase type II. (D) After digestion and centrifugation, three layers are formed; the beads bearing the glomeruli are in the middle layer, as indicated by the black arrow. (E) Separate the isolated beads bearing the glomeruli using the magnetic concentrator. (F) Representative image of freshly isolated glomeruli. (G) Growing cells from isolated glomeruli after 3 days. [Please click here to view a larger version of this figure.](#)

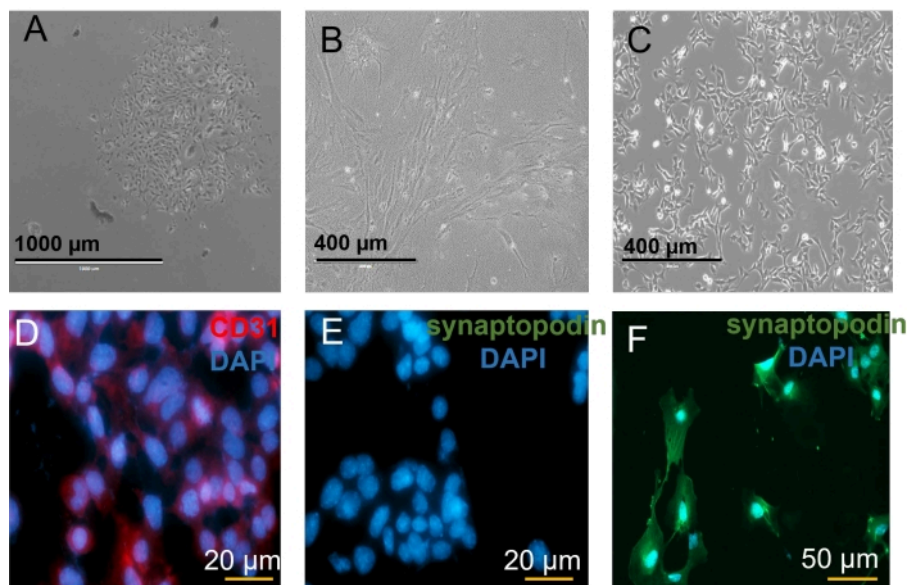


Figure 4: Mitochondrial assay *in vitro*. (A) Glomerular cells after 3 days of culture. (B) GEC after 7 days. (D) Purification of GEC with CD31-labeled beads. Microscopic imaging of CD31 staining. (E) GECs are negative for synaptopodin. (F) Podocytes stained with synaptopodin (488 nm, green fluorescence) as a negative control. [Please click here to view a larger version of this figure.](#)

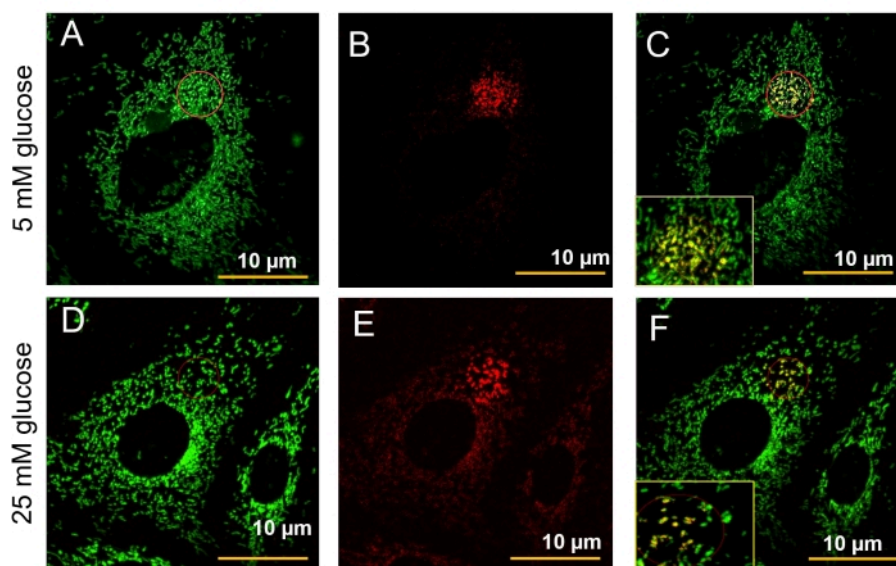


Figure 5: Microscopic imaging of fluorescent mitochondrial fusion *in vitro*. Images were acquired with a confocal microscope using a 40x water objective. **(A-B)** Selected area (red circle) was illuminated with a 405 nm line (4% laser power) for 300 bleaching iterations at a speed of 6.3-12.61/pixel, as previously described¹⁶. **(A-C)** Cells in growth media containing 5 mM glucose; green fluorescence (488 nm) shows healthy mitochondria. **(B,E)** Mitochondria were photo-switched into red (567 nm). **(C)** Yellow fluorescence detects an active fusion event of GEC treated with 5mM glucose. **(D)** Cells were treated with high glucose 25 mM for 30 min. Green fluorescence shows fragmented mitochondria and **(E)** a portion that was photo-switched into red. **(F)** The figure shows a decreased fusion event as shown by reduced yellow fluorescence. [Please click here to view a larger version of this figure.](#)

Discussion

Mitochondria are critical for cellular metabolism, homeostasis, and stress responses, and their dysfunction is linked to many diseases, including kidney disease. Mitochondria have a role in the pathologic generation of excessive reactive oxygen species (ROS), the regulation of intracellular calcium levels, cell death pathways, and cytoskeletal dynamics^{21,22,23}.

The isolation of murine GECs is challenging because of their small number, size, matrix factors, and interdependent nature with other glomerular cells that are essential for their survival. The current article presents a detailed protocol

for mouse GEC isolation. Specifically, we used transgenic mice as a tool to obtain conditionally immortalized cell lines with high proliferation potential. The transgenic mice, expressing simian virus 40 (SV40) large T antigen tsA58, enable the activation of continuous proliferation under permissive conditions at 33 °C and were here crossed with the *PHAM*^{excised} mice expressing mitochondrial-specific fluorescence. The double transgenic mice offered an excellent tool for studying mitochondria structure. Indeed, *PHAM*^{excised} mice bear mitochondrial-specific (subunit VIII of cytochrome c oxidase) Dendra2 green, allowing the live detection of fluorescent mitochondria and the observation

of fission and fusion events through green-red photo-switching¹⁶.

The protocol consists of three significant steps: extracting glomeruli using beads, the proliferation of glomerular cells, and the subsequent purification of GEC using CD31 beads once the cells are in culture. The use of only one digestion with collagenase is sufficient to isolate glomerular cells. Other protocols make use of a second digestion with DNase and proteinase²⁴. However, we have noticed better cell yields with only one digestion step. The DNase may remain active and slow down the proliferation of the cells. Furthermore, GEC could be isolated using differential sieving as previously described²⁰, which is not always easy when using mice kidneys.

It is crucial to autoclave all the surgical tools before starting the isolation of the kidneys and the glomeruli to avoid contamination. Moreover, it is important to filter and supplement the solutions with antibiotics to eliminate any source of contaminants. Indeed, mycoplasma infections may cause cytopathology that consequently interferes with every parameter measured in cell culture²⁵.

The isolation of glomeruli relies on the intra-cardiac perfusion of beads. Certainly, slow perfusion with 20 mL of 1x HBSS-containing beads is essential to harvest a high number of glomeruli. The beads will be trapped in the glomeruli, which eases their purification using a magnetic concentrator. The beads might be prepared and stored at 4 °C for up to 15 days before using them to ease the experimental procedure.

Furthermore, digesting glomeruli with continuous shaking is crucial for dissociating a good number of glomeruli from the kidney. On the other hand, the isolation of murine GECs is challenging because of their small size and the presence

of other glomerular cells. Therefore, the stepwise isolation of glomeruli, the growth of all glomerular cells in culture, and the subsequent use of CD31-coated beads to purify endothelial cells are vital, specifically for obtaining more significant numbers of viable and conditionally proliferative GECs. Nevertheless, validating the purity of cells with immunostaining and flow cytometry is needed.

In addition, the protocol describes the characterization of the GEC phenotype and the mitochondria fusion/fission assay *in vitro*. The mitoDendra2-GECs are an excellent tool for studying the cellular responses of GEC mitochondria to different stimuli *in vitro* without the need for transfection or staining.

Disclosures

Authors have no competing financial interests to declare.

Acknowledgments

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