MOLECULAR MECHANISMS OF PROTEINURIA, VOLUME II

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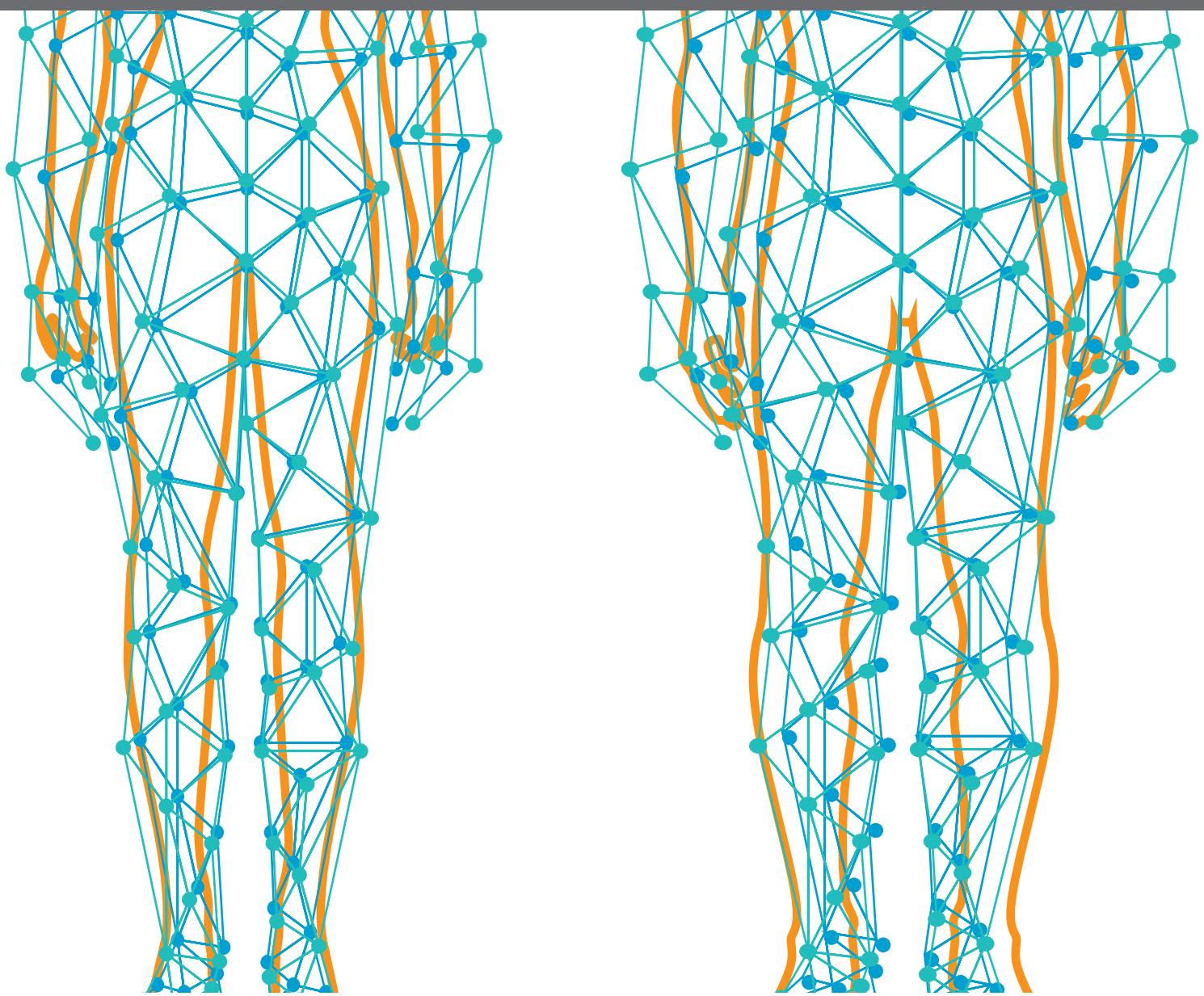

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MOLECULAR MECHANISMS OF PROTEINURIA, VOLUME II

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Editorial: Molecular mechanisms of proteinuria, volume II

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Editorial on the Research Topic
Molecular mechanisms of proteinuria, volume II

Proteinuria, which is defined as persistent and increased leakage of protein in the urine, is a consequence of an altered glomerular filtration and is considered a key marker for renal barrier dysfunction. In support, the recent recognition of the predictive link between proteinuria and disease progression to organ failure in kidney diseases has led to recommend proteinuria as an approvable primary endpoint by regulatory organizations, which will allow for the increased evaluation of investigational therapies in patients with kidney disease and accelerate the drug discovery process (1, 2). However, the molecular mechanisms leading to the development of proteinuria are complex, and there is a need to decipher these for a new generation of therapeutic drugs to treat the millions of people suffering from kidney disease.

This Research Topic represents a collection of several research articles, mini-reviews and reviews by our esteemed colleagues that are leaders in the field and put forth the current state-of-the-art of some of the key pathological mechanisms underlying proteinuric kidney diseases. The manuscripts in this Topic present exciting technologies aimed to uncover targets with therapeutic potential, as well as the glomerular cell specific contributions in the maintenance or in the breakdown of the glomerular filtration barrier. We are also thrilled that there is a diverse representation including female and minority researchers in STEM as first and senior corresponding authors participating in this Research Topic. We hope that this is a trend toward a more diverse and inclusive representation of emerging leaders in field.

In several original research articles of this collection, authors describe their discoveries of novel mechanisms that contribute to podocyte injury and development of proteinuria. Basgen et al. demonstrate that podocyte injury is the initiating event leading to albuminuria and mesangial expansion in the *Cd2ap* KO mouse model of focal segmental glomerulosclerosis (FSGS). The authors highlight that the sequence of events, starting with podocyte injury, has important implications, particularly in underlying proteinuric disease pathology. Li et al. describe a crucial role for microRNA-146a (miR-146a) in podocyte injury in non-diabetic and diabetic kidney disease. They demonstrate that

mice that lack podocyte miR-146a develop proteinuria and glomerular injury and confirmed its role in protecting podocytes in diabetic kidney disease (DKD) (Li et al.). Similarly, research by Song et al. unveils an essential role for podocyte MCC regulator of WNT signaling pathway (MCC), whereby reduced expression of MCC, which can be observed in glomeruli of diabetic mice and patients with FSGS, results in the loss of lamellipodia *in vitro*. Lastly, Azzam et al. investigated the interplay between reactive oxygen species (ROS) and sphingomyelin phosphodiesterase acid like 3b (SMPDL3B) in mediating the response to radiation-induced injury of podocytes and identify NOX-derived reactive oxygen species (ROS) as a novel upstream regulator of SMPDL3b.

Indeed, podocytes are central in the pathogenesis of proteinuria, and this collection also offers reviews and mini-reviews focusing on the role of other glomerular cells as critical players in the pathogenesis of glomerular injury and proteinuria. First, the role of the mesangial cells in glomerular function and intra-glomerular crosstalk is discussed in depth in a review by Ebefors et al. The authors highlight current literature that supports the active involvement of the mesangium in disease onset and progression. Next, the role of glomerular endothelial cells in proteinuria is reviewed by Ballermann et al. The review summarizes the vast literature that supports the importance of this monolayer in blocking large plasma proteins from passing into the glomerular filtrate and explains how the interaction of the glycocalyx with its endothelial surface layer contributes to the maintenance of the barrier function (Ballermann et al.). Further, the authors discuss the impact of glomerular endothelial cell dysfunction, de-differentiation and activation of endothelial cells in various diseases. The review by Gyarmati et al. centers on the role of endothelial cell injury and microthrombi in podocyte detachment and albumin leakage *via* hemodynamic and mechanical forces. The authors discuss how alterations of the glycocalyx and its interaction with the microenvironment represents a key pathogenic mechanism that results in proteinuria in various diseases (Gyarmati et al.), adding to our growing understanding of the glomerular cell specific roles in the pathogenesis of proteinuria.

Abnormalities of glomerular cell function due to mitochondrial defects is involved in the etiology of proteinuric glomerular diseases (3). In the review by Galvan et al., the authors discuss the latest discoveries that advance our understanding of the nature of mitochondrial dysfunction and how it could contribute to the progression of DKD. The authors highlight specific knowledge gaps as well as the potential for new therapeutics targeting mitochondrial dysfunction in DKD.

The mini-review by Wei et al. summarizes the role of urokinase-type plasminogen activator receptor (uPAR) in podocyte injury and in proteinuric kidney disease. The authors discuss the evidence supporting a role for elevated serum suPAR as a circulating risk factor for kidney diseases and how the complexity of suPAR derived from different enzymatic cleavage and other modifications may have confounded the determination of serum suPAR levels in patients in previous studies. In a review by Bisgaard and Christoffersen, the role of kidney-derived and plasma APOM is discussed in the context of kidney disease development. The authors highlight recent research suggesting that changes in the APOM/S1P axis contribute to the pathogenesis of kidney diseases and could be a potential therapeutic target (Bisgaard and Christoffersen). The molecular mechanisms of proteinuria in Minimal Change Disease (MCD) are reviewed by Purohit et al. and shed light on the complex interplay between the immune system, glomerular cells, and the genome, raising the possibility of distinct underlying mechanisms of proteinuria among patients with MCD. Hall and Wyatt review the mechanisms of proteinuria in HIV-associated nephropathy (HIVAN). The roles of injury induced podocyte dedifferentiation, hyperplasia, cytoskeletal dysregulation and apoptosis in HIVAN, and the role of apolipoprotein L1 (APOL1) risk variants are discussed. Indeed insights from HIVAN could help improve the management of COVID-19-associated kidney disease given the similar clinical presentations, pathological findings and potential disease mechanisms (4).

New and emerging technologies to study the molecular mechanisms of proteinuria are also presented in this Topic. The mini review by Latt et al. summarizes how single-cell and single-nucleus RNA sequencing approaches have improved our understanding of the pathophysiology of glomerular diseases at a cellular level. They argue that these methods could be useful in exploring non-invasive approaches to aid in the identification of precision therapeutics for proteinuric diseases (Latt et al.). Gong et al. discuss the opportunities for single cell RNA sequencing and bioinformatics-based spatial transcriptomics. In this context, the authors review how other novel approaches such as spatial transcriptomics, the development of the glomerulus-on-a-chip and of kidney organoids are contributing to our growing understanding of glomerular pathophysiology (Gong et al.). Advances in genetics have led to the remarkable discovery that the presence of two high-risk variants of Apolipoprotein L1 (APOL1) in people of recent African ancestry confers susceptibility to the development of several glomerular diseases, including FSGS and HIVAN. The mini review by Yoshida et al. discusses the advantages and limitations of several animal models, including mice, zebrafish and drosophila that have been used to study APOL1 function in health and disease. Forward thinking research will leverage these endeavors to unlock mechanistic insights that will open new avenues for discoveries in this space.

It is an exciting time in our field as in the past few years we have witnessed the development of new technologies and improved methods, that have led to the discovery of novel targets within the glomerular filtration barrier and application

of new drugs currently undergoing clinical evaluation (5). The number of compounds in all phases of clinical trials for kidney diseases has doubled in the past decade and we anticipate that the number will continue to increase in the next few years.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

SM is an inventor on pending or issued patents (PCT/US11/56272, PCT/US12/62594, PCT/US2019/041730, PCT/US2019/032215, PCT/US13/36484, and PCT 62/674,897) aimed at diagnosing or treating proteinuric kidney diseases and stands to gain royalties from the future commercialization of these patents. SM holds equity interest in L&F Research who has licensed worldwide rights to develop and commercialize hydroxypropyl-beta-cyclodextrin to ZyVersa Therapeutics, Inc.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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ORIGINAL RESEARCH
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Podocyte Foot Process Effacement Precedes Albuminuria and Glomerular Hypertrophy in CD2-Associated Protein Deficient Mice

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Background: Podocyte foot process effacement is a key histologic finding in proteinuric kidney disease. We previously showed that 3-week old CD2AP-deficient mice have significant proteinuria, glomerular hypertrophy and mesangial expansion. The goal of this study is to use morphometry to establish the temporal sequence of podocyte foot process effacement, glomerular volume expansion and albuminuria in *Cd2ap*$^{-/-}$ mice by measuring these parameters at the 2-week time point.

Methods: Wild-type mice age 14 ± 1 days with the Cd2ap gene (WT, $N = 5$) and mice deficient for Cd2ap (*Cd2ap* KO, $N = 5$) were generated. Kidneys were harvested and fixed in 2.5% glutaraldehyde and processed for examination by light and electron microscopy. An average of 415.2 (range 268–716) grid points were counted for all the glomeruli, and quantification of glomerular volume from each kidney. Urine was collected the day prior to sacrifice for urine albumin-to-creatinine ratio (ACR) measurements.

Results: There was no difference in albuminuria [median (range) mg/g] between WT [212.2 (177.6–388.4) mg/g] vs. *Cd2ap* KO mice [203.3 (164.7–910.2) mg/g], $P = 0.89$; or glomerular volume 68,307[10,931] vs. 66,844[13,022] μm^3, $p = 0.92$. The volume densities of glomerular components of the podocyte, capillary lumen and mesangium were not different for the two groups, $P = 0.14$, 0.14 and 0.17 respectively. However, foot process width was increased in *Cd2ap* KO 1128[286] vs. WT [374 ± 42] nm, $P = 0.02$.

Conclusion: Here we show that while 2-week old WT and *Cd2ap* KO mice have similar levels of albuminuria, glomerular and mesangial volume, *Cd2ap* KO mice have more extensive podocyte foot process effacement. The data suggests that podocyte injury is the initiating event leading to mesangial expansion and albuminuria in this model.

Keywords: CD2AP deficient mice, podocyte foot process effacement, albuminuria, kidney morphometry, glomerular volume, Cavalieri Principle, Delesse Principle

INTRODUCTION

Kidney podocytes are terminally differentiated epithelial cells with a complex cellular morphology that form the final barrier to urinary protein loss (1). Podocyte foot process effacement on electron microscopy is a common feature of proteinuric kidney diseases. Numerous disease-causing genes encoding functional components of the podocyte actin cytoskeleton and slit diaphragm have been identified in human familial focal segmental glomerulosclerosis (FSGS) and nephrotic syndrome (2–4). Direct evidence that podocyte loss causes glomerulosclerosis has been provided by elegant animal models where a 20% loss in podocyte number results in mild persistent proteinuria and FSGS while a depletion of >40% results in high-grade proteinuria and decreased renal function (5, 6).

The temporal relationship between the onset of albuminuria and the development of podocyte foot process effacement is unclear and inconsistent. Albuminuria has been reported without foot process effacement in mouse models of type IV collagen alpha3 deletion, in rats treated with an anti-nephrin antibody and in a rat model of diabetes (7–9). In glomerular basement membrane laminin β2 deficient mice, albuminuria occurs seven days before foot process effacement (10). The opposite relationship has also been reported. For example, the classic descriptions of rat puromycin aminonucleoside nephropathy showed foot process effacement occurring a few days before proteinuria (11–13). In humans with type 1 diabetes mellitus albuminuria preceded effacement (14, 15). More recently, foot process effacement was found in six out of eight children with Fabry's disease without albuminuria or other renal abnormalities who underwent research biopsies prior to starting enzyme replacement therapy (16).

Accurate measurements of podocytes and podocyte effacement require appreciation of the 3-dimensional nature of the glomerulus and glomerular components that are typically observed as 2-dimensional structures on microscopic images. Studies using limited 2-dimensional profiles may mis-report glomerular data when expressing the number of podocyte profiles per glomerular profile area as a surrogate for density or number of glomeruli per kidney. Importantly, glomerular profile number is not directly related to the number of podocytes contained in the 3-dimensional glomerulus. For example, large glomeruli may have a greater probability of intersecting the section than small glomeruli, thus a bias toward overcounting large glomeruli compared to smaller glomeruli (17), and overestimating average glomerular volumes. To avoid the bias of large glomeruli having a greater probability of intersecting the section and thus being over-represented, disector sampling of pairs of sections can facilitate selection of the glomeruli to be measured (17). Importantly, the distance between the two sections must be less than the smallest glomerular diameter and only glomeruli that intersect the second section and not the first should be selected. Using electron microscopy, Farquhar, Vernier, and Good were the first to describe a "smearing" and loss of foot processes in children with proteinuria (18, 19). Powell was the first to quantitate these changes by counting the number of slit pores per length of GBM (20). More recently, researchers have measured individual foot process and calculated the average width of the foot processes (21–23). Direct measurement of foot process width has the problem of measuring the width of the foot processes as they curve around the capillary wall. Instead of measuring individual foot process widths we measured length of slit diaphragm per area of GBM (14, 15, 24). This can be accomplished by counting the number of slit diaphragm profiles and the number of intersections between grid lines and GBM resulting in the parameter length density of slit diaphragm per area of GBM. The reciprocal of this parameter is mean foot process width. It is less time consuming to count the number of slit diaphragm profiles and the number of intersections between the grid lines and the interface than to measure the individual foot process widths. This method also eliminates the problem of measuring the width of foot processes when they curve around non-straight GBM.

We previously identified a dendrin-dependent podocyte-mesangial crosstalk axis in mice lacking CD2-associated protein (*Cd2ap* KO) (25). At three weeks of age, the animals had heavy proteinuria, podocyte foot process effacement and significant mesangial and glomerular volume expansion (25), but the sequence of events was not examined. Here, we sought to establish the temporal sequence of albuminuria, podocyte foot process effacement and glomerular volume expansion in *Cd2ap* KO mice by measuring these parameters at the 2-week time point. We used design-based stereological methods to make measurements on 2-dimensional images to obtain structural information about the 3-dimensional glomeruli.

MATERIALS AND METHODS

Animals

The study followed the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health using a protocol approved by the Institutional Animals Care and Usage Committee of the Icahn School of Medicine at Mount Sinai. Global *Cd2ap* KO mice were obtained from Dr. Andrey Shaw (previously at Washington University, St. Louis, MO) (26). Mice with and without the Cd2ap gene were generated and genotype confirmed by PCR and five WT and four *Cd2ap* KO mice were analyzed. The day prior to sacrifice, urine was collected for determination of albumin-to-creatinine ratio (ACR). At age 14 ± 1 days, mice were anesthetized with isoflurane and injected with ketamine/xylazine. Kidneys were harvested, cut transversely, and approximately one third of each kidney was placed in 2.5% glutaraldehyde in PBS and shipped to the Morphometry and Stereology Laboratory at Charles R. Drew University of Medicine and Science for morphometric analysis.

Albuminuria

Albuminuria was measured by enzyme-linked immunosorbent assay following the manufacturer's protocol (Bethyl Laboratories, Montgomery, TX). Creatinine was measured using the Creatinine Urinary Colorimetric Assay Kit (Cayman Chemicals, Ann Arbor, MI) following the manufacturer's protocol with the same urine samples for ACR.

Kidney Morphometry

One-millimeter cubes were cut from the fixed kidney cortex, rinsed with buffer, post-fixed in 1% osmium tetroxide, and embedded in epoxy resin (Polybed 812, Polysciences, Warrington, PA) for light and electron microscopy analysis for quantification of the following parameters.

In order to examine glomerular components individually, the glomerulus was divided into four components: podocytes, mesangium including mesangial cells and mesangial matrix, capillary lumens including endothelial cells, and "other" including Bowman's space, GBM, and non-resolvable areas. The areal fraction of each component was measured using point counting (27). The volume fraction of each component is equal to the areal fraction if the measurements were done on random sections through the cortex-the Delesse Principle (28).

Glomerular Volume

Serial 1-μm thick sections were cut from the resin blocks using an EM UC7 ultramicrotome (Leica Microsystems, Buffalo Grove, IL) fitted with a Histo Jumbo diamond knife (Diatome US, Hatfield, PA). Every fifth section was saved to a microscope slide and stained with 1% toluidine blue. A total of 21 sections were saved from each kidney and the slides were labeled 0, 5, 10, 15, . . . , and 100. A BX51 microscope with a DP71 digital camera and DP Controller software (Olympus America, Cypress, CA) was used to observe the sections. A map of the glomerular profiles present in section 0 was made using the 10X objective lens. Glomeruli present in section 0 could not be used for volume measurement since an unknown volume of those glomeruli was lost. The map and subsequent sections were used to identify newly appearing glomeruli. Only glomeruli appearing and then disappearing within the stack of 21 sections could be used for analysis. The new glomeruli were mapped, numbered 1–7 (average 6.2, range 5–7 glomeruli per mouse) and imaged using the 100X objective lens. All profiles from each of the numbered glomeruli were imaged (average of 9.7, range 5–14 profiles per glomerulus).

An iMac computer with a 24′ monitor (Apple Inc., Cupertino, CA) and Photoshop software (Adobe Systems, San Jose, CA) were used to analyze the images. Using the Photoshop Polygonal Lasso tool a minimum string polygon was drawn around each glomerular tuft defining the limits of the glomerular profile. The Cavalieri Principle (27, 29) was used to determine glomerular volume by superimposing a grid of points over each profile from a glomerulus using the Layers function of Photoshop (**Figure 1**). The number of grid points falling on all of the profiles from a glomerulus was counted and the volume for each glomerulus was calculated using the equation:

$$\text{Volume}_{\text{glomerulus}} = 5 \times \sum \text{P}_{\text{glomerulus}} \times (25,000/1734)^2 \ \mu\text{m}^3$$

where 5 was the distance in μm between the profiles of a glomerulus, $\sum \text{P}_{\text{glomerulus}}$ was the sum of grid points falling on all the profiles from the glomerulus, 25,000 was the distance between points on the grid in μm, and 1734 was the magnification of the images. A stage micrometer was imaged to document the magnification. The mean glomerular volume for each mouse was determined by calculating the average of the individual glomerular volumes from the mouse.

Volume of Glomerular Components: Podocyte, Mesangium, Capillary Lumen, Other

Using the same ultramicrotome fitted with an Ultra diamond knife (Diatome US), a 1-μm thick scout section was cut from an epoxy block and used to identify complete glomerular profiles at least one large glomerular diameter from the edge of the block. Silver-grey sections were then cut, placed on formvar coated slot grids (1 × 2 mm slot) and stained with uranyl acetate and lead citrate. These sections were observed with a JEM 1200-EX electron microscope (JEOL USA, Inc., Peabody, MA) fitted with a digital camera and DigitalMicrographs software (Gatan, Inc., Pleasanton, CA). At an initial magnification of 2500X the complete glomerular profile was imaged. Small profiles needed a single image, while larger profiles needed up to four images that were fitted together using the Photoshop software to make a montage of the complete glomerular profile. An average of 5.0, range 4–6 glomerular profiles were analyzed per mouse.

Using the Polygonal Lasso tool of Photoshop, a minimum string polygon was drawn around the tuft defining the limits of the glomerular profile. The glomeruli were divided into four components: podocyte, mesangium, capillary lumen (including endothelial and circulating cells) and other (including Bowman's space and glomerular basement membrane) (**Figure 2,** Left Panel). To measure the areal density of each of the four components, a grid of points was superimposed over the glomerular profile using the Layers function of Photoshop and the number of points falling on each of the components was counted (28, 30) (**Figure 2,** Right Panel). The areal density of a component per glomerular profile was calculated using the equation:

$$A_{\text{A (componentX/glomerulus)}} = \sum \text{P}_{\text{componentX}} / \sum \text{P}_{\text{fourcomponents}} \ \mu\text{m}^2/\mu\text{m}^2$$

where $\sum \text{P}_{\text{componentX}}$ represents the sum of points falling on Component X, either podocyte, mesangium, capillary lumen or other for all the glomerular profiles from a kidney, and $\sum \text{P}_{\text{fourcomponents}}$ is the sum of points falling on all the components from all glomerular profiles from a kidney. Because the areal densities were measured on random profiles from the glomeruli the measured areal densities equal the volume densities for each component according to the Delesse Principle (28):

$$V_{\text{V (componentX/glomerulus)}} = A_{\text{A (componentX/glomerulus)}} \ \mu\text{m}^3/\mu\text{m}^3$$

The volume density does not determine the volumes of the components but only the ratio between the component volume and the glomerular volume. The individual component volumes were calculated using the equation:

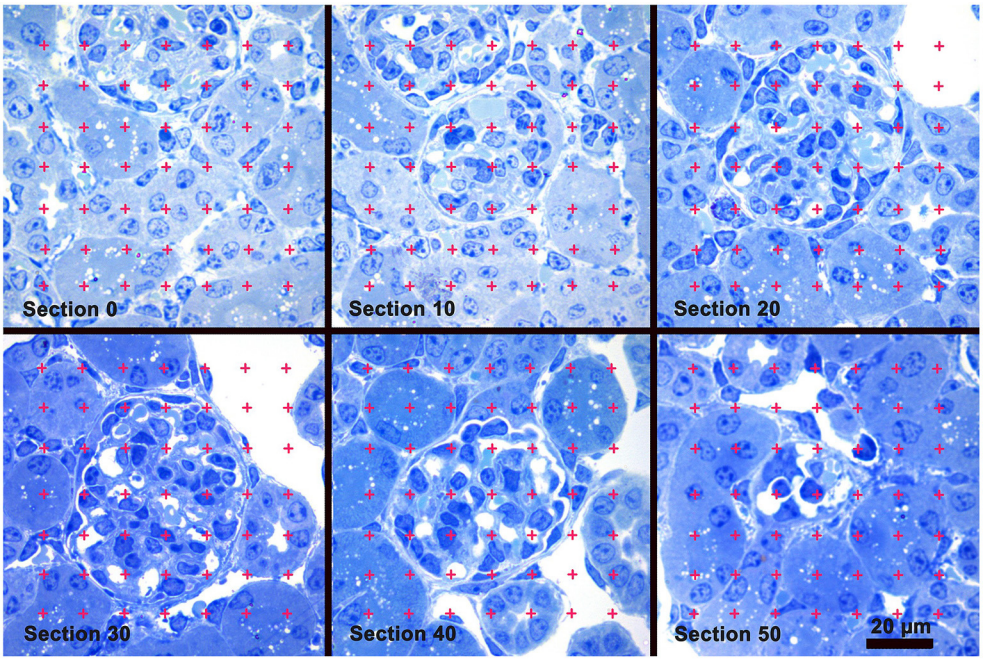

FIGURE 1 | Glomerular Volume by the Cavalieri Principle. A sample of images from a stack of sections used to measure glomerular volume. Section 0 precedes the appearance of the glomerulus present in sections 10 thru 50. A counting grid was randomly placed over each glomerular profile and the number of grid points falling on the profiles was counted. Glomerular volume was calculated by multiplying the distance between the sections by the sum of the points falling on all the profiles from the glomerulus, and then multiplied by the area represented by one grid point. Toluidine blue stain.

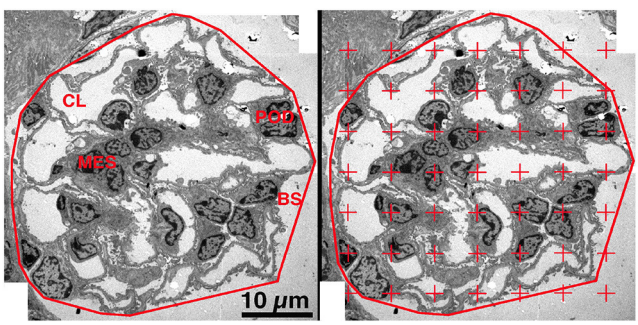

FIGURE 2 | Areal density of glomerular components. **Left Panel**. On a low magnification EM image the glomerulus was defined by drawing a minimum string polygon around the tuft. Four components were defined: podocyte (POD), mesangium (MES), capillary lumen (CL), and remainder which included Bowman's space (BS) and GBM. **Right Panel**. A counting grid was randomly placed over the image and the number of points falling on each component is counted. The areal density of component X was calculated by dividing the number of grid points falling on component X by the total number of points falling on all four components.

$$\text{Volume}_{\text{componentX}} = V_v(\text{componentX/glomerulus}) \times \text{Volume}_{\text{glomerulus}}\ \mu\text{m}^3.$$

Podocyte Effacement

The conventional 2-dimensional picture of the normal structure of the podocyte in transmission electron microscopy images has foot processes "sitting" on GBM with a profile of a slit diaphragm located between adjacent foot processes. Actually, in the 3-dimensional glomerulus the slit diaphragm, a specialized cell-cell junction, meanders between the foot processes of adjacent podocytes and thus has a length that is beautifully demonstrated in the classical paper by Rodewald and Karnovsky (31) and illustrated in **Figure 3A,** Left Panel. The length of the slit diaphragm per area of the glomerular basement membrane (GBM) it sits on can be measured as the stereological parameter, length of slit diaphragm per GBM area [L_s(slit/GBM)] (14, 32). The conventional characteristic of podocyte effacement is a widening of the foot processes. Another characteristic of podocyte effacement is a decrease of the L_s(slit/GBM) (**Figure 3A,** Right Panel). We measured L_s(slit/GBM) with the same glomeruli used to measure the volume of the glomerular components. A set of high magnification images was obtained systematically without bias from throughout each glomerular profile. An average of 36.0 (range 25–47) images per kidney were available for analysis. Using the Layers tool of Photoshop, a counting frame, consisting of inclusion, exclusion, and counting lines, and a guard zone surrounding the counting box was superimposed over each image. The guard zone eliminates ambiguous structures at the edge of the images. The number of intersections between the counting lines and the podocyte-GBM interface was counted as well as the number of slit diaphragm profiles within the counting frame and not touching the exclusion line (**Figure 3B**). The L_s(Slit/GBM) was calculated using the equation:

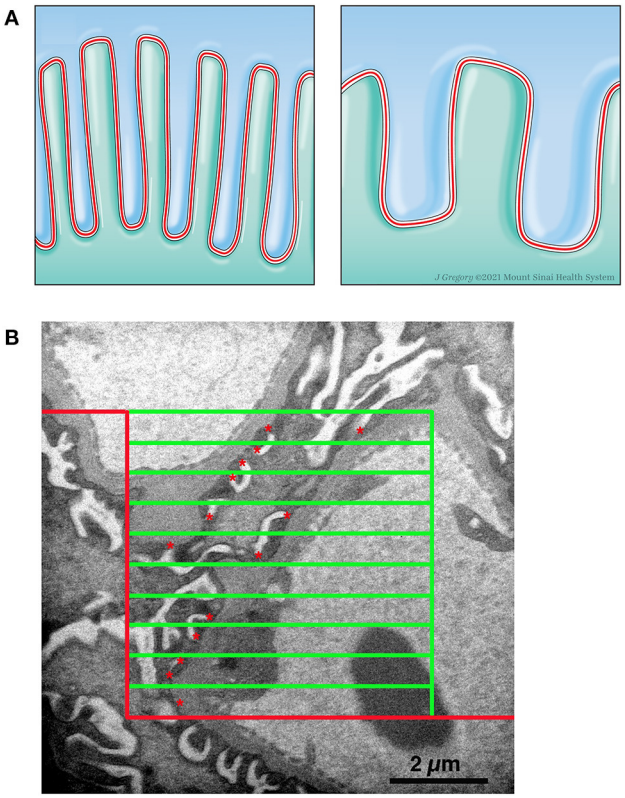

FIGURE 3 | Measurement of length density of slit diaphragm. **(A) Left Panel**. Schematic drawing of two podocytes (green and blue) with normal interdigitating foot processes and the slit diaphragm (red line) between them. **Right Panel**. Schematic drawing of effacement with shortening of slit diaphragm. **(B)** Electron micrograph with line grid used to measure Ls. A counting frame was superimposed over a high magnification image. The frame consists of a peripheral guard zone, inclusion lines (vertical green line and upper horizontal green line), exclusion lines (red lines), and 10 counting lines (green horizontal lines). The number of times a counting line intersects the podocytes-GBM interface was counted and the number of slit diaphragm profiles (red *) within the counting frame and not touching the exclusion line was counted.

$$L_s(\text{Slit/GBM}) = \sum Q_{\text{slit}} / (\sum I_{\text{Podo-GBM}} \times (22,500/37,500))\ \mu\text{m}/\mu\text{m}^2$$

where $\sum Q_{\text{slit}}$ was the sum of slit diaphragm profiles counted on all the images from a kidney, $\sum I_{\text{Podo-GBM}}$ was the sum of intercepts between the counting lines of the frame and the podocyte-GBM interface from all the images from a kidney, 22,500 was the distance in μm between frame lines, and 37,500 was the magnification of the images. A carbon replica was used to document the magnification of the images. The average foot process width is the reciprocal of the length density and was calculated using the equation:

$$\text{Foot Process Width} = 1/(L_s(\text{Slit/GBM}) \times 1,000)\ \text{nm}.$$

Statistics

Morphometry data are presented as mean (SD). Group differences were compared using the Mann-Whitney U test. $P < 0.05$ was set *a priori* as the level considered statistically significant.

RESULTS

No Differences in Albuminuria Levels

Albuminuria levels were similar in WT vs. *Cd2ap* KO mice at 2 weeks of age. The median albumin/creatine ratio was 212.2 mg/g in WT mice [interquartile range (177.6–388.4) mg/g] vs. 203.3 mg/g in *Cd2ap* KO mice [164.7–910.2], $P = 0.89$ (**Figure 4A**).

No Differences in Average Glomerular Volumes

At the time of sacrifice (14 ± 1days old) some glomeruli were not fully developed. To avoid measuring undeveloped podocytes only glomeruli at the capillary loop stage or older were used for analysis. Glomerular volume was measured by the Cavalieri Principle (25) using superimposed grid points. This method does not assume any particular shape of the glomeruli and measures the volume of individual glomeruli so that a distribution of glomerular volumes within a kidney can be obtained. A mean of 415.2 (range 268–716) grid points were counted for glomeruli from each kidney. The average glomerular volume was 68,307(10,931) μm^3 for WT and 66,844(13,022) μm^3 for *Cd2ap* KO mice, $P = 0.92$ (**Figure 4B**).

No Differences in the Volume of Glomerular Components

We next measured the volume densities of the podocyte, capillary lumen, and mesangium components which were not different for the two groups, $P = 0.17$, 0.17, 0.27, respectively (**Figure 4C**). An average of 592 (range 387–1048) points for all the glomeruli/kidney were counted for determination of the volume densities of glomerular components. The volumes of the podocyte, capillary lumen, and mesangium components were calculated by multiplying the glomerular volume by the appropriate component volume density. There was no significant difference between the two groups, $P = 0.41$, 0.41, and 0.41 respectively (**Figure 4D**).

Increased Podocyte Foot Process Width in Cd2ap$^{-/-}$ Compared to Cd2ap$^{+/+}$

L_s (slit/GBM) was determined on the same glomeruli used to measure the volume of the glomerular components. An average of 230 (range 65–382) intercepts was counted from all the glomeruli per kidney for measurement of the foot process effacement and an average of 260 (range 99–696) slit diaphragm profiles was counted from all the glomeruli per kidney. L_s(Slit/GBM) was 0.0027 (0.0003) $\mu m/\mu m^2$ for the WT and 0.0009 (0.0003) $\mu m/\mu m^2$ for the *Cd2ap* KO mice. The difference between the two groups was statistically significant, $P = 0.02$ (**Figure 4E**). The values for foot process width were 374 (42) nm for WT mice and 1128 (286) nm for Cd2ap KO mice (**Figure 4F**). Since the foot process width is the reciprocal of the L_s (Slit/GBM) the *P*-value was the same.

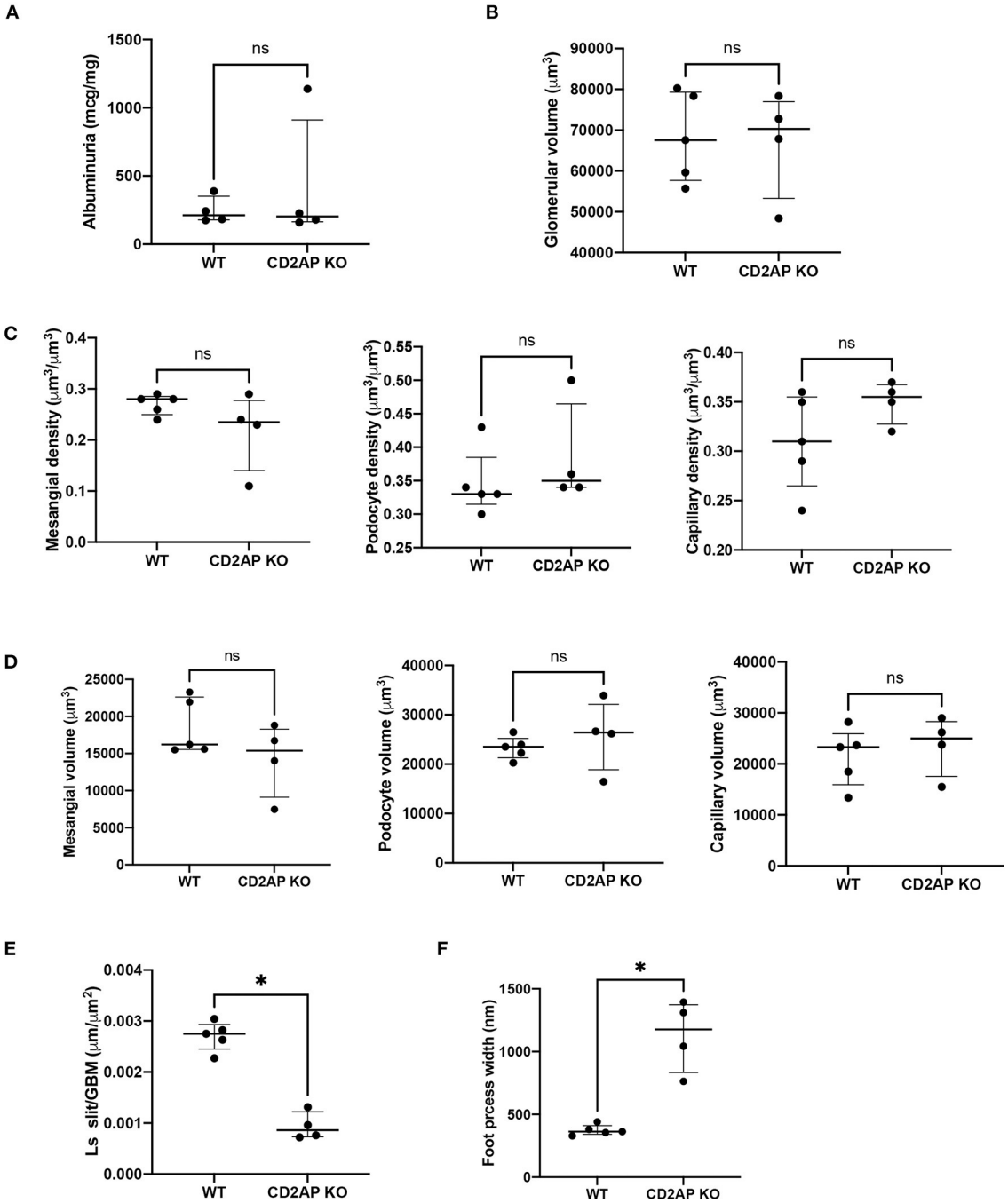

FIGURE 4 | Albuminuria and morphometric data. **(A)** Plot of albuminuria showing no difference between WT and CD2AP KO groups. **(B)** Plot of glomerular volumes showing no difference between WT and CD2AP KO groups. **(C)** Plot of glomerular component volume densities showing no difference between WT and CD2AP KO groups for mesangium, podocyte or capillary lumen. **(D)** Plot of glomerular component volumes showing no difference between WT and CD2AP KO groups for mesangium, podocyte or capillary lumen. **(E)** Plot of length density per GBM area of the slit diaphragm showing a statistical difference between WT and CD2AP KO groups. **(F)** Plot of foot process width showing a statistical difference between WT and CD2AP KO groups. $^{*}p = 0.02$.

DISCUSSION

This is the first study to report ACR and detailed kidney morphometric measurements for 2-week $Cd2ap^{+/+}$ and $Cd2ap^{-/-}$ mice. We showed similar results for ACR, volume of glomerular components but significantly increased podocyte foot process width in $Cd2ap^{-/-}$ compared to $Cd2ap^{+/+}$. The data indicates for the first time that podocyte injury is the initiating

event that subsequently leads to mesangial volume expansion and glomerular changes in proteinuric disease progression.

The development of albuminuria has been linked to changes in several structures of the glomerulus including the components that comprise the filtration barrier (33). The podocyte plays an essential role in establishing the filtration barrier which consists of slits between interdigitating podocyte foot processes surrounding the glomerular capillaries of fenestrated endothelium and glomerular basement membrane (34, 35). Injury to podocytes, resulting in podocyte effacement has been observed in several experimental albuminuric/proteinuric models induced by toxins and/or genetic mutations (36), such as mutations in slit-diaphragm associated proteins, including CD2AP (26). Maintaining the integrity of the filtration barrier is a critical part in maintaining overall renal function, but whether podocyte effacement preceded the development of expansion of mesangial volume, which has been shown to correlate with progressive renal dysfunction (37) during loss of excess albumin had not previously been examined. Understanding the sequence of events has important implications, particularly in underlying disease pathology, but also for therapeutic strategies. Thus, the use of detailed morphometric analyses, as conducted in this study was necessary to identify the chronology of structural changes that might predict albuminuria. Indeed, it is possible some albumin that crosses the barrier is absorbed by the proximal tubules and therefore doesn't appear in the urine but Oken (38) and colleagues showed that only a small amount of albumin is absorbed by the tubules.

We previously published that an early morphometric abnormality in CD2AP KO mice was glomerular volume expansion due to increases in the mesangial compartment at 3 weeks of age (25). This was an interesting finding since CD2AP expression in glomeruli is limited to the podocyte. Indeed, in that study, a reduction in podocyte number did not occur until week 5. Our findings here confirm the importance of CD2AP expression in maintaining podocyte integrity with CD2AP loss causing significant foot process effacement. Taken together, these results suggest that the loss of CD2AP in podocytes causes podocyte injury (identified by foot process effacement on electron microscopy), drives pathogenic podocyte-mesangial crosstalk, ultimately leading to glomerular volume expansion, podocyte depletion and progressive glomerular disease progression. Albuminuria occurs after podocyte foot process effacement, but before podocyte depletion and is first significantly increased at the time of glomerular volume expansion.

This study has its limitations. First, we did not distinguish superficial from juxtamedullary glomeruli. There is some evidence that though variable, morphologic changes related ischemia and hyperfiltration may be more severe in juxtamedullary glomeruli (39). Second, while we performed detailed glomerular morphometric analyses in this study as well as in our previously published work with older CD2AP KO mice (25), here we took the additional step of measuring foot process width. This enabled us to achieve our stated goal of defining the temporal relationship between the development of albuminuria and the development of podocyte foot process effacement. Third, depending on developmental stage podocytes possess a diverse repertoire of intercellular junctional components including tight, adherens and gap junctions (40). The slit diaphragm is a modified adherens junction (41). Under nephrotic conditions, tight junction complexes have been reported to appear at foot process interfaces to replace the diminished or displaced slit diaphragm (42). Our cellular junction analysis was limited to slit diaphragm length density per GBM area and did not include other cell-cell adhesion measurements. Finally, the sample size of 4 animals per group is small and limited to one glomerular disease model. Further studies will be needed to establish generalizability given the heterogeneity of proteinuric kidney disease.

While CD2AP is a disease causing gene for human FSGS, the temporal relationship between albuminuria and morphometric glomerular changes has implications beyond the CD2AP KO experimental model and genetic causes of nephrotic syndrome. Albuminuria is typically the first clinical sign that a patient has glomerular disease. Our data suggests that this could be a delayed finding after significant podocyte actin cytoskeletal injury has already occurred. Podocyte foot process effacement is a dynamic process that involves a redistribution of two actin networks: central actin bundles and the cortical actin network beneath the plasma membrane (43). Simplification and retraction of individual foot process, impaired adhesion properties and ultimately, detachment can result. The findings therefore highlight the need for assays that can better detect subclinical podocyte injury. A good example of this was recently shown in a small study where 6 of 8 patients with classic Fabry disease, but normal kidney function and no albuminuria were found to have podocyte foot process effacement on kidney biopsy (16). Foot process widening has also long been described in normoalbuminuric patents with type I diabetes (44). Since podocytes are terminally differentiated with a limited capacity to regenerate (45), with a critical reduction in podocyte number of >40% considered the "point of no return" beyond which proteinuric kidney disease progresses in a committed manner (5), early identification of podocyte injury is essential.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Icahn School of Medicine at Mount Sinai IACUC Committee.

AUTHOR CONTRIBUTIONS

JB, SN, and KC: conceptualization and supervision. JB: kidney morphometry. JW and JR: animal data collection and ELISA. JB, JW, JR, KC, and SN: manuscript draft writing, review, and

editing. All authors have read and agreed to the published version of the manuscript.

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frontiers in Medicine

REVIEW
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Mitochondrial Regulation of Diabetic Kidney Disease

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The role and nature of mitochondrial dysfunction in diabetic kidney disease (DKD) has been extensively studied. Yet, the molecular drivers of mitochondrial remodeling in DKD are poorly understood. Diabetic kidney cells exhibit a cascade of mitochondrial dysfunction ranging from changes in mitochondrial morphology to significant alterations in mitochondrial biogenesis, biosynthetic, bioenergetics and production of reactive oxygen species (ROS). How these changes individually or in aggregate contribute to progression of DKD remain to be fully elucidated. Nevertheless, because of the remarkable progress in our basic understanding of the role of mitochondrial biology and its dysfunction in DKD, there is great excitement on future targeted therapies based on improving mitochondrial function in DKD. This review will highlight the latest advances in understanding the nature of mitochondria dysfunction and its role in progression of DKD, and the development of mitochondrial targets that could be potentially used to prevent its progression.

Keywords: diabetic kidney disease, mitochondria, mitochondrial dynamics, oxidative phosphorylation, mitochondrial respiratory complexes, bioenergetics

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INTRODUCTION

The kidney contains a great diversity of cell types in order to perform all of its endocrine and exocrine functions. Importantly, several different cell types in the kidney must act harmoniously in diverse microenvironments for the kidneys to function properly. An early indication as to the importance of mitochondria to the kidney function derives not only from their relative abundance in the kidney, but also the relative distribution of mitochondria specific to the needs and function of the cell type of the kidney with mitochondria-rich cells predominantly distributed in highly metabolically active proximal tubular cells, while podocytes and tubular epithelial cells of thin limb of Henle and collecting ducts exhibit comparatively a lower number of mitochondria (1–5).

Mitochondria are organelles with an endosymbiotic origin critical to proper function of eukaryotic cells. Central to the diverse functions of mitochondria are their bioenergetics properties serving as "powerhouses" of the cell generating adenosine triphosphate (ATP), as well as playing key roles in producing intermediates metabolites, reactive oxygen species (ROS) production, calcium homeostasis and apoptosis (**Figure 1**). As the most important physiological system for producing chemical energy stored as ATP from glucose, it is not surprising that mitochondria gained early attention as a possible target of diabetes and its micro/macrovascular complications.

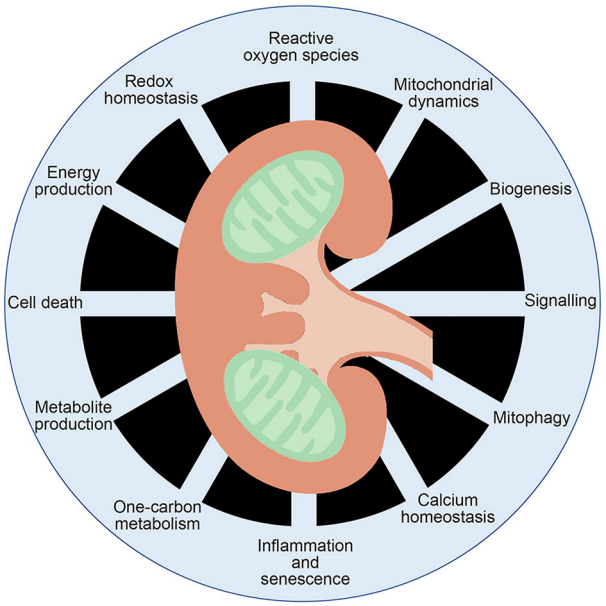

FIGURE 1 | Multifaceted functions of mitochondrial function. Mitochondria are known to generate ATP and metabolites critical for signal transduction, as well as playing key roles in inflammation, calcium homeostasis, redox homeostasis and cell death.

The time course of mitochondrial dysfunction in the kidney has been documented in several experimental models of diabetic kidney disease (DKD) (6, 7). For instance, it was found that mitochondrial changes in size and function preceded histological and biochemical changes associated with kidney damage and these mitochondrial changes evolved with DKD progression (6). Indeed, altered mitochondrial morphology, bioenergetics and increased mitochondrial transition pore opening and ROS were all apparent prior to the presence of albuminuria (6, 8–12). These results suggest that mitochondrial dysfunction could be contributing to diabetic associated kidney damage.

Direct evidence that mitochondrial dysfunction can be a cause of chronic kidney disease (CKD) and DKD can also be gleaned by evaluating renal function in the presence of known mutations of mitochondrial associated proteins. The evidence is strengthened by several studies evaluating mutations in mitochondrial associated proteins that led to kidney dysfunction (13–21). Several independent mutations, relevant to mitochondrial function, result in kidney dysfunction, including prenyl diphosphate synthase subunit 2 (PDSS2) (22–24), mitochondrial inner membrane protein (Mpv17) (25), required for meiotic nuclear division 1 homology (RMND1) (26–30), ATP-binding cassette A1 (ABCA1) (12), apoptosis-inducing factor 1 (AIF1) (31), and several mitochondrial tRNAs (32–36). Podocyte-specific knockout of pdss2 further suggested the possible cell type specific consequences of some of these genes since it resulted in podocyte-associate renal disease. However, kidney damage was not apparent with conditional knockout of pdss2 in tubules, monocytes, or hepatocytes (22, 23). Podocyte knockout of ABCA1 was also shown to predispose the mice to DKD (12). Altogether, the evidence suggests that mitochondrial dysfunction can be a driving and primary cause of CKD and DKD, potentially playing an intrinsic and early role in disease progression. However, despite much interest, the precise nature of the changes to mitochondria and its physiological or pathophysiological significance remains elusive in DKD.

Mitochondrial Function and DKD Progression

Due to the diverse pathways ascribed to mitochondria, there is not a single means to determine their function nor single biochemical assay to define their "health." However, due to their classically assigned and pivotal role in energy production, many investigators have evaluated alterations in mitochondrial respiratory complexes, oxygen consumption rates, and/or ATP production as "proxy" for mitochondrial dysfunction with DKD progression. The oxygen consumption rate (OCR) measurements in the early phase of DKD (1–4 weeks after diabetic induction) in animal models indicated that metabolic activity was increased in renal cortex and proximal tubular cells (37–40), but subsequently declined with progression of albuminuria in experimental models of DKD (41). This would seem to be consistent with reports of increased respiratory complex activities in early phases of DKD (42, 43). However, other studies report contrasting results indicating decreased mitochondrial respiratory complex activities likely representing later stages of DKD (44–48). Similarly, while ATP levels within the kidney cortex have frequently been found unchanged during progression of DKD (49, 50), significantly lower levels of ATP have also been reported in other studies (6, 51). Our interpretation of these studies is that these results may indicate a compensatory increase in mitochondrial respiration early in DKD which is lost during progression of DKD.

The observations on mitochondrial function during DKD progression focusing mainly on tubular cells seem to be in contrast to the glomerular region of kidney cortex. Since glomerular cells are not as mitochondrial rich as tubules, the reduction in mitochondrial densities may allow for enhanced metabolic plasticity in these cells. Indeed, a number of studies seem to indicate that glomerulus and specifically podocytes have decreased OCR or metabolic activity from early onset of DKD which persist with progression of DKD (9, 52–54). In support of these observations, other reports suggest that mitochondrial respiratory complex activity is also decreased early on with DKD (45, 55, 56). The effects on ATP levels, however, are less clear. While several reports seem to indicate that ATP in podocytes is decreased (6, 9, 52), others have reported no major or little change (49, 50). These paradoxical results are not unexpected since podocytes have been reported to readily utilize glycolysis, possibly exhibiting a more flexible approach to ATP production (3). The inherent tissue differences in mitochondrial number and function highlights some of the limitations in our ability to complete a wholistic picture.

While mitochondria have been clearly demonstrated to be important players in the development and progression

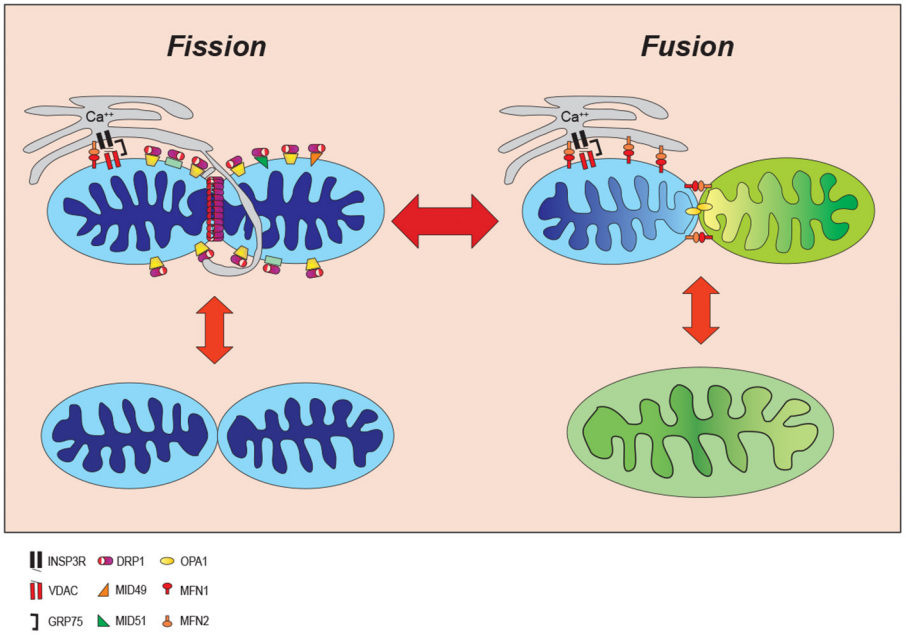

FIGURE 2 | Mitochondrial dynamic. Mitochondria continuously change their size and shape by two opposing processes: mitochondrial fission and fusion. During the mitochondrial fission, mitochondria become fragmented in response to cell stress whereas they form an elongated shape increasing ATP production to adjust to cellular stresses. INSP3R, Inositol trisphosphate receptor; DRP1, dynamin-related protein 1; OPA1, optic atrophy 1; VDAC, voltage-dependent anion channel; MID49/51, mitochondrial dynamics proteins of 49 and 51kD; MFN1/2, mitofusin proteins 1 and 2; GRP75, glucose-regulated protein 75.

of DKD, the intricacies and nature of their dysfunction is not fully understood (57, 58). We and others have reported enhanced mitochondrial fission, increased mitochondrial ROS, and decreased oxidative phosphorylation (OXPHOS) in mouse models of DKD, whereas others have reported conflicting results. It is unclear if differing reports are due to different means of diabetic induction in animal models, the renal cell type examined, species-specific differences, or time of observation in the disease process. It will be an important future goal to reach a consensus on these questions. We will further highlight some of the current knowledge and possible gaps in defining the nature of mitochondrial dysfunction in DKD.

Role of Mitochondrial Dynamics

Mitochondrial dynamics are the processes by which mitochondrial length, shape, and size are determined (59–61). Mitochondria have variable morphologies, even within the same cell, depending on the cell type, cellular needs and signaling cues. In its most basic and rudimentary understanding, mitochondrial morphology appears to be regulated by an ever changing and antagonistic intracellular balance between mitochondrial fission factors and mitochondrial fusion factors (62).

During mitochondrial fission, a mitochondrion is constricted to effectively divide a larger parent mitochondrion into smaller daughter organelles (59, 60). Mitochondrial fusion is the opposite process whereby smaller mitochondria have the outer and inner membranes joined to create a larger mitochondrion (59, 60). The balance between these factors of opposing action ultimately imparts characteristic size and shape of the mitochondria in a tissue-specific manner (**Figure 2**). Metabolic demands and signaling cues in a cell's microenvironment can push the balance toward mitochondrial fission to generate more fragmented and spherical mitochondria, or conversely toward mitochondrial fusion generating a more tubular and elongated morphology. Since this fluid process provides cells with rapidly responding metabolic flexibility, it is not surprising to realize that mitochondrial dynamics is highly regulated through a spatio-temporally precise cooperation among mitochondria, cytoskeleton, endoplasmic reticulum, and resident and recruited mitochondrial-associated proteins (11, 63–67).

While mitochondrial fission can be viewed as a process of sequential discrete steps, the order and independence of each step remains to be fully understood. An early step is marking of the site where the mitochondria will divide. The current model suggests that the endoplasmic reticulum (ER) initially marks fission furrows in the mitochondria where mitochondrial fission will ultimately occur (65, 66, 68). Increases in cytoplasmic calcium drive actin assembly around the ER protein inverted formin 2 (INF2) and the actin polymerization is believed to provide some force for constriction (64, 67). The association of INF2 with mitochondrial localized Spire 1C, links the mitochondrial, actin polymerization event between the two organelles (mitochondria and ER) (69) and will enhance calcium transfer from ER to mitochondria via mitochondrial calcium uniporter 1 (MCU1) initiating constriction of the mitochondrial inner membrane prior to outer membrane constriction in a process which requires activation of the electron transport complexes and the mitochondrial metalloendopeptidase, OMA1

(70). Mitochondrial fission will further proceed by recruitment of the cytoplasmic fission factor, dynamin-related protein 1 (DRP1) to the outer mitochondrial membrane (71–74). DRP1 is recruited to the mitochondrial outer membrane where it oligomerizes to form a ring around the mitochondria at the fission furrow. DRP1 is anchored to the mitochondria by interactions with its mitochondrial receptors including mitochondrial fission 1 (FIS1), mitochondrial fission factor (MFF), and mitochondrial dynamics proteins of 49 and 51 kD (MiD49/MiD51). Constriction of the mitochondrial membrane utilizes DRP1-driven GTP-hydrolysis for energy to drive mitochondrial fission.

DRP1 translocation to the mitochondria is further regulated by several posttranslational modifications including phosphorylation (52, 75–80), *O*-GlcNAcetylation (81), sumoylation (82–84), and S-nitrosylation (85–87). DRP1 activation is also enhanced by binding with actin (88), actin-related proteins (11, 89), AKAP1 (80), cardiolipin, and palmitic acid (90–92).

Mitochondrial fusion, on the other hand, is mediated by another dynamin related protein, optic atrophy 1 (OPA1), at the mitochondrial inner membrane and mitofusin proteins 1 and 2 (MFN1 and 2) at the outer mitochondrial membrane. MFN1/2 can interact both as homo- and hetero-dimers to mediate fusion of the outer mitochondrial membrane. OPA1 appears to be regulated in part by post-translational changes driven by the mitochondrial membrane potential and interactions with the mitochondrial OMA1 zinc metallopeptidase. In addition, to its function in mitochondrial fusion, OPA1 also plays a key role in maintaining mitochondrial cristae morphology and respiratory ETC function by sequestering cytochrome c within the cristae. The importance of these mitochondrial dynamic protein factors to life is evidenced by findings that knockout of several member proteins is embryologically lethal (93–96).

Enhanced mitochondrial fission is reported in multiple cell types of the kidney including tubules and podocytes in animal models of DKD (1, 11, 52, 97–103). In support of these preclinical studies, clinical evidence have revealed increased fragmented mitochondria in several cell types within the kidney cortex of diabetic patients as well (99, 104, 105). Our studies in the *db/db* model of DKD identified enhanced mitochondrial fission and increased expression of DRP1 in both glomerular endothelial cells and podocytes (8). Importantly, while podocyte-specific depletion of DRP1 had no effect on mitochondrial function, DRP1 deficiency specifically in podocytes in diabetic *db/db* mice improved DKD progression by improving mitochondrial function suggesting a role for cellular stress to unravel the effect of DRP1 on mitochondrial function (52). The tendency toward mitochondrially fragmented morphology has been tied most strongly to several proteins regulating mitochondrial fission (11, 52, 74, 80, 100, 106, 107). Other studies have confirmed these initial observations in other models of DKD. For example, Myo-inositol oxygenase (MIOX) expression was shown to be increased in kidneys of *db/db* mice and streptozotocin (STZ)-treated diabetic mice contributing to progression of DKD, and linked to enhanced DRP1 and FIS1 expression with decreased MFN2 expression (98, 108). The Src homologous-collagen homolog adaptor protein, p66Shc, expression and phosphorylation were also increased in kidneys of both *db/db* mice and STZ-treated diabetic mice, and were found to correlate with increased DRP1 and FIS1 expression and decreased MFN1 expression (99, 109). Knockdown of *Fis1* prevented mitochondrial fragmentation, restored MFN1 expression, and reduced p66Src binding to FIS1 under high glucose conditions (99). Dual-specificity protein phosphatase−1 (DUSP1) was shown to be decreased and JNK pathway activation increased in the kidneys of STZ diabetic mice and linked to increased DRP1 and MFF expression with decreased MFN1 and OPA1 expression (101). Finally, the expression of hypoxia inducible factor 1 (HIF1) was conditionally deleted in proximal tubular cells of STZ treated diabetic mice showed enhanced DKD progression with increased expression of DRP1 and FIS1 with decreased MFN1 expression. *In vitro* it was suggested that HIF1 modulates these changes by its target heme oxygenase-1 (HO-1) (110).

Post-translational modifications of DRP1 and specifically its phosphorylation also seem to play a critical role in pathogenesis of DKD. We and others have found that DRP1 phosphorylation at the human residue S637 and equivalent mouse residue serine 600 (S600 in mouse DRP1 isoform b), hereafter referred to as S600, enhances DRP1 activity and translocation to the mitochondria to mediate enhanced mitochondrial fragmentation (8). We have shown that Rho-associated, coiled-coil containing protein kinase 1 (ROCK1) activation in the diabetic kidney phosphorylates DRP1 at S600 both *in vivo* and *in vitro* triggering mitochondrial fragmentation (8). Recently, it was shown that S600 of DRP1 in renal tubules maybe phosphorylated by the compartment directing, A kinase (PRKA) anchor protein 1 (AKAP1), localizing protein kinase A (PKA) to the outer mitochondrial membrane and triggering mitochondrial fragmentation in a STZ model of type-1 diabetes (80). We also provided *in vivo* evidence indicating that knock-in diabetic *db/db* mice mutating S600 in DRP1 to the non-phosphorylable alanine at position 600 (S600A) exhibited marked improvement in DKD progression and protected mitochondrial morphology and bioenergetics of podocytes. Mechanistically, it was shown that phosphorylation of DRP1 at S600 enhanced its interaction with both MFF and the actin related protein 2/3 complex (ARP2/3) enhancing mitochondrial localization of DRP1 and triggering mitochondrial fission (11). Similarly, it has been reported that phosphorylated DRP1 was increased and the expression of MFN1 markedly decreased in proximal tubular cells isolated from *db/db* mice, while treatment of diabetic mice with a β2-agonist, formoterol, decreased phosphorylated DRP1 levels and restores MFN1 levels (51).

While there is a growing body of evidence indicating that mitochondrial fission is a key morphological indicator of kidney damage in DKD, hyperfused and large mitochondria may also have a role in DKD progression (111). Hyper-elongated mitochondria may be an indicator of cellular senescence and associated with mitochondrial DNA damage, loss of mitochondrial membrane potential, and enhanced ROS as well (112–115).

Overall, the evidence seems to indicate that renal damage in DKD is associated with a shift in mitochondrial dynamics

toward enhanced fission. The evidence is clear that DRP1 lies at the center of this dynamic and has been found to be increased and/or modified in multiple kidney cell types. These changes are frequently found in conjunction with increased expression of fission proteins such as FIS1 and MFF and decreased expression of MFN1. The functional consequences of tipping the mitochondrial dynamic balance toward fission seem to share deleterious end points such as enhanced ROS contributing to DKD progression.

Mitochondrial Bioenergetics and Oxidative Stress

Cellular ATP is maintained through two interconnected metabolic pathways, glycolysis and oxidative phosphorylation (OXPHOS). During glycolysis, glucose is transported into the cell cytoplasm and converted into 2 molecules of pyruvate to generate 2 ATP molecules. In the absence of oxygen, glycolysis will anaerobically ferment the pyruvate to lactate generating 2 NADH in the cytoplasm. However, in the presence of oxygen, pyruvate will be decarboxylated into acetyl coenzyme A (AcCoA) inside mitochondrial matrix and enter the tricarboxylic acid (TCA) cycle. The TCA cycle is an enzymatically controlled series of oxidation steps culminating in production of CO_2 and 8 NADH, 2 $FADH_2$, and 2 ATP molecules. Ultimately OXPHOS can harvest 30–36 ATP from the entry of the NADH and $FADH_2$ per glucose depending upon the amount of proton leak.

OXPHOS is comprised of 4 respiratory complexes (I-IV) within the inner mitochondrial membrane which are collectively referred to as the electron transport chain (ETC) in which a series of redox reactions are converted into a proton motive force by pumping protons into the mitochondrial intermembrane space (**Figure 3**). Complex I accepts electrons from NADH while complex II accepts electrons from $FADH_2$ and both transfer the electron to coenzyme Q (CoQ). Complex III in conjunction with cytochrome c can accept these electrons and pass them to complex IV with oxygen as the terminal electron acceptor. Complex V or ATP synthase allows passage of protons back to the matrix linked to generation of ATP. Electron escape during ETC reactions is capable of generating ROS which under physiological conditions are both quenched by endogenous antioxidant mechanisms and utilized as important cellular signaling molecules. It has been suggested that increased ROS generation and decreased ROS quenching result in oxidative damage to cellular components and mitochondria capable of resulting in cell death. This apparent paradox may exist with low levels of ROS serving as survival signaling during conditions of stress while once reaching a threshold become damaging to the cell and synergistically contribute to enhanced mitochondrial dysfunction.

Superoxide production during ETC transport was first reported in 1966 (116), and has been an area of interest ever since. Complex I (117–120) and complex III (118, 121–124) are believed to be the principle sites of mitochondrial ROS generation during ETC transport, of which complex I is believed to produce the majority of mitochondrial ROS (122, 125–127). Complex III can produce both intermembranous and matrix superoxide during transport of electrons through the quinol (Q)-cycle depending on the mitochondrial membrane potential and oxidation state of cytochrome c (122, 123). Complex I can produce superoxide by two distinguishable mechanisms. When the $NADH/NAD^+$ ratio is high and respiratory chain activity is inhibited, the matrix facing flavin mononucleotide (FMN) site can produce superoxide (119, 128–130). Alternatively, superoxide can be generated when mitochondrial potential drives reverse electron transport at complex I. Reverse transport occurs when the mitochondrial potential is high and CoQ is reduced forcing the reduction of NAD to NADH at the flavin mononucleotide (FMN) site (121, 131–133). In DKD, it has been demonstrated that both complex I (134) and complex III (135) can generate superoxide and increased mitochondrial ROS in the kidney (136–138).

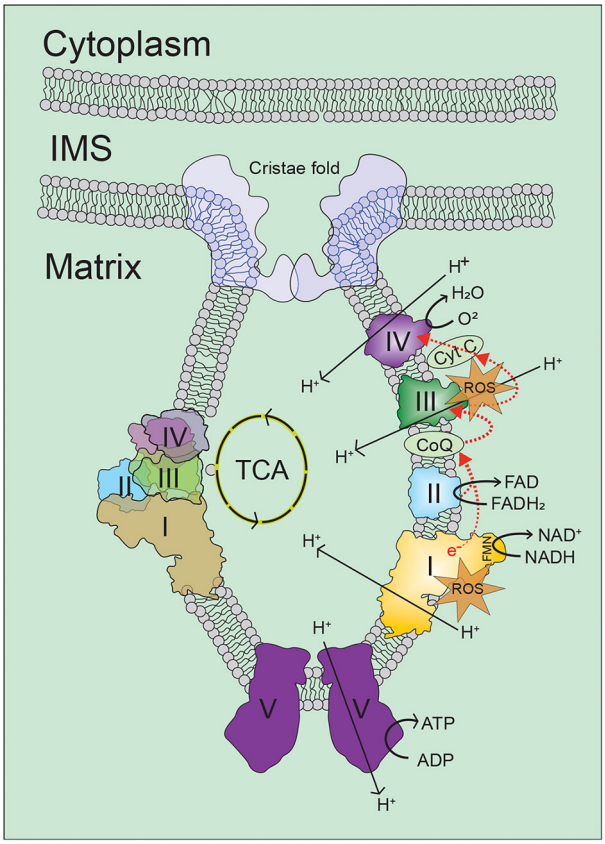

FIGURE 3 | Oxidative phosphorylation. Cellular energy in the form of ATP is mainly generated in mitochondria by the oxidative phosphorylation (OXPHOS) process, in which electrons on the inner-membrane of the mitochondria are passed through a series of mitochondrial complexes (Complexes I-V) in redox reactions. Energy released in these reactions is then coupled to ATP generation. Increase in intracellular levels of NADH and FADH2 drives oxidative phosphorylation, which leads to increase of oxygen consumption and ATP production by ATP synthesis. For more information, refer to the main text. ROS, reactive oxygen species; TCA, tricarboxylic acid; I/II/III/IV/V, mitochondrial respiratory complex I/II/III/IV/V; NADH, reduced nicotinamide adenine dinucleotide; NAD, nicotinamide adenine dinucleotide; $FADH_2$, reduced flavin adenine dinucleotide; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; CoQ, coenzyme Q; Cyt C, cytochrome C.

Transgenic expression of superoxide dismutase or thioredoxin protected the kidney in mouse models of DKD (139, 140). However, not all antioxidants were equally effective as transgenic glutathione peroxidase-1 expression in STZ-treated mice did not have renal protection (141).

Substantial evidence has accumulated in patients and animal models of DKD indicating that mitochondrial ROS is significantly increased in the kidney and generated the free radical theory of diabetic microvascular complications (142–144). The "Unifying Hypothesis" suggests that chronically driven glucose over production of mitochondrial ROS at the mitochondria leads to cellular and eventual end kidney failure. Increased mitochondrial ROS production has been demonstrated both *in vitro* and *in vivo* in multiple mouse models of DKD (39, 41, 56, 100, 109, 145–148). However, an important gap in our current understanding of the role of mitochondrial ROS in DKD pathogenesis is to identify the source(s) of enhanced mitochondrial ROS in DKD. The increased mitochondrial ROS production was initially proposed has been proposed to be linked to mitochondrial dynamics remodeling and biogenesis (149). This suggestion was supported experimentally by some recent studies indicating that overexpression of DRP1 or MFF, as well as knockdown of MFN1/2 together or alone in cultured cells, lead to mitochondrial fragmentation and increased mitochondrial ROS (149–154). Increased expression of p66Shc, NR4A1, ROCK1/DRP1, and HIF1 (hypoxia inducible factor 1) in DKD also caused fragmented mitochondria and increased mitochondrial ROS and apoptosis (8, 98, 99, 101, 110, 155). Decreased expression of DUSP1, MIOX, or PGC1α in the DKD were similarly reported to increase mitochondrial ROS and apoptosis (98, 101, 145). Increased production of mitochondrial ROS appears to be a central effector of cellular damage, but is inherently difficult to measure mitochondrial ROS *in vivo* due to their multiple species and frequently very short biological half-lives. Indeed, a central challenge in addressing the role of redox biology in DKD progression is to accurately measure mitochondrial ROS. Importantly, studies addressing mitochondrial ROS have resulted in conflicting interpretations mainly because of variations in the detection methods employed with a wide range of experimental approaches, including the use of fluorescent indicators of ROS, electron paramagnetic resonance (EPR), spectrophotometry, and high-performance liquid chromatography (HPLC); each method with its own limitations and advantages and generally specific to the ROS molecule attempting to be measured, cross reactivity, cellular permeability and localization.

We have recently used a transgenic, redox-sensitive GFP based biosensor specifically expressed in the mitochondrial matrix to determine mitochondrial generated ROS in a *db/db* mouse model *in vivo* (56). Kidney from transgenic control and diabetic mice were examined by 2-photon microscopy followed by ratio-metric determination of the redox state of the biosensor. Increased mitochondrial ROS in the diabetic kidneys was found which strongly implicated complex I as a key generator as the biosensor was matrix localized and the increase in ROS was prevented by a genetic bypass of complex I. This report and others have utilized mitochondrial targeted antioxidants such as mitoTEMPO, elamipretide, and others to demonstrated reduced mitochondrial ROS correlating with improved histological features of DKD in mouse models (12, 56, 156, 157).

Evidence determining mitochondrial ROS in the kidney of diabetic mice has also been obtained using dihydroethidium (DHE) as the redox sensor (45). Results in these mice were in contrast to the previous studies describing decreased mitochondrial ROS in the diabetic kidney. However, both studies were in agreement in regards to decreased activity of mitochondrial respiratory chain activity and found evidence of oxidative stress in the kidneys of diabetic animals (45, 56). These contrasting findings might be indicative of the difficulty in interpretating the cross-talk among different sources of ROS production (45, 56, 100). One such point of cross-talk in DKD could be derived from NADPH oxidases pathway. The NADPH oxidases of the NOX family are important enzymatic sources of ROS whose main biological function is electron transport across the plasma membrane and generate ROS by reducing oxygen to superoxide and/or hydrogen peroxide (158). At least seven homologs of NOX are present in the human genome: NOX1 to NOX5, DUOX1, and DUOX2. These mainly differ in their activation mechanisms, tissue distribution, and type of ROS production (157). Among different members of NOX family, NOX4 expression has been shown to be increased in the kidneys of diabetic mouse models, and capable of producing different types of ROS, mainly hydrogen peroxide (45, 159–163). However, under stress conditions, NOX4 might be translocated to mitochondria contributing to enhanced mitochondrial ROS by regulating mitochondrial respiratory complex I activity (164, 165). Consistent with this notion, deletion and pharmacological inhibition of NOX4 have been demonstrated to attenuate progression of DKD (161). NOX5 is also increased in the human diabetic kidney but not encoded by the mouse genome. Nevertheless, it has been shown that forced ectopic expression of NOX5 in mouse models leads to accelerated progression of DKD which could be ameliorated by pan-NOX inhibitors (161, 166–168).

While the specific contribution of mitochondrial generated superoxide remains an open question, it is clear that there is enhanced ROS in the kidneys of diabetic mouse models probably arising from multiple sources. The generated ROS is usually carefully balanced to stimulate stress abrogation responses, while not exceeding the cell ability to protect itself from damage through anti-oxidant enzymes. Once the balance is shifted such that the production of ROS exceeds the cells inherent antioxidant protections, an increasing cycle of cell damage is elicited resulting in compromised mitochondrial function, damaged mitochondrial DNA and proteins (169). If the cell cannot re-establish its balance, the end result is cellular death and kidney dysfunction.

Mitochondrial Biogenesis and Mitophagy

Mitochondrial biogenesis and degradation are highly regulated in order to maintain a healthy pool of mitochondria in the cell. However, both of the processes are dysregulated in DKD. Mitochondrial biogenesis refers to the cellular regulation of

mitochondrial abundance titrated through an interconnected set of transcription factors. Central among these transcription factors is the peroxisome proliferator-activated receptor gamma (PPAR) coactivator-1 family of transcriptional coactivators (PGC1α/β) and PGC-1-related coactivator (PRC), coined as "master regulators" of mitochondrial biogenesis.

PGC1α was initially identified by the Spiegelman group (170) as a binding partner of PPAR that is highly expressed in tissues with high energy demand such as the kidney. As a coactivator, PGC1 does not bind to DNA promoters directly, but in dimerization with a variety of transcription factors to modulate a series of mitochondrial active gene products (171, 172). A few of the better understood partners of PGC1α include nuclear respiratory factor 1 (NRF1), NRF2, and the estrogen-related receptors (ERR). These heteromeric dimers likely, at least in part, could explain why experimental results with modulating PGC1α appear so highly tissue-specific since the possible dimeric combinations and relative amounts could depend on a specific tissue's expression levels of PGC1α, its various binding partners, and posttranslational modifications. Regardless, the system allows for a high degree of specialization in the regulation of gene products impinging upon mitochondrial biogenesis, mitochondrial gene transcription, fatty acid oxidation, TCA cycle, and OXPHOS. The role of PGC1α as a transcriptional rheostat tuning metabolic cellular function to physiological energy demands has been experimentally demonstrated in a myriad of tissues.

A number of studies have provided strong evidence that decreased PGC1α and reduced mitochondrial biogenesis are key features in the development of DKD. PGC1α has been demonstrated to be significantly decreased in the diabetic kidneys (9, 45, 145, 173–175). STZ treated rats have decreased PGC1α in renal tubules. This is evident in several mouse models of DKD as well. Diabetic OVE26, AKT2, and *db/db* mouse models have all been illustrated to have decreased PGC1α in the kidneys (176, 177). PGC1α was demonstrated to play a key role in another study examining an enzyme believed to couple glycolysis to mitochondria bioenergetics, pyruvate kinase M2 (PKM2) (10). In this study, podocyte-specific depletion of PKM2 in diabetic mice exacerbated diabetic renal injury, while pharmacological activation of PKM2 protected diabetic mice from kidney damage. Importantly, increased levels of PKM2 were correlated to protection from DKD in diabetic patients. The underlying mechanism proposed was that the protection was due in part to PKM2 linked activation of PGC1α and improved mitochondrial function (10, 178).

Our group has demonstrated that PGC1α could also be regulated by a long non-coding RNA, Tug1 (taurine upregulated 1). We found that Tug1 overexpression protects *db/db* mice from DKD (9). The protection was linked *in vitro* to Tug1 binding to PGC1α and improved mitochondrial function. However, another report found that podocyte-specific inducible overexpression of PGC1α in mouse models of DKD failed to offer renal protection (179). High expression levels of PGC1a could potentially drive a mitochondrial substrate preference toward b-oxidation of lipids contributing to worsening phenotype of DKD in experimental models. These results may indicate that PGC1α levels must be regulated and maintained within a very limited range to be beneficial.

PGC1α offers renal protection, at least in part, by driving oxidized nicotinamide adenine dinucleotide (NAD+) biosynthesis (180, 181). The redox imbalance of NADH/NAD^+ (reduced/oxidized) is high in the diabetic kidney as electrons from the breakdown of nutrients become stored as NADH and metabolic pathways such as sirtuins consume NAD^+. Complex I or lactate dehydrogenase can then regenerate NAD^+ through oxidation of NADH (182–185). Modulation and the end balance of these processes determine the NADH/NAD^+ ratio and represents one intersection point of PGC1α with sirtuins in mitochondrial biogenesis and bioenergetics.

The family of NAD-dependent deacetylases known as Sirtuins (SIRT1-7) regulate mitochondrial biogenesis and function as a nutritional rheostat which effects mitochondrial function via protein acetylation and have been implicated in several pathologies, including DKD (186–189). Proximal tubular overexpression of SIRT1 protected diabetic mice from DKD. Knockout of SIRT1 exacerbated renal injury in two separate diabetic mouse models and induced albuminuria in non-diabetic animals (190). The SIRT1 agonist, resveratrol, reduced podocyte damage in diabetic mice by activating PGC1α as well as its targets NRF1 and mitochondrial transcription factor 1 (TFAM) to improve mitochondrial function and reduce oxidative stress. SIRT1 has been shown to play a protective role in both tubules and podocytes of diabetic mouse models. The renoprotection stems in part through deacetylation of transcription factors, including PGC1α and PPARy (181, 191, 192). Podocyte-specific overexpression of SIRT1, and several non-specific agonists of SIRT1 such puerarin have been shown to attenuate DKD in animal models (193, 194). A more specific agonist, BF175, was tested and was shown to protect the kidney in type 1 diabetic OVE26 mice (195).

Consistent with the interplay between PGC1α and SIRT1, it has been shown that PGC1α can also increase levels of the mitochondrially-localized, SIRT3 (175, 196, 197). SIRT3 has been demonstrated to regulate mitochondrial function through direct binding to ETC proteins, mitochondrial dynamics, redox protection, and TCA cycle modulation and is the main mitochondrial deacetylase regulating cellular ROS. The SIRT3 agonist, honokol, was tested in BTBR *ob/ob* mice with type 2 diabetes and determined to be protective in DKD (186). SIRT3 was determined to be significantly decreased in the kidney of BTBR *ob/ob* mice in conjunction with increased ROS levels. Treatment with Honokol, a Magnolia tree bark extract and SIRT3 activator, reduced albuminuria and podocyte injury in the diabetic mice and was found to restore PGC1α levels in glomerular cells. The protective role of SIRT3 on glomeruli was mediated in part through increased SIRT3 tubular expression and upregulation of tubular nicotinamide phosphoribosyl transferase (Nampt), suggesting a possible tubule-glomerulus retrograde signaling mechanism. The lack of regulation of SIRT3 in glomeruli and postulated tubular-glomerular signaling was also a finding of a study examining SIRT1 in the diabetic kidney (190) where diabetic glomerular damage was improved by selective upregulation of tubular SIRT1.

In contrast to mitochondrial biogenesis, the process of mitophagy is the physiological clearance mechanism for removal of damaged mitochondria from the cell which appears to become overwhelmed in DKD (198–200). Mitophagy appears to have both a ubiquitin-dependent and -independent pathway (201, 202). The ubiquitin-dependent pathway is dependent upon mitochondrial dynamics, energetics, transport, and autophagic factors. The phosphatase and tensin homolog (PTEN) induced putative kinase 1 (PINK1) and Parkin (PRKN) are key mediators of the pathway. Physiologically PINK1 is transported to the inner mitochondrial membrane and proteolytically degraded in a ubiquitin dependent manner. When mitochondria become damaged and depolarized PINK1 is autophosphorylated and stabilized on the outer mitochondrial membrane to recruit PRKN and its E3 ligase activity. Mitochondrial fate is determined by the balance of the ubiquitination/deubiquitination process whereby increased poly-Ub targets the mitochondrion for proteasome destruction. PINK1 can increase mitochondrial fission by indirectly increasing DRP1 activity while the PINK1/PRKN interaction enhances Mfn2 degradation (203–210). Ubiquitin-independent pathway involves several ubiquitin E3 ligases which can localize to mitochondria and recruit autophagic factors.

The kidney has been shown to have a high rate of mitophagy relative to other organs, as well as cell type dependent regulation where podocytes have greater levels of mitophagy relative to tubules (211, 212). Increased mitophagy has been shown to be protective in models of CKD, DKD, and AKI (213–220). The PINK1/PRKN pathway is activated by oxidative stress established in DKD whereas treatment with the mitochondrial antioxidant, MitoQ, has been suggested to protect from DKD by increasing mitophagy levels (218). Tissue-specific knockout of ATG5 in a STZ model of DKD revealed that podocyte deletion induced podocytopathy and glomerulosclerosis while endothelial-specific knockout accelerated progression of DKD and when deleted in both tissues together increased DKD (221). These observations in aggregate suggest a critical role of mitophagy in DKD progression.

CONCLUSION AND FUTURE PERSPECTIVES

In this review, we touched the surface of several possibilities by which mitochondrial dysfunction could contribute to the development and progression of DKD, but we recognize that there still much remains to be uncovered. We would like to underscore a few gaps in knowledge for future discoveries.

As of yet, it is difficult to reach a clear consensus on the time course of mitochondrial respiratory activity and OXPHOS changes during progression of DKD. We await the arrival of more specific bioreporters to evaluate specific sources of enhanced ROS in real time in living animals, which ideally could link ROS to their enzymatic source in mitochondria. Similarly, a complete understanding of how mitochondrial dynamics fidelity is regulated and an evaluation of the "coincident detection" to fully integrate multiple organelles and biological factors into a single framework remains to be fully accomplished. The mitochondrial biogenesis pathway, and PGC1α in particular, are attractive therapeutic targets for DKD, but likely await the ability of targeting this pathway selectively in the kidney within a narrow therapeutic window. Finally, mitophagy, the crossroads of diverse signaling pathways, has shown a great promise as a therapeutic target, but the molecular mechanisms by which mitochondrial packaging for mitophagy becomes uncoupled during DKD progression remains unclear.

In conclusion, there have been increasing efforts to better define the nature of mitochondrial dysfunction in DKD over the past two decades. Initial studies utilizing metabolic screening approaches to identify the best possible biomarkers for predicting DKD susceptibility and progression are currently on-going (222, 223). While these and other studies have identified several mitochondrially-derived molecules such as mitochondrial DNA in serum and/or urine as potentially useful markers for DKD progression, none has exceeded expectations and are not currently available for patients care. Looking forward, opportunities in mitochondrial medicine involve the use of "multi-omics" and proteogenomics to provide further insights into the role of mitochondrial biomarkers in predicting DKD progression. A quantitative assessment of mitochondrial dysfunction in patients with DKD could accelerate the identification and development of novel biomarkers and treatments, and improve the ability to assess the efficiency of new drugs by measuring mitochondrial function pre- and post-therapies. Finally, the genetic and hormonal environment of the male and female kidney is significantly different, and these differences have been implicated on the onset and progression of DKD in both Type1 and 2 diabetes (224–227). The impact of gender on mitochondrial bioenergetics and function in kidney diseases has recently been reported (228). While many questions still remain to be carefully addressed, it seems clear that sexually determined differences in mitochondrial biogenesis, bioenergetics, and ROS generation exist, and these differences may also contribute to differences in long-term prognosis in patients with DKD (229, 230). Further research is needed to conclude a causal association between differences in gender, mitochondrial dysfunction and progression of kidney disease in large diabetic population.

AUTHOR CONTRIBUTIONS

DG is responsible for writing the manuscript and literature research. KM and FD reviewed the manuscript and made significant revisions on the drafts. All authors have read and agreed to the final version of the manuscript.

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Crosstalk Between SMPDL3b and NADPH Oxidases Mediates Radiation-Induced Damage of Renal Podocytes

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Patients undergoing radiotherapy (RT) for various tumors localized in the abdomen or pelvis often suffer from radiation nephrotoxicity as collateral damage. Renal podocytes are vulnerable targets for ionizing radiation and contribute to radiation-induced nephropathies. Our prior work previously highlighted the importance of the lipid-modifying enzyme sphingomyelinase acid phosphodiesterase like 3b (SMPDL3b) in modulating the radiation response in podocytes and glomerular endothelial cells. Hereby, we investigated the interplay between SMPDL3b and oxidative stress in mediating radiation injury in podocytes. We demonstrated that the overexpression of SMPDL3b in cultured podocytes (OE) reduced superoxide anion generation and NADPH oxidase activity compared to wild-type cells (WT) post-irradiation. Furthermore, OE podocytes showed downregulated levels of NOX1 and NOX4 after RT. On the other hand, treatment with the NOX inhibitor GKT improved WTs' survival post-RT and restored SMPDL3b to basal levels. *in vivo*, the administration of GKT restored glomerular morphology and decreased proteinuria in 26-weeks irradiated mice. Taken together, these results suggest a novel role for NOX-derived reactive oxygen species (ROS) upstream of SMPDL3b in modulating the response of renal podocytes to radiation.

Keywords: ionizing radiation (IR), sphingolipids (SLs), reactive oxygen species (ROS), podocytes (MeSH: D050199), nephropathies, smpdl3b, NADPH oxidases (NOX)

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INTRODUCTION

Radiation nephropathy is defined as an irreversible and detrimental renal injury caused by ionizing radiation. Kidneys are one of the most radiosensitive tissues, often receiving excessive exposure upon the management of abdominal or paraspinal tumors or total body irradiation. Subsequently, irrevocable intracellular cascades happen within the various renal structures, manifesting first as an acute phase of proteinuria to chronic kidney failure requiring dialysis or even kidney transplantation. The clinical manifestations of radiation nephropathy are presented as hypertension, azotemia, and severe anemia long-lasting after radiotherapy (RT) which culminates in renal failure. Histopathological features are also noted, with mesangiolysis, tubular atrophy, and tubulointerstitial scarring (1).

Ionizing radiation induces tissue injury through direct damage to the DNA structure, and indirectly via overproduction of reactive oxygen species (ROS) by water radiolysis. Data from numerous studies have suggested that late effects of RT are caused by an acute and chronic production of oxidative stress. Excessive ROS generation has deleterious consequences that initiate cascades of molecular events that disrupt signaling pathways and result in cellular damage. The latter happens mainly through the oxidation of major macromolecules such as proteins, lipids, and nucleic acid, abrogating their functions (2). Multiple physiological cellular sources mediate ROS production. Amongst these, the NADPH oxidases (NOX) family has received particular attention because of their normal function in host defense and cellular signaling including those in the kidney (3).

The mechanisms behind radiation-induced nephropathies are still largely unknown. The disorders identify the glomerulus as the main culprit in orchestrating the damaging phenotype through complex and dynamic interactions between glomerular, tubular, and interstitial cells. Among those, podocytes are highly specialized epithelial cells that wrap around capillaries to mediate glomerular filtration, a pivotal renal function which filters excess fluids and waste products into urine. Podocytes are identified as critical players in numerous kidney diseases such as focal segmental glomerulosclerosis (FGSG) and diabetic nephropathy, pointing to the crucial function that these cells fulfill in renal homeostasis. However, it remains ambiguous how radiation affects podocytes at a molecular level.

Recent studies conducted by our group and others reveal the importance of sphingolipids in mediating normal renal function especially in podocytes (4, 5). In this context, the sphingomyelinase phosphodiesterase acid-like 3b (SMPDL3b) enzyme was found to play a pertinent role in focal segmental glomerulosclerosis (6) and diabetic nephropathy (7), where SMPDL3b was found to mediate insulin receptor signaling (8). Although the intrinsic enzymatic activity of this protein remains to be elucidated, a growing body of evidence suggests a ceramide-1-phosphate (C1P) lyase-like activity (8, 9).

In this article, we hypothesized that radiation injury in podocytes is conducted through a crosstalk between SMPDL3b and NADPH oxidases. Radiation podocytopathy was mediated through a ROS-dependent mechanism initiated by NOXs that led to downregulated levels of SMPDL3b. Overexpression of the lipid-modifying enzyme mitigated NADPH oxidases activity post-RT and conferred radioprotection to podocytes.

Abbreviations: C1P, ceramide-1-phosphate; DSBs, double-strand breaks; FGSG, focal segmental glomerulosclerosis; GenC, glomerular endothelial cells; γ-H2AX, phosphorylated histone H2AX; NAC, N-acetylcysteine; NOXs, NADPH oxidases; OE, overexpressors of SMPDL3b; ROS, reactive oxygen species; RT, radiotherapy; SMPDL3b, sphingomyelinase acid phosphodiesterase like 3b; WT, wild type; RAAS, renin-angiotensin-aldosterone system.

MATERIALS AND METHODS

Immortalized Human Podocytes Cell Culture, Irradiation and Treatment

Immortalized human podocytes, wild-type (WT) and SMPDL3b overexpressors (OE) were cultured on collagen-coated dishes and differentiated in RPMI-1640 medium containing 10% FBS (Sigma-Aldrich) and 5% penicillin/streptomycin (Biowest). Briefly, cells were propagated at 33°C in 1% insulin-transferrin-selenium 100x (Gibco, USA) containing media on T25 flasks and then thermoshifted for differentiation for 14 days at 37°C. A single dose of 8Gy was delivered from an RS2000 X-ray irradiator (225 kV) according to the manufacturer's specifications (Rad Source Technologies, Suwanee, GA, USA). The dose rate was adjusted to 265 cGy/min. Cells were then irradiated (8Gy) and treatment was stopped by removing the media and adding cold saline solution at the proper time points. For ROS scavenging and NOX1/4 inhibition, cells were treated with 100 μM of N-acetylcysteine (NAC) and 10 μM GKT137831 dissolved in DMSO and PBS respectively for 2 hours prior to irradiation. An equal quantity of DMSO or PBS was added to the control samples.

Immunofluorescence With DHE and DAPI

Superoxide anions were detected using dihydroethidium (DHE) stain by quantification of mean immunofluorescence (MIF). Briefly, cells were grown on 35 mm dishes and were stained with 5μM of DHE for 1 hour at 37°C, fixed with 4% of formaldehyde for 20 minutes, and stained with DAPI. Podocytes were visualized using Zeiss confocal microscope (LSM710 Meta, Carl Zeiss, Inc., Thornwood, NY, USA). Data were analyzed using the LSM Image Browser Software.

Quantitative RT-PCR

Cells were washed with ice-cold PBS and then scrapped from the plate with Trizol for RNA extraction. RNA was quantified by NanoDrop (Thermo Fischer Scientific) and converted to cDNA using iScript cDNA kit (Bio-Rad). cDNA was then diluted (1:10) and 2 μL were added per 20 μL of reaction. Using the iTaq Syber Green, the reaction was executed in real-time PCR system (CFX384 Touch Real-Time PCR Detection System, Bio-Rad, USA) as per the manufacturer′s instructions. Real-time and qualitative PCR was done for human NOX1 (F:5′-GCAGG GAGACAGGTGCCTTTTCC-3′; R: 5′-CTACAGACTTGGG GTGGGAGGT-3′), NOX2 (F:5′-TTCCAGTGCGTGCTGCT CAACA-3′; R:5′-CTGCGGTCTGCCCACGTACAA-3′), NOX3 (F:5′-CCATCCATGGGACGGGTCGGA-3′; R:5′-AGGGGTGC CACTCCAGCGAA-3′), NOX4 (F:5′-CTGGCTCGCCAACGAA GGGG-3′; R:5′-GCTTGGAACCTTCTGTGATCCTCGG-3′), NOX5 (F:5′-GGAGCAAGGTGTTCCAGAAAG-3′; R:5′-AAG GCTCCTCCAAGTAGCAAG-3′), SMPDL3b (F:5′-GCATG GTTCCGGGAGGGCTT-3′; R5′-TGCCCGAAGAACTGCCCT GC-3′), GAPDH (F:5′-TGCACCACCAACTGCTTAGC-3′; R:5′-GGCATGGACTGTGGTCATGAG-3′) and B-actin (F: 5′GCA TGGGTCAGAAGGATTCCT-3′; R: 5′-TCGTCCCAGTTGGTG ACGAT-3′).

MTT Assay

MTT kit (Abcam) was applied as per the manufacturer's recommendation. Briefly, cells were seeded at 15,000 cells/well on collagen-coated 24-well plates and incubated for 24 hrs at 33°C. Afterward, cells were shifted for differentiation at 37°C, and N-acetylcysteine or GKT pretreatment was administered as previously described before irradiation. The MTT assay was applied at 24 hrs post-irradiation. A microplate reader (Multiskan EX, Thermo-Fisher Scientific) was used to measure the absorbance at 590 nm by spectrophotometry.

Protein Extraction and Western Blotting

Podocytes were homogenized in cold RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 50 mM Tris pH 8) supplemented with 10 μL of protease and phosphatase inhibitor cocktail each (Biowest). Protein lysates were collected after centrifugation at 13500 rpm, for 30 min at 4°C. Protein quantification was done using a Lowry Reagent Assay kit from Bio-Rad. Samples were then prepared after quantification with 2X Laemmli sample buffer (Bio-Rad). An equal number of proteins (25-40 μg) were then loaded into 10-12-15% SDS-PAGE gels (Bio-Rad) and transferred on nitrocellulose membrane for 2hrs on ice at 300mA. Membranes were then blocked with 5% skimmed milk or BSA in Tris-saline solution for 1 hour at room temperature. The following primary antibodies were used, each according to the protocol suggested by the manufacturer: rabbit polyclonal anti-SMPDL3b (1:1000) (Genway Biotech, Inc., San Diego, CA, USA), mouse monoclonal anti-GAPDH (1:1000) (Abcam), rabbit monoclonal NOX1 and NOX4 antibodies (1:500) (Abcam), rabbit monoclonal caspase 3 (1:250) (Cell Signaling). Membranes were incubated with the primary antibodies overnight then washed 3 times for 10 minutes each in Tris-saline solution with 0.1% Tween 20. Horseradish peroxidase-conjugated secondary antibodies were used, and the images were developed using enhanced chemiluminescence (Bio-Rad). Densitometry was performed using the ImageJ software (National Institute of Health, Bethesda, MD, USA).

NADPH Oxidase Assay

The activity of the NADPH oxidases enzymes was assessed in cultured podocytes as previously described (10). Cells were washed with ice-cold PBS and scraped from the plate with a special lysis buffer (20 mM KH2PO4 pH 7.0, 1 mM EGTA, 10μL Protease Inhibitor). The homogenate was quantified using the Bio-Rad protein assay reagent. The assay was conducted on 25μg of homogenates which were added to 50 mM phosphate buffer (pH 7.0) containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin, and 100 μM NADPH. Light emission was measured after 30 seconds for 8 minutes in a luminometer. The first and last readings were discarded, and a buffer blank was subtracted from each reading. Superoxide production was averaged and expressed as relative light units/min.mg of protein.

Animal Studies

To assess podocyte cell damage in vivo, ten weeks old C57BL6 male mice were treated with normal saline or GKT137831 with or without a single dose of 14 Gy. GKT137831 treatment was administered by oral gavage 1 hr prior to irradiation at a prophylactic concentration of 20 and 40 mg/kg 1hr following irradiation. Mice were then sacrificed at 24 hrs or 26 weeks post-radiation. 26 weeks has been chosen as the time for phenotype analysis to assess late radiation sequelae (1, 11, 12) Both kidneys were harvested and processed for histological immunohistochemical and molecular studies. Mice were irradiated using RS2000 X-ray irradiator (225 kV) in a specialized lead jig that precisely delivered stereotactic doses to the kidneys. Mice were anesthetized with ketamine/xylazine (1:5) at a concentration of 80 and 8 mg/kg respectively.

Morphometric Glomerular Assessment and Histology

Right kidney was removed for histological analysis and the left kidney was collected for glomeruli extraction. Hematoxylin-eosin (H&E) and Masson Trichrome staining of paraffin-embedded kidney sections (5 μm thick) were performed using a standard protocol. Histological images were visualized using a light microscope (Olympus BX 41, Tokyo, Japan) at 20x magnification. Glomerular area surface was analyzed via ImageJ software. Twenty glomeruli per section were analyzed for collagen deposition by quantitating positive areas for Masson Trichrome staining along with morphometric glomerular parameters, both performed by two blinded independent investigators.

Blood Pressure Measurement

Blood pressure was measured daily by non-invasive determination of tail blood volume, flow, and pressure using a volume pressure-recording sensor and an occlusion tail-cuff (CODA System; Hakubatec Lifescience Solutions, Tokyo, Japan). This is a highly accurate system with the capability of measuring systolic and diastolic blood pressures and heart rate simultaneously and non-invasively. Before measurement, the mice were placed on a 37°C warming pad until the temperature of the tail region reached 37°C according to an infrared thermometer. Following warming, the mice were trained for 15-minute sessions each day for 7 days or until we obtained stable blood pressure recordings.

Statistical Analysis

Results were expressed as the means ± SEM. One-way or Two-way ANOVA along with t-test were used to compare groups, and results were considered statistically significant if $P < 0.05$ (GraphPad Prism software; La Jolla, CA, USA). Results shown are the mean SEM values of at least three independent experiments.

RESULTS

Overexpression of SMPDL3b Decreases Radiation-Induced Superoxide Anion Generation in Podocytes

Our prior work uncovered the differential expression of phosphorylated histone H2AX (γ-H2AX) between the wild-type (WT) and SMPDL3b overexpressing (OE) human podocytes cell lines. This finding suggested a radioprotective role for SMPDL3b against DNA damage (13). In fact, reactive oxygen species (ROS) are among the primary causes of DNA insults following low linear energy transfer (LET) ionizing radiations,

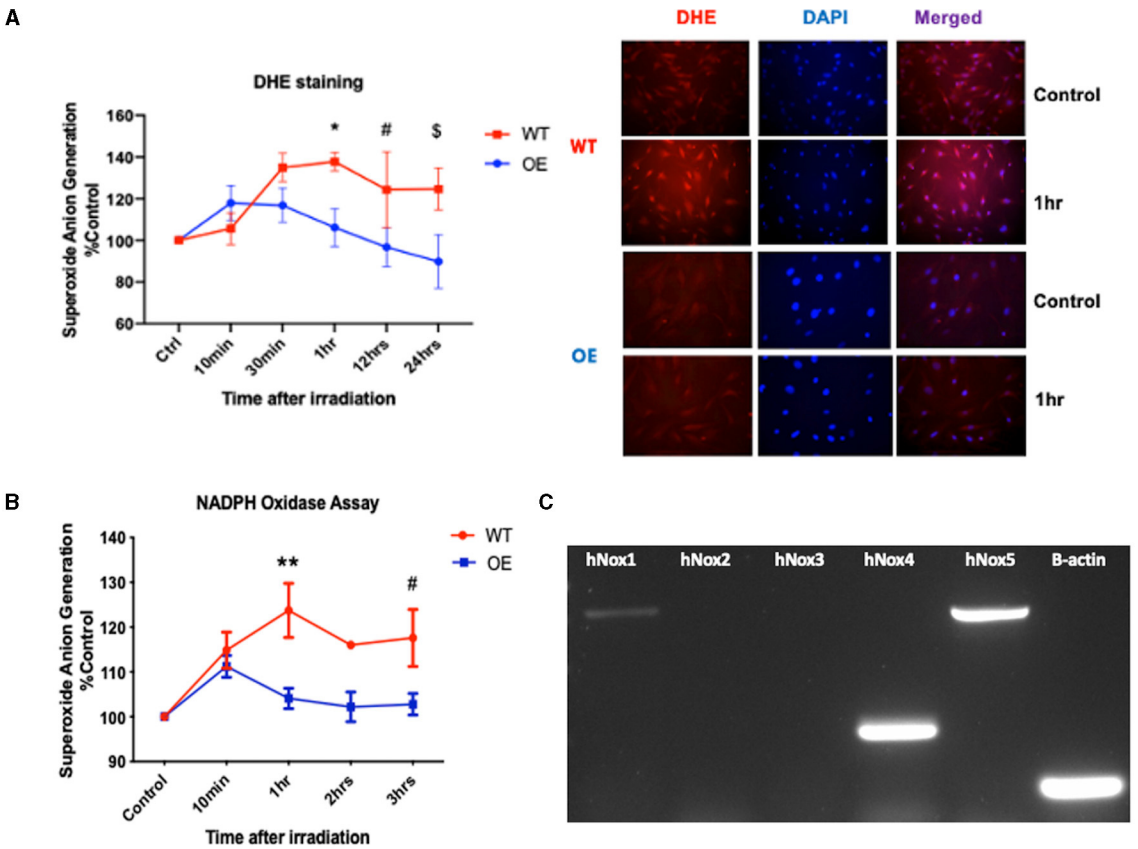

FIGURE 1 | Overexpression of SMPDL3b protects podocytes from radiation-induced reactive oxygen species. **(A)** Quantification of DHE mean immunofluorescence (left panel) at baseline (control), 10 and 30 minutes, 1hr, 12hrs, and 24 hours post-irradiation at 8 Gy (WT vs OE: *$p = 0.007$ at 1hr, #$p = 0.0156$ at 12 hrs, \$$p = 0.0039$ at 24hrs). Immunofluorescence staining with DHE and DAPI of podocytes (right panel) at baseline 0 Gy (control) and 8 Gy at 1hr post-irradiation. Objective 20x. **(B)** Superoxide anion generation measured via lucigenin mediated NADPH oxidase assay in both WT and OE human podocytes at baseline (control) and 10min, 1hr, 2hrs, 3hrs after 8 Gy irradiation (WT vs. OE: **$p = 0.0036$ at 1hr, #$p = 0.0340$ at 3hrs). **(C)** Conventional PCR analysing expression of NOX1 (730bp),−2,−3,−4 (253bp), and−5 (762bp), ß-actin (106bp) in WT human podocytes cell lysate. The results shown are the mean values of at least three independent experiments.

such as those used in conventional clinical radiotherapy. ROS are generated from radiation-induced water radiolysis and predominantly contributed to the formation of DNA double-strand breaks (DSBs). Passage through mitosis with unresolved radiation-induced DNA DSBs leads to cell death. The induction of DNA DSBs leads to phosphorylation of H2AX (γ-H2AX), and γ-H2AX foci are widely used as a DNA damage marker (4, 14). Thus, we investigated the potential interplay between SMPDL3b and ROS generation. To that end, we quantified the superoxide anion generation via DHE staining of both cell lines at different time points after irradiation (**Figure 1A**). OE podocytes showed a substantial decrease in the superoxide anion generation compared to WT starting at 1 hr post-irradiation.

Differential Expression of NOX1/NOX4 Is Observed Between OE SMPDL3b and WT Podocytes

NADPH oxidases are one of the main sources implicated in the generation of superoxide anions (3). We investigated the differential expression of NADPH oxidases in the two cell lines. For that purpose, we performed the NADPH oxidase assay to assess NOXs' enzymatic activity following irradiation. Our data demonstrated a differential increase in NADPH oxidase activity in WT podocytes compared to OE especially at 1hr post-irradiation (**Figure 1B**). Following conventional PCR (**Figure 1C**), we confirmed the detection of NOX1 (730bp), NOX4 (253bp), and NOX5 (763bp) mRNAs in our human podocytes' cell lines. This is consistent with prior studies confirming NOX1 and NOX4 as the major NOX isoforms expressed in podocytes (3).

Next, we examined whether radiation induces a differential NOX1/NOX4 expression in both cell lines. NOX1 mRNA levels in WT cells showed a time-dependent increase in contrast to OE cells (**Figure 2A**). A similar trend was observed in mRNA levels of NOX4 between both cell lines (**Figure 2B**). Comparable results were also found in NOX1 and NOX4 protein levels (**Figures 2C,D**).

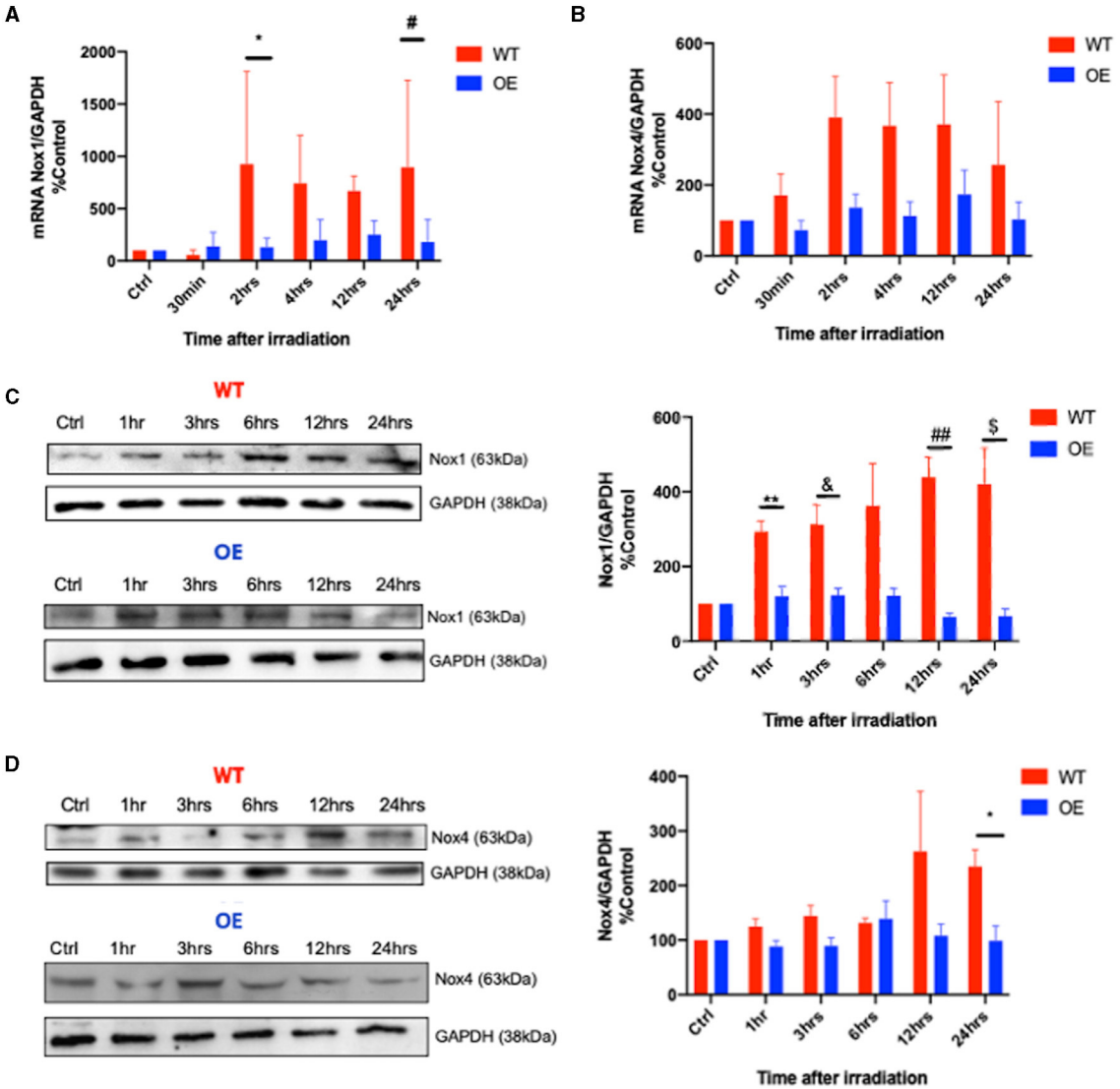

FIGURE 2 | Differential expression of NOX1 and NOX4 in WT and OE podocytes upon radiation. Transcriptional RT-PCR analysis of NOX1 **(A)** (*$p = 0.007$ for WT vs. OE at 2 hrs, #$p = 0.0142$ at 24hrs) and NOX4 **(B)** mRNA expression in WT and OE human podocytes at baseline, 30 min, 2 hrs, 4 hrs, 12 hrs, and 24 hrs after 8 Gy irradiation. Translational immunoblotting analysis of NOX1 **(C)** (WT vs. OE: 1hrs **$p < 0.0041$; 3 hrs &$p < 0.0303$; 12 hrs ##$p < 0.0052$; 24hrs \$$p < 0.0321$), and NOX4 **(D)** (WT vs. OE: 24 hrs *$p < 0.0245$) in WT and OE human podocytes at baseline, 1 hr, 3 hrs, 6 hrs, 12 hrs, 24 hrs following 8 Gy irradiation. The results shown are the mean values of at least three independent experiments.

Inhibition of NOX1/NOX4 via GKT137831 Restores SMPDL3b's Levels in WT Podocytes

These results led us to investigate the relationship between SMPDL3b and NADPH oxidases. To that end, we used the ROS scavenger N-acetylcysteine (NAC), and GKT137831, a dual inhibitor of NOX1 and NOX4. It has been previously demonstrated that SMPDL3b starts to decrease 4hrs post-irradiation in WT podocytes (13). Interestingly, administration of both NAC and GKT restored protein levels of SMPDL3b 24hrs following irradiation while it did not affect its transcriptional levels (**Figures 3A,B**). This was associated with improved cell viability as measured using MTT assay (**Figure 3C**).

Early and Late Radiation Sequelae Ameliorated Upon GKT Treatment in Irradiated C57BL6 Mice

Next, we proceeded to verify our results via an *in vivo* model. C57BL6 males at 10 weeks of age were treated with either normal saline or a full dose of GKT (40 mg/kg) 1 hr prior to focal renal radiation of 14 Gy. All mice were sacrificed 24 hours later to assess the acute radiation effects. A notable increase of protein expression of NOX1 and NOX4

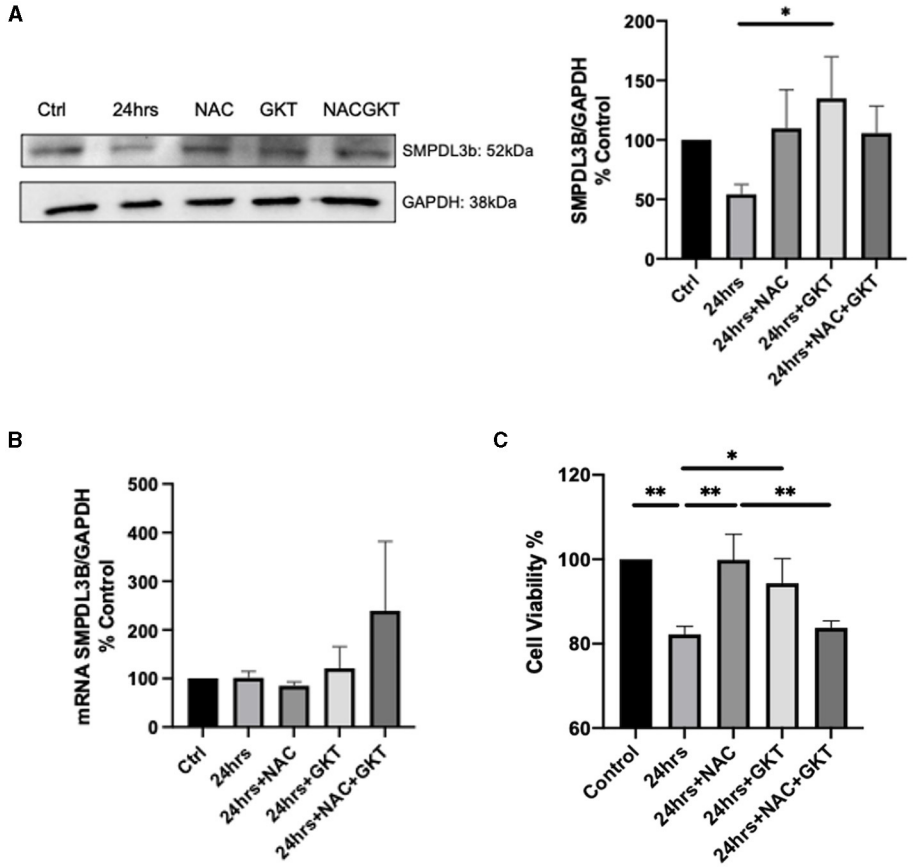

FIGURE 3 | SMPDL3b protein levels are restored upon administration of both NAC and GKT after 24 hours of radiation in podocytes. SMPDL3b protein **(A)** (24 hrs vs. 24 hrs+GKT: $^{*}p = 0.0382$) and mRNA expression **(B)** at baseline and 24 hrs after 8 Gy irradiation in pre-treated WT podocytes with ROS scavenger N-acetylcysteine (100 μM) and/or dual NOX1/4 inhibitor GKT (10 μM). **(C)** Survival profile of WT podocytes at baseline and 24hrs post-irradiation pre-treated with NAC and/or GKT via MTT assay detected by spectrophotometry (Ctrl vs. 24 hrs $^{**}p = 0.0045$; Ctrl vs. 24 hrs+NAC+GKT $^{**}p = 0.0083$; 24 hrs vs. 24 hrs+NAC $^{**}p = 0.0047$; 24 hrs vs. 24 hrs+GKT $^{*}p = 0.0401$; 24 hrs+NAC vs. 24 hrs+NAC+GKT $^{**}p = 0.0088$). The results shown are the mean values of at least three independent experiments.

was identified in the irradiated group compared to control littermates (**Figures 4A,B**). Furthermore, inhibition of NOX1/4 via GKT treatment in the irradiated group decreased cleavage of caspase 3, indicating improved survival at a cellular level (**Figure 4C**).

We were also interested in investigating late radiation sequelae. C57BL6 males of 10 weeks were treated with either normal saline or a prophylactic dose of GKT (20 mg/kg) 1 hr before focal renal radiation of 14 Gy. Following X-ray exposure, those who received a prophylactic dose were further treated with a full 40 mg/kg of GKT. All mice were sacrificed 26 weeks post-irradiation. Significant proteinuria was noted in the irradiated group compared to control, which was restored to normal levels upon administration of GKT (**Figure 5A**). High levels of systolic pressure were detectable in the irradiated group and alleviated in GKT treated littermates (**Figure 5B**).

To assess the impact of ionizing radiation on renal morphology, kidney sections were stained with hematoxylin and eosin. Quantification demonstrated irradiation-induced hypertrophy of glomeruli as evidence by increased glomerular area surface in irradiated mice which was partially alleviated upon administration of GKT (**Figure 5C**). We observed thinning of parietal cells of Bowman's capsule and capillary dilatation in glomerular tufts that were restored with GKT administration. Additionally, collagen staining increased in irradiated glomeruli which was downregulated with GKT treatment (**Figure 5D**). Furthermore, protein levels of SMPDL3b were downregulated in 26 weeks post-irradiated renal cortices homogenates with a tendency of being restored upon GKT treatment (**Figure 5E**). This is in line with a previous study of ours where SMPDL3b was decreased in 11 weeks of irradiated mice glomeruli (13).

DISCUSSION

Radiation nephrotoxicity remains a clinical concern and can be an obstacle to treatment in some cancer patients undergoing RT, especially those with compromised renal function. Despite advances in delivering precise radiation doses to the tumor, some normal tissues are unavoidably irradiated and other out-of-target normal tissues also receive a dose that results in acute and late

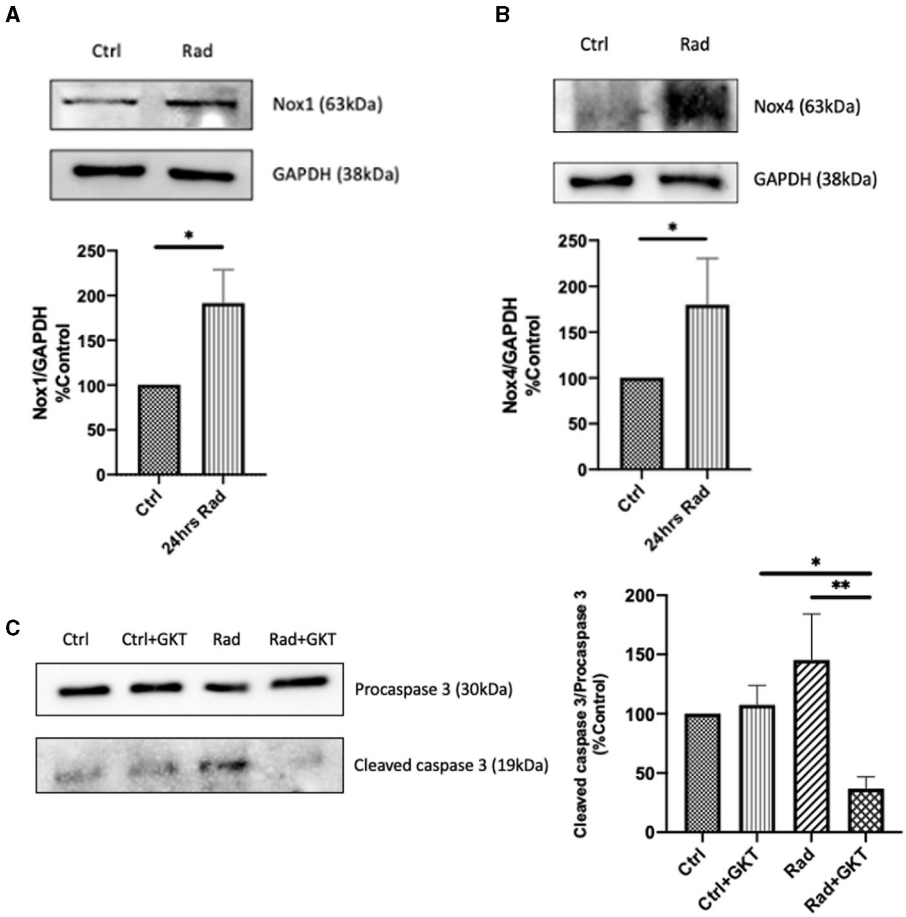

FIGURE 4 | Radiation causes acute upregulation of NADPH oxidases protein expression in kidneys of 24hrs post-RT C57BLC/6 mice. Increase of protein levels of NOX1 **(A)** (Ctrl vs. Rad $^{*}p = 0.0493$), and NOX4 **(B)** (Ctrl vs. Rad $^{*}p = 0.0196$) detected by immunoblotting in renal cortices at 24hrs post-irradiation. Post-irradiation cleavage of caspase 3 reduced upon administration of GKT (Ctrl+GKT vs. Rad+GKT $^{*}p = 0.0498$, Rad vs. Rad+GKT $^{**}p = 0.0075$) **(C).** The results shown are the mean values of at least three independent experiments.

RT side effects. Therefore, there is a pressing clinical need to investigate the molecular events underlying these acute and late normal tissue effects.

SMPDL3b was shown to play a protective role in a panel of diseases. Pioneering work suggested that treatment of patients with rituximab at the time of kidney transplant might prevent recurrent FSGS by modulating podocyte function in an SMPDL3b–dependent manner (6). Prior work has shed light on the radioprotective role of SMPDL3b in cultured podocytes. OE podocytes showed a reduced number of γ-H2AX foci. Moreover, these OE cells had abrogated actin cytoskeleton remodeling and caspase 3 cleavage post-RT, in contrast with WT cells. Intriguingly, SMPDL3b exhibited a time-dependent decrease following irradiation both *in vitro* and 3 months post-RT *in vivo* (13). Nevertheless, the molecular mechanisms underlying these events were not clear.

The current study examines the relationship between SMPDL3b and oxidative stress in the context of RT in podocytes. The novelty of our study suggests a crosstalk between SMPDL3b and NADPH oxidases. In wild-type cells, irradiation increased NOXs expression and enzymatic activity and thus upregulated ROS production. The administration of NAC and/or GKT restored levels of SMPDL3b, suggesting that NOX-derived ROS may account for the time-dependent decrease of the lipid-modifying enzyme after RT. Alternatively, overexpression of SMPDL3b led to decreased enzymatic activity and levels of NADPH oxidases, confirming a crosstalk.

It is plausible that SMPDL3b could be degraded in an oxidative-dependent manner, initiated by NOXs. In fact, protein oxidation facilitates both proteasomal and lysosomal-mediated degradation (15) and it remains ambiguous which pathway is involved in the loss of the enzyme. There are currently no known ROS-dependent modifications in the protein's configuration. Proteasomes and lysosomes which are the main cellular compartments responsible for proteolysis, are redox-sensitive (15) and could be activated by ionizing radiation to eliminate potentially damaging oxidized proteins (16, 17). Further experiments are warranted

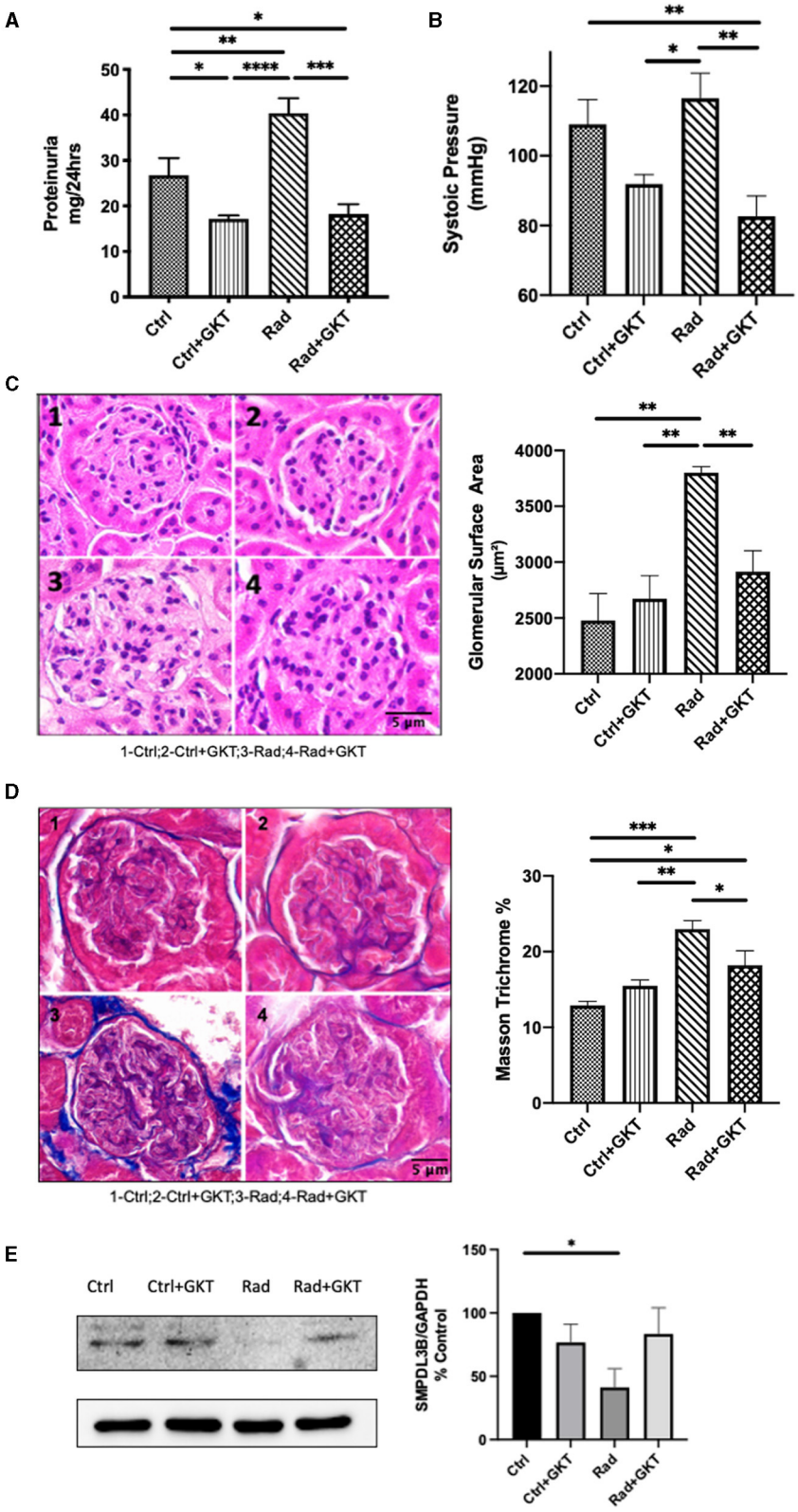

FIGURE 5 | Administration of GKT restores radiation injury markers in 26 weeks post-irradiation mice. **(A)** Proteinuria measured in C57BLC/6 mice at 22 weeks post-RT by urine collection (Ctrl vs. Ctrl+GKT $^{*}p = 0.0308$; Ctrl vs. Rad $^{**}p = 0.0452$; Ctrl vs. Rad+GKT $^{*}p = 0.0484$; Ctrl+GKT vs. Rad $^{****}p < 0.0001$; Rad vs. Rad+GKT $^{***}p = 0.0001$). **(B)** Systolic pressure measured by non-invasive tail-cuff method (Ctrl vs. Rad+GKT $^{**}p = 0.0089$; Ctrl+GKT vs .Rad $^{*}p = 0.0134$; Rad vs.

(Continued)

FIGURE 5 | Rad+GKT**$p = 0.0018$). GKT treatment ameliorates partially morphological parameters of irradiated glomeruli in mice. Representative photomicrographs of glomeruli from Ctrl (1), Ctrl+GKT (2), Rad (3), Rad+GKT (4) kidneys harvested from C57BLC/6 mice at 24 weeks post-radiation; Quantification of all parameters of at least 20 glomeruli in all groups **(C)** Paraffin-embedded sections, 5 μm thick, were stained with H&E (20x), scale bar 5 μm (Ctrl vs. Rad **$p = 0.0010$; Ctrl+GKT vs. Rad **$p = 0.0027$; Rad vs. Rad+GKT **$p = 0.0099$). **(D)** Paraffin-embedded sections, 5 μm thick, were stained with Masson Trichrome (blue stain) for collagen deposits (20x), scale bar 5 μm (Ctrl vs. Rad ***$p = 0.0008$; Ctrl vs. Rad+GKT *p=0.0222; Ctrl+GKT vs. Rad **p=0.0055; Rad vs. Rad+GKT *p=0.0347). **(E)** Immunoblotting of SMPDL3b in renal cortices homogenates of 26 weeks irradiated and non-irradiated C57BL6 mice. SMPDL3b is restored upon GKT treatment (Ctrl vs. Rad *$p = 0.0214$; Rad vs. Rad+GKT ns $p = 0.0724$). The results shown are the mean values of at least three independent experiments.

to investigate the possibility of redox modifications of SMPDL3b. The latter could be examined through recent omics-based approaches such as thiol redox proteome (18, 19). Thiol is a redox-sensitive group that allows cysteine to be subjected to all kinds of oxidative reactions. A growing body of evidence suggests that thiol redox modifications are not random cellular incidents but well-organized and coordinated events leaving a particular signature on the oxidized molecule.

Overexpression of SMPDL3b conferred cellular protection by alleviating radiation-induced ROS generation. Nevertheless, the mechanism behind this inhibition remains to be established. Emerging evidence suggested a C1P-lyase like activity for SMPDL3b (8, 9). Following that, we can speculate that the overexpression of the lipid-modifying enzyme leads to a shift in the lipidomic profile of the podocytes. Consequently, as second messengers and bioactive entities, sphingolipid metabolites play an essential role in regulating biological processes and might potentially alter signaling pathways (20, 21). Previous data analysis of mass spectrometry showed that irradiated OE SMPDL3b podocytes had downregulated levels of ceramide-1-phosphate, while interestingly, no changes in sphingosine and ceramides were noted, (8, 13). On the other hand, irradiated WT podocytes exhibited upregulation in ceramides and a decrease in sphingosine. Ceramides were shown to activate ROS generating entities like NADPH oxidases and to form lipid-rafts that assemble the corresponding subunits. On the other hand, sphingosines were found to inactivate the enzymatical activity of NADPH oxidases (22, 23). As for the decrease in transcriptional levels of NADPH oxidases, we could speculate that the shift of sphingolipids in the OE could impact signaling cascades influencing promoter regions of NOXs' genes. For instance, the 5'-region of the human NOX1 gene contains binding elements for signal transducers and activators of transcription (STATs), interferon regulatory factor (IRF) (3), which are regulated by different sphingolipids (20). Thus, via its C1P-lyase activity, overexpression of SMPDL3b might regulate the transcriptional levels of NADPH oxidases. Alternatively, SMPDL3b might be influencing NADPH oxidases through direct protein-protein interaction. Such hypothesis is worth examining in the future given that the two enzymes are transmembrane proteins with activity modulated by lipid-rafts (8, 24).

NADPH oxidases contribute to many normal physiological processes such as cell signaling, host defense, and metabolism (3). Nonetheless, this family of enzymes is involved in numerous ROS-derived pathologies of renal dysfunction targeting podocytes particularly. For instance, NOXs have their share in orchestrating diabetic nephropathy (3, 25–28). Moreover, NOXs are implicated in a plethora of podocyte injury models such as hyperhomocysteinemia (29), FSGS (30), renal hypertension (31), and renal inflammation (32). However, the role of NOXs in the context of renal RT remained unclear. It has been reported that NADPH oxidases mediated radiation insult in rat brain microvascular endothelial cells (33) and ROS production in radiation-induced senescent cells (34). Additionally, inactivation of both NOX4 and NOX5 abrogated radiation injury in human primary fibroblasts (35). While countless studies have investigated NOXs' implication in diverse podocyte injury models, our results are the first to show an upregulation of NADPH oxidases upon RT in these cells.

Our work also demonstrates the impact of pharmacologic inhibition of NOXs as an approach to reverse radiation. Reversal of the renal fibrosis, proteinuria, and systolic pressure through NOX inhibition reveals that this family of enzymes contributes to the pathophysiology observed in the glomeruli post-RT *in vivo*. Increased systolic pressure is indicative of renal impairment (1, 36). Kidneys are key players in regulating systemic blood pressure through the renin-angiotensin-aldosterone system (RAAS). It is well established that RAAS-inhibition via the angiotensin-converting enzyme (ACE) and angiotensinogen I (ATI) inhibitors alleviates the progression of many kidney diseases including radiation nephropathy (37). Elevated diastolic and systolic blood pressures were noted after 100 days of kidney irradiation in mice, which were further increased upon bilateral renal radiation, pointing out that radiation nephropathy has long-lasting functional and metabolic consequences (38). Administration of GKT in our irradiated C57BL6 mice showed a decrease in systolic blood pressure 26 weeks later compared to control mice. Molecularly, protein levels of NOX1 and NOX4 were upregulated within 24hrs in irradiated renal cortices. This reflects that oxidative stress generated by NADPH oxidases is an early radiation response in renal cells. Moreover, the administration of GKT alleviated the cleavage of caspase-3 upon radiation *in vivo*, thus conferring radioprotection to renal cells.

Ultimately, our results show that the crosstalk between SMPDL3b and oxidative stress is critical for predicting the response of podocytes to radiation injury.

Ionizing radiation can cause injury not only to targeted podocytes but also to neighboring non-targeted cells which exhibit similar molecular damages and disturbances in the oxidative metabolism. The spread of the insult happens essentially through intercellular mechanisms (2). Moreover, crosstalks have been identified between different renal cell types and these intercellular communications play major roles in both

healthy and diseased glomeruli, implicating oxidative stress, systemic chronic inflammation, and perturbations in the RAAS (11, 39, 40). While many studies have investigated this interplay in the context of diabetes especially (40, 41), none have examined this relationship in irradiated kidneys.

Glomerular endothelial cells (GenC), are key players in the onset and progression of numerous kidney diseases including radiation injury. Radiation-induced oxidative stress in glomerular endothelial cells has been examined by another study from our group (42). Irradiated GenC showed increased NOX activity and superoxide anion generation. Silencing NOX1 using NOX1-specific siRNA mitigated oxidative stress and cellular injury. Additionally, mice treated with GKT showed decreased apoptotic glomerular endothelial cells 24hrs post-irradiation. Ultimately, molecular interactions between glomerular endothelial cells and podocytes should be evaluated in the context of irradiation, ideally in a co-culture model or *in vivo*.

CONCLUSION

Radiation nephropathy remains one of the main hurdles faced by patients towards their path to full recovery. Albeit remarkable advancements in radiation dose delivery techniques, damages to healthy neighboring renal tissues still occur. Our study establishes a radioprotective role of SMPDL3b in mitigating injury in podocytes in a ROS-dependent manner. Furthermore, our work unmasked a crosstalk between the lipid-modifying enzyme and the NADPH oxidases. Additional research is warranted to understand the mechanisms behind this crosstalk. Deciphering these events on the physiological and molecular levels might help unfold future therapeutic aspects in treating the radiation-induced nephrotoxicity. Therefore, proposing SMPDL3b as a novel therapeutic target holds major clinical implications given the canonical importance of podocytes in regulating renal function.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and by the Institutional Animal Care and Use Committee (IACUC) of American University of Beirut.

AUTHOR CONTRIBUTIONS

YZ, AF, BM, AE, and PA designed the research. PA and YZ analyzed the data and wrote the paper. PA, MF, MM, TY, and AD performed the research. All authors contributed to editing the paper.

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Conflict of Interest: AF is an inventor on pending or issued patents (PCT/US11/56272, PCT/US12/62594, PCT/US2019/041730, PCT/US2019/032215, PCT/US13/36484, and PCT 62/674,897) aimed to diagnosing or treating proteinuric kidney diseases and stands to gain royalties from their 20 future commercialization of these patents. AF is Vice-President of L&F Health LLC and is a consultant for ZyVersa Therapeutics, Inc. ZyVersa Therapeutics, Inc has licensed worldwide rights to develop and commercialize hydroxypropyl-beta-cyclodextrin from LF Research for the treatment of kidney disease. AF is the founder of LipoNexT LLC. AF is supported by Hoffman-La Roche and by Boehringer Ingelheim.

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suPAR, a Circulating Kidney Disease Factor

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Urokinase plasminogen activator receptor (uPAR) is a multifaceted, GPI-anchored three-domain protein. Release of the receptor results in variable levels of soluble uPAR (suPAR) in the blood circulation. suPAR levels have been linked to many disease states. In this mini-review, we discuss suPAR as a key circulating molecule mediating kidney disease with a particular focus on differently spliced isoforms.

Keywords: suPAR, isoform, proteinuric disease, kidney, uPAR

THE IMPLICATION OF uPAR IN KIDNEY DISEASE

The urokinase-type plasminogen activator receptor (uPAR) is a GPI-anchored membrane bound protein involved in many physiological and pathological events. It acts as a receptor for urokinase-type plasminogen activator (uPA), facilitating the generation of activated plasmin, thus playing a role in the directional invasion of migrating cells. It is also implicated in a plethora of cellular responses that include cellular adhesion, differentiation, proliferation and migration in a non-proteolytic fashion as a signaling orchestrator (1, 2). uPAR is a member of the lymphocyte antigen 6 (Ly-6) superfamily proteins, containing three domains, namely DI-DIII, as numbered from the N terminus (3). Protein structure analyses show that uPAR packs into a concave structure with uPA binding to the central cleft, while vitronectin binding to the outside surface (4, 5). This special protein structure makes it possible for uPAR to bind different ligands simultaneously, allowing coordinated regulation of proteolysis, cell adhesion and signaling (6–9). Yet, the structure of the unoccupied human uPAR has not been determined, due to the difficulty in crystalizing the protein (10, 11).

uPAR is expressed on a variety of cells, including monocyte, lymphocyte, endothelial cells (12). The efforts to examine the expression of uPAR in normal kidney and its alterations in kidney disease started in the mid 1990 (13). Almus-Jacobs et al. found the stimulation of murine uPAR gene by endotoxin (14). Xu et al. observed the upregulation of uPAR expression in the glomeruli and in the arterial walls of thrombotic microangiopathy (15). Within the glomeruli of rejected kidney samples, Tang et al. reported positive immunostaining for uPAR in the mesangial cells, but not in the majority of endothelial cells (16). In a rat model of nephrotoxic nephritis, the induction of glomerular uPAR expression was observed as soon as 1 h after nephrotoxic serum injection (17). An unusual implication of uPAR in obstructive nephropathy was reported in unilateral ureteral obstruction (UUO) mouse model, whereby uPAR deficiency accelerated renal fibrosis (18, 19). These findings suggest that renal uPAR may have protective effects in attenuating the fibrogenic response to some renal injury. In the renal biopsy of acute renal allograft rejection, Roelofs et al. found both uPA and uPAR are upregulated (20). Our team identified podocyte uPAR as an important molecule mediating glomerular filtration barrier function in 2007 (21). We found the induction of glomerular uPAR in both human and rodent proteinuric kidney diseases. Gene transfer of uPAR to podocytes but not that to endothelial cells in uPAR deficiency mice induced proteinuria, suggesting the expression of uPAR from podocytes was required for proteinuria

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development. Mechanistically, uPAR activated αVβ3 integrin in podocytes, promoting cell motility and the activation of small GTPase Rac-1 (21).

THE IMPLICATION OF suPAR IN PROTEINURIC KIDNEY DISEASE

The presence of soluble form of uPAR or generally suPAR was first reported by Ploug et al. (22), when phorbol 12-myristate 13-acetate (PMA)-stimulated U937 cells were treated with bacterial phosphatidylinositol-specific phospholipase-C (PI-PLC). Subsequently, suPAR was detected in many body fluids, such as plasma, serum, urine, saliva, and cerebrospinal fluids. Since then, the elevation of circulating suPAR has been documented in many disease states, reflecting the activation state of the immune system (12).

The initial study of suPAR in proteinuric kidney disease was largely prompted by the concept of a circulating blood factor that causes primary or recurrent focal segmental glomerulosclerosis (FSGS). FSGS refers to a histologic pattern that involves different etiology yet shares a common theme of podocyte injury and/or depletion (23). Generally, FSGS is divided into two categories, primary and secondary. While many studies support the idea that primary FSGS is presumably caused by circulating permeability factor or factors, the identification and characterizing of such factor or factors have been painstakingly challenging. In 2011, our team published the findings that indicate suPAR contributes to primary and recurrent FSGS as a circulating factor (24). suPAR fulfills the criteria of a circulating FSGS factor such as: elevated concentration in patients and the ability to signal to podocytes thereby causing injury and disease. suPAR's injurious activity can be blocked by antibodies against integrin or by lowering suPAR through plasma exchange (25). Some other less characterized candidates for FSGS factor include active proteinases (26), cardiotrophin-like cytokine-1 (27, 28), and protein tyrosine phosphatase receptor O (29).

Using patient samples, we found elevated serum suPAR in two-thirds of primary FSGS, but not in other glomerular disease. In the transplantation subgroup, the mean levels of suPAR, both pre- and post-transplantation, were significantly higher in recurrent FSGS patients than in non-recurrent FSGS patients (24). Like the membrane-bound uPAR, suPAR activated β3 integrin in podocytes. Sustained expression of the secreted form of mouse uPAR induced proteinuria as well as kidney pathological changes in mice (24). In a follow-up study with two independent primary FSGS cohorts, 70 patients from the FSGS clinical trial (CT) and 94 patients from PodoNet, we found the increase of circulating suPAR in 84.3% of CT, 55.3% of PodoNet FSGS patients, compared with 6% of controls (30). Note that CT is a North-America based randomized study that compared the efficacy of cyclosporine A with the combination of mycophenolate mofetil and dexamethasone. Key inclusion criteria are age 2–40 years, eGFR $>$ 40 ml/min per 1.73 m^2, biopsy-proven FSGS, and resistance to corticosteroid therapy. PodoNet however is a Europe-based consortium for clinical, genetic, and experimental study of SRNS. The inclusion criteria are children (age 0–18 years) with steroid-resistant nephrotic syndrome (SRNS) based on management protocols at the participating medical centers and adults with familial SRNS. The difference in patient population could partially account for the difference in suPAR levels with these two primary FSGS cohorts.

Unsurprisingly, these reports on the implication of suPAR as an FSGS factor soon generated excitement and follow-up studies (31, 32). Case reports emerged showing that lowering circulating suPAR levels through plasmapheresis or immunoadsorption could reduce proteinuria in recurrent FSGS (33), making it an effective therapy for some transplant FSGS patients (34, 35). Conversely, transmission of elevated suPAR from a mother with FSGS to her child was correlated to the child being born with proteinuria (36). Morphologically, podocyte effacement could be closely linked to suPAR levels at the time of post-transplantation FSGS occurrence (37). In line with these clinical observations, our findings obtained from animal experiments support the causative correlation between suPAR levels and renal function (38). Using a series of experimental approaches, including bone marrow transplantation and adoptive cell transfer, we discovered that bone marrow (BM)-derived immature myeloid cells (IMCs) are likely a main source of circulating suPAR, thereby contributing to proteinuric kidney diseases. These findings, in agreement with early observations (39), suggest the functional contribution of BM to kidney function and BM-derived IMCs as the possible origin of circulating suPAR responsible for renal injury. As evidence, we have shown that suPAR-generating cells transferred from proteinuric mice are essential for the induction of proteinuria and a concomitant suPAR increase in healthy mice (38) (**Figure 1**).

Particularly, in the first 5 years after the initial discovery of suPAR as a FSGS factor, not all studies with suPAR have resulted in supporting conclusions. This could be attributed to technical reasons such as differences in biological models or assays or a lack of power in some cohort-based studies. In a single center study of idiopathic FSGS in children, Bock et al. did not find the difference in serum suPAR levels among FSGS, non-FSGS glomerular disease, non-glomerular kidney disease and healthy controls (40). Subsequently, there were several studies from different groups questioning the usefulness of serum suPAR as a diagnostic marker for FSGS. The possibility of retention of suPAR due to decreased glomerular filtration rate (GFR) has been raised as well (41–44). The main argument was the relative unspecific nature of an elevated circulating suPAR, which could be observed in FSGS, but also in non-FSGS kidney disease and in many non-kidney disease. This discrepancy was clarified in a large cohort study establishing suPAR as a risk factor for chronic kidney disease (CKD) (45). Conflicting conclusions were arisen from different animal studies as well. We showed that the injection of full-length mouse recombinant suPAR protein (derived from NS0 cells, Fc Chimera, R&D systems) caused a mild proteinuria in uPAR knockout ($Plaur^{-/-}$) mice. Whereas, Cathelin et al. did not detect proteinuria in wild type C57BL/6J mice injected with mouse full length suPAR protein purified from NS0 cells or a monomeric mouse full length uPAR isolated from eukaryotic S2 cells (46). In contrast to our experimental model where the secreted form of mouse uPAR was expressed via electroporation

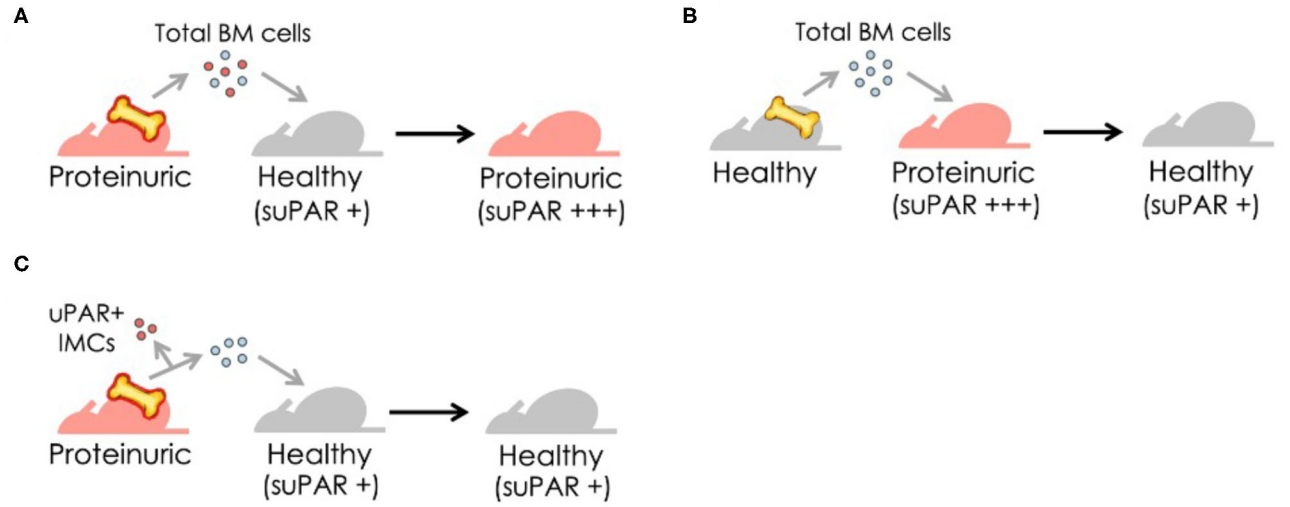

FIGURE 1 | Bone marrow (BM)-derived immature myeloid cells (IMCs), newly identified as a cellular source of suPAR, transfer disease from proteinuric to healthy mice. **(A)** Transfer of BM cells from proteinuric to healthy mice results in proteinuria and increases blood suPAR levels. **(B)** Replacement with BM cells from healthy mice significantly reduces suPAR levels and subsequently improves renal function in proteinuric mice. **(C)** The removal of uPAR-expressing IMCs prior to transfer of BM cells from proteinuric to healthy mice protects mice from proteinuria.

in wild type C57BL/6J mice (24), Spinale et al. utilized a full-length mouse suPAR transgenic floxed FVB mouse model. While the induction of mouse suPAR in the liver was achieved by retro-orbital injection of Adeno Associated Virus 8 particles that carry a hepatocyte-specific Cre recombinase, these mice did not develop proteinuria up to 44 days (47). These seemingly conflicting studies employed different models, methods and human patient cohorts, and If interpreted more carefully in their respective close context would have caused less confusion. As pointed out by late Schlondorff, these discrepancies should not discourage further research on the potential roles of suPAR in proteinuric kidney disease, including FSGS (48), and indeed they did not. In 2014, Delville et al. published a circulating antibody panel for pre-transplant prediction of FSGS recurrence after kidney transplantation. In their study, CD40 autoantibody alone had the best correlation (78% accuracy) with recurrent FSGS risk after transplantation; interestingly injection of CD40 autoantibody obtained from recurrent FSGS patients enhanced human suPAR-mediated proteinuria in wild type mice, suggesting the possible synergy between CD40 autoantibody and suPAR (49). Later, Alfano et al. found that full-length human suPAR down-regulated nephrin expression in human primary podocytes. Additionally they found infusion of this same human full length suPAR protein into uPAR knockout mice induces proteinuria (50). In individuals of recent African ancestry, variants in APOL1 have been associated with certain forms of CKD. In two large unrelated cohorts, Hayek et al. found that decline in kidney function associated with APOL1 risk variants is dependent on plasma suPAR levels. Their study suggested the synergy of circulating suPAR and APOL1 variant G1 or G2 on αVβ3 integrin activation is an underlying mechanism (51). Needless to say, the initial debate regarding suPAR and proteinuric kidney disease triggered and/or intensified the investigation of suPAR as a biomarker and risk factor for CKD and acute kidney injury (AKI), the details of which will be reviewed elsewhere.

To further understand the possible causative role of suPAR, we have created three different transgenic mouse models, constitutively expressing full-length mouse suPAR (muPAR1), secreted form of mouse suPAR (muPAR2) and mouse suPAR DIIDIII fragment under AP2 promoter, respectively. Compared to muPAR1, muPAR2 does not possess GPI anchor sequence and only have an intact DI. In certain experiments, the transgenic mice were fed with high fat diet to induce the expression of mouse suPAR. Interestingly, we observed different kidney pathologies with these transgenic mice: 4 months into high fat diet induction, muPAR1 transgenic mice developed significant low grade proteinuria in about one third of the mice (38); 6 months after high fat diet treatment, proteinuria became severe in some but not all muPAR1 transgenic mice (52). A small portion of muPAR1 transgenic mice developed spontaneous proteinuria by 1 year old without high fat diet treatment. In contrast, most muPAR2 transgenic mice developed spontaneous proteinuria by 2 months old without high fat diet. With high fat diet treatment, muPAR2 transgenic mice presented chronic and progressive proteinuria. By high fat diet induction for 6 months, some muPAR2 transgenic mice demonstrated a severe proteinuric kidney disease characteristic of FSGS changes. Mechanistically, msuPAR2 requires the presence of β3 integrin-Src signaling to generate proteinuria (52, 53). Collectively, these findings indicate that different forms of mouse suPAR generate kidney disease state with different severity, further reflecting the complexity of suPAR biology.

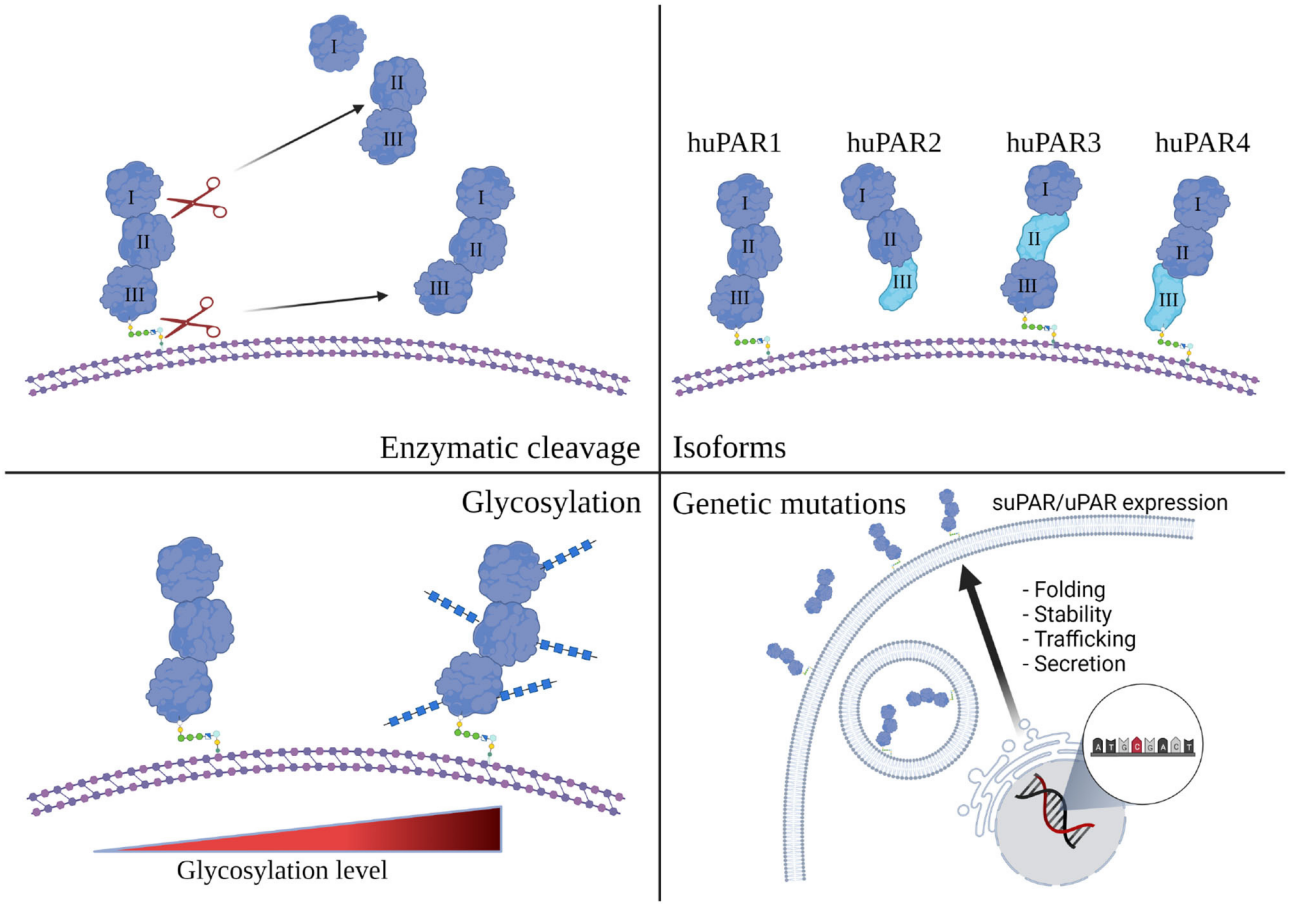

FIGURE 2 | Human suPAR, more than just one flavor. Schematic presentation of human uPAR and suPAR (Created with BioRender.com). While enzymatic cleavage has been considered to be the main source of suPAR, transcriptional splicing, genetic mutation, and post-translational glycosylation could also impact the composition and function of circulating suPAR in different human subjects.

suPAR/uPAR, MORE THAN JUST ONE LOOK

How can we understand the multifaceted role of suPAR in kidney disease? While generally known as suPAR, it clearly has more than one form. It has been documented that cleavage of GPI anchor releases full-length suPAR from membrane-bound uPAR (22). Numerous studies have indicated that full-length suPAR is functional (12). It retains uPAR's ability to bind to uPA, and suPAR binds vitronectin and integrins as well (9, 54, 55). As suPAR and uPAR can be cleaved at the linker region between DI and DII by a variety of enzymes (56), they generate DI fragment and DIIDIII fragment. Both fragments have been detected in body fluids (57, 58). DIIDIII fragment, while cannot efficiently bind uPA or vitronectin is active as it possess chemotactic properties shown by many studies (59–61). In addition to different suPAR fragments, there are other modifications that could impact circulating suPAR composition and function as well, including post-translational glycosylation, genetic mutation, and different isoforms derived from alternative splicing (**Figure 2**). The amino acid sequence for human uPAR/suPAR contains five N-linked glycosylation sites affecting the molecule mass and possibly the function of these proteins. Several different glycosylated variants have been reported in different cell types (62). Glycosylation profiling of a recombinant suPAR expressed in Chinese hamster ovary cells indicated that only four sites were utilized (63). How different glycosylated suPAR variants function in human body remains unknown. Recently, there are several studies presenting different uPAR genetic variants, correlating to circulating suPAR levels or not (64, 65). Understanding the role of these uPAR genetic mutations in human physiology and disease will be an exciting research avenue in the future.

uPAR ISOFORM AND PROTEINURIC KIDNEY DISEASE

Notably, uPAR has multiple isoforms both in human and in mice due to alternative splicing of the seven encoding exons (**Table 1**) (66, 67). About three decades ago, two alternatively spliced mouse uPAR mRNAs were identified in the gastrointestinal tract, with

TABLE 1 | Major uPAR isoforms in mouse and human.

	Isoform	Exon	Domains	GPI anchor	Length (Amino acid)	Nucleotide ID	Protein ID
Mouse	Isoform 1 canonical form, muPAR1	7 Exons (1–7)	Three intact domains (I, II, III)	Yes	327	NM_011113	NP_035243
	Isoform 2 secreted, muPAR2	Exons 5 to 7 missing	DIII and part of DII missing	No	222	BC010309	CAA44575
Human	Isoform 1 canonical form, huPAR1	7 Exons (1–7)	Three intact domains (I, II, III)	Yes	335	NM_002659	NP_002650
	Isoform 2 secreted, huPAR2	Exon 7 missing	C-terminal part of DIII missing	No	281	NM_001005376	NP_001005376
	Isoform 3, huPAR3	Exon 5 missing	Part of DII missing	Likely	290	NM_001005377	NP_001005377
	Isoform 4, huPAR4	Exon 6 missing	N-terminal part of DIII missing	Likely	286	NM_001301037	NP_001287966

the full length, canonical form (muPAR1) localized in the luminal epithelial cells, and the shorter secreted form (muPAR2) found in the basal epithelial cells (66). Unlike muPAR1, which has 3 intact domains (DI to DIII) and 7 predicted sites of glycosylation, muPAR2 has only intact DI, encoded by exons 2 and 3, and part of DII as encoded by exon 4, missing the rest of the native protein (whole DIII and part of DII, encoded by exons 5–7), including GPI anchor. Judged from its amino acid sequence, msuPAR2 was once considered to be unstable due to its number of cysteine residues (68). We originally cloned muPAR2 mRNA (GenBank ID, BC010309) from cultured mouse podocytes, and found its protein interacting with integrin β3; once expressed in C57BL/6 mice via electroporation, muPAR2 induced proteinuric kidney disease (24). The pathogenesis of muPAR2 was later confirmed in muPAR2 transgenic mice that developed a chronic kidney disease, resembling FSGS. In addition, we purified msuPAR2 protein from HEK cells and characterized it as a stable protein, forming a dimer comprising DI and part of DII. The single long strand of β-sheet in the DII region might pair with the strand from its dimer partner (52). Our studies indicate that some form of suPAR causes FSGS-like changes at least in mice (24, 52).

Compared to mouse uPAR, human uPAR has more splicing isoforms. So far, at least 4 major human uPAR isoforms have been documented in different human cells and tissues. While we detected these isoforms in human peripheral mononuclear cells (PBMC) by real-time quantitative PCR (52), Hagemann-Jensen et al. reported the presence of multiple uPAR isoforms in different T cells, monocytes and HEK cells by single cell RNA sequencing (69). Some of these uPAR isoforms could be detected in human glomeruli as well (personal communication with Dr. Mattias Kretzler from University of Michigan). Human isoform 1 (huPAR1) is equivalent to canonical muPAR1, with three intact Ly6/uPAR domains and a GPI anchor. Human isoform 2 (huPAR2) has a deletion of exon 7, and lacks a GPI anchor sequence. As with muPAR2, huPAR2 could provide the natural (secreted and uncleaved) source of suPAR. Human isoform 3 (huPAR3) has a deletion of exon 5, hence lacks the three C-terminal β-strands in DII. Human isoform 4 (huPAR4) has an in-frame deletion of exon 6, which contributes the N-terminal sheet assembly to DIII, but retains the 3 C-terminal strands of DIII and the GPI anchor. How do these splicing isoforms impact suPAR's composition and function remains unclear. Notably, the currently available ELISA kits are all utilizing different antibodies developed against huPAR1, the canonical form, yet they present different suPAR levels (70). While they can detect the full length human suPAR and DIIDIII fragments derived from huPAR1, they cannot efficiently detect most of alternative human uPAR isoforms.

What is the implication of different human uPAR isoforms in kidney? Since muPAR2 is associated with FSGS-like kidney changes in our mouse model, it is possible that overexpression of one or more of these human isoforms could be associated with the development of FSGS. Among these human uPAR isoforms, huPAR3 seems to be the closest to muPAR2 at least structurally. It likely forms the same dimer assembly as we observe in the msuPAR2 structure (52). Indeed, transient expression of huPAR3 in C57BL/6J mice induced proteinuria (unpublished data). In no doubt, further studies on alternative human uPAR isoforms are required to determine their respective roles in the pathogenesis in kidney disease.

In summary, elevation of suPAR is a circulating risk factor for kidney disease, including FSGS. Certain form of suPAR (i.e., muPAR2) causes FSGS-like changes in mice. The complexity of suPAR derived from different enzymatic cleavage, transcriptional splicing and post-translational modification may explain suPAR/uPAR's multifaceted roles. New technologies such as single cell based deep sequencing and proteomic analysis should help understand their respective underlying mechanisms in different disease settings. Detecting the different circulating uPAR isoforms in human samples could possibly provide differentiating diagnostic or prognostic value with different suPAR related disease.

AUTHOR CONTRIBUTIONS

CW, RS, EH, and JR wrote the manuscript. All authors contributed to the article and approved the submitted version.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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New Endothelial Mechanisms in Glomerular (Patho)biology and Proteinuria Development Captured by Intravital Multiphoton Imaging

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In the past two decades, intravital imaging using multiphoton microscopy has provided numerous new visual and mechanistic insights into glomerular biology and disease processes including the function of glomerular endothelial cells (GEnC), podocytes, and the development of proteinuria. Although glomerular endothelial injury is known to precede podocyte damage in several renal diseases, the primary role of GEnCs in proteinuria development received much less attention compared to the vast field of podocyte pathobiology. Consequently, our knowledge of GEnC mechanisms in glomerular diseases is still emerging. This review highlights new visual clues on molecular and cellular mechanisms of GEnCs and their crosstalk with podocytes and immune cells that were acquired recently by the application of multiphoton imaging of the intact glomerular microenvironment in various proteinuric disease models. New mechanisms of glomerular tissue remodeling and regeneration are discussed based on results of tracking the fate and function of individual GEnCs using serial intravital multiphoton imaging over several days and weeks. The three main topics of this review include (i) the role of endothelial injury and microthrombi in podocyte detachment and albumin leakage via hemodynamic and mechanical forces, (ii) the alterations of the endothelial surface layer (glycocalyx) and its interactions with circulating immune cells in lupus nephritis, and (iii) the structural and functional remodeling and regeneration of GEnCs in hypertension, diabetes, and other experimental injury conditions. By the comprehensive visual portrayal of GEnCs and the many other contributing glomerular cell types, this review emphasizes the complexity of pathogenic mechanisms that result in proteinuria development.

Keywords: proteinuria, podocyte, glomerular endothelium, glycocalyx, immune cells, lupus nephritis

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INTRODUCTION

Proteinuria is a key clinical marker of kidney dysfunction, and it is commonly due to the disruption of the glomerular filtration barrier (GFB). In the healthy kidney, several traditional and newly recognized layers of the GFB help to prevent the filtration of plasma macromolecules. These include the endothelial surface layer (glycocalyx) and fenestrations, the glomerular basement membrane (GBM) and the podocyte foot processes including their slit diaphragm (1, 2).

Research in the past 20 years centered mainly on the podocyte and led to major advances in understanding the numerous pathogenic molecular mechanisms in the slit diaphragm largely thanks to advances in mouse and human genetics (3). However, intercellular communications in the glomerulus including the role of glomerular endothelial cells (GEnCs) in proteinuria development received much less attention. Although GEnC injury is a known early event preceding podocyte pathomechanisms in several glomerular pathologies (4, 5), the primary roles and contributions of GEnCs and their crosstalk to podocytes in proteinuria development are less understood.

Intravital imaging using multiphoton microscopy (MPM) has become a revolutionary new research approach in the field that in the past 20 years contributed significantly to our understanding of glomerular and tubular mechanisms of proteinuria (6–9). It should be noted that the earliest kidney MPM studies were technically limited to imaging the most superficial glomeruli in mostly non-physiological models using specific strains (e.g., Munich-Wistar-Fromter rat) or experimental manipulations to generate superficial glomeruli in mice (9–13). A few studies discussed in this review used these earlier approaches (14–16), therefore those results on GFB function should be interpreted with caution. However, recent major improvements in laser and microscopy technologies have made it possible to routinely image deep cortical glomeruli in the intact mouse kidney (17). Most of the new knowledge and topics reviewed here were derived from using these recent state-of-the-art MPM imaging approaches in intact adult mouse kidneys up to 8-months of age (**Figures 1**, **2**) (18–22). Importantly, MPM has been able to directly visualize (patho)physiological processes of the entire glomerulus, the many elements of the GFB simultaneously as parts of the whole functional unit rather than just focusing on a single cell type (12, 14). This integrative and holistic pathophysiology approach has successfully identified multiple levels of cell-to-cell interactions between individual cells and cell types of the GFB. Intravital imaging was able to shed light on the consequences of manipulating a single GFB layer on overall glomerular structure and function including albumin leakage (10, 12, 14, 15, 18, 20, 21). This review highlights important functions and roles of GEnCs in the development of glomerular injury and proteinuria based on recent *in vivo* MPM studies in both physiological and disease models (**Figure 1**). In addition, the targeting of newly discovered pathophysiological mechanisms for potential new regenerative therapeutic developments for proteinuric kidney diseases is also explored.

ENDOTHELIAL INJURY, MICROTHROMBI, HEMODYNAMIC, AND MECHANICAL FACTORS IN ALBUMIN LEAKAGE

GEnCs play key roles in the physiological function and maintenance of the healthy GFB and in the development of glomerular pathologies. The unique morphological and functional features of GEnCs include their flat and fenestrated profile that enables the normally enormous rate of glomerular plasma ultrafiltration (23), and the presence of a dense and negatively charged endothelial surface layer (glycocalyx) that constitutes a newly recognized layer of the GFB (2, 24). Rich in proteoglycans and secreted glycosaminoglycans (e.g., heparan sulfate and hyaluronic acid), the GEnC glycocalyx has important roles in several processes including restricting albumin passage through the GFB, binding chemokines and growth factors, and immune cell adhesion (2, 24, 25). Intravital MPM imaging approaches have successfully visualized these GEnC structures and functions in the intact living kidney including the bulk fluid flow in fenestrated capillaries (11, 26) and the presence and alterations in GEnC glycocalyx in various disease models (15, 19–21).

Regardless of the type of GEnC injury, capillary occlusion and reduced plasma flow (based on internal vascular obstruction) were the common pathological and hemodynamic features of the glomerular capillaries observed by MPM imaging. In the rat puromycin (PAN)-induced focal segmental glomerulosclerosis (FSGS) model this was due to shedding of individual GEnCs (likely the result of direct GEnC cytotoxicity) and the instantaneous formation of localized microthrombi in the affected capillary area (14). Similarly, laser-induced mild injury of single GEnCs immediately led to localized microthrombi formation that reduced capillary segment perfusion, blocked red blood cell passage but allowed diminished plasma flow (12). Temporary or permanent adhesion of circulating immune cell types triggered similar hemodynamic alterations (21, 27). Importantly, capillary segment obstruction and altered local plasma flow in all injury models triggered the development of albumin leakage through the GFB and proteinuria (12, 14, 21). As described in these earlier reports and exemplified in **Figure 2A** (also in **Supplementary Video 1** at https://figshare.com/s/0ca6f8d0dae48415ca01), robust albumin leakage into the Bowman's space can develop within 1 min of thrombotic occlusion of a few glomerular capillary segments. On one hand, the rapidly increasing GFB albumin permeability was causatively linked to mechanical factors in the capillary segments that localized pre-occlusion, such as increased capillary pressure, fluid filtration and shear stress of podocyte foot processes, and increased podocyte mechanical strain that ultimately led to podocyte detachment (**Figure 2A**) (14). On the other hand, the increased GFB albumin permeability in capillary segments that localized after the occlusion can be explained by reduced GBM compression [according to the gel compression model (28)] and/or the reduced capillary blood flow causing locally reduced filtration, which in turn can diminish the electrical field (streaming potential) that normally helps to restrict albumin passage according to the electrokinetic model (29, 30). Regardless of the multiple mechanistic alternatives listed above, the primary event was always GEnC injury underscoring the major importance of the glomerular endothelium in proteinuria development.

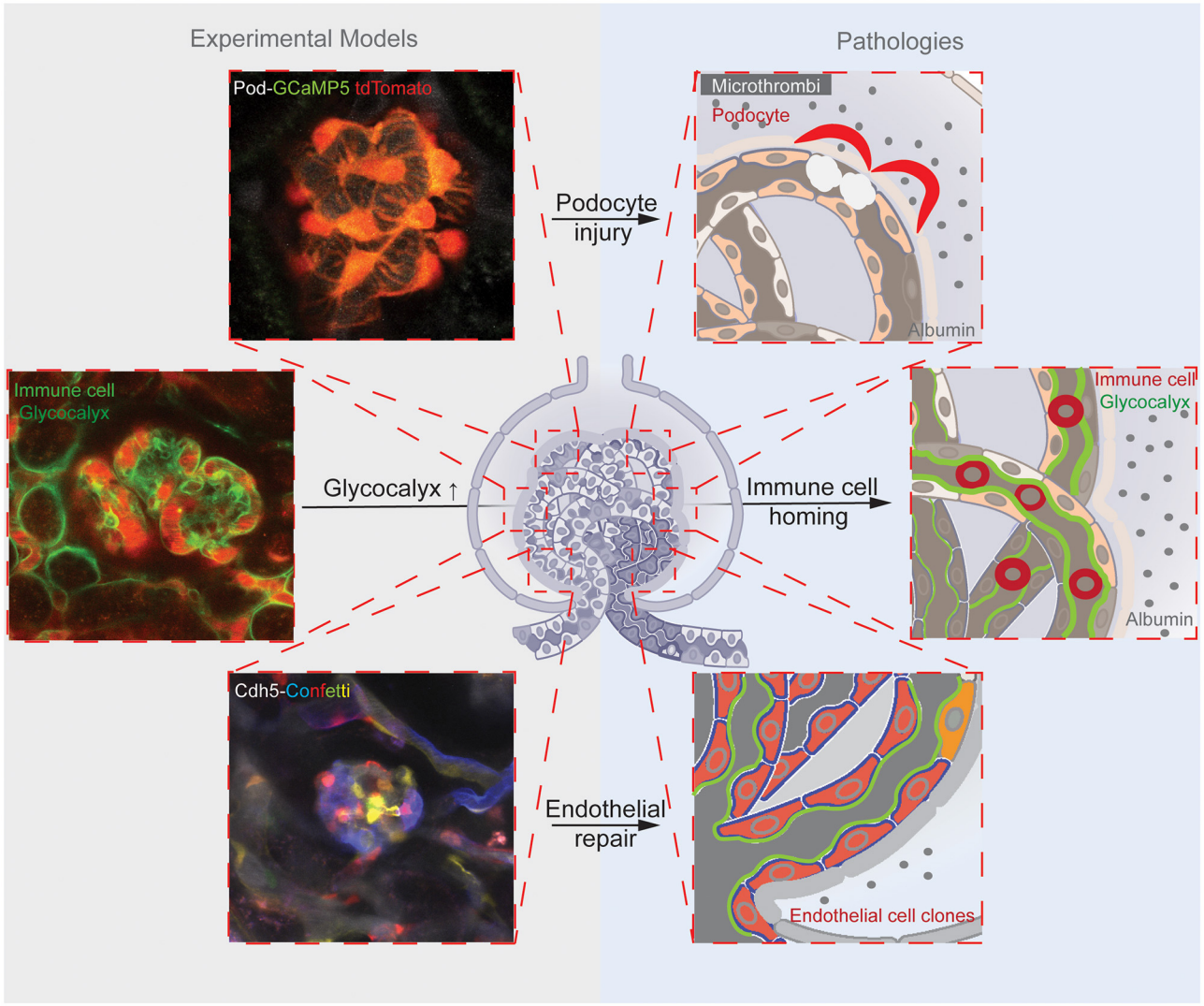

FIGURE 1 | Illustrations of the reviewed three main glomerular endothelial mechanisms (shown in the top, center, and bottom rows). Corresponding representative images of the experimental models (on the left) that were applied to study the pathological mechanisms (schematics on the right). The top panels illustrate the endothelial injury-induced formation of microthrombi and hemodynamic and mechanical GFB alterations leading to podocyte damage, and mice with podocyte-specific expression of the calcium reporter GCaMP5 (green)/tdTomato (red). The center panels show the endothelial surface layer (glycocalyx)-mediated glomerular homing of immune cells, and the labeling of the glomerular endothelial glycocalyx with FITC-WGA and immune cells with anti-CD44-Alexa Fluor 488 antibodies (green) and the circulating albumin with Alexa Fluor 594 (red). The bottom panels demonstrate clonal remodeling and functional regeneration of the glomerular endothelium by local endothelial precursor cells, and the genetic Cdh5-Confetti mouse model with multicolor fluorescent reporter (CFP/GFP/YFP/RFP, a.k.a. Confetti) expression in GEnCs.

ENDOTHELIAL SURFACE LAYER (GLYCOCALYX) CONTROLS GLOMERULAR HOMING OF IMMUNE CELLS AND PROTEINURIA

The glomerular endothelial surface layer (glycocalyx) is a newly recognized layer of the GFB and functions as a major determinant of GFB macromolecule permeability (2, 24). In addition, it is essential in immune cell adhesion (25). According to the existing paradigm, the negatively charged glycocalyx covering the GEnC fenestrations acts as a barrier against albumin filtration, and therefore treatment with glycocalyx degrading enzymes induces albumin passage across the endothelium (15, 31). On the other hand, components of the glycocalyx (e.g., heparan sulfate and hyaluronic acid) play well-known anchoring roles in immune cell homing, and therefore trigger local inflammation that increases albumin permeability (25). The key to understanding the ultimate role of the glomerular glycocalyx may be in the balance between these pro and anti-proteinuric functions.

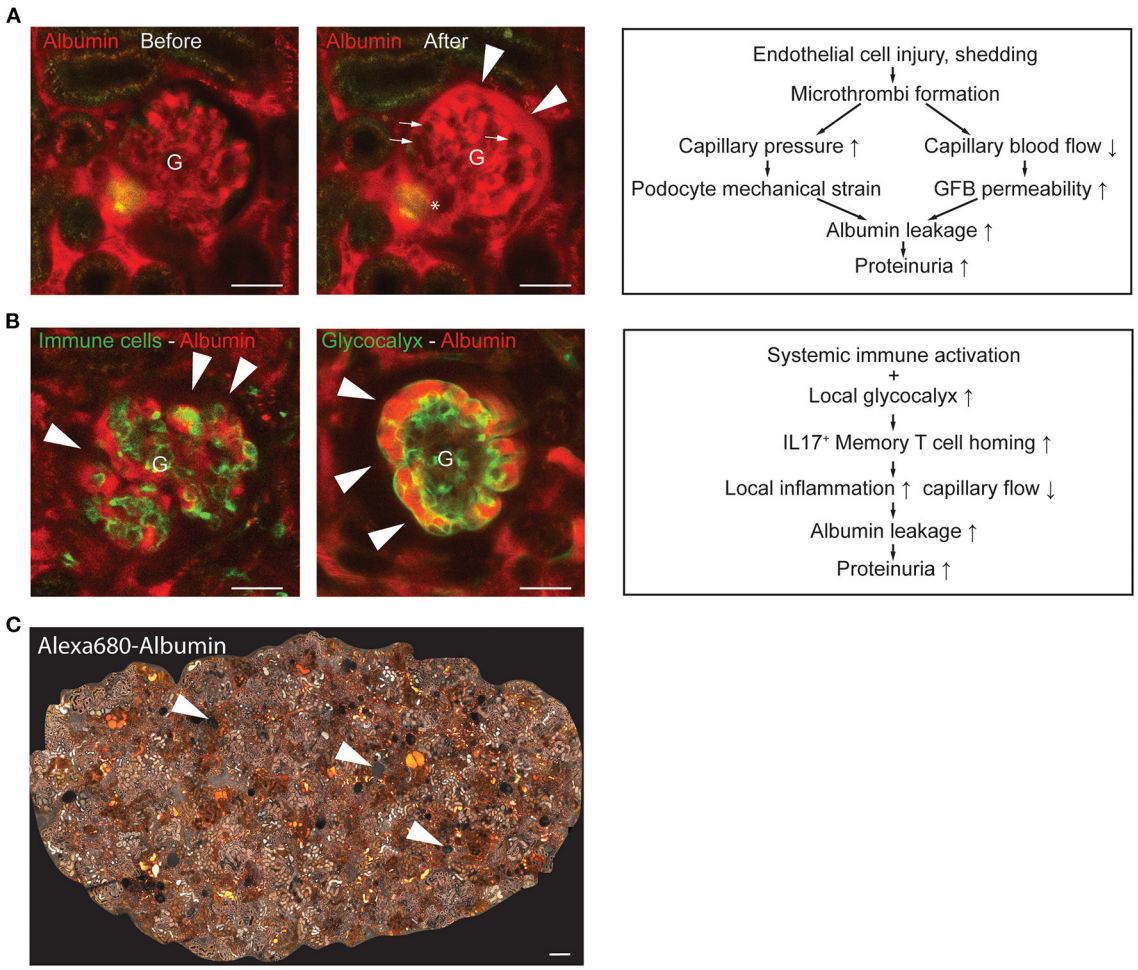

FIGURE 2 | Representative intravital MPM images of glomerular endothelial injury models and schematics of their underlying pathogenic mechanisms. **(A)** Laser-induced injury of GEnCs [injury site indicated by an asterisk (*)] triggered microthrombi formation in several glomerular capillary segments [dark, excluding the plasma marker Albumin-Alexa Fluor 594 (red), indicated by arrows] and robust albumin leakage into the Bowman's space (arrowheads). Note the normal flow of red blood cells (dark, plasma excluding) in glomerular (G) capillaries before GEnC laser injury (left panel), and the blockade of red blood cell passage due to microthrombi with only reduced plasma flow (intense red) in glomerular capillaries after laser injury (middle panel). The "after" image of the same glomerulus was acquired 1 min following the "before" image; the entire video sequence starting at the end of laser injury is shown in **Supplementary Video 1** available at https://figshare.com/s/0ca6f8d0dae48415ca01. Schematic illustration of the mechanisms involved in the development of GEnC injury-induced albumin leakage and proteinuria development (right panel). **(B)** Representative *in vivo* MPM images of the NZM.2328 BAFF transgenic mouse model of lupus nephritis (LN). Endogenous circulating $CD44^+$ immune cells were labeled with iv injected anti-CD44-Alexa Fluor 488 antibodies (green) and the circulating plasma albumin with Alexa Fluor 594 (red) (left panel). Note the leakage of plasma albumin into the Bowman's space (arrowheads) based on the light red color of the Bowman's space in contrast to the dark (albumin-free) Bowman's space shown in panel A above. Alternatively, the GEnC glycocalyx was labeled by iv injected FITC-WGA (green, arrowheads) (middle panel). Schematic illustration of the mechanisms involved in glomerular immune cell homing and proteinuria development in LN (right panel). **(C)** Representative tile scan image of the entire superficial cortical area of an 8-months old NZM.2328 mouse kidney injected with Albumin-Alexa Fluor 680 iv (shown in grayscale) illustrating the complexity of LN pathologies. These include glomerular albumin leakage (arrowheads, based on the light gray color of the Bowman's space), high albumin-containing tubule segments (intense white), and several focal fibrotic interstitial areas (lack of glomerular or tubular structures). Bars are 50 μm in **(A,B)** and 100 μm in **(C)**.

Our recent intravital MPM imaging study visually captured the essential role of the GEnC glycocalyx in the pathogenesis of lupus nephritis (21). Unexpectedly, MPM imaging found a robust accumulation rather than loss of the GEnC glycocalyx, and a high level of glomerulus-specific homing of $CD44^+$-$IL17^+$ activated memory T cells in two different proteinuric LN mouse models (**Figures 2B,C**) (21). This glycocalyx accumulation involved its hyaluronic acid component and appeared to be specific to LN, since other inflammatory conditions such as diabetes were associated with diminished glycocalyx as described before (16, 21). Glomerular immune cell homing, local inflammation, glomerular albumin leakage, and albuminuria observed in these LN mouse models were mediated via the binding of CD44 (expressed on the surface of activated memory T cells) to its ligand hyaluronic acid present in excess in the GEnC glycocalyx (21). Importantly, treatment with different glycocalyx degrading

enzymes reduced glycocalyx thickness back to normal levels (rather than completely eliminating it), disrupted immune cell homing, improved clinical LN including albuminuria and animal survival (21). One important conclusion from these studies was that GEnC glycocalyx thickness is not in linear relationship with proteinuria development, and too much of a generally protective mechanism (glycocalyx) can be pathogenic. In other words, the role of GEnC glycocalyx in proteinuria development is complex and involves both protective and pathogenic mechanisms. The other important outcome of this study was the efficient therapeutic targeting of the excess GEnC glycocalyx by hyaluronidase that may be further developed for LN.

ENDOTHELIAL REPAIR AND VASCULAR REGENERATION IMPROVE ALBUMIN LEAKAGE

Improving our understanding of the dynamics and mechanisms of GEnC turnover and repair after injury at the single-cell level in the intact living kidney over time is critically important for the development of mechanism-based regenerative therapeutic approaches for glomerular kidney diseases. The research technique of serial intravital MPM imaging of the same glomeruli in the same mouse kidney over several days and weeks has been developed and used earlier in combination with cell function and genetic cell fate tracking tools to examine glomerular tissue remodeling by podocytes (10) and cells of the renin lineage (22). This same approach was recently applied to GEnCs with single-cell resolution to shed light on the dynamic and functional endothelial remodeling mechanisms and vascular regeneration in healthy glomeruli and in disease models. Compared to the slow turnover observed in other organs and vascular beds, the rapid, and clonal expansion of single GEnC precursors were quantitatively visualized in response to hypertensive, diabetic, and laser-induced GEnC injuries (20). Interestingly, GEnC progenitor cells were locally residing at the glomerular vascular pole, mostly in the terminal afferent and/or efferent arteriole segments rather than derived from a circulating progenitor pool. Functionally, the newly and clonally remodeled (regenerated) glomerular capillary segments featured a lower amount of GEnC glycocalyx and a lower level of GFB albumin permeability compared to non-clonal segments. These morphological and functional features were consistent with functionally regenerated capillaries and/or with a less differentiated state of GEnCs. Activating and enhancing the function of this newly identified GEnC progenitor cell population may facilitate glomerular vascular regeneration in future therapeutic development.

DISCUSSION

The recent intravital MPM imaging studies reviewed here shed new light on the many important roles and contributions of GEnCs to glomerular pathobiology and proteinuria development. The numerous technical advances applied in these investigations were instrumental to successfully label and directly and quantitatively visualize using MPM imaging the glomerular endothelium at the single-cell level, their subcellular features and functions including glycocalyx output, immune cell interactions, fenestrations, ultrafiltration, and albumin permeability in the intact living kidney. The simultaneous imaging of the structure and function of all GFB layers provided visual evidence for the important and primary roles of the glomerular endothelium in several mechanisms of proteinuria development in addition to the well-known roles played by the GBM and podocytes. In addition, the many contributing factors that MPM imaging studies revealed, including microthrombi, locally altered hemodynamics and mechanical strain, glycocalyx, immune cell homing, and endothelial remodeling altogether emphasizes the complexity of pathogenic mechanisms that result in proteinuria. Finally, the newly identified GEnC molecular and cellular mechanisms are promising therapeutic targets for glomerular diseases.

AUTHOR CONTRIBUTIONS

GG, CJ, and JP-P wrote and prepared the manuscript for publication. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.765356/full#supplementary-material

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Mechanisms of Proteinuria in HIV

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Proteinuria is common in the setting of HIV infection, and may reflect comorbid kidney disease, treatment-related nephrotoxicity, and HIV-related glomerular diseases. The mechanisms of podocyte and tubulointerstial injury in HIV-associated nephropathy (HIVAN) have been the subject of intense investigation over the past four decades. The pathologic contributions of viral gene expression, dysregulated innate immune signaling, and ancestry-driven genetic risk modifiers have been explored in sophisticated cellular and whole animal models of disease. These studies provide evidence that injury-induced podocyte dedifferentiation, hyperplasia, cytoskeletal dysregulation, and apoptosis may cause the loss of glomerular filtration barrier integrity and slit diaphragm performance that facilitates proteinuria and tuft collapse in HIVAN. Although the incidence of HIVAN has declined with the introduction of antiretroviral therapy, the collapsing FSGS lesion has been observed in the context of other viral infections and chronic autoimmune disorders, and with the use of interferon-based therapies in genetically susceptible populations. This highlights the fact that the lesion is not specific to HIVAN and that the role of the immune system in aggravating podocyte injury warrants further exploration. This review will summarize our progress in characterizing the molecular mechanisms of podocyte dysfunction in HIVAN and other forms of HIV-associated kidney disease.

Keywords: podocyte, glomerular disease, HIVAN-associated nephropathy, APOL1, collapsing FSGS

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INTRODUCTION

In the four decades since the first cases of AIDS were reported in 1981, an estimated 77.5 million people have been infected with HIV and more than 34 million people have died from complications of HIV infection (1). Kidney disease emerged as an important complication of HIV in the early years of the epidemic, with the first reports of a unique pattern of collapsing focal segmental glomerulosclerosis (FSGS) with accompanying tubulointerstitial injury published in 1984 (2, 3). HIIV-associated nephropathy (HIVAN) quickly became the leading cause of end-stage kidney disease (ESKD) in people living with HIV (PLWH), demonstrating a marked predilection for individuals of African descent. Although the incidence of ESKD attributed to HIVAN plateaued in the United States following the widespread introduction of 3-drug antiretroviral therapy (ART) in 1997, HIVAN remains an important cause of kidney disease in the setting of untreated HIV infection (4). The original case series also reported a spectrum of immune complex glomerular lesions, and contemporary biopsy series continue to identify immune complex kidney diseases as one of the most common histologic diagnoses in PLWH. Other common causes of kidney disease in PLWH include ART toxicity and comorbid kidney disease due to traditional risk factors such as diabetes (4). As a result, kidney biopsy is often required for definitive diagnosis of proteinuric kidney disease in PLWH.

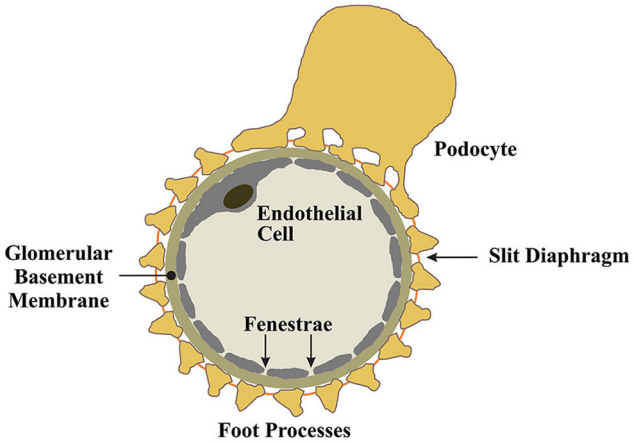

FIGURE 1 | Mechanisms of proteinuria in HIVAN.

The epidemiology of HIVAN suggested that both viral and host factors play a central role in pathogenesis. The development of HIVAN in HIV-transgenic mouse models has allowed for extensive investigation into the mechanisms of glomerular injury, proteinuria, and kidney failure in HIVAN, which will be the primary focus of this review.

HIV INFECTION OF THE KIDNEY

The emergence of HIVAN in the setting of AIDS and the decline in incidence of ESKD with the introduction of ART is consistent with a direct role for HIV in the pathogenesis of HIVAN. Early reports demonstrated the presence of HIV nucleic acids in renal epithelial cells (5–7); however, the absence of CD4, CXCR4, and CCR5 receptor expression on these cells (8, 9) implied the existence of a non-receptor mediated viral entry mechanism (10, 11). The subsequent identification of HIV-1 entry into human podocytes via lipid rafts (12) and by dynamin-mediated endocytosis (13–15) provided compelling evidence for non-canonical routes of viral particle entry. Although many questions remain, substantial progress has been made in characterizing the effects of HIV infection on podocyte physiology and function (**Figures 1**, **2**).

NORMAL PODOCYTE FUNCTION AT THE GLOMERULAR FILTRATION BARRIER

Podocytes are an essential cellular component of the tripartite glomerular filtration barrier (16). Podocytes are post-mitotic epithelial cells characterized by their highly specialized, actin-based membranous extentions known as foot processes (16). Podocytes envelop glomerular capillaries, adhering to the glomerular basement membrane (GBM) through a network of intermolecular interactions connecting foot processes to the GBM (16). Between interdigitating foot processes, a zipper-like assembly of proteins known as the slit-diaphragm functions as a molecular seive to provide charge and size selectivity for ultrafiltration (17–19). Podocytes produce the molecular constituents of the slit diaphragm and the expression of these proteins coincides with podocyte differentiation and maturation (20, 21). For example, the slit diaphragm protein nephrin, first identified in a Finnish cohort study of congenital nephrotic syndrome (17, 22), is produced by podocytes. The various roles of nephrin at the slit diaphram and as a modulator of prosurvival signaling in podocytes are well-documented (23–28) and will not be detailed in this review, however, it is clear that disease processes that impair podocyte nephrin expression, and other slit diaphragm components, result in podocyte dysfunction and drive the development of proteinuria and the FSGS lesion (29).

EFFECTS OF VIRAL GENE EXPRESSION ON PODOCYTE PHYISOLOGY AND FUNCTION

The collapsing FSGS lesion of HIVAN is characterized by podocyte dedifferentiation and hyperplasia, loss of podocyte maturity markers, foot process effacement and podocyte detachment, podocyte apoptosis, and heavy proteinuria (30–33). The cytotoxic effects of HIV gene expression in podocytes are well-established, and experimental models of HIVAN suggest that podocyte-restricted expression of viral proteins is sufficient to induce a dysegulated podocyte phenotype and the collapsing FSGS lesion (30, 31, 34–38). In particular, subtantial evidence exists for the roles of the HIV proteins Nef and Vpr in driving podocyte injury and dysfunction in HIVAN (**Figures 1**, **2**).

Nef

Nef is one of four accesory proteins (i.e., Nef, Vpr, Vif, and Vpu) expressed by HIV (39). Despite early descriptions of Nef as a negative regulatory factor of viral replication (40–42), subsequent studies demonstrated that Nef exerts a neutral or positive effect on viral replication in various cell types (39, 42). Although HIV does not appear to produce productive infection in podocytes (13, 43), Nef exerts a variety of deleterious effects on podocyte physiology and function that are unrelated to the enhancement of viral replication. In 2002, Husain et al. demonstrated that Nef expression induced the loss of maturity markers, proliferation and anchorage-independent growth in cultured human podocytes (36). These data were later validated in a murine model of podocyte-restricted Nef expression. Husain et al. showed that podocyte-specific expression of Nef caused the loss of maturity marker expression (i.e., synaptopodin and WT1), induction of STAT3 activtion, and expression of the proliferation marker Ki-67 (36). Notably, this model did not manifest the proteinuria or glomerular injury chracteristic of HIVAN, leading the authors to conclude that Nef may be responsible for the early molecular changes that drive podocyte injury in HIVAN. Sunamoto et al. demonstrated that Nef expression was necessary and sufficient to induce proliferation and dedifferentiation in murine podocytes (44). He et al. later provided mechanistic insights into the role of Nef in podocyte hyperplasia when they demonstrated that Nef stimulates pro-proliferative signaling through the Src tyrosine

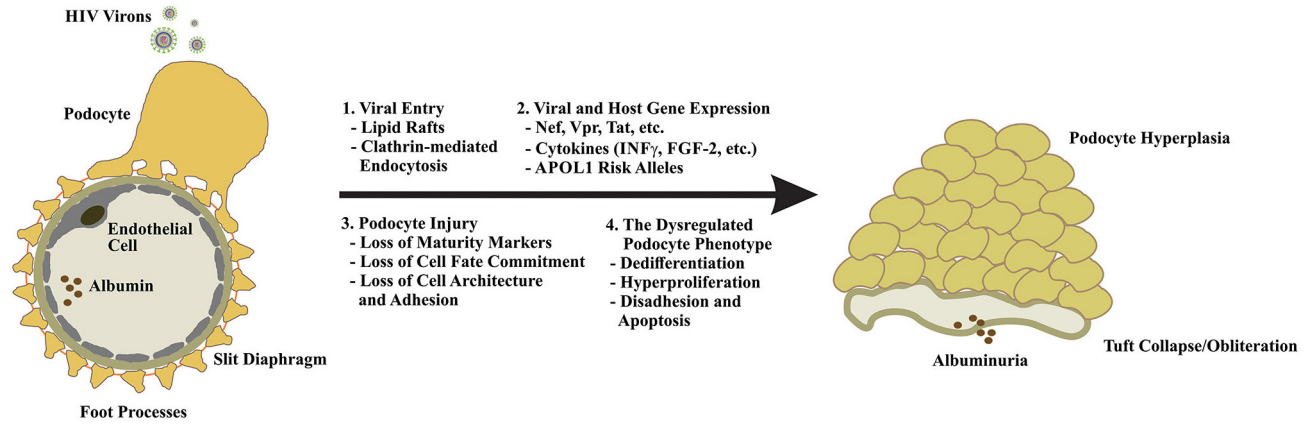

FIGURE 2 | Podocyte dysfunction in response to HIV infection is multifaceted. Because podocytes lack the receptors for canonical viral entry, viral particle entry via lipid rafts, and by clathrin-mediated endocytosis have been proposed (1). Upon entry, expression of viral genes and other mediators of innate immunity drive podocyte cytotoxicity and may enhance the expression of the high-risk APOL1 alleles in individuals of African descent to increase their risk of developing HIVAN (2). Podocyte injury manifests in the loss of maturity markers (e.g., nephrin, synaptopodin, WT1, CALLA, etc.), loss of cell fate commitment, and hyperplasia and the loss of cytoarchitecture and adhesion (3). The dysregulated podocyte phenotype appears to be irreversible and largely unresponsive to standard-of-care FSGS therapies highlighting the urgent need for a nuanced understanding of disease evolution, novel therapeutics, and early intervention (4).

kinase-dependent activation of Ras-c-Raf-MAPK1/2 and STAT3 signaling in conditionally immortalized human poocytes (45). The importance of STAT3 activation in podocyte hyperplasia was highlighted by the work of Feng et al. who demonstrated that reduction of STAT3 expression and activity ameliorated proteinuria, glomerulosclerosis, and tubulointersitial injury in a murine model of HIVAN (46). Similar findings were also reported by Gu et al. with STAT3 gene deletion in the same animal model (47). STAT3 is an established transcriptional regulator of molecules that drive cell-cycle re-entry and proliferation such as C-Myc, Cyclin D-1, CDC25A, and anillin (48, 49), supporting the hypothesis that STAT3 is a key regulator of podocyte proliferation in HIVAN. Several studies have also implicated Nef in the disruption of the podocyte cytoskeleton through various intermolecular interactions with actin and other key regulators of cytoskeletal dynamics (50–55). Other functions of Nef, such as its ability to interact with clathrin at the plasma membrane to disrupt endocytic trafficking, may also contribute to podocyte injury; however, this aspect of Nef signaling has not been documented in podocytes.

Vpr

The HIV accessory protein Viral Protein R (Vpr) has also been identified as a significant contributor to kidney injury in HIVAN. Like Nef, podocyte-restricted expression of Vpr in murine models established on the susceptible FVB/N background was sufficient to produce glomerular collapse and tubulointerstitial disease (38, 56, 57). Double transgenic expression of Vpr and Nef synergistically induced the full spectrum of podocyte injury, glomerular collapse, and tubulointerstitial diseased observed in human HIVAN (38, 57). In renal tubular epithelial cells (RTECs), Vpr has been shown to induce G2/M phase cell cycle arrest and dysregulation of cytokinesis (57–60). Vpr also induces apotosis in RTECs via the persistant activation of ERK MAP kinase and the upregulation of the ubiquitin-like protein FAT10 (61, 62). Less is known about the mechanisms of Vpr-induced podocyte injury. In 2014, Gbadegesin et al. demonstrated that the cytokinesis regulatory protein and pro-proliferative signaling molecule anillin, was upregulated in a murine model of podocyte-restricted Vpr expression (63). Anillin is an essential component of the cytokinetic ring and a driver of abnormal cellular proliferation in various malignacies (64–66). In the Vpr transgenic mouse, the upregulation of anillin in glomerular podocytes likely represents an accumulation of anillin in arrested cells or a cell-type specific derangement of cytokinetic drive and cell-cycle re-entry signaling.

Other Viral Proteins

The HIV regulatory protein Tat may also contribute to podocyte dysfunction in HIVAN. Tat is essential for HIV gene transactivation (67). In primary and conditionally immortalized podocytes, Conaldi et al. showed that Tat expression induced basic fibroblast growth factor (FGF-2)-driven hyperplasia, loss of maturity markers, cytoskeletal dysregulation, and impairment of permselectivity in a dose-dependent manner (43). Similar findings were later reported by Doublier et al. who showed that Tat exposure impaired the permeability of isolated glomeruli and reduced nephrin expression in conditionally immortalized human podocytes (68). Insights into the mechanisms of Tat-induced podocyte injury were provided by Xie et al., who reported that Tat targets to cholesterol-enriched lipid rafts, where it drives RhoA, matrix metalloproteinase-9 expression and FGF-2-mediated proproliferative signaling (69). Notably, murine models of podocyte-restricted Tat expression have failed to recapitulate the HIVAN phenotype (70). Overexpression of other HIV proteins such as Rev, Vif, and Vpu have not been associated with podocyte cytotoxicity and have not induced glomerular injury in murine models (70).

Contributions of the High-Risk Apolipoprotein L1 (APOL1) Alleles

The epidemiology of HIVAN is also consistent with a role for host genetic susceptibility, with a marked predilection for individuals of African descent. The discovery of high-risk variants in the *APOL1* gene provided evidence of a genetic contribution to the racial disparity (71). The G1 (rs4821481 and rs3752462) and G2 (rs71785313) *APOL1* variants were identified in an association analysis comparing 205 African-American individuals with non-familial, biopsy-proven FSGS and 180 healthy African-American controls. *APOL1* encodes apolipoprotein L1, a trypanolytic serum factor that confers resistance against the parasitic infection that causes African sleeping sickness (71). The G1 and G2 *APOL1* variants are found exclusively in individuals of recent African descent and confer resistance against a deadly subspecies of Trypanosoma that is normally resistant to lysis by wild-type APOL1. Carrying two *APOL1* variants significantly enhances the risk of developing HIVAN in untreated HIV-infected individuals and explains up to 35% of the disease (72, 73). Our understanding of the mechanisms of APOL1-mediated kidney injury is rapidly increasing. In 2016, Olabisi et al. identified direct cytotoxic effects of the APOL1 proteins via the formation of cation permeable pores that disrupt potassium flux and lead to cellular swelling and death (74). Subsequently, Jha et al showed that the APOL1-mediated enhancement of potassium efflux induces proinflammatory cytokine expression, activation of the NLRP3 inflammasome and cellular pyroptosis (75). Other mechanisms of APOL1-mediated cellular injury have been uncovered. For example, Ma et al demonstrated that the APOL1 renal risk variants induce mitochondrial fission, reduce mitochondrial repiratory capacity, respiration rate and membrane potential (76, 77). Expression of the G1 and G2 variants also induced dysregulation of endosomal trafficking and lysosomal acidification in Drosophila and Saccharomyces (78). Additionally, the G1 and G2 renal risk variants have been shown to enhance the expression of miR193a, a negative regulator of autophagy (79). Consistent with an impairment in autophagy, Wen et al showed that overexpression of the APOL1 risk alleles induce endoplasmic reticulum stress in culture human podocytes (80). Upregulation of miR193a has also been shown to impair adherens complex stability, disrupt actomyosin cytoskeletal organization, reduce nephrin expression and promote dedifferentiation in podocytes (79, 81).

In the context of HIVAN, elaboration of interferon-γ (INF-γ) and other circulating mediators of innate immunity signaling appear to drive *APOL1* gene transcription (82, 83). *In vitro*, the cytotoxicity of the high-risk *APOL1* variants is dose-dependent, suggesting that any process that enhances the expression of the *APOL1* renal risk alleles may provoke glomerular injury (84). This finding may, at least partially, explain why HIV is among the strongest promoters of glomerular disease in the setting of the high-risk *APOL1* genotype, which has been associated with up to 89-fold increase in odds of HIVAN (85). Notably, collapsing glomerulopathy has been observed in individuals of African descent treated with interferon therapies and following viral infection with Parvovirus B19, CMV, EBV, HTLV1, Coxsackie B, Dengue, Zika, and most recently, SARS-CoV-2 (86–88). Small studies have demonstrated an association between the high-risk *APOL1* genotype and the development of collapsing FSGS in the setting of COVID-19 (89) (**Figures 1**, **2**).

MECHANISMS OF PROTEINURIA IN OTHER KIDNEY DISEASES IN PLWH

HIV infection, by a variety of intracellular and systemic influences on podocyte physiology, perturbs cellular fate commitment, gene expression, and viability to promote development of the collapsing FSGS lesion of HIVAN. A nuanced understanding of the processes that drive podocyte injury in HIVAN may uncover novel therapeutic targets for treatment of other glomerular diseases. Prompted by epidemiologic studies demonstrating accelerated progression of kidney disease in the setting of HIV and diabetes (90), Mallipattu et al. demonstrated that the induction of diabetes with streptozotocin resulted in more prominent histologic changes in HIV-transgenic mice compared to wild-type littermates (91). These findings were confirmed in a subsequent study using podocyte-specific transgenic mice with low HIV transgene expression to more closely reflect the current clinical status of ART-treated individuals (92). In this model, HIV and diabetes had a synergistic effect on the expression of Sirtuin-1 deacetylase, suggesting a potential therapeutic role for Sirtuin-1 agonists.

A lack of animal models has slowed progress toward elucidating the pathogenesis of immune complex glomerular disease in PLWH. This has been compounded by the diverse spectrum of glomerular lesions that occur in this setting and that have been considered together in most clinical studies. Small but rigorous human studies have suggested a role for immune complexes directed against HIV antigens in the pathogenesis of immune complex kidney disease (93). Because podocytes have been shown to play a role in the clearance of immune deposits (94), it is possible that HIV-induced podoycte damage also promotes immune complex kidney disease in PLWH.

Kidney injury due to the antiretroviral agent tenofovir disoproxil fumarate (TDF) may also present with proteinuria, although this is typically low molecular weight proteinuria rather than albuminuria. Tenofovir is a nucleotide analog that is chemically related to the older antiviral agents cidofovir and adefovir, both of which are known to exhibit dose-limiting proximal tubular toxicity. The first approved tenofovir prodrug, TDF, has been associated with proximal tubulopathy and non-albumin proteinuria. Although the mechanism of proximal tubular cell injury has not been fully elucidated, it is thought to involve mitochondrial dysfunction as a result of the weak inhibition of mitochondrial DNA polymerase gamma (95, 96). Tenofovir is eliminated by glomerular filtration and active proximal tubular cell secretion, and an increase in intracellular concentration due to increased plasma concentration, decreased glomerular filtration, or impaired apical transport of tenofovir is thought to increase the risk of proximal tubular cell dysfunction or injury. Although it is possible that HIV-induced cell damage

promotes tenofovir toxicity, non-albumin proteinuria has also been observed with the use of TDF for HIV pre-exposure prophylaxis in HIV-negative individuals (97). A newer prodrug, tenofovir alafenamide, is effective at lower plasma concentrations and may reduce the risk of tenofovir toxicity, although longer followup is needed.

Despite some risk of nephrotoxicity with tenofovir and other antiretroviral agents, the use of ART for treatment and prevention of HIV infection is currently the most effective way to mitigate the myriad pathogenic effects of HIV on the kidneys. While the incidence of advanced kidney disease due to HIVAN has decreased with the use of ART, HIVAN remains a valuable model for the study of podocyte injury and APOL1-induced glomerular disease.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The apoM/S1P Complex—A Mediator in Kidney Biology and Disease?

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Kidney disease affects more than 10% of the population, can be both acute and chronic, and is linked to other diseases such as cardiovascular disease, diabetes, and sepsis. Despite the detrimental consequences for patients, no good treatment options directly targeting the kidney are available. Thus, a better understanding of the pathology and new treatment modalities are required. Accumulating evidence suggests that the apolipoprotein M/sphingosine−1-phosphate (apoM/S1P) axis is a likely drug target, but significant gaps in our knowledge remain. In this review, we present what has so far been elucidated about the role of apoM in normal kidney biology and describe how changes in the apoM/S1P axis are thought to affect the development of kidney disease. ApoM is primarily produced in the liver and kidneys. From the liver, apoM is secreted into circulation, where it is attached to lipoproteins (primarily HDL). Importantly, apoM is a carrier of the bioactive lipid S1P. S1P acts by binding to five different receptors. Together, apoM/S1P plays a role in several biological mechanisms, such as inflammation, endothelial cell permeability, and lipid turnover. In the kidney, apoM is primarily expressed in the proximal tubular cells. S1P can be produced locally in the kidney, and several of the five S1P receptors are present in the kidney. The functional role of kidney-derived apoM as well as plasma-derived apoM is far from elucidated and will be discussed based on both experimental and clinical studies. In summary, the current studies provide evidence that support a role for the apoM/S1P axis in kidney disease; however, additional pre-clinical and clinical studies are needed to reveal the mechanisms and target potential in the treatment of patients.

Keywords: apolipoprotein M, sphingosine-1-phosphate, kidney failure, proteinuria, chronic kidney disease, lipoproteins

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INTRODUCTION

The etiology leading to the development of kidney disease is diverse, but the disease can be divided into two categories, acute or chronic kidney disease, which are both characterized by a decrease in kidney function. Chronic kidney disease is estimated to affect more than 10% of the population worldwide, and up to 5% develop acute kidney injury during hospitalization. Unless resolved, the disease will result in a progressive loss of kidney function and, eventually, the need for dialysis or kidney transplantation. Kidney disease is also linked to other diseases, such as cardiovascular diseases, diabetes, and sepsis. Despite the detrimental consequences for patients, no good treatment options directly targeting kidney function are yet available. Thus, a better understanding of the pathology and new treatment modalities are required.

Apolipoproteins (apo)s are found associated with lipoproteins and assist with the structural stability of the particles as well as the formation, secretion, and uptake of lipoproteins via receptor-mediated pathways. Furthermore, some apos are also involved in inflammation and oxidation and function as chaperones of vitamins or bioactive lipids among others (1–5). So far, apoA through R have been discovered. ApoM was discovered in 1999 by Xu and Dahlbäck, and it was later revealed that apoM acts as the principal carrier of S1P in plasma (5, 6). Since its discovery, apoM has been suggested to be involved in mechanisms such as lipoprotein turnover, especially LDL, and to have anti-inflammatory and atheroprotective effects in part via an improved HDL cholesterol efflux capacity and anti-oxidative effect (7–10). Also, the apoM/S1P complex is involved in maintaining a normal endothelial cell barrier and participates in the regulation of triglyceride metabolism (5, 11). Thus, mice that do not express apoM have increased vessel permeability, increased amounts of brown adipose tissue, and improved triglyceride turnover (5, 11). In contrast, mice overexpressing apoM have delayed triglyceride turnover (12). In addition, apoM has been linked to diabetes [recently reviewed by Christoffersen (13)] and sepsis (14, 15). In a recent study, it was also shown that low apoM levels are associated with adverse outcomes in patients with heart failure (16). Finally, accumulating evidence points toward a role for apoM in fibrosis formation both in the liver and lungs (17, 18). However, the role of apoM in kidney biology and disease has received much less attention. The increasing knowledge from other studies related to different organs or diseases suggests that apoM might also play a role in the pathology of kidney disease. This will be discussed further in this review.

APOM AND LIPOPROTEINS

Regulation of ApoM Expression

ApoM is primarily expressed in the liver and kidneys but has also been detected in the intestine, and recent studies suggest that apoM is also expressed in brain endothelial cells and adipocytes (19–22). The apoM gene is localized within the MHC class III region of the genome containing genes involved in the immune system, and it is therefore speculated that apoM might be regulated by inflammatory stimuli. This link has been supported by a study that showed that inflammation results in decreased apoM expression (23). Furthermore, several cytokines [e.g., Transforming growth factor alpha (TGFα), Transforming growth factor beta (TGFβ), Epidermal growth factor (EGF), and Hepatocyte growth factor (HGF)] downregulate apoM expression in liver cells, with TGFβ having the most pronounced effect (24). In contrast, Interleukin 1 alpha (IL-1α) and tumor necrosis factor alpha (TNFα) did not affect apoM expression in HepG2 cells, whereas platelet-activating factor (PAF) stimulation increased apoM expression (25).

ApoM expression in the liver is also regulated by transcription factors and hormones [reviewed in detail by Ren (26)]. Stimulation of HepG2 cells with propofol, a commonly used anesthetic, resulted in a ∼3-fold upregulation of apoM expression. This increase is seen concomitantly with an increase in the transcription factors HNF-1α and FOXA2, suggesting that they are both involved in regulating apoM expression (27, 28). A role for HNF-1α is further supported by the finding that HNF-1α deficient mice have decreased apoM expression (29), while Wolfrum et al. have shown that inactivation of FOXA2 leads to lower apoM levels (30). In contrast, stimulation of the transcription factor LXR with the artificial ligand T0901317 results in decreased apoM expression in the liver both *in vivo* and *in vitro* (31, 32), although the same LXR activation leads to increased apoM expression in the intestine (20).

Leptin and insulin seem to be the two most important hormones in the regulation of apoM expression. In humans with hyperinsulinemia, plasma apoM is reduced by ∼10% (33, 34), while experimental models with hyperinsulinemia display a reduction in apoM levels of ∼50% (35). In addition, Xu et al. demonstrated that insulin stimulation of HepG2 cells leads to a dose- and time-dependent downregulation of apoM (36). It is plausible that this is mediated by insulin-regulated FOXA2 activation (30, 37). Leptin plays an important role in fat metabolism and appetite regulation, and mice with leptin deficiency develop experimental metabolic syndrome and insulin resistance. These mice also have markedly lower apoM levels compared to controls, which is normalized by leptin replenishment (38). In contrast, leptin stimulation of HepG2 cells *in vitro* decreases apoM expression (39). The reason for this apparent discrepancy between the *in vivo* and *in vitro* roles of leptin is unclear and requires further investigation. However, it could at least to some extent, reflect the fact that leptin is a multifunctional hormone that affects many different metabolic pathways *in vivo*, which indirectly modulates apoM expression.

The current knowledge on the regulation of apoM expression suggests the existence of a rather complex system with interactions between transcription factors and hormones. How the different modulators affect each other needs to be further assessed, as well as the potential differences between liver and kidney-derived apoM. To date, no knowledge is available on the specific regulatory mechanisms of apoM expression in the kidney.

ApoM and Triglyceride Rich Lipoproteins

ApoM is 25 kDa, a member of the lipocalin protein superfamily, and is characterized by an antiparallel β-barrel structure that forms a binding pocket for small hydrophobic molecules (40, 41). In addition, the first 20 amino acids of the apoM protein form a hydrophobic α-helix containing a signal peptide (41). The signal peptide anchors the apoM protein into the phospholipid layer of lipoproteins (42, 43). In 2011, Christoffersen et al. showed that apoM can bind and act as a transporter of S1P (4, 5). ApoM can also bind retinoic acid and retinol in the hydrophobic binding pocket, but the physiological relevance of this is still unexplored (4).

The liver is the primary source of plasma apoM, while the contribution of plasma apoM from the kidneys and other cell types is small, if present. Importantly, apoM is one of the few apolipoproteins, where the hydrophobic signal peptide is not cleaved off during protein maturation. This means that apoM is not present in its free form in the circulation but is instead

associated with lipoproteins via its retained signal peptide (42, 43). Thus, 95% of plasma apoM is associated with HDL, but apoM is also present in LDL, VLDL, and chylomicron particles. The relatively low concentration of apoM in plasma (∼0.9 μM) means that only ∼5% of the HDL particles carry an apoM molecule, while ∼2% of LDL particles carry them (44). ApoM is a promiscuous protein that exchanges between lipoproteins in the blood (7, 45). This contrasts with most apolipoproteins that are normally associated with exclusive lipoprotein class (es). The precise mechanism for the exchange of apoM is not clear, but seems to be driven by the availability of different lipoprotein subtypes (7, 45).

Plasma apoM is positively associated not only with the concentration of cholesterol in HDL particles but also with the cholesterol concentration of LDL particles in humans (34). In mouse studies, overexpression of apoM leads to an increased plasma cholesterol concentration, whereas the absence of apoM results in decreased cholesterol concentration (8). At the same time, LDL receptor knockout mice have increased plasma concentrations of apoM, which is also the case in patients carrying single nucleotide polymorphisms in the LDL receptor or apoB (7, 45). Thus, although only a minor portion of apoM is associated with LDL particles, apoM-containing lipoproteins are dependent on the LDL receptor for clearance. Such a link was supported by data reported by our group, who showed that the turnover of apoM-enriched HDL particles injected into LDL receptor knockout mice is slower than that in WT mice (7). In contrast, apoM-containing VLDL/LDL particles had reduced turnover compared to apoM-free VLDL/LDL particles in LDL-receptor-deficient mice, suggesting that apoM may regulate clearance of VLDL/LDL via pathways other than LDL-receptor-mediated uptake.

To date, the role of apoM in triglyceride-rich lipoprotein metabolism in organs other than the liver has not gained much attention. However, apoM is expressed in the intestine and may play a role in triglyceride and chylomicron processing (12, 20). Furthermore, the kidney expresses other apolipoproteins such as apoB100, an apolipoprotein important for LDL and VLDL formation (46). At this point, it is unknown whether apoM expressed in the kidney is similarly involved in the processing of triglycerides containing lipoproteins from the kidney and requires further investigation.

ApoM and HDL Particles

Due to the hydrophobic nature of the signal peptide, apoM most likely needs a close association with some form of phospholipid layer to be secreted, but the mechanism for this is still unclear. Studies in HEK293 cells and primary hepatocytes overexpressing apoM showed that the signal peptide retains apoM in the intracellular compartment, while cleavage of the signal peptide results in a higher secretion rate of apoM (47, 48). Furthermore, incubation of HEK293 cells overexpressing apoM with serum or HDL stimulates native apoM that are found localized in HDL particles in the medium (47). A similar mechanism was recently found in a study of brain endothelial cells (21). This suggests that apoM can be secreted from cells to lipoprotein particles already present in the plasma. However, how apoM is transported through different intracellular organelles and via the cell membrane to HDL particles, and whether stimulants other than HDL can mediate the secretion, are currently not clear.

While HDL seems to stimulate the secretion of apoM, conversely, apoM also affects HDL biology. However, the data published so far are somewhat contradictory. In brief, nascent HDL particles are primarily formed in the liver in a process where apoAI, the principal apolipoprotein constituent of HDL particles, forms a scaffold at which phospholipids and minor amounts of cholesterol can be associated. This results in the formation of a sub-population of HDL particles called pre-β-HDL (49), which are then secreted from the liver for further maturation in the circulation. These mature HDL particles differ in apoAI content, particle size, and electrophoretic mobility. A study by Wolfrum et al. showed that downregulation of apoM in the liver results in decreased apoAI levels and a lack of pre-β-HDL formation, while there is an accumulation of large HDL particles (10). A similar pattern was seen in HNF-1α deficient mice. These mice have no apoM expression and large buoyant HDL particles (29, 50). In contrast, a subsequent study by our group did not find any obvious difference in HDL particle size in either apoM knockout or apoM transgenic mice (8). In support of this, Mulya et al. have shown that apoM is not necessary for pre-β-HDL formation but is needed to form larger sized particles (51), while Liu et al. showed that the signal peptide is involved in the formation of larger HDL particles (48). Finally, overexpression of apoM in primary hepatocytes induces the formation of larger apoM-enriched HDL particles (52).

In summary, the mechanisms leading to apoM secretion are still poorly understood, but seem to be at least partially induced by HDL particles. At the same time, the predominant data available suggest that apoM plays a role in the formation of larger HDL particles; however, further studies are needed to clarify the mechanism. Finally, how these observations influence and modulate the release of apoM from the kidney remains unknown.

S1P EXPRESSION, SECRETION AND SIGNALING

S1P is synthesized from ceramide using sphingosine as an intermediate molecule. The final step of the synthesis is controlled by one of two sphingosine kinases (SPHK1 and 2), both of which are widely expressed in the body. Most cell types can produce S1P, but the S1P concentration is generally low in tissues except the blood. The plasma concentration of S1P ranges from 0.1 to 0.8 μM (53) and is primarily derived from the red blood cells but also from endothelial cells, thrombocytes, mast cells, macrophages, and thrombocytes (54–56). The secretion of S1P from red blood cells is facilitated mainly by the major facilitator superfamily transporter 2b (Mfsd2b). Plasma S1P levels are reduced by 50% in Mfsd2b knockout mice, while the S1P content in red blood cells is markedly increased (57). In addition, several other transporters, including spinster 2, PLTP, and several ABC transporters, have been shown to mediate the secretion of S1P [extensive reviewed by Thuy (58)].

In plasma, several candidates have been suggested as acceptors of S1P, such as apoM, apoAIV, and albumin. Thus, S1P is primarily associated with apoM (65%), and 30% is associated with albumin (5, 59, 60). A more recent study suggested that apoAIV can act as an S1P carrier in the absence of apoM and albumin (61). A study by Sutter et al. showed that apoM-enriched HDL particles enhance the S1P efflux from erythrocytes, while the efflux was comparable when stimulating the release of S1P by, respectively, HDL from apoM knockout mice and WT mice (62). Similar results were obtained by Christensen et al., showing that HDL is more potent than albumin in stimulating S1P release from erythrocytes, and this is enhanced when HDL contains apoM (63). Notably, apoM expression in tissues also seems to enhance S1P expression and secretion. Thus, overexpression of apoM in the liver leads to a higher S1P concentration in both liver tissue and plasma, without affecting the S1P levels in red blood cells, suggesting that S1P can be secreted with apoM from tissues expressing apoM (52, 64). This is supported by studies in HepG2, HeLA, and Raw264.7 cell lines where overexpression of apoM increased the intracellular content of S1P in all three cell types, while secretion was only induced in HepG2 and HeLa cells (64). Furthermore, introduction of a cleavage site for the signal peptide in apoM does not affect S1P expression in hepatocytes, but the secretion of the apoM/S1P complex is increased in cells expressing apoM with the cleavage site (48). Whether other apoM-independent mechanisms mediate S1P release from tissues, such as hepatocytes, remains undetermined. However, pharmacological inhibition of ceramide synthesis leading to increased S1P levels in hepatocytes results in the accumulation of S1P in the cells, suggesting that apoM is rate-limiting for S1P secretion, at least in hepatocytes (48, 52). However, additional studies are needed to reveal the precise mechanism of apoM-induced S1P expression and secretion.

S1P acts as both an intracellular and an extracellular signaling molecule. While the precise mechanism and signaling pathway of intracellular signaling are still only ambiguously described, extracellular signaling is mediated by binding to one of 5 G protein-coupled S1P receptors [sphingosine-1-phosphate receptor 1–5 (S1PR1-5)]. S1P is involved in multiple functions in the body, such as vascular maturation, lymphocyte trafficking, endothelial barrier functions, cell proliferation, and survival. This complexity is mainly thought to arise from the unique expression pattern of S1PRs in different organs and the Ga subunits they activate. Thus, S1PR1 acts via Gi, S1PR2, and 3 via Gi, Gq, or G12/13, while binding to S1P4–5 activates Gi or G12/13. In general, S1PR1 and 3 are believed to mediate similar responses, S1P2 counteracts S1PR1 signaling, while less is known about S1P4 and 5.

Notably, accumulating data suggest that the cellular response to S1P differs depending on whether S1P is bound to apoM or albumin (65). S1P bound to apoM promote a more sustained effect on barrier function compared to S1P bound to albumin, due to a reduction in S1P1 degradation (66). Further, only apoM bound S1P seems to attenuate the endothelial inflammatory response to tumor necrosis factor α, and while albumin bound S1P promote lymphocyte egress, apoM bound S1P inhibits this process (67, 68). The complexity is further highlighted by a study showing that while physiological concentrations of S1P preserve endothelial function, excess levels of S1P results in a dysfunctional endothelial barrier via activation of S1PR2 (69).

KIDNEY DERIVED APOM—A MEDIATOR OF S1P SEQUESTERING OR A MEDIATOR OF S1P SIGNALING IN THE KIDNEY?

In 2003, Zhang et al. showed that apoM is highly expressed in proximal tubular epithelial cells in kidneys (19). Despite this, the functional role of apoM in kidney biology remains unclear. However, it is hypothesized that apoM can be secreted from proximal tubular cells to the pre-urine. In the pre-urine, apoM may bind otherwise excreted molecules such as S1P and sequester them from excretion by re-uptake into the proximal tubular cells via binding to the megalin receptor (*Lrp2*). This hypothesis is supported by studies showing that apoM can bind to the megalin receptor (70) and that apoM is present in the urine of mice with proximal tubular-specific megalin receptor deficiency, but not in that of WT mice (62, 70). The importance of megalin as a central mediator of apoM uptake in the proximal tubular cells is further supported by a study showing that lack of either the chloride channel ClC-5 (*Clcn5*) or cystinosin (Ctns), which leads to aberrant megalin receptor function, results in excretion of apoM into the urine (62).

What happens to apoM and the molecules that have been sequestered after re-uptake into the proximal tubular cells is even less studied. Likely, molecules such as S1P, which are sequestered in the pre-urine, are released into the peritubular capillaries and thereby returned into the circulation. Whether apoM follows the same route into the circulation or is degraded upon re-uptake or re-shuffled to the pre-urine is unknown. Megalin deficiency in proximal tubular cells and, thereby, loss of apoM to the urine does not affect plasma apoM levels (62), suggesting that apoM is not secreted to the basolateral site of the proximal tubular cells facing the peritubular capillaries. However, these data are only based on western blotting, and could, thus, also reflect that the amounts secreted are below what is possible to detect with such techniques.

Due to the hydrophobic signal peptide, secretion of apoM from the proximal tubular cells would require that apoM is either intracellularly lipidated or that it associates with an intra- or extracellular soluble carrier protein. Faber et al. reported that apoM in the urine of megalin-deficient mice is found in particles that are larger than apoM alone but smaller than HDL particles and devoid of apoAI (70). However, what this particle consists of remains to be determined. Notably, apoM secreted from the liver is already present in the plasma. Thus, the apoM detected in urine from mice with aberrant megalin function could originate from apoM filtered from plasma via the glomeruli. Further supporting this hypothesis, ApoAI and pre-β HDL can be filtered by glomerular capillaries (71). However, apoM was not found to be associated with pre-β HDL in plasma but instead bound to larger HDL particles that likely do not pass the filtration barrier (8, 51, 72). In addition, apoM-containing particles in the urine from megalin-deficient mice do not contain apoAI, further

indicating that apoM detected in urine does not originate from plasma but likely from the proximal tubular cells or other cells in the nephron.

The role of kidney-derived apoM as a scavenger of hydrophobic molecules is the only hypothesis put forward for kidney-derived apoM, but other functions may also exist. The accumulation of lipids in non-adipose tissue can be detrimental. This is also the case for the kidney, where lipid accumulation may lead to kidney injury and disease (73). ApoB, the primary apolipoprotein in LDL particles, is expressed in the kidney by proximal tubular epithelial cells, and Krystanek et al. showed that the kidney is able to produce lipoprotein-like apoB-containing particles with a density similar to that of LDL (46). Importantly, knockout of apoB expression leads to the accumulation of triglycerides in the kidney cortex. As apoM is expressed in the same cells as apoB and play a role in metabolism of intestinal derived apoB- and triglyceride-rich lipoproteins (12), an obvious hypothesis is that apoM plays a role in the formation and/or secretion of these particles. If so, apoM could be an important player in regulating lipid metabolism in the kidney, and downregulation could lead to lipid accumulation in kidney cells. However, this is purely speculative and needs to be addressed.

In line with the elusive biological role of apoM in the kidney, several basic features of apoM expression and regulation are still unknown. Thus, whether apoM is expressed in other cell types in the kidney in addition to the expression in the proximal tubular cells have not yet been determined. One study reported that apoM is expressed by mesangial cells (74), but further studies are needed to confirm this finding and determine the level of expression as well as to evaluate its function. In addition, a systematic analysis of the possible expression of apoM in different kidney cells is required. Moreover, no studies have addressed whether the expression of apoM in the kidney is regulated by the same mechanisms as in the liver. The HNF1α transcription factor is expressed in the proximal tubular epithelial cells, as well as LXR, whereas FOXA2 is not expressed in the adult mouse kidney (75–77). This suggests that the regulation differs, at least to some degree, but this is purely speculative and needs to be investigated.

S1PR1-S1PR4 is expressed in the kidney, with 1 and 3 having the highest expression, followed by receptor 2, while 4 had the lowest expression level. Divergent data exist for receptor 5, with some studies finding it expressed while other studies do not (78, 79). As the kidney is a complex organ with many different cell types, structures, and functions, the location of the expression might be highly relevant for the functional role of S1P. So far, no systematic analysis of the expression pattern of the different receptors in the kidney and their relative expression has been published. However, *in vitro* studies have shown that S1PR1, S1PR2, S1PR3, and S1PR5 are expressed in glomerular mesangial cells (80, 81). All five S1PRs are expressed in glomerular endothelial cells (82) and proximal tubular cells (83, 84), while S1PR1 to S1PR4 are detected in immortalized mouse podocytes (85). Thus, while the expression of apoM seems to be cell-specific, S1PR1-S1PR3 is expressed in both the renal cortex as well as in the inner and outer medulla (summarized in **Figure 1**). Therefore, it could be speculated that if apoM carrying S1P is secreted locally in the kidney from the proximal tubular cells, it may have local effects in the kidney, even if the secretion is too low to affect the overall plasma concentration of apoM/S1P. However, further studies are required to clarify whether this is biologically relevant. Interestingly, increasing number of scRNAseq data on single cells are available in different databases such as the Kidney Interactive Transcriptomics initiative. These databases can provide new insight into which cell types express which of the S1P receptors as well as apoM and potentially also new biological understandings.

In summary, apoM is expressed in proximal tubular epithelial cells, where it is likely secreted to the pre-urine to sequester small lipophilic molecules such as S1P from excretion. However, the nature of such molecules and their faith, as well as the faith of apoM, remain unknown. Furthermore, it is unclear whether kidney-derived apoM plays a role in other physiological pathways. S1PRs are expressed throughout the kidney. Thus, it could be speculated that sequestered S1P in a complex with apoM has local effects on the kidney.

ASSOCIATION OF APOM AND S1P LEVELS WITH KIDNEY FUNCTION IN KIDNEY DISEASE

The plasma concentration of apoM is affected by different conditions and diseases. Thus, BMI and T2D are inversely associated with apoM levels, while familial hypercholesterolemia with an isolated increase in LDL-C level is associated with an increase in plasma levels of apoM (7, 13). The literature on apoM levels in patients with CKD is heterogeneous and is presented in **Table 1**. Patients with IgAV-induced nephritis have increased apoM and S1P levels compared to healthy controls but lower levels than those without nephritis (86). These results are supported by an animal study performed in HIGA mice (an experimental model of IgA nephropathy) that found that HIGA mice have increased apoM plasma levels compared to those in control mice (91). In contrast, apoM levels in patients with CKD of mixed etiology are negatively associated with CKD severity (87), while plasma apoM levels in patients with end-stage renal disease are lower than those in controls (88). In patients with primary nephrotic syndrome, plasma apoM is reduced, as well as in patients with hyperlipidemia (90). Furthermore, apoM levels were positively associated with albuminuria in patients with primary nephrotic syndrome (90), while no difference in apoM levels was found when comparing patients with micro-, normo-, and macroalbuminuria in a diabetic cohort (89). The discrepancies found for the association of apoM and kidney function in the different studies may, to some extent, reflect that the underlying etiology for developing CKD is very diverse and that conditions other than kidney disease itself are more important for determining apoM levels. Thus, diabetes is today one of the most common diseases leading to CKD, and patients with T2D have decreased apoM levels, which might at least partially explain the low apoM levels seen in patients with CKD. In line with this, Sorensen et al. found that plasma apoM levels are lower in patients with CKD combined with T2D

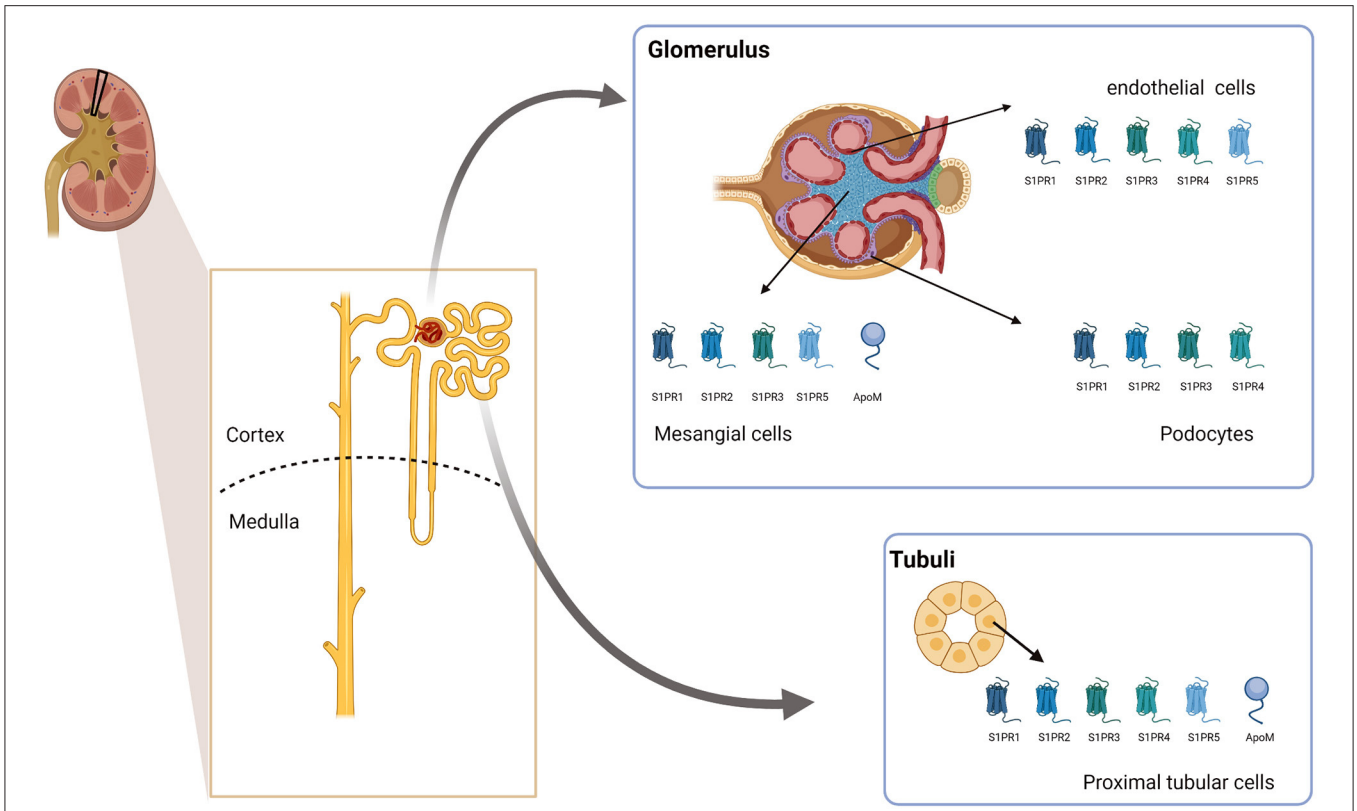

FIGURE 1 | ApoM and S1PR expression in the kidney. The schematic figure summarizes the current knowledge on which cell types in the kidney express apoM and different S1PRs. Created with BioRender.com. ApoM, apolipoprotein M; S1PR1-5, Sphingosine-1-phosphat receptor 1–5.

than in patients with CKD without diabetes (87). Furthermore, induction of mild kidney disease by 5/6-nephrectomy in mice resulted in a somewhat surprising increase in plasma apoM levels. However, the study was conducted in apoE-deficient mice, which are known to have aberrant lipoprotein metabolism and elevated plasma cholesterol levels. Elevated plasma cholesterol levels are as described earlier associated with an increase in apoM levels. Thus, the apoM levels observed in this study may be explained by the cholesterol levels and not the kidney disease, again supporting the idea that circumstances other than kidney disease itself determine apoM levels in patients with CKD (92). This is further supported by the observation that patients with CKD in contrast to the apoE-deficient 5/6-nephrectomized mice normally do not have elevated cholesterol levels (if anything it decreases with decreasing kidney function) and have decreased apoM levels. In addition, streptozotocin-induced type 1 diabetes resulted in increased apoM levels, which were reversed by insulin treatment, while age induced a decrease in apoM levels in mice (93). Together, this highlights the complexity of establishing the underlying cause for a change in plasma apoM levels. Of note, apoM is normally not detectable in the urine, but Svarrer et al. reported that apoM can be detected in urine from pediatric patients with acute kidney injury post heart surgery (94). This is believed to be caused by a direct injury to the proximal tubular cells that are then not able to re-uptake apoM secreted to the urinary site. The study did not measure plasma apoM levels, but it cannot be excluded that pathological changes in the kidney, can induce loss of apoM in the urine and potentially contribute to changes in plasma apoM levels. Further studies are however needed to clarify this.

Only a few studies have explored the association between S1P levels and kidney function in patients with CKD. One study has shown that plasma S1P levels in patients with type 2 diabetes decrease concomitantly with kidney function (89). In addition, S1P levels in HDL particles show a tendency to decrease with decreasing kidney function in patients with CKD (87), while S1P plasma levels in pediatric patients with CKD are higher than those in controls (95, 96). In streptozotocin-induced diabetic mice, plasma S1P levels are elevated globally (93) whereas (4 days) diabetic rats have elevated S1P levels locally in glomerular cells (97). Furthermore, berberine treatment of diabetic mice leads to less kidney injury, which is associated with decreased S1P signaling (98). Interestingly, patients with systemic lupus erythematosus had decreased S1P levels compared to controls, but the decrease did not correlate with the plasma albumin levels, suggesting that the dysregulation of S1P is mediated via changes in the apoM/HDL-bound S1P fraction and not the albumin fraction (99). In line with this, S1P decreased more in the HDL fraction than in the albumin fraction in the earlier stages of CKD (87).

TABLE 1 | Studies of patients or animals with kidney disease and effects on apoM levels.

Patient group	Number of participants	Method for apoM assessment	Plasma apoM	Lipid profile	Confounders	Albuminuria/ proteinuria	References
IgAV	185 (76)	Commercial elisa	↑	↑ TC ↑ LDL-C → HDL-C ↓ TG		+	(86)
CKD stage 1-5	409	In-house elisa	↓	↓ LDL-C ↓ HDL-C ↑ TG	Diabetes CVD	NA	(87)
ESRD	40 (20)	In-house elisa	↓	↓ TC ↓ LDL-C ↓ HDL-C ↑ TG	CVD	NA	(88)
T2D*	90	Commercial elisa	→	→ TC → LDL-C → HDL-C → TG	Diabetes CVD	+	(89)
PNS	205 (110)	Commercial elisa	↓	↑ TC ↑ LDL-C ↓ HDL-C ↑ TG		+	(90)
Old mice	–	Commercial elisa	↓	NA	–	NA	(18)
HIGA mice	–	Western blot	↑	NA	–	+	(91)
5/6 NX, mice	–	In-house elisa	↑	↑ TC	–	NA	(92)

ESRD, end-stage renal disease; IgAV, immunoglobulin A–associated vasculitis; CKD, chronic kidney disease; T2D, type 2 diabetes; PNS, primary nephrotic syndrome; 5/6 Nx, 5/6 nephrectomy; TC, total cholesterol; C, cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides. Plasma apoM is increased (↑), decreased (↓) or unchanged (→) compared to control groups. Number of participants are reported as total participants with the number of included healthy controls shown in ().

Changes in plasma S1P levels could be a result of excess loss via the urine. In support of this idea, results from a study in patients with T2D showed an inverse association between urinary albumin excretion and plasma S1P levels (89). In another study, albuminuria in patients with IgA nephropathy was found to have a positive correlation with both serum S1P and urinary S1P levels (100).

Altogether, the data available points toward a complex biology where the association of both apoM and S1P with kidney disease varies, likely depending on the underlying etiology of the disease. This highlights the complexity of kidney diseases in general and the possible role of apoM and S1P in the development of kidney disease and needs to be included in the interpretation of apoM and/or S1P as biomarkers for kidney disease in future studies. Further, measuring the total plasma S1P might not provide a full picture. Instead, more focus should be given on measuring S1P in albumin and apoM/HDL fractions, respectively.

THE APOM/S1P AXIS—A MEDIATOR OF KIDNEY DISEASE?

While the association between S1P and kidney function has been poorly studied, the mechanistic role of S1P signaling in kidney disease has been extensively addressed in animal models, recently reviewed in detail by Drexler et al. (65). Thus, in the present review, only a brief summary of the overall trends is provided. Solid evidence points toward a protective effect of S1P signaling in acute kidney disease. In experimental models of ischemia reperfusion (IR) injury, both S1P treatment, an agonist for S1PR in general (FTY720), or specifically, S1PR1 (SEW2871), reduces kidney injury (78, 84, 101–103). Furthermore, SEW2871 stimulation of proximal tubular epithelial cells *in vitro* protected the cells from IR-induced apoptosis, while mice with a proximal tubular specific knockout of S1PR1 have a greater decline in kidney function after IR injury (83, 84). Similarly, mice with a proximal tubular specific knockout of S1PR1 develop more kidney injury after cisplatin injury, which could not be rescued by FTY720 treatment, indicating that local S1P stimuli of the proximal tubular cells are essential for the protective effect (104). In addition, S1P signaling in endothelial cells appears to be important in the development of acute kidney disease as selective deletion of S1PR1 in endothelial cells exacerbates kidney injury upon IR (105).

Only a few studies have examined the role of receptors other than S1PR1, but it has been suggested that S1PR3 signaling aggravates kidney injury after IR (106). In addition, in contrast to the findings for S1PR1, inhibition of S1PR2 signaling is protective against IR injury (107).

Similar to what has been found for acute kidney disease, the literature in general supports a protective role for S1P signaling in chronic kidney disease, although fewer studies are available. In a chronic model of IR injury, mice with an endothelial knockout of S1PR1 develop a more severe injury than WT mice (108). In addition, FTY720 treatment alleviates kidney injury in a rat model of polycystic kidney disease (109), and

in a model of early stage diabetic nephropathy, both FTY720 and an S1PR1 specific agonist attenuates kidney injury (85). In contrast, suppression of S1PR2 signaling protects against experimental diabetic nephropathy (110). The protective role of S1PR1 signaling seems to be relevant both in the prevention of injury and in the recovery phase. Thus, knockout of endothelial S1PR1 expression before injury leads to exacerbation of kidney injury after IR, while knockout after IR results in increased tissue fibrosis and impaired healing (108). In humans, sphingosine-1-phosphate lyase (SGPL1) deficiency has been linked to nephrotic syndrome. SGLP1 mediates the degradation of S1P, meaning that deficiency results in accumulation of S1P and increased plasma levels, which further supports a causal role for S1P in kidney disease (111–113).

Little is known about the role of apoM in kidney diseases. However, accumulating data suggest that apoM is not only affected by kidney disease, but also has a causal role. In a recent study by Ding et al., reduced apoM levels resulted in increased kidney injury (18). Similarly, adenovirus-induced overexpression of apoM results in less kidney injury in HIGA mice and reduced expression of classical markers of fibrosis (incl. TGFβ and FN-1), while siRNA-induced apoM deletion results in accelerated injury compared to control animals (91). The mechanisms underlying the effect of apoM on the kidney are not clear; however, S1P bound to albumin results in increased proliferation of mesangial cells and increased expression of pro-fibrotic genes in the proximal epithelial cells; these effects were not observed when the cells were stimulated with S1P bound to apoM. To this point, only one study has addressed the role of kidney-derived apoM in kidney disease. This study suggests that silencing of apoM expression in mesangial cells leads to mitochondrial damage and apoptosis (74). However, apoM is mainly expressed in the proximal tubular cells. Therefore, it would be of great importance to unravel whether apoM derived from these cells has any role locally in the kidney during kidney injury.

Taken together, these data suggest that local S1P signaling in the kidney is of high importance for protecting the kidney against injury. What role both plasma and kidney-derived apoM play in connection to this, however, still needs to be addressed in future studies. Though highly speculative the data so far available could suggest that the apoM/S1P-complex act in two separate ways in the kidney—(1) being a systemic effect of circulating HDL-apoM/S1P or albumin-apoM/S1P on endothelial cells in the kidney and (2) a direct effect of kidney derived apoM on tubular cells. This is important to keep in mind as these two pathways might not have the same effect and regulation, which could influence how potential drug candidates targeting the apoM/S1P axis act in different diseases.

CONCLUSION

As this review suggests and highlights, significant gaps in our knowledge still need to be elucidated before we understand the role and function of kidney-produced apoM and S1P, but also whether the plasma lipoproteins containing apoM and S1P play a role in biological processes that affect kidney function and pathologies. Drugs targeting the S1P-receptor pathways are evolving as well as apoM-modulating drugs with a focus on cardiovascular diseases and inflammation; however, only a few of these studies have also reported data that are relevant to kidney biology. For example, pharmacological targeting of S1PR1 can improve renal microcirculation during sepsis in mice (114). Acute kidney failure or organ failure is a well-known consequence of severe sepsis. Likewise, treating animals with an artificial apoM-FC component can attenuate hypertension and IR injury (115). Whether any of these drugs will reach a level of clinical use will be very interesting to follow the years ahead. Hopefully, and in parallel, the community will gain further information from upcoming pre-clinical studies on potential side effects of such drugs related to, for example, lipid accumulation or compromised inflammatory response, both harmful to the kidney.

AUTHOR CONTRIBUTIONS

LB and CC designed and wrote this manuscript. All authors contributed to the article and approved the submitted version.

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Lessons From APOL1 Animal Models

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African-Americans have a three-fold higher rate of chronic kidney disease compared to European-Americans. Much of this excess risk is attributed to genetic variants in *APOL1*, encoding apolipoprotein L1, that are present only in individuals with sub-Saharan ancestry. Although 10 years have passed since the discovery of *APOL1* renal risk variants, the mechanisms by which *APOL1* risk allele gene products damage glomerular cells remain incompletely understood. Many mechanisms have been reported in cell culture models, but few have been demonstrated to be active in transgenic models. In this narrative review, we will review existing *APOL1* transgenic models, from flies to fish to mice; discuss findings and limitations from studies; and consider future research directions.

Keywords: APOL1, animal model, CKD—chronic kidney disease, glomerular diseases, podocyte

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INTRODUCTION

APOL1 genetic variants are an important cause of kidney disease, affecting individuals who have sub-Saharan African ancestry (1). Apolipoprotein L1 is a component of the innate immune system. It is produced primarily by the liver but also by brain, kidney, and other tissues. APOL1 is a component of HDL particles, which serve as delivery platforms for multiple proteins related to defense against infectious disease. When African trypanosomes circulating in blood ingest HDL particles, APOL1 is released from the HDL particles and trafficked to the lysosomes where it forms ion channels, thereby killing the parasite and thus preventing African sleeping sickness.

Trypanosomes developed a defense protein, serum resistance antigen (SRA), that binds APOL1 and prevents trypanocide (2). In response to the evolutionary pressure, human *APOL1* genetic variants, APOL1-G1 and APOL1-G2, arose that interfere with SRA binding. The APOL1 variants thereby restore trypanosomal killing but do so at the cost of increased risk for chronic kidney disease. This parallels the relationship between malaria and sickle cell hemoglobin, whereby the latter protects to some extent against malaria, particularly severe malaria, and consequently has been evolutionarily selected for in Mediterranean and sub-Saharan Africa regions (3). However, this protection comes at the cost of a disabling disorder, sickle cell anemia. While the molecular mechanism leading to sickle cell anemia is well understood, this has not been the case for the involvement of APOL1 in glomerular injury.

Histologic manifestations of APOL1 kidney disease including focal segmental glomerulosclerosis, collapsing glomerulopathy, arterionephrosclerosis (also termed hypertension-associated kidney disease), and advanced forms of lupus nephritis (4–6). Other manifestations of APOL1 kidney disease include shorter renal allograft survival among recipients with two *APOL1* risk alleles and a rare but concerning gradual loss of kidney function among living kidney donors (7, 8). These risks are generally confined to those who carry two *APOL1* renal risk alleles (high risk genotype). The one setting where there is an increased risk associated with carriage of one *APOL1* risk allele is HIV-associated collapsing glomerulopathy in South Africa (9).

Current therapies for chronic kidney disease are less effective at preventing progressive loss of kidney function in individuals with *APOL1* high risk genotype, compared to other forms of glomerular disease, even when there may be a reduction in proteinuria in a particular patient. APOL1 may not be an essential protein, as one individual who is genetically null for *APOL1* appears phenotypically normal (10). However, it is only one case report and we obviously need further confirmation to understand function and necessity of APOL1. Further, APOL1 is unique to humans, gorillas, and baboons (11). Interestingly, the gene is absent from the genome of chimpanzees and bonobos, our nearest neighbors in evolutionary terms; this remains a puzzle. Taken together, these observations suggest that APOL1-targeted therapy for APOL1-associated kidney disease may be well-tolerated, except in regions with endemic African trypanosomiasis. One such approach is to suppress APOL1 protein levels; this is now investigated in clinical trials (12, 13). Other therapeutic approaches are also needed, and experimental animal models will be of great utility in testing the efficacy of such therapies, as well as improving understanding of APOL1 disease pathogenesis.

Criteria that are frequently applied when animal models are considered in the study of human disease include similarity of the model to humans with respect to physiology, anatomy and genetics. But others such as the level at which a model system can be manipulated, controlled, and examined and the scalability so to achieve statistical power are equally important.

MICE

Mouse models are the most frequently used animal models and have been widely explored to investigate APOL1 kidney disease, even though mice do not have an ortholog to the human *APOL1* gene. The mouse apolipoprotein L family includes 12 genes and one pseudogene (*Apol6, Apol7a, Apol7b, Apol7c, Apol7e, Apol8, Apol9a, Apol9b, Apol10a, Apol10b, Apol11a, Apol11b,* and pseudogene *Apol10c*) clustered on chromosome 15 (14).

A primary aim of the *APOL1* transgenic mouse studies has been to characterize the molecular mechanism of kidney damage induced by *APOL1* renal risk alleles (G1 and G2) and to examine effects of human *APOL1* renal risk allele and non-risk allele (G0) *in vivo*. Several approaches have been taken to reproduce APOL1-associated human disease in mouse models, including various expression systems, gene promoters, gene variants, mouse background strain, and interventions that impose kidney stress or damage. Here we review published work in this area and consider what is missing or incomplete and what possible future approaches might be taken.

With regards to generating or selecting a transgenic mouse model, it is important to consider aspects of transgene expression such as tissue specificity of expression and regulation of expression. There are many available transgene promoters and enhancers. The transgene may be inserted randomly or may be inserted into a selected genetic locus. Random insertion is simpler, but tissue expression may be influenced by proximity to strong enhancer elements; this potential problem can be avoided by adding locus control regions to the transgene, but at the cost of a larger and more complex transgene (15). Further, the genetic background of the mouse strain must be considered, as outcomes may vary with genetic background.

In 2016, Bruggeman et al. reported the first transgenic *APOL1* mouse model, using the mouse *Nphs1* promotor (16). Both APOL1-G0 and -G2 mice showed decreased podocyte density and preeclampsia. Using the mouse *Nphs1* promoter, which is highly specific within kidney for podocytes, allowed targeting of APOL1-transgene expression to the cell where APOL1 is expressed in human kidneys and from where it is thought to drive APOL1-mediated kidney disease. One limitation was that even though these investigators showed that APOL1 expression affected podocyte function, this effect was not specific to the renal risk alleles but also to common variant APOL1-G0.

Beckerman et al. took a different approach, using a Tet-on activatable overexpression system to generate APOL1 transgenic mice (17). They showed that mice with *Nphs1* promotor-driven APOL1-G1 and -G2 overexpression developed global and segmental glomerulosclerosis, compared with -G0 mice, which had no kidney phenotype. In individual mice, albuminuria levels correlated with *APOL1* expression levels. Mechanistically, reduced autophagy resulted in pyroptosis *i.e.,* inflammatory cell death. Similarly, Kumar and others used the Tet-On3G inducible system to investigate APOL1 functions and showed that A*POL1* risk alleles de-stabilized the junction complex, which contributes to cell-cell contact and cell-matrix adhesion (18). One limitation to all these transgenic mice is the difficulty in relating transgene expression levels to those seen in humans.

In another approach, Okamoto et al. studied human *APOL1* gene locus transgenic mice, where the transgene is a 47 kb human cDNA that contains sequence from chromosome 22, included within a bacterial artificial chromosome (BAC). This BAC contains the exons and intron of APOL1, as well as portions of APOL2 and MYH9. This approach facilitates studies of gene regulation using physiologic stimuli, such as interferon or agents that promote interferon expression (19). The APOL1-G2 variant mouse showed significantly higher albuminuria compared to APOL1-G0 mice, following exposure to a combination of interferon-γ (to stimulate APOL1 gene transcription), basic fibroblast growth factor, and puromycin aminonucleoside (to induce podocyte injury). Importantly, the study showed that APOL1-variant mRNA activated protein kinase R and that this activation contributed to the observed podocyte injury.

To replicate physiological *APOL1* gene regulation and expression levels as seen in humans, Aghajan et al. used 32 Mb fosmid DNA vectors in the generation of transgenic mice (20). The authors used interferon-γ injections to induce transient albuminuria, which was most prominent in APOL1-G1 mice. They demonstrated that pre-treatment with an anti-sense oligonucleotide targeting APOL1 could block the induction of albuminuria. This proof-of-concept study indicated that anti-sense oligonucleotides might have a therapeutic role in APOL1 nephropathy. However, many steps remain toward a clinical application of this technology.

McCarthy et al. recently reported the results of *APOL1* BAC transgenic mice that received *via* hydrodynamic tail vein

injection an IFN-γ-expressing pCpGfree plasmid that lacks CpG motifs to achieve sustained IFN-γ levels in the animals. They showed robust induction of proteinuria and glomerulosclerosis in G1/G1 and G2/G2 mice but not in G0/G0 mice. They also showed that the heterozygous mice (G0/G1 or G0/G2) had greater proteinuria response than hemizygous mice (G1/- or G2/-), suggesting that APOL1-G0 does not rescue -G1 or -G2 allele toxicity. Further, mice with a multicopy G2 transgene ($G2^{multi}/G2^{multi}$) showed the greatest proteinuria response with worst prognosis, supporting the recessive nature of APOL1-nephropathy and the notion that disease is a function of the expression level of *APOL1* risk variant (21).

Ryu et al. used the BAC/APOL1 mouse model described above and showed that reduced cholesterol efflux and concomitant cholesterol accumulation may contribute to APOL1 nephropathy (22). In 2018, Kumar et al. generated a TetOn3G-APOL1 mouse model and identified a dual feedback loop in glomerular parietal epithelial cells, in which APOL1 suppresses miR-193a expression and miR-193a suppresses APOL1 expression (23).

Some models have involved dual-transgenic mice. Bruggeman et al. (24) studied the well-characterized Tg26 model of HIV-associated nephropathy, in which the presence of the six regulatory and accessory genes of HIV-1, under the control of viral long terminal repeats (LTR), develop FSGS and/or collapsing glomerulopathy (25). They reported that among Nphs1.APOL1 × Tg26 dual transgenic mice, APOL1-G0 × Tg26 mice showed less podocyte loss in compared with APOL1-G2 × Tg26 or Tg26 mice alone (24). These data suggest the intriguing idea that APOL-G0 protein might have some trophic or protective effect on podocytes, perhaps one that only manifests in certain injury settings.

Ge et al. studied a triple transgenic model, BAC/APOL1 × podocin-rtTA × TRE/NFATc1nuc mouse model (26). These mice manifest elevated levels of triglycerides and cholesterol in kidney, as well as glomerulosclerosis. The authors suggested that APOL1 risk variant expression increases the susceptibility to lipid-mediated podocyte injury, leading to mitochondrial dysfunction.

Another injury pathway was identified by Wakashin et al., who reported on a CAG-APOL1-B3 mouse model expressing the APOL1-B3 protein isoform under control of a CMV early enhancer/chicken β-actin promoter. The APOL1-B3 isoform, in contrast to the better characterized APOL1-A isoform, lacks a signal sequence for targeting to the secretory pathway and this results in cytosolic expression (27). These mice manifested podocyte injury and elevated IL-1β production for the G2 but not for the G0 variant. Further, APOL1-B3 interacted with NACHT, LRR, and PYD domains-containing protein 12 (NLRP12), a key regulator of toll-like receptor signaling. Through these pathways, APOL1-B3 and its risk variants seem to enhance inflammatory signaling in podocytes.

APOL1 is expressed most abundantly in liver, and this is the source of most plasma APOL1 (28). Targeted expression of APOL1 risk variants in murine livers by hydrodynamic gene delivery induced liver injury, demonstrating the cytotoxicity of the variants (11). Human data from kidney transplant studies suggest that only kidney-expressed, and not APOL1 expressed in other tissues has the potential to injure kidneys (29–31).

As shown above, mouse models have contributed in many ways to the understanding of molecular mechanisms of APOL1 kidney injury. Mouse kidney physiology shares many similarities to that of humans and diverse transgenic approaches are available, even though mice lack *APOL1*orthologs. Future research using existing and new mouse models will help to identify which mechanisms found *in vitro* are likely to be the relevant *in vivo* and relevant to human disease and may contribute to novel therapeutic approaches.

ZEBRAFISH

The zebrafish (*Danio rerio*) is the second most employed animal model in APOL1 research. Zebrafish express a protein with considerable homology to human APOL1 and this, together with its genetic tractability, has made it a useful model to study APOL1 function (32). Anderson et al. reported that translational suppression or CRISPR/Cas9 genome editing of *apol1* in zebrafish embryos results in podocyte loss and glomerular filtration defects (32). They also showed that complementation with the APOL1-G0 allele rescued the phenotype, but complementation with APOL-G1 or -G2 did not. Thus, the gene encoding APOL1 appears essential to glomerular function in fish, whereas this may not be the case in humans. A human subject who developed an unusual trypanosomal infection was found to be lacking in the *APOL1* gene but nevertheless had normal kidney function (10). However, the uniqueness of this intriguing finding warrants a cautious interpretation of the role of APOL1 or the lack of APOL1.

Kotb et al. confirmed that *apol1* knockdown causes podocyte damage in zebrafish (33). Olabisi et al. investigated zebrafish with G0, G1, or G2 *APOL1* variants, expressed using the Gal4-UAS system in podocytes or endothelial cells, using podocin and *Flk* (a receptor for vascular endothelial growth factor) promoters, respectively (34). Transgenic expression of *APOL1* G1 and G2, compared to the G0 allele, was associated with histologic abnormalities in zebrafish glomeruli but renal function remained normal. Bundy et al. investigated molecular pathways in zebrafish podocytes, using pathway analysis of differentially expressed transcripts, and showed enrichment for transcripts characterized by autophagy associated-terms, implicating autophagy pathways in APOL1 G2-associated kidney dysfunction (35).

The zebrafish model recapitulates certain aspects of molecular mechanisms acting downstream of human *APOL1* risk alleles, but the functional changes and the effects seen by knocking down APOL1 seem contradictory to observations in human subjects. Consequently, the specificities of zebrafish-APOL1 interactions have to be kept in mind and carefully further explored to advance our understanding of this animal model.

DROSOPHILA

Drosophila melanogaster, the common fruit fly, is a model system that offers ready access to genetic manipulation, enabling studies of protein function and protein-protein interaction. In 2017, two reports presented findings on effects of APOL1 expression

on nephrocytes in *Drosophila* (36, 37). These models exploit structural and functional similarities of *Drosophila* pericardial nephrocytes to those of human podocytes and proximal tubular cells. The accessibility of *Drosophila* nephrocytes facilitates high resolution *in vivo* analysis of renal cells (36).

Fu et al. reported that ubiquitous expression of human *APOL1*-G0 or -G1 variants in *Drosophila* induced lethal phenotypes, with -G1 being more toxic than -G0. Expressing the *APOL1* transgene in nephrocytes impaired the acidification of organelles. Kruzel-Davila et al. reported that ubiquitous expression of the human APOL1-G1 and -G2 variants caused near-complete lethality in the flies, with no effect observed for -G0 (37). These effects are more severe than those seen in humans, perhaps due to higher expression levels in the flies or

TABLE 1 | Summary of APOL1 animal models.

Species	Transgene	Variants	Cell	Method	Findings	Phenotype	Ref
Mouse	Nphs1.rTA × TRE-APOL1	G0, G1, G2	Podocytes	Tet-on	↑APOL1 expression, ↓autophagy, ↑pyroptosis	Glomerulosclerosis in G1 and G2	(17)
	Pax8.rTA × TRE-APOL1	G0, G1, G2	Proximal tubules	Tet-on	No	No kidney disease	(17)
	TetOn3G-APOL1	G0, G1, G2	Podocytes	Tet-on	De-stabilizes the adherens complex	Albuminuria in G1/G1 and G1/G2	(18, 23)
	Nphs1.APOL1	G0, G2	Podocytes		No change in necrosis, apoptosis, autophagy	↓podocyte density, Preeclampsia in G0 and G2	(16)
	BAC (Merck)	G0, G1, G2	Native	IFN-*g*/PAN/FGF	APOL1 mRNA activates PKR	Albuminuria in G2	(19)
	Fosmid (Ionis)	G0, G1, G2	Native	IFN-γ	ASO blocks APOL1 expression	Albuminuria in G1	(20)
	BAC	G0, G1, G2	Native	pCpG-Muγ	Phenotype severity: $G2^{multi}/G2^{multi}$ >G2/G2 >G2/G0>G2/-	Glomerulosclerosis in G1/G1 and G2/G2	(21)
	CAG-APOL1-B3	G0, G2	Systemic	uninephrectomy	↑IL-1β production	Albuminuria in G2	(27)
	TetOn3G-APOL1 × Tg26	G0, G1, G2	Podocytes	Tet-on	Not stated	Not stated	(23)
	Nphs1.APOL1 × Tg26	G0, G2	Podocytes		↑Podocyte in G0	No change	(25)
	BAC-APOL1 × Podocin-rtTA × NFATc1nuc	G0, G1	Podocytes	Tet-on	↑TG and Chol in G1	FSGS in G1	(22)
	pRG977-APOL1	G0, G1, G2	Liver-dominant	hydro- dynamic gene delivery	giant cell formation, macrophage infiltration: G1 > G2	severe liver necrosis, calcification: G1 > G2	(11)
Zebrafish	KO-zebrafish-apol1	G0, G1, G2	Systemic	Morpholino oligonucleotide-knockdown, CRISPR/Cas9	podocyte loss and glomerular filtration defects in KO, rescued by G0 only	Edema in KO	(32)
	KO-zebrafish-apol1	G0	Systemic	Morpholino oligonucleotide-knockdown	leakage of the filtration barrier in KO	Edema in KO	(33)
	APOL1	G0, G1, G2	Podocyte or endothelial cell	UAS-Gal4 system	Segmental podocyte FP effacement and irregularities in podocyte-G1	No dysfunction	(34)
	APOL1	G0, G2	Podocyte or endothelial cell	mRNA microinjection into the yolk	autophagy pathways upregulated in G2 podocytes	Not stated	(35)
Drosophila	APOL1	G0, G1	Systemic or nephrocyte	UAS-Gal4 system	impaired the acidification of organelles	Lethal in G0 and G1; G1 > G0	(36)
	APOL1	G0, G1, G2	Systemic or nephrocyte or eye-specific	UAS-Gal4 system	disruption of the endolysosomal processes	Lethal in G1 and G2	(37)

Shown are 18 transgenic models of APOL1 pathophysiology, involving three animal species. Methods used to generate models are shown.
BAC, bacterial artificial chromosome; KO, knock out; CAG, cytomegalovirus immediate early enhancer-chicken beta-actin hybrid promoter; FGF, fibroblast growth factor; PAN, puromycin aminonucleoside; rtTA, reverse tetracycline transactivator; TRE, tet-response element; UAS-GAL4, upstream activation sequence, which responds when GAL4 is expressed and activates the target gene; pCpG-Muγ, an IFN-γ-expressing pCpGfree plasmid that lacks CpG motifs.

the particular pattern of tissue expression, but this study does lend support to a gain-of-dysfunction model for APOL1 variant cell toxicity. These authors also observed differential toxicity of the APOL1 risk alleles compared with non-risk alleles including disruption of endolysosomal processes.

The fly models have proven useful in showing a lethal phenotype and endolysosomal disruption by APOL1 risk alleles, and to identify interacting partners of APOL1. These models to address specific mechanisms that may be shared by nephrocytes and podocytes, but these two cell types have important differences that limit definitive conclusions.

DISCUSSION

Several factors should be considered when designing or selecting an animal model system that can most effectively and efficiently address the selected research questions.

Promoter Selection

Promoter selection is of great importance, as it determines the specificity, kinetics, and dose of transgene expression. These parameters determine the types of questions that can be addressed. In the kidney, APOL1-mediated cytotoxicity is particularly prominent in podocytes. Consequently, many transgenic mouse studies have selected podocyte-specific promoters to interrogate podocyte-specific function of *APOL1*. However, APOL1 is not only expressed by podocytes but also expressed by renal endothelial cells (38). This finding warrants the application of endothelial cell-specific animal models to specifically investigate the effects of APOL1 on endothelial cells. It is true that APOL1 expression is still a controversial topic as Ma et al. reported the presence of APOL1 protein in kidney tubular cells suggesting a function of APOL1 there (39). Further, several studies have explored systemic expression of APOL1 to interrogate unbiased, systemic function of *APOL1*. Considering APOL1's ubiquitous expression in many organs plus its presence on HDL particles circulating in the bloodstream, systemic models offer avenues to uncover the remaining unknowns in APOL1 functions.

Intervention and Expression System

Establishing a robust transgene phenotype is critical in generating a persuasive animal model. In human APOL1 nephropathy, APOL1 variant risk status appears to be insufficient for disease onset and a second factor, generally increased APOL1 expression, appears to be required. Interventions to increase transgene expression are also required in several APOL1 transgenic animal models to increase gene expression. IFN-γ activates the APOL1 promoter (19, 20) and doxycycline activates the Tet-On promoter (17, 18). Puromycin aminonucleoside and/or basic fibroblast growth factor have been used to induce podocyte injury (19). Identifying effective interventions that result in overt phenotypes remain challenging.

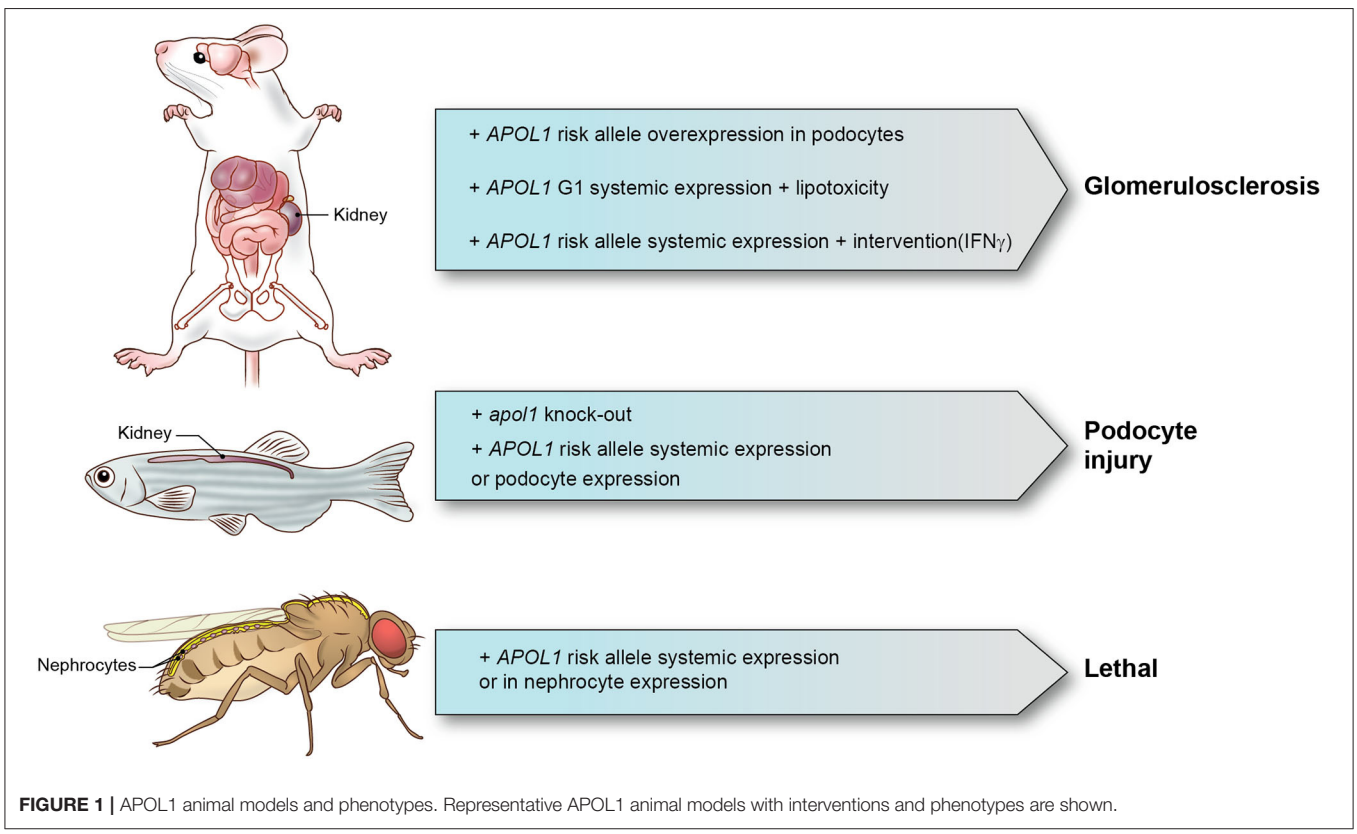

FIGURE 1 | APOL1 animal models and phenotypes. Representative APOL1 animal models with interventions and phenotypes are shown.

Background Strain of Mouse Models

Mouse background strain can have a substantial effect on susceptibility to disease or response to a disease-promoting intervention. A common background strains for transgenic mice is FVB/N, due to the relative ease of injecting the large pronuclei in the zygote. This strain has also proven very useful in kidney studies, as other strains including strains 129S1 or B6 turned out to be more resistant to kidney injury.

Findings From Animal Models

In **Table 1**, we summarize the models that have been reported. Although a range of mechanisms have been documented for *in vitro* models that investigate APOL1 function, only limited number of mechanisms have been identified and confirmed in animal models. We also summarized major animal models in **Figure 1**. Animal models come with a variety of limitations. Designing and generating an animal model for complex human genetic diseases is challenging especially if specific physiology or environmental factors are contributing elements. The APOL gene family arose in primates and diversified rapidly, by gene duplication, on the one hand, and by gene loss and pseudogenization on the other hand. This gene family is absent from all the commonly used research animal species, including mice. This raised the potential concern that other molecules that co-evolved in primates to interact with APOL1 and perhaps regulate or alter its activity would be missing in mice and other laboratory animals. Although modern molecular biology methods allow a gene or genomic fragments containing gene and extended flanking regions to be introduced in these model systems, it remains a challenge to decipher how the transgene expression affects the transgenic animal and how these effects relate to pathological processes and especially their kinetics observed in human subjects. The duty remains to carefully explore how far a model can inform on the human setting.

Future Directions for Animal Model Research

In patients, APOL1 kidney disease appears to follow a two-hit model, whereby a genetic predisposition (presence of two *APOL1* kidney risk alleles) is coupled with factors that increase *APOL1* gene expression (interferon being the most well-documented). Given an increase in *APOL1* gene expression, it remains unclear to what extent peak magnitude of expression vs. area under the curve of expression is more important in inducing glomerular injury. Also, there may be factors other than interferon that increase gene expression or alternatively synergize with what might otherwise be sub-pathogenic APOL1 expression levels to induce kidney injury. Future animal studies will be required to understand the relationship between the kinetics of gene expression and glomerular injury.

Animal studies offer the opportunity to examine what other factors, beyond APOL1 variant expression level, might alter the timing and degree of glomerular injury. These might include age, sex, body weight and various co-morbidities. Single cell methods that provide unprecedented insights into otherwise not recognizable changes in per-cell based transcriptomes may also shed light into molecular mechanisms by APOL1, together with novel animal models. Further APOL1 animal studies will be warranted to better understand to which factors have to be paid more attention in the population carrying *APOL1* risk alleles.

AUTHOR CONTRIBUTIONS

TY reviewed the literature and drafted the manuscript. KL and JK provided comments. TY, JH, and JK edited the manuscript. All authors read and approved the final draft.

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Glomerular Kidney Diseases in the Single-Cell Era

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Recent advances in single-cell technology have enabled investigation of genomic profiles and molecular crosstalk among individual cells obtained from tissues and biofluids at unprecedented resolution. Glomerular diseases, either primary or secondary to systemic diseases, often manifest elements of inflammation and of innate and adaptive immune responses. Application of single-cell methods have revealed cellular signatures of inflammation, cellular injury, and fibrosis. From these signatures, potential therapeutic targets can be inferred and in theory, this approach might facilitate identification of precision therapeutics for these diseases. Single-cell analyses of urine samples and skin lesions from patients with lupus nephritis and of urine samples from patients with diabetic nephropathy and focal segmental glomerulosclerosis have presented potential novel approaches for the diagnosis and monitoring of disease activity. These single-cell approaches, in contrast to kidney biopsy, are non-invasive and could be repeated multiple times as needed.

Keywords: glomerular diseases, single cell genomics, precision medicine, immunity and inflammation, urine biomarkers

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INTRODUCTION

Over the past decade, advances in technology have revolutionized our understanding of the genetic and molecular bases of glomerular diseases. For example, transcriptomics studies investigating the genome-wide gene expression profiles of diseased tissues identified candidate genes and pathways upregulated or activated in nephrotic diseases (1, 2). However, due to the lack of spatial and temporal resolution, it is usually not possible to define the exact cellular origins and states of transcriptomics signatures in tissues. Further, bulk transcriptomic results can potentially be confounded by transcriptomic signatures arising from other cells, particularly proximal tubular cells, which are the predominant cell type in kidney biopsy samples (2).

Single-cell and single-nucleus RNA sequencing (sc/sn RNA-seq) approaches overcome this limitation by providing transcriptomic data for individual cells, albeit with less depth of coverage than the bulk RNA sequencing (RNA-seq). Application of these single-cell methods to glomerular diseases has enabled investigators to identify cell types within tissues and to characterize the cell injury and activation states and the specific gene expression patterns. Recent studies of scRNA-seq performed on urinary cells and skin lesion biopsies in lupus nephritis reported gene expression profiles that were highly correlated with single-cell data from renal biopsy samples. These findings open up the possibility of alternatives to renal biopsy to establish diagnosis and monitor disease activity (3–5). However, compared to secondary glomerular diseases, relatively few single-cell studies have been published to date in the field of primary glomerular diseases (**Table 1**).

In this review, we will emphasize the recent progress made by single-cell studies of glomerular diseases, particularly lupus nephritis (LN), diabetic nephropathy (DN), focal segmental glomerulosclerosis (FSGS) and IgA nephropathy (IgAN), in understanding pathophysiology at the cellular level and in exploring non-invasive approaches for clinical application. We will also discuss how this approach could be applied to other primary glomerular diseases.

SINGLE-CELL/NUCLEUS STUDIES IN SECONDARY GLOMERULAR DISEASES

Secondary glomerular diseases arise as complications of systemic disease, such as systemic vasculitis, systemic lupus, and diabetes. Single-cell/nucleus RNA-seq studies, using kidney biopsy and/or urine samples, have been reported for lupus nephritis and diabetic nephropathy (3–7). ScRNA-seq captures the transcripts from whole cells but it requires enzymatic dissociation of tissue to yield a single-cell suspension, a process that typically causes cellular stress and with that, potentially, transcriptional alterations. Further, this approach may fail to include certain cell types, especially rare cell types, due to incomplete dissociation of kidney tissue (11, 12). Although snRNA-seq captures only nuclear transcripts, it usually covers all the different cell types in the tissue. Both single-cell RNA-seq and single-nucleus RNA-seq approaches yield insights into the tissue composition, cellular mechanisms and provide a detailed, high-resolution assessment of cellular transcriptomics, which goes beyond what can be attained from bulk tissue RNA studies.

LUPUS NEPHRITIS

Studies of bulk transcriptomic analysis in mouse and human LN have reported molecular signatures related to inflammation and fibrosis. Cross-species joint network analysis of human LN and mouse bulk transcriptomic data had identified shared pathways of immune cell infiltration and activation, macrophage and dendritic cell activation, endothelial cell activation, and damage and tissue remodeling/fibrosis in kidney (2, 13).

Details of the immune cell profile of LN were reported in 2019 by Arazi et al., in a single-cell RNA-seq study of immune cells from urine samples and kidney biopsy of LN subjects from the Accelerating Medicines Partnership rheumatoid arthritis/systemic lupus erythematosus (AMP RA/SLE) consortium (3). Stepwise clustering of immune cells identified finer subclusters including inflammatory, phagocytic and M2-like macrophages in the myeloid lineage; central and effector memory T cells, cytotoxic and memory T cells in the T cell lineage; and activated B cells and plasma cells in the B cell lineage. There was a high concordance of gene expression for particular leukocyte classes between urine and biopsy samples, which suggests that most types of immune cells infiltrating the kidneys may also be found in the urine. This concordance supports the potential utility of urine single-cell studies, as they can inform on ongoing inflammatory processes in the kidneys and offers a rationale to pursue similar studies of urine cells in other kidney diseases.

In other reports from the AMP RA/SLE consortium, Der et al. described the upregulation of type I interferon-responsive signature genes in renal tubular cells and in skin keratinocytes. These findings suggest that skin keratinocytes gene signatures could help with assessing the nephritis activity in lupus and serve, in addition to urine cells, as a potential surrogate approach to renal biopsy (4, 5). Moreover, the authors observed differences in the activated pathways between membranous and proliferative subtypes of LN in tubular cells and keratinocytes. In proliferative LN, these cells showed higher expression of genes in pathways of tumor necrosis factor (TNF)-related pathways and interferon response pathways, which were not observed in cells from membranous LN. Consequently, the potential utility of these cellular gene signatures in identifying disease subtypes should be further evaluated. Interferon response signatures and fibrotic signatures were also higher in tubular cells of treatment responder group compared to the non-responder group. These signatures suggest the presence of active innate immune pathways at the time of renal biopsy. This information could be useful to clinicians, as it suggests that these analyses could help verify when effective immunosuppression has been achieved in the kidney.

Interaction analysis showed possible interactions of cytokines across various cell types including cytokines from the TNF (tumor necrosis factor) superfamily, *TNF*, *TNFSF10* (encoding TNF-related apoptosis-inducing ligand or TRAIL) and *TNFSF14* (encoding LIGHT, homologous to **L**ymphotoxin, exhibits **I**nducible expression and competes with HSV **G**lycoprotein D for binding to **H**erpesvirus entry mediator, a receptor expressed on **T** lymphocytes). These cytokines and related pathways may be potential targets for immunotherapy in LN.

DIABETIC NEPHROPATHY

Bulk transcriptomic studies of DN have identified upregulation of genes that are also recognized in single-cell studies as markers of specific cell types and fibrotic pathways. Lindenmeyer et al. identified elevated tubulointerstitial expression levels of extracellular matrix related genes, including *COL1A2*, *COL4A1*, *FN1*, *VIM1*, and *TIMP1* (14). Similarly, Woroniecka et al. also reported the upregulated expression of collagen genes in renal tubules in a study of human glomeruli and tubules isolated from diabetic subjects. Further, expression of podocyte marker genes, such as *NPHS1*, *NPHS2*, *SYNPO*, *PODXL*, *WT1*, and *PLA2R1* were downregulated in the glomeruli, which could be due to podocyte dedifferentiation or podocyte loss as a result of inflammation and/or mechanical stress from hyper-filtering glomeruli (15).

Using single-nucleus RNA-seq on cryopreserved kidney biopsy samples, Wilson et al. reported gene expression profiles for various cell types in early human diabetic nephropathy (6). Differential expression tests showed modest values of log-fold changes but identified increased expression of extracellular matrix component collagen genes in mesangial and endothelial

TABLE 1 | Summary of published glomerular disease studies applying single-cell/nucleus technology.

Disease	Author (references)	Sample type	Sample number	Single-cell technology	Number of cells & genes/cell analyzed	Cell types focused in analysis	Main findings
Lupus nephritis	Arazi et al. (3)	Kidney biopsy and urine	24 lupus nephritis and 10 controls	Single-cell (CEL-seq2 and 10x)	2,736 leukocytes and 145 epithelial cells, 250–5,000 genes per cell	Immune cells	- Total 21 subsets of myeloid, T, NK and B cells with interferon response. - Gene expression of immune cells in urine and kidney was highly correlated.
Lupus nephritis	Der et al. (4, 5)*	Kidney biopsy, skin biopsy and urine	21 renal tissue samples and 17 skin biopsy samples from lupus nephritis subjects, three pairs of kidney and skin biopsy samples from controls	Single-cell (Fluidigm C1 mRNA seq HT IFC)	4,019 cells, >100 genes per cell	Tubular cells and skin keratinocytes	- Type I interferon-response signature in tubular cells and skin keratinocytes associated with failure to respond to treatment. - Proliferative subtype is associated with pathways of inflammation and fibrosis which were not observed for membranous subtype.
Diabetic nephropathy	Wilson et al. (6)	Nephrectomy samples	Three diabetic nephropathy and three control samples	Single-nucleus (10x)	23,980 cells, 2,541 genes per cell	Podocytes, mesangial, endothelial, leukocytes, proximal tubules	- Diabetic thick ascending limb, late distal convoluted tubule, and principal cells all adopt a gene expression signature consistent with increased potassium secretion and decreased paracellular calcium and magnesium reabsorption. - Strong angiogenic signatures in glomerular cell types, proximal convoluted tubule, distal convoluted tubule, and principal cells.
Diabetic nephropathy	Abedini et al. (7)	Urine	17 urine samples from five subjects	Single-cell (10x)	23,082 cells, >300 genes per cell	Podocytes, proximal tubules, loop of Henle and collecting duct cells, macrophages, and lymphocytes	- Urine contains the major kidney cell types and leukocytes. - Urinary cells have similar gene expression with kidney cells.
FSGS	Menon et al. (8)	healthy kidney single-cell samples and FSGS glomerular bulk transcriptomic data	24 healthy single-cell samples and 74 FSGS bulk samples	Single-cell (10x)	22,268 cells, 500–5,000 genes per cell	Endothelial cells	- Highest glomerular endothelial cell scores were observed in patients with FSGS. - Glomerular A2M transcript levels associated with lower proteinuria remission rates in FSGS.
FSGS	Latt et al. (9)	Urine from FSGS subjects and bulk transcriptomic data from FSGS and MCD	17 urine samples from 12 subjects captured into 23 single-cell samples	Single-cell (10x)	5,551 cells, 100–6,000 genes per cell	Immune cells and podocytes	- Urine single-cell data showed podocytes showing with inflammatory signatures suggesting EMT, and immune cells, mostly monocytes polarized into M1 and M2 subtypes. - Immune- and EMT-specific genes from FSGS urine single-cells showed higher levels of expression in FSGS compared to MCD samples in NEPTUNE biopsy transcriptomic data.
IgAN	Zheng et al. (10)	Kidney biopsy from IgAN, normal tissue from nephrectomy subjects and peripheral blood mononuclear cells	13 IgAN and six normal kidney samples, five PBMC samples from IgAN and five from healthy donors	Single-cell (STRT-seq)	2,785 kidney cells and 835 PBMCs, more than 3,000 genes/cell	Mesangial, intercalated and principal cells, PBMCs	- IgAN mesangial cells show upregulation of *JCHAIN* which is important for dimerization and transepithelial transportation of IgA. - Peripheral blood monocytes express type I interferon response encoding genes and CD8+ T cells show downregulation of cytotoxic marker genes. - A transitional cell type with intercalated and principal cell markers show EMT and fibrosis signatures.

**Since the main findings from two studies are similar, we focused on the later study, which has bigger sample size and contains control samples.*

cells. Endothelial cells also showed increased expression of glucose transporters and angiogenesis regulators. Notably, interaction analysis identified increased expression of *CCN1*, encoding cellular communication network factor 1, by mesangial cells, as well as possible interactions between integrins and their ligands, which were also upregulated in podocytes and endothelial cells. These predicted interaction pairs appear to suggest on-going cycles of cell injury, tissue repair and fibrosis in DN.

Moreover, gene expression data from these diabetic subjects suggested that tubules may be engaged in greater potassium secretion and decreased calcium and magnesium reabsorption in the thick ascending limb of the loop of Henle and late distal convoluted tubules. There were also infiltrating monocytes and lymphocytes, with monocytes showing an interferon-γ signaling signature.

In a recent report, Abedini et al. captured urinary cells from subjects with DN. They identified a variety of cell types including renal epithelial cells and immune cells, and showed that these cells retained transcriptomic signatures similar to the corresponding cell types in the kidneys (7). The authors found that expression of genes that had previously been nominated to mediate the effect of the polygenic genome-wide association study (GWAS) of estimated glomerular filtration rate (eGFR) were strongly enriched in urinary proximal tubules. These findings connecting the GWAS results with single-cell transcriptomic data may support the notion that proximal tubule activities may be related to eGFR decline in progressive kidney disease, whether as drivers or responders to that decline. As the urine of healthy individuals contains few immune or kidney epithelial cells, a comparison between the gene expression profiles of DN urinary cells and those of urinary cells from healthy individuals was not feasible. However, this study provides proof of the concept that the urine of DN subjects contain cells whose transcriptional profiles may reflect pathological process ongoing in the kidneys.

PRIMARY GLOMERULAR DISEASES

In primary glomerular diseases, intra-renal pathology initiates from within glomerular cells. There are four most common primary glomerular diseases: minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS), membranous nephropathy (MN), and IgA nephropathy (IgAN). Bulk transcriptomic studies have been reported for three of these diseases, (MN is currently an exception), and a few single-cell studies have been reported for FSGS and IgAN. Due to the similar clinical presentations and challenges in making a definitive diagnosis in pediatric patients, FSGS and MCD are often investigated comparatively in transcriptomic studies.

FOCAL SEGMENTAL GLOMERULOSCLEROSIS AND MINIMAL CHANGE DISEASE

FSGS and MCD are primary podocyte diseases that may share similar clinical presentations. In adults, the differential diagnosis is broader, as it includes membranous nephropathy and occasionally other diseases; therefore, establishing a definitive diagnosis usually requires a kidney biopsy. In children, the common diagnostic possibilities are most often limited to MCD and FSGS and so a trial of glucocorticoids is usually undertaken, as the former is uniformly treatment-sensitive and the latter is often treatment-resistant. Moreover, due to the focal nature of lesions in FSGS, early FSGS can be mistaken for MCD if the glomerular lesions are not captured in the biopsy samples. Single-cell and single-nucleus methods could be helpful to elucidate intraglomerular pathophysiology and to identify gene expression signatures of cellular injury, especially in podocytes.

Several bulk tissue transcriptomic studies investigating FSGS and MCD have been reported recently (1, 2, 16–20). The FSGS kidney transcriptomic studies showed the upregulation of inflammatory genes (*SPP1*, *VCAM1*, *THY1*, *CXCL1*, *CXCL2*, and *CXCL4*) and fibrotic genes (*COL1A1*), none of which were observed in MCD. Using transcriptomic data from the Nephrotic Syndrome Study Network (NEPTUNE) cohort, Sampson et al. reported upregulation of *CXCL9*, *CXCL11* and *UBD* in the glomerular compartment of subjects with two *APOL1* risk alleles, which are known to have a large effect size in inducing FSGS in African-descent populations, compared to subjects with one or zero risk alleles (1). This suggests that the *APOL1* high risk subjects have a higher degree of inflammation in the glomeruli, although this finding may well have been influenced by the fact that more than half of *APOL1* high risk samples in the analysis had FSGS and the majority of MCD samples, which typically lack glomerular inflammation, had low risk *APOL1* genotypes.

Using data from the NEPTUNE study, the Kidney Precision Medicine Project (KPMP) and the European Renal cDNA Bank (ERCB), Menon et al. derived cell-specific gene expression signatures of healthy human kidney tissue single-cell data and investigated those signatures in bulk kidney cortex transcriptomic data from NEPTUNE for correlation with clinical outcomes. High α-2 macroglobulin (A2M) gene expression from the glomerular endothelial cell signature at the time of initial biopsy was associated with lower FSGS remission rates (8).

In a recent single-cell study of urine samples from FSGS subjects, we reported inflammatory signatures in urine podocytes and immune cells (9). Among the most highly expressed genes in urine podocytes are the genes associated with epithelial-to-mesenchymal transition (EMT). The majority of immune cells were monocytes, polarized into M1 (inflammatory) and M2 (anti-inflammatory) subtypes. EMT is known to predispose to fibrosis, and together with inflammatory signatures from immune cells, could represent a profibrotic signature that could help to differentiate FSGS from MCD. We confirmed this hypothesis using gene expression data from NEPTUNE consortium, which showed higher expression of immune and EMT genes in FSGS compared to MCD samples. Immune genes were also found to be higher in NEPTUNE nephrotic syndrome cases without remission compared to cases with remission.

Cell-cell interaction analysis revealed possible interaction of *TNFSF12* (encoding tumor necrosis factor-like weak inducer of apoptosis or TWEAK) with *TNFRSF12A* (Fn14) and *TNFSF10* (TRAIL) with *TNFRSF10B* (DR5) and with *TNFRSF11B* (osteoprotegerin) receptors between the immune cells and

kidney epithelial cells. Both cytokines are known to be involved in apoptosis, chronic inflammation, organ remodeling and fibrosis (21–28), and their signaling pathways could be targets for immunotherapy. This study suggests that urine testing could provide valuable information for differential diagnosis of nephrotic syndrome in the pediatric population as a form of liquid biopsy and might contribute to disease activity monitoring in children and adults.

IgA NEPHROPATHY

IgAN is caused by overproduction of immunoglobulin A1 (IgA1) and deposition of IgA1-IgG immune complexes in glomeruli with subsequent mesangial proliferation and extracellular matrix deposition (29). Hodgin et al. compared the bulk transcriptomes of IgAN glomeruli with and without endocapillary proliferation. There was upregulation of genes related to classical complement activation (*C1QA*, *C1QB*, *C2*, and *VSIG4*), extracellular matrix degradation and turnover (*HSPE*, *TIMP1*) and *CD163*, which is a marker of M2 macrophage polarization, in glomeruli with endocapillary proliferation and these genes were inversely correlated with renal function (30).

Zheng et al. recently reported the first single-cell gene expression profile of kidney cells and peripheral blood monocytes from IgAN (10). The authors isolated glomerular cells, tubular epithelial cells and immune cells in a stepwise manner and captured mesangial cells and a smaller number of podocytes for single-cell analysis. The IgAN mesangial cells showed upregulation of *JCHAIN* which is essential for dimerization and transepithelial transportation of IgA, and the peripheral blood monocytes showed significant expression of type 1 interferon-encoding genes. Cell-cell interaction analysis showed increased interactions in IgAN mesangial and endothelial cells and reduced interactions in intercalated cells compared to their healthy counterparts. Further analysis of intercalated cells revealed a new transitional cell type which expressed marker genes of both intercalated and principal cells and genes associated with EMT.

DISCUSSION

For an increasing number of kidney diseases, single-cell technology has provided insights into the cellular landscape of inflammation, the molecular crosstalk among various immune, epithelial and mesenchymal cell types, and the gene expression profiles of these cells. Cellular injury patterns may well-reflect the effect of immune or inflammatory response superimposed on the primary disease mechanisms. Single-cell analysis of four different mouse kidney disease models (nephrotoxic serum nephritis, diabetes, doxorubicin toxicity, and CD2AP deficiency) showed cell type-specific and injury type-specific response in glomeruli (31). It remains to be seen whether and to what extent single-cell approaches can help to establish the primary etiology in particular cases of human primary glomerular diseases since there are no published single-cell/nucleus study on kidney biopsy samples from FSGS, MCD, or MN to date.

As discussed above, urine single-cell data from lupus nephritis, diabetic nephropathy and FSGS studies demonstrate that urine contains immune and kidney epithelial cells that reflect the intrarenal pathology. For the purpose of distinguishing FSGS from MCD in the diagnosis of pediatric nephrotic syndrome, studies with larger sample sizes will be needed to capture urine immune cells, possibly by fluorescence-activated cell sorting (FACS) and/or to quantify the protein products of key inflammatory genes from immune cells in serum and urine by enzyme-linked immunosorbent assay (ELISA) and determine the sensitivity and specificity of these approaches.

For some primary glomerular diseases, podocytes are the initial loci for key pathologic processes. The digestion of glomeruli from tissue in a single-cell approach is usually not complete and some kidney single-cell studies did not capture the podocytes (4, 32). In FSGS and membranous nephropathy biopsies, particularly when advanced, it will be more challenging to release cells from sclerosed or partially sclerosed glomeruli. In these cases, the single-nucleus RNA-seq approach is more effective than single-cell RNA-seq in enriching for podocytes (33). Technical challenges in designing single-cell/single-nucleus experiments with currently available protocols was discussed in detail in an informative recent review by Deleersnijder et al. (34).

For FSGS, characterizing differences in cell type composition and the transcriptional profiles among cells of a single type and comparing sclerosed and normal-appearing glomeruli could be important in understanding the focal and the segmental nature of the lesions. One strategy to address this could be spatial transcriptomics, which localizes RNA expression patterns within tissue. This technique has not yet been applied widely in the field of nephrology, but it may provide insight into the focal and segmental nature of FSGS, the origin of myofibroblasts and the molecular crosstalk among various cell types in the glomerular microenvironment even at the current resolution limits.

An important pathophysiological process that must be addressed in glomerular diseases is progressive renal fibrosis. IgAN and FSGS urine single-cell studies report expression of genes associated with EMT in epithelial cells, such as podocytes in FSGS and principal and intercalated cells in IgAN. However, a recent single-cell study of subjects with hypertensive nephrosclerosis with and without chronic kidney disease (CKD) found that the contribution of epithelial cells (de-differentiated proximal tubule cells) to fibrosis by EMT could be relatively minor compared to mesenchymal cells (35). Notably, the authors enriched non-proximal tubule cells to study them separately from proximal tubule cells, which are the major cell type in renal cortex samples. Myofibroblasts are defined in the study as the cell type that expressed the most extracellular matrix (ECM). Diffusion mapping analysis, which organizes single-cell data along complex pathways that may provide insight into cell lineages, suggests fibroblasts and pericytes as the major cellular source of myofibroblasts in hypertensive CKD. The extent of contribution of EMT to sclerosis and the origin of myofibroblasts in other glomerular diseases remain to be identified.

Another avenue to be explored for understanding primary glomerular diseases is to identify the immunological triggers that lead to renal diseases. Genetic studies indicate strong associations

with immune loci, especially the class II major histocompatibility complex (MHC) genes, for IgAN, idiopathic MN and childhood steroid sensitive nephrotic syndrome (36–42). Single-cell studies of peripheral blood mononuclear cells (PBMCs) may illuminate immune signatures associated with disease onset or relapse, and in the case of minimal change disease, may allow to identify putative plasma factors secreted by dysregulated or activated immune cells leading to podocyte injury. A recent study of PBMCs from subjects who received immunization used cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) to profile 82 surface proteins of these immune cells (43). The authors reported that differences in baseline blood transcriptional signatures are predictive of antibody response to influenza and yellow fever vaccination, and that these signatures reflect the extent of activation in plasmacytoid dendritic cells and lymphocytes. These same signatures were also associated with disease activity in patients with lupus with plasmablast-associated flares. CITE-seq is a powerful approach to identify novel immune cell activation states and to characterize cell subtypes with confidence; it offers opportunities to study the immunological basis of glomerular diseases.

Single-cell technology can also be used to identify culprit genes and cell types from among the wealth of candidates identified by GWAS studies. Many GWAS-identified disease loci are located in non-coding intronic or intergenic genomic regions and yet are associated with gene expression levels in tissues (44, 45). In this case, it can be challenging to identify the critical causal genes and cell types in order to fully interpret the results.

Single-nucleus assay for transposable chromatin with sequencing (ATAC-seq) can give information about regions of open chromatin state in a particular cell at a particular moment. Further, it can identify cell types that harbor enhancer elements in the regions encompassing the most highly disease-correlated single nucleotide polymorphisms (SNPs). Together with sc/sn RNA-seq data in high risk and low risk genotype groups, ATAC-seq might help to pinpoint the causal genes for those GWAS hits that confer disease risk by regulating gene expression.

Ongoing innovations in the single-cell technology and analysis offer better prospects to identify important cell types and states in kidney diseases. Using pseudotime analysis of single-nucleus RNA-seq and ATAC-seq data from developing and adult mouse kidneys, Miao et al. delineated the trajectory of the developmental process in mice from nephron progenitor cells to podocytes and renal tubular cells (46). Pseudotime analysis may be applied to human disease data to characterize the differentiation and transition processes from one cell state to another in the presence or absence of treatment or in longitudinal studies. Cell-cell interaction analysis can also provide insight into intercellular signaling in the glomerular microenvironment and identify potential biomarkers and therapeutic targets. In conclusion, the single-cell technology represents a powerful tool to dissect pathophysiology and can be exploited to provide new insights into glomerular disease pathogenesis.

AUTHOR CONTRIBUTIONS

KL reviewed the literature and drafted the manuscript. JK, JH, and TY provided comments. KL and JK edited the manuscript. All authors read and approved the final draft.

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MINI REVIEW
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Emerging Technologies to Study the Glomerular Filtration Barrier

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Kidney disease is characterized by loss of glomerular function with clinical manifestation of proteinuria. Identifying the cellular and molecular changes that lead to loss of protein in the urine is challenging due to the complexity of the filtration barrier, constituted by podocytes, glomerular endothelial cells, and glomerular basement membrane. In this review, we will discuss how technologies like single cell RNA sequencing and bioinformatics-based spatial transcriptomics, as well as *in vitro* systems like kidney organoids and the glomerulus-on-a-chip, have contributed to our understanding of glomerular pathophysiology. Knowledge gained from these studies will contribute toward the development of personalized therapeutic approaches for patients affected by proteinuric diseases.

Keywords: Proteinuria, kidney disease, single cell transcriptomics, spatial transcriptomics, kidney organoids, kidney-on-a-chip, glomerular filtration barrier

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BACKGROUND

The kidney performs filtration function of removing waste products and maintaining fluid balance in the body. The structural unit responsible for these processes in the kidney is the nephron constituted by the glomerulus and tubules (1). The filtration process occurs in the glomerulus, within the glomerular filtration barrier comprised of fenestrated glomerular endothelial cells, glomerular basement membrane [300–350 nm thick membrane containing type IV collagen, proteoglycans, laminin and nidogen (2)] and visceral epithelial cells (podocytes) (3). Parietal epithelial cells make up the structure of the Bowman's capsule. The ultrafiltrate that leaves the glomerulus passes through the Bowman's space on its way to entering the tubules where reabsorption and secretion processes lead to the production of urine. Proteinuria is defined as the presence of abnormal or increased amount of protein in the urine (such as neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), cystatin C, α1-microglobulin and Tamm-Horsfall proteins (4). Proteinuria has been recognized as a marker of kidney disease since Hippocrates in 400 B.C. (5, 6) and is currently considered a key diagnostic indicator of renal progression. Impairment and/or dysfunction of different nephron compartments, glomeruli and tubules, have been shown to strongly contribute to initiation of proteinuria (7). Inversely, recent studies have also confirmed that high levels of proteinuria can directly damage the kidney (8). Thus, understanding the diverse functional roles of the cells comprising the glomerular filtration apparatus and the tubular structure in contributing to the development of proteinuria is critically important to finding new avenues or pathways to inhibit these processes. Different *in vitro* and *in vivo* studies have been conducted both in humans as well as in animal models, leading to important advances in our understanding of physiological changes causing proteinuria (4, 7–9). However, the ability to perform mechanistic studies in patients is not practical, and insights gained from

animal studies are limiting due to differences between human and animal physiology. In this regard, emergent technologies, such as single cell and spatial transcriptomic as well as kidney organoids and microfluidic systems can greatly contribute to more in-depth understanding of the molecular origins of proteinuria, thus helping to elucidate the key molecular pathways responsible for the initiation and progression of these processes. Knowledge gained from these studies will help the identification of possible novel targets for therapeutic treatments for patients with chronic kidney disease. In this review we will describe these approaches, discuss their contribution to the field and pinpoint advantages and pitfalls.

SINGLE CELL TRANSCRIPTOMIC APPLICATIONS TO STUDY KIDNEY DISEASE AND PROTEINURIA

Bulk RNA-sequencing (RNA-seq) approaches have been instrumental in elucidating important characteristics about kidney pathophysiology, but it was the development of the single cell RNA-seq (scRNA-seq) that greatly expanded our knowledge on kidney cell heterogeneity, allowing the characterization of renal cell types based on gene expression of few cells. This also opened new avenues for the comparison of transcriptomic profiles of normal renal vs. diseased renal tissues, creating new opportunities to uncover early mechanisms leading to proteinuria and kidney damage.

Several reports provide in detail characterization of renal and/or glomerular specific cell types from mice and humans using the scRNA-seq approach. Through unbiased clustering of scRNA-seq data Chung et al. successfully identified different cell types in the C57BL/6J mouse glomerulus, with podocytes, glomerular endothelial cells, and mesangial cells comprising greater than 90% of the cells, indicating the highly successful isolation of glomeruli (10). The remaining 10% of cells were identified as vascular smooth muscle cells, immune cells, parietal epithelial cells, and proximal tubular (PT) cells. Novel marker genes and gene signatures for mesangial cells (*Plvap*, *Prkca*, *Art3*, and *Nt5e*), vascular smooth muscle cells of the afferent and efferent arterioles (*Cnn1*, *Cygb)*, and new population of endothelial cells (*Dlk1*, *Ednrb*) were identified. To find a genetic and cell specific link to proteinuria, Park et al. analyzed the expression pattern of 29 human gene homologs in mice, which have been associated with monogenic inheritance of proteinuria in humans (11). Unlike earlier studies that implicated defects in endothelial cells and PT in the development of proteinuria (12–16), they concluded that podocyte dysfunction is the primary reason for proteinuria and could be the focus for targeted therapeutics based on the observations that 21 out of the 29 genes were found in only one cell type – the podocyte (11).

scRNA-seq has also been instrumental for investigating the transcriptomic changes in cell-type specific responses to injury within the glomerulus. Analysis of three different glomerular injury models (BTBR ob/ob mice, doxorubicin treatment, CD2AP-deficient mice) by Chung et al. showed minimal overlap in terms of transcriptional responses and the cell types involved, with the exception for mesangial cells, which showed persistent induction of genes involved in wound healing, including distinct patterns of expression for matrix (*Col4a1*, *Col6a3*, *Col8a1*) and chemokine (*Ccl2*, *Cxcl1*, *Cxcl13*) associated genes in each model (10). In contrast, in a glomerular nephritis mouse model, injection of nephrotoxic serum induced cytoskeletal regulation, cell adhesion and inflammatory response in podocytes on day 1 (peak proteinuria) that largely normalized on day 5, when proteinuria was nearly resolved. In podocytes, the response to injury was shown to be mediated through the Hippo pathway with upregulation of *YAP* and *TAZ* genes, and their known targets, including *Ctgf*, *Cyr61*, and *Axl*. The effects of injury were more prominent in TAZ (Wwtr1-/-) or YAP (Yap1-/-) knock-out mice at day 1 and exhibited delayed resolution, indicating that Hippo pathway proteins are essential for podocyte recovery after immune injury. Identifying the genes and pathways involved in the cell's programmed injury response holds potential to finding new biomarkers of kidney injury and proteinuria. In addition, scRNA-seq has been applied to study the role of sex differences on kidney function in health as well as during disease progression. In this regard, important differences in the segment 3 (S3) of the PT were identified. It was found that males had high expression of the *Cyp7b1* transcript while females had high expression of the *Slc7c12* transcript (17). The differential expression of genes in male PT cells could possibly be linked to their increased susceptibility to ischemia compared to their female counterparts. While no clear evidence has been found yet to corroborate these mechanisms in humans, if these findings are confirmed they can inform about novel sex-specific targets for the treatment of proteinuria.

In human, applications of scRNA-seq have been widely used to study different etiologies of CKD, including IgA nephropathy (18), diabetic nephropathy (19), lupus nephritis (20, 21) and others (22). Studies in IgA nephropathy revealed increased mesangial expression of novel genes, including *MATAL1*, *GADD45B*, *SOX4*, and *EDIL3*, and upregulation of genes enriched in inflammatory pathways including *TN*, *IL17,* and *NOD-like receptor* signaling in the tubules (18). In early diabetic nephropathy single nucleus RNA-seq showed increased immune cell activation and revealed cell type specific transcriptional signature consistent with increased potassium secretion, driven by alterations in *Na+/K+-ATPase*, *WNK1*, *NEDD4L*, and mineralocorticoid receptor expression, and decreased paracellular calcium and magnesium reabsorption (19). In lupus nephritis kidneys, 21 disease specific gene subsets specific for leukocytes, including myeloid, natural killer (NK), T and B cells were identified (21). Independently, using scRNA-seq, Chen et al. analyzed kidney biopsies affected by IgA nephropathy, lupus nephritis and membranous nephropathy and showed that podocytes from all these etiologies had increased expression of *FXYD5*, *CD74*, *B2M* (beta-2 microglobulin, a component of MHC class I molecules) when compared with a healthy donor (20). Increased *CD74* (a trafficking regulator and cell membrane receptor for macrophage migration inhibitory factor) mRNA expression has been associated with increased interaction between podocytes and immune cells in glomerulonephritis. In addition, *B2M* has pro-inflammatory properties (20). Since

immune infiltration has been linked to proteinuria, further research into how the upregulation of CD74 expression may contribute to poor filtration would aid in our understanding of proteinuria development. Increased immune cell infiltration in the glomerulus in lupus nephritis has been shown to impair filtration and lead to proteinuria (23, 24) suggesting that these kind of studies could be pivotal for understanding the signaling cascades involved in the development of proteinuria.

While great advancements have been achieved with the use of scRNA-seq technology in the study of kidneys and acute and chronic renal diseases, there are technical limitations, ranging from depth of the sequencing and higher background noise that limits the ability to detect lowly expressed genes to high data variability that prevent more robust and in detail data analysis. There are also biological limitations that prevent comprehensive detection, analysis, and interpretation of scRNA-seq data. One such limitation is the so called "transcript dropout phenomenon", defined by loss of transcription in large fractions of cells that usually occurs for low or moderately expressed transcripts. This may be explained by temporal fluctuations in the transcription, when the expression of protein is there, but its transcript may not be detectable for a large fraction of the time following initial higher transcriptional activity [a phenomenon called transcriptional bursting (25)]. There are also limitations regarding differentiating different renal cell types, like for example glomerular mesangial cells from other stromal mesenchymal cell types. Since the early focus of much research on the glomerular mesangial cells in the 1970's, the lack of molecular definition and absence of genetic tools have been major hurdles to study their functional role in glomerular injury. This is because many genes typically expressed by mesangial cells, such as *PDGFRA*, *PDGFRB*, *DES*, *GATA3* and *ITGA8* are also highly expressed in other stromal cells (10, 26). These studies unraveled that mesangial cells do not only possess a pericyte-like signature, but also a prominent fibroblast-like and vascular smooth muscle cell-like profile, suggesting that they represent a hybrid of pericyte-vSMC (vascular smooth muscle cells) and fibroblast.

In addition, the availability and use of different scRNA-seq platforms and library preparation methodologies [such as CEL-seq2/C1, Drop-seq, MARS-seq, SCRB-seq, Smart-seq/C1 and Smart-seq2 (27)] makes it difficult to make valuable comparisons between different studies (28). Much needed improvements in technical and computational approaches that constitute the basis of scRNA-seq will in the near future promote our ability to take full advantage of single cell analysis and enable the discovery of subpopulations of interest, benefitting our understanding of kidney health and disease.

SPATIAL TRANSCRIPTOMIC APPLICATIONS TO STUDY KIDNEY DISEASE AND PROTEINURIA

Spatial transcriptomics (ST) is an emerging new technology that enables the study of gene expression profiles in tissue histology sections while retaining the morphological information of the transcripts and their originating cell types (29). ST integrates quantitative transcriptomics (30–32) with high-resolution tissue imaging, spatially resolved *in situ* hybridization technologies (33–35) and unbiased bioinformatics analysis to facilitate the molecular characterization of tissue and cell structures. This is accomplished using barcode-based approaches (29, 36, 37), which in contrast to tissue dissection followed by sequencing-based transcriptomic profiling and fluorescence *in situ* hybridization (FISH) approaches, allows for whole transcriptome spatial profiling.

The morphological context of gene expression is of critical importance when studying cell-to-cell interactions or signaling between different segments of a functional tissue to understand the molecular mechanisms of tissue homeostasis vs. pathological developments. The advent of ST creates tremendous opportunity for understanding proteinuric kidney disease. By retaining the positional information of cells and individual nephrons, ST enables the study of disease heterogeneity one nephron or nephron compartment at a time, which is critically essential for understanding how transcriptional diversity contributes to mosaicism of tissue phenotypes which ultimately progresses to the development of proteinuria and renal failure.

Over the last decade, the utility of ST in biological research has grown substantially. Many studies on different mouse models of human disease as well as human specimen have been performed, but mainly in the cancer field (38–40). Several powerful commercial platforms have since emerged, including the Visium by 10X Genomics (US), the NanoString Technologies (US), Akoya Biosciences (US), Fluidigm, Canopy Biosciences (a Bruker company), Lunaphore Technologies (Switzerland), Vizgen (US) and RareCyte (US) that offer whole genome as well as custom ST solutions (41). To this date, very few studies reported the use of ST in the kidney setting, mainly in the acute kidney injury (AKI) setting (42–44).

Different regions of the nephron, due to their specific functional and metabolic needs have different susceptibility to AKI, and are, therefore affected variably (42). ST represents a powerful tool that can contribute to our understanding of the spatial orientation and cellular interactions in AKI providing nephron-segment-specific characterization of the disease heterogeneity. Applying the 10x Genomics Visium ST platform, Melo Ferreira et al. (43) interrogated epithelial cell-immune crosstalk in two murine AKI models, ischemia/reperfusion injury (IRI) and cecal ligation puncture (CLP). The distribution of *Havcr1* (hepatitis A virus cellular receptor 1, that produces the kidney injury molecule-1 protein) expression was found to be localized to the outer stripe region of the medulla in the IRI model and remained unregulated in the LCP model. The outer stripe in the IRI model co-localized with the expression of *Atf3* (activating transcription factor 3, a regulator of neutrophil migration) in the PT S3 cells. Importantly, 52.2% of $Ly6G^{+}$ and $CD11b^{hi}$ neutrophils in the IRI model were found localized in the outer stripe by immunofluorescence imaging. In the CLP model, 80.2% of the $Ly6G^{-}$ and $CD11b^{hi}$ infiltrating macrophages co-localized with cortical PT epithelial regions, while the NK cells were found in the cortex and outer stripe of the kidney in the ST data, suggesting that a subpopulation of PT cells with

Atf3 expression may be responsible for neutrophils chemotaxis (43). A link between neutrophil recruitment and development of proteinuria has been suggested and is currently under intense investigation (44–46). These studies were done using human cells and mouse models.

In addition, areas of low-expression regions were identified when compared with sham control. In IRI, the low-expression regions had upregulated genes highly enriched in pathways associated with metabolism of amino acids and fatty acids, and injury response mechanisms including apoptosis, oxidative stress and the p38 MAPK cascade. Neutrophil migration and IL-17 signaling was associated with potential inflammatory response of neutrophils in the IRI model. In the CLP model in contrast, pathways enriched in low-expressing regions were associated with p53 signaling, cell cycle arrest, apoptosis, TNF signaling and macrophage differentiation (43).

In a mouse model of endotoxemia associated renal injury, Janosevic et al. used integration of Visium Spatial Gene Expression with scRNA-seq to map transcriptomic changes from early injury into the recovery phase of the disease (47). Using this method, spatial resolution of unique subclusters of endothelial cell populations were plotted. Subtypes of S3 PT called S3-Type 2 (S3T2) with unknown position in the nephron and characterized by expression of angiotensinogen (*Agt*) and other unique identifiers such as *Rnf24*, *Slc22a7*, and *Slc22a13* were localized to the outer stripe of the outer medulla. Similarly, the same group also identified unique features in macrophage subtypes relating to RNA kinetics and cell differentiation, which could not be identified by the traditional flow cytometry-based classification of M1/M2 phenotypes. Overall, combination of spatial and single cell RNA-seq methods and analytical approaches are powerful new tools that can provide new insight into the mechanisms of AKI pathogenesis and facilitate the development of potential new targets for treatment.

The study of proteinuria during CKD progression has been particularly challenging because the mechanisms underlying glomerular disease are many and include paracrine, inflammatory, immune, fibrotic, proliferative, metabolic, and apoptotic processes. Similarly, in the tubulointerstitial compartment multiple inflammatory and pro-fibrotic pathways contribute to the sustained fibrogenesis and renal damage progression. Historically, *in situ* hybridization methods have been applied for validation of bulk and/or scRNA seq data (48). The applications of ST, on the other hand, have the potential to advance the field; however, to date, no peer-reviewed data encompassing different etiologies of CKD and including both the glomerulus and tubules have been reported. Using ST, it will be possible to study different human glomerulopathies and key observations will presumably indicate the significance of interglomerular differences during injury that may prove to be essential for understanding how damage to the glomerulus is initiated and how these processes prime the kidney for the onset of proteinuria. Thus, the use of ST provides great potential to advance our understanding of CKD, and ultimately promote the development of personalized medicine approaches for the treatment of kidney diseases.

Different ST platforms have specific limitations; but they all share some drawbacks that include high processing costs, tissue processing requirements that often are challenging and laborious as well as region of interest resolution returning averaged transcriptional information from several cells within the specified area of interest (49), thus hindering the discovery of cell type-specific spatial patterns of localization and expression. In addition, formalin fixed paraffin embedded (FFPE) tissue preservation remains common practice, but most available ST methods only work satisfactorily on fresh frozen tissue, where morphological structure analysis is inferior to FFPE. Biological limitations in regard to glomerular mesangial cell identity crisis and molecular signature described for the scRNA-seq holds true also for ST but is mitigated by the possibility to spatially determine their localization. Lastly, a major drawback of all current techniques is that they only provide a snapshot in time of a dynamically active tissue (49). Therefore, samples derived at different time points would need to be studied independently to evaluate the spatiotemporal transcriptomic signatures (49, 50). Nonetheless, considering that ST is still in its infancy, future optimizations and development will provide us more precise and efficient approaches to study kidney disease.

IN VITRO APPROACHES

Despite the availability of many *in vitro* tools, 2D and tissue engineering based systems are yet functionally incomplete models of the filtration unit of the kidney, thus limiting studies aimed at understating mechanisms of proteinuria. Our chances of understanding glomerular mechanisms of injury have significantly improved thanks to the development of novel approaches such as kidney organoids or more recent microfluidic systems.

Kidney organoids are self-organized three-dimensional aggregations of cells that can be derived from embryonic stem cells as well as induced pluripotent stem cells (iPSCs). A wide range of protocols have been developed and perfectioned over the years to obtain kidney organoids that closely resemble the *in vivo* environment (51). Their potential to study the pathophysiology of the kidney has energized efforts for the development of more complex, better structured organoids that more closely mimic the *in vivo* development and maturation. They have quickly become a great platform to study podocyte damage (52, 53) and genetic defects, thanks to the use of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 technology or primary mutated iPSC lines (54–57). Despite the great advances in the field, kidney organoids still present several challenges for the study of the glomerular filtration barrier and for functional studies including limited or partial cell maturation (58), underrepresentation of specific renal cells (59–61) as well as lack of a proper glomerular basement membrane (GBM) (53). In fact, even though that glomeruli organoids show maturing GBM matrisome, detectable amounts of type IV collagen $\alpha 3$ and $\alpha 4$ chains, which are essential for the formation of the mature basement membrane, was never clearly presented (53). Moreover, no clear deposition of collagen

IVα3α4α5 trimer (the major constituent of the GBM), and proof that podocytes are fully mature, as the sole producer of this specific collagen chains were clearly shown by western blot. Scalability of kidney organoids for high-throughput studies was improved thanks to bioprinting approaches (62). Common to all organoids including kidney organoids is the lack of a proper vascularization which limits both their long-term viability as well as their usefulness to study organ functionality including permselectivity and tubular reabsorption. Different approaches have been employed to improve vascularization including the combination of organoids and microfluidic chips (63), as well as implantation of kidney organoids under the kidney capsule of mouse hosts (64). In conclusion, these novel systems hold great potential in promoting our understanding of renal development and pathophysiology, but their practical use is still elusive and further development is required to allow them to become a critical tool. In particular, the potential that kidney organoids could evolve to allow the *in vitro* study of proteinuria, or mechanisms of proteinuria remains highly limited.

While kidney organoids aim to comprehensively recreate all the renal compartments *in vitro*, the development of a kidney-on-a-chip system has focused on the *in vitro* generation of tubules and glomeruli. Successful development of PT structures has been confirmed by many groups (65, 66). However, while tubular proteinuria (inability of tubules to reabsorb proteins) could be an interesting target for mechanistic as well as therapeutic studies (67), kidney tubules-on-a-chip have not been used to investigate these phenomena, its mechanisms of action or potential drug treatments.

Establishment of glomeruli-on-a-chip has been for long time challenged by architectural and functional hurdles as well as by sourcing of cells. In fact, the generation of a glomerulus-on-a-chip was first reported in 2016, almost a decade later compared to the corresponding models for renal tubules (68). In a common fashion for glomerulus-on-a-chip systems, podocytes and endothelial cells were separated in the chip by a porous polycarbonate membrane coated with basement membrane extracts. Assessment of proteinuria was performed by applying mechanical force to the endothelial side, leading to cell damage, loss of junctions, and changes to the cell's cytoskeleton as well as leakage (69). Deleterious effects of mechanical stress on leakage were also studied by Musah et al. in their iPSC generated glomerulus-on-a-chip system (70). Mechanical strain, achieved by stretching the flexible PDMS (polydimethylsiloxane) membrane using vacuum, lead to a greater expression of nephrin in podocytes and promoted functionality, expressed as albumin retention (70). Moreover, albuminuria was also confirmed in an adriamycin mediated injury model, further supporting the microfluidic system as a platform for pathophysiological studies (70). At the same time, Wang at al. reported the effects of high glucose treatment on albumin leakage in another personalized glomerulus-on-a chip system accompanied by oxidative stress and podocyte damage (71). In 2019, our lab reported the development of a glomerulus-on-a-chip based on a new approach that allowed the generation of a filtration barrier

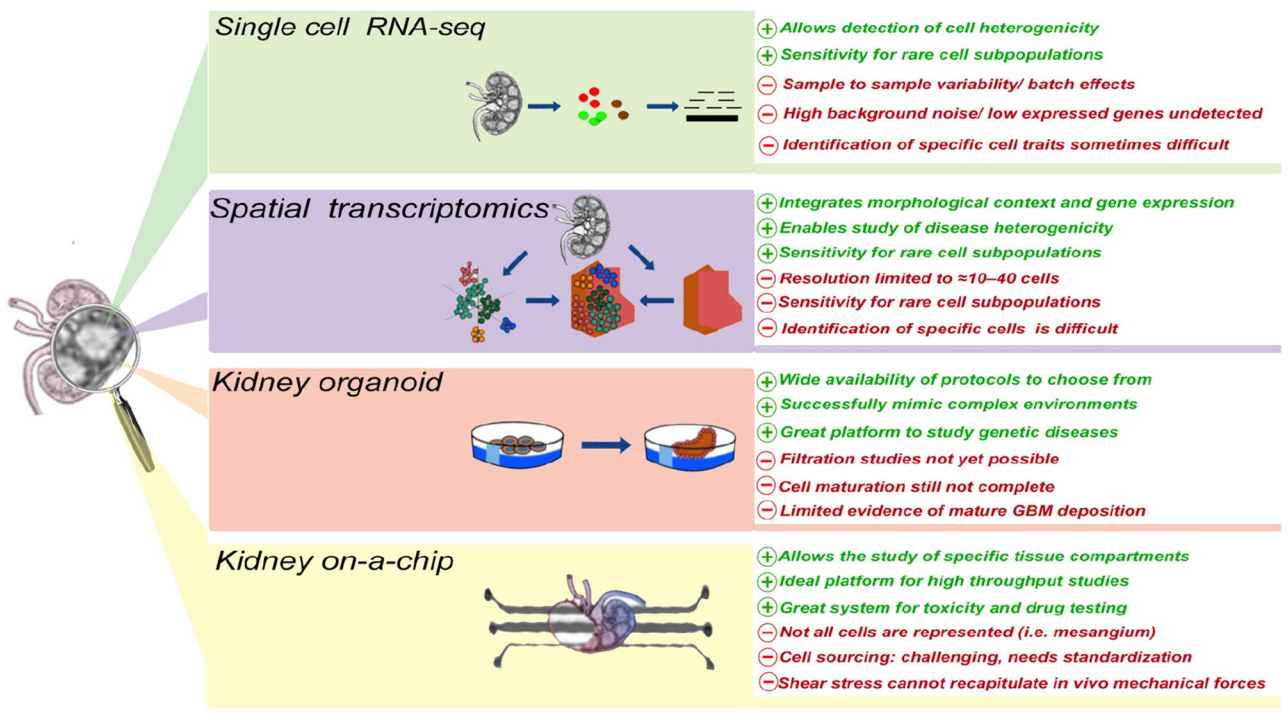

FIGURE 1 | The advantages and disadvantages of different technologies currently applied to study cellular and molecular mechanism(s) of proteinuria.

devoid of artificial membranes separating human podocytes and glomerular endothelial cells, thus allowing for improved interaction between the two cell layers as well as the *de novo* generation of GBM (72). Permselectivity under normal culture conditions as well as albuminuria following a variety of insults including puromycin aminonucleoside damage and high glucose exposure was confirmed. Notably, use of podocytes carrying a collagen IV mutation (typical of Alport Syndrome) lead to loss of permeability. We also established a model of autoimmune disease by using sera from membranous nephropathy patients, confirming albuminuria that was proportional to the clinical levels of proteinuria and PLA2R antibody titer in the donors (72). More recently, Xie et al. developed a glomerulus-on-a-chip using a novel approach based on extruded topographic hollow fibers and successfully confirmed permselectivity of their platform (73). Taken together, the exciting progress in the field of kidney microfluidic systems, while still in its initial stages, holds the promise for new discoveries based on molecular mechanisms of injury in the filtration barrier as well as the possibility to apply those findings for a more personalized therapeutic approach. The existing microfluidic systems reconstruct separate segments of the nephron, such as the glomerulus or the tubular compartment only and are thus limited in regard to creating a complete functional nephron that faithfully replicates the mechanical forces at play in the glomerular tuft and in the tubular compartment. Additional limitations of these *in vitro* systems include the absence of other key cells like, for example, mesangial cells or macula densa cells. The *in vitro* systems also lack immune cells, such as resident macrophages, which presumably play important maintenance role in the glomerulus (74, 75).

CONCLUSIONS AND FUTURE DIRECTIONS

In the last decade, the development of a wide array of research tools has significantly advanced our ability to better understand kidney disease, its origin, and its manifestations, including proteinuria. Single cell transcriptomics has the potential to enable the identification of the cell type (or types) initiating, promoting, or being damaged by proteinuria. Generation of these data is well complemented by the inclusion of spatial data. Additionally, development of kidney organoids and microfluidic systems has the potential to allow, for the first time, the *in vitro* study of proteinuric diseases, thus enabling both mechanistic and pre-clinical studies (**Figure 1**).

Despite the great promises, the use of these tools to study kidney disease and proteinuria is still in their infancy stage due to both cost and efficiency issues development of improved platforms, tailored and built upon the current needs in kidney research will bring, in the coming years, the much-needed tools to advance our knowledge of kidney disease.

AUTHOR CONTRIBUTIONS

EG, LP, SD, and SS conceptualized and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Glomerular Endothelium Restricts Albumin Filtration

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Inflammatory activation and/or dysfunction of the glomerular endothelium triggers proteinuria in many systemic and localized vascular disorders. Among them are the thrombotic microangiopathies, many forms of glomerulonephritis, and acute inflammatory episodes like sepsis and COVID-19 illness. Another example is the chronic endothelial dysfunction that develops in cardiovascular disease and in metabolic disorders like diabetes. While the glomerular endothelium is a porous sieve that filters prodigious amounts of water and small solutes, it also bars the bulk of albumin and large plasma proteins from passing into the glomerular filtrate. This endothelial barrier function is ascribed predominantly to the endothelial glycocalyx with its endothelial surface layer, that together form a relatively thick, mucinous coat composed of glycosaminoglycans, proteoglycans, glycolipids, sialomucins and other glycoproteins, as well as secreted and circulating proteins. The glycocalyx/endothelial surface layer not only covers the glomerular endothelium; it extends into the endothelial fenestrae. Some glycocalyx components span or are attached to the apical endothelial cell plasma membrane and form the formal glycocalyx. Other components, including small proteoglycans and circulating proteins like albumin and orosomucoid, form the endothelial surface layer and are bound to the glycocalyx due to weak intermolecular interactions. Indeed, bound plasma albumin is a major constituent of the endothelial surface layer and contributes to its barrier function. A role for glomerular endothelial cells in the barrier of the glomerular capillary wall to protein filtration has been demonstrated by many elegant studies. However, it can only be fully understood in the context of other components, including the glomerular basement membrane, the podocytes and reabsorption of proteins by tubule epithelial cells. Discovery of the precise mechanisms that lead to glycocalyx/endothelial surface layer disruption within glomerular capillaries will hopefully lead to pharmacological interventions that specifically target this important structure.

Keywords: endothelial surface layer, endothelial dysfunction, fenestrae, glycocalyx, hyaluronan, permselectivity, proteoglycans, thrombotic microangiopathy

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INTRODUCTION

Albuminuria is the hallmark of essentially all disorders affecting renal glomeruli. In some cases, endothelial cell (EC) injury predominates, for instance the hemolytic uremic syndrome (HUS) (1), thrombotic thrombocytopenic purpura (TTP) (2), pre-eclampsia (3, 4), and nephrotoxicity due to VEGF inhibitors (5). Transient proteinuria also accompanies generalized EC activation, in

the presence of sepsis (6) or viral infections (7, 8). Microalbuminuria is a feature of widespread EC dysfunction in diabetes (9, 10) and in cardiovascular disease, where it is a predictor of cardiovascular risk (11–19). EC activation and injury also contributes to proteinuria in many glomerulonephritides and vasculitides affecting glomeruli. This review will tackle the question to what extent the glomerular endothelium contributes to the glomerular capillary wall (GCW) barrier preventing filtration of albumin and other circulating macromolecules. It will review the molecular components of this part of the glomerular capillary barrier (GCB) followed by an exploration of some human diseases in which glomerular EC injury or dysfunction leads to proteinuria. Due to space limitations components of the endothelial glycocalyx that govern complement activation, coagulation and inflammatory cell adhesion and transmigration will not be reviewed in detail.

It is useful to recall that without a glomerular barrier to macromolecule filtration, the potential filtered load of albumin would be 3–5 g/min (∼4–7 kg/24 h) in human adults, assuming a plasma albumin concentration of 40 g/L and a glomerular filtration rate (GFR) in the range of 75–125 ml/min. Given the upper limit for urinary albumin excretion of 30 mg/24 h in normal adults, it follows that <0.001% (∼ 1/100,000) of the potential filtered load of albumin is excreted in the urine. Indeed, even with severe nephrotic syndrome, urinary albumin loss usually represents <1% of the potential filtered load. Thus, extraordinarily effective mechanisms prevent urinary loss of albumin and other circulating macromolecules.

THE GLOMERULAR CAPILLARY WALL BARRIER

The Glomerular Sieving Coefficient (θ) for Albumin ($_g\theta_{Alb}$) and Other Macromolecules

The GCW sieving coefficient, $_g\theta$, is defined as the ratio of the Bowman's space to plasma concentration for any given molecule. Since the GCW prevents macromolecule filtration despite its large hydraulic conductivity (water permeability), $_g\theta$ for macromolecules is usually much lower than 1 and depends on size, charge, and shape of the macromolecule. It should also be noted that proximal tubule albumin reabsorption contributes to the low urinary albumin concentration. Hence, $_g\theta_{Alb}$ can only be determined from urinary albumin levels if the modification of urine by proximal tubule cells is blocked. Alternatively, the albumin/tracer concentration in Bowman's space or early proximal tubule must be quantified. Functional models of the GCW (20–24), derived from experimental sieving data for infused tracers like ficoll (25–28), dextran (29–31), albumin (32–34) or endogenous circulating proteins (28, 35, 36), suggest that the GCW functions as a composite gel-like mesh with a high density of pores having a radius in the 45–50 Å range, a few large pores with radii of 75–155 Å, and a negatively charged layer at the blood/endothelial interface with a charge density of ∼35–45 mEq/L (22). Taking into account these experimentally derived parameters, a mathematical model predicted a $_g\theta_{Alb}$ of 2×10^{-3} (a ratio of ultrafiltrate: plasma albumin of 2: 1,000) (22). In fairly close agreement, the best measured estimate of $_g\theta_{Alb}$ obtained by micropuncture in rats was 6.2×10^{-4} (0.62: 1,000) (33), and $_g\theta_{Alb}$ derived from radiolabeled albumin tracer studies (34) was 6×10^{-4}. More recent quantification of $_g\theta_{Alb}$ by intravital two-photon fluorescence microscopy in rats has varied more widely: 0.034 (37), 0.014 (38), 0.002–0.004 (39) and 0.00044 (40). It appears that technical limitations account for some of the higher values by this approach (39, 41). Norden et al. (36) studied humans with the Fanconi syndrome due to Dent's disease, in whom proximal tubule albumin reabsorption is negligible, and found that $_g\theta_{Alb}$ averaged 7.7×10^{-5}. Similarly, when megalin and cubulin were conditionally deleted in mice (42, 43), $_g\theta_{Alb}$ was estimated at 7.5×10^{-5} and 1.7×10^{-5}, respectively. In such mice, streptozotocin diabetes (43) or superimposed podocin (42) deletion resulted in a significant increase in $_g\theta_{Alb}$. Since proximal tubule uptake of albumin was completely absent in megalin/cubulin deficient mice (42), these data, taken together with those from rats and humans, indicate that ∼0.01–0.1% of plasma albumin passes through the GCW into Bowman's space. In normal humans therefore, an estimated 500–5,000 mg of albumin are filtered each day (44). Proximal tubule uptake then reduces excretion to <30 mg/day.

Size and Charge Selectivity

Mathematical models of sieving data (20, 24, 25, 35, 45, 46) agree that the GCW is best described as a hydrated gel that hinders entry and movement of macromolecules based on size, shape, flexibility and charge (21, 22). Gaps in the gel that allow relatively free filtration of water and small solutes are modeled as abundant small "pores" with a molecular radius cutoff in the 45–50 Å range. The models include a small number of larger "pores" to account for the transit of a small fraction of large macromolecules. It turns out that the shape and flexibility of macromolecules influence movement through the small, abundant gaps given that large, elongated uncharged carbon nanotubes seem to be filtered relatively freely (47). Recent data furthermore suggest that compression of GCW components against intact podocytes may influence the size of gaps in the gel and therefore the molecular size cutoff (48). Abundant experimental data in animals (25–27, 30, 34, 49–52) and humans (20, 29) and ensuing mathematical models (20, 22, 27, 53–56) have concluded that for molecules like albumin whose size is close to the 45–50 Å radius cutoff, negative charge also impedes movement into and through the gel, compared to the same or similar neutral molecule. Conversely, neutralization of negative charges in the GCW with cationic protamine sulfate (57, 58), hexadimethrine (34, 59) or their removal with neuraminidase/sialidase (60, 61) which strip sialic acid from the GCW, all increase albumin excretion rapidly and reversibly. However, because these interventions also cause structural changes in podocytes and glomerular EC, the cause-effect relationship specifically between the reduction in GCW negative charge density and albuminuria was not proven. Nonetheless, infusion of enzymes to destroy negatively charged glycosaminoglycans (GAGs) also increase the fractional clearance of albumin across the GCW (62, 63), even without changes in EC or podocyte ultrastructure. By contrast, in isolated GBM, no change in albumin permeability was observed when

negative charges were neutralized with protamine (64), and the substantial reduction of GBM negative charges due podocyte-specific deletion of agrin ± perlecan (65), or the heparan sulfate glycosyltransferase EXT1 (66) raise albumin excretion only minimally. While a change in ${}_g\theta_{Alb}$ in the knockout mice could have been masked by proximal tubule albumin reabsorption, the results nevertheless cast some doubt on the possibility that "fixed negative charges" located in the GBM play a major role in charge selectivity.

Location of the GCW Albumin Barrier

The first detailed transmission electron microscopy (TEM) studies of glomeruli caused Farquhar (67) to rule out the glomerular endothelium as a component of the barrier because its fenestrae, lacking visible proteinaceous diaphragms, seemed simply too large to restrict anything smaller than circulating cells. Hence, the glomerular basement membrane (GBM) (68) and podocyte filtration slit diaphragms were held to be the main barrier to macromolecule filtration, with charge selectivity assigned to the GBM (46, 69, 70). This deduction was strengthened by findings of negatively charged sites within the GBM (71–73), congruent functional studies showing charge selectivity of the GCW (34, 51, 74), and the fact that disorders affecting podocytes or GBM all lead to proteinuria (75).

Nonetheless, the concept that the GBM and podocyte slit diaphragm constitute the main barrier to GCW protein flux cannot be reconciled with the fact that bulk convective transit of macromolecules through wide open glomerular endothelial fenestrae would rapidly clog the filter unless high-capacity mechanisms returned them, intact, to the circulation (76, 77). While podocytes endocytose and degrade albumin and other macromolecules (78), this mechanism does not have the capacity to deal with a daily load of albumin in the 4–7 kg range. Long albumin and immunoglobulin half-lives and a low renal albumin degradation rate (79) are also inconsistent with removal and degradation of massive quantities macromolecules by podocytes. Farquhar (67) suggested that macromolecules pass through the endothelium into the GBM and sub-podocyte space and then are swept into the mesangium. However, bulk transit of plasma proteins through the mesangium back into the circulation has never been demonstrated, and glomerular lymphatics that would be needed to clear them from the mesangium have not been found (80).

It turns out that under physiological conditions, endogenous albumin (81, 82), or infused gold-conjugated albumin (68), actually do NOT penetrate glomerular endothelial fenestrae, leading to the more attractive conclusion that a barrier covering the endothelium and extending into endothelial fenestrae retains all but a small fraction of albumin and other large proteins within the circulation. Indeed, disruption of glomerular EC adherens junctions by EC-specific notch1 activation or VE-cadherin deletion results in glomerular EC glycocalyx damage and significant proteinuria (83), implying that fully differentiated glomerular EC with intact adherens junctions and glycocalyx are critically important components of the GCW barrier. No doubt, as detailed by comprehensive models of GCW permselectivity (48, 84–87), one cannot consider any single GCW component in isolation (88), but the role of the glomerular endothelium in GCW permselectivity, for which data were already accumulating in the 1980's (9, 89) is only now becoming widely accepted (10, 53, 88, 90–95).

PHYSICAL STRUCTURE OF THE GLOMERULAR EC GLYCOCALYX AND SURFACE LAYER

The EC glycocalyx consists of proteoglycans, sialomucins, other glycoproteins and glycolipids, all anchored to EC plasma membrane. Molecules in the EC glycocalyx interact with and extend into the sub-endothelial GBM and into a luminal endothelial surface layer (ESL). The ESL is composed of secreted and circulating molecules that associate reversibly with the luminal EC glycocalyx, forming a hydrated, loose gel-like layer between blood and EC glycocalyx. These delicate EC surface components are destroyed by tissue processing for conventional electron microscopy (EM) due to their hygroscopic nature, and perfusion and oxygenation are required for their stability (81, 96). The luminal EC glycocalyx and the ESL were therefore not appreciated until appropriate techniques for their visualization and quantification were developed.

Visualization of the EC Glycocalyx

With conventional processing for transmission or scanning EM the glomerular endothelium has the appearance of a sieve, with fenestrae ∼60–80 nm (600–800 Å) in diameter accounting for ∼30% of the glomerular EC surface area. Glomerular EC fenestrae are plasma membrane-lined, transcellular pores that lack the proteinaceous PV-1-based diaphragms observed in most other fenestrated endothelia (97). The size and density of glomerular EC fenestrae accounts for the enormous hydraulic conductivity of the GCW (98). Any decrease in their density and/or size leads to a reduction in GFR, for instance in experimental models of uranyl nitrate (99) and gentamicin (100)-induced acute renal failure, streptozotocin induced diabetes (101), and in humans with diabetes (102) and preeclampsia (103).

The radius of glomerular EC fenestrae is much larger than the effective radius of circulating macromolecules that are not filtered, for instance orosomucoid (29 Å), albumin (36 Å), Transferrin (43 Å), IgG (55 Å), α2-macroglobulin (90 Å) and fibrinogen (108 Å) (35, 104), so they were initially assumed to allow their free convective movement into the GBM. Yet, studies in non-glomerular capillaries had suggested that EC fenestrae are impermeant to macromolecules (104), and Luft (105) found that EC do not present a "naked" surface to circulating plasma, given that perfused electron-dense ruthenium red accumulated on the EC luminal surface revealing an anionic coat. Avashi and Koshy (106) perfused kidneys with ferritin, a multimer ∼120 Å nm in diameter, so much smaller than glomerular EC fenestrae. Cationic ferritin densely decorated the glomerular EC surface and the core of fenestrae and did not penetrate into the GBM. Anionic ferritin was completely excluded from the EC surface and the GBM, indicating that the EC coat excludes negatively charged macromolecules. Furthermore, adhesion of

cationic ferritin was removed by neuraminidase and reduced by heparinase and hyaluronidase without change in EC or podocyte ultrastructure. The authors concluded that "*glomerular endothelial fenestrae are not empty holes" but "are occupied by an anionic matrix that is visualized only following the binding of an electron-dense tracer. In this respect the matrix in the fenestrae is similar to the glycocalyx at the external surface of cells which also remains invisible in unstained preparations*" (106). Rostgaard and Qvortrup (96) extended these observations using oxygen-carrying perfusion fixation and tannic acid/uranyl acetate staining. They observed "sieve plugs" in fenestrae of intestinal and peritubular capillary EC, and a similarly stained layer covering the EC. But in glomerular EC the same procedure revealed only a delicate ∼300 nm thick surface coat (96). Hjalmarsson et al. (107) reported a colloidal lanthanum labeled ∼60 nm thick EC glycocalyx that was revealed in oxygen-carrying perfusion fixed, tannic acid-stained tissue. They observed a thicker ∼200 nm coat ascribed to glycocalyx plus ESL. In their study, cupromeronic blue stained tissue showed a semi-ordered proteoglycan network within the fenestrae (107). In glomerular EC, Hegermann (108) recently visualized an amorphous 200–300 nm thick layer with alcian blue. With cationic colloidal thorium they observed an electron-dense layer that filled the fenestrae, extended from the EC surface by 50 to 300 nm and was organized into bundles that were about 50 nm wide at the EC surface, with sub-organization into wider and wider bundles as they moved away from the surface. They concluded that the glycocalyx proper represents bundles of proteoglycans that are anchored to the EC plasma membrane and extend vertically from the cells toward the capillary lumen (**Figure 1**). These findings are consistent with those by Squire et al. (109) in non-glomerular EC, who reported vertically organized bundles extending from the EC surface, intertwined with horizontal strands forming a lattice with gaps that could account for size-selectivity. Indeed, Fan et al. (110) were able to visualize hyaluronan (HA) and heparan sulfate (HS) at the single molecule level in cultured EC, using stochastic optical reconstruction microscopy (STORM), i.e. a super-resolution imaging technique with a resolution of 20 × 50 nm. They reported that HS bundles extend vertically from the EC surface and are intertwined with horizontally arranged, long HA strands to form an organized lattice-like network on the EC surface (110, 111).

Defining the Height of the ESL

It could still be argued that tissue processing and deposition of electron-dense material for transmission EM could produce artifact that might overestimate the dimensions of the EC glycocalyx and/or might remove the ESL. To assess the thickness of the EC glycocalyx/ESL the zone of exclusion for RBCs or fluorescently labeled tracers above the EC plasma membrane is therefore commonly determined. For instance, in hamster cremasteric muscle capillaries, the exclusion zone for dextran 70 and RBCs was found to be 400–500 nm (112), and was significantly reduced by hyaluronidase, and partially reconstituted hyaluronan or chondroitin sulfate infusion (113). In renal glomerular capillaries, the zone of exclusion for infused intralipid droplets was ∼200 nm and was also significantly reduced by enzymes that cleave glycosaminoglycans (114) and by elution of ESL components with hypertonic NaCl (115). Evaluation of the EC glycocalyx/ESL thickness is now a commonly used technique in human clinical research (116) and has helped define changes in the height of the glycocalyx/ESL in disease.

Hence, all EC surfaces are covered by an organized glycocalyx and an associated ESL. These form an anionic surface that results in repulsion of anionic macromolecules as well as the anionic glycocalyx of circulating cells. The anionic EC glycocalyx extends into the fenestrae, forming a semi-permeable matrix that allows rapid filtration of water and small solutes, but not macromolecules. The lattice-like arrangement of the EC coat furthermore suggests that it participates in size-selectivity of the GCW. It is already well-established that immune-activation of EC changes its glycocalyx, breaching the normal glycocalyx/ESL (116), allowing EC interactions with circulating cells and platelets and facilitating thrombosis (117). Under those conditions it is therefore expected that permselectivity is also reduced.

MOLECULAR COMPONENTS OF THE EC GLYCOCALYX

The EC glycocalyx is composed membrane-anchored proteoglycans and sialomucins that in conjunction with secreted, hyalectin-bound hyaluronan (HA) form an organized, extremely hydrated lattice-like gel. Many membrane-anchored glycoproteins embedded in the glycocalyx serve as receptors for cytokines, growth factors and as counter-receptors for circulating cells (117). The ESL, on the other hand is a concentrated layer of circulating and EC-secreted proteins, glycoproteins, small proteoglycans and other macromolecules, that is in dynamic equilibrium with the circulation (118).

Glycosaminoglycans

The structure and function of the EC glycocalyx depends critically on its glycosaminoglycan (GAG) composition. GAGs are long, unbranched polymers of repeating disaccharides, each consisting of an amino sugar (N-acetylglucosamine or N-acetylgalactosamine) and either a galactose or uronic acid sugar (**Figure 1A**). Due to their high hydroxyl and sulfate content, GAGs are negatively charged; they bind large amounts of water, critical for their viscoelastic properties; they repel negatively charged molecules like albumin and they often serve as co-receptors for growth factors and cytokines. In the luminal EC glycocalyx GAGs confer anti-coagulant properties, they repel circulating cells, and they impart the charge barrier to the endothelium. Heparan sulfate (HS), chondroitin sulfate (CS)/dermatan sulfate (DS) and keratan sulfate (KS) GAGs are all assembled on core proteins of distinct proteoglycans. Hyaluronic acid (HA; aka hyaluronan) (119) is the only GAG synthesized outside the Golgi as a stand-alone polysaccharide composed of non-sulfated N-acetylglucosamine/glucuronic acid disaccharide repeats. HS (120), the most abundant GAG in the EC glycocalyx, consists of N-acetylglucosamine/uronic acid repeats, CS GAGs (121) consist of N-acetylgalactosamine/glucuronic acid and

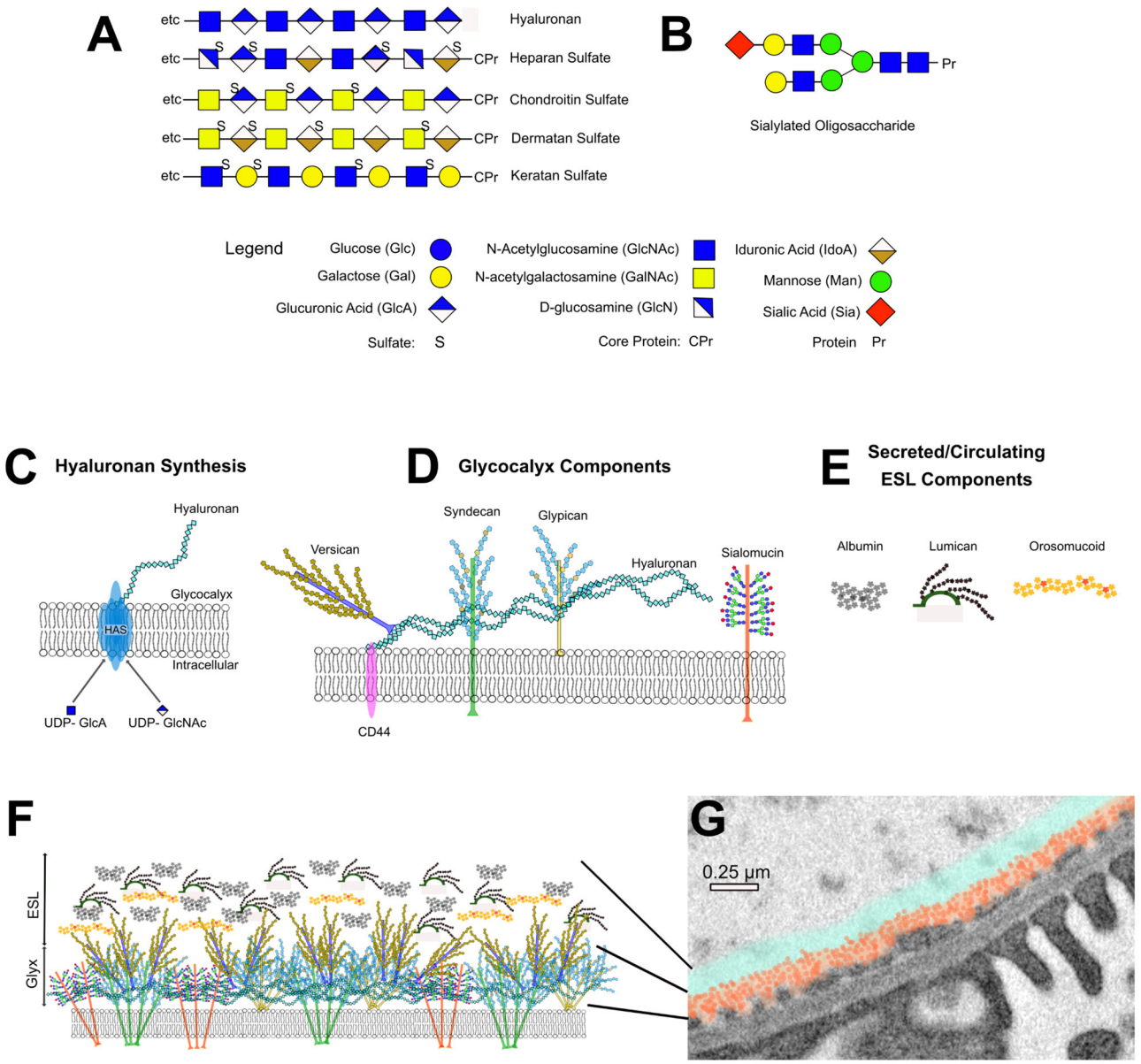

FIGURE 1 | Components of the EC glycocalyx and ESL. **(A)** Structure of glycosaminoglycan chains. **(B)** Example of a branched, sialylated oligosaccharide side-chain. **(C)** Schematic representation of hyaluronan synthase (HAS)-mediated hyaluronan synthesis at the cell membrane. **(D)** Schematic representation of major EC glycocalyx components. **(E)** Schematic representation of major ESL components. **(F)** Artistic representation of the dense, bundled glycocalyx component. **(G)** Transmission EM image of a glomerular capillary wall with superimposed artistic representation of the glycocalyx (orange) and ESL (blue) thickness.

KS (122). GAGs are composed of N-acetylglucosamine/galactose disaccharide repeats (**Figure 1A**). Addition of GAG chains to proteoglycans in the Golgi is initiated by specific glucuronyl transferases that covalently couple a bridging tetrasaccharide through O-linkage on serine or threonine or N-linkage on asparagine, followed by elongation of the polysaccharide chain and subsequent position-specific modifications through de-acetylation/sulfation and epimerization. For instance, within the initial HS polymer, epimerization converts some of the glucuronic acids to iduronic acid (123) and de-acetylation converts some of the N-acetylglucosamine to glucosamine. Similarly, CS is converted to DS through epimerization of glucuronic to iduronic acid. For proteoglycans, structural and functional diversity is therefore not only due to their different protein cores, but also the GAG type, the number and length of their GAGs, as well as position-specific epimerization and sulfation. Since enzymes involved in GAG-core protein attachment, chain elongation and modification serve multiple proteoglycans, any mutations or deletion in the many enzymes that carry out these functions tend to have different, and often

more severe phenotypes than mutations or deletion of any one proteoglycan core protein (124).

Cleavage by enzymes that are GAG-specific, namely hyaluronidases, heparinase and chondroitinase have been used extensively to define the functional role of GAGs in the GCW and EC glycocalyx, and shedding of HA and EC cell-surface proteoglycans (125) into the circulation due to endogenous enzymes is used as an indicator of glycocalyx damage (126, 127).

Hyaluronic Acid/Hyaluronan

In vertebrates HA is produced by HA synthases encoded by three distinct genes (HAS1-3). The HA synthases are integral plasma membrane proteins with multiple membrane-spanning domains (**Figure 1C**). The catalytic site within their hydrophilic core acts as a polymerase, converting soluble intracellular UDP-GLcNAc and UDP-GLcA to polymeric HA, simultaneously extruding the growing polymer it into the extracellular space (128). The length of the HA polymer varies from about 1,000 to 10,000 kDa. The rate of HA synthesis depends on the availability of substrate sugars (129), and conversely, high rates of HA synthesis are associated with a shift of cellular metabolism to glycolysis (130–132). HA binds to cell-surface receptors CD44 (133), RHAMM (receptor hyaluronan mediated motility) (134) and the lymphatic EC receptor LYVE-1. The cytoplasmic domain of CD44, a single pass membrane-spanning receptor, is coupled to cortical actin by ERM (ezrin radixin moesin) proteins (135). The interaction of CD44 with HA enhances EC barrier function (136) and is necessary for transmission of shear force signals that cause Rac1-dependent EC re-orientation (137), enhanced nitric oxide synthesis (138) and increased HAS2 expression (129, 139). In keeping with luminal HA/CD44 interactions, HA loss from the glycocalyx profoundly reduces shear-force induced NO synthesis (140).

HA turnover is rapid and regulated, in part, through HA degradation by hyal-1 and−2 (Hyaluronidase-1 and−2) and by Cemip-1 and−2 (Cell Migration-Inducing hyaluronidase-1 and−2; the latter also known as transmembrane protein 2/TMEM2) (131, 141). Hyal-2 is a GPI-anchored plasma membrane-associated enzyme that cleaves CD44-bound HA. The fragments are then internalized by the GAG scavenger receptor HARE (142). The cemid1 (143) and−2 (144) hyaluronidases are single-pass plasma membrane-spanning proteins that degrade extracellular HA into small, bioactive extracellular fragments (oHA). These oligosaccharides modify VEGF signaling in EC (144).

HA polymers are hydrated with 15 H_2O molecules per disaccharide unit (145). At the apical/luminal EC surface HA interweaves with other components of the glycocalyx/ESL (110, 134) and shear stress (129, 146) and inflammatory stimuli (147) augment HA accumulation in the EC glycocalyx. HA binds proteoglycans in the hyalectin family (see below) forming large, patterned aggregates. A HA-versican lattice may in fact account, at least in part, for the semi-ordered appearance of the apical EC glycocalyx (108, 109), and perhaps also contribute to size-selectivity of the GCW.

In systemic microvessels, destruction of HA by hyaluronidase markedly reduces the height of the EC glycocalyx and its macromolecular barrier function (113). Similarly, in glomerular capillaries the height of the EC glycocalyx is reduced by hyaluronidase infusion along with an increase in the fractional excretion of albumin (114). EC-specific, conditional HAS2 deletion in mice reduced glomerular EC HA and cationic ferritin labeling, along with progressive proteinuria, glomerular EC ultrastructure changes and capillary involution (148). Conversely, in the mouse streptozotocin model of diabetes deletion of the HYAL1 gene reduced hyaluronidase activity, preserved the EC glycocalyx and was associated with less glomerular barrier disruption than in wild-type mice (149). These findings are consistent with those in diabetic patients, where higher levels of circulating HA and hyaluronidase were found to be associated with the development of microalbuminuria (150, 151), and where endothelial glycocalyx disruption was associated with a substantial reduction in glomerular endothelial HA content (148).

Endothelial Proteoglycans

Among many glycosylated proteins, proteoglycans are distinguished by their very long, unbranched, sulfated GAG sidechains usually accounting for at least 60% of their molecular mass, the exception being perlecan, where the GAG chains are a minor component (see below). Classification of distinct proteoglycans is based on the structure of their protein core, the type and number of associated GAG chains, and their molecular interaction profile. Some proteoglycans are integral membrane-spanning proteins, some are covalently bound to the outer leaflet of the plasma membrane by GPI (glycosylphosphatidylinositol) anchors, and others are secreted. The principal structural glycocalyx proteoglycans in EC are membrane-spanning syndecans, GPI-anchored glypicans and secreted perlecan and versican. Other small, secreted proteoglycans are produced by EC and participate in defining the dynamic EC phenotype. In addition to their GAG chains, proteoglycans can also be modified by branched oligosaccharide side chains, some terminated by sialic acid. There currently is a paucity of data on potential effects of such modifications on the properties of the glycocalyx/ESL. Thus, future studies are needed to better understand their impact on glomerular permselectivity.

Syndecans

Syndecans 1–4 are ubiquitous single-pass type I membrane-spanning proteoglycans, with core proteins in the 20–45 kDa range. The extracellular domains of syndecan-1 and−3 are decorated by HS and CS GAGs, while syndecans-2 and−4 contain only HS GAGs. Their GAG-rich extracellular domains interact with many growth factors, cytokines and extracellular matrix proteins transmitting signals *via* their cytoplasmic domains down several intracellular pathways (152–155). In EC, syndecans act as co-receptors promoting angiogenesis (156–158) and the EC response to inflammation (158–160). At the basal surface of angiogenic EC, syndecan-1 is part of integrin/focal adhesion complex (157, 161) that promotes angiogenesis, and both syndecan-4 (162) and syndecan-1 (163) participate in the EC remodeling response to shear stress. All syndecans are expressed in cultured glomerular EC where they are part of the

luminal/apical EC glycocalyx (127, 159, 164). In Zebrafish *in vivo*, syndecan-3 is the main syndecan in glomerular EC (165).

The EC response to inflammation is characterized by upregulation of syndecan expression (158, 160) and shedding of syndecans from the glycocalyx into the circulation (125, 166), resulting in less syndecan in the ESL (167). The increased mRNA expression levels may be compensatory of the increased shedding of proteins (168). Syndecan shedding is observed in response to thrombin activation (166, 169), hypoxia, ischemia-reperfusion injury (170) and in preeclampsia (171). In glomerular EC, syndecan-4 shedding in response to IL-1β activation and was mediated by matrix metalloproteinase-9 (127). Protease-dependent syndecan shedding (126, 127, 160, 172, 173) produces bioactive soluble syndecan fragments generally inhibiting the inflammatory response (160). Syndecan shedding is now widely recognized as a biomarker of EC glycocalyx disruption (174) and is associated with a reduced glycocalyx thickness and a reduced barrier function resulting in edema formation and albuminuria (175). While cleavage of HS and CS GAGs reduces the size and barrier of the glycocalyx in glomerular and non-glomerular EC, endothelial-specific deletion of syndecan 1 alone only reduced the height of the glycocalyx but did not change its barrier function (176). In cultured EC, sphingosine-1-phosphate (S1P) rescued shedding of syndecan-1 and glycocalyx GAGs due to plasma protein depletion (177). Since S1P is presented to EC by albumin, this was taken to indicate that the effect of serum proteins on glycocalyx integrity may be mediated by S1P (178, 179). Substantial syndecan shedding along with thinning of the EC glycocalyx has also been reported in patients with CKD where it correlates with markers of EC dysfunction (180). Hence, the syndecans along with their HS GAGs are major contributors to the EC glycocalyx thickness. Their shedding signals EC glycocalyx dysfunction along with a reduction in the EC glycocalyx barrier to protein filtration.

Glypicans

Glypicans (124, 181) are proteoglycans composed of 60–70 kDa core proteins with heparan sulfate GAG side chains. The C-termini of glypicans are attached to the plasma membrane through GPI anchors. There are 6 glypican genes (GPC1-6), among these, glypican-1 is predominant in EC (182). Glypicans enhance fibroblast growth factor (FGF) (183), and VGEF-dependent (182) cell proliferation, in turn stimulating angiogenesis. Due to its GPI anchor, glypican-1 localizes to lipid microdomains often referred to as rafts, and clusters in response to shear stress in EC caveolae (184), where it activates NO synthesis in response to traction forces (163, 185, 186). In glypican-deficient mice, NO synthesis is markedly reduced, in keeping with a major role of EC glypican-1 in signaling NO synthesis (187). Conversely, the HS GAGs of glypican-1 undergo non-enzymatic cleavage from their core protein by NO (124), resulting in glypican-1 endocytosis and recycling (188). Reduced NO synthesis, a hallmark of EC dysfunction in inflammatory diseases and under conditions of increased oxidative stress, has therefore been attributed to reduced EC glycocalyx glypican-1 function (174).

Versican

Versican and aggrecan, both abundant in the vasculature, belong to the family of hyalectins (189), large, secreted CS-containing proteoglycans that bind hyaluronan with high affinity forming aggregates with substantial viscoelastic strength (190). Like other proteoglycans, hyalectin GAGs bind growth factors and cytokines, regulating their interaction with cell-surface receptors, and their cleavage by proteases releases bioactive fragments (189). In EC, synthesis of an HA-binding CS proteoglycan by EC was first demonstrated by Morita et al. (191) and versican was subsequently shown to be produced by EC (192), including glomerular EC (164). Aggrecan is synthesized by vascular smooth muscle cells and myofibroblasts (193) but evidence for its synthesis by EC is lacking so far. While versican is part of the subendothelial matrix where it binds the matrix protein fibulin (194), it also localizes to the apical/luminal EC surface where it binds hyaluronan which, in turn, attaches to cell-surface CD44 (189). Co-localization of CS GAGs and hyaluronan on the apical surface of immortalized glomerular EC in culture has been documented and removal of CS reduced the transendothelial resistance and increased apical to basal albumin flux (195) indicating a role for CS containing proteoglycans in the EC barrier function. In zebrafish, versican was observed in glomerular EC and podocytes, and its knockdown reduced the barrier function of the GCW (165). Versican synthesis by cultured glomerular EC is inhibited by puromycin aminonucleoside (164). Adriamycin *in vivo* similarly reduced glomerular versican expression along with a profound loss of glomerular EC glycocalyx/ESL thickness and an increase in the sieving coefficient for albumin due to a charge defect (196). A similar charge defect was associated with reduced glomerular versican expression in diabetic mice (197). In aggregate, these studies indicate that versican is part of the glycocalyx that surrounds EC, and that its GAGs participate in glomerular charge selectivity.

Perlecan

Perlecan is a massive proteoglycan whose protein core alone has a molecular mass of ∼470 kDa and is composed of 5 distinct functional domains (152, 198). Three GAG chains, which can be HS, CS or KS, decorate the N-terminal perlecan domain, each contributing another ∼40 kDa to the overall molecular mass. The C-terminus of perlecan interacts with transmembrane integrins. Produced by all EC (164, 199), perlecan carries only HS GAGs in EC, and is secreted into the subendothelial matrix and the EC apical/luminal surface layer (199, 200). A host of molecular interactions specific for each of the 5 perlecan domains have been described, and proteolytic cleavage of perlecan produces bioactive fragments (152, 198, 199). Relevant for EC is the pro-angiogenic action of intact perlecan and the anti-angiogenic function of endorepellin, the cleaved, soluble perlecan V domain that inhibits VEGFR2 in EC (199). Perlecan functions as a mechanosensor at the surface of chondrocytes where it transmits shear stress signals produced by compression-induced fluid flow in cartilage canaliculi. A similar function as a shear stress sensor has been proposed for EC (152, 198), though is no proven so far. Perlecan deletion in mice is lethal, but the knock-out mice

are viable when perlecan is selectively rescued in chondrocytes (201). In that model, EC perlecan is required for the appropriate formation of EC cel-cell junctions and pericyte recruitment by brain microvessels (201). In mice carrying a perlecan mutation that precludes attachment of its GAG chains, no abnormalities in glomerular structure or function were detected, and the macromolecular GCW barrier function remained intact (65). So, while perlecan is a major proteoglycan produced by EC, its GAG components do not seem to confer charge-selective properties to the GCW, and absence of perlecan GAGs do not impair glomerular EC ultrastructure. Nonetheless, perlecan shedding from the glycocalyx has been observed under conditions of EC dysfunction. For instance, in patients with severe preeclampsia circulating perlecan levels are significantly higher than in normal pregnant women (202).

Small Leucine-Rich Proteoglycan Family

The (SLRP) family (203) includes decorin, biglycan and lumican all produced by EC, including glomerular EC in culture (164) and *in vivo* (204, 205). These SLRPs are characterized by a small core protein (∼ 40 kDa) with few CS/DS or KS GAG chains (206). They are secreted into the subendothelial matrix where they interact directly with collagen, aiding in the structural matrix organization and EC adhesion and migration (207). Lumican is found in a high-salt eluate of renal vessels (115, 208), suggesting that it is a major component of the ESL. The SLRPs interact with, and regulate the function of TGF-β and its family members and other growth factors (203). Most recently decorin was shown to activate the autophagy pathway in EC (209). Decorin, biglycan and lumican null mice have been created, but so far roles in defining glomerular EC ultrastructure, thickness of the glomerular EC coat, or glomerular permselectivity have not been reported.

Endothelial Specific Molecule-1

Endothelial specific molecule-1 (ESM1, aka endocan) is a small, secreted EC-specific CS/DS proteoglycan (210) induced by TNF-α and IL-1β. It interacts with integrins and growth factors and is involved in regulating angiogenesis. Its circulating levels increase and correlate with microalbuminuria in patients with hypertension (211).

Serglycin

Serglycin is a small proteoglycan expressed by EC, and hematopoietic cells (212, 213) whose name refers to a serine/glycine repeat domain that supports attachment of several GAGs through O-linked glycation on Ser residues. At baseline, serglycin is sequestered in intracellular granules and participates in granule mobilization in response to inflammatory stimuli. In activated EC, serglycin promotes cell-surface localization of chemokine receptors (213, 214).

Endothelial Sialomucins

Sialomucins in the EC glycocalyx are integral plasma membrane glycoproteins each with a single membrane-spanning domain, a large extracellular "mucin" domain and a cytoplasmic domain that interacts with cortical actin *via* ERM (ezrin radixin moesin) proteins. Mucin domains are ser/thr/pro-rich regions densely decorated by O-glycans initiated by core 1 β1,3 galatosyltransferase (215) and containing terminal sialic acids. Silomucins largely accounting for the high sialic acid content of the EC glycocalyx. Several sialomucins, including podocalyxin (216, 217), endoglycan (218) (aka podocalyxin 2), CD34 (219), and endomucin (220, 221) are expressed by EC, while podoplanin is restricted to lymphatic EC (215). Sialomucins are sorted to the apical/luminal surface (216, 222) of EC where they play a repulsive role during embryonic vascular lumen formation (222, 223) and they repel circulating cells by virtue of their negative charge (219). EC sialomucins (219) play a role in hematopoietic precursor trafficking (219) and as counter-receptors for L-selectin, though this latter function requires modification of the O-glycan by carbohydrate 6-*O*-sulfotransferase restricted to high endothelial venules (224). The potential role of sialomucins the glomerular EC barrier to macromolecule flux has only been studied indirectly, through infusion of neuraminidase (60, 61, 95, 106, 225–227), which removes sialic acid from the GCW and consistently results in albuminuria. However, since podocytes also express the sialomucins podocalyxin and podoplanin, it is possible that the neuraminidase-induced GCW barrier results from stripping of sialic acid from both, EC and podocyte sialomucins. Even so, in cultured EC, podocalyxin knock-down markedly reduces the trans-endothelial resistance. EC-specific podocalyxin deletion in mice alters EC structure and reduces the EC barrier function in lung and brain in the presence of pro-inflammatory stimuli (228–230). Global podocalxyin deletion in mice is lethal due to a major podocyte defect, though in these mice glomerular EC are also thickened and lack fenestrae (231). Conditional deletion of the core 1 β1,3 galatosyltransferase, critical for sialylation of all sialomucins, results in marked albuminuria (232). Finally, in children with streptococcus pneumoniae associated HUS, neuraminidase-mediated removal of sialic acid from sialoglycoproteins in the EC glycocalyx likely plays a significant role in triggering intravascular coagulation, hemolysis, and acute renal failure accompanied by proteinuria (233).

CIRCULATING PROTEINS IN THE ENDOTHELIAL SURFACE LAYER

The ESL refers to a layer of macromolecules that merges with glycocalyx GAGs substantially increasing the separation of freely flowing plasma from the EC surfce (118). The height of glycocalyx with ESL is ∼250 nm in glomerular capillaries (114), and up to 500–1,000 nm in systemic vessels (113). The loosely bound macromolecules of the ESL, some secreted by EC, others derived from circulating blood (**Figure 1**), are in dynamic equilibrium with flowing plasma and are concentrated in the zone above the glycocalyx due to the sieving effect.

The precise composition of the ESL is not known, though it contains albumin, orosomucoid, lipoproteins, lipases, complement components, and small proteoglycans secreted by EC, like lumican (115). Removal of GAGs and terminal sialic acids disrupts the interactions of ESL components with the

glycocalyx proper, causing glycocalyx collapse and a reduction in the zone of exclusion.

Albumin

Produced by the liver at a rate of ∼10 g/day, albumin is the most abundant circulating protein, with normal plasma concentrations of 35–50 g/L and a half-life of 19–20 days. Encoded by a single gene, human albumin is secreted as a monomeric non-glycosylated polypeptide consisting of 585 amino acids (MW ∼66.5). A relatively high content of acidic amino acids and fatty acid binding result in an estimated isoelectric point of 4.7–5.8 (234). Hence, in physiologic solutions albumin is negatively charged. Structural analyses (235–238) show that albumin is not a simple sphere, but that it consists of 3 major domains, each containing subdomains, with 17 intramolecular disulfide bonds contributing to 3D folding. Normally, albumin assumes a heart-shaped triangular structure (237) with a hydrodynamic radius of 36.2 Å, though it can assume other conformations depending on pH, including an expanded cigar-like shape with a hydrodynamic radius of 61.5 Å (238). Were it not for its negative charge, the structure of albumin and its ability to take on different conformations suggest it could penetrate a meshwork with mean pore radii in the range of 40–60 Å, like the glomerular capillary wall (20). The albumin monomer contains hydrophobic pockets that bind many lipophilic substances, among them endogenous fatty acids, steroid hormones, thyroid hormone, bilirubin, vitamins, and phytochemicals. Its binding affinity for many drugs and its potential as drug carrier have been extensively investigated. Non-enzymatic glycation of albumin results in conformational changes that alter its interaction with endogenous substances and drugs, increase its half-life and reduce formation of albumin aggregates (38, 239).

While albumin flux across the endothelial glycocalyx and ESL is highly restricted (81), albumin also associates with the ESL and alters the endothelial barrier function. *In vitro* NMR studies show interactions between albumin and hyaluronan resulting in albumin/hyaluronan complexes that hinder the mobility of albumin in solution (240). Albumin binds to immobilized artificial glycocalyx composed of hyaluronan, heparan sulfate and chondroitin sulfate GAGS though its binding affinity is low (241). In cultured EC, albumin similarly associates with the EC cell surface in a reversible fashion (242), and in perfused frog mesenteric microvessels (243) endogenous albumin was observed in a ∼200 nm thick layer covering the EC surface (243). Likewise, albumin associates with lung EC glycocalyx; Lowering perfusate plasma protein/albumin content significantly increased penetration of endogenous, negatively charged ferritin into the vessel wall (244). Similarly, in isolated dog glomeruli, lowering perfusate albumin concentrations raised GFR not only due to a reduction in the colloid osmotic pressure, but also due to an increase in the hydraulic conductivity of the glomerular capillary wall (245). A similar effect of albumin on the hydraulic conductivity was also reported for non-glomerular vessels in frog (225) and rabbit (246). Finally, in the analbuminemic Nagase rats, the negative charge density of the glomerular EC coat was reduced, with enhanced penetration by macromolecules in the 60–90 kDa range both corrected by albumin infusion (247). In tracer studies, enhanced flux of glycated albumin across the EC layer has been reported (248), though by two-photon microscopy its GCW sieving coefficient was not different than that of native albumin (38). Instead, reduced uptake of glycated albumin by the neonatal Fc receptors (FcRn) in proximal tubule cells enhanced its renal excretion (38). Thus, *in vitro* and *in vivo* studies all indicate that albumin associates with the EC coat, reducing the filtration coefficient and the trans-endothelial flux of macromolecules (179). The relatively low affinity of albumin for glycocalyx/ESL components furthermore suggests that bound albumin is constantly exchanged with circulating albumin. Given that albumin binds the bioactive lipid S1P (178, 249), and that S1P protects the EC glycocalyx (250), it is likely that albumin not only changes the function of the endothelial glycocalyx/ESL through physical binding, but that it also delivers mediators to the EC that alter glycocalyx/ESL synthesis and degradation.

Orosomucoid

In humans, orosomucoids are produced by two distinct genes, ORM1 and 2. Orosomucoids are sialylated, negatively charged circulating glycoproteins produced mainly by the liver (251) but also by EC (252). Basal plasma concentrations are in the range of 1 g/L. Orosomucoid synthesis is strongly induced by inflammatory stimuli, like lysopolysaccharide (LPS), and interleukins-1 and−6; they are therefore considered to be acute phase reactants (251). Orosomucoid core proteins (∼21.5 kDa) undergo complex and variable glycosylation prior to secretion, increasing their molecular mass to ∼ 44 kDa, and resulting in a high sialic acid content. Orosomucoid glycosylation is modified in response to acute inflammatory stimuli, increasing the density of sialyl-Lewis × epitopes (sLe^x) that can interact with EC surface P- and E-selectins (253, 254). In cultured EC, orosomucoid 1 binds both high affinity, relatively low capacity cell surface receptors, and lower affinity, extremely high capacity binding sites (255). The former likely represent EC P- and L-selectin binding, the latter association with the ESL, increasing the ESL negative charge density (256). Pertinent to this discussion, orosomucoid reduces the flux of albumin across rat hindlimb microvessels (257), and lactalbumin flux across frog mesenteric vessels (258) and the blood brain barrier (259). In the kidney, perfusate containing orosomucoid reduces the fractional clearance of albumin (32, 260), and administration of of orosomucoid protects rats from puromycin aminonucleoside—induced albuminuria and GFR loss (261). Hence, orosomucoid, which is not filtered but associates with the surface of EC, reduces albumin flux across EC, by increasing the ESL negative charge density. Orosomucoid-dependent modulation of inflammatory cell recruitment and EC transmigration may also contribute to the renal response to injury. For instance, urinary excretion of orosomucoid increases in patients with type 2 diabetes and may be a biomarker for EC dysfunction due to low-grade inflammation (262). In triple (ORM1-3) knockout mice (unlike humans, mice have 3 ORM genes), enhanced inflammation and a greater susceptibility to renal fibrosis in the unilateral ureteral obstruction (263) and acute ischemia-reperfusion (264) models have been reported. At this time, quantitative

glomerular permselectivity studies in ORM deficient mice are lacking.

DISRUPTION OF THE EC MACROMOLECULAR BARRIER IN DISEASE

A thorough understanding of the GCW barrier not only requires knowledge of its composition, still incomplete, but also its dynamic regulation. The complete EC barrier consists not only of the glycocalyx/ESL covering the EC surface and filling the fenestrae, but also cell-cell junctions, and the subendothelial glycocalyx/matrix. Even at equilibrium, all constituents of the EC glycocalyx and ESL are continually turning over through tightly regulated mechanisms. They are subject to, and participate in responses to shear and compression forces, to soluble mediators and to signals from podocytes (265–267). Mechanisms disrupting the barrier can range from EC dysfunction observed in the metabolic syndrome and cardiovascular disease, to EC de-differentiation upon withdrawal of critical stimuli like VEGF, observed in preeclampsia, to EC activation by inflammatory stimuli in HUS, TTP and sepsis, all the way to destruction of the EC in some forms of glomerulonephritis and vasculitis.

Microalbuminuria Reflects Generalized EC Dysfunction

EC dysfunction, characterized by diminished flow-mediated vasodilation due to reduced endothelial NO production, signals generalized EC abnormalities in patients with cardiovascular disease, the metabolic syndrome, diabetes and chronic kidney disease (10, 174, 265, 268). Microalbuminuria is strongly associated with EC dysfunction (269), predicts cardiovascular morbidity (19) and is one of the earliest indicators of generalized, chronic EC injury (9). Note again that microalbuminuria tends to underestimate the GCW defect, due to proximal tubule reabsorption of albumin (79). EC glycocalyx disruption with a substantial reduction in glomerular EC HA content has been documented in patients with diabetic nephropathy (148). In generalized vascular disease, microalbuminuria is associated with a reduction in EC glycocalyx/ESL height and increase in circulating EC glycocalyx components, including hyaluronan (149, 270) and proteoglycans (126, 150, 151, 174). As EC glycocalyx glypican-1 is required to elicit shear-induced NO synthesis (184, 186), it seems likely that glycocalyx degradation is, in fact, the proximate cause of reduced flow-dependent NO synthesis in generalized EC dysfunction. In experimental diabetes, endomucin restored the EC glycocalyx (221, 271), and in human diabetic patients partial restoration of the EC glycocalyx with sulodexide, an orally administered mixture of GAGs, not only lowers blood pressure, but also reduces albuminuria and other diabetic complications (272–276). Hence, microalbuminuria reflects the endothelial barrier defect that accompanies glycocalyx disruption and EC dysfunction in cardiovascular disease, diabetes and chronic kidney disease. The use of GAGs to enhance EC glycocalyx function could well develop into new therapeutic approach. It is important to note that the massive increase in cardiovascular morbidity of dialysis patients is, at least in part, due to chronic EC glycocalyx/ESL dysfunction (277, 278).

Albuminuria Reflects Glomerular Endothelial Barrier Dysfunction in Preeclampsia

Preeclampsia affects 3–5% of all pregnant women and is associated with substantial risk to baby and mother. Albuminuria and hypertension are the earliest manifestations of preeclampsia. Marked glomerular EC swelling along with loss of glomerular EC fenestrae, also referred to as "glomerular endotheliosis" has long been recognized as the key glomerular abnormality in preeclampsia (279–281). EC abnormalities in preeclampsia, are not restricted to the glomerular endothelium, often involving the choroid plexus as well, and preeclampsia can progress to the full-blown thrombotic microangiopathy of pregnancy (282), the HELLP (hemolysis, elevated liver function tests, low platelets) syndrome. Even so, proteinuria is the main indicator of EC dysfunction in these patients. Glomerular EC differentiation and fenestration depend critically on podocyte-derived VEGF (283) and endotheliosis lesions are observed in mice with podocyte-specific VEGF haploinsufficiency (284). Also, bone morphogenetic protein-9 (BMP-9) signaling *via* the endothelial-specific ALK-1/endoglin receptor complex signals EC differentiation (285, 286). In patients with preeclampsia, placenta-derived, circulating soluble VEGF receptor and soluble endoglin inhibit VEGF- and BMP-9 signaling pathways leading to glomerular EC de-differentiation (3, 287, 288). Along with the ultrastructural EC changes, reduced EC glycocalyx/ESL height and shedding of glycocalyx components into the circulation have been documented in preeclampsia (171, 174, 202, 289). The use of VEGF inhibitors to reduce tumor angiogenesis (290) and macular degeneration (5) can evoke a similar syndrome of albuminuria, sometimes in the nephrotic range, and hypertension. It turns out that the human diacylglycerol kinase epsilon (DGKE) mutation (291), a cause of the hemolytic uremic syndrome, also reflects inhibition of VEGF signaling and consequent de-differentiation of glomerular EC (292). Hence, albuminuria in preeclampsia, and in patients treated with VEGF inhibitors, reflects EC de-differentiation resulting in a breach of the normal glomerular EC barrier to macromolecules.

The Thrombotic Microangiopathies

Characterized by a vicious cycle of intracapillary thrombus formation, platelet consumption and microangiopathic hemolytic anemia, the thrombotic microangiopathies all involve EC activation (293), whether by Shiga toxin (294), COVID-19 (295, 296), thrombin, complement components (297) and/or inflammatory cytokines (296). Normally, endogenous inhibitors prevent activation of the coagulation and complement cascades at the EC surface and soluble EC-derived mediators like NO and prostacyclin block platelet activation. As part of the luminal EC glycocalyx, integral membrane-spanning thrombomodulin binds and inhibits thrombin, and stimulates protein C, which actively cleaves components of the coagulation cascade (298), by binding

to the EC protein C receptor. Tissue factor pathway inhibitor (TFPI) (299) and complement factor H (CFH) (300) both bind HS GAGs in the EC glycocalyx/ESL, inhibiting local thrombin and complement activation, respectively. EC activation also results in reduced NO and prostacyclin production, de novo expression of membrane-anchored tissue factor, release of TFPI and CFH from the EC surface by heparinases (300), and mobilization of P-selectin and von Willebrand Factor (vWF) to capture platelets (301). It follows that even minor causes of EC activation, for instance a viral infection, can trigger run-away intravascular thrombosis in patients with genetic mutations or neutralizing antibodies to thrombomodulin (298), ADAMTS13 (297, 302), or to complement inhibitors (293). Loss of sialic acid EC glycocalyx by pneumococcal derived neuraminidase can also trigger the hemolytic uremic syndrome in children (233), as can reduced VEGF signaling due to loss of function mutations in diacylglycerol kinase (291, 292). While albuminuria is common in patients with these disorders, end-organ damage due to microvascular thrombosis are clinically more significant.

EC Glycocalyx Disruption in Critically ill Patients

Trauma, cardiovascular surgery, septic shock (303) and more recently in critical illness due to COVID-19 (7, 295), all are associated with generalized EC activation and EC glycocalyx disruption. While proteinuria is common in critically ill patients (6, 8), pulmonary and brain EC barrier disruption tend to have greater relevance for outcomes and therapy in these patients. Endothelial cell activation by inflammatory mediators, among them TNF-α and Il1-β, results in shedding of EC glycocalyx components exposing cell-surface adhesion molecules that enable the initial capture and rolling of leukocytes on the endothelium and integrin-dependent leukocyte transmigration (304). EC glycocalyx disruption also promotes platelet adhesion and reduces the anti-coagulant and fibrinolytic activity of the EC surface (305). Even so, a recent metanalysis concluded that while EC glycocalyx shedding is common in critically ill patients, it does not distinguish between various causes and is not consistently associated with "vascular leak" (116). Similarly, albuminuria in this setting is a non-specific marker of EC glycocalyx dysfunction.

SUMMARY AND FUTURE CONSIDERATIONS

The glomerular endothelium is a critically important component of the size- and charge-selective GCW barrier. Only a very small fraction of circulating albumin and other macromolecules can penetrate glomerular EC to reach the underlying GBM and sub-podocyte space. While glomerular EC fenestrae support filtration of massive volumes of water and small solutes, they are not permeable to larger plasma proteins due to a negatively charged, organized glycocalyx and ESL that covers the EC surface and fills the fenestrae. This pericellular environment not only serves as a physical barrier to macromolecules, it also controls the activity of many mediators, cytokines, growth factors, complement and coagulation cascades, and circulating cell and platelet repulsion/adhesion. Glycocalyx degradation in disorders that cause wide-spread EC dysfunction and/or activation, like the metabolic syndrome, diabetes, sepsis and other forms of systemic inflammation, result in glycocalyx degradation and proteinuria. More specific insults like VEGF pathway interruption and localized activation of complement and coagulation cascades can cause somewhat more restricted glomerular EC injury. Many components of the EC glycocalyx/ESL are known, but it is expected that there are unique aspects of its composition and organization in glomerular EC. To define these in health and disease represents a major, but important challenge for the future, given that most glycocalyx/ESL components are ubiquitous, and their function is not just defined by protein expression, but also by many position-specific polysaccharide modifications.

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BB performed the literature review and wrote this manuscript. JN and BH have contributed fundamental research and insights to this topic and reviewed/critiqued the final manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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MCC Regulator of WNT Signaling Pathway (MCC) Is a Podocyte Essential Gene

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Podocytes are an integral part of the glomerular filtration barrier. Many genes are already known to be essential for podocyte survival, structure and function, but there are more podocyte essential genes to be identified. By single-cell RNA-seq of mouse podocytes, we detected the expression of gene encoding MCC regulator of WNT signaling pathway (MCC) in majority of the podocytes and speculated that MCC is essential for podocytes. We confirmed MCC expression in mouse podocytes and further showed its expression in human podocytes. To experimentally prove the essentiality of MCC for podocytes, we knocked down MCC in cultured podocytes and found marked morphological change of cell shape, cytoskeletal F-actin stress fiber disruption, increased apoptosis, and downregulation of podocyte essential genes, CD2AP and WT1, demonstrating that MCC is essential for podocytes. Since MCC has been implicated in cell cycle and β-catenin signaling, we examined the expression of cell cycle related genes and activity of β-catenin in the MCC knockdown podocytes, but did not find significant changes. To further explore the mechanism underlying the role of MCC in podocytes, we performed RNA-sequencing and bioinformatics analysis of MCC knockdown podocytes and found a significant enrichment of the regulated genes in lamellipodia formation. Consistently, we found that MCC is present in lamellipodia and MCC knockdown resulted in loss of lamellipodia in the cells. Lastly, we found that MCC was downregulated in podocytes treated with puromycin aminonucleosides and in glomeruli of diabetic mice and FSGS patients, implicating MCC is involved in the development of podocytopathy and proteinuria. In conclusion, MCC is potentially essential for podocytes and its downregulation may be involved in podocytopathy.

Keywords: MCC, podocyte, podocytopathy, lamellipodia, RNA-seq

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INTRODUCTION

Podocytes attach to glomerular base membrane (GBM) to cover the capillaries. They have extensive foot processes which are interdigitally arranged to form slit diaphragms, allowing small molecules, e.g., salts and glucose, to pass through, while retaining the macromolecules, e.g., albumin in the blood vessels (1, 2). Therefore, podocytes are an integral part of the glomerular filtration barrier. Podocyte injury underlies and initiates focal segmental glomerulosclerosis (FSGS) (3). Podocyte injury is also involved in many other types of glomerular diseases, e.g., membranous diseases,

diabetic nephropathy, IgA nephropathy, etc. (4). It is essential to understand podocyte biology and pathogenesis.

Podocytes are also characterized by terminal differentiation and do not proliferate life-time (5, 6). Podocytes express a set of CDK inhibitors that may help maintain cellular quiescence and differentiation state, e.g., p21Cip1, p27Kip1, and p57Kip2 (7, 8). In many types of injury and disease, podocytes exhibit expression change of the cell cycle proteins, including the CDK inhibitors and cyclin molecules. The downregulation of CDK inhibitors could induce podocytes to re-enter cell cycle for proliferation (9); however, due to the intrinsic barrier to normal cell cycle in differentiated podocytes, the podocytes cannot complete mitosis, and the mitotic arrest results in mitotic catastrophe that leads to cell death (10, 11). On the other hand, the upregulation of CDK inhibitors can cause cell cycle arrest in podocytes in certain diseases, e.g., diabetic nephropathy, leading to hypertrophy and cell death (12–15). Therefore, it is essential for podocytes to possess a machinery that optimally maintains their differentiation and quiescence. However, the machinery has not been completely understood.

MCC regulator of WNT signaling pathway (MCC) is a potential colorectal tumor suppressor gene and may negatively regulate cell cycle progression. The gene encodes a phosphoprotein that is associated with the plasma membrane and membrane organelles. It has been shown that MCC overexpression can inhibit the entry into S phase in cell cycle (16). As a tumor suppressor, MCC inhibits Wnt/β-catenin signal transduction (17, 18). Interestingly, MCC was found to act as an oncogene in B cells and its knockdown induced apoptosis in human multiple myeloma (19).

We previously performed single-cell RNA-seq analysis of mouse podocytes and found a huge heterogeneity of gene expression between individual podocytes with a correlation coefficient of only 0.2. There were only a small proportion of genes that were commonly expressed in all individual podocytes (20). We speculated that commonly-expressed genes are indispensable for podocyte survival and structural and functional homeostasis; while the differentially expressed genes could be dispensable. We identified 335 genes that were expressed in all individual podocytes sequenced and demonstrated the essentiality of some genes for podocytes (20). In addition, we identified genes whose expression was detected in most of the podocytes sequenced. We speculated that these genes were also expressed in all the podocytes but the expression detection might have failed in some cells due to technical variations in the single-cell RNA-seq process; thus, they could be potential podocyte essential genes as well.

In the present study, we explored the list of genes expressed in majority of the sequenced podocytes and focused on MCC. This was because MCC expression was detected in 18 out of the 20 sequenced podocytes and, as described above, it regulates cell cycle and Wnt/β-catenin signaling, whose abnormality is known to be involved in podocyte injury. We knocked down MCC in cultured podocytes, followed by characterization of cell injury and RNA-seq analyses. The results showed that MCC deficiency induced podocyte injury. However, the injury did not involve deregulation of cell cycle and Wnt/β-catenin, unexpectedly, but instead loss of lamellipodia. This study has thus identified MCC as a potential podocyte essential gene.

MATERIALS AND METHODS

Database Mining and Bioinformatics Analysis

We extracted information of interest from following databases: data of MCC expression were obtained from the single podocyte sequencing (GSE88814) and bulk sequencing of mix mesangial and endothelial cells (GSE89263). MCC expression in mouse and human podocytes were also available in KIT database (http://humphreyslab.com/SingleCell/). We used the Human Protein Atlas (www.proteinatlas.org) to examine MCC protein expression in human glomeruli. To investigate MCC expression changes in diseases, we searched Nephroseq (www.nephroseq.org).

Reagents

The antibodies against MCC, CD2AP, total β-catenin, p16, p21, p27, p57, CDK1, Cyclin E1, Cyclin D2, PCNA, and GAPDH (Proteintech, USA); antibodies against active β-catenin, total ERK1/2 and phospho-ERK1/2, total AKT and phospho-AKT (Cell Signaling Technologies, USA); WT1 antibody (Abcam, USA); puromycin aminonucleosides (Sigma, USA); Alexa Fluor® 647 Annexin V and Propidium Iodide (Biolegend); Reverse transcription kit, DRR037A (Takara); quantitative PCR kit (Thermo Fisher Scientific); RIPA cell lysis buffer, BCA protein quantification kit (Beyotime, Shanghai); RNA extraction kit (Takara).

Culture of Immortalized Human Podocytes

The human podocyte cell line was provided by Dr. Saleem M (University of Bristol, United Kingdom). The cells were cultured in RPMI 1640 containing 10% fetal bovine serum and pennicilin/streptomycin (100 U/ml of each) (Gibco, USA) and 1% insulin-transferrin-selenium (ITS, Invitrogen). Podocytes were grown at 33°C and switched to 37°C, followed by incubation for ∼10 days for differentiation.

siRNA and PAN Treatment of Podocytes

The siRNA of MCC was synthesized by HanBio (Shanghai) according to the sequence, 5′-CAUCACUAAAGGGAGAUAU (sense strand). Podocytes were cultured at 37°C for differentiation in 6- or 12-well plates. Transfection of the siRNAs into the differentiated cells was performed using the Lipofectamine® RNAiMAX reagent (Invitrogen, CA) according to the instruction manual. The medium was changed after 6 h, and the cells were harvested 36 or 72 h after transfection. We used 50 or 100 μg/ml PAN (Sigma-Aldrich, USA) to treat podocytes followed by RNA and cell lysate preparation.

Immunofluorescence Staining

We followed the method described previously (21).

Phalloidin Staining of F-Actin

F-actin was stained using rhodamine-labeled phalloidin, and the resulting microscopy images were digitized. The images were converted to 8-bit and then inverted. Rhodamine-stained areas were quantified using ImageJ software (National Institutes of Health, Bethesda, MD). Mean actin per pixel and total actin content per cell were calculated and given as arbitrary units as previously described (21).

Quantification of the Actin Cytoskeleton

Rhodamine-labeled phalloidin was used to stain F-actin in the podocytes, and the resulting images were obtained by confocal microscopy and digitized. The rhodamine-stained areas of the actin fibers were converted to black pixels and then inverted, followed by quantification using ImageJ software (NIH). The grayscale values ranged from 0 (black) to 200 (white, the maximal actin content). The mean podocyte actin content per pixel and the total actin content per cell were calculated and expressed as AU.

RNA Extraction and qPCR Analysis

Cultured podocytes were subjected to total RNA extraction using the RNA Extraction Kit (Takara). Reverse transcription was performed using the kit from Takara (RR036A). The primers were: MCC, 5'-ACTCACTTCAGGACTGCTCCA-3′ (forward) and 5′-ATTCAGCCGTTCTGTTTCCAC-3′ (reverse); CD2AP, 5′-AAAAGCCCTTAATCCTACAGT-3′ (forward) and 5′-CCTTCTTTACCATTAAGTTCGC-3′ (reverse); WT1, 5′-AAGCAGCTAACAATGTCTGGT-3′ (forward) and 5′-TTCCATCCCCAGCGAAAACGA-3′ (reverse); p15, 5′-ACCCTGCCACTCTCACCCGAC-3′ (forward) and 5′-CCCAGGCATCGCGCACGTCCA-3′ (reverse); p16, 5′-GGGTTTTCGTGGTTCACATCC-3′ (forward) and 5′-CTAGACGCTGGCTCCTCAGTA-3′ (reverse); p21, 5′-AGGTGGACCTGGAGACTCTCAG-3′ (forward) and 5′-TCCTCTTGGAGAAGATCAGCCG-3′ (reverse); p27, 5′-CGGAGCACCCCAAGCCCTC-3′ (forward) and 5′-CAAGCTGCCCTTCTCCACCT-3′ (reverse); p57, 5′-CAAAGCCCAAAGAGCCCCGAG-3′ (forward) and 5′-CTGCTACATGAACGGTCCCAG-3′ (reverse); PCNA, 5′-ATTTGCACGTATATGCCGAGA-3′ (forward) and 5′-GCAGAAAATTTCACTCCGTCT-3′ (reverse); CDK1, 5′-GGAAACCAGGAAGCCTAGCATC3′ (forward) and 5′-GGATGATTCAGTGCCATTTTGCC-3′ (reverse); CDK4, 5′-CCATCAGCACAGTTCGTGAGGT-3′ (forward) and 5′-TCAGTTCGGGATGTGGCACAGA-3′ (reverse); Cyclin B1, 5′-GACCTGTGTCAGGCTTTCTCTG-3′ (forward) and 5′-GGTATTTTGGTCTGACTGCTTGC-3′ (reverse); Cyclin D2, 5′-GAGAAGCTGTCTCTGATCCGCA-3′ (forward) and 5′-CTTCCAGTTGCGATCATCGACG-3′ (reverse); Cyclin E1, 5′-TGTGTCCTGGATGTTGACTGCC-3′ (forward) and 5′-CTCTATGTCGCACCACTGATACC-3′ (reverse); Cyclin E2, 5′-CTTACGTCACTGATGGTGCTTGC-3′ (forward) and 5′- CTTGGAGAAAGAGATTTAGCCAGG-3′ (reverse); 18s rRNA, 5′-TTCTCGATTCCGTGGGTGG-3′ (forward) and 5′-AGCATGCCAGAGTCTCGTTC-3′ (reverse). SYBR Green dye was used in the qPCR. The thermal condition was 95°C/30 s for denature, followed by 40 cycles of 95°C/5 s−60°C/30 s on the ABI 7900HT Fast Real time System. Threshold cycle (CT) values were determined and the relative abundance of the mRNA was calculated with the formula $2^{-\Delta\Delta Ct}$.

Western Blot Analysis

Podocytes were washed with cold PBS and then lysed with 150 μl of RIPA buffer containing proteinase inhibitors cocktail (Roche) and phosphatase inhibitors. The lysates were incubated on ice and then centrifuged 12,000 g for 15 min at 4°C. The supernatants were transferred to fresh tubes and then subjected to protein concentration measurement with BCA protein kit (Bio-Rad). After mixed with loading buffer, the samples were boiled at 98°C for 5 min. 10 or 8% SDS-PAGE was used to fractionate the samples, and semi-dry transfer system (Bio-rad) was used to transfer the protein from the gel to PVDF membrane. The blot was blocked with 5% milk in TBST solution (20 mM Tris-HCl, PH 7.14, 150 mM NaCl, 0.1% Tween-20) for 60 min at room temperature, and then incubated with an antibody overnight at 4°C. After washed with TBST for 3 times, the blot was incubated with HRP-labeled secondary antibody for 1 h at room temperature. After washed, ECL system (Millipore) was used to detect the protein. Cell lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail (Roche) and a phosphatase inhibitor. The blot was incubated with a primary antibody of interest.

Flow Cytometric Analysis of Apoptosis via Annexin V Staining

After treatment, the podocytes were collected, washed twice with ice-cold PBS, resuspended in 200 μl binding buffer, and then incubated with FITC-conjugated annexin V at a final concentration of 0.5 μg/ml at room temperature for 15 min. Then, the cells were washed, centrifuged, and resuspended in 500 μl binding buffer. The cells were stained with 50 μg/ml propidium iodide at room temperature for 5 min, followed by flow cytometric analysis using a FACScan flow cytometer and CellQuest software (BD).

RNA-Sequencing

Total RNA was extracted from the cultured podocytes using Trizol (Invitrogen, USA) following the manual instruction. Paired-end libraries were made using the TruSeq® RNA Sample Preparation Kit (Illumina, USA) following the Sample Preparation Guide of the kit. Briefly, the poly-A containing mRNA molecules were incubated with oligo poly-T magnetic beads, followed by washing and elution to obtain purified mRNA molecules for library construction. Purified libraries were quantified by Qubit® 2.0Fluorometer (Life Technologies, USA) and examined with the Agilent 2100 bioanalyzer (Agilent Technologies, USA) to validate the insert size and measure the concentrations. Clusters were generated by cBot with the library diluted to 10 pM and then sequenced on the Illumina HiSeq X-ten (Illumina, USA). The library construction and sequencing were performed at Shanghai Biotechnology Corporation (China).

Data Analysis for Gene Expression

We preprocessed the sequencing raw reads by removing the reads of rRNA, sequencing adapters, short-fragments, as well as other low-quality reads. We used Hisat2 (version:2.0.4) (22) to map the cleaned reads to the human GRCh38 reference genome with two mismatches. After genome mapping, we used Stringtie (version:1.3.0) (23, 24) with a reference annotation to generate FPKM values for known gene models. Differentially expressed genes were determined using the edgeR (25). The p-values of significance in multiple tests were determined by the false discovery rate (FDR) (26, 27). The fold-changes were calculated based on the FPKM in each sample. The differentially expressed genes were determined by FDR $\leq$0.05 and fold-change $\geq$1.5.

Differential Expression Analysis

Differential expression analysis was performed with R package "DESeq2"[@LoveDESeq2]. The estimation steps were wrapped into a single function "DESeq". Results tables were generated with log2 fold changes, *p* values and adjusted *p* values. Finally, log fold change shrinkage was used to improve on the previous estimator.

Statistics

Data are presented as the mean ± SD. Differences between 2 groups were analyzed using a 2-tailed Student's *t* test and incorporated into GraphPad Prism 5 software (GraphPad Software). $P < 0.05$ was considered statistically significant.

RESULTS

MCC Is Expressed in Podocytes

We found that MCC expression was detected in 18 out of the 20 podocytes sequenced and > 5 fold in podocytes versus mesangial and endothelial cells [**Figure 1A**, (20)]. We also analyzed the bulk RNA-seq data of purified mouse podocytes, mesangial and endothelial cells (GEO database: GSE123179) (28), respectively, and confirmed the expression of MCC in mouse podocytes, which was higher than that in mesangial and endothelial cells (**Figure 1B**). We further confirmed MCC expression in podocytes in the single-cell RNA-seq database, KIT (Kidney Interactive Transcriptomics) (http://humphreyslab.com/SingleCell/) (**Figure 1C**). Furthermore, we performed immunohistochemical staining of MCC in mouse kidney and found expression of MCC in podocytes (**Figure 1D**). As gene expression conservation across species in a cell type reflects essentiality of the gene for the cell type, we investigated MCC expression in human podocytes. We found in above KIT database that MCC is expressed in human podocytes at a level higher than that in mesangial and endothelial cells (**Figure 1E**). Consistently, from the Human Protein Atlas (www.proteinatlas.org), MCC was found mainly expressed in podocytes as shown by the immunohistochemical staining pattern characteristic of podocytes along the periphery of glomerular tuft (**Figure 1F**). These results together demonstrate that MCC is expressed relatively specifically in podocytes in glomeruli in an evolutionarily conserved manner.

MCC Is Localized in Cytoplasm, Nucleus, and Lamellipodia of Podocytes

To determine the subcellular localization of MCC protein in podocytes, we performed fluorescence immunostaining of MCC and DAPI in cultured podocytes. The result showed that MCC protein was present in the cytoplasm and nucleus, as well as the periphery of the cells (**Figure 2A**). The treatment using siRNA of MCC (si-MCC) resulted in a marked decrease of intensity in these areas, demonstrating the specificity of staining. To further prove the cytoplasmic and nuclear localization of MCC in podocytes, we separated cytoplasmic and nuclear fractions of glomerular cells, and performed immunoblotting of MCC with cytoplasmic marker (GAPDH) and nucleic marker (Lamin B1). The result clearly showed MCC protein in both cytoplasmic and nucleic samples of glomerular cells (**Figure 2B**), confirming that MCC protein localizes in both cytoplasm and nucleus of podocytes.

MCC Deficiency Caused Podocyte Injury

To prove the essentiality of MCC for podocytes directly, we knocked down MCC by siRNA (si-MCC) in cultured podocytes. We tested four independent siRNAs against MCC, and they all showed excellent efficiency in MCC mRNA elimination at 24 h post si-MCC transfection. **Figure 3A** shows the knockdown efficiency for one of them by qPCR. Consistently, the protein level of MCC was accordingly reduced in the cells (**Figures 3B,C**). We then performed various experiments to examine podocyte changes in the absence of MCC.

We observed that the podocytes transfected with si-MCC became elongated in morphology 72 h after transfection (**Figure 4**). In phalloidin staining, si-MCC-treated cells exhibited marked cytoskeletal change with a significant reduction of F-actin stress fibers in the cells (**Figures 5A,B**). Reduction of actin stress fibers is a sensitive indicator of podocyte injury and is commonly used for assessing the injurious effect of a variety of treatments (20, 21). We next examined the expression of CD2AP and WT1, two podocyte essential genes whose downregulation is usually observed in various injuries, and found that they were downregulated at both mRNA and protein levels (**Figures 5C–F**). In the flow cytometry of Annexin V staining, si-MCC treated cells exhibited a significantly higher level of apoptosis than the control cells (**Figure 6**).

MCC Knockdown Had Limited Effects on Cell Cycle, Wnt/β-Catenin, AKT and ERK in Podocytes

Since MCC has been shown to regulate cell cycle positively or negatively as tumor suppressor gene or oncogene depending on cell types, we examined the expression of cell cycle related genes in podocytes treated with si-MCC by qPCR. Among CDK inhibitors, p21Cip1, p27Kip1 and p57Kip2, but not p15INK4b and p16INK4a, were slightly but significantly downregulated in the podocytes treated with si-MCC for 72 h (**Supplementary Figures 1A–E**). At the earlier time point of 36 h, p27Kip1 but not the others had already exhibited little reduction. We then examined the expression of other cell cycle genes and

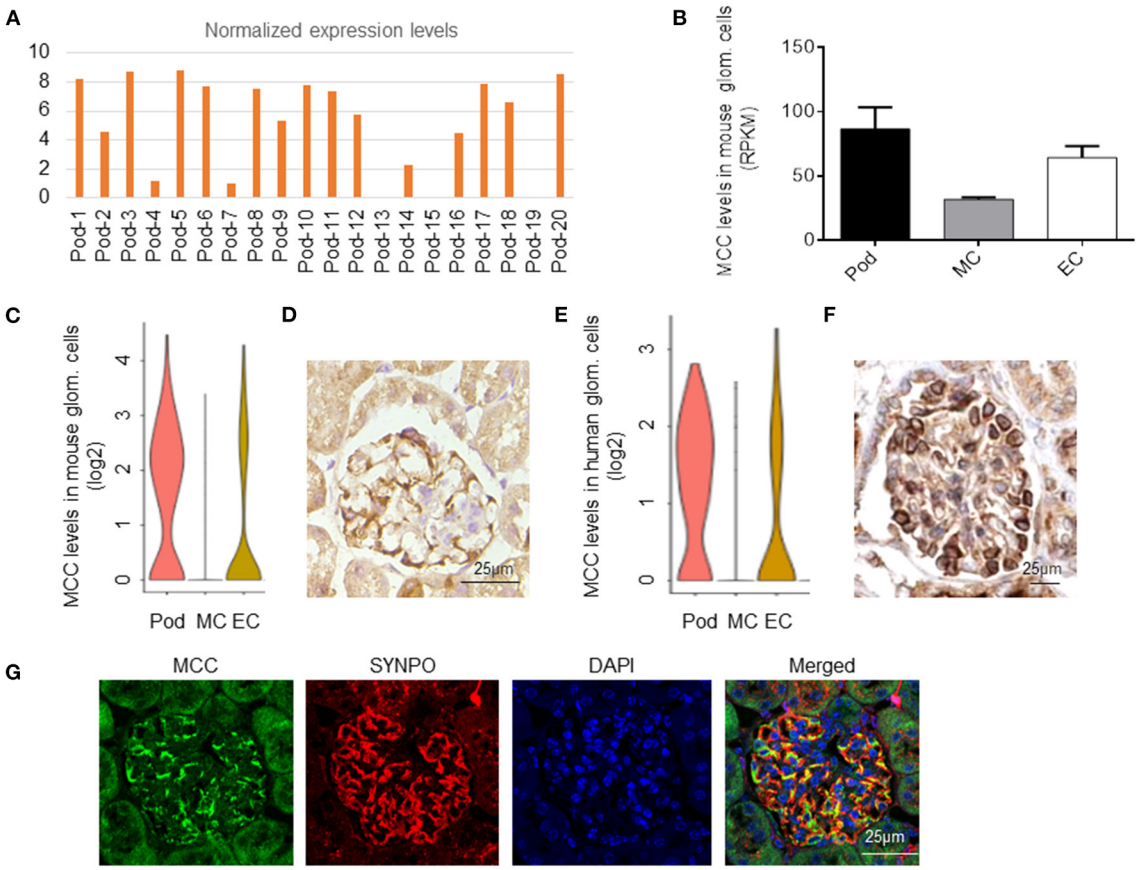

FIGURE 1 | MCC expression in podocytes in mouse and human. **(A)** MCC mRNA was detected in 18 out the 20 mouse podocytes subjected to single-cell RNA-seq analysis (GEO: GSE88814). **(B)** MCC expression in mouse podocytes, mesangial cells and glomerular endothelial cells from the bulk sequencing of mouse glomerular cells (GEO: GSE123179). **(C)** MCC expression in glomerular cell types according to the database, KIT (http://humphreyslab.com/SingleCell/). **(D)** Immunohistochemical staining of MCC in a mouse glomerulus, showing a staining pattern characteristic of podocytes. **(E)** MCC expression in human glomerular cell types, podocytes (Pod), mesangial cells (MC) and endothelial cells (EC) according to the KIT database above. **(F)** The immunohistochemical staining of MCC from the Human Protein Atlas (www.proteinatlas.org) shows that MCC expression is relatively specific to podocytes. **(G)** Fluorescence co-immunostaining of MCC and SYNPO in mouse kidney, showing the localization of MCC in foot processes of podocytes.

found downregulation of CDK1, CDK4, Cyclin B1, Cyclin D2, Cyclin E1 and Cyclin E2 in the cells at 72 h post-transfection of si-MCC (**Supplementary Figures 2A,B**). At 36 h post-transfection only cyclin E1 and E2 had started to be downregulated while the others remained unchanged. Consistently, downregulation of PCNA was also found in the cells at 72 h but not 36 h post-transfection (**Supplementary Figure 2C**). We then performed immunoblotting to examine the proteins of p16, p21, p27, p57, CDK1, Cyclin E1, and Cyclin D2, whose antibody was available in the lab. However, the protein levels of these genes were essentially not changed except for p57 and cyclin D2 (**Supplementary Figure 3**). We also examined PCNA, the marker of cell proliferation, which is upregulated in the transition of G1-S phase, and found that it was not changed. These results together suggested that MCC deficiency had little effect on cell cycle of podocytes.

Since MCC is known to regulate Wnt/β-catenin signaling and even reduces β-catenin expression level (18), we then examined β-catenin in the control and si-MCC cells, but no any difference was found at both 36 and 72 h post siRNA transfection (**Supplementary Figures 4A,B**). AKT and ERK activities were also examined and compared between si-MCC and si-scramble control cells. Slight reduction of both total AKT and p-AKT was found in the cells at 72 h but not 36 h post siRNA transfection; and no any change was found in ERK and p-ERK levels at any time point (**Supplementary Figures 5A–D**).

RNA-Sequencing and Gene set Enrichment Analysis (GSEA) of Genes Regulated in MCC Knockdown Podocytes

To further explore the mechanism underlying the effects of MCC knockdown on podocytes, we performed RNA-seq analysis of MCC knockdown podocytes 24 h post siRNA transfection when MCC mRNA was mostly eliminated in the cells and the cells did not exhibit any overt morphological change (**Figure 3**). Although

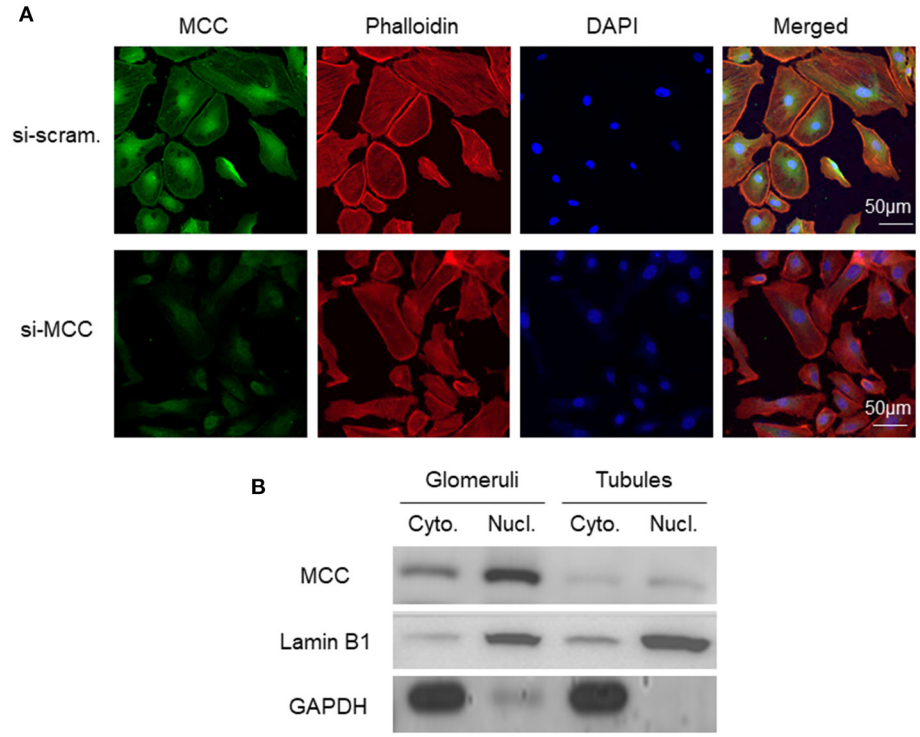

FIGURE 2 | Subcellular localization of MCC protein in podocytes in culture. **(A)** Fluorescence immunostaining of MCC and DAPI in cultured podocytes treated with scramble and si-MCC, respectively, which shows MCC protein in the cytoplasm, nucleus and periphery of the cells. **(B)** Representative immunoblotting of MCC in the cytoplasmic and nuclear fractions of human glomerular cells. GAPDH served the cytoplasmic marker, while Lamin B1 the nuclear marker. MCC expression in tubules was also examined, showing cytoplasmic and nuclear distribution; however, the protein levels were both significantly lower than that in glomerular cell cytoplasm and nuclei, respectively.

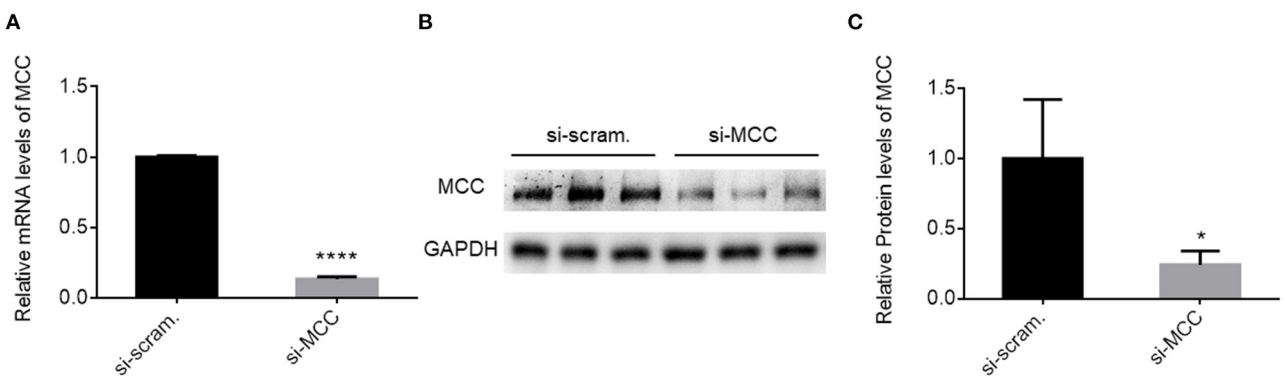

FIGURE 3 | MCC siRNA treatment resulted in marked reduction of MCC expression. **(A)** qPCR analysis of MCC mRNA in si-scramble control and si-MCC cells which were harvested for mRNA preparation 24 h post transfection. **(B)** Immunoblotting of the MCC protein in the cells in A. **(C)** Quantification of the MCC protein levels in the blot in B. Each column represents the mean ± SD of the triplicate samples. *$p < 0.05$, statistically significant. ****$p < 0.0001$, extremely significant.

the GSEA (GO_BP) showed the activation of "regulation of transcription involved in G1/S transition of mitotic cell cycle", the enrichment did not reach a statistical significance (**Figure 7**). We also found activation of inflammatory and immune responses (including TLR-4 signaling, T-cell activation, etc). On the other hand, several enrichments were significantly suppressed, including base-excision/mismatch repair, and mesenchymal to epithelial transition (MET). In the GO_MF analysis, many activities involve nuclei (**Supplementary Figure 6**), and this is consistent with nucleic localization of MCC as shown in **Figure 2** and suggestive of a role for MCC in gene expression regulation. In the GO_CC analysis (**Figure 8**), we found "lamellipodia

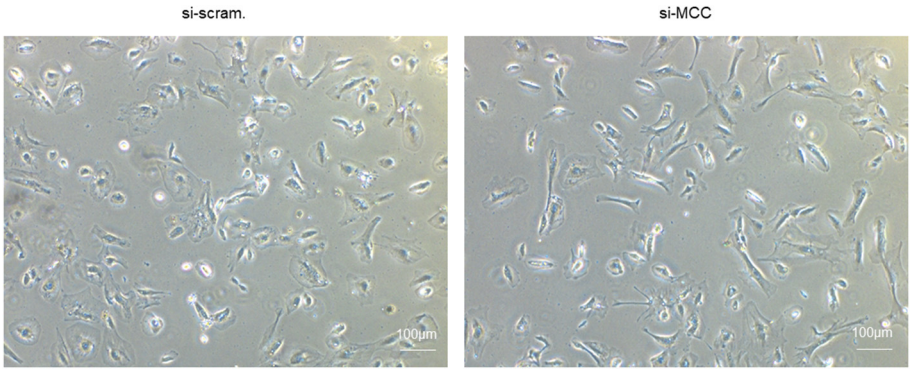

FIGURE 4 | Morphological change of podocytes treated with si-MCC. At 72 h post transfection of si-MCC, the podocytes exhibited an elongated shape compared with the scramble control cells.

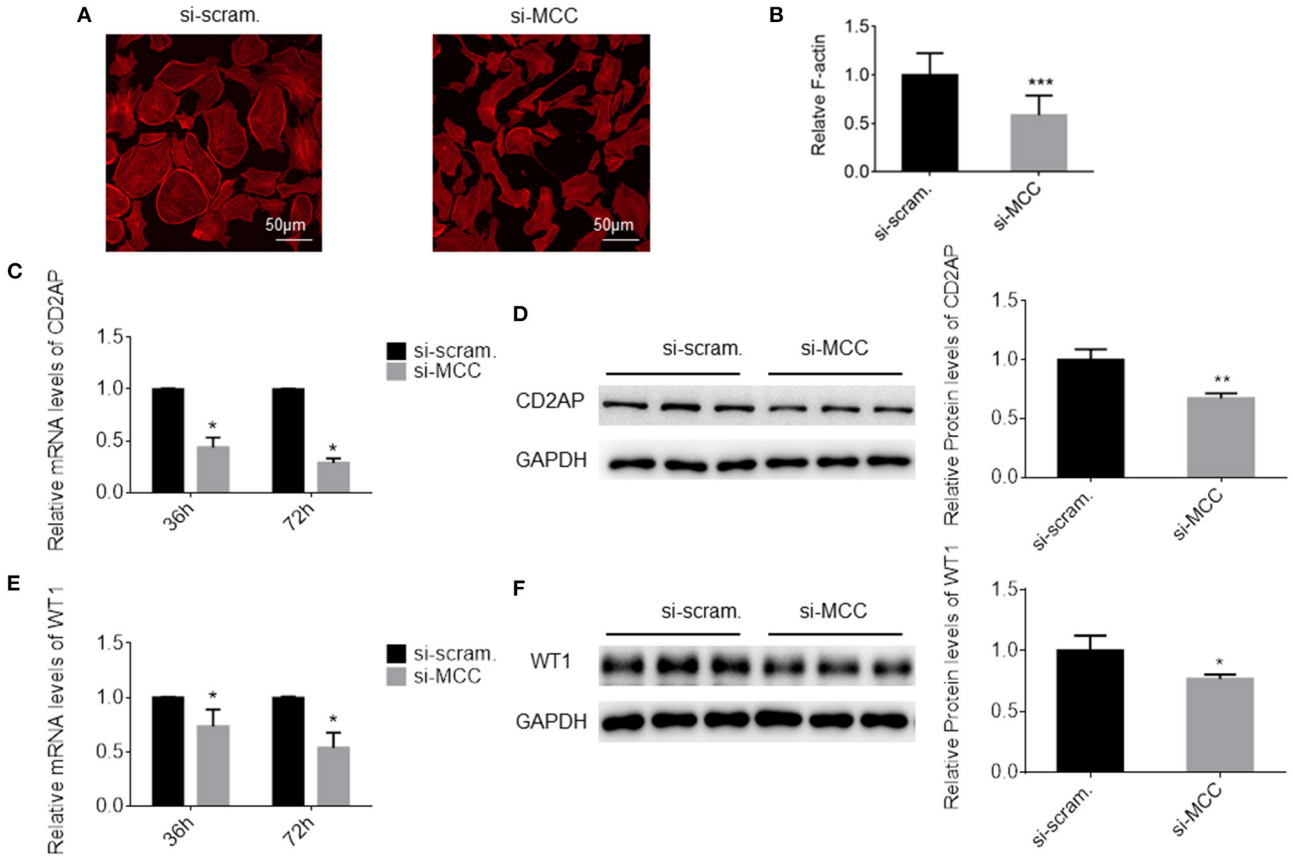

FIGURE 5 | MCC silencing resulted in injury in podocytes. **(A)** Phalloidin staining of the cells treated with scramble control and si-MCC for 72 h. **(B)** Quantification of the staining intensity using ImageJ showed a significant reduction of actin stress fibers in the cells treated with si-MCC compared with scramble control. **(C)** qPCR quantification of CD2AP mRNA in control cells and the cells treated with si-MCC at 36 h and 72 post transfection, respectively. **(D)** Immunoblotting of the CD2AP protein in the cell samples in C to confirm CD2AP downregulation at the protein level. Quantification of the blot is shown on the right. **(E)** qPCR analysis of WT1 in the cells treated with scramble control and si-MCC, respectively. **(F)** Immunoblotting of WT1 in the samples in **(E)** and the quantification shown on the right. The data represent the mean ± SD of three independent experiments. $^{*}p < 0.05$, statistically significant; $^{**}p < 0.01$ and $^{***}p < 0.001$, statistically very significant.

membrane", "lamellipodium" and "cell leading edge", suggesting a role of MCC in lamellipodia formation of podocytes. KEGG and Reactome analyses were consistent with the GO analysis (**Supplementary Figures 7**, **8**).

MCC Deficiency Disrupted Lamellipodia Formation

To determine the association of MCC with podocyte lamellipodium as revealed by RNA-seq and bioinformatics

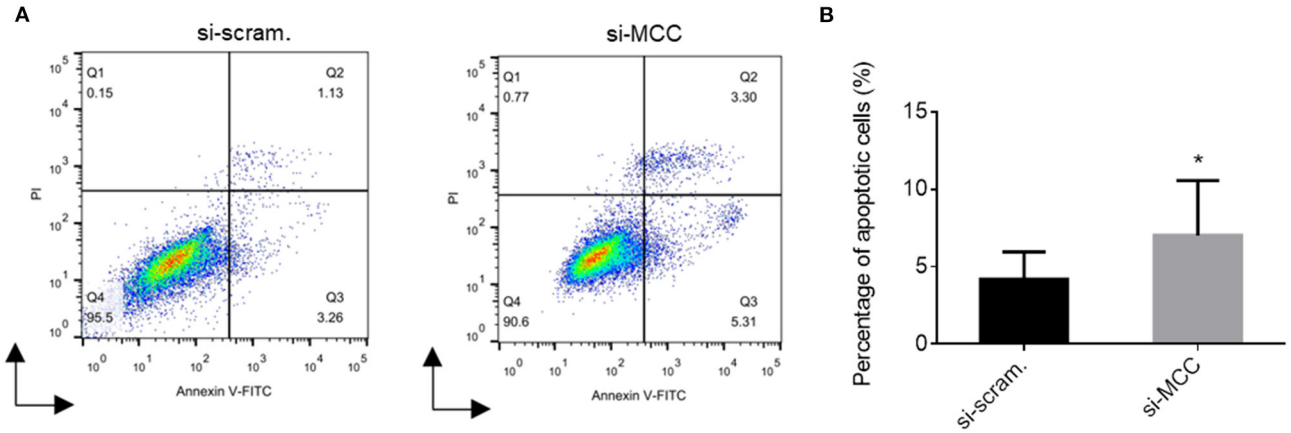

FIGURE 6 | Flow cytometry of Annexin V staining of podocytes treated with scramble control and si-MCC for 72h. **(A)** Histogram representation of the results of the flow cytometric assay. **(B)** Quantification of the percentages of the Annexin V positive cells in the samples. The results were obtained from three independent experiments ($n = 3$) and calculated as mean ± SD. $^*p < 0.05$, statistically significant.

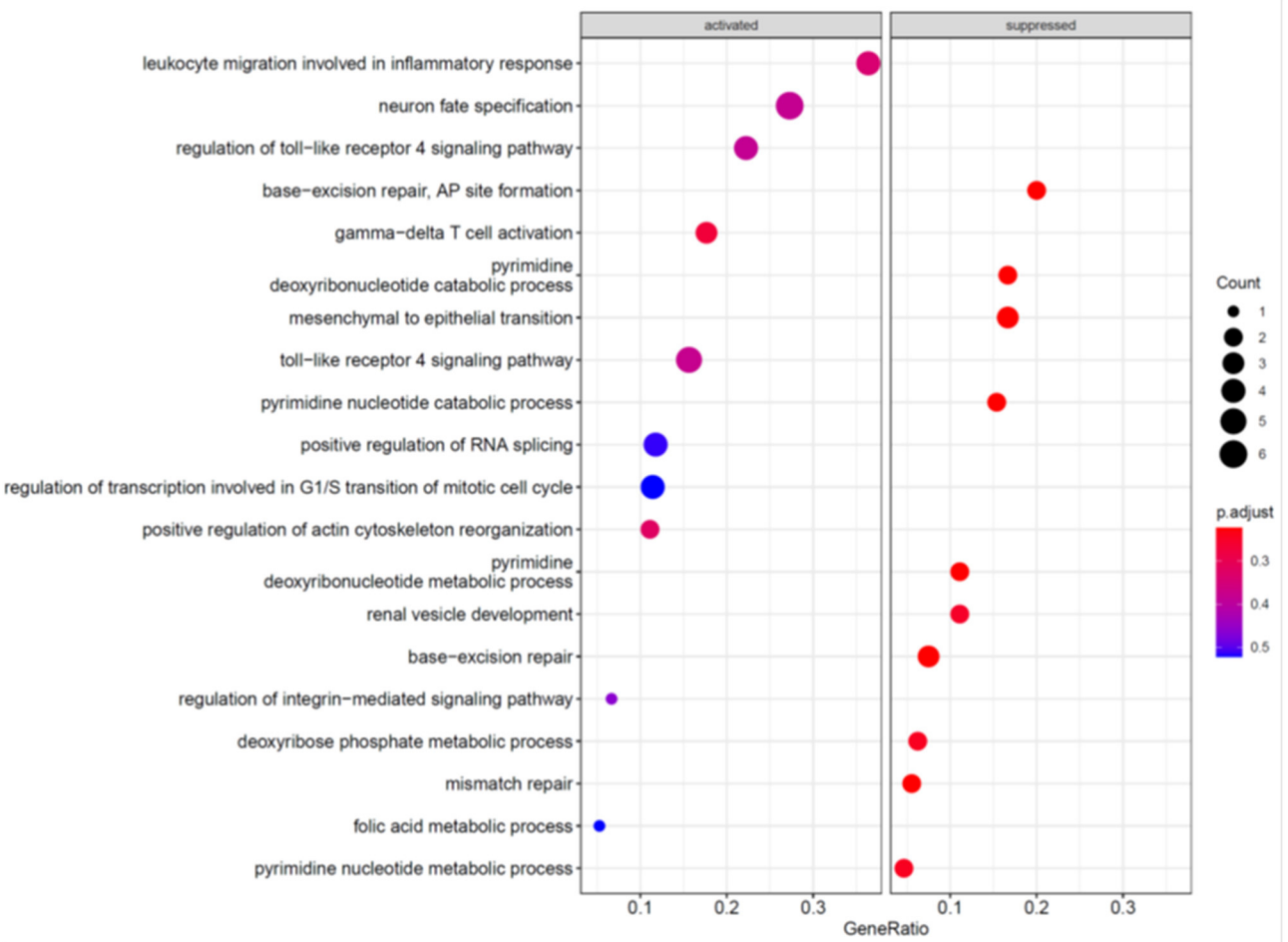

FIGURE 7 | GO_BP analyses of genes regulated in the podocytes deficient in MCC.

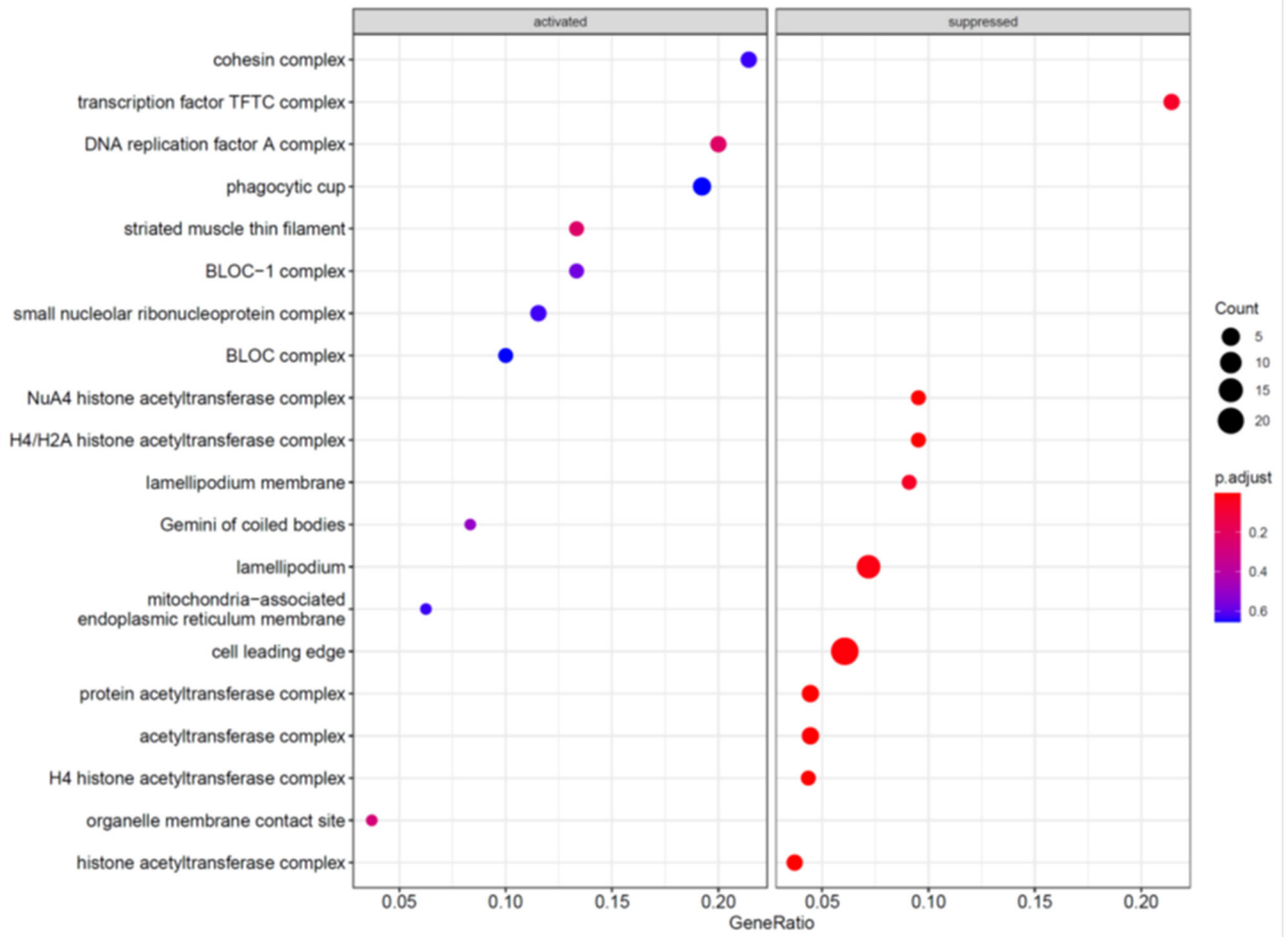

FIGURE 8 | GO_CC analyses of genes regulated in the podocytes deficient in MCC.

analysis above, we co-stained MCC with lamellipodium marker, phalloidin-stained F-actin, and observed that MCC was co-localized with F-actin at the periphery of podocytes, indicating that MCC is localized in lamellipodia (**Figure 9**). In the si-MCC treated podocytes, we were surprised to find that the cells lost most of the lamellipodia as shown by negative staining of phalloidin at the cell periphery (**Figure 9A**), indicating that MCC is involved in the formation of lamellipodium in podocytes. We examined the integrity of lamellipodia using another marker of lamellipodia, cortactin, which showed presence of lamellipodia in control cells but absence in the podocytes treated with si-MCC (**Figure 9B**).

MCC Is Downregulated in Injurious Podocytes and Glomerular Diseases

To explore the role of MCC expression changes in podocytopathy, we first explored whether MCC expression could be affected by injurious stimuli. In cultured podocytes treated with puromycin aminonucleosides (PAN), a commonly used podocyte injury model, we found a significant dose-dependent downregulation of MCC in the cells as assessed by qPCR (**Figure 10A**). Next, we searched Nephroseq database and found that MCC is significantly downregulated in glomeruli of diabetic mice (**Figure 10B**), as well as the patients with focal segmental glomerulosclerosis (FSGS) (**Figure 10C**). These results suggest that MCC downregulation may facilitate podocyte injury in the glomerular diseases.

DISCUSSION

In the present study, we pursued a previous finding that MCC expression was detected in majority of podocytes that underwent single-cell RNA-sequencing and speculated that MCC might be indispensable for podocytes. Further database mining confirmed MCC expression in mouse podocytes, as well as in human podocytes, supporting a role for MCC in podocytes. We performed siRNA silencing of MCC in cultured podocytes and observed cell injury. Thus, we have identified MCC as a novel podocyte essential gene. We further investigated the potential mechanism underlying the role of MCC and found that it is required for lamellipodia formation in podocytes.

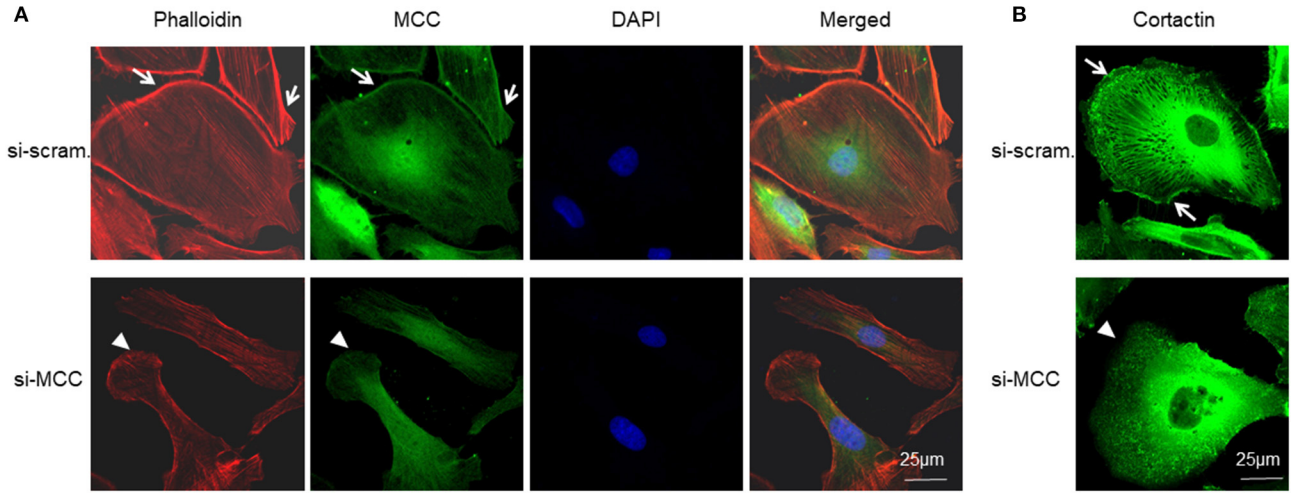

FIGURE 9 | Requirement of MCC in lamellipodium formation of podocytes. **(A)** Fluorescence immunostaining of MCC and F-actin (phalloidin) shows that MCC protein is abundant in lamellipodia (arrows), and its knockdown caused loss of lamellipodia (arrow head) in the podocytes. **(B)** Fluorescence immunostaining of lamellipodial marker, cortactin, further shows the loss of lamellipodia in the cells lacking MCC (arrow head).

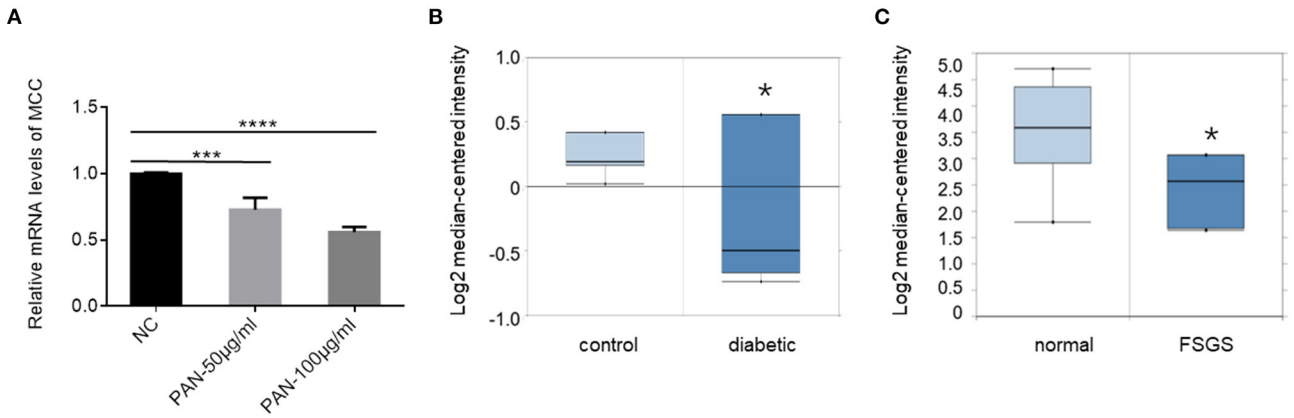

FIGURE 10 | MCC downregulation in podocyte injury and podocytopathy. **(A)** qPCR analysis of cultured podocytes treated with or without PAN for 24 h, showing a significant downregulation of MCC by PAN. The results represent the mean ± SD of three independent experiments; ***$p < 0.001$, ****$p < 0.0001$, statistically very significant; **(B)** Nephroseq database search showed MCC downregulation in glomeruli of diabetic mice (eNOS-deficient C57BLKS db/db) ($n = 7$) compared with the non-diabetic control mice ($n = 5$). *$p < 0.01$; **(C)** Nephroseq database search revealed downregulation of MCC in glomeruli of FSGS patients. Normal control, $n = 9$; FSGS patients, $n = 6$; *$p < 0.05$, statistically significant.

Single-cell RNA-seq is a powerful tool to dissect gene expression in single cells, providing many new insights into the biology and pathology of a given cell type. We previously performed single-cell RNA-seq of mouse podocytes and mesangial cells and identified a number of novel podocyte and mesangial essential genes (20, 29). Our approach for essential genes identification for a cell type is based on the speculation that genes expressed in all individual cells are likely indispensable for the cell type while genes expressed only in a proportion of cells could be dispensable. We performed ultra-deep sequencing of mouse podocytes and identified 335 genes whose expression was detected by RNA-seq at levels >0.5 RPKM. Among the 335 genes, 92 showed > 5-fold expression levels of that in mesangial and endothelial cells and considered to be podocyte specific essential genes. Among the 92 genes, about 30 were already known to be essential for podocytes, thus validating the approach. We took several other approaches to demonstrate the essentiality of the novel candidates (20). In addition to the genes expressed in all sequenced podocytes, there were many other genes whose expression was detected in majority of the cells and we supposed that these genes might be also essential for podocytes and their absence might have arisen from technical variations in the single-cell RNA-seq process. We examined the list of such genes and became interested in MCC which had been shown to act a tumor suppressor and prevent cell cycle progression.

Firstly, we analyzed phenotypes of the podocytes treated with MCC siRNA, including actin stress fiber formation, cell morphology, apoptosis, and the molecules in the injurious pathways. We found that MCC knockdown indeed caused podocyte injury as demonstrated in above assays. We then explored the mechanism underlying the role of MCC in podocytes.

Podocytes are characterized by their terminally differentiated property. Dependent of their nature, injurious stimuli are capable of positively or negatively regulating expression of genes involved in cell cycling, e.g., CDK inhibitor, p21, p27, and p57 (16, 19, 30), resulting in either cell cycle arrest or re-entry. Alteration of p21, p27, and p57 levels was observed in multiple podocyte injury models, including puromycin nephrosis, TGF-β and Angiotensin II, and facilitated podocyte apoptosis in the models (30). In the present study, MCC knockdown resulted in downregulation of p21, p27, and p57 at the mRNA level in the podocytes. However, at the protein level only p57 showed a slight reduction. Downregulation of cell cycle molecules were also observed at the mRNA level, but only cyclin D2 was reduced markedly at the protein level. Regardless of these changes, the cell cycle status of the cells deficient in MCC was maintained as evident by the unchange of PCNA protein, the marker of S-G1 phase transition. Therefore, the role for MCC in podocytes might not involve cell cycle regulation, at least, under normal condition as shown in the present study. It would be interesting to test whether MCC has a role in cell cycle regulation of podocytes under stresses or in diseases.

MCC has been shown to inhibit Wnt/β-catenin signaling (17) and could even directly reduce β-catenin level (18). In our present study with cultured podocytes, we did not observe the change of β-catenin protein level and activity in the podocytes treated with si-MCC. It appears that regulatory activity of MCC in Wnt/β-catenin signaling is also cell type dependent. However, it is necessary to examine β-catenin transcriptional activity in the podocytes to definitely conclude it. In addition, it could be more important to determine whether MCC is involved in β-catenin signaling in podocytes treated with injurious factors.

In the RNA-seq and GSEA analysis, we identified several earliest enrichments in the podocytes upon the elimination of MCC in the cells 24 h post siRNA transfection when there was no any apparent change that could be seen. Interestingly, both cell cycle and Wnt/β-catenin signaling were not significantly enriched, consistent with our experimental results. However, we found that MCC deficiency was associated with "lamellipodia", "lamellipodia membrane" and "cell leading edge". We then carefully investigated the issue and found that MCC protein was abundant in the lamellipodia and MCC knockdown led to loss of lamellipodia in the podocytes as shown by the absence of both F-actin and cortactin at the periphery of the cells. *In vivo*, we performed MCC and SYNPO co-staining with kidney and found that MCC co-localized along GBM, indicating that MCC is present in foot processes of podocytes (**Figure 1G**). Foot process localization of MCC *in vivo* is consistent with its localization in lamellipodia of cultured podocytes. Lamellipodia is essential for the foot process and slit diaphragm formation of podocytes (31, 32). Therefore, MCC may be also involved in these processes thereby underlying its essentiality for podocytes. In fact, involvement of MCC in lamellipodia formation has been reported in colon epithelial cells, and mechanistically, MCC binds to Scrib and its downstream partner Myosin-IIB in a multiprotein complex (33). Scrib and Myosin-IIB are both highly expressed in podocytes according to HPA and other sources of data (data not shown). It would be interesting to test whether MCC regulates lamellipodia in a similar way.

From a translational perspective, we found that MCC can be downregulated by PAN in podocytes. Furthermore, MCC was found significantly downregulated in glomeruli of diabetic mice and FSGS patients according to Nephroseq database. MCC downregulation may facilitate the process of podocyte injury and disease development. Further investigation of the exact molecular mechanism underlying MCC downregulation-mediated podocyte injury is warranted. There is a pitfall when one claims downregulation of a gene in podocytes based on quantification with glomerular material, e.g., RNA and protein. This is because podocyte loss contributes to the reduction of podocyte gene expression in the glomerular material, likely causing false downregulation of the podocyte gene. To definitely conclude MCC downregulation in podocytes of DKD and FSGS, we used Nephroseq data to compare the fold changes of MCC with that of podocyte marker genes, MAGI2, LMX1B, WT1, FOXC1, FOXC2, VEGFA, SYNPO, NPHS1, NPHS2, YAP1 and CD2AP, which are well known to be downregulated in the same diseases. We found that MCC had a similar reduction to those of the podocyte markers, confirming that MMC is downregulated in the diabetic nephropathy and FSGS (**Supplementary Figures 9**, **10**).

In conclusion, our present study has identified a novel podocyte essential gene, MCC, which plays an essential role in the formation of lamellipodia in the cultured podocytes, and likely in the formation of podocyte foot processes and slit diaphragms in animal. MCC downregulation may facilitate podocyte injury. Therefore, reversal of MCC downregulation in podocytes may be a potential therapeutic approach for podocytopathy. Although the present *in vitro* study has identified MCC as a novel essential gene of podocytes and shown some potential mechanistic insights into its role in lamellipodium formation, further studies, particularly *in vivo*, are required to better understand the function and mechanism of MCC in podocyte pathophysiology.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE186547.

AUTHOR CONTRIBUTIONS

SS conceived the study. HS, ZL, WH, and SS designed the experiments. HS, LZ, and XX performed the experiments. JS performed bioinformatics analysis. HS, SS, and ZL interpreted

data and wrote the manuscript. All authors have reviewed and approved the final version of the manuscript.

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Molecular Mechanisms of Proteinuria in Minimal Change Disease

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Minimal change disease (MCD) is the most common type of idiopathic nephrotic syndrome in childhood and represents about 15% cases in adults. It is characterized by massive proteinuria, edema, hypoalbuminemia, and podocyte foot process effacement on electron microscopy. Clinical and experimental studies have shown an association between MCD and immune dysregulation. Given the lack of inflammatory changes or immunocomplex deposits in the kidney tissue, MCD has been traditionally thought to be mediated by an unknown circulating factor(s), probably released by T cells that directly target podocytes leading to podocyte ultrastructural changes and proteinuria. Not surprisingly, research efforts have focused on the role of T cells and podocytes in the disease process. Nevertheless, the pathogenesis of the disease remains a mystery. More recently, B cells have been postulated as an important player in the disease either by activating T cells or by releasing circulating autoantibodies against podocyte targets. There are also few reports of endothelial injury in MCD, but whether glomerular endothelial cells play a role in the disease remains unexplored. Genome-wide association studies are providing insights into the genetic susceptibility to develop the disease and found a link between MCD and certain human haplotype antigen variants. Altogether, these findings emphasize the complex interplay between the immune system, glomerular cells, and the genome, raising the possibility of distinct underlying triggers and/or mechanisms of proteinuria among patients with MCD. The heterogeneity of the disease and the lack of good animal models of MCD remain major obstacles in the understanding of MCD. In this study, we will review the most relevant candidate mediators and mechanisms of proteinuria involved in MCD and the current models of MCD-like injury.

Keywords: minimal change disease, nephrotic syndrome, proteinuria, immune cell, podocyte, circulating factor

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INTRODUCTION

Minimal change disease (MCD) is the most common type of nephrotic syndrome in children, whereas it only accounts for 10–16% cases in adults (1, 2). The term MCD refers to a histological pattern characterized by the normal or near-normal appearance of glomeruli on light microscopy and immunofluorescence with podocyte foot process effacement (FPE) on electron microscopy as the sole abnormality observed in kidney biopsy (3). While histological findings are similar in

children and adults with MCD, the clinical response to steroids, considered as first-line therapy, is different. Most children achieve resolution of proteinuria within days, whereas it can take months in adults (4, 5). Therefore, a kidney biopsy is only performed in selected pediatric cases, whereas it is mandated in all the adults to rule out other forms of nephrotic syndrome including infections, malignancies, or other glomerular diseases. For children who respond to steroid therapy, namely steroid-sensitive nephrotic syndrome (SSNS), MCD represents the most common underlying histological pattern followed by focal segmental glomerulosclerosis (FSGS), a more severe form of nephrotic syndrome involving glomerular scarring (4).

The name MCD can be misleading. While kidney histology shows minor changes and MCD has been traditionally considered a benign disease, it is often associated to multiple relapses, important comorbidities, and serious complications [acute kidney injury (AKI), thrombotic disorders, infections, etc.] (6–8). In addition, the pediatric onset disease can persist in adulthood (9). Therefore, MCD represents an important burden to patients, families, and the healthcare system (10). Some patients also develop resistance to therapies and/or progression toward advanced stages of chronic kidney disease (CKD) and this is usually associated with a change in the glomerular histology from MCD to FSGS (11, 12). So, a diagnosis of MCD may not be definitive; but, whether MCD and FSGS are distinct diseases or a continuum of the same disease remains unclear (13).

The clinical hallmark of MCD is sudden-onset proteinuria and anasarca. However, the mechanisms of proteinuria remain poorly understood (14). The lack of inflammatory cells and immune complexes in the MCD glomerulus led to the hypothesis that some circulating factor(s), presumably released by T cells, may trigger proteinuria and podocyte injury (15–18). This assumption is widely accepted by the nephrology community and it is supported by some clinical observations; but the presence, nature, and the cell source for the presumed circulating factor(s) have remained elusive for decades. With the discovery of nephrin as a key protein in the podocyte slit diaphragm (SD) and the glomerular filtration barrier (GFB), podocyte biology has been the center of most research efforts in MCD over the last two decades (19–21). In fact, podocytes are key to maintain the integrity of the GFB as implied in forms of genetic nephrotic syndrome with abnormal or absent podocyte proteins and in knockout *in-vivo* models (19, 22–24). In MCD, there are changes in the expression, phosphorylation, and/or localization of podocyte-specific proteins such as synaptopodin and nephrin during relapse (25–27). In addition, the observations that some immunosuppressive drugs used in MCD may act directly on podocytes have also supported the concept of MCD as a podocyte disorder (28–32). In the recent years, there has been an increasing interest on the underpinning genetic architecture in MCD and several studies have identified gene variants that seem to confer susceptibility to the disease (33–36). Therefore, the pathogenesis of MCD seems to involve a complex interplay between immune cells, the glomerulus, and genetics (**Figure 1**). This complexity is reflected by the paucity of major breakthroughs in the understanding of the disease and lack of targeted therapies.

In this manuscript, we will provide an overview of the current experimental approaches available to study MCD and a review of candidate mediators and mechanisms of proteinuria involved in MCD and SSNS, that is commonly associated with MCD.

EXPERIMENTAL MODELS OF MCD

Table 1 shows a summary of current experimental models to study MCD. While these models have helped to advance, to some extent, our understanding of the disease, they still have significant limitations. Hence, there is an urgent need to develop better experimental approaches that mimic the natural course of human MCD.

Animal Models

Puromycin aminonucleoside (PAN) (rat model) and lipopolysaccharide (LPS) (mouse model) are the two most widely used models to induce sudden-onset proteinuria and MCD-like injury on kidney histology. The PAN model induces DNA damage via reactive oxygen species resulting in remarkable proteinuria, FPE, redistribution of proteins of the SD, changes in anionic charges in the glomerular basement membrane (GBM), and podocyte loss (37–41). In contrast, the LPS model is thought to directly activate the Toll-like receptor 4 (TLR-4) and downstream inflammatory pathways on podocytes resulting in mild and transient proteinuria, FPE, and changes in nephrin phosphorylation (20, 42, 43). Therefore, both models resemble some key features of MCD (sudden proteinuria, MCD-like injury on histology, and changes in podocyte proteins) (25–27), though apparently by different mechanisms. The main strength of the PAN model is the remarkable proteinuria and FPE, whereas that of the LPS model is the immune activation, likely relevant to MCD given its clinical association with infections (44, 45). However, both models have significant limitations. The PAN model often results in FSGS likely due to direct podocyte injury and loss rather than by a circulating factor (39). The LPS model is associated with a remarkable immune response resulting in sepsis, transient proteinuria, and AKI (46).

Other animal models have been described, but their use to study MCD is anecdotic. Polyinosinic: polycytidylic acid (Poly: IC) is a TLR-3 ligand that, when injected to mice, induces proteinuria and podocyte injury involving synaptopodin loss and FPE with no immune deposits on electron microscopy (47), mimicking features of MCD. Poly: IC is thought to mediate direct podocyte injury via TLR and activation of the inflammatory pathway nuclear factor-kappa B (NF-kB) (48). A weakness of this model is that proteinuria is mild and transient, but contrary to the LPS model, it is not associated with clinical sepsis (47). This model seems promising because it is well-tolerated by mice and results in glomerular changes that mimic MCD, but it remains to be determined whether variations from the original Poly: IC model of proteinuria may yield higher and/or sustained proteinuria like that observed in human MCD.

Sellier-Leclerc et al. developed a humanized mouse model of MCD by injecting CD34+ (a marker for hematopoietic stem cells) and CD34– peripheral blood mononuclear cells (PBMCs) from patients with MCD and FSGS into immunocompromised mice (49). Mice injected with CD34+ cells, but not with CD34–, developed albuminuria and partial FPE. Since CD34+ and CD34– induce the expansion of immature and mature T

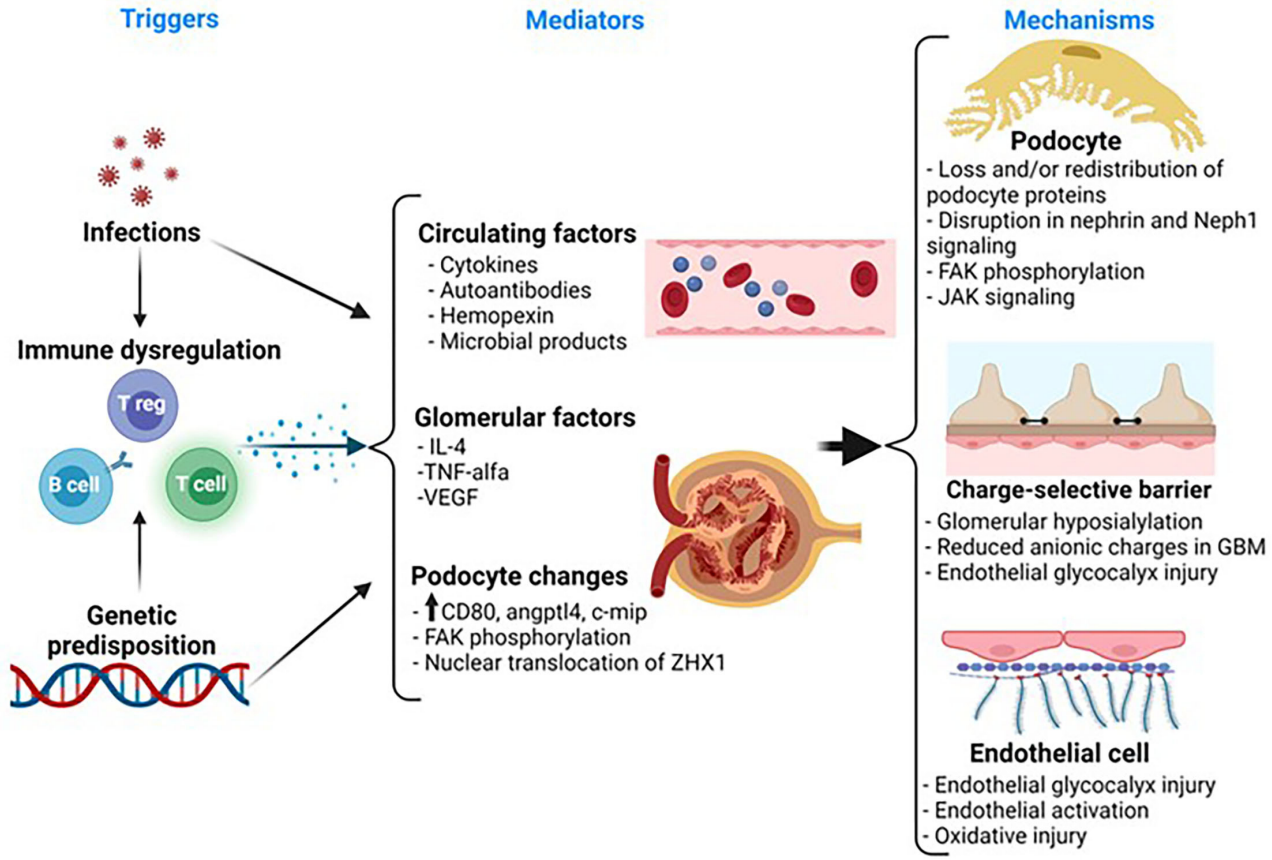

FIGURE 1 | Representative schematic of the pathogenesis in MCD. MCD is associated with immune dysregulation. Notably, there is a strong association between human leukocyte antigen (HLA) and susceptibility to idiopathic nephrotic syndrome. So, it is possible that children with MCD have a genetic predisposition toward immune dysregulation that, in turn, contributes to release of factors that may play a role in the pathogenesis of the disease. Also, infections often trigger relapse in these patients and this may be the result of an exaggerated immune response in a susceptible patient. Several candidate mediators, released by systemic T or B cells or by glomerular cells, have been suggested to play a role in the development of proteinuria in MCD, but to date, there is a no definitive mediator of MCD. The podocyte exhibits morphological and molecular changes involving key proteins such as synaptopodin, FAK, and nephrin, but these changes are not specific for MCD and the upstream pathways leading to these changes remain unknown. The glomerular basement membrane (GBM) and the endothelial cell also show subtle changes. Thus, there is a loss of anionic charges in the GBM, but this does not appear to be a key driver of proteinuria. More recently, there is evidence of endothelial cell activation and oxidative stress, but whether this may play a role in the disease or it may represent a paraphenomenon is unclear. MCD, minimal change disease; IL, interleukin; VEGF, vascular endothelial growth factor; Angptl4, angiopoietin-like 4; ZHX1, zinc fingers and homeoboxes; FAK, focal adhesion kinase; JAK, Janus kinase.

cells, respectively, authors postulated that proteinuria in this model could be mediated by immature T cells. The caveat of this model is that the degree of albuminuria was low and its use has not been reported since. It is unknown whether an additional insult (PAN, LPS, and Poly: IC) could have triggered a higher degree of proteinuria and still maintain MCD features.

A transgenic (TG) rat model characterized by the podocyte-specific overexpression of angiopoietin-like 4 (Angptl4) has also been associated to proteinuria and MCD-like changes on kidney histology (50). The main caveat of this model is the slow-onset proteinuria, contrary to the animal models described above and to the human disease. Therefore, these TG rats need to be exposed to an additional insult (puromycin, adriamycin, etc.) to accelerate proteinuria (50).

Another animal model of interest is the Buffalo/Mna rat. These rats spontaneously develop nephrotic syndrome with histological features of glomerulosclerosis. Notably, proteinuria and kidney lesions resolved when the Buffalo/Mna kidneys were transplanted into healthy rats, suggestive of a circulating factor(s) as driver of proteinuria (51). In addition, the Buffalo/Mna kidneys exhibited a greater infiltration of macrophages and T cells than control rats, along with an upregulation of macrophage and T-helper type 2 (Th2) cytokine transcripts before the progression of proteinuria (52). This model could serve to investigate potential mechanisms of disease progression and recurrence after transplantation.

In-vitro Models

Cell culture studies are a valuable tool to study podocyte biology and they remain as standard approach to study

TABLE 1 | Overview of experimental models of MCD.

Animal models (animal/route)	Strengths	Limitations
PAN (Rat, IP)	- MCD-like injury - Acute and severe proteinuria	- Can evolve to FSGS - Direct toxicity on podocytes
LPS (Mouse, IP)	- MCD-like injury - Systemic immune activation - Acute proteinuria	- Mild proteinuria - Sepsis - Acute kidney injury
Poly: IC (Mouse, IP)	- MCD-like injury - Acute proteinuria - Mimic viral infection	- Mild proteinuria - Anecdotical use
Humanized mouse	- MCD-like injury - Incorporate PBMC from patients	- Mild proteinuria
	- Acute proteinuria	- Anecdotical use
Angptl4 (Rat, TG)	- MCD-like injury	- Slow onset proteinuria - Requires second insult (puromycin, etc.)
Cell studies	**Strengths**	**Limitations**
Human podocytes	- Well-characterized - Mechanistic control - High throughput - Relatively easy and inexpensive	- Does not recapitulate microenvironment - No shear stress - Limited glycocalyx - Inability to test permselectivity
Kidney organoids	- Incorporate different glomerular cells - Ability to test permselectivity and study crosstalk	- Requires expertise and longer timeline for experiments - Expensive - Does not include all glomerular cells - Limited throughput
3D co-cultures	- Ability to integrate two cell types and extracellular matrix - Microfluidic system - Reproduce shear stress - Ability to test permselectivity and study crosstalk - Develops glycocalyx	- Requires expertise and longer timeline for experiments - Expensive - Does not include all glomerular cells - Limited throughput

PAN, puromycin aminonucleoside; IP, intraperitoneal; MCD, minimal change disease; FSGS, focal segmental glomerulosclerosis; LPS, lipopolysaccharide; Angptl4, angiopoietin-like 4; TG, transgenic. Poly: IC, Polyinosinic: polycytidylic acid.

mechanisms of disease in glomerulopathies. As such, different toxins (puromycin, LPS, and Poly: IC) and sera from patients with MCD have been used on cultured immortalized human podocytes in an attempt to replicate molecular changes triggered by the circulating factor(s) involved in MCD (43, 53). However, cultured human podocytes do not form secondary processes or slit diaphragms, show a variable expression of podocyte-specific proteins, and lack of cell-to-cell communication with glomerular endothelial cells (GEnC) and mesangial cells as in the human GFB (54). These are important limitations given the importance of the SD and the crosstalk between glomerular cells to maintain the integrity of the GFB. Another important consideration is that circulating toxins, at least those presumably present in the MCD sera or plasma, may not directly encounter podocytes in the human glomerulus as they do in culture systems.

To overcome the above limitations, new and promising *in-vitro* systems, such as kidney organoids and three-dimensional (3D) cocultures, have been developed over the last few years. These systems help to recapitulate the ontogeny of renal development and recapitulate features of the glomerular filtration barrier including cell-to-cell interactions and microfluid circulation. Proposed applications have included in-depth mechanistic studies and drug screening, gene knockouts and overexpression, and structural changes in response to sera of patient and various and sundry cytokines. These models have been reviewed in detail elsewhere and a detailed discussion is beyond the scope of this review (54, 55).

MEDIATORS AND MECHANISMS OF PROTEINURIA IN MCD

Table 2 shows a summary with some postulated mediators and mechanisms of proteinuria involved in MCD.

CIRCULATING FACTORS

Since early 1970s, MCD has been thought to be mediated by some circulating factor(s). While this remains to be proven, there are some clinical and experimental observations that support this: (1) therapeutic response to immunosuppression, (2) lack of immune complexes in glomeruli, (3) resolution of proteinuria after transplanting kidneys with active MCD into patients without MCD, and (4) development of MCD-like injury in rats that received supernatants of PBMCs from patients with active SSNS and supernatants from T-cell hybridomas derived from MCD in relapse (4, 56–58). However, the clinical observations do not demonstrate causality and the experimental studies remain to be

TABLE 2 | Summary of candidate mediators and mechanisms of proteinuria in MCD.

Candidate mediators	Potential mechanisms of podocyte injury
Circulating factors	
Cytokines	
IL-13	- Loss and redistribution of podocyte proteins (60) - Glomerular CD80 upregulation (60)
IL-8	- Loss anionic charges in GBM (61)
IL-4	- Podocyte JAK signaling (62)
Autoantibodies	
UCHL1	- Unknown
Anti-nephrin	- Disrupting nephrin signaling
Hemopexin	- Loss anionic charges in GBM (77) - Disrupting nephrin signaling (78)
Microbial products	- Podocyte TLR activation and NFkB signaling (43, 47)
Intraglomerular factors	
IL-4	- Podocyte JAK signaling (62)
TNF-α	- Nephrin loss (88) - Podocyte FAK phosphorylation (89) - Glomerular CD80 upregulation (91) - NFkB activation (91) - Podocyte syndecan 4 shedding and β3 integrin signaling (104)
VEGF-A	- Dysregulated endothelial-podocyte crosstalk (92)
Charge selective barrier	
Angptl4	- Loss anionic charges in GBM (50)
Hemopexin	- Loss anionic charges in GBM (77)
IL-8	- Loss anionic charges in GBM (61)
Podocyte dysfunction	
CD80	- Prevent β1 integrin and Neph1 signaling (31, 111) - Activates local inflammatory pathways (47)
Angptl4	- Loss anionic charges in GBM (50) - Oxidative injury in GEnC (50) - Podocyte-endothelial crosstalk (50)
C-mip	- Disrupt nephrin signaling (121)
FAK	- Actin cytoskeleton reorganization (123) - Enhance metalloprotease activity (122)
ZHX1	- Podocyte angptl4 upregulation (124)
Endothelial dysfunction	
EG degradation and release of EG products (syndecans, etc.)	- Loss of electrostatic charges (101) - Podocyte activation via β3 integrin signaling (104)
CD80	- Activates local inflammatory pathways (47)
Caveolin-1	- Facilitates albumin transcytosis (133)

IL, interleukin; GBM, glomerular basement membrane; UCHL1, ubiquitin carboxyl-terminal hydrolase L1; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor; Angptl4, angiopoietin-like 4; GEnC, glomerular endothelial cell; FAK, focal adhesion kinase; ZHX1, zinc fingers and homeoboxes; EG, endothelial glycocalyx.

validated decades after the original publication (57, 58). Here, we will review some of the candidates circulating mediators and their postulated mechanisms of proteinuria in MCD.

Circulating Cytokines

Several cytokines, predominantly from the Th2 subset, have been linked to MCD (59). A detailed review on systemic cytokine patterns in MCD is beyond the scope of this review. Here, we will specifically focus on those cytokines with a presumed pathogenic role in the development of proteinuria in MCD.

Interleukin-13 (IL-13)

The strongest evidence to support a role of IL-13 in MCD comes from a TG rat model characterized by high serum IL-13 levels (60). These rats developed nephrotic syndrome, FPE, loss and redistribution of some podocyte proteins, and CD80 upregulation in glomeruli, thereby mimicking some key features of human MCD (60).

Interleukin-8 (IL-8)

Garin et al. postulated that systemic IL-8 could play a role in MCD. Rats infused with IL-8, reaching serum levels similar to those observed in MCD, developed proteinuria due to an increased metabolism of glycosaminoglycans (GAGs) in the GBM, mimicking the anionic loss reported in the GBM of some patients with MCD (61). Nevertheless, IL-8 only caused mild proteinuria.

Interleukin-4 (IL-4)

High systemic IL-4 via liver overexpression caused proteinuria and podocyte FPE in mice, which was ameliorated with a JAK inhibitor, suggesting that IL-4 may mediate proteinuria by activating JAK signaling in podocytes (62). In a single-center study, 10 of 29 patients with active MCD had a positive staining for phosphorylated STAT6, a surrogate marker of IL-4 signaling in glomeruli, whereas it was positive in only 1 of 23 controls.

There is some evidence that IL-13, IL-8, and IL-4 could play a role in experimental models of proteinuria, but the significance of these studies remains unclear due to the lack of further validation by other research groups and by the heterogenous pattern of these cytokines in patients with MCD (53, 59, 63–65).

While T-cell effectors are the source of the above and other pro-inflammatory cytokines, a deficiency in regulatory T cells (Treg cells) has also been implicated in the pathogenesis of MCD (14, 66). An example is the association of MCD with immune dysregulation, polyendocrinopathy, enteropathy, and X-linked (IPEX) syndrome. This is an immunodeficiency syndrome characterized by a FOXP3 mutation that inactivates Treg cells (67). In addition, Treg cells express CTLA-4. This is an important modulator of the immune response by binding to CD80 on antigen presenting cells (APCs). Of note, patients with MCD have a high CD80/CTLA-4 ratio in urine, suggesting that an imbalance in these molecules may have a role in MCD (68).

In summary, experimental studies on animal models have shown a possible role for some cytokines in the pathogenesis of proteinuria. However, the results of these studies have not been confirmed in human disease. This may be due to the variable methodology among studies, the heterogeneity of the disease, the differences between human MCD and animal models, and the complex interplay among pro- and anti-inflammatory cytokines, immune cells, and glomerulus. To date, there is no evidence that a single cytokine is a key mediator of podocyte injury in MCD.

B Cells and Autoantibodies

While most studies have focused on T-cell-related cytokines, the efficacy of anti-CD20 therapy [rituximab (RTX)] to reduce the frequency of relapses in SSNS and MCD has brought an increasing interest on the potential role of B cells in the disease pathogenesis (69). It remains unclear whether B cells may contribute to the disease by promoting certain T-cell responses or by releasing autoantibodies against podocyte proteins (70–72). For instance, Oniszczuk et al. found higher circulating levels of plasmablasts and B-cell activating factor in the serum of adults with MCD during relapse (73), but whether these drive T-cell activation in MCD remains to be determined. Colucci et al. found that patients with idiopathic nephrotic syndrome (INS) and poor response to therapies carry higher levels of T cells with hyposialylated immunoglobulin M (IgM) on the surface (72). Supernatants from these T cells caused podocyte cytoskeletal rearrangements *in vitro*, but this was prevented when T cells were incubated with sialylated IgM, suggesting that IgM on the T-cell surface modulates T-cell responses and that B- and T-cell crosstalk may play an important role in the pathogenesis of MCD (72). Trachtman et al. recently showed that IgM can trigger the classical pathway of complement in glomeruli from patients with INS (74). These findings emphasize the potential pathogenic role of B cells in nephrotic syndrome.

Furthermore, B cells produce antibodies. Thus far, there have been two candidate autoantibodies proposed as mediators of MCD, but the lack of immune complexes on kidney tissue remains an argument against a key pathogenic role of these autoantibodies.

Antiubiquitin Carboxyl-Terminal Hydrolase L1 (UCHL1) Antibodies

Combining human, experimental, and animal studies, Jamin et al. found elevated anti-UCHL1 antibody titers in plasma in about half of children with SSNS in relapse compared to controls (71). These autoantibodies targeted podocytes causing cell detachment *in vitro* and proteinuria and MCD-like injury *in vivo*. Interestingly, antibody titers were not increased in adults with active MCD (71). If these findings are validated, this would support a pathogenic role of autoantibodies and suggest that childhood- and adulthood-onset MCD may have a different underlying pathophysiology.

Antinephrin Antibodies

Watts et al. recently found circulating antinephrin antibodies in 29% of patients with active MCD ["Autoantibodies against nephrin elucidate a novel autoimmune phenomenon in proteinuric kidney disease." Medrxiv (Preprint). Available at https://www.medrxiv.org/content/10.1101/2021.02.26.21251569v1.full]. In the MCD glomerulus, authors showed granular immunoglobulin G (IgG) deposits colocalizing specifically with nephrin, but not with other podocyte proteins. This is an attractive finding as antinephrin antibodies mediate recurrence of nephrotic syndrome in patients with congenital nephrotic syndrome. Watts et al. postulated that circulating antinephrin antibodies may bind to nephrin changing its localization in the slit diaphragm, thereby resulting in proteinuria. However, it remains unknown whether these antibodies play a causative role either as primary or secondary insult to the podocyte or whether they may represent a paraphenomenon. Of note, circulating antinephrin antibodies are also present in patients with diabetes, but they were not associated to a higher risk of proteinuria (75). While antinephrin antibodies cause proteinuria in rats, they only induce a partial retraction of podocytes; so, the exact mechanisms by which these autoantibodies may cause proteinuria remains unclear (76).

Future studies are needed to determine the pathogenic role of B cells, to assess for causality between circulating autoantibodies and proteinuria in MCD, and to screen for novel candidate autoantibodies.

Hemopexin

Hemopexin is a plasma glycoprotein with high affinity for heme and immunoregulatory properties. When infused into rats, hemopexin caused reversible proteinuria and podocyte FPE resembling MCD-like injury (77). Mechanistically, it is thought to contribute to proteinuria by reducing anionic charges in the GBM *in vivo* and by disrupting nephrin signaling *in vitro* (77, 78). Patients with MCD may carry an "active" form of hemopexin. While plasma levels were first reported low in patients with MCD compared to controls, a recent study found that serum and urine levels of hemopexin are high in children with active nephrotic syndrome (79, 80); so, larger studies by using standardized methods to quantify hemopexin could provide insights into the value of hemopexin as marker or mediator of disease. More recently, plasma hemopexin was found to discriminate among patients with SSNS and steroid-resistant nephrotic syndrome (SRNS) with lower levels noted in SSNS compared to SRNS (81).

Microbial Products

In children with MCD, proteinuria is often triggered by infections (44). In mice, TLR ligands induce transient proteinuria and podocyte injury. However, it remains unknown whether viral or bacterial products directly stimulate TLR on podocytes or whether proteinuria may be the result of an exaggerated systemic immune response in susceptible patients (42, 47).

Others

Over the last few years, proteomic and metabolic studies have identified candidate biomarkers in urine or plasma (α1-macroglobulin, adiponectin, etc.) to discriminate MCD from FSGS and SSNS from SRNS, but whether some of these may also have a pathogenic role in proteinuria is still unclear (81, 82). In 2021, there have been numerous cases reported of new onset or relapsing MCD following COVID-19 vaccine (83). These cases shared a strong temporal association between vaccine administration and onset of proteinuria, highly suggestive of an exaggerated and rapid T-cell-mediated immune response to viral messenger RNA (mRNA). Future studies are required to investigate a potential causal link between the COVID vaccines and MCD. On the other hand, Angeletti et al. recently reported that protein-based vaccines are not associated with a higher risk of relapse in patients with nephrotic syndrome (84).

LOCAL (INTRAGLOMERULAR) FACTORS

Most researchers have historically focused on the study of circulating cytokines, but recent studies have recognized the importance of the local microenvironment in the development of podocyte injury in the experimental models. Here, we will review some of the molecules, thought to be released within the glomerulus, that have been linked to the pathogenesis of MCD.

Interleukin-4

In an elegant study, Kim et al. planted a B-cell antigen (hen egg lysozyme) on the GBM and found that injection of polarized antigen-specific B cells led to transient proteinuria within 24 h and histological changes consistent with MCD-like injury. In contrast, transfer of polarized antigen-specific B cells that were IL-4 deficient did not cause proteinuria, suggesting that proteinuria was mediated by local activated B cells and release of IL-4 (62). Interestingly, circulating IL-4 was undetectable, suggesting that the local release of IL-4 was sufficient to induce proteinuria and podocyte injury *in vivo* (62).

Tumor Necrosis Factor-α (TNF-α)

Studies involving TNF-α have largely focused on FSGS rather than MCD. Because some patients with MCD eventually develop FSGS, intraglomerular TNF-α remains a target of interest as mediator of podocyte injury in MCD (85, 86). Sera from patients with FSGS are able to increase TNF-α expression in cultured human podocytes and consistent with this, glomerular TNF-α is increased in patients with FSGS (86, 87). While glomerular TNF-α is inversely correlated with estimated glomerular filtration rate (eGFR), it did not show a correlation with serum TNF-α, reflecting the discrepancy between the systemic and glomerular cytokine levels (86). Mechanistically, TNF-α downregulates nephrin expression and phosphorylates paxillin and focal adhesion kinase (FAK) leading to cytoskeletal rearrangement (88, 89). This may be relevant to MCD, as podocyte FAK is activated in these patients (90). In addition, TNF-α activates the inflammatory NF-kB pathway and can induce CD80 expression on podocytes (see CD80 section) (91).

Vascular Endothelial Growth Factor (VEGF)

Podocytes are a source of several factors that act as ligands of receptors expressed by GEnC and this cellular crosstalk is key to maintaining endothelial homeostasis and the integrity of the GFB as demonstrated by landmark studies in the field (92). VEGF-A is the most well-characterized molecule of the VEGF family and it has been implicated in the pathogenesis of diabetic nephropathy, preeclampsia, and thrombotic microangiopathy (92). Podocyte-specific loss of VEGF-A in mice prevents glomerular development and formation of glomerular endothelium and the inactivation of single VEGF-A allele leads to endothelial injury and end-stage renal disease. In contrast, the podocyte-specific overexpression of $VEGF_{164}$ results in collapsing glomerulosclerosis, suggesting that VEGF expression within the glomerulus is tightly regulated (92). In MCD, glomerular expression of VEGF has been reported high during relapse by some authors but not by others (93, 94). The discrepancy between studies could be related to methodology and the heterogeneity of the disease. Another consideration is that a kidney biopsy only reflects the molecular signature at a specific time point, whereas VEGF expression may fluctuate during different stages of relapse and remission.

Others

Several molecules involved in endothelial–podocyte crosstalk such as angiopoietins 1 and 2 and VEGF-C have been linked to proteinuric glomerular diseases such as preeclampsia and diabetic nephropathy, but their role in MCD remains to be investigated.

Further studies are needed to characterize the cytokine and inflammatory signature in the MCD glomerulus, the cellular source of these local cytokines (infiltrating T and/or B cells, podocytes, other glomerular cells, etc.) and the stimuli triggering such cell responses. Likewise, a reduced number of Treg cells has been reported in the MCD glomerulus (95), so it is possible that the imbalance between effectors T and Treg cells may be important to determine a pro- *vs.* anti-inflammatory microenvironment in the glomerulus.

ROLE OF THE GFB AS A CHARGE SELECTIVE BARRIER IN MCD

The GFB is a size and charge-selective functional unit that allows the free flow of water and small molecules while preventing the passage of plasma proteins into urine. It consists of three layers: GEnC and associated glycocalyx, the GBM and podocytes with their foot processes, SD, and glycocalyx. The disruption of the GFB at any layer can result in proteinuria, but podocytes play a critical role in the formation and integrity of the GFB (96). However, the cause of proteinuria in MCD was historically attributed to the loss of negative charges in the GBM based on the observation that anionic charges were reduced in the GBM in animal models of podocyte injury and in some patients with MCD (40, 97). Subsequent experimental models failed to demonstrate a causal link between the GBM charges and proteinuria *in vivo* (98), so this theory was nearly abandoned in the twenty first century. More recently, Huizing et al. found evidence of glomerular hyposialytation in 26% of patients with proteinuric glomerular disease including MCD and other glomerular diseases (99). These observations provided the rationale for an ongoing randomized trial to test whether N-acetylmannosamine (ManNAc), a sialic acid precursor, may ameliorate proteinuria in human glomerular disease (99). Further studies are warranted to identify the primary affected glomerular cell/protein with hyposialytation, the triggering insult, and whether ManNAc restores sialylation and ameliorates proteinuria in human MCD.

The GBM and, to a lesser extent, podocytes have been the focus of interest for researchers studying the role of charges in MCD, but it is notable that little attention has been paid to the endothelial glycocalyx (EG). This is a thick meshwork of GAG and proteoglycans negatively charged that covers the entire endothelium and its fenestrations. It has been postulated that

the EG is a major site for the generation of an electrokinetic field that prevents the passage of negatively-charged plasma proteins such as albumin (100, 101). In support to this, the loss of EG is associated to proteinuria in experimental models of diabetes and sepsis and human diseases (102). Of note, a study found an increase in circulating syndecan 1, as surrogate of EG degradation, in patients with MCD (103). So, further studies need to address whether the EG may be relevant for the development of proteinuria in MCD. It is possible that the EG injury favors the passage of albumin through the GFB and that EG products may modulate biological processes including podocyte response to injury. For example, syndecan 4, an important component of the EG, can activate podocyte via TRCPC6 and β3 integrin signaling (104).

PODOCYTE DYSFUNCTION

In MCD, podocytes experience alterations in shape known as FPE and changes in the regulation of key proteins such as nephrin and synaptopodin (25–27). These changes include nephrin downregulation, redistribution, dephosphorylation, and synaptopodin downregulation (25–27). However, these molecular dysregulations are not specific of MCD and it is still unclear whether they are the cause of the podocyte injury and/or FPE or whether they represent a non-specific adaptive response of podocytes to injury. Indeed, MCD has been associated with an increased in podocyte autophagic activity (105, 106) that is an important mechanism for stress adaptation. Notably, progression from MCD to FSGS on histology was associated with a decline of autophagic activity (106).

Podocytes are key to prevent proteinuria, but there is a poor correlation between the level of proteinuria and FPE in patients with MCD and other glomerular diseases (107), questioning a direct cause and effect between FPE and proteinuria and suggesting that other cell types may contribute to the disease.

Candidate Mediators of Proteinuria in Experimental MCD

Current *in-vivo* approaches to study MCD (PAN and LPS) result in a reduction and/or redistribution of podocyte proteins mimicking some key features of human MCD. Here, we will review some of the most studied and/or promising candidate mediators of podocyte injury in experimental models of MCD.

CD80

This is a costimulatory molecule expressed by APCs upon activation. In cultured podocytes, CD80 expression is upregulated upon injury with LPS and TNF-α and mediates actin rearrangements, a surrogate marker for FPE (42, 91). There is indirect evidence *in-vivo* supporting a role of CD80 as mediator of proteinuria in the LPS model. CD80 knockout mice do not develop proteinuria following LPS exposure and CD80 expression on non-hematopoietic cells is also necessary for TLR stimulation to cause albuminuria in mice (42, 91). Also, CD80 is excreted into urine following TLR stimulation (47). However, some groups could not identify CD80 expression in podocytes following LPS questioning the role of CD80 in proteinuria (108). More recently, we and others demonstrated that CD80 is upregulated by kidney endothelial cells following LPS by using immunofluorescence and endothelial-specific translating ribosome affinity purification (EC-TRAP) and high-throughput RNA sequencing analysis, respectively (109, 110). Mechanistically, CD80 is thought to prevent talin binding to β1 integrin and its downstream signaling and to prevent Neph1 binding to nephrin, thereby altering actin polymerization and organization (31, 111).

In humans, CD80 was initially found upregulated in podocytes in some patients with MCD during relapse. However, there have been contrasting results among studies questioning the validity of the CD80 staining in human kidney tissue and this has tamped down the initial enthusiasm for this molecular target (108, 112). Our group recently demonstrated that CD80 is indeed present in podocytes in MCD, but, surprisingly, most CD80 was lining the capillary lumens in an endothelial pattern (109). We and others also showed that urinary CD80 levels are consistently high in a subset of patients with MCD in relapse compared to controls and to patients with other proteinuric glomerular disease (68, 112–115). Interestingly, there are two cases reported in that patients with active MCD and high CD80 in urine underwent rapid transient and sustained remission following anti-CD80 therapy, respectively (116, 117). In contrast, the efficacy of anti-CD80 therapy in FSGS, usually associated to normal CD80 levels in urine, remains controversial (118). These observations suggest a potential link between CD80 or downstream pathways and proteinuria in selected patients with MCD (those with either high urinary or glomerular CD80).

Angiopoietin-Like 4

This is a glycoprotein highly expressed by the liver and adipose tissue. Podocyte-specific Angptl4 overexpression in rats caused albuminuria and FPE over time without immune complex deposition mimicking features of MCD. Proteinuria was exacerbated when these transgenic rats received a single dose of puromycin and it was partially ameliorated when animal received steroids or ManNAc, which is a sialic acid precursor (50). In this model, injured podocytes released hyposialylated Angptl4 that bound to the GBM neutralizing its negative charges and also enhanced oxidative injury to GEnC *in vitro*. In contrast, normosialylated Angptl4 is released into circulation and this mediates hyperlipidemia and ameliorates proteinuria by interacting with glomerular endothelial αvβ5 integrin in different animal models of proteinuria including FSGS and diabetic nephropathy (50, 119). Still, the mechanisms of proteinuria in the podocyte Angptl4 model are not fully understood. In humans, Angptl4 was found overexpressed in podocytes from few patients with MCD in relapse (50), but these findings were not validated in a larger clinical study (120). Angptl4 is excreted into urine at high levels in different proteinuric diseases, suggesting that Angptl4 may reflect a non-specific response of podocytes to various insults rather than being a specific marker or mediator of MCD (120). More clinical studies are necessary to determine whether Angptl-4 may contribute to human MCD.

c-Mip

This was initially found elevated in T cells of patients with MCD in relapse and subsequently in podocytes of patients with active MCD and other forms of proteinuric glomerular disease. In animal studies, c-mip overexpression in podocytes causes albuminuria, FPE, and loss of total and phosphorylated nephrin without evidence of inflammatory changes in the glomerulus, thereby mimicking key features of MCD (121). LPS triggers podocyte c-mip upregulation and this seems independent of the cellular or humoral immunity. Mechanistically, c-mip interferes with Fyn binding to nephrin preventing downstream signaling and nephrin phosphorylation, which is a critical for podocyte restoration following transient podocyte injury (121). To date, there is strong experimental data to support a potential role of c-mip as mediator of proteinuria in MCD and other glomerular disease, but further clinical studies are necessary.

Focal Adhesion Kinase

FAK is a non-receptor tyrosine kinase that resides at sites of integrin clustering serving as a link between the extracellular matrix and the acting cytoskeleton. Upon phosphorylation, FAK modulates cell motility and migration and also contributes to the secretion of matrix metalloproteinases (122). In podocytes, FAK activation is necessary for the development of proteinuria and FPE following LPS (123). In experimental models, nephrin phosphorylation is an upstream activator of the Cas-Crk pathway, involving FAK activation (90). In patients with MCD, but not FSGS, FAK activation is observed in podocytes during relapse. However, MCD is associated with a reduction in nephrin phosphorylation (26), so that the upstream signaling triggering FAK activation in MCD is not fully understood.

Zinc Fingers and Homeoboxes (ZHX)

This refers to a family of transcriptional factors (ZHX1, ZHX2, and ZHX3) that regulates the expression of key podocyte genes. ZHX proteins localize at the membrane as hetero- or homodimers. Using different injury models of proteinuria, Macé et al. showed that translocation of ZHX proteins from the membrane into the nucleus may result in distinct types of nephrotic syndrome (124). Nuclear ZHX3 alone or in combination with ZHX2 was associated to FSGS, whereas nuclear ZHX1 was associated to MCD-like injury and podocyte Anpgtl4 upregulation in culture systems. Thus, this study provided a mechanism by which podocyte Angptl4 expression may be regulated in MCD and it proposed a plausible pathway that may be involved in the development of several forms of proteinuric kidney disease (124). Data on human MCD are still scarce. The same group found an increase in ZHX1 expression in podocyte nuclei from patients with active MCD, whereas ZHX2 expression was downregulated compared to controls (124).

In summary, there are several candidate mediators of podocyte injury and proteinuria in models of MCD. Nevertheless, the lack of specificity to discriminate among models of proteinuria and the lack of validation in large clinical studies do question their clinical relevance for human MCD. This emphasizes the urgent need to develop novel experimental models and/or approaches to study MCD.

Candidate Targets Identified in Human Tissue

The use of novel approaches such as transcriptomics has helped to identify molecules and pathways relevant for the pathogenesis of the human disease. Sanchez-Niño et al. showed higher expression of fibroblast growth factor-inducible 14 (Fn14), monocyte chemotactic peptide-1 (MCP-1), and NF-kB in podocytes from kidney tissue of patients with FSGS, but not from MCD (125). Bennet et al. reported an increased expression of genes involved in inflammation and fibrosis (osteopontin, CD24, CCL3, CXCL2, CXCL14, SOX9, etc.) in FSGS glomeruli compared to controls. Likewise, authors found a decrease in podocyte-specific genes (NPHS1, WT1, VEGF, etc.) in patients with FSGS compared to controls (126). Hodgin et al. identified few differentially expressed genes in glomeruli from MCD in relapse *vs.* controls. Specifically, these genes are involved in amino acid and metabolic processes (BHMT, DDC, and XPNEP2) and cell adhesion (CDH11, MPZL2, OPCML, and TRO) (127). Using single cell transcriptomics, Menon et al. demonstrated an upregulation of alpha-2 macroglobulin in GEnC from patients with FSGS compared to living donors and this was associated to poor clinical outcomes (128).

These novel approaches and collaborative efforts are key to elucidate the different molecular signatures linked to MCD. In particular, single cell transcriptomics is important to identify the dysregulated cell type within the glomerulus. Likewise, future research should also address the upstream pathways leading to these intraglomerular changes.

ENDOTHELIAL DYSFUNCTION

Endothelial cells line the entire vasculature and, in the glomerulus, are in close proximity to podocytes. Endothelial–podocyte communication is critical for the maintenance of the GFB. In addition, endothelial cells also have the machinery to present and process antigens and are important modulators of the immune response and inflammation (129). These features along with the assumption of a circulating factor involved in MCD could make endothelial cells an attractive cell target for the disease pathogenesis. However, studies involving GEnC in MCD are anecdotical unlike in other glomerular diseases. There have been few reports that showed evidence of endothelial dysfunction, ultrastructural changes in the glomerular endothelium, and upregulation of markers of cell activation, such as CD80 and caveolin-1, in GEnC of patients with MCD during relapse (103, 109, 130–132). These findings suggest that the endothelium is injured and/or activated in MCD and raises the possibility that activated GEnC may contribute to a pro-inflammatory milieu in the glomerulus rather than being an innocent bystander. For instance, CD80 is associated with activation of downstream inflammatory pathways and caveolin-1 mediates albumin transcytosis and endothelial cell function (133). More recently, Trachtman et al. showed that IgM can bind to epitopes on injured GEnC and activate the complement pathway (74). In addition, injury to the endothelium may facilitate the passage of proteins through the GFB due to the loss

of the glycocalyx that serves as charge barrier (101). Therefore, further studies are warranted to determine whether activated GEnC plays a pathogenic role in proteinuria in MCD.

GENETICS

Epidemiological studies have shown cases of familial SSNS and certain ethnic differences across populations. For instance, SSNS is more common in Asian children and it may have a more difficult clinical course in patients of African-American or Hispanic descents (134). However, Mendelian mutations have been rarely described in SSNS or MCD contrary to that observed in SRNS or FSGS. These findings suggest that SSNS and MCD are polygenic diseases with a complex inheritance pattern influenced by individual genetic risk and environmental factors. Given the association of SSNS and MCD with immune dysregulation, HLA genes have been a target of interest for researchers (33, 135). Numerous groups used an HLA candidate approach to identify genetic risk loci for SSNS and identified several variants including HLA-DQB, HLA-DBA, HLA-DRB1, HLA-DQB1, HLA-DQW2, and HLA-DR7 (134). Using a non-biased approach such as genome-wide association studies (GWASs), Gbadegesin et al. identified HLA-DQA1 and PLCG2 missense variants as candidate risk loci for children with SSNS. HLA-DQ1 variants resulted in perturbation of protein secondary structure, which may alter the process of antigen presentation in these patients. Notably, HLA-DQA1 is also reported in IgA nephropathy and membranous nephropathy, suggesting a shared immune dysregulation among these proteinuric diseases (36). As previously mentioned, IL-4 and IL-13 have been linked to MCD/SSNS. Several studies have investigated the potential association between gene variants and MCD with contrasting results. Al Rushood et al. found no association between IL-4 and IL-13 gene polymorphisms and susceptibility to SSNS (136), whereas Acharya et al. reported a possible association of these gene variants and MCD (137). In a similar study, Ikeuchi et al. showed an association between STAT6 gene polymorphisms and MCD (138) contrary to that reported by others (137). Using GWAS, Jia et al. recently identified NPHS1 (nephrin) and TNFSF15 regions as susceptibility factors for childhood SSNS (139).

Genetics studies continue to shed light into potential targets and pathways relevant for the disease and also reinforce the role of immunity in MCD. Nevertheless, these studies demonstrate associations rather than causality.

CONCLUSION

Minimal change disease is a clinical–histopathological entity with variable clinical outcomes. Despite research efforts, the mechanisms of proteinuria remain poorly understood and this has hampered the development of targeted therapies. MCD involves a complex interplay between environmental factors, genetic susceptibility, immune dysregulation, and the glomerular microenvironment, suggesting that MCD is not simply an immune or podocyte disease. This may explain the heterogeneity of the human disease, which together with the lack of good animal models, remain major obstacles to elucidate the pathogenesis of MCD. Therefore, future research strategies should integrate analysis of timed human biosamples including large and well-characterized cohort of patients along with novel experimental models including animal studies and state-of-the-art *in-vitro* approaches to improve our understanding of cell-to-cell interactions in the disease.

AUTHOR CONTRIBUTIONS

SP, FP, and GC-F contributed to the design of the review paper. FP created figures and tables. All authors contributed to the revision and reading of the manuscript and approved the submitted manuscript.

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REVIEW
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The Glomerulus According to the Mesangium

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The glomerulus is the functional unit for filtration of blood and formation of primary urine. This intricate structure is composed of the endothelium with its glycocalyx facing the blood, the glomerular basement membrane and the podocytes facing the urinary space of Bowman's capsule. The mesangial cells are the central hub connecting and supporting all these structures. The components as a unit ensure a high permselectivity hindering large plasma proteins from passing into the urine while readily filtering water and small solutes. There has been a long-standing interest and discussion regarding the functional contribution of the different cellular components but the mesangial cells have been somewhat overlooked in this context. The mesangium is situated in close proximity to all other cellular components of the glomerulus and should be considered important in pathophysiological events leading to glomerular disease. This review will highlight the role of the mesangium in both glomerular function and intra-glomerular crosstalk. It also aims to explain the role of the mesangium as a central component involved in disease onset and progression as well as signaling to maintain the functions of other glomerular cells to uphold permselectivity and glomerular health.

Keywords: glomerulus, mesangial cells, crosstalk, glomerular barrier, glomerular diseases, IgAN, DKD

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INTRODUCTION

The glomerulus is made up of three cell types, the endothelial cells, the podocytes and the mesangial cells (MCs). All three cell types are necessary and dependent on each other for normal glomerular function. During the last two decades, attention has been on the functional properties of the podocytes and to some extent to the contribution of endothelial cells and their glycocalyx to glomerular function and the role of MCs has been less in focus. Herein, the current state of knowledge about the MCs and the mesangium will be reviewed and integrated with recent information about this important cell type having a central role in the glomerulus.

LOCATION OF THE MESANGIAL CELLS IN THE GLOMERULUS

The MCs make up about 30–40% of the glomerular cell population (1) and are situated between the capillary loops embedded in the mesangial matrix. The glomerular cells, including the MCs, originate from the metanephric mesenchyme during development. The S-shaped bodies organize the endothelium and the podocytes and their respective precursors migrate into the S-shaped bodies while associated stromal mesenchymal cells form the mesangium (2). It has been suggested that Platelet Derived Growth Factor Subunit B (PDGFB) secreted by the endothelial progenitors

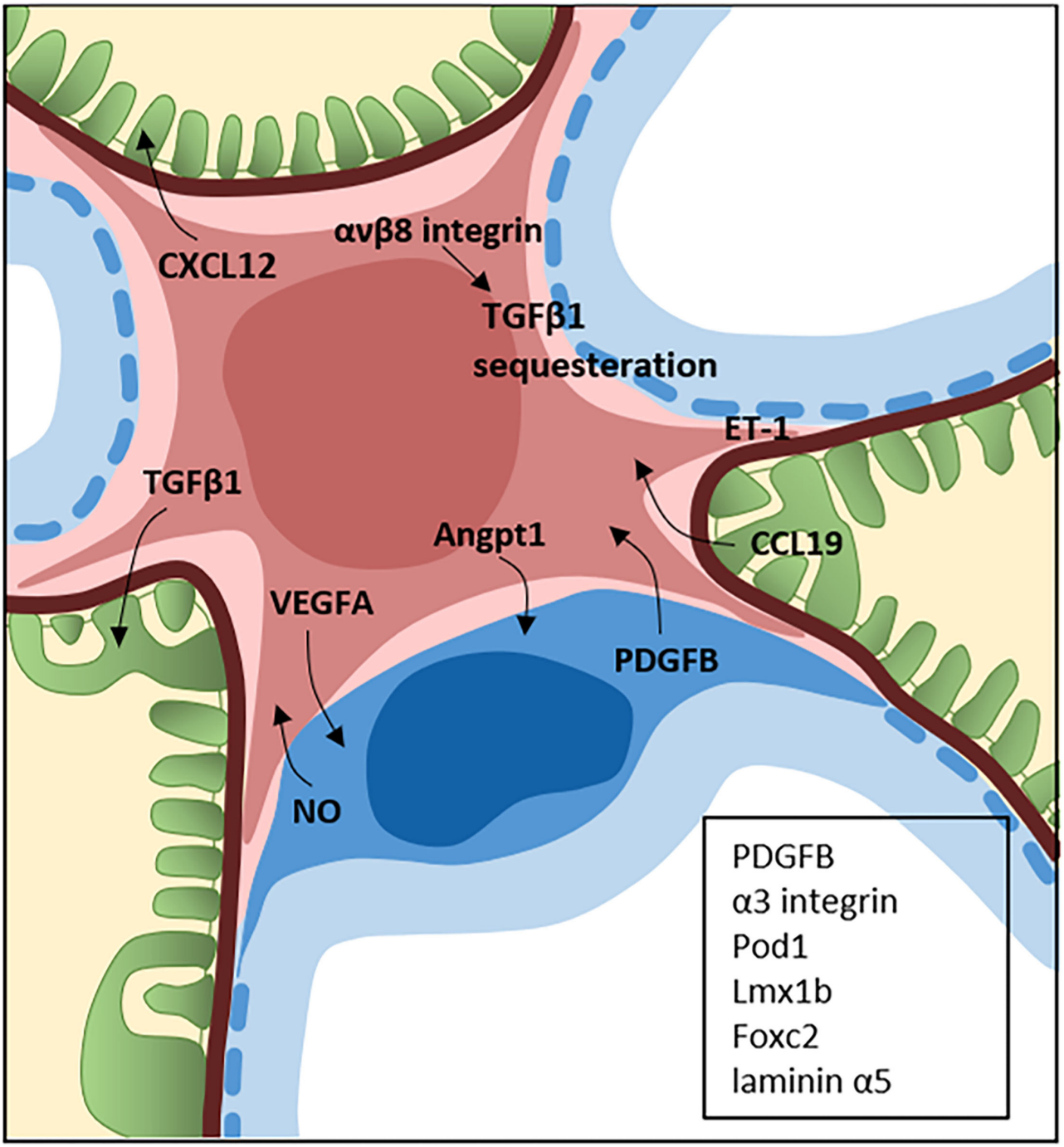

FIGURE 1 | Crosstalk between the mesangial cells, podocytes and glomerular endothelial cells. The central position of mesangial cells (pink) in the glomerulus is a perfect location for crosstalk with both the podocytes (green) and endothelial cells (blue). The field of glomerular crosstalk is emerging and the figure summarizes some of the known crosstalk signaling molecules between mesangial cells and the podocytes and endothelial cells in the normal and diseased state. Endothelin-1 (ET-1) is expressed by all glomerular cells and the crosstalk can occur in several directions. The proteins in the black box represents proteins expressed by the endothelial cells (PDGFB) and podocytes (PDGF, α3 integrin, Pod1, Lmx1b, Foxc2, and laminin α5) which are known to be necessary for normal development of the mesangium.

recruit the mesangial progenitor cells to migrate into the cleft where they promote glomerular tuft formation (3). In the mature glomerulus, the MCs are in direct contact with the endothelial cells but separated from the podocytes by the basement membrane. The MCs are connected to the basement membrane at the paramesangial angles (4). The MCs are also

in continuity with the extraglomerular mesangium and the juxtaglomerular apparatus. The MCs are not considered as being a direct part of the filtration barrier but are rather forming a central stalk of the glomerulus where they are important contributors to glomerular function.

OVERVIEW OF THE ROLE OF THE MESANGIAL CELLS IN THE GLOMERULUS

The MCs have multiple functions such as regulating the capillary surface filtration area, being a source of growth factors and cytokines and clearing the mesangial region from macromolecules entering from the endothelial layer. MCs are considered to be a form of microvascular pericytes with features resembling smooth muscle cells (5). However, the cells have also been shown to possess immune cell-like characteristics such as phagocytic and scavenging properties (6, 7). A recent single-cell transcriptomic study in mice identified the MCs as mesenchymal stromal cells, a class of cells that include fibroblasts, pericytes and vascular smooth muscle cells (8). It has been discussed whether all MCs have similar properties or if there are subclasses of MCs. He et al., using single cell sequencing of both mouse and human glomerular cells, propose that there are distinct subclasses of MCs in the mesangium, including both a prominent pericyte-like MC type and a more fibroblast-like MC type (9). These results also indicate that the MCs possess phagocytic properties as previously suggested (6). Several glomerular diseases affect the MCs such as IgA nephropathy (IgAN), diabetic kidney disease (DKD) and lupus nephritis, to mention a few. However, there is still a lack of knowledge about the exact contribution of the MCs for disease development and especially their role in glomerular crosstalk. The mesangial cells have an important role in clearing the glomerulus of pathogens and deposited extra-glomerular material, in cellular immune responses and in contribution to cell-to-cell signaling in the glomerulus. As the field of crosstalk is emerging, MCs with their location in the glomerulus are highly likely to be central to disease onset and progression (**Figure 1**).

MESANGIAL CELLS AND THE BASEMENT MEMBRANE

The mesangial connection to the glomerular basement membrane has been shown to be of importance for organization of the glomerular capillaries as well as for the contractile properties of MCs. Laminin α5 replacement of laminin α1 in the basement membrane during the capillary loop stage is required for glomerulogenesis. MC adhesion to laminin α5 is mediated by integrin α3β1 and the Lutheran glycoprotein and is necessary for MC organization of the glomerular capillaries (10). More recent work by Zimmerman et al. has shown that nephronectin produced by the podocytes and localized to the glomerular basement membrane may provide an anchoring point for MCs via integrin α8β1. Mice with a conditional deletion of nephronectin in nephron epithelial progenitors or mice with a podocyte specific deletion of nephronectin gave rise to increased numbers of MCs and mesangial matrix and loss of lateral adhesion of the MCs to the glomerular basement membrane (11). Since the mesangial cells are connected to the basement membrane (4) the mesangial cells have been suggested to have a role in regulating the dimension of the glomerular capillaries by changing the extent of the MC-glomerular basement membrane connection (12, 13). Decrease of the capillary lumen has been suggested to occur by pulling the peripheral sites of the glomerular basement membrane centripetally and increase of the capillary lumen by releasing the most peripheral anchoring points of the MCs, thereby regulating the length of the basement membrane (14).

THE ROLE OF THE MESANGIAL MATRIX IN NORMAL AND DISEASE CONDITIONS

The MCs form the central stalk of the glomerulus and are embedded in their own self-made mesangial matrix. The generation and turnover of the mesangial matrix is tightly regulated by the MCs themselves but in some glomerular diseases loss of the tight self-control leads to expansion of mesangial matrix and sclerosis. The mesangial matrix is not only important for structural support but is also involved in cell signaling and harboring signaling molecules, for example Transforming Growth Factor Beta 1 (TGFβ1) (15). The mesangial matrix is mainly composed of collagen type IV, collagen type V, laminin, fibronectin and proteoglycans (PGs) (16) but the exact composition of the mesangial matrix is unknown. Agrin and perlecan are two PGs that have been identified in the mesangial matrix (17). Perlecan has been shown to be upregulated in the mesangium in a rat model of chronic transplant dysfunction (17) and in patients with IgAN increased perlecan expression in the glomeruli correlates with slower progression of disease (18). Biglycan and decorin are two PGs that are normally expressed at low levels in the glomeruli but have been shown to be upregulated in renal disease with mesangial expansion resulting in sclerotic areas of the glomeruli (18, 19). Laminin is a major constituent of the mesangial matrix and increased laminin expression in the mesangium has been observed in DKD (20). Nidogens are glycoproteins also expressed in the mesangium in the normal physiological state and are upregulated in the mesangial matrix in patients with glomerular disease (21). Collagen III is normally absent from the glomerulus but has been found in the mesangium in patients with renal disease and correlates with increased mesangial matrix (22). Expansion of the mesangium (increased cell number and matrix) reduce the luminal space and filtration area leading to decreased kidney function. Since a change in the mesangial matrix leading to sclerosis is a major problem in several glomerular diseases, development of drugs targeting this process would be very beneficial in preserving renal function in this large patient group.

MESANGIAL CELLS HAVE CONTRACTILE PROPERTIES

Early studies on the vascular perfusion of amphibian glomeruli led to the observation that blood flow and its distribution

between glomeruli and independent capillary loops varied over time (23). These findings suggested the existence of an internal glomerular mechanism for the regulation of blood flow through the glomerular tuft. Subsequent research findings have largely supported this view although some controversy might exist regarding the nature of such regulation. In the 1960's, the first observations were made of the contractile properties in cultured isolated glomeruli (24). The results indicated a role for MCs as source of the contractile force generating the observed glomerular contraction. Later, observations were made showing that vasoactive substances regulating the ultrafiltration coefficient (K_f), a product of the capillary surface area and hydraulic permeability, also regulated MC contraction (25). These observations led to the suggestion that regulation of blood flow through the glomerular capillaries and regulation of the filtration surface area, was achieved through the contractile properties of MCs (26).

Studies performed both *in vivo* and *in vitro* have demonstrated the contractile properties of whole glomeruli and isolated MCs alike. Micropuncture studies have suggested that mesangial cells are involved in regulating the single nephron glomerular filtration rate (snGFR) by modulating glomerular haemodynamics (27, 28). A more recent study by Ziegler et al. have shown that MCs actively contribute to the regulation of snGFR. They found that MCs contract in response to AngII *in vivo*. By the use of Thy1.1 antibodies to deplete the MCs in rats they showed that the snGFR was reduced and it was no longer affected by Angiotensin II (AngII) stimulation (29).

MCs share certain similarities with smooth muscle cells as they contain a contractile unit consisting of actin and myosin as well as tropomyosin (30, 31). In similarity to the smooth muscle cells, the contraction of MCs is also dependent on an intracellular rise in Ca^{2+}, and the phosphorylation of myosin light chains promoting interaction with actin (32). Although evidence points to a role for the MCs as dynamic regulators of glomerular filtration, it should be noted that some findings emphasize that the contractile properties of the MCs mainly serve to stabilize normal glomerular function. Isometric contraction rather than dynamic contraction serves to counteract the forces generated by increased hydrostatic pressures in the glomerular capillaries to maintain glomerular filtration rate (GFR) (31, 33).

THE REGULATION OF THE CONTRACTILE PROPERTIES OF THE MESANGIAL CELLS

Contraction of MCs can be initiated by several vasoactive substances. AngII was first shown to cause MC contraction (34), an effect that has been observed repeatedly in both isolated glomeruli and MCs *in vitro*. The binding of AngII to the glomerulus was mainly located to the MC area (35). Arginine vasopressin (AVP) also stimulated MC contraction (34). Endothelin-1 (ET-1), derived from endothelial cells, was further found to cause MC contraction *in vitro* (36, 37). Besides these vasoactive substances, PDGF has also been shown to cause contraction of MCs (38). Conversely, atrial natriuretic peptide (ANP) and nitric oxide (NO) are two substances found to cause relaxation of MCs and isolated glomeruli *in vitro* (35, 39–41).

The importance of Ca^{2+} levels for biological activity and contraction of MCs was demonstrated early (42). Treatment of MCs with vasopressin or AngII altered the intracellular levels of Ca^{2+}. Subsequent studies later revealed that MC contraction was a result of the Ca^{2+} initiated activation of plasma membrane chloride channels that in turn generated a depolarization of the plasma membrane and activation of voltage gated Ca^{2+} channels (VOCC). Studies showed that AngII, vasopressin, endothelin and ATP all individually caused a Ca^{2+} mediated increase in chloride conductance (43–45). Further studies showed that the initial Ca^{2+} increase was a result of release from intracellular stores (35), later proven to be mediated by the phospholipase C-γ–inositol triphosphate pathway (36, 46–48). The chloride conductance of the plasma membrane was upheld by activation of voltage gated Ca^{2+} channels (49), further increasing intracellular Ca^{2+} concentration and generating contractile forces.

Large calcium activated potassium channels, known as BK_{Ca} channels, are the main regulators of relaxation in MCs. The channel consists of a core α-subunit forming the pore of the channel and one of four accessory β-subunits differently expressed depending on the cell type. In mesangial cells, it is the β_1-subunit that is expressed (50). When first identified in MCs (51), the BK_{Ca}–channel was shown to be activated by Ca^{2+} and membrane depolarization. In the same study, it was shown that the intracellular increase in Ca^{2+} following AngII stimulation could, at least in part, activate the channel. The opening of the BK_{Ca}–channel causes a hyperpolarization of the plasma membrane, resulting in a closure of VOCC and inhibition of the chloride and the VOCC positive circuit causing MCs to contract (50, 52). Further studies showed that relaxing hormones such as ANP and NO could activate the BK_{Ca}–channel in MCs through the action of cGMP and PKG. These second messengers sensitize the BK_{Ca}–channel and decrease its threshold for activation by Ca^{2+} and membrane depolarization (52, 53). Recent research concerning the contraction of MCs has focused on identifying ion channels and possible regulators of contraction, as well as further improving the understanding of the role of MCs in regulation of glomerular filtration. This research has identified a role for the Transient Receptor Potential Cation Channel Subfamily C Member 6 (TRPC6) in MC contraction as well as provided new methods for studying MC contraction *in vivo* in order to establish the role of MCs in regulation of glomerular filtration (29, 54–56).

MESANGIAL CELLS CAN PERFORM PHAGOCYTOSIS

MCs can perform receptor independent micro- and macro-pinocytosis and phagocytosis as well as receptor dependent uptake (6). However, some studies suggest that this process is not performed by the MCs *per se*, but rather by a population of cells in the mesangium that have a different phenotype and is responsible for the phagocytic properties of the mesangium (57). The initial report on phagocytosis by MCs was an electron microscopic study where it was observed that MCs could ingest large

molecules (58). It was subsequently shown that MCs can take up zymosan particles *in vitro* (59). Thereafter, it was observed that MCs *in vitro* actively take up serum-coated colloidal gold particles via a coated pit mechanism and that the particle ended up in endosomes and phagolysosomes (60). It has also been demonstrated that MCs in culture ingest neutrophils undergoing apoptosis (61, 62). In the anti-Thy1.1 model of mesangial proliferative glomerulonephritis in rats, apoptotic MCs were phagocytosed by healthy neighboring MCs as a mechanism for resolution of hypercellularity (63). Mice deficient in integrin α8 have a delayed healing of glomerulonephritis induced by Habu snake venom compared to wild type mice (64). Using MCs isolated from these mice, it was found that integrin α8 facilitates phagocytosis in MC, likely mediated by integrin α8-cytoskeleton interactions (65). MCs have also been shown to actively take up IgA1. This was determined by incubating MCs with TRITC-labeled IgA1 and after fixation visualizing them with confocal microscopy showing IgA1 in vesicles in the cells. Unfortunately, there was no information on how the IgA1 was taken up by the cells (66). In a recent single-cell RNA sequencing study using human and mouse glomeruli, the MC enriched genes were shown to display several pathways involved in phagocytosis. The results were confirmed *in vitro* by latex bead phagocytosis assays in human MCs as well as *in vitro* by injection of FITC-labeled bovine serum albumin (BSA) in mice showing that the labeled BSA ended up in the MCs (9).

MESANGIAL CELLS ARE A SOURCE OF GROWTH FACTORS AND CYTOKINES

Hyperproliferation of MCs and an increased deposition of mesangial matrix are common occurrences in glomerular disease. Inflammatory processes cause inevitable damage and eventually, as part of the healing process, glomerular sclerosis can ensue causing a decline in glomerular filtration function (67). Recent research has focused on identifying the underlying mechanisms for the above-mentioned events and it has been found that MCs themselves both respond to and secrete various cytokines and growth factors that contribute to these pathological events (68–71).

In the normal state, the MCs are relatively quiescent and secretion of growth factors and cytokines is tightly regulated. However, upon activation by certain stimuli, the MCs will increase their biological activity and secretion. Two of the main actions of these growth factors and cytokines are the initiation of MC proliferation and production and deposition of components of the extracellular matrix (ECM) (72).

TGF-β is a well-known regulator of fibrosis and known to be associated with glomerular disease and the progression of CKD (71, 73). Mesangial cells are known to both act as a target and a source of this important growth factor. In cultured MCs, the expression of TGF-β can, amongst others, be stimulated by mechanical stretch, high glucose, advanced glycation end products (AGEs), AngII, renin, PDGF and platelet activating factor (PAF) (74–78). Some of these factors have also been found to affect the expression of TGF-β receptors, all 3 of which are expressed by MCs (79, 80). *In vitro*, TGF-β has been found to mainly increase the production of ECM components such as fibronectin, collagen I, III and IV as well as proteoglycans (81–84). Concurrently, it has also been found to affect the expression of matrix metalloproteinases (MMPs) as well as increasing the expression of TIMP-2, a tissue inhibitor of MMPs which promotes the deposition of components of the ECM (83, 85). An additional effect of TGF-β in MCs is the induction of expression of PDGF and connective tissue growth factor (CTGF) (75).

CTGF is a growth factor that is implicated in the development of renal fibrosis and DKD (86, 87). MCs *in vitro* are known to upregulate their CTGF-expression in response to stimuli such as high glucose, mechanical strain, AngII, and TGF-β (88, 89). Secretion of CTGF from MCs has further been seen following stimulation with both high glucose and TGF-β for which CTGF acts as a downstream regulator of some of the previously mentioned TGF-β effects such as deposition of fibronectin (75, 90). Similar to TGF-β, CTGF is also known to induce collagen production in cultured MCs (91) and CTGF has also been found to cause MC hypertrophy, a commonly observed occurrence in DKD (92). In addition, mesangial CTGF has been suggested to have a role in enhancing macrophage chemotaxis and adhesion (93).

PDGF is a well-characterized growth factor expressed by MCs and a known stimulator of MC proliferation (70). PDGF is expressed in several different isoforms, A-D, and their receptors consists of dimers of α and β-chains. In MCs, the main receptors expressed are the PDGFR-αβ and PDGFR-ββ. These receptors are primarily activated by binding to dimers of PDGF-B, C and D. MCs are known to express both PDGF-A and B (94–96). Expression and secretion of PDGF from MCs can be stimulated by several factors such as epidermal growth factor (EGF), TGF-β and tumor necrosis factor alfa (TNF-α) as well as PDGF, creating an autocrine loop for growth stimulation (97). Besides proliferation, PDGF can also induce MC migration and production of components of the ECM making the PDGF system an important part of the mechanism underlying mesangioproliferative diseases and renal fibrosis (70).

The inflammatory processes observed in glomerular disease are partly driven by external cells infiltrating the glomerulus as well as by resident cells. MCs are known to secrete cytokines and chemokines that both attract immune cells and affect the MCs themselves (98). The common pro-inflammatory cytokines TNF-α, IL6, IL8, and IL1 are all secreted by MCs and some of these cytokines can also regulate the secretion of cytokines from MCs (99, 100). IL6 was early on shown to be secreted by and to have a mitogenic effect on MCs (101, 102) while having an inhibitory effect on the production of ECM (103). Similarly, IL1 is also known to have mitogenic effects on MCs (100). Besides inducing expression of other cytokines and chemokines, TNF-α is also known to stimulate the expression of CTGF and to regulate cell proliferation and cytotoxicity (104, 105). MCs are known to secrete chemoattractants under experimental settings simulating an inflammatory milieu in the glomerulus. Such chemoattractants are monocyte chemoattractant protein-1 (MCP1), regulated on activation, normal T-cell expressed and secreted (RANTES), IL8 and IP-10 as well as the leukocyte

adhesion molecule ICAM-1 (106, 107). The MCs also express chemokine receptors such as CC chemokine receptors type 1 and 7 (CCR1 and CCR7) (106, 108). This suggests that the chemoattractants secreted by MCs are not only serving to attract and recruit leukocytes and monocytes to sites of glomerular inflammation but that the MCs themselves also serve as targets for chemokines secreted during inflammatory processes.

MESANGIAL CELLS IN GLOMERULAR DISEASE

Several glomerular diseases involve the mesangium either as the entry point of the pathological process or later when the disease progresses. Since an extensive crosstalk is present in the glomerulus between the various glomerular cells, injury to the MCs will eventually lead to damage to the other cells in the glomerulus driving the injury process further with progression of disease ultimately leading to loss of renal function.

In IgAN, the MCs are activated by deposition of immune complexes containing galactose deficient IgA (gd-IgA1). The activation leads to increased production of cytokines, chemokines and complement resulting in MC proliferation and matrix expansion [for detailed review see ref (109)]. Several receptors for immune complexes have been suggested to be located on MCs: the transferrin receptor (CD71) (110), asialoglycoprotein receptor (111), Fc α/μ- (112) or Fc α-receptor (113), α1/β1 and α2/β1 integrin receptors (114) and the β-1,4-GalT1-receptor (115). However, a conclusive result as to a specific receptor is lacking. Recently a paper by Li et al. demonstrated that deletion of microRNA-23b-3p in mice gave rise to IgAN like phenotype with increased mucosal IgA synthesis and IgA depositions in the kidneys along with albuminuria, hypertension and elevated serum creatinine. They propose that microRNA-23b is a potential new therapeutic target for IgAN (116).

DKD is distinctly different from IgAN. It is classified as a microvascular complication of diabetes but eventually the entire glomerulus is affected. In DKD, the first changes seen in the glomerulus are thickening of the glomerular basement membrane followed by mesangial expansion including MC hypertrophy and matrix accumulation leading to sclerosis. Work in the 1970–80s identified that the expansion of the mesangium and the reduction in peripheral capillary surface acts as a constituting mechanism leading to reduced kidney function in DKD (117–120). Accumulating evidence from the last decades suggest that one of the initial pathological events in DKD is a phenotypic transdifferentiation, also known as activation, of mesangial cells into a myofibroblastic phenotype characterized by the expression of α-SMA and production of interstitial collagen. These early pathological cellular changes are associated with the sclerotic events observed in DKD and can be initiated by the common factors driving progression of DKD mentioned below (121–126). Factors that can activate the MCs in DKD include high glucose, dyslipidemia, increased AngII and mechanical stress induced by systemic hypertension. The progression of DKD is mediated by several pro-inflammatory and pro-sclerotic pathways such as the TGF-β and the TNF-α pathways (127). One of the key factors in the sclerotic events in DKD is increased production of TGFβ by the MCs which can be induced by hyperglycemia and AngII leading to increased matrix production by the MCs (128, 129). Another growth factor suggested to be involved in the MC sclerotic process is connective tissue growth factor (CTGF) (130) whose production by the MC is increased by TGFβ stimulation, high glucose and mechanical strain leading to increased matrix production by the MCs (88, 131).

MESANGIAL CELLS AND THE IMMUNE SYSTEM

The general view is that the mesangium has a role in the immune response in many glomerular diseases especially in glomerulonephritis (GN). There is always a question of the importance of factors produced by the cell itself in the onset of disease in relation to factors originating from other cells, tissues or organs. It is clear that the immune system in the most prevalent GN, IgAN, is heavily involved at the level of the B-cells that are known to produce increased amounts of IgA1 and gd-IgA1 (132). The gd-IgA1 has a tendency to form IgA-IgG immune complexes that when escaping clearance by the liver may deposit in the mesangium. It is not likely that the production of gd-IgA1 by the B-cells is the only triggering factor for onset of IgAN since it is known that B-cells may act the same way in healthy individuals without causing disease (109, 133). However, it is generally recognized that the gd-IgA1 immune complexes are a part of the pathogenesis of IgAN (134, 135). The deposits are thought to interact with potential IgA receptors on the surface of MCs triggering an intracellular cascade where cytokines and other pro-inflammatory molecules are released resulting in cellular proliferation and extracellular matrix expansion (136).

MESANGIAL CELLS AND COMPLEMENT ACTIVATION

The complement system is another system that is involved in MC pathology where it is believed to enhance and potentiate injury in glomerular disease. Dysregulation of the complement system is generally observed in many autoimmune disorders and plays a central role in systemic diseases but it is also activated locally in the glomerulus in disease states such as IgAN and DKD. The possibility to block C5 by the use of a monoclonal antibody against C5 in atypical hemolytic uremic syndrome (HUS) has significantly improved clinical outcomes for this patient group (137, 138). Preliminary data on other glomerular diseases are also promising pinpointing the importance of the complement system in glomerular disease onset and progression (139).

Among the three complement pathways, the alternative pathway seems to be the main pathway activated in MCs at least in IgAN. C3 deposits are present in over 90% of IgAN cases often along with properdin and factor H (140). The gd-IgA1 forming

complexes have been suggested as triggers of C3 along with IL-6 and proliferation of the mesangial cells (141). Hydrolysis of C3 leads to an increase in C3a and C3b. C3b causes formation of C3 convertase and thereafter C5 convertase. It was previously reported that alternative complement pathway components such as Factor P, Factor B and complement factor H can be detected in kidney tissue in IgAN, and elevated levels of Factors P and B are found in the circulation of patients with IgAN (142). In addition, it is known that the lectin pathway also can be activated by polymeric IgA in MCs in IgAN causing deposition of C4 but this pathway is activated to a lesser extent compared to the alternative pathway. Alternative pathway complement components (Factors B and P, CFB, and CFP) and complement regulatory protein (complement factor H, CFH) are widely present in the kidney tissues of patients with IgAN and there are also significantly increased CFB and CFP levels in the circulation of patients with IgAN. Terminally, C5b-9 [also called membrane attack complex (MAC)] is being formed and deposits of MAC are frequently seen in IgAN (143, 144). The lectin pathway is involved in both IgAN (145) and in IgAN vasculitis through upregulation of C3 acting on MCs (146).

Complement is also involved in the disease progression of the most common cause of end stage renal disease (ESRD); diabetic kidney disease. Less is known about the MCs and their involvement but there is clearly an upregulation of complement in DKD and glycated end products are thought to render glomerular cells prone to complement upregulation. Most studies mention complement upregulation in endothelial cells, podocytes and tubular cells. Of the three possible complement activation pathways, the alternative and the lectin pathways seem more upregulated (147, 148). Less is known about the MCs but C5a is upregulated along with many other complement molecules both systemically and locally in the mesangium (149). It has also been shown that inhibition of C5a could attenuate mesangial proliferation in rats with experimental DKD (150).

IN VIVO AND *IN VITRO* MODELS FOR INVESTIGATING MESANGIAL FUNCTION IN HEALTH AND DISEASE

The most commonly used *in vivo* model for studying mesangial function is the Anti-Thy1.1 model (151) and models of mesangial proliferation such as the Habu snake venom model (152). Administration of anti-thymocyte serum or anti-Thy1.1 antibody to rats causes mesangiolysis with following mesangial proliferation (Anti-Thy1.1 nephritis) and is a model of mesangial proliferative glomerulonephritis (151). Administration of Habu snake venom to rats gives rise to segmental mesangial proliferation (152). As there is no specific protein exclusively expressed by MCs, generation of mice knock out models specifically targeting mesangial genes is not possible. On the other hand, attempts to study MC gene function has been made using the *FoxD1-cre* mouse line (153, 154). FoxD1 is not exclusively expressed by MCs but is expressed by a population of progenitor cells that give rise to renal stroma, pericytes, vascular smooth muscle cells and MCs (155–157).

There are also *in vitro* models that are more specific for glomerular diseases affecting the MCs. For IgAN it has been difficult to establish a good mouse model. This is mainly due to the lack of IgA1 in species other than primates and galactose-deficient IgA is a form of IgA1 lacking sugars in the hinge region. Existing murine models of IgAN are excellently reviewed in detail elsewhere (158). In short, the two most recent murine IgAN models are the grouped ddY mouse and the α1KI-CD89Tg mouse. The grouped ddY mouse was established by inter-crossing early onset ddY mice (159). The ddY mouse strain is a spontaneous IgAN model where the mice develop mesangial IgA depositions with co-deposits of IgG, IgM and C3 (160). The α1KI-CD89Tg mouse expresses both human IgA1 and CD89 resulting in mesangial deposits of IgA1-sCD89 complexes resulting in kidney inflammation, hematuria and proteinuria similar to human IgAN (161).

For DKD, there are different models depending on whether the aim is to recapitulate DKD from type I or II diabetes but most models give rise to mesangial proliferation and mesangial matrix expansion at varying levels. The most commonly used type I diabetes model uses streptozotocin (STZ) as STZ leads to irreversible pancreatic beta cell apoptosis. There are also genetic models of type I diabetes in mice, e.g., the Akita $Ins2^{+/C96Y}$ model, but one of the drawbacks of this model is that only male mice develop hyperglycaemia. For type II diabetes (insulin resistance), the db/db or ob/ob has been widely used in combination with high-fat feeding. The problem with most mouse models of DKD is that renal damage is limited, usually takes a long time to establish and only partly recapitulates human disease. More information regarding mouse models of DKD is found in reference (162).

MCs are rather easy to culture from glomeruli obtained from animals and humans. They are usually characterized by the expression of smooth muscle actin, PDGF receptor β and vimentin and negative expression for markers of parietal cells, endothelial cells and podocytes (163). Recently is has been shown that PDGF receptor β is expressed not only by the MCs in the glomeruli and for identifying true MCs a set of genetic markers has been suggested (PDGFRB, PDGFRA, GATA3 and CNN1) (9). MCs can also be cultured from glomeruli obtained from needle biopsies of patients with IgAN (164). It is worthy to note that MCs cultured *in vitro* express smooth muscle actin, a marker that is not usually expressed in the mature healthy glomerulus *in vivo*. MCs expressing smooth muscle actin are considered to be activated and/or dedifferentiated as seen *in vivo* in disease states and have been described as a glomerular myofibroblast (122). If cultured for a longer time period, MCs may form nodular structures and these structures were shown to have less smooth muscle actin and a phenotype more resembling MCs *in vivo* (165).

To recapitulate mesangial disease MCs can be cultured in a diabetic milieu to mimic diabetic conditions or stimulated with gd-IgA1 to mimic IgAN. Cells can also be stimulated with growth factors important for mesangial proliferation and fibrosis such as PDGF and TGFβ or pro-inflammatory mediators like IL-1β. In addition, it is easy to knock down genes of interest in MCs in culture to investigate the role of different proteins for

mesangial function. For studies of mesangial crosstalk *in vitro*, the most common setting has been medium transfer or co-culture (please see crosstalk section for examples). New and exciting *in vitro* models for glomerular crosstalk include glomerulus-on-a-chip and organoids but unfortunately the glomerulus-on-a-chip models that have been developed do not include MCs (166) and MCs are so far missing or underrepresented in kidney organoid glomeruli (167–169), possibly due to the lack of vascularization of the organoids.

THE ROLE OF MESANGIAL CELLS IN GLOMERULAR CROSSTALK

The MCs are in direct contact with glomerular endothelial cells and separated from the podocytes by the basement membrane. Although MC crosstalk is understudied compared to podocyte and endothelial crosstalk, their central position in the glomerulus and close contact to the other cell types supports their role as a central hub in the glomerulus likely to contribute significantly to glomerular crosstalk. For example, angiopoeitin1 is expressed by both podocytes and MCs and the receptor, Tie1, is found on the endothelial cells. In mice with induced deletion of Angpt1 at E10.5, reduced numbers of MCs were observed (170).

MESANGIAL-ENDOTHELIAL CROSSTALK

MCs are dependent on PDGF-B from the endothelial cells for their development. This was demonstrated by genetically deleting PDGF-B production in glomerular endothelial cells rendering only a single vascular sack per glomerulus resulting in the death of the mice before birth (171). Knock down of PDGF receptor β gives a similar result with glomeruli lacking MCs and the mice die shortly after birth (172). In a co-incubation experiment of bovine aortic endothelial cells and rat MCs, it was found that stimulating NO release from the endothelial cells with bradykinin caused changes in cGMP in the MCs (173). These findings were also confirmed by others (39). Integrin αvβ8 is expressed by MCs and is known to reduce TGFβ signaling by sequestering it. In mice, deletion of integrin αvβ8 leads to glomerulopathy due to reduced latent TGFβ binding. This leads to increased bioavailability of TGFβ and induction of endothelial cell apoptosis suggesting that MCs impact TGFβ signaling which in turn influences endothelial cell function (174). Endothelial to MC crosstalk has also been shown by transferring exosomes from endothelial cells cultured in high glucose to MCs. The high glucose treated endothelial cells secreted a higher number of exosomes and they were highly enriched in TGF-β1 mRNA compared to cells cultured in normal glucose. The exosomes were taken in by the MCs and promoted cellular proliferation and extra cellular matrix production through the TGFβ1/Smad3 signaling pathway (175).

Co-culture of MCs and human umbilical vein endothelial cells (HuVEC), rendered a lower concentration of endothelin 1 (ET-1) in the cell culture media. It was demonstrated that this was due to down-regulation of endothelin converting enzyme 1 (ECE-1). Losartan abolished the downregulation of ECE-1 in the co-culture and AngII induced inhibition of ECE-1 expression in HuVECs suggesting AngII I can be one of the mediators involved in the ECE-1 down regulation. This shows that the bioactivity of ET-1 is regulated not only by the endothelial cells but also by the surrounding cells demonstrating crosstalk between the cells (176). ET-1 crosstalk between endothelial cells and MCs has also been demonstrated in a study investigating the role of the endothelin B receptor in diabetes. Using the streptozotocin model of diabetes in $ETBR^{-/-}$ mice, increased expression of ET-1 was found in these mice compared to controls. *In vitro* experiments showed that conditioned medium from high glucose treated $ETBR^{-/-}$ glomerular endothelial cells promoted MC proliferation and increased matrix related proteins. Similar effects on the MCs were achieved by ET-1 knock out in glomerular endothelial cells or inhibition of ET-1/endothelin A receptor in glomerular endothelial cells (177). Crosstalk has been demonstrated between MCs and endothelial cells in mesangial proliferative glomerulonephritis (MPGN) using the Anti-Thy1 nephritis model and co-culture of MC and endothelial cells. In the anti-Thy1 model, endothelial proliferation can be seen beside mesangial proliferation and the authors investigated the connection. They found that in anti-Thy1 nephritis mesangial cells express VEGFA and the endothelial cells increased their expression of angiopoietin 2 (Angp2). Using a co-culture system, it was confirmed that MCs activated by PDGF-BB expressed VEGFA leading to activation of VEGF receptor 2, Angp2 expression and endothelial cell proliferation. Increased Angp2 inhibited Tie2 phosphorylation and enhancing Tie2 phosphorylation by Vasculotide alleviated endothelial cell proliferation on day 7 of the anti-Thy1 model. This was suggested as a strategy to lessen the vascular lesions in MPGN (178).

MESANGIAL-PODOCYTE CROSSTALK

The relationship between podocytes and MCs has been described in the developing kidney where several knock out and mutation experiments have demonstrated that MC recruitment and adhesion is dependent on proteins expressed by the podocytes. Several genes expressed by podocytes (α3 integrin, Pod1, Lmx1b, Foxc2) are needed for proper formation of the glomerular capillary loops and mesangium and mice lacking these genes have defects in MC recruitment, glomerular capillary loops and podocytes (179–183). In addition, laminin α5 in the basement membrane is needed for adhesion of MCs to the glomerular basement membrane via the G domain of laminin α5 and this is crucial for normal glomerular capillary loop development and a normal mesangium (10). Another proof of MC and podocyte crosstalk is that mutations in Wilms tumor suppressor gene gives rise to mesangial sclerosis (184). Another way for podocytes and MCs to communicate is through chemokines. This has been demonstrated by MC expression of the chemokine receptor CCR7 and its ligand expressed by the podocytes (CCL19) and the receptor CXCR4 receptor expressed on podocytes and the ligand (CXCL12) expressed by MCs (108, 185). In DKD, endoplasmic reticulum (ER) stress has been suggested to be part

of the disease progress. Culturing MCs in high glucose and transferring the medium to podocytes led to inhibition of the endoplasmic-reticulum-associated protein degradation pathway (ERAD) and podocyte injury. In diabetic mice inhibition of ERAD resulted in increased albuminuria, podocyte apoptosis and reduced nephrin expression (186). The identities of the specific molecules produced by the MCs leading to podocyte damage are unknown.

Podocyte-MCs crosstalk has also been investigated in the setting of IgAN, where such crosstalk is important in driving the glomerular damage seen in IgAN. Podocytes do not bind IgA from patients with IgAN (gd-IgA1) and stimulation of podocytes with gd-IgA1 does not induce release of growth factors or cytokines. However, transferring medium from human MCs stimulated with gd-IgA1 lead to increased expression of TNF-α as well as CTGF and increased expression of the TNF-α receptors on podocytes reducing important podocyte markers and increasing podocyte apoptosis (187–189). Medium transfer from MCs treated with gd-IgA1 induced epithelial-to-mesenchymal transition in podocytes and the PI3-K/Akt pathway was involved in the process (190). gd-IgA1 stimulation of MCs has also been shown to upregulate TGFβ1 and CXCL1. Medium from MCs treated with gd-IgA1 or CXCL1 in combination with TGFβ1 reduced podocyte adhesion and increased podocyte cell death (191). Increased TGFβ1 expression after gd-IgA1 stimulation of MCs *in vitro* has been reported (164, 188) as well as in glomeruli from patients with IgAN (18).

In summary, there is an emerging view that crosstalk between the MCs and the other cells in the glomerulus is active and ongoing during development in the normally functioning glomerulus and during disease.

CONCLUSION

In conclusion, over the years the role of the MCs in the glomerulus has been extensively studied and existing data suggest a central, pivotal role for MCs in glomerular function. In some forms of glomerular disease the MCs are heavily involved and are likely to be central for disease onset and progression. There are still some areas, especially concerning the role of MCs in glomerular crosstalk, that are less well-studied both in the normal state and in disease conditions.

AUTHOR CONTRIBUTIONS

KE have taken the lead in the final editing of the review. JN has finalized and submitted the review. All authors have planned and written the manuscript and contributed to the review.

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Podocyte-specific deletion of miR-146a increases podocyte injury and diabetic kidney disease

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Diabetic glomerular injury is a major complication of diabetes mellitus and is the leading cause of end stage renal disease (ESRD). Healthy podocytes are essential for glomerular function and health. Injury or loss of these cells results in increased proteinuria and kidney dysfunction and is a common finding in various glomerulopathies. Thus, mechanistic understanding of pathways that protect podocytes from damage are essential for development of future therapeutics. MicroRNA-146a (miR-146a) is a negative regulator of inflammation and is highly expressed in myeloid cells and podocytes. We previously reported that miR-146a levels are significantly reduced in the glomeruli of patients with diabetic nephropathy (DN). Here we report generation of mice with selective deletion of miR-146a in podocytes and use of these mice in models of glomerular injury. Induction of glomerular injury in C57BL/6 wildtype mice (WT) and podocyte-specific miR-146a knockout (Pod-miR146a$^{-/-}$) animals *via* administration of low-dose lipopolysaccharide (LPS) or nephrotoxic serum (NTS) resulted in increased proteinuria in the knockout mice, suggesting that podocyte-expressed miR-146a protects these cells, and thus glomeruli, from damage. Furthermore, induction of hyperglycemia using streptozotocin (STZ) also resulted in an accelerated development of glomerulopathy and a rapid increase in proteinuria in the knockout animals, as compared to the WT animals, further confirming the protective role of podocyte-expressed miR-146a. We also confirmed that the direct miR-146a target, ErbB4, was significantly upregulated in the diseased glomeruli and erlotinib, an ErbB4 and EGFR inhibitor, reducedits upregulation

and the proteinuria in treated animals. Primary miR146$^{-/-}$ podocytes from these animals also showed a basally upregulated TGFβ-Smad3 signaling *in vitro*. Taken together, this study shows that podocyte-specific miR-146a is imperative for protecting podocytes from glomerular damage, *via* modulation of ErbB4/EGFR, TGFβ, and linked downstream signaling.

KEYWORDS

podocytes, MicroRNA, miR-146a, diabetic nephropathy, glomerular disease

Introduction

Diabetes mellitus affects more than 37 million Americans (>11% of the US population) and remains one of the most common medical conditions (1). Progressive glomerular kidney injury due to diabetes mellitus is the leading cause of end-stage renal disease (ESRD) in the US (2). The diabetic glomerulopathy is characterized by loss of glomerular podocytes, glomerular basement membrane (GBM) thickening, segmental glomerulosclerosis, and mesangial expansion. Podocytes are specialized cells located in the Bowman's capsule of the glomerulus and are essential for the formation and function of the urinary filtration barrier in the kidney. Thus, these cells are vital to a healthy glomerulus and normal kidney function (3, 4). Diabetes results in significant podocyte injury (2, 3), although the exact molecular mechanisms are unclear. Some disease-associated pathways have been elucidated in recent studies (2, 4–12), however, mechanisms that lead to podocyte damage during diabetic nephropathy (DN) pathogenesis are yet to be elucidated. Furthermore, targeted therapeutics are sorely needed in the clinic for those suffering from DN.

MicroRNAs (miRNAs) are a family of small, non-coding regulatory RNAs that are approximately 18–22 nucleotides (nt) in length. Among their key functions, they regulate post-transcriptional expression of their target genes by promoting messenger RNA (mRNA) degradation or suppressing mRNA translation into functional proteins by binding the 3' untranslated region (UTR) of target mRNAs in a sequence-dependent manner (5). Thus, miRNAs play a vital role in regulating cell biological functions. Like protein expressing mRNAs, the expression of miRNAs is also regulated in a tissue specific fashion. Different miRNAs are expressed in all stages of kidney development and are also differentially regulated in the glomeruli in response to various external stimuli, indicating their involvement in disease pathogenesis (6). miRNAs are essential in podocyte homeostasis, as conditional deletion of miRNA processing enzymes Drosha and Dicer results in significant proteinuria and accelerated glomerular injury (7–9). Similarly, deletion of specific miRNAs in podocytes results in increased proteinuria and glomerulosclerosis (7). Expression levels of multiple miRNAs change in early DN, resulting in a change in expression of a number of DN associated genes and pathways and playing an important role in the pathophysiology of diabetic glomerular injury (10).

MicroRNA-146a (miR-146a) is a negative regulator of the pro-inflammatory signaling in myeloid cells and thus a key molecular brake on the inflammatory innate immune cell responses (11, 12). It also modulates key adaptive immune cell functions and plays an important role in hematopoiesis and cancer cell proliferation, *via* targeting a different set of genes (11, 13). miR146a is also highly expressed in the podocytes and in all other in types of cells in the glomeruli, suggesting that it has important homeostatic and regulatory roles in podocytes, including regulating kidney function during diabetic injury (14–16). Myeloid cell-expressed miR-146a was recently shown to increase in expression in murine DN, where it plays an anti-inflammatory role by suppressing proinflammatory cytokines in macrophages (17). Conversely, we previously found that the glomerular miR-146a levels were dramatically reduced in the kidney sections of diabetic patients (18), and that the level of miR-146a expression in the kidneys inversely correlated with proteinuria in the patients, suggesting that podocytic miR-146a plays a protective role in DN. A recent study with 460 subjects (300 cases and 160 controls) also confirmed that there exits an inverse association of blood miR-146a levels with diabetes and its complications (19). Furthermore, miR-146a levels were found to be down-modulated in the kidneys of diabetic rats and mice, suggesting that it plays a reno-protective role (20). We previously showed that the absence of miR-146a in the global miR-146a knockout animals (miR-146a$^{-/-}$) increased susceptibility of these animals to diabetic kidney disease, *via* hyperglycemia-induced podocyte damage (18). However, given that these prior studies used global miR-146a$^{-/-}$ animals, the studies were not able to provide direct evidence of the podocyte specific role of miR-146a.

To address this, we report here generation of podocyte-specific miR-146a$^{-/-}$ animals (Pod-miR146a$^{-/-}$) to study the functional consequences of the absence of miR-146a specifically in the glomerular podocytes. We find that the Pod-miR146a$^{-/-}$ mice mimic the diabetic glomerular injury observed in the global miR-146a$^{-/-}$ animals (18). Additionally, given that miR-146a directly targets tyrosine receptor kinase

ErbB4, we again find that the absence of miR146a results in increased ErbB4 expression and signaling, thus driving a podocyte-damaging feed-forward loop. Altogether, the results presented here confirm the role of podocyte-specific miR-146a in protecting podocytes from damage during DN pathogenesis, *via* control of ErbB4 and downstream signaling.

Results

Animals with podocyte-specific deletion of miR-146a are more sensitive to lipopolysaccharide and nephrotoxic serum-induced renal injury

Healthy podocytes abundantly express miR-146a, where it plays an important role in cellular health and function (14, 15). We previously showed that miR-146a levels significantly decreased in the glomeruli of diabetic patients and that the decrease was associated with progressively increasing kidney damage (18). Furthermore, using animals with global deletion of miR-146a (miR146a$^{-/-}$), we also showed that the absence of miR-146a accelerated the progression of diabetic nephropathy (DN) in the knockout animals. However, miR-146a is also highly expressed in the immune cells, where it suppresses NFκB signaling pathways and has been shown to function as an anti-inflammatory miRNA (12, 13). Given our previous data with DN patient biopsies and the DN models in miR-146a$^{-/-}$ mice, we hypothesized that the podocyte-expressed miR-146a plays an essential role in protecting glomeruli from injury. To unambiguously explore this role of miR-146a, we developed a novel, podocyte-specific miR146a deleted transgenic mouse (referred to as Pod-miR146a$^{-/-}$) using published protocols (21) (**Figure 1A**). Briefly, we first crossed C57BL/6 miR146a$^{fl/fl}$ mice containing floxed sites around the miR146a exon with Flp-recombinase expressing mice to remove the selection cassette containing LacZ and neomycin. Next, we crossed homozygous progeny with Podocin-Cre recombinase-expressing mice (22), which recognize the loxP sites surrounding the miR146a gene and only cut these sites in cells under the control of the Podocin promoter (which is highly and selectively in podocytes), thus selectively deleting miR146a specifically in podocytes to obtain Pod-miR146a$^{-/-}$ mice. The resulting mice were backcrossed >6 times before use in experiments here. Quantitative RT-PCR (qRT-PCR) based analyses of isolated podocytes showed an almost complete loss of miR-146a expression, as compared to miR-146a levels in cells from the wild type C57BL/6 wild-type (WT) littermates, whereas miR-146a expression in spleen and whole kidney showed no significant difference, as compared to the WT animals (**Figure 1B**). These data confirm successful deletion of miR-146a in podocytes.

In order to evaluate the effect of podocyte-specific miR146a deletion in models of glomerular injury, we examined its role in low-dose lipopolysaccharide (LPS) induced glomerular injury model of focal segmental glomerulosclerosis (FSGS) (23). Expectedly, LPS administration into WT mice resulted in a strong induction of albuminuria after 24 h compared with vehicle controls (**Figure 2A**). LPS administration into the Pod-miR146a$^{-/-}$ animals produced a significantly higher rise in albuminuria as compared to the LPS-treated WT animals, suggesting the lack of miR-146a in podocytes exacerbates glomerular injury. Histochemical analyses confirmed significant glomerular damage in the LPS-treated groups compared to controls (**Figures 2B,C**). These results suggest that podocyte-specific miR146a is protective of acute glomerular injury.

We further investigated the role of miR146a in glomerular injury using an established model of anti-glomerular basement membrane (anti-GBM) nephritis (24). Administration of nephrotoxic serum (NTS) into WT and Pod-miR146a$^{-/-}$ mice resulted in progressively increased albuminuria, with the Pod-miR146a$^{-/-}$ animals showing significantly worse disease and peak proteinuria at 4 weeks post NTS administration (**Figure 3A**), suggesting that miR146a plays an important role in protecting glomeruli from chronic injury. Histochemical analyses further confirmed significantly higher glomerular damage, including increased mesangial matrix expansion, in the NTS-treated Pod-miR146a$^{-/-}$ mice (**Figures 3B,C**). Altogether, these data show that podocytic miR146a is protective and its loss exacerbates glomerular injury in acute and chronic settings.

Diabetic glomerular injury is exacerbated in Pod-miR146a$^{-/-}$ animals and is reduced by erlotinib

To further investigate the effect of podocyte-specific miR146a deletion on glomerular function *in vivo*, we induced hyperglycemia in WT or Pod-miR146a$^{-/-}$ animals using published STZ protocols (25). STZ treatment resulted in induction of hyperglycemia and body weight decline in WT animals and to a similar extent in the Pod-miR146a$^{-/-}$ mice (**Figure 4A**), confirming our recent findings with the global miR-146a$^{-/-}$ mice (17, 18). Both strains also showed diabetes induced increase in albuminuria, although, the WT animals showed a slower increase (25–27), whereas the Pod-miR146a$^{-/-}$ mice showed a more rapid increase (as soon as 6 weeks post STZ injection) and significantly higher albuminuria level over time (**Figure 4A**), in line with our recent report with the global miR-146a$^{-/-}$ (18) and suggesting that miR-146a is critical for podocyte health and that its loss in podocytes greatly accelerates glomerulopathy *in vivo*.

ErbB4 (v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4, also known as HER4) is a tyrosine kinase

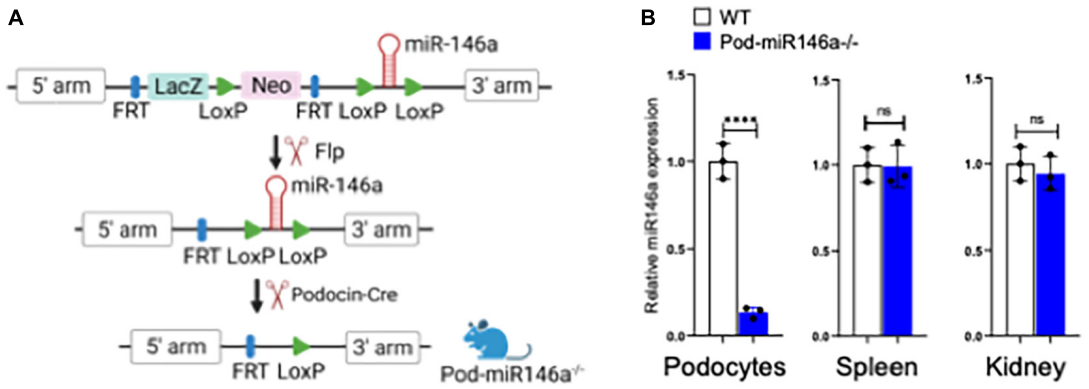

FIGURE 1
Generation of podocyte-specific miR-146a deleted mice. **(A)** Schematic showing steps used in the generation of podocyte-specific miR146a knockout mice by crossing miR146a$^{flox/flox}$ mice with Flp-mice and subsequently with podocin-cre mice. **(B)** miR-146a expression levels measured by quantitative RT-qPCR from primary podocytes, spleen and whole kidney isolated from WT or Pod-miR146a mice. Statistics were performed using the Student's *t*-test. Data shown are mean ± SEM (n = 3). ***$p < 0.0001$; ns, no significant difference.

receptor that is a member of the epidermal growth factor receptor (EGFR, also known as ErbB) family of receptors (28). ErbB4 often heterodimerizes with EGFR to form a functional receptor. ErbB4 mRNA is a direct molecular target of miR-146a, and binding of miR-146a to ErbB4 mRNA targets ErbB4 for degradation (29–32). Previously, we observed that ErbB4/EGFR expression and signaling was upregulated in the diabetic miR-146a$^{-/-}$ animals and that blockade of this pathway with clinically available ErbB4/EGFR inhibitor erlotinib was therapeutic, and erlotinib treatment reduced ErbB4 and EGFR expression and signaling (18, 33). To determine if this pathway was also therapeutic in the context of podocyte-specific miR-146a deletion, we also administered erlotinib to a group of diabetic WT and Pod-miR146a$^{-/-}$ animals. Results show that erlotinib treatment significantly reduced the level of albuminuria in both the diabetic WT and Pod-miR146a$^{-/-}$ animals (**Figure 4A**), without affecting the increased hyperglycemia or decreased body weight, as expected. This suggests that podocytic miR-146a protects cells *via* suppression of the ErbB4/EGFR pathway. Histopathologic analyses of kidney sections showed significant mesangial sclerosis in the glomeruli of the diabetic WT and Pod-miR146a$^{-/-}$ animals, and that erlotinib treatment significantly protects glomeruli from injury (**Figures 4B,C**). Immunofluorescence staining of the kidney sections showed a significant upregulation of ErbB4 in the glomeruli of non-diabetic Pod-miR146a$^{-/-}$ at baseline, as compared to the WT animals, suggesting that deletion of miR-146a in podocytes results in increased expression of its direct molecular target ErbB4 (**Figures 5A,B**). Data showed an increase in ErbB4 in podocytes, as visualized by colocalization of ErbB4 with synaptopodin *via* immunofluorescence (**Supplementary Figure 1**). Interestingly, no increase was observed for EGFR or Notch-1, unlike our previous findings with the global miR-146a$^{-/-}$ mice (18). Expectedly, STZ-induced diabetic injury resulted in significant increase in the expression of all three markers ErbB4, EGFR and Notch-1 in the diseased

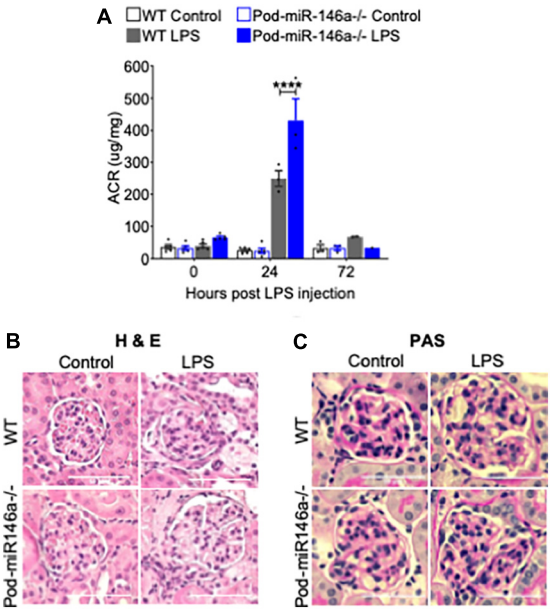

FIGURE 2
Pod-miR-146a mice show increased impairment of kidney function in response to LPS. **(A)** Graph showing the albumin to creatinine ratio (μg/mg) in the urine of WT (gray bars) and Pod-miR-146a (blue bars) mice after 0, 24, and 72 h post administration of either vehicle (open bars) or LPS (shaded bars). Statistics were performed using two-way ANOVA. Data shown are mean ± SEM (n = 5). ****$p < 0.0001$. **(B,C)** Representative images showing histochemical analyses with hematoxylin-eosin (H&E), periodic acid-Schiff (PAS) staining of kidney tissues from 24 h post LPS treatment. Scale bar, 50 μ m.

glomeruli. Additionally, treatment with kinase inhibitor erlotinib, which suppresses the kinase activity and a feed-forward suppression of kinase expression, showed significant reduction in the expression level of all three harmful proteins. Immunofluorescence imaging-based quantification of podocyte numbers also showed that STZ treatment resulted in a significant reduction in podocytes in both strains and that erlotinib significantly protected both strains from the loss of podocytes (**Supplementary Figure 2**).

Transforming growth factor beta 1 (TGFβ1), a member of the TGFβ superfamily, is a central regulator of diabetic glomerular injury and kidney fibrosis. TGFβ also mediates podocyte damage and diabetic glomerular injury in diabetes (34, 35). TGFβ1 imparts its intracellular effects by binding and activating TGFβ receptors (TGFBR), increasing phosphorylation and activation of the downstream Smad2/3 and MAPK signaling pathways (34–37). Given our previous finding that the TGFβ1 levels and signaling were increased in the diabetic miR-146a$^{-/-}$ podocytes (18), we investigated pSmad3 levels in the tissues of the animals here. Immunohistochemical (IHC) staining of kidney sections from untreated WT and Pod-miR-146a$^{-/-}$ mice showed a significant upregulation of pSmad3 staining under basal conditions in the glomeruli (no change in total Smad3) (**Figures 6A,B**). Additionally, STZ-induced diabetic injury resulted in further, significant increase in the expression of pSmad3 in the diseased glomeruli, and erlotinib treatment significantly suppressed the pSmad3 levels.

Taken together, these data suggest that ErbB4, a direct target of miR-146a, and the ErbB4/EGFR pathway are upregulated in the glomeruli of miR-146a-deficient and in diabetic animals. It also shows a basally upregulated TGFβ signaling pathway, that, together, predispose cells for injury. Podocyte-specific deletion of miR146a also results in worse diabetic glomerulopathy. Finally, our data strongly show that blocking ErbB4/EGFR signaling *via* erlotinib is therapeutic.

ErbB4 and its downstream signaling components show basal elevation in Pod-miR146a$^{-/-}$ podocytes

Next, we used western blot (WB) analysis of lysates from isolated WT and Pod-miR146a$^{-/-}$ primary podocytes to investigate levels of ErbB4, a direct target of miR-146a, and TGFβ signaling in these cells under basal conditions. The analyses showed increased ErbB4 expression as well as elevated levels of phospho-ErbB4 in the miR-146a deleted cells (**Figure 7** and **Supplementary Figure 3**), confirming the tissue immunofluorescence staining findings above. TGFβ1 is a central mediator of glomerular injury and fibrosis in DN *via* activation of mitogen activated protein kinase (MAPK) and Smad-based signaling pathways (38). WB analyses also showed a basal increase in phospho-p38 MAPK and increased levels of TGFβ1 and phospho-Smad2/3, suggesting that absence of miR-146a basally increases the harmful TGFBR/Smad signaling and predisposes podocytes to injury. Exact molecular details of how increased ErbB4/EGFR increases TGFb1 levels are not currently known and will need to investigated in the future.

Discussion

MicroRNAs (miRNAs) are a family of small regulatory RNAs that are central mediators of posttranscriptional repression in mammalian cells, thereby providing finer control of gene expression in cells (5). Thus, changes in expression of various miRNAs results in gene expression changes in cells, leading to different phenotypic outcomes. MicroRNA-146a (miR-146a) is a master regulatory RNA in myeloid cells, where it regulates TLR-dependent pro-inflammatory signaling *via* an NFκB-dependent negative feedback loop (11–13, 39). Recent studies have also shown that this miRNA is expressed widely in multiple types of mammalian tissues, where it regulates expression of target genes. We previously showed that miR-146a expression is significantly reduced in the glomeruli of diabetic patients, and that it negatively correlates with proteinuria in these patients (18). We also showed, using mice with global deletion of miR-146a, that the absence of miR-146a in podocytes drives podocyte damage *via* de-repression of its target genes ErbB4 and Notch-1. However, given our use of global miR-146a$^{-/-}$ animals, it wasn't clear of the podocyte-expressed miR-146a was the driver of the observed phenotypes. Here, we addressed this by generating mice with podocyte-specific deletion of miR-146a (Pod-miR146a$^{-/-}$) and using these animals in models of kidney disease.

Our studies show that the Pod-miR146a$^{-/-}$ are more sensitive to induction of kidney injury. We found that administration of LPS induced a significantly higher level of proteinuria in the Pod-miR146a$^{-/-}$ mice as compared to the C57BL/6 WT mice. Similarly, administration of NTS also resulted in significantly higher level of proteinuria and glomerular injury in the Pod-miR146a$^{-/-}$ mice. Additionally, as with the global miR-146$^{-/-}$ animals (18), induction of hyperglycemia *via* STZ administration produced a significantly higher level of proteinuria and increased glomerular sclerosis in the Pod-miR146a$^{-/-}$ animals. Given our previous findings that miR-146a suppresses expression of harmful ErbB4 gene expression, immunofluorescence imaging of the kidney sections confirmed basal increase in expression levels of ErbB4 in the otherwise healthy animals, suggesting that absence of miR-146a results in induction of ErbB4/EGFR pathway. WB based analyses of isolated primary podocytes also confirmed a basal increase in ErbB4 in the cells from Pod-miR146a$^{-/-}$ mice. Finally, previous studies had shown that ErbB4/EGFR signaling results in increased TGFβ-Smad pathway. WB with Pod-miR146a$^{-/-}$ podocytes also confirmed a basal increase in this

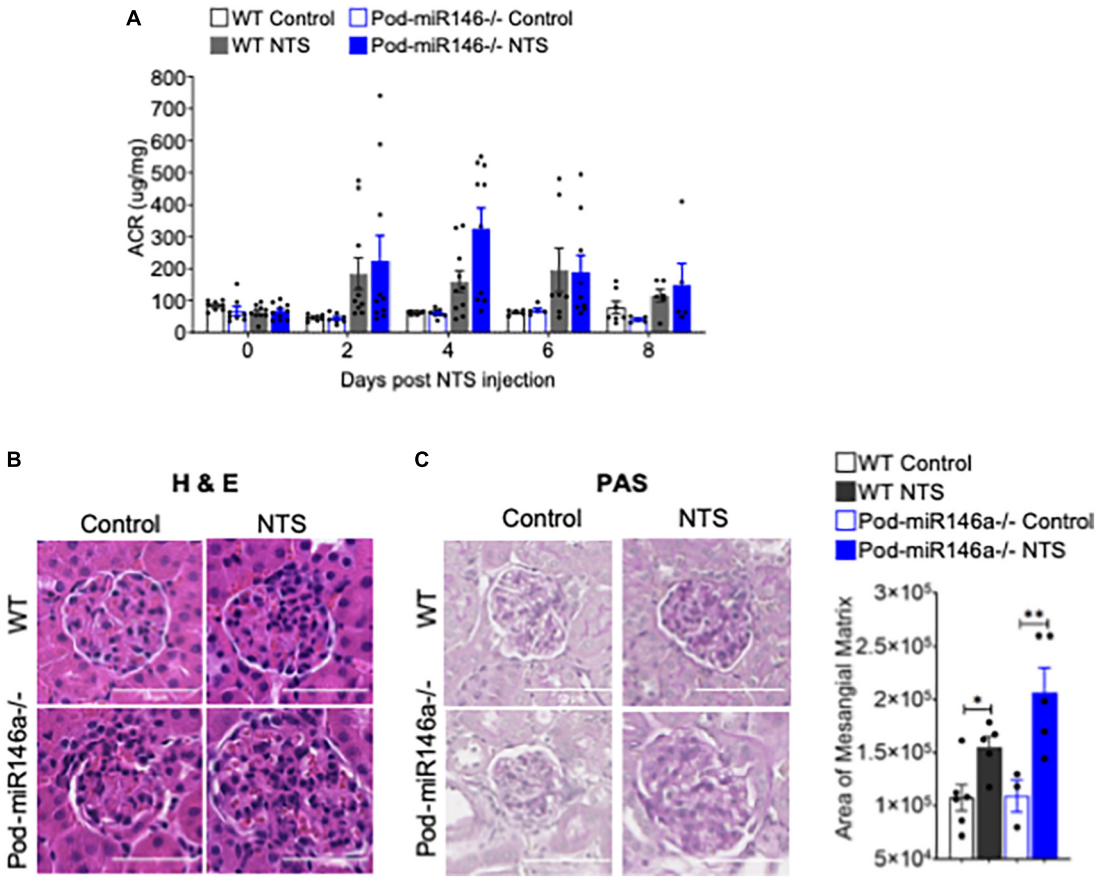

FIGURE 3
Increased albuminuria in Pod-miR-146a$^{-/-}$ mice after NTS treatment. **(A)** Graph showing the albumin to creatinine ratio (μg/mg) in the urine of WT (gray bars) and Pod-miR-146a (blue bars) mice post administration of either vehicle (open bars) or NTS (shaded bars) at various time-points, as indicated. Statistics were performed using two-way ANOVA. Data shown are mean ± SEM (n = 5). Representative images showing histochemical analyses with **(B)** H&E and **(C)** PAS staining of kidney tissues from each of the four groups of animals from 8th day post NTS treatment. Scale bar, 50 μm. Graph showing quantified mesangial matrix from the PAS-stained sections. Statistics were performed using the Mann-Whitney test. Data shown are normalized to the level of staining in control tissue and are mean ± SEM (n = 3). *$p < 0.05$; **$p < 0.01$.

pathway. Currently, in the absence of direct molecular evidence, it is unclear as to how blockade of ErbB4/EGFR *via* erlotinib suppresses Smad3 activation. We speculate that TGFβ, *via* an autocrine mechanism, elicits the EGFR-Smad3 pathway. Future studies are needed to further delineate the exact molecular mechanisms. Collectively, these data confirm that miR-146a in podocytes has a protective role and that its absence sensitizes podocytes to injury, *via* upregulation of ErbB4/EGFR pathway and induction of TGFβ signaling (**Figure 8**).

Several recent studies also highlight the role of miR-146a in protecting patients and animals from diabetic end-organ injury. A number of studies, including ours (18), show that reduced miR-146a levels are associated with injury. For example, serum miR-146a levels inversely and independently correlate with chronic complications of diabetes, including cardiovascular disease (CVD) and diabetic retinopathy, in a cohort of patients with type 1 diabetes (19). Similarly, serum miR-146a levels show significant age-related decline in type 2 diabetics and show inverse correlation with creatinine (40). Furthermore, miR-146a was also found to be present in urinary exosomes in hypertensive patients and its levels were reduced in patients with albuminuria (41). Similarly, miR-146a$^{-/-}$ mice showed age-dependent development of immune complex glomerulonephritis (42) and urinary exosomal miR-146a levels were significantly reduced in lupus nephritis patients and has been proposed as a disease biomarker (43). Conversely, there are other studies that suggest that increased miR-146a expression is associated with diabetic kidney injury (17, 44). However, all studies conclude that miR-146a has a protective role, albeit *via* a different mechanism, perhaps because it is widely expressed in many cell types and tissues, thus displaying multi-factorial effects.

Over-expression of miR-146a has also been shown to afford protection from diabetic kidney injury. STZ-induced diabetes in rats reduced expression of miR-146a in the kidney and the heart tissues, and exposure of endothelial cells

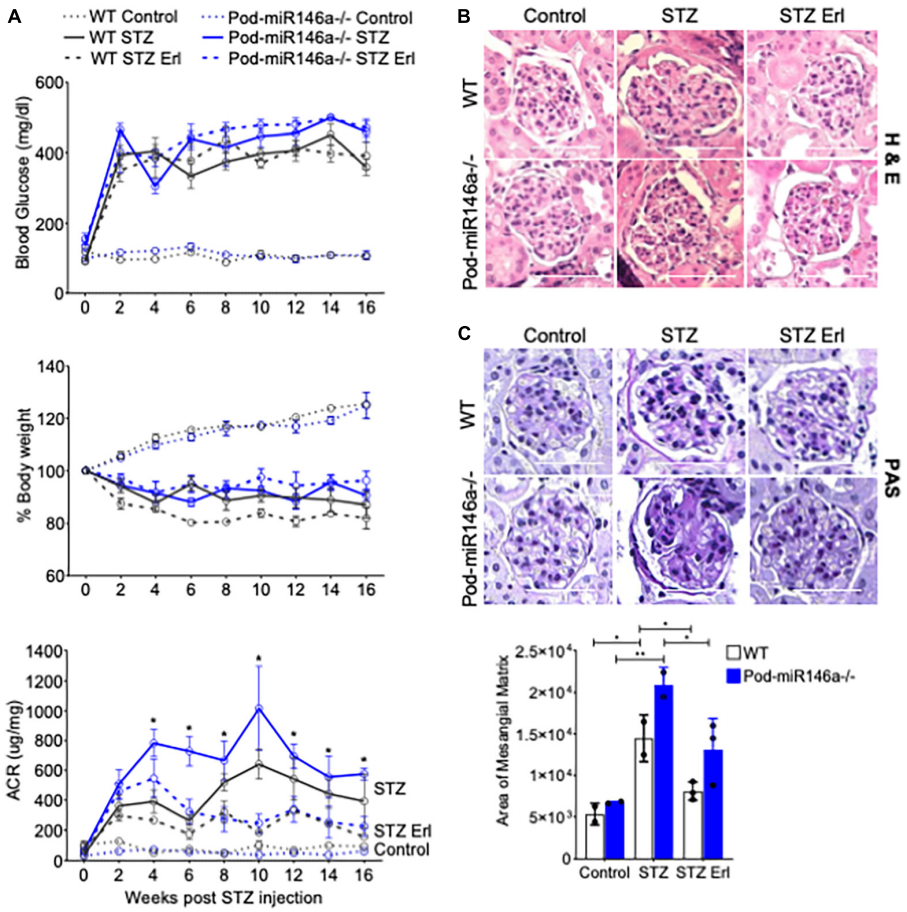

FIGURE 4
STZ accelerates glomerular injury in Pod-miR-146a$^{-/-}$ mice that is attenuated by erlotinib. **(A)** (Top) Graph showing levels of hyperglycemia in various animals, as measured by serum glucose levels (mg/dL) in each of the six groups, as indicated, post STZ administration and at various time-points, as indicated. Data shown are mean ± SEM (n = 5). Treatment with erlotinib starting at 4 weeks after STZ-induction did not result in any change in level of hyperglycemia in either strain. Blood glucose levels remained unchanged in the non-STZ treated mice; (Middle) Graph showing levels of weight loss upon in various animals in each of the six groups, as indicated, post STZ administration and at various time-points, as indicated. Data shown are mean ± SEM (n = 5). WT and Pod-miR146a animals displayed equal levels of weight loss upon STZ-induced hyperglycemia that was unaffected by treatment with erlotinib; (Bottom) Graph showing the albumin to creatinine ratio (μg/mg) in the urine of mice from each of the six groups, as indicated, post STZ administration and at various time-points, as indicated. Statistics were performed using one-way ANOVA. Data shown are mean ± SEM (n = 5). *$p < 0.05$. **(B)** Representative images showing histochemical analyses with H&E staining of kidney tissues from each of the six groups of animals at end point. Scale bar, 50 μm. **(C)** Representative images showing histochemical analyses with PAS staining of kidney tissues from each of the six groups of animals at end point. Scale bar, 50 μm. Graph showing quantified mesangial matrix from the PAS-stained sections. Data shown are normalized to the level of staining in control tissue and are mean ± SEM (n = 3). *$p < 0.05$; **$p < 0.01$.

to high glucose downregulated miR-146a in these cells (20). Furthermore, intravitreal administration of miR-146a mimic restored retinal miR-146a levels. STZ-induced hyperglycemia produced down-regulation of miR-146a in the kidneys of WT mice and resulted in increased kidney and retinal injury, and transgenic mice overexpressing miR-146a in endothelial cells are protected from diabetic retinal and kidney injury (45). Collectively, the published studies and our studies presented here show that miR-146a plays an important, homeostatic role in protecting kidney from injury and that such protection is mediated *via* many different cells present in the kidney. Using the podocyte-specific miR-146a deleted animals, we conclusively show that podocyte-expressed miR-146a is protective.

Materials and methods

Materials

Cell culture reagents were purchased from Thermo Fisher Scientific (Waltham, MA). Rat tail collagen I was purchased from Sigma (St. Louis, MO) and mouse recombinant interferon-γ was obtained from Cell Sciences (Canton, MA).

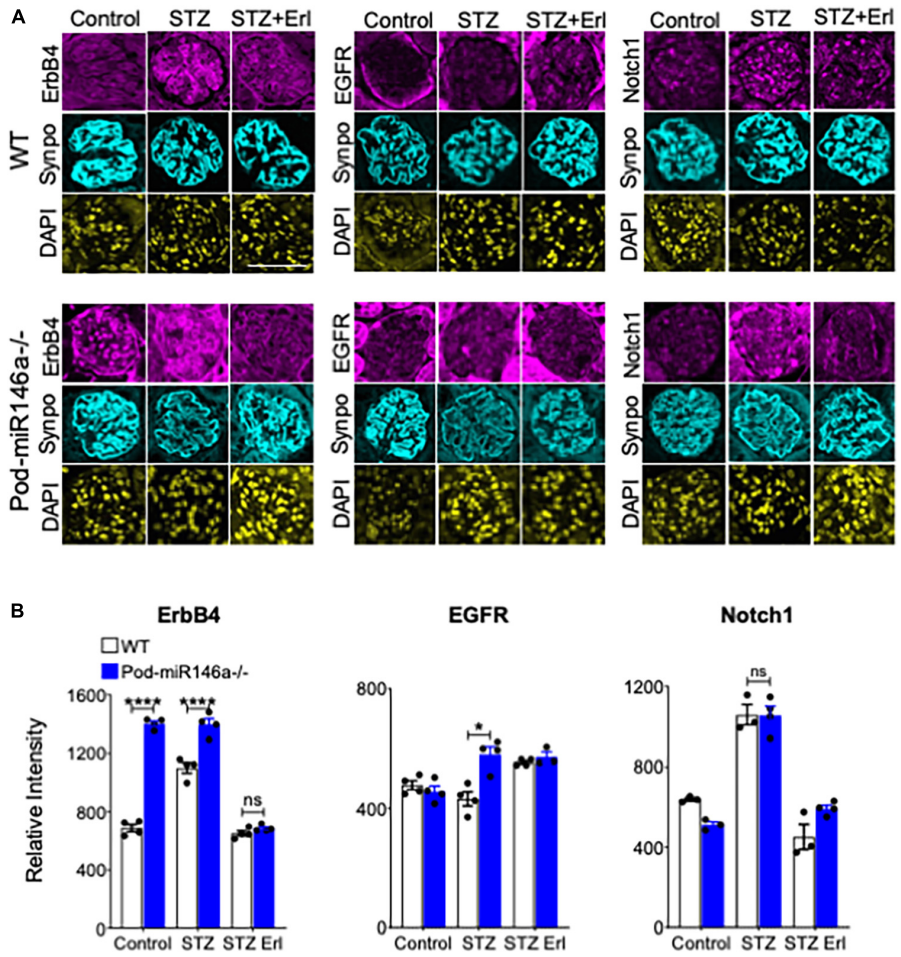

FIGURE 5
Immunofluorescence imaging-based analyses of glomerular sections shows erlotinib administration protects WT and Pod-miR-146a$^{-/-}$ mice from STZ injury via reduction in ErbB4 and EGFR. **(A)** Representative confocal microscopy images of immunofluorescently labeled glomeruli from WT (top three panels) and Pod-miR146a (bottom three panels) mice treated with vehicle alone (Control), with STZ and vehicle (STZ) or with STZ and erlotinib (STZ Erl). Tissue sections were imaged after staining with DAPI (nuclear marker) and antibodies against ErbB4, EGFR, Notch-1 and Synaptopodin (Synpo, podocyte marker) (as indicated). Scale bar, 50 μm. **(B)** Bar graphs showing quantification of relative glomerular signal intensity of ErbB4, EGFR and Notch-1 in tissue samples from A. Statistics were performed using two-way ANOVA. Data shown are mean ± SEM (n = 5/group). *$p < 0.05$; ***$p < 0.001$; ns, no significant difference.

Erlotinib was purchased from LC Laboratories (Woburn, MA). The polyclonal goat anti-synaptopodin antibody (P-19) and rabbit anti-ErbB4 antibody (C-18) were purchased from Santa Cruz (Santa Cruz, CA), rabbit anti-EGFR (06-847) from Millipore (Darmstadt, Germany), polyclonal rabbit anti-Notch1 (100-401-407) from Rockland (Limerick, PA), rabbit anti-podocin antibody from Sigma, anti-cleaved-Notch1 antibody (Val-1744) and anti-GAPDH (6C5) antibodies were from Abcam (Cambridge, MA), the anti-EGFR antibody (D38B1), anti-p-EGFR (Tyr-845) antibody, anti-p-EGFR (Tyr-1068) antibody (D7A5), anti-p-EGFR (Tyr-1173) antibody (53A5), anti-p-ErbB4 antibody (Tyr-984), anti-ERK antibody (137F5), anti-p-ERK antibody (Thr-202/Tyr-Y204), anti-p38 antibody, anti-phospho-p38 (Thr-180/Tyr-182) antibody (28B10), anti-Smad2/Smad3 antibody, anti-p-Smad2 (Ser-465/467)/Smad3 (Ser-423/425) antibody (D27F4), and anti-TGF-β1 antibodies were purchased from Cell Signaling (Danvers, MA).

Animals

Animal care and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Rush University Medical Center and were performed in accordance with institutional guidelines. The C57BL/6J wild-type (WT), and miR-146a$^{fl/fl}$ (034342) mice (21) were purchased from the Jackson Laboratory (Bar Harbor, ME). Flp recombinase and Podocin-Cre mice were generously provided by the laboratory of Dr. Jochen Reiser (Rush University Medical Center).

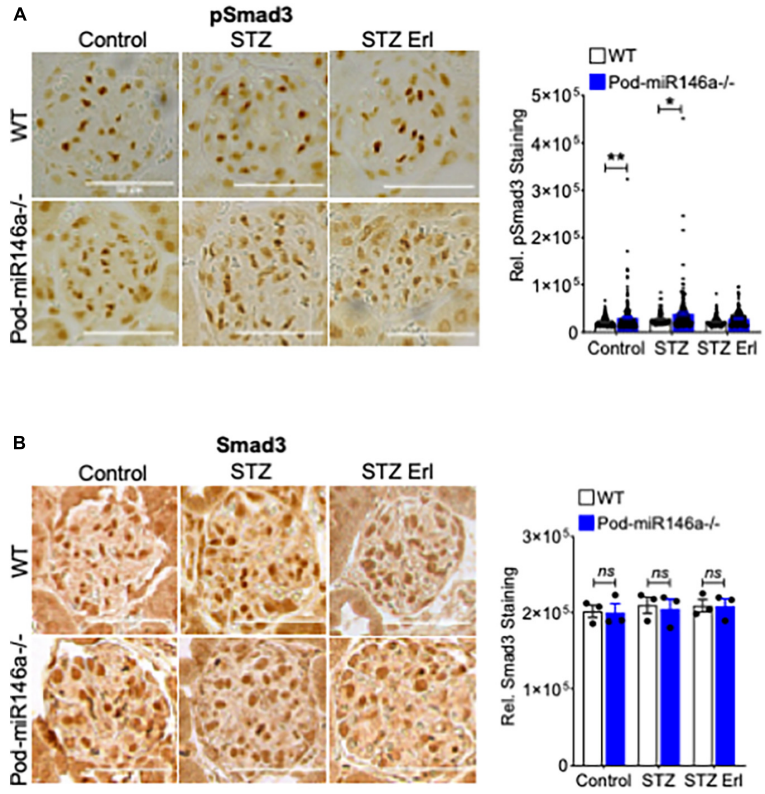

FIGURE 6
Increase in glomerular pSmad3 levels by STZ treatment is suppressed by erlotinib. **(A)** Representative immunohistochemical images of glomeruli stained with an antibody against pSmad3 from WT (top row) and Pod-miR146a mice (bottom row) treated with vehicle alone (Control), with STZ and vehicle (STZ) or with STZ and erlotinib (STZ Erl). Scale bar, 50 μm. Graph on the right shows quantification of pSmad3 positive cells per glomeruli in these samples. Statistics were performed using two-way ANOVA. Data shown are mean ± SEM (n = 3). *$p < 0.05$, **$p < 0.01$. **(B)** Representative immunohistochemical images of glomeruli stained with an antibody against total Smad3 from WT (top row) and Pod-miR146a mice (bottom row) treated with vehicle alone (Control), with STZ and vehicle (STZ) or with STZ and erlotinib (STZ Erl). Scale bar, 50 μm. Graph on the right shows quantification of total Smad3 positive cells per glomeruli in these samples. Statistics were performed using two-way ANOVA. Data shown are mean ± SEM (n = 3). ns, no significant difference.

Generation of podocyte specific miR-146a deleted mice (Pod-miR146a$^{-/-}$) was achieved by first crossing miR-146a$^{fl/fl}$ mice with Flp recombinase expressing mice to delete the selection cassette. Subsequently, the homozygous progeny were crossed with Podocin-Cre recombinase-expressing mice to delete miR-146a selectively in podocytes (22) (**Figure 1A**). Knock-out of miR-146a were confirmed by genotyping.

Blood glucose measurement and urinary albumin and creatinine assays

Blood glucose measurement and urinary albumin and creatinine assays were performed as described previously (18). Briefly, blood glucose was measured from tail vein blood by using a FreeStyle Freedom lite glucometer (Abbott, Abbott Park, IL). For urinary albumin and creatinine measurements, spot urine samples were collected non-invasively from mice. Urinary albumin and creatinine concentrations were measured using a mouse albumin ELISA (Bethyl Laboratories, Montgomery, TX) and a creatinine assay (Exocell, Philadelphia, PA), respectively. Subsequently, protein concentrations and urine albumin-to-creatinine ratios were calculated.

Primary podocyte isolation

Primary mouse podocytes were isolated as previously published (18). Briefly, mice were anesthetized and perfused with HBSS containing DynaBeads (Invitrogen, #14013). Mouse kidneys were collected from 8- to 16-week-old mice and minced into small pieces and placed in digestion buffer containing collagenase A (Roche) and DNase I (Sigma) and incubated shaking for 30 min at 37°C. After passing the digested kidneys twice through a 100 μm cell strainer (Thermo Fisher Scientific), they were spun down (200 × g for 5 min). After resuspending the beads (attached to kidney glomeruli), they were magnetically separated out of solution, washed, counted, and placed in a

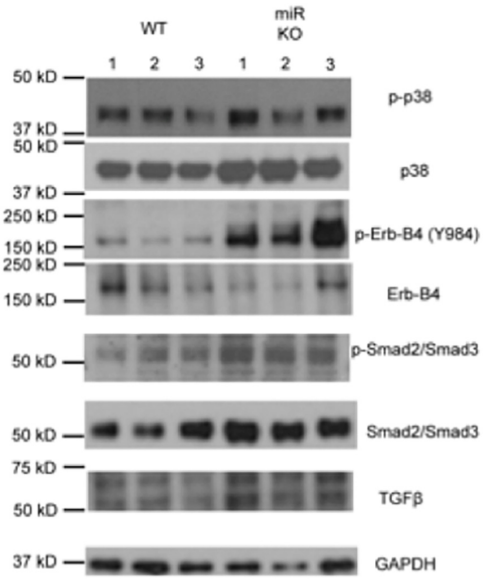

FIGURE 7
Deletion of miR146a in podocytes upregulates ErbB4/TGFβ /Smad3 signaling. Immunoblot analysis of various phosphorylated (p-) and total proteins in the lysates from primary podocytes isolated from the WT and Pod-miR146a mice. Data presented is from three independent samples from each group. GAPDH was used as the loading control. Relative position of the molecular weight markers is shown on the left.

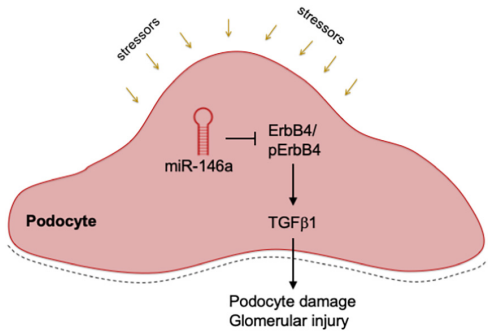

FIGURE 8
Mechanistic model. A diagram showing a mechanistic working model. Podocyte expressed miR-146a represses expression of ErbB4 and Notch-1 during homeostatic conditions, thereby controlling the ErbB4/EGFR and TGFβ 1 signaling pathways. Various external stressors or deletion of miR-146a result in de-repression of ErbB4 and Notch-1, thereby driving the harmful ErbB4/EGFR signaling and inducing TGFβ 1. An autocrine feed-forward loop via TGFβ 1 induces the downstream TGFR/Smad3 signaling, that result in podocyte damage, glomerular injury and proteinuria.

collaged-coated petri dish with RPMI 1640 medium containing 10% FBS. Podocytes were allowed to grow in culture for 4–5 days before use in downstream analyses (e.g., lysed in Trizol for RNA analysis).

Immunofluorescence

Immunofluorescence staining of EGFR, Notch-1, ErbB4 and synaptopodin mouse kidney tissues were performed as described previously (18). Briefly, mouse kidney tissues from the different treatment groups from WT and Pod-miR146$^{-/-}$ mice were fixed in formalin and embedded in paraffin for further processing. Tissue sections of 4 μm thickness were deparaffinized and hydrated through xylenes and graded alcohol series before antigen retrieval with citrate buffer (Vector Labs, #H-3300). Sections were incubated in 0.2% Triton-X for 10 min at RT, washed, and incubated with the blocking buffer (4% FBS, 4% BSA and 0.4% fish gelatin in PBS) for 1 h at RT. For EGFR, Notch-1 and ErbB4 staining, sections were then incubated with primary antibodies—Rabbit anti-mouse EGFR (Millipore, #06847), mouse anti-mouse ErbB4 (Santa Cruz, #sc-8050) and Rabbit anti-mouse Notch-1 (Rockland, #100-401-407) in the blocking buffer at 4°C, overnight. After incubation, sections were washed and incubated with 0.3% hydrogen peroxide for 30 min at RT to block endogenous peroxidase activity. Sections were then incubated with blocking buffer containing HRP labeled secondary antibody polymer (Vector Labs, #MP7401 or #MP-7402) for 30 min at room RT. Sections were washed, incubated with TSA reagent (Akoya Biosciences, #NEL744001KT) for 10 min, washed and stained with DAPI for 3 min at RT. For synaptopodin staining, serial sections were processed and blocked as described earlier. Sections were then incubated with anti-synaptopodin antibody (Santa Cruz, #sc-515482) and incubated overnight at 4°C. Sections were subsequently washed and incubated with goat anti-mouse AF488 (Thermo Fisher Scientific, #A-11001). Glomeruli from the stained tissues were imaged with a Zeiss 700 LSM confocal microscope (Zeiss) and the images were quantified and analyzed using Image J software (NIH, Bethesda, MD).

Tissue histochemical staining

Mouse kidneys were harvested after PBS wash. One section of the removed kidney was fixed in 10% formalin and embedded in paraffin and another part was immediately snap frozen in OCT embedding compound on liquid nitrogen and stored at 80°C. Paraffin-embedded sections (4 μm) were stained with hematoxylin-eosin (H&E), periodic acid-Schiff (PAS), or Masson's trichrome. Tissue processing, including fixation, dehydration, embedding, and histochemical staining was performed at University of Illinois at Chicago (UIC) Research Histology and Tissue Imaging Core. Stained slides were blindly evaluated by an experienced pathologist and scanned using Aperio software (Leica, Buffalo Grove, IL). Fibrosis was indicated as a percent of tissue area stained blue with Masson's trichrome. Quantification for glomerular volume and mesangial expansion was performed according to published

methods using ImageJ software (NIH, Bethesda, MD) (46). Glomerular expression of pSmad3 and Smad3 was analyzed by regular immunohistochemical staining using pSmad3 antibody 1:200 dilution, (Abcam, 52903) and Smad3 antibody 1:200 dilution (Proteintech, 25494-1-AP). Briefly, tissue sections were deparaffinized in xylene and rehydrated through descending concentrations of ethanol and subjected to antigen retrieval by steam heating in an acidic pH solution (Citrate-based, Vector Laboratories). Sections were incubated in 0.3% hydrogen peroxidase in water for 30 min and were blocked (4% FBS, 4% BSA, 0.4% fish gelatin) at room temperature for 1 h. Subsequently, sections were incubated with primary antibodies at 4°C overnight, followed by washing and incubation with appropriate secondary HRP-labeled secondary antibody polymer (Vector Labs) for 30 min at room temperature. Signal development was accomplished by using the DAB substrate kit (Vector Labs). pSmad3 and Smad3 quantification was done by counting marker positive cells in four independently, randomly chosen areas analyzed at 20x using a Zeiss 700 LSM confocal microscope and the images were quantified and analyzed using Image J software (NIH, Bethesda, MD) (46).

Western blotting

Primary mouse podocytes were isolated according to published protocols (18, 47). Briefly, kidneys were collected from 8 to 12 week old mice, mashed with cold PBS and sequentially passed through test sieves (Retsch, Newtown, PA) with pore diameters of 180, 75, and 52 μm, respectively. Glomeruli from the sieve of 52 μm pores were collected in PBS, spun down and resuspended in RPMI 1640 medium containing 10% FBS. Cells were plated on collagen I coated plates for 14 days. Subsequently, the adherent cells were trypsinized and filtered with 40 μm strainer. Filtered cells were spun down and seeded on collagen I coated 6-well plates. Subsequently, cells were washed with ice-cold PBS and lysed using cold lysis buffer (RIPA containing EDTA and EGTA, Boston Bioproducts, Ashland, MA) supplemented with protease inhibitor (Roche Life Science, Indianapolis, IN) and phosphatase inhibitor (Sigma). Cell lysates were incubated on ice for 30 min (with intermittent vortexing) and centrifuged using a tabletop microcentrifuge at 13,000 rpm for 15 min at 4°C. Supernatants were carefully transferred to new microcentrifuge tubes and protein concentrations were determined by Bradford assay (Bio-Rad). Equal amounts of protein from each sample were loaded to NuPAGE Novex 4–12% BisTris gels (Life Technologies) and transferred to an Immobilon-P PVDF membrane (EMD Millipore, Billerica, MA). The membranes were blocked with 5% BSA (Thermo Fisher Scientific) in TBS/Tween-20 (0.05%) (Boston Bioproducts, Ashland, MA). Membranes were then incubated with primary antibodies at 4°C overnight. Membranes were washed with TBS containing Tween 20, followed by incubation with a secondary antibody conjugated to horseradish peroxidase (Promega) for 1 h at room temperature. Blots were developed with Super- Signal West Pico chemiluminescent substrate (Thermo Fisher Scientific) and captured using X-ray films (Kodak, Rochester, NY) using an AX-700LE film processor (Alphatek, Houston, TX).

Quantitative RT-PCR

MicroRNA gene expression analysis was performed using quantitative reverse transcription polymerase chain reaction (qRT-PCR) as previously described (18). Briefly, total RNA or miRNA fractions were isolated from mouse kidney tissue or from mouse podocytes using miRNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer provided protocol and total RNA concentration was measured using NanoDrop (Thermo Fisher Scientific). Isolated RNA (0.5–2.0 μg) was used as template for cDNA synthesis using a TaqMan ^{TM} Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific). qRT-PCR was performed using CFX96 Real-time System (Bio-Rad) and the following TaqMan Gene expression assays (Thermo Fisher Scientific) were used: miR-146a (A25576, ID: 478399_mir), miR361 (A25576, ID: 478056_mir), and Gapdh (4331182, ID: Mm99999915_g1). For analysis, the fold-change in mRNA levels between various groups was determined after normalizing each mRNA expression level with Gapdh [$2^{(-\Delta\Delta Ct)}$ method]. The fold-change in miR-146a levels between various groups was determined after normalizing the miR-146a expression level in each group with levels of miR361.

Lipopolysaccharide-induced proteinuria mouse model

A model of transient proteinuria was used in the wild type C57B/L6 and the Pod-miR-146a$^{-/-}$ mice according to published protocols (48). Briefly, WT and Pod-miR146a$^{-/-}$ male mice (n = 5) were divided into two groups and given intraperitoneal (i.p.) injection of phosphate-buffered saline (PBS) or lipopolysaccharide (LPS) at 5 mg/kg (Sigma-Aldrich, #L3024). Spot urine samples were collected at 0, 24, and 72 h post i.p. injection for evaluation of albumin to creatine ratio (ACR). Some animals were sacrificed at 24 h after LPS injection for histologic analysis of kidney tissue. These data are representative of 3 independent experiments.

Streptozotocin-induced hyperglycemia

A type I diabetes model of inducing hyperglycemia was used in the wild type C57B/L6 and the Pod-miR-146a$^{-/-}$

mice according to published protocols (18, 25). Briefly, 8–10-week-old male mice were administered two doses of streptozotocin (STZ, 125 mg/kg body weight) (Sigma) in 50 mM sodium citrate buffer, pH 4.5, intraperitoneally (i.p.) on days 1 and 4. Glucose levels from tail blood were measured with an AccuCheck glucometer (Roche Life Science). Animals with glucose levels >400 mg/dl on two consecutive measurements were regarded as hyperglycemic. The mice received no insulin during the study period. Urinary albumin and creatinine were analyzed prior to STZ administration and subsequently at intervals shown in the graph. Erlotinib (37 mg/kg) was prepared in saline solution containing 1% Tween-20, 25% Kolliphor, and 2.5% DMSO and was administered intraperitoneally every other day starting at week 4 post-STZ to a group of mice, according to literature protocols (31, 49, 50). Kidneys were harvested and processed for histological and ultrastructural analyses after 16 weeks post-STZ administration.

Statistical analysis

Data were analyzed using Excel (Microsoft, Redmond, WA) and Prism (GraphPad, San Diego, CA) softwares and data were analyzed using one-way ANOVA, two-way ANOVA, or Student's *t*-test, where appropriate. $p < 0.05$ was considered statistically significant.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) at Rush University Medical Center.

Author contributions

VG managed the project and coordinated author activities. XL, IV, HW, and TG designed and performed experiments including *in vivo* assays. AR, VV, and HF helped with tissue acquisition and analyses. XL, RWH, and MA performed western blot analyses. XL, HW, and MA performed statistical analysis. VG, TG, and MA co-wrote the manuscript with input from all authors.

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Conflict of interest

VG was an inventor on patent applications related to these studies. AR recently completed her post-doctoral fellowship and was employed by Genentech.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.897188/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Representative confocal microscopy images of immunofluorescently labeled glomeruli from WT (top panels) and Pod-miR146a (bottom panels) mice treated with vehicle alone (Control), with STZ and vehicle (STZ) or with STZ and erlotinib (STZ + Erl). Tissue sections were imaged

after staining with DAPI (nuclear marker, blue) and antibodies against ErbB4 (red) and Synaptopodin (green). Colocalization between ErbB4 and synaptopodin is apparent as yellow. Scale bar, 50 μm.

SUPPLEMENTARY FIGURE 2
Representative confocal microscopy images of immunofluorescently labeled glomeruli from WT and Pod-miR146a mice treated with vehicle alone (Control), with STZ and vehicle (STZ) or with STZ and erlotinib (STZ + Erl). Tissue sections were imaged after staining with DAPI nuclear marker) and anti-Synaptopodin antibody (Synpo, podocyte marker) (as indicated) to identify and manually count podocytes. Scale bar, 50 μm. **(B)** Bar graphs showing quantification of podocyte numbers per glomerular section from at least five glomeruli from three independent samples per group. Data shown are mean ± SEM. Statistics were performed using two-way ANOVA. *$p < 0.05$; ****$p < 0.001$; ns, no significant difference.

SUPPLEMENTARY FIGURE 3
Original immunoblot images for the data presented in **Figure 7**.

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