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REVIEW ARTICLE

Cell biology of diabetic nephropathy: Roles of endothelial cells, tubulointerstitial cells and podocytes

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ABSTRACT

Diabetic nephropathy is the major cause of end-stage renal failure throughout the world in both developed and developing countries. Diabetes affects all cell types of the kidney, including endothelial cells, tubulointerstitial cells, podocytes and mesangial cells. During the past decade, the importance of podocyte injury in the formation and progression of diabetic nephropathy has been established and emphasized. However, recent findings provide additional perspectives on pathogenesis of diabetic nephropathy. Glomerular endothelial damage is already present in the normoalbuminuric stage of the disease when podocyte injury starts. Genetic targeting of mice that cause endothelial injury leads to accelerated diabetic nephropathy. Tubulointerstitial damage, previously considered to be a secondary effect of glomerular protein leakage, was shown to have a primary significance in the progression of diabetic nephropathy. Emerging evidence suggests that the glomerular filtration barrier and tubulointerstitial compartment is a composite, dynamic entity where any injury of one cell type spreads to other cell types, and leads to the dysfunction of the whole apparatus. Accumulation of novel knowledge would provide a better understanding of the pathogenesis of diabetic nephropathy, and might lead to a development of a new therapeutic strategy for the disease.

INTRODUCTION

Diabetic nephropathy is a potentially fatal diabetic vascular complication characterized by slowly increasing proteinuria and a gradual decrease in renal function. Diabetic nephropathy is the leading cause of end-stage renal failure in Japan and Western countries, affecting alarmingly large numbers of people worldwide[1]. In Japan, diabetic nephropathy accounts for 44% of newly-induced hemodialysis, and a governmental survey estimated that there are approximately 100,000 hemodialysis patients. The number of patients is increasing rapidly, making diabetic nephropathy a critical social and economical problem.

The pathogenesis of diabetic nephropathy has been intensely investigated, and the roles of various mechanisms has been established, and include the effect of high glucose, polyol pathway activation, renin–angiotensin system activation, reactive oxygen species (ROS), activation of the protein kinase C pathway, increase of advanced glycation end-product (AGE) and glomerular hyperfiltration[2,3]. These changes lead to various cellular responses, expression of secretory factors and extracellular matrices that ultimately result in disruption of the glomerular filtration barrier, and histological changes including mesangial expansion, nodular glomerular sclerosis and tubulointerstitial fibrosis (Figure 1)[4].

During the past decade, 'podocentric' experiments have accumulated a huge amount of knowledge on the roles of podocytes in diabetic nephropathy. In contrast, recent findings have provided an additional perspective that other cell types are also affected at very early stages of diabetic nephropathy and contribute to the progression of the disease. To emphasize this concept, the current review will highlight the emerging roles of glomerular endothelial cells and tubulointerstitial cells in the pathogenesis of diabetic nephropathy, and juxtapose them to the roles of podocytes.

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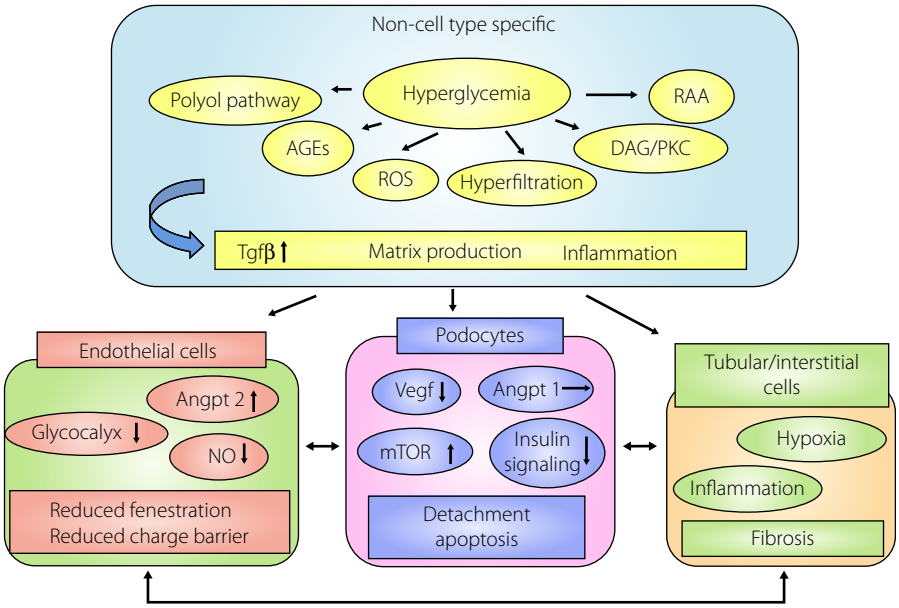

Figure 1 | Schematic diagram on pathogenesis of diabetic nephropathy. In diabetic conditions, high glucose, activation of the polyol pathway, glomerular hyperfiltration, activation of the renin–angiotensin–aldosterone system (RAA), increase of advanced glycation end-product (AGE), increased reactive oxygen species (ROS), activation of diacylglycerol (DAG)/protein kinase C (PKC) pathway and an increase in Tgfb lead to abnormal cellular responses, such as overproduction of extracellular matrices and inflammation in the kidney. Diabetes also affects the production of nitric oxide (NO), Angpt2 and glycocalyx in the glomerular endothelial cells, and leads to endothelial injury. In podocytes, reduced vascular endothelial growth factor (VEGF) A expression increased mechanistic target of rapamycin (mTOR) signaling, and insulin resistance leads to podocyte dysfunction resulting in podocyte detachment and apoptosis. Furthermore, recent findings show that tubulointerstitial fibrosis also plays significant roles. These cell types are tightly connected together, and dysfunctions in one compartment can spread to other cell types.

GLOMERULAR ENDOTHELIAL CELLS

Glomerular endothelial cells are highly fenestrated, with 50- to 80-nm pores that go through the cytoplasm[5]. The luminal surface of endothelial cells is covered by a thick layer of glycocalyx that includes proteoglycans (PGs), such as syndecan, glypican, perlecan and versican, as well as their glycosaminoglycan (GAG) side chains, heparan sulfate and chondroitin sulfate[6]. Endothelial GAGs maintain the negative charge of the endothelial glycocalyx, and are believed to be a significant component of the glomerular charge barrier[6].

Endothelial Cells are a Significant Part of the Glomerular Filtration Barrier

Because of the huge size of fenestration compared with that of circulating proteins, such as albumin, it has been believed that glomerular endothelial cells do not contribute to the filtration of macromolecules. However, emerging studies show its importance in the glomerular filtration barrier. Digestion of GAGs with heparanase, chondroitinase and hyaluronidase decrease the thickness of the glycocalyx layer, and the negative charge density of the glomerular filtration barrier, resulting in the increase of the fractional clearance of albumin[7,8]. In addition, elusion of non-covalently bound components of the endothelial glycocalyx by infusion of hypersonic sodium chloride into the renal artery leads to a 12-fold increase in the fractional clearance of albumin without detectable change of the ultrastructure[9]. In disease models, chronic infusion of hyaluronidase causes proteinuria in apolipoprotein E (ApoE) knockout mice[10], and Adriamycin injection largely decreases the thickness of the glycocalyx and proteoglycan expression in the glomeruli[11]. These experiments provide strong evidence that the endothelial glycocalyx forms a significant part of the glomerular filtration barrier.

Role of Endothelial Cells in Diabetic Nephropathy

Several diabetic animal models show reduced endothelial glycocalyx. In non-obese diabetic (NOD) mice, long-term diabetes causes a threefold increase in the fractional clearance for negatively charged albumin compared with controls. In contrast, the fractional clearance for neutral Ficoll that has a similar size to albumin is not increased. This change is accompanied by a decrease in glycocalyx proteins including versican and decorin[12]. Therefore, the authors concluded that the charge barrier, rather than the size barrier, is affected in NOD mice glomeruli. Disruption of the charge barrier before that of the size barrier might show that endothelial damage occurs before podocyte/glomerular basement membrane (GBM) damage in NOD mice. In addition, staining for lectin that binds to hyaluronic acid and heparin sulfates of the endothelial glycocalyx is attenuated in streptozotocin (STZ)-induced diabetic rats[13] and Zucker fatty rats[14].

How about human beings? It has long been known that endothelial injury, assessed by elevation of plasma von Willebrand factor, is seen in both type 1 and type 2 diabetic patients[15,16]. An extensive study using state-of-the-art technology showed that the volume of endothelial glycocalyx in the total body and the thickness of the glycocalyx layer in retinal and sublingual arteries are reduced in type 1 diabetic patients. In that study, the decrease of endothelial glycocalyx was more severe in the group with albuminuria[17]. A similar reduction of the endothelial glycocalyx was also observed in type 2 diabetic patients[18]. In addition, very important studies have shown morphological abnormalities of renal glomerular endothelium in diabetic patients. Toyoda *et al.*[19] showed that the fraction of fenestrated endothelium is reduced from 41% in controls to 32% in normo- and microalbuminuric patients, and further to 25% in macroalbuminuric patients with type 1 diabetes. Recently, Weil *et al.*[20] reported a similar decrease of fenestrated area in type 2 diabetes patients (Figure 2). Interestingly, podocyte detachment also starts in the normoalbuminuric stage and correlates to albuminuria[20]. In that study, the endothelial fenestration fraction correlated with the urinary albumin creatinine ratio more closely compared with podocyte detachment. Although podocyte detachment has been considered to be a critical event in various types of glomerular diseases, including diabetic nephropathy[21], these studies clearly showed that endothelial damage simultaneously occurs when podocytes are damaged. This notion could raise a question against the concept that podocyte injury is the primary event and endothelial damage occurs as a secondary reaction[22,23]. These findings rather support the concept that the glomerular filtration barrier is a composite multilayered structure, and injury in one layer might spread to any other layers and affect the whole function of the glomerular filtration barrier.

Endothelial Dysfunction and Diabetic Nephropathy: Lessons from Genetically Targeted Mice

Various cytokines and factors are secreted by/have effects on endothelial cells to maintain the glomerular filtration barrier. Emerging evidence using genetically targeted mice prove their importance in diabetic nephropathy.

Nitric Oxide

In diabetic animals and humans, bioavailability of the nitric oxide (NO) is reduced[24]. In cultured endothelial cells, high glucose inhibits endothelial NO synthase (eNOS) activity[25]. However, in type 2 diabetic patients, eNOS expression is increased in kidney glomeruli by immunohistochemistry, and the increase was correlated with more severe vascular complications[26]. Similarly, protein expression of eNOS in STZ-induced diabetic rats is increased in afferent arterioles and glomeruli[27]. In contrast, eNOS required cofactors including tetrahydrobiopterin (BH4) to produce NO. If eNOS is 'uncoupled', peroxynitrite (ONOO–), a putative reactive oxygen species, is produced instead of NO. In endothelial cells, high glucose results in a

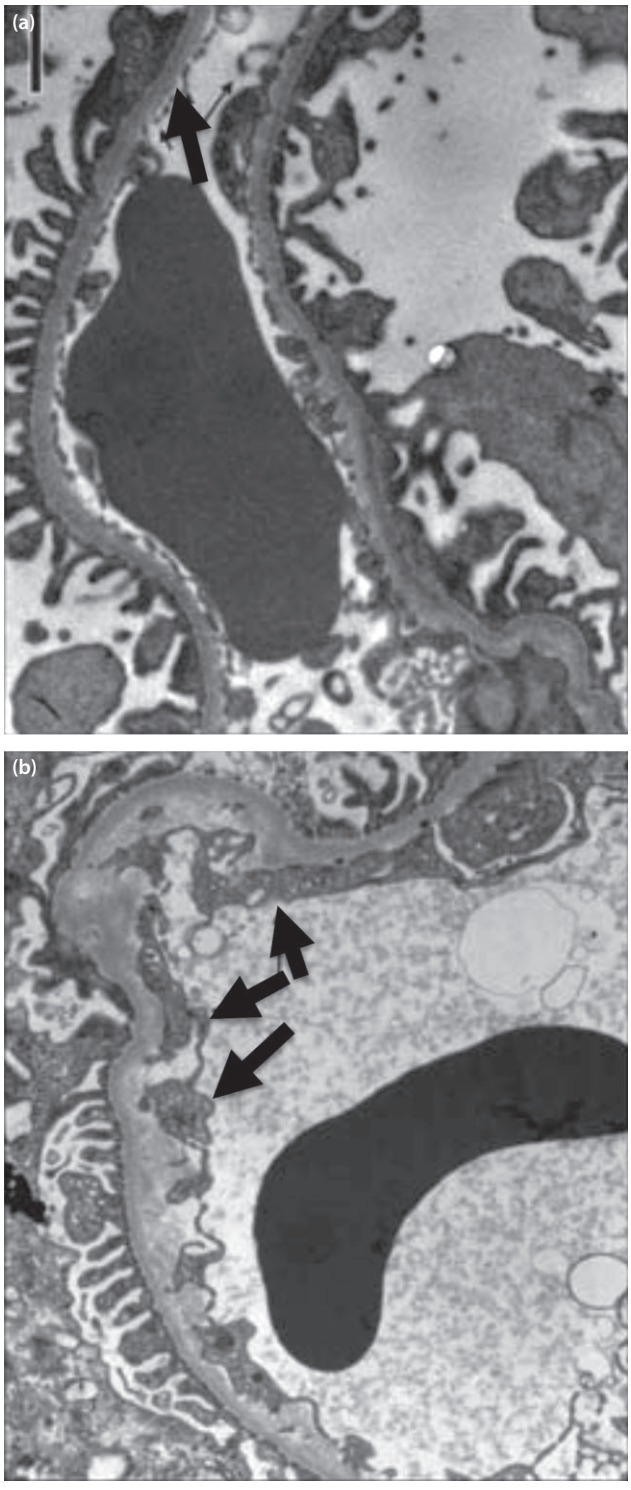

Figure 2 | Endothelial injury is already present in the normoalbuminuric stage. (a) The ultrastructure of glomerular filtration barrier from a normal subject, note intact endothelial fenestration. (b) Sample from a normoalbuminuric Pima Indian patient with type 2 diabetes showing intact podocyte foot processes, but normal endothelial fenestrae are absent (arrows; adapted from Weil *et al.*[20] with permission).

uncoupling of eNOS, reduction of NO production and increased superoxide production[28]. This uncoupling might explain the discrepancy between elevated expression of eNOS and reduced NO production in the diabetic condition.

The requirement of eNOS in the glomerular endothelium in diabetes was examined using two diabetic animal models combined with genetic deletion of eNOS. Both *db*/*db* and STZ mice bred with eNOS knockout mice showed dramatic albuminuria, increased glomerular basement membrane thickness, mesangial expansion, and focal segmental and nodular sclerosis[29,30]. The potential mechanism of the enhanced nephropathy was an uncoupling of the vascular endothelial growth factor A (VEGFA)–eNOS axis, with enhanced VEGF expression and impaired NO production, which led to excessive endothelial proliferation. These phenotypes were at least partially mediated by intraglomerular hypertension, because lowering blood pressure rescues the glomerular lesion in the diabetic eNOS deficient mice[31]. These results provide robust evidence that endothelial dysfunction results in enhancement of diabetic nephropathy and suggest NO as a potential therapeutic target.

VEGFA

A large amount of VEGFA is produced by podocytes. The secreted VEGFA go across the GBM and bind to kinase insert domain protein receptor (Kdr; also known as VEGF receptor 2) expressed on the endothelial cells. The VEGFA–Kdr axis is essential for the formation and maintenance of the glomerular filtration barrier[32,33]. Podocyte-specific deletion of VEGFA leads to impaired recruitment of endothelial cells into glomeruli, failure in formation of glomerular filtration barrier and congenital nephrotic syndrome[33]. Mice that carry haploinsufficient VEGFA allele in podocytes show an endothelial swelling and loss of fenestration in glomeruli known as endotheliosis – a feature seen in thrombotic microangiopathies (TMA)[33]. Overexpression of VEGFA in podocytes leads to a collapse of the glomerular tuft[33]. In addition, patients on anti-VEGF therapy sometimes develop proteinuria as a result of TMA of the glomeruli[34]. Indeed, deletion of VEGFA alleles from adult mice podocytes results in TMA[34].

The role of VEGFA in diabetic nephropathy has been a controversy. An increase of VEGFA expression was shown in the glomeruli and tubulointerstitium in STZ diabetic rats and in type 2 diabetic mice[35–37]. As VEGFA is a potent stimulator that destabilizes endothelial cells and induces vascular permeability, some investigators attempted to block VEGFA signaling to treat diabetic nephropathy in mice[38,39]. In *db*/*db* mice, an inhibitor for tyrosine kinase of Kdr reduced urinary albumin excretion (UAE)[40,41]. In Zucker diabetic rats, the neutralizing antibody for VEGF reduced glomerular hypertrophy, but did not improve UAE[40]. Therefore, these reports appear to support the notion that VEGF worsens diabetic nephropathy.

However, reports on VEGFA expression in human diabetic nephropathy are inconsistent. Hohenstein *et al.*[42] reported enhanced VEGFA expression in glomeruli of type 2 diabetes patients by immunostaining. In contrast, Baelde *et al.*[43] showed reduction of *Vegfa* messenger ribonucleic acid expression in human type 2 diabetic glomeruli by Affymetrix microarray[43]. Several additional reports showed that *Vegfa* expression was decreased in both the glomeruli and tubulointerstitium, and was correlated with reduced renal microvascular density, tubular epithelial atrophy, mesangial expansion and proteinuria[44,45]. These results rather support the notion that VEGF is protective.

Recently, two genetic mice models shed a new light onto this controversy. Veron *et al.*[46] produced a mouse model that carries podocyte-specific inducible overexpression of Vegf164, and rendered the mice diabetic by STZ injection. The results showed accelerated nephropathy with Kimmelstiel–Wilson like nodular glomerulosclerosis and massive proteinuria[46]. In contrast, Sivaskandarajah *et al.*[47] showed that inducible deletion of VEGFA in adult podocytes results in severe enhancement of diabetic nephropathy using a STZ-induced mice model. In that report, the diabetic mutant showed severe glomerulosclerosis, enhanced proteinuria and increased apoptosis in the kidneys[47]. These results clearly show that the levels of VEGFA in the glomeruli require 'fine tuning', and either overdose or suppression could result in exacerbation of diabetic nephropathy. This tight and subtle regulation is similar to that of VEGFA in glomerular development.

Angiopoietins

Another family of angiogenic factors required for maintenance of glomerular endothelial cells is angiopoietin–Tek signaling. Angiopoietin 1 (Angpt1) and angiopoietin 2 (Angpt2) are both ligands to Tek tyrosine kinase (Tek/Tie-2)[48,49]. Angiopoietin 1 binds to the Tek receptor expressed on endothelial cells, and cause its tyrosine phosphorylation, but Angpt2 works as an antagonist while not activating any intracellular signaling. However, some data suggest that Angpt2 activates phosphorylation of Tek in certain conditions[50]. Angpt1 is believed to stabilize the blood vessel, reduce vascular permeability and support the survival of endothelial cells. Angpt1 conventional knockout mice die at embryonic day 12.5[49] and Angpt2 knockout is also perinatal lethal[51].

Recent analysis using conditional alleles showed critical roles of Angpt1 in glomeruli. Jeansson *et al.* showed that deletion of Angpt1 at embryonic day 10.5 resulted in abnormal glomerular development with a single capillary loop without mesangial migration that is reminiscent to the phenotype of *Pdgfb*/*Pdgfrb* mutants[52,53]. In contrast, loss of the Angpt1 allele at e13.5 does not cause any phenotype, showing that Angpt1 is only required when the vasculature is undergoing dynamic remodeling.

The role of angiopoietins in diabetes has been shown by several reports. In diabetic patients, Angpt2 expression is increased[54]. On the other hand, diabetic animal models show a decrease of Angpt1 and increase of Angpt2. Furthermore, STZ-induced diabetic mice with whole-body or glomerular-specific Angpt1 deletion develop increased urinary albumin excretion, severe mesangial expansion, glomerular sclerosis and early

mortality[53]. A study using *db*/*db* mice showed that treatment with recombinant adenovirus-expressing cartilage oligomeric matrix protein (COMP)-Ang-1, a potent Angpt-1 variant, resulted in improvement in diabetic renal damage[55]. Recently, STZ diabetic mice with podocyte-specific overexpression of Angpt1 also showed a similar protective effect on diabetic nephropathy[56]. Taken together, the Angpt1–Tek axis plays an important protective role in glomerular endothelial cell function in the diabetic condition. Drugs that target Angpt1 and its downstream molecules might provide potentially useful therapeutic strategies.

TUBULOINTERSTITIAL CELLS

Although it has been widely accepted that glomerular injury is the main component of diabetic nephropathy, plenty of evidence has shown that tubulointerstitial changes are present and are involved in its progression[57]. Tubulointerstitium includes the tubular system, interstitial cells and vascular system, and accounts for as much as 90% of kidney volume[58].

Histological Abnormality of Tubulointerstitium Correlates with Progression of Diabetic Nephropathy

Histologically, early diabetic kidneys show tubular hypertrophy, thickening of the tubular basement membrane and interstitial inflammation with mononuclear cell infiltration[59]. When it progresses, they show tubular atrophy and tubulointerstitial fibrosis. Interestingly, tubulointerstitial expansion closely correlates with elevation of serum creatinine in both type 1 and type 2 diabetes[60–62]. Taft *et al.*[63] followed 47 patients with diabetes for 4 years, and carried out renal biopsies at the beginning and the end of the study. The results showed that cortical tubulointerstitial fibrosis more closely correlated to the decrease of creatinine clearance than glomerular changes[63]. This association seems to be weaker in the microalbuminuric stage[64], suggesting that tubulointerstitial fibrosis is a good determinant of moderate-to-severe renal injury rather than early diabetic nephropathy[59].

In addition, abnormalities in glomerulotubular junctions have been reported in diabetic kidneys. A study on renal biopsy samples of type 1 diabetes patients showed that 4% of glomeruli from microalbuminuric patients show some glomerulotubular junctional abnormalities, including connections of glomeruli to atrophic tubules. Furthermore, in the macroalbuminuric stage, 71% of glomeruli showed glomerulotubular junction abnormalities, including 8% glomeruli without any tubule connected (atubular glomeruli)[65,66]. Type 2 diabetic glomeruli also show atubular glomeruli and connection to atrophic tubules from the stage of microalbuminuria[67]. These data suggest that, even though tubulointerstitial changes are dominantly seen in the advanced stage, subtle abnormality is already present in the microalbuminuric time-point.

Increase of Tubular Markers in Urine of Early Diabetic Patients

Data of urinary biomarker also show that tubular damage starts at an early stage of diabetic nephropathy. Studies on type 1 diabetic children showed that urinary N-acetyl-b-D-glucosaminidase (NAG) is increased in diabetes patients compared with normal controls, and is correlated with urinary albumin excretion and glycemic control. This increase was already seen in the microalbuminuric stage[68,69]. Intriguingly, within microalbuminuric type 1 diabetic patients, a group that shows low levels of urinary NAG and kidney injury molecule 1 (KIM-1) tends to show regression of albuminuria after 2 years, suggesting that tubular dysfunction is a critical component of the early course of diabetic nephropathy[69].

Does Tubulointerstitial Change Affect Glomerular Structure and Function–Tubuloglomerular Feedback?

It has been considered that the glomeruli is the primary site of injury, and the tubulointerstitial change is a secondary reaction to elevated intratubular protein as a result of glomerular leakage. Recent studies suggested that tubular changes could lead to alteration in glomerular structure and function.

Using genetically modified mice, VEGFA was overexpressed in renal tubular cells on administration of doxycyline. The mutant mice showed an interstitial fibrosis and tubular cysts with dense network of peritubular capillaries. The mice did not show significant proteinuria; however, they developed glomerular changes including marked mesangial expansion similar to that seen in diabetic nephropathy. The authors observed downregulation of VEGFA expression in podocytes, and concluded that tubular overproduction of VEGFA resulted in suppression of VEGFA in podocytes that led to interference of cross-talk between podocytes and the endocapillary compartment (Figure 3)[70].

Another study also provides evidence for tubuloglomerular feedback. Deletion of sirtuin 1 (Sirt1), a NAD+ regulated deacetylase, specifically in tubules leads to increased albuminuria and effacement of podocyte foot processes. Additionally, STZ-induced diabetes aggravated the nephropathy of tubule-specific Sirt1 knockout mice. Furthermore, overexpression of Sirt1 in tubules protected the mice from the glomerular ultrastructural changes and albuminuria in STZ mice. In human diabetic nephropathy with proteinuria, Sirt1 was downregulated and Claudin1 was upregulated. These results suggest that Sirt1 in proximal tubules protects against diabetic nephropathy and influence podocyte function[71]. Taken together, these experiments clearly show that tubulointerstitial change can affect glomerular structure and function.

Epithelial to Mesenchymal Transformation in Diabetic Nephropathy

Epithelial to mesenchymal transformation (EMT) is a cellular process through which epithelial cells undergo 'de-differentiation'; lose epithelial characteristics; express mesenchymal markers, such as alpha-smooth muscle actin, desmin and vimentin; and acquire a matrix-producing fibroblast-like phenotype. A study using genetically tagged proximal tubules showed that up to 36% of the interstitial fibroblasts were derived from proximal

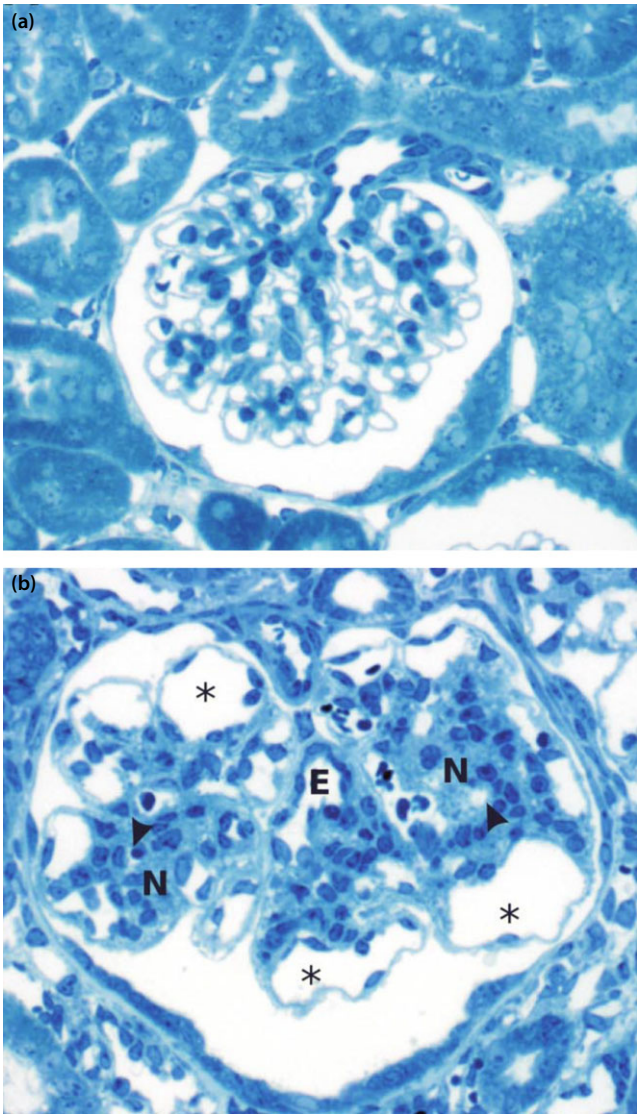

Figure 3 | Genetic overexpression of vascular endothelial growth factor A (VEGFA) in tubules results in glomerular disease. (a) A glomerulus from a control mouse. (b) A glomerulus from a tubular-specific VEGFA overexpression mouse that shows dilated capillaries at the periphery and nodule-like mesangial expansion, showing that tubular dysfunction results in glomerular disease (reproduced from Hakroush *et al.*[70] with permission).

tubules in a unilateral ureter obstruction mouse model[72]. A recent analysis reported that 35% of the myofibroblasts were derived from bone marrow, 10% from endothelial cells and 5% from tubular cells through an EMT program in a unilateral ureter obstruction model[73,74]. In diabetic nephropathy, EMT-like changes of the tubules, such as upregulation of vimentin and decrease of E-cadherin, have been observed both *in vitro* and *in vivo*[75,76]. In addition, tubular expressions of mesenchymal markers are also shown in renal biopsy samples from human diabetic nephropathy[77]. In contrast, however, overexpression of transforming growth factor beta (Tgfb) in tubular cells, a multifunctional cytokine that is believed to be the key mediator of EMT, resulted in total decomposition of tubular cells and fibrosis, but not mesenchymal transformation of tubular cells[78]. Therefore, there is no solid evidence that tubular cells transform into matrix-producing interstitial fibroblasts in diabetic nephropathy. In terms of endothelial to mesenchymal transformation, Li *et al.*[79] carried out genetic tag experiments using Tie2-Cre mice, and showed that infusion of AGE to mice resulted in fibroblasts derived from endothelial cells[79].

PODOCYTES

Morphology of Podocytes

Podocytes are terminally differentiated cells that wrap the glomerular capillary loops from outside, providing physical support and secretary cytokines. They develop microtubule-based thick primary foot processes and fine actin-based secondary foot processes that interdigitate each other. The foot processes from the neighboring podocytes are connected with each other by a specialized intercellular junction called a slit diaphragm (SD). SD consists of proteins including nephrin, podocin, Cd2-ap and actin-binding protein alpha-actinin 4. The mutations of these proteins cause focal and segmental glomerulosclerosis in mice and humans, thus SD is considered to be essential in macromolecular filtration[80].

Diabetic Nephropathy and Podocytes

The early key events in diabetic nephropathy include loss of podocytes in glomeruli. A landmark study was carried out by Pagtalunan *et al.*[81], and showed that the number of podocytes per glomeruli were reduced in renal biopsies from Pima Indian type 2 diabetes patients with macroproteinuria. The loss of podocytes was correlated with the flattening of podocyte foot processes[81]. Subsequent analysis showed that the decrease of podocyte number is a good predictor of progression of albuminuria[82]. Similar findings are also reported on the nephropathy of type 1 diabetic patients[19,83].

The mechanism of podocyte loss is considered to be detachment and apoptosis. Podocyte apoptosis is observed in many animal diabetic models including Akita mice, *db*/*db*[84] mice and STZ induced rats[85], and could at least partially be mediated by an increase of Tgfb, Smad7[86], AGE[87], angiotensin II[88] and reactive oxygen species[84]. In human type 2 diabetic kidneys, apoptosis was seen in both tubular and glomerular compartments, some was obviously in podocytes[89]. In contrast, under diabetic conditions, some podocytes detach from the GBM and fall into the urine. The detached 'urinary' podocytes are viable and can propagate *ex vivo*[90]. Indeed, patients of type 2 diabetes show urinary excretion of podocytes, and it worsens as the disease progresses from normoalbiminuria, microalbuminuria and to macroalbuminuria[91]. Interestingly, the urinary podocyte detachment can be reduced by administration of hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitors[92].

Factors Involved in Podocyte Function in Diabetic Nephropathy

Proteins of Slit Diaphragm

Nephrin is a 180-kDa transmembrane protein that belongs to the immunoglobulin superfamily. In 1998, Karl Triggvasson *et al.*[93] group identified the nephrin (*NPHS1*) gene, and showed that its mutations caused the congenital nephrotic syndrome of the Finnish type. The nephrin cytoplasmic tail includes three tyrosine-aspartic acid-x-valine (YDxV) residues. These residues are phosphorylated by Src family kinases, recruit SH2/SH3 containing Nck adaptor proteins resulting in reorganization of actin cytoskelton. Deletion of Nck1/2 in podocytes leads to massive congenital proteinuria[94,95]. Many reports have shown downregulation of nephrin in diabetic nephropathy in rodent models and human patients[96–98]. Some reports suggest that the decrease of nephrin is partially mediated by the renin–angiotensin system, AGE and protein kinase C pathway[98–101]. In contrast, there are also several reports showing upregulation of nephrin expression in mice diabetic kidneys[41,102]. The cause of this diversity is still unknown, but could be a difference of disease stages. In addition, other slit diaphragm proteins, such as podocin and synaptpodin, are also significantly reduced in human diabetic nephropathy[103].

Insulin Signaling

Podocytes express insulin receptor and increase their glucose uptake on insulin stimulation[104]. In addition, knockdown of nephrin in podocytes attenuates insulin-dependent glucose uptake in podocytes, showing that nephrin is required for insulin function in podocytes[105]. Recently, a study on a genetic mouse model that lacks insulin receptor in podcytes was reported. The mice showed massive proteinuria and histological abnormality similar to diabetic nephropathy. In addition, *in vitro* studies using an immortalized human podocyte cell line suggest that insulin sends signals through mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K), and regulates actin cytoskeleton remodeling in podocytes[106]. These studies show that local insulin resistance affects podocyte function and leads to progression of diabetic nephropathy, thus demonstrating the importance of the therapy that increases insulin sensitivity in podocytes.

mTor Signaling

The mechanistic target of rapamycin (mTor) is a ubiquitously expressed serine-threonine kinase. mTor binds to regulatory-associated protein of mTOR (Rptor), or Rptor independent companion of mTor (Rictor), and forms mTORC1 and mTORC2, respectively[107]. mTORC1 is activated by amino acids, stress, oxygen and growth factors, and regulates protein translation, ribosomal biogenesis, cell growth and autophagy. mTORC2 responds to growth factors, and regulates cell survival, growth, metabolism and cytoskeletal rearrangement[107]. Rapamycin acutely suppresses mTORC1 function[108]; however, in certain contexts, it also inhibits mTORC2[109]. Interestingly, organ transplant patients using rapamycin sometimes develop proteinuria, suggesting the importance of mTor signaling in glomeruli[108]. Indeed, mice studies showed that the mTor pathway is essential for podocyte function. Deletion of mTor or Rptor specifically from podocytes leads to massive proteinuria and renal failure[110,111].

In contrast, mTor activity was increased in renal biopsy samples of diabetic patients and in diabetic *db*/*db* mice[111,112]. Activation of mTORC1 by deletion of its suppressor, Tsc1, in podocytes results in proteinuria, podocyte loss, mesangial expansion and thickening of glomerular basement membrane[112]. Furthermore, haploinsufficiency of Rptor protected mice from diabetic nephropathy in STZ mice and *db*/*db* mice, showing a decrease in urinary albumin excretion and mesangial expansion[111,112]. These results show the critical roles of mTor signaling in diabetic nephropathy, and that either overactivation or elimination of the mTor pathway leads to podocyte dysfunction and glomerular disease. Recent analysis showed that podocyte-specific ablation of Rictor or Akt2, a potent target of mTORC2, results in accelerated nephropathy induced by heminephrectomy, showing that mTORC2 is also involved in progression of chronic kidney disease (CKD)[113].

Notch Signaling

The Notch pathway regulates cell proliferation, differentiation, cell fate specification and organ development, and is conserved in all metazoans[114]. Notch is a transmembrane receptor that binds to cell surface ligands, such as Delta-like or Jagged, on neighboring cells. On interaction of these ligands, Notch intracellular domain (NICD) is cleaved by gamma-secretase, and then NICD translocates to the nucleus where it forms transcriptional complexes with deoxyribonucleic acid (DNA) binding proteins including RBPj, CBF1 and Su[114].

Although the importance of the Notch pathway has been well documented in renal development, it is mostly silenced in adult normal kidneys[115]. However, NICD is increased in podocytes of human diabetic nephropathy and focal segmental glomerulosclerosis, and in *db*/*db* mice. Conditional overexpression of NICD in podocytes resulted in proteinuria and glomerulosclerosis. In addition, deletion of RBPj, an essential transcriptional partner of NICD, or administration of gamma-secretase inhibitor, protected rats from proteinuric renal models including diabetic nephropathy[115]. The Notch pathway might mediate high glucose-induced apoptosis in podocytes[116,117].

Wnt/beta-Catenin Signaling

Wnts are a family of highly-conserved secreted glycoproteins that regulate cell proliferation, cell fate determination, organogenesis and tumorgenesis. Beta-catenin is the central component of the canonical Wnt pathway. On binding of Wnts to their receptor, Frizzled, a series of downstream signalings including Dishvelled, Axin, adenomatous polyposis coli (APC) and glycogen synthase-3beta (GSK-3beta) are activated, and eventually lead to dephosphorylation of beta-catenin.

Dephosphorylated beta-catenin escapes the ubiquitin-mediated degradation, then the stabilized beta-catenin translocates to the nucleus where it binds to DNA and regulates transcription of various target genes.

Wnt beta-catenin signaling is activated in podocytes of diabetic nephropathy in humans and STZ-induced diabetic mice[118,119]. Pharmacological activation[118], as well as podocyte-specific stabilization of beta-catenin, leads to albuminuria[119]. Interestingly, deletion of beta-catenin in podocytes and expression of Wnt inhibitor Dickkopf-related protein 1 (Dkk1) result in enhancement of diabetic nephropathy induced by STZ[119], suggesting that both hypo- and hyperactivation of the beta-catenin pathway promotes proteinuric diseases. *In vitro* studies showed that Wnt/beta-catenin signaling regulates podocyte motility, adhesion, apoptosis and expression of podocyte differentiation markers including nephrin[119]. Interestingly, beta-catenin is a key molecule that regulates the cell fate determination when podocytes differentiate from renal vesicles[120].

Tgfb

Many reports show that Tgfb is a central factor that contributes to the progression of glomerulosclerosis. Increased expression of Tgfb1 is observed in human diabetic kidneys at early and late stages of nephropathy, and the expression of Tgfb is correlated with glycemic control[121,122]. In *db*/*db* mice, elevation of Tgfb, its receptor Tgf beta receptor 2 (Tgfbr2) and nuclear translocation of Smad3, a main signaling transducer of Tgfb signaling, were observed[123]. Tgfb level was elevated in the glomeruli of STZ-induced diabetic rats by micropuncture[124]. In addition, overexpression of Tgfb in hepatocytes results in multiple organ phenotype including glomerulonephritis and renal fibrosis[125]. Recently, overexpression of Tgfb receptor 1 specifically in podocytes resulted in massive glomerulosclerosis[126]. Interestingly, this phenotype was mediated by Endothelin1 release by podocytes, which leads to mitochondrial oxidative stress in adjacent endothelial cells in a paracrine manner, thus emphasizing the importance of cross-talk between podocytes and endothelial cells.

As mentioned earlier, Tgfb is reported to induce podocyte apoptosis through the P38-MAPK pathway[86]. Smad7 is one of the downstream targets of Tgfb signaling, and is also known as a potent inhibitor of the classical Smad signaling pathway, forming a negative feedback. Smad7 knockout mice show exacerbated diabetic nephropathy with increased albuminuria, enhanced renal fibrosis and increased expressions of inflammatory factors. In the diabetic Smad7 knockout kidneys, both the Tgfb–Smad pathway and nuclear factor-kappa B pathway are activated. Overexpression of Smad7 by gene transfer specifically to kidneys attenuated microalbuminuria[127]. Although Smad7 is reported to induce podocyte apoptosis[86], these data suggest that Smad7 plays a protective role in diabetic nephropathy, highly likely through suppression of Tgfb signaling pathways.

In the immortalized podocyte cell line, Tgfb decreased podocyte markers, such as nephrin and Zo-1, and increased mesenchymal markers, including alpha smooth muscle actin and vimentin, showing that Tgfb induces de-differentiation in podocytes similar to epithelial mesenchymal transformation[128]. In addition, it has been shown that Tgfb increases the expression of extracellular matrices including fibronectin and type IV collagen in podocytes, and contributes to the thickening of GBM[129,130].

Soluble Flt1

Soluble fms-related tyrosine kinase 1 (sFlt1) is an alternatively spliced soluble form of VEGF receptor 1 (VEGFR-1)/Flt-1, and works as a decoy ligand to Kdr. An increase of sFlt1 using adenovirus results in a marked decrease of endothelial fenestrae in mice glomeruli[131]. sFlt blood levels are higher in pre-eclampsia patients, and injection of sFlt1 to pregnant rats causes hypertension and proteinuria[132]. A recent report showed a surprising additional function of sFlt1 on non-endothelial cells through Kdr-independent mechanisms. Deletion of Flt1 in podocytes leads to reorganization of their cytoskeleton, massive proteinuria and renal failure. The allele that lacks the intracellular kinase domain of Flt1 rescues this proteinuric phenotype, suggesting that full-length protein is not critically required. The authors showed that sFlt binds to the lipid microdomain on the podocyte surface, and promote cellular adhesion and actin reorganization[133]. Interestingly, mice with overexpression of sFlt1 in podcytes were protected from STZ-induced changes in glomeruli. Transgenic diabetic mice show a decrease in urinary albumin excretion, mesangial expansion, podocyte foot processes fusion and glomerular basement membrane thickening[134]. These results show an interesting autocrine function of sFlt1 that protects podocytes in addition to its role as a decoy on endothelial VEGFA signaling. However, systemic overexpression of sFlt1 will not be useful, because intramuscular injection of adeno-associated virus 1 encoding human soluble Flt1 resulted in improvement in podocyte injury, but exacerbated tubulointerstitial damage, showing a conflicting effect of sFlt1 in podocytes and interstitial compartment[135].

Rho GTPases

Rho guanosine triphosphatases (GTPases) are known as master regulators of actin cytoskeleton. The classical members of this family are Rho, Rac1 and Cdc42. Activation of Cdc42 leads to fillipodia formation, Rac1 promotes lamellipodia formation and RhoA mainly regulates formation of stress fibers[136]. Many reports suggested its importance in podocyte biology. Podocyte-specific deletion of Cdc42 leads to a congenital nephrotic syndrome and renal failure with defect in actin polymerization at intracellular domain of nephrin[137], whereas the other two Rho guanosine triphosphatases did not show any proteinuria when deleted in podocytes. Activation of RhoA in podocytes results in focal segmental glomerulosclerosis[138]. It has been shown that RhoA is activated in the renal cortex of diabetic *db*/*db* mice[139]. Several trials have been carried out to suppress RhoA in diabetic rodent models. Administration of Fasudil, a Rho-kinase

inhibitor, resulted in attenuation of urinary albumin excretion, ultrastructural chances of glomerular filtration barrier and mesangial expansion in *db*/*db* mice[139]. A similar protective effect was also observed in STZ-induced diabetic mice and rats[140–142]. These effects included suppression of Tgfb1 and connective tissue growth factor, and hypoxia-inducible factor 1 alpha signaling[141,142].

CONCLUSION

Diabetic nephropathy affects many cellular functions, some of them are common across cell types and some are cell-type specific. Recent studies showed distinct roles of each compartment, and factors that are required for cross-talk between cell types. An accumulation of a better understanding on the pathogenesis of diabetic nephropathy would provide a novel opportunity for the development of a new therapeutic strategy.

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