CELL CROSS-TALK IN DIABETIC KIDNEY DISEASES

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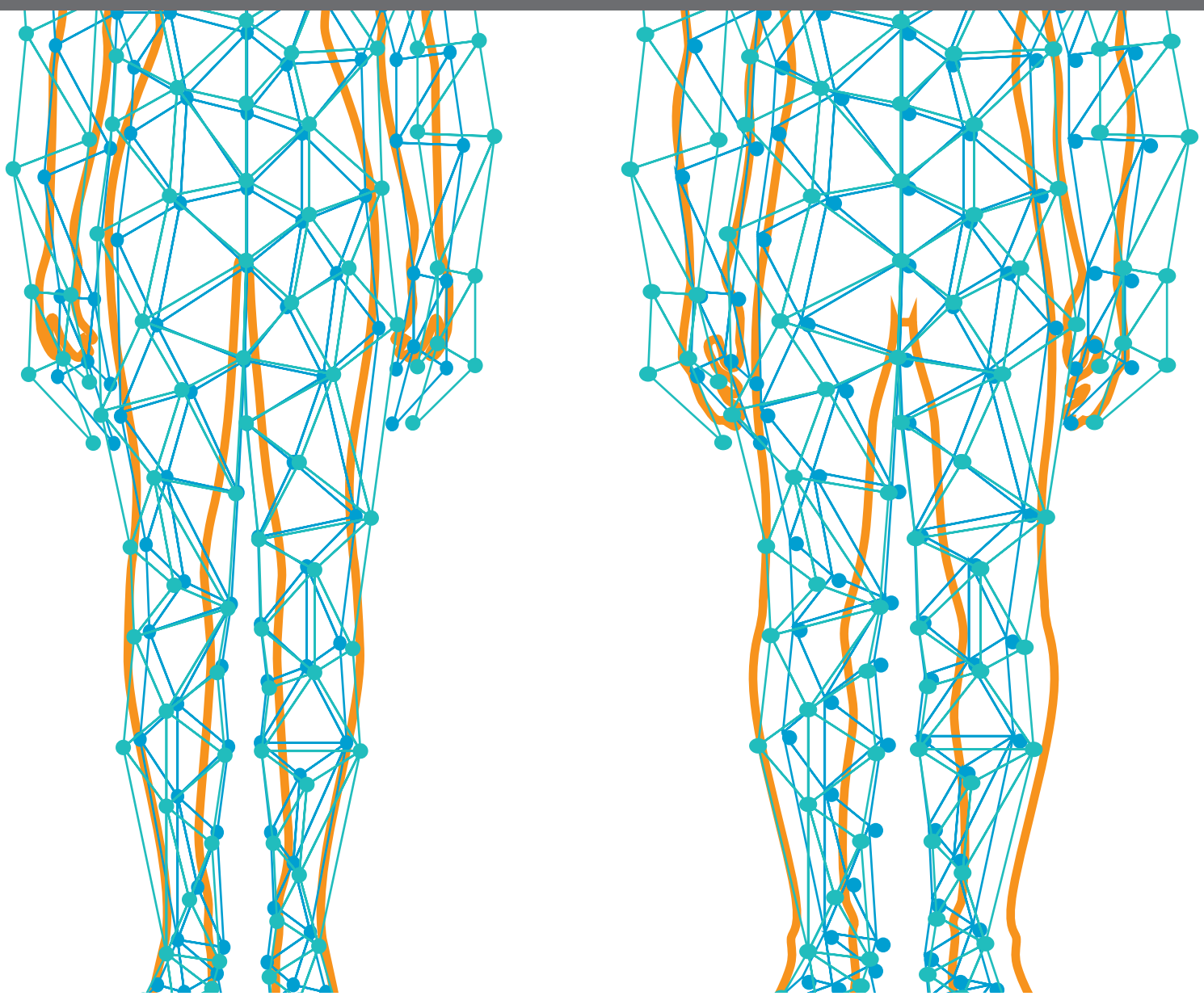

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CELL CROSS-TALK IN DIABETIC KIDNEY DISEASES

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EDITORIAL
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Editorial: Cell Cross-talk in Diabetic Kidney Diseases

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Keywords: diabetic nephropathy, cell crosstalk, renal fibrosis, diabetic kidney disease, chronic kidney disease

Editorial on the Research Topic

Cell Cross-talk in Diabetic Kidney Diseases

Diabetic kidney disease (DKD) is the leading cause of new-onset end-stage renal disease (ESRD). Although the development of clinical therapy for DKD has made great progress, the progression of DKD still cannot be controlled. Therefore, further study of the pathogenesis of DKD and improvements in DKD treatment are crucial for prognosis. Here, there are evidences suggest the cell crosstalk in the pathogenesis of DKD could provide mechanistic clues that underlie DKD and provide novel avenues for therapeutic intervention.

Liu et al. applied secreted protein comparison and verification experiments indicated that WFDC2 from the tubule could downregulate PEX19 levels at the glomeruli in diabetic kidney disease (DKD). This study revealed the distinctive crosstalk pathways of the tubules and glomeruli and identified interacted genes during kidney disease progression. Feng et al. demonstrated HIF-1α/Notch1 pathway of M1 macrophage could be activated by endothelial cell dysfunction in DKD mouse, and PPAR-α agonist fenofibrate had the protective effect on DKD by reducing M1 macrophage recruitment via inhibiting HIF-1α/Notch1 pathway (Liu et al.). Li Q. et al. uncovered S-nitrosylation of Myo9A, actin, and RhoA as an integrated signaling crosstalk that reversibly transduces metabolic cues to regulate actin dynamics and podocyte motility in DKD (Feng et al.). It suggested that dysregulation of the signal axis may contribute to the pathogenesis of advanced DKD and may be amenable to therapeutic targeting.

During diabetic nephropathy, endothelial cells, and podocytes are stressed and damaged. Besides, each can communicate with the other, directly affecting the progression of glomerular injury. Glomerular ECs are crucial actors of DKD pathophysiology, and cross-communications with podocytes constitute major events for diabetic renal disease progression. Mahtal et al. emphasized new treatments that aim to prevent microvascular injury or restore microvascular function could be an effective strategy for preventing; or even reversing DKD.

Single-cell RNA sequencing (scRNA-seq) technology provided new insight into cellular heterogeneity and genetic susceptibility regarding DKD at cell-specific level. Based on scRNA-seq it is enable a much deeper understanding of cell-specific processes such as interaction between cells.

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Du et al. highlighted scRNA-seq research on intra- or extra-glomerular cell crosstalk and cellular targets for DKD (Li T. et al.), including crosstalk between podocyte and GEC, podocyte and parietal epithelial cell (PECs), glomerular mesangial cell (GMC), and other glomerular cell types. In addition, Li T. et al. identified a subgroup of glomerular endothelial cells with pro-angiogenesis characteristics in DKD using an online single-cell RNA profile (Wei et al.). Also, immune cells such as macrophages, T lymphocytes, B lymphocytes, and plasma cells contribute to the disease progression. There is a complicated cellular crosstalk inside glomeruli. Dysfunction of glomerular endothelial cells and immature angiogenesis result from the activation of both paracrine and autocrine signals. Based on snRNA-seq data of DKD (He et al.), Wei et al. revealed cell-to-cell interactions via integrin pathways are increased, mesangial cells are stimulated and glomeruli-tubular communication is strongly enhanced in DKD progression. This work found the level of glomerular FGF1 is positively associated with the level of GFR, while the levels of glomerular NRP1, tubular COL4A1, and tubular NRP1 are negatively associated with the level of GFR. This study furthers our understanding of cell cross-talk in DKD and reveals novel mechanisms, new biomarkers, and potential therapeutic targets to benefit patients.

Recent studies have shown that ncRNAs play an important role in the occurrence and development of DKD and participate in the regulation of oxidative stress in DKD. He et al. summarized the functions and mechanisms of ncRNAs in DKD-related oxidative stress (Xu et al.). These ncRNAs would play a pivotal role in the cell crosstalk of DKD progression. Quercetin antagonizes glucose-induced renal injury by suppressing aerobic glycolysis via HIF-1α/miR-210/ISCU/FeS pathway in mesangial cells [9].

In summary, contents of our topic provided valuable insights into cell crosstalk in DKD. Effective strategy for preventing; or even reversing DKD, may consider crosstalk within the glomerular or/and tubular system.

AUTHOR CONTRIBUTIONS

QH prepared the draft. CH and FY revised it. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Integrative Informatics Analysis of Transcriptome and Identification of Interacted Genes in the Glomeruli and Tubules in CKD

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Chronic kidney disease (CKD) is a complex disease in which the renal function is compromised chronically. Many studies have indicated the crosstalk between the tubule and the glomerulus in CKD progression. However, our understanding of the interaction of tubular and glomerular injury remains incomplete. In this study, we applied a meta-analysis approach on the transcriptome of the tubules and glomeruli of CKD patients to identify differentially expressed genes (DEGs) signature. Functional analysis of pathways and Gene Ontology found that tubular DEGs were mainly involved in cell assembly and remodeling, glomerular DEGs in cell proliferation and apoptosis, and overlapping DEGs mainly in immune response. Correlation analysis was performed to identify the associated DEGs in the tubules and glomeruli. Secreted protein comparison and verification experiments indicated that WFDC2 from the tubule could downregulate PEX19 mRNA and protein levels at the glomeruli in diabetic kidney disease (DKD). This study revealed the distinctive pathways of the tubules and glomeruli and identified interacted genes during CKD progression.

Keywords: informatics analysis, CDK, DKD, glomeruli tubule, crosstalk

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INTRODUCTION

Chronic kidney disease (CKD) affects between 8 and 16% of the population worldwide and is often underrecognized by patients and clinicians (1, 2). Diabetic kidney disease (DKD) is the leading cause of CKD and is the single strongest predictor of mortality in patients with diabetes (3). In recent years, although the development of clinical therapy for DKD has made great progress, the progression of DKD still cannot be controlled (4). Therefore, more detailed study of CKD-associated mechanisms is needed to fully understand its clinical relevance and underlying pathophysiology, which is critical to identify predictors of the disease course and therapeutic targets.

Traditional studies have identified multiple individual factors involved in the pathogenesis of CKD (5–7). However, these candidate gene approaches have limited value toward the full understanding of the molecular mechanisms of these diseases. Recent studies have provided us new insights into the crosstalk between tubular and glomerular segments (8). Glomerulosclerosis

with resulting ischemia to the downstream tubules causes tubulointerstitial fibrosis (9). Tubulointerstitial injury may also lead to increased glomerular injury (10). A sequential tubular–glomerular injury model found that even mild preexisting tubulointerstitial injury sensitized the glomeruli to subsequent podocyte-specific injury (11). Many studies also indicated that tubular epithelial cells (TECs) and glomerular endothelial cells (GECs) can crosstalk with each other in the development of DKD. Studies have shown that TECs inflammatory response (12), Ang-1/Ang-2 Tie2 (13), and VEGF/VEGFR axis (14) contribute to the injury of GECs, whereas Kruppel-like factor (KLF) (15), HGF/c-MET (16), and IGFBPs (17) mediate injury from GECs to TECs. Improving injury and maintaining normal crosstalk between them may become a new strategy for the prevention and treatment of kidney diseases in the future.

In this study, we applied a meta-analysis and correlation analysis to identify genes and pathway signatures for the tubule and glomerulus and novel genes crosstalk between them. We used a meta-approach on CKD patients vs. healthy donors to identify differentially expressed genes (DEGs) signature. Functional analysis of pathways and Gene Ontology (GO) was performed to identify overlapping and distinguish pathways for the tubule and glomerulus in CKD. Correlation analysis was also performed with gene expression in both the tubule and glomerulus tissues to obtain the interaction genes. Secreted proteins were compared with the interaction gene pairs, and we identified that WFDC2 and PEX19 could be interacted from the tubules and glomeruli within the pathophysiological progression of CKD.

METHODS

Data Collection

Publicly available human microarray and next-generation sequencing datasets for all kidney diseases [lupus nephritis (LN), diabetic nephropathy (DN), focal segmental glomerulosclerosis (FSGS), membranous nephropathy (MN), IgA nephropathy (IgAN), and minimal change disease (MCD)] were obtained from Nephroseq (https://www.nephroseq.org/) and PubMed and downloaded from GEO (**Supplementary Table 1**). All the transcriptome data were downloaded from GEO, and the accession numbers were listed in **Supplementary Table 1**. We collected eight datasets for kidney diseases, which included high-throughput transcriptome data for 508 disease and control samples. Each dataset manually selected the samples with clinical information. There are two datasets that contained the transcriptome data for tubule tissue, two datasets for glomerular transcriptome, and four datasets for both tubule and glomerulus data for the same patient (**Supplementary Table 1**). For each study, we grouped the samples with the clinical and phenotypic information reported by the corresponding original studies. Then, for the raw microarray data, we performed quality assessment, and all the microarray platform data were re-annotated to the most recent NCBI Entrez Gene Identifiers (Gene IDs) by AILUN (http://ailun.ucsf.edu) (18). All the expression values were base-two log-transformed and normalized by quantile–quantile normalization.

Meta-Analysis

Meta-analysis methods were described in our previous paper (19). Briefly, we used two meta-analysis methods effect sizes and combining significance analysis of microarrays (SAM) q values to analyze all the transcriptome data. In the first method, we estimated the effect size and summarized the effect size with fixed effect inverse–variance model for all annotated genes in all datasets. We combined the study-specific effect sizes for each gene into one meta-effect size (f_{meta}) using a linear combination of effect sizes (fi) by weighting each effect size by the inverse of the variance (wi) in the corresponding study (19). We used false discovery rate (FDR) (20) to test the significant difference for each gene as FDR $\leq$5% was used for cutoff as significant. In the combining SAM (21) method, we used q <10% as cutoff for significantly expressed genes between healthy controls and CKD patients. Finally, for different datasets, we used Fisher's exact test to test whether the probability of obtaining was significant or not with $p \leq 0.05$ as cutoff.

Pathway Network, Generation, and Analyses

The DEGs for microarray and sequencing in kidney diseases compared with normal were identified by meta-analysis. Then, DEGs for the tubules and glomeruli were compared to obtain the unique and overlapping DEGs. We used two methods to perform gene enrichment analysis. DEGs with a fold change cutoff of $\geq$1.5 were used INGENUITY© IPA (www.ingenuity.com/products/ipa) and Enrichr (https://amp.pharm.mssm.edu/Enrichr/) for GO and pathways. The interaction of genes was visualized by Cytoscape (https://cytoscape.org/).

Crosstalk Between Tubular Cell and Podocyte in Disease Condition

Next, for the four datasets having both tubular and glomerular data from the same patient, we performed the gene expression correlation analysis with "pearson," "kendall," and "spearman" correlation coefficient methods. We identified specific correlated paired genes with correlation coefficient >0.7 and $p < 0.001$ as cutoff. Then, we compared the correlated DEG pairs and obtained 59 pairs of associated DEGs in all four datasets. The association between the tubules and the glomeruli was visualized with Cytoscape (http://www.cytoscape.org/). Then, we obtained the human secretome and membrane proteome list from Human Protein Atlas (www.proteinatlas.org) (22) and identified the secreted proteins in our associated gene pairs, which could be secreted and interacted with proteins in other cells.

Cell Culture, Real-Time PCR, and Western Blot

HK2 cell (ATCC CRL-2190) and glomerular epithelial cell (ATCC CRL-192) obtained from ATCC were cultured in RPMI-1640 medium (Corning). Human podocyte cell line (23) was cultured in RPMI-1640 medium (Corning) containing 10% fetal bovine serum (FBS; Corning) supplemented with 1% Insulin–Transferin–Selenium-A liquid media (Life

Technologies) and 100 U/ml penicillin. Cultures were incubated at a 33°C humidified incubator and transferred at 37°C for differentiation (23). Expression-ready lentiviral constructs for WFDC2 overexpression were purchased from Horizon Inspired Cell Solutions (MHS6278-202801004, Clone Id: 5186932, MGC Human WFDC2 Sequence-Verified cDNA). Negative control overexpression pCMV-SPORT6 was used as a negative experimental control. Lentivirus for WFDC2 and control plasmids has been produced by HEK 293T cells. TRIzol reagent (Thermo Fisher Scientific) was used to extract RNA following the manufacturer's protocol for cultured cell and mice kidneys. Quantitative real-time PCR and $2^{-\Delta\Delta CT}$ method were performed to quantity the gene expression. For western blot, cultured cells and mice kidney tissues were lysed with lysis buffer with phosphorylation protease and protease inhibitor cocktails. The following antibodies were used: WFDC2 (rabbit monoclonal HE4/WFDC2, Catalog # NBP2-66883; Novus Biologicals), PEX19 (PEX19 monoclonal antibody (GT554), Catalog # MA5-17266; Invitrogen), and GAPDH (mouse monoclonal antibody, Catalog # G8795-100UL; Sigma).

STZ-Induced Diabetic Mice Model and Glomeruli Isolation

$eNOS^{-/-}$ mice were purchased from Jackson Laboratory, and streptozotocin (STZ)-induced model and glomeruli isolation were described previously (24). For induction of diabetes, 8 weeks old male $eNOS^{-/-}$ mice were injected low-dose STZ (Sigma-Aldrich) for 5 consecutive days at 50 μg/g intraperitoneally. The same age male CL-$eNOS^{-/-}$ mice injected with vehicle were used as non-diabetic controls. The diabetes group model was considered successful when the fasting blood glucose level was higher than 300 mg/dl after 10 weeks of STZ injection. The glomeruli and tubules were separated using Dynabead perfusion as described in a previous paper (25). Briefly, mice were perfused with phosphate-buffered saline (PBS) for 2 min then with prewarmed 8 ml bead solution in enzymatic digestion buffer (Collagenase type II 300 U/ml, Proteinase E 1 mg/ml, and DNase I 50 U/ml). The kidneys were chopped to 1 mm^3 pieces and digested at 37°C for 15 min in a digestion buffer with continued rotation. A 100-μm cell strainer was used to get rid of the undigested tissue debris then centrifuged at 200 g to obtain the tubules and glomeruli. The cell pellet was resuspended in Hanks' balanced salt solution, and the glomeruli were collected using a magnet. The rest of the non-glomeruli part was collected for the tubule part. The separated glomeruli and tubules were resuspended in Hanks' buffer for further cell lysis for quantitative PCR (qPCR) and western blot.

RESULTS

Meta-Analysis of Transcriptome Reveals Different Molecular Mechanisms for Tubular and Glomerular Tissues of CKD

We obtained transcriptome data from eight studies with both tubular and glomerular samples of renal CKD patients (**Supplementary Table 1**). We used the clinical information reported from the corresponding studies. Finally, transcriptome data of 508 samples of healthy control and CKD patients were used for further analysis. Preprocessing analysis was performed for gene annotation, quantile–quantile normalization (18). We applied two meta-analysis methods (see the Methods section, **Figure 1**) to obtain the DEGs in CKD vs. normal samples in tubular and glomerular tissues across multiple datasets. Based on two meta-analysis methods, we identified a total of 619 (for the glomeruli, **Supplementary Table 2**) and 1,824 (for the tubules, **Supplementary Table 3**) overlapped genes to be significantly different. Meta-effect size, meta-SAM q values, effect size for each dataset, and SAM q values for each dataset were shown in **Supplementary Tables 2**, **3**. Of these DEGs, there are 196 overlapping genes for tubular and glomerular samples, and 1,628 and 423 DEGs unique for tubular and glomerular samples (**Supplementary Table 4**).

To compare the DEGs functions for the tubules and glomeruli in CKD, we performed pathway and network analysis for the tubular and glomerular common and unique genes using QIAGEN's Ingenuity Pathway Analysis. We found that tubular and glomerular DEGs were involved in distinct pathways and GO terms (**Figure 2A**). The common DEGs in tubular and glomerular samples were mainly involved in immune response (**Figure 2B**), with GO terms of defense response, immune response, and response to wounding, highlighted in **Figure 2B** (**Supplementary Table 5**). The glomerular DEGs regulated cell proliferation and apoptosis (**Figure 2C**, **Supplementary Table 7**) with many GO terms of regulation of cell proliferation and cell death, whereas the tubular DEGs were mainly involved in cell assembly and secretion (**Figure 2D**, **Supplementary Table 6**). The genes include FCN1, C1QB, ITGAM, and WIPF1, which are well-known to be involved in kidney injury (26–29).

The pathways for tubular and glomerular DEGs were also distinguished (**Figure 3A**). The pathways common for tubular and glomerular DEGs were involved in immune response, with dendritic cell maturation, altered T cell and B cell signaling in rheumatoid arthritis, and CD28 signaling in T helper cells (**Figure 3B**, **Supplementary Table 8**). The pathways for glomerular DEGs were involved in VEGF signaling, molecular mechanisms of cancer, and Myc-mediated apoptosis signaling (**Figure 3C**, **Supplementary Table 9**). The tubular DEGs were enriched in different pathways, such as integrin signaling, remodeling of epithelial adherents junctions, and SAPK/JNK signaling (**Figure 3D**, **Supplementary Table 10**). The pathways for tubular and glomerular DEGs are consistent with GO terms. The common DEGs pathways are involved in immune response, and the pathways for tubular DEGs are mainly involved in cell remodeling and assembly. The pathways for the glomeruli are involved in cell proliferation and apoptosis as pathways related to cancer and apoptosis signaling.

Gene Expression Correlation Analysis Between the Tubule and the Glomerulus

Some previous studies have delineated that glomerular injury causes tubulointerstitial injury, and that tubular injury sensitizes the glomeruli to injury (9, 11, 30). To identify the crosstalk between tubular and glomerular DEGs in CKD patients, we performed the gene co-expression correlation analysis for the four datasets with tubular and glomerular expression data in

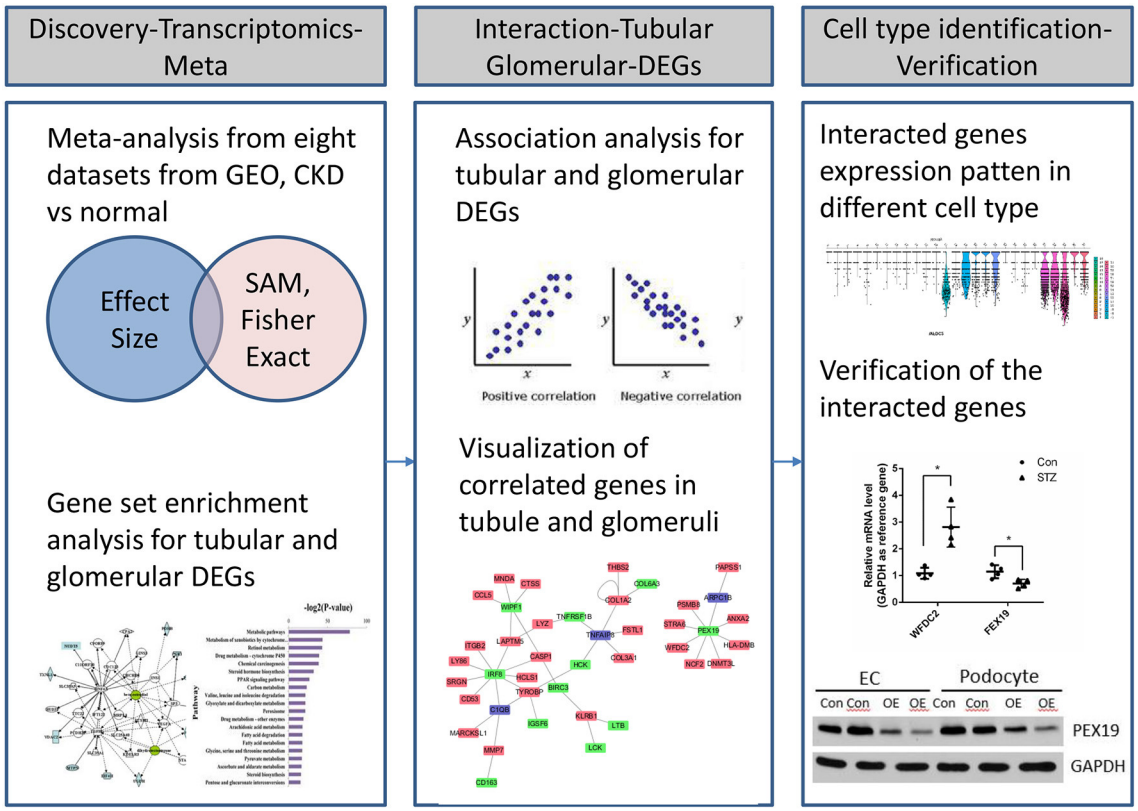

FIGURE 1 | Workflow of transcriptional profiling and identification of interacted genes through integrative informatics approach.

the same patient. We identified 59 specific correlated paired genes with a cutoff of correlation coefficient >0.7 and $p <$ 0.001 in all four datasets (**Figure 4A**, **Supplementary Table 11**). Visualization of the networks from these correlated DEG pairs was generated by Cytoscape (**Figure 4B**). We found that some interesting associated genes from the tubules and glomeruli reported could be interacted by previous studies. IRF8 from the glomerulus positively correlated with many genes in the tubule, such as C1QB, CASP1, CD53, HCLS1, ITGB2, LAPTM5, LY86, SRGN, and TYROBP. Studies have proven that IRF8, a pro-apoptotic factor, was a hypomethylated gene in acute kidney injury (AKI) and this hypomethylation was associated with a marked induction of Irf8 (31). Studies showed that IRF8 is the transcription factor that regulates C1QB (32), CASP1 (33), CD53 (34), LAPTM5 (35), and TYROBP (36) as binding to their promoter regions.

Identification and Verification of Secreted Proteins From Tubular and Glomerular Interaction

Next, we tried to identify the secreted proteins from our tubular and glomerular associated gene pairs as the secreted proteins can interact with proteins in other cells. From public data at Human Protein Atlas portal (www.proteinatlas.org), we obtained 1,708 predicted secreted proteins. Overlapping with our gene pairs, we identified 18 and 10 secreted proteins in tubular and glomerular samples in CKD (**Supplementary Table 12**). Many proteins are well-known to be important in kidney diseases, such as TGFBI (37), TNFRSF1B (also known as TNFR2) (38, 39), CXCL6 (40), and CCL5 (41). In the secreted proteins list, we found that WFDC2 from the tubule was negatively correlated with PEX19 from the glomeruli (**Supplementary Table 12**). WFDC2 is a molecular marker of tubulointerstitial fibrosis and tubular cell damage in patients with CKD (42–44). We then validated that WFDC2 was significantly upregulated in many kidney diseases in human and kidney disease models in mouse from Nephroseq database (**Figure 5A**). FEX19 was downregulated in FSGS in the glomeruli in Nephroseq (**Figure 5B**). We also validated that WFDC2 is mainly expressed in tubular cells in single-cell sequencing data from Nephrocell database (http://nephrocell.miktmc.org) (**Supplementary Figure 1**). Next, we found that WFDC2 was upregulated, whereas FEX19 was downregulated in STZ-induced eNOS depletion diabetic mice model by qPCR (**Figure 5C**) and western blot (**Figure 5D**). To further examine the regulation of WFDC2 to FEX19, we overexpressed WFDC2 gene in proximal tubular cell line (HK2) and collected its culture media (**Figure 5E**). Then, we used the culture media that contained secreted WFDC2 proteins to treat podocyte cells and

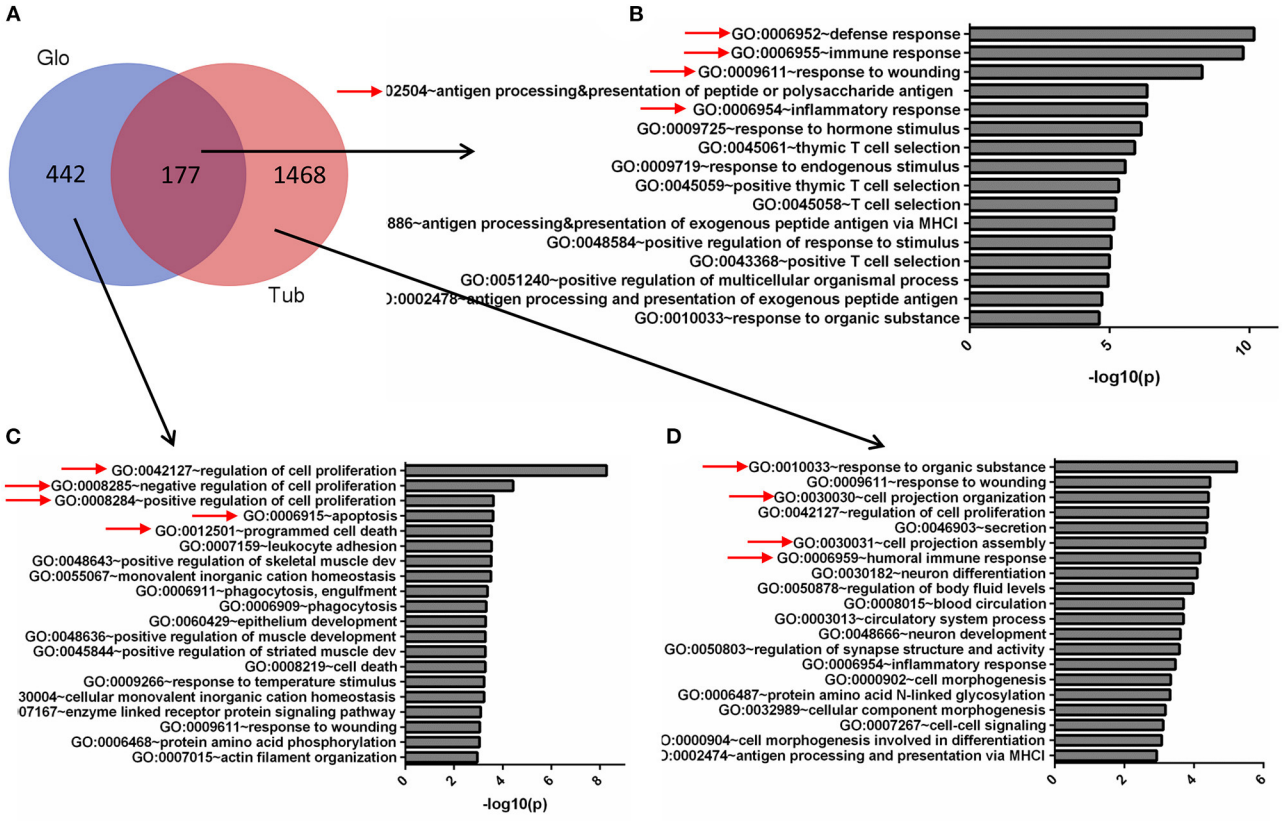

FIGURE 2 | Gene Ontology (GO) terms of glomerular and tubular DEGs. **(A)** Venn diagram for the overlapping comparison of glomerular and tubular DEGs. GO terms of **(B)** glomerular and tubular overlapping DEGs, **(C)** tubular unique DEGs, and **(D)** glomerular unique DEGs. Significance is expressed as a *p*-value calculated using Fisher's exact test ($p < 0.05$) and shown as $-\log_{10}$ (*p*-value).

GECs. We found that PEX19's mRNA and protein levels both decreased in podocyte cells and GECs with WFDC2 treatment (**Figure 5F**).

DISCUSSION

Integrative informatics approach is a powerful tool to explore the pathogenesis and to identify the therapeutic targets for complex diseases (45). The informatics approaches that combine high-throughput data with the identification of DEGs interacting networks and pathways could drive kidney diseases. Advances in omics biotechnology, such as next-generation DNA sequencing and protein mass spectrometry, let us study the complex CKD in genome, transcriptome, and proteome levels to identify the interaction between molecules that play synergistic roles (45). Here, in this study, we used integrative informatics analysis that identified the DEGs interactions from the tubules and glomeruli that play pathological roles in CKD processes. This pattern of study we used with the combination of experimental approaches and informatics approach is expected to provide us with a deeper understanding of the interaction of critical genes to elucidate CKD progression and could be new potential therapeutic targets.

The physiology of kidney function and the pathophysiology of kidney disease involve interactions of different cells from the tubules and glomeruli of the kidney. Many studies have shown that tubular injury can cause subsequent glomerular injury. Tubulointerstitial hypoxia caused by peritubular capillary loss stimulates fibrogenesis with increased collagen I and a-smooth muscle actin, indicators of increased myofibroblasts (46, 47). HIF-2a target genes are upregulated in sclerosing glomeruli, and there is a potential signaling interaction between transforming growth factor beta and hypoxia-inducible factors (HIFs) to promote renal fibrogenesis, even in normoxia (48). Meanwhile, other studies have delineated numerous mechanisms whereby glomerular injury causes tubulointerstitial injury, including ischemia, filtered proteins/cytokine elaboration, and so on (49). The tubular injury and glomerular injury feedbacks enhance CKD progression in all settings, whether there is initial isolated tubulointerstitial injury or combined glomerular/tubular injury. However, the initial or consequence of tubular or glomerular injury to the other component is not well-studied. The interaction of genes or proteins in the tubules and glomeruli is still less known. In this study, we performed correlation analysis to identify the associated genes in the tubules and glomeruli and verified their interaction *via* experiment, providing a

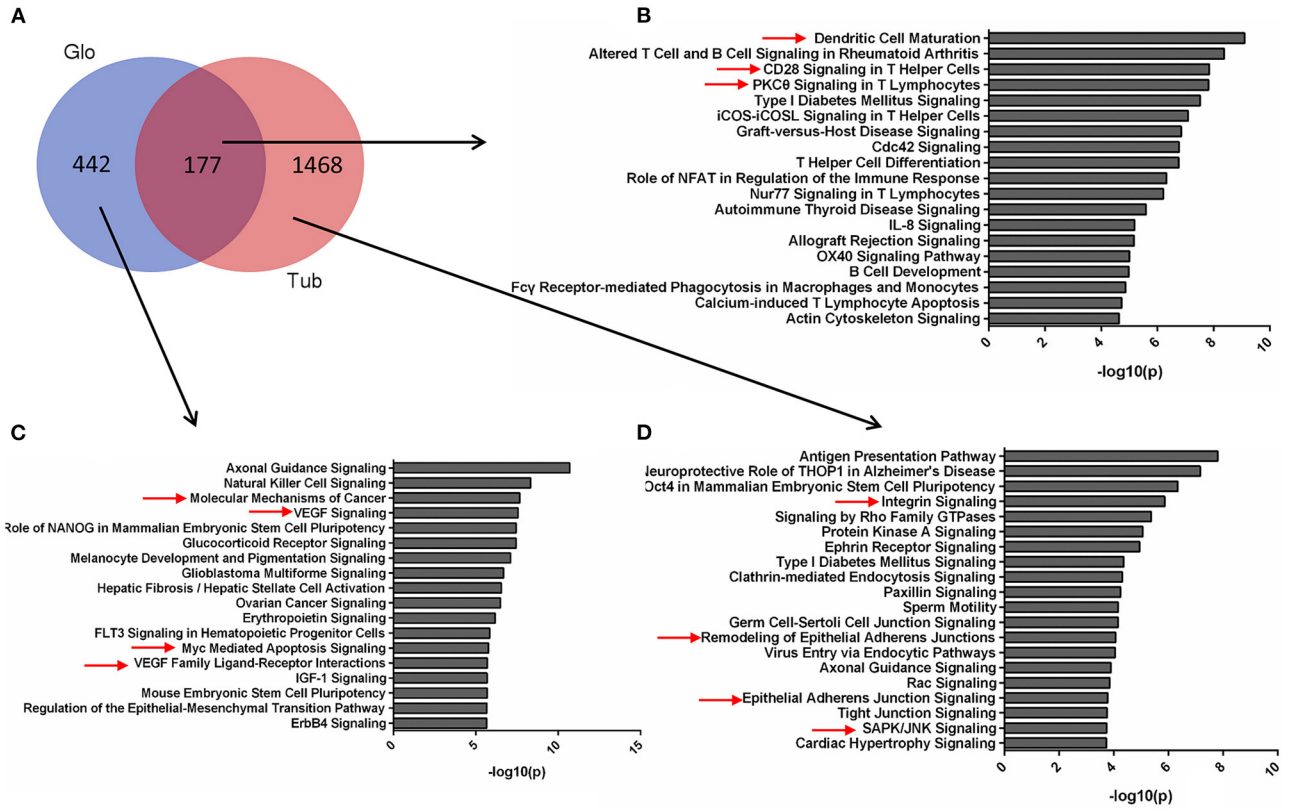

FIGURE 3 | Pathways for tubular and glomerular DEGs. **(A)** Venn diagram for the overlapping comparison of glomerular and tubular DEGs. Pathways of **(B)** glomerular and tubular overlapping DEGs, **(C)** tubular unique DEGs, and **(D)** glomerular unique DEGs. Significance is expressed as a *p*-value calculated using Fisher's exact test ($p < 0.05$) and shown as $-\log_{10}$ (*p*-value).

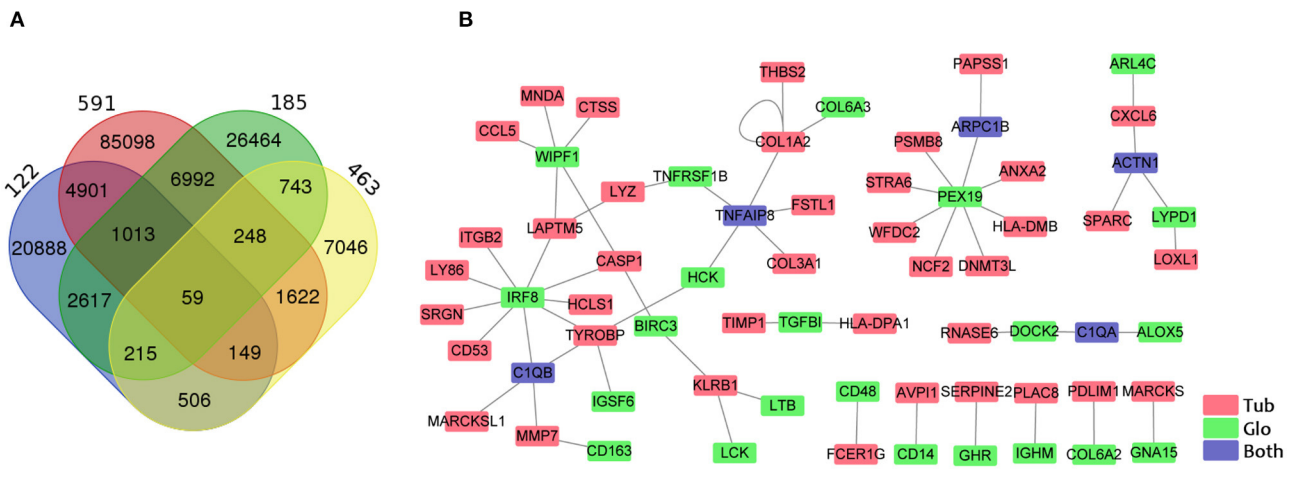

FIGURE 4 | Identification of genes correlated between the tubule and the glomerulus. **(A)** Venn diagram shows the overlapping of associated gene pairs in four datasets. **(B)** Visualization of 59 paired genes correlated in the tubules and glomeruli in all four datasets. Red, green, and blue colors indicate the GECs in the tubules, glomeruli, and both the tubules and the glomeruli.

methodology pipeline of the identification of interaction genes in the tubules and glomeruli.

DKD remains as the most common cause of end-stage renal disease (ESRD) in the US and most countries (50). DKD is most likely a disease with individual and temporal heterogeneity. Pathological and molecular understanding of this heterogeneity will be essential to make progress (50). HE4 (encoding human epididymis protein 4, also known as WAP

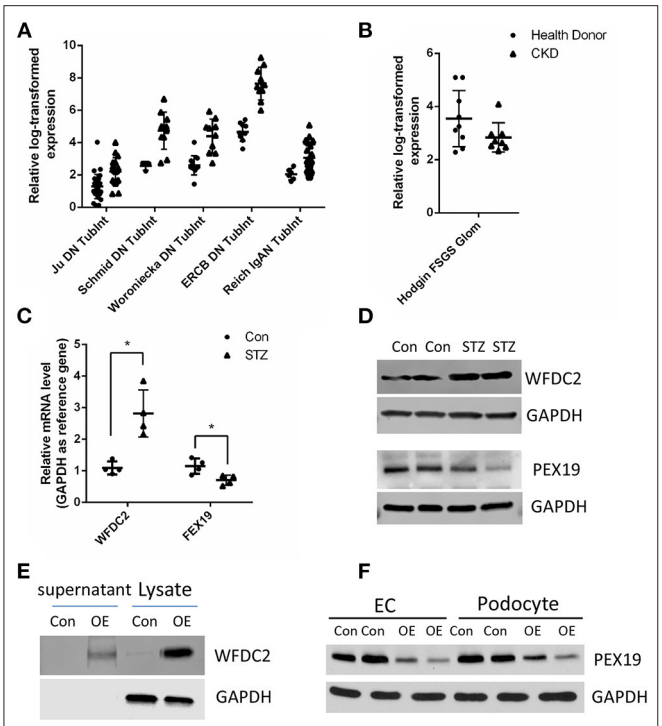

FIGURE 5 | Verification of WFDC2 and PEX19 interaction in HK2, podocyte, and glomerular endothelial cells. **(A)** Relative WFDC2 transcript levels were upregulated in many diabetic nephropathy human and mice datasets in the kidney tubule. **(B)** Relative FEX19 transcript levels were downregulated in FSGS in the kidney glomeruli in Nephroseq database. **(C,D)** WFDC2 in the tubule was upregulated, and FEX19 in the glomeruli was downregulated in STZ-induced diabetic mice by qPCR and western blot. **(E)** Western blot shows WFDC2 overexpression in HK2 cell. **(F)** PEX19 protein level was decreased with WFDC2 treatment in podocyte and glomerular endothelial cells by western blot. Con, WFDC2 empty vector; OE, WFDC2 overexpression vector; EC, glomerular endothelial cells. **(A–C)** Values are mean ± SEM; $^*p \leq 0.05$ between means. **(A,B)** All the genes were significantly changed in CKD compared with control with $p < 0.001$.

4-disulfide core domain-2 or WFDC2) is a secretory protein produced in normal glandular epithelium of the reproductive tract, renal tubules, and respiratory epithelium (51). A study showed that WFDC2 circulating WFDC2 is postulated to be a biomarker of renal fibrosis in DKD patients (52). Another study also showed that serum WFDC2 is associated with renal function and DKD in patients with type 2 diabetes mellitus (53). The overexpression of HE4 in serum from CKD patients was associated with decreased kidney function, and the serum concentrations of HE4 obviously increased with advanced renal fibrosis stage in patients with CKD (54). Increased HE4 in serum is closely associated with the development of LN or CKD in patients with systemic lupus erythematosus (55). Recently, a study identified HE4 as a fibroblast-derived mediator of fibrosis, as an inhibitor of multiple proteases, including serine proteases and matrix metalloproteinases, and as a specific inhibitor of their capacity to degrade type I collagen (43). Another study indicated that HE4 in TECs promotes extracellular matrix accumulation and renal fibrosis *via* nuclear factor kappa B (NF-kB) (31909536). WFDC2 was also reported to play important roles in diabetes and DKD. Our study found that activated WFDC2 in the tubules could interact with glomerular podocyte/endothelial cells, causing the downregulation of peroxisomal biogenesis factor 19 PEX19 mRNA and protein levels and effect downstream pathways. PEX19 undoubtedly is a key player in several steps of peroxisomal membrane proteins (PMPs) transport (56). Here, in this study, our data showed that WFDC2 was upregulated in the tubules causing PEX19 expression to decrease in the glomeruli, which provide a new mechanism of how WFDC2 regulates diabetes and DKD.

There are many other known genes associated between the tubules and the glomeruli that enhanced kidney diseases from identification. We identified that the mRNA level of C1QB in the tubules is correlated with the mRNA level of IRF8 in CKD patients. Other studies have shown that C1qB promoter was co-precipitated with PU.1 and IRF8. shRNA knockdown of PU.1 and IRF8 diminished C1qB promoter response to interferon gamma (IFNγ). STAT1 instead regulated C1qB promoter through IRF8 induction (32). We also identified that TIMP1 and TGFBI are associated in the tubules and glomeruli, and that the association is confirmed by other studies (57). However, our analysis showed that these two genes are positively correlated in the tubules and glomeruli in CKD. Meanwhile, the TGFBI overexpression in human corneal epithelial cells result in MMP1, MMP3 increasing, and TIMP1 decreasing. More experimental study needs to be performed to study their relationship.

Our study has limitations. Firstly, as there are more single-cell sequencing experiments performed by many groups, it is very direct to analyze the transcriptome of different cell types and identify the association genes. However, there are still issues that needed to be improved for single-cell sequencing technology, such as low depth of the sequencing and artificial bias caused by process steps for obtaining single cells. Therefore, the method of bulk sequencing data analysis for the tubules and glomeruli can obtain some information that single-cell sequencing cannot identify. Secondly, we used all kinds of CKD patients for analysis, including LN, DKD, FSGS, MN, IgAN, and MCD. We realize that there should be a huge variation between the mechanisms of different disease types. However, with more sample number and robust integrative informatics approach, we can identify the common critical genes or mechanistic pathways in all kinds of CKD progression.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Materials**.

ETHICS STATEMENT

The animal study was reviewed and approved by the First Hospital of Jilin University Ethics Committee.

AUTHOR CONTRIBUTIONS

JH and CW led the project, designed the study, analyzed and interpreted the data, and drafted the manuscript. ZY, LL, and FM performed the meta-analysis and bioinformatics analysis. LL, FM, YH, XY, and BX performed the experiments of qPCR, western blot, cell culture, and mice model. JH and FM performed other statistical analysis. JH and CW performed the study conception and design, along with drafting of the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.615306/full#supplementary-material

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Quercetin Antagonizes Glucose Fluctuation Induced Renal Injury by Inhibiting Aerobic Glycolysis via HIF-1α/miR-210/ISCU/FeS Pathway

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Background and Objective: Glucose fluctuation (GF) has been reported to induce renal injury and diabetic nephropathy (DN). However, the mechanism still remains ambiguous. Mitochondrial energy metabolism, especially aerobic glycolysis, has been a hotspot of DN research for decades. The activation of HIF-1α/miR210/ISCU/FeS axis has provided a new explanation for aerobic glycolysis. Our previous studies indicated quercetin as a potential therapeutic drug for DN. This study aims to evaluate levels of aerobic glycolysis and repressive effect of quercetin via HIF-1α/miR210/ISCU/FeS axis in a cell model of GF.

Methods: The mouse glomerular mesangial cells (MCs) were exposed in high or oscillating glucose with or without quercetin treatment. Cell viability was measured by CCK8 assay. Aerobic glycolysis flux was evaluated by lactate acid, pH activity of PFK. Apoptosis level was confirmed by Annexin V-APC/7-AAD double staining and activity of caspase-3. TNF-α and IL-1β were used to evaluate inflammation levels.

Results: GF deteriorated inflammation damage and apoptosis injury in MCs, while quercetin could alleviate this GF-triggered cytotoxicity. GF intensified aerobic glycolysis in MCs and quercetin could inhibit this intensification in a dose-dependent manner. Quercetin prevented activities of two FeS-dependent metabolic enzymes, aconitase, and complex I, under GF injury in MCs. The mRNA expression and protein contents of HIF-1α were increased after GF exposure, and these could be alleviated by quercetin treatment. Knockdown of ISCU by siRNA and Up-regulating of miR-210 by mimic could weaken the effects of quercetin that maintained protein levels of ISCU1/2, improved cell viability, relieved inflammation injury, decreased apoptosis, and reduced aerobic glycolysis switch in MCs.

Conclusion: Quercetin antagonizes GF-induced renal injury by suppressing aerobic glycolysis via HIF-1α/miR-210/ISCU/FeS pathway in MCs cell model. Our findings contribute to a new insight into understanding the mechanism of GF-induced renal injury and protective effects of quercetin.

Keywords: glucose fluctuation, quercetin, renal injury, aerobic glycolysis, HIF-1α/miR-210/ISCU/FeS axis

INTRODUCTION

The incidence of diabetes mellitus (DM) has been increasing worldwide and become a major public health problem in China (1). Diabetic nephropathy (DN) is the most common chronic microvascular complication triggered by DM, which is the leading cause of end-stage renal disease (2). Glucose fluctuation (GF) has been reported to induce renal injury and be involved in the pathogenesis of DN. It was demonstrated that the short-term glucose variability was closely associated with decreased estimated glomerular filtration rate and an increased risk of CKD in DM patients (3). Other vitro studies indicated that unstable blood glucose had apoptosis-triggering effects on cells, including glomerular mesangial cell (4) and vascular endothelial cell (5). However, it still remains ambiguous under the mechanism between glucose variability and DN.

Mitochondrial energy metabolism has been a hotspot in DN research for decades, including aerobic glycolysis (the "Warburg effect"). Aerobic glycolysis flux, indicated by glucose uptake and lactate production, was increased in DN rats and increasing aerobic glycolysis could remarkably induce myofibroblasts activation and affected the number and function of podocytes (6, 7). The activation of HIF-1α/miR210/ISCU/FeS axis has provided a new explanation for aerobic glycolysis (8). MiR-210 is a response binding element of HIF-1α and represses its downstream molecules, iron-sulfur cluster assembly scaffold protein (ISCU), which mediates FeS assembly (9). Disturbance of FeS assembly contributes to the development of DN via inactivation of FeS-dependent enzymes, such as complex I (10). HIF-1α is also considered to play roles among GF (11), DN (12), and Warburg effect (13). Mitochondria are the major sites for regulating glucose metabolism of cells. In the condition of glucose intermittent, hypoxia-inducible factor 1α (HIF-1α) enhances its transcriptional activity triggered by dysfunction of mitochondria (11). Thus, it is meaningful to investigate whether HIF-1α/miR210/ISCU/FeS axis underlies aerobic glycolysis in conditions of GF induced renal injury.

Quercetin distributes in various fruits and vegetables and is one of bioflavonoid compounds of Abelmoschus plants, which has been reported as a potential therapeutic herb for the treatment of DN in our previous studies (14, 15) and other studies (16, 17). Quercetin shows diverse pharmacological effects, including anti-oxidation and anti-inflammatory (18, 19). It is reported that Quercetin could inhibited proliferation in high glucose–treated mouse glomerular mesangial cells (MCs) and in early DN mouse (20). Our previous study showed that quercetin presented protective effects against the initiation and progression of DN in diabetic mice by improving the renal accumulation of lipid bodies (21). A network pharmacology study demonstrated that Quercetin had a good binding on factors of inflammatory response, angiogenesis and oxidative stress reaction, which all involved in DN (22). Quercetin also held the ability to inhibit Warburg effect in many cells (23, 24) and downregulate HIF-1α to reduce renal oxidative stress apoptosis (25). However, the protective mechanism of quercetin against aerobic glycolysis in the GF induced renal injury has not been reported.

In the present study, we first evaluated levels of aerobic glycolysis and repressive effect of quercetin in a mouse MCs model of intermittent high glucose. Then, we elucidated the roles of HIF-1α/miR210/ISCU/FeS axis underlying these effects of quercetin.

MATERIALS AND METHODS

Cell Culture and Treatment

The mouse glomerular MCs SV40 MES 13 was purchased from Cell Bank/Stem Cell bank (Shanghai Chinese Academy of Sciences). MCs were cultured in DMEM (5.56 mmol/L glucose) supplemented with 10% fetal bovine serum (FBS), antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin), in a 5% CO_2 humidified atmosphere at 37°C.

The cells were randomly divided into seven groups: normal glucose group (NG, 5.6 mmol/l glucose), high glucose group (HG, 50 mmol/l glucose), glucose fluctuation group (GF, alternated 5.6 mmol/l glucose and 50 mmol/l glucose every 8 h), GF+10 μmol/L quercetin group (GF+Q10, cells treated with GF in the presence of 10 μmol/L of quercetin), GF+20 μmol/L quercetin group (GF+Q20, cells treated with GF in the presence of 20 μmol/L of quercetin), GF+40 μmol/L quercetin group (GF+Q40, cells treated with GF in the presence of 40 μmol/L of quercetin), mannitol group (MG, 5.6 mM glucose plus 44.4 mM mannitol as an osmotic pressure control).

Cell Transfection

The oligonucleotides were transfected into cells according to the manufacturer's instructions. Briefly, cells were seeded 24 h before transection to make sure 70–80% cell density. Then, Opti-MEM medium without antibiotics and serum was used to dilute the oligonucleotides (200nM ISCU1/2 siRNA, 100 nM miR210 mimic or inhibitor) and Lipofectamine 3000 transfection reagent (Invitrogen, USA). Subsequently, mix these two diluents. After 48 h of incubation in a 5% CO_2 humidified atmosphere at 37°C, the transfection medium was replaced with fresh penicillin/streptomycin-free medium for 24 h before subsequent experiments. ISCU siRNA and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Both miR210 mimic and mimic control were purchased from Kaiji Biotech (Jiangsu, China).

Cell Counting Kit-8 (CCK-8) Assay

The cells were plated in 96-well plates with 5×10^3 cells per well. The cells were serum starved for 24 h after adherence, followed by different managements. Subsequently, cells were incubated in 10 μL CCK-8 (Dojindo Laboratories, Kumamoto, Japan) for 1 h. The optical density (OD) at 450 nm of each group was determined by a microplate reader (BioTek, USA). Mean OD value was calculated by triplicate repeats.

Measurements of Lactate Acid and pH in Cell Supernatant

Lactate acid (lac) level was measured by using Lac Colorimetric/Fluorometric Assay Kit (Jiancheng Biotech., A019-2-1). The pH was measured with pH instrument

(OHAUS STARTER 2C, USA) according to the manufacturer's instructions.

PFK Activity Assay

PFK Activity Colorimetric Assay Kit was applied to evaluate the activity of phosphofructokinase (PFK) (Sigma-Aldrich, USA). Treated cells were mixed with PFK Assay Buffer and under cell lysis with Reaction Mix according to the manufacturer's instructions. Microplate reader was used to test the OD value of the mixtures per 30 s. One unit of PFK mediates 1.0 μM per minute of NADH generation. A standard line of NADH was built for PFK activity calculation. After normalization to the protein concentration, the PFK activity was showed as milliunits/mg of protein.

Measurements of Aconitase and Complex I Activity Assays

Mitochondria were isolated using the Mitochondrial Isolation Kit for Cultured Cells from Abcam. The Complex I Enzyme Assay Kit (Abcam) and Aconitase Assay Kit (Sigma) were used to determine activity of Complex I and aconitase, respectively, according to the manufacturer's protocol. A Multi-Plate Reader was used to read the plate at a wavelength of 450 nm.

Measurements of TNF-α and IL-1β

Levels of tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-β) in culture supernatants were quantified using commercially available ELISA kits conducted in accordance with the manufacturer's instructions.

Annexin V-APC/7-AAD Double Staining

After treatment, MCs were harvested, washed and stained with Annexin-V APC/7-AAD cell apoptosis assay kit (Jiangsu Kaiji Biotech., KGA1024) according to the manufacturer's instructions. Four subpopulations were identified: normal cells (Annexin V-APC$^-$/7-AAD$^-$), necrotic cells (Annexin V-APC$^-$/7-AAD$^+$), early apoptotic (Annexin V-APC$^+$/7-AAD$^-$) and late apoptotic (Annexin V-APC$^+$/7-AAD$^+$). Apoptosis index was the total rates of early apoptotic and late apoptotic cells.

Western Blotting Analysis

After measuring the protein concentrations, the cell lysates and subcellular fractionation were separated by SDS-PAGE and then transferred to PVDF membranes (Millpore, Billerica, MA, USA). Then, the membranes were blocked with 5% fat-free milk and incubated with anti-caspase3 (1:500, proteintech, 19677-1-ap), cleaved caspase3 (1:1,000, CST, 9664), anti-PKM2 (1:1,000, Beijing Boaosen Biotechnology, bs-0102M), anti-p-PKM2 (1:1,000, CST, 3827), HIF-1α (1:1,000, CST, 14179), and anti-ISCU (1:1,000, proteintech, 14812-1-AP), respectively. The bound antibodies were detected with 1:5,000 diluted goat-anti-rabbit IgG-HRP (Jiangsu Kaiji Biotech., KGAA35) and the bands were developed using an enhanced chemiluminescence ECL kit (Applygen Technologies). The relative levels of each protein to beta-actin were determined by the G: BOX ChemiXR5 imaging system.

qRT-PCR Analysis

The first strand of cDNA was synthesized by M-MLV Reverse Transcriptase (Life Technologies). The RT-qPCR was performed as previously described (26). After normalizing with U6, the relative levels of target miRNAs were calculated by ΔΔCT method.

Immunofluorescence Analysis

MCs cultured on glass coverslips were washed and fixed with 4% paraformaldehyde for 30 min. After three times of PBS washing, the cells were blocked with 10% ready-to-use goat serum for 20 min at room temperature, incubated with primary antibodies (1:100) at 37°C for 2 h, followed by incubation with a secondary antibody with FITC (1:100) at 37°C protected from light for 1 h. ISCU antibody was purchased from Wuhan Sanying Biotechnology (Wuhan, China, 14812-1-AP). Then, the cells were counterstained with DAPI at 37°C protected from light for 5 min. The coverslips were mounted onto glass slides. Fluorescence microscope was used to observe the expression of protein and take images of three high expression areas.

Statistical Analysis

All experiments were repeated three times. Results were expressed as the mean ± standard deviation (SD). The difference among groups was analyzed by one-way ANOVA using SPSS 22.0 software. $P < 0.05$ was considered significantly different.

RESULTS

Quercetin Protected Glomerular MCs From GF-Induced Inflammation and Apoptosis Injuries

We first explore the cytotoxicity of GF in MCs. As shown in **Figure 1**, the viability of MCs was significantly decreased in the FG group compared with the NG and HG groups at 24, 48, and 72 h, while no significant difference was found between NG and HG group (**Figure 1A**). In comparison of NG and HG, FG could significantly increase inflammation levels (TNFα and IL-1β) at 24, 48, and 72 h (**Figure 1B**). Flow cytometry and WB test showed that the apoptosis index and rate of cleaved caspase-3/caspase-3 were significantly increased in the FG group compared with the NG and HG groups at 48 h, respectively (**Figures 1C,D**). Flow cytometry showed that the numbers of necrotic cell were significantly increased in the FG group compared with the NG and HG groups at 48 h (**Supplementary Figure 1A**). Taken together, these results indicated that GF deteriorated cell viability, inflammation and apoptosis injury in MCs.

Then, we evaluated the protective effects of quercetin against GF related cytotoxicity and selected 48 h as time point for further experiments. Both moderate (20 μmol/L) and high dose (40 μmol/L) of quercetin could significantly increase MCs viability and decreased inflammation damage under FG condition (**Figures 1E,F**). Quercetin could reverse GF-induced cell apoptosis by reducing index of apoptosis and activity of caspase-3 (**Figures 1G,H**). Quercetin could reduce GF-induced

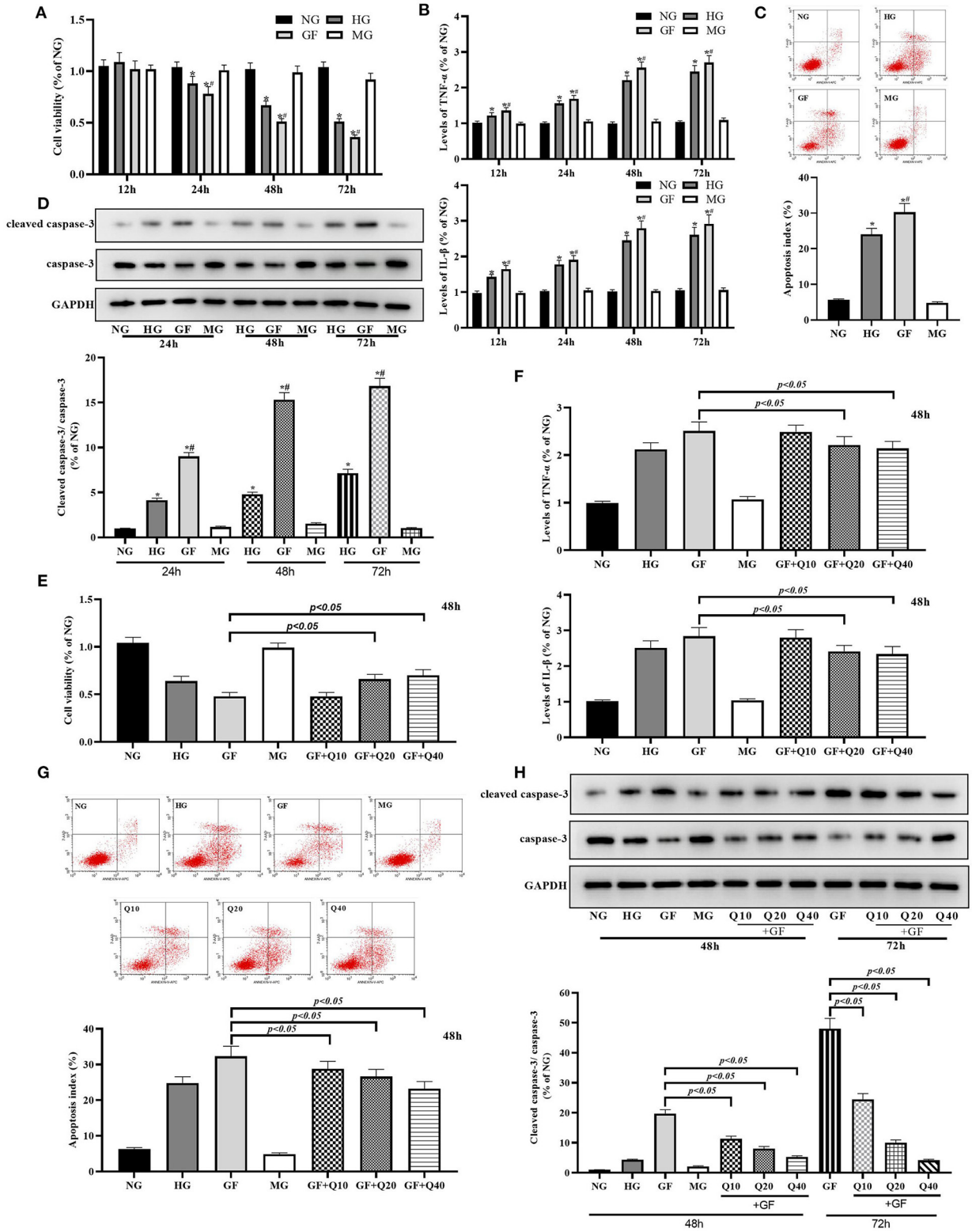

FIGURE 1 | Quercetin protected glomerular MCs from GF-induced inflammation and apoptosis injuries. **(A)** Viability of MCs was tested by CCK8 at 24, 48, and 72 h. **(B)** Inflammation levels of TNFα and IL-1β at 24, 48, and 72 h. **(C)** Apoptosis index was detected by flow cytometry under different glucose at 48 h. **(D)** Rates of cleaved caspase-3/caspase-3 measured by WB analysis at 48 h. **(E,F)** Moderate (20 μmol/L) and high dose (40 μmol/L) of quercetin treatment presented protection effects on viability of MCs and levels of inflammation damage. **(G,H)** Effects of quercetin on apoptosis index and caspase-3 activity. The error bar reflects the S.E.M. of at least three independent experiments. $^{*}P < 0.05$ vs. NG. $^{\#}P < 0.05$ vs. HG.

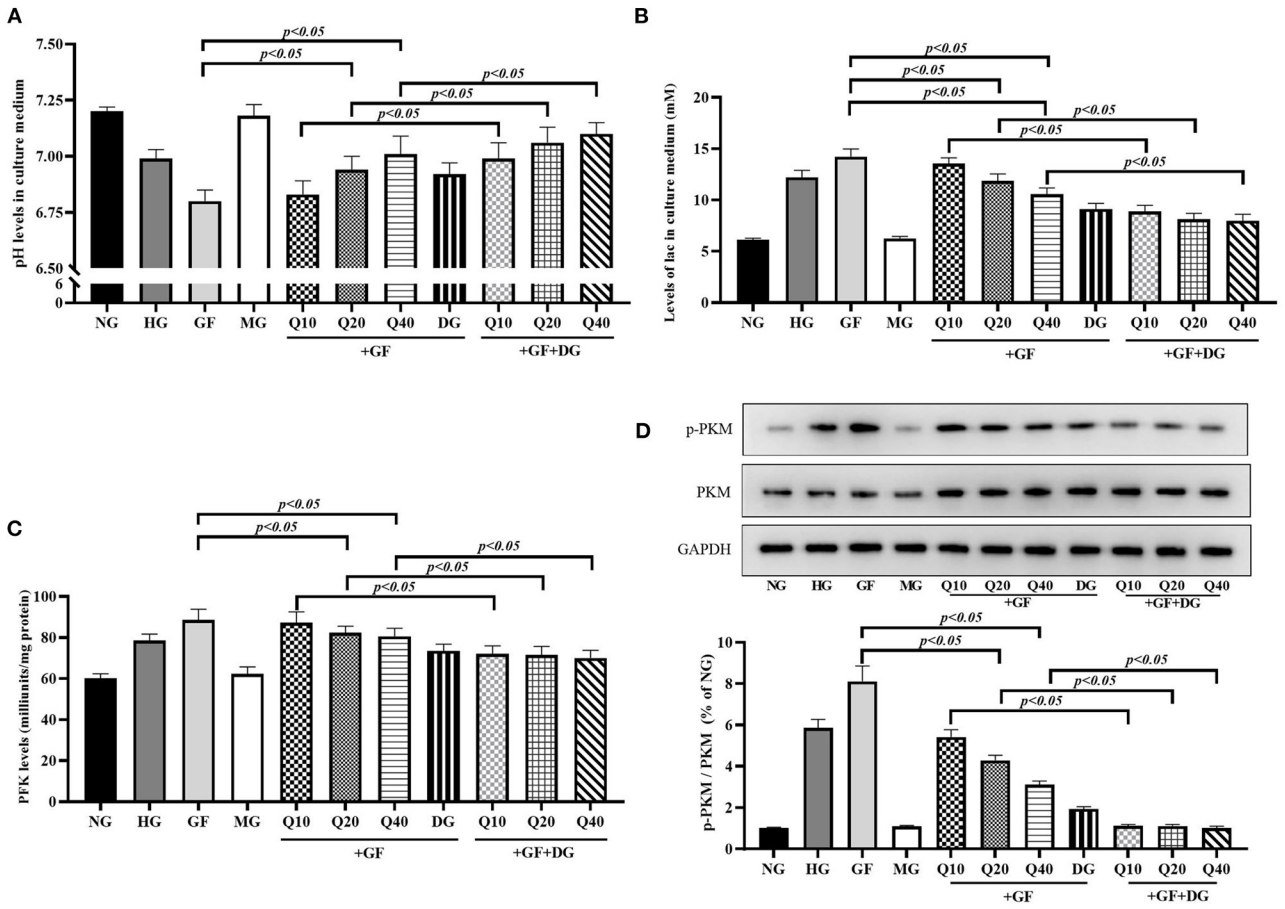

FIGURE 2 | Quercetin reversed GF-triggered aerobic glycolysis in glomerular MCs. **(A)** Levels of pH in each group. **(B)** Levels of lac in each group. **(C)** Activity of PFK tested by Colorimetric Assay. **(D)** Western blot analysis showing phosphorylation levels of PKM2 in each group. As expect, these GF-triggered increase of aerobic glycolysis could be dose-dependently blocked by quercetin and glycolytic pathway inhibitor 2-deoxyglucose (2-DG). The error bar reflects the S.E.M. of at least three independent experiments.

cell necrosis (**Supplementary Figure 1B**). In general, these data suggested that GF deteriorated inflammation damage and apoptosis injury in MCs, while quercetin could alleviate this GF-triggered cytotoxicity.

Quercetin Reversed GF-Triggered Aerobic Glycolysis in Glomerular MCs

Next, we focused on the levels of aerobic glycolysis under GF condition and the effects of quercetin against aerobic glycolysis in MCs. As presented in **Figure 2**, GF could remarkably induce abnormal of cellular energy metabolite levels, including reduction of pH (**Figure 2A**) and elevation of lac in cell culture medium (**Figure 2B**). Phosphofructokinase (PFK) catalyzes fructose-6-phosphate to fructose-1, 6-diphosphate and is the rate-limiting enzyme of glycolysis. The results showed that activity of PFK was enhanced under GF condition (**Figure 2C**). PKM2 serves as the final rate-limiting enzyme associated with cell reliance on aerobic glycolysis. We detected the level of PKM2 particularly phosphorylated PKM2 at Tyr105 (p-PKM2) in the MCs under different glucose administration. Western blot analysis showed both levels of p-PKM2/PKM2 were markedly increased (**Figure 2D**). As expect, these GF-triggered increase of aerobic glycolysis could be dose-dependently blocked by quercetin and glycolytic pathway inhibitor 2-deoxyglucose (2-DG). Totally, these results indicated that GF intensified aerobic glycolysis in MCs and quercetin could inhibit this intensification in a dose-dependent manner.

Quercetin Increased Activities of FeS-Dependent Metabolic Enzymes in Condition of GF

Then, we aimed to evaluate the influence of GF to oxidative phosphorylation (OXPHOS), which was an essential process for ATP generation. Cellular OXPHOS depends on a series of FeS-dependent metabolic enzymes, including aconitase and mitochondrial respiratory chain complex I. In this experiment, as shown in **Figure 3**, GF presented repression effects to the activity of aconitase and complex I and these suppressions were restored

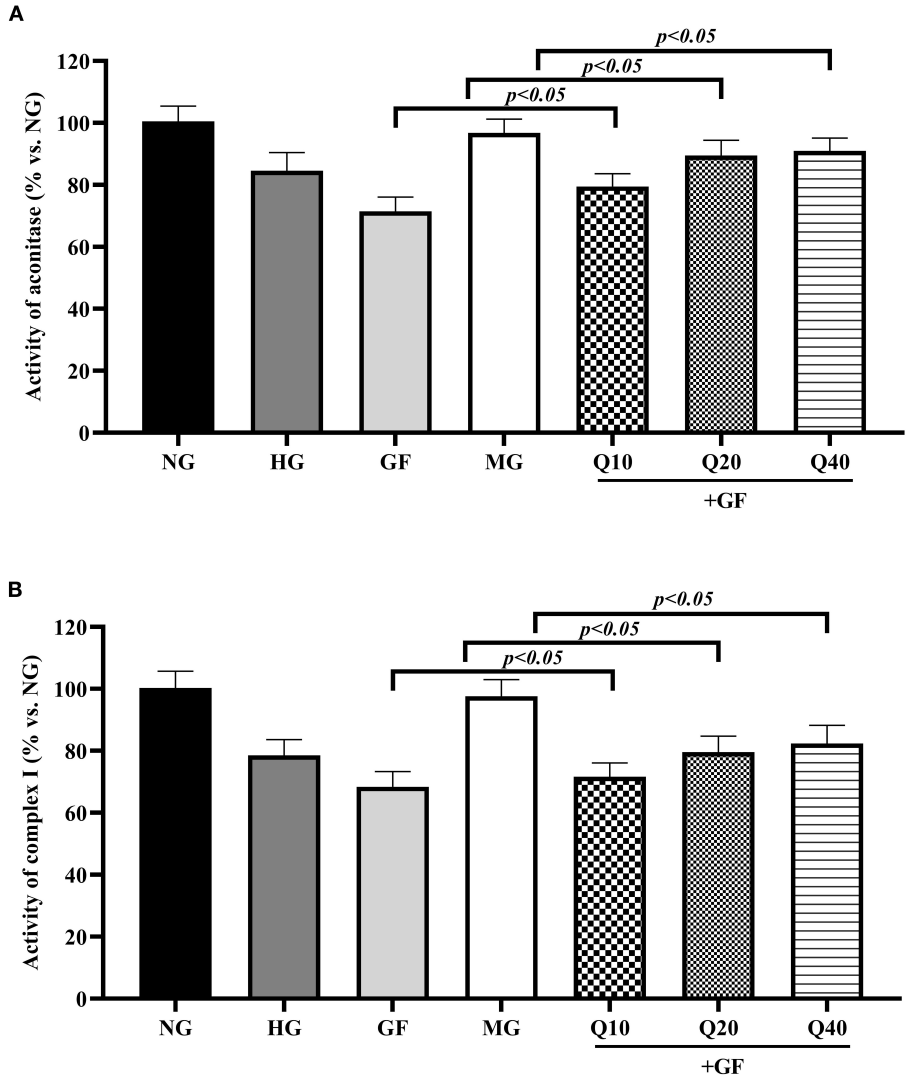

FIGURE 3 | Quercetin increased activities of FeS-dependent metabolic enzymes in condition of GF. The activities of two typical FeS-dependent metabolic enzymes, aconitase **(A)**, and complex I **(B)**, were measured with colorimetric assays. The error bar reflects the S.E.M. of at least three independent experiments.

in the presence of quercetin. Altogether, these results suggested that quercetin prevented activities of FeS-dependent metabolic enzymes under GF injury in MCs.

Quercetin Alleviated GF-Induced Alteration of ISCU1/2 Levels in MCs

To elucidate the protective mechanism of quercetin against GF injury via FeS assembly, we analyzed alteration of ISCU1/2 levels, which was the up-stream regulator of FeS. First, we used immunofluorescence staining to find the reduction of ISCU1/2 under GF injury in MCs (**Figure 4A**). Consistent with IF results, Western blot analysis showed that GF treatment could decrease the protein levels of ISCU1/2 and quercetin could alleviate these reductions (**Figure 4B**). Then, we used a specific siRNA to silence ISCU1/2 (**Figure 4C**). Knockdown of ISCU by siRNA could weaken the effects of quercetin that maintained protein levels of ISCU1/2 (**Figure 5D**). ISCU siRNA could also diminish protective effects of quercetin in improving cell viability (**Figure 4E**), relieving inflammation injury (**Figure 4F**), decreasing apoptosis (**Figures 4G,H**) and necrosis (**Supplementary Figure 1C**), and reducing aerobic glycolysis switch (**Figure 4I**) in MCs. Therefore, these results indicated that quercetin prevented against GF injury via ISCU/FeS axis in MCs.

Quercetin Inhibited GF-Induced Upregulation of HIF-1α/miR-210 Levels

Next, we confirmed whether HIF-1α/miR-210, as the regulator of ISCU/FeS axis, participated in the protective effects of quercetin

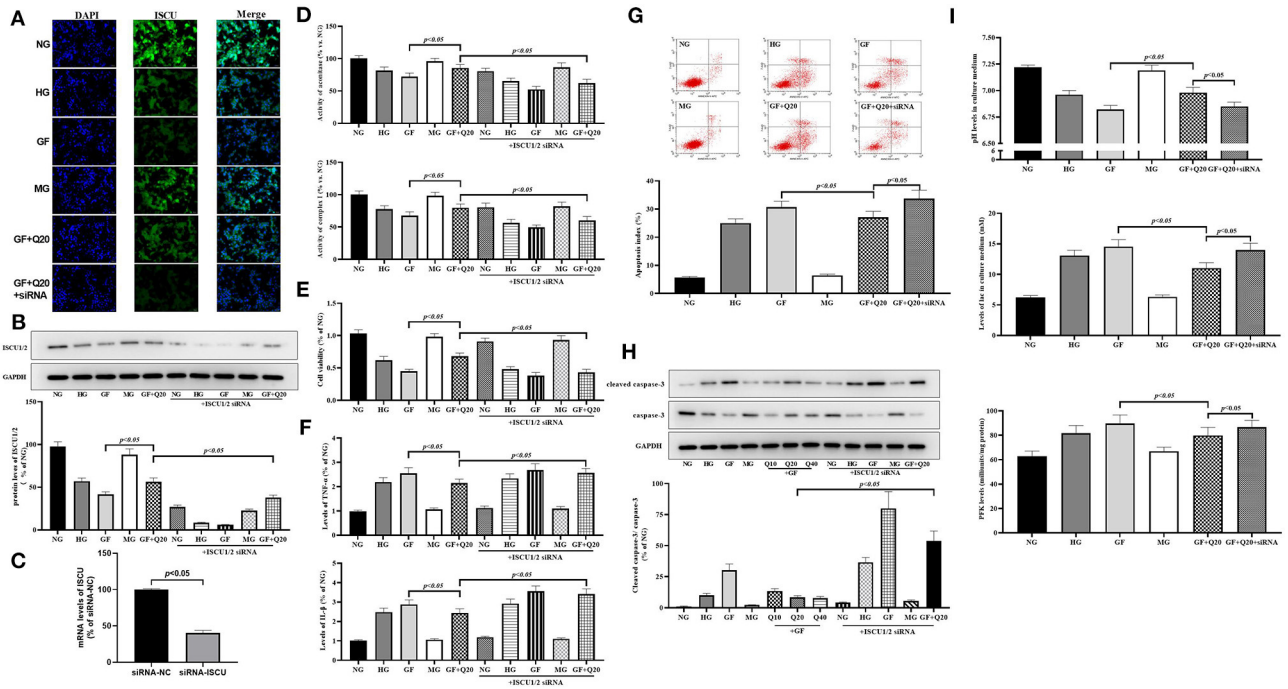

FIGURE 4 | Quercetin alleviated GF-induced alteration of ISCU1/2 levels in MCs. **(A)** Representative IF staining for ISCU1/2 in MCs under different glucose. Blue indicates DAPI staining and grean means ISCU1/2 staining. Scale bar = 50 μm. **(B)** Western blot analysis showing protein levels of ISCU1/2. **(C)** Silence effect of ISCU siRNA tested by RT-qPCR. **(D)** Activities of FeS-dependent metabolic enzymes (aconitase and complex I) measured by colorimetric assays. **(E)** Viability of MCs tested by CCK8. **(F)** The effects of quercetin and ISCU1/2 siRNA on Inflammation levels of TNFα and IL-1β in MCs under different glucose. **(G,H)** The effects of quercetin and ISCU1/2 siRNA on index of apoptosis and activity of caspase-3 in MCs. **(I)** Effects of quercetin and ISCU1/2 siRNA on aerobic glycolysis activity (levels of pH, lac, and PFK). The error bar reflects the S.E.M. of at least three independent experiments.

against GF-induced injury in MCs. First, we found the mRNA expression and protein contents of HIF-1α (**Figures 5A,B**) were increased after GF administration, and these could be alleviated by quercetin treatment. Subsequently, RT-qPCR analysis showed that GF could trigger a sharp increase in miR-210 expression and quercetin could repress this overexpression (**Figure 5C**). Up-regulation of miR-210 by mimic could weaken the effects of quercetin that maintained protein levels of ISCU1/2 (**Figure 4D**). Then, we found miR-210 mimic could inhibit protective effects of quercetin in improving cell viability (**Figure 5E**), relieving inflammation injury (**Figure 5F**), decreasing apoptosis (**Figures 5G,H**) and necrosis (**Supplementary Figure 1D**), and decreasing aerobic glycolysis levels (**Figure 5I**) in MCs. Taken together, these results indicated the inhibition of quercetin against GF-induced upregulation of HIF-1α/miR-210 levels.

DISCUSSION

Unstable blood glucose levels have been wildly accepted to trigger more inflammation and apoptosis damages than constant high or low glucose levels in our previous study and others (4, 5, 27). Oscillating high glucose has been regard to participate in the pathogenesis of DN (3). In the present study, we proposed a cellular model using primary cultured MCs exposed to GF which partly minics glucose oscillation *in vivo* in DM patients. We observed the GF-triggered cytotoxicity in MCs, which were consisted with previous study (28). We also found that quercetin could block these damages by reducing inflammation levels and apoptotic cell numbers in MCs. Total flavones of Abelmoschus manihot has been reported as a potential therapeutic herb for the treatment of DN in our previous studies (14, 15). Quercetin, one of the bioflavonoid compounds of Abelmoschus manihot, presented protective effect against the initiation and progression of DN in diabetic mice in our previous study (21). On the basis of our previous study, the present study was performed with a mouse glomerular MCs cell line. Our finding provided further evidence in support of quercetin's kidney protection.

Aerobic glycolysis has been confirmed to engage in a series of chronic kidney pathological processes, such as inflammation and fibrosis. Ding et al. (6) found that aerobic glycolysis was increased in mouse kidney with unilateral ureter obstruction related nephropathy or TGF-beta1-treated renal interstitial fibroblasts, which indicated that aerobic glycolysis was positively correlated with kidney fibrosis process. Another study found that the aerobic glycolysis was the vital recodification of cell energy metabolism in renal tubular epithelial cell fibrosis. The increasing flux of aerobic glycolysis affected the number and function of podocytes and aggravated renal interstitial fibrosis (7). It has been demonstrated the crosstalk interaction between inflammatory cytokine TNF-α and aerobic glycolysis (29). As is well-known, both inflammation and fibrosis are the key

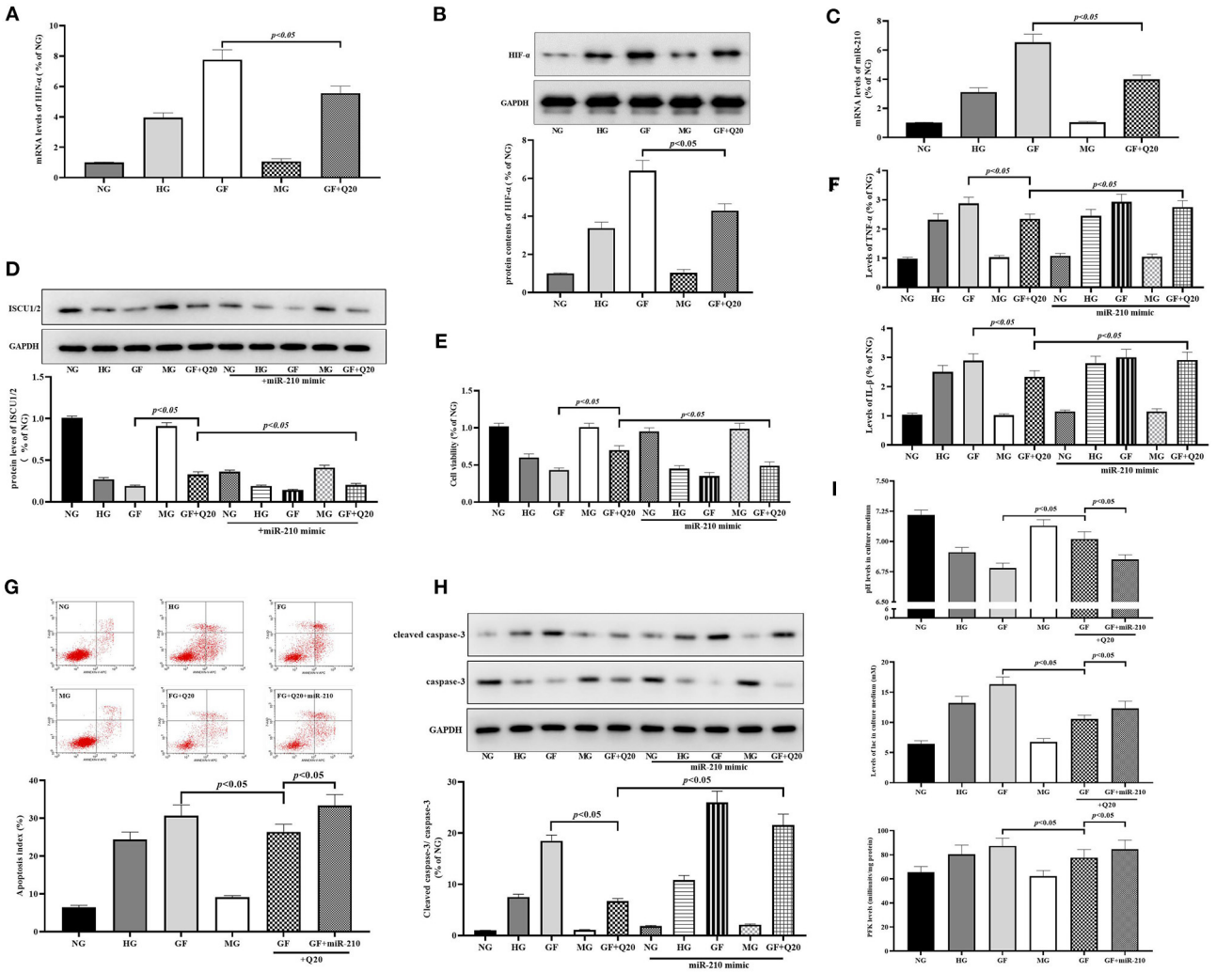

FIGURE 5 | Quercetin inhibited GF-induced upregulation of HIF-1α/miR-210 levels. **(A,B)** mRNA expression and protein contents of HIF-1α measured by RT-qPCR and WB, respectively. **(C)** RT-qPCR analysis presented expression of miR-210. **(D)** Western blot analysis showing levels of ISCU1/2. **(E)** Cell viability tested by CCK8. **(F)** Levels of inflammation factors. **(G,H)** The effects of quercetin and miR-210 mimic on index of apoptosis and activity of caspase-3 in MCs. **(I)** Effects of quercetin and miR-210 mimic on aerobic glycolysis activity (levels of pH, lac, and PFK). The error bar reflects the S.E.M. of at least three independent experiments.

features of DN. The results of our study showed that GF could intensify aerobic glycolysis in MCs, including reduction of pH and elevation of lac in MCs culture medium, and activation of PKM2 phosphorylation. PKM2 is the final rate-limiting enzyme associated with cell reliance on aerobic glycolysis. The recoding energy metabolism under oscillating glucose may lead a fire-new direction for research regarding GF-triggered renal injury of DN. Interestingly, our results showed quercetin could block GF-triggered increase of aerobic glycolysis. Quercetin has been reported to inhibit aerobic glycolysis levels in rat testis and some tumor cells (30, 31). Our results further proved quercetin played as an inhibitor of aerobic glycolysis in a cell model of GF-induce renal injury. The effect of quercetin to block aerobic glycolysis may increase the knowledge of quercetin in kidney protection.

To elucidate the inhibition molecular mechanism of quercetin against aerobic glycolysis, we select HIF-1α/miR210/ISCU/FeS axis. This axis has been reported as a classical regulator of aerobic glycolysis in multiple physiologic and pathologic processes (8, 32, 33). We confirmed that the expressions of HIF-1α/miR-210 were both sharply increased after GF administration and quercetin could repress these over-expressions. It has been demonstrated that oscillating glucose induced up-regulation of HIF-1α, which might play a pivotal role in the series of injuries triggered by unstable blood glucose (34). MiR-210 is a response binding element of HIF-1α. It has been reported that unstable blood glucose induced energy stress via up-regulating miR-210 in pancreatic cancer cells (35). Our finding provided the evidence that swing of glucose also induced abnormal expression of miR-210 in a mouse

glomerular MCs cell line. Considering HIF-1α/miR-210 are involving in GF (11), DN (12), and Warburg effect (13), it is reasonable to believe that this axis may be a vital target in the treatment of GF-related damage and the prevention of DN. ISCU/FeS pathway is the down-stream target of HIF-1α/miR-210. FeS-dependent metabolic enzymes are essential factors in cellular OXPHOS. Our results showed that GF presented repression effect to the activity of aconitase and complex I and these suppressions were restored in the presence of quercetin in MCs. We also found knockdown of ISCU by siRNA could weaken the effects of quercetin that maintained protein levels of ISCU1/2 and activities of FeS-dependent metabolic enzymes. ISCU1/2 is the vital enzyme in the progress of FeS assembly and is the down-stream target of miR-210. Overexpression of miR-210 has been reported to disturb cellular energy metabolism and induce mitochondrial dysfunction via inhibiting ISCU1/2 in rat brain and H9c2 cardiomyocyte (36, 37).

There are some shortcomings in this study. First, we did not elucidate whether HIF-1α/miR-210/ISCU/FeS were direct or indirect targets of quercetin. Secondly, we only tested in mouse MCs but not any other cell lines related to DN, such as podocytes and endothelial cells. Thirdly, our study only experimented *in vitro* without *in vivo* experiments.

In summary, our study demonstrated that quercetin antagonized GF-induced renal injury by suppressing aerobic glycolysis via HIF-1α/miR-210/ISCU/FeS pathway in MCs cell model. Although further studies are needed, our findings may contribute to a new insight into understanding the mechanism of GF-induced renal injury and protective effects of quercetin.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

W-lX and Y-hP: conceptualization. SL, NL, L-fY, MZ, C-yL, YZ, and QP: methodology and investigation. J-jB and X-jC: validation. Y-hP: writing—review and editing. W-lX and J-yY: supervision, project administration. 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.2021.656086/full#supplementary-material

Supplementary Figure 1 | Numbers of necrotic cell detected by flow cytometry. **(A)** Numbers of necrotic cell under different glucose at 48 h. **(B)** Effects of different quercetin doses on cell necrosis. **(C)** The effects of quercetin and ISCU1/2 siRNA on necrosis in MCs. **(D)** The effects of quercetin and miR-210 mimic on necrosis in MCs. The error bar reflects the S.E.M. of at least three independent experiments. $^{*}P < 0.05$ vs. NG. $^{\#}P < 0.05$ vs. HG.

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Glomerular Endothelial Cell Crosstalk With Podocytes in Diabetic Kidney Disease

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Diabetes is the main cause of renal failure worldwide. Complications of the kidney micro-and macro-circulation are common in diabetic patients, leading to proteinuria and can progress to end-stage renal disease. Across the complex interplays aggravating diabetes kidney disease progression, lesions of the glomerular filtration barrier appear crucial. Among its components, glomerular endothelial cells are known to be central safeguards of plasma filtration. An array of evidence has recently pinpointed its intricate relations with podocytes, highly specialized pericytes surrounding glomerular capillaries. During diabetic nephropathy, endothelial cells and podocytes are stressed and damaged. Besides, each can communicate with the other, directly affecting the progression of glomerular injury. Here, we review recent studies showing how *in vitro* and *in vivo* studies help to understand pathological endothelial cells-podocytes crosstalk in diabetic kidney disease.

Keywords: podocyte, endothelium/physiopathology, diabetes, glomerulosclerosis, disease module identification, angiocrine factors, glycocalyx (glycocalix)

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INTRODUCTION

Diabetes is a multifactorial disease and encompasses multi-organ complications, including kidney lesions leading to diabetic kidney disease (DKD). DKD is characterized by elevated urinary albumin excretion rate (UAER), increase in blood pressure, and decline in renal function leading to end-stage renal disease (ESRD). In addition, these patients have a high risk of cardiovascular disease, which further increases with deteriorating renal function. Although the methodologies to assess diabetes complications and consequences lack accuracy, possibly underestimating its burden, diabetes is recognized as major public health and economic plague (1). In parallel, the prevalence of diabetes in ESRD has been increasing constantly and diabetes is now the main cause of ESRD worldwide (2) and a rapidly increasing problem in the developing countries with the epidemic of type 2 diabetes. Albuminuria is an indicator of glomerular injury during diabetes, and a first step through ESRD (3).

The glomerular filtration barrier (GFB) is altered in DKD, a consequence of the combination of long-term hyperglycemia, advanced glycation end products through glycation reaction between reducing sugars, such as glucose, and proteins, lipids or nucleic acids; dysregulated insulinemia (with alternated hypo- and hyperinsulinemia), and frequently associated endothelial dysfunction and hypertension. Alterations of the GFB involve glomerular endothelial cells (ECs) and podocyte lesions. Endothelial dysfunction, increased extracellular matrix deposition, loss of podocyte permselectivity and progressive podocyte apoptosis occur along the time-course of DKD and contribute to the GFB dysfunction and progressive demise. Podocytes and ECs are physically

close and isolated from each other by the glomerular basal membrane (GBM). ECs form a fenestrated endothelium delimiting the vascular compartment, whereas on the other side of the GBM podocytes board the urinary pole in the glomerulus. Communications between ECs and podocytes are physiological and occur from development to adult. During DKD however, pathological mechanisms such as hyperglycemia and hypertension impact the GFB, and lesions of the glomerular endothelium and the podocyte monolayer are common in DKD patients (4). EC are central players of the GFB, and damages of ECs participate to glomerulosclerosis and albuminuria in various pathological contexts, including diabetes (5–7). Evidence of a negative loop taking place between podocytes and ECs has been reported and reviewed, where stressed ECs impair podocytes and *vice versa* (8, 9). In this review, we focus on recent *in vitro* and *in vivo* data illustrating ECs-podocytes crosstalk in diabetic conditions.

Endothelin-1 (ET-1) Is Detrimental for Podocytes and ECs in DKD

Endothelin-1 (ET-1) is a powerful vasoconstrictor and mitogen that has emerged as an interesting novel target for the treatment of DKD (10). ET-1 (EDN1) expression is increased in diabetic kidneys and higher plasmatic ET-1 levels are found in patients with diabetes as well as in animal models of DKD (11–14). ET-1 receptor blockers have renoprotective properties in several DKD (10, 15–17). ET-1 has a key role in regulating renal hemodynamics, salt and water homeostasis, and acid-base balance and in modulating cell proliferation, extracellular matrix accumulation, inflammation, and fibrosis. Consequently, any abnormality in the intrarenal ET system may result in renal dysfunction (e.g., salt sensitivity) and/or injury. Notably, the ET system is present in the renal areas targeted by diabetes, including the microvasculature, mesangial cells, and podocytes. To decipher whether the ET-1 system is a disease modifier beyond its role in the glomerular hemodynamics and sclerosis processes, our group investigated the roles of the ET receptors in podocytes in mice wherein podocyte-specific, double deletion of the ETA (EDNRA), and ETB (EDNRB) receptors was induced. These mice were protected against diabetes-induced podocyte loss and glomerulosclerosis but also provide evidence that the ETB receptor may play as important a role as does the ETA receptor. ETB receptor activation increased intracellular calcium and triggered the NF-κB and β-catenin signaling pathways, analogous to activation of the ETA receptor (**Figure 1**). The quantitative contribution of the ETB receptor may be substantial, as suggested by the fact that it is upregulated to a larger extent than the ETA receptors in the podocytes of diabetic mice. This study suggests an important role for it in mediating podocyte injury upon stimulation by ET-1, presumably produced by ECs during diabetes (18).

Recent publications from the Ilse Daehn group have also highlighted the role of ET-1 signaling in EC-podocyte crosstalk but with different mechanisms. They showed that when the forced expression of ET-1 by podocytes (and likely, in ECs) was induced through podocyte-specific activation of TGF-β signaling in transgenic mice and BALB/c mice with adriamycin-induced glomerulosclerosis, activation of ET-1 receptor type A in ECs induced mitochondrial oxidative stress and dysfunction, which in turn lead to release of yet unidentified factors mediating injury and depletion of podocytes in such experimental focal and segmental glomerulosclerosis (19). Glomerular endothelial mitochondrial dysfunction was also associated with increased glomerular ET-1 receptor type A expression and increased circulating ET-1 in experimental DKD. Moreover, pharmacological prevention of EC mitochondrial stress in this diabetes model prevented podocyte loss (20). Secreted factors from dysfunctional ECs were sufficient to cause podocyte apoptosis in supernatant transfer experiments or co-culture but this did not occur when ECs had been previously treated with mitoTEMPO, a mitochondrial antioxidant (21). Thus, ET-1 seems to be a key mediator in podocytes-to-ECs and ECs-to-podocytes communications promoting cell injury in several renal pathologies including DKD. In line with these experimental studies, the SONAR trial suggested that the ETA receptor antagonist atrasentan decreases albuminuria and the risk of major kidney outcomes when given to adults with type 2 diabetes, estimated glomerular filtration rate (eGFR) 25–75 mL/min per 1.73 m^2, and a urine albumin-to-creatinine ratio (UACR) of 300–5,000 mg/g who had received maximum labeled or tolerated renin–angiotensin system inhibition for at least 4 weeks (10). Interestingly, albuminuria decrease with atrasentan was consistent irrespective of sodium glucose cotransporter 2 inhibitor (SGLT2i) use before enrolment in the SONAR trial, suggesting that the effects of atrasentan are additive to SGLT2i (22).

Glomerular Glycocalyx Degradation in DKD

In glomeruli, the glycocalyx surrounding endothelial cells creates a space between the blood and the endothelium, controlling vessel permeability, restricting leukocyte and platelet adhesion, and allowing an appropriate endothelial response through mechanosensing. The negative charge of the glycocalyx on podocytes also repulses proteins, contributing to permselectivity towards negatively charged plasma proteins that limit their leakage in the urine. A study of Pima Indians with type 2 diabetes found that both podocyte damage and glomerular endothelial injury were commonly present in a cohort with macroalbuminuria. Interestingly, compared with podocyte injury, endothelial abnormalities were more closely associated with increased urine albumin excretion, suggesting that endothelial cell injury may be more critical to glomerular alterations in DKD compared with the commonly viewed importance of podocyte injury. Glycocalyx composition includes proteoglycans, glycoproteins, glycolipids, and glycosaminoglycans. Increased expression of proteolytic enzymes such as MMP9 (23–25), hyaluronidase (26), or heparanase [reviewed in van der Vlag and Buijsers (27)] was observed in diabetic patients and could participate in glycocalyx degradation in such pathological context, thus promoting proteinuria in diabetic patients (28, 29). MMP9 is mainly produced by podocytes and parietal epithelial cells in DKD where it participates in podocyte injury and promotes

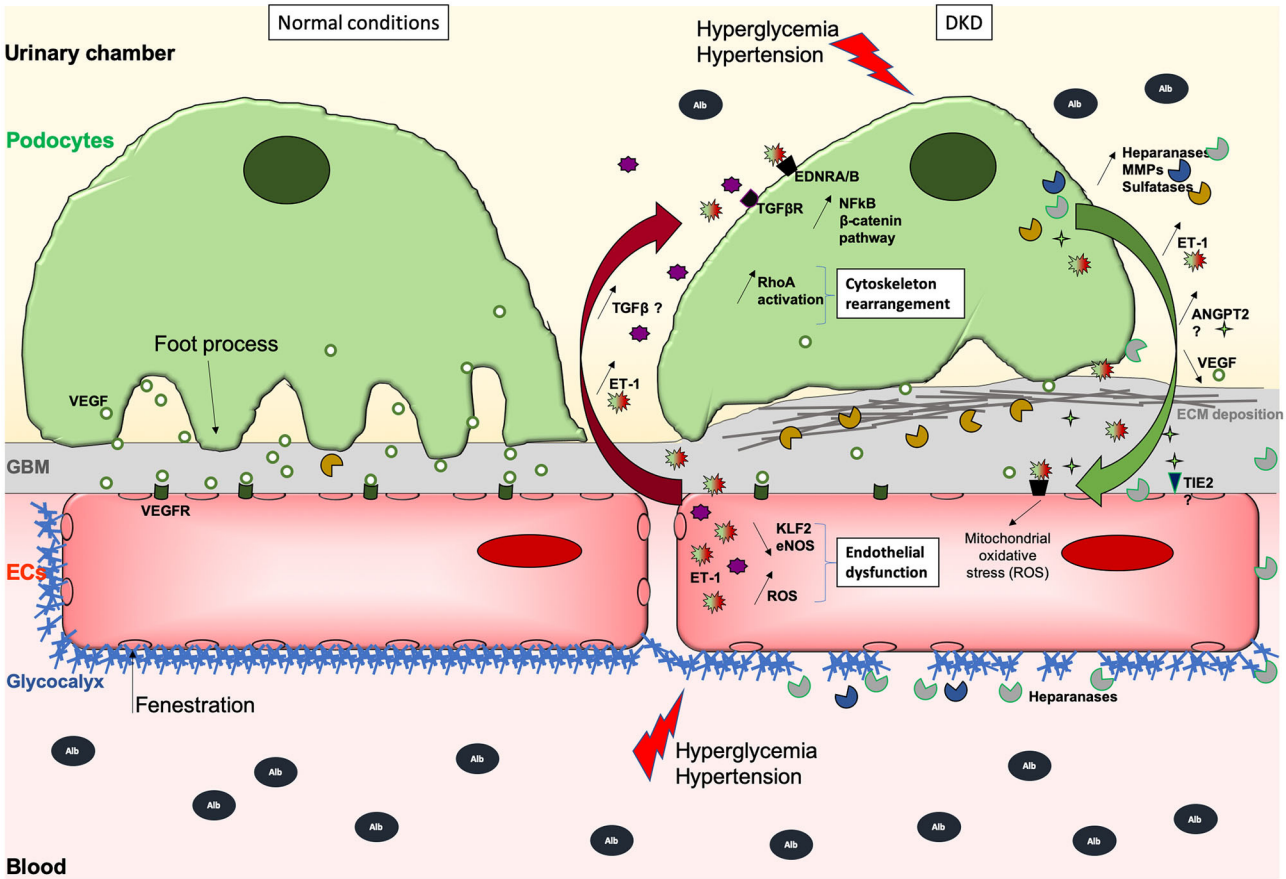

FIGURE 1 | Schematic illustration of the GFB during normal (left) and DKD (right) conditions. During DKD, podocytes lose their foot processes, dedifferentiate with transiently increased ANGPT2 and VEGF production, and then detach or die, which then leads to fewer VEGF secretion. Initial and sustained loss of endothelial permselectivity is also fostered by ANGPT2/ANGPT1 imbalance fostering EC lesions through binding to its receptor TIE2. This initially reduces ECs quiescence and impairs permselectivity then ECs viability, and stimulates ET-1 synthesis that in turn acts on the other side of the GFB, activating podocyte Wnt/beta-catenin and NFkB pathways, heparanase release, inappropriate cytoskeletal remodeling, and abnormal extracellular matrix synthesis causing GBM thickening. ET-1 produced by EC but also by podocytes may also contribute to increased oxidative stress and secretion of proteases, which can degrade the glycocalyx, creating a vicious loop. Hence, ECs are subjected to oxidative and mitochondrial stress, lose their fenestrations, do not exert their functions properly, and may die. Deleterious effects of (ECs-derived?) -TGFβ-mediated signaling on podocytes are suggested. Moreover, the overproduction of sulfatases in the GBM could decrease the bioavailability of growth factors needed by ECs. The GBM thickens due to extracellular matrix (ECM) protein deposits, such as collagen fibers and fibronectin which augments the distance between rarefied podocytes and ECs, potentially altering podocyte to EC bidirectional signal cross-talk.

extracellular matrix (ECM) synthesis (23). Interestingly, MMP9 also promotes syndecan-4 shedding at ECs cell surface (30) and MMPs inhibition in a mouse diabetic model prevents syndecan-4 degradation and glycocalyx disruption in glomeruli (31). Whether it is the production by podocytes or by other cell types that induce ECs glycocalyx degradation in this context remains to be explored.

Heparanase is strongly up-regulated in podocytes exposed to high glucose (32, 33) (**Figure 1**). The increased heparanase expression by podocytes in kidneys has been demonstrated in DN (32, 34), and is essential for the development of albuminuria DN in both animal models and likely, in human (35, 36). Mice that lack heparanase develop less proteinuria or structural injury in diabetes induced with streptozotocin. Notably, loss of glycocalyx has been suggested in patients with type I diabetes 1 (37). Further, the development of microalbuminuria in diabetic patients results in further reductions of the systemic glycocalyx, leading to systemic vascular dysfunction (38). In Pima Indians with type 2 diabetes, podocyte foot processes in microalbuminuric participants were not different from those in control subjects and although microalbuminuria in type 2 diabetic Pima Indians often heralds progressive glomerular injury, it is not the result of defects in the size permselectivity of the glomerular barrier but rather of changes in either glomerular charge selectivity or tubular handling of filtered proteins or of a combination of these two factors (39). Another interesting study confirmed more directly that glycocalyx is perturbed in individuals with type 2 diabetes mellitus, and oral glycocalyx precursor treatment improved glycocalyx properties (28).

Garsen et al. demonstrated that heparanase production by podocytes promotes heparan sulfate degradation and glycocalyx disruption at podocyte and the endothelial cell surface in

diabetic context by using *in vivo* mouse models and *in vitro* podocyte-to-EC supernatant transfer approaches (40) (**Figure 1**). Interestingly, in this latter article, the authors demonstrated that diabetes-mediated heparanase production in podocytes is mediated by endothelin pathway activation in podocytes in response to ET-1 production by ECs. Eberfors et al. also found that ET-1 signaling mediates degradation of the glomerular endothelial glycocalyx in non-diabetic kidney disease *via* pathological crosstalk between activated podocytes and glomerular endothelial cells but with a different mechanism. Indeed, here the authors found increased heparanase and hyaluronoglucosaminidase gene expression in glomerular ECs in response to podocyte-released factors and to ET-1 (41). Boels et al. further confirmed the crucial role of the endothelin pathway on heparanase expression and glycocalyx injury. Atrasentan, an antagonist of the endothelin receptor ETA, prevented glycocalyx degradation in DKD through reduction of glomerular and endothelial heparanase expression, although the production of heparanase by podocytes was not specifically explored in this context (42).

VEGF Family Pathway Dysregulation in Glomeruli During DKD

Podocytes act as pericyte-like cells to support ECs differentiation and notably produce VEGF which is crucial to maintain glomerular ECs differentiation. In a pioneer work published in 2008, Hirschberg et al. showed that VEGF is upregulated in podocytes after high glucose (HG) treatment (43). VEGF is sufficient to promote proliferation and tube formation of blood outgrowth EC (BOEC) through Flk-1 *in vitro*, and co-culture of podocytes and BOEC also enhances proliferation of the latter, hence emphasizing the role of podocyte-derived VEGF. These data suggested a podocyte role on angiogenesis during the early onset of diabetic nephropathy *in vivo* (44, 45). The role of the VEGF family *in vivo* has already been extensively reviewed elsewhere (46–51), and the growing evidence point to the role of VEGFA and VEGFC during DKD. VEGFA is mainly expressed by podocytes, can be alternatively spliced in different isoforms such as VEGFA165b, and bind the endothelial receptors VEGFR1 and VEGFR2 (**Figure 1**). Besides, other members of the family include VEGFB, VEGFC, VEGFD, and PlGF. VEGFC can act both on lymphatic and blood vessels through VEGFR3 and VEGFR2, respectively. *In vitro*, VEGFC protects glomerular ECs from the negative influence of VEGFA reducing their permeability, and podocyte VEGFC overexpression protects ECs during diabetes *in vivo* (52). Besides, VEGFA and diabetic conditions (in db/db mice) increase glomerular albumin permeability *ex vivo*, which is rescued by VEGFC treatment. VEGFA is increased in HG-cultured podocytes, and a direct axis TGFβ1/VEGFA/AP-1, terminating in Bcl2 reduction and podocyte apoptosis, has been described (53). In the same study, inhibition of VEGFA or AP-1 was beneficial for diabetic rats. However, VEGF-A165b improved the permeability of isolated diabetic human glomeruli, and diabetic mice treated with VEGF-A165b or having a specific podocyte-overexpression of it develop a less severe phenotype (54). Dysregulation of the VEGF pathway during DKD would be probably more complicated than just dysregulation of VEGF synthesis by podocytes (**Figure 1**). The Semaphorin 3-Neuropilin axis is an important regulator of podocyte-to-endothelial cells during development (55) and plasmatic and urinary Semaphorin 3 expressions are positively associated with DKD (56, 57). Furthermore, advanced glycation end-products suppress Neuropilin 1 expression in podocytes, thus promoting their migration *in vitro* (58). Podocyte-selective Semaphorin 3A overexpression exacerbates DKD in mice by remodeling podocyte cytoskeleton and ECM synthesis (59). In addition to Semaphorin 3A signaling, Neuropilin 1 plays an important role in endothelial cells *via* its binding to VEGFA. Nevertheless, Neuropilin 1 expression in glomeruli seems restrained to podocyte and decreased during diabetes in mice (58) and to our knowledge, no one has modulated Neuropilin 1 expression specifically in endothelial cells during DKD to explore its function in such pathological context. Neuropilin 1 receptor could function as an extracellular scaffold protein generating podocyte-endothelial cell cross-signaling during DKD. Together, these data illustrate the intertwined and multiple effects of the VEGF family during diabetes.

Other Influences of Glomerular ECs on Podocyte Injury During DKD

Physiologically, loops of glomerular capillaries are subjected to mean laminar shear stress (LSS) estimated at 10–20 dyne/cm^2 (60). Slater et al. have shown that glomerular ECs submitted to LSS have increased ERK5 pathway activation leading to high KLF2 expression, which promotes ET-1, NO, and eNOS secretion (61). High KLF2 expression has also been observed in human glomeruli *ex vivo*. However, using conditioned media transfer from ECs to podocytes, and a co-culture strategy, they also show that LSS-exposed ECs secrete factors reducing the resistance of a podocyte monolayer. Of note, KLF2 is reduced in ECs exposed to high glucose but increased by insulin (62). In the same work, the authors showed that mice deficient for KLF2 in ECs have an aggravated phenotype during diabetes, and more pronounced podocyte lesions associated with higher glomerular mRNA levels of *Vegfa*, *Flk1*, and *angiopoietin 2*, and lower *Flt1*, *Tie2*, and *angiopoietin 1* levels. These data highlight crosstalk between ECs and podocytes relying on KLF2 endothelial expression level, which promotes in basal conditions an anti-inflammatory phenotype and appears required for ECs-podocytes homeostasis (**Figure 1**).

One of the first works identifying EC-to-podocyte crosstalk in DKD came from Isermann et al. Indeed, they demonstrated that activated protein C (APC), which is regulated by endothelial thrombomodulin, is downregulated in DKD. They used gain-of-function and loss-of-function complementary approaches in diabetic mice to show that APC inhibits hyperglycemia-induced endothelial and podocyte mitochondrial-dependent apoptosis (63).

Yuen et al. unraveled the role of eNOS in the EC-podocyte crosstalk (64). Mice deficient in eNOS develop podocytopathy, although eNOS is expressed in endothelial cells, but not in podocytes (65, 66). Authors have observed a marked cytoskeleton

rearrangement of podocytes treated with the serum of diabetic eNOS-deficient mice, which suggests a modulation of the RhoA family that controls cytoskeleton dynamics. Moreover, increased activation of RhoA in podocytes treated with supernatants from glomerular ECs exposed to high glucose and/or angiotensin II isolated from diabetic eNOS-deficient mice was observed, despite the reduction of RhoA activity when ECs were from control mice. Nevertheless, the modulation of RhoA in podocytes has been reported detrimental (67).

Transforming growth factor-beta (TGF-β) is a well-described mediator of renal fibrosis in DKD with pleiotropic effects on glomerular cells. It promotes mesangial cell hypertrophy and extracellular matrix deposition and induces endothelial and podocyte dedifferentiation or death in mouse diabetic models [Reviewed in Chang et al. (68) and Ghayur and Margetts (69)]. TGF-β1 and TGF-β2 may originate from several cell types in DKD (in particular mesangial cells and EC) and as a secreted molecule, it would not be surprising that TGF-β could promote glomerulosclerosis and GFB dysfunction in DKD through paracrine mechanisms and participate in cross-communication within the GFB. The mechanisms for such deleterious effects of TGF-β on the GFB cellular components, podocytes and EC, are still unclear, whereas this growth factor displays contrasting hypertrophic and survival actions on other cell types such as fibroblast, mesangial cells or vascular smooth muscle cells.

A recent publication from regret Detlef Schlondorff's group highlighted the role of BAMBI a negative modulator of TGF-β1 in DKD with specific roles in ECs and podocytes (70). Interestingly the authors demonstrated that in diabetes, selective EC-*Bambi* deletion induced podocyte injury similarly to a selective podocyte-*Bambi* deletion. Diabetes-induced podocyte loss was even more pronounced in the EC-*Bambi* KO than in the podocyte-*Bambi* KO mice. Similarly, endothelial-selective autophagy inhibition also promotes podocyte injury in DN, supporting the concept that ECs injury in DKD may be a crucial mediator of podocyte injury and underscoring the importance of glomerular crosstalk in DKD (71).

ECs communicate with podocytes through secreted proteins, but also *via* exosomes during diabetes. To our knowledge, only one work from Wu et al. demonstrates that during diabetes, ECs could negatively affect podocytes by releasing exosomes (72). In HG conditions, ECs undergo endothelial-to-mesenchymal transition, secrete more exosomes, and the latter are internalized by podocytes which increased the TGF-β1/Wnt/β-catenin pathway. Consistently, podocytes treated with HG-cultured EC-derived exosomes are more permeable to albumin *in vitro*. Activation of the Wnt/β-catenin pathway in podocytes during diabetes and other proteinuric kidney diseases is known to be detrimental (73–75), and leads to oxidative stress (76).

Other Influences of Podocytes on Glomerular ECs

In recent work, Ngo et al. collected plasma samples to assess renal arteriovenous gradients (77). A positive correlation between testican-2 and eGFR, and an association between higher baseline testican-2 levels and slower decline of eGFR, were observed in cohorts of patients including diabetics. Interestingly, testican-2 is expressed by podocytes and glomerular basal membrane, and can increase glomerular EC tube formation and motility, but not proliferation, *in vitro*. Hence, secreted testican-2 from podocytes seems beneficial for glomerular ECs in a variety of chronic kidney diseases including diabetes. Of note, testican-2 is not expressed in mice (78), illustrating the need for *in vitro* models to study podocyte-EC crosstalks. More complex culture models have been investigated, being 3D tri-partite cell cultures or "glomerulus-on-chip" systems. Waters et al. used glomerular EC, mesangial cells, and podocyte to reproduce the glomerular filtration barrier in 3D cultures (79). They showed that TGFβ-induced glomerulosclerosis, as seen in DKD, was prevented in 3D tri-cultures by conjoint inhibition of ALK5 and CTGF, and differential effects of TGFβ on mesangial cells and glomerular ECs. TGFβ led to nodule formation and loss of ECs arborization in 3D tri-cultures, and in mono-cultures, it increased the mediator CTGF expression in podocytes and increased ALK5 expression in mesangial cells, which favors an upregulation of TGFβ pathway activation through SMAD2/3. Moreover, BMP7 appeared to modulate the effects of TGFβ on ECs but not in mesangial cells.

Another well-known angiogenic signaling is likely to be involved in podocyte-to-endothelial cell cross-communication in DKD, whereas direct evidence is missing. Angiopoietin 1 (ANGPT1) produced by podocytes promotes maturation and stabilization of glomerular capillaries *via* TIE2 receptor on endothelial cells (80), playing an important role in the regulation of angiogenesis, endothelial cell survival, proliferation, migration, adhesion, and spreading, but also maintenance of vascular quiescence. Angiopoietin 2 (ANGPT2) has opposite effects to ANGPT1 by destabilizing blood vessels and effects of ANGPT2 are dependent on VEGFA levels (81, 82). ANGPT2 levels are associated with indexes of endothelial dysfunction in clinical diabetes mellitus (83–85). A decreased circulating ANGPT1/ANGPT-2 ratio may contribute to the development of DKD after administration of STZ in mice (86) and STZ-induced DKD rats (87). Meanwhile, plasma ANGPT2, like VEGF, was found to be raised in human diabetes regardless of vascular disease. Whereas, both growth factors correlated with HbA1c and with each other, ANGPT2 levels did not correlate with carotid atherosclerosis, plasma von Willebrand factor (vWf), and urine albumin to creatinine ratio in humans with type 2 diabetes after multiple adjustment (88). This is not surprising as the ANGPT system is regulated locally in the microvessels. Regulation of glomerular angiopoietin levels is key in animal models of DKD. To further investigate the role of ANGPT1 in diabetes, Jeansson et al. compared diabetic controls and *Angpt1*-deleted mice induced with STZ. The *Angpt1* knockout kidney showed accelerated diabetes-mediated glomerular damage, suggesting that ANGPT1 could potentially protect the glomerular microvasculature from diabetes-induced injury (89). Mice with podocyte-specific inducible ANGPT-1 overexpression in the early stage of DKD led to a 70% reduction of albuminuria and prevented diabetes-induced GEC proliferation via increased

TIE-2 phosphorylation suggesting a critical role of ANGPT1-1/ANGPT-2 in early DKD. Meanwhile, hyperfiltration and renal morphology were unchanged, indicating a still limited role (90). The role of angiopoietins in DKD has been nicely reviewed elsewhere (91, 92). Overall, the authors consider that angiopoietins are produced by podocyte and signal *via* TIE2 in endothelial cells only, which is most likely the mechanism but has not been entirely demonstrated.

Finally, dysregulation of the GBM composition by podocytes could also impair ECs in DKD. Indeed, heparan sulfate, the major component of glomerular ECM, modulates growth factor signaling, notably modulating VEGFA availability to the surrounding ECs. Schumacher et al. showed that Wilms' Tumor 1 changes VEGFA and FGF2 signaling by increasing the expression of the 6-*O*-endosulfatases Sulf1 and Sulf2, which remodel the heparan sulfate 6-*O*-sulfation pattern in the GBM (93). Mice deficient in both *Sulf1* and *Sulf2* developed age-dependent proteinuria as a result of ultrastructural abnormalities in podocytes and endothelial cells. Sulf1 and Sulf2 double-knockout (DKO) mice also showed glomerular hypercellularity, matrix accumulation, and GBM irregularity. Platelet-derived growth factor (PDGF)-B and PDGF receptor-β were upregulated in Sulf1 and Sulf2 DKO mice. Diabetic mice showed an upregulation of glomerular Sulf1 and Sulf2 expression and diabetic Sulf1 and Sulf2 DKO mice showed an acceleration of the glomerular pathology without glomerular hypertrophy (94). Thus, Sulf1 and Sulf2 may play protective roles in DKD, probably by modulating growth factor availability to podocytes and ECs.

DISCUSSION

Across the years, researchers tried to model pathological conditions happening during DKD, by culturing EC and/or podocytes with HG. More recently, this EC-podocyte crosstalk has been explored with conditioned media transfers. This shed light on the crucial role of VEGF, produced by podocytes for EC survival and dysregulated during DKD (43, 50, 95), but also to signaling pathways modulated in EC or podocytes during the excess of glucose. In parallel, rodent DKD models were developed, confirmed both of these observations, and even more. Transgenic animals gave substances to the former hypothesis and opened new horizons, as integrated systems (96). Nevertheless, cell cultures remain powerful tools, they tend to naturally evolve from monolayered and mono-typed to multi-typed, organoids and even glomerulus-on-chip, to represent an alternative, or at least an intermediate, to *in vivo* studies. Recently, Wang et al. developed a tri-partite glomerular filtration barrier in a 3D culture from rat glomeruli and showed that high glucose conditions in the endothelial side increased barrier permeability and podocyte migration in this model (97). Zhou et al. developed a device composed of ECs and podocytes cultured together but physically separated by a porous membrane (98). Hypertensive conditions on the endothelial compartment led to increased barrier permeability and damages of both EC and podocytes. Hence, *in vitro* models constitute an efficient way to study glomerular ECs – podocytes crosstalk during DKD. More importantly, they are useful to dissect molecular pathways involved in pathophysiology. Recent advances in 3D cell cultures and microfluidics tend to combine the comfort and high throughput of *in vitro* assays with partial biological relevance of *in vivo* studies. A significant limitation of such 3D systems being obviously to poorly mimic the pathophysiological environment of glomerular cells as would occur in chronic diabetic condition.

ECs - podocytes crosstalk is crucial during DKD development, where ECs also dialogue with other renal cell types. As an example, the elevation of EC-secreted ET-1 directly enhances mesangial expansion and extracellular matrix deposits, characteristics of DKD (99). ECs injury could also participate in parietal epithelial cell activation, a condition seen in focal segmental glomerulosclerosis (FSGS), a rare-to-common renal complication of diabetic patients. Indeed, Luque et al. demonstrated that Hif2α pathway inhibition in endothelial cells only sensitized mice to the development of hypertension-induced FSGS, suggesting that signals from the ECs could be transferred to PEC (100). Finally, renal tubular cell injury in diabetes modulates ECs [reviewed in Chen et al. (101)].

Together, these studies showed through different methodologies, from cell cultures to human samples, that glomerular ECs are crucial actors of DKD pathophysiology, and cross-communications with podocytes constitute major events for diabetic renal disease progression. Intensive control of glucose and blood pressure along with RAS inhibition and SGLT2i (for patients with type 2 DM) remain the clinical gold standards to deter the progression of DKD. The armament may be complemented in the near future with glucagon-like peptide 1 (GLP-1) receptor agonists, non-steroidal selective mineralocorticoid receptor antagonists (MRAs) or ETRAs. In fact, metformin, RAAS and ET-1 inhibitors were shown to prevent endothelial dysfunction beyond their effect on insulin resistance. Recent anti-diabetic drugs also display clear effects on the microcirculation in animal models and patients (102–107). Additional work is needed to understand the mechanisms involved, and new treatments that aim to prevent microvascular injury or restore microvascular function could be an effective strategy for preventing; or even reversing DKD. Such strategies may consider crosstalk within the glomerular system. Fine tuning of angiogenic systems and cellular energetics, promotion of autophagy, of glycocalyx protection, alleviation of chronic sterile inflammation and senescence may offer promising perspectives. The variety of molecules involved represents as many potential therapeutic targets to better take charge of the DKD burden and improve patient lives. Future studies need to consolidate the concept of the glomerulus as an integrated functional unit.

AUTHOR CONTRIBUTIONS

NM, OL, and P-LT wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Role of Non-coding RNAs in Diabetic Nephropathy-Related Oxidative Stress

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Diabetic nephropathy (DN) is one of the main complications of diabetes and the main cause of diabetic end-stage renal disease, which is often fatal. DN is usually characterized by progressive renal interstitial fibrosis, which is closely related to the excessive accumulation of extracellular matrix and oxidative stress. Non-coding RNAs (ncRNAs) are RNA molecules expressed in eukaryotic cells that are not translated into proteins. They are widely involved in the regulation of biological processes, such as, chromatin remodeling, transcription, post-transcriptional modification, and signal transduction. Recent studies have shown that ncRNAs play an important role in the occurrence and development of DN and participate in the regulation of oxidative stress in DN. This review clarifies the functions and mechanisms of ncRNAs in DN-related oxidative stress, providing valuable insights into the prevention, early diagnosis, and molecular therapeutic targets of DN.

Keywords: diabetic nephropathy, oxidative stress, ncRNA, mircoRNA, therapeutic target

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INTRODUCTION

Diabetic nephropathy (DN) is one of the main complications of diabetes mellitus (DM) and the main cause of diabetic end-stage renal disease (ESRD), resulting in the disability and death of patients with DM (1, 2). DN is characterized by progressive renal interstitial fibrosis, which is accompanied by a series of pathological changes, including excessive accumulation of extracellular matrix (ECM) components, thickening of the glomerulus and tubular basement membrane, and increased formation of glomerular and tubular basement membrane matrix (3, 4). The increased prevalence of DM has led to an increase in the incidence of DN. DN is a leading cause of ESRD (5, 6), which is one of the major health problems worldwide.

The imbalance between oxidants and antioxidants is called oxidative stress (OS) and occurs when the body is subjected to various harmful stimuli, leading to the injury of tissue and cells (7). OS exists in all stages of DM development, and hyperglycemia is the main factor promoting OS (8–12). In addition, the advanced glycation end products (AGEs) associated with hyperglycemia (13), reactive oxygen species (ROS) (14, 15), the protein kinase C (PKC) pathway (16), and the renin-angiotensin system promote the occurrence of OS (17) and its maintenance, and cause the

development of DN (8). Furthermore, the abundance of mitochondria in kidney tissue renders it more vulnerable to OS (18). Intrarenal OS, which also plays a vital role in the pathogenesis of DN, causes chronic inflammation of the kidney, and glomeruli and tubular hypertrophy (19).

Ribonucleic acid (RNA) is divided into two categories according to its characteristics: coding RNA and non-coding RNA (ncRNA). Research on ncRNAs has demonstrated that these molecules are not simply "junk" transcription products but functional regulatory molecules that mediate cellular processes. Many ncRNAs affect specific cellular biological responses, and are key regulatory molecules in the course of disease (20). For example, small ncRNAs, such as, microRNAs (miRNAs), may act as proto-oncogenes or tumor suppressor genes in cancers (21, 22); circular RNAs (circRNAs) participate in the development of tumors, neurological diseases, and rheumatic diseases (23–25); small nucleolar RNAs (snoRNA) participate in tumors, metabolic stress, and other diseases through modification (26, 27); long-chain ncRNAs (lncRNAs) have been linked to cancer, diabetes, heart failure, hypertension, kidney disease, and other diseases (28–32). Thus, ncRNAs are a new hot spot in epigenetic research. Although, the role of several ncRNAs in disease pathogenesis has been revealed, their specific regulatory networks have yet to be studied.

CLASSIFICATION OF ncRNAs

The Human Genome Project led researchers to discover that protein-coding sequences only account for nearly 2% of the human genome, while the remaining non-coding regions were considered "junk areas." The development of computational biology and the popularization of genome sequencing technology showed that these "junk regions" are transcribed into large amounts of RNA, of which nearly 74% are ncRNAs (33, 34). According to their functions and sizes, ncRNAs are roughly divided into three categories: housekeeping ncRNAs, small RNAs (sRNAs), and lncRNAs; circRNAs constitute a special type of lncRNA. Housekeeping ncRNAs are essential for cellular activities, and include ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs), and snoRNAs (35). sRNAs generally refer to ncRNAs shorter than 200 nucleotides (nt). According to their species origin, they are divided into two categories: bacterial sRNAs and eukaryotic sRNAs. Eukaryotic sRNAs include miRNAs, small interfering RNAs (siRNAs), and PIWI-interacting RNAs (piRNAs). lncRNAs have lengths of more than 200 nt (36), and are widely transcribed in the genome. According to their transcriptional location, molecular characteristics, or position relative to mRNAs, lncRNAs are divided into long intervening/intergenic ncRNAs, which are located in the gap between two mRNAs and have an independent transcription; natural antisense transcripts, which reversely overlap with an mRNA exon; promoter upstream transcripts (prompts); enhancer RNAs, which are transcribed from enhancers of protein-coding genes; and circRNAs (37, 38).

BIOLOGICAL CHARACTERISTICS OF ncRNAs

ncRNAs are widely involved in important biological functions, such as, the development and differentiation of cell development and differentiation, reproduction, cell apoptosis, and cell reprogramming, and are closely associated with disease development and progression. To date, many studies on miRNAs, lncRNAs, and circRNAs have been conducted (**Figure 1**).

miRNA Function

It is generally believed that miRNAs bind to RNA-induced silencing complexes in the cytoplasm, recognizing, and binding their matching target sequence (usually in the 3′ untranslated region of the protein-coding gene) in a sequence-specific manner, thus, regulating the degradation of target gene mRNA and/or its translation to inhibit gene expression. However, some studies have suggested that some miRNAs, such as miR-320, miR-373, miR-122, and miR-483, may also promote or inhibit the expression of target genes in the nucleus (39). Nuclear miRNAs may recognize and bind target sequences in gene promoters and other DNA regulatory elements in a sequence-specific manner, and then recruit proteins or complexes responsible for epigenetic modification, which results in chromatin remodeling, resulting in transcriptional activation or gene silencing. Alternatively, nuclear miRNAs inhibit lncRNAs near target genes, thereby regulating gene expression (40). At present, the reasons, mechanisms, and functions of miRNA accumulation in the nucleus remain unclear and require further investigation. Based on the role of miRNAs in regulating target gene expression, the functions of miRNAs are determined by the functions of their target genes. If the target genes regulate cell proliferation, apoptosis, differentiation, and other important biological functions, the miRNAs will play an important role in these biological functions. miRNAs have obvious cell line specificity in the regulation of target genes. Different miRNAs in the same cell line have different roles, and the same miRNAs in different cell lines also have different functions. Hundreds of miRNAs in cells have a complex regulatory role in tens of thousands of protein-coding genes, forming a genome-wide expression regulatory network that keeps protein expression at normal levels. Hence, miRNA abnormality often affects important physiological processes and triggers the occurrence of major diseases. Studies have shown that the abnormal regulation of miRNAs is a common feature of many diseases (41), making them a novel interventional target. Due to the stability of miRNAs in organisms, their application in the diagnosis and prognosis of disease will be useful. In short, miRNAs have tremendous potential in disease treatment and translational medicine.

lncRNA Function

lncRNAs are linear ncRNA transcripts with complex structures, and are widely expressed in mammalian genomes. Compared with mRNA, lncRNAs have low expression levels, high tissue specificity, and strong temporal and spatial expression specificity (42). It is estimated that more than 10,000 lncRNAs exist

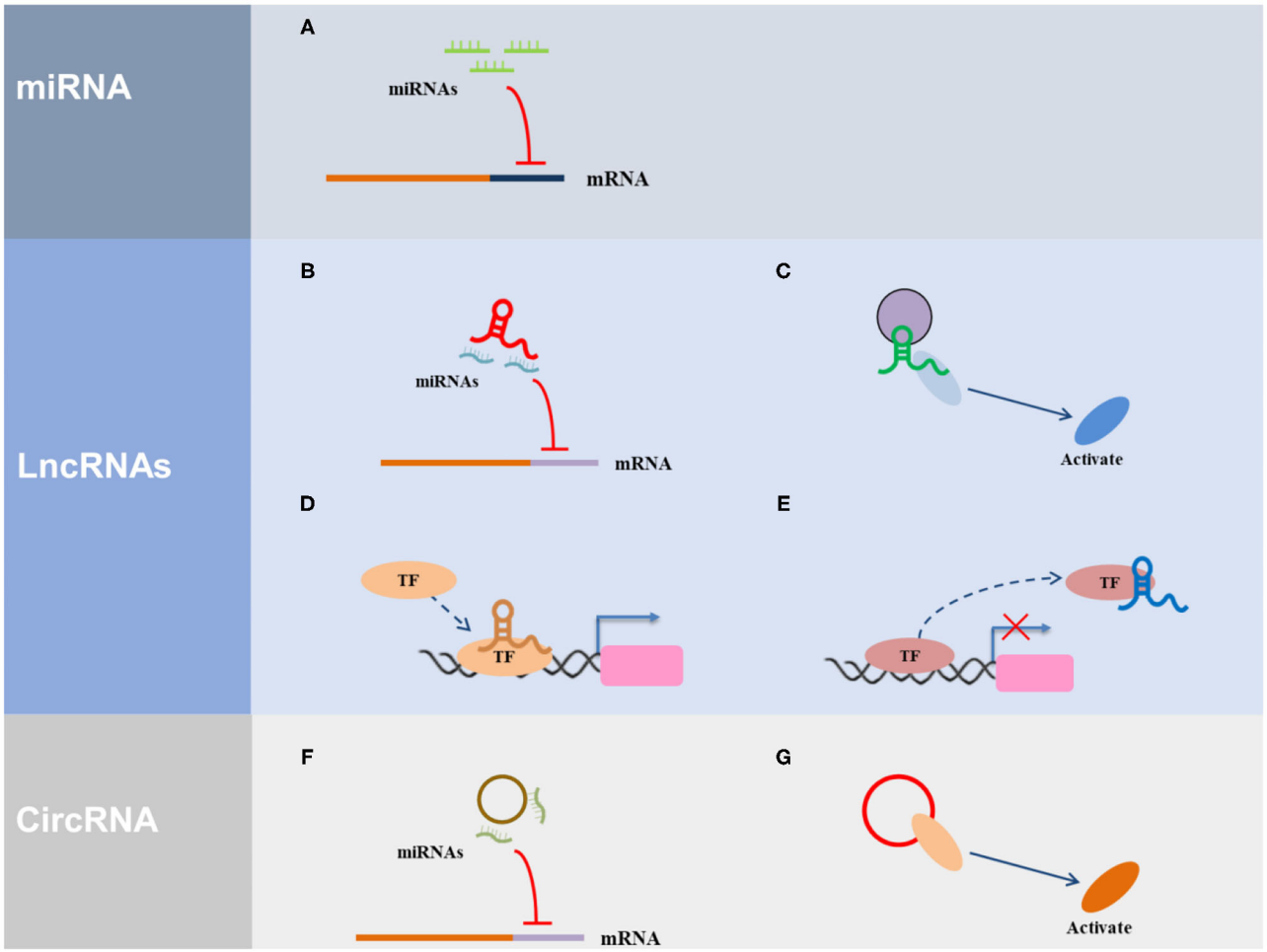

FIGURE 1 | The regulatory mechanism of ncRNAs. **(A)** miRNA can direct bind with the 3′ UTR of the target gene. **(B)** LncRNA serves as ceRNA to interact with miRNAs. **(C)** LncRNA acts as a scaffold *via* recruiting and interacting with proteins and regulate the activity of proteins. **(D)** LncRNA acts as a guide to promote the gene expression *via* recruiting transcription factors (TF) to the region of the gene promoter. **(E)** LncRNA acts as a decoy *via* interacting with TF to inhibit transcriptional regulation. **(F)** CircRNA serves as ceRNA to interact with miRNAs. **(G)** CircRNA acts as a scaffold *via* recruiting and interacting with proteins and regulate the activity of proteins.

in the human genome. However, biochemical identification and functional research is still in its infancy. At present, the functions of only ∼100 lncRNAs are understood. These lncRNAs regulate the expression or activity of target genes at the DNA, RNA, and protein levels, and are widely involved in a variety of important regulatory processes, such as, the inactivation of the X chromosome, genomic imprinting, stem cell pluripotency, somatic cell reprogramming, chromatin remodeling, the formation of nuclear substructures, and intranuclear transport, and are associated with the occurrence and development of many human diseases, such as, tumors, cardiovascular diseases, and neurological diseases (43–47). lncRNA regulation of target protein-coding genes occurs both during and after transcription, as lncRNAs may be located in the nucleus or cytoplasm. It has been shown that 30% of all lncRNAs are located in the nucleus, 15% in the cytoplasm, and the rest are found in both the nucleus and the cytoplasm.

Recently, with increasing research and the development of sequencing technology, an increasing number of lncRNAs and functions have been reported. Currently, there are over 20,000 lncRNA annotations, which is more than protein-coding gene annotations. For the whole genome of a cell, the expression of lncRNAs is much lower than that of protein-coding genes, and exhibits obvious tissue and cell line specificity. Some lncRNAs begin to function at certain stages of eukaryotic development. Because lncRNAs regulate the expression of protein-coding genes in different ways, lncRNAs are involved in many important biological processes, including heredity, development, cell cycle, and changes in chromosome structure. lncRNA

abnormalities are involved in the development of many common diseases (45, 47, 48).

circRNA Function

circRNAs are special ncRNAs that form closed loop structures through covalent bonds (49, 50). circRNAs are believed to mainly exist in the cytoplasm, with a small amount found in the nucleus (51, 52). circRNA expression is extremely abundant in eukaryotes and is evolutionarily conserved. Although, they do not encode proteins, they interact with proteins, regulate the variable splicing process of pre-mRNA, and regulate the maturation of rRNAs (53, 54). In addition, circRNAs play an indispensable role in the normal physiological processes of biological reproduction, growth, and aging, and are involved in the occurrence and development of neurological diseases, autoimmune diseases, cardiovascular diseases, and tumors (23, 55). Some circRNAs regulate the expression of protein-coding genes by competitively binding miRNAs (56). Furthermore, both lncRNAs and circRNAs are used as competitive endogenous RNAs to combine with miRNAs, forming an interactive regulatory network (57). The development of bioinformatics technology will allow further understanding of the functional role of these three types of ncRNAs, which will help us clarify the pathogenesis of certain diseases and develop therapeutic strategies and drugs.

Other ncRNAs Function

Other ncRNAs mainly contain snoRNAs, which can be divided into three categories based on their structural elements: box C/D snoRNA, box H/ACA snoRNA, and MRP RNA (58, 59). The main snoRNAs in cells are box C/D and box H/ACA snoRNAs. In addition, snoRNAs can be classified based on their gene organization, into independently coding snoRNA and intron coding snoRNA. snoRNAs can be classified based on their gene organization, ranging from independently transcribed genes under the control of independent promoters, to intronic coding units, which lack an independent promoter and are encoded in introns of protein-coding host genes. snoRNA participates in the biosynthesis of eukaryotic ribosomes, mainly to guide the modification of nucleotides at specific sites and participate in rRNA shearing (59, 60). Cajal body-specific small RNA (scaRNA) is similar to snoRNA and has box C/D or boxH/ACA structural elements (61). scaRNA guides the nucleotide modification of snRNA. Moreover, a special class of molecules including U85, U87, U88, U89, with both box C/D, and box H/ACA domains were found, which can simultaneously guide the ribose methylation and pseudouracilization of U5 and U4 snRNA. Vertebrate telomerase is a box H/ACA telomerase box, which may be essential for *in vivo* telomerase accumulation, 3′ end processing of the telomerase RNA precursor to ensure the stability of mature RNA, and telomerase activity. Mutations in human telomerase box H/ACA motif or the bound small nucleolar ribonucleoprotein (snoRNP) dyskerin cause multi-system genetic diseases (62). Although, some small RNAs have the typical structure of box C/D or box H/ACA, and primarily guide the chemical modification of other RNAs, including rRNAs, tRNAs, and snRNAs, a large subclass of snoRNAs called orphan snoRNAs cannot find complementary sequences that match rRNA or snRNA (63). It has been reported that many RNAs that are not directly related to ribosomal biosynthesis, including a small number of mRNAs, can temporarily stay in the nucleolus. Moreover, in yeast, the RNAse P-mediated 5′ end processing of some tRNA precursors occurs in the nucleolus. Therefore, it is possible that orphan snoRNAs act on RNAs other than rRNA and snRNA.

DN PATHOGENESIS

DN is characterized by pathological albumin excretion or albumin/creatinine ratio in the urine of patients with diabetes and a decrease in the glomerular filtration rate (64–66). Pathological changes in DN, such as, glomerulus enlargement, basement membrane thickening, and accumulation of glomerular mesangial and tubular ECM, lead to glomerular and tubular interstitial fibrosis, and even hardening (67, 68). DN is the main cause of ESRD worldwide and is related to the incidence and mortality of cardiovascular events (8, 69, 70). Risk factors for the occurrence and development of DN include increased inflammation, oxidation markers, AGEs, ROS, elevated levels of transforming growth factor-β (TGF-β), elevated PKC levels, abnormal polyol metabolism, uric acid levels, a long history of DM, age at diagnosis, race, systemic or glomerular hypertension, proteinuria, genetic susceptibility, insulin resistance, and diet composition (69, 71).

The pathogenesis of DN is complex process, involving in a series of signaling pathway changed (**Figure 2**). Moreover, the pathogenesis of DN depends on the following aspects: (1) Genetic susceptibility factors. Specific single nucleic acid polymorphisms in susceptibility genes have been associated with DN, and therefore, research on this area is helpful for the prevention of DN (72–74); (2) Abnormal glucose metabolism. Hyperglycemia promotes the occurrence of a variety of pathophysiological processes, including the activation of the polyol pathway (75, 76) and the generation and accumulation of AGEs (77–79); (3) Inflammatory reactions. DN is an inflammatory disease caused by a metabolic disorder As such, inflammatory reactions accompany the entire process of DN development, which result in the gradual scarring of the renal glomeruli, known as glomerulosclerosis (67, 80, 81); (4) Cytokines. Proinflammatory cytokines affect hemodynamics, promote cell proliferation, and increase ECM secretion and renal interstitial fibrosis, thus participating in the occurrence and development of DN (82); (5) OS. Excessive ROS production in the body activates PKC and the polyol pathway, leading to an increase in AGEs, and cytokine release, eventually resulting in severe pathological changes in the kidneys and promoting the occurrence and development of DN (83); (6) Endoplasmic reticulum stress (ERS). Excessive ERS may cause OS by promoting an increase in ROS, thereby damaging the kidneys (83–85) and participating in the development of DN; (7) Autophagy. This is the process whereby damaged proteins and organelles are decomposed after a stress response, and this plays an important role in maintaining cell homeostasis (86). The activation of the mechanistic target of rapamycin (mTOR) (87, 88) and the reduction of 5′ AMP-activated protein kinase (89)

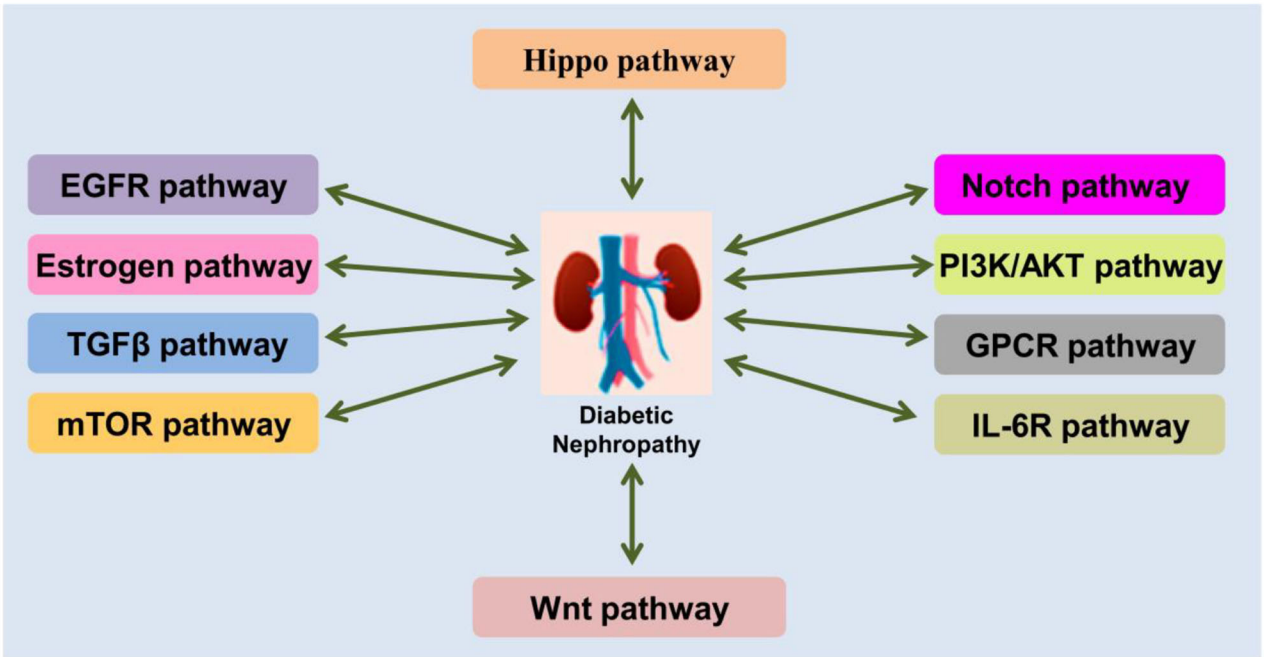

FIGURE 2 | Schematic demonstration of the crosstalks between DN and signaling pathways.

and Sirtuin 1 (SIRT1) (90) attenuate autophagy-related activities, and this attenuation has been linked to the pathogenesis of DN; (8) Exosomes and extracellular vesicles. Recently discovered, exosomes and extracellular vesicles are closely related to the occurrence and development of DN. The expression of exosomes is abnormal in DN, and the DNA, RNA, and protein contained in them are involved in the pathogenesis of DN and are used as molecular markers of DN (91, 92). In general, the pathogenesis of DN is complex, and what is currently known may just be the tip of the iceberg. Therefore, the integration of multiple aspects of the disease is crucial for the development of effective treatments.

Previous studies have reported that oxidative stress plays an important role in the occurrence and development of DN (3, 93–95). Hyperglycemia induces renal cells to produce large amounts of ROS, which increases oxidative stress. During oxidative stress, multiple signal pathways such as, glucose oxidation, production of advanced glycation end products (AGE), activation of protein kinase, hexosamine, and polyol pathways are involved in the metabolic regulation of glucose and lipids (96). Oxidative stress in the kidneys usually results in the massive production of ROS by peroxidase, which induces renal fibrosis and inflammation, and leads to tissue injury by promoting lipid peroxidation, DNA damage, and mitochondrial dysfunction (97). Under normal physiological conditions, ROS play a significant role in the regulation of cell proliferation, differentiation, apoptosis, and immune defense, while under the pathological conditions of diabetes, excessive ROS production in the kidneys stimulates the recruitment of inflammatory cells and the release of large amounts of inflammatory factors, growth factors, and transcription factors (71), thereby altering kidney structure and function, and promoting DN (98). Oxidative stress also accelerates the occurrence and development of DN by damaging the podocytes in the glomerular filtration barrier. The mechanisms whereby oxidative stress induces podocyte injury include ROS-induced mitochondrial dysfunction, activation of the mitogen-activated protein kinase (MAPK), and NF-κB signaling cascade, and oxidative damage of DNA (99). In addition, oxidative stress contributes to the development of glomerulosclerosis. ROS activate signaling pathways such as angiotensin II/transforming growth factor β1 (TGF-β1)/smad, protein kinase C (PKC), and NF-κB, inducing the deposition of extracellular matrix. However, signaling factors such as angiotensin II, TGF-β1, and PKC also facilitate the generation of ROS, aggravating DN and oxidative stress damage. Moreover, oxidative stress is involved in the development of renal tubular fibrosis. ROS stimulate the expression of a variety of pro-fibrotic growth factors such as TGF-β1, vascular endothelial growth factor (VEGF), and connective tissue growth factor, further boosting the deposition of extracellular matrix proteins and renal function damage (100). Therefore, it is extremely crucial to explore the mechanisms whereby oxidative stress participates in the occurrence of DN.

RELATIONSHIP BETWEEN ncRNAs AND DIABETIC NEPHROPATHY-RELATED OS

In recent years, various studies have confirmed that ncRNAs participate in the occurrence and development of DN by regulating OS. Among the ncRNAs that regulate OS in DN,

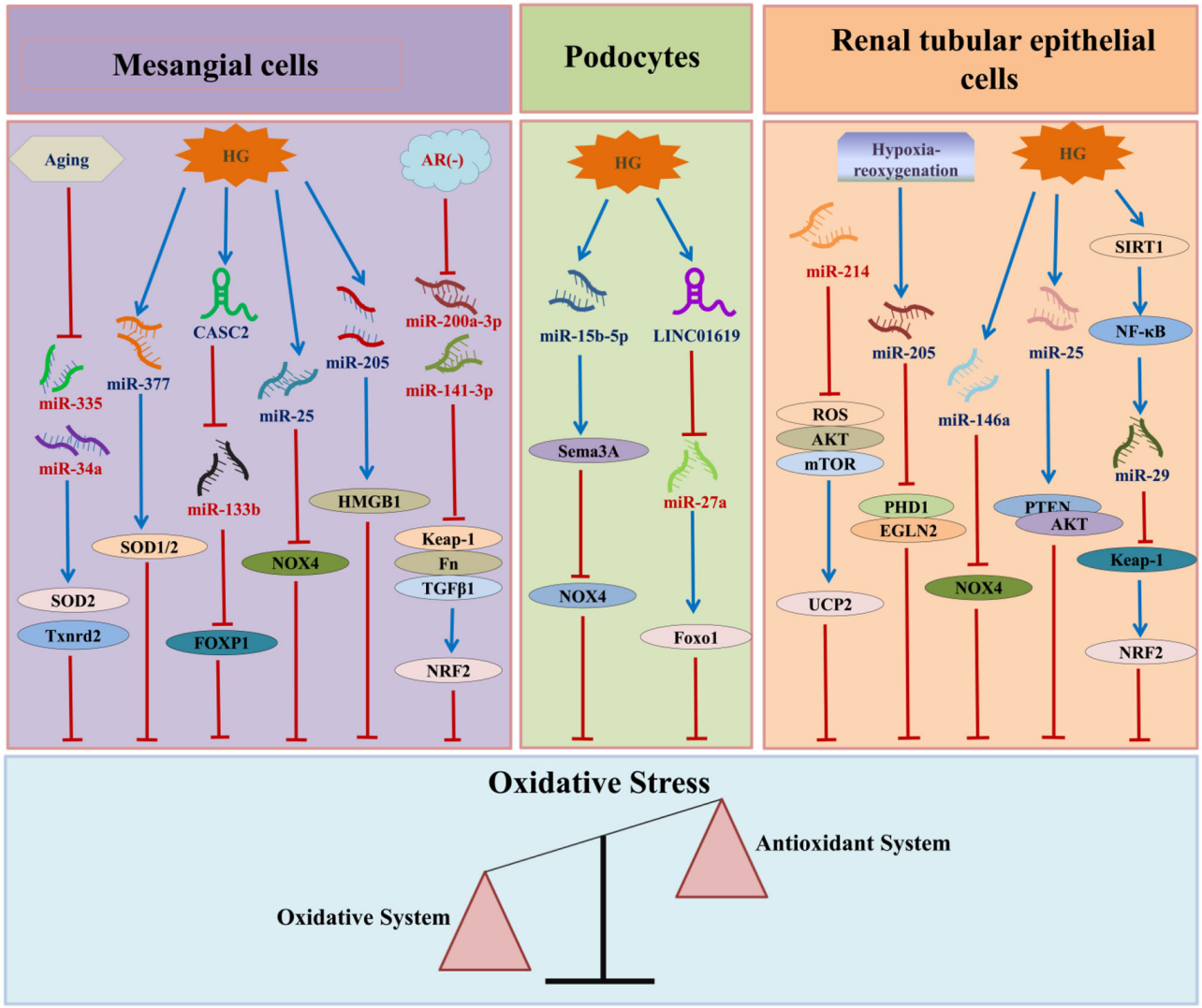

FIGURE 3 | The relationship between ncRNAs and DN-associated oxidative stress. HG, high glucose; AR, aldose reductase; CASC2, cancer susceptibility candidate 2; HMGB1, high mobility group box 1; SOD, superoxide dismutase; NOX4, nicotinamide adenine dinucleotide phosphate oxidase 4; FN, fibronectin; TGF-β1:transforming growth factor β1:TXNRD2, thioredoxin reductase 2; FOXP1, forkhead box P1; NRF2, nuclear factor erythroid E2-related factor 2; SEMA3A, semaphorin 3A; Foxo1, Forkhead box 1; ROS, reactive oxygen species; AKT, protein kinase B; mTOR, mechanistic target of rapamycin; UCP2, uncoupling protein 2; SIRT1, Sirtuin 1; NF-κB, nuclear factor-kappa B; PTEN, phosphatase and tensin homolog; PHD1, prolyl hydroxylase 1.

miRNAs are the most widely studied, followed by lncRNAs and circRNAs (**Figure 3**).

miRNAs and Diabetic Nephropathy-Related OS

miRNAs are post-transcriptional regulatory RNAs with a length of 18–23 nt that are widely present in eukaryotes, do not encode proteins, and are able to inhibit gene expression through specific interactions with target genes. Altered miRNA expression levels may generate OS and ultimately result in the development of disease. The relationship between miRNA and OS in the pathogenesis of DN has also become a research hotspot in recent years.

Up-Regulated miRNAs

The miR-23a/27a/24-2 cluster upregulates c-jun N-terminal kinases (JNKs) to induce caspase-dependent and caspase-independent cell death in human embryonic kidney cells (HEKT293), which is accompanied by an increase in ROS (101). In addition, overexpression of the miR-23a/27a/24-2 cluster results in changes in HEKT293 ERS and mitochondrial membrane permeability (102), suggesting that the cluster is closely related to OS in the kidney. Uncoupling protein 2 (UCP2), a negative regulator of ROS generation (103), is the target gene of miR-24 in the kidney; The downregulation of hsa-miR-24-3p results in UCP2 upregulation and subsequent reduction in ROS production (104). Shao et al. (105) found that miRNA-3550 is

upregulated in the kidneys of DN rats and is related to the Wnt/β-catenin signaling pathway. miR-27a directly targets nuclear factor erythroid 2-related factor 2 (NRF2), interfering with ROS homeostasis in DN, while adipokinin omentin 1 upregulates NRF2, and reduces OS by inhibiting miR-27a, restoring kidney function in type 2 diabetic db/db mice (106).

miRNA-452-5p expression is increased in high glucose (HG)-treated HK-2 cells (a renal tubular epithelial cell line), which is accompanied by an in increase in ROS and malondialdehyde (MDA) levels, and a decrease in SOD levels (107). However, these changes are reversed by interfering with miR-452-5p activity (108), which suggests that miR-452-5p is involved in the HG-induced OS response of renal tubular cells. Compared to controls, the levels of microRNA-377 have been shown to be consistently up-regulated in *in vitro* and mouse diabetic nephropathy models. The activity of miR-377 leads to reduced expression of p21-activated kinase and superoxide dismutase, which enhances fibronectin (FN) production. Thus, the overexpression of miR-377 in diabetic nephropathy indirectly leads to increased FN production. In addition, miR-377 targets SOD1 and SOD2 in human mesangial cells (HMCs) (109). Bioinformatics analysis and verification of the miRNA expression profiles in kidneys of young and old rats demonstrated that mitochondrial *Sod2* and thioredoxin reductase 2 (*Txnrd2*) are the targets of miR-335 and miR-34a, respectively. In aging MCs, miR-335 and miR-34a are significantly upregulated, while *Sod2* and *Txnrd2* are significantly downregulated, which is consistent with the production of ROS (110). Kato et al. (111) showed that TGF-β activates protein kinase B (AKT) in glomerular mesangial cells by inducing miR-216a and miR-217, leading to glomerular mesangial cell proliferation and hypertrophy. Zhang et al. (112) found that miR-133b is upregulated in the serum and HMCs of patients with DN, and that miR-133b targets forkhead box P1 (*FOXP1*). The upregulation of lncRNA CASC2 inhibits HG-induced HMC proliferation, ECM accumulation, and OS *via* the miR-133b/FOXP1 regulatory axis. Podocyte damage is a sign of DN, and is induced *via* ERS by the upregulation of miR-27a, which negatively targets forkhead box 1 (FOXO1) (113).

After analyzing miRNAs in the serum of healthy controls, patients with type 2 DM, and patients with DN, Regmi et al. (114) found that serum miR-99b and miR-122 levels are significantly increased in DM and DN group patients, while those of miR-20a, and miR-486 are decreased, and that the levels of these miRNAs are significantly related to albuminuria, glomerular filtration rate, blood sugar, and blood lipid levels. Moreover, target gene prediction of these four miRNAs revealed that they regulate OS, inflammation, and apoptosis (114). The emergence of RNAseq technology has facilitated the discovery of miRNAs related to OS in DN and the identification of their target genes, enabling in-depth research on the pathogenesis of DN.

Down-Regulated miRNAs

Renal tubular epithelial cells are one of the main cells that absorb glucose; however, long-term hyperglycemia directly causes their damage, and induces dysfunction through OS. In HG-treated HK-2 cells, the deacetylase activity of SIRT1 is weakened, resulting in a decrease in NF-κB activity. NF-κB regulates the expression of miR-29, which targets *KEAP1* by directly binding to its promoter. Downregulation of miR-29 in response to HG enhances the expression of KEAP1, and reduces NRF2 levels through ubiquitination, thereby inducing the damage of renal tubular epithelia (115). Moreover, miR-29 expression is negatively correlated with serum creatinine levels and creatinine clearance in diabetic rats (115). Yang et al. (116) found that miR-214 inhibits OS in DN and enhances the expression of UCP2 through the ROS/AKT/mTOR signaling pathway in HK-2 cells. The expression of UCP2 attenuates mitochondrial ROS activity, thereby exerting an antioxidant effect.

Superoxide derived from nicotinamide adenine dinucleotide phosphate oxidase (NOX) plays a key role in hyperglycemia-derived OS in DN; OS production by NOX mediates matrix accumulation and renal fibrosis in DN (117, 118). In HG-treated MCs and the kidneys of streptozotocin (STZ)-induced diabetic rats, the expression of miR-25 is significantly reduced, while the mRNA and protein levels of NOX4 are increased (119). MCs transfected with antagomiR-25 showed a considerable increase in the mRNA and protein levels of NOX4 (120). These results are consistent with the increased OS and diastolic dysfunction observed in the hearts of hypercholesterolemic rats when the expression of miR-25 is decreased (121). Thus, the miR-25-NOX4-OS axis seems to play a common role in kidney and heart diseases. In addition, miR-25 is downregulated in kidney biopsy tissue and serum of patients with DN, and is inversely proportional to proteinuria. Moreover, HG-treated HK-2 cells show decreased miR-25 levels in a time-dependent manner. Overexpression of miR-25 reduces the generation of ROS in HK-2 cells; the mechanism may be related to the activation of the phosphatase and tensin homolog/AKT pathway (122). Similarly, Wan et al. (123) confirmed that miR-146a expression is inhibited and NOX4 levels are increased in a DN mouse model. In addition, overexpression of miR-146a in HK-2 cells inhibits NOX4 expression, reducing ROS production, OS, and inflammation levels (123), which suggests that miR-146a has an anti-inflammatory and oxidation modulating effect in DN.

Wei et al. (124) reported that aldose reductase (AR) negatively regulates the expression of miR-200a-3p/miR-141-3p in MCs. In STZ-induced diabetic mice, AR deficiency significantly increases the miR-200a-3p/miR-141-3p levels in the renal cortex, which is accompanied by the significant downregulation of *Keap1*, *Tgfβ1/2*, and *fn1*, and the prominent upregulation of *Nrf2*. Therefore, the inhibition of AR and the restoration of the miR-200a-3p/miR-141-3p levels may be a potential research direction for the treatment of DN.

The expression of miR-506-3p is downregulated in HG-treated HK-2 cells and the serum of patients with DN, while overexpression of miR-506-3p inhibits inflammation, OS, and pyroptosis in HG-treated HK-2 cells (125). miR-15b-5p is significantly decreased in HG-treated podocytes, which is accompanied by increased levels of podocyte apoptosis and OS (112). Overexpression of miR-15b-5p inhibits cell apoptosis, decreases the expression of the OS-related markers *MDA* and *NOX4*, and increases the levels of *SOD* and hydrogen peroxide, which may occur by targeting *Semaphorin 3A* (*Sema3A*) (126). miR-124a expression in bone marrow

stromal stem cells (BMSCs) has a protective effect on OS-induced podocyte damage; transfection of BMSCs with miR-124a inhibits the phosphoinositide 3-kinase/mTOR signaling pathway, thus protecting podocytes (127). This suggests that the combined effects of BMSCs and miRNA may be beneficial for the treatment of DN.

lncRNAs and Diabetic Nephropathy-Related OS

lncRNA refers to a long-chain RNA molecule with a length >200 nt and no protein coding ability, and regulate the process of DN. The expression of the lncRNA KCNQ1OT1 is increased in the serum of patients with DN and in HG-treated HK-2 cells (125). Further, KCNQ1OT1 directly targets miR-506-3p, and therefore, the interference of KCNQ1OT1 expression promotes miR-506-3p expression, thereby inhibiting inflammation, OS, and pyroptosis in HG-treated HK-2 cells (125). Another study in HK-2 cells showed that the expression of the lncRNA GAS5 decreases in HG-treated HK-2 cells, while the overexpression of GAS5 reduces ROS and MDA levels, and increases SOD levels (108). miRNA-452-5p expression is increased in HG-treated HK-2 cells, and interference of GAS5 expression may reverse the effects of miRNA-452-5p on HG-induced inflammation, OS, and pyroptosis of renal tubular cells (108). Furthermore, lncRNA Blnc1 is highly expressed in the serum of patients with DN, STZ-induced DN models, and HG-treated HK-2 cells; it is involved in the occurrence and development of DN, and its interference significantly reduces renal fibrosis, inflammation, and OS (128). Wang et al. (129) found that Linc00462 is significantly upregulated in the kidneys of patients with DN, and that its level increases in a glucose concentration- and time-dependent manner in HG-treated HK-2 and HMC cells. Knockdown of Linc00462 significantly reduces the cell viability of HG-treated cells and the levels of ROS and MDA induced by HG, while increasing the levels of SOD and catalase. Therefore, it upregulates the antioxidant system against ROS, indicating that knocking out Linc00462 may be a potential treatment for DN.

In addition to renal tubular cells, impairment of lncRNA expression in glomerular cells also participates in DN-related OS. Zhang et al. (112) found that the expression of lncRNA CASC2 decreases in the serum of patients with DN and in HG-treated HMCs, while the upregulation of CASC2 inhibits HMC proliferation, ECM accumulation, and HG-induced OS. miR-133b, the target of CASC2, is highly expressed in the serum of patients with DN and in HG-treated HMCs; the enrichment in miR-133b reverses the effect of CASC2 upregulation. The study confirmed that the upregulation of CASC2 inhibits HMC cell proliferation, ECM accumulation, and HG-induced OS through the miR-133b/FOXP1 axis, suggesting that CASC2 may be used as a novel target for DN treatment (112). Linc01619 is downregulated in renal tissues of patients with DN, and is associated with proteinuria and decreased renal function (113). Further, *in vitro* experiments confirmed that Linc01619 is expressed in the cytoplasm of podocytes, participating in the ERS signaling pathway, where it may be used as a competitive endogenous RNA that regulates the ERS and podocyte damage mediated by miR-27a/FOXO1 in DN (113). These results indicate that lncRNAs play an important role in DN-related OS and may improve DN by affecting miRNA expression.

Other ncRNAs and Diabetic Nephropathy-Related OS

circRNA is a large ncRNA, which binds to miRNA and terminates the regulation of its target genes, namely, the circRNA-miRNA-mRNA regulatory network. circLRP6 regulates HG-induced MC proliferation, OS, ECM accumulation, and inflammation by competitively binding to miR-205 and *HMGB1*, and activating the Toll-like receptor 4/NF-κB pathway (130). These findings provide a better understanding of the pathogenesis of DN. In addition, antisense mitochondrial non-coding RNA-2 (ASncmtRNA-2) is expressed in experimental DN models and *in vitro* in human renal mesangial cells (HRMCs). Furthermore, it is significantly upregulated in mice with hereditary type 2 DM that also develop DN. When using the nitric oxide synthase inhibitor NG-nitro-L-arginine methyl ester (L-NAME) to inhibit ROS, the upregulation of ASncmtRNA-2 in DN is significantly reduced. In cultured HRMCs, HG treatment upregulates ASncmtRNA-2 expression in a time-dependent manner. Incubation of HRMCs with L-NAME also reduces the glucose-induced upregulation of ASncmtRNA-2. In addition, ROS upregulate ASncmtRNA-2 and may promote glomerular fibrosis in DN by actively regulating the expression of pro-fibrotic factors (131). This indicates that ASncmtRNA-2 is involved in the DN-related OS response and renal fibrosis development.

POTENTIAL CLINICAL APPLICATIONS OF OS-RELATED ncRNAs IN DN

The morbidity and mortality of DN remains high worldwide. Many studies have shown that early and timely intervention in DN significantly limits proteinuria, thereby preventing further development of DN (132). Therefore, the identification of novel molecular markers and drug targets for DN prevention and treatment is urgently needed. The application of next-generation sequencing technologies for RNAseq revealed that changes in the expression of miRNAs are very common in the pathogenesis of diabetes and DN (133). In addition, miRNAs are widely present in various parts of the cell, increasing their potential as DN-specific molecular markers.

The expression of many miRNAs related to OS is different in diabetic and healthy people (134, 135). For example, OS-related miR-21 has been proposed as a diagnostic marker for prediabetes (135), and receiver operating characteristic (ROC) curve analysis has shown that the expression of miR-21 is a suitable candidate marker for distinguishing diabetes from prediabetes (sensitivity, 93%; specificity, 35%). The ROC area under the curve (AUC) was 0.7 (**Table 1**). Moreover, the serum levels of miR-99b, miR-486-5p, miR-122-5p, and miR-20a in the serum, whose target genes are closely related to OS (114), are considered to have diagnostic value in diabetic kidney diseases (**Table 1**).

Although, ncRNAs are closely associated with DN-related OS, there are limited related clinical drug studies. Shao et al. (105)

TABLE 1 | Diagnostic index of miRNA in human DM-related OS.

miRNAs	Diseases	Sample numbers (control/diseases)	AUC	Sensitivity	Specificity	OR (95% CI*)	Ref
miR-21	Diabetes	44/27	0.7	93%	35%	1.05 (1.01–1.09)	(121)
miR-99b	DKD	25/42	0.895	-	-	-	(114)
miR-486-5p	DKD	25/42	0.853	-	-	-	(114)
miR-122-5p	DKD	25/42	0.8	-	-	-	(114)
miR-20a	DKD	25/42	0.697	-	-	-	(114)

DM, diabetes mellitus; AUC, area under the curve; OR, odds ratio; CI, confidence interval; Ref, reference; DKD, diabetic kidney disease.

treated DN rats with ginsenoside Rb1, triclosan, and ginsenoside Rb1 plus trigonelline and found that the combination of ginsenoside Rb1 and trigonelline significantly alleviates renal dysfunction, OS, and pathological changes. Further, studies have confirmed that ginsenoside Rb1 and trigonelline regulate the expression of miR-3550, which regulates the Wnt/β-catenin signaling pathway, therefore preventing the occurrence of diabetic kidney disease (105). Sitagliptin, a dipeptidyl peptidase 4 inhibitor used for the treatment of type 2 DM, has a protective effect on diabetic chronic kidney diseases. Civantos et al. (136) conducted proteomic and miRNA transcriptomic analysis of the renal cortex of wild-type (Wistar), diabetic Goto-Kakizaki (GK) rats, and rats treated with siglitine. Proteomic analysis of diabetic GK and Wistar rats showed differential expression of 39 proteins, and significant changes occurred among 15 miRNAs in GK rats, which are mainly related to OS and catabolism. Further studies have confirmed that treatment with sitagliptin improves OS in experimental DN *via* the mir-200a/*Keap-1*/*Nrf2* antioxidant pathway, thus, exerting renal-protective effects. Ochratoxin A, a mycotoxin with nephrotoxic and potentially carcinogenic activity, induces the expression of miR-200c and miR-132 in renal proximal epithelial cells. miR-200c and miR-132 target *NRF2* and *HO-1*, respectively, thereby promoting renal OS and inducing renal injury (137). Thus, ncRNAs are considered potential therapeutic targets based on their regulatory roles. Further, research is necessary to explore the diagnostic value of OS-related ncRNAs in DN, to identify novel drug targets, and prevent DN.

CONCLUSIONS

Recently, ncRNAs have been recognized as a "new star" in the field of DN. As a class of novel regulatory molecules, they participate in multiple steps of DN by modulating the expression of several related genes. The development of RNAseq and next generation sequencing technologies has allowed the identification of a large number of ncRNAs. The extraction and detection of ncRNAs has higher specificity and sensitivity than those of proteins. Thus, ncRNAs can potentially be used as potential diagnostic and prognostic biomarkers. In addition, we can silence or activate ncRNAs in DN patients by exogenous means. For example, we can wrap the silenced or active lentiviral vector of ncRNAs into exosomes or other vehicles *in vitro*, and perform a targeted injection into the corresponding organs *via* blood. This can be developed as a therapy for DN. Considering their roles in the progression of DN-related OS, ncRNAs have great potential as "biological tools" for the screening, diagnosis, and treatment of DN, and may ultimately cure DN. However, there is a long road from the scientific research of ncRNAs to their clinical application. Recently, the research on ncRNA and DN has faced a series of challenges and limitations: (1) many ncRNAs are yet to be discovered and identified in DN through the development of RNAseq and next generation sequencing technologies; (2) ncRNAs need to be further investigated to determine whether they are specifically related to one or more diseases, and to explore the underlying molecular mechanisms whereby ncRNAs contribute to the disease; (3) The exact and specific mechanisms whereby ncRNAs regulate DN-related OS remain to be discovered; (4) According to many animal models, ncRNAs play a role in DM and its complications; however, the lack of clinical trials confirming the accuracy and safety of these findings remains an issue; (5) Many endogenous and exogenous factors involved in ncRNA production have not yet been identified, which hinders the use of ncRNAs as clinical therapeutic targets for DN treatment.

In this review, the regulatory role of ncRNAs in DN-related OS has been summarized. These ncRNAs regulate individual target genes or constitute interaction networks, such as lncRNA-miRNA-mRNA or circRNA-miRNA-mRNA, and play an important role in the regulation of DN-related OS. As our understanding of the molecular mechanisms involved in ncRNA regulation and their function *in vitro* and *in vivo* increases, novel and more effective treatment methods will be developed, which may cure DN by targeting the corresponding key ncRNAs.

AUTHOR CONTRIBUTIONS

XH, GK, and YZ conceived the concept and wrote the manuscript. CO edited and improved the manuscript. All authors contributed to the article and approved the submitted version.

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Single-Nucleus Transcriptomic Analysis Reveals Important Cell Cross-Talk in Diabetic Kidney Disease

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Diabetic kidney disease (DKD) leads to the loss of renal function and cell cross-talk is one of the crucial mechanisms participating in the pathogenesis of DKD. However, the mechanisms of cell communication were not fully elucidated in previous studies. In this study, we performed cell cross-talk analysis using CellPhoneDB based on a single-nucleus transcriptomic dataset (GSE131882) and revealed the associations between cell communication-related genes and renal function, providing overall insight into cell communication in DKD. In addition, this study may facilitate the discovery of novel mechanisms, promising biomarkers, and therapeutic targets that are clinically beneficial to patients.

Keywords: single-cell sequencing, cell cross-talk, diabetic kidney disease, CellPhoneDB, glomerulotubular communication

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INTRODUCTION

Diabetic kidney disease (DKD) (1, 2) is one of the most important microvascular complications of diabetic mellitus and a leading cause of renal function loss and end-stage renal disease (ESRD). Nevertheless, the mechanism of DKD is complex and not fully elucidated.

Renal parenchymal cells, resident immune cells, and infiltrating immune cells orchestrate active cell-to-cell interactions, thereby contributing to the development of DKD. Previous studies (3, 4) have revealed the significance of cell communication in the pathogenesis of DKD. Dmike et al. (5) deciphered the tubulovascular cross-talk mediated by vascular endothelial growth factor A. Wu et al. (6) found that high glucose-induced glomerular endothelial cell-derived exosomes trigger the epithelial-mesenchymal transition and podocytes dysfunctions. Nespoux et al. (7) reviewed the renoprotective mechanism of sodium-glucose cotransporter 2 (SGLT2) inhibitors, which downregulate tubular reabsorption-induced early glomerular hyperfiltration. Garson et al. (8) revealed that podocytes mediate glomerular transendothelial albumin passage via endothelin-1-regulated heparanase expression. Lai et al. (9) revealed the importance of cell-to-cell communication between different glomerular cell types in DKD using podocyte and endothelial-specific elimination of bone morphogenetic protein and activin membrane-bound inhibitor (BAMBI) expression in streptozotocin-induced diabetic endothelial nitric oxide synthase (eNOS)-deficient and control eNOS-deficient mice. Unfortunately, these studies merely highlight the limited types of cell-to-cell interactions in DKD, and detailed insight into cell communication in DKD is lacking.

Single-cell sequencing (scRNA-seq) is a technological evolution and provides unprecedented insight into cell communication (10–12). In experimental studies of renal diseases (13–16), scRNA-seq technology furthers the understanding of the mechanisms and cell-to-cell interactions involved in disease pathogenesis. In human kidneys (17–19), scRNA-seq has helped to identify novel cell types, reveal potential mechanisms, and investigate cell communication from distinct aspects. Lake et al. (17) primarily deciphered the cell types, distributions, cell differentiation, and cell-to-cell interactions based on integrins in normal human kidneys. A study on lupus nephritis (18) highlighted the immune cells, immune-associated mechanisms, and cell-to-cell interactions based on the functions of chemokines and cytokines. These studies merely described the specific patterns of cell cross-talk. Moreover, cross-talk has not been fully elucidated in DKD (19).

In this study, we provided an overall perspective of cell communication in human DKD based on a single-nucleus transcriptomic dataset. In addition, the relationships between hub genes involved in cell communication and renal function were determined. This study of cell communication between individual cells based on ligand-receptor interactions in DKD may facilitate the discovery of novel mechanisms, biomarkers, and drug targets to better serve patients.

MATERIALS AND METHODS

Single-Nucleus Transcriptomic Data Preparation

First, we downloaded snRNA-seq data from the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo) dataset GSE131882, which contained the single-nucleus transcriptomic data of three nondiabetic controls and three patients with early DKD produced by 10× Genomics.

Cell Type Identification

The raw gene expression matrix was obtained and processed to align reads with the reference genome (Homo_sapiens_GRCh38_96) using Cell Ranger (version 4.0.0). Data filtration and normalization were performed using the R package Seurat (version 3.1.1) according to the manufacturer's manual (http://satijalab.org/seurat/) (20). Nuclei with at least 200 genes and percentage of mitochondrial DNA-derived gene expression <25% and genes expressed in at least one single nucleus were retained in the subsequent analysis; otherwise, they were removed. Only snRNA-seq data that met quality control criteria were analyzed in this study.

Further, t-distributed stochastic neighbor embedding (t-SNE) was performed for unsupervised clustering using the R package Seurat (version 3.1.1). Subclustering of specific cell types was performed using OmicStudio (https://www.omicstudio.cn/tool). Annotation of all clusters and subclusters was manually performed based on known cell-type marker genes (17, 18).

Deferentially Expressed Genes in Specified Clusters

After cell annotation, differentially expressed genes (DEGs) in specified cell-types were analyzed using the FindMarkers function based on the bimod algorithm of the R package Seurat (version 3.1.1). Fold changes $\geq$1.25 and $p < 0.05$ were considered significantly modulated.

Cell Cross-Talk Analysis

CellPhoneDB (21) is a public repository of curated receptors, ligands and their interactions. In this study, cell cross-talk interaction was performed using CellPhoneDB (version 2.1.1) according to the manufacturer's manual (https://www.cellphonedb.org/). The mean value represents the average ligand and receptor expression in a specific cell type, which is calculated based on the percentage of cells expressing the specific gene and the gene expression mean. The *P*-value is calculated based on the proportion of the means that are as high as or higher than the actual mean, which represents the likelihood of a specific cell type of a given receptor–ligand complex.

Protein Expression and Immunochemistry Analysis

The protein expression determined using immunochemistry was obtained from The Human Protein Atlas (https://www.proteinatlas.org/).

Clinicopathological Correlation Analysis

Nephroseq is a free platform for integrative data mining, including genotype data and phenotype data. The two datasets in Nephroseq, Woroniecka Diabetes Glom, and Woroniecka Diabetes TubInt (22), were analyzed in this study. Pearson's correlation analysis between hub genes and glomerular filtration rate (GFR) in patients with DKD was performed using Nephroseq v5 according to the manufacturer's manual (http://v5.nephroseq.org).

Statistical Analysis and Data Visualization

Statistical analysis was performed using SPSS 22.0 (SPSS Inc., USA). The figures were illustrated using OmicStudio, GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, USA), and Microsoft PowerPoint (Microsoft Inc., Redmond, USA).

RESULTS

Identifications of Renal Cells and Immune Cells

After data filtration, the number of nuclei analyzed in the current study was 21,529. According to the known markers, we manually identified proximal tubular convoluted cells, cells of the loop of Henle, connecting tubule cells, principal cells of the collecting duct, distal convoluted duct cells, intercalated cell A from the collecting duct, endothelial cells, parietal epithelial cells, podocytes, intercalated cell B from the collecting duct, mesangial cells, and immune cells (**Figure 1A**). The markers used in this study and the distributions of disparate cells in the different groups are shown in **Figures 1B,C**, respectively.

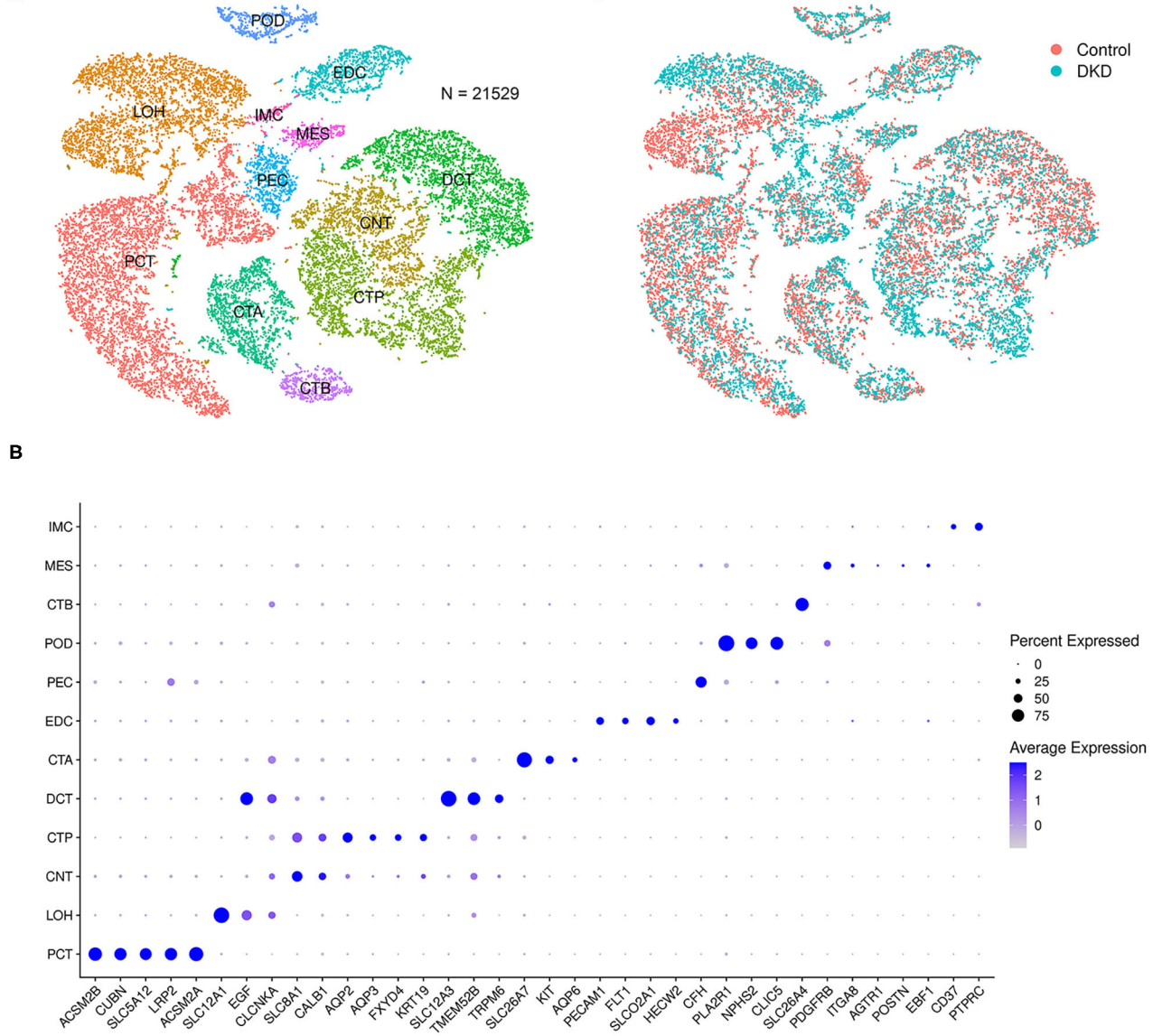

FIGURE 1 | Integrated snRNA-seq of nondiabetic and diabetic kidneys. We performed t-SNE analysis **(A)**, identified gene markers **(B)**, and determined the distributions **(C)** of the 21,529 nuclei. Proximal tubular convoluted cells (PCT), cells of the loop of Henle (LOH), connecting tubule cells (CNT), principal cells of the collecting duct (CTP), distal convoluted duct cells (DCT), intercalated cell A from the collecting duct (CTA), glomerular endothelial cells (EDC), parietal epithelial cells (PEC), podocytes (POD), intercalated cell B from the collecting duct (CTB), mesangial cells (MES), and immune cells (IMC) were manually identified.

Notably, the number of immune cells was significantly increased in the DKD group (DKD vs. control, 148 nuclei vs. 40 nuclei, $p < 0.05$). To determine the types of immune cells, we performed subcluster analysis using t-SNE in immune cells (188 nuclei) and found that renal immune cells comprise T cells, monocytes, dendritic cells, B cells, and plasma cells (**Figure 2A**) using reported marker genes (**Figure 2B**). In nondiabetic controls, T cells, monocytes, and dendritic cells consist of renal immune cells. In the DKD group, the total number of immune cells was increased, and numbers of T cells, monocytes, and dendritic cells were increased (**Supplementary Table 1**). Intriguingly, all B cells and plasma cells newly accumulated in the DKD group (**Figure 2C**).

DEGs in Specific Cell Types

Next, we analyzed the DEGs of specific cell types.

In the mesangial cells (399 nuclei), 88 upregulated genes (the top five genes were SLC2A3, RIPOR3, CCN1, RGS16, and HSPA1A) and 141 downregulated genes (the top five genes were TSC22D3, SPARCL1, ZFAND5, MT1X, and PIK3R1)

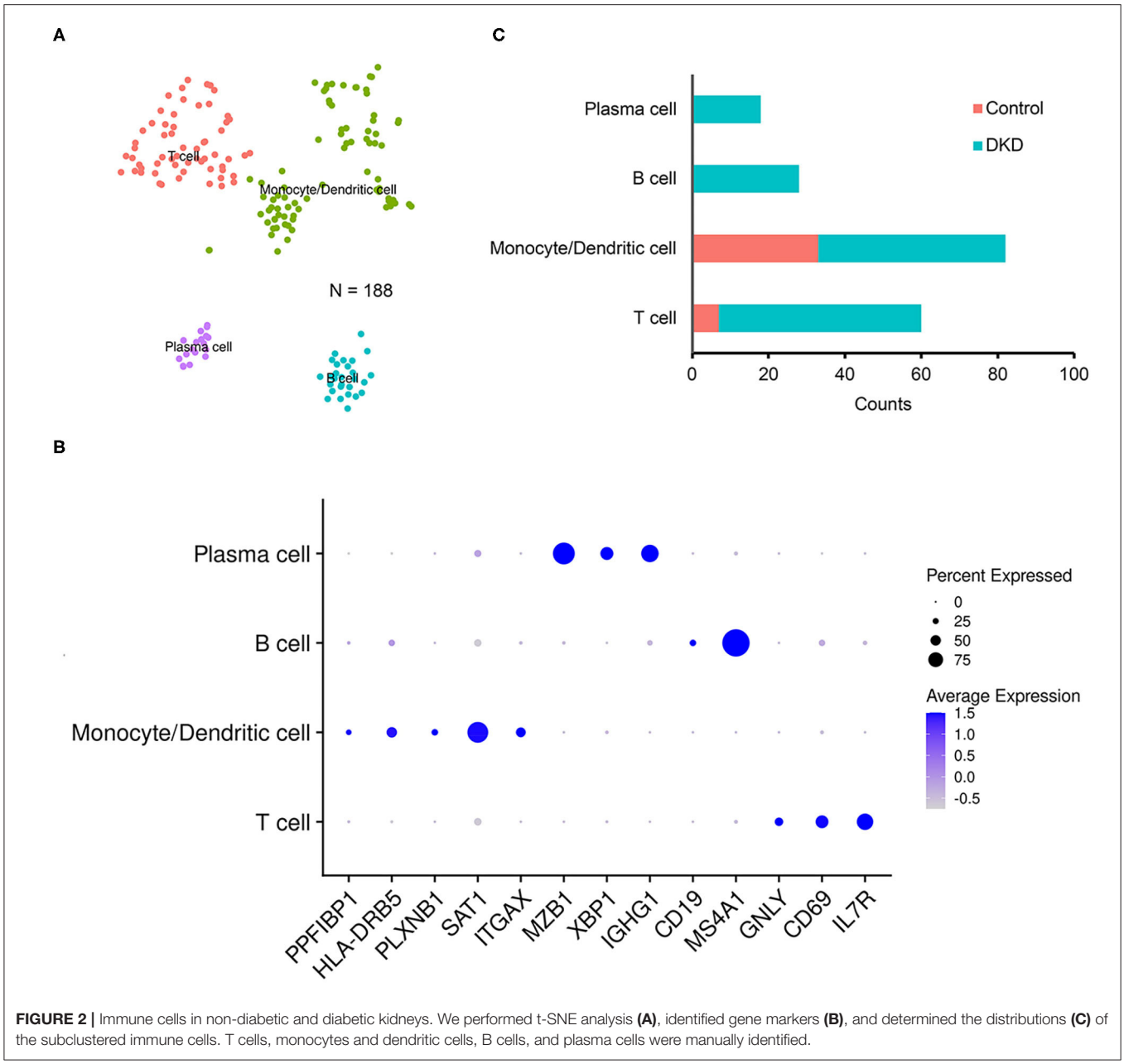

FIGURE 2 | Immune cells in non-diabetic and diabetic kidneys. We performed t-SNE analysis **(A)**, identified gene markers **(B)**, and determined the distributions **(C)** of the subclustered immune cells. T cells, monocytes and dendritic cells, B cells, and plasma cells were manually identified.

were identified (**Figure 3A**). Extracellular environment-related genes (THBS1, ITGA1, COLA1, and COL4A1) are upregulated in DKD.

Figure 3B shows the 102 upregulated genes (the top five genes were VCAM1, SLC2A3, FOS, EMP1, and ICAM1) and 105 downregulated genes (the top five genes were PDK4, TSC22D3, DDIT4, MT1M, and MT-CYB) in EDC (1,079 nuclei). Moreover, the levels of indicators of injury (IGFBP5 and SEMA6A) were increased.

A total of 663 podocytes were analyzed, and we determined that the levels of 39 genes were increased (the top five genes were FOS, EGR1, NR4A1, JUN, and MYADM), while the levels of 80 genes were reduced (the top five genes were GLUL, GPX3, GADD45B, MT-ATP6, and PRMT1; **Figure 3C**). In addition, PLA2R had no significant alteration according to our analysis.

We analyzed proximal tubular convoluted cells (5,474 nuclei) regarding its crucial roles in reabsorption and glomerulotubular balance and determined 84 upregulated genes (the top five genes were HSPA1A, SOX4, VCAM1, HIST1H2AC, and PROM1) and 134 downregulated genes (the top five genes were FKBP5, FTL, FTCD, TIPARP, CYP3A5; **Figure 3D**). The expression of HIF1A and NRP1 was increased, and the expression of ACE was decreased. Nevertheless, we did not find a significant change in the expression of ACE2.

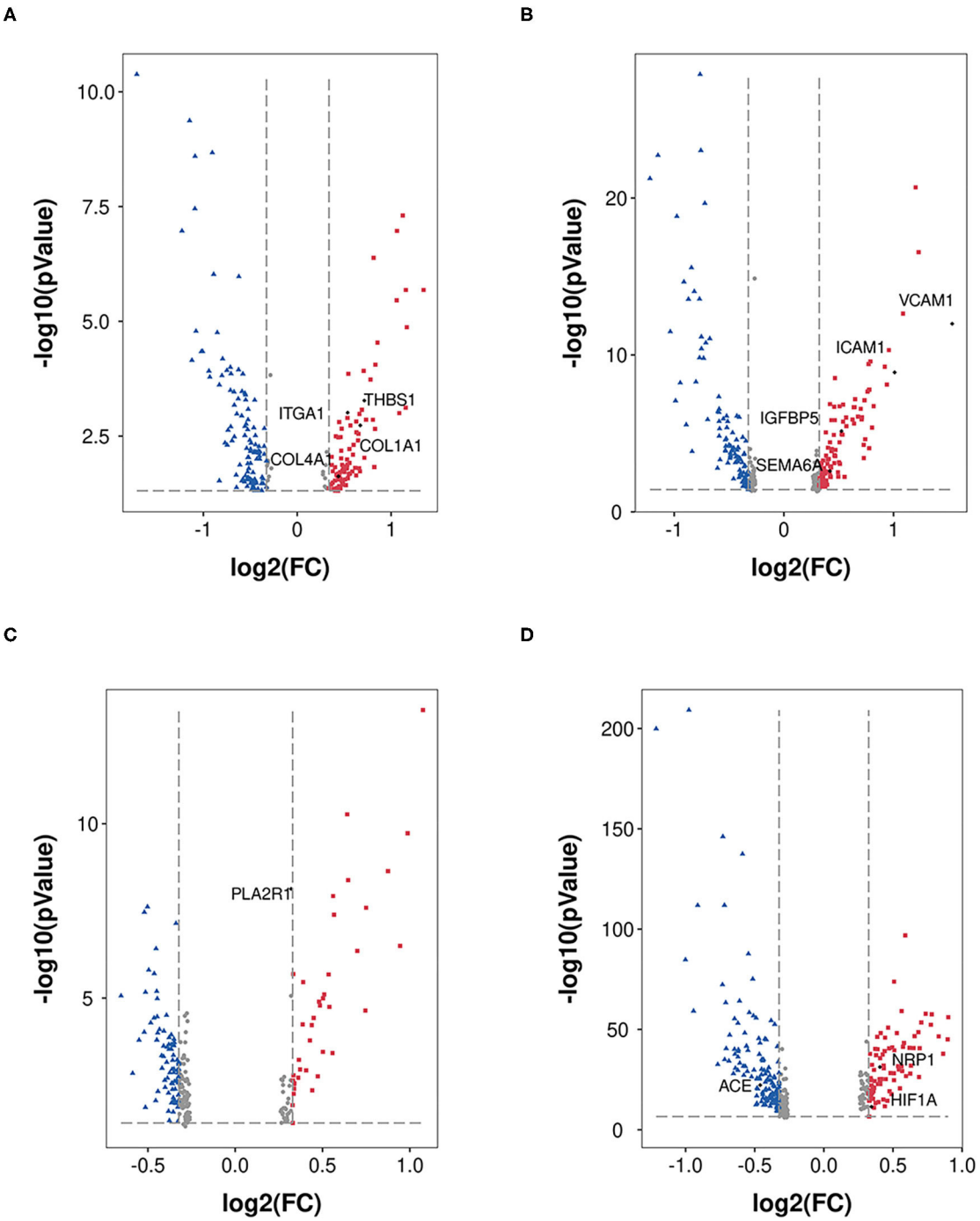

FIGURE 3 | DEGs in MES, EDC, POD, and PCT. DEGs (DKD vs. nondiabetic control) were analyzed in MES **(A)**, EDC **(B)**, POD **(C)**, and PCT **(D)**. The dots in red represent upregulated genes, and the dots in blue represent downregulated genes.

Cell Cross-Talk in DKD

To reveal the cellular communication in the kidney of DKD, we performed an analysis based on receptor-ligand interactions using CellPhoneDB.

Cell communication in nondiabetic kidneys is defined as basic cell communication that maintains normal renal function (**Figure 4**). We found that glomerular endothelial cell-expressed FLT1 and podocyte-expressed VEGFA and FGF1 are key molecules (**Figure 4A**) and that glomerular endothelial cells together with podocytes play crucial roles in glomerular and glomerulotubular cell cross-talk.

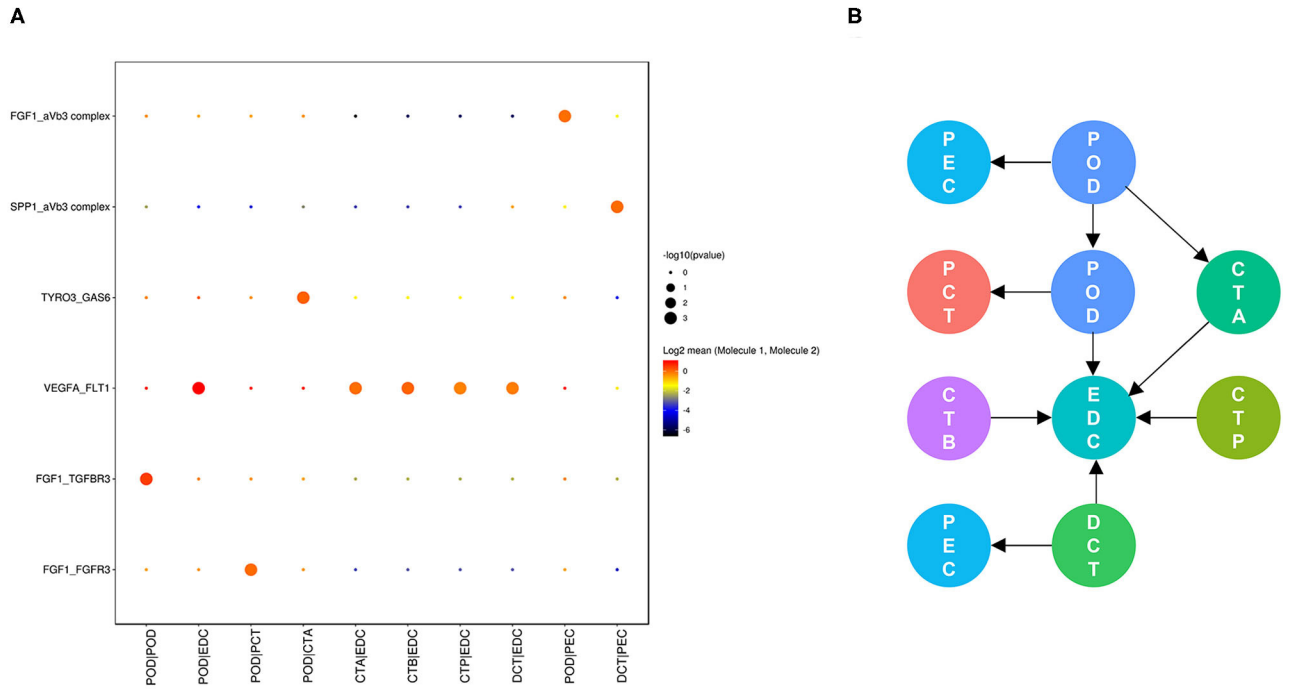

FIGURE 4 | Cell cross-talk in the kidneys of nondiabetic patients. We analyzed all individual cells based on ligand-receptor interactions to reveal the cell cross-talk in the kidneys of nondiabetic patients **(A)**. The involved cell types are summarized and the thickness of the arrow represents the number of interactions **(B)**.

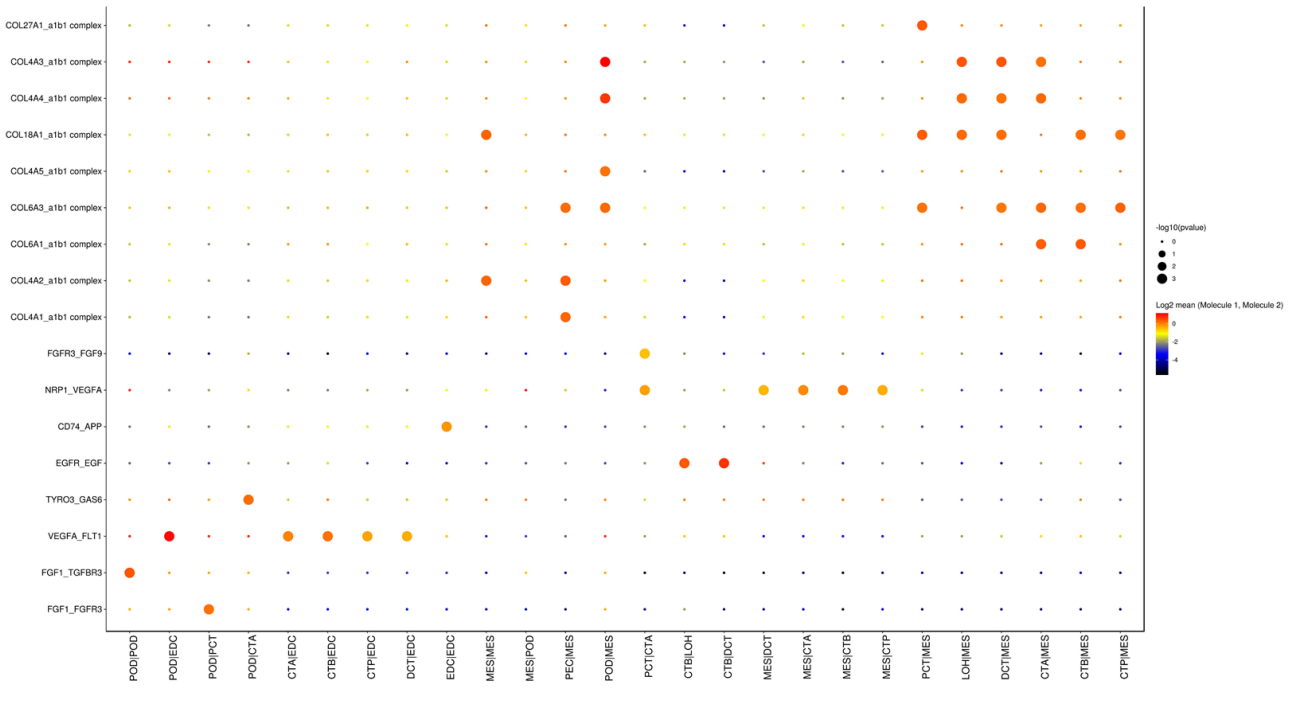

FIGURE 5 | Cell cross-talk in the kidneys of diabetic patients. We analyzed all individual cells based on ligand-receptor interactions to reveal the cell cross-talk in the kidneys of diabetic patients.

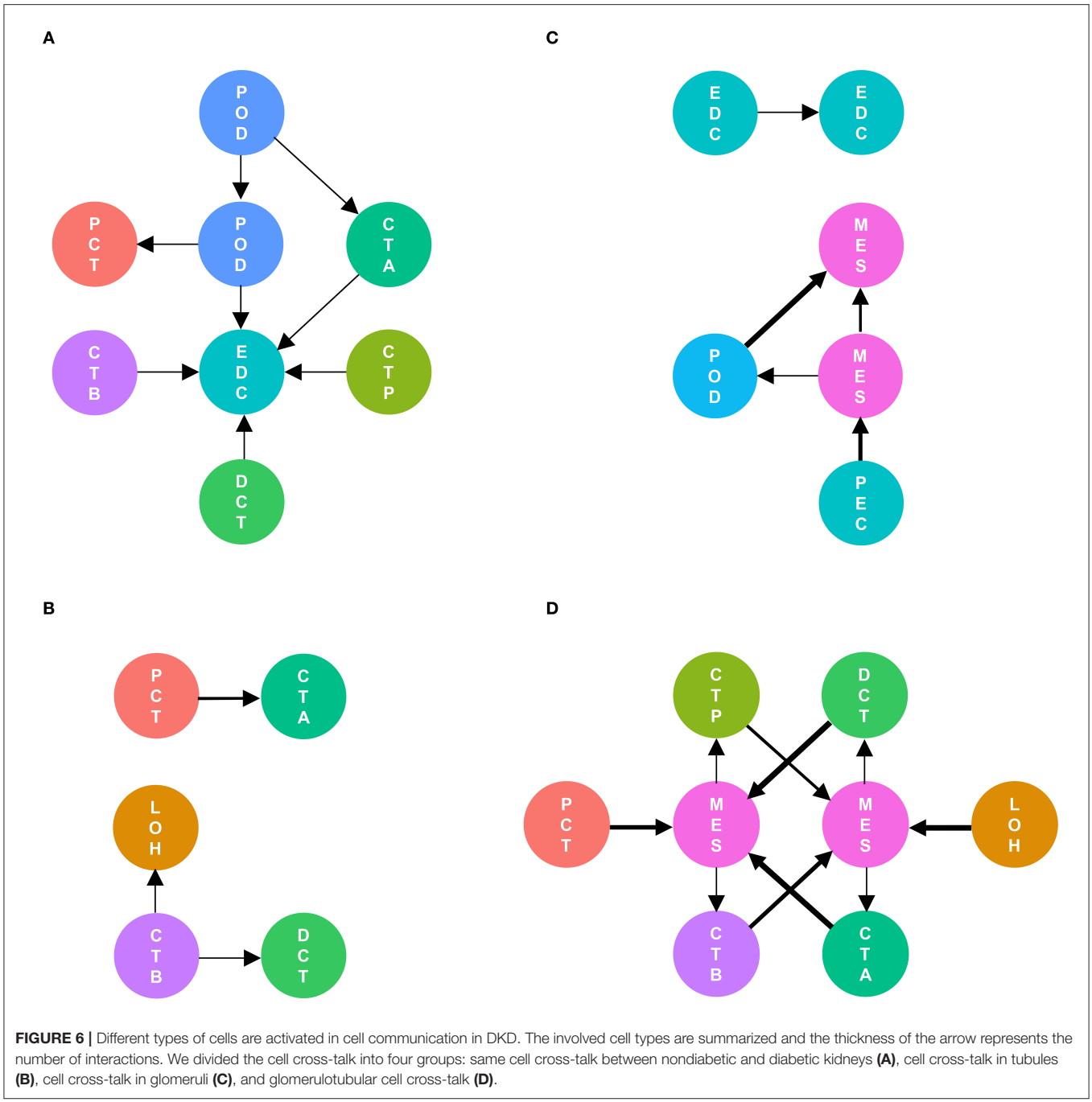

FIGURE 6 | Different types of cells are activated in cell communication in DKD. The involved cell types are summarized and the thickness of the arrow represents the number of interactions. We divided the cell cross-talk into four groups: same cell cross-talk between nondiabetic and diabetic kidneys **(A)**, cell cross-talk in tubules **(B)**, cell cross-talk in glomeruli **(C)**, and glomerulotubular cell cross-talk **(D)**.

As shown in **Figure 5**, cell communication was significantly altered in DKD conditions. The most noticeable change is the activation of integrin pathways in glomerular and glomerulotubular cell cross-talk. In addition, we noticed that both glomerular and tubular NRP1 participate in the enhanced cell cross-talk of DKD. As shown in **Figure 6**, we summarized different types of cell communications separately. **Figure 6A** shows the impairment of basic cell communication and reveals the reduction of the podocyte-expressed FGF1-to-PEC-expressed aVb3 complex and DCT-expressed SPP1-to-PEC-expressed aVb3 complex. Conversely, **Figure 6B** indicates that the cell cross-talk in the tubule was markedly enhanced. Moreover, we found that mesangial cells were strongly activated in both glomerular (**Figure 6C**) and glomerulotubular (**Figure 6D**) cell cross-talk.

Genes Involved in Cell Cross-Talk Are Associated With Renal Function

Finally, we investigated the relationship between hub genes involved in cell communication and renal function. Glomerular

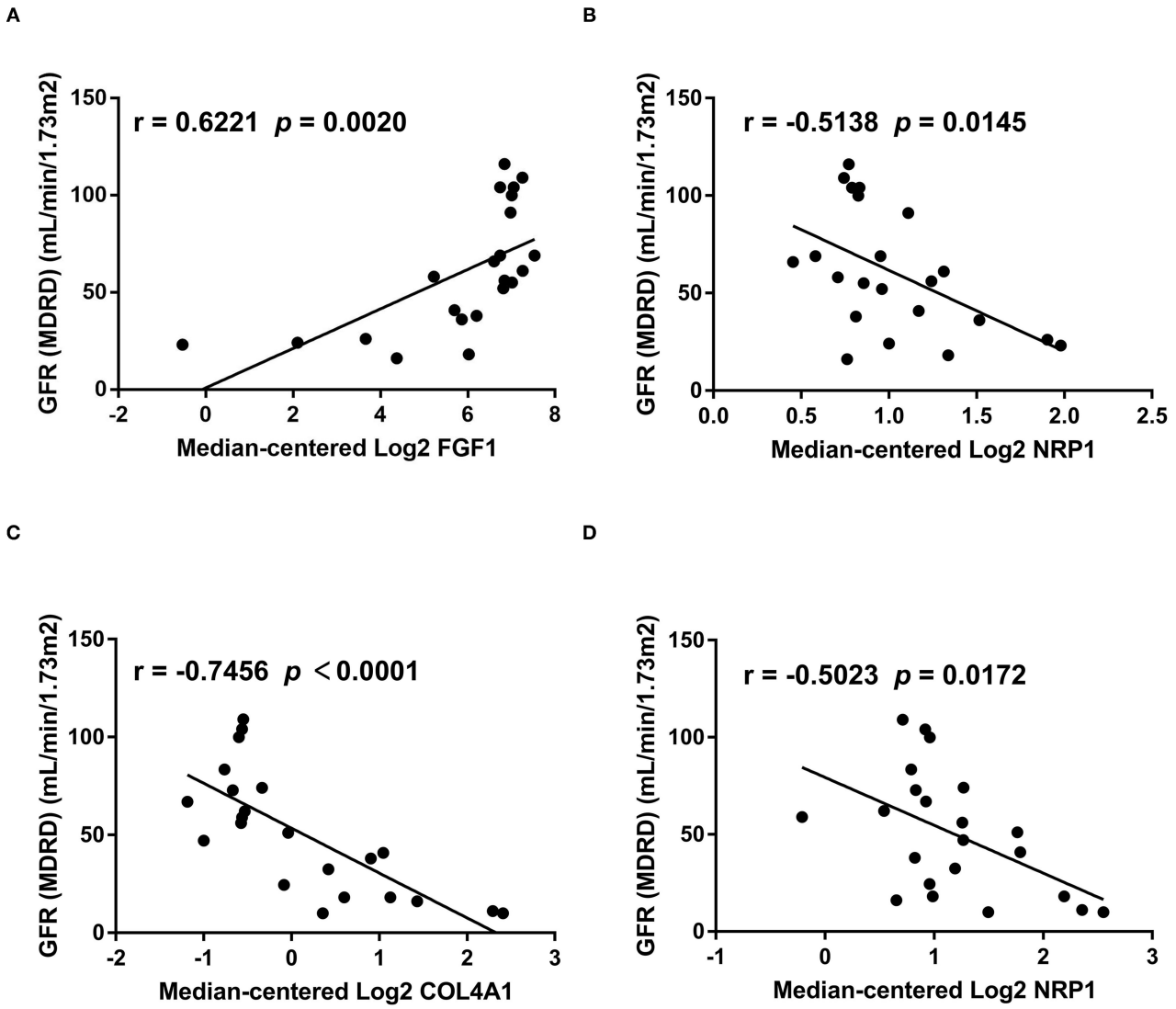

FIGURE 7 | The relationship between hub genes and GFR. We analyzed the relationship between genes involved in cell cross-talk and GFR based on bulk sequencing data. The levels of glomerular FGF1 **(A)** are positively associated with the levels of GFR, while the levels of glomerular NRP1 **(B)**, tubular COL4A1 **(C)**, and tubular NRP1 **(D)** are negatively related to the levels of GFR.

FGF1 expression (**Figure 7A**) was positively associated with the levels of GFR, while the levels of glomerular NRP1 (**Figure 7B**), tubular COL4A1 (**Figure 7C**), and tubular NRP1 (**Figure 7D**) were negatively related to the levels of GFR, suggesting that cell cross-talk-related mechanisms contribute to the development of DKD. These findings implied that the hub genes may have potential roles in the diagnosis and prevention of DKD.

DISCUSSION

Cell cross-talk participates in the development of DKD. Based on a snRNA-seq dataset and two bulk gene datasets, we provided new insight into cell communication and genes involved in DKD.

In 2019, Fu et al. (14) primarily performed scRNA-seq analysis in streptozotocin-induced diabetic endothelial nitric oxide synthase (eNOS)-deficient and control eNOS-deficient mice and revealed increased infiltrating macrophages in glomeruli, dynamic alterations in the pattern of expressed genes in glomerular endothelial cells and mesangial cells of DKD and control mice, and variable responses of individual cells. In addition, this study preliminarily analyzed the cell cross-talk between glomerular individual cells based on ligand-receptor analysis. In the same year, Wilson et al. (19) performed snRNA-seq analysis in DKD for the first time and revealed the significance of increased potassium secretion and angiogenic and possible ligand-receptor signaling pathways in glomerular individual cells. Regretfully, the former studies

have limitations. First, only the cell-to-cell interactions between glomerular individual cells were reported. Second, the subunit architecture of ligands and receptors, which accurately represents heteromeric complexes, was not taken into account. This is crucial, as cell cross-talk interacts mediated by multisubunit protein complexes instead of the binary representation used in the previous study (19). Third, the relationship between cell communication-related genes and clinical indicators was not elucidated. In this study, we analyzed cell cross-talk in all individual cells in human kidneys using a novel method (20), which accurately represents heteromeric complexes, and revealed the relationship between hub genes involved in cell cross-talk and renal function. This study reveals further mechanisms and indicates novel biomarkers and potential therapeutic targets.

Cell-to-cell interactions in the same cell type play important roles in both nondiabetic and diabetic kidneys. Podocyte-to-podocyte interactions possibly maintain the basic function of the kidney, which needs to be further studied. In DKD, mesangial cell proliferation contributes to increased internal communication via integrin pathways. Moreover, the internal communication of glomerular endothelial cells via CD74-APP is increased. CD74 (23) is upregulated in diabetic retinopathy with proliferative lesions, and APP (24, 25) is increased in diabetic microvascular complications, indicating a potential mechanism by which the angiogenesis mediated by CD74 and APP participates in DKD development and progression.

Cell-to-cell interactions limited into glomerular or tubular individual cell types are changed in DKD. In glomeruli, podocyte-expressed FGF1-mediated cell cross-talk is decreased, which is consistent with the former report that the protein levels of glomerular FGF1 are decreased in DKD (26). We found that the levels of glomerular FGF1 are positively related to the levels of GFR, suggesting that FGF1 may contribute to the development of DKD. Previous studies (26, 27) showed that FGF1 supplementation ameliorates DKD due to anti-inflammatory and antioxidative stress mechanisms, suggesting that FGF1 is a renoprotetctive factor and an encouraging therapeutic target in DKD. In tubules, PCT-expressed NRP1-mediated cell cross-talk was increased, and NRP1 expression was upregulated (**Supplementary Figure 1**). We also found that FGF1 expression is negatively associated with GFR, suggesting a potential NRP1-regulated mechanism in DKD. However, a previous study (28) showed a low density of NRP1 expression and downregulated NRP1 levels in renal fibrosis, which is contradictory to our findings. To elucidate the role of NRP1 in DKD, more samples including different disease statuses need to be collected, and further studies are needed.

In the DKD groups, we deciphered active glomerulotubular cell-to-cell interactions. In a tubule-centric view (29), the upregulation of SGLT1 and SGLT2 in PCT induced the alteration of glomerulotubular communication and hyperfiltration, explaining the renoprotective mechanisms of the novel agent SGLT2 inhibitor in DKD treatment (30–32). Nevertheless, the levels of SGLT1 and SGLT2 were not significantly altered in PCT in this study. Individual differences and different disease statuses may lead to contradictory results.

Finally, cell identification revealed immune cells in kidneys. Interestingly, DKD with high IFTA (interstitial fibrosis and tubular atrophy) samples contributed all identified B cells, suggesting the crucial role of B cells in DKD (**Supplementary Figures 2A,B**). We performed DEG analysis in immune cells and found that CD20 expression was significantly increased in the DKD groups (**Supplementary Figure 2C**). Some studies have revealed that targeting CD20 achieves therapeutic effects in renal diseases. An experimental study showed the protective effects of CD20 antibodies in lupus mice (33). Clinical evidence provides that CD20 antibodies achieve therapeutic effects in recurrent focal segmental glomerulosclerosis (34) and membranous nephropathy (35). However, the role of B cells in DKD has not been fully elucidated (36). Increased IgG+ B cells were found in the glomeruli of diabetic NOD mice when compared with those in nondiabetic mice, suggesting that B cells may contribute to the pathogenesis and prognosis of DKD (37). In DM patients, Zhang et al. (38) found increased CD38+CD19+ B cell counts in the peripheral blood. Moreover, the number of CD38+CD19+ B cells was positively correlated with the 24 h urinary protein concentration and was reduced after treatment. Taken together, these findings suggest that B cells may participate in the development of DKD. We speculate that agents targeting B cells or CD20 antibodies may have promising therapeutic effects in DKD, which needs to be further studied in future research.

Regretfully, snRNA-seq has its own limitation in capturing immune cell populations due to nanodrop technology, and frozen or optimal cutting temperature compounds may lead to the loss of information. Thus, the cross-talk between immune cells and renal parenchymal cells in DKD was not fully deciphered in this study. In addition, larger sample sizes and conditional knockout models are needed to better elucidate cell cross-talk and its further mechanism in DKD.

CONCLUSION

In summary, this study revealed cell cross-talk based on snRNA-seq and the associations between genes involved in cell communication and renal function in DKD. In DKD, cell-to-cell interactions via integrin pathways are increased, mesangial cells are stimulated and glomerulotubular communication is strongly enhanced. The level of glomerular FGF1 is positively associated with the level of GFR, while the levels of glomerular NRP1, tubular COL4A1, and tubular NRP1 are negatively associated with the level of GFR. This study furthers our understanding of cell cross-talk in DKD and reveals novel mechanisms, new biomarkers, and potential therapeutic targets to benefit patients.

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 in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

YW designed the study, performed the data analysis, and wrote the first draft. XG and ZJ revised the draft. AL and ML helped improve the methodology. 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.2021.657956/full#supplementary-material

Supplementary Figure 1 | The expression of NRP1 in tubules. The protein levels of NRP1 in normal human kidneys were obtained from The Human Protein Atlas **(A)**. Tubular NRP1 is upregulated in DKD based on bulk sequencing data **(B)**. $^{***}p < 0.001$.

Supplementary Figure 2 | The distribution and DEGs of IMC. The distribution of IMCs according to disease **(A)** and IFTA **(B)** is presented, and DEGs (DKD vs. nondiabetic control) in IMCs were calculated **(C)**. The dots in red represent upregulated genes, and the dots in blue represent downregulated genes.

Supplementary Table 1 | Number of immune cells in each sample.

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ORIGINAL RESEARCH
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Glomerular Endothelial Cells Are the Coordinator in the Development of Diabetic Nephropathy

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The prevalence of diabetes is consistently rising worldwide. Diabetic nephropathy is a leading cause of chronic renal failure. The present study aimed to explore the crosstalk among the different cell types inside diabetic glomeruli, including glomerular endothelial cells, mesangial cells, podocytes, and immune cells, by analyzing an online single-cell RNA profile (GSE131882) of patients with diabetic nephropathy. Differentially expressed genes in the glomeruli were processed by gene enrichment and protein-protein interactions analysis. Glomerular endothelial cells, as well as podocytes, play a critical role in diabetic nephropathy. A subgroup of glomerular endothelial cells possesses characteristic angiogenesis genes, indicating that angiogenesis takes place in the progress of diabetic nephropathy. Immune cells such as macrophages, T lymphocytes, B lymphocytes, and plasma cells also contribute to the disease progression. By using iTALK, the present study reports complicated cellular crosstalk inside glomeruli. Dysfunction of glomerular endothelial cells and immature angiogenesis result from the activation of both paracrine and autocrine signals. The present study reinforces the importance of glomerular endothelial cells in the development of diabetic nephropathy. The exploration of the signaling pathways involved in aberrant angiogenesis reported in the present study shed light on potential therapeutic target(s) for diabetic nephropathy.

Keywords: angiogenesis, glomerular endothelial dysfunction, single cell RNA analysis, diabetic nephropathy, cell crosstalk

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INTRODUCTION

The prevalence of diabetes keeps rising worldwide (1). Diabetes and diabetes-induced complications remarkably affect life quality and reduce life span compared with the non-diabetes population, although many advances have been made in the early diagnosis and clinical treatments (1, 2). Diabetes-induced complications include retinopathy, nephropathy, and neuropathy. Among them, diabetic nephropathy is a leading cause of chronic renal failure. Patients with diabetic nephropathy present albuminuria (<300 mg per day) at an early stage and later develop proteinuria, leading to renal failure (3, 4). Pathological changes in diabetic nephropathy include glomerular capillary widening, glomerular basement membrane thickening, mesangial matrix expansion, arteriolar hyalinosis, and glomerulosclerosis.

Glomeruli are a tight cluster of capillaries consisted of endothelial cells, podocytes, and mesangial cells. Inside the glomerulus, endothelial cells, podocytes, and glomerular basement membrane are fundamental structures for glomerular filtration. Mesangial cells are supporting cells functioning as pericytes and vascular smooth muscle cells. In diabetic patients, podocyte foot process changes are consistently observed. Since preservation of these changes reduces urinary protein excretion and improves kidney function (5), podocyte injury is considered to be a vital feature of diabetic nephropathy. On the other hand, the role of glomerular endothelial cells has been intensively studied in the last decade (6, 7). Diabetes-induced glomerular endothelial dysfunction presents the destruction of fenestrated endothelial integrity, increased cell proliferation, and immature angiogenesis, as well as an increased endothelial-to-mesenchymal transition (8). Of note, immune cells, including macrophages, T lymphocytes, B lymphocytes, plasma cells, and dendritic cells, are all involved in the development of diabetic nephropathy (9–16). Thus, it is critical to consider the importance of cellular crosstalk inside glomeruli in the progress of diabetic nephropathy since glomeruli are a fine-tuning functional unit.

Single-cell sequencing, the updated version of the next-generation sequencing technologies, provides a high resolution of cell differences in microenvironments. The use of single-cell sequencing have led to the identification of novel cells and a better understanding of specific cells in comprehensive microenvironments in developmental biology (17, 18), neurology (19), oncology (20), immunology (21, 22), cardiovascular research (23, 24), infectious disease (23, 25) as well as microbiomes (26). The online single-cell sequencing data (GSE131882) have identified fifteen types of cells, including parenchymal cells and immune cells, in the renal cortex of diabetes patients (27). The present study focused on crosstalk inside human diabetic glomeruli by subsetting the genomic data of glomerular endothelial cells, podocytes, mesangial cells, and immune cells. It was designed to investigate the role of glomerular endothelial cells under diabetic conditions, with special attention being paid to the interactions of endothelial cells with other cells inside glomeruli in the progress of diabetic nephropathy.

METHODS

Data Sources

The dataset (GSE131882) (28) were downloaded from the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/). As reported, the GSE131882 recruited three healthy and three patients with early diabetic nephropathy, among which two of the three patients presented proteinuria and glomerulosclerosis (28). The raw data were processed with zUMI (29). After gene name conversion, the Seurat package (version 3.1.2) with min.cells = 3 and min.features = 200 was used (30, 31). Quality control was performed in counts nuclei gene between 500 and 3,000, and mitochondrial gene percentage <20%. Uniform manifold approximation and projection (UMAP) presented 17 clusters of cells with dims = 1:30 and reduction = "pca." Run Principal Component Analysis (RunPCA) was set with npcs = 50. *t*-Distributed stochastic neighbor embedding (TSNE) was generated by RUNTSNE being set with dims = 1:30 and reduction = "pca." Nearest-neighbor search was run with FindNeighbors set with dims = 1:30. Clusters of cells were identified with the FindClusters being run with resolution = 0.1. Differential expressed genes (DEGs) among individual clusters were detected with FindAllMarkers function with the following settings: log-fold change.threshold = 0.25, min.pct = 0.1 and test.use = "wilcox." Highly expressed genes were identified by adjusted p-value < 0.05 with FDR <0.05. Cluster assignment was performed based on expressions of canonical marker genes. Cell identification was performed based on previous reports (32–34) and the CellMarker database (35). A total of 19,700 cells were identified in the renal cortex, including proximal convoluted tubule cells, cells in the loop of Henle, distal convoluted tubule cells, intercalated cells, principal cells, endothelial cells, podocytes, mesangial cells, and leukocytes (**Figure 1A**).

Genomic data of endothelial cells, podocytes, mesangial cells, and immune cells were extracted by the Subset function in Seurat since the present study focused on cellular crosstalk inside glomeruli. RunPCA function set with npcs = 50 was used to identify significant principal components (PCs). Significant PCs was then inputted for running the RUNTSNE and RUNUMAP. For endothelial cells, FindNeighbors was run with dims = 1:10 and FindClusters was run with resolution = 0.1. For glomerular endothelial cells (36, 37), FindNeighbors was run with dims = 1:10 and FindClusters was run with resolution = 0.3. For mesangial cells, FindNeighbors was run with dims = 1:10 and FindClusters was run with resolution = 0.05. For leukocytes, FindNeighbors was run with dims = 1:20 and FindClusters was run with resolution = 0.35. Highly expressed genes were identified by adjust p-value < 0.05 with FDR <0.05. Cluster assignment was performed based on expression of canonical marker genes (**Figure 1B**).

By using the FindMarkers function with log-fold change.theshold = 0.25, min.pct = 0.1 and test.use = "t," DEGs were defined when absolute foldchange was higher than 1.5 or lower than 0.67 with a p-value <0.05.

ClueGO (38), a plug-in in Cytoscape 3.8.3, was used for DEGs enrichment. Function clusters were calculated using kappa-score on their biological roles and presented in pie charts. The top sixty DEGs, including upregulated and downregulated ones, were visualized by heatmaps and processed using the *ComplexHeatmap* R package (version 2.2.0) (39).

To study cell-to-cell communications inside glomeruli, a ligand-receptor interaction analysis was performed using iTALK (40). DEGs described above were inputted to the FindLR function and presented by the LRPlot function.

RESULTS

Using the pan-endothelial markers EMCN and ENG, 1,070 cells were identified as endothelial cells (41). Among them, 294 endothelial cells had high expressions of EHD3 and HECW2 and they were defined as glomerular endothelial cells (36, 37). In addition, a total of 498 podocytes, 465 mesangial cells, and 336 immune cells were detected (**Table 1** and **Figures 1A,B**).

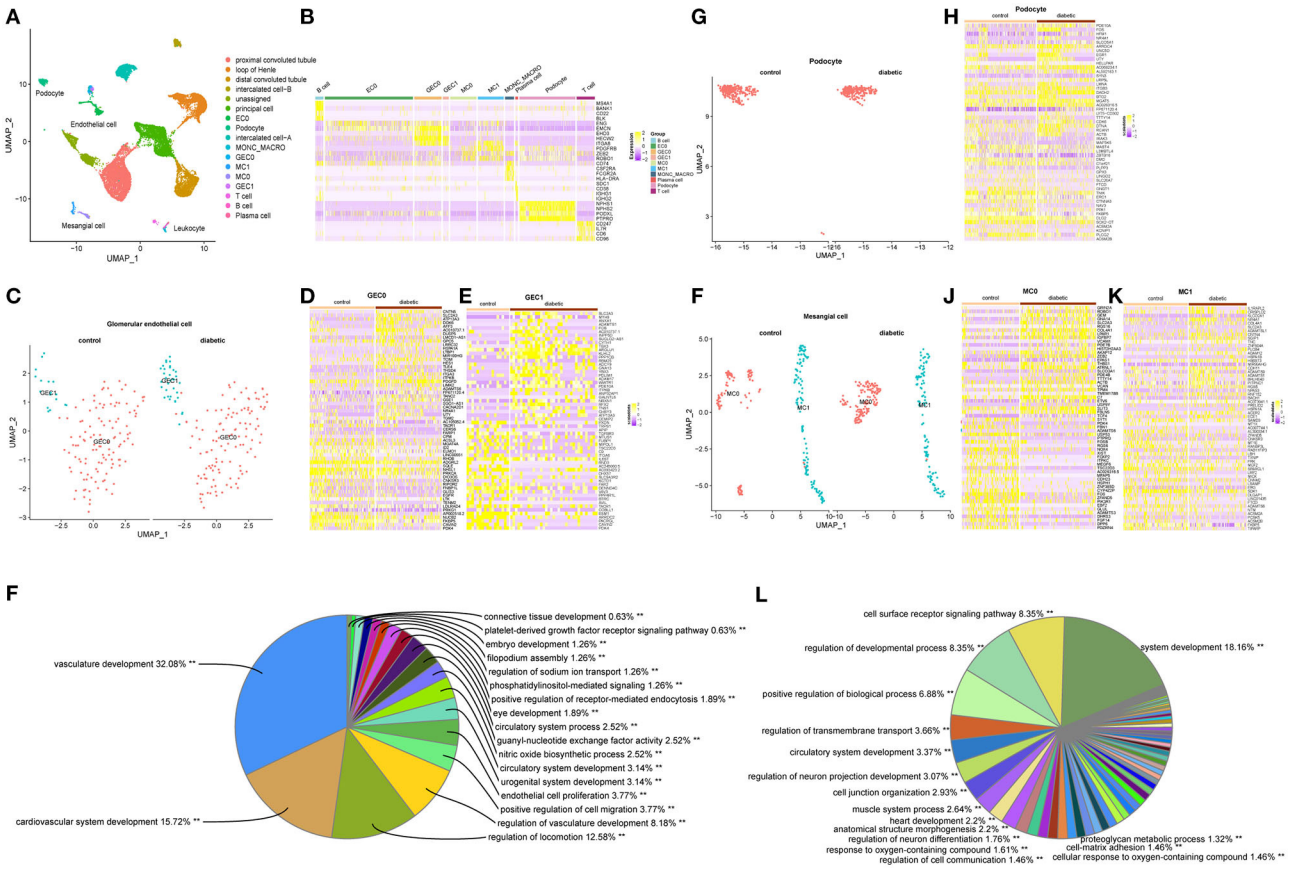

FIGURE 1 | (A) UMAP plot from unsupervised clustering of 23,980 cells from kidney cortex of healthy subjects and patients with diabetic nephropathy. **(B)** Expression levels of representative genes from each cluster. **(C)** UMAP plot of the two subclusters of glomerular endothelial cells. **(D)** Heatmap of top 30 up- and down-regulated DEGs in GEC0. **(E)** Heatmap of top 30 up- and down-regulated DEGs of GEC1. **(F)** Pie chart of significantly enriched GO terms group of GEC1. **(G)** UMAP plot of podocytes in healthy and diabetic conditions. **(H)** Heatmap of DEGs of podocytes. **(I)** UMAP plot of the two subclusters of mesangial cells. **(J)** Heatmap of top 30 up- and down-regulated DEGs in MC0. **(K)** Heatmap of top 30 up- and down-regulated DEGs in MC1. **(L)** Pie chart of significantly enriched GO terms group from DEGs of MC1. *P*-value < 0.05 were considered statistically different.

TABLE 1 | Cell counts in glomeruli from healthy subjects and patients with diabetic nephropathy.

		Healthy	Diabetic	Total
Endothelial cells	GEC0	120	122	242
	GEC1	17	35	52
Podocytes	Pod	274	224	498
Mesangial cells	MC0	101	134	235
	MC1	121	109	230
Immune cells	Monocyte	19	60	79
	T cell	16	145	161
	B cell	1	71	72
	Plasma cell	0	24	24

Among the 294 glomerular endothelial cells, 137 cells were from healthy subjects, and 157 cells were from diabetic patients. Glomerular endothelial cells were further classified into GEC0 and GEC1 subsets according to the function analysis: the former having the DEGs for the negative regulation of cell activation, cell adhesion and lymphocyte activation, and positive regulation of smooth muscle proliferation (**Supplementary Figure 1A**); and in the latter subset, the DEGs were in the modules of vasculature development, cell migration, and endothelial cell proliferation modules in the functional analysis (**Figures 1C–F** and **Supplementary Figure 1B**). In the GEC0 cluster, 120 cells were from control subjects, and 122 cells were from diabetic patients (122 cells). In GEC1 clusters, 17 endothelial cells were from the control subjects, and 35 were from diabetic patients.

All podocytes were in one group, 274 from control and 224 from diabetes. Fifty-two DEGs were identified by comparing podocytes from the control and diabetic groups. Functional analysis revealed that the DEGs were enriched in modules of structure constituent of postsynapse, striated muscle cell apoptotic process, and skeletal muscle cell differentiation (**Figures 1G,H** and **Supplementary Figure 1C**).

Mesangial cells were grouped into MC0 and MC1 subsets. Mesangial cells in MC0 (101 in control and 134 in

diabetes) were enriched for regulating anatomical structure morphogenesis, cell migration, and extracellular matrix organization (**Supplementary Figure 1D**), whereas MC1 cells (121 in control and 109 in diabetes) were enriched for the regulation of collagen biosynthetic process and vascular development (**Figures 1I–L** and **Supplementary Figure 1E**).

In the cluster of immune cells, 36 cells were from control subjects, and 300 cells were from diabetic patients. Subcluster analysis further grouped the immune cells into monocytes (macrophages) which showed high expression of CD74, CSF2RA, FCGR2A, and HLA-DRA; T lymphocytes, being highly expressed with CD247, IL7R, CD6, and CD96; B lymphocytes, with high expression of MS4A1, BANK1, CD22, and BLK; as well as plasma cells which were highly expressed with SDC1, CD38, IGHG1, and IGHG2. In the monocyte (macrophage) cluster, M1-like genes, including ITGAX and CD86, and M2-like genes, including CD163 and MRC1, were presented in both control and diabetic groups (**Figures 2A–E**).

There were 17 T lymphocytes from the control and 145 cells from the diabetic groups. DEGs in T lymphocytes were involved in gene expression, mRNA metabolic process, and T cell receptor signaling pathway.

Seventy-one B lymphocytes (only one of them were from the control group) and 24 plasma cells (all from diabetic patients) were observed in diabetic patients with proteinuria. Top genes in B lymphocytes encoded for proteins for regulating B cell differentiation and proliferation, and B receptor signaling pathway. Top genes in plasma cells were enriched in the modules of immunoglobulin biosynthetic process and B cell receptor signaling pathway (**Supplementary Figures 1F–I**).

Cell-to-Cell Communication

To study ligand-receptor interactions, DEGs of GEC1, MC1, monocytes (macrophages), T lymphocytes, as well as the top 500 genes from podocytes, B lymphocytes, and plasma cells were used. A total of 43 interactions, in autocrine and/or paracrine mechanism, were identified. In glomerular endothelial cells, TGFB2 were upregulated in diabetic group; it acted on TGFBR3 in podocytes and endothelial cells, TGFBR2 in B lymphocytes and LRP2 in mesangial cells. The growth factor PDGFB was also differentially upregulated in glomerular endothelial cells; it acted on ITGAV in podocytes and sphingosine 1-phosphate receptor 1 (S1PR1, also known as EDG1) in endothelial cells. The upregulated molecule ADAM17 in glomerular endothelial cells affected ERBB4 in podocytes, and the upregulated PSEN1 acted at CD44 of B lymphocytes and T lymphocytes and NOTCH2 of podocytes. Increased expression of ITGB8 were also observed in glomerular endothelial cells, and this protein responded to COL4A1 from mesangial cells. The receptor for TGFB2 from endothelial cells, LRP2, were downregulated in both endothelial and mesangial cells of the diabetic patients. ADAM28 was downregulated in plasma cells and B cells (**Figure 2F**).

DISCUSSION

By performing bioinformatics analysis on the online single-nucleus RNA sequencing dataset regarding glomerular cells in diabetic patients (28), the present study reports that (1) glomerular endothelial cells also play a critical role in the development of diabetic nephropathy; (2) apart from well-studied diabetes/high glucose-induced endothelial dysfunction, a group of glomerular endothelial cells possesses characteristic angiogenesis genes; and (3) immune cells such as macrophages, T lymphocytes, B lymphocytes, and plasma cells take part in the progress of diabetic nephropathy.

Podocytes and podocyte-released glomerular basement membrane are critical for preventing macro-molecular proteins from filtering out from the plasma to the kidney tubules. Moreover, podocytes are an important cell source of growth factors, which regulate endothelial cell proliferation and angiogenesis. In the present study, VEGFA, EGR1, and NOTCH2 genes are among the top 500 highly-expressed genes, supporting the critical role of podocytes in maintaining glomerular endothelial hemostasis. It is reported that podocyte counts increase in the early stage and decrease in the advanced stage of diabetes (42, 43). The present study identified comparable podocyte counts between the control and diabetic groups, with the latter showing fifty-two DEGs, in which none of them were enriched in modules of angiogenesis, vascular development, or glomerular development. The findings thus suggest that the molecular and functional changes in podocytes unlikely contribute to the progress, at least, not during the initiation phase of diabetic nephropathy.

ADAM metallopeptidase domain 17 (ADAM17) is a disintegrin and metalloprotease. By shedding tumor nuclear factor, platelet receptors glycoprotein 1, adhesion molecules, and angiotensin-converting enzyme converting enzyme 2 (ACE2), ADAM17 plays a critical role in the proinflammatory responses, thrombus formation, and renin-angiotensin system activation (44–47). ADAM17 and its shedding effects on ACE2 lead to glomerular area enlargement, glomerular and tubular basement membrane thickening, mesangial matrix expansion, and collagen deposition (48). Increased expressions of ADAM17 in kidneys are reported in diabetic patients (49) and experimental diabetic rodents (50–52). In the present study, ADAM17 in glomerular endothelial cells was upregulated, and it targeted at V-Erb-B2 avian erythroblastic leukemia viral oncogene homolog 4 (ERBB4), a member of the epidermal growth factor receptor family (EGFR), in the podocytes. Increased ERBB4 expressions phosphorylate EGFR, activates TGF-Smad-2/3 signaling, resulting in podocyte apoptosis in type 2 diabetic patients and diabetic mice (both type 1 and type 2) (53). Blockade of ERBB4 reduces glomerular damage and protects animals from the development of albuminuria (54, 55).

The role of glomerular endothelial dysfunction in the initiation and development of diabetic nephropathy has drawn attention recently. Besides well-studied endothelial dysfunction in macrocirculation in diabetes (56–59), characterized by reduced nitric oxide bioavailability, increased oxidative stress, and enhanced inflammatory responses, endothelial cells in the microcirculation also present upregulation of adhesion molecules, breakdown of endothelial barrier, and aberrant angiogenesis. Angiogenesis is a characteristic feature in diabetic microcirculation (57). The present analysis demonstrates that

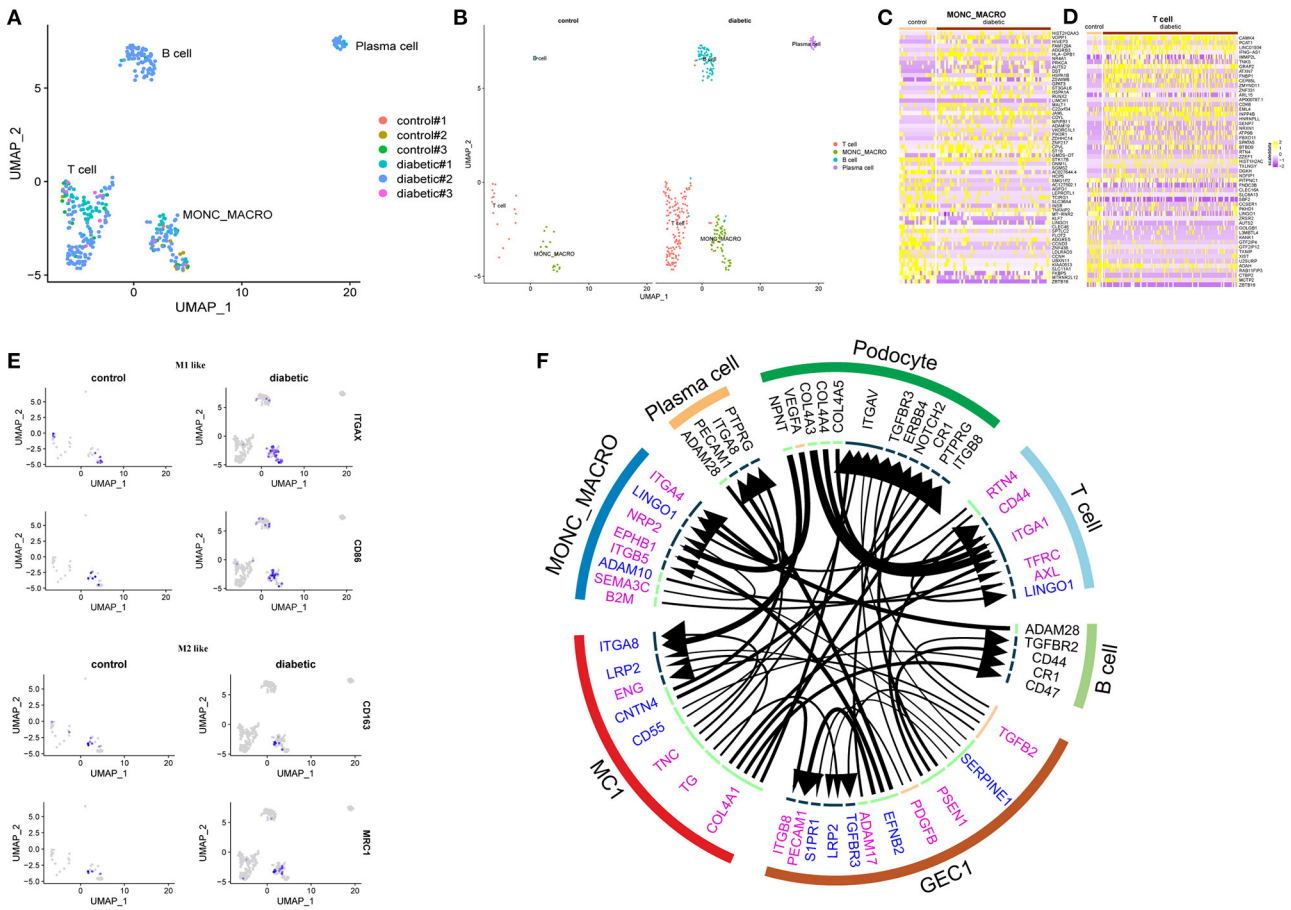

FIGURE 2 | (A) UMAP plot of the distribution of immune cells in different samples. **(B)** UMAP plot of four subclusters of immune cells. **(C)** Heatmap of top 30 up- and down-regulated DEGs in monocytes/macrophages. **(D)** Heatmap of top 30 up- and down-regulated DEGs in T lymphocytes. **(E)** Distribution of selected cell markers of monocyte/macrophage: (Upper) Presence of M1-like macrophage markers (ITGAX and CD86) and (Lower) M2-like macrophage markers (CD163 and MRC1). **(F)** Ligand-receptor interactions prediction network with DEGs from GEC1, MC1, monocytes/macrophages (MONO_MACRO), T lymphocytes, and top 500 expressed genes of podocytes, B lymphocytes, and plasma cells. In the circus, upregulated genes are labeled purple; downregulated genes labeled blue; top expressed genes in podocytes, B lymphocytes and plasma cells labeled black. The lines and arrowheads inside are scaled to indicate the correlations of the ligand and receptor. *P*-value < 0.05 were considered statistically different.

diabetic patients have a higher proportion of glomerular endothelial cells in the GEC1 group, the glomerular endothelial cells with high expression of angiogenetic genes, than control subjects (17/137 for healthy subjects, and 35/157 for diabetic patients), thus supporting that diabetes induces glomerular endothelial cell proliferation, and these proliferative endothelial cells are fundamental for immature angiogenesis, vessel leakage as well as glomerulosclerosis.

VEGF, an endothelial-specific growth factor, promotes endothelial cell proliferation and differentiation, resulting in increased endothelial permeability. Under physiological conditions, a low basal VEGF level is required for endothelial cell homeostasis (60). VEGF, mainly VEGFA, is produced by podocytes, and VEGFRs are present on glomerular endothelial cells. In the present study, VEGFA was highly expressed in podocytes while an upregulation of its canonical receptors in glomerular endothelial cells was not detected in diabetic patients; the finding thus suggests that other angiogenetic signaling pathways are involved in diabetes-induced aberrant angiogenesis.

Endothelial released-PDGFB, another angiogenetic factor, targeted S1PR1 in endothelial cells, which was downregulated in the present study. The S1PR1, a G-protein-coupled receptor family member, responds to sphingosine-1-phosphate (S1P) (61), VEGF (62), and PDGFB (63). S1PR1 is mainly expressed in microvascular endothelial cells and plays a critical role in promoting barrier integrity (64, 65), sproutings (62), angiogenesis maturation (66–68), and nitric oxide generation (69). Endothelium-specific S1PR1-knockout mice exhibit impaired blood-brain-barrier integrity and increased adhesion molecule expressions in a middle cerebral artery occlusion-induced stroke model (70–72). Cardiomyocyte-restricted deletion of S1PR1 shows progressive cardiomyopathy and premature death due to impaired activity of sarcolemmal Na^+/H^+ exchange and increased Ca^{2+} sensitivity

(73). S1PR1 signaling pathway controls the renal vasculature development in mouse early embryogenesis (74), and protects glycocalyx by shedding syndecan-1 (75). The uncoupled expressions of PDGFB and S1PR1, with the former being upregulated and the latter downregulated, in the present study and literatures (63) indicates that the overspilled PDFGB probably signals through other receptors, resulting in endothelial barrier leakage and immature angiogenesis.

Ephrin B2 (EFNB2) was decreased in endothelial cells, while its receptor EPHB1 was increased in monocytes/macrophages. Ephrin/Eph receptor interactions are bidirectional and play essential roles in vascular development. Mice with endothelial EFNB2-deletion display a severely compromised vascular system and die at mid-gestation. Inhibiting Ephrin B ligands prevents endothelial cell sprouting and induces endothelial cell assembly in disorder (76–79). Besides, Ephrin/Eph receptor interaction facilitates macrophage recognition of differentiating human erythroblasts (80).

Serpin family E member 1 (SERPINE1, also known as endothelial plasminogen activator inhibitor PAI-1) is the primary physiological inhibitor of tissue plasminogen activator and urokinase-type plasminogen (uPA) activator, and participates in preventing fibrinolysis and promoting angiogenesis as well as inhibiting matrix metalloproteinases (81, 82). SERPINE1 stimulates angiogenesis through its vitronectin-binding function. SERPINE1 promotes angiogenesis at physiological concentrations but inhibits vascularization at pharmacological concentrations (83). By combining with uPA receptor and LDL-receptor-associated protein (LRP), SERPINE1 affects monocyte/macrophage motility (84–86). In the present study, both glomerular endothelial cells and mesangial cells have reduced expressions of LRP2, an endocytic receptor for protein reabsorption from the glomerular filtrate. So far, the presence of LRP is mainly reported in tubular cells and podocytes, with few reports in mesangial cells and glomerular endothelial cells.

Presenilin-1 (PSEN1) is a component of synaptic and endothelial adherens junctions (87). Genetic mutation on presenilin-1 presents early-onset Alzheimer symptoms in mice, accompanied by decreases in capillary sprouting sites and increases in capillary diameter (88). It indicates that PSEN1 is involved in angiogenesis.

In the high-angiogenetic GEC1 group, increased expressions of PDGFB, TGFB2, ADAM17, and ITGB8, and reduced expression of S1PR1 are linked to glomerular angiogenesis and glomerulosclerosis, whereas the increased expression of presenilin-1 and the decreased expressions of SERPINE1 and EFNB2 in glomerular endothelial cells correlate to the downregulation of angiogenesis. The activity of glomerular endothelial cells, including immature angiogenesis, is regulated by podocytes, mesangial cells, glomerular endothelial cells, and immune cells in a paracrine and/or an autocrine way. The co-existence of pro-angiogenetic and anti-angiogenetic factors in glomeruli of diabetic patients indicates that diabetes-induced angiogenesis is counterbalanced by the compensatory mechanisms from the neighboring cells. It is further confirmed with their pathological changes that two of three diabetic patients presented with proteinuria and an increased proportion of global glomerulosclerosis (28). It is important to note that the progressive changes in diabetic nephropathy are hard to restore when compensatory works fade. Therefore, protecting endothelial cell function and preventing angiogenesis may have therapeutic potential since the two compensatory molecules have additional physiological roles.

In addition to aberrant angiogenesis, interstitial fibrosis is another characteristic feature of diabetic nephropathy. Integrins are a family of ubiquitous αβ heterodimeric receptors. Integrins form receptors for different ligands due to combinations of alpha and beta subunits; thus, one integrin binds several ligands while one ligand is recognized by several integrins (89–91). Integrins regulate a variety of biological processes, including cell growth, proliferation, migration, signaling, and cytokine activation, thereby playing important roles in inflammation, infection, and angiogenesis (92). In glomeruli, integrin α8 (ITGA8) is exclusively present in mesangial cells (93, 94). Increased ITGA8 expression has the potential to be a clinical marker of glomerular disease prognosis since ITGA8 supports adhesion of mesangial cells (95), reduces cell proliferation (96), protects against apoptosis (97), and facilitates phagocytosis (95, 98). Of importance, increased expressions of ITGB8 play a role in glomerular endothelial viability by controlling the release of bioactive TGF-β (99, 100), a potent inducer of endothelial-mesenchymal transition, especially TGF-β2 isoform (101). In the present study, ITGB8/ITGA8 was on the top list of the genes expressed in podocytes and plasma cells. While ITGB8 expression is increased in glomerular endothelial cells, ITGA8 expression is decreased in mesangial cells. Moreover, COL4A1 in mesangial cells, the expression of which was increased in diabetes, acted on ITGB8 in glomerular endothelial cells. The upregulated TGF-β2 in endothelial cells further activates its corresponding receptors, namely TGFBR2, TGFBR3 and ENG, on B lymphocytes, podocytes, glomerular endothelial cells, and mesangial cells, respectively, leading to epithelial-mesenchymal transition and fibrosis in the development of diabetic nephropathy.

In addition, ITGA1 expression was increased in T lymphocytes, and ITGA4 expression was on the top gene list of monocytes/macrophages. Blocking ITGA4 inhibits neutrophil migration into the glomerulus and reduces proteinuria in mice with glomerular basement membrane nephritis (102). Combined treatment of anti-ITGB2 and anti-ITGA4 antibodies reduces monocyte/macrophage infiltration into the glomeruli, while neither alone has significant effects (103).

Both integrin and CD44 respond to osteopontin, collagens, and matrix metalloproteinases (104). In the present study, CD44 expressions were upregulated in both T and B lymphocytes, suggesting that glomerular parenchymal cells, together with lymphocytes, participate in glomerulosclerosis.

Semaphorins are a large family of secreted and membrane-bound proteins. The class 3 secreted semaphorin, SEMA3, is present in human peripheral blood monocytes. In response to signals of cadherins (105, 106), and VEGFRs (107, 108), SEMA3 forms complexes with neuropilin (NRP) and integrins (109, 110) to regulate organ development, tissue repair, immune responses, and tumorigenesis processes (109, 111–113). Both

NRP1 and NRP2 expressions are reduced in M1 differentiation, while NRP1 and SEMA3A expression are increased in M2 phenotype (114). Concomitant upregulation of SEMA3A and NRP2 demonstrated in the present analysis indicates that diabetes induces M2-like macrophages through an autocrine mechanism (115).

ADAM28 expression is downregulated in both B lymphocytes and plasma cells. ADAM28 expression is positively related to B cell proliferation (116). Upregulated CD19 controls B cell differentiation by regulating ADAM28-mediated NOTCH2 cleavage (117). It indicates that these antibody-producing lymphocytes are inactivated or dysfunctional in diabetes, although presences of B lymphocytes and plasma cells were exclusively observed in diabetes.

By analyzing the online dataset GSE131882 (28), the present study focuses on exploring the interactions of parenchymal cells of glomeruli as well as the potential involvements of immune cells in the progress of diabetic nephropathy, and special attention is paid to the angiogenesis process. Both pro- and anti-angiogenetic genes are observed in the GEC1 and its neighboring cells, indicating a dynamic interplay between parenchymal cells and immune cells in the glomerulus during the early stage of the disease. A limitation of this study is a lack of confirmation in a cohort of diabetic kidneys. Given the reluctance of diabetic patients for biopsy, pertinent experimental animal models are an alternative. However, species differences are a critical issue, because species-specific genes and cell-type identification can affect the analysis. In human diabetes, endothelial cells with high expression of EHD3 and HECW2 are defined as glomerular endothelial cells; by contrast, in mouse diabetes, endothelial cells are identified with high expression of Emcn, Kdr, Flt1, and Pecam1 (36). Thus, cautions are warranted for the interpretation, as well as conclusion, of dataset from experimental animals, and a direct extrapolation of those dataset to human condition may not be feasible.

In brief, the present study reports comprehensive interactions in diabetic glomeruli. A subgroup of glomerular endothelial cells with pro-angiogenesis characteristics is identified, thereby providing an evidence for the critical contribution of immature angiogenesis to the vessel leakage, glomerular barrier dysfunction, and glomerulosclerosis in the progress of diabetic nephropathy. Furthermore, glomerular endothelial cells are not an independent player in the progress of diabetic nephropathy. Inside glomeruli, podocytes, mesangial cells, monocytes/macrophages, lymphocytes are all orchestrating in the scenario. The identification of glomerular endothelial cells with angiogenetic characters and the signaling pathways involved in the present study shed light on the therapeutic target for diabetic nephropathy.

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 in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

TL, KS, and JL analyzed the data. TZ, SL, and YS wrote the manuscript, read, edited/revised the manuscripts, and gave final content approval. 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.2021.655639/full#supplementary-material

Supplementary Figure 1 | (A) Pie chart of functional biological modules (left) and GO terms (right) of DEGs in GEC0. **(B)** Pie chart of functional biological modules (left) and GO terms (right) of DEGs in GEC1. **(C)** Pie chart of functional biological modules (left) and GO terms (right) of DEGs in podocytes. **(D)** Pie chart of functional biological modules (left) and GO terms (right) of DEGs in MC0. **(E)** Pie chart of functional biological modules (left) and GO terms (right) of DEGs in MC1. **(F)** Pie chart of functional biological modules (left) and GO terms (right) of DEGs in monocytes/macrophages. **(G)** Pie chart of functional biological modules (left) and GO terms (right) of DEGs in T lymphocytes. **(H)** Pie chart of functional biological modules (left) and GO terms (right) of top 200 genes in B lymphocytes. **(I)** Pie chart of functional biological modules (left) and GO terms (right) of top 200 genes in plasma cells.

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PPAR-α Agonist Fenofibrate Prevented Diabetic Nephropathy by Inhibiting M1 Macrophages via Improving Endothelial Cell Function in db/db Mice

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Background: Diabetic nephropathy (DN) is one of the major diabetic microvascular complications, and macrophage polarization plays a key role in the development of DN. Endothelial cells regulate macrophage polarization. Peroxisome proliferator-activated receptor (PPAR)-α agonists were demonstrated to prevent DN and improve endothelial function. In this study, we aimed to investigate whether PPAR-α agonists prevented DN through regulating macrophage phenotype via improving endothelial cell function.

Methods: Eight-week-old male C57BLKS/J db/m and db/db mice were given fenofibrate or 1% sodium carboxyl methylcellulose by gavage for 12 weeks.

Results: Db/db mice presented higher urinary albumin-to-creatinine ratio (UACR) than db/m mice, and fenofibrate decreased UACR in db/db mice. Fibrosis and collagen I were elevated in db/db mouse kidneys compared with db/m mouse kidneys; however, they were decreased after fenofibrate treatment in db/db mouse kidneys. Apoptosis and cleaved caspase-3 were enhanced in db/db mouse kidneys compared to db/m mouse kidneys, while fenofibrate decreased them in db/db mouse kidneys. Db/db mice had a suppression of p-endothelial nitric oxide synthase (eNOS)/t-eNOS and nitric oxide (NO), and an increase of angiopoietin-2 and reactive oxygen species (ROS) in kidneys compared with db/m mice, and fenofibrate increased p-eNOS/t-eNOS and NO, and decreased angiopoietin-2 and ROS in db/db mouse kidneys. Hypoxia-inducible factor (HIF)-1α and Notch1 were promoted in db/db mouse kidneys compared with db/m mouse kidneys, and were reduced after fenofibrate treatment in db/db mouse kidneys. Furthermore, the immunofluorescence staining indicated that M1 macrophage recruitment was enhanced in db/db mouse kidneys compared to db/m mouse kidneys, and this was accompanied by a significant increase of tumor necrosis factor (TNF)-α and interleukin (IL)-1β in kidneys and in serum of db/db mice compared with db/m

mice. However, fenofibrate inhibited the renal M1 macrophage recruitment and cytokines associated with M1 macrophages in db/db mice.

Conclusions: Our study indicated that M1 macrophage recruitment due to the upregulated HIF-1α/Notch1 pathway induced by endothelial cell dysfunction involved in type 2 diabetic mouse renal injury, and PPAR-α agonist fenofibrate prevented DN by reducing M1 macrophage recruitment via inhibiting HIF-1α/Notch1 pathway regulated by endothelial cell function in type 2 diabetic mouse kidneys.

Keywords: PPAR-α agonists, diabetic nephropathy, macrophages, endothelial function, HIF-1α, Notch1

INTRODUCTION

Diabetic nephropathy (DN), the main cause of end-stage renal disease, is one of the major microvascular complications of diabetes. The primary initiating mechanism in DN is hyperglycemia-induced vascular dysfunction (1). Nitric oxide (NO) is a major regulator of vascular tone. Diminution of NO has been considered as a major mechanism underlying development of diabetic complications involving the vasculature, especially DN (2). There are three distinct genes that encode three nitric oxide synthase (NOS) isoforms: neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS), and endothelial nitric oxide synthase (eNOS). Both nNOS and iNOS are weakly expressed in the kidney. Most eNOS is strongly expressed in renal endothelial cells, although tubular expression of eNOS also occurs (3). Thus, NO in kidneys is mainly generated by eNOS in endothelial cells. Recent studies have found that eNOS and NO were decreased, whereas iNOS and nNOS were increased in diabetic rats (4, 5). ENOS dysfunction in endothelial cells has been demonstrated to have a key role in the development of DN. Both type 1 and type 2 diabetic mice with eNOS deficiency are more susceptible to renal injury compared to wild type diabetic mice (6–8).

Renal vasoconstriction, induced by the deficiency of NO, likely contributes to renal injury due to renal tissue hypoxia, which leads to the increased expression of hypoxia-inducible factor (HIF)-1α. Renal vasodilatation function was improved through upregulating the activity of eNOS and consequently downregulating the expression of HIF-1α in septic shock rats, which could be antagonized by eNOS inhibitor (9), supporting that the activation of eNOS could regulate the level of HIF-1α. Our previous study has found that increased angiopoietin-2 (Ang-2), an indicator of endothelial dysfunction, was related to elevated HIF-α in mouse kidneys (10). Recent studies have also proven that the inhibition of HIF-1 protected against DN (11). Our previous study showed that endothelial-specific prolyl hydroxylase domain protein-2 (PHD2) knockout (PHD2ECKO) mice, with the upregulated expression of HIF-α in endothelial cells due to the deficient degradation of HIF, presented significant renal fibrosis through activating Notch (10).

Notch is a key regulator of cellular development, differentiation, survival and function, which is usually achieved by interacting with other pathways, including HIF-1α signaling. HIF-1α, which is induced by hypoxia, contributes to Notch increasing (12), and inhibiting HIF-1α decreases Notch activity (13). Notch signaling promotes the development of DN including accelerating pathological changes in glomerulus, tubules, interstitium, and blood vessels (14). Moreover, Notch has been proven to regulated macrophage polarization which further induces fibrosis in DN (15).

Accumulating evidence suggests the critical role of macrophage polarization in the development of fibrosis, and the effect of M1 macrophage polarization on accelerating renal fibrosis in DN (1, 15–17). In the progression of DN, monocytes are rapidly recruited to sites of diabetic complications and differentiate into macrophages, which leads to diabetic nephropathy, fibrosis, and proteinuria. Macrophage polarization can be regulated at least partially by endothelial cells. Endothelial-specific Ang-2 overexpressed mice showed increased macrophage infiltration (18, 19). Endothelial cells decrease M1 marker expression (20). Furthermore, endothelial cell senescence was in connection with renal M1 macrophage accumulation (21). These researches have suggested that endothelial cell function might regulate M1 macrophage accumulation in kidneys. Recent researches have found that hypoxia regulated macrophage polarization, and there was a significant relationship between HIF-1α and M1 macrophage polarization (22, 23). The regulation effects of Notch on macrophage polarization and fibrosis have been paid generally attention in renal injury (1, 15–17). Notch can promote renal fibrosis through inducing M1 macrophage polarization. However, whether endothelial function regulates macrophage polarization via HIF-1α/Notch1 pathway has been uncertain.

Peroxisome proliferator-activated receptor (PPAR)-α is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors and plays an important role in lipid metabolism (24). The consistency of clinical data from PPAR-α agonists studies have demonstrated consistent benefit with fenofibrate on preventing the progression of diabetic microvascular diseases, independent of lipid levels (25). The FIELD study showed significant beneficial effects on diabetic complications in micro-vascular (i.e., nephropathy, retinopathy, and non-traumatic amputations) (26). The DAIS study indicated that fenofibrate prevented the progression to microalbuminuria on a long-term basis in diabetic patients (27). Furthermore, our previous research also found that fenofibrate reduced microalbuminuria in patients with type 2 diabetes (28). In addition, PPAR-α agonists have been demonstrated to prevent

DN and reduce proteinuria in both type 1 and type 2 diabetic animals (29–32). Nevertheless, the mechanism of how PPAR-α agonist fenofibrate prevents DN has not been fully explored.

In our previous studies, high glucose induced endothelial dysfunction as indicated by an increased reactive oxygen species (ROS) generation and a decreased NO production in human umbilical vein endothelial cells (HUVECs) (33). However, fenofibrate recoupled eNOS and increased the secretion of NO in HUVECs (34). In addition, fenofibrate significantly improved coronary flow velocity reserve (CFVR) and arterial stiffness in patients with hypertriglyceridemia (35). Our previous results suggested that PPAR-α agonist fenofibrate could adjust endothelial function and vascular tone. In addition, studies *in vivo* and *vitro* have found that PPAR-α agonists had therapeutic effects on ischemic retina diseases, especially on diabetic retinopathy, through the downregulation of HIF-1α in endothelial cells (36, 37). PPAR-α agonists diminished hypoxia-induced HIF-1α expression and activity in cancer cells (38). Moreover, upregulating PPAR-α could suppress Notch-1 signaling (39).

PPAR-α agonists prevented renal fibrosis in DN (40). However, the effects of PPAR-α agonists on macrophage phenotype have been still unclear. Furthermore, whether PPAR-α agonists prevent DN through regulating macrophage phenotype through HIF-1α/Notch1 pathway adjusted by endothelial cell function has not been studied yet. In this study, we aimed to investigate the mechanism of preventing DN by PPAR-α agonist fenofibrate.

MATERIALS AND METHODS

The animal experiments were approved by the Animal Ethics Committee of Beijing Chao-Yang Hospital, Capital Medical University and were performed in accordance with animal care guidelines of Beijing Chao-Yang Hospital, Capital Medical University.

Experimental Animal Model and Treatment

Seven-week-old male C57BLKS/J db/m and db/db mice (t002407) were obtained from Nanjing Biomedical Research Institute of Nanjing University, Nanjing, China. They were divided into four groups, db/m group, db/m+F group, db/db group, and db/db+F group.

Db/m+F and db/db+F groups ($n = 6$ for each group) were given 100 mg/kg of fenofibrate (0.1%, w/w, Sigma, St Louis, MO, USA) dissolved in 1% sodium carboxyl methylcellulose (Na-CMC) by gavage once per day for 12 weeks starting at 8 weeks of age. Db/m and db/db groups ($n = 6$ for each group) were treated with 1% Na-CMC alone by gavage once per day for 12 weeks starting at 8 weeks of age (29).

All mice were housed in clear cages ($n = 3$/cage) and maintained on a 12-h light/dark cycle (lights on 08:00–20:00 h) at 22 ± 1°C with water and food available *ad libitum*. After 12-week administration, all mice were placed in metabolic cages separately. At week 20, all animals were anesthetized by intraperitoneal injection of a mixture of Rompun 10 mg/kg (Bayer Korea, Ansan, Gyeonggi-Do, Korea) and Zoletil 30 mg/kg (Virbac, Carros, France). Blood was obtained from the left ventricle and was stored at −80°C for subsequent analyses. The mouse kidneys were removed.

Measurements of Blood and Urinary Parameters

Blood was collected following an overnight fast for 12 h. A 24-h urine collection was obtained using metabolic cages. Blood glucose (GLU) level was detected by HemoCue B-Glucose kit (HemoCue AB, Angelholm, Sweden). Insulin (INS) level was detected by radioimmunoassay kit (Linco Reasearch, St Charles, MO, USA). Triglycerides (TG) and total cholesterol (TC) concentrations were measured by an auto-analyzer (Wako, Osaka, Japan). Blood urea nitrogen (BUN) was measured by iStat-Kit (HESKA, Fort Collins, MO, USA). Serum and urine creatinine concentrations were detected by HPLC (Beckman Instruments, Fullerton, CA, USA). Urinary albumin concentration was detected using an immunoassay (Bayer, Elkhart, IN, USA). Urinary albumin-to-creatinine ratio (UACR) was calculated as urine albumin/urine creatinine (μg/mg).

Light Microscopic Study

The renal tissues were fixed in neutral-buffered 10% formalin solution (SF93-20; Fisher Scientific, Pittsburgh, PA, USA). The histology was measured by Hematoxylin & Eosin (H&E) staining (ab245880, Abcam, Cambridge, MA, USA) and Periodic Acid Schiff (PAS) staining (ab150680, Abcam, Cambridge, MA, USA). The fibrosis score was based on the ratio of fibrotic area to total area determined by Sirius red staining (ab150686, Abcam, Cambridge, MA, USA) and Masson's trichrome staining (ab150686, Abcam, Cambridge, MA, USA). Renal apoptosis was detected by TUNEL (MK1018, Boster, Wuhan, China). The renal samples were also embedded in frozen optimal cutting temperature compound (4585; Fisher Health Care, Houston, TX, USA). Frozen sections were prepared (8 μm in thickness). Reactive oxygen species (ROS) in frozen sections was measured by dihydroethidium (DHE) staining. Immunostaining of F4/80 (1:100; Abcam, Cambridge, MA, USA), CD86 (1:100; R&D systems, MN, USA), and CD32/16 (1:100; R&D systems, MN, USA) antibodies were in fresh frozen sections. These immune-stained sections were incubated with donkey anti-rat IgG-FITC (Sigma-Aldrich, Shanghai, China) (1:500) or anti-goat IgG-Cy3 produced in rabbit (Sigma-Aldrich, Shanghai, China) (1:500). For the quantification of proportional areas of staining, 10 areas were used, which were randomly located in mouse kidneys. Image J software (NIH, Bethesda, MD, USA) was used for image-analysis.

Western Blot Analyses

The renal cortex tissues were homogenized in lysis buffer. The homogenates were centrifuged at 16,000 × g at 4°C for 10 min. A bicinchoninic acid protein assay kit (Pierce Co, Rockford, IL, USA) was used for measuring the protein concentrations. Equal amounts (20 μg) of the protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline and were incubated by the following

primary antibodies overnight: podocin (1:1,000; Sigma-Aldrich, Shanghai, China), collagen I (1:1,000; Abcam, Cambridge, MA, USA), cleaved caspase-3 (1:1,000; Abcam, Cambridge, MA, USA), phospho-endothelial nitric-oxide synthase (p-eNOS) (1:1,000; BD transduction, San Jose, CA, USA), total-endothelial nitric-oxide synthase (t-eNOS) (1:1,000; BD transduction, San Jose, CA, USA), Ang-2 (1:1,000; Santa Cruz, CA, USA), HIF-1α (1:1,000; Novus Bio, Littleton, CO, USA), Notch1 (1:1,000; Abcam, Cambridge, MA, USA), and β-actin (1:1,000; Cell Signaling, Danvers, MA, USA). After washing, the membranes were incubated for 2 h with an anti-rabbit or anti-mouse secondary antibody coupled to horseradish peroxidase (1:5,000; Santa Cruz, CA, USA). Luminol was used as substrate. Densitometric analyses were performed using image acquisition and analysis software (Bio-Rad).

RNA Extraction and Quantitative Reverse Transcriptase PCR (RT-PCR)

The total RNA was extracted from mouse renal tissue using TRIzol (Invitrogen, Carlsbad, CA, USA). RT -PCR was executed with QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA).

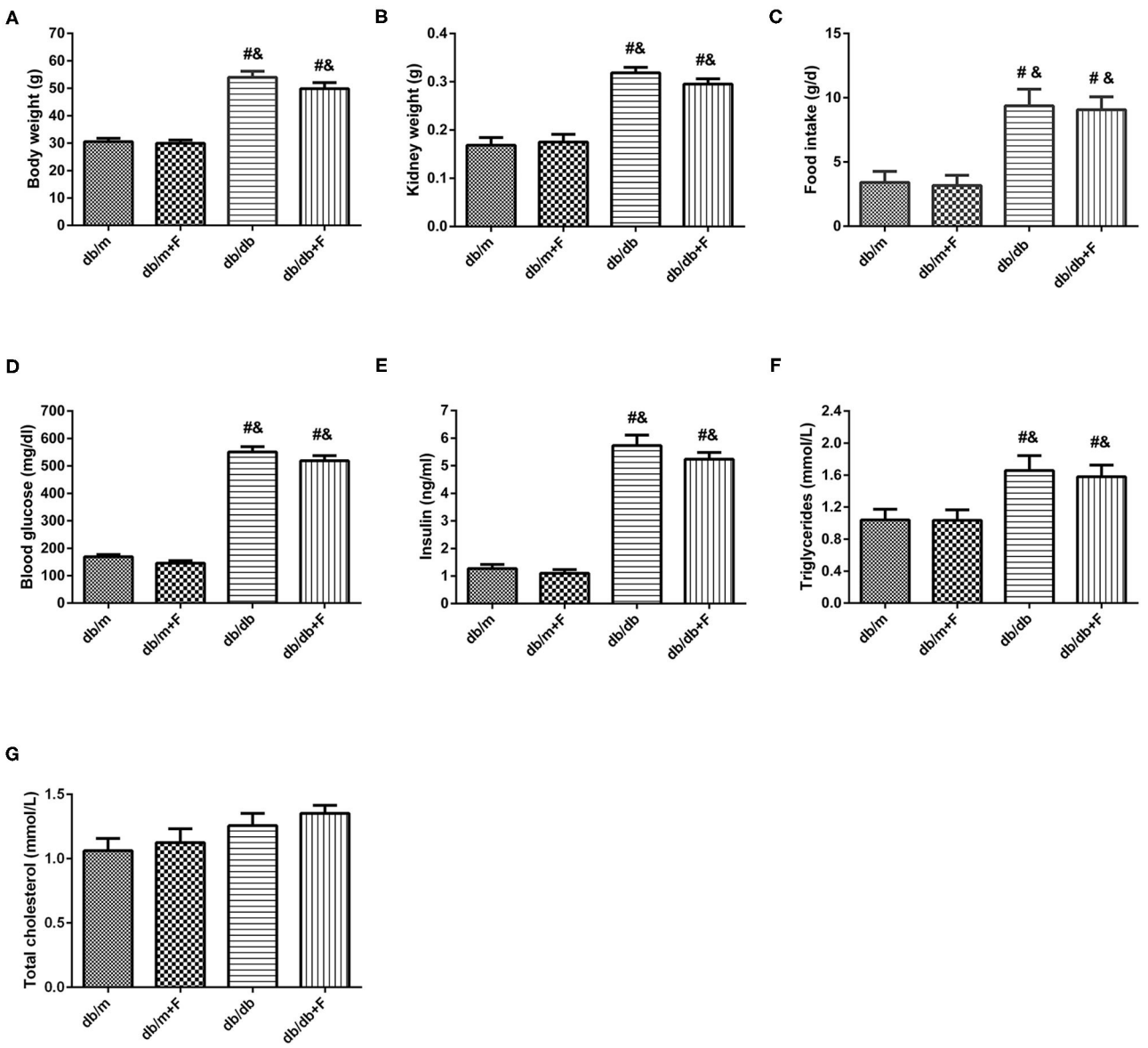

FIGURE 1 | Physical and biochemical characteristics in db/m, db/m+F, db/db, and db/db+F groups. **(A)** Body weight. **(B)** Kidney weight. **(C)** Food intake. **(D)** Fasting blood glucose level. **(E)** Fasting Insulin level. **(F)** Fasting triglycerides. **(G)** Fasting total cholesterol. $n = 6$ mice/group. $^{\#}P < 0.05$, vs. db/m group; $^{\&}P < 0.05$ vs. db/m+F group. Db/m, db/m mice without fenofibrate treatment; db/m+F, db/m mice with fenofibrate treatment; db/db, db/db mice without fenofibrate treatment; db/db+F, db/db mice with fenofibrate treatment. Data are means ± S.E.M.

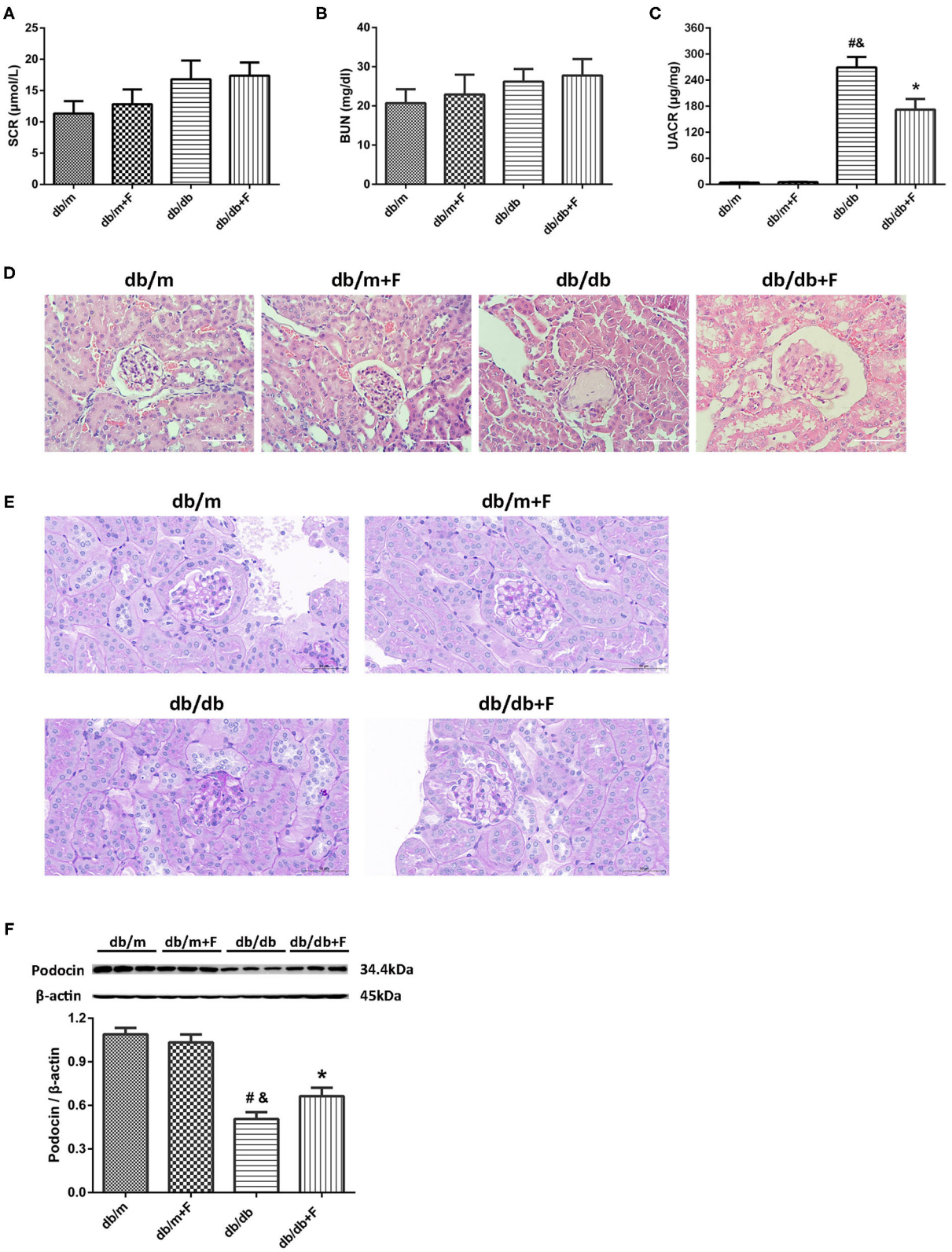

FIGURE 2 | Renal phenotype in db/m, db/m+F, db/db, and db/db+F groups. **(A)** Serum creatinine (SCr). **(B)** Blood urea nitrogen (BUN). **(C)** Urinary albumin-to-creatinine ratio (UACR). **(D)** Representative photographs of mouse kidneys by H&E staining. **(E)** Representative photographs of mouse kidneys by PAS

(Continued)

FIGURE 2 | staining. **(F)** Representative photographs and quantification of podocin in mouse kidneys detected by western blot. $n = 6$ mice/group. $^{\#}P < 0.05$ vs. db/m group; $^{\&}P < 0.05$ vs. db/m+F group; $^{*}P < 0.05$ vs. db/db group. Db/m, db/m mice without fenofibrate treatment; db/m+F, db/m mice with fenofibrate treatment; db/db, db/db mice without fenofibrate treatment; db/db+F, db/db mice with fenofibrate treatment. Data are means ± S.E.M.

Primer sequences of tumor necrosis factor (TNF)-α were 5′–CAG GAG GGA GAA CAG AAA CTC CA−3′ (sense) and 5′–CCT GGT TGG CTG CTT GCT T−3′ (antisense), primer sequences of IL-1β were 5′–GCA ACT GTT CCT GAA CTC AAC T−3′ (sense) and 5′–ATC TTT TGG GGT CCG TCA ACT−3′ (antisense), and primer sequences of β-actin were 5′-CAT CCG TAA AGA CCT CTA TGC CAA C−3′ (sense) and 5′-ATG GAG CCA CCG ATC CAC A−3′ (antisense).

NO Levels

Renal NO production was measured using commercial kits (Sigma-Aldrich, Shanghai, China), performed in accordance with the manufacturer's protocol.

Serum Inflammatory Cytokines

Serum inflammatory cytokines associated with M1 macrophages, TNF-α and interleukin (IL)-1β, were measured with ELISA (eBioscience, San Diego, CA, USA). All assays were performed according to the manufacturer's protocol.

Statistical Analyses

All data were analyzed using Statistical Package for Social Sciences version 22.0 (SPSS, Inc., Chicago, IL, USA). Data were expressed as means ± S.E.M. Comparisons of the means of corresponding values in four groups were performed using one-way ANOVA. All tests were two-sided, and a $P < 0.05$ was used to indicate statistical significance for the results.

RESULTS

Physical and Biochemical Characteristics

As presented in **Figure 1**, body weight (BW), kidney weight (KW), food intake, blood glucose (GLU), insulin (INS), and triglycerides (TG) were significantly higher for db/db and db/db+F groups than db/m and db/m+F groups. Db/m and db/m+F groups were similar in BW, KW, food intake, GLU, INS, and TG, and there was no difference in BW, KW, food intake, GLU, INS, and TG between db/db and db/db+F groups (**Figures 1A–F**). Moreover, the mice in four groups were similar in total cholesterol (TC) (**Figure 1G**).

Renal Phenotype

As shown in **Figure 2**, no significant difference was observed in serum creatinine (SCr) and blood urea nitrogen (BUN) of all mice (**Figures 2A,B**). Db/db group presented higher UACR level than db/m and db/m+F groups. However, db/db+F group had the significantly decreased level of UACR compared with db/db group (**Figure 2C**). H&E staining and PAS staining indicated that mice in db/db group presented glomerular mesangial expansion and glomerulosclerosis in kidneys, but fenofibrate improved these changes in kidneys of db/db+F group (**Figures 2D,E**). In addition, western blot indicated that renal podocyte marker-podocin was decreased in db/db group compared with db/m and db/m+F groups, and was increased in db/db+F group compared with db/db group (**Figure 2F**). These findings indicated that PPAR-α agonist fenofibrate prevented DN in type 2 diabetic mice.

Renal Fibrosis

Next, we examined whether fenofibrate alleviated renal fibrosis in diabetic mice. Sirius red staining and Masson's staining exhibited that renal fibrosis was promoted in db/db group compared to db/m and db/m+F groups, but was alleviated in db/db+F group compared with db/db group (**Figures 3A–C**). Western blot further indicated that collagen I was enhanced in the kidneys of db/db group compared to db/m and db/m+F groups, but was inhibited in the kidneys of db/db+F group compared with db/db group (**Figure 3D**). These findings suggested that there was more significant renal fibrosis in type 2 diabetic mice than non-diabetic mice, and fenofibrate would prevent renal fibrosis in type 2 diabetic mice.

Renal Apoptosis

Then, we detected mouse renal apoptosis. As shown in **Figure 4**, TUNEL assay showed that renal apoptosis was increased in db/db group compared to db/m and db/m+F groups, whereas renal apoptosis was decreased in db/db+F group compared with db/db group (**Figures 4A,B**). Moreover, western blot indicated that cleaved caspase-3 was promoted in kidneys of db/db group compared with db/m and db/m+F groups, and was inhibited in kidneys of db/db+F group compared with db/db group (**Figure 4C**). These findings indicated that type 2 diabetes enhanced apoptosis in mouse kidneys, but PPAR-α agonist fenofibrate treatment prevented apoptosis in mouse kidneys of type 2 diabetes.

Endothelial Function in Mouse Kidneys

Accumulating evidence suggests an involvement of endothelial dysfunction in the diabetic renal injury (6–8), and our previous study demonstrated that fenofibrate improved endothelial function (34). In current study, western blot showed that a significant suppression of p-eNOS/t-eNOS and a significant increase of Ang-2 in kidneys of db/db group compared to db/m and db/m+F groups, but fenofibrate treatment improved p-eNOS/t-eNOS and Ang-2 in kidneys of db/db+F group (**Figures 5A,B**). This was accompanied by a significant reduction of NO in renal tissues of db/db group compared to db/m and db/m+F groups, which was also improved by fenofibrate treatment in renal tissues of db/db+F group (**Figure 5C**). In addition, DHE staining showed that ROS formation was increased in renal tissues of db/db group compared to db/m and db/m+F groups, while ROS formation was decreased in db/db+F group compared to db/db group (**Figures 5D,E**). These results indicated that there was more significant endothelial dysfunction

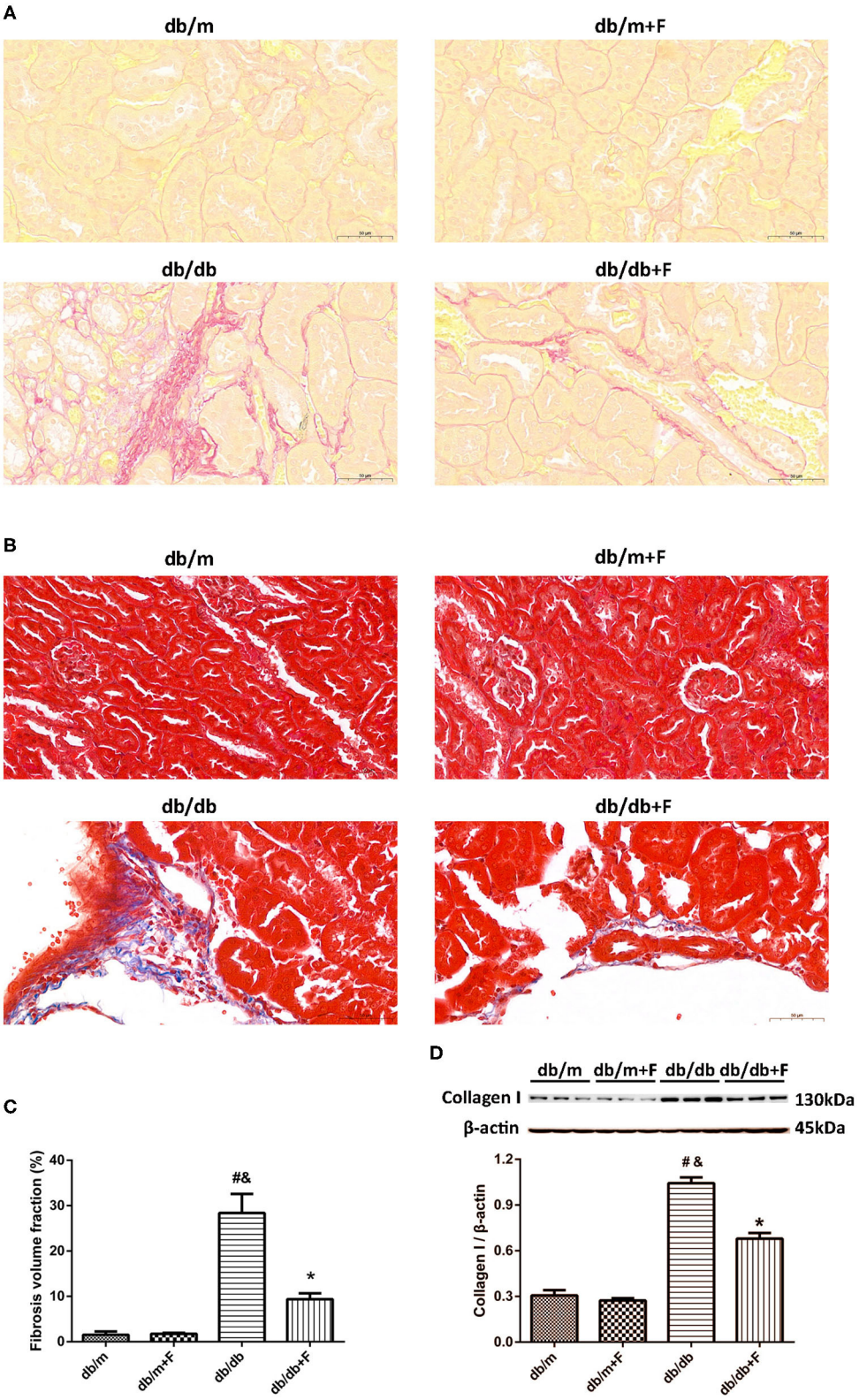

FIGURE 3 | Renal fibrosis in db/m, db/m+F, db/db, and db/db+F groups. **(A–C)** Representative photographs and quantification of renal fibrosis measured by Sirius red staining **(A)** and Masson's staining **(B)**. **(D)** Representative photographs and quantification of collagen I in mouse kidneys detected by western blot. $n = 6$ mice/group. $^{\#}P < 0.05$ vs. db/m group; $^{\&}P < 0.05$ vs. db/m+F group; $^{*}P < 0.05$ vs. db/db group. Db/m, db/m mice without fenofibrate treatment; db/m+F, db/m mice with fenofibrate treatment; db/db, db/db mice without fenofibrate treatment; db/db+F, db/db mice with fenofibrate treatment. Data are means ± S.E.M.

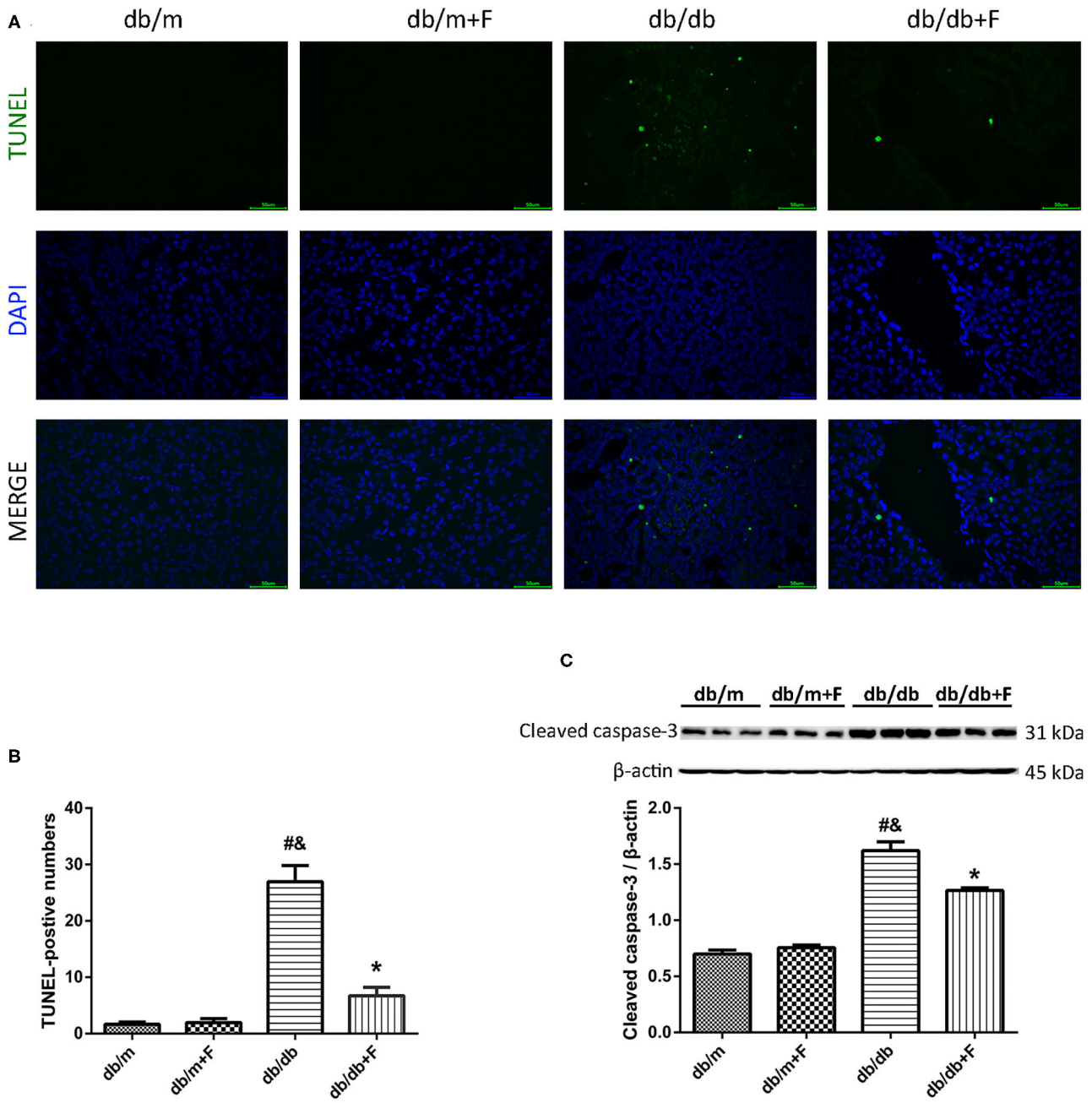

FIGURE 4 | Renal apoptosis in db/m, db/m+F, db/db, and db/db+F groups. **(A,B)** Representative photographs and quantification of renal apoptosis measured by TUNEL assay. **(C)** Representative photographs and quantification of cleaved caspase-3 in mouse kidneys detected by western blot. $n = 6$ mice/group. $^{\#}P < 0.05$ vs. db/m group; $^{\&}P < 0.05$ vs. db/m+F group; $^{*}P < 0.05$ vs. db/db group. Db/m, db/m mice without fenofibrate treatment; db/m+F, db/m mice with fenofibrate treatment; db/db, db/db mice without fenofibrate treatment; db/db+F, db/db mice with fenofibrate treatment. Data are means ± S.E.M.

in type 2 diabetic mouse kidneys compared to non-diabetic mouse kidneys, and fenofibrate improved endothelial function in type 2 diabetic mouse kidneys.

HIF-1α and Notch1 in Mouse Kidneys

Endothelial dysfunction might cause tissue hypoxia and the upregulation of HIF-1α expression (9, 11). In our previous study, increased HIF-α in endothelial cells was documented to exacerbate renal fibrosis by upregulating Notch (10). In present research, HIF-1α and Notch1 were elevated in kidneys of db/db group compared with db/m and db/m+F groups, and were reduced in kidneys of db/db+F group compared with db/db group after fenofibrate treatment (**Figures 6A,B**).

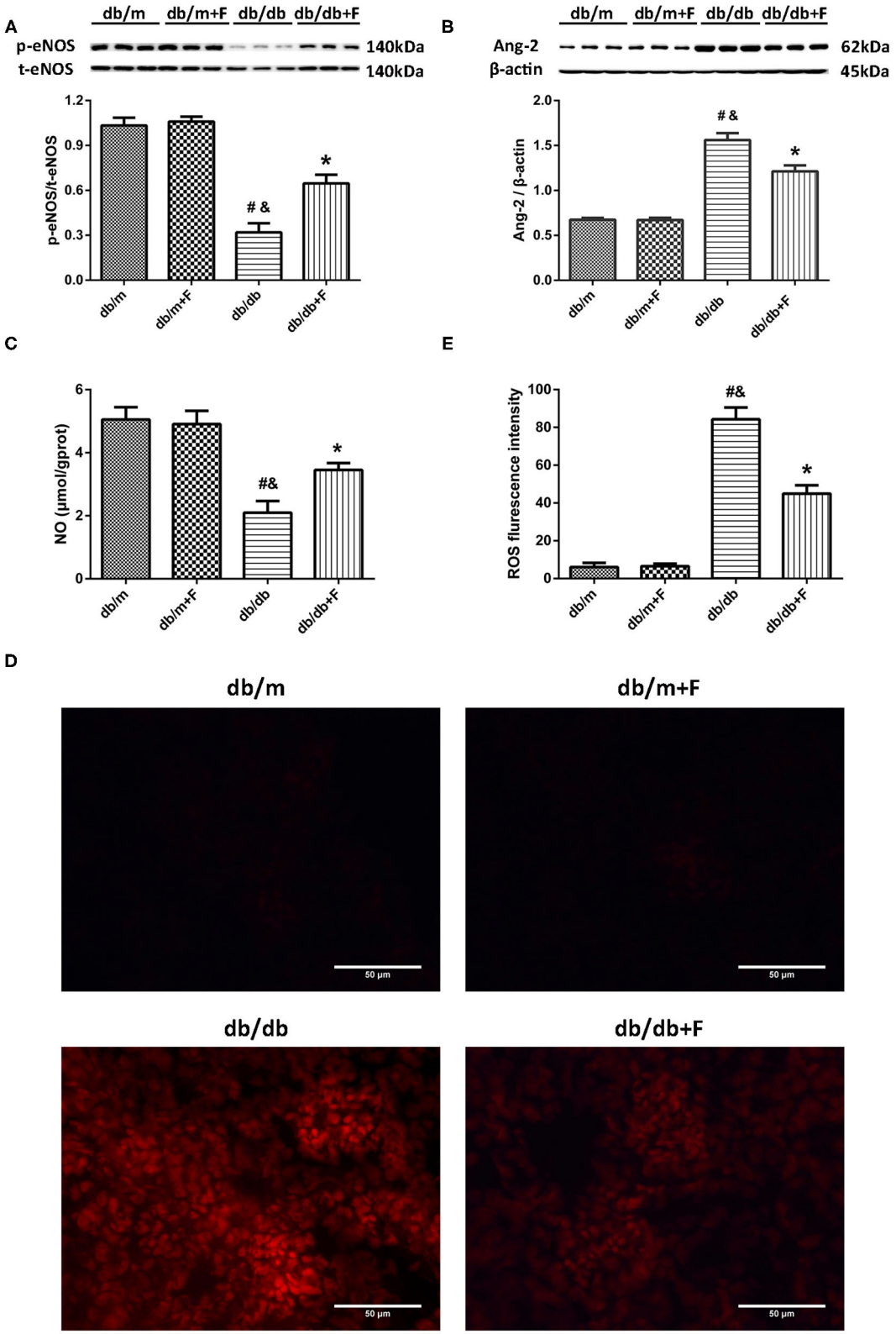

FIGURE 5 | Endothelial function in mouse kidneys. **(A)** Representative photographs and quantification of phospho-endothelial nitric-oxide synthase (p-eNOS)/total-endothelial nitric-oxide synthase (t-eNOS) in mouse kidneys measured by western blot. **(B)** Representative photographs and quantification of angiopoietin-2 (Ang-2) in mouse kidneys measured by western blot. **(C)** Quantification of nitric oxide (NO) level in mouse kidneys measured by Griess. **(D,E)**

(Continued)

FIGURE 5 | Representative photographs and quantification of reactive oxygen species (ROS) formation in mouse kidneys by dihydroethidium staining. $n = 6$ mice/group. $^{\#}P < 0.05$ vs. db/m group; $^{\&}P < 0.05$ vs. db/m+F group; $^{*}P < 0.05$ vs. db/db group. Db/m, db/m mice without fenofibrate treatment; db/m+F, db/m mice with fenofibrate treatment; db/db, db/db mice without fenofibrate treatment; db/db+F, db/db mice with fenofibrate treatment. Data are means ± S.E.M.

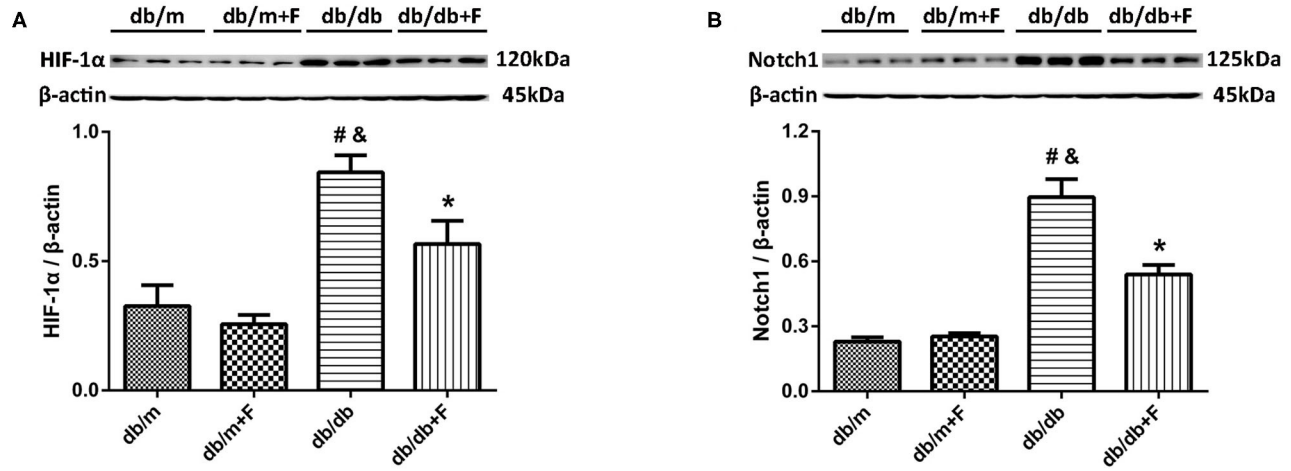

FIGURE 6 | Hypoxia-inducible factor (HIF)-1α and Notch1 in mouse kidneys. **(A,B)** Representative photographs and quantification of HIF-1α **(A)** and Notch1 **(B)** in mouse kidneys measured by western blot. $n = 6$ mice/group. $^{\#}P < 0.05$ vs. db/m group; $^{\&}P < 0.05$ vs. db/m+F group; $^{*}P < 0.05$ vs. db/db group. Db/m, db/m mice without fenofibrate treatment; db/m+F, db/m mice with fenofibrate treatment; db/db, db/db mice without fenofibrate treatment; db/db+F, db/db mice with fenofibrate treatment. Data are means ± S.E.M.

M1 Macrophage Phenotype

M1 macrophage polarization plays a critical role in fibrosis, which is regulated by endothelial cell function (18–20), associated with HIF-1α (22, 23) and controlled by Notch signal pathway (1, 15–17). Thus, we explored the M1 macrophage phenotype in mouse blood and kidneys. As shown in **Figure 7**, the immunostaining fraction of F4/80 was 5.55, 5.87, 36.38, and 18.35% in db/m group, db/m+F group, db/db group, and db/db+F group, respectively. The immunostaining fraction of CD86 was 1.88, 2.37, 26.09, and 11.63% in db/m group, db/m+F group, db/db group, and db/db+F group, respectively. Moreover, the immunostaining fraction of CD32/16 was 1.09, 1.05, 29.67, and 13.29% in db/m group, db/m+F group, db/db group, and db/db+F group, respectively. The co-staining of F4/80 and CD86 and the co-staining of F4/80 and CD32/16 showed that M1 macrophages were increased in kidneys of db/db group in comparison to db/m and db/m+F groups, whereas M1 macrophages was reduced in kidneys of db/db+F group compared with db/db group (**Figures 7A–E**). Similarly, mice in db/db group had higher cytokines associated with M1 macrophages, including TNF-α and IL-1β both in kidney measured by RT-PCR and in serum measured by ELISA, than mice in db/m and db/m+F groups, but these cytokines in kidney and serum were diminished in db/db+F group compared to db/db group (**Figures 7F–I**). These results indicated that M1 macrophage recruitment involved in the renal injury of type 2 diabetic mice, and fenofibrate suppressed M1 macrophage phenotype in type 2 diabetic mice.

DISCUSSION

In present study, we found that db/db mice presented higher UACR, more significant renal histological damage, and more significant podocyte injury than db/m mice, renal fibrosis and apoptosis were elevated in db/db mice compared with db/m mice, and the more significant endothelial dysfunction and the increased levels of HIF-1α and Notch1 in db/db mouse kidneys compared with db/m mouse kidneys. On the contrary, PPAR-α agonist fenofibrate decreased UACR, renal histological damage, and podocyte injury in db/db mice, and reduced fibrosis and apoptosis, ameliorated endothelial cell function, and depressed the expression of HIF-1α and Notch1 in db/db mouse kidneys. Importantly, we further demonstrated that type 2 diabetes led to promoted M1 macrophage recruitment in mouse kidneys, while fenofibrate treatment downregulated M1 macrophage recruitment in mouse kidneys of type 2 diabetes.

PPAR-α agonist fenofibrate is known as an important lipid-lowering drugs in clinical. Moreover, PPAR-α agonist fenofibrate has been proven to prevent DN and reduce urinary albumin in diabetic patients (26, 27) and in diabetic mice (29–32), which is independent of the effect on lipid-lowering. In current research, mice in different groups were similar in total cholesterol (TC). Body weight (BW), kidney weight (KW), food intake, blood glucose (GLU), insulin (INS), and triglycerides (TG) were increased in db/db and db/db+F groups compared with db/m and db/m+F groups. There were no differences in BW, KW, food intake, GLU, INS, and TG between db/m and db/m+F groups, and between db/db and db/db+F groups. Db/db group

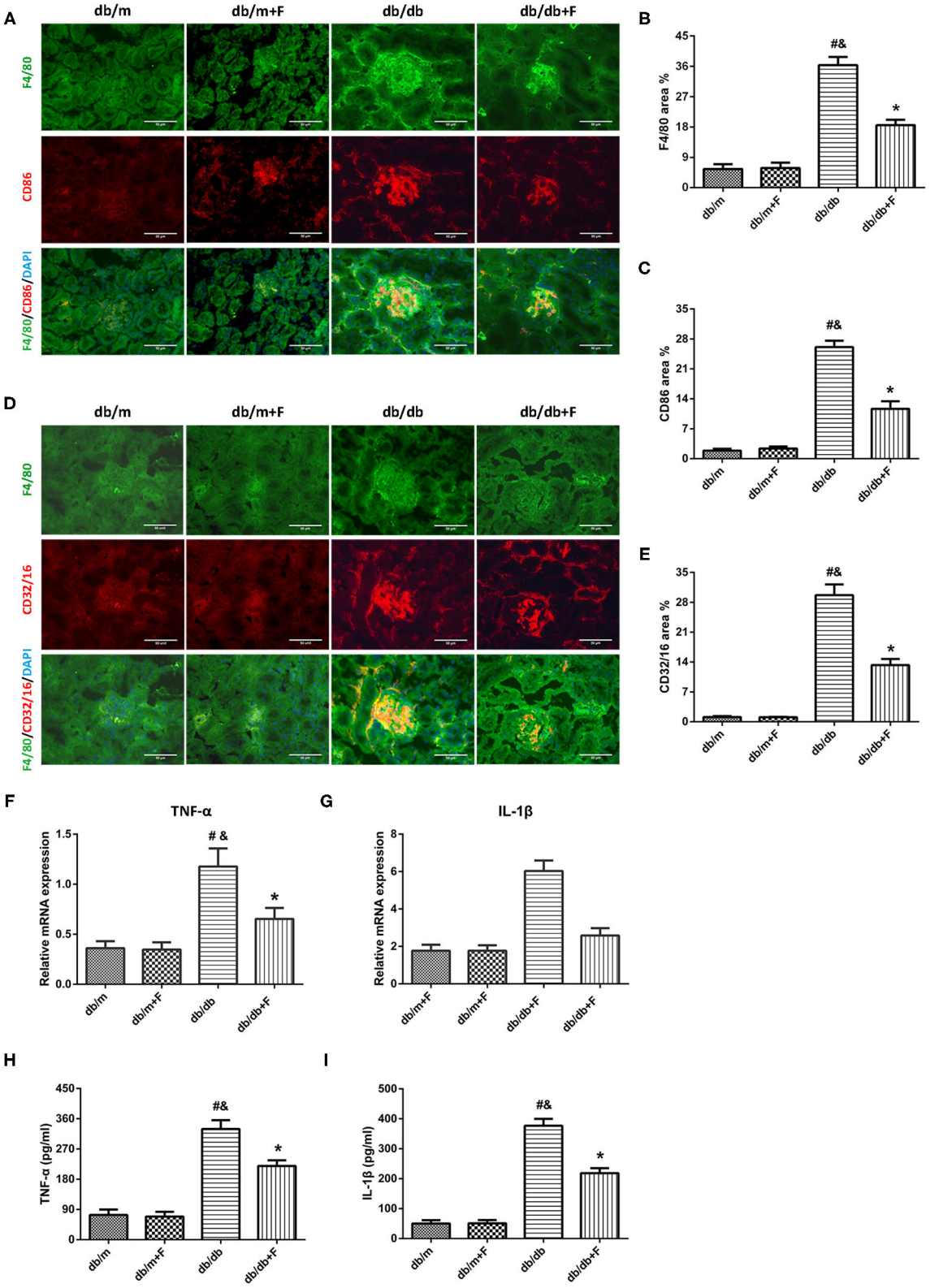

FIGURE 7 | M1 macrophages in mouse kidneys and serum. **(A–C)** Representative photographs and quantification of co-staining of F4/80 and CD86 in mouse kidneys. **(D,E)** Representative photographs and quantification of co-staining of F4/80 and CD32/16 in mouse kidneys. **(F,G)** MRNA expression analyses of tumor necrosis factor (TNF)-α **(F)** and interleukin (IL)-1β **(G)** by quantitative reverse transcriptase PCR (RT-PCR). **(H,I)** Serum TNF-α **(H)** and interleukin (IL)-1β **(I)**. $n = 6$

(Continued)

FIGURE 7 | mice/group. $^{\#}P < 0.05$ vs. db/m group; $^{\&}P < 0.05$ vs. db/m+F group; $^{*}P < 0.05$ vs. db/db group. Db/m, db/m mice without fenofibrate treatment; db/m+F, db/m mice with fenofibrate treatment; db/db, db/db mice without fenofibrate treatment; db/db+F, db/db mice with fenofibrate treatment. Data are means ± S.E.M.

presented higher UACR than db/m and db/m+F groups, and db/db+F group had the significantly lower UACR level than db/db group. Diabetes causes glomerular mesangial expansion and glomerulosclerosis in kidneys, and leads to glomerular podocyte injury (41). In current research, these pathological changes were found in mouse kidneys of db/db group, while fenofibrate improved these injuries in mouse kidneys of db/db+F group. These results provided a strong evidence of PPAR-α agonist fenofibrate to prevent DN.

Fibrosis is an important pathological manifestation of DN (42), and apoptosis plays a critical role in the pathogenesis of DN (43). In current study, no significant difference of renal fibrosis area was observed between db/m and db/m+F groups. In contrast, there was a significant increase in the renal fibrosis area in kidneys of db/db group as compared to db/m and db/m+F groups. Consistent with the changes of the fibrosis fractional area, the expression of collagen I was significantly increased in kidneys of db/db group as compared to db/m and db/m+F groups. Moreover, apoptosis detected by TUNEL and apoptosis associated protein-cleaved caspase-3 in kidneys of db/db group were exacerbated compared with db/m and db/m+F groups. However, these renal fibrosis and apoptosis changes were ameliorated by fenofibrate treatment in db/db+F group compared to db/db group. These findings suggested that PPAR-α agonist fenofibrate prevented type 2 diabetes-induced renal fibrosis and apoptosis.

DN is one of the diabetic microvascular complications. Vascular endothelial dysfunction plays a crucial role in diabetic renal injury. Ang-2, an indicator of endothelial injury, can be induced by hyperglycemia in endothelial cells (44), and can regulate macrophage polarization (18–20). Our previous study has found that Ang-2 was increased in PHD2 ECKO mice, which was associated with the elevated HIF-1α and fibrosis in mouse kidneys (10). ENOS knockout mice with both type 1 and type 2 diabetes are sensible to DN in comparison to wild type mice with diabetes (6–8). ENOS provides the principal means by which NO is generated in the kidneys (3). Decrease in NO is the major cause of diabetic vascular complications, including DN (2). In our previous study, high glucose caused endothelial dysfunction with reduced NO generation and elevated ROS production in HUVECs (33); however, fenofibrate recoupled eNOS and promoted NO in HUVECs (34). In addition, our previous study found that fenofibrate improved arterial stiffness and CFVR in patients with hypertriglyceridemia (35). Our previous findings suggested that PPAR-α agonist fenofibrate improved endothelial function and vascular tone. The current study showed a significant enhancement of Ang-2, and a significant inhibition of p-eNOS/t-eNOS and NO in mouse kidneys of db/db group compared with db/m and db/m+F groups, but fenofibrate treatment decreased Ang-2, and promoted p-eNOS/t-eNOS and NO in mouse kidneys of db/db+F group. This was accompanied by a significant elevation of ROS formation in mouse kidneys of db/db group compared with db/m and db/m+F groups, which was suppressed by fenofibrate treatment in mouse kidneys of db/db+F group compred to db/db group.

Interestingly, recent evidence has indicated that HIF-1α is associated with endothelial function. However, the data have been inconsistent. There have been some studies suggesting that low HIF-1α expression might be correlated with endothelial dysfunction (45, 46). Controversially, most studies have indicated that the upregulated expression of HIF-1α was induced by tissue hypoxia due to endothelial dysfunction and deficient NO production (47), and the inhibition of eNOS by N-nitro-L-arginine methyl ester (L-NAME) promoted the expression of HIF-1α (9). Recent studies have proven that the inhibition of HIF-1 protected against diabetic renal injury (11). In our previous research, elevated HIF-α in endothelial cells enhanced renal fibrosis (10). Moreover, PPARα agonists reduced hypoxia-induced HIF-1α expression and activity in cancer cells (38), and improved ischemic retina diseases through decreasing HIF-1α in endothelial cells (36, 37). In present study, the level of HIF-1α was increased in kidneys of db/db group compared with db/m and db/m+F groups, and was decreased after fenofibrate treatment in mouse kidneys of db/db+F group compared with db/db group. It might be hypothesized that the elevated HIF-1α expression could reflect the hypoxia on db/db mouse kidneys, which might be caused by endothelial dysfunction, decreased NO production, and endothelial-dependent vasodilation dysfunction induced by hyperglycemia. PPAR-α agonist fenofibrate could improve endothelial function and increase the production of NO, and could consequently improve hypoxia and suppress the HIF-1α level in kidneys of type 2 diabetic mice.

Notch accelerates fibrosis and apoptosis in diabetic renal injury (14, 47). Recent studies have proven that Notch could be activated by HIF-1α (12, 13), and our previous study demonstrated that increased HIF-α in endothelial cells accelerated renal fibrosis through Notch activating (10). Additionally, upregulating PPAR-α could suppress Notch-1 signaling (39). In present research, Notch1 was enhanced in kidneys of db/db group compared with db/m and db/m+F groups, and fenofibrate decreased the expression of Notch1 in mouse kidneys of db/db+F group.

Macrophage polarization plays an important role in the development of renal fibrosis (1). The effects of macrophage polarization in the progression of DN have not been adequately defined. However, some studies have supported that macrophage polarization was strongly correlated with the pathological mechanism of DN (48, 49). Furthermore, there have been studies which demonstrated that the upregulation of M1 macrophage polarization accelerated renal fibrosis (1). It has been proven that endothelial function regulates macrophage polarization (18–20). Recent researches have documented that hypoxia exerted

great effects on macrophage polarization, and there was a significant relationship between HIF-1α and M1 macrophage polarization (22, 23). Notch has also been proven to promote renal inflammation and regulate macrophage polarization (1, 15–17).

The co-staining of F4/80 and CD86 and the co-staining of F4/80 and CD32/16 showed increased M1 macrophage recruitment in kidneys of db/db group compared to db/m and db/m+F groups, and this recruitment was eliminated by fenofibrate in kidneys of db/db+F group compared with db/db group. Moreover, cytokines that were associated with M1 macrophages both in kidneys and in serum, such as TNF-α and IL-1β, were elevated in db/db group compared to db/m and db/m+F groups, and were repressed after fenofibrate treatment in db/db+F group compared with db/db group. These results indicated that M1 macrophage recruitment enhanced the development of DN in type 2 diabetic mice, while fenofibrate relieved M1 macrophage recruitment in type 2 diabetic mouse kidneys.

CONCLUSIONS

In summary, M1 macrophage recruitment due to the upregulated HIF-1α/Notch1 pathway induced by endothelial cell dysfunction involves in type 2 diabetic mouse renal injury, and PPAR-α agonist fenofibrate prevents DN by reducing M1 macrophage recruitment through inhibiting HIF-1α/Notch1 pathway caused by the improved endothelial cell function in type 2 diabetic mouse kidneys.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee of Beijing Chao-Yang Hospital, Capital Medical University.

AUTHOR CONTRIBUTIONS

XF and XG: design, experimentation, statistics, and article revision. SW: experimentation, statistics, and article revision. MH, ZS, HD, and HY: experimentation. GW: design, statistics, and article revision. All authors contributed to the article and approved the submitted version.

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S-Nitrosylation of RhoGAP Myosin9A Is Altered in Advanced Diabetic Kidney Disease

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The molecular pathogenesis of diabetic kidney disease progression is complex and remains unresolved. Rho-GAP *MYO9A* was recently identified as a novel podocyte protein and a candidate gene for monogenic FSGS. Myo9A involvement in diabetic kidney disease has been suggested. Here, we examined the effect of diabetic milieu on Myo9A expression *in vivo* and *in vitro*. We determined that Myo9A undergoes S-nitrosylation, a post-translational modification dependent on nitric oxide (NO) availability. Diabetic mice with nodular glomerulosclerosis and severe proteinuria associated with doxycycline-induced, podocyte-specific $VEGF_{164}$ gain-of-function showed markedly decreased glomerular Myo9A expression and S-nitrosylation, as compared to uninduced diabetic mice. Immortalized mouse podocytes exposed to high glucose revealed decreased *Myo9A* expression, assessed by qPCR, immunoblot and immunocytochemistry, and reduced Myo9A S-nitrosylation (SNO-Myo9A), assessed by proximity link assay and biotin switch test, functionally resulting in abnormal podocyte migration. These defects were abrogated by exposure to a NO donor and were not due to hyperosmolarity. Our data demonstrate that high-glucose induced decrease of both *Myo9A* expression and SNO-Myo9A is regulated by NO availability. We detected S-nitrosylation of Myo9A interacting proteins RhoA and actin, which was also altered by high glucose and NO dependent. RhoA activity inversely related to SNO-RhoA. Collectively, data suggest that dysregulation of SNO-Myo9A, SNO-RhoA and SNO-actin may contribute to the pathogenesis of advanced diabetic kidney disease and may be amenable to therapeutic targeting.

Keywords: diabetic kidney disease, cell cross-talk, RhoA, S-nitrosylation, *MYO9A*, actin

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INTRODUCTION

Diabetic kidney disease (DKD) is a major complication of both type 1 and type 2 diabetes that leads to renal failure, and the single most frequent cause of end-stage renal disease (ESRD) worldwide (1). In the last few years novel therapies led to remarkable improvement of metabolic control in diabetic patients (2, 3). However, the prevalence and progression of DKD have not decreased as yet (2, 3). Incomplete understanding of the molecular mechanisms involved in DKD progression has precluded the development of effective treatments to prevent, halt or reverse progression to ESRD (4–6). In an effort to address this, we investigated the role of a novel podocyte protein, Myosin 9A, in progression to advanced DKD.

MYO9A was recently identified as a novel candidate gene for monogenic FSGS (7). This study also suggested dysregulation of *Myo9A* expression in other experimental proteinuric diseases, such as nephrotic syndrome and diabetic nephropathy (7). The relevance of *Myo9A* involvement in diabetic kidney disease is presently unknown. Myosins are a super family of actin binding molecular motors that regulate cell shape and motility, organelle trafficking and signaling (8–10). Several non-muscle myosins regulate foot process actin dynamics in podocytes (11). The 40 gene members of the myosin family share a common structure consisting of head, neck and tail domains and have been grouped in 18 classes based on their distinctive features. Class 2 non-muscle myosin *MYH9* has been implicated in the pathogenesis of DKD (12) and MYO1E mutations cause monogenic FSGS (13). Class 9 myosins' unique features are their RhoGTPase-activating protein (Rho-GAP) tail domain and a loop insert in their head domain (14). Myosin 9A (Myo9A) crosslinks and bundles actin, inactivates RhoA and controls epithelial cell junction assembly (9, 10, 14). *Myo9A* is expressed by epithelial cells in brain, kidney, testis and lung (15). In the kidney Myo9A localizes to podocytes and proximal tubular cells (7). *Myo9A* loss-of-function increases kidney RhoA activity and alters podocyte function (7).

In diabetes, hyperglycemia induces uncoupling of nitric oxide synthase homodimers and overproduction of reactive oxygen species (ROS) relative to antioxidant molecules, resulting in low nitric oxide (NO) availability (16–18). NO signals through two distinct pathways: activation of guanylyl cyclase to produce cyclic GMP (cGMP) and protein S-nitrosylation. S-nitrosylation is the reversible, oxidative addition of NO to Cys residues to form S-nitrosothiols (SNOs) that modifies myriad proteins, providing a redox-based cellular signaling mechanism that conveys the ubiquitous influence of NO on cellular function (19). S-nitrosylation regulates protein activity of multiple proteins that play important roles in DKD, including all nitric oxide synthase (NOS) isoforms, guanylyl cyclase (GC), hypoxia-inducible factor1α (HiF1α), thioredoxin (20–23), as well as in cytoskeletal dynamics, such as actin and RhoA (24–26).

Since Myo9A directly interacts with both actin and RhoA (27, 28), we hypothesized that Myo9A might undergo S-nitrosylation and thereby participate in a transnitrosylation cascade to serve NO signaling. The goals of this study were to determine whether Myo9A dysregulation is involved in the severity of DKD and to assess whether the molecular mechanism involves Myo9A S-nitrosylation. We documented that Myo9A is S-nitrosylated *in vivo* and in cultured podocytes in control conditions. Diabetic mice with advanced DKD revealed downregulation of Myo9A expression and S-nitrosylation. Cultured podocytes showed that high glucose-induced Myo9A dysregulation is NO dependent and involves actin and RhoA S-nitrosylation. These findings uncover Myo9A relevance in advanced DKD and identify a targetable pathway that might influence DKD progression involving cross-talk among multiple nephron cell types.

MATERIALS AND METHODS

Animal Model

Experiments were performed using kidney tissue from *podocin*-rtTA:tet-O-$VEGF_{164}$ (i*VEGF*) diabetic mice, herein called DM-i*VEGF* mice, previously reported (29). *Podocin-rtTA:tet-O-*$VEGF_{164}$ are podocyte-specific inducible transgenic mice that overexpress $VEGF_{164}$ in podocytes upon induction with doxycycline, as described (30). Mice were crossbred on FVB/N background. Diabetes was induced using streptozotocin (50 mg/kg body weight i.p. for 5 consecutive days) following the Animal Models of Diabetic Complications Consortium (www.AMDCC.org) short protocol in 5.0 ± 0.6 week old i*VEGF* mice ($n = 15$). Diabetic i*VEGF* mice (DM-i*VEGF*) were fed doxycycline containing chow (0.625 mg/g chow; Harlan-Teklad) (DM-i*VEGF* +dox, $n = 8$), or fed standard chow (DM-i*VEGF* -dox, $n = 7$) for 12 weeks to induce $VEGF_{164}$ expression or serve as diabetic controls, respectively (29). At the end of 12 weeks, mice were anesthetized and kidneys were perfused with sterile PBS and excised prior to euthanasia. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Yale University School of Medicine.

Cell Culture

Immortalized mouse podocytes were cultured in RPMI-1640 medium (11875-093, Life Technologies), 1% Insulin-Transferrin-Selenium (41400-045, Life Technologies), 10% heat inactivated FBS (10438-026, Life Technologies), 1% Pen/Strep at 33°C with 5% CO_2. Podocyte differentiation was induced by incubation at 37°C for 7 days. Podocytes incubated in control medium (11 mM D-glucose), medium + 25 mM glucose, medium + 25 mM mannitol, or medium + 25 mM glucose + 10μM DETA NONOate (#82120,Cayman Chemical) for 24 h. For immunocytochemistry and proximity link assays, podocytes were cultured in 4-chamber slides; for cell migration assays, podocytes (1×10^5cell/ml) were cultured in 35 mm dishes.

Immunoblot/Immunoprecipitation

Kidneys were snap frozen in liquid nitrogen at the time of euthanasia, and podocytes were pelleted by centrifugation at the end of culture experiments. Both tissues and cells were lyzed in lysis buffer (1% NP-40, 1% Triton X, 50 mM Hepes, 150 mM NaCl, 0.1 mM EDTA, 0.1 mM Neocuproine, complete protease inhibitor, Roche) for immunoblot and co-immunoprecipitation analysis, as previously described (7, 31). Proteins were resolved by SDS-PAGE in 10% or 4–20% SDS–polyacrylamide gels (BioRad), transferred to polyvinylidene difluoride membranes, blocked with 5% dry-milk or 5% BSA in TBST and incubated with primary antibodies: actin (A2066, Sigma), Myo9A (Abnova, clone 4C11) and RhoA (67B9,Cell Signaling), followed by appropriate species specific HRP-conjugated secondary antibodies (Jackson Immuno Research Laboratories Inc.). Immunoblotted proteins were visualized with ECL. Co-immunoprecipitation was performed using podocyte lysates, as previously described (7). Briefly, following pre-clearing with prewashed protein A agarose beads, lysate supernatants were incubated anti-MYO9A rabbit polyclonal antibody (A305-702A-M, Bethyl) at 4°C, pre-washed agarose beads were added and incubated overnight. Agarose beads were washed with PBS+protease inhibitors (Roche), spun and resuspended in Laemmli sample buffer for western blot analysis as described above.

Histology/Immunohistochemistry/ Immunocytochemistry

Kidneys were perfused with sterile PBS for morphologic studies prior to euthanasia, incubated in 18% sucrose, embedded in optimal cutting temperature medium (OCT, Sakura Finetek USA), frozen in isopentane/dry ice and kept at −80°C for immunohistochemistry (IHC), as described (29) or processed for light microscopy. Histology was evaluated by periodic acid–Schiff's reagent (PAS) stain. Kidney frozen sections and podocytes were fixed in 4% PFA, permeabilized with 0.3% triton-X, blocked with 10% donkey serum, 5% BSA in PBST at room temperature, and incubated overnight at 4°C in primary antibodies: S-nitrosocysteine mouse monoclonal antibody (AG Scientific,1:100) and rabbit anti Myo9A (NBP1-92160, Novus, 1:50). Sections were washed, incubated with fluorescent-tagged secondary antibodies: goat anti-mouse Alexa Fluor 594 or goat anti-rabbit Alexa Fluor 488 (Life Technologies, 1:150) at room temperature. Coverslips were mounted with Vectashield + DAPI (Vector Labs). Stained sections and cells were examined using an Olympus IX 71 inverted fluorescence/phase and bright field microscope (Olympus, Tokyo, Japan) equipped with an Optronics (Goleta, CA) Microfire camera and Pictureframe version 3.00.30 software. Images were processed with Adobe Photoshop CC 2018 (Adobe Systems).

In situ Proximity Ligation Assay (PLA)

Myo9A S-nitrosylation was detected and localized using an *in situ* proximity link assay, as previously described (32, 33). Here, we used Myo9A rabbit polyclonal antibody (Novus) and S-nitrosocysteine mouse monoclonal antibody (AG Scientific), Duolink PLA probes and fluorescent labeled oligonucleotides to visualize the amplified reaction product attached to the antibody protein complex, following the Duolink® PLA fluorescence protocol (Sigma). Briefly, kidney frozen sections or podocytes were fixed, permeabilized and blocked as described above + 0.3% hydrogen peroxide in PBS and incubated overnight with primary antibodies Myo9A and S-nitrosocysteine at 4°C. Secondary antibodies (PLA probes) donkey anti-rabbit and anti-mouse conjugated with oligonucleotides were added and incubated at 37°C for 60 min. Sections were washed with PBS, incubated with ligation solution containing oligonucleotides for 30 min at 37°C. Ligation of oligonucleotides generates a circular DNA strand that serves as a template only if the probes are in close proximity. Then, sections were incubated at 37°C with DNA polymerase and fluorescently labeled oligonucleotides for 100 min. The amplification reaction product attaches to the antibody protein complex and is visualized as a fluorescent signal resulting from the hybridization of fluorescently labeled oligonucleotides. Kidney sections and podocytes were washed, and coverslips placed using mounting medium with DAPI (Vector). Cy3 and DAPI fluorescence signals were detected by inverted fluorescence microscopy at × 400 magnification and processed as described above.

Biotin Switch Assay (BST)

S-nitrosylation of Myo9A, RhoA and actin was measured using a biotin switch assay (34) (S-nitrosylated protein detection kit, Cayman Chemical Co.), following the manufacturer's instructions. Briefly, podocyte lysates (1,000 μg) were re-suspended in blocking buffer to block free thiols, acetone precipitated, S-NO bonds were reduced, and the resulting free thiols were labeled with maleimide-biotin. Proteins were acetone-precipitated, the pellets were re-suspended in equal volumes of HENS/10 + 1% SDS buffer. To pull-down the biotinylated proteins we added streptavidin-agarose beads (Fluka). Beads were washed 5 times and bound proteins were eluted in 2X sample buffer. Myo9A, RhoA and actin presence in the eluates was detected by immunoblotting.

RhoA Activity

Active RhoA was measured with Rho-activation pulldown assay (Millipore) following manufacturer's instructions. Active RhoA was detected by immunoblotting using RhoA antibody (67B9, Cell Signaling), as described (7).

Statistical Analysis

Data are analyzed with GraphPad-Prism-8 software (San Diego, CA) using unpaired Student's-*t*-test with Welch's correction, Welch's or Brown-Forsythe ANOVA, as appropriate. Non-parametric Kriskall-Wallis and Mann-Whitney test were used to analyze RhoA activity data. $P < 0.05$ was deemed statistically significant. Data are expressed as mean ± SD, unless otherwise indicated.

RESULTS

Kidney Myo9A Expression and S-Nitrosylation in Diabetic Kidney Disease

To begin to understand the role of *Myo9A* in DKD progression we compared Myo9A expression in diabetic mice with mild vs. advanced diabetic kidney disease (DKD). We examined Myo9A expression and distribution of S-nitrosylated proteins in kidneys from mice with streptozotocin-mediated diabetes and doxycycline-inducible, podocyte $VEGF_{164}$ overexpression (DM-i$VEGF_{164}$) (29, 30). As previously reported (29), uninduced diabetic mice (− dox) show discrete glomerular changes (**Figure 1A**) and mild albuminuria (ACR: 212 ± 18 μg/mg creatinine, **Figure 1C**), whereas doxycycline-induced diabetic mice overexpressing $VEGF_{164}$ (DM- i$VEGF_{164}$+dox) develop severe diabetic nodular glomerulosclerosis (**Figure 1B**) and nephrotic range proteinuria (ACR:1947 ± 708 μg/mg creatinine, **Figure 1C**), herein referred to as advanced DKD. Induced and uninduced diabetic mice developed similar hyperglycemia (29). Using immunoblotting we determined that kidney Myo9A expression is significantly decreased in mice with advanced DKD (+ dox) as compared to mice with mild DKD (- dox) (**Figure 1D**) and non-diabetic mice (7).

Dual immunofluorescence labeling (IF) revealed that Myo9A and S-nitrosylated proteins localize to glomeruli from all diabetic mice (**Figure 1E**). S-nitrosylated proteins partially co-localize with Myo9A. We observed a significant decrease of glomerular Myo9A and S-nitrosylated proteins in induced diabetic mice (+ dox) as compared to non-induced diabetic mice (- dox). Quantitation of Myo9A and nitroso-Cys IF signals

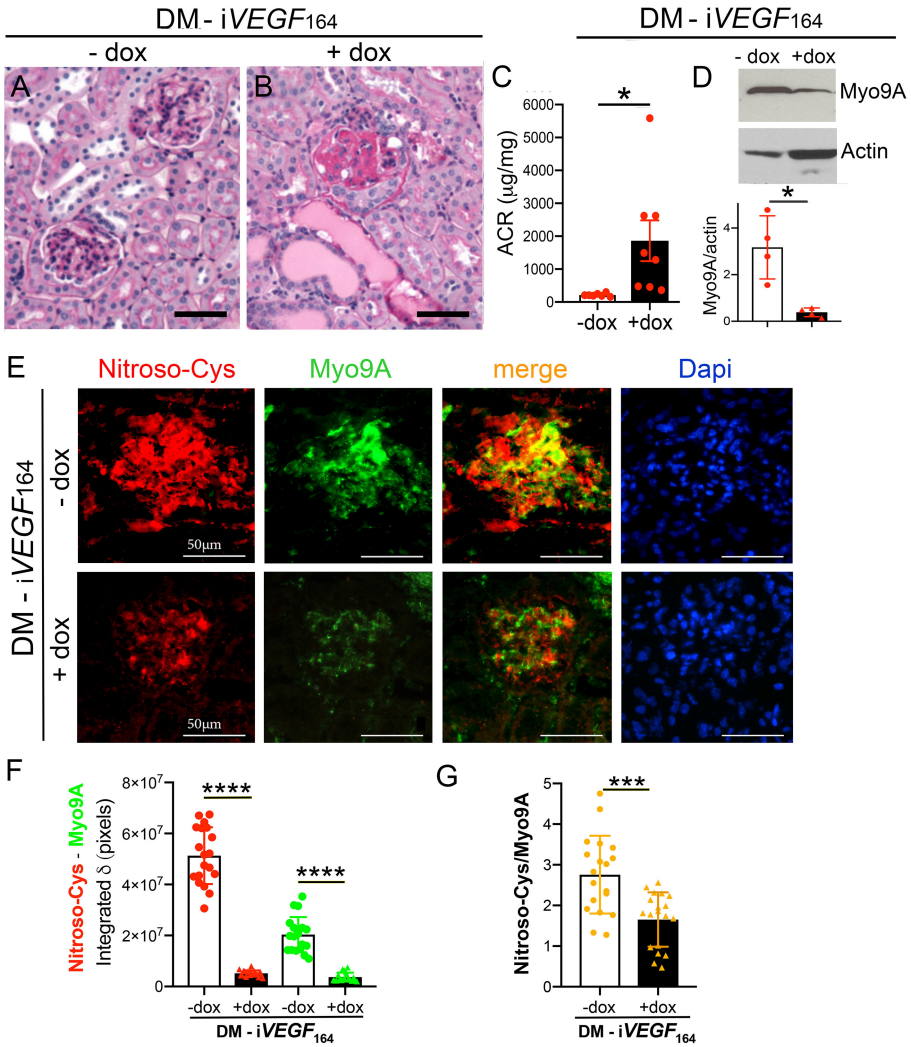

FIGURE 1 | Myo9A is downregulated in advanced diabetic kidney disease. **(A)** Kidney PAS stain from uninduced diabetic mouse (DM-i$VEGF_{164}$, - dox) showing mild mesangial proliferation; **(B)** kidney PAS stain from induced diabetic mouse (DM-i$VEGF_{164}$, + dox) showing nodular glomerulosclerosis and large protein casts; **(C)** Urine ACR (albumin:creatinine ratio, mg/mg) shows mild albuminuria in uninduced diabetic mice (DM-i$VEGF_{164}$, - dox, $n = 7$) and nephrotic range proteinuria in induced diabetic mice (DM-i$VEGF_{164}$, + dox, $n = 8$), unpaired t-test with Welch's correction, $P = 0.033$; **(D)** representative immunoblot shows decreased kidney Myo9A expression in mice with advanced DKD (DM-i$VEGF_{164}$, + dox); quantitation of Myo9A expression normalized to actin confirms significant Myo9A downregulation in $n = 4$ immunoblots (kidney lysates pooled from 4 to 6 mice/experimental group), mean ± SD, $P < 0.05$; **(E)** Fluorescence IHC shows S-nitrosylated proteins (red) and Myo9A (green) partially co-localized (merge) in glomeruli from uninduced diabetic kidneys (DM-i$VEGF_{164}$, - dox), both Myo9A and nitroso-Cys IF signals are reduced in glomeruli from kidneys with advanced DKD (DM-i$VEGF_{164}$, + dox); **(F)** quantitation of Myo9A and nitroso-Cys IF signals confirm a dramatic decrease in glomerular Myo9A expression and S-nitrosylated proteins in kidneys with advanced DKD (DM-i$VEGF_{164}$, + dox), mean ± SD, $n = 19$ glomeruli/experimental group (each from 3 to 5 mice), unpaired t-test with Welch's correction, $p < 0.0001$; **(G)** quantitation of the IF signals' ratio Nitroso-Cys/Myo9A shows significant decrease in kidneys with advanced DKD, mean ± SD, $n = 19$ glomeruli/experimental group, unpaired t-test with Welch's correction, $p = 0.0002$. Scale bars = 50µm. *$p < 0.05$, ***$p < 0.005$, ****$p < 0.0001$.

is shown in **Figure 1F** and the ratio of Nitroso-Cys/Myo9A IF signals is shown in **Figure 1G**. Together, IF data indicate that glomerular Myo9A and S-nitrosylated proteins partially co-localize and are decreased in the setting of advanced diabetic glomerulosclerosis, raising the possibility that Myo9A could be a S-nitrosylated protein.

To determine *in situ* whether glomerular Myo9A is S-nitrosylated we utilized a proximity link assay (PLA) (32, 33). Immunofluorescent PLA signals shown in **Figure 2A** demonstrate the presence of nitroso-Cys residues linked to Myo9A in uninduced glomeruli (- dox) and dramatically reduced SNO-Myo9A signal in glomeruli from induced (+ dox) diabetic mice. Quantitation of PLA signals is shown in **Figure 2B**. PLA data indicate that glomerular Myo9A is S-nitrosylated and that this post-translational modification is significantly downregulated in mouse kidneys with advanced DKD.

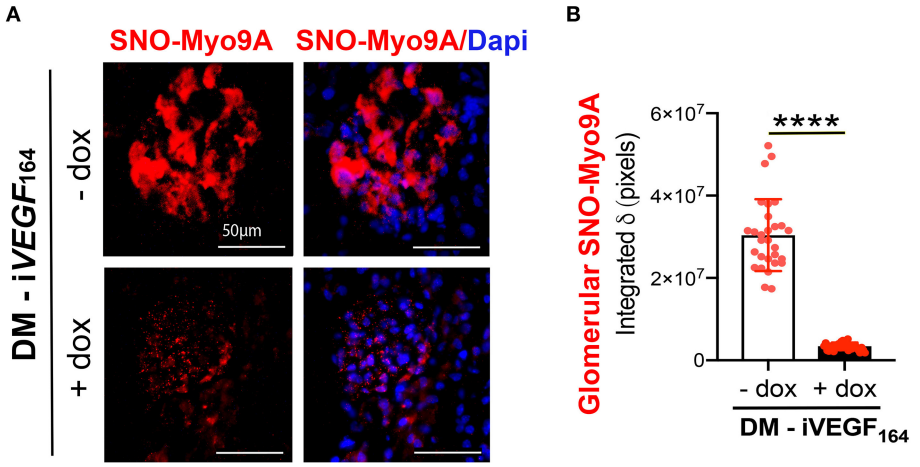

FIGURE 2 | Glomerular Myo9A is S-nitrosylated in diabetic mice. **(A)** Proximity link assay IF signal (red) identifies abundant S-nitrosylated Myo9A (SNO-Myo9a) in glomeruli from uninduced diabetic mice with mild DKD (DM-i*VEGF*$_{164}$ - dox), whereas SNO-Myo9A is clearly reduced in glomeruli from induced diabetic mice with advanced DKD (DM-i*VEGF*$_{164}$, + dox). Dapi (blue) identifies cell nuclei. Scale bars = 50 µm. **(B)** Quantification of PLA IF signals, mean ± SD, n = 29–31/experimental group, unpaired t-test with Welch's correction, ****$p < 0.0001$.

Podocyte Myo9A S-Nitrosylation Regulation by Glucose

A previous study demonstrated Myo9A expression in glomerular podocytes *in vivo* and in immortalized mouse and human podocytes (7). Therefore, we examined the effect of hyperglycemia on podocyte Myo9A expression and on S-nitrosylation. Immortalized mouse podocytes were exposed to normal glucose, mannitol, or high glucose, as described in the methods section. Mannitol was used as a control for hyperosmolarity-induced changes. Using IF dual labeling, we determined that Myo9A co-localizes with nitroso-Cys (SNO-Cys) signals in undifferentiated podocytes on normal glucose medium (**Figure 3A**, control, *top panels*) and differentiated podocytes exposed to mannitol (**Figure 3A**, *middle panels*), while differentiated podocytes exposed to high glucose showed reduced Myo9A and nitroso-Cys (SNO-Cys) proteins (**Figure 3A**, *bottom panels*). Quantification of IF signals demonstrating these highly significant changes are shown in **Figure 3B**.

Proximity linked assay (PLA) revealed *in situ* S-nitrosylated Myo9A (SNO-Myo9A) fluorescent signals in podocytes grown in normal glucose and in podocytes exposed to mannitol, whereas SNO-MyoA signals were barely detected in podocytes exposed to high glucose (**Figure 3C**). Quantitation of PLA IF signals confirmed that exposure to high glucose significantly decreases SNO-Myo9A in podocytes (**Figure 3D**), demonstrating that SNO-Myo9A is regulated by glucose in podocytes.

Podocyte Myo9A S-Nitrosylation Regulation by Nitric Oxide Availability

Since IF revealed decreased podocyte Myo9A protein expression upon exposure to high glucose, we performed qPCR and immunoblotting to quantitate this effect at both mRNA and protein levels. Immortalized differentiated mouse podocytes were exposed to normal glucose, mannitol, high glucose or high glucose + nitric oxide donor (DETA). Podocyte *Myo9A* mRNA and protein decreased significantly upon exposure to high glucose as compared to normal glucose or mannitol (**Figures 4A,B**). The long acting NO donor DETA abrogated the high glucose-induced defect in *Myo9A* expression (**Figures 4A,B**).

To examine further the regulation of Myo9A S-nitrosylation we performed biotin-switch test (BST). Consistent with the PLA results (**Figures 3C,D**), biotin-switch tests (BST) demonstrated that Myo9A S-nitrosylation (SNO-Myo9A) decreases ∼50% upon podocyte exposure to high glucose (**Figure 4C**). Addition of NO donor DETA partially improves the Myo9A S-nitrosylation defect induced by podocyte exposure to high glucose (**Figure 4C**). We evaluated the effect of high glucose on podocyte function using a migration assay. Upon exposure to high glucose podocyte migration was significantly reduced, as compared to normal glucose or mannitol (**Figure 4D**). The migration defect was partially abrogated by addition of DETA (**Figure 4D**).

S-Nitrosylation of Myo9A Interacting Proteins RhoA and Actin

We assessed whether high glucose and NO regulate S-nitrosylation of Myo9A interacting proteins, RhoA and actin (7, 14). We determined that Myo9A interacts with RhoA in podocytes using immunoprecipitation (**Figure 5A**). Then, we performed BST to evaluate SNO-RhoA and SNO-actin under the conditions described above. These experiments revealed that both RhoA and actin are S-nitrosylated in control podocytes (**Figures 5B,C**). Remarkably, high glucose decreased SNO-RhoA, while addition of NO donor DETA abrogated podocyte RhoA de-nitrosylation (**Figure 5B**). High glucose also induced >50% SNO-actin decrease in podocytes and exposure to NO donor partially prevented this defect (**Figure 5C**). We measured RhoA

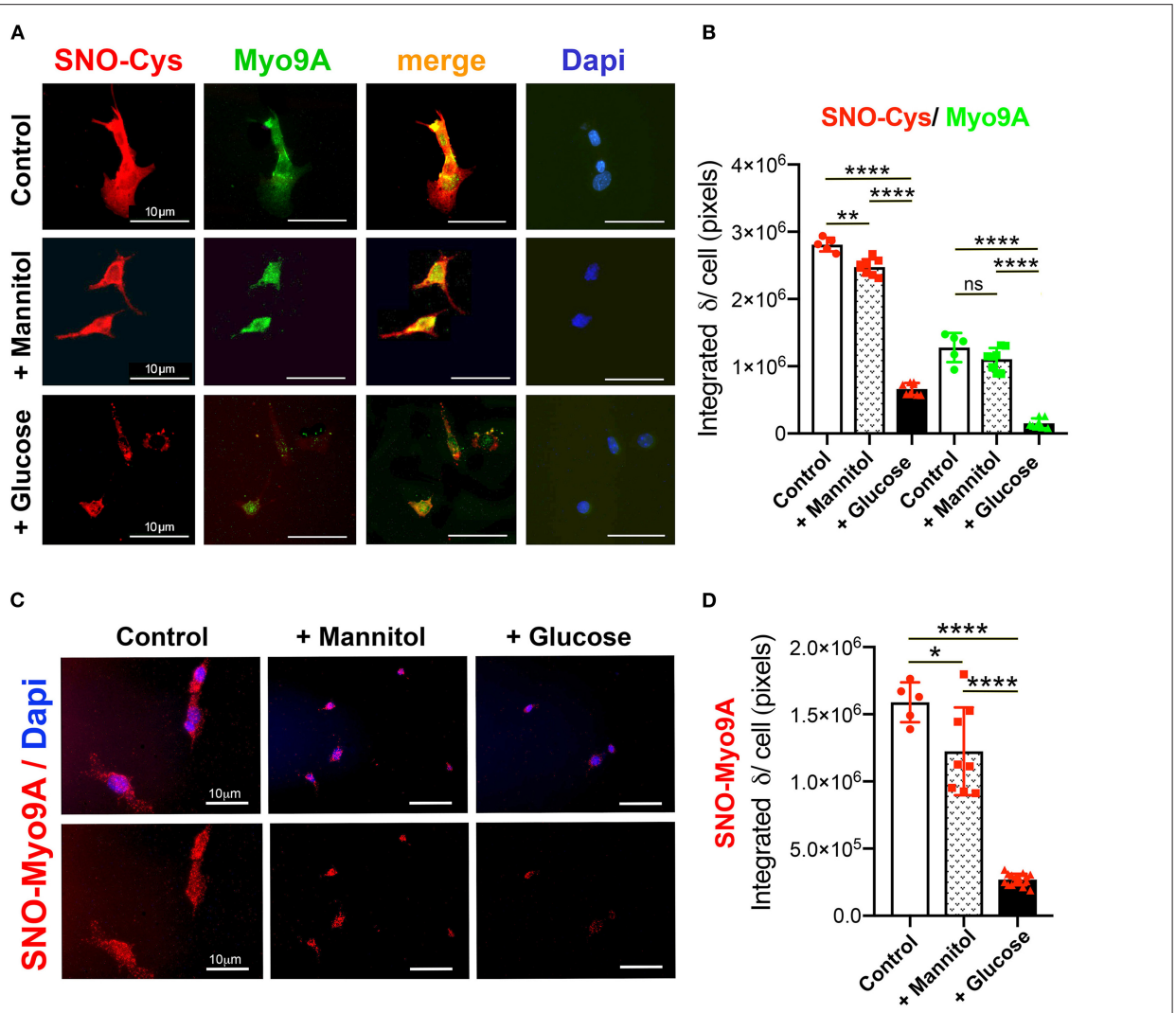

FIGURE 3 | Podocyte Myo9A expression and S-nitrosylation are downregulated by high glucose. **(A)** IHC shows abundant SNO-Cys proteins (red) and Myo9A (green) partially co-localized in normal podocytes (*top panels*) and in podocytes exposed to mannitol (*middle panels*), whereas both SNO-Cys and Myo9A signals are clearly reduced in podocytes exposed to high glucose (*bottom panels*). Scale bars= 10μm. **(B)** Quantitation of IHC IF signals demonstrate highly significant decrease in Myo9A and SNO-Cys proteins in podocytes exposed to high glucose, data expressed as mean ± SD, $n = 24$–32 cells/experimental group, Welch's ANOVA $p < 0.0001$, unpaired *t*-test with Welch's correction $p < 0.027$ or n.s. control vs. mannitol, $p < 0.0001$ mannitol vs. high glucose. **(C)** Proximity link assay IF signal (red) identifies SNO-Myo9A in control podocytes, a mild decrease in podocytes exposed to mannitol and barely detected SNO-Myo9A in podocytes exposed to high glucose; Dapi (blue) identifies cell nuclei. Scale bars = 10μm. **(D)** Quantification of PLA IF signals, mean ± SD, $n = 20$–31 cells/experimental group, Welch's ANOVA $p < 0.0001$, unpaired *t*-test with Welch's correction $p < 0.02$ control vs. mannitol, $p < 0.0001$ mannitol vs. high glucose. $^*p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

activity using a pull down assay (7) and determined that high glucose induces an increase in RhoA activity, which is partially abrogated by NO donor (**Figure 5D**).

Taken together, our findings indicate that in podocytes high glucose-induced downregulation of SNO-Myo9A is associated with similar decreases in SNO-actin and SNO-RhoA, as well as with increased RhoA activity, all of which are regulated by NO availability.

DISCUSSION

This study demonstrates that the unconventional myosin Myo9A is S-nitrosylated in normal podocytes and that diabetic milieu downregulates Myo9A expression and S-nitrosylation *in vivo*. Our findings revealed that Myo9A S-nitrosylation is regulated by glucose and nitric oxide availability in cultured podocytes, consistent with *in vivo* findings. Data uncover S-nitrosylation as an integrated signaling between Myo9A and its interacting proteins RhoA and actin that transduces metabolic cues (high glucose + low NO), modifies cytoskeletal effectors (RhoA) function and impacts podocyte behavior.

Using an experimental type 1 diabetes (T1D) mouse model we determined that Myo9A expression in the kidney is decreased in diabetic mice with advanced DKD, while in diabetic mice with mild DKD Myo9A expression is not different from non-diabetic mice (7). Glomerular Myo9A is S-nitrosylated in mice with mild DKD whereas SNO-Myo9A is significantly reduced in mice with advanced DKD. These findings suggest (but do not prove) that

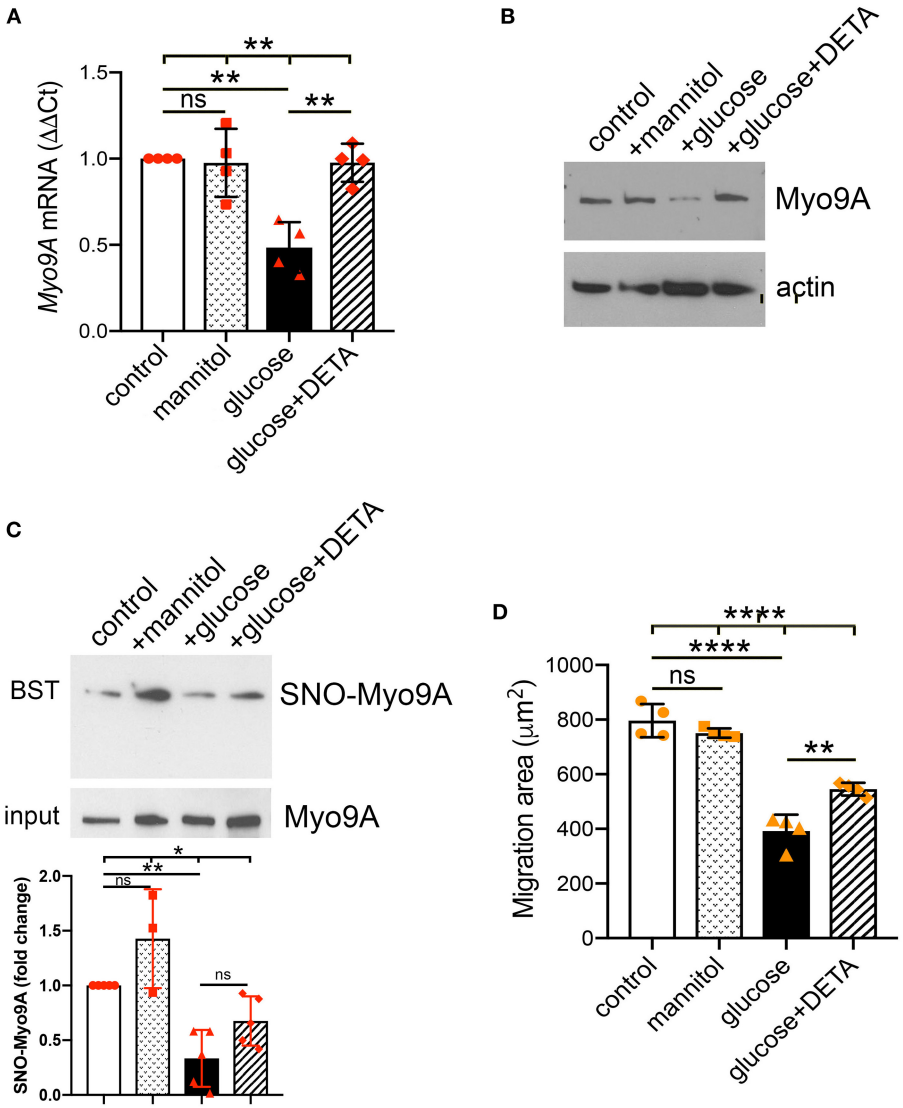

FIGURE 4 | Podocyte Myo9A expression and SNO-Myo9A are regulated by glucose and NO. **(A)** qPCR shows that *Myo9A* mRNA is not affected by mannitol, decreases ~50% in podocytes exposed to high glucose and addition of NO donor prevents *Myo9A* mRNA downregulation, mean ± SD, n = 4 independent experiments; Welch's ANOVA $p < 0.02$, unpaired *t*-test with Welch's correction: n.s. control vs. mannitol, $p < 0.02$ control vs. high glucose, $p < 0.02$ high glucose vs. high glucose + DETA. **(B)** Immunoblots show that Myo9A protein expression is not altered by mannitol, decreases ≥50% in podocytes exposed to high glucose and addition of NO donor prevents Myo9A downregulation. **(C)** BST shows SNO-Myo9A in control podocytes, SNO-Myo9A ~50% decrease in podocytes exposed to high glucose, addition of NO donor partially prevents Myo9A de-nitrosylation. Input shows total Myo9A loading, mean ± SD, $n = 3$–5 independent experiments, Brown-Forsythe ANOVA test, $p = 0.022$, unpaired *t*-test with Welch's correction non-significant (n.s.) control vs. mannitol, $^{**}p = 0.0046$ control vs. high glucose, $p = 0.0575$ (n.s.) high glucose vs. high glucose + DETA. **(D)** Migration 'wound' assay shows that podocyte migration is not affected by mannitol, whereas high glucose clearly reduces podocyte migration and addition of NO donor partially prevents this defect, mean ± SD, $n = 4$ independent experiments; Welch's ANOVA $p < 0.0001$, unpaired t-test with Welch's correction non-significant (n.s.) control vs. mannitol, $p < 0.005$ control vs. high glucose, $p < 0.02$ high glucose vs. high glucose + DETA. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

downregulation of Myo9A expression and S-nitrosylation are mechanistically involved in the progression or severity of DKD. In this experimental model the development of diabetic nodular glomerulosclerosis is driven by inducible podocyte $VEGF_{164}$ overexpression (29). Thus, the observed changes in Myo9A expression and SNO-Myo9A in induced mice with severe DKD could be a direct effect of excess glomerular VEGF-A and not mechanistically contributing to DKD progression. A previous study showed that VEGF-A cell autonomously decreases laminin S-nitrosylation in podocytes (33). Alternatively, hyperglycemia and $VEGF_{164}$-induced NOS uncoupling reduce NO availability (17), which in turn could influence *Myo9A* expression and SNO-Myo9A in diabetic glomeruli and thereby contribute to DKD progression. Results from a DNA array showing >2-fold decrease of *Myo9A* expression in diabetic Zucker rats, a model of type 2 diabetes (T2D) are consistent with the latter possibility (35).

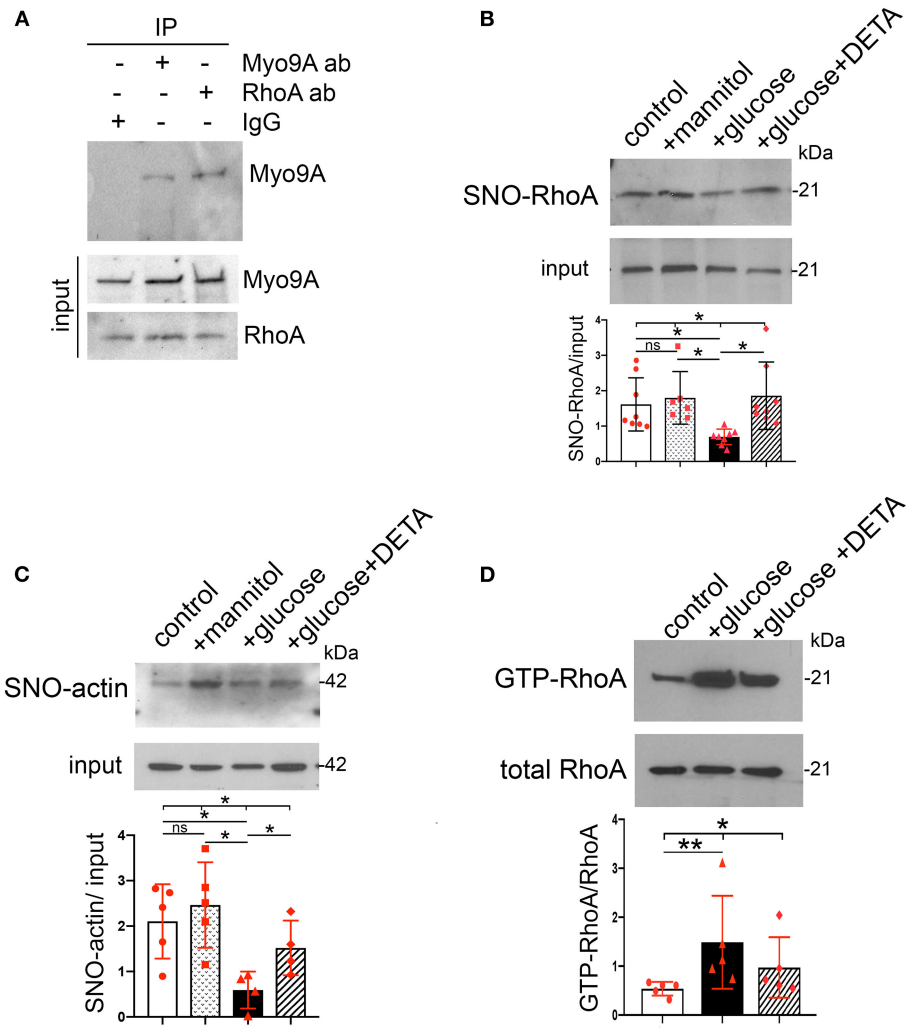

FIGURE 5 | Podocyte SNO-RhoA, SNO-actin and RhoA activity regulation by glucose and NO. **(A)** Immunoprecipitation (IP): Myo9A and RhoA, WB: RhoA and Myo9A demonstrate Myo9A-RhoA interaction in podocytes. **(B)** BST shows SNO-RhoA in normal podocytes, ~50% SNO-RhoA decrease in podocytes exposed to high glucose, addition of NO donor prevents RhoA de-nitrosylation. Input shows total RhoA, mean ± SD, $n = 6–8$ independent experiments, Welch's ANOVA test, $p = 0.002$, unpaired t-test with Welch's correction non-significant (n.s.) control vs. mannitol, $p < 0.01$ control vs. high glucose, $p < 0.01$ high glucose vs. high glucose + DETA. **(C)** BST shows SNO-actin >50% decrease induced by high glucose, partially prevented by the NO donor DETA. Input shows actin loading, mean ± SD, $n = 4–6$ independent experiments, Welch's ANOVA test, $p = 0.013$; unpaired t-test with Welch's correction n.s. control vs. mannitol, $p = 0.0011$ control vs. high glucose, $p < 0.05$ high glucose vs. high glucose + DETA. **(D)** RhoA activity assay shows that exposure to high glucose increases active GTP-RhoA and addition of NO donor partially prevents activation of RhoA. Total RhoA shows equal input, mean ± SD, $n = 5$ independent experiments, Kruskall-Wallis test, $p = 0.013$; Mann-Whitney test $p = 0.0079$ control vs. high glucose. $^*p < 0.05$ and $^{**}p < 0.01$.

Further studies assessing Myo9A role in DKD progression in other experimental mouse models of advanced DKD, e.g., T1D or T2D + *eNOS* KO, are warranted. Here we used podocytes to examine how the diabetic milieu influences Myo9A at the cellular level.

A key finding of this study is that Myo9A is S-nitrosylated in normal podocyte culture conditions and de-nitrosylates in diabetic milieu. Data indicate that this is not due to hyperosmolarity associated with high glucose and it is abrogated by addition of NO donor, demonstrating that SNO-Myo9A is glucose and NO dependent. *Myo9A* expression is also glucose and NO dependent, raising the intriguing possibility that *Myo9A* regulation is both transcriptional and post-translational in podocytes. High glucose-induced Myo9A downregulation and de-nitrosylation were associated with decreased podocyte migration, which was partially abrogated by a NO donor. Although this abnormal podocyte behavior in the diabetic milieu could be mediated via multiple pathways, it is remarkably similar to that reported in *Myo9A* knockdown podocytes (7), suggesting that Myo9A dysregulation is involved.

Myo9A binds actin at one of the two actin-binding sites in loop 2 of the catalytic domain forming crosslinks that bridge across actin filaments in parallel polarity at 36 nm regular intervals matching the actin helical repeat, thereby bundling actin filaments to form ordered networks (36). Experimental conditions such as calcium-calmodulin, ATP and redox status

influence Myo9A actin crosslinking activity *in vitro* (36). However, it is presently unknown whether SNO-Myo9A is required for actin crosslinking *in vivo*.

We report for the first time SNO-actin in normal podocytes, which is regulated by high glucose and NO dependent alike SNO-Myo9A. In physiological conditions all actin isoforms are S-nitrosylated on Cys374 and probably on additional Cys residues (37). Because actin is abundantly expressed and largely S-nitrosylated in most cells, it has been proposed that actin serves as a cell SNO-thiol reservoir that trans-nitrosylates with GSH-nitroso-glutathione (GSNO) (24, 26). S-nitrosylation affects actin polymerization and its interaction with proteins that are relevant for actin dynamics, including VASP, cofilin1, profilin and α-actinin (37).

An important finding of this work is that SNO-RhoA occurs in normal podocytes and inversely relates with RhoA activity. Myo9A interacts directly with RhoA through its tail RhoGAP domain (14, 28). Upon binding, Myo9A dephosphorylates RhoA GTPase rendering it inactive (14). We recently reported that *Myo9A* haploinsufficiency increases RhoA activity in kidneys and podocytes, consistent with loss of RhoGTPase function (7). Here we show that SNO-RhoA is regulated by high glucose and NO dependent, i.e., inversely related to high glucose and positively related to NO availability, and that RhoA activity is inversely related to SNO-RhoA in podocytes. Our results are consistent with a report showing that endothelial cell RhoA S-nitrosylation occurs in physiological conditions, is NO dependent and inhibited by increased intracellular Ca^{+2}, while RhoA activity is inversely correlated to SNO-RhoA (38). It is well-established that RhoA activity is elevated in T1D and T2D experimental models and that high glucose increases RhoA activity in endothelial cells (39), mesangial cells (40) and podocytes (41). Collectively, these findings suggest that RhoA de-nitrosylation induced by the diabetic milieu may mediate RhoA activation in all three glomerular cell types.

Our novel findings of podocyte Myo9A, actin and RhoA S-nitrosylation, which are regulated similarly, suggest that these post-translational modifications are linked. SNO transnitrosylation, i.e., the transfer of NO^- between Cys residues, occurs between interacting proteins or those closely adjacent within a cell compartment or microdomain, and its specificity is spatially determined (19, 42, 43). We have previously described Myo9A-actin interaction in podocytes (7). Here we report for the first time Myo9A-RhoA interaction in podocytes. We propose a model of transnitrosylation cascade involving Myo9A, RhoA and actin, three interacting proteins that are critical for podocyte cytoskeleton homeostasis (**Figure 6**). In this model, the three proteins are S-nitrosylated in control conditions (A) and Myo9A interacts physically and functionally with actin through its catalytic domain that hydrolyses ATP, crosslinks and bundles actin, as well as with RhoA via the tail RhoGAP domain that de-phosphorylates and inactivates the RhoA GTPase (14, 36). In the diabetic milieu (B), high glucose and low NO decrease SNO-Myo9A, SNO-actin and SNO-RhoA leading to increased RhoA activity and abnormal actin dynamics, which alter podocyte function, as assessed by decreased migration. Remarkably, these changes are at least partially reversible. SNO-actin changes upon high-glucose exposure are consistent with

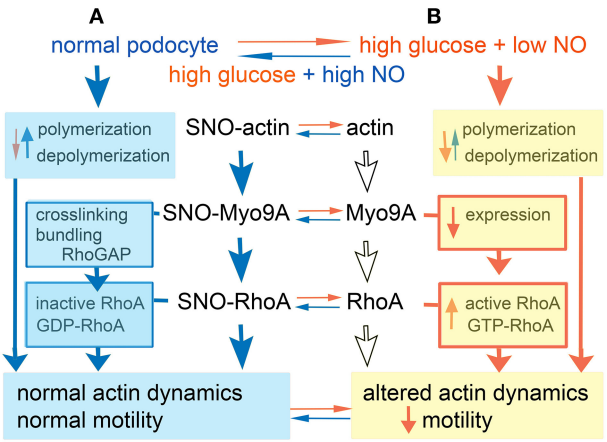

FIGURE 6 | Model. **(A)** In normal podocyte culture conditions actin, Myo9A and RhoA are S-nitrosylated (SNO), whereby F actin polymerization predominates, Myo9A crosslinks and bundles actin and inactivates RhoA leading to normal actin dynamics and podocyte motility. **(B)** In high glucose + low NO conditions podocyte SNO-Myo9A, SNO-RhoA and SNO-actin decrease significantly (de-nitrosylate), actin depolymerization may predominate, Myo9A expression decreases and RhoA activity increases, together resulting in altered actin dynamics and reduced podocyte motility. Most of these changes are partially reversible by addition of NO donor.

the hypothesis that it acts as a SNO reservoir (26). Alternatively, S-nitrosylation of actin isoforms may be regulated differently (24, 44). We speculate that SNO-RhoA may also be regulated by TRPC6-mediated increases in iCa^{+2}, known to be stimulated by AngII and VEGF-A and to mediate RhoA activation in the diabetic milieu (45–47). Akin to SNO inhibition of PKM2 (48–50), the reversible inhibitory S-nitrosylation of RhoA described herein may provide a novel mode of regulation amenable to therapeutic intervention in DKD.

Further studies are needed to provide detailed insight on the proposed model. For example, it is critical to elucidate whether SNO-MyoA is required for Myo9A's actin crosslinking activity and RhoGAP function and to determine how does SNO-actin influence the balance of actin polymerization-depolymerization dynamics in podocytes. Actin is also oxidized on Met44 and Met47 by MICAL, a flavo-oxygenase expressed in the kidney and in podocytes (51, 52) that leads to F-actin disassembly (52, 53). It is not known if oxidation of actin Met residues is regulated by glucose or NO dependent. Characterization of Myo9A post-translational modifications is limited (14). Identification of Myo9A Cys residues that undergo S-nitrosylation has been elusive as yet, precluding definitive experiments testing our model. Limitations of this study include not examining S-nitrosylation of Myo9A and its interacting partners in biopsy samples from DKD patients, in proximal tubular cells, glomerular endothelial and mesangial cells, known to be involved in DKD progression, nor in additional experimental T1D and T2D models. Further *in vivo* studies are needed to ascertain whether decreased SNO-RhoA and SNO-actin contribute to DKD progression and to evaluate the effect of NO donors on S-nitrosylation of Myo9A, RhoA and actin.

In summary, this work shows that Myo9A, RhoA and actin are S-nitrosylated in normal podocytes and that diabetic milieu

induces Myo9A, actin and RhoA de-nitrosylation, resulting in increased RhoA activity and impaired podocyte migration, which proved to be partially reversible, and therefore potentially targetable. Collectively, our findings uncover S-nitrosylation of Myo9A, actin and RhoA as an integrated signaling crosstalk that reversibly transduces metabolic cues to regulate actin dynamics and podocyte motility.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at the Yale University School of Medicine.

AUTHOR CONTRIBUTIONS

QL and DV performed experiments, analyzed data, and contributed to manuscript writing. AT designed and supervised experiments, analyzed data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Single Cell Transcriptome Helps Better Understanding Crosstalk in Diabetic Kidney Disease

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Years of research revealed that crosstalk extensively existed among kidney cells, cell factors and metabolites and played an important role in the development of diabetic kidney disease (DKD). In the last few years, single-cell RNA sequencing (scRNA-seq) technology provided new insight into cellular heterogeneity and genetic susceptibility regarding DKD at cell-specific level. The studies based on scRNA-seq enable a much deeper understanding of cell-specific processes such as interaction between cells. In this paper, we aim to review recent progress in single cell transcriptomic analyses of DKD, particularly highlighting on intra- or extra-glomerular cell crosstalk, cellular targets and potential therapeutic strategies for DKD.

Keywords: single-cell RNA sequencing, crosstalk, diabetic kidney disease, glomerulus, tubular epithelial cell

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INTRODUCTION

Diabetic Kidney Disease (DKD) is a microvascular complication associated with type I or type II diabetes. It has become a public issue and seriously threaten human health and lives. As the leading single cause of end-stage renal disease (ESRD) in many countries, such as the United States, DKD accounts for more than half of all patients enrolled in renal replacement therapy (RRT) programs (1). Although there has been a decline in the incidence of DKD over the past 30 years due to improved diabetes managements, the absolute risk of renal and cardiovascular morbidity and mortality remains overwhelmingly high (2–6). A deeper insight into the pathogenesis of DKD is required for innovative treatment strategies to prevent, arrest, and reverse DKD. Hyperglycemia is thought to be a major factor for diabetic complications and causes accumulation of toxic glucose derivatives (7, 8). However, hyperglycemia alone is not sufficient to the development of DKD since about only 30% of patients with type 1 diabetes mellitus (DM1) and 40% of patients with type 2 diabetes mellitus (DM2) develop this microvascular complication (1, 9). Family aggregation of DKD shown by independent familial studies in different populations suggests a genetic predisposition to DKD (10, 11). Moreover, patients with DKD are not always present with micro/macro-albuminuria. A large proportion of diabetic patients have declined renal function in absence of substantial proteinuria (12). The DKD heterogeneity suggested by the aforementioned evidences implies variant modulation of kidney function in diabetes and highlights the need for better biomarkers to predict the progressive kidney failure in the patients without heavy proteinuria.

Kidney is a highly complex organ consisting of about a million nephrons in humans which is composed of more than 40 different cell types (13, 14). The need for better understanding of the complex cell-to-cell interaction within or even beyond the heterogeneous kidney milieu comes naturally and rationally to reveal the complex mechanism underlying kidney organization, function and disease. The current clinical diagnoses for renal diseases as well as experimental researches on kidney depend largely on morphological cell identification and their known biomarkers. However, some important disease discriminative and prognostic features may not be effectively captured due to the highly operator-dependent microscopical observation and the limited biomarkers available. A single cell transcriptional profiling by a new set of technologies–single cell RNA-sequencing (scRNA-seq) has emerged in the last 10 years as a powerful approach helping to decipher complex information in cells and organs (15, 16). Here we aim to review cell-to-cell cross talk in DKD, particularly highlighting the latest insight gained by scRNA-seq researches.

BRIFE INTRODUCTION OF SINGLE CELL RNA SEQUENCING

scRNA-seq is a new set of technologies for genome wide RNA profiling of individual cells based on whole-genome-amplification (WGA) methods and next-generation sequencing (NGS) technologies (17–20). Before the invention of scRNA-seq, the genome-wide transcriptomic information primarily came from "bulk" RNA-seq, whose data represent an average of gene expression across individual cells and thus may mask some transcriptional information from less representative subpopulation. Compared with bulk RNA-seq, scRNA-seq provides more unbiased gene expression profiles at a single-cell resolution. The scRNA-seq methods have gained considerable progress over the last decade while the single cell DNA sequencing (scDNA-seq) has proven to be more challenging than RNA due to the fact that a single cell contains only two copies of each DNA molecule, but thousands of copies of most RNA molecules, which result in more technical error in scDNA-seq (15). All scRNA-seq techniques include several common steps: single cell isolation, cell lysis and RNA capture, reverse transcription and transcriptome amplification, cDNA library preparation, and sequencing and quantification. The most challenge for scRNA-seq is cell isolation and individualized RNA capture (individual transcriptome barcoding). Two barcoding strategies are suggested, either (1) the addition of a cell-specific barcode to each transcriptome following cell isolation (Microfluidics-based scRNA-seq), or alternatively (2) the addition of a unique index combination to each cell transcriptome without physical partitioning (e.g., Split-seq) (21–25) (**Figure 1**). Bio information from sequencing is intensively analyzed by computer and the final result is generation of a digital expression matrix including all detected gene expression in each individual cell. High throughput scRNA-seq data are processed to cluster cells and visualized by dimensionality reduction graph. Cell types are identified by examining known marker gene expression in each cluster and shown by the heatmap. Gene-gene correlation analysis helps to clarify the relationship between two marker genes within a cluster as well as the relationship of two marker genes from different clusters. Dynamic gene expression in single cell can be tracked along pseudotemporal trajectory corresponding to a biological process (e.g., development, differentiation, and disease progression). Key regulators for the dynamic gene expression can also be revealed by regulatory network analysis on transcription factors (26).

APPLICATIONS OF SINGLE CELL RNA SEQUENCING ON KIDNEY DISEASES

The knowledge regarding the transcriptional landscape of kidney in last 20 years was achieved largely from the "bulk" RNA-seq, which, though highly informative, is limited to describing an average transcriptome across a cell population in a bulk renal tissue or even in finely separated kidney compartments and thus masks or skews signals of interest (26–29). The comprehensive definition of cell types and states cooperating with examination of gene expression in specific cells by scRNA-seq makes it possible for determining specific disease-causal cells and genes. Park et al. performed scRNA-seq on kidneys from healthy male mice and unexpectedly identified a transitional cell type between intercalated cells (ICs) and principal cells (PCs) in collecting duct (30). They further demonstrated this IC-to-PC transition is mediated by Notch signaling and the shift toward the PC fate is the likely cause of metabolic acidosis in chronic kidney disease (CKD) (30). Recently Liao et al. delineated a transcriptomic map of human kidney cells basing on scRNA-seq analysis of normal human kidney (31). Another single-cell transcriptome profiling performed on human kidney allograft biopsy specimens (32) helped mapping previously defined rejection-associated genes to single cell types and revealed paracrine signaling pathway between infiltrating leukocytes and kidney parenchyma (33). A more recent scRNA-seq performed on purified glomeruli from four common kidney injury models (nephrotoxic serum nephritis, diabetes, doxorubicin toxicity and CD2AP deficiency) generated comprehensive snapshots of the altered genetic landscapes in multiple models (34). This research provided new insights into kidney injuries, such as that mesangial cell may shape the characters of the inflammation and wound healing programs in response to distinct types of injuries; persistent mesangial reaction may drive the chronic decline of kidney function in many disease; Hippo pathway is critical for podocyte repair in kidney injuries (34). In a scRNA-seq research on isolated glomerular cells from experimental diabetic mice, the unique trajectory analysis of scRNA-seq revealed dynamic changes of gene expression in endothelial and mesangial cells in diabetic mice (35). Subramanian et al. in a research regarding kidney organoids presented a comprehensive census of human organoids enabled by scRNA-seq in comparison to human adult and fetal kidneys (36). This census achieved some quantitative insight into organoid reproducibility and the data validated the faithfulness of kidney organoids from

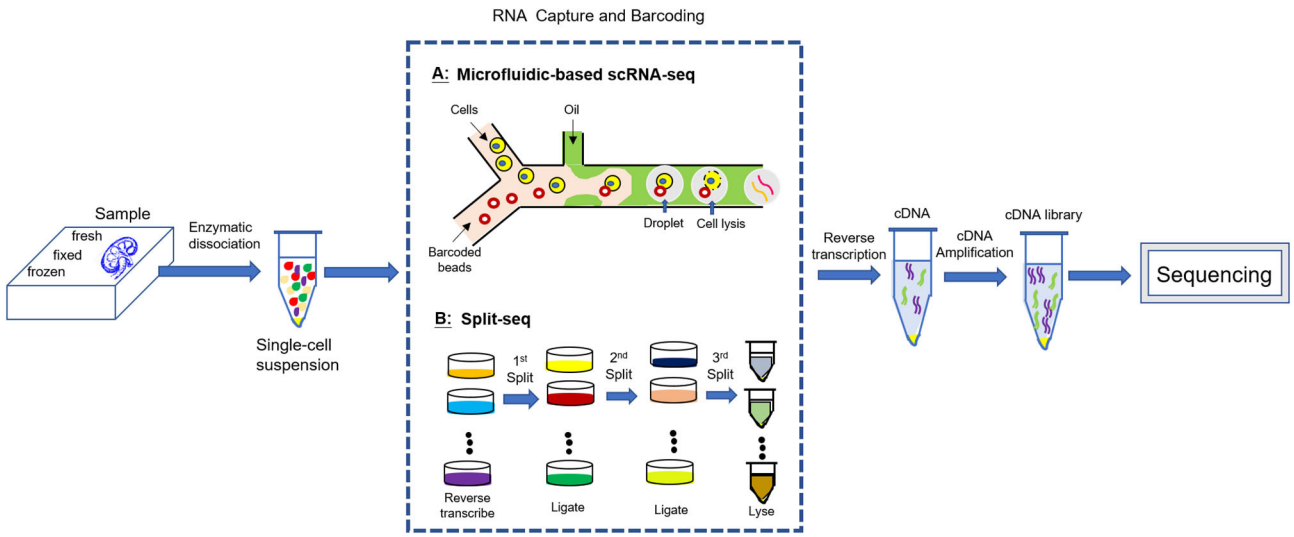

FIGURE 1 | scRNA-seq experimental workflow. All scRNA-seq techniques include several common steps: enzymatic dissociation of the sample into a single cell suspension; individualized RNA capture (individual transcriptome barcoding); reverse transcription and transcriptome amplification; cDNA library preparation; sequencing.

four different patient-derived induced pluripotent stem cell (iPSC) lines (AS, N1, N2, and ThF), which serve as surrogates of human kidney tissue for the study of a broad array of kidney diseases. The census data also addressed an issue of organoid quality, suggesting that the elimination of off-target cells may also benefit organoid maturity (36). In renal tumor research field, Young et al. studied Wilms' tumor, clear cell, and papillary renal cell carcinoma in relation to healthy fetal, pediatric, adolescent, and adult kidneys, as well as ureters (37). By analyzing tumor composition with scRNA-seq, they verified the hypothesis that Wilms' tumor cells are aberrant fetal cells and defined cancer-associated normal cells as well as delineated a complex VEGF signaling circuit (38). The power of scRNA-seq is not just to identify or catalog cells. It can help predict treatment outcomes and guide therapy. In a scRNA-seq research carried out by Park group, intratumoral heterogeneity was examined between a pair of primary renal cell carcinoma and its lung metastasis (37). The activation of drug target pathways demonstrated considerable variability between the primary and metastatic sites, as well as among individual cancer cells within each site. Guided by scRNA-seq analysis, a combinatorial regimen co-targeting two mutually exclusive pathways for the metastatic cancer cells gained better treatment efficacy over monotherapy (37).

More recently, Humphreys group (39) and Susztak group (40) both profiled kidney transcriptome and chromatin accessibility with sc/nRNA-seq and single nucleus assay for transposase-accessible chromatin using sequencing (snATAC-seq) respectively in their researches. These two multi-omics researches revealed the powerful potential of joint profiling with scRNA-seq in understanding kidney disease and development.

CHALLENGES TO SINGLE CELL RNA SEQUENCING ON KIDNEY RESEARCH

Despite the tremendous development of technology, scRNA-seq research is still facing many challenges. Cell isolation and individualized RNA capture remain to be the most challenges, since enzymatic dissociation protocols usually compromise cell viability and adult kidneys are relatively dense with matrix, thus the quality of single cell suspension does not accurately reflect the transcriptional state of each cell before dissociation (26). The possibility of selective cell loss during tissue dissociation and the transcriptional stress response induced by the proteolytic process as well as RNA degradation lead to bias. This may partly account for the failure in detecting about 25% of single kidney cells in sequencing in the work by Park et al. (30). The failure in detecting podocytes in transplant biopsy might be explained by the similar reason (33). The dissociation protocols need to be optimized responding to different kidney origins, since in some diseases, the injured podocytes are more susceptible to loss during enzymatic digestion whereas mesangial cells are less effectively isolated and captured due to the increased matrix. Cold dissociation was recommended by researchers as digestion on ice avoided stress and achieved more abundant cell types than warm dissociation at 37°C (41). The strategy adopted by Chen et al. and Karaiskos et al. in their experiments may partly correct some dissociative artifacts by dissecting specific portion of kidney tissues (e.g., proximal tubules or glomeruli) (42, 43). Human biopsy from patient is another challenge to scRNA-seq since scRNA-seq requires a relatively large number of cells for the automatic cell separation and capture system.

Recently, single nuclear sequencing (snRNASeq) rise popular as an alternative to scRNA-seq for its obvious advantages in

gaining good quality nuclei from snap frozen sample while bypassing the proteolytic process at 37°C (44–46). But there were researches implied T, B, and NK lymphocytes were underrepresented in the single-nucleus libraries (40, 42, 44). When interpreting the results of scRNA-seq, protocol-specific biases must be taken into consideration as cryopreservation of dissociated cells results in a major loss of epithelial cell types while methanol fixation maintains the cellular composition but suffers from ambient RNA leakage (41).

In addition to the crucial step of cell dissociation, a successful scRNA-seq is also challenged through the computational workflow. Depending on the platform of choice, researchers individualize their own procedural steps and choose specific analytic tools for data processing from the step of raw counts normalization to feature selection, dimensionality reduction, and clustering. When inferring cell-cell communication from transcriptomics, most of the researchers built the lists of ligand-receptor pairs from multiple databases and literature curation. Armingol et al. collated publicly available lists into a single ligand-receptor pair repository to facilitate further use and comparison (47). However, integrating multiple sources of data is challenging and requires reconciliation of the different ways ligand–receptor pair confidence was assessed or how orthologs were determined (47). To identify the genes associated with cell communication, the gene expression matrix generated by scRNA-seq is filtered by ligand-receptor pairs and a communication score for each ligand-receptor pair is computed using their gene expression levels [function f(L, R), where L and R are the expression values of the ligand and the receptor, respectively] (47). An aggregated communication score is also computed to estimate the overall state of interaction between respective samples or cells and the final results are visualized as Circos plot, network, Heatmap, etc. (47). There may exist some false positives or negatives in the inferred cell communication due to the data-driven inference process, which can lead to different results depending on the strategy adopted (47). Although some powerful computational tools such as CellChat, CellPhoneDB, NicheNet help to minimize the false discoveries (48–50), the findings derived from scRNA-seq need to be validated by experimental tests including immunohistochemistry, western blot and other functional assessment.

INTRAGLOMERULAR CROSSTALK IN DKD

Glomerulus is a highly organized complex with two major compartments, the glomerular capillary tuft and the so-called Bowman's capsule which surrounds the capillary tuft. Podocytes wrap around the glomerular capillary with foot processes, which are connected by slit diaphragms bridging the filtration slits. The intraglomerular mesangial cells reside between capillary loops in close contact with glomerular endothelium. Parietal epithelial cells (PECs) compose the outer layer of the capsule, directly connecting to proximal tubules. The formation and maintenance of the glomerular filtration barrier require the communication within glomerulus including a multidirectional crosstalk between podocytes, mesangial cells and endothelial cells as well as PECs (51–57). The normal glomerular structure is shown in **Figure 2** (9).

In DKD, the glomeruli are exposed to various noxious stimuli such as high glucose, fatty acids, uric acid, growth factors, cytokines, and hormones, which dysregulate the communication in glomerulus.

Crosstalk Between Podocyte and Glomerular Endothelial Cell (GEC)

Studies have shown that the severity of DKD is correlated with endothelial dysfunction in T1DM and T2DM (58, 59). The growth and survival of GECs are regulated by paracrine vascular endothelial growth factor alpha signaling (VEGFA-VEGFR) (60, 61). Podocyte VEGFA deletion results in the development of heavy proteinuria, marked glomerulosclerosis and glomerular cell apoptosis (62). Conversely, increased podocyte-derived VEGFA was shown to be deleterious in non-diabetic mice, and the injury was further exacerbated with diabetes induction, resulting in advanced glomerulopathy with massive proteinuria (63). Podocyte-produced angiopoietins, functioning as endothelial cell-protective factors in diabetes, mediate podocyte-to-endothelial crosstalk and are critical for modulating the vascular response after the onset of diabetes (61, 64). Angiopoietin-1 (Angpt1) is expressed by podocytes and mesangial cells and its cognate tyrosine kinase receptor, Tie2/Tek is expressed by the glomerular endothelial cells. Angpt1 and Angpt2 both bind to Tie2 receptor and have classically been viewed as having opposing effects on microvascular development, with Angpt1 stabilizing the vasculature, and Angpt2 antagonizing these effects by binding to the Tie2 receptor in a competitive manner (52). Endothelin-1 (Edn-1)/endothelin receptor type A (EdnRA) axis has been demonstrated to be a causative regulator in promoting endothelial cell dysfunction in human biopsies and experimental model of FSGS, and is responsible for the loss of glomerular endothelial glycocalyx by increased degradation of glycosaminoglycans (65–67). In diabetes, EdnRA in GECs is activated by increased circulating Edn-1 or local Edn-1, resulting in mitochondrial stress and endothelial dysfunction (68, 69).

A similar stressed endothelial-to-podocyte crosstalk *via* mitochondrial oxidative stress in endothelial cells downstream from Edn-1/EdnRA could also underlie segmental lesions in DKD and highlighted a potential mechanism for the proven renoprotective activities of EdnRA inhibitors (70, 71). Activated protein C (APC) formation, which is regulated by endothelial thrombomodulin, is required for podocyte viability (72). Loss of thrombomodulin-dependent APC formation interrupts crosstalk between the vascular compartment and podocytes, causing glomerular apoptosis and diabetic nephropathy (72). Krüppel-like factor 2 (KLF2) is a shear stress-inducible transcription factor and has endoprotective effects on podocytes. Endothelial cell knockout of KLF2 resulted in reduction of the endothelial glycocalyx and podocyte injury in diabetes (73). Recently, a study showed that endothelial derived exosomes, which are enriched with

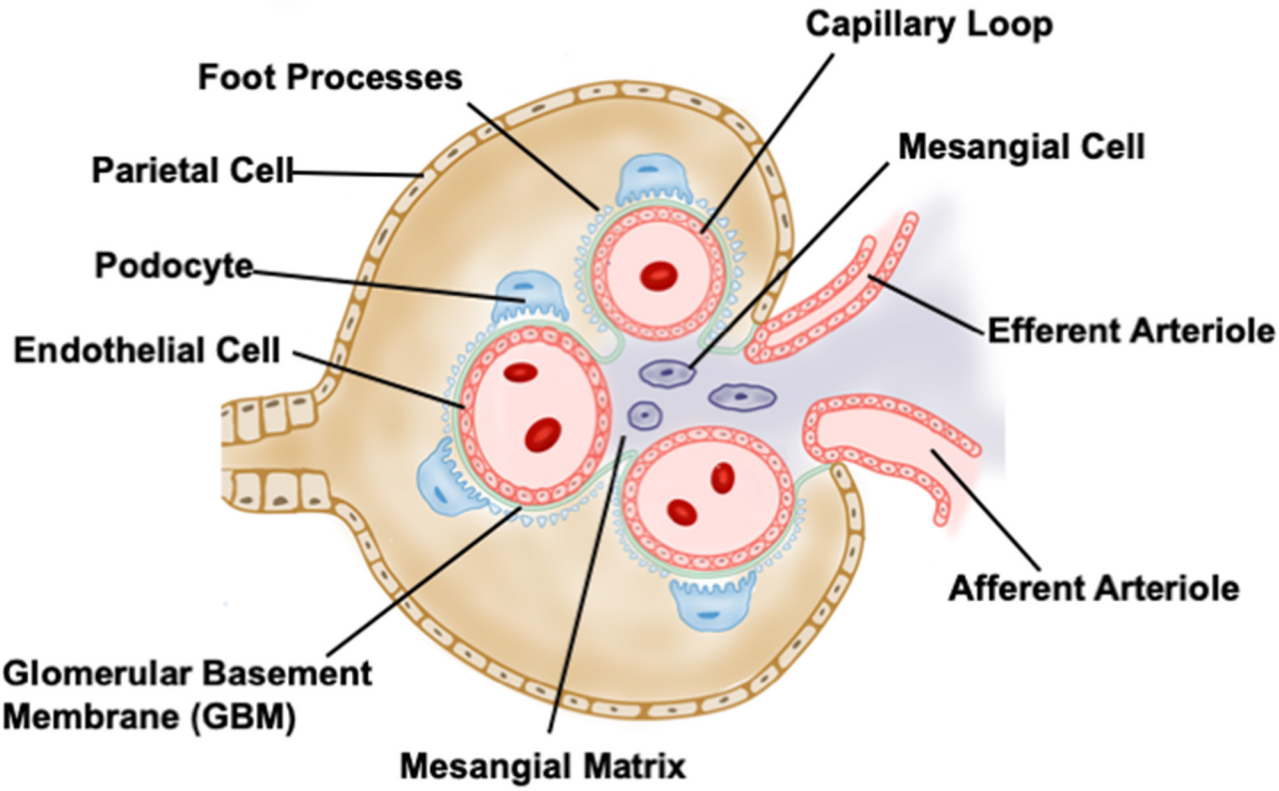

FIGURE 2 | Diagrammed normal glomerular structure.

TGF-β1 mRNA, could mediate epithelial-to-mesenchymal transition (EMT) and induce dysfunction of podocytes in a paracrine manner with activation of canonical Wnt/β-catenin signaling (74).

Crosstalk Between Podocyte and Parietal Epithelial Cell (PECs)

Enlightened by delicate balance between visceral and parietal epithelial cells across Bowman's space, the crosstalk between podocytes and PECs are thought to be as equally important as the tight interaction between endothelial cells and podocytes across the glomerular basement membrane (GBM) (75). Indeed, multiple studies have consistently corroborated that the depleted podocytes can be regenerated *via* differentiation of the adjacent PECs, which serve as the local progenitors of podocytes to reconstitute the podocyte population upon glomerular injury and podocyte loss (53, 76–83). Injured podocytes secrete heparin-binding epidermal growth factor-like growth factor (HB-EGF), which in turn stimulates and promotes the proliferation of PECs while disturbs their compensatory differentiation toward podocytes (77, 83). Another growth factor, insulin-like growth factor-1(IGF-1) has been proved more critical for promoting the differentiation of PECs into podocytes (83). A 3D multiscale modeling study suggested that promoting PECs differentiation are as equivalently important as amelioration of glomerulus stress for podocyte regeneration (83).

Crosstalk Between Glomerular Mesangial Cell (GMC) and Other Glomerular Cell Types

Mesangial cells are considered to be specialized pericytes due to their spatial intimacy with endothelial cells, thus functioning in stabilizing vasculature, synthesizing components of the basement membrane, and participating in controlling capillary vascular tone (84). The tight link between the fates of mesangial cell and endothelial cell has been well established by demonstrating the importance of platelet-derived growth factor B (PDGF-B) and its receptor PDGFR-β in the interaction of endothelial and mesangial cells (84–87). Recent evidences have revealed a significant role of exosomes as the messenger cargos for intercellular communications within glomerulus in DKD (88).

TABLE 1 | Summary of the mediators for intraglomerular crosstalk in DKD.

Crosstalk	Ligand/Receptor	Extracellular vesicles	Signal pathway	Pathological role in DKD	Reference
Podocyte-GEC	VEGFA–VEGFR2			The expression of VEGFA and VEGFR2 is increased in early DKD, but with the loss of podocytes at later stage of DKD, the expression of VEGFA is also significantly decreased. The VEGFA-VEGFR2 signaling contribute to vascular rarefication and renal fibrosis in the development of DKD.	(3, 60–63, 91)
	Angpt1/2–Tie2			Decreased ratio of Angpt-1/Angpt-2 contributes to the development of DKD. Angpt-1 could retard the development of albuminuria as well as glomerular endothelial cell proliferation, whereas Angpt-2 has the opposite effects in DKD.	(3, 64)
	Edn-1–EdnRA			The expression of Edn-1 is upregulated in DKD and combined with the receptor EdnRA, which contributes to the mitochondrial dysfunction of endothelial cell and podocyte apoptosis.	(64, 66–69)
	SDF-1–CXCR4			SDF-1/CXCR4 axis is involved in the pathogenesis of glomerulosclerosis in case of type 2 diabetes. Inhibition of SDF-1significantly reduced diffuse glomerulosclerosis and prevented albuminuria in the diabetic model.	(70)
			ANGPTL4	An upregulation of podocyte secreted Angptl4 has described in experimental diabetic animal, which contributed to proteinuria and endothelial injury.	(3)
GEC-Podocyte	APC–PAR1/ EPCR/S1PR1			A loss of thrombomodulin-dependent protein C activation and aggravated glomerular apoptosis is described in diabetic mice. Increased levels of APC formation prevent podocyte apoptosis and downregulates coagulation and inflammation in DKD.	(72)
			KLF2	The expression of KLF2 is reduced in diabetic kidneys and it lack aggravates endothelial and podocyte injury in diabetic nephropathy.	(73)
			eNOS	A tight relation has been found between eNOS deficiency and a podocyte-specific injury and heavy albuminuria in advanced DKD.	(75)
			Endothelial glycocalyx	The damage of endothelial glycocalyx and shear-stress is observed in early DKD, and this damage altered organization of extracellular matrix.	(67)
		TGF-β1		The increased secretion of exosomes enriched with TGF-β1 mRNA probably mediates the EMT and dysfunction of podocytes through the Wnt/β-catenin signaling pathway.	(74)
Podocyte-PEC	HB-EGF–c-MET			Injured podocytes secrete HB-EGF, which in turn stimulates and promotes the proliferation of PECs while disturbs their compensatory differentiation toward podocytes.	(77, 82)
	IGF–IGFBPs			Dysregulation of the growth hormone/IGF system is found in early DKD and is associated with both glomerular hypertrophy and microalbuminuria.	(82)

(Continued)

TABLE 1 | Continued

Crosstalk	Ligand/Receptor	Extracellular vesicles	signal pathway	Pathological role in DKD	Reference
GEC-GMC	PDGFB–PDGFR-β			PDGFR-β signaling is activated in glomeruli and tubule of diabetic mice. It may contribute to the progress of diabetic nephropathy, with an increase in oxidative stress and mesangial expansion.	(86, 87)
		TGF-β1		The increased secretion of exosomes enriched with TGF-β1 mRNA can promote α-SMA expression, proliferation and extracellular matrix protein overproduction in GMCs through the TGFβ1/Smad3 signaling pathway.	(88)
GMC-GEC			Integrin αvβ8	The integrin is expressed by mesangial cells, where it sequesters TGF-β, thereby reducing TGF-β signaling. Integrin αvβ8 and its ligand latent TGF-β protect kidney from glomerular dysfunction, endothelial apoptosis, and development of proteinuria, but the role of Integrin αvβ8 in DKD is unknown.	(91)
Podocyte-GMC			VEGF	The diabetic podocyte produces excessive VEGF in the setting of low endothelial NO and stimulates growth and proliferation of mesangial and endothelial cells, leading to increased extracellular matrix accumulation, hyperfiltration, and proteinuria.	(83)
GMC-Podocyte		TGF-β1		Exosomes derived from high-glucose-induced mesangial cells induced podocyte injury through the increased secretion of TGF-β and TGF-β1/PI3K-Akt signaling.	(83, 89)
			ERAD	ERAD-associated genes are downregulated in diabetic Glomeruli, and inhibition of ERAD processes could leading to the suppression of nephrin phosphorylation and podocytes injury under diabetic conditions.	(92)

DKD, diabetic kidney disease; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; Angpt1/2, angiopoietin-1/2; Tie2, angiopoietin 1 receptor; Edn-1, Endothelin-1; EdnRA, Endothelin receptor A; SDF-1, stromal cell-derived factor 1; CXCR4, C-X-C Chemokine Receptor Type 4; ANGPTL4, angiopoietin-like 4; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; HB-EGF, heparin-binding epidermal growth factor-like growth factor; c-MET, mesenchymal epithelial transition factor; APC, Activated protein C; PAR1, Protease-activated receptor 1; Sirt1, Sirtuin 1; EPCR, endothelial protein C receptor; S1PR1, Sphingosine 1-phosphate receptor 1; TGF-β1, Transforming growth factor β1; KLF2, Krüppel-like factor 2; eNOS, endothelial nitric oxide synthase; PDGFB, Platelet-derived growth factor B; PDGFRβ, platelet-derived growth factor receptor beta; IL-1β, Interleukin 1β; Integrin αvβ8, Integrin alphavbeta8; CCN1, Cellular communication network factor1; INSR, insulin receptors; ERAD, ER-associated protein degradation; NMN, nicotinamide mononucleotide; CCL2, C-C motif chemokine ligand 2; EMT, epithelial-mesenchymal transition; α-SMA, α-smooth muscle actin; GMC, glomerular mesangial cell; GEC, glomerular endothelial cell; PEC, parietal epithelial cell.

Wu et al. demonstrated exosomes released by high glucose-treated GECs could promote α-smooth muscle actin (α-SMA) expression, proliferation and extracellular matrix protein overproduction in GMCs through the TGF-β1/Smad3 signaling pathway (89). In respect of the crosstalk between podocyte and mesangial cell, several signaling pathways have been suggested to be involved including VEGF, Edn-1, CCR7, and its ligand SLC/CCL21, PDGF, connective tissue growth factor (CTGF), hepatocyte growth factor (HGF) and TGF-β (84, 90). Among these signalings, VEGFA and nitric oxide (NO) are considered to play a pivotal role in driving the development of typical DKD lesions, causing as important effects on GMCs as those on endothelial cells. (91). The diabetic podocyte produces excessive VEGF in the setting of low endothelial NO and stimulates growth and proliferation of mesangial and endothelial cells, leading to increased extracellular matrix accumulation, hyperfiltration, and proteinuria (92). A recent research has shown that an intraglomerular crosstalk between mesangial cells and podocytes can inhibit physiological endoplasmic reticulum stress-associated degradation (ERAD) and suppress the phosphorylation of nephrin in podocytes, which thereby lead to podocyte injury under diabetic conditions (92).

The intraglomerular crosstalks are summarized in **Table 1**.

Findings Based on scRNA-Seq

By comparing the differential gene expression detected by scRNA-seq between specific cell types with the existing ligand-receptor database (http://fantom.gsc.riken.jp/5/) or the potential paracrine secreted ligand-to-membrane receptor pair list obtained by using Human Protein Atlas (https://www.proteinatlas.org/humanproteome/ secretome) and BIOGRID v3.5.165 (https://thebiogrid.org), the researchers can identify cell-cell crosstalk between glomerular cell types. To date, crosstalk data from scRNA-seq research regarding DKD are

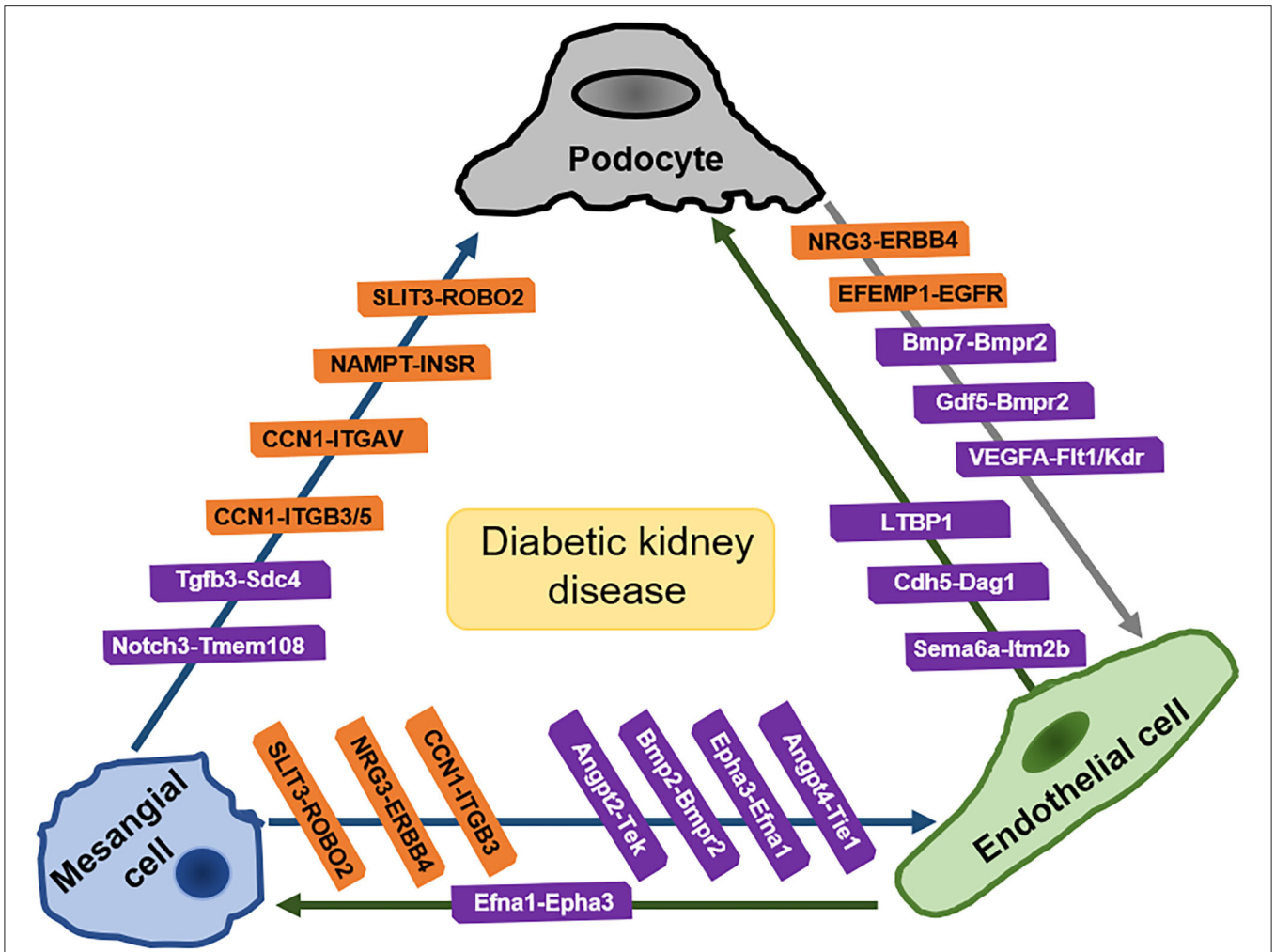

FIGURE 3 | Intraglomerular crosstalk based on ligand-receptor pair analysis of scRNA-seq. Pairs in orange frames come from the analysis of kidney of human with early diabetic nephropathy. Pairs in purple frames come from the analysis of kidney of diabetic mice. SLIT3, slit guidance ligand 3; ROBO2, roundabout guidance receptor 2; NAMPT, Nicotinamide phosphoribosyltransferase; INSR, insulin receptors; CCN1, cellular communication network factor1; ITGB3/5, integrin beta3/5; ITGAV, integrin subunit alpha-V; Tgfb3, Transforming growth factor 3; Sdc4, syndecan 4; Notch3, notch reporter 3; Tmem108, transmembrane protein 108; NRG3, neuregulin-3; ERBB4, Erb-B2 receptor tyrosine kinase 4; Bmp2, bone morphogenetic protein 2; Bmp7, bone morphogenetic protein 7; Bmpr2, bone morphogenetic protein receptor 2; Epha3, erythropoietin-producing hepatocellular carcinoma A3; VEGFA, vascular endothelial growth factor A; Flt1(VEGFR1, vascular endothelial growth factor receptor 1; Kdr (VEGFR2), vascular endothelial growth factor receptor 2; Angpt, angiopoietin; Tie (Tek): angiopoietin 1 receptor; Efnb2, ephrin B2; Efna1, ephrin A1; LTBP1, latent transforming growth factor (TGF)-beta binding protein-1; Cdh5, cadherin 5; Dag1, dystroglycan 1; Sema6a, semaphorin 6a; Itm2b, Integral membrane protein 2B; Cdf5, cycling dof factors 5; EFEMP1, Epidermal Growth Factor-containing Fibulin-like Extracellular Matrix Protein 1; EGFR, Epidermal Growth Factor Receptor. [Pairs in orange are summarized from Mitu et al. (93) and pairs in purple are summarized from Fu et al. (35)].

quite limited. Fu et al. performed scRNA-seq analysis on isolated glomerular cells from induced diabetic eNOS$^{-/-}$ mice (35). They analyzed a total of 644 cells (326 control and 318 diabetic) with a median of 3,457 genes per cell (3,417 control and 3,509 diabetic). With less cells capture but much greater sequencing depth per cell (five-fold) in a plate-based platform, as the researchers mentioned, compared with the microdroplet-based platform, several ligand-receptor pairs in the glomerular cell were identified, some of which are well established (e.g., podocyte VEGFA-endothelial Flt1 and Kdr) while the others are less well characterized in the glomerular homeostasis (e.g., mesangial Epha3-endothelial Efna1) (35). The ligand-receptor pair analysis in scRNA-seq is unprecedentedly informative to suggest almost all the potential direct cell-to-cell crosstalks. Taking Fu et al.'s research for example, this analysis not only identified the established crosstalks such as VEGFA pair, which can be subjected to cross validation with the existing literatures, but also guide future exploration for those less established interaction, such as podocyte BMP7-mesangial BMPR2 pair and mesangial Angpt4-endothelial Tie1 pair, which are implied by insufficient literature, yet to be validated (35). Unlike the above-mentioned crosstalk of podocyte VEGFA-endothelial Flt1 and Kdr having handful supportive evidences, the literatures in respect of the cellular crosstalk involving bone morphogenetic protein-7 (BMP-7) in DKD are limited. BMP-7 in podocytes was reported possibly having protective effects against renal

damage produced by hyperglycemia *via* the interaction with receptor BMPR2 (93, 94). But its cellular crosstalk in DKD *via* the interaction with its receptor needs more solid evidences. So does the pair of mesangial Angpt4- endothelial Tie1. Moreover, by comparing the diabetic ligand-receptor pairs with control, the changed crosstalk can be revealed, for example, the pairs of mesangial Angpt4-endothelia receptors were not detected in control, but showed in diabetes, even more prominent than other Angpt pairs (35).

In another scRNA-seq on cryopreserved human diabetic kidney samples, 23,980 single-nucleus transcriptomes were generated from three control and three early diabetic nephropathy samples (91). The researchers examined differentially expressed ligand–receptor pairs in glomerular cell types and found human diabetic mesangial cells had increased expression of CCN1 and SLIT3 (95). CCN1 responding extracellular protein, ITGAV, ITGB3, ITGB5 were expressed by podocytes, which interact with CCN1 and subsequently regulate tissue repair. Another CCN1 responding protein, ITGB3 was expressed by endothelial cells. ROBO2 was expressed by podocytes and endothelial cells, which interacted with SLIT3 to modulate cell migration. Diabetic endothelial cells also expressed increased LTBP1, which regulated targeting of latent TGF-β complexes (95). A scRNA-seq on mouse GMCs revealed GMCs having a high enrichment of genes involved in endothelial activity, supporting the long-existing notion that mesangial cells are specialized pericytes (96). Interestingly, the researchers also found that some mesangial cells express podocyte marker genes (e.g., Wt1) as well as endothelial cell marker genes (Tie2, Flk1, Flt1/ Vegfr1) (96).

When interpreting the data of ligand-receptor pair analysis, bias coming from misrepresented cell population must be taken into consideration. Podocytes, especially the injured podocytes are susceptible to loss during the dissociation process, which leads to limited podocytes detected, therefore results in the podocyte population underrepresented in most of the scRNA-seq research. It is also difficult to clearly identify mesangial cells due to the similarity between mesangial cells and stromal mesenchymal cells. Chung et al. found that several genes which were used in their study to identify mesangial cells are not specific while in some cases are specific to smooth muscle cells (SMCs) (Myh11, Rergl, Pln, and Olfr558) (34). They pointed that the study by Fu et al. was limited by the small number of cells, as the reason they referred were that the authors were unable to distinguish mesangial cells from SMCs/JG cells and neither obtain sufficient numbers of podocytes from diabetic mice for analysis (34).

Intraglomerular crosstalk based on ligand-receptor pair analysis of scRNA-seq are shown in **Figure 3**.

EXTRAGLOMERULAR CROSSTALK IN DKD

Tubular-glomerular interplay, which includes two well-known components, glomerular-tubular balance and tubuloglomerular feedback (TGF), has been demonstrated to play important roles in physiological renal function as well as in DKD (97). Proteins leak from glomeruli and arrive at tubular regions, then leading to further tubular injury, which is caused by the accumulation of proteinuria-inducing reactive oxygen species and various cytokines (98). Sirtuin 1 (SIRT1), a nuclear deacetylating enzyme, which mediates deacetylation of transcription factors and histone, is found being downregulated in proximal tubules preceding podocyte injury in DKD (99). Claudin-1 is a membrane protein involved in the formation of tight junctions and is normally expressed in parietal epithelial cells, which creates tight junctions that might prevent leakage from Bowman's capsule (100). In DKD, the downregulated proximal tubular SIRT1 decreases SIRT1 level in podocytes, thereby leading to the ectopic expression of claudin-1 in podocytes and causing albuminuria (101). Glomerular hyperfiltration is proposed to be resulted from tubular growth and upregulates sodium-glucose cotransporter 2 (SGLT2), which enhances proximal tubular reabsorption, leading a reduction of sodium chloride (NaCl) delivery to the macula densa, therefore increasing GFR *via* TGF response (SGLT2-NaCl pathway) (99–101).

Recently Hasegawa group also reported SGLT2 was elevated during early stages of DKD, which could upregulate intracellular glucose levels in proximal tubules and subsequently decrease SIRT1 expression whereas SGLT2 inhibitors preserved SIRT1 expression (102). SGLT2 inhibitors, suggested Hasegawa, might maintain the proximal tubule-podocyte communication. Other tubular-glomerular communications include a group of exosomes enriched with microRNA (miR) mediating podocyte or proximal tubular cell damage (103).

The communications between macrophages and kidney cells rely much on extracellular vesicles (EV). It was reported that miR-21-5p in macrophage-derived EVs regulated pyroptosis-mediated podocyte injury by A20 in DKD (104). It was also suggested that exosomal miR-19b-3p mediated the communication between injured tubular cells and macrophages, leading to M1 macrophage activation (105). Exosomes from high glucose-treated macrophages were implied to activate GMCs *via* TGF-β1/Smad3 pathway (106).

Findings Based on scRNA-Seq

A scRNA-seq performed on whole kidney cells from healthy mice revealed specific cell types responding to specific kidney related disorders (30). CKD related genes are strongly enriched in proximal tubules. The researchers identified a transitional cell type between principle cell (PC) and intercalated cell (IC) in collecting duct. Notch regulates the cellular identity of neighboring cells by the expression of either Notch ligands or Notch receptors. Genes encoding Notch ligands were highly expressed in ICs while Notch2 receptor and its transcriptional target Hes1 were shown in PCs with high expression level, suggesting that PCs are the Notch signal-receiving cells in the collecting duct. A higher ratio of PCs to ICs in human diabetic kidney biopsy with increased Notch signaling and HES1 expression suggested a shift toward PCs, which is likely the cause of metabolic acidosis in mouse models and patients with CKD (30). A human kidney scRNA-seq research identified

TABLE 2 | Summary of the mediators for extraglomerular crosstalk in DKD.

Crosstalk	Ligand/Receptor	Extracellular vesicles	Signal pathway	Pathological role in DKD	Reference
Podocyte-Tubular epithelial cell		miR-6538, miR-3474, miR-1981-3p, miR-7224-3p. Let-7f-2-3p		Upregulation of Let-7f-2-3p and downregulation of miR-1981-3p, miR-3474, miR-7224-3p and miR-6538 were detected by RT-qPCR in DKD. These EVs from podocyte may travel through the urinary tract and involved in the extrinsic apoptotic signaling pathway of TECs.	(88)
		miR-221		Podocyte-derived EVs in diabetes acted as key mediators of proximal tubule cell injury and the miR-221 in EVs mediated the cells damage through Wnt/β-catenin signaling.	(103)
Tubular epithelial cell-Podocyte			Sirt1	Sirt1 in tubular epithelial cell protects against albuminuria in diabetes by maintaining NMN concentrations around glomeruli, thus influencing podocyte function.	(102)
			SGLT2-NaCl	Glomerular hyperfiltration is proposed to be resulted from tubular growth and upregulates sodium-glucose cotransporter 2 (SGLT2), which enhances proximal tubular reabsorption, leading a reduction of sodium chloride (NaCl) delivery to the macula densa, therefore increasing GFR *via* TGF response.	(99–101)
Macrophage-Podocyte		miR-21-5p		EVs miR-21-5p secreted from macrophage through inhibition of A20 elevate the inflammasome NLRP3, caspases-1 and IL-1β related to pyroptosis, and augment the production of ROS, thereby causing podocyte injury.	(104)
Tubular epithelial cell- Macrophage		miR-19b-3p		Exosomes enriched with miR-19b-3p mediated the communication between injured TECs and macrophages, leading to M1 macrophage activation and tubulointerstitial inflammation though SOCS-1 pathway.	(105)
Intercalated cell-Principle cell	Notch2-Hes1			Genes encoding Notch ligands were highly expressed in ICs while Notch2 receptor and its transcriptional target Hes1 were shown in PCs with high expression level, suggesting that PCs are the Notch signal-receiving cells in the collecting duct.	(3)

DKD, diabetic kidney disease; TEC, Tubular epithelial cell; RT-qPCR, real-time polymerase chain reaction; EVs, Extracellular vesicles; IL-1β, Interleukin 1β; ROS, Reactive oxygen species; NLRP3, NACHT, LRR, and PYD domains-containing protein 3; SOCS-1, Suppressor of cytokine signaling-1; Sirt1, Silencing information regulator 2 related enzyme 1; NMN, nicotinamide mononucleotide; SGLT2, sodium-glucose cotransporter 2; NaCl, sodium chloride; Hes1, Hairy and enhancer of split homolog-1; PCs, principle cells; ICs, principle cell.

NAMPT expressed in mesangial cells, which regulates insulin secretion in pancreatic β-cells, while uncovered a decreased expression of insulin receptors in diabetic podocytes (95). Interestingly, a single-cell transcriptome profiling performed on BTBR ob/ob mice, which do not develop hypertension, showed those animals had no major changes in endothelial cell gene expression while surprisingly gave the vascular disease stereotype of diabetes (34). The researchers suggested that rather hypertension not diabetes induce transcriptional changes in endothelial cells given the prevalence of hypertension in patients with diabetes (34). They thought hypertension might be more important in injuring endothelial cells. However, there remains confusion since findings to date about kidney crosstalk much developed from researches set under the diabetic milieu. The extraglomerular crosstalks are summarized in **Table 2**.

DISCUSSION

scRNA-seq is a powerful tool providing unprecedented insight into cell transcriptome, including deciphering cell-to-cell communication in diseases such as DKD. With the aid of scRNA-seq, some new and complicated cellular interactive in DKD have been revealed as well as some new cell subpopulations have been identified in kidney, which imply some key regulators and therapeutic targets for DKD. Though the technology has achieved great advances, the researchers have to face several

challenges during seRNA-seq. The cell isolation protocol needs to be optimized to be more efficient when balancing between cell dissociative efficacy and viability since kidney has relatively dense matrix and some of kidney cells under abnormal conditions are susceptible to loss. The big discrepancy on kidney cell numbers and gene expressions gained in different kidney seRNA-seq researches by now is attributed much to their different dissociation protocols. The types of kidney cell identified in scRNA-seq researches primarily depend on the available cell markers, which may not be specific enough or even yet be revealed. Moreover, the huge volume of complex data generated by seRNA-seq needs appropriate analytical and statistical methods and the interpretation of raw data is determined by the choices of computational tools and databases. Of the most importance, the findings drawn from scRNA-seq need to be validated by subsequent experimental tests.

More researches of scRNA-seq coupled with multiomic approaches are expected in future to gain closer access to profound pathogenesis of DKD and contribute to develop new therapeutic strategies.

AUTHOR CONTRIBUTIONS

All literatures were reviewed by FY and CD. Materials were collected by YR, GL, YY, and ZY. The final manuscript was drafted by CD and FY, reviewed by FY and approved by all authors.

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