COMBATING DIABETES AND DIABETIC KIDNEY DISEASE

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COMBATING DIABETES AND DIABETIC KIDNEY DISEASE

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Editorial: Combating Diabetes and Diabetic Kidney Disease

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Editorial on the Research Topic

Combating Diabetes and Diabetic Kidney Disease

Diabetic kidney disease (DKD) is a leading cause of end-stage renal disease, resulting in more than 950,000 deaths each year globally (Thomas et al., 2015; Cooper and Warren, 2019). These patients carry a significantly increased risk of cardiovascular morbidity and mortality. The link between renal disease and cardiovascular disease is poorly understood and this knowledge gap contributes to the suboptimal treatment options available for these patients. Improved understanding of the pathogenesis of DKD and its association with the development of cardiovascular disease is urgently needed to catalyze the development of novel therapeutics and should be targeted to the early stages of these diseases, before kidney and/or cardiovascular damage becomes irreversible.

Currently approved therapeutic regimens include ACE inhibitors, angiotensin receptor blockers (ARBs), and statins which minimize, but do not prevent, the progression of cardiovascular morbidities and the incidence of ESRD (Srivastava et al., 2020a; Hartman et al., 2020). Moreover, these therapies are neither tissue- nor cell-specific and are ineffective in reversing kidney fibrosis and diabetic complications. In recent years, a number of reno-protective agents, including sodium glucose co-transporter (SGLT-2) inhibitors, mineralocorticoid receptor antagonists, endothelin A antagonists, dipeptidyl transferse-4 (DPP-4) inhibitors, and N-seryl-acetyl-lysyl-proline have been studied in both preclinical settings and in controlled clinical trials, some with promising outcomes (Kanasaki et al., 2014; Stavropoulos et al., 2018; Srivastava et al., 2020b). Still, more research is needed to validate their cell- and tissue-specific mechanisms to optimize their use in human disease. Understanding these critical pathways will guide future therapies to combat kidney fibrosis and cardiovascular complications in diabetes.

In this special issue of Frontiers in Pharmacology, we discuss new pathophysiologic mechanisms which are driving therapies to combat kidney fibrosis in diabetes. We focused on three major sections.

NEW LEADS TARGETED TO DKD

First, we discuss new leads targeted to DKD. In recent years SGLT-2 inhibitors are of significant importance in restoring kidney structure and fibrotic phenotypes in diabetes. SGLT-2 plays a key role in reabsorption of glucose filtered from the glomerulus. Nearly all (90–95%) filtered glucose in the urine is reabsorbed through SGLT2. The EMPA-REG trial demonstrated that the SGLT2 inhibitor empagliflozin reduced renal complications in high-risk diabetic patients and was also effective in patients with advanced kidney disease; this finding represents a key development which advances the clinical practice of diabetic medicine (Mayer et al., 2019). These researchers explain that the renal

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benefit of SGLT2 inhibition is based on hemodynamic alterations and the ability to lower blood glucose. However, SGLT2 inhibitors might also protect the kidneys from defective central metabolism as evidenced by their ability to mitigate abnormal glycolysis and improve lipid metabolism (Li et al., 2020b). In this issue, a meta-analysis and randomized clinical trials demonstrate the beneficial effect of SGLT-2 inhibitors on hemoglobin and hematocrit levels, suggesting that SGLT-2 inhibitors treatment may offer additional benefit in DKD (Qu et al.). Similarly, DPP-4 inhibitors such as linagliptin, and incretin analogs, which are known drugs for treatment of type II diabetes, are effective in improving kidney fibrosis in diabetes in preclinical settings. Various DPP-4 inhibitors have diverse effects in kidney health and are dependent on specific drug types and metabolic characteristics. Research led by Professor Kawanami discusses the beneficial effects and clinical efficacy of glucagon-like-peptide-1 (GLP-1) agonists in DKD (Kawanami and Takashi.). GLP-1 agonists have the potential to develop into a future class of medication for combating DKD. Another review article describes new therapeutic targets such as DPP-4, notch signaling, and sirtuins in DKD (Zoja et al.).

NEW CELLULAR MECHANISMS IN DKD

In recent years research by our group (Yale University, United States; Kanazawa Medical University, Japan) has focused on mesenchymal metabolic shifts that play a critical role in renal fibrosis (Li et al., 2020a; Srivastava et al., 2021a). Abrogation of both defective central metabolism and mesenchymal metabolic shifts through the use of small chemicals (glycolysis inhibitors and fatty acid oxidation activators) is effective in improving kidney structure and function (Kang et al., 2015; Srivastava et al., 2018). Glucocorticoid receptors (GR) are essential for endothelial cell homeostasis and regulate defective metabolism in endothelial cells. Endothelial GR regulates renal fibrogenesis by targeting Wnt signaling, defective fatty acid oxidation and associated mesenchymal activation in diabetic kidneys (Srivastava et al., 2021b). In this issue, the authors discuss new cellular mechanisms and cell signaling in the regulation of DKD pathogenesis. In this section, we describe the significance of mitochondrial control for the health and metabolism of the kidneys. Mitochondrial SIRT3 regulates cell-to-cell differentiation programs in kidney endothelial cells and its deficiency influences cellular *trans*-differentiation processes in neighboring cells, suggesting that SIRT3 is crucial for cellular homeostasis in diverse cell types in the kidney (Srivastava et al.). Sol et al., describe the importance of glomerular endothelial cells in sclerotic glomerular diseases such as focal segmental glomerulosclerosis and diabetic nephropathy (Sol et al.). Another article describes the differences in molecular and cellular mechanisms of ROCK1 and ROCK2 in DKD and discusses how targeting ROCK1 and ROCK2 have shown beneficial effects in treating other microvascular complications such as neuropathy and retinopathy (Matoba et al.). Sheng et al., describe the functional role of epidermal growth factor receptor (EGFR) in the development of DKD and discuss the therapeutic potential of EGFR inhibitors in the treatment of DKD (Sheng et al.). In brief, authors describe that the persistent activation of EGFR causes hemodynamic alterations, metabolic disturbances, inflammatory responses and parenchymal cellular dysfunction (Sheng et al.). Furthermore, an article describes the critical roles of FOXO1 in the regulation of cellular homeostasis and post-translational modifications (Wang et al.). The authors discuss how FOXO1 dysregulation contributes to the development of DKD and how improvement in FOXO1 dysregulation is associated with reversal of DKD phenotypes. Hence, FOXO1 is a potential therapeutic target in DKD (Wang et al.).

NON-CODING RNAS IN DKD

MiR-29 and miR-let-7 family clusters are the key antifibrotic microRNAs which are regulated by cross-talk mechanisms in endothelial cells, and this cross-talk regulation protects against endothelial-to-mesenchymal transition (Srivastava et al., 2016). Also, crosstalk regulation inhibits pro-fibrotic mechanisms (i.e. DPP-4 level and TGFβ signaling) and regulates health and disease processes of diverse type of kidneys cells. miR-29 and miR-let-7 family clusters require further exploration in diabetic nephropathy (Srivastava et al., 2019). In this issue, research led by Shi et al., adds further useful information about interactions between long-noncoding RNAs and microRNAs in endothelial cells (Shi et al.). Such interactions are physiologically important in renal health and disease processes under diabetic conditions, in which expression of LncRNA-H19 is higher and concomitantly inhibits the anti-mesenchymal and protective effect of miR-29a, resulting in more fibrosis. Under non-diabetic conditions, miR-29a binds to LncRNA-H19 and inhibits its profibrotic properties, resulting in less fibrosis. Moreover, lncRNAs-H19 function as sponges for miR-29a to regulate the expression of its target proteins. Another review describes new therapeutic strategies and the role of anti-fibrotic and pro-fibrotic microRNAs in DKD (Sakuma et al.). The authors discuss the antifibrotic roles of miR-29 and miR-let-7s and the pro-fibrotic roles of miR-21 and miR-214 in multiple dimensions of DKD. Further, Gu et al., add the functional importance of non-coding RNAs and discuss their potential as biomarkers in DKD (Gu et al.). Further research is needed to translate their potential to the clinical setting.

CONCLUSION

Diabetic kidney fibrosis is an important research topic for both clinicians and research scientists. In this special issue, we have discussed recent therapeutic advancements and new drug targets for combating kidney fibrosis and vasculopathy in diabetic nephropathy. We hope this special issue provides useful information for clinicians and basic science researchers to catalyze novel therapeutic approaches and future research directions.

AUTHOR CONTRIBUTIONS

SS has proposed the idea, conceptualized, contributed to writing, and provided intellectual input. KK provided intellectual output. JG performed final editing and provided intellectual output.

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Dexmedetomidine Enhances Autophagy *via* α2-AR/AMPK/mTOR Pathway to Inhibit the Activation of NLRP3 Inflammasome and Subsequently Alleviates Lipopolysaccharide-Induced Acute Kidney Injury

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Background: Acute kidney injury (AKI) is a severe complication of sepsis; however, no effective drugs have been found. Activation of the nucleotide-binding domain-like receptor protein 3 (NLRP3) inflammasome is a major pathogenic mechanism of AKI induced by lipopolysaccharide (LPS). Autophagy, a process of intracellular degradation related to renal homeostasis, effectively restricts inflammatory responses. Herein, we explored the potential protective mechanisms of dexmedetomidine (DEX), which has confirmed anti-inflammatory effects, on LPS-induced AKI.

Methods: AKI was induced in rats by injecting 10 mg/kg of LPS intraperitoneally (i.p.). Wistar rats received intraperitoneal injections of DEX (30 μg/kg) 30 min before an intraperitoneal injection of LPS. Atipamezole (ATI) (250 μg/kg) and 3-methyladenine (3-MA) (15 mg/kg) were intraperitoneally injected 30 min before the DEX injection.

Results: DEX significantly attenuated renal injury. Furthermore, DEX decreased activation of the NLRP3 inflammasome and expression of interleukins 1β and 18. In addition, autophagy-related protein and gene analysis indicated that DEX could significantly enhance autophagy. Finally, we verified the pharmacological effects of DEX on the 5′-adenosine monophosphate-activated protein kinase (AMPK)/mechanistic target of rapamycin (mTOR) pathway. Atip and 3-MA significantly reversed the protective effects of DEX.

Conclusions: Our results suggest that the protective effects of DEX were mediated by enhanced autophagy *via* the α_2-adrenoreceptor/AMPK/mTOR pathway, which decreased

activation of the NLRP3 inflammasome. Above all, we verified the renal protective effects of DEX and offer a new treatment strategy for AKI.

Keywords: acute kidney injury, dexmedetomidine, autophagy, NLRP3 inflammasome, α2-AR/AMPK/mTOR pathway

INTRODUCTION

Sepsis, a clinical syndrome that occurs in response to infection, is characterized by systemic hyperinflammation, dysregulation of the immune response, and multiple organ failure (Zarjou and Agarwal, 2011; Binkowska et al., 2015). Acute kidney injury (AKI) is one of the main effects observed with multiple organ dysfunction in patients suffering from sepsis; indeed, sepsis is responsible for 50% of all cases of AKI (Bagshaw et al., 2009; Plotnikov et al., 2018). The pathogenesis of severe AKI involves microcirculatory dysfunction, inflammation, and bio-energetic adaptive responses. In particular, inflammation plays an important role in the pathogenesis of AKI (Murugan et al., 2010; Zarbock et al., 2014). Because of its heterogeneous pathological processes, currently the only viable solution for AKI is renal replacement therapy (Gatward et al., 2008). However, the high price of renal replacement therapy and scarcity of kidney sources makes AKI a heavy burden to healthcare systems. At present, no drugs have been approved by the United States Food and Drug Administration for the treatment of AKI (Chen et al., 2019), and no optimal treatments are available for AKI resulting from sepsis (Wu et al., 2015). Thus, the mechanisms and treatment of AKI still need further elucidation.

Lipopolysaccharide (LPS) present within the cell wall of Gram-negative bacteria is one of the main causes of sepsis (Remick et al., 2000; Bhargava et al., 2013). As such, injection of LPS is widely used in animal studies to establish AKI models (Doi et al., 2009). LPS, a notable source of sepsis, plays an important role in the pathogenesis of AKI by causing excessive inflammatory responses and subsequent escalation of oxidative stress, renal hypoperfusion, and severe kidney injury (Chen et al., 2018). LPS can combine with toll-like receptor 4 (TLR4) to induce an intracellular response by recruiting transcription factors such as nuclear factor κB (NF-κB) in the nucleus, followed by secretion of chemokines and cytokines that regulate inflammatory processes and immune responses (Heinbockel et al., 2018). LPS is a typical member of pathogen-associated molecular patterns (PAMPs). The NLRP3 inflammasome functions as an innate sensor of several PAMPs and damage-associated molecular patterns (DAMPs), and acts an imperative mediator of inflammatory responses in various models of AKI (Shen et al., 2016). Previous studies reported a close relationship between the NLRP3 inflammasome and inflammatory responses during exacerbation of AKI (Kim et al., 2013; Wen et al., 2018). NLRP3 inflammasome activation is associated with caspase activation recruitment domain (ASC) and caspase-1, and promotes caspase-1 cleavage (Schroder and Tschopp, 2010). Activation of the NLRP3 inflammasome has been shown to regulate the maturation and excretion of inflammatory cytokines, especially interleukin 1β (IL-1β) and IL-18, leading to an inflammatory response (Sun et al., 2013). In addition, recent studies demonstrated a relationship between activation of the NLRP3 inflammasome and mitochondrial function (Deng et al., 2019). As mitochondrial membranes are involved in NLRP3 activation, the proximity of NLRP3 to mitochondria is a vital indicator of kidney injury (Lei et al., 2012). Some previous studies reported moderate renal-protective effects in NLRP3-knockout mice (Kim et al., 2013; Kim et al., 2018b). Therefore, the NLRP3 inflammasome is an important therapeutic target for preventing inflammatory responses associated with AKI.

Dexmedetomidine (DEX) is a selective α_2-adrenoreceptor (α_2-AR) agonist with sedative, analgesic, and anti-anxiety effects (Shen et al., 2017). In addition, several animal studies have noted antioxidant, anti-apoptosis, and anti-inflammatory effects of DEX, although some of the molecular pathways remain unclear (Kutanis et al., 2016; Wang et al., 2019). Many studies have demonstrated that DEX can decrease endotoxin-induced upregulation of inflammatory molecules and attenuate renal function associated with AKI (Lai et al., 2009; Liang et al., 2017; Qiu et al., 2018). In addition, recent studies have shown that DEX can decrease expression of the NLRP3 inflammasome and provide protective effects against renal injury (Kim et al., 2018a; Yin et al., 2018). However, the mechanism by which DEX downregulates the NLRP3 inflammasome to reduce inflammation has not been clearly identified.

Autophagy has been recognized as essential for maintaining cellular homeostasis and stress responses (Lenoir et al., 2016). Autophagy serves as a degradation system by which intracellular pathogens, damaged or long-lived proteins, and dysfunctional organelles are encased into autophagosomes and eliminated in lysosomes (Marino and Lopez-Otin, 2004; Wang et al., 2018a). Several studies have shown that autophagy can block activation of the NLRP3 inflammasome, subsequently inhibiting IL-1β and IL-18 (Shi et al., 2012b; Hong et al., 2019). Autophagy, which plays a protective role in the pathological processes of renal tubular injury, has been widely studied. The mechanistic target of the rapamycin (mTOR) pathway has been acknowledged as a key inhibitor of autophagy in response to a variety of intracellular disorders. As an upstream negative regulator of mTOR, 5′ adenosine monophosphate-activated protein kinase (AMPK) also plays a vital role in anti-inflammatory processes (White et al., 2015). However, it is unclear whether the mechanism by which DEX protects the kidney is related to autophagy. Hence, this potential relationship remains to be explored.

Our findings, which provide evidence that DEX has renal protective effects, explore the underlying mechanism by which

DEX enhances autophagy in response to AKI induced by sepsis. The results may provide a novel therapeutic strategy for AKI.

MATERIAL AND METHODS

Animals and Treatment

Thirty-six adult male Wistar rats were obtained from the Second Affiliated Hospital of Harbin Medical University (Harbin, China). Rats weighed 180-220g and were housed in a room that had a 12h light and dark cycle (lights on from 6:00-18:00) with temperature 20 ± 2°C and humidity 45%-55% for one week to adapt to the environment. Rats were divided randomly groups (3 per cage) and were fed ad libitum with standard food and fresh tap water. All experimental procedures in this study met the requirements of the Animal Experimental Committee of Northeast Agricultural University and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

The Wistar rats were randomly divided into six groups (n=6 per group):

1. CON group was injected intraperitoneally with saline.
2. CON+DEX group was injected intraperitoneally with DEX (30 μg/kg, American Pfizer).
3. LPS group was injected intraperitoneally with LPS (10 mg/kg, L2630-100MG, Sigma-Aldrich, USA) for 4h to establish the animal model of sepsis-induced AKI as described previously (Feng et al., 2019).
4. LPS+DEX group was injected intraperitoneally with DEX (30 μg/kg) 30 min before treatment with LPS (10 mg/kg).
5. LPS+DEX+ATI group was injected intraperitoneally with Atipamezole (ATI) (250 μg/kg, American Pfizer), an α2-receptor inhibitor, 30 min before treatment with DEX (30 μg/kg), and injected intraperitoneally with DEX 30 min before treatment with LPS (10 mg/kg).
6. LPS+DEX+3-MA group was injected intraperitoneally with 3-MA (15 mg/kg) 30 min before treatment with DEX (30 μg/kg), and injected intraperitoneally with DEX 30 min before treatment with LPS (10 mg/kg).

All rats initially received inhalation anesthesia with 1.5% isoflurane (Yipin Pharmaceutical, Co., Ltd., Hebei, China) and were sacrificed after 4h. Then, we collected blood, urine, and kidney samples.

Biochemical Indexes Analysis

The blood samples were collected quickly by heart puncture and kept at room temperature for 30 min. The serum was obtained by centrifugation at 3500 rpm for 10 min at 4°C from blood samples. The collected serum was measured for serum creatinine (Scr) and blood urea nitrogen (BUN) using a UniCel DxC800 Synchron (Beckman, USA). Urine samples were collected by bladder puncture for the analysis of kidney injury molecule-1 (KIM-1) using an ELISA kit (R&D Sytstems, Minneapolis, MN).

Histopathological Analysis

For analysis, kidneys were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5μm thickness sections. The sections were stained with hematoxylin and eosin stain. Images of stained tissues were visualized and captured using a light microscopy (BX-FM; Olympus Corp, Tokyo, Japan). The kidney histological scores were quantified by ten renal cortex regions from every section (400x magnification). The percentages of tubules that showed tubular dilatation and vacuolization, interstitial edema, brush border defect, and inflammatory cell infiltration were scored as follows: 0 = none, 1 = 0 - 20%, 2 = 20 - 50%, 3 = 50 - 70%, 4 = more than 70% (Brooks et al., 2009). And observers used a double-blinded approach to evaluate scores.

Immunohistochemistry Analysis

The 3μm thick paraffin-embedded kidney sections were dewaxed, and then dehydrated using graded concentrations of alcohol. To inhibit endogenous peroxidase, the sections were incubated with 3% H_2O_2. The sections were microwaved in citric acid for 15 min, and then treated with goat serum for 15 min at room temperature. Afterwards, the sections were incubated in blocking solution with primary antibody at 4°C overnight. After washing with PBS 3 times, the secondary antibody was added and immunostaining was performed using a DAB horseradish peroxidase color development kit (Beyotime, China), and then sections were counterstained with hematoxylin and made transparent with xylene. Finally, sections were observed with the PD37 type microscope (Olympus,Japan). Under 400 × magnification, pictures were taken in 5 random fields. Primary antibodies were used at the following dilutions: IL-1β diluted 1:100 (WL02257, Wanlei, Shenyang, China); IL-18 diluted 1:100 (WL01127, Wanlei, Shenyang, China).

ELISA Assay

The levels of IL-1β (H002) and IL-18 (H0015) in serum were detected with an ELISA kit according to the manufacturers' instructions (Nanjing Biotechnoloy Co., Ltd., Nanjing, China).

Real-Time Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was isolated from the kidney tissue with Trizol reagent (Invitrogen, Carlsbad, CA, American) according to the manufacturer's instructions, and reverse transcribed into cDNA using the PrimeScript RT reagent kit (DRR037A; Takara, Dalian, China). Then, quantitative real-time PCR detection of RNA copies were performed on a Light Cycler® 480 II Detection System (Roche) using IQ SYBR Supermix reagent (Bio-Red, San Diego, CA). The relative expression levels were normalized to GAPDH and analyzed by the $2^{-\Delta\Delta Ct}$ method. The primers for the detection of target mRNA are listed in **Table 1**.

Western Blot

Frozen kidney tissues were cut into small pieces and lysed with RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China). Phenylmethanesulfonyl fluoride (PMSF) (Beyotime

TABLE 1 | Primer sequence in this study.

Genes	Sequence (5′ - 3′)
p62	(F) CCCGTCTACAGGTGAACTCC
	(R) CTGGGAGAGGGACTCAATCA
Beclin1	(F) GTTGCCGTTATACTGT
	(R) TTTCCACCTCTTCTTTGA

Biotechnology, Shanghai, China) was added, and the tissue was homogenized through a Tissue Grinding instrument (Shanghai Jingxin Industrial Development Co., Ltd., Shanghai, China), and then centrifuged at 3000 rpm for 10 min at 4°C to collect the supernatant. Protein concentrations were quantified by a BCA Protein Assay kit (Beyotime Biotechnology, Shanghai, China). Equal amounts of protein sample were separated by standard Tris-glycine SDS-PAGE gel electrophoresis, and then transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% skimmed milk for 2h at room temperature, the PVDF membranes were incubated with primary antibodies at 4°C overnight. Primary antibodies and dilutions were as follows: AMPKα (WL02254, Wanlei, Shenyang, China) diluted 1:500; p-AMPKα2(Ser173) (bs-5575R, Bioss, Beijing, China) diluted 1:1000; mTOR (A2245,ABclonal,Wuhan,China) diluted 1:1000; p-mTOR (Ser2448) (AP0094, ABclonal, Wuhan, China) diluted 1:1000; LC3 (A5202, Bimake, Houston, American) diluted 1:1000; Beclin (D40C5, Cell Signaling Teghnology, American) diluted 1:1000; p62 (WL02385, Wanlei, Shenyang, China) diluted 1:500; NLRP3 (WL02635, Wanlei, Shenyang, China) diluted 1:1500, IL-1β (WL02385, Wanlei, Shenyang, China) diluted 1:500; IL-18 (WL01127, Wanlei, Shenyang, China) diluted 1:1000; caspase-1 (WL02996a, Wanlei, Shenyang, China) diluted 1:750; cleaved-caspase-1 (WL03450, Wanlei, Shenyang, China); ASC (A11433, ABclonal, Wuhan, China); GAPDH (WL01114, Wanlei, Shenyang, China). After washing five times with Tris-buffered saline containing Tween (TBST), the membranes were incubated with 1: 20000 horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (ZB-2301, ZSGB-BIO, Beijing, China) or anti-mouse IgG secondary antibody (ZB-2305, ZSGB-BIO, Beijing, China) at room temperature for 2h and then washed with TBST, followed by development using ECL reagent (WLA003, Wanlei, Shenyang, China), captured by the Amersham Imaher 600 software (GE, American), and analyzed using Image J software.

Transmission Electron Microscopy

The number of autolysosome and ultrastructural changes were detected by transmission electron microscopy. Kidney tissues were cut into about 1 mm × 1 mm × 1 mm pieces and placed in 4% glutaraldehyde at 4°C for 12h. The samples were post-fixed in 1% osmic acid for 90 min and washed by 0.1M PBS 3 times for 15 min each. After that, the samples were dehydrated in a graded series of ethanol (50%, 70%, 90%, 100%) and embedded in epoxy resin. The ultrathin sections were prepared and then stained with uranyl acetate and lead citrate. The sections were observed with transmission electron microscopy (Hitachi HT7700, Tokyo, Japan).

Immunofluorescence Staining

The 3μm thick paraffin sections were deparaffined, rehydrated, and prepared for immunofluorescence assays according to a standard protocol. The sections were incubated with primary antibodies as follows: anti-NLRP3 (WL02635, Wanlei, Shenyang, China) diluted 1:200 and anti-TOM20 (A19403, ABclonal, Wuhan, China) diluted 1:100 overnight at 4°C. After being washed with PBS 3 times, the sections were incubated with secondary antibody. The sections were washed with PBS 3 times again and then sealed with coverslips. Fluorescence images were acquired with a Nikon Eclipse Ni inverted microscope (TE2000; Nikon, Tokyo, Japan).

Statistical Analysis

Data were expressed as mean ± SD (standard deviation). All statistical analyses were performed using the PASW statistics 18 software (SPASS, IL, USA). Comparisons among multiple groups with measurement data obeying normal distribution were conducted using one-way analysis of variance (ANOVA), and comparisons between two groups were made using the least square method (LSD). Graphs were made using GraphPad Prism5 (San Diego, California). $p < 0.05$ was considered statistically significant. Statistical differences were considered to be extremely significant when $p < 0.01$.

RESULTS

DEX Improved Renal Function in Rats With Sepsis

To investigate whether DEX improved the kidney function of rats with sepsis, we assessed levels of renal function indicators: blood urea nitrogen (BUN, **Figure 1D**), creatinine (CRE, **Figure 1E**), and kidney injury molecule-1 (KIM-1, **Figure 1F**). All three indicators were significantly increased in the LPS group compared with the control (CON) group. However, treatment with DEX significantly decreased levels of all three markers, indicating that DEX improved the renal function of rats with sepsis. In addition, treatment with the α_2-AR inhibitor ATI or autophagy inhibitor 3-MA abolished the protection elicited by DEX against sepsis-induced renal dysfunction. The observed insignificant difference between CON and CON+DEX groups suggested that DEX had no effect on normal rats.

DEX Ameliorated Pathology in Rats With Sepsis

To determine the impact of DEX on renal tissue injury, we detected the pathological changes in the kidney by microscopy (**Figures 1B, C**). Normal kidney structures were observed in the CON group. After LPS injection, kidney tissues displayed renal tubular epithelial cell vacuolar degeneration, renal tubular cavity expansion, hemorrhage, and infiltration of intertubular inflammatory cells. However, DEX ameliorated this pathological damage. Furthermore, ATI and 3-MA reversed the effects of DEX.

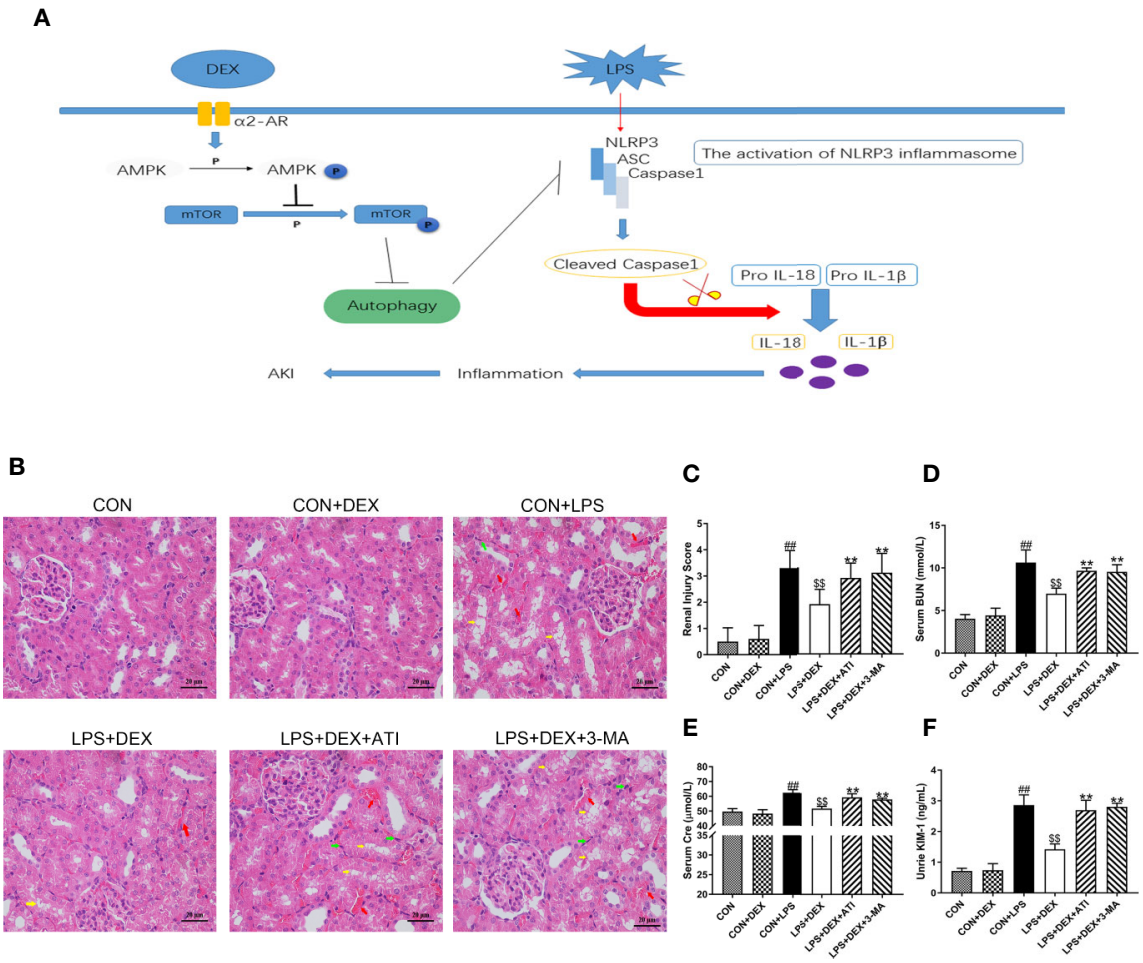

FIGURE 1 | DEX improved renal damage induced by LPS-induced AKI. **(A)** Sepsis-induced AKI is established by intraperitoneally injecting LPS (10mg/kg) into rats. The activation of NLRP3 inflammasome caused inflammatory responses that led to renal injury. DEX enhances autophagy through the α2-AR/AMPK/mTOR pathway to inhibit inflammation and protect the kidney. **(B)** Represented images of H&E staining (× 400) in the renal cortex. Red arrow indicates hemorrhage, yellow arrow indicates vacuolar degeneration, and black arrow indicates infiltration of intertubular inflammatory cells. Scale bars = 20μm. **(C)** The histopathological score of kidney damage. **(D)** The level of serum BUN in rats. **(E)** The level of serum Cre in rats. **(F)** The level of urine KIM-1 in rats. Data are expressed as mean ± SD (n = 6). $^{\#\#}p <$ 0.01 compared with CON group. $^{\$\$}p < 0.01$ compared with CON+LPS group. $^{**}p < 0.01$ compared with LPS+DEX group. CON: control; DEX: dexmedetomidine; LPS: lipopolysaccharide; ATI: atipamezole; 3-MA: autophagy inhibitor.

DEX Ameliorated Inflammatory Response by Reducing NLRP3 Inflammasome and Inflammatory Cytokines in Rats With Sepsis

To determine whether sepsis was successfully established, we examined changes in serum levels of inflammatory factors (**Figures 2I, J**). Enzyme-linked immunosorbent assay results revealed significantly upregulated serums levels of IL-1β and IL-18 level in response to LPS, while DEX obviously ameliorated these changes. However, ATI and 3-MA reversed the effect of DEX. By further evaluating the inflammatory response of renal tissue (**Figures 2A–H**), we found that LPS significantly increased expression of IL-1β, IL-18, NLRP3, ASC, caspase-1, and cleaved-caspase-1, which were all downregulated by DEX. Moreover, ATI and 3-MA could eliminate the effects of DEX. Immunohistochemical analysis to confirm the localization of inflammatory cytokines in the kidney tissue indicated the presence of IL-18 and IL-1β near the renal tubule, as well as significant increases in LPS, LPS+DEX+ATI, and LPS+DEX+3-MA groups. However, DEX could reverse these changes. Moreover, according to calculated IOD values, immunohistochemical results were consistent with western blot results (**Figures 3B–E**). To evaluate the localization of NLRP3 and mitochondria, NLRP3 and the mitochondrial membrane protein TOM20 were stained for co-immunofluorescence microscopy. The results showed that LPS increased NLRP3 expression, whereas DEX decreased NLRP3 expression, and ATI and 3-MA reversed the effect of DEX. Moreover, NLRP3 clearly localized with mitochondria (**Figure 3A**).

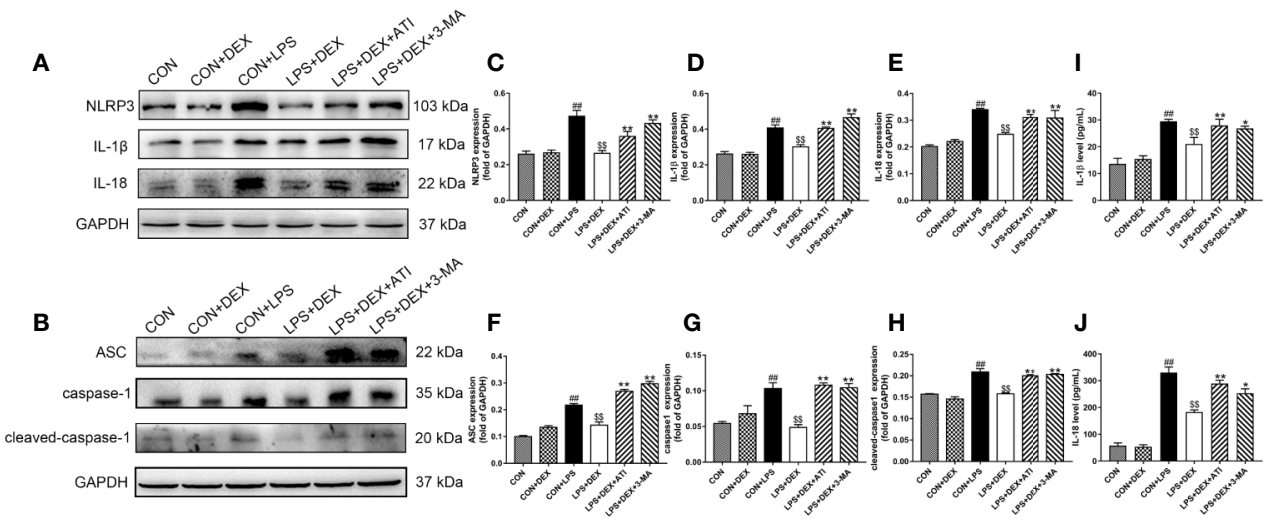

FIGURE 2 | DEX ameliorates inflammatory responses in kidney tissue and serum induced by LPS. **(A)** Protein levels of NLRP3, IL-1β, and IL-18. **(B)** Protein levels of ASC, caspase-1, cleaved-caspase-1. **(C–E)** The protein expression of NLRP3, IL-1β, and IL-18 were normalized to the level of GAPDH protein. (**F–H)** The protein expression of ASC, caspase-1, cleaved-caspase-1 were normalized to the level of GAPDH protein. **(I)** The content of IL-1β in serum. **(J)** The content of IL-18 in serum. Data are expressed as mean ± SD (n=6). $^{\#\#}p < 0.01$ compared with CON group. $^{\$\$}p < 0.01$ compared with CON+LPS group. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with LPS+DEX group. CON: control; DEX: dexmedetomidine; LPS: lipopolysaccharide; ATI: atipamezole; 3-MA: autophagy inhibitor.

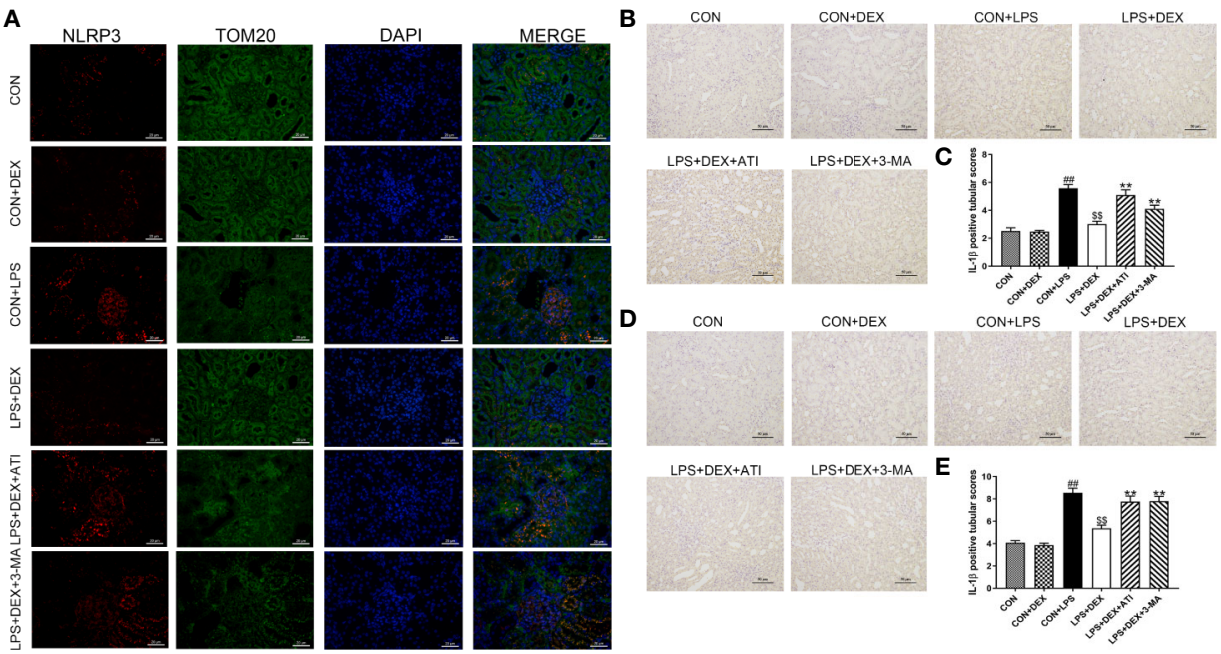

FIGURE 3 | (A) The positive expression of NLRP3 in kidney tissue was observed by immunofluorescence staining under a laser scanning confocal microscope. The NLRP3 cells were marked in red, the mitochondrial membranes were stained with TOM20 in green, and the nuclear was stain with DPAI in blue. Scale bars = 50 μm. **(B)** Immunohistochemistry analysis of IL-1β in kidney tissue. **(C)** Quantitative analysis of IL-1β in kidney tissue. **(D)** Immunohistochemistry analysis of IL-18 in kidney tissue. **(E)** Quantitative analysis of IL-18 in kidney tissue. Data are expressed as mean ± SD (n=6). $^{\#\#}p < 0.01$ compared with CON group. $^{\$\$}p < 0.01$ compared with CON+LPS group. $^{**}p < 0.01$ compared with LPS+DEX group. CON: control; DEX: dexmedetomidine; LPS: lipopolysaccharide; ATI: atipamezole; 3-MA: autophagy inhibitor.

DEX Ameliorate LPS-Induced NLRP3 Inflammasome Activation by Regulating Autophagy

To confirm the role of autophagy in DEX-regulated NLRP3 activation, we examined the autophagy-related proteins microtubule-associated protein light chain 3 (LC3), beclin-1, and p62. Our results showed that LPS decreased the expression of LC3-II and beclin-1, but increased expression of p62. With DEX intervention, the LC3-II/LC3-I ratio and expression of beclin-1 were significantly increased, while expression of p62 was decreased. However, ATI and 3-MA could suppress the effects of DEX, as they reduced the LC3-II/LC3-I ratio and beclin-1 expression, and increased p62 expression (**Figures 4A–D**). Immunohistochemical analysis of LC3, beclin-1, and p62 indicated expression levels consistent with western blot results. Moreover, autophagy was observed to occur near the renal tubule (**Figure 4G**). Ultrastructural observations of the kidney tissue indicated that LPS caused a large number of pathological changes, such as shrunken nuclei, mitochondrial disruption, and fuzzy mitochondrial cristae. DEX ameliorated these pathological changes and increased the number of autolysosomes compared with the LPS group. Using the autophagy inhibitor 3-MA, we observed obvious decreases in the number of autolysosomes, which were replaced by intracellular damage. Intervention with ATI produced the same effects as 3-MA (**Figure 4H**). Upon measuring transcription levels, we found that LPS significantly decreased LC3 and beclin-1 mRNA expression. After DEX treatment, LC3 and beclin-1 mRNA expression were obviously upregulated. However, both 3-MA and ATI could suppress the effect of DEX (**Figures 4E, F**).

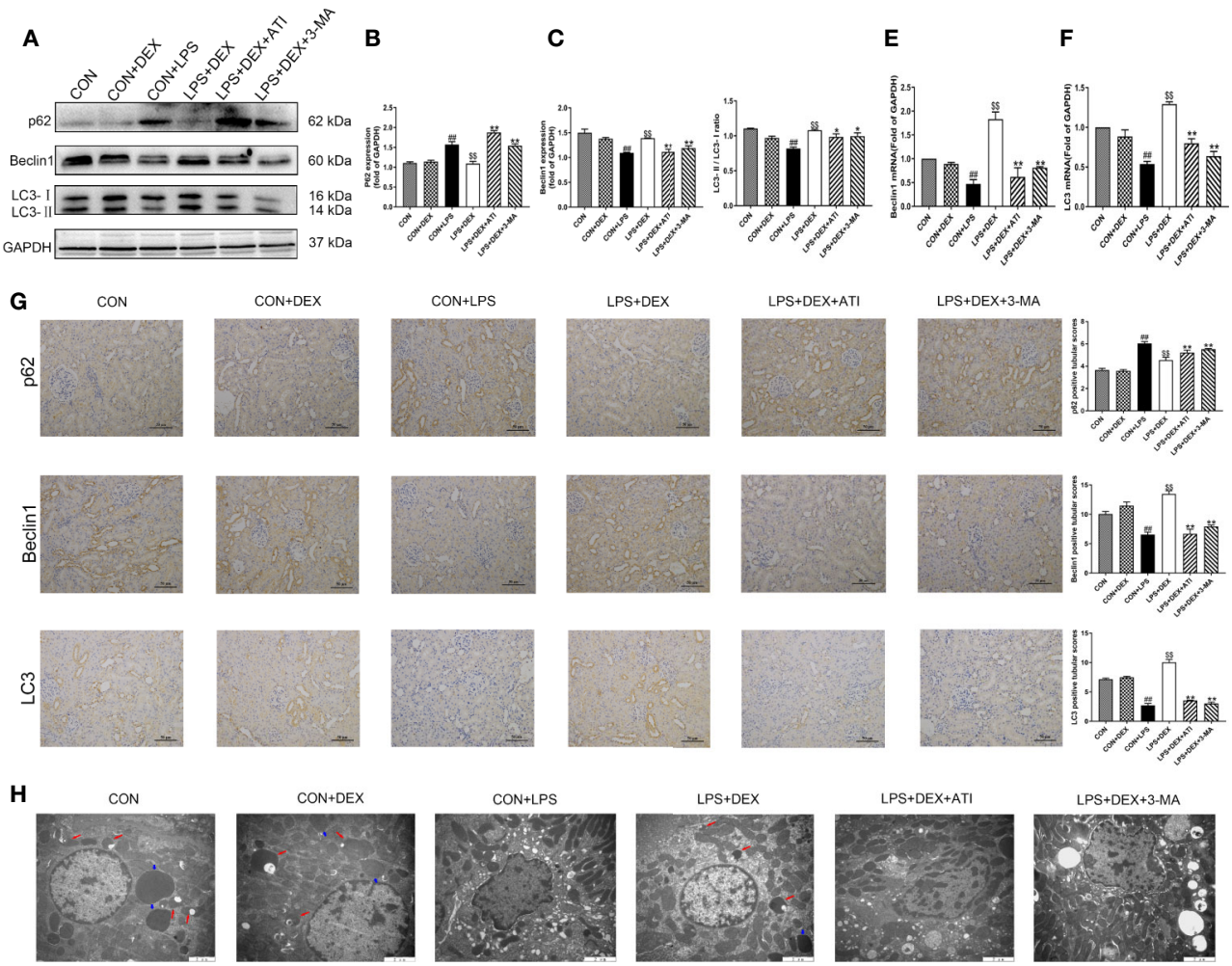

FIGURE 4 | DEX enhanced autophagy. **(A)** Protein levels of p62, Beclin1, LC3. **(B–D)** The protein expression of p62, Beclin1, LC3 were normalized to the level of GAPDH protein. **(E)** mRNA expression of Beclin1. **(F)** mRNA expression of LC3. **(G)** Immunohistochemistry analysis and quantitative analysis of p62, Beclin1, LC3 in kidney tissue. **(H)** Represented ultrastructure by transmission electron microscopy in kidney tissue. Red arrow indicated autolysosome and blue arrow indicated lysosome. Scale bars = 2 μm. Data are expressed as mean ± SD (n=6). $^{\#\#}p < 0.01$ compared with CON group. $^{\$\$}p < 0.01$ compared with CON+LPS group. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with LPS+DEX group. CON: control; DEX: dexmedetomidine; LPS: lipopolysaccharide; ATI: atipamezole; 3-MA: autophagy inhibitor.

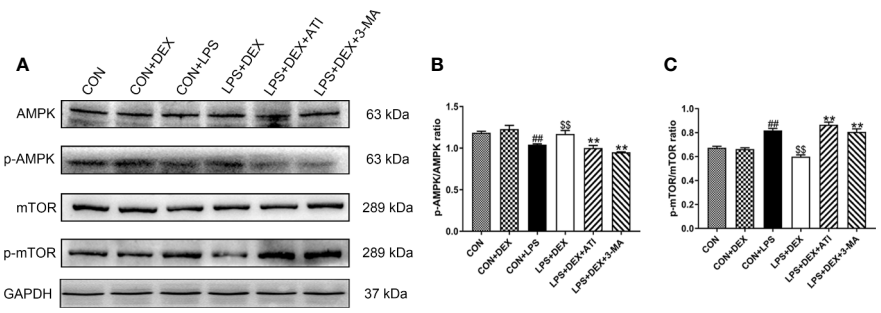

FIGURE 5 | Dex enhanced autophagy *via* AMPK/mTOR pathway. **(A)** Protein levels of AMPK, p-AMPK, mTOR and p-mTOR. **(B)** The protein expression of p-AMPK was normalized to the level of AMPK protein. **(C)** The protein expression of p-mTOR was normalized to the level of mTOR protein. Data are expressed as mean ± SD (n=6). $^{\#\#}p < 0.01$ compared with CON group. $^{\$\$}p < 0.01$ compared with CON+LPS group. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with LPS+DEX group. CON: control; DEX: dexmedetomidine; LPS: lipopolysaccharide; ATI: atipamezole; 3-MA: autophagy inhibitor.

DEX Ameliorate LPS-Induced NLRP3 Inflammasome Activation by Enhancing Autophagy *via* AMPK/mTOR Pathway

To verify the correlation between the AMPK/mTOR pathway and the pharmacological effects of DEX, we examined expression of AMPK, phosphorylated AMPK (p-AMPK), mTOR, and p-mTOR (**Figures 5A–C**). Our western blot results showed that after treatment with LPS, expression of p-AMPK tended to decrease while expression of p-mTOR increased. However, DEX reversed these effects of LPS, and ATI and 3-MA could block the effects of DEX.

DISCUSSION

AKI complicated by sepsis is a clinical syndrome associated with high mortality and morbidity. As a lack of optimal treatments is responsible for these results, effective treatments are urgently needed. In the present study, we first verified the establishment of our AKI model and the protective effects of DEX in the kidney. Next, we examined the inflammatory response induced by activation of the NLRP3 inflammasome. We then confirmed that NLRP3 inflammasome activation and inflammatory cytokines could be inhibited by DEX-enhanced autophagy. Finally, we determined that DEX enhanced autophagy *via* the α2-AR/AMPK/mTOR pathway.

AKI induced by sepsis is closely associated with excessive inflammatory responses and severe renal impairment. Indeed, the kidney is one of the earliest and most frequently affected organs during sepsis (Bagshaw et al., 2008; Jin et al., 2020). Therefore, early intervention can prevent further exacerbation of AKI and more serious damage caused by sepsis. According to previous studies, an early stage of AKI was established 4 h after intraperitoneal injection (Tunctan et al., 2018; Feng et al., 2019). Histopathological and biochemical analyses are classical techniques to evaluate kidney function. With LPS intervention, we observed infiltration of intertubular inflammatory cells, vacuolar degeneration of the tubular lining epithelium, tubular dilatation, and hemorrhaging. Biochemical indicators also reflected renal dysfunction, including significantly increased levels of BUN, CRE, and KIM-1. KIM-1 reportedly regulates renal function recovery and tubular degeneration, and thus serves as an indicator of tubular injury (Ichimura et al., 2008). Our results show that AKI can be initiated by LPS stimulation. However, pretreatment with DEX prevented the occurrence of disorders both pathologically and biochemically. Similar to the results of a previous study (Feng et al., 2019), our findings demonstrate that DEX exerted a renal protective capacity against AKI induced by LPS. However, potential underlying mechanisms still need to be explored.

LPS-induced inflammatory responses are the cause of severe renal dysfunction (Gomez et al., 2014). As a starting point for treatment, inhibition of inflammatory responses may be an effective strategy for sepsis-induced AKI. A previous report indicates that LPS can increase NLRP3 activation in AKI animal models (Chunzhi et al., 2016). Recently, more extensive studies demonstrated that activation of the NLRP3 inflammasome mediates maturation and secretion of IL-1β and IL-18, a process that prominently contributes to AKI (Shen et al., 2016; Sogawa et al., 2018). As an important proinflammatory factor, IL-1β not only stimulates the kidney to produce aggressive inflammatory responses, but can cause severe renal re-absorption disorders (Wang et al., 2015; Schett et al., 2016; Liu et al., 2019a). As a marker, IL-18 is more than 90% sensitive and specific to diagnosed AKI, and high expression of IL-18 can eventually lead to tubular damage (Parikh et al., 2004; Shi et al., 2012a). Our results showed that LPS can promote NLRP3 inflammasome activation, as well as IL-1β and IL-18 expression. Furthermore, serum levels of IL-1β and IL-18 indicated the presence of a widespread inflammatory response. A previous study confirmed that damage associated with LPS-induced AKI occurred in renal tubular epithelial cells (Li et al., 2019; Lu et al., 2019), consistent with our immunohistochemistry results. Thus, stimulation with LPS can induce activation of the NLRP3 inflammasome and promote an excessive inflammatory response in the kidney. Other previous studies indicated that mitochondrial dysfunction also plays a vital role in NLRP3

inflammasome activation. Both mitochondrial reactive oxygen species and membrane proteins are involved in activation of the NLRP3 inflammasome (Kim et al., 2018b; Chung et al., 2019). In our study, we observed that NLRP3 localized with TOM20, a mitochondrial protein, as well as ultrastructural damage in mitochondria. These results suggest a close relationship between NLRP3 and mitochondria. However, the process of NLRP3 inflammasome activation needs further exploration. Regardless, DEX pretreatment could inhibit activation of the NLRP3 inflammasome and largely alleviate the inflammatory response induced by LPS.

Autophagy, a highly dynamic process of intracellular degradation, is closely related to the elimination of damaged proteins and dysfunctional organelles (Marino and Lopez-Otin, 2004). Accumulating evidence indicates that NLRP3 inflammasome activation is inhibited by enhanced autophagy (Wong et al., 2018; Torp et al., 2019), which can also reduce inflammatory cytokines associated with LPS-induced AKI (Zhao et al., 2019). LC3 protein is imperative for initiating the formation of autophagosomal membranes. LC3-II arises from a combination of LC3-I and phosphatidyl ethanolamine upon initiation of autophagy (Li et al., 2020). The ratio of LC3-II/LC3-I expression, indicating the conversion of LC3-I to LC3-II, is a crucial indicator of autophagy (Chen et al., 2010). In addition, beclin-1 is essential for regulating autolysosome formation (Deretic et al., 2013). Expression of p62 protein, an autophagy adapter protein that binds to ubiquitinated protein aggregates and LC3-II (Guo et al., 2020), is contrary to that of LC3 and beclin-1. Surprisingly, the autophagy response to LPS in our study differed from previous studies. The low autophagy level presented in our study was consistent with Radovan Vasko's study (Vasko et al., 2013). However, after LPS intervention, this lack of autophagy enhanced NLRP3 inflammasome activation and inflammatory cytokine expression; perhaps this dosage of LPS destroys autophagy. Although the reason for this discrepancy in results is unclear, it may be caused by differences in experimental models. Autophagy-related genes LC3 and beclin-1 were decreased after LPS intervention; thus, the effect of LPS on autophagy at a transcriptional level was confirmed. LPS pretreatment could obviously suppress autophagy and induce intracellular injury. However, DEX significantly restored autophagy in our study, consistent with the results of Oh et al. (2019). According to these results, we confirmed that DEX can restore the lack of autophagy induced by LPS.

It is difficult to determine whether the observed reductions in injury elicited by DEX were related to its role in increasing autophagy. To solve this puzzle, we selected the autophagy inhibitor 3-MA to verify this relationship. As a class-III phosphoinositide 3 kinase inhibitor, 3-MA is widely used to inhibit autophagy, frequently at a dosage of 15 mg/kg (Wu et al., 2015; Bao et al., 2018; Zhao et al., 2019). Under the influence of 3-MA, autophagy was significantly decreased, whereas renal injury and expression of inflammatory cytokines, such as NLRP3, IL-1β, and IL-18, were increased. Hence, autophagy can alleviate LPS-induced renal injury by downregulating the inflammatory response elicited by NLRP3 inflammasome activation. The dosage of atipamezole (250 μg/kg), a complete α_2-AR antagonist, was based on previous studies in which DEX protected the kidney (Si et al., 2013; Li et al., 2018; Qiu et al., 2018). In the present study, ATI was used to confirm that DEX exerts its pharmacological role through the α_2-AR. However, we were still perplexed with regard to the potential mechanism by which DEX upregulates autophagy.

According to previous studies, mTOR is one of the most important negative regulators of autophagy. In addition, AMPK can reportedly upregulate autophagy by suppressing mTOR phosphorylation. The AMPK/mTOR signaling pathway has emerged as a crucial regulator of autophagy (Kim et al., 2011; Kim et al., 2016). AMPK activation generally plays a protective role in various renal injury models (Allouch and Munusamy, 2017; Bao et al., 2019; Liu et al., 2019b). Recently, DEX has been confirmed to exert protective effects by activating AMPK and suppressing inflammatory responses (Wang et al., 2018b). Our results indicate that DEX pretreatment can upregulate AMPK phosphorylation and suppress mTOR phosphorylation. Above all, these results indicate that DEX can enhance autophagy through the AMPK/mTOR pathway in acute kidney injury induced by LPS.

In summary, our results suggest an effective role of DEX in protecting against LPS-induced AKI *via* inhibited inflammation. This study also provides evidence that the inflammatory response induced by NLRP3 inflammasome activation can be significantly reduced by autophagy. Finally, we confirmed that DEX enhances autophagy *via* the α_2-AR/AMPK/mTOR pathway to inhibit activation of the NLRP3 inflammasome and subsequently alleviates LPS-induced AKI.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Experimental Committee of Northeast Agricultural University.

AUTHOR CONTRIBUTIONS

HF and TY contributed to the conception and design of the study. YZ, TY, and HY conducted experiments. TY organized the database. MW performed the statistical analysis. TY wrote the first draft of the manuscript. XF and HZ wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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GLP-1 Receptor Agonists in Diabetic Kidney Disease: From Clinical Outcomes to Mechanisms

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Diabetic Kidney Disease (DKD) is the leading cause of end stage renal disease (ESRD) worldwide. Glucagon-like peptide 1 receptor agonists (GLP-1RAs) are now widely used in the treatment of patients with type 2 diabetes (T2D). A series of clinical and experimental studies demonstrated that GLP-1RAs have beneficial effects on DKD, independent of their glucose-lowering abilities, which are mediated by natriuresis, anti-inflammatory and anti-oxidative stress properties. Furthermore, GLP-1RAs have been shown to suppress renal fibrosis. Recent clinical trials have demonstrated that GLP-1RAs have beneficial effects on renal outcomes, especially in patients with T2D who are at high risk for CVD. These findings suggest that GLP-1RAs hold great promise in preventing the onset and progression of DKD. However, GLP-1RAs have only been shown to reduce albuminuria, and their ability to reduce progression to ESRD remains to be elucidated. In this review article, we highlight the current understanding of the clinical efficacy and the mechanisms underlying the effects of GLP-1RAs in DKD.

Keywords: diabetic kidney disease, diabetic nephropathy, GLP-1 receptor agonists, liraglutide, semaglutide, dulaglutide

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INTRODUCTION

Diabetic kidney disease (DKD) is a global concern because it causes end stage renal disease (ESRD) and affects mortality in diabetic patients. The inhibition of the onset and progression of DKD is an urgent issue, and the development of therapeutic approaches against DKD is required. Furthermore, DKD is an established risk factor for cardiovascular disease (CVD) (Rawshani et al., 2018). Thus, anti-diabetic agents that can attenuate both DKD and CVD have been awaited. A recent meta-analysis demonstrated that SGLT2 inhibitors and glucagon-like 1 receptor agonists (GLP-1RAs) have favorable effects on the cardiorenal outcomes in type 2 diabetes (T2D) (Giugliano et al., 2019; Kristensen et al., 2019). GLP-1RAs improve glucose metabolism by increasing glucose-dependent insulin secretion and suppress the release of glucagon. They have also been shown to have beneficial effects on cardiovascular (CV) risk factors by improving obesity, hypertension, and the lipid profile (Drucker, 2018). Recent CV outcome trials utilizing GLP-1RAs have also investigated renal outcomes. In addition, the elucidation of basic mechanisms underlying the renoprotective effect of GLP-1RAs is progressing. In this review article, we

discuss the current understanding of the renoprotective effects of GLP-1RAs from clinical and mechanistic standpoints.

THERAPEUTIC TARGETS IN DIABETIC KIDNEY DISEASE

DKD is a risk factor for both ESRD and CVD. Intensive glycemic control is effective for preventing the onset and progression of the early-middle stage of DKD. However, its usefulness for progressed DKD and established CVD remains unclear. Recent clinical trials demonstrated that SGLT2 inhibitors and GLP-1RAs bring beneficial effects on the cardiorenal outcomes of T2D subjects who are at high risk for CVD. T2D patients with severe renal impairment are not eligible for SGLT2 inhibitors, and GLP-1RAs could be an important therapeutic option for these patients. DKD is developed by glucose-dependent and -independent mechanisms, including oxidative stress and inflammation. GLP-1RAs have been shown to have beneficial effects on these factors.

GLP-1RAS AND RENAL OUTCOMES

Accumulating clinical evidence demonstrates that GLP-1 RAs have beneficial effects on renal outcomes. The results of major trials are summarized in **Table 1**.

Liraglutide

In an observational study, 52 weeks of liraglutide treatment was shown to increase the glomerular filtration rate (GFR) (5.4 ml/min/1.73 m^2) and reduce albuminuria by 50% in overweight T2D patients with stage 3 CKD (De Lucas et al., 2017). A small size randomized controlled trial (RCT) demonstrated that treatment with liraglutide (1.8 mg) for 12 weeks resulted in a reduction of albuminuria by 32% in T2D patients (Von Scholten et al., 2017). In the Satiety and Clinical Adiposity–Liraglutide Evidence (SCALE) Diabetes trial, a total of 846 overweight and obese patients with T2D were randomly assigned to receive 3.0 or 1.8 mg of liraglutide or placebo for 56 weeks. At the end of the study period, the reductions in the albumin-to-creatine ratios (UACR) of the liraglutide (3.0 mg), liraglutide (1.8 mg), and placebo groups were 18.36, 10.79, 2.34%, respectively (Davies et al., 2015). In the LIRA-RENAL trial, 279 T2D subjects with moderate renal impairment [estimated glomerular filtration rate (eGFR) 30–59 ml/min/1.73 m^2] were randomly assigned to receive liraglutide (1.8 mg) or placebo for 26 weeks. However, liraglutide treatment failed to show significant improvement of the UACR and eGFR trajectory in comparison to placebo (Davies et al., 2016).

The Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) study assessed the CV outcome of liraglutide (1.8 mg) in comparison to placebo (Marso et al., 2016b). A total of 9340 participants with a high CV

TABLE 1 | Clinical effects of GLP-1RAs on DKD.

Trial	Agents, Follow-up Duration	Subjects	Renal Outcomes	Results
LEADER (n=9,340)	Liraglutide (1.8 mg) vs. placebo, 3.84 years	T2D with high CV risk	New-onset macroalbuminuria, doubling of the serum creatinine level, ESRD, renal death	HR 0.78 (95% CI: 0.67-0.92)
SUSTAIN-6 (n=3,297)	Semaglutide (0.5 mg, 1.0 mg) vs. placebo, 104 weeks	T2D Age >50 with established CVD or CKD stage 3-5 Age >60 with CV risk factors	New or worsening of nephropathy (persistent macroalbuminuria, doubling of the serum creatinine level and CCr < 45 mL/min/1.73 m2, RRT)	HR 0.64 (95% CI: 0.46-0.88)
REWIND (n=9,901)	Dulaglutide (1.5 mg) vs. placebo, 5.4 years	T2D with a previous CV event or CV risk factors	New onset of macroalbuminuria, sustained eGFR decline (≥30%) or RRT	HR 0.85 (95% CI: 0.77-0.93)
AWARD-7 (n=576)	Dulaglutide (0.75 mg, 1.5 mg) vs. placebo, 52 weeks	T2D with moderate to severe CKD (stage 3-4)	Changes in eGFR decline and UACR from baseline	eGFR decline: -1.1 (1.5 mg), -1.5 (0.75 mg), -2.9 (glargine) UACR: no significant differences among groups
ELIXA (n=6068)	Lixisenatide (10-20 μg) vs. placebo, 108 weeks	T2D with recent acute coronary syndrome	Percent change in UACR and eGFR from baseline	eGFR decline: no significant differences among groups UACR: -1.69% (95% CI: -11.69% to 8.30%) in patients with normoalbuminuria, -21.10% (95% CI: -42.25% to 0.04%) in patients with microalbuminuria, -39.18% (95% CI: -68.53% to -9.84%) in patients with macroalbuminuria

These effects are mainly driven by the reduction of albuminuria.

risk, who were ≥50 years of age, with HbA1c ≥7% were randomly assigned to receive placebo or liraglutide (1.8 mg). At baseline, 20.7% of the patients had an eGFR of 30–59 ml/min/1.73 m^2 and 2.4% had an eGFR of <30 ml/min/1.73 m^2. Microalbuminuria and macroalbuminuria were present in 26.3 and 10.5% of the participants, respectively. The definitions used for the renal outcomes in the LEADER study were a composite of new-onset persistent macroalbuminuria, persistent doubling of serum creatinine, ESRD, or death due to renal disease (Mann et al., 2017). Over a median follow-up of 3.8 years, liraglutide treatment resulted in less renal outcomes in comparison to placebo [HR 0.78 (95% CI: 0.67–0.92, p=0.03)] (Mann et al., 2017). This observation was largely driven by a reduction in new-onset macroalbuminuria in the liraglutide group in comparison to the placebo group [HR 0.74 (95% CI: 0.60–0.91, p=0.004)]. No significant differences in the doubling of the serum creatinine, initiation of renal replacement therapy (RRT), or renal death were observed between the liraglutide and placebo groups (Mann et al., 2017). In a *post-hoc* analysis of the LEADER trial, liraglutide was shown to reduce the risk of major adverse CV events and all-cause mortality in comparison to placebo in patients with chronic kidney disease (CKD), defined as eGFR < 60 ml/min/1.73 m^2 and albuminuria (UACR >30 mg/g) (Mann et al., 2018).

Semaglutide

The SUSTAIN-6 (trial to evaluate cardiovascular and other long-term outcomes with semaglutide in subjects with type 2 diabetes) was a double-blind trial in which T2D patients were randomized to receive either 0.5 or 1.0 mg of once-weekly subcutaneous semaglutide or placebo (Marso et al., 2016a). At baseline, 25.2% of the participants had an eGFR of 30–59 ml/min/1.73 m^2 and 2.9% had an eGFR of <30 ml/min/1.73 m^2. The composite renal outcome of this study was new or worsening nephropathy, defined as persistent macroalbuminuria, persistent doubling of the serum creatinine level and creatinine clearance <45 ml/min/1.73 m^2 or the need for RRT. After a median follow-up of 2 years, the incidence of new or worsening nephropathy in the semaglutide group was lower than that in the placebo group [HR 0.64 (95% CI: 0.46–0.88, p=0.05)]. This result was largely driven by a reduction in new onset macroalbuminuria. No significant changes were observed in ESRD or renal death (Marso et al., 2016a).

The PIONEER-6 trial primarily evaluated the cardiovascular safety of oral semaglutide (14 mg) in comparison to placebo (Husain et al., 2019). A total of 3,183 participants of ≥50 years of age with established CVD or CKD, or ≥60 years of age with CV risk factors were only observed for a median of 15.9 months. At baseline, 26.9% of participants had an eGFR of <60 ml/min/1.73 m^2. There was no significant reported difference in the eGFR decline from baseline to the end of treatment or in the rate of renal death (Husain et al., 2019). The PIONEER-5 trial showed that semaglutide use in T2D patients with renal impairment (eGFR 30–59 ml/min/1.73 m^2) was safe and effective (Mosenzon et al., 2019a). Further study is needed to elucidate whether the renoprotective effects of semaglutide are consistent in those individuals.

Currently, the ongoing FLOW is assessing whether or not semaglutide can inhibit worsening of CKD in patients with T2D (https://clinicaltrials.gov/ct2/show/NCT03819153). Renal impairment defined as either an eGFR 50–75 ml/min/1.73 m^2 and UACR 300–5,000 mg/g or an eGFR 25–50 ml/min/1.73 m^2 and UACR 100–5,000 mg/g are included in this study. An estimated 3,160 participants are to receive once-weekly subcutaneous semaglutide (starting with 0.25 mg and the dose will be increased to 0.5 mg at 4 weeks and 1 mg at 8 weeks) for up to 5 years. The primary endpoint is the time to the first occurrence of a composite primary outcome event, defined as a persistent eGFR decline (≥50% from baseline), reaching ESRD, renal death, or CV death. This study will elucidate the effects of semaglutide in detail.

Dulaglutide

The AWARD-7 study assessed the efficacy and safety of dulaglutide in T2D patients with moderate-to-severe CKD (Tuttle et al., 2018). The baseline cystatin C–based eGFR (eGFRcys) and creatinine-based eGFR (eGFRcre) values of the participants were 35.3 ml/min/1.73 m^2 and 36.0 ml/min/1.73 m^2, respectively. A total of 577 patients were randomly assigned (1:1:1) to receive once-weekly dulaglutide (1.5 mg), once-weekly dulaglutide (0.75 mg), or daily insulin glargine as basal therapy, all in combination with insulin lispro, for 52 weeks. The renal outcomes were changes in the eGFR and UACR. At 52 weeks, the eGFR decline was −1.1 in the dulaglutide (1.5 mg) group, −1.5 in the dulaglutide (0.75 mg) group, and −2.9 in the glargine group. However, the UACR reduction was not significantly different (Tuttle et al., 2018).

The REWIND study evaluated the cardiovascular safety of dulaglutide (1.5 mg) in comparison to placebo (Gerstein et al., 2019b). In total, 9,901 participants of ≥ 50 years of age with T2D and a history or a high risk of CVD were observed for a median of 5.4 years. The composite renal outcome (the first occurrence of new macroalbuminuria, a sustained decline in eGFR of ≥30% from baseline, or RRT) developed less frequently in participants using dulaglutide in comparison to those using placebo [HR 0.85 (95% CI: 0.77–0.93), p=0.0004]. This result was largely driven by a reduction of albuminuria [HR 0.77 (95% CI: 0.68–0.87), p < 0.001]. The rates of a sustained decline in eGFR [HR 0.89 (95% CI: 0.78–1.01), p=0.066] and the need for RRT showed a downward trend but were not statistically significant [HR 0.75 (95% CI: 0.39–1.44), p=0.39] (Gerstein et al., 2019a).

Exenatide

The EXSCEL (Exenatide Study of Cardiovascular Event Lowering) trial evaluated the CV safety of exenatide (2 mg weekly). In total, 14,752 participants with T2D (HbA1c 6.5–10.0%) with or without a history of CVD were observed for a median of 3.2 years (Holman et al., 2017). At baseline, 21.6% of the participants had an eGFR of <60 ml/min/1.73 m^2. Exenatide treatment did not change the eGFR significantly. Macroalbuminuria occurred less (2.2%) in exenatide group compared to placebo group (2.5%) [HR 0.87 (95% CI: 0.70–1.07)]. Neither renal composite 1 (40% eGFR decline, RRT, or renal death) nor composite 2 (composite 1 variables plus

macroalbuminuria) was reduced by exenatide in unadjusted analyses; however, renal composite 2 was reduced after adjustment [HR 0.85 (95% CI: 0.74–0.98)] (Bethel et al., 2020). In a *post hoc* analysis of a 52-week randomized trial, exenatide treatment did not alter the renal function (creatinine clearance or eGFR) or the onset/progression of albuminuria in comparison to titrated insulin glargine in overweight T2D patients (Muskiet et al., 2019). Finally, a pooled analysis of RCTs and open-label phase III studies showed that once-weekly exenatide reduced albuminuria 26% (95% CI%: −39.5 to −10%) compared with comparators (Van Der Aart-Van Der Beek et al., 2020). Furthermore, the change in the HbA1c value from baseline did not affect the result, suggesting that once-weekly exenatide reduced albuminuria independent of the glucose-lowering effect (Van Der Aart-Van Der Beek et al., 2020).

Lixisenatide

In the ELIXA (Evaluation of Lixisenatide in Acute Coronary Syndrome) study, T2D patients with a recent coronary artery event were randomly assigned (1:1) to a lixisenatide (10–20 μg) group or placebo group (Pfeffer et al., 2015). Baseline UACR data were available for 5,978 (99%) of the 6,068 patients who were included in the study. Among them, 19% of the participants had microalbuminuria, and 7% had macroalbuminuria. After 108 weeks, changes in UACR from baseline with lixisenatide were −1.69% [(95% CI: −11.69 to 8.30), p=0.7398] in patients with normoalbuminuria, −21.10% [(95% CI: −42.25 to 0.04), p=0.0502] in patients with microalbuminuria, and −39.18% [(95% CI: −68·53 to −9·84), p=0.0070] in patients with macroalbuminuria. No significant differences in eGFR decline were observed between the treatment groups (Muskiet et al., 2018).

Albiglutide

Harmony outcomes was a double-blind trial that included a total of 9,463 T2D participants of ≥40 years of age and a history of CVD, who were allocated to an albiglutide (30–50 mg weekly) group or placebo group (Hernandez et al., 2018). After a mean follow-up period of 1.6 years, no significant difference in eGFR decline was observed between the two groups (Hernandez et al., 2018).

RENOPROTECTION OF GLP-1RAS IS LARGELY DEPENDENT ON REDUCTION OF ALBUMINURIA

As described above, GLP-1RAs have no clinically important effect on eGFR and hard renal endpoints. In a meta-analysis of 60 studies involving 60,077 T2D patients, GLP-1RAs marginally reduced the UACR in comparison to placebo and other antidiabetic agents, but resulted in no clinically relevant changes in eGFR (Avgerinos et al., 2019). Consistently, a recent meta-analysis that included LEADER (liraglutide), SUSTAIN-6 (semaglutide), REWIND (dulaglutide), EXSCEL (exenatide), ELIXA (lixisenatide), Harmony outcomes (albiglutide), and PIONEER-6 (oral semaglutide), demonstrated that treatment with GLP-1 RAs reduced the composite kidney outcome (development of new-onset macroalbuminuria, decline in eGFR or increase in creatinine, ESRD, or renal death by 17% [HR0.83 (95% CI: 0.78–0.89, p < 0·0001)], mainly driven by a reduction in albuminuria (Kristensen et al., 2019).

RENOPROTECTIVE MECHANISMS OF GLP-1RAS

Experimental studies to elucidate the beneficial effects on DKD have been extensively reported. The inhibition of oxidative stress, inflammation, fibrosis, and induction of natriuresis have been mainly implicated as mechanisms underlying the attenuation of DKD by GLP-1RAs (**Figure 1**).

GLP-1 Receptors in the Kidney

The distribution of GLP-1R in the kidney is controversial. GLP-1R has been shown to be expressed in the renal cortex as well as the proximal tubules (Schlatter et al., 2007; Carraro-Lacroix et al., 2009). However, several investigations reported a lack of GLP-1R

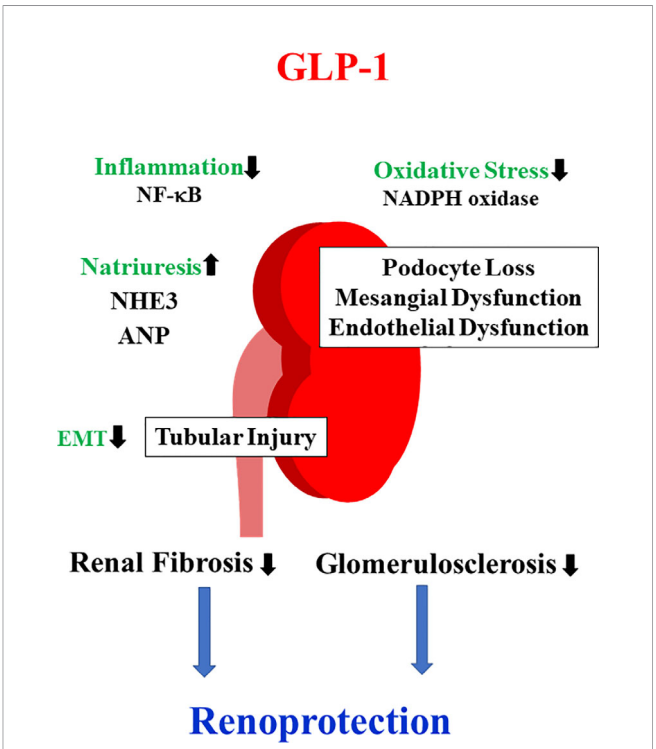

FIGURE 1 | Mechanisms of the renoprotective effects of GLP-1RAs. GLP-1RAs have been shown to activate PKA and increase the production of cyclic adenosine monophosphate (cAMP). As a consequence, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and NF-κB activity are inhibited, resulting in the attenuation of oxidative stress and inflammation. These favorable effects prevent podocyte loss as well as mesangial and endothelial dysfunction.. GLP-1RAs inactivate NHE3 and promote atrial natriuretic peptide (ANP) secretion, thereby inducing natriuresis. Furthermore, GLP-1RAs inhibit tubular injury and subsequent tubulointerstitial fibrosis.

in tubules (Pyke et al., 2014; Lee et al., 2015; Ronn et al., 2017). Studies using monoclonal antibodies against GLP-1R revealed that it is mainly present in the vasculature of the kidney (Pyke et al., 2014; Jensen et al., 2015; Ronn et al., 2017). To date, the presence of GLP-1R in the renal vasculature has been confirmed but not in the tubules (Hviid and Sorensen, 2020).

Oxidative Stress/Inflammation

GLP-1RAs have been shown to prevent renal oxidative stress by inhibiting nicotinamide adenine dinucleotide phosphate (NADPH) oxidase through the activation of PKA and the production of cyclic adenosine monophosphate (cAMP). Recombinant human GLP-1 inhibits protein kinase C (PKC)-β, but increases protein kinase A (PKA), which reduces oxidative stress in both glomeruli and tubules (Yin et al., 2019). Consistent with this observation, the combination of olmesartan and exenatide has been shown to attenuate the renal NADPH oxidase 4 (Nox4) expression in insulin-resistant Otsuka Long-Evans Tokushima Fatty (OLETF) rats (Rodriguez et al., 2020). Hendarto et. al. revealed that liraglutide attenuates oxidative stress and albuminuria in streptozotocin (STZ)-diabetic rats *via* the PKA-mediated inhibition of renal NADPH oxidases (Hendarto et al., 2012). Liljedahl et al. performed label-free shotgun mass spectrometry (MS) and demonstrated that liraglutide increased the abundance of structurally involved proteins as well as proteins involved in oxidative stress responses in the kidney of STZ-induced diabetic mice (Liljedahl et al., 2019). Moreover, it is reported that exendin-4 inhibits mesangial fibrotic responses (Li et al., 2012; Xu et al., 2014). Exendin-4 has been shown to reduce advanced glycation end product (AGE)-induced interleukin (IL)-6 and TNF-α production, the expression of receptor for AGE (RAGE), and cell death in mesangial cells (Chang et al., 2017). The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and Kelch-like ECH-associated protein1 (Keap1) signaling pathways play an important role in preventing oxidative stress (Wang et al., 2014). Nrf2 activator bardoxolone methyl is known to have renoprotective effects (Ito et al., 2020). Interestingly, exendin-4 has been shown to activate the Nrf2 signaling pathway in vascular smooth muscle cells (Zhou et al., 2016) and retinal pigment epithelial cells (Cui et al., 2019). A further study to investigate whether a similar mechanism exists in kidney under diabetic conditions would be intriguing.

NF-κB plays a central role in the inflammatory pathway in the development of DKD (Kawanami et al., 2016). The hyperglycemia-induced downregulation of GLP-1R is involved in NF-κB activation and the subsequent inflammatory response in mesangial cells (Kang et al., 2019). Liraglutide has been shown to increase renal endothelial nitric oxide synthase (eNOS) levels by downregulating NF-κB in STZ-induced diabetic rats (Zhou et al., 2014). Furthermore, liraglutide inhibits the expression levels of TNF-α-mediated NF-κB activation in podocytes (Ye et al., 2019). Kodera et. al. reported that exendin-4 attenuates albuminuria and glomerulosclerosis independent of the glucose-lowering effect in STZ-induced diabetic rats by inhibiting oxidative stress and NF-κB activation. From a mechanistic standpoint, these observations are mediated by GLP-1R in monocytes/macrophages and glomerular endothelial cells (Kodera et al., 2011). Ye et al. showed that liraglutide attenuated the morphology and structure damage of podocytes in obesity-related glomerulopathy model mice (Ye et al., 2019). Mechanistically, they found that liraglutide inhibited the renal TNF-α expression and NF-κB as well as the MAPK pathway activation in these mice (Ye et al., 2019). Similarly, liraglutide has been shown to reduce renal lipid accumulation and improve the mitochondrial function by activating the Sirt1/AMPK/PGC1α pathways in an obesity-induced rat CKD model (Wang et al., 2018). In addition to the MAPK pathway, the JAK/STAT signaling pathway is also involved in liraglutide-induced renoprotection. Zitman-Gal et al. revealed that liraglutide attenuated the phosphorylation of JAK2 and STAT3 in AGE-stimulated endothelial cells and the kidney of db/db mice (Zitman-Gal et al., 2019). Furthermore, it has been reported that the administration of exenatide attenuates the renal inflammation index, including reducing the TNF-α, IL-6, hsCRP, and CCL5 levels, in STZ-induced diabetic rats by increasing the superoxide dismutase and decreasing malondialdehyde levels (Wang et al., 2019). Taken together, the prevention of oxidative stress and inflammation is a key mechanism for the renoprotective effects of GLP-1RAs.

Natriuresis

Acute infusion of GLP-1 has been shown to stimulate diuresis and natriuresis in both experimental (Crajoinas et al., 2011; Jensen et al., 2015) and human studies (Gutzwiller et al., 2004; Skov et al., 2013; Muskiet et al., 2016). These observations seem to be associated with the inhibition of Na^+/H^+ exchanger 3 (NHE3) in the proximal tubules. NHE3 plays an important role in reabsorbing filtered Na^+ in the proximal tubules (Schultheis et al., 1998). Therefore, inactivation of NHE3 can result in natriuresis. GLP-1 RAs have been shown to induce phosphorylation and inactivation of NHE3 (Carraro-Lacroix et al., 2009; Crajoinas et al., 2011; Farah et al., 2016; Muskiet et al., 2017). The long-term administration of lixisenatide has been shown to decrease NHE3 activity in overweight T2D patients. In this study, 35 participants were randomly allocated to a lixisenatide (20 mg) group or once-daily insulin glulisine treatment group. After 8 weeks of follow-up, the administration of lixisenatide increased the phosphorylation of NHE3, which reduced its activity in urinary extracellular vesicles in comparison to once-daily insulin glulisine treatment (Tonneijck et al., 2019). However, it remains unclear whether these natriuretic responses are direct effects of GLP-1RA because a lack of GLP-1R in the proximal tubules has been reported (Pyke et al., 2014; Lee et al., 2015; Ronn et al., 2017). Furthermore, cardiomyocyte GLP-1R plays an important role in natriuresis. It has been shown that liraglutide promotes natriuresis by atrial natriuretic peptide (ANP) secretion from cardiomyocytes in an Epac2-depdenent manner (Kim et al., 2013).

Fibrosis

GLP-1RAs have been shown to attenuate renal fibrosis. For instance, exendin-4 has been shown to ameliorate the high glucose-induced fibronectin (FN) and type I collagen (Col1) expression in tubular epithelial cells by inhibiting the secretion of miR-192, an microRNA (miRNA) that is regulated by p53 and plays a role in renal fibrosis (Jia et al., 2018). Consistent with this observation, liraglutide has been shown to attenuate unilateral ureteral obstruction (UUO)-induced tubulointerstitial fibrosis by suppressing TGF-β and its downstream signaling pathways, including Smad3 and ERK1/2 (Li et al., 2018). These protective effects of GLP-1RAs for renal fibrosis are also mediated by attenuating the epithelial-to-mesenchymal transition (EMT) of tubular cells (Li et al., 2018; Yin et al., 2018).

The Endothelial Function

DKD is associated with endothelial dysfunction (Chen et al., 2020). Endothelial GLP-1R has been shown to be involved in endothelial dysfunction in a mouse angiotensin II-induced hypertension model (Helmstadter et al., 2020). Liraglutide was found to increase eNOS phosphorylation and nitric oxide (NO) production *via* AMPK-dependent pathways in endothelial cells (Li et al., 2016; Honda et al., 2018; Han et al., 2019). Lixisenatide has also been shown to prevent the free fatty acid-induced reduction of eNOS phosphorylation in endothelial cells (Zhao et al., 2019). Sukumaran et al. showed that liraglutide improves the renal endothelial dysfunction in obese Zucker rats on a high-salt diet by increasing the renal eNOS expression (Sukumaran et al., 2019). They also found that liraglutide increases the NO-mediated vasodilation of small intrarenal arteries using X-ray microangiography (Sukumaran et al., 2019). Furthermore, exendin-4 has been shown to attenuate lipotoxicity-induced glomerular endothelial cell dysfunction in diabetic ApoE-deficient mice by increasing the ABC transporter A1-mediated cholesterol efflux (Yin et al., 2016). Taken together, these findings highlight the improvement of the glomerular endothelial dysfunction as an important renoprotective effect of GLP-1RA.

Cleavage Products of GLP-1

Moellmann et al. reported that cleavage products derived from GLP-1 reduced tubulointerstitial renal damage, lowered the expression of tubular injury markers, and attenuated the renal accumulation of macrophages and T cells (Moellmann et al., 2018). These findings suggest that GLP-1R-independent renoprotective effects are mediated by GLP-1 cleavage products. Since distribution of GLP-1R in the kidney remains controversial, the renoprotective effects of GLP-1RAs may be partially explained by this mechanism.

Glycemic Control

Although the glucose-independent mechanisms are emphasized, glycemic control by GLP-1RAs is considered to be involved in its renoprotective effects. In LEADER, the use of liraglutide was associated with a 0.4% HbA1c reduction compared with the placebo (Marso et al., 2016b; Zinman et al., 2018). As a reduction of 0.5% in HbA1c is a clinically important difference (Zinman et al., 2018), a greater reduction by liraglutide may contribute to the renoprotective effects. Furthermore, liraglutide use was associated with a reduction in weight of 2.3 kg. In SUSTAIN-6, semaglutide use *vs.* placebo was associated with respective reductions in HbA1c of −0.66% (0.5 mg) *vs.* −1.05% (1.0 mg) and body weight of −2.9 kg (0.5 mg) *vs.* −4.4 kg (1.0 mg) (Kaul, 2018). In REWIND, dulaglutide use reduced the HbA1c value by −0.61% and body weight by −1.46 kg compared with placebo (Gerstein et al., 2019b). In these trails, GLP-1RAs exerted renoprotection, irrespective of the baseline HbA1c (Kristensen et al., 2019).

LIMITATIONS OF RENOPROTECTION BY GLP-1RAS

A series of experimental studies revealed that GLP-1RAs can exert renoprotective effects independent of their glucose-lowering activities; however, clinical evidence at present is insufficient to support these observations. For instance, there are no established methods for assessing the reduction in oxidative stress and inflammation by GLP-1RAs. It is difficult to evaluate the extent to which glucose-independent mechanisms are involved in renoprotection by GLP-1RAs. In clinical settings, glucose-lowering, weight loss, natriuresis, and blood pressure reduction may account for the renoprotective effects of GLP-1RAs. As described above, GLP-1RAs attenuate albuminuria and marginally reduce eGFR decline. However, whether or not albuminuria is a clinically relevant renal outcome remains unclear. Long-term clinical trials will be needed to address this question. The additive effects of combination treatment of GLP-1RAs and SGLT2 inhibitors on DKD are uncertain. In DELIGHT, the combination of saxagliptin and dapagliflozin showed potentially additive but marginal albuminuria-lowering effects in T2D subjects (Pollock et al., 2019). A meta-analysis showed that GLP-1RA and SGLT2 inhibitor combination therapy was associated with a greater reduction in HbA1c (−0.74%), body weight (−1.61 kg), and systolic blood pressure (−3.32 mmHg) than SGLT2 inhibitor monotherapy (Castellana et al., 2019), suggesting that this combination may induce additive renoprotective effects. Further studies will be required to address this issue.

CONCLUSION AND PERSPECTIVES

GLP-1RAs are widely used in the treatment of T2D. The treatment of DKD has been largely dependent on the management of hyperglycemia and hypertension. Thus, novel therapeutic approaches that exert renoprotective effects independently of these factors have been awaited. A series of clinical trials and experimental studies support the beneficial effects of GLP-1RAs on DKD. Lessons from clinical trials demonstrate these effects are mainly driven by reductions in albuminuria. In contrast, the beneficial effects of SGLT2 inhibitors on albuminuria and eGFR decline in DKD were demonstrated by EMPA-REG OUTCOME (Wanner et al., 2016), CANagliflozin cardioVascular Assessment Study (CANVAS) (Perkovic et al., 2018), DECLARE-TIMI58

(Mosenzon et al., 2019b), and Canagliflozin and Renal Events in Diabetes with Established Nephropathy Clinical Evaluation (CREDENCE) (Perkovic et al., 2019). It remains unclear why these differences were observed. The effects of SGLT2s inhibitors on hemodynamics and glomerular hyperfiltration seem to be robust whereas those of GLP-1RAs have not been established. In addition, the different distribution of SGLT2 and GLP-1R may be involved. Further studies are required to clarify the differences in their effects on the kidney and how to use them appropriately in clinical practice. Nevertheless, GLP-1RAs are a promising therapeutic option for DKD.

AUTHOR CONTRIBUTIONS

DK and YT wrote and revised the manuscript.

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MINI REVIEW
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The Physiology, Pathology, and Therapeutic Interventions for ROCK Isoforms in Diabetic Kidney Disease

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Rho-associated coiled-coil-containing protein kinase (ROCK) is a serine/threonine kinase that was originally identified as RhoA interacting protein. A diverse array of cellular functions, including migration, proliferation, and phenotypic modulation, are orchestrated by ROCK through a mechanism involving cytoskeletal rearrangement. Mammalian cells express two ROCK isoforms: ROCK1 (Rho-kinase β/ROKβ) and ROCK2 (Rho-kinase α/ROKα). While both isoforms have structural similarities and are widely expressed across multiple tissues, investigations in gene knockout animals and cell-based studies have revealed distinct functions of ROCK1 and ROCK2. With respect to the kidney, inhibiting ROCK activity has proven effective for the preventing diabetic kidney disease (DKD) in both type 1 and type 2 diabetic rodent models. However, despite significant progress in the understanding of the renal ROCK biology over the past decade, the pathogenic roles of the ROCK isoforms is only beginning to be elucidated. Recent studies have demonstrated the involvement of renal ROCK1 in mitochondrial dynamics and cellular transdifferentiation, whereas ROCK2 activation leads to inflammation, fibrosis, and cell death in the diabetic kidney. This review provides a conceptual framework for dissecting the molecular underpinnings of ROCK-driven renal injury, focusing on the differences between ROCK1 and ROCK2.

Keywords: notch, hypoxia, inflammation, Rho (Rho GTPase), ROCK1/ROCK2, diabetic kidney disease (DKD)

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INTRODUCTION

The World Health Organization estimates that, each year, around 1.2 million people worldwide die from end-stage renal disease (ESRD). Artificial kidneys and miniaturized dialysis save millions of lives, however dialysis requires cost up to US$91,000 per patient per year in the United States (End chronic kidney disease neglect, 2020), and fewer than half of those on dialysis survive for more than 5 years from the onset of ESRD. Diabetic kidney disease (DKD) in particular has had a devastating impact on the increasing frequency of ESRD.

One major breakthrough in the management of DKD came in the past two decades, when inhibitors of the renin-angiotensin system (RAS) were proven to attenuate the progressive impairment of the renal function. While cardiovascular outcome trials with sodium glucose co-transporter 2 (SGLT2) inhibitors demonstrated these agents' renoprotective actions (Zinman et al., 2015; Kosiborod et al., 2017; Neal et al., 2017), the details are undoubtedly much more complex, with key concerns that

current standards of care do not elicit complete remission. Given the limited drugs available to suppress DKD progression, there has been an ongoing effort to identify factors inducing renal injury and to develop effective therapeutic strategies.

Rho-associated protein kinase (ROCK) belongs to the family of serine/threonine kinases and is a major downstream effector of the small GTP-binding protein RhoA. ROCK signaling is involved in the regulation of a plethora of cellular functions. Due to its centrality in most cellular events, robust temporospatial and context-dependent regulation of ROCK is needed for cell homeostasis. In the kidney, over-activation of the ROCK pathway is clearly harmful; it promotes glomerular fibrosis and podocyte loss in the setting of a variety of diseases including but not limited to diabetes (Matoba et al., 2010; Meyer-Schwesinger et al., 2012; Matoba et al., 2013; Matoba et al., 2017). In addition, elevated ROCK activity results in the increase of oxidative stress, sodium retention, and vascular tone (Bussemaker et al., 2009; Calo et al., 2016; Calo et al., 2017). The beneficial effects of ROCK inhibition have been described in rodent models of DKD (Gojo et al., 2007; Kolavennu et al., 2008).

Two mammalian ROCK isoforms, ROCK1 (also known as Rho-kinase β/ROKβ) and ROCK2 (also referred to as Rho-kinase α/ROKα), have been identified (Nakagawa et al., 1996). The ROCK1 gene is located on chromosome 18 and consists of 1354 amino acids, while the ROCK2 gene is located on chromosome 2 and consists of 1388 amino acids. While these isoforms share 65% overall identity in amino acid sequence, ROCK1 and ROCK2 are differentially regulated, with distinct functions.

This review focuses on the pathophysiological functions of ROCK1 and ROCK2, and discusses the therapeutic effects of ROCK isoform inhibition in DKD.

THE STRUCTURE AND MOLECULAR FUNCTION OF ROCK ISOFORMS

Among protein kinase neighbors, ROCKs are closely associated with myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) and citron kinase. These kinases have the same domain structure, which consists of an N-terminal kinase domain, a central coiled-coil region, and various functional motifs at their respective C-terminal (**Figure 1**). In ROCKs, these functional motifs contain Rho-binding domain (RBD) and pleckstrin homology domain (PHD) that is split into two by an internal cysteine-rich C1 domain (CRD). Under natural conditions, PHD blunts ROCK activity by sequestering kinase interface (Wen et al., 2008). Supporting of this is the fact that deletion of the C-terminal region including the PHD results in constitutive activation *in vitro* (Wen et al., 2008). However, when the RBD binds to GTP-bound active RhoA, RhoB, or RhoC, or PHD is removed, ROCK is constitutively activated. Despite the high sequence homology in their kinase domains, different machinery is involved in the activation process, with ROCK1 activated through the cleavage of the C-terminal PHD by caspase-3 and ROCK2 activation mediated by granzyme B-regulated cleavage. In addition, the inactivation process differs between these two isoforms: ROCK1 is negatively controlled by Rad GTP-binding protein, whereas ROCK2 is inhibited by Gem GTP-binding protein (Ward et al., 2002).

While ROCK1 is predominantly distributed in non-neural tissues including the gastrointestinal tract and lung, ROCK2 is found in the brain, kidney, and bladder (Nakagawa et al., 1996; Iizuka et al., 2012), indicating distinct actions of each isoform in these tissues. At the cellular level, ROCK1 has been detected in the cell membrane (Glyn et al., 2003), actin filaments, and lysosomes (Iizuka et al., 2012); however, the subcellular distribution of ROCK1 has not been fully clarified. ROCK2 activates p300 acetyltransferase to mediate gene transcription *in vitro*, which might explain why ROCK2 is predominantly localized to the nuclei (Tanaka et al., 2006). Consistently, ROCK2 is detected in euchromatin, where transcriptional events take place. ROCK1 and ROCK2 thus have different tissue and cellular distributions, which may affect their functions.

Findings obtained from global knockout of ROCK1 or ROCK2 have expanded our understanding regarding the function of each isoform. Mice harboring systemic ROCK1 deletion display impaired eye closure and an abnormal umbilical ring (Shimizu et al., 2005), whereas ROCK2 deficiency leads to intrauterine growth retardation (Thumkeo et al., 2003). While these data, coupled with other findings,

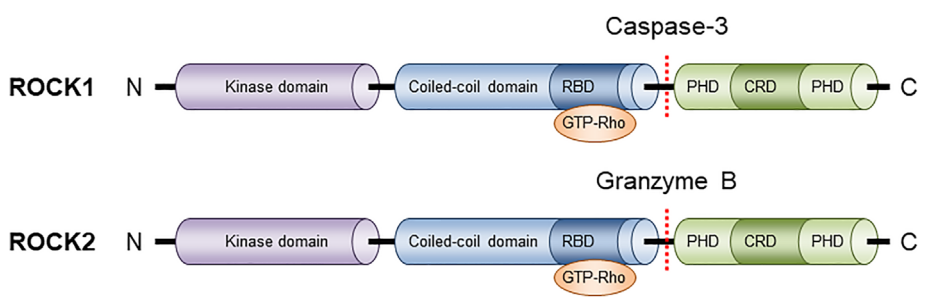

FIGURE 1 | Structure of ROCK isoforms. ROCK1 and ROCK2 are known as ROKβ and ROKα respectively. Both isoforms consist of three major domains: a kinase domain in the N-terminal domain, a coiled-coil domain that contains a Rho-binding domain (RBD), and a putative pleckstrin homology domain (PHD) at its C-terminal end.

suggest divergent physiological and pathological functions of ROCK isoforms, the specificity of those substrates has not been fully characterized (Hartmann et al., 2015).

MECHANISTIC INSIGHTS CONCERNING ROCK ISOFORM INHIBITION IN DKD

Renal ROCK signaling is activated in rodent models of diabetes, regardless of the diabetes type (Gojo et al., 2007; Matoba et al., 2013). The ROCK-mediated molecular basis of DKD progression has been shaped by researchers using pharmacological inhibitors of ROCK (Y27632 and fasudil). Both of these agents ameliorate ROCK activity by competitively combining the ATP sites of the ROCK catalytic domain. While these studies have expanded ROCK research in the field of renal biology, these compounds inhibit both ROCK1 and ROCK2 with equal potency and have non-specific targets, such as protein kinase C, A, and mitogen-activated protein kinases at higher doses (Liao et al., 2007). Some of these disadvantages have been overcome by gene silencing approach, such as with small interfering RNA (siRNA) and systemic or conditional knockout. The distinct actions of each ROCK isoform in DKD are summarized in **Figure 2**.

ROCK1-MEDIATED ALBUMIN TRANSPORT, MITOCHONDRIAL DYNAMICS, TRANSDIFFERENTIATION IN DKD

The upregulation of the ROCK1 isoform is detected in the glomerular endothelium and mesangium of db/db mice (Peng et al., 2016) as well as in the distal tubules of streptozotocin (STZ)-induced diabetic rats (Wu et al., 2013). In cell-based experiments, tubular ROCK1 is activated by the CXC chemokine ligand 16 (Liang et al., 2018), a cytokine produced by diabetic kidney (Ye et al., 2017), to drive production of pro-inflammatory cytokines including tumor necrosis factor α (TNF-α), interleukin 1β, and caspase-3 activation and apoptosis.

From a transcriptional standpoint, we previously showed that siRNA-mediated gene ablation of ROCK1 was sufficient to induce a reduction in hypoxia-inducible factor 1α (HIF-1α) under diabetic conditions (Matoba et al., 2013). In that study, the HIF-1α expression was also suppressed by ROCK2 inhibition, suggesting that both ROCK1 and ROCK2 are requisite for glomerular HIF-1α generation and downstream fibrotic reactions in mesangial cells. The specific action of mesangial ROCK1 has not yet been clarified.

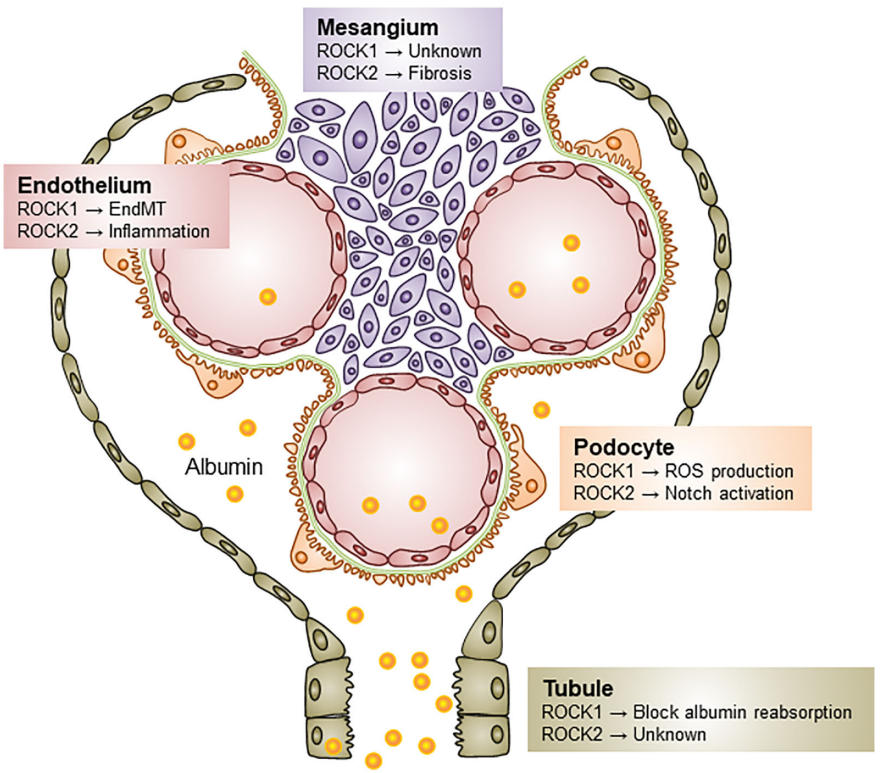

FIGURE 2 | Distinct roles of ROCK isoforms in diabetic kidney disease. Both ROCK1 and ROCK2 contribute to the pathogenesis of DKD *via* different mechanisms. ROCK1 activation induces podocyte ROS production, EndMT, and blocks albumin endocytosis in tubular epithelial cells. Little is known about the role of ROCK1 on the mesangial biology, but ROCK2 elevation induces as the progression of mesangial expansion, Notch activation in podocytes, and endothelial inflammation. ROS, Reactive oxygen species; EndMT, Endothelial-to-mesenchymal transition.

A series of elegant and comprehensive investigations from the Danesh laboratory identified ROCK1-mediated molecular events in DKD using gain- and loss-of-function studies in mice (Wang W. et al., 2012). Intriguingly, ROCK1-deficient mice showed attenuation of albuminuria and histological abnormalities in these models. Conversely, podocyte-specific ROCK1 knockin confers a phenotype that has many of the features of DKD. Mechanistically, they described an unexpected direct action of ROCK1 for regulating mitochondrial fission through phosphorylation and the recruitment of dynamin-related protein-1 (Drp1). The results of that study implicate ROCK1 as a critical regulator of the mitochondrial dynamics in diabetes and suggest that ROCK1 may be a relevant therapeutic target for the generation of oxidative stress in podocytes.

The permselectivity of the glomerular filtration barrier limits the passage of albumin into the Bowman's capsule, resulting in the loss of transport selectivity and culminating in albuminuria, as is common among individuals in DKD. Glomerular endothelium, a key component of the filtration barrier, is converted into the mesenchymal phenotype in cases of diabetes, a process termed endothelial-to-mesenchymal transition (EndMT). Peng et al. investigated the contribution of ROCK1 to EndMT using ROCK1-overexpressing glomerular endothelial cells (Peng et al., 2016). The authors performed quantitative polymerase chain reaction (qPCR) and Western blotting and observed the increased expression of mesenchymal markers (e.g. α-SMA and Snail), together with the loss of endothelial junctional molecules, particularly VE-cadherin. Collectively, they reported that the activation of ROCK1 triggers EndMT, resulting in the loss of cellular attachment to each other and vascular hyper-permeability. These data provide critical insights into the heretofore unclear functions of ROCK1 in the signaling pathway that mediates the damage to glomerular tight junctions and albuminuria in DKD.

Zhou et al. investigated the function of ROCK1 in STZ-induced DKD models (Zhou et al., 2011). To determine the pathological contribution of tubular ROCK1, the authors analyzed the phenotype of diabetic ROCK1-deficient mice. They found that genetic ablation of ROCK1 prevented the development of albuminuria, and this effect was associated with protection against the loss of megalin and cubulin, members of the low-density lipoprotein receptor family that mediate albumin endocytosis in proximal tubular epithelial cells (Zhai et al., 2000). That study provided novel insights into the role of ROCK1 in albumin reabsorption in tubules. Interestingly, benidipine, a calcium channel blocker, has been suggested to inhibit proteinuria by suppressing ROCK1 and the transdifferentiation of renal tubular epithelium without affecting the glucose metabolism or blood pressure (Wu et al., 2013).

The inhibition of both ROCK isoforms by Y27632 or fasudil is effective for preventing tubulointestinal fibrosis in unilateral ureteral obstruction (UUO) models (Nagatoya et al., 2002; Baba et al., 2015); however, the systemic deletion of ROCK1 did not protect against the obstructive kidney damage (Fu et al., 2006). There was no recovery of transforming growth factor β (TGF-β)/SMAD signaling or structural derangement in the kidney of ROCK1-deficient mice. As such, we may reasonably suggest that targeting ROCK1 alone may not be adequate for attenuating tubular fibrosis, at least in UUO models, and the pathological contribution of ROCK1 to tubules may differ between DKD and other renal disease.

Whether or not ROCK1 exerts other functions in DKD is not completely understood. Genome-wide screening approaches will be required to define ROCK1 targets and the precise mechanisms of action. Such analyses will also provide promising opportunities for the development of ROCK1 inhibitors and their translation into clinical medicine.

ROCK2-INDUCED FIBROSIS, NOTCH ACTIVATION, AND INFLAMMATION IN DKD

Initial insights linking ROCK2 to diseases were gleaned from studies implicating ROCK2 as a regulator of, among others, immunity, inflammation, and fibrosis (Yang et al., 2018; Stam et al., 2019; Ricker et al., 2020). With regard to the kidney, we provided the first evidence indicating ROCK2 to be a core component of signaling circuitry that governs DKD progression. Nagai et al. demonstrated the upregulation of ROCK2 in the renal cortex of type 2 diabetic db/db mice (Nagai et al., 2019). In that study, ROCK2 inhibitors were evaluated for their efficacy against glomerular expansion and albuminuria *in vivo*. As a result, the preventive effects of these histological and functional abnormalities were confirmed. The authors also performed a loss of function analysis and revealed that gene deletion of ROCK2, but not ROCK1, decreased the fibrogenic response, concomitant with the suppression of phosphorylation of JNK and Erk, which in turn blocks the nuclear translocation of nuclear factor κB (NF-κB). Hence, ROCK2 inhibition appears to be a promising pharmacological intervention against DKD.

The podocyte slit diaphragm proteins nephrin and podocin are critical component forming the filtration barrier. In the context of diabetes, these components are damaged, mainly by the activation of Notch signaling pathways (Mathieson, 2011; Loeffler and Wolf, 2014). After the binding of Notch receptors to Notch ligands, such as Jagged-like and Delta-like, the C-terminal Notch intracellular domain (NICD) is cleaved from the cell membrane by γ-secretase and translocates into the nucleus, where the formation of recombination signal binding protein for immunoglobulin κJ region (Rbpj) and mastermind-like (MAML) proteins occurs in order to induce the expression of gene sets important for the development of the kidney (Malashicheva et al., 2020). The Notch pathway is reactivated in renal tissue obtained from diabetic mice to regulate the expression of Notch ligands (Niranjan et al., 2008). High-glucose conditions, TGF-β, or vascular endothelial growth factor (VEGF) are postulated to be the molecular basis for the upregulation of Notch signaling (Bonegio and Susztak, 2012). Of note, ROCK2-deficient podocytes are characterized by a significant reduction in TGF-β-induced Notch ligand

expression (Matoba et al., 2017). In contrast, the induction of Notch ligand was not inhibited by ROCK1 gene deletion. These findings indicate the isoform-specific role of ROCK2 in podocytes and provide critical insights into potential strategies against albuminuria seen in DKD. Studies aimed at revealing the interdependency between ROCK2 and Notch modules through the generation of conditional knockout models are thus expected to be beneficial.

There is growing appreciation for the influence of vascular inflammation on regulating the progression of diabetic renal damage (Matoba et al., 2019). In addition to its effect in mesangial cells and podocytes, ROCK2 also plays important roles in endothelial cells. Takeda et al. conducted a series of studies to unravel the mechanisms by which ROCK2 activates vascular inflammation (Takeda et al., 2019). The qPCR array analysis of the mRNA expression profiles in ROCK2-null endothelium revealed differentially expressed genes related to vascular inflammation. Since chemokines and E-selectin production were downregulated in the endothelium, the authors examined monocyte migration and cell to cell adhesion, and found that these activities were abolished compared with those in endothelium with normal levels of ROCK2. These observations will need to be considered when establishing the contribution of ROCK2 to DKD, and when administering ROCK2 inhibitors to patients.

The impressive journey of ROCK2 inhibitors started with the development of KD-25 (formally SLx-2119), which is an orally available and selective inhibitor with a half maximal inhibitory concentration (IC_{50}) and an inhibitory constant (Ki) of 60 nM and 41 nM, respectively (Boerma et al., 2008). Since this drug is used in clinical trials for patients with graft versus host disease (GVHD) and psoriasis (Yiu and Warren, 2016; MacDonald et al., 2017) (**Table 1**), ROCK2 inhibitors may could be used to treat DKD. The success of ROCK2 inhibitor clinical trials will hopefully inspire researchers to redouble their efforts to determine the molecular profiles responsible for ROCK2-regulated events in DKD.

CONCLUSIONS AND FUTURE PERSPECTIVES

Cardiovascular events are pertinent to morbidity and mortality in patients with DKD. Therefore, elucidation of molecular circuitry that governs atherogenic changes remains a major area of research. Recently, critical roles of ROCK isoforms in vascular disease have been evaluated by researchers. James Liao from Chicago and Hiroaki Shimokawa from Sendai are leaders in this field. Liao et al. identified macrophage ROCK1 as an essential element in the development of atherosclerosis through the modulation of foam cell formation and macrophage chemotaxis (Wang et al., 2008). ROCK2 also influences foam cell formation by inhibiting peroxisome proliferator-activated receptor-γ-mediated reverse cholesterol transport in inflammatory cells (Zhou et al., 2012). In vascular smooth muscle cells, ROCK2 controls migration and proliferation activities (Shimizu et al., 2013). In addition, Shimizu et al. focused on the pathologic role of ROCK2 in heart disease and showed that ROCK2 regulates hypertrophy of cardiomyocyte and cell death through interaction with serum

TABLE 1 | Clinical trials of ROCK inhibitors.

Disease	Interventions	Target	Phase	Status	Identifier	Primary outcome
Psoriasis	KD025 (SLx-2119)	ROCK2	2	Completed	NCT02106195	Safety and tolerability
	KD025 (SLx-2119)	ROCK2	2	Completed	NCT02317627	Safety and tolerability
	KD025 (SLx-2119)	ROCK2	2	Completed	NCT02852967	Number of subjects with a 75% decrease in PASI
GVHD	KD025 (SLx-2119)	ROCK2	2	Active, not recruiting	NCT03640481	Overall response rate
Systemic sclerosis	KD025 (SLx-2119)	ROCK2	2	Recruiting	NCT03919799	CRISS response
	Fasudil	ROCK1/2	3	Completed	NCT00498615	Skin temperature
Autoimmune disease/Fibrosis	KD025 (SLx-2119)	ROCK2	1	Completed	NCT03907540	Absolute bioavailability
	KD025 (SLx-2119)	ROCK2	1	Completed	NCT03530995	PK profile
Hepatic Impairment	KD025 (SLx-2119)	ROCK2	1	Recruiting	NCT04166942	PK profile
Chronic kidney disease	SAR407899A	ROCK1/2	1	Completed	NCT01485900	Number of patients reporting adverse events
Atherosclerosis	Fasudil	ROCK1/2	2	Completed	NCT03404843	Blood flow responses
	Fasudil	ROCK1/2	2	Completed	NCT00120718	Vascular reactivity
Diabetic macular edema	Fasudil	ROCK1/2	3	Completed	NCT01823081	Best corrected visual acuity
Retinopathy of prematurity	Fasudil	ROCK1/2	2/3	Recruiting	NCT04191954	Retinal vascularization
Glaucoma	Netarsudil (AR-11324)	ROCK1/2	1	Recruiting	NCT04234932	Peripapillary capillary perfusion density
Fuchs' endothelial corneal dystrophy	Ripasudil (K-115)	ROCK1/2	4	Recruiting	NCT03249337	Corneal clearing
	Ripasudil (K-115)	ROCK1/2	2	Recruiting	NCT03813056	Time to corneal clearance
Amyotrophic lateral sclerosis	Fasudil	ROCK1/2	2	Recruiting	NCT03792490	Safety and tolerability
Erectile dysfunction	SAR407899	ROCK1/2	2	Completed	NCT00914277	Duration of penile rigidity during sexual stimulation

PASI, Psoriasis Area and Severity Index Score; CRISS, Combined Response Index in Diffuse Cutaneous Systemic Sclerosis (CRISS); GVHD, graft-versus-host-disease; PK, pharmacokinetics.

response factor and ERK (Shimizu and Liao, 2016). These important findings coupled with the work of others have led to an increasing appreciation for ROCK2 as a critical molecule for not only renal disease but also cardiovascular disease.

As discussed above, published data have added to a burgeoning body of evidence that ROCKs are critical therapeutic targets against DKD and its related cardiovascular events. However, some caveats must be considered before this concept is accepted. First, the development of ROCK1-specific inhibitors and prospective intervention studies using ROCK1 or ROCK2 inhibitors are required in order to justify targeting ROCK isoforms to treat DKD. Second, whether an isoform-specific approach or pan ROCK inhibition would provide a better therapeutic outcome has yet to be clarified. The comparison of circulating and tissue levels of ROCK1 and ROCK2 between DKD patients and healthy subjects would facilitate our understanding the contribution of each isoform to the pathogenesis of DKD. These studies will also help identify useful targets of DKD therapy, which may vary by clinical stage, and allow for the earlier recognition of patients with diabetes who are at risk of DKD. Third, an open and thorough discussion of the risks while balancing potential clinical benefits of ROCK isoform inhibition is warranted. RhoA activation as well as RhoA inhibition results in podocyte damage (Wang L. et al., 2012), indicating that there is likely a narrow therapeutic window for ROCK isoform activity. This information will provide important insights to consider before commencing with ROCK isoform-selective inhibition in patients. In addition, given the impairment of insulin signaling in skeletal muscle observed in ROCK1 knockout mice (Lee et al., 2009), drugs with limited access to the kidney may be beneficial for patients with diabetes. However, it should be noted that the feasibility of ROCK inhibition has already been established with fasudil, a pan ROCK inhibitor, in patients with stroke (Shibuya et al., 2005). Moreover, clinical data of statins, which inhibit both ROCK1 and ROCK2 through the regulation of RhoA prenylation, demonstrate this medication to be well tolerated and safe during long-term treatment (Ford et al., 2016). Considering these findings alongside cogent evidence that ROCK is critical in versatile pathological aspects of diabetes, targeting ROCK1 and/or ROCK2 is expected to have therapeutic value for not only DKD but also other microvascular complications (i.e. retinopathy, neuropathy) (Yokota et al., 2007; Kanazawa et al., 2013). A deeper understanding of both the divergent and redundant roles of each isoform is therefore considered to be important for the development of effective therapeutic strategies, and for improving the prognosis of patients with diabetes.

AUTHOR CONTRIBUTIONS

KM wrote the manuscript. YT, YN, KS, TY, KU, and RN helped edit and revised the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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REVIEW
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Glomerular Endothelial Cells as Instigators of Glomerular Sclerotic Diseases

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Glomerular endothelial cell (GEnC) dysfunction is important in the pathogenesis of glomerular sclerotic diseases, including Focal Segmental Glomerulosclerosis (FSGS) and overt diabetic nephropathy (DN). GEnCs form the first cellular barrier in direct contact with cells and factors circulating in the blood. Disturbances in these circulating factors can induce GEnC dysfunction. GEnC dysfunction occurs in early stages of FSGS and DN, and is characterized by a compromised endothelial glycocalyx, an inflammatory phenotype, mitochondrial damage and oxidative stress, aberrant cell signaling, and endothelial-to-mesenchymal transition (EndMT). GEnCs are in an interdependent relationship with podocytes and mesangial cells, which involves bidirectional cross-talk *via* intercellular signaling. Given that GEnC behavior directly influences podocyte function, it is conceivable that GEnC dysfunction may culminate in podocyte damage, proteinuria, subsequent mesangial activation, and ultimately glomerulosclerosis. Indeed, GEnC dysfunction is sufficient to cause podocyte injury, proteinuria and activation of mesangial cells. Aberrant gene expression patterns largely contribute to GEnC dysfunction and epigenetic changes seem to be involved in causing aberrant transcription. This review summarizes literature that uncovers the importance of cross-talk between GEnCs and podocytes, and GEnCs and mesangial cells in the context of the development of FSGS and DN, and the potential use of GEnCs as efficacious cellular target to pharmacologically halt development and progression of DN and FSGS.

Keywords: Kidney glomerulus (MeSH: D007678), Glycocalyx (MeSH: D019276), Endothelial cells (MeSH: D042783), Podocytes (MeSH: D050199), Proteinuria (MeSH: D011507), Diabetic Nephropathy (MeSH: D003928), Focal Segmental Glomerulosclerosis (MeSH: D005923)

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THE KIDNEY AND THE GLOMERULUS

The kidneys have a vital role in fluid homeostasis and osmoregulation. Additionally, the kidneys are important for control of blood pressure and mineral metabolism. By filtering blood in the glomeruli, the kidneys produce about 150 liter glomerular filtrate per day of which 99% is reabsorbed in the tubules, to eventually generate approximately 1 liter of urine per day. By blood

filtration and tubular excretion, waste products such as urea, minerals and toxic substances, are excreted from the body.

The glomerulus is a network of capillary loops, known as the glomerular tuft, and is enclosed by the Bowman's capsule. Blood flows into the glomerulus *via* the afferent arteriole and leaves the glomerulus *via* the efferent arteriole (Scott and Quaggin, 2015). The glomerulus is assembled by four different cell types: parietal epithelial cells, glomerular endothelial cells (GEnCs), podocytes (visceral epithelial cells), and mesangial cells (**Figures 1A, B**). Parietal epithelial cells line the Bowman's capsule, where the pre-urine is collected and forwarded to the proximal tubule. GEnCs cover the luminal surface of glomerular capillaries and are the cells of the glomerulus in direct contact with the blood. GEnCs are characterized by transcellular pores (i.e., fenestrae), essential for blood filtration. At the adluminal side, GEnCs are covered with the endothelial glycocalyx, filling the fenestrae (Satchell, 2013; Scott and Quaggin, 2015; Hegermann et al., 2016) (**Figure 1C**). The endothelial glycocalyx is a gel-like layer consisting of glycoproteins, proteoglycans with bound glycosaminoglycans (GAGs) (Reitsma et al., 2007; Slater et al., 2012; Garsen et al., 2014; Dane et al., 2015) and plasma proteins loosely adherent within the meshwork of the glycocalyx. The endothelial glycocalyx prevents leakage of circulating plasma proteins by size and steric hindrance and electrostatic repulsion (Ryan and Karnovsky, 1976; Singh et al., 2007; Patrakka and Tryggvason, 2010; Friden et al., 2011; Dane et al., 2013), and inhibits adhesion and extravasation of inflammatory cells.

The endothelial glycocalyx serves as the primary sensor of wall shear stress through the initiation of signal transduction in GEnCs (Tarbell and Ebong, 2008). Wall shear stress, the hydrodynamic frictional force created from blood flow, transmits through the endothelial glycocalyx into the GEnC, leading to signal transduction that subsequently regulates the expression of Krüppel Like Factor 2 (KLF2), KLF4 and the transcription of eNOS and the production of nitric oxide (NO) which are crucial to maintain GEnC function (Dekker et al., 2006; Weinbaum et al., 2007; Ohnesorge et al., 2010; Slater et al., 2012; Dogne et al., 2018). In addition, GEnCs also function as a sink for factors essential for the regulation of the vascular tone and cross-talk with other glomerular cell types, such as vasoactive factors (endothelin-1 (ET-1), and NO) (Feliers et al., 2005; Dhaun et al., 2012).

Podocytes are specialized perivascular epithelial cells with elaborate projections called foot processes that are intimately wrapped around the exterior of glomerular capillaries (**Figures 1B, C**). The foot processes leave slits between them, called slit diaphragms, which are instrumental for proper blood filtration. GEnCs and podocytes share a common extracellular matrix, referred to as the glomerular basement membrane (GBM), which separates the GEnCs from the podocytes. Together, the GEnCs and the endothelial glycocalyx, the GBM, and the podocytes constitute the glomerular filtration barrier (GFB). The GFB is responsible for size-selective and charge-dependent filtration of the blood. Small and positively charged molecules such as urea,

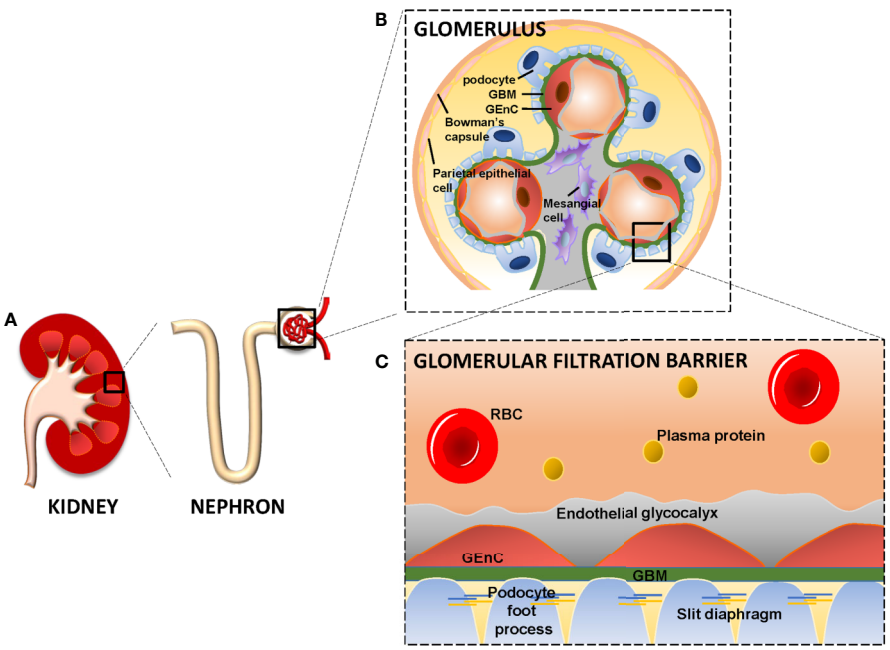

FIGURE 1 | The kidney, glomerulus, and the glomerular filtration barrier. Each kidney consists of about 1 million nephrons. Each nephron consists of a glomerulus and a tubular compartment **(A)**. The glomerulus is assembled by four different cell types, namely parietal epithelial cells, glomerular endothelial cells (GEnC), podocytes (visceral epithelial cells), and mesangial cells **(B)**. GEnC and podocytes share a common extracellular matrix, the glomerular basement membrane (GBM). GEnC and their fenestrae are covered by the endothelial glycocalyx. Podocytes contain foot processes with slit diaphragms that are wrapped around the exterior of glomerular capillaries. Together, the GEnC and the endothelial glycocalyx, GBM and podocytes comprise the glomerular filtration barrier to filter the blood and remaining essential plasma proteins in the circulation **(C)**. RBC, Red Blood Cell; GBM, Glomerular Basement Membrane; GEnC, Glomerular Endothelial Cell.

glucose, amino acids, and minerals can pass the GFB freely, whereas circulating cells and large and negatively charged proteins, including albumin, cannot pass the GFB. Mesangial cells are located in between the capillaries and form the mesangium together with their extracellular matrix (ECM). The mesangium provides structural stability to the glomerular vasculature and modulates capillary blood flow (Scott and Quaggin, 2015). The functionality and integrity of the GFB depends on proper function of GEnCs, podocytes and mesangial cells. Dysfunction of any of the cellular or extracellular components of the GFB culminates in a decreased filtration and eventually glomerulosclerosis (Haraldsson and Nystrom, 2012; Fu et al., 2015).

CROSS-TALK BETWEEN GLOMERULAR CELLS IS ESSENTIAL FOR GLOMERULAR INTEGRITY

There is a growing understanding of the interdependent relationship between GEnCs, podocytes and mesangial cells, which involves bidirectional cross-talk at a molecular level. To exemplify the importance of cross-talk between glomerular cells, the signaling of Vascular Endothelial Growth Factor A (VEGFA), Endothelin-1 (ET-1), and endothelial Nitric Oxide Synthase (eNOS) between GEnCs and podocytes are described. These molecules together form the VEGFA-eNOS/NO-ET-1 axis between GEnCs and podocytes.

VEGFA-eNOS/NO-ET-1 Axis

VEGFA is synthesized by podocytes and binds to its receptors VEGFR1 and VEGFR2 expressed on GEnCs (Eremina et al., 2008). Under physiological conditions, VEGFA induces eNOS activation in GEnCs and a subsequent increase in NO production. The increase of NO may negatively regulates the amount of VEGFA produced by podocytes (Mooyaart et al., 2011). *Via* this crosstalk, the glomerular cells ensure that sufficient VEGFA is produced to maintain viability of GEnCs, without VEGFA levels rising to a level that induces sprouting angiogenesis by GEnCs. In addition to NO, VEGFA also regulates ET-1 production by GEnCs, since VEGFA blockage in podocytes induces ET-1 release from GEnCs (Collino et al., 2008). GEnCs are considered the principal source of ET-1 within the glomeruli (Herman et al., 1998). ET-1 exerts its effect *via* ET-1 receptors (ETR) A and ETRB. Low levels of ET-1 induce an increase in NO, whereas high levels of ET-1 inhibit NO production (Watschinger et al., 1995; Dong et al., 2005; Sud and Black, 2009). ET-1 release from GEnCs associates with cytoskeleton redistribution with a decrease of nephrin in podocytes (Lenoir et al., 2014; Yuan et al., 2019). NO, in its turn, inhibits ET-1 expression (Khimji and Rockey, 2010) and exerts protective effects in podocytes (Sun et al., 2013). An illustration of cross-talk between GEnCs and podocytes in the VEGFA-eNOS/NO-ET-1 axis is provided in **Figure 2**. Next to the effect of ET-1 on podocytes, ET-1 also exerts effects on mesangial cells. ETRA signaling is associated with inflammation, contraction and proliferation of mesangial cells (Barton and Sorokin, 2015), and fibrosis. ETRB signaling has a reciprocal effect and is associated with vasorelaxation *via* eNOS-derived NO release (Barton and Yanagisawa, 2008).

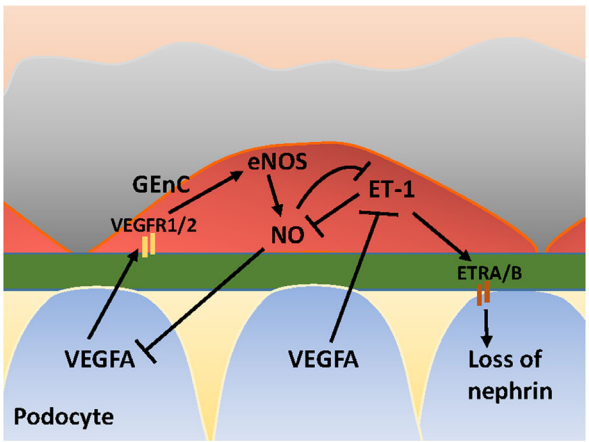

FIGURE 2 | Glomerular cross-talk between GEnC and podocytes *via* the VEGFA-eNOS/NO-ET-1 axis. VEGFA, Vascular Endothelial Growth Factor A; VEGFR1/2, VEGF Receptor 1 and 2; eNOS, endothelial Nitric Oxide Synthase; NO, Nitric Oxide; ET-1, Endothelin-1; ETRA/B, ET-1 Receptor A and B. Stimulating and inhibitory effects are indicated with arrows and blunt lines, respectively.

GLOMERULAR SCLEROTIC DISEASES: HISTOPATHOLOGY OF FSGS AND DN

DN is a long-term complication of both type 1 and type 2 diabetes mellitus and develops in 20%–40% of all diabetes mellitus patients (Rossing et al., 2018). DN, together with Focal Segmental Glomerulosclerosis (FSGS), is the most important cause of chronic kidney disease (CKD). Two types of FSGS exist: primary (or idiopathic) FSGS and secondary FSGS. In primary FSGS, which comprises 80% of all FSGS cases, the etiology is unknown. Secondary FSGS is induced by a preexisting pathologic condition, e.g., hypertension (Jefferson and Shankland, 2014), a viral infection, such as human immunodeficiency virus, drug-induced, or induced by genetic mutations (Lim et al., 2016). In case of primary or mutation-induced FSGS, mutations in genes encoding proteins expressed in podocytes, which are mostly related to slit diaphragm structure, the actin cytoskeleton, or foot processes, such as nephrin (NPHS1), podocin (NPHS2), actinin α4 (ACTN4), and TRPC6 are commonly observed (Lim et al., 2016). No mutations are known in GEnC-specific genes that would cause FSGS.

FSGS and overt diabetic nephropathy (DN) both are characterized by scarring (sclerosis) of the glomerular tuft, i.e., glomerulosclerosis (**Figure 3**). Glomerulosclerosis causes obliteration of the glomerular capillaries eventually (Fioretto and Mauer, 2007; De Vriese et al., 2018). In FSGS, only a fraction of the glomeruli (i.e., focal) is affected in a segmental manner, i.e., part of

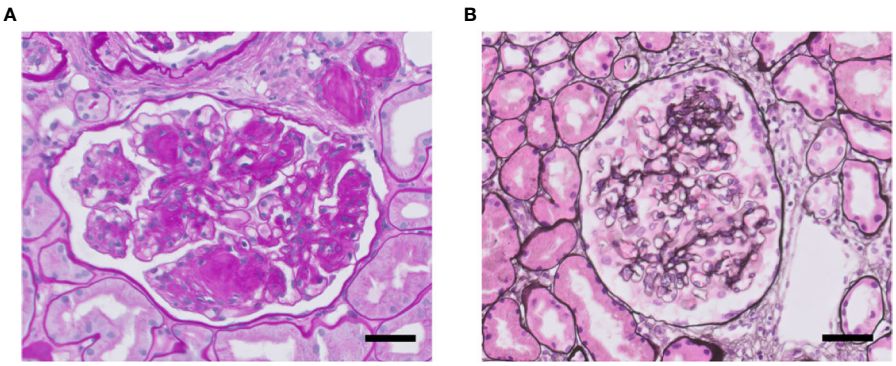

FIGURE 3 | Glomerulosclerosis in DN (A) and FSGS (B). Light microscopy photomicrographs of a glomerulus showing DN with characteristic nodular mesangial expansion (Kimmelstiel-Wilson lesions) (Periodic acid–Schiff staining) **(A)**, and of a glomerulus with mild FSGS (methenamine-silver staining) **(B)**. Nuclei are stained in blue. Both glomeruli presenting glomerulosclerosis with increased glomerular extracellular matrix deposition and obliteration of capillaries. Scale bars represent 50 μm.

a glomerulus is affected. Sclerosis in FSGS is characterized by deposition of extracellular matrix (ECM) at the capillary loops. DN is the specific histopathology associated with reduced renal function in patients suffering from diabetic kidney disease (Yamanouchi et al., 2020). Overt DN comprises diffuse and sometimes nodular glomerulosclerosis in many glomeruli, caused by mesangial cell proliferation and mesangial sclerosis, and develops primarily in patients with proteinuria. Of note, nonproteinuric diabetic kidney disease also exists and which is characterized by minor histopathological changes without DN and with better prognosis compared with proteinuric diabetic kidney disease (Yamanouchi et al., 2020). So, particularly FSGS but also DN are accompanied by proteinuria (macroalbuminuria: >300 mg/gr creatinine), as well as by glomerular hypertension and hyperfiltration, and activation of glomerular inflammatory pathways (Fioretto and Mauer, 2007; Reidy and Kaskel, 2007). At the ultrastructural level, damage to podocytes and GEnCs is observed. Podocyte injury is observed as extensive effacement of the foot processes, ultimately leading to detachment of podocytes from the GBM (podocyte loss). GEnC dysfunction is characterized morphologically as a reduction of the endothelial glycocalyx, loss of fenestrae, widening of the subendothelial space, and swelling of the cytoplasm (Weil et al., 2012; Eleftheriadis et al., 2013; Morita et al., 2015; Taneda et al., 2015; Boels et al., 2016). In many patients, DN and FSGS progresses into end-stage renal disease (ESRD). Therapy resistance and the failure to adequately treat proteinuria, a glomerular inflammatory phenotype and hypertension are the main reasons for progression towards ESRD (Kiffel et al., 2011; Collins et al., 2013). Renal replacement therapy (dialysis or kidney transplantation), is the only effective treatment to postpone premature death in ESRD patients (Collins et al., 2014).

GENC DYSFUNCTION IN DN AND FSGS

GEnC dysfunction is important in the pathogenesis of glomerular sclerotic diseases, including FSGS and overt DN. GEnCs, covered by a thick glycocalyx, form the first cellular barrier in direct contact with all circulating factors. Changes in these circulating factors, such as high glucose levels and advanced glycation end-products, can induce GEnC dysfunction (Singh et al., 2011; Singh et al., 2013; Peng et al., 2016). In FSGS, the development of mesangial matrix expansion and sclerosis by parietal epithelial cells appears to be secondary to podocyte injury, whereas in DN mesangial matrix expansion is the key morphologic finding (Najafian et al., 2015). It is likely that GEnC dysfunction precedes and possibly also contributes to podocyte damage and mesangial expansion. In the past decade, evidence has been provided that also GEnC dysfunction is present and plays an important role in FSGS and DN development. GEnC dysfunction occurs in the early stages of FSGS and DN, and is sufficient to cause podocyte injury, proteinuria and activation of mesangial cells, as will be discussed in detail below. An interdependent relationship between GEnCs, podocytes and mesangial cells exists, which involves bidirectional cross-talk with intercellular signaling. Disturbed molecular cross-talk involving for example endothelial nitric oxide synthase (eNOS) may result in reduced GEnC-derived NO exposure to podocytes and can induce podocyte damage, and eventually compromise glomerular integrity (Yuen et al., 2012). Therapies aiming to prevent endothelial injury have shown to reduce DN in animal models. For example ETRA blockers have shown to restore the endothelial glycocalyx and to reduce albuminuria in diabetic mice (Boels et al., 2016). In diabetic patients, the ETRA blocker atrasentan reduced urinary albumin to creatinine ratios (Lin et al., 2018). Furthermore, renal elevation of cGMP, a key messenger for NO signaling, resulted in a reduction of glomerulosclerosis in rats with DN (Boustany-Kari et al., 2016). Given that GEnCs are the first cells exposed to changes in circulating factors and that GEnC behavior directly influences podocyte function, it is conceivable that GEnC dysfunction may culminate in podocyte damage and mesangial activation. It is, however, elusive which molecular mechanisms underlie GEnC dysfunction and the subsequent altered cross-talk with podocytes and mesangial cells. To develop new treatment options in order to halt the progression

of glomerular sclerotic disease, a deeper understanding of the pathogenetic mechanisms underlying GEnC dysfunction and the disturbed cross-talk is required. Hereunder, it is described that GEnC dysfunction comprises multiple facets and is a pivotal and early factor in the development of glomerulosclerosis and is at the basis of developing proteinuria, podocyte dysfunction and mesangial expansion in FSGS and DN.

Compromised Endothelial Glycocalyx in DN and FSGS

Healthy GEnCs are covered with an endothelial glycocalyx. The endothelial glycocalyx consists of glycoproteins, glycolipids and proteoglycans with bound GAGs. Proteoglycans with bound GAGs, of which heparan sulphate and hyaluronan constitute up to 90%, are the main contributors to the function and structure of the endothelial glycocalyx (Reitsma et al., 2007; Slater et al., 2012; Garsen et al., 2014; Dane et al., 2015). In DN and FSGS, the endothelial glycocalyx is reduced, characterized by a loss of essential GAGs, including heparan sulphate and hyaluronan, and reduced thickness (Nieuwdorp et al., 2006a; Kuwabara et al., 2010; Satoh et al., 2010; van den Berg et al., 2019). Environmental factors, such as elevated levels of glucose, oxidative stress, or inflammatory stimuli, can modulate the endothelial glycocalyx (Singh et al., 2011; Singh et al., 2013; Kolarova et al., 2014). Inflammatory mediators like cytokines and chemokines cause degradation of the endothelial glycocalyx. Under physiological conditions, adhesion molecules on endothelial are covered by the endothelial glycocalyx, and only become accessible to leukocytes upon degradation of the glycocalyx (Kolarova et al., 2014). *In vivo*, intravenous administration of the bacterial heparan sulphate-degrading enzyme heparinase enhances leukocyte adherence to endothelial cells (Constantinescu et al., 2003). High glucose and oxidative stress cause a reduction of heparan sulphate in the endothelial glycocalyx on GEnCs *in vitro* (Singh et al., 2011; Singh et al., 2013). Furthermore, high glucose reduces GAG biosynthesis in GEnCs (Singh et al., 2011). Reduction of heparan sulphate culminates in increased passage of albumin across a GEnC monolayer (Singh et al., 2011; Singh et al., 2013). In line with these *in vitro* data, a reduced endothelial glycocalyx instantly causes proteinuria *in vivo* (Gil et al., 2012). Preservation of the endothelial glycocalyx by the genetic deletion of the heparan sulphate-degrading enzyme heparanase prevents proteinuria and kidney failure in experimental DN and glomerulonephritis (Gil et al., 2012; Garsen et al., 2016a). Loss of endothelial hyaluronan and thereby the endothelial glycocalyx induced by an endothelial-specific deletion of the hyaluronan synthesis enzyme hyaluronan synthase 2 (HAS2) (van den Berg et al., 2019) or by treatment with the hyaluronan-degrading enzyme hyaluronidase (Meuwese et al., 2010) also induces proteinuria (Meuwese et al., 2010; van den Berg et al., 2019) and progressive glomerulopathy (van den Berg et al., 2019), phenocopying the events in DN. In addition to the induction of leukocyte adherence and proteinuria, degradation of the endothelial glycocalyx also compromises GEnC signaling *via* the loss of mechanosensing. Fluid shear stress induces the production of NO in endothelial cells *via* activation of eNOS (Boo et al., 2002). Fluid shear stress-induced NO production is almost completely inhibited upon enzymatic removal of heparan sulphate in the endothelial glycocalyx (Florian et al., 2003), due to loss of eNOS activation. Impaired eNOS activation has negative effects on both GEnCs and podocytes *in vivo* as this results in GEnC dysfunction and disturbed cross-talk with podocytes (Yuen et al., 2012). A reduced endothelial glycocalyx on GEnCs, in response to noxious stimuli, clearly induces glomerular inflammation, proteinuria, and disturbs GEnC signaling. Loss of the endothelial glycocalyx coincides with coagulation activation (Nieuwdorp et al., 2006b) and could possibly also be linked with complement activation (Boels et al., 2013), which is described elsewhere (Nieuwdorp et al., 2006b; Boels et al., 2013) and will not further be addressed here.

Compromised Barrier Function by Endothelial Cell-Selective Adhesion Molecule (ESAM)

The barrier function of GEnCs in FSGS or DN is mainly compromised by a reduction of the endothelial glycocalyx but additional factors that contribute to an increased permeability have been described as well. The altered expression of endothelial cell-selective adhesion molecule (ESAM) has been implied in the loss of the endothelial cell barrier in DN. ESAM is a surface protein laterally expressed on GEnCs that is part of the endothelial tight junctions, and mediates the interaction between endothelial cells. ESAM expression is reduced in the early course of DN (4 weeks) and is associated with increased vascular permeability *in vitro*. *In vivo*, genetic ablation of ESAM causes proteinuria, a decrease in GEnC fenestrations and an increased space between GEnCs through expanded tight junctions, while no structural changes are observed in podocytes, the GBM and mesangium (Hara et al., 2009). Therefore, these observations provide evidence that solely GEnC dysfunction (induced by ESAM deficiency) already leads to glomerular paracellular albumin leakage with preserved podocyte structure (Hara et al., 2009).

Pro-Inflammatory Phenotype

GEnC dysfunction also contributes to glomerulosclerosis *via* obtaining a pro-inflammatory phenotype without having direct effects on podocytes and mesangial cells. Inflammatory pathways are involved in the pathogenesis of DN and FSGS (Navarro-Gonzalez et al., 2011; Wada and Makino, 2013; Moreno et al., 2018; Wilkening et al., 2020). Inflammation-related molecules and pathways (but without pronounced inflammation) may promote fibrotic and proliferative responses of mesangial cells, culminating in glomerulosclerosis (Furuta et al., 1993; Fogo, 2007). GEnC activation plays an important role in glomerular leukocyte infiltration as GEnC activation enables leukocyte rolling, adhesion, arrest and transmigration across the endothelial cell lining (Ley et al., 2007). Upon GEnC activation, the expression of chemokines and adhesion molecules on the cell surface of GEnCs, such as E-selectin (Hirata et al., 1998), intercellular adhesion molecule 1 (ICAM-1) and monocyte chemoattractant protein 1

(MCP-1), are increased (Rao et al., 2017). The high glucose-induced toxic metabolites advanced glycation end-products (AGEs), induce the expression of ICAM-1 and MCP-1 in a Rho-kinase dependent manner. AGE-induced activation of Rho-kinase could be a result of activation of the receptor for AGEs (RAGE) (Hirose et al., 2010). Also ET-1 can activate Rho-kinase in endothelial cells (Gien et al., 2013). Blockage of Rho-kinase in DN mice reduces the expression of ICAM-1 and MCP-1, and ablates concomitant glomerular infiltration of macrophages and glomerulosclerosis. Since macrophages also display Rho-kinase, an endothelial-specific inducible Rho-kinase gene targeting approach would be needed to confirm the role of endothelial Rho-kinase in the increased expression of ICAM-1 and MCP-1 in DN. This implies that AGEs-induced expression of adhesion molecules on GEnCs plays a key role in the development of diabetic glomerulosclerosis (Rao et al., 2017). Indeed, inhibition of AGEs reduces glomerulosclerosis in diabetic mice (Wilkinson-Berka et al., 2002; Forbes et al., 2003). In addition to the increased expression of adhesion molecules, GEnCs show a reduced expression of endothelial-specific molecule-1 (ESM-1), already in very early stages of DN. Under physiological conditions, GEnCs constitutively express ESM-1 that functions as an anti-inflammatory molecule and inhibits migration and rolling of leukocytes. Four weeks after the induction of diabetes, before the development of histological glomerular changes indicative of DN, ESM-1 expression was decreased in glomeruli of DN-susceptible mice compared to glomeruli of DN-resistant mice. These observations demonstrate that in early stages of DN, GEnCs display a pro-inflammatory phenotype which precedes glomerular damage (Zheng et al., 2017).

Mitochondrial Damage

In DN and FSGS, GEnCs display oxidative mitochondrial DNA lesions and mitochondrial oxidative stress, which is associated with loss of GEnC fenestrations (Daehn et al., 2014; Qi et al., 2017) and a loss of the endothelial glycocalyx (Ebefors et al., 2019). Mitochondrial oxidative stress in GEnCs was mediated by release of ET-1 by podocytes and the subsequent paracrine ETRA activation in GEnCs (Daehn et al., 2014; Ebefors et al., 2019). ET-1 induced an increase in heparanase mRNA expression in GEnCs *in vitro*, which could explain the loss of the endothelial glycocalyx upon release of ET-1 by podocytes *in vivo* (Ebefors et al., 2019). Mitochondrial oxidative stress was only observed in GEnCs and not in podocytes in streptozotocin (STZ)-induced DN (Qi et al., 2017). Interestingly, mitochondrial damage in GEnCs preceded podocyte loss, proteinuria, and glomerulosclerosis in adriamycin-induced FSGS and STZ-induced DN (Daehn et al., 2014; Qi et al., 2017). Scavenging of mitochondrial superoxide by systemic administration of the mitochondria-targeted potent antioxidant mitoTEMPO prevented GEnC mitochondrial oxidative stress (Daehn et al., 2014; Qi et al., 2017), the loss of fenestrations (Qi et al., 2017) and the loss of the endothelial glycocalyx (Ebefors et al., 2019). Attenuation of GEnC mitochondrial stress results in ameliorated podocyte loss, demonstrating that mitochondrial damage in GEnCs and the resulting production of mitochondrial superoxide are important triggers for podocyte loss (Daehn et al., 2014; Nagasu et al., 2016; Qi et al., 2017).

eNOS Inactivation

eNOS inactivation, due to impaired dimerization and phosphorylation, has been suggested to play an important role in experimental DN (Cheng et al., 2012). In mice, resistant for adriamycin-induced glomerulopathy, administration of adriamycin induced massive proteinuria and severe glomerulosclerosis upon eNOS deficiency. This observation shows that loss of eNOS increases the susceptibility for the development of adriamycin-induced nephropathy. GEnC dysfunction, observed as loss of CD31 and apoptosis, appeared 3 days after adriamycin administration. Notably, podocyte damage (i.e., loss of synaptopodin expression and apoptosis), occurred only after 7 days, demonstrating that GEnC dysfunction preceded podocyte damage in this model (Sun et al., 2013). Part of these *in vivo* results could be explained by adriamycin's ability to induce inflammatory effects (Abou El Hassan et al., 2003). In line with these findings it has been shown that eNOS prevents heparanase expression and the development of proteinuria in adriamycin-induced experimental FSGS (Garsen et al., 2016b). *In vitro*, conditioned medium from eNOS-overexpressing microvascular endothelial cells protected podocytes from TNF-α-induced synaptopodin loss, suggesting that "healthy" GEnCs protect podocytes from an inflammatory insult in a paracrine manner by secreting protective mediators. Which mediators are secreted by GEnCs and how these mediators affect podocytes is not known (Sun et al., 2013).

Disturbed Crosstalk in the VEGFA-eNOS/NO-ET-1

Disturbances in paracrine signaling of VEGFA, eNOS/NO, and ET-1 between podocytes to GEnCs are critical and may compromise glomerular integrity. Either increased or decreased VEGFA expression, decreased eNOS signaling and increased ET-1 signaling are all implicated in glomerular pathology. In mice, gain of VEGFA in podocytes and lack of eNOS causes the development of proteinuria and nodular glomerulosclerosis (Veron et al., 2014). Podocyte-specific deletion of VEGFA causes GEnC damage, observed as swelling of GEnCs, necrosis and culminating in capillary obliteration (Eremina et al., 2008) and loss of fenestrae (Eremina et al., 2003). Additionally, podocyte-specific deletion of VEGFA also causes a loss of GEnCs in diabetic mice (Sivaskandarajah et al., 2012). Whole-body deletion of VEGFR2 results in a loss of viable GEnCs (Sison et al., 2010). Also podocyte-specific VEGFA overexpression results in loss of GEnCs and collapse of capillary loops (Eremina et al., 2003) and causes advanced DN with endothelial swelling (Veron et al., 2011), suggesting the existence of a delicate balance between the protective and deleterious effects of VEGFA, depending on the strength of signaling. Deletion of eNOS causes GEnC dysfunction and subsequently podocyte damage (Yuen et al., 2012). Administration of NO to cultured podocytes increases the production of cyclic guanosine monophosphate (cGMP), which controls the cytoskeletal structure of podocytes and limits podocyte retraction (Sharma et al., 1992). Deletion of eNOS and decreased availability of NO probably causes

decreased cGMP production and subsequent podocyte retraction and foot process effacement. Maintenance of endothelial eNOS levels by the essential eNOS cofactor tetrahydrobiopterin ameliorates DN (Kidokoro et al., 2013). Furthermore, treatment with sepiapterin, a stable precursor of the eNOS cofactor tetrahydrobiopterin or L-arginine, the nitric oxide precursor induces a correction of eNOS dimerization and phosphorylation and decreases albuminuria (Cheng et al., 2012). In a recent paper, it was shown that ET-1 induces heparanase expression in podocytes, which was associated with a reduced glomerular endothelial glycocalyx in experimental diabetes and which could be prevented in a podocyte-specific ETR deficient mouse model nephropathy (Garsen et al., 2016c). The mechanisms underlying the trafficking of podocyte-derived VEGFA and heparanase against the filtration direction remain to be identified, but may involve heparan sulfate present in the GBM.

These studies demonstrate that the VEGFA-eNOS/NO-ET-1 signaling pathway is important for intraglomerular cross-talk between podocytes and GEnCs, and the strength and direction of signaling is critical for glomerular health. Disturbed cross-talk causes glomerular damage. GEnCs are the first cells in contact with all circulating factors in the blood. It is therefore likely that GEnC dysfunction, culminating in altered secretion of signaling molecules, occurs prior to, and is in fact (partly) responsible for podocyte damage and activation of mesangial cells. GEnC dysfunction might therefore be a leading initiating factor in the development of both FSGS and DN.

Other Aberrant Molecular Signaling and Expression Patterns

LRG1 and Enhancement of TGF-β/ALK1 Signaling

Recently, transcriptome profiling of GEnCs obtained from diabetic mice showed increased gene expression of leucine-rich α-2-glycoprotein (LRG1) in early stages of DN (Fu et al., 2018). LRG1 is a protein present in the glomeruli and is predominantly expressed by GEnCs. LRG1 is involved in angiogenesis and the pathogenesis of DN by enhancement of endothelial Tumor Growth Factor β (TGF-β)/activin receptor-like kinase 1 (ALK1) signaling. TGF-β signaling has previously been found to be involved in the pathogenesis of DN by promoting cell hypertrophy, ECM accumulation in the mesangium, and increasing glomerular permeability (Chang et al., 2016). Global genetic ablation of LRG1 led to a reduction of oxidative damage and glomerular angiogenesis in diabetic mice. Concomitantly, podocyte foot process effacement, podocyte loss, proteinuria, and glomerulosclerosis were attenuated. These results exemplify that alterations in GEnC gene expression and molecular pathways in early disease mediate podocyte damage and glomerulopathy (Hong et al., 2019). How increased LRG1 expression and TGF-β signaling in GEnCs specifically relate to podocyte damage was not addressed in these studies.

GEnC-Derived Exosomes

As a consequence of high glucose concentration, GEnCs show an increased secretion of exosomes containing TGF-β1 mRNA. *In vitro*, these exosomes induced mesangial cells to proliferate and produce ECM (Wu et al., 2016) and caused the induction of epithelial-mesenchymal-transition in podocytes (Wu et al., 2017). Injection of exosomes, derived from high glucose-treated GEnCs *in vitro*, caused glomerulosclerosis in mice (Wu et al., 2016). These studies together suggest that high glucose-induced GEnC dysfunction increases the production of GEnC exosomes, which induce phenotypic changes in mesangial cells and podocytes *in vitro*, and culminate in glomerulosclerosis *in vivo* (Wu et al., 2016).

Hypoxia-Induced Dysregulation of GEnCs

DN is associated with renal cortical hypoxia (O'Neill et al., 2015). Hypoxia and concomitant dysregulation of hypoxia-regulated transcriptional mechanisms in GEnCs are associated with the pathogenetic mechanisms involved in both FSGS and DN development. Endothelial PAS domain-containing protein 1 (EPAS1) is an isoform of hypoxia inducible factor (HIF), also known as HIF-2α. Endothelial-specific deletion of EPAS1 induced the loss of GEnC fenestrations and enhanced endothelial swelling in experimental hypertension-induced secondary FSGS. Additionally, GEnC dysfunction was associated with podocyte foot process effacement and worsening of proteinuria and glomerulosclerosis. In the presence of hypertension and EPAS1, podocyte lesions were not observed, demonstrating that aberrant EPAS1-mediated endothelial signaling associates with podocyte damage and exacerbates FSGS (Luque et al., 2017). Potential mechanisms for aforementioned results include a direct effect of EPAS1 on endothelial-dependent vasoreactivity and modulation of glomerular pressure resulting in hyperfiltration, as mechanical stress is thought to contribute to FSGS. Hyperfiltration results in glomerular hypertrophy, culminating in loss of podocytes and aggravation of mechanical stress and glomerular damage. Furthermore, EPAS1 was previously shown to associate with the assembly of intercellular adherens junctions and enhanced endothelial barrier integrity (Gong et al., 2015). The involvement of dysregulation of hypoxia-associated mechanisms in GEnCs in the pathogenetic pathways leading to glomerular disease is further substantiated by a study showing that endothelial-specific knockout of hypoxia inducible factor 1α (HIF1α) prevents the development of proteinuria and collagen deposition in hypertensive FSGS (Luo et al., 2015). These and the previous mentioned results show that HIF1α is detrimental, whereas EPAS1/HIF2α confers protection in glomerular disease. An explanation could be that the target genes of HIF1α and HIF2α differ in a context-dependent manner (Dengler et al., 2014). Collectively, the aforementioned studies show that disturbed hypoxia-driven signaling in GEnCs contributes to the pathogenesis of glomerular damage in FSGS and DN.

GEnC Plasticity: Endothelial-to-Mesenchymal Transition

GEnC dysfunction can induce the process of endothelial-to-mesenchymal transition (EndMT). Whether EndMT is an initiating event in glomerulosclerosis, and to which extent

EndMT contributes to glomerulosclerosis is not known. EndMT is a process in which endothelial cells show an abrogated endothelial phenotype (such as loss of the expression of endothelial cell markers CD31 and VE-cadherin) and loss of endothelial characteristics such as an increased vascular permeability. Loss of endothelial marker expression coincides with an increase of mesenchymal marker expression such as α-smooth muscle actin (αSMA) and fibroblast specific protein 1 (FSP-1), and the production of ECM proteins (Dejana et al., 2017). In general, endothelial cells are suggested to contribute to the number of activated fibroblasts *via* EndMT. EndMT most probably contributes to fibrosis and is observed in cardiac and cancer-related fibrosis (Zeisberg et al., 2007), fibro-proliferative vascular disease (Moonen et al., 2015), but also in experimental kidney disease as shown in streptozotocin (STZ)-induced DN, unilateral ureteral obstruction, and a mouse model for Alport's syndrome (Zeisberg et al., 2008). In these models, ~30%–50% of the activated fibroblasts co-express the endothelial cell marker CD31 and mesenchymal markers, such as αSMA and FSP-1 (Zeisberg et al., 2008). In lineage tracing experiments in STZ-induced diabetic mice, interstitial endothelial cells acquired a more mesenchymal-like phenotype by expressing αSMA, already early in development of renal interstitial fibrosis (Li et al., 2009). Also in glomeruli of DN patients, EndMT is observed as demonstrated by co-expression of endothelial and mesenchymal markers (Peng et al., 2016; Liu et al., 2018). High glucose conditions and advanced oxidation protein products will stimulate GEnCs to undergo EndMT (Liang et al., 2016; Peng et al., 2016; Shang et al., 2017). Together, aforementioned observations provide evidence that GEnCs can acquire a mesenchymal-like phenotype and may contribute to glomerular fibrosis in DN. The process of EndMT is shown to be controlled by autophagy in endothelial cells (Patschan et al., 2016; Wang et al., 2017). In diabetic mice, deletion of autophagy in endothelial cells induced by the endothelial-specific genetic deletion of Autophagy-Related Gene 5 (ATG5) caused endothelial cell lesions, podocyte foot process broadening and effacement, and an increase of microalbuminuria. These results exemplify the tight intercellular cross-talk between GEnC and podocytes, in which GEnC dysfunction (induced by ATG5 deficiency) leads to podocyte injury (Lenoir et al., 2015).

EPIGENETIC MODIFICATIONS: A POTENTIAL MECHANISM INVOLVED IN GENC DYSFUNCTION

The above mentioned facets of GEnC dysfunction in FSGS and DN associate with altered gene and protein expression. A quiescent endothelial phenotype is harbored by tight regulation of the endothelial transcriptome, i.e., the full array of mRNA transcripts produced (Brooks et al., 2002; Passerini et al., 2004; Gimbrone and Garcia-Cardena, 2013). Epigenetic mechanisms are involved in this regulation of the transcriptome of cells (Eccleston et al., 2013). Epigenetic modifications can cause changes in gene expression, without changing the DNA sequence (Gibney and Nolan, 2010) and are self-perpetuating, dynamic, and reversible in response to the environment (Beckerman et al., 2014). Many factors can influence epigenetic profiles, including hyperglycemia, hypoxia, and inflammation (Lu et al., 2017). Epigenetic modifications can either be beneficial, or hamper GEnC function by changing the transcriptome, resulting in GEnC dysfunction and potentially disturbed cross-talk and pathogenesis of FSGS and DN.

Epigenetic modifications include DNA methylation and histone modifications. In general, DNA methylation is associated with gene repression by changing the biophysical characteristics of the DNA to bind transcription factors. DNA methylation can also inhibit gene expression *via* methyl binding proteins, which in turn recruit transcriptional co-repressors. DNA methylation at genes can modulate transcriptional elongation and alternative splicing (Gibney and Nolan, 2010; Lu et al., 2017).

In addition to DNA methylation, epigenetic mechanisms also include modifications of histones. The best-characterized histone modifications involve methylation, acetylation, and phosphorylation. Histone modifications stably alter the conformation of chromatin, and thereby either enhance or inhibit gene transcriptional activity depending on the type of modification and the position of the modified residue within the histone (Kouzarides, 2007; Berger et al., 2009). DN is associated with aberrant DNA methylation in proximal tubules and peripheral blood cells (Maghbooli et al., 2014), and DNA methylation is recently shown to be present in GEnCs (Fu et al., 2018). Histone modifications have previously been shown to be involved in the pathogenesis of DN and FSGS (Sun et al., 2017; Majumder et al., 2018), but not much is known about altered histone modification patterns in GEnCs in DN or FSGS. Recently, transcriptome profiling of GEnCs obtained from diabetic mice with early DN, showed that many of the genes with decreased expression were involved in epigenetic regulation, suggesting altered epigenetic regulation in GEnCs in early stages of DN (Fu et al., 2018). Lysine-specific demethylase 6A (KDM6a), also known as Ubiquitously Transcribed Tetratricopeptide Repeat X Chromosome (UTX) was one of the genes found to be downregulated. KDM6a is a histone demethylase that specifically demethylates lysine 27 of histone 3. Methylation of lysine 27 of histone 3 (H3K27me3), mediated by the methyltransferase Enhancer of Zeste Homolog 2 (EZH2), is associated with gene repression (Tan et al., 2014). The role of EZH2 and H3K27me3 in GEnCs in DN and FSGS is yet unknown. In podocytes, H3K27me3 was previously shown to be decreased in DN, which associated with the extent of podocyte damage due to activation of Notch signaling and loss of quiescence (Majumder et al., 2018). Previous studies showed that EZH2 plays a role in endothelial homeostasis and is a modulator of a number of endothelial cell functions, such as endothelial-leukocytes interactions and angiogenesis (Dreger et al., 2012; Maleszewska et al., 2016). This is indicative for a role of altered epigenetic modifications in GEnCs resulting in aberrant and pathologic gene expression contributing to the pathogenesis of DN. Alteration of epigenetic modifications is shown to be beneficial. For example, inhibition of the demethylases Jumonji

C domain–containing demethylases (JMJD3) and UTX attenuated podocyte injury in diabetic mice (Majumder et al., 2018). Also in an unilateral ureteric obstruction mouse model, inhibition of EZH2 and H3K27me3 attenuated renal fibrosis (Zhou et al., 2016). Our current knowledge about the contribution of an altered epigenetic landscape to GEnC dysfunction and disturbed cross-talk in DN and FSGS is limited. Therefore, expanding our knowledge on the potential causative role of epigenetic modifications in GEnCs is highly needed. Herewith, specific mediators involved in epigenetic pathways involved in GEnC dysfunction and disturbed cross-talk can be considered potential targets for future therapies in the pathogenesis of DN and FSGS.

SUMMARY AND FUTURE PERSPECTIVES

As outlined above, podocytes and mesangial cells have previously received a lot of attention in research on the pathogenesis of FSGS and DN. However, the studies summarized in this review show that GEnC dysfunction occurs in the early stages of FSGS and DN, and contributes to podocyte damage and mesangial activation, eventually culminating in glomerulosclerosis. Several of the studies described here show that GEnC dysfunction precedes podocyte damage, and is sufficient to develop proteinuria. This provides a new insight on the role of GEnCs in the early phase in development of FSGS and DN. GEnC dysfunction is characterized by a compromised endothelial glycocalyx, an inflammatory phenotype, mitochondrial damage and oxidative stress, aberrant signaling and EndMT, resulting in proteinuria, podocyte damage or loss, mesangial activation, and ultimately glomerulosclerosis (**Figure 4**). The glomerular endothelium poses a potential efficacious cellular target to pharmacologically halt disease development and progression in DN and FSGS. Aberrant gene expression patterns largely contribute to GEnC dysfunction and altered epigenetic mechanisms seem involved in this aberrant transcriptome. To expand our understanding of the cross-talk between GEnCs and other glomerular cells in health and disease, isolated systems could be useful, such as co-cultured cells and organoids. Co-culture systems of differentiated GEnCs and podocytes (Li et al., 2016) and organoids (Hale et al., 2018) with subsequent endothelial genetic and epigenetic characterization and manipulation could be instrumental for understanding the pathways involved in GEnC-podocyte cross-talk. Until now, the knowledge of the epigenetic mechanisms involved in GEnC dysfunction in DN and FSGS is scarce and needs to be expanded.

Transcriptome profiling of GEnCs in DN and FSGS is of utmost importance to identify aberrantly expressed genes and associated regulatory pathways. Epigenomic databases, such as encyclopedia of DNA elements (ENCODE), in which chromatin modifications on both DNA and histone proteins are mapped in various cell lines (Consortium, 2012), could reveal potential epigenetic modifications responsible for aberrant expression patterns. Cell-specific delivery is needed to therapeutically intervene in the epigenetic mechanisms involved in GEnC dysfunction to avoid off-target cell effects. The identification of epigenetic mechanisms involved in GEnC dysfunction can effectively be studied with CRISPR-Cas9 technology *in vitro* (Adli, 2018). However, cell-specific delivery of CRISPR-Cas is still a huge challenge (Adli, 2018). The delivery of nucleotides, such as siRNAs therefore is an approach with great potential for intervention in GEnCs. As epigenetic modifications are regulated

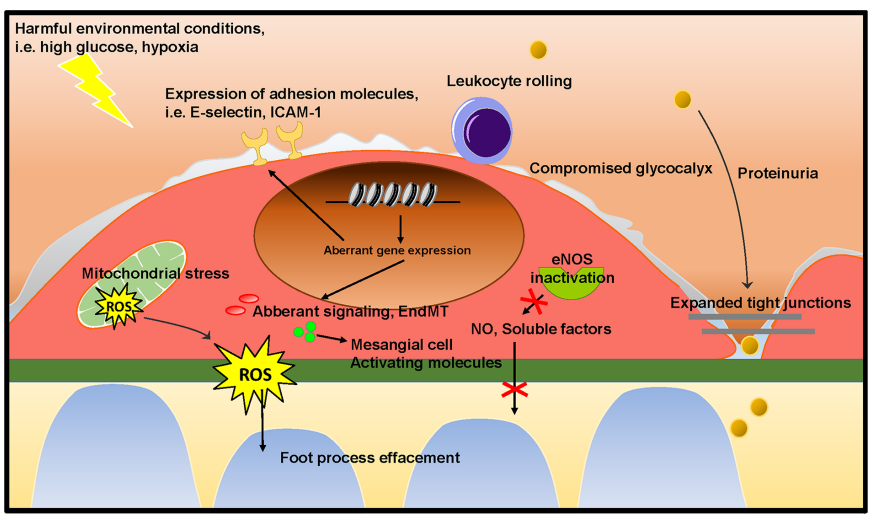

FIGURE 4 | Proposed mechanism on the role of GEnC in the development of glomerular sclerotic diseases. Harmful environmental conditions, such as hyperglycemia and hypoxia cause GEnC dysfunction. GEnC dysfunction is characterized by a compromised endothelial glycocalyx, an inflammatory phenotype, mitochondrial damage and oxidative stress, aberrant signaling and EndMT, resulting in proteinuria, podocyte damage or loss, mesangial activation, and ultimately glomerulosclerosis.

by epigenetic enzymes, intervening in the expression of epigenetic enzymes can influence the amount of epigenetic modifications. Endothelial cell-specific delivery of siRNA is feasible and this strategy has previously been used to successfully deliver siRNA to inflamed endothelial cells, including specifically GEnCs, and to decrease the expression of the target gene of interest (Kowalski et al., 2014; Choi et al., 2017).

AUTHOR CONTRIBUTIONS

MS searched articles, drafted and wrote the manuscript. MS, JK, GK, and J-LH created the outline of the manuscript. JK, JB, MH, JV, GK, and J-LH supervised the manuscript writing and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Potential Targeting of Renal Fibrosis in Diabetic Kidney Disease Using MicroRNAs

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Diabetic kidney disease (DKD) is a major health problem and one of the leading causes of end-stage renal disease worldwide. Despite recent advances, there exists an urgent need for the development of new treatments for DKD. DKD is characterized by the excessive synthesis and deposition of extracellular matrix proteins in glomeruli and the tubulointerstitium, ultimately leading to glomerulosclerosis as well as interstitial fibrosis. Renal fibrosis is the final common pathway at the histological level leading to an end-stage renal failure. In fact, activation of the nuclear factor erythroid 2-related factor 2 pathway by bardoxolone methyl and inhibition of transforming growth factor beta signaling by pirfenidone have been assumed to be effective therapeutic targets for DKD, and various basic and clinical studies are currently ongoing. MicroRNAs (miRNAs) are endogenously produced small RNA molecules of 18–22 nucleotides in length, which act as posttranscriptional repressors of gene expression. Studies have demonstrated that several miRNAs contribute to renal fibrosis. In this review, we outline the potential of using miRNAs as an antifibrosis treatment strategy and discuss their clinical application in DKD.

Keywords: diabetic kidney disease, microRNA, renal fibrosis, end-stage renal disease, antifibrosis treatment

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INTRODUCTION

The International Diabetes Federation reported 425 million subjects with diabetes worldwide in 2017. This number is predicted to reach 629 million by the year 2045. Diabetic kidney disease (DKD) is a major complication of diabetes and also one of the leading causes of end-stage renal disease (ESRD). Approximately 30–40% of patients with diabetes will eventually develop DKD. Although the exact mechanism underlying the development of DKD remains unknown, several causes in addition to hyperglycemia are known to contribute to its development, including genetic, environmental, and hemodynamic factors (such as hypertension, aging, arteriosclerosis, dyslipidemia, and proteinuria) (Brook, 2006; Gohda et al., 2019).

The complex pathophysiology of DKD is caused by changes in renal hemodynamics, increased oxidative stress as a result of glucose metabolic disorders, inflammatory processes, and enhanced activity of the renin-angiotensin-aldosterone system. However, the final common pathway of all these processes at the histological level is renal fibrosis, which inevitably results in ESRD.

Activated fibroblasts play a major role in the accumulation of extracellular matrix (ECM) under pathological conditions, subsequently leading to renal fibrosis. The origin of these activated fibroblasts has been extensively studied and understood to be derived from the differentiation

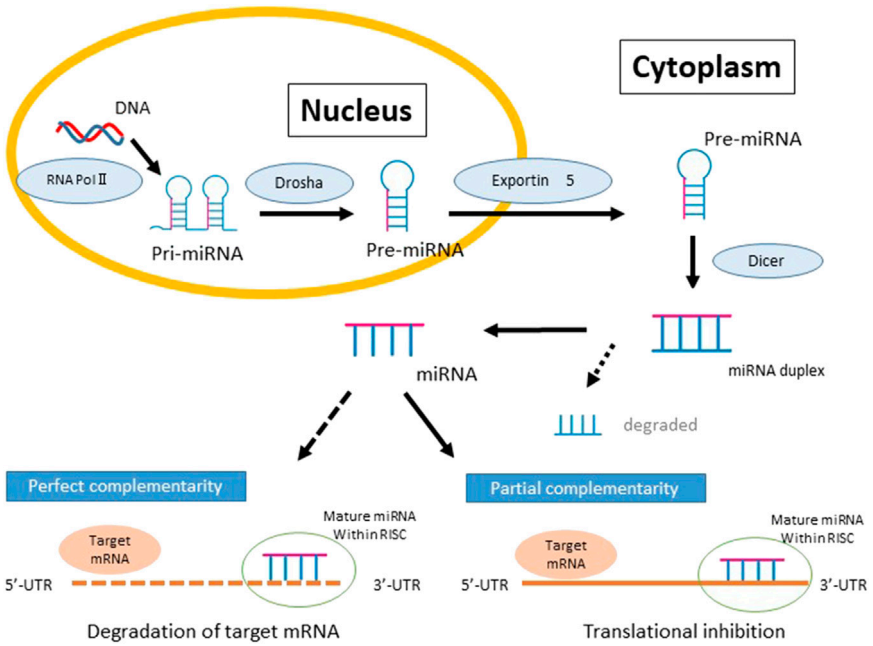

FIGURE 1 | The biogenesis and function of MicroRNAs (miRNA) and the repression of gene expression. Biosynthesis of miRNAs begins in the nucleus and RNA polymerase II-dependent transcription produces capped polyadenylated transcripts known as primary miRNAs (pri-miRNAs). Pri-miRNA is processed by the RNase III endonuclease, Drosha, into a stem-loop structure known as the precursor miRNA (pre-miRNA). Pre-miRNA is transported from the nucleus to the cytosol by Exportin 5 and further processed by the second RNase III, Dicer, to generate miRNA duplexes. Posttranscriptional gene silencing occurs when the mature miRNA is then loaded into the miRNA-induced silencing complex and binds to the 3′ UTR of target mRNAs with either complete or partial complementarity.

and proliferation of resident fibroblasts, recruited from the bone marrow, and via epithelial-to-mesenchymal transition (EMT) and endothelial-to mesenchymal transition (EndMT). EMT and EndMT are the processes by which renal tubular epithelial cells and glomerular endothelial cells lose certain specific characteristics while acquiring other phenotypic properties of mesenchymal and fibroblast-like cells (Carew et al., 2012; LeBleu et al., 2013).

In recent years, activation of the nuclear factor erythroid 2-related factor 2 pathway by bardoxolone methyl and inhibition of transforming growth factor beta (TGF-β) signaling by pirfenidone have been envisioned as therapeutic targets for DKD, with a number of clinical trials being currently underway (Chin et al., 2018; Isaka, 2018).

MiRNAs, which are small noncoding RNA molecules (18–22 nucleotides), are transcribed from genomic DNA as primary miRNA (pri-miRNA) transcripts. These molecules are subsequently processed by the microprocessor complex which consists of Drosha, a nuclear RNase III, and DGCR8 (DiGeorge syndrome critical region gene 8), to yield the precursor miRNA (pre-miRNA) molecule in the form of a hairpin-loop structure (Hagiwara et al., 2013). Pre-miRNAs are then exported from the nucleus to the cytoplasm via exportin 5 where they are further processed in the cytoplasm by the ribonuclease Dicer, leading to the removal of the terminal loop to generate a mature 22-bp miRNA duplex. Finally, one of the duplex strands is loaded into the RNA-induced silencing complex (RISC) while the other strand is degraded. The RISC-miRNA complex recognizes the 3′-UTR of the target mRNA through partially complementary nucleotide sequences, ultimately resulting in the degradation of the target mRNA (**Figure 1**) (Knight and Bass, 2001; Lee et al., 2002; Nagalakshmi et al., 2011). In addition to 3′-UTRs, there are some miRNAs that bind to 5′-UTRs or coding regions of mRNAs and induce gene repression (Patel and Noureddine, 2012). A single miRNA can potentially modulate the expression of several genes by targeting one or more genes in various signaling pathways and therefore impact multiple biological pathways and cell function, contributing to disease (Gomez et al., 2016; Cao et al., 2019). Studies have also demonstrated the nuclear accumulation of miRNAs and roles in gene regulation by binding to promotor regions and chromatin remodeling effects (Kim et al., 2008; Place et al., 2008; Younger and Corey, 2011; Huang et al., 2012).

More recently, single nucleotide polymorphisms (SNPs) in miRNAs and their connection to diabetes have also received much attention. The SNPs have been shown to impact on every aspect of miRNA biology, from transcription and biogenesis to altered targeting of miRNA to their binding sites. More specifically, some miRNA SNPs have been associated with type 1, type 2, and gestational diabetes, as well as diabetic complications (Gong et al., 2012; Li and Lei, 2015; Moszyńska et al., 2017; Zhuang and Wang, 2017; Chen et al., 2019; Zhang et al., 2019); however the impact of miRNA-related SNPs in DKD is beyond the scope of this review.

It is postulated that the interplays between metabolic and hemodynamic pathways such as hypertension, the renin-angiotensin-aldosterone system, and vasoactive hormones

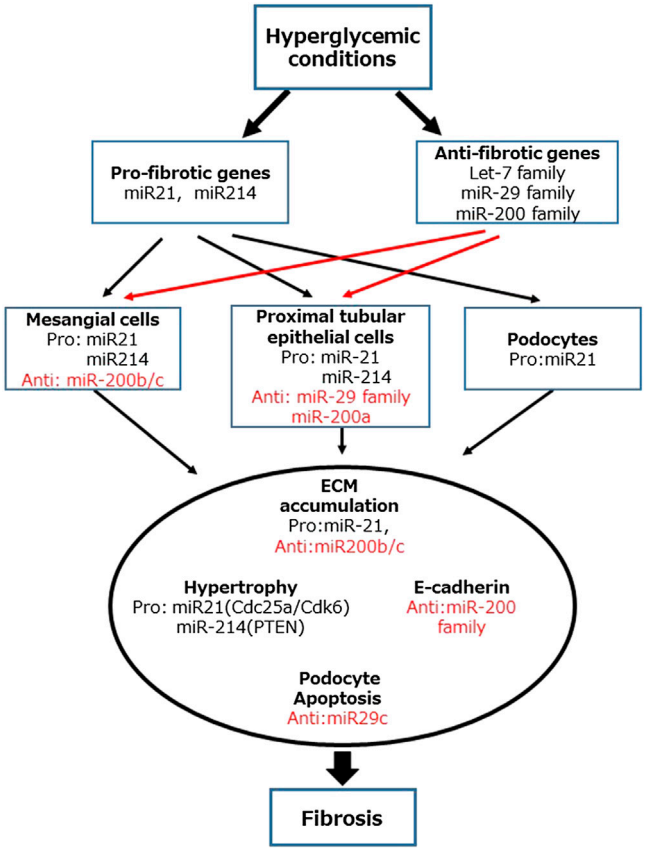

FIGURE 2 | Role of MicroRNAs (miRNAs) in the development and progression of diabetic kidney disease. MiR-21 and miR-214 are classified as fibrotic genes. In contrast, let-7, miR-29, and miR-200 families are classified as antifibrotic genes.

plays an important role in the development and progression of DKD (Cooper, 2001). We have previously reviewed the role of miRNA associated with the metabolic and hemodynamic pathways contributing to the progression of DKD (Hagiwara et al., 2013). In recent years, several miRNAs contributing to renal fibrosis and EMT have been reported and it is thought that targeting these could lead to novel antifibrotic therapeutic treatments in DKD (Reidy et al., 2014; Lin et al., 2018). We have provided a list of validated mature miRNAs and their targets relevant to DKD in **Table 1**. In this review, we focus on the role of miRNAs contributing to renal fibrosis in the context of DKD. Some of these miRNAs related to fibrosis are summarized in **Figure 2** and are outlined below.

Antifibrotic MicroRNAs in Diabetic Kidney Disease

Several miRNAs associated with DKD are considered to be negative regulators of fibrotic pathways.

【let-7】

Let-7, one of the first miRNAs to be discovered, was in *Caenorhabditis elegans* as an essential developmental gene (Tolonen et al., 2014). Since then, the let-7 family of miRNAs was found to be highly conserved in many species, playing a key role as inhibitory factors regulating stem cell reprogramming. This family also regulates the deposition of the ECM in breast, pancreatic, and oral cancer cells (Chang et al., 2011; Dangi-Garimella et al., 2011; Thornton et al., 2012). Moreover, the let-7 family has also been described as negative regulators of renal fibrosis. Renal let-7 expression levels were found to be decreased in a mouse unilateral ureteral obstruction (UUO) model, where upregulation of TGF-β expression is normally observed. Let-7b decreases ECM protein expression through a mechanism that involves the TGF-β mothers against decapentaplegic homolog (Smad) 3 pathway. This is probably due to the direct inhibition of let-7 on the TGF-β receptor-mediated signaling, as demonstrated in rat proximal tubular epithelial cells (NRK52E) (Brennan et al., 2013; Tolonen et al., 2014; Wang et al., 2014).

EndMT is also thought to be an important driver of renal fibrosis. The let-7 family has anti-EndMT effects, and interestingly, the fibroblast growth factor (FGF) receptor is involved in EndMT through the regulation of let-7 expression (Chang et al., 2011). The antifibrotic peptide N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) is one of the endogenous substrates of angiotensin-converting enzyme (ACE) and

TABLE 1 | Validated mature miRNAs relevant to DKD

	miRNA	Target gene
Antifibrotic	hsa-let-7b-5p	HMGA2, IGF2BP2, **TGFBR1**, JAG1, THBS1
	hsa-miR-29a-3p	**COL4A1**, **COL4A2**, **HDAC4**, LAMC2
	hsa-miR-29b-3p	SP1, **HDAC4**, TGFB1, IL6, LAMC2
	hsa-miR-200a-3p	**ZEB1**, **ZEB2**, KEAP1, TGFB2
	hsa-miR-200b-3p	**ZEB1**, **ZEB2**
	hsa-miR-200c-3p	**ZEB1**, **ZEB2**,
Profibrotic	mmu-miR-29c-3p	**Spry1**
	hsa-miR-21-5p	BCL2, **CDC25A**, PPARA, PDCD4, **PTEN**, **SMAD7**, TGFBR2, TIMP3
	hsa-miR-214-3p	**PTEN**

DKD, diabetic kidney disease; miRNAs, MicroRNAs. Source: from miRTarBase (http://mirtarbase.cuhk.edu.cn/php/index.php). Validated mRNA target genes relevant to fibrosis in DKD are shown in bold face and are discussed in this review.

hydrolyzed by it. Kanasaki et al. (Nagai et al., 2014) showed that dual treatment with ACE inhibitor (ACEi) and AcSDKP improved renal fibrosis by inhibiting EndMT more than ACEi treatment alone in diabetic CD-1 mice. The antifibrotic and anti-EndMT actions of AcSDKP have been associated with the upregulation of let-7 levels and reduced TGF-β signaling in these mice (Nagai et al., 2014; Nitta et al., 2016; Srivastava et al., 2020). Let-7 downregulated high mobility group A2 (HMGA2) which is involved in EMT in human pancreatic cancer cells. HMGA2 is a chromatin factor that is mainly expressed in undifferentiated tissues and mesenchymal tumors (Watanabe et al., 2009; Lamouille et al., 2014). Let-7 was significantly downregulated and HMGA2 was markedly upregulated in the tissue samples of DKD mice and renal mesangial cells (MCs) cultured under high glucose conditions (Wang et al., 2019). Let-7 also modulates the TGF-β pathway that is a potent driver of EMT in renal tubular epithelial cells (Wang et al., 2012a). Crosstalk between antifibrotic miRNA, in particular miR-29, and Let-7 is also important in endothelial cells homeostasis via a complex set of interactions involving FGF receptor phosphorylation and TGF-β receptor activation. This crosstalk is enhanced via the antifibrotic peptide AcSDKP, whose renoprotective action appears to be via maintenance of the cross-regulation between miR-29 and let-7 (Srivastava et al., 2019). Indeed, there is extensive crosstalk between many miRNAs and the pathways they regulate since each miRNA can target multiple genes, often in related pathways. The studying of individual miRNAs and isolated targets is often difficult because of this regulatory overlap.

【miR-29】

The human miR-29 family consists of hsa-miR-29a, 29b-1, 29b-2, and 29c. MiR-29b-1 and miR-29b-2 share the identical sequence and are both referred to as miR-29b. The miR-29 family shares a common seed sequence and is generally expected to act on the same target genes. The miR-29 family has been demonstrated to exert antifibrotic effects in various organs, such as the heart and kidney (van Rooij et al., 2008; Maurer et al., 2010; Cushing et al., 2011; Roderburg et al., 2011; Xiao et al., 2012). Its other effects include the promotion of apoptosis and the regulation of cell differentiation (Kriegel et al., 2012).

Podocyte dysfunction is one of the detrimental features of DKD. The depletion of nephrin integrity may be associated with the development of diabetic podocytopathy. Lin et al. (2014) demonstrated that the levels of the podocyte injury marker desmin were increased, whereas the number of Wilms' tumor-1-positive cells and the expression of nephrin were decreased in the glomeruli of streptozotocin- (STZ-) induced diabetic mice. Interestingly, the glomerular expression level of miR-29a, but not of miR-29b and miR-29c, was decreased in diabetic mice. When compared with diabetic wild-type mice, glomerular hyperfiltration and urinary protein levels in diabetic miR-29a-transgenic mice were significantly reduced, although blood glucose levels remained unaltered. Furthermore, miR-29a overexpression reduced nephrin loss and improved podocyte integrity probably through a mechanism involving reduction of histone deacetylase 4 levels and ubiquitination in these mice. Du et al. (2010) reported that miR-29a was downregulated by high glucose or TGF-β in human proximal tubule (HK-2) cells and that downregulated miR-29a increased the production of collagen IV protein by directly targeting the 3′UTR of *col4α1* and *col4α2*.

Renal expression of miR-29 family members was decreased with the progression of renal fibrosis in mice with UUO. However, Smad3-deficient mice with UUO were protected against renal fibrosis and increased renal miR-29 expression. Overexpression of miR-29b inhibited TGF-β-mediated induction of collagens I and III in tubular epithelial cells, whereas knockdown of miR-29b enhanced the expression of these genes, identifying miR-29b as a downstream inhibitor of TGF-β-/Smad3-mediated fibrosis (Qin et al., 2011).

Although the miR-29 family is generally considered to be protective against renal fibrosis, the data for miR-29c are discordant. Long et al. (2011) identified that Sprouty homolog 1 (Spry1), which plays a vital role in kidney development and remodeling, was targeted by miR-29c. Spry1 is considered to be a negative regulator of Rho kinase through the noncanonical Wnt signaling pathway. Several studies have reported that the inhibition of Rho kinase reduced albuminuria and mesangial matrix accumulation in experimental diabetes. High glucose downregulated Spry1 protein expression through the upregulation of miR-29c in podocytes, leading to apoptosis. Consistent with these observations, specific inhibition of miR-29c significantly reduced the high glucose-mediated induction of apoptosis in podocytes. In addition, miR-29c knockdown db/db mice exhibited decreased albuminuria through the inhibition of apoptosis, mesangial matrix accumulation, and increased fibronectin protein expression in glomeruli.

【miR-200】

The miR-200 family consists of five species (-200a, -200b, -200c, -429, and -141) encoded by two separate genomic loci on chromosome 1 (Bracken et al., 2015). The mechanism through which the miR-200 family protects against renal fibrosis may involve prevention of tubular epithelial-to-EMT in proximal tubule epithelial cells (pTECs). Several studies have focused on the role of miR-200 and tubular EMT (Korpal et al., 2008; Oba et al., 2010; Wang et al., 2011; Patel and Noureddine, 2012; Xiong et al., 2012).

MiR-200a and miR-141 levels were found to be downregulated very early in the kidney of UUO mice. TGF-β mediated downregulation of the miR-200 family members is dependent on Smad signaling in pTECs. The protection against EMT by the miR-200 family is achieved by the direct targeting the zinc finger E-box-binding homeobox (ZEB) 1 and ZEB2 genes, which are transcriptional repressors of E-cadherin (Xiong et al., 2012). In contrast, the miR-200 family was upregulated in the UUO model,

with the induction of miR-200b being the most pronounced. Intravenous administration of miR-200b precursor improved renal fibrosis in UUO and increased the expression of both ZEB-1 and ZEB-2 (Oba et al., 2010).

Profibrotic MiRNA in Diabetic Kidney Disease

【miR-21】

MiR-21 has been widely investigated because several of its targets that are relevant to DKD and especially related to TGF-β were found to induce the activation of phosphoinositide 3-kinase-(PI3K-) AKT signaling (Godwin et al., 2010; Zhong et al., 2011). Moreover, it has been reported that TGF-β upregulated miR-21 expression in the liver, heart, lung, and kidney in mice and was involved in TGF-β-induced fibrosis in these tissues (Zavadil et al., 2007; Davis et al., 2008; Zhong et al., 2011; Loboda et al., 2016). TGF-β stimulation upregulated the expression of miR-21 in pTECs. Interestingly, Smad3, but not Smad2, was involved in the induction of miR-21 in response to TGF-β. Furthermore, mice deficient in Smad3 were found to be protected against the upregulation of miR-21 and renal fibrosis in the UUO model. Indeed, miR-21 expression and renal fibrosis were promoted in Smad2-knockout UUO mice. Gene transfer of a miR-21-knockdown plasmid was found to cease the progression of renal fibrosis in the UUO model. These results demonstrated that Smad3 signaling promoted the expression of miR-21 in the UUO mice (Zhong et al., 2011).

Dey et al. (2012) showed that phosphatase and tensin homolog (PTEN) acts as a target gene of miR-21 in human glomerular MCs. Upregulation of miR-21 by TGF-β stimulation downregulated the expression of PTEN, resulting in the activation of AKT and mammalian target of rapamycin complex 1, which regulated MC hypertrophy (Mahimainathan et al., 2006; Kato et al., 2009; Dey et al., 2012).

McClelland et al. (2015) reported that upregulation of miR-21 in the kidney was positively associated with the severity of fibrosis and renal dysfunction in patients with DKD. Using rat pTECs, they demonstrated that TGF-β promoted renal fibrosis by inducing miR-21 which in turn targets Smad7 and PTEN, the negative regulators of Smad3 and PI3K, respectively.

In diabetic KK-A^y mice, the expression of miR-21 was observed predominantly in cortical glomerular and renal proximal tubular cells. The expression of miR-21 was positively correlated with the urine albumin–creatinine ratio, as well as TIMP1, collagen IV, and fibronectin protein levels, and negatively correlated with the creatinine clearance ratio and MMP-9 protein levels (Wang et al., 2013).

Cell division cycle 25a (Cdc25a) and cyclin-dependent kinase 6 (Cdk6) were identified as targets of miR-21 in mouse MCs. MiR-21 directed the inhibition of Cdc25a and Cdk6 and led to MC hypertrophy via a mechanism that impaired cell cycle progression. Furthermore, miR-21 antagonism in a STZ-induced diabetic mouse model resulted in reduced fibrotic and inflammatory gene expression, as well as reduced mesangial expansion, podocyte loss, interstitial fibrosis, macrophage infiltration, and proteinuria (Kolling et al., 2017).

Liu et al. (2019) reported that bone morphogenetic protein 7 (BMP-7), a human recombinant protein, inhibited EMT and ECM synthesis and accumulation in rat renal tubular epithelial (NRK-52E) cells cultured under high glucose conditions. Moreover, injection of a BMP-7-overexpressing plasmid to STZ-diabetic mice caused a significant decrease in miR-21 expression and upregulated Smad7 expression, thereby leading to the prevention of EMT and ECM accumulation. These data support the view that the protective effect of BMP-7 against renal fibrosis in DKD is in part via regulation of miR-21 and Smad7 signaling.

The bioactive saponin Astragaloside IV (AS-IV), which is extracted from astragalus root, is known to have therapeutic effects on conditions such as liver fibrosis, DKD, and chronic medical heart failure (Gui et al., 2006; Wang et al., 2012b; Guo et al., 2017). Wang et al. demonstrated that AS-IV decreased the expression of miR-21 in cultured mouse MCs, mouse primary podocytes, and serum and kidney of diabetic KK-A^y mouse. In MCs and podocytes, overexpression of miR-21 enhanced signaling via the TGF-β/Smad and the β-catenin signaling pathways, which was abolished by AS-IV treatment. It was reported that AS-IV improved renal function and fibrosis by a mechanism that involved prevented increased miR-21 expression and thereby preventing podocyte dedifferentiation and MC activation in mice with DKD (Wang et al., 2018).

【miR-214】

High expression levels of miR-214 have been detected in human and animal models of kidney disease (Gomez et al., 2016). MiR-214 is cotranscribed with miR-199a as a single long noncoding RNA from an intron on the complementary strand of the dynamin-3 gene. The upregulation of both miR-214 and miR-199a is driven by the TWIST transcription factor and HIF-1-mediated hypoxia (Lee et al., 2009; el Azzouzi et al., 2013; Chen et al., 2014).

The antifibrotic effect was observed when the anti-miR-214 drug was administered to mice before the induction of UUO. In the UUO model, inhibition of canonical TGF-β signaling did not change endogenous miR-214 expression but blocked Smad2/3 activation. In contrast, treatment with miR-214 antagonist in mice did not prevent the activation of Smad2/3. Moreover, TGF-β inhibition when combined with deletion of miR-214 resulted is superior renal protection than miR-214 deletion alone. It was demonstrated that miR-214 has a fibrotic effect independent of Smad2/Smad3 activation (Denby et al., 2014).

Gene profiling revealed a significant upregulation of renal cortical miR-214 expression in diabetic db/db mice. In human embryonic kidney cells 293, PTEN was identified as a target of miR-214. Inhibition of miR-214 was observed to significantly decrease the expression of collagen IV, α-SMA, and SM22. In the same study, miR-214 inhibition also partially restored PTEN protein levels in human MCs under high glucose conditions as well as in db/db mice. Furthermore, this inhibition attenuated albuminuria and mesangial expansion in diabetic mice. Moreover, overexpression of PTEN was found to ameliorate

MC hypertrophy, whereas knockdown of PTEN promoted MC hypertrophy (Wang et al., 2016).

【miR-199a】

As previously mentioned, miR-199a is cotranscribed with miR-214. While several studies have investigated miR-199a and its relevance to tissue fibrosis, the role of miR-199a in kidney disease and particularly in DKD has not yet been established.

The expression of miR-199a-5p was found to be increased in TGF-β-stimulated lung fibroblasts, UUO mice, and mice with CCl4-induced liver fibrosis, suggesting that dysregulation of miR-199a-5p contributes to the fibrogenesis. *In vitro* studies have demonstrated that miR-199a-5p is a key downstream mediator of TGF-β signaling in lung fibroblasts where it targets caveolin-1, an important mediator of pulmonary fibrosis (Lino Cardenas et al., 2013).

Sun et al. (2015) demonstrated that miR-199a-5p expression was dramatically increased in the renal tissue of patients with autosomal dominant polycystic kidney disease (ADPKD), in the renal tissue of the rat ADPKD model, and in human ADPKD in the epithelial cell lining. The target gene involved here was found to be cyclin-dependent kinase inhibitor 1C (CDKN1C)/p57. Increased expression of miR-199a in the ADPKD renal tissue may promote cell proliferation through the suppression of CDKN1C.

Therapeutic Strategies for Diabetic Kidney Disease Using MicroRNAs

Dysregulation of TGF-β by resident renal cells and infiltrating inflammatory cells that are subject to stress in response to high glucose, angiotensin II, and reactive oxygen species, is a key factor contributing to renal fibrosis. TGF-β causes MC hypertrophy and proliferation, the induction of podocyte apoptosis and detachment from the glomerular basement membrane, ECM synthesis and accumulation, and other structural and functional changes in the kidney (Wu and Derynck, 2009; Boor and Floege, 2011; Rask-Madsen and King, 2013; Meng et al., 2016; Ma et al., 2019). Drugs targeting signal transduction pathways such as TGF-β have been developed for the treatment of DKD with limited success due to the important functions these pathways exert in normal physiology. As detailed earlier, several miRNAs have been implicated in the development and progression of DKD. Recent efforts were focused on applying the current knowledge regarding miRNA structure and function to develop novel miRNA therapeutics for DKD. Novel strategies were focused on inhibiting those miRNAs that are induced by DKD or increasing the expression of renoprotective miRNA (Lennox and Behlke, 2011; Trionfini et al., 2015; Lima et al., 2018).

MiRNA mimics for therapeutic use are designed to mimic the endogenous miRNA. They are double-stranded synthetic oligonucleotides that are processed in cells to mimic the endogenous function of miRNA, with improved stability and chemical modifications that enable efficient delivery and entry into target cells. The inhibition of endogenous miRNA may be achieved by introducing anti-miRNA oligonucleotides that target pri-miRNA, pre-miRNA, or mature miRNA to sequester or remove endogenous miRNA (Weiler et al., 2006; Kato et al., 2016). Although miRNAs are generally considered to be stable, individual miRNAs can rapidly decay in certain cellular environments (Trionfini et al., 2015). Several modifications have been made to increase RNA stability *in vivo*, which include 1) replacing the phosphodiester backbone with a phosphorothioate backbone, 2) ribose 2′-OH group, 3) locked nucleic acid modifications, and 4) peptide nucleic acid modification (Lennox and Behlke, 2011; Cao et al., 2019).

MiRNA may be a novel and attractive target for the treatment of DKD; however, several obstacles must be overcome to move miRNA-based therapies into clinical trials. Targeting miRNAs to the kidney remains a significant challenge in order to avoid potential unwanted effects in other tissues and organs, as well as off-target effects. Using miRNA mimics or inhibitors *in vivo* is considered to be a promising therapeutic strategy for the treatment of DKD. In fact, successful delivery of mimics and inhibitors to the kidney has been achieved via intravenous and subcutaneous injections (Trionfini et al., 2015).

Miravirsen, an anti-miR-122, is the first drug targeted for miRNA, and a phase II trial in patients with hepatitis C virus infection has been conducted. The use of Miravirsen in patients with chronic HCV genotype 1 infection prolonged reduction of HCV RNA levels (van der Ree et al., 2016). With further developments in this area, it is envisaged that targeting various miRNAs would be introduced to clinical practice as a nephroprotective treatment approach in the future.

CONCLUSION

DKD is a major complication of diabetes and a leading cause of ESRD. It is a complex multifactorial disease, which involves several physiological pathways leading to fibrosis. In recent years, various therapeutic agents targeting fibrosis have been investigated for DKD treatment, and some clinical trials have been conducted; however, no useful therapeutic agent has been found till date. MiRNA profiling may provide a better understanding of the complex pathways of DKD progression, and inhibition or overexpression of miRNA may lead to miRNA-based therapeutics in the future.

AUTHOR CONTRIBUTIONS

HS wrote and edited the manuscript. SH drafted and wrote and edited the manuscript. PK edited and revised the manuscript. TG reviewed and edited and revised the manuscript. YS reviewed and edited the manuscript.

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Knockdown of LncRNA-H19 Ameliorates Kidney Fibrosis in Diabetic Mice by Suppressing miR-29a-Mediated EndMT

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Diabetic nephropathy is the leading cause of kidney fibrosis. Recently, altered expressed or dysfunction of some long non-coding RNAs (lncRNAs) has been linked to kidney fibrosis; however, the mechanisms of lncRNAs in kidney fibrosis remain unclear. We have shown that the DPP-4 inhibitor linagliptin can inhibit endothelial-mesenchymal transition (EndMT) and ameliorate diabetic kidney fibrosis associated with DPP-4 protein levels via the induction of miR-29. Here, we found that expression of the lncRNA H19 was significantly up-regulated in TGF-β2-induced fibrosis in human dermal microvascular endothelial cells (HMVECs) *in vitro*, and in kidney fibrosis of streptozotocin-induced diabetic CD-1 mice. We also detected up-regulated H19 expression and down-regulated miR-29a expression in the early and advanced mouse models of diabetic kidney fibrosis. H19 knockdown significantly attenuated kidney fibrosis *in vitro* and *in vivo*, which was associated with the inhibition of the EndMT-associated gene FSP-1. We also found that the up-regulation of H19 observed in fibrotic kidneys associated with the suppression of miR-29a in diabetic mice. H19, miR-29a, and EndMT contribute to a regulatory network involved in kidney fibrosis, and are associated with regulation of the TGF-β/SMAD3 singling pathway. This study indicates that inhibition of LncRNA H19 represents a novel anti-fibrotic treatment for diabetic kidney diseases.

Keywords: TGF-β/SMAD3 singling, kidney fibrosis, long non-coding ribonucleic acid-H19, endothelial-mesenchymal transition, microRNA-29a

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INTRODUCTION

Diabetic nephropathy (DN) is a major cause of morbidity and mortality in patients with both type I and type II diabetes mellitus and is the leading cause of end-stage renal disease worldwide (Loeffler and Wolf, 2015). Kidney fibrosis is usually the final outcome of many renal diseases, of which DN is the leading cause (Kanasaki et al., 2013). Many cellular and molecular events occur in kidney fibrosis such as the activation of interstitial fibroblasts, phenotypic conversion of tubular epithelial and endothelial cells, extracellular matrix (ECM) overproduction, and microvascular dysfunction (Eddy and Neilson, 2006). Our previous studies shown that the endogenous antifibrotic peptide N-acetyl-

seryl-aspartyl-lysyl-proline (AcSDKP), the substrate of angiotensin-converting enzyme (ACE), is an orally available peptide drug used to cure kidney fibrosis in diabetic mice. AcSDKP treatment can restore the level of anti fibrosis miRNAs in diabetic mice, such as miR-29s and let-7s (Nitta et al., 2016).

DPP-4 inhibitors have been introduced into the market as antidiabetic drugs. We have found that the DPP-4 inhibitor linagliptin ameliorated kidney fibrosis in diabetic mice without altering the blood glucose levels associated with the inhibition of EndMT and the restoration of microRNA (miR) -29s (Kanasaki et al., 2014). However, whether there are other RNA mechanisms underlying diabetic fibrosis remains largely unclear.

Long non-coding RNAs (lncRNAs) are defined as transcripts longer than 200 nucleotides with little or no protein-coding ability (Ma et al., 2013) and have been reported to participate in a lot of biological and pathological processes such as carcinogenesis and chronic diseases including DN (Briggs et al., 2015; Huarte, 2015; Uchida and Dimmeler, 2015; Li et al., 2018). It has been reported that lncRNAs might function as competing endogenous RNAs (ceRNAs) to regulate the expression of miRNAs (Ma et al., 2013). H19 is a 3 kb lncRNA expressed in the nucleus and cytoplasm and is highly expressed in embryogenesis. The expression of H19 is significantly increased in some diseased conditions (Bartolomei et al., 1991; Matouk et al., 2007; Dudek et al., 2010) and it has been reported to play an important role in renal development (Okamoto et al., 1997).

Xie found that H19 expression was significantly upregulated in TGF-β2-induced HK-2 cell fibrosis and in unilateral ureteral obstruction (Xie et al., 2016). Our preliminary study showed that EndMT and the restoration of miR-29s is associated with TGF-β2-induced kidney fibrosis (Kanasaki et al., 2014). Whether there is a further connection between H19, EndMT, and other signaling pathways remains unclear. Herein, we explored the therapeutic potential and possible mechanisms of H19 in kidney fibrosis in a streptozotocin (STZ) induced diabetic mouse model, examining the mechanism of H19 in kidney fibrosis in association with miR-29a-mediated EndMT.

MATERIALS AND METHODS

Animal Model and Treatment

All animal experimental procedures were approved by the Ethics Committee of the affiliated Hospital of Southwest Medical University. Eight-week-old male CD1 mice (Dossy Laboratory Animal Co. Ltd., Chengdu, China) were administered with a single intraperitoneal injection of streptozotocin (STZ) (200 mg/kg); control mice were injected with citrate buffer. Two weeks after the STZ injection, mice with blood glucose levels >16 mmol/L were confirmed as valid diabetic mice and used for this study. The mice were divided into the following three groups: control, DM and H19 knockdownt group.24 weeks after the initiation of diabetes, the mice were sacrificed. Kidney tissues were isolated and then stored at −80°C for histological, RNA and protein analysis.

Cell Culture

Human dermal microvascular endothelial cells (HMVECs, Lonza, Basel, Switzerland) were cultured in EGM (Lonza, Basel, Switzerland) containing 10% fetal bovine serum (FBS, Gibco) in a regular CO2 incubator at 37°C under 5% CO2/ 95% air. When HMVECs reached 70% confluence, they were treated with 5 ng/ml recombinant human TGF-β2 (Abcam, Cambridge, UK) for 48 h to induce fibrosis.

Transfection

A specific duplex small interfering RNA (siRNA) and a short hairpin RNA (shRNA) against H19, with their respective AAV vectors were synthesized by Vigene Biosciences (Jinan, Shandong, China). CD-1 mice were injected with AAV- shH19 at a dose of 2×10^{12} viral genome particles per animal through the tail vein using an insulin syringe and a 30-gauge needle. Mice were sacrificed 4 weeks later. The expression of H19 was analyzed using quantitative real time PCR (qPCR). For *in vitro* transfection studies, HMVECs were passaged in 6-well plates with growth medium; they were then transfected with 100 nM shRNA and an antagomiR against miR-29a using Lipofectamine 2000 transfection reagent (Jinan, Shandong, China), according to the manufacturer's instructions. HMVECs were transfected with shH19 followed by treatment with TGF-β2 (5 ng/ml) for 48 h to induce fibrosis. The sequences of shH19: GGATCCAGCAAGAGCAGAA. The sequences of mimetics for miR29s: 29a-3p: UAGCACCAUCUGAAAUCG GUUA, 29b-3p: UAGCACCAUUUGAAAUCAGUGUU, 29c-3p: UAGCACCAUUUGAAAUCGGUUA. The sequences of antagomiR for miR29s: 29a-3p: UAACCGAUUUCAGAUGGU GCUA, 29b-3p: AACACUGAUUUCAAAUGGUGCUA, 29c-3p: UAACCGAUUUCAAAUGGUGCUA.

Immunofluorescence

Frozen kidney sections (5 μm) were used for immunofluorescence and the number of double positive cells labeled for FSP-1 (cat. no. ab197896; Abcam) and CD31 (cat. no. ab9498; Abcam) were measured. Briefly, frozen sections were dried and placed in acetone for 10 min at −30°C. Once the sections were dried, they were washed twice in phosphate-buffered saline (PBS) for 5 min and then blocked in 2% bovine serum albumin/PBS for 30 min at room temperature. Thereafter, the sections were incubated in primary antibody (1:400) for 1 h and washed in PBS (5 min) three times. Next, the sections were incubated with the secondary antibodies (1: 600) for 30 min, washed with PBS three times (5 min each), and mounted with mounting medium containing DAPI. The immunolabeled sections were analyzed with an Olympus fluorescence microscope (Olympus Corporation, Beijing, China).

Histology

Mouse kidney specimens were processed for further investigation. The tissues were fixed in 4% paraformaldehyde solution, dehydrated with a series of graded ethanol and embedded in paraffin. Sections (10 μm thick) were stained with hematoxylin and eosin (H&E) and Masson's trichrome staining (MTS) then photographed under an optical microscope (Leica Imaging Systems, Cambridge, United Kingdom). Masson's trichrome labeled sections were imaged and analyzed with ImageJ software, and fibrotic areas were quantified.

RNA Isolation and Quantitative Real Time PCR

Total RNA was extracted from renal tissue or HMVECs using Trizol reagent (Foregene, Chengdu, China). Reverse transcription was performed using the Premix RT EasyTM II (With gDNase) (Foregene, Chengdu, China). All qPCR experiments were performed using SYBR Green real time qPCR Master Mix (Foregene, Chengdu, China) on a Bio-Rad CFX Connect Real Time qPCR Detection system (Bio-Rad Laboratories, Inc.). For the qPCR reactions, two ul cDNA was added to a 20 μl reaction mixture containing 10 μl of 2 × Power SYBR Green qPCR Master Mix with 0.8 μl of each primer. The comparative Ct method was used to detect target gene expression in the test samples relative to control samples. All primers were synthesized by RIBOBIO (Guangzhou, China). 18S RNA level was used as a reference. The primers sequences: H19: 5'-AAGCAGATGGAACAGGTG GC-3' (forward) and 5'-CACAGCCAAACTGCCCAAAG-3' (reverse); miR 29s: miR-29a-3p: 5 -UAGCACCAUCUGAAA UCGGUUA, miR-29b-3p: 5i UAGCACCAUUUGAAAUCA GUGUU, miR-29c-3p: 5 UAGCACCAUUUGAAAUCGGUUA.

Western Blotting

Protein from renal tissues and HMVECs was extracted using protein lysis buffer (Beyotime Biotechnology Co., Ltd., Shanghai, China). Approximately 20 μg of protein lysates were separated on SDS-PAGE and blotted onto PVDF membranes using semidry transfer. After blocking with 5% BSA/TBST, the membranes were incubated with primary antibodies (1:1000) at 4°C overnight. The membranes were washed thrice by TBST and incubated with secondary antibodies (1:10000) for 1 h at room temperature. The rabbit polyclonal to CD31 antibody (cat.:ab9498; Abcam), rabbit polyclonal to alpha smooth muscle actin (cat: ab5694; Abcam), rabbit polyclonal anti-GAPDH (cat:ab8245; Abcam), rabbit polyclonal anti-TGFβ-receptor I (TGFβ R1) antibody (cat:ab31013; Abcam), rabbit polyclonal anti-TGFβ-receptor-II (TGFβ R2) antibody (cat:ab269279; Abcam), rabbit monoclonal anti-fibroblast specific proteins (FSP1, sometimes displayed as S100A4) antibody (cat:ab197896; Abcam), and rabbit anti-SMAD3 (phospho S423 + S425) antibody (cat:ab40854; Abcam) were purchased from Abcam (Cambridge, UK). The IRDye 800CW goat anti-rabbit IgG secondary antibody (cat:926-32211; LI-COR) was purchased from LI-COR (Nebraska, USA).

Wound Healing Assay

Wound healing assays were performed to evaluate the migration rate of HMVECs transfected with or without H19 shRNA. HMVECs were placed in six-well plates and using a pipette tip at an angle of 30°, each well received a straight scratch simulating a wound. After 24 and 48 h, the number of cells that had migrated into the wounded area was counted under a light microscope (Leica Imaging Systems, Cambridge, UK).

Cell Migration Boyden Chamber Assay

The bottom side of the migration chamber (cell culture insert; BD Falcon, San Jose, CA) was coated with Matrigel (BD Biosciences, US), and 1,000 HMVECs were passaged in the upper migration chamber. Twenty-four h after passage, the medium was changed to medium containing the transfection reagents in both the upper and the bottom wells. After 48 h, the cells were washed with PBS, followed by fixation with formaldehyde (3.7% in PBS) at room temperature for 2 min. After washing twice with PBS, the cells were permeabilized with 100% methanol for 20 min at room temperature. Then, cells were washed twice with PBS and stained with H&E. After scraping off the nonmigratory cells (upper well) with a cotton swab, the number of migrated cells was counted under a light microscope (Leica Imaging Systems, Cambridge, UK).

Assessment of Urinary Albumin and Creatinine Concentrations

Urinary albumin concentration was measured using a Mouse Albumin ELISA quantitation kit (E90-134; Bethyl Laboratories Inc; Montgomery, TX, USA). Assay was conducted according to the manufacturer's protocol. Urinary creatinine levels were measured using a CREP2 kit (Roche Diagnostics, Meylan, France) according to an established protocol. The urinary albumin to creatinine ratio was calculated.

Assessment of Serum Creatinine

The concentration of serum creatinine was detected using a creatinine assay kit (cat. no. C011-1; Nanjing Jiancheng Bioengineering Institute). Assay was conducted according to the manufacturer's protocol.

Glomerular Filtration Rate

Mice were anesthetized with isoflurane and a miniaturized imager device (Mannheim Pharma and Diagnostics, Mannheim, Germany) was mounted onto the animald) back. The skin background signal was recorded for 5 min before intravenous injection of 150 mg/kg FITC-sinistrin (Mannheim Pharma and Diagnostics, Germany). Then, *trans*-cutaneous fluorescence was recorded for 1 h in conscious animals. GFR (ml/min.g.Kw) was calculated from the decrease in fluorescence intensity over time (ie, plasma half-life of FITC-sinistrin) and an empirical conversion factor using the MPD Lab software (Mannheim Pharma and Diagnostics, Germany). Results are means ± SEM.

Statistical Analysis

The data are expressed as means ± S.E.M. A one-way ANOVA followed by a Tukey's multiple comparison test was used to determine significance which was defined as $P < 0.05$, if not otherwise noted. GraphPad Prism software (Ver 7.0) was used for the statistical analysis.

RESULTS

H19 Expression Was Significantly Up-Regulated in TGF-β2-Induced HMVEC and in the Fibrotic Kidneys of Streptozotocin-Induced Diabetic CD-1 Mice

To determine the pathological significance of H19, we analyzed STZ-induced diabetic male CD-1 mice, a murine model with

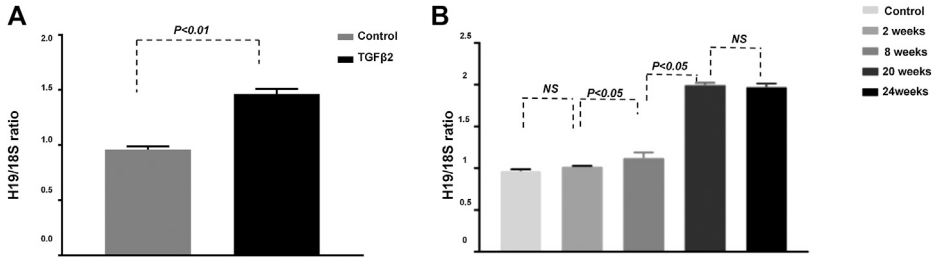

FIGURE 1 | H19 expression is significantly up-regulated in TGF-β2-induced fibrosis *in vitro* and in the kidney of streptozotocin-induced diabetic CD-1 mice. **(A)** qPCR analysis of H19 expression in HMVECs; **(B)** qPCR analysis of H19 kidney expression at different time points after the initiation of diabetes . The data are presented as mean ± SE in each group (n = 5) of three independent experiments.

extensive diabetes-associated kidney fibrosis, and TGF-β2-induced HMVECs (Sugimoto et al., 2007). Our qPCR analysis showed that H19 expression was significantly higher in HMVECs treated with TGF-β2 (**Figure 1A**). To further investigate the role of H19 in the progression of kidney fibrosis, we analyzed the expression of H19 at different time points after the initiation of diabetes (**Figure 1B**). We found that in the early period of fibrosis, there was no difference in H19 expression in the kidneys of control and STZ mice; however, after 8 weeks the expression of H19 was significantly higher in the kidneys of STZ mice when compared with control mice, which exhibiting a time dependence. We show that 20 weeks after the initiation of diabetes, the kidneys exhibited serious fibrosis; however, our data showed that the expression of H19 was not different between the 20 and 24 weeks. These data revealed that H19 expression was associated with the progress and severity of kidney fibrosis.

H19 Knockdown Significantly Attenuated Kidney Fibrosis in the Diabetic Kidney

To further examine the potential relationship between H19 and kidney fibrosis, we treated diabetic mice (DM) with H19 shRNA 20 weeks after the initiation of diabetes and 4 weeks later harvested their kidneys. Our qPCR results confirmed knockdown of H19 in DM treated with shRNA (**Figure 1A**). We performed H&E and MTS staining to evaluate fibrosis in the kidney. Twenty-four weeks after the initiation of diabetes mice exhibited severe fibrosis when compared with control mice and H19 shRNA-treated DM exhibited restored normal kidney structures (**Figure 1B**). Our morphometric analysis of the kidneys revealed that DM displayed significantly enlarged glomeruli (**Figure 1C**), mesangial expansion (D), and relatively large areas of Masson's trichrome–positive interstitial fibrosis (E), whereas restored normal kidney histology and normal architecture were seen in H19 shRNA treated DM mice. The glomerular functional assays such as Albumin Creatinine ratio (**Figure 1F**), Glomerular Filtration rate (G) and Serum Creatinine ratio (H) also support the result.

H19 Knockdown Ameliorated Kidney Fibrosis Was Associated With the Suppression of EndMT

Our previous study showed that EndMT plays an important role in kidney fibrosis (Kanasaki et al., 2014). The inhibition of the EndMT associated gene FSP-1 ameliorated kidney fibrosis *in vivo* and *in vitro*. To confirm the connection between H19 and EndMT, we analyzed EndMT in the kidney of H19 shRNA treated DM mice. Western blot analysis showed the expression of the endothelial marker CD31 was suppressed and the mesothelial cell marker FSP-1 was induced in DM compared with control mice, suggesting the induction of EndMT in the DM kidney; however, when the DM were treated with H19 shRNA, EndMT was repressed (**Figure 2A**). Immunofluorescence results for FSP-1 (green) and CD31 (red) were in agreement with the western blot data (**Figure 2B**). Furthermore, we found that TGF-β2 induced EndMT was suppressed by H19 knockdown in HMVECs (**Figure 2C**). These data revealed that H19 knockdown ameliorated kidney fibrosis is associated with the suppression of EndMT *in vivo* and *in vitro*.

We previously showed that EndMT in kidney fibrosis in mediated by miRNA-29 family members (Kanasaki et al., 2014). Whether the protective role of H19 knockdown in kidney fibrosis is related to miRNA-29 family member regulation remains unknown. We therefore confirmed the expression of the miRNA-29 family members *in vivo* and *in vitro* and found that their expression was suppressed in the diabetic kidney, in agreement with our previous research. However, only the expression of miR-29a was restored with H19 shRNA. There was no significant difference in miR-29b and miR-29c expression with or without H19 knockdown (**Figures 3A–C**). *In vitro*, we also found that TGF-β2 suppressed miRNA-29a could be restored by H19 shRNA in HMVECs, while miR-29b and miR-29c could not (**Figures 3D–F**). Furthermore, we confirmed the levels of H19 with individual miRNA-29 family member knockdowns; we found that TGF-β2 induced higher H19 expression could only be suppressed with knockdown of miR-29a in HMVECs but not with miR-29b and miR-29c knockdown (**Figures 3G–M**). These results confirmed that the H19 knockdown mediated kidney fibrosis was associated with miR-29a-mediated EndMT.

H19 Knockdown Inhibits TGFB/SMAD3 Signaling in the Diabetic Kidneys

Many researches have shown that targeting TGF-β/SMAD3 signaling may represent a specific and effective therapy for kidney fibrosis (Meng et al., 2015; Song et al., 2016). Our

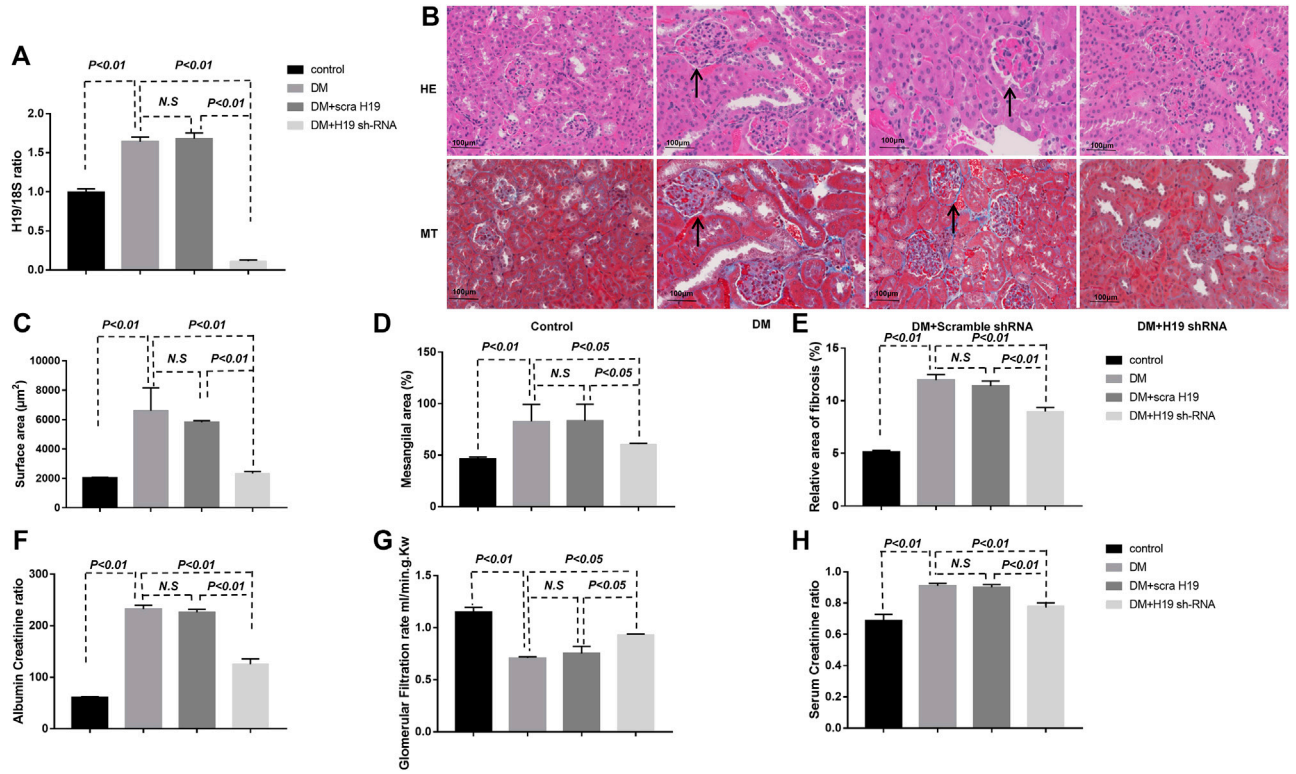

FIGURE 2 | H19 knockdown significantly attenuates fibrosis in the diabetic kidney. **(A)** qPCR analysis of H19 expression in diabetic mice treated with shRNA; **(B)** Representative images of hematoxylin and eosin, and Masson's trichrome staining used to evaluate fibrosis in the kidney. Scale bar: 100 μm. **(C)**–**(E)** Morphometric analysis of kidney histology. **(F)** Albumin Creatinine ratio, **(G)** Glomerular Filtration rate (ml/min.g.Kw), **(H)** Serum Creatinine ratio. The data are presented as mean ± SE in each group (n = 5) of three independent experiments.

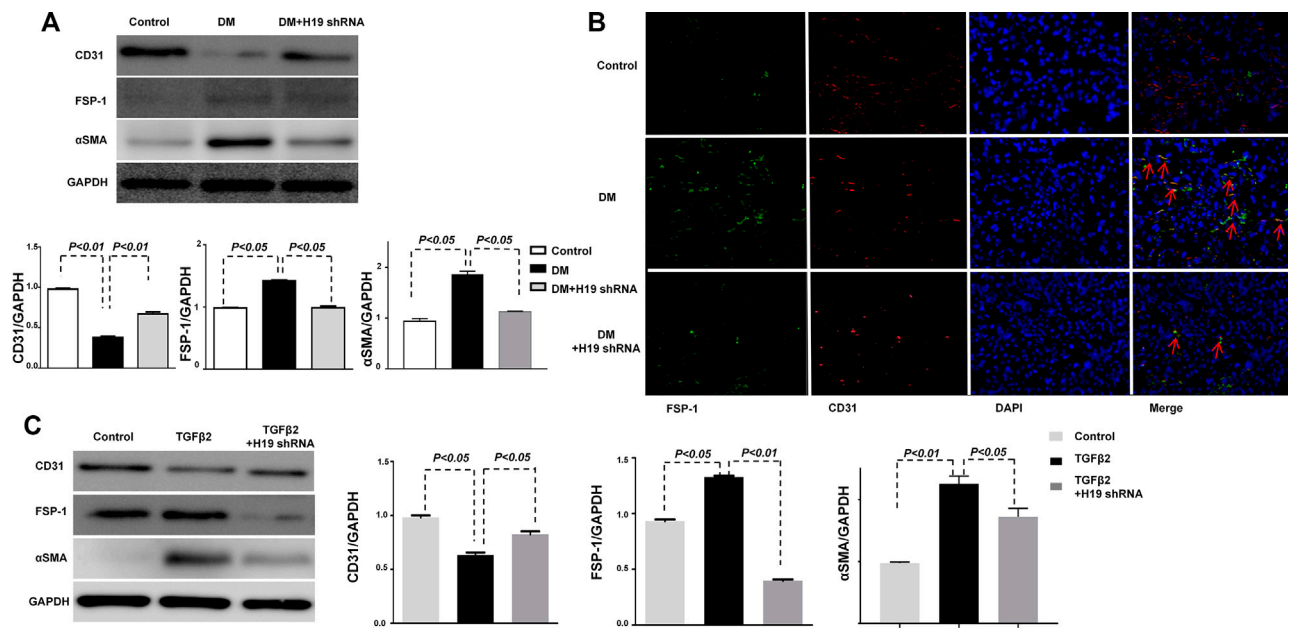

FIGURE 3 | Ameliorated kidney fibrosis by H19 knockdown is associated with the suppression of the EndMT associated gene FSP-1. **(A)** Western blot analysis of CD31, FSP-1, α-SMA, and GAPDH *in vivo*; **(B)** Immunofluorescence analysis of CD31 and FSP-1. The original magnification was ×400. Scale bar: 100 μm in each panel. **(C)** Western blot analysis of CD31, FSP-1, α-SMA and GAPDH *in vitro*. The data are presented as meanent in each group (n = 5) of three independent experiments.

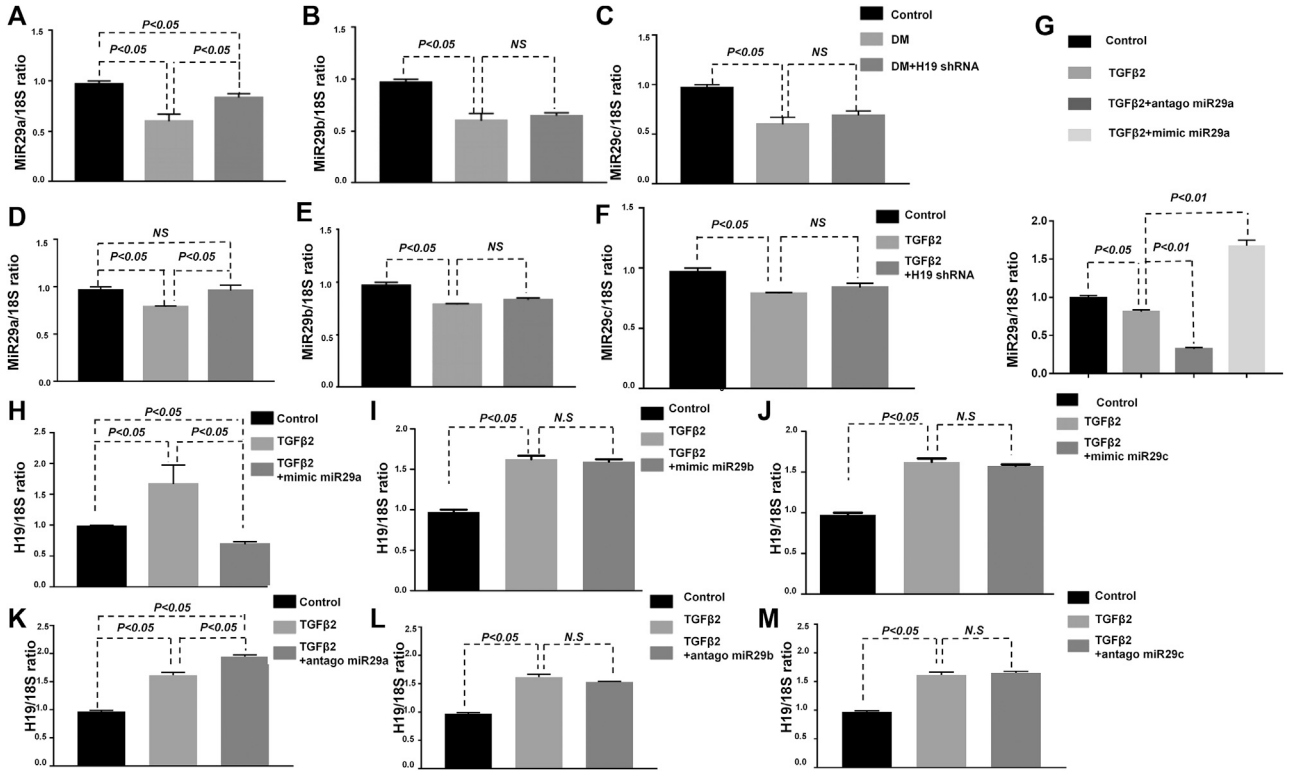

FIGURE 4 | H19 knockdown ameliorated kidney fibrosis was associated with miR-29a. **(A)–(C)** qPCR analysis of miRNA 29a/b/c expression *in vivo*; **(D)–(F)** qPCR analysis of miRNA 29a/b/c expression *in vitro*; **(G)** qPCR analysis of miRNA 29a expression with diffreent treated. qPCR analysis of lncRNA-H19 expression when **(H)–(J)** knockdown miRNA-29a/b/c (the antagonist of miRNA 29a/b/c) and **(K)–(M)** over expression miRNA-29a/b/c (the mimic of miRNA 29a/b/c) *in vitro*. The data are presented as meanent in each group (n = 5) of three independent experiments.

research also confirmed that DPP-4 inhibitors ameliorate kidney fibrosis via TGF-β/SMAD3 signaling modulation (Kanasaki et al., 2014; Shi et al., 2015; Shi et al., 2016). Here, we analyzed TGF-β/SMAD3 signaling *in vivo* and vitro. Western blot analysis revealed that the expression of TGFβR1, TGFβR2 and *p*-SMAD3 in the DM kidney were significantly induced when compared with control mice; however, expression was restored to control levels when DM were treated with H19 shRNA (**Figures 4A–D**), suggesting that STZ-induced TGF-β/SMAD3 signaling was suppressed by H19 knockdown. TGF-β2 induced TGF-β/SMAD3 was similarly suppressed by H19 knockdown in HMVECs (**Figures 4E–H**). Furthermore, wound healing cell invasion assays revealed that TGF-β2 induced the migration of HMVECs, while H19 knockdown inhibited their invasion (**Figures 4I,J**). The Boyden chamber cell migration assay also revealed that H19 knockdown inhibited endothelial cell transmigration through Matrigel (**Figures 4K,L**). These data reveal that TGF-β/SMAD3 signaling may be the key pathway in the protective role for kidney fibrosis in H19 knockdown in DM.

DISCUSSION

In this research, our preliminarily data confirmed that H19 expression was significantly up-regulated in TGF-β2-induced HMVEC fibrosis and in the fibrotic kidneys of STZ induced diabetic CD-1 mice. H19 knockdown significantly attenuated kidney fibrosis *in vitro* and *in vivo*, which was associated with the inhibition of the EndMT associated FSP-1. We also found that the up-regulated H19 observed in diabetic kidneys may be associated with suppressed levels of miR-29a in DM. H19, miR-29a, and EndMT contribute to a regulatory network involved in kidney fibrosis, all of which were associated with the regulation of the TGF-β/SMAD3 singling pathway.

LncRNA, initially thought to be transcriptional noise, have been intensely studied in recent years and they have been found to participate in gene expression, mammalian development, and various disease processes (Ponting et al., 2009; Caley et al., 2010). Several lines of evidence indicate that lncRNAs are responsible for renal cell apoptosis in DN (Kato et al., 2016; Long et al., 2016; Chen et al., 2017; Tsai et al., 2018). Recent evidence demonstrates that lncRNAs also mediate renal fibrosis in DN, such as the lncRNA NEAT1 and lncRNA ASncmtRNA-2 which induce kidney fibrosis in DN, and 1700020I14Rik and lncRNAGm4419 which attenuate kidney fibrosis in DN (Gao et al., 2017; Yi et al., 2017; Huang et al., 2019). In this study, we found that H19 knockdown can attenuate kidney fibrosis *in vivo* and *in vitro*. Xie et al. (2016) also found that H19, along with miR-17 and fibronectin, contributed to a regulatory network involved in renal fibrosis.

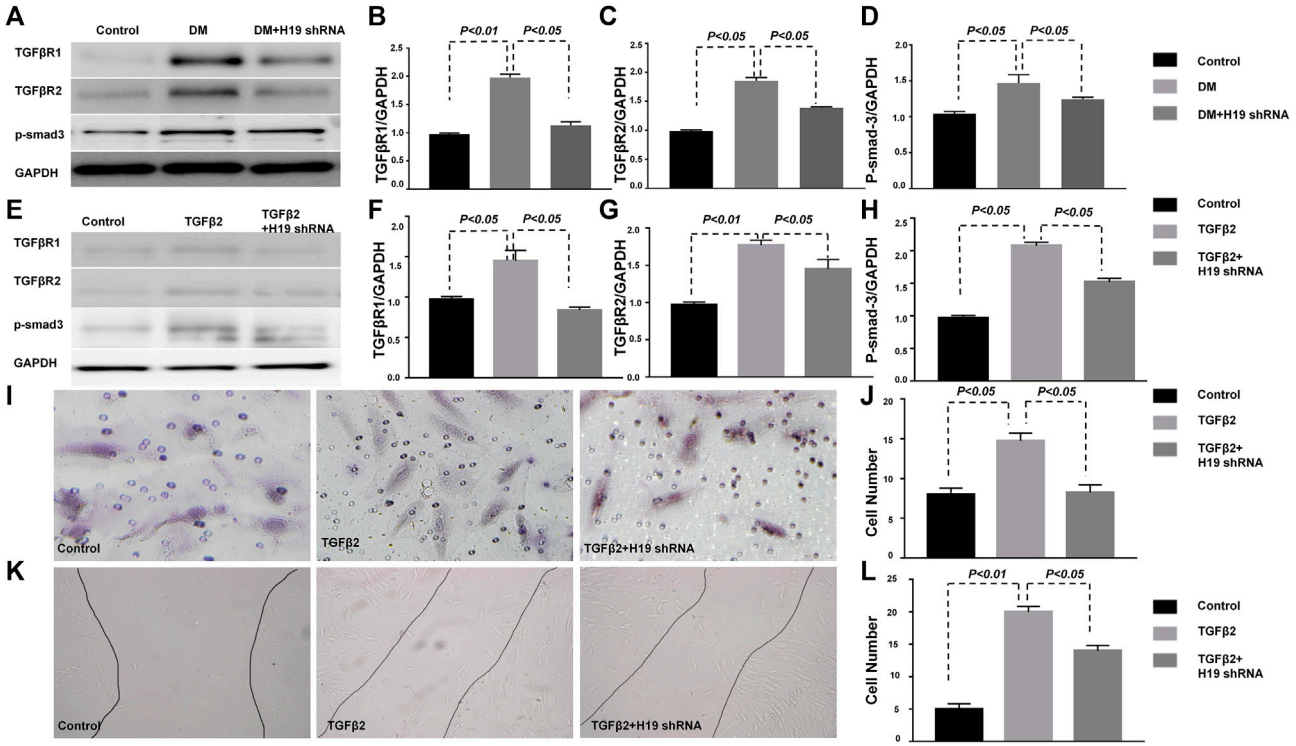

FIGURE 5 | TGF-β/SMAD signaling protects the kidney from fibrosis in H19 knockdown in HMVECs. **(A)–(D)** Western blot analysis of TGFβR1, TGFβR2, *p*-SMAD3 and GAPDH in the kidney; **(E)–(H)** Western blot analysis of TGFβR1, TGFβR2, *p*-SMAD3 and GAPDH in HMVECs; **(I)–(J)** Wound healing cell invasion assay; **(K)–(L)** The Boyden chamber cell migration assay. The data are presented as meanent in each group (n = 5) of three independent experiments.

Inhibition of kidney fibrosis is a fundamental process in research on developing therapies against kidney disease, although kidney fibroblasts have been implicated in kidney fibrosis pathogenesis, inoculating only kidney fibroblasts as therapeutic targets would be challenging. The inhibition of kidney fibrosis and the restoration of normal kidney structure are fundamental processes to combat the progression of DN. Our previous research found that EndMT is very important in the progression of kidney fibrosis (Kanasaki et al., 2014). In our analysis, the expression of H19 was induced in the diabetic kidney in a time dependent manner and was associated with the progress and severity of kidney fibrosis. H19 knockdown inhibited kidney fibrosis and restored normal kidney structure. These data confirmed that EndMT is a key factor in the progress of kidney fibrosis. We know that mRNA, miRNA, and lncRNAs can communicate with each other by competing for shared miRNA targets (Tay et al., 2014; Srivastava et al., 2016). To further examine the mechanism of H19 in fibrosis, we analyzed the miR-29 family members which have been shown to have an antifibrotic role in DN (Denzler et al., 2014; Kanasaki et al., 2014; Srivastava et al., 2016). We revealed that H19 knockdown can restore the suppressed miR-29a in the diabetic kidney and in TGF-β2-fibrosis-induced HMVECs. The TGF-β/SMAD signaling pathway being key pathway to both. Thus, H19, miR-29a, and EndMT contribute to a competing endogenous RNA regulatory network. This regulatory network maintained a relative balance to avoid abnormal kidney fibrosis. When H19 was induced in kidney fibrosis, elevated H19 expression could alleviate the repressive effects of miR-29a and lead to increased EndMT associated gene expression, which is a target gene of miRNA-29 family. Similar H19 regulatory mechanisms have previously been reported such as the finding that the H19/miR-675 pathway inhibited cell growth and Igf1r expression (Keniry et al., 2012); H19/Let-7-mediated inhibition on the target HMGA2-mediated epithelial to mesenchymal transition (Ma et al., 2014); and the H19/miR-675 axis inhibits prostate cancer metastasis via affecting TGF-β1 expression (Zhu et al., 2014). Thus, H19 may act as a competitive endogenous RNA. The regulatory network integrates the transcriptional and posttranscriptional regulatory network of kidney fibrosis.

In summary, our findings reveal high expression of the lncRNA H19 in the diabetic kidney and in TGF-β2 induced fibrosis in HMVECs. Inhibition of H19 attenuated kidney fibrosis and restored normal kidney structure (**Figure 5**). Interestingly, inhibition of H19 only altered miR-29a levels, not miR-29b or miR-29-c levels, inactived the TGF-β/SMAD pathway, in order to down-regulate EndMT, leading to the suppression of kidney fibrosis. All together our data suggest that suppression of H19 plays an anti-fibrotic role, which may serve as a novel therapeutic target for DN.

DATA AVAILABILITY STATEMENT

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

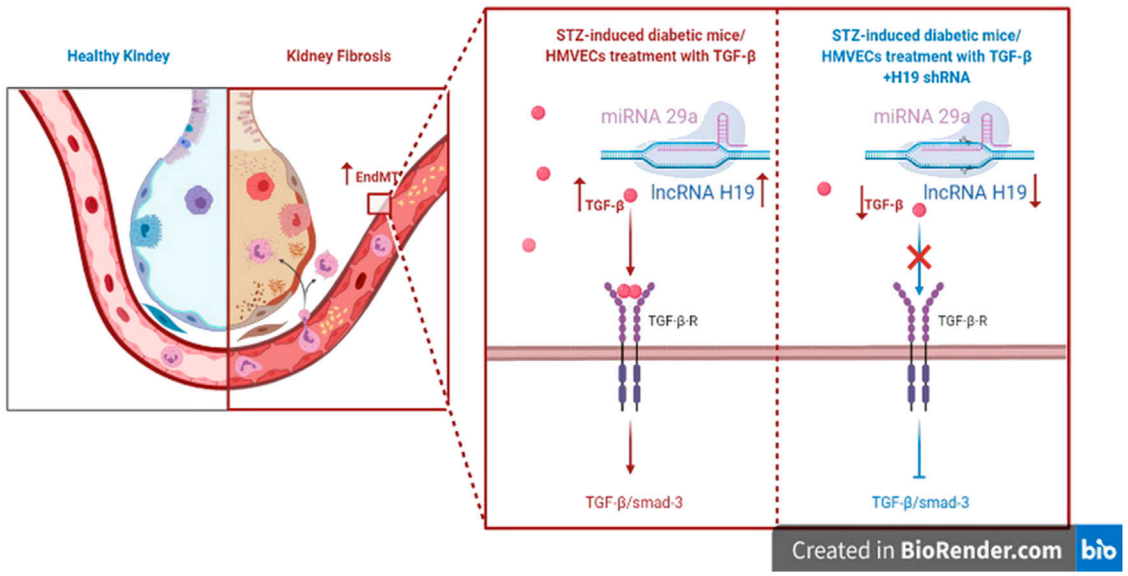

FIGURE 6 | Possible mechanism of inhibition of H19 attenuated kidney fibrosis. Inhibition of H19 up-regulate miR-29a levels, inactive the TGF-GFSMAD pathway and down-regulate EndMT, leading to the suppression of kidney fibrosis.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of the affiliated Hospital of Southwest Medical University.

AUTHOR CONTRIBUTIONS

SS performed the research and wrote the paper. LS contributed important reagents and assisted in writing the paper. HY, SF, JH, and YL contributed to the animal experiments, and collected and analyzed data. YH designed the research project.

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REVIEW
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Loss of Mitochondrial Control Impacts Renal Health

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Disruption of mitochondrial biosynthesis or dynamics, or loss of control over mitochondrial regulation leads to a significant alteration in fuel preference and metabolic shifts that potentially affect the health of kidney cells. Mitochondria regulate metabolic networks which affect multiple cellular processes. Indeed, mitochondria have established themselves as therapeutic targets in several diseases. The importance of mitochondria in regulating the pathogenesis of several diseases has been recognized, however, there is limited understanding of mitochondrial biology in the kidney. This review provides an overview of mitochondrial dysfunction in kidney diseases. We describe the importance of mitochondria and mitochondrial sirtuins in the regulation of renal metabolic shifts in diverse cells types, and review this loss of control leads to increased cell-to-cell transdifferentiation processes and myofibroblast-metabolic shifts, which affect the pathophysiology of several kidney diseases. In addition, we examine mitochondrial-targeted therapeutic agents that offer potential leads in combating kidney diseases.

Keywords: mitochdrial damage, mitochondrial sirtuins, renal damage, kidney fibrosis, diabetic kidney disease, glycolysis, fatty acid oxidation, polycystic kidney disease

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INTRODUCTION

Chronic kidney disease (CKD), which affects 10–15% of people, is a leading cause of death worldwide (Levin et al., 2017). Almost 75% of CKD incidents are related to diabetic kidney disease (DKD) and linked-hypertensive kidney disease (HKD) (Levin et al., 2017). Angiotensin-converting enzyme inhibitors (ACEis) and angiotensin II receptor blockers (ARBs) are two classes of anti-hypertensive agents that can effectively reduce the incidence of end-stage kidney disease and are first-line drugs for therapy in diabetic kidney disease (Laverman et al., 2004; Palmer et al., 2015; Gu et al., 2016; Srivastava et al., 2020a; Srivastava et al., 2020b). In addition, the renal protective nature of SGLT-2 inhibitors, DPP-4 inhibitors and statins has been studied in the mouse models and controlled clinical trials (Kanasaki et al., 2014; Edwards, 2016; Wanner et al., 2016; Bae et al., 2019; Hanssen and Jandeleit-Dahm, 2019). However, there remains a lack of efficacious drugs that can retard CKD or DKD (Zelnick et al., 2017). This lack of progress is likely due to poor understanding of the mechanisms of kidney diseases (Breyer and Susztak, 2016). Renal fibrosis is the final consequence of all types of progressive kidney disease, including DKD, that results in end-stage renal disease (ESRD) (Allison, 2019; Cooper and Warren, 2019). Renal fibrosis results in damage to normal cellular functions and structures and is a result of severe inflammation and loss of control over wound healing mechanisms which ultimately lead to an excess accumulation of extracellular matrix (ECM) and fibrosis-associated proteins (Srivastava et al., 2019b). Renal fibroblasts accumulation play a crucial role during fibrogenic processes however, the genesis of fibroblasts is not clear and is a matter of ongoing discussion (Srivastava et al., 2019b).

The primary functions of the kidneys are to balance electrolytes, acid-base status and to maintain water homeostasis and remove toxic substance from the body, all of which are highly energetic processes. Since catabolism of free fatty acids produces more ATP than does catabolism of glucose, kidney tubule segments are mostly dependent on fatty acid oxidation (FAO) and have enormous numbers of mitochondria; they utilize mitochondrial oxidative phosphorylation (OXPHOS) to supply their energy demands (Kang et al., 2015). Mitochondrial synthesis needs the expression of both nuclear- and mitochondrial-coded proteins (Tanaka et al., 2020). The production of cellular energy, in the form of ATP, is the primary function of this organelle (O'Rourke and Blatter, 2009; Kuhlbrandt, 2015). However, the mitochondrion also participates in calcium homeostasis, heat production, cell-signaling and apoptosis (O'Rourke and Blatter, 2009). The metabolic enzymes in the mitochondria are highly regulated by cellular energy status and play an important role in metabolic control (O'Rourke and Blatter, 2009).Doleris et al. reported mitochondrial cytopathy cases from patients who had glomerulosclerosis (Doleris et al., 2000). M2-to-M1 macrophages conversion had been observed in the ESRD patients and which are associated associated with metabolic shifts from oxidative phosphorylation to glycolysis, indicates that suppression of mitochondrial oxidative phosphorylation is positively linked with inflammation and CKD (Ravi et al., 2014; Quadri et al., 2019). Loss of control over mitochondrial biogenesis, mitochondrial function or regulation affects fibrogenic phenotypes in kidney cells (Qin et al., 2018; Srivastava et al., 2018; Chung et al., 2019). The association among renal function, FAO, and bio-energetics suggests that alterations in tubule cell metabolism lead to CKD, DKD and activation of fibrogenic events (Kang et al., 2015). Over-expression of peroxisome proliferator-activated receptor alpha (PPARa) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1a) in epithelial cells, or pharmacological activation of PPARa by fenofibrate were reported to be renal protective in several mouse models of renal fibrosis (Kang et al., 2015).

The Unifying hypothesis suggests that defects in mitochondrial oxidative phosphorylation is a shared pathway in the pathogenesis of microvascular complications of diabetes, including diabetic nephropathy and CKD progression (Brownlee, 2005) however, the various aspects and validity of this theory have been challenged and remain to be carefully addressed (Galvan et al., 2017).

The present review will asses the association between the loss of control over mitochondrial bioengergetics in CKD development and, provide new insights into the role of mitochondrial sirtuins in the regulation of kidney diseases. A comprehensive analysis and its underlying mechanisms offer future therapeutic approaches in the management of kidney diseases.

LOSS OF MITOCHONDRIAL CONTROL IN KIDNEY DISEASE

Evidence suggests that excessive production of mitochondrial ROS is linked to cellular damage and progression of renal disease (Galvan et al., 2017; Forbes and Thorburn, 2018). Autophagy plays an essential role in the homeostasis of diverse cell types including kidney endothelial cells. Autophagy defects in endothelial cells lead to IL-6 (interleukin 6)-dependent endothelial-to-mesenchymal transition (EndMT) and organ fibrosis with metabolic defects in mice (Takagaki et al., 2020). Mitophagy is the removal of damaged mitochondria and recycling of useful components. Identification of the Parkin–phosphoubiquitin complex, PINK–ubiquitin complex and prohibitin 2, a mitophagy receptor contribute to mitophagy (Kumar et al., 2017; Schubert et al., 2017; Wei et al., 2017). Mitophagy regulatory mechanisms can be ubiquitin-dependent or independent (Zachari and Ktistakis, 2020). In addition, mitochondrial dynamics are regulated by the PINK1–Parkin pathway for proteasomal degradation by targeting mitofusins (MFN) and Miro (outer mitochondrial membrane protein) (Shirihai et al., 2015). In receptor-mediated mitophagy, PHB2 (prohibitin 2) and cardiolipin interactiom play a crucial role in LC3 impairment (Wei et al., 2017; Zhou et al., 2020b). In diabetic nephropathy, mitochondrial debris accumulation has been observed in the kidneys, suggesting that defective clearance of abnormal mitochondria is associated with disease (Sheng et al., 2018; Zhang et al., 2018). Calpain10, which is a mitochondrial cysteine protease, is suppressed in streptozotocin-induced diabetic rats which activate PINK1 (Smith et al., 2012). Calpain10 suppression causes reduction in mitochondrial fusion and induction of mitochondrial fission and autophagy, suggesting that calpain10 negatively regulates mitochondrial autophagy in early diabetics (Smith et al., 2012). However, researchers believe that in early diabetes, removal of defective mitochondria takes place and mitochondrial autophagy is compensatorially increased, which is correlated with progression of diabetic nephropathy (Smith et al., 2012; Yamahara et al., 2013).

NLRP3 inflammatory bodies regulate the secretion of IL-1β and IL-18, which are the critical for the inflammatory response (Kelley et al., 2019). Activated NLRP3 is linked to the activation of caspase-1 (Kelley et al., 2019). This activated caspase-1 enhances the formation of IL-1β and IL-18 by cleaving pro-IL-1β and pro-IL-18 (Kelley et al., 2019). These interleukins are involved in both the inflammatory response and the innate immune response in the kidney cells. Higher ROS or mtDNA release activate NLRP3 inflammasome formation, whereas mitochondrial autophagy inhibits NLRP3 inflammasomes (Zhuang et al., 2015).

ER stress is characterized by alteration in calcium homeostasis, redox imbalance, impaired protein glycosylation and causes misfolded proteins to gather in the ER lumen (Molino et al., 2017). ER-mitochondria crosstalk and contact sites are crucial in autophagosome formation (Molino et al., 2017). The ER-derived mitochondria-associated membranes (MAMs) form contact sites between the ER and mitochondria (Molino et al., 2017). MAMs are involved in lipid biosynthesis, mitochondrial dynamics and bioenergetics, and autophagy (Molino et al., 2017). MAMs transmit stress signals from the ER to mitochondria (Molino et al., 2017). In MCD patients, ER stress and higher release of mitochondrial ROS accelerate the interstitial fibrosis (Lindenmeyer et al., 2008). The elevated ROS level, ER

dysregulation, and inflammasome are major factors in the development of renal fibrosis in diabetic nephropathy (Quadri et al., 2019).

LOSS OF MITOCHONDRIAL FUNCTIONS IN GLOMERULAR CELLS

Glomerular disease is often linked to mesangial cell proliferation and extracellular matrix deposition (Scindia et al., 2010). Increased mesangial proliferation leads to ECM accumulation and glomerular sclerosis (Scindia et al., 2010). Under high glucose conditions, excessive ROS level, is associated with a decrease in MnSOD activity, mtDNA copy number, mitochondrial membrane potential, and ATP production (Xu et al., 2012). Excessive ROS activate nuclear factor-κB signaling and activated protein-1 and induce TGFβ1 which is associated with inflammation, ECM synthesis, and glomerular sclerosis in diabetic kidneys (Jha et al., 2016). ROS promote mesangial cell proliferation and ECM synthesis by inducing ERK1/2) (Chen et al., 2018). Cyt bc1 complex inhibitor stigmatellin and the respiratory chain complex I inhibitor rotenone inhibit mesangial proliferation and its associated ECM synthesis by suppressing ROS production (Huang et al., 2009; Akool et al., 2012).

Podocytes, GBM and glomerular endothelial cells constitute the glomerular filtration barrier (Garg, 2018). Disruption in the permeability of the glomerular filtration barrier leads to proteinuria (Garg, 2018). The complex formed by nephrin, CD2AP, and podocin plays an important role in maintaining the homeostasis of the glomerular filtration barrier (Mallipattu and Kravets, 2020). Mutations in the mitochondrial gene A3243G cause podocyte injury such as abnormalities in podocyte mitochondria size and structure, aberrant podocyte cell bodies and foot process fusion (Hotta et al., 2001). The puromycin aminonucleoside-induced mouse model of glomerular sclerosis is associated with a defect in oxidative phosphorylation, suppressed mtDNA copy number, and downregulated expression of respiratory chain enzyme complex subunits (Hagiwara et al., 2006). Suppressed level of oxidative phosphorylation results in podocytes cell apoptosis (Zhou et al., 2019). Mitochondrial fission caused by high-glucose leads to effacement of podocyte foot processes, through Drp-1 phosphorylation by Rho-associated coiled-coil-containing protein kinase 1 (ROCK1) (Wang et al., 2012). In addition, podocytes have shown higher mTORC-associated autophagy levels while being unable to regenerate (Cinà et al., 2012) and, as a result of compensatory podocyte loss, parietal cells show fibrogenic responses (Hakroush et al., 2014).

LOSS OF MITOCHONDRIAL FUNCTION AND METABOLISM IN TUBULAR EPITHELIAL CELLS

Fibrosis in renal tubules is a final common outcome in all kinds of chronic kidney disease which lead to ESRD (Efstratiadis et al., 2009; Liu et al., 2018).TECs are highly susceptible to damage (Liu et al., 2018). Proteinuria, lipid loading, aberrant levels of cytokines, ischemia, hypoxia, and hyperglycemia can damage tubular functions (Liu et al., 2018). Injured TECs can undergo phenotypic transitions into mesenchymal cell phenotypes via epithelial-to-mesenchymal transition (EMT) (Grande et al., 2015; Lovisa et al., 2015; Srivastava et al., 2019a). During EMT events, altered sets of inflammatory cytokines disrupt normal TECs structure and lead to fibrosis (Grande et al., 2015; Lovisa et al., 2015; Srivastava et al., 2019a). mtDNA depletion and loss of control over mitochondrial function induce EMT process and the recovery of mtDNA and mitochondrial function can reverse the EMT phenotype via gain of endogenous E-cadherin, downregulation of α-SMA expression, and restoration of an epithelial cell phenotype (Yuan et al., 2012). In folic acid-induced and urinary obstruction renal fibrosis models, deterioration in mitochondrial structure and function can cause mitophagy and apoptotic necrosis, which lead to defective fatty acid metabolism and accelerate interstitial fibrosis (Kang et al., 2015; Bhargava and Schnellmann, 2017). Induction of transforming growth factor (TGF)-β signaling is involved in the pathogenesis of renal fibrosis (Meng et al., 2015; Chung et al., 2018). The TGFβ/Smad3 pathway plays an important role in EMT and EndMT events (Srivastava et al., 2013). EMT and EndMT processes are key phenomena in the formation of cancer-associated fibroblasts in diabetes (Amar et al., 2020; Srivastava and Goodwin, 2020).TGFβ impairs antioxidant status by enhancing pro-oxidant NADPH oxidase (Wan et al., 2016).

In contrary to previous findings (Hickey et al., 2011), found higher expression of key mitochondrial proteins from renal biopsies from diabetic nephropathy patients, suggesting mitochondrial biogenesis in renal fibrosis (Hickey et al., 2011). The level of c-AMP is positively related with mitochondrial copy numbers and ATP levels in the tubules (Ding et al., 2018). Restoring cAMP levels by rolipram, a phosphodiesterase (PDE4) inhibitor, improves kidney fibrosis by inhibiting the mitochondrial biogenesis pathway regulator C/EBP-β /PGC1-α (Ding et al., 2018). It was observed that monoallelic mutations in the gene encoding glycine amidinotransferase (GATM), a renal proximal tubular enzyme in the creatine biosynthetic pathway, caused the abnormal aggregation of GATM (Reichold et al., 2018).

The mitochondrial transcription factor A (TFAM) is the crucial human mtDNA binding protein which is involved in the expression and maintenance of mtDNA (Campbell et al., 2012). TFAM regulates metabolic activities by directly targeting PGC1a and PPARa (Scarpulla, 2008). Whole-body knockout of TFAM in mice is lethal (Larsson et al., 1998); however, tubule-specific knock out mice are associated with metabolic defects and kidney fibrosis (Chung et al., 2019). TFAM regulates the mitochondrial copy number and concentration of ATP (Chung et al., 2019). Loss of TFAM in the tubules results in cytokine activation and immune cell infiltration. TFAM deficiency leads the mtDNA to move into the cytoplasm and activate the stimulator of interferon genes (STING) pathway, which, in turn, potentially lead to tubular cell apoptosis, interstitial fibrosis and, renal failure. These results suggest that

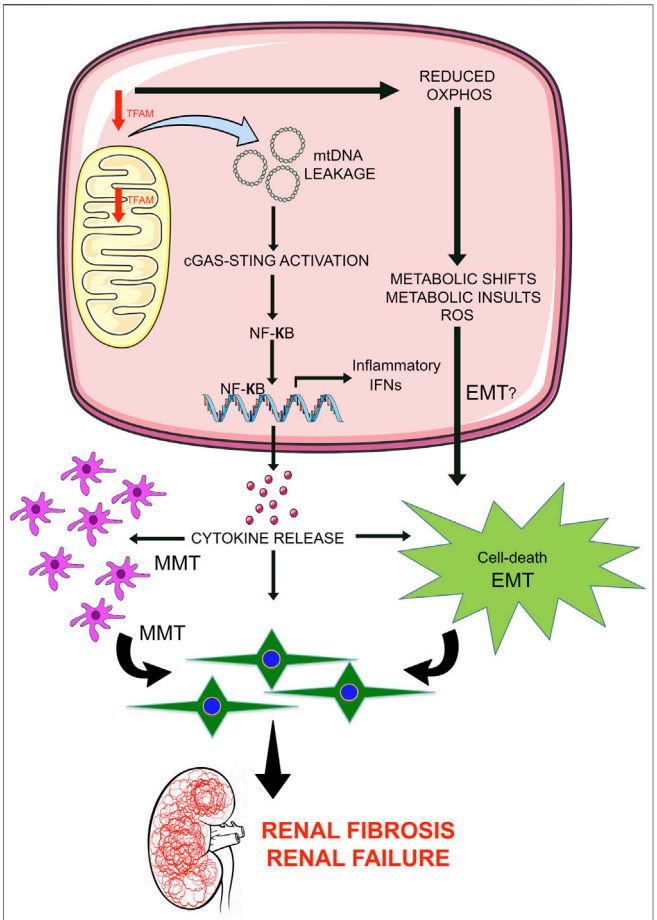

FIGURE 1 | Mitochondrial damage leads to renal inflammation and fibrosis. Mitochondrial transcription factor A (TFAM) is critical in the regulation of mtDNA structure, replication, and stability. Suppression of TFAM is a key event in renal fibrosis in 2 ways: 1) By leading to the leakage of mtDNA in the cytosol which activates the STING pathway and results in transcription of NFkB-associated cytokines and release of cytokines outside the cells, induces pathological inflammation in tubular cells and neighboring cell types i.e. macrophages. Higher release of cytokine may influence macrophage-to-mesenchymal transition and epithelial-to-mesenchymal transition and contribute to the accumulation of mesenchymal-like cells in the extracellular matrix; 2) Deficiency of TFAM causes reduced oxidative phosphorylation that is involved in the alteration of metabolic shifts and metabolic insults, influences the reactive oxygen species level; cumulative effects may lead to epithelial-to-mesenchymal transition program and accumulations of EMT-derived myofibroblasts in the extracellular matrix and contributes to renal injury and renal fibrosis.

tubule cell-specific loss of TFAM or mitochondrial damage leads to renal fibrosis not only by causing defective metabolism and energy deficits but also by leaking mtDNA into the cytoplasm, resulting in the activation of STING-dependent NF-kB pathways (Chung et al., 2019). Induction of STING-associated renal inflammation is an important downstream event in the development of kidney disease and inhibiting the STING pathway ameliorates disease development processes in the kidney. **Figure 1** depicts a schematic diagram showing the functional importance of TFAM in tubular epithelial cells.

LOSS OF MITOCHONDRIAL FUNCTIONS IN KIDNEY ENDOTHELIAL CELLS

Glomerular endothelial cells (GECc) regulate hemodynamic homeostasis, ROS levels and metabolic homeostasis (Jourde-Chiche et al., 2019). GECs affect the integrity of the filtration barrier. Injuries to the endothelial cells lead to microvascular occlusion, glomerular capillary function loss and glomerular sclerosis (Jourde-Chiche et al., 2019). Loss of cristae membranes in the mitochondria of endothelial cells have been observed after ischemic injury in rats (Liu et al., 2014). Restoring mitochondrial structure effectively reduces the loss of peritubular capillaries and cortical arterioles (Liu et al., 2014). Endothelial cell dysfunction results in microalbuminuria in early diabetic nephropathy (Daehn, 2018). High glucose increases mitochondrial superoxide anion production with resultant decreased membrane potential and respiratory chain enzyme complex I deactivation (Sivitz and Yorek, 2010). In the early stages of the rat model of 5/6 nephrectomy, which is an established model of chronic progressive renal injury, with glomerular sclerosis and interstitial fibrosis are observed as well as increased GECs proliferation and apoptosis (Kang et al., 2002). Inflammation contributes to endothelial cell damage through EndMT (Galle et al., 2003; Bogdanova and Castellon, 2016; Zhou et al., 2020a). TNF-α stimulates mitochondrial membrane permeability, thereby inducing cytoplasmic entry of cytochrome c, induction of the proapoptotic protein Bak and suppression of the anti-apoptotic protein Bcl-xL (Meßmer et al., 2000). In addition, mitochondrial oxidative stress induces EndMT (Lin et al., 2018b; Thuan et al., 2018).

Interstitial endothelial cells play critical roles in health and disease processes of the kidneys (Kanasaki et al., 2014; Shi et al., 2015; Chung et al., 2019). Mitochondrial biogenesis and dynamics are important events in determining endothelial cell homeostasis (Wada and Nakatsuka, 2016; Hu et al., 2018). and are central for stress responses, that includes cell-differentiation and organ fibrosis (Stallons et al., 2014; Hu et al., 2018; Srivastava et al., 2018). Das et al. reported that microRNAs regulate mitochondrial function by regulating mitochondrial gene expression (Das et al., 2017). MiR-let-7a regulates glucose catabolism by generating ROS in cancinoma cells (Serguienko et al., 2015). Downregulation of miR-let-7 genesis has critical roles in aerobic glycolysis (Ma et al., 2014). Additionally, the clusters of miR-let-7 have diverse and critical roles in endothelial cell function and metabolism (Srivastava et al., 2013; Srivastava et al., 2014; Hu et al., 2018). Targeting the miR-let-7 biogenesis pathway can affect mitochondrial structure and function (Hu et al., 2018) and among all clusters of the miR-let-7 family, miR-let-7b is well-known to contribute to mitochondrial biogenesis (Kuppusamy et al., 2015). The fibroblast growth factor (FGF)/FGFR1 signaling pathway plays a crucial role in regulating both mitochondrial biogenesis and dynamics and endothelial cell homeostasis (Li et al., 2017a; Hu et al., 2018).

N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP), an endogenous tetrapeptide, plays a crucial role in kidney cell homeostasis (Srivastava et al., 2016; Srivastava et al., 2020b).

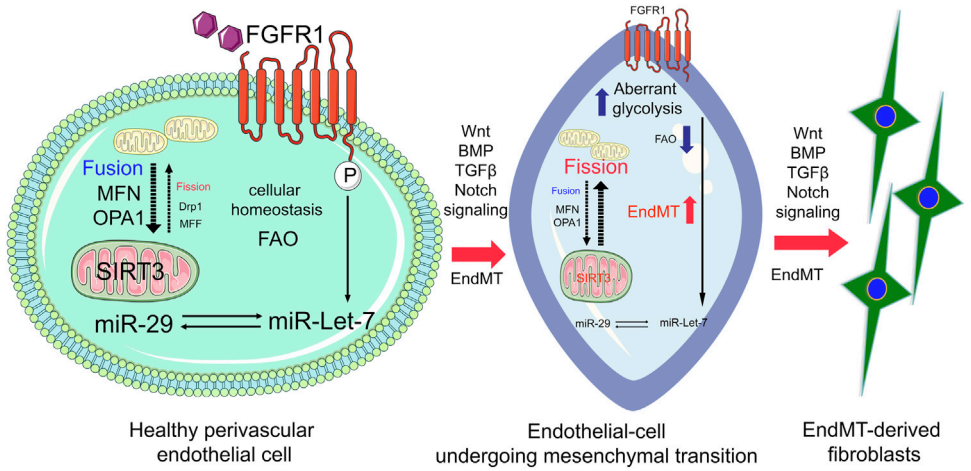

FIGURE 2 | FGFR1-miR-let-7 axis maintains endothelial cell homeostasis by regulating mitochondrial dynamics and integrity through modulating SIRT3. In healthy endothelial cells, FGFR1-miR-let-7 maintains mitochondrial dynamics and influences SIRT3; however, suppression of the FGFR1-miR-let-7 axis disrupts the miR-29 level and SIRT3 level, and ultimately disrupts mitochondrial biogenesis and integrity. Cumulative effects of suppressed levels of FGFR1-miR-let-7 axis and SIRT3 lead to the activation of pro-mesenchymal signaling, Wnt signaling, BMP, Notch, and TGF-β signaling; resulting in EndMT events and accumulation of EndMT-derived myofibroblasts.

AcSDKP induces the expression of FGFR1 and miR-let-7 in diabetic endothelium (Nagai et al., 2014; Nitta et al., 2016; Srivastava et al., 2016; Srivastava et al., 2019a). FGFR1 is required for the action of AcSDKP in regulating endothelial-mitochondrial dynamics by controlling miR-let-7b genesis (Hu et al., 2018). The FGF21/FGFR1 axis accelerates mitochondrial biogenesis in an AMP-activated-protein-kinase (AMPK)-dependent manner (Wang et al., 2016). miR-let-7a and miR-let-7b preserve endothelial cell-mitochondrial biogenesis and protect endothelial cells by mitigating ROS generation (Bao et al., 2014). In summary, mitochondrial dynamics, mitochondrial biogenesis, and mitophagy, play key roles in endothelial cell function and homeostasis (Sanchis-Gomar et al., 2014). Endothelial cell SIRT3 and endothelial cell glucocorticoid receptor deficiency is associated with endothelial-to-mesenchymal transition in the kidneys and endothelial FGFR1 deficiency results in severe organ fibrosis in both the kidney and heart via the induction of AcSDKP-resistant EndMT (Li et al., 2020a; Srivastava et al., 2020c). **Figure 2** depicts the functional importance of FGFR1-miR-let-7s axis in the regulation of mitochondrial dynamics and suppression of FGFR1-miR-let-7s axis is associated with activation of endothelial-to-mesenchymal transition in the kidney.

MITOCHONDRIA-TARGETTED THERAPEUTICS IN KIDNEY DISEASES

In vitro and *in vivo* studies confirm the involvement of microRNAs in the pathogenesis of kidney diseases (Srivastava et al., 2019a; Metzinger-Le Meuth et al., 2019; Nascimento and Domingueti, 2019). Several microRNAs play a crucial role in mitochondria (Gomez et al., 2013; Jaquenod De Giusti et al., 2018; Bai et al., 2019). MiR-30e is suppressed in renal fibrosis, and its antagonism exerts an antifibrotic effect by targeting mitochondrial protein UCP2 (Jiang et al., 2013a). miR-21 contributes widely to organ fibrosis by acting on energy metabolism. miR-21 antagonism suppresses ROS production and significantly reduces glomerular sclerosis, interstitial fibrosis, and inflammatory responses (Chau et al., 2012; Kolling et al., 2017). miR-17 is capable of mitochondrial metabolism and promotes the growth of polycystic kidney cysts (Hajarnis et al., 2017).

The renal cortex of db/db mice have reduced levels of total and oxidized forms of Coenzyme Q10 and intervention with Q10 ameliorated mitochondrial functions and suppressed collagen deposition in these diabetic kidneys (Sourris et al., 2012). Similarly, supplementation of Q10 suppressed ROS levels and ameliorated renal function in nephrectomized rats (Ishikawa et al., 2010). Q10 mitigates nicotine-induced oxidative stress in tubular epithelial cells through activating the non-mitochondrial fork protein p66shc (Arany et al., 2016). A clinical randomized trial showed that hemodialysis patients benefitted from daily use of 1,200 mg of Q10 per day to control oxidative stress (Rivara et al., 2017).

MitoQ is used as a mitochondria-targeted antioxidant, exerts a protective effect on lipid peroxidation and oxidative stress (Kelso et al., 2001). MitoQ is known to reduce oxidative stress and protect renal function in the ischemia-reperfusion-induced renal injury (Rouschop et al., 2005) and has antifibrotic effects in Ins2Akita mouse model of type I diabetic nephropathy (Chacko et al., 2010) and in db/db mice (Ward et al., 2017). MitoQ suppresses oxidative stress through inducing autophagy, inhibiting mitochondrial membrane potential, suppressing fission protein Drp1, and restoring fusion protein Mfn2 expression in tubular epithelial cells (Xiao et al., 2017). MitoQ prevents hypertension, stimulates endothelial NO bioavailability and improves kidney structure in spontaneously-hypertensive

rats (Graham et al., 2009). Oral MitoQ is in phase II clinical trials (Gane et al., 2010) (Snow et al., 2010).

SS-31 is a small peptide which suppresses excess ROS, stabilizes mitochondrial membrane potential, prevents cytochrome c translocation, and is associated with fibrogenesis in 5/6 nephrectomized rats (Zhao et al., 2017b). SS-31 significantly reduced tubular apoptosis, macrophage infiltration and maintained the integrity of mitochondrial function while inhibiting renal fibrosis in the UUO rat model (Mizuguchi et al., 2008) and in rat model of ischemia-reperfusion (Zhang et al., 2019). SS-31 restored mitochondrial function in podocytes (Sweetwyne et al., 2017) and parietal epithelial cells (Zhao et al., 2013), and reduced fibrosis in glomeruli and endothelial cells (Sweetwyne et al., 2017).

Rapamycin, an inhibitor of mTORC1, regulates mitochondrial autophagy (Bartolomé et al., 2017). Activation of the mTOR signaling pathway is the key pathogenic mechanism in diabetic kidney disease (Lloberas et al., 2006). Rapamycin inhibits kidney fibrosis, glomerulosclerosis, proteinuria and mesangial matrix deposition through mitigating the activation of mTOR (Lloberas et al., 2006; Li et al., 2019). Rapamycin can inhibit both mTORC1 and mTORC2 (Kawata et al., 2018). Rapamycin therapy is limited due to its side effects such as immunosuppression, and glucose intolerance in type II diabetic mice by reducing mTORC2 (Lamming et al., 2012) (Schreiber et al., 2019). mTORC2 regulates autophagy genes by FOXO3a phosphorylation and activation of the Akt pathway (Hung et al., 2012) (Chen et al., 2013). FOXO3a regulates mitochondrial autophagy through LC3, Bnip3, Nix, Atg4b, and Atg12l (Higgins and Coughlan, 2014). Further research is needed to establish rapamycin and its analogs as safe measures for treating fibrotic renal disease.

METABOLIC CONTROL BY POSTTRANSLATIONAL MODIFICATIONS IN MITOCHONDRIA

Metabolic control switches depend on the availability and scarcity of fuel. These regulatory mechanisms are highly conserved throughout evolution and affect many cellular signaling pathways linked to food intake and bioenergetics (Finkel et al., 2009; Morigi et al., 2018). Reversible acetylation is one important mechanism that regulates metabolic processes in mitochondria (Guan and Xiong, 2011). Reversible acetylation is regulated by the antagonistic activities of protein acetyltransferases (KATs) and deacetylases (HDACs) (Guan and Xiong, 2011). These proteins are encoded by multigene families, and are nuclearly-encoded (Guan and Xiong, 2011). In mammalian cells, thirty KATs and approximately eighteen HDACs are known (Guan and Xiong, 2011). The eighteen HDACs are classified into four types. Class I and II, which consist of ten members, are called "classical" HDACs; the enzyme activity of these classical HDACs can be repressed by trichostatin A, excluding HDAC11 that is unresponsive to trichostatin A. Class III HDACs, known as SIRTs (sirtuins), include 7 members and all are structurally different from HDACs. Most of the SIRTs need nicotinamide adenine dinucleotide (NAD+) as a co-substrate and are repressed by nicotinamide (NAM); however, these are unaffected by trichostatin A treatments (Guan and Xiong, 2011). Increasing evidence suggests metabolic function of these SIRTs whereas, the function of KATs and HDACs are less clear (Guan and Xiong, 2011). Out of the eleven HDACs, 4 (HDACs: 1, 2, 8, and 11) are localized in the nucleus and six (HDACs: 3, 4, 5, 7, 9, and 10) are either dispensed in or channeled between the nucleus and the cytosol (Seto and Yoshida, 2014). HDAC7 has been found to localize to the mitochondria (Seto and Yoshida, 2014).

Moreover, out of the seven SIRTs, SIRT3, 4, and 5 reside in mitochondria while SIRT2 is found in the cytosol and SIRT1 has been reported to be present in both the nucleus and the cytosol (Seto and Yoshida, 2014; Morigi et al., 2018). **Figure 3** demonstrates the subcellular localization and biological functions of these sirtuins. The presence of many situins in mitochondria suggests a crucial role of metabolic control by the mitochondria (Wakino et al., 2015; Hershberger et al., 2017). Studies of several sirtuins in different model organisms have suggested that sirtuin genes play a role in life span, caloric-restriction, nutrient responses and bioenergetics (Lin et al., 2000; Finkel et al., 2009).

Deacetylase and Autosomal Dominant Polycystic Ribosylase Activity

In humans, the deacetylase domain of SIRTs is distinct from class I and class II HDACs, which are zinc dependent. SIRTs utilize one NAD^+ to produce acetyl-ADP-ribose and NAM in the deacetylation process. Defective mitochondrial pathways can lead to metabolic diseases, oxidative damage, organ fibrosis and cancer (Pearce et al., 2009; Kang et al., 2015; Carrico et al., 2018; Srivastava et al., 2018). A proteomic approach revealed that twenty percent of mitochondrial proteins that are involved in life-span control and in metabolic control are present in the acetylated form (Kim et al., 2006). Reversible deacetylation of mitochondrial proteins is a crucial mechanism of metabolic control (Carrico et al., 2018). Acetyl-CoA and NAD^+ are critical markers of energy levels in cells. Acetyl CoA is the substrate for histone acyl transferases while NAD^+ is a co-substrate for SIRTs (Carrico et al., 2018). The deacetylation processes of mitochondrial SIRTs are known to have key roles in metabolic shifts in cancer cells (Carrico et al., 2018).

The deacetylation ability of the histone H4 peptide is different among the sirtuins. SIRT 1, 2, 3, 4, 5, 6, and 7 show increased tendencies toward histone H4 polypeptide whereas, SIRT 1,4,6 have mono-ADP-ribosylation activity (Lee et al., 2019). SIRT1 deacetylates histones, p53, Ku70 and FOXO. Among the sirtuins, only SIRT 2 deacetylates tubulin (Morigi et al., 2018). Similarly, the mono-ADP-ribosylating activity of sirtuins differs among different groups and is associated with SIRT4 and SIRT6. Mono-ADP-ribosylation is a process in which ADP-ribose from nicotinamide-adenine-dinucleotide is moved to the target acetylated protein (Carrico et al., 2018). This process is highly conserved from bacteria to humans (Saunders and Verdin, 2007). In addition to mono-ADP-ribosylation, SIRT4 has robust deacylase activity as well as substrate-dependent lipoamidase

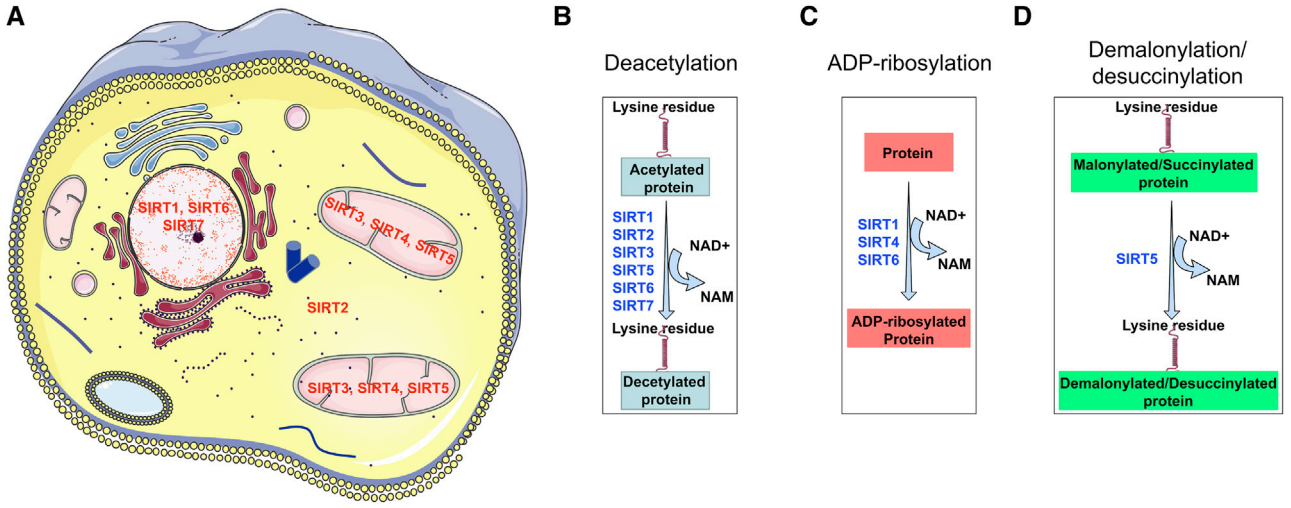

FIGURE 3 | Sub-cellular localization of diverse types of sirtuins and their functional properties. SIRT1, SIRT6, and SIRT7 localize in the nucleus; SIRT3, SIRT4, and SIRT5 localize in mitochondrial; however only SIRT4 is known to reside in the cytosol. SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7 have deacetylation properties; SIRT1, SIRT4 and, SIRT6 have ADP-ribosylation activity, however, SIRT5 has two unique properties of demalonylation and desuccinylation. These functional differences make each sirtuin of diverse biological importance in renal health.

and deacetylase properties (Lee et al., 2019; Tomaselli et al., 2020). Beside deacetylase activity, SIRT5 has also been found to have demalonylase and desuccinylase activity (Lee et al., 2019).

Regulation of Mitochondrial Sirtuins

Among all mitochondrial SIRTs, SIRT3 mainly targets those proteins that are involved in metabolic homeostasis (Lombard et al., 2007; Houtkooper et al., 2012). For example, SIRT3 targets long-chain acyl CoA dehydrogenase, a key protein in FAO in prolonged fasting conditions (Hirschey et al., 2010). Deficiency of *Sirt3* disrupts lipolysis, and lipid catabolism, and thus promotes diet-induced obesity (Hirschey et al., 2011). SIRT3 deacetylates 3-hydroxy-3-methylglutaryl-CoA-synthase 2, which controls the synthesis of ketone bodies, a crucial energy source for the brain under fasting conditions (Shimazu et al., 2010). During caloric restriction, SIRT3 induces the enzyme activitiy of isocitrate dehydrogenase (Someya et al., 2010), glutamate dehydrogenase and the enzymes of the TCA cycle (Lombard et al., 2007). SIRT3 deacetylates components of ETC such as complex I, complex II and complex III, and these are associated with oxidative-phosphorylation, the final stage of aerobic respiration (Ahn et al., 2008; Finley et al., 2011b; Jing et al., 2011). In addition, SIRT3 protects cells from oxidative stress by mitigating ROS levels (Someya et al., 2010; Jing et al., 2011) and activating the enzyme activity of superoxide-dismutase 2, a key antioxidant enzyme in mitochondria (Qiu et al., 2010). Caloric restriction increases SIRT3-associated deacetylation of IDH2, thereby increasing the reduced-to-oxidized glutathione ratio, and hence iinhibiting ROS (Someya et al., 2010).

SIRT4 primarily plays a role in metabolic control (Han et al., 2019). SIRT4 inhibits the GDH enzyme activity by ADP-ribosylation, and hence, blocks amino-acid-linked insulin secretion (Argmann and Auwerx, 2006). As a result, SIRT4 knock out mice have elevated levels of plasma insulin, in fed, as well as fasted,conditions (Argmann and Auwerx, 2006). In addition, SIRT4 controls FAO in cultured hepatocytes and myotubes, and knockdown of SIRT4 in the liver is associated with higher FAO (Nasrin et al., 2010). Interestingly, SIRT3 and SIRT4 have shown antagonistic properties in the regulation of GDH (Haigis et al., 2006; Lombard et al., 2007) and FAO (Hirschey et al., 2010; Nasrin et al., 2010). Further scientific advancement is required to analyze how SIRT3 and SIRT4 coordinate similar nutrient states to achieve opposite responses.

SIRT5 deacetylates CPS1 in a fasting state and promotes ammonia detoxification in the urea cycle (Nakagawa et al., 2009). SIRT5 may not primarily act as a deacetylase (Nakagawa et al., 2009) however, it acts as a demalonylase and desuccinylase (Peng et al., 2011), even for the described deacetylase target CPS1 (Du et al., 2011).

Sirtuin3 Regulates Mitochondrial Dynamics

In spite of the oval-shaped structure of mitochondria, these organelles can exist in an active, dynamic nexus and continuously go through fission and fusion phenomena (Lesnefsky et al., 2001; Otera and Mihara, 2011; Chan, 2012). Mitochondria divide by a simple binary fission process which requires only mtDNA for its function (Otera and Mihara, 2011; Chan, 2012). Both fission and fusion are highly linked to replication of mtDNA. Several proteins involved in the fission process have been implicated in mitochondrial diseases (Otera and Mihara, 2011; Chan, 2012).

Mitochondria maintain their active dynamic form by coordinating networks and sustaining a series of fusion (mitofusin-2, MFN-2; optic atrophy protein 1, OPA1) and fission cycles (dynamin-related protein-1, DRP1) (Dorn et al., 2015; Wada and Nakatsuka, 2016). The effector molecules differ in the outer and inner membranes of the mitochondria (Wada and Nakatsuka, 2016). Membrane-bound dynamins arbitrate

fusion between outer-membranes and are identified as MFN1 and MFN2 proteins (Wada and Nakatsuka, 2016). However, a single-dynamin OPA1 coordinates fusion processes between inner-membranes (Wada and Nakatsuka, 2016). This protein is well-conserved among mammals, flies, and yeast. OPA1 is anchored to the inner membrane of mitochondria. OPA1 assists in preserving cristae structure and protectscells from apoptosis (Frezza et al., 2006). SIRT3 can directly target OPA1 (Samant et al., 2014). OPA1 is hyper-acetylated under stress conditions, and hyperacetylated OPA1 reduces its GTPase enzyme activity, thereby mitigating its biological functions (Frezza et al., 2006). SIRT3 activates OPA1 by deacetyation and consequently alters mitochondrial dynamics (Samant et al., 2014). Therefore, SIRT3 activates the function of mitochondria not only by affecting the enzyme activity level but by directly regulating mitochondrial dynamics by activating OPA1 (Samant et al., 2014). The DRP1 protein, a dynamin protein of large GTPases, positively regulates the mitochondrial fission process by pinching off the membrane stalk between two forming daughter mitochondria (Dorn et al., 2015; Wada and Nakatsuka, 2016). SIRT3-loss is associated with activation of mitochondrial-fission by moldulating DRP1 protein level.

Sirtuin3 in Renal Health and Metabolism

SIRT3 plays a major role in kidney health. Available evidence suggests that SIRT3 maintains mitochondrial energy homeostasis in proximal and distal tubule compartments (Morigi et al., 2018). A role for SIRT3 in the regulation of tubular-cell homeostasis has also been demonstrated and suggests that SIRT3 regulates microtubule-dependent transport of mitochondria among tubular epithelial cells, which is a process that conserves cell bioenergetic profiles and antioxidant mechanisms (Morigi et al., 2018).

However, our recent studies suggest that SIRT3 has a protective role in renal fibrosis and diabetic kidney disease (Srivastava et al., 2018). Renal fibrosis is the dominant cause of end-stage renal disease across the world (Roxburgh et al., 2009). It is characterized by deposition of collagen, myofibroblasts, and pro-inflammatory cells (Zeisberg et al., 2003; LeBleu et al., 2013). Renal fibroblasts have a crucial role in such fibrotic events, but, the genesis of these fibroblasts is not clear (Grande and Lopez-Novoa, 2009; Zeisberg and Neilson, 2010; Liu, 2011; Schrimpf and Duffield, 2011; Grgic et al., 2012; Srivastava et al., 2013; Srivastava et al., 2019b).

Available data suggest that activated myofibroblasts and fibroblast formation are caused by activated resident fibroblasts and/or activation of mesenchymal cell differentiation processes in neighboring cells such as epithelial cells, endothelial cells, pericytes and M2-derived macrophages. (Srivastava et al., 2013; Srivastava et al., 2019b). SIRT3 acts as a tumour-suppressor, maintains stability in the genome (Kim et al., 2010) and inhibits the features of organ fibrosis by mitigating TGF-β/Smad signaling (Sundaresan et al., 2009; Chen et al., 2015; Sundaresan et al., 2015; Bindu et al., 2017; Sosulski et al., 2017). However, during the cell-to-cell transition process, the metabolic switch is altered (Jiang et al., 2013b; DeNicola and Cantley, 2015; Liu et al., 2016). Thus, the fuel choice or energy sources of these injured cells is a matter of ongoing debate (Jiang et al., 2013b; DeNicola and Cantley, 2015; Liu et al., 2016). These reprogrammed metabolic shifts cause production of myofibroblast precursors and may assist in fibroblast growth and survival (Zeisberg et al., 2003; Kalluri and Weinberg, 2009; Jiang et al., 2013b).

Sirtuin3, in Association With Activated STAT3, Regulates Aberrant Glycolysis

In response to cytokines, signal transducer and activator of transcription (STAT)3 phosphorylation on its tyrosine 705 residue (Y-P) is mediated by receptor-associated JAK kinases (Schindler et al., 2007). Tyrosine^{705} phosphorylation causes dimerization and translocation from the cytosol to the nucleus where it binds to gene promoters and modulates the transcription (Yu et al., 2014). Besides regulation by phosphorylation on Tyr^{705}, STAT3 is also regulated by phosphorylation on Ser^{727} by some members of the MAP kinases. Ser^{727} phosphorylation is a secondary step for enhancing transcriptional activity of STAT3 (Wen et al., 1995). STAT3 is mainly regulated by the cytokine IL-6 and other cytokines such as IL-11 and IL-27 which work through a gp130 signal transducer in their receptors (Zhong et al., 1994; Niemand et al., 2003). IL-10, IL-21 and leptin can also activate STAT3 which is independent of gp130 signal transduction (Niemand et al., 2003). STAT3 activation is also regulated by phosphatases and by the Suppressors of Cytokine Signaling (SOCS), which interfere with nuclear translocation or promote STAT3 degradation (Ward et al., 1994).

STAT3 affects energy metabolism by influencing the pathways both at nucleus and the mitochondrion, depending on specific post-transcriptional modifications (Y-P or S-P) triggered by diverse stimuli (Poli and Camporeale, 2015). Y-P nuclear STAT3 accumulation mediates transcriptional upregulation of HIF1α and the downregulation of mitochondrial genes (Poli and Camporeale, 2015). This leads to aberrant aerobic glycolysis, suppressed ETC activity, and decreased ROS generation, thus enhancing cell-proliferation and inhibiting apoptosis (Poli and Camporeale, 2015). S-P STAT3 mitochondrial activity also leads to increased cell-proliferation and limits apoptosis through retaining ETC activity, stimulating aerobic glycolysis, decreasing ROS generation, and inhibiting the opening of the mitochondrial permeability transition pore (Poli and Camporeale, 2015; Meier et al., 2017).

STAT3 promotes Complex I activity and mitochondrial respiration by binding on GRIM-19, which is a component of Complex I of the electron transport chain (ETC) (Lufei, 2003). In addition, STAT3 can bind to Complex II or Complex V (ATP synthase) and modulates ATP production (Gough et al., 2009). Mitochondrial STAT3 binds to cyclophilin D, and inhibits the opening of the mitochondrial permeability transition pore, hence reducing ROS generation (Meier et al., 2017). GRIM-19 functions as a chaperone which helps in recruiting STAT3 to the mitochondrial inner membrane complex and the mitochondrial importer Tom20 is involved in STAT3 recruitment into mitochondria (Boengler et al., 2010; Tammineni et al., 2013). Mitochondrial recruitment of Stat3 is

enhanced by its acetylation, but the mechanism remains unclear; mitochondrial sirtuins may be involved in this mechanism (Xu et al., 2016b). The role of mitochondrial STAT3 has been demonstrated primarily in cancer, cardiology, neuroscience, organ fibrosis and in diabetic kidney disease (Yang and Rincon, 2016). JAK inhibitors and STAT3 inhibitors may have the potential to develop a new generation of therapeutics (Rincon and Pereira, 2018).

Recent studies suggest that SIRT3 deficiency in diabetic kidneys leads to the induction of aberrant glycolysis and linked fibrogenic programming through activation of the TGFβ/Smad3 signaling pathway in tubular epithelial cells that promotes an epithelial-to-mesenchymal transition program (Srivastava et al., 2018). SIRT3 deficiency-linked abnormal glycolysis is due to higher PKM2-dimer formation, HIF1α and activated STAT3 signaling (Srivastava et al., 2018; Li et al., 2020b). Disruption in glucose metabolism is associated with metabolic reprogramming in damaged cells and mesenchymal activation and gain of fibrogenic properties in diabetic kidneys (Srivastava et al., 2018). Suppression of SIRT3, HIF-1α accumulation and STAT3 phosphorylation are linked with EMT phenotype and defective glucose metabolism (Finley et al., 2011a; Palmirotta et al., 2016). Inhibition of glycolysis by either by 2-deoxy-glucose (2-DG) or with dichloroacetate (DCA) leads to reduction in EMT processes and cancer cell metastasis (Sottnik et al., 2011; Lu et al., 2015; Zhao et al., 2017a). HIF-1α and STAT3 phosphorylation in renal epithelial cells is linked to mesenchymal activation and renal fibrogenesis (Higgins et al., 2007; Sun et al., 2009). Proximal-tubular-cells (PTCs) are widely unprotected to excessive glucose uptake from the urine in severe-diabetes; it may possible that the urinary glucose can be utilized as a substrate for glycose catabolism by TECs (Hato et al., 2016). Hence, cumulative effects of SIRT3 deficiency and HIF-1α accumulation and defective central metabolism, triggered by higher glucose reabsorption, stimulate PTCs to transform into an intermediate-type mesenchymal- and complete-mesenchymal cell phenotype (Srivastava et al., 2018). In our study, it was demostated that loss of SIRT3-linked PKM2 tetramer-to-dimerization occurred in diabetic kidneys and in cultured TECs exposed to high-glucose-stimulated cell media (Srivastava et al., 2018). PKM2-dimerization causes transactivation of HIF1α and is a crucial mechanism for abnormal glucose metabolism in cancer cells by enhancing the Warburg effect (Greer et al., 2012; Soga, 2013; Palsson-McDermott et al., 2015).

The pathogenic role of defective glucose metabolism has been demonstrated in diabetic kidney disease (Qi et al., 2017). Tubular interstitial pathology was improved with increased enzyme activity of the PKM2 tetramer (Qi et al., 2017). TEPP-46 induced PKM2 tetramer formation, suppresseed accumulation of fibronectin and type-I-collagen, and mitigated TGFβ1 levels in the injured tubules. Conversely, TEPP-46 has less effect on glomerular collagen deposition since TGFβ1 signaling is higher in damaged tubules and not induced in the glomeruli of diabetic kidneys (Qi et al., 2017). This study suggests that aberrant glycolysis in TECs confers a renal disease phenotype in diabetes. Higher SIRT3 expression levels inhibit glucose-stimulated cell-senescence through FOXO1-mediated signaling mechanisms (Zhang et al., 2013) and enhance cellular resistance to oxidative stress damage (Morigi et al., 2018).

SGLT2 inhibitors and glycolysis inhibitors act primarily on kidney proximal tubular cells. One recent study suggests that SGLT2 inhibition and glycolysis inhibition in diabetic tubules impact central metabolism through restorating SIRT3 protein level, by causing a reduction in EMT events reducing abnormal glycolysis attributed to STAT3-phosphorylation, HIF1α-transactivation and PKM2-dimerization (Li et al., 2020b). SGLT2 inhibition suppresses EMT events in proximal tubular cells and linked EndMT in perivascular endothelial cells (Li et al., 2020b) and has been the focus of intensive discussion as a potential source of myofibroblasts (Srivastava et al., 2019a). TECs are injured in diabetic kidneys. They undergo phenotypic changes and acquire the features of matrix-generating mesenchymal cells, and fibrogenesis markers such as αSMA, fibronectin and FSP1. Renal fibrosis is influenced by intercommunication among several cell types in the kidney (Srivastava et al., 2019b). EMT influences the mesenchymal activation of perivascular endothelial cells, pericytes and macrophages through soluble-factors. **Figure 4** represents the role of aberrant glucose metabolism in the induction of EMT events. EMT releases soluble factors that may accelerate EndMT events in diabetic kidneys.

Sirtuin3 Deficiency Disrupts Fatty Acid Oxidation

PTECs require excessive energy for proper function and have enormous quantities of functional mitochondria. Free fatty acids (FAs) are used as the most favored metabolic fuel for TECs, since catabolism of FAs synthesizes more ATP per molecule than does glucose catabolism (Kang et al., 2015). FA uptake is facilited by the transporter protein CD36, and fatty acids transporter proteins (Susztak et al., 2005). Catabolism of FFAs is dependent on transport into mitochondria that is catalyzed thorugh carnitine-palmitoyltransferase 1 (CPT1) (Schug and Li, 2011). The peroxisome-proliferator-activated-receptor-α (PPARα) and PPAR-γ-coactivator-1α (PGC1α) are crucial transcription factors that control the expression of genes/proteins in FA uptake and oxidation (Tran et al., 2011; Kang et al., 2015). In healthy TECs, FA uptake, FAO and FA biosynthesis are highly regulated to abstain from intra-cellular lipid deposition (Kang et al., 2015). The accumulation of lipids in TECs and their pathological role in acute and diabetic kidney disease is the subject of debate among researchers (Decleves et al., 2014; Srivastava et al., 2014). In addition, defective FA utilization and oxidations leads to mesenchymal activation and fibrogensis (Kang et al., 2015) since higher triglyceride accumulation in TECs catalyzes lipotoxicity, and augments the induction of mesenchymal activation and progression of kidney fibrosis (Decleves et al., 2014).

In diabetic kidneys with tubules undergoing mesenchymal cell formation, SIRT3 suppression is associated with defective FAO and concomitant induction of abnormal glycolysis (Srivastava et al., 2018; Srivastava et al., 2020b). Defective fatty acid oxidation

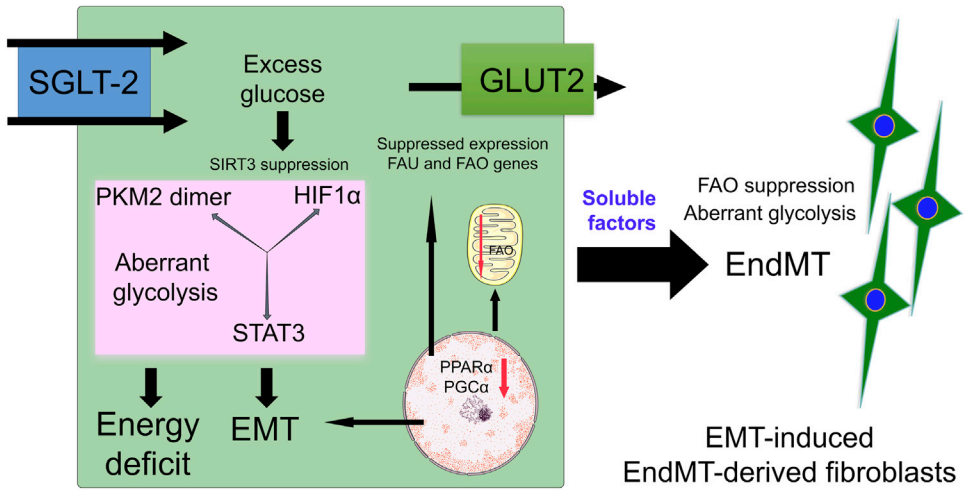

FIGURE 4 | Defective central metabolism in diabetic tubular epithelial cells. Diabetic tubular epithelial cells have higher expression of SGLT-2 which transports urine glucose into tubular cells. Excess glucose accumulates in the cytosol which results in activation of SIRT3 deficiency-linked disruption in central metabolism. This defective central metabolism is characterized by PKM2-and STAT3-linked aberrant glycolysis and suppression of fatty acid oxidation. Defective central metabolism contributes to EMT events. These EMT events produce and release soluble factors which affect the homeostasis of endothelial cells and induce EndMT events in the kidneys.

has been found to play a critical role in humans and in mouse models of tubulointerstitial fibrosis (Kang et al., 2015; Srivastava et al., 2020b). Injured tubulointerstitial cells have been shown to have suppressed levels of regulatory enzymes of FAO and accumulation of intracellular lipids (Kang et al., 2015). FAO inhibition in TECs by the small molecule etomoxir causes ATP-deficits, cell-death, cell-differentiation, and lipid-accumulation, mimicking features of renal fibrosis (Kang et al., 2015). However, normalizing FAO by genetic or pharmacological means using small chemicals protects against renal fibrosis, suggesting that normalization of the renal-metabolic abnormality might be utilized for the treatment of chronic kidney disease (Kang et al., 2015).

Sirtuin4 in Renal Health and Metabolism

SIRT4 is a critical molecule in mitochondrial physiology. Researchers have investigated a pathological connection between SIRT4 and diabetic nephropathy in high-glucose-stimulated cultured podocytes (Shi et al., 2017). Glucose stimulation remarkably induces podocyte apoptosis which is associated with diminished protein levels of SIRT4, suggesting that SIRT4 suppression is critical in diabetic nephropathy (Shi et al., 2017). Over-expression of SIRT4 suppresses podocyte apoptosis, stimulates mitochondrial-membrane potential and decreases ROS generation (Shi et al., 2017). SIRT4-overexpression suppresses the level of apoptosis-linked effector molecules such as NOX1, Bax and p38 phosphorylation and increases the Bcl-2 protein expression in high-glucose-treated cultured podocyte cells (Shi et al., 2017). These results demonstrate that SIRT4 overexpression protects against hyperglycemia-associated podocyte cell death and ROS generation and that deficiency of podocyte SIRT4 represents a critical development in the understanding of diabetic nephropathy (Shi et al., 2017).

SIRT4 inhibits renal tumor metabolism, especially glutamine metabolism, and therefore functions as a tumor suppressor gene in the kidneys (Jeong et al., 2013). It is thought to be a gatekeeper of glutamine metabolism energetics (Mathias et al., 2014). Indeed, a metabolic shift is a hallmark of all types of tumors (Faubert et al., 2013). Tumor cells typically show Warburg metabolism and are dependent on enhanced glucose and glutamine uptake and catabolism to meet the large energy demand for tumor development (Daye and Wellen, 2012). In the future, it will be desirable to determine the corelation between SIRT4 levels and the prognosis of renal clear cell carcinoma, pending the existence of a suitable number of patients, which would allow further analysis of the influence of SIRT4 on the biological behavior of these cancer cells.

Sirtuin5 in Renal Health and Metabolism

SIRT5 has diverse, unique functional properties in its substrate choice for succinyllysine, malonyllysine, and glutaryllysine; it enhances fatty acid oxidation in hepatcytes and cardiac myocytes (Rardin et al., 2013; Du et al., 2018). Intriguingly, SIRT5 has been shown to localize to peroxisomes as well (Chiba et al., 2019). In contrast to its effect on mitochondrial FAO, SIRT5 suppresses peroxisomal FAO *in vitro* and in rodent liver (Chiba et al., 2019). SIRT5 knock out mice are protected against ischemic- and cisplatin-mediated AKI (Chiba et al., 2019). Although the mitochondrial function is moderately suppressed in SIRT5 KO kidneys, the peroxisome function is increased in kidneys from mice subjected to acute kidney injury (Chiba et al., 2019). These results suggest that SIRT5 controls the balance of mitochondrial versus peroxisomal FAO in PTECs and protects from acute kidney injury (Chiba et al., 2019).

The loss-of-function of SIRT5 is renoprotective (Chiba et al., 2019). The role of SIRT5 is antagonistic to that of SIRT1 and SIRT3, as their loss promotes acute kidney injury. Knock-down of

SIRT3 in proximal tubular epithelial cells disrupts mitochondrial-fatty acid oxidation through hypersuccinylation and therefore, reduces the enzyme activity of key proteins involved in FAO (Chiba et al., 2019). Metabolic adaptation to blocked mitochondrial-FAO in proximal tubular epithelial cells reveals the compensatory FAO in the peroxisome, hence mitigating oxygen necessity, reducing reactive oxygen species, and protecting against kidney injury (Chiba et al., 2019).

METABOLIC AND MITOCHONDRIAL REPROGRAMMING IN POLYCYSTIC KIDNEY DISEASE

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common, monogenic disorders and is caused mostly by gene mutations in polycystic kidney disease 1 (*PKD1*) and 2 (*PKD2)*, which encode polycystin 1 and polycystin 2, respectively (Harris and Torres, 2009). ADPKD is characterized by bilateral renal cyst development that impairs kidney function, leading to ESRD (Torres et al., 2007). Multiple pathways are dysregulated in the cystic epithelium including alterations in cell metabolism which have emerged as a hallmark of ADPKD (Rowe et al., 2013; Menezes et al., 2016; Podrini et al., 2020). Evidence suggests that the cystic epithelial lining shares neoplastic features (Rowe et al., 2013). Impaired mitochondrial structure and function play a role in ADPKD disease progression (Cassina et al., 2020). Metabolic reprogramming in PKD is similar to that reported in cancer (Rowe et al., 2013). Studies suggest that aerobic glycolysis is present in the disease, along with other metabolic defects such as augmentation of the pentose phosphate pathway, and increases in glutamine anaplerosis and fatty acid biosynthesis,; fatty acid oxidation and mitochondrial metabolism are suppressed (Podrini et al., 2020). ADPKD cells alter their energy dependency from oxidative phosphorylation to glycolysis (Podrini et al., 2020).

The precise origin of metabolic shifts has not been clearly demonstrated, however, two hypotheses has been postulated (Podrini et al., 2020). First, the polycystins have the ability to regulate mitochondrial function and structure either by regulating Ca^{++} uptake in mitochondria, or by a direct translocation of a small fragment protein into the mitochondrial matrix (Kuo et al., 2019). Second, loss of mitochondrial functions in ADPKD is driven by multiple signaling pathways, which include AMPK, PPARα, PGC1α, mTORC1, cAMP and cystic fibrosis transmembrane conductance regulator (CFTR)-mediated ion transport as well as the expression of crucial components of the mitochondrial energy production apparatus (Hajarnis et al., 2017). PKD1-deficient mouse embryonic fibroblasts were found to have increased glucose uptake and glycolysis as their primary source of energy, even in normoxic conditions (Rowe et al., 2013). Importantly, these effects are dependent on the upregulation of mTORC1 signaling, resulting in the inhibition of AMP-activated protein kinase (AMPK), which led to the hypothesis that, in ADPKD, cells preferentially use aerobic glycolysis for energy production (Rowe et al., 2013). Menezes et al. found reduced oxidative phosphorylation in $Pkd^{-/-}$ cells that had fatty acids as their main energy source, suggesting that FAO is reduced (Menezes et al., 2016), and is accompanied by a compensatory glycolysis and administration of 2-deoxyglucose slowed disease progression (Riwanto et al., 2016).

In adition, a renal transcriptomic analysis and urine metabolomic analysis in a mouse model of ADPKD revealed altered metabolic pathways that are associated with cyst formation (Menezes et al., 2012). Among these altered pathways were high levels of acetylcarnitine in the urine of ADPKD mice, suggesting the presence of defective fatty acid metabolism in mitochondria. Transcriptional profiling and metabolomic analysis of progressive ADPKD found alterations in lipid metabolism (Menezes et al., 2016). The defective FAO in mice with renal tubule-specific *Pkd1* deletion occurs via signaling involving miR-17 and PPARα (Hajarnis et al., 2017). Inhibition of miR-17 restored defective FAO and suppressed cyst formation in PKD mouse models (Hajarnis et al., 2017). Moreover, mutations in genes encoding components of the OXPHOS and FAO pathways, as well as in PPARα target genes, result in clinical disorders that include cystic kidneys (Hackl et al., 2017). Renal cysts develop in patients with glutaric acidaemia type II (Whitfield et al., 1996), which is caused by gene mutations in electron transfer flavoprotein subunit-α (ETFα), ETFβ or ETF dehydrogenase (ETFDH), which are components of an OXPHOS enzyme, ETF complex. Polycystins affect the function and morphology of mitochondria (Padovano et al., 2017; Lin et al., 2018a). The clear mechanisms underlying these alterations identified in ADPKD will need further investigation.

PERSPECTIVES AND FUTURE DIRECTIONS

TFAM is important for mitochondrial integrity and its loss causes metabolic insults in tubular cells and cytosolic mtDNA leak, resulting in activation of an inflammatory response and kidney damage (Chung et al., 2019). Loss of TFAM causes tubular cell inflammation which is key for the activation of mesenchymal programming in epithelial cells and in neighboring cells. However, it is hard to determine the severity of mitochondrial DNA leakage in CKD or DKD subjects, and it would be striking to unravel the contribution of the upstream-effectors of STING that could have a potential impact on disease progression. Mitochondrial sirtuins and their association with TFAM may be a key link in the pathogenesis of kidney disease.

Mitochondrial sirtuins are a prominent class of metabolic regulators that exert effects on energy metabolism by protein acetylation and are linked to several biological effects on kidney health. Restoring SIRT3 has shown to have renal protective effects in age-associated renal fibrosis, as well as several models of kidney injury and diabetic models as well (Srivastava et al., 2018). However, dissecting the diverse functions of SIRT3 in different cell types remains a challenge. How SIRT3 expression levels change in different compartments and what the impact of the distribution of subcellular SIRT3 is on cellular health has not yet been fully investigated.

As compared to SIRT3, little is known about the role of SIRT4 and SIRT5 in the regulation of central metabolism in diverse cell types in the kidney. ADP-ribosylation activity by SIRT4, and demalonylation and desuccinylation activity by SIRT5, distinguish them from SIRT3 (Morigi et al., 2018). Available clinical data describing the effects of SIRT4 and SIRT5 on patients is not yet sufficient enough to decipher the role of SIRT4 and SIRT5 in the kidney (Morigi et al., 2018). The understanding of different sirtuins in renal physiology is still in its early stages. However, scientific advancement in evaluating the broad-spectrum of sirtuins targets involved in protective and developmental mechanisms has been studied but still needs more attention in the development of suitable therapeutics for combating kidney diseases.

In mouse models of diabetic kidney disease, SGLT-2 inhibitors and glycolysis inhibitors have shown protective effects on renal tubules; however, an unbiased study is needed to test FAO modulators, DPP-4 inhibitors, Wnt signaling inhibitors, antifibrotic peptides, ACE inhibitors and ARBs on their ability to restore SIRT3 levels in injured kidneys (Kanasaki et al., 2014; Srivastava et al., 2020a; Li et al., 2020b).

Further studies are required to identify small molecules that activate sirtuins activity, known as sirtuin-activating-compounds (STAC). Mostly, these STAC are related to naturally-occuring polyphenols (Morigi et al., 2018). Resveratrol was a well-known sirtuin activator discovered from these naturally-occurring polyphenols (Morigi et al., 2018). Resveratrol induces SIRT1 and SIRT3 and can act as an allosteric modulator, leading to conformational changes in the substrate, which can then influence the binding affinity for sirtuins (Xu et al., 2016a; Morigi et al., 2018). These studies suggest that there is a need to search for compounds that influence sirtuin level and activity in diabetic kidneys, such as flavonoids, chalcones, polyhydroquinolines, propiophenone derivatives, deoxyandrographolides, 2-methoxy-estradiol (2-ME) and thiazolidin-4-one derivatives; all of these compounds have shown protective effects in mouse models of diabetes mellitus (Kumar et al., 2010; Srivastava et al., 2010; Srivsatava et al., 2010; Shukla et al., 2011; Jaiswal et al., 2012; Kumar et al., 2012; Verma et al., 2012; Jaiswal, 2013; Mishra et al., 2013; Raza et al., 2013; Balaramnavar et al., 2014; Arha et al., 2015; Kanasaki et al., 2017), and can be further tested and potentially could be used in the treatment of diabetic kidney disease. The non-coding microRNAs play a critical role in the pathogenesis of diabetes mellitus; in spite of certain limitations about their specificity, the tissue-specific expression of these microRNAs is needs to be to analyzed (Kaur et al., 2011; Pandey et al., 2011; Srivastava et al., 2019a).

In current literature, it is reported that curcumin, silybin and AICAR induce SIRT3 levels, improve renal function and improve mitochondrial physiology in cisplatin-induced AKI (Li et al., 2017b; Ortega-Dominguez et al., 2017). Stanniocalcin reduces renal damage by SIRT3-linked activation of AMPK and UCP2 in a mouse model of renal ischemia-reperfusion injury (Pan et al., 2015). Honokiol decreases renal damage by activating SIRT3 in sepsis-associated AKI and hypertensive nephropathy (Kume et al., 2010; Li et al., 2014). Of great interest for future development would be new pharmacological strategies to target the effector molecules that control NAD + syntheses such as NAPMT and pharmacological compounds that reduce the activity of NAD + -depleting enzymes such as ADP-ribosyl-cyclases (Morigi et al., 2015; Camacho-Pereira et al., 2016). Further research and large double-blind clinical trials will be required to advance our understanding in this field. In summary, mitochondrial SIRT3 could be a novel candidate for treating renal diseases.

AUTHOR CONTRIBUTIONS

SS proposed the concept, designed the figures, wrote and edited the manuscript. KK provided intellectual input. JG edited the manuscript.

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REVIEW
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Diabetic Nephropathy: Novel Molecular Mechanisms and Therapeutic Targets

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Diabetic nephropathy (DN) is one of the major microvascular complications of diabetes mellitus and the leading cause of end-stage kidney disease. The standard treatments for diabetic patients are glucose and blood pressure control, lipid lowering, and renin-angiotensin system blockade; however, these therapeutic approaches can provide only partial renoprotection if started late in the course of the disease. One major limitation in developing efficient therapies for DN is the complex pathobiology of the diabetic kidney, which undergoes a set of profound structural, metabolic and functional changes. Despite these difficulties, experimental models of diabetes have revealed promising therapeutic targets by identifying pathways that modulate key functions of podocytes and glomerular endothelial cells. In this review we will describe recent advances in the field, analyze key molecular pathways that contribute to the pathogenesis of the disease, and discuss how they could be modulated to prevent or reverse DN.

Keywords: diabetic nephropathy, renin-angiotensin system, angiotensin 1–7, sirtuins, notch signaling, thyroid hormone signaling, sodium-glucose cotransporter 2, hypoxia inducible factor

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INTRODUCTION

Diabetes is a global epidemic that is creating an unsustainable strain on healthcare systems due to its rising incidence worldwide and the costs associated with its chronic complications (http://www.idf.org/diabetesatlas). About one-third of diabetic patients develop diabetic nephropathy (DN), which in patients with micro- and then macro-albuminuria tends to progress to end-stage renal disease (ESRD) (Remuzzi et al., 2002). In type 2 diabetes, albuminuria is now recognized not simply as a marker of renal dysfunction but also as a risk factor for cardiovascular disease, which is three times as high as that for diabetic patients with no evidence of renal disease (Fox et al., 2004; Zhou et al., 2009). Renin-angiotensin system (RAS) inhibitors reduce albuminuria and the cardiovascular complications of diabetes but may provide incomplete renoprotection if started late in the course of the disease (Perico et al., 1994; Ruggenenti et al., 2010). Developing efficient therapies for DN is extremely challenging because of the complex pathobiology of the diabetic organ, which undergoes a set of profound structural, metabolic and functional changes.

Glomerular visceral epithelial cells (podocytes). Podocytes are the main determinant of the maintenance of the perm-selective properties of the glomerular filtration barrier (Nagata, 2016; Conti et al., 2017), and podocyte dysfunction has been considered a major factor in the development of diabetic glomerular disease (Pagtalunan et al., 1997; Wolf et al., 2005). Podocytes are highly specialized cells located on the visceral side of the Bowman's capsule and exhibit podocyte foot processes, which are connected by a specialized intracellular junction, the slit diaphragm, which in turn forms a size-selective barrier for the passage of large molecules. Specific diaphragm proteins,

such as nephrin, form the filtration slits (Conti et al., 2017). At the slit diaphragm, podocin and other proteins provide structural and functional support to the filtration barrier and participate in signaling pathways by interacting with actin cytoskeleton components (Perico et al., 2016a; Schell and Huber, 2017). Reduced expression of podocyte proteins, which reflects podocyte dysfunction, and a reduced number of podocytes are characteristic features of DN, both in experimental models and humans (Pagtalunan et al., 1997; Aaltonen et al., 2001; Benigni et al., 2004; Lin and Susztak, 2016). A correlation between podocyte detachment/loss and the albumin excretion rate has been reported in DN patients (Pagtalunan et al., 1997; Meyer et al., 1999; Lemley et al., 2000). Recently, scanning electron microscopy (SEM) analysis of the podocyte cytoarchitecture in type 2 diabetic patients at different stages of kidney disease showed that in normoalbuminuric subjects, podocytes had intact cell bodies with normal interdigitating foot processes (Conti et al., 2018). In patients with micro-albuminuria, features of podocyte injury, consisting of podocyte hypertrophy with diffuse foot process effacement and occasional pseudocysts representing site of initial cell detachment from the GBM, were observed. In the late stages of proteinuric DN the structural integrity of the glomerular barrier was irreversibly compromised, with the occurrence of striking podocyte loss and extensively denuded glomerular basement membranes (Conti et al., 2018). These observations help explain why drugs may fail to affect renal disease progression in the latter circumstance while underlining the need for early therapeutic intervention to efficiently achieve renoprotection. Although podocytopathy has been considered the culprit in the development of diabetic glomerular disease, glomerular endothelial dysfunction also plays a key role in the pathogenesis and progression of DN (Toyoda et al., 2007; Broekhuizen et al., 2010; Kuwabara et al., 2010; Satchell, 2012; Weil et al., 2012).

Glomerular endothelium. The glomerular endothelium along with the glycocalyx–a negatively charged network of proteoglycans and glycoproteins that covers the luminal surface of fenestrated glomerular endothelial cells–has been recognized as crucial in restricting the passage of plasma proteins and preserving the glomerular filtration barrier (Fogo and Kon, 2010; Haraldsson and Nystrom, 2012; Salmon and Satchell, 2012). Loss of the endothelial glycocalyx is linked to increased vascular permeability in type 2 diabetic patients and to albuminuria in experimental DN (Broekhuizen et al., 2010; Kuwabara et al., 2010). Lower endothelial cell fenestrations are associated with macroalbuminuria and GFR decline (Weil et al., 2012) and glomerular capillary loss correlates with the degree of glomerulosclerosis (Hohenstein et al., 2006). Multiple pathways contribute to endothelial dysfunction in DN. Hyperglycemia and oxidative stress cause glycocalyx destruction through the induction of heparanase, a degrading enzyme of heparan sulfate, reduced synthesis of heparan sulfate, and uncoupling of the endothelial nitric oxide synthase (Fu et al., 2015; Jourde-Chiche et al., 2019). Studies have provided evidence that there is cross-talk between glomerular endothelial cells and podocytes that is important in regulating survival and function for both cells (Satchell, 2012; Fu et al., 2015; Lennon and Hosawi, 2016; Cassis et al., 2019b). Thus, glomerular endothelial dysfunction may cause injury in the neighboring podocytes and, *vice versa*, podocyte activation may foster endothelial damage through specific paracrine signals. Vascular endothelial growth factor (VEGF), angiopoietins, endothelin-1, transforming growth factor-β (TGF-β), to name a few, have all been implicated as major mediators of this vicious cycle (Fu et al., 2015; Garsen et al., 2016; Wu et al., 2017; Jourde-Chiche et al., 2019). Targeting the reciprocal interaction between endothelial cells and podocytes may be a therapeutic opportunity to limit DN progression.

Impairment of the glomerular filtration barrier, with the onset of overt proteinuria, accelerates the progression of diabetic kidney disease, and glomerular sclerosis and interstitial fibrosis is the final step toward ESRD. Several studies have elucidated the complexity of the fibrogenic process in the kidney, which involves the interplay among different cell types, the activation of several profibrotic pathways, including the most known TGF-β, and their epigenetic regulation (Srivastava et al., 2013; Lovisa et al., 2016; Macconi, et al., 2016; Zhao et al., 2020). Kidney fibrosis develops through intracellular mechanisms, that comprise glomerular and tubular epithelium distress, inflammation, dysregulated innate and adaptive immune response, tubular injury and atrophy, and microvasculature rarefaction (Liu, 2011; Tang and Yiu, 2020). In the last decade potential progenitors for myofibroblasts were identified which include proliferating resident interstitial fibroblasts, bone marrow-derived cells, perivascular mesenchymal stem cells, and epithelial and endothelial cells that acquire a myofibroblast phenotype in processes termed epithelial to mesenchymal transition (EMT) and endothelial-to-mesenchymal transition (EndMT) (Zeisberg et al., 2008; LeBleu et al., 2013; Srivastava et al., 2013; Falke et al., 2015; Kramann et al., 2015; Lovisa et al., 2016; Srivastava S. P. et al., 2019).

Experimental models of diabetes have revealed promising therapeutic targets by enabling the identification of pathways that modulate key functions of podocytes and glomerular endothelial cells. In this review, we describe recent advances in the field and discuss emerging therapeutic strategies. Specifically, we focus on the angiotensin converting enzyme 2 (ACE2)/Angiotensin-(1–7)/Mas receptor axis and the protective effects of cyclic Ang-(1–7) on both podocytes and glomerular endothelial cells in experimental type 2 DN. Morever, we discuss Sirtuins 1 and 3 as a therapeutic target for counteracting diabetes-induced oxidative stress and glomerular injury. The role of the developmental pathways Notch1 and thyroid hormone signaling in podocytes during diabetic disease is described as a mechanism that underlies podocyte de-differentiation and loss. Finally, the contributions of the sodium-glucose cotransporter 2 (SGLT2), hypoxia-inducible factor-1 (HIF-1) and dipeptidyl peptidase-4 (DPP-4) signaling pathways to the progression of DN are discussed.

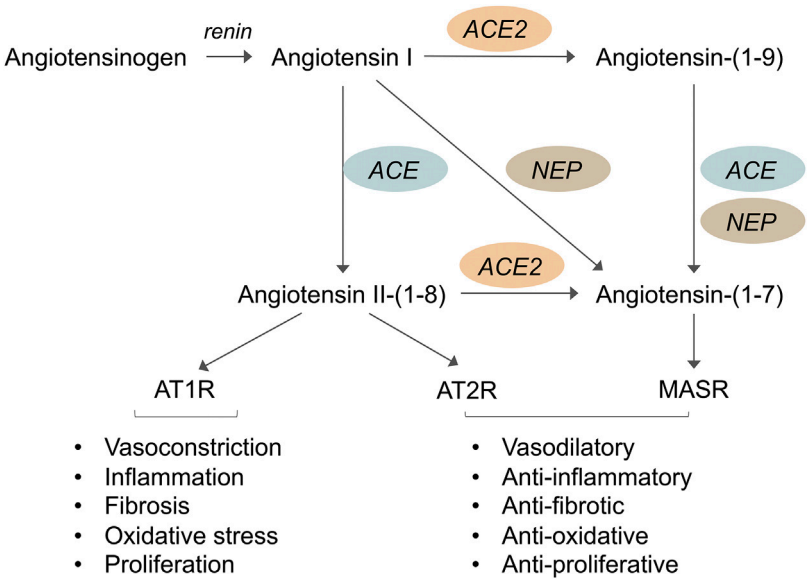

FIGURE 1 | Schematic representation of the renin-angiotensin system (RAS) showing that the angiotensin converting enzyme 2 (ACE2)/Angiotensin-(1–7)/Mas receptor axis exerts opposite effects to those of ACE/Angiotensin II/AT1 receptor axis. Renin cleaves hepatic angiotensinogen into Angiotensin I which is then cleaved via ACE into Angiotensin II. The effects of Angiotensin II are exerted mainly through the activation of the Angiotensin II type 1 receptor (AT1R) and includes vasoconstriction, inflammation, fibrosis, oxidative stress and cell growth. Angiotensin II also binds to Angiotensin II type 2 receptor (AT2R) which usually opposes the actions of AT1R. Angiotensin-(1–7), which is a specific Mas receptor (MasR) agonist, can be formed directly from Angiotensin II via ACE2, or it can be generated through the ACE2-catalyzed hydrolysis of Angiotensin I to the inactive Angiotensin-(1–9) which is then converted to Angiotensin-(1–7) by ACE or neprilysin (NEP). However, Angiotensin-(1–7) is mainly formed through the action of ACE2 on Angiotensin II which has more affinity to ACE2 than Angiotensin I. When levels of Ang II are not sufficiently elevated, Ang-(1–7) can also be formed directly from Ang I via NEP. Interaction of Angiotensin-(1–7) with MasR triggers intracellular signaling pathways leading to beneficial actions such as vasodilation, anti-inflammatory, anti-fibrotic and anti-oxidative effects, and inhibition of cell proliferation.

THE RENIN-ANGIOTENSIN SYSTEM

Angiotensin Converting Enzyme/ Angiotensin-II/Ang II Type 1-Ang II Type 2 Receptors

The RAS plays a key role in a variety of physiological and pathological processes. The RAS is activated by the secretion of renin by the juxtaglomerular cells of the kidney. Renin hydrolyzes liver-derived angiotensinogen into angiotensin (Ang) I, a decapeptide, which is then cleaved by ACE into the octapeptide Ang II (**Figure 1**). This occurs not only in the circulation but also locally in several organs, including the kidney, blood vessels, and heart. The effects of Ang II are exerted mainly through the activation of the G protein-coupled receptor Ang II type 1 receptor (AT1R) and include vasoconstriction, fluid retention, inflammation, fibrosis, oxidative stress, and cell growth and migration, to name a few (Forrester et al., 2018).

Ang II also binds to Ang II type 2 receptor (AT2R), which usually has the opposite effects of AT1R, in terms of blood pressure regulation, vascular remodeling and cell growth (Horiuchi et al., 1999; Jones et al., 2008; Kaschina et al., 2017). The local actions of Ang II depend on the combined net effect of AT1R and AT2R, so that the levels of AT2R expression relative to AT1R in different pathological states, including diabetes, may be crucial for determining the end-organ response (Jones et al., 2008). However, the signaling mechanisms of AT2R are not completely understood (Forrester et al., 2018). While several studies have reported beneficial effects of AT2R activation on organ protection (Naito et al., 2010; Padia and Carey, 2013; Chow and Allen, 2016; Sumners et al., 2019), some others have shown that increased activation of AT2R could have detrimental effects (Cao, 2002; Tejera et al., 2004). Most of the RAS inhibitors currently used to delay the progression of kidney injury in diabetes target the ACE/Ang II/AT1 receptor axis.

Angiotensin Converting Enzyme Substrate N-Acetyl-Seryl-Aspartyl-Lysyl-Proline has Antifibrotic Properties in Diabetic Nephropathy

ACE has two homologous N- and C-terminal active domains (Bernstein et al., 2011). While the ACE C-terminal catalytic domain is the main site of Ang I cleavage into Ang II *in vivo* (Fuchs et al., 2008), the N-domain specifically cleaves its natural substrate N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) into inactive fragments (Fuchs et al., 2004). Ac-SDKP is released by the nephron from its precursor thymosin β4 through two-step proteolytic cleavage that involves meprinα and the serine protease prolyl oligopeptidase (POP), also known as prolyl endopeptidase (Prep) (Kumar et al., 2016), and the thymosin β4-Ac-SDKP axis is a peptidergic system that prevents kidney fibrosis under normal conditions (Romero et al., 2019) and can reduce established

fibrosis during kidney injury (Zuo et al., 2013). The homeostatic role of Ac-SDKP in collagen balance is further supported by evidence that low endogenous levels of the peptide in the kidneys as a result of POP inhibition promote organ fibrosis, which is accelerated in the presence of profibrotic stimuli (Cavasin et al., 2007). Consistently, the epigenetic downregulation of POP/Prep by miR-324-3p contributed to kidney fibrosis in chronic kidney disease (CKD) by rendering renal tubular cells more prone to acquiring a mesenchymal phenotype in response to profibrotic stimuli (Macconi et al., 2012). The rise in plasma and urinary levels of Ac-SDKP caused by ACE inhibitors is part of their renoprotective effect (Macconi et al., 2012; Nagai et al., 2014; Srivastava et al., 2020a; Srivastava et al., 2020b). Differences in the renal protective ability of ACE inhibitors and AT1R blockers (ARB) have been described in experimental DN, which were attributable to the capacity of ACE inhibitor, but not ARB, to prevent ACE-induced Ac-SDKP degradation (Nagai et al., 2014; Srivastava et al., 2020a; Srivastava et al., 2020b). In a mouse model of type 1 DN, both ACE inhibitor and Ac-SDKP, but not ARB, ameliorated renal fibrosis by controlling the metabolic switch between glucose and fatty acid metabolism, thus suppressing glycolysis-related EMT (Srivastava et al., 2020b). Moreover, the ACE inhibitor alone or combined with Ac-SDKP inhibited the renal overexpression of the enzyme dipeptidyl peptidase-4 (DPP-4) and the activation of TGF-β signaling by restoring the expression of the anti-fibrotic microRNAs miR-29s and miR-let-7s, which targeted DDP-4 and the TGF-β receptor TβRI, respectively. This resulted in reduced EndMT and ECM deposition in diabetic kidneys (Srivastava et al., 2020a). Unlike ACEi, an ARB, which failed to protect the diabetic kidney against fibrosis, did not modulate miRNAs and DDP-4 expression (Nagai et al., 2014; Srivastava et al., 2020a).

ANGIOTENSIN CONVERTING ENZYME 2/ ANGIOTENSIN-(1–7)/MAS RECEPTOR AXIS IN DIABETIC NEPHROPATHY

Studies performed in the past few decades have revealed the great complexity of the RAS and demonstrated that, in addition to the classical ACE/Ang II/AT1R axis, the RAS comprises other important, biologically active enzymes, peptides and receptors (Simoes e Silva et al., 2013; Forrester et al., 2018; Povlsen et al., 2020). In 2000, the discovery of ACE2, a zinc metalloprotease homologous to ACE, revealed a new pathway for the Ang II peptide metabolism (Donoghue et al., 2000; Tipnis et al., 2000; Hamming et al., 2007). ACE2 hydrolyzes Ang II to the heptapeptide Ang-(1–7) and converts Ang I to the nonapeptide Ang-(1–9), which in turn can be converted to Ang-(1–7) by ACE, limiting Ang II production (**Figure 1**). Ang-(1–7) is produced mainly through the action of ACE2, which has a greater affinity for Ang II than Ang I; thus, Ang II is the major substrate for Ang-(1–7) synthesis. However, when levels of Ang II are not sufficiently elevated, Ang-(1–7) can also be formed directly from Ang I via neprilysin (NEP) (Rice et al., 2004). Ang-(1–7) is degraded to Ang-(1–5) by the action of ACE. Ang-(1–7) binds to a specific G protein-coupled receptor, the Mas receptor (Santos et al., 2003) triggering intracellular mechanisms and functional events that oppose many of the deleterious effects of Ang II, to the point that the ACE2/Ang-(1–7)/Mas receptor is considered the counterregulatory axis of ACE/Ang II/AT1R (Simoes E Silva and Teixeira, 2016; Rodrigues Prestes et al., 2017). In diabetes, an imbalance between the Ang II and Ang-(1–7) systems is indeed associated with vascular dysfunction, inflammation and fibrosis (Simoes e Silva et al., 2013; Srivastava P. et al., 2019).

Angiotensin Converting Enzyme 2

ACE2 is an 805 amino-acid type 1 integral membrane glycoprotein (110–120 kDa) that consists of an extracellular domain, a transmembrane region and an intracellular tail. The extracellular domain of ACE2 contains a single active catalytic domain, unlike ACE, which consists of two catalytic domains (Donoghue et al., 2000; Tipnis et al., 2000; Batlle et al., 2012). ACE2 is mainly a tissue enzyme that is expressed at high levels in the kidneys, testes, intestine and heart (Donoghue et al., 2000; Tipnis et al., 2000) but can also be found in the lungs, liver, brain and pancreas. Unlike ACE, its levels in plasma are relatively low. A soluble form of ACE2 has been found in the circulation, in urine and in cerebrospinal fluid.

Studies have suggested that ACE2 has a renoprotective role in experimental renal diseases, including DN, particularly in combination with decreased ACE activity (Ye et al., 2004), because it enhances the degradation of Ang II (Batlle et al., 2012). Reduced expression of glomerular ACE2, coupled with increased expression of ACE, has been found in type 2 diabetic *db/db* mice, which favors excessive Ang II accumulation and its deleterious effects (Ye et al., 2006). After diabetic mice were treated with a specific ACE2 inhibitor, ACE increased further. In the same study, immunogold electron microscopy, used to identify the ultrastructural localization of ACE2 and ACE in the glomeruli of diabetic mice, showed that ACE2 was predominantly localized in podocyte foot processes, whereas ACE was expressed in glomerular endothelial cells. This finding suggested that the presence of ACE2 in podocytes could play an important counterregulatory role by preventing glomerular Ang II accumulation and the Ang II-mediated increase in glomerular permeability that results in the development of albuminuria (Ye et al., 2006). In this regard, the selective overexpression of human ACE2 in the podocytes attenuated the development of nephropathy in mice with streptozotocin-induced type 1 diabetes, and compared with wild-type diabetic mice, these mice experienced less glomerular injury, a delay in developing albuminuria, a blunted decrease in the podocyte markers nephrin and synaptopodin, and protection against podocyte loss (Nadarajah et al., 2012). On the other hand, pharmacological ACE2 inhibition worsened albuminuria and glomerular mesangial matrix expansion in streptozotocin-induced diabetic mice, in association with increased glomerular and vascular ACE expression (Soler et al., 2007; Wysocki et al., 2017). Studies using recombinant ACE2 in rodents have demonstrated the ability of ACE2 to rapidly metabolize ANG II *in vivo* and to

promote Ang-(1–7) formation (Batlle et al., 2012). However, recombinant ACE2 induced an increase in plasma ACE2 activity but did not affect urinary ACE2, and failed to protect mice against the development of DN, indicating that when the augmentation of ACE2 activity is limited to the circulation, it is not sufficient to provide renoprotection because ACE2 needs to reach the urinary space to be effective (Wysocki et al., 2017). The fact that there was no increase in urinary ACE2 activity was attributed to the lack of glomerular filtration of recombinant ACE2, because of its large molecular size. Recently, shorter forms of ACE2, which are enzymatically active and can be filtered and delivered to the kidney, have been generated (Wysocki et al., 2019). They would enhance the formation of Ang-(1–7) from Ang II and could be a potential therapeutic approach for kidney diseases, including DN.

Angiotensin-(1–7)

A large body of studies has shown that the biological actions of Ang-(1–7) through the Mas receptor are generally the opposite of those exerted by Ang II through its AT1R (Simoes E Silva and Teixeira, 2016; Rodrigues Prestes et al., 2017). Ang-(1–7) is formed mainly by Ang II via ACE2 and the balance between these two peptides within the RAS is greatly dependent on this enzyme (Marquez and Batlle, 2019). Beneficial effects of Ang-(1–7) have consistently been reported in experimental DN (Giani et al., 2012; Mori et al., 2014; Shi Y. et al., 2015; Zhang et al., 2015). The delivery of Ang-(1–7) by osmotic minipumps to Zucker diabetic fatty rats, a model of type 2 DN, thus caused a reduction in proteinuria, systolic blood pressure, and renal fibrosis, in association with decreased production of oxidative stress and inflammatory markers (Giani et al., 2012). Similarly, Ang-(1–7) treatment reduced oxidative stress, fibrosis and lipotoxicity in the kidneys of *db/db* mice (Mori et al., 2014). The infusion of Ang-(1–7) also attenuated the progression of streptozotocin-induced diabetic injury, limiting glomerulosclerosis, oxidative stress and cell proliferation (Zhang et al., 2015). Enhancing the Ang-(1–7) axis led to remarkable anti-inflammatory effects, resulting in the reduction of diabetes-induced leukocyte recruitment (Bossi et al., 2016). All these reported effects therefore made Ang-(1–7) a candidate therapeutic agent for DN.

Cyclic Ang-(1–7) and Renoprotection in Diabetic Nephropathy

Ang-(1–7) has a short half-life in plasma, due to rapid *in vivo* catabolism by ACE and other proteases (Yamada et al., 1998), which is a limiting factor for its use for clinical purposes. Through the thioether cyclization method, a modified lanthipeptide cyclic (c)-Ang (1–7) was generated in which the amino acids Tyr4 and Pro7 were replaced with a D,L lanthionine (dAla-S-Ala) (Kluskens et al., 2009; Kuipers et al., 2019). The thioether-cyclized Ang-(1–7) provided enhanced resistance against proteolytic degradation in the circulation, with improved activity compared to the linear counterpart (Kluskens et al., 2009; de Vries et al., 2010). The higher resistance of cAng-(1–7) enables the use of lower doses and possibly less frequent administration than would be necessary with the linear peptide (Kluskens et al., 2009). Another advantage of the thioether-bridged cAng-(1–7) is that, unlike linear Ang-(1–7), it offers the possibility of oral and pulmonary delivery (de Vries et al., 2010). The lantipeptide cAng-(1–7) stimulates the Mas receptor, maintaining the receptor profile of the linear Ang-(1–7), specifically, as indicated by evidence that its vasodilating activity was abolished or decreased by the Mas receptor agonists D-Prot7 and D-Ala7 (Kluskens et al., 2009). In both mice with streptozotocin-induced diabetes and *db/db* mice, cAng-(1–7) caused an increase in insulin levels and reduced blood glucose levels, indicating the therapeutic potential that cAng-(1–7) has for treating type 1 and 2 diabetes (Kuipers et al., 2019).

Using BTBR *ob/ob* diabetic mice, a model that reproduces characteristic features of human type 2 DN better than other murine models, remarkable renoprotection was obtained after subcutaneous injections of cAng-(1–7) (Cassis et al., 2019a). Cyclic Ang (1–7) treatment, started when mice had already developed albuminuria, significantly limited the progressive increase in albuminuria that was observed in untreated BTBR *ob/ob* mice. Notably, we found that cAng (1–7) had as strong an antiproteinuric effect as the ACE inhibitor lisinopril, which was used for comparison, and limited glomerular fibrosis and inflammation even better than lisinopril (Cassis et al., 2019a). To uncover the mechanisms underlying the strong antiproteinuric effect of cAng-(1–7), we focused on podocytes and the glomerular endothelium because of their key role in maintaining an intact glomerular filtration barrier in DN (Weil et al., 2012; Siddiqi and Advani, 2013). cAng-(1–7) ameliorated the defective expression in podocytes of nephrin–the slit diaphragm protein that preserves slit pore integrity and renal filtration capacity–and nestin–a protein involved in the organization of the cytoskeleton–and limited podocyte loss, similar to the ACE inhibitor. cAng (1–7) was better at counteracting glomerular capillary rarefaction, a hallmark of advanced DN (Eleftheriadis et al., 2013), than lisinopril. The beneficial effects of cAng (1–7) on the glomerular endothelium were also revealed by electron microscopy analysis showing a reduction of vacuolization and improvement in the loss of endothelial fenestration. These data indicate that podocytes and glomerular endothelial cells are important targets of the renoprotective effects displayed by cAng-(1–7) in experimental diabetes. When cAng (1–7) was combined with lisinopril, the renoprotective action was additive, with a superior anti-proteinuric effect than ACE inhibitor had alone, along with better preservation of podocyte proteins and glomerular capillaries. Thus, cAng-(1–7), added to a background of chronic ACE inhibition, may provide a therapeutic opportunity for those diabetic patients who benefit less from ACE inhibitors.

EFFECTS OF SIRTUINS IN DIABETIC NEPHROPATHY

Sirtuins are an evolutionarily conserved family of seven NAD^+ -dependent deacetylases that reside in different subcellular compartments and regulate many physiological processes, including energy production, metabolism, mitochondrial

biogenesis, stress resistance, inflammation and longevity (Haigis and Sinclair, 2010; Guarente, 2011; Morigi et al., 2018).

Sirtuin-1

Of the seven sirtuins, sirtuin-1 has been one of the most extensively investigated in kidney diseases (Kong et al., 2015), and its renoprotective effects have been consistently demonstrated in experimental DN (Wang et al., 2019). It is localized in the nucleus and exerts its biological effects through the deacetylation of histones and transcription factors relevant for kidney disease progression, including p53, NF-kB, FOXO4, STAT3, PGC-1alpha, and consequently regulating their activities (Morigi et al., 2018; Wang et al., 2019). Sirtuin-1 protein expression was reduced in podocytes and in glomerular cells of human diabetic kidneys (Chuang et al., 2011).

Role of Sirtuin-1 on Podocytes

The podocyte-specific loss of sirtuin-1 reduced podocyte numbers, exacerbated albuminuria, and accelerated renal disease progression in diabetic mice (Chuang et al., 2014; Liu et al., 2014). There is evidence that sirtuin-1 is necessary for the preservation of cytoskeleton integrity and podocyte survival (Motonishi et al., 2015; Nakatani and Inagi, 2016). Analyses of isolated glomeruli from podocyte-specific sirtuin-1 knockout mice after the induction of a non-diabetic injury revealed severe morphological changes in podocytes, with foot process effacement and cytoskeleton derangement, in association with reduced expression of podocyte proteins, such as nephrin, nestin and Wilm's tumor 1 protein (WT-1) (Motonishi et al., 2015). The mechanisms responsible for podocyte dysfunction after the loss of sirtuin-1 were found to be dependent on the inactivation of cortactin, an actin-binding protein that regulates the assembly, polymerization and stabilization of F-actin in different cell types, including podocytes. Indeed, sirtuin-1 deacetylated cortactin and enhanced cortactin activity, favoring localization in the cytoplasm and interaction with actin fibers, which are essential for maintaining the actin cytoskeleton (Motonishi et al., 2015). The induction of sirtuin-1 overexpression, specifically in podocytes, or treatment with the specific sirtuin-1 agonist BF175, in OVE26 type 1 diabetic mice, reduced albuminuria and attenuated diabetes-induced podocyte loss and oxidative stress, providing evidence that sirtuin-1 protects against diabetic disease (Hong et al., 2018). Sirtuin-1 renoprotection was mediated through PGC-1 alpha, the master regulator of mitochondrial function that, once deacetylated, protects podocytes against high glucose-induced oxidation and mitochondrial dysfunction (Hong et al., 2018). Some data suggest that in DN there is complex functional interplay between proximal tubules and glomeruli, regulated by sirtuin-1 (Hasegawa et al., 2013). The targeted deletion of sirtuin-1 in the proximal tubules of diabetic mice led to reduced levels of sirtuin-1 and high expression of the tight junction protein claudin-1 in podocytes, which led to the initiation of albuminuria and the development of renal function impairment (Hasegawa et al., 2013). To provide one potential explanation for these results, it was shown that proximal tubular cells exposed to high glucose concentrations *in vitro* secrete less nicotinamide mononucleotide (NMN), which lowers sirtuin-1 in podocytes and upregulates claudin-1 expression (Hasegawa et al., 2013). There is also some evidence that sirtuin-1 and the RAS interact, and this supports the hypothesis that sirtuin-1 is an important therapeutic target in DN. Sirtuin-1 activates the ACE2 promoter, thus favoring the production of Ang-(1–7) and its positive effects (Clarke et al., 2014; Mori et al., 2014). Ang-(1–7) increases sirtuin-1 expression, whereas Ang II has the opposite effect. In podocytes exposed to Ang II the expression of sirtuin-1 actually decreased, concomitant with the acetylation of p53, a pathway involved in podocyte apoptosis. Treating diabetic mice with an ARB that reduced albuminuria and protected podocytes against apoptosis and loss was associated with increased sirtuin-1 activity and reduced p53 acetylation in the kidneys (Gu et al., 2016).

Role of Sirtuin-1 on Endothelial Cells

It has been shown that sirtuin-1 regulates the angiogenic activity of endothelial cells and a specific deletion of its deacetylase activity in endothelial cells aggravated capillary rarefaction in a model of renal interstitial fibrosis (Potente et al., 2007; Kida et al., 2016). There is evidence that hyperglycemia-induced endothelial dysfunction was associated with sirtuin-1 downregulation and overexpression of vasoactive and profibrotic factors, such as endothelin-1 and TGF-β (Mortuza et al., 2015). Sirtuin-1 overexpression prevented glucose-induced increased endothelial permeability and extracellular matrix protein production *in vitro*. In addition, sirtuin-1 overexpressing transgenic mice with diabetes exhibited ameliorated albuminuria and kidney fibrosis (Mortuza et al., 2015). As for the mechanism(s) underlying the protective role of sirtuin-1 in diabetes-induced endothelial dysfunction, a recent study showed that sirtuin-1, through its deacetylase activity, suppresses the capacity of the 66-kDa Src homology two domain-containing protein (p66Shc) to induce vascular oxidative stress (Kumar et al., 2017). The p66Shc is a member of the Shc family of the adaptor proteins that acts as a redox enzyme for intracellular ROS generation. There is evidence that p66Shc is upregulated in cultured endothelial cells exposed to high glucose and in the vascular endothelium of diabetic mice, and that it is responsible for the upregulation of miR-34a, an upstream epigenetic regulator of sirtuin-1 (Li et al., 2016). Actually, systemic infusion of miR-34a inhibitor or genetic ablation of endothelial miR-34a prevented downregulation of endothelial sirtuin-1 caused by hyperglycemia (Li et al., 2016). All the above findings indicate that interplay between sirtuin-1, p66Shc and miR-34a regulates oxidative stress-driven dysfunction of vascular endothelium in diabetes.

Sirtuin-3 and Diabetic Nephropathy

Sirtuin-3, localized in the mitochondrial matrix, is the main mitochondrial NAD+ -dependent deacetylase that affects key mitochondrial processes, such as respiratory chain activity, the tricarboxylic acid cycle, ATP production, and antioxidant pathways (Ahn et al., 2008; Perico et al., 2016b). Changes in sirtuin-3 expression have a profound impact on the pathophysiology of several diseases, including metabolic syndrome, diabetes, and the aging processes (Benigni et al.,

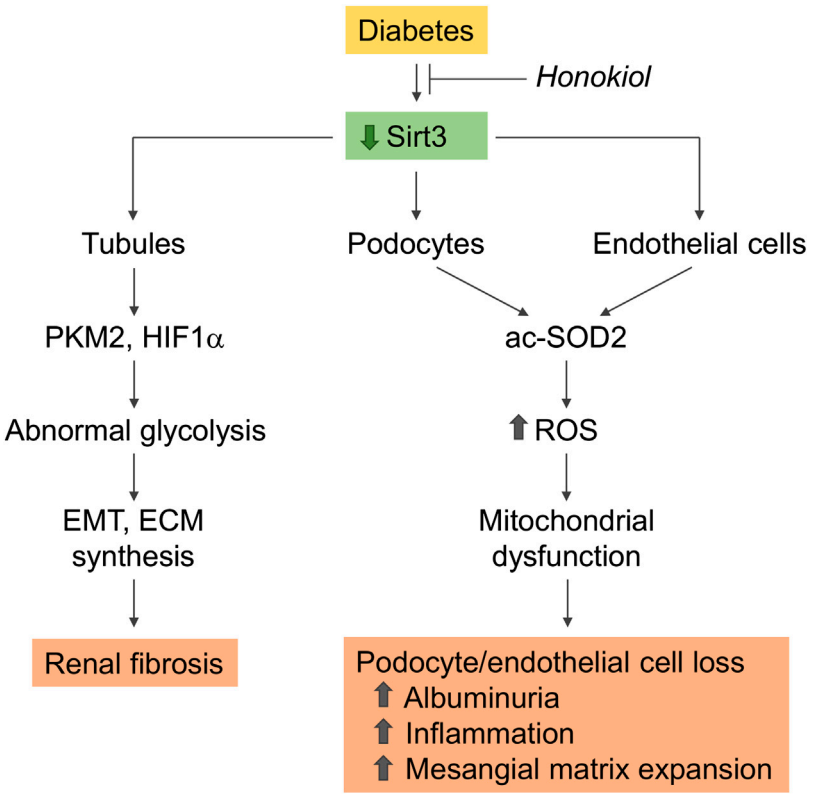

FIGURE 2 | Role of sirtuin-3 dysregulation in kidney disease progression in diabetes. Sirtuin-3 is downregulated in the diabetic kidney. Reduced expression in podocytes and glomerular endothelial cells impairs SOD2 antioxidant activity as a consequence of enzyme acetylation, resulting in increased ROS generation, which promotes mitochondrial dysfunction and cell loss. These changes contribute to the development of albuminuria associated with inflammation and mesangial matrix expansion. Renal tubules with reduced sirtuin-3 undergo a metabolic reprogramming with a shift toward abnormal glycolysis, display EMT, and acquire a profibrotic phenotype. Rescuing sirtuin-3 by the specific activator honokiol prevents glomerular and tubule dysfunction and ameliorates diabetic nephropathy. acSOD2, acetylated SOD2; PKM2, pyruvate kinase isozyme M2; EMT, epithelial to mesenchymal transition; ECM, extracellular matrix.

2016; Perico et al., 2016b; Benigni et al., 2019). In an *in vitro* model of diabetes, sirtuin-3 overexpression protected proximal tubular cells against high glucose-induced oxidative stress by enhancing the expression of antioxidant genes superoxide dismutase (SOD) and catalase (Jiao et al., 2016). Sirtuin-3 also protected endothelial cells from high glucose-induced cytotoxicity by modulating ROS production and oxidative stress through SOD deacetylation (Liu G. et al., 2015). There is evidence that sirtuin-3 mRNA expression is downregulated in kidney biopsies from DN patients (Wang X.X. et al., 2016). The kidneys of streptozotocin-induced diabetic CD-1 mice consistently exhibited a reduction in the sirtuin-3 protein, with the concomitant induction of a fibrogenic phenotype, which was exacerbated after sirtuin-3 suppression by the systemic administration of sirtuin-3 small interfering (si)RNA (Srivastava et al., 2018). The observation that in these mice the suppression of sirtuin-3 was associated with the induction of abnormal glycolysis, and that treatment with glycolysis inhibitors ameliorated renal fibrosis and restored sirtuin-3 levels as well, was taken to suggest that the restoration of sirtuin-3 could be a strategy for combating diabetes-associated kidney fibrosis through the inhibition of aberrant glycolysis (Srivastava et al., 2018) (**Figure 2**).

Honokiol and Renoprotection in Diabetic Nephropathy

We recently demonstrated that renal sirtuin-3 mRNA expression was lower in type 2 BTBR *ob/ob* diabetic mice, in association with an impairment in its deacetylase activity toward SOD2, a major target of sirtuin-3, and was also associated with increased ROS production (Locatelli et al., 2020) (**Figure 2**). The selective activation of sirtuin-3 through the administration of honokiol, a natural biphenolic compound isolated from magnolia bark that has antioxidant, anti-inflammatory and anti-fibrotic properties, resulted in the attenuation of albuminuria and amelioration of glomerular injury (Locatelli et al., 2020). The anti-albuminuric effect of honokiol was associated with the amelioration of podocyte dysfunction and loss. In addition, honokiol limited glomerular capillary rarefaction, as revealed by immunofluorescence for CD-31, an endothelial cell marker, and the accumulation of Mac-2 positive monocytes/macrophages in the glomeruli. Sirtuin-3 activation with

honokiol also translated into improvements in mitochondrial wellness in the glomeruli of diabetic mice through the activation of SOD2 and the restoration of the defective expression of PGC-1 alpha, a known regulator of mitochondrial homeostasis. The specific activation of sirtuin-3 is therefore effective in reducing diabetes-induced oxidative stress and providing protection for podocytes and more generally the glomerulus against diabetes-induced damage.

All the above evidence suggests that the pharmacological modulation of sirtuins is an attractive option for treating DN, and natural and synthetic sirtuin-activated compounds that have been tested in experimental kidney diseases (Morigi et al., 2018) are available for this purpose.

NOTCH SIGNALING IN DIABETIC NEPHROPATHY

The Notch signaling pathway comprises a family of four Notch transmembrane receptors (Notch1–4) and two different families of Notch ligands, namely Jagged (Jag1–2) and Delta-like (Dll1–4). The activation of this signaling requires cell–cell contact. Following ligand engagement, the Notch receptor is proteolytically cleaved by metalloproteases and the γ-secretase complex into the Notch intracellular domain (NICD), which enters the nucleus and associates with the DNA-binding protein CSL (CBF1/RBPjκ/Su(H)/Lag-1) and other transcriptional coactivators to trigger the transcription of target genes, such as hairy-enhancer of split (Hes) and Hes-related genes with the YRPW motif (Hey) (Kopan and Ilagan, 2009). Notch signaling is highly active during nephrogenesis, where it regulates nephron endowment and segmentation spatiotemporally through the differentiation of nephron progenitor cells into mature nephron cell types and patterning cell types within the collecting duct. Specifically, Notch2 signaling plays a key role early in nephrogenesis and is required in the acquisition of proximal nephron cell fates, including those of proximal tubules and podocytes (Mukherjee et al., 2019). Notch1 also contributes to nephrogenesis, albeit to a lesser extent than Notch2 (Surendran et al., 2010).

Reactivation of Notch1 Signaling in Diabetic Nephropathy

In the normal kidney, Notch signaling is attenuated after birth and is inactive in the mature glomeruli of the adult kidney. *De novo* expression of active Notch1 in mature podocytes has been shown to induce apoptosis, which translates *in vivo* into the development of proteinuria and glomerulosclerosis (Niranjan et al., 2008). Active Notch1 leads to an increase in TGF-β1 transcription, which activates the Notch1 signaling pathway through the upregulation of Jag1. *De novo* expression of Notch pathway-related transcripts and the active Notch1 intracellular domain have been observed in the glomeruli and podocytes of murine and human diabetic kidneys (Niranjan et al., 2008). On the other hand, TGF-β1 is stimulated by diabetic states and plays an important role in the pathogenesis of DN (Ziyadeh, 2004). Thus, the interplay between Notch and TGF-β pathways in disease conditions is crucial in the regulation of podocyte apoptosis and can contribute to maintaining the damage.

Notch1 vs. Notch2

Proof that podocyte Notch signaling activation in DN plays a detrimental role comes from studies on the genetic knockdown of Notch signaling components. Diabetic mice with a podocyte-specific deletion of RBPj, which is essential for canonical Notch signaling, were partially protected against renal damage, exhibiting lower levels of albuminuria and less podocyte dedifferentiation and loss, accompanied by reduced TGF-β and vascular endothelial growth factor (VEGF) expression compared with wild-type mice with DN (Niranjan et al., 2008). In addition, the relative role of Notch1 vs. Notch 2 in podocytes during DN development was investigated in studies based on the specific genetic deletion or overexpression of each receptor (Sweetwyne et al., 2015). Podocyte-specific Notch1 deletion ameliorated DN, reducing albuminuria and mesangial expansion by preventing podocyte dedifferentiation and loss (Sweetwyne et al., 2015). In contrast, mice with podocyte-specific deletion of Notch2 were not protected against diabetic kidney disease development. Notch1-null podocytes exhibited preserved nephrin and podocin expression after TGF-β1 stimulation and were protected against growth factor-induced apoptosis. Moreover, glomeruli with podocyte-specific Notch1 deletion exhibited enhanced Notch2 expression, whereas Notch2 levels were lower in TGF-β1-stimulated podocytes with active Notch1, indicating that Notch1 regulates Notch2 in podocytes, both at baseline and after TGF-β1 treatment (Sweetwyne et al., 2015). Consistent with previous findings, podocyte-specific expression of the active Notch1 intracellular domain caused albuminuria and glomerulosclerosis, while mice with overexpression of the Notch2 intracellular domain did not exhibit phenotypic alterations (Sweetwyne et al., 2015). These studies highlighted the harmful role that Notch1 plays in inducing podocyte injury in diabetic kidney disease, while suggesting that Notch2 has a protective effect. Given the differential role of Notch1 and 2, it is likely that the loss of Notch1 and maintenance of Notch 2 in podocytes has a superior effect on glomerulosclerosis and proteinuria than the podocyte-specific loss of the pan-Notch regulator Rbpjk. In this context, the findings that higher glomerular Notch2 expression from diabetic mice that overexpressed podocyte-specific Mafb–a transcription factor that is essential for podocyte differentiation and foot process formation–ameliorated DN (Morito et al., 2014), and that pharmacological activation of Notch2 by an agonist mAb was beneficial against adriamycin-induced nephrosis (Tanaka et al., 2014) further support the hypothesis that this receptor has a protective effect on podocyte function and survival.

Triggers of Notch1 Signaling and Downstream Effectors

The Notch 1 signaling pathway has been recognized as playing a pathogenic role in DN through the induction of podocyte dysfunction and the loss of integrity of the glomerular filtration barrier, eventually resulting in proteinuria. The

functional link between Notch1 activation and nephrin downregulation in podocytes, which is a hallmark of DN, is crucial to this event. Target transcripts that are induced by the active Notch1 intracellular domain include Snail, a transcriptional factor involved in EMT, which acts as a repressor of nephrin expression (Matsui et al., 2007). The Notch1/Snail pathway has been identified as the molecular mechanism underlying Ang II-induced nephrin downregulation in podocytes and the perpetuation of glomerular injury in experimental and human type 2 DN (Gagliardini et al., 2013). In Ang II-stimulated podocytes, the activation of Notch1 canonical signaling, through Hes1, upregulated the expression of Snail and its translocation into the nucleus, leading to nephrin downregulation. These effects were reversed by a γ-secretase inhibitor. In keeping with this, kidney specimens from either diabetic rats or humans exhibited a strong association between enhanced Snail protein signal and reduced nephrin protein expression. The Notch1/Snail pathway has clinical relevance, since its modulation by ACE inhibitors improved podocyte function and reduced overt proteinuria in diabetic patients (Gagliardini et al., 2013). *In vitro* studies have demonstrated that Ang II-induced Notch1 activation in podocytes was associated with the upregulation of TGF-β and VEGF, promoting apoptosis, and these effects were reversed by ARBs. Consistently, in the glomeruli of diabetic kidneys, the overexpression of TGF-β paralleled the increase in Jag1 and active Notch1 intracellular domain staining in podocytes. Ang II inhibition through telmisartan reduced albuminuria in Ins2 Akita diabetic mice by inhibiting TGF-β-associated activation of the Notch1 pathway (Koshizaka et al., 2012). In this context, another ARB, valsartan, also inhibited the activation of Notch, B-Cell CLL/Lymphoma 2 (Bcl-2) and p53 apoptotic pathways, and reduced apoptosis and podocyte detachment and loss in the glomeruli of mice with streptozotocin-induced diabetes (Gao et al., 2016). These findings suggest there is a link between Ang II and TGF-β in the activation of Notch1 signaling in podocyte loss in DN. Since TGF-β can also induce sustained Snail expression in podocytes via Notch1 (Sweetwyne et al., 2015), it is conceivable that the growth factor may also act as a mediator of Ang II-driven activation of the Notch1/Snail axis, leading to podocyte dedifferentiation. Another mechanism through which Notch triggers the onset of proteinuria is by promoting dynamin-dependent, raft-independent nephrin endocytosis (Waters et al., 2012).

Studies in cultured podocytes exposed to high glucose, mimicking diabetic conditions, enabled the identification of VEGF as a downstream effector of Notch1-induced podocyte dedifferentiation and apoptosis. VEGF, which is upregulated in the early stages of DN, is a direct trigger of nephrin repression and apoptosis in podocytes. The inhibition of Notch1 signaling by a γ-secretase inhibitor abrogated the high glucose-induced upregulation of VEGF, reduced nephrin expression and podocyte apoptosis and ameliorated proteinuria in diabetic rats (Lin et al., 2010). Another study has demonstrated there is interplay between the Notch1 and phosphatidylinositol 3-kinase (PI3K)/Akt pathways in regulating high glucose-induced podocyte apoptosis, suggesting that the balance between these two pathways may be important in the context of DN (Wang et al., 2014).

Notch1 Signaling: A Therapeutic Target for Podocyte Protection in Diabetic Nephropathy

Pharmacological Modulation of Notch1 Signaling

The reactivation of Notch1 signaling in podocytes contributes to diabetic glomerulopathy, and its modulation can be achieved through the pharmacological inhibition of RAS (Koshizaka et al., 2012; Gagliardini et al., 2013; Gao et al., 2016), the standard therapy for CKD, including DN, and of Rho kinase, which mediates TGF-β-induced Jag1 expression in podocytes (Matoba et al., 2017) (**Figure 3A**). Type 2 diabetic mice treated with fasudil exhibited reduced albuminuria, urinary nephrin excretion and podocyte loss, which was associated with the downregulation of Jag1 and apoptotic markers in podocytes and less glomerular apoptosis (Matoba et al., 2017).

Preclinical studies have demonstrated that targeting Notch signaling via the genetic deletion of its components or the γ-secretase inhibitors ameliorated DN by having a protective effect on podocyte function and survival (Lin et al., 2010). However, γ-secretase inhibitors are nonspecific because they inhibit all γ-secretase complex-regulated intramembrane proteolyses of different substrates and fail to distinguish between individual Notch receptors. Moreover, concerns have been raised regarding adverse side effects on the gastrointestinal, immune and cutaneous systems, especially with long-term treatment (Wong et al., 2004; van Es et al., 2005). Notch receptor-specific inhibitors may overcome γ-secretase inhibitor-associated side effects, such as gastrointestinal tract toxicity, which depends on the dual inhibition of Notch1 and 2 receptors (Wu et al., 2010). Two different classes of Notch1 monoclonal antibodies are now available (Aste-Amezaga et al., 2010). Nanoparticle-based delivery of Notch1 monoclonal antibodies, which represses Notch signaling by locking the Notch1 receptors in a ligand-unresponsive state, is emerging as a promising, more targeted and efficient therapeutic strategy for treating cancer (Valcourt et al., 2020). The translatability of this tool for treating aberrant Notch1 signaling in DN remains to be established.

Islet-Like/Islet-Based Cell Therapy

Notch1 signaling in DN is emerging as a molecular target of the beneficial effects of cell therapy based on the administration of bone marrow-derived stem cells, which differentiate into islet-like cells in combination with microRNA 124a. Bone marrow-derived stem cells combined with miR-124a inhibited high glucose-induced podocyte apoptosis, concomitant with the repression of Notch1 activation, and ameliorated DN in type 2 diabetic rats (Sun et al., 2018). Islet transplantation consistently ameliorated albuminuria and podocyte ultrastructural changes in DN (He et al., 2018). However, these beneficial effects on renal injury and podocyte restoration were limited by the aberrant activation of Notch1 despite glycemic control, suggesting that the activation of this pathway by multiple factors can promote podocytopathy and

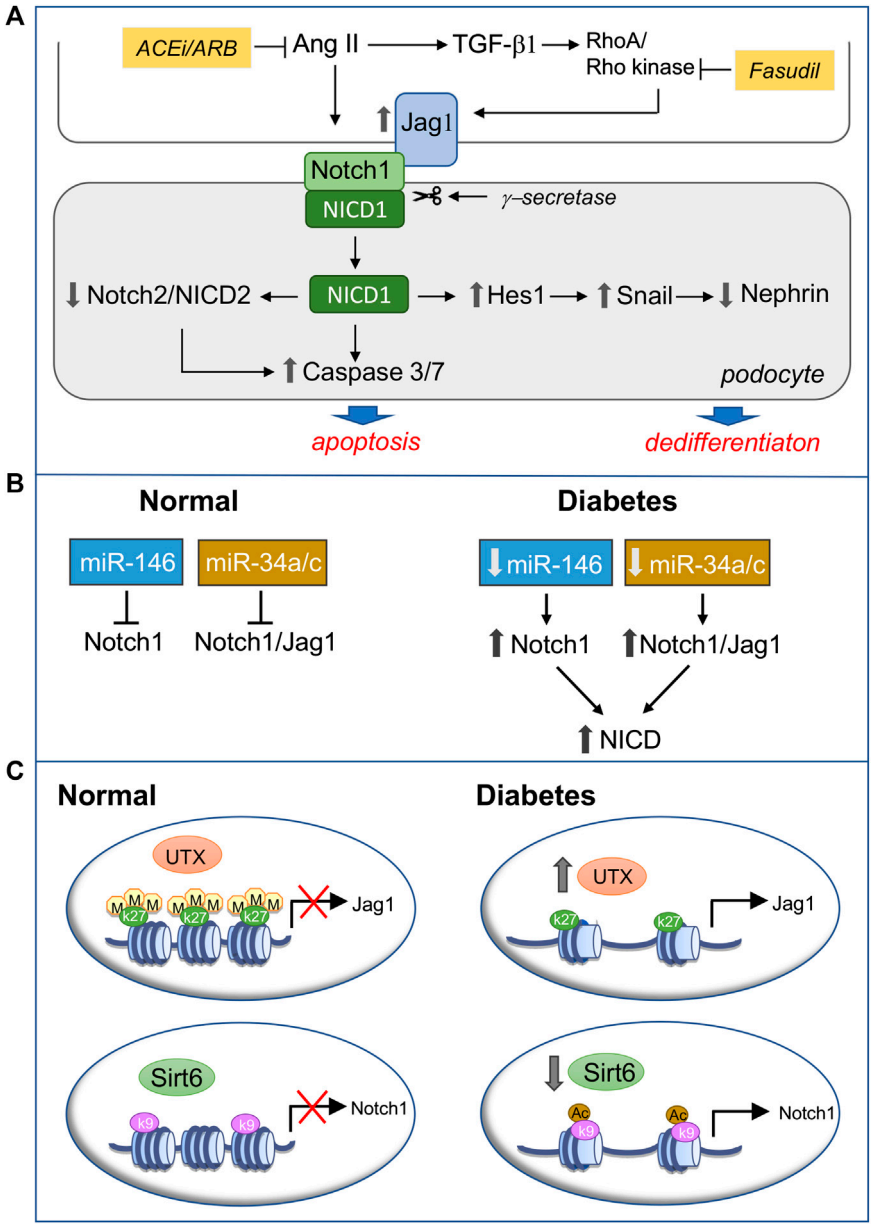

FIGURE 3 | Modulation of podocyte Notch1 signaling in diabetic nephropathy (DN). **(A)** Pharmacological inhibition of active Notch1 inducers. Ang II and TGF-β drive podocyte injury in diabetes through the activation of Notch1 signaling. Following Jag1 engagement Notch1 is cleaved by γ-secretase and the released Notch1 intracellular domain (NICD1), via Hes1, induces sustained Snail expression, which represses nephrin, leading to podocyte dedifferentiation. On the other hand, NICD1 downregulates Notch2 and NICD2 and activates proapoptotic pathways, promoting podocyte apoptosis. Targeting Ang II by RAS inhibition and TGF-β-induced upregulation of Jag1 with the Rho kinase inhibitor fasudil prevents Notch1-mediated podocyte phenotypic changes and loss, ameliorating DN **(B**,**C)**. Epigenetic regulation of Notch1 signaling through miRNAs **(B)** or posttranslational histone modification **(C). (B)** In normal, mature, differentiated podocytes miR-146a and miRNA 34a/c prevent Notch1 signaling activation by targeting the 3′UTR of Notch1 or both Notch1 and Jag1, thus decreasing their mRNA and protein expression. Conversely, diabetes and hyperglycemia-induced downregulation of miR-146a and miRNA 34a/c result in Notch1 signaling activation in podocytes **(C)** In healthy podocytes the trimethylation of lysine residue 27 on histone protein H3 (H3K27me3) in the Jag1 promoter and the Sirt6-mediated deacetylation of lysine residue nine on histone protein H3 (H3K9) in the Notch1 promoter keep the Notch1 signaling pathway silent. In diabetes, reduced H3K27me3 – dependent on the overexpression of the demethylase UTX–and increased H3K9ac due to Sirt6 downregulation relieve the repression of Jag1 and Notch1, respectively, switching on Notch1 signaling in podocytes. ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; UTX, Jumonji C domain-containing family: ubiquitously transcribed tetratricopeptide repeat on chromosome X.

disease progression and affect response to treatment. In this context, the overexpression of active Notch1 in podocytes is not limited to DN but is a common type of intracellular signaling underlying glomerulopathy in several proteinuric kidney diseases, where it strongly correlates with glomerulosclerosis (Murea et al., 2010).

Epigenetic Regulation of Notch1 Signaling

An alternative way of modulating Notch1 dysregulation in DN has been suggested by the recent discovery of the epigenetic regulation of Notch1 and/or its ligand Jag1 (**Figures 3B,C**).

MicroRNAs

Notch1 is a direct target of miR-146a, which is highly expressed in healthy podocytes, protecting them against diabetic injury. In contrast, miR-146a-deficient mice exhibited accelerated glomerulopathy and albuminuria following streptozotocin-induced hyperglycemia. Consistently, the downregulation of miR-146a in the glomeruli of both diabetic human and mouse kidneys correlated with glomerular damage and with a faster decline in renal function and paralleled the upregulation of Notch1 and ErbB4, a member of the epidermal growth factor receptor (Lee et al., 2017). Other miRNAs, including the miR-34 family members miR-34a and miR-34c, have been identified as upstream regulators of the Notch1 signaling pathway. Both miRNAs were downregulated in podocytes under hyperglycemic conditions, while their overexpression inhibited high glucose-induced podocyte apoptosis by directly targeting the 3′UTR of either Notch1 or Jag1, thus decreasing their mRNA and protein expression and blunting Notch1 signaling activation (Liu X. D. et al., 2015; Zhang et al., 2016).

Posttranslational Histone Modification

Chromatin dynamics control cell fate determination and the maintenance of a differentiated phenotype. Specifically, the trimethylation of lysine residue 27 on histone protein H3 (H3K27me3), is enriched at the promoter region of Jag1 and, by inhibiting Jag 1 transcription, restrains Notch pathway activity in adult differentiated podocytes (Majumder et al., 2018). The gain of the H3K27me3 mark is catalyzed by the histone methyltransferase enzyme, the enhancer of zeste homolog 2 (EZH2), while its loss depends on the activity of the Jumonji C domain-containing histone demethylases Jmjd3 and UTX. Notably, podocytes in glomeruli from humans with diabetic glomerulosclerosis exhibited reduced H3K27me3 concomitant with UTX overexpression, Jag1 upregulation, and nephrin loss. Moreover, the inhibition of Jmjd3 and UTX reduced albuminuria, podocyte foot process effacement, and Jag1 upregulation in diabetic mice, indicating that shifts in podocyte H3K27me3 levels may influence the development and outcomes of glomerular injury in DN (Majumder et al., 2018).

Chromatin remodeling and gene transcription are also regulated by histone acetylation/deacetylation, with deacetylated histones being associated with transcriptional repression. Sirtuin-6 is a member of the sirtuin family of class III NAD^+-dependent histone deacetylases, which inhibits Notch signaling by deacetylating lysine residue nine on histone protein H3 (H3K9). Sirtuin-6 expression was reduced in the kidneys of type 1 and 2 diabetic mice, mainly in the podocytes, and in renal biopsies from DN patients. It correlated positively with estimated glomerular filtration rate and negatively with proteinuria and was associated with increased H3K9ac levels. Lower Sirt6 expression in high glucose-treated podocytes consistently paralleled the increased levels of H3K9ac in the promoters of Notch1 and Notch4 and the overexpression of Notch downstream target genes Hes1 and Snail. Furthermore, the activation of Notch signaling is part of the mechanism through which podocyte-specific loss of sirtuin-6 exacerbates podocyte injury and proteinuria in DN (Liu et al., 2017).

Altogether these findings suggest that the epigenetic regulation of Notch1 signaling through the modulation of either miRNA or posttranslational histone modification could be a novel strategy for preventing the reactivation of this developmental pathway in podocytes during glomerular disease and a potential therapeutic intervention that confers protection against DN.

THYROID HORMONE SIGNALING

Thyroid Hormone Signaling: A Critical Player in Diabetes-Induced Fetal Reprogramming

Thyroid hormone signaling plays a critical role in physiological growth and organ development. It is mediated by two main classes of thyroid hormone receptors (TRs) that regulate gene transcription: TR alpha (TRα) and TR beta (TRβ). In mammals, the predominant TR isoforms include TRα1, TRβ1, TRβ2, TRβ3, TRβ4. Other TR variants lack T3-binding capacity, and these are TRα2, TRα3 and TRαΔE6. TRβ is the predominantly adult isoform and regulates TH levels and the liver and kidney metabolism, and is also critical for the normal development of auditory and visual systems (Brent, 2012; Mourouzis et al., 2020). TRα1 is highly expressed in developing organs, including the heart, brain and kidney, and plays a key role in cell proliferation prenatally, while after birth it regulates differentiation in various cell types (Horn and Heuer, 2010; Pantos and Mourouzis, 2014; Benedetti et al., 2019). In the fetus, when the levels of the active form of TH L-triiodothyronine (T3) are low, TRα1 mainly acts as an apo-receptor (unliganded state) to repress adult genes (thus protecting the embryo from premature differentiation) and enhances cell proliferation and organ growth. In contrast, after birth when T3 levels increase, TRα1 switches to the holo-receptor (liganded state) to induce the expression of adult genes, thus promoting cell differentiation, physiological organ maturation and function.

Compared to the healthy population, diabetic patients exhibit lower T3 plasma levels (Wu et al., 2015) and a higher prevalence of thyroid dysfunction, suggesting the recurrence of the fetal profile, with low T3 levels. Several clinical studies have also shown that thyroid dysfunction and low T_3 levels are strongly associated with worse renal clinical outcomes and increased mortality in diabetic patients (Zoccali et al., 2006; Lazzeri et al., 2012; Rhee, 2016). However, the etiogenesis underlying these phenomena remains poorly understood.

Recent studies in our lab have shown that podocytes and parietal epithelial cells in the glomeruli of patients and rats with DN re-expressed the fetal isoform TRα1, and that these cells were also positive for several fetal, mesenchymal and damage-related podocyte markers (Benedetti et al., 2019). Notably, the simultaneous re-expression of TRα1 and fetal markers in the glomerulus was observed in almost all of the common rodent

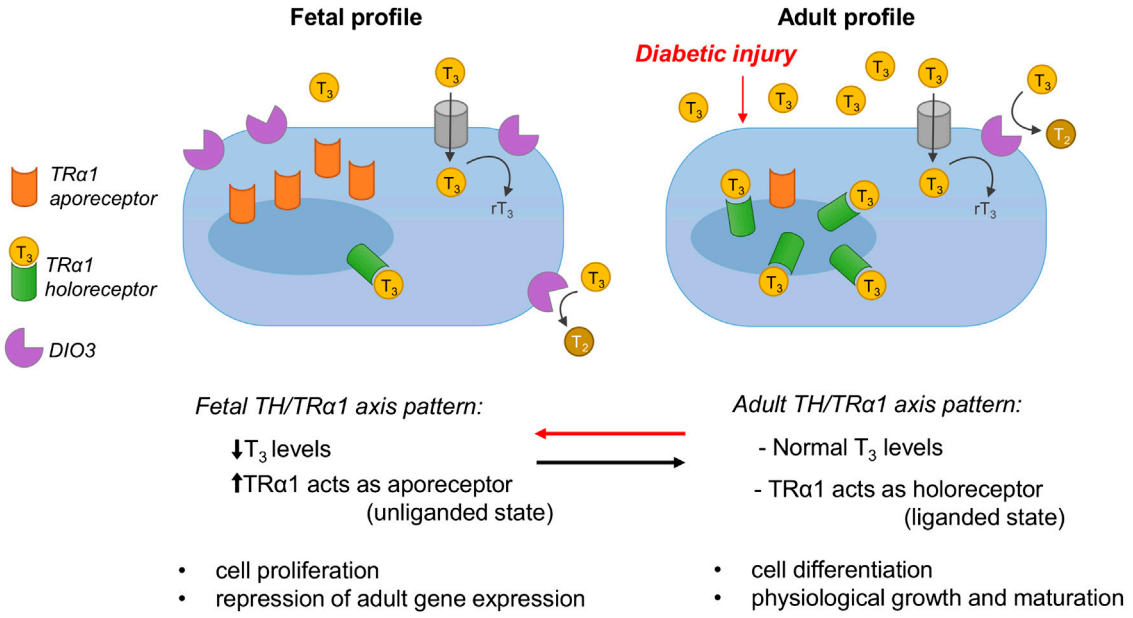

FIGURE 4 | In the fetus, when the levels of the active form of TH L-triiodothyronine (T3) are low, TRα1 mainly acts as an apo-receptor (unliganded state) to repress adult genes (thus protecting the embryo from premature differentiation) and enhances cell proliferation and organ growth. In contrast, after birth when T3 levels increase, TRα1 switches to the holo-receptor (liganded state) to induce the expression of adult genes, thus promoting cell differentiation, physiological organ maturation and function. In adult life, local T_3 availability is controlled by the T_3-inactivating enzyme DIO3, which converts excessive T_3 into rT_3 and T_2. In response to diabetic injury, systemic T_3 levels drop markedly, and TRα1 and DIO3 are overexpressed locally, resulting in the coordinated adoption of the fetal ligand/receptor relationship profile (i.e., low T_3 availability/high local TRα1). Apo-TRα1 binds DNA and represses the transcription of target adult genes (as happens in the fetus), leading to cell dedifferentiation, metabolic and structural remodeling, and cell cycle reactivation. The figure is modified from Benedetti et al., 2019.

models of DN (i.e. streptozotocin-induced type I diabetes, and the models of type II diabetes with a deficiency for leptin (*ob/ob* mice) or for leptin receptor (Zucker diabetic fatty rats). In rats with DN, we also observed that the glomerular expression of the TH-inactivating enzyme deiodinase 3 (DIO3) increased, and blood T3 levels decreased progressively, correlating inversely with the metabolic and renal disease worsening. In addition, human podocytes exposed to typical components of the diabetic milieu *in vitro* (high glucose and H_2O_2), exhibited markedly upregulated TRα1 and DIO3 expression. The adoption of this fetal profile of TH signaling was associated with cytoskeleton rearrangements, adult podocyte marker downregulation and fetal kidney marker upregulation, along with the induction of a maladaptive cell cycle, and TRα1-ERK1/2-mediated hypertrophy (Benedetti et al., 2019).

It is noteworthy that similar alterations in the TH-TRα1 axis are also observed in cardiomyocytes, another terminally differentiated and highly specialized cell type. In response to a wide range of stressful stimuli, cardiomyocytes adopt a fetal TH signaling profile and (at least partially) re-activate the fetal gene program, which eventually leads to structural alterations and the deterioration of organ function. It has been shown that during adrenergic injury the unliganded TRα1 induces the adoption of a fetal pattern of myosin isoform expression and radical phenotypical changes in the structure, shape and size of neonatal cardiomyocytes (Pantos et al., 2007). Similarly, inhibiting T3 binding to the TRα1 receptor delayed cardiac myoblast differentiation, while enabling the T3-TRα1 binding reversed all the aforementioned phenotypical changes (Pantos et al., 2008). Our ongoing studies have shown that cardiac TH signaling was also altered in diabetic rats, and these alterations were associated with molecular and phenotypical changes in the left ventricle.

In light of the above data, which demonstrate the existence of a causal link between the reduction in TH availability and the reactivation of developmental pathways in adulthood, and considering the crucial regulatory role of TH signaling in development and metabolism, we hypothesize that the TH/TRα1 axis is a key regulator of the reactivation of the cell developmental program (defined as fetal reprogramming, FR) in terminally differentiated and highly specialized cells (**Figure 4**).

Adopting a Fetal Thyroid Hormone Signaling Profile: Adaptation, Maladaptation or Therapeutic Opportunity?

So far, our data suggest that the fetal profile of TH signaling (characterized by low T3 systemic levels, TRα1 in the apo-receptor state and increased DIO3 activity) in the diabetic kidney triggers podocytes to dedifferentiate and re-activate fetal genes, re-enter the cell cycle and increase DNA content and cell size. Although the role of TH signaling in the pathobiology of the stressed kidney is clear, the biological

significance of this response, especially in humans, remains unknown.

One hypothesis we can put forward is that the reactivation of fetal TH signaling is an adaptive response of the tissue to diabetes-induced chronic stress to enable non-proliferating cells to reach a lower energy state and/or to allow for compensatory growth. Alternatively, it could be a maladaptive response of the tissue that leads to phenotypical changes that are more detrimental than beneficial. The insufficient availability of data in DN limits the formulation of robust hypotheses about the biological significance of the adoption of fetal TH signaling in the diabetic kidney. Nevertheless, analyzing data and paradigms from other organs may help us to better understand the causal rationale behind this response. Generally, low T3 levels initially provide a metabolic benefit to stressed organs. In the diabetic heart, for example, the local reduction of T3 levels triggers a metabolic switch that is associated with remodeling of the contractile machinery, which includes a switch from the expression of proteins that consume high levels of energy to energy-saving ones (Rajagopalan and Gerdes, 2015). In addition to the metabolic/energetic benefit, low T3 levels also enable the proliferation of damaged cells in some tissues that are endowed with high regenerative capacities. This is clearly observed in skeletal muscles, which in mice can regenerate through satellite cell amplification after acute injury (Dentice et al., 2010; Dentice et al., 2014). The regeneration process begins with a drastic DIO3-mediated reduction of local T3 concentrations (Dentice et al., 2014), which allows for cell proliferation and is immediately followed by a concomitant downregulation of DIO3 and upregulation of DIO2 expression, thus leading to a renewed increase in T3 levels, enabling cell differentiation (Dentice et al., 2010). This strategy is effective for organs that can regenerate through cell proliferation and differentiation (at least in response to acute injuries). However, for terminally differentiated and highly specialized cells, such as podocytes and cardiomyocytes, which cannot proliferate without affecting organ integrity and function, another strategy was selected. In order to cope with the increased workload that results from cell loss, these cells dedifferentiate and increase their genome content (polyploidization) and cell size (hypertrophy). For these coordinated phenotypical alterations to occur, the fetal profile of TH signaling (characterized by low T3 systemic levels, TRα1 in the apo-receptor state and/or increased DIO3 activity) must be recapitulated. Even though this is beneficial for organ function (at least in the early stages of the disease), the persistent lack of T3 induces extensive cell dedifferentiation and maladaptive proliferation, the reactivation of several developmental pathways, and pathological growth and structural remodeling in damaged tissue. Several studies in experimental hypothyroidism support this concept. In diabetes, hypothyroidism induces the dedifferentiation and/or transdifferentiation of pancreatic β-cells, and these phenomena (instead of apoptosis) have been proposed as putative explanations for pancreatic β-cell loss (Moin and Butler, 2019). Notably, the simultaneous overexpression of TRα1 and the administration of T3 enhanced cell cycle progression and proliferation, leading to the reprogramming of pancreatic cells into insulin-producing cells, in both the rat β-cell line and in an animal model of STZ-induced diabetes (Furuya et al., 2010). These findings are consistent with our studies on the diabetic kidney (Benedetti et al., 2019) and indicate that the adoption of a fetal TH signaling profile is associated with cell dedifferentiation and loss and pathological growth.

Regardless of whether these phenomena should be considered adaptations or maladaptations, they can be exploited as therapeutic opportunities: controlling these pathways spatiotemporally could in fact be a strategy for directing the regeneration of damaged tissues. Administering T3 to pharmacologically modulate the TH-TRα axis has indeed exhibited exceptional therapeutic potential in various diabetic organs (Furuya et al., 2010; Lin and Sun, 2011; Mourouzis et al., 2013) and in *in vitro* models (Furuya et al., 2010; Benedetti et al., 2019). In diabetic milieu-injured podocytes, T3 treatment completely reversed the fetal phenotype and subsequent pathological alterations by upturning changes in TH signaling, promoting re-differentiation, and restoring normal cellular morphology (Benedetti et al., 2019). In the kidneys of patients with chronic kidney disease, TH treatment improves renal function (Shin et al., 2012, Shin et al., 2013), while in our ongoing models of diabetes, T3 reverses FR by promoting re-differentiation and reducing hypertrophy, and improves renal structure.

Nevertheless, translating this strategy into clinical practice will not be straightforward: the high doses of T3 that need to be administered to achieve therapeutic effects under conditions of systemic hypothyroidism lead to various adverse effects (Collet et al., 2012; Ali Rajab et al., 2017; Pantos and Mourouzis, 2018). Thus, to maximize therapeutic efficacy while minimizing possible adverse effects, future therapeutic strategies should use drug delivery systems that can target and deliver the drug to injured cells only. Alternatively, new thyromimetics with a higher affinity for TRα1 and less susceptibility to inactivation need to be produced to allow for more efficient receptor activation and to drastically reduce the high dose-related adverse effects. We are currently working in both directions to ensure the success of the most clinically promising option.

SODIUM-GLUCOSE COTRANSPORTER 2

SGLT2, which is located on the apical membrane of renal tubular epithelial cells, is the principal contributor to the reabsorption of filtered glucose, and SGLT2 inhibitors are now a well-defined class of anti-hyperglycemic agents for type 2 diabetes. These drugs block renal reabsorption of glucose, promoting glycosuria and lowering blood glucose (Vallon and Thomson, 2017). In addition, SGLT2 inhibitors have direct effects on glomerular hemodynamics, which are important for renoprotection in DN. In diabetes, because of a

high filtered load of glucose, reabsorption of glucose and sodium is increased in the proximal tubule via SGLT2, with a resulting diminished delivery of sodium to the macula densa. These effects reduce the tubuloglomerular feedback signal, causing constriction of the adjacent efferent arteriole, and dilatation of the afferent arteriole, leading to increases in intraglomerular pressure and single nephron GFR. The inhibition of SGLT2 increases the delivery of sodium to the macula densa, restoring tubuloglomerular feedback and promoting afferent arteriolar constriction, which results in reduced intraglomerular pressure and hyperfiltration (Skrtic and Cherney, 2015; DeFronzo et al., 2017; Perico et al., 2017; Toto, 2017) would translate into lowered albuminuria and reduced progression of the diabetic kidney disease. Several kidney and cardiovascular outcome studies in type 2 diabetes have indeed demonstrated that there are important advantages to using SGLT2 inhibitor therapy, including mortality benefits (for review see (Alicic et al., 2019) (Kanduri et al., 2020). In experimental diabetes SGLT2 inhibitors controlled hyperglycemia and limited albuminuria and renal damage, including glomerular mesangial matrix accumulation and interstitial fibrosis, through combined effects on glomerular hemodynamics and the inhibition of inflammation and oxidative stress (Gembardt et al., 2014; Terami et al., 2014; Vallon et al., 2014; Wang et al., 2017). SGLT2 inhibition also prevented podocyte injury and loss (Wang et al., 2017). There is evidence that in addition to tubular epithelial cells, SGLT2 is expressed in glomerular cells, and that SGLT2 inhibitors may exert tubular SGLT2-independent reno-protective effects (Cassis et al., 2018; Maki et al., 2019). The expression of SGLT2 protein has been demonstrated in cultured mesangial cells, and was upregulated by exposure to high glucose (Maki et al., 2019). Moreover, in *db/db* mice with type 2 diabetes, a low dose of SGLT2 inhibitor–which, unlike a higher dose, did not affect hyperglycemia and glycosuria–was still able to reduce albuminuria and mesangial expansion in the same way as a higher dose (Maki et al., 2019). Through *in vitro* and *in vivo* experiments, we have shown that SGLT2 is also expressed in mouse podocytes and that its level was increased by albumin overload, depending on NF-kB activation (Cassis et al., 2018). Further, we showed that SGLT2 inhibitor limited proteinuria and protected mice with protein-overload proteinuria against podocyte dysfunction and loss, and that SGLT2 inhibitor directly targeted podocytes through the maintenance of actin cytoskeleton architecture (Cassis et al., 2018). All the above evidence indicates that SGLT2 inhibitors, through their pleiotropic effects, independently of their glucose-lowering property, may provide renoprotection not only in diabetic but also non-diabetic CKD.

HYPOXIA-INDUCIBLE FACTOR-1

Chronic hypoxia has been recognized as an important signaling pathway driving diabetic kidney disease (Hesp et al., 2020). Emerging evidence indicates that many of the renoprotective benefits of SGLT2 inhibitors may be due to their action on hypoxia-inducible factor (HIF)-1 (Bessho et al., 2019; Packer, 2020), a heterodimeric transcription factor that plays a key role in cellular adaptation to different oxygen concentrations (Patten et al., 2010; Packer, 2020). It is composed of an oxygen-sensitive α-subunit (HIF-1α) and a constitutively expressed ß-subunit (HIF-1β). In normoxic conditions, HIF-1α subunit is continuously produced in the cytosol but rapidly degraded; it is hydroxylated at specific proline residues by prolyl-4-hydroxylase domain (PHD) proteins, allowing for recognition by von Hippel-Lindau-(VHL)-E3 ubiquitin ligase complex, which targets HIF-1α for proteasomal degradation. During hypoxia, the degradation process is suppressed and HIF-1α is transferred into the nucleus to form, with the ß-subunit, an active heterodimer that binds to hypoxia response elements (HRE) in the promoter regions of target genes involved in different processes, including erythropoiesis, glycolysis, angiogenesis, oxidative stress and fibrogenesis (Haase, 2006; Packer, 2020). Notably, in addition to hypoxia, nonhypoxic factors such as high glucose, Ang II, TGF-β and ROS–all of which mediate renal damage in diabetes–promote HIF-1 activation (Macconi et al., 2014; Nayak et al., 2016). HIF-1 is *per se* implicated in the regulation of the above mediators, so it has been proposed that there is a feedback loop through which HIF-1 mediates the initiation and progression of diabetes-induced renal damage (Nayak et al., 2016). It has been reported that the activation of HIF-1 signaling by hypoxia promoted fibrosis. Thus HIF-1α enhanced EMT transition in renal epithelial cells *in vitro,* and genetic ablation of epithelial *Hif-1α* reduced tubulointerstitial fibrosis in a mouse model of kidney fibrosis (Higgins et al., 2007). Increased expression of HIF-1 and its target genes has been found in fibrotic areas of microdissected kidney tissues from DN patients (Higgins et al., 2007), and the upregulation of HIF-1α has been detected in hypertensive DN kidneys of mice with renal fibrosis (Jiao et al., 2018). Moreover, HIF-1α blockade through treatment with a HIF-1 inhibitor ameliorated glomerular hypertrophy, mesangial matrix expansion and fibrosis in diabetic OVE26 mice (Nayak et al., 2016). Based on *in vitro* and *in vivo* experiments, recent studies have proposed HIF-1 as a therapeutic target for an SGLT2 inhibitor for DN (Bessho et al., 2019; Packer, 2020). In cultured tubular epithelial cells, an SGLT2 inhibitor reduced hypoxia-induced HIF-1α protein expression and its target genes by reducing mitochondrial oxygen consumption (Bessho et al., 2019). In diabetic *db/db* mice, treatment with the SGLT2 inhibitor attenuated cortical tubular HIF-1α expression, tubular injury and interstitial fibrosis (Bessho et al., 2019). There is also evidence that in type 2 diabetes, SGLT2 inhibitors enhanced nutrient deprivation signaling through the upregulation of AMPK and SIRT1, which in turn act to suppress HIF-1α (Packer, 2020).

DIPEPTIDYL PEPTIDASE-4

Dipeptidyl peptidase-4, also known as CD26, is a ubiquitously expressed serine protease that cleaves several substrates,

TABLE 1 | Selected targetable signaling pathways in experimental diabetic nephropathy mentioned in this review.

Pathway	Intervention	Model	References
ACE2/Ang-(1–7)/MasR	Podocyte-specific hACE2 overexpression	STZ-induced diabetes in mice	Nadarajah et al. (2012)
	Ang-(1–7)	Zucker diabetic fatty rats, *db*/*db* mice, STZ-induced diabetes in mice	Giani et al. (2012), Mori et al. (2014), Zhang et al. (2015), Bossi et al. (2016)
	Cyclic Ang-(1–7)	BTBR *ob*/*ob* mice, type 1 and type 2 diabetes mouse models	Cassis et al. (2019a), Kuipers et al. (2019)
Sirtuin-1	Podocyte-specific sirtuin-1 overexpression	OVE 26 type 1 diabetic mice	Hong et al. (2018)
	Sirtuin-1 agonist BF175		
Sirtuin-3	Honokiol	BTBR *ob*/*ob* mice	Locatelli et al. (2020)
Notch	Podocyte-specific RBPj deletion	STZ-induced diabetes in mice	Niranjan et al. (2008)
	Podocyte-specific Notch 1 deletion	STZ-induced diabetes in mice	Sweetwyne et a. (2015)
	Podocyte-specific Mafb overexpression	STZ-induced diabetes in mice	Morito et al. (2014)
	γ-secretase inhibitor DAPT	STZ-induced diabetes in mice	Lin et al. (2010)
	histone demethylase inhibitor GSK-J4	*db*/*db* mice	Majumder et al. (2018)
Thyroid hormone	L-triiodothyronine (T3)	*db*/*db* mice	Lin and Sun (2011)
		high glucose-loaded podocytes	Benedetti et al. (2019)

including the incretin hormones, glucagon-like peptide 1 (GLP–1) and glucose-dependent insulinotropic polypeptide (GIP), which regulate post-prandial insulin secretion (Röhrborn, 2015). DPP-4 inhibitors have been approved as antihyperglycemic medication for type 2 diabetes. DPP-4 inhibitors are oral, weight neutral, well tolerated blood glucose-lowering drugs with a low risk of hypoglycemia and proven cardiovascular safety (Gallwitz, 2019). Clinical studies have reported that some DPP-4 inhibitors used as monotherapy or added to ACE inhibitors/ARBs reduced albuminuria in diabetic patients without affecting other renal outcomes (see reviews (Penno et al., 2016; Coppolino et al., 2018; Taylor and Lam, 2020). However, there are no definitive data that would make it possible to establish whether DPP-4 inhibitors confer renoprotection on type 2 patients (Hanssen and Jandeleit-Dahm, 2019).

The role of DPP-4 and the effects of DPP-4 inhibitors in diabetic kidney disease have been reviewed recently, with a focus on linagliptin (see (Kanasaki, 2018; Gupta and Sen, 2019). In the healthy rat kidney, DPP-4 is expressed in proximal tubular cells and in the glomerulus, mainly in podocytes. In humans, glomerular expression of DDP-4 was only detected under pathological conditions. Consistent with this, *in vitro* studies have reported DPP-4 induction in human podocytes and glomerular endothelial cells in response to inflammatory cytokines and high glucose (Kanasaki, 2018). In experimental DN the DPP-4 inhibitor linagliptin reduced albuminuria and ameliorated glomerulosclerosis and interstitial fibrosis, independently of glucose control (Kanasaki, 2018). The renoprotective effects were associated with the attenuation of podocyte dysfunction and loss (Sharkovska et al., 2014; Takashima et al., 2016) and the inhibition of EndMT (Kanasaki et al., 2014). The molecular mechanisms underlying DPP4-induced EndMT have been elucidated by *in vitro* studies that showed that DPP-4 interacts with the integrin β1, causing TGF-βR heterodimer formation and the consequent activation of TGF-β signaling. The DPP-4/integrin β1 complex can also downregulate VEGFR2 while upregulating VEGFR1, thus favoring EndMT (Shi S. et al., 2015). In diabetic kidneys DPP-4 is overexpressed in endothelial cells with a mesenchymal phenotype, concomitant with the downregulation of miR-29s. By restoring miR-29s, which target DPP-4, linagliptin inhibited DPP-4 overexpression and its interaction with integrin β1, thus reducing TGF-β-induced EndMT (Kanasaki et al., 2014; Shi S. et al., 2015). This effect is unique to linagliptin and not shared by other members of the gliptin family (Shi et al., 2016). Similarly to what has been observed in endothelial cells, linagliptin is able to reduce TGF-β signaling in proximal tubular cells under hyperglycemic conditions by inhibiting the interaction of DPP-4 with the cation-independent mannose 6-phosphate receptor (Gangadharan Komala et al., 2015). Altogether these findings suggest that the DPP-4 inhibitor linagliptin has a pleiotropic effect that is incretin- and glucose-lowering- independent, and which confers protection against kidney fibrosis in experimental DN through miRNA modulation and the inhibition of DPP-4 interaction with other proteins.

CONCLUSION

Diabetes is a global health concern of epidemic proportions. About one-third of affected people develop diabetic nephropathy, a leading cause of end-stage kidney disease worldwide. There is an imperative need to identify novel therapeutic interventions with renoprotective effects for those diabetic patients who do not respond completely to standard therapy. In this review we first described four major signaling pathways that have emerged as mediators of podocyte/endothelial cell injury that contribute crucially to the pathogenesis of DN and can be targets for therapeutic interventions (**Table 1**). The development of cAng-(1–7), a modified peptide that is more peptidase resistant than the linear peptide, is particularly attractive for long-term treatment and has potential suitability for clinical use. The availability of natural compounds that increase sirtuin expression/activity in the diabetic kidney makes

pharmacological modulation of sirtuins a novel strategy for treating DN. Notch1 and TH signaling pathways, which are abnormally activated in podocytes in DN, are targets for podocyte-directed therapy. Future drug delivery systems that can target and deliver the TH to injured cells, or new thyromimetics with a higher affinity for TRα1, may allow us to maximize the regenerative potential of TH signaling and minimize the high dose-related adverse effects. In addition, there are a number of different experimental therapies that could directly or indirectly target other discussed signalings. Actually, drugs that target podocytes or vasculature, such as SGLT2 inhibitors and DPP-4 inhibitors, as well as drugs that can modulate HIF activity, may lead to next-generation therapeutics that can efficiently mitigate diabetes complications in the kidney. Finally silencing of miRNAs that are found to directly contribute to the pathogenesis of DN, e.g., miR-21 (Kölling et al., 2017), miR-214 (Wang X. et al., 2016) or miR-184 (Zanchi et al., 2017), to name a few, or to induce changes in TH signaling (e.g., induction of the DIO3 (Di Girolamo et al., 2016), may provide a solid basis for the development of therapeutic solutions that can arrest or even reverse the structural and functional alterations of the diabetic kidney.

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

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REVIEW
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Non-Coding RNAs as Biomarkers and Therapeutic Targets for Diabetic Kidney Disease

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Diabetic kidney disease (DKD) is the most common diabetic complication and is a leading cause of end-stage kidney disease. Increasing evidence shows that DKD is regulated not only by many classical signaling pathways but also by epigenetic mechanisms involving chromatin histone modifications, DNA methylation, and non-coding RNA (ncRNAs). In this review, we focus on our current understanding of the role and mechanisms of ncRNAs, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) in the pathogenesis of DKD. Of them, the regulatory role of TGF-β/Smad3-dependent miRNAs and lncRNAs in DKD is highlighted. Importantly, miRNAs and lncRNAs as biomarkers and therapeutic targets for DKD are also described, and the perspective of ncRNAs as a novel therapeutic approach for combating diabetic nephropathy is also discussed.

Keywords: diabetic kidney disease, micro RNAs, long non-coding RNAs, TGF-β, fibrosis, inflammation, biomarker, therapeutic target

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INTRODUCTION

Diabetic kidney disease (DKD) is one of the most predominant diabetic complications and is a leading cause of chronic kidney disease (CKD). It is reported that up to 20–50% of living diabetes, including type 1 (T1DM) and type 2 (T2DM) diabetes, would eventually develop into DKD (Selby and Taal, 2020), which contributes to the high mortality of patients with DKD (Braunwald, 2019). The established DKD is characterized by the onset of persistent albuminuria and progressive decline of estimated glomerular filtration rate (eGFR) (Magee et al., 2017). Pathologically, the histological features of DKD include the thickening of the glomerular basement membrane (GBM), glomerular capillary hypertension, mesangial expansion, nodular sclerosis, glomerulosclerosis, interstitial fibrosis, inflammation, and tubular atrophy (Raval et al., 2020).

In patients with diabetes, hyperglycemia may trigger oxidative stress, renal inflammation, and fibrosis in kidneys (Matoba et al., 2019; Patel et al., 2020). Among those pathogenic factors, renal fibrogenesis is the major driving force in the development of DKD (Hills and Squires, 2011; Lan, 2012a). It is well-established that transforming growth factor β (TGF-β) as the master regulator for the fibrotic and inflammatory process in CKD (Meng et al., 2016). Hyperglycemic factors such as advanced glycation end products (AGEs) and angiotensin II (AngII) may trigger the activation of TGF-β signaling *via* Smad dependent or independent pathway, therefore promoting fibrosis in kidneys (Lan, 2011; Meng et al., 2016; Gu et al., 2020) (**Figure 1**).

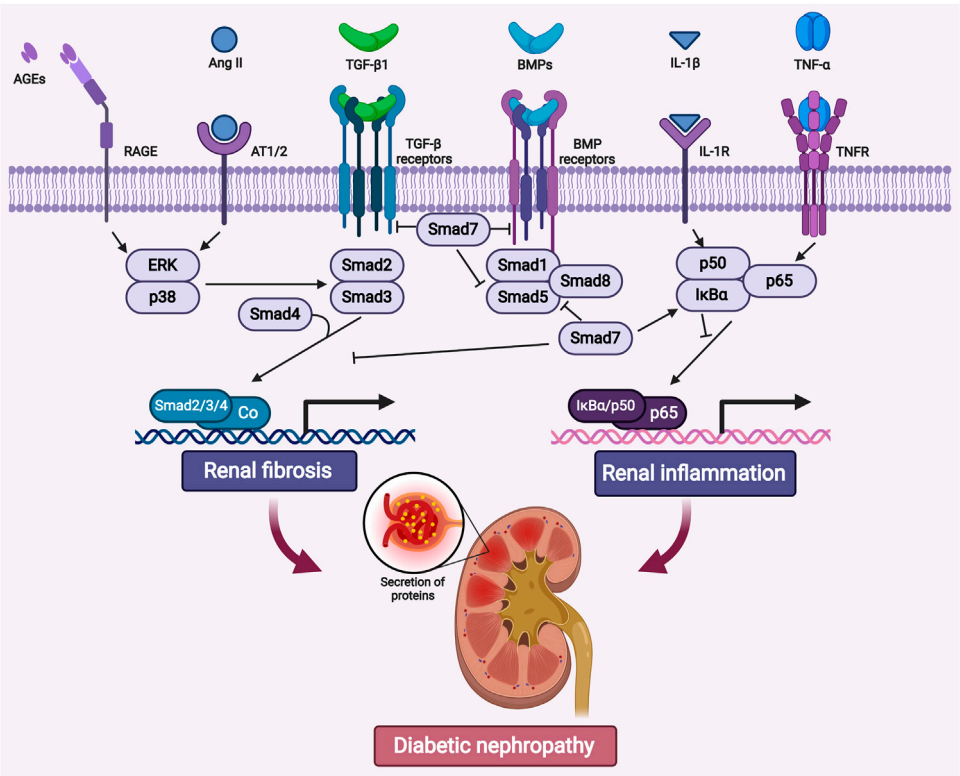

FIGURE 1 | The crosstalk of canonical and noncanonical TGF-β signaling pathways associated with renal fibrosis and inflammation in diabetic nephropathy. TGF-β/Smad and NF-κB signaling pathway are highly activated under hyperglycemic conditions. AGEs, Ang II, IL-1β and TNF-α etc, may trigger these two pathways to promote fibrosis and inflammation in diabetic kidneys. Abbreviations: AGEs, advanced glycation end products; RAGE, receptor for AGE; Ang II, angiotensin II; AT1/2, Ang II receptor 1 and 2; BMP, bone morphogenic protein; TNF-α, tumor necrosis factor α; TNFR, TNF receptor; IL-1β, interleukin 1β; IL-1R, IL-1 receptor; ERK, extracellular-signal regulated kinase; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α. (Figure created with BioRender.com).

The emerging field of epigenetic regulation by ncRNAs has focused on the pathogenic pathways to halt the progression of DKD. With no function in protein-coding, ncRNAs were implicated as therapeutic targets or biomarkers for DKD (Loganathan et al., 2020). Interestingly, these ncRNAs could also be regulated by TGF-β (Meng et al., 2015). In this review, we will focus on the regulatory role of miRNAs and lncRNAs in the progression of DKD, and their potentials as therapeutic targets and biomarkers for DKD are highlighted. Moreover, the mechanisms of ncRNAs on renal fibrosis and inflammation in DKD based on the TGF-β/Smad-mediated signaling pathway will also be discussed.

THE EMERGING ROLE OF NON-CODING RNAS IN DKD

miRNAs are single-stranded endogenous RNAs (20–22 nucleotides in length) that regulate gene expression on the post-transcriptional or transcriptional level (Wahid et al., 2010). LncRNAs are RNA transcripts over 200 nucleotides in length, which are able to modulate gene expression by binding to either DNAs, RNAs, or proteins (Yao et al., 2019). The roles of miRNAs and lncRNAs in kidney development and disease have been reviewed (Kaucsár et al., 2010; Moghaddas Sani et al., 2018; Zhou et al., 2019). Thus, we mainly focus on the roles and underlying mechanisms of miRNAs and lncRNAs relevant to DKD pathogenesis (as shown in **Figure 2**).

Non-Smad-dependent miRNAs in DKD

The functional relevance of miRNA in renal diseases has caught our attention since the rapid development of RNA sequencing strategy. In most cases, miRNAs hybridize to the 3'UTRs (untranslated regions) of the target mRNAs and hence silencing the expression of target genes. Up to date, the function and underlying mechanisms of many miRNAs in renal diseases have been well-demonstrated and reviewed (Hou and Zhao, 2013). These miRNAs are of great importance to the epigenetic regulation on DKD.

Renal tubulointerstitial fibrosis (TIF) is one of the predominant features of DKD. A group of miRNAs have been shown to be profibrotic in DKD (**Table 1**). The expression of

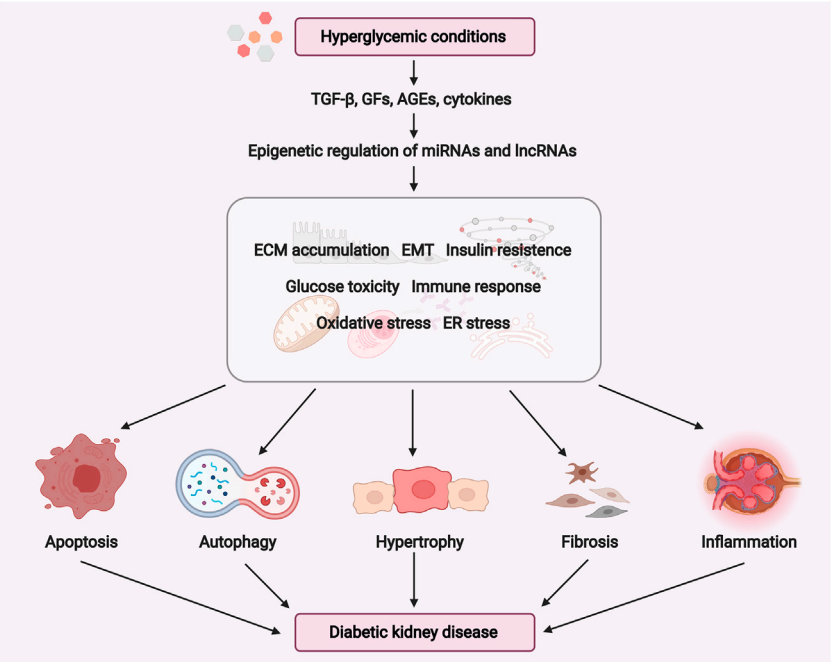

FIGURE 2 | Potential role of miRNAs and lncRNAs in the pathogenesis of diabetic kidney disease. Under hyperglycemic conditions, the expression of TGF-β, growth factors such as CTGF, FGF, and cytokines may induce ECM accumulation, EMT, ER stress, oxidative stress, insulin resistance, glucose toxicity, fibrosis, and inflammatory response. These pathogenic processes are positively or negatively regulated by ncRNAs (miRNAs and lncRNAs) to promote cell apoptosis, autophagy, hypertrophy, fibrosis, inflammation in the diabetic kidney. Abbreviations: GFs, growth factors; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; ER, endoplasmic reticulum. (Figure created with BioRender.com).

TABLE 1 | Non-Smad-dependent miRNAs in DKD.

miRNA	Target	Pathological output(s)	References
miR-22	PTEN	Pro-fibrosis	(Zhang et al., 2018)
miR-23a	SnoN		(Xu et al., 2018a)
miR-34a-5p	SIRT1		(Xue et al., 2018)
miR-133b	SIRT1		(Sun et al., 2018b)
miR-199b			
miR-135a	TRPC1		(He et al., 2014)
miR-184	LPP3		(Zanchi et al., 2017)
miR-370	CNPY1		(Yu et al., 2019)
miR-30c	JAK1; Snail1	Anti-fibrosis	(Zhao et al., 2017; Gao et al., 2020)
miR-98-5p	HMGA2		(Zhu et al., 2019c)
miR-302a-3p	ZEB1		(Tang et al., 2018b)
miR-342	SOX6		(Jiang et al., 2020b)
miR-379-5p	LIN28B		(Li et al., 2019b)
miR-455-3p	ROCK2	Anti-fibrosis	(Wu et al., 2018a; Zhu et al., 2019b)
miR-544	FASN	Anti-inflammation	(Sun et al., 2020)
miR-217	SIRT1/HIF-1α	Pro-fibrosis Pro-inflammation	(Shao et al., 2016)
miR-770-5p	TIMP3	Pro-inflammation	(Zhang et al., 2019c; Wang and Li, 2020)
miR-15b-5p	Sema3A	Anti-inflammation	(Fu et al., 2019)
miR-34b	IL-6R		(Lv et al., 2019)
miR-140-5p	TLR4		(Su et al., 2020)
miR-146a	NOX4		(Wan and Li, 2018)
miR-218	IKK-β		(Li et al., 2020a)
miR-374a	MCP-1		(Yang et al., 2018)
miR-423-5p	NOX4		(Xu et al., 2018c)
miR-451	LMP7		(Sun et al., 2016b)
miR-485	NOX5		(Wu et al., 2020)
miR-874	TLR4		(Yao et al., 2018)

miR-22 was increased in streptozotocin (STZ)-induced DKD model and in high glucose (HG)-treated tubular epithelial cells (TECs). miR-22 targets phosphatase and tensin homolog (PTEN), therefore suppressing autophagy and inducing the expression of collagen IV and α-smooth muscle actin (α-SMA) (Zhang et al., 2018). A high level of miR-23a was also observed in diabetic patients and HG-cultured TECs. It directly targets the nuclear transcription co-repressor Ski-related novel protein N (SnoN) (Tan et al., 2006), a crucial negative regulator to TGF-β/Smad3-mediated signaling pathway, to induce fibrosis in DKD (Xu et al., 2018a). Sirtuin 1 (SIRT1) expression in the nucleus and the cytoplasm has also been shown as a renoprotective regulator by inhibiting TGF-β/Smad-induced fibrosis and downstream hypoxia-inducible factor-1α (HIF-1α). miR-34a-5p, miR-217, miR-133b, and miR-199b may dcirectly or indirectly target and suppress the expression of SIRT1 under hyperglycemic conditions (Shao et al., 2016; Sun et al., 2018b; Xue et al., 2018). The transient receptor potential cation channel subfamily C member 1 (TRPC1) is downregulated in diabetic patients and animal models, which may contribute to the development of DKD (Zhang et al., 2009a). miR-135a targets TRPC1 to promote the fibrotic process in diabetic renal injury (He et al., 2014). Interestingly, diabetic-induced albumin triggers the expression of miR-184 in the tubular cells to promote TIF, which is associated with decreased expression of lipid phosphate phosphatase 3 (LPP3) (Zanchi et al., 2017). The canopy 1 (CNPY1) is a target of miR-370 to modulate fibroblast growth element signaling (Matsui et al., 2011). Overexpression of miR-370 significantly increases the accumulation of extracellular matrix (ECM) and promotes the proliferation of mesangial cells (MCs) (Yu et al., 2019). On the other hand, the anti-fibrotic miR-342 binds to the 3'UTR of SRY-box 6 (SOX6), therefore inhibiting SOX6 expression and the level of fibrotic biomarkers (Jiang et al., 2020b). miR-379 is also involved in the pathogenesis of DKD. It is reported that miR-379 triggers miR-let-7, which prevents ECM accumulation and proliferation of MCs (Li et al., 2019b). Nevertheless, some miRNAs exert protective effects by inhibiting the epithelial-to-mesenchymal transition (EMT). Notably, miR-30c, miR-98-5p and miR-302a-3p target the fibrosis-related JAK1, Snail1, HMGA2, and ZEB1, respectively, thus blocking the fibrotic process in DKD by inhibiting EMT (Zhao et al., 2017; Tang et al., 2018b; Zhu et al., 2019c; Gao et al., 2020). Furthermore, miR-455-3p also inhibits renal fibrosis by targeting ROCK2, together with the reduction of anti-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and monocyte chemotactic protein 1 (MCP-1) (Wu et al., 2018a). Interestingly, miR-455-3p also serves as a sponge for pathogenic lncRNA Hottip. Hottip is upregulated under HG conditions, while miR-455-3p may reverse Hottip-mediated fibrosis and inflammation (Zhu et al., 2019b). Fatty acid accumulation (FAC) was also induced by DKD, fatty acid synthase (FASN) is not only the vital lipogenic enzyme to FAC, but also an upregulated molecule that contributes to glomerulosclerosis and renal inflammation. miR-544 binds to the 3'UTR of FASN thus attenuating the infiltration of inflammatory cells, the activation of NF-κB signaling and renal fibrosis (Sun et al., 2020). All these findings have suggested a crucial role of miRNAs in DKD-induced renal fibrosis based on the epigenetic regulation level.

Hyperglycemia triggers the inflammatory response by recruiting immune infiltration and inducing the production of pro-inflammatory cytokines. Of note, podocyte is the barrier to maintain glomerular filtration, and it also functions as the receptor and producer of various cytokines. The dysfunction of podocyte is an essential event in lesion development and glomerulonephritis. This process promotes the progression of DKD (Lal and Patrakka, 2018). Stimulated by HG, miR-770-5p is upregulated and promotes podocyte injury by targeting metalloproteinase 3 (TIMP3), and Tp53 regulated inhibitor of apoptosis 1 (TRIAP1), knocking down of miR-770-5p reverse the apoptosis and inflammation induced by HG in kidney biopsy and mouse podocytes (Zhang et al., 2019c; Wang and Li, 2020). On the other hand, more anti-inflammation related miRNAs have been identified. Overexpression of miR-15b-5p significantly restrained HG-induced apoptosis, oxidative stress and inflammation in podocytes, it also directly targets Sema3A, suggesting that miR-15b-5p could be a therapeutic target for DKD (Fu et al., 2019). miR-34b targets to the interleukin-6 (IL-6) receptor and downstream JAK2/STAT3 signaling, thus reducing the expression of TNF-α, IL-6, interleukin-1β (IL-1β), and caspase-3 in TECs (Lv et al., 2019). The nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase (NOX)-derived reactive oxygen species (ROS) may induce inflammation, implying that NOX enzymes as novel targets for DKD (Lambeth et al., 2008). Of note, miR-146a (Wan and Li, 2018), miR-423-5p (Xu et al., 2018c), and miR-485 (Wu et al., 2020) target NOX4 and NOX5, respectively, to reduce the production of pro-inflammatory cytokines. NF-κB signaling pathway is the classical player in inflammation, which is activated in a wide range of kidney diseases, including DKD. miR-218 targets the IKK-β to regulate NF-κB signaling, as well as reducing the expression of TNF-α, IL-6, IL-1β, and MCP-1 (Li et al., 2020a). miR-451 also targets large multifunctional protease (LMP7) to modulate NF-κB-mediated renal inflammation, which is confirmed by the downregulating level of pro-inflammatory molecules (Sun et al., 2016a). In addition, miR-140-5p and miR-874 also function as anti-inflammatory modulators in suppressing the expression of TNF-α, IL-6, IL-1β in TECs by directly binding to toll like receptor 4 (TLR4), the upstream molecule of NF-κB signaling (Yao et al., 2018; Su et al., 2020). These reports suggest that miRNA-mediated renal fibrosis and inflammation have critical functions in DKD.

Non-Smad-dependent lncRNAs in DKD

As promising candidates, some miRNA drugs have been approved to proceed toward phase III or IV trials in the coming future. However, the toxicity and off-target effects of miRNA are somehow inevitable (Seok et al., 2018; Hanna et al., 2019). The emerging studies on lncRNAs have shed light on their characteristics of tissue-and-cell-type-specificity and regulation on both transcriptional and translational levels, making lncRNA as the promising therapeutic targets and attractive drugs for DKD treatment **(Table 2)** (Kato, 2018; Guo et al., 2019).

TABLE 2 | Non-Smad-dependent lncRNAs and their mechanisms in DKD.

lncRNA	Target	Pathological output(s)	References
ZEB1-AS1	miR-216a-5p; MLL1; p53	Anti-fibrosis	(Wang et al., 2018a; Meng et al., 2020)
NR_038323	miR-324-3p; DUSP1		(Ge et al., 2019b)
1700020I14Rik	miR-34a-5p		(Li et al., 2018a)
CYP4B1-PS1-001	Nucleolin		(Wang et al., 2016b; Wang et al., 2018c)
ENSMUST00000147869	*Cyp4a12a*		(Wang et al., 2016c)
XIST	miR-93-5p; CDKN1A	Pro-fibrosis	(Yang et al., 2019a)
PVT1	miR-23b-3p; WT1		(Zhong et al., 2020)
SNHG16	miR-141-3p; CCND1		(Jiang et al., 2020a)
OIP5-AS1	miR-30c-5p		(Fu et al., 2020)
LINC00968	p21/EZH2		(Li et al., 2018b)
ASncmtRNA-2	ROS		(Gao et al., 2017a)
MEG3	miR-181a; Egr-1; TLR4; miR-145	Pro-fibrosis	(Li et al., 2019a; Zha et al., 2019)
BLNC1	NRF2/HO-1; NF-κB		(Feng et al., 2019)
NEAT1	Klotho/ERK1/2; miR-23c; Akt/mTOR; miR-27b-3p/ZEB1		(Ma et al., 2019a; Huang et al., 2019b; Wang et al., 2019b; Li et al., 2020b; Yang et al., 2020)
MALAT1	Wnt/β-catenin; miR-145/ZEB2; SRSF1; IL-6; TNF-α		(Puthanveetil et al., 2015; Hu et al., 2017; Liu et al., 2019a; Zhang et al., 2019a)
Hottip	miR-455-3p; Wnt2B		(Zhu et al., 2019b)
Gm4419	NF-κB/NLRP3; p50		(Yi et al., 2017)
GAS5	MMP9; miR-221; SIRT1	Anti-fibrosis Anti-inflammation	(Ge et al., 2019a; Zhang et al., 2020)
Rpph1	Gal-3/Mek/Erk	Pro-inflammation	(Zhang et al., 2019b)
HOXA-AS2	miR-302b-3p; TIMP3	Anti-inflammation	(Li and Yu, 2020)

LncRNA zinc finger E-box binding homeobox 1 antisense 1 (ZEB1-AS1) plays a protective role in DKD by targeting profibrotic miR-216a-5p to inhibit HK-induced EMT and renal fibrosis. Besides, the anti-fibrotic function of ZEB1-AS1 is also verified that it may bind to H3K4 methyltransferase myeloid and lymphoid or mixed-lineage leukemia 1 (MLL1) and p53 in patients with DKD (Wang et al., 2018a; Meng et al., 2020). lncRNA NR_038323 exerts an anti-fibrotic effect by interacting with miR-324-3p. miR-324-3p is verified to induce dual-specificity protein phosphatase-1 (DUSP1) and the activation of p38/MAPK and ERK1/2 signaling (Ge et al., 2019b). Moreover, the expression of lncRNA 1700020I14Rik is decreased in db/db mice. Bioinformatic method and RNA binding protein immunoprecipitation assay have confirmed the interaction of 1700020I14Rik and miR-34a-5p, which may then modulate the SIRT1/HIF-1α signaling to prohibit renal fibrosis (Li et al., 2018a). Nucleolin is a nuclear protein that expresses on the surface of endothelial cells. CYP4B1-PS1-001 is the lncRNA that upregulated in early DKD. By direct interaction with Nucleolin, CYP4B1-PS1-001 inhibits fibrosis in MCs (Wang et al., 2016b; Wang et al., 2018c). Nevertheless, some lncRNAs interact with miRNAs to trigger and promote the fibrotic process. (Yang et al., 2019a; Jiang et al., 2020a; Fu et al., 2020; Zhong et al., 2020). Interestingly, LINC00968 inhibits p21 by recruiting EZH2 to enhance proliferation and fibrosis of MCs (Li et al., 2018b). ASncmtRNA-2 is upregulated by ROS, and it promotes the expression of TGF-β1 and other fibrotic factors (Gao et al., 2017b).

As shown in **Table 2**, by direct interaction with miRNAs or inflammatory molecules, lncRNAs play as sponges, inhibitors, or activators to influence either fibrogenesis or inflammatory response. All these findings have demonstrated a critical role of lncRNAs therapeutic targets in the pathogenesis of DKD.

TGF-β/SMAD-DEPENDENT NON-CODING RNAS IN DKD

TGF-β signaling is highly activated under diabetic conditions and has been shown to be a major pathway leading to DKD. It has been well established that DKD-associated fibrosis and inflammation are mediated by TGF-β via Smad-dependent or -independent signaling pathways (Chung and Lan, 2015; Tang et al., 2018a). Active TGF-β1 binds and activates TGF-β receptor II (TβRII) and receptor I (TβRI) which induces phosphorylation of Smad2/3 to form a complex with Smad4 that translocate into the nucleus to regulate transcription of target genes. In general, Smad3 is pathogenic, while Smad2 and Smad7 are protective. Smad4 plays diverse roles in renal fibrosis and inflammation, suggesting Smad4 may not serve as the ideal therapeutic target for DKD (Chung et al., 2013; Li et al., 2014). Many ncRNAs are induced by TGF-β to regulate renal fibrosis and inflammation via Smad-dependent mechanisms in DKD as highlighted in **Table 3**.

TGF-β/Smad-dependent miRNAs in Renal Fibrosis and Inflammation in DKD

miR-192 is the first landmark found in DKD (Kato et al., 2007). TGF-β upregulated miR-192 in MCs and glomeruli from db/db mice, STZ-induced mice model as well as in DKD patients (Kato et al., 2007; Krupa et al., 2010; Putta et al., 2012; Ma et al., 2016; Liu et al., 2018). Indeed, these studies have shown the high correlation between miR-192 and diabetic kidneys.

TABLE 3 | TGF-β/Smad3-dependent miRNAs and lncRNAs in renal fibrotic and inflammatory response of DKD.

miRNA	Mechanism/target	Pathological output(s)	References
miR-192	p53; Zeb1/2; E-cadherin; Egr1	Anti/pro-fibrosis	(Kato et al., 2007; Chung et al., 2010; Krupa et al., 2010; Kato et al., 2011b; Putta et al., 2012; Deshpande et al., 2013; Ma et al., 2016; Liu et al., 2018)
miR-200	TGF-β1/2		(Kato et al., 2011a; Wang et al., 2011)
miR-29c	Spry1; TPM1		(Long et al., 2011; Shao et al., 2019; Huang et al., 2020)
miR-21	Smad7; Spry; PPARα; PTEN; CDC25a; CDK6; MMP9; TIMP1; TIMP3	Pro-fibrosis Pro-inflammation	(Zhong et al., 2011; Wang et al., 2013; Zhong et al., 2013; Wang et al., 2014; Lai et al., 2015; Mcclelland et al., 2015; Kölling et al., 2017; Chen et al., 2018)
miR-27a	SFRP1; PRKAA2; PPARγ	Pro-fibrosis	(Hou et al., 2016; Wu et al., 2018b; Shi et al., 2020)
miR-130b	TGF-β1; Smad2/3; Smad4		(Castro et al., 2014; Lv et al., 2015; Liu et al., 2019b; Ma et al., 2019b)
miR-215	CTNNBIP1		(Mu et al., 2013a)
miR-216a	Ybx1; FoxO1		(Huang et al., 2019a; Meng et al., 2020)
miR-382	HSPD1; FoxO1		(Fang et al., 2017; Wang et al., 2018d)
miR-488	TGF-β1		(Sun et al., 2019)
miR-26a	CTGF; Smad4	Anti-fibrosis	(Koga et al., 2015; Cai et al., 2018; Dong, 2019; Gao et al., 2019)
miR-29a,b	TGF-β1/2; Spry; Col; MMP; Fos; Adams; HDAC4		(Qin et al., 2011; Winbanks et al., 2011; Lan, 2012b; Wang et al., 2012; Chen et al., 2014; Srivastava et al., 2019; Tung et al., 2019)
miR-93	Orai1		(Ma et al., 2018; Yang et al., 2019a; Yang et al., 2019b)
miR-136	SYK; TGF-β/Smad3		(Liu et al., 2020)
miR-let-7	TβR1		(Srivastava et al., 2020)
lncRNA			
Erbb4-IR	miR-29b; Smad7	Pro-fibrosis	(Sun et al., 2018a; Feng et al., 2018; Xu et al., 2020)
NR_033515	miR-743b-5p		(Gao et al., 2018)
Arid2-IR	Egr1; Smad3	Pro-fibrosis Pro-inflammation	(Zhou et al., 2015; Yang et al., 2019c)
	Egr-1	Pro-inflammation	(Peng et al., 2019)
NONHSAG053901			
LRNA9884	MCP-1		(Zhang et al., 2019d)
TUG1	TGF-β1; PI3K/AKT; miR-21; miR-377; PGC-1α; TRAF5;	Anti-fibrosis	(Li and Susztak, 2016; Long et al., 2016; Duan et al., 2017; Lei et al., 2018; Wang et al., 2019a; Shen et al., 2019; Zang et al., 2019)
PRINS	Smad7	Anti-fibrosis Anti-inflammation	(Jiao et al., 2019)

Mechanistically, miR-192 may promote the expression of collagens by targeting the E-box repressor Smad-1 interacting protein (SIP1 or Zeb2) (Kato et al., 2007; Putta et al., 2012). Also, activation of Akt may lead to MCs proliferation and hypertrophy in DKD. miR-192 upregulates miR-216a and miR-217, inhibiting PTEN to induce Akt activation under diabetic conditions. Nevertheless, miR-192 also plays a complex and diverse role in DKD depending on different models or time points. One study has observed a correlation between miR-192 level, tubulointerstitial fibrosis, and eGFR. TGF-β treatment decreases the expression of miR-192 in TECs, resulting in the promotion of fibrosis and the decline of eGFR (Krupa et al., 2010). Similarly, by targeting Egr1, miR-192 decreases the expression of TGF-β1 and fibronectin in glucose-treated TECs and Otsuka-Long-Evans-Tokushima-Fatty rats, a diabetic murine model (Liu et al., 2018). These studies have reported the complexity of miRNA in mediating the fibrotic process in DKD.

miR-200 family (miR-200a, miR-200b, miR-200c) is well-studied miRNA clusters that maintain the epithelial differentiation in cells. Induced by TGF-β or hyperglycemia, the expression of miR-200a are downregulated in TECs. miR-200a functions as a suppressor to EMT, thus protecting kidney from diabetic insults by inhibiting the TGF-β-mediated fibrotic process. Mechanistic study has further revealed that miR-200a downregulates TGF-β2 expression by directly targeting the 3'UTR of TGF-β2 (Wang et al., 2011). However, the expression of miR-200b/c are elevated in glomeruli from type 1 diabetes (T1DM) and type 2 diabetes (T2DM) mice model and in MCs treated with TGF-β1 (Kato et al., 2011b), implying that difference on the miR-200 expression may due to cell type specificity and individual variability. miR-200 family may serve as the therapeutic targets specific to certain cell types response to DKD process.

miR-21 is another well-studied miRNA in renal disease. Although the expression of miR-21 is downregulated in early DKD (Zhang et al., 2009b), it is upregulated in TECs and MCs stimulated by TGF-β1 or HG and in the renal biopsies of DKD patients (Zhong et al., 2011; Wang et al., 2013; Zhong et al., 2013; Wang et al., 2014; Lai et al., 2015; Mcclelland et al., 2015; Kölling et al., 2017; Chen et al., 2018). The mechanism of miR-21 participates in DKD may be related to its activation on both canonical and noncanonical TGF-β signaling. miR-21 not only suppresses the inhibitory Smad7 of TGF-β signaling to promote fibrosis (Zhong et al., 2013; Wang et al., 2014) but also targeting the Sprouty (SPRY) to activate the Ras/MEK/ERK signaling to activate fibrogenesis of TGF-β signaling (Xu et al., 2014). In addition, miR-21 also exerts profibrotic and pro-inflammatory

effects by targeting PTEN, tissue inhibitor of matrix metalloproteinases (TIMPs), and other molecules, as shown in **Table 3**.

miR-29 family is demonstrated to be protective miRNAs that are highly expressed in kidneys but significantly reduced under diabetic conditions. The expression of miR-29 family in various renal cells is decreased when they are stimulated with TGF-β1 or treated with HG (Qin et al., 2011; Chen et al., 2014). The protective role of miR-29 family has been supported by the evidence that overexpression of miR-29 may inhibit the transcription of collagen genes while suppression of miR-29 promotes ECM accumulation. Many studies have identified fibrosis-related targets of miR-29 under hyperglycemic conditions, demonstrating the anti-fibrotic role of miR-29 in DKD. Insterestingly, studies also revealed that miR-29c, serves as a signature miRNA that promotes the progression of DN and fibrosis (Long et al., 2011; Shao et al., 2019; Huang et al., 2020). More and more studies are revealing the functions and mechanisms of miRNAs in fibrosis and inflammation during diabetic conditions, these miRNAs may play as potential therapeutic targets to combat DKD.

TGF-β/Smad-Dependent lncRNAs in Renal Fibrosis and Inflammation in DKD

Under hyperglycemic condition, the expression of profibrotic and pro-inflammatory lncRNAs are usually upregulated, suggesting their regulatory role in DKD. TGF-β-mediated lncRNA Erbb4-IR is highly expressed in diabetic db/db mice and AGEs-treated MCs. It is regulated by Smad3 as Smad3 deficiency inhibits the transcription of Erbb4 (Feng et al., 2018; Xu et al., 2020). The upregulation of Erbb4-IR is consistent with the elevation of albuminuria, serum creatinine, and fibrotic biomarkers. The mechanistic role of Erbb4-IR may be the binding of Erbb4-IR with the 3'UTR of miR-29b, therefore suppressing anti-fibrotic miR-29b expression. Moreover, Erbb4-IR may also bind with Smad7 to promote renal fibrosis (Sun et al., 2018a; Feng et al., 2018).

lncRNA NR_033515 is found to be significantly increased in the serum of DKD patients, which has shown a positive correlation with KIM-1 and NGAL, diagnostic markers of DKD. The mechanistic study has further confirmed the fibrotic role of NR_033515 by revealing the binding of NR_033515 and miR-743b-5p, resulting in the proliferation, EMT, and fibrosis increasing level of proliferation-related proliferating cell nuclear antigen (PCNA), Cyclin D1, and the fibrotic proteins during DKD (Gao et al., 2018).

Arid2-IR is regulated by Smad3, knockdown of Arid2-IR in TECs has no effect on TGF-β/Smad-mediated fibrosis but promotes IL-1β-induced NF-κB-driven renal inflammation in obstructive kidney disease (Zhou et al., 2015). However, a recent study has reported the profibrotic effect of Arid2-IR by interacting with early growth response protein-1 (Egr1) in high-fat-diet and STZ-induced mice. Arid2-IR induces the expression of collagens and α-SMA in mouse MCs, contributing to the ECM accumulation in DKD (Yang et al., 2019c).

Interestingly, lncRNA NONHSAG053901 also targets Egr1 in mouse MCs, but their interaction has promoted inflammation by upregulating pro-inflammatory cytokines (Peng et al., 2019). The pathogenic role of Smad3-regulated LRNA9884 is observed in db/db mice with more severe albuminuria, histological injuries, and a decline of eGFR. LRNA9884 is induced by AGEs, and it targets MCP-1 to promote MCP-1-driven renal inflammation (Zhang et al., 2019d).

lncRNAs taurine upregulated gene 1 (TUG1) is an anti-fibrotic lncRNA mediated by TGF-β with multiple functions in DKD. In response to metabolic alterations of DKD, the expression of TUG1 is downregulated in podocytes. Overexpression of TUG1 can reverse the mitochondrial dysfunction in podocytes by targeting the transcription factor peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α (PGC-1α) (Li and Susztak, 2016; Shen et al., 2019). In consistence with previous results, TUG1 can also modulate mitochondrial bioenergetics in podocytes by binding with PGC-1α (Long et al., 2016). These findings have highlighted the connection between lncRNAs and DKD. By interacting with TNF receptor-associated factor 5 (TRAF5), TUG1 can suppress TRAF5-mediated podocyte apoptosis (Lei et al., 2018) and negatively downregulate the PI3K/Akt signaling to inhibit proliferation and ECM deposit in MCs (Zang et al., 2019). TUG1 is also able to interact with miR-21, thus promoting the expression of TIMP3 to alleviate renal fibrosis in HG-stimulated TECs and in db/db mice (Wang et al., 2019a). Furthermore, TUG1 sponges for miR-377 to regulate PPARγ and ECM in MCs (Duan et al., 2017). All these protective effects of lncRNA TUG1 in various cell types has supported its therapeutic potential in treating DKD. Besides, some lncRNAs may play diverse roles in the pathogenesis of DKD. lncRNA psoriasis-susceptibility related RNA gene induced by stress (PRINS) may exert both anti-fibrotic, anti-inflammatory but pro-apoptotic effects by regulating Smad7 in DKD. It has been demonstrated that there is a positive correlation between PRINS and Smad7 in DKD patients. As overexpression of Smad7 inhibits renal fibrosis and inflammation but also induces apoptosis in podocytes (Schiffer et al., 2001; Ka et al., 2012), thus, overexpression of PRINS upregulates Smad7 expression and promotes apoptosis in mouse podocytes (Jiao et al., 2019). lncRNA PRINS may be a therapeutic target of DKD-induced renal fibrosis and inflammation. But the underlying mechanisms of interaction between PRINS and Smad7 remain unexplored. In conclusion, the connection of TGF-β-mediated lncRNA and DKD is well-defined. Further studies on revealing the therapeutic targets and underlying mechanisms of these lncRNAs remain to be further explored.

NON-CODING RNAS AS NOVEL BIOMARKERS FOR DKD

The diagnosis and monitoring of renal injuries in DKD are now dependent on the detection of urinary albumin or serum creatinine. However, some patients may not present microalbuminuria or creatinine alterations during the progression of DKD, suggesting that none of these measures

can accurately indicate the severity and type of injury induced by hyperglycemia (Magee et al., 2017; Lin et al., 2018). In addition, urinary albumin is not specific to DKD, which may also occur in other diseases. Besides, the diagnostic and prognostic test of renal biopsy is invasive and may not be a reliable way to establish the full patterns of DKD. Thus, the availability of sensitive and specific biomarkers will provide therapeutic benefits in the control of DKD.

Non-coding RNAs in body fluids could facilitate communication between cells. Non-coding RNAs may exist in a stable form in serum and urine. As biomarkers, they may form a complex with proteins or be stored in transporters, including exosomes, microparticles, and apoptotic bodies. Based on the tissue- and cell type-specific characteristics of lncRNAs, significant differences in expression of novel lncRNAs in DKD (as shown in **Tables 2** and **3**) have mapped the signaling pathways in the pathogenesis of diabetic nephropathy (Guo et al., 2019). Indeed, a recent study has reported a novel lncRNA, PANDAR, related to T2DM DKD patients. The expression of PANDAR is upregulated in diabetic patients and higher in DKD patients with massive proteinuria, demonstrating its potential as biomarker and predictor for prognosis and progression of DKD (Zhao et al., 2020). The expression of lncRNA CASC2 is downregulated in T2DM patients with chronic renal failure but not T2DM patients with other complications, suggesting that lncRNA cancer susceptibility candidate 2 (CASC2) could also serve as a renal specific biomarker for DKD. Moreover, the study has further followed up for 5 years and found out that serum level of lncRNA CASC2 is negatively correlated with the incidence of chronic renal failure, supporting that serum level of lncRNA CASC2 may be a specific and reliable biomarker for diagnosis in DKD progression (Wang et al., 2018b). These studies have shown that lncRNAs are of high relevance in the development and progression of DKD, however, further mechanistic investigations on lncRNAs as therapeutic targets are warranted.

Some circulating miRNAs may also serve as sensitive and useful biomarkers for early detection and diagnosis for DKD (Zhang et al., 2016; Nascimento and Domingueti, 2019). For instance, in the early stage of T2DM DKD, the expression of miR-377 is positive, while miR-192 is negatively correlated with renal function (Tayel et al., 2020). In addition, circulating miRNA of miR-1246, miR-642a-3p, let-7c-5p, miR-1255b-5p, let-7i-3p, miR-5010-5p, and miR-150-3p are significantly upregulated in DKD patients compared with healthy volunteers (Kim et al., 2019). Moreover, the expression of miR-126 is decreased in DKD patients, which is negatively associated with albuminuria, level of fasting glucose, and glycated hemoglobin but positively correlated with eGFR (Al-Kafaji et al., 2016). The level of serum miR-21 is also consistent with tissue miR-21 that closely reflects renal function in DKD (Wang et al., 2016a). Up to date, many studies have reported the expression profiles of circulating miRNAs in diabetic nephropathy, making miRNAs as one of the promising candidates for DKD diagnosis and therapeutic targets.

The urinary exosomal miRNAs are called as "liquid biopsy" (La Marca and Fierabracci, 2017), which are typically secreted by cells from renal segments. They carry proteins, RNAs, and biomarkers that may reflect renal injury and dysfunction (Xu et al., 2018b). For example, miR-200b is a novel urinary biomarker that negatively correlates with the degree of renal fibrosis in CKD and DKD (Yu et al., 2018). One study has suggested that the expression of miR-27b-3p and miR-1228-3p in urine may be useful indicators for the degrees of renal fibrosis of DKD patients (Conserva et al., 2019). Notably, the expression of miR-126 in urine is increased in DKD patients compared to diabetic patients without renal disease. Interestingly, the urinary level of miR-126 is significantly decreased in DKD patients with a better diabetic control, implying that miR-126 may be a biomarker in DKD and monitor for DKD treatment response (Liu et al., 2014).

Although the clinical relevance in urinary miRNAs have been well studied (Lv et al., 2013; Cheng et al., 2014), there is still no consensus on the normalization of miRNAs isolated from urine, as the levels of urinary miRNAs may be high veriable and affected by urinary contents and concentrations. Neverthless, the better normalizer strategies should be encouraged (Blondal et al., 2013; Lekchnov et al., 2016; Corral-Vazquez et al., 2017), as the normalization of the validated data may help to provide statistically significant results without causing unwanted bias.

NON-CODING RNAS AS PROMISING THERAPEUTIC TARGETS FOR DKD

The regulatory role of non-coding RNAs in the pathogenesis of DKD has highlighted their potential as therapeutic targets for DKD. Restoring expression or inhibition of non-coding RNAs in renal or inflammatory cells under diabetic conditions may halt renal fibrosis and inflammation (**Figure 3**). Besides, rebalancing the overactivated TGF-β signaling induced by hyperglycemia could be another strategy that controls renal complication.

The delivery of synthetic non-coding RNA oligonucleotides, plasmids, or inhibitors may alter pathogenic signaling pathways related to DKD. Antagonism of miR-21 not only reduces the loss of podocytes and albuminuria but also inhibits renal fibrotic response by inhibition of collagen and fibronectin *in vivo* and *in vitro* (Wang et al., 2013; Kölling et al., 2017; Roy et al., 2020). Silencing miR-215 with specific antagomir increases the expression of CTNNBIP1, reduces of β-catenin activity, and accumulation of fibrotic proteins in db/db mice (Mu et al., 2013b). We have established the non-invasive ultrasound microbubble-mediated gene transfer to knock down renal expression of miR-21, thus suppressing the activation of the TGF-β and NF-κB signaling pathways by targeting Smad7 in the diabetic mouse model (Zhong et al., 2013). In addition, restoring the expression of miR-29b by delivery of doxycycline-inducible pre-miR-29b into the kidney, could significantly reverse the pathological changes of progressive DKD (Chen et al., 2014). Moreover, kidney-specific silencing of lncRNA Erbb4-IR and LRNA9884 with ultrasound technique can convert plasmids into the damaged kidney to ameliorate injuries, albuminuria, fibrosis, and inflammation (Sun et al., 2018a; Zhang et al., 2019d). Notably, exosomes secreted by cells contain non-coding RNAs that may have a regulatory

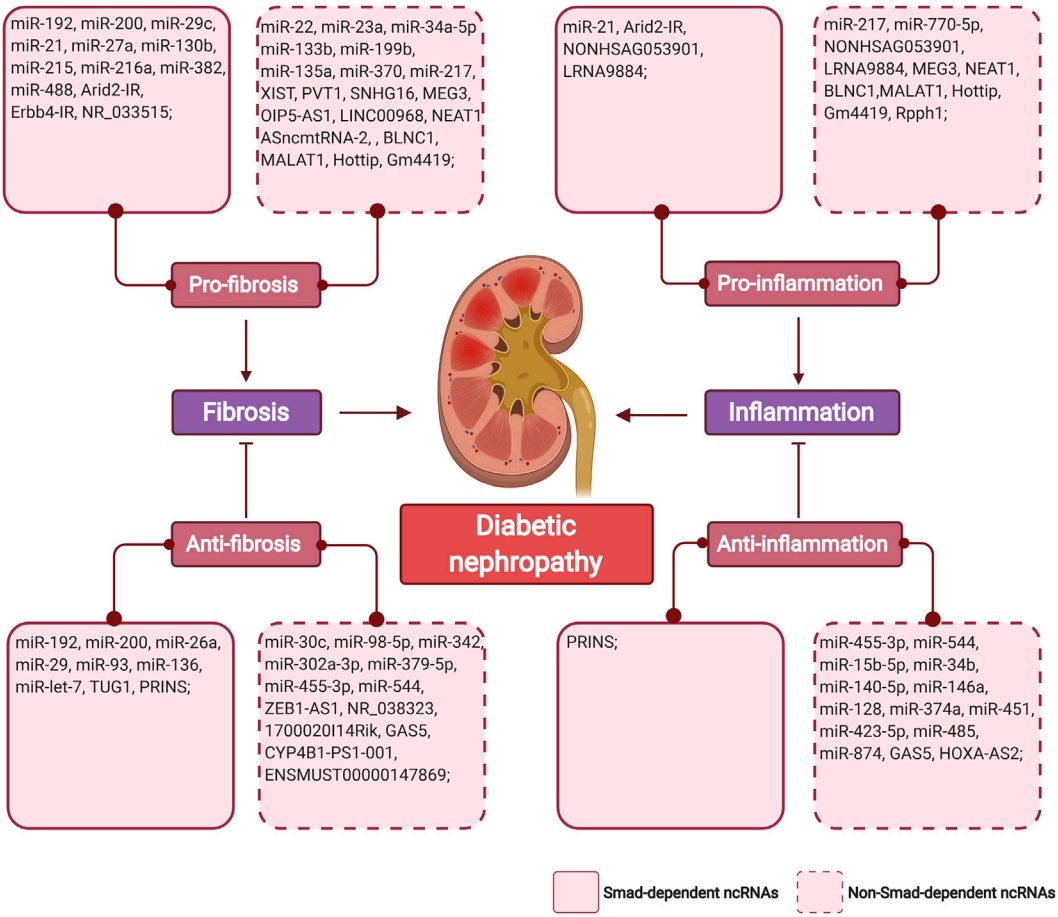

FIGURE 3 | The summary of TGF-β/Smad-dependent and non-TGF-β/Smad-dependent miRNAs and lncRNAs in diabetic renal fibrosis and inflammation. Non-coding RNAs are classified as pro/anti-fibrosis, pro/anti-inflammation in regard with their mechanistic funcions in diabetic nephropathy. (Figure created with BioRender.com).

role in DKD. Injection of exosomes from HG-treated macrophages induces MCs proliferation, fibrotic, and inflammatory factors activation *in vivo* as well as *in vitro*. Intriguingly, exosomes from TGF-β1 knockdown macrophages may reverse pathogenic changes in MCs (Zhu et al., 2019a), underscoring the importance of TGF-β signaling in the pathogenesis of DKD.

The rapid development of the field of non-coding RNAs has helped these RNA-based biopharmaceuticals to enter clinical trials before market approval. However, non-coding RNA treatments remain to be explored. The low expression, low conservation between species, time specificity, toxicity, and off-target effect of non-coding RNA are obstacles waiting to be solved in the development of RNA therapy (Yang et al., 2014; Ard et al., 2017). Up to date, the number of non-coding RNAs related to clinical trials on DKD is limited (Sankrityayan et al., 2019). Nevertheless, some ongoing miRNA-based therapies may be the potential next-generation medicine for DKD (Chakraborty et al., 2017). For example, Remlarsen, a miR-29 mimic that is undergoing in the clinical test (https://clinicaltrials.gov/ct2/show/NCT03601052) and could be the promising drug to combat renal fibrosis in DKD. Hopefully, new technologies such as clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) gene editing may represent novel strategies to modulate the expression and function of non-coding RNAs in DKD (Miano et al., 2019). Further studies are needed to reveal the therapeutic potential of ncRNAs in the clinical treatment of DKD.

CONCLUSION AND FUTURE PERSPECTIVES

Non-coding RNAs have garnered the major attention of researchers in the past few decades. We are now shifting toward their regulatory role and mutual relationship in the pathogenesis of DKD. Reports in this review and available literature have drawn the patterns of ncRNAs profiles in the process of diabetic nephropathy, but further investigation into the crucial mechanisms of ncRNAs in epigenetic regulation is warranted. Moreover, as biomarkers, the expression of renal ncRNAs may reflect the cellular response to hyperglycemic injuries, thus contributing to the early diagnosis and prognosis of DKD. The discovery of miRNAs and lncRNAs also represents a

new field of molecular therapy into DKD treatment. Together these findings are expected to yield novel insights into the complex pathogenesis of DKD and could be incorporated in the clinical settings.

AUTHOR CONTRIBUTIONS

Y-YG wrote and revised the manuscript. F-HL, X-RH, WM, and LZ revised the manuscript. X-SL, X-QY, and H-YL revised and edited the manuscript. All authors contributed to the manuscript conception development, data collection and analysis, and discussion on the manuscript writing and revising.

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REVIEW
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Epidermal Growth Factor Receptor: A Potential Therapeutic Target for Diabetic Kidney Disease

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Diabetic kidney disease (DKD) is a leading cause of end-stage renal disease worldwide and the major cause of renal failure among patients on hemodialysis. Numerous studies have demonstrated that transient activation of epidermal growth factor receptor (EGFR) pathway is required for promoting kidney recovery from acute injury whereas its persistent activation is involved in the progression of various chronic kidney diseases including DKD. EGFR-mediated pathogenesis of DKD is involved in hemodynamic alteration, metabolic disturbance, inflammatory response and parenchymal cellular dysfunction. Therapeutic intervention of this receptor has been available in the oncology setting. Targeting EGFR might also hold a therapeutic potential for DKD. Here we review the functional role of EGFR in the development of DKD, mechanisms involved and the perspective about use of EGFR inhibitors as a treatment for DKD.

Keywords: Epidermal growth factor receptor, diabetic nephropathy, hemodynamic alternation, metabolic disturbance, inflammation, multicellular dysfunction

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INTRODUCTION

Diabetic kidney disease (DKD) is a complication of diabetes mellitus and one of the leading causes of end-stage renal disease (ESRD) worldwide. DKD places a heavy personal burden on the many people world-wide who need hemodialysis and heavy economic burden on health care systems. It is urgent to find ways to slow the progression of DKD. However, its pathogenesis is complex and the mechanism is still poorly understood.

Increasing evidence indicates that various signaling pathways are activated and involved in the pathogenesis of DKD. Among them, the role of epidermal growth factor receptor (EGFR) has been extensively studied (Matrougui 2010; Advani et al., 2011; Zhang et al., 2014; Koya 2015). The EGFR belongs to a family of receptors that harbor tyrosine kinase activity and is composed of four members: EGFR (ErbB1), ErbB2, ErbB3, and ErbB4. They can be activated by several ligands, including epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), betacellulin, epigulin and epigen (Higashiyama et al., 2008; Schneider and Wolf 2009; Rayego-Mateos et al., 2018b). Upon ligand binding, the receptors form homodimers or heterodimers, leading to phosphorylation of some specific tyrosine residues in intracellular domains. These residues act as docking sites for initiating activation of multiple intracellular signaling pathways (Forrester et al., 2016).

Activation of EGFR signaling has been implicated in numerous physiological and pathophysiological processes, including embryonic development, cell proliferation, cell survival, and tumorigenesis. In the mammalian kidney, EGFR is widely expressed in glomeruli and proximal tubules, including renal epithelial cells, glomerular endothelial cells, podocytes, tubular cells,

mesangial cells and medullary interstitial cells (Gesualdo et al., 1996; Harskamp et al., 2016). In the past decade, many studies have investigated the role of EGFR signaling in the progression of chronic kidney disease (CKD) (Chen et al., 2012; Harskamp et al., 2016; Rayego-Mateos et al., 2018b). In this review, we will discuss the role and the mechanism of EGFR in the development of DKD and consider the potential use of EGFR inhibitors as a treatment of this disease.

Diabetic Kidney Disease

Chronic hyperglycemia in diabetes mellitus can induce dysfunction of all types of cells in the kidney. DKD is mainly manifested by proteinuria, which varies in several stages, including the silent stage, the microalbuminuria stage (30–300 mg/day) and the macroalbuminuria stage (>300 mg/day) (Papadopoulou-Marketou et al., 2017). Proteinuria occurs along with morphological changes in the glomerulus and interstitium (Qi H. et al., 2017.). From the onset of diabetes mellitus to nearly 5 years, kidney size increases along with an increase in renal plasma flow and hyperfiltration, with thickened glomerular basement membrane and mild or severe mesangial expansion. After 5–10 years, glomerular damage progresses with the occurrence of microalbuminuria and nodular accumulation of mesangial matrix. As glomerulosclerosis advances, extra-glomerular lesions also form. Proteinuria is irreversible at this stage as the glomerular filtration rate (GRF) drops below 60 ml/min/1.73 m^2 and ultimately reaches end-stage levels below 15 ml/min/1.73 m^2 (Sulaiman 2019; Han et al., 2017; Kanwar et al., 2011; Papadopoulou-Marketou et al., 2017; Qi C. et al., 2017). During the progression of DKD, mitochondria generate excess reactive oxidative species (ROS) or reactive nitrogen species (RNS), resulting in the activation of several signaling pathways, transcription factors and cytokines, such as TGF-β/smad/MAPK signaling, JAK/STAT signaling, VEGF, EGFR. Activation of these signaling pathways and transcription factors is associated with cell growth, angiogenesis, and apoptosis, leading to DKD ultimately (Kanwar et al., 2011; Magee et al., 2017; Papadopoulou-Marketou et al., 2017; VR et al., 2019).

Epidermal Growth Factor Receptor Transactivation in Diabetic Kidney Disease

Renal EGFR phosphorylation levels were significantly increased in animal models of diabetes mellitus and in cultured cells treated with high glucose (Konishi and Berk 2003; Saad et al., 2005; Portik-Dobos et al., 2006; Uttarwar et al., 2011; Li R. et al., 2015). EGFR inhibition slowed the progression of DKD, including the improvement of proteinuria and morphologic changes (Wassef et al., 2004; Chen et al., 2012; Zhang et al., 2014). The concentration of EGFR ligands in plasma and kidneys, such as EGF, TGF-α and HB-EGF, was also increased in DKD (Uttarwar et al., 2011; Miyazawa et al., 2013; Perlman et al., 2015). Some reports suggested that connective tissue growth factor (CTGF) is a novel EGFR ligand and that blocking CTGF-mediated profibrotic effects could also be a potential therapeutic option to treat fibrotic renal diseases (Rayego-Mateos et al., 2013; Rayego-Mateos et al., 2018a).

Besides direct activation by its ligands, EGFR transactivation has been recognized as another important mechanism for signal transduction. The process of EGFR transactivation is not mediated through direct ligand binding, but through other second messengers. Several stimuli known to be involved in the pathogenesis of DKD were found capable of transactivating EGFR, such as ROS, TGF-β and PKC. In streptozotocin-induced diabetes and in cultured cells exposed to high glucose, ROS inhibition with superoxide dismutase (SOD) or an NADPH oxidase inhibitor attenuated the upregulation of EGFR phosphorylation (Chen et al., 2015; Sheng et al., 2016). Endothelin-1 (ET-1) mediated activation of endothelin A (ETA) receptor also contributed to EGFR transactivation in diabetic animals (Portik-Dobos et al., 2006). The mechanism by which EGFR transactivation occurs upon stimulation with these active factors remains unclear. A well-accepted hypothesis is that these substances act on their own receptors and then induce release of EGFR ligands (Konishi and Berk 2003; Higashiyama et al., 2008; Chen et al., 2012). EGFR ligands including EGF, HB-EGF and TNF-α are synthesized as precursors anchored on the cell membrane. Upon stimulation, they are released from the membrane in soluble bioactive forms by specific metalloproteases such as ADAM17 (Ohtsu et al., 2006; Uttarwar et al., 2011) (Uttarwar et al., 2011; Li T. et al., 2015; Morgado-Pascual et al., 2015). In diabetes, several second messengers, such as ROS and protein kinases can induce activation of ADAMs, leading to shedding of EGFR ligands. The shed ligands can bind to EGFR in an autocrine or paracrine-dependent manner (Schreier et al., 2014). In addition, Src, a non-receptor tyrosine kinase, can also mediate EGFR transactivation initiated by activation of G-protein-coupled receptors (GPCRs) (Taniguchi et al., 2013; Forrester et al., 2016).

Epidermal Growth Factor Receptor and the Pathogenesis of Diabetic Kidney Disease

The pathogenesis of DKD is a complex process involving many factors, including hemodynamic alteration, metabolic disturbance, inflammatory response and parenchymal cellular dysfunction (Tung et al., 2018; VR et al., 2019). EGFR transactivation by high glucose causes multicellular dysfunction, which initiates and accelerates kidney injury. Studies have found that EGFR inhibition can reduce kidney size after in STZ treated diabetic mice, without affecting body weight, blood glucose or blood pressure (Wassef et al., 2004). Inhibition of EGFR with erlotinib also markedly reduces albuminuria and renal expression of CTGF, collagen I, collagen IV in diabetic mice (Zhang et al., 2014).

Hemodynamic Alteration

Hemodynamic alteration plays an important role in the pathogenesis of DKD. Chronic hyperglycemia induces metabolic alteration and dysfunction in endothelial and vascular smooth muscle cells, leading to vascular dysfunction and hemodynamic alteration in kidneys and other organs (Matrougui 2010; Li T. et al., 2015). Glomerular hemodynamic

alterations such as hyperfiltration and hyper-perfusion are found in the early stages of DKD. Hyperfiltration is a result of a decrease in glomerular afferent and efferent arteriolar resistance; dilation of the efferent arteriole is relatively less than dilation of the afferent arteriole, causing a relative increase in glomerular transcapillary hydraulic pressure (Hostetter 2003; Wolf and Ziyadeh 2007; Ziyadeh and Wolf 2008). This facilitates the development of albumin leakage from the glomerular capillary compartment to Bowman's space. Many factors, especially angiotensin II (AngII), have been implicated as important biologically active agents that cause hyperperfusion and hyperfiltration. (Cooper 2001; Wolf 2004; Forbes et al., 2007). Since Ang II can induce transactivation of EGFR, it has been suggested that blockade of EGFR can reduce Ang II-mediated hemodynamic alteration (Krishna et al., 2007; Akhtar et al., 2012).

In diabetic animal models, treatment with EGFR inhibitors results in a significant normalization of the altered vasoconstrictor and vasodilator response without effecting blood glucose levels (Benter et al., 2005; Yousif et al., 2005; Benter et al., 2009; Akhtar et al., 2012; Schreier et al., 2014). Mechanistic studies showed that EGFR inhibition mediated vascular response to different stimuli occurs through reduction of ROS generation in mesenteric resistance arteries (Kassan et al., 2015). This may involve the correction of diabetes-induced reduction in nitric oxide synthase (eNOS) activity and nitric oxide (NO) generation in vascular smooth muscle cells (VSMC) (Benter et al., 2015). Despite disturbed vascular response, EGFR also mediates vascular remolding in diabetes. Akhtar and others found that inhibition of EGFR activation results in a remarkable reduction in blood vessels thickening both in intima and media, and attenuates vascular hyper-responsiveness via ERK1/2-ROCK pathway (Palen and Matrougui 2008; Akhtar et al., 2019). Thus, EGFR inhibition could help restore some vascular endothelial functions, independent of glucose lowering, providing considerable therapeutic strategy for vascular protection in DKD.

Metabolic Disturbance

Diabetic kidneys are highly sensitive to metabolic alteration. Patients with diabetes mellitus experience chronic hyperglycemia. Glucose was translocated into cells by various transporters including glucose transporter (GLUT)-1, GLUT-4, and sodium-glucose-linked transporters. Excess glucose influx into cells leads to glucose transport along various metabolic pathways, along with the generation of reactive oxygen species (ROS) and advanced glycation end product (AGEs) (Magee et al., 2017). These metabolic derangements induced activation of several signaling pathways related to proliferation and fibrosis, such as the transforming growth factor-β (TGF-β) and the protein kinase C (PKC) signaling pathways (Cooper 2001; Forbes et al., 2007; Kanwar et al., 2011). In addition, researchers found activation of GLUT1 synthesis itself was associated with growth factor upregulation and extracellular matrix secretion (Heilig et al., 2013).

EGFR has been implied in the regulation of the metabolic pathways. In a diabetes model with eNOS knockout, inhibition of EGFR attenuated albuminuria, glomerulosclerosis and tubulointerstitial fibrosis, along with a decreased urinary excretion of F2-isoprostane, a marker of oxidative stress (Portero-Otin et al., 2002; Li et al., 2018). In addition, inhibition of EGFR tyrosine increased glucose tolerance and ameliorated insulin resistance (Li et al., 2018). In STZ-induced diabetes models, EGFR inhibition markedly reduced renal oxidative stress and endoplasmic reticulum stress (ERS), and attenuated renal fibrosis and apoptosis (Xu et al., 2017). In another study, inhibition of EGFR reversed the accumulation of ROS and superoxide levels, probably by improving *p*-eNOS expression and inhibiting Nox4 expression (Wang et al., 2020). Advanced glycation end product receptors (AGERs) are receptors that mediate AGEs-induced toxicity to cells. AGER can interact with EGFR and mediate oxidative species generation, as characterized by H_2O_2 formation in mesangial cells and in human embryonic kidney epithelium-like cells (Cai et al., 2006). AGE product precursors could also impair EGFR signaling (Portero-Otin et al., 2002). EGFR activation along with alteration of these metabolic pathways leads to disturbed signaling and mediates kidney injury.

Inflammation

Low-grade systemic inflammation seems to play a critical role in the pathogenesis of DKD (Rivero et al., 2009; Matoba et al., 2019; Vasanth et al., 2019). Scurt et al. found that serum markers of inflammation such as CXCL-16, MCP-1, ANGP-2 could predict the onset of microalbminuria in patients with diabetes mellitus type 2 (Scurt et al., 2019). Other studies indicate that serum IL-18 and TNF-α levels were increased in diabetic patients, especially in those with kidney impairment (Moriwaki et al., 2003; Mora and Navarro 2004). In the onset of diabetic mellitus, excess AGEs and ROS, and activation of several signaling pathways, induced transcription of various adhesion molecules and pro-inflammatory cytokines, and mediated macrophage infiltration and the progression of DKD (Matoba et al., 2019). EGFR inhibition decreases renal T-cell infiltration and islet macrophage infiltration in diabetic glomeruli and the interstitium (Li et al., 2018). Aldosterone-induced proinflammatory gene (CCL-2 and CCL-5) expression in cultured tubular epithelial cells was also shown to occur through the ADAM-17/TGF-α/EGFR pathway (Morgado-Pascual et al., 2015). Zhang *et al.* also found that treatment with erlotinib, an EGFR inhibitor, reduced kidney macrophage infiltration and oxidative stress in the tubular interstitium (Zhang et al., 2014). Thus, EGFR may be involved in renal inflammatory responses in DKD.

Multicellular Dysfunction in Diabetic Kidney Disease

It has been thought that pathologic changes to mesangial cells represent the central feature of glomerulosclerosis in DKD. However, damage to other cell types, including endothelial cells, podocytes, tubular epithelial cells and fibroblasts, also

contributes to progression of DKD (Qian et al., 2008; Magee et al., 2017).

Mesangial Cell

Mesangial proliferation and expansion is considered the hallmark of DKD. Mesangial cells are mesenchymal in origin, and are easily activated to undergo proliferation and matrix secretion. Activation of growth factors promotes different signaling pathways that mediate proliferation of mesangial cells. Research by Wu and colleagues indicated that high glucose induced collagen production in mesangial cells through EGFR-mediated activation of the PI3K-Akt signaling pathway (Wu et al., 2007; Wu et al., 2009). Another study also indicated that high glucose induced collagen accumulation in mesangial cells through the Src/TACE/HB-EGF signaling pathway (Taniguchi et al., 2013). EGFR transactivation by high glucose does not require PKC, ROS, or AngII, but HB-EGF release is essential for transactivation of EGFR in mesangial cells to induce mesangial cell proliferation and matrix secretion (Uttarwar et al., 2011).

Endothelial Cell

Endothelial cells can produce NO and regulate platelet adhesion, immune function, control of volume. Endothelial dysfunction is the inability of vasculature to dilate in response to certain stimuli acting on the endothelium (Goligorsky 2015). It is always associated with a deficiency of eNOS activity and NO release (Rivero et al., 2009; Kolluru et al., 2012; Sharma et al., 2012). Wang et al. applied a novel rhynchophylline analogue, Y396, to study the role of EGFR on endothelial function. They found that Y396 inhibits the tyrosine kinase activity of EGFR by directly targeting EGFR and restores endothelium-dependent vascular relaxation without affecting vascular structure (Wang et al., 2020). This effect of EGFR inhibition may be mediated by downregulation of Nox2 and Nox4 expression and ROS suppression (Galan et al., 2012). Similarly, Belmadani et al. also demonstrated that EGFR activation is elevated and induces resistance artery dysfunction and endothelium-dependent relaxation in diabetic mice without interfering blood pressure (Belmadani et al., 2008).

Another mechanism that regulates vascular dysfunction is the endothelial-to- mesenchymal transition (EndoMT) (Piera-Velazquez et al., 2016; Pardali et al., 2017; Piera-Velazquez and Jimenez 2019). EndoMT is known as endothelial cells transition into a mesenchymal cell type. In 2013, LeBleu et al. employed cell linage tracing and found that approximately 10–15% of myofibroblasts were derived from EndoMT (LeBleu et al., 2013). Since then, much attention has been paid to the EndoMT in DKD, especially the renal interstitial fibrosis (Li et al., 2009; Li and Bertram 2010). The role of EGFR in EndoMT in the kidney was less extensively investigated. In an animal model of cardiac fibrosis, Liu et al. observed that EGFR mediated EndoMT promotes several fibrosis-related events post myocardial infarction, (Liu et al., 2020). These events include acquisition by endothelial cells of a spindle-like shape and the ability to migrate. Although glomerular endothelial mitochondrial dysfunction plays a key role in the pathogenesis of DKD as evidenced by podocyte depletion and proteinuria (Qi H. et al., 2017), the role of EGFR in glomerular endothelial cell pathophysiology has not been well investigated.

Podocyte

Podocyte injury is an early event in DKD and is a hallmark of glomerulopathy. Studies have suggested that podocyte injury is associated with the early stage of proteinuria in patients with diabetes (Wolf and Ziyadeh 2007; Bose et al., 2017; Dai et al., 2017). As terminally differentiated cells, podocytes are vulnerable to injury and may not be able to regenerate or repair themselves after injury. They could undergo hypertrophy, epithelial-mesenchymal transition, detachment and apoptosis under certain stimuli, leading to depletion of these cells within the glomerulus, characterized by foot process effacement on biopsy (Han et al., 2017). In DKD, podocytes are involved in the development of glomerular hypertrophy, proteinuria and glomerulosclerosis (Li et al., 2007; Dai et al., 2017; Maestroni and Zerbini 2018) and promote the development of interstitial fibrosis. High concentrations of glucose induce the production of ROS and initiate podocyte apoptosis and podocyte depletion, which may be an early pathological change in DKD (Susztak et al., 2006). EGFR also plays an important role in podocyte injury in DKD. This is evident by the observations that EGFR inhibition led to less podocyte loss in models of diabetic nephropathy while podocyte-specific deletion of EGFR attenuated albuminuria and podocyte loss induced by hyperglycemia (Taniguchi et al., 2013; Li et al., 2018). This may be mediated by activation of TGF-β-SMAD2/3 signaling pathway and enhanced ability of mitochondrial NADPH oxidase to increase ROS production (Chen et al., 2015).

Tubular Epithelial Cell

The tubular epithelial cell has been implicated in interstitial fibrosis. Tubular epithelial cells are also vulnerable to pathologic stress due to high glucose levels and can undergo epithelial-mesenchymal-transition and apoptosis (VR et al., 2019). Upon injury, epithelial cells can secrete growth factors and inflammatory cytokines to induce fibroblast activation and renal fibrosis (Sheng and Zhuang 2020). EGFR is highly expressed in proximal tubules. Its transactivation mediates sodium and water transport by regulation of NHE3 and serum glucocorticoid regulated kinase-1 (sgk1) (Panchapakesan et al., 2011). Erlotinib treatment decreased tubular injury and tubulointerstitial fibrosis in db/db mice (Li et al., 2018). EGFR inhibition also attenuated renal tubular epithelial cell proliferation and apoptosis in diabetic rats (Wassef et al., 2004). In addition, erlotinib treatment decreased ER stress and increased autophagy in tubular cells in diabetes (Zhang et al., 2014). The protective effect of some other interventions may be also partly through EGFR. For example, histone deacetylase inhibition can attenuate tubular cell proliferation and early diabetic renal enlargement in response to high glucose by downregulation of EGFR (Gilbert et al., 2011).

EGFR also participated in epithelial-mesenchymal transition (EMT). EMT, a process by which injured renal tubular cells undergo a phenotype change and acquire mesenchymal

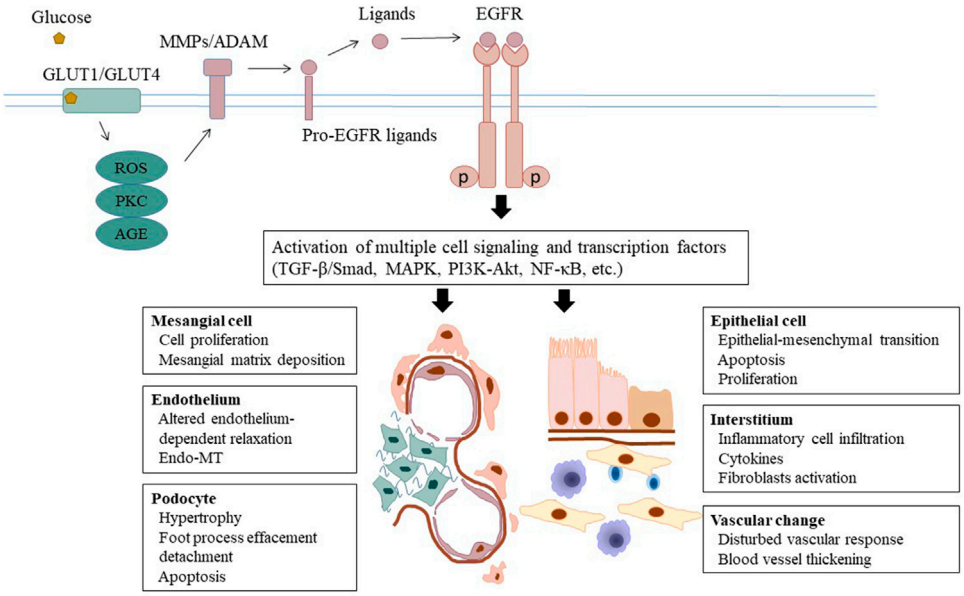

FIGURE 1 | Epidermal growth factor receptor and the pathogenesis of DKD. Under the circumstance of diabetes, glucose triggers activation of ADAMs through several second messengers including ROS and protein kinase C. ADAM-mediated shedding of ligands induces phosphorylation of EGFR and subsequent activation of cell several signaling pathways and transcription factors. As a result, multiple cellular and tissue responses are initiated and implicated in the pathogenesis of DKD progression, including hemodynamic alteration, metabolic disturbance, inflammatory response and parenchymal cellular dysfunction. EGFR, epidermal growth factor receptor; DKD, diabetic kidney disease; ADAM, a disintegrin and metalloprotease; ROS, reactive oxidative species.

characteristics, is widely recognized as a critical mediator of fibrogenesis in chronic kidney diseases. EGFR has been thought to mediate EMT. Sustained EGFR activation in the tubule induces epithelial dedifferentiation and cell cycle arrest with an increase in the mesenchymal marker and decreases in the epithelial marker (Overstreet et al., 2017). Administration of CTGF in cultured tubular epithelial cells caused G2/M cell cycle arrest and EMT via EGFR pathways. The cells lost the typical cobblestone pattern and showed a spindle-shaped pattern, along with the elevation of mesenchymal marker and a decrease in epithelial marker. EGFR inhibition attenuated these changes (Rayego-Mateos et al., 2018a). It is possible that the activation of EGFR in diabetic kidney disease may mediate EMT, thus promoting interstitial fibrosis.

Non-Renal Effects of Epidermal Growth Factor Receptor Inhibitor in Diabetes

Two groups have reported that patients who suffered from non-small-cell lung cancer (NSCLC) experienced an improvement in diabetes after erlotinib (an EGFR inhibitor) treatment (Portero-Otin et al., 2002; Costa and Huberman 2006; Brooks 2012). This improvement may be due to erlotinib-elicited reduction of insulin resistance by inhibition of TNF-α and the T-cell mediated immune response (Brooks 2012). These interesting clinical reports are supported by a striking finding in animal studies showing that erlotinib-treated mice had a relatively slow increase in body weight, a decrease in fasting blood glucose levels, and improved glucose disposition and insulin sensitivity. EGFR inhibition with erlotinib also decreased islet macrophage infiltration and increased autophagy, leading to preservation of pancreatic β-cell function and subsequent improvement of metabolic status. Moreover, EGFR blockade increases circulating levels of the adipokine adiponectin, an adipocyte-derived hormone that has insulin-sensitizing, anti-inflammatory, and kidney-protective effects (Fang et al., 2015; Ding et al., 2016; Li et al., 2018). In addition, treatment with EGFR inhibitor PD153035 reduces low-grade inflammation, macrophage infiltration in adipocytes and improves glucose tolerance and insulin actions (Prada et al., 2009). These studies suggest that EGFR inhibitors may also ameliorate the progression of DKD through improving insulin sensitivity and pancreatic beta cell functions.

Role of Other Epidermal Growth Factor Receptor Family Members in Diabetic Kidney Disease

Other EGFR tyrosine kinase family members, such as the ErbB2 and ErbB4, may also contribute to the progression of CKD and the pathogenesis of DKD (Zeng et al., 2018a). It relies on heterodimerization with other EGFR family members for signaling. Akhtar et al. investigated the phosphorylation of ErbB2 in diabetes. They found that high glucose exposure enhanced activation of ErbB2, induced vascular dysfunction in VSMCs (Akhtar et al., 2013). ErbB4 expression was increased in the mild fibrotic kidneys, and decreased as fibrosis progressed (Zeng et al., 2018a). ErbB4 suppression significantly attenuated diabetic glomerular injury and albuminuria. Mesangial expansion

TABLE 1 | EGFR and the pathogenesis of DKD.

Factors		Main findings	References
Hemodynamic alternations		Altered vasoconstrictor and vasodilator response	Benter et al. (2015); Akhtar et al. (2019); Palen and Matrougui (2008); Kassan et al. (2015)
Metabolic disturbance		Generation of reactive oxygen species (ROS) and advanced glycation end product (AGEs)	Li et al. (2018); Wang et al. (2020); Cai et al. (2006); Portero-Otin et al. (2002)
Inflammatory response		Inflammatory cell infiltration and proinflammatory cytokine expression	Li et al. (2018); Morgado- Pascual et al. (2015); Zhang et al. (2014)
Parenchymal cellular dysfunction	Mesangial cell	Mesangial cell proliferation and mesangial expansion	Wu et al. (2007); Wu et al. (2009); Taniguchi et al. (2013); Uttarwar et al. (2011)
	Endothelial cell	Altered endothelium-dependent relaxation	Wang et al. (2020); Galan et al. (2012); Belmadani et al. (2008)
		Endothelial-to-mesenchymal transition	Liu et al. (2020)
	Podocyte	Podocyte hypertrophy, detachment and apoptosis	Li et al. (2018); Taniguchi et al (2013); Chen et al. (2015)
	Tubular epithelial cell	Increased ER stress, decreased autophagy; cell proliferation and apoptosis	Li et al. (2018); Wassef et al. (2004); Gilbert et al. (2011)
		EMT	Rayego-Mateos et al. (2013), Morgado-Pascual et al. (2015)

and sclerosis were reduced with ErbB4 inhibition, as well as STZ-induced podocyte foot process effacement and podocyte loss. TGF-β1 induced MCP-1 expression in podocytes was also suppressed by ErbB4 inhibition (Lee et al., 2017). ErbB4 may also play an important role in glucose homeostasis and lipogenesis. ErbB4 deficiency-related obesity and adipose tissue inflammation may contribute to the development of metabolic syndrome (Zeng et al., 2018b). Some researchers suggest that increased expression of ErbB4 may actually reflect a compensatory effort to prevent development of tubulointerstitial injury (Zeng et al., 2018a).

Treatment of Diabetes and Diabetic Kidney Disease by Targeting Epidermal Growth Factor Receptor

In the past decades, much attention has been paid on application of tyrosine kinase inhibitors to treat diabetes, including EGFR inhibitors in animal models (Fountas et al., 2015; Malek and Davis 2016). With EGFR inhibitors being extensively used to treat non-small-cell lung cancer (NSCLC), their efficacy in treating CKD and DKD have also been explored in animal models and culture systems. Numerous animal studies and *in vitro* studies have provided evidence that EGFR inhibition could attenuate or prevent development and progression of DKD. This effect may associate with improvement in β cell function and insulin resistance (Li et al., 2018).

Although there are no clinical trials designed for treatment of human DKD by targeting EGFR, there are two case reports about the application of EGFR inhibitor erlotinib in diabetes. In 2006, Costa et al. observed that administration of erlotinib to a lung cancer patient improved his type 2 diabetes (Costa and Huberman 2006). When given chemotherapy with erlotinib 100 mg daily, the patient felt frequent episodes of hypoglycemia, and her fasting glucose level was stabilized as well. After 8 months, her HbA1c had dropped to 6.5% from 8.2%. In another case report, a 73-year-old man with history of metabolic syndrome, CKD and insulin-dependent type 2 diabetes received erlotinib 150 mg daily after being with metastatic NSCLC. Four weeks after starting erlotinib, the patient's insulin requirement began to decline from 90 units daily. After 10 weeks he was off insulin completely. His HbA1c decreased from to 6.6% from 7.4% in six months. At the same time, an abrupt increase in his serum creatinine slowed down (Brooks 2012).

The first EGFR tyrosine kinase inhibitor (TKI) was approved for clinical use in 2003 and was mostly used in patients with non-small-cell lung cancer (NSCLC) carrying EGFR-activating mutations and in patients with breast and pancreatic cancers. Nevertheless, EGFR-TKIs may cause adverse effects. Since EGFR plays a role in epithelial maintenance, the most frequent and severe side effects are dermatological reactions and diarrhea. Other adverse effects include hepatotoxicity, stomatitis, interstitial lung disease, ocular toxicity and hypomagnesaemia (Shah and Shah 2019; Xu et al., 2019; Huang et al., 2020). Most of the data come from patients with cancer. In addition, seven patients were reported in the literature to develop anti-EGFR-induced nephrotic/nephritic syndrome after 2–24 weeks of therapy. All the cases of kidney disease associated with EGFR inhibitor treatment were identified in patients with cancers and shown by the variable and often prolonged time course between drug exposure (2 weeks–6 months) and clinical recognition of kidney injury (Izzedine and Perazella 2017). Since DKD treatment needs a long-term application of drugs, it is anticipated that use of EGFR-TKI in DKD patients would have additional safety concerns. As such, future clinical observations and/or clinical trials are needed to determine the benefit and side effect of EGFR-TKI in those population of patients.

CONCLUSION

Nearly one third of patients with diabetes develop DKD, which in many cases progress to end-stage renal disease and the need for dialysis or kidney transplantation. The underlying mechanisms mediating DKD remain incompletely understood. *In vitro* and *in vivo* studies have demonstrated that EGFR activation can initiate multiple pathological processes leading to DKD, such as hemodynamic and metabolic alterations, chronic inflammation, and multicellular dysfunction (**Figure 1** and **Table 1**). Given the

importance of EGFR in mediating the pathogenesis of DKD, much work has gone into studying whether EGR inhibition could slow or stop the development of DKD. EGFR inhibitors have been extensively used to treat various tumors, in particular lung carcinoma. This suggests an interesting possibility that EGFR inhibitors may be repurposed as a treatment for DKD and CKD caused by other etiologies. Nevertheless, beside their benefit effects, long-term use of EGFR inhibitors may result in some adverse effects including kidney problems. Most of side effects of EGFR inhibitors in patients with tumor are tolerable. But it is uncertain whether they are also applicable and tolerable in patients with CKD, in particular DKD. Therefore, clinical trials are needed to determine the efficacy and adverse effects of EGFR inhibitors in patients with DKD.

AUTHOR CONTRIBUTIONS

LS drafted the article, and GB and SZ edited the manuscript. All the authors reviewed the manuscript and approved is for publication.

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Effects of Sodium-Glucose Co-transporter 2 Inhibitors on Hemoglobin Levels: A Meta-analysis of Randomized Controlled Trials

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Background: This study aimed to explore the effects of sodium-glucose co-transporter 2 (SGLT2) on hemoglobin levels in patients with type 2 diabetes mellitus (T2DM) and chronic kidney disease.

Methods: PubMed, EMBASE, the Cochrane Central Register of Controlled Trials, the China National Knowledge Infrastructure database, Wanfang Digital Periodicals Database (WFDP) and the Chinese Biological and Medical database (CBM) were searched for randomized trials of SGLT2 inhibitors in patients with T2DM and chronic kidney disease up to July 25, 2020. A total of four studies that included 19,259 patients were identified.

Results: Compared to control patients, SGLT2 inhibitors were shown to increase hemoglobin levels in patients with T2DM and chronic kidney disease (standard mean difference = 0.70, 95% CI, 0.59–0.82, $p < 0.0001$).

Conclusion: SGLT2 inhibitors may bring additional benefits to patients with T2DM and chronic kidney disease.

Keywords: meta-analysis, SGLT2 inhibitors, type 2 diabetes, chronic kidney disease, hemoglobin

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INTRODUCTION

In the past ten years, the incidence of type 2 diabetes mellitus (T2DM) has been increasing (International Diabetes Federation, 2017) which indicates a massive increase in end-stage renal disease on a global scale. One of the most common complications of chronic kidney disease is renal anemia (Sugahara et al., 2017). The presence of anemia significantly increases the risk of micro- and macrovascular complications. Patients who are not properly treated have significantly reduced quality of life and a poor prognosis (Sugahara et al., 2017).

Sodium-glucose co-transporter 2 (SGLT2) inhibitors are a newly approved class of oral hypoglycemic agents that increase the excretion of glucose in the urine by inhibiting the reabsorption of urine glucose in the proximal tubules of the kidney, thereby reducing blood glucose levels, weight and blood pressure (Polidori et al., 2014; Inagaki et al., 2015). In addition, SGLT2 inhibitors also have a protective effect on the kidney (Xu et al., 2017) as evidence has shown that after treatment with SGLT2, hemoglobin levels are increased (Maruyama et al., 2019).

In this study, we aimed to ascertain the effects of SGLT2 inhibitors on the hemoglobin levels in patients with T2DM and chronic kidney disease.

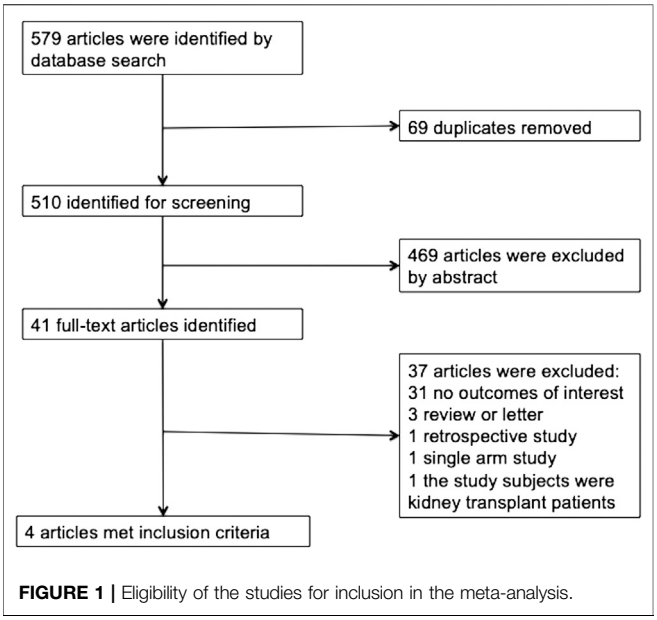

FIGURE 1 | Eligibility of the studies for inclusion in the meta-analysis.

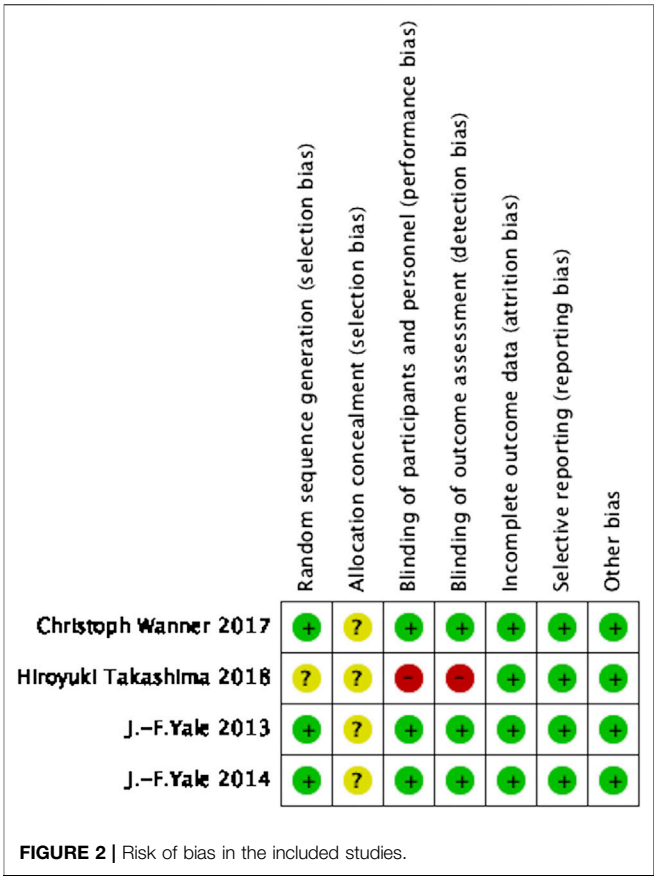

FIGURE 2 | Risk of bias in the included studies.

METHODS

Data Sources and Search Strategies

The PubMed, EMBASE, the Cochrane Central Register of Controlled Trials, the China National Knowledge Infrastructure (CNKI) database, Wanfang Digital Periodicals database (WFDP), the Chinese Biological and Medical database (CBM) were searched. The following Medical Subject Headings (MeSH) terms and free-text terms were applied: "Sodium-Glucose Transporter 2 Inhibitors", "Sodium Glucose Transporter 2 Inhibitors", "SGLT2 Inhibitors", "SGLT-2 Inhibitors", "SGLT 2 Inhibitors", "Gliflozins", "Renal Insufficiency, Chronic", "Chronic Renal Insufficiencies", "Renal Insufficiencies, Chronic", "Chronic Renal Insufficiency", "Kidney Insufficiency, Chronic", "Chronic Kidney Insufficiency", "Chronic Kidney Insufficiencies", "Kidney Insufficiencies, Chronic", "Chronic Kidney Diseases", "Chronic Kidney Disease", "Disease, Chronic Kidney", "Diseases, Chronic Kidney", "Kidney Disease, Chronic", "Kidney Diseases, Chronic", "Chronic Renal Diseases", "Chronic Renal Disease", "Disease, Chronic Renal", "Diseases, Chronic Renal", "Renal Disease, Chronic", "Renal Diseases, Chronic". All publications up to July 25, 2020 were selected without the restriction of origins, countries, languages or article types.

Selection Standards

Published articles that were included in the analysis were required to meet the following criteria: 1) the eligible subjects were men and women with T2DM and chronic kidney disease; 2) interventions involved treatment with SGLT2 inhibitors alone or with other hypoglycemic agents; 3) studies compared placebo control or standard of care; 4) outcomes reported changes in hemoglobin levels from baseline; 5) studies that were randomized controlled trials (RCTs); 6) studies with follow-up times of 12 weeks or longer. Observational studies, non-randomized trials and uncontrolled trials were excluded from the analysis.

Data Extraction and Quality Assessment

Two investigators extracted the following data independently from eligible publications: first author, publication year, study design, inclusion criteria, sample size, patient characteristics, interventions (types and doses of SGLT2 inhibitors), comparison (placebo control or standard care), follow-up duration and outcomes (changes in hemoglobin levels from baseline). The unit of hemoglobin levels was uniformly converted into g/l. Discrepancies were resolved by the discussion between two investigators. The Cochrane risk-of-bias tool was adopted to assess randomization, masking of treatment allocation, blinding, adherence and withdrawals for each of the RCTs (Higgins et al., 2011).

Statistical Analysis

Data analysis was performed using Stata version 12.0 software. The effect sizes on scores were presented as the standard mean difference (SMD) and 95% confidence intervals (CIs). The Chi-squared test based on Q-statistic and I2 statistics was used to estimate the heterogeneity ($I2 \leq 25\%$, low heterogeneity; $25\% < I2 < 50\%$, moderate heterogeneity; $I2 \geq 50\%$, high heterogeneity) (Higgins et al., 2003). A fixed-effects model was used to pool the results when heterogeneity was ≤50%, while a random-effects

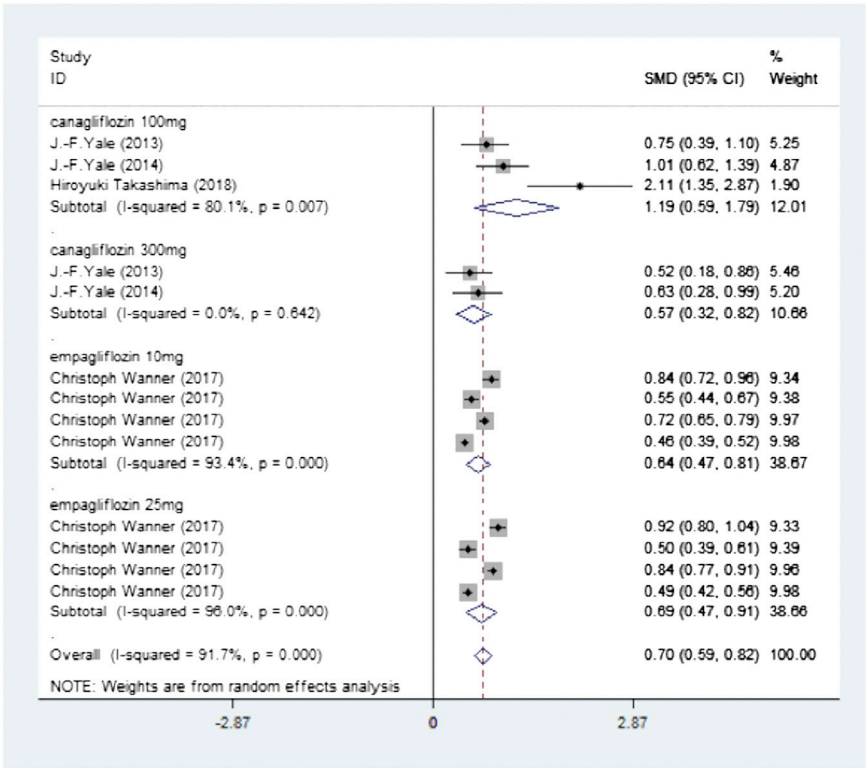

FIGURE 3 | Meta-analysis and forest plot of hemoglobin levels in the experimental and the control groups.

model was used when heterogeneity was >50% (Mantel and Haenszel, 1959; DerSimonian and Laird, 1986). A sensitivity analysis was performed to reveal the influence of a single study on the overall pooled estimates by deleting one study in each turn. Publication bias was evaluated using the Begg's and Egger's tests (Begg and Mazumdar, 1994; Egger et al., 1997). *p* values (<0.05) were considered to represent statistically significant publication bias.

RESULTS

Description of the Studies

A total of 579 references were retrieved and finally, four studies (Yale et al., 2013; Yale et al., 2014; Wanner et al., 2018; Takashima et al., 2018) met the inclusion criteria for the meta-analysis (**Figure 1**). The sample size included 19,259 patients with T2DM and chronic kidney disease in this meta-analysis (**Table 1** Characteristics of the included studies). Three of the studies were multi-center, double-blind, placebo-controlled trials (Yale et al., 2013; Yale et al., 2014; Wanner et al., 2018) in which white people took up the majority of patients and one was a single-center, open-label, parallel-group trial conducted in Japan (Takashima et al., 2018). The types of SGLT2 inhibitors included canagliflozin (100 mg/300 mg) and enpagliflozin (10 mg/25 mg). Follow-up duration ranged from 12 to 164 weeks. All included studies were evaluated in terms of the risk of bias using the Cochrane risk of bias tool and the details are illustrated in **Figure 2** (Risk of bias in the included studies).

Risk of Bias

With the exception of one open-label study, the other three studies on random sequence generation were fully considered. The studies were all double-blind trials but there was no further explanation on the details of allocation concealment. There were no incomplete outcomes and selective reporting in the four studies. Based on the characteristics, we believe that the included studies had a low risk of bias.

Effects of Interventions on Hemoglobin Levels

Four studies (Four publications) investigated a total of 19,259 participants (experimental group: 9,668, control group: 9,591) and reported hemoglobin levels. There was high heterogeneity ($I2 = 91.7\%$, $p < 0.0001$) and so the random-effects model was used. The pooled effect size showed significant differences in hemoglobin levels (SMD = 0.70, 95% CI, 0.59–0.82, $p < 0.0001$) in favor of the experimental groups compared to the control groups (**Figure 3** Meta-analysis and forest plot of hemoglobin levels for experimental group compared with the control group).

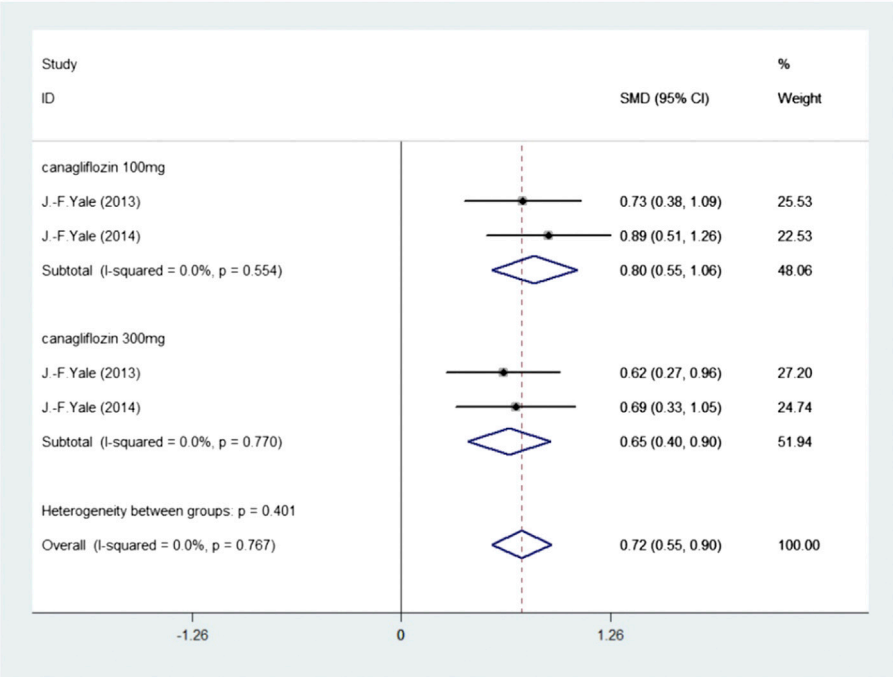

FIGURE 4 | Meta-analysis and forest plot of Hematocrit for experimental group compared with control group.

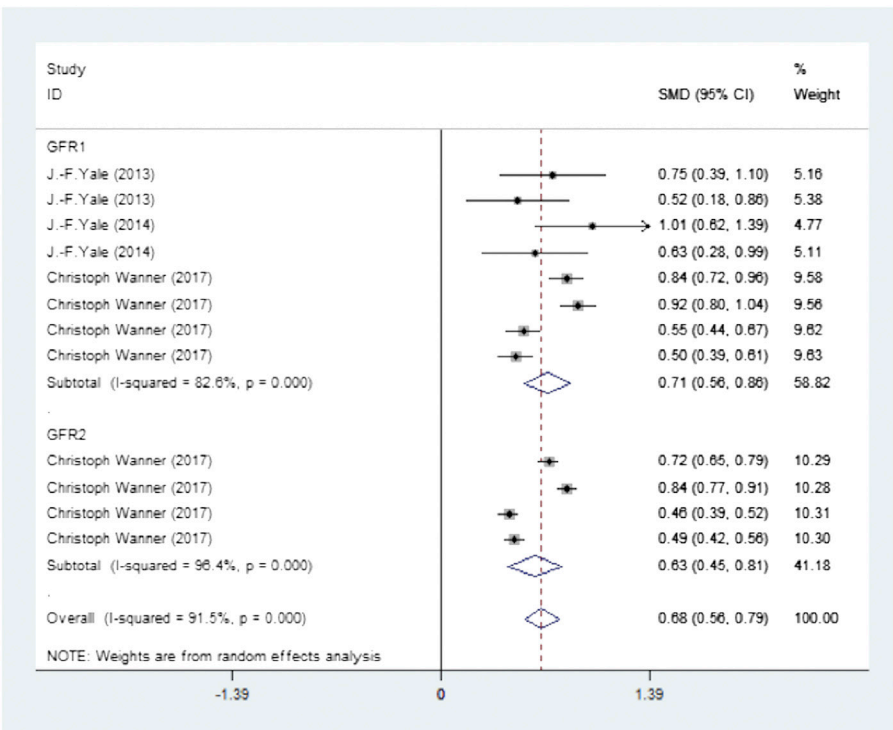

FIGURE 5 | Meta-analysis and the forest plot data of hemoglobin levels in the subgroup analysis.

TABLE 1 | Summary of the characteristics of the included studies.

First author, Year	RCT or not	Inclusion criteria	Age, Years (T/C)	SGLT2i dosing	Comparison	Period of treatment	Treatment group			Control group		
							Sample size (n)	Hemoglobin outcome (M ± SD)	Hematocrit outcome (M±SD)	Sample size (n)	Hemoglobin outcome (M ± SD)	Hematocrit Outcome (M±SD)
Yale et al. (2013)	Y	T2DM; HbA1c ≥ 7.0, while ≤10.5%; eGFR ≥30, while <50 ml/min/1.73 m^2	69.5/68.2	Canagliflozin 100 mg QD	Placebo control	26-weeks	69	5.3 ± 7.4	6 ± 7.6	62	−0.5 ± 8.1	−0.1 ± 9.1
		T2DM; HbA1c ≥ 7.0, while ≤10.5%; eGFR ≥30, while <50 ml/min/1.73 m^2	67.9/68.2	Canagliflozin 300 mg QD	Placebo control	26-weeks	76	3.1 ± 5.9	4.8 ± 6.9	62	−0.5 ± 8.1	−0.1 ± 9.1
Yale et al. (2014)	Y	T2DM; HbA1c ≥ 7.0, while ≤10.5%; eGFR ≥30, while <50 ml/min/1.73 m^2	69.5/68.2	Canagliflozin 100 mg QD	Placebo control	52-weeks	62	6.5 ± 7.9	6.6 ± 8.5	57	−1.4 ± 7.8	−0.9 ± 8.4
		T2DM; HbA1c ≥ 7.0, while ≤10.5%; eGFR ≥30, while <50 ml/min/1.73 m^2	67.9/68.2	Canagliflozin 300 mg QD	Placebo control	52-weeks	70	4.2 ± 9.6	5.9 ± 10.9	57	−1.4 ± 7.8	−0.9 ± 8.4
Wanner et al. (2017)	Y	T2DM; HbA1c ≥ 7.0, while ≤10%; eGFR ≥30, while <60 ml/min/1.73 m^2	66.2/66.0	Empagliflozin 10 mg QD	Placebo control	12-weeks	605	5.7 ± 7.4	——	607	−0.5 ± 7.4	——
		T2DM; HbA1c ≥ 7.0, while ≤10%; eGFR ≥30, while <60 ml/min/1.73 m^2		Empagliflozin 25 mg QD	Placebo control	12-weeks	607	6.3 ± 7.4	——	607	−0.5 ± 7.4	——
		T2DM; HbA1c ≥ 7.0, while ≤10%; eGFR ≥30, while <60 ml/min/1.73 m^2	66.2/66.0	Empagliflozin 10 mg QD	Placebo control	164- week	605	6.2 ± 14.8	——	607	−2 ± 14.8	——
		T2DM; HbA1c ≥ 7.0, while ≤10%; eGFR ≥30, while <60 ml/min/1.73 m^2		Empagliflozin 25 mg QD	Placebo control	164- week	607	5.4 ± 14.8	——	607	−2 ± 14.8	——
		T2DM; HbA1c ≥ 7.0, while ≤10%; eGFR ≥60 ml/min/ 1.73 m^2	61.6/61.9	Empagliflozin 10 mg QD	Placebo control	12- week	1740	6 ± 8.3	——	1726	0 ± 8.3	——
		T2DM; HbA1c ≥ 7.0, while ≤10%; eGFR ≥60 ml/min/ 1.73 m^2		Empagliflozin 25 mg QD	Placebo control	12- week	1733	7 ± 8.3	——	1726	0 ± 8.3	——
		T2DM; HbA1c ≥ 7.0, while ≤10%; eGFR ≥60 ml/min/ 1.73 m^2	61.6/61.9	Empagliflozin 10 mg QD	Placebo control	164- week	1740	5.3 ± 16.7	——	1726	−2.3 ± 16.6	——
		T2DM; HbA1c ≥ 7.0, while ≤10%; eGFR ≥60 ml/min/ 1.73 m^2		Empagliflozin 25 mg QD	Placebo control	164- week	1733	5.9 ± 16.7	——	1726	−2.3 ± 16.6	——
Takashima et al. (2018)	Y	T2DM; HbA1c <10.0%; eGFR ≥45, while <90 ml/min/1.73 m^2	64.7/65.4	Canagliflozin 100 mg QD	Usual care	52-weeks	21	8 ± 6	——	21	−2 ± 3	——

T2DM, type 2 diabetes mellitus; eGFR, estimated glomerular filtration rate; RCT, randomized controlled trial; C, control group; T, treatment group; QD, once a day; SGLT2i, sodium glucose co-transporter 2 inhibitors; HbA1c, hemoglobin A1c; M ± SD, mean ± standard deviation.

Effects of Interventions on Hematocrit

We further analyzed the effect of SGLT2 inhibitors on hematocrit. Two publications investigated 515 participants (experimental group: 277, control group: 238) reported Hematocrit. There was no heterogeneity ($I2 = 0$, $p = 0.767$); thus, the fixed-effects model was used. The pooled effect size showed a significant difference in Hematocrit (SMD = 0.72, 95% CI 0.55–0.90, $p < 0.05$) in favor of experimental group, compared with the control group (**Figure 4** Meta-analysis and forest plot of Hematocrit for experimental group compared with control group).

Subgroup Analysis

To explore the sources of heterogeneity, we conducted a subgroup analysis of the type and dosage of SGLT2 inhibitors and eGFR. According to the type and dosage of the drug, treatments were divided into four subgroups (canagliflozin 100 mg, canagliflozin 300 mg, empagliflozin 10 mg and empagliflozin 25 mg). Subgroups were divided as to eGFR based on 30 ≤ eGFR <60 ml/min/1.73 m^2 and eGFR ≥60 ml/min/1.73 m^2. The results are summarized in **Figure 5**. All of the results in the subgroups were statistically significant compared to those in the control group, however, heterogeneity was not significantly reduced following subgroup analysis.

Sensitivity Analysis

A sensitivity analysis was conducted by sequentially removing one study to observe the influence of each of the included studies on the overall pooled SMD. No single study was found to significantly influence the overall pooled SMD indicating that the results were stable.

Publication Bias

In assessing publication bias, a funnel plot for the four studies analyzed was constructed. The shape of the funnel plot was symmetrical indicating the absence of publication bias. No significant bias was observed using the Begg's rank correlation test ($Z = 1.4$, $p = 0.161$ (>0.05)) and Egger's linear regression test ($t = 0.78$, $p = 0.451$ (>0.05)).

DISCUSSION

This article conducted a meta-analysis of four randomized controlled studies to explore the effects of SGLT2 inhibitors on hemoglobin levels in diabetic patients with chronic kidney disease. The results showed that the hemoglobin levels of patients after treatment with SGLT2 inhibitors increased from baseline and the differences were statistically significant. The hematocrit levels of patients after treatment with SGLT2 inhibitors increased from baseline and the differences were statistically significant. Whether it was different type and dosage of the drug (canagliflozin 100 mg, canagliflozin 300 mg, empagliflozin 10 mg and empagliflozin 25 mg), or different eGFR (30 ≤ eGFR <60 ml/min/1.73 m^2 and eGFR ≥60 ml/min/1.73 m^2), the differences were statistically significant.

Comparison With Other Published Studies

Most of the observations from previous reports in the literature and meta-analysis demonstrate the effects of SGLT2 on blood sugar levels, cardiovascular events and renal outcomes. There are very few studies that have analyzed the effects of SGLT2 inhibitors on hemoglobin levels or have performed meta-analyses of these effects across multiple RCTs.

Mechanisms

SGLT2 inhibitors protect patients with T2DM and chronic kidney disease through several different mechanisms. First, in diabetic patients, upregulation of SGLT2 increases the reabsorption of sodium and glucose by the proximal tubules, SGLT2 inhibitors lower blood sugar by blocking the glucose reabsorption of SGLT2 in the proximal renal tubules. Second, SGLT2 inhibitors also have a certain effect on renal hemodynamics. SGLT2 inhibitors block the reabsorption of glucose and sodium in the proximal tubules and increase the transport of sodium to the macula densa, thereby restoring impaired tubuloglomerular feedback. Thus, SGLT2 inhibitors can alleviate glomerular filtration in the early stage of diabetic nephropathy, reduce albuminuria, and delay the decline of renal function for a long time (Cherney et al., 2014; Škrtić and Cherney, 2015). Besides, the protective effects of SGLT2 inhibitors are also manifested in the reduction of blood pressure, weight loss, osmotic diuresis, reduction of inflammation, fibrosis, and proliferation of proximal renal tubular cells (Panchapakesan et al., 2013).

The mechanisms by which SGLT2 inhibitors improve hemoglobin levels in patients with diabetes and chronic kidney disease are not fully understood. It has been reported that SGLT2 inhibitors have diuretic-like effects and reduce plasma volume (Lambers Heerspink et al., 2013). It has also been reported that in diabetic patients with normal renal function, SGLT2 inhibitors can reduce the load caused by excessive glucose reabsorption in the proximal tubules, and can improve renal tubular interstitial hypoxia and restore fibroblasts to produce erythropoietin (EPO) causing hemoglobin levels to increase (Lambers Heerspink et al., 2013). In diabetic patients with chronic kidney disease, SGLT2 inhibitors can also increase hemoglobin levels by promoting the production of EPO. Studies have also shown that SGLT2 inhibitors can upregulate AMPK and SIRT1 (Swe et al., 2019; Packer 2020), thereby inhibiting HIF-1α and activating HIF-2α (Treins et al., 2006; Dioum et al., 2009; Lim et al., 2010). HIF-2α is the isoform responsible for the synthesis of EPO (Eckardt and Kurtz, 2005). The increase in hematocrit may be due to the decrease in plasma volume caused by SGLT2 inhibitor-related diuresis and natriuresis, or it may be due to increased erythropoiesis after the treatment of SGLT2 inhibitor. The increase in hematocrit during treatment with SGLT2 inhibitors may indicate the improvement of hypoxia and oxidative stress in the tubular interstitial area of the renal cortex, as well as the recovery of EPO production by interstitial fibroblast-like cells. SGLT2 inhibitors also inhibit hepcidin, which may lead to increased iron bioavailability and utilization and increased red blood cell production (Ghanim et al., 2020). These effects on erythropoiesis suggest that SGLT2 inhibitors may reduce the

incidence of anemia. The post-hoc analysis of the CREDENCE trial by Megumi Oshima et al. found that the risk of anemia or the risk of starting anemia treatment in the anemia group of patients with type 2 diabetes and chronic kidney disease was significantly lower than that of the placebo group (Oshima et al., 2020). In the exploratory analysis of EMPA-REG test data, the increase in hematocrit during empagliflozin treatment was closely related to beneficial cardiovascular outcomes (Inzucchi et al., 2018). Studies have shown that increased expression of HIF-2α in cardiomyocytes can protect mitochondrial integrity and prevent experimental ischemic damage (Bautista et al., 2009; Mastrocola et al., 2016). For the same blood flow, a higher hematocrit is expected to deliver more oxygen to the tissue (Testani et al., 2010). It has been suggested that the increase in hematocrit may contribute to the cardioprotective effect of these drugs by increasing the oxygen-carrying capacity (Ferrannini et al., 2016; Lytvyn et al., 2017).

Limitations

Several limitations of this study should be noted. Firstly, a total of four articles were included in the analysis which is a small sample size, however, the total number of patients included was nor large. Secondly, the majority of the subjects were Caucasian and so the applicability of the data to other races including Asians requires further investigation. Thirdly, although we concluded that hemoglobin levels increased after treatment with SGLT2 inhibitors, we did not observe differences in the effects of different types of SGLT2 inhibitors on hemoglobin levels, the relationship between the increase in hemoglobin level and the duration of medication. Fourthly, among the populations included in the study, some had eGFR ≥ 60 ml/min/1.73 m^2. This meant that a small number of patients with normal renal function may have been included. In addition, the population included in the study did not have obvious renal anemia before SGLT2 inhibitors treatment. Therefore, for patients with significant renal anemia, the benefits of SGLT2 inhibitors need to be further investigated.

In summary, patients with T2DM and chronic kidney disease have increased hemoglobin and hematocrit levels after treatment with SGLT2 inhibitors. SGLT2 inhibitors may bring additional benefits to patients with T2DM and chronic kidney disease.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

WQ and LY designed the study. TX and BT identified and acquired reports of trials, WQ extracted the data and performed all data analyses. XL contributed to data interpretation. WQ drafted the report and all other authors critically reviewed and approved the final manuscript.

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frontiers in Pharmacology

MINI REVIEW
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Improving the Dysregulation of FoxO1 Activity Is a Potential Therapy for Alleviating Diabetic Kidney Disease

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A substantial proportion of patients with diabetes will develop kidney disease. Diabetic kidney disease (DKD) is one of the most serious complications in diabetic patients and the leading cause of end-stage kidney disease worldwide. Although some mechanisms have been revealed to contribute to the understanding of the pathogenesis of DKD and some drugs currently in use have been shown to be beneficial, prevention and management of DKD remain tricky and challenging. FoxO1 transcriptional factor is a crucial regulator of cellular homeostasis and posttranslational modification is a major mechanism to alter FoxO1 activity. There is increasing evidence that FoxO1 is involved in the regulation of various cellular processes such as stress resistance, autophagy, cell cycle arrest, and apoptosis, thereby playing an important role in the pathogenesis of DKD. Improving the dysregulation of FoxO1 activity by natural compounds, synthetic drugs, or manipulation of gene expression may attenuate renal cell injury and kidney lesion in the cells cultured under a high-glucose environment and in diabetic animal models. The available data imply that FoxO1 may be a potential clinical target for the prevention and treatment of DKD.

Keywords: forkhead box O1, diabetic kidney disease, posttranslational modification, sirtuin-1, oxidative stress

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INTRODUCTION

Diabetic kidney disease (DKD), one of the common complications related to both types of diabetes, occurs in approximately 30–40% of diabetic patients and is the main cause of end-stage renal disease worldwide (Gnudi et al., 2016; Bonner et al., 2020; Chen et al., 2020). Renal enlargement and increased glomerular filtration rate are the initial changes of kidneys in diabetes. The earliest symptom is often albuminuria, which can develop into nephrotic-range proteinuria with morphological abnormalities such as glomerular hypertrophy, glomerular basement membrane (GBM) thickening, and extracellular matrix (ECM) expansion. Progressive glomerulosclerosis from nodular (Kimmelstiel-Wilson lesion) to global and tubulointerstitial fibrosis contributes to progressive loss of renal function in advanced DKD (Fioretto and Mauer, 2010; Tervaert et al., 2010; Badal and Danesh, 2014). The pathogenesis of DKD is multifactorial. The major pathophysiologic mechanisms contributing to glomerulopathy and tubulointerstitial lesions and the related morphological alterations are exhibited in **Figure 1**. Intensive management of patients with DKD including control of blood glucose and blood pressure, blockade of the renin-angiotensin-aldosterone system (RAAS), and inhibition of the sodium-glucose cotransporter 2 (SGLT2) may slow the progression of the disease. However, owing to the intricate pathogenesis of DKD, there is still no effective treatment to prevent the onset and to arrest the progression of the disease (Stanton, 2014; Thomas et al., 2015; Kidney Disease:

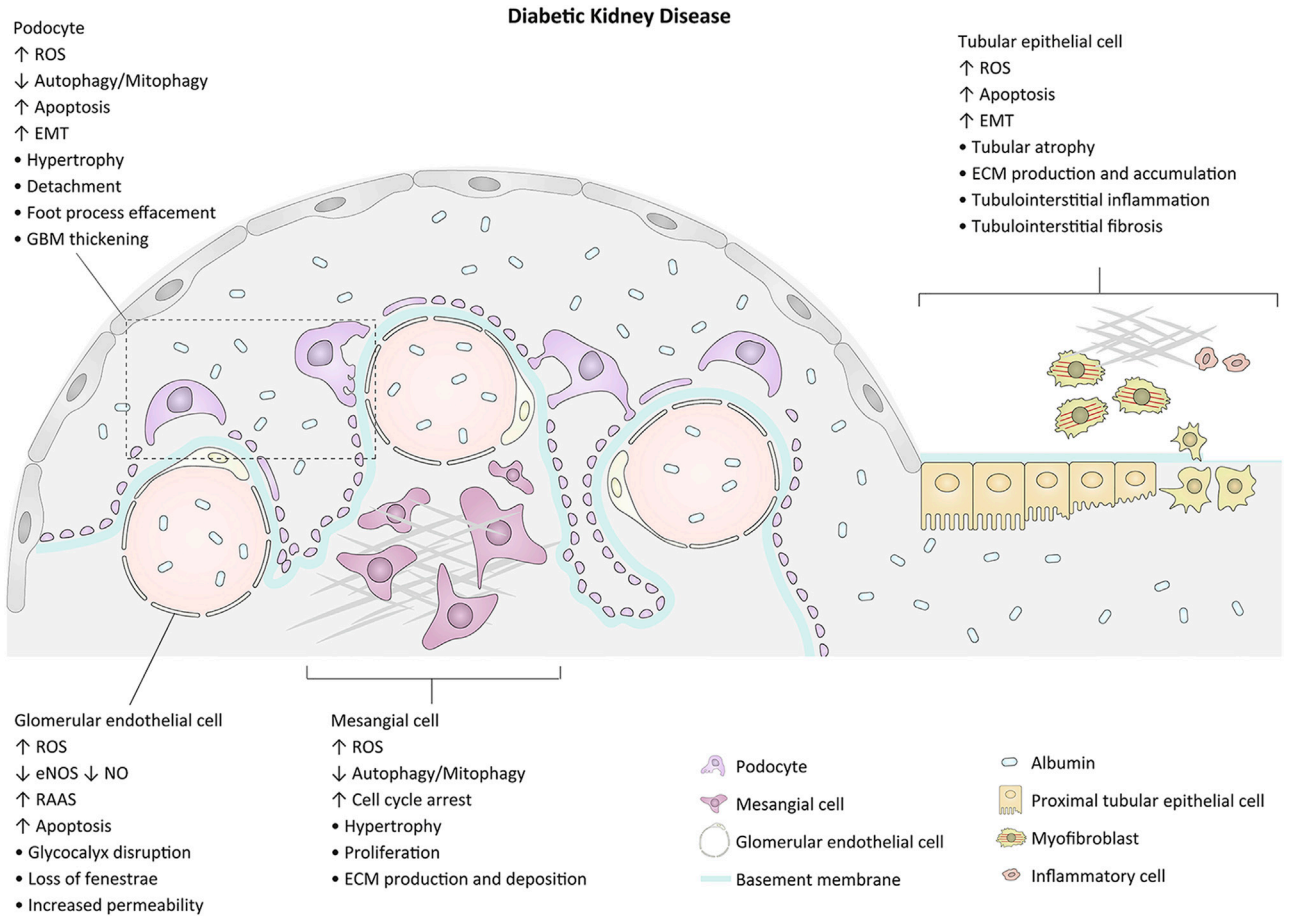

FIGURE 1 | Major events and morphological changes related to the pathogenesis of glomerulopathy and tubulointerstitial lesion in diabetes. GBM, glomerular basement membrane; EMT, epithelial-mesenchymal cell transformation; ROS, reactive oxygen species; ECM, extracellular matrix.

Improving Global Outcomes Diabetes Work, 2020). Therefore, exploring the underlying mechanisms of renal impairment in the pathophysiological state of diabetes will be helpful to identify possible intervention targets and develop promising therapeutic strategies for DKD.

Forkhead box O (FoxO) transcription factors are essential modulators of cellular homeostasis. FoxO proteins respond to various external stimuli, including nutrient deprivation, growth factor signaling, oxidative stress (OS), and genotoxic stress. These input signals influence FoxOs intracellular localization, DNA binding, and interactions with other cofactors via a series of posttranslational modifications (PTM) containing phosphorylation, acetylation, ubiquitination, and methylation. Through integrating these modifications, FoxOs regulate cell-type-specific gene expression programs to respond to stress, maintain metabolic homeostasis, and balance redox (Tothova et al., 2007; Link and Fernandez-Marcos, 2017; Murtaza et al., 2017; Brown and Webb, 2018). There is growing evidence that, via the downstream target genes that are involved in the regulation of a variety of cellular processes such as energy metabolism, stress resistance, apoptosis, autophagy, and cell cycle arrest, FoxOs play a crucial role in the molecular mechanisms of DKD development, among which FoxO1 is the most extensively studied. This review summarizes our current perspectives on the regulation of FoxOs activity and the physiological functions of FoxO1, highlighting evidence to support the notion that dysregulated FoxO1 activity contributes toward renal parenchymal cell damage in the pathogenesis of DKD.

The Regulation of FoxO Activity

There are four different FoxO transcription factors in mammals including FoxO1, FoxO3a, FoxO4, and FoxO6, which belong to the family of forkhead proteins. Each FoxO protein consists of four regions: a DNA-binding domain at N-terminal, a transactivation domain at C-terminal, a nuclear export sequence, and a nuclear localization sequence. All FoxO proteins share a common highly conserved DNA-binding

domain, while other domains are enriched in sites for PTM and protein-protein interactions and are specific for the unique members (Tothova et al., 2007; Arden, 2008; Link and Fernandez-Marcos, 2017). In cells receiving survival or growth factor signal, the activity of FoxO protein is downregulated, primarily by being sequestered in the cytoplasm of the cell (Brunet et al., 1999; Brunet et al., 2002). The decrease in the level of FoxO protein may result from increased proteasomal degradation (Aoki et al., 2004). Upregulation of FoxO activity is by increased mRNA stability and expression as well as chromosomal rearrangement causing fusion of FoxO transactivation domain with DNA-binding domain of other transcription factors (Fritz and Radziwill, 2011).

The activity of FoxO protein is largely regulated by PTM that has been recognized as a crucial mechanism for the alteration of FoxO activity. Phosphorylation of FoxO by serine/threonine kinase Akt or serum- and glucocorticoid-induced kinase (SGK) exposes the nuclear export sequence and increases FoxO translocation to the cytoplasm, and the cytoplasmic sequestration or ubiquitination and subsequent proteasomal degradation inhibit FoxO activity (Brunet et al., 1999; Zhao et al., 2004; Huang et al., 2005; Huang and Tindall, 2011; Tzivion et al., 2011; Saline et al., 2019). Conversely, specific phosphorylation of FoxO by kinase mammalian sterile 20-like kinase 1 (MST1) or c-Jun N-terminal kinase (JNK) regulates FoxO activity in the opposite direction (Lehtinen et al., 2006; Densham et al., 2009; Brown and Webb, 2018). Reversible acetylation of FoxO regulates its activity as a second modulation layer. The acetylation of FoxO by cAMP-response element-binding protein (CREB)-binding protein (CBP), p300 or p300/CBP-associated factors (PCAF), and the subsequent deacetylation by class I and II histone deacetylases including the nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase sirtuin-1 (Sirt1) alter the transcriptional activity of FoxO (Imai et al., 2000; Perrot and Rechler, 2005; Haigis and Sinclair, 2010). Acetylated FoxO is retained in the nucleus for engaging Sirt1, while deacetylation of FoxO by Sirt1 promotes FoxO transcriptional activity and accelerates FoxO degradation (Kitamura et al., 2005). Acetylation reduces the DNA-binding capacity of FoxO protein and enhances Akt-dependent phosphorylation of FoxO, suggesting the interplay between different PTM in regulating FoxO activity. Phosphorylation-dependent nuclear exclusion and deacetylation-dependent nuclear retention synergistically regulate the activity of FoxO protein, and each PTM may affect another (Matsuzaki et al., 2005; Qiang et al., 2010).

The Physiological Function of FoxO1

FoxO binds via the DNA-binding domain to the same consensus binding site (5'-*TTGTTTAC*-3') within the promoter of its target gene. FoxO-DNA affinity differs between response element and PTM (Brent et al., 2008). The combination of FoxO with different sets of genes in different tissues results in a diversity of FoxO-mediated biological effects. The physiological functions of different FoxO proteins are not identical (Link and Fernandez-Marcos, 2017; Murtaza et al., 2017; Brown and Webb, 2018).

FoxO1, highly expressed in insulin-responsive tissues such as liver, adipose tissue, skeletal muscle, and pancreas, coordinates transcriptional cascades to modulate glucose metabolism and is therefore considered as a major governor of insulin signaling and glucose homeostasis. As a final effector of the insulin signaling pathway, FoxO1 responds in general to decreased nutrients by inducing gluconeogenesis in the liver, inhibiting adipocyte and myocyte differentiation, or shifting fuel utilization in muscle from glucose to lipids (Dong et al., 2008; Kousteni, 2012). In the absence of growth factor or insulin signaling or with stress stimuli, FoxO1 resides in the nucleus and is active as a transcription factor that governs apoptosis, autophagy, cell cycle arrest, stress resistance, and immune response. The program of gene expression transcriptionally regulated by FoxO1 ordinarily protects cells from the life-threatening consequences of nutrient, oxidative, or genotoxic stress (Dong et al., 2008; Murtaza et al., 2017).

Dysregulation of FoxO1 Activity Is Involved in the Pathogenesis of Diabetic Kidney Disease

Several studies suggest that genetic variation in the FoxO1 gene is a predisposing factor for type 2 diabetes (T2D) or DKD in humans, revealing that FoxO1 may be involved in the initiation and development of DKD in patients with T2D, which provides new insight into the etiology of DKD (Müssig et al., 2009; Gong et al., 2017; Zhao et al., 2017).

Studies for investigating the protective effects of certain oral hypoglycemic drugs or natural compounds on the kidneys in diabetic animal models demonstrate that FoxO1 is an important target. Xu et al. reported that puerarin, a natural isoflavone from *Pueraria lobata* (Wild.), upregulated the expression of Sirt1, peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), and FoxO1 in renal cortex from type 1 diabetic (T1D) mice. Puerarin reduced reactive oxygen species (ROS) and increased the activity of manganese superoxide dismutase (Mn-SOD) and catalase (CAT), accompanied by attenuated kidney tissue damage. These findings suggest that puerarin exerts renal protection effect on DKD through the Sirt1-PGC-1α/FoxO1 pathway (Xu et al., 2016). In a T2D rat model, liraglutide, a glucagon-like peptide-1 agonist, markedly reduced renal damage including the production of ECM proteins. Liraglutide inhibited the phosphorylation of FoxO1 and increased Mn-SOD expression in the diabetic kidneys. It seems that liraglutide exerts a protective effect on early DKD by a FoxO1-mediated upregulation of renal Mn-SOD (Chen et al., 2018). Hussein et al. reported that treatment of DKD rats with the Sirt1 agonist resveratrol increased superoxide dismutase (SOD) activity and reduced malondialdehyde (MDA), collagen (Col) IV, and fibronectin (FN) expression by increasing FoxO1 activity (Hussein and Mahfouz, 2016).

In addition to these *in vivo* studies, many studies have investigated the effect of the change in FoxO1 activity on renal parenchymal cell injury in diabetic conditions by *in vitro* and *in vivo* experiments, as shown below by the different cell types studied.

Podocyte

Podocyte is one of the components of the glomerular filtration barrier (GFB) and plays an essential role in maintaining the integrity of GFB. As a terminal differentiated atypical epithelial cell, podocyte cannot regenerate after suffering from injury and apoptosis (Quaggin and Kreidberg, 2008). Under a diabetic environment, the podocyte often undergoes hypertrophy, epithelial-mesenchymal cell transformation (EMT), apoptosis, and detachment, leading to the impairment and destruction of GFB, which becomes a crucial constituent in the development of DKD (**Figure 1**) (Fioretto and Mauer, 2010; Tervaert et al., 2010; Oh et al., 2012; Dai et al., 2017).

The glomerular insulin signaling is critical for GFB integrity and normal kidney function (Welsh et al., 2010), and podocyte is a unique insulin-responsive cell in the GFB (Coward et al., 2005). The insulin-dependent phosphorylation of Akt was impaired in the podocytes from diabetic mice at the onset of albuminuria. Dysregulation of Akt phosphorylation and subsequent FoxO1 phosphorylation in podocytes was associated with its susceptibility to apoptosis, suggesting that the inability of podocyte to respond to insulin partially accounts for the decreased podocyte number seen in early DKD (Tejada et al., 2008; Katsoulieris et al., 2016).

Several studies investigated the effect of FoxO1 on protecting podocytes from injury in diabetes. The transcriptional activity of FoxO1 decreased in the kidney from type 1 diabetic rodents induced by streptozotocin (STZ) and the podocytes cultured under high-glucose (HG) condition (Guo et al., 2015; Du et al., 2016; Li et al., 2016; Li et al., 2017). Guo et al. found that overexpressing FoxO1 by injection of recombinant lentivirus into the renal cortex decreased albuminuria and serum urea nitrogen and creatinine levels, preserved podocalyxin and nephrin expression, and ameliorated pathological changes in the glomerulus of diabetic kidneys, suggesting the protective effect of FoxO1 on podocyte injury (Guo et al., 2015). Li et al. reported that upregulation of FoxO1 activity reversed HG-dependent downregulation of PTEN-induced putative kinase 1 (PINK1), an important functional protein in mitophagy, which suggests that, through downstream PINK1/Parkin pathway, FoxO1 limits the production of ROS under HG conditions and maintains mitochondrial morphology and stability, thus playing a crucial role in the protection against mitochondrial dysfunction and podocyte apoptosis (Li et al., 2016; Li et al., 2017). Du et al. found that constitutive FoxO1 activation suppressed HG-induced activation of the transforming growth factor (TGF)-β1/Smad3/integrin-linked kinase (ILK) pathway and thus partially reversed podocyte EMT (Du et al., 2016).

A number of studies have revealed that the renoprotective effect of certain drugs used to treat diabetes or some protein molecules is FoxO1-mediated. For example, FoxO1 was identified as a target of microRNA (miR)-21, and the upregulation of miR-21 in podocytes cultured under HG conditions inhibited the expression of FoxO1, thus attenuating autophagy and promoting apoptosis (Wang et al., 2019a). While Atrasentan, an endothelin-1 receptor antagonist (Egido et al., 2017), could enhance FoxO1 expression by downregulating miR-21 and thereby attenuate HG-induced podocyte injury and hamper the progression of DKD (Wang et al., 2019a). In another study, progranulin (PGRN), a secreted glycoprotein, attenuated mitochondrial damage and dysfunction in the podocytes treated with HG. Since PGRN induced the expression of Sirt1 and reduced the acetylation levels of PGC-1α and FoxO1 in HG-treated podocytes, it suggests that PGRN modulates mitochondrial biogenesis and mitophagy through Sirt1-PGC-1α/FoxO1 signaling and thus protects against podocyte injury in DKD (Zhou et al., 2019).

Mesangial Cell

Mesangial cell (MC) plays an important role in maintaining the structural integrity of glomerular capillary and mesangial matrix homeostasis. MC also can regulate filtration surface area and phagocytose apoptotic cells or immune-complexes. MC hypertrophy and mesangial matrix expansion are among the earliest pathological features of DKD. MCs are primary targets of diabetes and they respond differently to a diabetic environment, where some of them acquire an activated phenotype undergoing hypertrophy and proliferation with excessive production of matrix proteins, growth factors, chemokines, and cytokines, whereas others undergo apoptosis (**Figure 1**) (Abboud, 2012).

Das et al. found that HG induced Akt-dependent phosphorylation of FoxO1, and dominant-negative FoxO1 increased the phosphorylation of Akt. CAT blocks HG-stimulated Akt phosphorylation to inhibit the inactivation of FoxO1 and PRAS40, leading to the inhibition of mTORC1 activity. In contrast, HG-inactivated FoxO1 decreased CAT expression, leading to an increase in ROS production, mTORC1 activation, MC hypertrophy, and FN and PAI-1 expression. These findings suggest the existence of a positive feedback loop involving sustained Akt activation, FoxO1 inactivation, decreased CAT expression, and increased ROS, resulting in mTORC1 activation, MC hypertrophy, and matrix excessive production (Das et al., 2014).

Wu et al. reported that HG-induced FoxO1 inhibition and relevant PGC-1α downregulation were accompanied by mitochondrial dysfunction and increased ROS generation, whereas constitutive FoxO1 activation increased PGC-1α expression and partially reversed these changes in MCs. PGC-1α was identified as a direct transcriptional target of FoxO1. Overexpression of FoxO1 in diabetic rat kidneys significantly increased the expression of PGC-1α, mitochondrial-related transcription factor (Nrf1), and mitochondrial fusion protein (Mfn2) and decreased MDA production and proteinuria. These findings suggest that the activation of FoxO1/PGC-1α attenuated HG-induced mitochondrial dysfunction and MC injury (Wu et al., 2015).

Guo et al. found that HG-elevated p-Akt level and subsequent alleviation of FoxO1 activity were accompanied by the downregulation of CAT and SOD2 mRNA expression, activation of TGF-β/Smad signaling, and increases in the protein expression of FN and Col I in MCs. Conversely, overexpression of nucleus-localized FoxO1 upregulated the expression of antioxidative enzymes, accompanied by inhibition of TGF-β/Smad3 signaling and a decrease in the expression of ECM proteins. This study suggests that the antioxidative effect mediated by FoxO1 may play a crucial role in attenuating TGF-β-induced ECM production in MCs under an HG environment (Guo et al., 2016).

Fiorentino et al. found that tissue inhibitors of metalloproteinase3 (TIMP3), an inhibitor of ADAM metallopeptidase domain 17 (ADAM17), were reduced in the kidneys from type 1 diabetic mice. In the kidneys of diabetic *Timp3*-deficient mice, the expression of FoxO1 and FoxO1-targeted autophagy-related genes, including Atg5, Atg8, LC3, and Beclin1, was decreased, and the expression of signal transducers and activator of transcription 1 (STAT1), a repressor of FoxO1 transcription, was increased. Renal biopsy of patients with DKD showed similar data. Knockdown of TIMP3 in the MCs cultured under an HG environment led to the downregulation of FoxO1 and FoxO1-targeted autophagy-related genes and an increase in the LC3II/I ratio. This study suggests that the reduction of autophagy, especially in MCs, caused by TIMP3 deficiency may deteriorate DKD (Fiorentino et al., 2013).

Liu et al. reported that overexpression of FoxO1 in MCs caused upregulation of p27 and downregulation of cyclin D1 and CDK4, which promoted cell cycle arrest at the G0/G1 phase and attenuated proliferation induced by HG. Degradation of FoxO1 caused a decrease in p27 and stimulated MCs proliferation. These findings suggest that FoxO1 is involved in regulating MCs proliferation induced by HG via FoxO1/p27 signaling (Liu et al., 2014).

In a recent study, metformin effectively attenuated glycolipid metabolic disorders as well as renal damage in a T2D rat model. Mechanistically, metformin relieved OS, enhanced autophagy, and suppressed cell proliferation in cultured MCs stimulated by HG through AMPK/Sirt1-FoxO1 signaling pathway (Ren et al., 2020).

Glomerular Endothelial Cell

The glomerular endothelial cell (GEC), which is highly fenestrated and covered by a rich glycocalyx, participates in the sieving properties of GFB and in the maintenance of podocyte structure. Both a reduction in the thickness of the glycocalyx and a reduction in the fenestration of endothelium are early characteristics of DKD. GEC injury can occur via hemodynamic stimuli that cause reduced nitric oxide (NO) bioavailability via suppression of endothelial nitric oxide synthase (eNOS), or it can result from growth factor driven altered metabolism. As GEC is the first cell encountered by any circulating stimulus relevant to diabetes, it not only is a direct target of diabetes but also serves as cell sending paracrine signals to adjacent MC and podocyte (**Figure 1**) (Dane et al., 2013; Jourde-Chiche et al., 2019).

Carota et al. reported that the expression of vascular endothelial protein tyrosine phosphatase (VE-PTP), which can dephosphorylate tyrosine kinase with Ig and EGF homology domains 2 (TIE2), was robustly upregulated in the GECs in a diabetic mouse model. The reduction of TIE2 signaling due to increased VE-PTP expression under diabetic conditions resulted in decreased eNOS phosphorylation, as well as increased FoxO1 levels and its downstream profibrotic and proinflammatory targets (Carota et al., 2019). In this study, FoxO1 appears to play an opposite role in GECs than in other renal parenchymal cells and reduced transcriptional activity of FoxO1 and subsequent downstream target genes expression may ameliorate GECs injury in diabetic rodents. The mechanism of FoxO1 activity regulation in GEC and the effect of FoxO1 on GEC injury under diabetic conditions need further studies.

Proximal Tubular Epithelial Cell

Although glomerulosclerosis is a major feature of DKD, the severity of tubulointerstitial lesions ultimately determines the extent of renal impairment. Albuminuria, a hallmark of DKD, can activate proximal tubular epithelial cell (PTEC) to evoke tubulointerstitial inflammation (Tang et al., 2003). In addition to albumin, several diabetes-related substrates such as HG, advanced glycation end-products, and angiotensin II may activate a number of signaling pathways including nuclear factor kappa B, extracellular signal-regulated kinase 1/2, p38 mitogen-activated protein kinases, protein kinase C, STAT1, and ROS generation, leading to the accumulation of numerous growth factors, cytokines, chemokines, and adhesion molecules in the interstitium to orchestrate further inflammation and fibrosis (**Figure 1**) (Donadelli et al., 2003; Tang et al., 2003; Tang and Lai, 2012).

Thioredoxin-interacting protein (TXNIP) is a negative regulator of thioredoxin (TRX). TXNIP-TRX has been shown to be an important contributor to the enzyme system involved in ROS production and renal OS (Li et al., 2009; Kibbe et al., 2013). Ji et al. reported that TXNIP and TXN were identified as the direct FoxO1 transcriptional targets, and kidney-specific overexpression of FoxO1 attenuated renal tubular injury by restraining the increase in TXNIP and the decrease in TRX levels in diabetic mice. The study suggests that FoxO1 protects against HG-induced renal PTECs injury through regulating TXNIP-TRX-mediated ROS generation (Ji et al., 2019).

The activation of STAT1, the phosphorylated form of STAT1 (p-STAT1), has been shown to be involved in tubular EMT and tubulointerstitial fibrosis (TIF) in animal models including diabetes (Nakajima et al., 2004; Nightingale et al., 2004). Huang et al. reported that kidney-specific overexpression of FoxO1 significantly downregulated p-STAT1, accompanied by reduced renal damage, apoptosis, and TIF in diabetic mice. Knockdown of FoxO1 in PTECs enhanced the expression of p-STAT1, resulting in EMT and apoptosis, whereas overexpression of FoxO1 markedly inhibited EMT and apoptosis in PTECs under an HG environment. These findings suggest that, partially through STAT1 signaling, FoxO1 plays a protective role against PTECs injury in DKD (Huang et al., 2019).

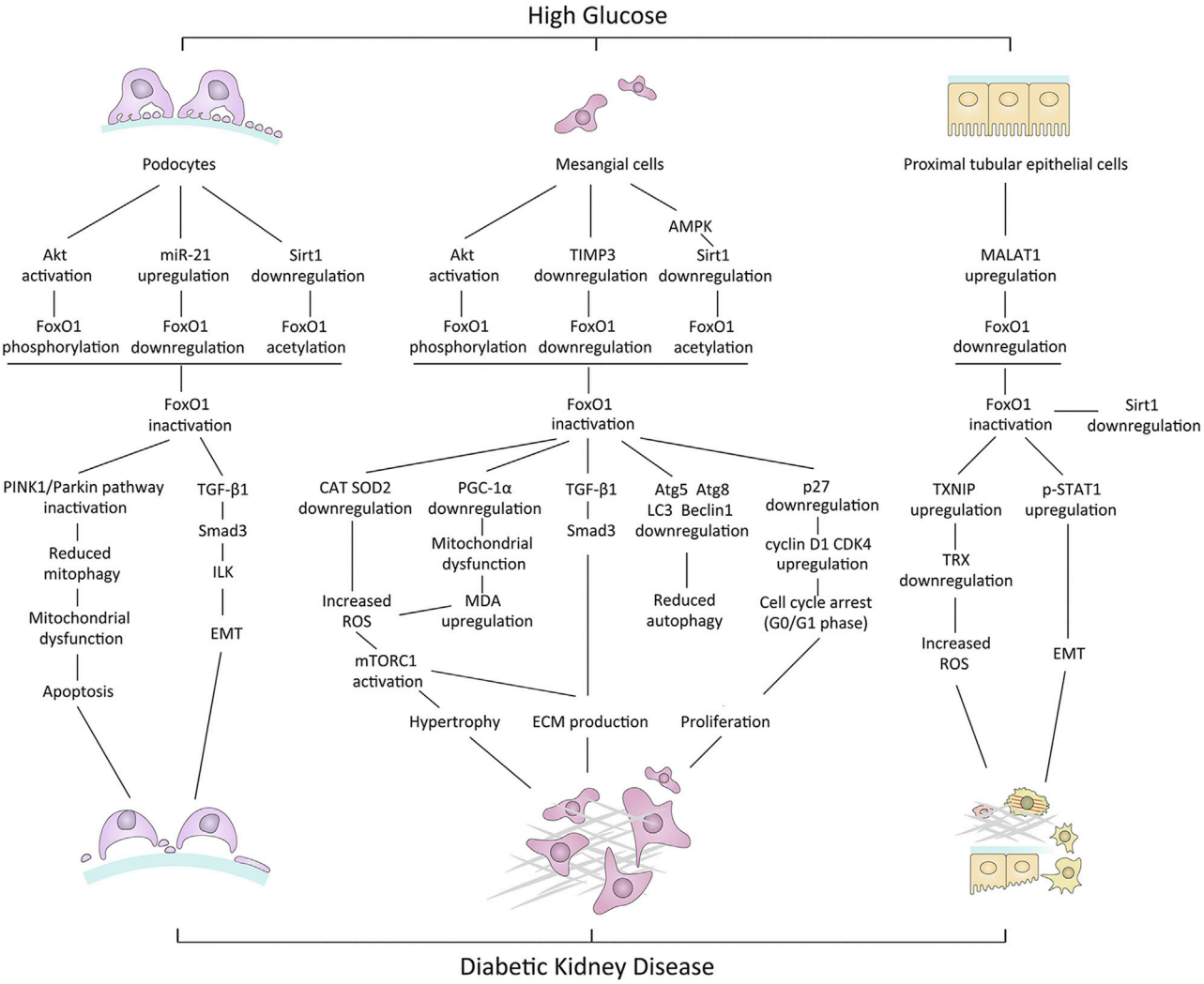

FIGURE 2 | Mechanisms underlying renal cell damage associated with dysregulation of FoxO1 activity in high-glucose conditions. PINK1, PTEN-induced putative kinase 1; TGF-β1, transforming growth factor-beta1; ILK, integrin-linked kinase; EMT, epithelial-mesenchymal cell transformation; TIMP3, tissue inhibitors of metalloproteinase3; CAT, catalase; SOD, superoxide dismutase; MDA, malondialdehyde; ROS, reactive oxygen species; ECM, extracellular matrix; TXNIP, thioredoxin-interacting protein; TRX, thioredoxin; p-STAT1, phosphorylated STAT1.

The effects of Sirt1 on suppressing apoptosis induced by kidney cell injuries, alleviating renal inflammation, improving mitochondrial function, and repressing OS indicate that it is involved in the development of DKD (Dong et al., 2014; Wang et al., 2019b). Zhou et al. reported that HG induced PTECs injury by attenuating the deacetylase activity of Sirt1 (Zhou et al., 2015). Further study showed that metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), which belongs to the long noncoding RNA (lncRNA), was upregulated in the kidney from diabetic mice and in the PTECs cultured with HG, and the expression of Sirt1 was decreased. The interaction between MALAT1 and FoxO1 was promoted by HG. By combination with the promoter of Sirt1, FoxO1 induced Sirt1 transcription, whereas MALAT1 repressed Sirt1 expression by targeting FoxO1. These findings suggest that the interaction between lncRNA MALAT1 and FoxO1 represses the transcription of Sirt1 in PTECs treated with HG and thus promotes HG-induced PTECs injury (Zhou et al., 2018).

DISCUSSION

Metabolic disturbance, mitochondrial dysfunction, OS, inflammation, impaired autophagy, and apoptosis may contribute to diabetic renal cell injury. Data from animal models and cell experiments suggest that the dysregulation of FoxO1 activity may be associated with these cellular processes, leading to kidney damage in the diabetic environment, thereby being involved in the pathogenesis of DKD. Mechanisms underlying renal cell damage associated with dysregulation of FoxO1 activity in HG conditions are summarized in **Figure 2**.

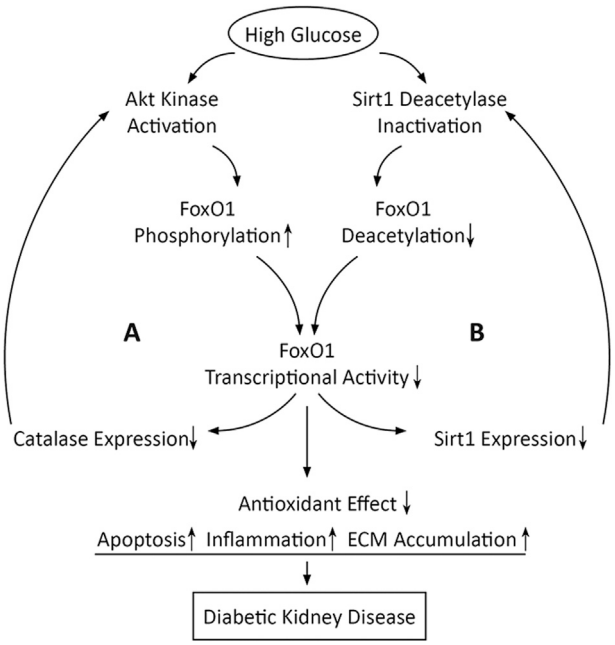

FIGURE 3 | Schematic diagram shows that high glucose may induce two positive feedback loops in which FoxO1 inactivation is involved. Loop A is positive feedback between activation of Akt and inactivation of FoxO1 and loop B is positive feedback between inactivation of Sirt1 and inactivation of FoxO1.

FoxO1 phosphorylation and subcellular localization, leading to improper FoxO1 activity and its target genes transcription (Katsoulieris et al., 2016). Furthermore, the inactivation of FoxO1 owing to Akt-dependent phosphorylation and the phosphorylation of Akt owing to FoxO1 inactivation are mutually reinforcing, which results in a positive feedback between activation of Akt and inactivation of FoxO1 in diabetic conditions (Das et al., 2014). On the other hand, the reduced deacetylase activity of Sirt1 by HG inhibits transcriptional activity of FoxO1 (Wang et al., 2019b), while the decreased transcriptional activity of FoxO1 causes the reduced Sirt1 transcription and expression, which seems also to be positive feedback in diabetic conditions (Zhou et al., 2018) (**Figure 3**).

Based on the majority of the available evidence, dysregulation of FoxO1 activity may reduce the antioxidant effect to respond to OS, leading to apoptosis, inflammation, and ECM accumulation in diabetic kidneys. Given that FoxO1 inactivation induced by high glucose may be enhanced unceasingly even if hyperglycemia is controlled, it is conceivable that the self-reinforcement of the FoxO1 activity dysregulation could be one of the reasons for the progression of kidney damage in patients with DKD who have well levels of blood glucose. Therefore, natural compounds or synthetic drugs that can modulate the activity of FoxO1 could be a novel therapeutic option for alleviating DKD. Potential FoxO1 modulators, their cellular targets, and their effects on cell physiology are summarized in **Table 1**.

TABLE 1 | Potential FoxO1 modulators and their effects in DKD.

Modulators	Cellular targets	Effects on FoxO1-mediated pathway and cell physiology	Experimental model of DKD	References
Puerarin	↑Sirt1 expression	↑PGC-1α/FoxO1 deacetylation ↓ROS ↑Mn-SOD and CAT activity	T1D mice	Xu et al. (2016)
Liraglutide	↓FoxO1 phosphorylation	↑FoxO1 activity ↑Mn - SOD expression ↓ECM production	T2D rats	Chen et al. (2018)
Resveratrol	↑Sirt1 activity	↑FoxO1 deacetylation ↑SOD activity ↓MDA expression ↓Col IV and FN expression	T2D rats	Hussein and Mahfouz (2016)
Atrasentan	↓miR-21 expression	↑FoxO1 expression	Podocytes cultured in HG, T2D (KK-Ay) mice	Wang et al. (2019a)
Progranulin	↑Sirt1 expression	↑PGC-1α/FoxO1 deacetylation ↓Mitophagy ↑Mitochondrial biogenesis	Podocytes cultured in HG, T1D mice	Zhou et al. (2019)
Metformin	↑AMPK/Sirt1	↑FoxO1 activity ↓ROS ↑Autophagy ↓Cell proliferation	Mesangial cells cultured in HG, T2D rats	Ren et al. (2020)

T1D, type 1 diabetes; T2D, type 2 diabetes; Mn-SOD, manganese superoxide dismutase; CAT, catalase; SOD, superoxide dismutase; MDA, malondialdehyde; ROS, reactive oxygen species; Col, collagen; FN, fibronectin.

PTM is an important mechanism for regulating FoxO activity, and the abnormality in PTM in diabetic conditions is the common reason for FoxO1 dysfunction. Kidney is an important target organ of insulin action (Lay and Coward, 2018). The disruption of normal insulin signaling owing to hyperinsulinemia, insulin resistance, or absolute insulin deficiency associated with diabetes causes dysregulation of

AUTHOR CONTRIBUTIONS

YW wrote the manuscript. WH conceived, wrote, and revised the manuscript.

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