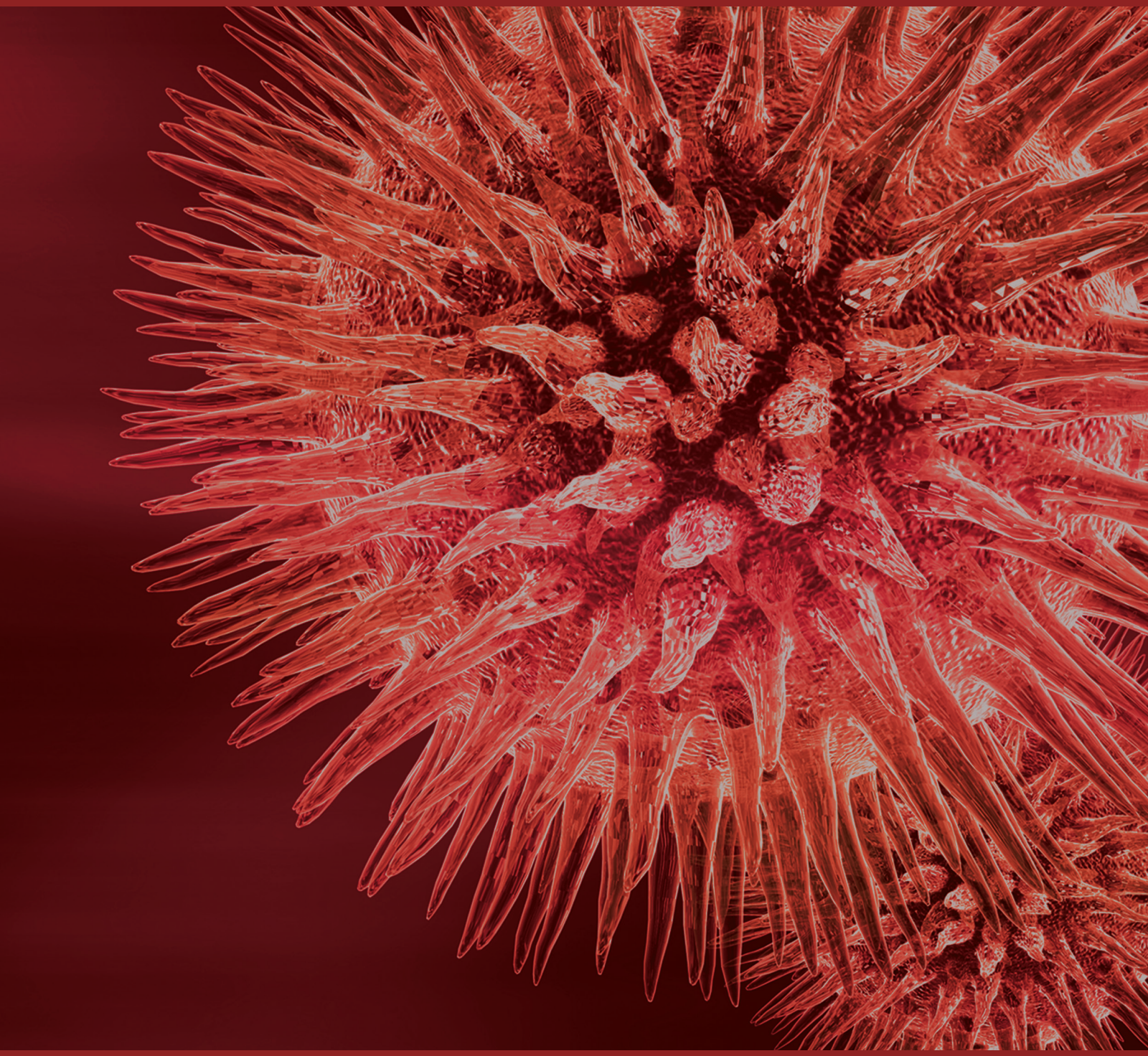
BioMed Research International

Combating Kidney Fibrosis

Guest Editors: Keizo Kanasaki, Akito Maeshima, Gangadhar Taduri, and Ignacio Revuelta



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Contents

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Editorial

Combating Kidney Fibrosis

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An estimated 10% of the world population has some form of kidney disease. Kidney fibrosis is the final common pathway of progressive kidney diseases, resulting in subsequent massive destruction of normal kidney structure and diminishing the function. Currently approved therapies are neither pathway nor cell specific in nature, due to which these therapies became ineffective in reducing the fibrosis and are associated with side effects. The understanding of the pathways and cells that are involved in the fibrosis will guide the future therapies to combat the kidney fibrosis.

In this special issue of the BioMed Research International, we have designed to invite original as well as review articles regarding the pathophysiologic clue to combat kidney fibrosis in various diseases.

In a research article Takako Nagai et al. (Kanazawa Medical University, Japan) focused on the endogenous antifibrotic peptide, N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP), one of the substrates of angiotensin converting enzyme (ACE), and found that AcSDKP suppressed kidney fibrosis in diabetes or even restored normal kidney structure from damaged kidney associated with the inhibition of endothelial mesenchymal transition and the induction of fibroblast growth factor receptor-microRNA let-7 axis.

There are two other research articles in the topic of diabetic nephropathy from the research team led by Yong Xu (Luzhou Medical College, China). Huang et al. found that proteasome inhibitor MG132 inhibited profibrotic cytokine transforming growth factor (TGF)-β signaling via endonuclear transcription corepressor SnoN degradation and ameliorated diabetic nephropathy. Another paper from Yong's research team by Zhou et al. found that high glucose in mesangial cells induced sumoylation (by SUMO 2/3) of smad4, the co-smad essential for TGF-β-induced signal transduction, and such sumoylation would be important for enhanced TGF-β signal transduction in the cells exposed to high glucose condition and diabetic kidney.

Focusing on TGF-β-induced profibrotic signaling pathway on the ligand-receptor complex level, there are two exciting papers included in the special issue. Maeshima et al. (Gunma University Graduate School of Medicine, Japan) found that activin A, a member of TGF-β superfamily, exhibited profibrotic action in unilateral ureteral obstruction (UUO) model. They showed that UUO kidney displayed significant induction of activin A in the interstitial αSMA-positive fibroblasts and follistatin, an activin antagonist, significantly reduced the fibrotic area in the UUO kidney, suggesting the essential role of activin A signaling in the development of interstitial fibrosis in this model, and its antagonist could be a novel approach for the prevention of kidney fibrosis.

Another paper by Rodrigues-Diez et al. (Universidad Autonoma Madrid, Spain) focused on gremlin, a well-known bone morphogenetic proteins (BMPs) antagonist. They found that gremlin induced early activation of smad2/3 signal transduction via TGF-β independent manner in human tubular epithelial cells and long-term exposure of gremlin induced epithelial mesenchymal transition (EMT). Such long-term exposure of gremlin-induced EMT was diminished by TGF-β neutralizing antibody, suggesting that, different from early

effects, TGF-β induction was involved in the long-term exposure.

UUO is the model that researchers frequently used for kidney fibrosis research. Therefore the biology of UUO would provide meaningful information for future kidney fibrosis research. In regard to this, Rodrigues-Pena et al. (Universidad de Salamanca, Spain) focused on early fibrotic changes of UUO and found that activation of RAS pathway would be the clue to inhibit fibrotic changes of UUO by confirming the potent inhibitor of this pathway utilizing angiotensin II, losartan, atorvastatin, and farnesyl transferase inhibitors.

Banon-Maneus et al. (Ludwig-Maximilians-University, Germany, and laboratorio Experimental de Nefrologia Transplant, Spain) focused on Wnt/β-catenin pathway for the therapeutic target to combat kidney fibrosis. It is well known the significance of Wnt/β-catenin pathway in several human diseases and abnormal activation of Wnt/β-catenin pathway is associated with progressive damage and organ failures. In their paper, authors confirmed that activation of Wnt/β-catenin pathway was involved in 5/6 renal mass reduction model (RMR) and suggested that RMR is nice animal model for aberrant activation of Wnt/β-catenin pathway to perform experimental therapy by various molecules.

Finally, the special issue included exceptional 5 review articles. Maeshima et al. (Gunma University Graduate School of Medicine, Japan) summarized recent advance in regenerative medicine for kidney. Renotropic factors, renal stem/progenitor cells, and stem cell therapies are examined in this review, and the authors discussed the issues to be solved to realize regenerative therapy for kidney diseases in humans. Kim et al. (Pusan National University School of Medicine, Korea) well described the role of uric acid in kidney fibrosis and also suggested future direction of interventional research to proof causal relationship between uric acid and kidney fibrosis development. Kume et al. (Shiga University of Medical Science, Shiga, Japan) discussed the roles of nutrient sensing pathway, such as mTORC1, AMPK, and sirt1, in the development of diabetic nephropathy. They provided a excellent summary of recent advances in this field and made very informative tables for other researchers who investigate the signaling pathway associated with nutritional responses. Torres et al. (Autonomous University of Barcelona, Spain) wrote very unique, informative, and educative review about the interaction between inflammation and fibrosis in transplanted kidney. In spite of previous hypothesis, early subclinical inflammation may trigger the chronic sequence of kidney fibrosis, resulting in kidney graft failure. The authors reviewed the different immunosuppressive protocols used in kidney transplantation, focusing in minimization protocols and their impact in kidney transplant fibrosis emphasizing the benefit to use calcineurin inhibitors, in particular tacrolimus. Takiyama et al. proposed hypoxia and hypoxia inducible factors (HIFs) involvements in diabetic kidney and discussed their pathophysiological relevance. In this review they summarized the diverse roles of HIFs from the several aspects in diabetic nephropathy well including kidney protective roles of antidiabetic drug metformin and sirtuins.

Kidney fibrosis is important research topic for both clinicians and research scientists. Today we have neither magic drugs nor miracle method to cure kidney fibrosis. Despite such limitation in real world, as shown in this special issue, recent advance in the kidney fibrosis research would provide some clues for combating kidney fibrosis. We hope this special issue provides sufficient and useful information for clinical/basic science researchers to design the therapeutic approach and the future research directions.

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Review Article

Role of Nutrient-Sensing Signals in the Pathogenesis of Diabetic Nephropathy

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Diabetic nephropathy is the leading cause of end-stage renal disease worldwide. The multipronged drug approach still fails to fully prevent the onset and progression of diabetic nephropathy. Therefore, a new therapeutic target to improve the prognosis of diabetic nephropathy is urgently required. Nutrient-sensing signals and their related intracellular machinery have evolved to combat prolonged periods of starvation in mammals; and these systems are conserved in the kidney. Recent studies have suggested that the activity of three nutrient-sensing signals, mTORC1, AMPK, and Sirt1, is altered in the diabetic kidney. Furthermore, autophagy activity, which is regulated by the above-mentioned nutrient-sensing signals, is also altered in both podocytes and proximal tubular cells under diabetic conditions. Under diabetic conditions, an altered nutritional state owing to nutrient excess may disturb cellular homeostasis regulated by nutrient-responsible systems, leading to exacerbation of organelle dysfunction and diabetic nephropathy. In this review, we discuss new findings showing relationships between nutrient-sensing signals, autophagy, and diabetic nephropathy and suggest the therapeutic potential of nutrient-sensing signals in diabetic nephropathy.

1. Introduction

Nutrient-sensing pathways dependent on extracellular nutrient conditions are well conserved among eukaryotes from yeasts to mammals. Recently, the importance of three nutrient-sensing pathways in metabolism development has become clear as the study of diabetes and obesity advances (Figure 1). Generally, in excessive nutrient conditions, the mammalian target of rapamycin (mTOR) is activated by increases in glucose, amino acid, and insulin levels [1, 2]. However, in nutrient-depleted conditions, AMP-activated protein kinase (AMPK) and oxidized NAD- (NAD^+-) dependent histone deacetylase (Sirt1) are activated by increases in intracellular AMP and NAD^+ levels, respectively [3–5]. These nutrient-sensing pathways use posttranslational phosphorylation and acetylation modification of proteins to regulate energy homeostasis in metabolic organs under both excess and restricted nutrient conditions [1–5]. Dysregulation of these pathways is associated with the development of diabetes [1–5].

Interestingly, these pathways also exist in the kidney [6–9], and tissue concentrations of glucose, amino acid, AMP, and NAD^+ are modified by particular nutrient states, such as diabetes, and diet therapies, such as protein or dietary restriction. These findings led us to consider whether these signaling pathways are involved in the pathogenesis of renal diseases and whether they may be potential therapeutic targets for treating renal diseases associated with diabetes and obesity [9].

In the multiple organs involved in diabetes and obesity, cells experience stress with nutrient excesses, leading to organelle dysfunction, including mitochondrial stress [10], peroxisomal oxidative stress [11, 12], and endoplasmic reticulum (ER) stress [2], which are thought to be pathological factors in diabetic nephropathy [11–14]. We considered how it might be possible to protect organelles against stresses mediated by excessive nutrient conditions.

The study of "autophagy" in mammalian systems is advancing rapidly, and many researchers are entering this new and exciting field. Autophagy plays a critical role in

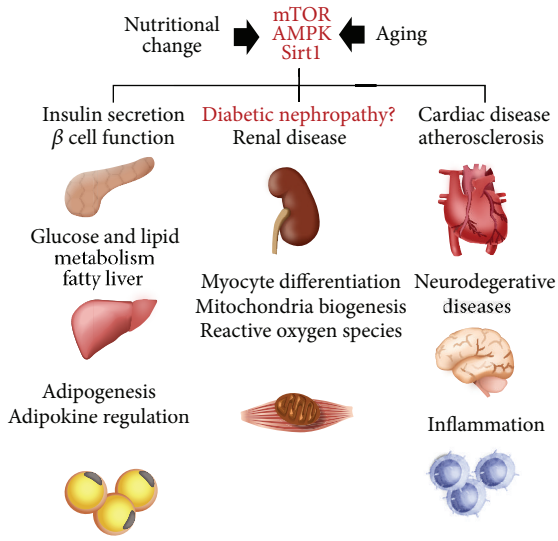

Figure 1: Nutrient-sensing signal and diseases. The three nutrients-sensing pathways, mTOR, AMPK, and Sirt1, independently and coordinately regulate organ metabolism in multiple organs. Their alterations are involved in the pathogenesis of obesity-related and age-related diseases.

removing damaged organelles for the maintenance of intracellular homeostasis [15]. Interestingly, this process is also regulated by the above-mentioned nutrient-sensing pathways [16, 17].

In this paper, we review the roles of nutrient-sensing pathways in renal diseases associated with diabetes and obesity and provide a perspective that may assist future research in this field.

2. mTOR in Diabetic Nephropathy

Of the three nutrient-sensing pathways, the pathological roles of mTOR and the therapeutic potential of rapamycin, an inhibitor of mTOR, in diabetic nephropathy are increasingly being examined in experimental animal models (Table 1).

mTOR is a nutrient-sensing signal which was first identified in yeast mutants resistant to the effects of rapamycin, an immunosuppressive agent. mTOR associates with the nonenzymatic regulatory-associated scaffold proteins of TOR in mTOR complex 1 (mTORC1) and the rapamycin-insensitive companion of mTOR in mTOR complex 2 (mTORC2) [1]. Rapamycin can inhibit the function of predominantly mTORC1 but not mTORC2.

Activation of mTORC1 most prominently results in phosphorylation of two downstream targets, ribosomal S6 kinase and eukaryotic translation-initiation factor 4E-binding protein [1]. These proteins stimulate ribosome biogenesis and protein translation, leading to increases in cell mass [1]. However, current information on the exact role and regulatory mechanisms of mTORC2 is still limited. Thus, knowledge of the role of mTOR in diabetic nephropathy is largely limited to the role of mTORC1.

Over the past few years, the role of mTOR in the pathogenesis of diabetic nephropathy has received increased attention. Studies using rapamycin were important in defining the role of the mTORC1 pathway. After being investigated primarily for its antiproliferative effects, it was increasingly clear that the mTOR pathway has broad implications in both normal and diseased physiologies, including regulating cell and organ size [1]. Additionally, the role of rapamycin has been studied in a wide spectrum of kidney diseases, including diabetic nephropathy [6, 27]. It has long been known that typical features of diabetic nephropathy include renal and glomerular hypertrophy, proteinuria, and advanced fibrosis and inflammation. In addition to inhibiting renal and glomerular hypertrophy present in the early stages of diabetic nephropathy [20], rapamycin seems to ameliorate mesangial expansion, glomerular basement membrane thickening, renal fibrosis and macrophage recruitment, and the development of proteinuria in diabetic rodent models [18, 19, 21–25] (Table 1). These findings suggest that mTOR activation may contribute to the pathogenesis of typical lesions, such as glomerular sclerosis and renal hypertrophy, in diabetic nephropathy.

Two recent studies using heterozygous-raptor knockout mice revealed that hyperactivation of the mTORC1 signal is strongly associated with the progression of podocyte injury and proteinuria in diabetic animal models [27, 28]. In the podocyte of heterozygous-raptor knockout mice showed a resistance to diabetes-induced dysregulation of foot process formation and apoptosis leading to podocyte loss. Furthermore, we recently reported that obesity-mediated hyperactivation of the mTORC1 signal led to the suppression of autophagy activity in proximal tubules resulting in the development of cell vulnerability, which were ameliorated by calorie restriction and rapamycin [26]. These findings suggest that mTORC1 activation in all kinds of renal component cells may be involved in the pathogenesis of diabetic nephropathy.

mTOR is activated by both insulin and nutrients such as glucose and amino acids in renal cells in diabetic and obese states. Because hyperglycemia occurs along with hyperinsulinemia in obese type 2 diabetic patients, combined hyperinsulinemic and hyperglycemia would be expected to activate the mTOR pathway in obese and type 2 diabetic patients. However, hyperglycemia, rather than growth factors such as insulin, may contribute more to mTOR activation in diabetes because mTOR is activated even in the kidneys of insulin-independent type 1 diabetic models [6].

In summary, mTOR induces the synthesis of matrix proteins associated with basement membrane thickening and mesangial matrix accumulation. Additionally, mTOR enhances fibrosis through fibroblast proliferation, epithelial-to-mesenchymal transition, and the expression of profibrotic cytokines such as transforming growth factor-β1 and connective tissue growth factor. mTOR-dependent infiltration of macrophages and production of proinflammatory cytokines, such as MCP-1, might support inflammation during diabetic nephropathy [18, 19, 21–25]. Additionally, hyperactivity of mTORC1 signal is strongly related to podocyte dysfunction characterized by dysregulation of nephrin protein and

Table 1: The roles of mTOR (mTORC1) in diabetic nephropathy.

Experimental type	Renal outcome/phenotype	Mechanism	Reference
S6 Kinase $1^{-/-}$ mice	Renal hypertrophy↓	Inhibition of p70S6 kinase	Chen et al. [18]
Rapamycin (db/db mice)	Renal and glomerular hypertrophy↓	eEF2 kinase phosphorylation and laminin β1 expression	Sataranatarajan et al. [19]
Rapamycin (STZ-diabetic mice)	Renal hypertrophy↓	Inhibition of p70S6 kinase	Sakaguchi et al. [20]
Sirolimus (STZ-diabetic rats)	Glomerular hypertrophy↓, podocyte loss↓	Decreases of TGF-β and VEGF expression	Wittmann et al. [21]
Rapamycin (db/db mice)	Albuminuria↓, glomerular lesion↓	Inhibition of p70S6 kinase	Mori et al. [22]
Rapamycin (STZ-diabetic rats)	Albuminuria↓, glomerular lesion↓, and inflammation↓	Decreases of TGF-β, VEGF, and MCP-1 expression	Yang et al. [23]
Rapamycin (STZ-diabetic rats)	Albuminuria↓, glomerular lesion↓	Decreases of TGF-β, CTGF, and α-SMA expression	Lloberas et al. [24]
$Gas6^{-/-}$ mice	Glomerular hypertrophy↓, mesangial expansion↓	Inhibition of akt and p70S6 kinase	Nagai et al. [25]
Rapamycin (HFD-induced obesity)	Proximal tubular cell damage↓	Increase of autophagy activity	Yamahara et al. [26]
Podocyte-specific raptor-heterozygous mice (db/db mice)	Albuminuria↓, glomerular lesion↓	Inhibition of mislocalization of nephrin	Inoki et al. [27]
Podocyte-specific raptor-heterozygous mice (STZ-diabetic mice)	Albuminuria↓, glomerular lesion↓	Inhibition of podocyte loss	Godel et al. [28]

STZ; streptozotocin, PTECs: proximal tubular epithelial cells, TGFβ: transforming growth factor β, VEGF: vascular endothelial growth factor, MCP-1: monocyte chemoattractant protein-1, CTGF: connective tissue growth factor, α-SMA: α-smooth muscle actin, and PARP: p(ADP-ribose) polymerases.

podocyte loss [27, 28]. Finally, more recent reports demonstrated that mTOR contributes to proximal tubular cell apoptosis and damage in diabetes [26, 29]. Thus, it is evident that mTOR plays a central role in the development of the major features of diabetic nephropathy, although additional mechanisms are likely to be involved.

Caution is necessary when interpreting the role of mTOR in diabetic nephropathy because many of the current insights regarding mTOR are derived from pharmacological inhibition of mTOR by rapamycin [30]. Additionally, as mentioned above, our understanding of the regulatory mechanisms and substrates of mTORC2 is very limited. Thus, kidney tissue-specific analysis of mTORC1- and mTORC2-dependent signaling in mice is required to further understand the functional role of mTOR in diabetic nephropathy.

3. AMPK in Kidney Diseases

AMPK is a ubiquitously expressed kinase that also acts as an energy sensor [3]. Kinase activity is strictly regulated by extracellular nutrient conditions, which are detected as rising concentrations of AMP, and increases in AMP/ATP ratios [3]. Additionally, AMPK activity is positively regulated by an adipokine, adiponectin [31]. During energy-depleted conditions, there is a marked increase in both intracellular concentrations of AMP and plasma adiponectin levels, leading to activation of AMPK. Activated AMPK acts to restore energy homeostasis by phosphorylating multiple substrates to enhance energy production and suppress energy consumption [3]. In metabolic organs such as skeletal muscle, brown adipose tissue, and the liver, AMPK enhances lipolysis through phosphorylation of acetyl-CoA carboxylase (ACC) and mitochondrial biogenesis and suppresses hepatic gluconeogenesis [3]. Thus, AMPK acts as a nutrient sensor to maintain cell and tissue homeostasis under energy-depleted conditions.

Several studies on whole renal cell lysates have demonstrated that AMPK expression and activity in the kidney are determined by phosphorylation at Thr^{172} [32–36]. Immunoelectron microscopy analysis of its exact localization in glomeruli revealed that AMPK is expressed in podocytes, mesangial cells, and glomerular endothelial cells, although its expression pattern in different renal tubular epithelial cells remains uncertain.

Recently, our understanding of the pathophysiological role of AMPK in the kidney has increased [8]. The expression and activity of AMPK have been examined in several types of diabetic rodent models (Table 2). In streptozotocin-(STZ-) induced type 1 diabetic models, AMPK expression and activity decreased [32–34]. The exact mechanism underlying the decrease in renal AMPK activity in this model is unknown; however, it is possible that decreases in plasma adiponectin, in addition to hyperglycemia, could contribute to this decrease [33]. Pharmacological activation of AMPK by 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) and metformin ameliorated renal hypertrophy in STZ-induced diabetic rats [34]. In diet-induced obese mouse models and type 2 diabetic db/db mice, renal AMPK activity was suppressed and plasma adiponectin levels were reduced [35, 36]. Interestingly, adiponectin was able to stimulate AMPK activity in podocytes, mesangial cells, and glomerular endothelial cells [39], and adiponectin-deficient mice showed development of albuminuria together with decreased AMPK activity in podocytes [38]. These

Table 2: The activity and pathophysiological roles of AMPK in kidney disease.

Experimental type	AMPKα activity and Renal outcome	Mechanism	Reference
Diabetic models			
STZ-diabetic rats	AMPKα expression↑, AMPKα activity↓	Unclear	Cammisotto et al. [32]
STZ-diabetic rats	AMPKα activity↓	Plasma adiponectin↓	Guo and Zhao [33]
Treatment of AICAR and metformin (STZ-diabetic rats)	AMPKα activity↑, renal hypertrophy↓	Unclear	Lee et al. [34]
Db/db mice	AMPKα activity↓	Unclear	Kitada et al. [35]
High-fat diet-induced obese mice	AMPKα activity↓, renal lipogenesis↑	Unclear	Kume et al. [36]
Treatment of AICAR	AMPKα activity↑, Albuminuria↓, and glomerular lesion↓	Improvement of mitochondria dysfunction	Dugan et al. [37]
Nondiabetic models			
Adiponectin$^{-/-}$ mice	AMPKα activity↓ in podocytes	Adiponectin deficiency	Sharma et al. [38]
Treatment of adiponectin and AICAR	AMPKα activity↑ (podocytes, mesangial cells, and glomerular endothelial cells)	Adiponectin receptor-dependent	Cammisotto and Bendayan [39]

STZ; streptozotocin, AICAR: 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside.

findings suggest that activation of the adiponectin-AMPK pathway may be a therapeutic target for diabetes- and obesity-related kidney diseases.

It is likely that AMPK activity decreases in the kidneys of diabetic and obese animals, which raises the question of how decreases in AMPK activity are involved in the pathogenesis of renal diseases. A study has reported that, in the kidney of obese mice, intrarenal lipid metabolism was altered and was characterized by enhanced renal lipogenesis and suppressed lipolysis [36]. AMPK-mediated phosphorylation inactivates a lipogenic enzyme, ACC, which results in decreased lipogenesis and enhanced lipolysis [3]. Decreases in kidney AMPK activity in these mouse models may be involved in altered renal lipid metabolism and subsequent lipotoxicity-associated renal damage. Recent reports suggest that altered mitochondrial biogenesis and subsequent ROS production under excessive nutrient conditions contribute to the pathogenesis of metabolic diseases such as diabetes and obesity. Proximal tubular cells contain a large number of mitochondria because the energy demand in these cells is relatively higher than those of other cells. Thus, the altered mitochondrial biogenesis observed under diabetic conditions might be associated with ROS production and subsequent tubular damage [35]. Collectively, decreases in renal AMPK activity may cause the typical features of renal disease in obese and diabetic patients. Actually, pharmacological activation of AMPK by AICAR significantly improved renal injury including albuminuria in diabetic Akita mice [37].

4. Sirt1 in Kidney Disease

Calorie restriction slows aging and increases life span in different species. Even if calorie restriction is shown to increase life expectancy in humans, it is unlikely that such restrictions will be widely adopted because of the difficulty in maintaining long-term calorie restriction in modern society. Therefore, there has been increased interest in identifying molecules that act as "calorie restriction mimetics." Interestingly, several recent studies have revealed that Sirt1, one of seven mammalian sirtuin/Sir2 genes in the NAD^+-dependent deacetylase family, could be a common mediator that explains the health benefits under calorie restriction [4, 5]. Furthermore, several recent reports have shown that Sirt1 regulates several biological functions, including cell survival, mitochondrial biogenesis, insulin secretion, and glucose and lipid metabolism in various tissues [5].

Reduced forms of nicotinamide adenine dinucleotide (NADH) are metabolites of glucose and fatty acids. Thus, NAD^+/NADH ratios decrease in cells under nutrient excess. Sirt1 senses intracellular NAD^+ levels and acts as a deacetylase in a NAD^+-dependent manner. Protein acetylation can be regulated in a metabolically responsive manner, and this process may also contribute to adaptation to metabolic stress. Collectively, these findings suggest that Sirt1's deacetylase activity decreases in nutrient excess conditions, and its activation, which acts as a calorie restriction mimetic, may be a new therapy for renal disease in obese and diabetic patients. However, the relationship between Sirt1 and diabetic complications is not well known. We discuss here the role of Sirt1, and speculate on the possible involvement of Sirt1, in renal diseases in patients with diabetes and obesity.

The effects of nutrient conditions on Sirt1 expression levels in the kidney have been determined (Table 3). Dietary restriction in rats increases Sirt1 expression, which is associated with decreases in plasma growth factors such as insulin/IGF-1 [42]. In STZ-induced type 1 diabetic kidneys, Sirt1 expression decreases [40, 41], but Sirt1 expression does

Table 3: The activity and pathophysiological roles of Sirt1 in kidney disease.

Experimental type	Renal outcome/phenotype	Mechanism	Reference
Activity/expression			
STZ-diabetic rats	Sirt1 expression↓	Unclear	Tikoo et al. [40]
STZ-diabetic rats	Sirt1 expression↓	Unclear	Li et al. [41]
Db/db mice	Sirt1 expression →	Unclear	Kitada et al. [35]
Calorie-restricted rats	Sirt1 expression↑	Insulin/IGF-1↓	Cohen et al. [42]
STZ- and db/db mice	Sirt1 expression↓	NMN depletion	Hasegawa et al. [43]
Pathophysiological roles			
$Sirt1^{+/-}$ mice (PTECs)	Renal aging↑	Autophagy deficiency	Kume et al. [16]
$Sirt1^{+/-}$ mice (Medullary cells)	UUO-induced renal fibrosis↑	Decrease of Cox2 expression	He et al. [44]
PTECs-specific Sirt1-TG mice	ROS- and cisplatin-induced PTECs damage↓	Increase of catalase expression	Hasegawa et al. [45]
Treatment with resveratrol	UUO-induced fibrosis↓	Suppression of TGFβ-Smad3 pathway	Li et al. [46]
Sirt1 overexpression (mesangial cells)	ROS-induced apoptosis↓	Inactivation of p53	Kume et al. [47]
Sirt1 overexpression (mesangial cells)	TGFβ-induced apoptosis↓	Inactivation of Smad7	Kume et al. [48]
Treatment of SRT1720 (PTECs)	Mitochondrial biogenesis↑, ROS↓	Activation of PGC-1α	Funk et al. [49]
PTECs-specific Sirt1-TG mice	Diabetes-induced podocyte injury↓	Epigenetic mechanism	Hasegawa et al. [43]

STZ; streptozotocin, PTECs: proximal tubular epithelial cells, ROS: reactive oxygen species, UUO: unilateral ureteral obstruction, TGFβ: transforming growth factor β, IGF-1: insulin-like growth factor 1, Cox2: cyclooxygenase 2, PGC-1α: peroxisome proliferator-activated receptor γ coactivator-1α, and NMN; nicotinamide mononucleotide.

not change in kidneys of type 2 diabetic db/db mice [35]. Sirt1 activity is strictly regulated by intracellular NAD^+ concentrations [50], which are likely to decrease in diabetic organs. Thus, to determine whether Sirt1 activity is altered in diabetic kidneys, it is necessary to assess the deacetylation of Sirt1 substrates such as NFκB (p65), p53, and forkhead proteins [4].

Many researchers are entering this exciting field, and interesting findings showing the renoprotective effects of Sirt1 have been reported (Table 3). Sirt1 expression and activity determined by deacetylase activity on the Foxo3a substrate were significantly altered in aging kidneys [16]. Furthermore, heterozygous Sirt1-deficient mice showed dietary restriction-resistant premature renal aging [16]. This evidence suggests that Sirt1 may be involved in renal aging and dietary restriction-mediated renoprotection.

Tubulointerstitial fibrosis is a common final pathway for end-stage renal diseases, including diabetic nephropathy. Several recent reports have shown the antifibrotic effects of Sirt1 in mouse experimental models [44, 46]. In those reports, mechanisms of Sirt1's antifibrotic effects were explained by suppression of both cyclooxygenase 2 expression [44] and activation of TGFβ-induced Smad3 [46], both of which are recognized as classical pathogenic factors in diabetic nephropathy. Additionally, in vivo studies by Hasegawa et al. showed direct renoprotective effects of Sirt1 overexpression in proximal tubular epithelial cells (PTEC) [45]. Proximal tubular cells with specific Sirt1 overexpression were resistant to cisplatin-mediated PTEC damage because of overexpression of catalase and subsequent peroxisome protection [45]. Funk et al. showed that the specific Sirt1 activator, SRT1720, enhanced mitochondrial biogenesis through deacetylation of PGC1 and subsequently reduced ROS in cultured proximal tubular cells [49]. The role of Sirt1 in glomerular cells is still small. In cultured mesangial cells, Sirt1 showed antiapoptotic effects against ROS and TGFβ [47, 48].

Based on more recent reports, Sirt1 activity is likely to be suppressed in the kidney of diabetic animal models [43, 51]. Interestingly, Hasegawa et al. reported that Sirt1 activity in both proximal tubular cells and podocytes was associated with diabetic nephropathy [43]. Interestingly, proximal tubular cell-specific Sirt1 transgenic mice showed a resistance to diabetes-related progression of podocyte damage and subsequent proteinuria [43]. Collectively, these results strongly support Sirt1 activation being considered as a new therapy to improve the prognosis of diabetic nephropathy.

5. Organelle Dysfunction and Renal Disease in Diabetes and Obesity

In addition to the classical pathogenesis of diabetic nephropathy, organelle dysfunction, such as in mitochondria [35], peroxisomal oxidative stress [11], and ER stress [13], has been proposed as a theory to explain the emergence of pathological features in diabetic nephropathy (Figure 2). ROS are an inevitable by-product of mitochondrial and peroxisomal metabolism. And excess ROS is implicated with classical pathogenesis by initiating events in diabetic nephropathy [11–14, 35]. In nutrient excess conditions, such as diabetes

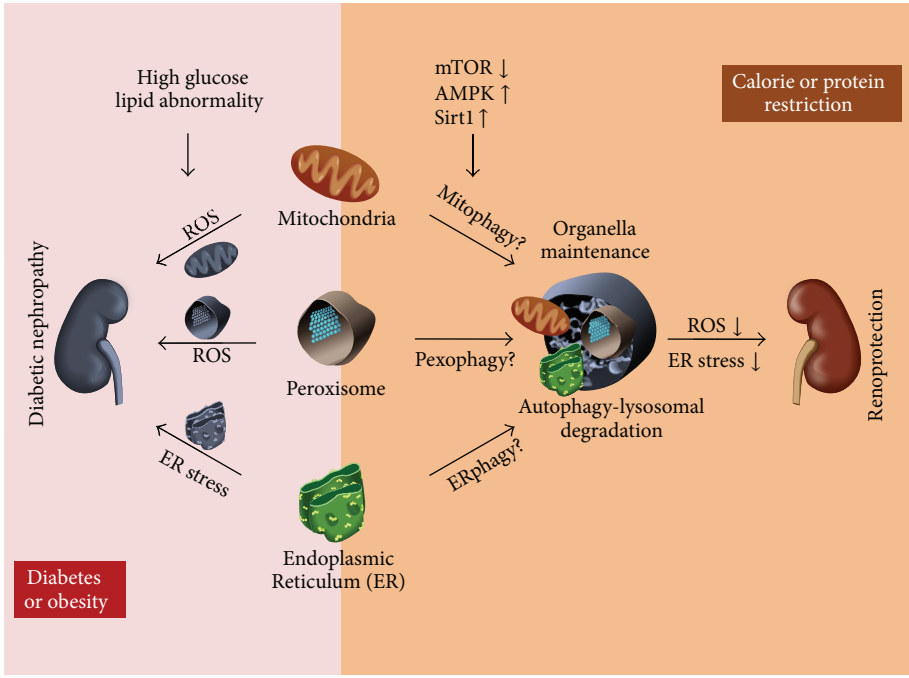

Figure 2: Organelles, such as mitochondria, peroxisome, and ER, dysfunction causes accumulation of reactive oxygen species (ROS) and ER stress in diabetic kidney. Dietary restriction enhances autophagy-lysosomal degradation system, leading to cell or tissue homeostasis. Nutrient-sensing signal and organella maintenance in diabetic nephropathy (DN).

and obesity, high glucose levels enhance the generation of mitochondrial ROS, and high plasma levels of free fatty acids can also cause ROS overproduction from mitochondria and peroxisomes. Therefore, maintaining healthy populations of functional mitochondria and peroxisomes in different renal cells under nutrient excess conditions is essential for the well-being of cells. This maintenance may prevent the development of renal disease in cases of diabetes and obesity.

ER contains quality-control mechanisms to handle misfolded proteins, and thus ER stress refers to physiological or pathological states that result in the accumulation of misfolded proteins. ER stress in renal pathophysiology is a relatively new area of research in proteinuric kidney diseases, including diabetic nephropathy. Hyperglycemia and high levels of free fatty acids cause ER stress in podocytes [52], which undergo apoptosis and the subsequent initiation of proteinuria in diabetic nephropathy. Additionally, in proteinuric kidney diseases, including overt stages of diabetic nephropathy, proteinuria filtered from glomeruli enhances ER stress responses in proximal tubules, leading to progression of tubulointerstitial lesions [53]. Thus, maintaining the ER's capacity for handling misfolded proteins in podocytes and tubular cells could be considered a new therapeutic target for protecting kidneys from proteinuria-related pathologies.

If this is the case, it remains to be determined how to protect organelles from stresses derived from nutrient excesses. Autophagy is an intracellular process for the degradation of proteins and organelles via lysosomes for the control of cell homeostasis [15]. This degradation system maintains cell homeostasis under nutrient-depleted conditions and has been generally well conserved among all types of eukaryotes. Nutrient excesses inactivate autophagy, but once nutrients are depleted, autophagy is activated to provide energy resources for cells. Interestingly, autophagy is closely regulated by nutrient-sensing pathways and by cellular stresses [16, 17, 54, 55]. Inhibition of mTOR and activation of AMPK and Sirt1 activate nonselective autophagy in response to nutrient depletion [16, 17]. Furthermore, oxidative stress derived from mitochondria and peroxisomes or ER stress can cause autophagy to degrade the damaged organelle itself (organelle-selective autophagy: mitophagy, pexophagy, and ERphagy) [2, 54, 56].

Recent reports have shown that autophagy deficiencies under nutrient excess conditions are related to the pathogenesis of obesity- or aging-associated diseases [15]. Currently, nephrologists are also entering this field of study. Although direct evidence showing the relationship between autophagy and diabetic nephropathy is unfortunately still weak, several interesting findings regarding autophagy have been recently demonstrated in the field of nephrology. Age-associated declines in autophagy in podocytes and proximal tubular cells are part of the development of key features in renal aging, albuminuria, glomerulosclerosis, and ROS production as a result of the accumulation of damaged mitochondria in proximal tubular cells [16, 57, 58]. Interestingly, Sirt1-mediated activation of autophagy under hypoxic conditions in proximal tubular cells is essential for dietary restriction-mediated antirenal aging [16]. Additionally, autophagy was activated in a renoprotective manner in ischemic-reperfusion and cisplatin-induced kidney injury mouse models [58–61].

Thus, autophagy is required for maintaining homeostasis in podocytes and proximal tubular cells and is activated under stress conditions to protect renal cells.

If autophagy is inactivated by mTOR activation and AMPK and Sirt1 inactivation in obesity and diabetic renal diseases, autophagy deficiency could enhance organelle dysfunction mediated by nutrient excess, hypoxia, and proteinuria. Organelle degradation systems regulated by nutrient conditions may be involved in the pathogenesis of renal diseases associated with obesity and diabetes. We previously found that autophagy activity was suppressed in an mTORC1-dependent manner in proximal tubular cells of obese mice, leading to more severe proteinuria-induced tubular interstitial lesions [26].

Autophagy could be a new therapeutic strategy for the treatment of diabetic nephropathy, although solid evidence of the involvement of autophagy in the pathogenesis of podocyte injury is lacking.

6. Diet Therapy in Renal Diseases in Diabetes and Obesity

Diet or calorie restriction is essential for strict glycemic control in diabetic patients without advanced nephropathy. Based on results from a large number of experimental studies on diabetes and obesity, ameliorating the above-mentioned nutrient pathways could be a mechanism underlying dietary restriction-mediated improvement of obesity and diabetes. Additionally, recent human clinical studies have shown that the quality of diet affects the development of insulin resistance and new onset of diabetes. A high fructose intake rather than glucose [62], and ingestion of high concentrations of plasma branched-chain amino acids [63], and saturated fatty acids rather than polyunsaturated fatty acids [64] are independent risk factors for developing insulin resistance and diabetes.

In patients with diabetic nephropathy, diet therapies, such as protein restriction, have been used. However, the efficacy of protein restriction is now controversial. If altered nutrient-sensing pathways, lipotoxicity, and organelle dysfunction in kidneys under excess nutrient stresses are involved in the pathogenesis of renal diseases, a high energy intake along with a low-protein diet may cause an insufficiency of protein restriction in the treatment of diabetic nephropathy. It may, therefore, be time to reconsider whether energy excess along with protein restriction is really beneficial. Also, we need to identify what types of carbohydrates, amino acids, and fatty acids in different diet regimens lead to a better prognosis in renal diseases associated with diabetes and obesity. Amelioration of nutrient-sensing pathways altered in the kidney of diabetic and obese animals may be a possible experimental target to examine the beneficial effects of diet therapy.

7. Concluding Comments

Currently, the incidence of obesity and diabetes associated with nutrient excess is increasing worldwide. Accordingly, diabetic nephropathy is the leading cause of end-stage renal disease, despite the application of various intensive therapy programs, such as hypoglycemic and antihypertensive therapy. The identification of additional new therapeutic targets for the prevention of renal diseases associated with diabetes and obesity is urgently needed.

This paper has provided a perspective on whether nutrient-sensing pathways are involved in the pathogenesis of diabetic nephropathy and whether they are acceptable new therapeutic targets. In the next few years, additional studies in conditional knockout mice, transgenic mice, and new disease models will elucidate this possibility. These studies will ultimately provide a clearer perspective on whether nutrient-sensing pathways and organelle maintenance by autophagy should be considered novel therapeutic targets for the treatment of renal diseases in diabetes and obesity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Effect of Angiotensin II and Small GTPase Ras Signaling Pathway Inhibition on Early Renal Changes in a Murine Model of Obstructive Nephropathy

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Tubulointerstitial fibrosis is a major feature of chronic kidney disease. Unilateral ureteral obstruction (UUO) in rodents leads to the development of renal tubulointerstitial fibrosis consistent with histopathological changes observed in advanced chronic kidney disease in humans. The purpose of this study was to assess the effect of inhibiting angiotensin II receptors or Ras activation on early renal fibrotic changes induced by UUO. Animals either received angiotensin II or underwent UUO. UUO animals received either losartan, atorvastatin, and farnesyl transferase inhibitor (FTI) L-744,832, or chaetomellic acid A (ChA). Levels of activated Ras, phospho-ERK1/2, phospho-Akt, fibronectin, and α-smooth muscle actin were subsequently quantified in renal tissue by ELISA, Western blot, and/or immunohistochemistry. Our results demonstrate that administration of angiotensin II induces activation of the small GTPase Ras/Erk/Akt signaling system, suggesting an involvement of angiotensin II in the early obstruction-induced activation of renal Ras. Furthermore, upstream inhibition of Ras signalling by blocking either angiotensin AT1 type receptor or by inhibiting Ras prenylation (atorvastatin, FTI o ChA) reduced the activation of the Ras/Erk/Akt signaling system and decreased the early fibrotic response in the obstructed kidney. This study points out that pharmacological inhibition of Ras activation may hold promise as a future strategy in the prevention of renal fibrosis.

1. Introduction

Renal interstitial fibrosis is a common histopathological endpoint in all forms of progressive renal diseases independently of their etiology. Unilateral ureteral obstruction (UUO) is a well-established experimental model in mice leading to tubulointerstitial fibrosis in the ligated kidney [1, 2]. Increased synthesis of angiotensin II (Ang II) has been implicated as playing a causative role in progression of kidney damage in obstructive nephropathy [3–5]. Ang II is directly profibrotic but also acts as a proinflammatory cytokine in the kidney through the activation of the nuclear factor-κB family of transcription factors, which in turn, induces an autocrine response enhancing both Ang II and tumor necrosis factor

alpha production [6]. By promoting tubulointerstitial infiltration of inflammatory cells, Ang II also contributes to the onset and progression of renal damage in UUO [6]. However, studies examining the effect of directly targeting Ang II in UUO-induced renal damage have yielded contradictory results. Thus, whereas it has been reported that Ang II receptor blockade or angiotensin-converting enzyme inhibition abrogates fibrosis deposition and myofibroblast proliferation in some studies of obstructive nephropathy [7–9], in other studies these interventions have been shown to aggravate renal damage after UUO [10–12]. These differences may be due to whether obstruction was partial or complete but they also suggest that optimal strategies may be better targeted downstream of the Ang II receptor complex, focused on those pathways that are implicated in the proinflammatory and profibrotic responses to Ang II.

Actions of Ang II are mediated via the Ang II type I (AT1) and type II G-protein coupled receptors (GPCR), as well as the receptor tyrosine kinases (RTK)s [13, 14]. Specific profibrotic actions of Ang II are implicated in the appearance of alpha-smooth muscle actin (α-SMA)-positive fibroblasts or myofibroblasts and fibronectin accumulation which are considered prominent features of fibrosis development in the obstructed kidney [15, 16].

Ras monomeric GTPases play a major role in the control of proliferation, differentiation, and cell death, connecting activated RTKs and GPCRs to the effector pathways Raf/mitogen-activated protein kinase (MAPK)/extracellular signal regulated kinases 1 and 2 (ERK1/2) and phosphatidylinositol-3 kinase (PI3 K)-Akt [17]. Many growth factors are known to activate intracellular signaling pathways that converge on Ras activation, including Ang II which has been reported to stimulate renal Ras/MAPK pathway *in vivo* and *in vitro* [18, 19]. Thus, Muthalif et al. [18] have demonstrated that Ang II infusion for 6 days induces hypertension and renal Ras activation, and both phenomena are reversed by administration of a farnesyl transferase inhibitor (FTI). However, the effects of acute Ang II administration on Ras activation have not been assessed.

Activation of Ras and its effectors ERK1/2 and PI3 K/Akt has been reported as mediators in progressive renal damage [20, 21]. Activation of Ras signaling pathway occurs after early UUO [22, 23], demonstrating a contribution of Ras downstream effectors to renal injury with a main involvement of ERK1/2 in apoptotic events and Akt in proliferative and fibrotic response [23]. There are several Ras isoforms (H-, N- and K-Ras) with different functional properties in fibrotic processes and in fibroblast biology [24–26]. Thus, we have observed that H-Ras knock-out (KO) mice show lower fibrosis after UUO [27], whereas in embryonic fibroblasts obtained from H-Ras or N-Ras KO mice, fibronectin and collagen synthesis were higher and proliferation and migration were lower than in wild type fibroblasts [24, 25]. Moreover, K-Ras knock-down decreases stimulated proliferation in renal fibroblasts [28] and inhibits fibrosis in a rat experimental model [29]. It is known that activation of Ras requires several posttranslational modifications that include prenylation, the addition of either the 15-carbon isoprenoid farnesyl or the 20-carbon isoprenoid geranylgeranyl to cysteine residue(s) at or near the C-termini of Ras proteins, allowing their anchorage to the cell membrane and subsequent activation [30]. Some evidence exists to demonstrate that inhibition of prenylation reduces extracellular matrix production by fibroblasts "*in vitro*" [31]. Thus, we aimed to interrogate the effects of decreasing prenylation on early UUO-induced Ras pathway activation and associated fibrotic responses in the mouse kidney. We used multiple strategies to address the question: (1) by inhibiting the synthesis of farnesyl groups using atorvastatin, an inhibitor of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase-involved in the synthesis of isoprenoid groups required for Ras prenylation [32, 33], (2) by directly inhibiting Ras farnesylation using a farnesyl transferase inhibitor [34], and (3) by treating with chaetomellic acid A, which selectively blocks H-Ras farnesylation [35] with reduced off-target toxicity relative to FTIs [36].

2. Methods

2.1. Animals and Disease Model. C57BL/6J male mice were kept in a germ-free facility, under controlled environmental conditions (Unidad de Experimentación Animal, University of Salamanca, Spain). Mice were reared on standard chow (Panlab, Barcelona, Spain) and provided with water *ad libitum*. Surgical techniques to produce UUO were performed as previously described on animals at 2 months of age [23]. All procedures were approved by the Committee for Animal Care and Use of the University of Salamanca and complied with the Guide for the Care and Use of Laboratory Animals of National Research Council.

2.2. Pharmacological Groups and Drug Administration

Angiotensin (Ang II) Group. A group of mice was treated with a single intraperitoneal dose of Ang II (0.8 mg/kg; Sigma, Saint Louis, MO, USA; $n = 3$ per time point) or saline vehicle (NaCl 0.9%; $n = 3$). No surgery was performed in this group of animals and kidneys were removed either 30 minutes, 4, or 12 hours after Ang II administration.

Losartan Group. A group of animals received a daily intraperitoneal injection of losartan (40 mg/kg; Du Pont, Wilmington, DE, USA; $n = 5$), whereas the corresponding control group received the vehicle isotonic saline (NaCl 0.9%; $n = 3$), for 4 days. UUO was carried out on the second day of treatment.

Atorvastatin Group. A group of mice were treated with atorvastatin calcium (70 mg/kg/day; Pfizer, Madrid, Spain; $n = 4$) by oral gavage, and the corresponding control mice group were treated with carboxymethylcellulose vehicle (Sigma, Saint Louis, MO, USA; $n = 3$), once daily for 6 days. UUO was performed at the fourth day after initiating the treatment.

Farnesyl Transferase Inhibitor (FTI) Group. A group of mice received a subcutaneous injection of L-744,832 (40 mg/kg; Biomol Inc, Plymouth Meeting, PA, USA; $n = 5$) while a control group received the vehicle solution (17 mM sodium citrate, 94 mM sodium chloride; pH 5.4; $n = 3$), daily for 6 days. UUO was performed on the fourth day of treatment.

Chaetomellic Acid A Group. A group of mice received subcutaneously injected chaetomellic acid A (3 mg/kg/day; Santa Cruz Biotechnology, CA, USA; $n = 4$) during 6 days, whereas a control group received the vehicle solution (17 mM sodium citrate, 94 mM sodium chloride; pH 5.4; $n = 4$). UUO was performed on the fourth day of treatment.

2.3. Preparation of Kidney Tissue and Protein Analysis. At endpoint in each group, kidneys were removed under terminal anaesthesia. Methods used for protein analysis, including affinity precipitation of Ras-GTP or ELISA Ras activation Kit (Upstate Biotechnology, MA, USA) and immunodetection of proteins by Western blot and immunohistochemistry, have been already described [23, 25, 27]. As we have previously reported, the amount of loading controls for WB such as tubulin or GAPDH change after UUO [23], and thus we have decided to control strictly the amount of protein loaded instead to perform WB for these proteins.

2.4. Statistical Analysis. One-way analysis of variance (ANOVA) was applied for statistical analysis (NCSS 2000 program, Utah, USA). Bonferroni's or Kruskal-Wallis multiple-comparison tests were, respectively, employed for analysis of data with or without normal distribution. Data were expressed as mean ± Standard Error of the Mean (SEM). $P < 0.05$ or $Z > 1.96$ were considered statistically significant.

3. Results

3.1. Renal Activation of Ras Signaling Pathway after Short-Term Ang II Infusion. Renal Ras activation, measured by ELISA, was higher both at 4 and 12 hours after single dose Ang II administration than in saline-treated control group (Ctrl; Figure 1).

Western blot analysis also detected an Ang II-induced increase in renal activation of Ras signaling effectors, ERK1/2 and Akt, as demonstrated by measuring the ratio phosphorylated (p)/total protein at 4 hours after Ang II administration (Figures 1(b) and 1(c), resp.).

3.2. Effect of AT1 Receptor Antagonist on Ras Activation and Renal Changes after UUO. We aimed to determine the effect of AT1 receptor antagonist losartan on the activation of Ras/ERK/Akt signaling pathway and the expression of fibronectin and α-SMA in kidneys submitted to 3 days of UUO. Western blot analyses showed that levels of Ras-GTP, pERK, pAkt, total Akt, fibronectin, and α-SMA were significantly higher in obstructed (O) kidneys than in nonobstructed (NO) kidneys of vehicle-treated animals (Figures 2(a)–2(f)). The increase observed in total Akt levels in kidneys submitted to ureteral obstruction (Figure 2(d)) has been previously described [23]. After receiving losartan treatment, no significant differences were found for levels of activated RAS, fibronectin, and α-SMA in O kidneys compared with NO kidneys (Figures 2(a), 2(e) and 2(f), resp.), and a similar pattern was observed for pERK1/2 and pAkt (Figures 2(b) and 2(c), resp.). No differences between NO groups of treated and untreated groups were observed for any of the analyzed proteins.

3.3. Effect of Ras Activation Inhibitors on ERK1/2 and Akt Activation and Fibrotic Markers after UUO. Our next objective was to assess whether inhibition of Ras activation after UUO could modulate activation of its downstream effectors, ERK1/2, and Akt, as well as expression of fibronectin and α-SMA in ligated kidneys. For this purpose we treated the animals with either the HMG-CoA reductase inhibitor atorvastatin or the farnesyl transferase inhibitor (FTI) L-744,832 or chaetomellic acid A.

Western blot analysis revealed that whereas O kidneys of vehicle-treated mice showed higher levels of Ras-GTP, pERK1/2, pAkt, and total Akt proteins than NO kidneys, the differences between O and NO kidneys for Ras-GTP, pERK1/2, pAkt, and total Akt abundance were not significant (Figures 5(a)–5(d)) in atorvastatin-treated animals (Figures 5(a)–5(d)). Similar results were obtained for fibronectin and α-SMA, with lower amounts for both proteins detected in the O kidneys of animals treated with atorvastatin than in O kidneys of vehicle-treated mice (Figures 5(e) and 5(f), resp.). In agreement with these results, immunohistochemistry studies revealed reduced amount of fibronectin (Figures 3(a), 3(b), 3(e) and 3(f)) and interstitial α-SMA (Figures 4(a), 4(b), 4(e) and 4(f)) in the O kidneys of atorvastatin-treated mice than in the O kidneys of vehicle-treated animals.

Pull-down and Western blot analysis showed that O kidneys of FTI-treated mice presented significantly lower Ras activation than kidneys of vehicle-treated animals (Figure 6(a)). Administration of FTI significantly blunted the obstruction-induced increase in pERK1/2 when compared to control vehicle group, with lower levels of activated protein also found in NO kidneys of FTI (Figure 6(b)). A slight, nonsignificant decrease in the expression of pAkt and total Akt was observed in O kidneys from FTI-treated animals (Figures 6(c) and 6(d), resp.). We observed a marked and significant reduction in fibronectin levels of O kidneys after FTI treatment when compared with kidneys of animals receiving vehicle administration (Figure 6(e)). In agreement with these results, immunohistochemical analysis revealed lower fibronectin staining in the renal cortical interstitium of O kidneys from FTI-treated mice compared with mice receiving the vehicle (Figures 3(a), 3(b), 3(g) and 3(h)). Compared with untreated mice, O kidneys of FTI group showed a mild although nonsignificant reduction in α-SMA levels (Figure 6(f)), which correlates with the interstitial amount detected by Figures 4(a), 4(b), 4(g) and 4(h).

Western blot analysis showed significantly lower pERK1/2 and pAkt levels in O kidneys from mice that received chaetomellic acid A with respect to O kidneys of control mice (Figures 7(a) and 7(b)). As measured by Western blotting, the expression of α-SMA, but not that of fibronectin was significantly lower in O kidneys of mice treated with chaetomellic acid compared with O kidneys of mice treated with vehicle (Figures 7(c) and 7(d), resp.). Immunohistochemistry analysis revealed that in the animals receiving chaetomellic acid, O kidneys showed lower staining for α-SMA but not for

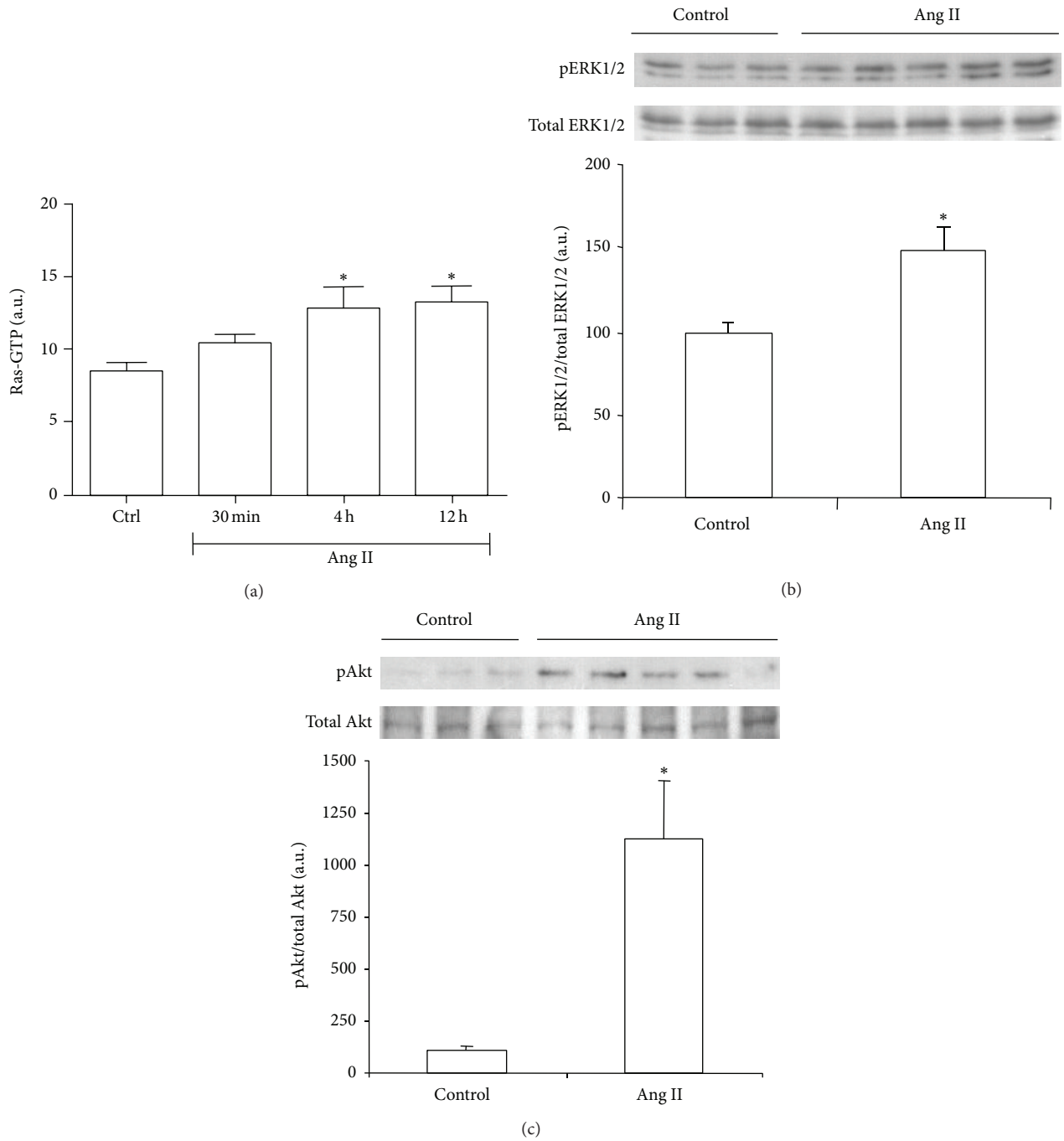

Figure 1: Effect of systemically administrated angiotensin II (Ang II) on Ras signaling pathway. Ras activation was evaluated as Ras GTP by ELISA (a). Phosphorylated (p)-ERK1/2 and p-Akt protein expression were evaluated as the ratio p/total protein by Western blot ((b) and (c), resp.). Bars represent the mean ± SEM of the optical density measured in kidney samples of control saline group (Ctrl; $n = 3$) and angiotensin II-treated animals (Ang II, 0.8 mg/kg; $n = 3$–5/per time point). $^{*}P < 0.05$ versus control group.

fibronectin than O kidneys from animals receiving the vehicle alone (Figure 8).

4. Discussion

Increased levels of Ang II have been suggested to play a major role in the progression of renal disease induced by experimental UUO [3]. Previous studies have demonstrated that both mRNA and protein levels are increased for renin, angiotensin converting enzyme activity, and Ang II content in the obstructed kidney 1 day after UUO [4]. Additionally, *in vivo* studies have shown renal Ras activation induced by infusion of Ang II for 6 days [18].

Having previously demonstrated that Ras signaling pathway is activated in obstructed kidneys after 3 days of UUO [22, 23, 37], we now demonstrate that systemic Ang II

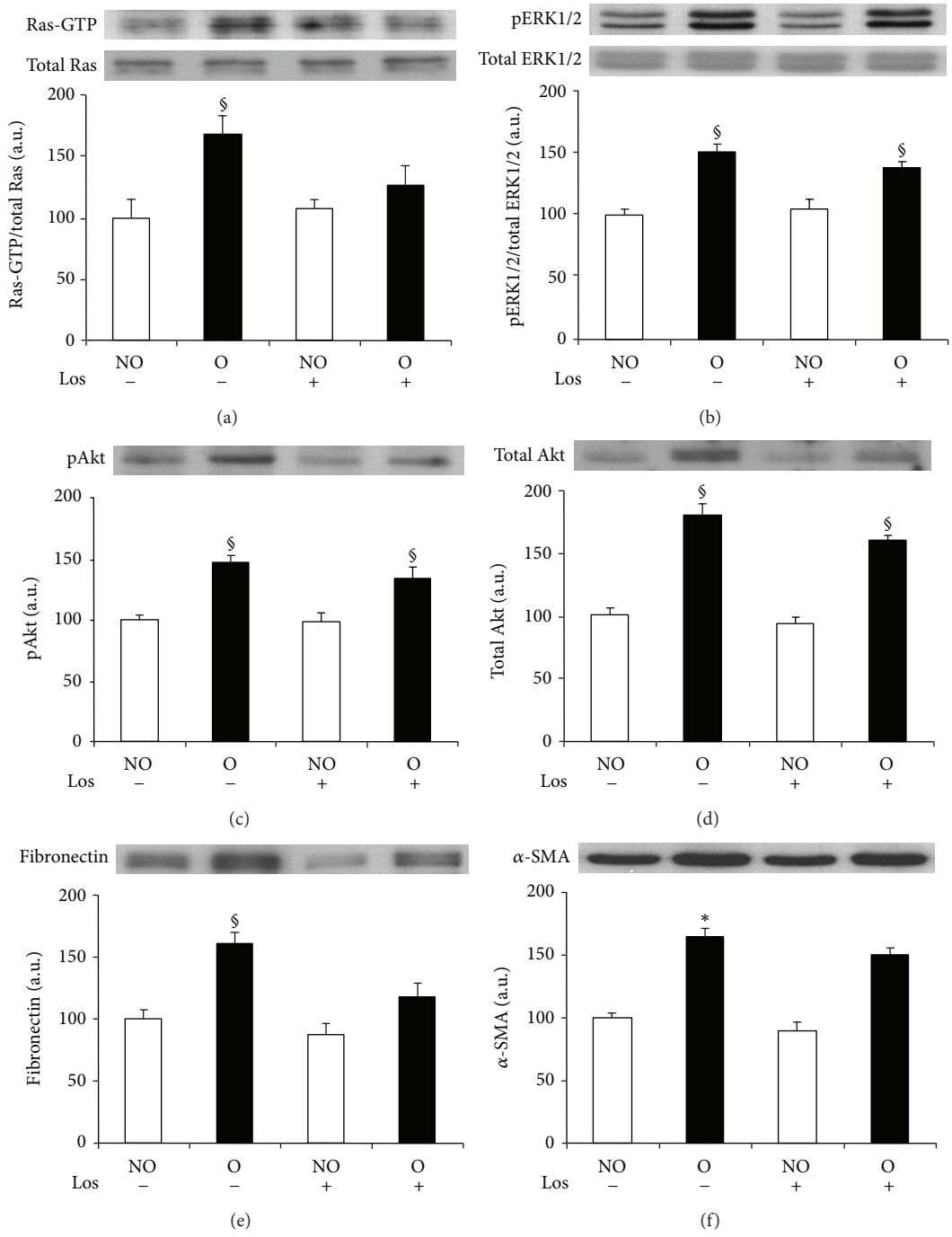

Figure 2: Effect of losartan administration on UUO-induced Ras pathway activation and fibrotic changes analyzed by Western blot. Protein expression of Ras (a), ERK1/2 (b), Akt ((c) and (d)), fibronectin (e), and α-SMA (f) detected by immunoblotting. Activation of Ras and ERK1/2 were measured as the ratio phosphorylated (p)/total proteins. Bars represent the mean ± SEM of the optical density measured in nonobstructed (NO) and obstructed (O) kidney samples of saline ($n = 3$) and losartan- (Los 40 mg/kg; $n = 5$) treated animals. $^{\S}P < 0.05$ and $^{*}Z > 2.6383$ versus NO vehicle-treated kidneys of UUO mice.

administration in normal mice results in a marked increase of the renal Ras signaling pathway as early as 30 minutes, with larger increases observed 4 hours after administration. Thus, our data demonstrates a rapid activation of the Ras pathway induced by UUO or Ang II administration. Furthermore, by blocking the AT1 receptor by losartan administration, levels of activated Ras in obstructed kidneys were markedly reduced. Taken together, these results suggest that Ang II leads to AT1 receptor dependent stimulation of Ras in early renal injury post-UUO.

Fibronectin is a major component of the pathological extracellular matrix (ECM) in tubulointerstitial fibrosis that

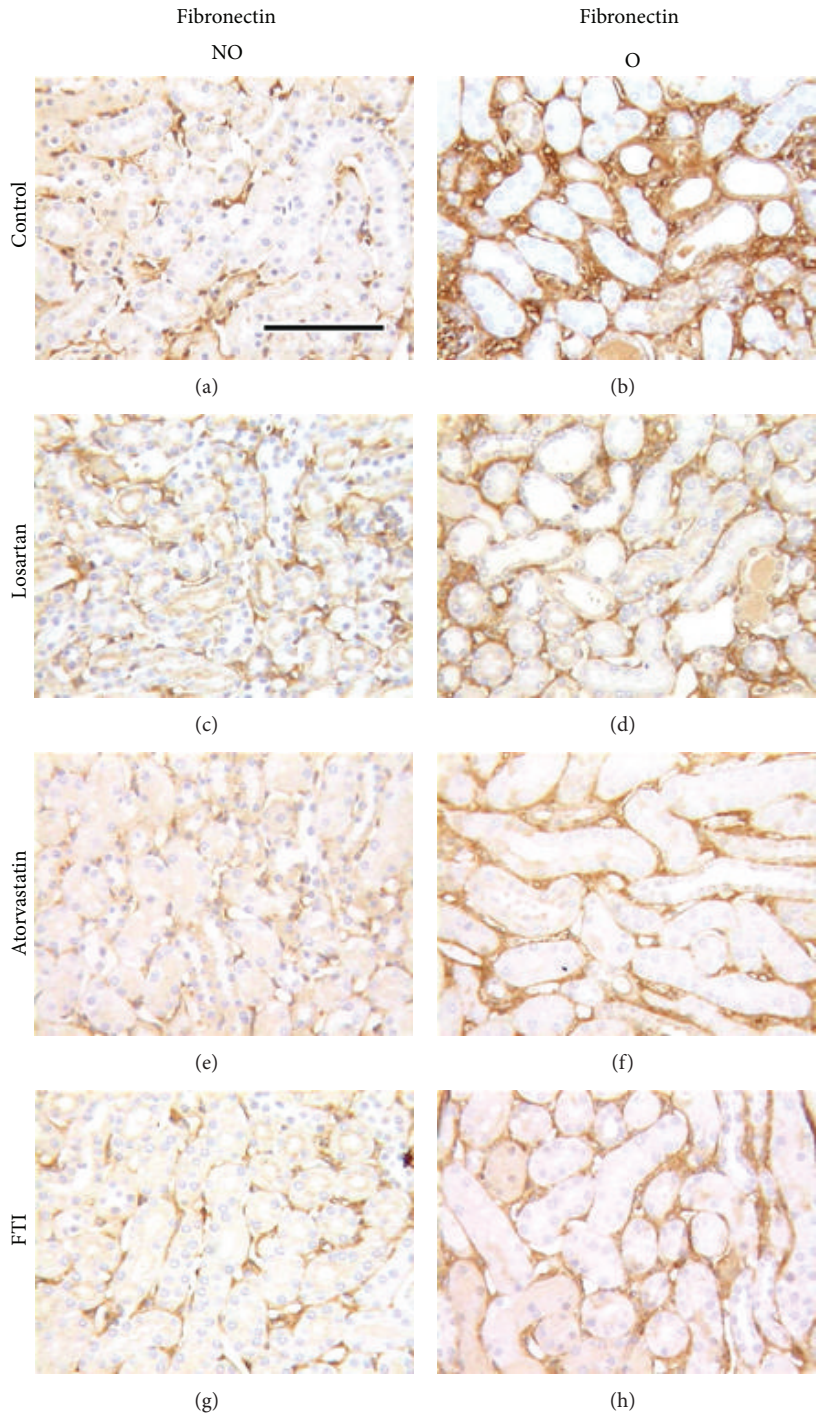

Figure 3: Effect of losartan, atorvastatin, or farnesyl transferase inhibitor (FTI) administration on renal fibronectin expression detected by immunohistochemistry in UUO mice. Representative interstitial sections from nonobstructed (NO) and obstructed (O) kidneys of UUO untreated control mice ((a) and (b)) and UUO mice treated with losartan ((c) and (d)), atorvastatin ((e) and (f)), or FTI ((g) and (h)). Black bar indicates 100 microns in all panels.

serves as a fibroblast chemoattractant and scaffold protein for the deposition of other ECM proteins [38]. Furthermore, the fibronectin scaffold has been demonstrated to be involved in the differentiation of fibroblasts to the α-SMA positive myofibroblasts [39], a key step in UUO-induced renal fibrosis [16]. Since inhibition of UUO-induced Ras activation by losartan treatment attenuates increases in fibronectin and α-SMA expression in the obstructed kidney, a potential role for Ras beyond mere association could be suggested in relation to the early stages of Ang II-mediated renal fibrosis.

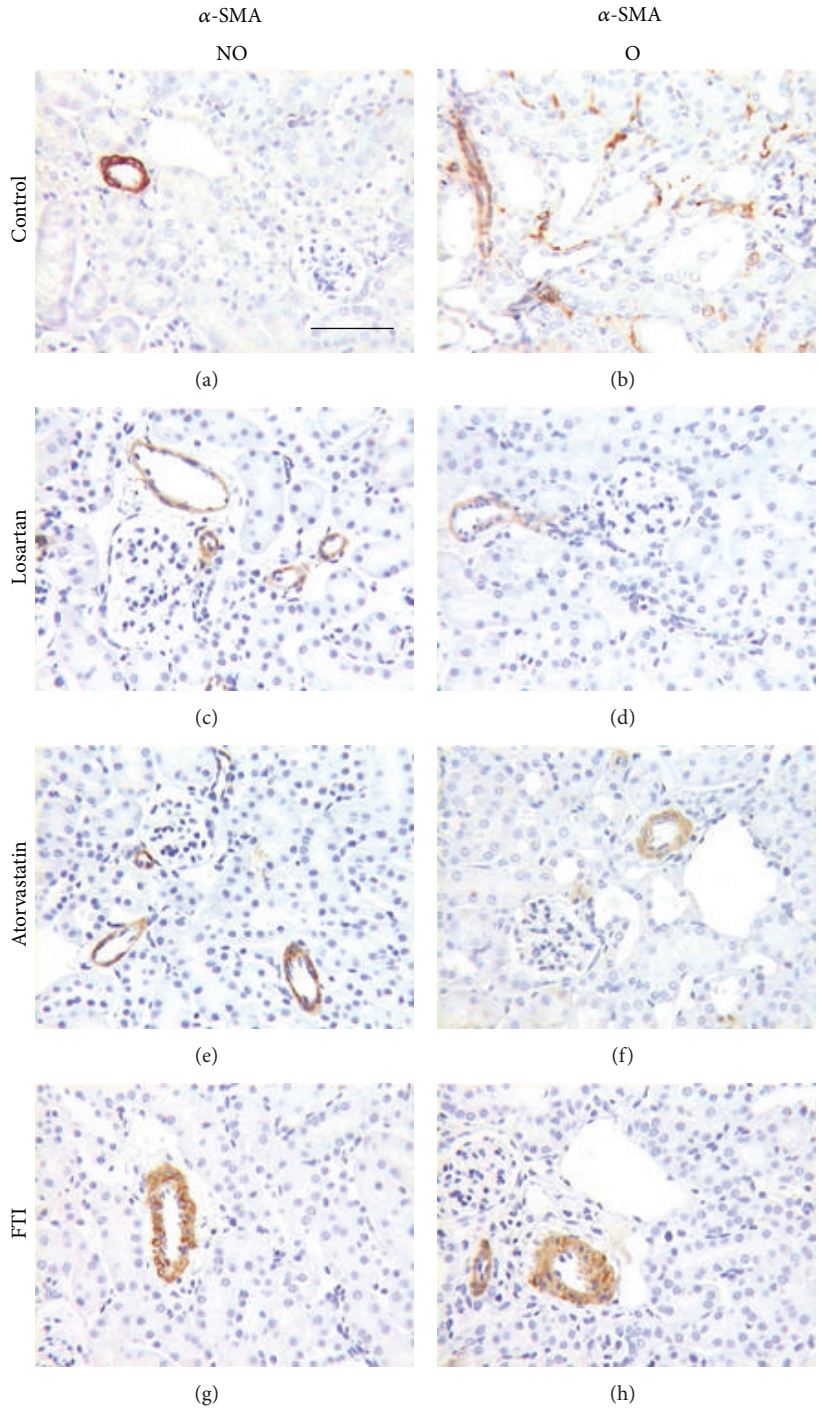

Figure 4: Effect of losartan, atorvastatin, or farnesyl transferase inhibitor (FTI) administration on renal alpha-smooth muscle actin (α-SMA) expression detected by immunohistochemistry in UUO mice. Representative interstitial sections from nonobstructed (NO) and obstructed (O) kidneys of UUO untreated control mice ((a) and (b)) and UUO mice treated with losartan ((c) and (d)), atorvastatin ((e) and (f)) or FTI ((g) and (h)). Black bar indicates 100 microns in all panels.

Considering all this data, it could be suggested that in the early stages of UUO the profibrotic effects of AT1 receptor-mediated Ang II are regulated, at least in part, via a Ras-dependent pathway.

Both downstream effectors of Ras, ERK1/2, and Akt have been implicated in renal damage response in obstructive nephropathy. We have previously reported that activation of Ras and ERK1/2 is significantly higher in the

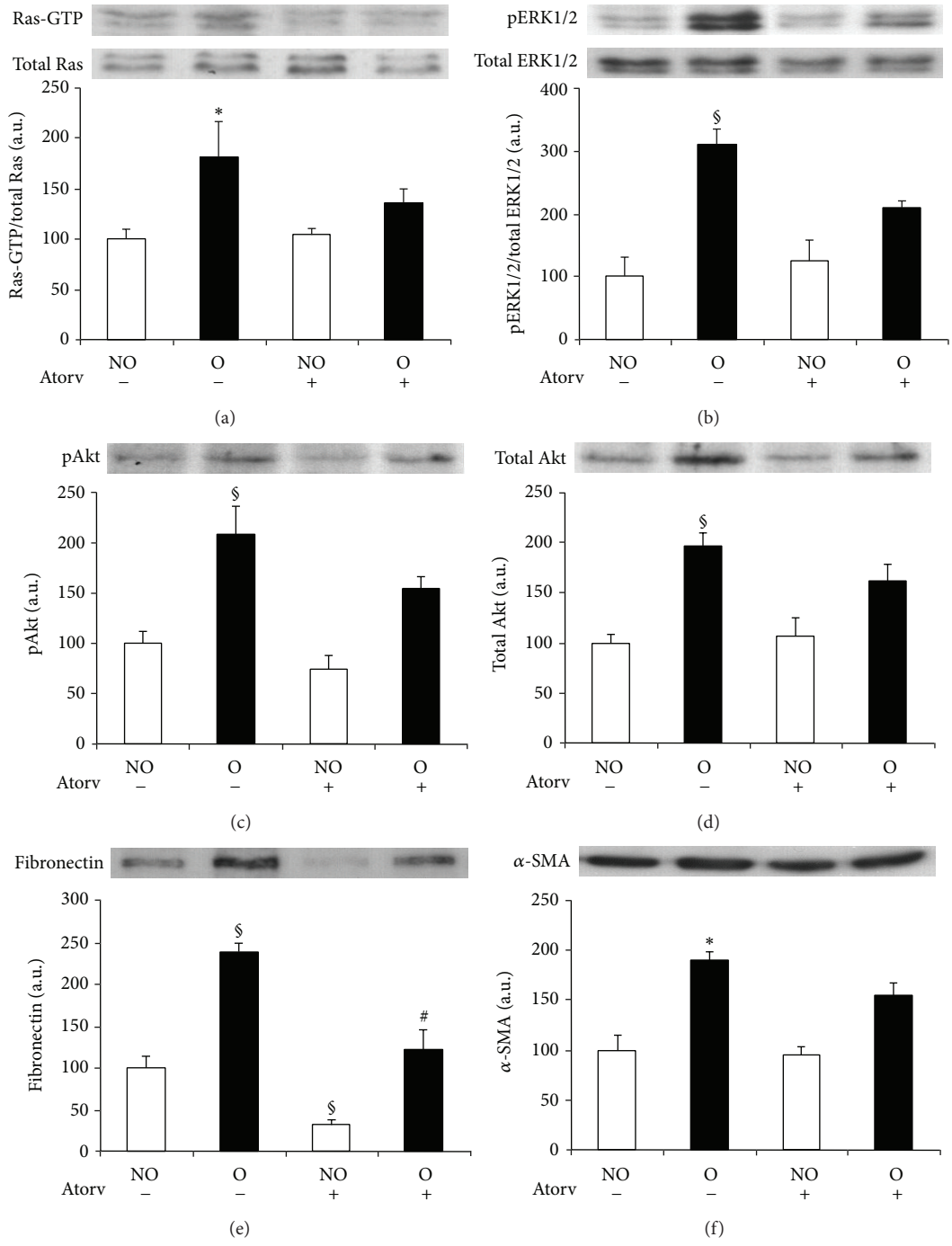

Figure 5: Effect of atorvastatin administration on UUO-induced Ras pathway activation and fibrotic changes analyzed by Western blot. Protein expression of Ras (a), ERK1/2 (b), Akt ((c) and (d)), fibronectin (e), and alpha-smooth muscle actin (α-SMA) (f) was detected by immunoblotting. Activation of Ras and ERK1/2 was measured as the ratio phosphorylated/total proteins. Bars represent the mean ± SEM of the optical density measured in nonobstructed (NO) and obstructed (O) kidney samples of vehicle ($n = 3$) and atorvastatin-treated animals (Atorv, 70 mg/kg; $n = 4$). $^{\S}P < 0.05$ and $^{*}Z > 1.9600$ versus NO vehicle-treated kidneys of UUO mice. $^{\#}P < 0.05$ versus O vehicle-treated kidneys.

obstructed (O) kidneys than in nonobstructed (NO) kidneys 3 days after UUO. The same pattern was observed in UUO-induced expression of p-Akt and total Akt, as demonstrated by Western and Northern blot analysis [22, 23]. Activation of ERK1/2 has been related to interstitial apoptosis and proliferation of tubular cells [23, 40, 41], whereas a role for activated Akt has been reported in early tubulointerstitial cell proliferation and profibrotic events in

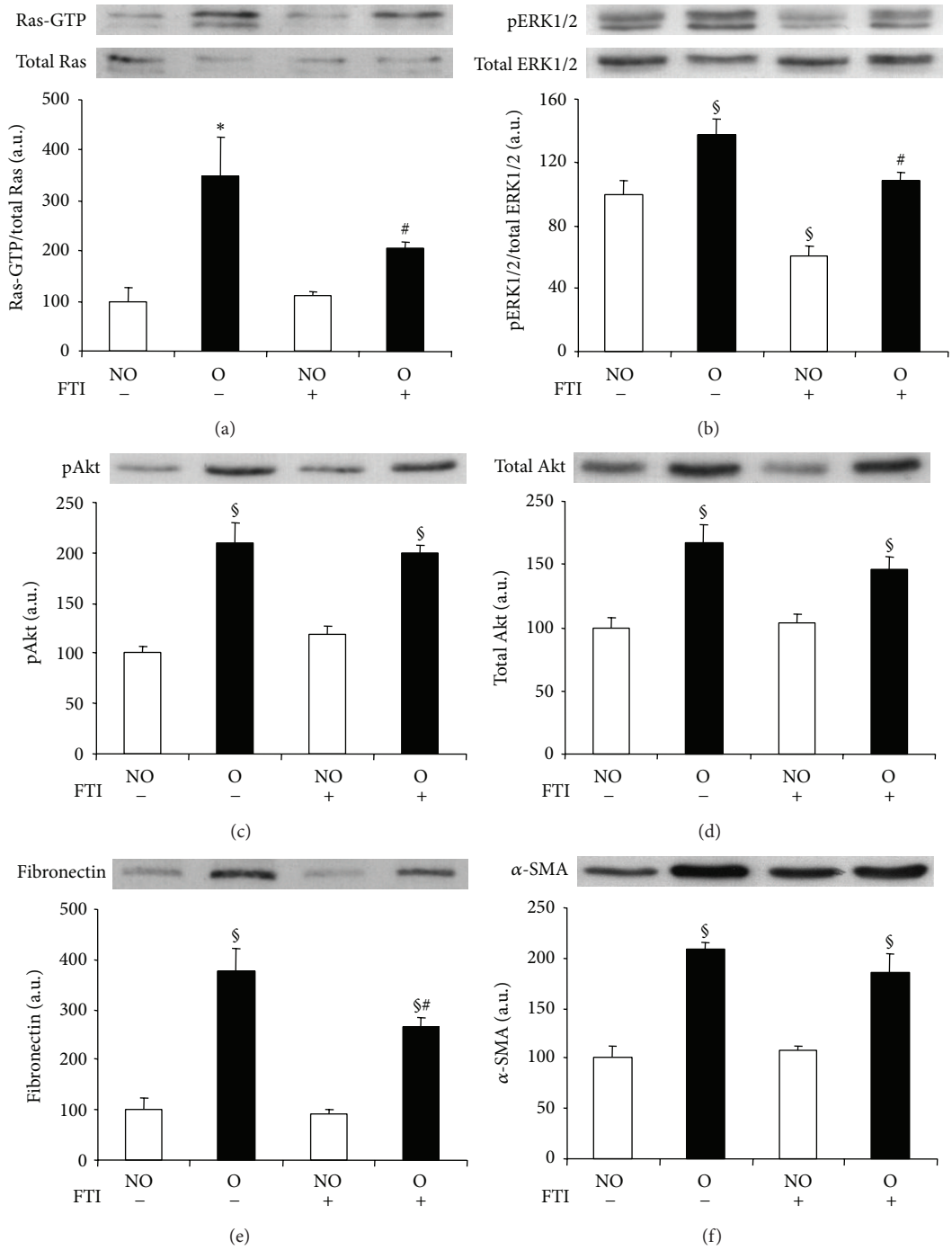

Figure 6: Effect of farnesyl transferase inhibitor (FTI) administration on UUO-induced Ras pathway activation and fibrotic changes analyzed by Western blot. Protein expression of Ras (a), ERK1/2 (b), Akt ((c) and (d)), fibronectin (e), and alpha-smooth muscle actin (α-SMA) (f) was detected by immunoblotting. Activation of Ras and ERK1/2 was measured as the ratio phosphorylated/total proteins. Bars represent the mean ± SEM of the optical density measured in nonobstructed (NO) and obstructed (O) kidney samples of vehicle ($n = 3$) and FTI-treated animals (40 mg/kg; $n = 5$). $^{\S}P < 0.05$ and $^{*}Z > 2.6383$ versus NO vehicle-treated kidneys of UUO mice. $^{\#}P < 0.05$ versus O vehicle-treated kidneys.

UUO [23]. Results presented in this study have also shown that inhibition of Ras activation by blocking either the farnesyl transferase or HMG-CoA reductase enzymes is able to reduce UUO-induced MAPK-ERK1/2 signaling pathway in the obstructed kidney. Additionally, administration of atorvastatin or chaetomellic acid A also induced a marked downregulation of PI3 K/Akt pathway. Thus, blockade of Ras activation can provide a new strategy to reduce the renal

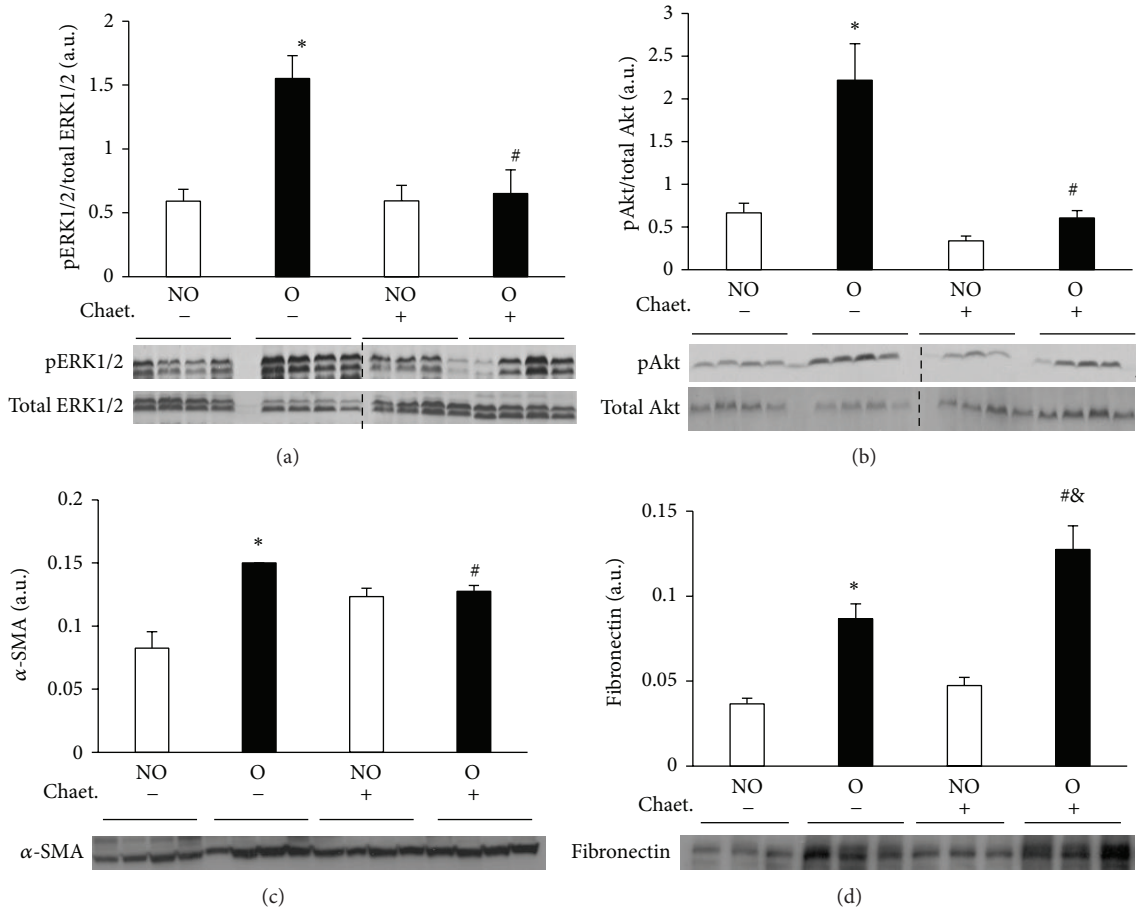

Figure 7: Effect of chaetomellic acid A administration on UUO-induced Ras pathway activation and fibrotic changes analyzed by Western blot. Protein expression of pERK1/2 and ERK1/2 (a), pAkt and Akt (b), alpha-smooth muscle actin (α-SMA) (c), and fibronectin (d) was detected by immunoblotting. Bars represent the mean ± SEM of the optical density measured in nonobstructed (NO) and obstructed (O) kidney samples of vehicle ($n = 4$) and chaetomellic acid A-treated animals (Chaet.; 1.5 mg/kg; $n = 4$). $^{*}P < 0.01$ versus NO vehicle-treated kidneys of UUO mice. $^{\#}P < 0.01$ versus O vehicle-treated kidneys. $^{\&}P < 0.01$ versus NO Chaet-treated kidney.

damage events elicited by activation of ERK1/2 and Akt signaling pathways.

Moreover, our data demonstrate that inhibition of Ras activation by atorvastatin or FTI administration reduces UUO-induced accumulation of fibronectin in the mouse kidney. Atorvastatin data are in agreement with previous studies reporting protective effect of statins in UUO-induced renal fibrosis [42–46].

In addition, atorvastatin, and chaetomellic acid A reduced the increased amount of the marker for myofibroblasts, α-SMA, after UUO. At least a part of myofibroblast present in the kidney after UUO may derive from epithelial cells by a process called epithelial-mesenchymal transition (EMT) [16] and there are enough evidences that Ras activation participates in EMT [47, 48]. These results suggest that Ras participates in the initiating molecular events are involved in renal interstitial fibrogenesis induced by ureteral obstruction in mice. We have already reported that H-Ras isoform is able to modulate renal fibrosis and myofibroblast activation following ureteral obstruction in mice [24, 27]. Notably Ras activation has been shown to be involved in EMT of tubular cells to myofibroblasts [49], and specifically H-Ras is involved in TGF-β1-induced EMT [50]. In the kidney, chaetomellic acid A selectively inhibits the membrane-bound of H-Ras without affecting the membrane-bound of Ki-Ras or other prenylated intracellular proteins like Rab [51]. Since H-Ras isoform only can be farnesylated, we have used chaetomellic acid A specifically to inhibit the activation of H-Ras isoform. Our results have demonstrated that chaetomellic acid A administration decreased Ras downstream signaling pathway as well as α-SMA accumulation in UUO kidneys. Previous studies of our group have shown that H-Ras KO mice show reduced numbers of myofibroblasts in the kidney following UUO [27]. It has been reported that in acute renal ischemia-reperfusion injury in rats the inhibition of the Ras pathway by chaetomellic acid A resulted in a beneficial effect, preserving both renal function and structure [51]. Furthermore, in an experimental murine model of ischemic stroke, chaetomellic acid A administration increased the intracellular concentration of inactive H-Ras, leading to a marked decrease of both superoxide anion production and volume of cerebral necrotic tissue, with the subsequent

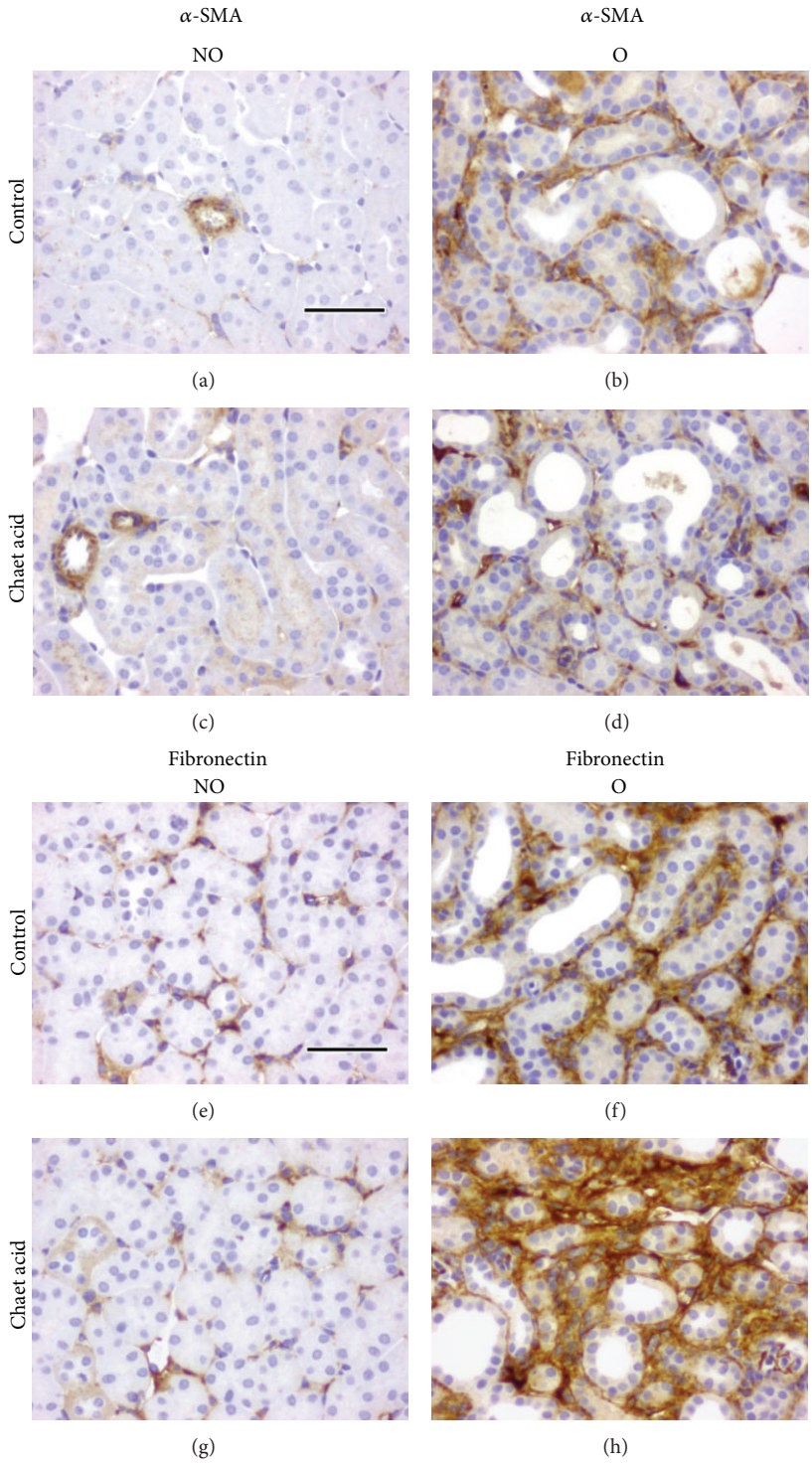

Figure 8: Effect of chaetomellic acid A administration on renal alpha-smooth muscle actin (α-SMA) ((a)–(d)) and fibronectin ((e)–(h)) expression detected by immunohistochemistry in UUO mice. Representative cortical interstitial sections from nonobstructed (NO) and obstructed (O) kidneys of UUO mice treated with vehicle (Control) or chaetomellic acid A (Chaet Acid). Black bar indicates 100 microns in all panels.

improved survival of hypoxic neuronal cells [35]. Thus, inhibition of H-Ras isoform specifically by chaetomellic acid A could be used as a potential therapeutic strategy to reduce fibrosis development.

5. Conclusions

In summary, our results suggest that increased Ang II production in the obstructed kidney could play a role in Ras/ERK/Akt pathway activation, which in turn, can be involved in early renal fibrosis induced by UUO. Our data also offer evidence of the pharmacological potential of Ras pathway inhibition in preventing the progression of renal tubulo-interstitial fibrosis. Chaetomellic acid A is of particular interest in this regard in relation to the arrest or reversal of renal fibrosis as it acts more specifically than statins and is less toxic than synthetic FTIs.

Conflict of Interests

The authors declare not to have any conflict of interests.

Authors' Contribution

Ana B. Rodríguez-Peña and Isabel Fuentes-Calvo contributed equally to this paper.

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Review Article

Hypoxia in Diabetic Kidneys

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Diabetic nephropathy (DN) is now a leading cause of end-stage renal disease. In addition, DN accounts for the increased mortality in type 1 and type 2 diabetes, and then patients without DN achieve long-term survival compatible with general population. Hypoxia represents an early event in the development and progression of DN, and hypoxia-inducible factor- (HIF-) 1 mediates the metabolic responses to renal hypoxia. Diabetes induces the "fraternal twins" of hypoxia, that is, pseudohypoxia and hypoxia. The kidneys are susceptible to hyperoxia because they accept 20% of the cardiac output. Therefore, the kidneys have specific vasculature to avoid hyperoxia, that is, AV oxygen shunting. The NAD-dependent histone deacetylases (HDACs) sirtuins are seven mammalian proteins, SIRTs 1–7, which are known to modulate longevity and metabolism. Recent studies demonstrated that some isoforms of sirtuins inhibit the activation of HIF by deacetylation or noncatalyzing effects. The kidneys, which have a vascular system that protects them against hyperoxia, unfortunately experience extraordinary hypernutrition today. Then, an unexpected overload of glucose augments the oxygen consumption, which ironically results in hypoxia. This review highlights the primary role of HIF in diabetic kidneys for the metabolic adaptation to diabetes-induced hypoxia.

1. Hypoxia in Diabetic Kidney

Diabetic nephropathy (DN) is now a leading cause of end-stage renal disease (ESRD) and therefore constitutes a major factor in progressive kidney disease. In addition, in absence of DN, diabetes is not associated with a large increase in mortality risk in type 1 or type 2 diabetes [1, 2]. Chronic hypoxia and tubulointerstitial fibrosis are presently considered to be a common pathway for various progressive kidney diseases including DN, and hypoxia inducible factor- (HIF-) 1α plays an important role in these pathological mechanisms [3–6].

Earlier studies have demonstrated that hypoxia represents an early event in the development and progression of experimental DN [7, 8] and an increased HIF-1α expression in diabetic kidneys compared to the kidneys of control rats [9] and normal human kidneys [10]. Intrarenal oxygenation can be assessed noninvasively in subjects with type 1 or type 2 diabetes by blood oxygenation level dependent magnetic resonance imaging (BOLD MRI) [11–13]. Inoue et al., especially, demonstrated hypoxia in the renal parenchyma of chronic kidney disease (CKD) patients with or without diabetes, using diffusion-weighted (DW) MRI and BOLDMRI [13], and confirmed intrarenal hypoxia in patients with diabetes, suggesting that factors other than tubulointerstitial alteration (such as loss of peritubular capillaries) determine the degree of hypoxia in the renal cortex [13]. The kidneys are less than 1% of total body weight and use 10% of total oxygen consumption. Na^+/K^+-ATPase in cortical proximal tubular cells consumes 80% of the oxygen for kidneys [14], and kidney needs 3%–18% of the total oxygen consumption for its basal metabolism [15].

Usually, tissue oxygen tension is determined by the balance between the blood flow and tissue oxygen consumption. However, the kidneys do not fall under such a simple calculation, because the kidneys receive blood to regulate the blood volume and composition, not for their own benefit. Increased renal blood flow increases the glomerular filtration rate, which in turn increases the rate of sodium reabsorption, which increases the tissue oxygen consumption.

Additionally, diabetes induces glomerular hyperfiltration [16] and increases tubular sodium and glucose reabsorption through sodium glucose cotransporters (SGLTs), which

enhances Na^+/K^+-ATPase activity, resulting in an increased ouabain-sensitive oxygen consumption [17]. Therefore, in diabetic kidney, hyperfiltration triggers a vicious circle between increasing oxygen delivery and increasing oxygen consumption that leads to more needs for oxygen supply.

Previous studies have shown that the loop diuretic furosemide, which inhibits reabsorption in the medullary thick ascending limb, increased medullary oxygen tension by reducing oxygen consumption rather than by increasing medullary blood flow [18, 19]. Intriguingly, the novel antidiabetic drug and SGLT2 inhibitor empagliflozin attenuate renal hyperfiltration in subjects with type 1 diabetes [20], suggesting the potential role of SGLT2 inhibitors in renoprotection in diabetic kidneys by inhibiting proximal tubular oxygen consumption.

In an in vitro study, high glucose failed to induce or enhance the expression of HIF-1α protein in human renal proximal tubular cells (HRPTECs) in normoxia or in hypoxia, respectively [21]. In addition, it was found in the same study that high glucose abolished the Pasteur effects, which are adaptive responses to hypoxic stress by decreased oxidative phosphorylation and an increase in anaerobic fermentation, and that high glucose decreased mitochondrial efficiency by uncoupling oxygen consumption from ATP production [21]. Furthermore, in another cultured system, high glucose blunted the vascular endothelial growth factor (VEGF) response to hypoxia in immortalized rat proximal tubular cells via the oxidative stress-regulated HIF/hypoxia-responsible element (HRE) pathway [22]. Collectively, hyperglycemia itself fails to enhance HIF-1 expression, whereas it attenuates HIF-1 mediated responses to hypoxia. Thus, hyperglycemia induces hypoxia and hypoxia-induced HIF-1 expression in the diabetic kidneys through hemodynamic or/and metabolic changes in vivo, not by a direct effect.

It is well known that there are some pathways that are activated by hyperglycemia, such as the polyol pathway, the hexosamine pathway, the protein kinase C activation, and the AGE pathway [23]. However, every therapeutic challenge using the inhibitors for these pathways failed to cure diabetic complications including DN, indicating the inappropriate therapeutic strategy and needs for an alternative approach. Recently, Friederich-Persson et al. studied the effects of the mitochondrial uncoupler dinitrophenol (DNP) in rats [24]. DNP did not affect renal blood flow, glomerular filtration, blood glucose, or oxidative stress but increased the kidney oxygen consumption and resulted in intrarenal tissue hypoxia. In addition, DNP increased urinary protein excretion, kidney vimentin expression, and infiltration of inflammatory cells. Friederich-Persson et al. thus confirmed that kidney tissue hypoxia, without confounding hyperglycemia or oxidative stress, may be sufficient to initiate the development of nephropathy.

We previously showed that the antidiabetes drug metformin suppresses mitochondrial respiratory function, suggesting that the redistribution of intracellular oxygen is involved in the inhibitory effects of metformin on HIF-1α expression (Figure 1) [21]. Our results also suggest that metformin inhibits mitochondrial respiration, indicated as the inhibition of oxygen consumption and intracellular ATP levels, which subsequently activates AMPK pathways, suggesting that AMPKα is a downstream regulator of the mitochondrial respiratory chain, and metformin-induced AMPK phosphorylation is not related to HIF-1α inhibition (Figure 1). Moreover, our preclinical study using type 2 diabetic model Zucker diabetic fatty (ZDF) rats confirmed the renoprotective effects of metformin in vivo. Therefore, the therapeutic target for diabetic kidneys should be to prevent excessive mitochondrial oxygen consumption or to restore renal oxygen availability, not the accompanying oxidative stress or downstream HIF activation.

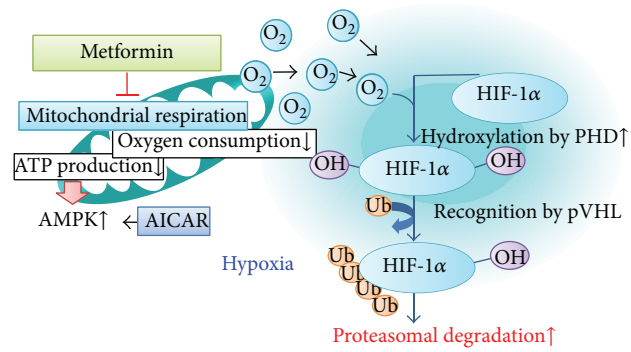

Figure 1: Metformin redistributes the intracellular oxygen. Metformin inhibits oxygen consumption and ATP production by inhibiting mitochondrial complex I. Subsequently, intracellular oxygen redistribution supplies oxygen for prolyl hydroxylase, which promotes the degradation of HIF-1α in the proteasome. ATP depletion caused by mitochondrial inhibition activates AMPK, which is a downstream signaling pathway of mitochondrial respiratory chain [21].

2. Sirtuins, Pseudohypoxia, and NAD^+-Related HIF

Sirtuins (SIRTs) are NAD-dependent Class III HDACs and their deacetylase activity is controlled by the cellular $[NAD^+]/[NADH]$ ratio, and age-, hypoxia-, and hyperglycemia-related NAD^+ deficiency lead to a reduction of SIRTs activity [25]. Previous studies, especially, proposed that SIRT1 has been linked to diabetes [26–28]. SIRT1 mRNA expression may be associated with energy expenditure and insulin sensitivity in the offspring of type 2 diabetic patients [26]. Moreover, a SIRT1 mutation was identified in a family with type 1 diabetes [27, 28].

In 1993, Williamson et al. first referred to pseudohypoxia as an increase in cytosolic $NADH/NAD^+$, caused by an increased rate of reduction of NAD^+ to NADH [29]. They proposed that the tissue lactate/pyruvate ratio is a more reliable parameter of the cytosolic ratio of free $NADH/NAD^+$, because the oxidation of NADH to NAD^+ by LDH is coupled to the reduction of pyruvate to lactate. They also stated that the increased metabolism of glucose via the sorbitol pathway is the most important mechanism for pseudohypoxia [29]. Interestingly, cells with an increased $NADH/NAD^+$ ratio attributable to hyperglycemic pseudohypoxia require less

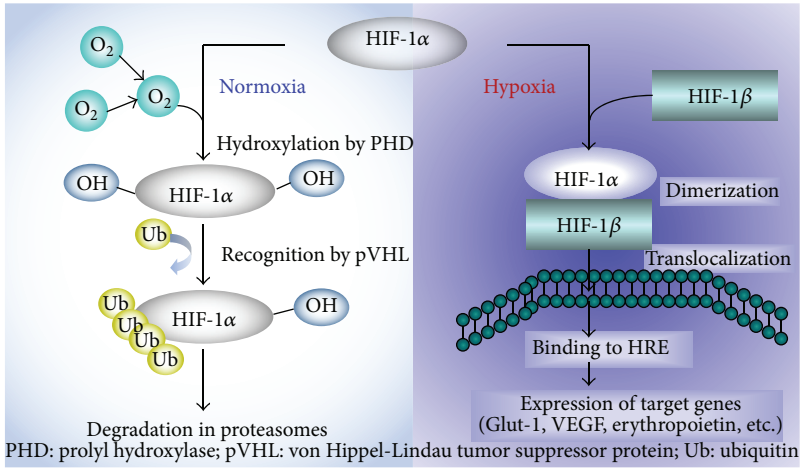

Figure 2: Oxygen regulates HIF-1α protein expression. HIF-1 is a heterodimeric transcription factor complex that is composed of an oxygen-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit; HIF-1 activity depends on the degradation of HIF-1α subunit; the half-life of HIF-1α protein on reoxygenation is less than one minutes [31].

severe hypoxia or ischemia to increase NADH/NAD^+ to the same level caused by hypoxia or ischemia alone. The relationship between pseudohypoxia and hypoxia has not yet been determined. Recent findings indicate that a number of nonhistone substrates, such as HIF-1, are deacetylated by sirtuin family members, expanding the physiological and pathophysiological roles of sirtuins, more than longevity and metabolism.

HIF-1 is a heterodimeric transcription factor complex composed of an oxygen-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit, and HIF-1 activity depends on the degradation of the HIF-1α subunit. In normoxia, specific Fe^{2+}- and oxoglutarate-dependent prolyl 4-hydroxylases (PHDs) hydroxylate HIF-1α at two prolyl sites of the oxygen-dependent domain (ODD). The hydroxylated HIF-1α binds to the von Hippel-Lindau (VHL) tumor suppressor protein that is part of an E3 ubiquitin ligase complex targeting HIF-1 for proteasomal degradation [30] (Figure 2).

The expression of HIF-1α protein is tightly coupled to the intracellular oxygen concentration, and the half-life of HIF-1α protein on reoxygenation is less than 1 min [31]. Accumulating evidence demonstrates that HIF-1 is a target of epigenetic regulation such as the DNA hypermethylation of gene promoters, histone modifications, small interfering RNAs and microRNAs [32]. In light of the inhibitory effects of histone deacetylase inhibitors (HDAIs), HIF function apparently requires deacetylase-dependent transactivation [33].

Interestingly, some isoforms of sirtuins have been known to modulate HIF-1 activity [34–38] (Figure 3). SIRTs consist of seven mammalian proteins, SIRTs 1–7. During hypoxia, decreased NAD^+ levels downregulated SIRT1, which allowed the acetylation and activation of HIF-1α [34]. SIRT1 inactivates HIF-1α by binding to HIF-1α and deacetylating it and by blocking coactivator p300 recruitment and consequently repressed HIF-1 targeted genes [34]. SIRT2 also regulates the autoacetylation of p300, in vitro and in cells [35], and thus it might modulate HIF-1 activation. Mitochondrial SIRT3 destabilizes HIF-1α, which controls glycolytic gene expressions, opposing the reprogramming of cancer cell metabolism, known as the Warburg effect [36]. SIRT6-deficient mice develop normally but die early due to lethal hypoglycemia [37]. As SIRT6 functions as a corepressor of HIF-1α and controls the expressions of multiple glycolytic genes, SIRT6-deficient cells exhibit increased HIF-1α activity and show increased glucose uptake with an upregulation of glycolysis and diminished mitochondrial respiration [37]. At last, SIRT7 negatively affects HIF-1α and HIF-2α protein levels by a mechanism that is independent of prolyl hydroxylation and that does not involve proteasomal or lysosomal degradation [38]. Then the mechanism by which SIRT7 regulates HIF activity differs from those of the other sirtuins because of the independence of its deacetylase activity. SIRT7 could regulate HIF through protein-protein interactions, not by enzymatic activity [38].

Although it is due to a complex metabolic scenario of pathological mechanisms, pseudohypoxia might be defined as a paucity of NAD^+, leading to sirtuins-HIF interaction (Figure 3). Indeed, pseudohypoxia should be considered hyperglycemia-induced metabolic hypoxia, presenting the activation of HIF, a pathophysiological state in which, although oxygen is present, oxygen is less used in mitochondrial respiration by secondary mitochondrial dysfunction, similar to the Warburg effect in cancer cells. Therefore, cells with pseudohypoxia, which has already induced HIF-1, do not need severe hypoxia for inducing HIF-1, and this fraternal hypoxia results in the preference for glycolysis.

The pyruvate/lactate ratio during normoxic conditions in early diabetes may be explained by either increased gluconeogenesis or by glycolysis, not by making an effect on oxidative phosphorylation. In addition, hyperglycemia activates NADPH oxidase (NOX), which critically provides additional NAD^+ for glycolysis. NOXs are one of the many sources of reactive oxygen species (ROS) [39]. NOX4, which

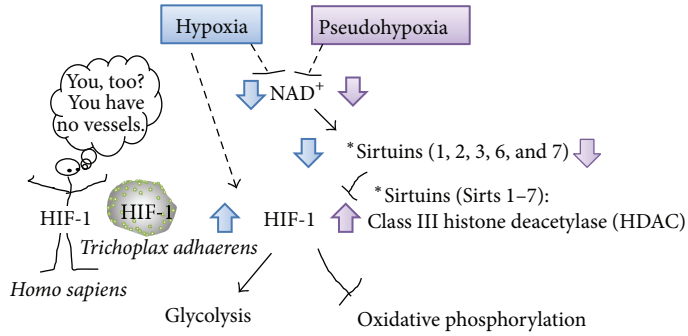

FIGURE 3: NAD^+-sirtuins modulate glucose metabolism via HIF-1 in all metazoan species from the simplest animal *Trichoplax adhaerens* to human. Sirtuins (SIRT) consist of seven mammalian proteins, SIRTs 1–7. Some isoforms of sirtuins inhibit HIF-1 activation by deacetylation or noncatalyzing effects [34–38]. Under hypoxia or pseudohypoxia, decreased NAD^+ levels downregulated SIRT, leading to upregulated HIF-1 activation which shift the glucose metabolism by promoting glycolysis and by inhibiting oxidative phosphorylation.

is highly expressed by human vascular endothelial cells, is the predominant form in the kidneys. NOX4 is known to be a novel target for HIF-1 [40] and specifically inhibits mitochondrial complex I [41], leading to mitochondrial dysfunction. Therefore, in diabetic kidney, hyperglycemia and hypoxia may induce glycolysis partly via activation of NOX4 by providing NAD^+ for fuel and by suppressing mitochondrial oxidative phosphorylation.

3. AV Shunting and Polar Vasculosis

Originally, the kidneys are away from hypoxia, because they receive approximately 25% of the cardiac output at only less than 1% of the total body weight. Oxygen delivery to the human kidney is 84 mL/min/100 g, and oxygen consumption in the kidney is 6.8 mL/min/100 g [15]. The renal oxygen delivery thus exceeds the renal oxygen consumption. However, the kidneys normally do not suffer from hyperoxia; rather they are susceptible to hypoxia as shown in DN as well as acute kidney injury (AKI), which is the paradox of oxygen physiology in the kidneys.

One of the reasons for the sensitivity of the kidneys to hypoxia is diffusional arterial-to-venous (AV) oxygen shunting [42, 43]. It is well known that the pressure of oxygen (pO_2) in renal veins is above those in the efferent arterioles of the outer cortex, proximal tubules, and distal tubules [44]. In addition, the fractional extraction of oxygen falls with increased renal blood flow (RBF), but renal parenchymal pO_2 remains unchanged [43]. These findings indicate preglomerular AV oxygen shunting by a countercurrent arrangement of mammalian arteries and veins [45] and indicate that AV oxygen shunting contributes to the dynamic regulation of intrarenal oxygenation [43].

However, AV oxygen shunting limits the change in oxygen delivery to renal tissue and stabilizes tissue pO_2 when arterial pO_2 changes but renders renal parenchyma susceptible to hypoxia when oxygen delivery falls or parenchymal oxygen consumption increases [46]. In physiologically normal conditions, this AV oxygen shunting preserves the antioxidant defense mechanisms in the kidneys from hyperoxia-induced reactive oxygen superoxide production, by which the kidneys could cope with superfluous oxygen as like in the lung with its pO_2 close to atmospheric pO_2 (159 mmHg) [45]. The kidneys are second to the heart in terms of oxygen consumption, especially for sodium transport. For the first time in the evolution of humans, the kidneys are experiencing exceeded metabolic demand, because of hypernutrition. Since hyperglycemia increases the renal blood flow and then the tubular reabsorption of glucose and erythrocytes (which need oxygen consumption), leading to decreased oxygen tension of peritubular capillaries, the increased oxygen gradient between arterioles and veins augments the AV oxygen shunting, promoting renal parenchymal hypoxia (Figure 4). Then, the AV oxygen shunting preferentially promotes hypoxia-induced injury in diabetic kidney rather than an escape from hyperoxia.

Interestingly, retinal venous oxygen saturation is increased in diabetic patients with the severity of the diabetic retinopathy, although no significant differences in the retinal arterial oxygen saturation were observed between controls and patients with diabetic retinopathy at any stage, suggesting the involvement of the occlusions and obliterations in the retinal capillary bed, leading to the formation of AV shunting vessels [47]. Similarly, physiological preglomerular AV shunting might make an important contribution to the sequential decline of the glomerular filtration rate (GFR) caused by the glomerular hypertension in DN.

Although cortical tissue oxygenation is independent of medullary blood flow and oxygenation, medullary tissue pO_2 appears to be dependent on the levels of both cortical and medullary perfusion [48], which seem to have equal effects on renal perfusion pressure (RPP). Kidneys have an autoregulation system in which the total renal blood flow (RBF) is autoregulated between the RPP values of 90 and 200 mmHg. However, the blood flow to the medulla is not autoregulated [49]. Three decades ago, Casellas and Mimran demonstrated that one-tenth of all juxtamedullary glomeruli have anatomical shunts with preglomerular vessels to postglomerular vasa recta capillaries [50]. Since these shunts

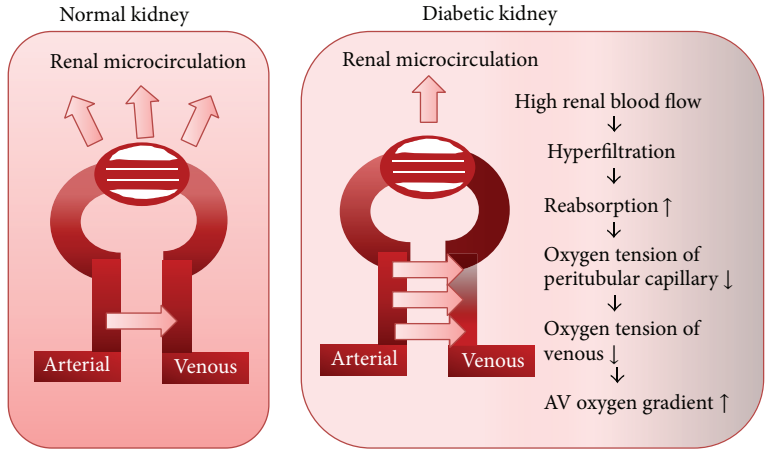

Figure 4: Diabetic kidney exhibits more AV oxygen shunting. Intrarenal oxygen tension is maintained at stable levels by hemodynamic and metabolic interactions of renal blood flow, GFR, oxygen consumption, and arteriovenous (AV) oxygen shunting. Originally, AV oxygen shunting develops as a unique system to rescue kidney from hyperoxia. However, in diabetic kidney, hyperglycemia induces hyperfiltration leading to more AV oxygen gradient which turns in increased AV oxygen shunting. The figure was modified from O'Connor et al. [42].

arise proximally to the afferent arterioles, these would not be subject to tubuloglomerular feedback and autoregulation. Although the medullas of the kidneys receive less than 10% of the total RBF, the medullary blood flow responding to an elevation of systemic arterial pressure might contribute to the high oxygen tension in the outer stripe of the outer medulla, as in the superficial cortex [51].

Intriguingly, there is another specific vascular architecture involved in the pathogenesis of diabetic kidneys, that is, polar vasculosis [52–54]. Like the new vessel formation in diabetic retinopathy, neovascularization at the glomerular vascular pole was described in diabetic nephropathy [52–54]. Min and Yamanaka demonstrated that 72% of 73 autopsy cases and 21 biopsy cases of diabetic glomerulonephropathy had multiple small vessels around the polar vascular pole which connect at a point after two or three branches from the afferent arteriole, and their opposite ends outside the glomerulus anastomose to peritubular capillaries [53]. Those authors proposed that these vessels probably compensate for the decreased function of efferent arterioles because of sclerosis, which is a characteristic finding in diabetic nephropathy [55]. Although this vascular abnormality was specific to diabetic kidneys, the pathological role of polar vasculosis remains obscure. The neovascularization might lower the intraglomerular pressure by shunting afferent and efferent arteries, and it may reveal a compensatory response for glomerular hypertension. However, the diameters of these abnormal arterioles are so small that they might be affected by arteriosclerosis and are obstructed, leading to the progression of renal dysfunction.

4. Roles of HIF in Diabetic Kidneys

HIF-1 was discovered in an investigation of the regulation of human erythropoietin gene, which encodes erythropoietin [56], and it was later shown to regulate VEGF expression [57]. These earlier studies indicated the pivotal roles of HIF-1 in hematogenesis and angiogenesis. All metazoan species including the simplest animal *Trichoplax adhaerens* which totally has no specialized systems for oxygen delivery, such as blood cells or vessels, possess HIF-1 [58]. HIF targets in *T. adhaerens* include glycolytic and metabolic enzymes, suggesting a primary role for HIF in the regulation of oxygen consumption as an adaptation of basal multicellular animals to fluctuating oxygen levels. Of note, *taPDK* is among the strongest hypoxically induced genes in *T. adhaerens*, and human pyruvate dehydrogenase (PDH) kinase 1 (PDK1) is also an HIF target gene [58]. PDK1 inhibits the PDH-catalyzed conversion of pyruvate to acetyl-coenzyme A (acetyl-CoA) for the entry into mitochondrial tricarboxylic acid (TCA) cycle (Figure 5). Therefore, HIF in *T. adhaerens* also switches from oxidative phosphorylation to glycolysis. Since HIF originally functions as master regulator of oxygen homeostasis in all metazoan species, HIF also regulates glucose and energy metabolism to adapt to reduced oxygen availability in hypoxia-susceptible diabetic kidneys. Under a sustained hypoxic condition, HIF subsequently remodels the hemodynamics by producing blood cells and by inducing new vessels, via, respectively, targeted erythropoietin and VEGF. Even after this new HIF-induced microenvironmental reprogramming, hypoxia still exists, and then HIF would eliminate these hypoxic lesions as seen in wounds or infections distant from healthy tissues by surrounding fibrotic capsules.

Using HIF-1α knockout mice, Higgins et al. demonstrated that HIF-1α enhanced the epithelial-to-mesenchymal transition (EMT) in vitro and that the genetic ablation of renal epithelial HIF-1α inhibited the development of tubulointerstitial fibrosis [10]. Sun et al. further provided a novel explanation for the EMT of hypoxic renal tubular cells through the upregulation of Twist induced by HIF-1α [59]. Kimura et al. showed that the stable expression of HIF-1α in tubular epithelial cells promotes interstitial fibrosis in

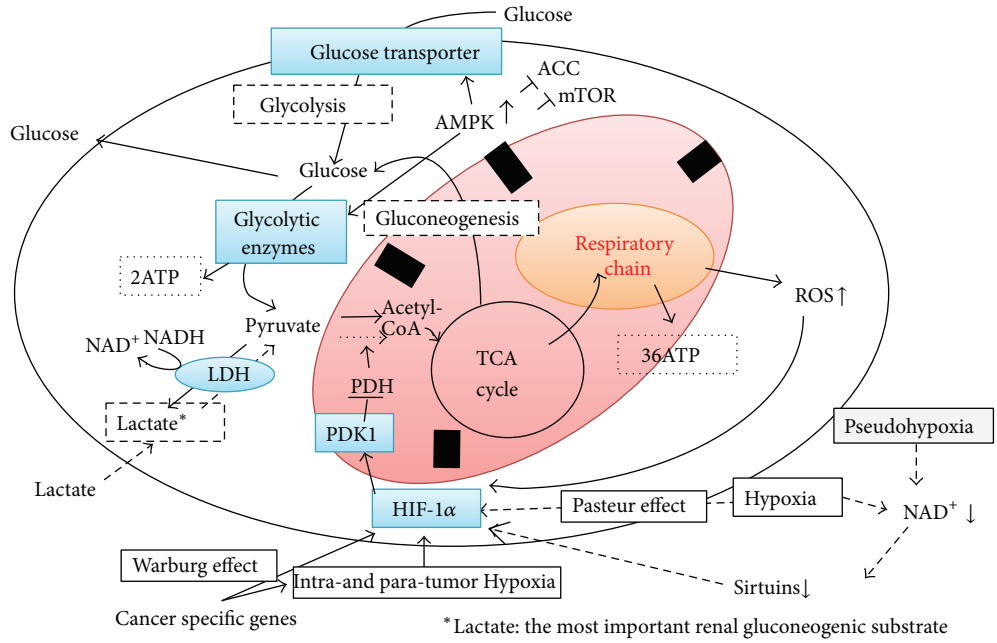

FIGURE 5: The essential role of HIF-1 is to switch the glucose metabolism from oxidative phosphorylation to glycolysis under hypoxic (Pasteur effect) or normoxic (pseudohypoxia or Warburg effect) conditions. Glycolysis: glucose + $2P_i$ + 2ADP + $2NAD^+$ → 2pyruvate + 2ATP + 2NADH + $2H_2O$, 2pyruvate + 2NADH → 2lactate + $2NAD^+$. Aerobic respiration: glucose + $6O_2$ + $36P_i$ + 36ADP → $6CO_2$ + 36ATP + $42H_2O$.

knockout mice with VHL tumor suppressor, which acts as an ubiquitin ligase to promote the proteolysis of HIF-1α [60]. If HIF can play a renoprotective role in diabetic kidneys, HIF expression would not be observed in diabetic kidneys, because HIF rescues the kidneys from hypoxia, and its signal will vanish within a few minutes. Therefore, when HIF is observed in diabetic kidneys, it means that HIF cannot conquer persistent hypoxia. The long-standing expression of HIF induces deleterious phenomena such as renal fibrosis. Two recent studies also clarified that the continuous activation of HIF-1 or HIF-1-targeted molecules contributed to chronic kidney diseases [61, 62]. Wang et al. showed that silencing of HIF-1α gene attenuates chronic ischemic renal injury in two-kidney one-clip rats [61]. Gobe et al. demonstrated that recombinant human erythropoietin protected the tubular epithelium from apoptosis but stimulated EMT, and the supraphysiological dose needed for renoprotection contributed to fibrogenesis and stimulated chronic kidney disease in the long term [62]. This adverse effects of erythropoietin remind us of the potential disadvantage of the HIF stabilizer, FG-4592, which is an oral compound in Phase III trials for the treatment of anemia in ESRD patients. FG-4592, an inhibitor of HIF prolyl hydroxylase (HIF-PH), prevents HIF degradation and stimulates the erythropoietin production [63]. The therapeutic short activation of HIF may be protective for kidneys by switching from oxidative phosphorylation to glycolysis, accompanied by decreased oxygen consumption, suppressed ROS production, and minimum ATP production. On the contrary, the chronic activation of HIF does not seem to have potential in the treatment of diabetic kidneys, because HIF would consistently mediate hypoxia-caused cell injury via the HIF-downstream molecules. In addition, chronically enhanced HIF expression implies abnormal cell metabolism as is common in cancer. We cannot exclude the possibility that HIF promotes carcinogenesis. Further studies are required to clarify the mechanisms underlying diabetic hypoxic kidneys and to understand the over- and sustained expressions of HIF-promoted cell metabolism. Furthermore, it has been recently shown that the therapeutic target for diabetic kidneys could be simply hypoxia, not HIF. This point was brought into focus by the findings of Friederich-Persson and colleagues [24].

5. Why Hypoxia in Diabetic Kidneys?

As described above, the kidneys are very sensitive to hypoxia, because of the energy demand for its function, and this is the reason why hypoxia contributes to the glucose metabolism. The kidney is unique organ that uptakes, uses, and produces glucose. Kidneys need oxygen for the reabsorption of filtrated glucose and electrolytes, and they produce ATP via oxidative phosphorylation and glycolysis. Under hypoxic conditions, to produce ATP to maintain kidney function, the kidneys use anaerobic fermentation by Pasteur effects. Even in normoxia, hyperglycemia causes an increased glucose influx along with activated glycolysis, which leads to other glucose submetabolic pathways such as polyol pathways. Therefore, hyperglycemia promotes glycolysis and other metabolic pathways rather than mitochondrial oxidative phosphorylation in normoxia, which resembles the Warburg effect that plays an important role in cancer metabolism.

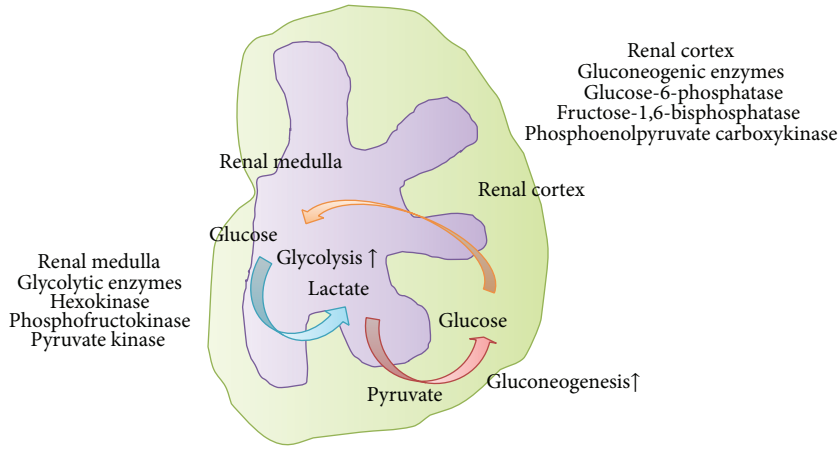

Figure 6: Glycolysis and gluconeogenesis in the kidney. A lactate shuttle may operate in the kidney between medulla and cortex as a main source of energy to sustain renal physiology. In diabetic kidneys, HIF may augment the medullary glycolysis rather than oxidative phosphorylation, leading to the production of lactate, which could be taken up by the cortical proximal tubules for gluconeogenesis. Then, renal glucose release in diabetic kidney is increased.

HIF plays a key role in the glycolytic switch by the regulation of genes encoding most glycolytic transporters and enzymes and then enhancing renal medullary glycolysis. By using hyperpolarized [1-^{13}C] pyruvate MRI, Laustsen et al. demonstrated that reduced oxygen availability in streptozotocin diabetic rat kidney altered the energy metabolism by increasing lactate and alanine formation [64]. In addition to diabetes-induced pseudohypoxia, reduced oxygen content in inspired air increased the pyruvate-to-lactate and pyruvate-to-alanine formation accompanied by a reduced NAD^+/NADH ratio. Both increased the mRNA expression of lactate dehydrogenase A (LDHA) which metabolites pyruvate to lactate and alanine aminotransferase (ALT) which converts pyruvate to alanine, suggesting that these two pathways may contribute to reducing substrate flux into the TCA cycle and limiting oxidative phosphorylation [64]. The similar pyruvate depletion in kidney is known during AKI in mice [65]. Although lactate levels in AKI mice kidneys fell below control levels, the pyruvate levels remained depressed, because AKI increases gluconeogenesis, which results in pyruvate consumption [65].

The kidney is known to be a significant gluconeogenic organ, and in overnight-fasted normal humans, proximal tubule gluconeogenesis is responsible for approximately 40% of whole-body gluconeogenesis [66]. Renal and hepatic glucose release in human diabetic subjects with type 2 diabetes are increased [67]. A study of ZDF rats also showed that renal gluconeogenesis is upregulated accompanied by an increase in the activities and mRNA levels of the key gluconeogenic enzymes [68].

The proximal tubule reabsorbs glucose and synthesizes glucose, known as "gluconeogenesis," but does not metabolize glucose. The renal medulla utilizes glucose and generates lactate for its glycolysis. Simultaneously, the cortical proximal tubules take up the lactate released by the medulla, use it for oxidative phosphorylation, and then generate and release glucose for the energy production by the medullary glycolysis (Figure 6). A similar lactate shuttle may operate in the brain between astrocytes and neurons as a main source of energy to sustain neuronal physiology [69]. In diabetic kidneys, HIF may augment the medullary glycolysis rather than oxidative phosphorylation, leading to the production of lactate, which could be taken up by the cortical proximal tubules for gluconeogenesis. HIF thus could remodel the energy production system for diabetic kidneys under hypoxia. In contrast, in AKI, HIF fails to work, which might result in deficits of medullary glycolysis and subsequent cortical gluconeogenesis, because the oxygen-sensitive medulla does not suffer severe hypoxia by relative lack of oxygen consumption for sodium reabsorption, due to a marked decrease in GFR [70].

In conclusion, if human beings do not develop diabetes mellitus, under a blood supply that is one-fourth of the cardiac output, AV oxygen shunting and high capacity for production of energy by rich mitochondria could protect the kidneys from hyperoxia-induced ROS. Diabetes mellitus increases the renal blood flow, glomerular filtration, sodium absorption, need for ATP production, and oxygen consumption. Diabetes mellitus thus induces hypoxia in the kidneys. The systems that protect the kidneys from hyperoxia ironically promote the susceptibility of diabetic kidneys to hypoxic injury.

Conflict of Interests

The authors declare there is no conflict of interests in this work.

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Research Article

The Proteasome Inhibitor, MG132, Attenuates Diabetic Nephropathy by Inhibiting SnoN Degradation *In Vivo* and *In Vitro*

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Transforming growth factor-β (TGF-β) has been shown to be involved in diabetic nephropathy (DN). The SnoN protein can regulate TGF-β signaling through interaction with Smad proteins. Recent studies have shown that SnoN is mainly degraded by the ubiquitin-proteasome pathway. However, the role of SnoN in the regulation of TGF-β/Smad signaling in DN is still unclear. In this study, diabetic rats were randomly divided into a diabetic control group (DC group) and a proteasome inhibitor (MG132) diabetes therapy group (DT group). Kidney damage parameters and the expression of SnoN, Smurf2, and TGF-β were observed. Simultaneously, we cultured rat glomerular mesangial cells (GMCs) stimulated with high glucose, and SnoN and Arkadia expression were measured. Results demonstrated that 24-hour urine protein, ACR, BUN, and the expression of Smurf2 and TGF-β were significantly increased ($P < 0.05$), whereas SnoN was significantly decreased in the DC group ($P < 0.05$). However, these changes diminished after treatment with MG132. SnoN expression in GMCs decreased significantly ($P < 0.05$), but Arkadia expression gradually increased due to high glucose stimulation ($P < 0.05$), which could be almost completely reversed by MG132 ($P < 0.05$). The present results support the hypothesis that MG132 may alleviate kidney damage by inhibiting SnoN degradation and TGF-β activation, suggesting that the ubiquitin-proteasome pathway may become a new therapeutic target for DN.

1. Introduction

Diabetic nephropathy (DN) is one of the most prevalent and serious microvascular complications of diabetes mellitus (DM) [1]. Early pathological characteristics are basement membrane thickening, increased mesangial matrix production, and extracellular matrix accumulation, with subsequent development of glomerulosclerosis and tubulointerstitial fibrosis, eventually leading to irreversible renal damage [2–4]. Currently, the pathogenesis of DN remains unclear and treatments such as strict glucose and blood pressure control are limited in their effectiveness [5]. Further investigations into molecular mechanisms are required, in order to develop new therapeutical approaches for DN.

As a key mediator of fibrogenesis, transforming growth factor-β (TGF-β) plays a critical role in the development of DN [6]. Many fibrogenic cytokines, such as advanced glycation end products (AGEs), due to hyperglycemia, may activate TGF-β signaling by a Smad-dependent pathway, resulting in fibrosis [7].

The transcriptional coregulator, SnoN, is a critical and versatile regulator of TGF-β-induced transcription and responses. SnoN controls TGF-β-mediated responses by acting as a transcriptional corepressor or transcriptional coactivator [8]. SnoN associates with Smad2/3 and Smad4 and is recruited to TGF-β responsive genes, thus influencing their transcription [9]. Remarkably, as well as inducing SnoN degradation, TGF-β stimulates SnoN transcription; once expressed, SnoN acts as a negative feedback inhibitor of TGF-β signaling. When overexpressed, SnoN inhibits transcription of genes regulated by the TGF-β/Smad signaling pathway [9, 10]. To counteract

SnoN inhibition of transcription, TGF-β signaling induces the degradation of SnoN by the ubiquitin-proteasome pathway (UPP) [11, 12]. SnoN expression is altered under many pathological conditions including wound healing, liver regeneration, and obstructive nephropathy [13].

Ubiquitin is well known for its function in targeting proteins for degradation by the 26S proteasome, which is important for the removal of abnormal and damaged proteins and many regulated processes. Ubiquitin ligases, such as Smurf2 and Arkadia, mediate the ability of TGF-β to induce ubiquitination and consequent degradation of SnoN [14]. Smad ubiquitin regulatory factor 2 (Smurf2) is an E3 ubiquitin ligase that regulates transforming growth factor-β (TGF-β)/Smad signaling and is implicated in a wide variety of cellular responses [15]. Arkadia is a nuclear protein with 989 amino acid residues, with a characteristic C-terminal RING domain [16]. Arkadia appears to effectively enhance TGF-β signaling through simultaneous downregulation of two distinct types of negative regulators, namely, Smad7 and SnoN, which are critical substrates of Arkadia and may play an important role in determining the intensity of TGF-β family signaling in target cells [17].

Previous studies have demonstrated that AGEs, formed as a result of hyperglycemia, can activate TGF-β signaling in DN. SnoN, as a negative regulator of TGF-β signaling, can be degraded by the UPP. However, whether ubiquitin degradation of SnoN, by TGF-β signaling, is involved in the development of DN still remains to be elucidated. Here, we established a rat model of DN by using STZ and selected MG132 as the specific ubiquitin-proteasome inhibitor to block the UPP, in order to explore the relationship between the UPP and the TGF-β/Smad signaling pathway *in vivo*. We also investigated whether SnoN is degraded and if UPP is activated in cultured rat glomerular mesangial cells (GMCs) stimulated by high glucose *in vitro*.

2. Materials and Methods

2.1. Establishing the Animal Model. Male Wistar rats weighing 200 g were purchased from the Biotechnology Corporation of Teng Xing (Chongqing, China). Rats were brought into a special room with a stable ambient temperature of 18°C–22°C and housed in wire cages with free access to a standard diet and tap water. Blood glucose levels of all rats were measured prior to the start of the experiment.

The rats were randomly allocated into two groups: a control group (NC group, $n = 20$) and an experimental group. Rats in the experimental group were rendered diabetic by intraperitoneal injection of Streptozocin (STZ, Sigma, USA), at a dose of 60 mg/kg. STZ was dissolved in 0.1 M citrate buffer at pH 4.5. Meanwhile, rats in the NC group received an intraperitoneal injection of the same volume of citrate buffer. After 3 days following the STZ injection, fasting glycemic measurements were performed in blood samples from tail veins, and blood glucose levels of ≥16.7 mmol/L lasting 3 days were confirmed as being "diabetic." Diabetic rats that presented mild microalbuminuria (an early sign of DN) were all included in the study and were further randomly divided into two groups: a diabetic control group (DC group, $n = 20$) and a diabetes therapy group (DT group, $n = 20$), treated with MG132 (0.05 mg/kg daily, CALBIOCHEM, USA). Meanwhile, the NC and DC groups received daily injections of equivalent volumes of citrate buffer.

2.2. Cell Culture. Rat GMCs (HBZY-1) were purchased from the Preservation Center at Wuhan University and maintained in low-glucose Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (Hyclone) at 37°C and 5% CO_2. GMCs were used for all experiments and randomly divided into the following five groups: normal control group (NC group, with medium containing 5.6 mmol/L glucose), 20 mmol/L glucose group (20 M group, with medium containing 20 mmol/L glucose), 30 mmol/L glucose group (30 M group, with medium containing 30 mmol/L glucose), osmotic pressure control group (OP group, with medium containing 5.6 mmol/L glucose + 24.6 mmol/L mannitol), and MG132 therapy group (MT group, with medium containing 30 mmol/L glucose + 0.5 μmol/L MG132) to block the UPP. Cells in each group were cultured for 12 h, 24 h, and 48 h to detect SnoN and Arkadia expression by Western blotting, RT-PCR, and immunofluorescence.

2.3. Sample Collection and Body Weight: Biochemical Measurements. All rats were weighed and 24-hour urinary microalbumin (mAlb) was collected every day. Urinary protein and urinary creatinine concentrations were measured according to the manufacturers' procedures described in the kits, and urine albumin-creatinine ratios (ACR) were calculated. After 6 or 8 weeks of MG132 (DT group) or citrate buffer (NC and DC group) injections, all rats were sacrificed and heart blood was collected to measure BUN levels and fasting blood glucose (FBG) levels, using an automatic biochemistry analyzer. Both kidneys were weighed and cut along the coronal plane; upper poles of the right kidneys were used for pathology, and the left renal tissues were preserved at −80°C until required for Western blot analysis and RT-PCR.

2.4. Morphological Analysis: Mesangial Expansion and Collagen. After 6 or 8 weeks, the animals were killed and the upper poles of the right kidneys were rapidly removed, fixed in 10% formaldehyde, dehydrated with gradient ethanol, embedded in paraffin, and sectioned at 4 μm thickness. Renal sections were stained with HE and Masson staining. All sections were evaluated under a light microscope. The glomerular cross-sectional area (Ag), mesangial area (Am), and tuft area (At) were measured in 20 glomerular profiles per rat by using Image-Pro Plus 6.0 software. The values of semiquantitative analysis for the positive areas are expressed as the means ± SD from rat at each group. All measurements were done in a masked fashion.

2.5. Immunohistochemical Staining. Sections were incubated with the following primary antibodies: SnoN (mouse, 1 : 100 dilution, Santa Cruz, USA) and Smurf2 (rabbit, 1 : 200 dilution, Beijing biosynthesis biotechnology, China) overnight at 4°C. After sections were washed with PBS, they were incubated with horseradish peroxidase-conjugated secondary antibodies (1 : 200 dilution) for 2 h at room temperature.

For visualizing the signals, sections were treated with peroxidase substrate DAB (3,3-diaminobenzidine) and counterstained with hematoxylin.

2.6. Western Blotting. Renal tissues and rGMCs were homogenized in lysis buffer (Kaiji, Shanghai, China). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Immunoblot analysis was performed using SnoN antibody (mouse, 1 : 1000; Santa Cruz, USA), TGF-β antibody (rabbit, 1 : 1000; CST, USA), β-actin antibody (rabbit, 1 : 1000; Abcam, USA), and GAPDH antibody (mouse, 1 : 2000; Beyotime China). Horseradish peroxidase-conjugated secondary antibodies were obtained from the Beyotime Institute of Biotechnology (Shanghai, China). Proteins were detected using the enhanced chemiluminescence system and ECL Hyperfilm (Amersham, England).

2.7. RNA Extraction and Reverse-Transcription Polymerase Chain Reaction. Total RNA was extracted from renal tissues and rGMCs using an RNA extraction kit (Tiangen Biotech, Beijing, China). Total RNA was reverse-transcribed (RT) using a Takara RNA PCR kit (Baoshengwu, Dalian, China). cDNA was amplified in a gradient thermal cycler (Eppendorf, Germany) using polymerase chain reaction (PCR) Master Mix (Baoshengwu, Dalian, China). The results were determined using an ultraviolet transilluminator and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression. The primer sequences were the following: SnoN (forward, 5′-GAAGAAAAGAAACTGAAGAT-3′, reverse, 5′-CTGGGGTGTAAAAATGAAT-3′) and GAPDH (forward, 5′-CCT CAA GAT TGT CAG CAA T-3′, reverse, 5′-CCA TCC ACA GTC TTC TGA GT-3′).

2.8. Immunofluorescence. Mesangial cells were grown on coverslips in 6-well plates. After overnight adherence, cells were treated with media that contained high glucose and MG132 for 24 h. Cells were then fixed in 4% paraformaldehyde (Pierce Biotechnology, Rockford, IL, USA) and blocked with 5% goat serum, followed by incubation with anti-SnoN antibody or anti-Arkadia antibody, overnight at 4°C. After washing, cells were incubated with fluorescein isothiocyanate-(FITC-) conjugated secondary antibodies (BioSynthesis) for 45 min in the dark. Images were taken with a DMIRE2 laser scanning confocal microscope (Leica, Germany).

2.9. Statistical Analyses. Each experiment was repeated at least twice. Data are expressed as mean ± standard deviation (SD). Differences were statistically analyzed using one-way analysis of variance (ANOVA), followed by the Least Significant Difference post hoc test for multiple comparisons. A probability value, P, of <0.05 was considered significant.

3. Results

3.1. MG132 Reversed STZ-Induced Changes in 24-Hour Urine Protein and Renal Function of Diabetic Rats. Compared to the NC group ($P < 0.05$), fasting blood glucose (FBG, Figure 1(a)) levels were increased in the DC and DT groups, and the body weight (Figure 1(b)) was significantly decreased ($P < 0.05$). There were, however, no obvious differences in FBG levels and body weight between the DT and DC groups. These data suggest that diabetic rat models were successfully achieved by using STZ and that MG132 was unable to influence STZ-induced changes in FBG and body weight. Conversely, compared to the NC group ($P < 0.05$), 24-hour urine protein (Figure 1(c)), urinary albumin-to-creatinine ratios (ACR, Figure 1(d)), and BUN levels (Figure 1(e)) increased in the DC and DT groups, all important features of DN. Moreover, compared with the DC group ($P < 0.05$), STZ-induced changes were partially reversed by MG132.

3.2. MG132 Attenuates STZ-Induced Downregulation of SnoN and Activation of TGF-β In Vivo. Renal tissue immunohistochemistry (Figure 2(a)) showed that, compared with the NC group, there was a decreased SnoN expression that was particularly evident in the DC group, which was partially reversed by MG132 in the DT group. However, an E3 ubiquitin ligase, Smurf2, express (Figure 2(b)) in the DC group was increased and practically returned to normal in DT group ($P < 0.05$). Renal tissue Western blotting and RT-PCR (Figure 2(c)) demonstrated that the expression of SnoN protein was reduced in the DC and DT groups; however, compared with the DC group ($P < 0.05$), SnoN degradation in the DT group was partially reversed by MG132, although the mRNA levels of SnoN were not statistically different in each group ($P > 0.05$).

To investigate the therapeutic effect of MG132 on the kidney, renal pathology was examined with HE staining (Figure 2(d)). Pathological changes in the kidney of diabetic rats were obvious; the glomerular tuft and mesangial area were increased at 6 or 8 weeks. There was a trend for an increase of glomerular volume in DC group compared with NC group. MG132 treatment ameliorated the increase of both tuft area and mesangial area. Collagen plays a critical structural role in renal fibrosis of DN. Observation with the light microscope, following Masson staining, (Figure 2(e)) demonstrated that accumulation of collagen in the kidney of the DC groups was greater than the NC group in gross appearance; this effect was significantly decreased by MG132 treatment. These experiments and figures showed that induction of DN by STZ was evident in the mesangial area, with deposition of abnormal substances, with the DT group being less pronounced than the DC group.

Consistent with the Masson staining patterns, Western blotting (Figure 2(f)) suggested that TGF-β expression, an important factor for regulation of fibrosis, was increased in the DC and DT groups ($P < 0.01$), but expression was significantly decreased in the DT group, due to the action of the proteasome inhibitor, MG132, compared with the DC group ($P < 0.05$).

3.3. MG132 Partially Reversed High Glucose-Induced Degradation of SnoN In Vitro. The relative expression of SnoN (SNON-to-GAPDH protein ratio) decreased as the glucose concentrations and time increased ($P < 0.05$). The most significant changes were observed with 30 mmol/L glucose

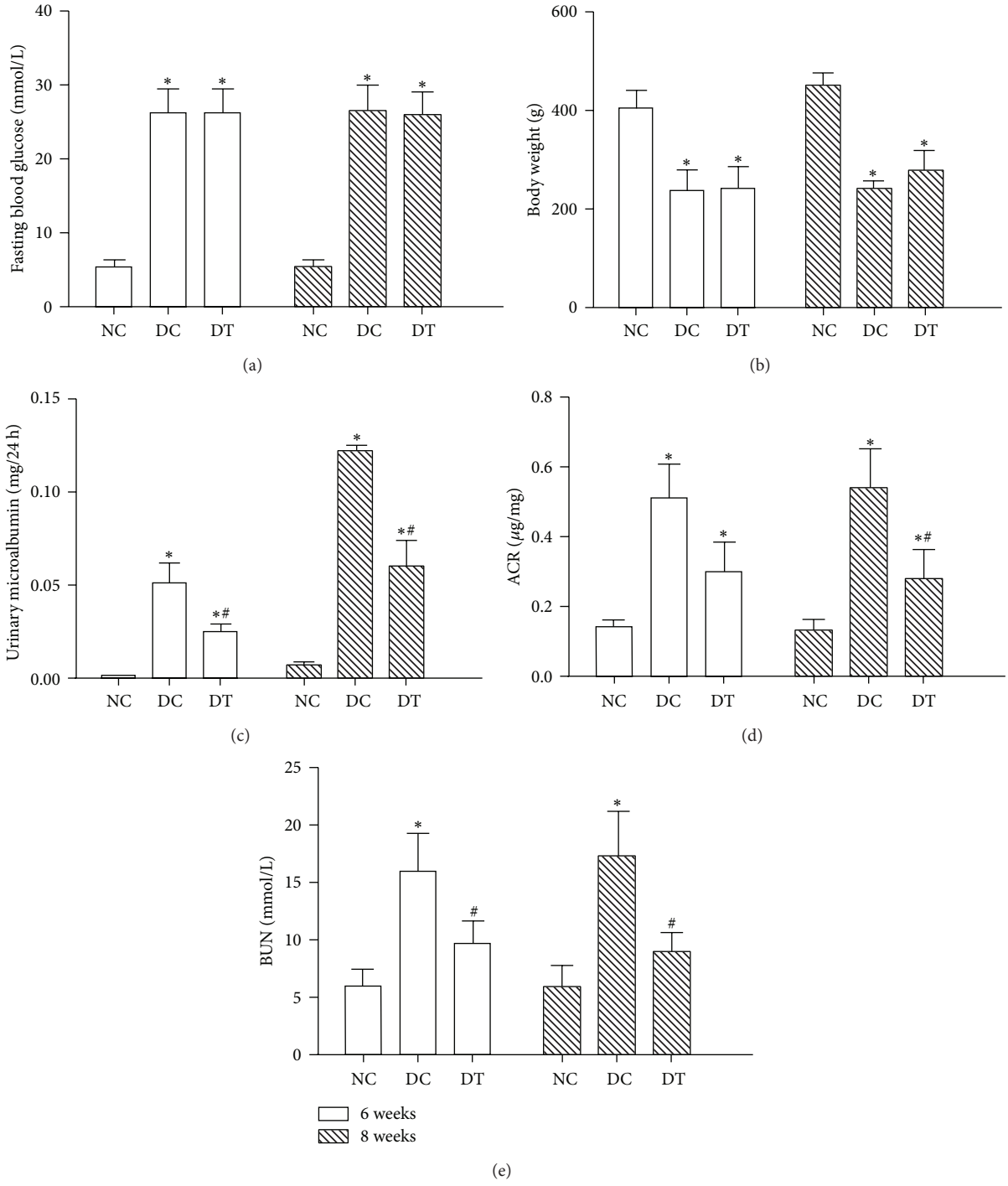
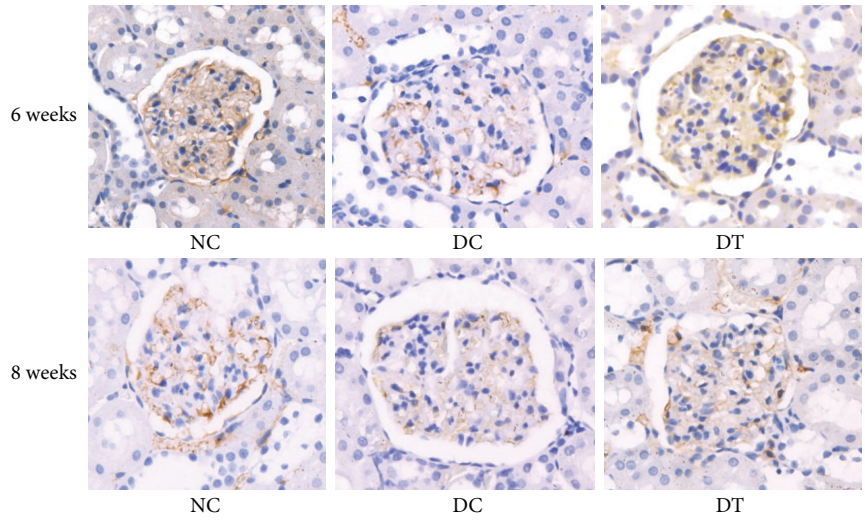
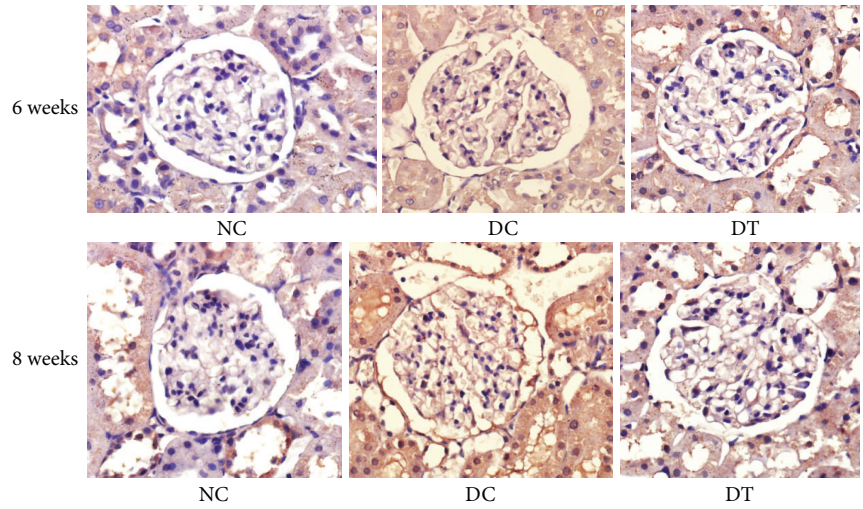


Figure 1: Therapeutic effects of the proteasome inhibitor, MG132, on diabetes-induced general changes and renal function. Diabetic rats were divided into two groups: a diabetic control group (DC group) and a diabetes therapy group (DT group) treated daily with MG132 (0.05 mg/kg). Meanwhile, the NC and DC groups received daily injections of equivalent volumes of citrate buffer. After 6 or 8 weeks, fasting blood glucose (a), body weight (b), 2-hour urine protein (c), ACR (d), and BUN (e) levels were examined before and after treatment with MG132. Data are presented as means ± SD. $^{*}P < 0.05$ versus NC group; $^{\#}P < 0.05$ versus DC group.

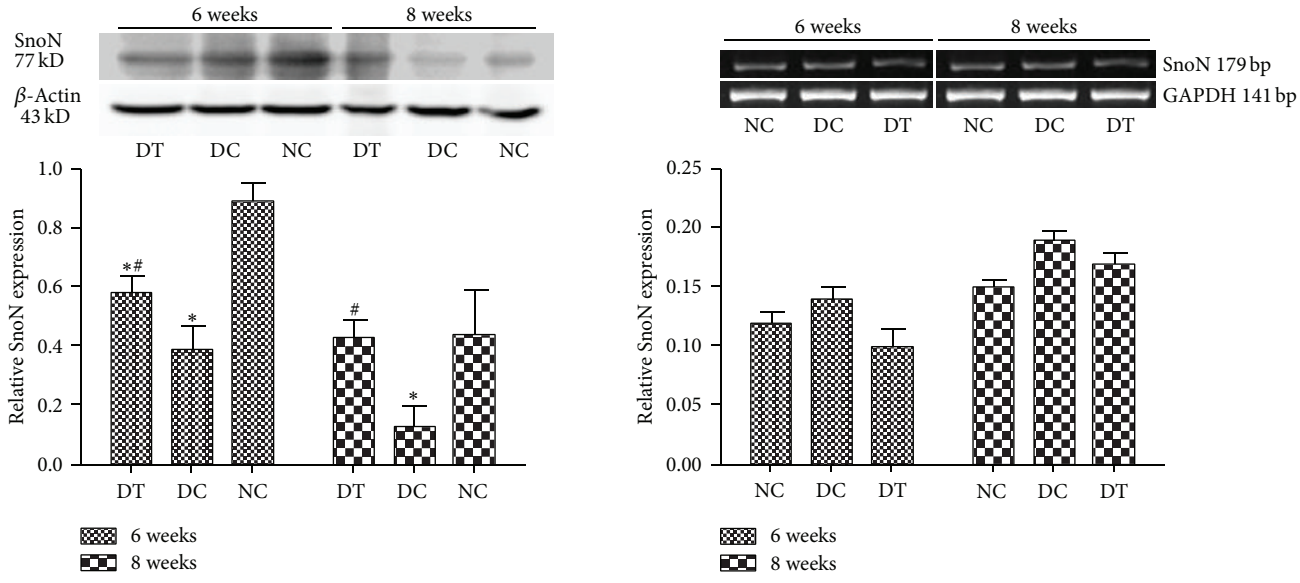
stimulation after 48 h (Figure 3(a)). SnoN was significantly degraded by different glucose concentrations, especially in the 30 mmol/L glucose group (Figure 3(b)). There was no significant difference between the NC and OP groups with regard to SnoN expression ($P > 0.05$), indicating that the high glucose-induced changes of SnoN were not an osmotic effect. However, the proteasome inhibitor, MG132, partially reversed SnoN degradation. After MG132 treatment, SnoN protein levels in the MT group were partially reversed compared with the 30 mmol/L glucose group. In addition,



(a)



(b)



(c)

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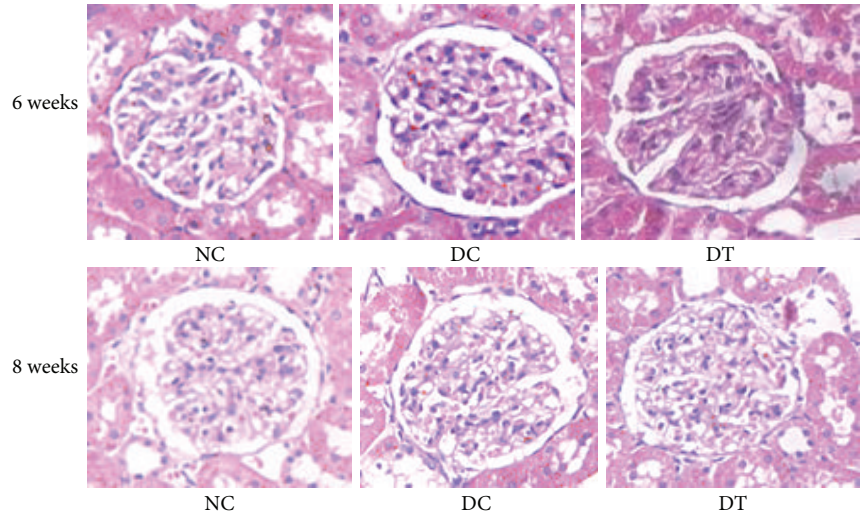
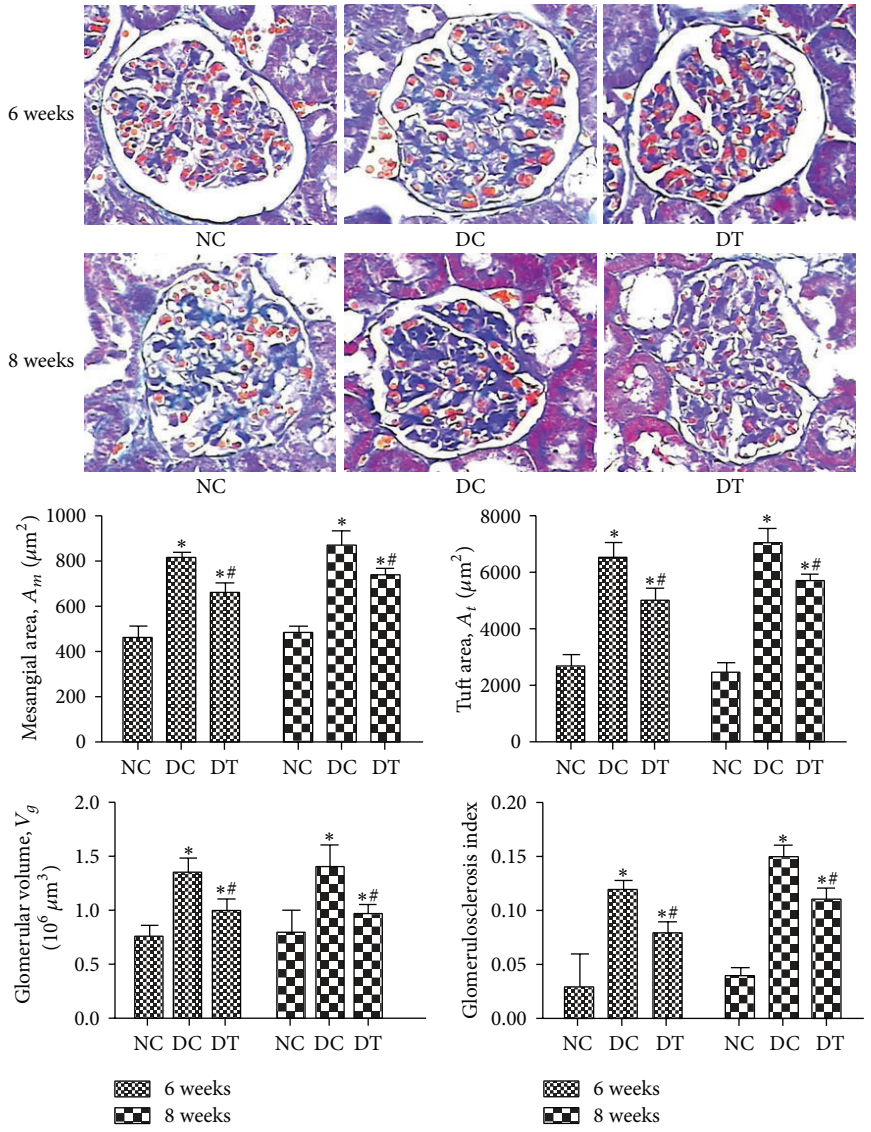


(d)



(e)

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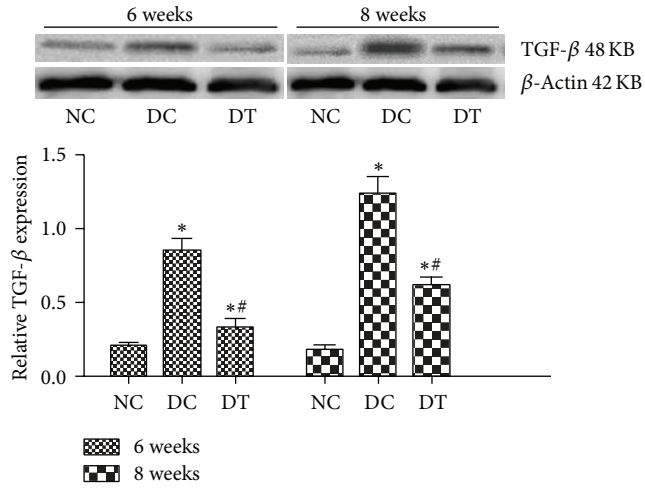

(f)

Figure 2: MG132 attenuates STZ-induced downregulation of SnoN and TGF-β activation *in vivo*. Representative images of immunohistochemistry staining showing SnoN (a) and Smurf2 (b) expression in renal tissue. SnoN and Smurf2 expression are represented as the positive yellow-brown stained area (200x). SnoN expression in renal tissues was detected by Western blotting and RT-PCR (c): SnoN expression decreased in the DC and DT groups after injection of STZ and MG132 partially reversed SnoN degradation in the DT group; the gray graph confirmed these trends. However, the mRNA levels of SnoN were not statistically different in each group ($P > 0.05$). Morphologic parameters of kidney pathology were examined with HE ((d), 200x) and Masson staining ((e), 200x). The gray graph shows the values of semiquantitative analysis for mesangial area (Am), tuft area (At), glomerular cross-sectional area (Ag), and glomerulosclerosis index. TGF-β (f) protein expression in each group by Western blotting and the gray graph show the relative statistical values of TGF-β for each group. Data are presented as means ± SD. $^{*}P < 0.05$ versus NC group; $^{\#}P < 0.05$ versus DC group.

similar to the experiment *in vivo*, there was no significant difference in each group involving the SnoN mRNA expression. These data suggest that high glucose led to decreased SnoN expression through the UPP.

Immunofluorescence (Figure 3(c)) showed that SnoN is predominantly expressed in the cytoplasm. After 30 mmol/L high glucose intervention, SnoN expression was significantly decreased, but this trend was strongly reversed in the MG132 intervention group, supporting the Western blotting response to high glucose and MG132. Furthermore, Arkadia (Figure 3(d)) was weakly expressed in the NC group but was prominent in the 30 mmol/L high glucose group and did not significantly change as the result of high osmotic pressure after culturing for 24 h, suggesting that the high glucose-induced activation of the UPP was not an osmotic effect. However, elevated Arkadia expression in the nucleus was significantly decreased by the addition of MG132.

4. Discussion

4.1. The Role of SnoN and MG132 in an STZ-Induced Diabetic Nephropathy (DN) Rat Model. The mechanism of diabetic nephropathy is multifactorial. The important role of the TGF-β signaling pathway in diabetic nephropathy (DN) has been recognized. The transcriptional regulator SnoN plays a fundamental role as a modulator of TGF-β-induced signal transduction and subsequent biological responses. Accumulating evidence suggests that SnoN plays a dual role as a corepressor or a coactivator of TGF-β-induced transcription [18].

The ubiquitin-proteasome pathway (UPP) is an important nonlysosomal protein degradation pathway. It is able to degrade intracellular proteins efficiently and in a highly selective manner and; in particular, it is able to upregulate or downregulate signaling pathways by degrading the intracellular inhibitor or activating factor of each signaling pathway [18]. Recent studies have shown that SnoN is mainly degraded by the UPP, and regulation of SnoN expression in obstructive nephropathy has also been shown to involve the UPP. Smurf2 is localized in the nucleus and physically associates with SnoN, strongly suggesting that Smurf2 is a ubiquitin E3 ligase that targets nuclear SnoN for proteasome-dependent degradation [19].

However, whether degradation of SnoN is involved in the development of DN is unknown. The most common way to block the UPP is by using small peptides or peptide analogues that bind and inhibit the activity of the 20S core protease, thereby blocking the whole pathway. MG132 is one example of this type of inhibitor; one concern, however, for the use of MG132 is nonspecific proteasome inhibition, and whether global proteasome inhibition has other undesirable effects. In general, effective proteasome inhibition by high doses of MG132 induces apoptotic cell death. By contrast, low doses of MG132 mediate a protective response against oxidative stress [20]. Therefore, MG132 at low daily doses (0.05 mg/kg *in vivo* or 0.5 μmol/L *in vitro*) may predominantly inhibit the elevated proteasomal activity that is caused by diabetes in multiple organs, without inhibiting proteasome activity in normal tissues [21].

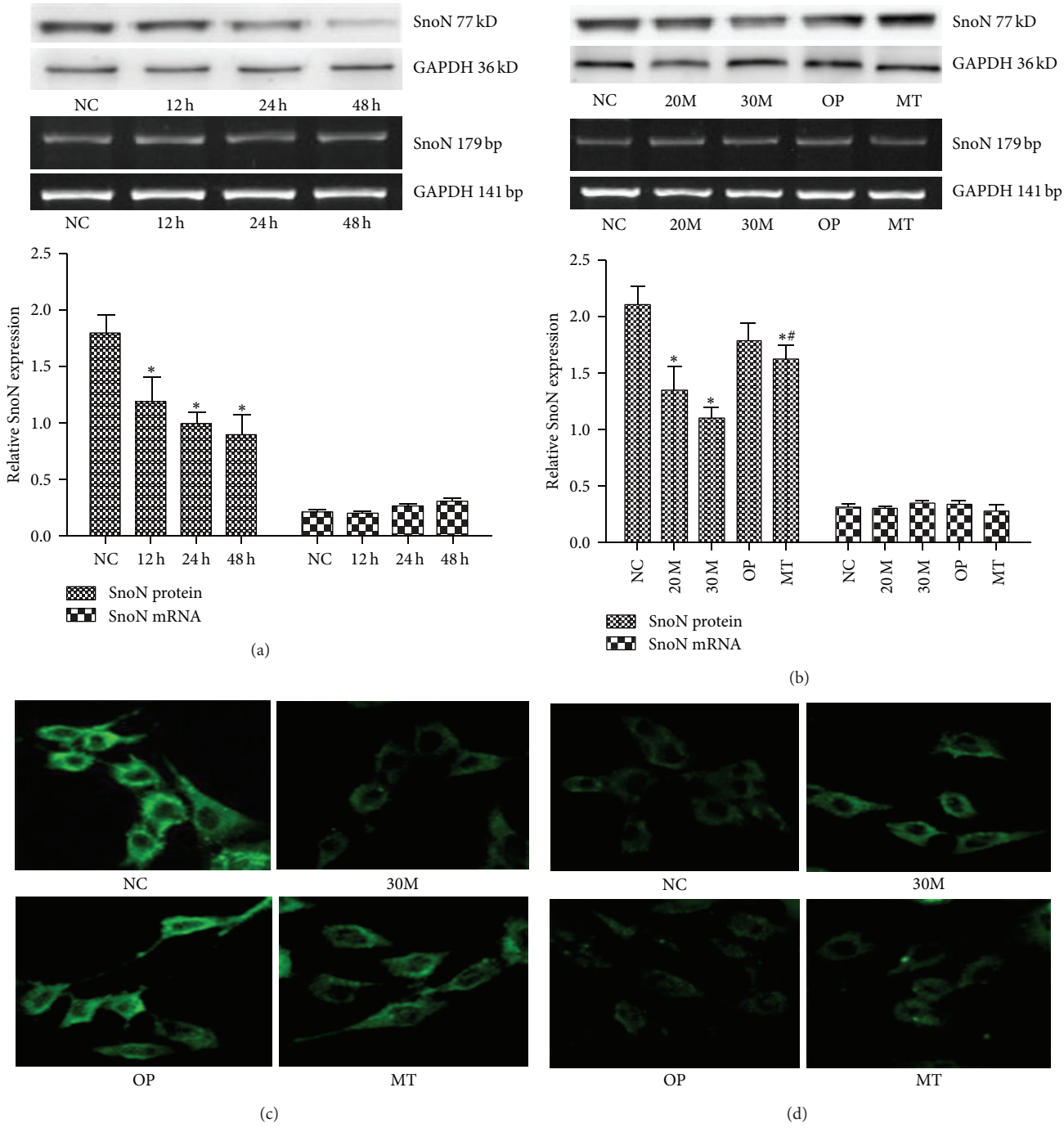

Figure 3: MG132 partially reversed high glucose-induced degradation of SnoN *in vitro*. rGMCs were treated with 30 mmol/L high glucose for 12 h, 24 h, and 48 h (a). Cells were treated with the indicated concentrations of glucose, mannitol, or MG132 for 48 h (b). SnoN expression after high glucose challenge for various times and various glucose concentrations was determined by Western blotting and RT-PCR. The gray graph shows the relative statistical values for SnoN protein and mRNA expression in each group. The data were normalized and are expressed as means ± SD. $^{*}P < 0.05$ versus NC group; $^{\#}P < 0.05$ versus 30M group; the expressions of SnoN (c) and Arkadia (d) of rGMCs were detected by immunofluorescence and laser scanning confocal microscopy (630x). SnoN and Arkadia were detected in the cytoplasm as green fluorescence.

This study was therefore carried out in order to clarify the discrepancy in the literature regarding the respective roles played by SnoN and by the UPP in the regulation of diabetic nephropathy (DN). Streptozocin (STZ) is toxic to pancreatic β cells and has been widely used to induce diabetes in animal models. The STZ-injected rats exhibited the main characteristics of diabetes mellitus and the changes in the DN markers in our study were similar to those

previously reported [22, 23]. Our data support the idea that the transcriptional regulation of SnoN could be considered as a negative regulator of the TGF-β signaling pathway in DN as there appears to be a decreased expression of SnoN in STZ-induced renal tissue, with a concomitant increase in TGF-β expression. However, upon protease inhibition with MG132, these changes were reduced. The results demonstrated that SnoN was involved in the TGF-β signaling pathway in the development of DN.

In this study, we did not examine changes in the mRNA levels of SnoN as a consequence of proteins stimulation by STZ or MG132. This implies that there is no difference in the gene order of SnoN, except for posttranslational modifications, including ubiquitination. In accordance with this, the upregulation of Smurf2 in the kidney is closely correlated with reduction of SnoN after stimulation by STZ. It was also demonstrated that the UPP played a role in activation of the TGF-β pathway and induced the progress of DN by ubiquitin degradation of SnoN. Consistent with our observations, Yang et al. identified SnoN as being a negative regulator of TGF-β signaling, and SnoN is also utilized as a prognostic marker in estrogen receptor-positive breast carcinomas [24, 25]. Several *in vivo* and *in vitro* studies have provided evidence for the increase in proteasomes in diabetes. For example, exposure of human umbilical vein endothelial cells to high glucose significantly increased the 26S proteasome activity. Proteasomal activity was also increased in skeletal muscle and hearts of STZ-induced diabetic rats and in gastrocnemius muscle of spontaneously diabetic (db/db) mice [26, 27].

The features associated with DN progression are glomerular hypertrophy, thickening of the GBM and mesangial expansion, and eventual loss of glomerular filtration and glomerulosclerosis. Microalbuminuria in diabetic patients predicts the onset of proteinuria, as well as an increased risk of death and cardiovascular events [28]. Our results showed that 24-hour urine protein, urinary albumin-to-creatinine ratios (ACR), and serum BUN levels increased in diabetic control groups; the increasing levels of BUN may indicate progressive renal damage. Recent studies have found that MG132 can protect the kidney against diabetes-induced oxidative damage, inflammation, and fibrosis [29], but the exact pathogenesis has not been completely clarified. Renal fibrosis in DN was induced by the activation of the TGF-β signaling pathway, but whether MG132 could treat DN by blocking ubiquitin degradation of SnoN has not been reported. Our research found that, on comparison with the DC group, levels of 24-hour urine protein, ACR, BUN, and collagen content tended to decrease after MG132 intervention. Meanwhile, the pathological changes upon light microscopy observation showed similar trends. This suggests that MG132, by acting as a UPP inhibitor, can protect rat renal tissue from damage, maintain the basement membrane permeability, and reduce urinary protein.

The present study demonstrated that MG132 positively affected these parameters (ACR, BUN, etc.) of DN, but MG132 treatment did not significantly improve blood glucose levels or body weight. Our findings are inconsistent with a previous study that showed a systemic improvement with MG132 when it was used for prevention of diabetes-induced renal pathological changes [30]. We assume that the discrepancy between our study and the previous one is due mainly to the differences of animal models and MG132 administration times [31]. Our results suggest that the therapeutic effect of chronic treatment with MG132 on diabetes-induced renal damage cannot be attributed to systemic improvement, at least not in the Wistar rat diabetic model.

4.2. The Role of SnoN and MG132 in Rat Glomerular Mesangial Cells (GMCs) Inducted by High Glucose. SnoN acts as a Smad corepressor by interacting with Smad complexes to inhibit their transcriptional abilities and by recruiting other corepressors and sequestering Smad proteins to prevent their translocation to the nucleus [11]. However, the role of SnoN in rat glomerular mesangial cells (GMCs) stimulated with high glucose is not fully understood. We found that glucose stimulation correlated in a time- and concentration-dependent manner with decreased SnoN expression, but osmotic stress had little effect on the expression of SnoN compared with high glucose. The decrease in SnoN was inhibited following MG132 intervention. These results suggest that high glucose concentration mediates SnoN degradation by means of the UPP and that MG132 may have a positive function in the treatment of DN by inhibiting SnoN ubiquitination, which affects TGF-β signaling.

Studies have previously found that Arkadia, as an E3 ubiquitin ligase, associates with SnoN proteins in their free forms, as well as when they are bound to Smad proteins. These findings suggest that Arkadia induces constitutive degradation of SnoN; Arkadia protein expression levels thus appear to determine the intensity of TGF-β signaling that is permitted in target cells [15]. However, whether Arkadia enhances TGF-β signaling responses in high glucose conditions is unknown. Our experiments show that Arkadia expression was gradually increased in GMCs stimulated with high glucose, compared with the NC group, the effect of which was almost completely reversed by adding MG132. Our results suggest that Arkadia enhances TGF-β signaling by inducing degradation of SnoN, which is a negative regulator of TGF-β signaling that acts in different ways, and MG132 may have a positive function in the treatment of diabetic nephropathy by inhibiting the disorders involving SnoN ubiquitination.

5. Conclusion

In conclusion, the present study has demonstrated that expression of SnoN protein in rats with early DN is downregulated by UPP and that the proteasome inhibitor, MG132, can reduce degradation of SnoN, thus inhibiting activation of the TGF-β pathway and conferring a therapeutical effect for DN *in vivo*. SnoN degradation, mediated by Smurf2 or Arkadia, may play an important role in activation of TGF-β pathway *in vivo* and *in vitro*. The present results support the hypothesis that ubiquitin degradation of SnoN may be involved in the pathogenesis of DN by specifically activating TGF-β/Smad signaling. Components of the ubiquitin-proteasome pathway may be potential therapeutic targets for the treatment of DN.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Wnt Pathway Activation in Long Term Remnant Rat Model

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Progression of chronic kidney disease (CKD) is characterized by deposition of extracellular matrix. This is an irreversible process that leads to tubulointerstitial fibrosis and finally loss of kidney function. Wnt/β-catenin pathway was reported to be aberrantly activated in the progressive damage associated with chronic organ failure. Extensive renal ablation is an experimental model widely used to gain insight into the mechanisms responsible for the development of CKD, but it was not evaluated for Wnt/β-catenin pathway. This study aimed to elucidate if the rat 5/6 renal mass reduction model (RMR) is a good model for the Wnt/β-catenin activation and possible next modulation. RMR model was evaluated at 12 and 18 weeks after the surgery, when CKD is close to end-stage kidney disease demonstrated by molecular and histological studies. Wnt pathway components were analyzed at mRNA and protein level. Our results demonstrate that Wnt pathway is active by increase of β-catenin at mRNA level and nuclear translocation in tubular epithelium as well as some target genes. These results validate the RMR model for future modulation of Wnt pathway, starting at shorter time after the surgery.

1. Introduction

Progression of chronic kidney disease (CKD) is characterized by deposition of extracellular matrix (ECM). This is an irreversible process that leads to tubulointerstitial fibrosis and finally loss of kidney function. It is widely accepted that epithelial-to-mesenchymal transition (EMT) is the main factor responsible for organ fibrosis. EMT is defined as phenotypic change of fully differentiated epithelial cells to matrix-producing fibroblasts [1].

Several studies have suggested that specific signal pathways may affect the development of fibrosis in the kidney and contribute to epithelial cell injury and fibroplasia. Some of the signaling mediators involved include proinflammatory and profibrotic cytokines produced by infiltrating and resident cells, such as interferon-γ, tumor necrosis factor-α, interleukin-1-β, and transforming growth factor-β-1 (TGF-β) [2].

The TGF-β signaling pathway plays a pivotal role in embryonic development and organogenesis and is a key fibrogenic cytokine in several fibrotic diseases of the kidney, lung, and other organs. Campistol et al. have previously demonstrated that TGF-β serum levels were significantly higher in kidney transplant patients with interstitial fibrosis and tubular atrophy than in those with normal renal function [3, 4].

TGF-β has been shown to be critical for extracellular matrix turnover and for cellular plasticity, such as epithelial-to-mesenchymal transition. This transition represents the phenotypic, reversible switching of epithelial to fibroblast-like cells and may play a major role in the pathogenesis of CKD, increasing the number of activated fibroblasts derived from epithelial cells [5–7]. The role of TGF-β in fibrogenic processes and renal fibrosis has been widely described, as has been the role of crosstalk between TGF-β and Wnt/β-catenin

pathways in cell fate determination during embryonic development and in the adult [8]. Crosstalk between two pathways could be carried out by transregulation, whereby one pathway regulates specific components of the other or through cross-communication between components of the pathways. For example, the stability of the intracellular signal transducers of the TGF-β pathway, the SMAD proteins, can also be regulated by axin, a regulator of Wnt signaling. However, there is little evidence of interdependence or retroregulation between the components of the two pathways in transcriptional regulation of target genes in mammals, particularly in the context of epithelial-to-mesenchymal transition and organ fibrosis [8].

Over the last years, the Wnt/β-catenin pathway was reported to be aberrantly activated in the progressive damage associated with chronic organ failure [9, 10]. Wnts are a family of 19 proteins essential for organ development, a process that has been described to be recapitulated in organ failure. Canonical Wnt pathway involves the cytoplasmic protein β-catenin. In the absence of Frizzled (FZD) receptor binding of Wnt ligands, β-catenin is constitutively phosphorylated by glycogen sintase kinase-3β (GSK-3β) and subsequently degrades by the complex composed of the adenomatous polyposis coli protein (APC) and axin. In the presence of Wnt ligand binding to a FZD receptor and a low density receptor (LRP) coreceptor leads to the phosphorylation of disheveled protein in the cytoplasm (DVL). The activated DVL binds to axin in order to antagonize the action of GSK-3β; subsequently β-catenin phosphorylation and ubiquitination are inhibited. This causes a citoplasmatic accumulation of β-catenin in the cytoplasm and translocation to the nucleus where it regulates target gene expression through interaction with members of the TCF/LEF family of HGM-domain transcription factors [9, 10].

Increased Wnt/β-catenin signaling has been shown to be involved in epithelial cell injury and hyperplasia, as well as in impaired epithelial-mesenchymal crosstalk in fibrosis. Modulation of Wnt signaling led to an attenuation of lung fibrosis *in vivo* [9], while altered expression of Wnt signaling components has been reported in several renal diseases [11] and animal models, such as rat chronic allograft dysfunction model [2], unilateral ureteral obstruction model [12], lupus nephritis [13], or diabetic nephropathy [14].

Several CKD animal models have been used to study the mechanisms responsible for the disease progression. Extensive renal ablation is an experimental model widely used to gain insight into the mechanisms responsible for the development of CKD since it causes a combination of glomerular hemodynamic alterations and nonhemodynamic events that result in progressive glomerulosclerosis, tubulointerstitial damage, proteinuria, and renal functional impairment in the remnant kidney. There is early glomerulosclerosis by week 4, with segmental sclerosis with tubulointerstitial fibrosis by week 8 [15, 16].

This study aimed to elucidate if the 5/6 renal mass reduction model is a good model for the Wnt/β-catenin activation and possible next modulation. To do this, we used 5/6 renal mass reduction model animals and we sacrificed animals at 12 and 18 weeks after the surgery, when CKD is well established, and then we characterized the fibrosis establishment and the Wnt activation.

2. Methods

2.1. Antibodies and Reagents. In this study, the following antibodies were used: total β-catenin (number 9562), phospho-S9- and total GSK-3β (numbers 9336 and 9315), phospho- and total LRP6 (numbers 2568 and 2560), and phospho-SMAD3 (C25A9) (all from Cell Signaling Technology, Beverly, MA); Wnt1 (600-401-A37) (Rockland, Gilbertsville, PA); α-SMA (A2547); Ca^{2+}ATPase (A7952); cyclin D1 (C7464); GADPH (G9545); β-actin (A3854); Pan CK (F3418); Desmin (D1033) and SIS3 (S0447) (all from Sigma-Aldrich, Saint Louis, MO); FBPase (sc-166097) and factor VIII (sc-27647) (from Santa Cruz Biotechnology, Santa Cruz, CA); Alexa Fluor 568 phalloidin (A-12380) (Invitrogen); vimentin (AB1620) and TGF-β (MAB1032) (from Millipore, Billerica, MA).

2.2. Animal Model: The 5/6 Renal Mass Reduction

2.2.1. Animals. Male Wistar rats (Charles River Laboratories España, Barcelona, Spain) weighing approximately 225 g were used. The rats were kept at a constant temperature and humidity and at a 12 h light/dark cycle. The animals had free access to standard rat chow (Harlan Interfauna Iberica, S.L., Barcelona, Spain) and water.

This study was approved by and conducted according to the guidelines of the Local Animal Ethics Committee (Comitè Ètic d'Experimentació Animal, CEEA, Decret 214/97, Catalunya, Spain).

2.2.2. Experimental Design. Animals ($n = 33$) were randomly assigned to two groups according to the surgical intervention. One group was assigned to undergo renal mass reduction (nephrectomized group (Nx)) and the other to have a sham operation (sham group (S)). Surgical procedures were performed under general anesthesia with isoflurane (Forane; Abbott Laboratories, S.A., Madrid, Spain). After an abdominal incision, the left kidney was exposed and separated from the adrenal gland. The lower and upper poles of the left kidney were frozen by application of a cylinder of dry ice of standard size for 2 minutes on each pole. After one week, the right kidney was removed. The sham group rats underwent the same abdominal incision and manipulation of the left and the right kidneys without tissue destruction as described by Rovira et al. [15, 16].

2.3. Renal Function Parameters. Serum creatinine, BUN, proteinuria, urinary creatinine, and proteinuria/creatinine ratios were determined 1-2 days before sacrifice. To collect urine samples the rats were housed in metabolic cages separately for 24 h.

2.4. Tissue Collection. At the end of the study, the rats were killed and kidney samples were harvested. Central slide from kidney was fixed in formalin and embedded in paraffin by

Table 1: Biochemical parameters for renal function evaluation.

		Diuresis (mL)	Proteinuria (mg/24 h)	Urinary creatinine (mg/24 h)	Creatinine (mg/dL)	BUN (mg/dL)	Creatinine clearance
12 weeks	Sham	18.13 ± 8.25	19.88 ± 6.33	18.75 ± 2.25	0.57 ± 0.06	16.88 ± 1.25	2.32 ± 0.37
	5/6 Nx	34.88 ± 11.13	150.75 ± 65.97	12.75 ± 2.43	1.29 ± 0.31	66.63 ± 18.62	0.73 ± 0.24
18 weeks	Sham	15.37 ± 4.00	13.37 ± 4.21	19.5 ± 2.00	0.6 ± 0.06	18.38 ± 2.50	2.21 ± 0.25
	5/6 Nx	27.85 ± 6.96	143 ± 37.40	10.71 ± 3.95	1.63 ± 0.44	92.28 ± 31.77	0.53 ± 0.36

routine methods. The remaining tissue was snap-frozen and stored at −80° until use.

2.5. Reverse Transcription and Quantitative Real-Time PCR. Total RNA was extracted by using a Perfect Pure RNA fibrous Tissue Kit (5PRIME, Hamburg, Germany) from renal tissue (human and rat) and by using RNeasy Mini Kit (Qiagen, Hilden, Germany) extraction kits according to the manufacturer's protocol. cDNAs were generated by reverse transcription using MuLV reverse transcriptase (Roche, Indianapolis). Quantitative (q)RT-PCR was performed using Light Cycler 480 SyBr Green I master (Roche), as previously described. r_RPL19, ubiquitously and equally expressed gene free of pseudogenes, was used as a reference gene in all qRT-PCR reactions. PCR was performed using the primers at a final concentration of 200 nM. The relative transcript abundance of a gene is expressed in DCt values ($DCt = Ct_{reference} - Ct_{target}$). Relative changes in transcript levels compared with controls are expressed in DDCt values ($DDCt = DCt_{treated} - DCt_{control}$). All DDCt values correspond approximately to the binary logarithm of the fold change, as mentioned in Section 3.

2.6. Protein Extraction from Tissue. Renal rat tissue was homogenized in extraction buffer QProteome Mammalian Protein Preparation Kit (QIAGEN), and the whole proteins were extracted by centrifugation (14,000 rpm) for 10 min at 4°C, as recommended by the supplier. Proteins were quantified using the Micro BCA Protein Assay Kit (PIERCE).

2.7. Western Blot. Samples containing 30 μg of protein were separated by electrophoresis on SDS polyacrylamide gels. The separated proteins were transferred to PVDF membranes (BioRad), blocked with 5% skimmed milk or BSA depending on customer indication, and incubated with the indicated antibodies. The proteins were then visualized by enhanced chemiluminescence detection (ECL, Pierce) with Image Quant LASS 4000 (GE Healthcare). Densitometry quantification was done using Image J.

2.8. Histology. Human biopsies and rat kidneys were placed in 4% (w/v) paraformaldehyde after explantation and were processed for paraffin embedding. Sections (3 μm) were cut and mounted on slides. Light microscopic evaluation was performed on sections stained by Masson trichrome to evaluate fibrotic changes in representative renal allografts [3].

2.9. Immunohistochemistry. The 3 μm sections were subjected to antigen retrieval and quenching of endogenous peroxidase activity using 3% (v/v) H_2O_2 for 20 minutes. Immune complexes were visualized using suitable peroxidase-coupled secondary antibodies, according to the manufacturer's protocol (Histostain Plus Kit; Zymed/Invitrogen). FBPase staining was made to discriminate the proximal tubule, and double staining with β-catenin was performed. Additionally, to exclude false-positive results for β-catenin nuclear translocation, we used histological slides of β-catenin from the next section to perform single staining with Vulcan fast red. The whole area of the biopsy samples was examined to detect cells with nuclear localization of β-catenin.

2.10. Collagen Assay. Protein extracts from rat kidneys were used. Total collagen content was determined using the Sircol Collagen Assay kit (Biocolor, Belfast, Northern Ireland) following the enhanced protocol. Samples and collagen standards were then read at 540 nm. Collagen concentrations were calculated using a standard curve generated by using acid-soluble type 1 collagen.

3. Results

3.1. Renal Function Analysis. All animals that undergo renal mass reduction (RMR) showed clear loss of renal function that it is increasing across the time. Diuresis, proteinuria, BUN, and creatinine were highly increased in nephrectomized animals as well as urinary creatinine and creatinine clearance was markedly reduced after renal mass reduction compared with sham animals (Table 1).

3.2. Assessment of Renal Fibrosis in RMR Model. Left kidney from sham (S) and 5/6 nephrectomized (Nx) rats was explanted at 12 or 18 weeks after surgery and underwent qRT-PCR analysis to assess renal fibrosis. General markers for fibroblast activation including α-smooth muscle actin (α-SMA), fibroblast-specific protein-1 (FSP1), collagen 1a1 (col1a1), and vimentin (Vim) were analyzed. In addition, mRNA expression of the profibrotic mediators transforming growth factor-β (TGF-β) and plasminogen activator inhibitor-1 (Pai1) as well as the antifibrotic bone morphogenic proteins 4 and 7 (BMP4 and BMP7) were analyzed.

First, we analyzed the fibroblast activation and we found a progressive induction of FSP1 across time; Col1a1 and Vim showed upregulation trend; however, only Vim became statistically significant at 18 weeks (Figure 1(a)). Otherwise, α-SMA and Fn1 did not show expression regulation. The

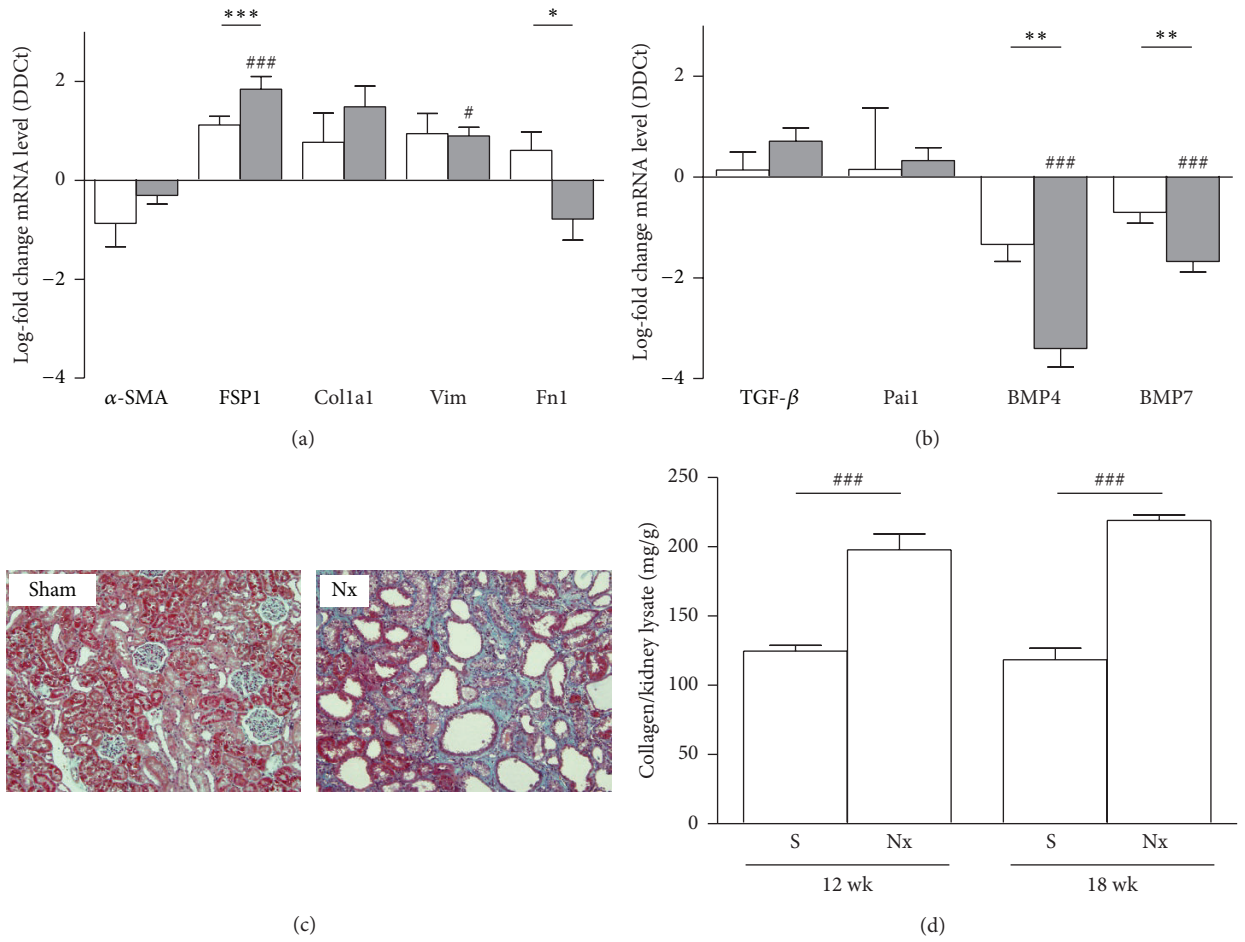

Figure 1: Assessment of fibrosis in rat 5/6 renal mass reduction model. (a) mRNA expression of general markers of fibroblast activation: alpha smooth muscle actin (α-SMA), fibroblast-specific protein-1 (FSP1), collagen 1a1 (Col1a1), vimentin (Vim), and fibronectin 1 (Fn1). (b) Profibrogenic mediators transforming growth factor-β (TGF-β), plasminogen activator inhibitor-1 (Pai1), and the antifibrotic bone morphogenetic proteins 4 and 7 (BMP4 and BMP7), and in rat kidneys of sham animals and 5/6 mass reduction animals by quantitative real-time PCR (qRT-PCR). Results are derived from 8-9 animals per group after 12 weeks (white columns) and 18 weeks (grey columns) after the surgery. Columns show median of log-fold change for each gene. $^{\#}P > 0.05$ and $^{\#\#\#}P < 0.001$, two-tailed Student's t-test for unpaired observations between S and Nx groups. $^{**}P > 0.01$ and $^{***}P < 0.001$, two-tailed Student's t-test for unpaired observations between 12 and 18 weeks. (c) Masson's trichrome of representative kidneys from sham or nephrectomized (Nx) rats. Representative pictures with focus on the cortex area are given. Green: collagen deposition. (d) Total collagen content was determined using the Sircol Collagen Assay. Columns are showing show median of mg collagen/g kidney lysate. $^{\#\#\#}P < 0.001$, two-tailed Student's t-test for unpaired observations between S and Nx groups.

antifibrotic gene analysis did not show significative changes of either TGF-β or Pai1, while the antifibrotic proteins BMP4 and BMP7 were downregulated (Figure 1(b)).

Next we determined kidney fibrosis through quantification of collagen deposition using Masson's trichrome stain and Sircol assay. 5/6 Nx animals showed a significant increase of total collagen content compared with sham animals at 12 and 18 weeks after the surgery (Figure 1(c)).

3.3. Expression of the Canonical Wnt Signaling Components in RMR Model. Wnt/β-catenin activity was assessed during the development of fibrotic remodeling in the rat 5/6 renal mass reduction model.

At mRNA level Wnt expression of individual Wnt ligands, receptors, intracellular signal transducers, and target genes was assessed by qRT-PCR. Global analysis showed a tendency to upregulation of the pathway, but only the intracellular signal transducer β-catenin was significantly upregulated, whereas Wnt ligands Wnt10a and Wnt5a and receptors FZD1 and FZD8 were significantly downregulated; all other Wnt components analyzed showed tendencies (Figure 2).

To assess Wnt/β-catenin signal activity, Western blot analysis was performed to LRP6, GSK-3β, β-catenin, cyclinD1, and Wnt1 for the 18 weeks' animal groups. Phosphorylation of both LRP6 and GSK-3β was increased (Figure 3), indicating activation of Wnt/β-catenin signaling. This finding correlated with increased expression of total β-catenin and of the Wnt target gene, cyclin D1, both of which were upregulated in Nx rat samples as compared with kidney tissue samples from sham animals. The Wnt ligand

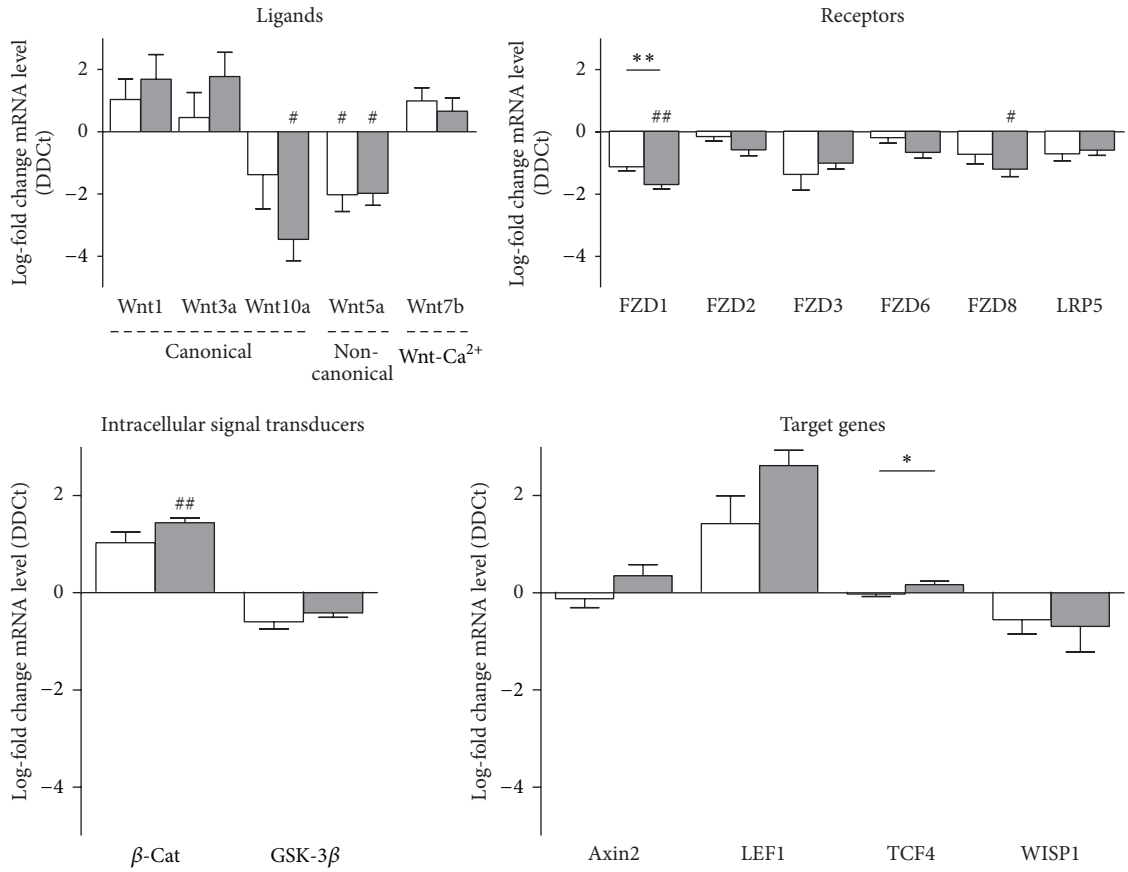

Figure 2: Expression of the canonical Wnt signaling components in rat 5/6 renal mass reduction model. mRNA levels of the Wnt ligands Wnt1, Wnt3a, and Wnt10a (canonical), Wnt5a (noncanonical), and Wnt7b (Ca^{2+} pathway); the Wnt receptors FZD1, 2, 3, 6, and 8 and LRP5; intracellular signal transducers β-catenin (β-Cat) and glucose sintase kinase3-β (GSK-3β); and the target genes axin2, lymphoid enhancer-binding factor 1 (LEF1), TCF4, and WISP1 were assessed in rat kidneys of sham animals (S) and 5/6 mass reduction animals (Nx) by quantitative real-time PCR (qRT-PCR). Results are derived from 8-9 animals per group after 12 weeks (white columns) and 18 weeks (grey columns) after the surgery. Columns show median of log-fold change for each gene. $^{\#}P > 0.05$ and $^{\#\#\#}P < 0.001$, two-tailed Student's t-test for unpaired observations between S and Nx groups. $^{**}P > 0.01$ and $^{***}P < 0.001$, two-tailed Student's t-test for unpaired observations between 12 and 18 weeks.

Wnt1 at mRNA level showed a tendency to upregulation (log-fold of 2.39 ± 0.87, not statistically significant), while the Wnt1 protein level detected by Western blotting was significantly increased (Figure 4).

To further confirm pathway activation in the animal model, the β-catenin distribution pattern was analyzed in rat kidney. After discrimination of proximal and distal tubules (Figure 5(a)), β-catenin was localized to both the proximal and distal tubules (Figures 5(b)–5(d)). Notably, positive staining was limited to cell-cell junctions and the basal layer in the proximal tubule (Figure 5(e)), while the distal tubule exhibited cytoplasmic (but not nuclear) localization in sham animals. Importantly, nuclear localization of β-catenin was found in epithelial cells mainly on the distal tubule in Nx animals (Figures 5(c), 5(e), and 5(f)).

4. Discussion

Renal fibrosis is one of the most common forms in chronic kidney diseases (CKD). The goal of this study was to characterize and validate the utility of the remnant kidney model with 5/6 nephrectomy (5/6 Nx) for the study of the Wnt pathway. Histological and molecular examination of the model demonstrated the damage and the Wnt pathway activation at 12 and 18 weeks after the surgery where we can mimic CKD. This confirms to us that 5/6 Nx is an easy, reliable, and good tool where Wnt pathway is activated.

Different models of renal mass reduction have been used to study the mechanisms of the progression of CKD in humans. In 5/6 Nx, the kidney's adaptive response to this surgical reduction in nephron number appears to be close enough to the pathophysiologic characteristics of human progressive nephropathies. 5/6 Nx is a well-described low nephron number model of chronic progressive renal disease with renal function impairment, proteinuria, glomerular sclerosis, and interstitial fibrosis [15, 16]. Our group previously described histologically the model, which is characterized for arteriolar hyalinosis and glomerulosclerosis. Additionally we described glomerular hypertrophy as well as severe tubular atrophy, interstitial fibrosis, interstitial

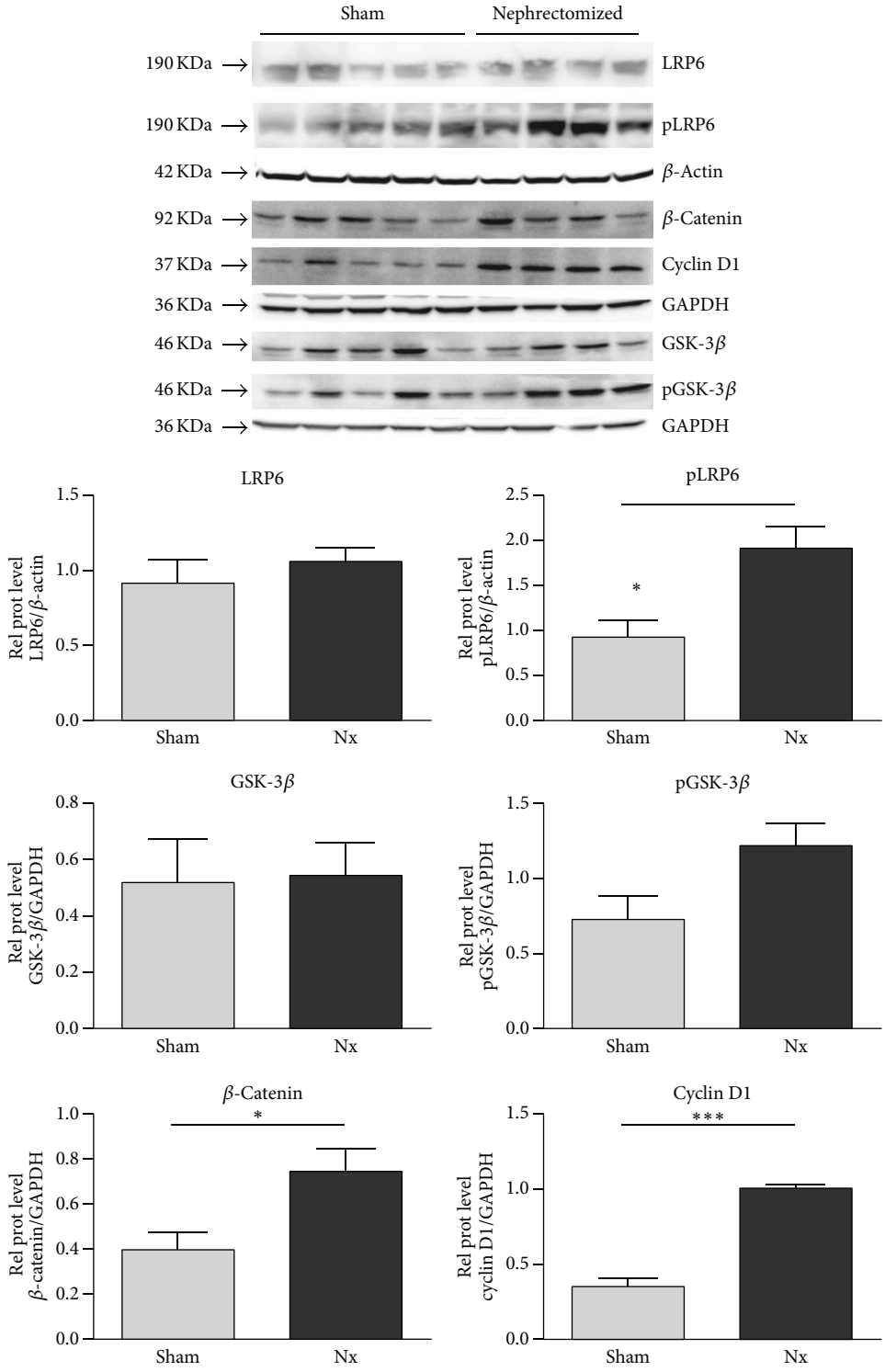

Figure 3: Activity of the canonical Wnt signal pathway in kidney homogenates of sham and nephrectomized rats. The expression of active Wnt components in kidney homogenates of sham and nephrectomized rats 18 weeks after the surgery was analyzed by immunoblotting of LRP6, phospho-LRP6, total β-catenin, cyclin D1, GSK-3β, and phospho-GSK-3β. Blotting of GDPH and β-actin served as loading controls. Results are derived from 4-5 animals per group.

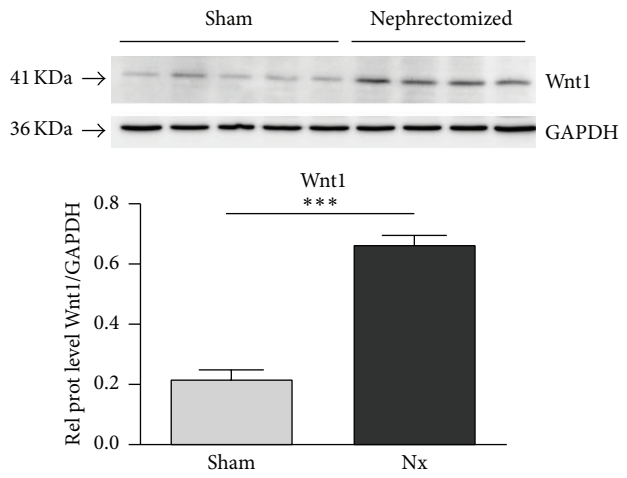

Figure 4: Expression of Wnt1 in kidney homogenates of sham and nephrectomized rats. The expression of Wnt1 in kidney homogenates of sham and nephrectomized rats 18 weeks after the surgery was analyzed by immunoblotting. Blotting of GDPH served as loading control. Results are derived from 4-5 animals per group.

inflammation, and glomerulosclerosis [16]. Fibrosis can result from an excessive synthesis of interstitial collagens such as collagens types I and III confirmed by trichrome stain and Sircol analysis in the present study. General markers for fibroblast activation, profibrotic mediators, and antifibrotic molecules were analyzed. The differentially regulated markers of fibrosis are summarized in Table 2. Upregulation of FSP1, Col1a1, and Vim has been implicated in renal fibrosis and the downregulation of the antifibrotic BMP4 and BMP7 confirmed the fibrosis establishment, while central role of TGF-β in human and experimental models for renal fibrosis has been well described [3, 4, 17, 18].

It is described that stimulation of canonical Wnt pathway led to β-catenin accumulation, nuclear translocation, and interaction with TCF/LEF complex resulting in target gene regulation. We found upregulation at mRNA level of β-catenin and LEF1. The Wnt/β-catenin canonical pathway plays an essential role during development, driving to branching nephrogenesis in fetal kidney. In the adult, Wnt signaling plays role in the control of tissue homeostasis. Wnt pathway promotes cell proliferation, tissue expansion, cell fate determination, and terminal differentiation. mRNA analysis of Wnt ligands showed no significant upregulation of the canonical Wnt ligands Wnt1 and Wnt3a and downregulation of Wnt10b. Königshoff et al. described upregulation of Wnt1 on day 7 after obstructive injury in mice followed by a decline in mRNA levels [10]. To further confirm pathway activation at mRNA level some target genes were analyzed, and we showed they are upregulated. Table 2 shows summary of regulated genes.

When the expression distribution of β-catenin was analyzed in the rat renal tissue, β-catenin was expressed in proximal and distal tubules, but the proximal tubules showed basal localization and rarely citoplasmatic distribution, while the distal tubules showed strong cytoplasmic signal. Some nuclei of both tubules showed nuclear translocation but less

Table 2: Summary of differentially regulated genes.

Gene symbol	12 weeks	18 weeks	12 weeks versus 18 weeks
Fibrosis related genes			
BMP4	↓n.s.	↓	↓
BMP7	↑n.s.	↔	↔
Col1a1	↑n.s.	↑n.s.	↑n.s.
FSP1	↑n.s.	↑	↑
Vim	↑n.s.	↑	↔
Wnt pathway ligands			
Wnt1	↑n.s.	↑n.s.	↑n.s.
Wnt3a	↑n.s.	↑n.s.	↑n.s.
Wnt5a	↓n.s.	↓n.s.	↔
Wnt7b	↑n.s.	↑n.s.	↔
Wnt10a	↓n.s.	↓n.s.	↔
Receptors			
FZD1	↓n.s.	↓	↓
FZD3	↓n.s.	↓n.s.	↔
FZD6	↔	↓n.s.	↔
FZD8	↓n.s.	↓	↔
LRP5	↓n.s.	↓n.s.	↔
Intracellular signal transducers			
β-Cat	↑n.s.	↑	↑
GSK-3β	↓n.s.	↓n.s.	↑n.s.
Target			
Axin2	↑n.s.	↔	↔
LEF1	↑n.s.	↑	↔
WISP1	↔	↔	↑n.s.

↑: statistically significant upregulation.
↓: statistically significant downregulation.
↑n.s.: no statistically significant upregulation.
↓n.s.: no statistically significant downregulation.

frequently than in human samples. To confirm Wnt signaling activation in fibrotic rat kidneys, the key components of canonical Wnt signaling were analyzed at the protein level. Our data confirm Wnt activation, as demonstrated by an increase in the total content of β-catenin, LRP6, and GSK-3β phosphorylation, the target gene cyclin D1, and the Wnt1 ligand, indicating that Wnt1 plays a role in IFTA progression in the kidney, as previously described in heart and lung animal models. Duan et al. reported that Wnt1 was upregulated eightfold in the heart within 48 h after acute ischemic cardiac injury. These authors describe Wnt1 fibroblast proliferation and profibrotic gene induction [19]. As in the kidney, in the heart, there is low expression of most Wnt ligands, and Wnt1 enhances the profibrotic function of cardiac fibroblasts, inducing their proliferation and expression of profibrotic genes.

In our model we observed an activation of the Wnt/β-catenin pathway but not as strong as we expected. The 5/6 Nx model with the sacrifice at 12 and 18 weeks is a really

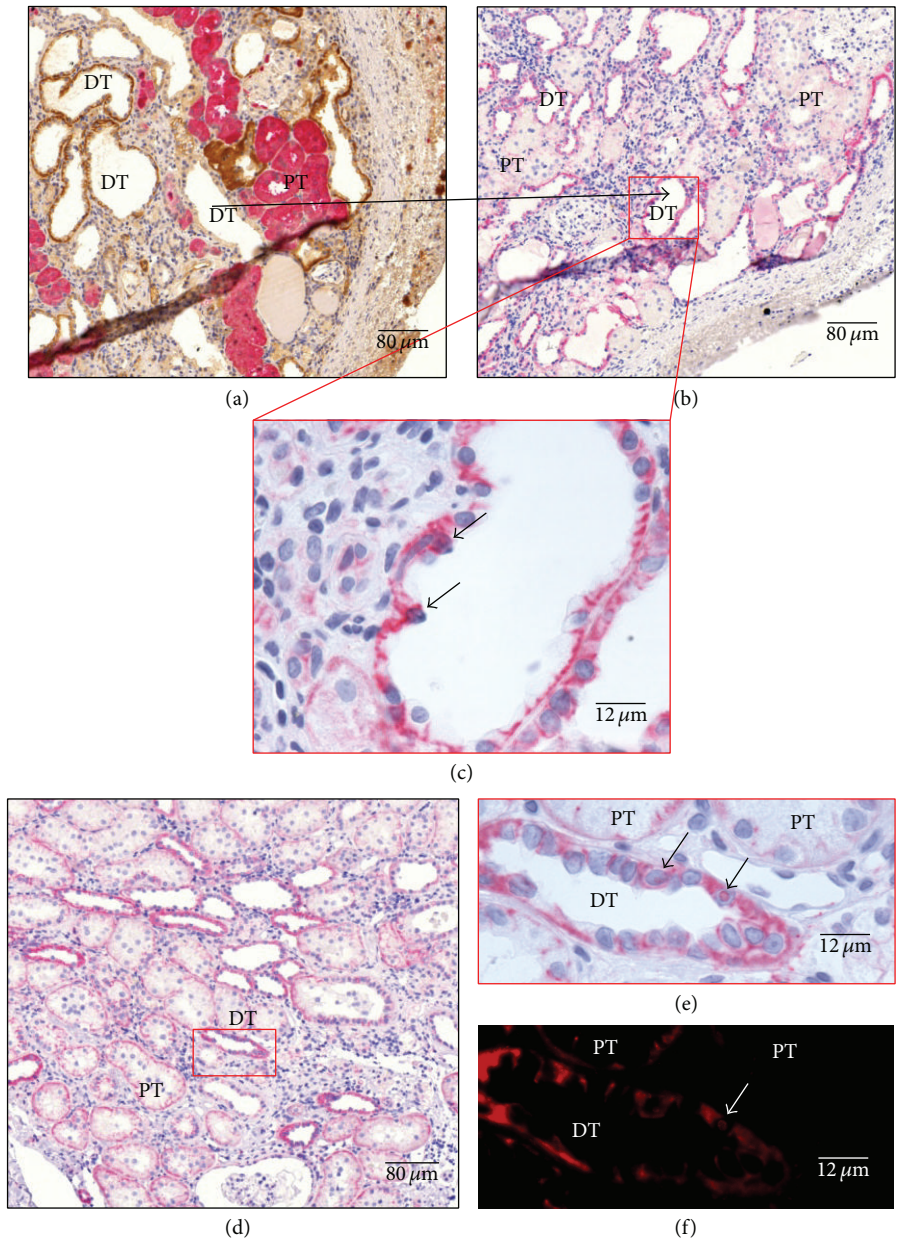

Figure 5: Expression and localization of β-catenin of representative kidneys in Nx rats. (a) Costain against FBPase in pink (Vulcan fast red), specific marker of proximal tubule (PT), and ATPase in brown (DAB), specific marker of distal tubule (DT), are made in serial cuts to discriminate between tubules; in (b) and (d) β-catenin stain was made in rat kidneys in pink (Vulcan fast red) and nuclei in blue (hematoxylin). Higher magnifications of tubular are shown in (c) and (e); (d) showing distal tubules with the translocated nuclei (arrows); (e) showing magnification of two proximal tubules with apical localization of β-catenin and one distal tubule with membrane localization, and short arrows indicate β-catenin translocated nucleus. As the β-catenin stain was made with Vulcan fast red, when slices were observed with a Texas Red filter clear image of translocated nuclei was obtained and false positives could be discarded; short arrow shows the confirmed 29 β-catenin translocated nucleus (f). Representative pictures with focus on the cortex area are given. Quantification of nuclei translocated β-catenin was done, related to proximal and distal epithelium surface. Stains are representative of using at least six different animals per group (magnification as indicated).

advanced chronic damage. von Toerne et al. described CAD model and they analyzed the Wnt pathway activation across CAD development but until 8 weeks after the transplantation, and they observed upregulation across the time [2]; however this model is useful only in the transplant context. There are different types of CKD that mimic different types of CKD, glomerulosclerosis models (5/6 Nx), or interstitial fibrosis models (UUO, Cyclosporine A nephropathy, etc.). The advantages of 5/6 Nx model include robust functional readouts (such as proteinuria, GFR, and secondary hypertension), reliable induction in rodents, and comparison to the kidney removed at induction of injury. This model was

useful for the elucidation of mechanisms that translate to CKD secondary to nephron loss in humans and glomerular disease [20]. He et al. described upregulation of the pathway in a UUO model but to 2 weeks after the surgery [12]. For studying tubulointerstitial fibrosis, UUO is the most widely used model. The intact nonobstructed kidney serves as an excellent control. However, this model lacks functional readouts in that serum creatinine is normal, there is no proteinuria, and 15 days could not be considered as chronic damage.

To our knowledge there are no reports about the Wnt regulation in a long term CKD. Our results demonstrate that pathway is active, but this is not a dramatic upregulation. That could be attributed to the functional tissue loss, because of the tubular and glomerular atrophy, indicating that time points analyzed could be too late for pathway modulation. Shorter time points will be necessary to set Wnt activation, in order to modulate its activation and couls study their effect on CKD activation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

E. Banon-Maneus, O. Eickelberg, J. M. Campistol, and M. Königshoff designed research; E. Banon-Maneus, J. Rovira, M. J. Ramirez-Bajo, D. Moya-Rull, N. Hierro-Garcia, and S. Takenaka performed research; E. Banon-Maneus, S. Takenaka, F. Diekmann, and M. Königshoff analyzed data; E. Banon-Maneus, J. M. Campistol, and M. Königshoff wrote the paper.

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Review Article

The Interplay between Inflammation and Fibrosis in Kidney Transplantation

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Serial surveillance renal allograft biopsies have shown that early subclinical inflammation constitutes a risk factor for the development of interstitial fibrosis. More recently, it has been observed that persistent inflammation is also associated with fibrosis progression and chronic humoral rejection, two histological conditions associated with poor allograft survival. Treatment of subclinical inflammation with steroid boluses prevents progression of fibrosis and preserves renal function in patients treated with a cyclosporine-based regimen. Subclinical inflammation has been reduced after the introduction of tacrolimus based regimens, and it has been shown that immunosuppressive schedules that are effective in preventing acute rejection and subclinical inflammation may prevent the progression of fibrosis and chronic humoral rejection. On the other hand, minimization protocols are associated with progression of fibrosis, and noncompliance with the immunosuppressive regime constitutes a major risk factor for chronic humoral rejection. Thus, adequate immunosuppressive treatment, avoiding minimization strategies and reinforcing educational actions to prevent noncompliance, is at present an effective approach to combat the progression of fibrosis.

1. Introduction

Progressive renal fibrosis, regardless of the underlying aetiology, is the final common manifestation of a wide variety of chronic kidney diseases (CKD) that lead to end-stage renal disease. Fibrosis is a process of normal wound healing and repair that is activated in response to injury to maintain the original tissue architecture and functional integrity. However, prolonged chronic injurious stimuli may cause deregulation of normal processes and result in an excess deposition of extracellular matrix (ECM) [1]. Continuous deposition of ECM results in fibrous scars and distorts the architecture of kidney tissues, leading to the collapse of renal parenchyma and the loss of kidney function [2]. Chronic injury involves a complex multistage inflammatory process with inflammatory cell infiltration, mesangial and fibroblast activation, tubular-epithelial to mesenchymal transition, endothelial to mesenchymal transition, cell apoptosis, and extracellular matrix expansion that is orchestrated by a network of cytokines/chemokines, growth factors, adhesion molecules, and signalling processes [3, 4]. These events include several phases summarized in Figure 1: (i) tissue injury and activation, (ii) recruitment of inflammatory cells, (iii) release of fibrogenic cytokines, and (iv) activation of collagen-producing cells. However, it should be stressed that renal fibrogenesis is a dynamic process in which many of these events occur simultaneously, often in a mutually stimulating fashion [2]. The injury phase, which can be induced by a variety of noxious stimuli including immunological, metabolic, hemodynamic, ischemic, and toxic assaults, results in the production and release of proinflammatory molecules caused by cytokine-mediated endocytosis/phagocytosis [5–8]. Neutrophils are the first cells recruited, as they uptake cell debris and phagocytose apoptotic bodies facilitating the repair of

the lost tissue components, resulting in a reconstitution of the original tissue architecture and function. This beneficial repairing process can be detrimental when proceeding in an uncontrolled manner, then leading to progressive fibrosis with a loss of function [9]. Thus, controlling excessive inflammation would be of great potential therapeutic benefit for inhibiting progressive fibrosis of kidney.

2. Molecular Mechanisms Leading to Fibrosis Progression

The pathogenesis of inflammation is complex and multifactorial, involving the interaction of cytokines, chemokines, and adhesion molecules. The participation and interaction of infiltrated cells with different cell types in the kidney is required to promote renal fibrosis. Depending on the aetiology of renal injury, tubular, glomerular, or interstitial infiltrated inflammatory cells become activated and produce fibrogenic and inflammatory cytokines. Inflammatory infiltrates, including neutrophils, macrophages, and lymphocytes, are evident in experimental models of renal disease and human renal biopsy specimens [10]. Activation of peritubular capillary endothelial cells may facilitate the recruitment of interstitial mononuclear cells. Following neutrophils, macrophages infiltrate damaged tissues and phagocytose and secrete fibrogenic cytokines. Macrophages are a major source of transforming growth factor-β 1 (TGF-β1) in fibrosing organs. T and B lymphocytes are also recruited to the site of injury and further facilitate secretion of fibrogenic cytokines [11]. At the same time, TGF-β1 is a potent chemoattractant for cells of macrophage-monocytic lineage. In addition to TGF-β1, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 (MIP-1), and macrophage inflammatory protein-2 (MIP-2) are also involved in recruitment of inflammatory cells [12]. The gradients of chemoattractant cytokines released by damaged tubular cells provide a directional signal for guiding the infiltration of inflammatory monocytes/macrophages and T cells to the injured sites and are thought to play an important role in this inflammatory process.

Members of the TGF-β superfamily are the most extensively studied growth factors that have been linked to renal fibrosis [13]. Macrophages, tubular epithelial cells, and myofibroblasts are all capable of synthesizing TGF-β at different stages during the development of renal fibrotic lesions [14]. However, the observation that macrophage ablation markedly attenuates fibrosis in various conditions suggests that these cells are among the main producers of this growth factor [15, 16]. Macrophages are heterogeneous and can be classified by distinct phenotypic markers that correspond to different subsets with distinct functional capabilities, including important roles in tissue repair and remodelling [17].

Although different fibrogenic factors have been documented, including various cytokines and hormonal, metabolic, and hemodynamic factors, it is widely accepted that TGF-β and its downstream Smad signalling play an essential role. Upregulation of TGF-β is a universal finding in virtually every type of CKD, both in animal models and in humans. Despite the well documented role of TGF-β in renal fibrosis, long-term inhibition of TGF-β action, in an attempt to hamper the progression of renal fibrosis, does not seem to be an optimal approach provided that TGF-β is also an anti-inflammatory cytokine. The profibrotic and anti-inflammatory properties of TGF-β pose a dilemma for the therapeutic application of TGF-β inhibition and this is one of the reasons that novel antifibrotic targets are under active investigation [18].

In renal fibrosis, the activation of the renin-angiotensin-aldosterone system and its main effector angiotensin II (AngII) stimulates vascular inflammation, upregulation of reactive oxygen species, cytokines, chemokines, and growth factors, and recruitment of infiltrating cells into the kidney [19, 20]. The relevance of AngII to renal fibrosis has immediate clinical relevance due to the availability of orally active inhibitory drugs. AngII has been shown to stimulate TGF-β production by various cells including renal tubular cells and fibroblasts and several studies have demonstrated that the use of either AngII receptor (AT1 and AT2) antagonists or angiotensin converting enzyme (ACE) inhibitors in experimental renal disease models reduces TGF-β production and attenuates renal interstitial fibrosis [21, 22].

In human kidney diseases, the activated renal renin-angiotensin system has been described. In diabetic nephropathy, elevated AngII generation did correlate with the presence of inflammatory cell infiltration, the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), and proinflammatory gene overexpression [23]. The inhibition of the NF-κB pathway has also shown the prevention of inflammation in experimental renal damage. These observations emphasize the importance of treatments that block the AngII-induced inflammatory process in human renal diseases and provide a rationale to investigate further the involvement of the AT2/NF-κB pathway in the inflammatory response in kidney diseases [20].

3. Molecular Mechanisms Leading to Fibrosis in Renal Transplantation

Inflammation has also been pinpointed as a hallmark for renal transplant functional decline. Inflammation, especially when it is associated with fibrosis in surveillance kidney biopsies, is a risk factor for long-term transplant failure. Park et al. [24] have shown that one-year surveillance biopsies with normal histology or fibrosis had stable renal function between 1 and 5 years, whereas those with both fibrosis and inflammation exhibited a decline in GFR and reduced graft survival. Immunohistochemistry confirmed increased interstitial T cells and macrophages/dendritic cells in the group with both fibrosis and inflammation, and there was increased expression of transcripts related to innate and cognate immunity. These authors demonstrated elevated expression of multiple innate and adaptive immune mediators consistent with tissue injury response, Th1-type T cell response, and suppression of counterregulatory pathways. Microarray analyses confirmed and extended this profile,

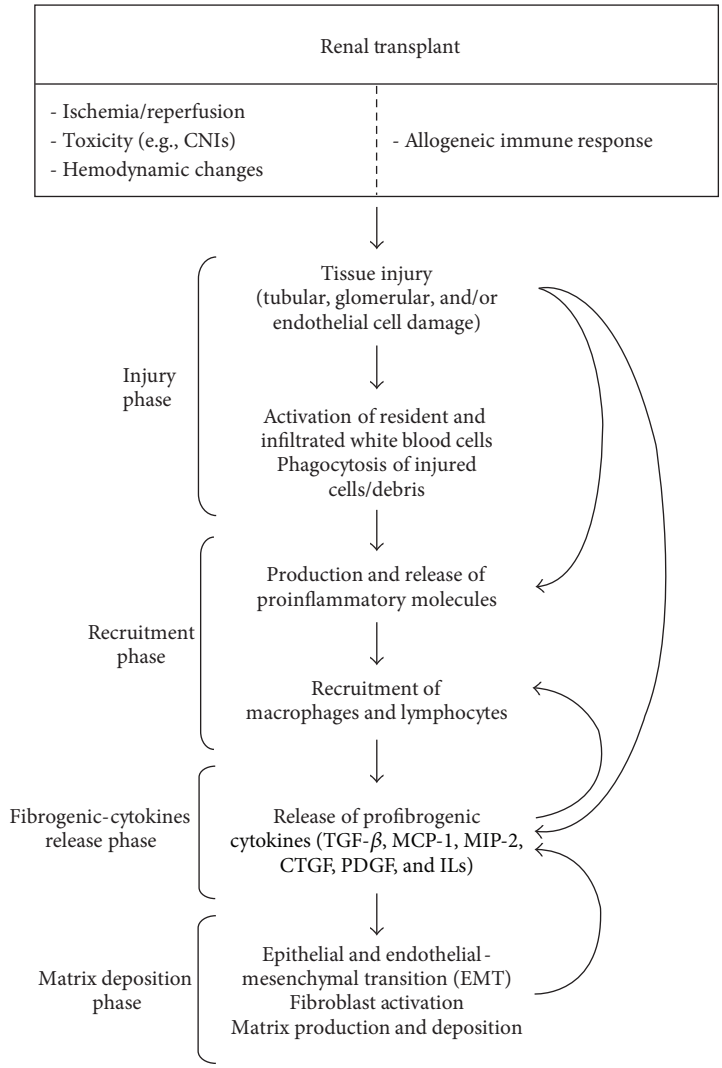

Figure 1: Renal transplant-induced fibrosis involves a complex multifactorial inflammatory process with the participation and interaction of infiltrated cells with different cell types in the kidney and is orchestrated by a network of cytokines/chemokines, growth factors, adhesion molecules, and signalling processes. These events include several phases in a dynamic process in which many of these events occur simultaneously, often in a mutually stimulating fashion.

revealing the overexpressed pathways and gene clusters in the interstitial fibrosis and inflammation group to be heavily enriched for immune activation and identifying the process as being closely linked with IFN-γ-induced, cytotoxic T lymphocyte-associated, and acute rejection signatures. Pathway analysis of microarray data for the interstitial fibrosis and inflammation group also provided evidence of active participation of a range of immunologic cell types, including T cells, B cells, monocyte/macrophages, dendritic cells, and natural killer cells. Results of this study indicate that early surveillance histology with or without targeted molecular analysis provides important prognostic information. It has been suggested that analysis of intragraft innate and adaptive immune pathways during early posttransplantation years may provide the basis for early interventions aimed at altering rejection-like inflammation improving long-term survival of kidney allografts [24–26].

4. Preexisting Kidney Fibrosis and Graft Outcome

Despite the fact that the use of new immunosuppressants has allowed reduction in the incidence of acute rejection and an improvement of short-term results in renal transplantation, long-term graft survival has been only marginally increased [27]. Among immune and nonimmune mechanisms influencing graft survival, donor related factors are one of the major determinants of graft outcome [28, 29].

The increased utilization of the so-called expanded criteria donors during the last years, that is, donors older than 60 years or donors older than 50 years with two of the following conditions, death due to stroke and history of hypertension and serum creatinine > 1.5 mg/dL, implies that a high proportion of kidneys already display interstitial fibrosis, tubular atrophy, vascular intimal thickening, and glomerulosclerosis at the time of transplantation. The severity

of these lesions is associated with delayed graft function, decreased glomerular filtration rate, and decreased allograft survival. Accordingly, different scores to evaluate the severity of preexisting damage have been proposed [30–32]. Although intra- and interobserver reproducibility of these measures is not ideal, the majority of studies have shown a close association between the severity of fibrosis and graft outcome [33, 34]. It has been proposed that the modest improvement on long-term allograft survival despite decreased incidence of acute rejection with actual immunosuppression is mainly explained by the increased use of kidneys already displaying fibrosis.

5. Ischemia/Reperfusion Injury and Kidney Fibrosis

Ischemia/reperfusion injury (IRI) is a key event in organ transplantation since restoration of blood flow to ischemic tissue exacerbates tissue damage by initiating a cascade of inflammatory events including release of proinflammatory cytokines and chemokines, recruitment of leukocytes, and activation of the complement system [35]. Different experimental and clinical studies have shown that transplant IRI may impact short- and long-term graft survival following kidney transplantation and is strongly associated with delayed graft function [36]. Acute kidney injury is associated with an extensive loss of the corticomedullary proximal tubular epithelial cells and with a reduction in the number of peritubular capillaries [37]. Moreover, delayed graft function increases the immunogenicity of the allograft and the risk of acute rejection episodes [36]. The initiation of profibrotic pathways is also relevant as shown by the increased expression of TGF-β and activation of NF-κB in allografts that developed chronic changes subsequent to the occurrence of acute tubular injury [38]. These phenomena, inherently present in the majority of the grafts, can be more pronounced in expanded criteria donors since these allografts have a limited capacity to repair parenchymal damage and could exhaust the ability of tubular epithelial cells to regenerate. Additionally, these processes could lead to accelerated senescence and aggravate the progression of interstitial fibrosis and tubular atrophy [39].

6. Fibrosis Progression in Surveillance Biopsies

Preexisting chronic donor damage can progress after transplantation due to the different immunologic and nonimmunologic insults to which the kidney is exposed. To evaluate the progression of fibrosis after transplantation different groups have performed surveillance biopsies at different time points after transplantation. From the initial reports, it became clear that chronic histological damage in the tubulointerstitial, vascular, and glomerular compartments rapidly progresses during the initial months after transplantation while renal function remains stable. In different studies it has been shown that the presence of interstitial fibrosis/tubular atrophy (IF/TA) involved about 40% of transplants at 3–6 months [39, 40], 50% at 1 year [41], and 65% at 2 years [42]. The progression of IF/TA was associated with an increased incidence of acute rejection before performing the surveillance biopsy and with a lower immunosuppressive treatment. Furthermore, it has been consistently shown that the presence of IF/TA adjusted for renal function at the time of biopsy is closely associated with long-term graft survival. However, since IF/TA is a nonspecific lesion that can be related with different immune and nonimmune injuries to the graft, during the last years a big effort has been done to characterize causes of late graft failure. In these studies, it has been shown that specific disease entities may be identified in more than 90% of cases, antibody-mediated rejection and glomerular disease being the leading causes of late graft failure [43, 44]. Recently, to integrate this apparent discrepancy, it has been shown that early chronic histological damage was an independent risk factor for late graft loss, irrespective of whether a specific, progressive disease was diagnosed or not [45]. Thus, the burden of fibrosis modulates outcome in different renal allograft diseases.

7. Inflammation as a Risk Factor for Progression of Kidney Fibrosis

The largest study contributing to describing the natural history of the evolution of inflammation and chronic damage in stable grafts was conducted on 120 recipients receiving simultaneous kidney-pancreas transplantation in whom near 1.000 surveillance biopsies were done during 10 years of follow-up. Most severe inflammation was already observed during the first months after transplantation and tended to decrease during the first year although the inflammation persists after the first year in a proportion of patients. At the same time, interstitial fibrosis rapidly progressed during the first months after transplant. Beyond one year, glomerulosclerosis and intimal thickening slowly progressed as well as the severity of IF/TA. The presence of severe chronic lesions was associated with declining renal function and graft failure [46]. It has been shown that early inflammation observed in surveillance biopsies is associated with the progression of IF/TA [47, 48] and with decreased renal allograft survival [49]. However, the classification of surveillance biopsies as (i) normal histology, (ii) fibrosis without inflammation, (iii) inflammation without fibrosis, and (iv) inflammation associated with fibrosis leads to the observation that only patients with inflammation associated with fibrosis showed a decreased renal allograft survival [24, 50]. Additionally, it has been shown that the presence of interstitial inflammation in areas of fibrosis (i-IFTA) in diagnostic biopsies is especially harmful for the graft [51]. Studies conducted on sequential biopsies have shown that acute cellular rejection, BK nephropathy, increasing number of HLA mismatches, retransplantation, and delayed graft function were risk factors for the presence of i-IFTA in one-year surveillance biopsies [48, 52]. More recently, it has been described that early inflammation after transplantation evaluated by means of surveillance biopsies is associated with an increased risk to develop de novo donor HLA specific

antibodies and chronic antibody-mediated rejection [53–55]. In summary, early inflammation is associated with three different conditions, interstitial fibrosis, interstitial fibrosis associated with inflammation, and chronic allograft rejection. However, graft survival is shortened in patients with i-IF/TA and chronic humoral rejection in comparison to patients with quiescent fibrosis. In Figure 2, the relationship between events modulating early inflammation after transplant and late different histological phenotypes is shown.

8. Treatment of Subclinical Rejection to Slow Kidney Fibrosis Progression

Since subclinical inflammation is indistinguishable from inflammation observed in episodes of acute cellular rejection, it was tempting to propose that treatment of subclinical inflammation with steroid boluses may improve outcome after renal transplantation. The first to test this hypothesis was Rush et al. [56] in an elegant prospective randomized clinical trial in which patients were randomized to be biopsied at 1, 2, and 3 months and treated with steroid boluses in case they showed subclinical inflammation. The control group was not biopsied at these time points and, accordingly, not treated for subclinical inflammation. Fibrosis at 6 months was less severe in patients that were biopsied and treated for subclinical inflammation. This was the first study to show, as a proof of concept, that treatment of subclinical inflammation prevents progression of fibrosis. It is important to remark that patients enrolled in this study were treated with cyclosporine, azathioprine, and prednisone, a regimen associated with a high prevalence of acute rejection and subclinical inflammation. In this study, over 50% of patients showed subclinical inflammation at the time of surveillance biopsy. A similar study was done more recently [57] in which patients were randomized to be biopsied at 1 and 4 months and treated with steroid boluses in case they presented subclinical inflammation. The control group was again not biopsied and accordingly not treated. Baseline immunosuppression consisted in a cyclosporine or tacrolimus based regimen. The prevalence of subclinical inflammation was 39% at 1 month and 26% at 4 months, a lower figure than in the previous study. Estimated glomerular filtration rate at 6 months and 1 year was better in patients that were biopsied and treated, suggesting that treatment of subclinical inflammation was associated with preservation of renal function.

Rush et al. published in 2007 the results of a multicentre trial in which patients treated with tacrolimus, mycophenolate, and prednisone were randomized, as in his previous study, to be biopsied at 1, 2, and 3 months and treated with steroid boluses in case they presented subclinical inflammation. The control group was again not biopsied and, accordingly, not treated. There were no differences between groups in the progression of fibrosis evaluated by means of a 6- and 24-month surveillance biopsy and the evolution of renal function was also not different between groups. Most remarkably, overall incidence of subclinical inflammation was less than 10% at 1, 2, and 3 months, suggesting that treatment with tacrolimus, mycophenolate mofetil, and prednisone may efficiently prevent early inflammation [58]. After this study the interest shifted from treatment to prevention of subclinical inflammation.

9. Prevention of Subclinical Inflammation to Avoid Kidney Fibrosis

The prevalence of subclinical inflammation in three-month surveillance biopsies is lower in tacrolimus than in cyclosporine treated patients [59–61]. Quantification of the severity of inflammatory infiltrates with monoclonal antibodies confirmed that patients receiving tacrolimus showed less severe glomerular and interstitial inflammation than patients treated with cyclosporine [62]. These data suggested that the type of immunosuppressive treatment modulates the severity of inflammation after transplantation. Since inflammation is associated with progression of fibrosis, the question whether prevention of early inflammation by treatment may delay the progression of fibrosis was raised.

In a prospective trial in which patients were randomized to receive 4 different immunosuppressive schedules: cyclosporine associated with mycophenolate mofetil, tacrolimus associated with mycophenolate mofetil, cyclosporine associated with sirolimus, and tacrolimus associated with sirolimus, it was observed that regimens combining a calcineurin inhibitor with sirolimus showed a lower prevalence of acute rejection during the first year, a lower prevalence of subclinical inflammation at 1-year protocol biopsy, and less severe fibrosis evaluated by means of a surveillance biopsy at 5 years, suggesting that immunosuppressive schedules that are effective in preventing acute rejection and subclinical inflammation are also effective in preventing the progression of fibrosis [63]. At the time this paper was published, it was assumed that the combination of a calcineurin inhibitor and an inhibitor of the mammalian target of rapamycin (i-mTOR) was a nephrotoxic combination [64]. Thus, this study challenged the idea that avoidance of anticalcineurin treatments was the best strategy to prevent the progression of fibrosis [65]. In the Concept trial, patients receiving cyclosporine, mycophenolate mofetil, and prednisone were randomized to continue with the same schedule or to be switched from cyclosporine to sirolimus. At one year, the surveillance biopsy showed that the severity of fibrosis quantified by means of an image analysis technique was not different between groups [66], while the presence of subclinical inflammation was 45% in sirolimus and 15% in cyclosporine treated patients, suggesting that sirolimus is less effective in preventing inflammation than cyclosporine [67]. More recently, it has been shown that the early switch from cyclosporine to everolimus is associated with an increased risk of appearance of the novo HLA donor specific antibodies and chronic humoral rejection [68], reinforcing the notion that an i-mTOR based regimen may be less effective than a calcineurin inhibitor regimen to control the immune response after transplantation.

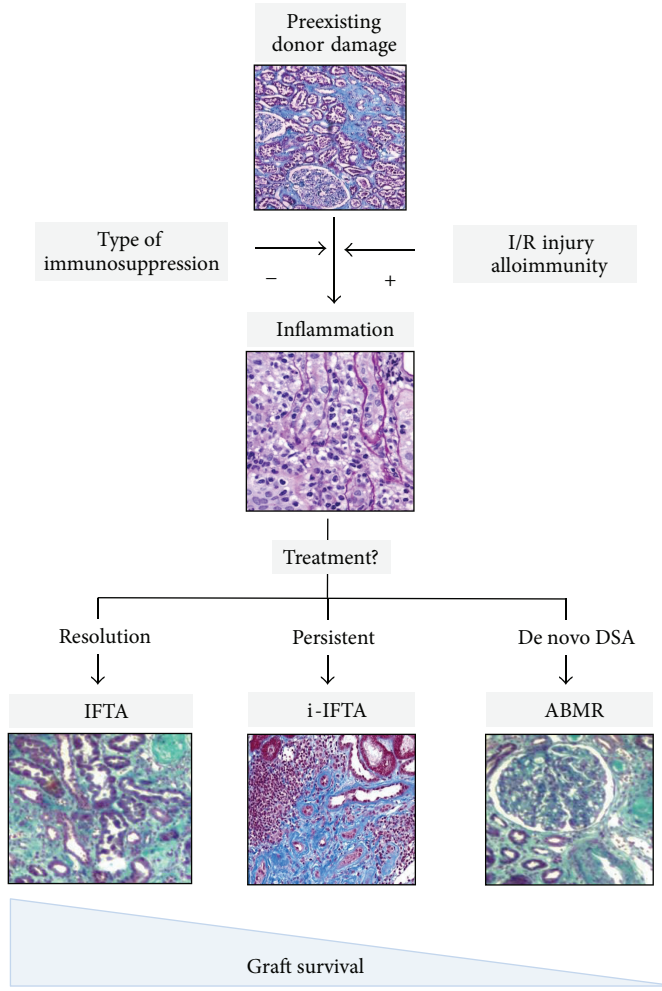

Figure 2: Progression of fibrosis after kidney transplantation. Fibrosis is already present in a proportion of grafts, especially in renal allograft obtained from expanded criteria donors. Ischemia/reperfusion (I/R) injury and alloimmune response trigger inflammation and its severity is modulated by immunosuppressive treatment. Subclinical inflammation can be ameliorated by treatment with steroid boluses or by increasing exposure to immunosuppressive drugs. Quiescent interstitial fibrosis/tubular atrophy (IF/TA) may represent the healing of the inflammatory insult while inflammation in areas of fibrosis (i-F/TA) and antibody-mediated rejection (ABMR) due to the appearance of de novo donor specific antibodies (DSA) may represent an ongoing inflammatory response that is associated with decreased allograft survival.

In the last two decades, the immunosuppressive schedule has changed from cyclosporine to tacrolimus based regimens. Thus, it is interesting to compare the prevalence of chronic lesions in surveillance biopsies obtained late after transplantation in these different periods, the cyclosporine and tacrolimus era. In 2003, in the paper published by Nankivell et al. [46], the prevalence of moderate or severe interstitial fibrosis at 5 years was 66% and in the Stegall et al. [69] paper published in 2011 it was 17%. Of note, hyaline changes were 90% in the first and 19% in the second study. Although such a comparison should be considered with caution, since patients characteristics between studies were different, it again suggests that the introduction of more powerful immunosuppressive schedules better controlling early inflammation may have changed the rate of progression of fibrosis after transplantation.

Further support for the role of immunosuppression in the prevention of early inflammation and progression of fibrosis comes from the observation that minimization of cyclosporine treatment was associated with progression of fibrosis when evaluated by means of 3- and 12-month surveillance biopsies [41]. Similarly, lower exposure to tacrolimus was also associated with accelerated progression of fibrosis evaluated again by means of surveillance biopsies done at 3 and 12 months. In this last study, low tacrolimus was also associated with higher prevalence of acute rejection, but high exposure to tacrolimus was not associated with lesions considered to represent anticalcineurin associated nephrotoxicity [70]. These results argue against minimization of immunosuppression, at least during the first months. Moreover, in the last years there is increasing evidence supporting a major role of patient's compliance in renal allograft survival [44, 71], and it has been also shown that patients enrolled in a special program aiming to improve treatment compliance have a better outcome than patients followed in the standard way [72].

10. Conclusions

Taken together, these data point out that inflammation early after transplantation is a major determinant of the progression of fibrosis, appearance of HLA donor specific antibodies, and graft outcome. On the other hand, an adequate immunosuppressive treatment, avoiding minimization strategies and reinforcing educational actions to prevent noncompliance, is at present an effective approach to combat the progression of fibrosis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

High Glucose Induces Sumoylation of Smad4 via SUMO2/3 in Mesangial Cells

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Recent studies have shown that sumoylation is a posttranslational modification involved in regulation of the transforming growth factor-β (TGF-β) signaling pathway, which plays a critical role in renal fibrosis in diabetic nephropathy (DN). However, the role of sumoylation in the regulation of TGF-β signaling in DN is still unclear. In the present study, we investigated the expression of SUMO (SUMO1 and SUMO2/3) and Smad4 and the interaction between SUMO and Smad4 in cultured rat mesangial cells induced by high glucose. We found that SUMO1 and SUMO2/3 expression was significantly increased in the high glucose groups compared to the normal group ($P < 0.05$). Smad4 and fibronectin (FN) levels were also increased in the high glucose groups in a dose-dependent manner. Coimmunoprecipitation and confocal laser scanning revealed that Smad4 interacted and colocalized with SUMO2/3, but not with SUMO1 in mesangial cells. Sumoylation (SUMO2/3) of Smad4 under high glucose condition was strongly enhanced compared to normal control ($P < 0.05$). These results suggest that high glucose may activate TGF-β/Smad signaling through sumoylation of Samd4 by SUMO2/3 in mesangial cells.

1. Introduction

Diabetic nephropathy (DN) is a serious microvascular complication of diabetes and a leading cause of end-stage renal disease in developed countries. Early DN is characterized by mesangial expansion, thickened glomerular and tubular basement membranes, and accumulation of the extracellular matrix (ECM) and can progress to glomerulosclerosis and tubulointerstitial fibrosis in later stages [1–3]. At present, the pathogenesis of DN remains unclear, and then further study of molecular mechanisms to develop new treatment approaches for DN is required.

As a key mediator of fibrogenesis, transforming growth factor-β (TGF-β) plays a critical role in diabetic nephropathy, and Smad4 is a common mediator in TGF-β signaling [4–6]. The TGF-β/Smad pathway is modulated by several posttranslational modifications, including phosphorylation, ubiquitination, and acetylation [7, 8]. In a previous study, we demonstrated that ubiquitination of histones H2A and H2B is involved in diabetic nephropathy by activating the TGF-β signaling pathway [9]. Recent studies have demonstrated that sumoylation is a reversible posttranslational modification involved in regulation of the TGF-β signaling pathway [10, 11].

SUMO is a type of small ubiquitin-like molecule primarily involved in posttranslational modification of proteins, similar to ubiquitination. In mammals, there are four SUMO paralogues, SUMO1, SUMO2, SUMO3, and SUMO4 [12]. SUMO2 and SUMO3 share 95% homology with each other and are collectively referred to as SUMO2/3 [13]. Sumoylation is the covalent attachment of SUMO to specific target proteins via an ATP-dependent enzyme cascade, including E1 activating enzyme, E2 conjugating enzyme (Ubc9), and several E3 ligases [14]. Sumoylation plays an important role in multiple biological processes, such as protein interactions, protein stability, nuclear-cytoplasmic trafficking, transcriptional regulation, DNA repair, and cellular signaling pathways [15, 16]. Recent studies have shown that sumoylation

regulates the TGF-β pathway by modifying several important signaling molecules, such as type I TGF-β receptor (TβRI) and Smad4 [17]. However, whether sumoylation is involved in the pathogenesis of DN is unknown.

In this study, we detected the levels of SUMO and Smad in each group to investigate whether they were regulated by high glucose and whether high glucose could induce sumoylation of Smad4 in rat mesangial cells. And we explored the role of sumoylation in regulating TGF-β/Smad signaling in DN.

2. Materials and Methods

2.1. Cell Culture. Rat glomerular mesangial cells (HBZY-1) were purchased from the Preservation Center at Wuhan University and cultured in low glucose DMEM (Hyclone) medium with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin. Mesangial cells were cultured with serum free medium for 24 h before treatment, followed by addition of 5.6 mmol/L glucose as a normal control, 10, 20, and 30 mmol/L glucose as experimental groups, and mannitol as an osmotic control for 6 h, 12 h, and 24 h.

2.2. Cell Lysis and Western Blotting. Cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer (3.0 M NaCl, 1.0 M Tris PH 7.5, 1% TritonX-100, 10% SDS and protease inhibitor). Cell lysates were centrifuged at 4°C, 15,000 ×g for 10 min to pellet cell debris and protein samples were extracted. Supernatants were separated on SDS-PAGE and transferred onto PVDF membranes (Millipore). Membranes were blocked in 5% nonfat milk for 1 h and incubated with primary antibodies overnight at 4°C. Primary antibodies included mouse monoclonal anti-Smad4 (1 : 500, Santa Cruz), rabbit polyclonal anti-SUMO2/3 (1 : 600, Millipore), rabbit monoclonal anti-SUMO1 (1 : 800, Abcam), and mouse monoclonal anti-GAPDH (1 : 1,000, Beyotime Biotechnology). Membranes were incubated with horseradish peroxidase-linked anti-mouse and anti-rabbit secondary antibodies (Beyotime) at room temperature for 1 h. Proteins were detected using ECL reagents (Millipore) and the Bio-Rad chemiluminescence system and quantitatively analyzed by Quantity One software.

2.3. Immunoprecipitation and Immunoblotting. Cells were lysed in ice-cold IP lysis buffer (150 mM NaCl, 25 mM Tris PH 7.4, 1 mM EDTA, 1% NP-40, 5% glycerol and protease inhibitor). Cell lysates were centrifuged at 15,000 ×g at 4°C for 15 min, and the supernatants were subjected to immunoprecipitation with mouse anti-Smad4 antibody or mouse IgG as negative control overnight at 4°C. Immune complexes were precipitated with protein G agarose beads for 1-2 h under rotary agitation, and the agarose beads were washed four times in wash buffer. Bound proteins were eluted in 2x reducing sample buffer, and samples were analyzed by Western blotting as described using mouse anti-Smad4 antibody for Smad4 and rabbit anti-SUMO1 or SUMO2/3 antibody for SUMO-conjugated Smad4 protein.

2.4. Reverse Transcription-PCR. Cells were washed twice in ice-cold PBS and lysed in TRIzol reagent, followed by total RNA extraction using the RNAsimple Total RNA kit (Tiangen Biotech). Samples were reverse transcribed (RT) to cDNA using a kit according to the manufacturer's instructions (Bio-BRK). PCR was performed using the cDNA and the following primers: SUMO1, forward 5′-TATGGACAG-GACAGCAG-3′, reverse 5′-CCATTCCCAGTTCTTTT G-3′; SUMO2/3, forward 5′-GACGAGAAACCCAAGGA-3′, reverse 5′-CTGCCGTTCACAATAGG-3′; FN, forward 5′-CAGCCTACGGATGACTC-3′, reverse 5′-CTCTTTCTG-CCACTGTTCT-3′; and GAPDH, forward 5′-GGTCAT-GAGTCCTTCCACGATA-3′, reverse 5′-ATGCTGGCG-CTGAGTACGTC-3′. PCR products were analyzed using 2% agarose gels and visualized by a UV transilluminator.

2.5. Immunofluorescence. Mesangial cells were grown on coverslips in 6-well plates. Cells were fixed in 4% paraformaldehyde (Pierce) and permeabilized in 0.25% Triton X-100 (Sigma) for 10 min. Cells were washed twice in PBS and blocked in 5% goat serum for 1 h at room temperature, followed by incubation with anti-Smad4 and anti-SUMO2/3 antibodies overnight at 4°C. After washing, cells were incubated with rhodamine and fluorescein isothiocyanate-conjugated secondary antibodies (Bio-Synthesis) for 45 min in the dark. The coverslips were washed and mounted onto slides using mounting medium (Beyotime) and imaged with a DMIRE2 laser scanning confocal microscope (Leica, Germany).

2.6. Statistical Analyses. All data obtained from at least three independent experiments were expressed as the mean ± standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA), followed by the LSD post hoc test for multiple comparisons (SPSS 11.5 statistical software). $P < 0.05$ was considered significant.

3. Results

3.1. High Glucose Induced SUMO1 and SUMO2/3 Expression in Mesangial Cells. To determine whether SUMO is regulated by glucose in mesangial cells, we first detected SUMO proteins by Western blot. As shown in Figure 1(a) SUMO1 and SUMO2/3 expression in mesangial cells gradually increased after treatment with 30 mmol/L glucose for 12 h to 24 h and was highest at 24 h. There was no significant increase at 6 h compared to the normal control. SUMO1 and SUMO2/3 were highly expressed in the high glucose groups, particularly in the 20 mmol/L glucose group, compared to normal control ($P < 0.05$, Figure 1(b)). A significant difference was found between the mannitol and normal control groups. However, the expressions of SUMO1 and SUMO2/3 were significantly decreased in the mannitol group compared with the 20 mmol/L and 30 mmol/L glucose groups ($P < 0.05$), suggesting that osmotic pressure had a little effect on the high glucose-induced SUMO expression. Reverse transcription-PCR (RT-PCR) was performed to assess glucose-induced

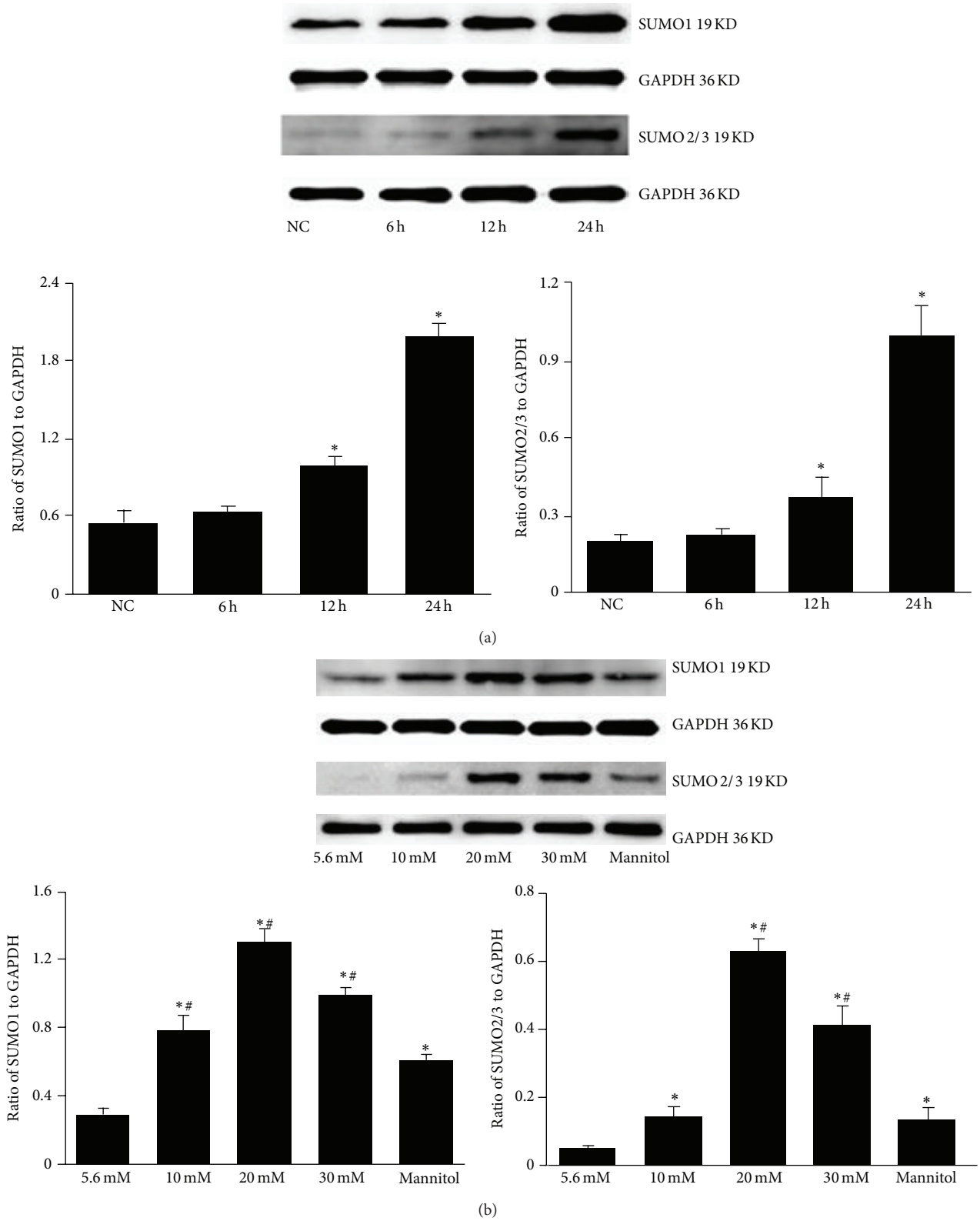

Figure 1: SUMO protein expression in mesangial cells detected by Western blot. (a) Cells were treated with 30 mmol/L high glucose for 6, 12, and 24 h, and Western blot was performed to detect SUMO1 and SUMO2/3 protein levels. $^{*}P < 0.05$ compared to normal control (NC) group. (b) Cells were treated with the indicated concentrations of glucose or mannitol for 24 h, with 5.6 mmol/L glucose as a normal control and 30 mmol/L mannitol as an osmotic control. $^{*}P < 0.05$ compared to normal control; $^{\#}P < 0.05$ compared to mannitol control. GAPDH was used as a loading control in (a) and (b).

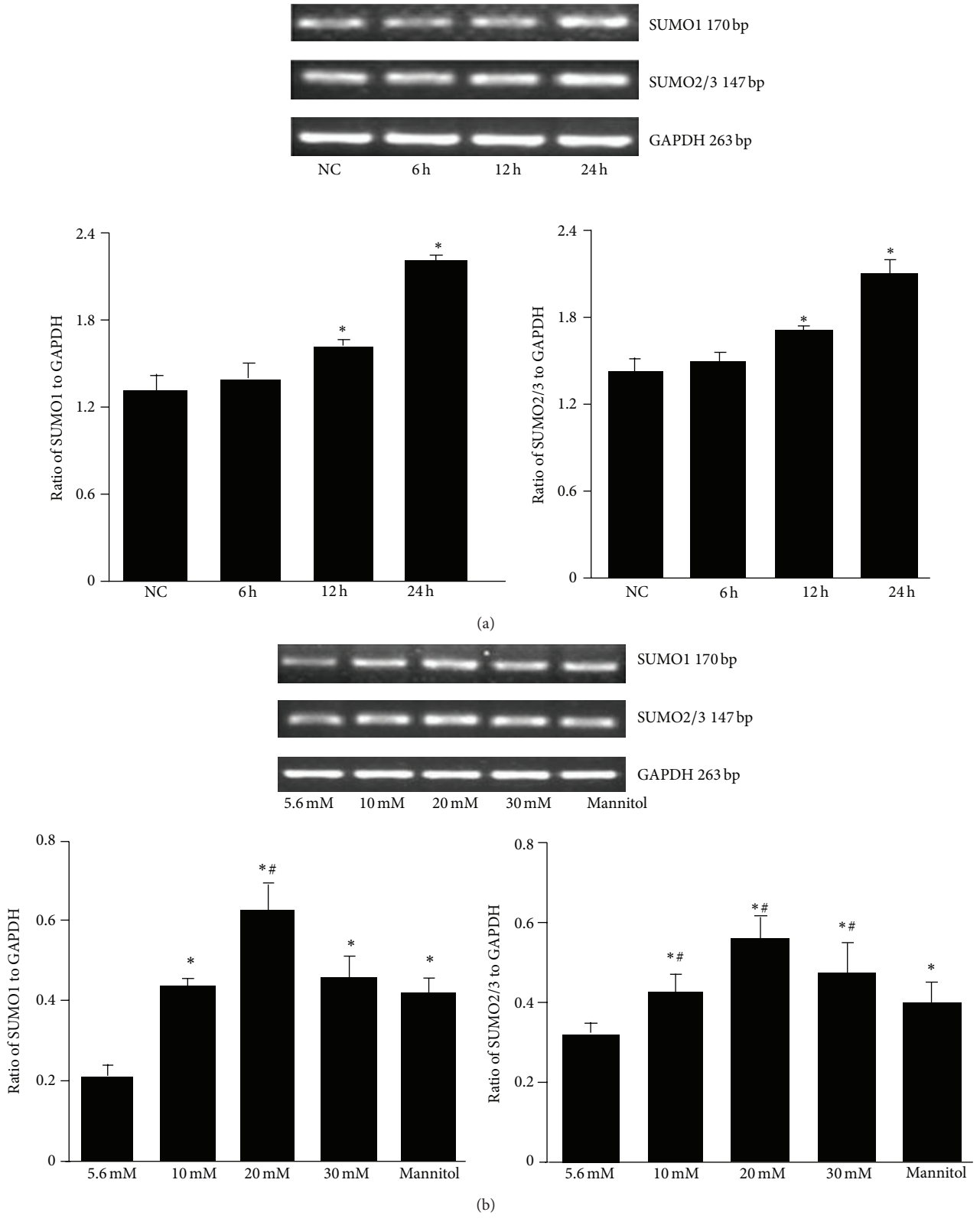

Figure 2: Reverse transcription-PCR analysis of SUMO mRNA expression. (a) Mesangial cells were treated with 30 mmol/L high glucose for 6, 12, and 24 h. SUMO1 and SUMO2/3 mRNA levels were assessed by RT-PCR and normalized to GAPDH. $^{*}P < 0.05$ compared to normal control (NC) group. (b) Cells were treated with the indicated concentrations of glucose or mannitol for 24 h, with 5.6 mmol/L glucose as a normal control and 30 mmol/L mannitol as an osmotic control. GAPDH was used to confirm equal loading. $^{*}P < 0.05$ compared to normal control; $^{\#}P < 0.05$ compared to mannitol control.

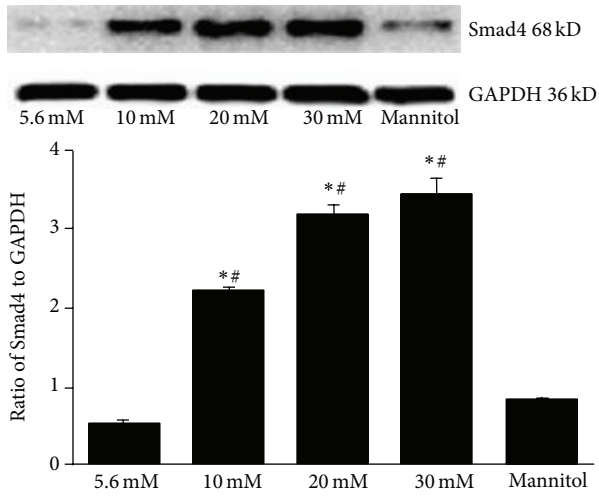

Figure 3: Smad4 protein expression in mesangial cells induced by high glucose. Mesangial cells were treated with the indicated concentrations of glucose or mannitol for 24 h, and Western blot was performed to detect Smad4 protein levels with 5.6 mmol/L glucose as a normal control and 30 mmol/L mannitol as an osmotic control. $^{*}P < 0.05$ compared to normal control; $^{\#}P < 0.05$ compared to mannitol control.

SUMO1 and SUMO2/3 mRNA expression. Results showed similar trend with SUMO protein expression (Figure 2).

3.2. High Glucose Induces Smad4 Expression in Mesangial Cells. Western blot was used to detect Smad4 protein levels in mesangial cells exposed to glucose or mannitol for 24 h. GAPDH was used as a loading control. As shown in Figure 3, Smad4 was abundantly expressed in the high glucose groups in a concentration dependent manner compared to normal control. In addition, there was no significant difference between the mannitol osmotic and normal controls, confirming that glucose, but not osmotic stress, increased Smad4 expression in mesangial cells.

3.3. Smad4 is Sumoylated by SUMO2/3 in Mesangial Cells. To determine whether Smad4 is sumoylated in mesangial cells, cell lysates were subjected to immunoprecipitation with anti-Smad4 antibody. Normal mouse IgG was used as a control antibody and immunoprecipitates were immunoblotted with anti-SUMO2/3 or anti-SUMO1 antibodies. An approximately 90 kDa band representing SUMO2/3-Smad4 conjugates was detected, and its size was compatible with the addition of a single SUMO2/3 molecule to Smad4 (Figure 4(a)). In contrast, the mouse IgG control antibody did not detect sumoylated Smad4 protein. Figure 4(b) shows that a specific band for SUMO1-Smad4 conjugated protein (about 90 kDa) was not detected, indicating that Smad4 is not sumoylated by SUMO1 in mesangial cells. To further assess high glucose-induced sumoylation of Smad4, anti-Smad4 immunoprecipitates were immunoblotted with anti-Smad4 or anti-SUMO2/3 antibodies after treating mesangial cells with various concentrations of glucose or mannitol for 24 h. As shown in Figure 4(c), a SUMO2/3-Smad4 conjugated protein was strongly expressed in the 20 mmol/L glucose group compared to normal and mannitol controls ($P < 0.01$). These results suggest that high glucose but not osmotic stress activates sumoylation of Smad4 via SUMO2/3 in mesangial cells.

3.4. Colocalization of SUMO2/3 and Smad4 in Mesangial Cells. Immunofluorescence was performed to determine subcellular localization of SUMO2/3 and Smad4 after mesangial cells were treated with high glucose for 24 h. Cells were incubated with anti-Smad4 and anti-SUMO2/3 primary antibodies and stained with fluorescein isothiocyanate and rhodamine-conjugated secondary antibodies. Under confocal immunofluorescence microscopy, Smad4 showed diffuse cytoplasmic and nuclear distribution (Figures 5(a), 5(d), and 5(g)), while SUMO2/3 was localized primarily in the nucleus with a punctated pattern in each group (Figures 5(b), 5(e), and 5(h)). The merged images showed that SUMO2/3 colocalized with Smad4 primarily in the nucleus and was strongly enhanced in the high glucose group (Figure 5(f)) compared with normal (Figure 5(c)) and mannitol controls (Figure 5(i)).

3.5. High Glucose Induced Fibronectin (FN) mRNA Expression in Mesangial Cells. Fibronectin (FN) is an ECM protein in diabetic kidney. RT-PCR analysis of FN mRNA in mesangial cells showed that the expression of FN mRNA increased in high glucose groups compared to the normal control group ($P < 0.05$) in a concentration and time dependent manner (Figure 6). A significant difference between the mannitol and normal control groups was observed. However, the expression of FN mRNA was lower in the mannitol group compared with the 20 mmol/L and 30 mmol/L glucose groups ($P < 0.05$; Figure 6(b)), indicating that high glucose-induced the increase of FN mRNA expression was not an osmotic effect.

4. Discussion

TGF-β has been considered a key mediator in multiple organ fibrosis, including the heart, liver, lung, and kidney [18–20]. TGF-β signaling is transduced by transmembrane receptors (TβRII, TβRI) and intracellular signals called Smads (Smad1-8) [21]. Hyperglycemia, AGE, and angiotensin II have been shown to activate TGF-β signaling in mesangial and tubular cells [5, 6]. Diabetic glomerulosclerosis and tubulointerstitial fibrosis are caused by accumulation of ECM proteins and glomerular basement membrane thickening [22, 23]. FN is one of the major components of the ECM and is useful in evaluating pathological conditions in DN [24]. Our data show that high glucose increased the expression of FN in mesangial cells, further confirming that high glucose promotes the fibrosis in DN.

Many studies have indicated that phosphorylation and activation of Smad2/3 contribute to diabetic renal fibrosis [25, 26]. However, the role of Smad4 in TGF-β-mediated renal fibrosis is largely unclear. Therefore, we investigated whether Smad4 is regulated by high glucose in cultured mesangial

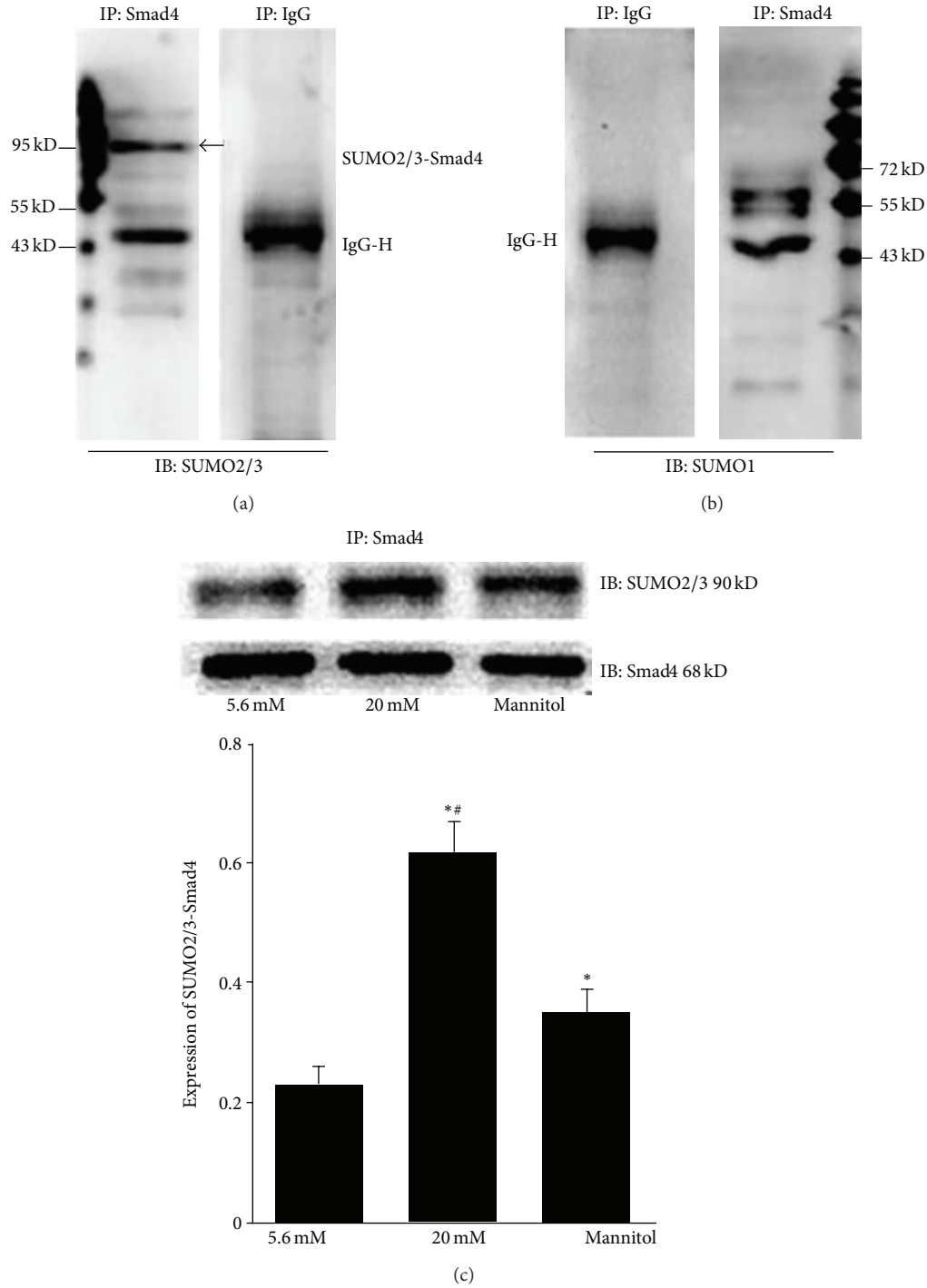

Figure 4: Smad4 is sumoylated by SUMO2/3 in mesangial cells. (a) and (b) Cell lysates were subjected to immunoprecipitation (IP) with anti-Smad4 antibody or normal mouse IgG as a negative control, followed by immunoblot (IB) with anti-SUMO2/3 or anti-SUMO1 antibody to detect the interaction between SUMO2/3 or SUMO1 with Smad4. The arrow marked band indicates SUMO2/3-Smad4 conjugates (a). No specific band was detected for SUMO1-Smad4 conjugates in mesangial cells (b). IgG-H marks the IgG heavy chain in (a) and (b). (c) Cells were treated with 5.6 mmol/L and 20 mmol/L glucose and equimolar concentration of mannitol for 24 h. Anti-Smad4 immunoprecipitates were subjected to immunoblotting with anti-Smad4 or anti-SUMO2/3 antibodies for Smad4 and SUMO2/3-Smad4 proteins. Relative expression of SUMO2/3-Smad4 conjugates was detected by Western blot and normalized to Smad4. $^{*}P < 0.05$ compared to normal control; $^{\#}P < 0.05$ compared to mannitol control.

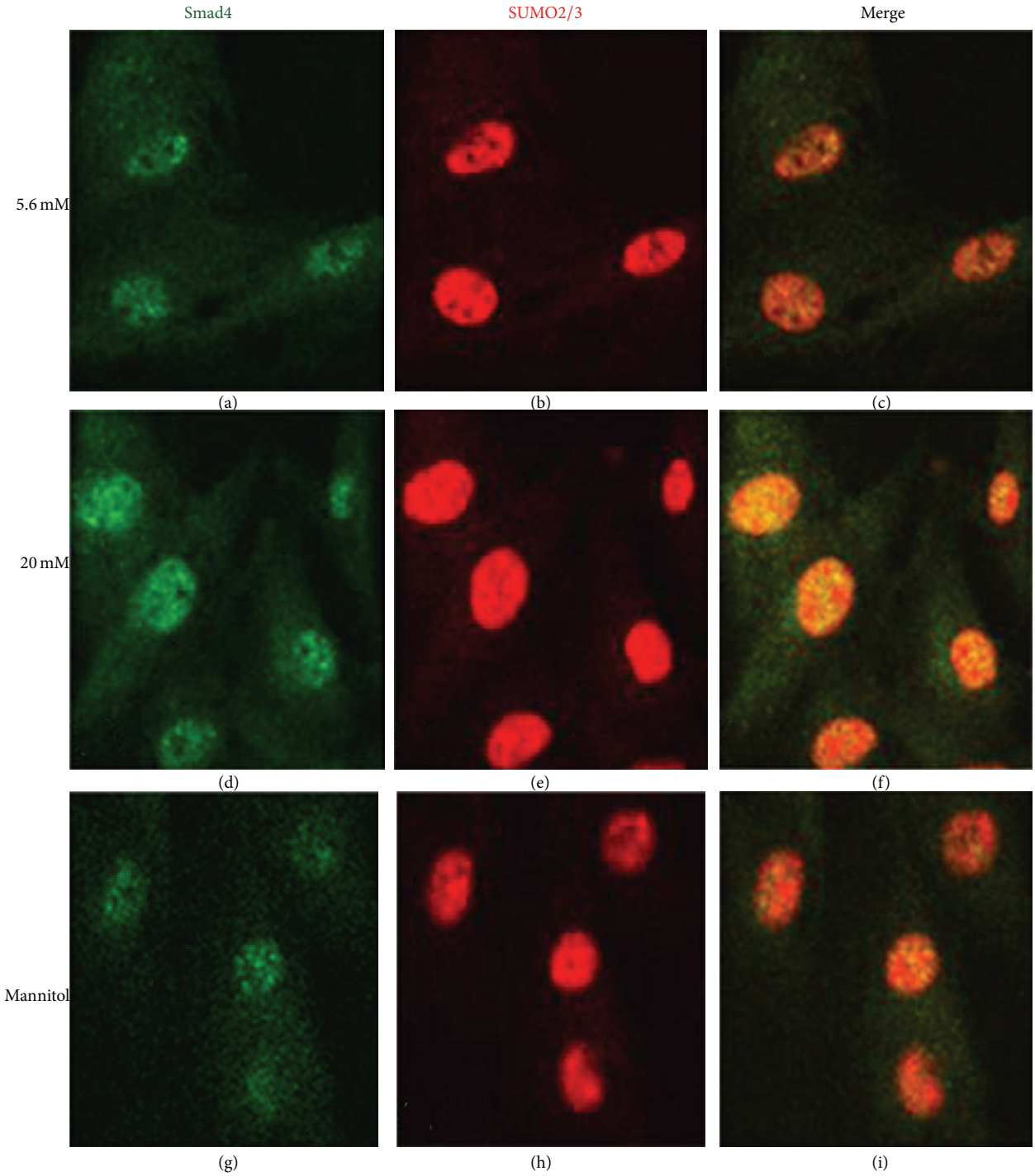

Figure 5: Colocalization of SUMO2/3 and Smad4 in mesangial cells. Cells were incubated with mouse anti-Smad4 monoclonal antibody and rabbit anti-SUMO2/3 polyclonal antibody and visualized with fluorescein isothiocyanate-conjugated anti-mouse IgG for Smad4 ((a), (d), (g)) and rhodamine-conjugated anti-rabbit IgG for SUMO2/3 ((b), (e), (h)). The merged images of Smad4 and SUMO2/3 stainings in each group are shown in (c), (f), and (i).

cells. As expected, our results showed that high glucose increased Smad4 expression in a concentration dependent manner, indicating that Smad4 is positively involved in TGF-β-mediated progression of DN. This finding is similar to that of Meng et al. [27], who revealed that deletion of Smad4 inhibited progression of renal fibrosis.

Our previous study showed that ubiquitination of histones H2A and H2B could activate the TGF-β signaling pathway in diabetic nephropathy [9]. However, several studies have suggested that use of the specific ubiquitin proteasome inhibitor MG132 cannot completely block activation of the TGF-β signaling pathway [28, 29]. This indicates that other

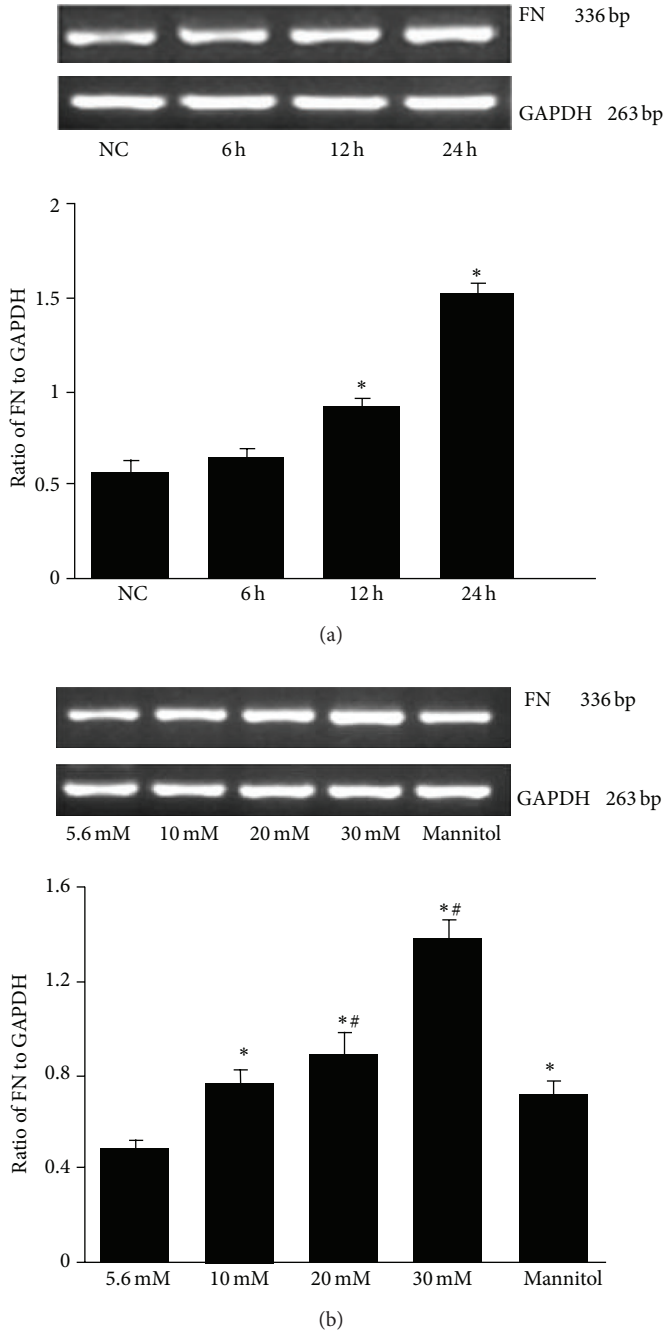

Figure 6: Reverse transcription-PCR analysis of FN mRNA expression in mesangial cells. (a) Mesangial cells were treated with 30 mmol/L high glucose for 6, 12, and 24 h, and FN mRNA expression levels were detected by RT-PCR. $^{*}P < 0.05$ compared to normal control (NC) group. (b) Cells were treated with different concentrations of glucose or mannitol for 24 h, with 5.6 mmol/L glucose as a normal control and 30 mmol/L mannitol as an osmotic control. $^{*}P < 0.05$ compared to normal control; $^{\#}P < 0.05$ compared to mannitol control.

protein modifications are also involved in TGF-β pathway activation in DN. For example, sumoylation has been demonstrated to play an important role in a wide range of biological processes and several human diseases, such as inflammation, cancer, heart disease, and neurodegenerative diseases [30–33]. However, it is not known whether sumoylation is involved in DN.

SUMO1 and SUMO2/3 are expressed in most tissues, whereas SUMO4 expression appears to be limited to the kidney, lymph node, and spleen [34]. In addition, various cellular stresses, such as osmotic, oxidative stress, and heat shock, have been shown to increase sumoylation by SUMO2/3 but have little effect on SUMO1 modification. Interestingly, a previous study reported that M55V substitution in the

SUMO4 gene (163A → G) was strongly associated with type 1 diabetes [35]. To determine whether SUMOs are expressed and regulated by glucose in mesangial cells, we used Western blot and RT-PCR for detection. Our data show that SUMO1 and SUMO2/3 are expressed in mesangial cells, and high glucose increased the expression of SUMO. Meanwhile, we found that osmotic stress had little effect on the expression of SUMO1 and SUMO2/3 compared with high glucose. These results suggest that SUMO1 and SUMO2/3 may be involved in the progression of DN. The detailed mechanisms involved will require further study.

More than 120 SUMO substrate proteins have been identified. The majority are nuclear proteins, and most of nonnuclear substrates are signal transduction proteins, including NEMO, IκBα, Smads, Glut4, and TβRI [36, 37]. Sumoylation changes the activity, subcellular localization, or stability of these proteins to influence signal transduction [15]. Sumoylation has been demonstrated to regulate the canonical TGF-β/Smad pathway through TβRI, Smad3, and Smad4 [10, 11, 38]. Several studies have shown that Smad4 is sumoylated by SUMO1 or SUMO2/3. Interestingly, these findings indicate an opposite effect of sumoylation on Smad4 activity, which can play a positive [39–41] or negative [42] role in regulating TGF-β/Smad signaling.

Given the important role of sumoylation in the TGF-β/Smad pathway and previously described results, we hypothesized that sumoylation of Smad4 may have a role in TGF-β-mediated DN. Therefore, we determined whether Smad4 is sumoylated and the effect of high glucose on covalent modification. As expected, our data showed that Smad4 interacted and colocalized with SUMO2/3 in mesangial cells and the expression was strongly enhanced by glucose, and osmotic stress has been shown to have little effect on sumoylation by SUMO2/3. Unfortunately, we found that Smad4 is not sumoylated by SUMO1 in mesangial cells. The results suggest that high glucose stimulates sumoylation of Smad4 through SUMO2/3 but not SUMO1 in mesangial cells, resulting in activation of the TGF-β signaling pathway. The mechanisms involved may include the sumoylation of Smad4, preventing the degradation of Smad4, increasing the level of Smad4, and enhancing nuclear accumulation and stability of Smad4. We did not analyze other regulators, such as several R-Smads and TGF-β receptors in this study. Additional studies are necessary to explore the diverse roles of sumoylation in TGF-β/Smad signaling, which mediates pathogenesis of DN.

A recent in vivo study first reported that inhibition of sumoylation by knockdown of Ubc9 suppresses canonical TGF-β/Smad signaling and prevents development of fibrosis in systemic sclerosis [43]. Netherton and Bonni reported that sumoylation plays an important role in TGFβ-induced epithelial mesenchymal transition (EMT), which contributes to fibrotic and neoplastic diseases [44]. Moreover, another study demonstrated that sumoylation was involved in the progression of glomerulonephritis by regulating α-smooth muscle actin transcription in mesangial cells [45]. All of these findings suggest that sumoylation may be a new therapeutic target for fibrotic diseases and is likely to provide new insights into the pathogenesis of these diseases.

In summary, our study found that high glucose significantly increased the expression of SUMO1 and SUMO2/3 and stimulated Smad4 sumoylation through SUMO2/3 but not SUMO1. We demonstrated that sumoylation of Smad4 may be involved in the pathogenesis of DN by regulating the TGF-β/Smad signaling pathway. Further studies on sumoylation will be necessary to find potential therapeutic targets for DN.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Gremlin Activates the Smad Pathway Linked to Epithelial Mesenchymal Transdifferentiation in Cultured Tubular Epithelial Cells

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Gremlin is a developmental gene upregulated in human chronic kidney disease and in renal cells in response to transforming growth factor-β (TGF-β). Epithelial mesenchymal transition (EMT) is one process involved in renal fibrosis. In tubular epithelial cells we have recently described that Gremlin induces EMT and acts as a downstream TGF-β mediator. Our aim was to investigate whether Gremlin participates in EMT by the regulation of the Smad pathway. Stimulation of human tubular epithelial cells (HK2) with Gremlin caused an early activation of the Smad signaling pathway (Smad 2/3 phosphorylation, nuclear translocation, and Smad-dependent gene transcription). The blockade of TGF-β, by a neutralizing antibody against active TGF-β, did not modify Gremlin-induced early Smad activation. These data show that Gremlin directly, by a TGF-β independent process, activates the Smad pathway. In tubular epithelial cells long-term incubation with Gremlin increased TGF-β production and caused a sustained Smad activation and a phenotype conversion into myofibroblasts-like cells. Smad 7 overexpression, which blocks Smad 2/3 activation, diminished EMT changes observed in Gremlin-transfected tubuloepithelial cells. TGF-β neutralization also diminished Gremlin-induced EMT changes. In conclusion, we propose that Gremlin could participate in renal fibrosis by inducing EMT in tubular epithelial cells through activation of Smad pathway and induction of TGF-β.

1. Introduction

Many embryological expressed genes regulate morphogenesis and then become quiescent in the normal adult kidney. Recent studies have shown that some developmental genes are reactivated in the adult diseased kidneys [1]. The reemergence of these genes appears to be linked to tissue repair, but when an imprecise interaction of developmental and inflammatory signals occurs, complete healing is not achieved. Instead, there is an excessive production of matrix proteins leading to a scar formation. Gremlin was identified as one of the developmental genes induced in cultured human mesangial cells exposed to high glucose, initially known as induced in high glucose-2 (IHG-2) and

also called downregulated by mos (Drm) [2]. Gremlin is a member of cysteine knot superfamily [3] that includes transforming growth factor-β (TGF-β) proteins and acts as a bone morphogenetic protein (BMP) antagonist [4]. Analysis of the predicted amino acid sequence indicated the presence of several significant features, including potential nuclear localization signals near the C-terminus, potential N-linked glycosylation sites, and multiple potential sites for phosphorylation. The signalling peptide and a predicted glycosylation site have been identified. Gremlin is a glycosylated, phosphorylated, secreted protein present both on the external cell surface and within the ER-Golgi compartments [3]. In many human renal diseases induction of Gremlin has been described [5–8]. Several experimental studies have shown that Gremlin participates in renal damage [9, 10]. Therefore, some authors have suggested that Gremlin could be considered as a mediator of renal injury.

Chronic progressive fibrosis of the kidney remains an unresolved challenge. Irrespective of the underlying cause, chronic kidney disease is linked to the development of tubulointerstitial fibrosis, characterized by accumulation of extracellular matrix (ECM). The mechanisms of renal fibrosis are complex and our therapeutic armamentarium is limited. The key cellular mediator of fibrosis is the myofibroblast. There are different sources of myofibroblasts, including activation of tissue fibroblasts and migration of circulating mesenchymal progenitors or cell transitions, including epithelial-mesenchymal transition (EMT) or endothelial mesenchymal transition [11, 12]. The investigation of the mechanisms involved in renal fibrosis and the identification of novel mediators with potential therapeutic application is an important open question in chronic kidney disease.

TGF-β1, signaling mainly through Smad proteins, is a key player in fibrosis and EMT [13–17]. Because of its pleiotropic actions, TGF-β blockade is not an ideal therapeutic tool; therefore, novel targets are needed. Among them, Gremlin may be an interesting candidate in progressive renal diseases. Recent *in vitro* studies developed by our group have shown that Gremlin gene silencing inhibited TGF-β-mediated matrix production and EMT [18]. However, the involvement of TGF-β in Gremlin responses has not been investigated. We have also reported the presence of Gremlin in glomerular crescents of human pauci-immune glomerulonephritis and in the tubulointerstitium of chronic allograft nephropathy. In these human diseases Gremlin correlated with the degree of tubulointerstitial fibrosis and was associated with TGF-β1 overexpression and Smad pathway activation [7, 8]. These studies suggest that Gremlin may activate the Smad pathway; therefore, the aim of this work was to evaluate whether Gremlin could directly activate the Smad pathway in tubular epithelial cells, evaluating whether this activation is linked to Gremlin-induced EMT, the main fibrotic effect observed in response to Gremlin stimulation in these cells [18].

2. Materials and Methods

2.1. Cell Cultures. Human renal proximal tubuloepithelial cells (HK2 cell line, ATCC CRL-2190) were grown in RPMI with 10% fetal bovine serum (FBS), 1% nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin, insulin transferrin selenite (ITS) (5 μg/mL), and hydrocortisone (36 ng/mL) in 5% CO_2 at 37°C. At 60–70% of confluence, cells were growth-arrested in serum-free medium for 24 hours before the experiments. Then, cells were stimulated for different times with recombinant Gremlin (50 ng/mL) (R&D) or human recombinant TGF-β1 (1 ng/mL, Peprotech). Cell culture reagents were obtained from Life Technologies Inc. TGF-β was targeted by a pan-specific polyclonal anti-TGF-β neutralizing antibody, which recognizes bovine, mouse, and human TGF-β1 and β2 isoforms (1 μg/mL) (R&D).

2.2. Transfection, cDNA Constructs, and Promoter Studies. HK2 cells were transiently transfected for 24–48 hours with FuGENE (Roche), pCDNA3-Gremlin-myc-IRES2-eGFP plasmid (GREM-GFP) and/or pCDNA-FLAG-Smad7 expression vector (kindly donated by Dr. Massagué, Memorial Sloan-Keternig Cancer Center, USA) or empty vector (pCDNA). The GREM-GFP was generated as follows: GREM1 cDNA was purchased from the Mammalian Gene NIH Collection (Bethesda, Maryland, USA). We added a c-myc tag to the 3′ portion of GREM1 using PCR with the forward primer 5′AGTGCGGCGGCTGAGGACCC GCCGCACTGACAT-3′ and the reverse primer 5′-ATAGCCGCCGCTTACAGATCCTCTTCTGAGATG-AGTTTTTGTTCATCCAAATCGATGGATATGC-3′. We also inserted an e-GFP sequence downstream of human Gremlin as follows. The IRES-eGFP sequence was obtained by PCR using a pIRES2-EGFP plasmid (Clontech Mountain View, CA, USA) as the template with the following primers: IRES-eGFP-F (5′-TACATTAAT-GGGCCCGGGATCCGCCCCTC-3′) and IRES-eGFP-R (5′-GGCCATATGCGCCTTAAGATACATTGATG-3′). The GREM1-c-myc and IRES-eGFP fragments were independently cloned into a pGEMT-Easy vector and then sequenced (Macrogen, Seoul, Korea) to confirm the modifications and absence of additional mutations. Next, both the GREM1-c-myc and IRES-eGFP fragments were subcloned into a modified pCDNA3 vector using the *Eco*RI and *Not*I restriction sites, respectively. In Gremlin-transfected cells, Gremlin production was confirmed by immunofluorescence (not shown).

To demonstrate Smad 7 transfection efficacy an anti-FLAG antibody was used (not shown). Smad-dependent promoter activation was evaluated by transfection of Smad/luc (kindly donated by Dr. Volgestein, Baltimore, USA) and TK-renilla as internal control, as described [19].

2.3. Protein Studies. Total cellular protein extracts (10–50 μg/lane) obtained in lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton X-100, 0.3% NP40, 100 μM phenylmethylsulphonylfluoride, 1 mM dithiothreitol, 100 μM Na_3VO_4, and 1 mM protease-inhibitor cocktail (Sigma)] were separated on 8–12% polyacrylamide-SDS gels under reducing conditions. Samples were then transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA), blocked with 5% nonfat dry milk, in 50 mM

Tris-HCl, pH 7.5, 150 mM NaCl with 0.05% Tween-20, and incubated overnight at 4°C with the primary antibodies and subsequently incubated with peroxidase-conjugated IgG (Amersham), and developed by ECL chemiluminescence (GE Healthcare, Buckinghamshire, UK).

Immunocytochemistry studies were performed in cells growing on coverslips. After the experiments, cells were fixed in Merckofix (Merck) and permeabilized with 0.2% Triton-X100 for 10 min (except for E-cadherin staining). After blocking with 4% BSA and 8% serum for 1 hour, samples were incubated with primary antibodies overnight at 4°C, and then 1 hour at room temperature with fluorescein isothiocyanate (FITC) [1/200] or AlexaFluor 633 [1/300] conjugated antibodies (Amersham). Nuclei were stained with 1 μg/mL propidium iodide (PI) or 4′,6-Diamidino-2-phenyindole dilactate (DAPI) (Sigma-Aldrich), as control of equal cell density. Absence of primary antibody was used as negative control. Samples were mounted in Mowiol 40–88 (Sigma-Aldrich) and examined by a Leica DM-IRB confocal microscope.

The antibodies employed were: p-Smad 3 (Abcam) (WB: 1/1000), Smad 2 and Smad 4 (Sta. Cruz) (IF: 1/300), Smad3 (Sta Cruz) (IF: 1/300, WB: 1/1000), Vimentin (BD Pharmingen) (IF: 1/200; WB: 1/1000), E-cadherin (R&D) (IF: 1/200, WB: 1/1000), Slug (Cell signaling) (WB: 1/1000), pan-Cytokeratin, and α -SMA (Sigma Aldrich) (IF: 1/200).

TGF-β1 protein was measured in the cell-conditioned medium using a commercial enzyme-linked immunoassay (ELISA) (BD Sciences, San Diego, USA) following the manufacturer's instructions. TGF-β1 levels were quantified by comparison with a standard curve using increasing concentrations of human TGF-β1. Protein content was determined by the BCA method (Pierce).

2.4. Gene Expression. Total RNA was isolated from cells with Trizol (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized from 2 μg of total RNA with random hexamer primers using the High capacity cDNA Archive Kit (Applied). Real-time PCR was performed using human FAM TaqMan MGB probes designed by assay-on-demand gene expression products (Applied): TGF-β1: Hs99999918_m1; connective tissue growth factor (CTGF): Hs00170014_m1: plasminogen activator inhibitor 1 (PAI1): Hs 00167155_m1. Data weres normalized to 18S eukaryotic ribosomal RNA: 4210893E (VIC). The mRNA copy numbers were calculated for each sample by the instrument software using Ct value ("arithmetic fit point analysis for the lightcycler"). Results were expressed in copy numbers and calculated relative to unstimulated cells after normalization against 18S.

2.5. Statistical Analysis. Results throughout the text are expressed as mean ± SEM. Differences between agonist-treated groups and controls were assessed by one-way analysis of variance, followed by post hoc Bonferroni or Dunnett test or Mann-Whitney test, as appropriate. $P < 0.05$ was considered significant. Statistical analysis was conducted using the SPSS statistical software, version 11.0 (SPSS).

3. Results

3.1. Gremlin Activates Smad Pathway in Human Cultured Tubuloepithelial Cells. Receptor mediated activation of Smad proteins (R-Smads 2 and 3) occurs by direct C-terminal phosphorylation. Smad 2/3 then form complexes with Smad 4 and translocate into the nucleus, where they associate and cooperate with DNA binding transcription factors to activate or repress target gene transcription [17]. In cultured HK2 cells, stimulation with recombinant Gremlin increased phosphorylation levels of Smad 3 as early as 5 minutes, and it was maintained until 15 minutes (Figure 1(a)).

Although Smad is the main signaling mechanism of TGF-β, several factors involved in renal damage, such as angiotensin II, can directly activate the Smad pathway, independent of endogenous TGF-β [17]. Therefore, to evaluate whether early Smad activation caused by Gremlin was mediated or not by TGF-β, cells were preincubated with a neutralizing antibody against active TGF-β. Gremlin-induced Smad activation (evaluated as p-Smad 3 levels) was not modified in the presence of the TGF-β antibody (Figure 1(b)). Similar lack of response was found in the presence of decorin (a proteoglycan that neutralizes active TGF-β, not shown). These data indicates that Gremlin directly activates the Smad pathway.

Some actions of Gremlin are due to its effect as BMP antagonist [4]. To determine the contribution of BMPs in Gremlin-induced Smad activation, HK2 cells were preincubated with BMP-2 or BMP-4 and then stimulated with Gremlin during 10 minutes. Phosphorylation of Smad 3 was not modified in the presence of any of these BMPs (Figure 1(c)), suggesting that Gremlin-induced Smad activation is independent of BMPs in tubular epithelial cells.

By confocal microscopy, we have confirmed that Gremlin rapidly increased Smad 3 translocation to the nucleus; the latter demonstrated by the yellow nuclear staining observed in the merge of Figure 2, while in untreated cells, the nuclei are red. Gremlin also increased nuclear localization of Smad 2 and Smad 4, observed at 15 minutes (Figure 2).

To further confirm that Gremlin activates the Smad pathway, cells were transfected with a Gremlin expression vector (GREM-GFP) for 24 hours. By confocal microscopy we observed that in positive Gremlin-transfected cells (GFP-green staining) there was a nuclear immunostaining showing the translocation of Smad 3 and Smad 2 (characteristic of Smad activation), compared to cells transfected with empty vector (Figures 3(a) and 3(b)). In the merge of Figure 3, a Gremlin expressing-cell marked by a yellow rectangle presented a positive nuclear staining, corresponding to the presence of Smad 3 or 2. In contrast, in cells transfected with empty vector, there are no nuclear Smad 2 and Smad 3 immunostaining (all cells present blue nuclei), as observed in some nontransfected cells.

To investigate whether Gremlin regulates Smad-mediated gene expression, cells were cotransfected with a Gremlin expression vector (GREM-GFP) and a luciferase Smad reporter plasmid. Gremlin transfected cells expressed higher Smad-dependent luciferase activity (SBE) than control cells (Figure 4). To demonstrate further the involvement of Smad

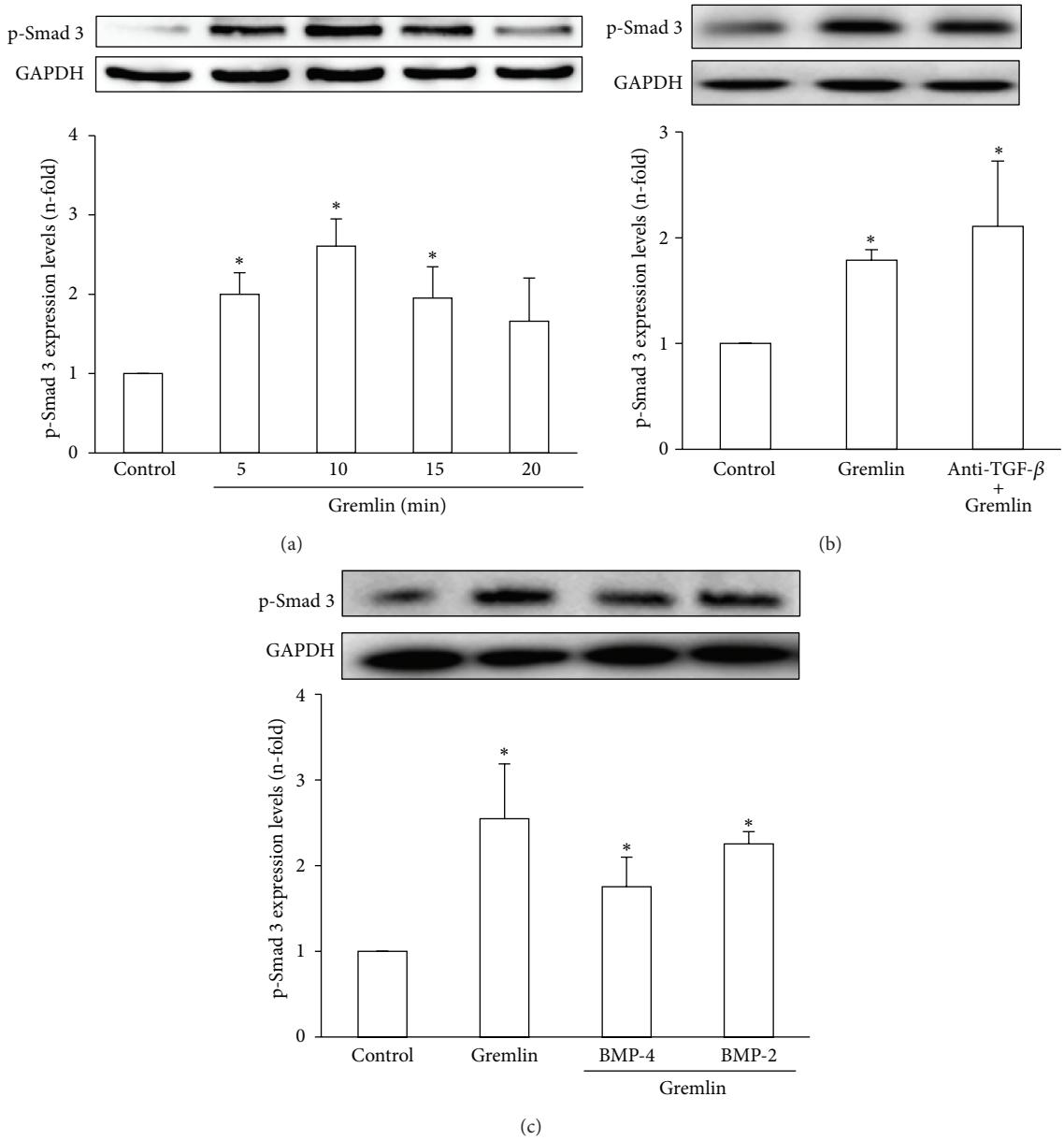

Figure 1: *(a) Stimulation with Gremlin rapidly increased Smad 3 phosphorylation in cultured human tubuloepithelial cells*. HK2 cells were stimulated with Gremlin (50 ng/mL) for different times. *(b) Early Smad 3 phosphorylation induced by stimulation with Gremlin was not mediated by TGF-β*. TGF-β was blocked or not (control) by pretreatment of cells for 1 hour with an anti-TGF-β neutralizing antibody and then treated with Gremlin for 10 minutes. (c) In some points, HK2 cells were preincubated with BMP-2 or BMP-4 and then treated with Gremlin for 10 minutes. Total proteins were isolated and protein levels were evaluated by western blot. GAPDH or Smad 3 were used as loading controls. Figures show a representative western blot of phosphorylated levels of Smad 3 and data are expressed as n-fold over control (considered as 1), as the mean ± SEM of 3-4 independent experiments. $^{*}P < 0.05$ versus control.

pathway in Gremlin-induced responses, a Smad 7 expression vector, that inhibits Smad-mediated transcriptional effects by interfering with receptor-mediated activation of R-Smad, was used [17, 19]. In HK2 cells cotransfected with GREM-GFP and Smad 7 expression vectors, the Smad-mediated luciferase activity was significantly lower than cells transfected with GREM-GFP alone, showing the specific Smad 7 blockade of Gremlin-mediated Smad activation (Figure 4).

3.2. Gremlin-Induced EMT Is Mediated by Smad Activation. We have previously demonstrated that in tubular epithelial cells long-term stimulation with recombinant Gremlin induced EMT [18]. Now, we have observed that transfection of HK2 cells with GREM-GFP induced EMT-related phenotypic changes observed by confocal microscopy after 48 hours (Figure 5). Cells transfected with empty-vector showed epithelial morphology, including the presence of

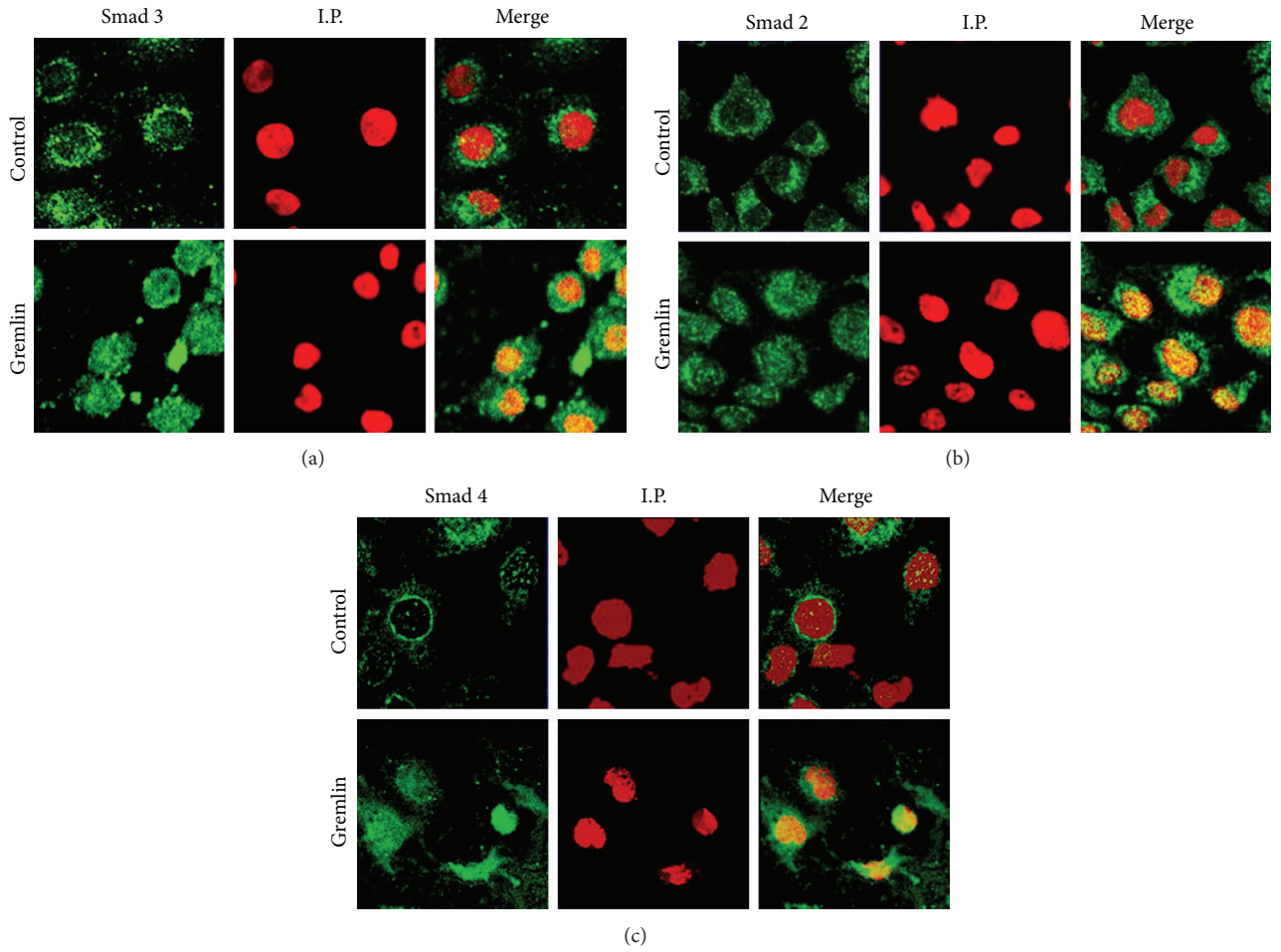

Figure 2: *Stimulation with Gremlin induces a rapid activation of the Smad pathway in cultured human tubuloepithelial cells.* HK2 cells were stimulated with Gremlin (50 ng/mL) for 15 minutes. The localization of R-Smad 3 (a) and 2 (b) and Smad 4 (c) was evaluated by confocal microscopy with FITC-secondary antibodies (green staining). Nuclei were stained with propidium iodide (I.P.) (red). In the merge, the yellow staining indicates the nuclear localization of Smad proteins. The results are representative of 3 independent confocal microscopy experiments.

epithelial markers, such as cytokeratin (red staining), and there is no positive staining for mesenchymal marker α-SMA (Figure 5). In contrast, overexpression of Gremlin caused changes in morphology to fibroblast-like shape and induction of α-SMA (see the GREM-GFP positive cell that presents yellow staining and elongated shape). Moreover, in Gremlin expressing cells cytokeratin staining was markedly diminished (absence of red staining in an area with several GREM-GFP positive green cells). The blockade of Smad activation, by cotransfection with Gremlin and Smad 7, diminished these EMT changes (Figure 5), as shown by restoration of the cytokeratin immunostaining and the epithelial morphology and diminution of α-SMA as observed in the green positive cell. These data suggest that Gremlin regulates EMT through the Smad pathway.

3.3. Role of Endogenous TGF-β on Gremlin-Induced EMT. Previously, we have reported that Gremlin acts as a downstream mediator of TGF-β-induced fibrosis in cultured renal cells and incubation with Gremlin for 24 hours induced a significant upregulation of TGF-β1 mRNA levels in cultured tubuloepithelial cells [18]. We have further investigated the relation between Gremlin/TGF-β, evaluating whether Gremlin could regulate TGF-β1 synthesis. In HK2 cells, active TGF-β1 protein increased in the supernatants of Gremlin-stimulated cells after 48 hours, but not at 24 hours (Figure 6(a)), suggesting that some of the profibrotic actions of Gremlin could be mediated by endogenous TGF-β1 synthesis. Therefore, we blocked TGF-β before HK-2 stimulation with Gremlin by adding a neutralizing antibody against active TGF-β, which is able to block angiotensin II-induced ECM production and EMT [19, 20]. TGF-β neutralization inhibited Gremlin-induced gene upregulation of profibrotic factors observed after 24 hours, including TGF-β, CTGF, and PAI-1 (Figure 6(b)). Moreover, TGF-β blockade antagonized several EMT-related changes induced by Gremlin after 48 hours, as shown by immunofluorescence (Figure 7(a)). We also observed by western blot that TGF-β neutralization diminished Vimentin and Slug induction caused by Gremlin

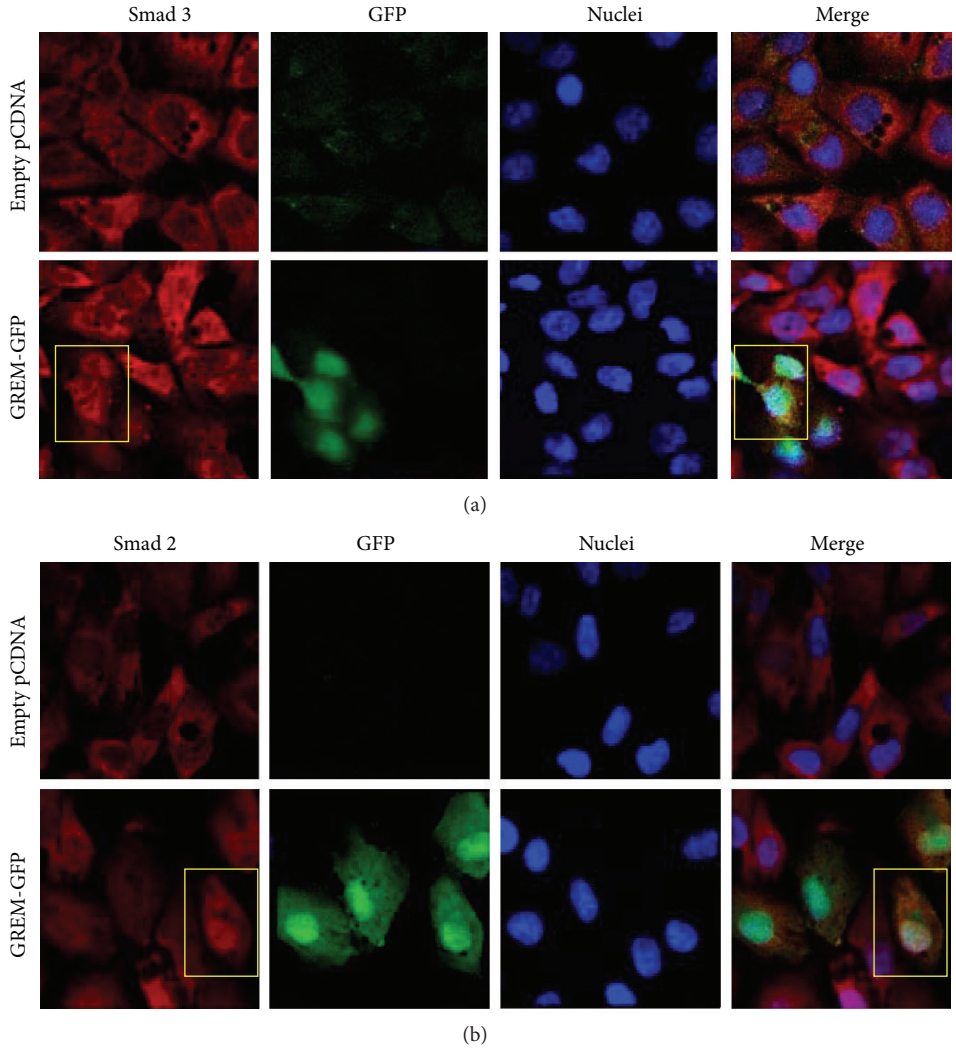

Figure 3: *Gremlin overexpression causes a sustained Smad activation in cultured human tubuloepithelial cells*. HK2 cells were transiently transfected with a Gremlin expression vector (GREM-GFP; green) or empty vector for 24 hours. The levels and localization of R-Smad 3 (a) and R-Smad 2 (b) were evaluated by confocal microscopy with Alexa-633 secondary IgG (red). Nuclei were stained using 4′,6-diamino-2-phenylindole dihydrochloride (DAPI; blue). In Gremlin-transfected cells (green staining by GFP), the Smad 2 and Smad 3 were found in the nuclei (white staining in the merge). Figures show representative images out of 3 independent observations.

and restored E-cadherin levels decreased by Gremlin (Figure 7(b)). These data suggest that TGF-β is a mediator of long-term responses of Gremlin in tubuloepithelial cells, including regulation of profibrotic factors and EMT changes.

4. Discussion

Our *in vitro* studies in cultured tubuloepithelial cells show that Gremlin directly activates the Smad pathway and participates in the EMT process, via Smad signalling. These data suggest that Gremlin could be a mediator of renal fibrosis.

Our study reveals that in cultured human tubuloepithelial cells Gremlin induces a rapid activation of the Smad pathway (observed after 5 min of stimulation) characterized by increased phosphorylation of the receptor-Smad (R-Smad), Smad 3, a critical downstream mediator of fibrosis [17], and Smad 2 proteins. Once R-Smad is phosphorylated it dimerises with Smad 4 and then shuttles to the nucleus to regulate gene expression. By confocal microscopy, we have found that Gremlin caused a rapid translocation to the nucleus of R-Smad/Smad 4 proteins. In several cells types Gremlin-induced TGF-β production [18, 21], as we have observed here after 48 hours of incubation. However, Gremlin-induced early Smad activation is independent of endogenous TGF-β, as we have demonstrated using TGF-β blockers (Figure 8). Other important profibrotic factors, such as angiotensin II, also activates the Smad pathway, rapidly and independent of endogenous TGF-β [17].

Previous studies in tubular epithelial cells have shown that the Smad route regulates EMT induced by key factors involved in renal fibrosis, such as TGF-β and angiotensin II [20]. The activation of Smad pathway has been described

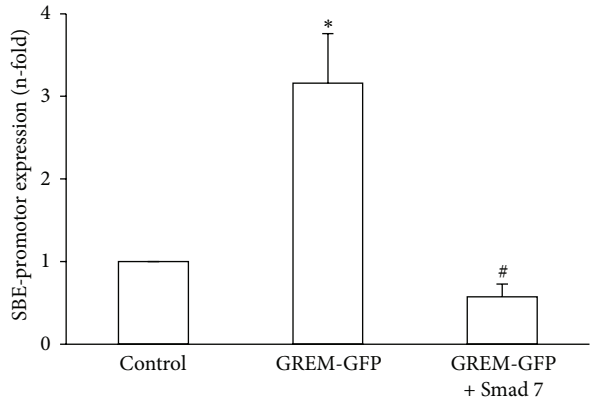

Figure 4: *Gremlin overexpression induces Smad-dependent gene transcription*. HK2 cells were transfected with GREM-GFP or empty vector, Smad/luc promoter, and TK-renilla for 24 hours. In some points, cells were cotransfected with Smad 7. Then, luciferase/renilla activity was measured. Data are expressed as increase in Smad binding element (SBE) promoter-luciferase dependent expression. Data are expressed as n-fold over control (considered as 1), as the mean ± SEM of 5 experiments. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus Gremlin.

in experimental renal fibrotic diseases, including glomerulosclerosis, tubulointerstitial fibrosis, hypertensive-induced renal damage, and diabetic nephropathy [20–26], as well as in renal tumor progression [27]. In angiotensin II-induced renal damage, renal activation of the Smad pathway was associated to EMT changes [20]. Moreover, Smad 7 overexpression ameliorates renal damage and fibrosis caused by unilateral ureteral obstruction, angiotensin II, and diabetes [22–24, 28]. We have observed that in tubuloepithelial cells Smad 7 overexpression blocked Gremlin-induced EMT changes. The involvement of Smad pathway in Gremlin-mediated fibrosis has been also described in other cell types *in vitro*. In optic nerve head astrocytes and lamina cribrosa cells recombinant Gremlin stimulates ECM production through the activation of TGF-β receptor and Smad 3 phosphorylation, suggesting a role for Gremlin in glaucoma [29]. In healthy dermal fibroblasts IL-6 mediated induction of collagen is dependent on Gremlin production and activation of TGF-β/Smad signalling [30]. Besides the regulation of renal EMT and fibrosis, Gremlin/Smad pathway could also be involved in the onset of proteinuria by modulating podocyte injury and changing the distribution of nephrin and synaptopodin [21].

Recent evidences suggest that Gremlin could be an important promoter of fibrosis in different pathologies, including liver fibrosis, lung diseases, particularly pulmonary hypertension and idiopathic pulmonary fibrosis, and myocardial fibrosis [31–35]. In several human renal diseases Gremlin overexpression was found, mainly in areas of tubule interstitial fibrosis [5–8]. Experimental studies in mice have shown that Gremlin blockade diminished renal fibrosis, as observed in streptozotocin-induced diabetes in knockout mice heterozygous for grem1 [9] and by Gremlin gene silencing [10]. Recent studies have demonstrated direct fibrogenic effect of Gremlin in renal cells. In mesangial cells Gremlin increased cell proliferation and ECM accumulation, via ERK [36]. In renal fibroblasts Gremlin increased ECM production [18], including type I collagen. In tubular epithelial cells Gremlin upregulates profibrotic genes, such as TGF-β and CTGF, and caused EMT changes [18]. Gremlin also induces EMT in airway epithelial cells [37] and in cancer cells [38]. Although the contribution of EMT to renal fibrosis is a matter of intense debate [39, 40], the lost of epithelial properties of the tubular epithelial cells, including permeability and polarity, may result in decreased viability and contribute to renal injury [40, 41]. Therefore, EMT-related changes are an initial step in renal damage and an important potential therapeutic target. Our data demonstrate that Gremlin via Smad pathway regulates EMT, showing a novel mechanism of Gremlin action in renal cells.

TGF-β is known as the major promoter of EMT during embryogenesis, cancer, and fibrosis [13–17]. In a mesothelioma cell line Gremlin-silencing inhibited cell proliferation, associated with downregulation of the transcription factor slug as well as mesenchymal proteins linked to cancer EMT [38]. We have recently demonstrated that Gremlin gene silencing blocked TGF-β-induced EMT in tubular epithelial cells [18]. Now, we have observed that Gremlin increased TGF-β production at 48 hours, and this endogenous autocrine TGF-β acts as a downstream mediator of Gremlin-induced profibrotic and EMT related factors in cultured human tubuloepithelial cells (Figure 8). All these findings reveal the complex relationship between Gremlin and TGF-β in the kidney, disclosing a positive feedback loop connection between them in promoting EMT and fibrosis.

Gremlin exerts a potent inhibitory action via binding to and forming heterodimers with BMP-2, BMP-4, and BMP-7. The binding of Gremlin to selective BMPs prevents ligand-receptor interaction and subsequent downstream signalling. Gremlin acting as a BMPs antagonist plays a critical role during the process of nephrogenesis [4]. BMP-7 is the antagonist of TGF-β1 signalling and has been found to inhibit TGF-β1-induced renal fibrosis by reversing EMT process [42, 43]. In experimental lungs and pulmonary fibrosis upregulation of Gremlin was associated with downregulation of BMP signalling [31, 32]. Gremlin overexpression has been found to inhibit BMP-4 thus leading to enhance TGF-β signalling and ECM deposition in primary open angle glaucoma [44]. However, BMP-independent mechanisms may mediate some actions of Gremlin. Exogenous Gremlin may bind to and act directly on endothelial cells to modulate angiogenesis including endothelial cell migration [45, 46]. We have found that BMPs did no inhibit Gremlin-induced early Smad 3 activation. Thus a receptor-mediated mechanism of action may exist for Gremlin. Therefore, future studies investigating the receptor involved in Gremlin responses in renal cells are needed.

5. Conclusion

Chronic progressive fibrosis of the kidney remains an unsolved challenge. The investigation of the mediators and mechanisms involved in renal fibrosis could lead to better

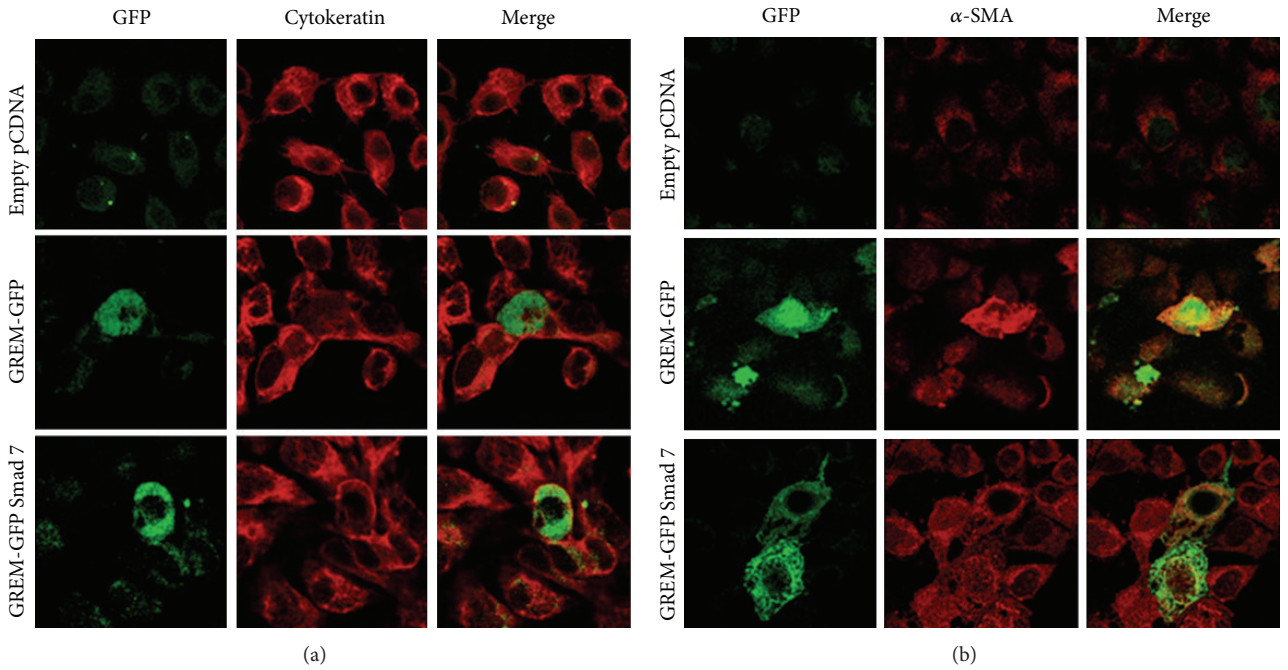

Figure 5: *Gremlin-induced EMT via the Smad pathway*. HK2 cells were transiently transfected with empty, Gremlin (GREM-GFP) alone or cotransfected with Smad 7 expression vectors. EMT markers were evaluated after 48 hours. Gremlin transfected cells express GFP (green staining). Confocal microscopy analysis of cytokeratin and α-SMA immunofluorescence was performed using specific primary antibodies and Alexa-633 secondary IgG (red staining). Representative image out of 3 experiments.

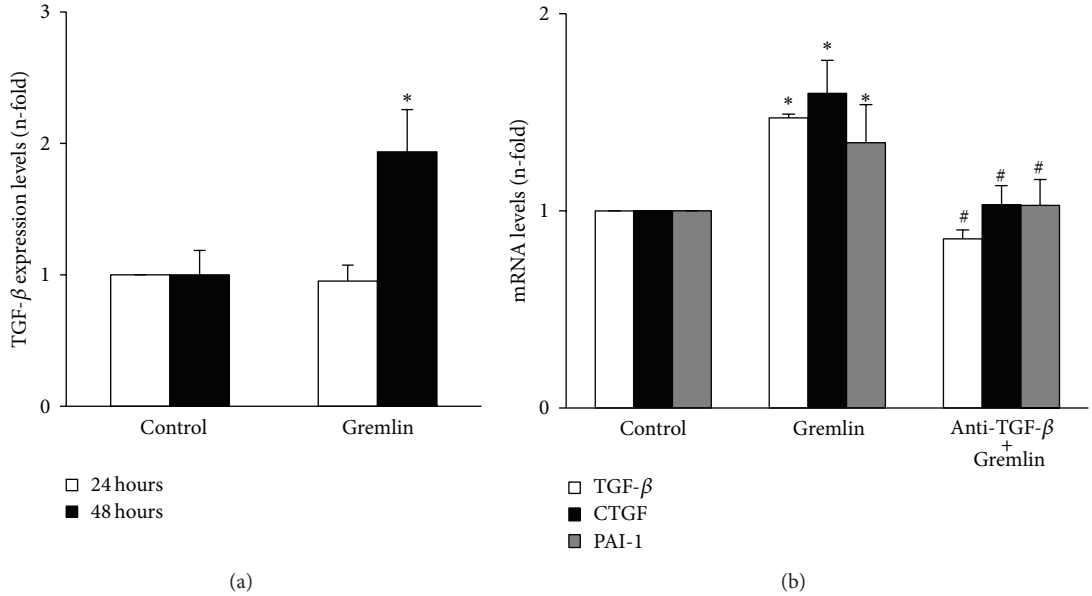

Figure 6: *(a) Gremlin increased TGF-β production*. HK2 cells were stimulated with Gremlin (50 ng/mL) for 24 and 48 hours in serum-free medium. TGF-β1 protein levels were measured in the cell-conditioned medium using a specific ELISA. Data are expressed as mean ± SEM of 6 independent experiments. $^{*}P < 0.05$ versus control. *(b) The late increase in gene expression of profibrotic factors caused by Gremlin is mediated by endogenous TGF-β production*. HK2 cells were stimulated with Gremlin (50 ng/mL) for 24 hours in serum-free medium. TGF-β was blocked or not (control) by pretreatment of cells for 1 hour with an anti-TGF-β neutralizing antibody. Total cell RNA was isolated to assess mRNA levels by real-time PCR. Data are expressed as n-fold over control (considered as 1), as the mean ± SEM of 3 experiments. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus Gremlin.

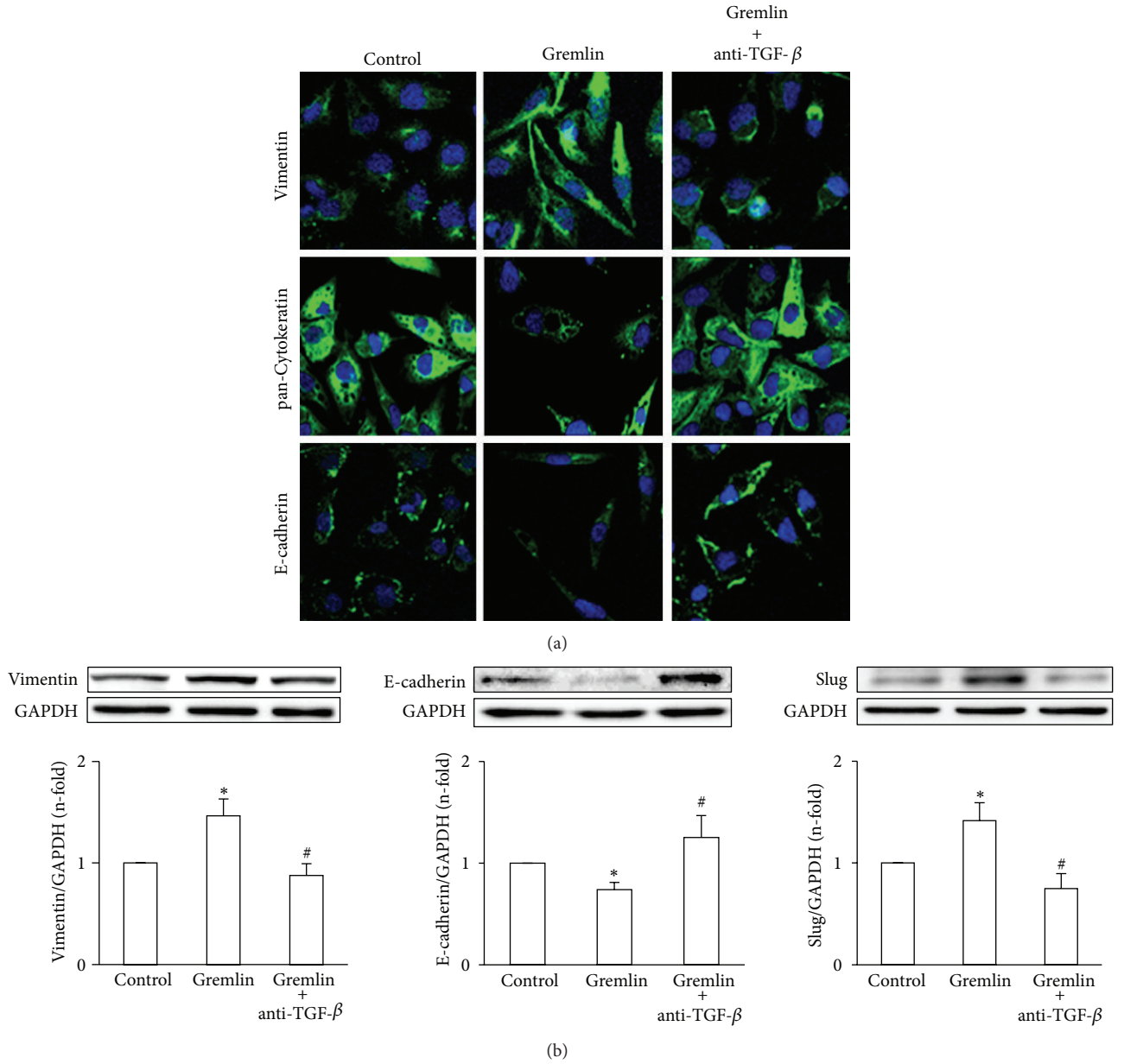

FIGURE 7: *TGF-β is a mediator of EMT-related changes following stimulation with Gremlin*. HK2 cells were stimulated with Gremlin (50 ng/mL) for 48 hours in serum-free medium. TGF-β was blocked or not (control) by pretreatment of cells for 1 hour with an anti-TGF-β neutralizing antibody. (a) EMT changes were evaluated by confocal microscopy. E-cadherin, pan-Cytokeratin, and Vimentin were studied by indirect immunofluorescence using FITC-secondary IgG (green) and confocal microscopy. Nuclei are shown in blue. Figure shows a representative image out of 3 independent observations. (b)Total proteins were isolated and Vimentin, E-cadherin, and Slug levels were analyzed by western blot. Data are expressed as n-fold over control (considered as 1), as the mean ± SEM of 3 experiments. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus Gremlin.

diagnostic tools and novel therapeutics approaches. Many studies have shown that renal expression of Gremlin is induced in diabetic nephropathy and in other progressive renal diseases, associated with tubulointerstitial fibrosis and Smad activation [5–8]. We show here that Gremlin activates the Smad signaling pathway and induces TGF-β and other related factors involved in EMT and fibrotic events in renal cells. All these data suggest that Gremlin could be a potential novel molecular antifibrotic target and biomarker useful for prognostication, disease monitoring, and therapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

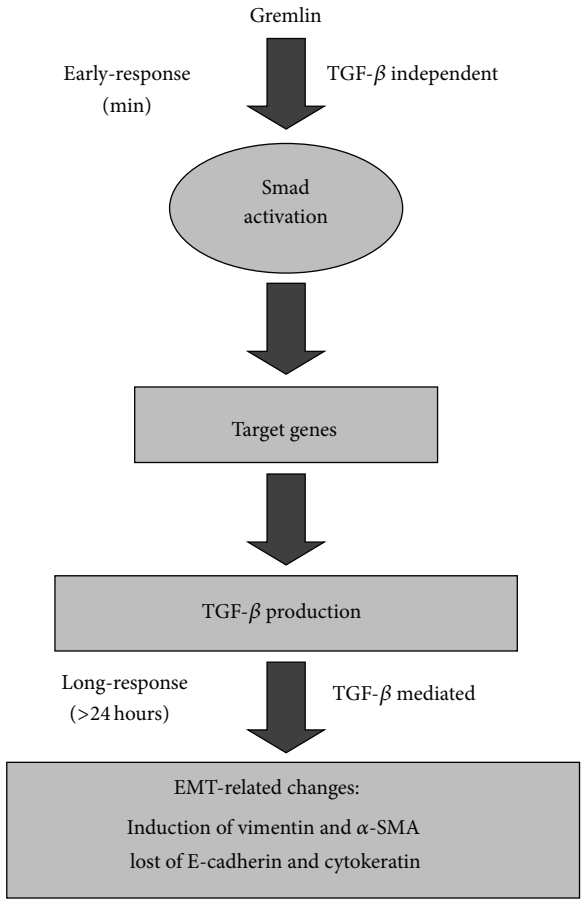

Figure 8: *Dual effects of Gremlin on Smad activation*. Gremlin induces an early (minutes) and direct, TGF-β-independent, Smad pathway activation. After 24 hours Gremlin increased several profibrotic genes, including TGF-β, and after 48 hours increased TGF-β production and induced EMT features. These long-term Gremlin-induced profibrotic events require autocrine TGF-β.

Authors' Contribution

Raquel Rodrigues-Diez and Raúl R. Rodrigues-Diez equally contributed to this work.

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Review Article

Regenerative Medicine for the Kidney: Renotropic Factors, Renal Stem/Progenitor Cells, and Stem Cell Therapy

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The kidney has the capacity for regeneration and repair after a variety of insults. Over the past few decades, factors that promote repair of the injured kidney have been extensively investigated. By using kidney injury animal models, the role of intrinsic and extrinsic growth factors, transcription factors, and extracellular matrix in this process has been examined. The identification of renal stem cells in the adult kidney as well as in the embryonic kidney is an active area of research. Cell populations expressing putative stem cell markers or possessing stem cell properties have been found in the tubules, interstitium, and glomeruli of the normal kidney. Cell therapies with bone marrow-derived hematopoietic stem cells, mesenchymal stem cells, endothelial progenitor cells, and amniotic fluid-derived stem cells have been highly effective for the treatment of acute or chronic renal failure in animals. Embryonic stem cells and induced pluripotent stem cells are also utilized for the construction of artificial kidneys or renal components. In this review, we highlight the advances in regenerative medicine for the kidney from the perspective of renotropic factors, renal stem/progenitor cells, and stem cell therapies and discuss the issues to be solved to realize regenerative therapy for kidney diseases in humans.

1. Introduction

The kidney is indispensable for tissue homeostasis as well as regeneration. Renal tubular epithelium composed of polarized mature cells has the capacity to regenerate following acute kidney injury. After the insult occurs, these cells rapidly lose their brush border and dedifferentiate into a more mesenchymal phenotype. The dedifferentiated cells migrate into the regions where cell necrosis, apoptosis, or detachment has resulted in denudation of the tubular basement membrane. They proliferate and eventually redifferentiate into an epithelial phenotype, completing the repair process [1]. Recent studies suggest that renal stem/progenitor system is present in the tubules, interstitium, and glomeruli of the adult kidney and functions as the main drivers of kidney regenerative responses after injury. Understanding the mechanisms that drive renal progenitor growth and differentiation represents the key step for modulating this potential for therapeutic purposes [2]. However, renal fibrosis, the inevitable consequence of an excessive accumulation of extracellular matrix, is irreversible. Patients with chronic renal disease show a progressive decline in renal function with time, finally leading to end-stage renal failure that requires lifelong dialysis or renal transplantation. Many therapeutic interventions seem to be effective in animal models of acute or chronic kidney injury. Nonetheless, it is still difficult to translate these promising results into humans in the clinical setting. As a new therapeutic option, regenerative therapies for the kidney have been extensively investigated from the aspect of stem cell biology, developmental biology, and tissue engineering. The four major strategies of regenerative medicine for the kidney are as follows: (1) identification of renotropic factors; (2) identification of renal stem/progenitor cells in embryonic kidney or adult kidney; (3) cell therapies with bone marrow-derived cells (BMDCs), namely, hematopoietic stem cells (HSCs) or mesenchymal stem cells (MSCs), endothelial

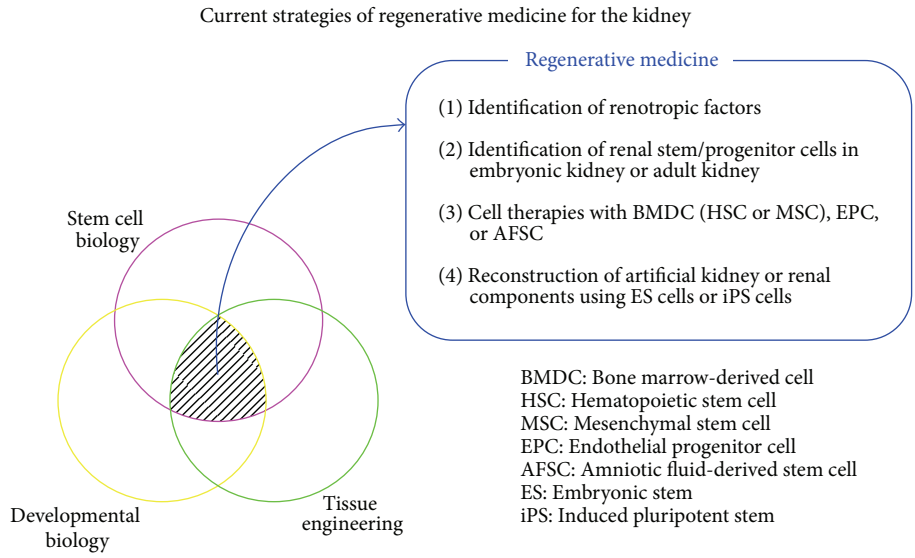

Figure 1

progenitor cells, and amniotic fluid stem cells; and (4) reconstruction of artificial kidney or renal components by using embryonic stem (ES) cells or induced pluripotent stem (iPS) cells (Figure 1). In this review, we highlight the recent advances of regenerative medicine for the kidney from the perspective of renotropic factors, renal stem/progenitor cells, and stem cell therapies and clarify the issues to be solved for the establishment of regenerative therapy.

2. Renotropic Factors

The regeneration process resembles the developmental paradigm. The remodeling and maturation of restored epithelium after renal injury have many parallels with the growth and maturation that occur during kidney organogenesis. Soluble factors involved in kidney development have been identified by gene targeting techniques, in vitro tubulogenesis models, and organ culture systems. By using animal kidney injury models, most of these factors also have been proved to regulate kidney recovery as potential renotropic factors. These factors include hepatocyte growth factor (HGF) [3], epidermal growth factor [4], insulin-like growth factor-I (IGF-I) [5, 6], heparin-binding EGF-like growth factor (HB-EGF) [7, 8], platelet-derived growth factor (PDGF) [9], bone morphogenetic protein-7 (BMP-7) [10, 11], and uterine sensitization-associated gene-1 (USAG1), a novel BMP antagonist [12]. Recently, the essential role of their receptors in kidney injury also has been demonstrated. Mice with a specific EGF receptor deletion in renal proximal tubules showed the importance of EGF receptor activation in the recovery phase after acute kidney injury [13]. Conditional knockout mice lacking the HGF receptor, *c-met*, specifically in renal tubules demonstrated the antiapoptotic or anti-inflammatory role of *c-met* signaling in renal protection after acute kidney injury [14]. Deletion of the BMP receptor activin-like kinase 3 (Alk3) in the tubular epithelium enhances TGF-beta signaling, epithelial damage, and fibrosis [15].

A negative regulator of kidney repair has also been identified. Data from transgenic mice expressing truncated activin type II receptor [16], an in vitro tubulogenesis model [17], the Wolffian duct culture [18–21], and isolated rat embryonic kidney culture [20] indicate that activin A is an endogenous inhibitor of renal organogenesis [22, 23]. Additionally, activin A is a potent inhibitor of renal regeneration after injury [24].

Key regulatory molecules required for renal organogenesis are reactivated in regenerating tubular cells after ischemic injury. These factors include Pax-2 [25–27], leukemia inhibitory factor [28], and Wnt4 [29].

Although many renotropic factors or signaling pathways have been identified, the mechanism by which these growth factors mediate recovery from renal injury is not totally understood. Most of these factors are epithelial cell mitogens in vitro, and they induce tubular cell proliferation after injury when exogenously administered. However, it remains unknown if these factors are involved in cell maturation, restoration of polarity, modulation of renal blood flow, and neutrophil infiltration. It is of great interest to examine if these renotropic factors promote renal regeneration via the activation of intrinsic renal stem cells. Recently, a critical role of peritubular capillary endothelium as a source of factors required for tubular recovery after injury has been reported [30]. Mechanisms of cell-cell interactions such as tubular epithelium and peritubular capillary endothelium or interstitial fibroblasts need to be clarified.

3. Renal Stem/Progenitor Cells

Despite the structural complexity of the adult kidney, attempts to identify adult kidney stem cells have been made based on the broad principles of stem cell biology, such as prolonged cell-cycling time (label-retaining cells), Hoechst

dye extrusion (side population cells), by growth in restrictive cell culture conditions, or expression of markers for other tissue stem cells or embryonic kidney.

3.1. Identification of Renal Stem/Progenitor Cells Based on Cell Behavior. Stem cells are considered to have an inexhaustible capacity for self-renewal and differentiation to ensure the lifelong maintenance of tissue homeostasis. To conserve growth potential and prevent genetic injury during mitosis, stem cells cycle slowly and are recruited only as demanded by tissue turnover.

One of the most common methods to identify stem cells is to search for slow-cycling cells by labeling their DNA with 5-bromo-2-deoxyuridine (BrdU). A pulse of BrdU labeling followed by a chase period allows the detection of slow-cycling label-retaining cells (LRCs), which represent the stem cell compartment. LRCs were identified in renal tubules of normal rat kidneys, and regenerating cells during tubular repair were essentially derived from LRCs [31]. Interestingly, tubular LRCs were involved in the epithelial to mesenchymal transition during renal fibrosis [32]. In vitro characterization revealed that LRCs are a multipotent cell population with tubulogenic capacity [33]. The number of these LRCs declines with age, leading to reduced regenerative capacity after injury in the aging kidney [34]. Other groups also found LRCs in tubules [35, 36], papilla [37], or renal capsules [38]. The location, properties, and behavior of LRCs after injury differ or remain controversial. This lack of consistency is probably due to differences in the timing or duration of the pulse and the length of the chase. Every tubular cell shares the capacity to retain BrdU and proliferate after injury. Nonetheless, the patterns of growth and differentiation of LRCs should be clarified in detail, because factors that can activate LRCs may possess renoprotective effects.

The ability of hematopoietic stem cells to efflux dyes such as Hoechst 33342 and Rhodamine 123 has been used as the basis of a single-step HSC isolation protocol [39]. These side population cells with the same efflux profile were found in the adult rodent kidney. Renal side population cells possess multilineage capacity [40, 41], and the introduction of side population cells into a model of acute experimental renal damage was therapeutically beneficial [40, 41]. In contrast, renal side population cells have no capacity to transdifferentiate into renal cells in vivo [42]. These data remain contradictory in terms of the relative size, origin, and lineage capacity of the renal side population cells. The definition of a marker phenotype that allows isolation without the assessment of dye efflux will be needed.

3.2. Identification of Renal Stem/Progenitor Cells Based on Specific Marker Expression. A subset of parietal epithelial cells localized to the urinary pole of Bowman's capsule was identified in human adult kidneys based on coexpression of CD24 and CD133, which are both used as markers of adult tissue stem cells. These cells exhibited multidifferentiation potential and long-term proliferative capacity in vitro. Injection of CD24/CD133 double-positive cells into mice with acute renal failure induced a complete recovery of renal function and restoration of tubular structures [43]. CD24/CD133 double-positive cells with stem cell properties were also found in embryonic kidney [44] as well as in proximal tubules [45]. Their proliferation rate and differentiation capacity into renal epithelial cells seem to be regulated by Toll-like receptor 2 [46, 47]. It is unknown whether these cells elicit repair via functional integration or humoral induction when delivered into the recipient animal. Glomerular hyperplastic lesions have been shown to be derived from the proliferation of CD133/CD24 double-positive cells [48].

CD133 is mainly known as a marker of HSC and endothelial progenitors [49], but recent reports indicate its expression in adult tissue stem cells. A rare population of CD133-positive cells was found in the interstitium, glomeruli, and tubules. When injected into mice with glycerol-induced acute renal injury, CD133-positive cells homed to the kidney and integrated into proximal and distal tubules during the repair process [50].

A nontubular multipotent stem/progenitor cell population was isolated from the adult mouse kidney and characterized as Sca-1 positive [51]. These cells were capable of differentiation into myogenic, adipogenic, and neural lineages. When injected directly into the renal parenchyma after ischemic injury, renal Sca-1-positive cells adopt a tubular phenotype and potentially could contribute to kidney repair.

3.3. Identification of Renal Stem/Progenitor Cells Based on Selective Culture Conditions. A unique population of cells that show self-renewal for more than 200 population doublings without evidence of senescence was isolated from rat kidneys. These cells express endothelial, hepatocyte, and neural markers, suggesting the plasticity of these cells. When injected intra-arterially after renal ischemia, these cells differentiate into renal tubules [52]. Screening of stem cell potential in nephron segments revealed that a cell line derived from the S3 segment of the proximal tubules could be maintained for a long term without transformation and replaced partly in injured tubules when engrafted to the kidney after renal ischemia [53]. A rare population of cells expressing several stem cell markers was selectively identified in the interstitium of the medulla. Intrarenal injection of this population into mice with ischemic injury repaired renal damage [54].

4. Stem Cell Therapy

BMDCs have a surprising degree of plasticity and differentiate into cell types of multiple organs of the body [55, 56]. Bone marrow (BM) transplantation is commonly used to study BM cell plasticity. The host BM is replaced by donor BM, and after BM chimerism is established, donor cells are tracked in the target tissues. The donor BMDCs are distinguished from host cells by virtue of their chromosome content (male Y chromosome-positive cells in a female host), the expression of a reporter molecule (beta-galactosidase, luciferase, and enhanced GFP), or the performance of a function (reestablishment of a function in a knockout mouse model). BMDCs have the ability to move to distant sites within the body. As in most organs, BMDCs appear in the kidney in response to

renal injury. BMDCs can transdifferentiate into renal tubular epithelial cells [57–59], mesangial cells [60–63], glomerular endothelial cells [64, 65], and even podocytes [66, 67]. Based on these data, cell therapy with BMDCs (HSCs and MSCs) has been extensively examined and reported to be effective. In light of their ease of accessibility, BMDCs are strong candidates for the cell source in stem cell therapy.

4.1. HSCs. HSCs are undifferentiated cells capable of self-renewal and stepwise differentiation into fully specialized cells of the blood such as erythrocytes, thrombocytes, and leukocytes. BMDCs significantly contribute to the regeneration of the renal tubular epithelium, differentiate into renal tubules [57–59], or promote proliferation of both endothelial and epithelial cells after injury [68]. These data suggest that the enhancements of the mobilization, propagation, and delivery of BMDCs to the kidney hold potential as entirely new approaches for the treatment of acute kidney injury. Stem cell factor and granulocyte colony-stimulating factor (G-CSF) induced HSC homing to the injured kidney, leading to the significant enhancement of the functional recovery of the kidney [69, 70]. In contrast, data against the use of granulocytosis-inducing HSC mobilization protocols for the treatment of ischemic injury was also reported. Unlike the reports above, the boosting of peripheral stem cell numbers was associated with increased severity of renal failure and mortality. High numbers of activated granulocytes seem to obscure the potential renoprotective effects of HSC [71]. There are several reports against the potential of BMDCs to transdifferentiate into tubular cells after injury [72]. Based on the data from transgenic mice that express GFP in BMDCs [73], in mature renal tubular epithelial cells [74], or in all mesenchyme-derived renal epithelial cells [75], it was suggested that, while BMDC recruitment occurs, kidney repair is predominantly elicited via proliferation of endogenous renal cells. BMDCs might contribute to the regenerative process by producing protective and regenerative factors, rather than by differentiating to directly replace damaged cells [75].

The contradictory results in the localization of BMDCs and the degree of the BMDC contribution to kidney regeneration after injury may be due to methodological limitations in tracking BMDCs, particularly in injured tissues. There are differences in the protocols used in these studies (species, type of injury, transplantation methods, type of cells used for transplantation, and specificity and sensitivity of the detection methods for BM cell origin). Cell fusion may also explain this discrepancy [76, 77]. In the reports of bone marrow recruitment to damaged kidneys, the lineage of the recruited BMDCs has not been established. Whether the recruitment of BMDCs has a beneficial effect on chronic renal damage remains unsolved.

4.2. MSCs. The other possible candidate for the BM cell responsible for ameliorating renal damage is the MSC. MSCs are undifferentiated adult stem cells of mesodermal origin that have the capacity to differentiate into a range of mesenchymal tissue types, including cartilage, bone, muscle, stroma, fat, tendon, and other connective tissues. MSCs represent a very small fraction of BM cells, but they can be isolated and expanded with high efficiency in culture as plastic adherent cells.

The therapeutic effect of MSC delivery has been demonstrated in animal models of renal damage [78] such as acute kidney injury induced by cisplatin [79, 80], gentamicin [81], intramuscular injection of glycerol [82], or ischemia [83], Adriamycin-induced nephrotic syndrome [84], mesangioproliferative anti-Thy1.1 glomerulonephritis [85], a mouse model of Alport disease [86], glomerular injured athymic mice [87], a rat remnant kidney [88], and a rat kidney transplantation model of chronic allograft nephropathy [89]. Beneficial effects of MSC are primarily mediated via paracrine factors [90] such as VEGF, HGF, IGF-I [91–93], and erythropoietin [94]. Heme oxygenase-1 (HO-1) [95], the SDF-1-CXCR4/CXCR7 axis [96], and CD44/hyaluronic acid interactions [97] play an important role in MSC-mediated protection. On the other hand, maldifferentiation of intraglomerular MSC into adipocytes accompanied by glomerular sclerosis was observed [98].

Adipose tissue-derived stem cells are an attractive source of stem cells with regenerative properties that are similar to those of BMDCs. Adipose tissue-derived stem cell therapy minimized kidney damage or improved renal dysfunction after renal damages such as ischemic injury [99], a mouse progressive renal fibrosis model [100], acute kidney injury induced by cisplatin [101] or folic acid [102], atherosclerotic renal artery stenosis in pigs [103, 104] or swine [105], and a rat antiglomerular basement membrane disease [106]. Beneficial effects of kidney-derived MSCs [107], allogeneic fetal membrane-derived MSCs [108], and human embryonic MSCs [109, 110] against renal damage have been reported.

4.3. Endothelial Progenitor Cells (EPCs). EPCs participate in the repair of tissues, including the kidney, under diverse physiological and pathological conditions. Renal ischemia rapidly mobilizes EPCs, and transplantation of EPC-enriched cells from the medullopapillary parenchyma provided partial renoprotection after renal ischemia [111]. Acute but not chronic elevation of uric acid acts as an endogenous mediator of EPC mobilization and renoprotection [112]. In a chronic renal artery stenosis model, a single intrarenal infusion of autologous EPCs preserved microvascular architecture and decreased microvascular remodeling by preserving hemodynamics [113]. Manipulation of homing signals may potentially allow therapeutic opportunities to increase endogenous EPC recruitment [114].

4.4. ES Cells. ES cells with unlimited developmental potential have been induced to differentiate in vitro into a broad spectrum of specialized cell types and are regarded as new tools for the elucidation of disease mechanisms. The generation of ES cell-derived progenitors offers the potential for regenerative therapies. Although kidney structures are complex, differentiation of ES cells into renal epithelial cell lineages has been successfully demonstrated [115–118]. Recently, it was reported that decellularization of intact rat kidneys in a manner that preserved the intricate architecture

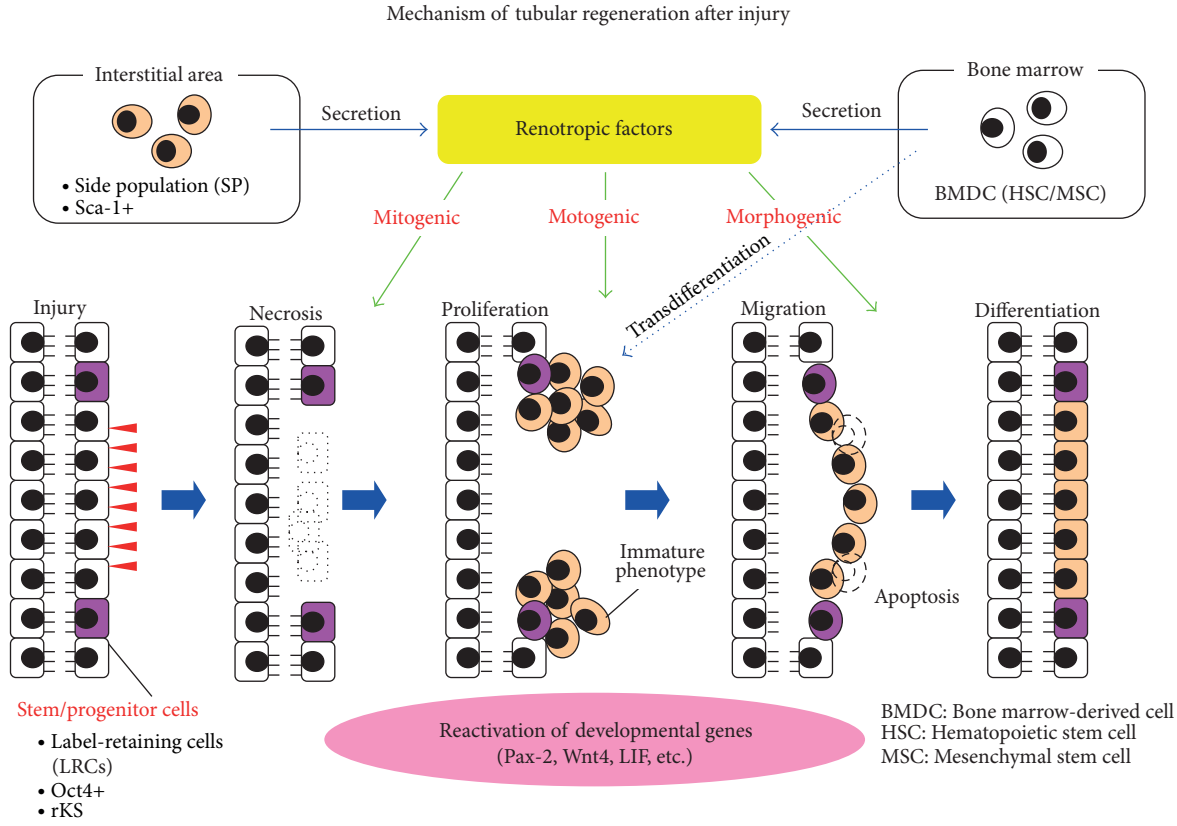

Figure 2

allowed seeded ES cells to populate and proliferate within the glomerular, vascular, and tubular structures [119].

4.5. iPS Cells. Forced expression of selected transcription factors can transform somatic cells into ES cell-like cells, termed iPS cells [120], which have the potential for multilineage differentiation and provide a resource for stem cell-based treatment. Recently, unique methods for stimulating the differentiation of human iPS cells into kidney lineages [121–123] or three-dimensional structures of the kidney [124] have been developed.

iPS cells from normal human mesangial cells [125], renal tubular cells present in urine [126, 127], and fibroblasts of patients with autosomal dominant polycystic kidney disease [128] have been established. Reprogrammed kidney iPS cells may aid the study of genetic kidney diseases and lead to the development of novel therapies.

The therapeutic effect of iPS cells on renal ischemia was also reported. Transplantation of iPS cells reduced the expression of oxidative substances, proinflammatory cytokines, and apoptotic factors and eventually improved survival in rats with ischemic acute kidney injury [129].

4.6. Human Amniotic Fluid Stem Cells. Human amniotic fluid stem cells, a novel class of broadly multipotent stem cells that exhibit characteristics of both embryonic and adult stem cells, have been regarded as a promising candidate for stem cell therapy [130]. Beneficial therapeutic effects of amniotic fluid stem cells have been shown in kidney injury models including acute kidney injury induced by glycerol [131, 132] or cisplatin [133], a mouse model of Alport syndrome [134], and a mouse unilateral ureteral obstruction (UUO) model [135].

5. Conclusion

In this review, the role of renotropic factors and intra- or extrarenal stem cells in kidney regeneration after injury is summarized (Figure 2). Compared to other organs, data regarding renal stem/progenitor cells remain at a preliminary stage. The precise location, size of the pool, and cellular morphology are either unknown or controversial.

The delivery of soluble factors with the potential to improve the ability of the tissue to repair itself is the most pharmacologically attractive strategy for organ repair in situ. In this regard, clarification of the factors or the signaling pathways that enhance the regenerative capacity of stem/progenitor cells will lead to a better understanding of the mechanisms of kidney regeneration, as well as to the identification of novel therapeutic strategies to facilitate renal repair after acute kidney injury in humans. Considering the recapitulation of the developmental process in kidney regeneration, such factors may be produced by the embryonic kidney. Understanding the molecular basis of kidney development will help us to develop regenerative therapies for kidney diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Follistatin, an Activin Antagonist, Ameliorates Renal Interstitial Fibrosis in a Rat Model of Unilateral Ureteral Obstruction

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Activin, a member of the TGF-β superfamily, regulates cell growth and differentiation in various cell types. Activin A acts as a negative regulator of renal development as well as tubular regeneration after renal injury. However, it remains unknown whether activin A is involved in renal fibrosis. To clarify this issue, we utilized a rat model of unilateral ureteral obstruction (UUO). The expression of activin A was significantly increased in the UUO kidneys compared to that in contralateral kidneys. Activin A was detected in glomerular mesangial cells and interstitial fibroblasts in normal kidneys. In UUO kidneys, activin A was abundantly expressed by interstitial α-SMA-positive myofibroblasts. Administration of recombinant follistatin, an activin antagonist, reduced the fibrotic area in the UUO kidneys. The number of proliferating cells in the interstitium, but not in the tubules, was significantly lower in the follistatin-treated kidneys. Expression of α-SMA, deposition of type I collagen and fibronectin, and CD68-positive macrophage infiltration were significantly suppressed in the follistatin-treated kidneys. These data suggest that activin A produced by interstitial fibroblasts acts as a potent profibrotic factor during renal fibrosis. Blockade of activin A action may be a novel approach for the prevention of renal fibrosis progression.

1. Introduction

Renal interstitial fibrosis is a common feature in various kidney diseases and correlates with renal dysfunction. The histological characteristics of renal fibrosis are excessive deposition of extracellular matrix (ECM) and accumulation of interstitial fibroblasts that proliferate, differentiate into myofibroblasts, and actively synthesize ECM [1]. During renal fibrosis, tubular epithelial cells were considered to transdifferentiate into interstitial fibroblasts via epithelial to mesenchymal transition [2, 3]. Transforming growth factor-beta 1 (TGF-β1), which shows enhanced expression in human fibrotic kidneys and in animal models of renal fibrosis, promotes renal fibrosis through the activation of interstitial fibroblasts and acts as a potent inducer of EMT [3, 4]. Blockade of TGF-β1 signals has been shown to ameliorate renal interstitial fibrosis in several experimental models [5]. However, the factors that contribute to renal fibrosis have not been fully identified.

Activin A, a member of the TGF-β superfamily, is a dimeric protein composed of two βA subunits and modulates cell growth and differentiation in various tissues. Activin exerts its biological effects by interacting with two types of transmembrane receptors (type I and type II) with intrinsic serine/threonine kinase activity [6]. A key regulatory factor that modulates activin A action is follistatin. Follistatin binds to activin A with high affinity and blocks its action [7]. Follistatin is synthesized in the target cells of activin A

and remains in the extracellular matrix [8], while activin A is trapped by follistatin, internalized by endocytosis, and subsequently degraded by proteolysis [9].

Activin A acts as a negative regulator of renal organogenesis [10]. In the embryonic kidney, activin A suppresses branching of the ureteric bud and induces cell differentiation in the metanephric mesenchyme. Activin A is an endogenous inhibitor of ureteric bud formation from the Wolffian duct. Cancellation of the autocrine action of activin A may be critical for the initiation of this process. Transgenic mice expressing mutant activin receptor had an increased number of glomeruli in the kidney. Activin A inhibited three-dimensional tubular formation in an in vitro tubulogenesis model using MDCK cells. Activin A is also involved in the recovery process of the kidney after injury [11]. Expression of activin A was undetectable in normal kidney but was upregulated in tubular cells of the kidneys after renal ischemia. Blockade of activin action by follistatin promoted tubular recovery after injury, thus suggesting that activin A is an endogenous inhibitor of tubular regeneration after injury.

Similarly to TGF-β, activin signaling is mediated by Smad2 and Smad3 [6]. Mice lacking Smad3 are protected against tubulointerstitial fibrosis following unilateral ureteral obstruction (UUO) by blocking of EMT and abrogation of monocyte influx and collagen accumulation [12], which suggests the involvement of activin signaling pathway in renal fibrosis. The present study demonstrated that the expression of activin A was significantly upregulated in the UUO kidneys and that recombinant follistatin prevented renal fibrosis in vivo. Blockade of activin action may therefore be a new strategy for the prevention of renal fibrosis progression.

2. Materials and Methods

2.1. Reagents. Recombinant human follistatin was provided by Dr. Y. Eto (Central Research Laboratory, Ajinomoto, Kawasaki, Japan). Antibodies used in this study were as follows: goat anti-type I collagen antibody (1 : 100), goat anti-type III collagen antibody (1 : 100) (Southern BioTech, Birmingham, AL), mouse anti-α-SMA antibody (1 : 100) (Sigma, St. Louis, MO), mouse anti-CD68 antibody (1 : 100) (Abcam, Cambridge, UK), rabbit anti-inhibin βA antibody (1 : 100) (Thermo Fisher Scientific, Yokohama, Japan), rabbit anti-fibronectin antibody (1 : 100), goat anti-vimentin antibody (1 : 100) (Santa Cruz biotechnology, Inc., CA), rabbit anti-CD3 antibody (1 : 100) (Vector Labs, Burlingame, CA), Alexa Fluor 488 goat anti-mouse IgG (1 : 2000), and Alexa Fluor 488 goat anti-rabbit IgG (1 : 2000) (Invitrogen, Carlsbad, CA).

2.2. Experimental Protocol. Male Wistar rats (200 g) were purchased from Nihon SLC Inc. (Hamamatsu, Japan). UUO was performed as described previously [13]. Briefly, after induction of general anesthesia by intraperitoneal injection of pentobarbital (50 mg/kg body wt), the abdominal cavity was exposed via a midline incision and the left ureter was ligated at three points with 4-0 silk. Recombinant human follistatin (1 μg) or saline was administered intraperitoneally into rats at 1, 3, 5, and 7 days after UUO. At the indicated times after UUO, rats were sacrificed and the kidneys were removed for RNA extraction or histologic examination. UUO was confirmed by observation of dilation of the pelvis and proximal ureter and collapse of the distal ureter. Sham-operated kidneys without ligation were used as controls. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Gunma University.

2.3. Histological Examination. Kidneys were fixed in 10% formaldehyde and were embedded in paraffin. Sections (4 μm) were stained with periodic acid-Schiff (PAS) and Masson-trichrome (MT). MT-stained sections were microscopically examined and the changes observed were limited to the outer medulla, where fibrotic change is most obvious. For semiquantitative analysis, renal interstitial fibrosis was graded as follows: 0, 0%; 1, 0% to 25%; 2, 25% to 50%; 3, 50% to 75%; 4, 75% to 100% of involvement of microscopic field at ×400 magnification. Five sections from five rats (a total of 25 sections) were used for each condition. Data are expressed as mean ± SE ($n = 5$).

2.4. Cell Proliferation. Cell proliferation was assessed by in vivo DNA labeling with bromodeoxyuridine (BrdU), an analogue of thymidine. BrdU (100 mg/kg), which is incorporated into DNA during S phase of the cell cycle, was injected intraperitoneally into rats at 1 h before sacrifice. Kidneys were removed, fixed with formaldehyde, and embedded into paraffin. Sections were deparaffinized with xylene, rehydrated with graded ethanol solutions (100, 100, 90, 70, and 50%) for 10 min each, and washed twice with distilled water. BrdU-positive cells were detected using a Cell Proliferation Kit (Amersham Biosciences Corp., Piscataway, NJ), in accordance with the manufacturer's instructions.

Quantitative analysis of BrdU-positive cells was performed by counting the number of BrdU-positive cells in tubules and interstitium separately in 10 randomly selected fields at ×400 magnification.

2.5. Reverse-Transcription PCR (RT-PCR). Whole kidneys were suspended in TRI reagent (Molecular Research Center Inc., Cincinnati, OH) and homogenized. Total RNA was extracted, and first-strand cDNA was prepared by reverse transcription with the Omniscript RT Kit (Qiagen Inc., Valencia, CA) using Oligo (dT) primer (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Reverse-transcription PCR (RT-PCR) was performed as described previously [13]. Sequences of primers used in this study were as follows: βA subunit (sense, 5′-GGACCTAACTCTCAGCCAGAGATG-3′; antisense, 5′-TCTCAAAATGCAGTGTCTTCCTGG-3′), activin receptor type I (sense, 5′-AGTCGTGGTTCAGGGAGACA-3′; antisense, 5′-GAGTGGTGAGCTGAAGGTAG-3′), activin receptor type II (sense, 5′-TGTGAAATGAGTAGGGTGCC-3′; antisense, 5′-CCTTCATATCCGTGTTGCAG-3′), follistatin (sense, 5′-AAAACCTACCGCAACGAATG-3′; antisense, 5′-AGGCATTATTGGTCTGATCC-3′), and GAPDH

(sense, 5′-CTACCCACGGCAAGTTCAAT-3′; antisense, 5′-TACTCAGCACCAGCATCACC-3′). Quantitative real-time PCR was performed as described previously [14].

2.6. Immunohistochemical Analysis. Immunostaining with the avidin-biotin coupling immunoperoxidase technique was performed using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) in accordance with the manufacturer's instructions. Briefly, sections were deparaffinized and rehydrated using standard methods. After inactivation of endogenous peroxidase with 1% metaperiodic acid in phosphate-buffered saline (PBS) for 10 min at room temperature, sections were preincubated with 3% BSA-PBS for 1 h. Sections were then incubated with primary antibody for 2 h, washed with PBS, and reacted with a biotinylated secondary antibody for 1 h. After washing with PBS, sections were reacted with Vectastain Elite ABC reagent. Antibody was detected with diaminobenzidine tetrahydrochloride in PBS, and sections were counterstained with hematoxylin. For immunohistochemical controls, primary antibody was replaced with 3% BSA-PBS, which did not exhibit positive staining, thus confirming specificity.

Indirect immunofluorescence staining was performed as described previously [14]. Briefly, frozen sections were washed in PBS, pretreated with 3% BSA-PBS for 1 h, and covered with primary antibody at room temperature for 1 h. After washing in PBS, sections were covered with a mixture of a fluorescence-labeled secondary antibodies and 4′-diamidino-2-phenylindole (DAPI). Immunofluorescence images were recorded with a Spot RT Slider digital camera attached to a Nikon Eclipse 80i fluorescence microscope.

Quantification of the positive area for α-SMA, type I collagen, type III collagen, and fibronectin was calculated by image analysis using Image J software (NIH, Bethesda, MD, USA). The mean value of the positive area was obtained by evaluating 10 randomly selected fields at ×200 magnification per kidney.

2.7. Statistical Analysis. Differences in means between groups were compared by Student's t-test, and P values of <0.05 were considered to be significant.

3. Results

3.1. Expression of Activin A, Activin Receptors, and Follistatin in Kidneys after UUO. We first examined the expression of the βA subunit for activin A, activin receptors, and follistatin in UUO kidneys by RT-PCR. Expression of βA subunit mRNA was undetectable in normal, sham-operated (data not shown), and contralateral kidneys (Figure 1(a)). In contrast, βA subunit expression was observed in the UUO kidneys at 1 day and thereafter. Activin signals are known to be mediated through two types of activin receptors, type I (ActRI) and type II (ActRII) [6]. Consistent with previous data showing that both of these activin receptors are localized in the tubular cells of kidneys [15], expression of both ActRI and ActRII is detectable in normal, sham-operated, contralateral, and UUO kidneys. Expression of follistatin, an activin antagonist, was also present in normal, sham-operated, contralateral, and UUO kidneys. Quantitative real-time PCR confirmed that the expression levels of βA subunit mRNA were significantly elevated in UUO kidneys, as compared to those in normal or contralateral kidneys (Figure 1(b)). There were no significant differences in the expression levels of activin receptors or follistatin among normal, sham-operated, contralateral, and UUO kidneys (data not shown).

We also investigated the localization of activin A in the UUO kidney by immunostaining. Immunoreactive activin A was detected in vimentin-positive glomerular mesangial cells in normal (Figure 1(c), upper panels), sham-operated, and contralateral kidneys (data not shown). Vimentin-positive interstitial fibroblasts also produce activin A in normal kidneys (Figure 1(c), middle panels). The expression of α-SMA was undetectable in normal kidneys (Figure 1(c), bottom panels) except in vascular smooth muscle cells (data not shown). In contrast, numerous α-SMA-positive cells were observed in the interstitium of UUO kidneys (Figure 1(d)). In UUO kidneys, activin A was colocalized with interstitial vimentin-positive fibroblasts or α-SMA-positive myofibroblasts, but not with CD3-positive T lymphocytes or CD68-positive macrophages (Figure 1(d)). These results suggest that interstitial fibroblasts are the activin-producing cells in the kidney after UUO.

3.2. Effects of Follistatin on Fibrotic Change of the Kidneys after UUO. In order to examine the role of endogenous activin A in this model, we administered recombinant human follistatin into the UUO-operated rats and assessed the effects of activin blockade on histological changes of the kidneys after UUO (Figure 2). PAS staining demonstrated normal architecture in normal, sham-operated (data not shown), and contralateral kidneys (Figure 2(a), panels (A) and (D)). Tubular dilation and atrophy were observed in the saline-treated UUO kidneys (Figure 2(a), panels (B) and (E)). In the follistatin-treated UUO kidneys, the renal parenchyma was markedly preserved (Figure 2(a), panels (C) and (F)). Masson-trichrome staining revealed the presence of interstitial fibrotic changes in the saline-treated UUO kidneys (Figure 2(b), panels (A) and (C)), but not in normal or contralateral kidneys (data not shown). The interstitial fibrotic area in the follistatin-treated kidneys was slightly reduced when compared to that in the saline-treated kidneys (Figure 2(b), panels (B) and (D)). Semiquantitative analysis showed that the fibrotic score of follistatin-treated kidneys was significantly lower than that of saline-treated kidneys (Figure 2(c)).

3.3. Effects of Follistatin on Cell Proliferation in UUO Kidneys. Cell proliferation was assessed by BrdU incorporation (Figure 3). BrdU-positive cells were rarely observed in normal (data not shown) or contralateral kidneys (Figure 3(a), panel (A)). In contrast, a large number of BrdU-positive cells were observed in UUO kidneys on day 3 (Figure 3(a), panel (B)). Most BrdU-positive cells were localized in tubular cells (Figure 3(a), panel (C)) and some were present in the interstitium of UUO kidneys (Figure 3(a), panel (D)). Quantitative

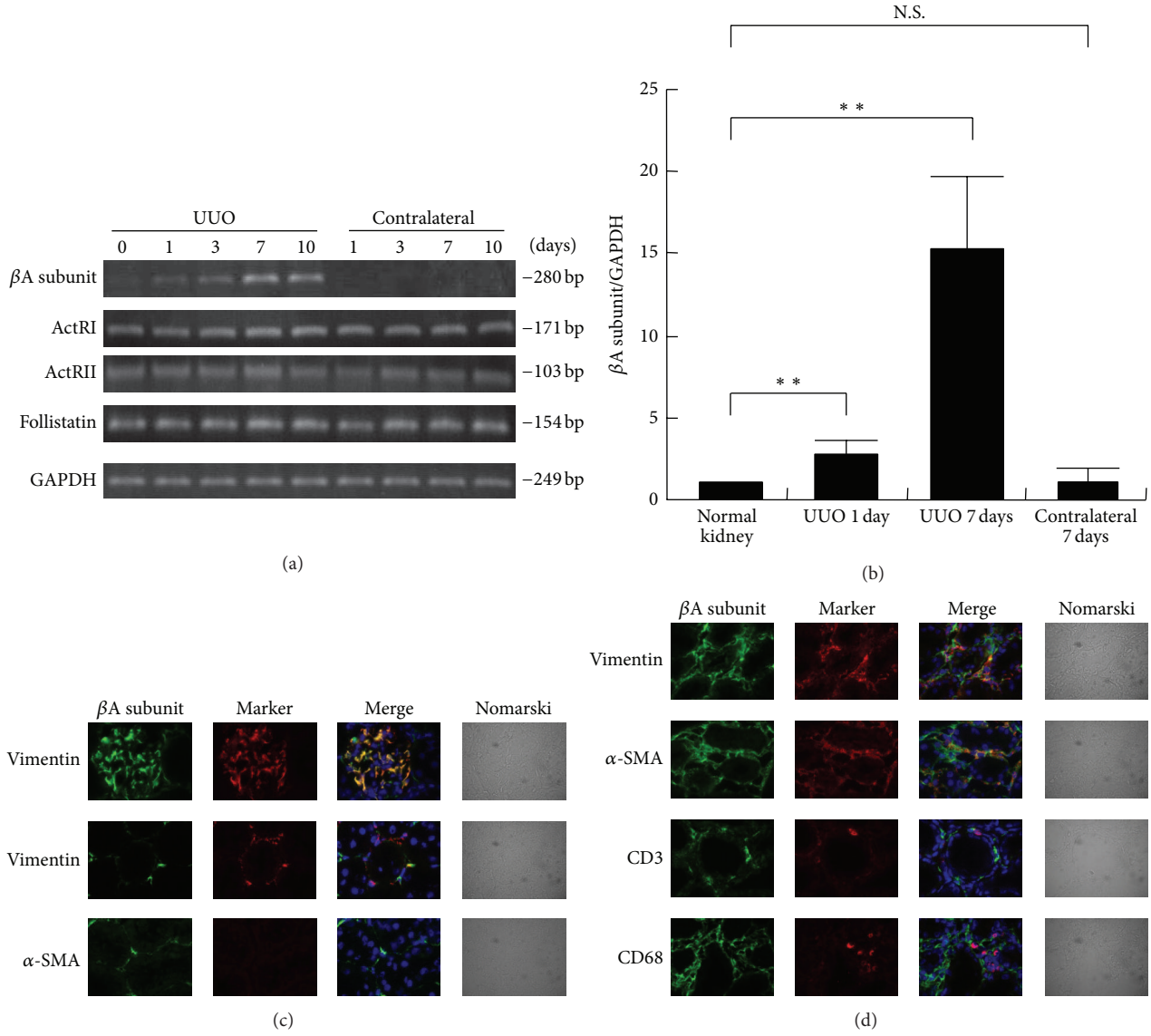

Figure 1: Expression of activin A, activin receptors, and follistatin in kidneys after UUO. (a) Total RNA was extracted from contralateral and UUO kidneys at the indicated times after surgery. Expression of βA subunit for activin A, activin type I receptor (ActRI), activin type II receptor (ActRII), and follistatin was examined by RT-PCR. (b) Expression of βA subunit for activin A in kidneys after UUO was measured by real-time PCR. Data are presented as mean ± SE ($n = 3$). $^{**}P < 0.01$ versus normal kidney. N.S., not significant. (c) Localization of βA subunit for activin A in normal kidneys was examined by immunostaining. Magnification: ×1000. (d) Localization of βA subunit for activin A in the kidneys at 7 days after UUO was examined by immunostaining. Magnification: ×1000.

analysis showed that there was no significant difference in the number of BrdU-positive tubular cells between saline-treated and follistatin-treated kidneys (Figure 3(b)). Interestingly, the number of BrdU-positive interstitial cells was significantly lower in the follistatin-treated kidneys, as compared to saline-treated kidneys (Figure 3(c)).

3.4. Effects of Follistatin on the Expression of α-SMA in UUO Kidneys. Transdifferentiation of interstitial fibroblasts into myofibroblasts is one of the critical processes involved in renal fibrosis [3]. We next investigated the expression of α-SMA, a marker of myofibroblasts, in UUO kidneys. No expression of α-SMA was observed in normal (Figure 4(a), panel (A)) and contralateral kidneys (Figure 4(a), panel (B)) except in vascular smooth muscle cells. In contrast, expression of α-SMA was abundantly detected in the interstitium of the saline-treated UUO kidneys (Figure 4(a), panels (C) and (E)). In the follistatin-treated kidneys, α-SMA expression was also observed (Figure 4(a), panels (D) and (F)), but its positive area was significantly smaller than that in the saline-treated kidneys at day 7 after UUO (Figure 4(b)).

3.5. Effects of Follistatin on Extracellular Matrix Production in UUO Kidneys. Myofibroblasts produce various types of

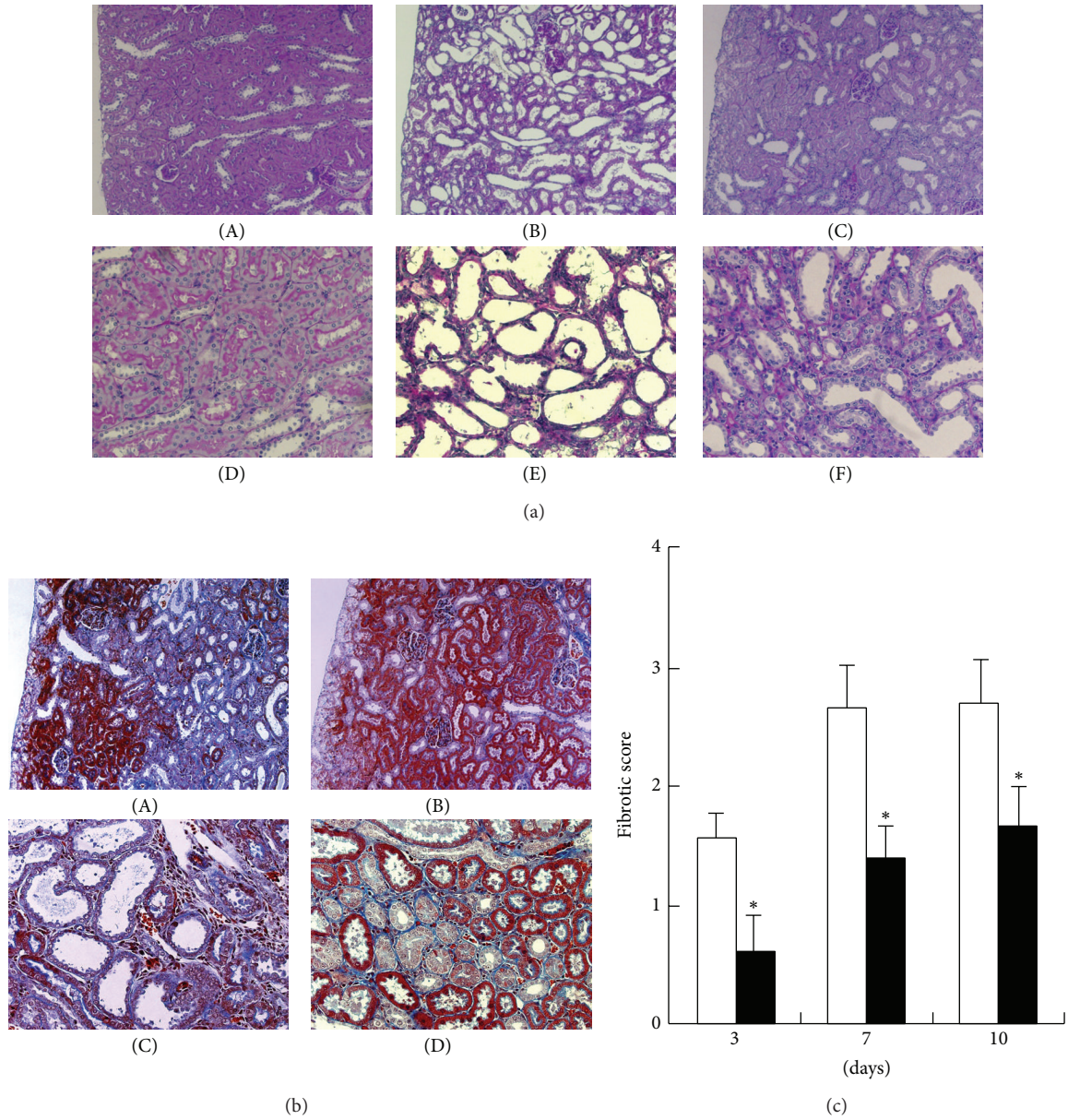

Figure 2: Effects of follistatin on fibrotic changes in kidneys after UUO. (a) Histological changes in kidneys after UUO were assessed by PAS staining. (A, D) contralateral kidneys, 7 days. (B, E) saline-treated UUO kidneys, 7 days. (C, F) follistatin-treated UUO kidneys, 7 days. Magnification: ×100 (A–C), ×400. (b) Fibrotic changes in kidneys after UUO were assessed by Masson-trichrome staining. (A, C) saline-treated UUO kidneys, 7 days. (B, D) follistatin-treated UUO kidneys, 7 days. Magnification: ×100 (A, B), ×400 (C, D). (c) Semiquantitative analysis of fibrotic changes in UUO kidneys. Fibrotic score was measured as described in Section 2. Data are presented as mean ± SE ($n = 5$). Saline (white bars), follistatin (black bars). $^*P < 0.05$ versus saline.

extracellular matrix (ECM), leading to the deposition of ECM during renal fibrosis. We next examined the effects of follistatin on the production of ECM by immunostaining (Figure 5). The deposition of type I collagen (Figure 5, panels (A) to (D)), type III collagen (Figure 5, panels (E) to (H)), and fibronectin (Figure 5, panels (I) to (L)) was observed in both saline-treated (Figure 5, panels (C), (G), and (K)) and follistatin-treated kidneys (Figure 5, panels (D), (H), and (L)), but not in normal (Figure 5, panels (A), (E), and (I)) or contralateral kidneys (Figure 5, panels (B), (F), and (J)). Quantitative analysis showed a significant decrease in type I collagen-positive area as well as fibronectin-positive area, but not in type III-positive area in the follistatin-treated kidneys (Figure 5(b)).

3.6. Effects of Follistatin on Macrophage Infiltration in UUO Kidneys. Macrophage infiltration is often correlated with degree of renal fibrosis, and the depletion of macrophages reduces fibrosis in several disease models [16]. We then investigated macrophage infiltration in UUO kidneys by

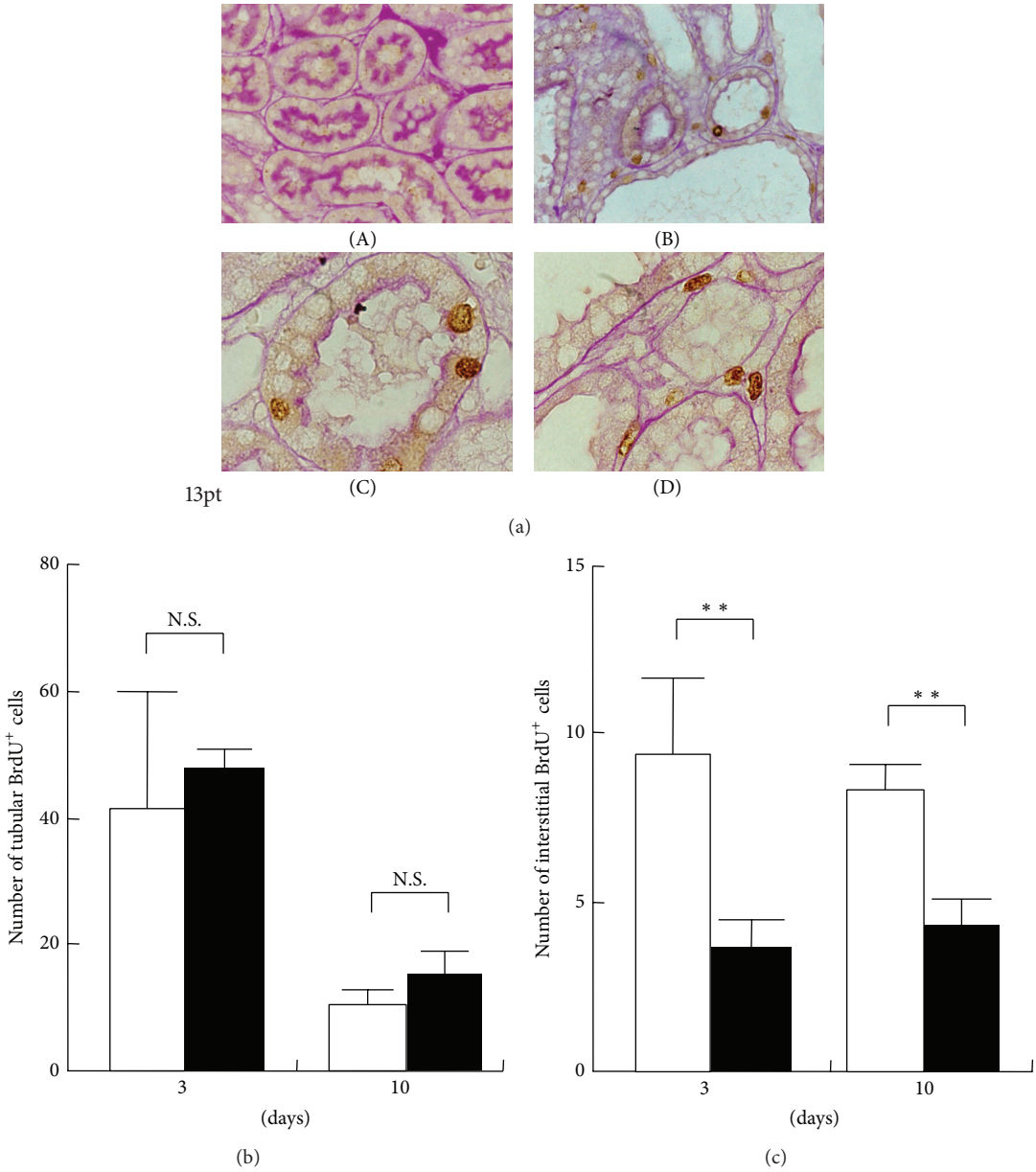

Figure 3: Effects of follistatin on cell proliferation in kidneys after UUO. (a) BrdU was intraperitoneally injected into UUO rats at 1 h before sacrifice. Cell proliferation was assessed by BrdU incorporation. (A) Contralateral kidneys, 3 days. UUO kidneys, 3 days. Magnification: ×200 (A, B), ×1000 (C, D). BrdU-positive nuclei (brown). (b), (c) Quantitative analysis of the number of tubular (b) and interstitial (c) BrdU-positive cells. BrdU-positive cells in the tubules and interstitium of the kidneys were separately counted in 10 randomly selected fields per rat at ×400 magnification. Values are mean ± SE ($n = 5$). Saline (open circle), follistatin (closed circle). $^{**}P < 0.01$ versus saline. N.S., not significant.

immunostaining. The expression of CD68, a macrophage marker, was not observed in normal (Figure 6(a), panel (A)) or contralateral kidneys (Figure 6(a), panel (B)). In contrast, CD68-positive cells were observed in the interstitium of saline-treated UUO kidneys (Figure 6(a), panels (C) and (E)) and follistatin-treated UUO kidneys (Figure 6(a), panels (D) and (F)). Semiquantitative analysis demonstrated that the number of CD68-positive cells in follistatin-treated kidneys was significantly lower when compared to that in the saline-treated kidneys at day 3, but not at day 7, after UUO (Figure 6(b)).

4. Discussion

Activin A is involved in tissue fibrosis in various organs [17]. Expression of activin A is upregulated in the fibrotic process in several tissues, including the lung [18, 19], pancreas [20], liver [21], and skin [22, 23]. Follistatin attenuated early events in fibrogenesis by constraining hepatic satellite cell proliferation and inhibiting hepatocyte apoptosis [24]. Furthermore, follistatin exerted antifibrotic effects in bleomycin-induced pulmonary fibrosis [25]. In transgenic mice overexpressing follistatin in the epidermis, scar formation was

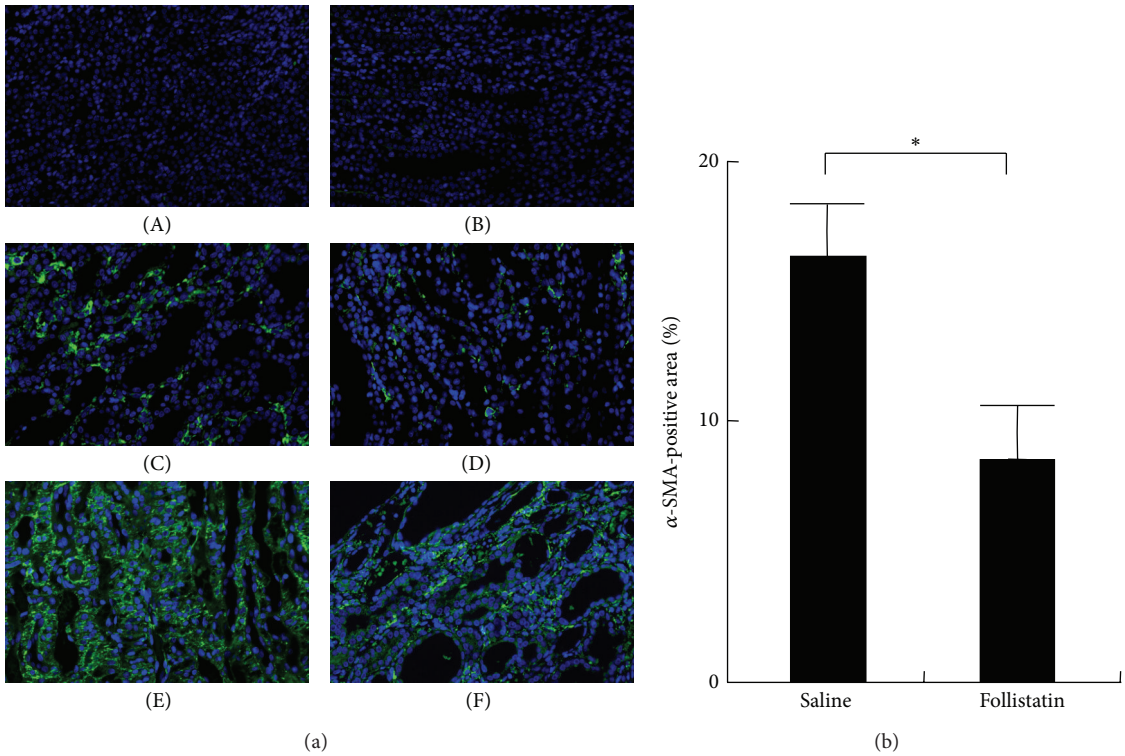

Figure 4: Effects of follistatin on expression of α-SMA in kidneys after UUO. (a) Expression of α-SMA, a marker for myofibroblasts, in the UUO kidneys, was examined by immunostaining. (A) Normal kidney. (B) Contralateral kidneys, 10 days. (C) Saline-treated UUO kidneys, 3 days. (D) Follistatin-treated UUO kidneys, 3 days. (E) Saline-treated UUO kidneys, 10 days. (F) Follistatin-treated UUO kidneys, 10 days. Magnification: ×200. α-SMA (green), DAPI (blue). (b) Quantitative analysis of α-SMA-positive area. α-SMA-positive area in the kidneys at 10 days after UUO was assessed as described in Section 2. Values are mean ± SE ($n = 5$). $^*P < 0.05$.

decreased after wounding the skin [26]. However, the role of activin A in renal fibrosis is unclear. We demonstrated here the upregulation of activin A in the UUO kidneys. Immunoreactive activin A was abundantly expressed by α-SMA-positive interstitial myofibroblasts in the UUO kidneys (Figure 1). Blockade of activin by follistatin reduced the fibrotic changes (Figure 2) and reduced the production of type I collagen and fibronectin (Figure 5) in the kidneys after UUO. Furthermore, follistatin inhibited the number of interstitial proliferating cells (Figure 3) and significantly reduced α-SMA-positive area in the UUO kidneys (Figure 4). It was previously demonstrated that activin A promoted cell proliferation and increased the production of both type I collagen and α-SMA expression in primary renal fibroblasts in vitro [13]. Renal interstitial fibroblasts express activin receptors [13]. These data suggest that activin A serves as an autocrine inducer of cell proliferation or activator of interstitial fibroblasts. The activin signaling pathway may be a novel therapeutic target for the prevention of renal fibrosis.

The precise mechanism by which follistatin reduced renal fibrosis remains unclear in this study. Follistatin may antagonize activin A action by blocking interaction with activin receptors on fibroblasts and preventing downstream signaling cascades leading to extracellular matrix synthesis. In addition to antagonizing the profibrotic action of activin A, two mechanisms may explain the therapeutic effects of follistatin on renal fibrosis. First, follistatin attenuates renal fibrosis by blocking the action of TGF-β. It was reported previously that activin A expression is induced by TGF-β1 and that blockade of activin by follistatin or transfection with truncated type II activin receptor reduces type I collagen expression induced by TGF-β1 in rat primary renal fibroblasts [13]. Similarly, TGF-β1 activity was inhibited by blockade of activin in rat hepatic stellate cells [24], pancreatic stellate cells [20], and human fetal lung fibroblasts [25]. It is therefore likely that TGF-β1 induces tissue fibrosis partly via activin A. Second, the action of follistatin is mediated through other members of the TGF-β superfamily. Follistatin binds to activin with high affinity and also binds to several BMP proteins [27]. Follistatin enhanced BMP-7 action to induce muscle growth during chick limb development [28]. Systemic administration of recombinant human BMP-7 leads to the repair of severely damaged renal tubular epithelial cells, in association with reversal of chronic renal injury [29]. Therefore, amelioration of renal fibrosis by follistatin may be obtained by the enhancement of the antifibrotic effects of BMP-7.

Emerging evidence has suggested activin A as a key mediator in inflammation [30]. Activin A exhibits proinflammatory actions in several tissues [17], is secreted from activated immune cells recruited to sites of inflammation by mast cell progenitors [31], and induces the directional migration

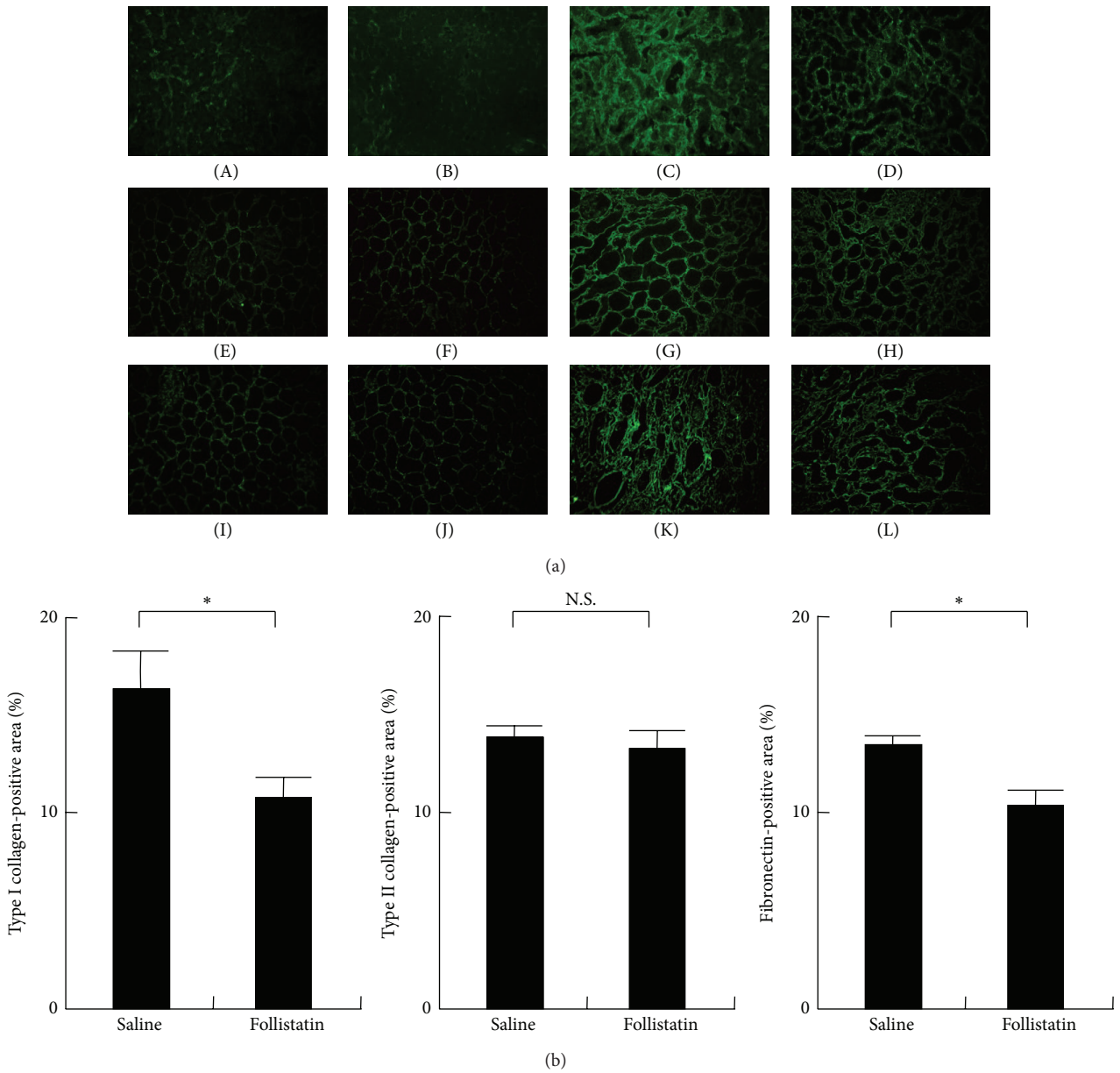

Figure 5: Effects of follistatin on the production of extracellular matrix in kidneys after UUO. (a) Production of type I collagen (A–D), type III collagen (E–H), and fibronectin (I–L) in the UUO kidneys was examined by immunostaining. (A, E, I) normal kidney. (B, F, J) contralateral kidney. (C, G, K) saline-treated UUO kidney, 7 days. (D, H, L) follistatin-treated UUO kidney, 7 days. Type I collagen, type III collagen, and fibronectin (green). Magnification: ×100. (b) Quantitative analysis of extracellular matrix production. Type I collagen, type III collagen, and fibronectin-positive area in kidneys at 7 days after UUO was measured as described in Section 2. Values are mean ± SE ($n = 5$). $^{*}P < 0.05$. N.S., not significant.

of immature myeloid dendritic cells through the activation of activin receptors [32]. In the colitis model, infiltrating macrophages were found to produce excess activin βA [33]. Immunoreactive activin A was abundantly expressed in the infiltrated macrophages in bleomycin-treated rat lung [25]. CD68-positive macrophage-lineage cells have been identified as activin-producing cells in rheumatoid synovium [34]. In an inflammatory state, activin A may be involved in the infiltration of macrophages by stimulating the gelatinolytic activity of matrix metalloproteinase-2 (MMP-2), which can degrade basement membrane collagens [35]. In this study, as an early inflammatory response after UUO, the infiltration of CD68-positive macrophages was observed in the UUO kidneys. Although it is unknown whether this inflammatory response directly contributes to the fibrotic process in this UUO model, follistatin significantly reduced the number of infiltrating CD68-positive macrophages (Figure 6). Activin A was expressed in the interstitial fibroblasts but not colocalized with infiltrating macrophages (Figure 1(d)). This raises the possibility that activin A, as a chemoattractant, may help

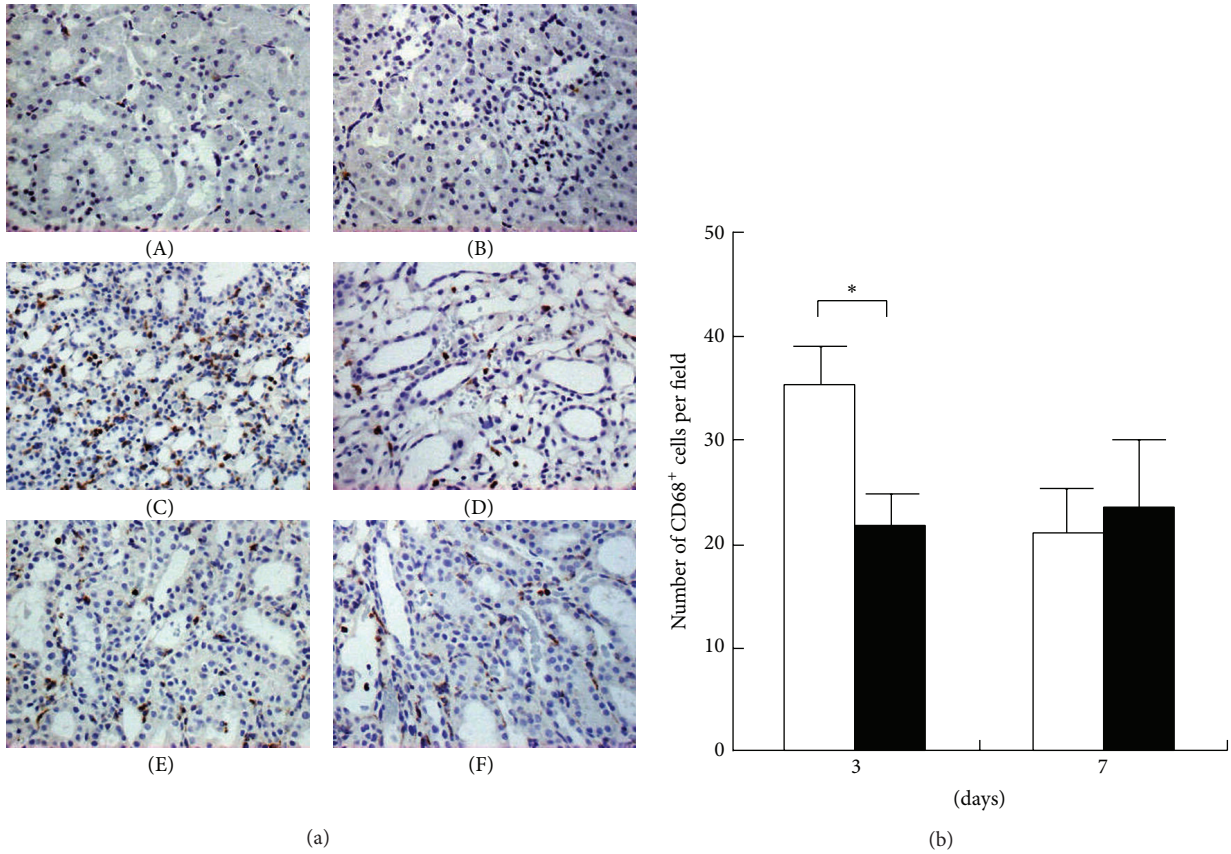

FIGURE 6: Effects of follistatin on macrophage infiltration in kidneys after UUO. (a) Expression of CD68, a marker for macrophages, in UUO kidneys, was examined by immunostaining. (A) Normal kidney. (B) Contralateral kidneys, 3 days. (C) Saline-treated UUO kidneys, 3 days. (D) Follistatin-treated UUO kidneys, 3 days. (E) Saline-treated UUO kidneys, 7 days. (F) Follistatin-treated UUO kidneys, 7 days. CD68-positive cells (brown). Magnification: ×400. (b) Quantitative analysis of CD68-positive cell number. CD68-positive cells were counted in 10 randomly selected fields per rat at ×400 magnification. Values are mean ± SE ($n = 5$). Saline (white bars), follistatin (black bars). $^*P < 0.05$ versus saline.

macrophages infiltrate the interstitium during renal fibrosis. Decrease in the number of CD68-positive cells by follistatin could be observed at 3 days but not at 7 days after UUO (Figure 6), which might suggest the presence of chemoattractants other than activin A or activin-independent mechanism. Further study is necessary to clarify this issue.

Conflict of Interests

All the authors have declared no conflict of interests.

Acknowledgments

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Review Article

The Role of Uric Acid in Kidney Fibrosis: Experimental Evidences for the Causal Relationship

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Hyperuricemia is a common finding in chronic kidney disease due to decreased uric acid clearance. The role of uric acid as a risk factor for chronic kidney disease has been largely debated, and recent studies suggested a role of uric acid in the causation and progression of kidney fibrosis, a final common pathway in chronic kidney disease. Uric acid and xanthine oxidase may contribute to kidney fibrosis mainly by inducing inflammation, endothelial dysfunction, oxidative stress, and activation of the renin-angiotensin system. Besides, hyperuricemia induces alterations in renal hemodynamics via afferent arteriolopathy and contributes to the onset and progression of kidney fibrosis. Xanthine oxidase inhibitors may prevent kidney damage via lowering uric acid and/or inhibiting xanthine oxidase. However, there is still no sufficient evidence from interventional clinical researches supporting the causal relationship between uric acid and kidney fibrosis. The effect and role of xanthine oxidase inhibitors in preventing kidney fibrosis and chronic kidney disease progression must be further explored by performing future large scale clinical trials.

1. Introduction

Regardless of the underlying etiology, most forms of chronic kidney disease (CKD) are characterized by progressive fibrosis as a final common pathway, which eventually affects all substructures of the kidney leading to a final consequence of end-stage renal disease. Although there has been a great deal of research, a comprehensive understanding of the pathogenetic mechanisms of kidney fibrosis remains uncertain and this hampers the development of effective therapeutic strategies [1].

Uric acid (UA) is the final breakdown product of purine degradation in humans, and elevated serum UA level, hyperuricemia, is causative in gout and urolithiasis due to the formation and deposition of monosodium urate crystals. Hyperuricemia is a common finding in CKD due to decreased UA clearance. Its role as a risk factor for CKD progression has been largely debated, and it was primarily considered as a marker or epiphenomenon of kidney damage [2, 3]. However, during the last 2 decades, accumulating evidences have suggested a role of UA in the causation or progression of cardiovascular diseases and CKD [3–9]. Therefore, UA lowering therapy with xanthine oxidase (XO) inhibitors, which are already being widely used in the treatment of gout, could be promising for preventing the progression of CKD even in patients without hyperuricemia; however, solid clinical evidence is still lacking. To promote large scale prospective clinical trials, it is essential to accumulate experimental evidences for the cause-effect relationship between UA and kidney fibrosis.

In this review, after providing a brief background concerning UA physiopathology, we will focus on the mechanistic role of UA in kidney fibrosis. We will also review the role of XO and the effect of XO inhibitors in preventing kidney fibrosis and their associated mechanisms.

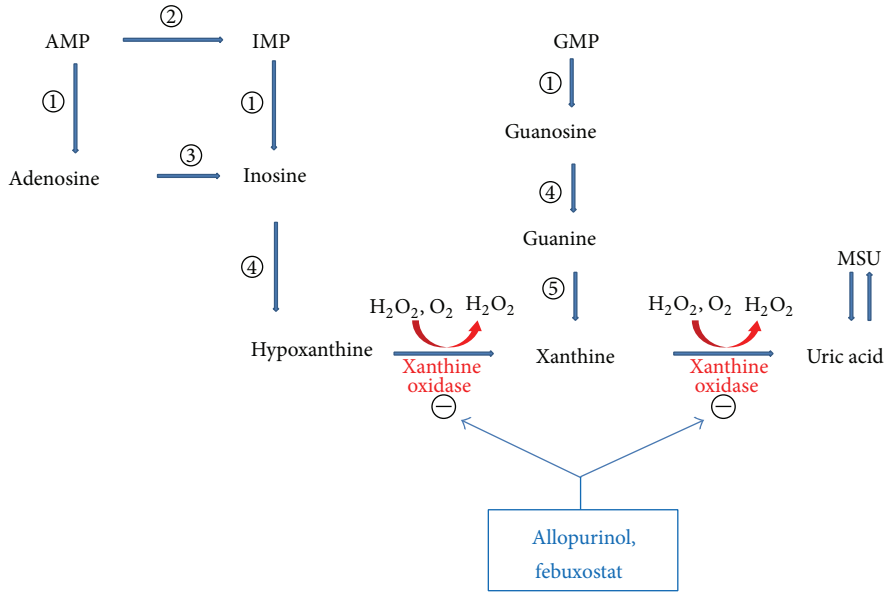

Figure 1: The pathway of purine nucleotides degradation in humans showing the competitive inhibition of uric acid formation by xanthine oxidase inhibitors and the site of action. AMP: adenosine monophosphate; GMP: guanosine monophosphate; IMP: inosine monophosphate; MSU: monosodium urate; ①: 5′-nucleotidase; ②: AMP deaminase; ③: adenosine deaminase; ④: purine nucleoside phosphorylase; ⑤: guanine deaminase.

2. Physiopathology of Uric Acid

Cell turnover leads to the production of adenosine, inosine, and guanosine. They degrade to hypoxanthine and xanthine, which are the substrates for the widely distributed XO in the formation of UA. XO catalyzes the oxidation of purine substrates, xanthine and hypoxanthine, producing both UA and reactive oxygen species (ROS). Thus, XO is one of the major enzymatic sources of ROS. Allopurinol and febuxostat are inhibitors of XO, and they reduce uric acid and ROS formation (Figure 1) [10].

UA is the oxidation end-product of purine metabolism in humans and higher primates. Most other mammals, except for the Dalmatian dogs, can degrade UA further to water-soluble allantoin with the enzyme uricase, and as a result serum urate levels are about 10% of those in humans [9, 11]. However, in humans and higher primates, mutations in the uricase gene occurred during evolution and, making the enzyme nonfunctional, resulted in higher levels of serum UA than in other mammals [12].

Urates are the ionized form of UA, and, at a physiologic pH of 7.4, over 95% of UA dissociates into urates, with 98% existing as monosodium urate. The serum urate level depends on dietary purines, the breakdown of endogenous purines, and the renal and intestinal excretion of urate. Hyperuricemia is defined as the accumulation of serum UA beyond its solubility point in water (6.8 mg/dL), and it develops due to UA overproduction, undersecretion, or both [13]. The dominating factor contributing to hyperuricemia is underexcretion of urate [11]. Under normal conditions, 70% of the UA produced is eliminated in the urine and the remaining UA is removed via biliary secretion. In the kidney, urate is easily filtered through the glomerulus and subsequently reabsorbed by the proximal tubule cells of the kidney and after further absorption, about 10% of urate is finally excreted [14]. An anion exchanger and a voltage-dependent pathway seem to be the mechanisms involved in urate transport [15].

Allopurinol, a purine inhibitor of XO, has been conventionally used for urate-lowering therapy to inhibit UA synthesis. A novel urate-lowering drug, febuxostat, is a potent nonpurine selective inhibitor of XO, and it inhibits both the reduced and oxidized forms of the enzyme in contrast to allopurinol that inhibits the reduced form of the enzyme only [16, 17]. Febuxostat is metabolized mainly by glucuronidation and oxidation in the liver, has its dual (urinary and fecal) pathways in excretion (urinary and fecal excretion rates: 49.1% and 44.9%, resp.), and is effective and well tolerated in patients with mild to moderate renal and hepatic impairment [18, 19]. Animal studies have demonstrated that febuxostat has a greater UA-lowering effect than allopurinol [20, 21]. Febuxostat's chemical structure does not resemble a pyrimidine or purine and is unlike that of allopurinol [22]. It does not inhibit other enzymes involved in purine or pyrimidine metabolism [23].

Studying the role of UA in kidney fibrosis, a process which eventually leads to CKD, is very difficult since uric acid is excreted primarily by the kidney, and hence a decrease in the glomerular filtration rate (GFR) is inevitably accompanied by a rise in the serum UA level. As such, studies in experimental animals in which serum UA can be modulated are critical to understand whether there is a role for UA in the causation or progression of CKD [7].

3. Experimental Studies Supporting the Roles of Uric Acid and Xanthine Oxidase in Kidney Fibrosis

In the past, hyperuricemia was thought to cause CKD, the so-called urate nephropathy, by the deposition of urate crystals in the renal interstitium. This results in a chronic inflammatory response and in progressive tubulointerstitial injury in a similar manner as seen with tophi in gouty arthritis [24]. However, the pathologic role of hyperuricemia in kidney disease by a crystal-independent mechanism is somewhat less clear.

3.1. Hyperuricemic Rat Models and Types of Kidney Injury. Generating hyperuricemia in laboratory animals proved to be difficult due to the fact that most mammals have the uricase enzyme. Rodent models in which the uricase gene was knocked out showed renal failure due to extensive tubular crystal deposition and finally death [25]. An alternative model with milder degree of hyperuricemia without crystal deposition, which is more applicable to human disease, was developed using uricase inhibitor, oxonic acid (OA) [26–29]. It is also possible to lower serum UA levels using the XO inhibitors such as allopurinol and febuxostat.

After the year 2001, when the hyperuricemic rat model was developed by using OA [26] and until recently, there have been accumulating experimental evidences that hyperuricemia induced renal injury, which may be prevented by lowering serum UA levels with XO inhibitors. The first study using OA-induced hyperuricemic rat model demonstrated that hyperuricemia induced systemic hypertension as well as ischemic type of kidney injury with collagen deposition, macrophage infiltration, and increase in tubular expression of osteopontin documented via immunohistochemical stains. The kidneys were devoid of urate crystals and were normal by light microscopy. Blood pressure was lowered by reducing serum UA levels with allopurinol. Hyperuricemic rats treated with OA also showed an increase in juxtaglomerular renin and a decrease in macula densa neuronal nitric oxide (NO) synthase. Both the kidney injury and hypertension were attenuated by treatment with renin-angiotensin system (RAS) blocker (enalapril) or a substrate for endothelial NO synthase (L-arginine). This study suggested that UA induced hypertension and renal injury via a crystal-independent mechanism with the activation of RAS and inhibition of NO synthase [26]. Another study demonstrated that hyperuricemia induced arteriolopathy of the afferent arteriole by a blood pressure-independent mechanism. In this study, hyperuricemic rats fed OA showed hypertension and afferent arteriolar thickening. Allopurinol prevented hyperuricemia, hypertension, and arteriolopathy. Controlling blood pressure with hydrochlorothiazide did not prevent hyperuricemia and arteriolopathy, suggesting that hyperuricemia-induced arteriolopathy was not mediated by blood pressure. This study also showed that arteriolopathy was mediated by the direct effect of UA on proliferation of vascular smooth muscle cells with activation of RAS [27].

In another rat model with dietary intake of adenine, which may be the source of the UA as a purine base, adenine-fed rats showed hyperuricemia [30, 31]. Adenine-fed rats also showed increased kidney inflammation (TNF-α), fibrotic (TGF-β), and oxidative (HO-1) markers, along with pathologically confirmed kidney fibrosis. Lowering of UA levels with allopurinol reversed the kidney damage, suggesting that UA played a major role in the pathogenesis of kidney fibrosis [30]. Another animal model of tubulointerstitial nephritis (TIN) induced by excessive adenine intake exhibited significant renal dysfunction and enhanced cellular infiltration accompanied by collagen deposition. It also showed higher gene and protein expression of proinflammatory cytokines. Treatment with allopurinol led to reduced levels of uric acid, oxidative stress, and collagen deposition and a downregulation of the nuclear factor-kB (NF-kB) signaling pathway [31].

Based on the growing evidence that lowering UA levels with allopurinol prevented renal injury induced by hyperuricemia, the role of febuxostat in preventing kidney injury was investigated. In OA-induced hyperuricemic rats, febuxostat lowered UA levels and ameliorated systemic and glomerular hypertension as well as preglomerular arteriolopathy. In normal rats without hyperuricemia, febuxostat tended to lower UA levels and had no effect on blood pressure, glomerular pressure, and afferent arteriole morphology [32].

3.2. Systemic and Glomerular Hypertension in Hyperuricemia. There are some experimental studies that demonstrated the relationship between UA and renal hemodynamics. In one study, hyperuricemic rats fed OA not only developed systemic hypertension but also glomerular hypertension. Hyperuricemic rats showed increased glomerular capillary pressure with afferent arteriole thickening. Allopurinol prevented hyperuricemia, systemic and glomerular hypertension, and arteriolopathy. Glomerular capillary pressure and arteriolar thickening correlated with serum UA and systolic blood pressure. This study suggested that glomerular hypertension might be mediated by insufficient vasoconstriction of the afferent arteriole to systemic hypertension, allowing the transmission of systemic pressure to the glomerular capillary tuft [33]. In another study, hyperuricemia induced by OA resulted in renal cortical vasoconstriction and glomerular hypertension due to afferent arteriole thickening in normal and remnant kidney rats. Allopurinol prevented structural and functional alterations in both normal and remnant kidney rats. This study suggested that hyperuricemia-induced glomerular alterations caused renal ischemia, which in turn induced tubulointerstitial inflammation and fibrosis [28].

3.3. Renal Progression in Animal Models of Chronic Kidney Disease. Hyperuricemia also contributed to renal progression in animal models of CKD. In 5/6 nephrectomy remnant kidney model, remnant kidney rats fed OA showed more severe renal failure, proteinuria, and histologic findings (thickening of the preglomerular arteries, glomerulosclerosis, and interstitial fibrosis) compared to remnant kidney rats without hyperuricemia. Allopurinol reduced serum UA levels

and prevented the functional and histologic changes in remnant kidney rats fed OA. This study also demonstrated that hyperuricemia accelerated renal progression by increasing renin expression in the renal cortex and cyclooxygenase-2 (COX-2) expression in the afferent arteriole. In particular, this study showed that increased COX-2 expression induced by hyperuricemia was associated with proliferation of vascular smooth muscle cells in preglomerular arteries [29]. In another 5/6 nephrectomy rat model, remnant kidney rats treated with OA developed hyperuricemia, renal vasoconstriction, and glomerular hypertension in association with further aggravation of afferent arteriolopathy compared to remnant kidney rats that were not treated with OA. Febuxostat prevented hyperuricemia and ameliorated renal injury in remnant kidney rats treated with OA. Interestingly, febuxostat had a comparable beneficial effect in both remnant kidney rats with hyperuricemia (treated with OA) and remnant kidney rats without hyperuricemia (not treated with OA) [34].

3.4. Exacerbation of Renal Injury in Animal Models of Cyclosporine and Diabetic Nephropathy. Hyperuricemia has also been known to exacerbate renal injury in some animal disease models including cyclosporine (CsA) and diabetic nephropathy. Hyperuricemia frequently complicated CsA therapy. In one study using a model of CsA nephropathy, the rats developed hyperuricemia with arteriolar hyalinosis, tubular injury, and interstitial fibrosis. CsA nephropathy rats fed OA showed higher UA levels with more severe histologic findings compared to CsA nephropathy rats that were not treated with OA. This study also demonstrated that the mechanism did not involve intrarenal urate crystal deposition and appeared to involve activation of RAS and inhibition of intrarenal NO production [35]. In another study, CsA-treated rats developed hyperuricemia with arteriolar hyalinosis, tubular atrophy, interstitial fibrosis, increased cell proliferation, and decreased vascular endothelial growth factor (VEGF). Treatment with allopurinol or a uricosuric, benzbromarone, reduced the severity of the kidney injury. Both drugs provided comparable protection and the similar protection observed with both drugs suggests that the effect is associated more with lowering UA levels than the antioxidant effect of allopurinol [36]. Hyperuricemia has recently been recognized to be a risk factor for nephropathy in the diabetic subject. Diabetic (db/db) mice developed hyperuricemia, albuminuria, mesangial matrix expansion, and mild tubulointerstitial disease. Allopurinol treatment not only reduced UA levels but also reduced albuminuria and ameliorated tubulointerstitial injury. The mechanism for protection was shown to be due to a reduction in inflammatory cells mediated by a reduction in ICAM-1 expression by tubular epithelial cells [37]. In another diabetic nephropathy model using KK-A(y)Ta mice, lowering UA levels with allopurinol attenuated transforming growth factor- (TGF-) β1-induced profibrogenic progression in the mice, suggesting that lowering serum UA may be an effective therapeutic intervention to prevent the progression of diabetic nephropathy [38].

3.5. Oxidative Stress and Endothelial Dysfunction in Hyperuricemia. While UA has been reported to be a potent antioxidant in the extracellular fluid [39], it has prooxidative effect once inside the cell [40, 41]. According to a hypothesis [39], the silencing of the uricase gene with an increase in the blood level of UA provided an evolutionary advantage for ancestors of *Homo sapiens*. This hypothesis was based on *in vitro* experiments which showed that UA is a powerful scavenger of singlet oxygen, peroxyl radicals, and hydroxyl radicals. UA circulating at an elevated level was proposed to be one of the major antioxidants of the plasma that protects cells from oxidative damage, thereby contributing to an increase in life span of human species and decreasing the risk of cancer [42]. On the other hand, a vast literature on the epidemiology of cardiovascular disease, hypertension, and metabolic syndrome overwhelmingly shows that, at least among modern *Homo sapiens*, a high level of UA is strongly associated with and in many cases predicts development of hypertension, visceral obesity, insulin resistance, dyslipidemia, diabetes, kidney disease, and cardiovascular and cerebrovascular events [42]. Antioxidant effect of UA varies according to the presence of specific components, in different physiochemical circumstances and in various compartments of the human body. UA is an antioxidant only in the hydrophilic environment and even in the plasma UA can prevent lipid peroxidation only as long as ascorbic acid is present [43]. Major sites where the antioxidant effects have been proposed are the central nervous system [44, 45], liver [46], and heart [47].

In OA-induced hyperuricemic rat model, hyperuricemia caused intrarenal oxidative stress, increased expression of NOX-4 subunit of renal NADPH oxidase and angiotensin II, and decreased NO bioavailability. Hyperuricemic rats also showed systemic hypertension, renal vasoconstriction, and arteriolopathy. Tempol (superoxide scavenger) attenuated the adverse effect induced by hyperuricemia despite equivalent hyperuricemia, suggesting that UA might cause oxidative stress [48]. In another study, hyperuricemic rats treated with OA showed decreased urinary NO metabolites (NO_2^-/NO_3^-), systemic hypertension, renal vasoconstriction, and preglomerular arteriolopathy. Chronic administration of L-arginine, a substrate for endothelial NO synthase, increased the urinary excretion of NO_2^-/NO_3^- and preserved arteriolar structures probably mediated by the antiproliferative effect of NO on vascular smooth muscle cells, suggesting the role of endothelial dysfunction as a mediator of renal injury induced by hyperuricemia [49]. A recent study reported that UA-induced endothelial dysfunction was associated with mitochondrial alterations and decreased intracellular ATP concentrations [50]. An experimental model of streptozotocin-induced diabetic rats showed that febuxostat improved endothelial dysfunction via attenuating oxidative stress by XO inhibition [51].

3.6. Hyperuricemia and Inflammatory Responses. UA induces inflammatory responses. Hyperuricemia-induced inflammatory response mediates kidney injury via alteration of vascular and tubular cells in kidney. UA has the ability to induce

monocyte chemoattractant protein- (MCP-) 1 in vascular smooth muscle cells, suggesting that it may have a role in the vascular changes associated with hypertension and vascular disease [52]. UA also contributes to kidney damage through vascular cell proliferation induced by activation of COX-2 [29] and increased expression of C-reactive protein (CRP) [53]. UA has been known to inhibit renal proximal tubule cell proliferation via activation of NF-κB and cytoplasmic phospholipase A_2 [54]. Hyperuricemia also increases extracellular matrix (ECM) synthesis through upregulation of lysyl oxidase (LOX) expression in renal tubular epithelial cells [55]. UA contributes to tubulointerstitial inflammation by inducing expression of intracellular adhesion molecule- (ICAM-) 1 in renal tubular epithelial cells [37].

XO has been reported to be upregulated by various inflammatory stimuli such as lipopolysaccharide (LPS), hypoxia, and cytokines [56–60]. Augmented XO eventually causes excess ROS formation, leading to tissue damage. Pharmacological inhibitors of XO, such as allopurinol and febuxostat, have been reported to have an anti-inflammatory effect in various diseases such as atherosclerosis, congestive heart failure, acute lung injury, renal interstitial fibrosis, and ischemia-reperfusion injury [61–66]. A recent study suggested a molecular mechanism underlying the involvement of XO in inflammatory pathways, and it also suggested that XO mediates LPS-induced phosphorylation of JNK through ROS production and MKP-1 inactivation, leading to MCP-1 production in macrophages. Febuxostat significantly suppressed LPS-induced MCP-1 production in human macrophages and *in vivo* in mice [56].

3.7. Hyperuricemia and Epithelial-Mesenchymal Transition. In the last decade, epithelial-mesenchymal transition (EMT), a process by which fully differentiated epithelial cells lose their epithelial characteristics and undergo phenotypic conversion to mesenchymal cells, has emerged as an important pathway leading to generation of matrix-producing fibroblasts and myofibroblasts in kidney fibrosis. In addition to kidney fibrosis, EMT has been known to play a pivotal role in embryonic development, wound healing, tissue regeneration, and cancer progression [67, 68].

A recent study showed that UA exerted a direct effect on renal tubular cells by inducing EMT [69]. OA-induced hyperuricemic rats showed evidence of EMT before the development of significant tubulointerstitial fibrosis at 4 weeks, as indicated by decreased E-cadherin expression and an increased α-smooth muscle actin (α-SMA). Allopurinol significantly inhibited UA-induced changes in E-cadherin and α-SMA with an amelioration of kidney fibrosis at 6 weeks. In cultured rat renal tubular epithelial cells (NRK cells), UA induced EMT, which was blocked by the organic acid transport inhibitor, probenecid. UA increased expression of transcriptional factors associated with decreased synthesis of E-cadherin. UA also increased the degradation of E-cadherin via ubiquitination, which is of importance since downregulation of E-cadherin is considered to be a triggering mechanism for EMT. This study suggested that UA-induced EMT of renal tubular cells might be a novel mechanism explaining the association of hyperuricemia and renal progression after taking into account that EMT is an early phenomenon in kidney fibrosis [69–71]. Further research is required to assess the role of UA in other mesenchymal cell generation pathways.

4. Perspective

The underlying mechanisms by which UA could cause kidney fibrosis have been reported in animal models since 2001, and there has been a growing interest in this topic and numerous retrospective and prospective observational studies have been performed to assess the relationship between UA and CKD. The majority of data support the role of UA as a cause or exacerbating factor for kidney fibrosis and progressive CKD. Although, over the last several years, some clinical intervention trials including randomized controlled trials (RCTs) have further supported this mechanistic role of UA [72–78], clinical evidence demonstrating the beneficial effect of UA lowering therapy with XO inhibitors on renoprotection and prevention of CKD progression is not certain yet and cannot be easily generalized. Most of the clinical studies are limited by their retrospective study design, small sample size, short follow-up duration, or lack of randomization, and no adequately powered RCT has yet demonstrated the beneficial effect of UA-lowering therapy on renal and cardiovascular outcomes in CKD [5]. Currently, there is no published clinical trial in which febuxostat was used as a XO inhibitor for evaluating the efficacy of UA reduction on progression of CKD.

With regard to the animal models in this research field, the uricase inhibitor OA-induced hyperuricemic rat model [26–29] was mainly used during the initial several years and all of the studies used allopurinol as a XO inhibitor. Thereafter, the research was performed using animal models of various kidney disease conditions, such as CsA nephropathy [36], diabetic nephropathy [38, 51], obstructive nephropathy [61], ischemia-reperfusion injury [62], and 5/6 nephrectomy rat model [34]. Further research using diverse animal models of CKD [79, 80] is essential not only for gaining a better understanding of molecular mechanisms of kidney fibrosis but also for designing more appropriate clinical studies.

Febuxostat, a chemically engineered nonpurine selective inhibitor of XO, received approval in February 2009 from the Food and Drug Administration for the chronic management of hyperuricemia in patients with gout [81]. Although its clinical use is increasing, febuxostat is still generally considered as a second-line option for patients with gout who are unable to take allopurinol due to hypersensitivity, intolerance, renal insufficiency, or lack of efficacy in achieving a target serum UA level of <6.0 mg/dL. However, recently in basic research, febuxostat has largely replaced allopurinol as a XO inhibitor, and several animal studies have shown various beneficial effects of febuxostat in reducing inflammation, oxidative stress, and kidney fibrosis [34, 61, 62]. Febuxostat prevented renal injury in 5/6 nephrectomy rats with and without coexisting hyperuricemia [34]. But still there is little clinical experience and there are no clinical trials with febuxostat

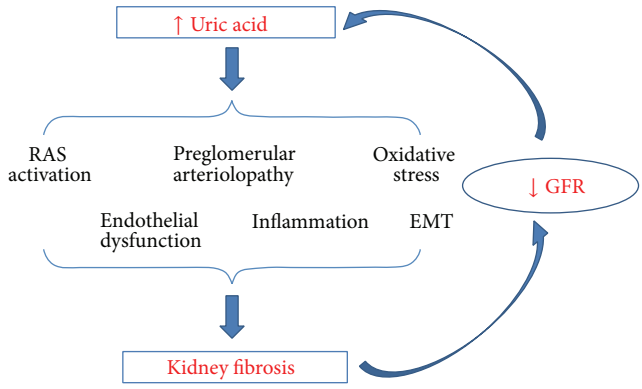

Figure 2: Mechanisms by which uric acid may cause kidney fibrosis based on experimental animal studies. EMT: epithelial-mesenchymal transition; RAS: renin-angiotensin system.

assessing the efficacy of lowering UA on CKD progression, and it is also more expensive than allopurinol.

At present, drug therapy for asymptomatic hyperuricemia is not actively recommended, and this negative approach is mainly due to the absence of evidence from adequately powered RCTs on the causality between hyperuricemia and the onset or progression of CKD. Considering the large sample size required for an adequately powered trial, an international collaboration is necessary.

Current research interests focus on developing new effective antifibrotic drugs to slow progression or even reverse chronic kidney injury, and several compounds which target various components of the fibrotic pathway, from signaling molecules that include TGF-β, phosphatidylinositide-3-kinase, and chemokines to microRNAs, are undergoing clinical trials [82, 83]. Although, at present, UA may be one of the ignored risk factors for CKD and the clinical use of UA-lowering drugs which include allopurinol and febuxostat is largely confined to gout management, discovering the potential value of XO inhibitors for preventing kidney fibrosis and CKD progression will provide additional valuable tools for managing CKD.

5. Conclusion

After taking into account the results of all the important experimental studies mentioned above, UA and XO may contribute to kidney fibrosis mainly by inducing inflammation, endothelial dysfunction, oxidative stress, and activation of RAS (Figure 2). Besides, hyperuricemia induces alterations of renal hemodynamics via afferent arteriolopathy and contributes to the onset and progression of kidney fibrosis. Many experimental studies have shown that XO inhibitors may prevent kidney damage by lowering UA and inhibiting XO. However, there is no sufficient evidence from interventional clinical researches supporting the causal relationship between UA and kidney fibrosis, a final common pathway of CKD progression. Thus, the time is not quite appropriate for recommending the widespread clinical use of XO inhibitors for preventing CKD progression in patients with hyperuricemia and more so in patients without hyperuricemia. The effect and role of XO inhibitors in preventing kidney fibrosis and CKD progression must be further explored by performing future large scale clinical trials.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

N-acetyl-seryl-aspartyl-lysyl-proline Inhibits Diabetes-Associated Kidney Fibrosis and Endothelial-Mesenchymal Transition

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Endothelial-to-mesenchymal transition (EndMT) emerges as an important source of fibroblasts. MicroRNA let-7 exhibits anti-EndMT effects and fibroblast growth factor (FGF) receptor has been shown to be an important in microRNA let-7 expression. The endogenous antifibrotic peptide N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) is a substrate of angiotensin-converting enzyme (ACE). Here, we found that AcSDKP inhibited the EndMT and exhibited fibrotic effects that were associated with FGF receptor-mediated anti-fibrotic program. Conventional ACE inhibitor plus AcSDKP ameliorated kidney fibrosis and inhibited EndMT compared to therapy with the ACE inhibitor alone in diabetic CD-1 mice. The endogenous AcSDKP levels were suppressed in diabetic animals. Cytokines induced cultured endothelial cells into EndMT; coincubation with AcSDKP inhibited EndMT. Expression of microRNA let-7 family was suppressed in the diabetic kidney; antifibrotic and anti-EndMT effects of AcSDKP were associated with the restoration of microRNA let-7 levels. AcSDKP restored diabetes- or cytokines-suppressed FGF receptor expression/phosphorylation into normal levels both in vivo and in vitro. These results suggest that AcSDKP is an endogenous antifibrotic molecule that has the potential to cure diabetic kidney fibrosis via an inhibition of the EndMT associated with the restoration of FGF receptor and microRNA let-7.

1. Introduction

Diabetic nephropathy is leading course of end-stage kidney disease and kidney fibrosis is the final common pathway in progressive kidney diseases. The fibroblasts that play a role in kidney fibrosis are believed to be heterogeneous [1]. Recently, the endothelial-to-mesenchymal transition (EndMT) has emerged as an important source of myofibroblasts or activated fibroblasts [2].

N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) is a tetrapeptide that is normally present in human plasma and is hydrolyzed by angiotensin-converting enzyme (ACE); ACE-inhibitor (ACE-I) treatment increases the plasma level of AcSDKP by fivefold [3]. We demonstrated that AcSDKP has an antifibrotic activity; that is, AcSDKP inhibits the transforming growth factor (TGF)-β-induced fibrogenic gene expression in human mesangial cells by inhibiting the smad 2/3 signaling [4] and rescues glomerular damage in db/db mice [5]. AcSDKP reportedly exhibits antifibrotic and organ protective effects in various experimental models [6–14].

We aimed to investigate whether antifibrotic peptide AcSDKP exerts additive antifibrotic effects associated with the inhibition of EndMT on top of the conventional ACE-I based therapy in fibrotic kidney model of diabetic mice.

2. Materials and Methods

2.1. Reagents and Antibodies. The AcSDKP was a gift from Dr. Omata from Asabio Bio Technology (Osaka, Japan). Imidapril (ACE-I) and TA-606 (ARB) were provided by

Mitsubishi Tanabe Pharma (Osaka Japan) through an MTA agreement. The mouse monoclonal anti-human CD31 antibody was purchased from R&D Systems (Minneapolis, MN, USA), and the rat polyclonal anti-mouse CD31 antibody was purchased from EMFRET Analytics GmbH & Co. KG (Eibelstadt, Germany). The polyclonal rabbit anti-αSMA antibody was obtained from Gene Tex (Irvine, CA, USA). The rabbit polyclonal anti-SM22α antibody and monoclonal anti-VE-cadherin antibody were obtained from Novus Biological (Littleton, CO, USA). The polyclonal anti-GAPDH and anti-TGF-β-receptor I antibodies were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fluorescein-, rhodamine-, and Alexa 647-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA). Antifibroblast growth factor (FGF) receptor, anti-phospho-FGF receptor, and the HRP-conjugated secondary antibodies for Western blot detection were purchased from Cell Signaling Technology (Danvers, MA, USA). TGF-β2, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β were purchased from PeproTech (Rocky Hill, NJ, USA).

2.2. Animal Experiments. We utilized a fibrotic diabetic kidney disease model, that is, streptozotocin- (STZ-) treated CD-1 mice [15]. Eight-week-old male CD-1 mice were obtained from Sankyo Lab Service (Tokyo, Japan). A single intraperitoneal injection of streptozotocin (STZ) (200 mg/kg) was given to the mice. We confirmed the induction of diabetes by a blood glucose level >16 mM at 2 weeks after the STZ injection. Sixteen weeks after the induction of diabetes, the diabetic mice were divided into the following four groups: (imidapril [2.5 mg/kg BW/day], AcSDKP [500 μg/kg BW/day using an osmotic mini-pump], AcSDKP+imidapril, TA-606 [3 mg/kg BW/day], and nontreatment). Imidapril or TA-606 was provided in drinking water. All of the mice were euthanized 24 weeks after the induction of diabetes, and their blood pressure was monitored using the tail-cuff method with a BP-98A instrument (Softron Co. Beijing, China) within a week before euthanasia.

2.3. AcSDKP Measurements. Blood was harvested into a heparinized tube containing captopril (final concentration 10 μmol/L) and centrifuged at 3,000 × g for 15 min at 4°C. We obtained estimated plasma and urine Ac-SDKP concentrations using a competitive enzyme immunoassay kit (SPI-BIO, Massy, France) according to the manufacturer's instruction. Urine AcSDKP was normalized at the urine creatinine level.

2.4. EndMT Detection In Vivo. EndMT were determined by double-labeling with antibodies against CD31 and αSMA, or with antibodies against CD31 and FSP1 on frozen sections (5-μm). The immunolabeled sections were analyzed using fluorescence microscopy (Axio Vert.A1, Carl Zeiss Microscopy GmbH, Jena, Germany). We obtained images of six different fields of view at 300x magnification and performed quantification. All immunolabelings were analyzed with appropriate negative control, including isotype IgG.

2.5. Morphological Evaluation. We determined the surface area of 10 glomeruli in each mouse using ImageJ software. A point-counting method was utilized to evaluate the relative area of the mesangial matrix (%). We analyzed 10 PAS-stained glomeruli from each mouse using a digital microscope screen grid containing 540 (27 × 20) points and employing Adobe Photoshop Element 6.0. The number of grid points on the mesangial tissue was divided by the total number of points in the glomerulus to obtain the mesangial area in a given glomerulus as the percentage of the total area of the glomerulus. Images of Masson's trichrome-stained tissue were analyzed using ImageJ software, and the fibrotic areas were quantified. For each mouse, images of six different fields of view at 100x magnification were evaluated.

2.6. In Vitro EndMT. Human umbilical vein endothelial cells (HUVEC) (Kurabo Industries Ltd., Osaka, Japan) cultured in HuMedia-EG2 medium and human dermal microvascular endothelial cells (HMVEC) (Lonza, Basel, Switzerland) cultured in EGM medium were used for the experiments. When cells grown on an adhesion reagent (Kurabo Medical, Osaka, Japan) reached 70% confluence, a combination of TGF-β2 (2.5 ng/mL), TNF-α (1.0 ng/mL), and IL-1β (2.0 ng/mL) was added to the experimental medium (a mixture of Humedia-EG2 in serum-free RPMI, 1 : 3 ratio) for an indicated interval, with or without a 2 h preincubation in AcSDKP (100 nM).

2.7. Western Blotting. Protein lysates were denatured in a SDS sample buffer at 100°C for 5 min. After centrifugation (17,000 ×g for 10 min at 4°C), supernatants were separated on SDS-polyacrylamide gels and blotted onto PVDF membranes (Pall Corporation, Pensacola, FL, USA) using the semidry method. The immunoreactive bands were developed using an enhanced chemiluminescence (ECL) detection system (Pierce Biotechnology, Rockford, IL, USA) and detected using an ImageQuant LAS 400 digital biomolecular imaging system (GE Healthcare Life Sciences, Uppsala, Sweden).

2.8. MicroRNA Array Analysis. Total RNA was isolated using a miRNeasy kit (Qiagen). After dephosphorylation and denaturation, the total RNA was labeled with cyanine 3-pCp and subsequently hybridized to an Agilent mouse microRNA microarray (release version 15) using the microRNA Complete Labeling and Hyb Kit (Agilent Technologies, Inc.). After hybridization for 20 h, the slides were washed using the Gene Expression Wash Buffer (Agilent Technologies, Inc.), scanned using an Agilent Scanner G2565BA, and processed and analyzed using Agilent Feature Extraction Software version 9.5.1. The raw data were analyzed using GeneSpring GX software version 12.5 (Agilent Technologies, Inc.).

2.9. MicroRNA Isolation and qPCR. The kidney tissues that had been maintained at −80°C were first incubated in RNA*later*^{R}-ICE (Life Technologies) for 16 h at −20°C before homogenization. The tissues were homogenized on ice and the microRNA was extracted. Total cDNA was synthesized

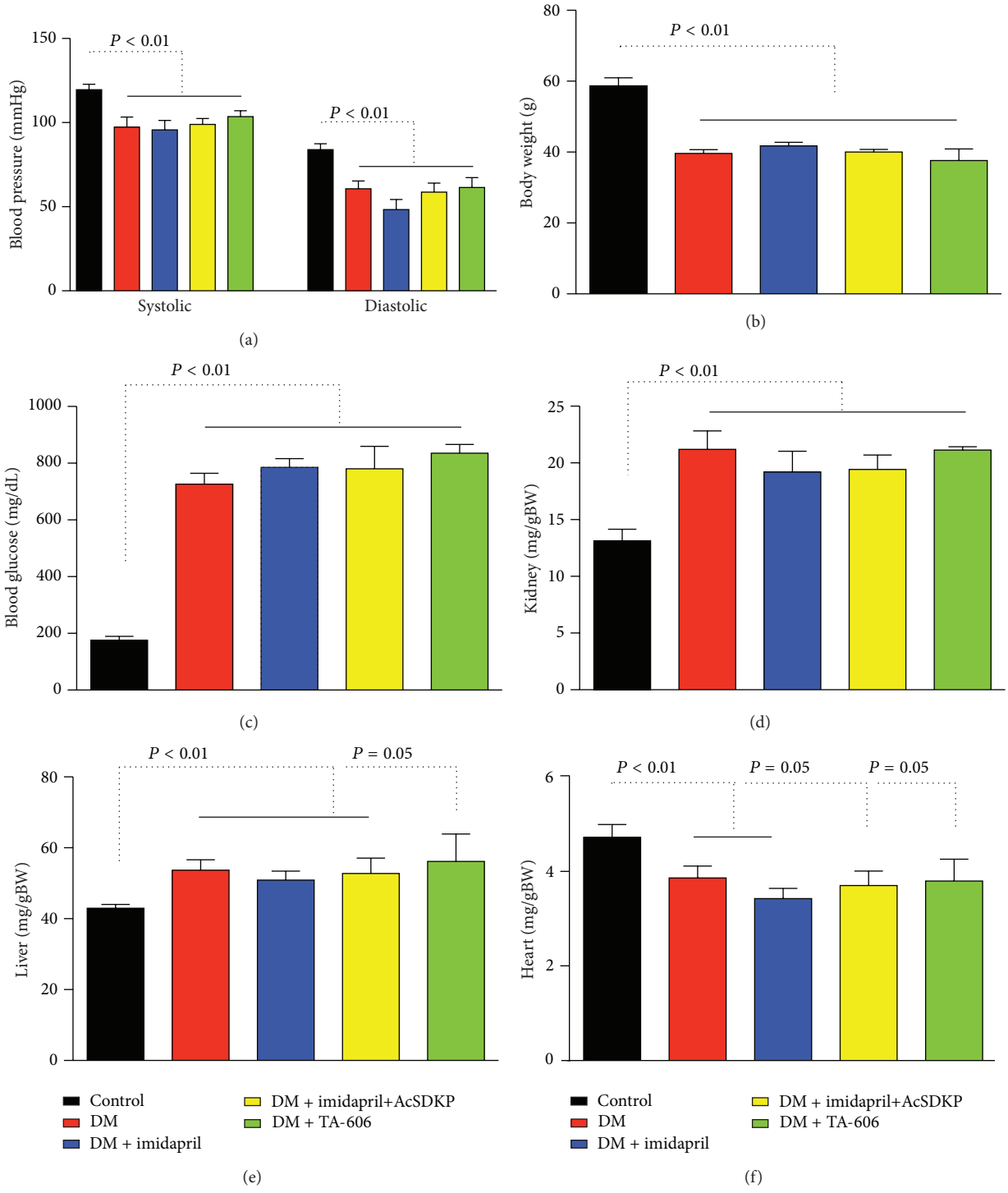

Figure 1: Characteristics of the experimental animals. (a) Blood pressure was measured within 1 week before the mice were euthanized. (b) Body weight. (c) Blood glucose level at the time of euthanasia. (d–f) Organ weights. Kidney (d), liver (e), and heart (f) weights relative to body weight (g) are shown. The data are expressed as the mean ± SEM values. Control: $n = 7$, STZ-induced diabetes: $n = 5$, diabetes treated with imidapril: $n = 6$, diabetes treated with imidapril+AcSDKP: $n = 5$, diabetes treated with TA-606: $n = 3$. Diabetic mice are designated as DM.

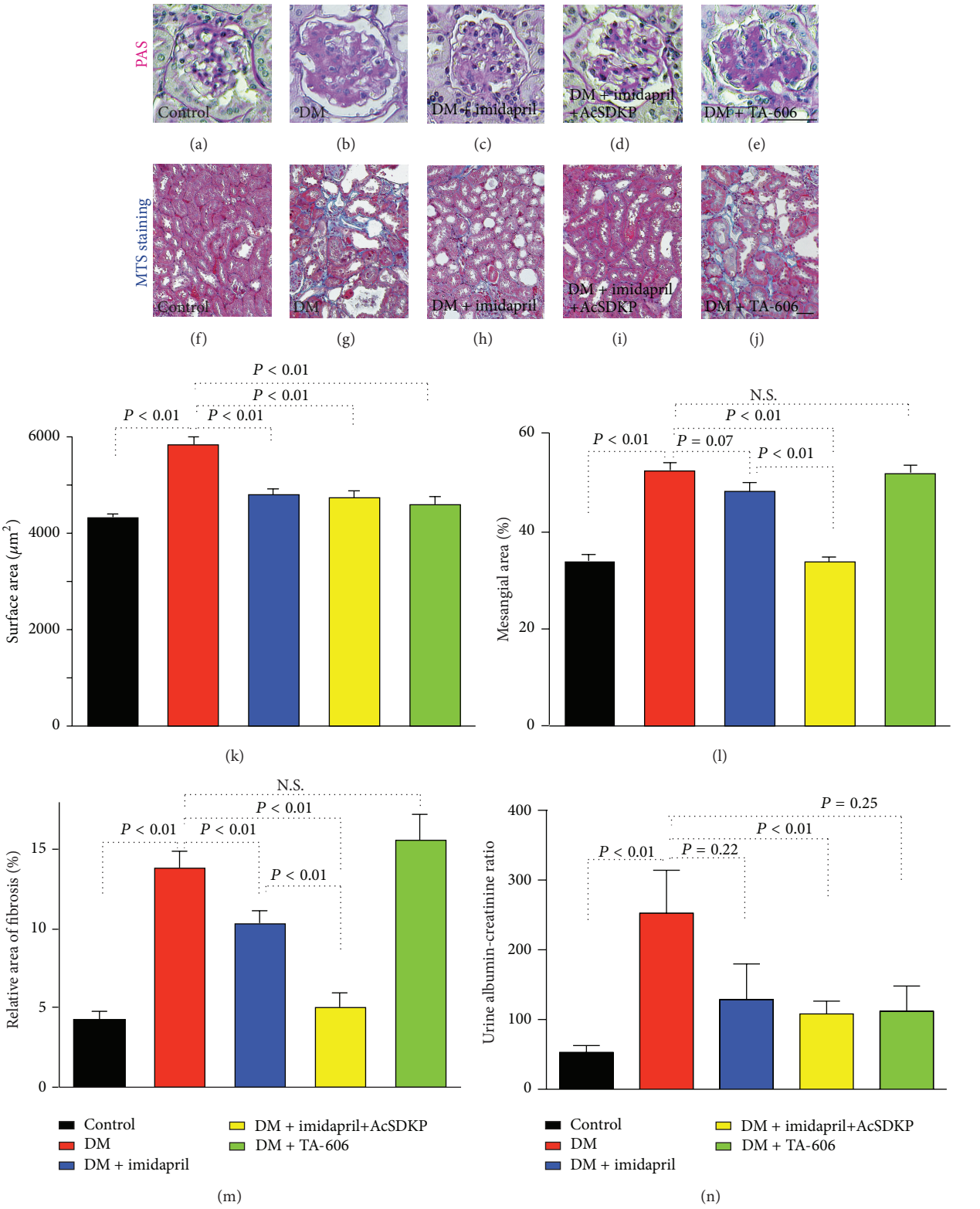

Figure 2: AcSDKP exerts antifibrotic effects on diabetic kidney disease. ((a)–(e)) Representative periodic acid Schiff- (PAS-) stained kidney samples from the indicated groups of mice. The original magnification of the images was 300x. ((f)–(j)) Representative images of Masson's trichrome-stained (MTS) samples from the indicated groups of mice are shown. The original magnification of the images was 200x. ((k)–(m)) Morphometric analysis. The glomerular surface area (k), relative mesangial area (l), and relative area of fibrosis (m) were analyzed by the method described in the Methods section. (n) Urinary albumin/creatinine ratio. The data are expressed as the mean ± SEM values. Control: $n = 7$, STZ-induced diabetes: $n = 5$, diabetes treated with imidapril: $n = 6$, diabetes treated with imidapril+AcSDKP: $n = 5$, diabetes treated with TA-606: $n = 3$. Diabetic mice are designated as DM. Scale bar: 50 μm.

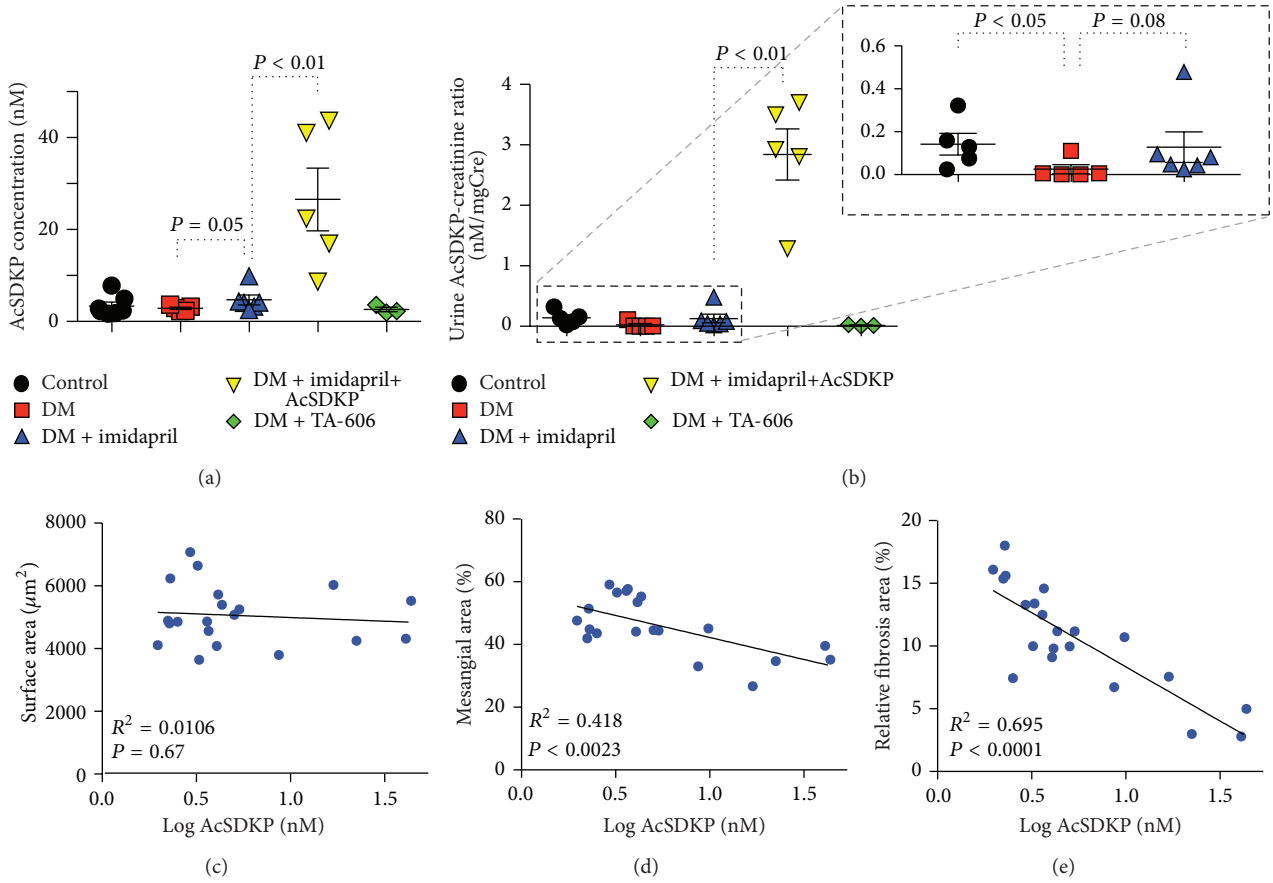

Figure 3: Negative correlation between the concentration of AcSDKP and mesangial area or relative fibrosis area. (a) Plasma concentration of AcSDKP in the indicated group of mice. (b) Urinary AcSDKP/creatinine ratio. Inset in (b) shows an enlargement of the dotted square area of the graph. The data are expressed as the mean ± SEM values. Diabetes is designated as DM. Control: $n = 7$ (urine, $n = 5$), STZ-induced diabetes: $n = 5$, diabetes treated with imidapril: $n = 6$, diabetes treated with imidapril+AcSDKP: $n = 5$, diabetes treated with TA-606: $n = 3$. ((c)–(e)) Linear regression analysis of the relationship between the plasma AcSDKP concentration and the values for the morphometric parameters. Glomerular surface area (c), relative mesangial area (d), and relative fibrosis area (e) are shown. The AcSDKP levels were plotted using a log conversion. Only diabetic animals were analyzed.

using a miScript II RT kit (Qiagen) and the real-time quantification of microRNA expression was performed using a miScript SYBR Green PCR kit (Qiagen). Samples of 3 ng of cDNA were used in the qPCR experiment. The primers for Mm_let-7f-1, Mm_let-7 g-1, and Mm_let-7i-1 were from the miScript Primer Assay designed by Qiagen. The mature microRNA sequences were 5′CUAUACAAUCUAUUGCCUUCCC for Mm_let-7f-1, 5′ACUGUACAGGCCACUGCCUUGC for Mm_let-7 g-1, and 5′CUGCGCAAGCUACUGCCUUGCU for Mm_let-7i-1. All of the experiments were performed in triplicate, and Hs_RNU6-2_1 (Qiagen) was used as an endogenous control for normalization.

2.10. Statistical Analysis. The data are expressed as the mean ± SEM values. The Mann-Whitney U-test was used to determine the significance. Statistical significance was defined as $P < 0.05$. GraphPad Prism software (ver. 5.0f) was used for the statistical analyses.

3. Results

3.1. Antifibrotic Effect of AcSDKP on the Top of ACE-I. The characteristics of the mice in each group are shown in Figure 1. Compared to the control mice, the diabetic mice had lower blood pressure, weighed less, and had higher blood glucose; their kidneys and livers weighed more, and their hearts weighed less. Treatment with imidapril, imidapril+AcSDKP, or the angiotensin II receptor blocker (ARB) TA-606 [16] did not alter the blood pressure, body weight, or organ weights of the diabetic mice (Figure 1).

The diabetic CD-1 mice exhibited glomerulomegaly and the accumulation of a PAS-positive matrix in the glomeruli (Figures 2(a), 2(b), 2(k), and 2(l)). Imidapril, imidapril+AcSDKP, and TA-606 inhibited the expansion of the glomerular surface area (Figures 2(b), 2(c), 2(d), 2(e), and 2(k)). The expansion of the mesangial area was partially inhibited by treatment with imidapril, and imidapril+AcSDKP treatment nearly completely inhibited the

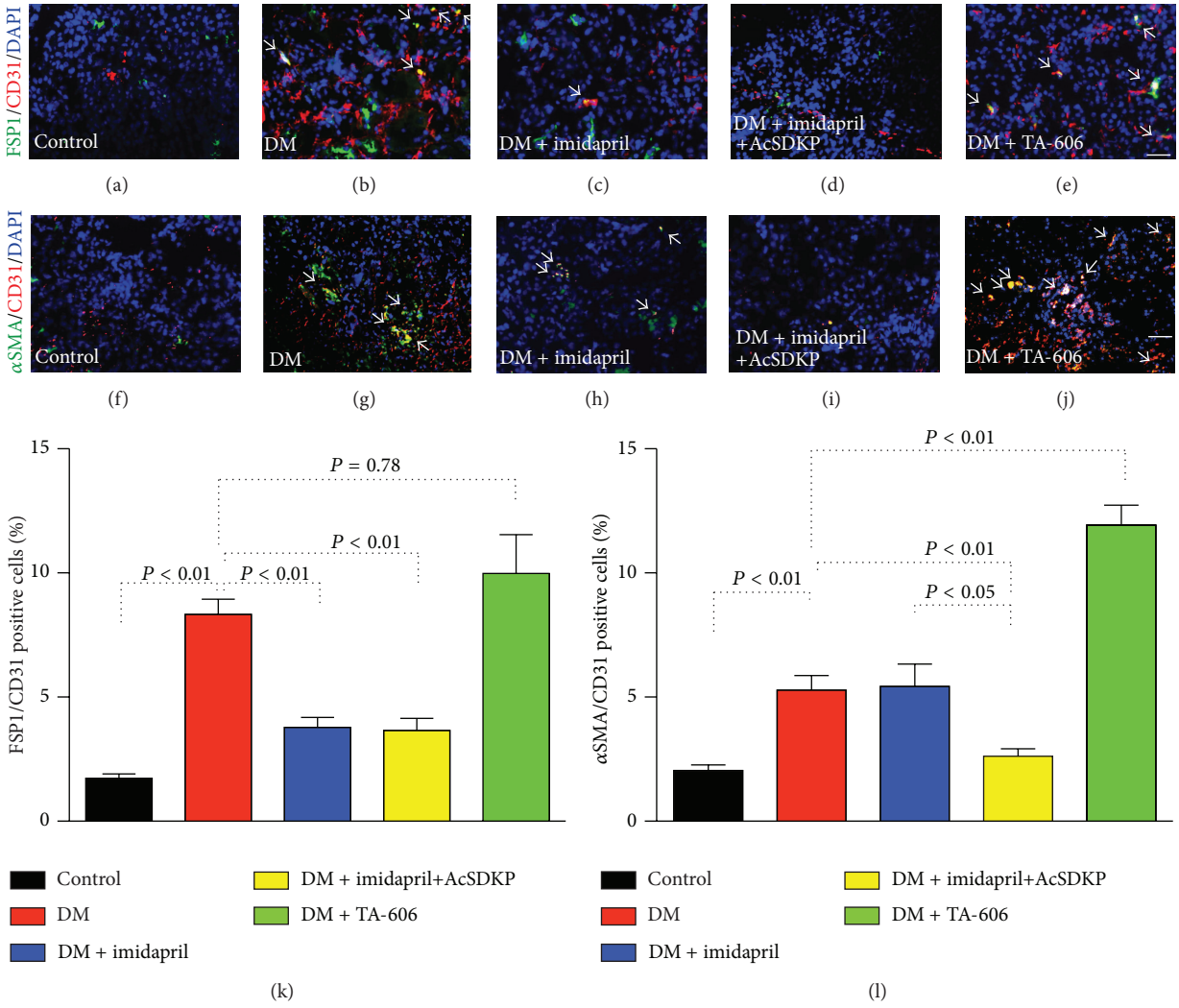

Figure 4: AcSDKP inhibits EndMT in the diabetic kidney. ((a)–(e)) Immunolabeling for FSP1 and CD31 in a kidney from each group of mice. Arrows indicate cells that are double-labeled for FSP1 and CD31. ((f)–(j)) Immunolabeling for αSMA and CD31 in a kidney from each group of mice. Arrows indicate cells that are double-labeled for αSMA and CD31. Merged images with DAPI-stained nuclei are shown. Scale bar: 25 μm. ((k), (l)) Quantification of cells undergoing EndMT. FSP1 and CD31 double-labeled cells (k) and αSMA and CD31 double-labeled cells (l) in each visual field were imaged using fluorescence microscopy and quantified. The data are expressed as the mean ± SEM values. Diabetes is designated as DM. For all of the groups except the group that received TA-606 ($n = 3$), $n = 5$ mice were analyzed.

matrix expansion (Figures 2(b), 2(c), 2(d), and 2(l)). However, TA-606 did not inhibit the expansion of the mesangial area in the diabetic kidney (Figures 2(b), 2(e), and 2(l)).

MTS staining revealed massive tubulointerstitial fibrosis in the diabetic mice that was not exhibited in the control mice (Figures 2(f), 2(g), and 2(m)). A comparison to the untreated diabetic mice showed that imidapril partially decreased interstitial fibrosis (Figures 2(g), 2(h), and 2(m)) and that imidapril+AcSDKP combination nearly completely inhibited the interstitial fibrosis (Figures 2(g), 2(i), and 2(m)). However, TA-606 treatment did not suppress kidney fibrosis (Figures 2(g), 2(h), 2(i), 2(j), and 2(m)). Compared to the control mice, the diabetic mice exhibited enhanced urinary albumin excretion (Figure 2(n)). Imidapril and TA-606 treatment inhibited the trend of increased urinary albumin excretion; imidapril+AcSDKP significantly inhibited urine albumin excretion in the diabetic mice (Figure 2(n)).

3.2. The Levels of AcSDKP and Kidney Fibrosis. The plasma AcSDKP concentration demonstrated a decreased trend in the diabetic mice (Figure 3(a)). The AcSDKP concentration was high in the mice treated with imidapril (Figure 3(a): $P = 0.05$). When AcSDKP was added to the imidapril treatment, the concentration of AcSDKP was additionally increased by 5.5-fold (Figure 3(a)). The mice treated with AcSDKP alone exhibited several antifibrotic effects of diabetic mice and displayed higher plasma levels of AcSDKP compared to the diabetic mice, as shown in several fibrotic animal models (see Supplementary Figure 1 available online at http://dx.doi.org/10.1155/2014/696475) [6–14]. The ratio of

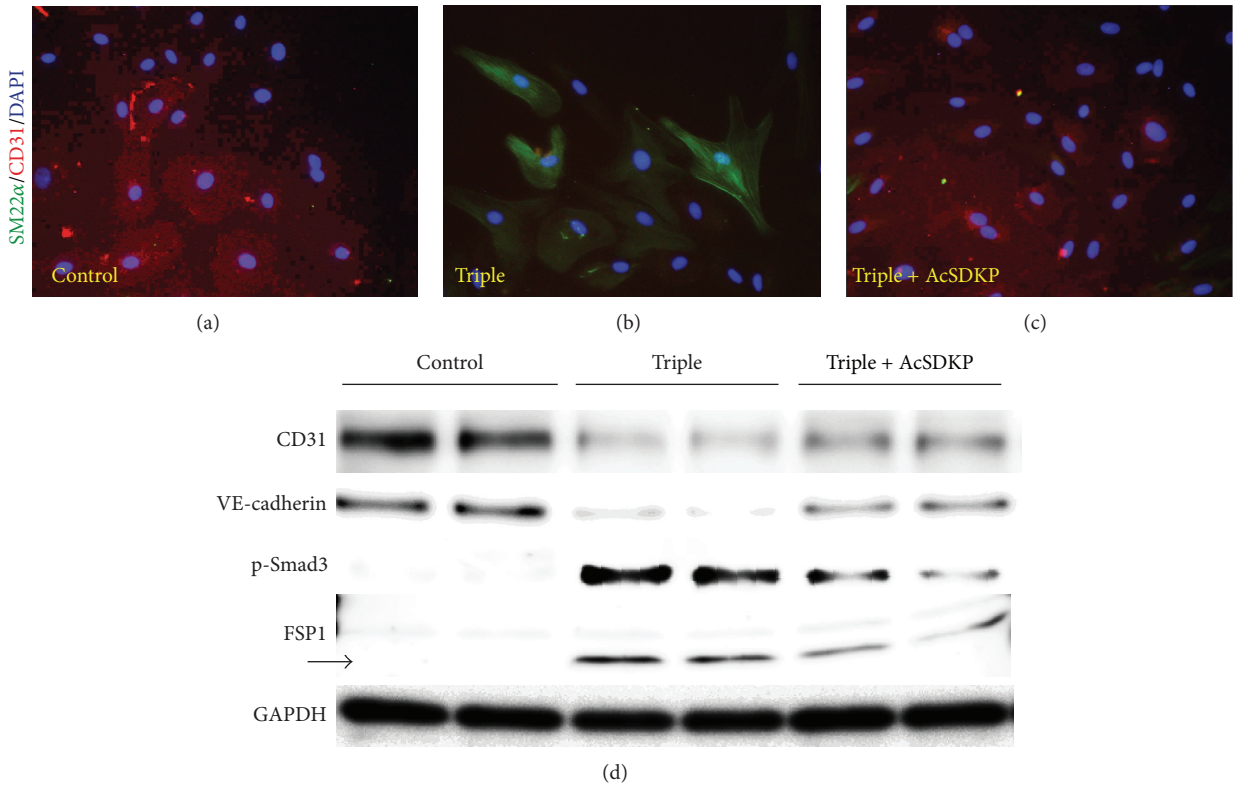

Figure 5: AcSDKP inhibits EndMT in vitro. HMVEC were exposed to a combination of cytokines (TGF-β2 at 2.5 ng/mL, IL-1β at 4 ng/mL, and TNF-α at 2 ng/mL) in the presence or absence of AcSDKP (100 nM) for 72 h. ((a)–(c)) Immunofluorescent microscopic analysis. The original magnification of the images was 200x. Merged images with DAPI-stained nuclei are shown. (d) Western blot analysis of the EndMT. HMVEC were stimulated with the triple cytokine mixture in the presence or absence of AcSDKP for 72 h. The cells were harvested, and the proteins were immunoblotted onto PVDF membranes. Chemiluminescence-detected bands were visualized. $n = 2$ in each group. GAPDH was used as the loading control.

the AcSDKP concentration to the creatinine concentration in urine exhibited a trend similar to that of the plasma AcSDKP levels, except that the urinary AcSDKP levels of diabetic mice were significantly lower than those of control mice (Figure 3(b)). TA-606 treatment in diabetic mice did not alter the level of AcSDKP either in plasma or in urine (Figures 3(a) and 3(b)). There was no correlation between the plasma AcSDKP concentration and the glomerular surface area in the diabetic mice (Figure 3(c)). In contrast, we found a negative correlation between the plasma concentration of AcSDKP and the mesangial area (Figure 3(d)) or the relative fibrotic area of the kidneys (Figure 3(e)).

3.3. AcSDKP Inhibited EndMT. An analysis of cells undergoing EndMT, which were identified by double-labeling with FSP1 and CD31 antibodies [FSP1(+); CD31(+)] or with αSMA and CD31 antibodies [αSMA(+); CD31(+)], showed that the diabetic kidneys contained significantly more cells undergoing EndMT than did the control kidneys (Figures 4(a), 4(b), 4(f), 4(g), 4(k), and 4(l)). Imidapril treatment decreased the number of FSP1(+); CD31(+) cells but did not affect the number of αSMA(+); CD31(+) cells compared to the untreated diabetic mice (Figures 4(b), 4(c), 4(g), 4(h), 4(k), and 4(l)). Imidapril+AcSDKP combination therapy nearly completely inhibited the induction of cells undergoing EndMT (Figures 4(b), 4(d), 4(g), 4(i), 4(k), and 4(l)). However, TA-606 treatment did not reduce the number of FSP1(+); CD31(+) cells; furthermore, the number of αSMA(+); CD31(+) cells was increased by TA-606 treatment relative to the diabetic mice (Figures 4(b), 4(e), 4(g), 4(j), 4(k), and 4(l)).

When HMVEC were stimulated with a triple mixture of cytokines (TGF-β2, IL-1β, and TNF-α), the expression of the endothelial marker CD31 or VE-cadherin was suppressed; the expression of the mesenchymal marker FSP1 or SM22α was induced, suggesting induction of EndMT (Figure 5). AcSDKP preincubation inhibited cytokines-stimulated EndMT associated with the suppression of smad3 phosphorylation (Figure 5).

3.4. Endogenous Antifibrosis Program by AcSDKP through FGF Receptor. Chen et al. [19] reported that FGF receptor-mediated induction of microRNA let-7 family members, which exhibits kidney protective roles [17, 18], acts as negative regulators of the EndMT program via inhibition of the TGF-β signaling pathway [19]. FGF receptor phosphorylation and protein levels were suppressed in diabetic kidney [20]; imidapril treatment increased both the phosphorylation and

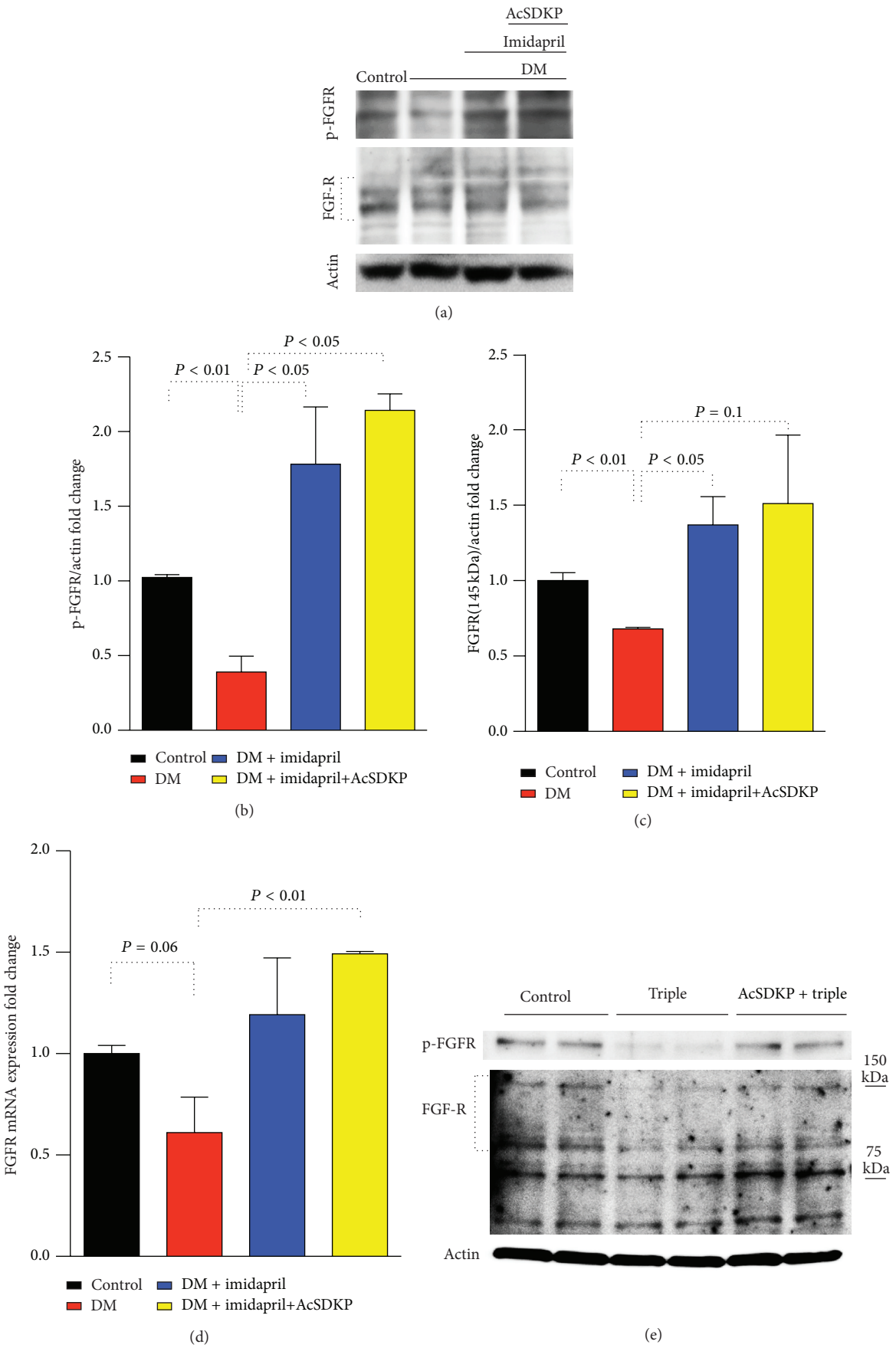

Figure 6: Continued.

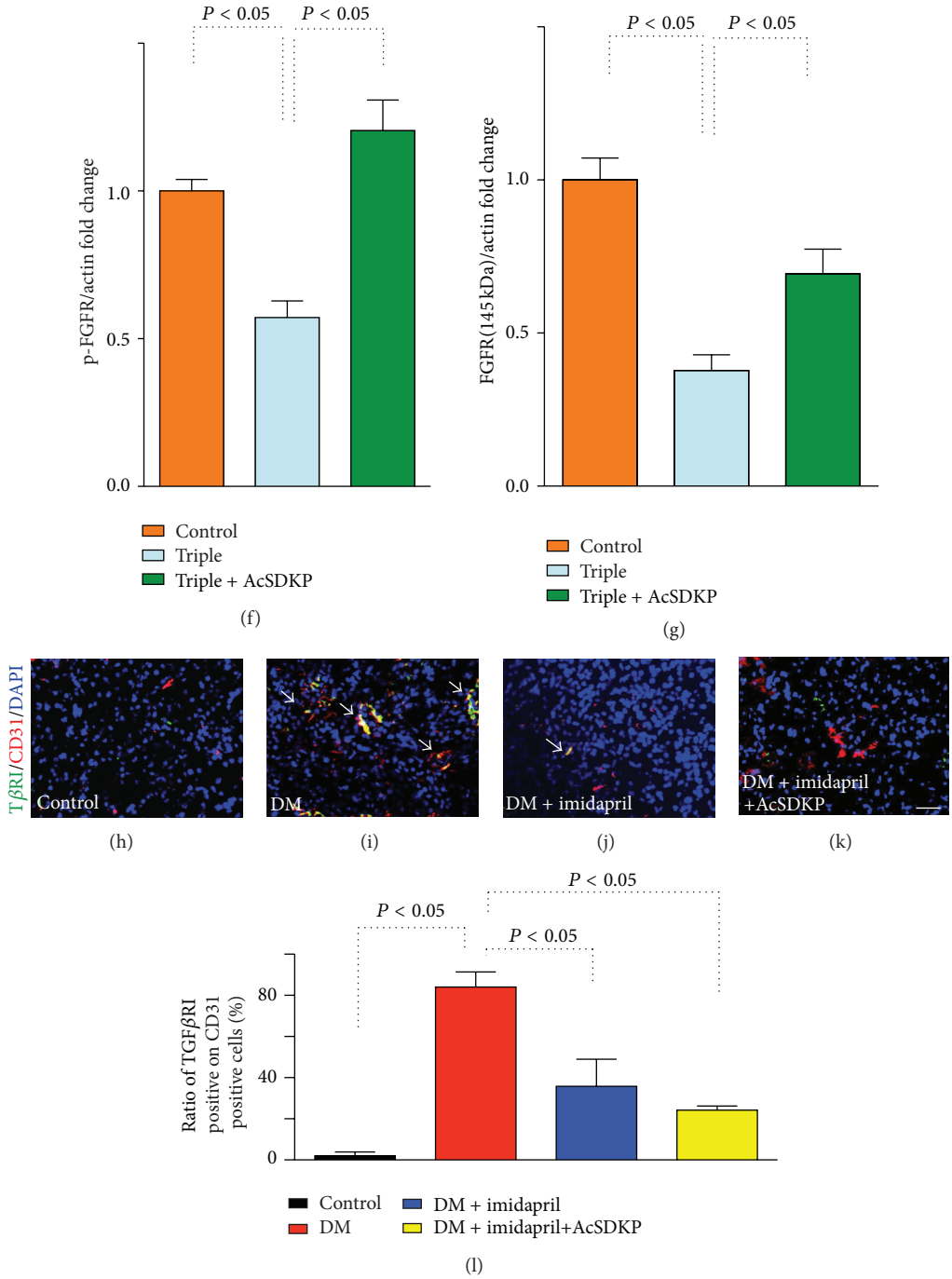

Figure 6: Antifibrotic effect of AcSDKP is associated with restoration of FGFR levels. (a) Western blot analysis of FGF receptor phosphorylation and total protein levels are shown. Actin is shown as a loading control. ((b)-(c)) Densitometric analysis of phospho-FGF receptor and total FGF receptor levels normalized to actin. The results are shown as the relative expression against the control animal values. The data are shown as the mean ± SEM values ($n = 3$). (d) qPCR analysis for FGF receptor in kidney. The data are shown as the mean ± SEM values. $n = 3$ in each group were analyzed. (e) Western blot analysis for FGF receptor levels and its phosphorylation. The same sample as Figure 5(d) was analyzed for FGF-R status. Representative results ($n = 2$) were shown. ((f), (g)) Densitometric analysis of phospho-FGF receptor and total FGF receptor levels normalized to actin. The results are shown as the relative expression against the control value. The data are shown as the mean ± SEM values ($n = 4$). TGF-β receptor I on endothelial cells in diabetic mice. ((h)–(k)). Immunolabeling for TGF-β receptor I (TβRI) and CD31 in a kidney from indicated group of mice. Arrows indicate cells that are double-labeled for TβRI and CD31. Scale bar: 25 μm. (l) Quantification of cells expressing TβRI and CD31. Ratio of TβRI positive on all CD31 positive cells in each visual field among 4 images from one animal using fluorescence microscopy and quantified. The data are expressed as the mean ± SEM values. Diabetes is designated as DM. For all of the groups $n = 4$ mice were analyzed.

protein levels of the FGF receptor in the diabetic kidney (Figures 6(a), 6(b), and 6(c)). A combination therapy exhibited stronger effects on the FGF receptor levels and phosphorylation (Figures 6(a), 6(b), and 6(c)). Such effects of intervention on the FGF receptor in diabetic mice were likely transcription-dependent (Figure 6(d)). When HMVEC was stimulated by mixture of cytokines, the protein levels and phosphorylation of the FGF receptor were significantly diminished; AcSDKP preincubation restored the FGF receptor levels (Figures 6(e), 6(f), and 6(g)).

Our microRNA array analysis of kidney samples revealed that expression of the microRNA mmu-let-7 family was suppressed in the diabetic kidney; we found that expression of most of the microRNA let-7 family members was restored by therapy with the combination of imidapril+AcSDKP (Supplementary Figure 2). qPCR analysis also confirmed that certain sets of microRNA let-7 were indeed inhibited in diabetic mice (Supplementary Figure 2); treatment with the imidapril+AcSDKP combination therapy completely restored their levels. The FGF receptor-microRNA let-7 family axis can suppress TGF-β receptor I levels [19, 21]. In agreement with this report, we observed that endothelial cells in diabetic mice exhibited strong expression of TGF-β receptor I; the combination therapy group nearly completely abolished such TGF-β receptor I expression on endothelial cells in the diabetic kidney (Figures 6(h), 6(i), 6(j), 6(k), and 6(l)).

4. Discussion

4.1. AcSDKP Inhibited EndMT in Fibrotic Kidney in Diabetes. EndMT has emerged as an important source of activated fibroblasts or myofibroblasts [1, 2, 22–24]. The EndMT has been shown to be associated with glomerulosclerosis in early diabetic kidney disease and tubulointerstitial fibrosis in a chronic type 1 diabetes kidney disease model [25]. The TGF-β-induced Smad signaling pathway plays an essential role in the EndMT [26]. In our analysis, AcSDKP inhibited both EndMT in diabetic kidney and in vitro culture cells associated with the inhibition of TGF-β/Smad signal transduction. It needs to be mentioned that we rather focused on EndMT in our analysis; there is a possibility that AcSDKP inhibits other fibroblast activation pathways, as reported elsewhere [27]. Also anti-inflammatory, antiapoptotic, and enhanced normal angiogenesis pathways would be contributed in the beneficial effects of AcSDKP [3].

4.2. Two Catalytic Sites of ACE and Endogenous Antifibrotic Program via AcSDKP. Mammalian ACE has two catalytic sites, the N- and C-terminal catalytic domains. These two catalytic domains may have different substrate selectivity. Although angiotensin-I can be converted to angiotensin-II by either catalytic domain, the C-terminal domain has a threefold higher affinity for angiotensin-I. Interestingly, only the N-terminal catalytic domain hydrolyzes AcSDKP [3]. Notably, each ACE-I exhibits a distinct affinity for one of the catalytic domains; for example, captopril exhibits a higher affinity for the N-terminal catalytic domain. Li et al. recently reported that mice deficient in the N-terminal catalytic domain of ACE exhibited an antifibrotic effect because of the accumulation of AcSDKP [28], which reveals the importance of the N-terminal domain for the antifibrotic activity of ACE-I [29–32].

4.3. AcSDKP Stimulates Antifibrotic Program. In our analysis, the concentration of AcSDKP was negatively associated with mesangial expansion and kidney fibrosis. Moreover, we found that endogenous AcSDKP levels were lower in the urine of diabetic animals with fibrotic kidneys. Similar to this observation, suppressed levels in other antifibrotic molecules, such as bone morphogenic protein 7 or its receptor-mediated signaling, in fibrotic kidney diseases have been reported elsewhere [33]. Apart from diabetic kidney disease model, the association between the levels of AcSDKP, other fibrotic kidney disease, and human kidney diseases needs further investigation. These results suggest that high intrarenal ACE activity in the diabetic kidney reduced the level of endogenous AcSDKP; this reduction of the antifibrotic peptide AcSDKP accelerated the fibrotic process in the kidney because of the imbalance between profibrotic and antifibrotic molecules. The antifibrotic/anti-EndMT effects of AcSDKP were associated with restoration of the FGF receptor's levels and associated induction of microRNA let-7. Regard with this, microRNA let-7 family has been shown to protect kidney from fibrotic stimuli [17, 18].

5. Conclusion

In conclusion, AcSDKP is potentially a valuable endogenous antifibrotic molecule that inhibits the EndMT and restores the expression of the let-7 microRNA family through FGFR restoration at least in part. AcSDKP may therefore be useful for the clinical therapy for kidney fibrosis in diabetes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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